



Mechanistic Studies of Extrinsic Apoptosis by Therapeutic Molecules in Cancer Cells

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Mechanistic Studies of Extrinsic Apoptosis by Therapeutic Molecules in Cancer Cells

A dissertation presented

by

Yijie Hua

to

The Division of Medical Sciences

in partial fulfilment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

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Cambridge, Massachusetts

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Abstract

Death receptor (DR) activation by ligands including TRAIL is the initiating step in extrinsic apoptosis. Despite considerable effort, the clinical benefits from DR agonists have been limited, and no such drug has demonstrated good efficacy in phase II clinical trials. This may be linked to observations that differential cell fate decisions can arise both between cell lines and among individual cells within the same cell line, contributing to resistance to TRAIL and DR therapeutic antibodies in general.

Here, I show that DISC activity and caspase-8 dynamics play an important role in extrinsic apoptotic fate decisions for newly engineered ligands. Experiments in cells treated with TRAIL and two novel synthetic TRAIL analogues, T191 and T202, demonstrate that the maximum caspase-8 activation rate threshold is a critical barrier that has to be overcome. In HeLa, DU145, and HT29 cells, this is the major barrier of apoptosis. In HCT116 cells, other factors may also play critical roles in determining cell fate.

I also explored potential sensitization strategies in this work in the hope of overcoming pro-survival barriers in TRAIL-resistant cells, and I found that glutamine depletion greatly increased receptor-mediated apoptosis in cells of various cancer types, and this sensitization effect may result from increased caspase-8 activity induced by changes in FLIP and/or TRAF2 levels. I have also determined that the combination of T202 with a proteasome inhibitor (bortezomib) or SMAC mimetic (LCL161) can produce a consistent synergistic effect in multiple breast cancer and colorectal cancer cell lines, pointing to a potentially promising strategy for T202-based treatment.

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1. Chapter 1 - Introduction

Apoptosis, also known as programmed cell death, is an essential biological process both during development and throughout adult life. During development, cells undergo apoptosis for tissue remodeling purposes, whereas apoptosis in adulthood helps preserve tissue homeostasis and removes damaged, dysfunctional, and infected cells. There are two types of apoptosis: intrinsic apoptosis induced by intracellular stress signals, and extrinsic apoptosis induced by activation of cell-surface death receptors (DRs) by ligands such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Kiraz et al. 2016).

TRAIL-induced signal transduction starts with the binding of TRAIL to DR4 and DR5, which triggers the formation of the death-inducing signaling complex (DISC) and the activation of initiator caspase-8. In type I cells, activated caspase-8 then cleaves and activates effector caspase-3, which leads to eventual cell death (Ozören and El-Deiry 2002). In type II cells, due to the presence of high levels of inhibitor of apoptosis proteins (IAPs)—particularly X-linked IAPs (XIAPs)—activated caspase-8 must first induce mitochondrial outer membrane permeabilization (MOMP) before caspase-3 can reach a critical mass to trigger final cell death (Ozören and El-Deiry 2002; Rudner et al. 2005; Jost et al. 2009; Aldridge et al. 2011). The induction of MOMP is controlled by the BCL-2 protein family, and cell death is believed to be inevitable after the onset of MOMP (Davids and Letai 2012).

The TRAIL-induced apoptotic pathway is selectively activated in cancer cells while sparing normal cells (A. Ashkenazi et al. 1999), and has therefore been regarded as a promising target for cancer therapy. A series of therapeutic human monoclonal

antibodies have been designed that target DR4 and DR5 as agonists. The most well-studied of these include DR4-agonist mapatumumab (Tolcher et al. 2007), DR5 agonist Apomab (Adams et al. 2008) and conatumumab (AMG655) (Kaplan-Lefko et al. 2010). TRAIL-like ligands have also been developed for the same purpose, with recent examples including T191 and T202, developed by Merrimack Pharmaceuticals, and ABBV-621, developed by AbbVie.

Although most players in the TRAIL-induced apoptotic pathway have been identified and the signaling cascade is well studied, a heterogeneous response can be observed both within and between various cancer cell lines after treatment with TRAIL or DR-agonist antibodies, resulting in differing degrees of fractional cell death (Flusberg and Sorger 2015). Multiple factors have been identified that account for the inter-cell line variation in TRAIL response, such as the abundance and modification of DR4 and DR5 (Wagner et al. 2007); caspase-8 and cellular FADD-like ICE/caspase 8 inhibitory protein (FLIP) (J.-J. Chen et al. 2012); or BCL-2, BCL-XL, and XIAP (Passante et al. 2013). In terms of intra-cell line variation, our lab has demonstrated that the activation rate of caspase-8 can predict fate decision in HeLa cells (Roux et al. 2015).

Several small-molecule drugs have also been developed that can antagonize pro-survival proteins such as the various anti-apoptotic BCL-2 proteins and IAPs. One strategy to overcome the limited efficacy of single-agent treatment would be combination treatment targeting both upstream caspase-8 activation and downstream anti-apoptotic BCL-2 proteins and IAPs. Both Bcl-2 inhibitors such as ABT199 and ABT263 and IAP inhibitors such as the SMAC mimetics have been shown to have

TRAIL-dependent sensitizing effect in multiple cancer types (Hao et al. 2004; Simone Fulda 2018; Kang and Reynolds 2009). Proteasome inhibitors such as bortezomib can also act as TRAIL-sensitizing agents, and proposed mechanisms underlying this sensitization include increased expression of DR4/DR5 and the pro-apoptotic BCL-2 family proteins BAX, BAK, BIM, BIK, NOXA, and PUMA, and reduction in FLIP and IAPs (de Wilt et al. 2013; Sayers and Murphy 2006; Sayers 2011).

1.1 Apoptosis and TRAIL-induced apoptotic pathway

1.1.1 Apoptosis

Programmed cell death was first defined by Lockshin and Williams in 1965 in the context of the development of silkworms (Lockshin and Williams 1965). Kerr, Wyllie and Currie subsequently introduced the term 'apoptosis' to distinguish this process as a morphologically different type of cell death from necrosis (Kerr, Wyllie, and Currie 1972). In necrosis, the endoplasmic reticulum (ER) and mitochondria swell, followed by the rupture of the cell membrane and release of cellular contents. In apoptosis, on the other hand, nuclei become condensed and fragmented, after which the cell breaks into small apoptotic bodies that are engulfed by macrophages.

Apoptosis plays a critical role throughout the human lifespan. This process is essential during development for the establishment and maintenance of tissue architecture, and for maintaining homeostatic balance throughout adult life. For example, at early developmental stages, excessive numbers of cells are produced in the nervous and immune systems. Apoptosis subsequently ensures that these systems

achieve proper function by removing non-functional neurons and immune cells (Nijhawan, Honarpour, and Wang 2000; Opferman and Korsmeyer 2003). Apoptosis is also essential to digital formation and sexual differentiation during development. In adult life, apoptosis is responsible for removing infected or damaged cells and clearing self-antigen-recognizing immune cells that might otherwise produce an auto-immune response (Shigekazu Nagata 2010). Apoptosis also plays an important role in tissue remodeling processes such as mammary gland involution and wound healing (Lund et al. 1996; Elmore 2007). It has been estimated that each day adult human body needs to produce around 10 billion cells to replenish the cell loss due to apoptosis (Renehan, Booth, and Potten 2001).

There are two major apoptosis pathways: the intrinsic apoptotic pathway initiated by MOMP, and the extrinsic apoptotic pathway initiated by the activation of cell membrane-bound receptors (Russo et al. 2006). Both pathways lead to the activation of the classical caspase signaling cascade. Intrinsic apoptosis is triggered by intracellular stimuli such as ER stress and DNA damage, and is often down-regulated in cancer cells (Czabotar et al. 2014). The extrinsic apoptosis pathway starts with the binding of tumor necrosis factor (TNF) family death ligands to cell-surface receptors. There are three main death ligands: TNF- α , FasL, and TRAIL (S. Nagata 1997). All three are type II membrane proteins that can be cleaved and released from cells as homotrimers (Marsters et al. 1999). FasL, TRAIL, and TNF- α respectively bind to Fas receptor, TRAIL-R1 and -R2 (also known as DR4 and 5, respectively), and TNF receptor 1 (TNF-R1) (Krammer 2000; Henning Walczak 2013). DR4 and 5 contain a death domain (DD) in their cytoplasmic tails that, upon activation, recruits Fas-associated protein with death

domain (FADD), procaspase-8, and FLIP to form DISC (Lavrik and Krammer 2012; Sessler et al. 2013). Procaspase-8 is then activated by proximity-induced autocleavage (Dickens et al. 2012). In intrinsic apoptosis the essential step is MOMP, mitochondrial outer membrane permeabilization, which releases cytochrome c and Smac/DIABLO (Avi Ashkenazi 2008). Cytochrome c forms apoptosome, an activator of caspase-9, with protein apoptotic protease activating factor 1 (Apaf-1), while Smac promotes apoptotic signaling cascade (Baliga and Kumar 2003). MOMP is the link between the extrinsic and intrinsic apoptotic pathways. It initiates intrinsic apoptosis, while also serving as a signal augmentation step in extrinsic apoptosis in some cell types (known as type II cells). In these cells, activated caspase-8 released from DISC is not sufficient to directly trigger cell death, and MOMP is necessary to overcome the anti-apoptotic threshold (Jost et al. 2009; Aldridge et al. 2011).

Given its central role in maintaining homeostatic balance in multiple organs and systems, too much or too little apoptosis can both have serious pathological consequences. In neural degenerative disorders, for example, apoptosis is the underlying mechanism of death in neuronal cells (Agostini, Tucci, and Melino 2011). In Alzheimer's disease, caspase-3 and -6 trigger early synaptic dysfunction (Avila 2010; D'Amelio et al. 2011), and it has been shown that neuritic beading—a pathological condition of focal swelling in the dendrites and axons in Alzheimer's patients—is also caspase-6-dependent (Sivananthan et al. 2010). And in the immune system, infected or damaged cells are killed by cytotoxic T cells and natural killer cells via apoptosis. Apoptosis also helps to eliminate immature T cells that are either nonfunctional or which target self-antigens during positive and negative selection in the thymus. Lack of

apoptosis can lead to autoimmune diseases arising from over-production of autoreactive T cells. For example, Fas-mediated activation-induced cell death (AICD) curtails the immune response after the stimulus is removed and promotes immune privilege in certain tissues, and deficiency in AICD has been shown to contribute to the autoimmune disease systemic lupus erythematosus (Cheng et al. 1994). In contrast, induction of excessive apoptosis in active immune cells is one of the hallmarks of human immunodeficiency virus (HIV) infection, and directly leads to systematic immune deficiency (Rothen et al. 1997). In immune-mediated cardiomyopathy, both mitochondria- and receptor (TNF- α and Fas)-mediated apoptosis contribute to the loss of cardiomyocytes (Doyama et al. 1996; Ishiyama et al. 1998; Twu et al. 2002; Kim and Kang 2010).

Evasion of apoptosis is an essential step in cancer development, and numerous studies have focused on the mechanisms underlying this phenomenon. Unlike healthy cells, cancer cells are routinely subject to various stressful stimuli such as DNA damage, hypoxia, reactive oxygen species (ROS) stress, and glutamine starvation. In order to survive under such conditions, the apoptotic pathways in cancer cells are usually altered at both the transcriptional and post-translational levels (Wong 2011). For example, anti-apoptotic proteins like BCL-2, BCL-XL, MCL-1, cIAPs, and XIAP are commonly up-regulated in tumor cells, while pro-apoptotic proteins like the DRs, caspases, APAF-1, BAX, BIM, PUMA, and NOXA are down-regulated (Letai 2017).

1.1.2 The TRAIL-induced extrinsic apoptotic pathway

In 1995, TRAIL, also known as Apo2L, was identified by the Human Genome Project due to its sequence similarity to the extracellular domain of TNF and FAS/Apo1, a TNF family protein expressed by cytotoxic T lymphocytes (Wiley et al. 1995; Pitti et al. 1996). Like other members of the TNF superfamily, TRAIL is a type II transmembrane protein that exists as preformed trimers (Idriss and Naismith 2000). Shortly after the discovery of TRAIL, genomic sequencing identified its two functional receptors: DR4/TRAIL-R1 and DR5/TRAIL-R2, as well as three non-functional decoy receptors: decoy receptor 1 (DcR1, also called TRAIL-R3), decoy receptor 2 (DcR2, also called TRAIL-R4) and the soluble osteoprotegerin protein (OPG) (Pan et al. 1997; H. Walczak et al. 1997; Wagner et al. 2007). By activating DR4 and/or DR5, TRAIL triggers the onset of cell death via caspase-mediated apoptosis.

TRAIL-mediated apoptosis occurs through a signal transduction cascade that involves multiple players (Figure 1.1). The signaling process starts with the trimerization of DR4 and DR5 receptors, induced by the binding of TRAIL or TRAIL-like molecules, and the formation of DISCs (Kaufmann and Earnshaw 2000). These complexes assemble at the cytoplasmic tails of DR proteins and contain important apoptotic proteins such as FADD and FLIP. The most critical component of the DISC is caspase-8, the initiator caspase, which triggers downstream signaling upon auto-cleavage. In type I cells, the cleavage of procaspase-3 by caspase-8 is sufficient to induce cell death (Ozören and El-Deiry 2002). In type II cells, apoptosis requires MOMP before cell death

can occur (Ozören and El-Deiry 2002; Rudner et al. 2005), and this process is controlled by the BCL-2 protein family, which are described in more detail below.

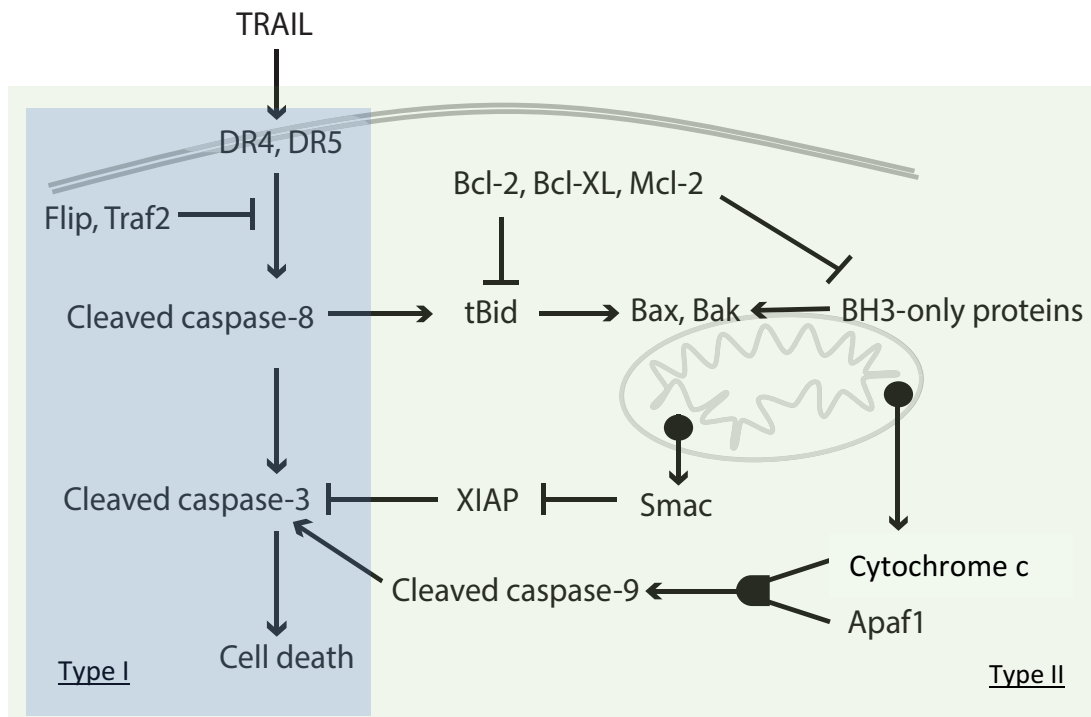


Figure 1.1 Schematic diagram of TRAIL-mediated apoptotic signaling cascade. Blue box highlights the apoptotic singling pathway of type I cells, green box highlights the additional components in the signaling pathway of type II cells.

In TRAIL-induced apoptosis, the initiator caspases cleave the BCL-2 family protein BID to generate a truncated form of this protein (tBID) that promotes oligomerization of two other BCL-2 proteins, BAX and BAK. BAX/BAK oligomers form pores in the mitochondrial outer membrane, which results in the release of mitochondrial inter-membrane space proteins including SMAC and cytochrome c, which promote caspase-3 activation (Tait et al. 2010). Cell death is believed to be inevitable after MOMP, with

rare exceptions in proliferating cells and some types of neurons where caspase activity is inhibited (Gama et al. 2014; Wright et al. 2004; Colell et al. 2007).

1.1.3 Regulation of TRAIL induced apoptosis

FLIP is an important apoptotic regulator within the DISC complex, which has three isoforms: FLIP-L (55 kDa), FLIP-S (27 kDa), and FLIP-R (25 kDa) (Thome et al. 1997; Irmeler et al. 1997). Like pro-caspase-8, these proteins have two death-effector domains (DEDs) at their N-termini, which are responsible for recruiting proteins to DISC (Li et al. 2006). Since the shorter FLIP-S and FLIP-R isoforms do not contain caspase-like domains in their C-termini, they are catalytically dead and inhibit pro-caspase-8 activation by blocking its auto-cleavage. FLIP-L, however, contains this additional caspase-like domain, and one study has suggested that FLIP-L may have a pro-apoptotic role under conditions of strong receptor stimulation or high levels of FLIP-S and FLIP-R (Fricker et al. 2010).

The decoy receptors DcR1, DcR2, and OPG also play a major role in the regulation of DISC assembly and caspase-8 activation. Since these receptors lack the conserved DD that recruits downstream proteins, they exhibit no catalytic activity (Pan et al. 1997; H. Walczak et al. 1997; Wagner et al. 2007). However, DcR1 and DcR2 can still bind to TRAIL and thus compete with and inhibit the activation of DR4 and DR5. It is unclear if OPG has a similar effect because of its lower binding affinity to TRAIL at physiological temperature (Emery et al. 1998; Pritzker, Scatena, and Giachelli 2004).

The BCL-2 family has more than fifteen members (Ryan and Letai 2013), which can be classified as pro-apoptotic or anti-apoptotic, and these collectively determine whether MOMP induction occurs (Itoh, Tsujimoto, and Nagata 1993, 2; Reed 1997; Davids and Letai 2012). The pro-apoptotic BCL-2 proteins include the BH1/2/3 multidomain effector proteins BAK and BAX, which directly trigger the permeabilization of mitochondria; the BH3-only activator proteins BID and BIM, which activate BAK and BAX; and the BH3-only sensitizer proteins BAD, BIK, HRK, BMF, PUMA and NOXA, which in turn inhibit the anti-apoptotic multidomain BCL-2 family members BCL-2, BCL-XL, MCL1, BCL-W, and BFL-1 (Taylor, Cullen, and Martin 2008). The overall balance between pro- and anti-apoptotic BCL-2 family proteins sets a threshold for cell fate, which establishes apoptotic 'priming' conditions, and this can be measured by BH3 profiling (Fraser, Ryan, and Sarosiek 2019).

IAPs are another family of negative regulators of TRAIL-induced apoptosis. The members of this family all have a conserved baculovirus IAP repeat (BIR) domain that can bind to the active site of caspases, and thereby either inhibit or promote their degradation (LaCasse et al. 2008). There are eight known members in the IAP family: neuronal apoptosis inhibitory protein (NAIP), cellular IAP1 (cIAP1), cellular IAP2 (cIAP2), XIAP, survivin, BRUCE/apollon, livin, and testis-specific IAP (Ts-IAP) (Owens et al. 2013). XIAP was shown to be able to bind and inhibit caspases via its conserved BIR domain, and can also ubiquitinate caspases via its E3 ubiquitin ligase domain, leading to their degradation (Morizane et al. 2005; Galbán and Duckett 2010). The cIAPs, on the other hand, only ubiquitinate caspases but do not inhibit them (H. k Huang et al. 2000; Eckelman and Salvesen 2006). Survivin binds to and inhibits

procaspase-9 and prevents it from forming the apoptosome with APAF-1 (Marusawa et al. 2003). In type II cells, high levels of IAPs, particularly XIAP, bind to and ubiquitinate caspases and thereby prevent cell death (Ozören and El-Deiry 2002; Rudner et al. 2005; Jost et al. 2009; Aldridge et al. 2011). As a consequence, the IAP inhibitor SMAC must first be released from the mitochondrial inter-membrane space via the induction of MOMP in order to induce apoptosis (Jost et al. 2009; Aldridge et al. 2011).

1.2 Cancer therapies targeting the TRAIL-induced apoptosis pathway

1.2.1 Apoptosis evasion in cancer cells

Cancer cells frequently face stressful stimuli such as DNA damage, cytokine deprivation, oxidative stress, and nutrient starvation, which usually trigger cell death in normal cells. Suppression of the apoptotic response is therefore essential to the survival of cancer cells. There are five general mechanisms underlying evasion of apoptosis in cancer cells: overexpression of anti-apoptotic BCL-2 family proteins, mutation or down-regulation of pro-apoptotic BCL-2 family genes and proteins, overexpression of IAPs, down-regulation of DR proteins, and decreased expression of caspases.

BCL-2 was one of the first apoptosis-related proteins found to be overexpressed in cancer cells. In 1986, Cleary, Smith, and Sklar found that the translocation of BCL-2 increased its expression in human acute lymphoblastic leukemia cells (Cleary, Smith, and Sklar 1986). BCL-2 overexpression has since been identified in multiple cancer types as protecting tumor cells from apoptosis. For example, BCL-2 was found to be expressed at high levels in neoplastic human prostate tissues, whereas this protein was

not expressed in normal prostatic cells (Raffo et al. 1995). In another study, overexpression of Bcl-2 was shown to confer protection against apoptosis in neuroblastoma, glioblastoma or breast carcinoma cell lines (Simone Fulda, Meyer, and Debatin 2002). BCL-2 overexpression has also been reported in various other cancers such as lymphoma, leukemia, melanoma, renal, and lung cancer (Miyashita and Reed 1993; Allsopp et al. 1993; Levine et al. 1993; Reed 1994). Similarly, BCL-XL amplification was later found in non-small cell lung cancer, pancreatic cancer, giant-cell tumor of bone, breast cancer, and ovarian cancer (Tonon et al. 2005; Smith et al. 2006; Beroukhim et al. 2010).

MCL-1 is another important anti-apoptotic BCL-2 protein that is overexpressed in many cancers, such as multiple myeloma, breast, and lung cancer (Wuillème-Toumi et al. 2005; Kendall et al. 2007; Beroukhim et al. 2010; De Blasio, Vento, and Di Fiore 2018). Under pro-survival conditions, MCL-1 is transactivated by the cAMP response element-binding (CREB) and signal transducer and activator of transcription 3 (STAT3) transcription factors (Wang et al. 1999; H. Liu et al. 2003), while under stressed conditions, MCL-1 is down-regulated by E2F1 (Croxtton et al. 2002). Studies have suggested that the normal regulation of MCL-1 by CREB and STAT3 may be disrupted in cancer cells via the PI3K/AKT pathway (Wang et al. 1999; H. M. Huang, Huang, and Yen 2000; Mills et al. 2008; Mohammad et al. 2015).

In contrast, the pro-apoptotic BCL-2 family proteins are often down-regulated in cancer cells. Decreased functional BAX levels have been found in many cancer types, arising either from gene mutation or transcriptional down-regulation. Frameshift and

point mutations in the *Bax* gene have been reported in an array of cancers, such as colorectal cancer, gastric cancer, breast cancer, lung cancer, renal cancer, glioblastoma, and melanoma (Pepper, Hoy, and Bentley 1997; Goolsby et al. 2005; Miquel et al. 2005; Ionov et al. 2000; Kholoussi et al. 2014; M. Zhang et al. 2016; Passante et al. 2013; Sjöblom et al. 2006). On the other hand, *Bax* is transactivated by P53 (Miyashita and Reed 1995), a well-known tumor suppressor that is mutated or inactivated in more than 50% of human cancers (Donehower et al. 1992).

In addition to BAX, P53 is also responsible for the activation many other pro-death BCL-2 genes; thus, the loss of P53 lowers the ratio of pro- versus anti-apoptotic BCL-2 proteins in cancer cells. Other pro-apoptotic BCL-2 proteins known to be directly induced by P53 include PUMA, NOXA, and potentially BID, and it has been shown that deficiency in these proteins contributes to resistance to apoptosis in cancer cells lacking P53 (Oda et al. 2000; Nakano and Vousden 2001; Sax et al. 2002; Yu et al. 2003). Loss of function of forkhead box O (FOXO) transcription factors also contributes to the imbalance between pro-and anti-apoptotic BCL-2 proteins by decreasing the expression of BIM (potentially via the ERK pathway) and PUMA (via the PI3K-AKT pathway) (Stahl et al. 2002; Sunter et al. 2003; You et al. 2006; Bean et al. 2013).

As their name indicates, IAPs are another important family of proteins that prevent cells from undergoing apoptosis and often contribute to the survival of cancer cells. For example, XIAP overexpression was found as a resistance biomarker in cervical cancer, rectal cancer, and acute myeloid leukemia (S. Liu et al. 2013; Tamm et al. 2004; Moussata et al. 2012). Together with another IAP protein, survivin, XIAP overexpression

also promotes resistance to apoptotic stimuli in non-small cell lung cancer (Krepela et al. 2009). Overexpression of cIAP2, survivin, livin, and XIAP has been observed in pancreatic cancer patient biopsies, where the amount of cIAP2 correlated most significantly with a multi-drug resistant phenotype (Lopes et al. 2007).

The functional death receptors DR4 and DR5 are often overexpressed in tumors, providing a rationale for targeting receptor-mediated apoptosis in cancer, but TRAIL resistance can sometimes arise from the loss or downregulation of these receptors. Deletion of DR4 and DR5 genes has been found in metastatic breast cancer patients, and DR5 deficiency has also been observed in colorectal cancer patients positive for human papillomavirus infection (Shin et al. 2001; Karbasi et al. 2015). In the colorectal cancer cell line SW480, a TRAIL-resistant subpopulation exhibited a deficiency in the transport of DRs to the cell surface (Yuneva et al. 2007). Cancer cells can also evade apoptosis by altering the production of decoy receptors, which compete with DR4 and DR5 for ligands but cannot trigger the apoptotic cascade due to their lack of a functional death domain (Jin et al. 2004; Fulda 2010). For example, in primary tumors of the gastrointestinal tract, decoy receptor DcR1/TRAIL-R3 was found to be overexpressed, potentially mediated by loss of function in P53 (Sheikh et al. 1999).

