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Acquired resistance of ER-positive breast cancer to endocrine treatment confers an adaptive sensitivity to TRAIL through post-translational downregulation of c-FLIP

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ABSTRACT

Purpose
One-third of ER-positive breast cancer patients who initially respond to endocrine therapy become resistant to treatment. Such treatment failure is associated with poor prognosis and remains an area of unmet clinical need. Here we identify a specific post-translational modification that occurs during endocrine resistance and which results in tumour susceptibility to the apoptosis inducer TNF-Related Apoptosis-Inducing Ligand (TRAIL). This potentially offers a novel stratified approach to targeting endocrine resistant breast cancer.

Experimental Design
Cell line and primary-derived xenograft models of endocrine resistance were investigated for susceptibility to TRAIL. Tumour viability, cancer stem cell (CSC) viability (tumourspheres), tumour growth kinetics and metastatic burden were assessed. Western blots for the TRAIL-pathway inhibitor, c-FLIP, and upstream regulators were performed. Results were confirmed in primary culture of 26 endocrine-resistant and endocrine-naïve breast tumours.

Results
Breast cancer cell lines with acquired resistance to tamoxifen-(TAMR) or faslodex were more sensitive to TRAIL than their endocrine-sensitive controls. Moreover, TRAIL eliminated CSC-like activity in TAMR cells, resulting in prolonged remission of xenografts *in vivo*. In primary culture, TRAIL significantly depleted CSCs in 85% endocrine-resistant, compared with 8% endocrine-naïve tumours, while systemic administration of TRAIL in endocrine-resistant patient-derived xenografts reduced tumour growth, CSC-like activity and metastases. Acquired TRAIL sensitivity correlated with a reduction in intra-cellular levels of c-FLIP, and an increase in Jnk-mediated phosphorylation of E3-ligase, ITCH, which degrades c-FLIP.

Conclusions
These results identify a novel mechanism of acquired vulnerability to an extrinsic cell death stimulus, in endocrine resistant breast cancers, which has both therapeutic and prognostic potential.

Translational Relevance

Current options for breast cancer patients who relapse on endocrine therapy are limited and mostly rely on re-targeting of endocrine pathways or non-specific chemotherapy, neither of which address the molecular changes that occur during transition to drug resistance. Here we identified one such acquired change, which resulted in endocrine-resistant tumours becoming sensitive to the pro-apoptotic effects of the targeted agent, TRAIL. Moreover, this acquired sensitivity was particularly acute in the cancer stem-like cell population, leading to long-term regression of tumours *in vivo*. Identification of the mechanism underlying this acquired sensitivity provides a companion predictive biomarker of this response. This data supports future clinical investigation into the stratified use of TRAIL agonists for patients who develop resistance to endocrine-therapy, a key area of clinical unmet need that affects more than 8 million breast cancer patients worldwide.

INTRODUCTION

Over 70% of breast tumours express estrogen receptor (ER) and it is clear that estrogen itself plays a vital role in tumour development and progression (1). As a result, the non-steroidal anti-estrogen tamoxifen has provided improved survival and quality of life for many early and advanced ER+ve disease sufferers (2,3).

Since the introduction of tamoxifen to the clinic nearly 30 years ago, further endocrine agents have been developed, notably the steroidal anti-estrogen fulvestrant (Faslodex) that also promotes estrogen receptor degradation (4), and aromatase inhibitors (AIs) that suppress estrogen production in the body (5,6). Although AIs are now the gold standard endocrine strategy in the ER+ve postmenopausal setting (7), tamoxifen remains a pivotal treatment option in ER+ve premenopausal breast cancer patients with 67% of patients responding to tamoxifen as first-line therapy (8–10). Fulvestrant in turn can prove valuable under conditions where tamoxifen or aromatase inhibitors fail (11).

Unfortunately a large proportion of patients acquire resistance to endocrine treatment following initial responsiveness and this is associated with re-instigation of tumour growth and disease progression (12,13). Thus, the majority of patients with advanced disease and up to 30% of ER+ve patients in the adjuvant setting will acquire resistance to the inhibitory effects of their endocrine treatment (12,13). The acquisition of resistance manifests with a partial epithelial- mesenchymal transition and increased migratory and invasive activity both in anti-estrogen resistant cell line models (14) and in clinical samples (14–17). Furthermore, a subset of tumour-initiating cells with stem-like characteristics (cancer stem cells: CSCs) is enriched in tamoxifen-resistant breast cancers and may demonstrate resistance to endocrine therapies, thus contributing to disease recurrence and metastatic progression (18–21).

It has been suggested that ER expression remains a stable phenotype for many acquired resistance tumours that initially respond to endocrine agents (22) which is corroborated by data showing that a significant proportion of acquired resistance tumours remain sensitive to alternative endocrine treatment. However, as a therapeutic avenue this too has its limitations, as response to additional endocrine therapies declines over time while some patients also lose ER expression during endocrine treatment (23–25). *In vitro* and xenograft model studies have elucidated that endocrine agents upregulate various "compensatory" growth factor signalling pathways that sustain cell survival and residual proliferative activity, culminating in resistant growth (26,27). A number of approaches have been examined in patients targeting such signalling mechanisms to overcome endocrine resistance (28),

