PRE CLINICAL AND CLINICAL STUDIES OF THE EFFECTS OF RESVERATROL, A PHYTOCHEMICAL WITH POTENTIAL CHEMOPREVENTIVE EFFICACY

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ABSTRACT

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Victoria Alison Brown

Resveratrol, found in grape skins and red wine, has potential chemopreventive activity *in vitro* at concentrations ≥5μM. This project explored the tolerability, pharmacokinetics and pharmacodynamics of resveratrol in two clinical phase 1 studies. *In vivo* data were supported by *in vitro* studies designed to mimic the daily dosing protocol, using concentrations observed clinically. Forty healthy volunteers received 29 oral daily doses of 0.5, 1.0, 2.5 and 5.0g resveratrol and 20 colorectal cancer patients 8 oral daily doses of 0.5 and 1.0g prior to surgical resection. The pharmacodynamics of resveratrol were assessed by measuring changes in plasma levels of proteins involved in the IGF system, oxidative DNA damage in whole blood and colorectal tissue (M_1dG), effects on inflammatory pathways in plasma (PGE_2) and colorectal tissue (COX-2), as well as colorectal tissue proliferation (Ki-67).

No serious adverse events were reported. In volunteers, mean peak plasma levels of resveratrol across the dose groups ranged from 44.7-954ng/mL (0.20-4.20μM), and for the main metabolite, resveratrol-3-sulfate, were 4-13 fold-higher. Despite low systemic bioavailability, resveratrol concentrations associated with potential chemopreventive efficacy were observed in colorectal tumour tissue with a mean of 44.0nmol/g detected in patients receiving the 1.0g dose (range 0.30-195nmol/g).

Post dosing, IGF-1 levels were reduced by 8% (P=0.03) in volunteers and by 33% (P<0.001) in colorectal cancer patients. In tissue, a reduction in cell proliferation of 5.5% (P=0.05) was observed, whilst there was an increase in COX-2 staining (P=0.004). Apart from the 2.5g dose in volunteers, where a significant increase was observed in blood M_1dG (21.6%, P=0.02), resveratrol did not significantly affect plasma PGE_2 or markers of DNA damage in either study. In cultured colon cancer cells, daily exposure to resveratrol was associated with increased antiproliferative activity compared to an equivalent single dose, supporting the indication that chronic administration may cause pharmacodynamic changes in humans. The work presented here suggests resveratrol has potential as a cancer chemopreventive agent and controlled clinical trials are now warranted.

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INTRODUCTION

1.1 Chemoprevention

1.1.1 Defining chemoprevention

Cancer continues to be a major cause of death throughout the world and only modest progress has been achieved in reducing the morbidity and mortality of this dreadful disease (Hail *et al*., 2008). Cancer is the result of a multistage, multi-mechanism carcinogenesis process involving mutagenic and epigenetic mechanisms (Trosko and Upham, 2005) which can take years to reach a final, invasive stage in humans (Sporn *et al*., 1976). Therefore, the concept of delaying or preventing epithelial transformation remains a potentially attainable goal for the future (Brenner and Gescher, 2005). Cancer chemoprevention is defined as using natural or synthetic compounds to reverse, suppress, delay or prevent carcinogenesis and refers to the administration of agents on a pre-determined schedule (Smith *et al*., 2005).

The field of cancer chemoprevention is emerging from its pioneer stage (De Flora and Ferguson, 2005) and if successfully deployed, could be a promising approach in reducing the incidence and burden of this disease (Brenner, 2008). An essential aspect of chemoprevention is to identify the appropriate target population who would benefit most from these strategies, for example, people at high-risk due to familial and genetic factors. Although carriers of gene mutations generally represent a small proportion of all at-risk cohorts, their risk is particularly high of developing one or more malignancies, usually at a much younger age than is the case for sporadic cancers (Veronesi and Bonanni, 2005). The risk/benefit ratio and ethical considerations are

lower in this population and such an approach is likely to be more cost effective than a chemopreventive intervention for the general population.

1.1.2 Mechanisms of chemoprevention

Tumour development is thought to consist of three separate, but closely linked stages of tumour initiation, promotion and progression. Initiation is a rapid and irreversible process involving a series of extracellular and intracellular events, including exposure to or uptake of a carcinogenic agent, followed by its distribution and transport to organs and tissues, where metabolic activation and detoxification can occur (Surh, 2003). The covalent interaction of reactive species with target-cell DNA can lead to the formation of DNA adducts, oxidised lesions or strand breaks (Surh, 2003). The presence of DNA adducts does not necessarily mean that mutations will result, as lesions may be repaired before cell division takes place or the DNA adduct may not be promutagenic (Singh and Farmer, 2006). However, if adducts are not repaired, alterations in the DNA sequence may occur upon DNA replication and become permanently fixed, which could lead to the synthesis of altered proteins (Singh and Farmer, 2006). In contrast to initiation, tumour promotion is a relatively lengthy and reversible process. During this stage actively proliferating preneoplastic cells accumulate. Progression, the final stage of neoplastic transformation, involves the growth of a tumour with invasive and metastatic potential (Surh, 2003).

The mechanisms of action of most inhibitors of carcinogenesis, both synthetic and naturally occurring, are generally multitargeted and currently not well understood.

Therefore, absolute classification of known chemopreventive compounds is difficult. A classification scheme developed by Wattenberg (Wattenberg, 1985) separates compounds into `blocking' or `suppressing' agents depending on the stage of the carcinogenic process on which they exert their effect (Figure 1.1.1). Blocking agents are effective at the initiation stage by preventing or reducing damage to DNA, particularly when administered before the carcinogen. Mechanisms of blocking agents include alterations to the profile of both phase I and II drug metabolising enzymes and rates of DNA repair as well as scavenging of reactive oxygen and other free radical species. Even if DNA has been damaged, blocking agents can limit further damage and mutation accumulation (Manson *et al*., 2000). Examples of blocking agents include certain polyphenols and isothiocyanates which act via induction of phase II enzymes; isothiocyanates can also inhibit cytochrome P450s involved in carcinogen activation (Hecht, 1999; Scalbert *et al*., 2005).

Suppressing agents act later in the carcinogenesis process, during the promotion and/or progression stages. They have been shown to mitigate the consequences of altered gene expression by reducing proliferation of initiated cells and restoring apoptosis to normal levels, thereby preventing damaged cells from accumulating (Manson *et al*., 2000). Examples of suppressing agents include aspirin and curcumin, which inhibit arachidonic acid (AA) metabolism, and epigallocatechin gallate (EGCG) and selenium, which can act as suppressing agents by inhibition of oxidative DNA damage (Morse and Stoner, 1993). Some inhibitors possess both blocking and suppressing properties (Greenwald et al., 1995; Manson et al., 2000; Wattenberg, 1985), including many natural products contained in fruits, vegetables and tea beverages, such as sulforaphane from broccoli,

resveratrol from grapes, genistein from soy, curcumin from turmeric and EGCG from green tea (Chen and Kong, 2005).

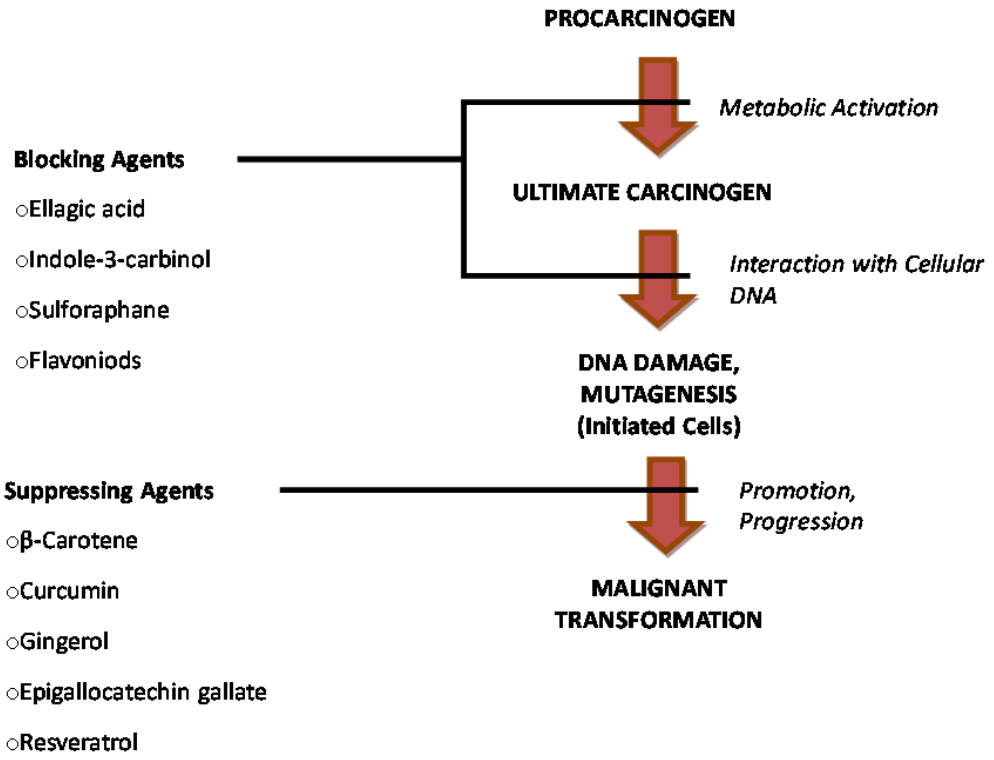


Figure 1.1.1 Classification of chemopreventive phytochemicals based on their mechanisms of action. Conventional classification of chemopreventive agents is based on the underlying mechanisms by which they exert protective effects in a specific stage of multistep carcinogenesis. Agents are subdivided into two major categories, blocking agents and suppressing agents (Section 1.1.2). Many chemopreventive agents e.g. curcumin and resveratrol have more than one defined mechanism of action, and hence, possess both blocking and suppressive properties. Modified from Surh (1999).

1.1.3 Preclinical development of chemopreventive agents

The US National Cancer Institute's Chemoprevention Programme uses efficacy and safety data to aid the preclinical and clinical development of potential chemopreventive agents. Data from epidemiological, laboratory and clinical research are surveyed for compounds that demonstrate possible cancer protective activity. These agents are then evaluated in a series of *in vitro* and *in vivo* assays to assess their biological effects in order to evaluate their potential in preventing the onset or progression of neoplasia in humans (Greenwald *et al*., 1995). For example, *in vitro* mechanistic assays and animal models of carcinogenesis including carcinogen induced and genetic models, to reflect various mechanisms of carcinogenesis are frequently conducted (Kelloff *et al*., 1994). The most promising agents undergo preclinical toxicity evaluation and pharmacokinetic parameters are determined. Clinical development is then planned only for agents that show efficacy and meet acceptable toxicity criteria. Additional testing is often undertaken to determine alternative routes of agent delivery, dosing regimens, new molecular targets and target tissues (Steele and Kelloff, 2005). An outline of the developmental approach for potential cancer chemopreventive compounds present in the diet is shown in Figure 1.1.2.

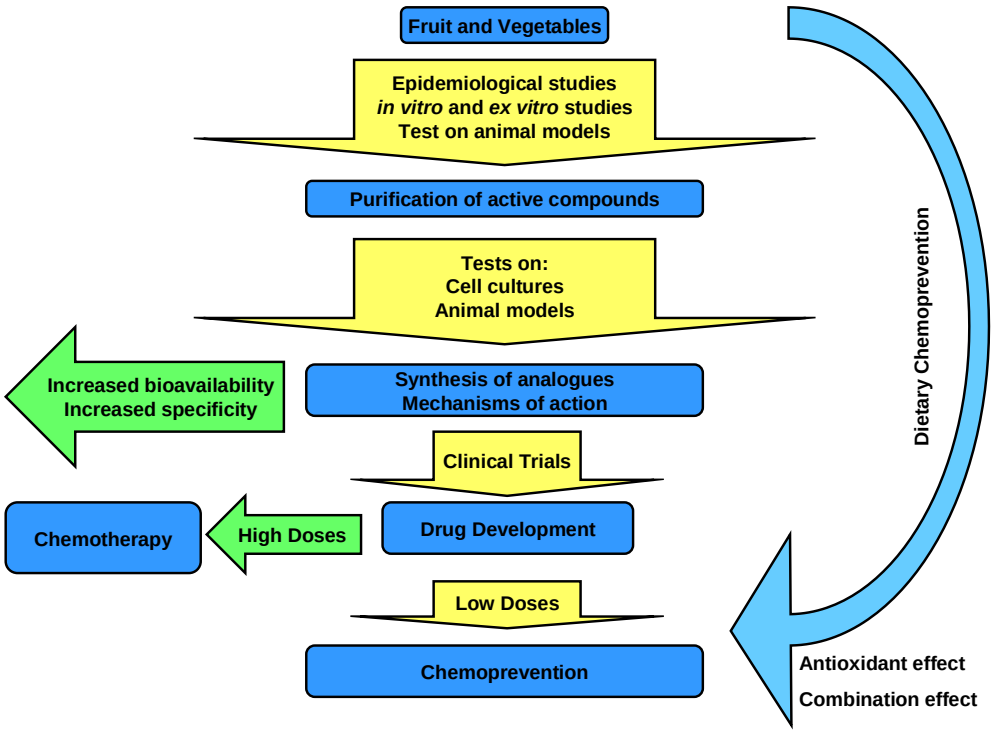


Figure 1.1.2 Approach to cancer prevention based on potential chemopreventive compounds present in the diet. Based on epidemiological and experimental studies, natural products, such as phytochemicals, can be isolated from their natural sources, purified and assayed to test their ability in killing precancerous and cancerous cells, with *in vitro* models. The procedure should continue with preclinical studies on animal models and phase I–III clinical trials. Synthetic analogues may be designed with improved bioavailability or greater activity towards a specific molecular target. Modified from Russo (2007).

1.1.4 Clinical development of chemopreventive agents

Chemoprevention studies differ from cancer chemotherapy treatment trials in several important aspects, including study populations, agents and endpoints.

Chemoprevention studies target individuals who are seemingly healthy but may be at

increased risk for developing cancer due to genetic susceptibility, premalignant conditions (e.g. oral leukoplakia) or carcinogenic exposure. In addition they may have been treated for a malignancy in the past and are at increased risk of developing a recurrence or a second primary tumour (Greenwald *et al*., 1995; Smith *et al*., 2005). These studies often present challenges in recruitment, retention, adherence and long-term follow up. Healthy individuals may be less likely to comply with protocol requirements that include the administration of an agent for a considerable period of time without knowledge of benefit and may find the slightest toxicity intolerable (Smith *et al*., 2005).

As cancer has a long latency, reduced incidence is not a practical endpoint for clinical evaluation of chemopreventive agents. Therefore intermediate biomarkers of carcinogenesis are being evaluated for use in preclinical and clinical studies as endpoints in chemopreventive agent development (Kelloff, *et al*., 2004; O'Shaughnessy *et al*., 2002). A primary criterion for endpoint biomarkers is that they fit expected biological mechanisms, including differential expression in normal and high-risk tissue and being closely linked to the causal pathway for the cancer. A further use of biomarkers reflecting mechanistic activity is to identify individuals who will benefit from treatment with specific chemopreventive agents (Steele and Kelloff, 2005).

Clinical chemoprevention studies are generally divided into 3 phases. Phase I trials are generally short term pilot projects to evaluate the pharmacokinetics (drug metabolism and bioavailability) and pharmacodynamics (target tissue and surrogate biomarkers) of the agent under evaluation. They are also designed to assess tolerance to escalating doses of the agent, whilst the subject is carefully observed using clinical assessment and

blood assays for toxicity, and include single and repeat dose studies. Repeat dose studies are generally conducted for a period of 1–3 months in normal subjects or longer in subjects at increased risk of cancer (Einspahr *et al*., 1997).

In contrast to small phase 1 pilot studies, phase II chemoprevention trials generally use randomized, blinded, placebo-controlled design to evaluate dose response and common toxicities likely to result from prolonged administration, preferably of 3 months or longer. In addition, there is the evaluation of intermediate biomarkers that may be correlated to cancer incidence or mechanisms of carcinogenesis, in order to determine any potential clinical effect of the putative chemopreventive agent. If safety and biomarker results are judged to be promising in these trials, agents proceed to phase III studies which are randomized, blinded, placebo-controlled. These trials test the efficacy of an agent in preventing cancer and assess compliance and toxicity. Studies are typically large and have the objectives of measuring the incidence of primary tumours in addition to the interim assessment of preneoplastic biomarkers. The long time frames involved necessitate assurances of the reproducibility of the formulation administered and patient compliance (Fisher *et al*., 1998; Lee *et al*., 2001a).

1.1.5 Biomarkers as chemopreventive targets and efficacy endpoints

Studies of chemopreventive approaches have been hindered by the long latency of the development of cancer which requires as a study endpoint observing many individuals over a longer period of time than required in standard phase III clinical trials of chemotherapy agents. Therefore, there is great interest in determining the biomarkers associated with specific stages of the carcinogenic process as intermediate endpoints

(Lee *et al*., 2001a), in order to reduce sample size, time and costs of chemoprevention studies. Schatzkin *et al.,* identified such biomarkers as a biological event which takes place between a carcinogen or external exposure and the subsequent development of cancer, for example a discrete event, such as the formation of colonic adenomas, or a quantitative change, such as an increase in cell proliferation (Schatzkin *et al*., 1990).

Validation of a biomarker requires establishing its relationship with the subsequent risk of cancer (Einspahr *et al*., 1997). Laboratory or assay validation is undertaken to assess the reliability and accuracy of the method, optimize sample acquisition and storage and determine potential confounders (Einspahr *et al*., 1997). To date, no single biomarker has been thoroughly validated as a surrogate for cancer incidence (Lippman *et al*., 1994). Examples of potential efficacy biomarkers for chemopreventive agents include specific molecular pathways, levels of circulating proteins and expression of histological markers (Veronesi and Bonanni, 2005). A summary of the ideal characteristics of biomarkers for use as intermediate endpoints in cancer chemoprevention efficacy assessment is shown in Table 1.1.1.

1.1.1 Ideal characteristics of biomarkers for use as intermediate endpoints in cancer chemoprevention efficacy assessment. Modified from Brenner (2008).

Ideal characteristics of biomarkers
Variability of expression between phases of the carcinogenesis process
Detected early in the carcinogenesis process
Genetic progression or protein pathway-based
Mechanistic target of modulation by preventive interventions
Changes in biomarker linked to reduction in incident cancer of epithelial target
Changes in biomarker linked to clinical benefit
Can be quantified directly or via closely related activity such as a downstream target or upstream kinase
Measurable in an accessible biosample (preferably urine, serum, saliva, stool)
High throughput, technically feasible, analytical procedure with strong quality assurance/quality control procedures
Statistical accuracy, precision and relevance of results to chemoprevention
Cost effective

1.1.6 Large scale randomised clinical chemopreventive studies

A number of agents with potential chemopreventive efficacy have been highlighted in pre clinical and early phase clinical data but important practical advances have only been made in a few cases. For example, tamoxifen has gained considerable interest in the chemoprevention of breast cancer. A meta-analysis (Cuzick *et al*., 2003) of four large primary prevention trials of tamoxifen (20 mg daily compared to placebo) in healthy individuals (Investigators and Cuzick, 2002; Fisher *et al*., 1998; Powles *et al*., 1998; Veronesi *et al*., 2002) revealed a 38% reduction in total breast cancer incidence; when considered separately, tamoxifen had no effect on the numbers of cancers

negative for oestrogen receptor (ER) but dramatically decreased the occurrence of ER-positive cancers by almost half. Despite these encouraging results, it is currently not clear if tamoxifen actually prevents or delays the onset of breast cancer, although recent findings have shown that the beneficial effects persist well beyond the 5-year treatment period (Cuzick *et al*., 2007). Unfortunately, tamoxifen use is complicated by an increase in endometrial cancer and venous thromboembolic events (VTE), and therefore a debate about how to provide a better risk/benefit ratio arises (Brown, 2002). Use of a lower dose of tamoxifen has been suggested as phase II trials have shown beneficial effects on markers of breast carcinogenesis, such as cell proliferation by Ki-67 and the IGF (insulin-like growth factor) system even at low doses (Decensi *et al*., 1998, Decensi *et al*., 2003). Currently the US Food and Drug Administration (US FDA) approves the use of tamoxifen in three settings, for prevention of contralateral cancer and ductal carcinoma in situ (DCIS) and as a chemopreventive agent in women at high risk for breast cancer. However, the use of tamoxifen in healthy women has been somewhat less than expected, in part because of the variability of results from the individual trials and the associated toxicity, but also because of uncertainty about the durability of the beneficial effect.

Other chemoprevention trials have also created much debate, including the Prostate Cancer Prevention Trial of Finasteride (Thompson *et al*., 2003), which to date, is the only reported phase III randomized clinical trial to evaluate the role of 5-alpha reductase inhibitors in the prevention and treatment of prostate cancer. Finasteride, a drug that is usually used in men for benign prostatic hypertrophy, reduced prostate cancer risk by 25% in comparison with placebo (Thompson *et al*., 2003). However, the original study data showed that patients who received finasteride had a greater incidence of high-grade

tumours and therefore potentially more aggressive tumours, which has currently limited the acceptance of finasteride as a chemopreventive agent by urologists.

After non-steroidal anti inflammatory drugs (NSAIDs) showed efficacy in reducing sporadic and hereditary colorectal adenomas and carcinomas, the US FDA has approved the use of celecoxib as adjuvant treatment of familial adenomatous polyposis (FAP) (Steinbach *et al*., 2000). Unfortunately its use has also been associated with an increased risk of cardiovascular disease (Baron *et al*., 2008).

Unfortunately not all chemoprevention studies have shown a benefit in cancer reduction. For example vitamins have generally been disappointing in trials. A precursor of vitamin A, β-carotene, actually increased rather than decreased the risk of lung cancer and lung cancer death in smokers and former smokers (Omenn *et al*., 1996). In addition vitamin E supplementation failed to prevent prostate cancer (Herbst *et al*., 2006). Therefore, although some large scale trials have shown a benefit in cancer reduction, a number of issues have been highlighted, which currently limit the widespread clinical acceptance of these agents. The complex aspects of the risk/benefit ratio, selection of individuals at high risk of cancer who may benefit most, and the appropriate dose and duration of treatment are still unresolved issues in many settings requiring additional research.

1.2 Colorectal cancer

1.2.1 Colorectal cancer chemoprevention

Colorectal cancer, a major cause of morbidity and mortality, is the second leading cause of cancer death, representing a major public health concern in all developed countries (Jemal *et al*., 2006). In the UK alone in 2006 there were 15,957 deaths from colorectal cancer, comprising 10,119 from colon and 5,838 from rectal cancer (Cancer Research UK, cancer statistics). Colorectal cancer is thought to result from a series of molecular, biochemical and histopathological changes through which normal colonic epithelial cells are transformed into a neoplasm (Fearon and Vogelstein, 1990). This is a chronic process, with an adenomatous polyp as an intermediate step (Fearon and Vogelstein, 1990). Age represents a major risk factor for colorectal cancer. Thirty to fifty percent of individuals over the age of 50 will develop adenomatous polyps and 1-10% of these polyps will progress to cancer in 5 to 10 years. The risk of a polyp developing into a cancer depends on characteristics of the polyp including size, architecture, degree of dysplasia as well as the total number of polyps present. Therefore, colorectal cancer represents an excellent model for primary and secondary prevention, given the availability of effective screening procedures with the adenomatous polyp as a good intermediate biomarker (Atkin and Saunders, 2002).

As surgical resection remains the only curative treatment, early detection is the goal of screening programs that use routine examination of stool for occult blood, with or without intermittent endoscopic examination of the bowel. Randomized studies have shown a reduction in mortality in the order of 15 to 33 % in those who undergo routine

screening (Hardcastle *et al*., 1996; Kronborg *et al*., 1996; Mandel *et al*., 2000). Although this seems encouraging, the optimal method for early detection is currently unclear and compliance is also a concern (Vernon, 1997). Therefore an alternative approach to reducing mortality from colorectal cancer involves the long-term use of a variety of oral agents that can delay, prevent or even reverse the development of adenomas in the large bowel, thus interfering with the multi-step progression from adenoma to carcinoma (Arber, 2005). Evidence from epidemiological and animal studies have shown that several chemopreventive agents exert their effect at different stages in the colon carcinogenesis pathway (Figure 1.2.1). This intervention is of particular importance to individuals with a hereditary predisposition to colorectal neoplasia.

Patients at high risk of colorectal cancer include those with a family history, who have 2 or more first or second-degree relatives (or both) with colorectal cancer and represent approximately 20% of all people with colorectal cancer (Lynch and de la Chapelle, 2003). About 5 to 10% of all cases of colorectal cancer develop in people with FAP, which is caused by a germline mutation in the adenomatosis polyposis coli (*APC*) gene located on chromosome 5, in which numerous colonic adenomas appear during childhood. Ninety percent of affected subjects will develop colonic cancer by the age of 45. Hereditary nonpolyposis colorectal cancer (HNPCC) accounts for approximately 1 to 5 % of all colonic adenocarcinomas caused by mutations in one of the mismatch repair genes, hMLH1, hMSH2, hMSH6, or hPMS2 and is primarily characterized by predominant involvement of the right colon. The mean age at initial colorectal cancer diagnosis is 45 years and the overall risk is 80 % in gene carriers (Lynch and de la Chapelle, 2003).

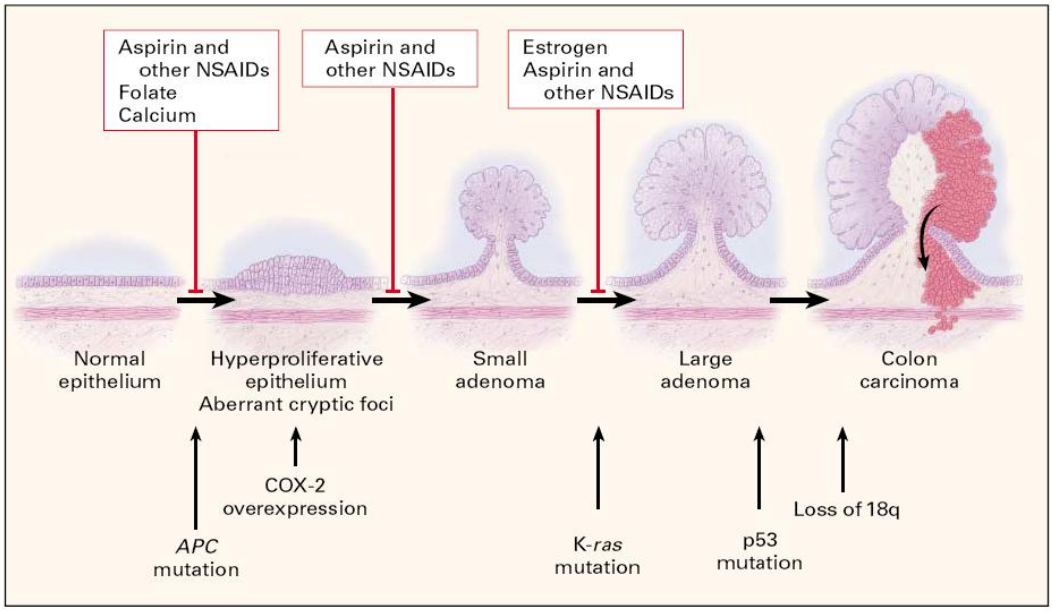


Figure 1.2.1 Colon carcinogenesis and the effects of chemopreventive agents. Colon cancers result from a series of pathologic changes that transform normal colonic epithelium into invasive carcinoma. The early stage of adenoma is marked by *APC* gene mutation and hypermethylation followed by K-*ras* mutation. In later stages of the process of adenoma transformation, p53 is lost as the adenoma develops towards carcinoma. Various chemopreventive agents exert their effects at different steps in this pathway, depicted on the basis of the available epidemiologic evidence, the results of studies in animals, and the known mechanisms of action of the agents. COX-2 refers to cyclooxygenase-2 (Janne and Mayer, 2000).

1.2.2 Studies of colorectal cancer chemopreventive agents

1.2.2.1 Clinical studies

Considerable research based on animal models and human data has suggested that aspirin and NSAIDs inhibit colorectal carcinogenesis. The potential role of aspirin as a chemopreventive agent was initially highlighted in humans in a large case-control study of colorectal cancer. This study explored a number of potential associations between

various medications (Kune *et al*., 1988) and found an inverse association between aspirin use and the risk of colorectal cancer. Since then, a large number of studies (Dube *et al*., 2007) have been conducted to explore this association and the results have demonstrated that aspirin leads to a reduction of 20 to 40% in the risk of colonic adenomas, depending on dose, duration and study design. For example, a randomized double blinded trial in patients with a history of colonic adenomas showed aspirin, at the low dose of 81 mg/day for 3 years, reduced the incidence of recurrent adenomas (Baron *et al*., 2003), with the risk reduction greatest for advanced lesions. For uncertain reasons the higher dose of 325 mg was not associated with a significant reduction in adenoma recurrence. However, patients with a history of colorectal cancer, who were randomly assigned to aspirin (325 mg/day) or placebo for a median of 12 months (Sandler *et al*., 2003) had significantly less recurrent adenomas in the aspirin group.

Regarding colorectal cancer incidence, two randomized controlled trials, showed that aspirin administered as either 325 mg or 100 mg on alternate days for 5 or 10 years respectively had no effect (Cook *et al*., 2005: Gann *et al*., 1993). The Nurses' Health Study, a cohort study, found no significant benefit in colorectal cancer reduction, until more than a decade of use and the maximum risk reduction occurred at a high dose of aspirin, more than fourteen 325 mg aspirin tablets per week (Chan *et al*., 2005). Two case control studies support the finding that doses of at least 300 mg per day are required for a statistically significant reduction in colorectal cancer incidence (Rodrıguez and Huerta-Alvarez, 2001; Rosenberg *et al*., 1998).

NSAIDs have also been evaluated in preventing colorectal cancer. Two controlled trials involving patients with FAP showed that COX-2 selective NSAIDs, led to a 28 %

reduction in rectal polyps with celecoxib (Steinbach *et al*., 2000) and almost a 10 % reduction with rofecoxib (Higuchi *et al*., 2003). From this data the US FDA approved the use of celecoxib in patients with phenotypic expression of FAP. The NSAID sulindac has also been associated with regression of colorectal adenomas in FAP, either as 300 mg/day for 4 months as part of a cross over design trial (Labayle *et al*., 1991) or 150 mg twice daily for 9 months (Giardiello *et al*., 1993). However, in contrast with the previous study, standard doses of sulindac of 75 or 150 mg twice daily for 48 months were ineffective in preventing the development of adenomas in FAP gene carriers (Giardiello *et al*., 2002). Individuals (non FAP) with a prior history of adenoma were randomly assigned to either 200 or 400 mg celecoxib twice daily or placebo for three years (Bertagnolli *et al*., 2006). Compared to placebo, there was a 45 and 33 % lower risk of recurrent adenomas at the higher and lower doses respectively and the effect was particularly pronounced for recurrent advanced adenomas. However, patients randomized to celecoxib had a significantly higher risk of cardiovascular events, a 2.6-fold increase at the lower dose and 3.4-fold for the higher dose (Bertagnolli *et al*., 2006) and other studies have also confirmed an increased risk of cardiovascular events with these agents (Baron *et al*., 2006; Bresalier *et al*., 2005). Although there are some case control studies that suggest regular use of NSAIDs is associated with a reduction in colorectal cancer incidence (Friedman *et al*., 1998; Kune *et al*., 1988; Reeves *et al*., 1996; Rodrıguez and Huerta-Alvarez, 2001) so far there have been no randomised control trials to examine this effect in either healthy individuals or subjects with a history of colorectal adenomas.

Although the evidence that aspirin and other NSAIDs have beneficial effects on carcinogenesis in the large bowel, several issues have arisen which currently limit their

clinical application as chemopreventive agents. These include determining the dose and duration of aspirin administration required for optimal chemopreventive efficacy, which is likely to be higher than recommended for the prevention of cardiovascular disease (Dube *et al*., 2007). A dose effect has been suggested for aspirin-induced gastrointestinal toxicity and the incidence of gastrointestinal bleeding among large cardiovascular studies was 2.5% in patients taking more than 100 mg/day of aspirin compared with 1.1% of those taking less (Serebruany *et al*., 2004). Many clinical trials suggest that aspirin administration for less than 3 years can be beneficial for adenoma recurrence, but an effect on cancer incidence appears to require a much longer period of use, at least 10 years (Flossmann and Rothwell, 2007; Giovannucci *et al*., 1995). It also appears that risk reduction requires consistent use, as shown in the Health Professionals Follow-up Study, when a benefit from aspirin was no longer evident four years after discontinuing use (Chan *et al*., 2008).

The hope of lower incidence of gastrointestinal side effects and a superior therapeutic index led to evaluation of selective COX-2 inhibitors in the prevention of adenomatous polyps and although COX-2 inhibitors have shown efficacy for colorectal neoplasia prevention (Baron *et al*., 2006; Bertagnolli *et al*., 2006), they are associated with increased cardiovascular risk and it is likely that the traditional NSAIDs that lack COX-2 selectivity may also have this adverse event. The US Preventive Services Task Force (USPSTF, 2007) has concluded that, overall, the harms outweigh the benefits of aspirin and NSAIDs for use in the prevention of colorectal cancer in asymptomatic adults at average risk for colorectal cancer, including those with a family history of colorectal cancer. However these recommendations do not apply to individuals with FAP, HNPCC, or a personal history of colorectal cancer or adenomas (Dube *et al*., 2007).

Although specific dietary changes, such as reduced fat intake or supplementation with fibre, β-carotene, vitamin C and vitamin E, have not yet shown any significant effect on the recurrence of adenoma or colorectal cancer (DeCosse *et al*., 1989; MacLennan *et al*., 1995; Schatzkin *et al*., 2000), calcium, and selenium have demonstrated significant efficacy. Calcium carbonate supplementation has shown a moderate, but consistent reducing effect on adenoma recurrence in two randomised trials (Baron *et al*., 1999; Bonithon-Kopp *et al*., 2000). The Nutritional Prevention of Cancer Trial (Clark *et al*., 1996) revealed a significant reduction in colorectal cancer incidence with selenium supplementation and prompted further investigation with other studies such as the SELECT trial (Lippman *et al*., 2009). Unfortunately, data from the Selenium and Vitamin E Cancer Prevention Trial (SELECT trial), have so far not shown any benefit with selenium and vitamin E supplements, taken either alone or together for an average of five years, on the incidence of prostate or other cancers (Lippman *et al*., 2009). Lippman *et al*., suggested that a limitation of the study was that it did not definitively assess results in subgroups of men who may have responded differently from the overall population (Lippman *et al*., 2009).

1.2.2.2 Preclinical *in vivo* carcinogenesis models

Hypotheses generated in epidemiological studies are ideally tested in humans in controlled studies, but these tend to be long and costly and there may be a risk to health, therefore animal studies are initially required (Hawk and Levin, 2005). Although many animal tumour models have similarities to their corresponding human cancers in regard to cell of origin, morphogenesis, phenotype markers and genetic alteration, interpolating

data from animal studies to humans can be problematic (Das *et al*., 2004). Major differences between animals and humans include lifespan, body weight, intestinal morphology and gene regulation (Corpet and Pierre, 2005).

Most human colorectal adenocarcinomas develop from aberrant crypt foci (*ACF*) and adenomas. The majority of these cancers are sporadic (90%), but share with FAP tumours the early *APC* mutations in 50-80% of cases (Corpet and Pierre, 2005). Because of the low spontaneous rate of colon cancer in rats, chemical carcinogens such as demethylhydrazine (DMH) or its metabolite azoxymethane (AOM) are frequently used to induce colorectal adenocarcinomas to provide a model of tumour development. AOM-induced tumours in rats share many histopathological similarities with human tumours; they go through the *ACF*, adenoma and carcinoma sequence and often carry mutations in K-*ras* and *β-catenin* genes. However, unlike human tumours, the *APC* gene (15%) is less frequently mutated, and rodent tumours never have a *p53* mutation (Corpet and Pierre, 2005). The APC^{Min} mouse carries an autosomal dominant heterozygous mutation of the *APC* gene, similar to that in patients with FAP and many sporadic cancers, which leads to spontaneous intestinal neoplasia. This model resembles human FAP, but the tumours are predominantly located in the small rather than in the large intestine as in humans, and *ACF* and adenocarcinomas are rarely found (Corpet and Pierre, 2005).

Observations in human studies with aspirin and NSAIDs confirm the findings in animal models. More than 90 % of 110 published animal studies reported an anti neoplastic effect and reduction of the occurrence of intestinal neoplasia with NSAIDs (Hawk and Levin, 2005). Initial experiments in animal models involved induction of cancer using

high doses of carcinogens, in which anti-inflammatory agents including indomethacin (Narisawa *et al*., 1981; Pollard and Luckert, 1980), piroxicam (Pollard *et al*., 1983; Reddy *et al*., 1987) and sulindac (Moorghen *et al*., 1988) reduced the number of animals with tumours and the number of tumours per animal. Aspirin inhibited intestinal carcinogenesis, although some studies (Craven and deRubertis, 1992; Mereto *et al*., 1994) found that the benefits were limited to early stages. In one review, a series of meta-analyses was conducted on different agents and confirmed a significant reduction in tumour incidence in carcinogen induced rodents with aspirin, β-carotene, calcium and wheat bran (Corpet and Pierre, 2005). In APC^{Min} mice aspirin, at a dose equivalent to 10 or 20 tablets (each 325 mg) per day in humans significantly reduced tumour multiplicity and total tumour load (Barnes and Lee, 1998). In a meta analysis of studies of APC^{Min} mice a protective effect of aspirin and wheat bran was confirmed with a significant reduction in the number of intestinal adenomas in treated mice (Corpet and Pierre, 2005).

Some mechanisms have been suggested whereby NSAIDs may inhibit colorectal carcinogenesis including, inhibition of angiogenesis (Tsujii *et al*., 1998), modulation of the insulin-related neoplastic pathways (Slattery *et al*., 2004) and increased apoptosis (Pasricha *et al*., 1995). NSAIDs inhibit cyclooxygenase enzymes, which catalyze prostaglandin production (Bennett *et al*., 1987). Higher levels of prostaglandins and COX-2 expression are found in colorectal tumours compared with normal tissue, and this observation is associated with tumour angiogenesis, cell proliferation, and inhibition of immune surveillance and apoptosis (Dannenberg *et al*., 2005). In patients with colorectal cancer, ten-year survival was significantly higher in patients with tumours with the lowest levels of COX-2 staining (68 versus 35%) (Sheehan *et al*.,

1999). The authors demonstrated that expression of COX-2 in tumour epithelial cells was related to lymph node metastasis, advanced Dukes staging, and poorer long-term outcome. When human colon cancer cells that expressed high levels of COX-2 (HCA7) were implanted into nude mice, treatment with a selective COX-2 inhibitor reduced tumour formation by 85 to 90% and inhibited colony formation of cultured cells (Sheng et al., 1997b), but this benefit was not seen with tumour cells that lacked COX-2 (HCT-116). Furthermore, in two large prospective cohorts, the protective effect of aspirin appeared to be limited to colorectal cancers that over expressed COX-2 (Chan *et al*., 2007).

NSAIDs may increase the rate of apoptosis by an increase in AA, a prostaglandin precursor (Rigau *et al*., 1991), which promotes the conversion of sphingomyelin to ceramide, a known mediator of apoptosis. In addition, sulindac induced p21 expression, which is associated with cell-cycle arrest and apoptosis (Yang *et al*., 2001). The *ras* mutation is found in 50 % of colorectal carcinomas and *ras*-transformed cells have been shown to possess increased expression of COX-2 which is thought to contribute to tumourigenicity of these cells (Sheng *et al*., 1997a). Selective inhibition of COX-2 activity has been shown to inhibit growth of these ras-transformed intestinal epithelial cells through induction of apoptosis (Sheng *et al*., 1997a). However, COX-2 selective antagonists have been shown to induce apoptosis in a colorectal cancer cell line that lacked detectable COX-2 expression, suggesting that COX-2 selective NSAIDs may induce apoptosis via mechanisms that do not involve COX-2 (Elder *et al*., 1997). A further mechanism suggested whereby NSAIDs may inhibit carcinogenesis is by their potential to influence epidermal growth factor receptor expression (EGFR) which may

be beneficial as animal models have demonstrated an association between the EGFR pathway and the development of colorectal cancer (Roberts *et al*., 2002).

1.3 Resveratrol: chemistry and pharmacokinetics

1.3.1 Sources

Resveratrol (trans-3'5'4-trihydroxystilbene) was first identified in 1940 in the roots of white hellebore (Veratrum grandiflorum O. Loes), and later in the dried roots of Polygonum cuspidatum, referred to as Ko-jo-kon in Japanese, which is used in traditional Chinese and Japanese medicine to treat a number of different conditions, including suppurative dermatitis, tinea pedis (athletes foot) and hyperlipidaemia (Aggarwal *et al*., 2004). Resveratrol attracted further interest after epidemiological data from Southern France revealed an inverse relationship between the consumption of red wine and the incidence of cardiovascular disease, an effect termed the "French paradox" (Renaud and DeLorgeril, 1992). In support of these findings, wine and grape extracts have since been shown to decrease platelet aggregation (Demrow *et al*., 1995), promote vasorelaxation (Fitzpatrick *et al*., 1993), suppress atherosclerosis (Wang *et al*., 2005), reduce lipid peroxidation (Fuhrman *et al*., 1995), and improve serum cholesterol and triglyceride concentrations (Frankel *et al*., 1993). Resveratrol's cancer chemopreventive potential was first appreciated when Jang *et al*. (Jang *et al*., 1997) showed that resveratrol was able to inhibit diverse cellular events associated with the three major stages of carcinogenesis (initiation, promotion, and progression). Since then, there have been many reports on the effects of resveratrol on critical events that regulate cellular proliferation and growth (Pervaiz., 2004).

Resveratrol is a naturally occurring phytoalexin, produced by a wide variety of plants such as grapes, peanuts, and mulberries in response to stress, injury, ultraviolet irradiation, and fungal (e.g. Botrytis cinerea) infection (Ignatowicz, *et al* 2001). Resveratrol is synthesized in the leaf epidermis, the skin of grape berries, lignified plant tissue (stalks and kernels of the berries), but not in the flesh (Langcake and Pryce, 1976). This polyphenol reaches concentrations of 50–400 µg/g fresh weight in the leaves with lower amounts in the fresh grape skin (50–100 µg/g) (Jeandet *et al*., 1991). The amount of resveratrol varies considerably in different types of grape juice and wine depending on the grape variety, environmental factors in the vineyard, juice extraction, and wine processing techniques. In general the concentration of resveratrol in red wine is between 0.2 and 5.8 mg/L with higher concentrations (4.9 to 13.4 mg/L) found in wines produced from muscadine grapes (Lamikanra *et al*., 1996). The length of contact time between the berry skin and must increases the concentration of this stilbene in the wine. Must from red wine is fermented with the berry skins for several days, whereas must for white wine is immediately separated from berry residues after mashing and explains the lower concentration of resveratrol in white wines. Taking a resveratrol content of red wine of ~5 mg/L and assuming moderate wine consumption (250 mL in a 70 kg person), the intake of resveratrol with red wine in humans is ~18 µg/kg/day (Gescher and Steward, 2003).

1.3.2 Chemical properties and synthesis

Resveratrol is a member of the stilbene family, a group of compounds that consists of 2 aromatic rings joined by a methylene bridge. Although the presence of the double bond

facilitates *trans*- and *cis*-isomeric forms [(*E*) - and (*Z*)-diasteromers, respectively], the *trans*-isomer is sterically more stable (Trela and Waterhouse., 1996). Reports of *cis*-resveratrol in certain wines has been attributed to photoisomeric conversion and enzyme action during fermentation. It is the *trans*-isomer of resveratrol that is commonly studied for its biological effects (Aggarwal *et al*., 2004) and unless stated, when resveratrol is discussed in this thesis, it refers to the *trans* form. The structures of resveratrol and its major metabolites are shown in Figure 1.3.1.

Several plants, including grapevine, synthesize resveratrol when attacked by pathogens (Aggarwal *et al*., 2004). Resveratrol is found in at least 72 plant species (Aggarwal *et al*., 2004) and is formed via a condensation reaction between 3 molecules of malonyl CoA and 1 molecule of 4-coumaroyl CoA (Soleas *et al*., 1997). Resveratrol synthase facilitates this condensation reaction, which also produces CO_2. In plants, resveratrol mostly exists in glycosylated piceid forms (3-*O*-β-D-glucosides) (Athar *et al*., 2007) and there are a number of naturally occurring analogues which have also been shown to have potential chemopreventive activity. Pterostilbene, the methoxylated analogue of resveratrol which has antioxidant properties, can prevent carcinogen induced preneoplastic lesion formation, however, unlike resveratrol, which is a potent inhibitor of COX-1 and 2, pterostilbene is only a moderate inhibitor of the former and a weak inhibitor of the latter (Rimando *et al*., 2002). In addition piceatannol (*trans*-3,4,3',5'-tetrahydroxystilbene) is another naturally occurring analogue of resveratrol with antileukaemic and tyrosine kinase inhibitory activities. CYP1B1, a cytochrome P450 enzyme is over expressed in a wide variety of human tumours and converts resveratrol to piceatannol by hydroxylation of the 3-position on the less substituted aromatic ring (Potter *et al*., 2002; Wolter *et al*., 2002). Furthermore, oxyresveratrol (2',3,4',5-

tetrahydroxystilbene), found in white mulberry, is a also a potent inhibitor of tyrosinase (Kim *et al*., 2002).

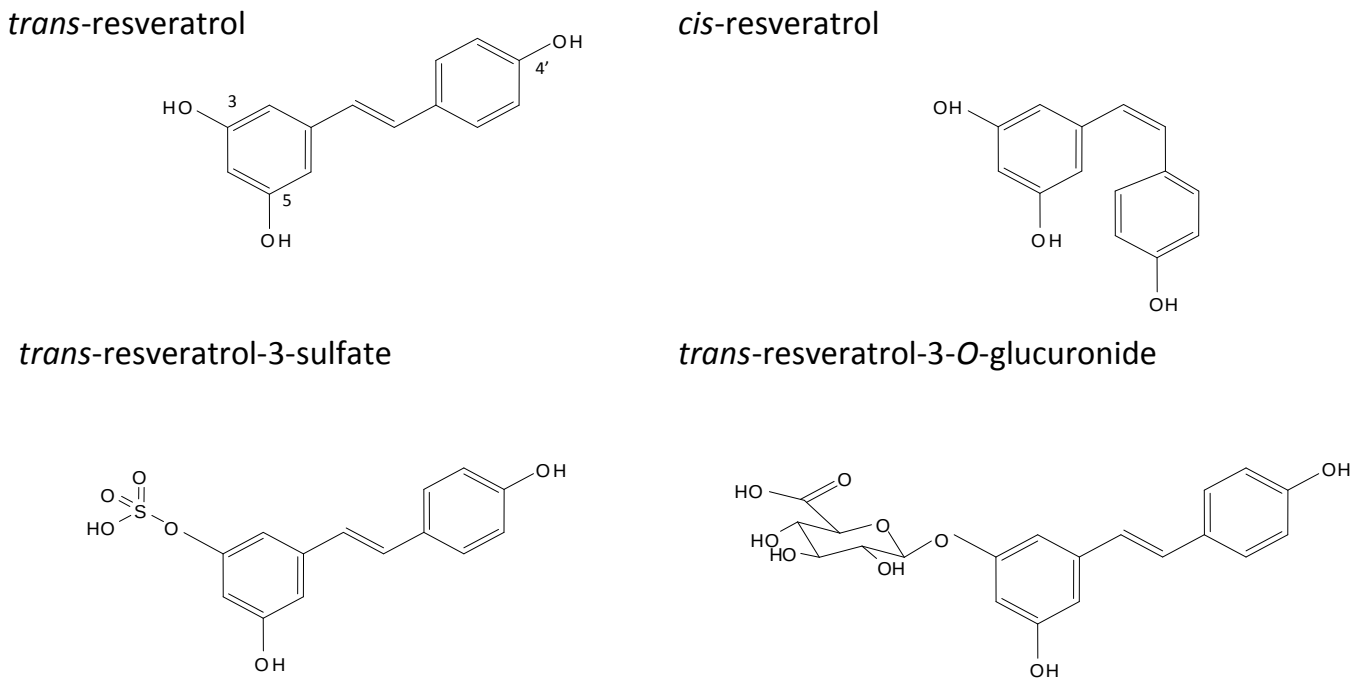


Figure 1.3.1 Structures of resveratrol and its main metabolites. Resveratrol exists as *cis* and *trans* isomeric forms. Resveratrol-3-sulfate and resveratrol-3-*O*-glucuronide are the main metabolites of resveratrol. The positions of the three hydroxyl groups are indicated on the parent molecule.

1.3.3 Pharmacokinetics, absorption and metabolism of resveratrol

The bioavailability of a pharmacological agent is defined by the degree to which it becomes available to the target tissue after administration. Knowledge of absorption, distribution, and metabolism of a compound *in vivo* is essential to determine its bioavailability (Wenzel and Somoza, 2005). To date, several *in vivo* studies in animals and humans have shown that when given orally, resveratrol is rapidly absorbed from the gut, with low plasma concentrations and short half life of the parent compound due to extensive metabolism in the gut and liver (Andlauer *et al*., 2000; Bertelli *et al*., 1996; Marier *et al*., 2002; Soleas *et al*., 2001).

In one of the first studies to evaluate the pharmacokinetics of resveratrol *in vivo*, red wine was administered to rats by gavage either as a single dose (80 μg/kg body weight) or as a daily dose for 15 days (40 μg/kg body weight). Resveratrol was rapidly absorbed and detected in the plasma 30 min after administration (Bertelli *et al*., 1996) with peak plasma concentrations (C_{max}) reached at approximately 60 min after ingestion and peak concentrations in the liver and kidneys observed slightly later. The concentration of resveratrol in the kidneys seemed to decrease with time, and the kidneys appeared to be the preferential organ of excretion. After 4 h, resveratrol was no longer detected in the plasma, but tissue levels were still measurable. Following repeated daily dosing the mean concentrations attained were reported as 0.033 μM in plasma, 0.23 μM in liver and 0.19 μM in kidney tissue, these levels being much higher than after single dose administration.

Since then, plasma pharmacokinetics of resveratrol after oral administration have been investigated in several other studies in animals (Asensi *et al*., 2002; Bertelli *et al*., 1996; Juan *et al*., 2002; Marier *et al*., 2002; Meng *et al*., 2004; Sale *et al*., 2004; Soleas *et al*., 2001; Vitrac *et al*., 2003) and in humans (Boocock et al., 2007; Goldberg *et al*., 2003; Vitaglione *et al*., 2005; Walle *et al*., 2004). The results of these studies support the observation that plasma resveratrol concentrations are consistently lower (ranging from <1% to 10%) than plasma levels of resveratrol metabolites, suggesting a very fast and extensive metabolic conversion (Crowell *et al*., 2007; Goldberg *et al*., 2003; Marier *et al*., 2002; Meng *et al*., 2004). The peak plasma concentration of resveratrol observed differs between studies and depends on route of administration, duration of dosing and species.

After a single oral dose of 20 mg/kg in mice, rats and rabbits, plasma peak concentrations of 2.6, 1.2 and 1.1 μM were achieved respectively (Asensi *et al*., 2002). Twenty eight daily oral doses of 2 mg/kg led to peak plasma concentrations of 2.4 μM in rats (Juan et al., 2002). In healthy human volunteers (Boocock et al., 2007) resveratrol administered as a single oral dose of 0.5, 1.0, 2.5 and 5.0 g generated peak resveratrol concentrations of 0.33, 0.51, 1.18 and 2.36 μM respectively. Three resveratrol metabolites were identified (resveratrol-3-sulfate and two monoglucuronides) of which resveratrol-3-sulfate was the most abundant and up to 20-fold higher than the parent compound. The plasma half lives of the three resveratrol conjugates were similar to those of parent resveratrol (2.9-8.9 h). In healthy volunteers who received 25, 50, 100 or 150 mg of oral resveratrol, 6 times/day, mean peak plasma concentrations (C_{max}) were 0.017, 0.032, 0.10 and 0.28 μM respectively (Almeida *et al*., 2009). Two monoglucuronides and a sulfate conjugate were also detected, and their

levels were 3 to 8 fold higher than the resveratrol plasma C_{max}, with the sulfate being the predominant metabolite.

The absorption of resveratrol was investigated using a CaCo-2 human intestinal cell model (Kaldas *et al*., 2003) and the metabolites identified in these cells included a resveratrol sulfate and resveratrol glucuronide, with the former more predominant. Passive diffusion of resveratrol in CaCo-2 cells was observed (Henry *et al*., 2005), however, the transport of *trans*-piceid required the sodium dependent glucose co-transporter SGLT1. In a rat small intestine model, resveratrol was absorbed on the serosal side of the jejunum with only small amounts absorbed across the enterocytes of the jejunum and ileum unmetabolized. The principal agent derived species detected on the serosal side was the glucuronide conjugate (Kuhnle *et al*., 2000).

Several studies have examined resveratrol absorption *in vivo*. Using radiolabelled resveratrol administered orally, an appreciable fraction, 50–75% of the dose, was absorbed in rats (Soleas *et al*., 2001) and at least 70%, was absorbed in human subjects (Walle *et al*., 2004). Resveratrol absorption and bioavailability varies between individuals, but is not affected by meal type or quantity of lipids in the meal (Vitaglione *et al*., 2005). The effect of a matrix on resveratrol absorption in humans was investigated by comparing plasma levels attained after ingestion of 25 mg/70 kg of resveratrol in 3 different media (white grape juice, white wine or vegetable juice) (Goldberg *et al*., 2003). There was no difference between matrices in resultant resveratrol levels, although white wine rather than red wine was used.

Conjugation of resveratrol was shown to occur initially during absorption into intestinal cells (Andlauer *et al*., 2000; Kuhnle *et al*., 2000). Resveratrol also readily undergoes glucuronidation and sulfation in human and rodent liver cells (Brill *et al*., 2006; de Santi *et al*., 2000a, de Santi *et al*., 2000b; Yu *et al*., 2002). Resveratrol and its metabolites seem to accumulate in rodent liver (Sale *et al*., 2004; Vitrac, *et al*., 2003; Yu *et al*., 2002). Such accumulation of metabolites probably results from two processes: metabolism of resveratrol in the small intestine and its subsequent absorption, and metabolism *in situ* (Soleas, *et al*., 2001). Similar to CaCo-2 cells, normal and tumour human hepatic cell lines utilize both passive diffusion and active transport for resveratrol uptake (Lancon *et al*., 2004). Resveratrol metabolism has been studied in human hepatocytes, and resveratrol-3-sulfate, resveratrol-4´-glucuronide, resveratrol-3-glucuronide and *cis*-resveratrol-4´-glucuronide were identified (Yu *et al*., 2002). Resveratrol sulfate seemed to be a minor human hepatic metabolite compared with the formation of the glucuronide in the incubations with human hepatocytes.

Resveratrol conjugation predominantly takes place at one of the three hydroxyl groups to form mono-conjugates, with the major conjugates in humans being resveratrol-3-sulfate, resveratrol-3-glucuronide and resveratrol-4´-glucuronide (Boocock *et al*., 2007; Walle *et al*., 2004; Yu *et al*., 2002). Phase II conjugation seems to be the major metabolic transformation of resveratrol, and other than conjugation at a single site, some studies have identified resveratrol-di- and tri-sulfates, and resveratrol-di-glucuronides in the plasma and urine of humans and rats (Burkon and Somoza, 2008; Wenzel *et al*., 2005) (Figure 1.3.2). In addition, dihydro-resveratrol, a phase I metabolic product has been identified in the urine of humans (Walle *et al*., 2004).

Resveratrol glucuronide conjugation is catalyzed by uridine diphosphate glucuronosyltransferases (UGTs) (Aumont, *et al*., 2001; Brill, *et al*., 2006), and sulfate conjugation by sulfotransferases (SULTs) (De Santi, *et al*., 2000b; De Santi, *et al*., 2000c; Walle *et al*., 2004). The biotransformation of resveratrol has been shown to occur in two phases, the first being glucuronidation and the second sulfation at higher doses (Wenzel *et al*, 2005a; Wenzel *et al*., 2005b). Although *in vitro* studies have shown that resveratrol can be hydroxylated by CYP1B1 to piceatannol (3,4,3',5'-tetrahydroxystilbene) (Potter *et al*., 2002), and piceatannol is formed at higher rates in human liver than in intestine, recent clinical studies *in vivo* have not detected this metabolite after administration of resveratrol (Walle *et al*., 2004). The presence of a second peak of resveratrol in the plasma drug concentration-versus-time profile after oral administration in humans and the predominant amount of resveratrol compared with its metabolites in the faeces suggest that resveratrol undergoes enterohepatic recirculation (Boocock *et al*., 2007).

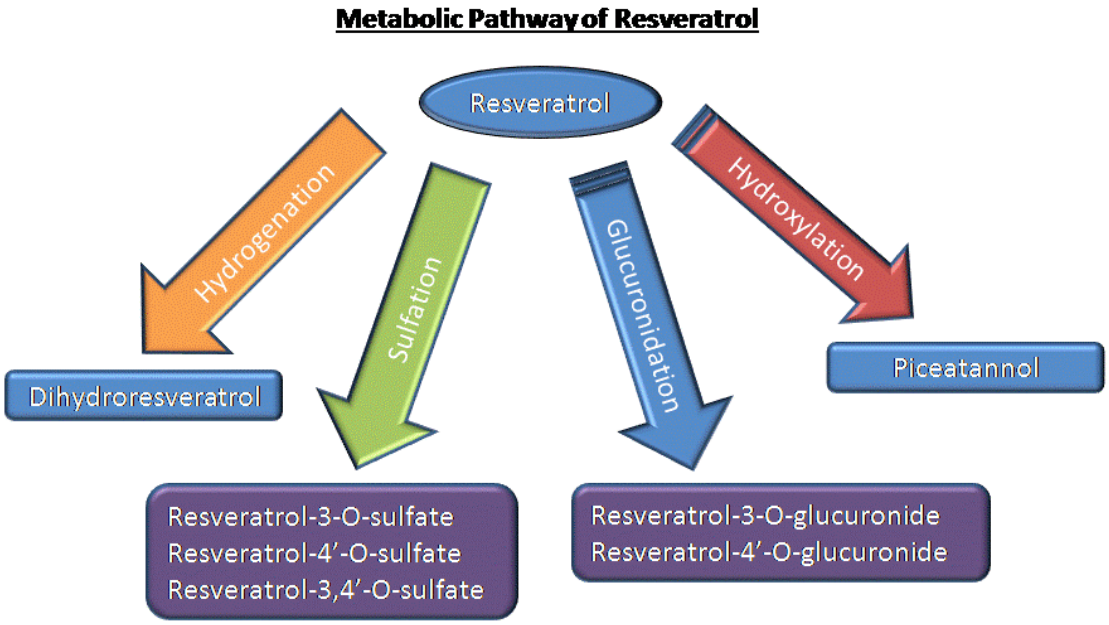


Figure 1.3.2 Metabolism of resveratrol. The metabolism of resveratrol involves several pathways with significant phase II conjugation to glucuronides and sulfates. Resveratrol undergoes glucuronidation to form two corresponding glucuronides (3-*O*-glucuronide and 4´-*O*-glucuronide) and sulfate conjugation yielding resveratrol 3-*O*-sulfate in humans. In addition, several other sulfate conjugates (4´-sulfate, 3,5-disulfate,3,4´-disulfate, 3,4´,5-trisulfate) have been identified in rats. *In vitro* studies have shown that resveratrol can be hydroxylated by CYP1B1 to piceatannol (3,4,3´,5´-tetrahydroxystilbene). Modified from Shankar *et al.*, (2007).

In animals enteric recirculation of conjugated metabolites by reabsorption after intestinal hydrolysis has been suggested (Marier *et al*., 2002). Enterohepatic recirculation in rats has been shown to be governed by the transit time of a drug to reach the caecum, after it has been released from the bile (Walsh and Levine, 1975), where the glucuronide metabolites may undergo enzymatic cleavage by glucuronidase enzyme, and reabsorption of the aglycone parent compound may occur (Figure 1.3.3).

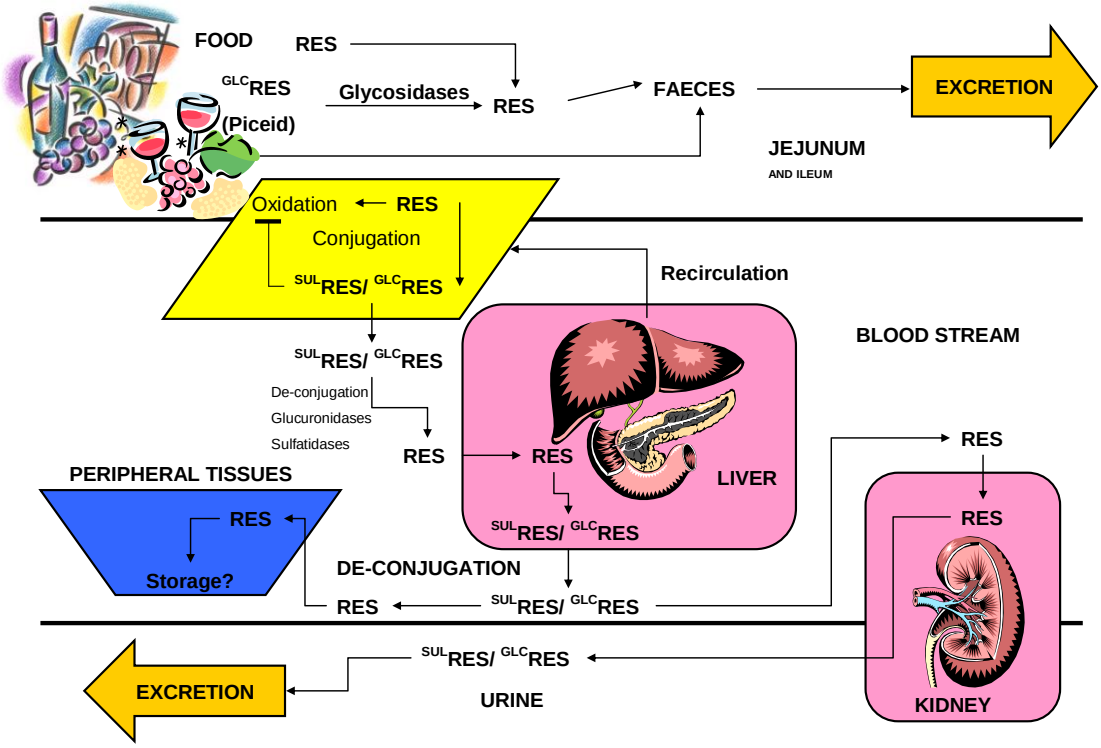


Figure 1.3.3 Pathways of resveratrol absorption, transport, metabolism and excretion. The absorption and transport of resveratrol have been studied in several models: isolated rat intestine, rats and mice after oral administration, human colon carcinoma CaCo-2 cell line, human hepatocytes and healthy human subjects as discussed in Section 1.3.3. Modified from Signorelli and Ghidoni (2005). ^{GLC}RES, resveratrol-3-*O*-β-glucoside (piceid), ^{SUL}RES, resveratrol-3-sulfate, ^{GLU}RES, resveratrol-3-*O*-glucuronide.

Only a small number of studies have investigated the tissue distribution of resveratrol. After oral administration of 20 mg/kg of resveratrol to rats (Asensi, *et al*., 2002), resveratrol was detected in lung, liver, kidney and brain in which the concentration was below 1 nmol/g tissue, and no metabolites were detected in the tissues or in plasma with only resveratrol glucuronide detected in urine. After intragastric resveratrol (1 mmol/kg) was administered to mice (Sale *et al*., 2004), peak parent compound concentrations were 32 μM in plasma and 51, 16, 50, 1.2, 75, 960 and 30 nmol/g tissue in the liver, kidney, lung, brain, heart, small intestinal and colonic mucosa, respectively.

High performance liquid chromatography (HPLC) analysis of liver and kidney samples displayed two peaks in addition to the parent compound, which were identified as a resveratrol glucuronide and resveratrol-3-sulfate. A single oral administration of ^{14}C-resveratrol to mice (Vitrac *et al*., 2003) showed that, whilst the concentration of radioactivity in whole blood was relatively low and constant during the experimental period (1.5 μM), the highest accumulation of the absorbed ^{14}C was found in the liver. HPLC analysis of the radioactive compounds in liver extracts 3 h after administration showed the presence of ^{14}C resveratrol, together with a relatively high concentration of unidentified radioactive glucuronide or sulfated conjugate, which were thought to reflect some active metabolic accumulation process, either as a result of metabolism of resveratrol in the small intestine and its subsequent absorption, or metabolism in the liver. Radioactivity was recovered from the gastrointestinal tract, blood, bile, and urine, as well as from liver, kidney, lung, spleen, heart, brain, and testis.

Renal excretion has been shown to be a major route of elimination after oral administration of resveratrol, resulting in high levels of resveratrol glucuronides and resveratrol sulfates in urine of rats as well as humans (Asensi *et al*., 2002; Goldberg *et al*., 2003; Meng *et al*., 2004; Soleas *et al*., 2001; Vitrac *et al*., 2003; Walle *et al*., 2004; Yu *et al*., 2002). In humans (Boocock *et al*., 2007), the amount of unchanged resveratrol excreted in the urine within 24 h post administration of oral resveratrol was low (<0.04% of the dose). Urinary excretion of the three resveratrol conjugates ranged from 0.51% (one of the glucuronides) to 11.4% of the dose (resveratrol-3-sulfate). Excretion rates were highest during the initial 4 h post-dose collection period and 77% of all urinary species derived from the low dose resveratrol was passed during this time period (Boocock *et al*., 2007).

From the *in vitro* and *in vivo* (animal and human) data available, resveratrol seems to be rapidly absorbed after oral administration and efficiently metabolized to glucuronide and sulfate conjugates. Although the human studies seem to support the results of the animal studies, there are observed differences, suggesting a certain degree of species specific metabolism. The pharmacokinetic analysis after a single oral dose of resveratrol in humans (Boocock *et al*., 2007) suggests that ingestion of an amount equivalent to that contained in several hundred bottles of red wine produced peak plasma levels below the resveratrol concentrations typically required for *in vitro* experiments to elicit pharmacologic effects associated with cancer chemoprevention (≥5 μM). Therefore, further evaluation of the bioavailability and putative beneficial effects of resveratrol in humans, in particular with repeated daily dosing is warranted to evaluate whether higher plasma concentrations of resveratrol can be achieved than after a single dose.

1.3.4 Adverse effects

To date, few studies have evaluated the toxicity of resveratrol in animals or humans and the maximum tolerated dose of resveratrol has not been thoroughly determined (Baur and Sinclair, 2006). Juan *et al*. administered resveratrol (20 mg/kg body weight) to rats orally for 28 days and reported no treatment related side effects except mild changes in serum liver enzymes (Juan *et al*., 2002) and an increase in brain and testicular weight. No histopathological changes were seen in these organs (Juan *et al*., 2002). In a study conducted by Crowell *et al*., rats were gavaged with 0, 300, 1000, and 3000 mg/kg/day resveratrol for 4 weeks. No adverse effects (AE) were observed in rats exposed to 300

mg/kg/day resveratrol, and only minor effects such as reduced body weight in females and elevated leukocyte counts in males were observed at 1000 mg/kg/day resveratrol. At 3000 mg/kg/day reduced body weight and food consumption, elevated kidney weight and increased incidences of kidney lesions were observed in male and female rats. It was concluded by the authors that oral administration of up to 300 mg/kg/day resveratrol for 4 weeks is non-toxic in rats (Crowell *et al*., 2004). In Beagle dogs doses up to 1000 mg/kg for 28 days did not cause any toxic effects (Crowell *et al*., 2007).

In the single dose study of resveratrol in healthy volunteers (Boocock *et al*., 2007) oral doses of up to 5.0 g were well tolerated with follow up revealing no serious AE. Only 2 participants, both on the 1.0 g dose, had AE potentially related to resveratrol administration, a slight increase in serum bilirubin and a minor rise in liver enzymes (alanine aminotransferase). Both events occurred on day 4 post-administration and resolved within 7 days. In a double-blind, randomised, placebo-controlled study in healthy volunteers, doses of 25, 50, 100 or 150 mg resveratrol, six times/day, for thirteen doses showed that repeat dosing was well-tolerated. The AE reported and considered possibly related to treatment were graded as mild in severity and included headache and lethargy (Almeida *et al*., 2009), but there was no increase in AE as the dose was increased.