Compared with other mechanisms, reduced caspase activity is less common in cancer. Devarajan et al. found that caspase-3 levels were either undetectable or greatly reduced in breast, ovarian, and cervical cancer biopsies compared to healthy tissue (Devarajan et al. 2002). Caspase-1 and -3 were also reported to be expressed at lower levels in prostate cancer (Winter et al. 2001). Additionally, downregulation of caspase-9

was found in patients with stage 2 colorectal cancer, and this was significantly correlated with poor clinical outcome in these patients (Shen et al. 2010). Reduced expression of initiator caspase-8 and caspase-10 has also been observed in choriocarcinoma cell lines (Fong et al. 2006).

Given these various anti-apoptotic mechanisms exploited by cancer cells, multiple families of therapeutic agents have been developed that target the TRAIL pathway, with the aim of specifically inducing apoptosis in cancer cells as a means to achieve tumor clearance. However resistance is still a problem and companies like Merrimack Pharmaceuticals have been trying to find potential biomarkers for resistance or sensitivity.

1.2.2 DR agonists: TRAIL, Apomab, mapatumumab, conatumumab and new molecules

The molecules designed to bind and activate DR4 and DR5 can be grouped into two categories: TRAIL-like molecules and humanized DR antibodies. Dulanermin is the recombinant form of human TRAIL. To date, dulanermin has been tested in combination with chemotherapy agents like paclitaxel, carboplatin, and bevacizumab in patients with colorectal cancer and non-small cell lung cancer (Quintavalle and Condorelli 2012). However, the combination of dulanermin with these agents did not produce superior anti-tumor activity relative to chemotherapy-only control groups (Herbst et al. 2010; Soria et al. 2010, 2011; Lim et al. 2013; Wainberg et al. 2013).

Recent research has also focused on engineering TRAIL-like molecules that achieve longer half-life and higher cell-killing effect than the endogenous protein. T191

and T202 are two pre-clinical stage molecules developed by Merrimack Pharmaceuticals. These fusion polypeptides consist of an IgG1 Fc region followed by two successive single-chain TRAIL trimers with specific point mutations (see next chapter). It has been shown that both T191 and T202 increase apoptotic activity in several cancer cell lines compared to recombinant human TRAIL and agonistic antibodies of DR4 and DR5 (unpublished data). In addition, these molecules induce apoptosis in TRAIL-resistant cancer cell lines SKU-LU-1 and PANC-1 (unpublished data). AbbVie has also developed an engineered TRAIL-like molecule ABBV-621 by linking two single-chain TRAIL binding-domain trimers to a human IgG1 Fc arm (Morgan-Lappe 2017).

Several therapeutic DR human monoclonal antibodies have also been developed in the hope that binding to the DRs will effectively trigger cell death in cancer cells. The most well-studied DR antibodies include mapatumumab, a DR4 agonist; Apomab, a DR5 agonist; and conatumumab (AMG655), a new DR5 agonist that also has a synergistic effect with TRAIL due to a proposed clustering effect (Graves, 2014). However, none of these therapeutic antibodies has exhibited meaningful efficacy in phase II trials. More recently, Medimmune reported a new multivalent DR5 agonist, MEDI3039, which has been shown to be able to kill multiple breast cancer cell lines, suppress outgrowth of surface and microscopic metastases, and to extend animal survival (Greer et al. 2019; Swers et al. 2013).

1.2.3 BCL-2 inhibitors – ABT199, ABT263 and S63845

The onset of MOMP is determined by the interaction between pro- and anti-apoptotic proteins. Thus, one strategy to induce apoptosis is by antagonizing anti-apoptotic BCL-2 family proteins. Several small-molecule drugs have been developed and tested in clinical trials; among the most advanced are ABT199 (Venclexta) and ABT263 (Navitoclax). Both drugs belong to a series of BH3-mimetic small molecules developed by AbbVie, and were designed to inhibit the anti-apoptotic protein BCL-2. The difference is that ABT263 binds to both BCL-2 and BCL-XL, while ABT199 is a highly potent and selective inhibitor of BCL-2 only. Due to its off-target effects, ABT263 has been associated with dose-limiting effects of thrombocytopenia, resulting in less clinical activity comparing with ABT199.

ABT199 has been approved for chronic lymphocytic leukemia in patients bearing a chromosome 17p deletion in 2016, based on successful phase III testing of ABT199 in combination with rituximab (Rituxan) in relapsed or refractory chronic lymphocytic leukemia patients (ClinicalTrials.gov, trial ID: NCT01889186). In 2018, the same combination was also approved for chronic lymphocytic leukemia or small lymphocytic lymphoma patients who have received one prior therapy (ClinicalTrials.gov, trial ID: NCT02005471). Later in the same year, FDA approved ABT199 in combination with azacitidine or decitabine or low-dose cytarabine for the treatment of newly-diagnosed acute myeloid leukemia (AML) in adults who are age 75 years or older, or who have comorbidities that preclude use of intensive induction chemotherapy (www.fda.gov). ABT199 is now being tested in several ongoing phase I and phase II trials in leukemia, lymphoma, and multiple myeloma patients. ABT263 has been tested in various ongoing

phase I or phase II trials for leukemia, lung cancer, lymphoma, ovarian cancer, and prostate cancer (Cang et al. 2015). When combined with TRAIL, BCL-2 inhibitors have also shown a sensitizing effect in cell culture, suggesting a potential avenue for combination therapy in multiple cancer types (Hao et al. 2004; Simone Fulda 2018; Kang and Reynolds 2009).

MCL-1 is another important anti-apoptotic BCL-2 protein that is overexpressed in many cancers (De Blasio et al. 2018). In 2016, Servier discovered a potent and specific MCL-1 inhibitor, S63845, which can induce BAX/BAK-dependent selective killing in MCL-1-dependent cancer cells (Kotschy et al. 2016). A derivative of this molecule is now being tested in a Phase I clinical trial (ClinicalTrials.gov, trial ID: NCT02992483), which is currently recruiting patients with relapsed lymphoma or multiple myeloma.

1.2.4 SMAC mimetics – LCL161 and birinipant

Given the IAPs' role in inhibiting apoptosis in cancer cell, plus the observation that increased expression of IAPs often correlates with treatment resistance, therapeutic agents inhibiting IAPs have been regarded as another promising treatment option. There are two broad categories of IAP inhibitors: antisense oligonucleotides targeting IAP genes, and small molecule inhibitors mimicking endogenous IAP inhibitors like SMAC (Owens et al. 2013). The antisense oligonucleotides act by binding to and inducing RNAase-mediated degradation of IAP-encoding mRNA transcripts (Dean et al. 2009). The SMAC mimetics, on the other hand, are composed of the N-terminal sequence of SMAC and can bind to the BIR domains of IAPs, preventing them from inhibiting pro-apoptotic proteins (Fulda 2017).

However, many IAP inhibitor programs have been terminated after either phase I or II clinical trials. For example, a phase I trial of the antisense XIAP-targeting oligonucleotide AEG35156 in combination with carboplatin and paclitaxel in patients with advanced non-small cell lung cancer was terminated due to serious neurotoxicity (ClinicalTrials.gov, trial ID: NCT00558922). Serious adverse events were likewise observed in a phase II trial of the same molecule in combination with sorafenib in patients with advanced hepatocellular carcinoma (Lee et al. 2016). The survivin antisense oligonucleotide candidate LY2181308, developed by Eli Lilly, failed two phase II trials in patients with prostate cancer or non-small cell lung cancer due to no significant difference in response between treatment and control groups (Natale et al. 2014; Wiechno et al. 2014).

Several other drugs are now being actively investigated, including two SMAC mimetics, LCL161 and birinipant (TL32711). Other new IAP inhibitors currently being clinically tested include the cIAP1, cIAP2, and XIAP inhibitors DEBIO1143 (AT-406) and ASTX660 (DiPersio et al. 2015; Ward et al. 2018).

1.2.5 Sensitization by glutamine starvation in cancer cell lines

Since the discovery of the 'Warburg effect' in 1924 (Warburg, Wind, and Negelein 1927), numerous studies have shown distinct metabolic traits in tumor cells, including reliance on a high rate of glycolysis to maintain survival and growth, even in oxygen-sufficient conditions. Inhibition of glycolysis has been shown to be a potential cancer therapy both *in vitro* and *in vivo* (Granja et al. 2015). Interestingly, some cancer cell lines also show strong dependency on glutamine, despite the fact that glutamine is one

of the nonessential amino acids and can be synthesized from glucose (Wise and Thompson 2010). As a nitrogen donor, glutamine is required for the synthesis of nucleotides and other nonessential amino acids such as alanine, serine, and aspartate in rapidly-dividing cancer cells (Hensley et al. 2013). Another study has suggested that in addition to contributing to the production of nonessential amino acids, some glutamine is also transported out of the cell in exchange for the intake of essential amino acids (Nicklin et al. 2009). These essential amino acids contribute to protein synthesis, but also directly activate the mammalian target of rapamycin complex (mTORC) 1, a master regulator that promotes protein translation and growth in cancer cells (Nicklin et al. 2009). Moreover, the conversion of glutamine to α -ketoglutarate in mitochondria replenishes the mitochondrial carbon pool and provides NADPH, which in turn contributes to the maintenance of mitochondrial membrane integrity and potential, and macromolecular synthesis in the cell (Yang et al. 2014; Hensley et al. 2013). In 2000, Lobo et al. discovered that specific genetic inhibition of glutaminase slows tumor cell growth (Lobo et al. 2000), and in 2007, it was found that MYC overexpression may lead to glutamine addiction in cancer cells (Yuneva et al. 2007).

In keeping with the accumulating evidence that glutamine promotes cancer cell survival and growth, inhibition of glutamine metabolism has shown great potential as an anti-cancer therapy. CB-839 is a potent inhibitor that binds to both splice variants of glutaminase, which was developed by Calithera Biosciences in 2014. This molecule has shown anti-proliferative activity in triple-negative breast cancer cells (Gross et al. 2014), and is now being tested in phase Ib trials in patients with advanced/metastatic melanoma, non-small cell lung cancer, and renal cell carcinoma, and in phase II trials in

patients with triple-negative breast cancer and advanced solid tumors (ClinicalTrials.gov). The established chemotherapy agent L-asparaginase, which was originally designed to eliminate cancer cells by its asparagine hydrolysis activity, also induces apoptosis in acute lymphoblastic leukemia and acute myeloid leukemia by lowering glutamine levels in the intracellular environment (Samudio and Konopleva 2013; Sugimoto et al. 2015).

Given the effect of glutamine addiction in cancer cells, some studies have been conducted to investigate the crosstalk between glutamine metabolism and apoptosis. In one study, the authors compared cancer cells' response to etoposide and cisplatin under glutamine-starved conditions. They found that glutamine deprivation suppressed etoposide-induced apoptosis by downregulation of BID via the P53 pathway, but stimulated cisplatin-induced apoptosis by suppressing expression of FLIP-S via the AKT pathway (Valter et al. 2017). Jacque et al. showed that glutamine levels in acute myeloid leukemia cells control mitochondrial oxidative phosphorylation. Inhibition of the glutaminase isoform GLS-1 in these cells synergized with the BCL-2 inhibitor ABT-199 (Jacque et al. 2015). In the context of TRAIL-mediated apoptosis, depletion of glutamine sensitized previously-resistant melanoma cell lines to TRAIL, producing a reduction in MCL-1 levels and an increase in NOXA (Qin et al. 2010). Supplying additional glutamine in the media, on the other hand, was shown to protect human colonic epithelial cells against the effects of TRAIL (Evans et al. 2005). A more recent study showed that glutamine starvation sensitizes triple-negative breast cancer lines to TRAIL (Mauro-Lizcano and López-Rivas 2018). In this study, the authors argued that although the

amount of both DR5 and FLIP-L was altered after glutamine starvation, the reduction in FLIP-L was the major factor that drove this sensitization effect.

1.3 Cell fate decision and single-cell heterogeneity

1.3.1 Extrinsic apoptosis markers and measurement

There are varying assays to score the onset of extrinsic apoptosis in single cells or to measure apoptosis at the population level. Depending on the purpose of the experiment, measurement can be done in either fixed- or live-cell setups. In general, these methods work by monitoring the activity or changes in the main components in the apoptotic pathway. Common apoptosis scoring methods include detection of phosphatidylserine (PS) exposure, caspase activation, caspase substrate cleavage, mitochondrial protein release, nuclear condensation, DNA fragmentation, and membrane blebbing.

In normal cells, PS is located in the inner leaflet of the plasma membrane. In the process of apoptosis, when the cell membrane is disrupted, PS translocates to the outer membrane. Using fluorophore-conjugated annexin V, which preferentially binds to PS, the onset of apoptosis can be detected by the fluorophore signal. Caspase activation, on the other hand, can be detected based on the cleavage of caspase substrates. For example, in Promega's Caspase-Glo assay, the activation of certain types of caspases in the cell population can be measured by the induction of the luciferase reaction by cleaved luminogenic caspase substrates in the cell lysate. Cleaved caspase substrates can also be measured by western blot. At the single-cell level, microscopy or flow

cytometry can be employed with apoptotic cells marked using antibodies that specifically recognize their cleaved substrates or the active forms of these caspases. MOMP leads to the release of proteins that normally reside inside of the mitochondria, such as cytochrome C and SMAC, and thus the occurrence of apoptosis can also be determined by detecting these proteins in the cytosol via western blot or microscopy. At the end stages of apoptosis, phenotypes such as nuclear condensation and DNA membrane blebbing can be recognized by imaging. The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay can also be used to detect DNA fragmentation at the end stage of apoptosis, with the caveat that this assay also marks cells experiencing DNA damage for reasons other than apoptosis.

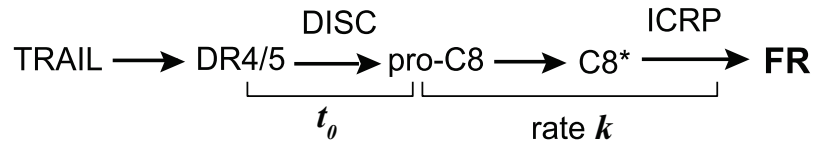
1.3.2 Fractional killing arising from single-cell caspase-8 threshold

In TRAIL-mediated apoptosis, it has been observed that even cells originating from the same clone have different cell fates upon TRAIL treatment, which is reflected at the population level as fractional killing. It has been shown that such cell-to-cell variability is due to naturally occurring differences in the levels or states of the proteins involved in the extrinsic apoptotic pathway in each cell. This process is multifactorial, and no single protein can predict the fate decision (Spencer et al. 2009; Gaudet et al. 2012; Aldridge et al. 2011).

However, in HeLa cells the caspase activity threshold can distinguish cells that survive from those that undergo apoptosis. For example, in HeLa cells, it has been shown that a threshold set by the rate of caspase-8 activation and the time to reach maximum caspase-8 activation rate plays a dominant role in determining cell fate in

receptor-induced apoptosis (Figure 1.2) (Roux et al. 2015). In this work, to characterize the features responsible for caspase-8 dynamics, a two-step model was developed to describe the simplified biochemical reactions underlying caspase-8 activation. The assumption is that the binding of DR4 and DR5 induces the assembly of DISC and activates caspase-8, which then cleaves a FRET (Förster resonance energy transfer)-based caspase-8 reporter, resulting in an increase in the readout variable, FRET ratio (1/FRET signal). With these assumptions, the FRET ratio can be modeled with a quadratic function dependent on time: $FR(t) = k \cdot (t - t_0)^2$. Caspase-8 activity or activation rate is the derivative of the quadratic function: $C8(t) \sim \frac{dFR(t)}{dt} = 2k \cdot (t - t_0)$, where k represents the rate of caspase-8 activation at DISC and t_0 is the time between the start of the treatment and the first detectable ICRP signal (Roux et al. 2015). One thing to note is that this model only captures the signal transduction process until maximum caspase-8 activity is reached, beyond which point the FRET signal may be affected by other biochemical reactions that are unaccounted for in this model, such as protein degradation and the activity of effector caspases.

A



B

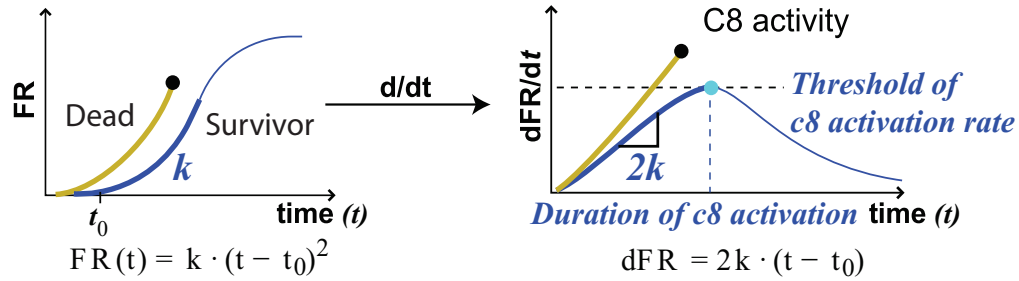


Figure 1.2 Caspase-8 dynamics separate surviving population from dying population in TRAIL-treated HeLa cells. (adapted from Roux et al. 2015).

A. Schematic representation of caspase-8 activation cascade and the cleavage of ICRP FRET probe (FR: FRET ratio). t_0 denotes the delay before caspase-8 is activated in DISC and k denotes caspase-8 activation rate.

B. After taking derivative of FR, a threshold of caspase-8 activation rate separates apoptotic cells (yellow) from surviving cells (blue).

1.3.3 Other factors controlling single-cell fate decision in TRAIL-induced apoptosis

More broadly speaking, given the natural variance in levels of different apoptotic proteins in different cancer cell lines, factors other than caspase-8 activity also contribute to the fate decision depending on the cell type. For example, feature-based sensitivity analysis with ordinary differential equation (ODE)-based mass-action ensemble extrinsic apoptosis reaction models (EARMs) showed that accurate prediction of the time of MOMP in individual cells requires knowledge of the levels of multiple proteins, where small changes in FLIP, MCL-2, and BCL-2 have a larger effect (Gaudet et al. 2012). Additionally, the amount of key proteins in the cells, such as pro-caspase-3

and XIAP, may have an impact on cell fate decision by pushing cancer cells towards either type I or type II behavior (Aldridge et al. 2011).

In my work here, I first characterized the caspase-8 dynamics in different cancer cell lines treated with two new molecules and found that caspase-8 activity plays an important role in cell fate decision in HeLa, DU145 and HT29 cells (Chapter 2). Next, I investigated sensitization mechanism of extrinsic apoptosis including glutamine starvation (Chapter 3), and co-treatment of bortezomib and LCL161 (Chapter 4).

**2. Chapter 2 - Caspase-8 dynamics separate apoptotic and surviving populations
in multiple cancer cell lines treated with TRAIL family agents**

2.1 Introduction

A heterogeneous response to TRAIL has been observed in various cancer cell lines, resulting in fractional cell death of different degrees (Flusberg and Sorger 2015). For example, in HeLa cells, under a saturating dose of TRAIL, only ~90% of HeLa cells receiving a saturating dose of TRAIL undergo apoptosis while ~10% survive (Roux et al. 2015). In a more resistant cell line like HCT116, more than 60% of cells die while the rest survive (Figure 2.2).

Multiple studies have identified factors contributing to TRAIL sensitivity in different cancer types. These include the abundance and modification of DR4 and DR5 (Wagner et al. 2007), death-inducing signaling complex (DISC) -associated proteins like caspase-8 and c-FLIP (Chen et al. 2012), and Bcl2 family proteins including BCL-2, BCL-XL, and XIAP (Passante et al. 2013). In HeLa cells, the apoptotic decision in individual cells can be predicted by the maximum caspase-8 activation rate, which can be derived from the dynamics of a caspase-8 reporter signal (Albeck et al. 2008; Roux et al. 2015). A two-step model can be used to describe the biochemical reactions happening during caspase-8 activation in which the binding of ligand to DR4/5 activates caspase-8 at DISC, and then caspase-8 cleaves itself together with the caspase-8 reporter (Roux et al. 2015).

Following on these findings, it would be valuable to measure live-cell caspase-8 activity in more cell lines, since different responses to TRAIL have been observed in various cancer cell lines. Chen et. al. (2012) have reported that among the 60 cancer cell lines in the NCI60 panel, which represent leukemia, melanoma, brain, ovarian,

breast, colon, kidney, and prostate cancers, 60% (35/60) of the cell lines are highly or moderately sensitive to TRAIL with a dose that inhibits 50% growth (GI_{50}) of less than 100 ng/mL, while the other 40% are resistant, with $GI_{50} > 100$ ng/mL (Chen et al. 2012).

In this study, I first characterized the caspase-8 dynamics induced by TRAIL and the synthetic TRAIL molecule; T191 and T202 in HeLa cells. I then tested whether the maximum caspase-8 activation rate and the two-step model proposed by our lab can still be applied to HeLa cells' response to the two new molecules. I extended the model to three other cell lines: DU145, HCT116, and HT29. The DU145 prostate cancer cell line was chosen because preliminary results in this cell line suggested that at the population level, accumulated caspase-8 activity may also be important for cell fate decision. The HCT116 and HT29 colorectal cancer cells were chosen because colorectal cancer is one of the main indications for which TRAIL-targeted therapy is an appealing option, due to a great unmet medical need and higher overall response rate to TRAIL (Tsikalakis et al. 2018).

2.2 Materials and methods

2.2.1 Cell lines and materials

Parental wildtype HeLa, DU145, HCT116, and HT29 cells were obtained from ATCC and cultured according to the manufacturer's instruction or common practice in lab:

HeLa cells were cultured in DMEM (Gibco, Montgomery County, MD), DU145 cells were cultured in EMEM (Gibco, Montgomery County, MD), and HCT116 and HT29 cells were cultured in RPMI (Gibco, Montgomery County, MD). All media were supplemented with

10% fetal bovine serum and penicillin/streptomycin (Gibco, Montgomery County, MD). For live-cell imaging, 5 mM L-glutamine was also added since the phenol red-free imaging media do not contain glutamine. The stable cell lines harboring initiator caspase activity reporter (ICRP) and mitochondrial outer membrane permeabilization (MOMP) reporter were derived as described in our lab's other publications (Roux et al. 2015; Albeck et al. 2008; Spencer et al. 2009). Hela cells overexpressing FLIP-L and FLIP-S were constructed by previous lab members (Roux et al. 2015). Recombinant human TRAIL was obtained from R&D Systems (Minneapolis, MN). T191 and T202 were gracious gifts from Merrimack Pharmaceuticals, Inc. (Cambridge, MA).

2.2.2 Dye-drop assay measurement of fraction of death

Cells were seeded 24 hours prior to treatment in 384-well plates by Multidrop Combi cell dispenser (Thermo Fisher Scientific). The seeding concentration is $1 - 1.5 \times 10^6/\text{mL}$ depending on cell types and the dispensing volume is 80 μL each well. Drug treatment were dispensed via HP D300 Digital Dispenser (Tecan, Mannedorf, Switzerland). After 15-hour treatment, the cells were stained with Live Dead Red (Life Technology, Carlsbad, CA) dye and Hoechst (Life Technology, Carlsbad, CA) in 10% Optiprep (Sigma, St. Louis, MO) in PBS for 30 minutes, and fixed with 3.7% formaldehyde solution in 20% Optiprep in PBS for 30 minutes. The plates were then washed with plate washer, aspirating all but 15ul from each well and dispensing 100ul of PBS. The plates were sealed afterwards and imaged. The image processing was performed in PerkinElmer's Columbus Image Data Storage and Analysis System.

2.2.3 Live-cell imaging and analysis

Clonal HeLa, DU145, HCT116 and HT29 cells expressing ICRP and MOMP reporters were seeded in 96-well plates coated with rat-tail collagen (BD, Franklin Lakes, NJ) 24 hours before imaging. All cell lines were cultured in the same types of media as described above, but without phenol red. The cells were pre-imaged for one hour before treatment and were continuously imaged for up to 12 hours in the live-cell chamber of an Operetta robotic microscope (Perkin Elmer, Waltham, MA) with either 3 minutes or 5 minutes time interval. Cell segmentation, tracking, intensity extraction, and cell death scoring were all done with in-house developed MATLAB (Natick, MA) codes.

2.2.4 Trace fitting and determining maximum caspase-8 activation rate thresholds

The averages of the FRET ratio from pre-imaged traces were first subtracted from the FRET ratio trace of each cells after treatment. The traces were then filtered by MATLAB *filtfilt* function with a moving window of 11 frames (33 minutes or 55 minutes) to reduce noise. Fitting of the traces was based on the model $FR(t) = k \cdot (t - t_0)^2$ where FR represents the FRET ratio, k represents caspase-8 activation rate and t_0 is the time between the start of the treatment and the first detectable ICRP signal (Roux et al. 2015). Traces that are too short (loss of tracking less than 30 min after treatment), with R^2 less than 0.5, or with a F-test p -value above 0.05 when comparing to a flat model, were discarded assuming these traces were results from other types of cell death.

Maximum caspase-8 activation rate threshold θ was determined using the error function:

$$Err(\theta) = \sum_{Surviving\ cells} f(Max(c8) - \theta) \cdot (Max(c8) - \theta) + \sum_{Dead\ cells} f(\theta - Max(c8)) \cdot (\theta - Max(c8))$$

, where f is the Heaviside function:

$$f(x) = \begin{cases} 0 & \text{if } x \leq 0 \\ 1 & \text{if } x > 0 \end{cases}$$

, where $c8$ represents the caspase-8 activation rate which was obtained from taking the derivatives using finite differences of the FRET ratios. By minimizing the error function, we can obtain the optimal thresholds (e.g. the red lines in Figure 2.4 and Figure 2.6) that best separate the surviving versus dying subpopulations.

2.3 Results

2.3.1 Engineered synthetic TRAIL molecules T191 and T202 induced a comparable or higher fraction of death in cancer cell lines relative to TRAIL

It has been shown that single-chain TRAIL trimer connected by peptide linkers can improve the plasma half-life of TRAIL (Schneider et al. 2010). The TRAIL group from Merrimack Pharmaceuticals converted a TRAIL trimer to single-chain polypeptide. The polypeptides were then fused to the carboxyl terminus of human IgG1 Fc to improve the pharmacokinetic properties such as thermostability and binding affinity. The resulting fusion protein was termed Fc-single chain TRAIL (Fc-scTRAIL) (Figure 2.1A). It has been shown that both T191 and T202 increase apoptotic activity in several cancer cell lines compared to recombinant human TRAIL and agonistic antibodies of DR4 and DR, and these molecules induce apoptosis in TRAIL-resistant cancer cell lines SKU-LU-1

and PANC-1 (unpublished data). This advantage is likely due to the fact that Fc-scTRAIL is homotrimeric. However, despite its increased activity, Fc-scTRAIL was not stable, with melting temperature (T_M) of 52 °C versus TRAIL's T_M of 70 °C.