however, responses to such targeted approaches are short-lived for many patients (29,30). Of these agents, CDK4/6 (palbociclib, ribociclib) and mTOR inhibitors (everolimus) have shown particular promise, however, toxicities associated with these therapies must be managed carefully and predictive biomarkers for response are currently being investigated to help aid in patient selection (31–33). There thus remains a critical need for superior treatments guided by predictive biomarkers with greater long-term benefits in the management of endocrine resistance in the clinic. Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) is an immune-related apoptotic protein that has been shown to specifically target cancer cells whilst sparing normal cells. Unfortunately, several studies have now shown that despite this cancer specificity, many tumour cell sub-types are inherently resistant to treatment with TRAIL agonists, with no improvement in overall survival rates in clinical trials (34). In breast cancer for example, epithelial-like ER-positive tumour cells are resistant to the pro-apoptotic effects of TRAIL, while cells with a mesenchymal phenotype, including CSCs within epithelial-like tumours, exhibit some sensitivity to TRAIL (35–37). Yet despite these observations TRAIL agonists have not progressed well in the clinical setting (34,38). Whilst tumour resistance has been largely attributed to poor agonist potency, it is probable that a scarcity of adequately stratified patient populations also partly contributes to resistance through a lack of understanding of the underlying mechanisms of resistance (39). The intrinsic TRAIL-mediated apoptosis inhibitor c-FLIP is believed to be one contributing factor behind TRAIL resistance of breast cancer cells as mesenchymal CSC and non-CSCs exhibit redistribution of c-FLIP (37) and removal of c-FLIP blockade hypersensitized both the bulk tumour cell population (40,41) and breast CSCs to TRAIL *in vitro* and significantly reduced metastasis in *in vivo* models (36).

Given the evidence above that endocrine resistance was also associated with mesenchymal-like traits (including CSCs), we postulated that endocrine-resistant breast tumours may respond better to TRAIL therapy than their endocrine naïve counterparts. If this were the case, we proposed that the increased TRAIL sensitivity may be mediated by c-FLIP. Here*,* we tested these hypotheses using *in vitro* and *in vivo* cell line models and clinical samples of acquired endocrine-resistant breast cancer. We showed that acquired endocrine-resistant breast cancer cell lines and their constituent CSC subpopulation were indeed sensitive to TRAIL treatment, and that this sensitivity correlated with the reduction of c-FLIP levels through a post-translational mechanism. Furthermore, the TRAIL-mediated reduction in endocrine-resistant CSCs *in vitro* was replicated *in vivo* with a marked reduction in metastatic disease. These findings were supported by patient-derived breast cancer tissue models leading to the conclusion that the selective use of TRAIL agonists to target the CSC compartment in breast tumours that have acquired endocrine resistance may improve clinical outcome with potential long-term benefits to patients.

MATERIALS AND METHODS

Cell Culture. Parental breast cancer cell lines MCF-7$^{ER+HER2-}$ and T47D$^{ER+HER2-}$ (obtained from ATCC) were maintained in RPMI 1640 medium supplemented with 5% foetal bovine serum, 1% penicillin-streptomycin and 0.5% L-glutamine. Tamoxifen- and Faslodex-resistant sub-clones of these cell lines were derived as described (22,42,43) and maintained in phenol red-free RPMI 1640 supplemented with 5% foetal bovine serum (charcoal stripped for MCF-7 derivatives) 1% penicillin-streptomycin, 0.5% L- glutamine and 100nM of 4-OH-tamoxifen (Sigma) or fulvestrant (AstraZeneca).

siRNA. Small interfering RNAs (siRNA) targeting two unique sequences in human c-FLIP (FLIPi - Sense: GGAUAAAUCUGAUGUGUCCUCAUUA, Anti-Sense: UAAUGAGGACACAUCAGAUUUAUCC) and a non-specific scrambled control(SCi- Sense: GGACUAAUAGUUGUGCUCCAAUUUA, Anti-Sense: UAAAUUGGAGCACAACUAUUAGUCC) RNA were used in reverse transfections (Invitrogen). Cells were trypsinised and resuspended at a density of 1x10^5 cells/ml and seeded into wells containing 20µl of 100nM siRNA in serum free Optimem (GIBCO) in a volume of 100µl per well together with 0.3µl of Lipofectamine (Invitrogen). Cells were cultured in the presence of siRNA for 48 hours prior to subsequent assay.

TRAIL Treatment of Target Cells. Cells were treated with soluble human recombinant TRAIL (SuperKillerTRAIL, Enzo Life Sciences) at a concentration of 20ng/ml or 100ng/ml for 18 hours at 37°C in 5% CO_2.

Western blot assays. Western blots of cell lysates were performed as described (44). The following antibodies were used: c-FLIP (Enzo Life Sciences - 7F10, ALX-8040428, Santa Cruz – 5D8, sc-136160), ER-alpha (Santa Cruz, sc-7207), ErbB2 (Abcam, ab2428), GAPDH (Santa Cruz, sc365062), ITCH (AbCam, 99087) and pITCH (Millipore, 10050).

Cell viability and cell death assays. CellTiter blue cell viability assay (CTB) (Promega) was performed according to manufacturer's instructions in a 96-well plate format and fluorescence assessed using a ClarioStar (BMG Labtech) plate reader, while Invitrogen live/dead labelled cells were stained for 15 minutes at 4°C in the dark and subsequently analysed by flow cytometry (BD Accuri).

