CHARACTERISATION OF TRAIL RECEPTOR SIGNALLING TO APOPTOSIS IN PRE-CLINICAL MODELS OF BREAST CANCER

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Characterisation of TRAIL Receptor Signalling To Apoptosis

in Pre-clinical Models of Breast Cancer

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TNF-Related Apoptosis-Inducing Ligand (TRAIL) belongs to the TNF cytokine family and can signal to apoptosis by binding to either of two membrane-bound death receptors, TRAIL-R1 or TRAIL-R2. Using ligands specific for TRAIL-R1 (R1L) or TRAIL-R2 (R2L), our laboratory has previously shown that combining a histone deacetylase inhibitor with R1L, but not R2L, induces apoptosis in primary chronic lymphocytic leukaemia cells. The aim of this project was to extend the profiling of TRAIL-Receptor signalling to breast cancer, using breast cancer cell lines and importantly primary breast tumours as model systems.

A 3-dimensional explant culturing technique was employed to maintain the primary tumour architecture and mimic the breast tumour microenvironment. In addition, tumour-initiating cells from advanced metastatic breast cancer patients were also tested for their sensitivity to TRAIL. The results obtained from breast cancer cell lines, primary mucinous carcinomas and advanced metastatic breast cancer cells suggest that in breast cancer, TRAIL-R1 is the predominant functional TRAIL death receptor independent of oestrogen receptor status. In contrast, invasive ductal/lobular carcinomas (IDC/ILC) were resistant to TRAIL-induced apoptosis and required the breast cancer chemotherapeutic, doxorubicin as a sensitising agent. Studies using the TRAIL-resistant cell line, T47D, demonstrated that doxorubicin sensitised tumour cells to TRAIL-induced apoptosis *via* enhanced TRAIL DISC formation. Importantly, in primary tumour explants, the combination of doxorubicin and TRAIL signalled to apoptosis exclusively in the tumour cells, but not in normal cells. Significantly, in four IDC/ILC tumours, doxorubicin sensitised breast tumour cells to R1L more efficiently than R2L. Therefore, using R1L in combination with sub-lethal doses of chemotherapeutic agents could improve the benefit of conventional therapy whilst reducing drug-associated side-effects and potential TRAIL-mediated cell proliferation/survival in apoptosis-resistant tumour cells. My data suggest that using a TRAIL-R1-selective agonist with an appropriate sensitising agent (example, doxorubicin), offers a promising therapeutic approach for treatment of breast cancer.

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Abbreviations

µg	Microgram
µl	Microlitre
APAF-1	Apoptosis Protease Activating Factor-1
Apo2L	Apoptosis 2 Ligand
ATCC	American Typed Cell Collection
ATM kinase	Ataxia Telangiectasia Mutated kinase
ATP	Adenosine TriPhosphate
ATR kinase	ATM and Rad-3 related kinase
b-TRAIL/R1L/R2L	Biotinylated-TRAIL/R1L/R2L
BIR	Baculoviral IAP Repeat
BH	Bcl-2 Homology
BSA	Bovine Serum Albumin
CAM-DR	Cell Adhesion Mediated Drug Resistance
CARD	CAspase Recruitment Domain
Caspase	Cysteine aspartate-specific protease
CDK	Cyclin Dependent Kinase
CO_2	Carbon dioxide
CLL	Chronic Lymphocytic Leukaemia
CRD	Cysteine Rich Domain
DAB	Diaminobenzidine tetrahydrochloride
DCF-DA	2'-7'-dichloroflourescein diacetate
DCIS	Ductal Carcinoma *in situ*
DED	Death Effector Domain
DD	Death Domain
DIABLO	Direct IAP-Binding protein with Low pI
DISC	Death-Inducing Signalling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribose Nucleic Acid
Dox	Doxorubicin
ECL	Enhanced Chemiluminscent
ECM	ExtraCellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptor

ESA	Epithelial Surface Antigen
EST	Expressed Tag Sequence
FAC	Fluorouracil, Adriamycin, Cyclophosphamide
FACS	Fluorescence Activated Cell Sorter
FADD	Fas-Associated Death Domain protein
FCS	Foetal Calf Serum
FEC	Fluorouracil, Epirubicin, Cyclophosphamide
FITC	Fluroescein isothiocyanate
FLIP	FADD-like interleukin-1β converting enzyme-like Protease (FLICE/C8)-inhibitory protein.
GPI	Glycosylphosphatidylinositol
h	Hours
H & E	Haematoxylin and Eosin
HDACi	Histone DeACetylase inhibitor
HGS	Human Genome Sciences
HRP	Horseradish Peroxidase
IAP	Inhibitor of APoptosis
ICC	Immunocytochemistry
IDC	Invasive Ductal Carcinoma
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
IMS	Industrial Methylated Spirits
LCIS	Lobular Carcinoma *in situ*
LOB	Lobule
MAPK	Mitogen Activated Protein Kinases
MFE	Mammosphere Forming Efficiency
min	Minutes
ml	Millilitres
mm	Millimetres
mM	MiliMolar
MCL	Mantle Cell Lymphoma
MMP	Mitochondrial Membrane Permeabilisation
NBF	Neutralised Buffer Formalin
NK cells	Natural Killer cells
NPI	Nottingham Prognostic Index
OPG	Osteoprotegerin
OPGL	Osteoprotegerin Ligand

PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PE	Phycoerythrin
PgR	Progesterone Receptor
PI	Propidium Iodide
PLAD	Pre-Ligand Assembly Domain
Poly-HEMA	Poly-2-hydroxyethyl methacrylate
PS	Phosphatidylserine
R1L	TRAIL-R1-specific mutant ligand of TRAIL
R2L	TRAIL-R2-specific mutant ligand of TRAIL
RIP	Receptor Interacting Protein
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SAHA	Suberoyl anilide hydroxamic acid
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
Smac	Second Mitochondria-derived Activator of Caspases
STS	Staurosporine
tBid	Truncated Bid
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween
TEB	Terminal End Buds
TD	Terminal Duct
TDLU	Terminal Ductu-Lobular Unit
TMRE	Tetramethylrhodamine, ethyl ester, perchlorate
TNF	Tumour Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAIL-R	TRAIL-Receptor
v/v	volume/volume
VEGF	Vascular Endothelial Growth Factor
XIAP	X-linked Inhibitor of APoptosis
zVAD.FMK	Carbobenzoxy-Val-Ala-Asp-(O-methyl)-fluromethylketone

Chapter 1

Introduction

1.1 Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (World Health Organization Update, 2008). Evasion of apoptosis is a common hallmark of cancer cells (Hanahan and Weinberg, 2011) and consequently most current anti-cancer therapies are targeted to facilitate cell death in cancer cells. However, the drawbacks of currently used therapies include adverse side effects and acquired drug resistance. The focus of this study is to investigate the potency of the promising cancer biotherapeutic, TRAIL, in epithelial tumours using breast cancer as a model system.

1.2 Breast cancer

Breast cancer is the most common cancer in the UK across all individuals (Figure 1.1). Over 45,000 cases of breast cancer are diagnosed in women each year in the UK alone, with over a million new cases each year worldwide. In the UK, around 300 men are also diagnosed each year with breast cancer (Table 1.1). In 2008, breast cancer was listed as the third most common cause of death from cancer (Figure 1.1). Each year approximately 12,000 women and 70 men are estimated to die from breast cancer in the UK. However, there has been a rise in patient survival in the past decade, primarily because of increased screening programmes resulting in early detection of breast cancer and the development of effective treatment regimes including combination therapies. For example, in the UK, for women whose breast cancer was detected during screening, 82% lived for at least 5 years and 77% lived for at least 10 years after the initial diagnosis. Additionally, improved adjuvant therapy, which ensures the removal of micrometastases, has resulted in a decrease in the likelihood of tumour relapse.

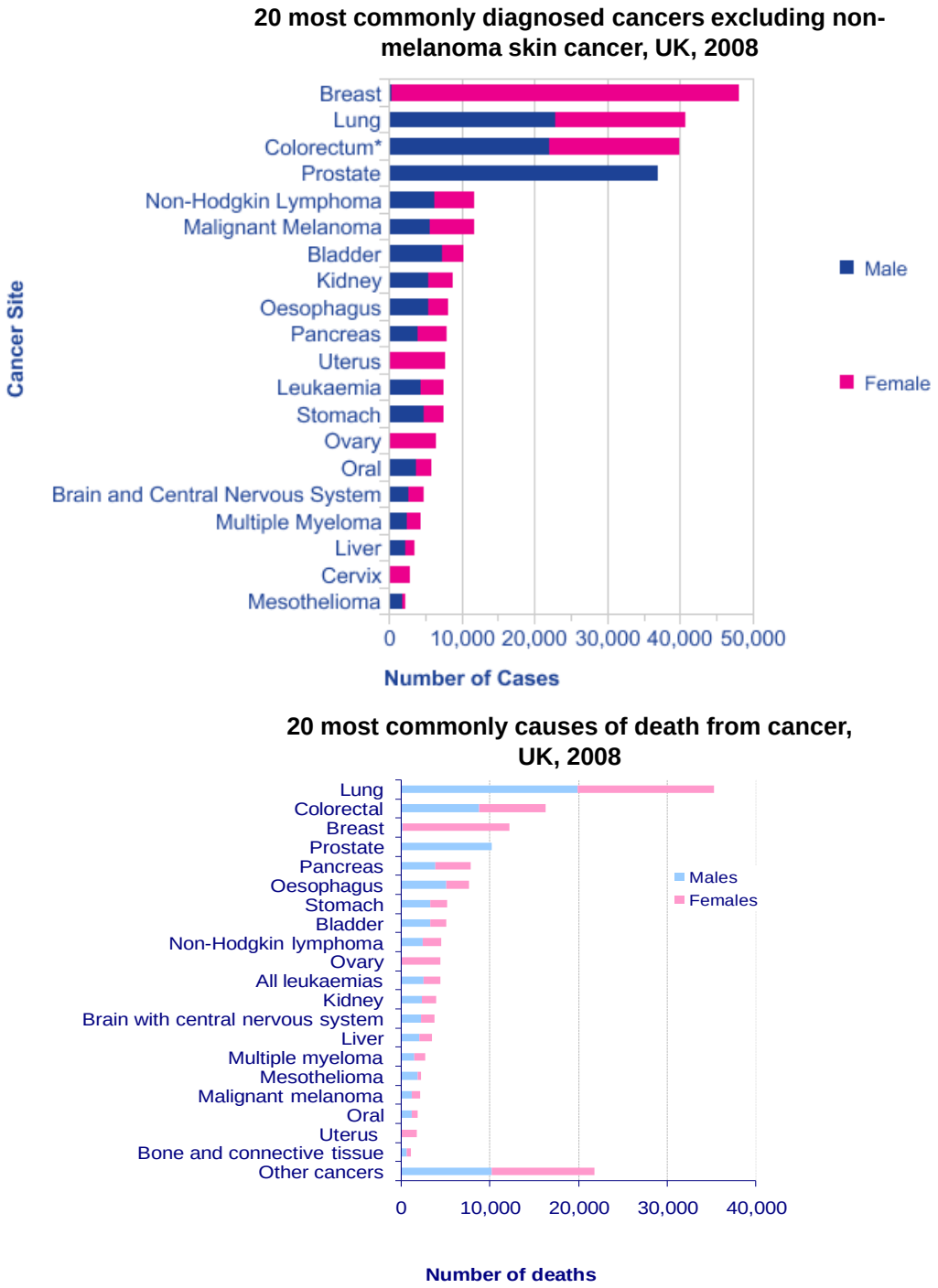


Figure 1.1 Breast cancer incidence and mortality in UK

Breast cancer is the most common cancer in the UK in all individuals and the third most common cause of death from cancer in the UK (http://info.cancerresearchuk.org/cancerstats/incidence/commoncancers/ ; http://info.cancerresearchuk.org/cancerstats/mortality/cancerdeaths/)

Table 1.1 Incidence, mortality and predicted survival rates for breast cancer in UK

Breast Cancer (UK)	Males	Females	Persons
Number of new cases (2007)	277	45,695	45,972
Number of deaths (2008)	69	12,047	12,116
Five-year survival rate (for patients diagnosed 2001-06, England)	N.D	82%	N.D
Ten-year survival rate (predicted survival for patients diagnosed in 2007, England and Wales)	N.D	77%	N.D

(http://info.cancerresearchuk.org/cancerstats/keyfacts/breast-cancer/)

N.D = Not Determined

1.3 Development of normal breast

The mammary gland parenchyma arises from a single epithelial ectodermal bud which forms the basic structures including ducts and short ductules during pre-natal growth. Post-natal growth of the breast tissue is minimal until puberty sets in. Under hormonal influences, the basic structures divide dichotomously to form the Terminal End Buds (TEB; Russo and Russo, 2004). The TEBs then further differentiate and grow into newer branches or they may sprout into smaller ductules which surround one Terminal Duct (TD). This structural unit of the breast tissue consisting of the TD and its ductules is called LOB1 (TDLU; Terminal Ductu-Lobular Unit). LOB1 then differentiates into LOB2 which has more ductules surrounding the TD. Further differentiation of LOB2 into LOB3 and LOB4 (secretory acini) occurs during pregnancy and lactation (Figure 1.2; Russo and Russo, 2004).

The development of breast cancer has been hypothesised to be a multi-step process beginning with normal cells within the breast undergoing hyperplasia (with or without atypia), followed by carcinoma *in situ*, invasive carcinoma and ultimately ending in metastasis. However, recent advances have challenged this concept and parallel pathways of development leading to the formation of low or high grade tumours have been recognised (reviewed in Simpson *et al.,* 2005). Various factors increase the risk for breast cancer development: these include age, presence of BRCA1/2 or p53 mutations, older age at first pregnancy and obesity (Walker, 2009).

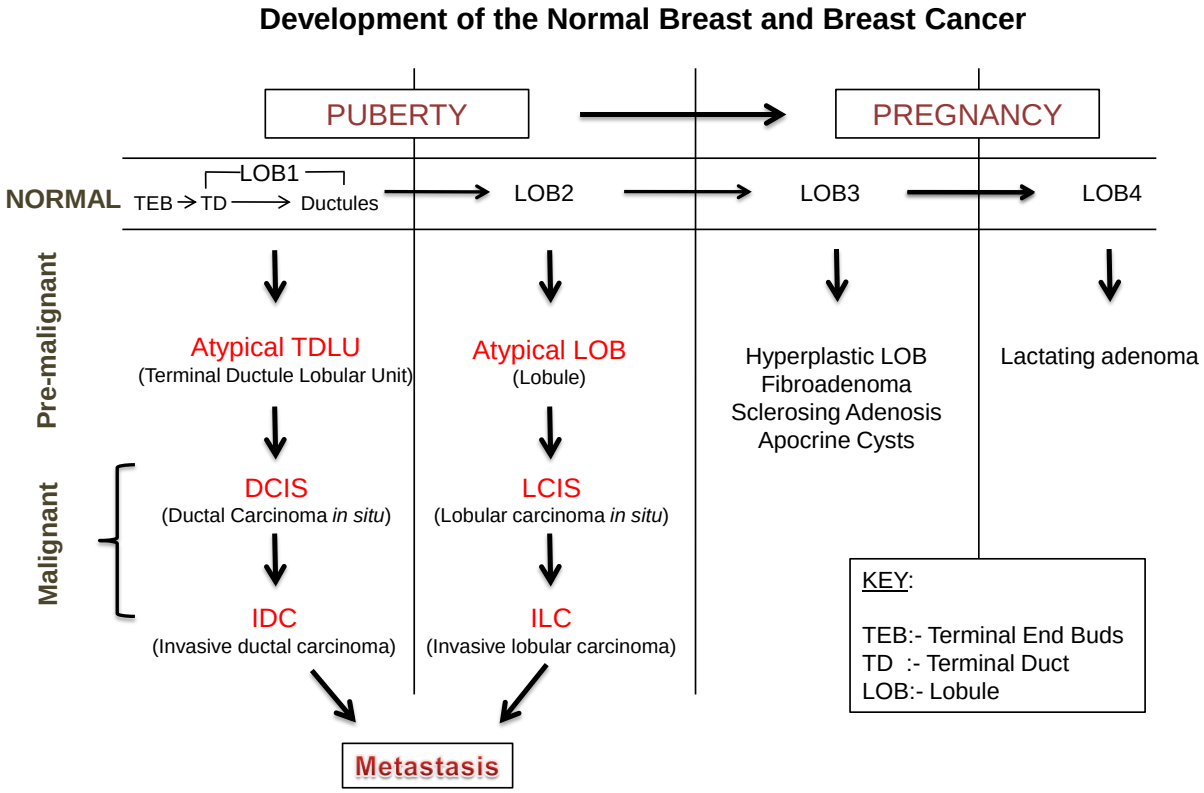


Figure 1.2 Development of normal and malignant breast tissue

Breast tissue undergoes cycles of differentiation depending on hormonal stimuli. The stage of differentiation of the tissue may also play a role in the type of tumour that may evolve from it. (Adapted from Russo and Russo 2004).

1.4 Types of breast cancer

Breast tumours can be classified as invasive or non-invasive based on their histology. Based on their pathology and molecular profile, invasive tumours can then be further categorised into different sub-types. Molecular profiling of invasive breast cancers also enables determination of appropriate treatment regimes following removal of the primary breast cancer. The same principles can additionally be applied to core biopsies to aid in the selection of neoadjuvant therapy.

1.4.1 Morphology-based classification

a) Ductal carcinoma *in situ* (DCIS)

DCIS is a tumour of the ducts and ductules of the breast, characterised by proliferative malignant breast epithelial cells, which remain within the basement membrane (Kalluri and Zeisberg, 2006). This is considered to form a boundary, preventing tumour cells invading into the surrounding stroma and hence into the blood and lymphatic vessels, thus preventing tumour metastasis. However, if tumour cells do cross the basement membrane, they can become an invasive carcinoma and have the potential to subsequently metastasise. DCIS can be graded as low, intermediate or high; the higher the grade of the tumour, the more aggressive is the phenotype and probability of it developing into an invasive tumour.

b) Lobular carcinoma *in situ* (LCIS)

LCIS like DCIS arises from the TDLU, but instead of being pre-cancerous, it is considered as a risk factor to invasive breast cancer. LCIS and indeed lobular carcinoma cells, are characterised by the total absence of E-cadherin on their cell surface (Cleton-Jansen, 2002). E-cadherin is already absent in LCIS without an invasive component, indicating that loss of E-cadherin occurs early during tumour development (Vos *et al.,* 1997). Inactivation of the E-cadherin gene occurs either through a truncation mutation (Berx *et al.,* 1995; Berx *et al.,* 1996) or methylation of its promoter (Droufakou *et al.,* 2001). E-cadherin plays an important role in maintaining cell-cell adhesion and the lack of this protein in lobular carcinomas causes lack of cohesion between the tumour cells, accounting for their growth pattern.

c) Invasive ductal carcinoma (IDC)

IDC is the most common invasive breast cancer, accounting for 70% of all cases. It arises from DCIS, following the escape of malignant cells from the basement membrane barrier and their spread to other regions through lymph/blood vessels (Ellis *et al.,* 1992). The prognosis of IDCs can depend on the sub-type, stage and grade of the tumour.

d) Infiltrating lobular carcinoma (ILC)

Infiltrating lobular carcinomas account for only about 5-10% of invasive cancers and tend to be positive for oestrogen and progesterone receptors (ER and PgR respectively). Although IDC and ILC differ phenotypically, there is no difference in the clinical outcome of either type of cancer. However, as mentioned before, ILC completely lacks the E-cadherin protein and the tumour cells are not as cohesive as cells from IDC.

e) Special types of invasive cancer

i) Mucinous/colloid carcinoma

This is a rare type of invasive ductal carcinoma accounting for only about 3% of breast cancer cases. Mucinous carcinomas are characterised by a high production of gel-forming mucin mainly transcribed from the MUC2 and MUC5 genes (O'Connell *et al.,* 1998), and usually have an ER positive and HER2 negative receptor status. Patients with mucinous carcinomas tend to have a good prognosis and a high long-term survival rate (Ellis *et al.,* 1992).

ii) Medullary carcinoma

This type of carcinoma is only seen in 5% of all breast cancer patients but a pure medullary carcinoma has a better prognosis than an invasive ductal carcinoma (Pedersen *et al.,* 1995).

iii) Metaplastic carcinoma

This is one of the rarest invasive sub-types comprising less than 1% of breast cancers. Metaplastic carcinomas can be very aggressive and a majority of tumours show the phenomenon of epithelial-mesenchymal transformation (Gilles and Thompson, 1996).

iv) Tubular carcinoma

This is also a type of invasive carcinoma characterised by tumour cells forming small tubules. Tubular carcinoma makes up less than 1% of all breast cancer cases. Tubular carcinomas are ER positive and HER2 negative.

v) Invasive papillary carcinoma

This class comprises approximately 3% of all breast cancer cases. Invasive papillary carcinoma is so called because the tumour cells resemble papules or finger-like projections.

1.4.2 Classification of tumours based on molecular profile

a) Luminal cancers

Luminal cancers usually express cytokeratin 8, 18 and are ER positive (Perou *et al.*, 2000). Luminal cancers are the most common breast cancer type and they can be further sub-divided into luminal A and luminal B subgroups.

Luminal A tumours have higher expression of ER-related genes and less expression of proliferative genes than Luminal B tumours and therefore tend to be less aggressive and have a lower grade (Brenton *et al.*, 2005). Expression of ER is considered as a good prognostic marker and it has been long known that the absence of ER from breast tumours can be associated with early tumour recurrence (Knight *et al.*, 1977). Thus, it is not very surprising that luminal A tumours have a better prognosis than other types of breast cancer.

b) Basal-like tumours

Basal-like tumours show a similar gene expression pattern to basal epithelial cells and usually do not express any hormone receptors. They typically possess cytokeratin 5, 6 and 17 and integrin β4 (Perou *et al.*, 2000). Basal-like tumours tend to be highly aggressive and spread more to the brain and bone than other breast tumours (Lin *et al.*, 2008).

c) HER2-amplified tumours

Not all HER2 positive tumours are categorised as HER2-amplified tumours. Those tumours, which show increased HER2 expression and also are negative for ER

expression, are classified under this category. HER2-amplified tumours are likely to be of higher grade and show increased rates of p53 gene mutations (Brenton *et al.*, 2005).

d) Normal breast-like tumours

Breast cancers, which cannot be classified into any of the above groups, are classified as normal breast-like tumours. These tumours have a similar gene expression pattern to those of basal epithelial cells and adipose cells.

1.5 Prognostic factors and breast cancer

An important prognostic factor in breast cancer is the tumour stage, which reflects the spread of the tumour in the body and it is usually determined by using the TNM system.

T stands for the size of the primary tumour and this can range from T1-T4

N ranges from N0-N3 and is used to describe the spread of tumour cells to lymph nodes

M is used to indicate whether or not the tumour has metastasised.

There are four stages of breast cancer which are classified as below.

Table 1.2 Classification of stages of breast cancer

Stage	Tumour size (cm)	Metastasis in lymph nodes	Distant Metastasis
I	<2	-	-
IIa	<2	+	-
	<5	-	-
IIb	<5	+	-
	>5	-	-
IIIa	>5	+	-
IIIb	Tumour fixed to chest wall/skin	+/-	-
IIIc	Any size	+	-
IV	Any size	+/-	+

(http://info.cancerresearchuk.org/cancerstats/types/breast/symptomsandtreatment/)

On the other hand, tumour grade is representative of the overall aggressiveness of tumours with Grade I being least aggressive and Grade III being the most. Grade I tumours are also well differentiated compared to Grade III tumours.

The tumour grade, size and degree of spread to lymph nodes are also important in determining the Nottingham Prognostic Index (NPI), which is used in breast cancer to determine 5-year survival rates following surgery (Lee and Ellis, 2008). Other assays to predict the benefit of chemotherapy and long term survival rates are based on the personalised study of specific gene expression in individual tumours. These assays include Oncotype Dx® and Mammaprint® which encompass the study of 21 and 70 gene signatures respectively and have been validated for their prognostic use in breast cancer (Paik *et al.,* 2004; Glas *et al.,* 2006).

1.6 Therapy for breast cancer

The primary treatment for solid tumours such as breast cancer is surgical removal of the tumour mass. Other treatments administered to breast cancer patients are usually directed at increasing long-term patient survival by targeting residual tumour cells (adjuvant therapy). Neoadjuvant therapy can also be administered before surgery in order to target metastatic disease and to aid in tumour shrinkage.

a) Surgery

Lumpectomy involves removing the tumour with an adequate excision margin to ensure maximal elimination of tumour cells. However, if the tumour is of a large size or if there are multiple cancers including DCIS, the whole breast is removed (mastectomy). During surgery, axillary lymph nodes are removed and assessed histologically for staging. If cancer cells are present in the lymph nodes, it is common practice to remove the surrounding lymph nodes completely to prevent tumour metastases.

b) Radiotherapy

Radiotherapy is usually used after surgery to kill cancer cells in the vicinity of the tumour region or to treat the chest wall in case of lymph node metastasis. It is, however, very important to restrict the use of radiotherapy only to the areas possibly containing tumour cells since exposure to radiation can be carcinogenic itself. Radiation to the left side of the chest may also result in damage to the heart muscle leading to increased fatigue.

c) Chemotherapy

Adjuvant chemotherapy is generally given after surgery to reduce the risk of tumour recurrence in case there are any surviving tumour cells. The most common chemotherapy given in case of breast cancer is adjuvant FEC/FAC (Fluorouracil, Epirubicin/Adriamycin, Cyclophosphamide) or CMF (Cyclophosphamide, Methotrexate, Fluorouracil). Administration of anthracyclines, like doxorubicin or epirubicin, is also frequent in the treatment of breast cancer. It should be noted that adjuvant chemotherapy can cause reduction in the risk of breast cancer recurrence by 30% and death by about 20% (Early Breast Cancer Trialist's Collaborative Group, 2005).

d) Endocrine therapy

Adjuvant endocrine therapy is given to patients presenting with ER positive tumours (~70% of all breast cancer cases; Clark *et al.,* 1984). In pre-menopausal women tamoxifen is administered, whereas the use of aromatase inhibitors is quite common in post-menopausal women (Coates *et al.,* 2007). Tamoxifen binds to the oestrogen receptor and thus interferes with oestrogen-mediated proliferative signalling. Aromatase inhibitors on the other hand, prevent the synthesis of oestrogen and subsequent activation of the oestrogen receptors (Brodie *et al.,* 1981). Adjuvant endocrine therapy can cause a reduction in breast cancer recurrence by about 40% and death at 5 years by 30% (Early Breast Cancer Trialist's Collaborative Group, 2005).

e) Targeted therapies

Targeted therapies for breast cancer include the use of an anti-HER2 antibody. HER2 is amplified in nearly 15% of breast cancers and these cancers respond favourably to treatment with Trastuzumab/Herceptin treatment (Dawood *et al.,* 2010). Herceptin can be used with adjuvant chemotherapy (like taxanes) and it has also been licensed for use in metastatic breast cancer in combination with paclitaxel.

The exact form of adjuvant or neo-adjuvant therapy given to a patient depends on the type of breast cancer. Luminal A and B tumours (ER positive) are usually treated with tamoxifen or aromatase inhibitors depending on the menopausal status of the individual. Adjuvant chemotherapy has been shown to be more efficient in the ER negative tumours as compared with the luminal sub-types. Trastuzumab can be given to patients in the HER2-amplified sub-type and its effects have been reported to be

enhanced when it is used in conjunction with chemotherapy (reviewed in Guarneri *et al.,* 2010). Treatment for basal-like cancer is restricted to the use of adjuvant chemotherapy and the lack of therapy options has been attributed to be one of the reasons for the aggressiveness and poor prognosis of this tumour sub-type. Therapy options for basal-like cancers include inhibition of EGFR (Epidermal Growth Factor Receptor), inhibition of c-kit (Imatinib; Nielsen *et al.,* 2004), inhibition of Src (Dasatinib) and inhibitors of PARP (De Soto *et al.,* 2006).

1.7 Drawbacks of current therapies

Despite the availability of a wide range of therapeutic options, breast cancer metastasis is still a common cause of death in the UK. It is also important to note that some of the therapies are restricted to a cohort of patients which display the relevant molecular markers. For example, patients belonging to the amplified HER2 group are treated with Herceptin, but this is only around 15% of cases. Also, while two-thirds of breast cancers show ER expression, treatment with tamoxifen is hindered by the presence of *de novo* or acquired endocrine resistance, ultimately resulting in oestrogen-independent tumour cell survival (reviewed in Osborne & Schiff, 2011).

In addition, there are various molecular determinants, like p53 status, which determine the response of tumour cells to therapy. Radiotherapy and many chemotherapeutic drugs are dependent on the activation of cell death pathways by p53 to kill tumour cells. However, most tumour cells evade apoptosis either by inactivating these pathways (for example, mutational inactivation of p53) or activating other cell survival pathways (such as MAPK pathways), leading eventually to drug resistance (reviewed in Longley and Johnston, 2005).

Drug resistance to a certain therapy may actually render an advantage to transformed cells making the tumour more aggressive. In addition, since none of the widely used therapies can distinguish between normal and tumour cells, the resultant death of normal cells is the cause of many side-effects including myelosuppression, nausea, alopecia and heart disease. Hence, sustained use of a single therapy may not be ideal for cancer therapy and therefore, most treatment regimes employ a combinational approach to circumvent the problems caused by acquired drug resistance in tumour cells. Usually, two or more therapies which have different modes of action are administered either sequentially or together to cause maximum tumour remission through synergistic or additive effects of drug use. Nonetheless, it is still common to see tumour relapse even after multiple therapies, which justifies the need for ongoing

research in the field of breast cancer therapy. Hence, despite advances in the use of different therapies, there still is a need for a drug that can select for transformed cells and which works through a mechanism that is minimally dependent on key molecular determinants like p53, which is mutated in approximately 20% of breast cancer cases (Pharoah *et al.,* 1999).

1.8 TRAIL

The exciting discovery of the natural cytokine, TNF-Related Apoptosis-Inducing Ligand (TRAIL; Wiley *et al.,* 1995) opened new avenues in the search for a new cancer therapeutic. TRAIL/Apo2L (Apoptosis 2-Ligand) belongs to the Tumour Necrosis Factor (TNF) superfamily along with other death-inducing ligands like CD95L/FasL and TNFα and was identified *via* a sequence motif characteristic to this superfamily using an EST (Expressed Sequence Tag) database (Wiley *et al.,* 1995; Pitti *et al.,* 1996).

Although CD95L and TNFα were discovered before TRAIL, further research showed that they caused severe adverse effects *in vivo*, thus preventing their future use in the clinic. While CD95L caused severe liver toxicity (Ogasawara *et al.,* 1993; Tanaka *et al.,* 1997), TNFα induced a strong inflammatory response in pre-clinical cancer models (van Molle *et al.,* 2002). On the contrary, TRAIL is perceived as an effective biotherapeutic agent because it is selective for transformed cells and a number of studies have shown that it is not toxic to normal cells (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999; Ichikawa *et al.*, 2001; Kelley *et al.,* 2001; Lawrence *et al.*, 2001). However, it is important to note that while most of these early studies used cancer cell lines, a number of primary tumour cells are resistant to TRAIL-induced apoptosis and require co-treatment with a sensitising agent (Ehrhardt *et al.,* 2003; MacFarlane *et al.,* 2005a; Herbst *et al.,* 2006; Todaro *et al.,* 2008).

Initial reports using tagged forms of TRAIL suggested that TRAIL could be potentially hepatotoxic (Jo *et al.*, 2000); however, subsequent work has shown that untagged recombinant TRAIL is not toxic to human hepatocytes *in vitro* or *in vivo*. Furthermore, TRAIL did not show any hepatotoxic effects when tested on chimeric mice (Hao *et al.,* 2004). It has also been further proven that tagged versions of TRAIL (His, Leucine zipper, or FLAG-tagged) may be hepatotoxic because they tend to form higher molecular weight aggregates which can surpass the normal apoptotic threshold in healthy cells (Lawrence *et al.*, 2001; Ganten *et al.,* 2006). Improper preparation of TRAIL and/or inappropriate isolation and culture of human hepatocytes have also been cited as potential causes of TRAIL-mediated hepatocyte toxicity (Volkmann *et al.*,

2007). The apparently consistent specificity of TRAIL in preferentially targeting transformed cells over normal cells has given it a colossal edge over many commonly used chemotherapeutics. Furthermore, TRAIL is minimally dependent on expression of p53 protein thus ensuring effective TRAIL activity across a variety of tumour cells irrespective of their p53 status. These combined advantages of TRAIL has thus, made it a novel and exciting approach to improving cancer therapy.

Like other members of the TNF cytokine family, TRAIL is a naturally occurring death-inducing ligand and is expressed on the cell surface as a 281 amino acid long Type II transmembrane protein. Metalloproteinase-mediated cleavage of the carboxy terminal region of the transmembrane protein results in formation of a soluble ligand (reviewed in Held and Schulze-Osthoff, 2001). Both the membrane-bound and soluble forms of TRAIL are potent against tumour cell lines and appear to have minimal activity against normal cells (reviewed in deVries *et al.,* 2006).

The structural conformation of TRAIL is important for its death-inducing ability; TRAIL is made up of three identical subunits, each of which possesses a cysteine residue at position 230, which coordinates a zinc ion. This coordination geometry is essential for trimer stability and hence is required for optimal TRAIL activity (Bodmer *et al.,* 2000b). TRAIL induces apoptosis in its target cells *via* binding to its receptors and triggering the extrinsic apoptotic pathway.

1.9 Physiological functions of TRAIL

The physiological role of TRAIL has been well studied using mouse models. It is important to note that mice contain only one TRAIL receptor (TRAIL-R) as opposed to humans who have five receptors for TRAIL. The role of TRAIL in normal mouse development does not appear to be critical, as TRAIL knock-out mice do not show any major developmental defects. Importantly though, TRAIL-deficient mice do exhibit defects in the innate immune system and phenotypically also show larger thymuses as compared with wild type mice (Lamhamedi-Cherradi *et al.*, 2003). The significance of TRAIL in susceptibility to autoimmune diseases is debatable, with some studies reporting that its deficiency causes an increase in autoimmune diseases (Lamhamedi-Cherradi *et al.*, 2003), whereas another study argues that TRAIL does not play a major role in thymocyte deletion (Simon *et al.*, 2001). Crucially, TRAIL-deficient mice also show increased susceptibility to tumour formation, especially haematological malignancies, and metastasis, underlining its importance as a tumour suppressor gene *in vivo* (Cretney *et al., 2002)*.

The use of TRAIL as a lethal agent is best exploited by cells of the immune system. Various types of immune cells including Natural Killer (NK) cells, monocytes and CD8+ T cells have been reported to recruit TRAIL as they continually perform anti-tumour surveillance within the body (Griffith *et al.,* 1999; Kayagaki *et al.,* 1999a; Kayagaki *et al.,* 1999b; Washburn *et al.*, 2003). TRAIL has also been implicated in mediating the apoptotic activity of Type I interferons in certain types of malignancies and also in retinoic acid-induced apoptosis of leukaemia cells (Altucci *et al.,* 2001; Chen *et al.,* 2001).

In addition, TRAIL receptor deficiency has also been shown to promote primary tumour formation and metastasis in mice (Finnberg *et al.*, 2008). On the other hand, another study reported that the absence of TRAIL-R does not affect primary tumour formation or initiation, but confirmed that TRAIL-R played a significant role in inhibiting tumour metastasis (Grosse-Wilde *et al.,* 2008). This discrepancy might be explained by differences in oncogene expression between the tumours as well as different tumour types and mouse models used in the above two studies. Importantly, both studies agree that an important role is played by the mouse TRAIL receptor in influencing tumour progression *in vivo*.

1.10 TRAIL receptors

There are four known human TRAIL receptors expressed on the cell surface, namely TRAIL-R1 (Death Receptor 4; DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR-1), TRAIL-R4 (DcR-2) along with one soluble TRAIL receptor (Osteoprotegerin; OPG). The genes encoding the cell surface TRAIL receptors are located on chromosome 8p21/22. TRAIL-R1/-R2/-R3/-R4 belong to the TNF-Receptor (TNFR) superfamily and are Type I transmembrane proteins characterised by the presence of cysteine rich sub-domains (CRD) in their extracellular region. CRDs help in the binding of TRAIL to its receptors and also in the ligand-independent association of the receptors through their pre-ligand assembly domain (PLAD). TRAIL-R2 and TRAIL-R4 have been shown to interact *via* PLADs and strikingly, the depletion of PLADs can lead to abrogation of the ligand-binding ability of the receptors (Clancy *et al.*, 2005).

1.10.1 TRAIL-R1/TRAIL-R2

Out of the five known TRAIL receptors, TRAIL-R1 and TRAIL-R2 possess the complete signal transducing structure and are similar in protein sequence to other death-inducing receptors belonging to the TNF-receptor superfamily (Figure 1.3; MacFarlane *et al.,*

1997; Sheridan *et al.,* 1997; Pan *et al.*, 1997a; Pan *et al.*, 1997b; Screaton *et al.*, 1997; Walczak *et al.*, 1997). TRAIL-R2 is expressed as both long and short forms, which are splice variants transcribed from the TRAIL-R2 gene and are functionally similar.

TRAIL-R1 and TRAIL-R2 both comprise the cysteine-rich extracellular domain, transmembrane domain and an intracellular domain containing an 80 amino acid long death domain (DD). The DD is crucial for the association of the death receptors to their downstream binding partners. Also, post-translational modifications including palmitoylation for TRAIL-R1 and glycosylation for TRAIL-R1 and -R2 have been described and are known to be important for receptor localisation and function (Wagner *et al.,* 2007; Rossin *et al.*, 2009). Interestingly, both TRAIL-R1 and TRAIL-R2 are up-regulated in response to a variety of chemotherapeutic agents and this up-regulation is known to occur *via* either an intronic p53 binding site or an NFκB binding element in the promoter regions of these genes (Wu *et al.,* 1997; Guan *et al.,* 2001; Song *et al.,* 2008).

TRAIL-induced apoptotic signalling is mediated by binding of at least three TRAIL molecules to TRAIL-R1 and/or TRAIL-R2 on the cell surface, resulting in cross-linking of the receptors and formation of either a receptor homotrimer or a TRAIL-R1/-R2 heterotrimer. This ligand-receptor complex then recruits Fas-Associated Death Domain (FADD) and procaspase-8 to the cell surface leading to formation of the TRAIL DISC (Death-Inducing Signalling Complex) which is the apical signalling platform involved in the TRAIL apoptotic pathway (Chinnaiyan *et al.*, 1995; Kischkel *et al.,* 1995; Bodmer *et al.*, 2000; Kischkel *et al.*, 2000; Sprick *et al.*, 2000). Signalling through the two death receptors appears to be different and for TRAIL-R2 particularly, oligomerisation of the receptor by ligation of highly aggregated, cross-linked antibody seems essential for apoptosis induction (Natoni *et al.*, 2007). On the other hand, TRAIL-R1 can be stimulated by TRAIL ligand and does not require cross-linked antibodies (MacFarlane *et al.,* 2005a).

Although both TRAIL-R1 and TRAIL-R2 can signal to apoptosis, the relative contributions of these two receptors to TRAIL-mediated apoptosis has been a topic of much debate. Early studies showed that TRAIL has a higher binding affinity to TRAIL-R2 as compared with TRAIL-R1 under physiological conditions (Truneh *et al.*, 2000). Furthermore, studies with mutant forms of TRAIL or agonistic antibodies specific to either TRAIL-R1 or TRAIL-R2 have suggested that TRAIL-R2 is more potent in certain cancers, including breast cancer cell lines (Kelley *et al.*, 2005).

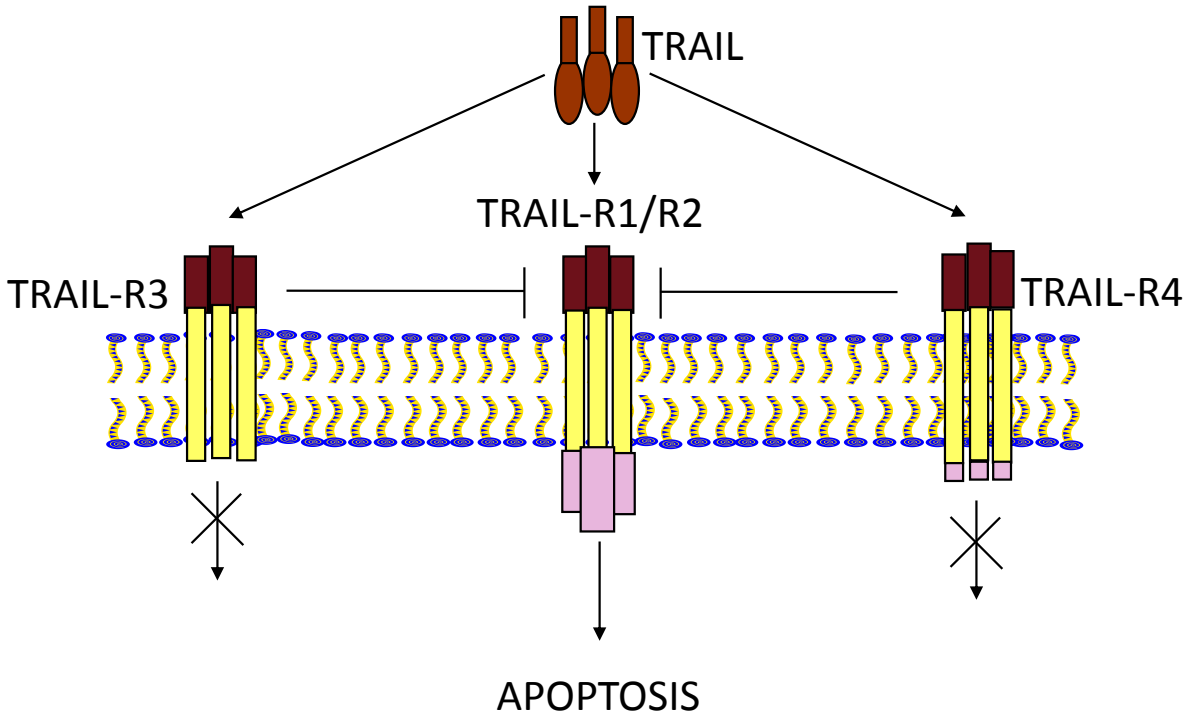


Figure 1.3 Cell Surface TRAIL Receptors

TRAIL has four main cell surface receptors. TRAIL-R1 and TRAIL-R2 have a cytoplasmic domain, a transmembrane domain and an intracellular domain and they are efficient in signalling the death stimulus. TRAIL-R3 lacks the intracellular domain, while TRAIL-R4 has a truncated intracellular domain. Hence TRAIL-R3 and TRAIL-R4 cannot signal a death stimulus when TRAIL binds to them (MacFarlane *et al.,* 1997; Pan *et al.,* 1998; Sheridan *et al.,* 1997; Adapted from MacFarlane et al., 2003)

Conversely, research done in our laboratory has provided evidence that when TRAIL-resistant primary CLL (chronic lymphocytic leukaemia) and MCL (mantle cell lymphoma) tumour cells are sensitised to TRAIL using Histone DeAcetylase Inhibitors (HDACi), TRAIL-R1 is the major TRAIL death receptor (MacFarlane *et al.*, 2005a; MacFarlane *et al.,* 2005b). Similarly another group has shown that TRAIL-R1 is the more important TRAIL death receptor in pancreatic carcinoma (Lemke *et al.*, 2010) and in transformed keratinoctyes (Leverkus *et al.*, 2003). However, TRAIL-R2 is widely believed to be the dominant TRAIL death receptor in numerous cancers and this has resulted in the development of five TRAIL-R2 targeting ligands/antibodies in clinical trials versus only one antibody targeted to TRAIL-R1. TRAIL-R2 is also up-regulated by most chemotherapeutic drugs and this up-regulation has been deemed as the mechanism of sensitisation of most TRAIL-resistant cells to TRAIL-induced apoptosis. As a result of these early studies, exploiting TRAIL-R2 as a therapeutic agent has gained more strategic importance that targeting TRAIL-R1.

1.10.2 TRAIL-R3/TRAIL-R4

Unlike TRAIL-R1 and TRAIL-R2, TRAIL-R3 lacks a death domain containing cytoplasmic tail and remains tethered to the cell membrane through a glycosyl-phosphatidylinositol (GPI) tail, while TRAIL-R4 has a truncated cytoplasmic domain that is inefficient in transducing the apoptotic signal (Figure 1.3; Degli-Esposti *et al.*, 1997b; MacFarlane *et al.,* 1997; Marsters *et al.*, 1997; Mongkolsapaya *et al.*, 1998; Pan *et al.*, 1997b; Pan *et al.*, 1998). Thus, both TRAIL-R3 and TRAIL-R4 can bind to TRAIL at the cell surface, but cannot transmit a death stimulus and hence, they are also commonly known as TRAIL decoy receptors (MacFarlane *et al.,* 1997; Pan *et al.,* 1998). Increased expression of TRAIL-R3 or TRAIL-R4 in cancer cells has been known to cause resistance to TRAIL-induced apoptosis (Zhang *et al.,* 2000; Bouralexis *et al.,* 2003). While both TRAIL-R3 and TRAIL-R4 can sequester TRAIL and thereby reduce its efficiency, TRAIL-R4 alone has also been implicated in TRAIL-mediated signalling to proliferative pathways, especially through activation of NFκB (Degli-Esposti *et al.*, 1997a). It has further been reported that TRAIL-R4 can associate with TRAIL-R2 and reduce stability of the TRAIL DISC, thus affecting TRAIL signalling (Merino *et al.,* 2006). Like TRAIL-R1 and TRAIL-R2, TRAIL-R3 also contains an intronic p53 binding site in its promoter region and its expression can be up-regulated in response to p53 activation (Ruiz de Almodóvar *et al.,* 2004).

1.10.3 OPG

OPG is normally known as the receptor for RANKL/OPGL and plays a regulatory role during the process of osteoclastogenesis (reviewed in deVries *et al*., 2006). It is a soluble receptor and since it does not possess a membrane anchor, it cannot be expressed on the cell surface. However, in addition to binding to OPGL, OPG has been implicated in binding to TRAIL, albeit with very low binding affinity at physiological temperatures (Emery *et al*., 1998; Truneh *et al.*, 2000). The physiological importance of this interaction, if any, is however unclear though it has been speculated that OPG may play an important role in bone cancer metastasis and development through binding to/sequestering TRAIL and preventing its apoptotic activity (Vitovski *et al.,* 2007).

1.11 TRAIL apoptotic pathway

Apoptosis (Greek for falling of leaves from a tree) is a form of programmed cell death (PCD) which plays an indispensable role in physiological development and maintenance of tissue haemostasis (Wyllie *et al.*, 1980). Apoptosis is an innate mechanism that cells employ to undergo cell death without causing inflammation or tissue injury and disruption of this process can have fatal consequences. Indeed, evasion of apoptosis is an important hallmark of cancer and increased apoptosis is responsible for a number of neurodegenerative diseases like Parkinson's disease and AIDS (Acquired Immuno Deficiency Syndrome; Thompson, 1995). The two major signalling mechanisms which culminate in apoptosis are the extrinsic apoptotic pathway and the intrinsic apoptotic pathway (Figure 1.4). The initial employment of either of these apoptotic pathways depends on the original stimulus; however, both pathways eventually coalesce at the level of effector caspase activation (See 1.12.2). TRAIL upon binding to its death receptors, engages the extrinsic apoptotic pathway, but it can also further engage the intrinsic pathway.

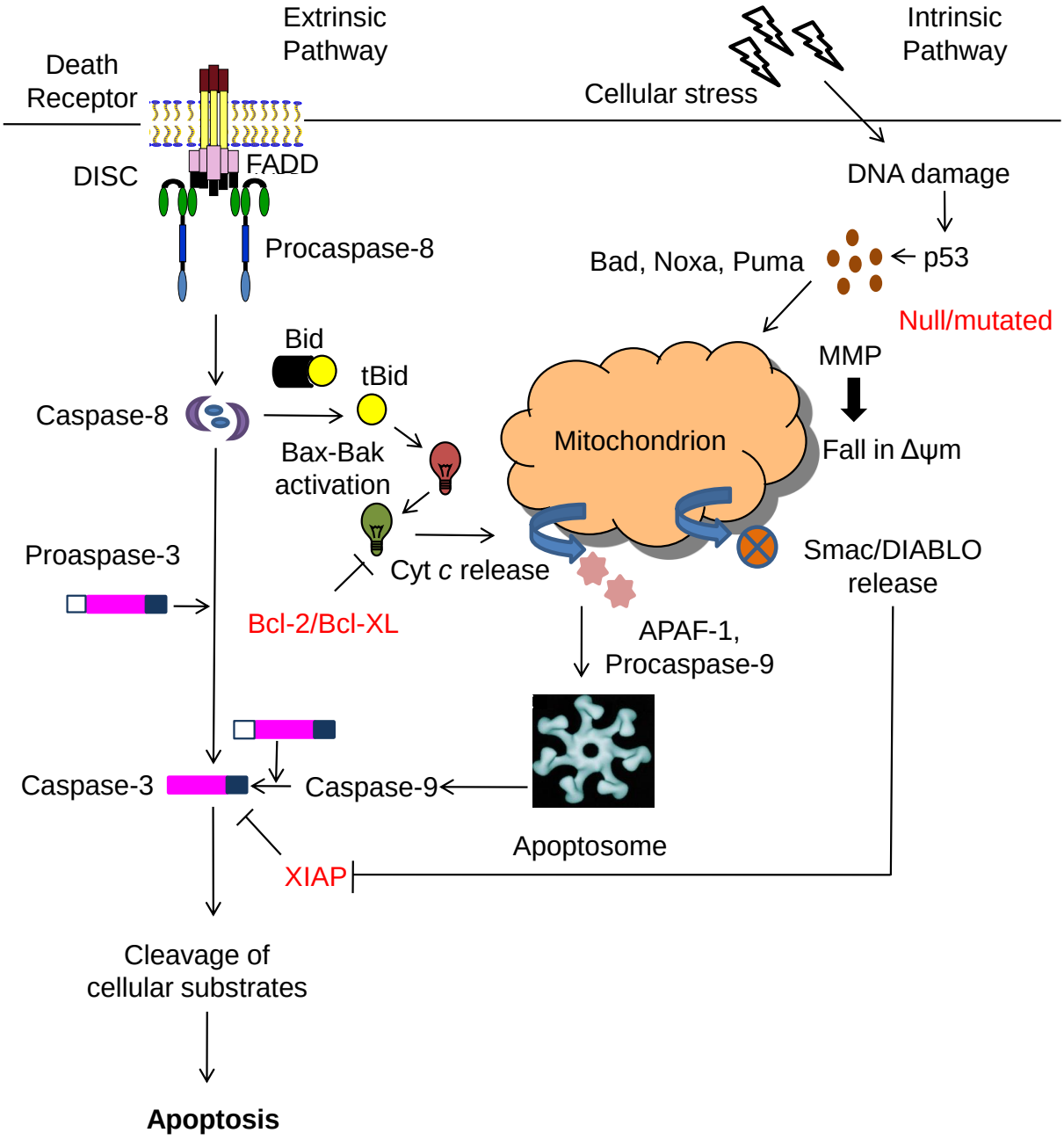


Figure 1.4 Apoptotic pathways

There are two apoptotic pathways-the extrinsic and the intrinsic pathway. The extrinsic pathway is employed by death-inducing ligands and it functions via signalling through death receptors on the cell surface. The intrinsic pathway is triggered by DNA damage, hypoxia, growth factor deprivation and oncogene induction and in many cases is dependent on p53 activation (reviewed in Ashkenazi *et al.,* 2002; Okada *et al.,* 2004).

1.12 The Extrinsic Apoptotic Pathway

1.12.1 Formation of the TRAIL DISC

Upon TRAIL binding to its death receptors, formation of an active TRAIL DISC is the primary step of TRAIL apoptotic signalling (Figure 1.4). At a minimum, an active TRAIL DISC consists of TRAIL, its cognate death receptors, FADD and procaspase-8 (Chinnaiyan *et al.,* 1995; Scaffidi *et al.,* 1997). Other proteins also known to be recruited into the TRAIL DISC are FADD-like interleukin-1β converting enzyme-like Protease (FLICE/C8)-inhibitory protein (c-FLIP), Receptor Interacting Protein 1 (RIP1), and caspase-10 (Harper *et al.,* 2001; Kischkel *et al.,* 2001; Wang *et al.,* 2001; Sprick *et al.,* 2002; Golks *et al.,* 2005;). The finding of these proteins in the DISC has often been cell-type dependent and their molecular functions have not been as clearly defined as those of FADD and caspase-8.

It has been suggested that following binding of TRAIL, a conformational change in the TRAIL receptors, leads to the recruitment of FADD through a DD interaction (Ashkenazi, 2002). FADD adaptor protein contains not only a death domain, but also a death effector domain (DED). The role of FADD in TRAIL-induced apoptosis was initially disputed, but increasing evidence has shown that FADD plays an indispensable role in the TRAIL apoptotic pathway (Marsters *et al.*, 1996; Pan *et al.*, 1997a). FADD null cells are resistant to TRAIL and the restoration of FADD expression sensitises these cells to TRAIL-induced apoptosis (Kuang *et al.,* 2000). Through its DED, FADD can bind with other DED containing molecules including other FADD molecules, procaspase-8/10 and c-FLIP (Boldin *et al.*, 1996).

Procaspase-8 is a key initiator caspase (Also see 1.12.2) and is expressed in cells as two splice variants- procaspase-8a (55 kDa) and procaspase-8b (53 kDa). Both these forms are recruited into the TRAIL DISC and cleaved to give fragments of 43 and 41 kDa, respectively. Further processing of caspase-8 leads to the formation and subsequent release of its active p18/p10 subunit into the cytoplasm. An increased local concentration of caspase-8 within the DISC has long been seen as the reason for proximity-induced caspase-8 autoactivation (Muzio *et al.*, 1998). However, studies from our laboratory have shown that proteolytic cleavage of caspase-8 is also imperative for its efficient activation (Hughes *et al.,* 2009). It has also been reported that the ubiquitination of caspase-8 by the E3 ligase, Cullin 3, may be important for the further stabilisation of active caspase-8 (Jin *et al.,* 2009). Procaspase-8 is critical for

TRAIL-mediated signalling; caspase-8 mutations are common in tumour cells and may be a mechanism to escape death receptor-mediated apoptosis.

The other caspase identified to be present in the TRAIL DISC is procaspase-10 (Wang *et al.,* 2001). Recruitment of procaspase-10 to the DISC leads to its activation and proteolytic cleavage followed by the release of its catalytic forms from the DISC (Engels *et al.,* 2005). The significance/function of caspase-10 in the TRAIL apoptotic pathway remains controversial, mainly because it cannot completely substitute for caspase-8. Although there have been reports stating the importance of caspase-10, caspase-8 is usually considered to be the crucial apical caspase of TRAIL-induced apoptosis. However, the recruitment of either caspases to the DISC can be inhibited by the caspase-8 homologue, c-FLIP.

Like its name suggests, c-FLIP was initially thought to be an inhibitor of caspase-8. There are many known splice variants of c-FLIP, but only three are found to be expressed in cells and these are c-FLIP Long (c-FLIP$_L$; 55 kDa), c-FLIP Short (c-FLIP$_S$; 26 kDa) and c-FLIP Raji (c-FLIP$_R$; 24kDa; Golks *et al.,* 2005). Like procaspase-8, all three c-FLIP isoforms have two DEDs; however, only c-FLIP$_L$ has been reported to be recruited to the DISC under physiological conditions. On the other hand, c-FLIP$_S$ can be found in the DISC but only in cells which strongly over-express it. Although procaspase-8 and c-FLIP$_L$ share significant homology, c-FLIP$_L$ does not possess any catalytic activity and has been suggested to act as a competitive binding partner of caspase-8, preventing its recruitment to FADD and subsequent activation (Krueger *et al.,* 2001; Golks *et al.,* 2005). However, other studies have implicated c-FLIP$_L$ to have a pro-apoptotic function and while no consensus has been achieved on the role of c-FLIP$_L$, it is widely agreed that the ratio of caspase-8 and c-FLIP$_L$ in a cell is an important determinant of the TRAIL apoptotic response. When highly expressed, c-FLIP$_L$ can prevent apoptosis by interfering with procaspase-8 binding to FADD, while at low levels of expression c-FLIP$_L$ can associate with procaspase-8 and facilitate caspase-8 dimerisation and consequent activation.

RIP1, on the other hand, is known to induce activation of the NFκB pathway and is believed to play an important role in TRAIL-mediated tumour cell proliferation (Lin *et al.,* 2000). Like FADD, RIP1 contains both a DD through which it can interact directly with the DD of the receptors. RIP1 can be cleaved by caspase-8 during apoptosis and this cleavage is believed to be important for inactivation of the pro-apoptotic NFκB signalling (Lin *et al.,* 1999). Recently, RIP1 has also been implicated in signalling to

necroptosis, a form of caspase-independent cell death (Holler *et al.,* 2000; Degterev *et al.,* 2005).

Irrespective of the presence of the other DISC components, the activation of caspase-8 in the DISC is the key apical event in the TRAIL apoptotic pathway.

1.12.2 Activation of caspases

The term 'caspase' stands for a family of cysteine-containing, aspartate-specific proteases, which play an indispensible role during the execution of apoptosis. Caspases are produced as inactive zymogens, which require proteolytic cleavage at specific aspartate residues to convert them to their active forms (Figure 1.5; Alnemri *et al.,* 1996). Caspases have been classified into two families, the initiator caspases and the effector caspases. Initiator caspases (caspase-2, -8, -9 and -10) function upstream in the pathway to relay the apoptotic stimulus to other pro-apoptotic moieties and/or the effector caspases (reviewed in Cohen, 1997). Initiator caspases are characterised by long pro-domains (>90 amino acids) and contrary to effector caspases, undergo autoactivation. Effector caspases (caspase-3, -6 and -7), on the other hand, have short prodomains (20-30 amino acids) and are activated through initiator caspase-mediated cleavage. Once activated, effector caspases cleave a variety of cellular substrates including PARP [{Poly (ADP-Ribose) Polymerase}; Teewari *et al*., 1995], and cytoskeletal proteins and induce morphological changes like DNA fragmentation (Jänicke *et al.*, 1998) or the externalisation of phosphatidylserine (PS), which marks the cell for phagocytic clearance (reviewed in Fadok *et al.,* 1998).

Caspase-8 is an initiator caspase, which as highlighted earlier acts as the key apical caspase of the TRAIL apoptotic pathway and gets activated within an active TRAIL DISC (Figure 1.4).

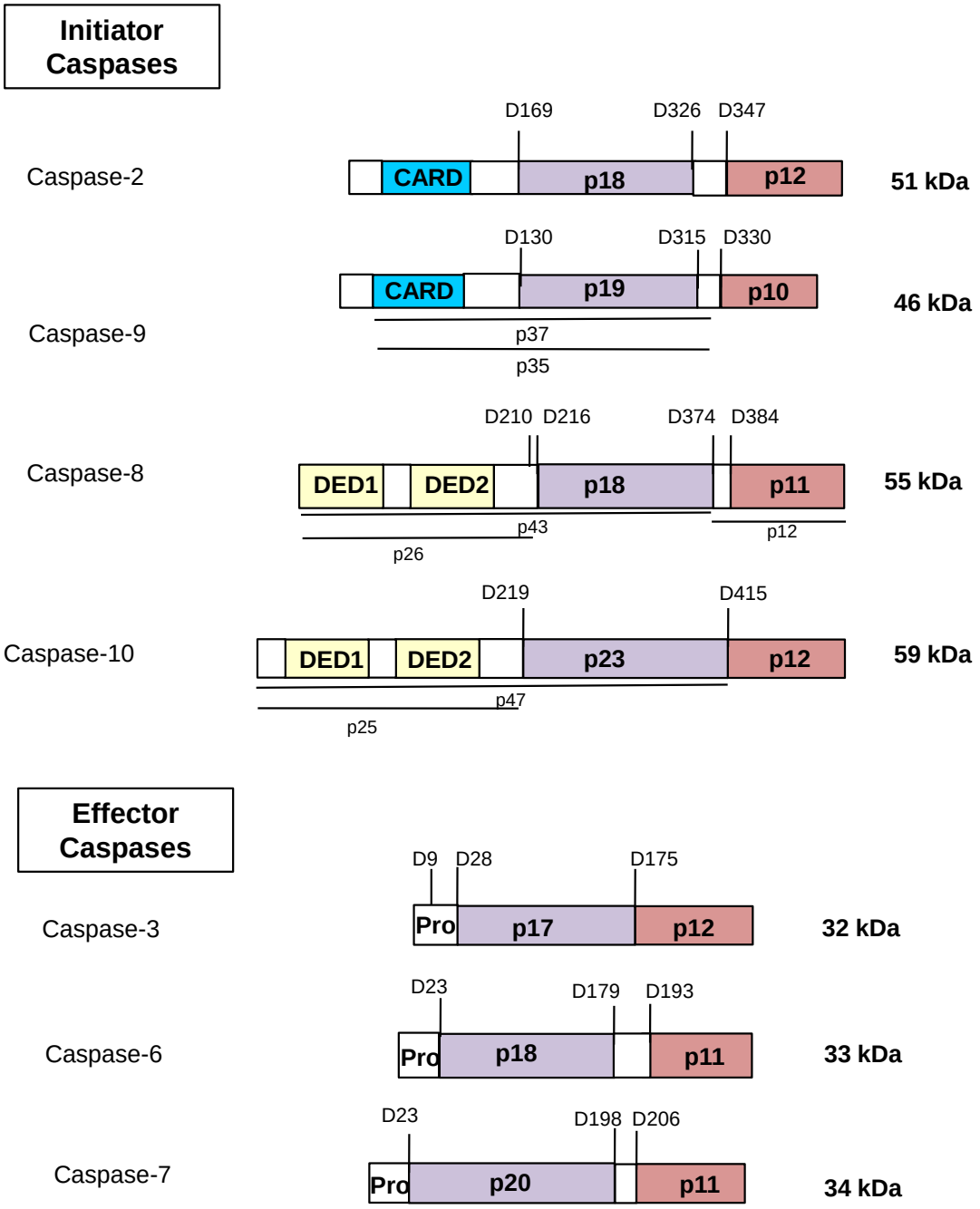


Figure 1.5 Activation of caspases

Caspases can be divided into two groups- initiator caspases and effector caspases. Initiator caspases are characterized by long pro-domains and undergo autoactivation. Effector caspases have short pro-domains and are activated by initiator caspase-dependent cleavage (Reviewed in Cohen, 1997; Adapted from Dickens, 2009).

1.12.3 Type I/II cells

Upon activation, caspase-8 can either directly cleave and activate the major effector caspase, procaspase-3 or cleave another caspase-8 substrate and Bcl-2 family member, Bid (Figure 1.4). Upon cleavage Bid forms tBid (truncated Bid) which then goes on to activate the mitochondrial arm of the extrinsic pathway *via* activation of Bax and Bak (Luo *et al.*, 1998; Korsmeyer *et al.,* 2000). In some cells, caspase-8-mediated procaspase-3 activation and subsequent degradation of the different caspase substrates is sufficient to ensure complete apoptosis. Those cells, which are not dependent on the mitochondrial amplification arm of the extrinsic pathway, have been classified as Type I cells (Figure 1.6; Scaffidi *et al.,* 1998). On the contrary, cells which require the tBid-mediated mitochondrial amplification loop to facilitate the progression of death receptor-mediated apoptosis are classified as Type II cells. Although Type I/II cell studies are useful in understanding the mechanisms of TRAIL-induced apoptosis, it is important to note that there is no strong correlation between the cell Type I/II status and sensitivity to TRAIL.

1.12.4 Bcl-2 protein family

The Bcl-2 family contains both pro- and anti-apoptotic proteins characterised by the presence of any of the four Bcl-2 homology (BH1-4) domains. BH1, 2 and 4 together comprise a hydrophobic groove within the molecule, whereas BH3 forms a short 8-12 amino acid long region which can bind into the groove (Sattler *et al.,* 1997; Muchmore *et al.,* 1996; Liu *et al.,* 2003). Some of the family members are multi-domain proteins sharing sequence homology in any of the four BH1-4 domains. Multi-domain anti-apoptotic proteins include Bcl-2, Bcl-xl, Bcl-w, Mcl-1, Bcl2l10, Bfl-1 and Bcl2l12, while multi-domain pro-apoptotic proteins include Bax, Bak, Bok, Bcl-G and Bcl-Rambo. Another important subgroup of the Bcl-2 family is the pro-apoptotic BH3-only domain containing proteins like Bid, Bik, Bad, Bim, Bmf, Noxa and Puma.

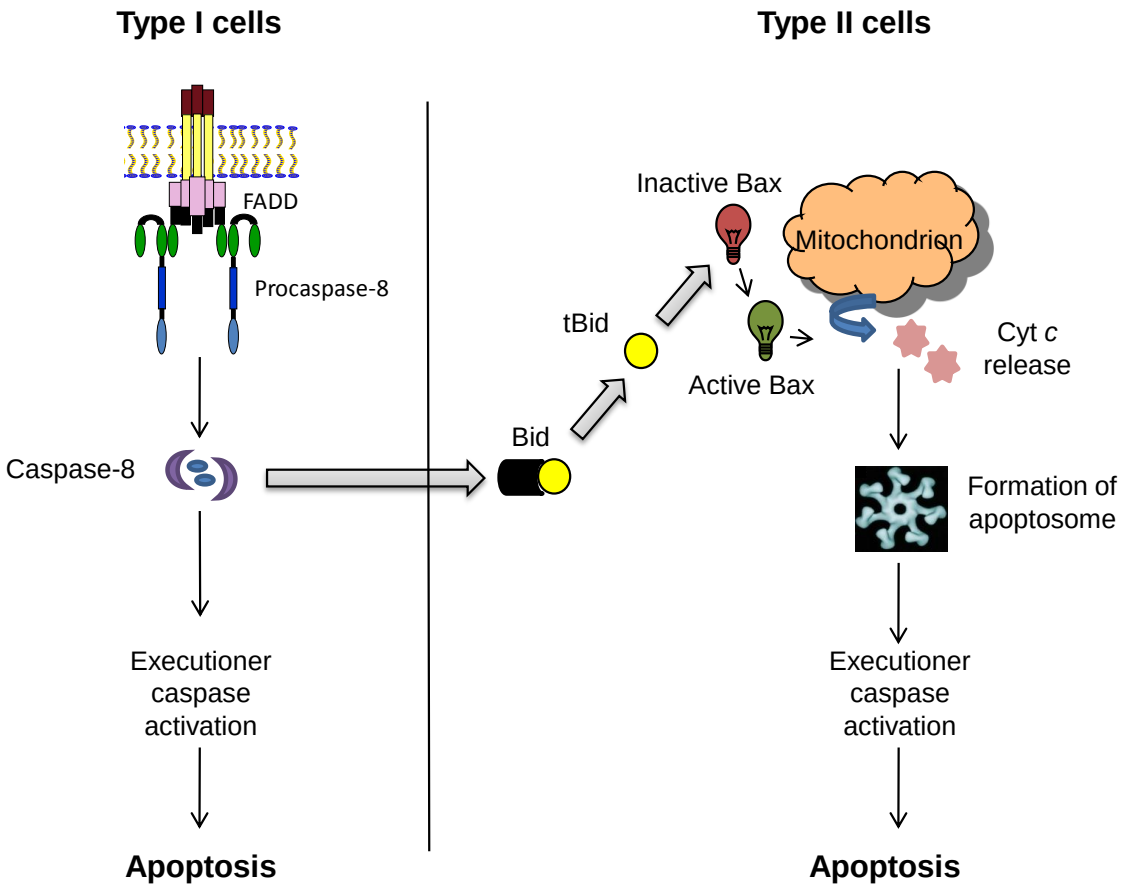


Figure 1.6 Type I and Type II cells

In Type I cells, the activation of procaspase-8 by the DISC is sufficient to culminate in effector caspase activation and cell death. In Type II cells, however, a mitochondrial amplification of the death signal is required to efficiently trigger the caspase cascade eventually leading to the demise of the cell (Scaffidi *et al.,*1998).

The pro- and anti-apoptotic Bcl-2 proteins regulate each other *via* BH-domain dependent interactions and hence, the relative ratio of these proteins in a cell plays a significant role in determining cellular sensitivity to cell death stimuli. Activation of Bax and Bak is critical for apoptotic signalling through the extrinsic apoptotic pathway in Type II cells. Bax and Bak can homodimerise and this association leads to formation of pores in the mitochondrial membrane and the resulting fall in mitochondrial membrane potential (MMP; ΔΨM) is hailed as a 'point of no return' for the cell (Figure 1.4; Letai *et al.,* 2002; reviewed in Kroemer and Martin, 2005). However, in normal cells, Bax and Bak are bound by the anti-apoptotic Bcl-2 family members, especially Bcl-2, Bcl-XL and Mcl-1 and are thus maintained in an inactive state. In fact, many tumour cells over express these anti-apoptotic Bcl-2 proteins in a bid to evade apoptosis and inhibition of these proteins can sensitise tumour cells to TRAIL-induced apoptosis. Conversely, Bax/Bak null or double knock out cells are resistant to TRAIL-induced apoptosis (LeBlanc *et al.*, 2002). The Bax/Bak-dependent mitochondrial membrane pore formation is a critical step ensuring the release of major pro-apoptotic proteins from the mitochondria in the extrinsic as well as intrinsic apoptotic pathways (Wei *et al.,* 2001).