Since lower thermostability often leads to shortened plasma half-life, several mutations were selected from a random mutagenesis library in order to improve the thermostability. This led to the identification of two clones: T191 has 5 mutations in each monomer (R103G/N228S/I247V/Y213W/S215D), and T202 has 3 mutations in each monomer (R103G/N228S/I247V) (Figure 2.1). These mutations greatly improved T_M and serum stability compared to Fc-scTRAIL (Table 1). Importantly, the three mutations also increased the binding affinity of T202 to DR4/5. The mechanism by which these mutations affect the properties of T191 and T202 is unclear, but it was speculated that

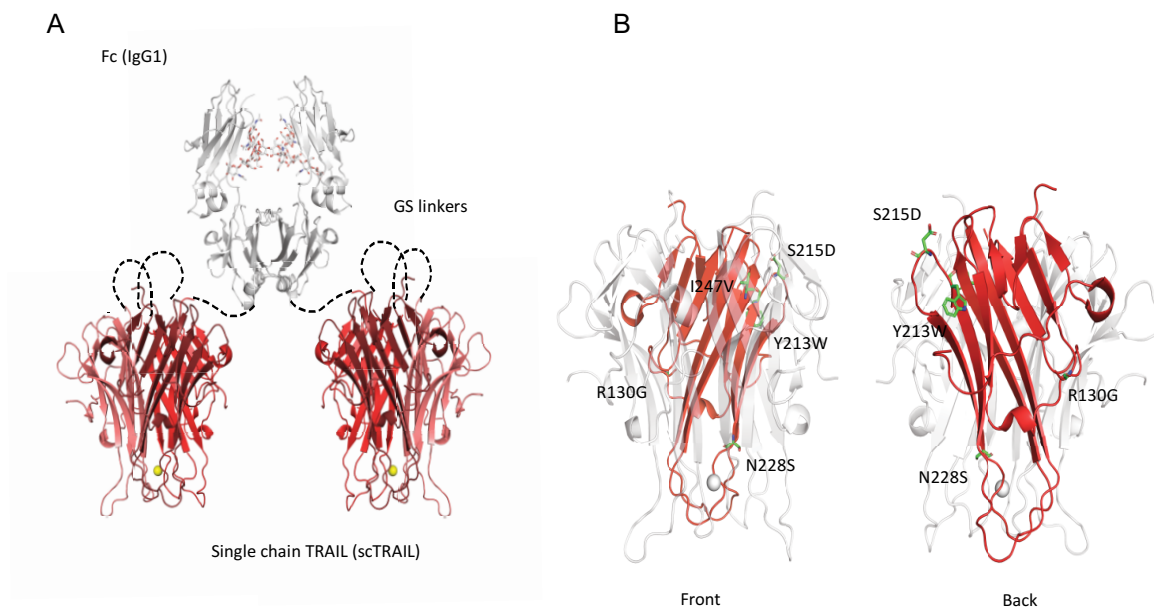


Figure 2.1 Engineered single chain TRAIL fusion proteins Fc-scTRAIL, T191 and T202
A. Format of the Fc-scTRAIL fusion protein
B. Representation of the mutations (see Table 1) introduced in TRAIL monomer domains to increase stability, expression, and DR binding

N288S and I247V could potentially stabilize the trimers since N228 is located close to C220, which is responsible for Zn coordination and I247 is close to the other trimer.

Table 1 Comparison of the mutations, T_M values, stability and DR-binding affinity of Fc-scTRAIL, T191 and T202.

	Fc-scTRAIL	T191	T202
Mutations	None	R103G/N228S/ I247/Y213W/S215D	R130G/N228S/ I247V
T _M (°C)	52	70.8	68.7
Fold loss after 7 days in serum	30-40	3.9	6.03
Binding to DR4/DR5 (nM)	8.8/4.8	11.0/9.0	3.7/2.9

To test the activity of the two molecules, I measured apoptosis in several cancer cell lines after treatment with TRAIL, T191, or T202 for 15 hours. As can be seen from Figure 2.2, in most (10/11) of the cell lines, T202 induced a higher fraction of cell death than TRAIL. T191, on the other hand, exhibited similar activity to TRAIL.

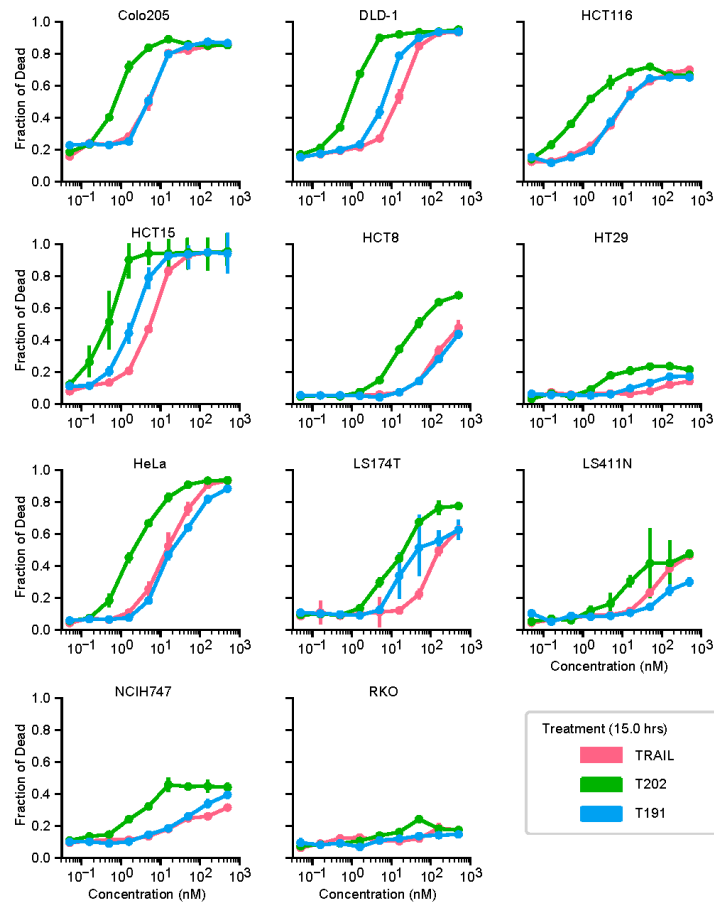


Figure 2.2 T202 triggered more cell death in multiple cancer cell lines. Dose-response curves of the indicated cancer cell lines treated with TRAIL (pink), T202 (green), or T191 (blue). Fraction of death was measured after 15 hours of treatment. Error bars represent the standard deviation of $n = 3$ or 5 technical replicates.

2.3.2 Compared to TRAIL, T191 induced similar caspase-8 activity and T202 induced stronger caspase-8 activity in HeLa cells

Following on these findings, I sought to find out if the cell killing effect of the new molecules comes from changes in DISC dynamics. Specifically, I examined caspase-8 activity, because according to Roux and Hafner (2015), cell-to-cell variability in

overcoming the caspase-8 activity threshold is an important determinant for fractional killing in HeLa cells.

Using ICRP FRET reporters, I visualized and measured this activity in individual cells by taking the inverse of the FRET signal ($1/\text{FRET}$) after the cleavage of the linker between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in the reporter by activated caspase-8. Figure 2.3A shows traces from two representative cells: the red trace was obtained from a cell with activated caspase-8 and the blue trace from a cell where no caspase-8 activity was observed. Side-by-side comparison of the single-cell and average level of $1/\text{FRET}$ signal from TRAIL-, T191- and T202-treated HeLa cells showed that in general, TRAIL and T191 triggered similar levels of caspase-8 activity, while T202 triggered higher caspase-8 activity (Figure 2.3B). T202 consistently induced considerably higher caspase-8 activity relative to the same dose of TRAIL and T191, especially in cells receiving lower doses of 2.5 nM or 1.3 nM treatment, as reflected in both the single-cell trace (blue curve) and population mean trace (red curve). For all three treatments, caspase-8 activity decreased in a dose-dependent fashion.

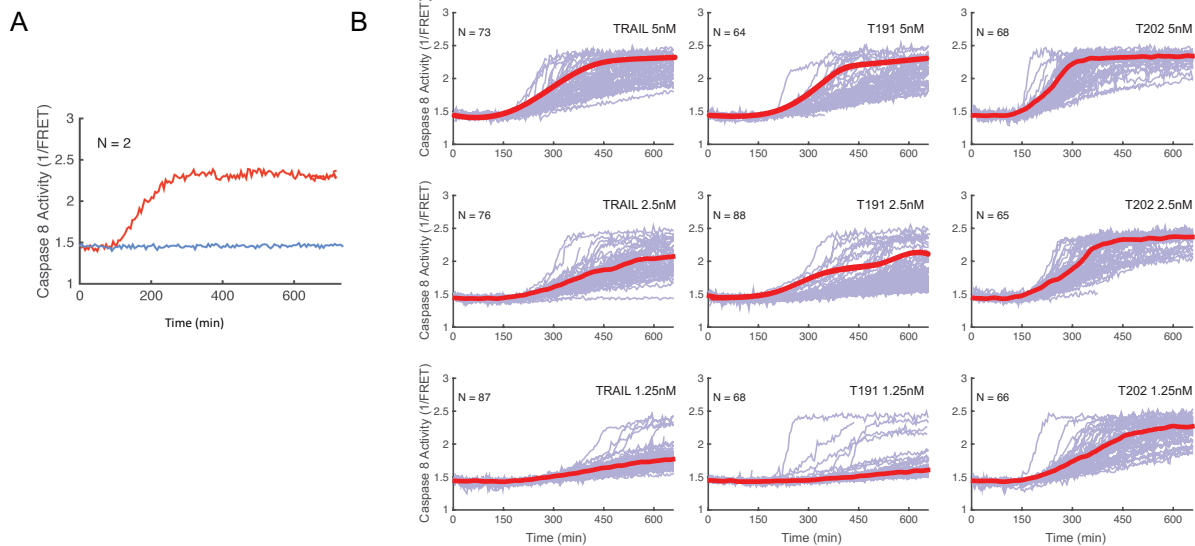


Figure 2.3 Single-cell caspase-8 activity measurements show that T202 induces stronger caspase-8 activity than recombinant TRAIL.

A. Representative ICRP FRET ratio traces of a HeLa cell with activated caspase-8 with ICRP cleavage (red trace) and a HeLa cell with no caspase-8 activity (blue trace).

B. caspase-8 activity traces of individual cells (blue traces) and the population average (red traces) with varying doses of TRAIL, T191, and T202 treatment.

2.3.3 Apoptotic decision in T191- and T202-treated HeLa cells can be separated by maximum caspase-8 activation rate

After measuring the response of caspase-8 activity induced by T191 and T202, the FRET ratio traces were analyzed to determine if the apoptotic population can be separated from the surviving population by the maximum caspase-8 activation rate k , which is the maximum of the derivative of the trace (see section 1.3.2).

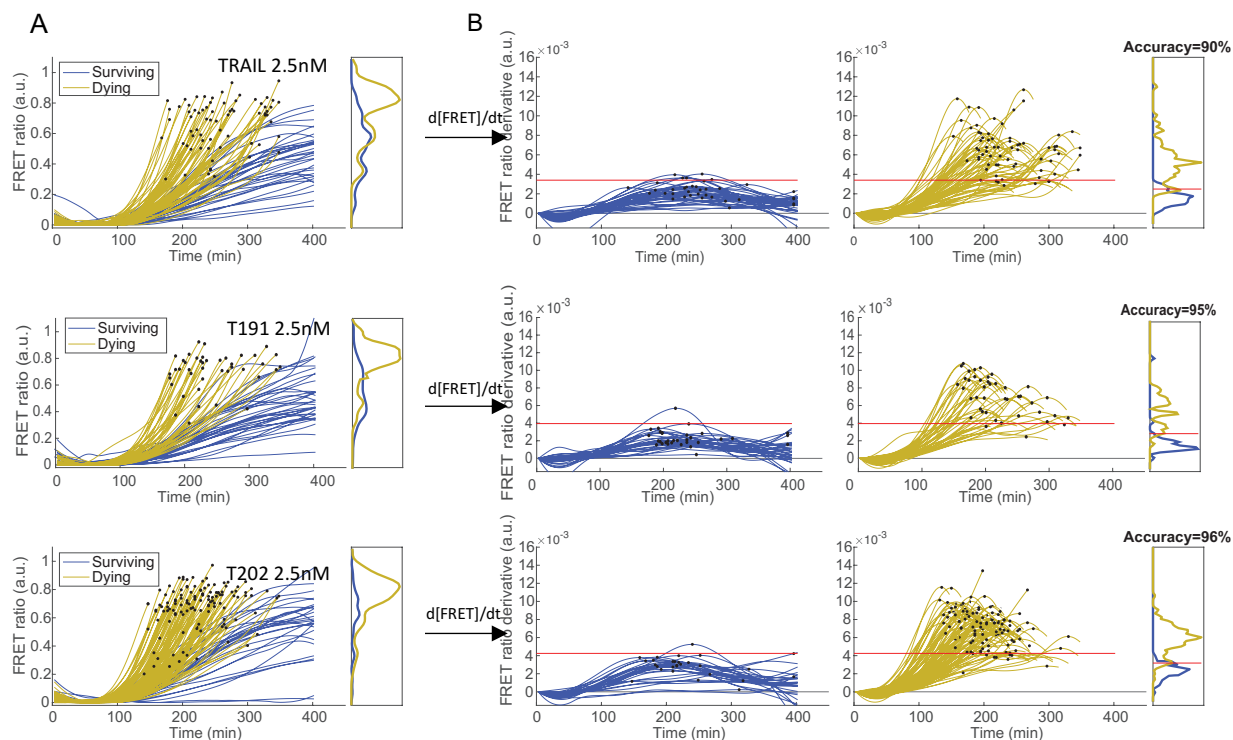


Figure 2.4 The apoptotic decision in HeLa cells treated with TRAIL, T191 or T202 can be predicted by their maximum caspase-8 activation rate.

A. FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) HeLa cells following treatment with 2.5 nM TRAIL, T191, and T202. Black dots indicated cell death, where traces were truncated. The right panel shows the distribution of maximal FRET ratio levels of the cells.

B. Derivative of the FRET ratio traces for surviving cells (blue, left panel) and dying cells (yellow, middle panel) for the same traces shown in A. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The right-most panel shows the distribution of maximal point values of the derivatives of the FRET ratio. The red lines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

It has been shown that after TRAIL treatment, the fate of HeLa cells can be determined based a threshold in the maximum caspase-8 activation rate, but not the level of accumulated caspase-8 activity (Roux, 2015). As is shown in Figure 2.4, similar to the case of TRAIL treatment, dying (yellow traces) and surviving populations (blue traces) cannot be distinguished by the raw FRET ratio, which indicates accumulated caspase-8 activity after T191 or T202 treatment (Figure 2.4A). After taking the derivatives of the raw FRET ratio signals, the surviving and dead cells can be separated

by thresholds of the maximum FRET derivatives (red line in the derivative plots), which represent the maximum caspase-8 activation rates. These thresholds were calculated separately by finding the optimal boundary that separates the surviving and dying cells after treatment with various molecules. The accuracy scores, defined as the number of true-positive classifications divided by the total number of samples, were $\geq 90\%$ in all three cases. In general, the accuracy scores of the maximum caspase-8 activation rate thresholds were $> 85\%$ at all doses of TRAIL, T191, and T202 tested in HeLa cells (see Appendix 6.1).

2.3.4 Increased doses of T191 and T202 pushed cells across the fate boundary by increasing caspase-8 activation rate

To further investigate changes in caspase-8 dynamics after treatment with T191 and T202, I used two-dimensional caspase-8 activity landscape plots to visualize τ and k values derived from single-cell traces, with boundary curves separating the dead and surviving populations.

The boundaries were calculated based on the two-step model of simplified caspase-8 activation process. As schematically described in Figure 2.5A, the assumption is that the binding of DR4 and DR5 induces the assembly of DISC and activates caspase-8. Then the activated caspase-8 cleaves the FRET reporter ICRP resulting in an increase in the inverse of FRET ratio ($1/\text{FRET}$). With these assumptions, FRET ratio can be modeled with a quadratic function that depends on time:

$$FR(t) = k \cdot (t - t_0)^2$$

, caspase-8 activity then is the derivative of the quadratic function:

$$C8(t) \sim \frac{dFR(t)}{dt} = 2k \cdot (t - t_0)$$

, where k represents the rate of the caspase-8 activation at DISC and t_0 is the time between the start of the treatment and the first detectable ICRP signal. As was shown in the previous section, HeLa cells treated with T191 and T202 undergo apoptosis when the maximum caspase-8 activation rate is above a threshold (denoted by θ). This boundary can be described as

$$\tau = \frac{\theta}{2k} + t_0.$$

In the two-dimensional landscape plots in Figure 2.5B, the red curves represent boundaries that separate the living and dying cell populations. The surviving cells fell on the left side of the boundaries with low caspase-8 activation rate k , and apoptotic cells fell on the right side of the boundaries with higher k . As is shown in these plots, the effect of increasing doses of T191 and T202 is to shift cells to the right side of the boundaries which represents a faster caspase-8 activation rate k . In the cases for both molecules however, the time to reach maximum caspase-8 activation rate τ also decreased with the increase in treatment doses which is at odd with the original model where shorter time to reach maximum caspase-8 activation rate duration leads to decrease in cell death. Here the shorter τ may be the outcome of the quick depletion of pro-caspase-8 resulted from the strong activation step. The two molecules may have

had such strong effect on the activation rate that the combined effect on the two axes still shifts the cells towards the apoptotic side of the landscape.

The boundary lines for T191 and T202 treatment groups were calculated separately, but the parameters θ and t_0 fell within a close range of values (table 2).

Table 2 Parameter comparison of the two step models fitted by FRET traces obtained from HeLa cells with TRAIL family molecules.

Cell line	Treatment	θ (a.u.)	t_0 (min)
HeLa	TRAIL	2.10×10^{-3}	33.42
	T191	2.47×10^{-3}	34.78
	T202	2.30×10^{-3}	34.20

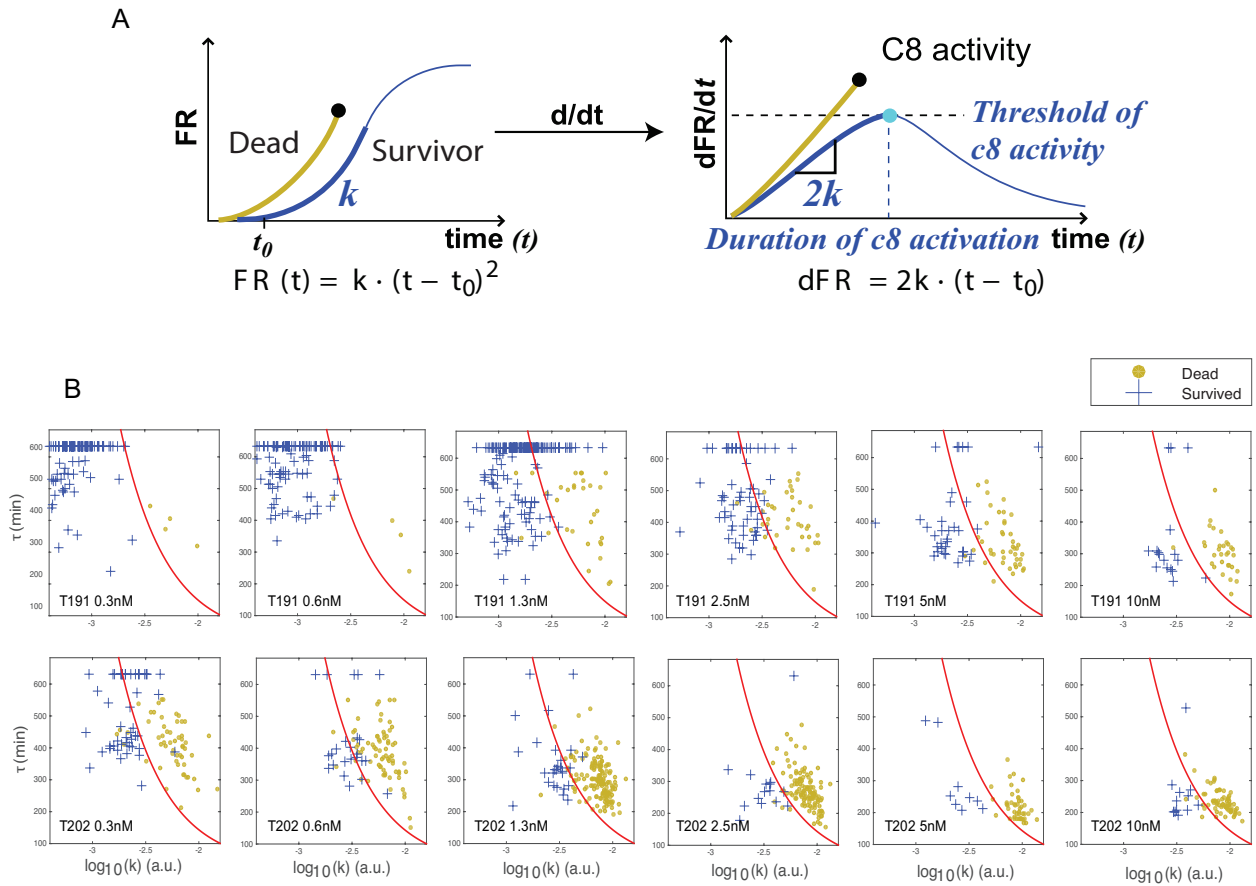


Figure 2.5 Increased doses of T191 and T202 pushed cells across the fate boundary by increasing caspase-8 activation rate

A. Schematic representation of caspase-8 activation cascade and the cleavage of ICPR FRET probe (FR: FRET ratio). t_0 denotes the delay before caspase-8 is activated in DISC and k denotes caspase-8 activation rate.

B. Caspase-8 landscape plots of HeLa cells after T191 and T202 treatment with varying doses. k represents caspase-8 activation rate and τ represents the time to reach maximum caspase-8 activation rate. Cells located at the top of the landscape with identical τ are the surviving individuals with increasing caspase-8 activity till the end of the experiment and thus have τ equal to the length of the experiment.

2.3.5 Maximum caspase-8 activation rates determine cell fate decision in TRAIL treated

DU145 and HT29 cells

One natural extension of the single-cell caspase-8 activity assay and two-step model is to apply the pipeline to cancer cell lines other than HeLa, measure their caspase-8 activity after TRAIL treatment, link it to the apoptotic decision and test whether maximum caspase-8 activation rate is still the major determinant of cell fate or not. To achieve this, DU145, HT29 and HCT116 stable cell lines harboring the FRET ICRP reporter and SMAC-mCherry MOMP reporters were constructed by me and previous members of the Sorger lab. I chose DU145 because preliminary results suggested that on the populational level, accumulated caspase-8 activity seemed to be also important for cell fate decision. HCT116 and HT29 colorectal cancer cells were chosen because colorectal cancer is one of the main indications for TRAIL, due to a great unmet medical needs and higher overall response.

As is shown in Figure 2.6A, DU145 cells exhibited similar single-cell caspase-8 dynamics after TRAIL treatment to HeLa cells. The dying (traces colored in yellow) and surviving populations (traces colored in blue) cannot be distinguished by raw FRET ratio traces which represent accumulated caspase-8 activity after TRAIL treatment. But after taking the derivatives of the raw FRET ratio signals, the surviving and dead cells can be separated by thresholds of the maximum FRET derivatives (red lines in the derivative plots), which represent the maximum caspase-8 activation rates. The accuracy scores of separating the two populations based on the thresholds of maximum caspase-8 activation rate were $\geq 89\%$ for all the three doses of TRAIL treatment. It should be noted that contrary to expectations from prior preliminary evidence, the single-cell caspase-8 activity measurements indicate that caspase-8 activation rate plays a more important role in determining cell fate than the accumulated caspase-8 activity. This could be due

to differences in assay choice and experimental setup. The earlier experiments used the Caspase-Glo 8 assay for caspase-8 activity measurement and scored apoptosis based on the fraction of death at the populational level, and thus lacked the granularity of single-cell level data.

In the two-dimensional landscape plots in Figure 2.6B where the red curves represent boundaries that separate the living and dying cell populations, TRAIL shifted DU145 cells to the lower-right corner of the landscape, representing a fast caspase-8 activation rate k and lower time to maximum caspase-8 activity τ . Although the shorter time to reach maximum caspase-8 activation rate may have a pro-survival effect, it could be that because of the strong caspase-8 activity, duration plays a minor role in terms of the commitment to apoptosis, or that fast caspase-8 activation depletes pro-caspase-8 quickly and thus results in a shorter time to reach maximum caspase-8 activation rate.

Similar to DU145, maximum caspase-8 activation rate separated surviving and dying population in HT29 cells with accuracy > 85% after TRAIL treatment (Figure 2.7). The lower accuracy scores suggest that factors other than caspase-8 activity may play an important role in determining cell fate. However, it needs to be noted that for HT29 cells, the live-cell caspase-8 experiments were performed under the glutamine-depleted sensitizing condition because HT29 cells are resistant to TRAIL under normal culture conditions and very few cells undergo apoptosis, thus making it impossible to analyze caspase-8 activity. The sensitizing effect of glutamine starvation on TRAIL will be further discussed in the next chapter.