1.4 Cancer chemopreventive properties of resveratrol in colorectal cancer

1.4.1 Pre clinical studies of resveratrol in colorectal cancer models

From preclinical data, a wide range of concentrations and doses have been reported at which resveratrol has pharmacological efficacy, with values ranging from ~32 nM to100 μM for *in vitro* experiments and ~100 ng/kg to 1,500 mg/kg *in vivo* in animals. As resveratrol has a short half-life, for example ~8 to 14 min in rodents and rabbits (Asensi *et al*., 2002; Marier *et al*., 2002) due to extensive metabolism, this raises many questions about the resveratrol concentration that is achievable *in vivo* and designing new studies can be problematic (Baur and Sinclair, 2006). To optimize the evaluation of resveratrol as a potential chemopreventive agent in colorectal cancer in humans, information on mechanisms and preclinical efficacy at clinically achievable doses is essential.

1.4.1.1 *In vitro* studies

Several reports suggest that at concentrations of ≥5 μM, resveratrol suppresses proliferation of colon cancer cells *in vitro* (Delmas *et al*., 2003; Ito *et al*., 2002; Liang *et al*., 2003; Mahyar-Roemer *et al*., 2001; Nam *et al*., 2001; Sale *et al*., 2004; Wolter *et al*., 2001). Treatment of the human colonic adenocarcinoma CaCo-2 cells with 25 μM resveratrol was found to cause a 70% growth inhibition and only minor inhibition was seen at doses of 5-20 μM. The effect of 25 μM resveratrol on CaCo-2 cell cycle phase distribution was assessed and growth arrest occurred at the S/G2 phase transition 16 and 24 h after addition of resveratrol to the medium. At 40 h the growth inhibitory effect of

resveratrol had disappeared and the normal cell cycle was restored. The culture medium and resveratrol were replaced at 48 h and then 16 h later the cells accumulated again at the S/G2 phase transition suggesting that the effects of resveratrol on the cell cycle were reversible. Also at this dose of resveratrol, there was a significant decrease of ornithine decarboxylase activity, a key enzyme of polyamine biosynthesis, which is enhanced in cancer growth (Schneider *et al*., 2000).

Wolter *et al*., also showed inhibition of the growth and proliferation of CaCo-2 cells with resveratrol and found these effects were dose dependent at 12.5-200 μM (Wolter *et al*., 2001). Furthermore, resveratrol (200 μM) increased caspase-3 activity at 24 and 48 h post-treatment; concentrations up to 50 μM led to S phase cell cycle arrest, whereas higher concentrations (100 and 200 μM) caused reversal of the S phase arrest. Levels of cyclin D1 and cyclin dependent kinase (CDK) 4 proteins were decreased by resveratrol treatment (Wolter *et al*., 2001). The investigators found similar effects on the cell cycle and CDKs with the same doses of resveratrol on HCT-116 cells, a different human adenocarcinoma cell line. CaCo-2 cells express little or no COX-1, and HCT-116 cells lack COX-1 or COX-2 activity. The investigators therefore concluded that the observed effects were not mediated by COX inhibition.

The *p53* gene is mutated in about half of all human tumours and is a transcription factor, whose activity gives rise to a variety of cellular outcomes, most notably cell cycle arrest and apoptosis. As CaCo-2 cells possess mutated *p53* and HCT-116 cells express wild-type p53, this suggests that inhibition of cell cycle progression by resveratrol can function independently of p53 (Wolter *et al*., 2001). Bax is an essential regulator of proapoptotic signaling, and the disruption of apoptosis is linked to the development of

cancer. In HCT-116 colon cells, 100 μM resveratrol has been shown to up regulate Bax regardless of p53 status (Mahyar-Roemer *et al*., 2001) and induce a Bax-mediated and a Bax-independent mitochondrial apoptosis (Mahyar-Roemer *et al*., 2002).

1.4.1.2 *In vivo* studies

The *in vivo* efficacy of resveratrol has been tested in rodent models of colorectal cancer, including DMH or AOM-induced ones and APC^{Min} mice. Administered at 200 μg/kg/day in the drinking water for 100 days, resveratrol significantly reduced the number and multiplicity of AOM-induced ACF and completely abolished large ACF , in the colon of rats. These changes were linked to changes in Bax and p21 expression (Tessitore *et al*., 2000). In Wistar rats resveratrol (8 mg/kg/day) markedly reduced the number of DMH induced ACF and incidence and size of DMH induced tumours. Resveratrol was administered in 3 different regimens, either pre or post carcinogen exposure or during the entire carcinogen exposure period of 15 weeks. The most pronounced inhibition of ACF development was noted in rats fed resveratrol for the entire period, although inhibition was also seen during the post exposure period. Mechanisms involved modulation of antioxidant defence status and the activities of carcinogen-detoxifying enzymes (Sengottuvelan *et al*., 2006a; Sengottuvelan *et al*., 2006b, Sengottuvelan *et al*., 2006c).

APC^{Min} mice receiving resveratrol (0.01% in the drinking water for 7 weeks) showed a 70% reduction in the formation of small intestinal tumours and prevention of colon tumour development (Schneider *et al*., 2001). Resveratrol treatment led to the down regulation of genes that are directly involved in cell cycle progression or cell

proliferation (cyclins D1 and D2, DP-1 transcription factor, and Y-box binding protein). In addition, it also caused up regulation of genes that are involved in the activation of immune cells (cytotoxic T lymphocyte Ag-4, leukaemia inhibitory factor receptor, and monocyte chemotactic protein 3) and in the inhibition of the carcinogenic process and tumour expansion (tumour susceptibility protein TSG101, transforming growth factor-beta, inhibin-beta A subunit, and desmocollin 2), suggesting a multiplicity of molecular targets (Schneider *et al*., 2001). Resveratrol and its synthetic analogue *trans*-3,4,5,4'-tetramethoxystilbene (DMU-212) were administered to APC^{Min} mice in their diet at concentrations of 60 or 240 mg/kg/day (Sale *et al*., 2005). Whilst the lower dose had no effect for either stilbene, at the higher dose both were efficacious in reducing the mean number of intestinal adenomas in the small and large intestine, with a reduction in number of ~25-30% , compared to controls.

This chemopreventive effect of resveratrol in APC^{Min} mice has been associated with inhibition of COX enzymes and interference with prostaglandin E_2 (PGE_2) generation (Sale *et al*., 2005). Despite some of the beneficial effects of resveratrol on reducing intestinal adenomas, some of the data generated in APC^{Min} mice have been conflicting (Athar *et al*., 2007). When resveratrol was given at 0, 4, 20, or 90 mg/kg as a powdered admixture in the diet for 7 weeks, there was no effect on intestinal tumourigenesis or COX-2 expression (Ziegler *et al*., 2004). Although poor bioavailability may explain the lack of effect of the resveratrol powder (Ziegler *et al*., 2004), the reasons for the discrepancy between this result and those obtained by Sale *et al*., (Sale *et al*., 2005) is not clear. Additional experiments are required to resolve these inconsistencies.

1.5 Molecular mechanisms of resveratrol as a chemopreventive agent

1.5.1 Overview

In 1997 Jang and colleagues published a paper reporting the ability of resveratrol to inhibit carcinogenesis at multiple stages (Jang *et al*., 1997). Their finding that topical application of resveratrol reduced the number of skin tumours per mouse by up to 98% led to global interest in this compound (Baur and Sinclair, 2006). Since then resveratrol has been shown to possess a wide range of activities considered important for cancer prevention, primarily from human cell culture systems. Emerging results of studies in laboratory animal models provide convincing evidence that resveratrol can inhibit carcinogenesis in several organ sites. The antitumour activities of resveratrol are mediated through several cell signaling pathways and include the reduction of inflammation via inhibition of prostaglandin production, COX-2 (Martinez and Moreno, 2000; Subbaramaiah *et al*., 1998), and nuclear factor κB activity (Holmes-McNary and Baldwin, 2000; Manna *et al*., 2000). Resveratrol suppressed tumour cell proliferation through induction of cell cycle arrest at G1 and G1/S phases of the cell cycle by inducing the expression of CDK inhibitors p21/WAF1/CIP1 and p27/KIP1 (Adhami *et al*., 2001; Ahmad *et al*., 2001; She *et al*., 2002). It induced apoptosis and differentiation through up regulation of Bax, Bak, p53, TRAIL, TRAIL-R1/DR4 and TRAIL-R2/DR5 and down regulated Bcl-2, Bcl-XL, Mcl-1 and survivin. Resveratrol also has antioxidation properties through acting as a free radical scavenger and by inhibition of hydroperoxidases, reactive oxygen species (ROS) production (Burkitt and Duncan, 2000; Jang *et al*., 1999) and NADPH and ADP-Fe^{3+}-dependent lipid peroxidation (Miura *et al*., 2000). Other beneficial effects for chemoprevention include reduction of

angiogenesis (Garvin *et al*., 2006), inhibition of adhesion, invasion, and metastasis (Aggarwal *et al*., 2004) and inhibition of cytochrome P450 activation of polycyclic aromatic hydrocarbon carcinogens (Chun *et al*., 1999; Ciolino and Yeh, 1999; Gusman *et al*., 2001).

Resveratrol's effects on cell signaling pathways may also be beneficial for a wide variety of other age-related illnesses, including diabetes, arthritis, and coronary, neurodegenerative, and pulmonary diseases (Baur and Sinclair, 2006; Harikumar and Aggarwal, 2008; Saiko *et al*., 2008) (Figure 1.5.1). Resveratrol mimics caloric restriction, improves health, and interferes with the aging process, all linked to its ability to activate sirtuin proteins (Baur and Sinclair, 2006). Sirtuins are a conserved family of NAD^+ dependent protein deacetylases that are involved in gene silencing processes related to aging, blockade of apoptosis and promotion of cell survival. It is thought that the caloric restriction mimetic and anti aging mechanisms of resveratrol may contribute to its effects against cancer. Although *in vitro*, resveratrol interacts with multiple molecular targets, the single dose study of pharmacokinetics of resveratrol in humans (Boocock *et al*., 2007), suggests that even high oral doses of resveratrol might be insufficient to achieve resveratrol concentrations required for the systemic prevention of cancer (Boocock *et al*., 2007). Therefore the chemopreventive effects of resveratrol may be best targeted at tissues where high levels can be achieved due to direct contact, such as skin and gastrointestinal tract tumours.

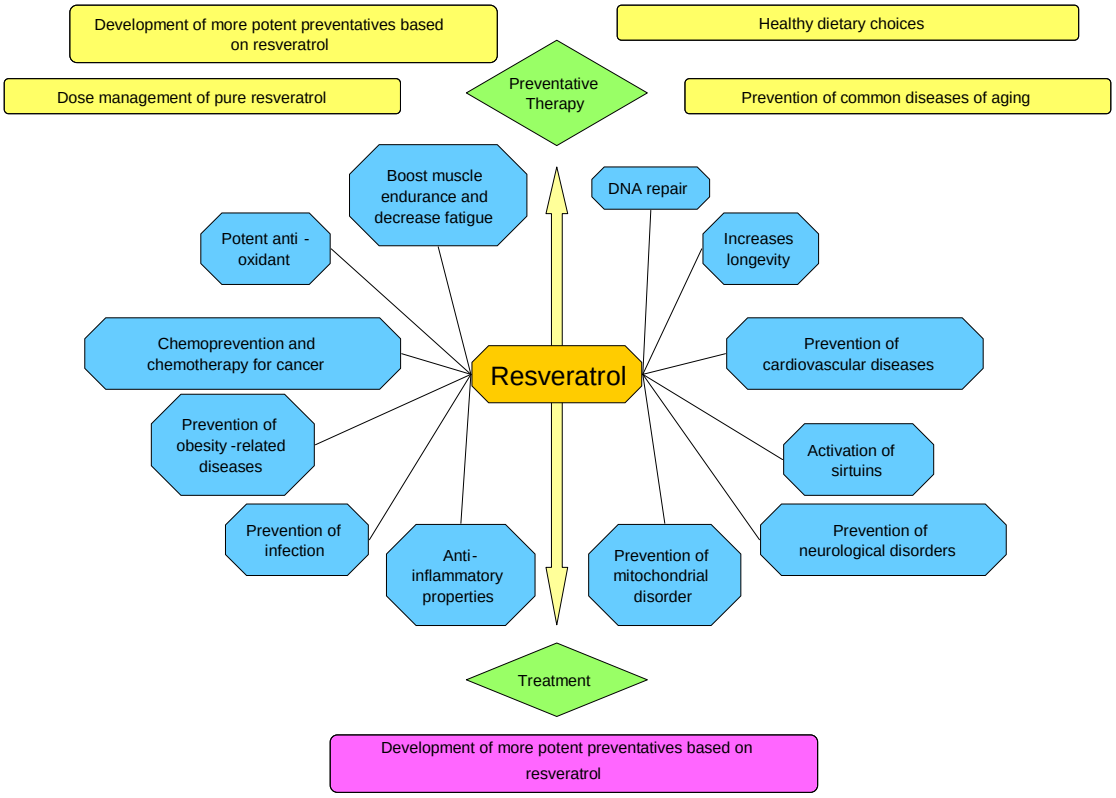


Figure 1.5.1 Examples of biological actions and possible clinical applications of resveratrol. Resveratrol has attracted wide attention due to its health benefits, especially preventing or slowing the progression of common age-related diseases such as cancer, cardiovascular disease, and neurological conditions as well as extending the lifespan of various organisms from yeast to vertebrates. The mechanism by which resveratrol exerts such a range of beneficial effects is not yet clear, but resveratrol can interact with multiple molecular targets involved in diverse intracellular pathways. Resveratrol binds to various cell-signaling molecules and modulates cell-cycle regulatory genes. It activates a number of transcription factors, inhibits protein kinases, suppresses the expression of antiapoptotic genes, as well as angiogenic and metastatic gene products and inflammatory biomarkers. In addition it induces antioxidant enzymes, and alters the expression of enzymes such as cytochrome P450s that are involved in drug metabolism. Calorie restriction is thought to be a way of extending longevity and resveratrol is considered to be a caloric restriction mimetic primarily through the activation of sirtuin proteins. Resveratrol undergoes extensive metabolism in the intestine and liver of animals and humans after oral administration and therefore the development of analogues with improved bioavailability or more potent compounds that mimic its effects may be required. Modified from Marques *et al.*, (2009).

1.5.2 Apoptosis

Programmed cell death (apoptosis) is a natural process for removing unwanted cells, such as those with potentially harmful mutations, or alterations in cell-cycle control. The mechanisms of apoptosis are highly complex and sophisticated and only a overview of the process will be presented here. There are two main apoptotic pathways: the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway), however, the two pathways are thought to be linked as molecules in one pathway can influence the other (Igney and Krammer, 2002).

Apoptosis is tightly regulated by the BCl-2 family of proteins, consisting of pro- and anti-apoptotic proteins which include the multi domain pro apoptotic Bax and Bak (Kuwana *et al*., 2002), activated by the pro-apoptotic BH3 only family members (such as Bid, Bim, Bad, Noxa and Puma) (Certo *et al*., 2006; Kuwana *et al*., 2005). Cytochrome c is then released leading to the formation of a complex called the apoptosome complex, containing cytochrome c, Apaf-1 and initiator caspase-9. Caspase-9 is auto-activated in the apoptosome (Boatright *et al*., 2003; Pop *et al*., 2006) and cleaves and activates executioner caspases.

External triggering involves the ligation of death receptor cell-associated ligands (Rathmell and Thompson, 1999) such as Fas, TNFa or TRAIL (TNF-related apoptosis-inducing ligand) and leads the intracellular death domains of these receptors to recruit adaptor proteins (such as FADD and TRADD) which together comprise the death-inducing signaling complex (DISC) (Kischkel *et al*., 1995). Here, there is activation of caspase -8 and -10 (Boatright *et al*., 2003) and this activation is controlled by c-FLIP

(cellular FLICE inhibitory proteins). Once activated, caspase-8 propagates apoptosis via direct cleavage of executioner caspases. The extracellular and intracellular apoptotic pathways cross at the level of the mitochondria given that caspase -8 can also cleave the protein Bid into its active form tBid. Being pro-apoptotic, tBid induces Bax/Bak-dependent permeabilization of the outer mitochondrial membrane and release of cytochrome c (Li *et al*., 1998). Both apoptotic pathways lead to activation of the executioner caspases, (caspases -3, 6 and 7), causing the cell to degrade which is regulated by inhibitor of Apoptosis Proteins (IAPs) (Roy *et al*., 1997). Figure 1.5.2 is a schematic representative of the intrinsic and extrinsic apoptotic pathways.

Several physiological growth control mechanisms that govern cell proliferation and tissue homeostasis are related to apoptosis (Igney and Krammer, 2002). Resistance to apoptosis is common in many human cancers (Johnstone *et al*., 2002), whereby tumours acquire resistance through several ways, such as loss of function mutations of the p53 tumour suppressor protein leading to failure of p53 to activate transcription of pro-apoptotic Bcl-2 proteins (Vousden and Lane, 2007), loss of functional pro-apoptotic Bax and Bak (Rampino *et al*., 1997) or high expression of anti-apoptotic proteins. In a subtype of B-cell lymphomas, Bcl-2 is highly expressed as a consequence of a Bcl-2 gene translocation next to an immunoglobulin gene (Tsujimoto *et al*., 1984). This translocation increases the incidence of spontaneous B-cell tumours in mice (McDonnell *et al*., 1989). Fas receptor expression is high in normal colon mucosa, but is reduced or even lost in colon carcinomas (Moller *et al*., 1994) and absence of Fas allows tumour cells to evade the immune destruction mediated by cytotoxic lymphocytes via this pathway.

Resveratrol induces apoptosis in various human cancer cells including colon cancer cell lines (Alkhalaf, 2007; Bode and Dong., 2000; Clement *et al*., 1998; Huang *et al*., 1999; Shankar *et al*., 2007; Surh *et al*., 1999). Resveratrol has been shown to induce apoptosis through p53 dependent and independent mechanisms (Mahyar-Roemer *et al*., 2001) dependent on cell type. It also induces the expression of pro-apoptotic Bax, Bak, PUMA, Noxa, and Bim, and inhibits the expression of anti-apoptotic Bcl2, Bcl- XL, and Mcl-1, directly affecting the mitochondrial death pathway (Shankar *et al*., 2007). Resveratrol mediated apoptosis also occurs via the death receptor Fas/CD95/APO-1 (Clement *et al*., 1998). Resveratrol can redistribute FAS/CD95 into lipid rafts in a ligand-independent way, enhancing the efficacy of signaling by FAS/CD95 and other death receptors in colon cancer cells (Delmas *et al*., 2003), and it can induce the redistribution of FAS/CD95 and other death receptors in lipid rafts and sensitize cells to death receptor agonists (Delmas *et al*., 2004).

Survivin is expressed at high levels in many tumours and is a member of the IAPs, which may contribute to resistance of tumours by facilitating both evasion from apoptosis and aberrant mitotic progression (Altieri, 2003). Treatment with resveratrol resulted in down regulation of survivin expression through transcriptional and post transcriptional mechanisms by inhibiting promoter activity, decreasing survivin protein stability and also by enhancing proteasomal degradation of survivin (Fulda and Debatin, 2004). The transcription factor NF-kappaB (NF-kB) plays an important role in tumour formation and progression and is involved in the control of activation of certain proapoptotic molecules, e.g. CD95 ligand, TRAIL-R1, TRAIL-R2 or TRAIL (Fulda and Debatin, 2006). The inhibitory effect of resveratrol on NF-kB-dependent signaling has been ascribed to its Sirtuin-activatory property (Howitz *et al*., 2003). SIRT1, the

mammalian ortholog of the yeast Silencing Information Regulator (SIR2) and a member of the Sirtuin family, has been implicated in modulating transcriptional silencing and cell survival, thereby promoting mammalian longevity (Blander and Guarente., 2004).

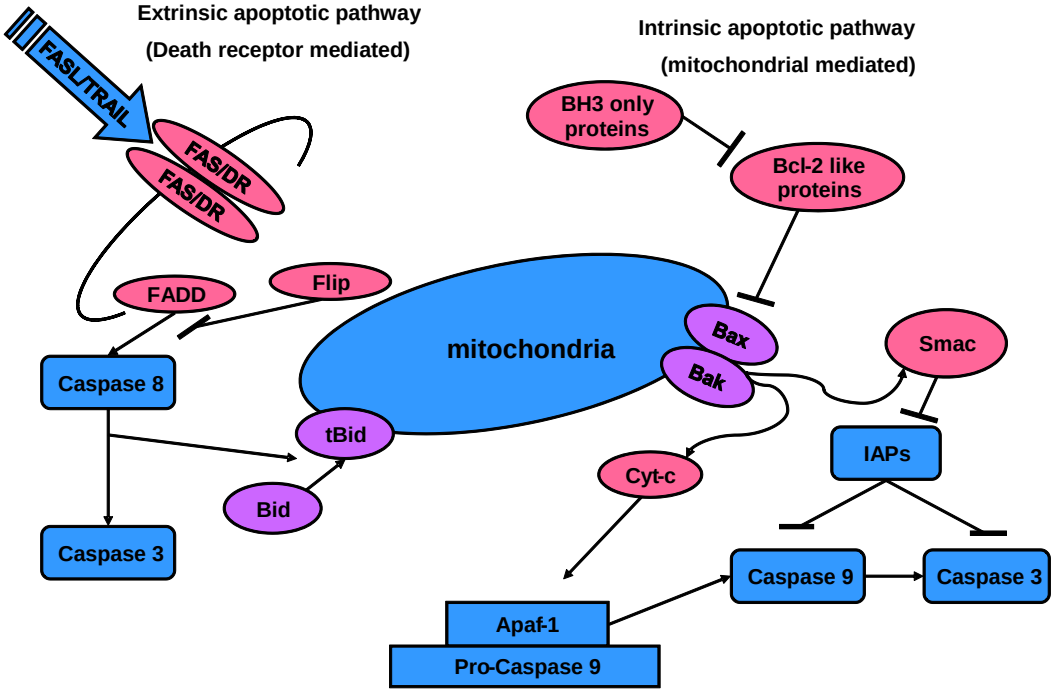


Figure 1.5.2 Mechanisms of induction of apoptosis, the extrinsic and intrinsic pathways. Apoptosis can be triggered by either the extrinsic (death receptor) or intrinsic (mitochondrial) pathway. The death receptor pathway is exemplified by events that occur following engagement of Fas (CD95) by its ligand FasL. The mitochondrial pathway is triggered in response to a number of stimuli including mitogenic signals and DNA damage. Activation of the tumour suppressor gene and transcriptional factor p53 in response to cellular stress leads to transcriptional activations of several pro apoptotic genes including Bax. Modified from Hector and Prehn (2009).

1.5.3 Cyclooxygenase activity

Arachidonic acid, *cis*- 5,8,11,14-eicosatetraenoic acid, (AA) is a 20-carbon polyunsaturated fatty acid and the main eicosanoid precursor in mammalian cells. Since it cannot be synthesised *de novo* from animal cells, most AA in the human body is

derived from linoleic acid, obtained from dietary sources. The first step in the cascade is cleavage and release of AA from the phospholipid-bound form. This may be achieved with the aid of at least one of three different enzymes, namely phospholipase A_2 (PLA_2), phospholipase C and phospholipase D (Farooqui and Horrocks, 2005). Free AA is metabolized by 5-lipoxygenase to leukotriene A_4 or may be channeled through the prostaglandin system which is composed of cyclooxygenase enzymes. The majority of AA metabolites can act both as pro- and anti-inflammatory mediators (Harris *et al*., 2009), modulating gene expression, cytokine signaling and other immune regulatory factors.

Three isoforms of the membrane bound enzyme COX have been identified, COX-1, COX-2 and COX-3. Although they differ in their pattern of expression and tissue distribution in human cells (Chandrasekharan *et al*., 2002; Williams *et al*., 1999), collectively they are responsible for the conversion of AA to the three classes of prostanoids. Whilst COX-1 is ubiquitous and produced constitutively in most mammalian cells and tissues, COX-2 is normally absent, but found readily at the sites of inflammation. COX-2 production is induced by a variety of stimuli such as cytokines and growth factors (Morita, 2002). The first step in the COX metabolic pathway is oxygenation of AA by the cyclooxygenase activity of the enzyme to give PGG_2, followed by rapid conversion of PGG_2 by its peroxidise activity into PGH_2. PGH_2 is an unstable endoperoxide, that functions as an intermediate for all further synthetic steps in the COX pathway; these subsequent steps are catalyzed by a number of cell-specific isomerases and lead to the formation of the prostaglandins (PGs) (Williams *et al*., 1999), including prostacyclin D_2 (PGD_2), prostacyclin E_2 (PGE_2), prostacyclin $PGF_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin I_2 (PGI_2) and thromboxane A_2 (TXA_2).

Since COX-2 expression is highly induced at sites of inflammation it was proposed that the anti-inflammatory and analgesic properties of traditional NSAIDs, which inhibit both COX-1 and COX-2, are largely due to their ability to inhibit COX-2. In addition, NSAIDs can cause gastric irritation, thought to be due to their ability to inhibit COX-1-mediated PG production in the gastric epithelium. This has led to the design and synthesis of a new class of NSAIDs that specifically inhibit the COX-2 isoform.

Mediators of inflammation, such as cyclooxygenase-2 (COX-2), prostaglandins (PG), inducible nitric oxide synthase (iNOS), and pro-inflammatory cytokines have been shown to be involved in carcinogenesis (Surh and Kundu, 2005). Elevated expression and/or activity of COX-2 and production of certain PGs have been found in various cancers such as cancer of the lung (Krysan *et al*., 2004), breast (Barnes *et al*., 2006), colorectal (Chan *et al*., 2007), prostate (Lee *et al*., 2001b), head and neck (Tang *et al*., 2003). Significant elevations of 85% and 50% in COX-2 expression were observed in human colorectal carcinomas and colorectal adenomas respectively, whilst levels of COX-1 were unchanged between normal mucosa and carcinoma. COX-2 has also been shown to be over expressed in adenomas from APC^{Min} mice (Rao *et al*., 2000). Identified regulatory factors controlling COX-2 expression include reactive oxygen species (ROS) mediated NF-κB activation (Surh *et al*., 2001), suggesting a positive feedback mechanism between expression levels of NF-κB and COX.

Resveratrol directly inhibits COX-2 activity (Subbaramaiah *et al*., 1998) and blocks the expression of COX-2 in lipopolysacharide (LPS)-, TPA- or H_2O_2-stimulated mouse peritoneal macrophages (Martinez and Moreno, 2000). It blocked TPA-induced NF-κB

activation and COX-2 expression in mouse skin *in vivo* (Kundu *et al*., 2006). Resveratrol also down regulated the expression of COX-2 mRNA transcript in *N*-nitrosomethylbenzylamine (NMBA)-induced oesophageal tumours in F344 rats (Li *et al*., 2002). MKP5, is a member of the dual-specificity MKP family of phosphatases that dephosphorylate mitogen-activated protein kinases (MAPKs) (Tanoue *et al*., 1999). MKP5 is a potent anti-inflammatory mediator and causes decreased NF-kB activation and reduced COX-2, IL-6, and IL-8 levels in normal prostatic epithelial cells. Resveratrol has been shown to induce MKP5 in a number of prostate cancer cells (Nonn *et al*., 2007).

1.5.4 Cell cycle progression

The cell cycle is divided into four phases. During two of these, cells carry out two basic events of cell division: generation of a single copy of its genetic material (the synthetic or S phase) and partitioning of the cellular components between two identical daughter cells (mitosis or M phase). The remaining phases of the cycle represent 'gap' periods, (G_1 and G_2), during which cells prepare themselves for the completion of the S and M phases, respectively (Figure 1.5.3). When cells cease proliferation, they exit the cycle and enter a non-dividing, quiescent state known as G_0 (Malumbres and Barbacid, 2001).

Cells have a series of checkpoints that prevent them from entering into a new phase, until they have completed the previous one (Hartwell and Weinert, 1989). The CDKs, a group of serine/threonine kinases, form active heterodimeric complexes after binding to cyclins. It is thought that CDK4, CDK6 and CDK2 regulate cells through G_1 into S phase and CDK4 and CDK6 are involved in early G_1 phase. CDKs also are regulated

by phosphorylation on a threonine residue, carried out by the CDK7–cyclin-H complex (Nigg, 1996).

Cell-cycle regulators have been shown to be frequently mutated in many human tumours. Changes include over expression of cyclins (mainly D1 and E1) and CDKs mainly CDK4 and CDK6, as well as loss of cyclin kinase inhibitors (CKI) mainly INK4A, INK4B and KIP1 and (retinoblastoma) protein (RB) expression. Tumour associated changes in the expression of these regulators often result from chromosome alterations, such as amplification of cyclin D1 or CDK4, translocation of CDK6 and deletions of INK4 proteins or RB or epigenetic inactivation, for example methylation of INK4 or RB promoters (Malumbres and Barbacid, 2001).

Resveratrol can inhibit cell proliferation by blocking cell cycle progression (Ahmad *et al*., 2001; Estrov *et al*., 2003; Hsieh *et al*., 1999a; Liang *et al*., 2003; Wolter *et al*., 2001; Yu *et al*., 2003) at S/G_2 (Hsieh *et al*., 1999b) and G_2 phases (Liang *et al*., 2003) of the cell-cycle. The anti-proliferative activity of resveratrol involves the induction of p21WAF1 and down regulation of cyclins D1/D2/E, CDKs 2/4/6 and hyperphosphorylated pRb (Adhami *et al*., 2001; Ahmad *et al*., 2001). Wolter *et al*., showed that resveratrol caused down regulation of the cyclin D1/CDK4 complex in colon cancer cell lines (Wolter, *et al*., 2001). Resveratrol induced G_2 arrest through the inhibition of Cdk7 and Cdc2 kinases in colon carcinoma HT-29 cells (Liang *et al*., 2003) and induced S- phase arrest and up regulation of cyclins A, E, and B1 in human SK-Mel-28 melanoma cells (Larrosa *et al*., 2003).

Resveratrol induces the expression of p53-responsive genes (p21WAF1, p300/CBP, and Apaf-1) and causes Bcl-2 down regulation (Narayanan, *et al*., 2003). In addition, p53-independent induction of p21WAF1 and subsequent cell cycle arrest in cells lacking wild-type p53 protein has been documented (Ahmad *et al*., 2001). Resveratrol down regulated c-MYC in medulloblastomas, in which 73% of tumour tissues expressed this oncogene, and its down regulation was accompanied by S phase arrest (Zhang *et al*., 2006). Upstream of MYC, inhibition of CDK1 rapidly down regulates survivin expression and induces MYC-dependent apoptosis in cell over expressing MYC (Goga *et al*., 2007). In prostate cancer cells, resveratrol decreases cyclin B and CDK1 kinase activity in both androgen-sensitive and androgen-insensitive manners (Benitez *et al*., 2007).

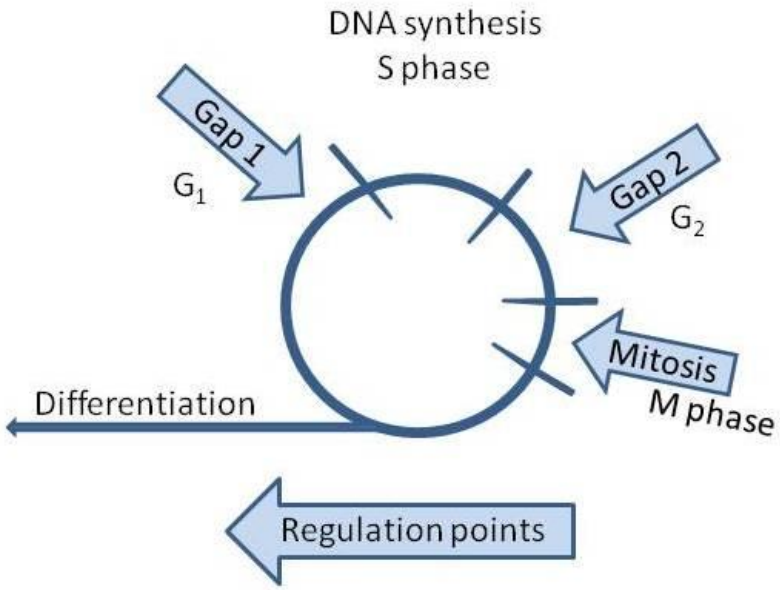


Figure 1.5.3 The cell cycle. The cell cycle is divided into four distinct phases G_1, S, G_2, and M. Cells can be recognised by morphological criteria in mitosis (M) and DNA synthesis takes place only in a specified period of the cell cycle termed the synthesis (S phase). The gaps (G) between M and S phase and between S phase and M are called the G_1 and G_2 phases respectively. Progression through the cell cycle is regulated by cell cycle regulatory complexes comprising the cyclins and CDKs (Section 1.5.4). Modified from King (2000).

1.5.5 Malondialdehyde and M_1dG adducts

Cellular oxidants, called reactive oxygen species (ROS), are constantly produced in animal and human cells. Several *in vitro* experiments report that ROS damages DNA, inducing potentially mutagenic modifications of nucleotides and promoting oxidation of proteins and lipid peroxidation. ROS are formed through a variety of events and pathways and one human cell could potentially be exposed to 1.5×10^5 oxidative hits per day from hydroxyl radicals and other reactive species (Valko *et al*., 2006). ROS-induced DNA damage encompasses single- or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. Currently, more than 100 structurally distinct products have been identified from the oxidation of DNA. An increase in ROS in the cell, through either physiological modification or through chemical carcinogen exposure, is thought to contribute to the processes of carcinogenesis (Klaunig and Kamendulis, 2004). The resulting DNA damage may cause cell cycle arrest, induction of transcription, induction of signal transduction pathways, replication errors and genomic instability (Cooke *et al*., 2003; Marnett, 2000). Lipid peroxidation is probably the most extensively investigated free radical–induced process (Sodergren, 2000). The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination (Pinchuk *et al*., 1998). Once formed, peroxyl radicals (ROO·) can be rearranged via a cyclization reaction to endoperoxides, with the final product of the peroxidation process being malondialdehyde (MDA) (Marnett, 1999). Malondialdehyde (MDA) an endogenous by-product of PG biosynthesis, is mutagenic and carcinogenic in rats. Under physiological conditions, MDA can react with multiple sites in DNA, with the guanine adduct M_1dG (malondialdehyde-deoxyguanosine, 3-(2'-deoxy-β-D-erythro-pentofuranosyl)-pyrimido-[1,2α]purin-10(3*H*)-one) being the major product formed

(Marnett, 2000). This lesion is mutagenic in bacterial and mammalian cells, where it primarily causes G→T and G→A base substitutions. In addition to MDA as a source of M_1dG, the DNA oxidation product, base propenal, can also react with deoxyguanosine to generate the same adduct (Otteneder *et al*., 2006). It has been widely proposed that M_1dG and the products of its *in vivo* oxidative metabolism may be valuable as biomarkers of endogenous DNA damage associated with oxidative stress or disease states (Otteneder *et al*., 2006).

One mechanism through which resveratrol may exert its beneficial effects is its ability to attenuate oxidative DNA damage, as has been shown in a wide variety of cells (Blond *et al*., 1995; Cai *et al*., 2003; Guo *et al*., 2007; Leonard *et al*., 2003; Sgambato *et al*., 2001; Yen *et al*., 2003). However, whether resveratrol acts by enhancing DNA repair, or by preventing oxidative DNA damage from occurring or a combination of both actions is currently unknown (Fauconneau *et al*., 1997). Resveratrol strongly inhibited NADPH- and ADP-Fe^{3+}dependent lipid peroxidation in rat liver microsomes at the initial and propagation stages (Miura *et al*., 2000). Tadolini *et al.*, showed that resveratrol inhibited lipid peroxidation mainly by scavenging lipid peroxyl radicals within the membrane (Tadolini *et al*., 2000). In human myeloid cells cultured *in vitro*, resveratrol (5 μM) decreased tumour necrosis factor induced lipid peroxidation, as reflected by MDA levels (Manna *et al*., 2000).

Sengottuvelan *et al.*, showed that oral resveratrol (8 mg/kg/day) protected colonic tissues against DMH induced colon carcinogenesis in rats. Analysis of the intestines and colon showed that resveratrol supplementation resulted in significant modulation of lipid peroxidation markers, which paralleled ACF suppression, as compared to control

rats administered DMH alone (Sengottuvelan, *et al*., 2006a; Sengottuvelan, *et al*., 2006b). In a randomized, crossover study, the effect of red wine polyphenols on postprandial levels of plasma and urine lipid peroxidation products (MDA) was investigated in healthy volunteers (Gorelik *et al*., 2008). After a meal of turkey meat cutlets, plasma MDA levels rose by 160 nM. After the same meal was soaked in red wine after cooking, there was a 75% reduction in the absorption of MDA, and after the meal was soaked in red wine prior to heating, the elevation of plasma MDA was completely prevented. Similar decreases were obtained for MDA levels measured in urine (Gorelik *et al*., 2008).

1.5.6 Insulin-like growth factor pathway

The IGF signaling system, which consists of IGFs, IGF binding proteins (IGFBPs) and IGF receptors, crucially influences malignant development. IGFs possess antiapoptotic and mitogenic properties (Butt *et al*., 1999; Ibrahim and Yee, 2004) and affect cell differentiation, neoplastic transformation and metastasis (Samani *et al*., 2004). The IGF system is regulated by IGFBPs, prominently IGFBP-3, which bind IGFs in the extracellular milieu with high affinity and specificity, thus reducing circulating levels of IGFs. Individuals with acromegaly, who have abnormally high levels of growth hormone and IGF-I (Jenkins and Besser, 2001) and healthy males with IGF-I levels in the highest quintile, compared with those in the lowest, have been shown to have an increased risk of colorectal cancer (Ma *et al*., 1999). In addition, several studies have suggested a direct relationship between levels of IGF-1, and an inverse relationship between levels of IGFBP-3, and risk of colorectal, prostate, breast or lung cancer (Renehan *et al*., 2004; Sandhu *et al*., 2002).

This association was strengthened for prostate cancer and premenopausal breast cancer by a meta-regression analysis of 26 different data sets from 21 separate trials (Renehan *et al*., 2004). IGF-1 has also been suggested to contribute to the development of adenomatous polyps (Schoen *et al*., 2005). The anti-carcinogenic activity of dietary restriction in preclinical models of carcinogenicity is thought to be mediated, at least in part, via reduction of circulating IGF-1 (Kari *et al*., 1999). Modulation of the IGF system has been proposed as a mechanism by which certain agents, for example 9-*cis* - retinoic acid, may prevent cancer (Lee *et al*., 2005). In addition, resveratrol can retard parameters linked to aging and acts as a calorie restriction-mimetic in mice (Baur *et al*., 2006; Pearson *et al*., 2008). Resveratrol lowered circulating IGF-1 in diabetic mice on a high calorie diet (Baur *et al*., 2006) and in prostate tumour tissue of TRAMP mice (transgenic adenocarcinoma mouse prostate), a genetic model of prostate carcinogenesis (Harper *et al*., 2007). Information on the effect of resveratrol on IGFBP-3 was not provided in these 2 studies. Whilst the potential of resveratrol as a cancer chemopreventive agent and/or calorie restriction mimetic in humans is a topic of considerable interest (Baur and Sinclair, 2006; Grifantini, 2008), potential biomarkers of its efficacy, such as levels of components of the IGF signaling pathway, in humans are virtually unknown.

The type 1 insulin-like growth factor receptor (IGF-1R) consists of two half-receptors, each comprising one extracellular subunit and one transmembrane subunit that possess tyrosine kinase activity (Adams *et al*., 2000; Ullrich *et al*., 1986). The IGF-1R is activated by its ligands IGF-I and IGF-II, which are produced by the liver and also by many extra hepatic sites, including tumour cells (Baserga *et al*., 2003). Ligand binding leads to signaling via phosphatidylinositol-3-kinase (PI3K)-AKT and

RAS/RAF/mitogen-activated protein kinase (MAPK) pathways (Manning and Cantley, 2007). The IGF-1R has been documented to be involved in cancer development and displays potent antiapoptotic effects (Werner and Le Roith, 1997).

A range of human cancers has been shown to over express this receptor or to have increased IGF-1R kinase activity (Rubin and Baserga, 1995). For example, during progression of colorectal adenoma to carcinoma an increase of IGF-1R expression was detected in human tissue samples and immunohistochemical analysis showed that strong IGF-1R staining correlated with a higher grade and stage of the tumour (Hakam *et al*., 1999). Inhibition of the IGF-I receptor using antisense or antibodies inhibited *in vitro* and *in vivo* growth of many tumours (Yu and Rohan, 2000). Furthermore, colon cancer growth and metastasis was found to be reduced in mice with decreased circulating IGF-1 levels due to liver-specific IGF-1 gene deletion (Wu *et al*., 2002). The IGF-1R seems to be required for cellular transformation by most oncogenes and mediates the combination of proliferation and survival signaling required for anchorage-independent growth which could enable transformed cells to form macroscopic tumours and survive the process of detachment required for metastasis (Sell *et al*., 1994).

Resveratrol is able to reduce many consequences of a high-calorie diet in mice and to increase their survival (Baur *et al*., 2006); mice on a high-fat diet consuming resveratrol remained lean and had improved muscle parameters (Lagouge *et al*., 2006) and resveratrol protects animals against insulin resistance (Baur *et al*., 2006; Lagouge *et al*., 2006). An insulin-like effect of resveratrol was also noticed in diabetic rats (Chi *et al*., 2007; Su *et al*., 2006) and resveratrol was able to affect blood insulin concentrations

(Baur *et al*., 2006; Chen *et al*., 2007; Chi *et al*., 2007; Su *et al*., 2006), including decreasing insulin secretion from pancreatic islets of normal rats (Szkudelski, 2006).

1.6 Aims

The polyphenol resveratrol is thought to possess cancer chemopreventive properties (Sections 1.4 and 1.5). The overall aim of this project was to assess the pharmacokinetics and pharmacodynamics of resveratrol in humans in order to determine whether further development of resveratrol as a potential chemopreventive agent is warranted. Two phase 1 studies were conducted, one in healthy volunteers and the second in patients with colorectal cancer. To our knowledge this is the first clinical study to measure resveratrol concentrations in colorectal tissue in humans.

In order to achieve this aim the project has 4 main objectives:

1) To assess resveratrol pharmacokinetics by measuring the concentration of resveratrol and its metabolites in the plasma, urine and stools of healthy volunteers, after at least 21 days of daily oral dosing. The hypothesis was tested that concentrations of resveratrol achieved would be in the same order of magnitude as those associated with pharmacological effects *in vitro* and in animal models (Sections 1.4 and 1.5).

2) To gain information on the pharmacodynamics of resveratrol by measuring changes in potential intermediate biomarkers of chemopreventive activity which were selected, based on existing knowledge of the mechanistic activity of resveratrol (Sections 1.4 and 1.5). The hypothesis was tested that ingestion of resveratrol for at least 21 oral daily doses in healthy volunteers and 8 oral daily doses in colorectal cancer patients would affect;

a) plasma levels of the insulin-like growth factor system (IGF-1, IGFBP-3, IGF-1/IGFBP-3 ratio). The IGF system has been shown to influence malignant development of various tumours and thus provides a potential mechanism for the effect of chemopreventive agents (Section 1.5.6).

b) oxidative DNA damage (M_1dG). Oxidative DNA damage has been implicated in carcinogenesis and one mechanism by which resveratrol exerts its beneficial effects may be its ability to attenuate oxidative DNA damage (Section 1.5.5).

c) biomarkers of inflammation in plasma (PGE_2) and in colorectal tissue (COX-2). The enzyme COX-2 and the arachadonic acid metabolite PGE_2 are important regulators of colon carcinogenesis and interference with their biological properties and actions has been considered an important cancer chemopreventive mechanism (Section 1.5.3).

d) colorectal tissue cell proliferation (Ki-67). As shown in Sections 1.5.2 and 1.5.4, resveratrol engages in antiproliferative mechanisms and anti-survival mechanisms.

3) To obtain a preliminary toxicity profile of resveratrol given at oral doses up to 5 g per day and to test the hypothesis that daily dosing with resveratrol is safe and has no or minimal toxicity.

4) To establish the relationship between colon tissue levels and plasma concentrations of resveratrol and its metabolites after 8 days of daily dosing. The hypothesis was tested that concentrations of resveratrol achieved in colorectal tissue would be of a similar order of magnitude to those associated with pharmacological effects *in vitro* and in animal models (Section 1.4).

CHAPTER 2

METHODS

2.1 Clinical studies

2.1.1 Summary

The safety, pharmacokinetics and pharmacodynamics of resveratrol were investigated in two separate phase 1 studies, the first involving healthy volunteers and the second including patients with colorectal cancer. Recruitment of colorectal cancer patients was carried out at the University Hospitals of Leicester, UK and the recruitment of healthy volunteers was conducted jointly at the University of Leicester, UK and the University of Michigan, USA. Approval for the running of both studies was obtained from Local Ethics Committee, Medicines and Healthcare Products Regulatory Agency (MHRA), University Hospitals of Leicester Trust and University of Michigan Institutional Review Board (IRBMED, USA). The studies were conducted in accordance with the applicable Guideline for Good Clinical Practice (ICH, 2002). The Eudract number for both studies is 2007-005692-34.

Forty healthy volunteers received resveratrol orally once daily for 29 days at 4 dose levels of 0.5, 1.0, 2.5, 5.0 g with 10 volunteers per level. Blood and urine were collected prior to the first dose and then after at least 20 daily doses (Figure 2.1.1). In the second, separate study, 20 patients with resectable colorectal cancer received 8 oral daily doses, prior to surgical resection, at 2 dose levels of 0.5 and 1.0 g with 10 patients per level. Blood and colorectal tissue were collected before dosing at diagnostic endoscopy and post dose at resection surgery (Figure 2.1.2). The healthy volunteers and colorectal cancer patients were administered uncoated, immediate release caplets

containing 0.5 g of resveratrol, supplied by Pharmascience Inc., Montreal, Quebec, Canada.

Analysis of all samples collected from both studies was carried out at the University of Leicester, UK. Concentrations of resveratrol and its metabolites were determined in the plasma, urine and stools of the healthy volunteers and in the plasma and colorectal tissue of patients with colorectal cancer, using a validated UV-HPLC assay described in Section 2.2.1. An assessment was carried out on the ability of resveratrol to modulate the insulin-like growth factor system (IGF-1, IGFBP-3, IGF-1/IGFBP-3 ratio) through measurement of proteins in plasma, oxidative DNA damage (M_1dG) levels in DNA extracted from whole blood and colorectal tissue. Markers of inflammation in plasma (PGE_2) and colorectal tissue (COX-2) were also assessed, together with a measure of cell proliferation in colorectal tissue (Ki-67) (Section 2.2.3 and 2.2.4). Study participants were assessed for adverse events, graded in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0) (Section 2.1.13).

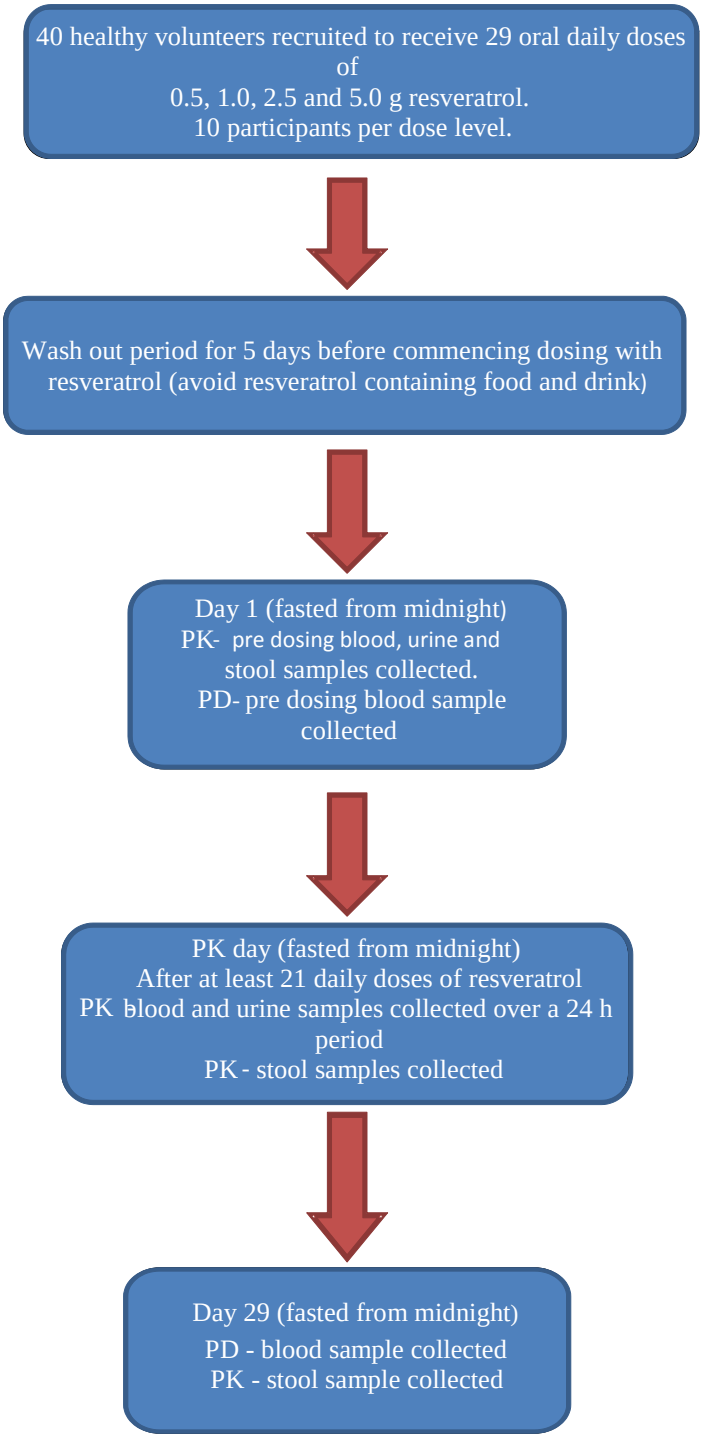


Figure 2.1.1 Schematic representation of the healthy volunteer study in which 40 healthy volunteers were recruited to receive 29 daily doses of resveratrol. The diagram shows a summary of when samples were collected for Pharmacokinetic (PK) and Pharmacodynamic (PD) analysis during the study period.

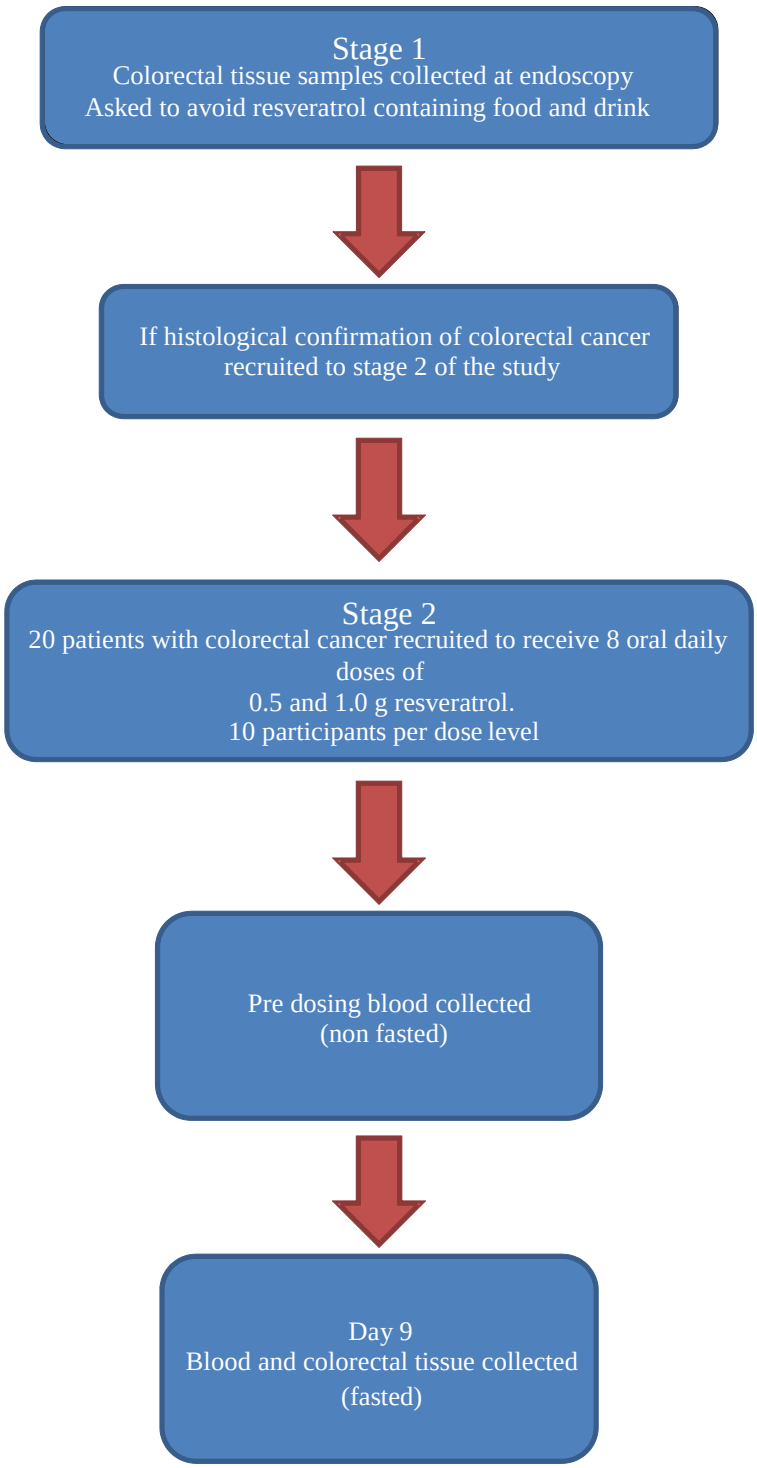


Figure 2.1.2 Schematic representation of the colorectal cancer study in which 20 colorectal cancer patients were recruited to receive 8 daily doses of resveratrol. The diagram shows a summary of when blood and tissue samples were collected for the detection of resveratrol and its metabolites and PD analysis during the study period.

2.1.2 Study population

For the healthy volunteer study attempts were made to match recruitment of males and females on each dose level in the order of 40-60%. It was also attempted to include a range of different ages, but to avoid problems with recruitment in the older population, a minimum of 30% of participants per dose level was required to be over the age of 40. As Leicester and Michigan both have racially mixed populations, this permitted recruitment of a range of people with different ethnicities. Although the colorectal cancer study was not controlled or stratified on the basis of gender or age, as it was considered that this would be difficult to achieve and restrict recruitment, attempts were made to match gender where possible. For the healthy volunteer study, participants were selected who had no active or chronic diseases and were not taking medication and/or vitamin supplements.

2.1.3 Methods of recruitment

For the healthy volunteer study, participants were recruited through advertisements posted on the University campus at Michigan and Leicester. Interested participants were sent an information leaflet, detailing what the study involved and a screening visit was arranged. At this visit the study was discussed and informed consent obtained. Participants agreed to take 29 oral daily doses of resveratrol and to the collection of blood, urine and faecal samples. Compensation was given to volunteers to cover any possible costs, such as childcare, incurred while participating in the study.

Participants for the colorectal cancer study were recruited from the Leicester Royal Infirmary and Leicester General Hospital (University Hospitals of Leicester Trust) in a two stage process. Patients with clinical or radiological suspicion of a colorectal cancer, who required an endoscopy for histological diagnosis, were recruited at Stage 1 of the study. Patients were sent a Stage 1 information leaflet along with their appointment, outlining what the study involved and were then approached in the Endoscopy Department, prior to the procedure, where participation in the study was discussed. Participant consent was required to obtain biopsies of colorectal tissue for the purposes of the study, but biopsies were only taken if colorectal cancer was detected during the endoscopy.

Patients recruited at Stage 1, who had histological confirmation of colorectal cancer and required surgical resection, were then recruited at Stage 2. Patients were approached at their surgical outpatient clinic appointment after diagnosis had been relayed. A Stage 2 information leaflet outlining the study was supplied. Patients were screened and consented to the study at their pre surgical assessment visit. They consented to take 8 daily doses of resveratrol prior to surgical resection and to the collection of blood and tissue samples. Patients were identified at Stage 1 and 2 from multidisciplinary team meetings. After several months of the study being open, there were difficulties in recruitment to Stage 2. This was felt to be due to a change in treatment policy, whereby many more patients with rectal cancer were treated with pre operative radiotherapy, making them ineligible for the study. A substantial amendment was therefore submitted to and approved by the Local Ethics Committee, MHRA, University Hospitals of Leicester Trust and University of Michigan Institutional Review Board, USA. This amendment enabled patients to be approached at Stage 2, who had not been recruited at

Stage 1. Patients were consented for colorectal biopsies, already obtained for diagnostic purposes and stored in the Pathology Department, to be used for the study.

2.1.4 Informed consent

All potential study participants were given the detailed information leaflet to read at home in their own time. In order to obtain informed consent, any questions regarding the trial were addressed at a screening visit. Participants were made aware of the purpose of the research, the practicalities and procedures involved in participating, length of participation, any potential benefits and risks and how data about them would be managed. It was also discussed that participation was voluntary and that the participant could withdraw from the study at any time, without giving any reason or compromising their future treatment. It was also mentioned that the conduct of the research had been approved by a research ethics committee.

All study participants were given contact details should they have further questions or wish to withdraw and details of the research sponsor and research funding body. No research procedures, including recording any demographic data, were performed, until after the informed consent had been completed and then a copy of the information leaflet and the informed consent was given to the participant and sent to their GP.

2.1.5 Inclusion and exclusion criteria

A number of inclusion and exclusion criteria to assess the eligibility of volunteers and patients were required for each study. Tables 2.1.1 and 2.1.2 show these criteria for the healthy volunteers. For the colorectal study there were 2 stages for recruitment and therefore separate inclusion and exclusion criteria at each stage (Table 2.1.3, 2.1.4 and 2.1.5).

Table 2.1.1 Inclusion criteria for the healthy volunteer study

Inclusion Criteria
• Men and women between the ages of 18 and 80
• Properly informed of the study and having signed the Informed Consent.
• Willing to abstain from ingesting large quantities of resveratrol-containing food and drink.
• Premenopausal women with intact female reproductive organs must be using a reliable contraceptive method and have a negative pregnancy test within 2 weeks of beginning resveratrol dosing. Post-menopausal is defined as no menses for the previous 12 months. If cessation of menses is within 18 months, then the participant should be treated as pre-menopausal and a pregnancy test performed.
• The following laboratory tests must be within the normal limits of the Institutional Clinical Laboratory: Haematologic parameters: white blood cell count, haemoglobin, platelet count. Hepatocellular function: total bilirubin, ALT and AST. Renal function: Serum creatinine, urine analysis.
• Performance status of World Health Organisation (WHO) 0-1.
• Must remain local to the study.

Table 2.1.2 Exclusion criteria for the healthy volunteer study

Exclusion Criteria
• Pregnant or lactating women. • Women contemplating pregnancy for the duration of the protocol. • Any chronic medications except for oral or depot contraceptives and hormone replacement therapy (HRT). Vitamin supplements not allowed, but acceptable if stopped 2 weeks prior to dosing. • Excessive alcohol intake (more than UK recommended limit, 21 and 14 units per week for men or women respectively). • Any cancer diagnosis currently under treatment, clinically detectable or treated within 5 years (basal cell and squamous cell carcinomas exempt). • Concurrent participation in any drug studies or studies that require sampling of a body fluid (or having finished within the past 6 months).

Table 2.1.3 Inclusion criteria for Stage 1 of the colorectal cancer study

Stage 1 Inclusion Criteria
• Over 18 years of age. • Properly informed of the study and having signed the Informed Consent. • Clinical suspicion of having a primary colorectal adenocarcinoma. • Willing to abstain from ingesting large quantities of resveratrol-containing food and drink. • Willing to abstain from ingesting vitamin supplements for the duration of the study. • Willing to abstain from non essential medications and NSAIDs at least 24 h prior to dosing with resveratrol and to refrain from their use until after colonic resection. • Performance status of WHO 0-2.

Table 2.1.4 Exclusion criteria for Stage 1 of the colorectal cancer study

Stage 1 Exclusion Criteria
• Unfit for general anaesthesia. • Active peptic ulcer disease. • Unwilling or unable to comply with the protocol. • Undergone radiotherapy or chemotherapy within 4 weeks of endoscopic tissue sampling. • Pregnant or lactating women and women contemplating pregnancy for the duration of the study. • Excessive alcohol intake (more than UK recommended limit, 21 and 14 units per week for men or women respectively). • A second (i.e. not the colorectal primary) cancer diagnosis currently under treatment, clinically detectable or treated within 5 years (basal cell and squamous cell carcinomas exempt). • Concurrent participation in other invasive or drug studies in the past 6 months. • Medication that could interfere with biomarker assay, anticoagulants including warfarin and low molecular weight heparins, NSAIDs including aspirin, (occasional use of NSAIDs allowed if participant has stopped the use of the drug at least 24 h prior to dosing and refrains from use until after colonic resection) and steroids.

Table 2.1.5 Inclusion criteria for Stage 2 of the colorectal cancer study

Stage 2 Inclusion Criteria **Identical to Stage 1 inclusion criteria with the addition of -**
• Histological diagnosis of colorectal adenocarcinoma. • Have disease amenable to surgical resection. • Adequate liver function 1) ALT ≤ 2.5 x ULN. 2) Serum bilirubin ≤1.5 x ULN • Adequate renal function: serum creatinine ≤140 μmol/L. • Adequate bone marrow function. • Haemoglobin ≥10g/dl. Patients with a lower haemoglobin included if the cause of anaemia due to bleeding from colorectal tumour and post transfusion haemoglobin ≥10g/dl prior to dosing. • Premenopausal women with intact female reproductive organs must be using a reliable contraceptive method and have a negative pregnancy test within 2 weeks of starting resveratrol dosing. Post menopausal status is defined as no menses for the last 18 months.

Stage 2 exclusion criteria for the colorectal study were identical to the Stage 1 exclusion criteria, with the addition of exclusion of patients who had undergone radiotherapy or chemotherapy before surgical resection.

2.1.6 Manufacturer and supplier of resveratrol

Study participants received uncoated immediate-release caplets containing 0.5 g resveratrol. The caplets were manufactured by Pharmascience Inc., Quebec, Montreal, Canada, using material synthesized under good manufacturing practice. Resveratrol in these caplets was stable under normal conditions (60% relative humidity, 25ºC) for the period of time covering the duration of the trial and under conditions of accelerated

decomposition (75% relative humidity, 40ºC) for at least 180 days (Certificate of Analysis, 2008). The formulation was shipped from Pharmascience Inc. to the National Cancer Institute's Drug Repository (Fisher BioServices) and then directly to the Pharmacy Department at each study site. Resveratrol was dispensed by the University of Michigan's Investigational Drug Service or by the University Hospitals of Leicester Research Pharmacy with appropriate multidose packaging. Each container held the precise number of caplets necessary to deliver the dose required for the assigned dose level for the duration of the study. The resveratrol caplets were stored at room temperature and dispensed by the Pharmacy at each site, who maintained drug and shipment logs.

2.1.7 Dosing of study participants

Participants recruited to the healthy volunteer study were required to take 29 single oral daily doses of resveratrol and were asked to take their dose every morning, as close to 8.30 am as possible. Participants recruited to the colorectal cancer study were asked to take a single oral dose of resveratrol every evening between 6 and 10 pm at a similar time each day, for 8 days up to and including the day prior to the surgical resection. In this case, evening dosing was chosen to avoid potential problems with compliance on the morning of surgery, when the patient would be nil by mouth for the procedure. On the evening prior to surgical resection, after the patient had been admitted to the ward, administration of the last dose of resveratrol was supervised, thus assuring compliance and enabling the shortest amount of time possible to elapse between the last dose and surgical resection.

For both studies, participants were advised to take their dose with as much water and food as required. Once the dose had been taken, a diary sheet was completed by the participant stating the time of ingestion, number of caplets taken, any problems with dosing or any AE. Each entry was initialed by the participant.

2.1.7.1 Method of dose selection

Each study was conducted separately with ten subjects entered at each dose level, beginning at 0.5 g and then escalating sequentially to the next dose level (Table 2.1.6). Recruitment to the next level occurred only when a lack of unacceptable toxicity at the previous dose had been established within a 14 day waiting period, after the final subject on that particular level had completed dosing with resveratrol.

Table 2.1.6 Method of dose selection for the healthy volunteer and colorectal cancer study. Each study was conducted separately with 10 subjects entered at each dose level, beginning at 0.5 g and then escalating sequentially to the next dose level as discussed in Section 2.1.7.1

			Exposure duration (number of days of dosing)	
Dose level	**Dose (g)**	**Number of caplets to be taken orally**	**Healthy volunteer study**	**Colorectal cancer study**
1	0.5	1	29	8
2	1.0	2	29	8
3	2.5	5	29	
4	5.0	10	29	

An unacceptable drug-related AE was defined as any possible, probable or definite drug related grade 2 or higher toxicity not resolving within 3 days, according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3 (Section 2.1.13). If this occurred in 2 or more study participants at a particular dose level, dose escalation would cease, the previous dose level would expand to a total of 16 participants, then the study would be stopped.

2.1.8 Study visits

2.1.8.1 Healthy volunteers

There was a maximum of 5 scheduled visits required for each study volunteer (Table 2.1.7). Participants were screened (screening visit) prior to recruitment to the study to ensure they were eligible. Each participant had been given a study information leaflet at least 24 hours prior to this visit. At screening the study was discussed and consent obtained. A medical and medication history was obtained, the subject given a physical examination, including recording height, weight and blood pressure and a blood test was drawn for haematological and biochemical parameters (clinical lab bloods, Table 2.1.8). Day 1, PK and day 29 visits were scheduled for early morning. Participants came to the research centre having fasted from midnight, then took the required dose, as close to 8:30 am as possible.

Table 2.1.7 Procedures performed and information documented at each scheduled study visit for the healthy volunteer study.

Visit	Samples obtained	Information obtained
Screening	• Clinical lab bloods • Dipstick urinalysis	• Year of birth • Gender and ethnicity • Medical/surgical history • Concomitant medication • Enrolment in any other experimental studies • Physical examination including blood pressure, pulse, respiratory rate, and temperature • Height and weight • Performance status (WHO scale)
Day 1	• Pre dose • PK stool (collected within 3 days of visit), blood and urine. Biomarker blood	• Concomitant medication
PK day	• PK blood: pre-dose, 0.25h, 1.0, 1.5h, 5h, 12h and 24h • PK urine: pre-dose, 0-2, 2-4, 4-8, 8-12, and 12-24 h collections • PK stool: pre (within 3 days of visit) and 1st stool after dose on the PK visit	• Physical examination • Height and weight • Performance status • Concomitant medication • Adherence • Adverse events
Day 29	• PK stool: pre (within 3 days of visit) • Clinical lab bloods • Dipstick urinalysis • Biomarker blood • PK blood sample and urine	• Physical examination including blood pressure, pulse, respiratory rate, and temperature • Height and weight • Performance status • Concomitant medication • Adherence • Adverse events

Table 2.1.8 Blood tests required for haematological and biochemical analysis for the healthy volunteer study

Blood tests	Screening and day 29 visits
Haematology	Haemoglobin, haematocrit, red blood count, white blood count, platelet count, differential white blood cell count
Clotting	PT, PTT, INR
Biochemistry	Sodium, chloride, potassium, blood urea nitrogen, creatinine, glucose, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, total protein, albumin, and alkaline phosphatase, total, conjugated and unconjugated bilirubin, calcium, phosphorus, triglycerides and cholesterol.
Pregnancy test	Premenopausal or postmenopausal within 18 months of last menses

As well as scheduled visits, participants were monitored on days 8, 15, 37 and 44 (± 3 days) of dosing. They were contacted by telephone, email or in person and asked about AE and concomitant medications. On days 8 and 15, participants were also asked about adherence to the study.

2.1.8.2 Colorectal cancer patients

For the colorectal cancer patients there was a maximum of 4 scheduled visits to the hospital required for the study. Where possible, these visits were arranged to coincide with their hospital appointment (Table 2.1.9). Participants were screened prior to recruitment at the pre surgical assessment clinic to ensure they were eligible. At this visit the study was discussed and consent obtained. A medical and medication history

was obtained, the subject given a physical examination, including height, weight and blood pressure (vital observations) and a blood test was drawn for haematological and biochemical parameters (clinical lab bloods, Table 2.1.10). Along with the study visits patients were monitored on day 2 of dosing, when they were contacted by phone to discuss adherence, concomitant medication, medication and AE.