1.13 Activation of the intrinsic apoptotic pathway

The intrinsic apoptotic pathway can be activated by the extrinsic pathway *via* tBid but also by exogenous stimuli such as growth factor deprivation, DNA damage or hypoxia (Okada and Mak, 2004). Most of the currently used cancer therapies target activation of the intrinsic pathway. p53 is the major tumour suppressor gene involved in engaging the intrinsic pathway in response to cancer therapy-mediated DNA damage. The stress on the cell is relayed to the p53 gene *via* activation of different moieties like ATM (Ataxia-Telangiectasia Mutated) or ATR (ATM and Rad Related). The p53 gene product is a transcription factor which leads to the activation of several DNA repair genes and also induces cell cycle arrest. If, however, the cell receives unsustainable damage, the p53 protein triggers apoptosis (reviewed in Brown and Attardi, 2005). One of the potential ways p53 can signal to apoptosis includes activating the synthesis of key apoptotic members like Noxa and PUMA, which in turn leads to activation of Bax and Bak (Figure 1.4; Oda *et al.,* 2000; Nakano and Vousden, 2001).

The conformationally active Bax protein aids in the formation of pores in the mitochondrial membrane and release of the resident mitochondrial protein cytochrome *c* (Wang *et al.,* 1996; Li *et al.,* 1998; Korsmeyer *et al.*, 2000). Cytochrome *c*, which is a key component of the oxygen respiratory chain inside the mitochondria, behaves as a

pro-apoptotic moiety upon its release from the mitochondria by inducing the formation of the apoptosome complex composed of APAF-1 (Apoptosis Protease Activating Factor-1) and the initiator caspase, procaspase-9 (Figure 1.4; Li *et al.,* 1997). Within the apoptosome complex, procaspase-9 is auto-activated to form active caspase-9 and this activation is dependent on the formation of a central ring by the CARD (CAspase Recruitment Domain) of APAF-1. Thus, caspase-9 is the apical caspase of the intrinsic apoptotic pathway. Once activated, caspase-9 can then cleave and activate procaspase-3, which subsequently leads to apoptosis.

Other mitochondrial proteins also released include Smac (Second mitochondrial activator of caspases)/DIABLO (Direct IAP-binding Protein with Low pI) and Omi/HtrA2. Smac and Omi are pro-apoptotic molecules known to antagonise the potent caspase-inhibitory protein, XIAP (Figure 1.4; X-Linked Inhibitor of APoptosis; Du *et al.,* 2000; Verhagen *et al.,* 2000; Martins *et al.,* 2002). Inactivation of XIAP is an important event essential for the unrestrained progress of apoptosis (Deveraux *et al.,* 1997; Deng *et al.*, 2002) and for manifestation of the apoptotic phenotype. XIAP is a prominent member of the IAP (Inhibitor of APoptosis) family of proteins which play an important role in apoptosis and can function to inhibit caspases under physiological conditions.

1.14 IAP family of proteins

The IAP family of proteins is characterised by the presence of at least one domain of the 70 amino acid long baculoviral IAP repeat (BIR). Proteins containing these BIR domains are well-conserved in eukaryotes and in humans this family consists of XIAP, cIAP1/2, NAIP, BRUCE and survivin. Of these, XIAP, cIAP1 and cIAP2 have been shown to bind the effector caspases-3 and -7 as well as the initiator caspase-9 (Deveraux *et al.,* 1997; Roy *et al.,* 1997). However, binding of XIAP, but not cIAP1/2 can inhibit caspase activity (Eckelman *et al.,* 2006). Furthermore, IAPs do not inhibit caspase-6, -8 or -10. XIAP, cIAP1 and cIAP2 contain three BIR domains; however, only BIR2 appears to be important for the anti-caspase ability of these proteins (Takahashi *et al.,* 1998). BIR1 and BIR3 share striking sequence homology with BIR2, but are incapable of interacting with any of the caspases. The presence of only one BIR domain in survivin also supports the suggestion that only one BIR domain is enough to bind to and inhibit caspase activation.

1.15 Regulation of apoptosis in tumour cells

The complete execution of apoptosis depends on the balance between pro- and anti-apoptotic proteins within the cell. Pro-apoptotic proteins including caspases, Bcl-2 family members like Bax and Bak, and Smac/Omi aid in the smooth progress of apoptosis, while, anti-apoptotic proteins like c-FLIP, Bcl-2 family members including Bcl-2 and Bcl-XL, and the IAPs regulate the process of apoptosis (reviewed in Green, 2002). Clearly, inhibition of anti-apoptotic proteins leading to enhancement of the pro-apoptotic stimulus is essential for the proper execution of apoptosis.

Most transformed cells evade apoptosis either through the down-regulation of pro-apoptotic proteins or through up-regulation or sustained activity of anti-apoptotic proteins. Many chemotherapeutics and radiation therapies used for cancer seek to restore the pro- and anti-apoptotic protein balance and assist in full apoptosis induction in the transformed cells.

Since most cancer cells evade apoptosis by harbouring mutations in the various pro- and anti-apoptotic proteins, activating both the intrinsic and extrinsic apoptotic pathways together would be an ideal approach in achieving an effective cancer therapy. The intrinsic and extrinsic apoptotic pathways can cross talk *via* Bid-dependent activation of Bax/Bak or caspase-3-dependent cleavage of caspase-8, thus further potentiating the apoptotic stimulus. Activating different cell death/signalling pathways forms the basis of combination therapy and TRAIL therefore, harbours great potential as a death-inducing ligand which can be used along with chemotherapy/radiotherapy.

1.16 TRAIL and TRAIL receptors in breast cancer

TRAIL and TRAIL-receptor expression are important factors that determine the sensitivity of any tumour cell to TRAIL-induced apoptosis. Since the exciting potential of TRAIL as a cancer therapeutic was recognised, many studies have focussed on determining the expression of TRAIL and its receptors in various cancers, including breast cancer.

Using immunohistochemistry, TRAIL itself was found to be expressed in a significant number of primary breast tumour tissues (52.2%). On the other hand, only one out of seven fibroadenoma cases and none of the normal breast tissue samples or lymphocytes in this study showed any TRAIL expression (Herrnring *et al.*, 2000). Interestingly, all the fibroadenomas and normal breast tissues specimens expressed

TRAIL at the mRNA level, suggesting that post-translational modifications play a crucial role in regulating the expression of TRAIL in normal cells.

The expression of TRAIL-receptors in primary breast cancer not only seems to vary with patient cohorts, but also appears to be dependent on the tumour grade and progression. For example, one study reported that TRAIL-R1 expression correlated positively with tumour grade in IDC (Sanlioglu *et al.*, 2007), but not tumour stage, indicating that tumour cell differentiation was an important governing factor for TRAIL-R1 expression. Also, another study showed that an increase in expression of TRAIL-R1 was seen in better differentiated tumours and inversely correlated with HER2 status and lymph node metastases of the tumours (Ganten *et al.*, 2009).

On the other hand, McCarthy *et al* reported that an increase in TRAIL-R2 expression was seen in lymph node metastases as compared to the primary tumours and that the increased TRAIL-R2 expression could be positively correlated with decreased patient survival (McCarthy *et al.*, 2005). In contrast to this study, Seitz *et al* showed that in metastatic breast tumour samples from six lymph nodes tested, there was a 2.4-fold increase in TRAIL-R1 and a 3-fold decrease in TRAIL-R2 (Seitz *et al.*, 2002). Importantly, Ganten *et al* provided evidence that although a significant number of breast tumours express TRAIL-R2, it is mainly localised to the cytoplasm (on the basis of immunohistochemical staining), where it cannot functionally bind TRAIL. This study also suggested that a profile of low TRAIL-R1 expression, high TRAIL-R2 expression and low Bcl-2 expression could show possible pre-disposition to metastasis (Ganten *et al.,* 2009).

Furthermore, while one study has argued that ER expression does not significantly affect TRAIL receptor expression (Sanlioglu *et al.*, 2007); another study showed that TRAIL-R1 expression positively correlates with ER status (Ganten *et al.*, 2009). Also, both TRAIL-R1 and TRAIL-R2 have been shown to positively correlate with progesterone status.

The discrepancies between the above studies might be due to differences in patient cohort, the size of the sample population tested and the techniques used to determine TRAIL-receptor expression. It is important to note that many studies only measure the mRNA or gene expression levels using microarray analysis and this measurement does not necessarily correlate to protein expression. A number of post-translational modifications are involved in the proper localisation and functioning of the receptors and hence, only the cell surface receptor expression levels can be a deciding factor of

TRAIL sensitivity. However overall, these studies do show that increased TRAIL-R2 expression can be correlated to tumour progression and a decrease in patient survival.

Mutations in the genes encoding TRAIL-R1 and TRAIL-R2 are also common in breast cancer and may represent a mechanism to evade TRAIL-mediated apoptosis by breast cancer cells. Allelic loss of chromosome 8p21-22 (where the genes for TRAIL receptors are located) was found to be significantly increased in metastatic breast cancer cells (Shin *et al.*, 2001). Seven mutations (three in the gene encoding TRAIL-R1 and four in the gene encoding TRAIL-R2) were shown to be present in metastatic breast cancer and have been reported to suppress apoptosis when expressed in cell lines (Shin *et al.*, 2001).

Importantly, many studies have previously tried to ascertain the potential of TRAIL in breast cancer therapy (Keane *et al.*, 1999; Singh *et al.*, 2003; Kelley *et al.*, 2005; Zinonos *et al.,* 2009). All of these studies have been based on breast cancer cell lines using different formulations of TRAIL and using a variety of measures to confirm the activation of apoptosis. All the studies classify the hormone receptor negative breast cancer cell line MDA-MB-231, as the most TRAIL-sensitive cell line, while a majority of the other cell lines, including MCF7, MDA-MB-468 and T47D cell lines have been classified as semi-sensitive or TRAIL-resistant cell lines (reviewed in Rahman *et al.*, 2009). TRAIL resistance of breast cancer cell lines has been attributed to defective endocytosis of the receptors, sustained expression of c-FLIP$_L$ and XIAP and activation of pro-survival pathways by TRAIL (Lee *et al.*, 2006; Zhang and Zhang 2008; Yoshida *et al.*, 2009). Efforts have also been made to sensitise resistant breast cancer cell lines to TRAIL-induced apoptosis by using ionising radiations as well as chemotherapy (Keane *et al.*, 1999; Chinnaiyan 2000; Singh *et al.*, 2003). In this regard, both HDACi and doxorubicin have been shown to sensitise breast cancer cell lines to TRAIL-induced apoptosis at varying levels (Keane *et al.,* 1999; Chinnaiyan *et al.,* 2000; Singh *et al.,* 2003; Singh *et al.*, 2005).

A previous study that used a receptor-specific mutant form of TRAIL has suggested that TRAIL-R2 might be the more potent TRAIL death receptor in breast cancer cell lines (Kelley *et al.*, 2005; see also 1.16). Also, a fully human agonistic antibody to TRAIL-R2 has been shown to be effective in xenograft models of breast cancer (Zinonos *et al.,* 2009). However, another study also provided evidence that increased expression of TRAIL-R1 can cause ligand-independent apoptosis in breast cancer cell lines (Kazhdan and Marciniak, 2004). However, all of these studies have been carried

out using breast cancer cell lines. It is therefore critical to determine the importance of the relative contribution of the TRAIL death receptors in primary breast tumours.

TRAIL is not only known to cause apoptosis in cancer cell lines, but it has also been reported to aid in cell proliferation in apoptosis-resistant cells (Mühlenbeck *et al.*, 1998; Hu *et al.*, 1999; Ehrhardt *et al.*, 2003; Baader *et al.*, 2005; Trauzold *et al.*, 2006). This is also true in breast cancer cell lines, where sub-toxic concentrations of TRAIL have been reported to drive proliferative pathways (Kazhdan and Marciniak, 2004). Interestingly TRAIL was also identified as one of the genes whose expression negatively correlated with survival in breast cancers that had metastasised to the brain (Bos *et al.*, 2009). Therefore, it is critical to determine the TRAIL sensitivity of tumour cells prior to therapy, and the co-administration of sensitising agents in the case of TRAIL-resistant tumours would be highly recommended.

Considering the selectivity of TRAIL in killing tumour cells and its efficiency in triggering the extrinsic apoptotic pathway, many pharmaceutical companies have developed agents to target the TRAIL receptors *in vivo* and these agents are currently in clinical trials either alone or with chemotherapy (Pukac *et al.*, 2005; Trarbach *et al.*, 2010).

1.17 Receptor-selective mutant TRAIL ligands; Previous findings in primary lymphoid malignancies

To study the relative contributions of the TRAIL death receptors, our laboratory has generated mutant ligands of TRAIL specific to TRAIL-R1 (R1L) or TRAIL-R2 (R2L) and validated their specificity and activity in both tumour cell lines and primary tumours of lymphoid origin (MacFarlane *et al*., 2005b; Harper and MacFarlane, 2008).

Though a majority of cell lines are responsive to TRAIL therapy, there is little evidence of TRAIL-induced apoptosis in primary tumour cells. Consistent with this observation, previous work done in our laboratory showed that, primary chronic lymphocytic leukaemia (CLL) cells are resistant to TRAIL as a single agent. However, primary CLL cells can be sensitised to TRAIL-induced apoptosis using HDACi as sensitising agents. Upon sensitisation, it was found that R1L induced significantly more apoptosis in primary CLL cells as compared to R2L (MacFarlane *et al*., 2005b).

This result is in stark contrast to other studies, which have reported that TRAIL-R2 is the more important death-inducing receptor involved in TRAIL-mediated apoptosis (Kelley *et al*., 2005). This key finding, therefore, highlighted the importance of investigating which functional TRAIL death receptor(s) are involved in TRAIL-induced

apoptosis prior to dispensing any treatment. For example, it is clear that TRAIL-R2 activating antibodies would initiate minimal, if any, response in primary CLL cells with a functional TRAIL-R1. Based on these earlier findings in primary lymphoid malignancies, it was thus, highly critical to assess the TRAIL-receptor profile of different tumour types to try and determine the most beneficial TRAIL treatment regime.

In this study we have used breast cancer cell lines and primary tumour tissues to comprehensively determine TRAIL sensitivity and TRAIL-receptor contribution to apoptosis in breast cancer.

1.18 Model system for breast cancer research: breast tumour cell lines

Established tumour cell lines are frequently used tools in cancer research. They are very easy to handle and culture, thus providing an opportunity to reproduce results and conduct mechanistic studies in the laboratory setting. Many breast cancer cell lines have been derived and are currently in use worldwide. Out of the most commonly used breast cancer cell lines, six cell lines were chosen for this study (Table 1.3), forming a panel which would be representative of breast cancer. These cell lines have different origins, ER and p53 status which should aid in determining the influence, if any, of these important prognostic markers on the sensitivity of breast cancer cells to TRAIL-induced apoptosis.

It is, however, important to note that cancer cell lines in monolayer culture do not possess the 3-dimensional (3D) architecture that is characteristic of solid tumours like breast cancer. Both the 3D architecture and the tumour microenvironment are significant factors governing tumour progression and drug resistance.

The effects of the 3D architecture of tissues on TRAIL sensitivity have been reported in an elegant study carried out by Weaver *et al* (Weaver *et al.,* 2002). This study effectively proves that an organised 3D architecture confers TRAIL resistance to cells through the ligation of β4 integrins, which regulate NFκB activation.

The importance of the tumour microenvironment in the development and progression of cancer was first highlighted by Stephen Paget in his ‘seed and soil’ theory. His observation that specific cancers could only metastasise to specific tissues in the body (for example, breast cancers metastasise mostly to the brain or bone) fuelled speculation that interactions between the host tissue and tumour cells may be crucial to tumour development (Paget, 1889).

Table 1.3 Panel of breast cancer cell lines

Cell line	ER	HER2 over-expression	p53	Origin	Pathology	Molecular Profile
MCF7 F43	+	-	Wild type	Pleural effusion	Invasive ductal carcinoma	Luminal
ZR-75.1	+	-	Wild type	Ascites	Invasive ductal carcinoma	Luminal
T47D	+	-	Mutated	Pleural effusion	Invasive ductal carcinoma	Luminal
MDA-MB-231	-	-	Mutated	Pleural effusion	Adeno-carcinoma	Basal B
MDA-MB-468	-	-	Mutated	Pleural effusion	Adeno-carcinoma	Basal A
BT20	-	-	Mutated	Breast	Invasive ductal carcinoma	Basal A

(Adapted from Burdall *et al*., 2003; Neve *et al.*, 2006)

The tumour microenvironment is a heterogeneous mixture of tumour cells, residential normal cells (epithelial cells, blood vessels and stromal fibroblasts), infiltrating normal cells (lymphocytes and macrophages) and a plethora of molecules (like cytokines, chemokines and antibodies) secreted by the cells. The extracellular matrix (ECM) forms the structural scaffold for the residing cells and is also responsible for the movement of different signalling molecules. The molecular components of the ECM, like the proteoglycans or collagen, are synthesised by the resident cells and then secreted out.

Like tumour cells, stromal cells can also accumulate mutations leading to increased expression of chemokines and cytokines, which affect the growth of tumours. For example, a study performed with breast tumour myoepithelial and myostromal cells showed that these cell types consistently over-expressed CXCL12 and CXCL14 as compared with normal breast cells (Orimo *et al.,* 2005). Furthermore changes in the levels of microenvironment signalling molecules can affect tumour progression by increasing genetic instability of tumour cells, putting selective pressures for the growth of drug-resistant cells and causing increased cell proliferation by activating survival pathways in the tumour cells. *In vitro* and *in vivo* studies in breast cancer have confirmed the influence of stromal cells on the survival and invasiveness of tumour cells by secretion of growth factors and metalloproteinases.

Not only can the tumour microenvironment affect the progression of the tumour, but it can also interfere in the response of tumour cells to drugs. Different areas in the tumour will have varying degrees of acidity, hypoxia and cell proliferation rate, all of which affect tumour cell uptake and sensitivity to drugs. Diffusion of drug to the tumour cells, far from the tumour vasculature, is a key factor in determining drug resistance. In fact, the development of tumour vasculature through angiogenesis is essential for tumour progression and is heavily influenced by the secretion of angiogenic molecules (e.g. Vascular Endothelial Growth Factor; VEGF) into the microenvironment. Overall, the tumour microenvironment exerts its effects on two major hallmarks of cancer-angiogenesis and metastasis (reviewed in Hu and Polyak, 2008).

The use of cell lines in monolayer culture fails to recapitulate the indispensable influence of the tumour microenvironment on tumour cells and therefore, it is important to design better models for solid tumours that are capable of mimicking the tumour microenvironment-tumour cell interactions.

1.19 Model system for breast cancer research: mammospheres

One of the ways of recapitulating the 3D architecture in cell lines is to culture the cells in spheroid culture which promotes strong cell-cell interactions. Different methods have been described to achieve the spheroid growth of cells including the use of matrix-forming gels like Matrigel, scaffolding supports and the culturing of cells in non-adherent plates.

When breast cancer cells are cultured in a plate with a non-adherent coating, like poly-2-hydroxyethyl methacrylate (poly-HEMA), they tend to float in the media and most of the cells undergo anoikis. Anoikis is a cell death pathway which is triggered by the lack of attachment to a basement membrane (Frisch and Screaton, 2001). The anoikis-resistant cells, however then divide and grow over time and form spheroid cultures known as mammospheres. Mammospheres from breast epithelial cells have been described previously and are commonly used to understand the mechanisms of anoikis as well as drug resistance in breast cancers (Farnie *et al.,* 2007).

However, even mammosphere cultures do not address the question of the effects of the tumour microenvironment on TRAIL sensitivity of breast cancer cells. Also, although tumour cell lines are the most commonly used *in vitro* models for cancer research, they cannot be used as a complete representative of primary tumour cells. Since cell lines are derived from only part of the tumour, they only represent the properties characteristic to that particular section. Thus, although cell lines are more

convenient to work with, results obtained from cell line-based experiments should be scrutinised carefully. To more directly address the question of the potential application of TRAIL-based therapies in patients with breast cancer, a modified approach to culture primary breast tumour tissue from patients was employed.

5.20 Model system for breast cancer research: primary tumour breast tissue

A number of techniques have been described in the literature to culture primary breast cells in the laboratory. One of these involves separating breast epithelial cells using enzymatic digestion followed by differential centrifugation and then culturing them *in vitro* (reviewed in Burdall *et al*., 2003). This method is used to prevent interference from other cell types like fibroblasts on the epithelial cells. However, using this method does not preserve the 3D architecture of the primary tissue. Hence, instead of separating the epithelial cells, in this project, small sections of the tissue explants were cultured *in situ* on Millipore Discs floating in culture media. The tissue viability was assessed after 48 hours and cells within the explants were seen to be healthy as assessed by Haematoxylin and Eosin (H & E) staining. After preliminary validation of this system, the tissue explant culture method was opted for, thus retaining the 3D architecture of the breast tumour tissue, since it can provide the most comprehensive *in vitro* representation of breast cancer tissue *in vivo*. This approach allowed us to study the potency of TRAIL in primary solid tumour tissue of varying grades and pathological types.

However, it is important to note that tumour metastasis to the lung, brain and bone is the major cause of death in a large number of breast cancer patients. Adjuvant therapy is mainly aimed at targeting metastatic cells and improving patient survival post-surgery and therefore it is also imperative to study the effect of TRAIL on metastatic breast cancer cells.

1.21 Model system for breast cancer research: primary metastatic cells

The invasive ductal carcinoma cells found within the primary tumour tissue explants have the potential to metastasise. However, it is difficult to determine whether these cells will metastasise and form secondary tumours outside the breast tissue. To study the effect of TRAIL on advanced metastatic breast cancer cells, we obtained primary tumour cells collected from ascites of breast cancer patients. These tumour cells were enriched for the tumour-initiating cell populations which have been shown to form secondary tumours when implanted in mouse models (Harrison *et al.,* 2010). To

assess the potency of TRAIL on these primary tumour cells, we used the mammosphere technique to culture the cells and observe the effect of TRAIL on the self-renewal capability of these tumour-initiating cells *in vitro*.

Thus, overall, we have used four different models for comprehensively assessing the efficacy of TRAIL treatment, in the presence or absence of potential TRAIL sensitsers (Table 1.4), in breast cancer. The information gained from this study will help establish the TRAIL-Receptor signalling profile of breast cancer, the most common cancer in the UK and may help provide information to improve the therapies used for its treatment.

Table 1.4 Potential TRAIL sensitisers

Class of Sensitiser	Name of Sensitiser	Potential mechanism of action	Reference
Anthracycline	Doxorubicin	• Up-regulation of TRAIL receptors • Modulation of Bcl-2 family protein levels	Singh *et al*., 2003
HDAC inhibitor	SAHA	• Up-regulation of TRAIL receptors • Modulation of Bcl-2 family protein levels	Butler *et al*., 2006
CDK inhibitor	Flavopiridol	•Down-regulation of c-$FLIP_L$	Palacios *et al*., 2006
ER inhibitor	Tamoxifen	• Modulation of Bcl-2 family protein levels	Lagadec *et al.,* 2007
Bcl-2 family inhibitor	ABT-737	• Down-regulation of anti-apoptotic Bcl-2 proteins • Up-regulation of TRAIL-R2	Huang and Sinicrope, 2008; Song *et al.,* 2008
Akt inhibitor/ p38 inhibitor	PI103/ SB203580	• Inhibition of Akt/p38	Opel *et al.,* 2008; Xu *et al.,* 2010
Smac mimetic	LBW242	• Inhibition of IAP family members	Bockbrader *et al.,* 2005; Fandy *et al.,* 2008; Vogler *et al.,* 2008; Vogler *et al.,* 2009

5.22 Specific Aims of the Project

- The primary aim of this project is to determine the relative contribution of TRAIL-R1 and TRAIL-R2 in TRAIL-induced apoptosis in epithelial-derived tumours using breast cancer as a representative model. The relative contribution of TRAIL-R's will be determined using the receptor-specific mutant ligands previously developed within our laboratory and/or agonistic Mabs that target TRAIL-R1/-R2 (either alone or in combination with a suitable sensitiser).

- TRAIL-mutants specific to TRAIL-R1 or TRAIL-R2 will be used on a panel of ER positive and negative breast cancer cell lines (Table 1.3) to identify the major TRAIL death receptor that signals to apoptosis. This study will also investigate the effect, if any, of ER and/or p53 status, on the relative contribution of TRAIL-R1/TRAIL-R2 to TRAIL-induced apoptosis.

- The project will also aim to identify and characterise potential chemosensitisers for TRAIL-resistant cell lines and primary tumour tissue. The effect of these different sensitisers on the relative contribution of TRAIL-R1/-R2 to apoptosis will help elucidate the potential mechanism of sensitisation and may also help identify new approaches to be used in breast cancer treatment.

- Ultimately the aim is to extend these studies to primary breast tumour tissue and profile the TRAIL-R's involved in TRAIL-induced apoptosis in primary tumour cells. Optimising conditions for the efficient culturing and treatment of primary breast tissue explants will provide us with a 3-dimensional *ex vivo* culture model, which is more clinically relevant and allows us to assess the profile of TRAIL-R signalling to apoptosis in breast cancer.

Chapter 2

Materials and Methods

All chemicals used were of the highest quality and were from Sigma-Aldrich (UK) unless otherwise stated.

Cell Culture Methods

2.1 Cell culture

The oestrogen receptor (ER) negative breast cancer cell lines, MDA-MB-231, MDA-MB-468 and BT20 were obtained from ATCC (American Typed Cell Collection, USA), while the ER positive cell lines, T47D and ZR-75.1 were obtained from the European Collection of Animal Cell Cultures (ECACC, UK). All the above cell lines were cultured in phenol red-free RPMI 1640 medium (Invitrogen, UK) which was supplemented with 10% (v/v) foetal calf serum (FCS, Life Technologies, UK) and 2 mM glutamax (L-glutathione) in 5% CO_2 at 37 °C. The breast cancer cell lines were grown in phenol-red free media as phenol-red is a weak activator of ER and may interfere with drug sensitivity (Berthois *et al.,* 1986).

The ER positive MCF7 F43 cells were a kind gift from M. Jäättelä (Danish Cancer Society Research Centre, Denmark) and were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS and 2 mM glutamax in 5% CO_2 at 37 °C. All cell lines were passaged every 3-4 days to maintain optimal confluency.

All the aforementioned cell lines have been tested for possible mycoplasma contamination and have been declared 'mycoplasma negative' (Mycoplasma Experience, UK). All cell lines were maintained for experimental use till they reached passage number 30 and were subsequently discarded.

2.2 Cell plating and treatments

To perform experiments, cells were plated in 6-well plates, 12-well plates (for immunocytochemistry), 24-well plates (for Bax activation assay) or T-175 flasks (DISC immunoprecipitations) at appropriate densities and allowed at least 24 h to adhere before treatment. For the mammosphere assay, cells were treated immediately after plating.

For treating adherent cells, the original medium was removed and replaced with 1 ml of warm medium to ensure removal of any dead cells and to maintain equal concentration of the treatments across all wells. Treatments conditions are as shown in Table 2.1.

For experiments requiring cross-linked Human Genome Sciences (HGS) ETR1/2 treatment, the antibodies were first incubated with the appropriate quantities of the $F(ab')_2$ fragment (goat anti-human IgG Fcγ) specific cross-linking antibody (Jackson ImmunoResearch Laboratories, USA) for 30 min at room temperature (Table 2.2) before addition to the cells.

2.3 Culturing and treatment of primary normal or tumour breast tissue

Normal breast tissue was obtained from consented patients after reduction surgery (Table 2.3), whilst breast tumour tissue was obtained from consented cancer patients with no prior exposure to chemotherapy (Table 2.4; Consenting done by Dr. Chris Neal; Ethical Approval # 10056; 06/Q2501/41; Project Title: Chemoprevention studies of human tumours; Appendix II). The tissue was kept in holding medium (phenol red-free DMEM F12 supplemented with 10% FCS and 2 mM Glutamax) till further processing. The tissue was then sectioned into small pieces (1 mm^3) using a pair of skin graft blades (Fisher Scientific, UK) on a base of dental wax (Agar Scientific, UK; Figure 2.1 for overview of explant culture method). The sections were then placed into fresh holding medium and tissue explants which sank to the bottom of the tube (generally containing more protein; the more fat-rich sections float at the top of the medium) were used for culturing. The protein-rich tissue explants were placed on Millicell organotypic inserts (0.4 µM pore size; Millipore, UK) floating on 1.5 ml culture media (phenol red-free DMEM supplemented with 10% FCS; 2 mM Glutamax and 5 ng/ml insulin) in 6-well plates.

Table 2.1 Death stimuli, their source and final working concentrations

Death stimuli	Source	Concentration used at
TRAIL (His tagged; wild-type)	MRC Toxicology Unit, UK (Harper and MacFarlane, 2008)	10-1000 ng/ml
TRAIL-R1-specific ligand (His tagged; R1L)	MRC Toxicology Unit	10-1000 ng/
TRAIL-R2-specific ligand (His tagged; R2L)	MRC Toxicology Unit	10-1000 ng/ml
Biotinylated versions of TRAIL/R1L/R2L	MRC Toxicology Unit	500 ng/ml
HGS-ETR1/ Mapatumumab (ETR1)	Kind gift from Human Genome Sciences, USA	10-2000 ng/ml
HGS-ETR2/ Lexatumumab (ETR2)	Kind gift from Human Genome Sciences	10-2000 ng/ml
Doxorubicin	Sigma-Aldrich (D1515)	0.1-10 µM
Flavopiridol	Alexis (ALX-430-161)	0.1-10 µM
ABT-737	Selleck Chemicals, USA (S1002)	10 µM
Tamoxifen	Merck, USA (579002)	10 µM
Suberoylanilide hydroxamic acid (SAHA)	Exclusive Chemistry, Russia	10 µM
Staurosporine	Sigma-Aldrich (S4400)	1 µM
PI103	Torcis Bioscience, UK (2930)	1-10 µM
SB203580	Cell Signaling Technology, USA (#5633)	10 µM
LBW242	Novartis, UK	1-20 µM
zVAD	MP Biomedical, UK	50 µM

Table 2.2 Amounts of antibody and cross-linker added for different ratio mixtures

ETR1/2:XL ratio ⟶	**1:1**	**1:3**	**1:5**
PBS (µl)	12.5	9.4	6.3
ETR1/2 (µl of 2 µg/ µl stock)	1	1	1
Cross-linker Ab (µl)	1.5	4.6	7.7
Total (µl)	15	15	15

Table 2.3 Summary of clinical information for the normal breast tissue samples obtained from breast reduction surgery

Sample#	**Date**	**Age (yr)**
N1	16/05/07	53
N2	21/05/07	19
N3	03/10/07	18
N4	15/10/07	54
N5	15/11/07	26
N6	22/11/07	45
N7	06/03/08	46

Table 2.4 Summary of clinical information for primary breast tumour samples. ER (SP1 clone, Dako, UK), PgR (Dako) and HER2 (HercepTest, Dako) staining was performed in the Specials Laboratory, Department of Histopathology, University Hospitals of Leicester and interpreted by Prof Rosemary Walker.

#	Date	Age (yr)	BC Type	Grade	ER status	PgR status	HER2 status	Lymph node (+/Total)
T1	**22/08/08**	42	Mucinous	II	High	Neg	Neg	0/9
T2	**24/06/08**	47	IDC	III	High	High	Neg	1/16
T3	**09/07/08**	79	IDC	III	High	Low	Neg	5/17
T4	**15/07/08**	46	IDC	III	Neg	Neg	Neg	0/13
T5	**18/08/08**	42	Mucinous	I	High	High	Neg	0/13
T6	**20/10/08**	49	IDC	III	High	Neg	Neg	1/10
T7	**03/11/08**	71	IDC	I	High	High	Neg	0/5
T8	**04/12/08**	59	IDC	III	Neg	Neg	Neg	2/24
T9	**27/01/09**	52	IDC	II	High	High	Neg	2/11
T10	**23/03/09**	71	IDC	II	High	High	Neg	8/20
T11	**22/07/09**	44	ILC	II	High	-	Neg	1/7
T12	**13/08/09**	61	IDC	III	Neg	-	Neg	2/14
T13	**24/09/09**	46	IDC	III	High	-	Neg	0/10
T14	**09/11/09**	66	IDC/ILC	III	Low	-	Neg	15/16
T15	**10/12/09**	46	IDC	II	High	-	Neg	0/9
T16	**11/02/10**	48	IDC/ILC	II	High	-	Neg	0/14

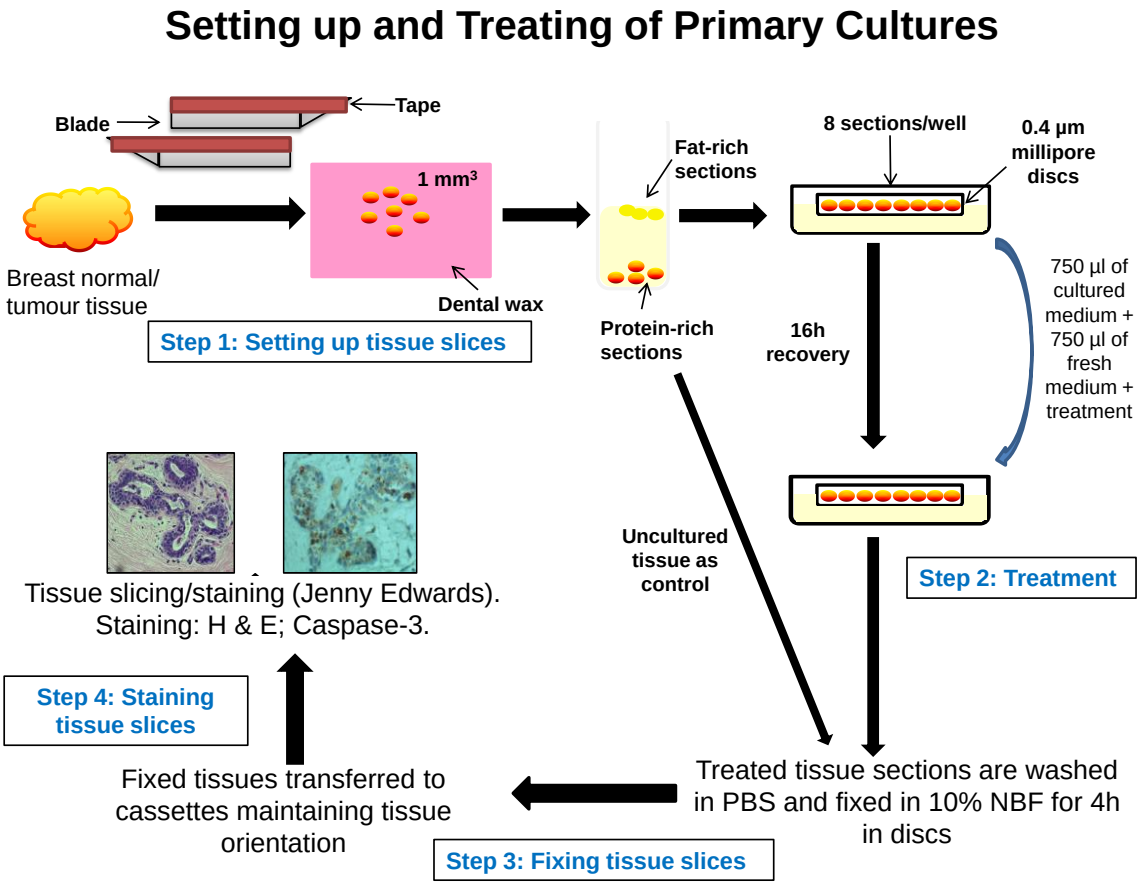


Figure 2.1 Culturing and Treatment of Primary Breast Normal and Tumour Tissue

The breast tissue explants were cultured overnight at 37 °C in 5% CO_2. After overnight recovery, tissue explants were treated with the appropriate death stimuli. For treatment, fresh 6-well plates were used and 750 µl of fresh culture media was added to these wells. The total volume of media in the wells was made up to 1.5 ml with 750 µl of conditioned media from the overnight culture of tissue explants. The media used from the previous cultures contains growth factors released by the tissue which might play an important role in maintaining the viability of the tissue or determining the sensitivity of tissue to death stimuli. The appropriate treatments were then administered and the Millicell inserts containing the tissue explants were transferred to the fresh plates. After 24 h of treatment, tissue explants were washed with PBS and fixed using 10% NBF (Neutralised Buffer Formalin) fixative for 4 h. The tumour tissue explants were then placed in IMS and transferred to the Histopathology Department (MRC Toxicology Unit).

2.4 Immunostaining of primary tissue explants

Primary tissue explants were then embedded in paraffin and sectioned into 4 µm slices (performed by Jenny Edwards; Imaging and Pathology Group; MRC Toxicology Unit). These sections were deparaffinised in xylene for either Haematoxylin and Eosin (H & E) staining or for immunostaining purposes (performed in collaboration with Jenny Edwards). Primary tissue sections were immunostained using the Zymed immunostaining method (Zymed Laboratories, USA; Catalogue No. 85-9043) following heat-based antigen retrieval. The Zymed system consists of a three step labelling method which includes a blocking step (with non-immune serum), primary antibody (Table 2.5) and biotinylated secondary antibody. Streptavidin-HRP (horse-radish peroxidase) conjugate was next added which binds specifically to the biotin of the secondary antibody and the peroxidase catalyses hydrogen peroxide and converts the chromogen (DAB; diaminobenzidine tetrahydrochloride) to a brown deposit which allows the visualisation of the staining. Alternatively, tissue sections were also stained with Hoechst 33342 (Molecular Probes). Briefly, following antigen retrieval, samples were incubated with 500 ng/ml of Hoechst for 20 min at room temperature and then covered with silver foil until imaging.

Table 2.5 Table of primary antibodies for immunohistochemistry

Antibody	Source	Concentration
Cleaved caspase-3	Cell Signaling (#9661)	1/200
Cleaved PARP	Abcam, UK (ES1; ab32064)	1/200-1/500
Rabbit Monoclonal IgG	Epitomics, USA (ISO-1639)	1/200-1/500

2.5 Mammosphere culture

For mammosphere culture of breast cancer cell lines (Figure 2.2), single cell suspensions were made through trypsinisation and by passing the cells through a fine needle at least 10 times. Cells were seeded in mammosphere media (DMEM F12 supplemented with B27 (GIBCO 1164), Invitrogen; 100 pg/ml Epidermal Growth Factor (EGF); R & D Systems, UK) at a density of 2500 cells/ml/well in poly-2-hydroxyethyl methacrylate (poly-HEMA) coated 6-well plates. Cells were treated at the same time as seeding and left for 7 days before colonies equal to or greater than 60 mm in diameter (mammospheres) were counted. All treatments were set up in triplicates and experiments repeated thrice. Mammosphere forming efficiency (% MFE) was calculated as below:

$$\text{Mammosphere forming efficiency} = \frac{\text{Mammosphere number/well}}{\text{Cell number plated/well}} \times 100$$

For isolation of anoikis-resistant cells, T47D cells were allowed to grow in poly-HEMA-coated flasks for 16 h and then collected. The cells were then passed through a viability column (MACS) following the manufacturer's instructions. The subsequently obtained anoikis-resistant cells were then counted and seeded in poly-HEMA-coated plates, treated with TRAIL and the mammospheres were counted after 7 days of treatment.

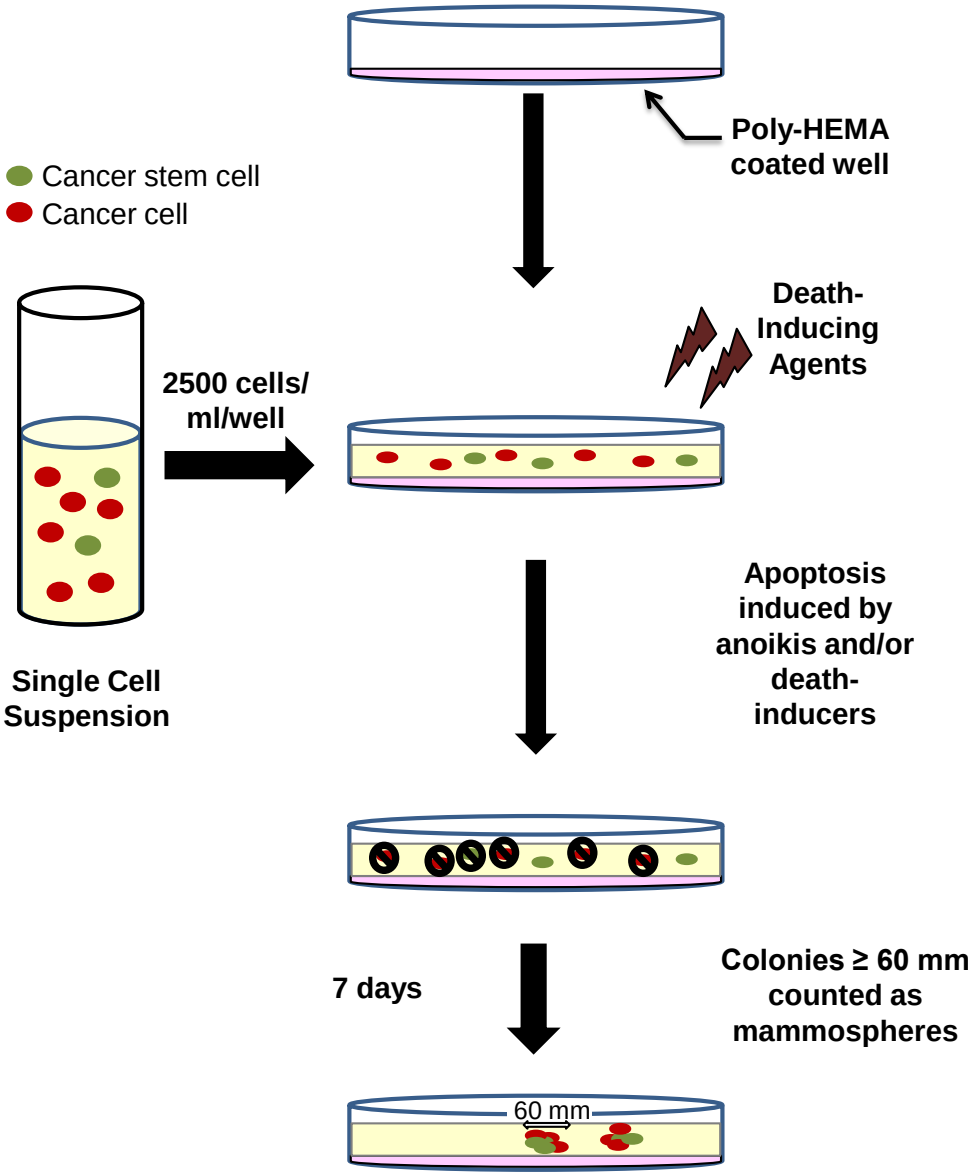


Figure 2.2 Diagrammatic representation of the formation of mammospheres

2.6 Isolation and culture of primary metastatic breast cancer cells

Ascites samples were collected from consented breast cancer patients and the tumour-initiating cells were identified and selected using a Cell Sorter (Table 2.6; sample collection and cell sorting performed by Dr. Ciara O'Brien, Paterson Institute for Cancer Research, UK). The tumour-initiating cell population can be distinguished by its characteristic $ESA^{+}/CD44^{high}/CD24^{low}$ receptor profile (Harrison *et al.,* 2010). The enriched tumour-initiating cell population was seeded in modified mammosphere media (mammosphere media including insulin (Clonetics SingleQuots, Lonza, UK), hydrocortisone (Clonetics SingleQuots, Lonza) and penicillin-streptomycin) on poly-HEMA-coated 6-well plates. Cells were treated at the time of seeding and 6 wells were used per treatment. Colonies equal to or greater than 60 mm in diameter were counted after 10 days and % MFE was calculated as mentioned before.

Table 2.6 Summary of primary metastatic breast tumour samples

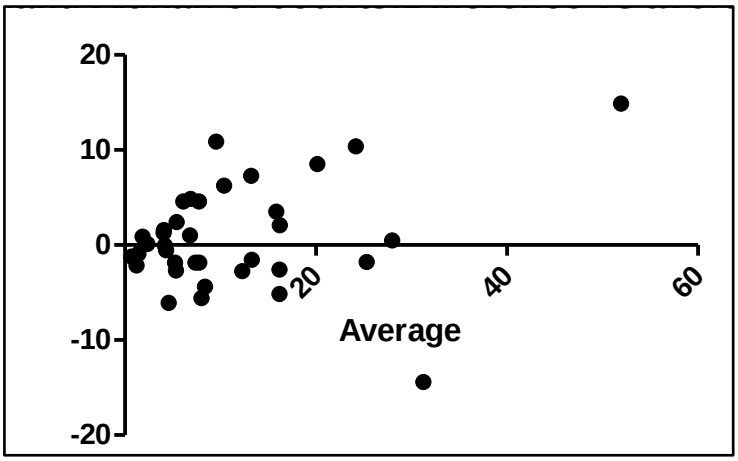
	BB7	**BB15**
Year of Birth	1940	1934
Grade	II	II
Size (of original tumour)	Not determined	37mm
ER	95%	7
PR	25%	5
HER2	Negative	Negative
Lymph nodes	Positive	Positive
Administered Chemotherapy	Adjuvant Fluorouracil/ Epirubicin/ Cyclophosphamide Paclitaxel Epirubicin Adjuvant Anastrozole Exemestane Fulvestrant Letrozole	Adjuvant Fluorouracil/ Epirubicin/ Cyclophosphamide Adjuvant Anastrozole Docataxel (after metastasis)

Apoptosis Assays

2.7 Imaging and counting of primary tumour cells

All immunostained tissue images were taken on an Axiovert S100 immunofluorescence microscope equipped with an Axiocam digital camera and software (Carl Zeiss Microimaging, NY). The Hoechst stained sections were imaged on a confocal microscope LSM 510 (Zeiss) at 630x magnification using UV light.

Adobe Photoshop CS4 Extended was used to manually quantify the live and apoptotic primary tumour cells. For mucinous samples, Hoechst staining was performed and condensed nuclei were counted as a marker of apoptosis. All tumour cells in each tissue block were counted and percentage of apoptotic cells was obtained for each block. The data were then pooled together and statistically analysed using Student's t-test. For all other carcinomas, cleaved PARP was used as a marker of apoptosis. Counts were made of the cells expressing strong cleaved PARP staining; the cells with weak cleaved PARP staining and of the cells that were unstained. Alternatively, images of cleaved PARP staining were analysed using ImmunoRatio, a publicly available web application for quantitative image analysis which runs in concert with ImageJ. The apoptosis analysis by the two different methods was subsequently subjected to correlation tests and gave a significant correlation of 0.812 indicating that there was no statistical difference between the apoptosis assessed using the two methods. Similarly the Bland-Altman plot (see inset) suggested that there was no significant difference between the two assessment methods.



2.8 Phosphatidylserine (PS) exposure

Normal cells maintain a certain degree of lipid asymmetry in their cell membrane. PS is a phospholipid which under normal conditions resides on the inner leaflet of the plasma membrane. However, when cells undergo apoptosis, they lose this cell membrane asymmetry and PS also begins to appear on the outer leaflet of the membrane. The

translocation of PS allows apoptotic cells to be recognised by phagocytes and is a relatively early event in apoptosis. Therefore, the exposure of PS can be used (commonly in combination with the vital dye, propidium iodide (PI)) as a measure of apoptotic cell death. Cells in early apoptosis will expose PS, and thus bind Annexin V-Fluroescein isothiocyanate (FITC), but will exclude PI. However, with the passage of time, the apoptotic cells lose their membrane integrity and also take up PI. Necrotic cells, on the other hand, do not expose PS and thus, only stain with PI. The PS and PI profile of cells can be observed using FACS (Fluorescence Activated Cell Sorter) analysis (reviewed in Fadok *et al.,* 1998; Figure 2.3; Part A). To measure PS externalisation, cells were harvested from each well by trypsinisation. The cell pellet, obtained following centrifugation, was re-suspended in 800 µl of fresh medium; 200 µl was aliquoted into a FACS tube and left to recover at 37 °C in 5% CO_2 for 20 min. After recovery the cells were centrifuged in a bench top centrifuge at 200 g for 3 min at 4 °C. The medium was aspirated and the cells were re-suspended in 1 ml Annexin buffer (10 mM HEPES-NaOH (Fisher Scientific) pH 7.4, 150 mM NaCl, 5 mM KCl (Fisher Scientific), 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) and incubated for 8 min at room temperature with 1 µl Annexin V-FITC labelled antibody (Bender Medsystems, UK). Then, 10 µl PI was added; the tube was briefly vortexed and put on ice. The percentage of PS exposure in the cell population was then measured using a FACSCalibur™ (Becton Dickenson, UK) using excitation/emission wavelengths of 488/525 nm (FITC) and 488/525 nm (PI) respectively and analysed using FACSDiva™ (BD Biosciences).

2.9 Loss of mitochondrial membrane potential (MMP; ΔΨm)

Tetramethylrhodamine, ethyl ester, perchlorate (TMRE) is used to detect the fall in MMP which is characteristic of cells undergoing apoptosis. Healthy cells with intact MMP take up TMRE and retain it within their mitochondria. However, apoptotic cells cannot do this and the subsequent loss in TMRE fluorescence can be used to distinguish the apoptotic and healthy cells (Figure 2.3; Part B).

To measure the loss in MMP, cells were harvested by trypsinisation and re-suspended in 800 µl of cell culture medium. A 250 µl aliquot was left to recover for 20 min at 37 °C in a 5% CO_2 incubator. 750 µl of pre-warmed culture medium was added to the cells followed by 0.5 µl of 100 µM TMRE (Molecular Probes). The tubes were placed back in the 37 C incubator for 10 min. FACS analysis was performed immediately after incubation at excitation/emission wavelengths of 549/574 nm respectively.

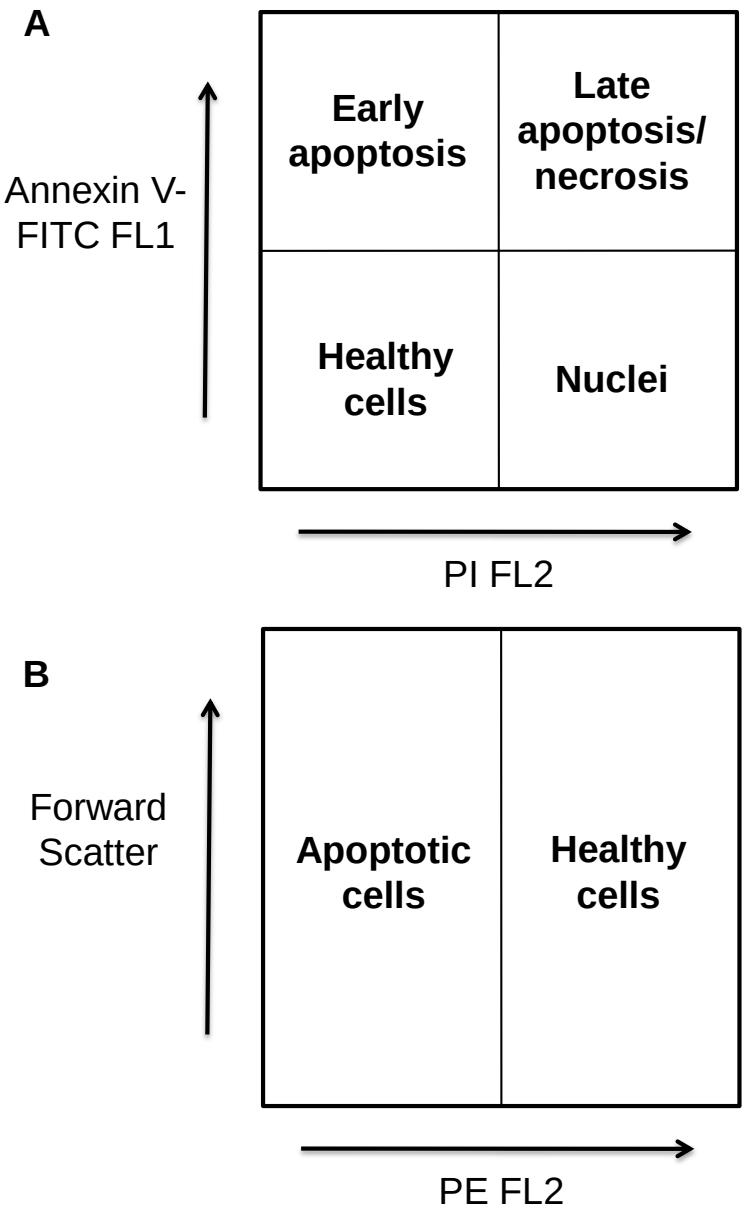


Figure 2.3 Diagrammatic representation of the positions of cells undergoing apoptosis as seen on FACS scan using A) Annexin V-FITC staining or B) TMRE

2.10 SDS-PAGE/Western Blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)/Western blotting was done to observe the cleavage and activation of various caspases in response to drug treatment as previously described (MacFarlane *et al.*, 1997). It was also used to compare the levels of various pro- and anti-apoptotic proteins present in the cells.

For SDS-PAGE/Western blot analysis, the harvested cell samples were re-suspended in chilled PBS and centrifuged again at 200 g for 3 min at room temperature. The PBS was aspirated and the cells were snap frozen on dry ice and stored at -80 °C until further use. Samples were prepared in 2X sample buffer (0.5 mM Tris, pH 6.8, 5% glycerol, 2% SDS and β-mercaptoethanol), sonicated and boiled before loading them on to SDS-PAGE gels.

BioRad Mini Protean II/III sets were used and gels were cast at an appropriate percentage (7% - 15%) according to the molecular weight of the protein of interest. Proteins were detected by immunoblotting after their transfer onto nitrocellulose membranes (Hybond C Extra, Amersham, UK). The blots were blocked with 5% Marvel/TBS-T (Tris Buffer Saline, 0.5% Tween) and incubated with primary antibodies (Table 2.7) for 1 h at room temperature. Following this, the blots were incubated with the appropriate HRP-conjugated secondary antibody (Table 2.8) for 1 h at room temperature. Antibody binding was detected using ECL (Enhanced Chemiluminscent, Amersham) and X-ray film (XOMAR, Kodak, UK).

All antibodies used in this study have been validated previously either by the source company or by experiments done in our and other laboratories (Rasper *et al.,* 1998; Rogakou *et al.,* 1999; Harper *et al.,* 2001; Inoue *et al.,* 2004).

Table 2.7 Primary antibodies used for western blotting

Antibody	Host	Source	Concentration
Caspase-8	Rabbit	MRC Toxicology Unit (Sun *et al.,* 1999)	1/2,000
Caspase-9	Mouse	MBL, USA	1/2000
Caspase-3	Rabbit	Merck, gift from Dr. D Nicholson	1/10,000
Caspase-7	Mouse, Rabbit	Cell Signaling (#9491); +BD Pharmingen, UK	1/1000
PARP	Mouse	Alexis Clone C2-10	1/2,500
Bid	Rabbit	Cell Signaling (#2002)	1/1000
Bcl-2	Mouse	Dako	1/2000
BAK	Rabbit	Upstate, UK	1/1000
BAX	Rabbit	Upstate	1/500
Bcl-XL	Rabbit	BD Pharmingen	1/1000
Mcl-1	Rabbit	Santa Cruz Biotechnology (sc-819)	1/500
XIAP	Mouse	Transduction Labs	1/2000
cIAP1	Goat	R & D Systems	1/500
cIAP2	Goat	R & D Systems	1/500
TRAIL-R1	Rabbit	Prosci Incorporated	1/1000
TRAIL-R2	Rabbit	Cell Signaling	1/1000
FADD	Mouse	Transduction labs	1/250
$FLIP_L$	Rabbit	Merck	1/1000
RIP1	Mouse	BD Pharmingen	1/400
p-Akt	Mouse	Cell Signaling (#4051)	1/1000
p-p38	Rabbit	Cell Signaling (#9211)	1,1000
p-ERK	Rabbit	Cell Signaling (#4377)	1/1000
p65	Rabbit	Cell Signaling (#4764)	1/500
γH2AX	Rabbit	Cell Signaling (#9718)	1/1000
Myc	Mouse	Sigma	1/500
Actin	Mouse	Sigma	1/4000

Table 2.8 Secondary antibodies used for western blotting

Antibody	Host	Source	Concentration
Anti-mouse	Goat	Sigma	1/2,000
Anti-rabbit	Goat	Dako	1/2,000
Anti-Goat	Rabbit	Dako	1/2000

2.11 Measurement of active Bax

Treated cells were fixed by incubating with 0.5 ml Sigma Fixative (10% formalin) per well for 20 min at room temperature. The fixative was removed and cells were washed in PBS and permeabilised using permeabilisation buffer (0.1% saponin, 3% BSA in PBS; 5 min at room temperature). The cells were placed in blocking buffer (3% BSA in PBS) for 1 h and then incubated overnight with the primary antibody (BD Biosciences, mouse monoclonal, clone 3; 1 µg/ml) at 4 ºC on a rocker. Following incubation with the primary antibody, the cells were washed with permeabilisation buffer and incubated with 200 µl secondary antibody [Goat anti-mouse Alexa Flour® 568 (Alexa Flour® 488 for experiments involving doxorubicin treatment), Molecular Probes] for 50 min in the dark at room temperature on a rocker. The cells were washed in blocking buffer followed by three washes in PBS. Hoechst 33342 (Molecular Probes, UK) was diluted to 250 ng/ml in PBS and cells were incubated with 1 µl of the diluted Hoechst in the dark for 15 min at room temperature. The cells were washed three times with PBS and stored at 4 ºC until the immunoflorescence (excitation/emission wavelengths of 578/603 nm (Alexa Flour® 568) or 495/519 (Alexa Flour® 488)) was read and analysed by the Cellomics Array Scan VTI (ThermoScientific, UK).

2.12 DISC precipitation assay

Unstimulated receptor and stimulated DISC precipitation were performed as previously described (Harper *et al.,* 2001). Cells were seeded at 6.25 X 10^6 cells per T-175 flask and two large flasks were set up. For doxorubicin pre-treatments, T47D cells were seeded at 6.5 X 10^6 cells per T-175 flask and two large flasks were used for each condition. For the stimulated DISC precipitation, the cells were first treated with biotinylated variants of TRAIL (b-TR), TRAIL-R1-specific ligand (b-R1L) or TRAIL-R2-specific ligand (b-R2L; 500 ng/ml) for 25 min at 37 ºC. Cells were lysed with 3 ml of DISC lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100 + complete protease inhibitors; 30 min on ice) and the lysate cleared by centrifugation before incubation with 50 µl of streptavidin Dynabeads ® (Invitrogen). For

unstimulated receptor precipitation, biotinylated TRAIL (500 ng/ml) was added post-lysis. After overnight incubation, the receptor or DISC was eluted from the beads through incubation with 75 µl of 2X SDS sample buffer at 99 ºC for 5 min. An aliquot of the eluted sample (20 µl) was then loaded onto a 10% SDS-PAGE gel and then immunoblotted for the various DISC components.

2.13 Electron microscopy

Electron micrographs were used to analyse the type of cell death occurring in T47D cells treated with doxorubicin and TRAIL.

T47D cells post-treatment were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) at 4 °C overnight and post-fixed with 1% osmium tetroxide/1% potassium ferrocyanide for 1 h at room temperature. After fixation, cells were stained *en bloc* with 5% aqueous uranyl acetate overnight at room temperature, dehydrated, and embedded in Taab epoxy resin (Taab Laboratories Equipment Ltd., UK). Electron micrographs of ultrathin sections were recorded using a QICAM 12-bit Mono Fast 1394 digital camera and QCapture-Pro software (MAG, Pleasanton, USA) in a Zeiss 902A electron microscope (performed by David Dinsdale, Imaging and Pathology Group, MRC Toxicology Unit).

General Methods

2.14 Cell surface TRAIL receptor expression

Phycoerythrin (PE)-conjugated TRAIL receptor antibodies (Insight Biotechnology, UK) were used to assess the cell surface expression levels of TRAIL-R1 and TRAIL-R2. For each cell line, 1 X 10^6 cells were re-suspended in 1 ml of fresh medium. The cells were allowed to rest for 15 min at 37 ºC in a 5% CO_2 incubator and then separated into four 250 µl aliquots. The cells were pelleted at 200 g for 3 min at 4 ºC and then incubated in 10% goat sera (diluted in PBS; Table 2.9) for 5 min at room temperature. The conjugated antibody was added (Table 2.9) and incubated with the cells for 1 h on ice in the dark. The cells were washed with PBS and fluorescence (575 nm) was read on the BD FACSCalibur™.

Addition of doxorubicin (Emission wavelength = 559/593 nm) to cells strongly interfered with the PE signal (Emission wavelength = 575 nm). Hence, to study the effect of doxorubicin treatment on cell surface receptor expression, unlabelled primary

antibodies specific for either of the TRAIL death receptors were used. Briefly, untreated and doxorubicin treated cells were harvested by trypsinisation and re-suspended in 1 ml of fresh medium. After a 15 min recovery period at 37 ºC in a 5% CO_2 incubator, 250 µl were aliquoted into four additional FACS tubes. The cells were spun down at 200 g for 3 min at 4 ºC and re-suspended in goat sera. 2 µl of the appropriate unlabelled antibody (eBiosciences) was then added to the FACS tube and the cells incubated for 1 h on ice. After the incubation time, the cells were washed once with PBS and FITC-labelled secondary antibody (Polyclonal goat anti-mouse Immunoglobulin/FITC Goat $F(ab')_2$; Emission wavelength= 518nm; DakoCytomation) was added and the samples were incubated for 1 h on ice in the dark. For every treatment, a sample labelled with the secondary antibody only was included as a control. The samples were then washed with PBS (three times) and fluorescence read at 518 nm on the BD FACSCanto™ II (BD Biosciences).

Table 2.9 Amounts of goat sera and antibody added per antibody type

Antibody type	Amount of goat sera	Amount of antibody
None (-)	50 µl	-
Isotype Control (Anti-IgG1)	40 µl	10 µl
Anti-TRAIL-R1 (CD261, clone DJR-1)	40 µl	10 µl
Anti-TRAIL-R2 (CD262, clone DJR2-4)	45 µl	5 µl

2.15 siRNA transfections

For MCF7 F43 cells, effectene (Qiagen, UK) was used as the transfection agent. The cells were first seeded at 2.0 X 10^5 cells per well of a 6-well plate and left for 4 h to adhere. The RNAi mixture was then prepared according to manufacturer's instructions and the cells were transfected for 48 h prior to any treatment. siRNA oligonucleotides were used at a final concentration of 20 nM per well.

For T47D cells, effectene did not facilitate a significant protein knock down (data not shown) and hence, lipofectamine RNAiMAX (Invitrogen) was the preferred transfection agent. T47D cells were seeded in a similar manner as the MCF7 F43 cells and transfections were carried out according to manufacturer's instructions. Cells and the

transfection mixture were incubated for at least 24 h before treatment. siRNA oligonucleotides were used at a final concentration of 10 nM per well.

Both cell lines were transfected with siRNA oligonucleotides for TRAIL-R1 and TRAIL-R2 (Ren *et al.,* 2004; Table 2.10). In addition a non-targeting oligonucleotide (Ambion, USA) served as negative control in the MCF7 F43 cells whilst in the T47D cell line (which does not express Bcl-2) an oligonucleotide targeting Bcl-2 was used as a negative control. Knockdown of the TRAIL receptors was confirmed by the determination of cell surface receptor expression using FACS analysis. Myc oligonucleotide from Dharmacon SMARTpool was used in T47D cells and knockdown was confirmed by western blotting.

Table 2.10 Primer sequences for siRNA oligonucleotides

Oligonucleotide Name	Primer Sequence (5'→3')
TRAIL-R1 (Ambion)	CACCAAUGCUUCCAACAAUtt
TRAIL-R2 (Ambion)	AUGAGAUAAAGGUGGCUAAtt
Bcl-2 (Ambion)	GGAUCCAGGAUAACGGAGGtt

2.16 Reactive Oxygen Species (ROS) formation

Doxorubicin has been reported to cause formation of reactive oxygen species in target cells; however, the physiological importance of ROS formation is unknown (reviewed in Gewirtz, 1999).

DCF-DA (2', 7'-dichloroflourescein diacetate; Molecular Probes) is oxidised by ROS to form the fluorescent moiety DCF and thus can be used to detect the presence of ROS in the doxorubicin treated T47D cells. T47D cells were seeded at 4.0 X 10^5 cells per well in a 6-well plate and treated with doxorubicin for the indicated time. Treated cells were harvested by trypsinisation and re-suspended in 1 ml of PBS. Samples for each treatment were split into two aliquots (500 µL in each FACS tube) and 10 µM DCF-DA was added to one aliquot. The other aliquot was used as a negative control to check for any interference in DCF fluorescence from doxorubicin. The tubes were then covered with foil and placed in an incubator at 37 ºC for 30 min. The samples were later washed with PBS (three times) and fluorescence was read at excitation and emission wavelengths of 492/495 nm and 517/527 nm respectively on the FACSCanto™. Doxorubicin did not interfere with DCF fluorescence and hence there was no requirement of fluorescence normalisation.

Hydrogen peroxide was used as a positive control for this experiment and 10 µM H_2O_2 was added to the cells following trypisinisation and just before addition of DCF-DA.

2.17 Clonogenic assay

A clonogenic assay is used to study the effect of drugs on the growth potential of tumour cells. Briefly, T47D cells were seeded at 4.75 X 10^4 cells per well of a 6-well plate and left to adhere overnight. The following day, the cells were treated with TRAIL for 24 h, following which the media was removed and cells were washed once with PBS at room temperature. Fresh media was then added to the cells and they were left to incubate for 7 days at 37 ºC in 5% CO_2. Following incubation, the cells were washed with PBS once and then stained with 0.75% crystal violet, 50% ethanol, 0.25% NaCl and 1.57% formaldehyde. Excess stain was removed by careful washing with PBS and visually analysed for detection of cell proliferation.

2.18 Detection of p65 translocation

TRAIL has been known to activate the pro-survival NFκB pathway in TRAIL-apoptosis resistant tumour cells. The activation of this pathways results in the translocation of the p65 subunit of NFκB from the cellular cytoplasm to the nucleus where it binds to DNA and acts as a transcription factor. Immunocytochemistry (ICC) was used for the detection of NFκB activation.

For ICC, T47D cells were seeded at 1.0 X 10^5 cells per well on coverslips in a 12-well plate and left to recover overnight. Next day the cells were treated with TRAIL (1000 ng/ml) or TNF (positive control; 200 ng/ml) and following treatment, the media was removed and cells were fixed using 0.5 ml Sigma Fixative for 20 min. The fixative was removed and cells were washed in PBS and permeabilised for 5 min in permeabilisation buffer. The cells were placed in blocking buffer (3% BSA in PBS) for 1 h and then incubated for 1 h with the primary antibody (NFκB p65 (C22B4), Cell Signaling Technology, #4764; 1:100 dilution) at room temperature. Following incubation with the primary antibody, the cells were washed with blocking buffer and incubated with 200 µl secondary antibody (Goat anti-rabbit Alexa Flour® 488, Molecular Probes, diluted 1:100 with 3% BSA) for 50 min in the dark at room temperature. The cells were washed in blocking buffer and then in PBS. Hoechst 33342 (Molecular Probes) was diluted to 250 ng/ml in PBS and cells were incubated with 1 µl of the diluted Hoechst in the dark for 15 min at room temperature. After staining the cells, the

coverslips were mounted on to slides and fluorescence was analysed using a confocal microscope (Zeiss).

2.19 Determination of protein concentration

Protein concentration of various samples was determined by performing the Bradford assay (Bradford, 1976).

To perform the Bradford assay, a standard curve for Bovine Serum Albumin (BSA) was first obtained. This was carried out by diluting the Bradford protein assay reagent (BioRad) 1:5 in ultrapure water. The diluted reagent was then aliquoted (1 ml) into cuvettes and triplicates were set up to generate a BSA concentration curve (0-8 µg/ml). To analyse the samples, 10 µl was added to 990 µl of diluted Bradford reagent, the absorbance measured on a spectrophotometer (Perkin Elmer, USA) at 595 nm and the concentration calculated using the BSA standard curve.

2.20 Statistical analysis

Statistical analysis was performed using the Students unpaired t-test in Microsoft Excel 2007 ($p \leq 0.05$ *, $p \leq 0.01$ **, $p \leq 0.001$ ***). Data are expressed as the mean of at least three independent determinations ± standard error of the mean (SEM).

Chapter 3
Profile of TRAIL-Receptor Signalling to Apoptosis in Breast Tumour Cell Lines

3.1 Introduction

Cell lines are the most routinely available tools for the study of signalling pathways and mechanisms in an intact cellular system. A plethora of different cancer cell lines have been derived from primary breast tumours and breast-related pleural efflusions. These cell lines have been studied worldwide as a representation of the potential events occurring in breast cancer *in vivo*. In this study, we have used a panel of breast cancer cell lines to explore the efficiency of the death-inducing agent, TRAIL, in the presence or absence of chemotherapeutic drugs. The primary advantage of breast cancer cell lines is that they provide a robust, reproducible experimental setup, and hence we have used the established cell lines to optimise treatment conditions which could be subsequently applied to primary breast tumour tissue. Experiments done with cell lines could also help provide proof of principle for the presence of a dominant functional TRAIL death receptor; however, it is important to note that primary breast tumour tissue could behave quite differently to established cell lines in culture. In this regard, the failure to take into account the effect of the tumour microenvironment on drug sensitivity of tumour cells is a significant drawback of using cell lines.