Primary-derived tumour cell processing and xenografts. Primary and local relapse human breast tumour pathological and post-surgical core biopsies were obtained following consent from the Cardiff and Vale Breast Cancer Centre, Llandough Hospital under NISCHR ethical approval (12/WA/0252) and pleural effusions/ ascitic fluid metastatic samples obtained through Manchester Cancer Research Center Biobank and The Christie NHS Foundation Trust (studies 12/ ROCL/01, 05/Q1402/25, respectively). Cells were processed, maintained and stored according to the Human Tissue Act with local (Cardiff University) research ethics approval. All animal xenograft procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the UK Home Office (PPL 30/2849). Tumours excised from mice or received from patients were finely minced then processed in a MACs tissue dissociator using MACs tissue dissociation kit according to the manufacturers

protocol (Miltenyi Biotec). Primary cells were maintained in 5% CO_2, 5% O_2 at 37°C. The patient-derived xenograft (PDX) models were generated either as previously described (45) or for the PDX 151 model, 3mm chunks of primary tumour material from a 72 year old patient with local relapse previously receiving endocrine treatment (see supplementary figure 4A) were serially passaged by subcutaneous implantation in JAX^{TM} NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wj} (NSG) mice.

Tumoursphere culture. Cell lines and primary tumour samples were initially treated in adherent culture conditions as described then dissociated into single cell suspensions and plated (P1) in ultra-low attachment 96 well plates (Corning) at a density of 20,000 cells/ml in a serum-free epithelial growth medium (DMEM/F12, Gibco), supplemented with 2% B27 (Invitrogen), 55 µM β-mercaptoethanol, 20ng/ml EGF (Sigma), 5µg/ml Insulin (Sigma) and 1µg/ml hydrocortisone. After 7 days tumourspheres were collected by gentle centrifugation (300 x g), dissociated in 0.05% trypsin, 0.25% EDTA and re-seeded (P2) at 20,000 cells/ml for subsequent passages. %TFU was calculated by dividing the number of spheres formed by the total number of single cells seeded.

Fluorescent activated cell sorting (FACS). The directly conjugated mouse FITC anti-CD24 antibody (BD, 555427), mouse allophycocyanin (APC) anti-CD44 antibody (BD, 559942), mouse APC anti-DR5 (biolegend, DjR2-4), mouse FITC anti-DR4 (AbCam, ab59047) were used to stain single cell suspensions at 4°C in the dark for 30 minutes. Control samples were stained with isotype-matched control antibodies and samples analysed using BD Accuri flow cytometer.

Aldefluor Assay. To measure ALDH activity, Aldefluor assay (Stem Cell Technologies) was performed on surviving cell populations as previously described (46). Briefly, dissociated single cells were suspended in assay buffer containing ALDH substrate, bodipyaminoacetaldehyde (BAAA) at 1.5 mM, and incubated for 45 min at 37°C. To distinguish between ALDH+ve and –ve cells, cells were also incubated under identical conditions in the presence of a 2-fold molar excess of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB).

Xenograft assays of MCF-7-TAMR cells. Tumour initiation and growth was assessed by orthotopic mammary fat pad transplantation of dissociated MCF-7 and MCF-7-TAMR cells. Cells were harvested using 1mM EDTA, washed and resuspended at a density of $5x10^6$ cells/ml in serum-free L15 media. A 1.5mg, 90-day slow release 17-β estradiol or Tamoxifen pellet (Innovative Research of America, Sarasota FLA) for parental and TAMR cells respectively was inserted subcutaneously above the right scapula of anaesthetised NOD-SCID mice (Harlan). $1x10^6$ cells were orthotopically injected directly into the abdominal mammary fat pad and when tumours reached 5mm in diameter the mice were treated with 16mg/kg TRAIL (IP) daily for 4 days twice, separated by a 6 day treatment holiday. Mice were then monitored and tumour volume measured twice weekly. Metastatic tumour burden within lung tissue was then assessed histologically in serial H&E sections.

RNA extraction and cDNA synthesis. Cultured cells were pelleted via centrifuge at 300rcf for 5 min before being resuspended in 350 µL RLT buffer (Qiagen) and placed on ice for immediate extraction or frozen at -80°C for future extraction. RNA extraction was performed using the Qiagen RNEasy kit following the manufacturer's instructions. The concentration

and quality of RNA was analysed using a nanodrop 3000 spectrophotometer (Thermo Scientific). Previously isolated RNA was synthesized into cDNA using the QuantiTect Reverse Transcription kit (Qiagen) according to manufacturer's instructions.

Quantitative PCR (QPCR). All qRT-PCR experiments were designed to include both target gene probes and ACTB control probe. For each experiment TaqMan Universal Master Mix II, with UNG (ThermoFischer Scientific) was used. TaqMan master mix was added to target and ACTB control probes together with RNase free H_2O to make individual target master mixes containing all reaction components and plated in 384-well qPCR plates (Applied Biosystems). Plates were then run on a QuantStudio 7 Real-Time PCR machine (Applied Biosystems) set to the following protocol: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec (denaturation), and 60°C for 1 min (annealing/elongation). Relative quantity (RQ) was determined using ΔΔCt values calculated from the reference sample.

Statistical methods. Data are represented as mean +/- standard error of the mean for a minimum of three independent experiments, unless otherwise stated. Statistical significance between means was measured using parametric testing, assuming equal variance, by standard T-test for two paired samples. The chi-squared test was used to determine the significance of deviation from expected ratios of responders to non-responders in the clinical cohort.