Table 2.1.9 Procedures performed and information documented at each scheduled study visit for the colorectal cancer study.

Visit	Samples obtained	Information obtained
Stage 1 endoscopy visit	• Clinical lab bloods • Six colorectal tissue samples and six colorectal tumour tissue samples	• Adverse events • Concomitant medication • Medical/ surgical history • Physical examination, vital observations
Surgical pre assessment clinic (pre dosing)	• Clinical lab bloods • PK and biomarker bloods (pre dose)	• Adverse events • Concomitant medication
Day 3-5 of dosing	• Clinical lab bloods	• Adverse events • Concomitant medication
Day 8 of dosing (surgical ward)	• Clinical lab bloods	• Adverse events • Concomitant medication • Physical examination, vital observations
Colorectal surgery	• PK and biomarker bloods (post dose) • Samples of malignant colorectal tissue and samples from non malignant colorectal tissue	• Adverse events

PK bloods refers to bloods obtained for detection of resveratrol and its metabolites

Table 2.1.10 Blood tests required for haematological and biochemical analysis for the colorectal cancer study.

Blood tests	Screening, day 3* and day 8 visits
*Haematology	Haemoglobin, haematocrit, red blood count, white blood count, platelet count, differential white blood cell count
Clotting	INR
Biochemistry	*Sodium, *potassium, *blood urea nitrogen, *creatinine, glucose, *alanine aminotransferase, *albumin, and *alkaline phosphatase, *total bilirubin, calcium, phosphorus.
Pregnancy test	Premenopausal or postmenopausal within 18 months of last menses

*Day 3 only certain blood tests performed

2.1.9 Washout period and restricted diet

All study participants were provided with a list of food and drink known to contain resveratrol (Appendix 9.2) which needed to be avoided for the entire study period and for 5 days prior to commencing dosing. Because surgical operation dates could be changed, sometimes at short notice due to availability of operating theatre times, the colorectal cancer patients recruited at Stage 1 of the study were asked to follow the restrictions until surgical resection. For participants not recruited until Stage 2, the restrictions applied once consent had been obtained. The aim was to eliminate as far as possible the intake of other sources of resveratrol.

Each healthy volunteer on the PK day was provided with 2 meals during the visit; a lunch, jacket potato with a choice of beans or cheese, after 12:30 pm and an evening

meal, pizza with a topping of their own choice, after 5:30 pm with water to drink. Both meals were selected to avoid food and drink containing resveratrol, thereby minimizing the dietary bias by controlling intake during the PK sampling.

2.1.10 Sample collection and processing

Analysis of resveratrol and its metabolites, subsequent PK analysis and biomarker measurements were conducted in the Department of Cancer Studies and Molecular Medicine, University of Leicester. All samples collected were processed, stored or frozen, as soon as possible after being obtained. All samples from the University of Michigan site were shipped on dry ice to Leicester, where they were processed and analysed.

2.1.10.1 Healthy volunteer study

Venous blood samples for pharmacokinetic analysis were collected in 7.5 mL lithium heparin vacutainers (Sarstedt, Leicestershire, UK) at 7 separate time points, pre dose and then 0.25, 1.0, 1.5, 5.0, 12 and 24 h post dose administration. Once collected, the tubes were chilled on ice and protected from light. During the 13 h the participant was in the department, a cannula was used for blood collections. Each blood draw from the cannula consisted of an initial 3 mL of wasted blood and then 30 mL of blood (4 x 7.5mL). After each blood draw the cannula was flushed with a minimal amount of normal saline (2-5 mL). As soon as possible post collection, 3 of the blood samples were centrifuged (3000 g, 4°C for 15 min), then the supernatant was removed and aliquoted into 1.2 mL cryovials for PK and biomarker analysis. Blood in the remaining

lithium heparin tube was aliquoted into labeled cryovials for further biomarker analysis. All samples were then immediately transferred to a -80°C freezer until shipment or analysis.

On the pharmacokinetic visit, prior to dosing, the participant was asked to urinate to void any residual urine. This was collected as the time 0/baseline sample and subsequent collections were at 0-2, 2-4, 4-8, 8-12 and 12-24 h post dose administration. In the 10 min preceding the end of each collection period, the participant was asked to empty their bladder. Each batch was collected in a clean plastic container over a specified time period and the containers were protected from light, either being amber coloured or by covering in foil and kept chilled on ice or in a refrigerator. The participant was provided with an insulated container with cold packs, in which to store the 12-24 h sample. On days 1 and 29, before the participant took their dose of resveratrol, a single void of urine was collected. From each urine sample collected during each study visit, four 5 mL aliquots were placed in a –80ºC freezer and stored until shipment or analysis.

A maximum of 4 stool samples were collected from each participant during the study period. Vials containing an integrated spatula in the lid were provided along with instructions on how to collect at least 3 spatulas for each sample required. The first sample collected was pre dose and within 3 days of commencing dosing, the second during the 3 days preceding the PK day and the third was obtained as the first stool sample produced on the PK day. If possible, a fourth and final sample was also collected within 3 days of day 29. Samples were stored in the dark, preferably frozen or

refrigerated, if possible, then transported to the laboratory, as soon as achievable, where they were stored, protected from light at –80ºC until shipment or analysis.

2.1.10.2 Colorectal cancer study

Patients recruited at Stage 1 of the study were consented for the collection of twelve biopsies, taken in addition to that required for standard care, with 6 biopsies of potentially malignant tissue and the remainder constituting macroscopically normal colorectal tissue. The latter were taken ~10 cm away from the malignant tissue and all biopsies were acquired by the medical or surgical consultant carrying out the procedure. Half of the number of biopsies from malignant and normal colorectal tissue were snap frozen in liquid nitrogen and stored at -80 ºC until analysis, whilst the remainder were placed in formalin for 24 h and then embedded in wax.

During surgery (Stage 2), the resected bowel was transported to the Pathology Department as quickly as possible in a sealed container on ice packs, protected from light. Dr Kevin West, Consultant Gastroenterology Pathologist, provided the tissue samples for the study according to pathology guidelines. Colorectal tissue including the mucosa was taken from several areas of the resected specimen. These sites included the tumour and, where possible, depending on the type of resection and site of the colorectal cancer, 5 cm and 10 cm proximal and distal to the tumour and from the proximal and distal resection margins. For each sample the distance from the tumour edge was recorded. Up to 1.0 g of normal and malignant tissue was harvested from resection specimens. Individual tissue samples were divided, with half being snap

frozen in liquid nitrogen and kept at -80 ºC until analysis and the remainder placed in formalin for 24 h, then embedded in paraffin wax.

Venous blood (3 x 7.5 mL) was collected in lithium heparin tubes pre dose at the time of colorectal cancer resection, normally within 1 h of the operation starting. The tubes were chilled on ice and protected from light. As soon as possible post collection, 2 of the blood samples were centrifuged (3000 g, 4°C for 15 min), then the supernatant was removed and aliquoted into 1.2 mL cryovials for PK and biomarker analysis. Blood in the remaining lithium heparin tube was aliquoted into labeled cryovials for further biomarker analysis. All samples were then immediately transferred to a -80°C freezer until analysis.

2.1.11 Data management

A set of case report forms for each participant was completed at the initial screening visit and then at each contact with the participant throughout the study. The report forms confirmed eligibility for the study, documented medical history and physical examination details, including height, weight and blood pressure, as well as biochemical and haematological indices. The forms recorded date of collection and storage of blood, urine, faeces and tissue samples and any AE during the study. A record of medication or vitamins taken prior to and during the study period and adherence to study agent was also required. An off study form confirmed that the participant had completed dosing on the study and that no further follow up was required. In addition, a form was signed by the Principal Investigator to verify that all the case report forms were complete. All data relating to each study participant were stored in a locked secure room and the

completion of case report forms was subject to quality control procedures and on site audits with respect to completeness and accuracy of the data recorded.

2.1.12 Adherence and compliance with resveratrol dosing

Participant adherence to resveratrol dosing was assessed through self-reporting, caplet counts performed at each study visit, and determination of resveratrol levels by UV-HPLC analysis. Each participant was given the exact number of caplets needed for the dosing period and instructed to return to the investigator any caplets, which for any reason had not been taken. Study participants were given a diary sheet, on which to record the date and time each dose was taken and initialed. They were asked to bring the form with them each time they returned for a scheduled visit, when research personnel reviewed the reports for compliance.

For the healthy volunteer study all participants with ≥ 75% adherence, as determined by the quantity of unused caplets returned to the site, were deemed evaluable. Participants who took less than 75% over the 29 days were replaced in the pharmacokinetic analyses, but were retained for the purpose of toxicity assessment. All colorectal cancer patients with ≥ 75% adherence as determined by the quantity of unused resveratrol returned to the study were evaluable for determining the effect of the study agent. Participants who did not take their final dose on the evening prior to surgery were considered non-compliant, as it was felt that this could bias the biomarker and PK endpoints in the surgical sample, and they were replaced.

2.1.13 Adverse events

An adverse event (AE) is defined as any condition that appears or worsens after the participant is enrolled in an investigational study. All AE were noted on the relevant case report form, whether or not it was considered related to the study agent. Information collected for all AE included start and stop dates of event and the severity was graded by a numerical score, according to the NCI Common Terminology Criteria for Adverse Events (CTCAE, version 3, 2006) (Table 2.1.11) (www.ctep.cancer.gov/protocolDevelopment/electronic_aplications/docs/ctcaev3.pdf) with a description of the CTCAE category. It was also made clear whether the event was classified as a serious adverse event (SAE), in which case the event would need to be reported urgently to the appropriate regulatory bodies. The study investigators also decided on the relationship to study agent (attribution). The AE outcome and whether or not the participant withdrew from the study due to the AE was also recorded. Participants were asked about AE at each study visit and were encouraged to contact the study team if they encountered any problems during the study period. Their GP was also made aware of their participation in the study and information leaflets with contact details were sent on enrolment.

Table 2.1.11 NCI Common Terminology Criteria for Adverse Events. CTCAE, version 3, 2006. Severity for each AE is graded by a numerical score (1-5).

Grade	Severity	Description
1	Mild	Barely noticeable, does not influence functioning Causes no limitations of usual activities
2	Moderate	Makes participant uncomfortable, influences functioning Causes some limitation of usual activities
3	Severe	Severe discomfort, treatment needed Severe and undesirable, causing inability to carry out usual activities
4	Life threatening	Immediate risk of death Life threatening or disabling
5	Fatal	Causes death of participant

2.2 Laboratory methods

2.2.1 Resveratrol and metabolite extraction methods

Extraction methods for resveratrol and its metabolites in plasma, urine and faeces were previously developed and validated within the Department of Cancer Studies and Molecular Medicine, University of Leicester and are described in brief below (Boocock *et al*., 2005).

2.2.1.1 Liquid extraction method from plasma and urine

Plasma (250 µL) was acidified with 4.4 µL of concentrated HCL, then methanol (MeOH) (250 µL) was added and each sample vortex-mixed for 1 min and placed at −20 ˚C to precipitate the protein (10 min). The samples were then centrifuged (13,000×g, 4ºC for 15 min) to remove denatured proteins and the supernatant was concentrated to dryness under a stream of nitrogen at room temperature in the dark. The residue was reconstituted in 200 µL of 50:50 MeOH: H_2O and mixed well. A final centrifugation (13,000 x g, 4 ˚C for 15 min) was performed prior to aliquoting into the appropriate HPLC vials, and if not analysed immediately, samples were kept in the dark at 4 ˚C in the autosampler chamber until injection. For urine, the extraction procedure was essentially the same as that conducted for plasma, except that urine was acidified with 1.3 µL of concentrated HCL and there was no need to dry down the extracted sample prior to analysis. Unless otherwise stated all chemicals in this methods chapter were purchased from Sigma-Aldrich, Dorset, UK and from Fisher Scientific, Leicestershire, UK.

2.2.1.2 Solid phase extraction of faeces

Faecal matter was homogenized in 5 mL of H_2O containing 2% phosphoric acid and then centrifuged (2,900 x g, 4˚C for 15 min). Resveratrol and related metabolites were isolated from the supernatant by solid-phase extraction using Oasis HLB cartridges (3mL) (Waters, Hertfordshire, UK). The columns were primed with 1 mL acidified MeOH (2% acetic acid) followed by 1 mL H_2O and washed with 1 mL acidified MeOH, (2% acetic acid) then the analytes were eluted with 100% MeOH. There was no need to dry down the extracted sample prior to analysis. The resveratrol stool content was related to dry faeces weight, which was obtained by drying stool samples at 100˚C, until constant weight was attained (24 h).

2.2.1.3 Extraction of resveratrol from colorectal tissue

Colorectal tissue (~200 mg) was prepared by grinding samples in liquid nitrogen, followed by homogenization in HEPES buffer (300 µL per 100 mg tissue). Extraction of resveratrol from colorectal tissue was performed with the help of Ketan Patel (Cancer Studies and Molecular Medicine, University of Leicester). Aliquots of homogenate (95 µL) were spiked with 5 µL of naringenin (25 ng /µL) as an internal standard. Following this, tissue samples were acidified with 1.75 µL concentrated HCL, then MeOH (100 µL) was added, before vortexing for 1 min. Samples were kept at -20˚C for 10 min, then centrifuged and the supernatant removed. The following liquid extraction procedures were the same as for the plasma samples, except tissue samples were reconstituted in 130 µL 50:50 MeOH: H_2O and 100 µL of each sample was injected onto the HPLC.

2.2.1.4 HPLC analysis of resveratrol and metabolites

Resveratrol and its metabolites were separated and quantified using a gradient UV-HPLC system (Waters Ltd, Elstree, Hertfordshire), as described by Boocock *et al.*, (Cancer Studies and Molecular Medicine, University of Leicester), in which the retention time for resveratrol was found to be ~18.6 min and the limit of detection in plasma and urine had previously been determined as 2.0 ng/mL (Boocock *et al.*, 2005). The method has been validated for resveratrol in terms of inter-day and intra-day variability, recovery, accuracy, and precision (Boocock *et al.*, 2005). Analysis was performed on a Waters Atlantis C_{18} column in combination with a Waters Atlantic C_{18} guard column (Waters Ltd, Elstree, Hertfordshire). The column oven temperature was 35˚C and the flow rate was 1 mL/min. The gradient elution system (A: 5 mmol/L ammonium acetate; B: 98% MeOH, both with 2% propan-2-ol) was as follows with respect to B: 0 to 7 min 20%, 7 to 16 min 50%, 16 to 18 min 55%, 18 to 23 min 95%, followed by re-equilibration to 0% B for 6 min before the next injection. Unless stated otherwise, typically a volume of 50 µL for each sample was injected onto the HPLC system.

As characterised resveratrol metabolite standards were not available in sufficient quantities for method development, their quantities were calculated based on the assumption that recovery characteristics and the relationship between peak area ratios and concentrations were the same as those for resveratrol. Metabolite concentrations are therefore described as resveratrol equivalents. A small amount of authentic resveratrol-3-sulfate was provided by Pharmascience Inc and was used to identify the HPLC peak corresponding to this metabolite. Additionally, metabolites were assigned

where possible, on the basis of previous structural characterization by LC-MS/MS performed in the Department by Boocock *et al.,* using chromatographic conditions identical to those employed in the present study (Boocock *et al*., 2005).

2.2.2 Cell culture

2.2.2.1 Maintenance of cell lines and routine passaging

HT-29 and HCA7 (American Type Culture Collection, Middlesex, UK) malignant colorectal carcinoma cell lines were grown in Dulbecco's modified eagle's media (4500 mg glucose/L), supplemented with 10% foetal calf serum (FCS). Cells were maintained in a warm (37˚C) humidified atmosphere (100%) supplemented with 5% CO_2. Cells were grown to approximately 70% confluence. Medium was aspirated and cells were washed twice in warm (37˚C) sterile phosphate buffered saline (PBS). Sterile trypsin in PBS (5%) was added (5 mL) and after a 5 min incubation at 37°C and gentle agitation, cell detachment was confirmed by microscopy. Warm sterile media (10 mL) was added to the flasks and the cell suspension centrifuged (13,000 rpm, 4ºC for 3 min). The supernatant was discarded and the cell pellet resuspended in 10 mL of media. Cells were counted and seeded into new flasks at various densities depending on the requirements. Cells were used with passage numbers between 5 and 15 for each experiment. All plastic ware for cell culture was purchased from Nunc, Denmark.

Cells were treated with resveratrol at doses ranging from 1 to 10 μM. Initial stock solutions were prepared in dimethyl sulfoxide (DMSO) such that the final DMSO concentration in the media would be <0.2% (v:v). Control cells were incubated with an

equivalent concentration of DMSO in culture media. All final working solutions were in media + 10% FCS

2.2.2.2 Stability of resveratrol under cell culture conditions

Media (10% FCS) containing 5 μM resveratrol (20 mL) was placed in 175 mL cell culture flasks and transferred to an incubator. At 0, 0.5, 1, 2, 4, 6, 12 and 24, 48, 72, 96, 120, 144 and 168 h, 1 mL of media was removed from each flask and aliquoted into a clean eppendorf. The media was stored at -80°C until analysis (<1 week). Analysis of the resveratrol content was carried out by HPLC (using the method described for plasma (Section 2.2.1.1) for each time point. Stability experiment was undertaken in duplicate, on 3 separate occasions. Gibco phenol red free media (purchased from Invitrogen Life Technologies, Paisley, Scotland) was used for this experiment, as phenol red interfered with the detection of resveratrol due to coelution under the HPLC conditions employed.

2.2.2.3 Effect of resveratrol on colon cancer cell growth

Cells were seeded in 24-well plates, at a density of 2500 and 7500 cells per well for HT-29 and HCA7 cells respectively. After an incubation period of 24 h, the media was removed and cells were treated with concentrations of resveratrol of 0, 1, 5 and 10 μM. Cells were exposed to either single or daily doses of resveratrol. For the latter, media was removed from each well and replaced with fresh media containing resveratrol each day. Cells were harvested by trypsinisation at time points of 72, 96, 120, 144 and 168 h following the first treatment with resveratrol and counted using a particle counter and

size analyzer (Beckman Coulter, Buckinghamshire, UK). Incubations were performed in triplicate on 3 separate occasions.

2.2.2.4 Cell cycle analysis

HT-29 and HCA7 cells were seeded in petri dishes at a range of densities to give the required number of cells for analysis at the appropriate time points (Table 2.2.1). Cells were allowed to adhere overnight, prior to dosing with fresh media containing resveratrol at concentrations of 0, 1, 5 and 10 μM. Cells were exposed to either single or daily doses of resveratrol as described above (Section 2.2.2.3). After incubation with resveratrol for 24, 48, 72, 96 and 120 h, the media was discarded, adherent cells were harvested by trypsinisation, washed twice in PBS, then resuspended in PBS (200 μL). Ice cold 70% ethanol (EtOH) in PBS (2 mL) was then added to each sample, whilst being vortexed vigorously. Cells were then stored at 4˚C for up to a week or -20ºC for 2 weeks. The day prior to analysis, cells were centrifuged (600 x g, for 10 min) and resuspended in 800 μL PBS. Following this, 100 μL of RNase (1 mg/mL in PBS) (Merck Biosciences, Nottinghamshire, UK, stock concentration10 mg/mL) and propidium iodide (50 μg/mL) were added and the samples stored at 4˚C overnight, before being analyzed by flow cytometry (Becton Dickinson, Oxfordshire, UK) and Modfit LT software (Verity Software House, Topsham, USA). Cell cycle analysis was undertaken in duplicate, on 3 separate occasions.

Table 2.2.1 Seeding densities of colon cell lines for cell cycle analysis

Time between treatment with resveratrol and harvesting (h)	Seeding densities (million cells)	
	HT-29 cells	HCA7 cells
24	1	1
48	0.5	0.5
72	0.2	0.3
96	0.2	0.3
120	0.1	0.2

2.2.3 Pharmacodynamic markers

2.2.3.1 Measurement of IGF-1 in plasma

Analysis of IGF-1 was undertaken using a quantitative enzyme immunoassay technique (Human IGF-1 ELISA kit, R&D Systems, Oxfordshire, UK). The assay had a monoclonal antibody specific for human IGF-1, pre coated on a microplate. For analysis 20 µL of each plasma sample was required. Reagents, working standards and samples were prepared as per manufacturer's instructions. Standards and plasma samples were pipetted into wells and incubated at 2-9˚C for 2 h, during which any IGF-1 present was bound by the immobilized antibody. After incubation, wash buffer supplied with the kit was used to remove any unbound substances, then an enzyme-linked polyclonal antibody specific for IGF-1 (200 µL) was added to the wells and the plate incubated at 2-8˚C for 1 h.

Following another wash step, 200 µL of a substrate solution (colour reagent) were added and colour developed in proportion to the amount of IGF-1 bound in the initial step. After 30 min of incubation at room temperature in the dark, 50 µL of a stop solution was added to quench the reaction. The optical density of each well was determined at 450 nm using a microplate reader (Fluostar Optima, BMG Labtech, Buckinghamshire, UK). Standards were assayed, allowing generation of a calibration curve, from which IGF-1 concentrations were extrapolated. Samples were analyzed in triplicate and standards in duplicate. To minimize potential variation between each plate processed, pre and post samples from each participant were analysed on the same plate and a selection of participants' samples were rerun on different days to confirm the results were consistent.

2.2.3.2 Measurement of IGFBP-3 in plasma

Analysis of IGFBP-3 was undertaken using a quantitative enzyme immunoassay technique (Human IGF-1 ELISA kit, R&D systems, Oxfordshire, UK) and the kit was analogous to the kit used for IGF-1. The assay has a monoclonal antibody specific for IGFBP-3 pre coated on a microplate. 10 µL of each plasma sample was required for analysis. Reagents, working standards and samples were prepared as per manufacturer's instructions. Standards and plasma samples were pipetted into wells (samples analyzed in triplicate and standards in duplicate) and incubated at 2-9˚C for 2 h. Any IGFBP-3 present was bound to the immobilized antibody. After incubation, washing was carried out to remove any unbound substances. An enzyme-linked polyclonal antibody specific for IGFBP-3 (200 µL) was then added to the wells and the

plate incubated at 2-8˚C for 2 h. The remaining steps in the procedure are the same as those for the IGF-1 assay after the 2 h incubation (Section 2.2.3.1).

2.2.3.3 Calculation of the plasma IGF-1/ IGFBP-3 ratio

Plasma IGF-I and IGFBP-3 levels were determined and the IGF-I/IGFBP-3 molar ratio was calculated based on 1 ng/mL IGF-I = 0.130 nmol IGF-I and 1 ng/mL IGFBP-3 = 0.036 nmol IGFBP-3 (Max *et al*., 2008).

The IGF-1/ IGFBP-3 ratio was calculated by using the equation:

(IGF-1 concentration ng/mL x 0.130) / (IFGBP-3 concentration ng/mL x 0.036)

2.2.3.4 Measurement of plasma PGE_2 levels

Plasma PGE_2 concentration was determined by the use of a Prostaglandin E_2 EIA monoclonal kit as per the manufacturer's instructions (Cayman, Michigan, USA). The procedure was optimized by Ankur Karmokar for human plasma samples (Department of Cancer Studies and Molecular Medicine, University of Leicester). Prior to using the kit, plasma samples were purified using a PGE_2 affinity sorbent (Cayman, Michigan, USA). The sorbent was gently mixed before use, then 30 µL was added to 1 mL of each plasma sample to bind any PGE_2 present. The samples were then mixed gently for 60 min. The samples were centrifuged (1500 x g, for 10 min) to sediment the sorbent and the supernatant carefully removed with a pipette and discarded. Care was taken to retain all sorbent as it contained PGE_2. The sorbent was washed with ultrapure water (1 mL) (Cayman, Michigan, USA), the samples centrifuged (1500 x g, for 15 min) and the

supernatant discarded again. The sorbent pellet was then resuspended in 0.5 mL of Elution buffer (95% EtOH: 5% ultrapure water) and vortex-mixed for 1 min. After the samples were centrifuged again (1500 x g, for 15 min), the supernatant was carefully removed and retained. A further 0.5 mL of Elution buffer was added to the sorbent and centrifuged (1500 x g, 2 min) and the supernatant again removed and retained. The corresponding supernatants were combined, placed under a stream of nitrogen and evaporated to dryness, to remove all organic solvents which could interfere with the assay. Dried samples were dissolved in 250µL EIA buffer and vortex-mixed for 2 min then loaded onto the ELISA plate.

The prostaglandin E_2 EIA monoclonal kit assay is based on the competition between PGE_2 in the sample and added PGE_2 acetylcholinsterase (AChE) conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody. Because the concentration of the PGE_2 tracer remains constant, while the concentration of PGE_2 present in the sample varies, the amount of the PGE_2 tracer, able to bind to PGE_2 monoclonal antibody is inversely proportional to the concentration of PGE_2 in the sample. This antibody-PGE_2 complex binds to goat polyclonal anti mouse IgG_2 attached to the well.

Reagents, working standards and samples were prepared as per manufacturer's instructions. EIA buffer (100 µL) was added to the non specific binding wells. Standards were assayed, allowing generation of a calibration curve from which PGE_2 concentrations were extrapolated and 50 µL of each standard and plasma sample were added to the appropriate wells. Samples were analyzed in triplicate and standards in duplicate. PGE_2 AChE tracer was then added (50 µL) to each well, except the total activity and blank wells. PGE_2 monoclonal antibody was added (50 µL) to each well,

except the total activity, non specific binding and blank wells. After covering the plate with a plastic film and incubating for 18 h at 4ºC, it was then washed in buffer provided with the kit, to remove any unbound material and Ellman's reagent (200 µL), which contains the substrate to AChE, was added to the wells and a tracer (5 µL) added to the total activity wells. The plate was covered with a plastic film and placed on an orbital shaker for 90 min and left to develop in the dark. The product of the enzyme reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, as determined spectrophotometrically using a plate reader (FLUOstar OPTIMA, BMG Labtech, Buckinghamshire, UK), is proportional to the amount of PGE_2 tracer bound to the well and is inversely proportional to the amount of free PGE_2 present in the well during the incubation. Each participant's pre and post samples were run on the same plate to minimize variability between plates. The calculation of concentrations of PGE_2 in the plasma samples and standards required the data to be plotted as logit (B/B_o) versus log concentration (as per manufacturer's instructions). The B/B_o represents the ratio of the absorbance of a particular sample of standard well to that of the B_o well. The B_o represents the maximum amount of tracer that the PGE_2 antibody can bind in absence of free analyte, logit (B/B_o) = In [average B/B_o /(1-average B/B_o)].

2.2.3.5 Analysis of M_1dG adduct levels

2.2.3.5.1 DNA extraction from whole blood

The method of DNA extraction from whole blood for analysis of M_1dG was optimized by Dr Raj Singh in the Department and is based on the Qiagen standard protocol, with some additional precautions to prevent artefactual formation of oxidised DNA lesions

during processing (Qiagen Crawley, West Sussex); all DNA extractions were done using kits and related buffers supplied by Qiagen. To lyse the cells and stabilize and preserve nuclei, 3 mL of ice-cold C1 buffer, spiked with 5 mM of deferoxamine was added to whole blood samples (3 mL). Deferoxamine binds Fe^{2+} ions preventing the formation of free radicals. Ice-cold HPLC grade water (9 mL) was then added, and the samples mixed by inversion, until the suspension became translucent, before incubating on ice for 10 min.

The lysed blood samples were centrifuged (3000 rpm, 4˚C for 15 min) and the supernatant discarded. Ice-cold C1 buffer (1 mL), spiked with 5 mM deferoxamine and ice cold HPLC water (3mL) was added to the pellet. The pellet was resuspended by vortexing and then centrifuged (3000 rpm, 4˚C for 15 min). The supernatant was discarded to leave a pellet of cell nuclei and then resuspended in 1 mL of G2 buffer spiked with 5 mM deferoxamine. Samples were vortexed for 10 sec and mixed on a rotating wheel for 5 min. G2 buffer (8.5 mL), spiked with 5mM deferoxamine was added, along with 100 µL of proteinase K (25 mg/mL in water) (Roche Diagnostics, East Sussex, UK). The samples were incubated at 37˚C for 4 h and vortexed every 30 min. They were then incubated overnight on a rotating wheel at 4°C.

After an overnight incubation, 25 µL of RNase A (10 µg/µL) and 10 µL of RNase T_1 (5U/µL in PBS) (Roche Diagnostics, East Sussex, UK) were added. The RNase had been heat activated for 10 min at 80˚C prior to adding to the samples. Samples were incubated at 37˚C for 60 min, with mixing after 30 min. A midi column 100/G (Qiagen, Crawley West Sussex) was equilibrated with 4 mL of QBT buffer. Each sample was vortexed for 5 min and loaded onto the column, which was then washed twice with 7.5

mL of QC buffer. The DNA was eluted with 5 mL of QF buffer warmed to 50˚C and precipitated by adding 3.5 mL of cold isopropanol. Samples were left overnight at 4˚C to encourage precipitation.

Samples were centrifuged (4000 rpm, 4˚C for 20 min) and the supernatant discarded, leaving a DNA pellet which was washed with 1 mL of cold 70% EtOH, followed by cold absolute EtOH. After the final wash the DNA was left to air dry for 10 min. The pellet was resuspended in 0.5 mL of HPLC grade water and stored at 4˚C overnight to allow the DNA to dissolve. The concentration of DNA in each sample was measured by determining the absorbance at 260 nm using a GeneQuant spectrophotometer (Pharmacia, Biotech, Cambridge, UK), assuming that one absorbance unit at 260 nm is equal to 50 µg/mL DNA. Samples were stored at -20˚C until used for M_1dG analysis.

2.2.3.5.2 Isolation of DNA from colorectal tissue samples

The method of DNA extraction from colorectal tissue for analysis of M_1dG was optimized by Dr Raj Singh within the Department (Singh *et al*., 2001), based on Qiagen standard protocol and all DNA extractions were done using kits and related buffers supplied by Qiagen. Tissue samples were homogenized mechanically in 9.5 mL of Qiagen buffer G2 using a glass tissue homogenizer (Jencons, West Sussex, UK) and then transferred to a 15 mL polypropylene tube. To each sample was added 0.5 mL of Proteinase K (20mL/mL in water) and 7 µL of RNase A (61 mg/mL). Each sample was mixed by vortexing and incubated at 45 ˚C for 2 h. Following this a Qiagen genomic column (midi column 100/G) was equilibrated using 4 mL of buffer QBT and the column allowed to empty by gravity flow. Each sample was vortexed for 30 sec and

loaded on to a column, which was then washed twice with 5 mL of buffer QC. The DNA was eluted with buffer QF warmed to 45˚C and precipitated by addition of ice cold isopropanol (3.5 mL) and overnight storage at -20˚C. DNA was washed and redissolved in 100 µL water as described above (Section 2.2.3.5.1).

2.2.3.5.3 Immunoslot blot assay for M_1dG

M_1dG levels in DNA extracted from whole blood samples were analyzed by an immunoslot blot assay validated and performed by Ankur Karmokar (Department of Cancer Studies and Molecular Medicine, University of Leicester) according to the method developed by Dr Raj Singh within the Department (Singh *et al*., 2001). Calf thymus DNA was treated with MDA (both provided by Dr Raj Singh) to provide a level of 10 fmol M_1dG/µg DNA and then used to prepare a series of standards to construct a calibration curve by diluting the stock with control thymus DNA. The standards were then vortex mixed and centrifuged (14,000 rpm for 1 min). Nine standards (in triplicate) were used to provide a calibration curve for quantification of adduct levels ranging from 0 to 10.0 fmol M_1dG /µg DNA. To each sample/standard was added 65 µL of KP buffer (10 mM dipotassium hydrogen orthophosphate, pH 7.0). To break up the DNA into approximately 100 base pair long strands, each sample/standard was sonicated for 20 min and then 150 µL of PBS was added. To form single stranded DNA, the samples/standards were heated at 100˚C for 5 min and then cooled on ice for 10 min. Following this, samples/standards were vortex mixed and centrifuged (14,000 rpm for 2 min) before 2M ammonium acetate (250 µL) was added.

Two gel blotting papers (Schleicher and Schuell, Surrey, UK) were bathed (6.3 x 22.8 cm) in 1M ammonium acetate, before putting on the immunoslot blot apparatus and a nitrocellulose filter (Schleicher and Schuell, Surrey, UK) was bathed in distilled water followed by 1M ammonium acetate, before being placed on top of the gel blotting papers. A volume containing 1 µg of DNA from each sample/standard (in triplicate) was applied to the nitrocellulose filter, while the aspirator was running. The wells were allowed to run dry and then 200 µL of 1M ammonium acetate was pipetted onto each well and allowed to dry for 30 min. The filter was carefully placed between filter paper and heated to 80˚C for 1.5 h in an oven. Following this, the filter was placed in a plastic tray and bathed in 100 mL of PBS-T (PBS plus 0.1% Tween-20) plus 5% non fat milk powder (Marvell, Nestle) and allowed to rock gently at room temperature (20˚C) for 1 h. The filter was then washed twice with 50 mL of PBS-T for 5 min.

The primary antibody (M_1dG) (supplied by Prof. Lawrence Marnett, Vanderbilt University, USA) (Singh *et al*., 2001) was diluted with PBS-T plus 0.5% milk powder to give a final concentration of 6.25 ng/mL. In a plastic tray, the filter was incubated with the primary antibody and rocked gently at room temperature for 2 h, followed by overnight incubation at 4˚C. The filter was then washed twice with PBS-T (50mL) for 5 min. The filter, whilst in a plastic tray, was incubated with the secondary antibody (goat anti mouse immunoglobulin horse radish peroxidase conjugated, Dako, A/S Denmark). The antibody was diluted 4000 x in 32 mL of PBS-T plus 0.5% milk powder and the filter gently rocked at 20˚C for 2 h. The filter was again washed in PBS-T for 15 min followed by 2 further washes of 5 min duration. Care was taken not to let the filter dry out after the final wash.

Ultra luminal/enhancer solution (Pierce, Illinois, USA) (4 mL) was mixed with 4 mL of ultra stable peroxide solution and the filter bathed in the chemiluminescent reagents for 5 min, then gently blotted on a paper towel and wrapped in cling film. The level of M_1dG adducts in each DNA sample was determined by obtaining a chemiluminescent image of the filter, using the ChemiGenius^2 image acquisition system (Syngene, Cambridgeshire, UK). Values were corrected according to the amount of DNA bound to the filter, as determined by propidium iodide staining. The limits of detection for the method previously determined was 2.5 adducts per 10^8 nucleotides (Singh *et al*., 2001) A standard curve was constructed by plotting the optimal density versus M_1dG adduct level (fmol/µg DNA) for the standards. Corrections were made for the endogenous level of adducts in control calf thymus for each sample. Samples were analysed in triplicate. A quality control (QC) sample was included in each set of blots and the data was accepted if the QC adduct level was within 30% of the expected value.

2.2.4 Immunohistochemistry in colorectal tissue

Sections of paraffin embedded colorectal tissue were cut and mounted on slides by Angie Gillies and Linda Potter (Department of Cancer Studies and Molecular Medicine, University of Leicester).

2.2.4.1 Ki-67 and COX-2 immunohistochemistry technique

Tissue sections were deparaffinized in xylene (3 min incubation x 2) and rehydrated through graded alcohols, 99% industrial methylated spirits (IMS) (3 min x 2), and 95% IMS (3 min x 2). Slides were then washed in running water for 5 min. Antigen

retrieval was performed by microwaving the slides for 20 min on full power (750 watts), whilst immersed in 500 mL of either TE buffer pH 9.0 (Ki-67) or citric acid buffer pH 6.0 (COX-2). The slides were left to cool at room temperature for 30 min in the buffer, then rinsed for 5 min in PBS buffer. The NovoLink Polymer Detection Kit was used in accordance with the manufacturer's instructions for the remaining steps. (NovoLink Polymer Detection Kit, Vision Biosystems, Tyne and Wear, UK). Peroxidase block (3% hydrogen peroxide) was applied to the slides for 5 min, followed by two 5 min washes in PBS. Slides were incubated with protein block (0.4% casein in PBS) for 5 min and washed again in PBS (5 min x 2). Slides were incubated with 100 µL of optimally diluted primary antibody (monoclonal mouse anti human Ki-67 or COX-2). Antibodies were diluted in blocking solution (3 % bovine serum albumin and 0.1 % triton-X-100 in PBS) 1:100 or 1:50 for Ki-67 and COX-2 respectively. Slides were incubated on a covered slide tray overnight at 4ºC with monoclonal mouse anti-human Ki-67 antibody or monoclonal mouse anti-human COX-2 antibody (Dako, Cambridgeshire, UK). The TE buffer was prepared using 60.57 g of tris(hydroxymethyl)aminomethane (TRIS) and 18.6 g of ethylenediaminetetraacetic (EDTA) in 500 mL of water, pH 9.0 and citrate buffer was prepared using 2.1 g of citric acid in 1 L of water, pH 6.0).

The next morning, the slides were washed in PBS (5 min x 2) and incubated with post primary block (polymer penetration enhancer) for 30 min. The slides were washed again in PBS (5 min x 2), then incubated with NovoLink Polymer (anti-mouse/rabbit IgG poly-HRP) for 30 min and washed (PBS 5 min x 2). Peroxidase activity was developed with DAB working solution (3, 3'-diaminobenzidine) applied to the slides for 5 min. The slides were washed in tap water for 5 min and then counter stained with

0.02% haematoxylin for 30 sec, followed by a further 5 min wash in tap water. Slides were then dehydrated by immersion in 95% IMS (3 min x 2), 99 % IMS (3 min x 2) and finally xylene (3 min x 2) and mounted using DPX (dibutyl phthalate xylene). All slides were given specific identification numbers to keep the sample runs as unbiased as possible. Participants' pre and post samples were run together to avoid variability between slides.

2.2.4.2 Immunohistochemistry technique for control tissue

To confirm that tissues had been processed and stained correctly, one positive tissue control was included for each set of primary antibodies in each run. HCT cells and HCA7 human colon cancer cells, grown under standard cell culture conditions, were used as positive controls for Ki-67 and COX-2 respectively. To verify the labeling specificity of the target antigen by the primary antibody, a negative control was also included in each run and DakoCytomation Mouse IgG1 (Dako, Cambridgeshire, UK), which is recommended by Dako as a negative control for this type of immunohistochemistry, was diluted to the same mouse IgG concentration as the primary antibody.

2.2.4.3 Interpretation of the slides

Interpretation of the slides included examination of the study participants' tissue samples and the negative and positive control tissue. Ki-67 staining was assessed by counting the total number of positive and negatively stained epithelial cells. At a magnification of x 400, six adjacent representative areas of each tissue section were

analysed (microscope supplied by Leitz, Germany and digital camera supplied by Leica, Buckinghamshire, UK). The scoring of COX-2 expression in tumour epithelial cells was carried out according to the method of Remmele and Stegner (Remmele and Stegner, 1987). An estimate of the staining intensity (cytoplasmic staining) was given after evaluation of all the 6 tissue sections and as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). All results were independently reviewed by Dr K West (Consultant Gastroenterology Pathologist, University Hospitals of Leicester).

2.2.5 Statistical analysis

Statistical analyses were undertaken using SPSS for Windows (Version 16.0). A $p < 0.05$ was considered statistically significant. Assessment of normal distribution was carried out by performing histograms and the Kolmogorov-Smirnov statistic. Scatter plots and box plots were used to compare the distribution of variables. Where appropriate, results were subjected to analysis by one or more of the following; Pearson Correlation, multiple regression, paired sample T test, independent T test, one way ANOVA and 2 way ANOVA. *Post hoc* comparisons were performed by using Fisher exact test or Dunnett's test. The non parametric test, Mann Whitney U Test was performed where applicable. Advice was provided by Dr Maria Viskaduraki, Statistical Department, University of Leicester.

CHAPTER 3

STUDY PARTICIPANT DEMOGRAPHIC RESULTS

3.1 Study participant demographics

3.1.1 Healthy volunteers

3.1.1.1 Screening and recruitment

From September 2006 to October 2008, 53 volunteers were screened for the trial (26 in Leicester, UK and 27 in Michigan, USA), of which 44 were recruited and received resveratrol. Four volunteers, 3 in Michigan and 1 in Leicester, did not complete the study due to adverse events, for personal reasons or because they were found to be non compliant and therefore were replaced. In total 40 volunteers, 10 per dose level (0.5, 1.0, 2.5 and 5.0 g), completed the study, 5 recruited at each site for the first 3 dose levels, whilst for the highest dose (5.0 g) 6 were based in Michigan and 4 in Leicester. For the remainder of the results section, unless otherwise stated, only volunteers who completed the trial are included in the analyses.

3.1.1.2 Demographics

The mean age of all healthy volunteers was 37.0 years (SD 11.7, range 20–73), with the vast majority of the study population being younger than 50 (Figure 3.1.1). The mean age of study participants in Leicester was 35.7 years (SD 12.6, range 20-73) and 38.6 years (SD 10.6, range 20-58) in Michigan. There was no real difference in the age range of volunteers at each dose level (Figure 3.1.1).

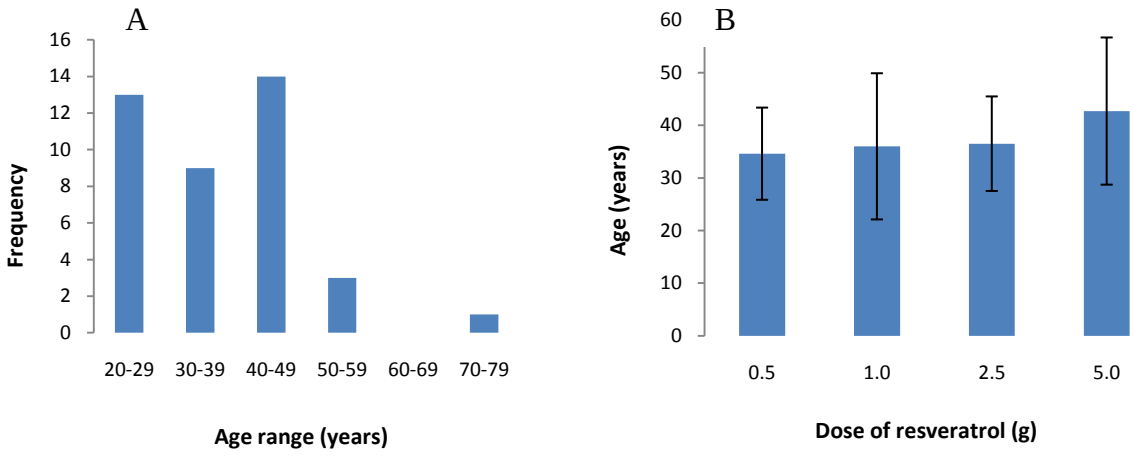


Figure 3.1.1 Age range of healthy volunteers who received 29 daily doses of resveratrol; (A) total study population (n =40) and (B) mean age of healthy volunteers at dose levels of 0.5, 1.0, 2.5 and 5.0 g (± SD). Values represent the mean of *n* = 10 for each dose level and age range (years) was 20-49 (0.5 g), 20-58 (1.0 g), 24-51 (2.5 g) and 21-73 (5.0 g).

Overall, 55% of the study participants were male, with 60, 50, 40 and 50% on the 0.5, 1.0, 2.5 and 5.0 g dose levels respectively. The ethnic origin of the study population consisted of Caucasian (65%), Asian (15%), Afro-Caribbean (12.5%) and other (biracial and Hispanic) 7.5%. The majority of participants on the 0.5, 2.5 and 5.0 g dose were Caucasian, with an equal number of Asian and Caucasian participants on the 1.0 g dose level (Figure 3.1.2). Gender and ethnic origin in regards to study site are illustrated in Table 3.1.1 and shows there was greater ethnic diversity in the Michigan site. The mean body mass index (BMI) of the total study population was 26.6 kg/m^2 (SD 6.08, range 18.6-42.4) and no real difference was seen in the mean and range of BMI across the 4 dose levels (Figure 3.1.3). Mean BMI of all Leicester study participants was 25.8 kg/m^2 (SD 4.00, range 18.61-34.10) and 27.2 kg/m^2 (SD 7.51, range 19.3-42.4) for all participants in Michigan.

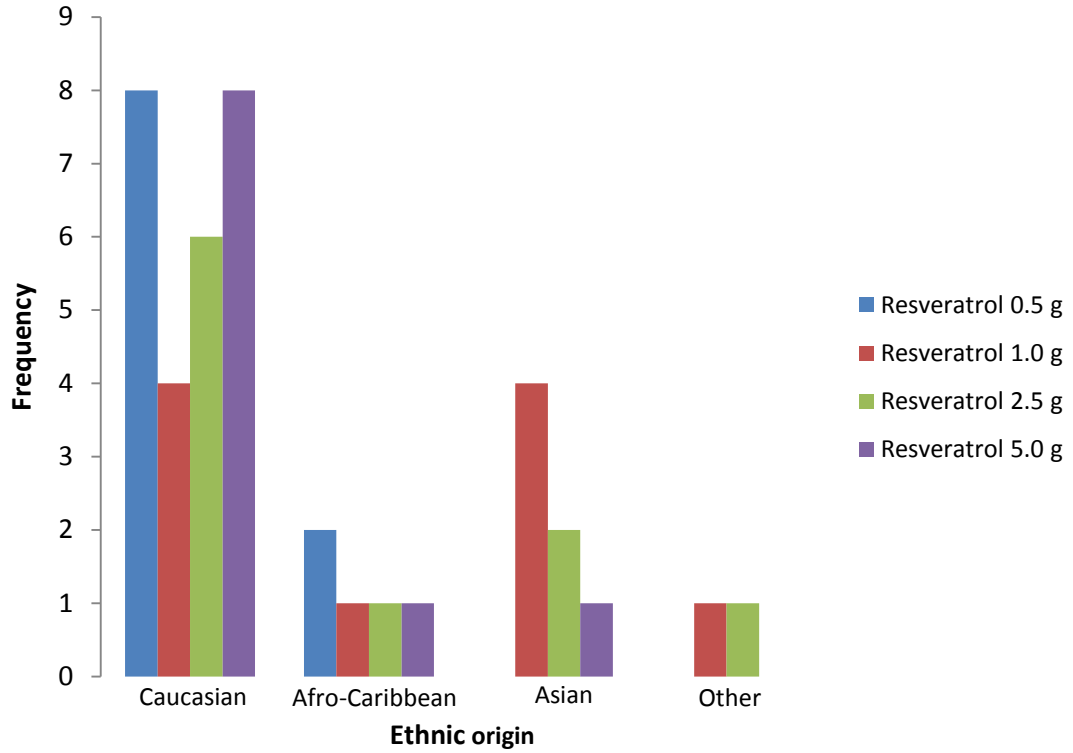


Figure 3.1.2 Ethnic origin of healthy volunteers who received 29 daily doses of resveratrol at dose levels of 0.5, 1.0, 2.5 and 5.0 g (*n*=10 per dose level).

Table 3.1.1 Gender and ethnic origin of study participants at each study site

Study Site	Males	Females	Caucasian	Asian	Afro-Caribbean	Other*
Leicester (*n*=19)	13	6	15	4	0	0
Michigan (*n*=21)	9	12	11	2	5	3

*Other refers to biracial and Hispanic ethnic origin.

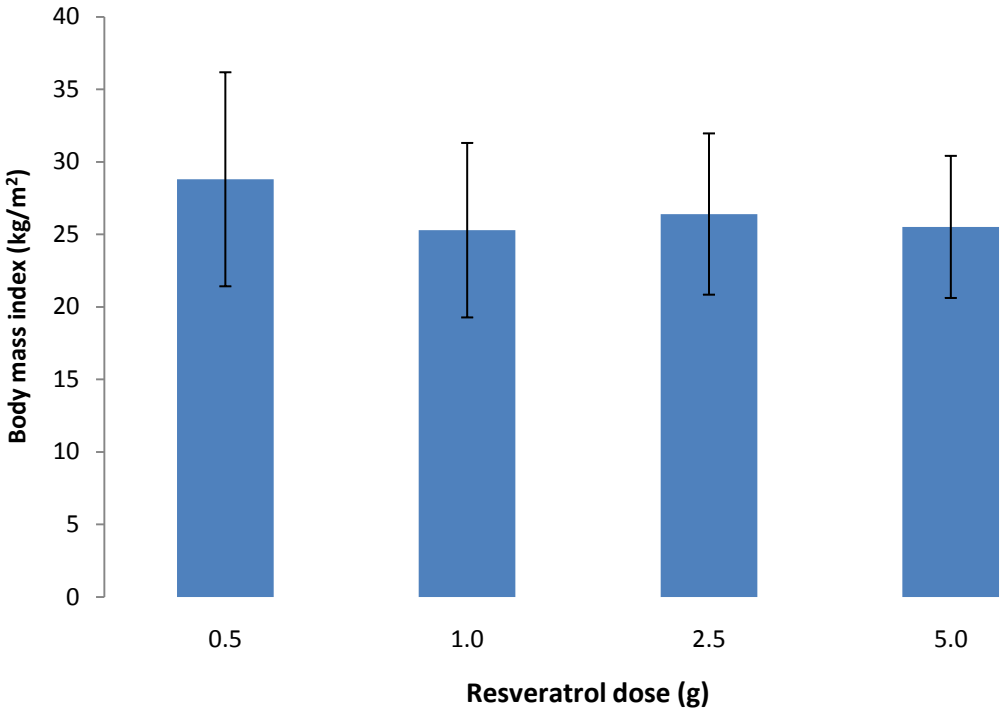


Figure 3.1.3 Mean body mass index of healthy volunteers who received 29 daily doses of resveratrol at dose levels of 0.5, 1.0, 2.5 and 5.0 g (± SD). Values are the mean of 10 volunteers for each dose level. Individual BMI (kg/m^2) values ranged from 20.0 - 42.4 (0.5 g), 18.6 - 39.4 (1.0 g), 19.3 - 39.0 (2.5 g) and 19.2 - 32.8 (5.0 g)

3.1.1.3 Medical and surgical history and concomitant medication

At the pre dosing screening visit a medical and surgical history was recorded, including the regular or occasional use of medication and vitamins as illustrated in Table 3.1.2. Participants were encouraged to avoid any unnecessary medication or supplements, where possible, for the whole study period and requested to report back if any were taken. Of the 40 volunteers, 26 reported a medical (previous clinical diagnosis) or surgical history (operations), distributed as follows; 8 participants on the 0.5 g dose level, 6 on the 1.0 g, 6 on the 2.5 g and 6 on the 5.0 g dose level. The remainder had no previous medical or surgical history. Thirteen volunteers described the use of

medication or vitamin supplements, 5 on the 0.5 g, 5 on the 1.0 g, 5 on the 2.5 g and 3 on the 5.0 g dose level with the remainder reporting no use of medication or vitamin supplements (Table 3.1.2). Smoking and alcohol intake was also documented. Eight of the volunteers were either current (4) or ex smokers (4) with a mean duration of 4.4 ± 4.2 pack years, (range 2-14) and 57% drank alcohol with an average consumption of 6.5 ± 6.1 units per week (range 0.5-21). Four of the volunteers had participated in a previous clinical study, but this was at least 12 months prior to dosing with resveratrol and 2 of these studies were observational only. All study participants were documented to have a WHO performance status of 0 (fully active).

Table 3.1.2 Medical and surgical history of healthy volunteers, including use of medication and/or vitamin supplements (occasional or regular use) prior to dosing with resveratrol.

Medical /surgical history	Number of volunteers
Abdominal hernia repair	2
Acne	1
Asthma	4
Breast fibroids	1
Coeliac sprue	1
C section	1
Dermatitis	1
Gastro oesophageal reflux	1
Lipomas	2
Haemorrhoidectomy	1
Keloid scars	2
Kidney stones	1
Knee operation	1
Migraine	2
Osteoarthritis	2
Rosacea	1
Vasectomy	2
White coat hypertension	1
Medication and supplements (occasional use)	
Aspirin	1
Antihistamine	2
Multivitamins	2
Naproxen	2
Salbutamol inhaler	2
Sinus Hyaluronan	1
Cod liver oil	1
Medication (regular use)	
Contraceptive hormone	2

Medical/surgical history reported by 26 participants and use of medication or vitamin supplements reported by 13 participants.

Of the 40 participants who completed the study, 5 (on the first three dose levels) reported occasional consumption of medication, whilst dosing with resveratrol as shown in Table 3.1.3. In addition, 2 volunteers on dose levels 0.5 and 2.5 g were taking hormone contraception throughout the study period.

Table 3.1.3 Concomitant medication taken by healthy volunteers whilst participating in the study

Dose level (g)	Medication/vitamin supplement	Number of days	Reason for use
0.5	Penicillin	4	Sore throat
	Ibuprofen	4	Headache
1.0	Frovatriptan	2	Headache
	Paracetamol	2	Headache
	Cough syrup	2	Sore throat
2.5	Paracetamol	1	Cramp
	Cephalexin	7	Cellulitis
	Fentanyl	1	Colonoscopy
	Loperamide	2	Diarrhoea
5.0	None		

Medication and vitamin use reported by 5 participants

3.1.1.4 Compliance and tolerability

In total, 40 participants completed the study and all were over 95% compliant with dosing, as judged by interviews, caplet counting and diary sheet, and therefore their samples have been included in the analyses. All participants took the required dose of resveratrol between 7 and 9 am with food and drink if required. Three volunteers in

Michigan received resveratrol (1.0 g), but did not complete the required 29 daily doses. One volunteer stopped dosing due to the death of a sibling, a second developed one episode of diarrhoea (mild) and decided not to continue, although it was felt on discussion between the participant and the study team that this was related to food intake. A third participant was advised to stop dosing by the study team after the appearance of red discoloration of the skin, noticed after the first dose, which reoccurred after the second dose. The participant was physically well and the rash was graded as mild in severity, but it was felt by the study team that an allergic reaction to resveratrol could not be ruled out. Although this event occurred in the USA, the UK regulatory bodies, the local Ethics Committee, MHRA and University of Leicester Research and Development Department were all informed. In Leicester, 1 volunteer who reported to be compliant (5.0 g) had no levels of resveratrol or any of its metabolites in the samples collected throughout the study period (plasma, urine and stools), when analysed by UV-HPLC. All 4 study participants were replaced and only data regarding AE events have been included in the analyses.

Resveratrol was found to be safe with doses of up to 5.0 g taken orally for 29 days. No SAE were reported at either site during the study, which consisted of the dosing period plus a subsequent two week follow up, and only 1 participant had to discontinue because of possible side effects, as mentioned above (skin rash, 1.0 g dose level). Of the 44 volunteers who received resveratrol, including those who were dosed but did not complete the study, 31 (12 Leicester and 19 Michigan) reported one or more AE, whilst on the study agent (Table 3.1.4). An AE is any unfavourable and unintended sign, symptom or disease temporally associated with the use of a medical treatment or procedure that may or may not be considered related to this (Section 2.1.13). The

National Cancer Institute CTCAE version 3.0 is a descriptive terminology utilized for AE reporting, using a grading (severity) scale provided for each AE term. Attribution of the AE to resveratrol was determined by the site study team after obtaining a detailed description of the symptoms, duration and other factors, such as medication or food intake that could have contributed to the symptoms described.

Table 3.1.4 Number of volunteers who reported adverse events during the study period at each study site. The total number of participants included in the analysis for AE reporting (including those who did not complete the study) includes 10 on the 0.5 g, 13 on the 1.0 g, 10 on the 2.5 g and 11 on the 5.0 g dose levels.

Dose level	Leicester	Michigan	Total
0.5 g	3	4	7
1.0 g	1	4	5
2.5 g	4	5	9
5.0 g	4	6	10

On the top two dose levels (2.5 and 5.0 g), 90% of volunteers reported one or more AE, which the study team felt were probably resveratrol related. The majority of these were gastrointestinal symptoms, including nausea, flatulence, abdominal discomfort and diarrhoea. All were graded as mild in severity (grade 1) apart from 4 participants (1 on the 2.5 g and 3 on the 5.0 g dose levels), who had symptoms of nausea and/or diarrhoea, graded as moderate in severity (grade 2). Only 1 participant (2.5 g), required intervention for the symptoms with 2 doses of loperamide being sufficient to control the diarrhoea.

Most of the gastrointestinal side effects started within a few days of commencing resveratrol and occurred 30-60 min after the morning dose. The symptoms improved throughout the day, but returned the following morning. Participants found that taking resveratrol after breakfast rather than on an empty stomach improved their symptoms. All the gastrointestinal symptoms resolved within 2 days of completing the 29 day course with no weight loss recorded in any participant (measured at baseline screening visit, PK visit and on day 29). All volunteers felt well throughout the study period.

Over the 4 dose levels, as well as gastrointestinal, a range of other symptoms were reported and all are shown in Table 3.1.5. These included urinary symptoms (2), worsening of acne (1) and fatigue (4). The 2 male participants who reported urinary symptoms intermittently throughout dosing (dark urine and /or cystitis) mentioned that the symptoms seemed to be worse in the morning after ingesting resveratrol, then improved throughout the day. Both had urine dipsticks performed, mid stream urine samples sent to microbiology and an extra blood test to monitor urea and creatinine. The results of these tests were all within the normal range and all symptoms resolved within 2 days of completing dosing. The participants had not experienced these symptoms prior to recruitment to the study.

Study participants had a panel of haematological and biochemical indices taken at baseline (screening) and day 29 visits. Apart from 2 participants, screening blood tests performed were all in the normal range. One participant had a raised cholesterol and another a low neutrophil count at screening, both were grade 1 in severity and both participants were recruited to the study after agreement from the site Principal

Investigator. Out of the 40 volunteers who completed the study, 36 had blood values outside the normal range at the day 29 visit (Table 3.1.6). All these AE were graded as mild in severity except for 3 laboratory abnormalities, which were all grade 2; a raised cholesterol and low neutrophil count, which were abnormal at screening as described above and low haemoglobin. For the majority of the study participants, the blood indices documented as out of normal range were felt not to be related to the study agent, but to other factors, such as fasting as part of the study protocol, blood draw and normal variation over time. Three participants (0.5 and 1.0 g dose levels) had raised bilirubin concentrations on day 29, reported by the study team as possibly related to the study agent.

Table 3.1.5 Adverse events (symptoms) reported by healthy volunteers during the study. The National Cancer Institute CTCAE version 3.0 was utilized for Adverse Event reporting.

Dose level	National Cancer Institute CTCAE Term (version 3.0)	Number of study participants	Severity (grade)*	Attribution to resveratrol**
0.5 g	Fatigue	3	1	-
	Headache	2	1	-
	Hypertension	1	2	-
	Viral throat infection	2	1	-
	Viral throat infection	1	2	-
1.0 g	Backache	1	1	-
	Diarrhoea	1	1	-
	Headache	1	2	-
	Nausea and vomiting	2	1	-
	Red discoloration of the skin	1	1	++
	Cystitis	1	1	+
2.5 g	Abdominal pain	4	1	+
	Acne	1	1	+
	Cellulitis	1	1	-
	Cramp	1	1	+
	Diarrhoea	1	1	-
	Diarrhoea	1	1	+
	Diarrhoea	1	2	+
	Discomfort on passing faeces	1	1	+
	Eczema	1	1	-
	Fatigue	1	1	+
	Flatulence	1	1	+
	Hunger feelings	1	1	+
	Light-headedness	1	1	-
	Mucositis (oral cavity)	1	1	-
	Nausea	1	1	-
	Nausea	2	1	+
	Pruritis	1	1	+
5.0 g	Abdominal pain	3	1	+
	Chest pain	1	1	+
	Diarrhoea	4	1	+
	Diarrhoea	2	2	+
	Diarrhoea	1	2	+
	Dizziness	1	1	+
	Dry mouth	1	1	+
	Flatulence	2	1	+
	Nausea	3	1	+
	Red, puffy and itchy eyes	1	1	+
	Urine colour change (dark)	1	1	+

A grading (severity) scale which is provided for each AE term. *Severity- 1 mild, 2 moderate, 3 severe, 4 life threatening, 5 fatal.
**Attribution of the AE to resveratrol was determined by the site study team – unrelated/ unlikely, + possible/probable, ++ definite

Table 3.1.6 Adverse events of haematological and biochemical blood indices in healthy volunteers after 29 daily doses of resveratrol.

Severity Grade of adverse event	Nature of event	No. of individuals with blood indices outside the normal range			
		Dose level			
CTCAE Version 3*		**0.5 g n=8**	**1.0 g n=10**	**2.5 g n=9**	**5.0 g n=9**
Grade 1	Raised alanine aminotransferase	2	1	0	1
	Raised triglycerides	0	1	1	1
	Low prothrombin	1	0	0	0
	Raised cholesterol	3	2	2	2
	Raised uric acid	1	0	0	0
	Raised urea	0	1	0	1
	Raised total protein	0	1	1	0
	Raised aspartate transaminase	1	0	1	1
	Raised chloride	0	1	3	3
	Raised potassium	0	0	0	1
	Low glucose	1	0	0	0
	Raised glucose	1	0	0	1
	Low inorganic phosphate	0	0	1	0
	Raised lactate dehydrogenase	0	3	0	0
	Low lactate dehydrogenase	0	0	1	0
	Low white cell count	2	0	2	1
	Low alkaline phosphatase	1	0	0	0
	Raised eosinophil count	1	1	1	2
	Low eosinophil count	0	1	1	0
	Raised total bilirubin**	1	1	0	0
	Raised conjugated bilirubin**	0	2	0	0
	Raised unconjugated bilirubin**	1	1	0	0
	Raised basophil	0	0	1	0
	Low basophil count	0	2	1	0
	Low haematocrit	2	3	3	2
	Low haemoglobin	0	2	2	1
	Low red blood count	2	3	1	4
	Low lymphocyte count	0	1	0	0
	Raised monocyte count	0	2	1	0
	Raised APTT (Activated Partial Thromboplastin Time)	1	0	0	0
	Low APTT	0	1	0	0
Grade 2	Raised cholesterol[+]	0	0	0	1
	Low haemoglobin	0	0	0	1
	Low neutrophil count[+]	1	0	0	0

*National Cancer Institute Common Toxicity Criteria version 3
A grading (severity) scale which is provided for each AE term.
Severity grade - 1 mild, 2 moderate, 3 severe, 4 life threatening, 5 fatal. (left hand column)
Attribution of the AE to resveratrol was determined by the site study team, no star (unrelated/ unlikely), **possible/probable, ***definite.
[+]Grade 1 at screening visit.

3.1.2 Colorectal cancer patients

3.1.2.1 Screening and recruitment

From January 2007 to October 2008, 21 patients with a histological diagnosis of colorectal adenocarcinoma, amenable to surgical resection were recruited and dosed on Stage 2 of the study. Patients were recruited from the clinics of 7 colorectal surgeons based at the University Hospitals of Leicester. After receiving the required 8 daily doses of resveratrol, 1 patient had her operation date postponed by 2 weeks and was replaced in the study. Five of the patients who completed dosing at Stage 2 had also been recruited at Stage 1 and had biopsies at the time of diagnostic endoscopy from normal and malignant colorectal tissue.

3.1.2.2 Demographics

Of the 20 patients who completed the study, 9 were males and 11 females with a mean age of 66.8 years (SD 17.2, range 46-83). On the 0.5 and 1.0 g dose levels, there were 20% and 70% of males respectively. The mean BMI of all patients was 25.3 kg/m^2 (SD 2.68, range 17.4-29.2) and the majority were Caucasian (90%) with the remainder of Asian ethnicity. A summary of the patient demographics is shown in Table 3.1.7.

Table 3.1.7 Colorectal cancer patient demographics.

	Patient characteristics (*n*= 20)	**Resveratrol dose level**	
		0.5 g (*n*=10)	**1.0 g (*n*= 10)**
Mean age, years (± SD)	66.8 (17.2)	70.4 (8.1)	63.7 (22.6)
Age range, years	46-83	60-80	46-83
Males	9	2	7
Females	11	8	3
Caucasian	18	9	9
Asian	2	1	1
Mean BMI, kg/m^2 (± SD)	25.3 (2.68)	24.2 (3.2)	26.3 (1.5)
BMI range, kg/m^2	17.4-29.2	17.4-29.2	22.8-27.6

3.1.2.3 Medical and surgical history and concomitant medication

At the screening visit (pre dosing), a medical and surgical history was obtained which included a list of medication as shown in Tables 3.1.8 and 3.1.9. The majority of patients reported a history of a medical condition or operation (95%) and the use of one or more medications (70%). Smoking and alcohol intake were also documented; 30% were ex smokers with an average duration of 0.6 ± 15.6 pack years, (range 8-51), with none of the patients currently smoking. Forty five percent drank alcohol, reporting an average intake of 11.4 ± 7.2 units/week (range 2-25). None of the study participants had been a volunteer or patient in a previous clinical study and all were documented to have a WHO performance status of 0.

Two patients had previously been diagnosed with cancer; one had a surgical resection for a rectal adenocarcinoma 16 years prior to recruitment and the other, a local surgical excision for a basal cell carcinoma of the skin 20 years previously. Neither had

received chemotherapy or radiotherapy. One patient had been diagnosed with idiopathic thrombocytopenic purpura 3 months before starting dosing with resveratrol and was under supervision by the Haematology Department. This participant was taking a daily low dose of prednisolone (10mg a day) and continued on the steroids throughout dosing with resveratrol. The inclusion of this individual in the study was approved by the study Principle Investigator. None of the patients received neoadjuvant radiotherapy or chemotherapy prior to surgery.

All patients continued their current medication up to surgical resection. Aspirin was the only medication discontinued 8-10 days prior to the operation on recommendation of the surgical team as standard care to reduce the risks of operative bleeding. One patient was found to have raised blood pressure at the pre operative screening visit and after being rechecked by the GP, an antihypertensive was prescribed which commenced whilst dosing with resveratrol. No other participants were required to commence any new medication.

Table 3.1.8 Medical and surgical history of colorectal cancer patients

Medical history	Both dose levels (*n*=19)	Resveratrol dose	
		0.5 g (*n*=10)	1.0 (*n*=9)
Hypertension	4	1	3
Diabetes	2	1	1
Ischaemic heart disease	3	0	3
Osteoarthritis	2	1	1
Asthma	1	1	0
Hypothyroidism	1	1	0
Sarcoidosis	1	0	1
Sickle cell trait	1	0	1
Idiopathic thrombocytopenic purpura	1	0	1
Previous abdominal surgery	6	3	3
History of cancer	2	2	0
Anxiety/depression	2	2	0
Irritable bowel syndrome	2	1	1

Table 3.1.9 Current medication taken by colorectal cancer patients

Medication	Both dose levels (n=14)	Resveratrol dose	
		0.5 g (*n*=7)	1.0 g (*n*=7)
Insulin	1	1	0
Anti hypertensive	7	4	3
Inhaler	1	1	0
HRT	1	1	0
Diuretics	2	0	2
Ferrous sulfate	1	0	1
Aspirin	2	0	2
Thyroxine	1	0	1
Statin	3	0	3
Isosorbide mononitrate	1	0	1
Proton pump inhibitor	2	0	2
Anti spasmodic	2	1	1
Steroid	1	0	1
No medication	6	3	3

3.1.2.4 Colorectal cancer histology

One of the 20 patients who completed the study had 2 colorectal adenocarcinomas identified at diagnostic colonoscopy, one located in the caecum and the other in the sigmoid colon. Both tumours were resected at the same operation with a right hemicolectomy and sigmoid colectomy respectively and both were reported as Dukes A adenocarcinomas on the histopathology report. All other study patients had only 1 colorectal tumour.

Of the 21 adenocarcinomas 10 were located in the sigmoid and 6 in the caecum, with the other tumours in the hepatic flexure/transverse colon (2) or recto-sigmoid (3). Eight of the resections were performed as a laparoscopic procedure with the remaining 13 carried out as a laparotomy. The histology of the resected specimens was reported as moderately (19) or poorly (2) differentiated with the majority classified as Dukes C1 (9) or B (7) with the remaining 5 as Dukes A. The range of colorectal cancer characteristics, such as tumour location and histology were reasonably similar on each dose level as shown in Table 3.1.10, and Figure 3.1.4 shows a diagram of the tumour locations. For all patients the mean time from diagnostic biopsy (colonoscopy) to colorectal resection was 9.5 ± 5.1 weeks (range 4.5-21). The mean time was 8.1 ± 4.3 weeks (range 4.5-19) and 10.8 ± 5.5 weeks (range 5-21) for the 0.5 and 1.0 g respectively. Three patients (one on the 0.5 and 2 on the 1.0 g dose level) required a cardiovascular opinion prior to having surgery, therefore time from diagnostic biopsy to surgical resection was delayed by a few months. If these particular patients are not included in the overall mean, the mean time from biopsy to resection is reduced to 7.3 ± 2.4 weeks (range 4.5-11) and for the 0.5 and 1.0 g dose levels, 6.9 ± 2.2 weeks (range 5-11) and 7.8 ± 2.5 weeks (range 5-11), respectively.