We have used six different breast cancer cell lines during the course of this project (Table 1.3). The cell lines differ in their p53, oestrogen receptor (ER) status and pathology and hence are useful tools to study the potential effect of these prognostic markers on TRAIL sensitivity. None of the breast cancer cell lines had HER2 amplification and therefore, correlation between TRAIL sensitivity and HER2 could not be observed.

3.2 Classification of breast cancer cell lines based on TRAIL sensitivity

The sensitivity of the six breast cancer cell lines to TRAIL-induced apoptosis was assessed alongside the potent apoptosis inducer and protein kinase inhibitor, Staurosporine (STS; 1 µM, 6 h) as a positive control. Cell lines were classified according to their ER status with a view to see if the presence of these receptors had any effect on TRAIL sensitivity (Appendix 1; performed in collaboration with Drs. Davina Twiddy and Roshna Mistry). Signalling through ER is known to promote cell proliferation (reviewed in Moggs and Orphanides, 2001) and hence the presence of ER may interfere with the sensitivity of breast tumour cells to TRAIL-induced apoptosis.

Table 3.1 TRAIL sensitivities of breast cancer cell lines is independent of pathology, p53 and ER status

Cell line	ER status	HER2 status	p53 status	Pathology	TRAIL sensitivity
MCF7 F43	+	-	Wild type	Invasive ductal carcinoma	**+++**
ZR-75.1	+	-	Wild type	Invasive ductal carcinoma	**+**
T47D	+	-	Mutated	Invasive ductal carcinoma	**-**
MDA-MB-231	-	-	Mutated	Adenocarcinoma	**+++**
MDA-MB-468	-	-	Mutated	Adenocarcinoma	**++**
BT20	-	-	Mutated	Invasive ductal carcinoma	**++**

Key: + to +++ represents increasing TRAIL sensitivity; - represents resistance to TRAIL

The breast cancer cell lines, MCF7 F43 (Appendix 1; Part A) and MDA-MB-231 (Appendix 1; Part D) were extremely sensitive to TRAIL as assessed by efficient cleavage of both initiator (caspase-8) and effector caspases (caspase-7 and -3 respectively), as well as the caspase substrate PARP. In contrast, MDA-MB-468 (Appendix 1; Part E), BT20 (Appendix 1; Part F) and ZR-75.1 (Appendix 1; Part B) cells were only semi-sensitive to TRAIL with low levels of caspase-8 activation and PARP cleavage. The cell line, T47D (Appendix 1; Part C) was the only cell line that was completely resistant to TRAIL-induced apoptosis. From this initial analysis, it is important to note that TRAIL sensitivity in breast cancer cell lines is independent of their ER status and pathology (Table 3.1). It is evident that the presence of wild-type or mutated variants of p53 also does not affect tumour cell sensitivity to TRAIL-induced apoptosis (Table 3.1).

The sensitivity of any cell to TRAIL is most likely in part dependent on the expression of functional TRAIL death receptors on the cell surface. Hence, we studied if there was any correlation between the sensitivities of the different breast cancer cell lines to TRAIL and expression of the death receptors, TRAIL-R1 and TRAIL-R2 on the cell

surface (Appendix 2; performed in collaboration with Drs. Davina Twiddy and Roshna Mistry).

The surface expression of TRAIL-R2 was highest in the TRAIL-resistant T47D cell line followed by ZR-75.1 cells and then the TRAIL-sensitive, MCF7 F43 and MDA-MB-231 cells. ZR-75.1 cells also had higher surface expression of TRAIL-R1 than MCF7 F43 and MDA-MB-231 cell lines. The TRAIL semi-sensitive MDA-MB-468 and BT20 cell lines appeared to express low levels of both TRAIL-R1 and TRAIL-R2 on the cell surface. However, there appeared to be no strong correlation between the cell surface expression of the TRAIL death receptors and the sensitivity of the cell lines to TRAIL-induced apoptosis.

We further checked whether there were any significant trends in terms of the relative expression of pro- and anti-apoptotic proteins in the cell lines which could in turn determine their response to TRAIL (Appendix 3; performed in collaboration with Drs. Davina Twiddy and Roshna Mistry). For this, we tested the major initiator and effector caspases, members of the Bcl-2 family and the anti-apoptotic protein XIAP. Though the expression of these proteins across the panel of breast cancer cell lines varied, we did not observe any significant correlation between TRAIL sensitivity and expression of any one key protein.

3.3 R1L, but not R2L, induces apoptosis in MCF7 F43 cells

MCF7 F43 (Appendix 1; Part A) and MDA-MB-231 cells (Appendix 1; Part D) were found to be the most sensitive to TRAIL-induced apoptosis amongst all the breast cancer cell lines tested. We, therefore, aimed to study the profile of TRAIL-receptor signalling to apoptosis in these two cell lines. MCF7 F43 cells do not express functional caspase-3 protein (Jänicke *et al.,* 1998 & Appendix 3); hence in these cells a decrease in mitochondrial membrane potential (MMP), processing of caspase-8, caspase-7 and cleavage of PARP were used as measures of apoptosis. The MCF7 F43 cells were treated with 500 ng/ml of wild-type TRAIL (TRAIL), TRAIL-R1-specific ligand (R1L) or TRAIL-R2-specific ligand (R2L) for 3 h and untreated cells were used as control.

Consistent with earlier data (Appendix 1; Part A), MCF7 F43 cells were highly sensitive to TRAIL with ~50% cells undergoing apoptosis (Figure 3.1; Part A; Lane 2). R1L induced significantly greater loss in MMP, cleavage of effector caspase-7 and PARP (Figure 3.1; Parts A & B; Lane 3) in MCF7 F43 cells as compared with R2L. The lack

of apoptosis induction by R2L was represented by no loss in MMP and no processing of caspase-7 and/or PARP (Figure 3.1; Parts A & B; Lane 4).

Although R1L was more potent than R2L at inducing apoptosis, it was not as efficient as TRAIL in inducing cell death. Hence, we decided to use varying concentrations of TRAIL and R1L at a later time point in order to see if R1L could induce a similar level of apoptosis as TRAIL. Since some of the TRAIL being added may be sequestered by TRAIL-R2/R3/R4, we speculated that at lower concentrations, R1L may be more potent than TRAIL. Surprisingly however, even at concentrations as low as 100 ng/ml, R1L was not more potent than TRAIL (Figure 3.1; Part C; Red arrows).

DISC formation is one of the key preliminary steps in apoptosis induction and therefore, it can be used to identify TRAIL binding to which death receptor was inducing apoptosis. To confirm that TRAIL-R1 was the most potent TRAIL death signalling receptor in MCF7 F43 cells, the TRAIL DISC was immunoprecipitated using biotinylated versions of receptor-specific TRAIL. An unstimulated ligand-receptor precipitation was used as a negative control (Figure 3.2; Part A; Lane 1), while the DISC stimulated by biotinylated TRAIL (b-TR) was used as a positive control (Figure 3.2; Part A; Lane 2). For effective signalling of apoptosis, a fully functional stimulated DISC should contain the signalling TRAIL death receptor(s), FADD and caspase-8. The addition of b-TR to pre-lysed MCF7 F43 cells resulted in a ligand-receptor complex which failed to recruit any FADD or capsase-8 (Figure 3.2; Part A; Lane 1).

Importantly, addition of b-TR to intact MCF7 F43 cells caused the recruitment of both FADD and caspase-8 to complex with the ligand and both TRAIL death receptors (Figure 3.2; Part A; Lane 2). Thus, TRAIL binds to both TRAIL-R1 and TRAIL-R2 leading to the formation of an effective TRAIL DISC by engaging FADD and caspase-8. b-R1L appeared to recruit both TRAIL-R1 and TRAIL-R2 to the DISC (Figure 3.2; Part B; Lane 2), whilst b-R2L recruited only TRAIL-R2 (Figure 3.2; Part B; Lane 5). The recruitment of both TRAIL death receptors with b-R1L indicates the formation of a TRAIL-R1/R2 heterocomplex after TRAIL-R1 recruitment. Importantly, the amount of FADD and caspase-8 recruited by b-R1L to the receptor complex was significantly more than that recruited by b-R2L (Figure 3.2; Part B; Lanes 2 & 5). Subsequently, b-R1L also induced more processing of procaspase-8 to its p43 and p41 fragments than b-R2L. This confirms that in MCF7 F43 cells, binding of R1L signals to apoptosis *via* formation of the DISC, while binding of R2L leads to only very low levels of DISC formation consistent with the relatively lower levels of apoptosis induction seen with R2L.

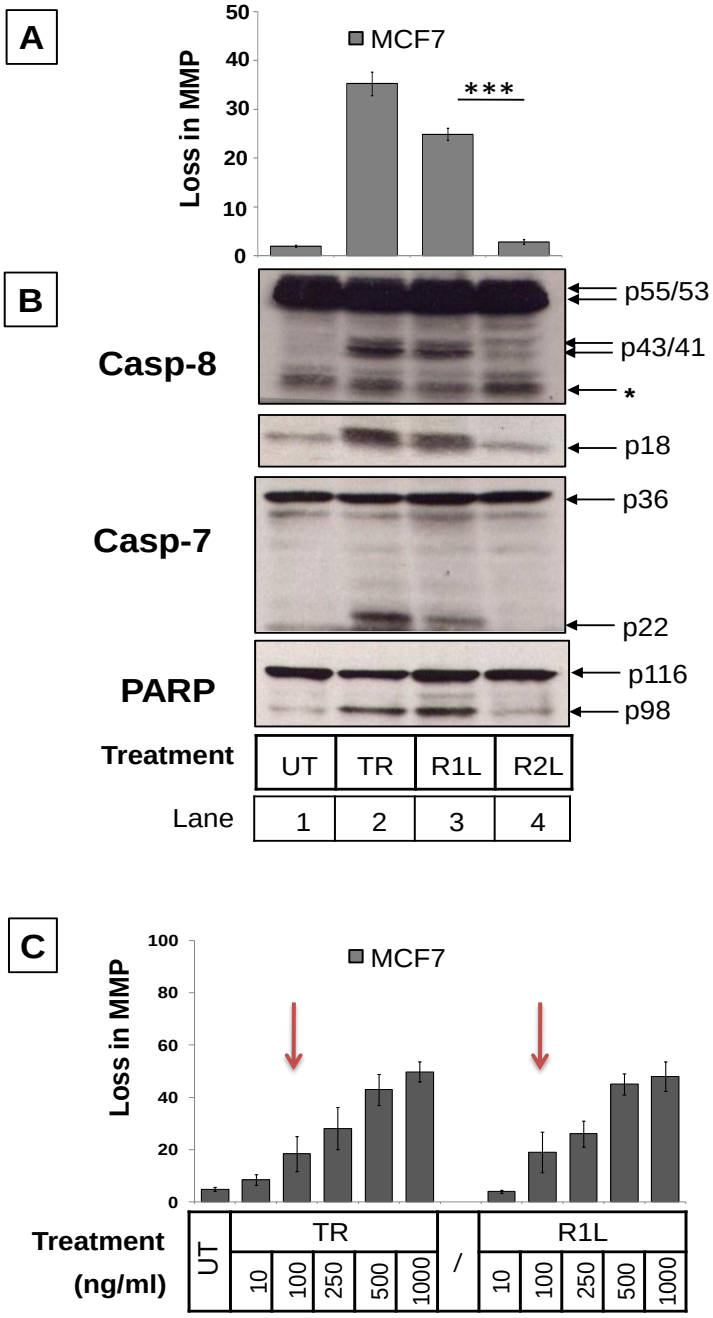


Figure 3.1 TRAIL induces apoptosis in MCF7 F43 cells *via* ligation of TRAIL-R1

MCF7 F43 cells were seeded at 4.0 X 10^5 cells/well and treated with 500 ng/ml of TRAIL (TR)/R1L/R2L for 3 h, following which cells were harvested and one third of the sample was used for TMRE analysis ((A); data represented as mean ± SEM (n=3)) and remaining sample was used for SDS-PAGE/Western blotting ((B); data shown is representative of two independently performed experiments). Alternatively, MCF7 F43 cells were treated with indicated concentrations of TRAIL or R1L for 6 h and TMRE analysis was performed ((C); data represented as mean ± SEM (n=4)). *** indicates significant difference ($P<0.001$). * indicates non-specific band and UT indicates untreated cells.

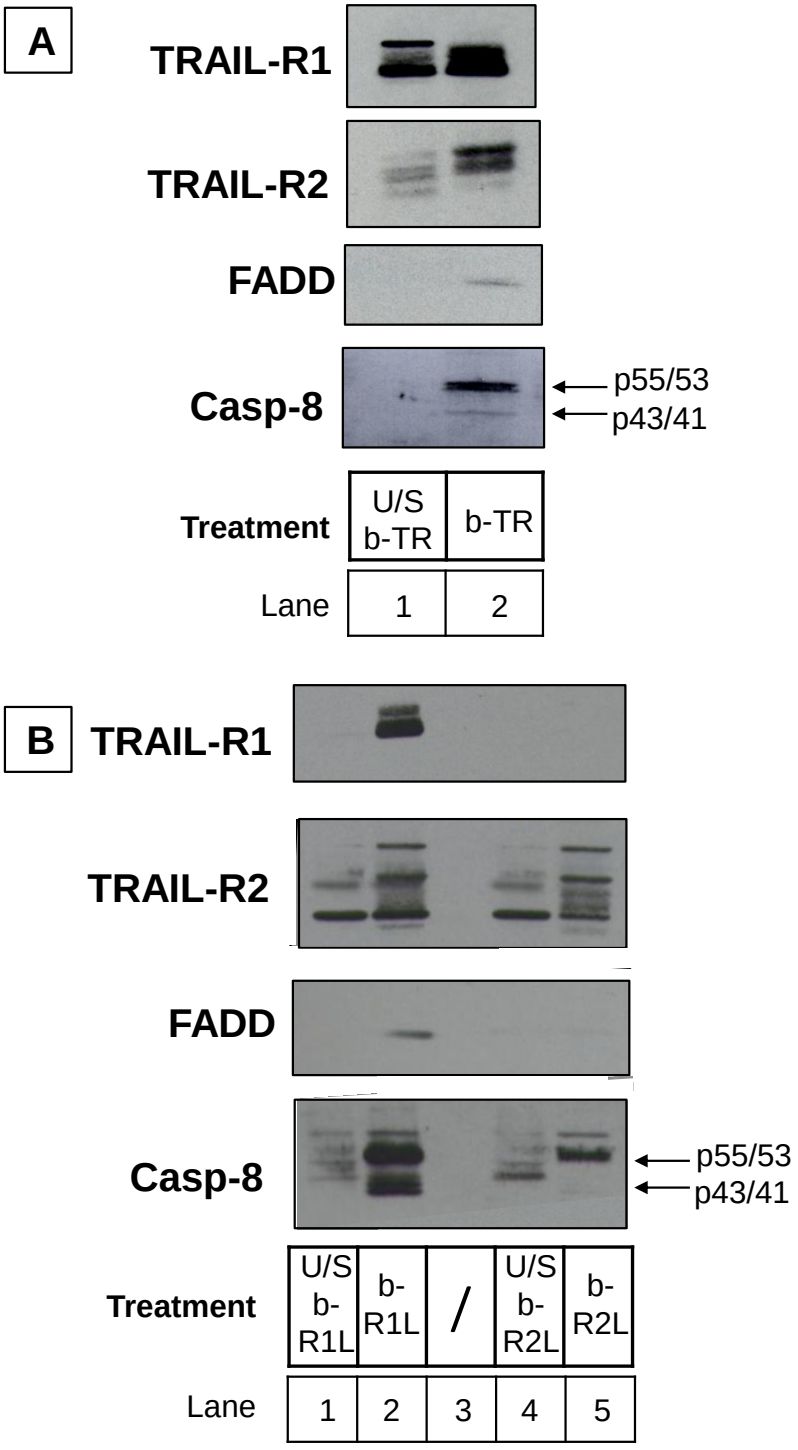


Figure 3.2 TRAIL and R1L, but not R2L, can recruit FADD and caspase8 to form an active DISC complex in MCF7 F43 cells

MCF7 F43 cells were used to analyse of TRAIL DISC formation using biotinylated TRAIL (b-TR; (A)), TRAIL-R1 (b-R1L; (B)) and TRAIL-R2 (b-R2L; (B))-specific ligands. TRAIL DISC was immunoprecipitated as mentioned in Materials and Methods and the samples were run using SDS-PAGE for western blotting. Data shown here is representative of two independently performed experiments.

3.4 In MDA-MB-231 cells, TRAIL-R1 is the predominant TRAIL receptor signalling to TRAIL-induced apoptosis

Consistent with MCF7 F43 cells, MDA-MB-231 cells also showed high sensitivity to TRAIL (Figure 3.3; Part A; Lane 2) as assessed by phosphatidylserine (PS) exposure. Again, in MDA-MB-231 cells, R2L (Figure 3.3; Part A; Lane 4) failed to induce any apoptosis, while R1L caused a massive increase in apoptotic cell death, efficient cleavage of the initiator caspase-8, the effector caspase-3 and PARP (Figure 3.3; Parts A & B; Lane 3). However, in contrast to MCF7 F43 cells, R2L did induce slight processing of caspase-8 and caspase-3, which appeared to be blocked prior to full caspase-3 activation. This blockage of caspase-3 cleavage might be due to the low levels of active caspase-8 formed which are then insufficient to induce efficient caspase-3 cleavage.

DISC immunoprecipitation experiments were then performed to confirm whether in MDA-MB-231 cells, R1L forms a more active DISC than R2L. Similar to MCF7 F43 cells, b-TR formed an active DISC in MDA-MB-231 cells *via* efficient recruitment of TRAIL-R1, TRAIL-R2, FADD and caspase-8 (Figure 3.4; Part A; Lane 2). Similarly, b-R1L appeared to recruit both TRAIL-R1 and TRAIL-R2 to the DISC (Figure 3.4; Part B; Lane 2), while b-R2L exclusively recruited TRAIL-R2 (Figure 3.4; Part B; Lane 5). The presence of both death receptors in the TRAIL-R1 DISC may be indicative of formation of a heterocomplex between both TRAIL death receptors upon ligand stimulation. Nonetheless, in MDA-MB-231 cells, it was b-R1L which formed an active TRAIL DISC as evidenced by the cleavage and activation of caspase-8 (Figure 3.4; Part B; Lane 2). Thus, in MDA-MB-231 cells as in the MCF7 F43 cells, TRAIL-R1 is the functional death receptor capable of transmitting the apoptotic signal *via* formation of an active DISC.

Taken together the above data show that in the TRAIL-sensitive cell lines, MCF7 F43 and MDA-MB-231, R1L induces significantly more apoptosis than R2L, thereby indicating that the main functional TRAIL death receptor in both these cell lines is TRAIL-R1.

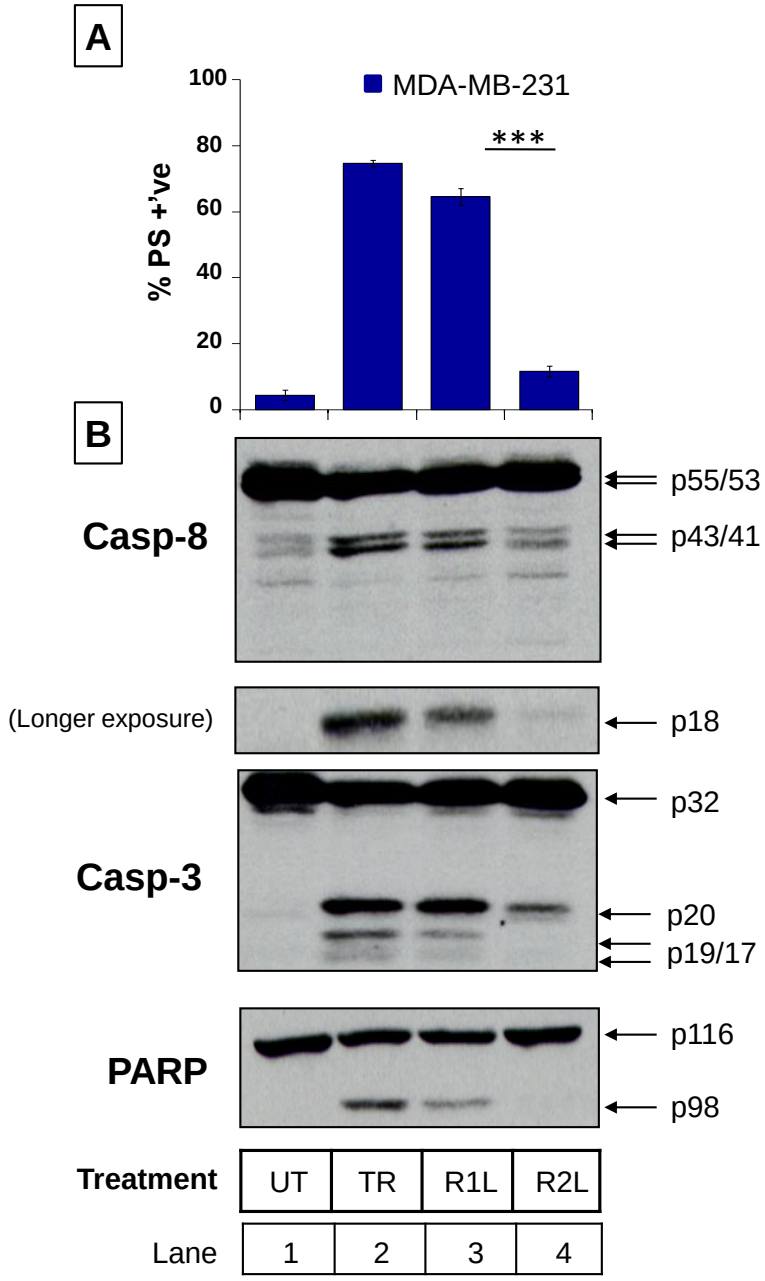


Figure 3.3 R1L induces significantly more apoptosis in MDA-MB-231 cells than R2L

MDA-MB-231 cells were seeded at 4.0 X 10^5 cells/well and treated with 500 ng/ml of TRAIL (TR)/R1L/R2L for 3 h and then harvested for Annexin V-FITC ((A); data represented as mean ± SEM (n=3)) staining as mentioned in Materials and Methods and SDS-PAGE/Western blotting ((B); data shown is representative of two independently performed experiments). *** indicates significant difference (P<0.001).

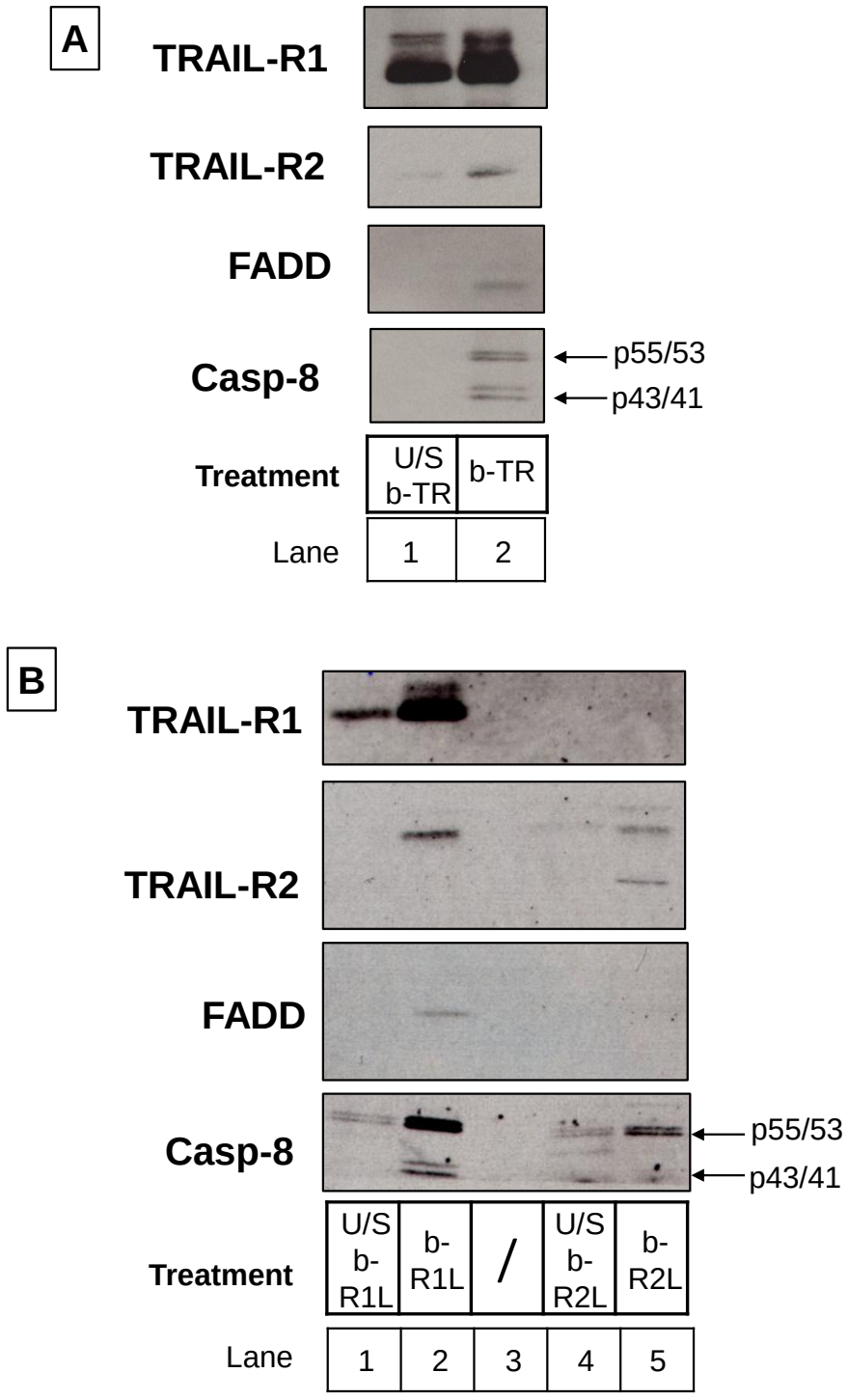


Figure 3.4 In the MDA-MB-231 cells, b-R1L forms an active TRAIL DISC

MDA-MB-231 cells were seeded and the DISC was immunoprecipitated as mentioned in Materials and Methods using either b-TRAIL (b-TR; (A)), b-R1L (B) or b-R2L (B) and samples were run using SDS-PAGE to determine presence of TRAIL receptors, FADD and caspase-8. Data shown here is representative of two independently performed experiments.

3.5 TRAIL-R1-specific ligand induces more apoptosis in MDA-MB-468 cells than TRAIL-R2-specific ligand

MDA-MB-468, BT20 and ZR-75.1 cell lines responded to treatment with TRAIL (Appendix 1; Parts E, F & B), however, the extent of apoptosis induced in these cell lines was much lower than that seen in MCF7 F43 or MDA-MB-231 cells.

To obtain a measurable level of apoptosis, MDA-MB-468 cells were treated with TRAIL, R1L or R2L at 500 ng/ml for 6 h and exposure of PS was used as a marker of apoptosis. TRAIL induced ~40% apoptosis in MDA-MB-468 cells (Figure 3.5; Part A; Lane 2) and lead to the activation of caspase-8, caspase-3 and cleavage of PARP (Figure 3.5; Part B; Lane 2).

There was significantly more apoptosis induced with R1L (Figure 3.5; Part A; Lane 3) in the MDA-MB-468 cells than with R2L (Figure 3.5; Part A; Lane 4). Similarly, R1L caused much more activation of caspase-8 and caspase-3 than R2L (Figure 3.5; Part B; Lanes 3 & 4). Thus, once again, in the MDA-MB-468 cell line, TRAIL-R1 is the major functional death-inducing TRAIL receptor.

3.6 In the BT20 cells, R1L induces more apoptosis than R2L

The BT20 cell line is the only cell line used in this study that has been derived from primary breast tumour tissue and not from a pleural efflusion. Therefore, BT20 cells might be, in fact, more representative of primary breast tumour tissue than the other cell lines we have tested.

Due to the high resistance shown by the BT20 cell line in initial experiments (Appendix 1; Part B), TRAIL-induced apoptosis in these cells was measured either by determining loss in MMP after TRAIL addition for 3 h or PS exposure 6 h after TRAIL. Following 3 h of TRAIL treatment, ~30% of the BT20 cells showed a fall in MMP (Figure 3.6; Part A; Lane 2). Similarly, R1L induced a loss in MMP (Figure 3.6; Part A; Lane 3), however R2L failed to induce a significant level of apoptosis (Figure 3.6; Part A; Lane 4). Consistently, after 6 h of treatment, TRAIL and R1L induced apoptosis *via* activation of the initiator caspase-8 (Figure 3.6; Part B; Lanes 2 & 3). Importantly, even at this later time point, R2L did not induce activation of caspase-8 and hence could not induce apoptosis in BT20 cells (Figure 3.6; Part B; Lane 4). Thus, in the BT20 cell line, TRAIL-R1 is once again, the more potent TRAIL death receptor signalling to apoptosis.

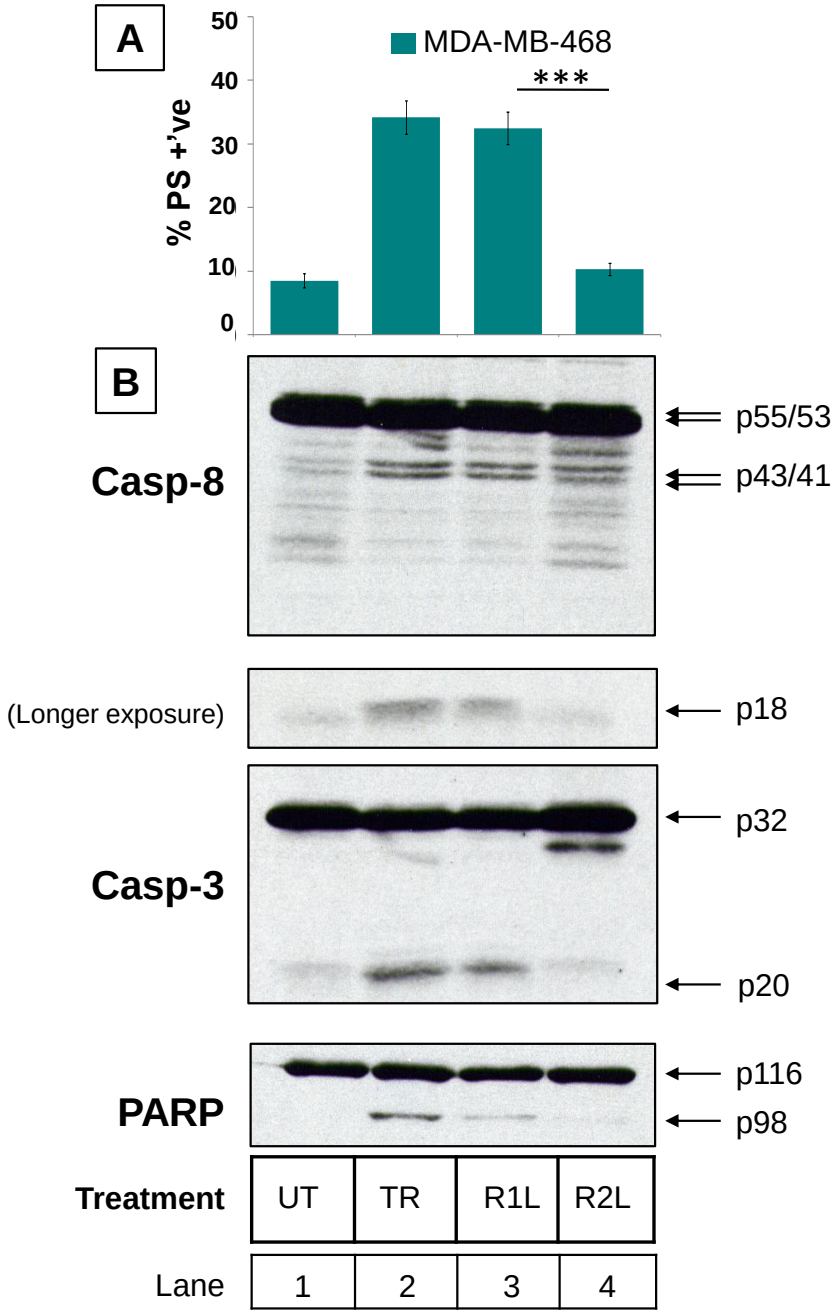


Figure 3.5 TRAIL binding to TRAIL-R1 induced apoptosis in MDA-MB-468 cells
MDA-MB-468 cells were seeded at 4.0 X 10^5 cells/well and treated with 500 ng/ml of TRAIL/R1L/R2L for 6 h, following which cells were harvested and one third of the sample was used for Annexin V-FITC analysis ((A); data represented as mean ± SEM (n=3)) and remaining sample was used for SDS-PAGE/Western blotting ((B); data shown is representative of two independently performed experiments). *** indicates significant difference (P<0.001).

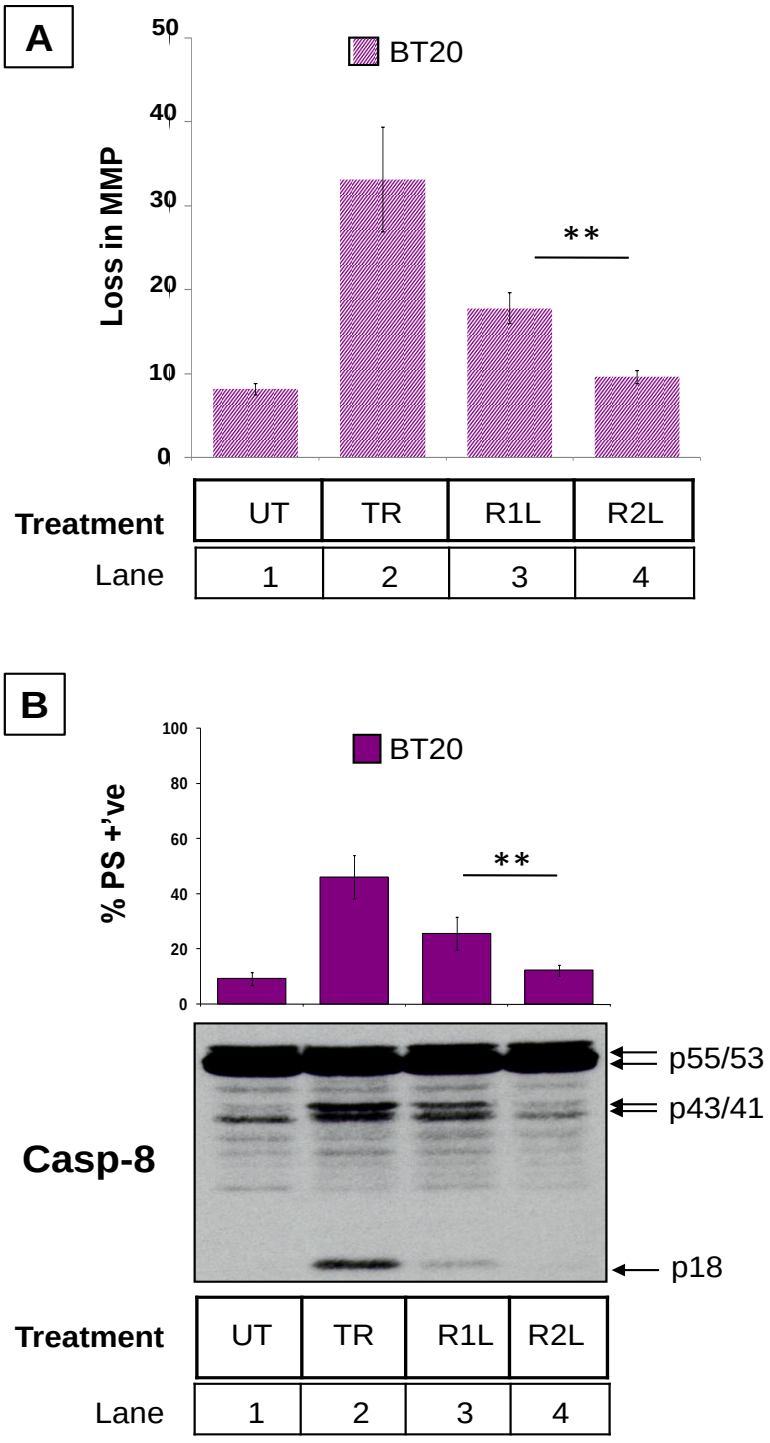


Figure 3.6 TRAIL-R1 is the major death-inducing receptor in BT20 cells

BT20 cells were seeded and treated with 500 ng/ml for either 3 h, following which cells were harvested and used for TMRE analysis ((A); data shown represented as mean ± SEM (n=3)) or for 6 h following which cells were analysed by Annexin V-FITC labelling ((B); data represented as mean ± SEM (n=3)) and SDS-PAGE/Western blotting (data shown is representative of two independently performed experiments). ** indicates significant difference ($P<0.01$).

Preliminary experiments indicated that the ZR-75.1 cell line was the least sensitive of the TRAIL-responsive breast cancer cell lines. TRAIL-induced apoptosis was not seen when these cells were treated with 500 ng/ml TRAIL for 6 h (data not shown), hence the concentration of TRAIL had to be increased to 1000 ng/ml. Importantly, even in the ZR-75.1 cell line, at this higher concentration, TRAIL and R1L induced apoptosis after 6 h of treatment, while again R2L did not signal to apoptosis (data not shown).

3.7 IAPs regulate TRAIL-induced apoptosis in TRAIL semi-sensitive cell line

Smac mimetic compounds are being implicated as promising anti-tumour therapeutic agents (Oost *et al.,* 2004). Similar to endogenous Smac, the mimetics bind to and inhibit the activity of XIAP and can also lead to degradation of cIAP1 and cIAP2 (Yang and Du, 2004). Inhibition of these anti-apoptotic proteins can render the target tumour cells susceptible to TRAIL-induced apoptosis (Bockbrader *et al.,* 2005; Fandy *et al.,* 2008; Vogler *et al.,* 2008; Vogler *et al.,* 2009). We were therefore interested to examine whether the inhibition of the IAPs would sensitise the TRAIL semi-sensitive cell line, MDA-MB-468 to a sub-lethal concentration of TRAIL. For this purpose, we used the monovalent Smac-mimetic compound, LBW242 which has already been shown to inhibit the activity of XIAP and cIAP1 (Chauhan *et al.,* 2007; Gaither *et al.,* 2007). The activity of LBW242 in MDA-MB-468 cells was confirmed by examining LBW242-induced degradation of cIAP1 following treatment (Figure 3.7; Part B). Consistent with earlier reports, Smac mimetic treatment for 22 h did not cause degradation of XIAP or cIAP2 (Chauhan *et al.,* 2007; Darding *et al.,* 2011). Instead there was a slight up-regulation of cIAP2 expression, which may be a result of NFκB activation triggered by cIAP1 degradation (Darding *et al.,* 2011). Exposure to LBW242 alone did induce a slight amount of apoptosis in MDA-MB-468 cells (Figure 3.8; Part A; Lanes 2-4). This apoptosis, in most instances, can be attributed to increased autocrine TNF signalling caused by down-regulation of IAPs (Peterson *et al.,* 2007; Varfolomeev *et al.,* 2007; Vince *et al.,* 2007). Importantly, at a concentration of 50 ng/ml, TRAIL did not induce apoptosis in MDA-MB-468 cells (Figure 3.7; Part A; Lane 5). However, when MDA-MB-468 cells were pre-treated with LBW242 for 16 h, they became susceptible to this sub-toxic concentration of TRAIL. LBW242, particularly when used at concentrations either 10 or 20 µM, caused significant sensitisation to TRAIL-induced apoptosis as evidenced by both a loss in MMP and an increase in PS exposure (Figure 3.7; Part A; Lanes 7 & 8). The apoptosis observed was also accompanied by a concomitant activation of caspases and cleavage of PARP (Figure 3.7; Part B).

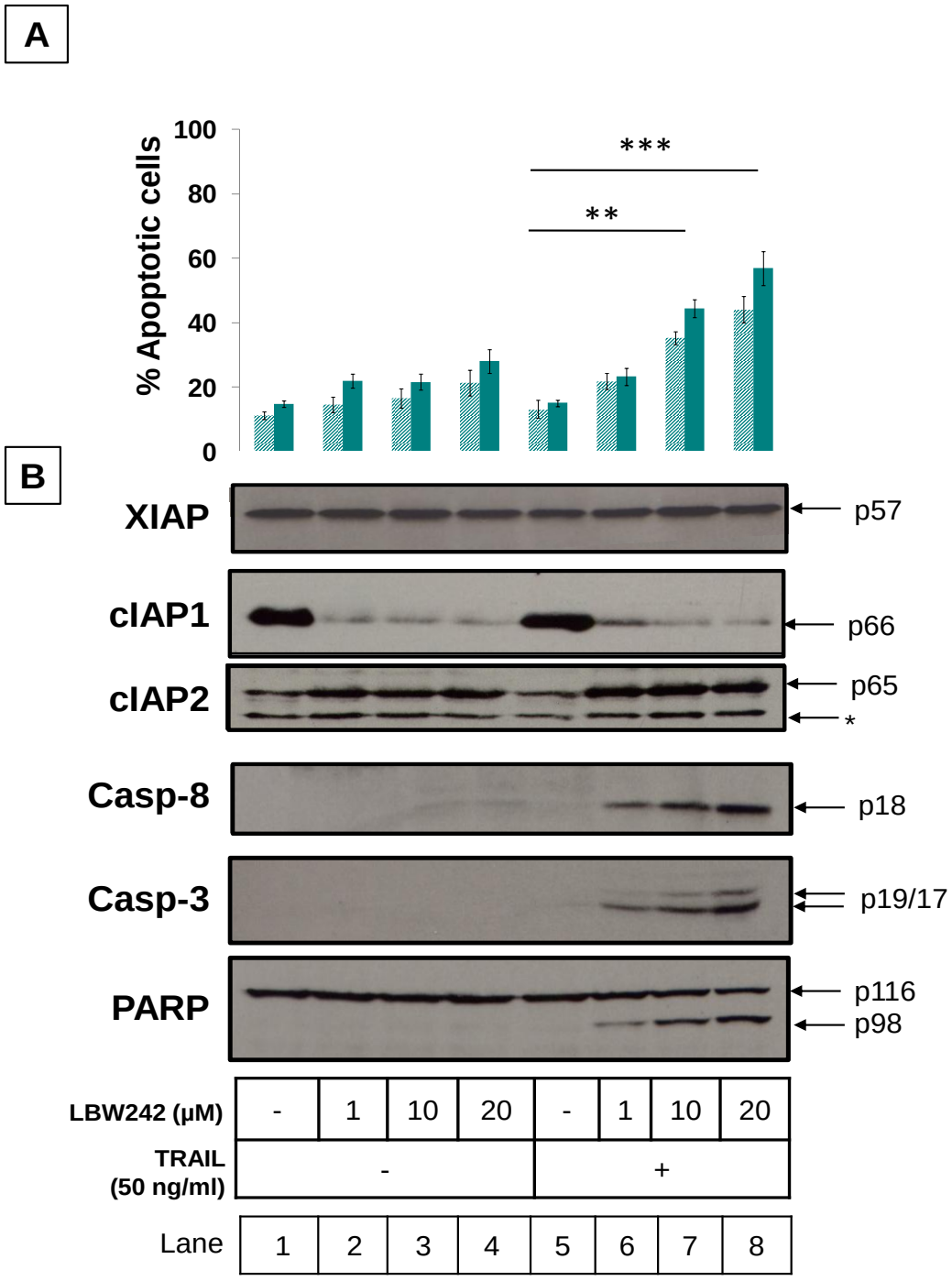


Figure 3.7 LBW242 sensitises MDA-MB-468 cells to sub-toxic a concentration of TRAIL

MDA-MB-468 cells were seeded and treated with indicated concentrations of LBW242 for 16 h, followed by TRAIL (50 ng/ml) for 6 h. Cell were then harvested and used for TMRE (stripped bars) and Annexin V-FITC labelling ((A); filled bars, data represented as mean ± SEM (n=4)) and SDS-PAGE/Western blotting ((B); data shown is representative of two independently performed experiments). *** indicates significant difference P<0.001 and ** indicates P<0.01. * indicates non-specific band.

These data thus suggest that in the MDA-MB-468 cells IAPs can regulate TRAIL-induced apoptosis. Furthermore, inhibition of the IAPs can facilitate the efficient activation of the extrinsic apoptotic pathway in these cells.

3.8 Cross-linked TRAIL-R2 can signal to apoptosis in MCF7 F43 and MDA-MB-231 cells

The data obtained in this study clearly shows that soluble TRAIL signals to apoptosis *via* TRAIL-R1 in breast cancer cell lines. However, we also wanted to explore whether other forms of TRAIL-receptor agonists would also signal through TRAIL-R1 to induce apoptosis in breast cancer cell lines. Currently, the TRAIL antibodies from Human Genome Sciences, Mapatumumab (HGS-ETR1; specific to TRAIL-R1) and Lexatumumab (HGS-ETR2; specific to TRAIL-R2) are in clinical trials for various forms of cancer. In addition, our laboratory previously reported that enforced aggregation of TRAIL-R2 can activate apoptosis in the usually TRAIL-resistant primary chronic lymphocytic leukaemia (CLL) cells (Natoni *et al.,* 2007). We therefore tested the potency of HGS-ETR1 and HGS-ETR2, in the presence or absence of pre-incubation with a cross-linking antibody, in the TRAIL-sensitive cell lines, MCF7 F43 and MDA-MB-231.

Initially, different concentrations of antibody versus cross-linker were tested to obtain the optimum ratio to be used for further experiments. Based on preliminary studies, antibody:cross-linker ratios of 1:1, 1:3 and 1:5 were used. The HGS antibodies, ETR1 and ETR2 (2000 ng/ml) were first incubated with cross-linker for 30 min at room temperature before addition to MCF7 F43 cells. Cells were then treated for 6 h and the extent of apoptosis measured by a decrease in MMP.

Consistent with the data obtained with R1L and R2L, ETR1 induced apoptosis in MCF7 F43 cells, while ETR2 did not (Figure 3.8; Part A; Lanes 4 & 8). Surprisingly, pre-aggregation of ETR1 by a cross-linking agent caused a reduction in the extent of apoptosis induced by ETR1 (Figure 3.8; Part A; Lanes 5-7). Importantly, and consistent with previous reports in primary CLL cells (Natoni *et al.,* 2007), cross-linked ETR2 induced massive apoptosis in MCF7 F43 cells (Figure 3.8; Part A; Lanes 9 & 10).

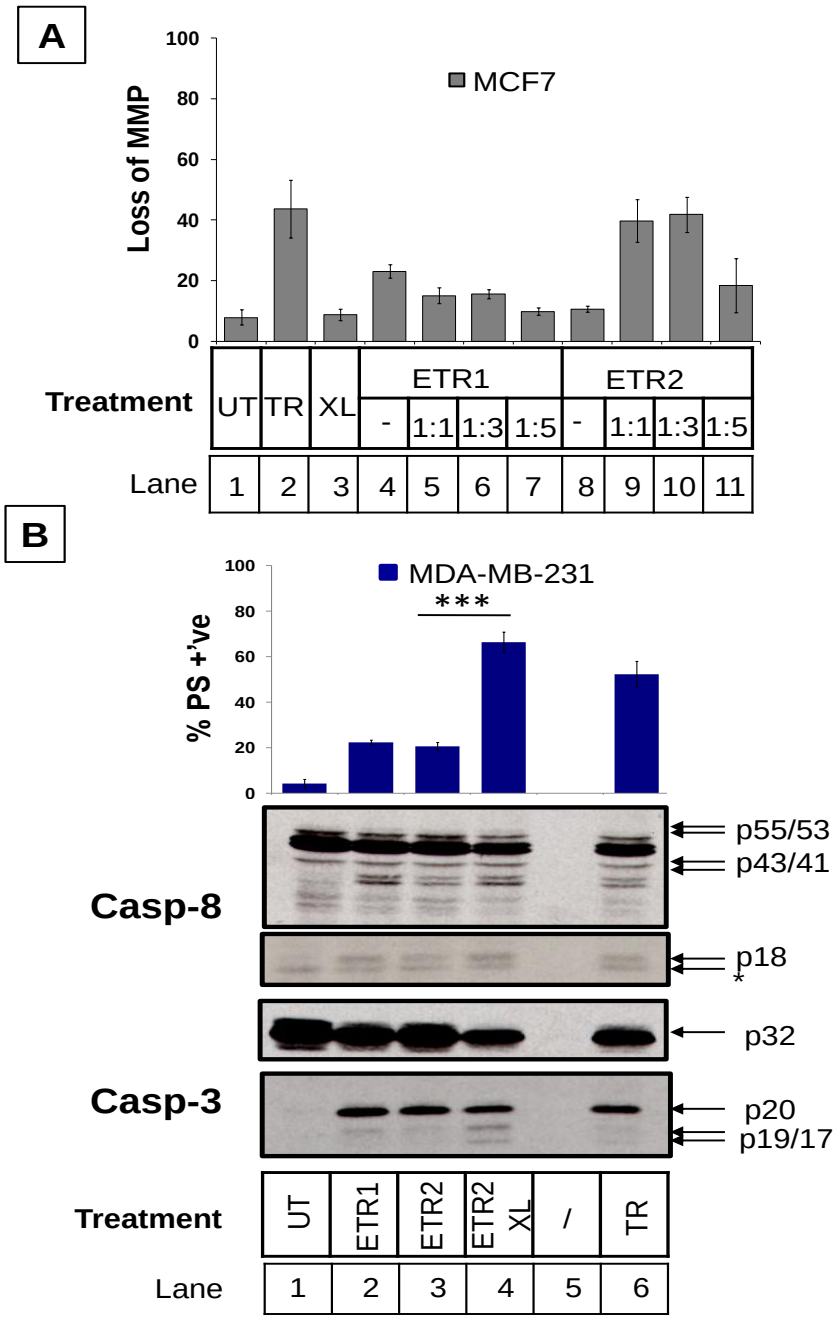


Figure 3.8 TRAIL-R2 can be activated by cross-linked antibody in breast cancer cell lines

MCF7 F43 cells were seeded and treated with either monomeric or cross-linked HGS TRAIL-R1 and TRAIL-R2-specific antibodies (ETR1 and ETR2 respectively) at indicated antibody:cross-linker ratios and used for TMRE analysis ((A); data represented as mean ± SEM (n=4)). Cross-linked ETR2 (2000 ng/ml; antibody:cross-linker = 1:3) was also used to treat MDA-MB-231 cells and the samples were analysed for Annexin V-FITC ((B); data represented as mean ± SEM (n=3)) and SDS-PAGE/Western blotting ((C); data shown is representative of two independently performed experiments). *** indicates significant difference (P<0.001). * indicates non-specific band.

Thus, we can conclude that TRAIL-R2 is functional in MCF7 F43 cells and can efficiently signal to apoptosis when presented with a highly aggregated antibody complex. This data points to the possibility of the presence of different activation thresholds for the death receptors, TRAIL-R1 and TRAIL-R2.

In order to extend the findings from MCF7 F43 cells, MDA-MB-231 cells were also treated with ETR1/2. Both ETR1 and ETR2 induced a modest level of apoptosis in MDA-MB-231 cells, and in the case of ETR2, apoptosis was greatly enhanced by pre-aggregation of ETR2 (Figure 3.8; Part B; Lanes 2-4). Thus, consistent with the data obtained in MCF7 F43 cells, cross-linking of ETR2 significantly increased its potency in MDA-MB-231 cells (Figure 3.8; Part B; Lane 4). The increased apoptosis was further evidenced by the presence of extensive cleavage of caspase-8 and almost complete processing of caspase-3 (Figure 3.8; Part B; Lane 4). We further investigated whether TRAIL-R1 had any role in cross-linked ETR2-mediated apoptosis. To study this, we performed siRNA transfection to silence the expression of TRAIL-R1 or TRAIL-R2 in MCF7 F43 cells (Figure 3.9; Part A) before exposure to cross-linked ETR2. Neither the transfection agent (Figure 3.9; Part B; Lane 2) nor non-targeting oligonucleotide (Figure 3.9; Part B; Lane 5) had any effect on cross-linked ETR2 driven apoptosis. Oligonucleotide directed towards TRAIL-R1 did not result in any attenuation of apoptosis as seen by efficient caspase-8 cleavage and loss of MMP (Figure 3.9; Parts B & C; Lane 8), thus ruling out any contribution of TRAIL-R1 in cross-linked ETR2-mediated apoptosis. Consistent with this, silencing of TRAIL-R2 completely abrogated cross-linked ETR2 induced apoptosis (Figure 3.9; Part B; Lane 11).

The above data combined with the earlier TRAIL DISC analysis of MCF7 F43 and MDA-MB-231 cells (Figures 3.2 and 3.4 respectively) suggests that while TRAIL-R1 forms an active TRAIL DISC without the need for aggregation, TRAIL-R2 requires ‘clustering’ to form higher molecular weight aggregates to trigger apoptosis.

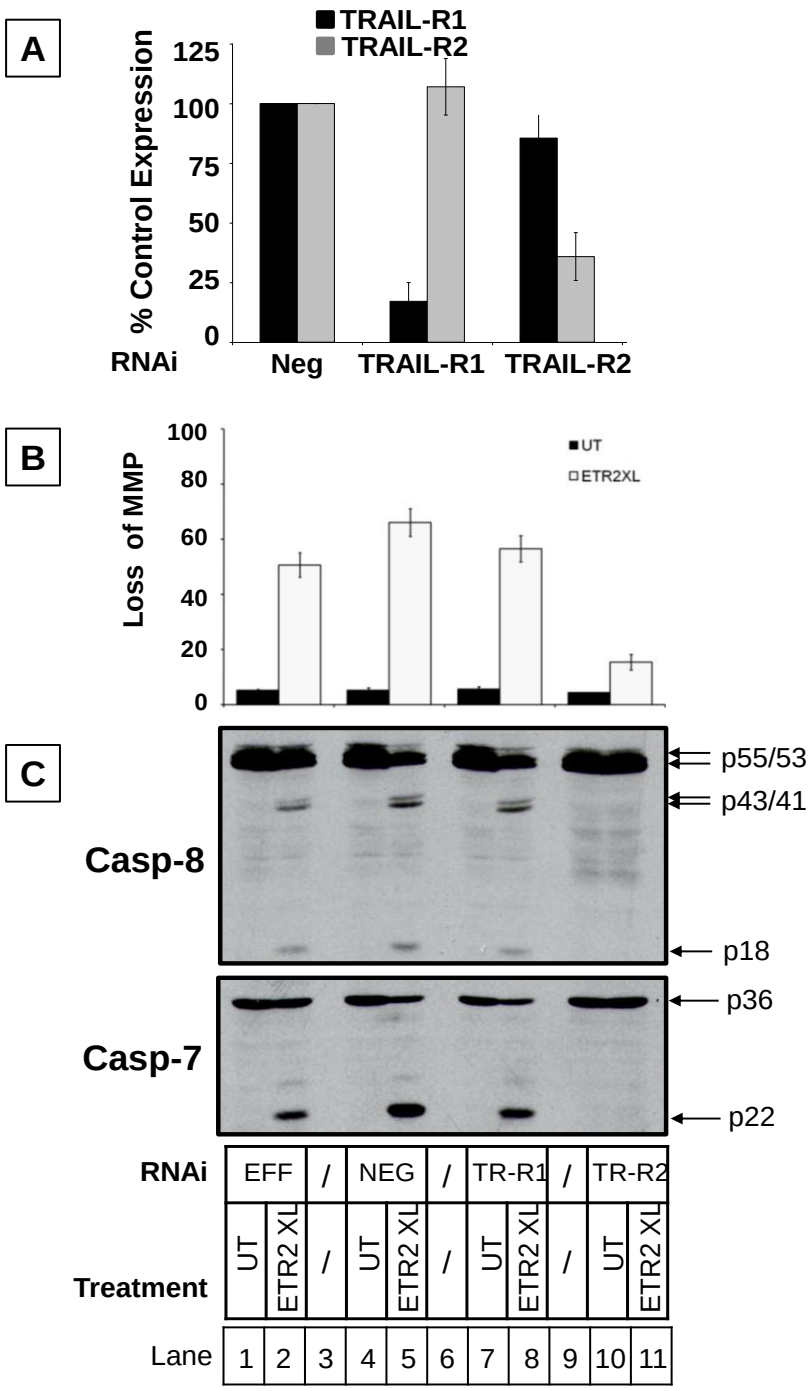


Figure 3.9 Cross-linked HGS-ETR2 signals exclusively through TRAIL-R2 to induce apoptosis in MCF7 F43 cells

Oligonucleotides were used to knock down the expression of either TRAIL-R1 or TRAIL-R2 from MCF7 F43 cells (A) and they were subsequently treated with cross-linked ETR2 (2000 ng/ml; antibody:cross-linker = 1:3) for 6 h. Cells were then harvested for TMRE analysis ((B); data represented as mean ± SEM (n=3)) and SDS-PAGE/Western blotting ((C); data shown is representative of two independently performed experiments).

3.9 Discussion

Of the six breast cancer cell lines chosen for this study, five cell lines were sensitive to TRAIL-induced apoptosis to varying extents (Table 3.2). MCF7 F43 and MDA-MB-231 cells were the most sensitive to TRAIL-induced apoptosis, whereas by comparison MDA-MB-468, BT20 and ZR-75.1 were semi-sensitive to TRAIL.

MCF7 F43 cells showed a strong apoptotic response to TRAIL despite the absence of caspase-3. This finding is particularly important because down-regulation of caspase-3 is seen in majority of primary breast tumours (Devarajan *et al.,* 2002) as a potential mechanism of chemoresistance. Thus, it may be possible to overcome chemoresistance of tumours lacking caspase-3 by incorporation of TRAIL within the anti-tumour therapy.

It is interesting to note that our findings suggest the formation of a TRAIL-R1/-R2 heterocomplex in both MCF7 F43 and MDA-MB-231 cells exposed to R1L (Figure 3.2; Part B; Lane 2 & Figure 3.4; Part B; Lane 2). Previous literature also supports the formation of TRAIL-R1/-R2 heterocomplex upon TRAIL binding (Schneider *et al.,* 1997; Kischkel *et al.,* 2000). Promiscuous binding of R1L to TRAIL-R2 might be another possible reason for the presence of TRAIL-R2 in the R1L stimulated DISC. However, if this were the case, TRAIL-R2 would have been pulled into the unstimulated TRAIL-R1 DISC (Figures 3.2 & 3.4). Similarly, if this association between the two death receptors is mediated *via* their PLAD domains, TRAIL-R2 would have been recruited into the unstimulated TRAIL-R1L DISC. The absence of TRAIL-R2 in the unstimulated TRAIL-R1 DISC provides evidence of the specificity of R1L to TRAIL-R1 (Figure 3.2; Part B; Lane 1 & Figure 3.4; Part B; Lane 1) and further supports the possible existence of TRAIL-R1/R2 heterocomplex. This data indicates that upon efficient activation of TRAIL-R1, a complex can be formed with TRAIL-R2 and this heterocomplex can efficiently recruit FADD and caspase-8. RNAi studies in the MCF7 F43 cells to deplete TRAIL-R2 showed that a lack of TRAIL-R2 did not inhibit TRAIL sensitivity of MCF7 F43 cells (data not shown). These findings argue that whilst upon activation, TRAIL-R1 can form a heterocomplex with TRAIL-R2, this heterocomplex is not absolutely required for efficient apoptosis induction.

Table 3.2 Profile of TRAIL-R signalling to apoptosis in breast cancer cell lines

Cell line	ER status	HER2 status	p53 status	TRAIL sensitivity	R1 vs. R2
MCF7 F43	+	-	Wild type	**+++**	**R1**
ZR-75.1	+	-	Wild type	**+**	**R1**
T47D	+	-	Mutated	**-**	**-**
MDA-MB-231	-	-	Mutated	**+++**	**R1**
MDA-MB-468	-	-	Mutated	**++**	**R1**
BT20	-	-	Mutated	**++**	**R1**

The relatively reduced expression of TRAIL-R1 and TRAIL-R2 on the cell surface of MDA-MB-468 and BT20 cells is a possible reason for the lower potency of TRAIL in these cells. Reduced surface expression of TRAIL receptors has been previously indicated to be a mechanism of TRAIL resistance in breast cancer cells (Yoshida *et al.,* 2009). However, despite the low receptor expression, TRAIL induced caspase-8 activation in both the MDA-MB-468 and BT20 cell lines, while apoptosis as assessed by PS exposure was further delayed, possibly due to the high expression of XIAP. Incidentally, both these cell lines have been previously described to be resistant to TRAIL-induced apoptosis, but in both these studies apoptotic markers downstream of mitochondrial perturbation/effector caspase activation were used (Singh *et al.,* 2003; Chopin *et al.,* 2004). Data presented in this study shows that in these cell lines, TRAIL can signal to apoptosis, however the subsequent apoptosis appears to be regulated by IAPs.

As shown here, Smac-mimetic compounds like LBW242 can sensitise the cell line MDA-MB-468 to sub-toxic concentrations of TRAIL; thus, confirming that regulation of caspases by IAP family plays an important role in governing TRAIL-induced apoptosis in breast cancer cells. It has been reported that Smac mimetics cause apoptosis in target cells *via* autocrine TNF signalling (Peterson *et al.,* 2007; Varfolomeev *et al.,* 2007; Vince *et al.,* 2007). However, in MDA-MB-468 cells, LBW242 caused only modest apoptosis at its maximal concentration ruling out a strong contribution of TNF signalling in Smac mimetic-mediated sensitisation to TRAIL. The efficient cleavage of

caspase-8 in cells treated with LBW242 suggests that inhibiting IAPs also had an effect on caspase-8 processing at the level of the DISC. It is known that XIAP does not inhibit caspase-8 processing, whereas cIAP1 and cIAP2 can bind to but not inhibit caspases. In this regard, it has been reported that cIAP1 can form an anti-apoptotic complex with GSK3 and DDX3 which can then inhibit death receptor-mediated caspase-8 activation (Sun *et al.,* 2008). However, while the authors showed that depleting GSK3 or DDX3 restored the apoptotic activity of TRAIL, they did not provide any evidence for the role of cIAP1 in the complex. However, this report suggests that cIAP1 might be able to indirectly regulate activation of caspase-8 at the DISC and hence, its down-regulation by LBW242 can lead to TRAIL sensitisation. Nonetheless, the data indicates that the combination of Smac mimetics and TRAIL can be successfully used to trigger apoptosis in breast cancer cells.

Although there were differences in the level of apoptosis induced by TRAIL in the breast cancer cell lines, both soluble TRAIL as well as TRAIL-receptor-specific antibodies mainly induced apoptosis *via* signalling through TRAIL-R1 (Table 3.2). Only highly aggregated form of TRAIL-receptor agonists could induce apoptosis through TRAIL-R2. This result is consistent with earlier reports which suggest that while TRAIL-R1 responds equally to cross-linked and monomeric soluble TRAIL; TRAIL-R2 signals in response to only cross-linked or membrane-bound TRAIL (Wajant *et al.,* 2001; Natoni *et al.,* 2007). It would have been interesting to investigate the effects of cross-linking the His-tagged TRAIL used in this study using biotin and comparing its apoptotic potency to cross-lined TRAIL antibodies.

Evidence supporting the apoptotic signalling through TRAIL-R1 includes DISC formation, caspase activation and activation of the mitochondrial amplification arm. Supporting evidence for the role of TRAIL-R1 can also be gained from a previous study which shows that over-expression of TRAIL-R1 induced apoptosis in breast cancer cell lines (Kazhdan and Marciniak*,* 2004). However, these authors did not investigate the effects of over-expression of TRAIL-R2 and it can be speculated that over-expression of TRAIL-R2 might cause apoptosis in the breast cancer cell lines, possibly through PLAD-dependent receptor cross-linking.

It has been previously reported, that a TRAIL-R1-specific ligand does not cause apoptosis in MDA-MB-231 cells (Kelley *et al.,* 2005). A likely reason for this discrepancy could be that although the TRAIL-R1-specific ligand used by these authors did bind to TRAIL-R1, it had no functional apoptosis-inducing activity (MacFarlane *et al.,* 2005b). Thus, while Kelley *et al* did not use an active form of a TRAIL-R1-specific

ligand, in this study I have used a fully functional mutant form of TRAIL that can bind TRAIL-R1 and induce apoptosis in breast cancer cell lines (MacFarlane *et al.,* 2005b).

Overall, we can conclude that five of the six breast cancer cell lines used in this project, respond to TRAIL as a single agent. Importantly, in all the five breast cancer cell lines, R1L is more potent than R2L, inducing significantly more apoptosis at equimolar concentrations. Importantly, the consistent predominance of TRAIL-R1 as the major functional TRAIL receptor is also independent of the ER and p53 status of breast cancer cells. Surprisingly, R1L was not more potent than TRAIL in any of the cell lines. A possible reason for this is that TRAIL, even at a low concentration, is sufficient to saturate all the TRAIL receptors displayed on the cell surface. It is still important to note that although TRAIL and R1L are equipotent, binding of TRAIL to TRAIL-R2 and TRAIL-R4 could still lead to activation of pro-survival pathways and hence using a specific TRAIL-R1 targeting agent rather than wild-type TRAIL in cancer therapy could be beneficial.

Chapter 4

Chemosensitisation of resistant breast tumour cells to TRAIL-induced apoptosis

4.1 Introduction

In contrast to breast cancer cell lines, it has been reported most primary tumour cells are resistant to TRAIL-induced apoptosis (Ehrhardt *et al.,* 2003; MacFarlane *et al.,* 2005a; Herbst *et al.,* 2006; Todaro *et al.,* 2008) and in these apoptosis-resistant tumour cells TRAIL can actually activate pro-survival pathways (Ehrhardt *et al.,* 2003; Baader *et al.,* 2005). The observation that TRAIL can drive tumour cell proliferation emphasises the need for development of a combinational therapeutic approach to ensure absolute tumour cell death. In this context, we examined the use of the breast cancer cell line, T47D as a pertinent model system to study combinations of TRAIL with different chemotherapeutic agents that could be further employed to treat primary breast tumour cells. In addition, this cell line model could also provide preliminary information on the profile of TRAIL-receptor signalling to apoptosis in the presence of sensitising agents.

Similar to primary tumour cells, the T47D cells were completely resistant to the apoptotic effects of TRAIL/R1L/R2L (Figure 4.1; Part A; Lanes 2-4) and instead, TRAIL treatment induced an increase in the proliferation of T47D cells (Figure 4.1; Part B). Particularly, in T47D cells, TRAIL activated the Akt and p38 MAPK pathways but failed to activate the NFκB pathway (Figure 4.1; Parts C & D and Appendix 4). This suggests that the cell line T47D mimics the primary tumour response to TRAIL and thus is a relevant model system to test TRAIL combinations with chemotherapeutic drugs which can be further applied to primary tumours. Based on previous literature, we compiled a panel of both novel and conventional chemotherapeutic agents (Table 1.4) to be used in combination with TRAIL in the TRAIL-resistant breast tumour cell line, T47D.

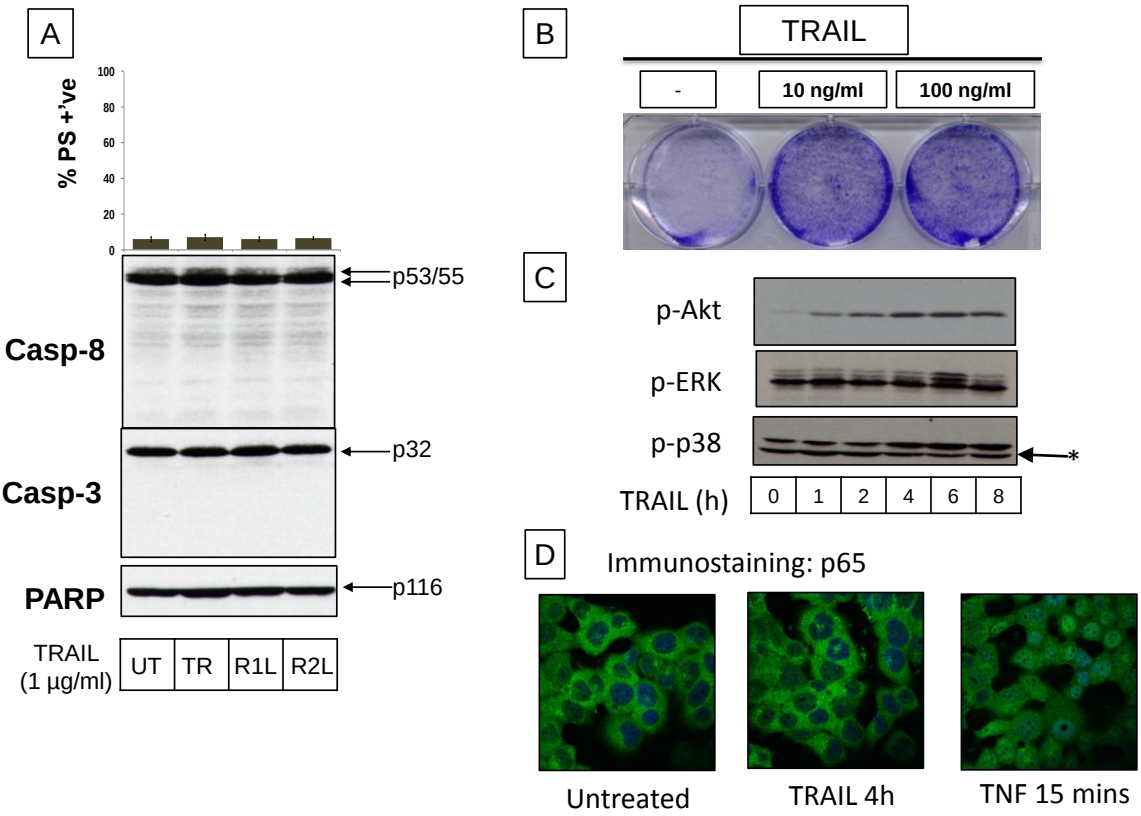


Figure 4.1 TRAIL signals to cell proliferation instead of apoptosis inT47D cells
T47D cells were seeded at 4.0 X 10^5 cells/well and treated with TRAIL(TR)/R1L/R2L (1000 ng/ml) for 24 h, following which cells were harvested and one third of the sample was used for Annexin V-FITC analysis (data represented as mean ± SEM (n=3)) and remaining sample was used for SDS-PAGE/Western blotting (A). Alternatively, T47D cells were seeded at 4.75 X 10^4 cells/well and treated with TRAIL at indicated concentrations for 24 h and analysed for colonogenic capacity ((B); data representative of three independently performed experiments) or seeded at 4.0 X 10^5 cells/well, treated for indicated time points with 1000 ng/ml TRAIL and harvested for SDS-PAGE/Western blotting (C). For p65 translocation, cells were treated with TRAIL (1000 ng/ml) or TNF (200 ng/ml) for indicated time and translocation was studied by ICC as outlined in Materials and Methods (D). * indicates non-phosphorylated p38.