RESULTS

Acquired endocrine-resistant breast cancer cell lines develop hypersensitivity to TRAIL-induced cell death. The development of endocrine therapy resistance in primary breast cancer is associated with poor prognosis and, in cell line models and clinical samples of endocrine resistance, by a transition to more aggressive tumour phenotypes that have undergone partial EMT (14–17). The cancer therapeutic TRAIL has been shown to preferentially induce cell death in breast cancer cell lines that exhibit a more mesenchymal phenotype (35). We wished to investigate therefore whether the adaptation to endocrine treatment also increased the sensitivity of breast cancer cells to TRAIL. Initially 4 models of acquired endocrine-resistance, derived from continuous endocrine treatment with tamoxifen or Faslodex of the luminal ER+ve breast cancer cell lines MCF-7 (42) and T47D (43) were investigated. Acquired tamoxifen-resistant (TAMR) and acquired Faslodex-resistant (FASR) cell lines were significantly more sensitive to the cytotoxic effects of TRAIL than their parental controls. (Figure 1A, B and Figure S1A). Sensitivity was particularly prominent in the TAMR derivatives. The gain in TRAIL sensitivity was then confirmed in an independent MCF-7-derived tamoxifen-resistant model (20) (Figure S1B). This marked susceptibility to TRAIL could not be explained by the combinatorial effect of TRAIL with either endocrine agent and was a feature confined to the resistant state, as the combination of tamoxifen or Faslodex with TRAIL treatment on the previously untreated MCF-7 cells (co-treated) or on cells pre-treated with these endocrine agents for up to 7 days (pre- treatment) did not confer TRAIL sensitivity (Figure 1C). Furthermore, tamoxifen withdrawal (TAMR-W) for 7 days prior TRAIL treatment also had no effect on the sensitivity of TAMR cells to TRAIL (Figure 1D). Combined, these results suggested that the substantially-increased TAMR sensitivity to TRAIL was due to a stable adaptation resulting from acquired resistance to tamoxifen.

TRAIL treatment of acquired endocrine-resistant cells targets cancer stem cell- like traits *in vitro* According to the cancer stem cell hypothesis, a drug resistant sub-population of stem/progenitor-like tumour cells is likely to contribute to promotion of resistance to conventional therapies (18,21,47,48). To determine if these cells demonstrated a comparable sensitivity to TRAIL to the bulk cell cultures in the acquired endocrine-resistant lines, the effect of TRAIL on stem-like activity was assessed by tumoursphere assay while cancer stem/progenitor-cell numbers were measured using cell surface markers and ALDEFLUOR^{TM} assay. Both the MCF-7-derived TAMR and FASR cell lines exhibited an increased capacity to form tumourspheres, that were similar in morphology and size to their parental MCF-7 cell line. However, in contrast to the parental line, these self-renewing tumoursphere-forming cells were significantly more sensitive to TRAIL, with a complete ablation of tumoursphere forming units (TFU) and colony forming units (CFU) in the TAMR cells following treatment with TRAIL (Figure 2A & 2B, Figure S2C). Furthermore, this TRAIL sensitivity was independent of the presence of tamoxifen in the media (Figure 2C), implying that the hyper-sensitization to TRAIL was inherent to the acquired endocrine (tamoxifen) resistance phenotype. The effect of TRAIL on the cancer stem cell compartment in the TAMR line was further supported by flow cytometry using surrogate markers of stem/progenitor cells. Both CD44^{Hi}CD24^{Lo} and ALDH^{+ve} sub-populations were significantly elevated in tamoxifen-resistant cell lines yet remained sensitive to TRAIL-mediated apoptosis (Figure 2D), although TRAIL failed to further deplete the CD44 population in TAMR cells. A significant reduction in CSC-like activity was also observed in a second cell line model of acquired tamoxifen resistance (T47D-derived), although the sensitivity to TRAIL was not as profound, TRAIL treated TAMR cells were still significantly reduced compared to their TRAIL-treated parental counterparts (Figure S2A & S2B) with a 50% reduction in tumoursphere forming capacity and a 4-fold reduction in CD44+ve cells in T47D-TAMR cells following TRAIL treatment. In summary, this data suggests that sensitivity of breast cancer stem-like cells to TRAIL develops following the acquisition of resistance to anti-estrogens, most acutely with tamoxifen resistance.

Systemic TRAIL administration results in long-term regression of TAMR tumours *in vivo* In order to determine if the TRAIL-mediated reduction in overall cell viability and CSC-like properties *in vitro* was reflected in tumour growth *in vivo*, mice bearing established MCF-7 parental or TAMR orthotopic tumours of approximately 5mm in diameter were subjected to eight intraperitoneal injections of TRAIL over a period of 2 weeks, then monitored for tumour response. Along with the previously observed elevation in CSC-like and proliferative activity of TAMR cells *in vitro* (Figure 2B), orthotopic TAMR tumours were found to be more aggressive than parental MCF-7 controls under vehicle control conditions, with an elevated growth rate and appearance of spontaneous metastases to the lung (Figure 2E, F). MCF-7 tumours responded transiently to TRAIL treatment (20% reduction in tumour volume) but within two weeks of treatment, had regained similar growth kinetics to the vehicle treated cohort (Figure 2E). Treatment of TAMR tumours with TRAIL on the other hand resulted in a complete (100%) and sustained regression of all tumours (n=8) for up to 15 weeks (Figure 2E). Furthermore, following TRAIL treatment both the number and size of TAMR metastatic lesions in the lungs were decreased by more than 95% (Figure 2F), correlating with the profound decrease in CSC-like activity observed *in vitro* (Figure 2B). Taken together these

data suggested that tamoxifen-resistant cell response to TRAIL *in vivo* correlated with the observed *in vitro* responses whereby tumour regression occured by de-bulking tumour mass and in turn metastatic tumour burden and tumour relapse were significantly reduced through the elimination of the cancer stem/progenitor pool.