Table 3.1.10 Colorectal cancer characteristics (histology and tumour location) and type of operation.

	Tumour characteristics	All tumours (*n*=21)*	Resveratrol dose	
			0.5 g (*n*=10)	1.0 g (*n*=11)*
Tumour location	Caecum	6	2	4
	Hepatic flexure /transverse colon	2	1	1
	Sigmoid	10	5	5
	Recto-sigmoid	3	2	1
Operation	Laparoscopy	8	3	5
	Laparotomy	13	7	6
Resection	Right hemicolectomy	8	3	5
	Left colectomy/sigmoid colectomy	2	0	2
	Anterior resection	11	7	4
Histology	Moderate differentiation	19	8	11
	Poor differentiation	2	2	0
	Lymphocytic invasion	4	3	1
	Extra vascular invasion	5	3	2
	Excision complete (R0)	20	10	10
	Incomplete excision (R1)	1	0	1
Dukes staging	A	5	3	2
	B	7	2	5
	C1	9	4	5

*In one patient two colorectal adenocarcinomas were identified in the colorectal resection specimen.

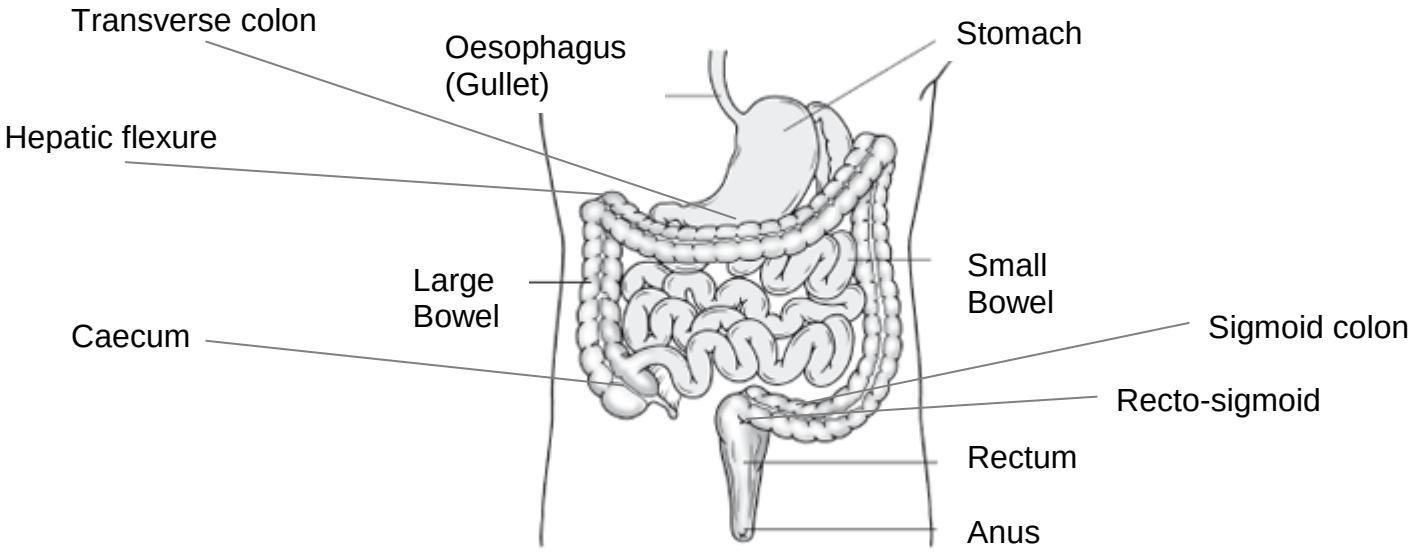


Figure 3.1.4 Location of colorectal tumours resected in the study participants

3.1.2.5 Compliance and tolerability (Stage 2 of the study)

All participants who completed the study took the required 8 daily doses of resveratrol between 5 and 10 pm, at a similar time each day with food and drink if required, but flexibility was given for two reasons. Firstly, many patients were taking other medication in the evening and to minimize the potential effects of this on the absorption or metabolism of resveratrol, were asked to take the resveratrol on its own and at least 2 h before or after any other medication. Secondly, this enabled them to fit the dosing into their daily routine and bed time, when most convenient for them, thus aiding compliance. On the evening prior to surgery patients took their last dose, which was observed by the study team on the ward, close to the time they had been dosing at home. The average time the last dose was taken was 18.36 ± 70 min (range 17.00–20.30).

Apart from one participant already mentioned who had her surgery delayed by 2 weeks and was therefore replaced, all had their operation as planned the following day, 60% in morning sessions with the remainder during the afternoon.

Of the 21 patients on the study, including the patient who did not complete, 4 on the 0.5 g dose level reported symptoms. A female developed a urinary tract infection on day 8 of dosing (grade 1) and 2 other patients developed a headache on day 2 (grade 1). As mentioned previously, the participant noted to be hypertensive (grade 2) prior to dosing with resveratrol was monitored closely by their GP. All AE were documented by the study team as unrelated to resveratrol.

From the blood drawn on either day 3 or day 8 of the study period, 95% of patients were reported to have one or more haematological and biochemical indices out of the normal range as shown in Table 3.1.11. Only one patient, on the 1.0 g dose, had no blood values outside the normal range. The majority of blood abnormalities were felt not to be related to resveratrol and more likely related to chronic bleeding from the bowel tumour or nutritional status prior to surgery. To prepare the bowel for surgery, most patients were advised by their surgical teams to avoid fibrous foods and green leafy vegetables for a few days prior to the procedure. A strict liquid only diet (broth and tea) was then followed for 48 h pre surgery. This change in fluid and nutritional intake could have caused the observed change in blood indices such as serum glucose, urea, creatinine and albumin. In addition, all patients with raised glucose concentrations were known to be diabetic pre dosing. One patient (0.5 g) had a raised bilirubin concentration on day 8, when the rest of the liver function tests were normal, documented as possibly related to resveratrol intake by the study team.

Table 3.1.11 Adverse events of haematological and biochemical blood indices in colorectal cancer patients on day 3 and day 8 of dosing.

Severity Grade of adverse event	Nature of events	Number of individuals with blood indices outside the normal range	
		Dose level	
CTCAE version 3*	**Unlikely to be drug related**	**0.5 g *n*=10**	**1.0 g *n*=10[+]**
Grade 1	Low sodium	2	0
	Raised platelet count	2	1
	Low platelet count	1	0
	Raised glucose	2	1
	Raised alkaline phosphatase	1	0
	Raised inorganic phosphate	3	1
	Raised eosinophil count	1	0
	Low eosinophil count	0	1
	High urea	4	2
	Low urea	0	1
	Low creatinine	2	1
	Low haematocrit	5	7
	Low red blood count	0	5
	Low lymphocyte count	2	1
	Low basophil count	1	0
	Low prothrombin	1	0
	Raised prothrombin	1	1
	Raised potassium	1	0
	Low haemoglobin	2	5
	Low potassium	0	1
	Raised neutrophil count	1	2
	Raised white cell count	0	1
	Raised monocyte count	1	0
	Low basophil count	0	1
	Raised bilirubin**	1	0
Grade 2	Low haemoglobin	2	0
	Raised glucose	0	1
	Low glucose	1	0
	Raised bilirubin	0	1
	Low albumin	1	0
Grade 3	Low sodium	1	0
	Low potassium	1	0
	Low haemoglobin	0	1

*National Cancer Institute Common Toxicity Criteria version 3
A grading (severity) scale which is provided for each AE term.
Severity grade - 1 mild, 2 moderate, 3 severe, 4 life threatening, 5 fatal. (left hand column)
Attribution of the adverse event to resveratrol was determined by the site study team, no star (unrelated/ unlikely), **possible/probable, ***definite.
[+] One of the 10 patients on this dose level had no blood abnormalities.

CHAPTER 4

PHARMACOKINETIC RESULTS

4.1 Analytical methods and data analysis of resveratrol and its metabolites

Pharmacokinetic blood, urine and faecal samples were analysed using a validated UV-HPLC method previously established in the Department of Cancer Studies and Molecular Medicine, University of Leicester (Boocock *et al*., 2005). This assay has been determined to have a limit of detection for resveratrol of 2.0 ng/mL and a limit of quantification of 5 ng/mL in plasma and urine (Boocock *et al*., 2005). The peak corresponding to parent resveratrol was identified based on the retention time of an authentic standard run at the start and end of each batch of samples. Individual resveratrol metabolite peaks were assigned according to the previous characterization performed by Boocock *et al*., using the same chromatographic system, but with LC-MS/MS detection. As shown by Boocock *et al*., administration of a single dose of resveratrol to humans results in a characteristic profile of metabolites in the plasma, urine and faeces with five conjugated metabolites. The metabolites were characterized as two monosulfates, one disulfate and two monoglucuronides.

Since a similar pattern was observed in the present study, it was possible to putatively assign the metabolite peaks depending on their retention time and relative order of elution. Towards the end of the study, a synthetic standard for resveratrol-3-sulfate (the most abundant metabolite in plasma) became available and therefore it was possible to confirm assignment of this metabolite by co-elution. Quantification of resveratrol was achieved using standard curves spanning the range 0-10,000 ng/mL. Neither resveratrol nor any of its metabolites analysed were found to degrade under the conditions of the assay for at least 48 h post-sample preparation (protected from light at 4º C to mimic the auto sampler conditions) as determined by Boocock *et al*., (Boocock *et al*., 2005).

Resveratrol is not an endogenous compound, but may be ingested and absorbed from a variety of foods. Although there was a 5 day restriction of known dietary sources of resveratrol, there is a possibility of non compliance if resveratrol containing foods were ingested unknowingly. The concentrations of resveratrol and its metabolites in plasma, urine and stools were not adjusted for measurable pre dosing (day 1) concentrations because there were at least 21 days between pre dosing (day 1) and the PK sampling day.

Non compartmental PK parameters for resveratrol and its five metabolites in plasma and urine were calculated using Winnonlin 5.2 software (Pharsight Products). Parameters determined include the area under the plasma concentration versus time curve (AUC by the trapezoidal method), maximal plasma concentration (C_{max}) and time of maximal plasma concentration (T_{max}). Other parameters calculated were; average concentration over the total collection period ($C_{av} = AUC_{0-24}/24$ h), apparent elimination half-life ($\ln 2/k_{el}$), where k_{el} is apparent elimination rate constant, apparent total clearance (CL/F = dose/AUC_{inf}), apparent renal clearance of resveratrol (CL_R; approximated by Ae_{0-24}/AUC_{0-24}, where Ae_{0-24} is estimated total amount excreted in urine over the total collection period), and apparent volume of distribution of resveratrol [V/F = dose/(k_{el} x AUC_{inf})].

4.1.1 Healthy volunteers

Plasma, urine and faeces were obtained from healthy volunteers and analysed for the presence of resveratrol and its metabolites by UV-HPLC. Overall, samples from 40 volunteers comprising 10 participants on each resveratrol dose level (0.5, 1.0, 2.5 and 5.0 g) were assayed. PK plasma and urine samples were collected after at least 21 daily doses of resveratrol. Sampling times for plasma were 0, 15 min and 1, 1.5, 5, 12 and 24 h post oral administration. Urine was collected in batches at 0, 0-2, 2-4, 4-8, 8-12 and 12-24 h post administration. One stool sample was collected within 3 days of starting dosing with resveratrol (before day 1) and then up to 3 samples were collected whilst dosing on the study.

In this chapter, where appropriate, the PK results from the healthy volunteers have been compared to the published single dose study of resveratrol (Boocock *et al*., 2007), which was used as a basis for the design of the present repeat dose study. Both were conducted jointly by the Leicester (UK) and Michigan (USA) study teams, with all sample analysis carried out in the Department of Cancer Studies and Molecular Medicine, University of Leicester (UK). The method of recruitment, source and formulation of resveratrol and PK sample analysis, in terms of validated method and analytical system used, were the same for both studies. In the single dose study, 40 healthy volunteers were recruited to take a single oral dose of resveratrol (0.5, 1.0, 2.5 and 5.0 g) with 10 participants per dose level. Blood and urine samples were collected pre dosing and then at intervals for up to 24 h post administration. Plasma collection time points were 0, 10, 20, 30, 40 min and 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h post administration. The urine was collected in batches at exactly the same time points as for the repeat dose study. Typically 3 stool samples were collected for the single dose

study, 1 pre dosing and 2 post administration of resveratrol. Participants on both studies were asked to avoid resveratrol containing foodstuffs for 5 days and to arrive fasted from midnight on the morning of the PK day.

4.1.1.1 Plasma pharmacokinetics

Resveratrol and metabolites were identified in the peripheral plasma samples of trial participants on the 0.5, 1.0, 2.5 and 5.0 g dose levels. To illustrate the typical results obtained, Figure 4.1.1 shows a series of chromatograms from the analysis of plasma samples taken during the study period from one participant who received 2.5 g of resveratrol each day. The retention time for resveratrol was 17.2 min, with the metabolites eluting prior to the parent compound at ~14.5 (resveratrol-3-sulfate), 8.8 (resveratrol monoglucuronide 1), 11.5 (resveratrol monoglucuronide 2), 9.6 (resveratrol disulfate) and 13.5 min (resveratrol-4´-sulfate). Post administration of resveratrol, during the PK day, every participant in the study had detectable levels of resveratrol and 4 of its metabolites (resveratrol-3-sulfate, disulfate, monoglucuronide 1 and monoglucuronide 2) in the majority of their plasma samples. In contrast, for resveratrol-4´-sulfate, several participants (7 on the 0.5 and 1 on the 2.5 g dose levels) had either no detectable levels or levels which were below the limit of quantification in all of their plasma samples taken.

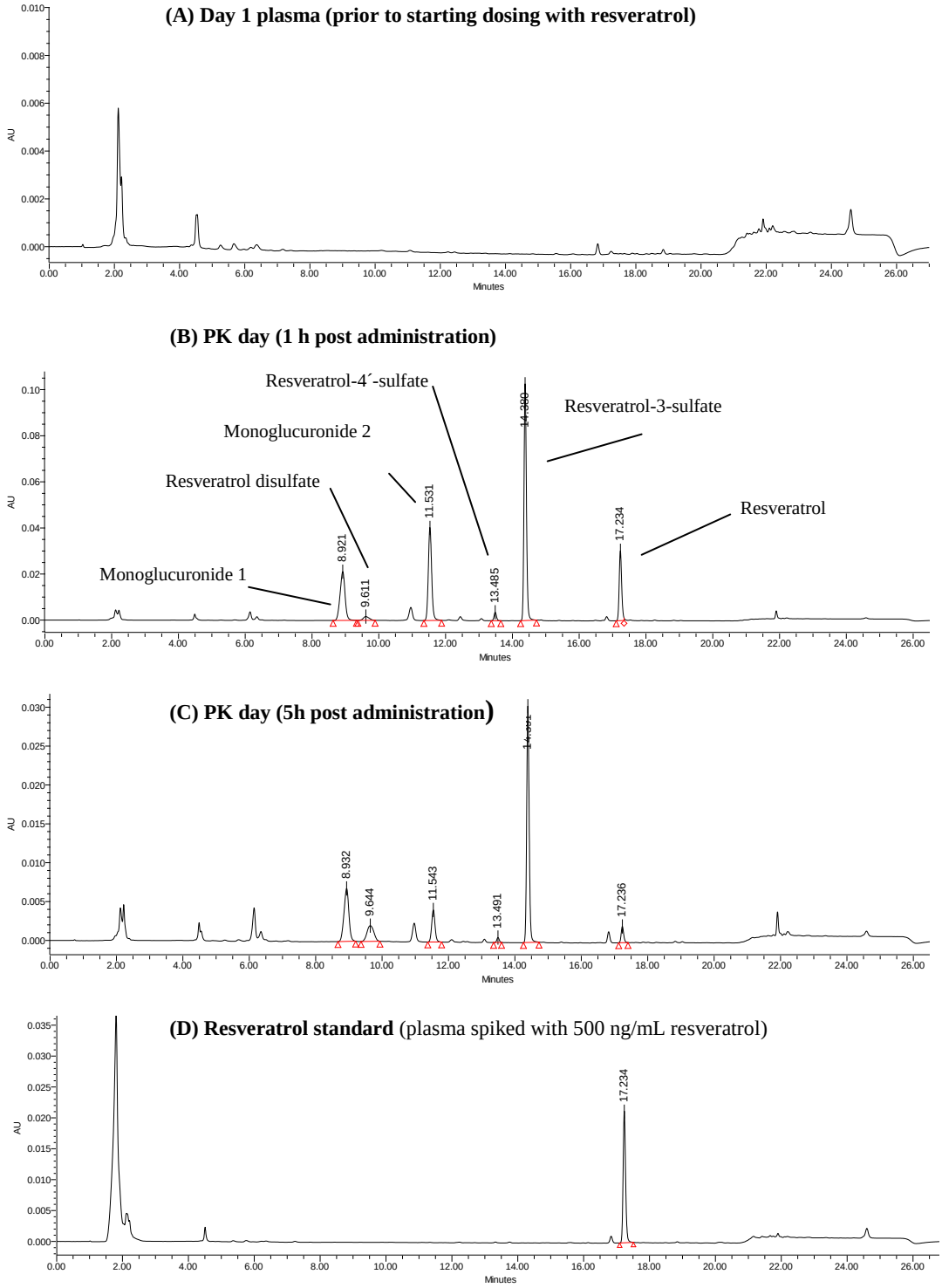


Figure 4.1.1 Representative HPLC chromatograms of resveratrol and its metabolites in peripheral plasma samples obtained from a healthy volunteer. The HPLC traces represent (**A**) plasma collected pre dosing (day 1) and then following at least 21 oral doses, (**B**) 1 h and (**C**) 5 h after administration of 2.5 g resveratrol. Identification of resveratrol and metabolites was based on retention times of standards (**D**) analysed under identical conditions as described in Section 2.2.1.1. Retention times of the major peaks are shown (min).

Figure 4.1.2 shows the mean plasma concentration versus time curves for resveratrol and its 5 metabolites, and PK variables derived from these plots are summarized in Tables 4.1.1- 4.1.5. The PK variables reported in the equivalent single dose study (Boocock *et al*., 2007) are also shown in these Tables, whilst a comparison of the 2 studies is presented in Section 4.1.1.3. After repeat oral dosing with resveratrol, there seems to be rapid absorption across all four dose levels, yielding peak plasma resveratrol concentrations (C_{max}) between 0.88 and 3.43 h post ingestion (Figure 4.1.2). The mean average (C_{av}) and peak plasma concentrations (C_{max}) across the dose levels ranged from 7.64 to 173.4 ng/mL (0.03-0.76 μM) and from 44.68 to 954.2 ng/mL (0.20-4.20 μM) respectively. The corresponding concentrations of the main resveratrol conjugates (resveratrol-3-sulfate, monoglucuronide 1 and monoglucuronide 2) exceeded those of their progenitor molecule by between 9.3-19.5 times for plasma C_{av} and between 4.4-12.6-fold for plasma C_{max}. Of the metabolites, resveratrol-3-sulfate displayed the highest C_{av} and C_{max} concentrations with ranges of 149.0 to 1607 ng/mL (0.48-5.20 μM) and 562.2 to 4154 ng/mL (1.80-13.50 μM) respectively across the 4 dose levels. Resveratrol-4´-sulfate had the lowest peak and mean average plasma concentration of all the compounds, ranging from 1.65 to 35.67 ng/mL (C_{av}) and 4.57 to 108.3 ng/mL (C_{max}). These values were up to 4.9-fold (C_{av}) and 9.8-fold lower (C_{max}) than the parent molecule.

The plasma half lives of the three main resveratrol conjugates ranged from 3.93 to 8.88 h for resveratrol-3-sulfate and 3.90 to 8.52 h for the monoglucuronides, in comparison, the mean half life of resveratrol was 6.14-18.01 h (Tables 4.1.1- 4.1.5). The values for the mean area under the plasma concentration versus time curve to time infinity (AUC_{inf}) for resveratrol was 225.6 ng h/mL at the lowest dose and 7304 ng h/mL at the

highest. The AUC_{inf} for resveratrol-3-sulfate and the glucuronides ranged from 3644 to 54,280 ng h/mL and 986.9 to 42,309 ng h/mL respectively (Tables 4.1.1-4.1.5). The mean apparent whole body clearance (2007-2755 L/h) and mean volume of distribution (17,568-31,205 L) of parent resveratrol were consistent with rapid metabolism of resveratrol into its metabolites and subsequent low bioavailability. The PK parameters for resveratrol and its metabolites presented in Tables 4.1.1-4.1.5 show that there was reasonably high inter-individual variability which is reflected by the coefficient of variance being generally greater than 40%.

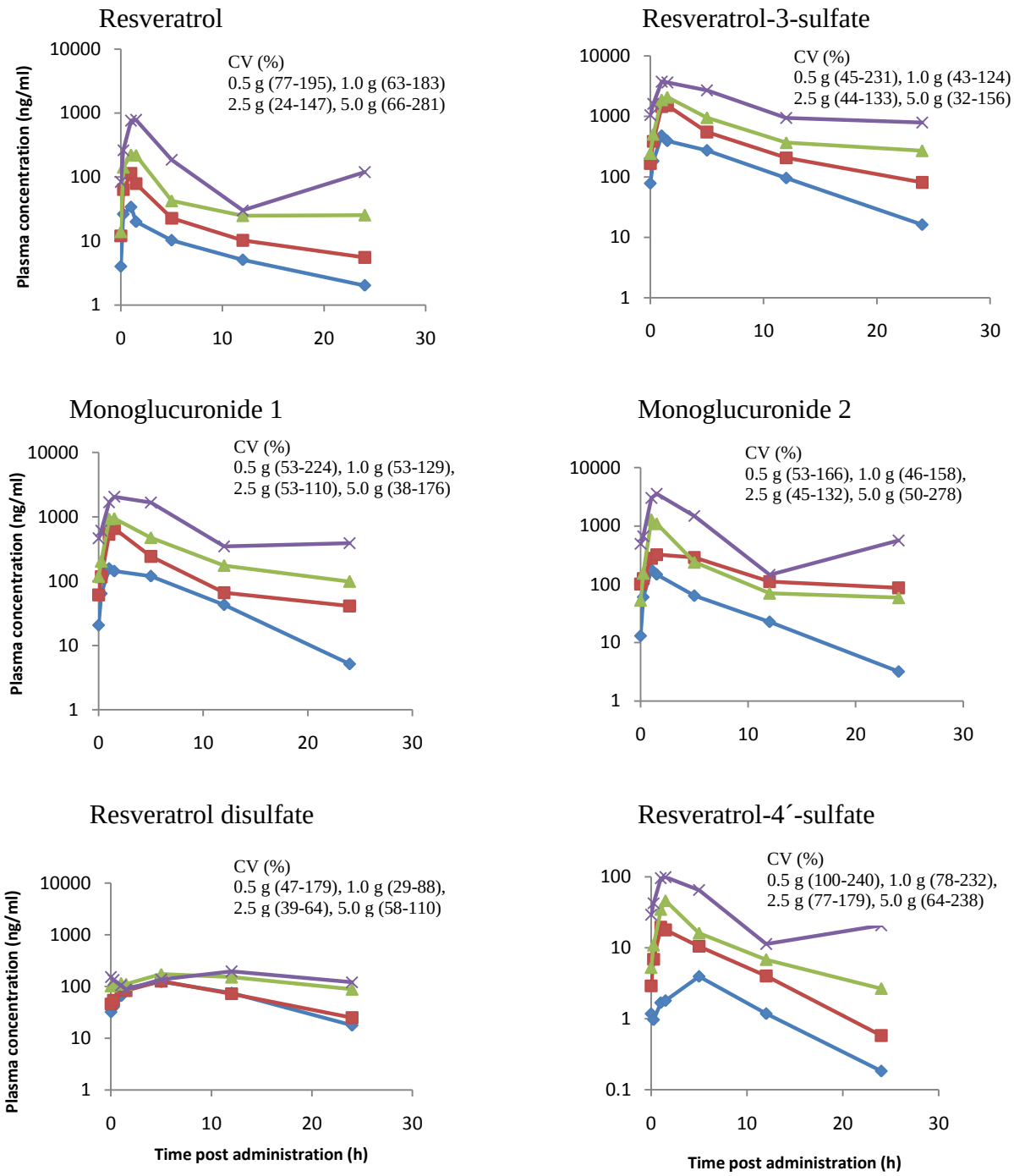


Figure 4.1.2 Plasma concentration versus time curves (log scales) for resveratrol and its main metabolites in healthy volunteers. Values represent the mean of 10 volunteers per dose level, insets show the coefficient of variation as a percent (CV)

0.5 g, 1.0 g, 2.5 g, 5.0 g resveratrol

Table 4.1.1 Comparison of the pharmacokinetics of resveratrol in the plasma of healthy volunteers after a single dose (Boocock *et al*., 2007) or after at least 21 daily doses of resveratrol for the 4 dose levels. Values represent the mean with coefficient of variation (CV) in % or range in brackets for 10 volunteers per dose level. Statistical analysis (Independent T test) has been performed to compare the single dose to the daily dose parameters (*P* values are given below the corresponding values to enable identification of any significant differences between parameters).

Variable	Dose of resveratrol (g)							
	0.5		1.0		2.5		5.0	
	Single	Repeat	Single	Repeat	Single	Repeat	Single	Repeat
AUC_{inf} (ng h/mL)	223.7*	225.6 (71.45)	544.8 (57.20)	1183 (164.4)	786.5 (36.20)	2071 (96.47)	1319 (59.10)	7304 (188.0)
P value			*0.45*		*0.18*		*0.36*	
C_{max} (ng/mL)	72.60 (48.90)	44.68 (86.39)	117.0 (73.10)	140.0 (69.40)	268.0 (55.30)	331.2 (59.17)	538.8 (72.50)	954.2 (53.24)
P value	*0.18*		*0.58*		*0.43*		***0.03***	
T_{max} (h)	0.83 (0.50-1.50)	3.43 (0.00-24.00)	0.75 (0.50-4.00)	0.88 (0.25-1.25)	1.38 (0.50-4.00)	1.38 (0.25-5.00)	1.50 (0.67-5.00)	1.25 (1.00-1.50)
P value	*0.30*		*0.16*		*0.65*		*0.38*	
C_{av} (ng/mL)	8.36 (57.80)	7.64 (77.16)	18.04 (51.60)	21.36 (74.84)	32.25 (43.00)	50.93 (41.64)	51.90 (80.70)	173.4 (103.1)
P value	*0.77*		*0.58*		***0.03***		*0.06*	
Half-life (h)	2.85*	7.75 (67.24)	8.87 (91.1)	18.01 (194.4)	4.22 (51.6)	15.13 (105.2)	8.52 (95.8)	6.14 (83.78)
P value			*0.55*		*0.16*		*0.50*	
CL/F (L/h)	2235*	2755 (34.72)	2593 (65.10)	2384 (70.05)	3471 (29.90)	2007 (63.55)	4930 (50.00)	2421 (109.5)
CLR (L/h)	1.17 (102.50)	0.85 (0.89)	0.70 (71.50)	1.05 (163.97)	0.66 (53.10)	0.22 (53.05)	1.44 (139.20)	0.23 (98.96)
V/F (L)	9198*	31,205 (76.95)	19,298 (54.30)	20,568 (49.67)	22,226 (67.30)	30,876 (61.23)	66,991 (112.0)	17,568 (144.6)

Abbreviations: AUC_{inf}, area under the concentration versus time curve to time infinity; C_{max}, maximal plasma concentration; T_{max}, median time of maximal plasma concentration; C_{av}, average plasma concentration; CL/F , apparent total body clearance (calculated as dose/AUC_{inf});CL R, apparent renal clearance approximated by amount excreted with urine within 24 h over AUC_{0-24};V/F, apparent volume of distribution *n = 1, value for AUC_{inf} at the lowest dose could be established in only one participant. Results were deemed significant if a *P* value of <0.05 was reached.

Table 4.1.2 Comparison of the pharmacokinetics of resveratrol monoglucuronide 1 in the plasma of healthy volunteers after a single dose (Boocock *et al.*, 2007) or after at least 21 daily doses of resveratrol for the 4 dose levels. Values represent mean with CV in % or range in brackets 10 volunteers per dose level. Statistical analysis (Independent T test) has been performed to compare the single dose to the daily dose parameters (*P* values are given below the corresponding values)

Variable	Dose of resveratrol (g)							
	0.5		1.0		2.5		5.0	
	Single	Repeat	Single	Repeat	Single	Repeat	Single	Repeat
AUC_{inf} (ng h/mL)	1919 (33.60)	1559 (55.01)	3059 (60.90)	4304 (52.93)	5664 (27.70)	8441 (52.03)	9923 (40.90)	31,603 (122.1)
P value	*0.34*		*0.20*		*0.14*		*0.14*	
C_{max} (ng/mL)	404.6 (35.30)	185.9 (56.77)	473.6 (76.80)	707.5 (57.58)	874.4 (37.50)	1139 (88.19)	1285 (55.40)	2310 (45.54)
P value	***0.001***		*0.19*		*0.44*		*0.10*	
T_{max} (h)	2.00 (1.00-6.00)	2.65 (1.00-5.00)	2.25 (1.00-6.00)	1.65 (1.00-5.00)	2.37 (1.00-8.00)	1.58 (1.00-5.00)	2.00 (1.50-5.00)	2.10 (1.00-5.00)
P value	*0.90*		*0.10*		*0.16*		*0.34*	
C_{av} (ng/mL)	76.90 (37.20)	61.00 (46.83)	110.3 (56.10)	161.0 (50.96)	215.5 (43.50)	298.4 (54.82)	344.1 (51.50)	829.5 (57.88)
P value	*0.23*		*0.14*		*0.18*		***0.01***	
Half-life (h)	2.85 (48.60)	5.26 (45.06)	7.27 (93.90)	5.10 (53.37)	10.60 (92.90)	7.57 (38.21)	7.90 (39.10)	8.52 (92.42)
P value	***0.02***		*0.37*		*0.45*		*0.86*	
CL/F (L/h)	282.7 (27.30)	407.2 (48.00)	493.5 (74.70)	307.0 (56.03)	469.5 (25.70)	392.8 (64.34)	590.6 (45.20)	348.4 (93.27)

Abbreviations: AUC_{inf}, area under the concentration versus time curve to time infinity; C_{max}, maximal plasma concentration; T_{max}, median time of maximal plasma concentration; C_{av}, average plasma concentration; CL/F , apparent total body clearance (calculated as dose/AUC_{inf}). Results were deemed significant if a *P* value of <0.05 was reached

Table 4.1.3 Comparison of the pharmacokinetics of resveratrol monoglucuronide 2 in the plasma of healthy volunteers after a single dose (Boocock *et al.*, 2007) or after at least 21 daily doses of resveratrol for the 4 dose levels. Values represent mean with CV in % or range in brackets for 10 volunteers per dose level. Statistical analysis (Independent T test) has been performed to compare the single dose to the daily dose parameters (*P* values are given below the corresponding values)

Variable	Dose of resveratrol (g)							
	0.5		1.0		2.5		5.0	
	Single	Repeat	Single	Repeat	Single	Repeat	Single	Repeat
AUC_{inf} (ng h/mL)	1287 (21.70)	986.9 (56.60)	2589 (66.40)	2349 (33.25)	4320 (32.90)	5977 (56.86)	8546 (62.30)	42,309 (193.0)
P value	*0.20*		*0.71*		*0.22*		*0.30*	
C_{max} (ng/mL)	369.5 (39.60)	184.2 (86.40)	672.6 (81.10)	587.0 (48.59)	1626 (71.50)	1548 (106.8)	1735 (66.40)	3853 (48.99)
P value	***0.01***		*0.65*		*0.90*		***0.01***	
T_{max} (h)	1.50 (1.00-5.00)	2.00 (1.00-5.00)	1.75 (1.00-5.10)	1.65 (1.00-5.00)	2.00 (1.00-6.00)	1.30 (1.00-1.50)	2.52 (1.50-8.00)	1.30 (1.00-1.50)
P value	*0.93*		*0.32*		***0.04***		*0.05*	
C_{av} (ng/mL)	51.00 (27.60)	39.59 (47.37)	99.90 (66.20)	89.27 (35.20)	193.8 (39.30)	215.9 (46.90)	317.8 (65.60)	916.5 (92.79)
P value	*0.14*		*0.65*		*0.59*		*0.05*	
Half-life (h)	3.09 (69.80)	4.19 (60.90)	6.64 (92.10)	3.90 (44.05)	8.42 (88.90)	6.52 (45.84)	5.83 (51.20)	6.59 (115.2)
P value	*0.35*		*0.21*		*0.52*		*0.80*	
CL/F (L/h)	408.8 (26.70)	656.7 (48.77)	642.5 (83.00)	469.3 (31.81)	636.9 (32.60)	608.9 (74.47)	1017 (94.60)	456.7 (124.3)

Abbreviations: AUC_{inf}, area under the concentration versus time curve to time infinity; C_{max}, maximal plasma concentration; T_{max}, median time of maximal plasma concentration; C_{av}, average plasma concentration; CL/F , apparent total body clearance (calculated as dose/AUC_{inf}). Results were deemed significant if a *P* value of <0.05 was reached

Table 4.1.4 Comparison of the pharmacokinetics of resveratrol-3-sulfate in the plasma of healthy volunteers after a single dose (Boocock *et al.*, 2007) or after at least 21 daily doses of resveratrol for the 4 dose levels. Values represent mean with CV in % or range in brackets for 10 volunteers per dose level. Statistical analysis (Independent T test) has been performed to compare the single dose to the daily dose parameters (*P* values are given below the corresponding values).

Variable	Dose of resveratrol (g)							
	0.5		1.0		2.5		5.0	
	Single	Repeat	Single	Repeat	Single	Repeat	Single	Repeat
AUC_{inf} (ng h/mL)	4049 (26.60)	3644 (61.10)	10,053 (73.20)	10,368 (44.99)	16,984 (41.70)	20,784 (68.11)	30,898 (46.10)	54,280 (89.40)
P value	*0.63*		*0.91*		*0.53*		*0.24*	
C_{max} (ng/mL)	1135 (25.70)	562.2 (35.05)	2102 (81.30)	1692 (35.09)	2786 (27.20)	2295 (50.75)	4294 (48.00)	4155 (40.58)
P value	***<0.0001***		*0.48*		*0.28*		*0.87*	
T_{max} (h)	1.50 (1.00-5.00)	2.60 (1.00-5.00)	2.00 (1.00-5.00)	1.70 (1.00-5.00)	2.00 (1.00-5.20)	1.25 (1.00-1.50)	2.05 (1.00-6.00)	1.53 (0.25-5.00)
P value	*0.40*		*0.27*		*0.05*		*0.38*	
C_{av} (ng/mL)	172.0 (23.20)	149.0 (51.80)	402.6 (70.50)	396.2 (42.09)	597.0 (27.00)	646.2 (39.63)	1089 (49.40)	1607 (49.12)
P value	*0.41*		*0.95*		*0.61*		*0.12*	
Half-life (h)	3.21 (56.60)	3.93 (45.18)	4.51 (82.80)	5.56 (53.00)	11.50 (95.50)	8.88 (54.16)	7.71 (42.30)	8.57 (51.60)
P value	*0.41*		*0.50*		*0.51*		*0.67*	
CL/F (L/h)	131.2 (25.80)	170.3 (40.46)	151.8 (62.70)	114.6 (41.08)	171.2 (40.00)	162.9 (54.76)	207.8 (63.90)	146.5 (74.01)

Abbreviations: AUC_{inf}, area under the concentration versus time curve to time infinity; C_{max}, maximal plasma concentration; T_{max}, median time of maximal plasma concentration; C_{av}, average plasma concentration; CL/F , apparent total body clearance (calculated as dose/AUC_{inf}). Results were deemed significant if a *P* value of <0.05 was reached

Table 4.1.5 Pharmacokinetics of resveratrol-4´-sulfate and resveratrol disulfate in the plasma of healthy volunteers after at least 21 daily doses of resveratrol for the 4 dose levels. Values represent mean with CV in % or range in brackets for 10 volunteers per dose level. Data are not available from the single dose study for these metabolites.

Variable	Dose of resveratrol (g)							
	0.5		1.0		2.5		5.0	
	Res-4´-sulfate	Res disulfate	Res-4´-sulfate	Res disulfate	Res-4´-sulfate	Res disulfate	Res-4´-sulfate	Res disulfate
AUC_{inf} (ng h/mL)	79.01 (146.5)	2288 (75.16)	328.5 (72.34)	4706 (113.3)	405.3 (54.32)	6323 (33.14)	1364 (148.2)	7033 (70.44)
C_{max} (ng/mL)	4.57 (115.8)	136.6 (42.50)	25.46 (79.20)	148.3 (45.61)	46.60 (74.96)	199.6 (49.22)	108.3 (69.42)	259.9 (59.41)
T_{max} (h)	2.64 (0.00-5.00)	5.00 (1.50-12.00)	1.65 (1.00-5.00)	5.53 (0.25-12.00)	1.64 (0.25-5.00)	5.75 (0.00-12.00)	1.28 (0.25-1.50)	7.50 (0.00-24.00)
C_{av} (ng/mL)	1.65 (154.7)	72.36 (66.53)	6.18 (107.1)	73.58 (35.35)	11.54 (80.01)	136.1 (50.80)	35.67 (105.4)	151.5 (44.62)
Half-life (h)	4.07 (56.07)	7.33 (29.66)	6.97 (70.99)	41.27 (179.0)	8.57 (59.23)	17.61 (73.93)	6.19 (63.44)	21.05 (29.26)
CL/F (L/h)	30,412 (89.80)	294.5 (44.26)	11,140 (164.7)	381.2 (56.25)	7759 (45.91)	444.0 (44.67)	8574 (65.71)	965.1 (105.9)

Abbreviations: AUC_{inf}, area under the concentration versus time curve to time infinity; C_{max}, maximal plasma concentration; T_{max}, median time of maximal plasma concentration; C_{av}, average plasma concentration; CL/F , apparent total body clearance (calculated as dose/AUC_{inf}).

Figures 4.1.3- 4.1.7 show the time dependent change in plasma concentration of resveratrol and its metabolites for each individual (up to 24 h post administration) for each dose level (0.5, 1.0, 2.5 and 5.0 g). Across the 4 levels, the shape of the plasma concentration versus time graphs for resveratrol were similar, with the C_{max} being reached within 2 h from administration followed by a sharp fall in plasma concentration within 5 h. In comparison to resveratrol, the monoglucuronides, resveratrol-3-sufate and resveratrol-4´-sulfate concentration versus time graphs generally showed a similar pattern for each dose level. However, in some individuals, the C_{max} for these metabolites was reached at 5 h post administration and even later for resveratrol-4´-sulfate, with concentrations then falling more gradually over the PK collection period. In contrast, resveratrol disulfate had more consistent levels over the 24 h collection period, reflected in its relatively long half life.

Table 9.1.1 (Appendix 9.1) shows the concentrations of resveratrol and its metabolites remaining at 24 h post administration for each dose level. This information was used in an attempt to assess whether these compounds could be accumulating in the body with repeat dosing and therefore producing detectable levels 24 h post administration, before the next dose was taken. The main compounds detected at 24 h were the metabolites monoglucuronide 1 (ranging from 5.17-390.4 ng/mL across the 4 dose levels), monoglucuronide 2 (3.19-565.9 ng/mL), resveratrol disulfate (17.86-120.9 ng/mL) and resveratrol-3-sulfate (16.19-790.7 ng/mL). In contrast, there were only small amounts of resveratrol, ranging from 2.02-25.44 ng/mL, for the 0.5-2.5 g dose levels and resveratrol-4'-sulfate, ranging from 0.18-20.67 across the 4 dose levels still present at 24 h. On the 5 g dose, one participant had a resveratrol concentration of 1072 ng/mL measured at 24 h (4.7 μM). This individual also had higher concentrations at 24 h of the

monoglucuronides, resveratrol-3-sulfate and resveratrol-4´-sulfate than the other participants on this dose level, but not of the disulfate. Interestingly, the concentrations of resveratrol and the 5 metabolites detected in the previous PK sampling time points (post administration of resveratrol) for this individual did not differ significantly from the other participants on the same dose. The plasma concentrations for resveratrol and its metabolites for this individual at the pre dosing (PK) and 24 h time points were very similar. All time points on this individual were re analysed, using different vials collected at the time, and the concentrations were found to be consistent with the original analysis indicating there was no problem with sample preparation or with the analysis, and that the concentrations measured were a true reflection of the actual plasma levels. Therefore the data from this study participant were included in the overall analysis.

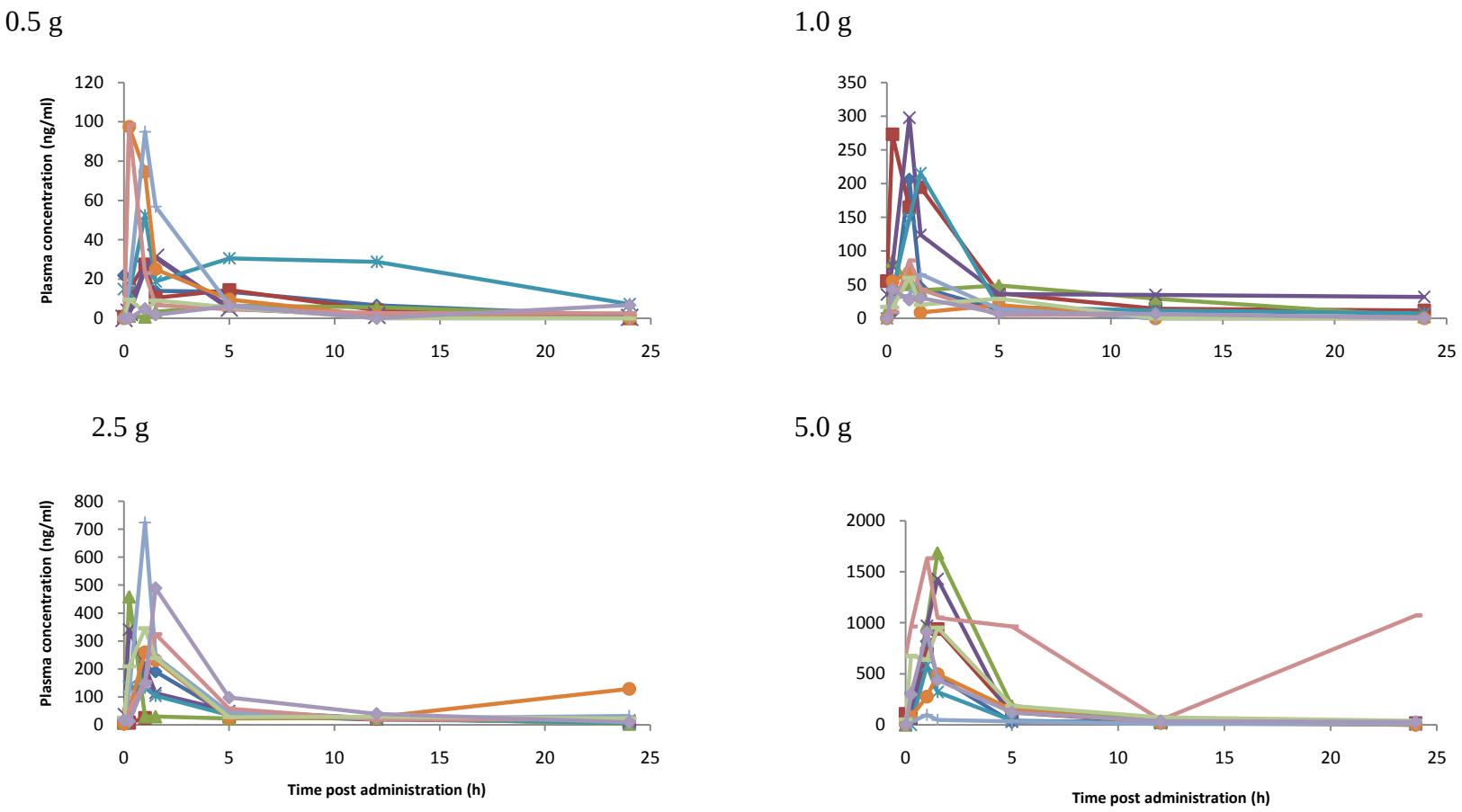


Figure 4.1.3 Plasma concentration of resveratrol versus time for each healthy volunteer (0.5, 1.0, 2.5 and 5.0 g dose levels); n=10 per dose level.

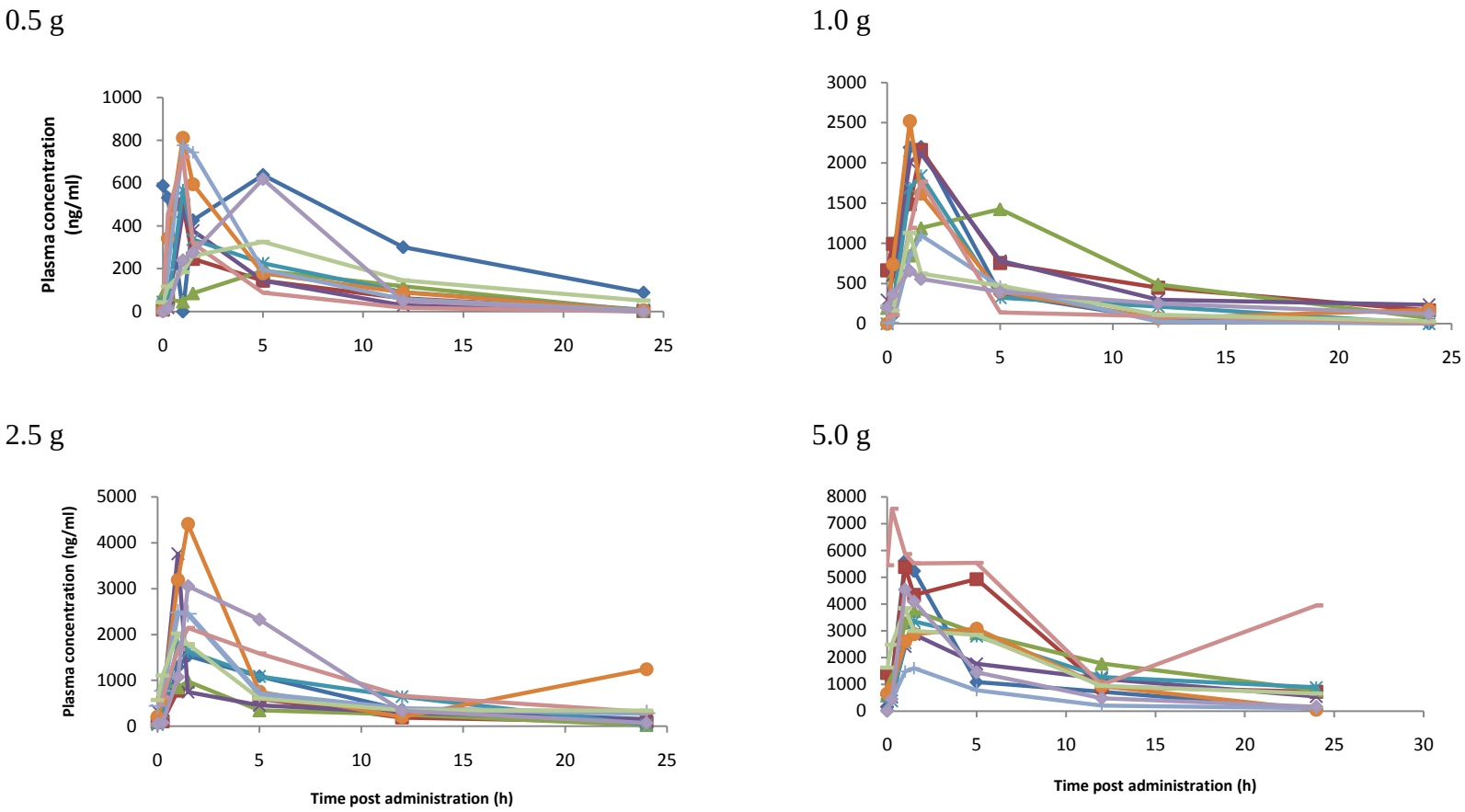


Figure 4.1.4 Plasma concentration of resveratrol-3-sulfate versus time for each healthy volunteer (0.5, 1.0, 2.5 and 5.0 g dose levels); n=10 per dose level.

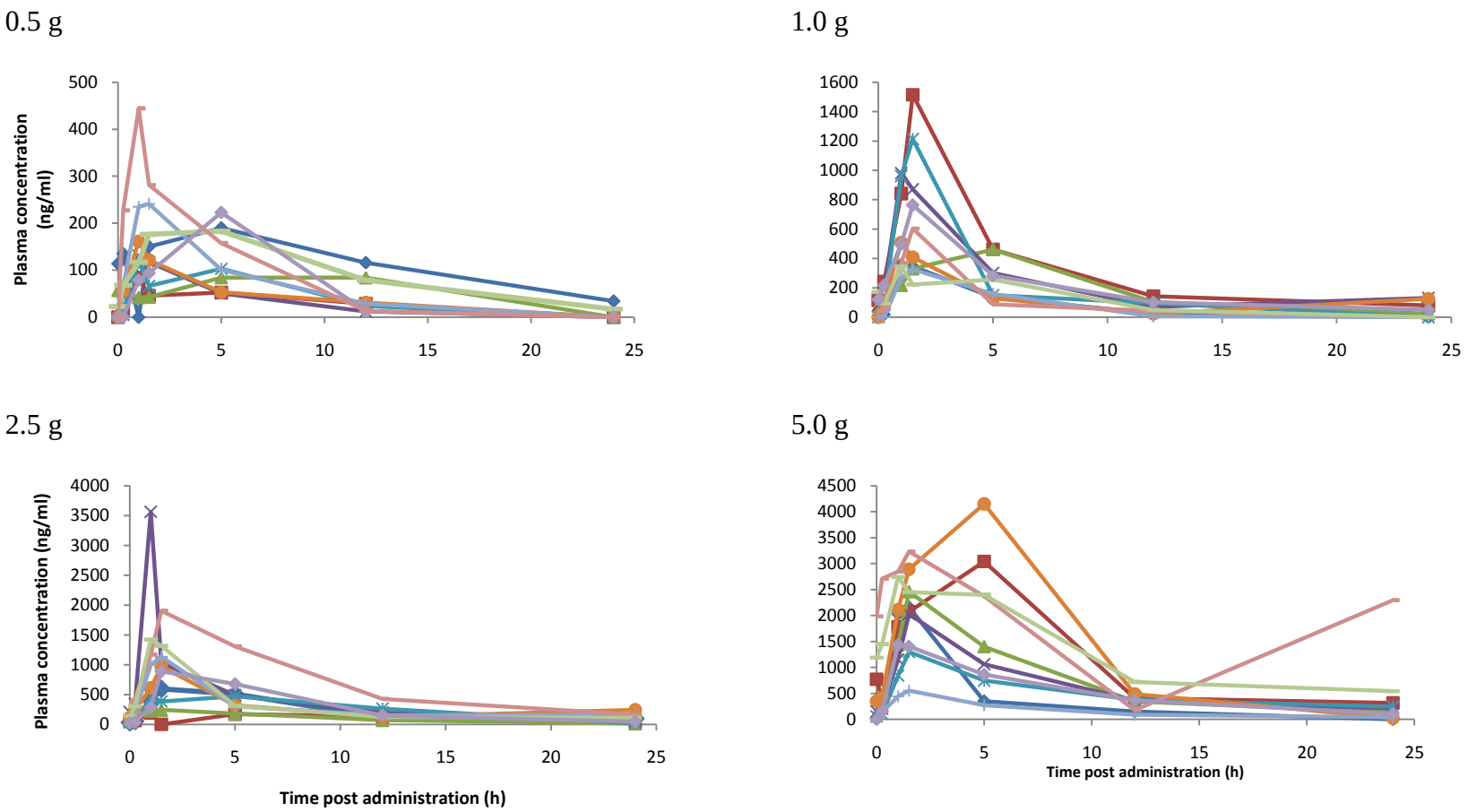
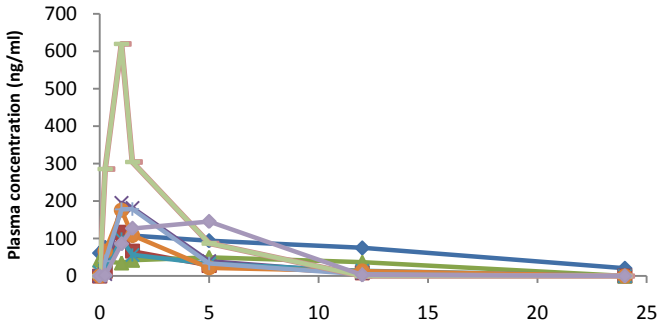
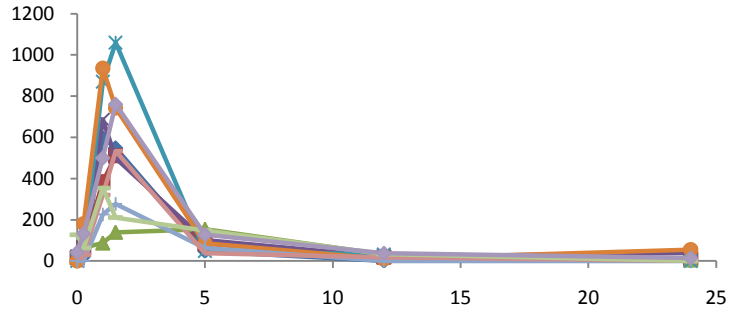


Figure 4.1.5 Plasma concentration of monoglucuronide 1 versus time for each healthy volunteer (0.5, 1.0, 2.5 and 5.0 g dose levels); n=10 per dose level.

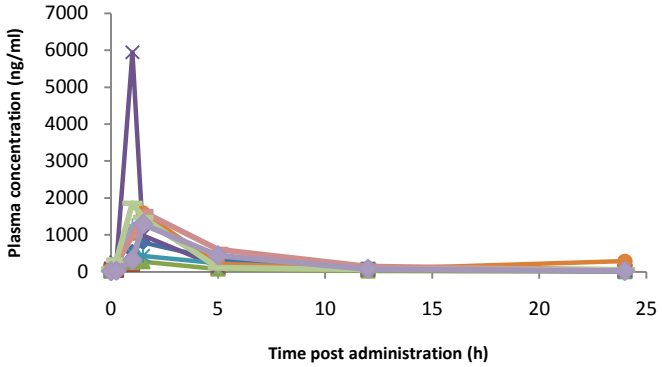
0.5 g



1.0 g



2.5 g



5.0 g

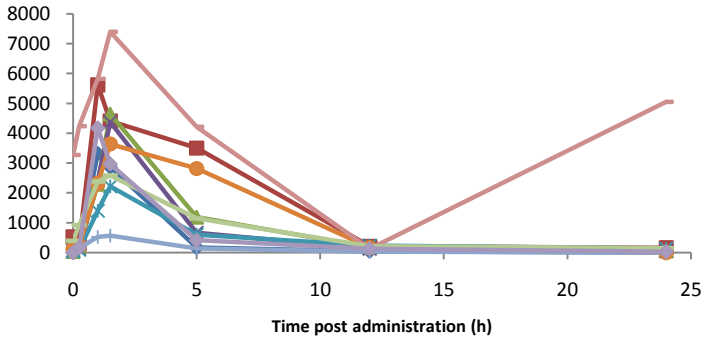
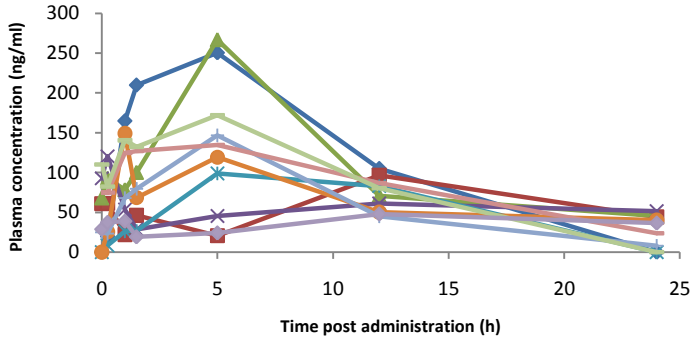


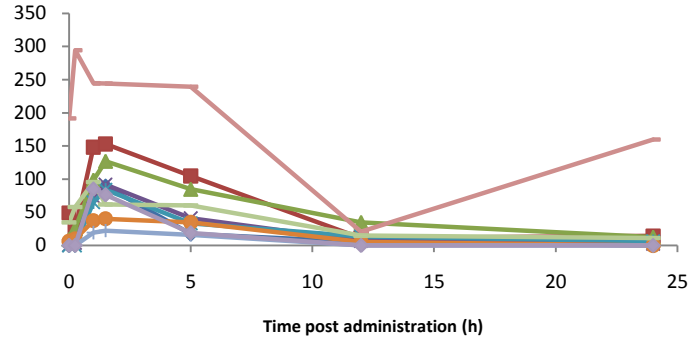
Figure 4.1.6 Plasma concentration of monoglucuronide 2 versus time for each healthy volunteer (0.5, 1.0, 2.5 and 5.0 g dose level); n=10 per dose level.

Resveratrol disulfate

1.0 g

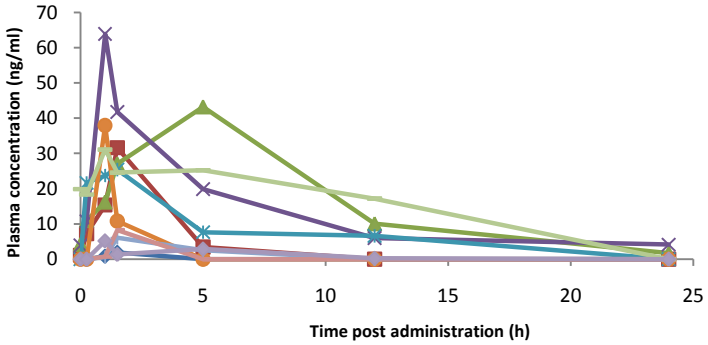


5.0 g



Resveratrol-4´-sulfate

1.0 g



5.0 g

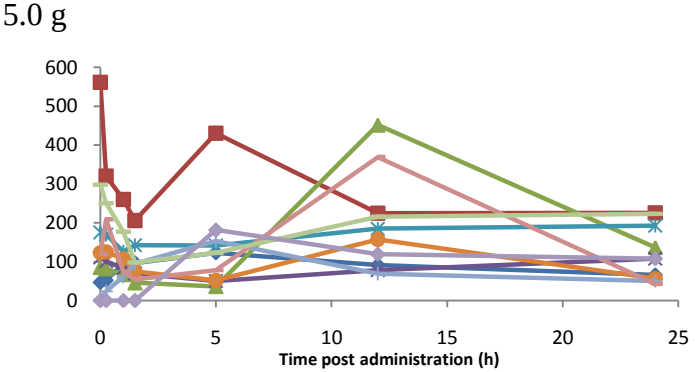


Figure 4.1.7 Plasma concentration of resveratrol disulfate and resveratrol-4´-sulfate versus time for each healthy volunteer (1.0 and 5.0 g); n=10 per dose level. Only the 1.0 and 5.0 g graphs are shown as representative examples of these metabolites after daily dosing.

4.1.1.2 Dose proportionality assessment of resveratrol

Dose proportionality for resveratrol and its metabolites was assessed based on the PK parameters C_{max} and AUC_{0-t} (Figures 4.1.8 and 4.1.9). When plotted versus dose, mean C_{max} and AUC_{0-t} values for these compounds increased over the dose range of 0.5 to 5.0 g. Whilst the total number of subjects per dose level was small and there was high inter-subject variation for these parameters, resveratrol and its metabolites (the monoglucuronides, resveratrol-3-sulfate and resveratrol-4´-sulfate) showed a near linear increase in mean C_{max} and AUC_{0-t} across the 4 dose levels, suggesting dose proportionality. Although resveratrol disulfate showed an increase over the 4 levels, it was not in a dose proportionate manner.

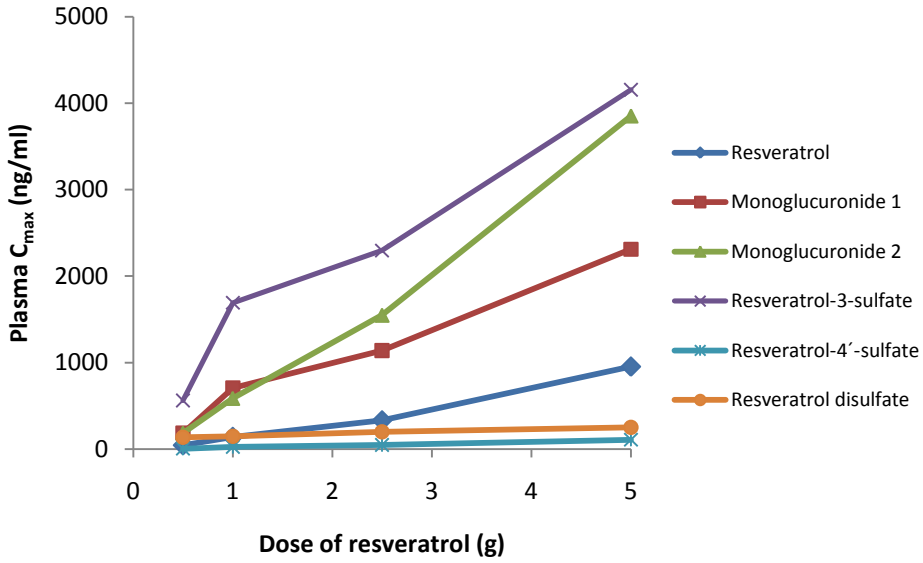


Figure 4.1.8 Relationship between dose of resveratrol and mean plasma C_{max} of resveratrol and its main metabolites in healthy volunteers. Values represent the mean for 10 volunteers per dose level.

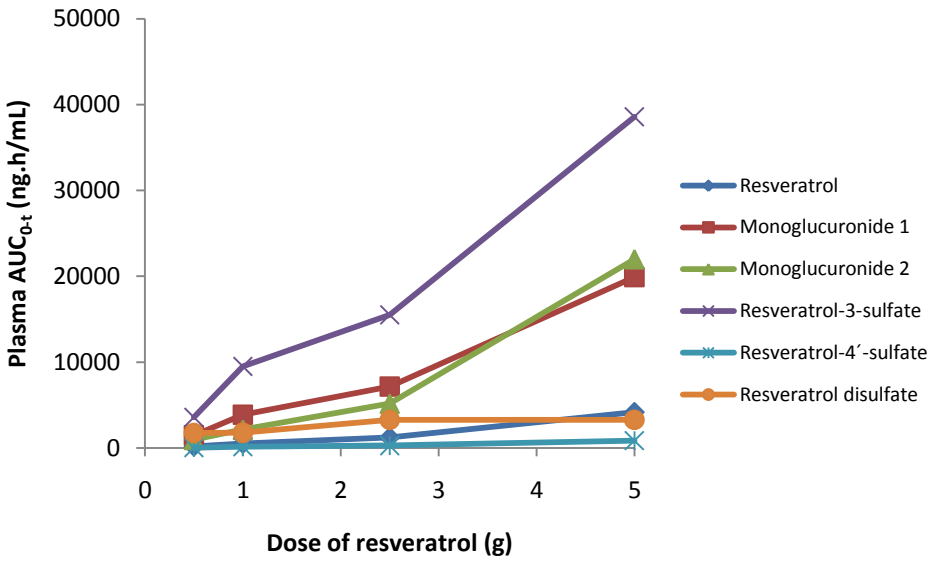


Figure 4.1.9 Relationship between dose of resveratrol and mean plasma AUC_{0-t} for resveratrol and its main metabolites in healthy volunteers. Values represent the mean for 10 volunteers per dose level.

4.1.1.3 Comparison of the pharmacokinetics of resveratrol after single versus repeat dosing

From the single dose study PK analysis (Boocock *et al*., 2007), the plasma C_{max} concentrations of resveratrol achieved were markedly below the concentrations required in the majority of published *in vitro* experiments to elicit pharmacologic effects associated with cancer chemoprevention (≥5 μM). Therefore, a comparison between the single and repeat dose studies was undertaken to assess whether repeat dosing achieved higher systemic concentrations. The PK plasma parameters for resveratrol, resveratrol-3-sulfate and the monoglucuronides for both studies over the dose range 0.5-5.0 g are shown in Tables 4.1.1-4.1.5. Resveratrol and its main metabolites (resveratrol-3-sulfate and the monoglucuronides), showed a dose-dependent increase in mean plasma C_{max}, C_{av} and

AUC_{inf} in both studies. An increase in these PK parameters across the dose levels was also observed for resveratrol-4´-sulfate and resveratrol disulfate with repeated dosing, although the latter showed only a modest rise in mean plasma C_{max} and C_{av} concentrations as the dose increased from 0.5 to 1.0 g. For resveratrol and monoglucuronide 1, on the top 3 dose levels, the plasma C_{max}, C_{av} and AUC_{inf} values for the repeat dose study were consistently higher than the single dose study. The C_{max} and AUC_{inf} for resveratrol and its metabolites for the 2 studies are compared in Figures 4.1.10-4.1.17. For resveratrol, the difference between the 2 studies, was statistically significant for C_{av} on the 2.5 g dose level (P=0.03) and for C_{max} on the 5.0 g dose level (P=0.03) and for monoglucuronide 1 was statistically significant for C_{av} on the 5.0 g dose level (P=0.01). Monoglucuronide 2 and resveratrol-3-sulfate showed consistently higher values with repeat dosing for AUC_{inf} and C_{av} on the top 2 dose levels, higher monoglucuronide 2 C_{max} values were present on the 5.0 g dose, with the latter reaching statistical significance (P=0.01).

Interestingly, for resveratrol, lower mean values for plasma C_{max}, and C_{av} were observed in volunteers receiving 0.5 g resveratrol repeatedly compared to after a single dose but this was not statistically significant (Figure 4.1.10 and Table 4.1.1). A comparison of the AUC_{inf} for resveratrol on the 0.5 g dose was not possible, as it was only calculated for one participant on the single dose study. In addition, on this dose lower mean values for plasma C_{max}, C_{av} and AUC_{inf} were also observed for the individual metabolites in volunteers on the repeat dose study, with a significantly reduced C_{max} for resveratrol-3-sulfate (P<0.0001), monoglucuronide 1 (P=0.001) and monoglucuronide 2 (P=0.01) compared to the mean value reported after a single equivalent dose. Two other statistically significant findings

were observed: monoglucuronide 1 (0.5 g) had a longer half life with repeat compared to single dosing (P=0.02) and the plasma T_{max} for monoglucuronide 2 was shorter on the highest 2 dose levels with repeat dosing and was statistically significant on the 2.5 g (P=0.04).

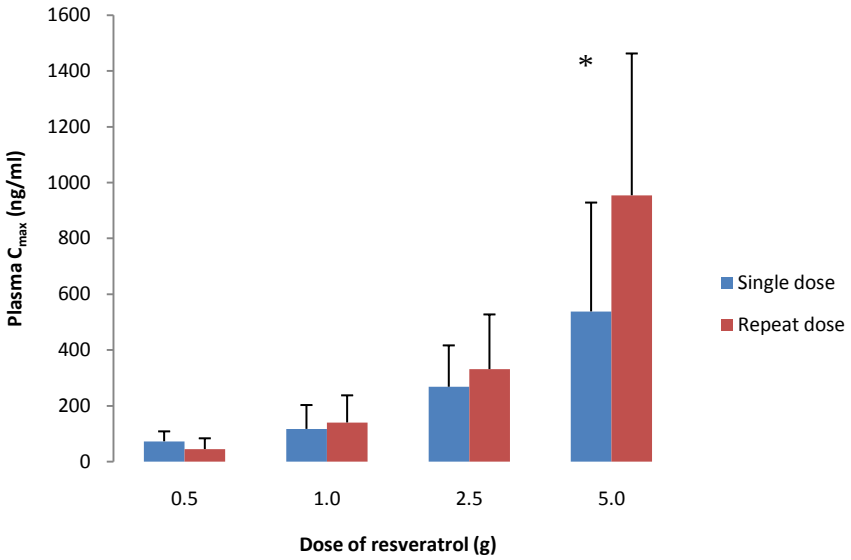


Figure 4.1.10 Relationship between dose administered and plasma C_{max} of resveratrol following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level. *P<0.01, **P<0.001.