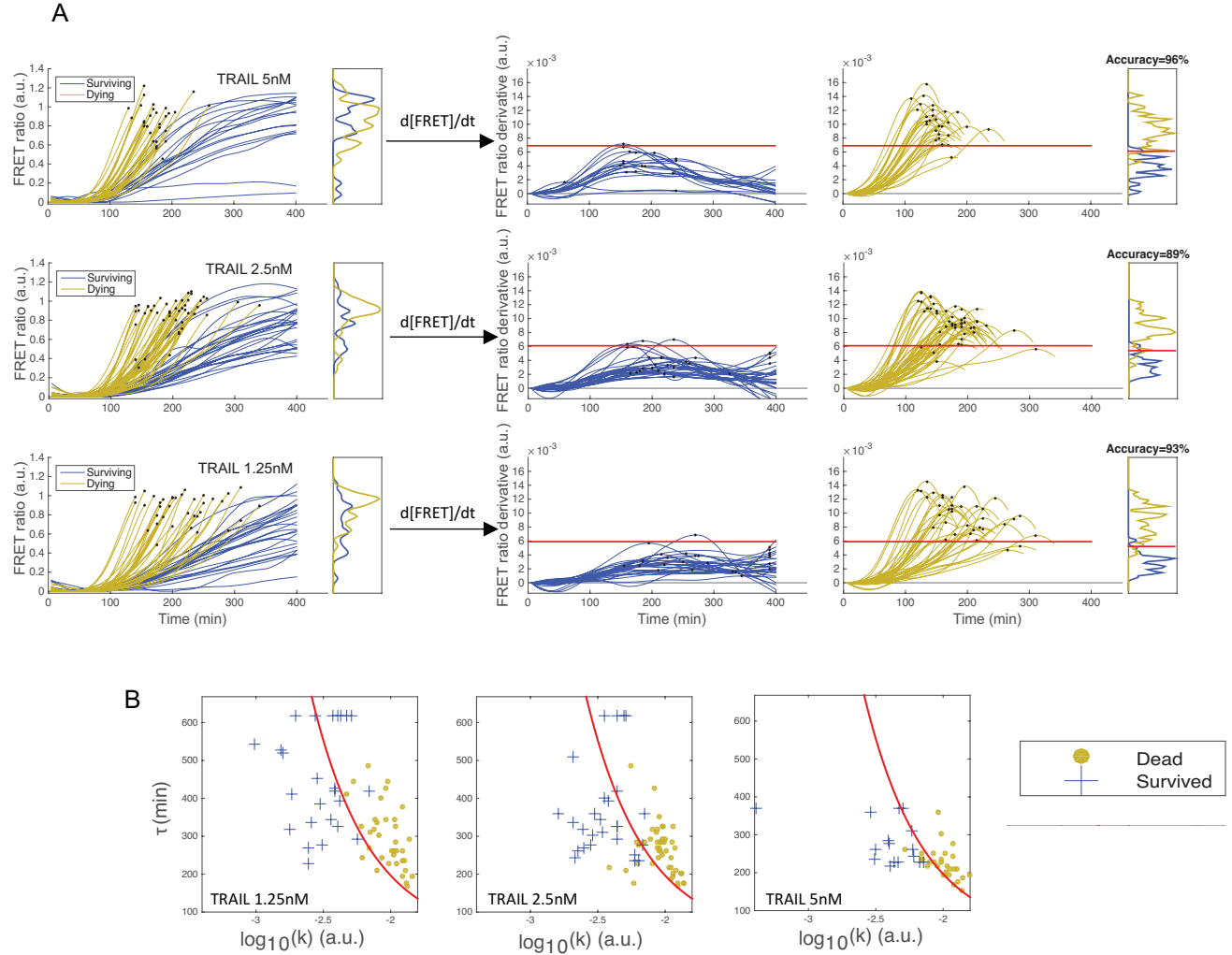


Figure 2.6 Maximum caspase-8 activation rates determine cell fate decision in TRAIL treated DU145 cells
A. Left panel: FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) DU145 cells following treatment with 5nM, 2.5nM and 1.25nM TRAIL. Black dots indicated cell death, where traces were truncated. The histograms show the distribution of maximal FRET ratio levels of the cells. Middle and right panels: derivative of the FRET ratio traces for surviving cells (blue, middle panel) and dying cells (yellow, right panel) for the same traces shown in left panel. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The histograms on the far right show the distribution of maximal point values of the derivatives of the FRET ratio. The redlines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

B. Caspase-8 landscape plots of DU145 cells after varying doses of TRAIL treatment. k represents caspase-8 activation rate and τ represents the time to reach maximum caspase-8 activation rate. Cells located at the top of the landscape with identical τ are the surviving individuals with increasing caspase-8 activity till the end of the experiment and thus have τ equal to the length of the experiment.

The boundary lines (red curves in Figure 2.6B and Figure 2.7B) that separated the dying and surviving subpopulations in the landscape were calculated based on the two-step model as described in the previous subchapter. As can be seen in Table 3, the fitted parameters θ and t_0 fell within a close range of values among the three cell lines. This suggested that these cells shared similar caspase-8 dynamics induced by TRAIL (HT29 cells in the sensitizing condition).

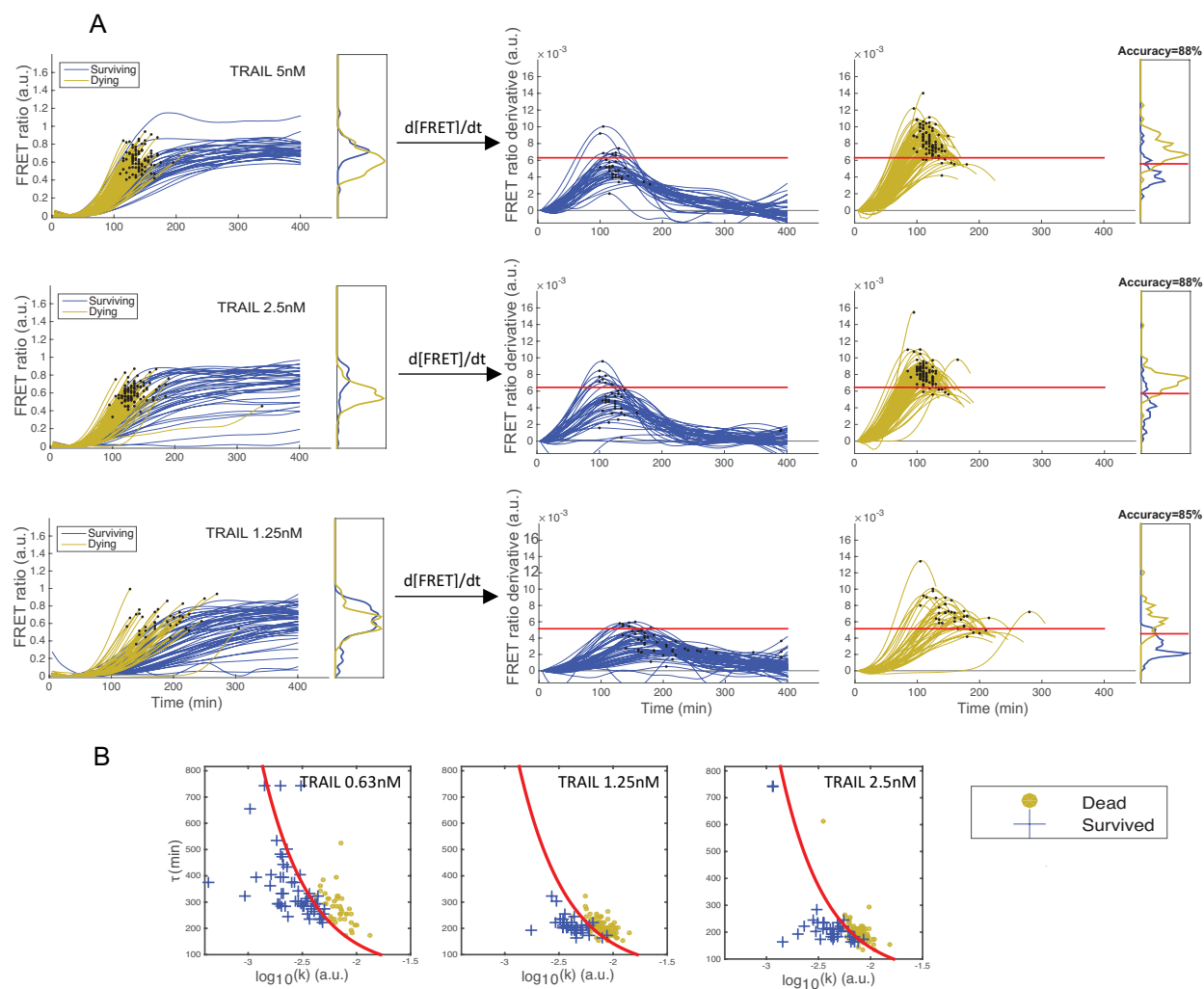


Figure 2.7 Maximum caspase-8 activation rates determine cell fate decision in TRAIL treated HT29 cells

in sensitizing condition (glutamine-depleted)

A. Left panel: FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) HT29 cells following treatment with 5nM, 2.5nM and 1.25nM TRAIL. Black dots indicated cell death, where traces were truncated. The histograms show the distribution of maximal FRET ratio levels of the cells. Middle and right panels: derivative of the FRET ratio traces for surviving cells (blue, middle panel) and dying cells (yellow, right panel) for the same traces shown in left panel. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The histograms on the far right show the distribution of maximal point values of the derivatives of the FRET ratio. The redlines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

B. Caspase-8 landscape plots of HT29 cells after varying doses of TRAIL treatment. k represents caspase-8 activation rate and τ represents the time to reach maximum caspase-8 activation rate. Cells located at the top of the landscape with identical τ are the surviving individuals with increasing caspase-8 activity till the end of the experiment and thus have τ equal to the length of the experiment.

Table 3 Parameter comparison of the two step models fitted by FRET traces obtained from HeLa, DU145 and HT29 cells treated TRAIL

Cell line	Treatment	θ (a.u.)	t_0 (min)
HeLa	TRAIL	2.10×10^{-3}	33.42
DU145	TRAIL	2.13×10^{-3}	37.36
HT29	TRAIL	2.02×10^{-3}	35.66

2.3.6 Maximum caspase-8 activation rate only partially separated HCT116 cells after TRAIL treatment

The same single-cell caspase-8 measurement assay was carried out in HCT116 cells with TRAIL treatment. However, as shown in Figure 2.8, neither accumulated caspase-8 activity or maximum caspase-8 activation rate can distinguish surviving HCT116 cells from cells committed to apoptosis. The maximum activation rate threshold failed to be predictive due to the presence of surviving cells with high caspase-8 activity and a high caspase-8 activation rate.

It seems that that there may be two subpopulations in the surviving HCT116 cells. In one population, there was minimal caspase-8 activity as the FRET ratio traces remained flat during the entire experiment. Meanwhile the other population showed caspase-8 activity very much comparable to the apoptotic cells, but without commitment to apoptosis. The lack of caspase-8 activity in the first subpopulation suggest that there may be a resistance mechanism at play in the DRs or DISC, such as a lack of functional DR4/5 or caspase-8 itself, or increased amounts of pro-survival components like FLIP

or TRAF2. In the second population, on the other hand, the resistance mechanism may come from the inhibition by BCL-2 protein family at the mitochondrial level, such that despite the presence of high levels of activated caspase-8, MOMP-blocking anti-apoptotic BCL-2 proteins still prevented these cells from undergoing apoptosis.

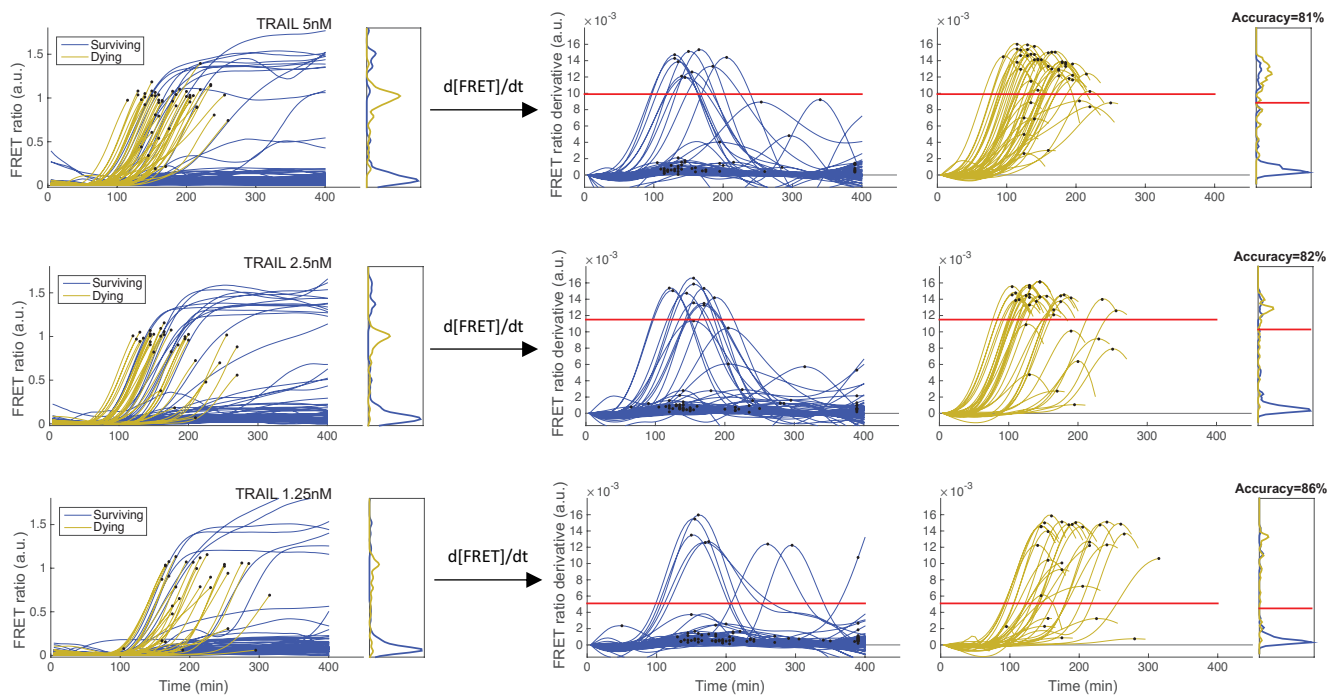


Figure 2.8 Maximum caspase-8 activation rate only partially separated HCT116 cells after TRAIL treatment
Left panel: FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) HCT116 cells following treatment with 5nM, 2.5nM and 1.25nM TRAIL. Black dots indicated cell death, where traces were truncated. The histograms show the distribution of maximal FRET ratio levels of the cells. Middle and right panels: derivative of the FRET ratio traces for surviving cells (blue, middle panel) and dying cells (yellow, right panel) for the same traces shown in left panel. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The histograms on the far right show the distribution of maximal point values of the derivatives of the FRET ratio. The redlines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

2.4 Conclusion

Motivated by the similar or superior potency of the new TRAIL-like molecules T191 and T202 in multiple cancer cell lines compared to TRAIL, I have characterized cancer cells' response to these agents. Since the two new molecules are essentially synthetic TRAIL homotrimers, my hypothesis was that their mechanism of action should be similar to that of TRAIL, and that the maximum caspase-8 action rate model can be extended to HeLa cells treated with the two new molecules. The results confirmed that HeLa cells' response to both molecules can indeed be predicted by the maximum caspase-8 rate with > 85% accuracy on average. This prediction accuracy is comparable to the 83%-86% accuracy obtained when this cell line was treated with TRAIL.

I then extended the model to three additional cell lines: DU145, HCT116 and HT29. All three lines stably express both the caspase-8 activity reporter ICRP and a MOMP reporter. These live-cell experiment results showed that cell fate decision in DU145 and HT29 cells treated with TRAIL can be separated by maximum caspase-8 activation rate. In HCT116 cells, factors other than caspase 8 activity may also play an important role in determining cell death.

2.5 Chapter Contributions

I have been fortunate enough to be able to collaborate with the TRAIL group from Merrimack Pharmaceuticals (Cambridge, MA): Eric Tam contributed Figure 2.1. T191 and T202 molecules used in this chapter and the following chapters are all generous gifts from Merrimack.

3. Chapter 3 – Caspase 8-dependent TRAIL sensitizing effect by glutamine starvation in cancer cells

3.1 Introduction

Although glucose has been in the center of cancer studies for decades, glutamine metabolism has only re-entered into the spotlight in recent years. Glutamine was identified as a major source for oxidative metabolism in lymphoma and HeLa cells in the 1970s (Reitzer, Wice, and Kennell 1979; Eagle 1955). In 2000, Lobo et al. discovered that specific genetic inhibition of glutaminase slows tumor cell growth (Lobo et al. 2000), and in 2007, it was found that MYC overexpression leads to glutamine addiction in cancer cells (Yuneva et al. 2007). In 2014 the first glutaminase inhibitor CB-839 was discovered by Calithera Biosciences and showed antitumor activity in triple negative breast cancer cells (Gross et al. 2014).

Despite the recent advancement in the knowledge of the importance of glutamine addiction and glutamine starvation's importance in cancer treatment, little is known about the crosstalk between glutamine metabolism and extrinsic apoptosis. In 2015, Jacque et al. reported that the glutaminase inhibitor CB-839 synergized with the BCL-2 inhibitor ABT199 in leukemia cells (Jacque et al. 2015). A more recent study showed that glutamine starvation sensitized the triple-negative breast cancer lines MDA-MB468 and MDA-MB231 to TRAIL, and the authors suggested the reduction of FLIP-L is responsible for this sensitization (Mauro-Lizcano and López-Rivas 2018).

I also observed that glutamine depletion sensitized certain cancer cell lines' response to TRAIL and T191, but not to BCL-2 and MCL-1 inhibitors, or intrinsic apoptosis-inducing agents. In this study, I explored the potential factors that may contribute to this sensitization effect. Live-cell caspase-8 measurements, and protein and RNA level

evidence suggested that the sensitization effect of glutamine starvation is caspase-8 activity-dependent, and may be due to the changes in FLIP and/or TRAF2 levels when glutamine is depleted from the environment.

3.2 Materials and methods

3.2.1 Cell lines and materials

Wildtype A549, CAL 51, CAL 85-1, DU145, HCC1954, HeLa, HT29, RKO cells were obtained from ATCC and cultured according to the manufacturer's instruction or common practice: CAL 51, CAL 85-1, and HeLa cells were cultured in DMEM (Gibco, Gaithersburg, MD), DU145 and RKO cells were cultured in EMEM (Gibco, Gaithersburg, MD), and A549, HCC1954, and HT29 cells were cultured in RMPI (Gibco, Gaithersburg, MD). All normal culturing media contain 5mL L-glutamine, and were supplemented with 10% fetal bovine serum, and penicillin/streptomycin (Gibco, Gaithersburg, MD). In glutamine depleted experiment, the same types of culturing media without pre-added glutamine were used.

3.2.2 Dye-drop assay measurement of fraction of death

Cells were seeded 24 hours prior to treatment in 384-well plates by Multidrop Combi cell dispenser (Thermo Fisher Scientific, Waltham, MA). The seeding concentration is $1 - 1.5 \times 10^6$ /mL depending on cell types and the dispensing volume is 80 μ L each well. Drugs were dispensed via HP D300 Digital Dispenser (Tecan, Mannedorf, Switzerland). After 15-hour incubation, the cells were stained with Live Dead Red (Life Technology, Carlsbad, CA) dye and Hoechst (Life Technology, Carlsbad, CA) in 10% Optiprep

(Sigma, St. Louis, MO) in PBS for 30 minutes, and fixed with 3.7% formaldehyde solution in 20% Optiprep in PBS for 30 minutes. The plates were then washed with plate washer, aspirating all but 15ul from each well and dispensing 100ul of PBS. The plates were sealed afterwards and imaged. The image processing was performed in PerkinElmer's Columbus Image Data Storage and Analysis System. The analysis was performed using in-house developed python code.

3.2.3 Immunoblotting

Cells were lysed in cell lysis buffer (Cell Signaling, Danvers, MA). Proteins were separated by SDS-PAGE using NuPAGE4-12% Bis-Tris mini gel, and transferred to Immobilon-P PVDF membrane (Millipore, Burlington, MA). The membranes were blocked with Odyssey Blocking Buffer and probed with primary antibodies: FLIP (human) monoclonal antibody (7F10) (Enzo Life Sciences, Farmingdale, NY), TRAF2 antibody (H-10) (Santa Cruz Biotechnology, Dallas, TX), Mcl-1 Antibody (22) (Santa Cruz Biotechnology), anti-GAPDH antibody [6C5] (Abcam, Cambridge, MA), and anti-clAP1 antibody [EPR4673] (Abcam). HRP-linked anti-rabbit IgG and anti-mouse IgG secondary antibodies and Immobilon Western Chemiluminescent HRP substrate (Millipore, Burlington, MA) were used for protein detection.

3.2.4 RNA extraction, RT-PCR and real-time PCR

Total RNA was extracted using miRNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Sequences of PCR primers are as follows: FLIP 5'/3': TTGCCCCAGATCAACTGGATT/

GCTGCTTGGAGAACATTCCTG, TRAF-2 5'/3': GTACTGCTCCTTCTGCCTGG/
GAACACAGGCAGCACAGTTC, Mcl-1 5'/3': GCATGCTTCGGAAACTGGAC/
GAGAGTCACAATCCTGCCCC, Actin 5'/3': CACCAACTGGGACGACAT/
ACAGCCTGGATAGCAACG, and Tubulin 5'/3': TTTTCAGCGAGACTGGCAATG/GTT
GGCTGCATCCTCCTTTC. RT-PCR was performed using PowerUp SYBR Green
Master Mix (Thermo Fisher Scientific, Waltham, MA).

3.3 Results

3.3.1 Glutamine starvation sensitizes cancer cell lines' response to TRAIL

I initially observed the sensitizing effect of glutamine starvation on TRAIL response when I was conducting live-cell experiments in HeLa cells. The imaging media was the same as normal DMEM media, except that it lacked phenol-red and glutamine. I found that culturing HeLa cells in the imaging media without pre-added glutamine for 24 hours did not affect their survival or cause any morphological changes, but significantly increased the fraction of cell death induced by TRAIL compared to cells cultured in normal DMEM with 5 mM L-glutamine.

I then tested the effect of glutamine starvation on TRAIL response in a variety of other cancer cell lines. As shown in Figure 3.1A, glutamine starvation can either increase the treatment's potency (i.e. lower IC_{50}), as is the case for HeLa cells, or increase the treatment's efficacy (i.e. higher E_{max}), as is the case for A549, CAL 85-1, DU145, HT29 and RKO cells. In the following sections, the cell lines that can be sensitized by glutamine starvation will be termed 'sensitive' cell lines. Among the 17 cancer cell lines I

tested, 8 showed an increase in TRAIL-induced cell death after glutamine starvation for 24 hours (see Appendix 6.2).

Figure 3.1B shows two examples of cell lines resistant to the sensitizing effect of glutamine starvation (termed 'resistant' cell lines in this chapter): CAL 51 was only sensitive (i.e. more cell death) to glutamine deprivation but not to TRAIL, while HCC1954 cells were resistant to both glutamine deprivation and TRAIL.

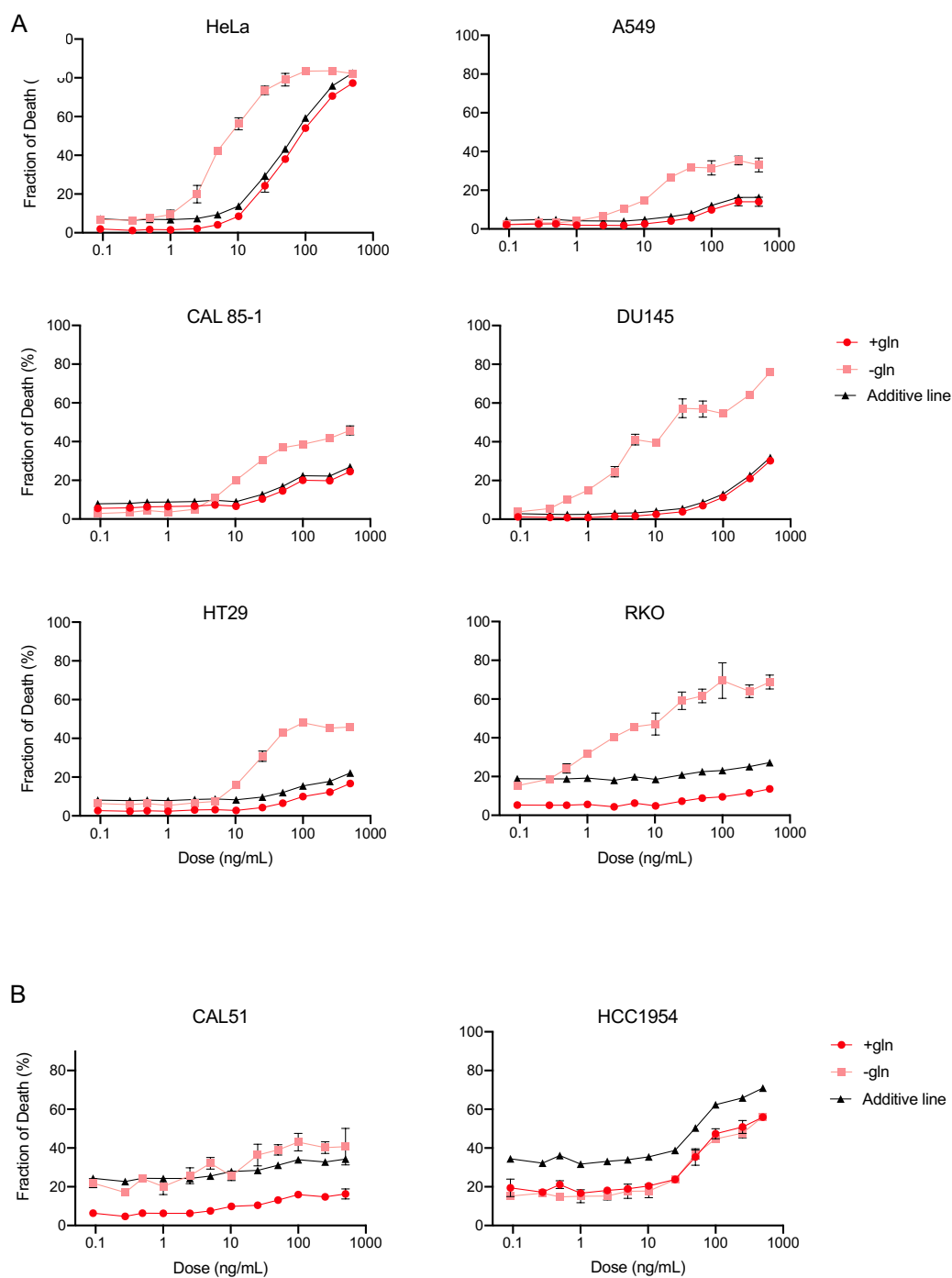


Figure 3.1 Multiple cancer cell lines showed changes in sensitivity of TRAIL with 24-hour pre-culturing in glutamine depleted media.

(A-B) Dose response curves of cell lines sensitive (A), or resistant (B) to the sensitizing effect of 24-hour glutamine starvation before TRAIL treatment; median with glutamine (red), or with glutamine depletion (pink). The black curves ('Additive line') represent the expected fraction of death if glutamine starvation has only additive effect to TRAIL response. Error bars represent the standard deviation of $n = 3$ technical replicates.

3.3.2 Glutamine starvation induced stronger caspase-8 activity and more cell death in HeLa cells treated with TRAIL

I hypothesized that the lack of glutamine produces a change in DISC dynamics that increases caspase-8 activity. To explore this hypothesis, I measured TRAIL-induced caspase-8 activity in HeLa cells with and without 24-hours of pre-treatment glutamine starvation. As shown in Figure 3.2, after being treated with the same dose of TRAIL, larger fractions of cells in the glutamine-deprived group had high caspase-8 activity and crossed maximum caspase-8 activation rate boundaries (red lines in the derivative plots, Figure 3.2B) compared to the control groups (Figure 3.2A). It should be noted that since the boundaries were calculated separately, only the maximum caspase-8 activation rate boundaries in the glutamine-deprived groups receiving 0.63 or 1.25 nM treatment should be used for comparison with the control groups. The boundaries of the lowest (0.31 nM) and highest (2.5 nM) doses were not representative, because there were not enough dead or surviving cells to accurately calculate the true boundary.