Clinical samples from multiple endocrine drug-resistant patients exhibit TRAIL sensitivity.
Having seen clear benefits of TRAIL treatment in cell line models of anti- estrogen-resistance *in vitro* and *in vivo*, the efficacy of TRAIL on endocrine-resistant breast cancer was determined in clinical samples *ex vivo*. Primary cell cultures were derived from pleural effusions or ascites of 13 patients with advanced ER+ve breast cancer, 11 having received endocrine treatment and 2 endocrine-naïve samples (Figure S3A). Cells were plated into tumoursphere culture prior to treatment with TRAIL *ex vivo* (Figure 3A). 9 out of 11 (82%) of the clinical samples from the advanced metastatic endocrine-resistant patients exhibited a significant reduction in tumoursphere forming units (TFU) following treatment with TRAIL (Figure 3A) while neither of the endocrine-naïve metastatic patient samples (90 and 94) responded to TRAIL. Similarly, 7 out of 8 (88%) of the endocrine-resistant patient samples cultured as an adherent monolayer were also sensitive to TRAIL, with only one of the two endocrine-naive samples exhibiting a significant response to TRAIL (Figure S3B).

A similar relationship was observed in primary cell cultures derived from 13 fresh core biopsies of local relapse ER-positive breast cancer (Figure 3A). Of two tumour biopsies originating from patients who had relapsed following treatment with tamoxifen (samples 127 and 188) both demonstrated a significant reduction in tumoursphere forming capacity, while only 1 of 11 (9%) of endocrine treatment-naïve primary tumours exhibited such a response to TRAIL.

Overall, 11 of 13 samples (85%) from all clinical groups that had relapsed following endocrine treatment responded to TRAIL *ex vivo*, compared to just 1 of 13 tumours that had not previously seen endocrine therapy (Figure 3A, B & C). Taken together, the findings from these *ex vivo* models provide pre-clinical evidence that TRAIL may be valuable in controlling endocrine-resistant breast cancers and potentially provide long-term benefits by targeting the CSC subset.

***In vivo* PDX models of endocrine-resistant breast cancer demonstrate sensitivity to TRAIL administration.** The potential for TRAIL as a further therapeutic option following acquisition of endocrine resistance was subsequently tested *in vivo* using two distinct patient-derived xenograft (PDX) models of endocrine-resistant breast cancer.

For the first PDX model, a primary ER-positive tumour was maintained orthotopically by serially passaging in immunocompromised mice receiving either estradiol (MaCa 3366) or tamoxifen (MaCa 3366 TAMR) for up to 3 years, generating estradiol-dependent and tamoxifen-resistant models respectively from the same parental tumour tissue (45). Mice were subjected to systemic administration of 8 intraperitoneal injections of TRAIL over 2 weeks (treatment windows highlighted in pink, Figure 4) and tumour growth kinetics monitored from the beginning of treatment (100mm^3). In the tamoxifen-sensitive estrogen-dependent control tumours, systemic *in vivo* TRAIL treatment failed to elicit a significant growth inhibitory response compared with vehicle only (Figure 4A). There was also no alteration in the CSC compartment within the tumours (Figure 4A). In contrast, a transient (10 day) yet significant regression of MaCa 3366 TAMR tumours was observed following first

TRAIL administration, compared to a doubling in tumour volume in the vehicle control arm over the same time period, which after a second dose of TRAIL culminated in a sustained and significant reduction in tumour size at 4 weeks compared to untreated controls (p=0.0045) (Figure 4B). Thus while TRAIL-treated naïve 3366 tumours exhibited a 4-fold increase in tumour volume over 4 weeks, TRAIL treated endocrine-resistant tumours exhibited a 1.8-fold increase over the same time period (Figure 4A & 4B). Furthermore, consistent with observations in our cell line models of tamoxifen resistance there was a significantly higher basal level of CSC- like cells in MaCa 3366 TAMR tumours compared with its endocrine responsive, estrogen-dependent, counterpart, and TRAIL treatment *in vivo* significantly depleted the CSC-like activity in the tamoxifen-resistant model (Figure 4B) whereas no significant difference was observed in the endocrine-sensitive model. Additionally, tumourspheres generated from the TRAIL-treated MaCa 3366-TAMR tumours also maintained their sensitivity to TRAIL when treated *ex vivo*, demonstrating that the tumoursphere-forming subset surviving *in vivo* TRAIL administration was not a consequence of acquired TRAIL resistance (Figure S4B).