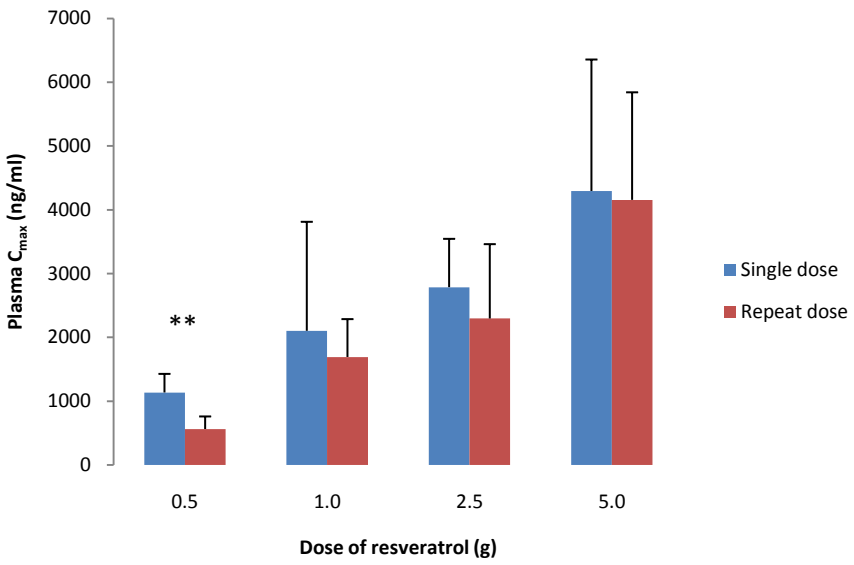


Figure 4.1.11 Relationship between dose administered and plasma C_{max} of resveratrol-3-sulfate following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level. **P*<0.01, ***P*<0.001.

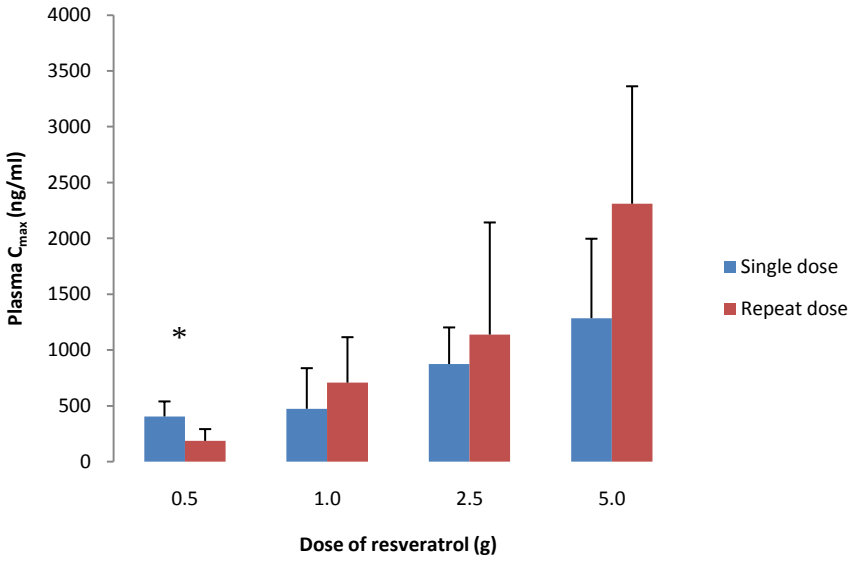


Figure 4.1.12 Relationship between dose administered and plasma C_{max} of resveratrol monoglucuronide 1 following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for10 volunteers per dose level. **P*<0.01, ***P*<0.001.

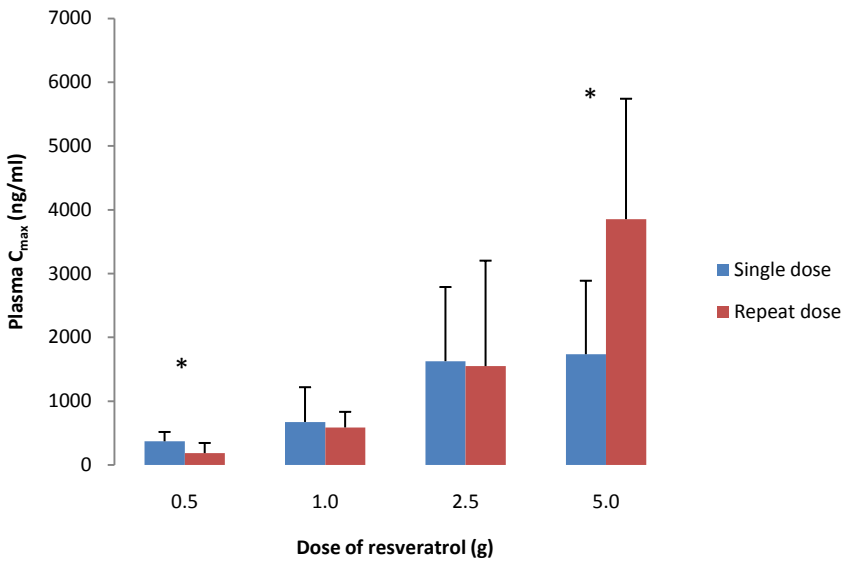


Figure 4.1.13 Relationship between dose administered and plasma C_{max} of resveratrol monoglucuronide 2 following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level. **P*<0.01, ***P*<0.001.

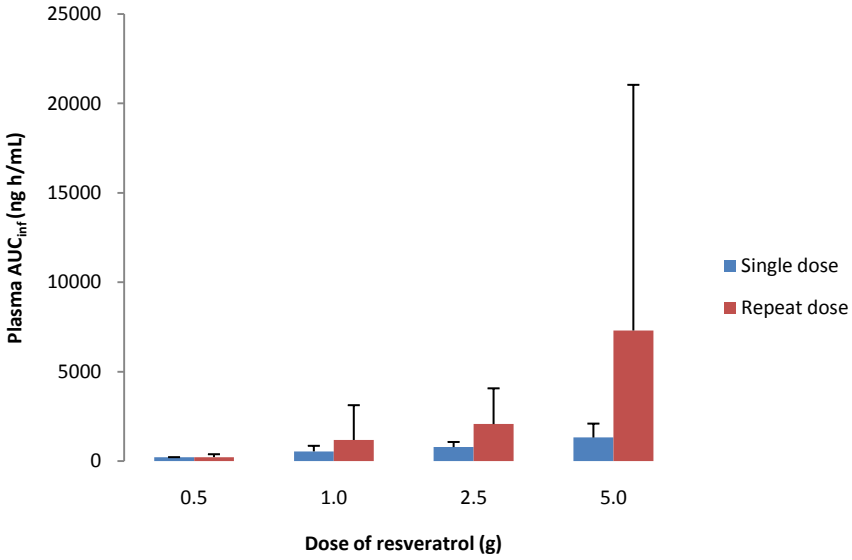


Figure 4.1.14 Relationship between dose administered and plasma AUC_{inf} of resveratrol following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level.

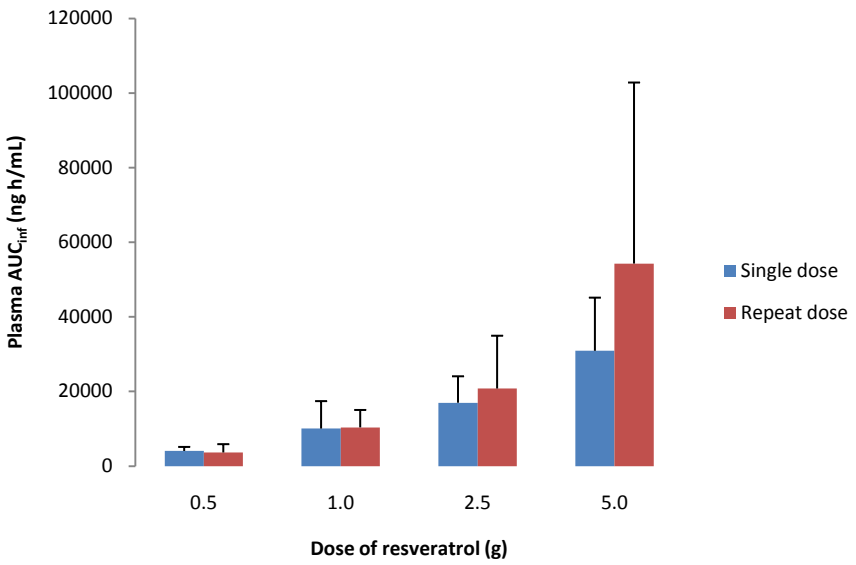


Figure 4.1.15 Relationship between dose administered and plasma AUC_{inf} of resveratrol- 3-sulfate following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level.

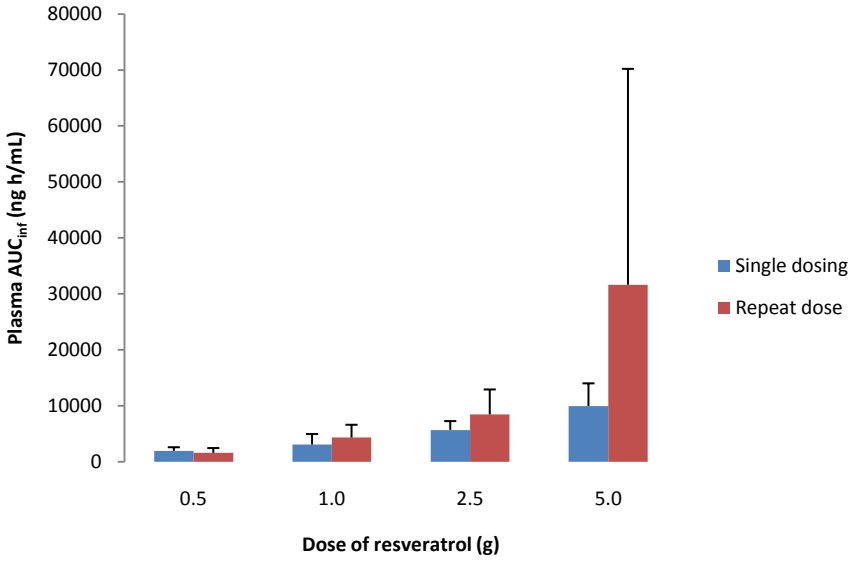


Figure 4.1.16 Relationship between dose administered and plasma AUC_{inf} of resveratrol monoglucuronide 1 following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level.

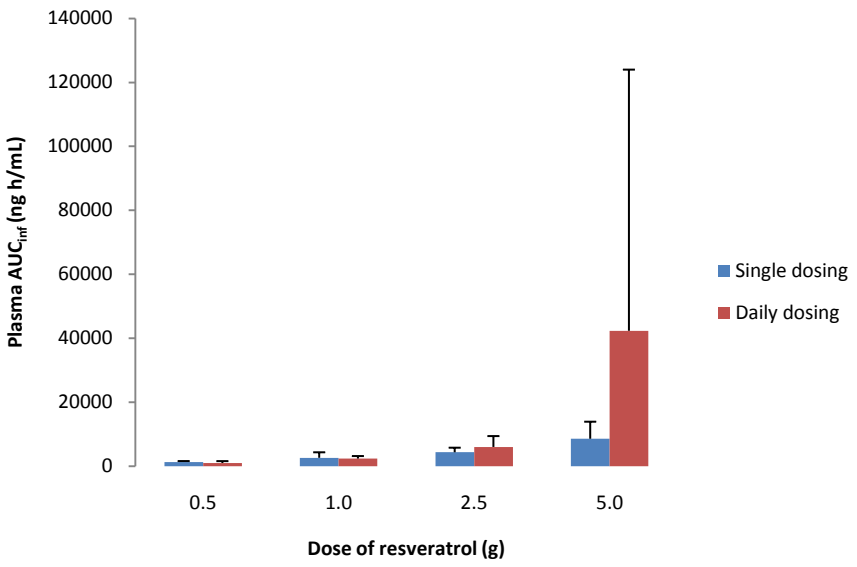


Figure 4.1.17 Relationship between dose administered and plasma AUC_{inf} of resveratrol monoglucuronide 2 following single and repeat dosing in healthy volunteers. Values represent mean (+SD) for 10 volunteers per dose level.

4.1.1.4 Assessment of relationships between participant demographic variables and pharmacokinetic parameters after repeat dosing

Potential relationships between the demographic parameters of age, gender and BMI and plasma PK parameters of C_{max}, $C_{av,}$ AUC_{inf} and half life for resveratrol and C_{max}, $C_{av,}$ AUC_{inf} for its metabolites were examined for each dose level as shown in Tables 9.1.2-9.1.7 (Appendix 9.1). Although there was high inter-individual variability for the PK parameters and only 10 participants per dose level, this analysis was carried out to assess if demographic parameters could affect the main plasma PK parameters for these compounds. For the continuous variables of age and BMI, comparisons with PK parameters were carried out by linear regression analysis and for gender, the Independent T test was performed.

Although no statistically significant correlation was found between age, gender or BMI and PK parameters (C_{max}, C_{av} and half life) of resveratrol, a positive correlation was observed between BMI and plasma AUC_{inf} across all 4 dose levels. In addition, a positive correlation for plasma resveratrol-3-sulfate was seen between age and both C_{max} and AUC_{inf} across the dose levels (excluding AUC_{inf} on the 0.5 g dose level) and for AUC_{inf}, this was statistically significant at the 2.5 g dose (r 0.66, *P*=0.04). Also for this metabolite, on the highest 2 dose levels, a positive correlation was observed between BMI and AUC_{inf}, which reached statistical significance at the 2.5 g dose (r 0.68, *P*=0.03) (Tables 9.1.2 and 9.1.7, Appendix 9.1).

Interestingly, there appeared to be a potential gender difference in PK parameters calculated for the monoglucuronides and resveratrol-3-sulfate, as shown in Tables 9.1.3 - 9.1.6 (Appendix 9.1). Apart from the 2.5 g dose level, in general, males had a higher plasma C_{max} and AUC_{inf} for resveratrol-3-sulfate; for example, at the 5.0 g dose the mean plasma C_{max} for males was 4964 ng/mL and for females 2942 ng/mL (*P*=0.06). In contrast, generally, females had higher mean plasma C_{max} and AUC_{inf} values for the monoglucuronides, for the first 3 dose levels. At the 0.5 g dose, the mean C_{max} for monoglucuronide 1 was higher for females (274.5 ng/mL) compared to males (126.9 ng/mL) (*P*=0.02). In addition, the mean C_{max} for monoglucuronide 2 on the 1.0 g dose was higher in females (766.7 ng/mL) than males (459.8 ng/mL) (*P*=0.08). Also at this dose level, the AUC_{inf} for monoglucuronide 2 in females was higher (2887 ng h/mL) compared to males (1917 ng h/mL) (*P*=0.06).

4.1.1.5 Excretion of resveratrol and its metabolites in urine

Representative HPLC chromatograms are shown in Figure 4.1.18 illustrating the analysis of urine collected from a healthy volunteer before commencing dosing and after at least 21 daily doses of 2.5 g resveratrol (batch collection over 2 h post administration). Over the 24 h collection period post resveratrol administration, only small amounts of unchanged parent compound, ranging from 0.05-0.18% of the dose, were excreted in the urine (Table 4.1.6). The main compounds excreted were resveratrol-3-sulfate, resveratrol disulfate and monoglucuronide 2. The urinary excretion of these metabolites expressed as a percentage of the dose eliminated over 24 h, ranged from 10.43% to 12.85% (resveratrol-3-sulfate), from 3.95% to 12.64% (resveratrol disulfate) and 5.70% to 9.50% (monoglucuronide 2) over the 4 dose levels, as illustrated in Table 4.1.6. In comparison with the single dose study the total fraction of resveratrol excreted in the urine over 24 h was higher with repeat dosing across all four dose levels and this difference was statistically significant at the 2.5 and 5.0 g doses (P <0.0001 and P=0.04 respectively). Apart from the lowest dose level, where the monoglucuronide fraction was lower with repeat dosing, all the metabolites showed a higher fraction of excretion after daily dosing. This was statistically significant for monoglucuronide 2 for the 1.0, 2.5 and 5.0 g doses (P=0.03, 0.004 and 0.04 respectively). After daily dosing, the excretion rate for resveratrol was highest during the initial 4 h post dose collection period (Table 4.1.7 and Figures 4.1.19- 4.1.23). After a single dose of resveratrol, 77% of all urinary species derived were passed during the first 4

h after administration, whereas repeat dosing led to elimination of the urinary species over a longer period of time, with more than 56% excreted in 8 h.

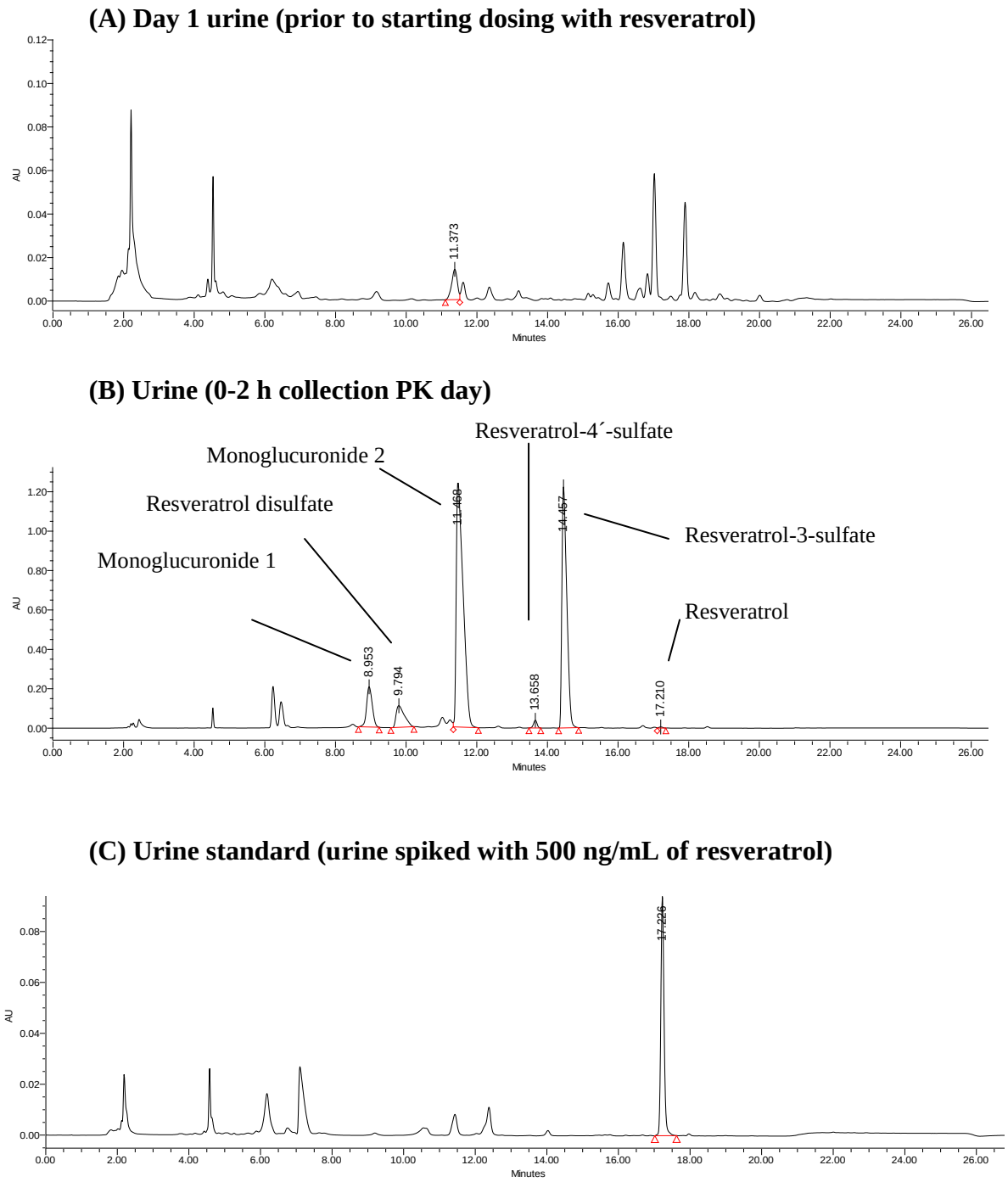


Figure 4.1.18 Representative HPLC chromatograms of resveratrol and its metabolites detected in urine samples obtained from a healthy volunteer. The HPLC traces represent (**A**) urine collected pre dosing (day 1) and then (**B**) following at least 21 oral doses, as a urine batch collection over 0-2 h after administration of 2.5 g resveratrol. Identification of resveratrol and metabolites was based on retention times of standards (**C**) analysed under identical conditions, as described in Section 2.2.1.1. Typical retention times are shown (min).

Table 4.1.6 Comparison of the total fraction excreted in the urine over the 24 h collection period (% of dose) of resveratrol and its metabolites following single (Boocock *et al*., 2007) and repeat dosing. Values represent mean (± SD) for 10 volunteers per dose level. Statistical analysis (Independent T test) has been performed to compare the single dose to the daily dose parameters (*P* values are given below the corresponding values to enable identification of any significant differences between parameters)**.** Results are deemed significant if a *P* value of <0.05 was reached.

	Resveratrol		**Monoglucuronide 1**		**Monoglucuronide 2**		**Resveratrol-3-sulfate**		**Resveratrol -4´-sulfate**	**Disulfate**
Dose (g)	**Single**	**Repeat**	**Single**	**Repeat**	**Single**	**Repeat**	**Single**	**Repeat**	**Repeat**	**Repeat**
0.5	0.03 (0.01)	0.18 (0.31)	1.96 (0.41)	1.64 (0.77)	6.85 (1.89)	6.70 (2.73)	11.36 (2.10)	12.85 (7.67)	0.31 (0.32)	12.64 (7.50)
***P* value**	*0.16*		*0.28*		*0.89*		*0.56*			
1.0	0.03 (0.03)	0.09 (0.15)	2.03 (1.16)	2.20 (1.96)	3.17 (1.62)	5.70 (2.96)	7.28 (2.61)	11.37 (5.78)	0.22 (0.22)	5.38 (2.18)
***P* value**	*0.22*		*0.81*		***0.03***		*0.06*			
2.5	0.02 (0.01)	0.05 (0.02)	1.51 (1.65)	1.65 (0.83)	2.95 (1.39)	6.37 (2.96)	5.10 (2.42)	9.13 (3.13)	0.16 (0.11)	4.60 (2.39)
***P* value**	***<0.0001***		*0.82*		***0.004***		***0.005***			
5.0	0.02 (0.01)	0.06 (0.06)	0.51 (0.22)	1.86 (2.16)	2.92 (1.42)	9.50 (8.68)	4.86 (1.47)	10.43 (8.72)	0.18 (0.16)	3.95 (4.65)
***P* value**	***0.04***		*0.08*		***0.04***		*0.08*			

Table 4.1.7 Time after dose administration of maximum excretion (T_{max}) for resveratrol and its metabolites in the urine of healthy volunteers. Values represent the mean (± SD) for 10 volunteers per dose level.

Dose (g)		Glucuronide 1	Resveratrol disulfate	Glucuronide 2	Resveratrol 4´-sulfate	Resveratrol 3-sulfate	Resveratrol
0.5	**T_{max}**	3.60	4.8	2.70	4.70	3.40	3.70
	(h)	(2.63)	(3.12)	(1.49)	(2.95)	(1.51)	(2.63)
1.0	**T_{max}**	2.90	3.90	1.90	2.50	2.40	2.1
	(h)	(1.37)	(3.07)	(1.20)	(1.78)	(1.71)	(1.20)
2.5	**T_{max}**	3.6	3.9	3.3	3.6	3.6	3.6
	(h)	(1.27)	(4.41)	(0.95)	(1.26)	(1.26)	(1.26)
5.0	**T_{max}**	2.5	4.9	2.2	3.8	2.3	2.5
	(h)	(1.58)	(7.13)	(1.03)	(5.25)	(1.64)	(1.58)

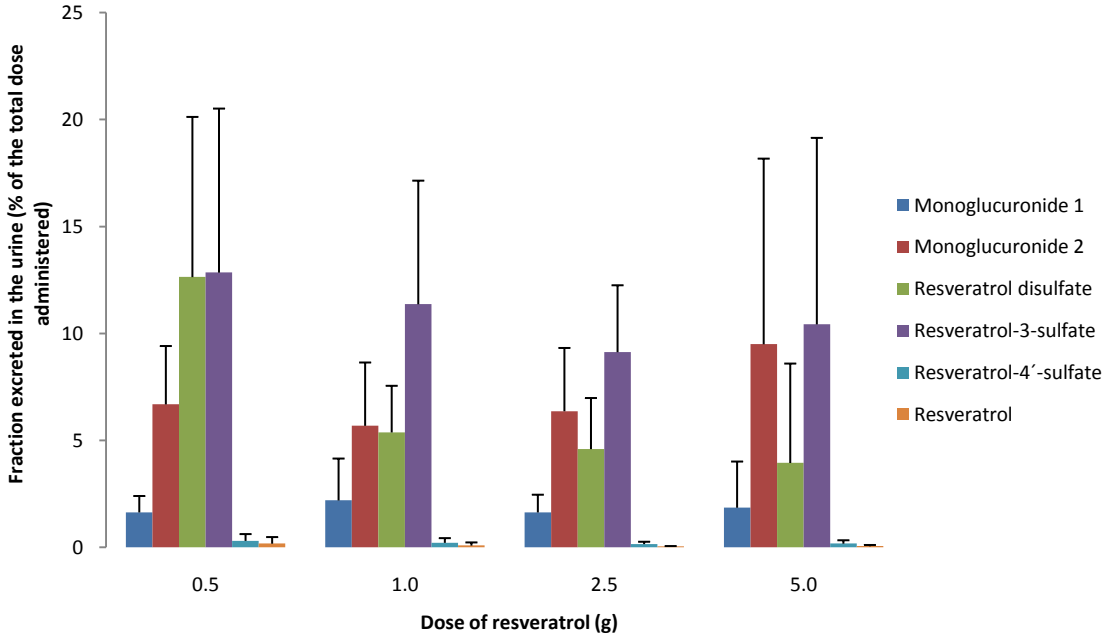


Figure 4.1.19 Excretion of resveratrol and its metabolites in the urine of healthy volunteers. Cumulative excretion over 24 h after ingestion of resveratrol at 0.5, 1.0, 2.5, or 5.0 g. Values represent mean (+SD) for 10 volunteers per dose level.

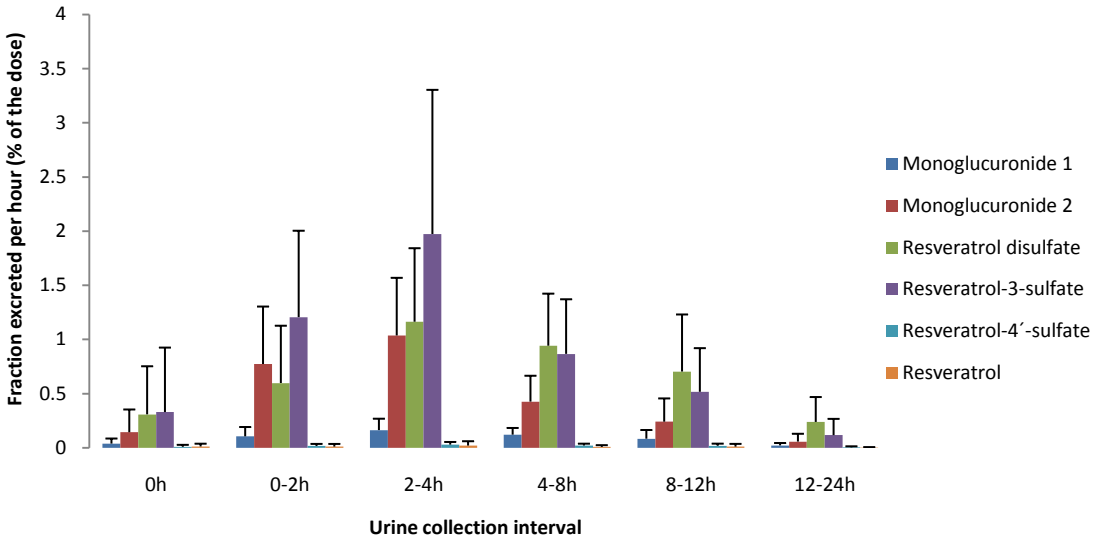


Figure 4.1.20 Excretion of resveratrol and its metabolites in the urine of healthy volunteers. Rate of excretion (per h) during 5 collection intervals within 24 h post administration of 0.5 g resveratrol, expressed as a percentage of the dose. Values represent mean (+SD) for 10 volunteers.

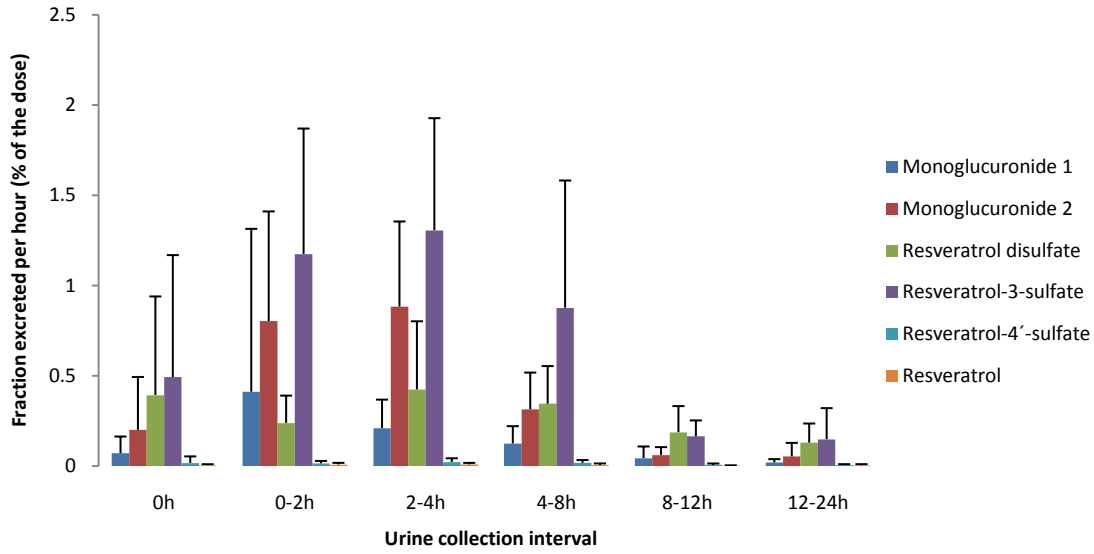


Figure 4.1.21 Excretion of resveratrol and its metabolites in the urine of healthy volunteers. Rate of excretion (per h) during 5 collection intervals within 24 h post administration of 1.0 g resveratrol, expressed as a percentage of the dose. Values represent mean (+SD) for10 volunteers.

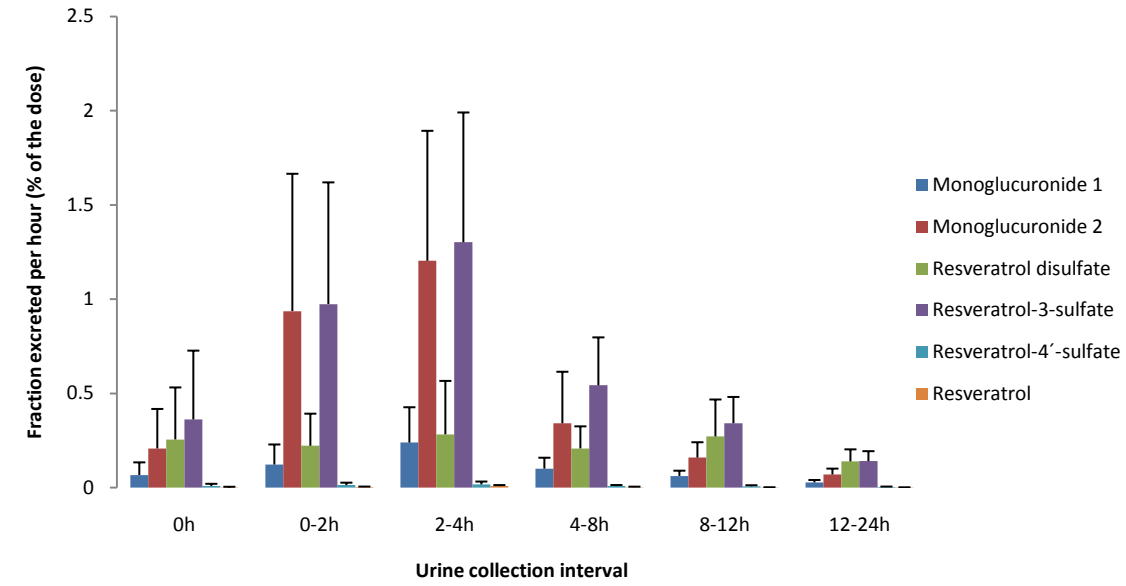


Figure 4.1.22 Excretion of resveratrol and its metabolites in the urine of healthy volunteers. Rate of excretion (per h) during 5 collection intervals within 24 h post administration of 2.5 g resveratrol, expressed as a percentage of the dose. Values represent mean (+SD) for 10 volunteers.

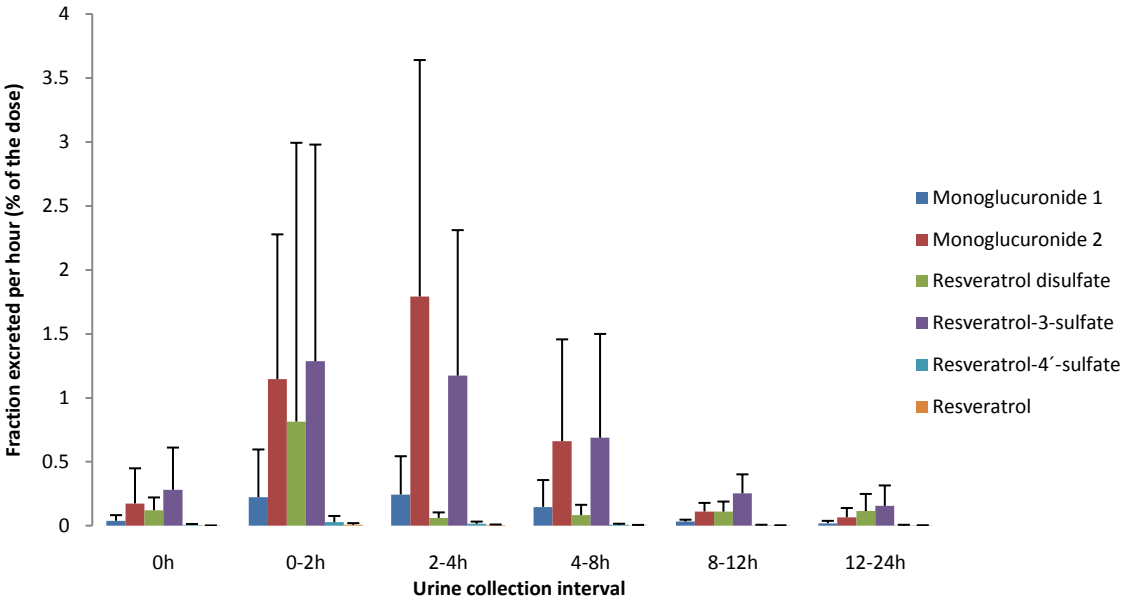


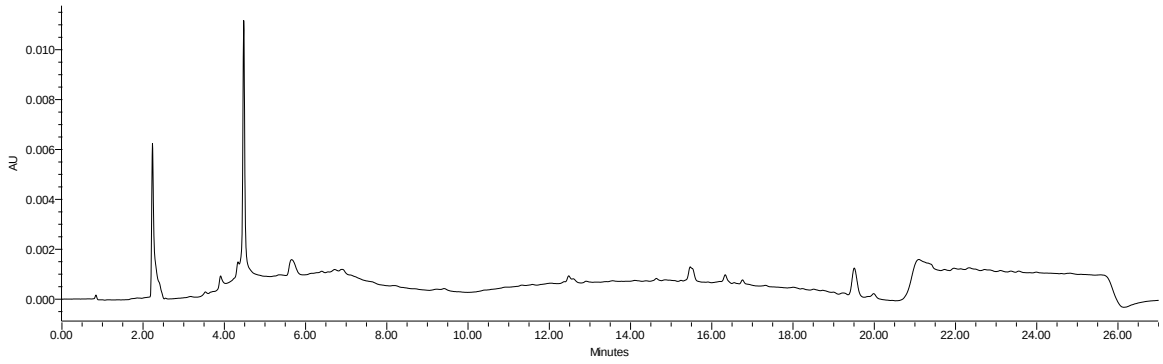
Figure 4.1.23 Excretion of resveratrol and its metabolites in the urine of healthy volunteers. Rate of excretion (per h) during 5 collection intervals within 24 h post administration of 5.0 g resveratrol, expressed as a percentage of the dose. Values represent mean (+SD) for 10 volunteers.

4.1.1.6 Concentration of resveratrol and its metabolites in faeces

Individual chromatographic peak areas of resveratrol and its metabolites in faeces were measured in stool samples as shown in Figure 4.1.24. Volunteers were asked to provide up to 4 faecal samples during the study period, a pre dosing sample and 3 during the dosing period, 1 provided within 3 days prior to the PK day, the first stool passed on the PK day and a final stool collection prior to the day 29 visit. If the PK visit was within a few days of, or the same day as, the day 29 visit it was not always possible to collect a 4^{th} sample. The dry mass of the stool sample collected was recorded. The vast majority of compound measured in the stools was resveratrol with calculated concentrations ranging from 0.00 to 155,556 ng/g dry weight of faeces; a value of 0.00 means that it was either undetectable or below the lower limit of quantification in that sample. The main metabolite detected was resveratrol-3-sulfate, which ranged from 0.00 to 3820 ng/g dry weight of faeces. In total, from all volunteers on the study, there were 84 faecal samples collected post administration of resveratrol. Of these 61% had detectable levels of resveratrol. The percentage of samples (post administration) containing metabolites were: resveratrol-3-sulfate (64%) monoglucuronide 1 (2%), monoglucuronide 2 (8%), resveratrol-4´-sulfate (30%) and resveratrol disulfate (6%). Table 4.1.8 shows the mean concentration for resveratrol and its metabolites per dry weight of faeces for each of the 4 stool sample collections and highlights that in many participants the only compounds detected were resveratrol and resveratrol-3-sulfate. Furthermore, where other metabolites were present, very few had any detectable levels of monoglucuronide 1. The concentration of resveratrol measured in faeces after repeat dosing with a minimum of 18 daily doses, was up to ~7 times higher

than that measured after a single dose of resveratrol. In addition the concentration of resveratrol metabolites in the repeat dose study, were up to ~17-fold higher than after a single dose.

(A) Pre-dose faecal sample (within 3 days of starting dosing with resveratrol)



(B) Faecal sample (PK day)

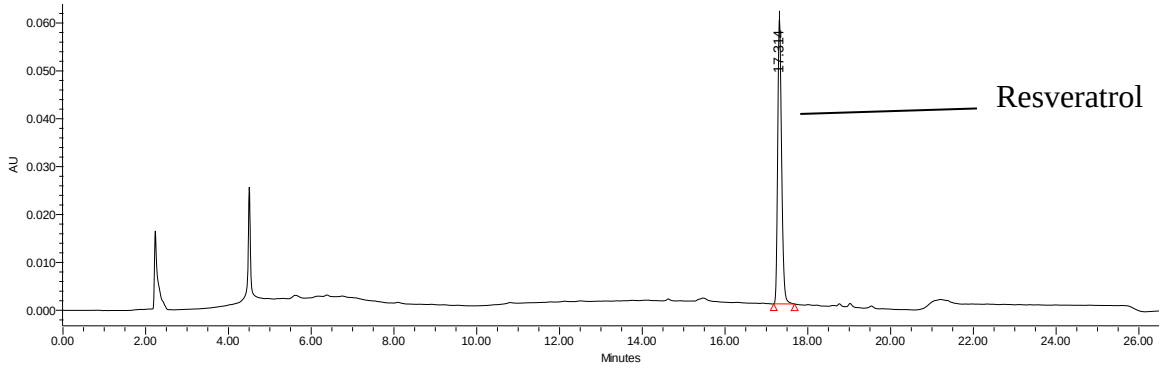


Figure 4.1.24 Representative HPLC chromatograms from the analysis of faecal samples obtained from a healthy volunteer. The HPLC traces represent faecal samples collected within the 3 days preceding commencement of resveratrol dosing (**A**) and a sample collected on the PK day, after at least 21 oral doses of 2.5 g (**B**). Identification of resveratrol and any metabolites was based on retention times of standards under identical conditions as described in Section 2.2.1.2. Only resveratrol was observed in this particular sample, which eluted at ~17.3 min.

Table 4.1.8 Estimated mean concentration of resveratrol and its metabolites in the faeces of healthy volunteers for the 4 dose levels. Values represent mean (± SD), **(**ng/g dry weight of faeces).

Dose (g)		Resveratrol	Resveratrol-3-sulfate	Glucuronide 1	Glucuronide 2	Resveratrol - 4´-sulfate	Resveratrol disulfate
0.5	Pre dosing (n=10)	0	2.40 (7.35)	0	0	0	0
	Post dosing 1 (n=10)	3.61 (11.90)	4.65 (10.41)	0	0	0	0
	Post dosing 2 (n=9)	549.6 (1077)	8.86 (20.73)	0	0	0	0
	Post dosing 3 (n=7)	49.8 (49.80)	3.94 (7.35)	0	0	0	0
1.0	Pre dosing (n=9)	233.0 (20.15)	10.92 (1.23)	0	0	0	0
	Post dosing 1 (n=10)	1232 (1456)	122.4 (34.22)	0	22.59 (31.86)	6.30 (68.78)	9.62 (0.90)
	Post dosing 2 (n=4)	0	25.12 (39.42)	0	10.55 (1.16)	21.34 (21.13)	0
	Post dosing 3 (n=2)	0	0	0	0	0	0
2.5	Pre dosing (n=10)	0	2.49 (7.52)	0	0	2.39 (10.93)	0
	Post dosing 1 (n=10)	1285 (1097)	69.57 (93.12)	0	0	24.93 (45.82)	1.00 (6.56)
	Post dosing 2 (n=10)	2787 (3305)	178.1 (378.2)	1.04 (8.38)	237.5 (756.1)	57.32 (121.96)	73.77 (238.3)
	Post dosing 3 (n=4)	2094 (2704)	69.23 (75.46)	0	0	32.64 (42.73)	0
5.0	Pre dosing (n=8)	0	3.03 (9.80)	0	0	0	0
	Post dosing 1 (n=8)	5549 (5592)	193.6 (450.6)	0	1.17 (9.93)	105.4 (253.4)	0
	Post dosing 2 (n=8)	2266 (1918)	492.0 (1345)	0	0	65.52 (171.1)	0
	Post dosing 3 (n=2)	6098 (8301)	19.16 (35.19)	0	412.9 (717.1)	45.56 (80.93)	78.5 (138.0)

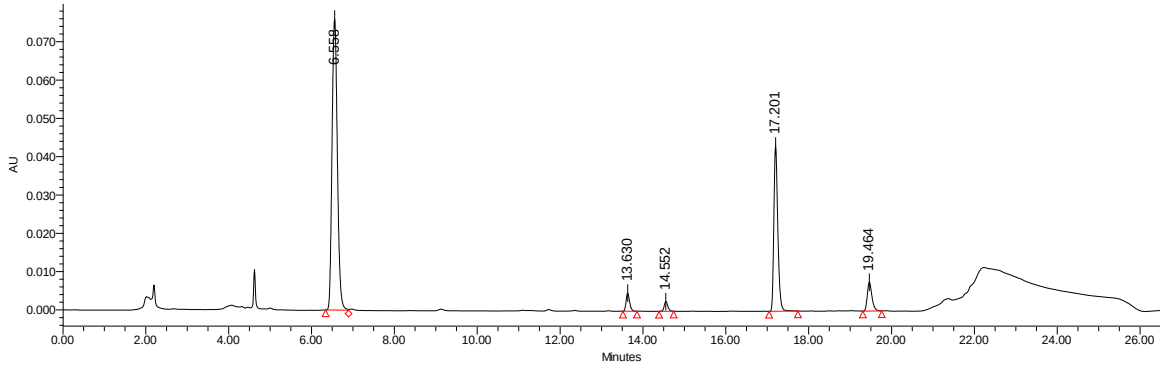
All values represent the mean ng/g of dry weight of faeces (± SD). On the 5.0 g dose level, 2 participants dosed in Michigan did not have any stool samples collected for analysis.

4.1.2 Colorectal cancer patients

Plasma and colorectal tissue samples were collected from 20 patients with colorectal cancer (10 per dose level of 0.5 and 1.0 g resveratrol). Pre dosing blood samples (pre day 1) and post dosing blood and tissue samples, collected in the operating theatre, were analysed for the presence of resveratrol and its metabolites by UV-HPLC. Figure 4.1.25 shows a representative chromatogram from the analysis of extracts of colorectal cancer and macroscopically normal colorectal tissue from a patient on the 1.0 g dose level. In all participants, blood samples were drawn as close to the time of surgical resection as possible to enable a comparison to be made between the concentration of resveratrol and its metabolites in the plasma with those in the colorectal specimen.

Time from oral administration of the last (8^{th}) oral dose of resveratrol to the time of surgical resection of the colorectal tissue ranged from 15.00 to 23.00 h (mean 18.39 h) and time to collection of plasma ranged from 11.00 to 21.00 h (mean 17.80 h). The resected colorectal specimen was taken immediately to the Pathology Department where samples were obtained from the tumour and from macroscopically normal colorectal tissue, 5 and 10 cm proximal and distal to the tumour and from the proximal and distal resection margins. The availability of normal colorectal tissue at these distances from the tumour depended on the location of the tumour within the resected specimen as well as the total length of resected bowel. Therefore, in some patients it was not possible to collect the samples 5 and 10 cm proximal and distal to the tumour. This was particularly the case for distal tissue samples.

(A)Tumour tissue



(B) Distal resection margin

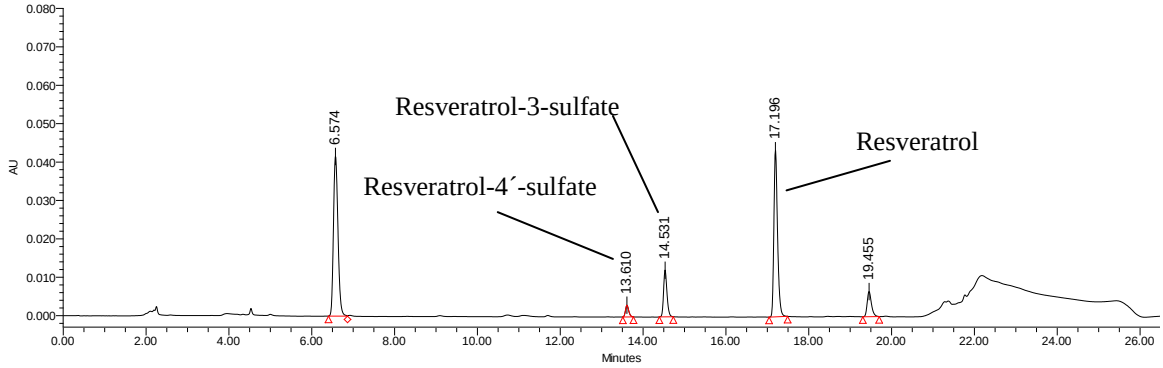


Figure 4.1.25 Representative HPLC chromatograms of resveratrol and its metabolites in (A) tumour and (B) distal resection margin from a resection specimen in a study participant who consumed 8 daily doses of 1.0 g resveratrol. The tumour was located in the left side of the colon (sigmoid colon). Identification of resveratrol and metabolites was based on retention times of standards under identical conditions as described in Section 2.2.1.3.

The concentration of resveratrol detected in tumour tissue ranged from 0.00 to 14.97 nmol/g (mean 2.94 ± 4.72 nmol/g) and 0.30 to 194.8 nmol/g (mean 43.92 ± 74.15) for the 0.5 and 1.0 g dose respectively, where a value of 0.00 signifies concentrations less than the lower limit of quantification. The main metabolite found in the tumour tissue was resveratrol-3-sulfate, which was up to 8-fold lower than the concentration of the parent compound and ranged from 0.00-2.85 nmol/g (mean 0.91 ± 1.28 nmol/g) and 0.00 to 24.69 nmol/g (mean 3.58 ± 7.49 nmol/g) in samples from patients on the 0.5 and 1.0 g dose respectively, as shown in Table 4.1.9.

In the macroscopically normal colorectal tissue, including all tissue sections proximal and distal to the tumour, the concentration of resveratrol in those on the 0.5 g dose ranged from 0.00 to 45.93 nmol/g (mean 18.96 ± 38.97 nmol/g) and on the 1.0 g dose from 0.00 to 3774 nmol/g (mean 867.8 ± 2564 nmol/g). The corresponding concentration of resveratrol-3-sulfate was 0.00-94.54 nmol/g (mean 22.75 ± 63.05 nmol/g) on the 0.5 g dose and 0.00-219.5 nmol/g (mean 64.70 ± 163.3 nmol/g) on the 1.0 g dose level (Table 4.1.10). Although resveratrol-3-sulfate was the main metabolite in tumour tissue, it was only the main metabolite in the 1.0 g dose level in normal tissue with monoglucuronide 2, the predominant metabolite in samples from patients on the 0.5 g dose.

In both the tumour and the normal colorectal tissue there was large inter-individual variability apparent in the concentration of resveratrol and its metabolites detectable at each dose level. Figures 4.1.26-4.1.31 show the mean concentration of resveratrol and its metabolites across the different colorectal tissue samples collected from the resected

specimen for each dose level. Each macroscopically normal tissue section represents a certain distance either proximal or distal to the tumour. These figures also illustrate the wide range of concentrations for these compounds in the different tissue sections with relatively large standard deviations and seem to suggest higher concentrations of resveratrol and its metabolites in the proximal tissue sections compared to the tumour and distal sections. However, for most participants due to location of the tumour, it was not possible to obtain as much tissue distal as proximal to the tumour. In addition, although most of the levels of these compounds generally showed an increase across the 2 dose levels in the tissue sections (tumour and normal tissue), in contrast resveratrol disulfate and the monoglucuronides showed a reduction in many of the sections.

Table 4.1.9 Concentration of resveratrol and its metabolites in colorectal tumour tissue (nmol/g) after consumption of 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) and range for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected.

	Dose							
	0.5 g				1.0 g			
	Range (nmol/g)				Range (nmol/g)			
Compound	Lower limit	Upper limit	Mean (nmol/g)	SD	Lower limit	Upper limit	Mean (nmol/g)	SD
Resveratrol	0.00	14.97	2.94	4.72	0.30	194.8	43.92	74.15
Resveratrol-3-sulfate	0.00	2.85	0.91	1.28	0.00	24.69	3.58	7.49
Resveratrol disulfate	0.00	1.33	0.44	0.59	0.00	0.00	0.00	0.00
Resveratrol-4´-sulfate	0.00	0.75	0.19	0.30	0.00	8.39	1.04	2.51
Monoglucuronide 1	0.00	0.49	0.09	0.19	0.00	1.00	0.25	0.34
Monoglucuronide 2	0.00	1.23	0.12	0.39	0.00	1.05	0.19	0.34

Table 4.1.10 Concentration of resveratrol and its metabolites in macroscopically normal colorectal tissue (nmol/g) after consumption of 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) and range for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected.

	Dose							
	0.5 g				1.0 g			
	Range (nmol/g)				Range (nmol/g)			
Compound	Lower limit	Upper limit	Mean (nmol/g)	SD	Lower limit	Upper limit	Mean (nmol/g)	SD
Resveratrol	0.00	45.93	18.96	38.97	0.00	3774.3	867.8	2564
Resveratrol-3-sulfate	0.00	94.54	22.75	63.05	0.00	219.5	64.70	163.2
Resveratrol disulfate	0.00	18.91	7.69	16.32	0.00	2.02	0.45	1.02
Resveratrol-4´-sulfate	0.00	3.34	0.84	2.46	0.00	9.31	3.76	5.90
Monoglucuronide 1	0.00	15.92	3.96	11.22	0.00	3.00	2.20	2.61
Monoglucuronide 2	0.00	178.7	39.19	122.8	0.00	2.02	7.95	18.69

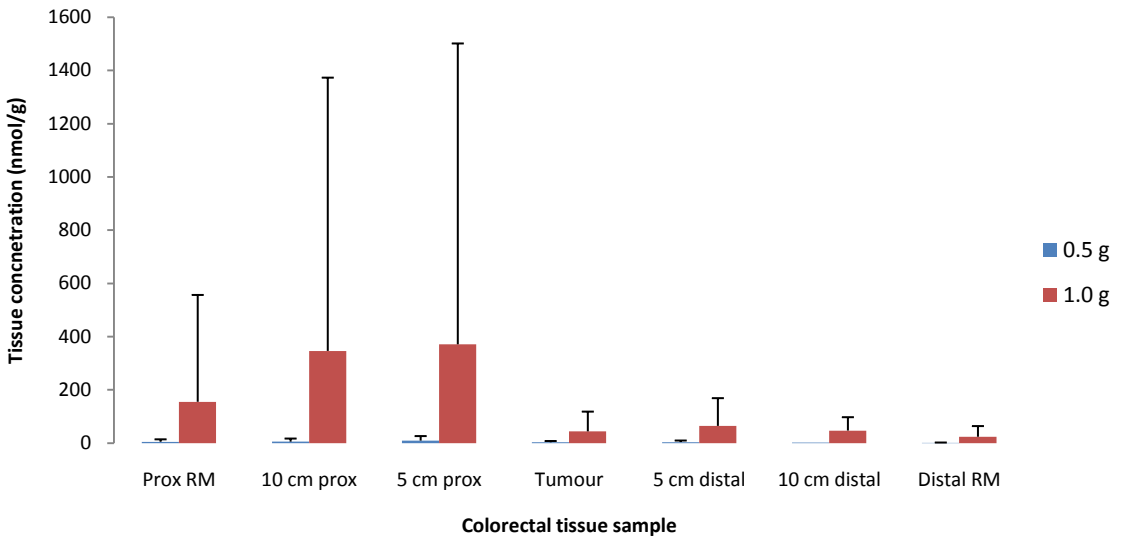


Figure 4.1.26 Mean concentration of resveratrol in different sections of colorectal and tumour tissue taken from patients who received 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected. Each tissue section corresponds to a certain distance from the tumour (prox RM represents proximal resection margin and distal RM represents distal resection margin).

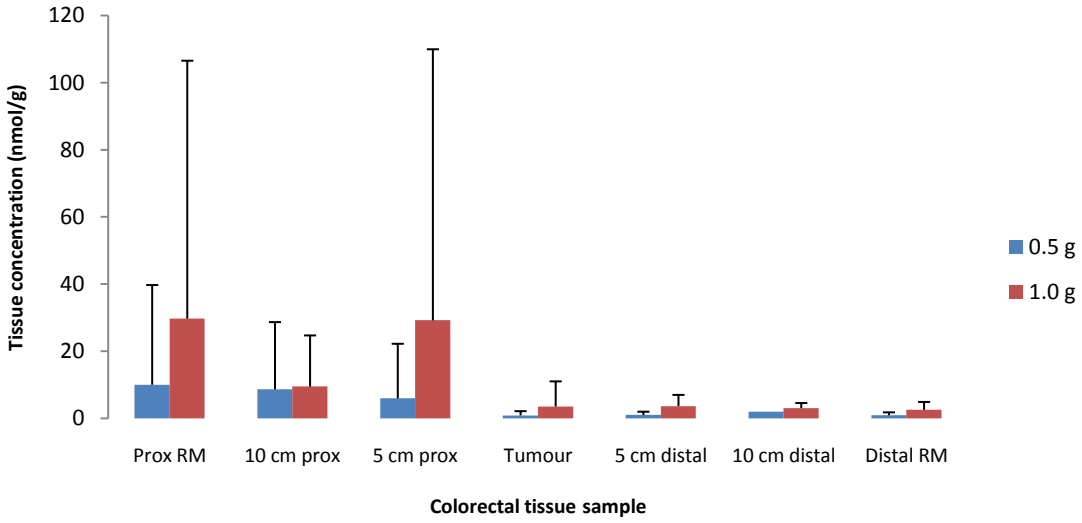


Figure 4.1.27 Mean concentration of resveratrol-3-sulfate in different sections of colorectal and tumour tissue taken from patients who received 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected. Each tissue section represents distance from the tumour (prox RM represents proximal resection margin and distal RM represents distal resection margin).

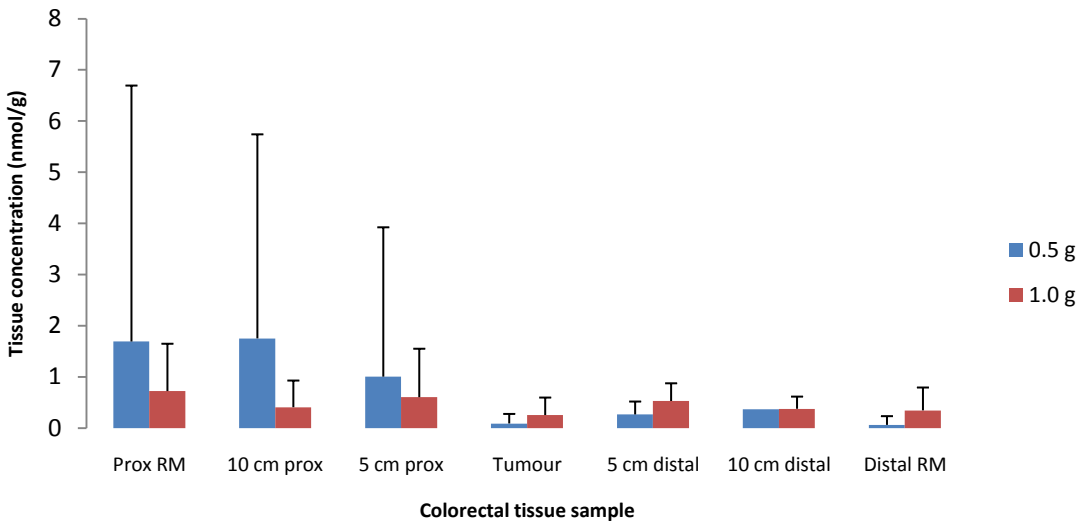


Figure 4.1.28 Mean concentration of monoglucuronide 1 in different sections of colorectal and tumour tissue taken from patients who received 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected. Each tissue section represents distance from the tumour (prox RM represents proximal resection margin and distal RM represents distal resection margin).

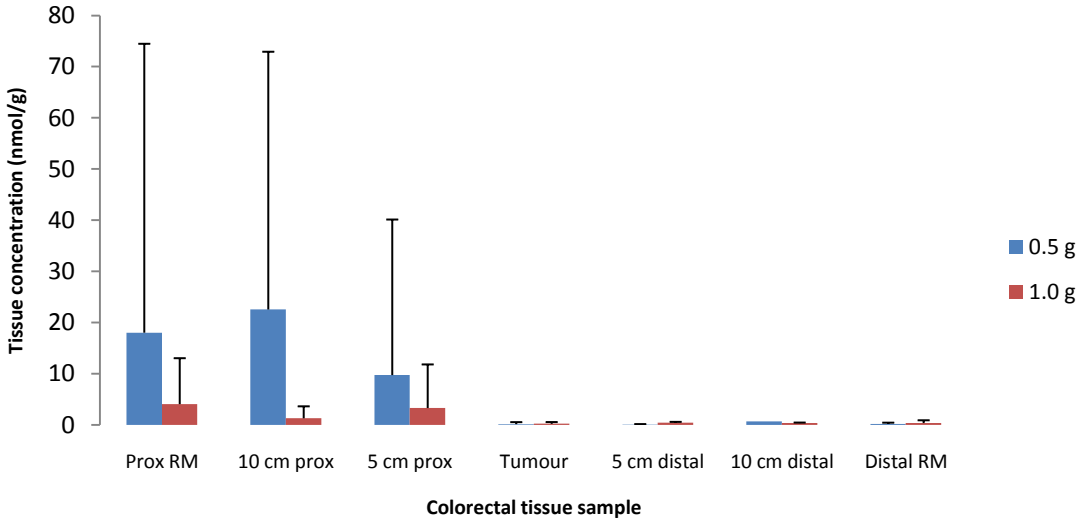


Figure 4.1.29 Mean concentration of monoglucuronide 2 in different sections of colorectal and tumour tissue taken from patients who received 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected. Each tissue section represents distance from the tumour (prox RM represents proximal resection margin and distal RM represents distal resection margin).

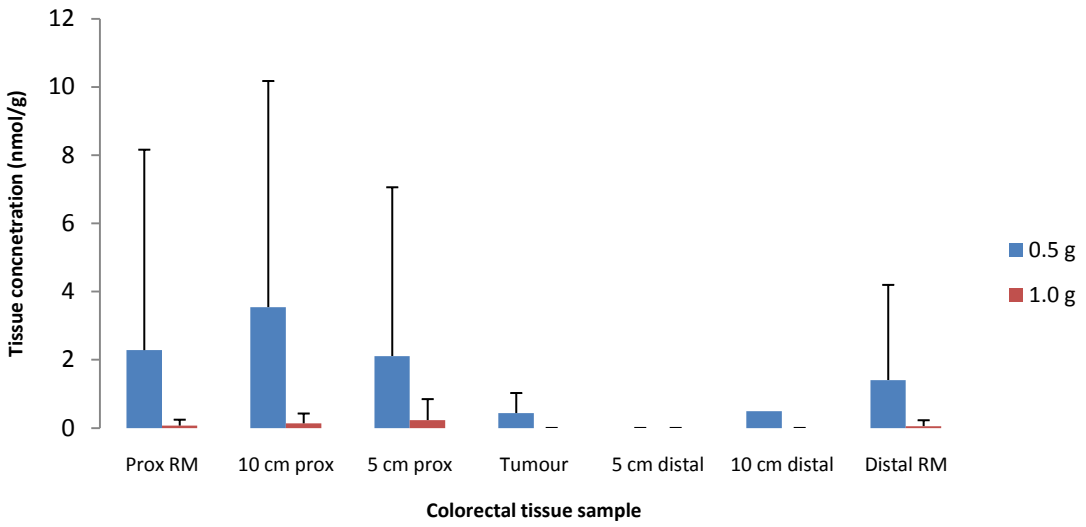


Figure 4.1.30 Mean concentration of disulfate in different sections of colorectal and tumour tissue taken from patients who received 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected. Each tissue section represents distance from the tumour (prox RM represents proximal resection margin and distal RM represents distal resection margin).

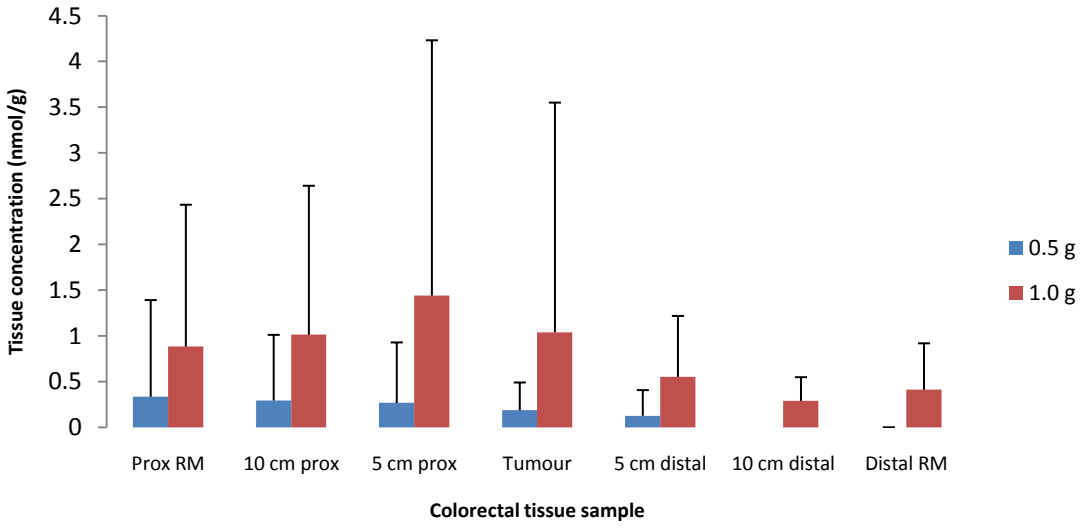


Figure 4.1.31 Mean concentration of resveratrol-4´-sulfate in different sections of colorectal and tumour tissue taken from patients who received 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers per dose level. One participant (1.0 g) had 2 colorectal tumours resected. Each tissue section represents distance from the tumour (prox RM represents proximal resection margin and distal RM represents distal resection margin).

The colorectal tumours can be separated according to their location, with right sided tumours including those in the caecum, ascending colon and hepatic flexure/transverse colon and left sided tumours encompassing those at the splenic flexure, descending colon, sigmoid colon and rectum. Right sided tumours were all resected by a right hemicolectomy and left sided tumours as an anterior resection or a sigmoid colectomy. Generally, much higher concentrations of resveratrol and its metabolites were seen in right sided tumours and the corresponding colorectal tissue.

Tables 4.1.11 and 4.1.12 show the mean concentration of resveratrol and its metabolites in tumour and normal colorectal tissue for left and right sided resections. Mean concentrations for the normal tissue are shown for all the proximal samples (5 and 10 cm to the tumour and the resection margin) and distal samples collected (5 and 10 cm to the tumour and the resection margin). The concentration of resveratrol in left sided colorectal samples, both normal and tumour, ranged from 0.00 to 92.49 nmol/g across the two dose levels compared to 0.00 to 3774 nmol/g for right sided. The corresponding concentration for resveratrol-3-sulfate was 0.00 to 8.70 nmol/g (left sided) and 0.49 to 219.5 nmol/g (right sided). Tables 4.1.11 and 4.1.12, illustrate the noticeable difference in concentrations (mean and range) of resveratrol and its metabolites dependent on whether the tumour and related colon tissue is situated on the right or left side of the large intestine. Higher concentrations were present in tissue located in the caecum, hepatic flexure and transverse colon (right sided) compared to sigmoid, recto-sigmoid and rectum (left sided).

Table 4.1.11 Comparison of colorectal tissue concentrations of resveratrol and its metabolites in right versus left sided cancer and normal colorectal tissue after 8 daily doses of 0.5 g resveratrol. Values represent mean (± SD) and range for n=7 (left sided) and n=3 (right sided). Normal tissue represents the mean of all sections either proximal or distal to the tumour. Right sided tissue includes caecum, ascending colon and hepatic flexure. Left sided tumours represent splenic flexure, descending colon, sigmoid colon and rectum.

0.5 g		Left sided (n=7)			Right sided (n=3)		
		Proximal to tumour	Tumour	Distal to tumour	Proximal to tumour	Tumour	Distal to tumour
Monoglucuronide 1	Range (nmol/g)	0.00 to 0.52	0.00 to 0.39	0.00 to 0.56	0.00 to 15.92	0.00 to 0.49	0.00 to 0.37
	Mean (nmol/g)	0.10	0.06	0.14	4.46	0.16	0.15
	SD	0.19	0.15	0.24	6.31	0.28	0.20
Monoglucuronide 2	Range (nmol/g)	0.00 to 0.43	0.00 to 0.00	0.00 to 0.40	0.00 to 178.72	0.00 to 1.23	0.00 to 0.64
	Mean (nmol/g)	0.07	0.00	0.04	4856	0.41	0.36
	SD	0.16	0.00	0.13	70.75	0.71	0.26
Resveratrol disulfate	Range (nmol/g)	0.00 to 2.22	0.00 to 1.33	0.00 to 7.50	0.00 to 18.91	0.00 to 1.28	0.00 to 0.49
	Mean (nmol/g)	0.52	0.34	1.25	6.97	0.66	0.10
	SD	0.80	0.59	2.65	8.60	0.64	0.22
Resveratrol-3-sulfate	Range (nmol/g)	0.00 to 1.15	0.00 to 1.41	0.00 to 2.51	0.00 to 94.54	0.51 to 3.51	1.37 to 2.17
	Mean (nmol/g)	0.61	0.32	0.70	25.19	2.29	1.75
	SD	0.57	0.52	0.87	35.96	1.58	0.32
Resveratrol-4´-sulfate	Range (nmol/g)	0.00 to 0.58	0.00 to 0.75	0.00 to 0.00	0.00 to 3.34	0.00 to 0.58	0.00 to 0.63
	Mean (nmol/g)	0.03	0.18	0.00	0.90	0.19	0.13
	SD	0.14	0.32	0.00	1.32	0.34	0.28
Resveratrol	Range (nmol/g)	0.00 to 3.01	0.00 to 1.80	0.00 to 1.04	0.00 to 45.93	3.11 to 14.97	2.47 to 13.45
	Mean (nmol/g)	0.71	0.63	0.48	18.58	8.33	4.94
	SD	0.72	0.69	0.47	17.39	6.06	4.82

Table 4.1.12 Comparison of colorectal tissue concentrations of resveratrol and its metabolites in right versus left sided cancer and normal colorectal tissue after 8 daily doses of 1.0 g resveratrol. Values represent mean (± SD) and range for n=6 (left sided) and n=5 (right sided). Normal tissue represents the mean of all sections either proximal or distal to the tumour. Right sided tissue includes caecum, ascending colon and hepatic flexure. Left sided tumours represent splenic flexure, descending colon, sigmoid colon and rectum.