I also observed that in the glutamine-depleted groups, not only did the dead subpopulation pass the caspase-8 activation rate threshold, but the time to reach maximum caspase-8 activation rate also seems to have been longer—especially in the medium-dose groups receiving 0.63 nM or 1.25 nM TRAIL (Figure 3.2A and B). This suggests that the effects of glutamine starvation may be two-fold, increasing caspase-8

activation rate while also increasing the time to reach maximum caspase-8 activation rate.

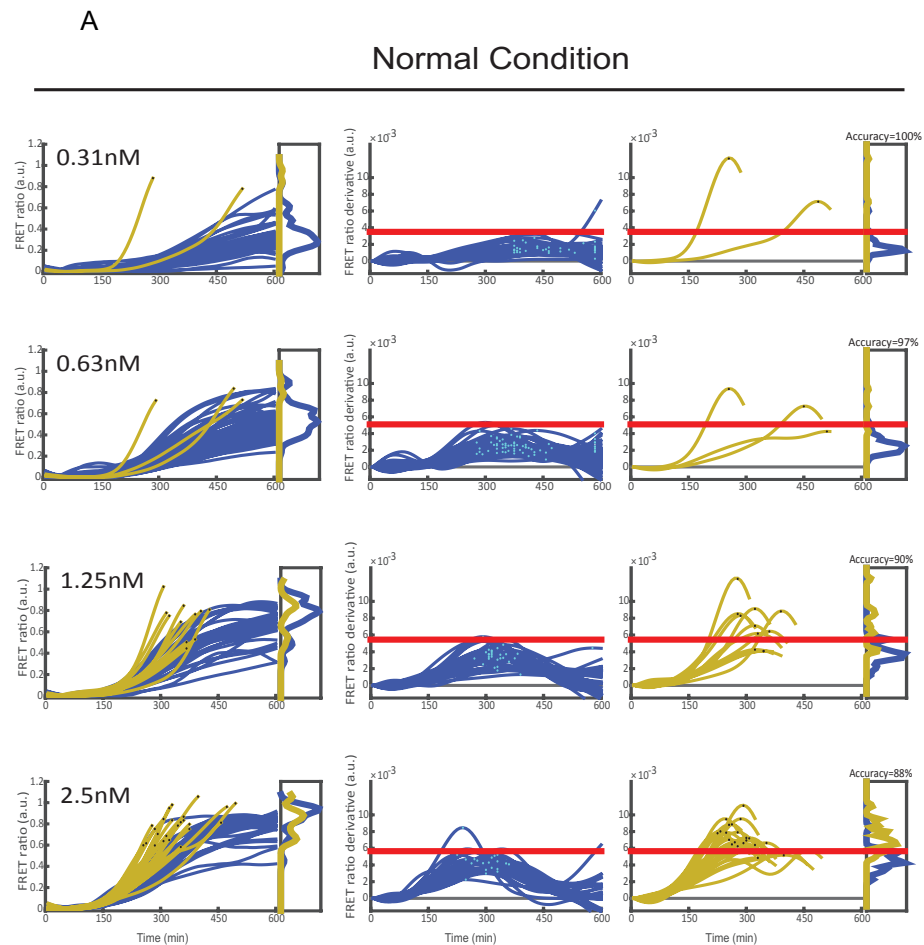
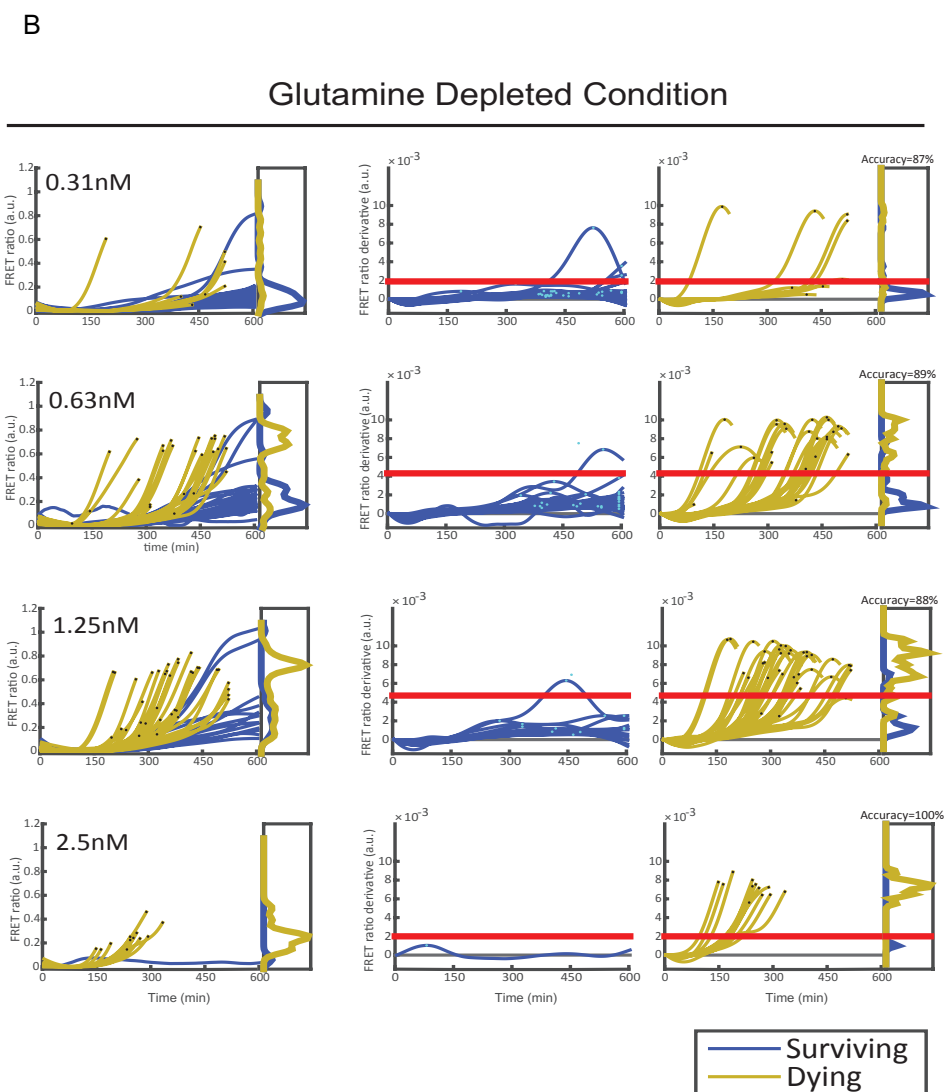


Figure 3.2 Glutamine starvation induced stronger caspase-8 activity and more cell death in HeLa cells treated with TRAIL

A. Left panel: FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) HeLa cells following treatment with varying doses of TRAIL. The histograms show the distribution of accumulated FRET ratio levels of the cells. Middle and right panels: derivatives of the FRET ratio traces for surviving cells (blue, middle panel) and dying cells (yellow, right panel) for the same traces shown in left panel. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The histograms on the far right show the distribution of maximal point values of the derivatives of the FRET ratio. The redlines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

B. Same types of figures as A, showing results from the glutamine depleted condition.

Figure 3.2 (Continued).



3.3.3 Glutamine deprivation produces a similar sensitizing response to T191

Next, I examined the sensitizing effect of glutamine starvation on T191 response in the same cancer cell lines. Since T191 is a synthetic TRAIL analogue, I predicted a similar sensitization effect from glutamine starvation. As shown in Figure 3.3A, cell lines cultured in glutamine-depleted media showed a similar increase in the fraction of death

after T191 treatment. Glutamine starvation increased the treatment's potency in HeLa and A549 cells, and increased the treatment's efficacy in the case of CAL 85-1, DU145, HT29 and RKO cells. The cell lines resistant to TRAIL and glutamine starvation showed similar responses here as well. As shown in Figure 3.3B, CAL 51 is only sensitive to glutamine deprivation but not to T191, and HCC1954 cells are resistant to both glutamine deprivation and T191.

Among the 14 cancer cell lines tested, the same 9 cell lines that responded in the TRAIL assay described above in Section 3.3 also showed an increase in T191-induced death after glutamine starvation for 24 hours (see Appendix 6.2). This indicates that the factors contributing to the sensitizing effects of glutamine starvation may indeed involve players from the extrinsic apoptotic pathway. T202 was not tested in this setting due to time limit, but because of its similar mechanism of action compared to TRAIL and T191, glutamine starvation may have very similar effect on these cell lines' response to T202.

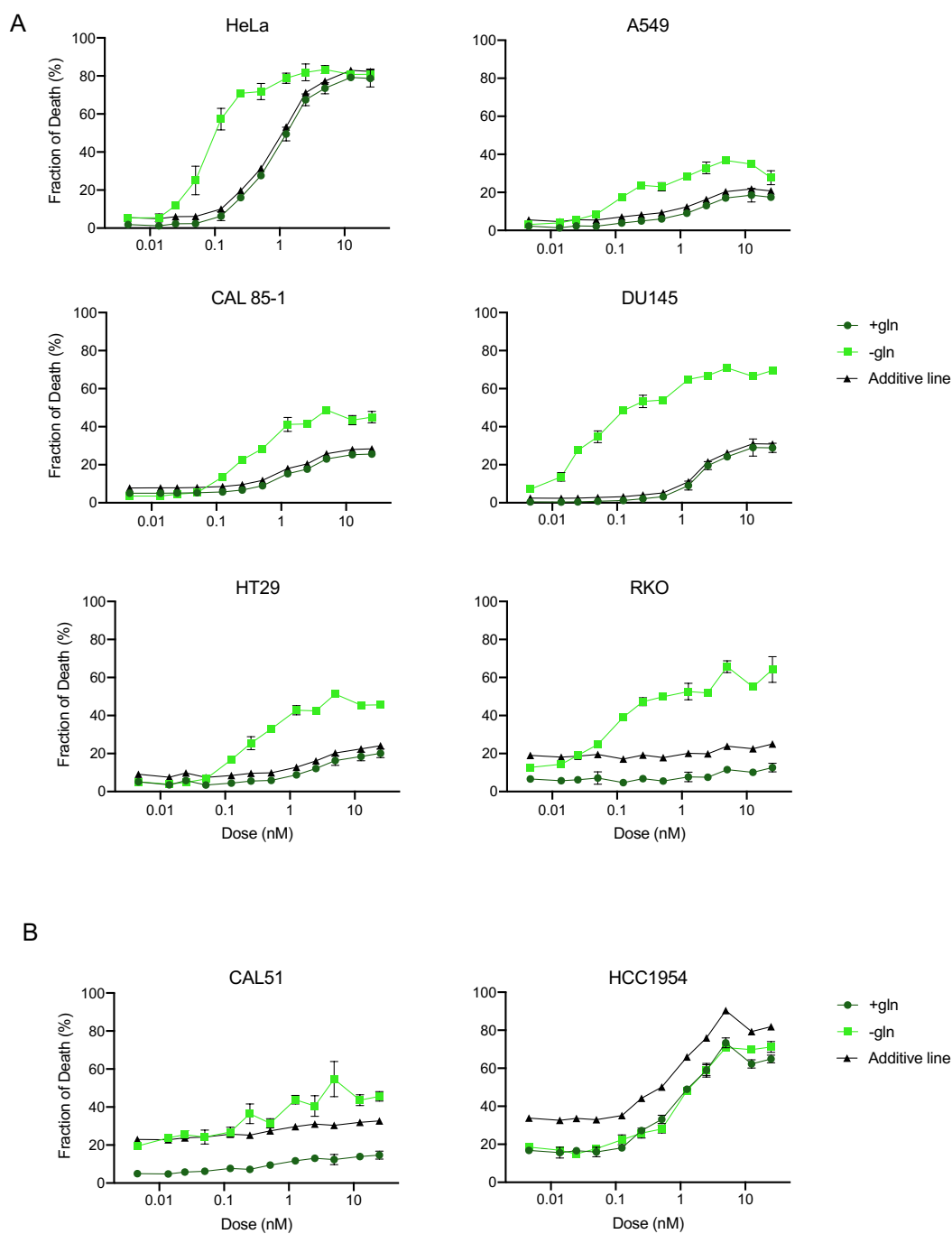


Figure 3.3 Multiple cancer cell lines showed changes in sensitivity of T191 with 24-hour pre-culturing in glutamine depleted media.

(A-B) Dose response curves of cell lines sensitive (A), or resistant (B) to the sensitizing effect of 24-hour glutamine starvation before T191 treatment; median with glutamine (green), or with glutamine depletion (light green). The black curves ('Additive line') represent the expected fraction of death if glutamine starvation has only additive effect to T191 response. Error bars represent the standard deviation of $n = 3$ technical replicates.

3.3.4 Glutamine starvation induces stronger caspase-8 activity and more cell death in HeLa cells treated with T191

To test whether this sensitization effect was reflected in caspase-8 dynamics, I measured caspase-8 activity in T191-treated HeLa cells cultured in both normal and glutamine-depleted conditions. As with TRAIL, glutamine-deprived HeLa cells showed higher caspase-8 activity, with more cells crossing the maximum caspase-8 activation rate boundary (Figure 3.4). However, in the surviving subpopulation of glutamine-starved cells, a few cells also showed high caspase-8 activity with maximum caspase-8 activation rate above the boundary (Figure 3.4B). This suggests that in these individual cells, DISC-independent barriers in other steps of the extrinsic pathway may play a role in preventing them from undergoing apoptosis. The fact that this was not seen in cells treated with TRAIL may relate to differences between the two molecules and merits further investigation.

Figure 3.4 Single-cell FRET ratio traces and FRET ratio derivatives in HeLa cells treated with T191 with or without glutamine in media

A. Left panel: FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) HeLa cells following treatment with varying doses of T191. The histograms show the distribution of maximal FRET ratio levels of the cells. Middle and right panels: derivative of the FRET ratio traces for surviving cells (blue, middle panel) and dying cells (yellow, right panel) for the same traces shown in left panel. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The histograms on the far right show the distribution of maximal point values of the derivatives of the FRET ratio. The redlines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

B. Same types of figures as A, showing results from the glutamine depleted condition.

Figure 3.4 (Continued).

A

Normal Condition

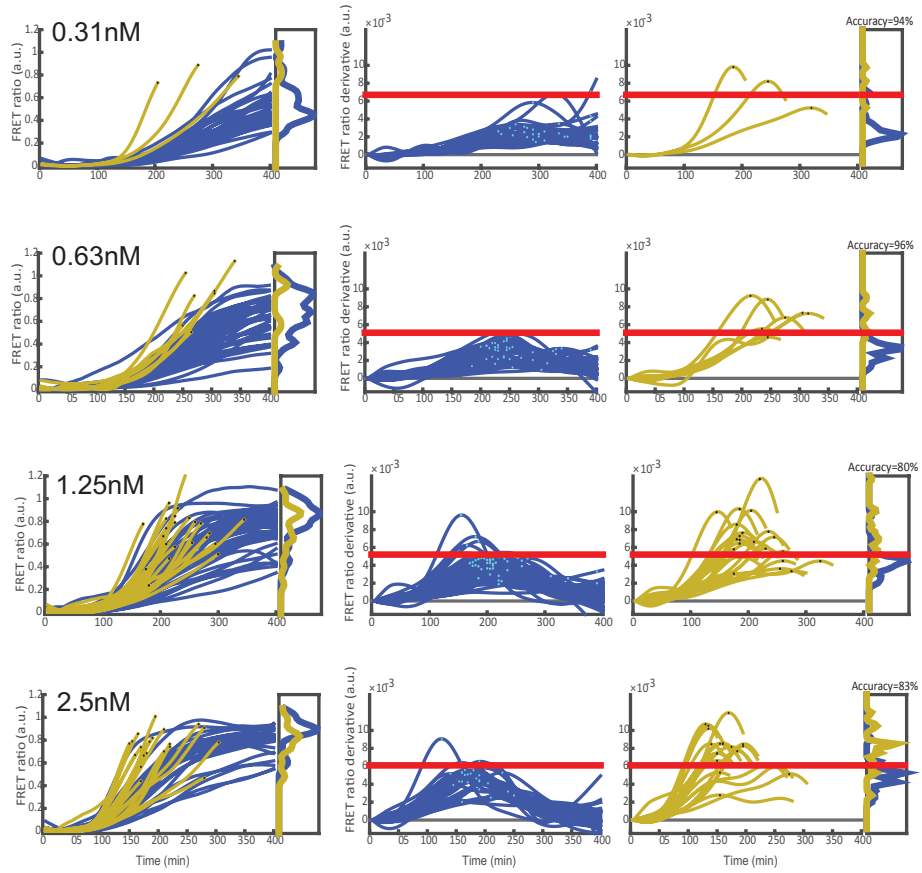
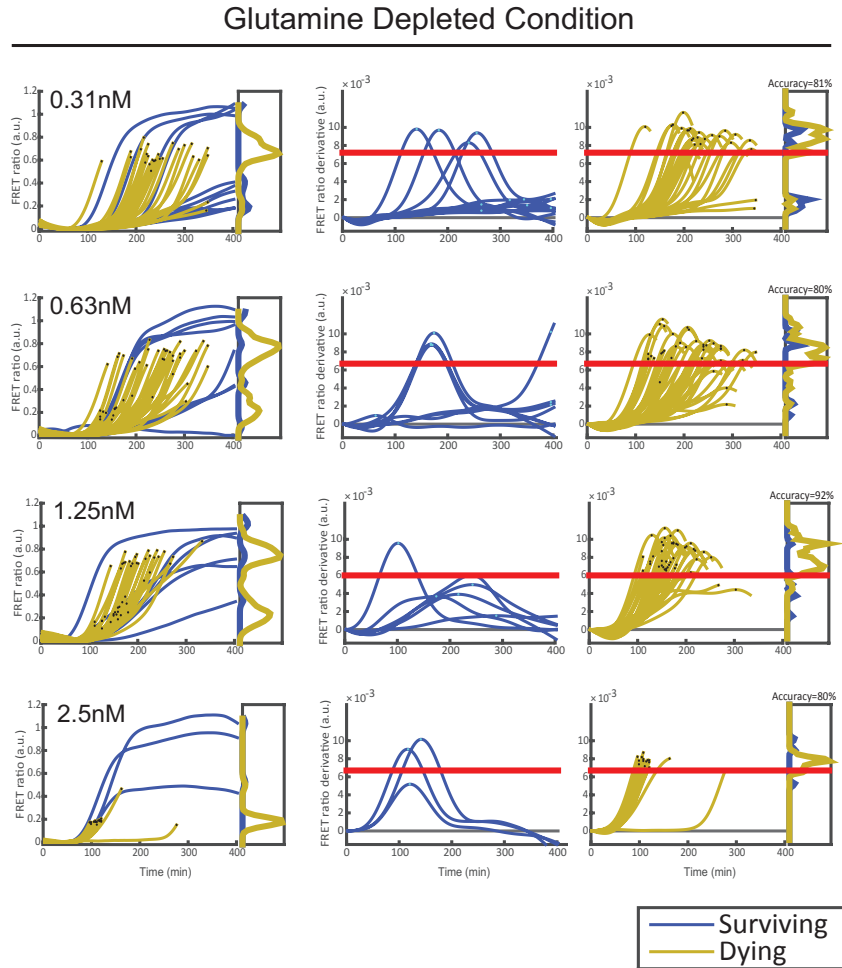


Figure 3.4 (Continued).

B



3.3.5 Glutamine starvation has little effect in the same sensitive cell lines treated with BCL-2 family inhibitors and intrinsic apoptosis inducers

Since changes in glutamine level are likely to have a global effect on protein expression (Mauro-Lizcano and López-Rivas 2018; Valter et al. 2017), it is worth

investigating whether glutamine starvation can sensitize cancer cell lines to other types of treatment, such as intrinsic apoptosis-inducing agents.

To probe this question, I measured the fraction of cell death in the same sensitive cell lines treated with the small-molecule drugs ABT199, S63845, etoposide, and staurosporine when cultured in the presence or absence of glutamine. As shown in Figure 3.5, glutamine starvation may have little effect on the priming state of sensitive cell lines. The fractions of dead cells were very similar in the ABT199- and S63845-treated cultures both with and without pre-treatment glutamine starvation. Similarly, pre-treatment glutamine starvation did not sensitize the cell lines to the intrinsic apoptosis inducer etoposide. For staurosporine, however, there was a certain level of sensitization observed after glutamine-depletion, although the magnitude of the effect was considerably smaller than for TRAIL or T191. One potential explanation is that although staurosporine is an intrinsic apoptosis-inducing agent, it can also induce receptor-independent caspase-8 activation (Tang et al. 2000; Zhang et al. 2004).

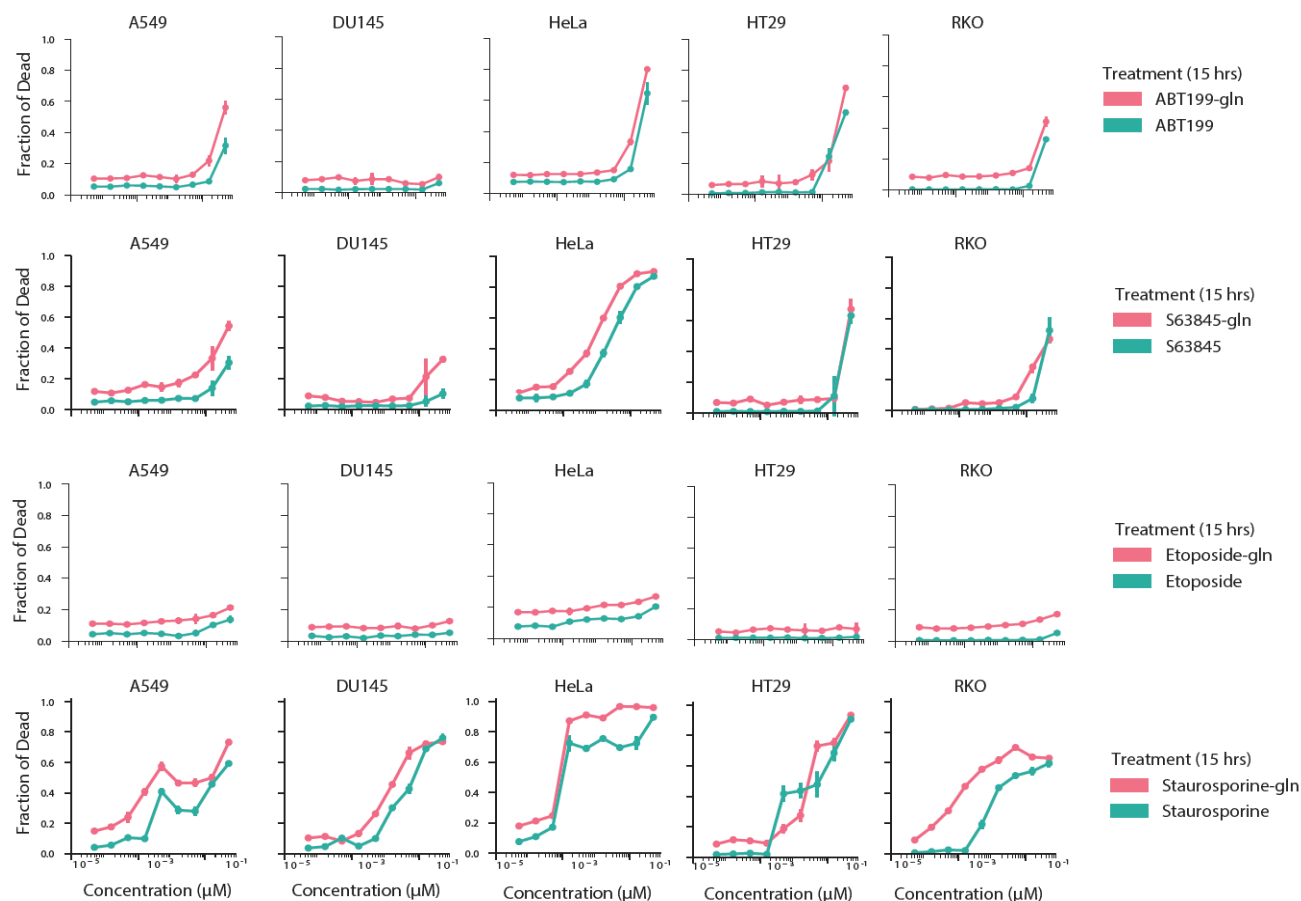


Figure 3.5 Glutamine starvation has little effect in the same sensitive cell lines treated with BCL-2 family inhibitors and intrinsic apoptosis inducers. From the top to bottom panels: fraction of death induced by ABT188, S63845, etoposide or staurosporine with (green curves) and without glutamine (red curves) starvation for 24 hours before treatment.

3.3.6 TRAF2, FLIP and MCL-1 decreased in sensitive lines in glutamine-depleted condition

To find out why glutamine starvation sensitizes cancer cell lines' response to extrinsic apoptosis, I measured changes in protein levels in cells via western blot after 24 hours of glutamine starvation. As shown in Figure 3.6A, TRAF2 and MCL-1 levels decreased after glutamine starvation in the sensitive cell lines CAL85-1, DU145, HeLa, and HT29, while no such pattern was observed in resistant cell lines CAL-51 and HCC1954. There is evidence that cIAP1 may be an important determinant for T191 sensitivity in colorectal cell lines (data from Merrimack, manuscript in preparation). However, it may not contribute to the increase in TRAIL and T191 sensitivity induced by glutamine starvation, as cIAP1 levels did not significantly change after glutamine depletion in either sensitive or resistant cell lines. FLIP is another DISC component that plays an important role in regulating caspase-8 dynamics. However, due to low expression levels, I was not able to obtain conclusive western blot results for FLIP.

I then measured mRNA levels of TRAF2, MCL-1, and FLIP by qPCR in order to confirm the observations from the western blots (Figure 3.6B). In Figure 3.6B, the y-axis is the foldchange in mRNA level after glutamine starvation for 24 hours. The mRNA level of all three genes decreased after glutamine depletion. A comparison of the fold-changes in expression revealed a sharper decrease in TRAF2 and FLIP mRNA levels in

sensitive cell lines than in resistant cell lines after glutamine starvation, while the difference in MCL-1 mRNA levels was not significant.