For the second PDX model, a core biopsy (CUbbs 151) obtained from a locally relapsed ER-negative tumour from an ER-positive (original status prior to endocrine treatment) breast cancer patient who had relapsed on the aromatase inhibitor (AI) anastrazole, was used to propagate tumours in recipient immunocompromised mice to model AI-resistant breast cancer (histopathologically characterised in Figure S4A). AIs have become the gold standard first-line therapy for the majority of postmenopausal ER-positive breast cancer patients, and many of the endocrine-resistant clinical samples used in this study (Figure 3) had at some point in their treatment received an AI (Figure S3A). In order to determine AI- resistant breast cancer sensitivity to TRAIL, mice bearing CUbbs 151 tumours (PDX 151) were administrated 8 intraperitoneal injections of TRAIL over 2 weeks. This resulted in a significant reduction in AI-resistant tumour growth compared to vehicle control (Figure 4C) and *ex vivo* control PDX 151 tumour cells also demonstrated significant sensitivity of CSC-like cells to treatment with TRAIL (Figure 4D). Furthermore, the sensitivity of PDX 151 CSCs *ex vivo* was consistent with the sensitivity of the patient biopsy (CUbbs 151) treated *ex vivo* prior to transplantation (i.e. original biopsy straight from the patient) (Figure S4C). This was paralleled by a profound reduction in both the number and size of spontaneous lung metastases *in vivo* (Figure 4E) adding additional confirmation that the CSC-like compartment within these tumours was particularly sensitive to TRAIL treatment.

c-FLIP degradation via Jnk activation leads to the observed sensitivity of tamoxifen-resistant breast cancer cells In order to elucidate the underlying cause of the TRAIL hypersensitivity gained both in endocrine-resistant breast cancer cell lines and clinical tumours, key cellular components of TRAIL-mediated cytotoxicity were assessed by western blot and FACS analysis in the TAMR and FASR cell lines. These included the TRAIL receptors DR4 and DR5 and the endogenous TRAIL-receptor complex inhibitor c-FLIP, all of which have been shown to influence TRAIL sensitivity in other cancer cell types (49–52). Comparative levels of DR4 and DR5 cell surface receptor expression did not reflect the relative sensitivity of each cell line to TRAIL (Figure S5A). The relative protein levels of the TRAIL pathway inhibitor, c-FLIP (55kDa isoform identified with two independent antibodies), however, were found to be lower in TAMR cells compared to parental MCF-7 cells (Figure 5A). Similarly, c-FLIP protein levels were also significantly reduced in TAMR and FASR derivatives of the T47D cell line (Figure 5A). This reduction in c-FLIP protein levels is attributed to post-transcription regulation of c-FLIP, as no correlation between c-FLIP mRNA levels in either the cell lines or

the primary samples tested correlated with TRAIL response (Figure S5B).

Previously a Jnk-mediated degradation of c-FLIP via the E3 ubiquitin ligase ITCH has been described to explain regulation of c-FLIP protein in primary mouse cells (53)(Figure S5C). Accordingly, phosphorylated ITCH (pITCH) was upregulated in MCF-7 and T47D- derived TAMR and FASR cells (Figure 5A) compared to parental controls with no parallel increase in total ITCH levels. The proposed link between Jnk and suppressed c-FLIP levels was confirmed by pre-treatment of the MCF-7-derived TAMR cells with the Jnk inhibitor SP600125 which after 4 hour exposure partially rescued TAMR cell and endocrine-resistant primary sample viability in the presence of TRAIL (Figure 5B, Figure S5D and Figure S5E). Furthermore, Jnk inhibition decreased ITCH activation (reduced phospho-ITCH levels) and subsequently restored c- FLIP protein expression of TAMR cells to parental MCF-7 levels (Figure 5C).

Similarly, untreated MaCa 3366-TAMR tumours exhibited a significantly lower level of c-FLIP protein expression and increased ITCH and pITCH expression compared to its endocrine sensitive, estrogen-dependent control (Figure 5D). Western blot analysis of 8 of the advanced metastatic endocrine-resistant clinical samples *ex vivo* with varying TRAIL response was performed in order to determine if the mechanism of sensitivity in these primary cells was consistent with that of the endocrine-resistant cell lines (Figure 5E). The activation (phosphorylation) of ITCH and relative c-FLIP levels were subsequently determined by densitometry normalised to GAPDH controls. The activation of ITCH and c-FLIP protein levels were inversely correlated such that increased activation of ITCH in endocrine-resistant samples correlated with a reduction in c-FLIP levels and, when averaged, cells sensitive to TRAIL had significantly lower levels of c-FLIP and higher levels of ITCH activation (Figure 5F, Figure S5F). Additionally, reduction of c-FLIP protein levels and increased ITCH activation correlated with response to TRAIL (Figure 5F, Figure S3B). Furthermore, the advanced metastatic endocrine-resistant clinical sample with the lowest activation of ITCH (sample 81, Figure 5E) and the sample with the highest level of c-FLIP protein (sample 29, Figure 5E), both of which demonstrated no significant response to TRAIL under adherent conditions, could be substantially sensitised to TRAIL by siRNA inhibition of c-FLIP, supporting the role for c-FLIP in TRAIL sensitivity (Figure 5G, Figure S5G and S5H). Finally, the endocrine-naïve, TRAIL-resistant, clinical sample 90, could also be sensitised to TRAIL by siRNA suppression of c-FLIP (Figure 5H, Figure S5I). These data demonstrate that the activation of ITCH and reduction of c-FLIP is one potential mechanism of hypersensitivity of endocrine-resistant clinical samples *ex vivo*.

DISCUSSION

There is a clear unmet need for alternative treatments for patients who relapse on endocrine therapy. Relapse rates are high and following multiple lines of endocrine therapy there is higher risk of metastasis and poorer overall survival with limited treatment options.