1.0 g		Left sided (n=6)			Right sided (n=5)		
		Proximal to tumour	Tumour	Distal to tumour	Proximal to tumour	Tumour	Distal to Tumour
Monoglucuronide 1	Range (nmol/g)	0.00 to 0.69	0.00 to 0.59	0.00 to 0.76	0.00 to 3.00	0.00 to 1.00	0.00 to 1.34
	Mean (nmol/g)	0.26	0.25	0.29	1.15	0.25	0.51
	SD	0.24	0.26	0.27	1.28	0.50	0.45
Monoglucuronide 2	Range (nmol/g)	0.00 to 0.56	0.00 to 0.00	0.00 to 0.61	0.33 to 28.67	0.33 to 1.05	0.20 to 1.83
	Mean (nmol/g)	0.15	0.00	0.19	7.80	0.53	0.50
	SD	0.18	0.00	0.12	11.62	0.37	0.48
Resveratrol disulfate	Range (nmol/g)	0.00 to 0.62	0.00 to 0.52	0.00 to 0.54	0.00 to 2.02	0.00 to 0.00	0.00 to 0.00
	Mean (nmol/g)	0.90	0.00	0.06	0.28	0.00	0.00
	SD	0.20	0.00	0.18	0.81	0.00	0.00
Resveratrol-3-sulfate	Range (nmol/g)	0.00 to 8.70	0.00 to 4.31	0.00 to 4.89	3.81 to 219.5	1.39 to 24.69	1.18 to 8.82
	Mean (nmol/g)	1.25	0.29	1.70	63.09	9.33	4.21
	SD	2.10	0.53	1.93	118.09	10.82	2.46
Resveratrol-4´-sulfate	Range (nmol/g)	0.00 to 5.08	0.00 to 1.99	0.00 to 1.61	0.24 to 9.31	0.00 to 8.39	0.00 to 0.96
	Mean (nmol/g)	0.41	0.27	0.38	2.53	2.36	0.49
	SD	1.21	0.75	0.68	3.08	4.03	0.34
Resveratrol	Range (nmol/g)	0.00 to 92.49	0.00 to 36.69	0.88 to 21.88	10.12 to 3774	12.74 to 149.8	10.65 to 272.2
	Mean (nmol/g)	8.51	4.90	11.12	862.0	122.2	66.40
	SD	22.24	8.06	20.50	1684	91.82	83.18

On the 1.0 g dose level, a participant had 2 colorectal tumours, 1 in the caecum, the other in the sigmoid colon. The histopathology report produced by a Consultant Pathologist at the University Hospitals of Leicester, stated that both tumours were the same size and classified as Dukes A colon cancers, which means the cancer involved the inside wall of the colon but had not spread to the outer wall or outside the colon. In this individual there were no samples collected 10 cm proximal and distal to the tumour due to the location of the tumour and the length of the resection specimen. The colorectal samples collected enabled a direct comparison of left versus right sided levels of resveratrol and its metabolites in the same patient to determine whether the effect suggested from the analysis of all patients was a real phenomenon or a consequence of inter-individual variability. In this individual, in all the resection tissue samples collected from the right hemicolectomy, there were higher concentrations of resveratrol, resveratrol-3-sulfate and the monoglucuronides compared to concentrations of these compounds in the tissue samples collected from the sigmoid colectomy (Figures 4.1.32 and 4.1.33). Resveratrol disulfate was not detected in any of the tissue samples from this participant (left or right sided) and no resveratrol-4´-sulfate was observed in the left sided specimen. The concentration of resveratrol-4´-sulfate in the right sided tissue samples was 1.05 nmol/g (PRM), 2.83 nmol/g (5 cm proximal), 0.77 nmol/g (tumour), 0.80 nmol/g (5 cm distal), 0.66 nmol/g (distal resection margin).

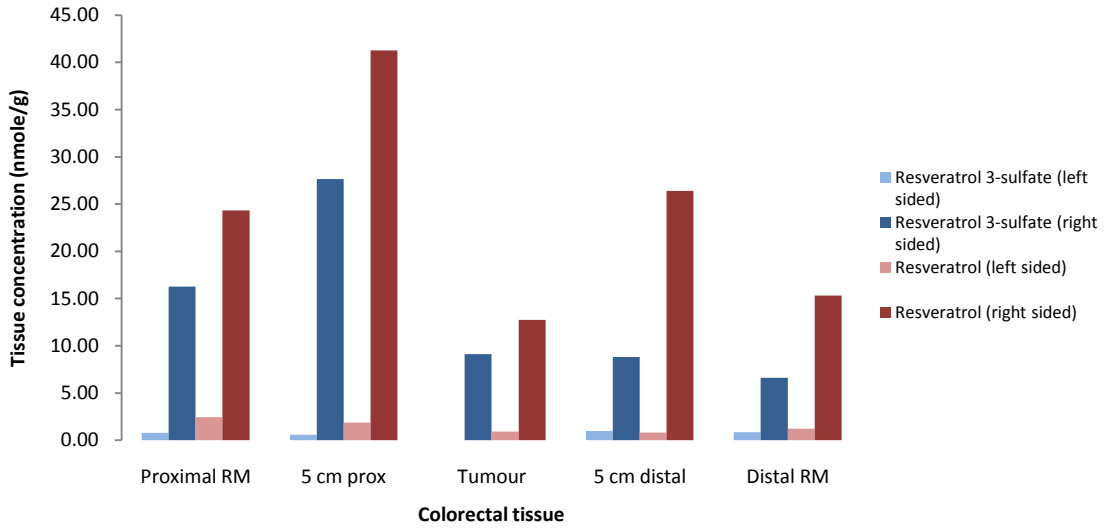


Figure 4.1.32 Comparison of the concentration of resveratrol and resveratrol-3-sulfate in left and right sided colorectal tissue in one individual who consumed 8 daily doses of 1.0 g resveratrol.

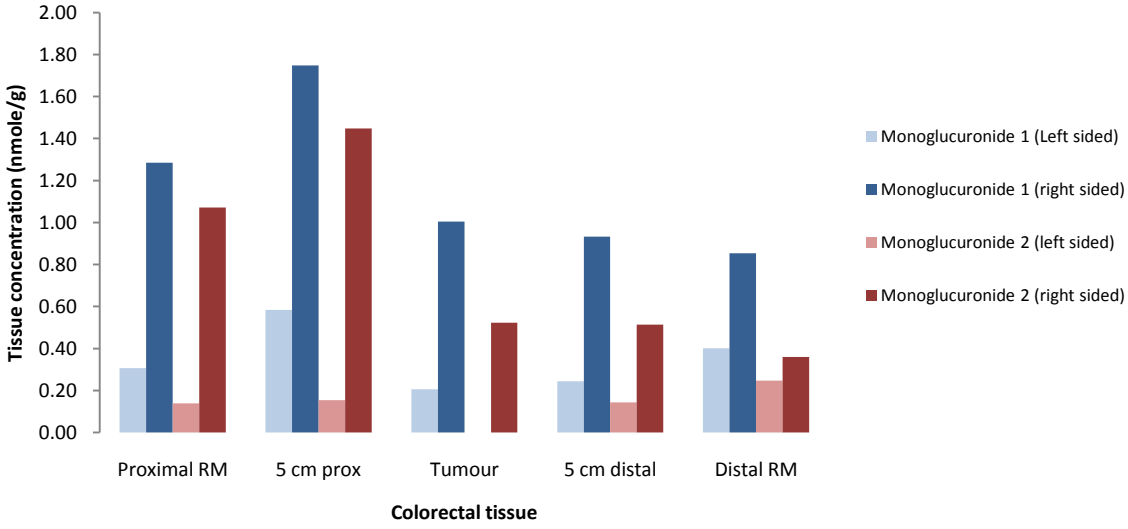


Figure 4.1.33 Comparison of the concentration of resveratrol glucuronide 1 and 2 in left and right sided colorectal tissue in one individual who consumed 8 daily doses of 1.0 g resveratrol.

4.1.2.1 Concentration of resveratrol and its metabolites in plasma

Bloods were drawn from all patients pre dosing and as close to surgical resection of the colorectal tumour as possible on day 9, after 8 daily doses of resveratrol. As observed for the healthy volunteers, only small amounts of the parent compound were detectable in the plasma. The metabolites, resveratrol disulfate and resveratrol-3-sulfate were the main compounds observed, with the former having the highest concentrations in both dose levels, up to 101 times the concentration of resveratrol (Figures 4.1.34 and 4.1.35). As would be expected, there was no significant difference seen in the plasma concentrations between participants with right and left sided tumours.

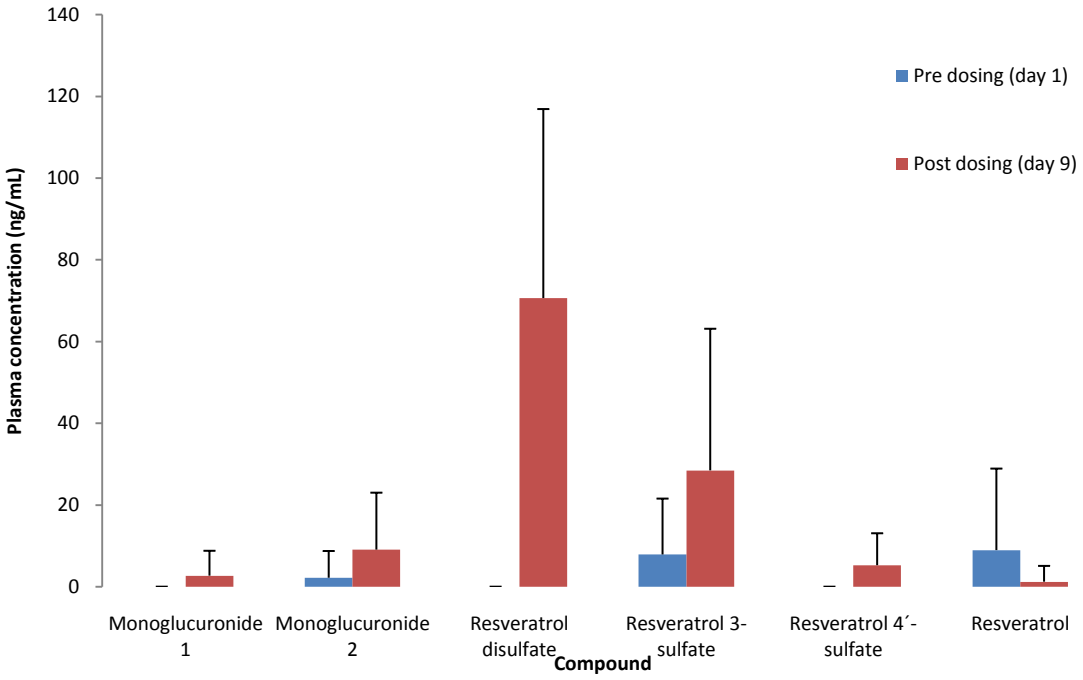


Figure 4.1.34 Plasma concentration of resveratrol and its metabolites in participants who consumed 8 daily doses of 0.5 g resveratrol. Values represent mean (+SD) for 10 volunteers**.** Pre dosing represents blood drawn prior to dosing and post dosing refers to blood drawn during the operation on day 9.

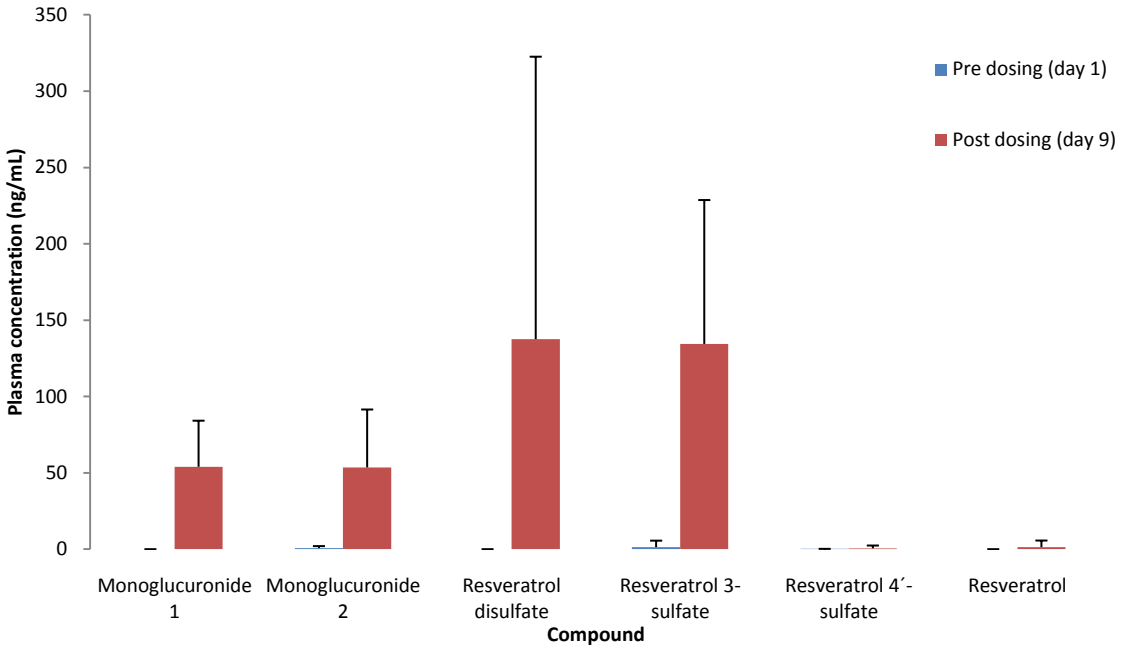


Figure 4.1.35 Plasma concentration of resveratrol and its metabolites in participants who consumed 8 daily doses of 1.0 g resveratrol. Values represent mean (+SD) for 10 volunteers**.** Pre dosing represents blood drawn prior to dosing and post dosing refers to blood drawn during the operation on day 9.

4.1.2.2 Relationship between plasma and tissue concentrations

At the time of tumour resection, there was very little resveratrol detectable in the plasma with concentrations of up to 0.06 µM. In contrast, much higher concentrations were present in the tissue (up to 3774 nmol/g). The main metabolite in plasma was resveratrol disulfate but this was not the main metabolite in the tissue. There was no obvious direct correlation between the concentration of resveratrol and its metabolites in colorectal tissue and plasma but it is difficult to draw definite conclusions due to the small number of patients on each dose level, high inter-individual variation in plasma and tissue concentrations as well as differences in the tumour location. A comparison of tissue concentration (nmol/g) was compared to plasma concentration (µM) on the assumption that 1g of tissue equals 1 mL and therefore nmol/g can be considered equivalent to µM. Repeat dosing with resveratrol gave plasma concentrations of up to 0.28 µM (monoglucuronide 1), 0.32 µM (monoglucuronide 2), 1.68 µM (disulfate), 0.98 µM (resveratrol-3-sulfate), 0.07 µM (resveratrol-4´-sulfate) and 0.06 µM (resveratrol) across the two dose levels. The corresponding concentrations detected in the colorectal tissue were much higher at 15.92, 178.7, 18.91, 219.5, 9.31 and 3774 nmol/g. Although these levels are the upper range achieved, no obvious correlation was seen when comparing the mean values for plasma and tissue for these compounds, and comparing samples from each participant.

CHAPTER 5

TOLERABILITY AND PHARMACOKINETIC

DISCUSSION

5.1 Discussion

Given the high probability of developing cancer during a normal human lifespan, cancer chemoprevention, for the delay or reversal of this process, provides an attractive therapeutic strategy (Pezzuto, 2008). For chemopreventive interventions to be successful they must be provided in doses that are effective, but be virtually free of toxicities and be acceptable to the individual to ensure compliance. In the healthy volunteer and colorectal studies presented in this thesis, no serious adverse events were reported following daily oral doses of up to 5.0 g resveratrol, with adverse events graded as 1 or 2 (mild or moderate) in severity. The safety profile observed here for resveratrol administered repeatedly at very high doses seems encouraging. The lack of serious adverse effects is consistent with the safety of resveratrol observed in previous clinical and preclinical studies (Almeida *et al*., 2009; Boocock *et al*., 2007; Sale *et al*., 2005; Williams *et al*., 2009), although the work presented here is unique in terms of dose and dosing schedule. The main side effects reported were gastrointestinal, including diarrhoea, abdominal pain and nausea, which occurred predominately at the top two dose levels in the healthy volunteer study (2.5 and 5.0 g). Although total faecal excretion of resveratrol was not calculated, the mean resveratrol concentrations for all faecal samples collected whilst dosing were 206.0 ± 663.8, 1005 ± 2154, 2046 ± 2488 and 4253 ± 4931 ng/g for the 0.5, 1.0, 2 5 and 5.0 g dose levels, respectively. Large inter-individual variations in concentrations of resveratrol detected at each dose level were apparent, as well as variation between samples collected

from each individual, nevertheless, overall there was a trend towards an increase in faecal levels of resveratrol, as the dose increased from 0.5 to 5.0 g.

Severe diarrhoea has been reported in patients who have been administered other plant polyphenols, and it has been mechanistically associated with an increase in intracellular cyclic AMP (cAMP) (Jatoi *et al*., 2003; Laurie *et al*., 2005). Resveratrol itself has been shown to raise intracellular cAMP levels in breast cancer cells *in vitro* (El-Mowafy and Alkhalaf, 2003), and resveratrol at concentrations of 100 μM was found to have significant effects on cAMP-dependent chloride secretion in T84 colon cancer cells and mouse jejunal epithelial membrane (Blumenstein *et al*., 2005). It was suggested that the underlying mechanism was related to the reduced expression of adenyl cyclase and decreased expression of the basolateral Na-K-2Cl cotransporter, which can lead to an impairment of basolateral chloride entry into the cell. Most of the GI side effects reported were at the top two dose levels of resveratrol, but interestingly, there was no increase in severity of diarrhoea as the dose increased from 2.5 to 5.0 g. With the 2 dose levels combined, participants who reported diarrhoea symptoms had a significantly higher mean faecal resveratrol concentration (4337.5 ± 4219.1 ng/g, n=10) compared to those participants without these symptoms (1192.2 ± 1518.2 ng/g, n=8, P=0.02, Independent T test). Across the dose levels, all diarrhoea symptoms were graded as mild to moderate in severity and all individuals affected were able to continue dosing with resveratrol.

In a recently reported clinical double-blind study, two subjects in each of four groups of ten healthy volunteers were randomized to receive placebo or resveratrol at doses of up to 150

mg, six times/day, for thirteen doses (Almeida *et al*., 2009). Adverse events possibly related to resveratrol included headache, myalgia of lower extremities, dizziness and one episode of epididymitis. All clinical adverse events were classified as mild and resveratrol was considered well tolerated with no dose-related differences apparent. Although in this particular study conducted by Almeida *et al*., the individual doses ingested were lower than in the healthy volunteer study presented in this thesis, doses were taken six times a day, making the total daily intake at the top 2 dose levels (Almerida *et al*., 2009), close to that ingested by healthy volunteers in the 0.5 and 1.0 g (lower doses) in the present study. This comparison supports the conclusion that repeat oral daily dosing with resveratrol is probably well tolerated and safe.

Although several studies have described the pharmacokinetics of resveratrol in animal model systems, there are few such studies in humans to date. In the present healthy volunteer study, resveratrol was shown to be quickly absorbed following oral administration with maximum plasma concentrations being reached on average between 0.88 and 3.43 h after administration. Peak plasma concentrations of up to 0.43, 1.31, 3.18 and 7.40 μM of resveratrol were detected in study participants on the 0.5, 1.0, 2.5 and 5.0 g dose levels, respectively. Although circulating levels of parent resveratrol were low, there were relatively high concentrations of the metabolites. The observation that oral bioavailability for the parent resveratrol is low, is consistent with previous studies in both animals and humans (Section 1.3.3). In the study of resveratrol described above by Almeida *et al.,* (Ameida et al., 2009), following daily dosing with 25, 50, 100 and 150 mg resveratrol, maximum plasma concentrations of resveratrol were reached within 0.8–1.5 h

post dose. Relatively low concentrations of resveratrol were detected in the plasma, with mean peak plasma concentration (C_{max}) of 3.89, 7.39, 23.1 and 63.8 ng/mL respectively. Taking the total daily dose consumed as 900 and 600 mg of resveratrol for the top 2 dose levels, the mean C_{max} values reported are lower than those presented in this thesis, where mean plasma C_{max} values of 44.7 and 140 ng/mL were detected in healthy volunteers in the 0.5 and 1.0 g dose levels respectively. After the administration of ^{14}C-labelled resveratrol (25 mg) to humans, mean peak plasma levels of resveratrol equivalents (parent compound plus metabolites) of 2.0 ± 0.4 μM were detected (Walle *et al*., 2004). However, only trace amounts of unchanged resveratrol (<5 ng/ml) were present in the plasma in this study.

The plasma kinetics of resveratrol after oral administration have been investigated in rats and mice (Section 1.3.3) (Asensi *et al*., 2002; Bertelli *et al*., 1996; Juan *et al*., 2002; Marier *et al*., 2002; Meng *et al*., 2004; Sale *et al*., 2004; Soleas *et al*., 2001; Vitrac *et al*., 2003). The results of these studies are generally in agreement with human studies, including the present healthy volunteer study. They all show efficient and fast absorption of resveratrol, with plasma concentrations of resveratrol consistently lower than its metabolites. Mean peak plasma levels of 1.2 and 6.6 μM were achieved in rats after resveratrol doses (i.g) of 20 mg/kg (Asensei *et al*., 2002) and 50 mg/kg (Marier *et al*., 2002), respectively.

Oral bioavailability is thought to be dependent on a number of factors, including the aqueous solubility, membrane permeability and metabolic stability of the drug under study (Hurst *et al*., 2007). Intestinal absorption of resveratrol has been investigated in several models, including isolated rat small intestine (Andlauer *et al*., 2000; Kuhnle *et al*., 2000),

rodents after oral administration (Asensi *et al*., 2002; Bertelli *et al*., 1998; Marier *et al*., 2002; Soleas *et al*., 2001; Vitrac *et al*, 2003), human intestinal CaCo-2 cells (Kaldas *et al*., 2003) and healthy volunteers (Goldberg, *et al*., 2003) as discussed in Section 1.3.3. Although the high lipophilicity of resveratrol and corresponding low aqueous solubility may restrict oral bioavailability, studies in animals and humans suggest that absorption is not a major problem, since ~70% is absorbed orally (Walle *et al*., 2004). More importantly, rapid metabolic removal of resveratrol with efficient glucuronidation and sulfation of the parent compound in both liver and intestinal epithelial cells is most likely the primary reason for the restricted oral bioavailability of resveratrol itself (Kuhnle *et al*., 2000; Marier *et al*., 2002). Whilst the mechanism of resveratrol movement across the intestinal epithelium appears to be rapid passive diffusion (Li *et al*., 2003), the uptake of resveratrol in hepatic cells probably results from the contribution of two processes, a passive one and a carrier-mediated one. Vitrac *et al*., studied the distribution of ^{14}C-resveratrol in mouse tissues after oral administration and showed that the highest accumulation of radioactivity was found in the liver. HPLC analysis of the radioactive compounds in liver extracts 3 h after administration showed the presence of ^{14}C-resveratrol, together with a relatively high concentration of unidentified radioactive glucuronide or sulfated conjugate, which was considered by the authors to be due to some active metabolic accumulation process, either as a result of metabolism of resveratrol in the small intestine and its subsequent absorption, or metabolism *in situ*, in the liver (Vitrac *et al*., 2003).

Consistent with many animal and human studies in the literature, the present study demonstrates that resveratrol is metabolised primarily to glucuronide and sulfate

conjugates. For example, after Yu and co-workers (Yu *et al*., 2002) administered resveratrol (60 mg/kg) to mice, via the i.g. route, resveratrol glucuronide and sulfate were detected as the only resveratrol metabolites in serum samples. The resveratrol sulfate concentration reached a maximal value in mouse serum after 30 min, and serum resveratrol disappeared after 30 min, with resveratrol sulfate and resveratrol glucuronide still detectable 3 h after dosage. In humans (Boocock *et al*., 2007; Goldberg *et al*., 2003; Walle *et al*., 2004) and in animals (Abd El-Mohsen *et al*., 2006; Marier *et al*., 2002; Wenzel *et al*., 2005) after oral administration of resveratrol, the amount of free resveratrol in plasma and serum accounted for less than 5% of total resveratrol (resveratrol and its metabolites) with higher concentrations of sulfate and glucuronide conjugates. The present healthy volunteer study supports these findings with the amount of free resveratrol (parent compound) in the plasma across the dose levels accounting for between 3.6 and 8.0% of the total resveratrol.

In the present healthy volunteer study, two isomeric glucuronic acid conjugates and three sulfate conjugates were identified in the plasma. The main metabolite observed was resveratrol-3-sulfate, which was clearly detectable in plasma samples within 2 h after dosing. The rapid formation of this metabolite suggests that sulfation may be one of the main factors limiting the bioavailability of resveratrol. After daily dosing with resveratrol, the mean peak concentration of the parent compound was 44.68, 140.0, 331.2 and 954.2 ng/mL on the 0.5, 1.0, 2.5 and 5.0 g, respectively. Apart from resveratrol-4´-sulfate and the disulfate, the concentration of all other metabolites formed was much higher than the parent compound. For the 3-sulfate, the major product formed, the mean peak plasma concentrations were 13, 12, 7 and 4 fold higher than the parent compound in humans on the

0.5, 1.0, 2.5 and 5.0 g doses, respectively. In addition, across the dose levels, monoglucuronide 1 mean peak concentrations were 2 to 5-fold higher and monoglucuronide 2 levels were 4 to 5-fold higher than the parent compound. In contrast, the relative mean peak concentration of resveratrol-4´-sulfate was between 2 to 5-fold lower than parent resveratrol across the 4 doses. Interestingly, the mean peak concentration of resveratrol disulfate in volunteers who took the lowest dose of 0.5 g, was 3-fold higher than that observed for resveratrol. In contrast, at the 1.0 g dose, similar concentrations were obtained, 148.3 ng/mL for the disulfate and 140.0 ng/mL for resveratrol. Only at the top two doses were concentrations of this metabolite lower than the parent compound, with levels 2 and 4-fold lower following intervention with 2.5 and 5.0 g resveratrol, respectively.

Meng *et al*., suggested that glucuronidation was a predominant metabolic pathway for resveratrol in humans and rats after oral administration (Meng *et al*., 2004). There is evidence *in vitro* using an isolated preparation of luminally and vascularly perfused rat small intestine, that the major form of resveratrol transferred across the rat intestinal epithelium into the blood stream is resveratrol glucuronide (Andlauer *et al*., 2000). Kuhnle *et al*., investigated the extent of the conjugation of resveratrol in rat intestinal epithelium, and the components transferred were identified and quantified by HPLC and mass spectrometry. Resveratrol was detected on the serosal side of the enterocytes, but the amount which was transferred unmetabolized, was very small (0.03 nmol/cm jejunum). In contrast, significant amounts (1.19 nmol/cm jejunum) of the glucuronide conjugate were detected in the serosal side, which represented 96.5% of the amount absorbed, suggesting

the susceptibility of resveratrol to glucuronidation during transfer across the rat jejunum (Kuhnle *et al*., 2000).

Although clinical trials involving large sample sizes are needed to assess factors influencing resveratrol pharmacokinetics and metabolism, the results from the healthy volunteer study presented in this thesis, suggest potential differences between males and females. On some of the dose levels, statistically significant differences between plasma metabolite concentrations in males and females were seen with the former having higher concentrations of resveratrol-3-sulfate and the latter, higher levels of the monoglucuronides. In humans, resveratrol rapidly undergoes phase II conjugation in the form of glucuronidation and sulfation at several sites of the molecule. These reactions are mediated by specific UDP-glucuronosyltransferase (UGT) and sulfotransferases (SULTS) isoforms (Brill *et al*., 2006; Miksits *et al*., 2005) and these enzymes exist in polymorphic states.

Compared to other enzyme families, such as cytochrome P450s, studies of human sulfotransferases (SULTs) in relation to drug response are more limited. SULTs belong to a superfamily of genes, and SULT enzymes have a wide tissue distribution being detected in human liver, lung, brain, skin platelets, breast, kidney and GI tract. At least 13 different SULTs have been characterized; many of the substrates metabolized by these enzymes are also substrates for UDP glucuronosyltransferases with the former generally considered a high affinity/low capacity pathway and the latter a low affinity, but high capacity pathway (Gamage *et al*., 2006).

Regarding transcriptional regulation of SULT genes, recent studies suggest there is marked inter-individual variation in enzyme activity in the human population (5-36 fold), which can be explained by polymorphisms in the coding regions of the SULT genes (Hebbring *et al*., 2008; Lindsay *et al*., 2008). Some of these polymorphisms have a significant influence on drug metabolism in the individual, and account for differential drug responses in different ethnic groups. There are differences between animal and human SULTs, which complicate extrapolation of animal data to humans, for example, humans have 4 members of the SULT1A subfamily, whilst rodents have just one member identified to date. Rats have 4 members of the SULT2a subfamily and humans only one. Other forms of mouse SULT3a1 and SULT5a1 have not been identified in humans (Blanchard *et al*., 2004). Marked differences between the way human and rodent SULT genes are regulated have been reported with many of the rodent forms exhibiting dramatic sexual dimorphisms (Coughtrei and Johnston, 2001).

The sulfation of resveratrol in human liver cytosol has been examined (Miksits *et al*., 2005). In the presence of 3´-phosphoadenosine-5´-phosphosulfate, three metabolites were formed, the structures of which were identified by mass spectrometry and NMR as *trans*-resveratrol-3-*O*-4´-*O*-disulfate (S1), *trans*-resveratrol-4´-*O*-sulfate (S2), and *trans*-resveratrol-3-*O*-sulfate (S3), respectively. Incubation of resveratrol with human recombinant SULTs demonstrated that S1 generation is almost exclusively catalyzed by SULT1A1 and only to a minor extent by SULT 1A2, 1A3 and 1E1, whereas S2 is selectively formed by SULT1E1. In addition, the production of S3 is catalyzed by

SULT1A1, SULT1E1, SULT1A2 and 1A3, depending on the resveratrol concentration (Miksits *et al*., 2005).

UDP-glucuronosyltransferase (UGT) enzymes comprise a superfamily of proteins that catalyze glucuronidation of a broad range of structurally diverse endogenous and exogenous chemicals (Guillemette, 2003). Glucuronidation is one of the major phase II drug-metabolizing reactions, with virtually all classes of hydroxy moiety-containing drugs serving as substrates for UGTs (Guillemette, 2003). At least two additional biological functions are attributed to UGTs: (i) they contribute to protection against certain toxic dietary components, tobacco smoke carcinogens and environmental pollutants (Nowell *et al*., 1999); (ii) they represent key elements in the homeostasis of a number of endogenous molecules, including bilirubin, steroid and thyroid hormones (Guillemette, 2003).

Over recent years, there has been significant progress in the identification of human UGTs, study of their tissue distribution and substrate specificities (Guillemette, 2003). Due to the diverse biological functions of UGTs, alterations in a metabolic pathway involving these enzymes could significantly modify the pharmacokinetics of a given drug, carcinogen or endogenous molecule (Guillemette, 2003). To date, only a few clinically relevant genetic polymorphisms in UGTs have been described, although knowledge of the genetic mechanisms underlying variability in glucuronidation capacity is being explored (Guillemette, 2003). The degree of allelic diversity has also been revealed for several human UGT genes. Some polymorphic UGTs have shown a significant pharmacological impact including associations with drug-induced adverse reactions as well as cancer

susceptibility. The glucuronidation rate of resveratrol by human liver microsomes was evaluated by Aumont *et al.*, (Aumont, *et al*., 2001) who showed that *in vitro* glucuronidation by human liver microsomes generated the 3- and 4´-monoglucuronides, with the reaction being mainly catalyzed by UGT1A1, UGT1A9, and UGT1A10 (Aumont, *et al*., 2001). Meng *et al*., suggested differences between human and mouse metabolism of resveratrol, as two resveratrol glucuronides were detected as major metabolic products in human urine samples after ingestion of resveratrol, but only a single glucuronide was observed following incubation of resveratrol with mouse liver microsomes, suggesting that the enzymes involved (glucuronosyltransferases) may differ in their specificity or activity and therefore their efficiency of glucuronidation (Meng *et al*., 2004). In the healthy volunteer study presented in this thesis, there were differences in the profile of resveratrol metabolites detected in the plasma compared to urine. Therefore metabolites present in the urine may not be entirely representative of the metabolism of resveratrol in the liver, making comparison of resveratrol glucuronidation in human urine and mouse liver samples difficult.

Various studies have shown the potential for resveratrol to modulate the expression of enzymes potentially involved in its metabolism at the mRNA level. In the human hepatoblastoma cell line, HepG2, Lancon *et al*., detected an increase in mRNA expression levels of three metabolizing enzymes, two isoforms of UDP-glucuronosyltransferases, UGT1A1 and UGT2B7 and a sulfotransferase, ST1E1, in cells pretreated for 24 h with 10 μM resveratrol (Lancon *et al*., 2007). Murias *et al*., investigated the uptake and metabolism of resveratrol (5-100 μM) in hormone-dependent ZR-75-1 and independent MB-MDA-231

human breast cancer cells. Resveratrol was extensively metabolized by ZR-75-1 cells, but only marginally by MDA-MB-231 cells, both forming only one metabolite, resveratrol-3-sulfate (Murias *et al*., 2008)

Using RT-PCR, the expression of SULT1A1 mRNA was shown to correlate with resveratrol-3-sulfate formation, with expression much higher in ZR-75-1 than in MDA-MD-231 cells. Metabolism of resveratrol was shown to be concentration dependent; while intracellular sulfation prevailed at concentrations of 10-30 μM, there was a reduction of up to ~60% and ~82% with higher resveratrol concentrations (40–100 μM) in ZR-75-1 and SULT1A1-transfected MDA-MB-231 cells, respectively, after 72 h incubation. Kinetic analysis determined non-competitive substrate inhibition as the main mechanism of the observed decrease in sulfate formation at higher resveratrol concentrations (Murias *et al*., 2008).

Many potential chemopreventive agents have demonstrated the ability to up regulate Phase II enzymes, and induction of these pathways is thought to be a promising strategy for cancer prevention (Baur and Sinclair, 2006). For example, enzymes involved in the phase II metabolic activation of heterocyclic amines to genotoxic carcinogens include UDPGT, SULTs, and glutathione-S-transferase (GST). Various polyphenols, including those in tea, have been reported to induce UDPGT (Bu-Abbas *et al*., 1995). In contrast, Phase I enzymes, and CYPs (cytochrome P450's), have consistently been implicated in the activation of procarcinogens (Baur and Sinclair, 2006).

Resveratrol has been shown to modulate the expression and activity of multiple drug-metabolizing enzymes. For example, *in vitro*, the compound inhibits the enzymatic activity of various CYPs (Chan and Delucchi, 2000; Chang *et al*., 2000; Piver *et al*., 2001; Yu *et al*., 2003) and blocks their transcription through antagonism of the aryl hydrocarbon receptor (Ciolino *et al*., 1998). In addition, resveratrol has been shown to protect cells from the DNA damaging effects of activated chemical carcinogens (Baur and Sinclair, 2006). Coadministration of resveratrol with the carcinogen benzo[*a*]pyrene, which is activated by the CYP isoform CYP1A1, was found to reduce the expression of this enzyme at the protein level and abrogate the DNA damaging effects of benzo[*a*]pyrene in lung tissue in mice (Baur and Sinclair, 2006; Revel *et al*., 2003).

Resveratrol has been demonstrated to induce expression of Phase II enzymes *in vitro* (Cao *et al*., 2004), and haem oxygenase 1 (Kaga *et al*., 2005) and quinone reductase 1 (QR1) (Floreani, *et al*., 2003) *in vivo*, indicating that resveratrol exerts cardioprotection against ROS-mediated menadione toxicity and improved tolerance of ischaemia (Baur and Sinclair, 2006). Most of the reported cytotoxic effects of menadione are thought to be the consequence of oxidative damage induced by reactive oxygen species (Chiuo and Tzeng, 2000). NAD(P)H:quinone oxidoreductase, also known as DT-diaphorase plays a critical role in cellular defences against quinone-containing substances, such as menadione by competing with one-electron reductase for quinone metabolism and, by converting it to the more stable hydroquinone form, protects cells from ROS-mediated toxicity. Therefore, any substance capable of increasing the activity of DT-diaphorase is expected to antagonize the action of menadione. Resveratrol was shown to scavenge peroxyl radicals, by a direct

ROS-scavenging effect, and by induction of DT-diaphorase in the protection of heart from oxidative damage (Floreani *et al*., 2003). The authors concluded that the induction of DT-diaphorase may be very important in the prevention of carcinogenesis, since many quinone-containing substances may be detoxified before starting the initiation process.

In contrast to its induction of QR1 *in vivo and in vitro*, resveratrol inhibited QR2 *in vitro* (Buryanovskyy *et al*., 2004). Although the biological function of QR2 is not well understood, it has been postulated that it might function to control endogenous electrophile concentrations and that an increase in the concentration of electrophilic species could induce expression of Phase II enzymes (Baur and Sinclair, 2006). In support of this, QR2-deficient cells and mice show enhanced resistance to menadione toxicity (Floreani *et al*., 2003; Long *et al*., 2002), whereas QR1-deficient mice exhibit increased sensitivity to quinone toxicity. A general down regulation of genes that encode Phase I drug-metabolizing enzymes and up regulation of the Phase II response was confirmed by cDNA arrays and RT-PCR analysis using the livers of rats treated with either 0.3, 1.0 or 3.0 mg/kg/day of resveratrol for 28 days (Hebbar *et al*., 2005). Induction of Phase II detoxifying enzymes, with a 2-fold up regulation of gene expression, was most pronounced at the highest dose. It was observed, when compared to control rats, that CYP2E1 enzyme activity was unchanged among the male rats, but there was very slight induction in enzyme activity among the female rats with the highest induction (1.5 fold) observed in the highest dose group. In the case of CYP1A1 there was a slight repression of enzyme activity seen among all dose groups, however, the repression was not significant.

In the healthy volunteer daily dose study presented in this thesis, between-subject variability in the PK parameters determined was relatively high, which is in line with other human and animal studies (Almeida *et al*., 2009; Boocock *et al*., 2007; Juan *et al*., 2002; Sale *et al*., 2005; Vaz-Da-Silva *et al*., 2008; Walle *et al*., 2004). In general, in both the healthy volunteer single and daily dose studies, there was an increase in inter-individual variability for plasma PK parameters for resveratrol and its metabolites as the dose increased. Additionally, on each dose level the variability was generally higher with repeated daily dosing compared to an equivalent single dose. The extent of variation was assessed by calculating the coefficient of variance, which ranged between 53 and 86% for resveratrol plasma C_{max} across the four dose levels. In comparison, in the single dose study the coefficient of variance was found to be between 49 to 73% across the dose levels. In both studies there was similarly high inter-individual variability in the metabolite parameters calculated with average coefficient of variance across the dose levels for single and daily dose studies respectively of 50% and 61% for the monoglucuronide 1, 64% and 72% for monoglucuronide 2, 45.5% and 40% for resveratrol-3-sulfate. Coefficient of variance across the four doses was 84% for resveratrol-4´-sulfate and 48% for resveratrol disulfate, which were only determined in the daily dose study presented here.

High plasma and urine inter-individual variability has previously been described for other related compounds such as green tea and tea polyphenols (Lee *et al*., 2002). Manach and Donovan, examined data from 97 published studies to investigate the kinetics among adults, after ingestion of a single dose of polyphenol provided as pure compound, plant

extract, or whole food/beverage (Manach and Donovan, 2004). They reviewed various classes of polyphenols, namely anthocyanins, flavonols, flavanones, flavanol monomers, proanthocyanidins, isoflavones, hydroxycinnamic acids, and hydroxybenzoic acids, in order to calculate mean values for several bioavailability measures, including the maximal plasma concentration (C_{max}), time to reach C_{max}, area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion. The results showed wide variability in the bioavailability of the different polyphenols. They reported extensive variability among the studies with a tenfold variation in the C_{max} values observed for most compounds. Although several factors may explain the wide range, such as the food matrix or background diet, inter-individual variation was considered to be of particular importance and the authors concluded that a possible explanation could be different levels or activity of metabolizing enzymes or transporters, the latter enabling more efficient absorption of polyphenols (Manach and Donovan, 2004).

For the healthy volunteer daily dose study a number of plasma PK parameters were calculated. Of these, the mean C_{max}, AUC_{inf}, and C_{av} for resveratrol increased across the four dose levels, consistent with the single dose healthy volunteer study (Boocock *et al*., 2007). In both studies, there was high inter-individual variability in PK parameters on each dose level, when plotted versus dose. Mean AUC_{inf} and C_{max} values for resveratrol increased with dose, but in a slightly less than dose-proportional manner with no evidence of saturation kinetics. When both the single and daily dose studies were compared, the top 3 dose levels (1.0, 2.5 and 5.0 g) showed higher mean values for resveratrol C_{max}, AUC_{inf}, and C_{av} with repeat dosing. The difference in PK parameters for resveratrol between the

two studies was statistically significant on the 2.5 g dose level for C_{av} (*P*=0.03) and on the 5 g dose level for C_{max} *(P*=0.03). The small increase in C_{max} and C_{av} reflects the fact that repeated dosing for longer than ~5 times the half life has resulted in steady state concentrations being attained. However, since the dosing regimen was not optimized to achieve a certain plasma concentration, the increase is relatively small and more frequent dosing would allow higher concentrations to be reached.

As seen for the parent compound and for the metabolites in the single dose study, the monoglucuronides and resveratrol-3-sulfate showed an increase in mean plasma C_{max}, C_{av} and AUC_{inf} with increasing dose. A similar effect was observed across the dose levels for these PK parameters for resveratrol-4´-sulfate and the disulfate, with repeated daily dosing, with neither derivative evaluated in the single dose study. When the two studies were compared, daily dosing led to an increase in C_{max}, C_{av} and AUC_{inf} at the top three dose levels for the monoglucuronide 1 and at the 5.0 g (C_{max}, AUC_{inf} and C_{av}) and 2.5 g doses (AUC_{inf} and C_{av}) for monoglucuronide 2. For monoglucuronide 1, the difference was statistically significant for C_{av} *(P*=0.01) and for monoglucuronide 2 for C_{max} *(P*=0.01), both on the 5.0 g dose. In contrast, the mean C_{max} for resveratrol-3-sulfate was lower at each dose level with daily dosing compared to a single dose of resveratrol. Although daily dosing led to lower peak plasma levels of resveratrol-3-sulfate, the AUC_{inf} (at the top 3 dose levels) and the C_{av} (at the top 2 dose levels) were higher compared to a single dose, but none of these findings were statistically significant. These data may suggest that after continued daily dosing, glucuronidation becomes a more predominant route of metabolism.

One of the most striking observations related to the plasma PK parameters is that on the 0.5 g dose level only, repeat dosing led to a reduction in mean plasma C_{max}, AUC_{inf} and C_{av} for resveratrol and its main metabolites, the monoglucuronides and resveratrol-3-sulfate, compared to an equivalent single dose. The decrease observed in plasma C_{max} with daily dosing was statistically significant for monoglucuronide 1 (P=0.001), monoglucuronide 2 (P=0.01) and resveratrol-3-sulfate (P<0.0001). On the basis of all these findings it appears that with daily dosing there may be dose-dependent differences in absorption and metabolism. For example, at lower doses of resveratrol there may be inhibition of metabolism, while the higher doses may induce metabolism of the parent compound.

When comparing the healthy volunteer study reported here with the single dose study published previously (Boocock *et al*., 2007), the half life of resveratrol was between 6.14 and 18.01 h across the four dose levels after 28 daily doses, whereas after a single dose the half life of the parent compound was shorter, between 2.85 and 8.87 h. However, this difference was not statistically significant. The longer half life with daily dosing may reflect an alteration in the metabolism or renal excretion rate with repeat dosing. A difference was seen, when comparing the half lives and T_{max} of resveratrol and its metabolites in the daily and single dose studies, between the lowest dose level and three higher doses. In the 1.0 and 2.5 g dose groups, the monoglucuronides had both a shorter half life and T_{max} with repeat dosing, compared to the equivalent single dose, whereas for these parameters in the 0.5 g group, the opposite was observed. This was statistically significant for the half life of monoglucuronide 1 after the 0.5 g dose (P=0.02), and for T_{max} of monoglucuronide 2 after the 2.5 g dose (P=0.04). Although the half life of resveratrol-3-

sulfate across the four dose levels was very similar in both studies, the T_{max} was longer with repeat dosing on the 0.5 g dose, but longer on the three higher doses with single dosing. No significant difference was seen between the two studies in the half life and T_{max} of resveratrol. These findings suggest a potential dose-dependent difference in the metabolism of resveratrol.

In previous studies it has been shown that the main route of resveratrol excretion is renal (Goldberg *et al*., 2003; Marier *et al*., 2002; Meng *et al*., 2004; Soleas *et al*., 2001; Vitrac *et al*., 2003; Walle *et al*., 2004; Yu *et al*., 2002). In the healthy volunteer daily dose study described here, the main compounds detected were resveratrol-3-sulfate, resveratrol disulfate and monoglucuronide 2, with only small amounts of the unchanged parent compound present. When comparing to the single dose study, in general, significantly higher levels of resveratrol and its metabolites were detected in the urine after repeat dosing. Interestingly, while there were relatively low concentrations of resveratrol disulfate in the plasma, significant levels were present in the urine. After a single or repeat doses, monoglucuronide 2 showed higher levels in the urine compared to monoglucuronide 1, which is in contrast to the plasma, where in general the concentrations of the two monoglucuronides were either similar or higher concentrations of monoglucuronide 1 were present.

The predominant compound in the faeces was resveratrol, much of which was probably non-absorbed material, with the 3-sulfate being the main metabolite detected. In many of the volunteers there were significant levels of monoglucuronide 2, resveratrol-4´-sulfate

and resveratrol disulfate, whereas the majority of individuals had no detectable monoglucuronide 1 in the faeces. When the daily and single dose healthy volunteer studies are compared, much higher levels of resveratrol were detected in the faeces on the repeat dose study. Although it is reasonable to suggest that daily dosing leads to higher levels of this compound in the faeces, it is also possible that after several daily doses of resveratrol, there may be more time for the compound to mix with the stool compared to just after a single dose. As only a small sample of stool is collected from each participant at each collection point, single dosing may decrease the chance of obtaining a sample containing compound.

The presence of metabolites in the faeces could be explained by a number of factors including intestinal metabolism of resveratrol and enterohepatic circulation. Enterohepatic recycling of resveratrol has been observed previously, as evidenced by an increase in plasma concentrations 4–8 h post administration, after a rapid decline in the plasma level immediately after administration (Boocock *et al*., 2007; Marier *et al*., 2002). Pharmacokinetic studies with other stilbenes structurally related to resveratrol such as pterostilbene, piceatannol, pinosylvin, and rhapontigenin have shown that these compounds are well absorbed and also undergo enterohepatic recirculation (Remsberg *et al*., 2008; Roupe *et al*., 2006). Similar to resveratrol, these compounds are also glucuronidated but mainly excreted via non-renal routes, predominantly via biliary elimination. The serum concentration of pterostilbene glucuronide was found to initially decrease followed by an increase 1–2 h post administration, which indicated enterohepatic recirculation of this metabolite (Remsberg *et al*., 2008).

In the colorectal cancer study presented in this thesis the mean concentrations of resveratrol detected in tumour tissue was 2.94 ± 4.72 nmol/g in patients on the 0.5 g dose and 43.92 ± 74.15 nmol/g in those on the 1.0 g dose. Although there was high inter-individual variability in tissue levels of resveratrol, the mean concentrations of this compound were generally higher in normal colorectal tissue compared to tumour tissue. For example, the corresponding concentrations of resveratrol in normal tissue were 18.96 ± 38.97 and 867.8 ± 2564 nmol/g in patients on the 0.5 and 1.0 g dose levels respectively. The main metabolite detected in both normal and malignant tissue was resveratrol-3-sulfate, with the only exception being at the 0.5 g dose level in normal colorectal tissue in which monoglucuronide 2 was the main metabolite detected in the majority of patients. As with the parent compound, higher levels of the metabolites were generally detected in normal, compared to the tumour tissue at both dose levels. The observed difference in the levels of resveratrol and its metabolites in the normal compared to malignant colorectal tissue may represent a difference in the uptake of resveratrol between malignant and normal tissue. As there were many more samples collected from normal colonic tissue, with samples collected at distances along the resection specimen, than collected from tumour tissue, the comparison may be affected by a sample size bias.

The most striking observation in the colorectal study was the difference in concentration of resveratrol and its metabolites in relation to the anatomical site of the tissue sample analysed. There were much higher concentrations of these compounds in samples from patients with right sided tumours (caecum, transverse colon) compared to left sided ones

(sigmoid and rectum). For example, compared to left sided tumour tissue, after dosing with 0.5 g resveratrol, mean concentrations were 7 (resveratrol-3-sulfate) and 13 (resveratrol) fold higher in right sided tissue. After 1.0 g dosing, mean concentrations 32 (resveratrol-3-sulfate) and 24 (resveratrol) fold higher were detected in right sided tissue. Importantly, this finding was reinforced in the one patient who had two Dukes A colon cancers, one located in the caecum (right sided) and the other in the sigmoid colon (left sided). The resveratrol tumour concentration was 0.92 nmol/g (sigmoid) and 12.74 (caecal) and the resveratrol-3-sulfate tumour concentration was 0 nmol/g (sigmoid) and 9.12 nmol/g (caecal). The concentrations of resveratrol in normal tissue, taken 5 cm proximal to the tumour, were 1.89 nmol/g (sigmoid) and 41.27 nmol/g (caecal). A possible explanation for this finding is the difference in faecal consistency, as the faecal material travels from the caecum across the transverse colon and into the sigmoid and rectum. In general, the faecal material is fluid on the right side of the colon and becomes more solid, as it passes onto the left side of the colon. Tissue levels of resveratrol are likely to be related to resveratrol concentrations in the faeces passing through the intestine in direct contact with the mucosa, so that the consistency of the faeces may influence uptake of the compound into the tissue. Another possible explanation is the transport of resveratrol in the human intestine. Although passive transport of resveratrol has been demonstrated to occur in the rat intestine, this has not yet been investigated in great detail in humans. It is possible that there are differences in transport mechanisms in different sections of the bowel, such as the large and small intestine, which could affect the levels of resveratrol and its metabolites present in the colorectal tissue. Resveratrol-3-sulfate remained the main metabolite irrespective of anatomical location. The tissue and plasma were collected many hours after

the dose was administered and therefore only very small amounts of unchanged resveratrol were detected in the plasma. The low plasma concentrations are consistent with the PK data from the healthy volunteer study in which only low levels of the parent compound remained at all time points 5 h post administration. The maximal concentration detected in plasma was 0.06 μM, whilst the concentrations in the tissue were up to 3774.33 nmol/g. The main metabolite detected in the plasma on both dose levels was the disulfate, with resveratrol-3-sulfate the next most prominent compound. With the data available, no direct relationship between plasma and colorectal tissue levels of these compounds could be established.

Blood was drawn in theatre at a mean of 17.6 and 18 h after oral administration of 0.5 g and 1.0 g resveratrol, respectively. In healthy volunteers, the last two time points of blood collection were 12 and 24 h after dosing. Therefore a direct comparison of plasma concentrations for these compounds in the two studies is difficult. The major metabolites in plasma detected at 12 and 24 h post dosing with 0.5 and 1.0 g resveratrol in healthy volunteers were resveratrol-3-sulfate, the disulfate and monoglucuronide 1. The mean concentration of the disulfate in healthy volunteers 12 hours after administration of resveratrol was 75.18 ng/mL on the 0.5 g dose, which is similar to the concentration detected in colorectal cancer patients (70.00 ng/mL). However, on the 1.0 g dose, there were lower levels of the disulfate in the volunteers, with mean concentration of 47.91 ng/mL compared to 137.52 ng/mL in colorectal cancer patients. The presence of metabolites in the colonic tissue may be primarily related to the presence of metabolites in the faeces rather than due to metabolism of the parent compound in colorectal cells. It is

impossible to distinguish in this study between resveratrol species that were excreted unchanged, not absorbed, or formed *in situ* in the GI tract.

The anticarcinogenic potential of resveratrol in preclinical models has been documented in several organ sites. Levels of resveratrol observed in the colorectal study presented in this thesis were up to ~1.2 (malignant tissue) or ~24.1-fold higher (normal colorectal tissue) than pharmacologically efficacious levels attained in the intestinal mucosa of APC^{Min} mice following dietary supplementation with reseveratrol (0.2%). In these mice, mean adenoma numbers were 27% lower than those in untreated mice. Polyp reduction was observed in both the small intestine and colon (Sale *et al*., 2005). Relatively low daily doses of resveratrol (between 0.2-2 mg/kg), which give peak plasma concentrations of unmetabolized resveratrol of up to 2 μM, have been shown to have pharmacodynamic activity in three chemical-induced rat carcinogenesis models (Banerjee *et al*., 2002; Li *et al*., 2002; Tessitore *et al*., 2000). Tessitore *et al*., suggested that in the azoxymethane-induced rat colon carcinogenesis model, resveratrol dissolved in the drinking water at the low dose of 200 μg/kg/day decreased the number of colonic aberrant crypt foci by 40% and their multiplicity by 50%. This efficacy was accompanied by an increase in the expression of the proapoptotic protein Bax in the foci and expression of the cell cycle inhibitory protein p21Cip1 in normal colonic mucosa in these rats (Tessitore *et al*., 2000).

In contrast, many studies *in vitro* suggest that carcinogenesis-modulating effects of resveratrol require the presence of concentrations in the range of 5-100 μM. The PK analysis of the healthy volunteer study suggests that even after 28 daily doses of

resveratrol, only 3 participants (7.5% of the healthy volunteers) reached plasma concentrations of resveratrol in this range (max 7.4 μM), and all 3 volunteers were on the highest dose of resveratrol. In contrast, 45% of the colorectal cancer patients achieved colorectal tissue concentrations of resveratrol ≥5 nmol/g. This represents 20 and 60% of participants achieving these concentrations in malignant and normal colorectal tissue on the 0.5 g and 1.0 g dose, respectively. In addition two participants on the 1.0 g dose level had levels of resveratrol >100 nmol/g in both their tumour and normal colorectal tissue. Therefore, even with relatively low plasma concentrations of resveratrol, pharmacologically efficacious levels of the parent compound are achievable in both normal and malignant colorectal tissue. These results support the case for colorectal tissue as a target site for resveratrol.

CHAPTER 6

PHARMACODYNAMIC RESULTS

6.1 Effect of resveratrol on biomarkers in blood and colorectal tissue

The IGF signaling system, consisting of IGFs, IGFBPs and IGF receptors, is thought to influence malignant development and it has been suggested that IGF-1 contributes to the development of adenomatous polyps (Section 1.5.6). Modulation of the IGF system has been proposed as a mechanism by which certain agents, for example 9-*cis* -retinoic acid, may prevent cancer, and resveratrol has been shown to lower circulating IGF-1 in diabetic mice on a high calorie diet (Section 1.5.6). Malondialdehyde, an endogenous product of peroxidised lipids and by product of prostaglandin biosynthesis is mutagenic in bacterial and mammalian cells and carcinogenic in rats (Section 1.5.5). One mechanism by which resveratrol exerts its beneficial effects may be its ability to attenuate oxidative damage to DNA, as has been shown in a wide variety of cells (Section 1.5.5). Mediators of inflammation, such as COX-2 and prostaglandins have been shown to be involved in carcinogenesis (Section 1.5.3). The anti tumour activities of resveratrol are mediated through several cell signaling pathways and include the reduction of inflammation via inhibition of prostaglandin production and COX-2 (Section 1.5.3).

Increased cell proliferation seems to be important in the development of cancer and the proliferation rate is a prognostic indicator for various malignancies, including colon cancer (Dai *et al*., 2005; Rosenwald *et al*., 2003). The anticancer property of resveratrol has been supported by its ability to inhibit proliferation of a wide variety of human tumour cells *in vitro* and *in vivo*, in APC^{Min} mice (Section 1.4.1, 1.5.1, 1.5.2 and 1.5.4). In addition, resveratrol prevented the formation of colon and small intestine tumours by down regulating genes that are directly involved in cell cycle progression or cell proliferation in

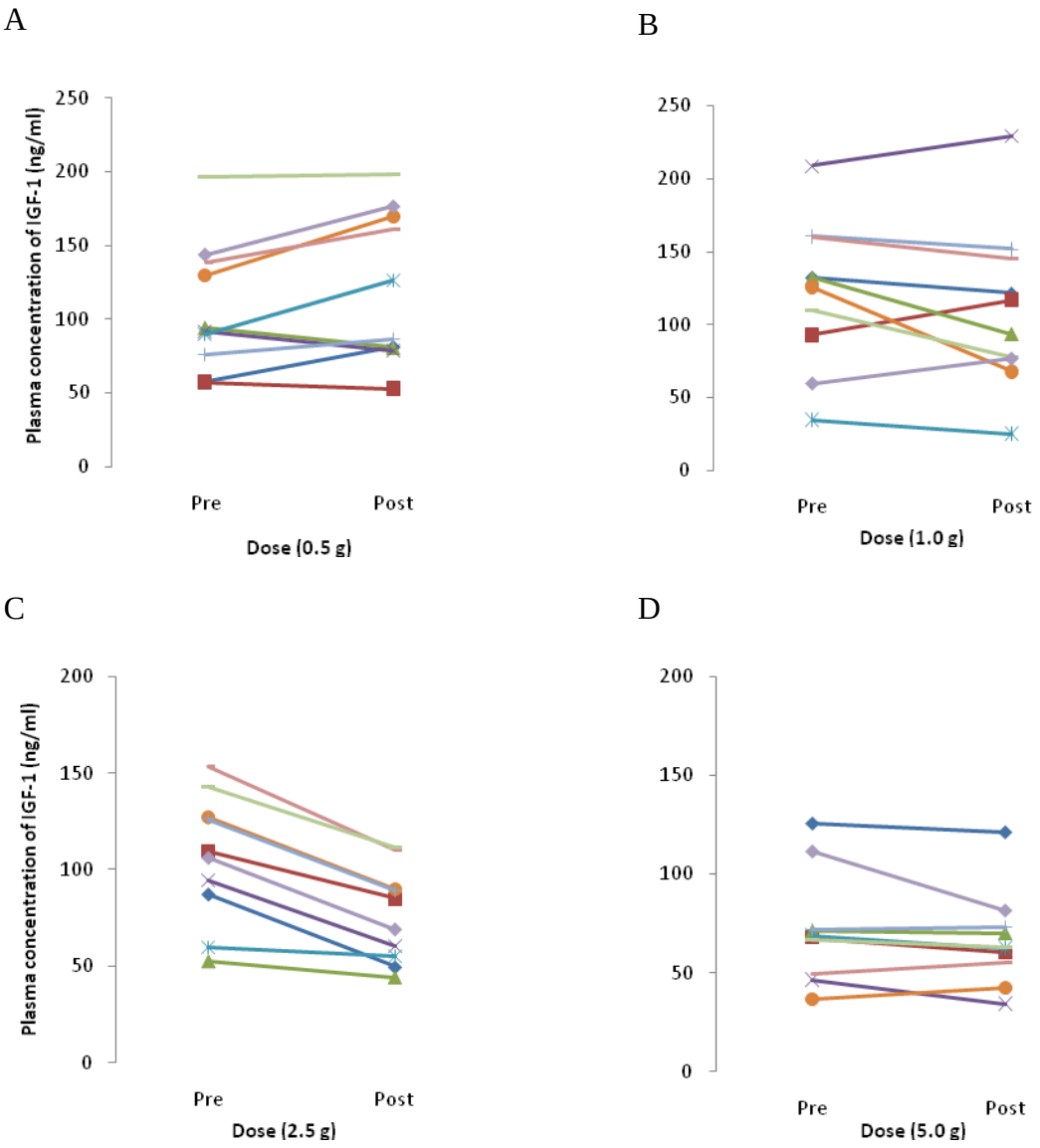
APC^{Min} mice (Schneider *et al*., 2001). The COX enzyme has been shown to play an integral role in the development of colorectal cancer (Section 1.5.3) and the chemopreventive effect of resveratrol in *APC*^{Min} mice has been associated with inhibition of COX enzymes and the interference with PGE_2 generation (Section 1.5.3).

The hypotheses were tested that the consumption of resveratrol leads to (1) an increase in plasma IGFBP-3 levels, (2) a reduction in IGF-1 levels and IGF-1/IGFBP-3 ratio in plasma, (3) alterations of malondialdehyde DNA adducts levels in whole blood and colorectal tissue, (4) reduced plasma PGE_2 concentration and colorectal tissue COX-2 staining and (5) inhibition of colorectal tissue proliferation. For analysis of these biomarkers, blood samples (pre and post dosing) were drawn between 7 and 8 am from the healthy volunteers who had fasted from midnight. Blood was drawn on day 1 of the dosing period prior to consumption of any resveratrol; post-dosing blood was taken on day 29 of the daily dosing period prior to the final dose. Blood from colorectal cancer patients was obtained up to 48 h prior to the first dose of resveratrol, when they were not fasted, and post-dosing on day 9, after fasting from midnight. Post dosing bloods were taken in theatre after the patient had been anaesthetized before resection of the tumour. Colorectal tissue was collected pre dosing (endoscopy) and after 8 daily doses of resveratrol at resection.

6.1.1 IGF-1 and IGFBP-3 in healthy volunteers

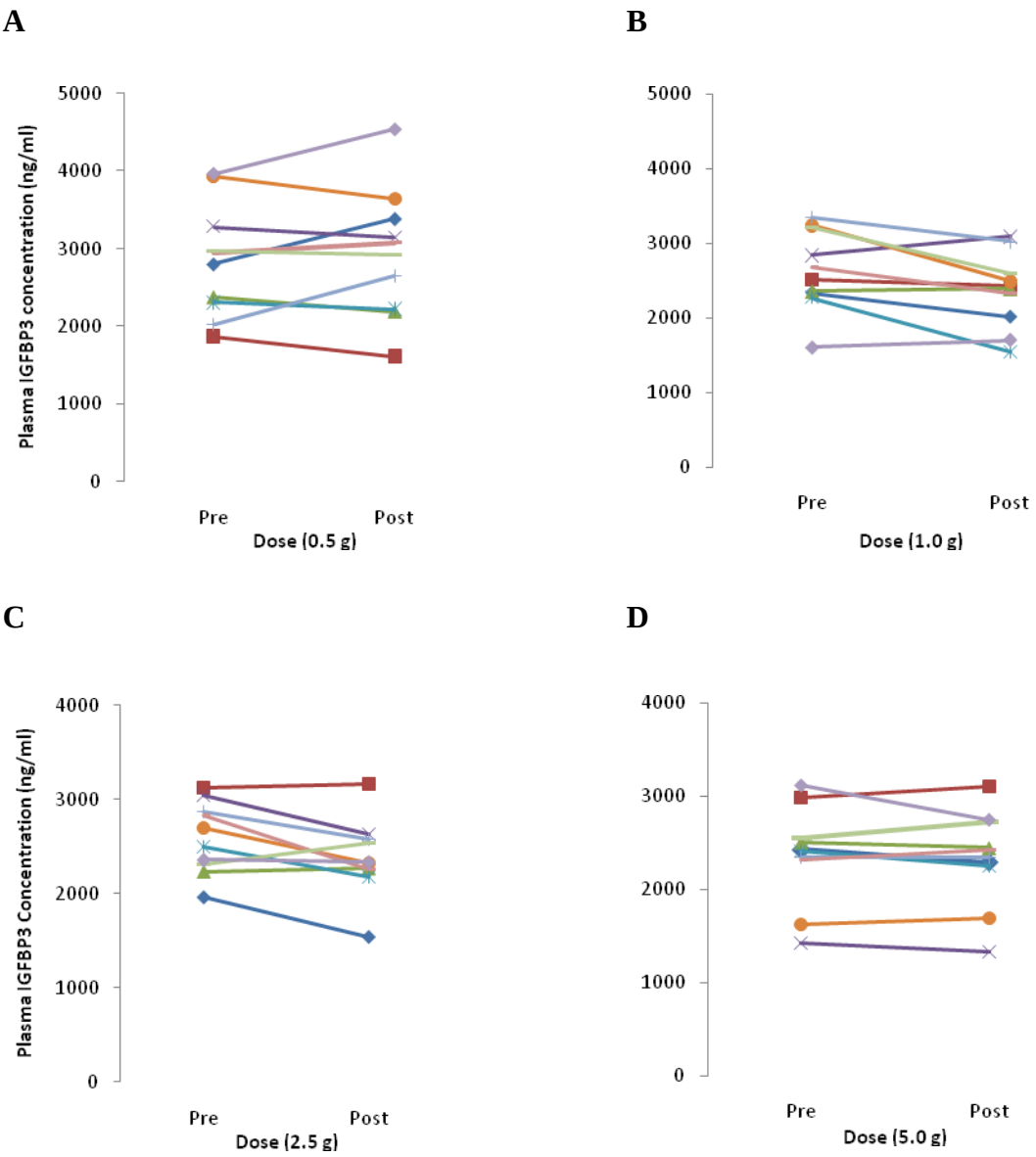
IGF-1 and IGFBP-3 levels in plasma samples obtained from the healthy volunteers on day 29 were compared with those taken before the first dose of resveratrol. When all study

participants were considered collectively there was a significant reduction in the mean IGF-1 and IGFBP-3 levels by 7.9% (P=0.03) and 4.2% (P=0.03), respectively, but the ratio IGF-1/IGFBP-3 was not affected. The modulating effect of resveratrol on the IGF system was most prominent in the 2.5 g dose group, in which mean IGF-1 was decreased by 28% (Figure 6.1.1), IGFBP-3 by 8.1% (Figure 6.1.2) and the ratio IGF-1/IGFBP-3 by 21.6% (Figure 6.1.3), compared to pre-dose values. At 5.0 g there was no significant effect on the IGF system, and the only significant change observed in individuals on 1.0 g resveratrol was a reduction in mean IGFBP-3 by 10.5%, compared to pre-dose value (Figure 6.1.2).