4.1.1 Doxorubicin

Doxorubicin/Adriamycin is an anthracycline commonly used for the treatment of breast cancer both as a monotherapy and in combination therapy. For example, Myocet®, a form of liposomal doxorubicin, is already in use in combination with another chemotherapeutic agent, cyclophosphamide, to treat metastatic breast cancer.

Doxorubicin induces a variety of effects in target cells; as a result, the exact mechanism of its anti-tumour activity is unclear. The primary effects of doxorubicin are exerted *via* its intercalation with the host cell DNA which results in its interference with transcription and replication processes (Momparier *et al.,* 1976). Subsequent DNA damage in turn triggers p53 activation and DNA repair systems. Another mechanism for doxorubicin-induced DNA damage is the formation of free radicals (Sinha *et al.,* 1987). Doxorubicin has additionally been shown to cause DNA alkylation, DNA cross-linking and adduct formation, and inhibition of helicase and topoisomerase II, resulting in specific single strand or double strand breaks (Tewey *et al.,* 1984; Goldenberg *et al.,* 1986; Bachur *et al.,* 1992). However, many of these changes have been reported at supra-clinical concentrations of doxorubicin and under non-physiological conditions; hence the validity of these latter mechanisms *in vivo* is questionable (reviewed in Gewirtz, 1999).

Doxorubicin has previously been shown to enhance TRAIL-mediated apoptosis in several cell lines, including breast cancer cell lines (Keane *et al.,* 1999; Singh *et al.,* 2003; Aroui *et al.,* 2009). This enhancement has been attributed to several doxorubicin-mediated effects, including the up-regulation of TRAIL-R1/-R2 in target cells and changes in the protein expression of various Bcl-2 family proteins and/or potential caspase-8 regulators such as $FLIP_L$ (Singh *et al.,* 2003; Wang *et al.,* 2010).

Doxorubicin was therefore our first choice as a potential TRAIL sensitiser in breast cancer cells because of the advantage that doxorubicin is already in clinical use coupled with previous reports showing its strong sensitising ability.

4.1.2 SAHA

SAHA (Suberoylanilide hydroxamic acid) is a pan-histone deacetylase inhibitor (HDACi) and is in Phase I clinical trials for use in solid tumours (Fakih *et al.,* 2010). As a potent HDACi, SAHA can regulate the expression of a variety of genes which may interfere with TRAIL sensitivity; however, its exact mechanism of action is unknown.

Previously, HDAC inhibitors have been used in our laboratory to sensitise primary CLL cells to TRAIL and therefore, we chose to examine an HDACi as a likely sensitising agent. It has also been shown that TRAIL in combination with HDACi can selectively kill tumour cells over normal cells (Dzieran *et al.,* 2008). The possible reasons reported for TRAIL sensitisation by HDAC inhibitors, include up-regulation of TRAIL-R2 on the cell surface and modulation of both anti- and pro-apoptotic family members (Singh *et al.,* 2005; Butler *et al.,* 2006; Frew *et al.,* 2008). However, it should be highlighted that previously our laboratory has proven that HDACi-mediated sensitisation to TRAIL occurs independently of HDACi-mediated up-regulation of TRAIL-R2 on the cell surface (Inoue *et al.,* 2006).

4.1.3 Flavopiridol

Flavopiridol is the first cyclin-dependent kinase (CDK) inhibitor to enter clinical trials for cancer treatment (reviewed in Senderowicz, 1999). Flavopiridol belongs to the flavone family and at low concentrations inhibits CDK-1, -2, -4, -5 and -7, thus interfering with cell cycle progression (Kaur *et al.,* 1992; Carlson *et al.,* 1996).

Flavopiridol has been previously reported to sensitise cancer cells to TRAIL-induced apoptosis by down-regulating $FLIP_L$ and/or XIAP (Palacios *et al.,* 2006; Fandy *et al.,* 2007). Thus, based on previous literature, we chose this promising CDK inhibitor as a potential sensitising agent for T47D cells.

4.1.4 Tamoxifen

Tamoxifen is the most commonly administered hormonal therapy to pre-menopausal women suffering from breast cancer. Oestrogen signalling through the oestrogen receptors has been identified as a potent driver of tumour cell proliferation and therefore, inhibitors of oestrogen like tamoxifen are now widely used in breast cancer therapy. Binding of tamoxifen to the oestrogen receptors results in an altered receptor conformation, which is not capable of transducing the growth signal to transcription factors downstream in the cascade.

Tamoxifen has also been previously reported to enhance the TRAIL sensitivity of breast cancer cell lines (Lagadec *et al.,* 2008) and hence, we sought to study whether tamoxifen could also sensitise T47D cells to TRAIL.

4.1.5 ABT-737

ABT-737 is a BH3-mimetic and is a potent inhibitor of key anti-apoptotic members of the Bcl-2 family (Oltersdorf *et al.,* 2005). Currently its orally bioavailable variant, ABT-263 is in clinical trials for its use as an anti-cancer therapy (Gandhi *et al.,* 2011). ABT-737 binds and antagonises Bcl-2 family members including Bcl-2, Bcl-XL and Bcl-w. It however, does not inhibit the anti-apoptotic activity of Mcl-1 and therefore high expression of Mcl-1 can lead to ABT-737 resistance (Yecies *et al.,* 2010).

ABT-737 has been previously reported to sensitise cancer cell lines to TRAIL (Huang and Sinicrope, 2008; Song *et al.,* 2008). Due to its future potential as a novel anti-tumour therapy, we chose ABT-737 as a possible TRAIL sensitising agent for this study.

4.1.6 PI103 and SB203580

As preliminary data from T47D cells exposed to TRAIL had shown that TRAIL can strongly activate the Akt and p38 MAPK signalling pathways, we wished to examine whether the inhibitors of these pathways were able to sensitise T47D cells to TRAIL. Inhibitors of proliferation pathways are in clinical trials for various cancers, thus justifying their use in combination therapy with TRAIL. Inhibition of Akt has already been shown to sensitise TRAIL-resistant tumour cells to TRAIL-induced apoptosis (Opel *et al.,* 2008; Xu *et al.,* 2010). Additionally breast cancer has been shown to harbour PI3K mutations (Benvenuti *et al.,* 2008), activating Akt mutations (Bleeker *et al.,* 2008) or lack of the Akt regulator, PTEN (Yonemri *et al.,* 2009), thus justifying the development of Akt inhibitors for breast cancer therapy.

4.1.7 LBW242

The Smac mimetic LBW242 was chosen as a potential sensitising agent for TRAIL-induced apoptosis in T47D cells based on our previous findings where it successfully sensitised MDA-MB-468 cells to sub-toxic concentrations of TRAIL (Figure 3.8).

4.2 TRAIL combined with chemotherapeutic agents induces varying levels of apoptosis in the TRAIL-resistant cell line, T47D

T47D cells were co-treated with TRAIL and individual chemotherapeutic agents (that had been previously tested for their activity; Figure 4.2) for 24 h. Combined treatment of TRAIL (1000 ng/ml) and doxorubicin (10 µM) for 24 h induced massive apoptosis in T47D cells (Figure 4.3). Similarly, co-treatment with the CDK inhibitor flavopiridol (10 µM) and TRAIL (1000 ng/ml) also induced a substantial amount of apoptosis in T47D cells (Figure 4.3), while ABT-737 (10 µM) together with TRAIL (1000 ng/ml) caused ~40% apoptosis in the cells (Figure 4.3). By comparison, the Akt inhibitor, PI103 (10 µM) sensitised T47D cells only slightly to TRAIL-induced apoptosis. In contrast the p38 inhibitor, SB203580 (10 µM), tamoxifen (10 µM) and SAHA (10 µM) did not facilitate the induction of any apoptosis when used in conjunction with TRAIL. In addition and in stark contrast to the data obtained previously in MDA-MB-468 cells (Figure 3.8), the Smac mimetic LBW242 (20 µM) failed to sensitise T47D cells to TRAIL-induced apoptosis. Based on this data, doxorubicin was chosen as the major TRAIL sensitising agent to be used for further experiments in the TRAIL-resistant breast tumour cell line, T47D and more importantly, for treating primary breast tumour explants in combination with TRAIL (see later).

4.3 Co-treatment with doxorubicin and TRAIL induces apoptosis in T47D cells

T47D cells were either left untreated (Figure 4.4; Part A; Lane 1) or treated with TRAIL alone (1000 ng/ml; Figure 4.4; Part A; Lane 2) or doxorubicin (10 µM; Figure 4.4; Part A; Lane 8) alone for 24 h. From this experiment, it was confirmed that T47D cells are resistant to both TRAIL and doxorubicin when used as single agents.

Co-treatment of T47D cells with TRAIL and doxorubicin induced apoptosis which increased with increasing concentrations of doxorubicin (with TRAIL maintained at a constant concentration of 1000 ng/ml). Co-administration of TRAIL with doxorubicin at 0.1 µM (Figure 4.4; Part A; Lane 3) failed to induce apoptosis; however, apoptosis was triggered by co-treating T47D cells with TRAIL and doxorubicin at a concentration of 0.5 µM (Figure 4.4; Part A; Lane 4). Further increases in doxorubicin concentration up to 1 µM (Figure 4.4; Part A; Lane 5) and 5 µM caused a significant enhancement in the extent of apoptosis, which was maximal at doxorubicin concentrations of 5 µM and 10 µM. As maximal sensitisation to TRAIL was observed with a co-administration of 5 µM doxorubicin, this concentration was chosen for further optimisation experiments.

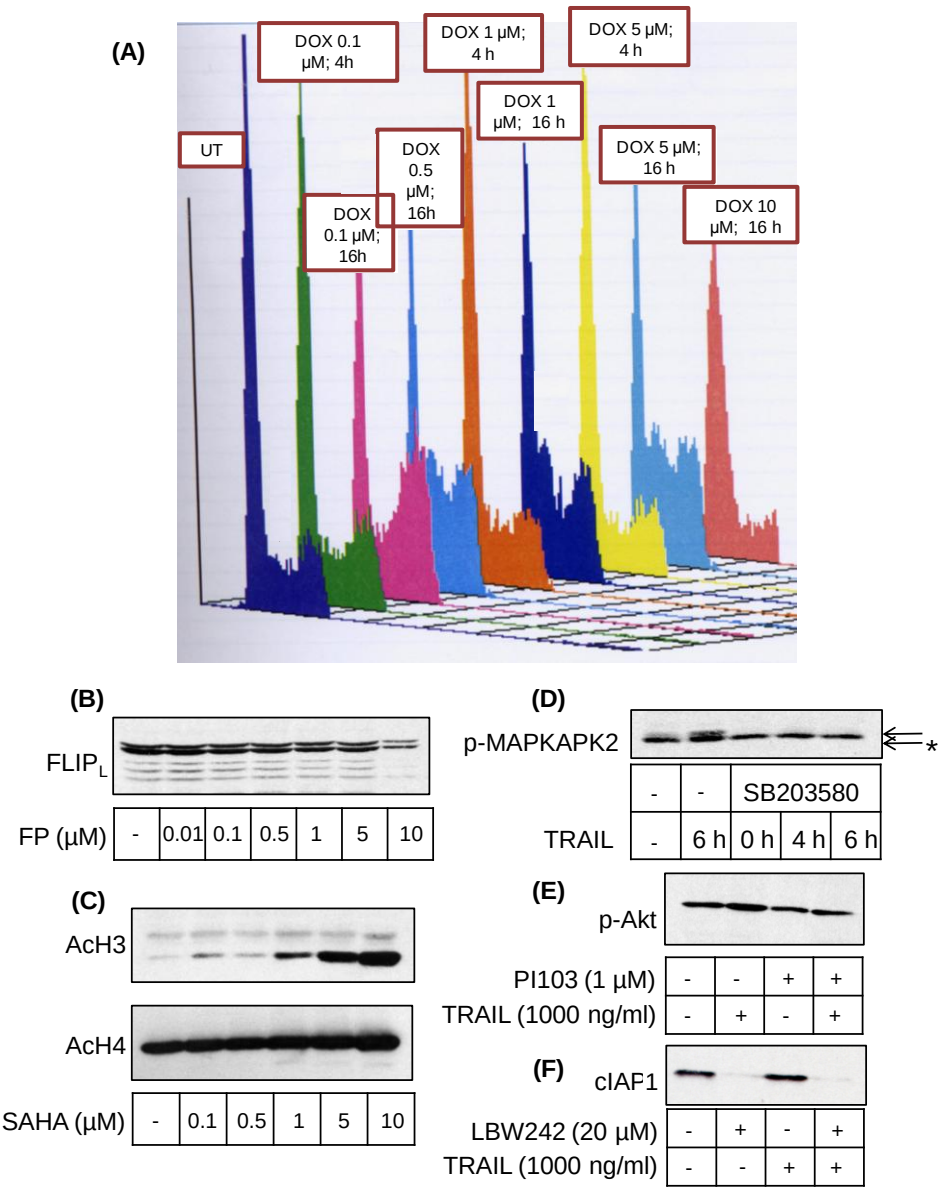


Figure 4.2 Verification of functional activity of the sensitisers used in the study

To check if doxorubicin used was active, cell cycle analysis was performed on treated T47D cells (A). Briefly, cells were treated for indicated times and then harvested by trypsinisation following which they were incubated in 70% ethanol/30% PBS at 4 ºC for 30 mins. After centrifugation at 3000 rpm, the samples were re-suspended in 800 µl PBS, 100 µl RNAse and 100 µl PI and left overnight at 4 ºC. Following day samples were washed with PBS and fluorescence was measured on FACSCanto™. For the other sensitising agents, cells were harvested post-treatment and changes in the relevant proteins were detected: Flavopiridol caused down-regulation of $FLIP_L$ (B); SAHA induced up-regulation of acetylated histones 3 and 4 (AcH3/4 respectively; (C)), SB203580 inhibited TRAIL-mediated up-regulation of MAPKAPK2 ((D); * indicates non-phosphorylated MAPKAPK2); PI103 inhibited TRAIL-mediated up-regulation of p-Akt (E) and LBW242 induced degradation of cIAP2 (F) .

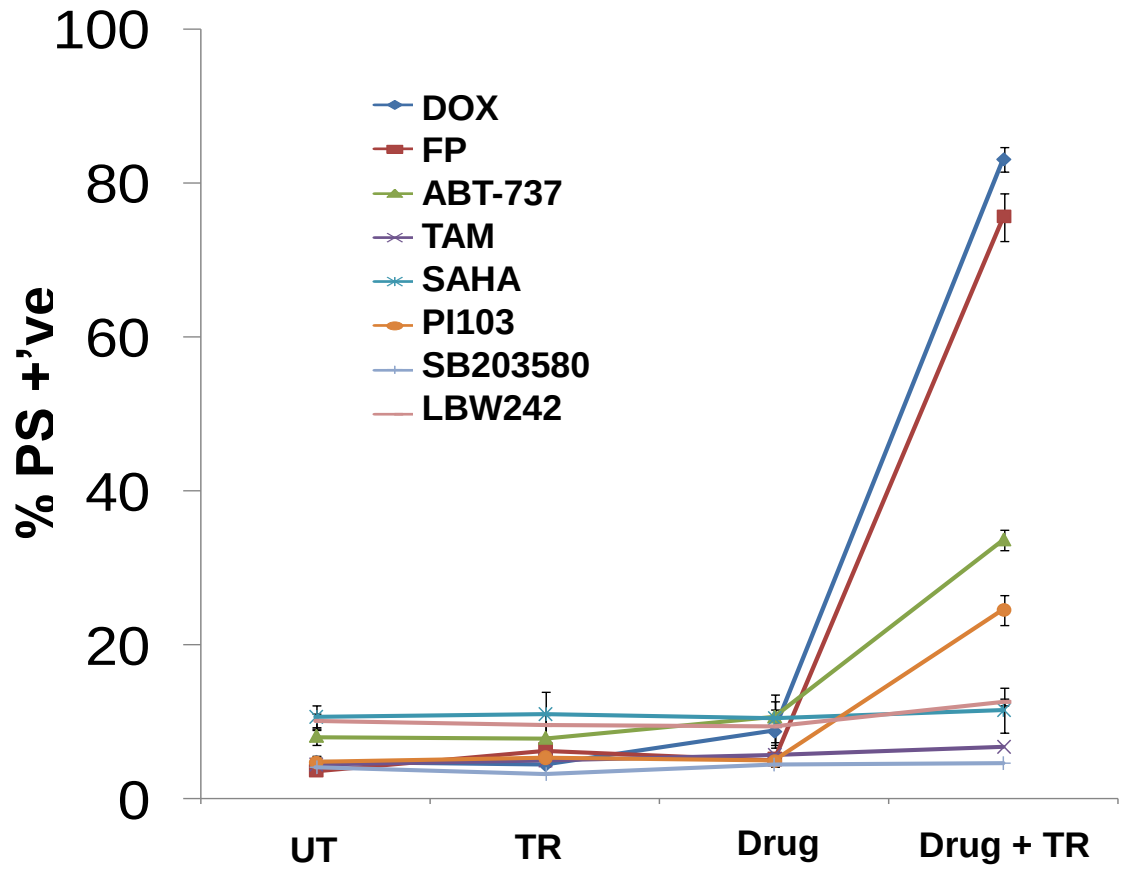


Figure 4.3 T47D cells can be sensitised to TRAIL-induced apoptosis using chemotherapeutic agents

T47D cells were seeded and treated with TRAIL (1000 ng/ml) in combination with different potential sensitizing agents; doxorubicin (DOX; 10 µM), flavopiridol (FP; 10 µM), ABT-737 (ABT-737; 10 µM), tamoxifen (TAM; 10 µM), SAHA (10 µM), PI103 (1 µM), SB203580 (10 µM) or LBW242 (20 µM) for 24 h and the samples were harvested and used for Annexin V-FITC labelling (data represented as mean ± SEM (n=3)).

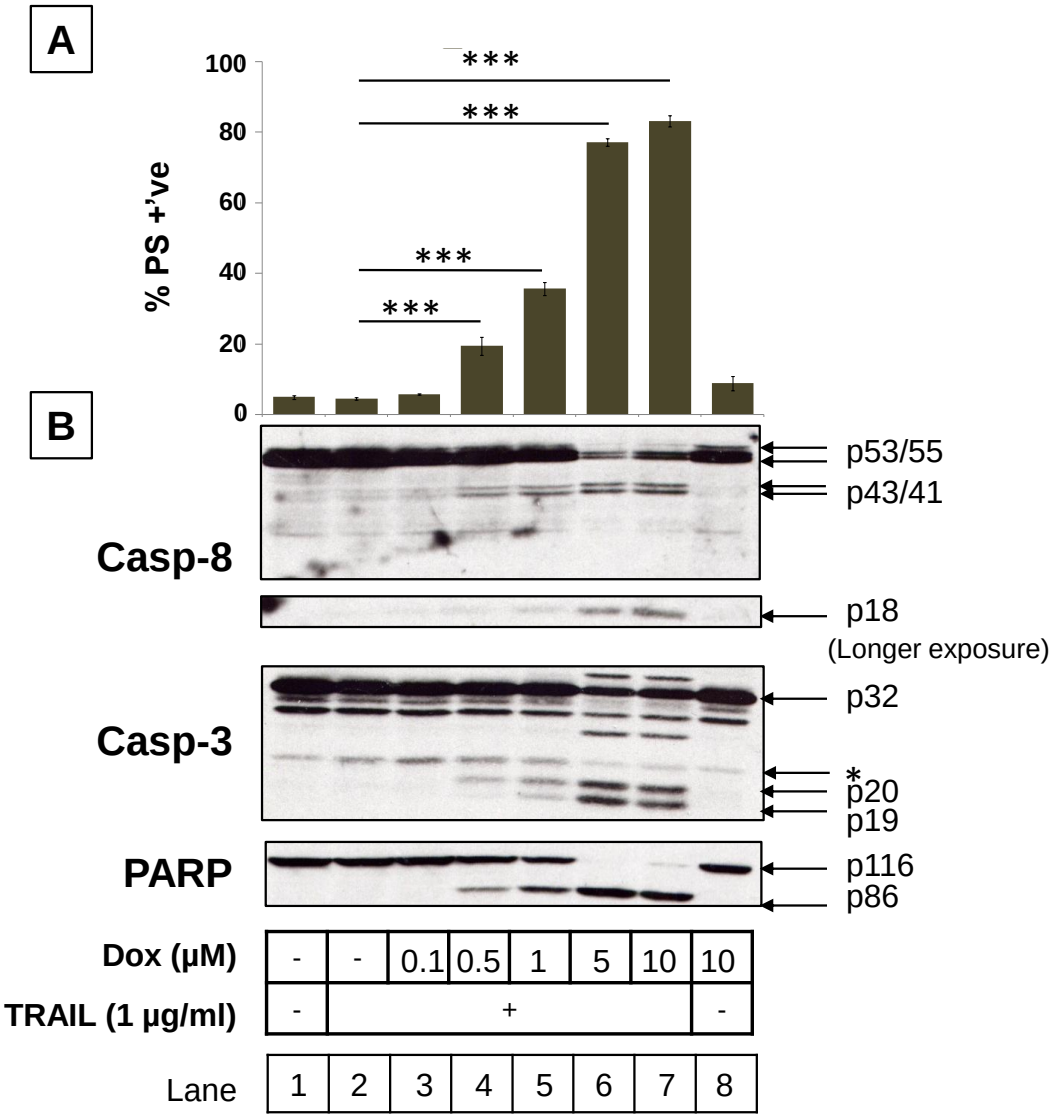


Figure 4.4 Co-treatment with doxorubicin and TRAIL induces apoptosis in T47D cells

T47D cells were co-treated with TRAIL (1 μg/ml) and increasing concentrations of doxorubicin for 24 h and apoptosis was assessed by analysis of PS exposure ((A); data represented as mean ± SEM (n=3)). The remaining sample was used for SDS-PAGE/Western blot analysis to detect cleaved caspase-8 and caspase-3 ((B); data shown as a representative of two independently performed experiments). *** indicates significant difference ($P<0.001$).

4.4 Doxorubicin sensitises T47D cells to TRAIL-induced apoptosis

We further wanted to study whether in co-treated T47D cells, doxorubicin was sensitising to TRAIL-mediated apoptosis or vice versa. Based on previous work done in our laboratory, we chose a pre-treatment time of 16 h with the sensitising agent followed by 6 h treatment with the apoptosis-inducing agent as our initial sensitisation treatment regime. T47D cells were left untreated (Figure 4.5; Parts A & B; Lane 1) or treated with TRAIL alone (1000 ng/ml; Figure 4.5; Parts A & B; Lane 2) or doxorubicin alone (5 µM; Figure 4.5; Parts A & B; Lane 3) for 22 h. Once again, no apoptosis was seen in T47D cells on treatment with either of these agents alone.

Cells pre-treated with doxorubicin for 16 h followed by TRAIL for 6 h (Figure 4.5; Parts A & B; Lane 4) or pre-treated with TRAIL for 16 h followed by doxorubicin for 6 h (Figure 4.5; Parts A & B; Lane 5) were then examined. No apoptosis was seen in cells pre-treated with TRAIL for 16 h followed by doxorubicin treatment as assessed by PS exposure or caspase activation (Figure 4.5; Parts A & B; Lane 5). However pre-treatment with doxorubicin for 16 h followed by TRAIL for 6 h induced caspase activation and apoptosis which was comparable with the extent of apoptosis achieved by co-treatment with doxorubicin and TRAIL (Figure 4.5; Parts A & B; Lanes 4 & 6).

It is thus evident that the apoptosis induced by co-treatment with doxorubicin and TRAIL in T47D cells is mediated by TRAIL but that doxorubicin is required for the initial sensitisation. Based of the above experiments and previous work done in our laboratory, the optimal TRAIL sensitisation regime for T47D cells was chosen as 16 h pre-treatment with doxorubicin, followed by 6 h treatment with TRAIL.

4.5 Supra-clinical concentrations of doxorubicin sensitise T47D cells to signal to TRAIL-induced apoptosis *via* both TRAIL-R1 and TRAIL-R2

Using the previously optimised treatment regime, we then wished to study the profile of TRAIL-receptor signalling to apoptosis in T47D cells.

T47D cells were either left alone or pre-treated with doxorubicin (5 µM) for 16 h and then treated with TRAIL (1000 ng/ml), R1L (1000 ng/ml) or R2L (1000 ng/ml) for 6 h. T47D cells treated with TRAIL (Figure 4.5; Parts A & B; Lane 2) or the mutant TRAIL ligands (Figure 4.6; Parts A & B; Lanes 3 & 4) alone did not undergo apoptosis as assessed by PS exposure and caspase activation. Exposure to doxorubicin alone (5 µM; Figure 4.6; Parts A & B; Lane 5), also failed to induce apoptosis which is consistent with earlier data (Figure 4.5).

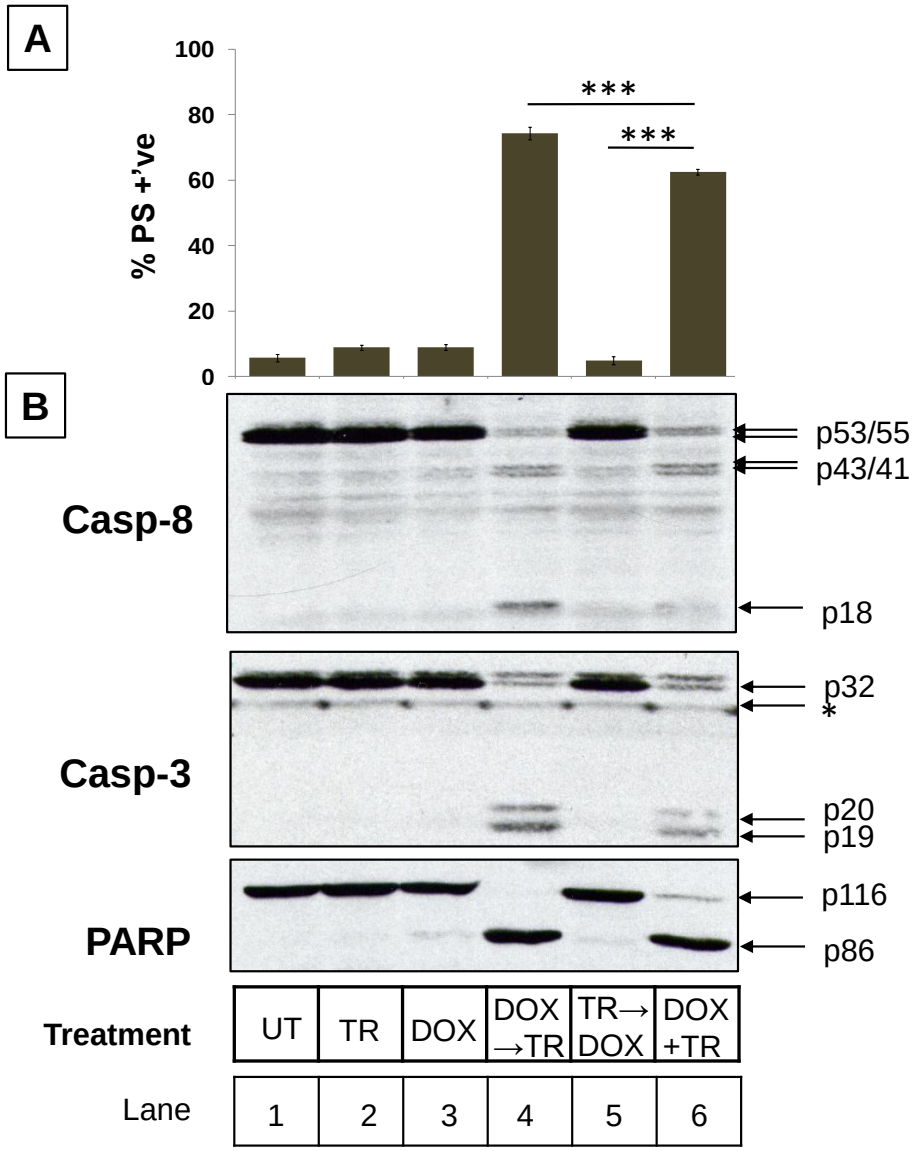


Figure 4.5 Doxorubicin sensitizes T47D cells to TRAIL-induced apoptosis

T47D cells were seeded and either pre-treated with doxorubicin (5 µM) for 16 h followed by TRAIL (1 µg/ml) for 6 h or pre-treated with TRAIL (1 µg/ml) for 16 h followed by doxorubicin (5 µM) for 6 h along with the appropriate controls. After harvesting, one fourth of the sample was used for Annexin V-FITC binding assay ((A); data represented as mean ± SEM (n=3)). The remaining sample was used for SDS-PAGE/Western blot analysis ((B); data shown is representative of two independent experiments). *** indicates significant difference ($P<0.001$).

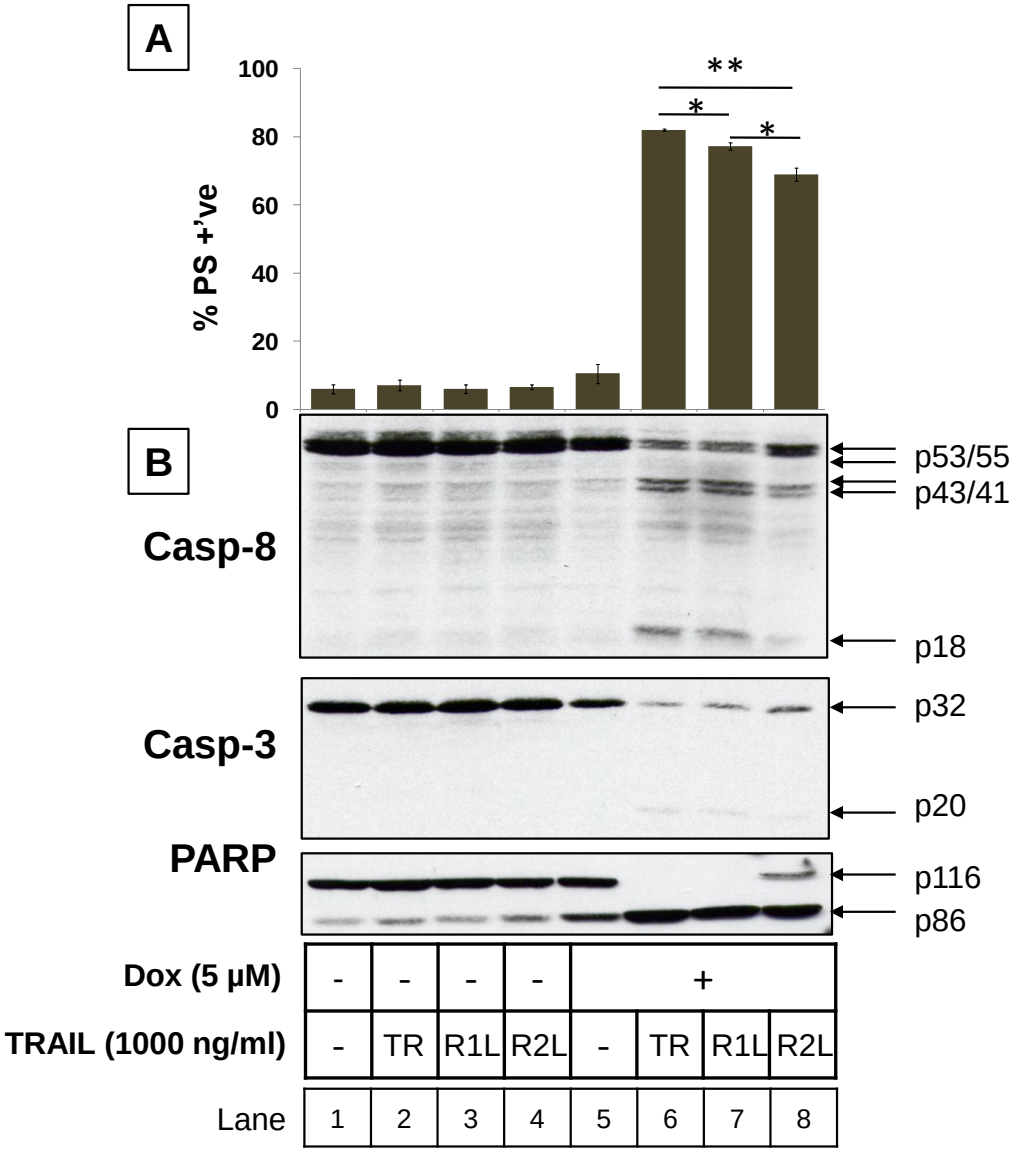


Figure 4.6 Doxorubicin pre-treated T47D cells are sensitive to R1L and R2L
T47D cells were seeded at 4.0 X 10^5 cells/well and then treated with TRAIL (TR), R1L or R2L at 1 µg/ml for 6 h in the presence or absence of doxorubicin (5 µM) and apoptosis was assessed using Annexin V-FITC binding assay ((A); data represents mean ± SEM (n=3)) and SDS-PAGE/Western blotting ((B); data representative of two independently done experiments). ** and * indicate significant difference ($P<0.01$ and $P < 0.05$ respectively).

However, pre-treatment with doxorubicin at 5 µM followed by treatment with TRAIL induced ~80% apoptosis (Figure 4.6; Part A; Lane 6). Strong caspase-8 processing and caspase-3 activation (Figure 4.6; Part B; Lane 6) provided further evidence for TRAIL-mediated apoptosis. Doxorubicin pre-treated T47D cells also underwent apoptosis with both the TRAIL-receptor selective ligands, R1L and R2L (Figure 4.6; Part A; Lanes 7 & 8). High PS exposure, activation of caspase-8, caspase-3 and cleavage of PARP with both R1L and R2L provided proof of high TRAIL potency mediated through both TRAIL-R1 and TRAIL-R2.

From these experiments, it is important to note that the extent of apoptosis triggered by R1L was significantly higher than that triggered by R2L, thus providing a hint that there may be a window of difference between the signalling thresholds of the two death receptors which could be further explored. Also, although our preliminary data indicated that 5 µM doxorubicin was required to maximally sensitise T47D cells to TRAIL (Figure 4.4; Part A; Lane 6), it has been argued that this concentration of doxorubicin is supra-clinical and is unlikely to be achieved in a clinical setting (reviewed in Gewirtz, 1999).

Thus, as we had shown previously (Figure 4.4; Part A; Lane 5), a concentration of only 1 µM doxorubicin was, in fact, sufficient to sensitise T47D cells to TRAIL, it was decided to use this more clinically relevant concentration of 1 µM for all future experiments.

4.6 Doxorubicin (1 µM) sensitises T47D cells to increasing concentrations of TRAIL

Having established a clinically relevant concentration of doxorubicin (1 µM), we next determined the optimum concentration of TRAIL to be used in combination with doxorubicin in T47D cells. Untreated T47D cells were used as a negative control for this experiment and TRAIL at a maximal concentration of 1000 ng/ml with doxorubicin was used as a positive control (Figure 4.7).

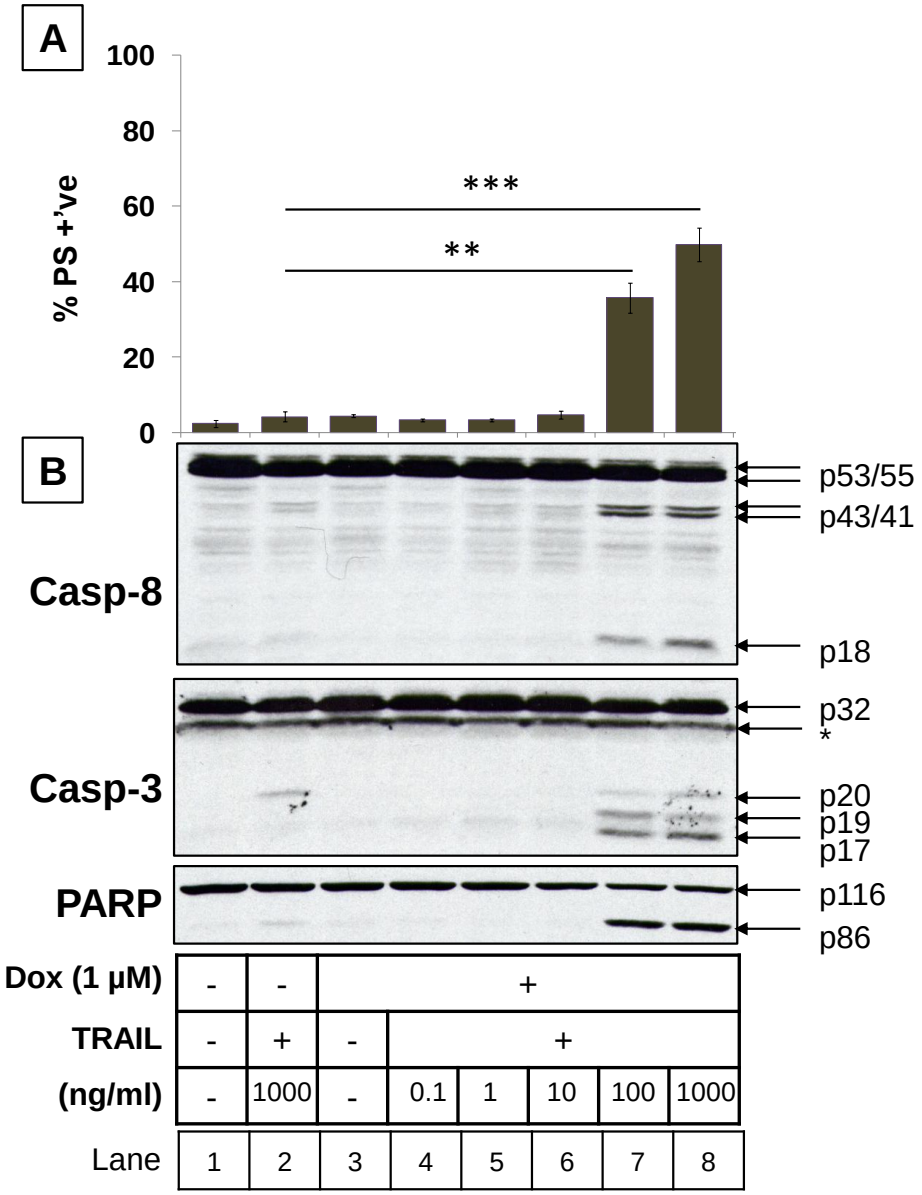


Figure 4.7 Doxorubicin (1 μM) sensitises T47D cells to increasing concentrations of TRAIL

T47D cells were seeded and pre-treated with doxorubicin (1 μM) for 16 h followed by TRAIL at indicated concentrations for 6 h and subsequently samples were harvested for Annexin V-FITC analysis ((A); data represented as mean ± SEM (n=3)) and SDS-PAGE/Western blotting ((B); data representative of two independently done experiments). *** and ** indicate significant difference (P<0.001 and P<0.01 respectively).

Keeping the pre-treatment concentration of doxorubicin constant at 1 µM, the T47D cells were subsequently treated with varying TRAIL concentrations from 0.1 ng/ml to 1000 ng/ml. TRAIL at 0.1, 1 and 10 ng/ml in combination with doxorubicin failed to induce any apoptosis (Figure 4.7; Part A; Lanes 4-6). However, when doxorubicin was used in conjunction with TRAIL at 100 ng/ml, T47D cells showed ~40% apoptosis (Figure 4.7; Part A; Lane 7). T47D cells showed maximal apoptosis when doxorubicin pre-treated cells were treated with TRAIL at 1000 ng/ml. This was also reflected by the higher exposure of PS and increased cleavage of caspase-8 and caspase-3 when TRAIL was used at 1000 ng/ml (Figure 4.7; Parts A & B; Lane 8). Based on the above experiments, pre-treated T47D cells with doxorubicin (1 µM) for 16 h followed by TRAIL (1000 ng/ml) for 6 h was chosen as the optimal treatment regime for further investigation of the mechanism of cell death in these cells.

4.7 The combination of doxorubicin and TRAIL induces apoptotic cell death in T47D cells which is protected by the addition of zVAD

There are various modes of cell death (reviewed in Kroemer *et al.,* 2009) and it was therefore, crucial to confirm that the cell death being induced by doxorubicin and TRAIL in T47D cells was indeed apoptosis.

For this purpose, zVAD-FMK was used to study whether the inhibition of caspase activity would affect the extent of cell death induced by doxorubicin and TRAIL combination in T47D cells. As seen before, T47D cells were resistant to either TRAIL (1000 ng/ml) or doxorubicin at a clinically relevant concentration of 1 µM when used as single agents (Figure 4.8; Part A; Lanes 2 & 3). zVAD (50 µM) alone did not induce any apoptosis in T47D cells after 22 h of treatment (Figure 4.8; Part A; Lane 4), nor did it sensitise the cells to TRAIL or doxorubicin-mediated apoptosis (Figure 4.8; Part A; Lanes 5 & 6). Doxorubicin and TRAIL combination induced efficient caspase-8 and caspase-3 cleavage in T47D cells (Figure 4.8; Part B; Lane 9), which was completely abrogated by the addition of zVAD (Figure 4.8; Part B; Lanes 7 & 8). Interestingly, complete abrogation of caspase cleavage did not cause a complete attenuation of PS externalisation induced by doxorubicin and TRAIL combination in T47D cells (Figure 4.8; Parts A & B; Lanes 7-9). It can be suggested that the small component of cells that undergo caspase-independent cell death in presence of zVAD reflect the necroptotic cells which exhibit permeabilised cell membranes (Vercammen *et al.,* 1998).

We then employed electron microscopy to further confirm whether apoptosis was the major form of cell death in doxorubicin and TRAIL treated T47D cells. There were no significant differences in the morphology of cells treated with TRAIL (1000 ng/ml; 22 h) or doxorubicin (1 µM; 22 h) as compared with untreated control T47D cells (Figure 4.9; Panels i-iii). All these samples contained a few apoptotic cells. Samples pre-treated with doxorubicin for 16 h followed by TRAIL for 6 h contained more cells showing classical signs of apoptosis including the formation of apoptotic bodies and dense inclusions, probably of cytokeratin (Figure 4.9; Panels iv & v; MacFarlane *et al.,* 2000). There was no clear morphological evidence for the presence of any other mode of cell death occurring in these cells.

To further confirm this observation, electron micrographs of T47D cells treated with doxorubicin and TRAIL in the presence of zVAD were examined. The caspase-dependent condensation of chromatin and formation of cytoplasmic inclusions could not be observed in this sample, thus verifying that zVAD inhibited caspase-dependent apoptosis in these cells (Figure 4.9; Panel vi). However, even in these cells, there was no obvious proof of any other mode of cell death taking place, thus indicating that apoptosis was in fact the major cell death pathway triggered by the combination therapy of doxorubicin and TRAIL.

Having established the mode of cell death as apoptosis, it was then important to profile the TRAIL-receptor signalling to apoptosis in T47D cells pre-treated with the clinically relevant concentration of doxorubicin (1 µM).

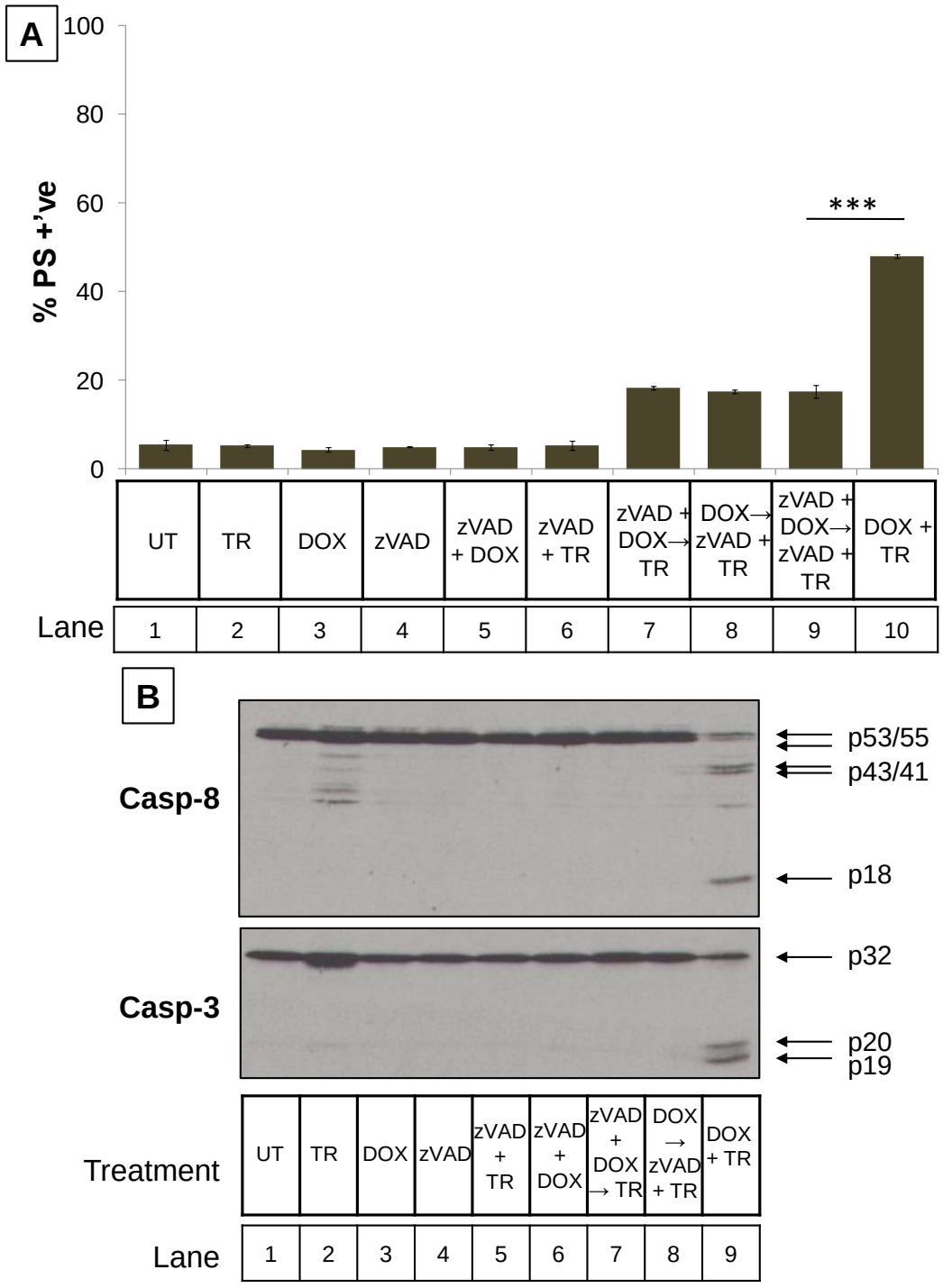


Figure 4.8 zVAD partially protects T47D cells from doxorubicin and TRAIL induced apoptosis

T47D cells were seeded at 4.0 X 10^5 cells/well and treated with either doxorubicin (1 µM), TRAIL (1000 ng/ml) or zVAD (50 µM) alone for 22 h or in their indicated combinations. zVAD was always added 1 h prior to doxorubicin or TRAIL treatment. Cells were harvested and used for Annexin V-FITC binding assay ((A); data shown as mean ± SEM (n=3)) and SDS-PAGE/Western blotting ((B); data representative of two independently done experiments). *** indicates significant difference (P<0.001).

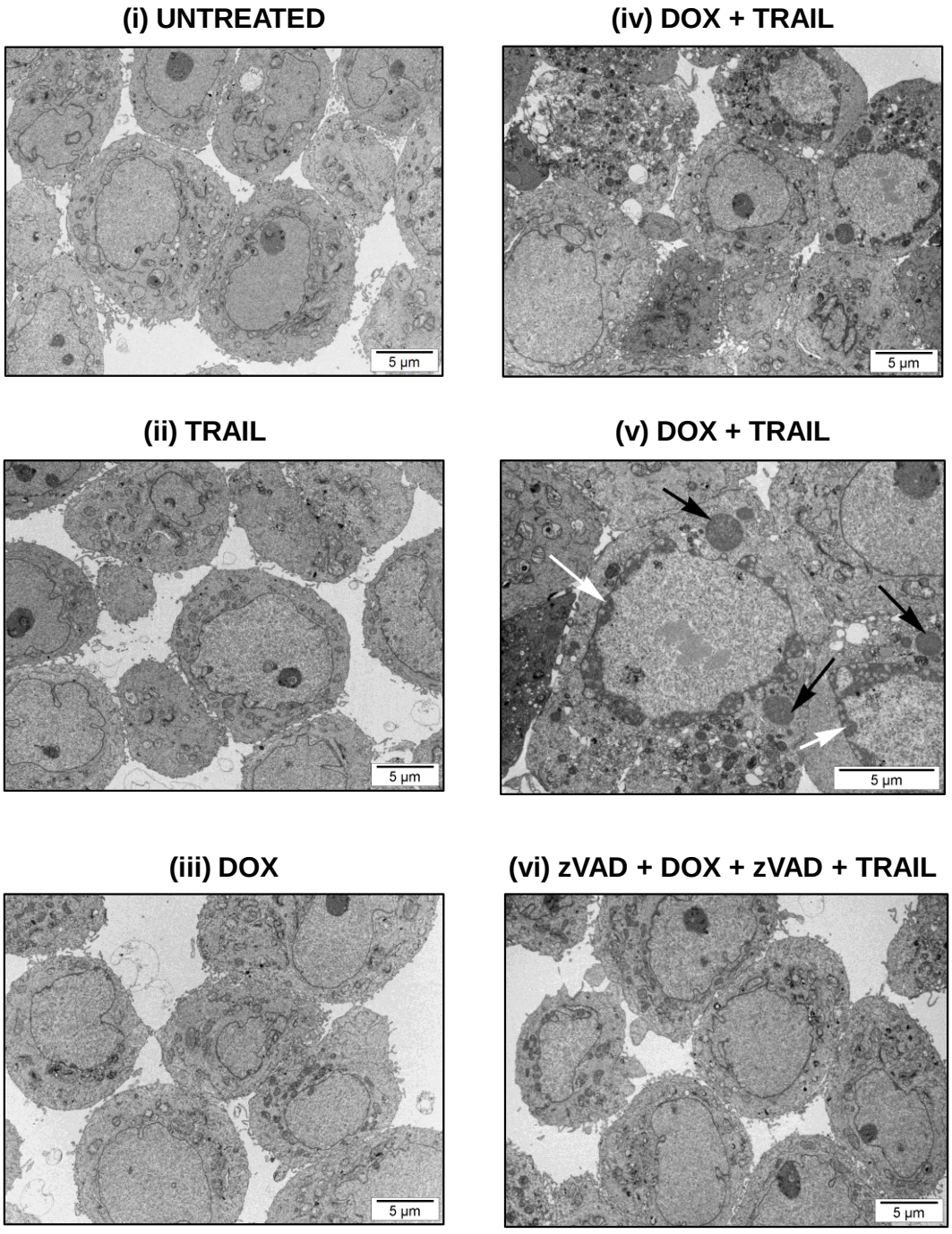


Figure 4.9 Electron micrographs confirm apoptosis to be the major cell death pathway triggered by TRAIL in doxorubicin pre-treated T47D cells

T47D cells were pre-treated with doxorubicin (1 µM) followed by TRAIL (1000 ng/ml) for 16 h in the absence or presence of zVAD and then fixed. Electron micrographs were taken and representative images are shown (Electron microscopy performed by David Dinsdale). Black arrows indicate cytoplasmic inclusions and white arrows indicate condensed chromatin.

4.8 T47D cells pre-treated with a clinically relevant concentration of doxorubicin (1 μM) signal to TRAIL-induced apoptosis predominantly through TRAIL-R1

T47D cells were pre-treated with doxorubicin (1 μM) for 16 h followed by addition of TRAIL, R1L or R2L. In these experiments, TRAIL and more importantly, R1L and R2L were used at different concentrations to explore the possibility of achieving a significant difference between the apoptosis induced by these agents at lower concentrations.

In T47D cells pre-treated with doxorubicin (1 μM), both TRAIL and R1L induced apoptosis from concentrations as low as 100 ng/ml (Figure 4.10; Part A; Blue & Red lines). There was a steady, yet comparable increase in apoptosis induced by increasing concentrations of both TRAIL and R1L. Importantly, R2L was significantly less potent than R1L and failed to induce significant apoptosis at similar concentrations of 100 ng/ml (Figure 4.10; Part A; Green line). Even at the maximum concentration, R2L (1000 ng/ml) induced only ~20% apoptosis in T47D cells as compared to the ~50% apoptosis induction by R1L (Figure 4.10; Part A; Red & Green lines).

The inefficiency of R2L to signal to apoptosis was also reflected in the lesser extent of processing of caspase-8 and caspase-3 as compared with R1L (Figure 4.10; Part B; Lanes 9-12 & 16-19). The lack of equivalent caspase-8 activation by R2L indicates that the DISC formed by R2L is not as active as the R1L-induced DISC. Lack of effective caspase-3 cleavage could further be attributed to the inability of the low levels of active caspase-8 formed to activate the mitochondrial amplification loop *via* cleavage of Bid. The relatively lower cleavage of the caspase substrate PARP is also an indicator of the lower potency of R2L than R1L.

Thus, it can be concluded that pre-treatment with doxorubicin at a clinically relevant concentration of 1 μM sensitises T47D cells to undergo TRAIL-induced apoptosis and signalling to apoptosis is predominantly mediated *via* TRAIL-R1.

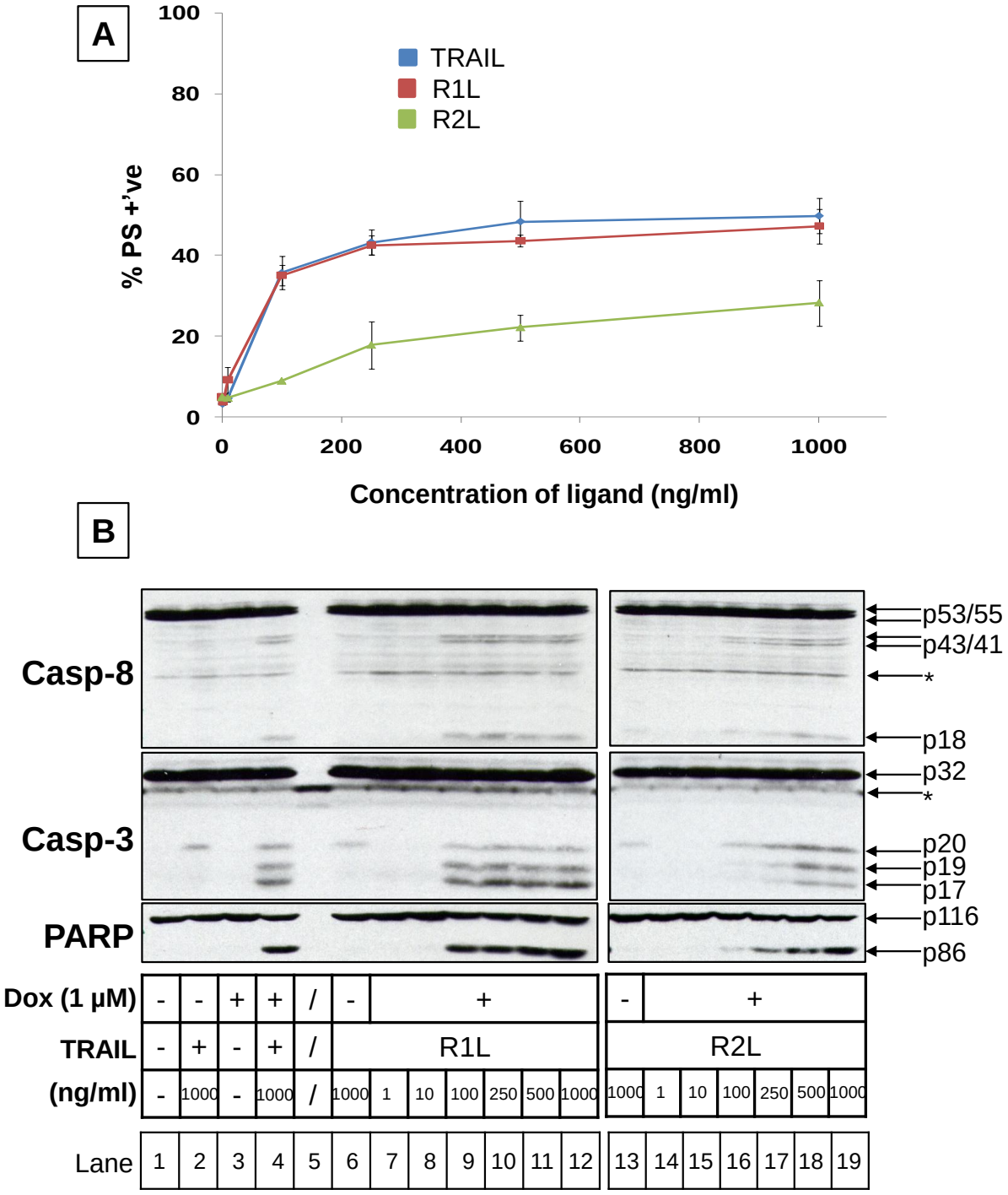


Figure 4.10 Doxorubicin (1 µM) sensitises T47D cells more to TRAIL and R1L than R2L

T47D cells were seeded and following pre-treatment with doxorubicin (1 µM) were treated with TRAIL, R1L and R2L at increasing concentrations for 6 h. Cells were harvested and used for Annexin V-FITC binding assay ((A); data shown as mean ± SEM (n=3)) and SDS-PAGE/Western blotting ((B); data representative of two independently done experiments). * indicates non-specific band.

4.9 R1L-induced apoptosis is initiated more rapidly than R2L-induced apoptosis

We further decided to study the efficiency of R1L at inducing apoptosis in T47D cells at shorter treatment times. Untreated T47D cells served as a negative control, while T47D cells pre-treated for 16 h with doxorubicin followed by 6 h with TRAIL at 1000 ng/ml were used as positive control.

The doxorubicin pre-treatment time of 16 h was kept constant and subsequently T47D cells were treated with R1L or R2L for 1 h, 2 h, 4 h or 6 h. As seen earlier, T47D cells were resistant to both doxorubicin and R1L/R2L as single agents (Figure 4.11; Lanes 2, 4 & 10). Similarly, when doxorubicin pre-treated T47D cells were treated with R1L or R2L for 1 h, both agents failed to induce apoptosis (Figure 4.11; Lanes 5 & 11).

When the TRAIL mutant ligand treatment time was increased to 2 h, R1L induced ~20% apoptosis, while R2L did not have any apoptotic effect on T47D cells (Figure 4.11; Lanes 6 & 12). This indicates that the kinetics of apoptotic signalling *via* TRAIL-R1 is faster than *via* TRAIL-R2. This suggestion is further confirmed by the observation that R2L induced a significant amount of apoptosis only after 6 h of addition to doxorubicin pre-treated cells (Figure 4.11; Lane 14). In contrast, R1L induced a modest amount of apoptosis only after 2 h of addition to doxorubicin pre-treated T47D cells and the extent of apoptosis induced increased significantly with treatment time (Figure 4.11; Lanes 6-8).

This experiment thus shows that TRAIL-R1 is the more potent TRAIL death receptor in T47D cells exposed to soluble TRAIL and that apoptotic signalling through TRAIL-R1 is more rapid than *via* TRAIL-R2.

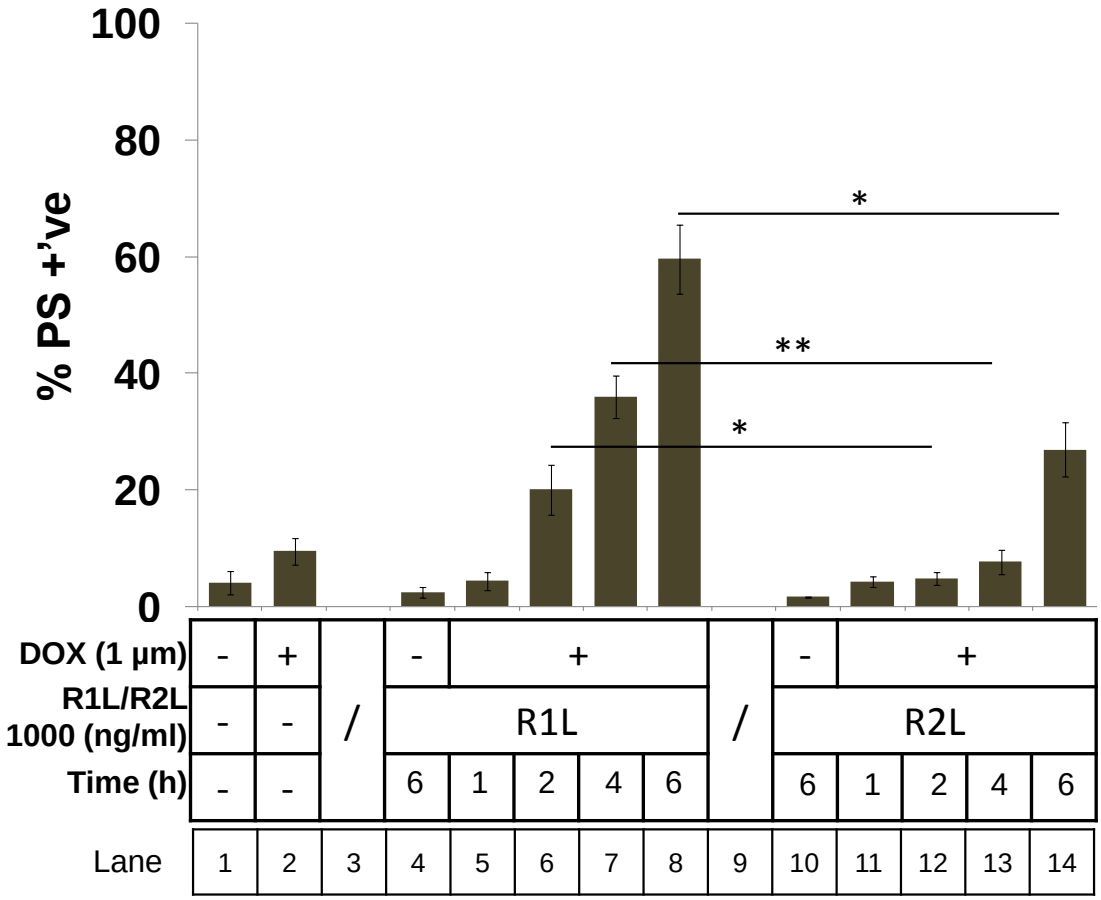


Figure 4.11 R1L signals to apoptosis in doxorubicin pre-treated T47D cells more rapidly than R2L

T47D cells were seeded and treated with doxorubicin for 16 h, followed R1L or R2L (1000 ng/ml) for 6 h. Subsequently the cells were harvested and one third of the sample was used for Annexin V-FITC analysis (data represented as mean ± SEM (n=3)) ** and * indicate significant difference ($P<0.01$ and $P<0.05$ respectively).

4.10 R1L causes increased activation of the mitochondrial amplification arm as compared with R2L in doxorubicin pre-treated T47D cells

We next sought to study activation of the mitochondrial amplification arm of the apoptotic pathway by the mutant TRAIL ligands, R1L and R2L. In this case, loss of MMP could not be used as a measure of apoptosis due to interference in TMRE fluorescence by doxorubicin. Hence we instead chose to study the activation of Bax as an indicator of the activation of the intrinsic pathway.

Since activation of Bax is a relatively upstream event in the apoptotic pathway, a shorter treatment time was used for this particular assay. T47D cells were pre-treated with 1 µM doxorubicin for 16 h followed by TRAIL/R1L/R2L at 1000 ng/ml for 3 h. Bax activation assay was then performed as described in Materials and Methods; Section 2.11. As expected, neither TRAIL nor the TRAIL mutant ligands induced any Bax activation (Figure 4.12; Panels ii-iv). Similarly, doxorubicin itself did not lead to any significant activation of Bax (Figure 4.12; Panel v). Importantly, and consistent with the data obtained so far, doxorubicin and TRAIL combination treatment induced significant activation of Bax (Figure 4.11; Panel vi) and characteristic nuclear changes including condensation and blebbing. R1L also caused a change in Bax conformation when used in combination with doxorubicin (Figure 4.12; Panel vii). Notably, the treatment of doxorubicin and R2L did not lead to any increase in Bax activation compared to that seen in the untreated T47D cells (Figure 4.12; Panel viii).

The results obtained from this experiment further emphasise the importance of TRAIL-R1 as the major TRAIL death-signalling receptor in T47D cells. The more efficient activation of the mitochondrial amplification loop observed with R1L could also possibly contribute to the faster signalling to apoptosis previously seen with R1L (Figure 4.11). It is also interesting to note that in T47D cells, even in the presence of doxorubicin, R1L does not show significantly higher potency than TRAIL. This trend has remained consistent across all the breast cancer cell lines we have tested. Importantly though, R1L has remained significantly more potent than R2L across all the six breast cancer cell lines.

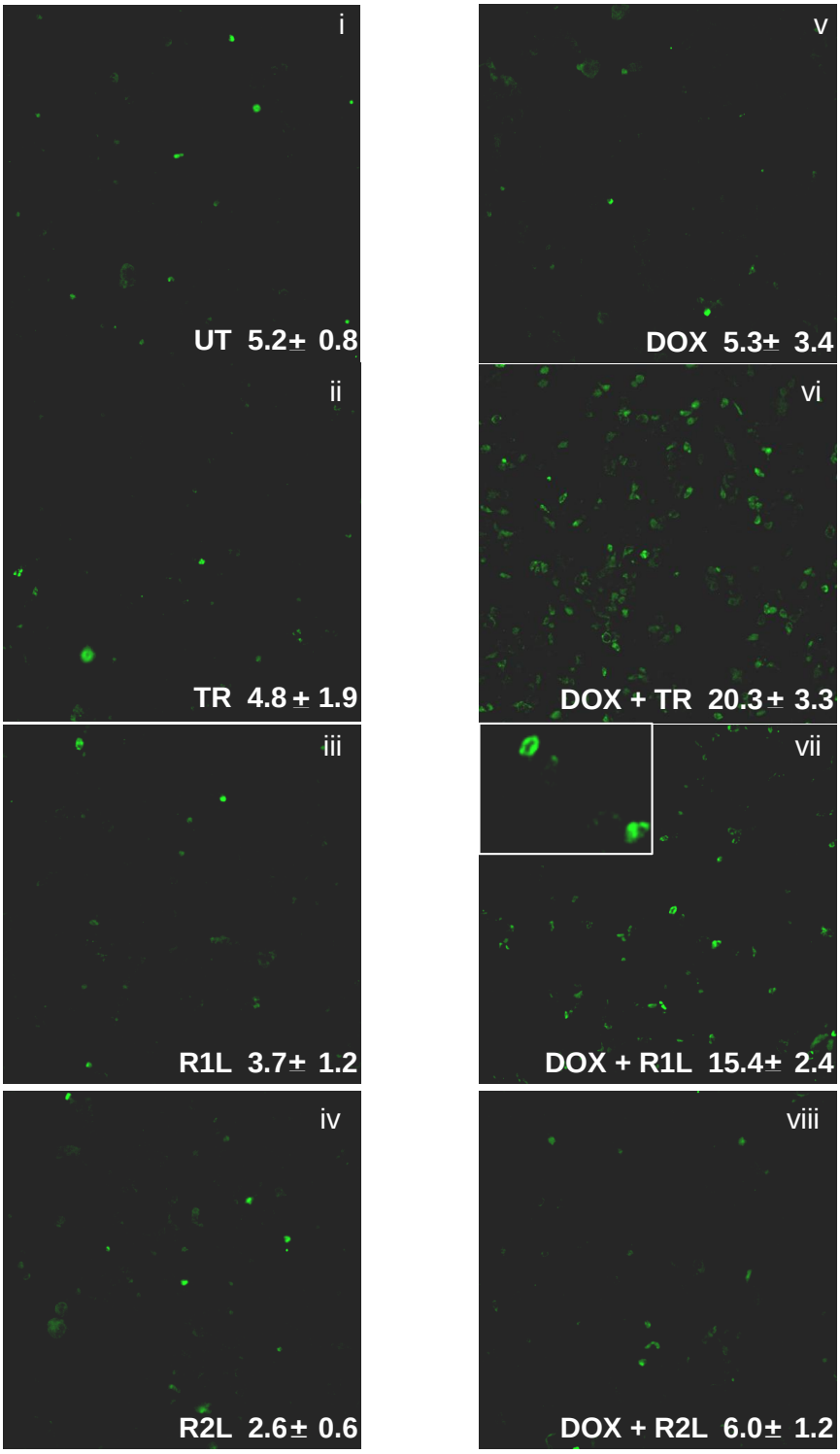


Figure 4.12 Doxorubicin facilitates efficient activation of Bax by TRAIL in T47D cells

T47D cells were seeded and treated with doxorubicin (1 µM) alone, TRAIL/R1L/R2L (1000 ng/ml) or doxorubicin for 16 h followed by TRAIL/R1L/R2L for 3 h. After treatment time the T47D cells were immunostained for active Bax (inset) and fluorescence was measured (data shown represented as mean ± SEM (n=3)).