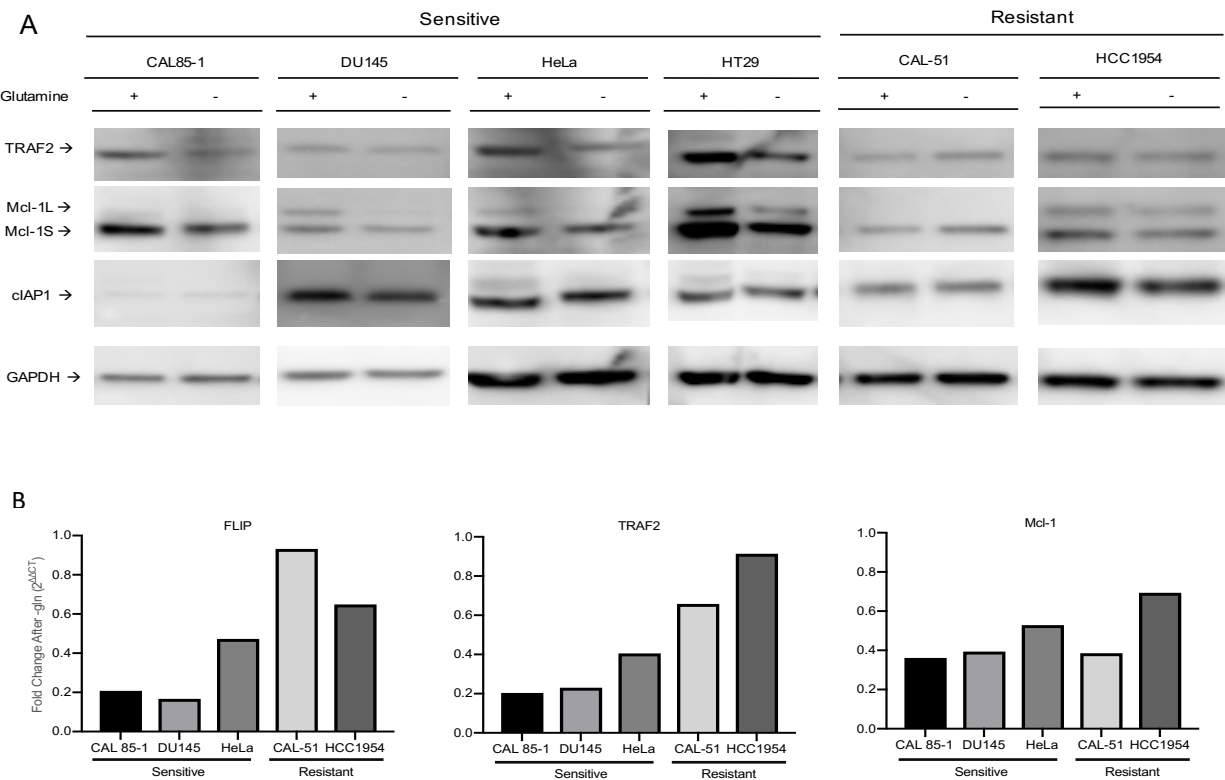


Figure 3.6 Changes in protein and mRNA levels in sensitive and resistant cell lines after glutamine starvation for 24 hours

A. Western blot analysis of TRAF2, MCL-1, cIAP1 after glutamine depleted treatment of CAL85-1, DU145, HeLa, CAL-51, and HCC1954.

B. qPCR analysis of FLIP (both isoforms), TRAF2 and MCL-1 after glutamine depleted treatment of CAL85-1, DU145, HeLa, CAL-51, and HCC1954.

3.3.7 FLIP overexpression protected HeLa cells from TRAIL-induced death in glutamine-depleted conditions

Given the reduction in FLIP levels after glutamine starvation, as well as previous studies pointing to the importance of FLIP in glutamine starvation-induced TRAIL sensitization (Mauro-Lizcano and López-Rivas 2018), I next measured the TRAIL-

induced apoptotic fraction in HeLa cells overexpressing FLIP-L or FLIP-S with and without glutamine depletion.

The overexpression system was developed in lab for imaging purpose. These HeLa cells stably express mCherry-tagged FLIP-L or FLIP-S driven by a CMV promoter. As can be seen from Figure 3.7A, these proteins were indeed expressed at a high level. Both cell lines were protected from TRAIL in the glutamine deprived culture conditions (Figure 3.7B). FLIP-S overexpression completely prevented cell death in both conditions after TRAIL treatment, while FLIP-L overexpression had a partially protective effect, where $E_{\max}$ increased from near zero to around 30% after glutamine starvation. This represents a roughly 62% reduction in fraction of death compared to parental HeLa cells treated with the same dose of TRAIL after glutamine starvation. There are two possible reasons for the different results from FLIP-L and FLIP-S-overexpressing cells. On one hand, as seen from the western blot results in Figure 3.7A, FLIP-S may be expressed at even higher level than FLIP-L, resulting in a more complete block of caspase-8 activity. Alternately, FLIP-L may have a dual function of both activating and inhibiting caspase-8, depending on the ratio between FLIP and procaspase-8 (Hughes et al. 2016). The protective effect of FLIP overexpression suggests that the sensitizing effect of glutamine starvation on TRAIL is caspase-8-dependent. But this phenomenon could be induced by multiple factors such as the reduction level in FLIP, and/or in TRAF2.

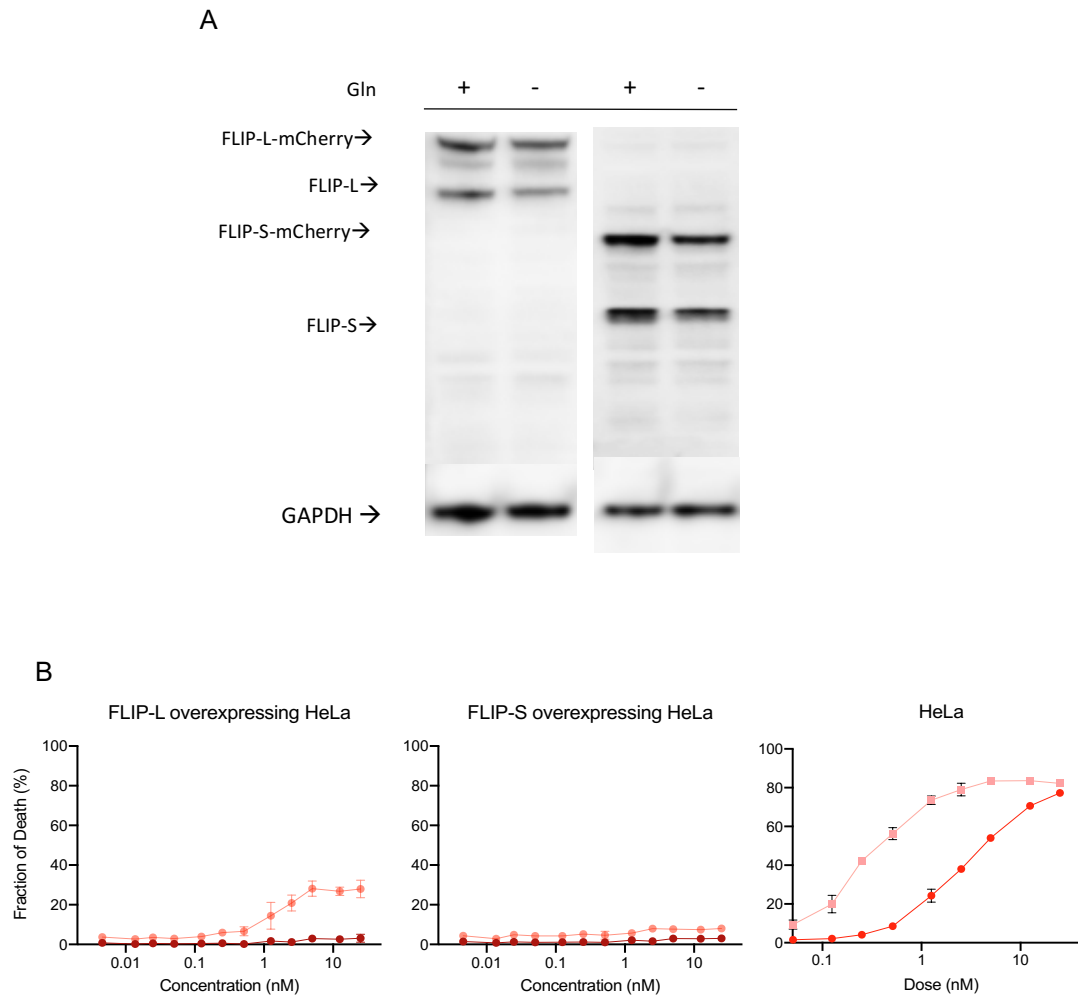


Figure 3.7 FLIP overexpression protected HeLa cells from TRAIL-induced apoptosis in both normal ● and ⁺gln glutamine-depleted conditions. ■ -gln

A. Western blot analysis of the two FLIP-overexpressing stable HeLa cell lines. Since the both FLIP-L and FLIP-S are tagged with mCherry, the molecular weight is higher than normal FLIP proteins.

B. Dose response curves of the two FLIP-overexpressing HeLa cell lines treated with TRAIL in normal (red) and glutamine-depleted (pink) conditions. Error bars represent the standard deviation of n = 3 technical replicates.

3.4 Conclusion

I first observed that glutamine starvation enhanced TRAIL response in HeLa cells. In the absence of glutamine, both T191 and TRAIL induced stronger caspase-8 activity

and more cell death in HeLa cells, compared to normal culture conditions with 5 mM L-glutamine. 24 hours of glutamine depletion likewise increased the fraction of cell death induced by TRAIL and T191 in multiple other cell lines from different cancer types. However, such a pattern was not observed following treatment with the BCL-2 family inhibitors ABT199 or S63845, or intrinsic apoptosis-inducing agent etoposide. Glutamine starvation had a weak sensitizing effect in these cell lines when treated with staurosporine, and this phenomenon could be due to the activity of caspase-8. In cell lines that showed an increased extrinsic apoptotic response after glutamine depletion, I also saw a decrease in FLIP, TRAF2 and MCL-1 levels. Together with the observation that overexpression of FLIP-L and FLIP-S protected cells from TRAIL-induced apoptosis in glutamine-depleted conditions, these findings suggest that glutamine deprivation's sensitization effect is the result of increased caspase-8 activity, potentially induced by changes in FLIP and/or TRAF2 levels. The effect of the changes in MCL-1 level may have a lesser impact on the increased sensitivity, given FLIP proteins rescued glutamine starvation's sensitizing effect.

4. Chapter 4 - Co-treatment with bortezomib and LCL161 enhances T191- and T202-induced apoptosis in breast cancer and colon cancer cell lines

4.1 Introduction

In addition to caspase-8 and other death-inducing signaling complex (DISC) - associated proteins, BCL-2 family proteins, inhibitors of apoptosis proteins (IAPs) and proteasome components also play important roles in determining cell fate.

The BCL-2 family of proteins, classified as pro- or anti-apoptotic, control mitochondrial outer membrane integrity and MOMP. The interactions between pro- and anti-apoptotic members of the Bcl-2 family ultimately determine whether MOMP happens. Thus, one strategy to induce apoptosis is by antagonizing anti-apoptotic BCL-2 family proteins. Several small-molecule drugs targeting these proteins have been developed and tested in clinical trials, among which the most advanced are ABT199 (Venclexta) and ABT263 (Navitoclax). To date, ABT199 has been approved for chronic lymphocytic leukemia in patients bearing a chromosome 17p deletion in 2016, ABT199 has been approved for chronic lymphocytic leukemia in patients bearing a chromosome 17p deletion in 2016, based on successful phase III testing of ABT199 in combination with rituximab (Rituxan) in relapsed or refractory chronic lymphocytic leukemia patients (ClinicalTrials.gov, trial ID: NCT01889186). In 2018, the same combination was also approved for chronic lymphocytic leukemia or small lymphocytic lymphoma patients who have received one prior therapy (ClinicalTrials.gov, trial ID: NCT02005471), and for the treatment of newly-diagnosed acute myeloid leukemia (AML) in adults who are age 75 years or older, or who have comorbidities that preclude use of intensive induction chemotherapy in combination with azacitidine or decitabine or low-dose cytarabine (www.fda.gov). ABT263 has been tested for leukemia, lung cancer, lymphoma, ovarian cancer, and prostate cancer, including a phase II trial for women with platinum-

resistant/refractory recurrent ovarian cancer (Cang et al. 2015). S63845 is a newer molecule targeting the anti-apoptotic BCL-2 family protein MCL-1. It has been shown that S63845 can induce BAX/BAK-dependent selective killing in MCL-1-dependent cells (Kotschy et al. 2016). A derivative of this molecule is now being tested in a Phase I clinical trial (ClinicalTrials.gov, trial ID: NCT02992483), which is currently recruiting patients with relapsed lymphoma or multiple myeloma.

BCL-2 family inhibitors have also shown sensitizing effects that could make them potentially suitable for use in TRAIL combination therapy for multiple cancer types (Hao et al. 2004; Fulda 2018; Kang and Reynolds 2009).

IAPs are another anti-apoptotic family of proteins that is often overexpressed in cancer cells (Fulda 2015), and small-molecule SMAC mimetic drugs have been designed to antagonize the IAPs. In a recent study by Merrimack Pharmaceuticals (Cambridge, MA), inhibition of cIAP1 by LCL161 and birinapant was shown to sensitize resistant colorectal cancer cells' response to T191 and reduce tumor size *in vivo* (unpublished data).

Another promising family of TRAIL-sensitizing agents is the proteasome inhibitors, including bortezomib (Velcade) and carfilzomib (Kyprolis) (Hellwig and Rehm 2012). Our lab and others have shown that bortezomib can greatly enhance TRAIL-induced cell death (Chen et al. 2011; de Wilt et al. 2013; Kao et al. 2014). The proposed mechanisms underlying bortezomib-dependent TRAIL sensitization include increased expression of death receptors and pro-apoptotic Bcl-2 family proteins, and reduction in FLIP and IAPs (de Wilt et al. 2013; Sayers and Murphy 2006; Sayers 2011).

Here, I have measured the effects of treating breast and colorectal cancer cells with combinations of T191 or T202 and the TRAIL-sensitizing agents bortezomib, ABT199, ABT263, S63845, and LCL161. The goal was to investigate the effect of T191- and T202-based combination treatments in different cell lines, and whether there are any general trends in the cell lines' response.

4.2 Materials and methods

4.2.1 Cell lines and materials

All cell lines were purchased from ATCC and cultured according to the manufacturer's instructions or common practice: CAL-51, CAL-85-1, CAL120, Hs578T cells were cultured in DMEM (Corning, Corning, NY), CAMA-1, MCF7, Ls174T, and RKO cells were cultured in EMEM (Corning, Corning, NY), Colo205, DLD-1, HCC1954, HCT116, HCT16, HCT8, HT29, LS411N, NCIH747, SKBR3, and T47D cells were cultured in RPMI (Corning, Corning, NY). All media was supplemented with 5 ml L-glutamine (Corning, Corning, NY), 10% fetal bovine serum (Corning, Corning, NY), and 1% penicillin/streptomycin (Gibco, Montgomery County, MD).

4.2.2 Dye-drop assay measurement of fraction of cell death

Cells were seeded 24 hours prior to treatment in 384-well plates by Multidrop Combi cell dispenser (Thermo Fisher Scientific, Waltham, MA). The seeding concentration is $1 - 1.5 \times 10^6$ /mL depending on cell types and the dispensing volume is 80 μ L each well. Drug treatment were dispensed via HP D300 Digital Dispenser (Tecan Life Sciences, Switzerland). After 15-hour treatments, the cells were stained with Live

Dead Red dye (Life Technology, Carlsbad, CA) and Hoechst 33342 (Life Technology, Carlsbad, CA) in 10% Optiprep (Sigma, St. Louis, MO) in PBS for 30 min, and fixed with 3.7% formaldehyde solution in 20% Optiprep in PBS for 30 min. The plates were then washed once with an ELx405 plate washer (BioTek, Winooski, VT) aspirating all but 15 ul from each well and dispensing 100 ul of PBS. The plates were then sealed and imaged. Image processing was performed with PerkinElmer's Columbus Image Data Storage and Analysis System.

4.2.3 Combination treatment and isobologram computation

The treatment files used for D300 drug treatments were generated with in-house python scripts for complete randomization (<https://github.com/datarail>). Cell seeding, treatment, staining, imaging and analysis were performed as described above for the dye-drop assay. After the fraction of death data was obtained, the data points were transformed into matrix forms where each axis represents varying doses of one of the two treatments. Isobolograms were calculated by first filtering the matrix with a Gaussian filter, then taking interpolation and creating surface plots in MATLAB (Natick, MA).

4.3 Results

4.3.1 Bortezomib plus T191 increased fraction of death in breast cancer and colorectal cancer cell lines

I first tested the response to T191 and bortezomib co-treatment in six breast cancer cell lines and eight colorectal cancer cell lines. Breast cancer was chosen because

these cells are generally resistant to TRAIL-induced apoptosis, and I sought to test if this was also the case for T191, and whether bortezomib can sensitize breast cancer cell lines to this treatment. Colorectal cancer cell lines, on the other hand, are generally more sensitive and have been regarded as a promising target for TRAIL family molecules. The question in this case was whether the addition of bortezomib can further increase their response to T191.

In these experiments, I measured the fraction of cell death after treatment with increasing doses of T191 at a fixed 100 nM dose of bortezomib. As a single agent, 100 nM of bortezomib did not induce significant cell killing (<10% fraction of death) in any of the cell lines tested.

As shown in Figure 4.1A, breast cancer cell lines showed variable responses to the co-treatment of T191 and bortezomib. In the partially sensitive CAMA-1 cell line, bortezomib only had an additive effect, as the dose-response curves of single-agent T191 and T191 + bortezomib co-treatment were almost parallel to each other (Figure 4.1A, left panel). In the more resistant lines CAL-51, T47D and SKBR3, 100 nM of bortezomib increased $E_{\max}$ 2-4-fold (Figure 4.1A middle panel). CAL 85-1 was resistant to both T191 alone and T191 + bortezomib co-treatment, as were the negative control MCF7 cells, which lack procaspase-3 (Figure 4.1A right panel) (Devarajan et al. 2002; Y. Zhang and Zhang 2008; Jänicke 2009).

In colorectal cancer cell lines, the effect of T191 and bortezomib co-treatment can be categorized into two types: 1) decreased IC_{50} in sensitive lines and 2) increased $E_{\max}$ in resistant lines. In cells that are sensitive to T191 as a single agent, such as Colo205,

DLD-1, HCT15, and HCT116, the addition of 100 nM bortezomib resulted in increased T191 potency, shifting the IC_{50} down to ~5 nM (Figure 4.1B, left panel). In the resistant HCT8, HT29, RKO, and NCIH747 cell lines, co-treatment produced a higher E_{max} , indicating that this proteasome inhibitor may help reduce some apoptotic barriers in these resistant cells.

Figure 4.1 Bortezomib plus T191 increased fraction of death in breast cancer and colorectal cancer cell lines

A-B Fraction of death in breast cancer cells (A) and colorectal cancer cells (B) treated with increasing doses of T191 and 100 nM bortezomib for 15 hours. Error bars represent the standard deviation of $n = 3$ technical replicates.

Figure 4.1 (Continued).

A

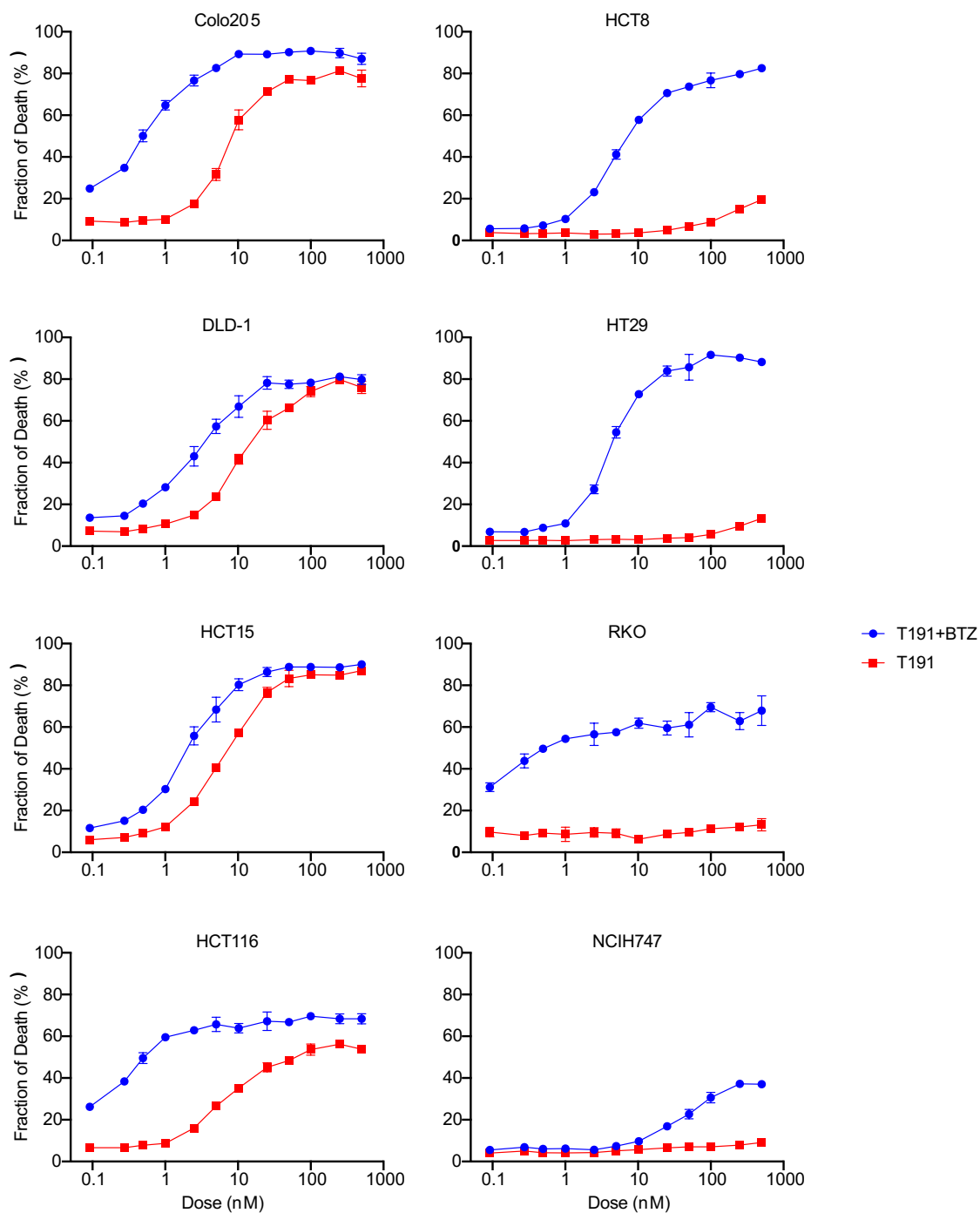
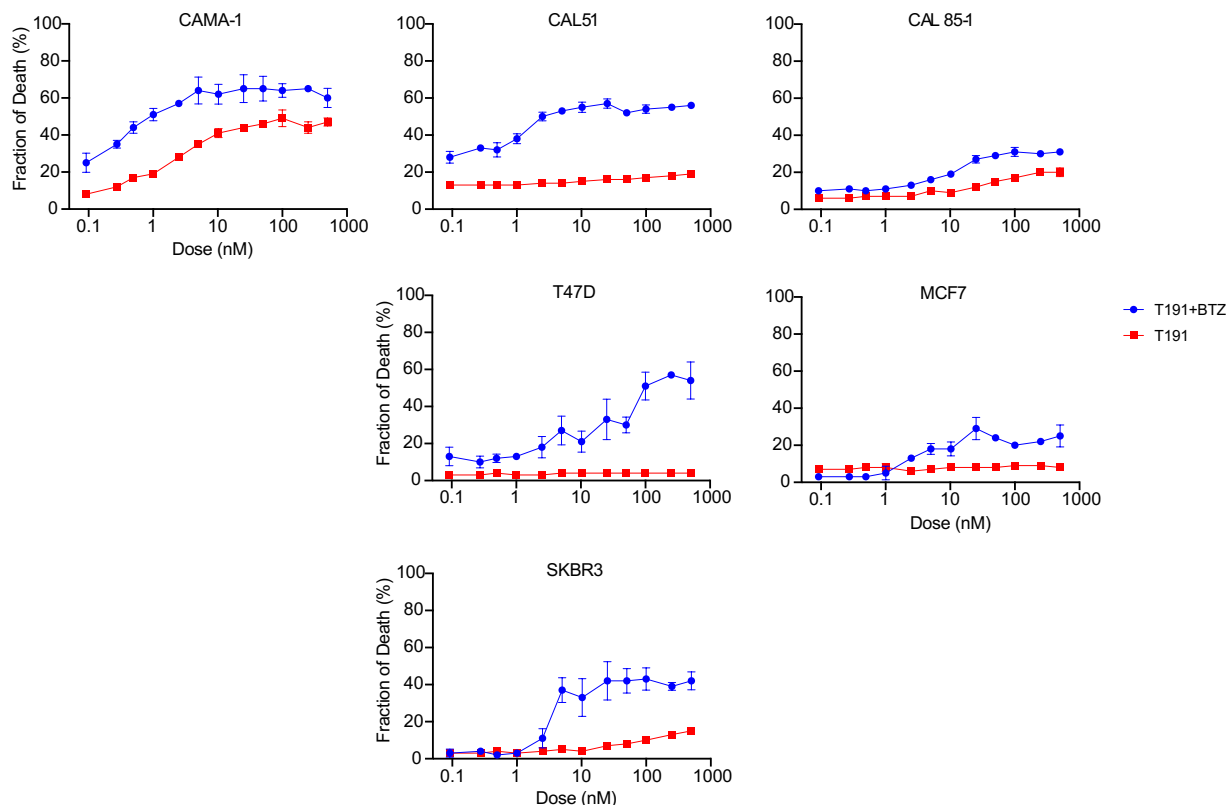


Figure 4.1 (Continued).

B



4.3.2 Synergistic effects of bortezomib and T202 co-treatment in breast and colorectal cancer cell lines

Given the broad sensitizing effect in breast and colorectal cancer cell lines, I next tested whether similar effects hold for T202 treatment. For a more comprehensive characterization of the combination effect, I measured the fraction of death at varying doses of both T202 and bortezomib. The resulting isobolograms—surface plots in which each line represents equality in the fraction of cell death, and colors represent the

gradient of the fraction of cell death—were used to visualize the synergistic effects of combination treatment. If the two treatments are independent of each other, there will only be an additive effect, and the lines in the isobolograms should be diagonal. When one of the two treatment is more dominant than the other, the lines will be either vertical or horizontal, depending on the axis used to plot the doses of that dominant treatment. Concave (inward bending) curves in the isobologram indicate synergism, while convex (outward bending) curves indicate antagonism.