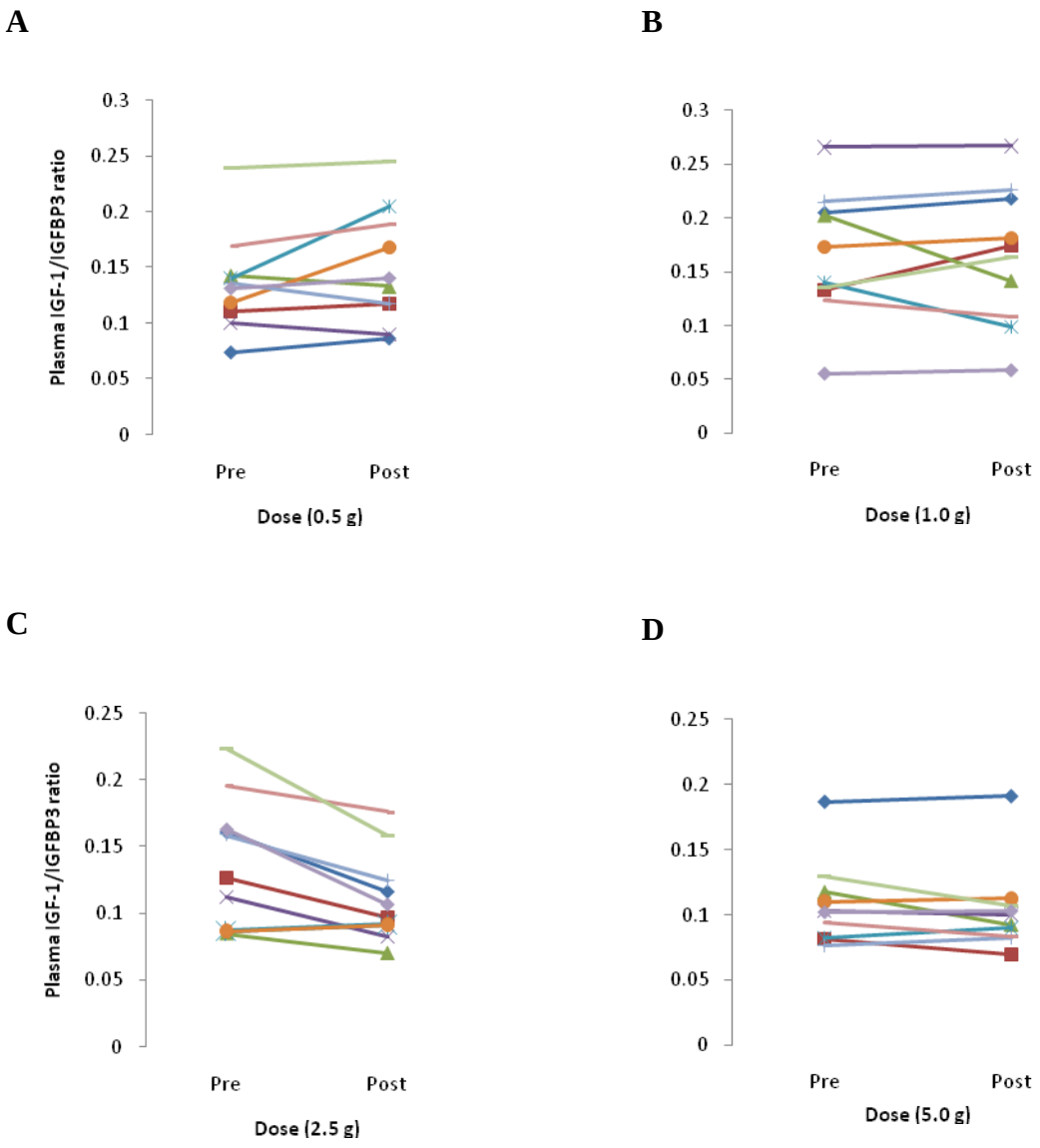
Dose (g)	Pre ng/mL Mean ± SD	Post ng/mL Mean ± SD	*P* *Paired students T test*
0.5	108 ± 44.0	121 ± 52.6	*0.06*
1.0	122 ± 50.6	111 ± 56.5	*0.22*
2.5	106 ± 33.3	76.4 ± 24.2	*<0.001*
5.0	71.6 ± 27.7	66.4 ± 23.7	*0.15*
All dose levels	102 ± 42.6	93.6 ± 46.4	*0.03*

Figure 6.1.1 Effect of resveratrol on plasma IGF-1 concentration in healthy volunteers after 28 daily doses of 0.5 (A), 1.0 (B), 2.5 (C) and 5.0 g (D). Changes shown are for each individual volunteer in all figures and the values in the table show the mean levels (± SD) with n=10 volunteers per dose.



Dose (g)	Pre ng/mL Mean ± SD	Post ng/mL Mean ± SD	*P Paired students T test*
0.5	2848 ± 728	2948 ± 832	*0.45*
1.0	2637 ± 540	2358 ± 503	*0.03*
2.5	2592 ± 380	2382 ± 410	*0.03*
5.0	2373 ± 522	2336 ± 511	*0.49*
All dose levels	2612 ± 561	2503 ± 618	*0.03*

Figure 6.1.2 Effect of resveratrol on plasma IGFBP-3 concentration in healthy volunteers after 28 daily doses of 0.5 (A), 1.0 (B), 2.5 (C) and 5.0 g (D) (n=10 per dose level). Changes shown are for each individual volunteer in all figures and the values in the table show the mean levels (± SD) with n=10 volunteers per dose.

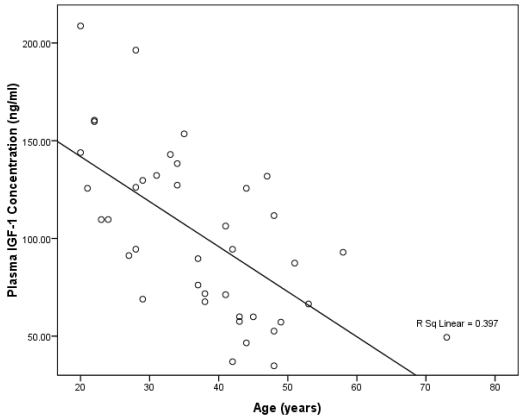


Dose (g)	Pre Mean ± SD	Post Mean ± SD	*P* *Paired students T test*
0.5	0.12 ± 0.05	0.45 ± 0.05	*0.14*
1.0	0.16 ± 0.06	0.16 ± 0.06	*0.91*
2.5	0.15 ± 0.05	0.12 ± 0.03	*0.001*
5.0	0.12 ± 0.03	0.10 ± 0.03	*0.20*
All dose levels	0.14 ± 0.05	0.13 ± 0.05	*0.16*

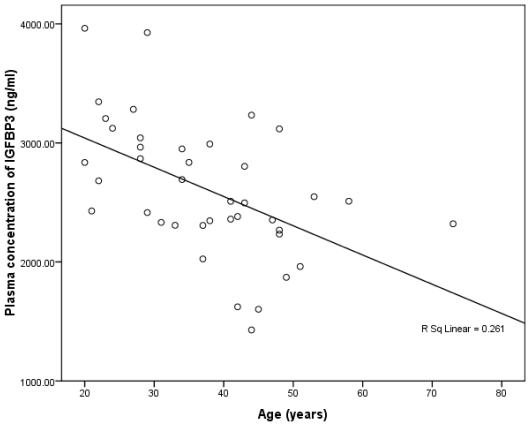
Figure 6.1.3 Effect of resveratrol on plasma IGF-1/IGFBP-3 ratio in healthy volunteers after 28 daily doses of 0.5 (A), 1.0 (B), 2.5 (C) and 5.0 g (D) (n=10 per dose level). Changes shown are for each individual volunteer in all figures and the values in the table show the mean ratio (± SD) with n=10 volunteers per dose.

For all dose levels combined there was a significant negative correlation between pre dosing plasma levels of IGF-1, IGFBP-3 or of IGF-1/IGFBP-3 ratio on the one side and age on the other (Figure 6.1.4), and between pre dosing IGFBP-3 and BMI (Figure 6.1.5). No significant gender difference was seen (Figure 6.1.6). Furthermore, there was no significant association between demographics (age, gender and BMI) and change in concentration of IGF-1 or IGFBP-3 or of IGF-1/IGFBP-3 ratio when individual values were compared with pre dosing values (Table 6.1.1). When the 4 dose levels were assessed separately, there was a gender difference in the effect on IGFBP-3, for individuals on the 0.5 g dose. In this dose level, for males there was an increase in the mean IGFBP-3 concentration of 286.58 ± 345.35 ng/mL, whilst a reduction of 204.47 ± 88.69 ng/mL was observed in females (Independent T test, P=0.02). In individuals on the 1.0 g dose level, the mean IGF-1/IGFBP-3 ratio increased by 0.016 ± 0.030 in males and by 0.021 ± 0.016 in females (independent T test, P=0.03). In volunteers on the 2.5 g dose level, a positive correlation was seen between BMI and IGFBP-3 concentration and between BMI and the IGF-1/IGFBP-3 ratio with r values of 1.6, (P=0.04) and r 2.07, (P=0.03), respectively.

A



B



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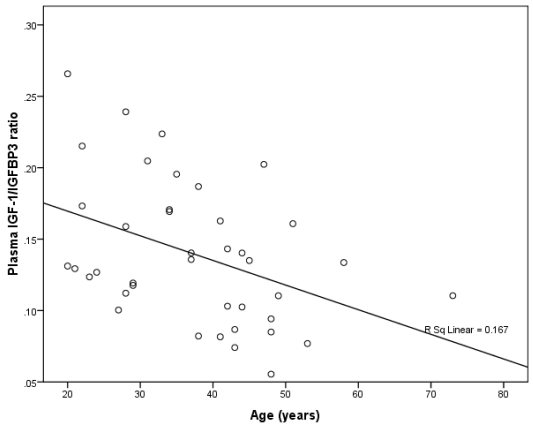
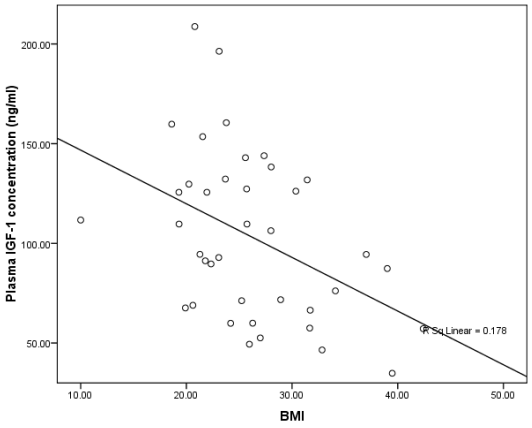


Parameter	*Pearsons coefficient (r)	*P*
IGF-1	-0.630	*<0.0001*
IGFBP-3	-0.511	*0.001*
IGF-1/IGFBP-3 ratio	-0.408	*0.009*

Figure 6.1.4 Relationship (linear regression) between age and pre dosing plasma levels of IGF-1 (A), IGFBP-3 (B) and the IGF-1/IGFBP-3 ratio (C) in all healthy volunteers who received 28 daily doses of resveratrol. Values represent n=40 volunteers. *Pearsons coefficient denoted by r, is a measure of the linear association between two variables.

A



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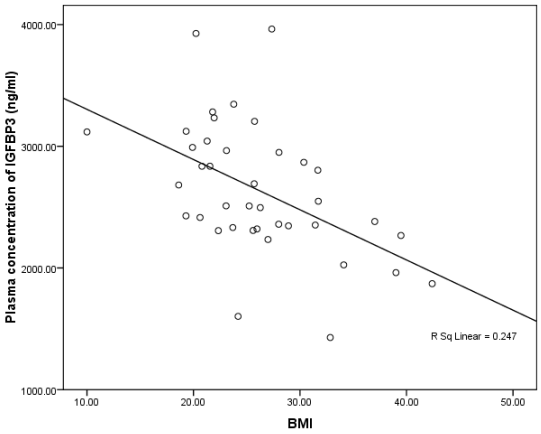

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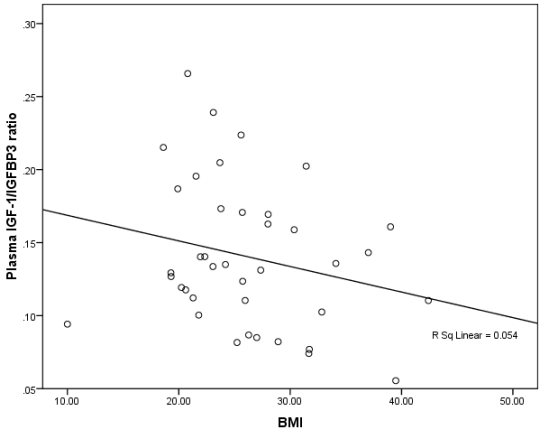
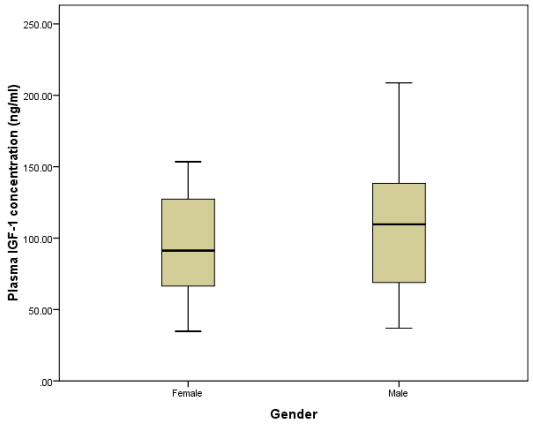


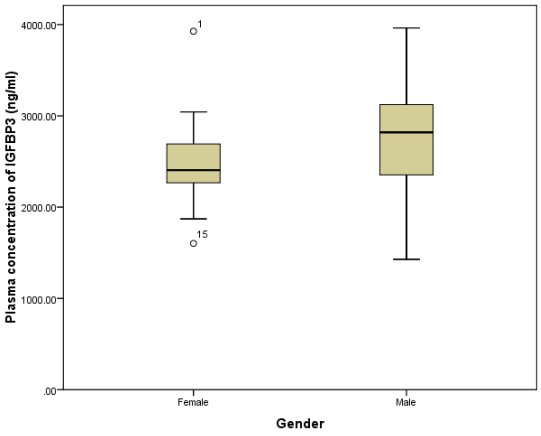
Parameter	*Pearsons coefficient (r)	*P*
IGF-1	-0.422	*0.08*
IGFBP-3	-0.497	*0.001*
IGF-1/IGFBP-3 ratio	-0.232	*0.155*

Figure 6.1.5 Relationship (linear regression) between BMI and pre dosing plasma levels of IGF-1 (A), IGFBP-3 (B) and the IGF-1/IGFBP-3 ratio (C) in all healthy volunteers who received 28 daily doses of resveratrol. Values represent n=40 volunteers. *Pearsons coefficient denoted by r, is a measure of the linear association between two variables.

A



B



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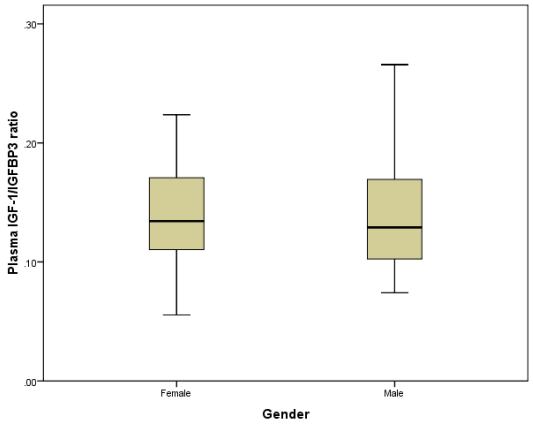


Parameter	Female ng/mL Mean ± SD	Male ng/mL Mean ± SD	*P Independent T test*
IGF-1	93.0 ± 35.0	108 ± 47.0	*0.30*
IGFBP-3	2486 ± 512	2715 ± 589	*0.20*
IGF-1/IGFBP-3 ratio	0.13 ± 0.47	0.14 ± 0.52	*0.77*

Figure 6.1.6 Box plots and table showing the relationship between gender and pre dosing levels of plasma IGF-1 (A), IGFBP-3 (B) and the IGF-1/IGFBP-3 (C) ratio in all healthy volunteers who received 28 daily doses of resveratrol. The table shows the mean values (± SD) for males and females. Values represent n=40 volunteers. In box plot (B), outliers are represented by circles and were not excluded from the overall analysis.

Table 6.1.1 Relationship between age (A), gender (B) and BMI (C) on the one side and mean change in plasma levels on the other of IGF-1, IGFBP-3 and the IGF-1/IGFBP-3 ratio in healthy volunteers. Values represent n=40 volunteers. * Independent T test. Participants were divided into 2 age ranges, 20-39 and 40-80 years, in order to represent the younger in comparison to the older participants. In addition, participants were divided into BMI ranges of < 25 and ≥25 kg/m^2. A BMI of ≥25 kg/ m^2 is considered to indicate the participant is overweight (WHO, 2000) and therefore these participants were compared to those in the normal weight range.

A

	Number of volunteers	Age (years)	Mean change (ng/mL)	SD	*P**
IGF-1	**22**	**20-39**	**6.33**	**25.18**	***0.61***
	18	**40-80**	**10.17**	**22.66**	
IGFBP-3	**22**	**20-39**	**164.4**	**435.9**	***0.73***
	18	**40-80**	**122.3**	**320.9**	
IGF-1/IGFBP-3 ratio	**22**	**20-39**	**0.005**	**0.029**	***0.76***
	18	**40-80**	**0.027**	**0.027**	

B

	Number of volunteers	Gender	Mean change (ng/mL)	SD	*P**
IGF-1	**22**	**Male**	**8.72**	**23.75**	***0.85***
	18	**Female**	**7.25**	**24.64**	
IGFBP-3	**22**	**Male**	**115.7**	**464.7**	***0.59***
	18	**Female**	**181.8**	**263.1**	
IGF-1 /IGFBP-3 ratio	**22**	**Male**	**0.01**	**0.02**	***0.32***
	18	**Gender**	**0.0018**	**0.33**	

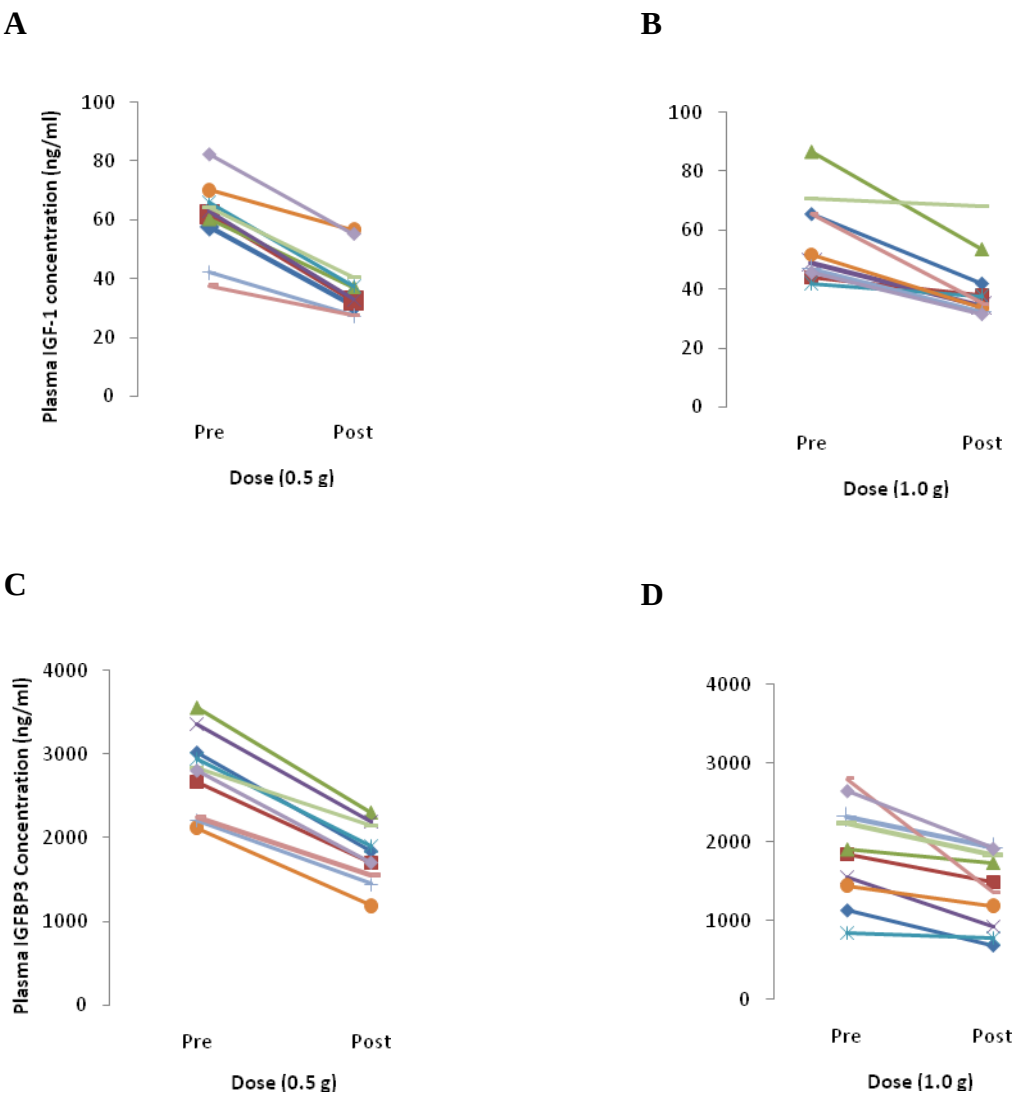
C

	Number of volunteers	BMI (kg/m^2)	Mean change (ng/mL)	SD	*P**
IGF-1	**19**	**<25**	**5.79**	**26.03**	***0.57***
	21	**≥25**	**10.11**	**22.14**	
IGFBP-3	**19**	**<25**	**183.1**	**253.9**	***0.56***
	21	**≥25**	**111.4**	**476.9**	
IGF-1 /IGFBP-3 ratio	**19**	**<25**	**-0.001**	**0.03**	***0.06***
	21	**≥25**	**0.014**	**0.0.3**	

6.1.2 IGF-1 and IGFBP-3 in colorectal cancer patients

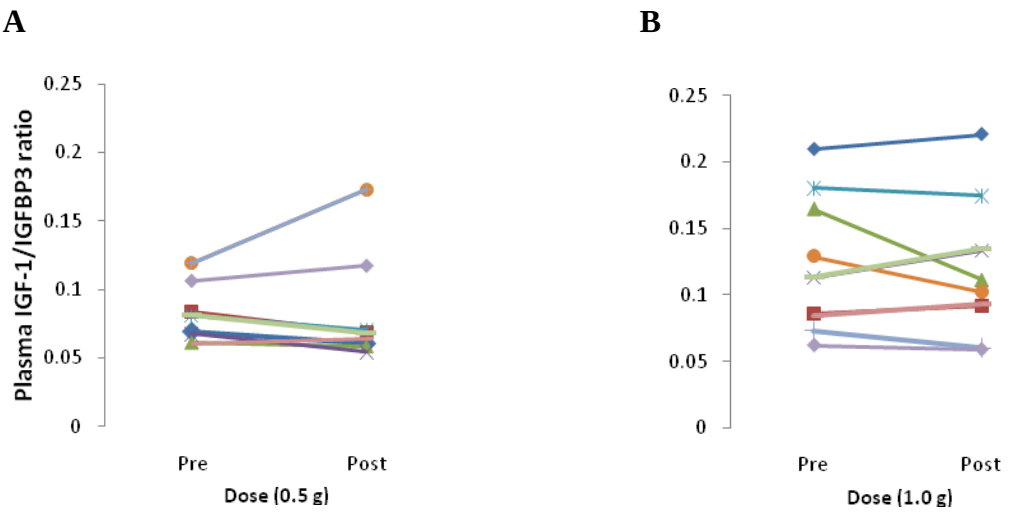
Blood was taken from the colorectal cancer patients prior to the first dose of resveratrol and on day 9 prior to surgical resection of the tumour, when they had ingested 8 daily resveratrol doses. When all study participants were considered collectively there was a significant reduction in the mean IGF-1 and IGFBP-3 by 33.2 and 31.6 %, respectively, when compared to pre-intervention values (Figure 6.1.7). There was no significant effect on the mean IGF-1/IGFBP-3 ratio in the patients (Figure 6.1.8). There was a greater protein lowering effect in those on 0.5 g resveratrol compared to the 1.0 g dose level with a 37.6 and 28.4 % reduction in mean IGF-1 and a 35.0 and 26.0 % reduction of IGFBP-3 respectively.

Most of the 20 patients were on a clear liquid diet for 48 h prior to the day of surgery, as instructed by their surgical team, to prepare for bowel surgery. Furthermore, the post dosing blood sample was taken after anesthesia had been initiated and therefore these factors may have influenced the results.



	Dose (g)	Pre ng/mL Mean ± SD	Post ng/mL Mean ± SD	*P* *Paired students T test*
IGF-1	0.5	60.6 ± 12.9	37.8 ± 10.5	*<0.001*
	1.0	56.7 ± 14.7	40.6 ± 11.6	*<0.001*
	Both dose levels	58.6 ± 13.6	39.2 ± 10.9	*<0.001*
IGFBP-3	0.5	2776 ± 483	1796 ± 352	*<0.001*
	1.0	1874 ± 642	1385 ± 474	*0.003*
	Both dose levels	2324 ± 721	1590 ± 457	*<0.001*

Figure 6.1.7 Effect of resveratrol on plasma IGF-1 (A and B) and IGFBP-3 (C and D) concentration in colorectal cancer patients after 8 daily doses of 0.5 and 1.0 g. Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD) for n=10 volunteers per dose level.



Dose (g)	Pre Mean ± SD	Post Mean ± SD	*P* Paired students T test
0.5	0.08 ± 0.02	0.08 ± 0.04	*0.95*
1.0	0.12 ± 0.05	0.12 ± 0.05	*0.63*
Both dose levels	0.01 ± 0.04	0.10 ± 0.05	*0.75*

Figure 6.1.8 Effect of resveratrol on plasma IGF-1/IGFBP-3 ratio in colorectal cancer patients after 8 daily doses of 0.5 (A) and 1.0 g (B). Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD), n=10 volunteers per dose level.

6.1.3 M_1dG adducts in healthy volunteers

M_1dG levels in whole blood obtained from the healthy volunteers on day 28 were compared with damage levels present in samples taken before the first dose of resveratrol. In the study participants collectively, consumption of resveratrol had no effect on M_1dG levels, which were 61.54 ± 58.1 and 61.1 ± 38.9 adducts per 10^8 nucleotides, before and after 28 daily doses of resveratrol respectively (*P*=0.95). When individual dose groups were analysed separately, there was an increase in mean M_1dG levels of 8.0, 9.0 and 21.6 % in individuals on the 0.5, 1.0 or 2.5 g, respectively, with the effect at 2.5 g being statistically significant (*P*=0.02). Only in the humans on 5.0 g was there a reduction in average M_1dG levels (31.0%), however this was not statistically significant (Figure 6.1.9).

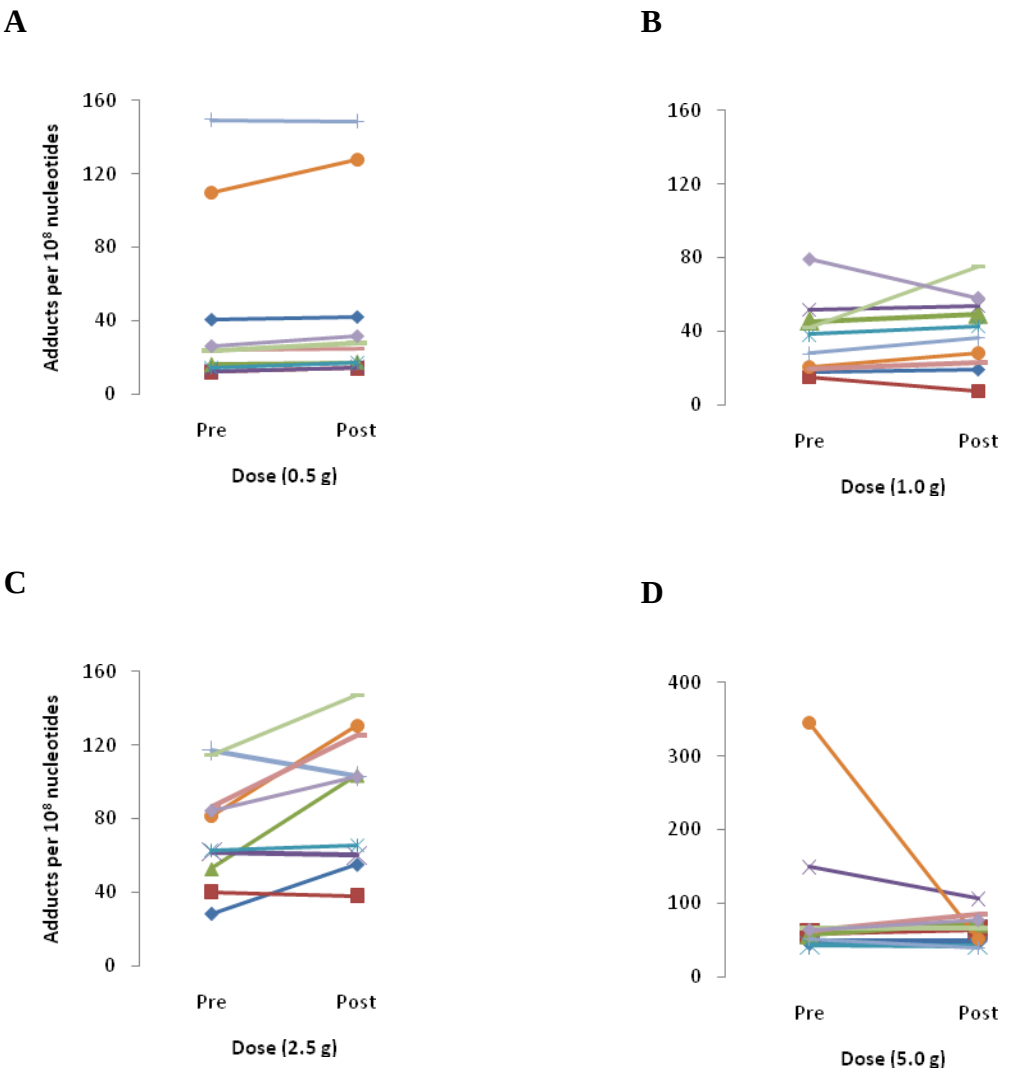
No relationship was observed between study participant demographics (age and BMI) and pre dosing levels of M_1dG. Although females had higher pre dosing levels of M_1dG (73.75 ± 75.78) compared to males (51.54 ± 37.30 adducts per 10^8 nucleotides), this difference was not statistically significant (Independent T test, *P*=0.23). For all study participants as a whole, no relationship was found between change in M_1dG levels (post compared to pre dosing) and demographics (age, BMI and gender). However, when each dose level was analysed separately, females on the 2.5 g dose had a mean increase of M_1dG levels of 32.60 ± 19.30 adducts per 10^8 nucleotides) compared to males who had a mean increase of 1.32 ± 13.25 adducts per 10^8 nucleotides) and this difference between females and males was statistically significant *(*Independent T test, *P*=0.01). In addition, in the 1.0 g dose group

collectively, there was a significant positive correlation between BMI and change in M_1dG levels (post compared to pre levels) (r 0.656, *P*=0.04).

6.1.4 M_1dG adducts in colorectal cancer patients

In colorectal cancer patients collectively there was a non-significant reduction in whole blood M_1dG levels of 11.9 % (*P*=0.09) after 8 daily doses of resveratrol. In individuals on the 0.5 and 1.0 g dose levels, M_1dG levels were reduced by 3.5 and 18.2 %, respectively but did not reach statistical significance (Figure 6.1.10). In one patient on the 0.5 g dose level there was insufficient blood for analysis, therefore the results include only 9 participants for the lower dose level.

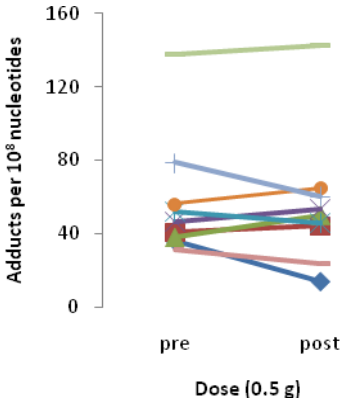
M_1dG levels were assessed in colorectal tissue in 5 participants on the 0.5 g dose level, whose colorectal tissue (tumour and macroscopically normal) was obtained from endoscopic biopsies (pre dosing) and colorectal resection specimens (post dosing). After 8 daily doses of resveratrol, there was a rise in M_1dG levels by 24.6 and 57.5 % in malignant and normal tissue, respectively. In both cases the increase in levels was not statistically significant (Figure 6.1.11).



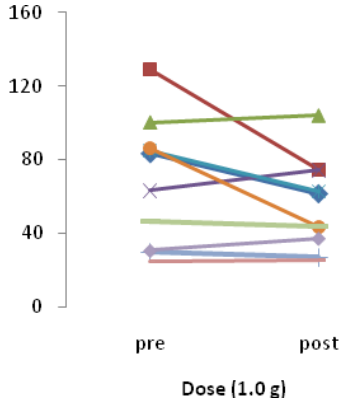
Dose (g)	Pre Adducts per 10^8 nucleotides Mean ± SD	Post Adducts per 10^8 Nucleotides Mean ± SD	*P* Paired students T test
0.5	42.8 ± 47.4	46.6 ± 49.4	*0.05*
1.0	35.7 ± 19.9	39.2 ± 20.5	*0.43*
2.5	73.0 ± 29.3	93.1 ± 36.5	*0.02*
5.0	94.6 ± 92.9	65.4 ± 20.8	*0.36*
All dose levels	61.4 ± 58.1	61.1 ± 38.9	*0.95*

Figure 6.1.9 Effect of resveratrol on whole blood M_1dG in healthy volunteers after 28 daily doses of 0.5 (A), 1.0 (B), 2.5 (C) and 5.0 g (D). Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD), n=10 volunteers per dose level.

A



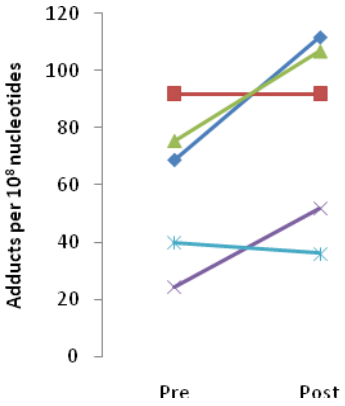
B



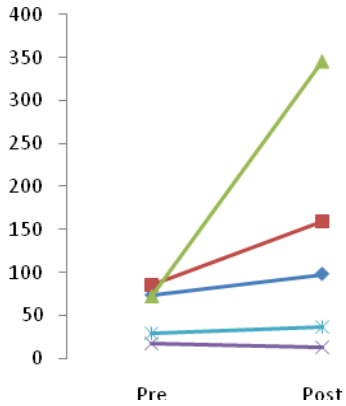
Dose (g)	Pre Adducts per 10^8 nucleotides Mean ± SD	Post Adducts per 10^8 Nucleotides Mean ± SD	*P* Paired students T test
0.5	57.5 ± 33.3	55.4 ± 36.6	*0.63*
1.0	67.8 ± 34.8	55.5 ± 24.7	*0.11*
Both dose levels	62.9 ± 33.5	55.4 ± 30.0	*0.09*

Figure 6.1.10 Effect of resveratrol on whole blood M_1dG in colorectal cancer patients after 8 daily doses of 0.5 (A) and 1.0 g (B) resveratrol. The values represent n=9 volunteers (0.5 g) and n=10 volunteers (1.0 g). Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD).

A



B



Colorectal tissue	Pre Adducts per 10^8 nucleotides Mean ± SD	Post Adducts per 10^8 nucleotides Mean ± SD	*P* Wilcoxon-Mann-Whitney test
Tumour	60.0 ± 27.4	79.7 ± 33.9	*0.09*
Normal	55.6 ± 29.9	131 ± 133	*0.21*

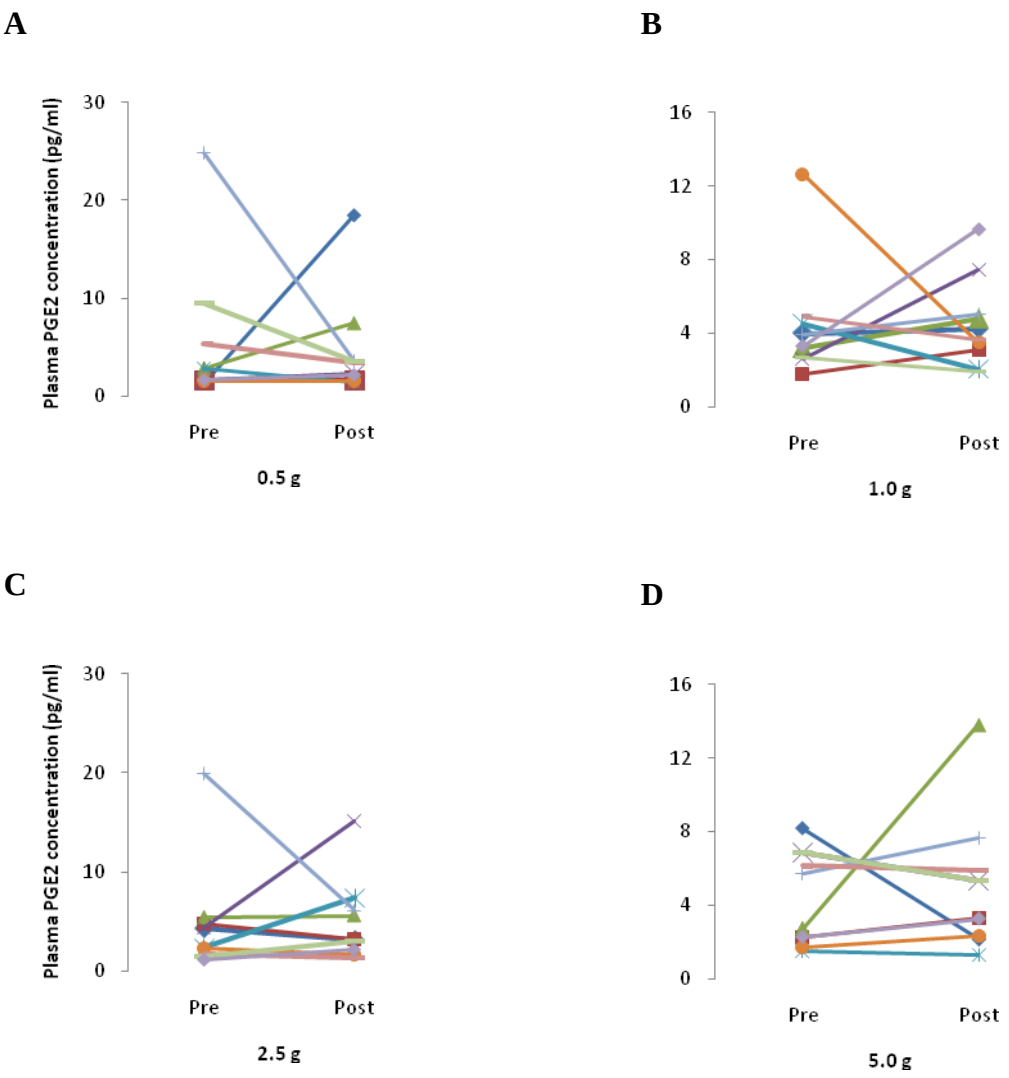
Figure 6.1.11 Effect of resveratrol on M_1dG in colorectal tumour tissue (A) and colorectal normal tissue (B) in colorectal cancer patients before and after 8 daily doses of 0.5 g resveratrol. Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD), n=5 volunteers per dose level.

6.1.5 PGE_2 levels in healthy volunteers

Plasma PGE_2 levels in individuals after 28 daily doses of resveratrol were not significantly different compared to pre dosing concentrations (Figure 6.1.12). There was no association between age or BMI and pre dosing concentrations of PGE_2, nor was there a difference in mean pre dosing concentration between males (4.9 pg/mL) and females (4.5 pg/mL) (Independent T test, P=0.80). In addition, no relationship was established between age, BMI or gender and change in plasma PGE_2 concentration (post compared to pre dosing).

6.1.6 PGE_2 levels in colorectal cancer patients

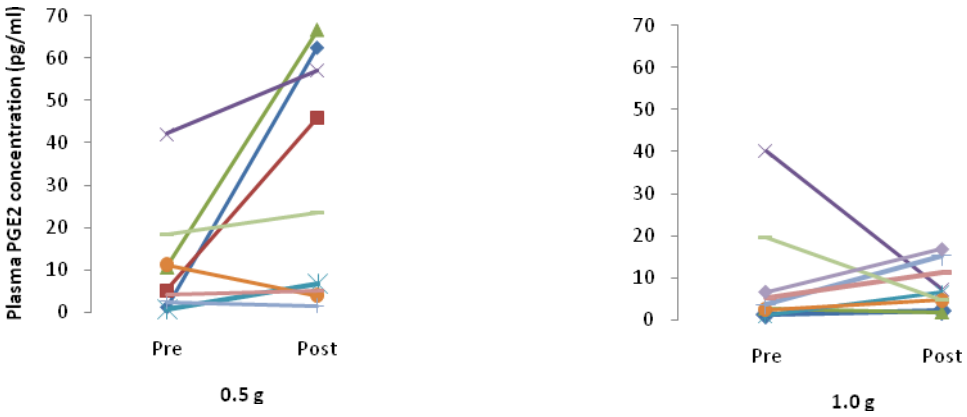
Plasma concentrations of PGE_2 after 8 daily doses of resveratrol were compared to pre dosing concentrations and in most participants an increase was observed. On the lowest dose level, there was a 2.8 fold increase in mean PGE_2 levels, whilst after 1.0 g daily dosing, a slight reduction was seen. Neither change on either dose level was statistically significant (Figure 6.1.13). In two participants (one on each dose level) there was insufficient plasma to perform the analysis and therefore the results represent 9 participants per dose level.



Dose (g)	Pre pg/mL Mean ± SD	Post pg/mL Mean ± SD	*P Paired students T test*
0.5	5.28 ± 7.33	4.55 ± 5.21	*0.81*
1.0	4.35 ± 3.04	4.54 ± 2.41	*0.89*
2.5	4.77 ± 5.53	4.89 ± 4.12	*0.95*
5.0	4.43 ± 2.55	5.04 ± 3.66	*0.66*
All dose levels	4.71 ± 4.82	4.75 ± 3.83	*0.66*

Figure 6.1.12 Effect of resveratrol on plasma PGE_2 concentration in healthy volunteers after 28 daily doses of 0.5 (A), 1.0 (B), 2.5 (C) and 5.0 g (D). Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD), n=10 volunteers per dose levels.

A **B**



Dose (g)	Pre pg/mL Mean ± SD	Post pg/mL Mean ± SD	*P Paired students T test*
0.5	10.7 ± 13.1	30.3 ± 26.7	*0.05*
1.0	9.2 ± 12.9	7.8 ± 5.4	*0.78*
Both dose levels	9.9 ± 12.7	19.1 ± 22.5	*0.11*

Figure 6.1.13 Effect of resveratrol on plasma PGE_2 concentration in colorectal cancer patients after 8 daily doses of 0.5 (A) and 1.0 g (B). Changes shown are for each individual volunteer in all figures and the values in the table show the mean (± SD), n=9 volunteers per dose level**.**

6.1.7 Cell proliferation and inflammation

Immunohistochemistry was utilised to test the hypothesis that consumption of resveratrol reduces cell proliferation, as reflected by Ki-67 and inflammation, as demonstrated by COX-2 in colorectal tumour tissue. Colorectal tumour sections were collected pre and post dosing with resveratrol at endoscopy (biopsy) and at surgical resection, respectively. For 5 patients on the 0.5 g dose level, pre and post dosing samples included sections of normal colorectal tissue obtained at least 10 cm away from the tumour. The proliferation rate of colorectal tumours has been monitored using the Ki-67 antigen (Suzuki *et al*., 1992). The Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but it is absent from resting cells (G_0). During interphase the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. In contrast, COX-2 staining pattern is cytoplasmic rather than nuclear.

6.1.7.1 Cell proliferation (Ki-67)

Colorectal tissue sections obtained pre and post dosing with resveratrol were stained for Ki-67 and the number of positive epithelial cells in 6 representative adjacent high power fields (x 400) were counted in each sample. The average number of epithelial cells counted for each section, including all 6 adjacent high power fields, was 1651 (range 1110 to 3386). When all study participants are considered collectively, the proportion of tumour epithelial cells which stained positive for Ki-67 was 88.00 ± 6.64 % (pre) and 83.17 ± 10.03 % (post dosing) with P=0.05 (Paired Students T test). With the 2 dose levels assessed separately,

the average decrease in Ki-67 expression was 5.64% (P=0.34) and 1.85% (P=0.43) for the 0.5 and 1.0 g dose levels, respectively (Figure 6.1.14). A participant on the 1.0 g dose level had two colorectal cancers, a sigmoid and a caecal adenocarcinoma, resected at the same operation, and therefore the 1.0 g dose represents 11 pre and post tissue samples. Figure 6.1.15 shows representative immunohistochemistry staining for Ki-67 in colorectal tissue pre and post dosing with resveratrol.

In the 5 participants who had normal colorectal tissue collected pre and post dosing with 0.5 g resveratrol, the proportion of tissue epithelial cells which stained positive for Ki-67 was 74.58 ± 20.60 (pre) and 67.63 ± 15.38% (post dosing) P=0.05 (Wilcoxon-Mann Whitney test). When comparing pre dosing biopsies of normal tissue with pre dosing biopsies of malignant tissue, less proliferation in the former was noted. In both tumour and normal colorectal tissue there was a trend towards a reduction in Ki-67 positive cells post intervention.

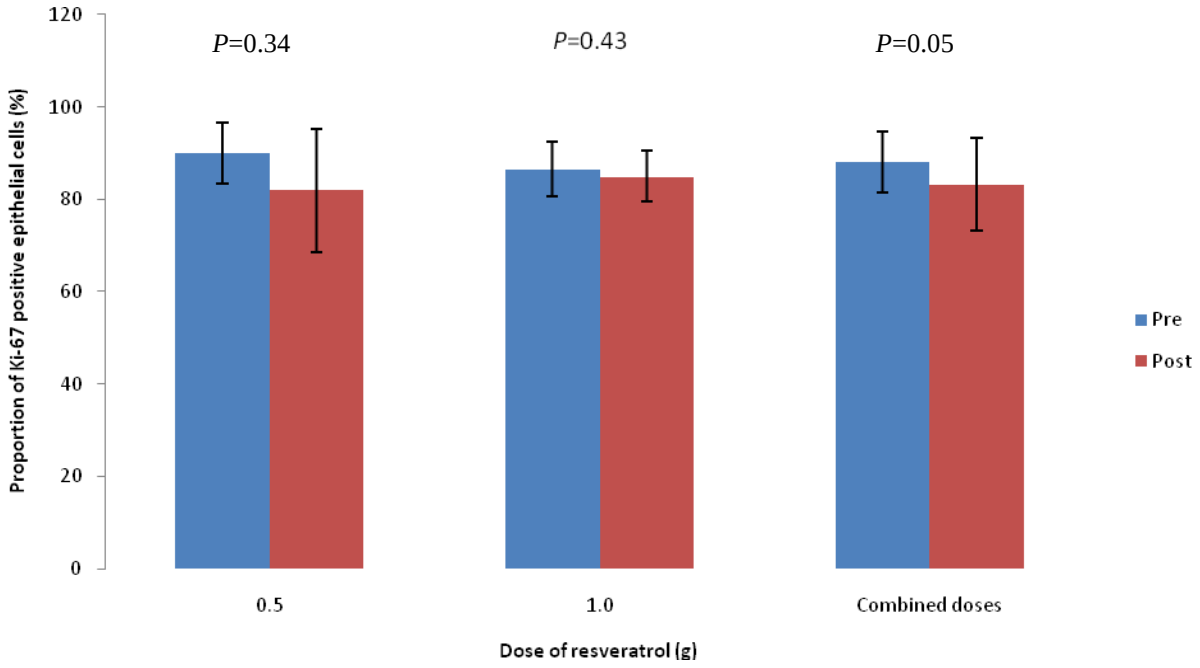
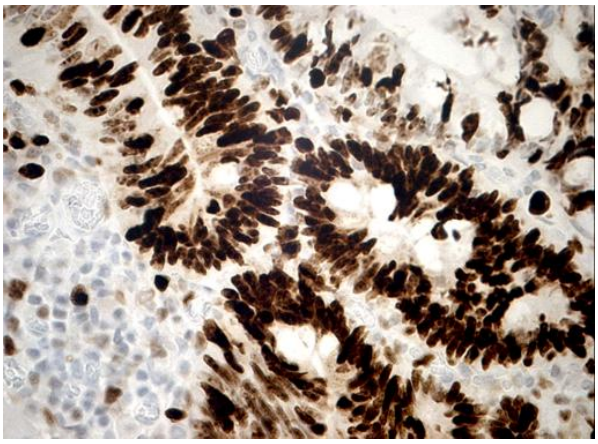


Figure 6.1.14 Proportion of Ki-67 positive epithelial cells (%) in colorectal cancer samples obtained pre (biopsy) and post (resection specimen) after 8 daily doses of 0.5 and 1.0 g resveratrol. Values represent mean ± SD with n=10 volunteers on the 0.5 g and n=11 volunteers on the 1.0 g dose levels.

A



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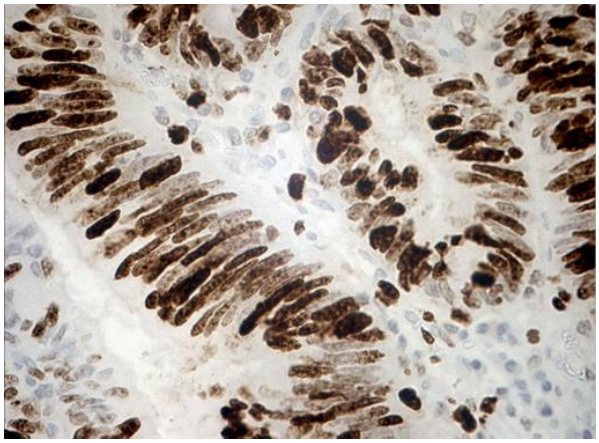


Figure 6.1.15 Representativeimmunohistochemical staining for Ki-67 in colorectal tumour tissue before (A) and after (B) 8 daily doses of resveratrol (x400). The sections illustrated are from one participant who consumed 1.0 g resveratrol.Ki-67 positive cells stain brown.

6.1.7.2 Inflammation (COX-2)

The scoring of COX-2 expression in tumour epithelial cells was carried out according to the method of Remmele and Stegner (Remmele and Stegner, 1987). Intensity of staining (cytoplasmic staining) was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong) (Figure 6.1.16). The scoring system was carried out as described in section 2.2.4.3**.** For all participants combined the extent of staining of tumour was scored as 0 (0%), 1 (42.8%), 2 (57.1%), 3 (0%) for pre dosing biopsies and scored as 0 (0%), 1 (14.3%), 2 (57.1%), 3 (28.6%) for the post dosing tumour sections, indicating a significant increase in staining post treatment (P=0.004) (Table 6.1.2). For 6 participants on the 0.5 g dose level the intensity of COX-2 staining showed an increase in the post treatment specimens with the remaining 4 participants showing no change. On the 1.0 g dose level, 6 participants showed an increase, 3 stayed the same and 2 showed a decrease in the intensity of COX-2 staining in the post resection samples (compared to pre treatment biopsies). The overall staining intensity increased on both dose levels but was only statistically significant on the 0.5 g dose (P=0.009, 0.5 g and P=0.14, 1.0 g) (Table 6.1.3). Normal colorectal tissue (pre and post treatment) was obtained for 5 participants on the 0.5 g dose. In 2 of them there was an increase, 1 stayed the same and 2 had a reduction in staining intensity (compared to pre treatment biopsy samples). Overall no significant effect was seen (P=0.59) (Table 6.1.2).

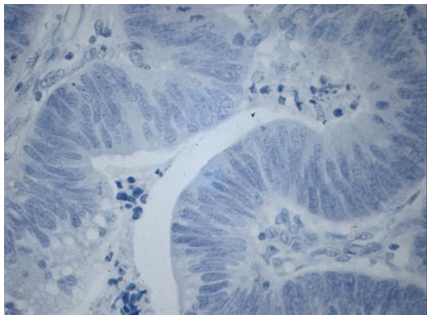
Table 6.1.2 Intensity of COX-2 staining in colorectal tumour tissue and normal colorectal tissue before and after the consumption of 8 daily doses of resveratrol. Tumour tissue values represent n=21 participants (one participant had two colorectal cancers) on the 0.5 and 1.0 g doses and normal colorectal tissue values represent n=5 participants on the 0.5 g dose.

	COX intensity	Negative	Weak	Medium	Strong
Tumour tissue (n=21)	Pre treatment (biopsy)	0	9	12	0
	Post treatment (resection)	0	3	12	6
Normal tissue (n=5)	Pre treatment (biopsy)	1	1	3	0
	Post treatment (resection)	2	1	2	0

Table 6.1.3 Intensity of COX-2 staining in colorectal tumour tissue before and after the consumption of 8 daily doses for each dose level of 0.5 and 1.0 g resveratrol. Values represent n=10 volunteers on the 0.5 g and n=11 volunteers on the 1.0 g dose levels (one participant had two colorectal cancers on the 1.0 g dose level).

Dose	COX intensity	Negative	Weak	Medium	Strong
0.5 g	Pre treatment (biopsy)	0	5	5	0
	Post treatment (resection)	0	1	6	3
1.0 g	Pre treatment (biopsy)	0	4	7	0
	Post treatment (resection)	0	2	6	3

A



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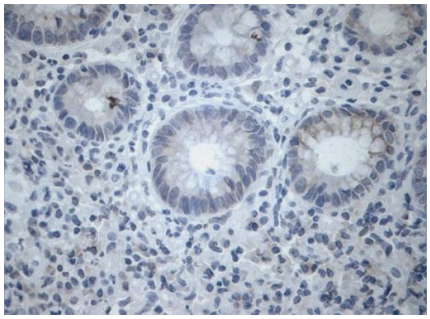

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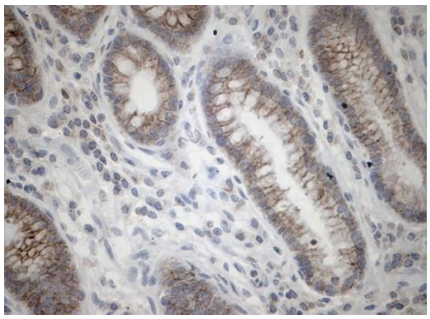

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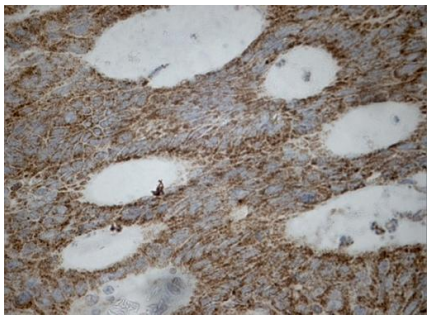

Figure 6.1.16 Representative immunohistochemical staining for COX-2 in colorectal tumour tissue . Intensity of staining (brown cytoplasmic staining) was scored as negative (A), weak (B), medium (C) and strong (D) (x400). The sections illustrated are from different study participants and represent pre dosing (B) or post dosing (A,C,D) with 8 daily doses of resveratrol (0.5 or 1.0 g).

6.2 Effect of resveratrol on colorectal cancer cells *in vitro*

6.2.1 Effect of single and repeated doses of resveratrol on cell growth

Several reports suggest that resveratrol suppresses proliferation of a variety of colon cancer cells *in vitro* at concentrations of $\geq$5μM, which are higher than systemic concentrations achieved *in vivo* in humans after oral administration of a single dose (Section 1.4.1). Therefore the hypothesis was tested that exposure to daily doses of resveratrol for 7 days would cause greater growth inhibition than exposure to a single dose. The daily dosing schedule was utilised to represent the repeat dose clinical protocol followed in this project. The effect of a single dose on HT-29 and HCA7 human colon adenocarcinoma cells was compared to repeated daily doses, where the resveratrol containing media was replenished each day. Daily dosing seemed to exert a greater inhibitory effect on the growth of both cell lines. The IC_{50} values, computed from the growth curves after exposure for 168 h, were 8.05 ± 0.21 or 5.11 ± 0.19 μM when HT-29 cells were exposed to single or daily doses, respectively (Figures 6.2.1 and 6.2.2). The corresponding IC_{50} for HCA7 cell were 9.70 ± 0.65 and 6.86 ± 0.21 μM. The greater inhibitory effect with daily dosing and hence the difference in IC_{50} values observed was statistically significant for both HT-29 and HCA7 cells (P<0.001 and P=0.001 respectively). The amount of DMSO vehicle added to each incubation did not exceed 0.1%, which on its own failed to affect the cell growth. Figures 6.2.3-6.2.6 show the effect of a single or repeated exposure, with resveratrol concentrations of 1, 5 or 10 μM, on the growth of HT-29 and HCA7 cells compared to the growth of control cells.

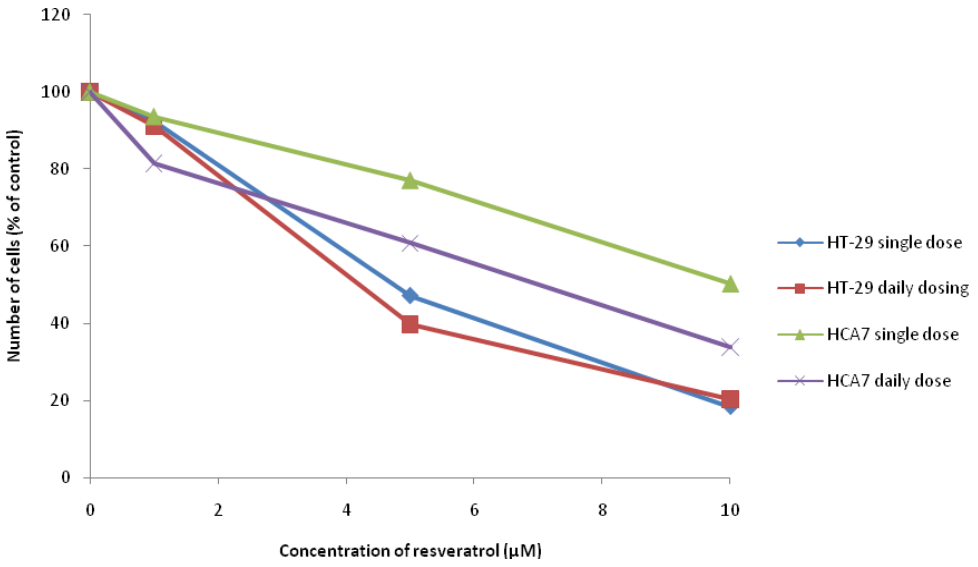

Figure 6.2.1 Effect of increasing concentrations of resveratrol on HT-29 and HCA7 cells (repeated compared to single dosing). Cells were harvested 144 h after plating. Results are the mean (± SD) of three separate experiments each performed in triplicate.

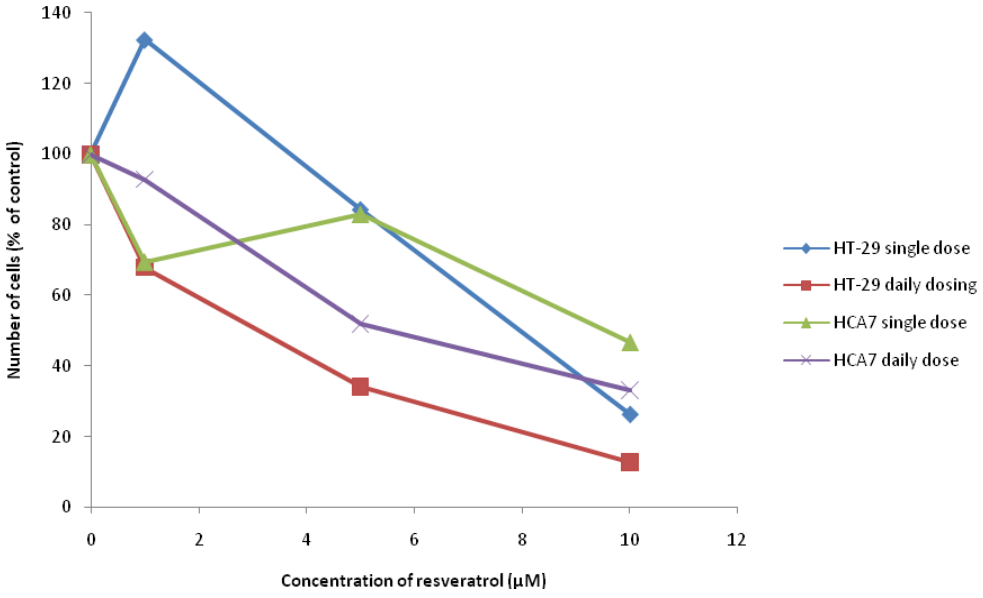

Figure 6.2.2 Effect of increasing concentrations of resveratrol on HT-29 and HCA7 cells (repeated compared to single dosing). Cells were harvested 168 h after plating. Results are the mean (± SD) of three separate experiments each performed in triplicate.

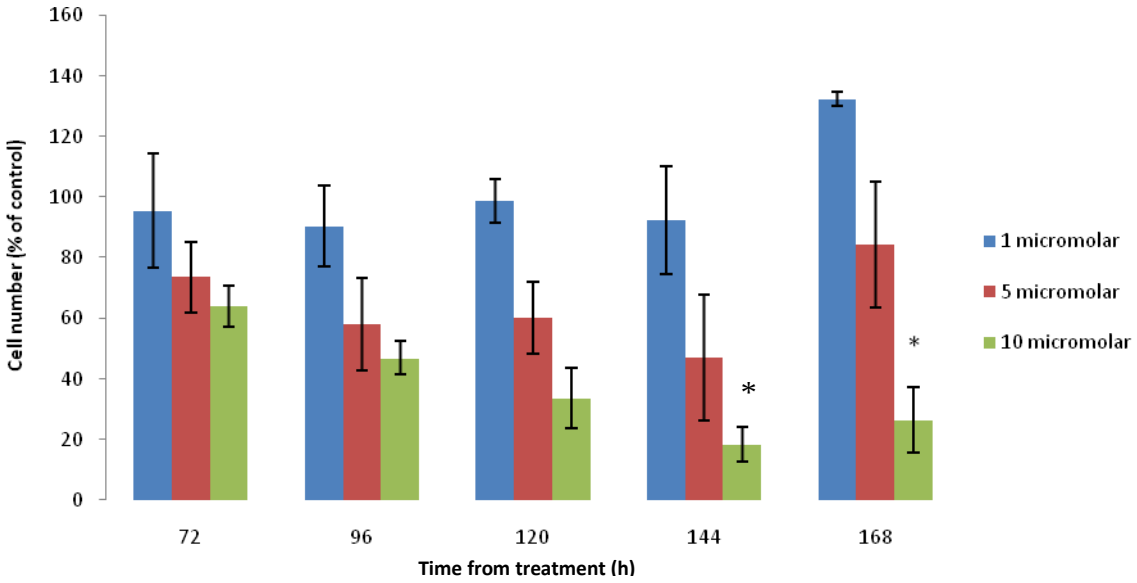

Figure 6.2.3 Effect of a single dose of resveratrol (1, 5 or 10 μM) on the growth of HT-29 cells compared to the growth of control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in triplicate. Asterisks indicate a significant difference from control cells, where *P=<0.05 and **P=<0.001.

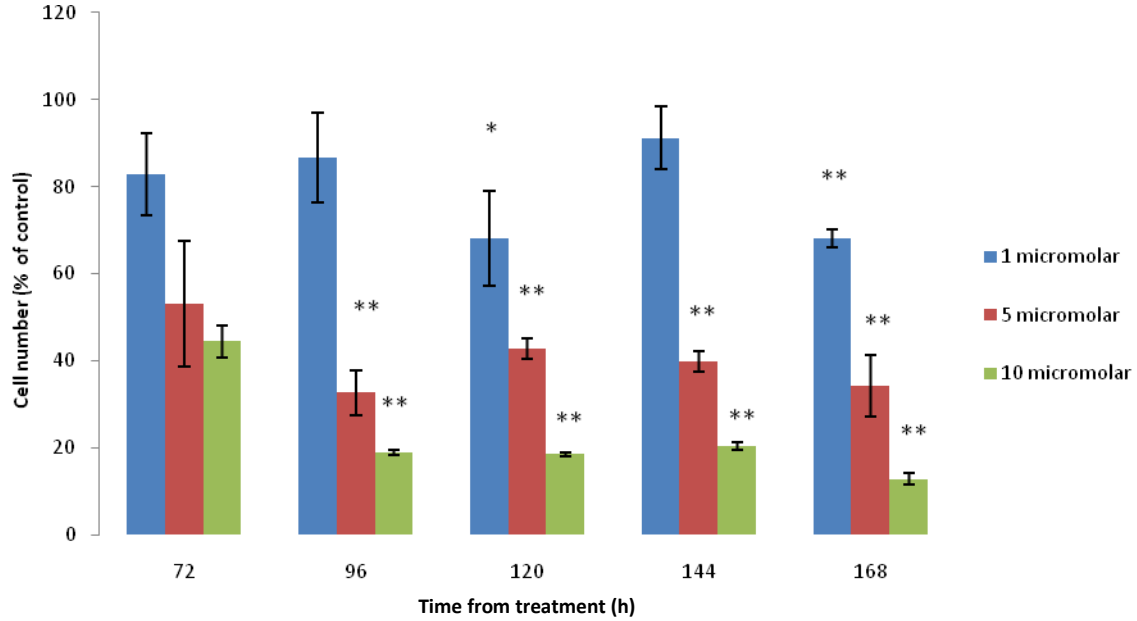

Figure 6.2.4 Effect of repeated dosing with resveratrol (1, 5 or 10 μM) on the growth of HT-29 cells compared to the growth of control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in triplicate. Asterisks indicate a significant difference from control cells, where *P=<0.05 and **P=<0.001. Time from treatment represents time from the first resveratrol treatment.

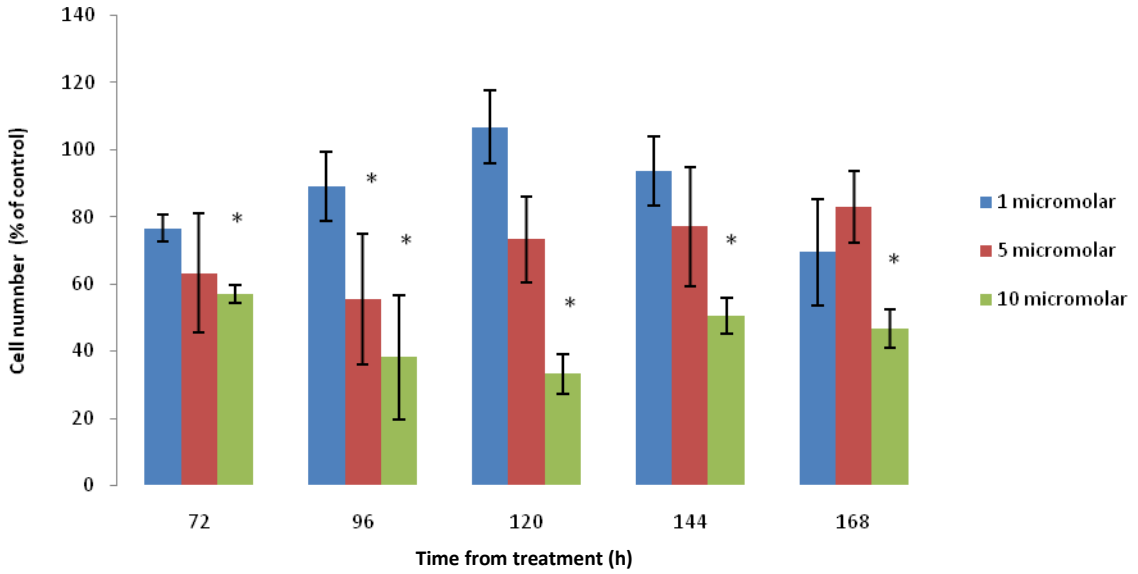

Figure 6.2.5 Effect of a single dose of resveratrol (1, 5 or 10 μM) on the growth of HCA7 cells compared to the growth of control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in triplicate. Asterisks indicate a significant difference from control cells, where **P*=<0.05 and ***P*=<0.001.

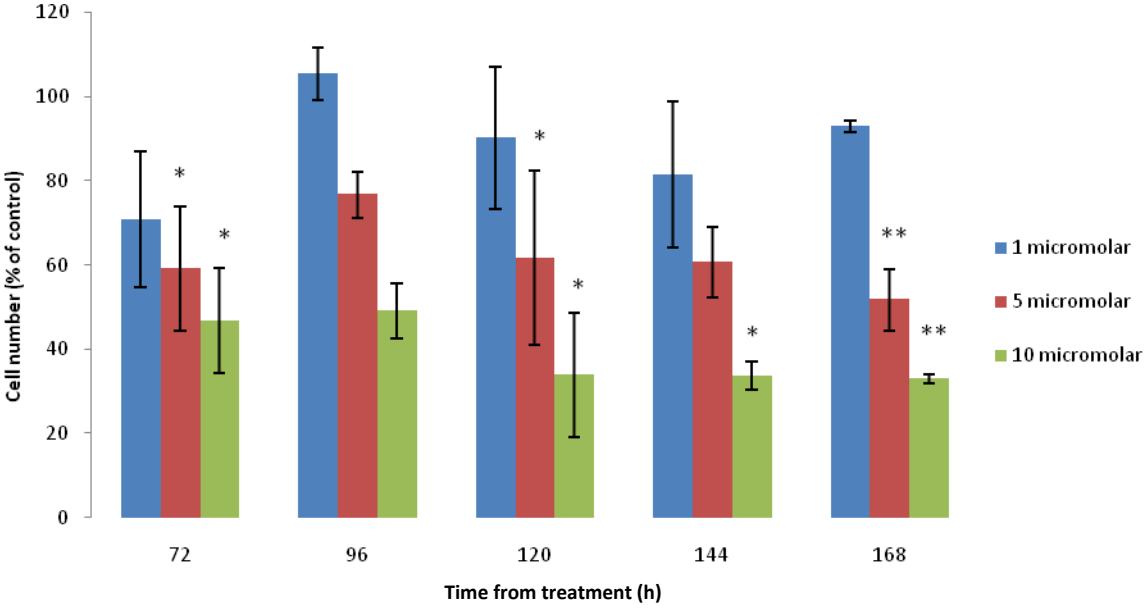

Figure 6.2.6 Effect of repeated dosing with resveratrol (1, 5 or 10 μM) on the growth of HCA7 cells compared to the growth of control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in triplicate. Asterisks indicate a significant difference from control cells, where **P*=<0.05 and ***P*=<0.001. Time from treatment represents time from the first resveratrol treatment.