4.11 Doxorubicin induces an up-regulation of TRAIL-R2 on T47D cell surface

It has been reported previously that treatment with doxorubicin results in an up-regulation of TRAIL-R2 and this up-regulation has often been attributed as the mechanism of doxorubicin-mediated TRAIL sensitisation (Keane *et al.,* 1999; Singh *et al.,* 2003; Wu *et al.,* 2007). However, these studies have only checked the total protein or mRNA levels of TRAIL-R2 pre- and post- doxorubicin treatment. In contrast, our view is that up-regulation of death receptors on the cell surface is key in terms of potentially influencing the binding of TRAIL and consequent signalling through the receptor. Hence, we studied the effect of doxorubicin pre-treatment on the cell surface level of both TRAIL-R1 and TRAIL-R2 in T47D cells.

We chose both the clinically relevant concentration of 1 µM and the supra-clinical concentration of 5 µM doxorubicin to study whether there were also any concentration-dependent variations in doxorubicin-mediated up-regulation of TRAIL-R2. In addition, T47D cells were treated with these two concentrations of doxorubicin for two different time points, 4 h and 16 h. Importantly, our preliminary studies had shown that treating T47D cells with doxorubicin for 4 h was incapable of sensitising the cells to TRAIL (data not shown). We therefore chose this time point as a control to rule out any changes caused by doxorubicin at this time as these are insufficient to cause sensitisation on their own. Cell surface receptor expression was measured using unlabelled TRAIL-R1 and TRAIL-R2 antibodies as described in Materials and Methods; Section 2.14. The fluorescence values for TRAIL-R1 and TRAIL-R2 for the various treatments were normalised to their corresponding secondary antibody fluorescence values.

None of the different treatment regimes of doxorubicin induced any changes to the level of TRAIL-R1 on the cell surface of T47D cells (Figure 4.13; Red bars). However, consistent with earlier reports, doxorubicin caused a significant up-regulation of the cell surface levels of TRAIL-R2 (Figure 4.13; Blue bars). This up-regulation of TRAIL-R2 was induced by both clinical and supra-clinical concentrations of doxorubicin after 16 h of doxorubicin treatment.

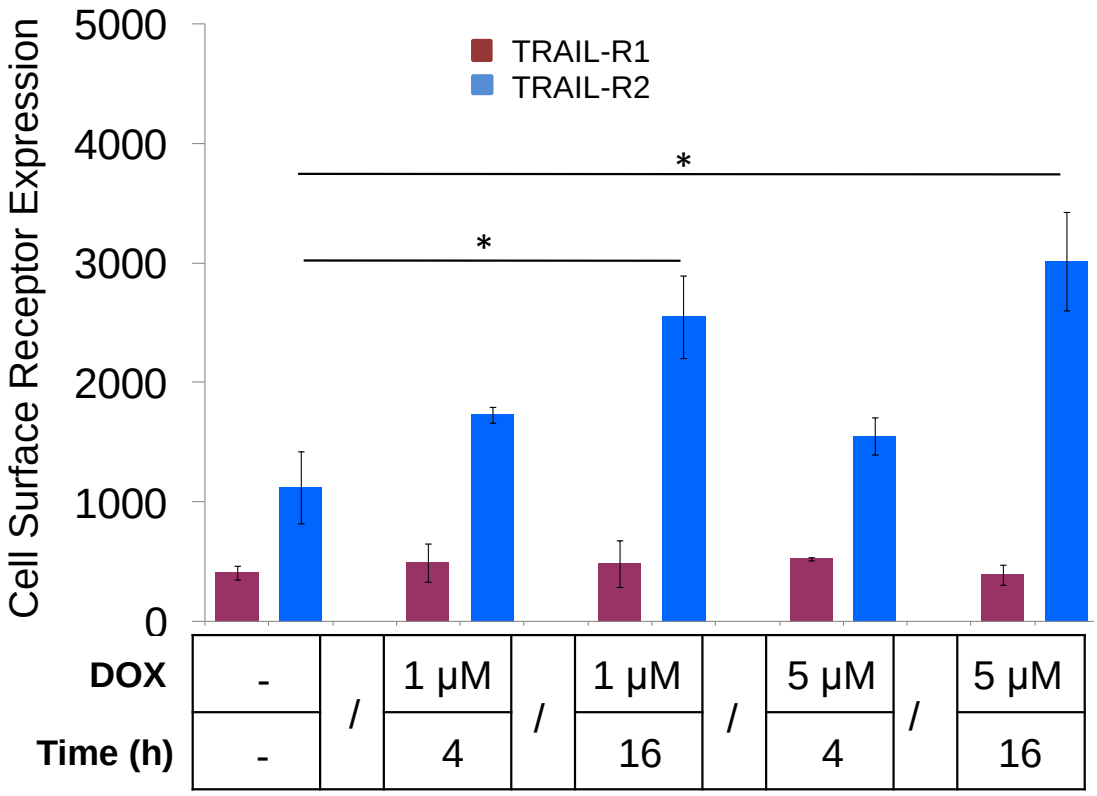


Figure 4.13 Doxorubicin causes an up-regulation of TRAIL-R2 in T47D cells

T47D cells were seeded and treated with doxorubicin at indicated concentrations for 0 h, 4 h or 16 h and samples were collected to determine cell surface receptor expression of the TRAIL receptors by flow cytometry as mentioned in Materials and Methods (data shown represented as mean ± SEM (n=3)). * indicates significant difference ($P<0.05$).

4.12 Doxorubicin enhances TRAIL DISC formation in T47D cells

Enhanced TRAIL DISC formation upon treatment with DNA damaging agents or HDAC inhibitors has been widely reported (Lacour *et al.,* 2003; Ganten *et al.,* 2004; Inoue *et al.,* 2004; MacFarlane *et al.,* 2005a; Inoue *et al.,* 2009). The increase in cell surface TRAIL receptor expression levels might be a potential reason for the formation of this stronger DISC. We therefore, studied the formation of TRAIL DISC in the presence or absence of doxorubicin. In these experiments, unstimulated ligand immunoprecipitations were used as negative controls.

In stimulated control T47D cells, biotinylated TRAIL recruited both TRAIL-R1 and TRAIL-R2, a slight amount of FADD and caspase-8 (Figure 4.14; Lane 2). Importantly, pre-treating T47D cells with doxorubicin, lead to increased recruitment of both splice variants of TRAIL-R2 to the DISC (Figure 4.14; Lane 5). This increased recruitment of TRAIL-R2 is concomitant with the doxorubicin-induced up-regulation of the cell surface expression of TRAIL-R2 (Figure 4.13). The level of TRAIL-R1 observed in the DISC remained similar irrespective of doxorubicin pre-treatment (Figure 4.14; Lanes 2 & 5). Crucially though, there was a dramatic increase in the amount of FADD and caspase-8 recruited to the DISC following doxorubicin pre-treatment (Figure 4.14; Lanes 2 & 5). This was also reflected by a subsequent increase in caspase-8 cleavage. These data suggest that the formation of a stronger DISC could be a key event mediating doxorubicin-induced TRAIL sensitisation of T47D cells. Concomitant with the increase in FADD, there was also an increase in recruitment of $FLIP_L$ and RIP1 to the DISC (Figure 4.14; Lanes 2 & 5). However, the increased recruitment of $FLIP_L$ was accompanied by an increase in $FLIP_L$ cleavage to its p43 fragment by active caspase-8 and the cleavage thus, reflects the increased extent of caspase-8 activation. $FLIP_L$ also appeared to be recruited into the unstimulated TRAIL DISC but the extent of this recruitment was not affected by pre-treatment with doxorubicin (Figure 4.14; Lanes 1 & 4). The recruitment of $FLIP_L$ to the unstimulated ligand-receptor complex has been observed previously, however its exact mechanism is unclear (Harper *et al.,* 2001). It is interesting to observe that though doxorubicin induced an increase in the recruitment of FADD, RIP, caspase-8 and $FLIP_L$ to the DISC, it had no significant effect on the expression of these proteins (Figure 4.14 and Figure 4.18). These data suggest that the effect of the doxorubicin which mediates its sensitisation ability is receptor-proximal and not dependent on the transcriptional up-regulation of these proteins.

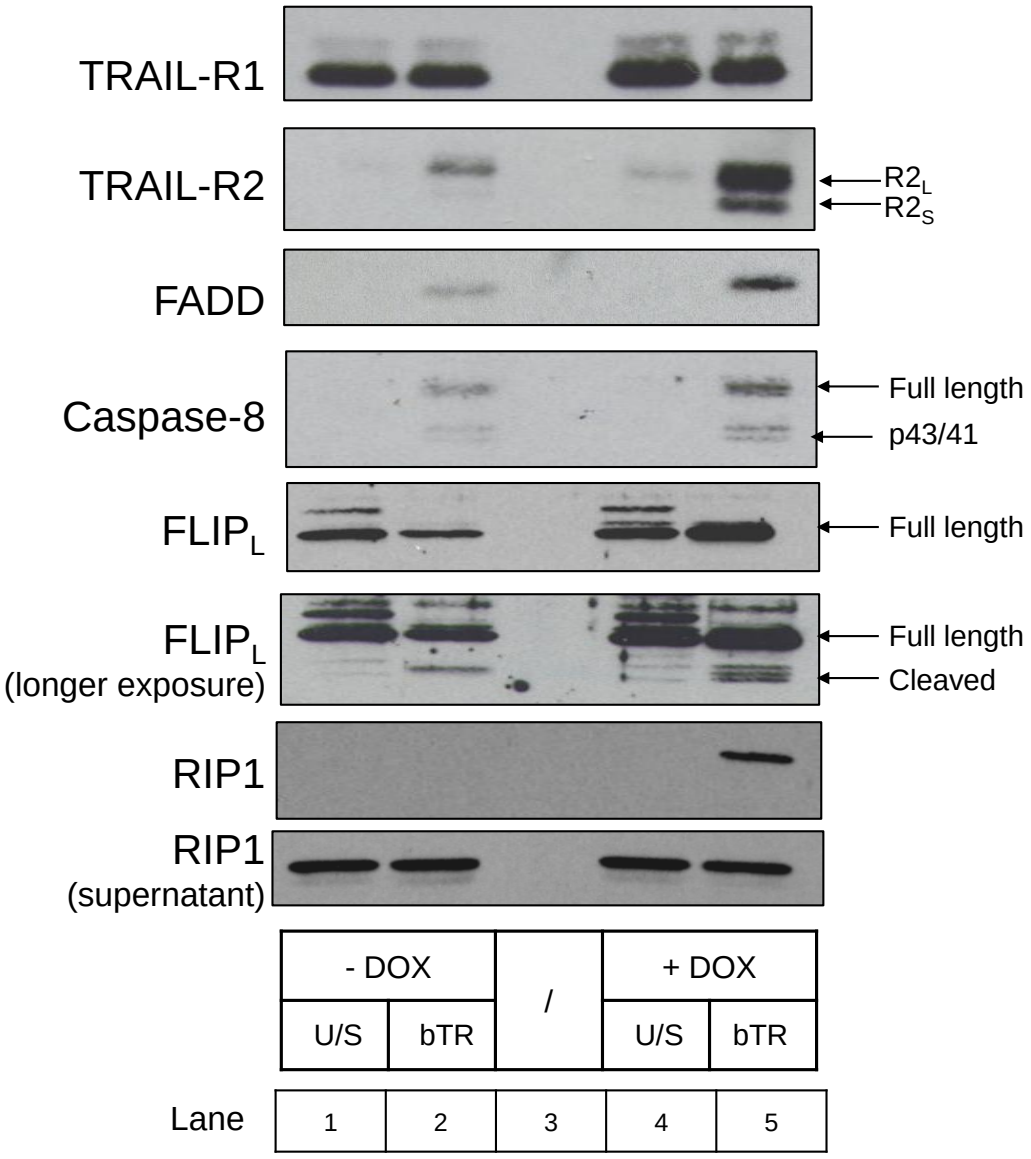


Figure 4.14 Doxorubicin causes formation of an active TRAIL DISC in T47D cells
T47D cells were seeded at a density of 6.25 X 10^6 cells/flask for untreated cells and 6.5 X 10^6 cells/flask for doxorubicin treatment. Following treatment, DISC was immunoprecipitated using biotinylated TRAIL (b-TR) and samples were collected for SDS-PAGE/Western blotting as mentioned in Materials and Methods (data shown representative of three independently performed experiments).

Based on the above data, it could be suggested that the doxorubicin-dependent up-regulation of TRAIL-R2 on the surface of T47D cells may contribute to the more efficient DISC formation and would thus be an important mechanism of sensitisation. However, this would argue against earlier data which showed TRAIL-R1 to be the main apoptosis-signalling receptor in these cells (Figure 4.10). We therefore, decided to further explore this question using biotinylated versions of the mutant variants of TRAIL to identify which of the two TRAIL death receptors was involved in forming an active TRAIL DISC.

4.13 Exposure to R1L but not R2L results in formation of an active TRAIL DISC

DISC immunoprecipitations carried out using biotinylated versions of mutant TRAIL specific to TRAIL-R2 further provided confirmation of the strong up-regulation of TRAIL-R2 by doxorubicin (Figure 4.15; Lanes 4 & 9). However, in contrast to previous reports, the increased TRAIL-R2 failed to recruit any FADD or caspase-8 and thus did not form an active TRAIL DISC (Figure 4.15; Lane 9).

On the other hand, doxorubicin did not cause any changes in the levels of TRAIL-R1 recruited to the DISC (Figure 4.15; Lanes 2 & 7). Significantly though, in the presence of doxorubicin, TRAIL-R1 recruited an increased amount of FADD and subsequently caspase-8 to form a more stable TRAIL DISC (Figure 4.15; Lane 7). There was also more processing of caspase-8 to its p43/41 subunits indicating the activation of caspase-8, which can then activate the caspase cascade downstream. The results from this experiment comprehensively show that in the presence of doxorubicin, only TRAIL-R1 is capable of forming an active TRAIL DISC *via* recruitment of FADD and caspase-8.

As seen previously with the MCF7 F43 and MDA-MB-231 cells (Figures 3.2 & 3.4), in the T47D cells, R1L also pulled in TRAIL-R2 to a small extent (Figure 4.15; Lane 7). Importantly, this TRAIL-R2 recruitment was not seen with the unstimulated R1L pull down, thus confirming that it is not the result of non-specific binding of R1L to TRAIL-R2.

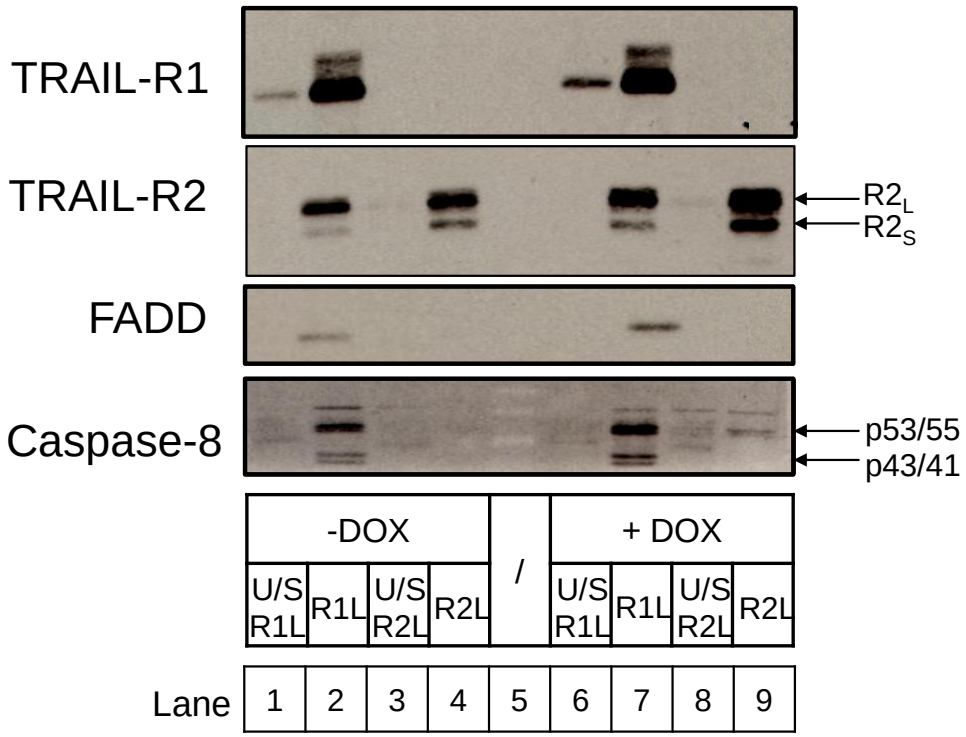


Figure 4.15 Caspase-8 and FADD are recruited exclusively to TRAIL-R1 in doxorubicin pre-treated T47D cells

T47D cells were seeded at a densityof 6.0 X 10^6 cells/flask for untreated cells and 6.25 X10^6 cells/flask for doxorubicin treatment. Following treatment, DISC was immunoprecipitated using biotinylated R1L or R2L and samples were collected for SDS-PAGE/Western blotting as mentioned in Materials and Methods (data shown representative of three independently performed experiments).

In this regard, it is important to note that the formation of a TRAIL-R1/-R2 heterocomplex has been consistently observed in the MCF7 F43, MDA-MB-231 and T47D cells in response to R1L. It is therefore possible that after stimulation, TRAIL-R1 associates with TRAIL-R2 and the formed heterocomplex might be more active at signalling to apoptosis than either receptor alone. If this is the case, then although TRAIL-R2 alone fails to recruit any FADD and caspase-8, it might still play an important role in TRAIL and even in R1L-mediated apoptosis. We therefore, decided to specifically deplete TRAIL-R2 from T47D cells and determine whether this had any effect on TRAIL-induced apoptosis following exposure to doxorubicin.

4.14 RNAi targeting of TRAIL-R1 and TRAIL-R2 in T47D cells

Effectene failed to induce efficient knockdown of proteins in T47D cells (data not shown) and hence, lipofectamine was used as transfection agent for carrying out siRNA based protein knock down in these cells (Materials and Methods; Section 2.15).

In the untreated samples, RNAi targeting of TRAIL-R1 or TRAIL-R2 resulted in significant decrease of the cell surface expression of the respective receptors (Figure 4.16; Part A). Similar to previous data, exposure to doxorubicin caused an increase in expression of TRAIL-R2, but not TRAIL-R1. Importantly, an efficient RNAi knockdown of even the elevated TRAIL-R2 levels could be achieved. Surprisingly, when TRAIL-R2 expression on the surface was decreased, this resulted in an increase in the TRAIL-R1 surface expression. The reason for this increase is unclear, but it could possibly be a compensatory mechanism to maintain a TRAIL receptor concentration threshold on the cell surface.

As expected, the knockdown of TRAIL-R1 or TRAIL-R2 did not affect the resistance of T47D cell line to doxorubicin (data not shown). Interestingly though, the knockdown of TRAIL-R2 induced a slight sensitisation of T47D cells to TRAIL, even in the absence of doxorubicin (Figure 4.15; Part B).

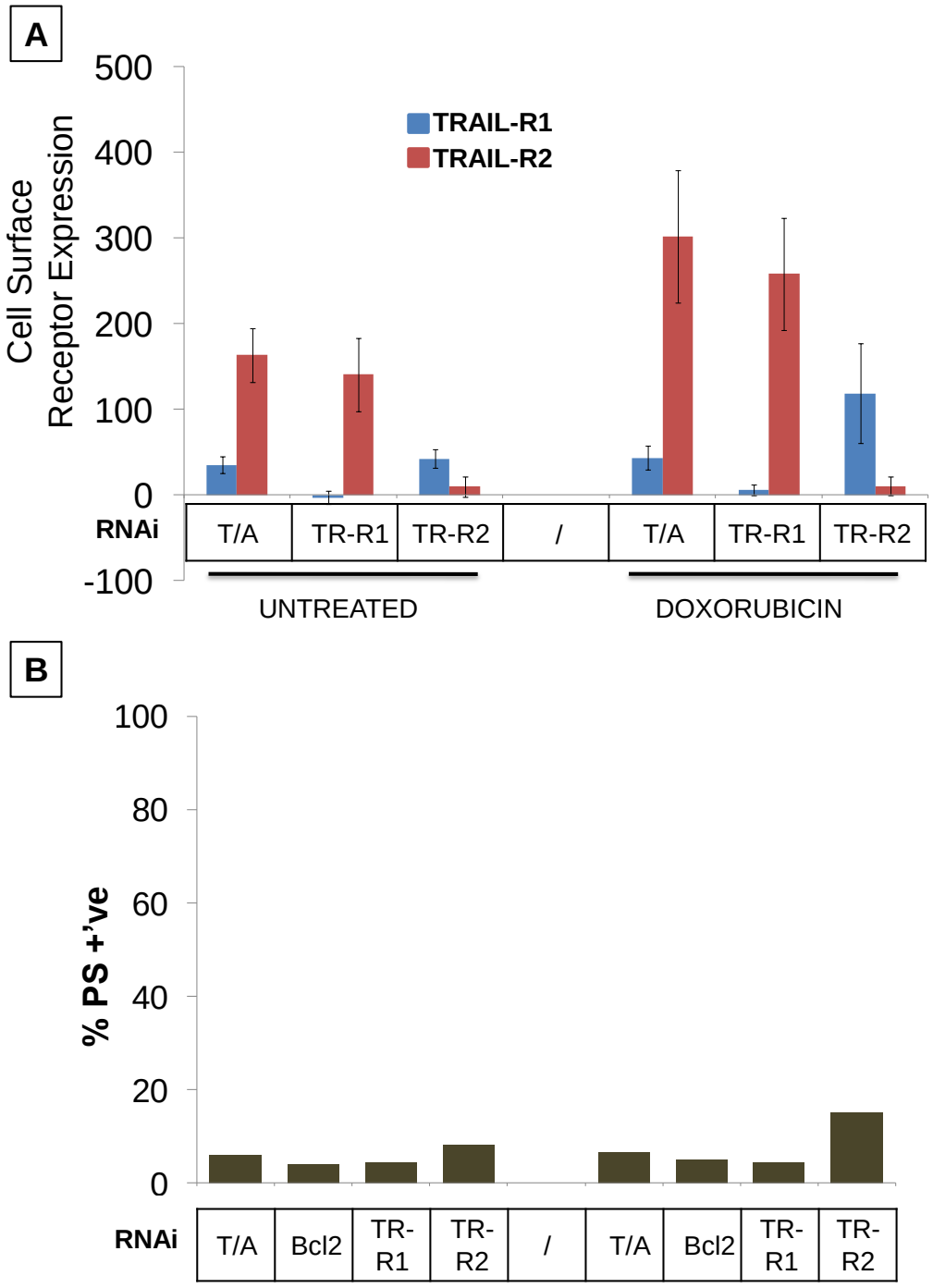


Figure 4.16 Knockdown of TRAIL-R2 slightly sensitises T47D cells to TRAIL-induced apoptosis

T47D cells were seeded and RNAi knockdown of the TRAIL death receptors was performed and confirmed using flow cytometry ((A); data represented as mean ± SEM (n=3)). Subsequently the cells were treated with TRAIL (1000 ng/ml; 6 h) and samples were analysed for Annexin V-FITC labelling ((B); data represented as average (n=2)). T/A = Transfection agent; TR-R1/R2 = TRAIL-R1/TRAIL/R2.

This modest sensitisation might be because of the increased TRAIL-R1 expression (Figure 4.16; Part A) in response to TRAIL-R2 depletion or inhibition of an alternative pro-survival signalling mediated through TRAIL-R2. Irrespective of the cause, it is important to note that this TRAIL-induced apoptotic signalling is mediated *via* TRAIL-R1 and this again emphasises that indeed TRAIL-R1 is the pre-dominant TRAIL death receptor signalling to apoptosis in T47D cells.

4.15 Doxorubicin-mediated TRAIL sensitisation in T47D cells occurs primarily through TRAIL-R1

We further studied the effect of RNAi targeting of TRAIL-R1 or TRAIL-R2 on doxorubicin-mediated TRAIL signalling to apoptosis. siRNA transfections were performed 24 h before treatment with doxorubicin to ensure maximal depletion of endogenous and doxorubicin-induced TRAIL-R2 expression. Since T47D cells lack Bcl-2 expression (Appendix 3), an oligonucleotide to Bcl-2 was chosen as an appropriate negative control for the experiments. As mentioned before, the transfections did not have any effect on untreated T47D cells (Figure 4.17; Part A; Lanes 1-4). Lipofectamine alone or transfection with siRNA for Bcl-2 did not prevent doxorubicin-mediated TRAIL sensitisation of T47D cells (Figure 4.17; Part A; Lanes 6 & 7). Significantly, knockdown of TRAIL-R1 abrogated TRAIL-mediated apoptosis, as indicated by lack of PS exposure, caspase-8 processing and PARP cleavage (Figure 4.17; Parts A & B; Lane 8). These data, thus provide further confirmation that doxorubicin sensitises T47D cells to TRAIL-induced apoptosis *via* TRAIL-R1.

Also, in T47D cells, the knockdown of TRAIL-R2 failed to inhibit TRAIL-induced apoptosis in the presence of doxorubicin (Figure 4.17; Part A; Lane 9). This further provides proof that TRAIL-R1 is the dominant death-inducing TRAIL-receptor in T47D cells. It can thus, be concluded that in all the breast cancer cell lines we have tested so far, TRAIL-R1 is the pre-dominant TRAIL death receptor involved in signalling to apoptosis.

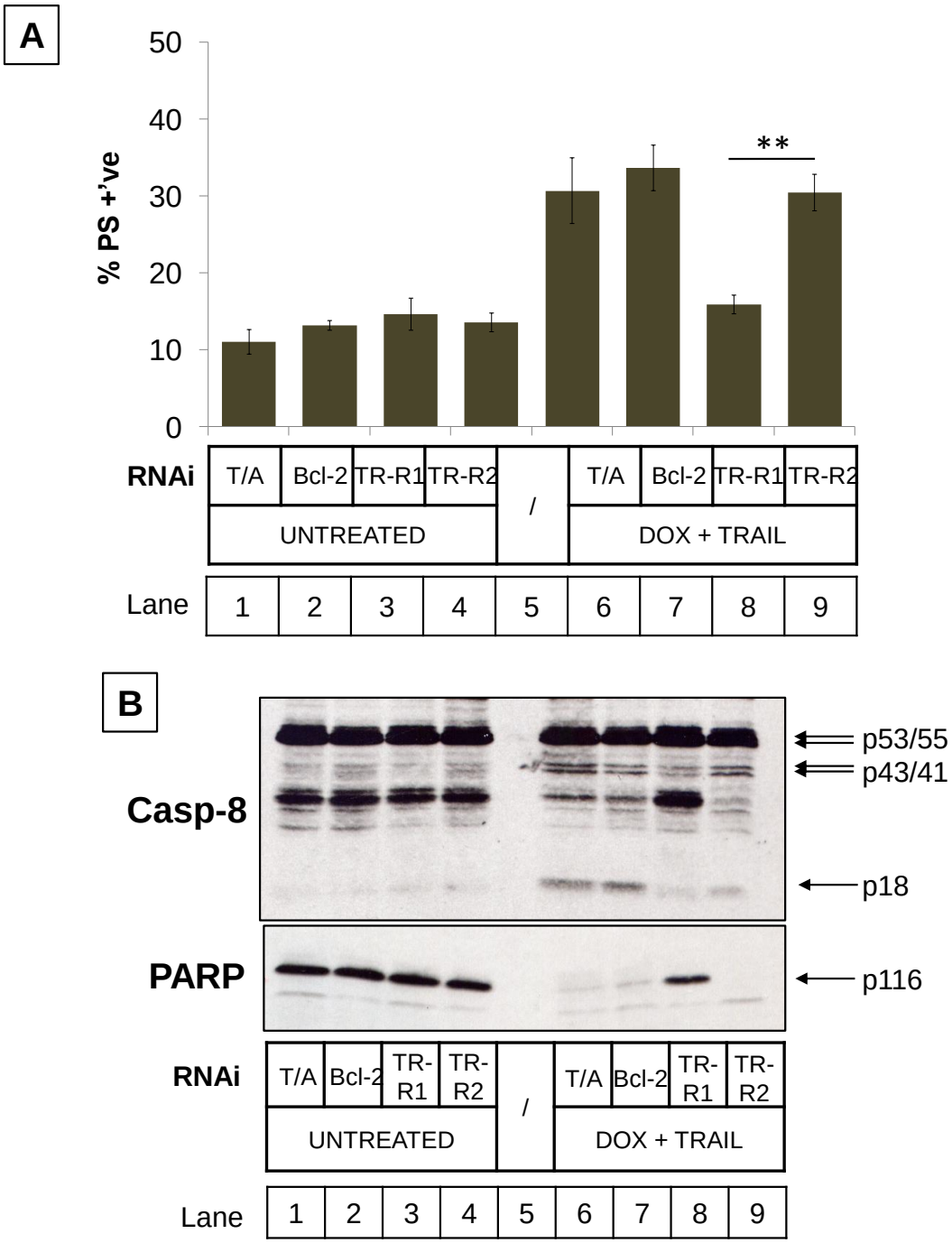


Figure 4.17 TRAIL-induced apoptosis in doxorubicin pre-treated T47D cells occurs *via* TRAIL-R1

RNAi knockdown of TRAIL death receptors was carried out in T47D cells and subsequently the cells were treated with doxorubicin (1 µM) and TRAIL (1000 ng/ml) and samples were analysed for Annexin V-FITC labelling ((A); data represented as mean ± SEM (n=4)) and SDS-PAGE/Western blotting ((B); data shown is representative of two independently performed experiments). ** indicates significant difference ($P<0.01$). T/A = Transfection agent; TR-R1/R2 = TRAIL-R1/TRAIL/R2.

4.16 Protein expression changes induced by doxorubicin

Doxorubicin can affect the expression of many pro- and anti-apoptotic proteins (Singh *et al.,* 2003). It was therefore, important to check the effect of doxorubicin on many of the key molecules regulating the TRAIL apoptotic pathway. Doxorubicin induced an increase in the phosphorylation of H2AX (γH2AX) which is an indicator of DNA damage (Figure 4.18; Part A; Rogakou *et al.,* 1998). γH2AX formation was observed 16 h after doxorubicin addition, but not after 4 h, which is consistent with earlier data showing that doxorubicin caused cell cycle arrest after 16 h, but not 4 h of treatment (Appendix 5). However, since T47D cells harbour mutant p53 (Neve *et al.,* 2006), the observed DNA damage could not induce activation of the p53 pathway which was reflected by the lack of up-regulation of p21 (Figure 4.18; Part A; El-Deiry *et al.,* 1993). However, the addition of doxorubicin did not significantly affect any of the known proteins involved in the extrinsic/intrinsic apoptotic pathway (Figure 4.18; Part A). Importantly, it has been suggested that doxorubicin modulates the expression levels of Bax and $FLIP_L$ and thus, affects TRAIL sensitisation (Leung and Wang, 1999; Kelly *et al.,* 2002). However, at the clinically relevant concentration of 1 μM, doxorubicin did not affect the expression of either of these proteins in T47D cells.

Further, it has been reported that doxorubicin can cause down-regulation of Myc protein in MCF7 cells (Fornari *et al.,* 1996). Recent data has suggested that this decrease in expression may be possibly mediated *via* miR-34c up-regulation (Cannell *et al.,* 2010; Cannell and Bushell, 2010). We therefore, examined the effect of doxorubicin on expression of miR-34c in T47D cells. Expectedly, doxorubicin also caused an up-regulation of miR-34c in T47D cells (Appendix 6) and a subsequent down regulation of Myc expression (Figure 4.18; Part A). To study whether doxorubicin-mediated Myc down-regulation was important for TRAIL sensitisation, oligonucleotide knock down of Myc protein was performed (Figure 4.18; Part B), but this did not sensitise T47D cells to TRAIL-induced apoptosis (Figure 4.18; Part C). These data indicate that Myc down-regulation is not the sole important sensitising event, but it does not provide absolute evidence that Myc down-regulation does not play any role in the sensitisation.

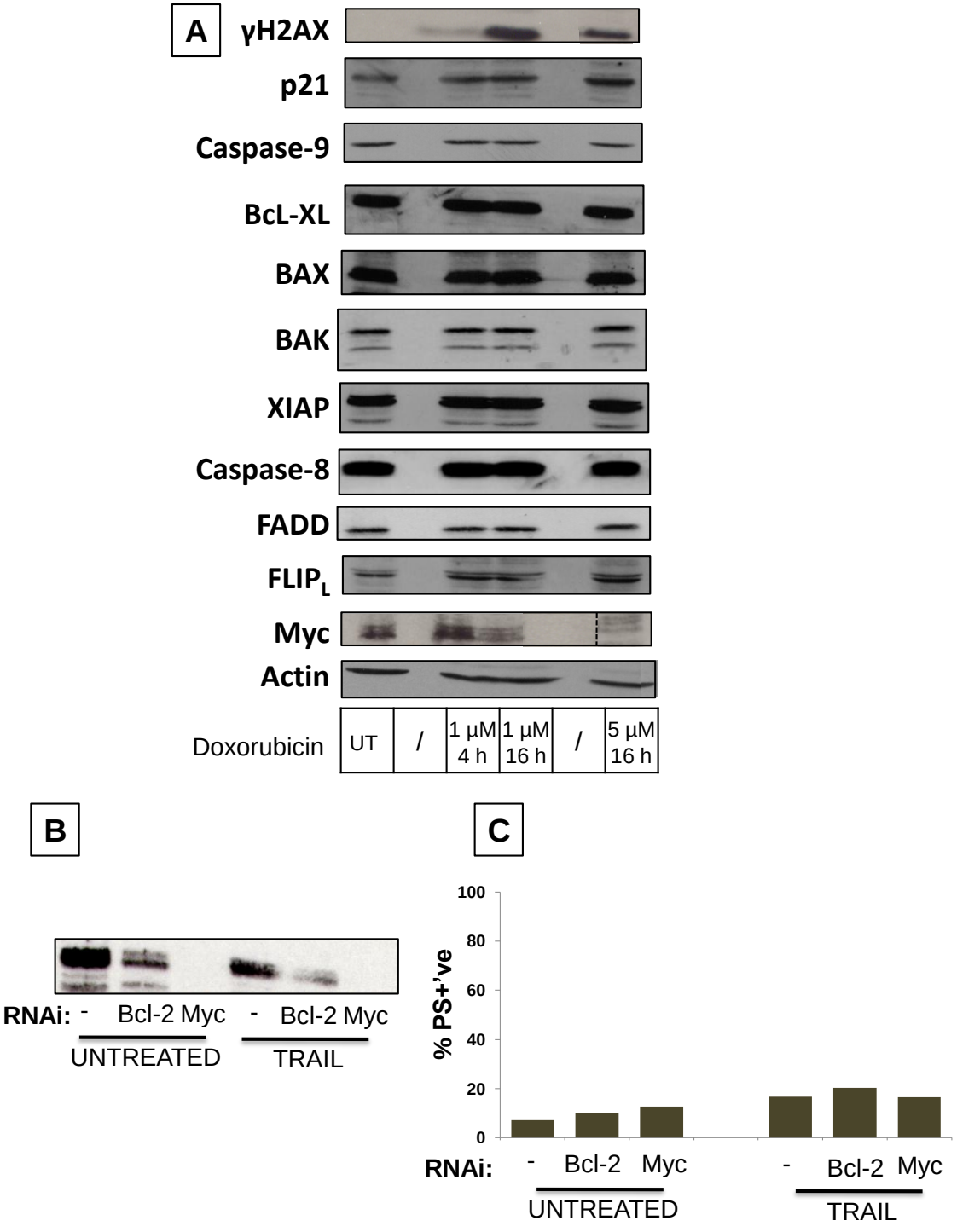


Figure 4.18 Doxorubicin causes down regulation of Myc in T47D cells

T47D cells were treated with doxorubicin (1 µM or 5 µM) for 4 h or 16 h and subsequently harvested in 2X SDS sample buffer and loaded onto SDS-PAGE gels \ western blotting (A) of different pro- and anti-apoptotic proteins. T47D cells were seeded and siRNA knockdown of Myc (B) was performed and cells were treated with TRAIL (1000 ng/ml) for 6 h and samples were taken for Annexin V-FITC ((C); data represented as average (n=2)).

4.17 Doxorubicin, at 1 µM, does not cause reactive oxygen species (ROS) formation in T47D cells

Doxorubicin has also been known to cause formation of ROS *in vitro* at supra-clinical concentrations; however, the importance of ROS formation under physiological concentrations of doxorubicin is unknown (reviewed in Gewirtz, 1999). There has been speculation that ROS formation might also play a significant role in TRAIL sensitisation of tumour cells (Jung *et al.,* 2005). It was therefore decided to test whether doxorubicin at 1 µM induced any ROS formation in T47D cells.

T47D cells were treated with doxorubicin at 1 µM for 16 h and ROS formation was measured using the DCFDA assay as outlined in Materials and Methods; Section 2.16. Hydrogen peroxide was used as a positive control and induced a significant increase in ROS formation (Figure 4.19). Moreover, doxorubicin did induce a modest increase in ROS formation at the supra-clinical concentration of 5 µM (data not shown). However, doxorubicin at 1 µM did not induce any ROS formation after 16 h (Figure 4.19), indicating that there was no influence of ROS in doxorubicin-mediated sensitisation of T47D cells to TRAIL-induced apoptosis at clinically relevant concentrations.

4.18 Flavopiridol sensitises T47D cells to TRAIL-induced apoptosis *via* TRAIL-R1

To confirm the dominance of TRAIL-R1 in T47D cells, we next used flavopiridol to sensitise the cells to apoptosis. As observed in Figure 4.2, the CDK-inhibitor flavopiridol and TRAIL combination caused a high level of apoptosis in T47D cells.

Co-treatment of TRAIL (1000 ng/ml) with increasing concentrations of flavopiridol lead to apoptosis which could be observed at a concentration as low as 0.5 µM (Figure 4.20; Part A). Further increase in the concentration of flavopiridol did not cause a significant increase in the amount of apoptosis caused by the combination. As a result, flavopiridol was used at a concentration of 0.5 µM for all further experiments.

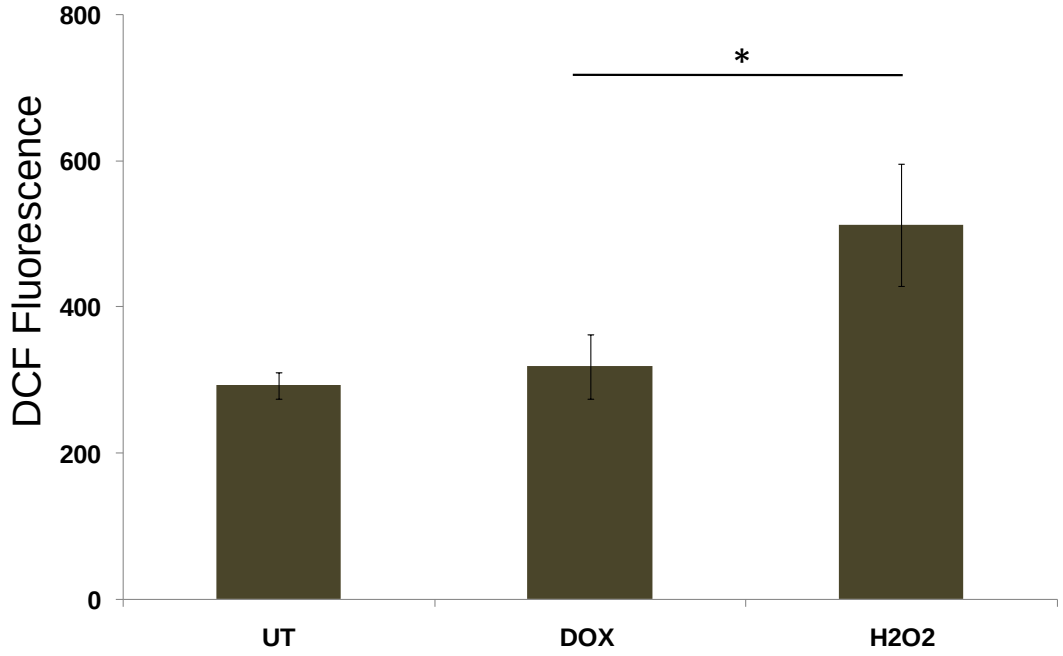


Figure 4.19 Doxorubicin (1 µM) does not result in formation of ROS in T47D cells
T47D cells were treated with doxorubicin (1 µM) for 16 h and ROS formation was measured as outlined in Materials and Methods. Hydrogen peroxide (10 µM) was used as a positive control for the experiment. Data represented as mean ± SEM (n=4). * indicates significant difference ($P<0.05$).

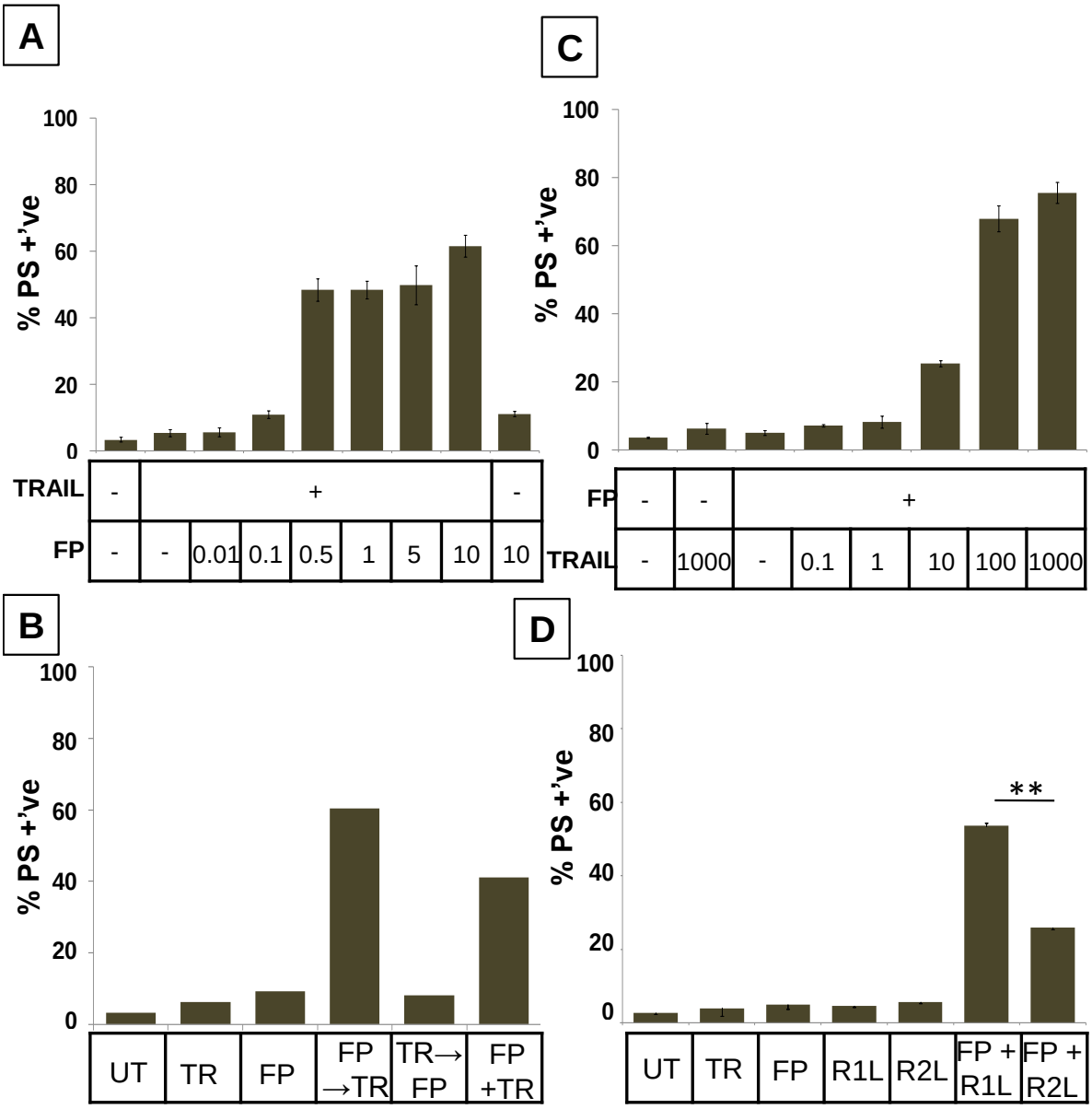


Figure 4.20 Flavopiridol also sensitises T47D cells to TRAIL-induced apoptosis *via* TRAIL-R1

T47D cells were seeded and treated with increasing concentrations of flavopiridol (FP) in combination with TRAIL (1000 ng/ml; (A); data represented as mean ± SEM (n=3)) for 24 h or pre-treated with flavopiridol (0.5 µM) for 16 h followed by TRAIL (1000 ng/ml) for 6 h or pre-treated with TRAIL for 16 h followed by flavopiridol for 6 h ((B); data represented as average (n=2)). T47D cells were also pre-treated with flavopiridol (0.5 µM) for 16 h followed by treatment with TRAIL at indicated concentrations ((C); data represented as mean ± SEM (n=3)). Alternatively seeded T47D cells were pre-treated with flavopiridol (0.5 µM) for 16 h followed by R1L/R2L for 6 h and samples were collected for Annexin V-FITC labelling ((D); data represented as mean ± SEM (n=3)). ** indicates significant difference ($P<0.01$).

Similar to doxorubicin, flavopiridol pre-treatment for 16 h followed by addition of TRAIL induced apoptosis in T47D cells, whereas TRAIL pre-treatment for 16 h followed by flavopiridol did not cause any cell death (Figure 4.20; Part B). This data provides evidence that flavopiridol sensitises T47D cells to TRAIL-induced apoptosis and not vice versa.

The optimal concentration of TRAIL to be used with flavopiridol was deduced by treating T47D cells with increasing concentrations of TRAIL in combination with flavopiridol at 0.5 µM. Based on this experiment, TRAIL at 100 ng/ml appeared to be the most effective and was therefore used for subsequent experiments (Figure 4.20; Part C).

In order to examine the generality of dominance of TRAIL-R1 in these cells, T47D cells were pre-treated with flavopiridol at 0.5 µM followed by R1L or R2L at 100 ng/ml. Consistent with that seen previously with doxorubicin treatment, flavopiridol sensitised T47D cells to R1L (Figure 4.20; Part D). Importantly, flavopiridol, like doxorubicin, failed to facilitate TRAIL sensitisation through TRAIL-R2 (Figure 4.20; Part D).

This data combined with the results obtained previously with doxorubicin conclusively prove that in T47D cells, TRAIL-R1 is the more potent TRAIL death signalling receptor and TRAIL-R2 does not play a major role in soluble TRAIL-mediated apoptosis.

4.19 In T47D cells, ETR1 shows higher potency than ETR2 in combination with doxorubicin

We also further wanted to confirm the relative contributions of TRAIL-R1 and TRAIL-R2 to TRAIL-induced apoptosis in T47D cells using the HGS agonistic antibodies, HGS-ETR1 and HGS-ETR2 that are currently in under clinical evaluation in Phase I/II trials.

Importantly, both antibodies were initially used in the presence or absence of their cross-linker. Both untreated and cross-linker treated T47D cells did not show any apoptosis (Figure 4.21; Part A; Lanes 1 & 2). ETR1 and ETR2 (2000 ng/ml) on their own failed to induce any apoptosis in the T47D cells (Figure 4.20; Part A; Lanes 5 & 10). Pre-aggregated ETR1 (antibody:cross-linker = 1:3) also did not cause any apoptosis (Figure 4.21; Part A; Lane 6); however, surprisingly, cells treated with pre-cross-linked ETR2 (antibody:cross-linker = 1:3) showed a modest amount of apoptosis (Figure 4.21; Part A; Lane 11).

T47D cells were also pre-treated with doxorubicin at 1 μM for 16 h and then treated with ETR1/2 for 6 h. Doxorubicin sensitised T47D cells to both ETR1 and ETR2 to a similar extent (Figure 4.21; Part A; Lanes 7 & 12). As shown earlier (Figure 4.12), addition of doxorubicin causes a massive up-regulation of TRAIL-R2 on the cell surface and the increased density of surface TRAIL-R2 may facilitate effective apoptotic signalling by ETR2.

Intriguingly, in doxorubicin pre-treated T47D cells, cross-linking ETR1 inhibited its potency (Figure 4.21; Part A; Lane 8), whereas cross-linking ETR2 lead to a significant increase in the amount of apoptosis caused (Figure 4.21; Part A; Lane 13). This data would also argue that higher order aggregates of soluble TRAIL would be required to efficiently activate TRAIL-R2 while it would appear that TRAIL-R1 can easily be activated without the need of ligand/antibody pre-aggregation. This would thus indicate that in T47D cells (and similar to the MCF7 F43 and MDA-MB-231 cells tested previously; Figure 3.9), TRAIL-R2 is functional and can be activated by an appropriate preparation of TRAIL/TRAIL agonists.

It was also important to study whether the lower activation threshold of TRAIL-R1 signalling was maintained if TRAIL-R1/R2 were instead stimulated using the HGS antibodies. Hence, T47D cells were treated with varying concentration of ETR1 or ETR2 after an initial pre-treatment of doxorubicin (1 μM) for 16 h. Similar to the results obtained with R1L and R2L, ETR1 induced modest apoptosis when used at a concentration of 100 ng/ml and the apoptosis induced significantly increased with increased concentrations of ETR1 (Figure 4.21; Part B; Lanes 8-10). ETR2 also caused apoptosis within the concentration range of 100 – 1000 ng/ml in presence of doxorubicin (Figure 4.21; Part B; Lanes 17-19). Importantly though, the amount of apoptosis caused by ETR1 was higher than that induced by ETR2, again emphasising that irrespective of the use of ligand or antibody, TRAIL-R1 is the more potent TRAIL death receptor signalling to apoptosis in T47D cells. The difference in the potency of ETR1 and ETR2 was not as dramatic as the difference between R1L and R2L; however, this might be due to variations in the mechanism of binding of ligand versus antibody to the death receptor or the differences in the interactions between the individual HGS-antibodies and the TRAIL receptors.

Nonetheless, it is crucial to note that all of the above data taken together suggest that TRAIL-R1 is the potent TRAIL death receptor which signals to apoptosis. Furthermore, the apoptotic signalling through TRAIL-R1 does not require pre-aggregation of the ligand/antibody and shows significantly faster kinetics than signalling *via* TRAIL-R2.

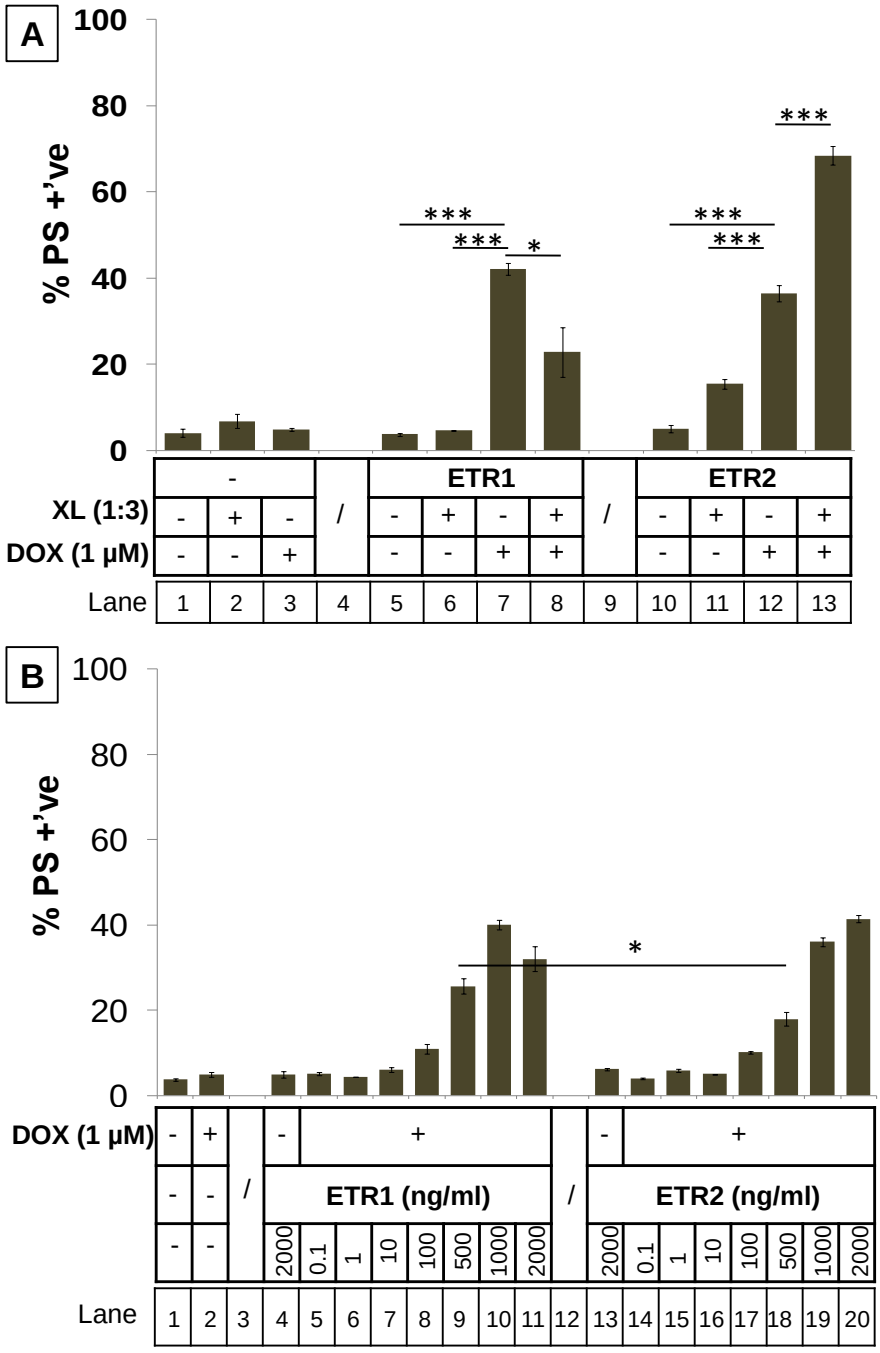


Figure 4.21 HGS-ETR1 and HGS-ETR2 can signal to apoptosis in doxorubicin treated T47D cells

T47D cells were seeded at 4.0 X 10^5 cells/well and treated with monomeric or cross-linked ETR1 and ETR2 antibodies in the presence or absence of doxorubicin and apoptosis was assessed by PS exposure ((A); data represented as mean ± SEM (n=3)). T47D cells were pre-treated with doxorubicin (16 h) followed by increasing concentrations of ETR1 and ETR2 antibodies (6 h) and apoptosis was assessed by PS exposure ((B); data represented as mean ± SEM (n=3)). *** and * indicate significant difference ($P<0.001$ and $P<0.05$ respectively).

4.20 Discussion

Most primary tumour cells are resistant to TRAIL monotherapy (Ehrhardt *et al.,* 2003; MacFarlane *et al.,* 2005a; Herbst *et al.,* 2006; Todaro *et al.,* 2008) and thus require a sensitising agent to facilitate apoptosis. In this regard, the cell line T47D provided a useful model to optimise a combinational therapeutic approach for breast cancer cells. However, it is important to remember that the T47D cells cannot be used as a substitute for primary breast tumour tissue and have been only used as a tool to perform preliminary experiments. The TRAIL resistance of T47D cells may be due to constitutive activation of pro-survival pathways or defects in the TRAIL apoptotic pathway. In T47D cells, binding of TRAIL to its receptors forms a ligand-receptor complex which does not efficiently recruit FADD (Figure 4.14). This data indicates that receptor proximal defects are responsible for the observed lack of apoptotic signalling by TRAIL. One of the potential reasons for TRAIL resistance could be the sequestering of TRAIL by the decoy receptors, TRAIL-R3 and TRAIL-R4, thus lowering the overall efficiency of TRAIL. However, if this were the case, R1L and R2L, which do not bind TRAIL-R3 or TRAIL-R4, should have been able to induce apoptosis, thus ruling out the expression of decoy receptors as a mechanism of resistance. Nonetheless addition of TRAIL alone to T47D cells did not cause activation of caspase-8 and therefore, did not signal to apoptosis.

Addition of doxorubicin and flavopiridol strongly sensitised T47D cells to TRAIL-induced apoptosis. In contrast, combining TRAIL with ABT-737 or PI103 caused moderate apoptosis in T47D cells while its combination with tamoxifen, SAHA, SB203580 and LBW242 did not cause any apoptosis. The inability of SAHA to facilitate TRAIL-induced apoptosis was surprising, since our laboratory has already shown that HDACi can sensitise TRAIL-resistant primary CLL cells to TRAIL (MacFarlane *et al.*, 2005a). These data indicate that there might be differences between the mechanism of action of different HDACi and resistance mechanisms of different types of tumours (for example, haematological malignancies versus epithelial tumours). Inhibition of the Akt pathway, but not the p38 pathway, caused moderate apoptosis in T47D cells suggesting that the constitutive activation of Akt but not p38 may contribute to the TRAIL resistance of T47D cells. Based on the data obtained previously in MDA-MB-468 cells (Figure 3.8), we also examined LBW242 in combination with TRAIL. However, this combination did not cause apoptosis in T47D cells indicating that caspase regulation by IAP family members is not the major cause of TRAIL resistance in these cells. Taken together these data suggest that combinations of certain chemotherapeutic agents and TRAIL can kill some tumour cell

lines but not others, thus emphasising that there may be key differences in treatment responses observed between individual tumours even *in vivo*.

In our TRAIL-resistant breast cancer cell line model, doxorubicin appeared to be the best TRAIL sensitising agent and hence, we used doxorubicin to further dissect the regulation of TRAIL-induced apoptosis in T47D cells. Interestingly, pre-treatment with doxorubicin followed by TRAIL caused more cell death than doxorubicin and TRAIL co-treatment. Based on preliminary experiments, it could be suggested that doxorubicin takes at least 8-12 h to induce the changes necessary to sensitise T47D cells to TRAIL-induced apoptosis (data not shown). Thus, when used in combination, TRAIL binding to its receptors within the first few hours of doxorubicin addition cannot signal to apoptosis, thus reducing its overall potency. However, cells pre-treated with doxorubicin prior to TRAIL addition are already sensitised to TRAIL and hence, this regime induces more apoptosis than simultaneous co-treatment of cells. The major mode of cell death caused by doxorubicin and TRAIL combination was apoptosis. The other possible mode of cell death that could explain the approximately 20% caspase-independent cell death seen in the presence of zVAD is necroptosis. The recruitment of RIP1 into the TRAIL DISC in the doxorubicin pre-treated T47D cells suggests the possibility for RIP1-mediated signalling to necroptosis. Thus, it is possible that doxorubicin and TRAIL combination causes mainly apoptotic cell death in T47D cells, but in the presence of zVAD, some of the cells undergo necroptosis. This might also be similar to the programmed necrosis mediated by RIP1/RIP3 in capsase-8 deficient mice (Oberst *et al.,* 2011; Kaiser *et al.,* 2011; Zhang *et al.,* 2011). However, electron micrographs could not confirm any mode of cell death other than apoptosis and further investigation into the caspase-independent death seen in the presence of zVAD is required to identify its exact mechanism. Using oligonucleotides against RIP1 or the RIP1 inhibitor, Necrostatin-1 could help reveal whether the caspase-independent cell death is indeed necroptosis (Degterev *et al.,* 2005; Degterev *et al.,* 2008).

Having established that apoptosis was the major model of cell death we then demonstrated that doxorubicin sensitised T47D cells to TRAIL in a concentration-dependent manner. The relative contributions of TRAIL-R1 and TRAIL-R2 to TRAIL-induced apoptosis also seemed to vary with increasing doxorubicin concentration. At a supra-clinical concentration of 5 µM, doxorubicin sensitised T47D cells to signal through both TRAIL-R1 and TRAIL-R2, while at the more clinically relevant concentration of 1 µM, the contribution of TRAIL-R1 to TRAIL-mediated apoptosis was significantly higher than that of TRAIL-R2. The exact mechanism of doxorubicin-mediated TRAIL sensitisation in T47D cells is still unclear, however it is possible that

doxorubicin at a supra-clinical concentration of 5 µM causes additional changes, as compared with 1 µM, which sensitise T47D cells to R2L. Importantly, however, using doxorubicin at the clinically relevant concentration of 1 µM preferentially sensitised T47D cells to a form of TRAIL that signals *via* TRAIL-R1 and not TRAIL-R2. The predominance of TRAIL-R1 signalling is seen by increased activation of the initiator caspase-8, Bax and the effector caspase-3. Furthermore, flavopiridol also sensitised T47D cells to TRAIL-induced apoptosis and this was again mediated predominantly *via* TRAIL-R1. Our data also show that TRAIL-R1 is the major death-signalling receptor not only for soluble form of TRAIL, but also TRAIL-receptor-specific agonistic antibodies. Overall, the data presented here conclusively show that TRAIL-resistant breast tumour cells can be sensitised to TRAIL-induced apoptosis using chemotherapeutic drugs and that in this model, signalling to apoptosis occurs mainly *via* TRAIL-R1.

It has been argued that up-regulation of TRAIL-R2 by chemotherapeutic agents is the main mechanism of TRAIL sensitisation. In this context, p53-dependent and independent mechanisms of up-regulation of TRAIL-R2 by DNA damaging agents have been described in the literature (Sheikh *et al.,* 1998). The TRAIL-R2 gene harbours an intronic p53 binding site which might explain its up-regulation following p53 activation by doxorubicin treatment (Wu *et al.,* 1997). However, as T47D cells harbour a mutant form of p53 (Neve *et al.,* 2006), a p53-dependent increase in TRAIL-R2 transcription is possibly not the main cause of the observed up-regulation. The TRAIL-R2 gene also contains an NFkB binding site and possible activation of NFkB by doxorubicin might also contribute to the increase in the levels of TRAIL-R2. Irrespective of the cause, it is important to note that doxorubicin indeed induced a rapid and significant increase in the cell surface expression level of TRAIL-R2. The presence of a TRAIL-R1/TRAIL-R2 heteroxomplex in the R1L DISC suggested that TRAIL-R2 might be important to facilitate R1L-mediated apoptosis. However, this is unlikely as the knockdown of TRAIL-R2 did not affect doxorubicin-mediated TRAIL sensitisation, thus indicating that sensitisation to TRAIL in this case is completely independent of TRAIL-R2 up-regulation. This could be further confirmed by performing an immunoprecipitation with the biotinylated R1L in T47D cells which have been depleted of TRAIL-R2 and then treated with doxorubicin. The data from this experiment would prove that the presence of TRAIL-R2 in a heterocomplex with TRAIL-R1 upon TRAIL stimulation is redundant and is not required for effective TRAIL signalling to apoptosis. Alternatively blocking antibodies to TRAIL-R1 or TRAIL-R2 which would prevent the apoptotic signalling

through the respective receptor could also be used to verify that TRAIL-induced apoptosis mainly occurs through TRAIL-R1 in the breast cancer cells.

There has also been a report that up-regulation of TRAIL-R1 by doxorubicin sensitises prostate cancer cells to TRAIL-R1-mediated apoptosis (Voelkel-Johnson, 2003). However, in T47D cells, doxorubicin did not affect the cell surface expression of TRAIL-R1 and thus, this cannot be a mechanism of sensitisation. As described earlier, doxorubicin does cause increased DISC formation. A possible reason for this could be the relocation of TRAIL-receptors into the lipid rafts by doxorubicin. Studies have shown that doxorubicin can up-regulate ceramide and cause the redistribution of death receptors into lipid rafts leading to their clustering and more efficient activation by TRAIL (Dumitru *et al.,* 2007; Aroui *et al.,* 2009). Other reported mechanisms of sensitisation to TRAIL include up-regulation of Bax, down regulation of FLIP and formation of ROS (Leung and Wang 1999; Kelly *et al.,* 2002; Koschny *et al.,* 2010). However, none of these changes occurred in T47D cells treated with a clinically relevant concentration of doxorubicin (1 µM), thus ruling out these as potential sensitisation mechanisms. Based on our data so far, it would appear that the major mechanism of doxorubicin sensitisation is enhanced TRAIL DISC formation and preferential signalling *via* TRAIL-R1.

Irrespective of the any other potential mechanisms of sensitisation, doxorubicin and TRAIL combination caused substantial apoptosis in T47D cells. Doxorubicin and TRAIL combination has already been reported to cause apoptosis in different breast cancer models (Keane *et al.,* 1999; Buchsbaum *et al.,* 2003; Lin *et al.,* 2003; Singh *et al.,* 2003; Wu *et al.,* 2007) and in other cancer types (Tomek *et al.,* 2004; Shankar *et al.,* 2005; El-Zawahry *et al.,* 2005; Koehler *et al.,* 2009; Vaculova *et al.,* 2010; Wang *et al.,* 2010). It has been reported in that doxorubicin and TRAIL combination is lethal to both normal and cancerous bone cells (Van Valen *et al.,* 2003). However, the loss of selectively for tumour cells was seen at doxorubicin concentration greater than 1 µM, thus suggesting that the clinically relevant concentration of doxorubicin used here may be safely used with TRAIL. Incidentally, it has also been shown that the combination of doxorubicin and a TRAIL-R1 targeting antibody can selectively kill prostate tumour cells, but not normal cells (Voelkel-Johnson, 2003). This further supports the clinical importance of using doxorubicin at a clinically relevant concentration in combination with a TRAIL-R1 targeting agent. To examine the validity of TRAIL-R1 and doxorubicin combination for use in the clinic, we next wanted to test this optimal combination on primary human breast tumour explants from affected patients.

Chapter 5

Profile of TRAIL-Receptor signalling to apoptosis in *ex vivo* primary human breast tumour cells

5.1 Introduction

Breast cancer is characterised by its heterogeneity, with carcinomas not only varying in the nature of their neoplastic component but also in the extent of associated non-neoplastic cellular components. As mentioned earlier, both normal and malignant epithelial cells, blood vessels, fibroblasts, lymphocytes, macrophages and adipose tissue can be present together with an interacting and extensive array of signalling molecules, including cytokines and growth factors. The inherent mutations within tumour cells coupled with the influence of the tumour microenvironment (TME) can therefore determine the sensitivity of the tumour to anti-cancer therapy. Thus, it is possible that chemotherapy/biotherapy has different potencies in cultured cell lines as compared to primary breast tumours *in vivo*. Indeed, as demonstrated by Weaver *et al* even culturing breast cancer cell lines as spheres can change their response to death-inducing ligands (Weaver *et al.,* 2002). The aim of developing TRAIL-R1/-R2 targeting agents would be to administer them to patients with breast cancer and therefore, it is critical to assess the response of primary human breast tumours to R1L and R2L.

5.2 Culture of normal breast tissue explants

Breast tissues were cultured as organ explants to ensure that the 3-dimensional (3D) architecture and TME of the tissue were conserved. The organ explant culture method (See Materials and Methods; Section 2.3) was first validated using normal breast tissue from breast reduction surgery. Viability of breast tissue after 48 h culture in media (DMEM F12; 10% FCS; 2 mM Glutamax and Penicillin-Streptomycin) was assessed using Haematoxylin and Eosin (H & E) staining and evaluated by Prof. Rosemary Walker (Breast Pathologist; Department of Cancer Studies and Molecular Medicine, University of Leicester). Additives previously described to be potentially beneficial to breast tissue growth in culture were included for testing in this setup. The effects of insulin (5 ng/ml), EGF (Epidermal Growth Factor; 10 ng/ml) and hydrocortisone (10 ng/ml) on the viability of healthy breast tissue explants were observed by assessing tissue viability on H & E stained sections. From these experiments it was deduced that the addition of insulin benefited the culture of the explants while neither EGF nor hydrocortisone had any significant effect on tissue viability (data not shown). It was, therefore, decided to incorporate insulin into the culture media for all further explant cultures.

Normal breast tissue explants were also used to validate potential treatment regimes and immunostaining techniques. Normal cells have been reported to be resistant to

TRAIL both *in vivo* and *in vitro* (Ashkenazi *et al.,* 1999; Roth *et al.,* 1999; Walczak *et al.,* 1999; Kelley *et al.,* 2001; Bouralexis *et al.,* 2005). The potency of TRAIL, R1L and R2L on normal tissue was assessed by immunostaining serial sections for cleaved caspase-3 and cleaved PARP. As with the cell line experiments, normal breast explants treated with staurosporine were used as a positive control. Tissue samples from seven different patients were tested for their response to TRAIL or staurosporine treatment. Expectedly, all seven healthy tissue samples were sensitive to staurosporine (Table 5.1 & Figure 5.1; Panel iii) and showed strong staining for both cleaved caspase-3 (data not shown) and cleaved PARP (Corresponding IgG staining – Appendix 7).