T202 and bortezomib co-treatment was synergistic in 6 out of 8 breast cancer cell lines measured, as the isobolograms of fraction of death of HCC1954, CAL85-1, SKBR3, MCF7, T47D and CAL 51 cells showed inward curved lines (Figure 4.2A). In Hs578T cells bortezomib seemed to play a more dominant role in cell killing giving vertical lines in the isobologram, while CAL120 cells had little response at all doses tested (Figure 4.2A).

Interestingly, two cell lines that did not respond to the co-treatment of T191 and 100 nM of bortezomib, MCF7 and CAL 85-1, both had a synergistic response in this experiment. This indicates that 1) T202 might trigger stronger caspase-8 activity and help overcome apoptotic barriers in resistant cancer cells, and 2) varying doses of both agents in the combination treatment is important as it can give a more comprehensive view of the effect of both drugs.

As with T191, colorectal cancer cells were generally more sensitive to T202 than breast cancer cells. Synergy was observed in 6 out of the 10 cell lines tested: LS411N, LS174T, NCIH747, HCT15, HCT8, and HT29 (Figure 4.2B). In HCT116 and RKO,

bortezomib showed a dominant effect, while in Colo205 and DLD-1, T202 showed a dominant effect (Figure 4.2B).

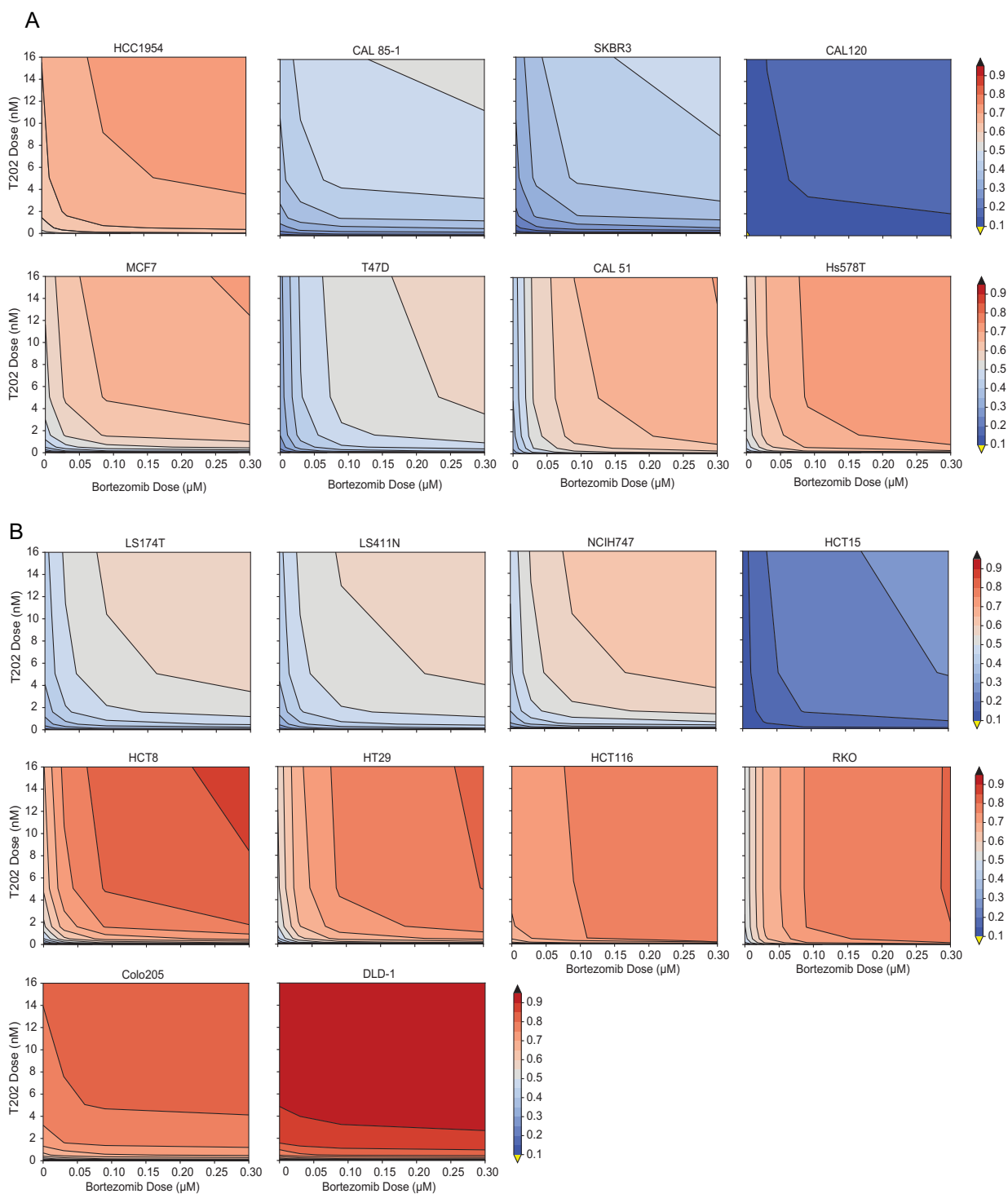


Figure 4.2 Synergy of bortezomib and T202 co-treatment in breast and colorectal cancer cell lines
 A. Isobolograms of the fraction of cell death in breast cancer cells treated with varying doses of T202 and bortezomib for 15 hours. Colors indicate the fraction of cell death.
 B. Isobolograms of the fraction of cell death in colorectal cancer cells treated with varying doses of T202 and bortezomib for 15 hours. Colors indicate the fraction of cell death.

4.3.3 Dose-response results suggest that LCL161 might be a candidate for T202-based combination treatment in breast cancer and colorectal cancer

In order to find other possible combination treatments based on T202, I tested the responses of breast cancer and colorectal cancer cell lines to the co-treatment with T202 and the Bcl-2 inhibitor ABT199 (Venetoclax), the Bcl-2/Bcl-XL inhibitor ABT263 (Navitoclax), the Mcl1 inhibitor S63845, and the IAP inhibiting SMAC mimetic LCL161.

Cancer cells were treated with increasing doses of the Bcl-2 inhibitors and SMAC mimetic, in combination with a fixed-dose of 1.25 nM T202 which is close to the IC_{50} doses in most T202 semi-sensitive cells. The aim was to test whether T202-induced caspase-8 activation can synergize with the removal of downstream blocks of apoptosis by increasing doses of Bcl-2 family inhibitors (ABT199, ABT263, S63845) or a SMAC mimetic (LCL161). Interpretation of the dose response curves for these experiments is as follows: if the two curves overlap, the fixed-dose of T202 has no additional effect; if the two curves are parallel and the difference between the two curves are close to the fraction of death induced by 1.25 nM T202 alone, then there is an additive effect of the fixed-dose T202; if the two curves separate more with increased doses of the Bcl-2 inhibitor or SMAC mimetic, the combination effect is more than additive and may be synergistic.

It has to be noted that except for CAMA-1 which has a fraction of death around 40% at 1.25 nM T202, all the other breast cancer cell lines tested here are resistant to T202 and have less than 10% fraction of death at 1.25 nM dose of T202. Thus, in these cell

lines when the difference in fraction of death induced by single-agent and the combination treatment are larger than 10%, there may be potential synergy.

Little synergy was observed between T202 and the Bcl-2 family inhibitors in the breast cancer cells tested (Figure 4.3). However, there were exceptions: CAL 120 and MCF7 cells showed some level of synergy when T202 was combined with ABT199, ABT263 or S63845. In five (CAL 120, HCC1954, Hs578T, MCF7, and SKBR3) out of nine cell lines, however, there was an increasingly higher fraction of cell death in the LCL161 co-treatment groups compared to the single-agent groups, suggesting potential synergism.

Almost all of the colorectal cancer cell lines had either overlapping or parallel response curves after co-treatment with T202 and BCL-2 family inhibitors, indicating either resistance to both drugs or a dominant effect of T202 (Figure 4.4). For the cell lines that have parallel curves Colo 205, DLD-1, HCT116, HCT8, LS174T and NCIH747, the difference between the two curves are very close to the fraction of death induced by 1.25 nM T202 as a single agent. As was the case in the breast cancer cell lines, there were exceptions here too, notably the HT29, HCT8 and RKO cell lines showed some degree of synergy (Figure 4.4). When treated with 1.25 nM T202 and increasing doses of LCL161, 6 out of 10 cell lines (HCT8, HT29, LS174T, LS411N, NCIH747, and RKO) showed an increasing fraction of cell death, suggesting some level of synergism. Thus, in both indications, co-treatment with T202 and LCL161 seems to confer the broadest effect. However, it is worth noting that only one dose of T202 was

tested here, and varying doses of both drugs may show a more comprehensive picture of cellular response and the synergy between the two agents.

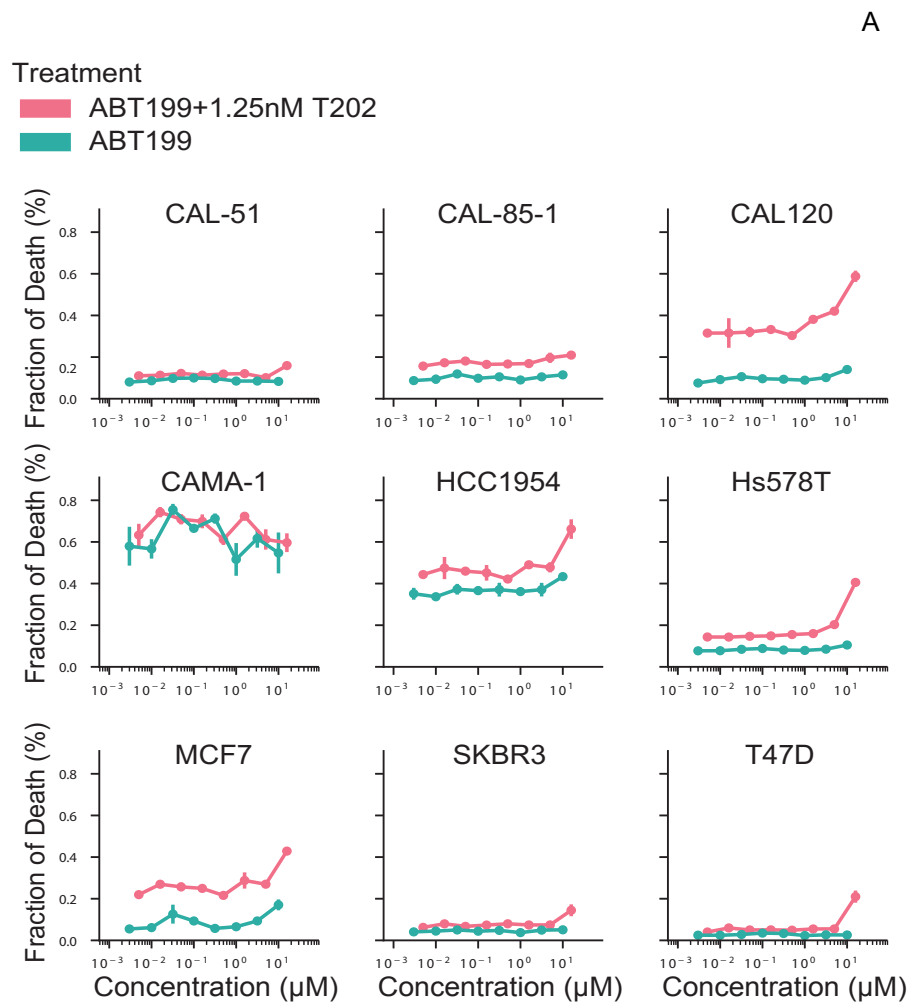


Figure 4.3 Dose-response results suggest that LCL161 might be a candidate for T202-based combination treatment in breast cancer cells
A-D Fraction of death in breast cancer cells treated with ABT199 (A), ABT263 (B), S63845 (C), and LCL161 (D) single-agent or in combination with T202. Error bars represent the standard deviation of n = 5 technical replicates.

Figure 4.3 (Continued).

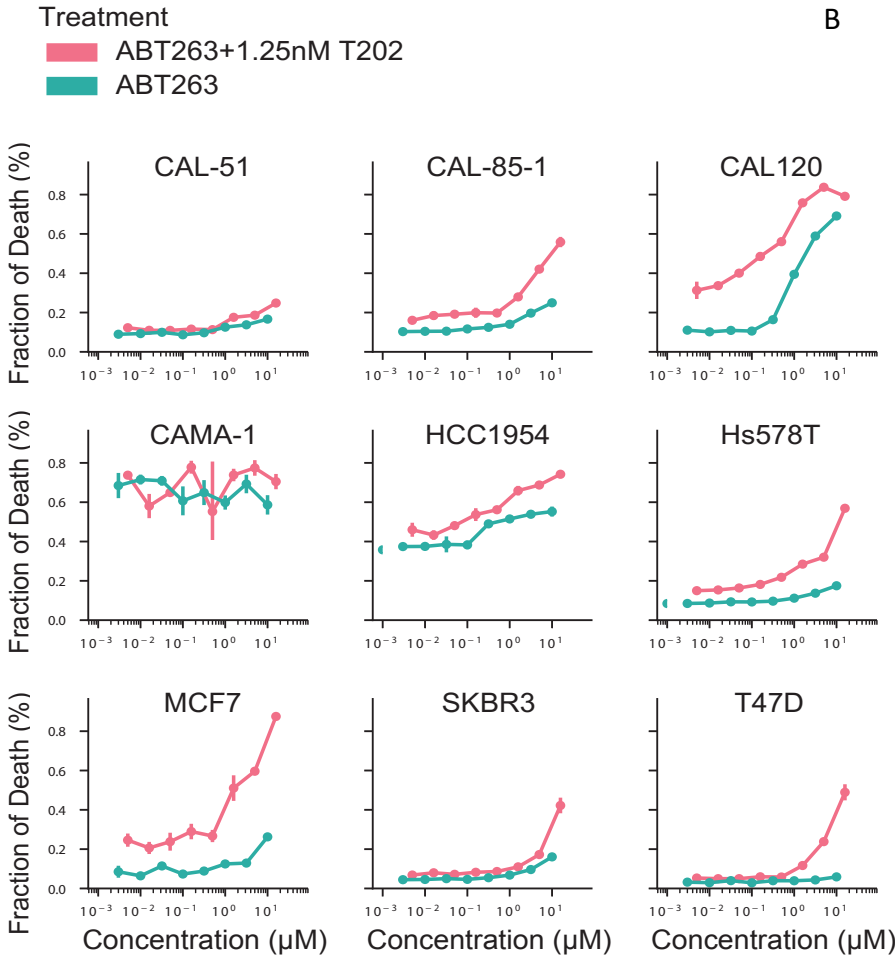


Figure 4.3 (Continued).

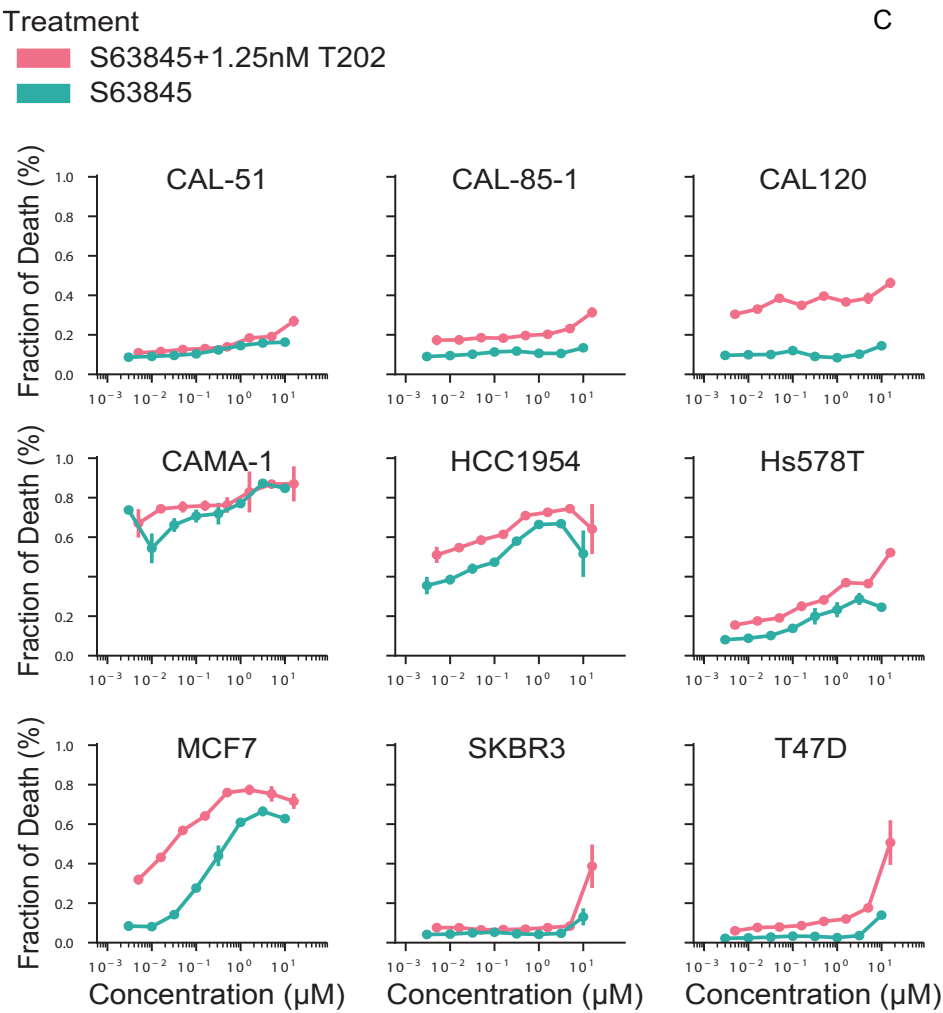
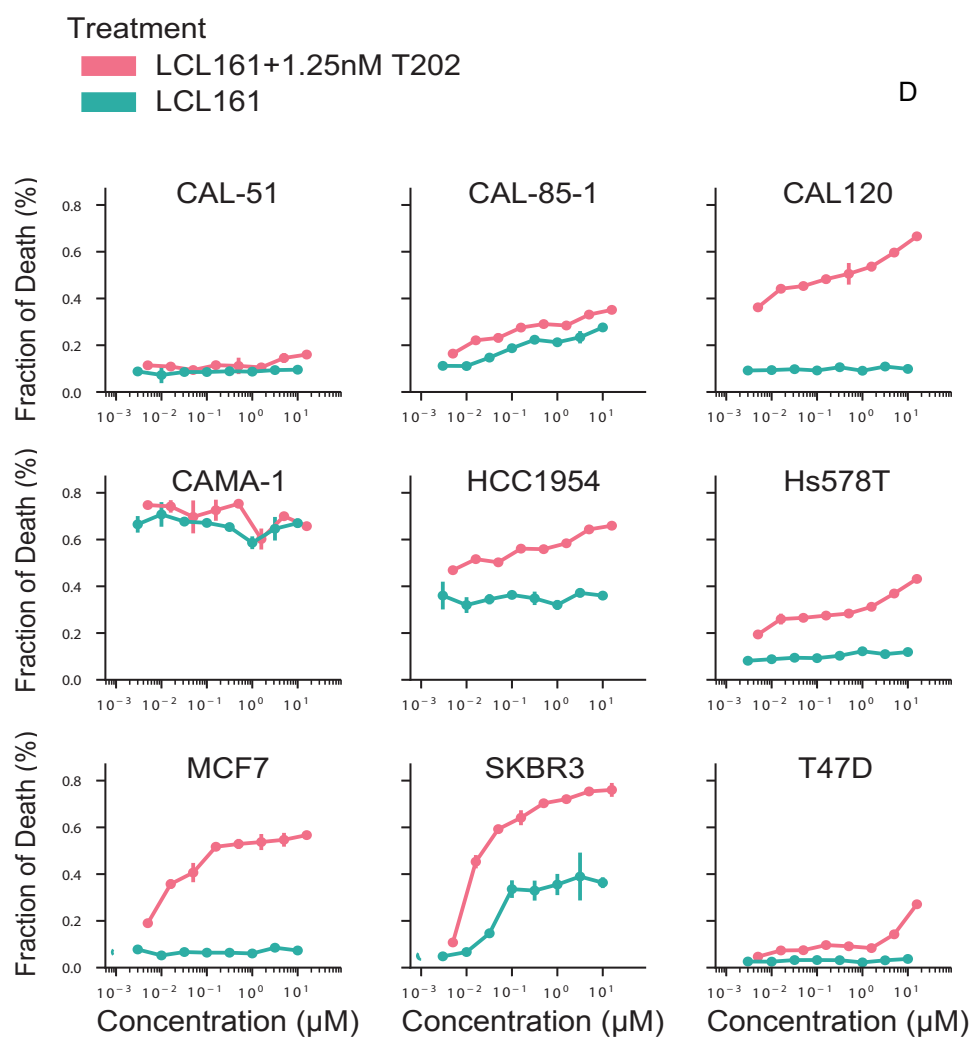


Figure 4.3 (Continued).



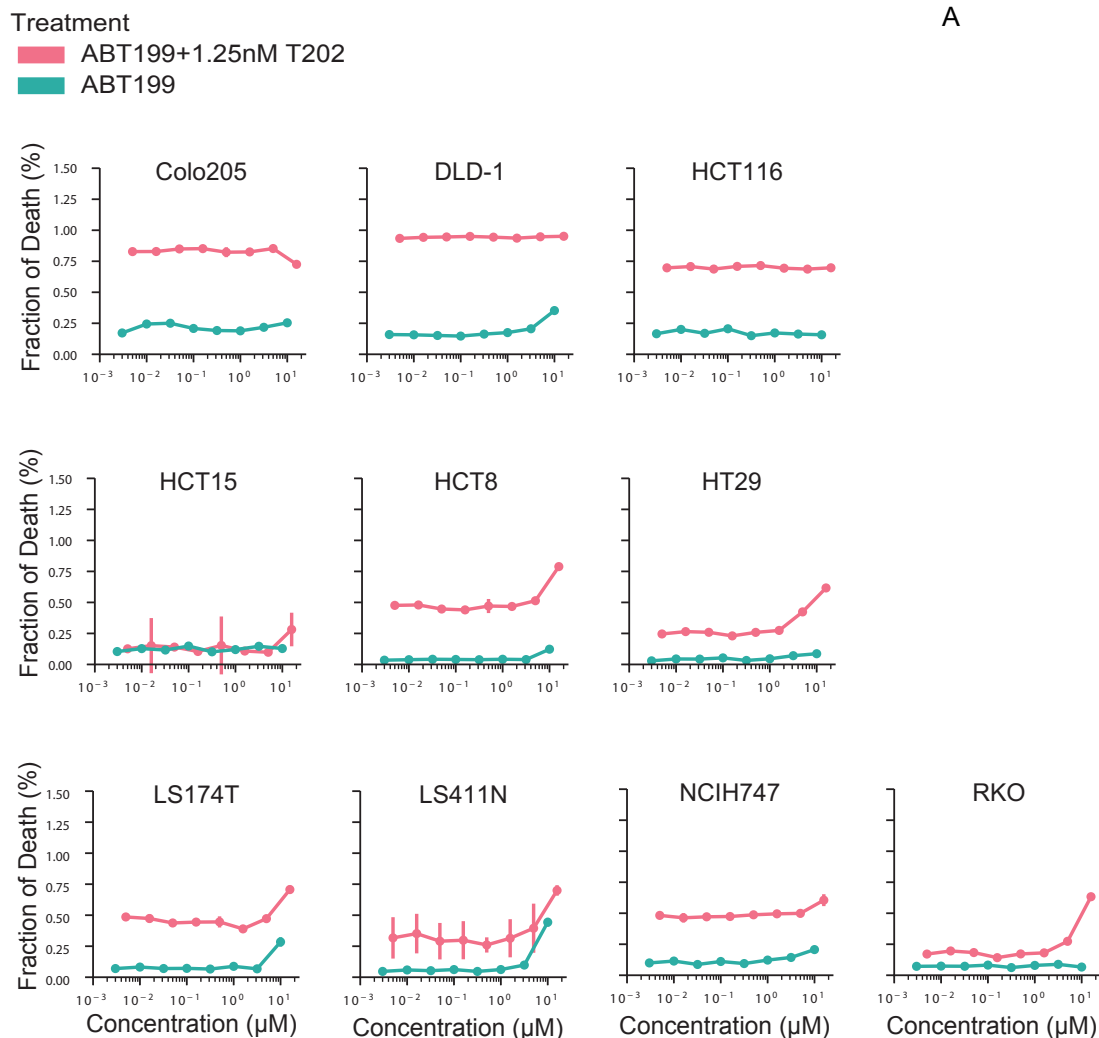


Figure 4.4 Dose-response results suggest that LCL161 might be a candidate for T202-based combination treatment in colorectal cancer cells
A-D Fraction of death in colorectal cancer cells treated with ABT199 (A), ABT263 (B), S63845 (C), and LCL161 (D) single-agent or in combination with T202. Error bars represent the standard deviation of n = 5 technical replicates.

Figure 4.4 (Continued).