6.2.2 Effect of resveratrol on the cell cycle

Molecular analysis of human tumours has shown that cell-cycle regulators are frequently mutated, which underscores how important the maintenance of cell-cycle is in the prevention of human cancer (Section 1.5.4). The hypothesis was tested that exposure to daily doses of resveratrol would cause greater inhibitory effects on cell cycle progression than after exposure to a single dose. DNA flow cytometry was performed to ascertain the cell cycle distribution of exponentially growing HT-29 cells and HCA7 cells after treatment with resveratrol either as a single dose or daily doses of 1, 5 and 10 μM for 120 h (Section 2.2.2.4).

Cells were harvested at 24, 48, 72, 96 and 120 h after the first dose of resveratrol. The amount of DMSO added to the incubate did not exceed 0.1%, and no statistically significant differences were seen between the DMSO and the media control. When cells incubated with single or repeated doses of resveratrol were compared with the corresponding control cells, there were no statistically significant differences in either cell line observed in cell cycle distribution. Figures 6.2.7- 6.2.15 show the cell cycle distribution of HT-29 cells harvested at 24, 48, 72, 96 and 120 h after the first dose of resveratrol.

For HT-29 cells, significant differences were observed when comparing single and daily dosing. At 72 h significantly more cells were observed in the G_2/M phase with repeated doses of 5 and 10 μM resveratrol and at the higher dose (10 μM) there was also a higher proportion of cells in S phase. For example, at 72 h, the percentage of cells in G_2 were 14.6

± 1.9% (daily) and 10.1 ± 2.1% (single dosing) with 5 μM resveratrol (2 way ANOVA, *P*=0.009) and 18.6 ± 6.4% (daily dosing) and 12.5 ± 4.2% (single dosing) with 10 μM resveratrol (*P*=0.04). At this time point, the corresponding percentages of cells in S phase after dosing with 10 μM resveratrol were 36.6 ± 4.02 and 29.3 ± 1.66% (*P*=0.04). Repeat exposure to 5 and 10 μM resveratrol for 72 h led to significantly fewer cells in G_1. Following dosing with 5 μM resveratrol the number of cells in G_1 was 45.67 ± 6.74 (daily) compared to 56.4 ± 6.2% (single dosing) (*P*=0.0008) and the corresponding percentages for 10 μM resveratrol were 43.90 ± 4.09 and 58.20 ± 3.97% (*P*=0.0002). For HCA7 cells no significant differences in the cell cycle were observed when comparing single with repeated dosing schedules.

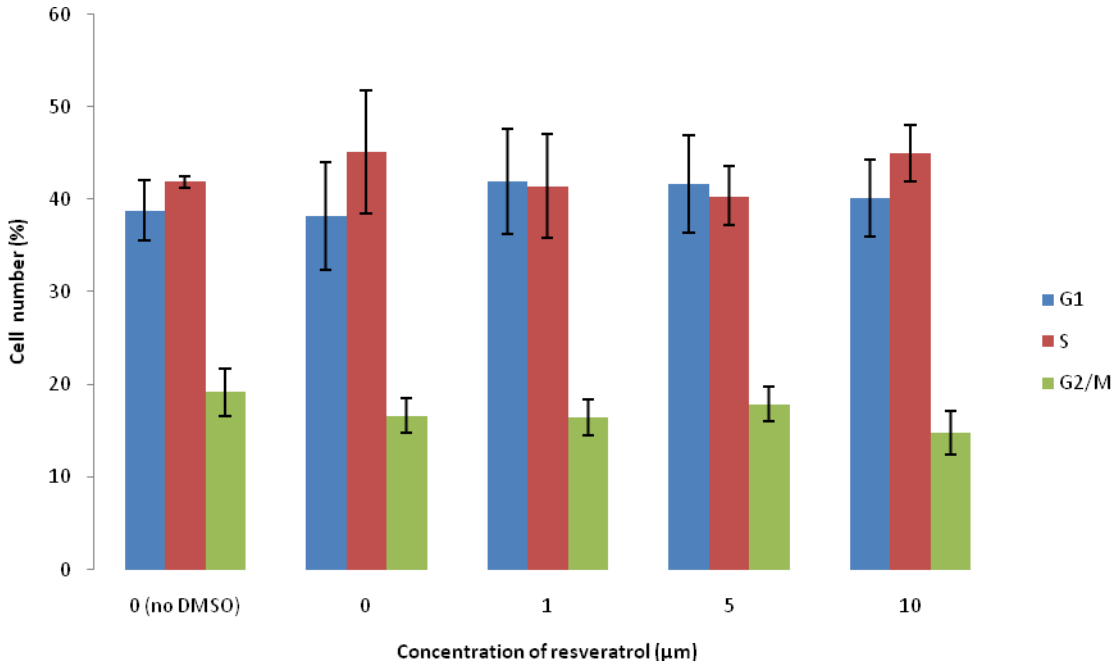

Figure 6.2.7 Effect of incubation with a single dose of resveratrol (1, 5 or 10 µM) for 24 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

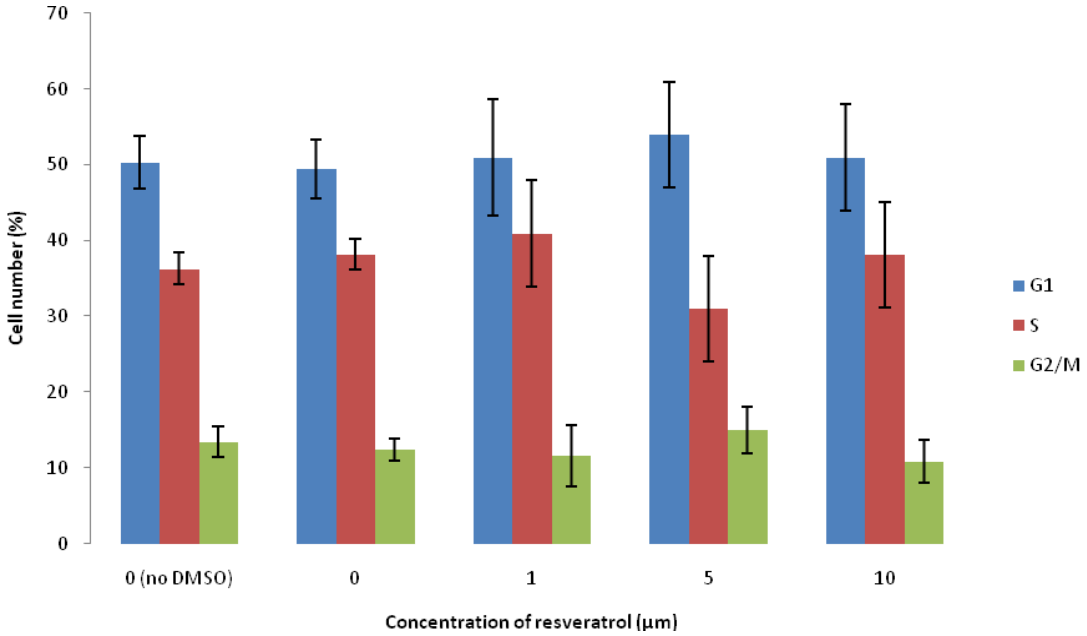

Figure 6.2.8 Effect of incubation with a single dose of resveratrol (1, 5 or 10 µM) for 48 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

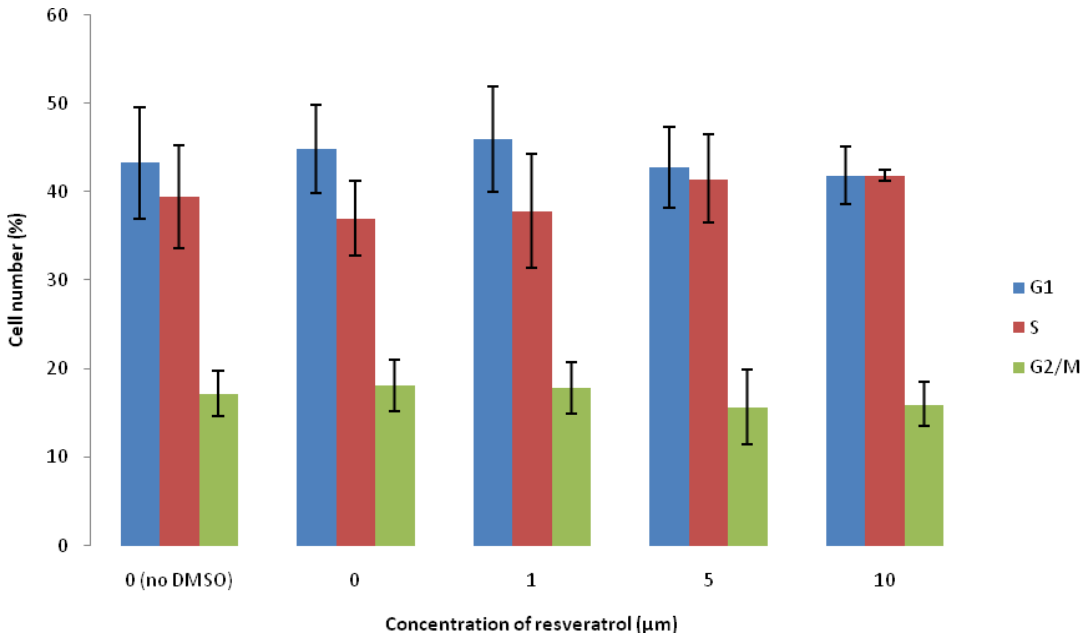

Figure 6.2.9 Effect of incubation of repeated dosing with resveratrol (1, 5 or 10 µM) for 48 h on the cell cycle of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

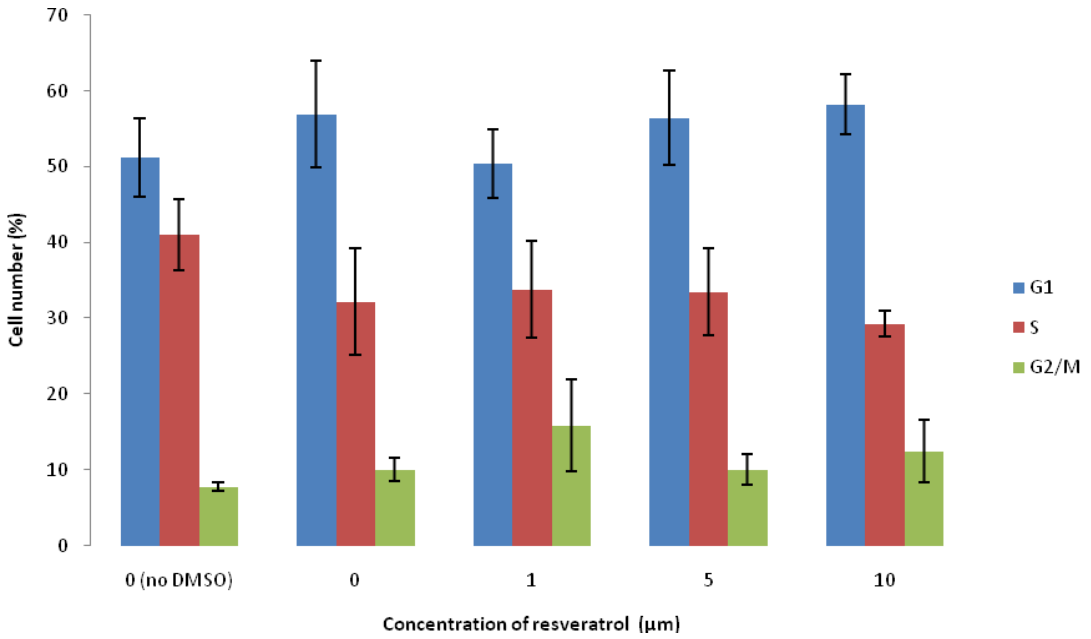

Figure 6.2.10 Effect of incubation with a single dose of resveratrol (1, 5 or 10 µM) for 72 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

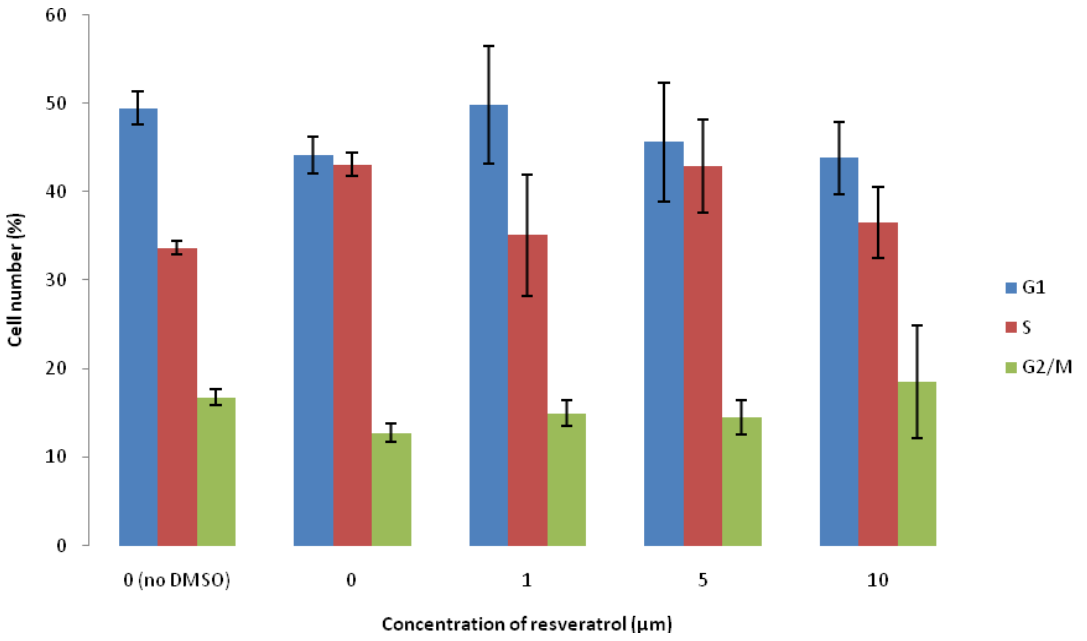

Figure 6.2.11 Effect of incubation of repeated dosing with resveratrol (1, 5 or 10 µM) for 72 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

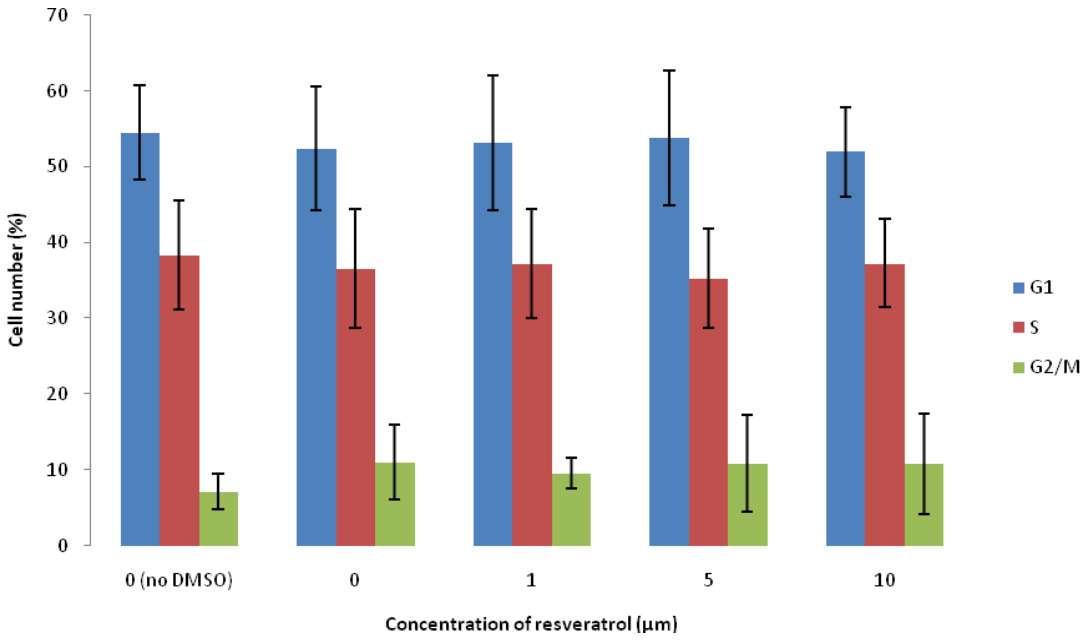

Figure 6.2.12 Effect of incubation with a single dose of resveratrol (1, 5 or 10 µM) for 96 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

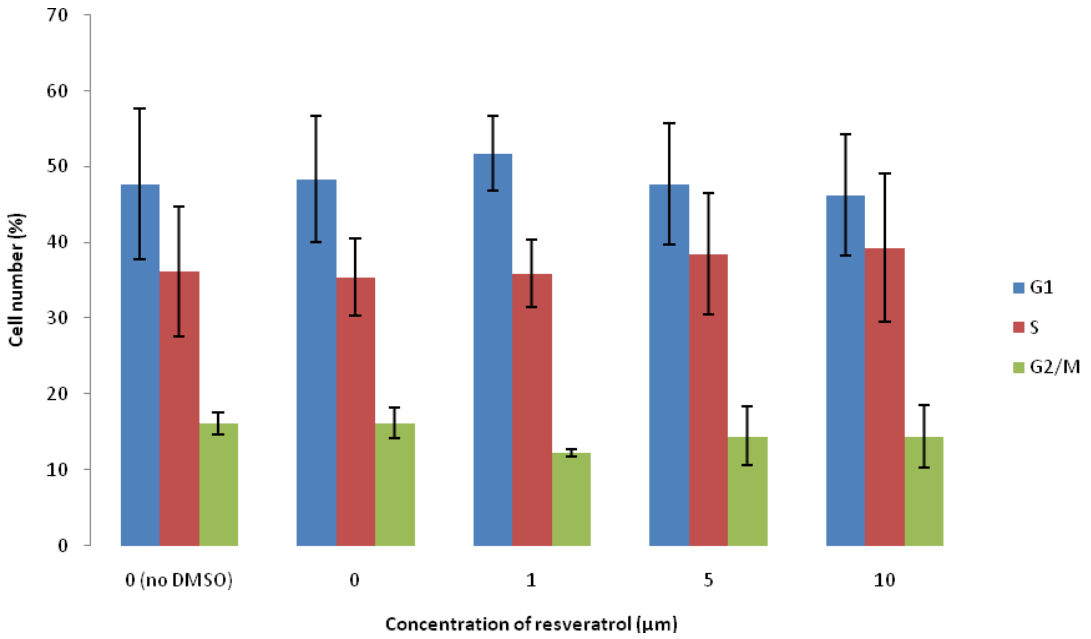

Figure 6.2.13 Effect of incubation of repeated dosing with resveratrol (1, 5 or 10 µM) for 96 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

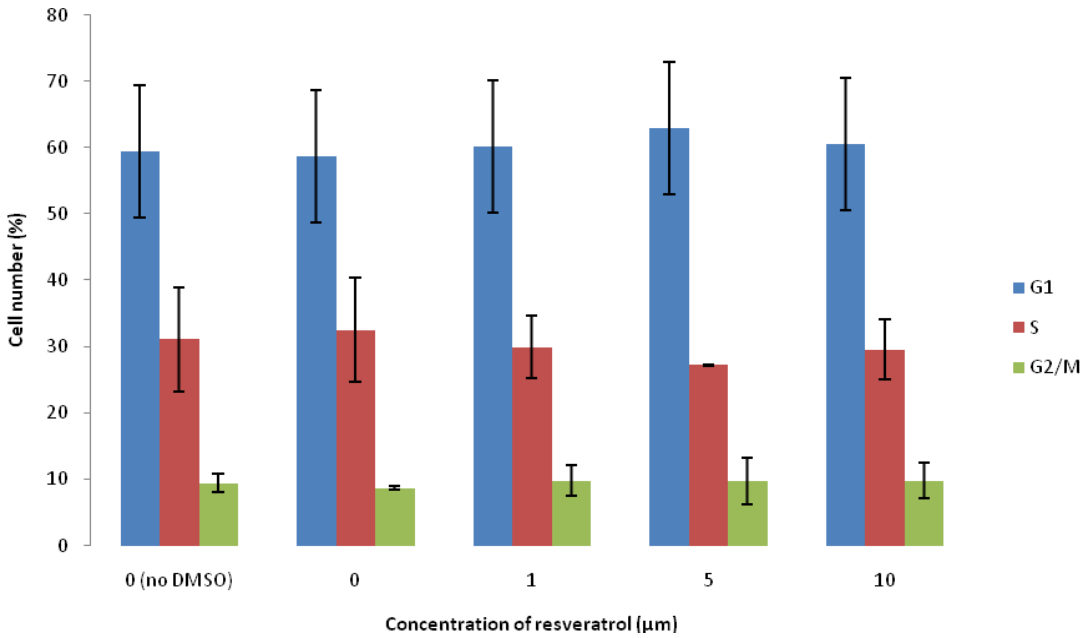

Figure 6.2.14 Effect of incubation with a single dose of resveratrol (1, 5 or 10 µM) for 120 h on the cell cycle distribution of HT-29 cells compared to control cells. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

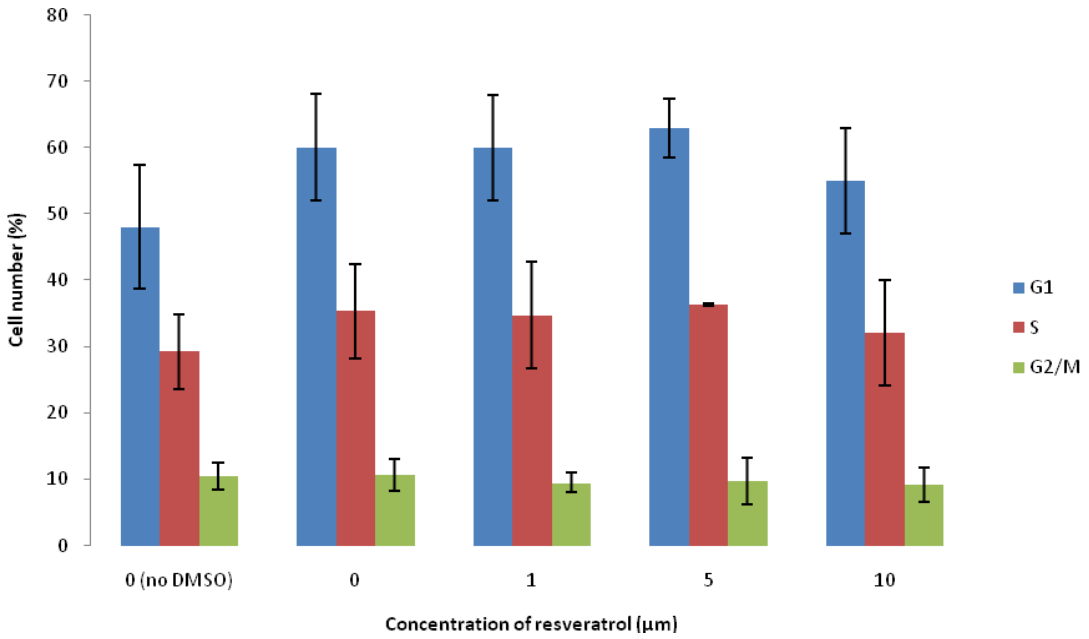

Figure 6.2.15 Effect of incubation of repeated dosing with resveratrol (1, 5 or 10 µM) for 120 h on the cell cycle distribution of HT-29 cells compared to control cells.
Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

6.2.3 Stability of resveratrol in cell culture conditions

Prior to assessing the effect of resveratrol on cell growth and the cell cycle, a preliminary study was conducted to determine the stability of resveratrol under standard cell culture conditions to see whether the loss of resveratrol over time due to instability may contribute to the lower antiproliferative effect of a single dose, which is added to the media and left for the duration of the experiment. The stability of 5 μM resveratrol was assessed in media over 168 h. Within 140 h the resveratrol concentration in media alone had reduced to 50% of its original concentration (Figure 6.2.16).

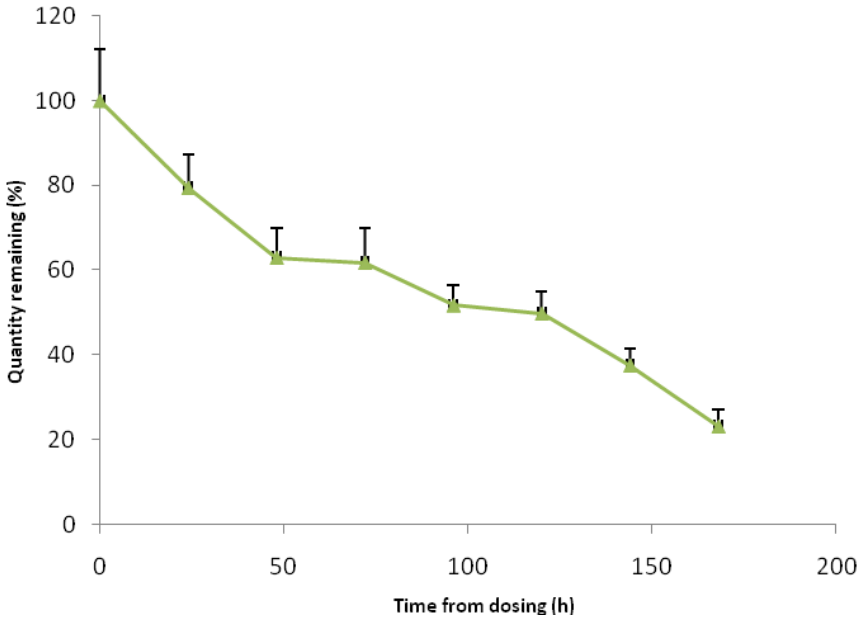

Figure 6.2.16 Stability of resveratrol in media under standard cell culture conditions for 168 h. The concentration of resveratrol was determined using the UV-HPLC method described in Section 2.2.1.1. Results are the mean (± SD) of 3 separate experiments, each conducted in duplicate.

CHAPTER 7

PHARMACODYNAMIC DISCUSSION

7.1 Discussion

As colorectal cancer has a long latency, reduced incidence is usually an impractical endpoint for clinical evaluation of chemopreventive agents. Thus intermediate biomarkers of carcinogenesis are vital as endpoints in chemopreventive agent development (Kelloff *et al*., 2004; O'Shaughnessy *et al*., 2002). Intermediate biomarkers such as abnormal cell proliferation, differentiation, and gene expression have been identified and have served to measure effects of chemopreventive agents in rodent models and in short-term human clinical trials. The understanding of intermediate endpoint biomarker expression in relation to the sequential events in colorectal tumourigenesis establishes a useful approach for evaluating chemopreventive agents (Scalmati and Lipkin, 1993).

Insulin-like growth factor-I (IGF-1) was selected as a potential intermediate biomarker, as high levels of IGF-1 may play an important role in colorectal carcinogenesis. Epidemiological studies have shown that IGF-I is positively associated with the risk of colorectal cancer, and experimental studies have shown that IGF-I has mitogenic and anti-apoptotic actions on colorectal cancer cells (Singh and Rubin, 1993; Tricoli *et al*., 1986). Therefore, the ability to decrease IGF-1 may be an important anticarcinogenic mechanism.

The work presented in this thesis suggests that consumption of resveratrol for a relatively short period of time could decrease circulating levels of IGF-1 and IGFBP-3 in humans. The lowering effect of resveratrol on the concentrations of these proteins was observed in both healthy individuals, who consumed resveratrol for 28 days and in colorectal cancer

patients, who received resveratrol for 8 days. In the healthy volunteers and cancer patients on resveratrol the mean observed IGF-1 reduction was by 8 and 33% respectively. In healthy volunteers, both the pre dosing and post dosing blood collection were fasted samples and are therefore comparable. However, in the colorectal cancer patients the pre dosing bloods were drawn as non fasted samples and post dosing bloods were taken in theatre, whilst the patient was fasted. Logistically it could be difficult to get fasting pre dose blood samples from patients without asking them to come back for an additional visit, which as well as inconveniencing the patient could have a significant adverse effect on recruitment. In addition, most patients followed a restricted diet for a couple of days prior to the procedure, which could potentially have affected nutritional status. Therefore, it is possible, that for the colorectal cancer population, differences in fasting and nutritional status and/or the use of anaesthetic during surgery contributed to the effects seen in the reduction of IGF-1. Patients undergoing surgery are often met with various restrictions regarding nutritional intake, and even brief fasting or hypocaloric nutrition for 1 to 3 days, can result in a marked reduction in insulin sensitivity, as measured by hyperinsulinaemic/normoglycaemic clamps in healthy subjects (Nygren *et al*., 1997; Svanfeldt *et al*., 2003).

The effects on IGF-1 observed in the present study are comparable to the size of IGF-1 decrease reportedly caused by anticancer drugs. Adjuvant or palliative treatment of breast cancer for 2-3 months with tamoxifen reduced IGF-1 by 35-47% (Corsello *et al*., 1998; Pollak *et al*., 1990), whilst in healthy women, where tamoxifen was used as a chemopreventive agent, one month intervention decreased IGF-1 levels by 23% (Decensi *et al*., 1999). Oestrogens and IGF-I have been found to have synergistic effects on breast

cancer cell growth *in vitro* (Parisot *et al*., 1999; Stewart *et al*., 1990) and the cytostatic effect of tamoxifen on the oestrogen-dependent MCF-7 breast cancer cell line has been shown to be associated with modulation of the IGF-1R signaling pathway (Guvakova and Surmacz, 1997). The somatostatin analogue octreotide administered for 4.5 months to patients with gastroenteropancreatic endocrine tumours reduced IGF-1 by 26% (Ronchi *et al*., 2007). In contrast, diet-derived agents have so far produced only negligible changes in the IGF system. Supplementation of the diet for 8 weeks with isolated isoflavones (Vrieling *et al*., 2007a) or the tomato constituent lycopene (Vrieling *et al*., 2007b) failed to affect levels of IGFs or IGF binding proteins in men at increased risk of colorectal cancer, although IGFBP1 and 2 were raised by lycopene. Also of interest was the observation in our healthy volunteer study that pre dosing plasma levels of IGF-1, IGFBP3 and the IGF-1/IGFBP3 ratio are independently and negatively associated with age and BMI. This supports the finding of a similar correlation between IGF-1 and age, documented in previous studies (Kaklamani *et al*., 1999; Lee et al., 2005).

Intervention with 9-*cis*-retinoic acid for 3 months decreased circulating IGF-1 in former smokers (Lee *et al*., 2005). Reduction in IGF-1 is often the corollary of elevation of IGFBP-3 concentrations, which sequester IGF-1 and decrease its bioavailability and thus its interaction with IGF receptors, the process through which it engages mitogenic and anti-apoptotic actions. Consistent with this notion IGFBP-3 levels were found to be elevated in the clinical trial of 9-*cis*-retinoic acid (Lee *et al*., 2005). Similarly, in immune-deprived mice bearing the DU145 prostate tumour xenograft, the putative cancer chemopreventive agent silibinin augmented circulating IGFBP-3 (Singh *et al*., 2004).

The results presented here show that exposure to resveratrol did not elevate IGFBP-3 levels. In contrast, there was actually some reduction in the majority of the volunteers and colorectal patients. In the healthy volunteers a reduction of 10 % (P=0.03) and 8 % (P=0.03) was observed in those on the 1.0 and 2.5 g dose, respectively. In colorectal cancer patients, there was a more pronounced significant reduction of 35% and 26% in those receiving 0.5 and 1.0 g of resveratrol respectively. It is difficult to interpret this finding in terms of mechanisms, by which resveratrol may exert chemoprevention, as the evidence for the relationship between IGFBP-3 levels and risk of cancer is mixed and somewhat inconclusive. In the case of malignancies of the lung or prostate, IGFBP-3 levels seem to be inversely related with cancer risk (Ali *et al*., 2003). The evidence for a protective role of IGFBP3 in colorectal cancer is equivocal, as two studies support a protective role (Giovanucci *et al*., 2000; Ma *et al*., 1999), whilst three do not (Kaaks, 2002: Palmqvist *et al*., 2002; Probst-Hensch *et al*., 2001). In the case of breast cancer a number of studies failed to demonstrate a link between IGFBP-3 levels and cancer risk (Hankinson *et al*., 1998; Toniolo *et al*., 2000), whilst others indicate that the risk may be increased with higher IGFBP-3 levels (Del Giudice *et al*., 1998; Krajcik *et al*., 2002).

The calorie restriction mimesis caused by resveratrol is thought to be mediated via activation of the mammalian sirtuin deacetylase enzyme SIRT1, which in turn stimulates mitochondrial growth (Lagouge *et al*., 2006). Activation of SIRT1 by resveratrol may be relevant to cancer chemopreventive activity, as SIRT1 exerts tumour suppressor functions. For example, in the APC^{Min} mouse model of colon cancer, elevated SIRT1 levels, as a result of ectopic SIRT1 expression or calorie restriction, resulted in reduced tumour formation (Firestein *et al*., 2008). IGF-1 and SIRT1 are mechanistically linked, in that

SIRT1 expression and activity are attenuated by IGF-1-mediated signaling (Tang, 2006). Consistent with this link, the decrease in IGF-1 caused by resveratrol presented in this thesis suggests that it may potentially activate SIRT1 in humans as well.

The effects of resveratrol on levels of the DNA adduct M_1dG presented in this thesis support the notion that resveratrol may possess dual activity with regard to its antioxidative properties (De Salvia *et al*., 2002). After only 8 daily doses of 0.5 and 1.0 g, a reduction in M_1dG was observed in DNA extracted from peripheral blood samples of colorectal cancer patients presented in this thesis. In contrast, after a longer period of dosing (29 days) an increase in levels was seen in healthy volunteers, which was significant at the 2.5 g dose. In addition, in colorectal cancer patients resveratrol seemed to have contrasting effects on M_1dG levels in peripheral blood and colorectal tissue. Whilst there was a reduction in the former, an increase was observed in the latter, which was of particular interest, as both sets of samples were collected from each patient at a similar time point. In order for the levels of M_1dG in the blood to be used as a surrogate biomarker, ideally the levels of M_1dG adducts in the blood should accurately reflect what is happening in the target tissue. This study was designed to compare target tissue damage levels with those in a more accessible surrogate sample and in this case opposing effects were observed. This suggests that changes in M_1dG levels in DNA extracted from blood are not a good indication of what is happening in the target tissue, although further research is needed to clarify this. An additional point to consider in interpreting the results obtained is that anaesthesia and the surgical procedure may have influenced blood and tissue levels of M_1dG. Recruitment of a placebo controlled group of patients, as discussed later, could help to establish the effects

of resveratrol alone. However, based on our data, M_1dG does not seem to be a promising biomarker for resveratrol.

Although several investigations *in vitro* have cited the possible role and protective effects of resveratrol against DNA damage and lipid peroxidation in cell membranes (Leonard *et al*., 2003), resveratrol has been shown to have both an antioxidant and a prooxidant activity (De Salvia *et al*., 2002). De Salvia and co workers studied the antioxidant activity of resveratrol against reactive oxygen species induced by H_2O_2 treatment in CHO cells, with attention on two major potential mechanisms, scavenging activity and interference with oxidative metabolism. Effects were assessed through the analysis of three important parameters: intracellular oxidation (Dichlorofluorescein Test), primary DNA damage (Comet Assay) and fixed DNA damage (chromosomal aberrations). Cells were treated with a single H_2O_2 dose (2×10^{-4} M) in order to induce reactive oxygen species and then challenged with resveratrol. Two experimental protocols were applied, simultaneous treatment and a 3 hour resveratrol pre treatment. Resveratrol alone appeared to induce 'per se' a slight increase in endogenous oxidation, a simultaneous supply of both resveratrol and H_2O_2 led to a slight but significant reduction in induced endogenous oxidation and pre treatment with resveratrol appeared to enhance the H_2O_2 oxidation damage. The authors therefore concluded that in combined treatments resveratrol appears to differently affect primary and fixed DNA damage and suggested that the mechanism of resveratrol action appears to be very complex depending on doses applied, supply conditions, test systems employed and endpoints observed (De Salvia *et al*., 2002).

It is conceivable that the increase in tissue M_1dG levels in the colorectal cancer study presented in this thesis may be due to effects of the surgical procedure rather than being directly related to the intake of resveratrol. In support of this possibility is the observation in previous studies that free radical induced damage in the form of increased serum lipid peroxide level is associated with surgical stress (Sane *et al*., 1993; Yukioka *et al*., 1996). Furthermore, rat small intestine has been shown to be highly susceptible to injury during surgery. Mild handling of the intestine by itself is capable of inducing oxidative stress in the enterocyte, which leads to increased permeability of the intestine. The damage has been shown to be maximal at one hour after surgery with recovery of the tissue by 24 hours (Anup *et al*., 1999). The reversibility may be a result of the minimum time taken for the surgical procedure and it is likely that in major abdominal surgery the time taken may be much longer, so that the structural and functional alterations in the intestine may be more extensive and sometimes irreversible (Anup *et al*., 1999). These data suggest that the effects of surgical stress occur rapidly and could influence the results in the colorectal study presented in this thesis.

Mediators of inflammation, such as COX-2, prostaglandins, inducible nitric oxide synthase (iNOS), NO, and pro-inflammatory cytokines are considered to be involved in carcinogenesis, especially in the promotion and progression stages (Hussain and Harris, 2007). A number of factors support the roles of COX-2 and prostanoids in carcinogenesis: the elevated expression and/or activity of COX-2 and production of certain prostaglandins in various cancers (Kundu *et al*., 2006), the increased susceptibility of COX-2 transgenic mice to chemically induced carcinogenesis (Muller-Decker *et al*., 2002) and the abrogation of experimental tumourigenesis in COX-2 knock out animals (Tiano *et al*., 2002).

Resveratrol has been reported to inhibit COX-2 at both transcriptional and post transcriptional levels (Li *et al*., 2002; Jang and Pezzuto 1998), prevent mobilization of arachadonic acid and decrease PGE_2 production (Martinez and Moreno, 2000), which were observed *in vitro.*

Resveratrol administered at doses of up to 5.0 g presented in this thesis failed to significantly affect plasma PGE_2. In addition, COX-2 staining intensity increased, rather than decreased, in colorectal tissue of patients who received both 0.5 and 1.0 g resveratrol, and this increase was statistically significant (P=0.004). It is probable that colorectal tissue ischaemia was present during the operation and this could have caused the change in COX-2 staining observed. In support of this notion are the observations in previous studies of an induction of COX-2 expression immediately after global (Koistinaho *et al*., 1999) or focal ischaemia (Collaco-Moraes *et al*., 1996) of the brain. Prolonged COX-2 expression has been reported in vulnerable neurons after global ischaemia (Matsuoka *et al*., 1999) and in neurons of the ischaemic penumbra region after focal ischaemia (Nogawa *et al*., 1997). The effects of genetic manipulation of COX-2 on focal ischaemia have also been reported. Mice lacking COX-1 are more susceptible to focal cerebral ischaemia, an effect that can be attributed to a more severe cerebral blood flow reduction in vulnerable regions at the periphery of the ischaemic territory (Ladecola *et al*., 2001) and neuronal over expression of COX-2 enhanced cerebral infarction (Dore *et al*., 2003).

It is therefore possible that the increase in COX-2 staining observed in the colorectal tissue presented in this thesis was related to bowel ischaemia at the time of surgical resection. The effect of ischaemia is likely to be greater on the resected colon specimen, than after

endoscopic biopsy, as in the former there is clamping of the vascular supply intra operatively. Unfortunately options were limited for obtaining the post dosing colorectal tissue required for the pharmacodynamic analysis in the colorectal cancer patient study. One possible way of limiting the effects of bowel ischaemia on the tissue samples collected, would be for the patients to undergo a further endoscopic procedure either prior to or during the surgical procedure, but before the resection of the tumour. However, this further procedure, with potential risks for the patient, is not part of standard surgical care and sections of tissue obtained endoscopically are usually small. A second, more realistic option would be to include a placebo control group of patients, who had not received resveratrol, so that tissue samples could be compared (Section 8.1).

The changes observed in colorectal tissue proliferation (Ki-67) presented here support previous published findings that resveratrol inhibits proliferation in colorectal cells *in vitro* (Fuggetta *et al.*, 2006) and in animals *in vivo* (Sengottuvelan *et al*., 2006c). It is conceivable that there are limitations in comparing biopsies (pre dosing) with the resected tissue samples; less tissue is obtained at a biopsy than after the surgical resection and therefore the tissue available for immunohistochemistry and analysis was more limited. However, as far as possible efforts were made to ensure that samples were comparable; tissue sections, stained for Ki-67 and COX-2, were approved by a consultant gastrointestinal pathologist (Section 2.2.4.3), who verified that the biopsies were suitable for analysis and comparison and had been prepared and processed correctly and included a review of the positive and negative control slides.

The aim of the cell work presented in this thesis was to explore the effect of achievable concentrations of resveratrol on cell proliferation, when administered on a daily basis to mimic the clinical scenario under investigation. The doses of resveratrol used in the cell work were chosen to represent concentrations potentially attainable in the plasma of the healthy volunteers. These doses are generally much lower than pharmacologically active concentrations reported in previous studies, which have been shown to arrest proliferation and cause cell cycle arrest. The results obtained indicate that even at these lower concentrations of resveratrol, there was a reduction in growth of the colon cells and daily dosing had a greater effect on cell proliferation than a single dose. These findings suggest that despite low systemic concentrations of resveratrol after repeat dosing in healthy volunteers, daily ingestion could possibly produce pharmacological efficacious effects for chemoprevention.

CHAPTER 8

FINAL DISCUSSION

8.1 Discussion

Over the last few years the potential cancer chemopreventive properties of dietary constituents have gained increasing interest as possible alternatives to drugs such as tamoxifen and aspirin. Resveratrol is representative of a group of diet-derived putative cancer chemopreventive agents encompassing curcumin, tea polyphenols and apigenin amongst others. These agents have been shown to engage a range of anticarcinogenic mechanisms in cellular studies *in vitro*, although hardly any of these processes have been explored as potential efficacy biomarkers in humans. Cancer chemoprevention frequently involves administering agents to relatively healthy individuals for prolonged periods of time, and therefore safety is of paramount importance. The results of the studies presented in this thesis suggest that oral daily dosing with resveratrol is safe and generally well tolerated.

To our knowledge this is the first study in which concentrations of resveratrol have been measured in colorectal, or indeed any tissue, in humans. The results indicate that consumption of up to 1.0 g daily achieves levels in the colorectal tissue which are of the order of magnitude commensurate with pharmacologically active doses in APC^{Min} mice (Sale *et al*., 2005). Although there was a reduction in cell proliferation in the tissue samples, as assessed by Ki-67, an increase in M_1dG and COX-2 tissue levels was also observed. In all study participants collectively, there appeared to be a 5.5 % and 9.3% reduction post treatment with resveratrol in the proportion of tumour epithelial cells and normal epithelial cells which stained positive for Ki-67 respectively. Both of these

approached statistical significance (P=0.05). For all participants combined, the extent of staining of COX-2 in tumour cells increased post treatment with resveratrol and was statistically significant (P=0.004), but there was no significant effect observed in the normal colorectal tissue. In addition, there was a rise in M_1dG levels after 8 daily doses of resveratrol by 24.6 and 57.5 % in malignant and normal tissue, respectively, but neither change was statistically significant.

The observed effect of resveratrol on IGF proteins in study participants indicates that modulation of the IGF axis may contribute to the activity of resveratrol *in vivo*. This is especially illustrated in the healthy volunteers, in whom the IGF system-modulating effect was most prominent in the 2.5 g dose group. The results tentatively suggest the possibility that IGF-1 and /or IGFBP-3 may serve as potential biomarkers of chemopreventive efficacy of resveratrol. They should be assessed as candidate markers when resveratrol is eventually evaluated in definitive clinical chemoprevention studies. To our knowledge, this is the first evidence in support of a potential biomarker of pharmacological activity of resveratrol in humans. Future work is required to verify and validate the effects of resveratrol on the IGF axis in humans, particularly in target tissues, and to establish whether resveratrol causes calorie restriction mimesis in humans, similar to reports in lower organisms and mice (Baur *et al*., 2006: Pearson *et al*., 2008). It is intriguing that on the 2.5 g dose level in healthy volunteers, whilst there was a significant reduction in IGF-1 concentration, there was also a significant increase in M_1dG levels. From the results presented in this thesis, resveratrol does not appear to have antioxidant properties, as reflected by this particular lesion at the doses administered, but these types of compounds can be both pro and antioxidants depending on the system and concentrations employed (Van Helden *et al*., 2009).

It has to be acknowledged that the changes in plasma and tissue levels of IGF proteins, PGE_2, M_1dG, COX-2 and Ki-67 observed in the colorectal cancer patient study could have been affected by one or more factors other than the agent administered, including surgical procedure, administration of an anaesthetic and fasting state. In both the colorectal cancer and healthy volunteer study, all participants were asked to discontinue the ingestion of multivitamins and foodstuffs containing resveratrol for the whole duration, including a 5 day wash out period prior to dosing. Changes in diet and vitamin intake could therefore be a possible explanation for the rise in blood M_1dG levels observed in the healthy volunteer study. In addition, it is possible that there are natural variations in plasma and tissue levels of these potential biomarkers over time, which are not yet fully understood and need to be established in order to fully appreciate any intervention-associated changes.

To assess more accurately the effects of resveratrol on tissue and blood samples, a different study design would be needed involving recruitment of a second control group of healthy volunteers and colorectal cancer patients, who did not receive resveratrol, but were still under identical study conditions. Ideally this group of participants should be matched to the group of participants taking the study drug, in regards to demographics (e.g. age and sex) and for the colorectal cancer patients, matched in terms of planned surgical procedure (e.g. type of colorectal resection) and radiological staging of the tumour. Although this would not eliminate the effects of fasting, surgery or anaesthetic on biomarkers, any observed changes in the samples collected from the corresponding study groups could be compared.

Taking the average resveratrol content of wine as ~5 mg/L, the volunteers and colorectal patients on the clinical studies presented in this thesis were administered resveratrol equivalent to drinking ~133, 267, 677 and 1334 bottles of wine per day on the 0.5, 1.0, 2.5 and 5.0 g dose levels respectively. Despite the high doses of resveratrol administered, the mean plasma C_{max} concentrations attained (up to 4.19 μM), although higher than after a single dose of the parent compound (Boocock *et al*., 2007), were still below the concentrations required to elicit pharmacologic effects associated with cancer chemoprevention in the majority of *in vitro* experiments (≥5 μM). However, *in vitro* studies tend to investigate concentrations which are much higher than those achieved in plasma in the present healthy volunteer study. It is possible that resveratrol could have effects at lower concentrations, if a different model was adopted, as illustrated by the greater inhibitory effect of repeat dosing on cell proliferation compared to the more typical experimental scenario of a single dose, presented in this thesis. As chemoprevention is ultimately aimed at humans, future *in vitro* experiments should include concentrations which mimic achievable and realistic systemic concentrations in humans in their study design. Despite the low systemic availability of parent agent, consumption of high-dose resveratrol by cancer patients results in relatively high colorectal tissue concentrations, of up to 3774 nmol/g. Therefore, low systemic concentrations of resveratrol may actually be advantageous for the chemoprevention of colorectal cancers, in that it could reduce potential systemic toxicity, whilst attaining efficacious concentrations in the target tissue.

Low plasma concentrations of resveratrol are primarily due to rapid phase II metabolism, which produces high systemic concentrations of resveratrol metabolites, particularly, resveratrol-3-sulfate and the two monoglucuronides, and it is conceivable that these

derivatives may themselves have chemopreventive properties. Whereas the pharmacological properties of resveratrol conjugates are largely unknown, conjugated metabolites of naturally occurring flavonoids, polyphenols chemically resembling resveratrol, have been suggested to be responsible for, or contribute to, the pharmacological activity of the parent molecule. For example, in vascular smooth muscle cells, quercetin 3-*O*-glucuronide inhibited both activity of c-Jun NH_2-terminal kinase and binding of transcription factor activator protein-1 to DNA (Yoshizumi *et al*., 2002) and quercetin conjugates seem to retain the antioxidant properties of the parent molecule (Morand *et al*., 1998). The pharmacokinetic data presented in our healthy volunteer study has suggested that metabolism of resveratrol may be affected to a certain extent by a number of factors including gender, dose administered and frequency of dosing. Gender dimorphisms have been previously documented in animals but not so well established in humans (Coughtrie and Johnston, 2001).

8.2 Conclusion

In summary, the results presented in this thesis confirm that resveratrol has low bioavailability, due to rapid metabolism in humans. Despite the repeat dosing schedules not achieving significantly higher systemic concentrations of resveratrol than after an equivalent single dose, pharmacological efficacious concentrations were observed in the colorectal tissue. Although there are numerous pitfalls in the development of new chemopreventive drugs, which include the possibility that the compound modulates surrogate endpoints, but fails to confer clinical benefit, has less specificity clinically than expected from preclinical data or has unexpected toxicity, resveratrol holds promise as a chemopreventive agent. More work is needed to identify pathways through which resveratrol may function and to develop mechanism based biomarkers for evaluating clinical outcome. Controlled clinical trials are now necessary to aid the development of resveratrol to become a standard clinical agent. The preclinical and clinical data presented in this thesis suggest that resveratrol is a promising candidate in chemopreventive and chemotherapeutic strategies.

CHAPTER 9

APPENDIX

9.1 Assessment of relationships between participant demographic variables and pharmacokinetic parameters after repeat dosing tables

Table 9.1.1 Concentration of resveratrol and its metabolites in the plasma of healthy volunteers drawn at 24 h post administration of resveratrol across the 4 dose levels of 0.5, 1.0, 2.5 and 5.0 g, (n=10 per dose level).

Compound		Dose (g) 0.5	1.0	2.5	5.0
Monoglucuronide 1	**Mean (ng/mL)**	5.17	41.03	98.66	390.4
	SD	11.61	53.14	72.89	689.1
Monoglucuonide 2	**Mean (ng/mL)**	3.19	12.54	59.04	565.9
	SD	7.08	19.42	82.82	1575
Resveratrol disulfate	**Mean (ng/mL)**	17.86	24.54	88.95	120.9
	SD	24.50	21.15	35.10	71.01
Resveratrol 3-sulfate	**Mean (ng/mL)**	16.19	81.19	270.6	790.7
	SD	29.81	87.68	360.3	1154
Resveratrol 4´-sufalte	**Mean (ng/mL)**	0.18	0.58	2.66	20.67
	SD	0.58	1.36	4.79	49.23
Resveratrol	**Mean (ng/mL)**	2.02	5.54	25.44	119.2
	SD	2.77	10.12	37.28	335.1

9.1.2 Linear regression comparing age and plasma PK parameters for resveratrol and its main metabolites in healthy volunteers for the 4 dose levels. Values represent Pearsons coefficient and P values in brackets)

		Dose of resveratrol (g) 0.5	1.0	2.5	5.0
Resveratrol	**C_{max} (ng/mL)**	0.61 (*P*=0.60)	-0.33 (*P*=0.36)	-0.21 (*P*=0.56)	0.36 (*P*=0.31)
	AUC_{inf} (ng h/mL)	-0.56 (*P*=0.22)	-0.35 (*P*=0.36)	0.48 (*P*=0.16)	0.68 (*P*=0.85)
	Half life (h)	-0.30 (*P*=0.44)	-0.27 (*P*=0.48)	-0.12 (*P*=0.77)	-0.45 (*P*=0.90)
Resveratrol 3-sulfate	**C_{max} (ng/mL)**	0.20 (*P*=0.59)	0.08 (*P*=0.83)	0.46 (*P*=0.18)	0.26 (*P*=0.47)
	AUC_{inf} (ng h/mL)	-0.22 (*P*=0.58)	0.56 (*P*=0.09)	0.66 **(*P*=0.04)**	0.11 (*P*=0.75)
Monoglucuronide 1	**C_{max} (ng/mL)**	0.45 (*P*=0.19)	-0.42 (*P*=0.23)	0.02 (*P*=0.96)	0.27 (*P*=0.46)
	AUC_{inf} (ng h/mL)	-0.13 (*P*=0.76)	-0.45 (*P*=0.19)	0.50 (*P*=0.14)	0.06 (*P*=0.86)
Monoglucuronide 2	**C_{max} (ng/mL)**	0.38 (*P*=0.28)	0.32 (*P*=0.36)	-0.43 (*P*=0.91)	0.55 (*P*=0.09)
	AUC_{inf} (ng h/mL)	-0.15 (*P*=0.72)	0.42 (*P*=0.26)	0.60 (*P*=0.07)	0.10 (*P*=0.80)

Table 9.1.3 Comparison of gender and plasma PK parameters for resveratrol and its main metabolites in healthy volunteers (0.5 g dose level). Mean +/-SD

0.5 g			Mean	SD	*Independent T test (P =)*
Resveratrol	**C_{max} (ng/mL)**	**Male**	37.43	33.77	*0.48*
		Female	56.67	49.29	
	AUC_{inf} (ng h/mL)	**Male**	237.3	202.6	*0.78*
		Female	202.1	5.94	
	Half life (h)	**Male**	7.61	3.98	*0.92*
		Female	8.02	8.30	
Resveratrol 3-sulfate	**C_{max} (ng/mL)**	**Male**	477.4	212.2	*0.77*
		Female	526.9	310.5	
	AUC_{inf} (ng h/mL)	**Male**	3026	1008	*0.48*
		Female	4878	3721	
Monoglucuronide 1	**C_{max} (ng/mL)**	**Male**	126.9	40.14	***0.02***
		Female	274.5	115.3	
	AUC_{inf} (ng h/mL)	**Male**	1204	690.0	*0.14*
		Female	2148	885.3	
Monoglucuronide 2	**C_{max} (ng/mL)**	**Male**	131.8	53.86	*0.36*
		Female	262.8	239.6	
	AUC_{inf} (ng h/mL)	**Male**	756.3	418.9	*0.14*
		Female	1371	621.7	

Table 9.1.4 Comparison of gender and plasma PK parameters for resveratrol and its main metabolites in healthy volunteers (1.0 g dose level). Mean +/-SD

1.0 g			Mean	SD	*Independent T test (P =)*
Resveratrol	**Cmax (ng/mL)**	**Male**	169.0	103.4	*0.25*
		Female	96.66	79.85	
	AUC_{inf} (ng h/mL)	**Male**	1732	2575	*0.38*
		Female	496.3	338.0	
	Half life (h)	**Male**	25.20	47.80	*0.53*
		Female	9.02	5.24	
Resveratrol-3-sulfate	**Cmax (ng/mL)**	**Male**	1794	451.4	*0.60*
		Female	1537	815.1	
	AUC_{inf} (ng h/mL)	**Male**	11,638	5680	*0.25*
		Female	8463	1848	
Monoglucuronide 1	**Cmax (ng/mL)**	**Male**	707.3	461.5	*0.10*
		Female	708.0	378.3	
	AUC_{inf} (ng h/mL)	**Male**	4313	2911	*0.99*
		Female	4289	1199	
Monoglucuronide 2	**Cmax (ng/mL)**	**Male**	459.8	202.5	*0.08*
		Female	766.7	295.0	
	AUC_{inf} (ng h/mL)	**Male**	1917	510.0	*0.06*
		Female	2887	763.1	

Table 9.1.5 Comparison of gender and plasma PK parameters for resveratrol and its main metabolites in healthy volunteers (2.5 g dose level). Mean +/-SD

2.5 g			Mean	SD	*Independent T test (P =)*
Resveratrol	**C_{max} (ng/mL)**	**Male**	344.8	319.7	*0.90*
		Female	322.0	86.41	
	AUC_{inf} (ng h/mL)	**Male**	1365	764.9	*0.39*
		Female	2542	2485	
	Half life (h)	**Male**	6.97	1.41	*0.14*
		Female	20.57	19.14	
Resveratrol-3-sulfate	**C_{max} (ng/mL)**	**Male**	2026	980.0	*0.58*
		Female	2474	1330	
	AUC_{inf} (ng h/mL)	**Male**	17,671	6689	*0.60*
		Female	22,858	17,914	
Monoglucuronide 1	**C_{max} (ng/mL)**	**Male**	670.0	423.3	*0.25*
		Female	1450	1189	
	AUC_{inf} (ng h/mL)	**Male**	6570	2311	*0.31*
		Female	9620	5216	
Monoglucuronide 2	**C_{max} (ng/mL)**	**Male**	848.4	587.9	*0.30*
		Female	2014	2015	
	AUC_{inf} (ng h/mL)	**Male**	4319	1911	*0.23*
		Female	7083	3865	

Table 9.1.6 Comparison of gender and plasma PK parameters for resveratrol and its main metabolites in healthy volunteers (5.0 g dose level). Mean +/-SD

5.0 g			Mean	SD	*Independent T test (P=)*
Resveratrol	**C_{max} (ng/mL)**	**Male**	1113	332.6	*0.25*
		Female	716.36	681.3	
	AUC_{inf} (ng h/mL)	**Male**	10,570	17454	*0.39*
		Female	2406	2174	
	Half life (h)	**Male**	7.46	6.36	*0.35*
		Female	4.16	1.80	
Resveratrol 3-sulfate	**C_{max} (ng/mL)**	**Male**	4964	1622	*0.06*
		Female	2942	933.1	
	AUC_{inf} (ng h/mL)	**Male**	64,101	60,600	*0.47*
		Female	39,547	21,462	
Monoglucuronide 1	**C_{max} (ng/mL)**	**Male**	2441	683.3	*0.66*
		Female	2115	1568	
	AUC_{inf} (ng h/mL)	**Male**	40,643	48,411	*0.40*
		Female	18,045	12,237	
Monoglucuronide 2	**C_{max} (ng/mL)**	**Male**	4576	1721	*0.15*
		Female	2768	1770	
	AUC_{inf} (ng h/mL)	**Male**	60,522	10,4675	*0.42*
		Female	14,990	9547	

Table 9.1.7 Linear regression comparing BMI and plasma PK parameters for resveratrol and its main metabolites in healthy volunteers for the 4 dose levels.
Values represent Pearsons coefficient and P values in brackets.

		Dose of resveratrol (g)			
		0.5	**1.0**	**2.5**	**5.0**
Resveratrol	**C_{max} (ng/mL)**	0.89 (*P*=0.08)	-0.21 (*P*=0.56)	-0.60 (*P*=0.07)	0.22 (*P*=0.56)
	AUC_{inf} (ng h/mL)	0.01 (*P*=0.99)	0.08 (*P*=0.88)	0.61 (*P*=0.06)	0.40 (*P*=0.20)
	Half life (h)	-0.20 (*P*=0.62)	0.11 (*P*=0.77)	0.45 (*P*=0.89)	0.39 (*P*=0.30)
Resveratrol 3-sulfate	**C_{max} (ng/mL)**	0.45 (*P*=0.90)	-0.40 (*P*=0.56)	0.44 (*P*=0.21)	0.60 (*P*=0.09)
	AUC_{inf} (ng h/mL)	-0.48 (*P*=0.19)	-0.43 (*P*=0.22)	0.68 **(*P*=0.03)**	0.46 (*P*=0.22)
Monoglucuronide 1	**C_{max} (ng/mL)**	0.36 (*P*=0.31)	-0.25 (*P*=0.49)	-0.10 (*P*=0.78)	0.13 (*P*=0.75)
	AUC_{inf} (ng h/mL)	-0.38 (*P*=0.36)	-0.27 (*P*=0.45)	0.21 (*P*=0.56)	0.40 *P*=0.28)
Monoglucuronide 2	**C_{max} (ng/mL)**	0.42 *P*=0.23)	-0.23 (*P*=0.52)	-0.05 (*P*=0.90)	0.35 (*P*=0.36)
	AUC_{inf} (ng h/mL)	-0.43 (*P*=0.28)	-0.03 (*P*=0.94)	0.54 (*P*=0.11)	0.47 (*P*=0.21)

9.2 Resveratrol food and drink restrictions

Date of dosing: _____

Start resveratrol-restricted diet on:_____

Restricted ingredients: Wine (red or white), peanuts, mulberries, grapes (seeds, skin, stalks), cranberries, blueberries, huckleberries, products containing resveratrol or any restricted ingredients.

Please avoid the following for the 5 days prior to the day of your treatment:

1. **Wine**

2. **Peanuts** and anything made with or from peanuts (peanut butter, candies/sweets made with peanuts, peanut flavored items)

3. **Mulberries** and anything made with mulberries (jams, jellies, wines, pastries, etc)

4. **Grapes**, **grape juices** (especially pay attention to jams, jellies, canned fruit and canned fruit cocktails, fruit juice combinations which may contain grape juice, also any food items sweetened with grape juice)

5. **Blueberries, cranberries, huckleberries** or products made with these fruits.

6. Supplements/Pills that contain **resveratrol**. The following are some of the names of supplements on the market:
    - Supplements that say they contain:
        - Wine extract
        - Grape juice powder
        - Grape seed or extract
        - Resveratrol
    - Resveratrol/Trans-resveratrol tablets or capsules
    - Cell Stat
    - Resveratrol Synergy
    - French Paradox with Resveratrol and Arkopharma
    - Resveratrol

If you are unsure of the contents of any food substance, please contact a member of the study team who can advise you further. If you should happen to eat anything on the above list be sure to tell the researcher before you take your first dose of Resveratrol for the study.

9.3 List of abbreviations

AA	arachidonic acid
ACF	aberrant crypt foci
AChE	acetylcholinsterase
ADP	adenosine diphosphate
AE	adverse event
ANOVA	analysis of variance
AOM	azoxymethane
Apaf-1	apoptotic protease-activating factor 1
APC	adenomatous polyposis coli
APTT	Activated Partial Thromboplastin Time
AUC_{inf}	area under the concentration versus time curve to infinity
Bcl-2	B-cell lymphoma-2
BMI	Body Mass Index
cAMP	cyclic adenosine monophosphate
Caspase	cysteinyl aspartate-specific proteinase
C_{av}	average concentration over the total collection period
CDC	cell division control
CDKs	cyclin-dependent kinases
CKI	cyclin-dependent kinase inhibitor
CHO	chinese hamster ovary
CL/F	apparent total body clearance
CLR	apparent renal clearance
C_{max}	maximal plasma concentration

CoA	coenzyme A
CO_2	carbon dioxide
COX	cyclooxygenase
CTCAE	Common Terminology Criteria for Adverse Events
CV	coefficient of variation
CYP	cytochrome P450
DAB	3',3'-diaminobenzidine
DCIS	ductal carcinoma in situ
DISC	death-inducing signalling complex
DMH	1,2-dimethylhydrazine
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
DPX	dibutyl phthalate xylene
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin gallate
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	oestrogen receptors
EtOH	ethanol
FADD	fas-associated death domain
FAP	familial adenomatous polyposis
FCS	foetal calf serum
FLIP	FADD-like interleukin 1-converting enzyme-inhibitory protein
GP	General Practitioner

GST	glutathione S-transferase
GMP	Good Manufacturing Practice
Hep G2	human hepatocellular carcinoma cell line
HCA-7	human epithelial colorectal adenocarinoma cell line
HCT-116	human epithelial colorectal adenocarinoma cell line
HNPCC	hereditary nonpolyposis colorectal cancer
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
HT-29	human epithelial colorectal adenocarinoma cell line
IAP	inhibitor of apoptosis proteins
IC_{50}	half maximal inhibitory concentration
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IMS	industrial methylated spirit
iNOS	inducible nitric oxide synthases
INR	International Normalized Ratio
K-ras	kirsten-ras
LC/MS/MS	liquid chromatography-tandem mass spectrometry
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MeOH	methanol
MDA	malondialdehyde
M1dG	malondialdehyde-deoxyguanosine adduct
MHRA	The Medicines and Healthcare Products Regulatory Agency

Min	multiple intestinal neoplasia
NAD	nicotinamide-adenine dinucleotide
NADPH	nicotinamide-adenine dinucleotide phosphate
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMBA	N-nitrosomethylbenzylamine
NMR	nuclear magnetic resonance
NO	nitric oxide
NOS	nitric-oxide synthase
NSAID	non steroidal anti-inflammatory drug
PBS	phosphate buffered saline
PD	pharmacodynamics
PG	prostaglandin
PK	pharmacokinetics
QC	quality control
QR	quinone reductase
Rb	retinoblastoma
RM	resection margin
RNA	ribonucleic acid
ROO	peroxyl radicals
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SAE	serious adverse event
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SD	standard deviation
SGLT1	sodium-dependent glucose cotransporter member 1

SIR1	silencing information regulator
SIRT 1	sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)
SULTS	sulfotransferases
T_{max}	median time of maximal plasma concentration
TRADD	tumour necrosis factor receptor-associated death domain protein
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
TRAMP	transgenic adenocarcinoma mouse prostate
Tris	tris(hydroxymethyl)aminomethane
TXA	thromboxane A
UDP	uridine diphosphate
UGT	uridine 5'-diphospho-glucuronosyltransferase
UV-HPLC	high performance liquid chromatography
US FDA	US food and drug administration
USPSTF	U.S. Preventive Services Task Force
V/F	apparent volume distribution
VTE	venous thromboembolism
WHO	World Health Organisation

9.4 Publications

Papers

Boocock, D.J., Faust, G.E.S., Patel, K.R., Schinas, A.M., Brown, V.A., Ducharme, M.P., Booth, T.D., Crowell, J.A., Perloff, M., Gescher, A.J., Steward, W.P. and Brenner, D.E. (2007). Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiology Biomarkers & Prevention*, 16, 1246-1252.

Hoh, C.S.L., Boocock, D.J., Marczylo, T.H., Brown, V.A., Cai, H., Steward, W.P., Berry, D.P., and Gescher, A.J. (2007). Quantitation of Silibinin, a Putative Cancer Chemopreventive Agent Derived from Milk Thistle (Silybum marianum), in Human Plasma by High-Performance Liquid Chromatography and Identification of Possible Metabolites. *Journal of Agricultural and Food Chemistry 55*(7), 2532-2535

Publication during PhD research period – unrelated to the PhD research presented in this thesis

Brown, V.A., Parker, P., Furber, L., Thomas. A. (2009). Patient Preferences for the Communication of bad news: A UK Cancer Centre. *European Journal of Cancer Care*. *In press*

Abstracts

Brown, V.A., Patel, K., Booth, T., Crowell, J., Perloff, M., Tuck, M., Deyampert, Brown, K., Gescher, A., Steward, W., Brenner. D. (2007). Clinical evaluation of the putative cancer chemopreventive agent resveratrol. The National Cancer Research Institute Meeting Abstracts, Birmingham, UK. Short listed for BACR/Gordon Hamilton award.

Brown, V.A., Patel, K., Booth, T., Crowell, J., Perloff, M., Tuck, M., Deyampert, A., Brown K., Gescher, A., Steward, W., Brenner. D. (2008). Phase I repeat-dose clinical study of safety, pharmacokinetics and pharmacodynamics of resveratrol**.** The American Association of Cancer Research Meeting Abstracts, Apr 2008: 497. San Diego, California, USA

Brown, V.A., Patel, K., Booth, T., Crowell, J., Perloff, M., Tuck, M., Deyampert, A., Karmokar, A., Brown, K., Gescher, A., Steward, W., Brenner. D. (2008). Phase I repeat-dose clinical study of safety, pharmacokinetics and pharmacodynamics of the putative cancer chemopreventive agent resveratrol. The National Cancer Research Institute Meeting Abstracts, Birmingham, UK.

Abstract during PhD registration period – unrelated to the PhD research presented in this thesis

Brown, V. A., Parker, P., Furber, L., Thomas. (2007). Patient Preferences for the Delivery of Bad News – Experience of a UK Cancer Centre. The National Cancer Research Institute Meeting Abstracts, Birmingham, UK

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