Table 5.1 Normal breast cells are resistant to TRAIL, but not staurosporine

Sample No	Age (years)	TRAIL	Staurosporine
N1	53	-	++
N2	19	-	++
N3	18	-	++
N4	54	-	++
N5	26	-	++
N6	45	-	++
N7	46	-	++

Key: - No increase in cleaved PARP staining over untreated cells
++ Increase in cleaved PARP staining over untreated cells.

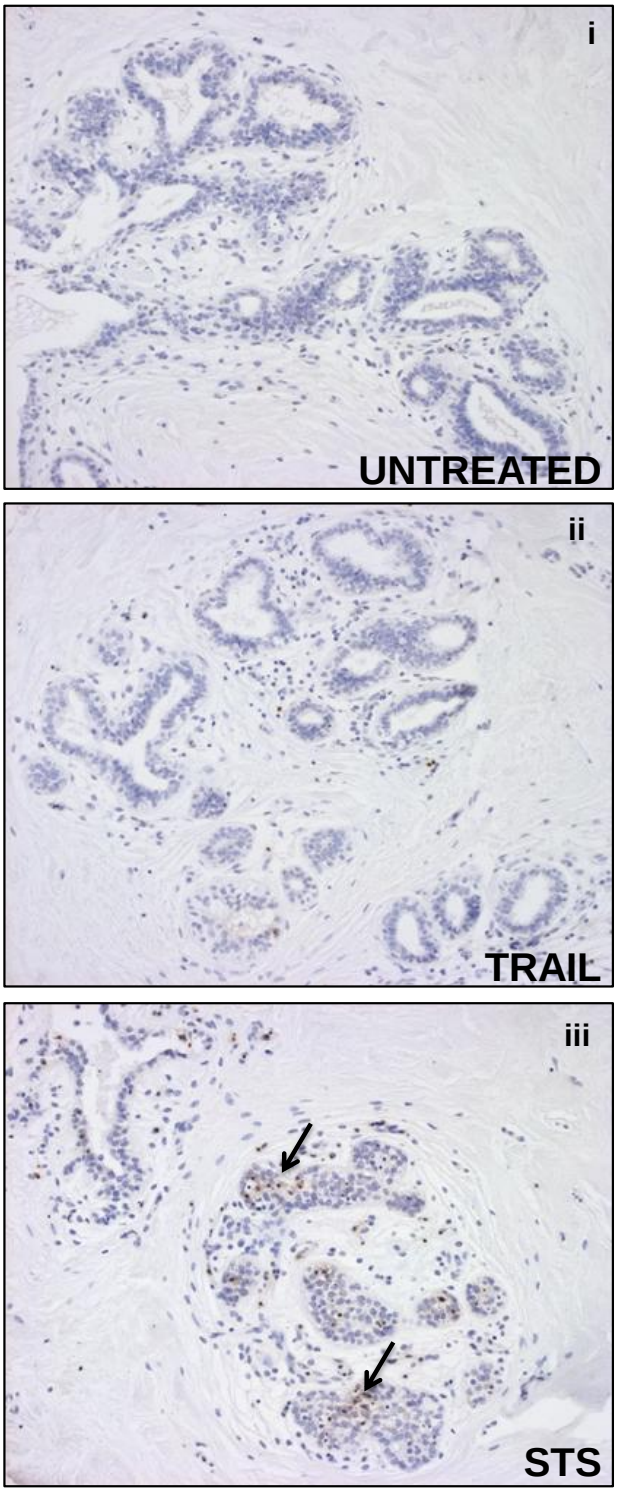


Figure 5.1 Normal breast tissue is resistant to TRAIL but not to Staurosporine (STS) Primary normal breast tissue explants were cultured and treated with indicated death stimuli (TRAIL; 1 µg/ml and STS; 1 µM) for 24 h. Images are samples stained with cleaved PARP and counter-stained with Haematoxylin (Magnification 200x). Arrows indicate apoptotic cells.

Interestingly, TRAIL and its variants failed to induce any apoptosis in the normal breast tissue explants (Figure 5.1; Panel ii & data not shown). This is indicated by the low level of staining for apoptotic markers (cleaved PARP) in TRAIL-treated samples, which is similar to the staining seen in the untreated controls (Figure 5.1; compare Panels i & ii). Thus, it appears that normal breast tissue is consistently resistant to TRAIL-induced apoptosis.

Importantly, the use of normal breast tissue explants also helped establish the appropriate culture media and helped validate the culture conditions and optimal immunostaining methods. Based on these data, the optimised techniques were then applied to the culture and assessment of the response of primary breast tumour tissue to cytotoxic agents.

5.3 Culture of breast tumour explants

A total of sixteen breast tumour samples were received from consented patients who had had no prior chemotherapy (Table 2.4). Out of the sixteen, two were mucinous carcinomas, eleven were the commonly occurring invasive ductal carcinoma (IDC), one was an infiltrating lobular carcinoma (ILC) and two had features of both IDC and ILC. Initially, the breast tumour samples were cultured in similar conditions to those used previously for culturing normal breast tissue explants.

Mucinous carcinomas represent a rare sub-type of invasive carcinomas and occur in less than 3% of all breast cancer cases. They are classically characterised by a high mucin content, which often gives the tumour a jelly-like appearance. We received two mucinous carcinoma samples (T1 and T5) and both these samples showed excellent viability following culture for 48 h in media containing 10% FCS. However, in comparison to the rarer mucinous carcinomas, the more common invasive ductal carcinomas showed poor viability following 48 h culture under the same conditions. Thus, IDC samples T2, T3 and T4 did not remain viable post-culture and therefore were discarded from further analysis (Figure 5.2).

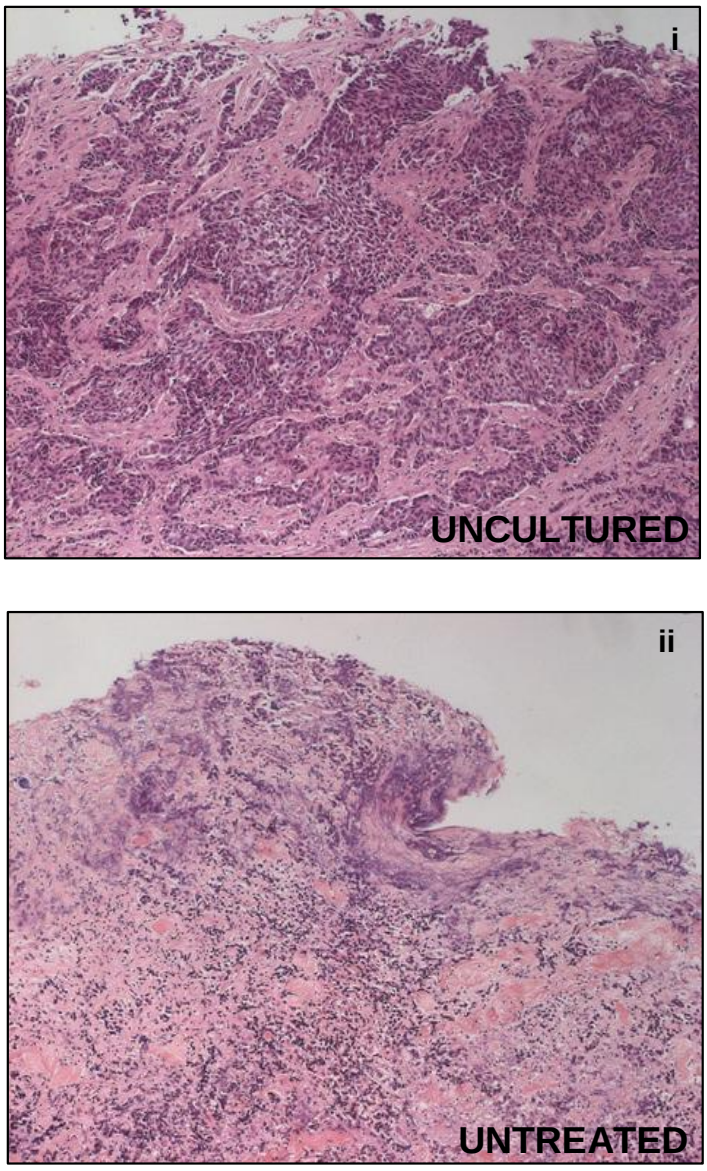


Figure 5.2 Invasive ductal carcinoma samples do not remain viable when cultured in media containing 10% FCS

Haematoxylin and Eosing (H & E) of breast tumour sample #T3 without culture (i) and after culture in media containing 10% FCS for 48 h (ii). The tissue viability in (ii) was very poor (Magnification 100x).

Profile of TRAIL-Receptor signalling to apoptosis in primary breast tumour cells

We hypothesised that the lack of viability observed in the IDC samples might be due to stress caused by the presence of high levels of serum factors in the media coupled with growth factors secretion by the tumour cells themselves. The next two samples were therefore used to directly assess whether the tumour cell viability could be better retained by decreasing the concentration of FCS used in the culture media.

The IDC tumour tissue, T6, was cultured in media of identical composition as before except for the concentration of FCS. Three different concentrations of FCS, 1%, 5% and 10% were used to check whether this had any major effect on maintaining tumour tissue viability during culture.

Interestingly, after 48 h culture, the tumour samples that were maintained in media containing 1% and 5% FCS remained viable, while those cultured in 10% FCS were not viable and showed clear signs of degeneration (Figure 5.3; Panel iii; indicated by black arrows). However, as mentioned earlier, the IDC tumour tissue may comprise a mixture of normal and tumour cells and it is essential to retain the viability of all cells in order to completely mimic the TME. Although culturing the tumour explants in media containing 1% FCS did not affect the viability of tumour cells, it is possible that this concentration of FCS is detrimental for the non-cancerous tissue components. Hence, it was initially decided to culture subsequent breast tumour samples in media containing 5% FCS. However, it was also later observed that some of the more aggressive tumours (Grade III) did not culture well in media containing 5% FCS and the FCS concentration was therefore lowered to 1% to aid culture of these samples.

Once the culture conditions were established, the following ten primary breast tumours were assessed for their apoptotic response to TRAIL either alone or in combination with doxorubicin. Mucinous carcinomas and invasive ductal or lobular carcinomas were analysed separately and the data obtained for each tumour sub-type is detailed below.

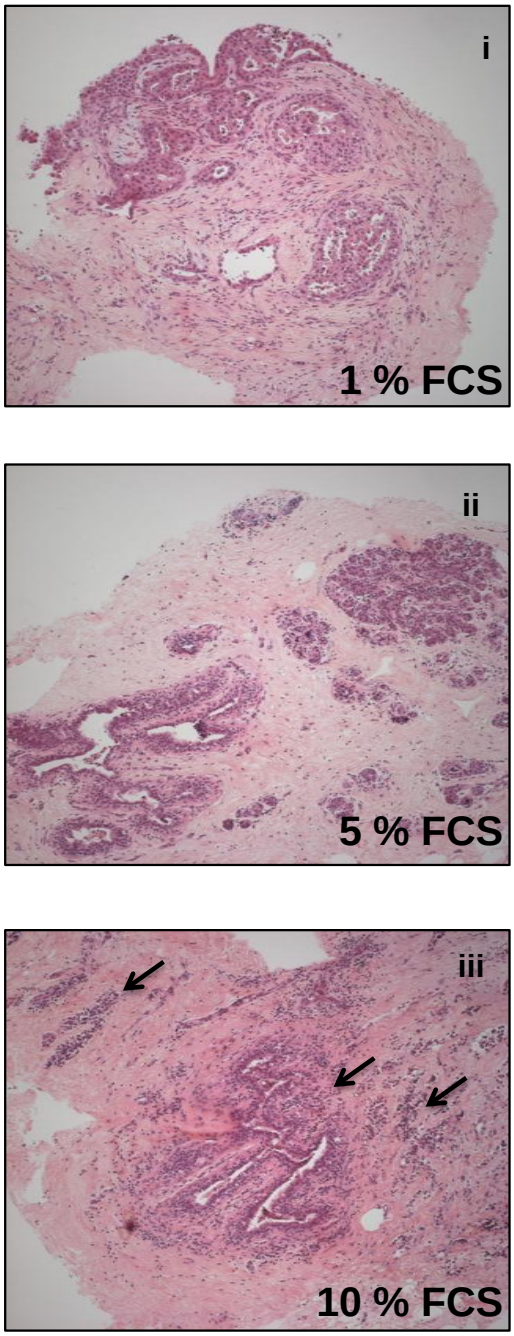


Figure 5.3 Media containing 5% FCS supports the culture of primary human breast tumour explants

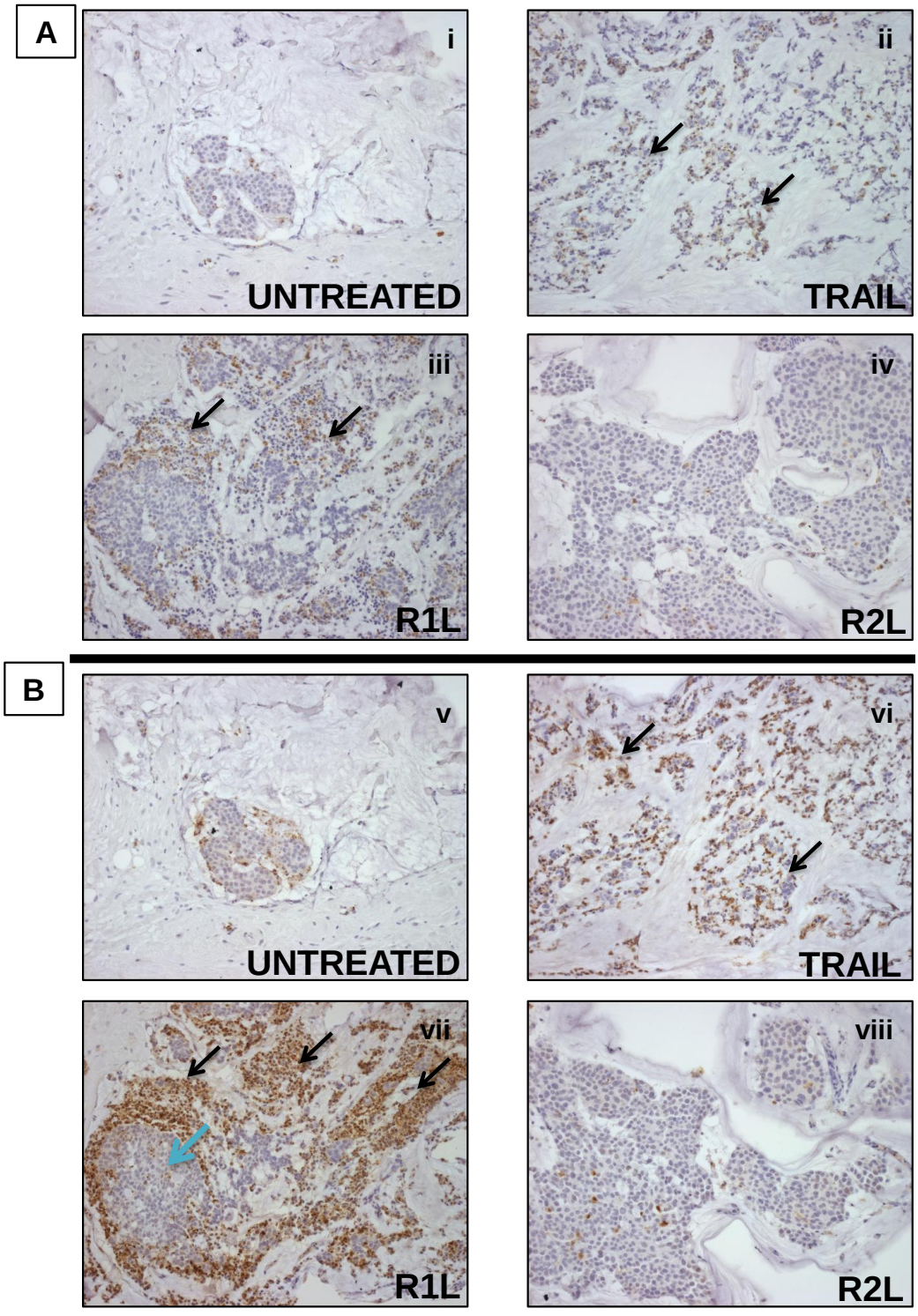
H& E of tumour tissue sections (IDC #6) after culture with media containing different concentrations of FCS showing that there is poor viability with 10% FCS (Magnification 100x). Arrows indicate areas of tissue degeneration. In contrast both 5% and 10% FCS show good viability.

5.4 Mucinous carcinomas T1 and T5

Mucinous carcinomas are typically ER positive and HER2 negative and consistent with this, both samples T1 and T5 showed the same receptor profile (Materials and Methods; Table 2.4).

Both carcinoma explants (T1, T5) retained viability on culturing as assessed by H & E staining and showed characteristic mucinous morphology. Surprisingly, both mucinous carcinomas were sensitive to TRAIL as a single agent (Figure 5.4; Panels ii and vi & Figure 5.5; Panel iii; Corresponding IgG staining – Appendix 8/9). TRAIL induced significant apoptosis in both the samples, as assessed by multiple markers of apoptosis including immunostaining for cleaved caspase-3, cleaved PARP and Hoechst staining.

The relative contribution of the TRAIL death receptors, TRAIL-R1 and TRAIL-R2 to TRAIL-induced apoptosis was studied in mucinous tumour sample T1 by treating with the TRAIL-R1/-R2-specific mutant ligands and subsequent analysis of immunostaining for cleaved caspase-3, cleaved PARP and Hoechst staining. Immunostaining showed conclusively that R1L was much more potent than R2L in inducing apoptosis in mucinous carcinomas (Figure 5.4; Parts A & B; Compare Panels iii & vii with Panels iv & viii respectively). Further analysis of the cleaved PARP staining using the software, Volocity®, quantitatively indicated that R1L induced much more apoptosis than R2L (data not shown). To confirm the Volocity®-based analysis, serial sections were stained with Hoechst in order to count the number of apoptotic cells. Nuclear blebbing is a characteristic feature of apoptosis and can therefore be used as a marker. Hoechst was used to label the cell nuclei and disintegrating nuclei were counted as apoptotic cells. Based on manual counting of Hoechst stained cells (Figure 5.4; Part D), it can concluded that significantly more apoptosis was induced by R1L than either TRAIL or R2L.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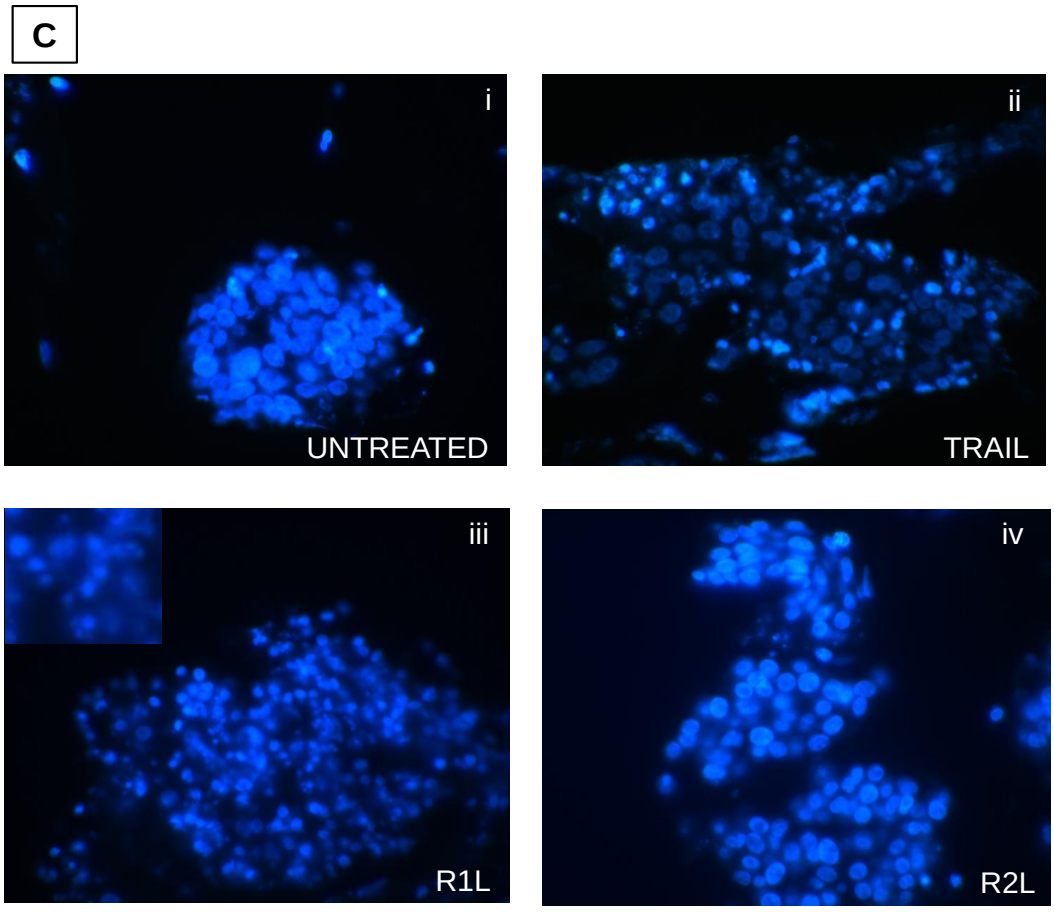


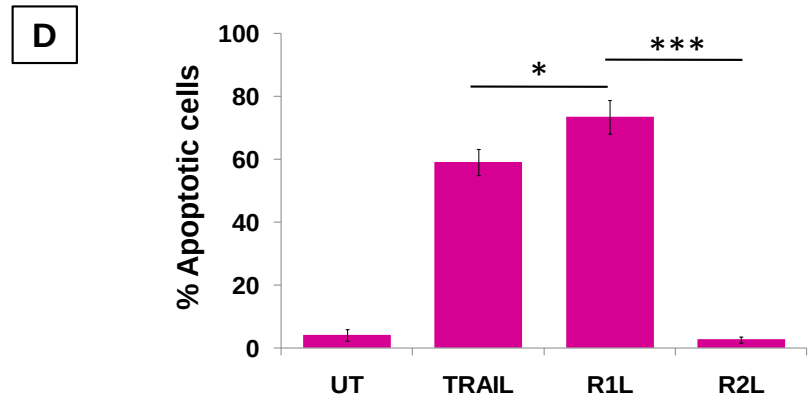


Figure 5.4 In mucinous carcinoma TRAIL signals to apoptosis *via* TRAIL-R1
Tumour explants from sample #T1 treated with TRAIL or the receptor-specific ligands (R1L, R2L) were assessed for apoptosis using cleaved caspase-3 (A); cleaved PARP (B) and Hoechst (C). Black arrows indicate apoptotic cells, blue arrow indicates area of no apoptotic response (D) shows the extent of apoptosis quantified on the basis of nuclear blebbing (inset), showing little evidence of apoptosis with R2L (Magnification 200x – Cleaved caspase-3 and cleaved PARP immunostaining; Magnification 630x – Hoechst staining; data shown are represented as mean ± SEM).

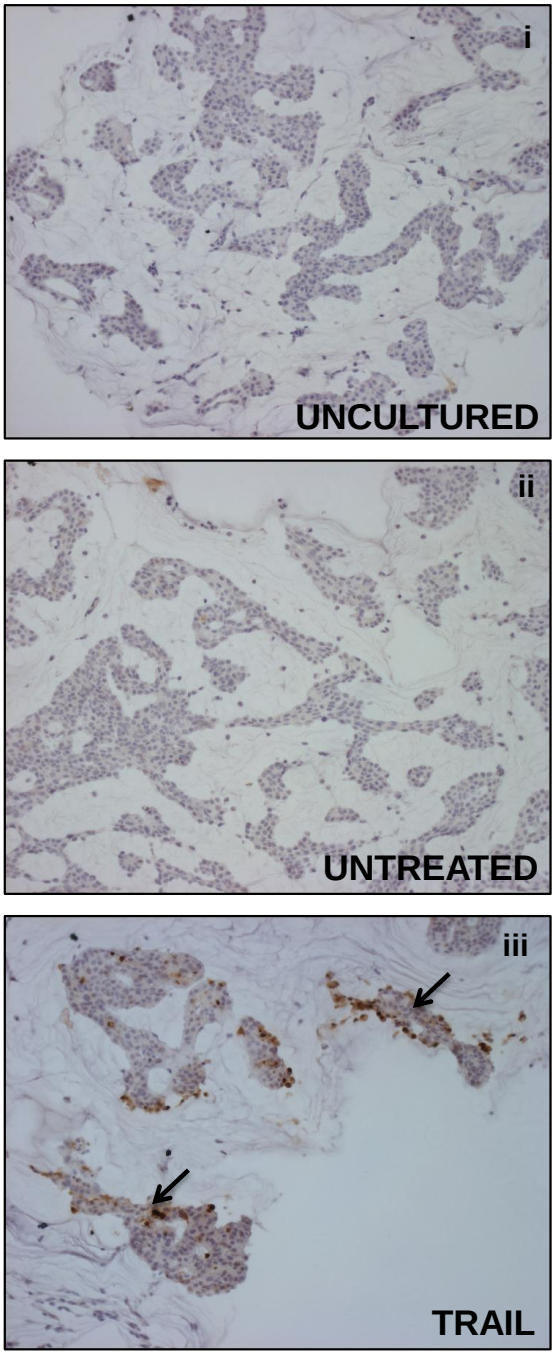


Figure 5.5 Mucinous carcinoma #T5 is sensitive to TRAIL-induced apoptosis

Immunostaining for cleaved PARP of sample #T5 shows that there are groups of brown stained nuclei indicative of apoptosis in the explants treated with TRAIL alone (representative images shown; Magnification 200x). Arrows indicate apoptotic cells.

Similar to results obtained with mucinous carcinoma T1, T5 also showed sensitivity to TRAIL as a single agent (Figure 5.5). However, owing to the small tumour sample size, the potency of R1L and R2L could not be directly tested on this sample. Nonetheless the data obtained from this sample reiterates the inherent sensitivity of mucinous carcinomas to TRAIL-induced apoptosis. This finding has important implications for the treatment of at least this particular type of breast cancer. Furthermore, in this tumour type, TRAIL-R1 is the major TRAIL death receptor signalling to apoptosis and not TRAIL-R2.

5.5 Invasive ductal carcinomas/Infiltrating lobular carcinomas/Mixed carcinomas

Following initial modification of the culture media composition to facilitate tumour tissue viability, the subsequent IDC tumour samples were assessed for their response to TRAIL. Despite the optimisation of culture conditions some carcinomas either did not retain their viability (T7, T11) or were intrinsically necrotic (T8, T14) and therefore could not be used for this study. In the other IDC and mixed carcinoma samples, unlike the mucinous carcinomas, TRAIL as a single agent failed to have an impact on tumour cell viability.

5.5.1 Carcinoma T6

Explants from tumour sample T6, belonging to the IDC tumour type, were cultured in the optimised DMEM media supplemented with 5% FCS, 2 mM Glutamax, insulin and penicillin/streptomycin. After the recovery period, the tumour tissue was treated with TRAIL (1000 ng/ml) alone, doxorubicin (5 μM) alone or co-treated with doxorubicin and TRAIL for 24 h. Analysis of cleaved caspase-3 and cleaved PARP immunostaining revealed that both the normal and tumour cells in the IDC tumour sample were resistant to doxorubicin as a single agent (Figure 5.6; Part A; Panel iii & Part B; Panel vii and Appendix 10). Interestingly, the DCIS component of the tumour appeared to be largely unaffected by TRAIL, whereas the IDC cells did appear to respond slightly to TRAIL alone (Figure 5.6; Part A; Panel ii & Part B; Panel vi).

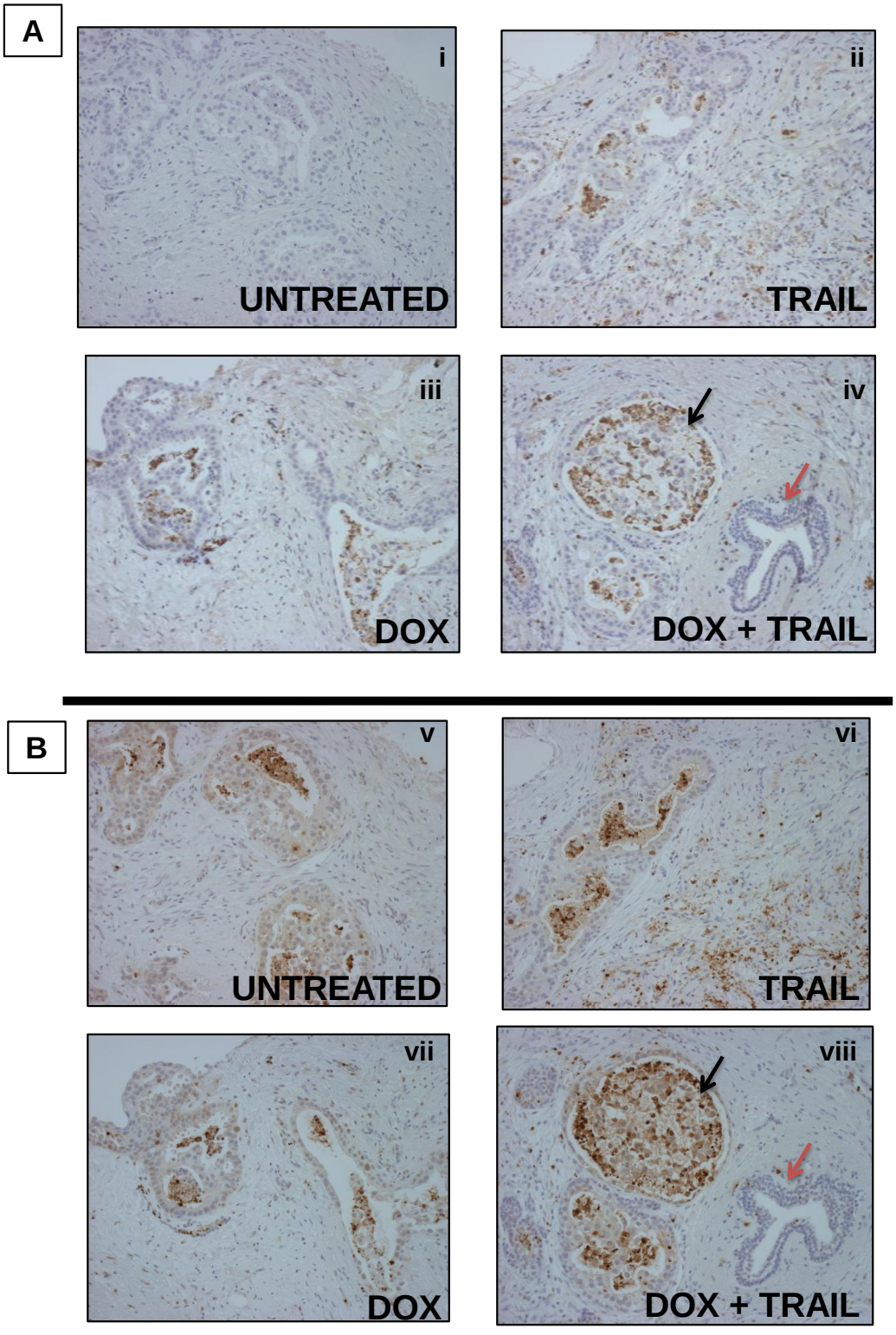


Figure 5.6 Doxorubicin selectively sensitises breast tumour cells to TRAIL-induced apoptosis

Sample #T6 treated with the specified treatments for 24 h and stained for cleaved caspase-3 (A) or cleaved PARP ((B); representative images; Magnification 200x). Black arrows indicate apoptotic tumour cells and red arrows indicate healthy normal breast cells.

Importantly, the co-treatment of TRAIL and doxorubicin induced a significant level of apoptosis exclusively in the tumour cells (Figure 5.6; Part A; Panel iv & Part B; Panel viii). The addition of doxorubicin also facilitated sensitisation of the DCIS tumour cells to TRAIL-mediated apoptosis. Crucially, co-treatment with doxorubicin and TRAIL had no apoptotic effect on the adjoining normal cells (indicated by red arrow) present within the tumour sample, thus indicating that TRAIL retained its selectivity for targeting tumour cells even in the presence of doxorubicin (Figure 5.6). These results suggest that TRAIL in combination with conventional chemotherapy might not be detrimental at least to normal breast epithelial cells.

5.5.2 Carcinoma T9

Sample T9 was a Grade II IDC which was ER and PgR positive and HER2 negative. Carcinoma T9 showed remarkable viability after 48 h culture *in vitro* with only a slight increase in apoptosis in the untreated tissue sections as compared to the 0 h control (Figure 5.7; Part B and Appendix 11a;11b). Interestingly, in this sample neither TRAIL nor its receptor-selective variants induced any significant apoptosis over that seen in the 48 h control. On closer inspection, TRAIL and R1L did cause a very modest level of cell death, primarily in the invasive component of the tissue; but R2L failed to induce any apoptosis (Figure 5.7; Part A; Panels ii-iv).

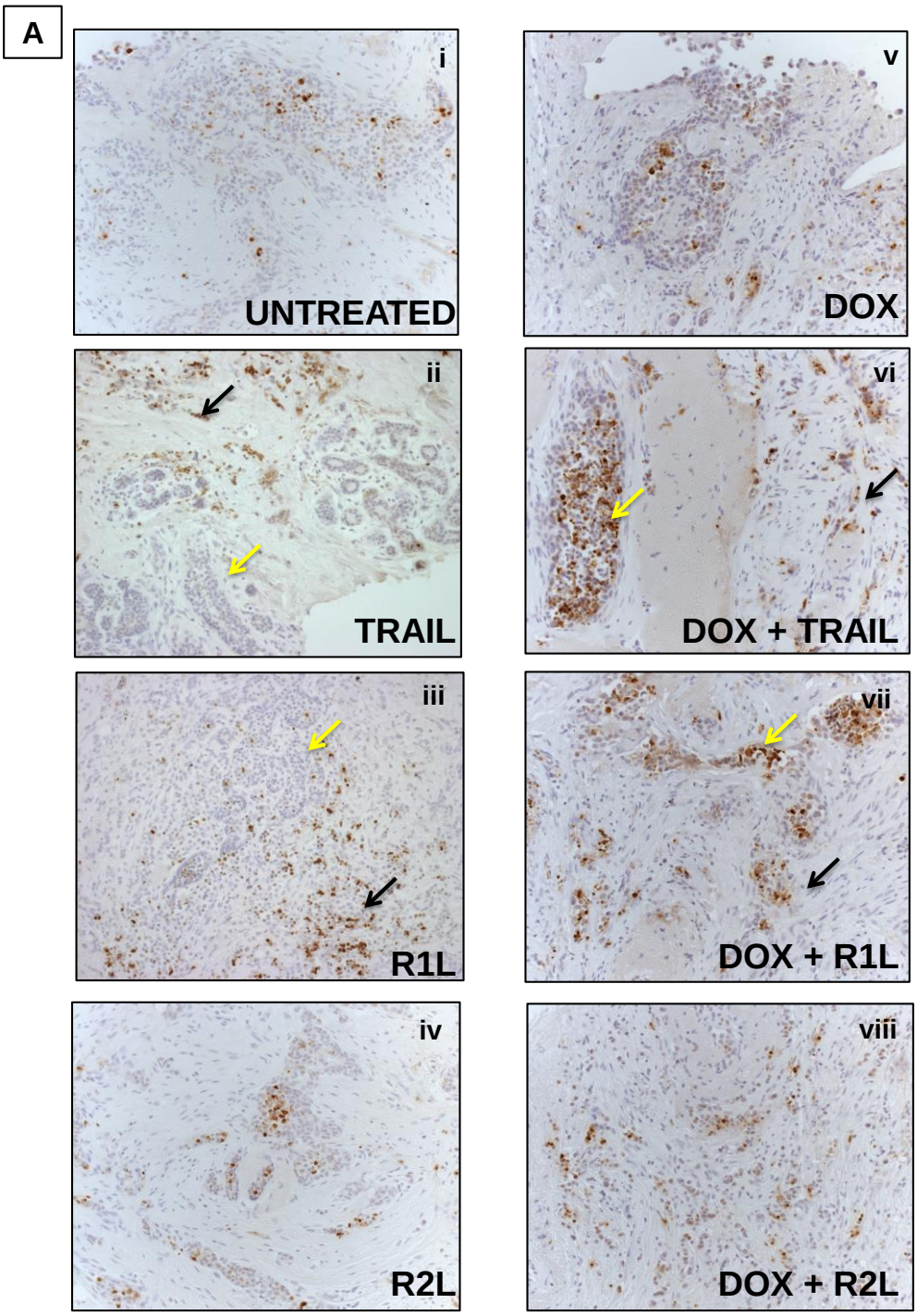
Doxorubicin, as a single agent, also did not have any effect on tumour tissue viability when used at the sub-lethal concentration of 1 μM (Figure 5.7; Part A; Panel v). Importantly, the combination of TRAIL and doxorubicin induced a significant level of apoptosis in the carcinoma (Figure 5.7; Part A; Panel vi). Considering both TRAIL and doxorubicin had no apoptotic effects as single agents, it can be assumed that the two drugs have acted synergistically to induce apoptosis. Based on the previous data shown with the cell line T47D (Figure 4.4), it can be postulated that doxorubicin sensitises the primary tumour cells to TRAIL-induced apoptosis. Importantly, the apoptotic effect of this combination could be seen in both the DCIS as well as the invasive components of the carcinoma sample (Figure 5.7; Panel vi).

To quantify the extent of apoptosis, both apoptotic and healthy cells were counted based on cleaved PARP immunostaining as described in Materials and Methods; Section 2.7. Strongly stained cells were counted as completely apoptotic (Figure 5.7; Part B; Red bars) while cells with weaker staining were judged to be still undergoing apoptosis when the tissue was fixed (Figure 5.7; Part B; Blue bars). Tissue sections

containing both the DCIS and IDC components were counted and data collated and analysed as described in Materials and Methods. Based on this quantification, it is clear that the combination of doxorubicin and TRAIL is considerably more effective than either agent alone at causing cell death.

R1L along with doxorubicin caused massive apoptosis in the *in situ* as well as the invasive tumour. Strikingly, in the presence of doxorubicin, R1L was more potent than TRAIL at inducing apoptosis (Figure 5.7; Part B). The slight increase in apoptosis in this carcinoma sample caused by R1L alone is also further evidence of its potency as compared to TRAIL. Interestingly, based on a more detailed quantification of apoptosis, it can be observed that R1L induces apoptosis in more tumour cells, however, the number of completely apoptotic tumour cells is similar in TRAIL and R1L treated tissue. A possible reason for this phenomenon maybe that TRAIL binding to its receptors can trigger both the apoptotic and survival pathways (Mühlenbeck *et al.*, 1998; Hu *et al.*, 1999; Ehrhardt *et al.*, 2003; Baader *et al.*, 2005; Trauzold *et al.*, 2006). Under this scenario, while a number of tumour cells undergo TRAIL-induced apoptosis, some of the dying tumour cells might still be rescued by the simultaneous activation of pro-survival pathways. On the other hand, R1L may signal predominantly to apoptosis and therefore, cell death can more easily be induced in a large cohort of tumour cells. An effective way of establishing whether this is indeed the case would be to culture the tissue explants for longer time points. However, due to the limited availability of tumour tissue, it was not feasible to undertake these additional experiments as a part of this study.

Significantly, doxorubicin did not sensitise the carcinoma to R2L as much as to R1L. This was reflected in the fewer number of completely apoptotic cells and those showing weak staining for cleaved PARP in the tissues treated with the R2L and doxorubicin combination. Importantly, the data from manual counting of cleaved PARP stained cells was further verified using the ImmunoRatio Plugin in ImageJ (Figure 5.7; Part C). Thus, it can be concluded that in this carcinoma sample, doxorubicin sensitised the tumour cells more strongly to R1L than to TRAIL or R2L.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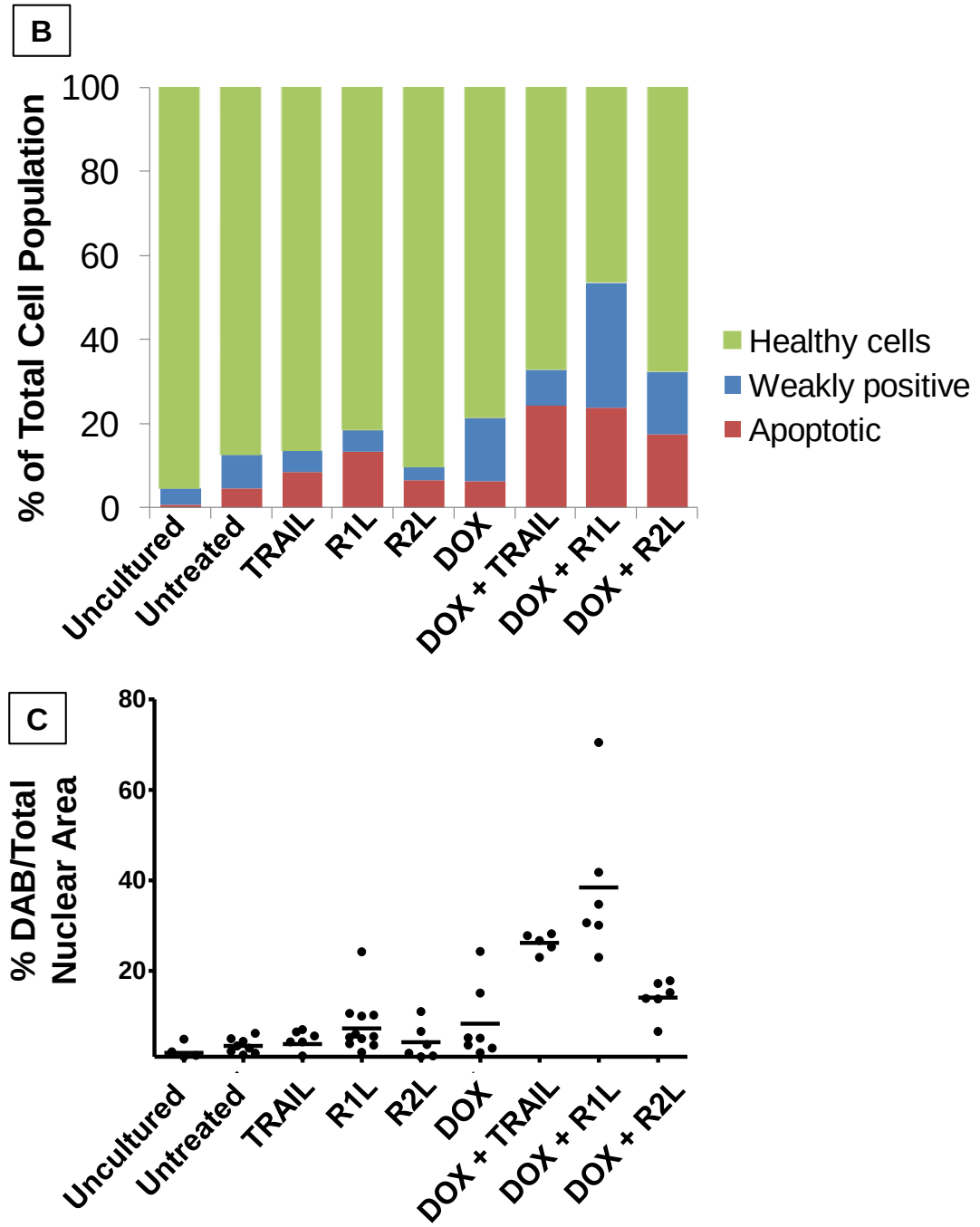


Figure 5.7 R1L is more potent than R2L in carcinoma #T9 in the presence of doxorubicin

Sample #T9 (IDC) treated with appropriate agents was stained with cleaved PARP as a marker of apoptosis. (A) shows representative images of explants exposed to different treatments (Magnification 200x). Yellow arrows indicate DCIS and black arrows indicate invasive cells. (B) is a graphical representation of the number of tumour cells counted as completely apoptotic, undergoing apoptosis or healthy for all treatments. (C) is the corresponding ImmunoRatio analysis of the extent of apoptosis.

5.5.3 Male breast carcinoma

Sample T10 was a Grade II invasive ductal carcinoma from a male patient. Male breast carcinoma (MBC) is extremely rare (less than 1% of all mammary carcinomas), with approximately 300 new cases detected in the UK per year. As with women, the incidence of MBC increases dramatically with age and presentation of the disease at an older age results in decreased survival rates. Relatively small size of the mammary gland in males allows unhindered access for the tumour cells to other tissues, but also makes the tumour more obvious resulting in early detection. Risk factors for MBC include hormonal imbalances (relative oestrogen excess or lack of androgen), Klinefelter syndrome (presence of 47 XXY karyotype instead of 46 XY) and a family history of breast cancer (particularly BRCA2 mutations). Male breast carcinomas generally express both ER and PgR but are less likely to express HER2. Treatment for MBC is similar to that for female carcinomas and the presence of ER in most male carcinomas makes tamoxifen the gold standard for hormonal therapy in men. The general lack of clinical trials for MBC precludes development of effective treatment regimes and research into the improvement of therapies is ongoing.

On histological assessment, it was observed that this carcinoma had a lot of stroma and correspondingly showed poor viability (Figure 5.8). There were areas of necrosis visible, which made assessment of this tumour difficult. Though TRAIL and doxorubicin combination did appear to have an effect on this sample, the lack of comparable viable controls ruled out further detailed analysis of this carcinoma.

5.5.4 Carcinoma T12

Sample T12 was a Grade III IDC which showed generally poor viability on subsequent culturing (Figure 5.9; Appendix 12a, 12b). Although both TRAIL and doxorubicin appeared to cause apoptosis when used as single agents, the tumour cells primarily showed weak cytoplasmic and nuclear staining for cleaved PARP instead of the more typical strong nuclear stain. This phenomenon made the quantification of this tumour difficult and therefore, it was not included in the final analysis. However, it could be observed that TRAIL, R1L and doxorubicin caused widespread apoptosis in the tumour when used as single agents. Use of combination therapy appeared to further enhance the apoptosis, but whether this effect was synergistic or additive cannot be determined. Irrespective of this, R2L failed to induce a strong level of apoptosis and therefore, it can be concluded that TRAIL-R1 is once again the predominant TRAIL death receptor signalling to apoptosis in this carcinoma sample.

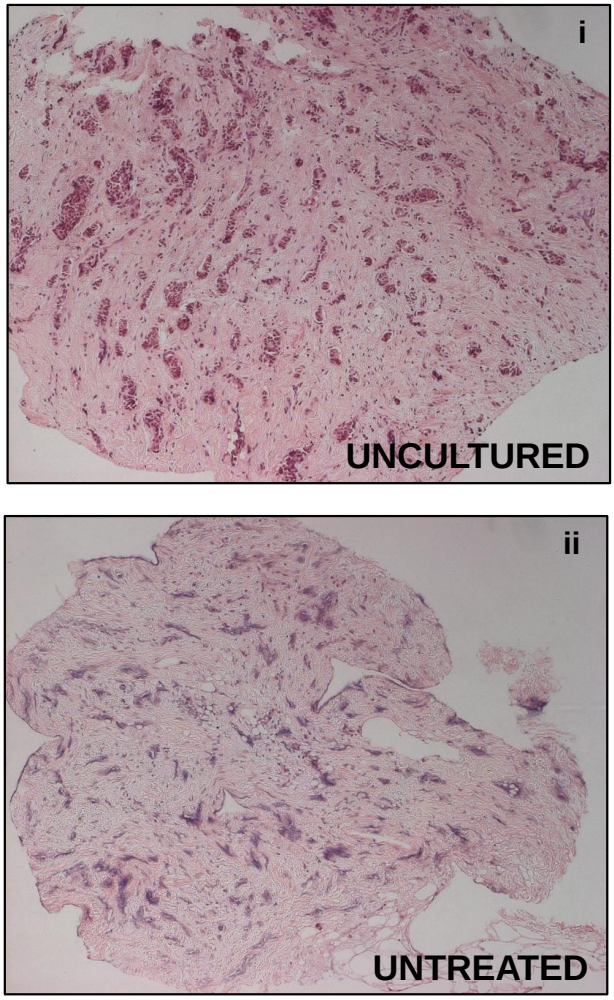


Figure 5.8 Male breast carcinoma is characterized by a large amount of stroma and poor tumour viability

Explants from the male breast carcinoma #T10 (IDC) were analysed for viability based on H & E staining either before culture (i) or after 48 h of culture (ii; representative images shown; Magnification 100x).

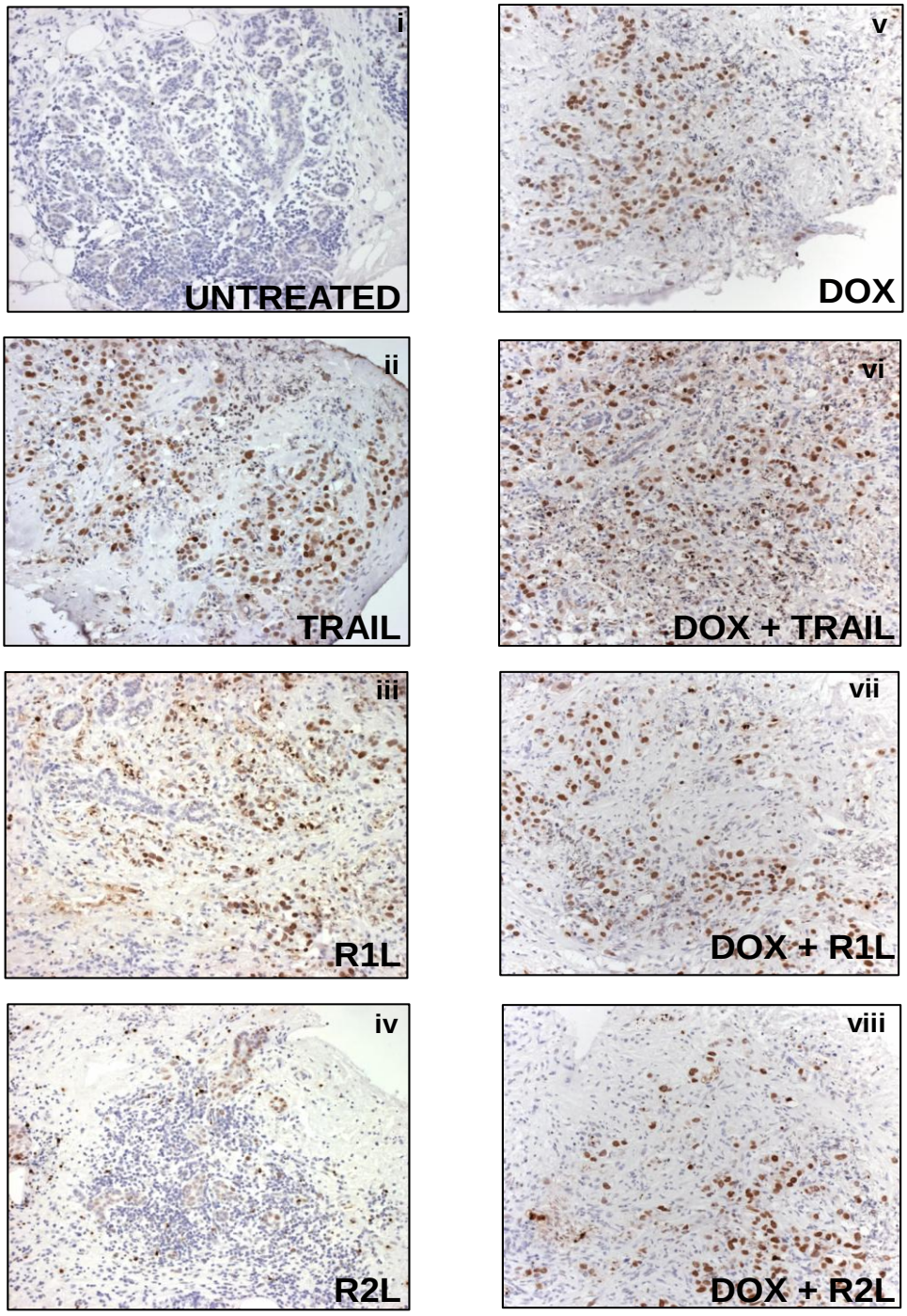
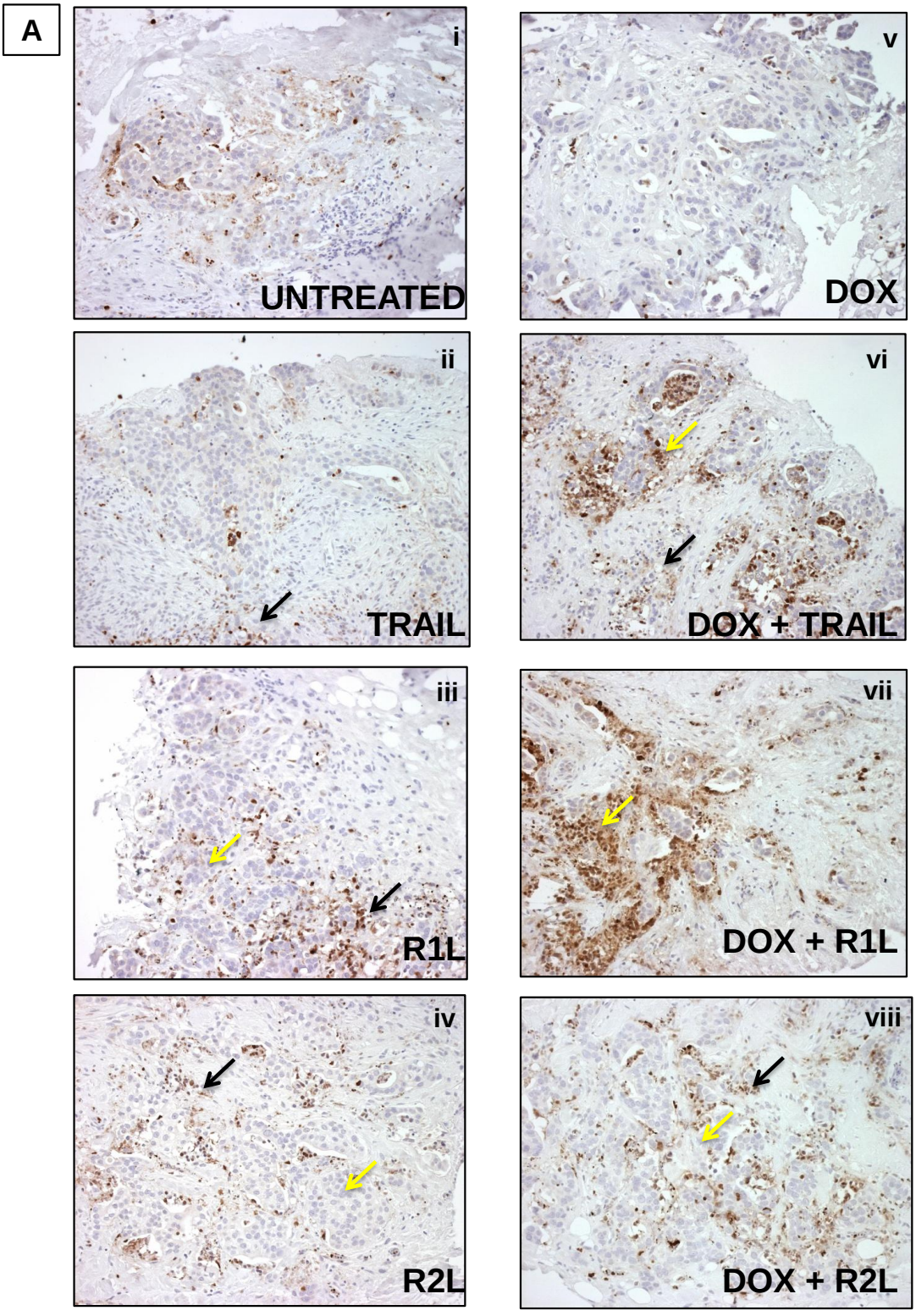


Figure 5.9 Cleaved PARP shows diffuse staining in all sections from sample #T12 Sample #T12 (IDC) was treated with the specified treatments and fixed with 10% NBF as described in Materials and Methods. Immunostaining for cleaved PARP was used as a marker of apoptosis (representative images shown; Magnification 200x)

5.5.5 Carcinoma T13

Carcinoma sample T13 was a Grade III IDC sample with a high ER expression and no HER2 amplification. In comparison to the other Grade III tumours, this carcinoma showed good viability after 48 h culture in DMEM supplemented with 1% FCS (Figure 5.10; Part B and Appendix 13a, 13b). Consistent with the earlier carcinoma samples, TRAIL did not induce any significant level of apoptosis in the tumour cells. In contrast, R1L did cause a rise in the level of apoptosis, again indicating that in primary tumour cells, R1L is more potent than TRAIL. Interestingly even in this sample, the slight apoptosis caused by R1L (and R2L) was restricted to the invasive tumour cells of the sample. The *in situ* component of the sample remained invariably resistant to the effects of TRAIL or its variants. While doxorubicin had no apoptotic effect on its own, its co-treatment with TRAIL did induce considerable apoptosis (~40%) in the carcinoma sample (Figure 5.10; Part A; Panels v-vi & Part B).

Importantly, the synergistic actions of doxorubicin and R1L led to a massive amount of apoptosis (~60%) throughout the tumour (Figure 5.10; Part B). There was strong cleaved PARP staining in both the DCIS and invasive components of the treated tumour samples. However, when R2L was used instead in the presence of doxorubicin, R2L induced only about 30% apoptosis (Figure 5.10; Part B). Intriguingly, even in the presence of doxorubicin, most of the apoptotic cells were in the invasive component rather than the DCIS, suggesting that the IDC cells have a lower threshold of apoptosis as compared to the DCIS cells. It might therefore be of clinical relevance that not only can R1L cause more apoptosis in combination with doxorubicin, but it can apparently target breast cancer cells in different stages of progression.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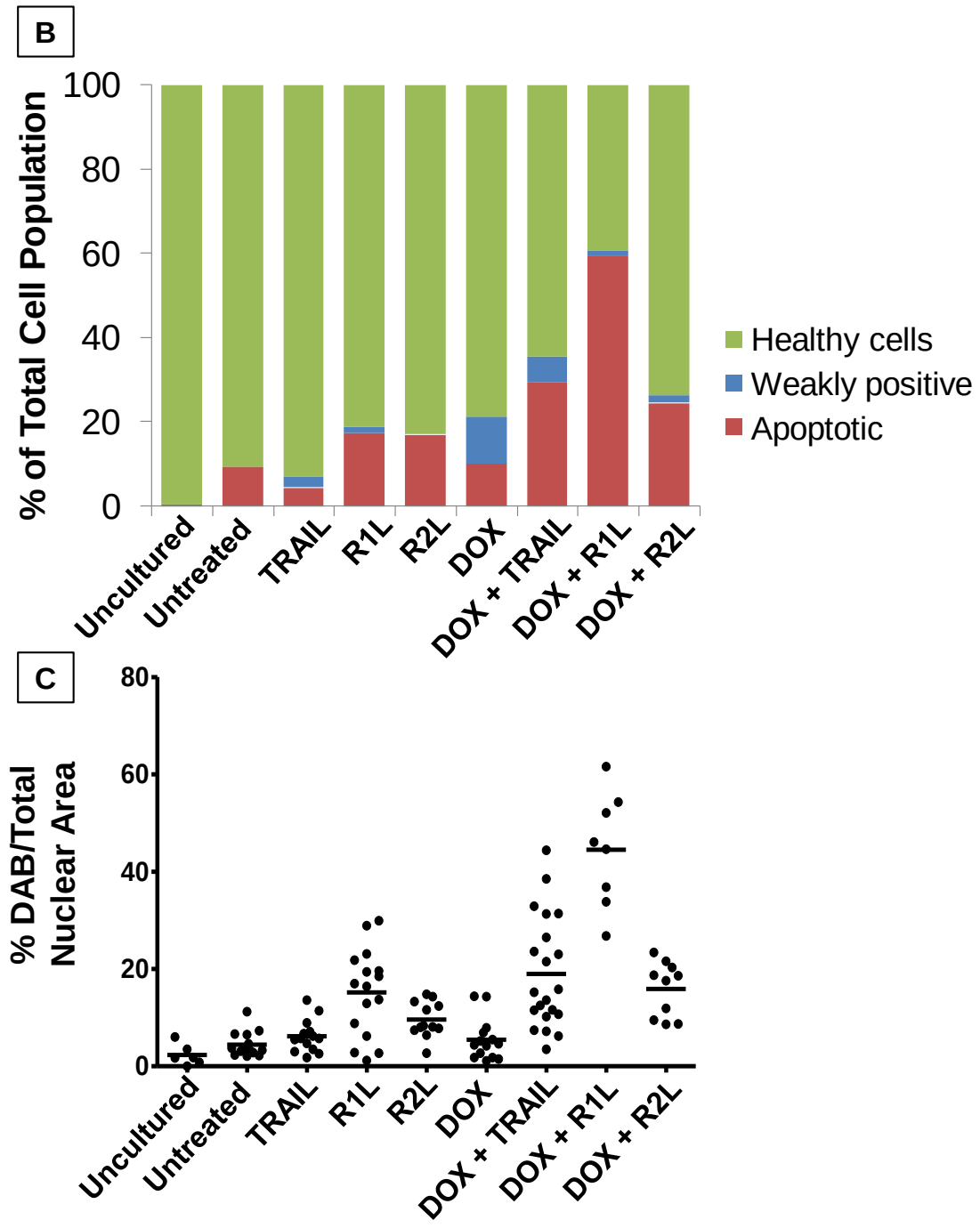


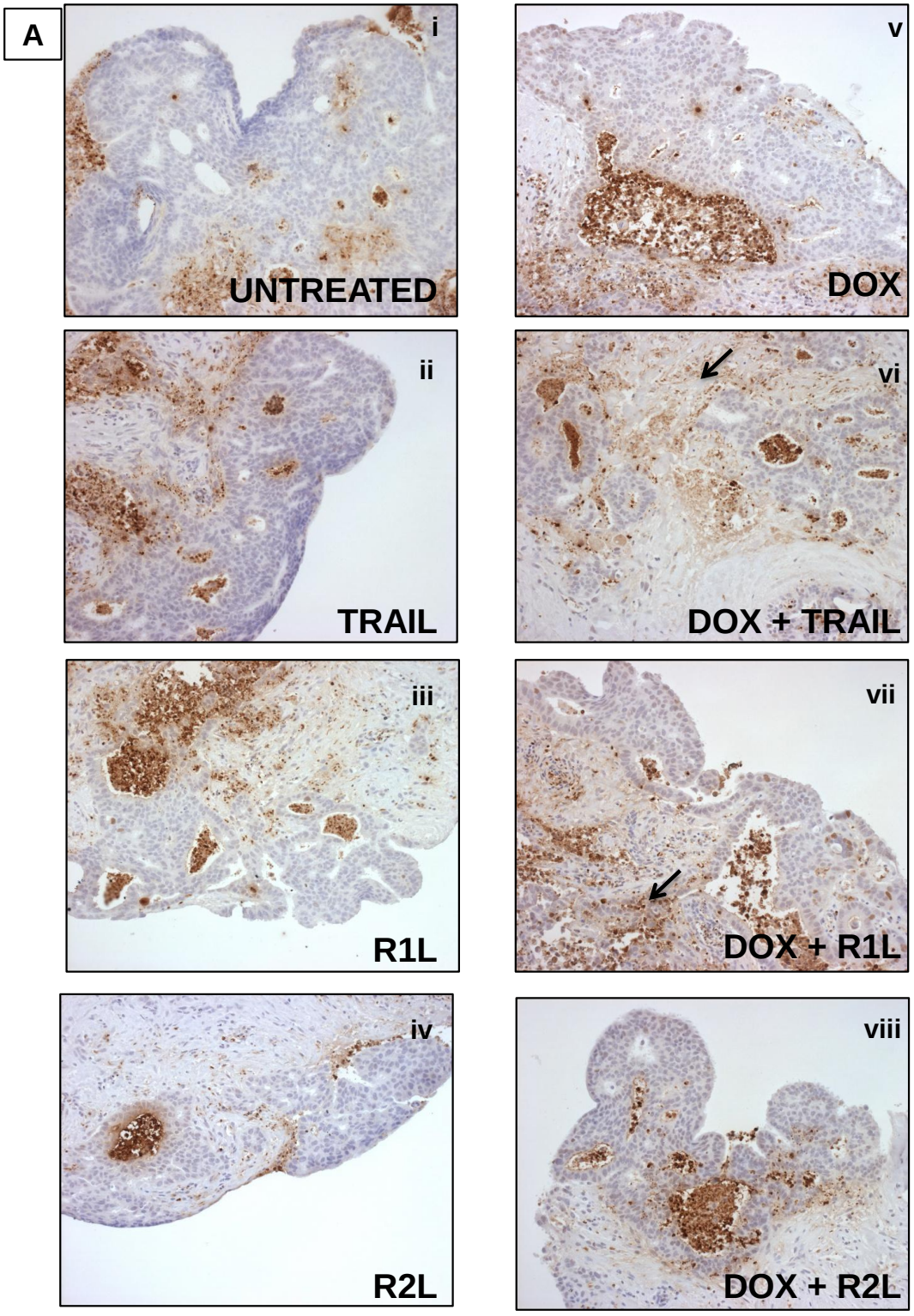
Figure 5.10 TRAIL-R1 is the major apoptotic signalling death receptor in sample #T13 Tumour explants from sample #T13 (IDC) were treated with appropriate agents and cleaved PARP was used as a marker of apoptosis. Yellow arrows indicate *in situ* tumour cells while black arrows indicate invasive cells. Data shown here as representative images for cleaved PARP staining ((A); Magnification 200x) or tumour cells categorised as completely apoptotic, undergoing apoptosis or healthy (B) or apoptosis analysed using ImmunoRatio (C).

5.5.6 Carcinoma T15

Carcinoma T15 was a Grade II IDC which had a cribiform and papillary architecture with small tubular structures in solid groups of cells. On H & E and cleaved PARP staining of untreated tumour some of the spaces and tubular structures showed evidence of necrosis (Figure 5.11; Part A; Panel I and Appendix 14a, 14b). The rest of the tumour appeared to be well differentiated, so the observed necrosis is probably not intrinsic. Possible reasons for the observed necrosis are the initial dicing of tissue or the time delay from surgery to setting up of the explant cultures. Therefore, while assessing the sample's response to the different treatments, the necrotic centre of this tumour was excluded from analysis.

In this particular sample, TRAIL, R1L and R2L were completely ineffective when used as single agents (Figure 5.11; Parts A, B & C). Unlike other IDC samples, there was no firm evidence of apoptosis induced by TRAIL or the receptor-specific ligands even in the invasive component of the sample. Importantly, not only did doxorubicin fail to cause any apoptosis on its own, but it was also unsuccessful at strongly sensitising the tumour cells to TRAIL-induced apoptosis. The doxorubicin and TRAIL combination did induce some apoptosis, but it was not as effective as that seen in the other tumours (Figure 5.11; Part A; Panel vi & Parts B & C).