Here we demonstrate that acquired endocrine-resistant breast cancer cells gain sensitivity to TRAIL treatment compared with their endocrine responsive parental counterparts. This appears to manifest following the acquisition of resistance and is a stable sensitivity, as administration of endocrine agents in ER-responsive cells does not increase response to TRAIL.

Although other changes in signalling pathways have been identified as a consequence of endocrine-resistance – such as EGFR/HER2, MAPK, CDK4/6, mTOR and Src (54–59) – the data

reported here represents to our knowledge the first demonstration of a sensitization to a single agent targeting a pro-apoptotic pathway. Previous studies have demonstrated increased Jnk signalling following the long-term acquisition of resistance to endocrine therapies (12,60). Here we demonstrate that one consequence of this Jnk activation is the ITCH-dependent destabilisation of c-FLIP protein, which correlates with TRAIL sensitivity both in cell line models *in vitro* and in clinical samples *ex vivo*.

Previously, modest outcomes in phase II clinical trials of TRAIL agonists in terms of overall survival has been partly attributed to the lack of potency of existing TRAIL receptor agonists (34), however, it could also be postulated that a lack of stratification and suitable biomarkers for response also contribute to the poor results seen to date. The data presented here suggests that acquired resistance to endocrine therapy, could represent a key subgroup of patients that might exhibit improved responses to TRAIL agonists and that monitoring of c-FLIP, pITCH and/or p-Jnk levels in tumours could further potentiate the selection process by identifying at least a proportion of TRAIL-responsive tumours. Further exploration of these molecular markers by immunohistochemical analysis of pathological samples with access to fresh tissue material would be required to determine if these could be used as predictive biomarkers of TRAIL response.

We recently reported that TRAIL can selectively target the CSC-like cells in breast cancer cell lines *in vitro* following suppression of c-FLIP. Here we demonstrate that acquired endocrine resistance potentiates sensitivity of the CSC-like compartment to TRAIL in primary-derived breast cancer samples, and that this significantly correlates with a reduction in c-FLIP levels. These observations are particularly significant given that CSCs have been demonstrated to evade conventional chemotherapy agents and potentially endocrine agents, either due to cell quiescence, through the prevention of drug uptake or modulation of intracellular signalling (18,21,61–63). Targeted therapies that initiate cell death through cell surface receptors circumvent these resistance mechanisms and therefore may prove to be a more effective strategy for targeting CSCs (64–66). Indeed, this was confirmed in xenograft models of endocrine-resistant tumours, whereby systemic TRAIL treatment resulted in a profound suppression of tumour recurrence (Figures 2 & 4) and a marked decrease in spontaneous metastasis (Figures 2F & 4E). Interestingly, response to TRAIL was observed regardless of cellular ER status. *In vitro* models of TAMR maintain ER-positivity following acquisition of resistance and active ER signalling remains important in cell proliferation. Similarly, MaCa 3366 also maintains ER positivity following acquisition of resistance, however, FASR cell lines and PDX 151 do not maintain their ER positivity. Despite these important differences in cellular signalling, all these models demonstrated sensitivity to TRAIL treatment *in vitro* and *in vivo*. Taken together, these data have important implications for potential long-term survival benefit patients who ultimately relapse on any form of endocrine therapy.

Previous studies have proposed that the acquisition of endocrine resistance is accompanied by the appearance of mesenchymal-like properties, which might also explain the increased risk of metastatic disease. This was the premise on which we initially tested for TRAIL sensitivity in endocrine-resistant breast cancer cells, as TRAIL had previously been shown to selectively target breast cancer cell types with a mesenchymal-like phenotype (35). The question still remains as to whether a partial epithelial-mesenchymal transition sufficiently explains the strong correlation between endocrine resistance and TRAIL sensitivity in clinical samples observed here. Further studies into the mechanism of Jnk activation and the role of EMT-like processes in TRAIL sensitivity will help elucidate its relationship with acquired

endocrine resistance.

In summary, we have shown that acquired endocrine resistance confers sensitivity of breast cancer cells and particularly breast cancer stem cells to TRAIL-mediated apoptosis. These data support further investigation into the selective use of TRAIL agonists as a subsequent treatment for ER+ve patients who relapse on endocrine therapy regardless of their ER status following relapse.

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Figure 1: Acquisition of endocrine resistance in breast cancer lines confers sensitivity to TRAIL. A) Acquired endocrine (faslodex or tamoxifen) resistant MCF-7 and B) T47D cell lines together with their parental endocrine responsive lines were treated with 20ng/ml TRAIL for 18 hours and viability assessed by CTB assay relative to their respective untreated control (*p <0.01). C) MCF-7 cells were either co-treated with endocrine treatment (100nM tamoxifen or faslodex) plus 20ng/ml TRAIL for 18 hours, or pre- treated for 7 days with endocrine agent and TRAIL for 18 hours, and viability was assessed by CTB assay (*p <0.01). D) MCF-7 TAMR cells were cultured as normal or with tamoxifen withdrawn for 7 days (TAMR-W) prior to 20ng/ml or 50ng/ml TRAIL treatment for 18 hours and viability assessed by CTB assay (*p<0.01).

Figure 2: CSCs in MCF-7-derived endocrine-resistant lines demonstrate hypersensitivity to TRAIL. A) Representative tumourspheres formed from surviving cell population following 20ng/ml TRAIL treatment for 18 hours. B) MCF-7 cells and their derived endocrine-resistant lines were treated with TRAIL or vehicle for 18 hrs in adherent culture, before transferring to tumoursphere culture conditions for one week and colonies counted as a % of viable cells seeded (*p<0.03, **p<0.01). C) MCF-7 TAMR cells previously maintained in adherent culture with tamoxifen or tamoxifen withdrawn (TAMR-W) for 1 week and then treated with 20ng/ml TRAIL for 18 hours were transferred to tumoursphere assay as above (*p<0.01). D) Expression of CSC surrogate markers (CD44, CD24, ALDH) in the MCF-7 parental and TAMR cell lines following 18 hours TRAIL treatment compared with their respective untreated control (*p<0.01). E) 10^7 MCF-7 parental or TAMR cells were transplanted into the 4th inguinal mammary gland and 16mg/kg TRAIL administered 8 times over 2 weeks (4 x daily, 6 days treatment holiday then 4 x daily) once tumours had reached a volume of 100mm^3 and growth kinetics monitored following final treatment by caliper measurement relative to starting tumour volume (*p<0.03, ***p<0.001 n=8 per group). F) Lung metastatic burden in vehicle or TRAIL treated TAMR transplanted animals following sacrifice at week 16 (*p<0.03, **p<0.01).

Figure 3: Endocrine-resistant clinical relapse tumour samples display increased tumoursphere sensitivity to TRAIL compared to endocrine-naïve samples. A) Tumourspheres isolated from anti-estrogen (AE)-resistant metastatic BC (red hashed), endocrine resistant local BC (red), endocrine-naïve metastatic BC (white hashed) and endocrine naïve local BC were treated with 100ng/ml TRAIL for 18 hours and fold change in %TFU from paired untreated control calculated for each sample 2 weeks later *p<0.05 vs paired control. **B)** Mean endocrine-naïve BC sample (metastatic and local combined) and endocrine-resistant (metastatic and local combined) %TFU following TRAIL treatment compared to their untreated controls (*p<0.01, n=13). C) Comparison of mean TRAIL response in endocrine naïve and resistant samples (* p<0.03, n=13).

Figure 4: Endocrine-resistant PDX models of BC demonstrate sensitivity to TRAIL. A) Established Tamoxifen-sensitive and B) Acquired Tamoxifen- resistant MaCa 3366 PDX tumours (n=8 each) were treated 8 times in 2 weeks with 16mg/kg of TRAIL I.P (pink = 4 x daily treatment windows) and growth kinetics monitored weekly by caliper measurement compared with vehicle control (* p<0.03, n=8). Vehicle and TRAIL- treated tumours excised 24 hours following the final TRAIL administration were dissociated and tumoursphere forming capacity assessed (P1). TFUs were then disaggregated and passaged to assess self-renewal capacity (P2) (* p<0.03, n=3) C) AI-resistant PDX model of BC (PDX 151) was treated and monitored as in (A) and tumour growth kinetics monitored by caliper measurement (p<0.03, n=3). D) Vehicle control PDX 151 AI-resistant tumours were excised, dissociated and %TFU assessed following treatment with 100ng/ml TRAIL for 18 hours *ex vivo* (*p<0.03). E) Assessment of lung metastatic burden (mean number and size of metastasis +/- SEM) in H+E serial sections from vehicle control and TRAIL treated PDX 151 tumours 2 weeks following last TRAIL treatment (* p<0.03).

Figure 5: Endocrine-resistant breast cancer cell sensitivity to TRAIL correlates with inherent increased ITCH activation (via Jnk activation) and c-FLIP protein reduction. A) Pan-ITCH phosphorylated-ITCH, and c-FLIP (long, 55kDa) protein levels in endocrine-resistant MCF-7 and T47D cell lines as assessed by western blot versus parental control. Two antibodies to the long form of cFLIP are used in MCF-7 cells to confirm specificity. B) MCF-7 TAMR cells and primary endocrine-resistant BB3RC46 cells were incubated with or without 10μM SP600125 Jnk inhibitor (JNKi) for 4 hours and then treated with or without 20ng/ml (TAMR)/ 100ng/ml (BB3RC46) TRAIL for 18 hours and mean cell viability/cell death was assessed by CTB (*p<0.03, n=3). C) MCF-7 TAMR cells were incubated with or without JNKi for up to 4 hours and ITCH, pITCH and c-FLIP

protein levels assessed by western blot. D). Levels of ITCH expression/activation and c-FLIP protein were assessed by western blotting protein extracted from MaCa 3366 (A6 and A7) and MaCa 3366 TAMR tumours (A1 and A6). E) ITCH activation and c-FLIP protein levels in endocrine-resistant metastatic tumour samples as determined by western blot. F) Correlation of c-FLIP protein level and ITCH activation to each other and to sample response to TRAIL (from Figure S3B). G) A TRAIL-resistant, endocrine-resistant, clinical sample from a metastatic breast cancer patient, BB3RC81, was incubated with either scrambled control (SCi) or FLIP targeted (FTi) siRNA and then treated with 100ng/ml TRAIL for 18 hours and cell viability assessed by CTB (**p<0.01). H) A TRAIL-resistant, endocrine-naïve, clinical sample from a metastatic breast cancer patient, BB3RC90, was incubated with either scrambled control (SCi) or FLIP targeted (FTi) siRNA and then treated with 100ng/ml TRAIL for 18 hours and cell viability assessed by CTB (**p<0.01).