Again, the co-treatment of doxorubicin and R1L was more potent than the doxorubicin and TRAIL combination (Figure 5.11; Part A; Panel vii & Parts B & C). TRAIL-mediated apoptosis again appeared to be restricted to invasive tumour cells lying around the groups of tumour cells. R2L also induced a significant amount of apoptosis in the presence of doxorubicin, but the majority of these cells showed only weak cleaved PARP staining (Figure 5.11; Part A; Panel viii & Part B). In this case, it would have been interesting to observe whether the weakly stained tumour cells would have eventually escaped apoptosis and remained viable. Overall, while even in this sample, doxorubicin sensitised the tumour cells predominantly to a TRAIL-R1 agonist, the potency of TRAIL or R1L was significantly lower when compared with all other samples in this study.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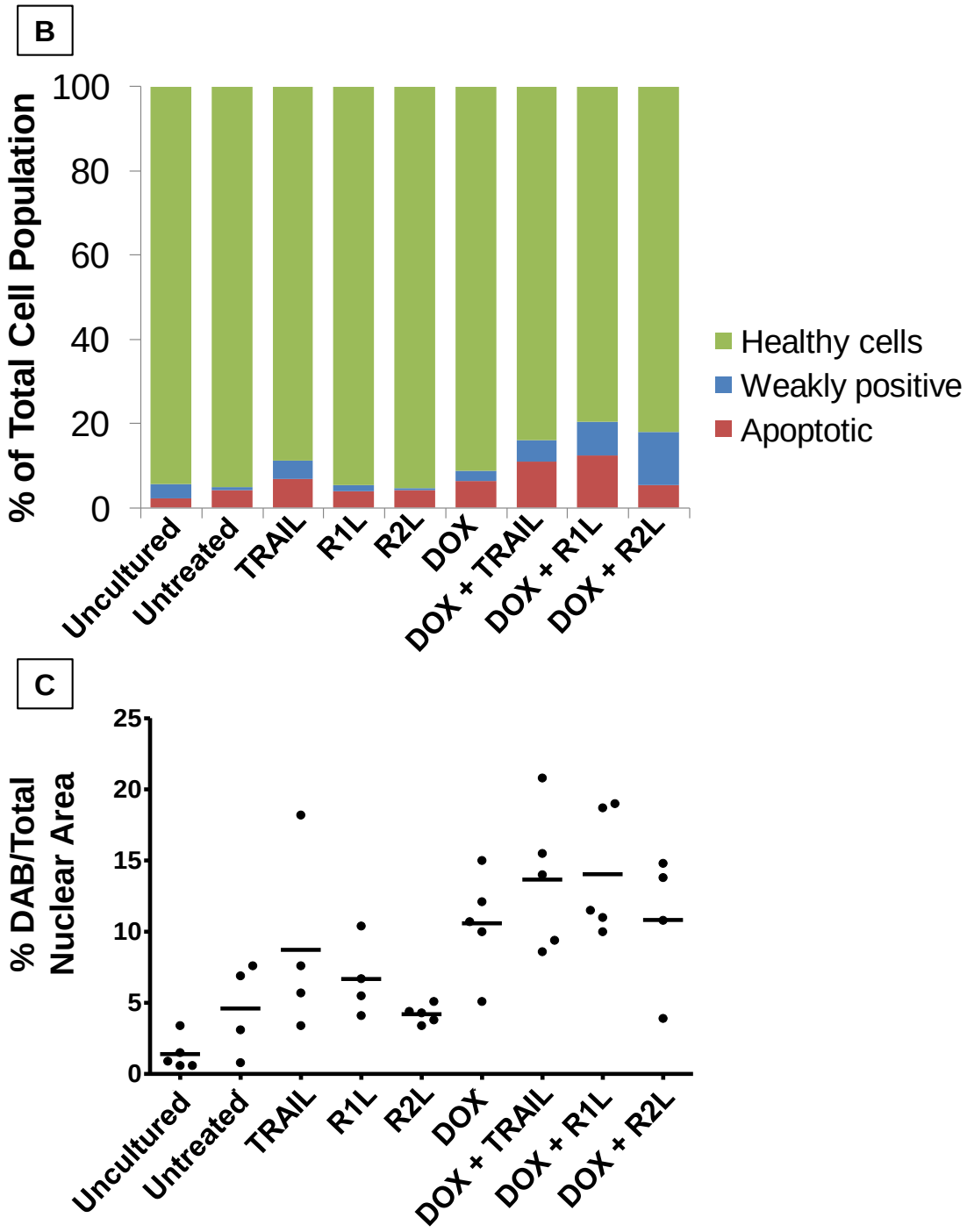


Figure 5.11 The combination of doxorubicin and TRAIL does not induce extensive apoptosis in sample #T15

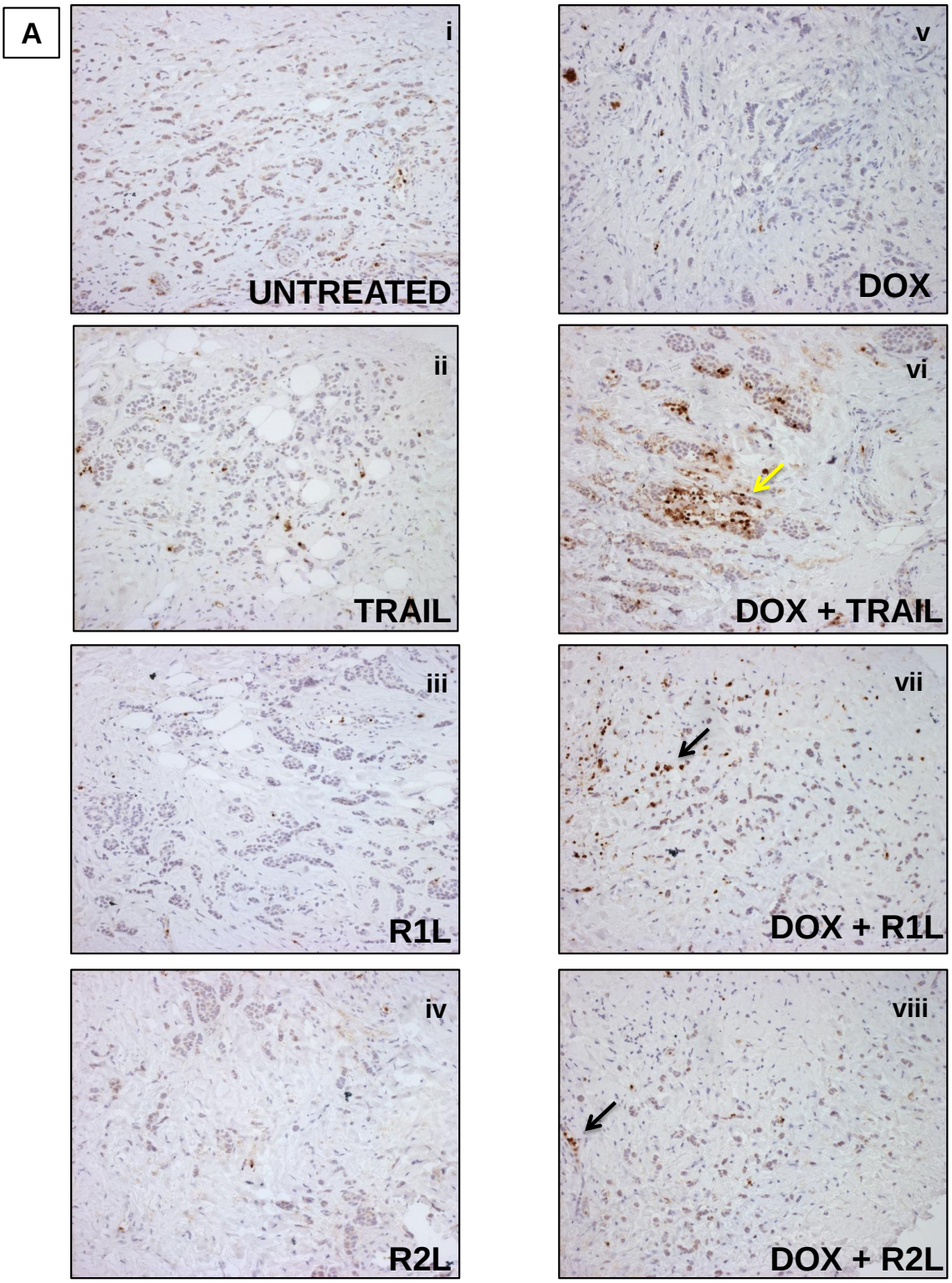
Sample #T15 (IDC) was treated with the specified treatments and subsequently stained for cleaved PARP ((A); representative images; Magnification 200x). There is some evidence of apoptosis in all samples (black arrows); counting of cells confirms that there is no significant effect seen with any treatment as quantified using Adobe Photoshop (B) or ImmunoRatio (C).

5.5.10 Carcinoma T16

This carcinoma sample was a Grade II tumour with a mixed pattern of an IDC and ILC. There was a slightly high level of background apoptosis in the uncultured tumour itself (Figure 5.12; Parts B & C and Appendix 15a, 15b). However culturing the tissue for 48 h did not cause a significant increase over this background apoptosis (Figure 5.12; Part A; Panel i & Parts B & C).

In this carcinoma, TRAIL was more potent that R1L when used as single agents and consistent with the other carcinomas, apoptotic cells were seen in the invasive component (Figure 5.12; Part A; Panels ii & iii & Parts B & C). Doxorubicin had little apoptotic effect when used as a single agent at 1 μM (Figure 5.12; Parts B & C). Doxorubicin and TRAIL combination caused cell death in both the invasive lobular and ductal components (Figure 5.12; Part A; Panel vi). When combined with doxorubicin, R1L caused much more apoptosis than TRAIL or R2L as assessed by strong staining for cleaved PARP (Figure 5.12; Part B). However, the potency of all the three ligands was comparable when the more weakly stained tumour cells were analysed separately (Figure 5.12; Part B). In this regard, it is important to note that the background apoptosis could be a contributing factor in the high levels of weakly stained tumour cells seen in this carcinoma.

Thus for this carcinoma, and especially for the doxorubicin and R2L combination treatment, it might not be appropriate to include the contribution of the weakly stained tumour cells in the overall apoptosis value. When considering total apoptosis, R1L and R2L appear to have similar potencies in this case, but if only the completely apoptotic cells are assessed, it can be concluded that R1L is more potent than R2L in the presence of doxorubicin.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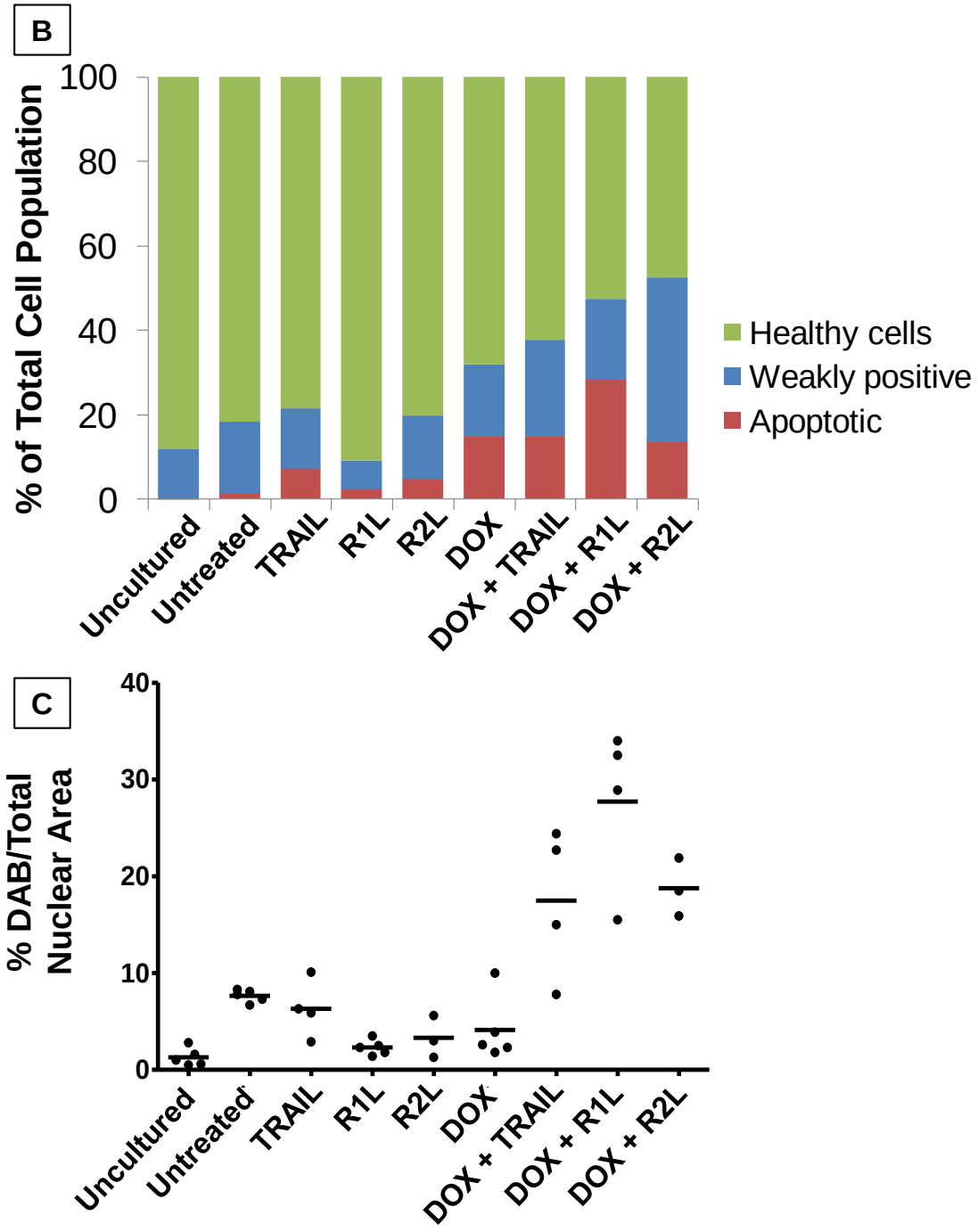


Figure 5.12 Doxorubicin sensitizes sample #T16 more to R1L than R2L

Cleaved PARP immunostaining was performed for sample #T16 (IDC/ILC) to assess apoptotic response for each treatment condition. Yellow arrow indicates apoptosis within lobular carcinoma cells while black arrows indicate apoptotic ductal carincoma cells ((A); representative images; Magnification 200x) and further analysed on Adobe Photoshop (B) or ImmunoRatio (C) to quantify level of apoptosis.

5.6 Anoikis sensitises resistant cells to TRAIL-induced apoptosis in a model for breast cancer metastatic cells

Cells from solid tumours like breast cancer can penetrate through their basement membrane and invade surrounding tissues or gain access to lymph nodes and metastasise to different areas of the body. It is therefore important for these cells to gain resistance to anoikis (cell death due to loss of attachment to a substrate), to enable them to survive in the lymph or blood.

These anoikis-resistant cells then subsequently attach to new substrates and form secondary tumours. It is hence, critical to understand the effects of anoikis on TRAIL sensitivity of tumour cells and more importantly, to target the anoikis-resistant subset of the tumour population to prevent the spread of the tumour to different parts of the body. We therefore cultured the cell lines MCF7 F43 and T47D on poly-HEMA coated plates to recapitulate the phenomenon of anoikis (Materials and Methods; Section 2.5). The cells were treated at the same time as sub-culturing and the cell colonies formed after seven days were then counted as a measure of the resistant cells.

As expected, both TRAIL (500 ng/ml) and R1L (500 ng/ml) inhibited mammosphere formation in MCF7 F43 cells (Figure 5.13; Part A). Surprisingly, even R2L induced apoptosis, albeit not as much as R1L, in MCF7 F43 cells when grown under anoikis (Figure 5.13; Part A). This result is contrary to earlier data showing that R2L is unable to trigger an apoptosis in MCF7 F43 cells grown as a monolayer (Figure 3.1). The different experimental setup used here thus suggests that anoikis may play a role in sensitising MCF7 F43 cells to R2L. To further confirm this, the TRAIL-resistant cell line, T47D was grown under anoikis conditions and its response to TRAIL was then monitored.

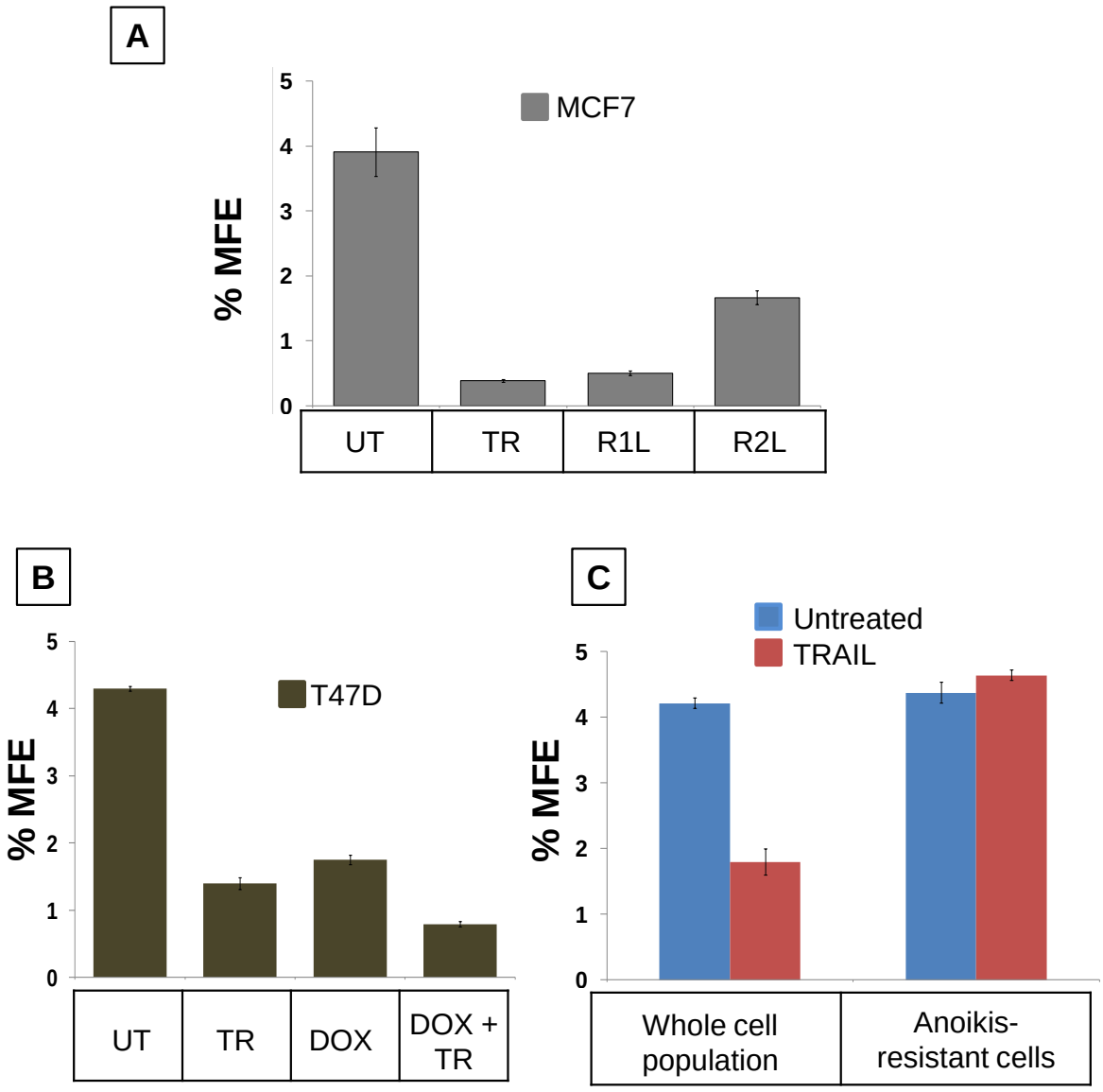


Figure 5.13 Anoikis, in combination with TRAIL, inhibits the mammosphere forming efficiency of breast tumour cell lines MCF7 F43 and T47D cells

MCF7 ((A); n=3) and T47D cells ((B), n=3) were seeded and either left untreated (UT) or treated with 0.5 µg/ml of TRAIL (TR), R1L, R2L or 0.05 µM of doxorubicin (DOX) as indicated and % MFE was calculated as described in Materials and Methods. Anoikis-resistant T47D cells were obtained and % MFE ((C); n=2) was calculated following treatment with 0.5 µg/ml TRAIL for 7 days.

Consistent with the data from MCF7 F43 cells, under conditions of anoikis, T47D cells were sensitised to TRAIL (500 ng/ml) and even doxorubicin (0.05 μM; Figure 5.13; Part B). This data reaffirms that the phenomenon of anoikis acts as a stress-inducing stimulus, which helps sensitise the cells to agents that trigger both the extrinsic and intrinsic apoptotic pathways. To confirm whether sensitisation to TRAIL was indeed related to anoikis, the anoikis-resistant T47D cell population was isolated as described in Materials and Methods; Section 2.5.

Remarkably, the anoikis-resistant T47D cells lost their sensitivity to TRAIL-induced apoptosis (500 ng/ml; Figure 5.13; Part C) and gave a similarly resistant response to TRAIL as that seen with T47D cells in monolayer culture (Figure 4.1). This experiment emphasises that indeed anoikis plays an important role in sensitising T47D cells to TRAIL-induced apoptosis. In this context, it is important to remember that *in vivo* metastasising tumour cells also undergo the phenomenon of anoikis and it is likely that during this phase they are most sensitive to TRAIL-mediated apoptosis. Administering TRAIL to patients with invasive carcinomas might therefore actually help lower the chances of tumour metastasis by targeting the metastasising tumour cells more effectively.

5.7 Primary metastatic breast tumour cells

Tumour metastases to lung, bone or brain are the major causes of death in breast cancer patients. Of the subset of tumour cells that metastasise, the tumour-initiating cells can localise in different tissues and form secondary tumours (Al-Hajj *et al.,* 2003). Hence it is of utmost importance to target this particular type of tumour cell in order to prevent both metastases and recurrence of the tumour. It is therefore, critical to study the apoptotic effects of TRAIL on the tumour-initiating stem cells of primary breast tumours, as this cell population will be a key target of any new bio/chemotherapy.

Ascites from two patients with metastatic breast cancer (BB7 and BB15) were collected and the tumour-initiating cells were selected as described in the Materials and Methods; Section 2.6. The resulting primary tumour samples were left either untreated or treated with TRAIL, R1L or R2L (1000 ng/ml). Interestingly both samples responded to TRAIL as evidenced by more than 50% inhibition of their mammosphere forming efficiency (% MFE; Figure 5.14; Parts A & B; Bar 2). Significantly, R1L showed remarkable potency in both BB7 and BB15 tumour samples (Figure 5.14; Parts A & B; Bar 3). R1L induced a higher inhibition of % MFE as compared to TRAIL or R2L (Figure 5.14; Parts A & B; compare bars 2, 3 & 4). Consistent with the data obtained in

the other breast cancer models (cell lines and primary tumour explants), this study also emphasises the importance of TRAIL-R1 as the dominant TRAIL death receptor in breast cancer. We also used TRAIL in combination with doxorubicin on these two tumour samples; however, the tumour cells were resistant to doxorubicin as a single agent (data not shown). This resistance might have been acquired during the previous treatment with epirubicin since these drugs have similar mechanisms of action. Consequently, doxorubicin also failed to cause any further sensitisation of the tumour cells to TRAIL-induced apoptosis. Nonetheless, the study on the BB7 and BB15 primary tumour cells indicate that in pre-treated advanced metastatic breast cancers, treatment with R1L would be of more value than TRAIL or R2L.

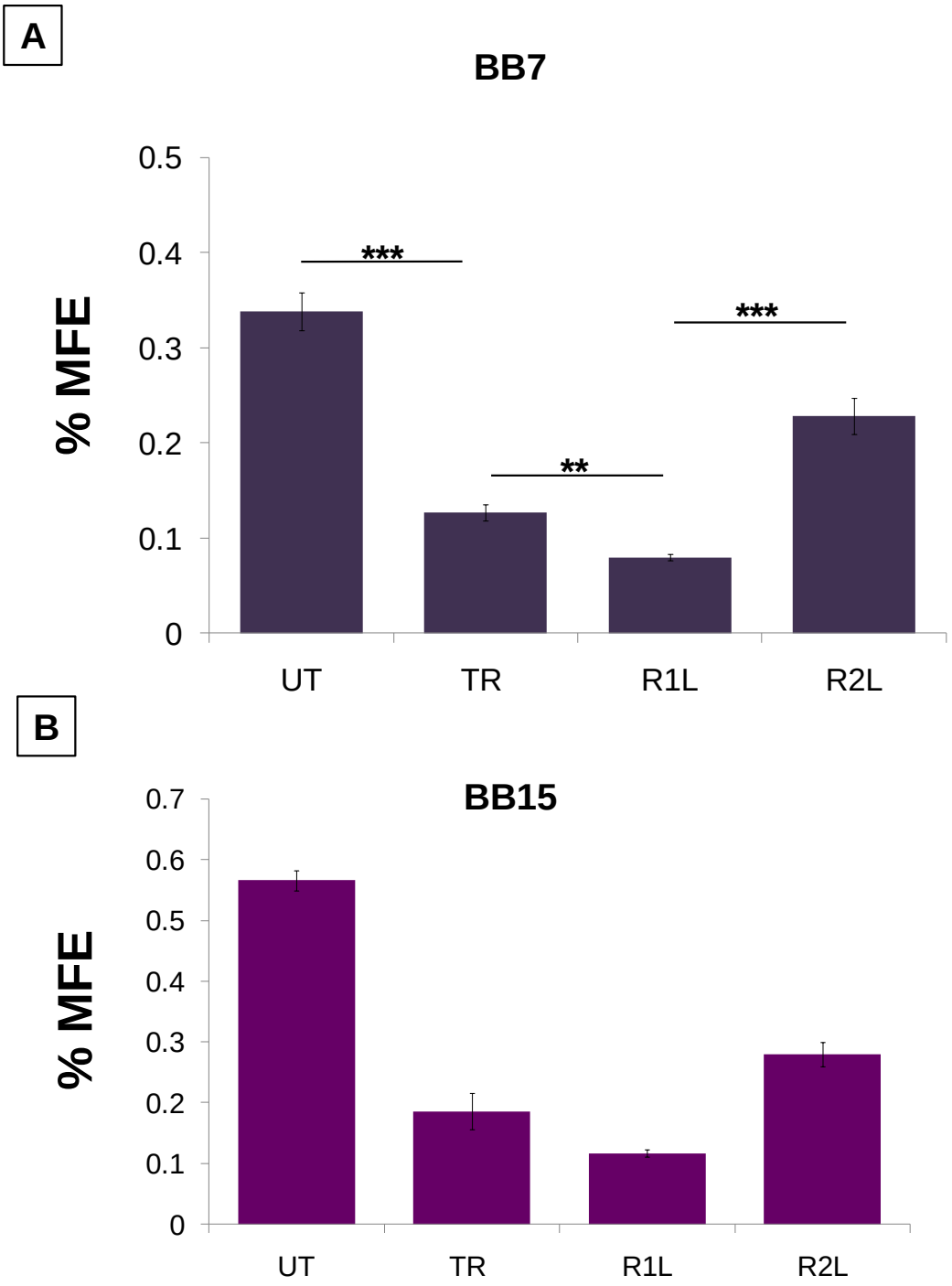


Figure 5.14 R1L is a more potent inhibitor of MFE than TRAIL or R2L in pre-treated advanced metastatic breast tumour-initiating cells

Tumour initiating cells ($ESA^{+}/CD44^{high}/CD24^{low}$) from Sample # BB7 ((A); data are represented as mean ± SEM (n=3)) or Sample # BB15 ((B); data are represented as mean ± range (n=2)) were isolated and treated with 1 µg/ml of TRAIL (TR), R1L or R2L. Mammospheres were counted after 10 days. ** and *** indicate significant difference ($P<0.01$ and $P<0.001$ respectively).

5.8 Discussion

A major drawback in the development of successful new therapies for solid tumours is the lack of relevant 3D model systems. As mentioned earlier, the TME plays an important role in tumour progression and drug resistance; an effect which cannot be recapitulated by cell lines. Hence, we used the more pertinent tumour explant model to examine the effect of combination treatment on breast cancer. It is clearly evident that while most breast cancer cell lines we tested were sensitive to TRAIL-induced apoptosis, most of the primary tumours were resistant. These data illustrate that primary human tumours and tumour cell lines established in culture respond very differently to TRAIL, thus again emphasising the need for better representative 3D tumour models for solid tumours.

5.8.1 Optimisation of tissue culture and immunostaining

The explant culture model, originally described by Hood and Parham (Hood and Parham, 1998), had to be modified to sustain the growth of breast tumour tissue. It was clear from the culturing of normal and tumour breast tissue that there were key differences between the growth requirements of these two tissue types; the tumour tissue was clearly more self-sufficient at producing the necessary growth factors and therefore, was less reliant on the presence of FCS than the normal breast tissue. Differences in the microenvironment of normal tissue and tumour tissue would also likely contribute to the variation in culture requirements. The reduced viability of more aggressive tumours in our culture system indicates that as the tumour grade increases, the dependence of tumour cells on external growth supplements decreases. Based on these data it would be recommended that higher grade tumours should be cultured in media containing FCS at concentrations of 1% or less. Indeed, evidence for this comes from the finding that the viability of the Grade III carcinomas, T6 and T13 could be retained when cultured in media supplemented with 1% FCS, but not 5% FCS (data not shown).

Post-culturing, two well established markers of apoptotic cell death – cleaved caspase-3 and cleaved PARP – were used to assess the potency of various treatments. Immunostaining for cleaved PARP was stronger than the staining obtained for cleaved caspase-3 which might reflect different affinities of the individual antibodies. However, a more likely explanation is that cleaved caspase-3 is rapidly degraded, whereas cleaved PARP is more stable within the tissue explants. As a result, cleaved PARP

would accumulate to a greater extent within the apoptotic cells. Based on its apparent increased sensitivity, we chose to use cleaved PARP as our primary marker of apoptosis while corresponding cleaved caspase-3 immunostaining was used to confirm the observed apoptosis (Appendices A9a-A15a).

5.8.2 Overview of apoptosis data from primary human breast tumours

As mentioned earlier, drug-associated side effects are a major drawback of currently used anti-cancer therapies, and this optimised explant model system is ideal to determine the potential benefit to risk ratio of candidate drugs prior to their use *in vivo*. If the candidate drug does not cause apoptosis in the explant model, there is obviously no benefit in administering it to the patient. However, it is important to remember that the explant model system cannot account for the effect of the drug on other tissues (for example, hepatocytes) and therefore, should not be used as a substitute for pre-clinical animal models for the study of drug safety. However, this model system can be used to evaluate the potential benefits of anti-cancer agents like TRAIL and doxorubicin which have already been approved in terms of initial safety testing in relevant preclinical animal models and Phase I clinical trials.

While the data from the seven normal breast samples established that normal breast tissue cells are resistant to both TRAIL and TRAIL-receptor-specific ligands (Table 5.1), the data from the quantifiable tumour samples suggested that TRAIL sensitivity of breast cancer depends on the type of cancer. Since none of the special sub-types of invasive carcinomas, such as medullary or papillary carcinomas were available during the course of this study, determination of their sensitivity to TRAIL was precluded. Importantly and consistent with previous reports, the majority of primary breast tumours tested were resistant to the apoptotic effects of TRAIL. However, the mucinous carcinomas, which represent more than 40,000 cases of breast carcinomas detected every year worldwide, were sensitive to TRAIL-induced apoptosis. Importantly, mucinous carcinomas appeared to be susceptible to TRAIL and R1L, but not R2L; however, this striking result would need to be further confirmed using a larger cohort of patient samples.

Interestingly, in the mucinous carcinoma T1, there were pockets of tumour cells (Figure 5.4; Part B; Panel vii; indicated by blue arrow) which did not show any apoptotic response to R1L. This lack of response could be the result of poor diffusion of the ligand to the tumour core due to the relatively short treatment time used (24 h). In this

regard, the limited penetration of anti-cancer drugs through solid tumours has already been highlighted as an important cause of clinical drug resistance (Tannock *et. al.,* 2002). However, this scenario may not be a major problem *in vivo* because as the peripheral tumour cells die, they would undergo phagocytosis, thus allowing R1L to access the cells at the core of the tumour. Alternatively the lack of response could mean that the central tumour cells are inherently resistant to TRAIL-induced apoptosis. Indeed, previous studies have postulated that hypoxic tumour cells are resistant to anti-cancer drugs (reviewed in Teicher, 1994). If the cells at the centre of a tumour mass are indeed TRAIL-resistant, it would be useful to combine a suitable chemotherapeutic agent as a possible sensitiser along with TRAIL therapy. However, due to the limited availability of patient samples and the fact that mucinous carcinoma is a relatively rare tumour sub-type, it was not possible to study the effect of combination therapy on mucinous carcinomas.

Differences in the sensitivities of the mucinous and other invasive sub-types to TRAIL-induced apoptosis could be due to the presence of varying mutations in the apoptotic pathways arising during tumour progression. It is also possible that the differing patterns of stroma between the carcinoma sub-types affect the sensitivity of the tumour cells to TRAIL. While in mucinous carcinomas, the jelly like stroma might aid TRAIL sensitivity, it is possible that the stroma in the other invasive tumours does not allow TRAIL to efficiently diffuse and thus, induce apoptosis.

The IDC and IDC/ILC mixed carcinomas showed varying sensitivity to TRAIL, with the invasive component being more responsive to TRAIL or R1L than the *in situ* component. In some carcinoma samples (T9 and T13) TRAIL and R1L induced a modest extent of apoptosis on their own, while R2L failed to induce any cell death. The differences in responses between the *in situ* and invasive tumour cells might be because the ligands failed to penetrate the membrane bound assembly of *in situ* tumour cells. This situation would be similar to that seen in carcinoma T1 and as explained before, should not be an issue *in vivo* as phagocytic clearance of peripheral cells should allow ligand access the tumour cells at the centre of the tumour mass.

Another possible explanation is that the 3D architecture of the *in situ* tumour and the associated cell adhesion confers TRAIL resistance to these cells. This occurrence would be similar to that reported by Weaver *et al* where TRAIL-sensitive tumour cells cultured in spheroids gained resistance to TRAIL-induced apoptosis (Weaver *et al.,* 2003). It has already been reported earlier that in colon carcinogenesis, the adenoma stage is less sensitive to TRAIL-induced apoptosis as compared to the carcinoma stage (Hague *et al.,* 2005). Additionally, the difference in the sensitivities might be the

result of extra mutations that occur during tumour progression which facilitate apoptosis induced by extrinsic stimuli.

Importantly, doxorubicin, which is already used in the clinic as a breast cancer therapy, sensitised the previously resistant *in situ* carcinoma components to TRAIL and R1L, while also enhancing ligand-induced apoptosis in the invasive tumour cells. Importantly, in the four quantifiable carcinoma samples, exposed to doxorubicin, R1L was significantly more potent than either TRAIL or R2L. Amongst the four carcinomas, sample T15 was the least responsive to the combination of doxorubicin and TRAIL. Possible reasons for this reduced potency of the combination therapy are the influence of the stroma, cell adhesion-mediated drug resistance and a complete lack of and/or mutations in the individual TRAIL receptors on the tumour cell surface. As mentioned earlier, the tumour cells in this sample appear to be clumped together, and similar to DCIS, are resistant to TRAIL-mediated apoptosis. However, while the DCIS in the other carcinomas could be sensitised to TRAIL using doxorubicin, the combination treatment failed to have any strong effect on this particular sample. This, thus, hints at the presence of an additional mechanism of resistance, possibly the ineffective induction of apoptosis due to specific defects in the apoptotic pathway. Mutations in TRAIL-R1 and TRAIL-R2 as well as caspase-8 which would affect apoptosis signalling have been described in the literature (Shin *et al.,* 2001; Soung *et al.,* 2004). Investigation of the reason for the lack of response shown by this particular carcinoma would aid in dissecting the resistance mechanisms shown by primary tumour samples and would thus help improve tumour therapy. Overall, in the four quantifiable primary tumour samples, the doxorubicin and R1L combination was the most potent of all the treatments used (Figure 5.15).

In addition to the primary tumour explants, R1L alone was also the most potent inhibitor of mammosphere forming efficiency in the two advanced metastatic breast cancer samples. However, it is important to remember that both the patients had received prior chemotherapy and this possibly would also help sensitise the tumour cells to TRAIL-induced apoptosis. Particularly, both patients had previously received epirubicin treatment, which has similar mechanisms of action as doxorubicin and therefore, might play a role in sensitising the tumour cells to TRAIL. Also, as shown previously, tumour cells undergoing anoikis can be sensitised to TRAIL (Figure 5.13) and this could also be a contributing factor to the observed TRAIL sensitivity of the non-adherent mammospheres.

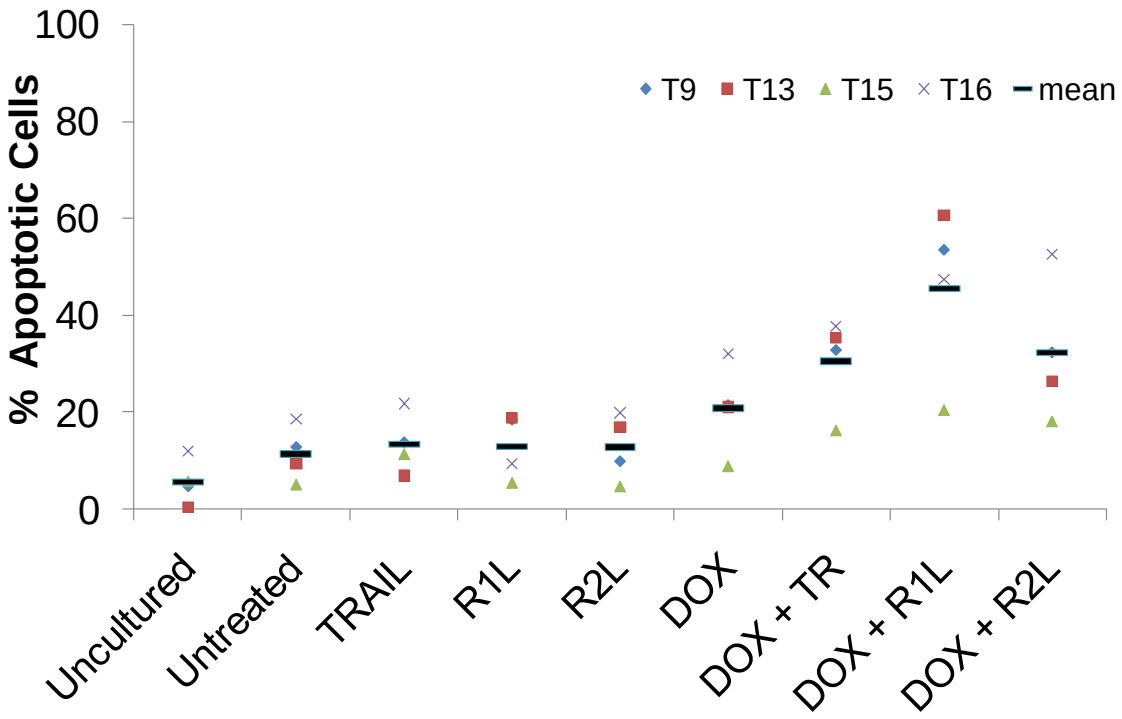


Figure 5.15 Doxorubicin sensitises primary breast tumour cells mainly to a TRAIL-R1-specific ligand

The apoptotic response to combination therapy in breast tumour samples from four patients diagnosed with invasive breast tumour (T9, T13, T15 and T16) was assessed based solely on extent of cleaved PARP immunostaining.

However, for tumour cells to metastasise, they need to survive detachment from the basement membrane and it is possible that the metastatic cells are inherently anoikis-resistant. Thus, while both BB7 and BB15 tumour samples responded to TRAIL and R1L, it is difficult to distinguish whether the sensitivity observed is inherent or a result of anoikis and/or previous chemotherapy.

Thus, the results of this study provide a strong basis for the development of TRAIL variants targeting TRAIL-R1 as potential anti-tumour agents for the treatment of breast cancer, especially to be used in combination with pre-existing chemotherapy (for example, doxorubicin) or more selective agents currently under evaluation. However, the sample size for this study was limited due to the restricted number of breast tumours of sufficient size from patients who had not received prior therapy to allow vital prognostic assessment as well as research. Several of the samples had to be used for optimisation of tumour culture methods, while a number of others showed poor viability in culture. Therefore, a larger study using an increased sample size is warranted to verify the results obtained from this preliminary work.

Importantly though and considering the potency of TRAIL and R1L in all the models of breast cancer we have used, it would be reasonable to expect that TRAIL-R1 is the predominant TRAIL death signalling receptor in breast cancer. In addition, combining a TRAIL-R1-targeting agent with sub-lethal concentrations of doxorubicin will not only improve tumour response but also decrease drug-associated side effects caused by high doses of doxorubicin when used as a monotherapy.

Chapter 6

Discussion

6.1 Introduction

Breast cancer is a characteristically heterogeneous disease and is the leading cause of death amongst women worldwide. Commonly used treatment measures for breast cancer are surgery, hormonal therapy, standard chemotherapy (FAC/FEC) or targeted therapy (Herceptin/Trastuzumab). Although there has been a surge in survival rates, most current therapies have a significant detrimental effect on patient health and despite intensive research the heterogeneous nature of the disease has made development of new therapies difficult. A major obstacle in the use of current treatments, and in the development of new therapies, is ensuring selectivity between normal and tumour cells. A critical drawback of contemporary treatment regimes are the drug associated side-effects which have severe implications for the standard of living of patients.

6.2 TRAIL as a cancer biotherapeutic

The use of TRAIL as a cancer treatment has received much attention, especially because of its apparent unique ability to specifically target tumour cells (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999; Ichikawa *et al.*, 2001; Lawrence *et al.*, 2001). Furthermore, the TRAIL apoptotic pathway is neither reliant on activation of p53 nor does TRAIL need to accumulate in target cells in order to be active. This in itself successfully circumvents two important resistance mechanisms of tumour cells – p53 mutations or deletion (reviewed in Brown and Attardi, 2005) and the up-regulation of transporter proteins (for example, P-glycoprotein) to prevent intracellular drug accumulation (reviewed in Gottesman *et al.,* 2002). In a number of studies, TRAIL has shown impressive pre-clinical potential as an effective and selective cancer therapeutic. However, the majority of primary human tumour cells have been shown to be resistant to TRAIL-induced apoptosis (Ehrhardt *et al.,* 2003; MacFarlane *et al.,* 2005a; Herbst *et al.,* 2006; Todaro *et al.,* 2008). This problem can be easily resolved through the use of agents that sensitise tumour cells to the apoptotic effects of TRAIL.

In our laboratory, we have previously shown that in primary CLL cells, sensitised to TRAIL by an HDACi, TRAIL-R1 is the predominant functional death receptor signalling to apoptosis (MacFarlane *et al.*, 2005a; MacFarlane *et al.,* 2005b). Antibodies specific for TRAIL-R1 or TRAIL-R2 are in clinical trials thus, making it imperative to profile functional TRAIL receptor signalling in primary tumours before administering a specific antibody therapy. Clearly, using TRAIL-R2-specific signalling agents in primary CLL cells would not yield any favourable results and may even actually trigger cell survival

pathways in the target tumour cells. Therefore, the primary aim of this project was to determine the major functional TRAIL death receptor in breast cancer in a bid to improve the efficacy of future TRAIL-based therapy against this highly prevalent epithelial cancer.

6.3 Use of explant culture as a pre-clinical model for testing drug efficacy

An important limitation to therapy development for solid tumours is the lack of suitable pre-clinical models for drug testing. Most screens for choosing novel drugs are performed using breast cancer cell lines, which at the most likely represent metastatic tumour cells. The influence of the tumour micro-environment (TME) on drug resistance in primary tumours has been discussed before and the complete lack of any 3D architecture/TME in cell line models can provide misleading results. The primary tumour explant culture described in this study is a pertinent model for testing of drugs prior to administering new/combination therapies. The explant model is a quick and easy *ex-vivo* tool which can be used to get reliable results about drug efficacies, the role of the tumour microenvironment in drug sensitivity and any related toxicity of drugs on normal cells within the same tissue. However, it is important to note that in the case of new drug combinations pre-clinical testing for any hepatocyte toxicity would still be required. Probably the only drawback for the explant culture method is that drug metabolism and thus, the effects of the drug and its intermediate products on healthy tissue (for example, hepatocytes) cannot be assessed. Thus, although this method offers an excellent platform for preliminary drug testing, care should still be taken before applying the drug in the clinic.

In addition, there has been a growing argument for personalised therapy for cancer and a relevant model which could assess drug sensitivity of individual tumours within a short time is wanting. In this regard, the research done in this study provides a reliable method for assessing primary tumour responses to drugs *in vitro* whilst also suggesting a potential drug combination which would be optimal for patient therapy.

6.4 Efficacy of TRAIL and TRAIL-receptor-specific ligands in breast cancer

Consistent with previous studies, most of the established breast cancer cell lines used in this project were responsive to TRAIL and significantly, all the responsive cell lines were sensitive to R1L but not R2L (Table 3.2; Figure 6.1). The cell line T47D, however, was completely TRAIL-resistant and required a sensitising agent to facilitate apoptosis induction (Figure 4.2). Amongst the primary breast tumour samples, only the

rare mucinous carcinomas were remarkably sensitive to TRAIL-induced apoptosis (Figures 5.4 & 5.5). As proposed earlier, TRAIL sensitivity of mucinous carcinomas might be as a result of the influence of their mucin-rich stroma. Nonetheless, similar to the compelling cell line data, even the mucinous carcinoma was sensitive to R1L but not to R2L (Figure 5.4 & 6.1).

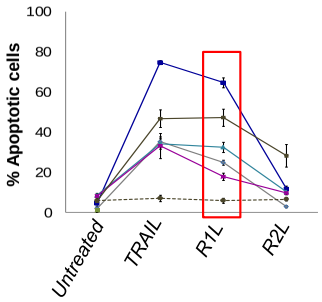
It is important to note that the IDC and carcinomas of mixed type were made up of tumour cells at different stages of progression. Breast cancer develops from a pre-invasive stage (DCIS), to an invasive stage (IDC/ILC) and ultimately can manifest as metastatic disease. During this progression, tumour cells acquire mutations that are required for their transition to the next stage and the resultant cohort of cells may have different drug sensitivities from their parent cells. Thus, even within the same patient, breast tumour cells can display different phenotypes and consequently, have varying sensitivities to chemotherapeutic drugs. Therefore, it is of utmost important to target all the different tumour cells to prevent any disease progression or relapse.

Targeting of pre-invasive tumours using chemotherapy is essential during neo-adjuvant therapy or for the removal of any residual tumour cells post-surgery; whilst effective killing of invasive and metastatic tumour cells is necessary to prevent secondary tumour growth. Failure to affect any of the different stages of tumour cells would undermine the efficacy of the treatment and the risk of relapse would remain, causing a decrease in the likelihood of survival. It is therefore important to understand the effect of TRAIL on the individual tumour stages while evaluating its potential clinical efficacy.

The data presented here have shown that the non-invasive component of primary tumours was consistently resistant to TRAIL as a single agent and showed negligible apoptosis across the quantifiable samples. However, the response of the invasive carcinomas varied, with modest apoptosis observed in some tumour samples. This variation in response is likely to be the result of differences in the genetic background of the samples but may also be caused by tumour heterogeneity within the sample. In the invasive carcinomas that did respond to TRAIL as a single agent, R1L seemed to cause more cell death than R2L. As pointed out earlier, these differences observed in the sensitivity of the tumour stages (non-invasive versus invasive) could be attributed to cell-adhesion mediated drug resistance as well as to the presence of differing genetic mutations.

TRAIL-Receptor Signalling To Apoptosis In Breast Cancer

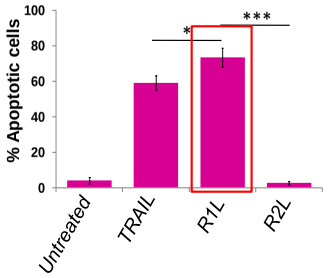
Breast cancer cell lines



TRAIL signals to apoptosis through TRAIL-R1, irrespective of p53 and ER status

Mucinous carcinomas

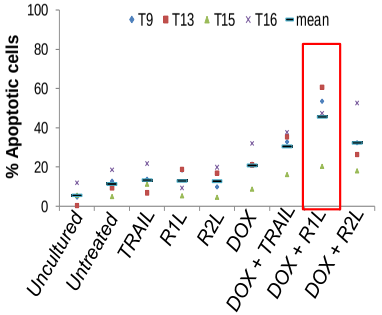
More than 1000 cases detected per year in the UK



Mucinous carcinomas are sensitive to TRAIL and R1L-induced apoptosis

Invasive ductal/lobular carcinoma

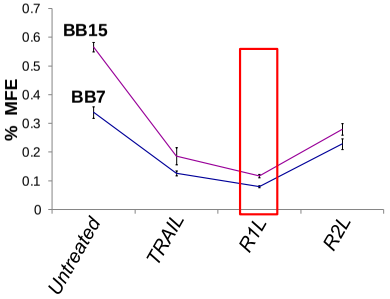
Generally resistant to TRAIL-induced apoptosis



Doxorubicin sensitises invasive carcinomas to R1L

Metastatic breast cancer cells

Potentially sensitised to TRAIL-induced apoptosis by anoikis/chemotherapy



R1L is a potent inhibitor of MFE in metastatic breast cancer cells

TRAIL-R1 is the major TRAIL death receptor signalling to apoptosis in Breast Cancer

Figure 6.1 Summary of the major findings of my thesis

The advanced metastatic breast tumour cells (mammospheres) came from heavily pre-treated patients and therefore, it is not possible to conclude whether the observed cell sensitivity to TRAIL is inherent to these cells or acquired as a result of adjuvant therapy.

Collectively, the data from breast cancer cell lines, mammospheres and primary tumours suggests that while most cell lines are TRAIL sensitive, primary tumours give a varied response. The data shown here demonstrate that anoikis and chemotherapy can potentiate the potency of TRAIL in tumour cells and this can be exploited in order to achieve maximal tumour remission while restricting drug associated side-effects to a minimum. Even in the case of sensitive cell lines/primary tumours, TRAIL is not absolutely effective and therefore, targeting these tumours with combination therapy might aid in complete eradication of the disease.

6.5 Efficacy of combination therapy on breast cancer

In this study, I used the TRAIL-resistant cell line T47D as a model for testing the ability of chemotherapeutic drugs to induce sensitisation to TRAIL. Importantly, the absence of HER2 amplification in T47D cells precluded the use of Herceptin/Trastuzumab as a sensitising agent for TRAIL. However, amongst the different hormonal and chemotherapeutic agents used, doxorubicin was the most promising agent to act as a TRAIL sensitiser (Figure 4.2).

Doxorubicin is commonly used as a breast cancer therapeutic, but its dosage is limited by its adverse side-effects including alopecia, amenorrhea and cardiotoxicity. Nonetheless, doxorubicin at a clinically relevant concentration (1 μM) sensitised T47D cells to TRAIL-induced apoptosis through an enhancement in the extent of TRAIL DISC formation. Interestingly, doxorubicin induced a greater level of sensitisation in T47D cells to R1L than R2L and it can thus be concluded, that even in the presence of a sensitising agent, TRAIL-R1 is the pre-dominant receptor signalling to death in breast tumour cells (Figure 6.1). Addition of doxorubicin also lead to the enhancement of TRAIL-induced apoptosis in the TRAIL-responsive cell lines, MDA-MB-231 and ZR-75.1 (data not shown). More importantly, in the presence of doxorubicin, the concentration of TRAIL required to induce apoptosis in these cell lines was reduced by 10-fold, thus further indicating the effective synergism between doxorubicin and TRAIL.

Unfortunately, due to the limited sample size (n=2), the effect of combination therapy on mucinous carcinomas could not be tested. As mentioned earlier, combination

therapy might be specifically useful in targeting the hypoxic cores of tumour groups within the mucinous carcinomas.

In the invasive carcinomas, on the other hand, doxorubicin facilitated a strong sensitisation effect in most of the TRAIL-resistant tumour cells. Significantly, doxorubicin-mediated TRAIL sensitisation occurred exclusively in tumour cells, whilst sparing adjacent normal breast cells. The ability of TRAIL to selectively target tumour cells even in the presence of low doses of a suitable sensitising agent is therefore of a definite advantage in the clinic.

The co-treatment of doxorubicin and TRAIL induced apoptosis in the TRAIL-resistant DCIS components of the tumours. Furthermore, with the exception of the highly resistant IDC sample T15, doxorubicin and TRAIL/R1L induced a significant extent of apoptosis in the IDC/ILC components of all viable samples tested (Figure 6.1). As discussed earlier, there are many potential reasons for the difference in response between DCIS and invasive tumour components within the same carcinoma and also for inter-patient variations. Further investigations into these reasons could aid in dissection of the exact mechanisms of drug resistance, which could then be exploited for further improvement of TRAIL therapy.

The sensitivity of metastatic breast tumour cells to TRAIL may be intrinsic or could be the results of sensitisation through anoikis or prior hormonal/chemotherapy. Nonetheless, it was again R1L which more strongly inhibited the tumour forming capacity of the metastatic breast cancer stem cells than R2L (Figure 6.1).

Thus, overall I can conclude that in the breast cancer cell lines, the mammospheres, primary breast tumour tissue and metastatic breast cancer stem cells, TRAIL-R1 is the predominant TRAIL death receptor that signals to death and therefore, TRAIL-R1 should be ideally targeted in the clinic (Figure 6.1).

6.6 TRAIL *vs.* TRAIL-R1-selective agents in cell lines and primary breast tumour cells

It is interesting to observe that whilst in breast cancer cell lines, TRAIL and R1L were consistently equipotent, in the primary tumour setting (tumour explants and primary tumour mammospheres) R1L was more potent that TRAIL (Figure 6.1).

A probable reason for this is that in monolayer culture, the amount of TRAIL added is in excess of the number of TRAIL receptors present. Therefore, even after competitive

binding of TRAIL to TRAIL-R2/R3/R4, enough ligand would still be available to bind and fully activate TRAIL-R1. However, the same amount of TRAIL, when added to tissue explants, is exposed to a much larger cell population and a complex extracellular matrix, which can itself act as a sieve and also contains a large number of factors capable of binding TRAIL. Hence, it is likely that TRAIL is not available in excess and therefore, after binding to TRAIL-R2 and the decoy receptors, not enough ligand is then left to saturate TRAIL-R1. This may also explain the increased potency of R1L, because R1L will bind only TRAIL-R1 and thus, will not be sequestered by TRAIL-R2, -R3 or -R4, thus allowing a much larger number of cells to be ligated with R1L.

Contrary to conventional apoptosis assays, the mammosphere assay is an excellent indicator of the pro-apoptotic as well as pro-survival signalling capabilities of TRAIL. The % MFE is an assessment of the number of tumour cells that have not only escaped apoptosis, but which can also proliferate to a minimum colony size. It is, therefore probable in this assay that under conditions of anoikis, TRAIL kills the sensitised tumour cells through TRAIL-R1, but then stimulates the proliferation of the remaining apoptosis-resistant cells through the other TRAIL receptors. It has previously been suggested that the signalling of TRAIL to proliferation occurs *via* binding to TRAIL-R1, TRAIL-R2 and TRAIL-R4 (Degli-Esposti *et al.*, 1997a; Mühlenbeck *et al.*, 1998; Hu *et al.*, 1999; Ehrhardt *et al.*, 2003; Baader *et al.*, 2005; Trauzold *et al.*, 2006). Consequently TRAIL can activate a number of survival pathways including NFκB, MAPK pathways (ERK, JNK and p38) and the PI3K/Akt pathway. Thus, while both TRAIL and R1L can induce apoptosis in the metastatic tumour cells, it is possible that TRAIL is a more active driver of proliferation and therefore, has a lower effect on % MFE than R1L.

On the whole, it can be concluded from all the data I have obtained that in the primary breast tumour setting, R1L is more potent than either TRAIL or R2L.

6.7 Supporting evidence for the significance of TRAIL-R1 in breast cancer

Overall the data I have obtained during the course of this study has shown that TRAIL-R1 is the key TRAIL death signalling receptor in primary breast cancer cells. This finding is also indirectly supported by earlier reports which suggested that over expression of TRAIL-R2 in breast cancer cells bears a significant correlation with poor prognosis and decreased patient survival (McCarthy *et al.,* 2005). A possible reason for this observation could be that increased TRAIL-R2 expression could be signalling to cell proliferation following binding of TRAIL. This further emphasises the advantage of

using a TRAIL-R1-specific agent in breast cancer therapy, as this form of TRAIL would not bind to any other TRAIL receptors. The reported over expression of TRAIL-R2 in primary cancer tissue (Ichikawa *et al.,* 2001) might therefore be a mechanism employed by the malignant cells to evade TRAIL-induced apoptosis *in vivo*. Additionally, it has been reported that metastases incidence is lower in patients with a high expression of TRAIL-R1 and Bcl-2 and low expression of TRAIL-R2 ($R1^{high}/R2^{low}/Bcl\text{-}2^{high}$) than with patients with low expression of TRAIL-R1 and Bcl-2 and high expression of TRAIL-R2 ($R1^{low}/R2^{high}/Bcl\text{-}2^{low}$; Ganten *et al.,* 2009). This study highlights that that the lower the expression of TRAIL-R1, the higher the chances of breast cancer progression into a metastatic stage. Overall, these primary tumour studies indirectly emphasise the critical role played by TRAIL-R1 *in vivo* and therefore, support the use of a TRAIL-R1-targeting agent in breast cancer.

6.8 Improving TRAIL and doxorubicin therapy

Tumour cell resistance to TRAIL is very different from other forms of drug resistance because TRAIL can drive aggressive proliferation in tumour cells that are resistant to the apoptotic effects of TRAIL. Clinically, various antibodies/ligands to the TRAIL death receptors are being developed. Results from Phase I studies have indicated that TRAIL antibodies can be safely administered without any major toxicity and have recommended the combination of TRAIL with chemotherapeutic agents for use as anti-tumour therapy (Table 6.1; Tolcher *et al.,* 2007; Plummer *et al.,* 2007; Mom *et al.,* 2009; Leong *et al.,* 2009; Wakelee *et al.,* 2010). While HGS-ETR1 (Mapatumumab) is the only antibody currently being developed which activates TRAIL-R1, there are many antibodies under development that target TRAIL-R2 (for example, HGS-ETR2/Lexatumumab, Apomab, LBY135). The focus on developing a number of TRAIL-R2 signalling antibodies is based on previous reports claiming that TRAIL-R2 is the predominant functional TRAIL death receptor in tumour cells (Kelley *et al.*, 2005; Ichikawa *et al.,* 2001). However, it is important to note that these studies were restricted to a few tumour cell lines and thus, cannot be used as a representation of primary tumours. In addition, the TRAIL-R1-specific ligand generated by Kelley *et al* was biologically inactive (MacFarlane *et al.,* 2005b) and in this context, my data effectively shows that at least in breast cancer, efforts should be made to target TRAIL-R1 by anti-cancer agents and not TRAIL-R2.

Table 6.1 Examples of TRAIL in clinical trials ± chemotherapy for solid tumours

Clinical Trial	TRAIL variant	Chemotherapeutic agent	Tumour type
Phase II	AMG 951 (Amgen)	Bevacizumab Carboplatin Paclitaxel	NSCLC
Phase II	Mapatumumab (HGS)	-	NSCLC
Phase Ib/II	Mapatumumab	Cisplatin	Cervical cancer
Phase I	Mapatumumab	Paclitaxel Cisplatin	Solid tumours
Phase I	Lexatumumab	-	Solid tumours

NSCLC = Non-small cell lung cancer; (http://clinicaltrials.gov/)

On the other hand, the major limiting factor for doxorubicin is its severe side-effects, the most dangerous being cardiotoxicity. Recent studies have focussed on improving doxorubicin delivery so as to improve its anti-cancer activity while reducing its associated cardiotoxicity. Liposomal doxorubicin is an effective way of delivering doxorubicin *in vivo* and has shown to be less cardiotoxic than unconjugated doxorubicin (reviewed in O'Brien, 2008). Similarly, epirubicin is another anthracycline which has been derived from doxorubicin and shares a similar mechanism of action, but is significantly less damaging to the heart. Another upcoming method of drug delivery is targeted nanoparticles which in theory should be almost free of any overt toxicity (Chen *et al.,* 2010). Recently, nanoparticles for the co-delivery of doxorubicin and TRAIL to tumour cells have also been described (Lee *et al.,* 2011).

In addition to improved drug delivery methods, it has also been widely recognised that personalised therapy would be the most efficient treatment for cancer. Based on the results of this study, it can be proposed that using doxorubicin and TRAIL simultaneously or doxorubicin treatment followed sequentially with TRAIL, or more specifically a TRAIL-R1-specific agent, would be of positive clinical consequence. Taking into account the current therapies being used for breast cancer, R1L or its antibody equivalent could be combined with routine FEC/FAC therapy. However, it is important to note that doxorubicin and TRAIL combination did not work successfully in the highly resistant IDC T15, indicating that different tumours may require different drug combinations. It would also be interesting to see the effect of other agents including tamoxifen, aromatase inhibitors and Herceptin on TRAIL-induced apoptosis.

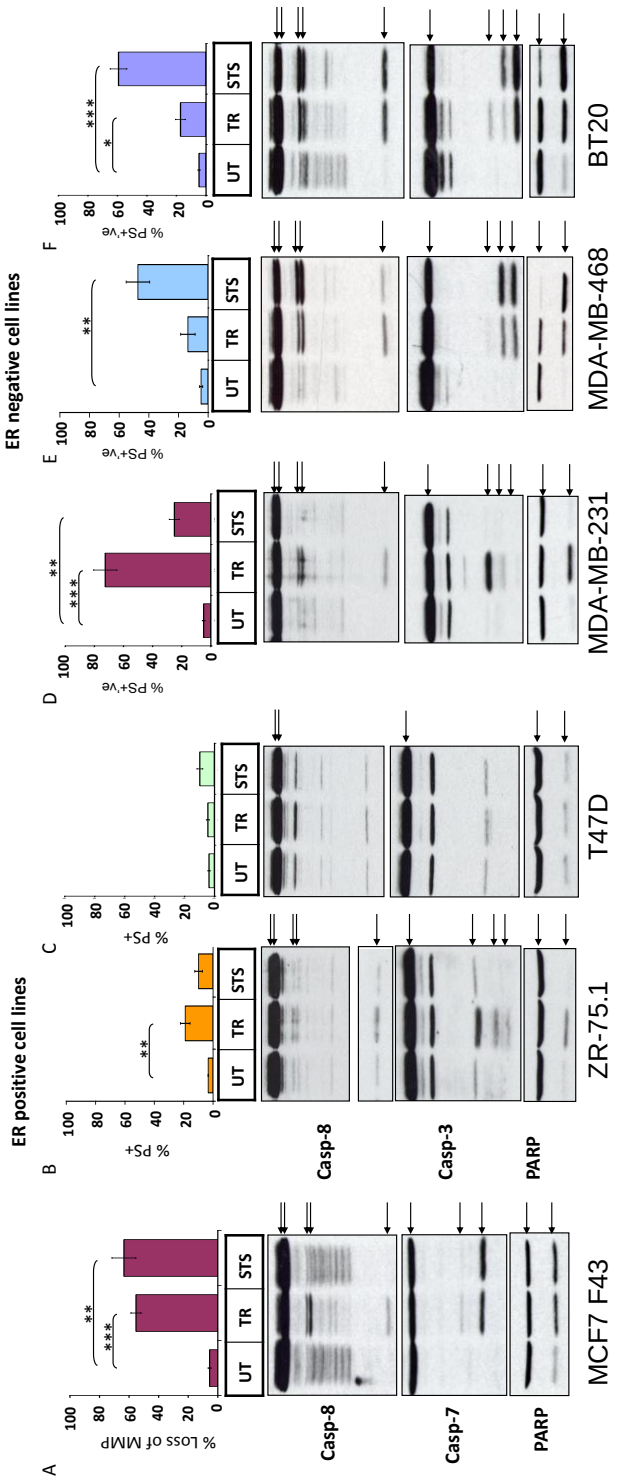
Unfortunately, these effects could not be studied during this project, due to the lack of a relevant cell line model system and the limited availability of suitable breast cancer samples.

6.8 Conclusion

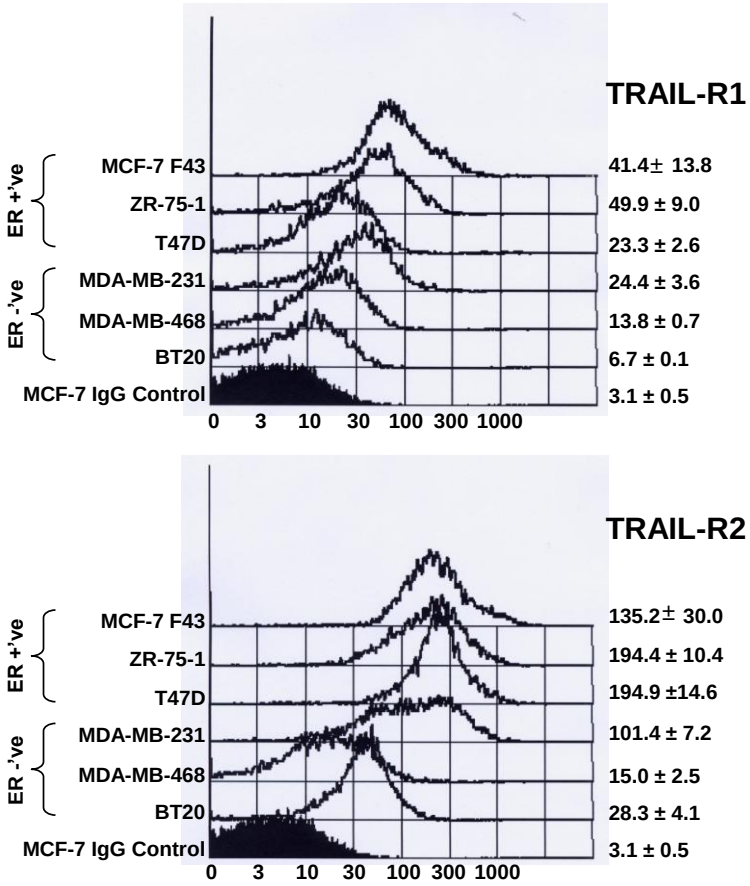
Positive data from Phase I clinical trials of TRAIL coupled with the data from this study effectively show that TRAIL can be used as a successful biotherapy in conjunction with current available therapies for the treatment of breast cancer patients. Agents targeting TRAIL-R1, like Mapatumumab, may be significantly more potent in breast cancer than agents targeting TRAIL-R2 and combining such agents with pre-existing chemotherapy is recommended. In addition, the explant culture model used in this study could be applied to profile the responses of other solid tumours (for example, lung carcinomas) to combinations of TRAIL and relevant chemotherapeutic agents. The information gained from this study could thus be used in improving the efficacy of therapies being used to treat the most common cancer in the UK, namely breast cancer.

Appendix I

Data



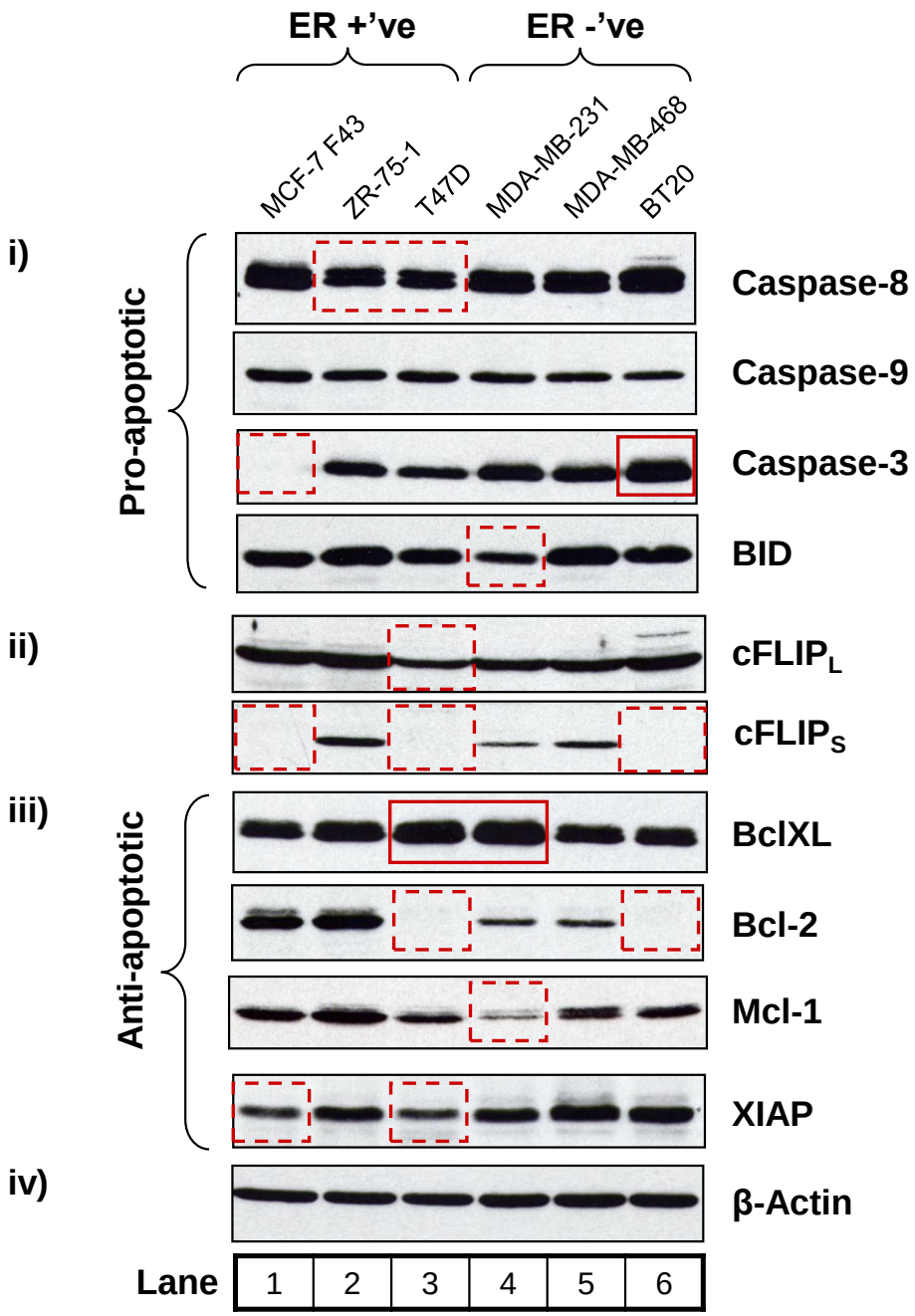
Appendix 1 TRAIL sensitivity of breast cancer cell lines does not correlate with their ER status
(performed in collaboration with Drs. Davina Twiddy and Roshna Mistry)
Cells were treated with appropriate stimuli {TRAIL (1 µg/ml) or STS (1 µM)} for 6h and apoptosis was measured using Annexin V-FITC labelling and SDS-PAGE/Western blotting.



Appendix 2 Cell surface receptor expression of TRAIL-R1 and TRAIL-R2 on breast cancer cell lines

(Performed in collaboration with Drs. Davina Twiddy and Roshna Mistry)

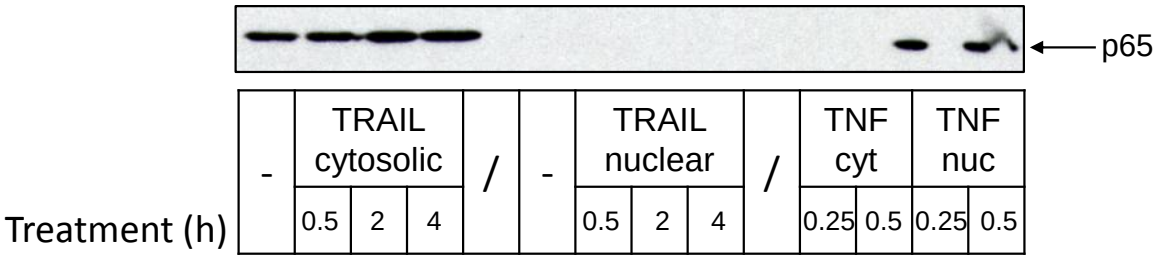
Expression of TRAIL-R1 and TRAIL-R2 on the cell surface was analysed using PE-conjugated antibodies on FACS Calibur. Data (Median Fluorescence Intensity) shown as mean ± SEM



Appendix 3 Expression of proteins across breast cancer cell lines

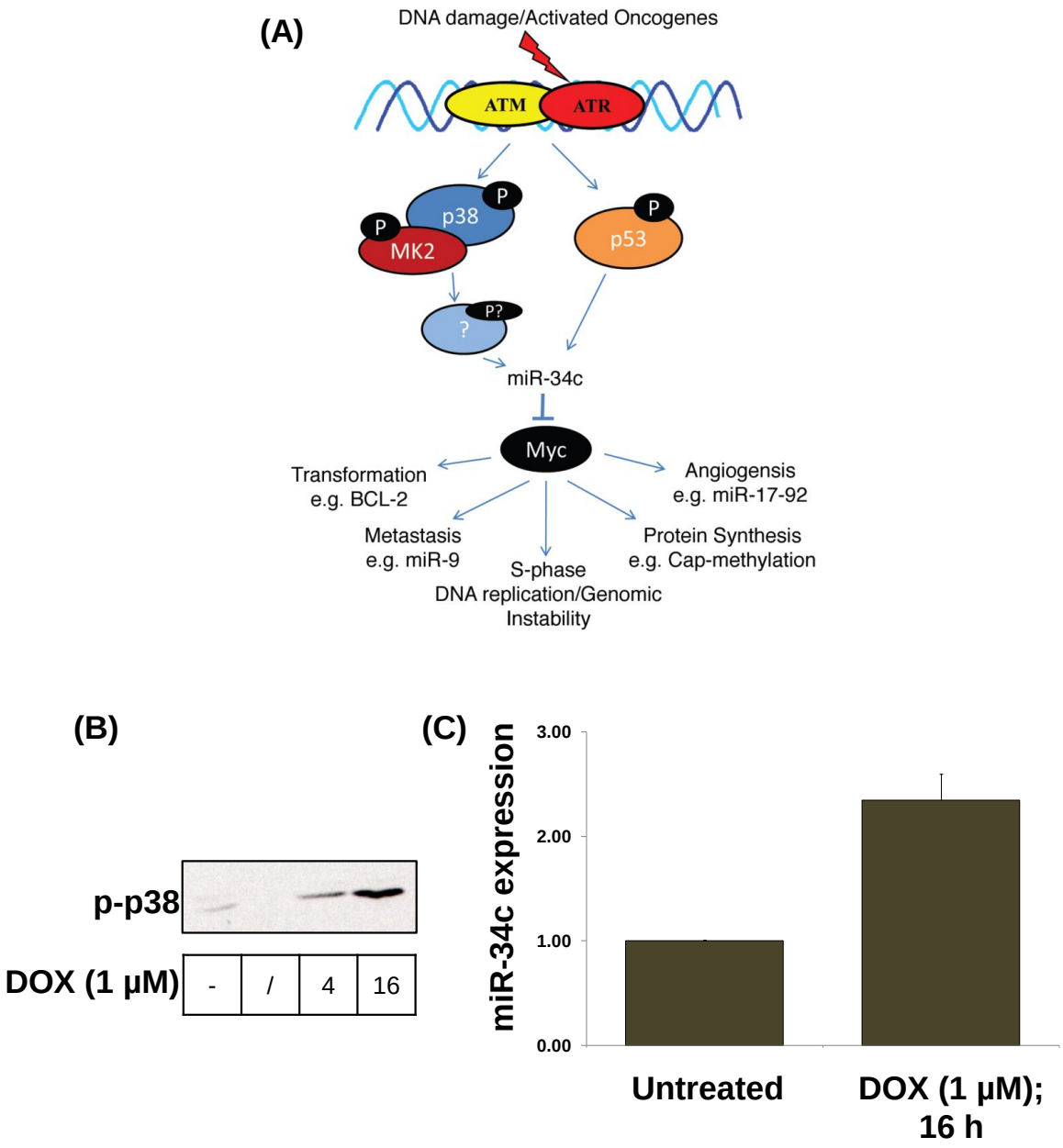
(performed in collaboration with Drs. Davina Twiddy and Roshna Mistry)

Expression of proteins across breast cancer cell lines was detected using SDS-PAGE/Western Blotting. Bradford's assay was used to ensure equal protein was loaded across all the cell lines.



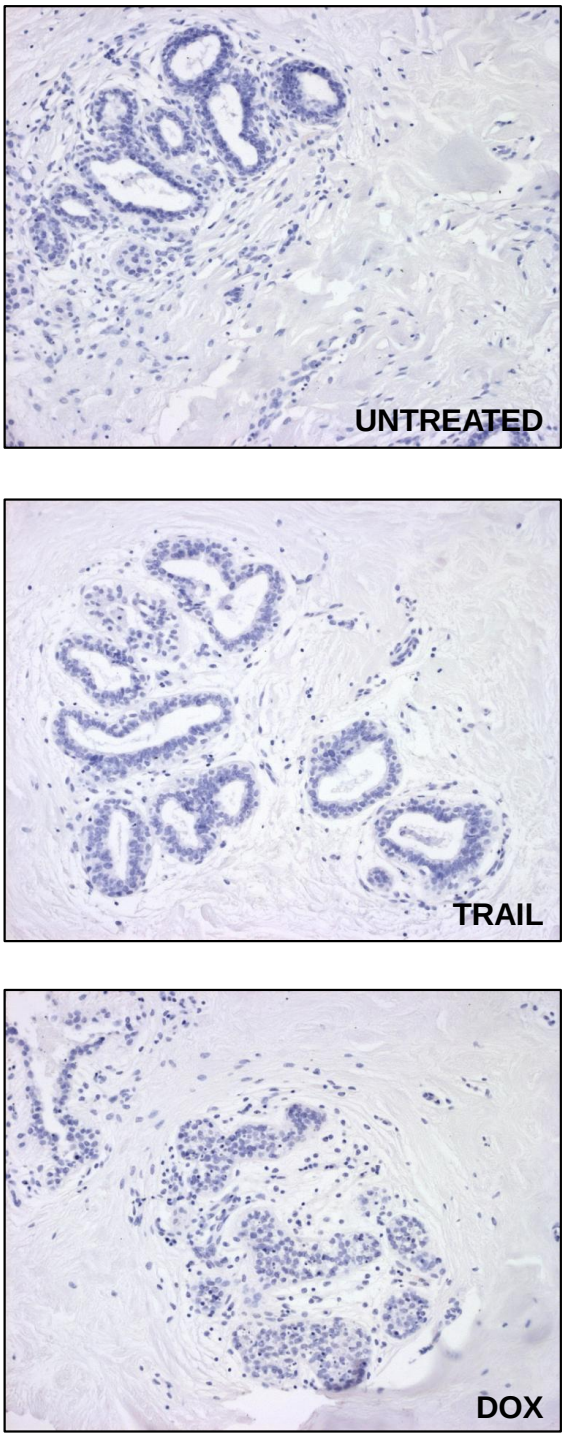
Appendix 4 TRAIL does not induce activation of NFκB activation in T47D cells

For cell fractionation, treated with TRAIL (1000 ng/ml) or TNF (200 ng/ml) for indicated times and subsequently harvested by trypsinisation and re-suspended in 400 µl cold lysis buffer (10 mM HEPES pH7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) and left on ice for 15 min following which 25 µl of Nonidet NP-40 was added. The lysed cells were then centrifuged for 30 sec at 3000 rpm and the supernatant was transferred to a fresh tube as the cytosolic fraction. The nuclear pellet was re-suspended in 50 µl ice cold buffer (20 mM HEPES pH 7.9; 4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) and the tube was vigorously shaken for 15 min at 4 ºC. Subsequently the extract was centrifuged for 10 min at 3000 rpm and supernatant represented the nuclear fraction. The samples were then loaded onto SDS-PAGE gels and blotted for the p65 subunit.



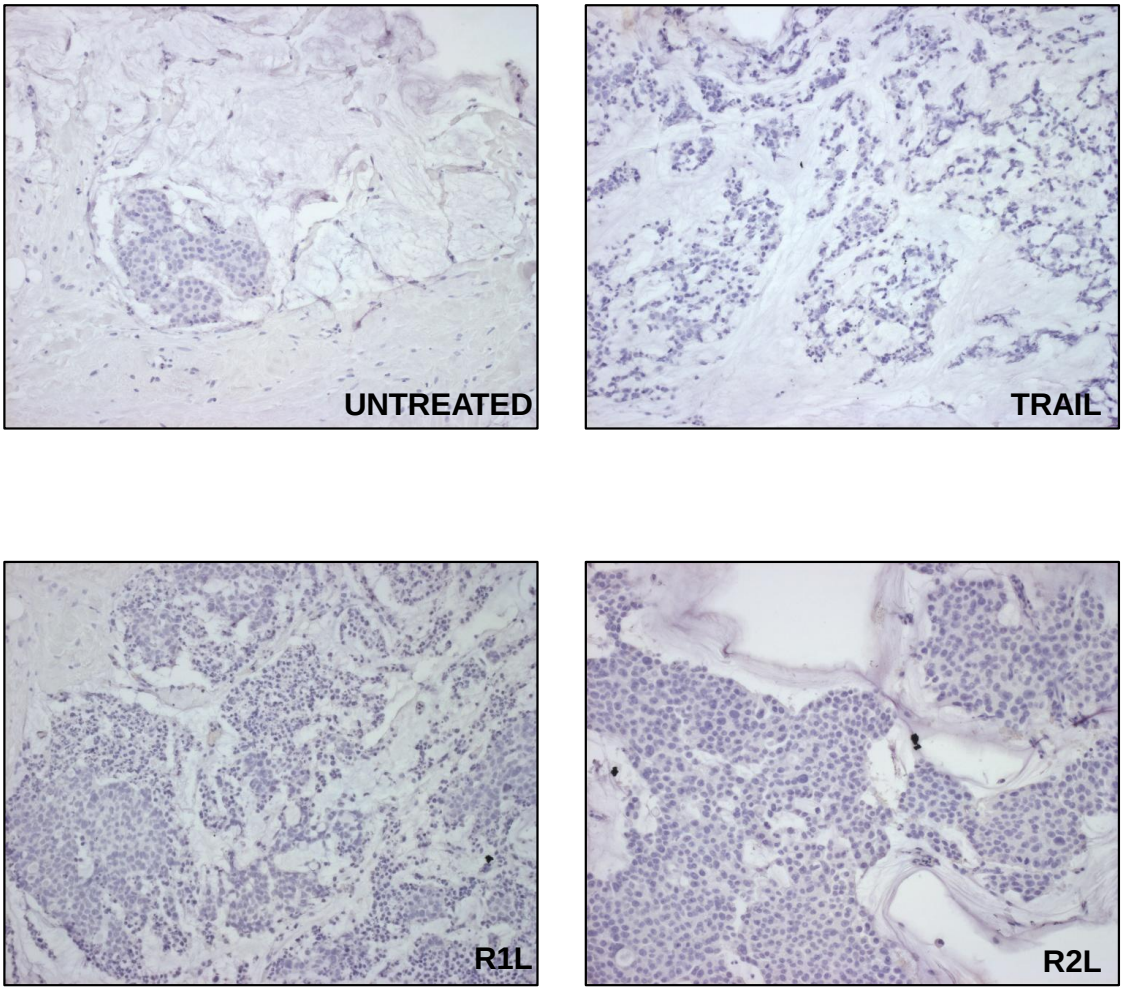
Appendix 5 Doxorubicin causes up-regulation of miR-34c

Doxorubicin has been reported to cause p38-dependent up-regulation of miR-34c ((A); Cannell and Bushell, 2010). In T47D cells, doxorubicin caused a time dependent increase in phosphorylation of p38 (B) and a corresponding up-regulation of miR-34c (C). miR-34c up-regulation was detected by performing qPCR analysis on isolated RNA using Taqman probes. Data represented as mean ± SEM.



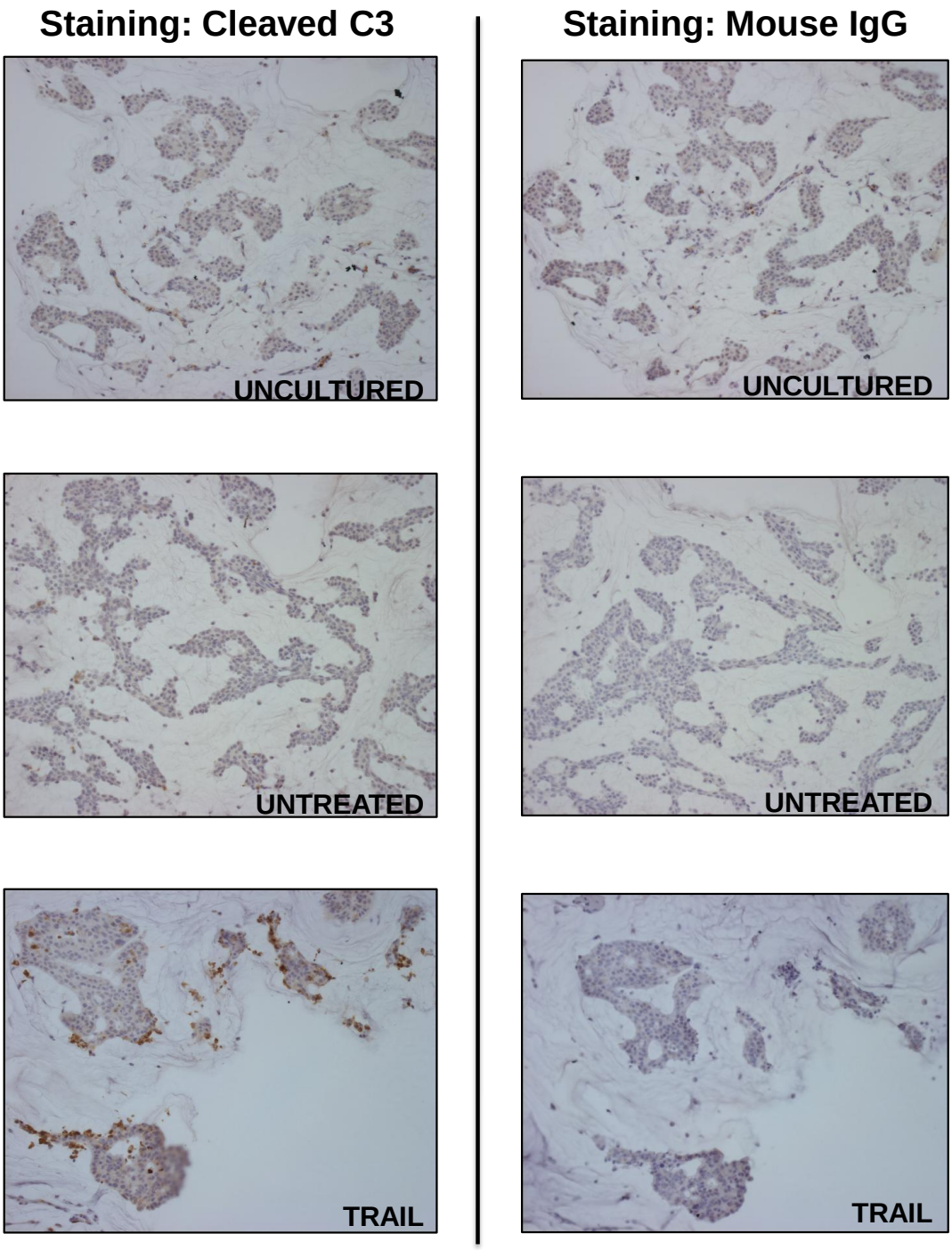
Appendix 6 Rabbit monoclonal IgG immunostaining for normal breast tissue

Primary normal breast tissue explants were cultured and treated with indicated death stimuli (TRAIL; 1 µg/ml and STS; 1 µM) for 24 h. Images are samples stained with rabbit monoclonal IgG and counter-stained with Haematoxylin (Magnification 200x).



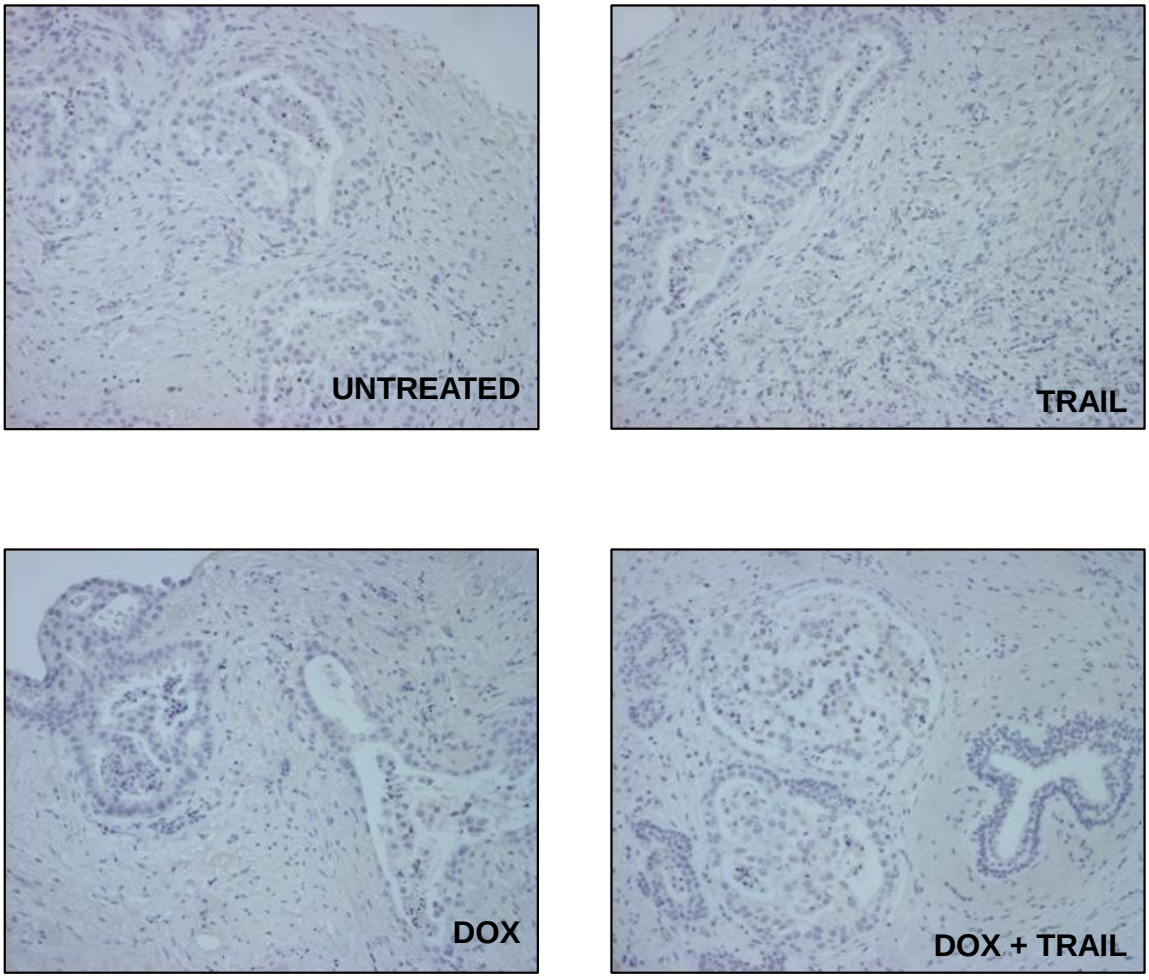
Appendix 7 Rabbit Monoclonal IgG staining for sample #T1

Tumour explants from sample #T1 treated with TRAIL or the receptor-specific ligands (R1L, R2L) and immunostained with rabbit monoclonal IgG (Magnification 200x).



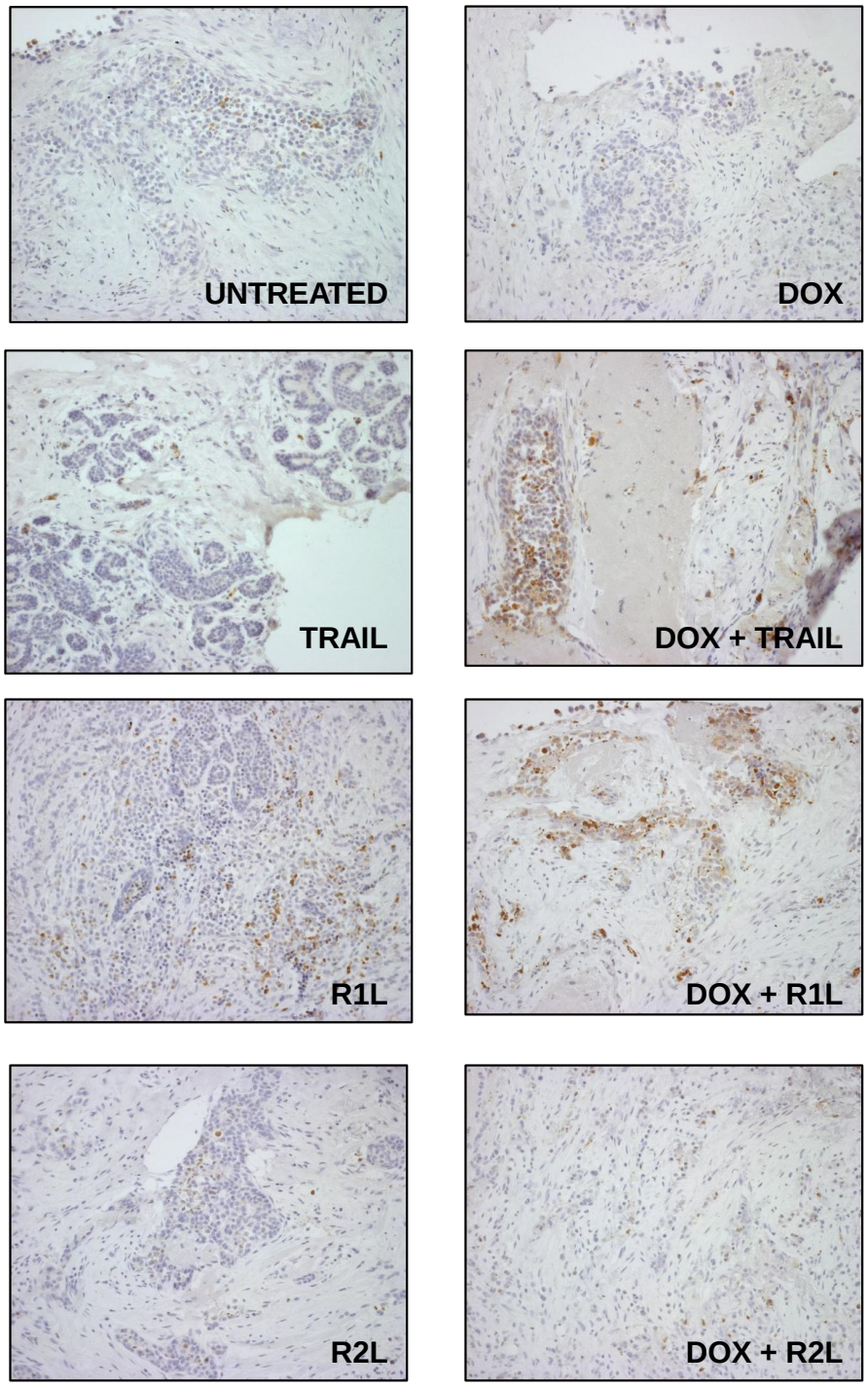
Appendix8 Cleaved caspase-3 and rabbit monoclonal IgG immunostaining of serial sections of sample #T5

Immunostaining for cleaved caspase-3 staining and rabbit monoclonal staining of sample #T5 (representative images shown; Magnification 200x).



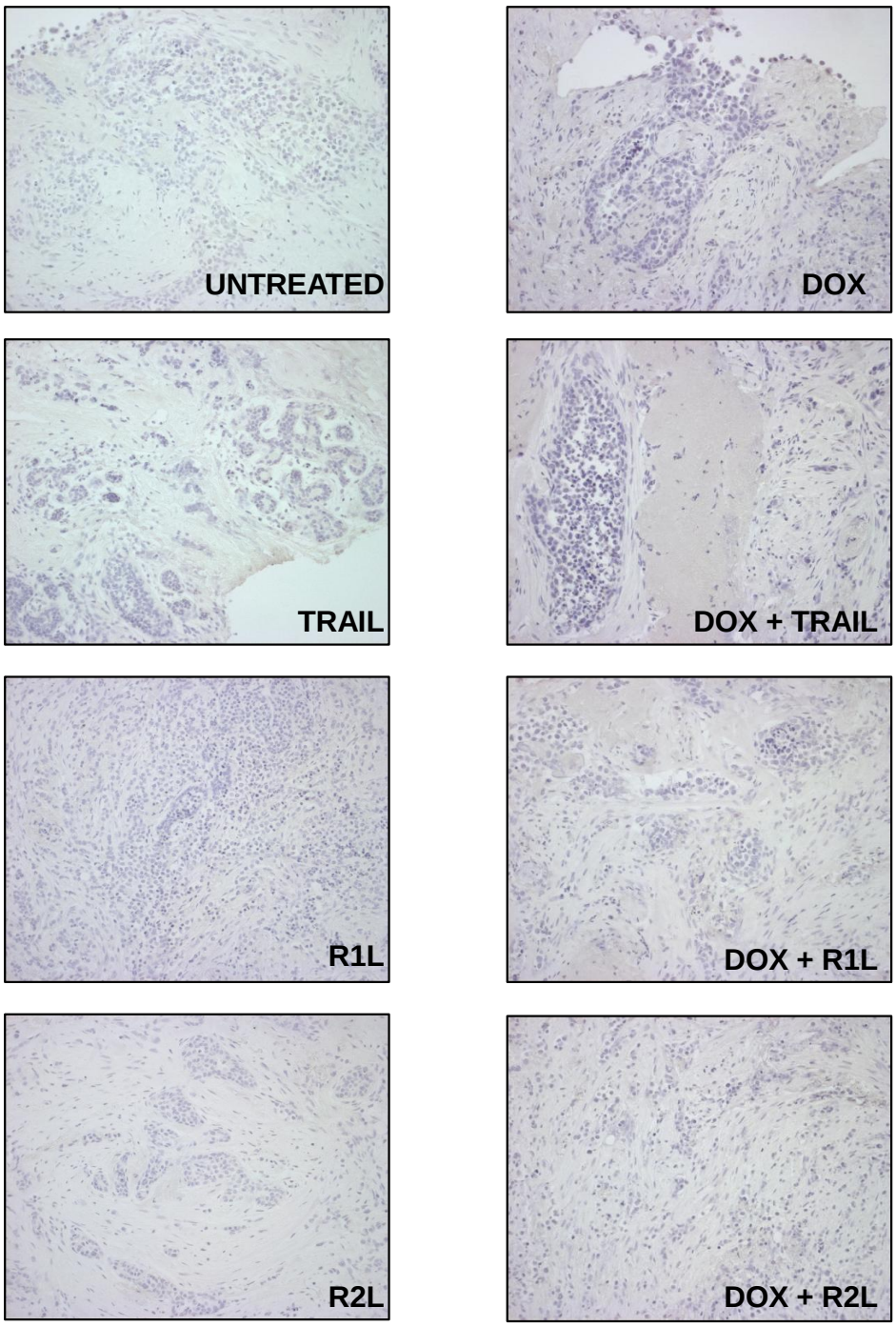
Appendix 9 Rabbit monoclonal immunostaining for sample #T6

Sample #T6 treated with the specified treatments for 24 h and stained with rabbit monoclonal IgG; representative images; Magnification 200x).



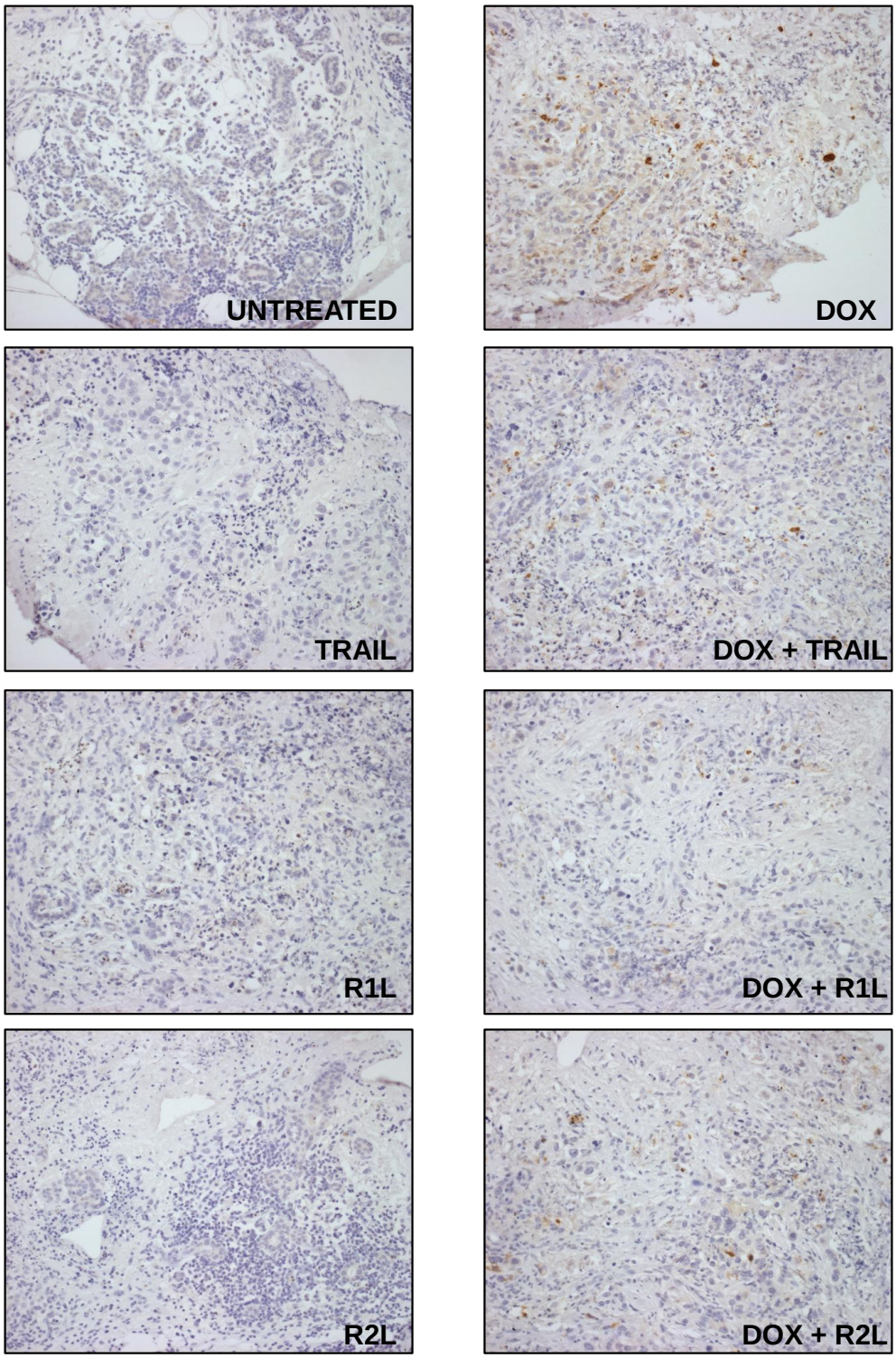
Appendix 10a Cleaved caspase-3 staining for sample #T9

Sample #T9 (IDC) treated with appropriate agents was stained with cleaved caspase-3; representative images of explants exposed to different treatments (Magnification 200x).



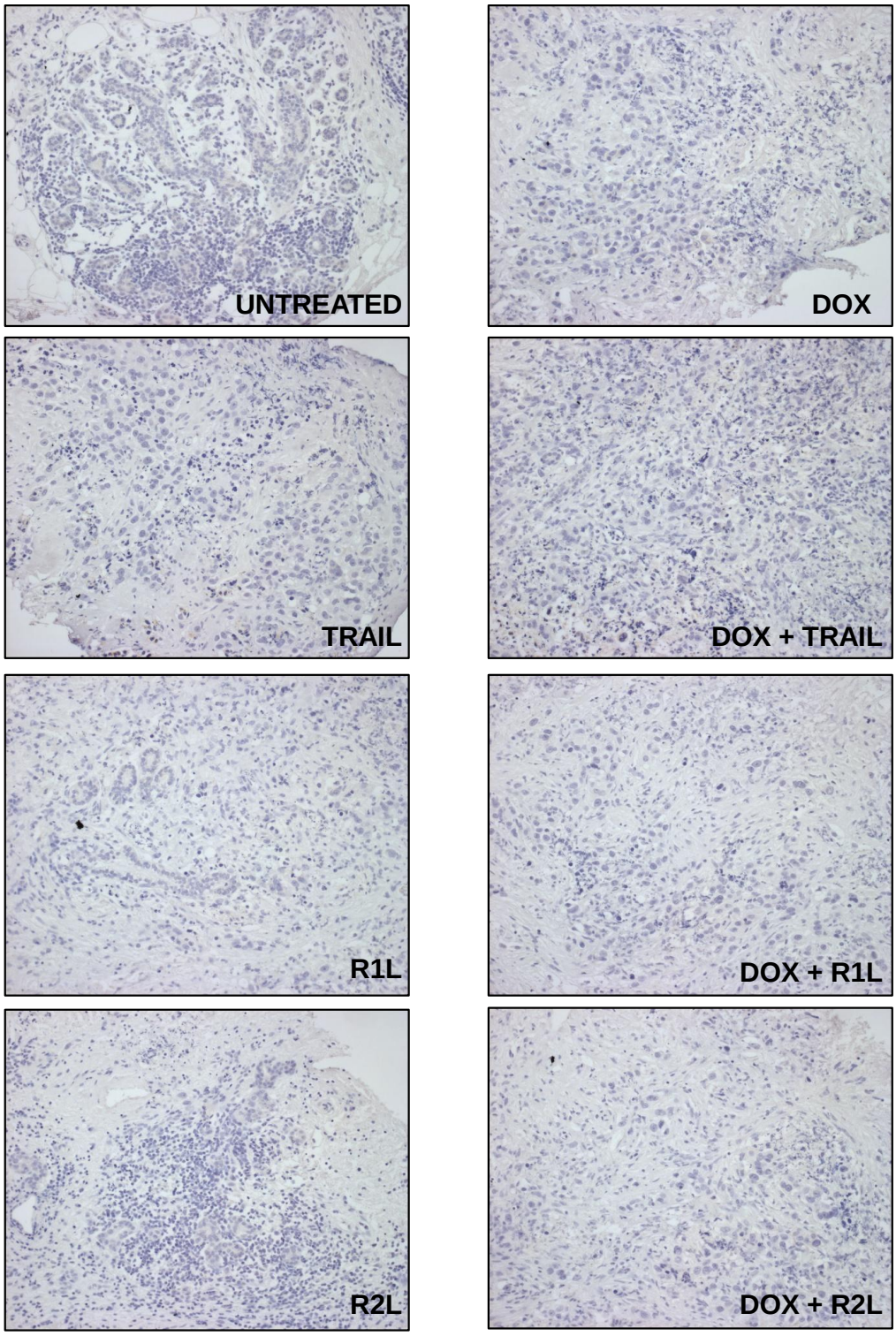
Appendix 10b Rabbit monoclonal IgM staining for sample #T9

Sample #T9 (IDC) treated with appropriate agents was stained with rabbit monoclonal IgG; representative images of explants exposed to different treatments (Magnification 200x).



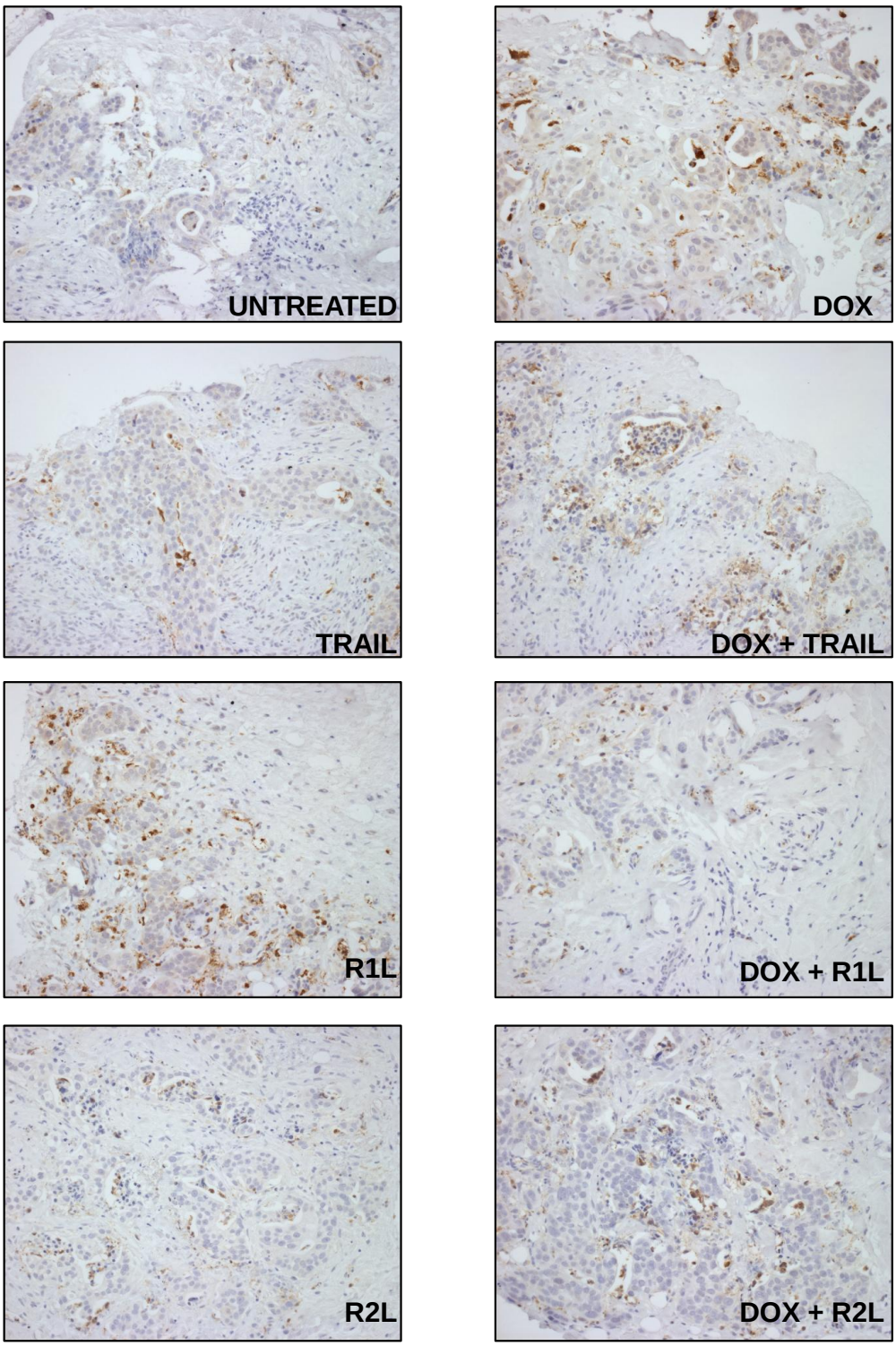
Appendix 11a Cleaved caspase-3 immunostaining for sample #T12

Sample #T12 (IDC) was treated with the specified treatments and fixed with 10% NBF as described in Materials and Methods. Immunostaining for cleaved caspase-3 was used as a marker of apoptosis (representative images shown; Magnification 200x)



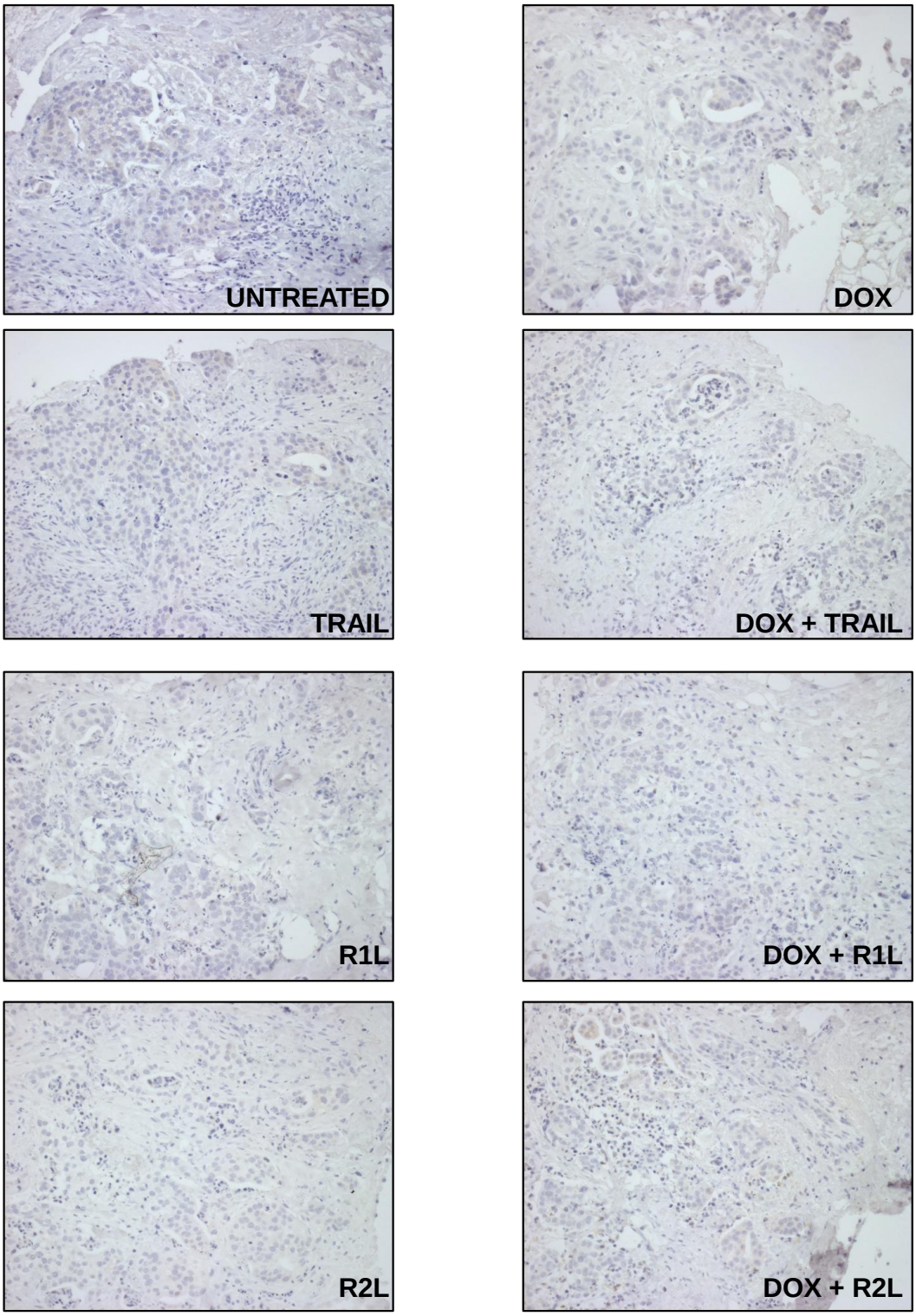
Appendix 11b Rabbit monoclonal IgG images for sample #T12

Sample #T12 (IDC) was treated with the specified treatments and fixed with 10% NBF as described in Materials and Methods. Immunostaining for rabbit monoclonal IgG (representative images shown; Magnification 200x)



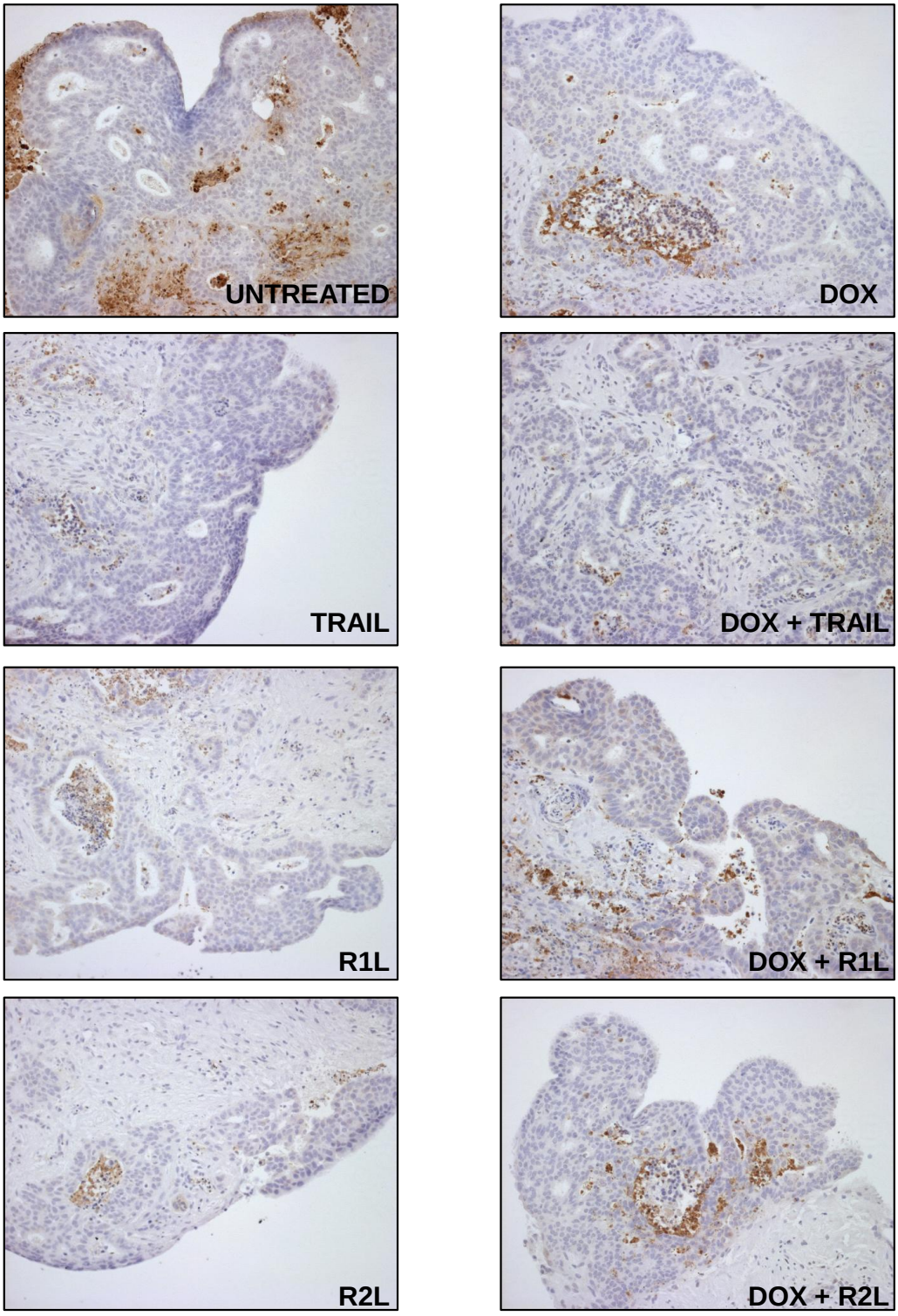
Appendix 12a Cleaved caspase-3 immunostaining for sample #T13

Tumour explants from sample #T13 (IDC) were treated with appropriate agents and cleaved caspase-3 was used as a marker of apoptosis. . Data shown here as representative images; Magnification 200x)



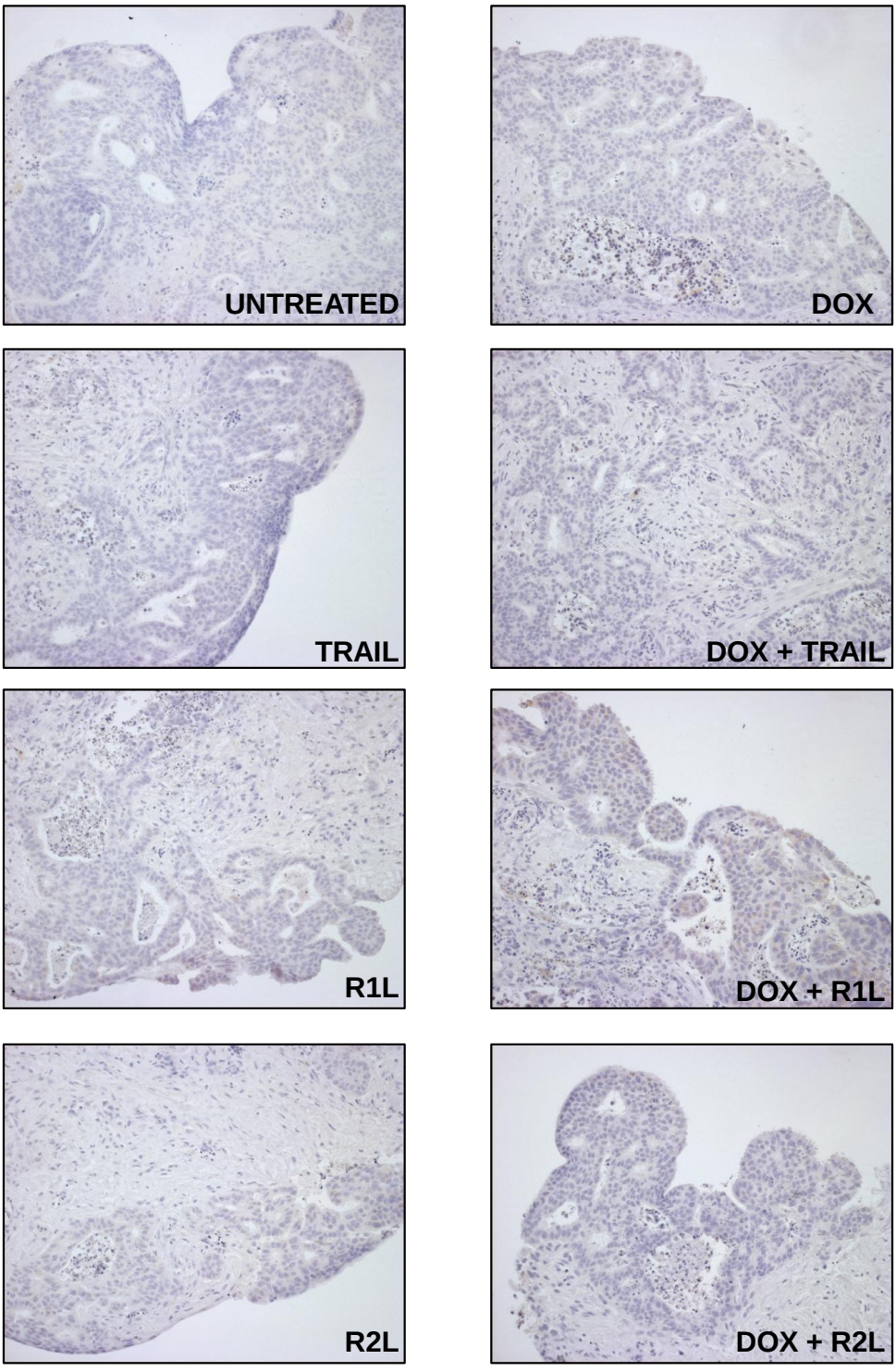
Appendix 12b Rabbit monoclonal IgG images for sample #T13

Tumour explants from sample #T13 (IDC) were treated with appropriate agents and stained with rabbit monoclonal IgG. . Data shown here as representative images; Magnification 200x)



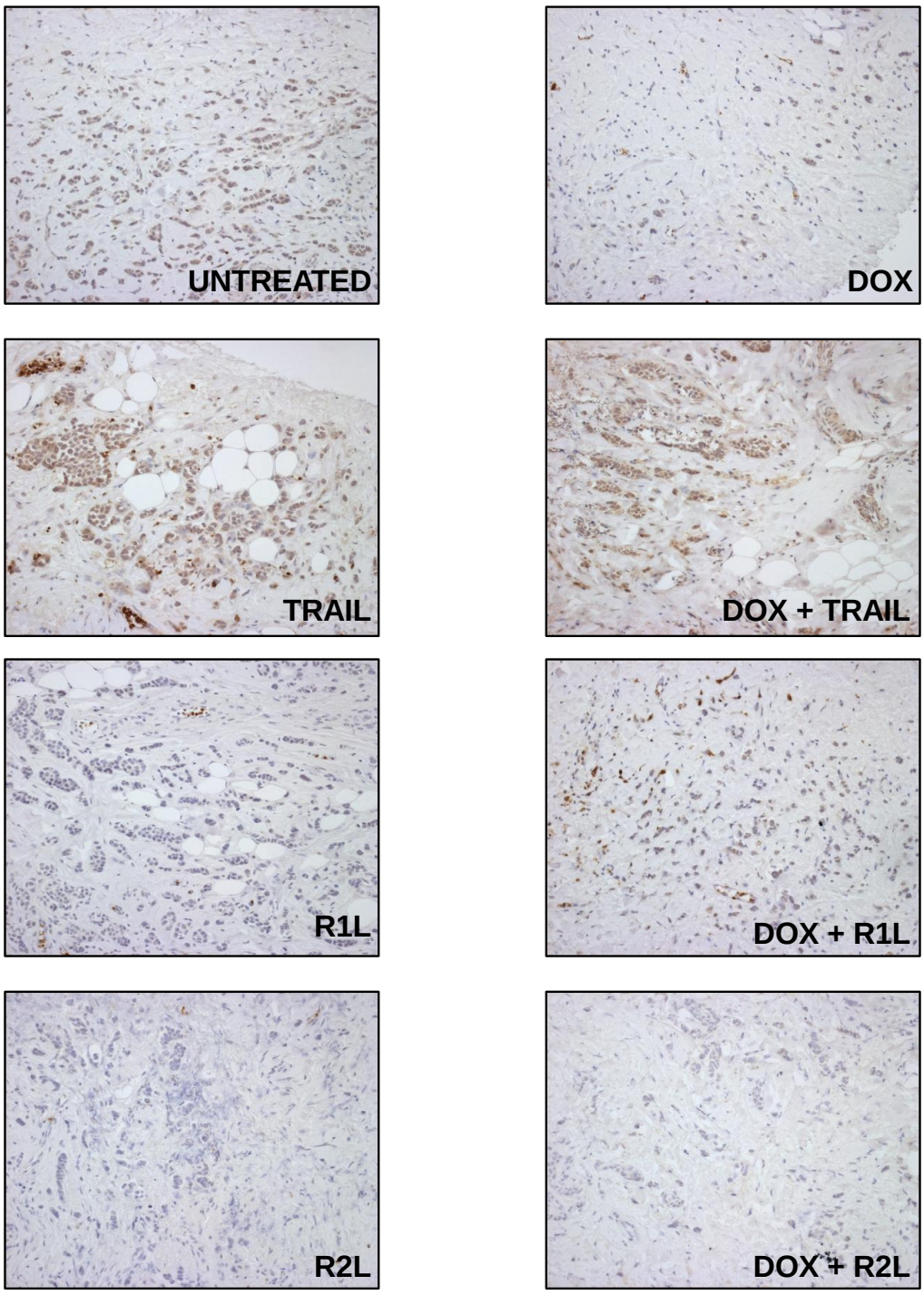
Appendix 13a Cleaved caspase-3 immunostaining for sample #T15

Sample #T15 (IDC) was treated with the specified treatments and subsequently stained for cleaved caspase-3; representative images shown ; Magnification 200x).



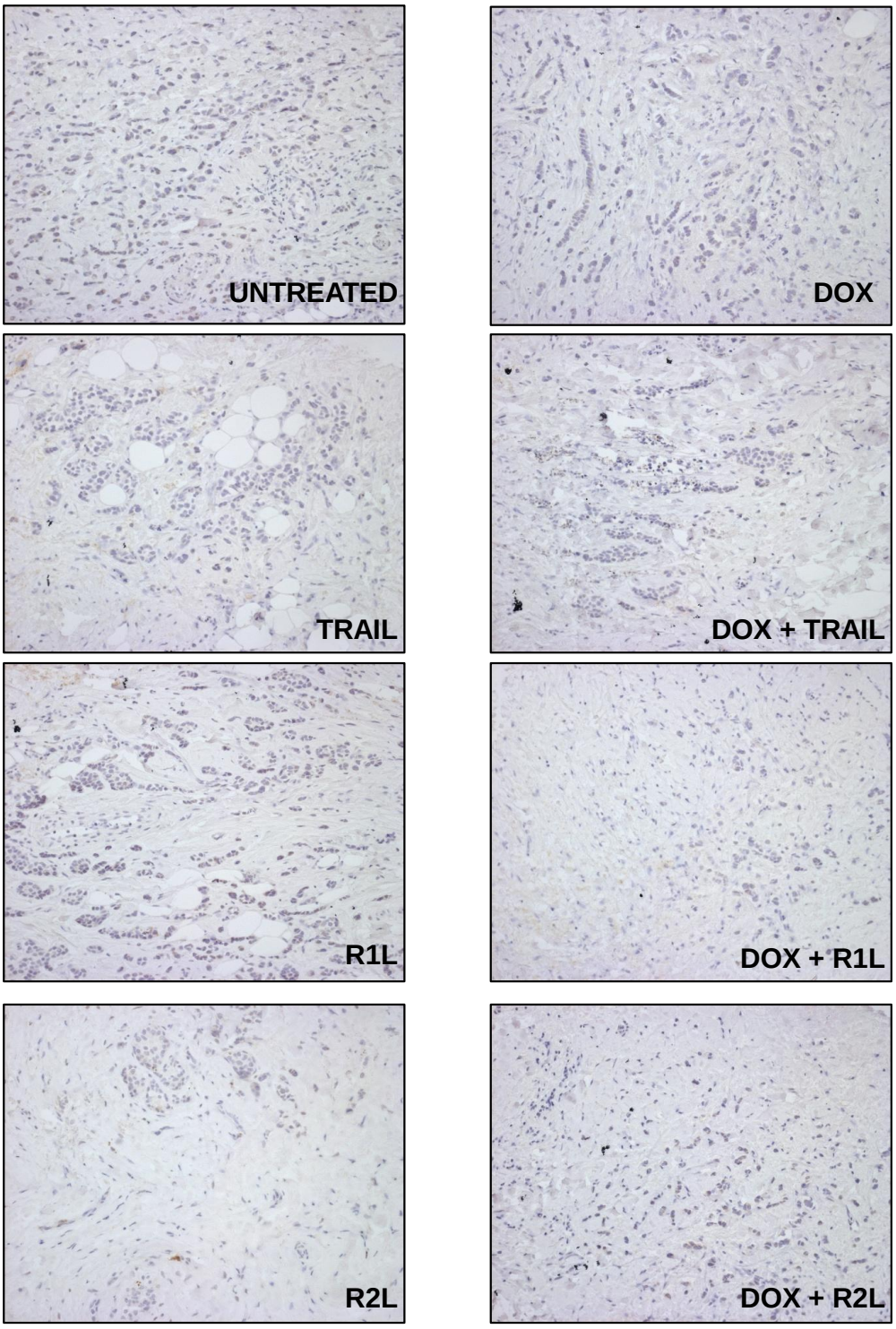
Appendix 13b Rabbit monoclonal IgG immunostaining for sample #T15

Sample #T15 (IDC) was treated with the specified treatments and subsequently stained for rabbit monoclonal IgG; representative images shown; Magnification 200x).



Appendix 14a Cleaved caspase-3 staining for sample #T16

Cleaved caspase-3 immunostaining was performed for sample #T16 (IDC/ILC) to assess apoptotic response for each treatment condition. Data shown are representative images; Magnification 200x



Appendix 14b Rabbit monoclonal IgG staining for sample # T16

Rabbit monoclonal IgG immunostaining was performed for sample #T16 (IDC/ILC). Data shown are representative images; Magnification 200x

Appendix II

Ethical Approval

University Hospitals of Leicester NHS
NHS Trust

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11th November 2008

Miss Shambhavi Naik
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Dear Miss Naik

Chemoprevention studies of Human Tumours

This letter confirms your right of access to conduct research through **University Hospitals of Leicester NHS Trust** for the purpose and on the terms and conditions set out below. This right of access commences on **1st September 2008** and ends on **31st August 2011** unless terminated earlier in accordance with the clauses below.

You have a right of access to conduct such research as confirmed in writing in the letter of permission for research from this NHS organisation. Please note that you cannot start the research until the Principal Investigator for the research project has received a letter from us giving permission to conduct the project.

The information supplied about your role in research at **University Hospitals of Leicester NHS Trust** has been reviewed and you do not require an honorary research contract with this NHS organisation. We are satisfied that such pre-engagement checks as we consider necessary have been carried out.

You are considered to be a legal visitor to **University Hospitals of Leicester NHS Trust** premises. You are not entitled to any form of payment or access to other benefits provided by this NHS organisation to employees and this letter does not give rise to any other relationship between you and this NHS organisation, in particular that of an employee.

While undertaking research through University Hospitals of Leicester NHS Trust, you will remain accountable to your employer **The University of Leicester** but you are required to follow the reasonable instructions of **Professor David Rowbotham** in this NHS organisation or those given on her/his behalf in relation to the terms of this right of access.

Where any third party claim is made, whether or not legal proceedings are issued, arising out of or in connection with your right of access, you are required to co-operate fully with any investigation by this NHS organisation in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any legal proceedings.

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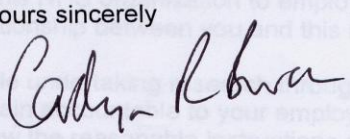

Carolyn Burden
R&D Manager

cc: **MRC Toxicology Unit – c/o Cathy Houghton**
John Whittingham, Asst. Dir HR UHL
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Appendix III

Publications

A TRAIL-R1-specific ligand in combination with doxorubicin selectively targets primary breast tumour cells for apoptosis

D Twiddy, S Naik, R Mistry, J Edwards, RA Walker, GM Cohen and M MacFarlane

Breast Cancer Research 2010, 12(Suppl 1):P58

Introduction

Although the majority of tumour cell lines, including breast cancer cell lines, are sensitive to the death-inducing ligand and potential cancer biotherapeutic TNF-related apoptosis-inducing ligand (TRAIL), most primary tumours are TRAIL-resistant. Importantly, doxorubicin, a chemotherapeutic agent commonly used in breast cancer, has previously been shown to sensitize TRAIL-resistant breast cancer cell lines to TRAIL. Furthermore, using receptor-selective ligands (patent filed by MRC Technology) specific for the TRAIL death receptors, TRAIL-R1/TRAIL-R2, we have previously shown that primary leukaemia cells isolated from patients with chronic lymphocytic leukaemia can be selectively sensitized to apoptosis by combining an a histone deacetylase inhibitor (HDACi) with a TRAIL-R1-specific form of TRAIL/TRAIL-R1 mAb.

Methods and results

To examine the potency of TRAIL-R1/TRAIL-R2-specific ligands in breast cancer, a panel of breast tumour cell lines was employed, which included the TRAIL-resistant breast cancer cell line, T47D. In addition, a modified approach of culturing primary breast tumour explants *ex vivo* to maintain their three-dimensional architecture provided a more clinically relevant breast tumour model. Importantly, all TRAIL-sensitive breast tumour cell lines responded *only* to a TRAIL-R1-specific form of TRAIL. Despite expressing TRAIL-R1/TRAIL-R2, the T47D cell line required initial sensitization by doxorubicin and again exhibited selectivity towards apoptosis induced by a TRAIL-R1-selective ligand. Crucially, we show that doxorubicin can also sensitize TRAIL-resistant primary breast tumour explants to TRAIL-induced apoptosis, while having no effect on normal breast tissue. Furthermore, in this *ex vivo* model, TRAIL combined with doxorubicin induced significantly more apoptosis via TRAIL-R1 than TRAIL-R2.

Conclusions

Our results have important implications for the potential treatment of breast cancer with TRAIL-based therapeutic agents. We propose that using a TRAIL-R1-specific ligand/mAb combined with subtoxic concentrations of doxorubicin will selectively target tumour cells and minimise potential side effects, such as triggering of TRAIL-induced prosurvival pathways in TRAIL-resistant primary tumour cells or cardiotoxicity induced by higher concentrations of doxorubicin used in monotherapy.

Doxorubicin Synergises With A TRAIL-R1-Specific Ligand To Selectively Target TRAIL-Resistant Breast Tumour Cells For Apoptosis

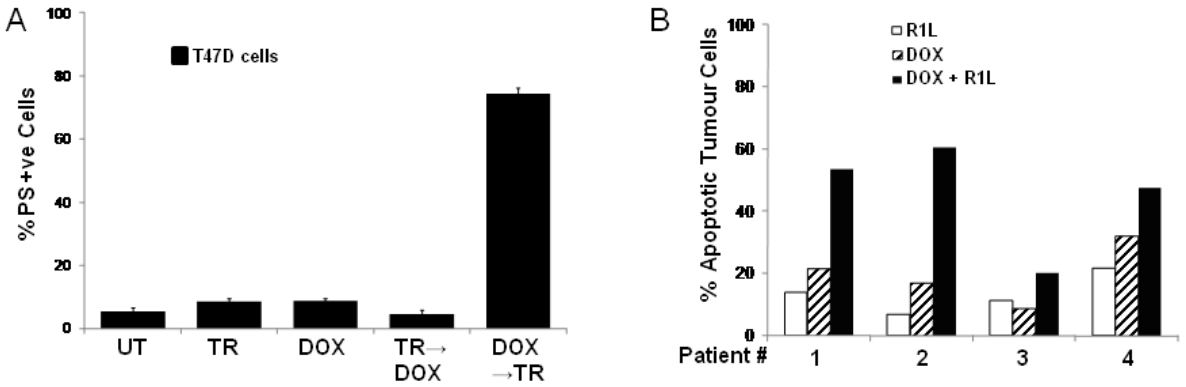
Shambhavi Naik, Davina Twiddy, Roshna Mistry, Jennifer M Edwards, Gerald M Cohen, Rosemary A Walker and Marion MacFarlane

(Toxicology, in press; Presented at the BTS Annual Congress, 2011 – BTS oral prize for best student presentation)

Breast cancer is the most common cancer in the UK. Doxorubicin is a commonly used chemotherapeutic agent for breast cancer, but is associated with several toxic side-effects, the most severe being cardiotoxicity. A way of minimising potential side-effects is to use Doxorubicin at lower clinical concentrations in combination with another targeted agent. For this purpose, TRAIL (TNF-Related Apoptosis-Inducing Ligand) which can selectively kill tumour cells and is currently under clinical evaluation as a targeted cancer biotherapeutic is an exciting option. However, though the majority of breast cancer cell lines are sensitive to TRAIL, most primary tumours are TRAIL-resistant. In addition, TRAIL has been reported to cause proliferation in tumour cells which are resistant to TRAIL-induced apoptosis, thus highlighting the need to identify agents that can be used in combination with TRAIL, while still retaining TRAIL tumour selectivity. Importantly, we have previously shown that primary Chronic Lymphocytic Leukaemia (CLL) cells, which are resistant to TRAIL alone, can be selectively sensitized to apoptosis by combining an HDACi with a TRAIL-R1-specific form of TRAIL (TRAIL-R1/R2-specific ligands generated in-house – patent filed by MRCT).

This study examined the potential for doxorubicin to be used in combination with TRAIL-R1/R2-specfic ligands in breast cancer. We employed a breast tumour cell line, T47D, and a modified approach of culturing primary breast tumour explants *ex-vivo*, thus providing a clinically relevant tumour model and the option to assess the toxicity of agent combinations on normal breast tissue.

Fig. 1: Doxorubicin combined with TRAIL -targets Breast Tumour cells for Apoptosis *via* TRAIL-R1



The results from this study show that the breast tumour cell line T47D is resistant to TRAIL-induced apoptosis (Fig 1A; Mean ± SEM, n=3) (UT, untreated; TR, TRAIL, 1 µg/ml, 6 h). However, on pre-incubation with doxorubicin (DOX, 1 µM; 16 h), T47D cells undergo TRAIL-induced apoptosis mediated predominantly *via* TRAIL-R1. Importantly we show that DOX also sensitizes TRAIL-resistant primary breast tumour explants to TRAIL-induced apoptosis, while having no effect on normal breast tissue (data not shown). In this *ex-vivo* model, a TRAIL-R1-specific ligand (R1L) in combination with DOX (1 µM; 24 h) induced tumour cell apoptosis *via* TRAIL-R1 (Fig 1B).

Our results have important implications in the treatment of breast cancer, as using a TRAIL-R1-specific ligand/mAb in combination with sub-toxic concentrations of doxorubicin could reduce potential adverse side-effects, such as triggering of TRAIL-induced pro-survival pathways or cardiotoxicity induced by higher concentrations of doxorubicin currently used in monotherapy.

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