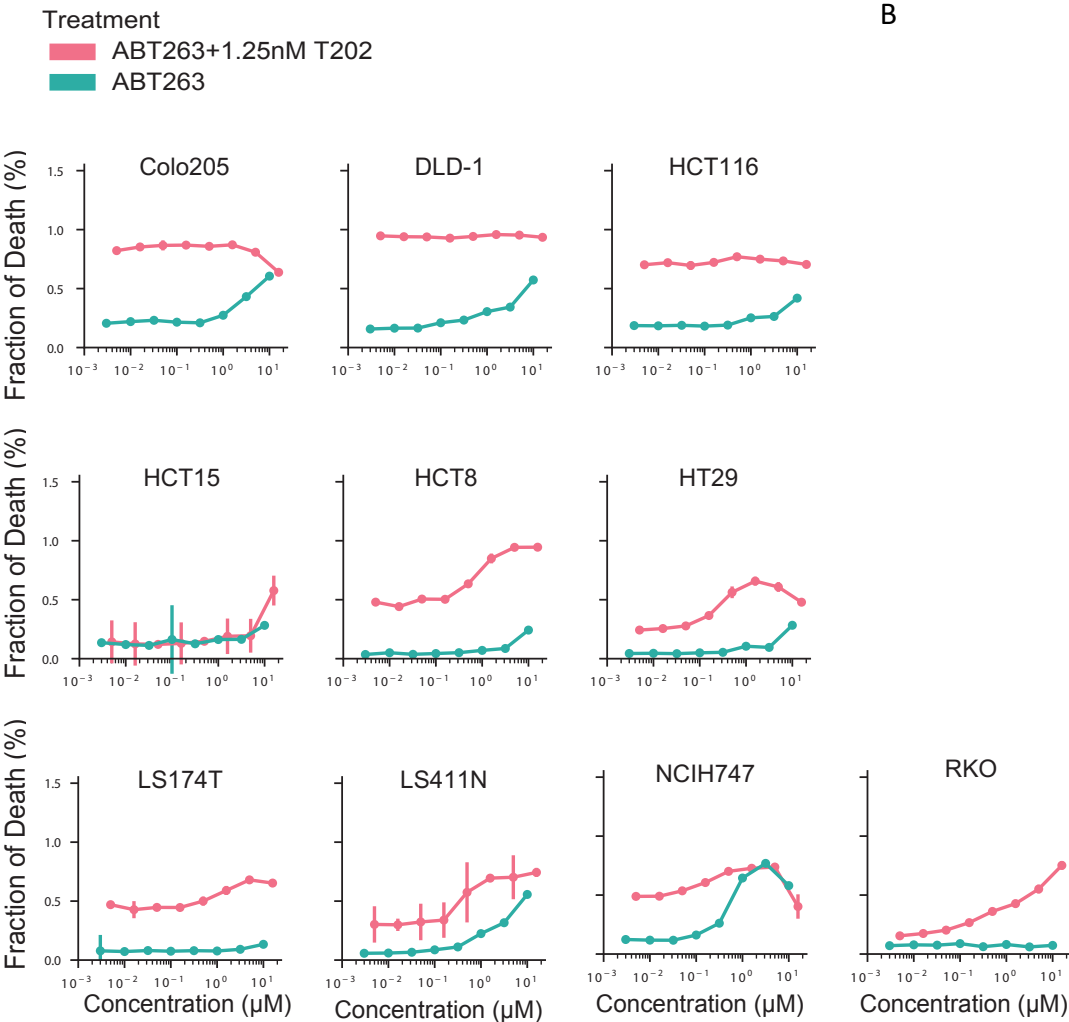


Figure 4.4 (Continued).

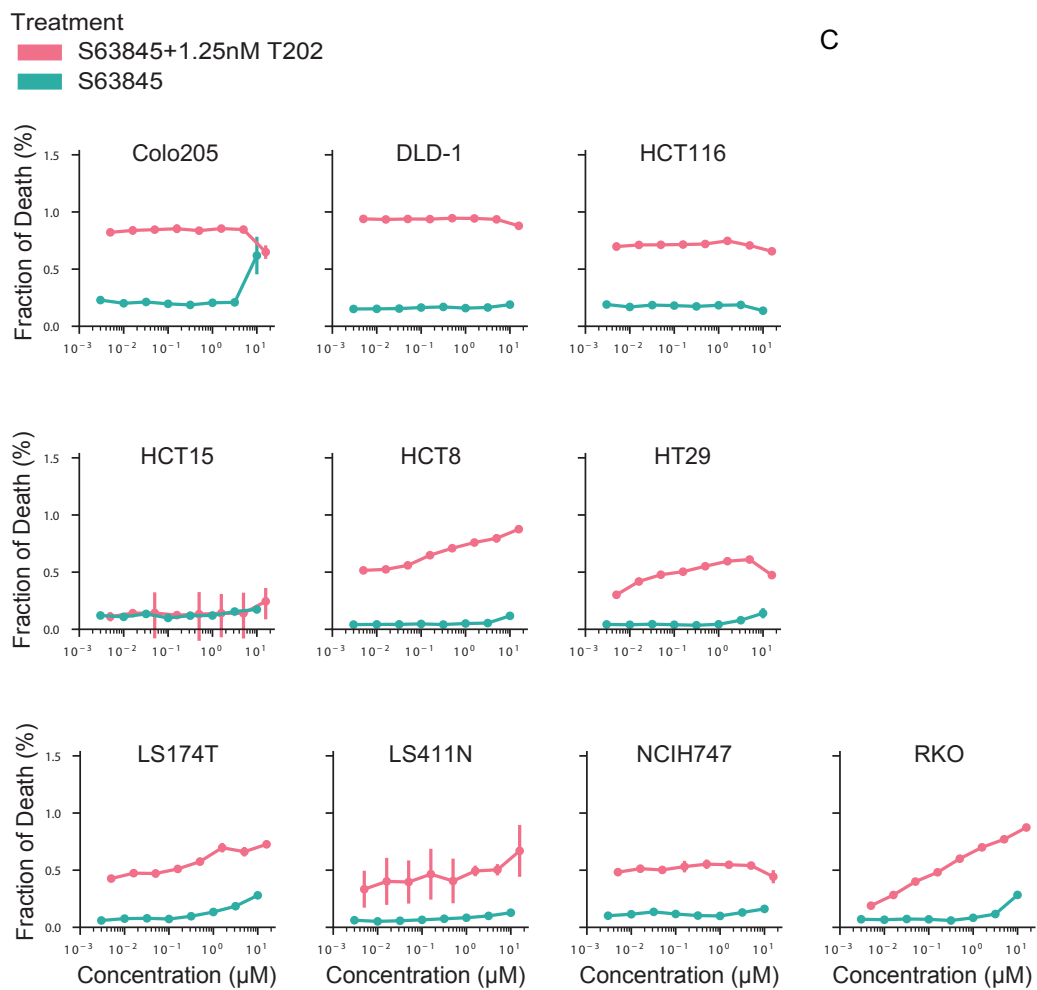
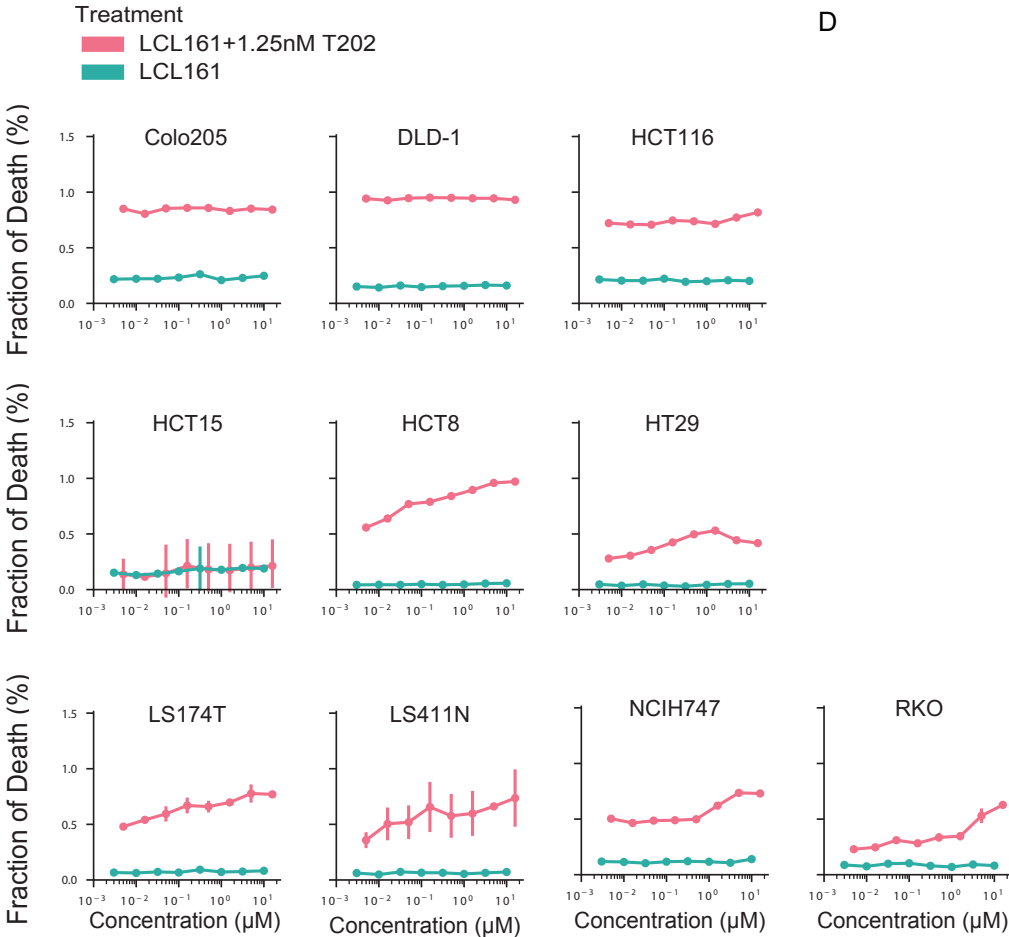


Figure 4.4 (Continued).



4.4 Conclusion

In this study, I characterized the effect of several T191- and T202-based combination treatments in breast cancer and colorectal cancer cell lines, with the rationale that if the extrinsic apoptosis pathway can be activated by using TRAIL family molecules, and at the same time, remove downstream blockades of apoptosis using BCL-2 family protein inhibitors or IAP inhibitors, a higher fraction of cell death may be achieved.

Among the secondary agents tested in combination with T191 and T202, bortezomib and LCL161 had the broadest synergistic effects. The exact target, or targets, of this phenomenon is worth further investigation. Previous pilot studies in our lab have shown that overexpression of XIAP may protect some cancer cells from bortezomib plus TRAIL co-treatment. This may point to a potential overlap between the two molecules, and more generally, between proteasome inhibitors and SMAC mimetics.

5. Chapter 5 - Discussion

5.1 Summary of main findings

This thesis summarizes my work on receptor-mediated apoptosis in cancer cells triggered by TRAIL family molecules. The fundamental question that I was hoping to answer was how heterogeneity in TRAIL response arises among individual cells within the same cancer cell line, and between different cell lines. Previous work in our laboratory has demonstrated that cell-to-cell variability contributes to both the cell fate decision and timing to death (Spencer et al. 2009). In HeLa cells, caspase-8 activity plays an important role in determining cell fate after TRAIL treatment, and a two-step caspase-8 activation model can separate surviving cells from dying cells (Roux et al. 2015). Between different cell lines, however, TRAIL-induced apoptosis can also be affected by other factors such as XIAP and other IAPs (Aldridge et al. 2011; Finlay et al. 2014).

In the first part of my study, I tested HeLa cells' response to two new synthetic TRAIL molecules, T191 and T202, and showed that the two-step model holds for these two new treatments and that caspase-8 activation rates separated surviving versus dying HeLa cells with more than 85% accuracy. Then I extended the assay to three new cell lines, DU145, HCT116, and HT29. DU145 and HT29 showed similar TRAIL-induced caspase-8 activation profiles, and maximum caspase-8 activation rates separated live versus dead subpopulations. In HCT116 cells, on the other hand, cell fate may be determined by the combination of caspase-8 activation and other factors.

I then studied conditions and treatment combinations that can sensitize cancer cells to TRAIL family molecules. 24-hour glutamine depletion increased the fraction of cell

death in HeLa cells and multiple other cell lines treated with TRAIL and T191, but not with BCL-2 family inhibitors ABT199 or S63845, or intrinsic apoptosis-inducing agents such as etoposide or staurosporine. In HeLa cells, TRAIL- and T191-induced caspase-8 activity increased after glutamine starvation. In cell lines that showed increased TRAIL response after glutamine depletion, I observed decreased protein or mRNA levels of FLIP, TRAF2 and MCL-1. Together with the observation that overexpression of FLIP-L and FLIP-S protected cells from TRAIL-induced apoptosis in glutamine-depleted conditions, these findings suggest that the sensitization effect of glutamine deprivation could result from increased caspase-8 activity induced by changes in FLIP and/or TRAF2 levels.

Finally, I explored the potential of T191- and T202-based combination treatment in breast and colorectal cancer cell lines. I tested several secondary agents, including the protease inhibitor bortezomib, BCL-2 family inhibitors ABT199, ABT263, and S63845, and the SMAC mimetic LCL161. In general, colorectal cancer cell lines proved more sensitive to T191 and T202 treatment. I observed synergy between T191/T202 and bortezomib in multiple cell lines from both cancer types. Among the other four more targeted molecules, only the combination of LCL161 with T191/T202 showed some level of consistent synergistic effect in multiple cell lines in both indications.

5.2 Relevance and significance

DR activation is the first step in triggering extrinsic apoptosis. Several groups have sought to develop DR agonists for treatment of cancer, but the clinical benefit demonstrated to date has been limited, and no DR agonists have made it past phase II

clinical trials. Recent research has also focused on engineered TRAIL-like molecules with longer half-life. In this work, I have characterized two such molecules, T191 and T202. The results showed that compared to recombinant TRAIL, T191 achieves comparable activity in multiple cell lines, while T202 had significantly higher activity. Moreover, the potency and efficacy of the two novel molecules could be increased by combination with bortezomib or LCL161, and in glutamine-depleted conditions in various breast and colorectal cancer cell lines.

More broadly, I set out to ask how heterogeneity in cell fate decision arises both between cell lines and among individual cells within the same cell line, and I have demonstrated that DISC activity and caspase-8 dynamics play an important role here. The maximum caspase-8 activation rate threshold is the first barrier that must be overcome in individual cells, and in HeLa, DU145, and HT29 cell lines, this is the major barrier for apoptosis. In HCT116 cells, on the other hand, the priming state, which is the overall balance between pro- and anti-apoptotic BCL-2 family proteins, may also play a critical role in determining cell fate as some HCT116 cells had caspase-8 activity above the cell death threshold, but still survived TRAIL treatment. However, as can be seen from the result of the combination treatment in HCT116, there is little synergy between 1.25 nM T202 and ABT199/263. There could be several reasons for this: 1) at 1.25 nM T202 we may not be able to see great synergistic effect as can be seen in Figure 2.8 there are relatively small number of resistant cells at this dose range; 2) the resistant gene(s) responsible for the high priming state may not be the targets of ABT199 and ABT263 which are Bcl-2 and Bcl-xL.

Another potential explanation of the surviving subpopulation is anastasis. Anastasis is the phenomenon that cells survive effector caspase (caspase-3) activation and DNA damage following the transient apoptotic stimuli (Tang et al. 2012). It has been shown that in HeLa cells that survived cell death express multiple factors that promote proliferation, survival, migration, and angiogenesis (Sun et al. 2017). Specifically, TGF β signaling and transcription factor Snail are responsible for the recovery from apoptosis in HeLa cells (Sun et al. 2017).

With knowledge of the two barriers of cell death, caspase-8 activation and mitochondria priming state, it is possible to design combination treatments that enhance cancer cells' response to TRAIL-family drugs. This work briefly examined potential combination strategies including protease inhibition, anti-apoptotic BCL-2 family protein inhibition, IAP inhibition, and glutamine starvation. Bortezomib and LCL161 both showed synergistic effects when combined with T202, which points to a potential strategy for TRAIL-based treatment of breast and colorectal cancer. In a paper previously published by our lab, the authors observed that bortezomib co-treatment increased TRAIL-dependent time to reach maximum caspase-8 activation rate and pushed HeLa cells up to the 'cell death zone' in the 2D landscape plots (similar to the landscapes shown in Figure 2.5B) (Roux et al. 2015). The catalytically active form of caspase-8 (p18) accumulated in HeLa cells for up to 12 hours with 100 nM bortezomib in combination with 25 ng/mL TRAIL, while in cells treated with TRAIL alone p18 peaked after 6 hours and dropped to baseline level after 12 hours (Roux et al. 2015). It has been seen in human non-small cell lung cancer cells that bortezomib up-regulates DR5 expression, leading to increased TRAIL sensitivity (Liu et al. 2007). In T-cell

lymphoma cells, bortezomib induces upregulation of Noxa and downregulation of Mcl-1 (Ri et al. 2009). Collectively these studies suggest that the sensitizing effect of bortezomib may come from both increasing the caspase-8 activity induced by TRAIL and increasing the priming state of the cancer cells. However, the specific targets of the bortezomib co-treatment and the degree of the sensitization may depend on cell types.

5.3 Future Work

The caspase-8 live-cell assay and the two-step model could be expanded to more types of treatment in HeLa cells, and to more cell lines in order to 1) characterize the caspase-8 dynamics induced by different drugs in different cell lines; 2) investigate how differences in caspase-8 dynamics translate to cell fate decision and how broadly applicable the two-step model is; and 3) explore in cell lines where maximum caspase-8 activation rate does not predict apoptotic decisions whether other factors like caspase-8 activation duration, cumulative caspase-8 activation, or priming state help determine the eventual cell fate.

Another future direction worth investigating is the sensitization effect of bortezomib and glutamine starvation on TRAIL-family molecules. This study and previous ones (Ri et al. 2009) have shown that both these mechanisms sensitize TRAIL response by altering DISC activity. However, given the broad impact of these two perturbations on global protein levels in cells, more than one factor could be contributing to this sensitizing effect. Systematic measurement of protein level changes after bortezomib treatment or glutamine starvation in sensitive and resistant cell lines, together with modeling and genetic/pharmacologic perturbation of key players should give a more

precise view on how bortezomib and glutamine depletion sensitize cells to receptor-mediated apoptosis.

5.4 Closing remarks

In conclusion, I have demonstrated that caspase-8 activity is an important cell fate determinant in TRAIL-induced apoptosis in multiple cell lines. Specifically, the maximum caspase-8 activation rate separated surviving and apoptotic HeLa cells treated with the synthetic TRAIL analogues T191 and T202, and the same model holds for DU145 and HT29 cells treated with TRAIL. Glutamine starvation increased multiple cancer cell lines' response to TRAIL, possible due to reduction in FLIP and TRAF2 levels. Finally, combination treatment with the protease inhibitor bortezomib or the SMAC mimetic LCL161 may potentially increase the potency and efficacy of both T191 and T202 against breast and colorectal cancer.

6. Appendix

6.1 Maximum caspase-8 activation rates separate apoptotic and surviving HeLa cells treated with varying doses of TRAIL, T191, and T202.

As can be seen from Figure 6.1 A, B, and C, in HeLa cells the accuracy scores of the maximum caspase-8 activation rate thresholds were > 85% at most doses of TRAIL, T191, and T202 with two exceptions: T191 at 10 nM (75% accuracy) and T202 at 0.63nM (84% accuracy). The lower scores in these two cases could be due to technical factors, such as lower cell number (e.g. T191 at 10 nM), and/or potential errors in cell death scoring. Thus, I conclude that the maximum caspase-8 activation rate is still a good metric to separate the apoptotic and surviving HeLa cells treated with all three TRAIL agonists.

Figure 1 Maximum caspase-8 activation rates separate apoptotic and surviving HeLa cells treated with varying doses of TRAIL (A), T191 (B), and T202 (C).

Left panel: FRET ratio traces for caspase-8 activity and surviving (blue) or dying (yellow) HeLa cells following treatment with 0.31nM - 10nM TRAIL, T191 and T202. Black dots indicated cell death, where traces were truncated. The histograms show the distribution of maximal FRET ratio levels of the cells. Middle and right panels: derivative of the FRET ratio traces for surviving cells (blue, middle panel) and dying cells (yellow, right panel) for the same traces shown in left panel. Black dots indicate the maximum point value of the derivative. The derivative traces of the dying cells were truncated after commitment of apoptosis. The histograms on the far right show the distribution of maximal point values of the derivatives of the FRET ratio. The redlines indicate the optimal values that separate the two populations with accuracy score shown on top of the panel.

Figure 1 (Continued).

A

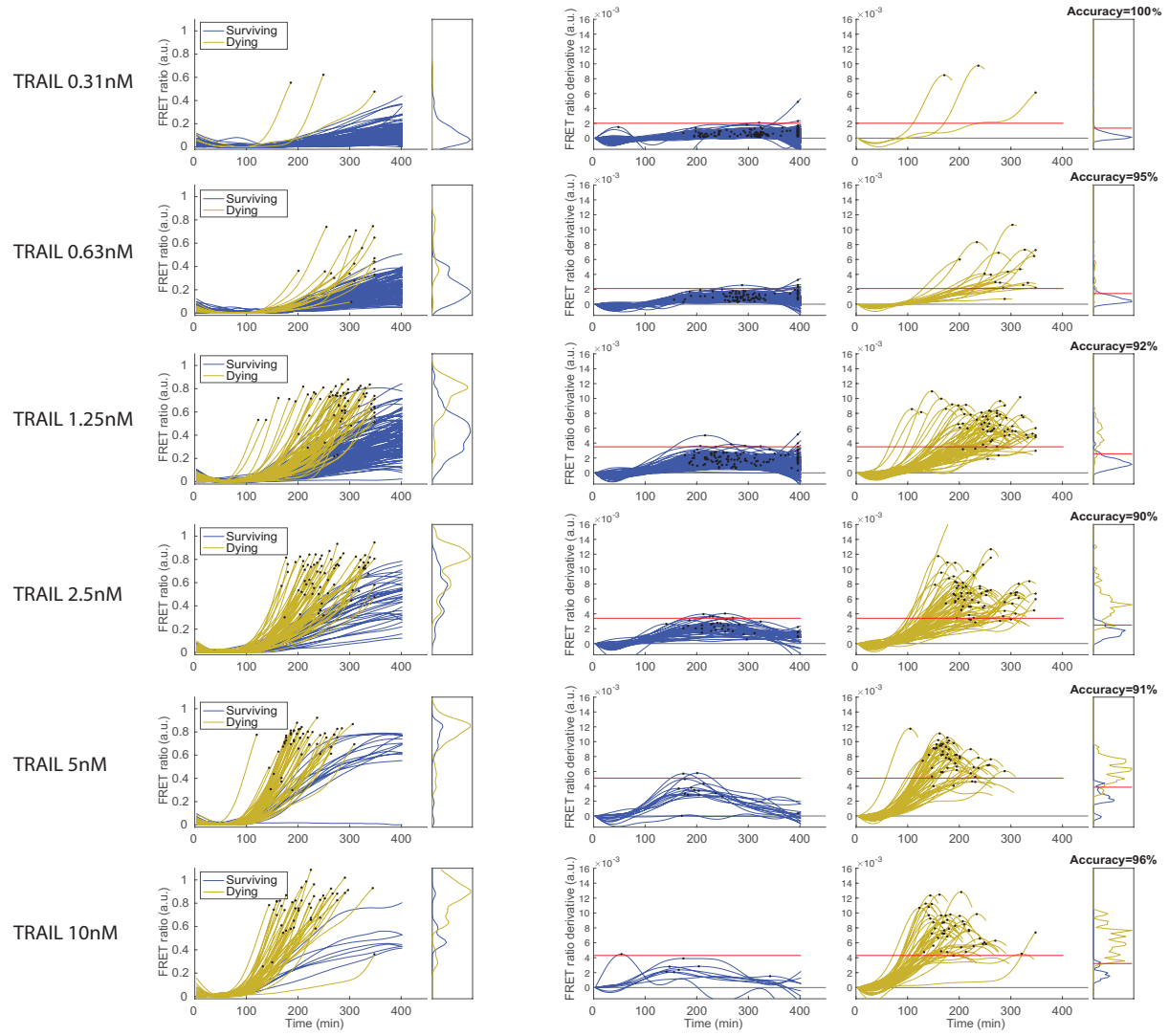


Figure 1 (Continued).

B

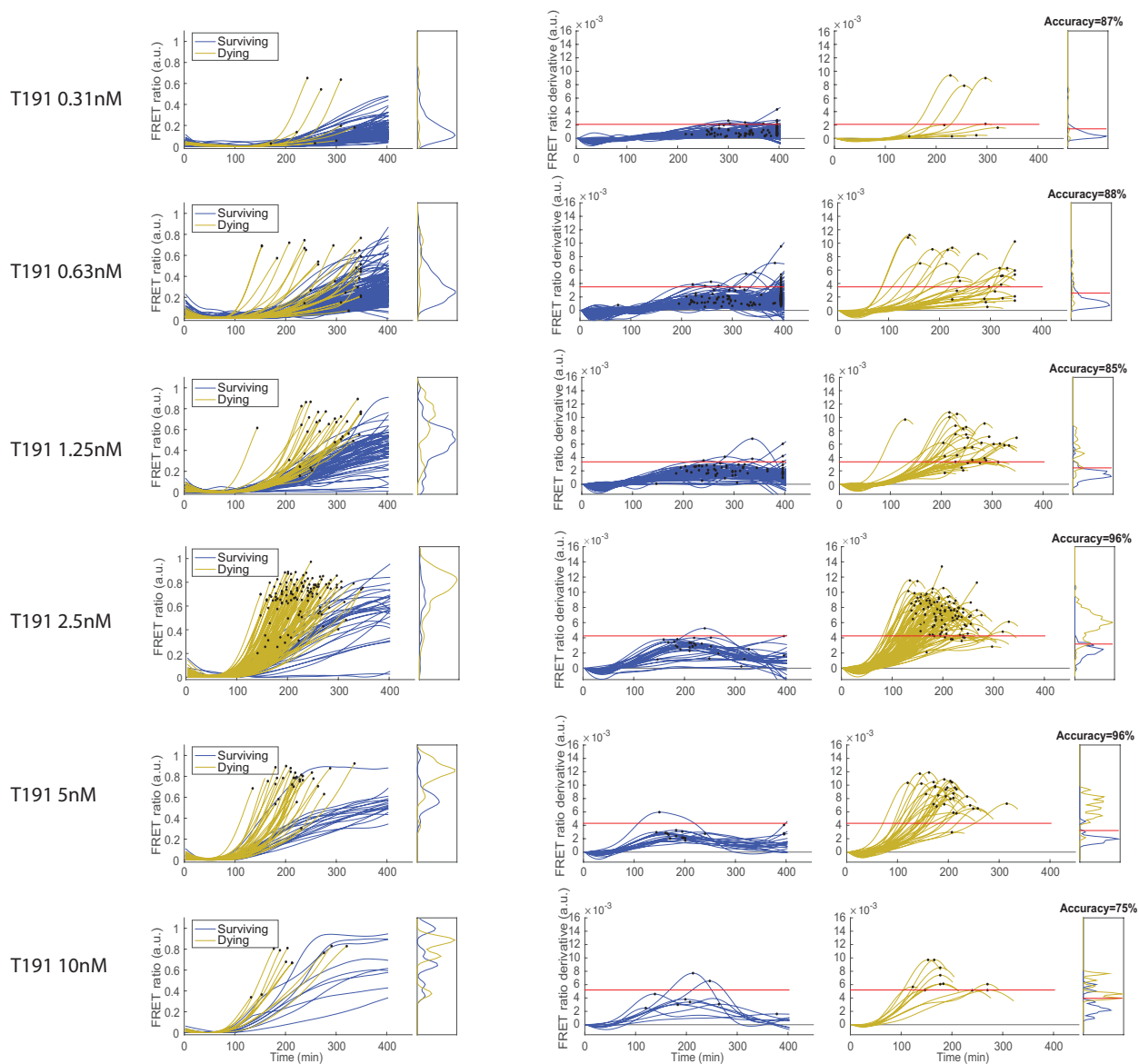
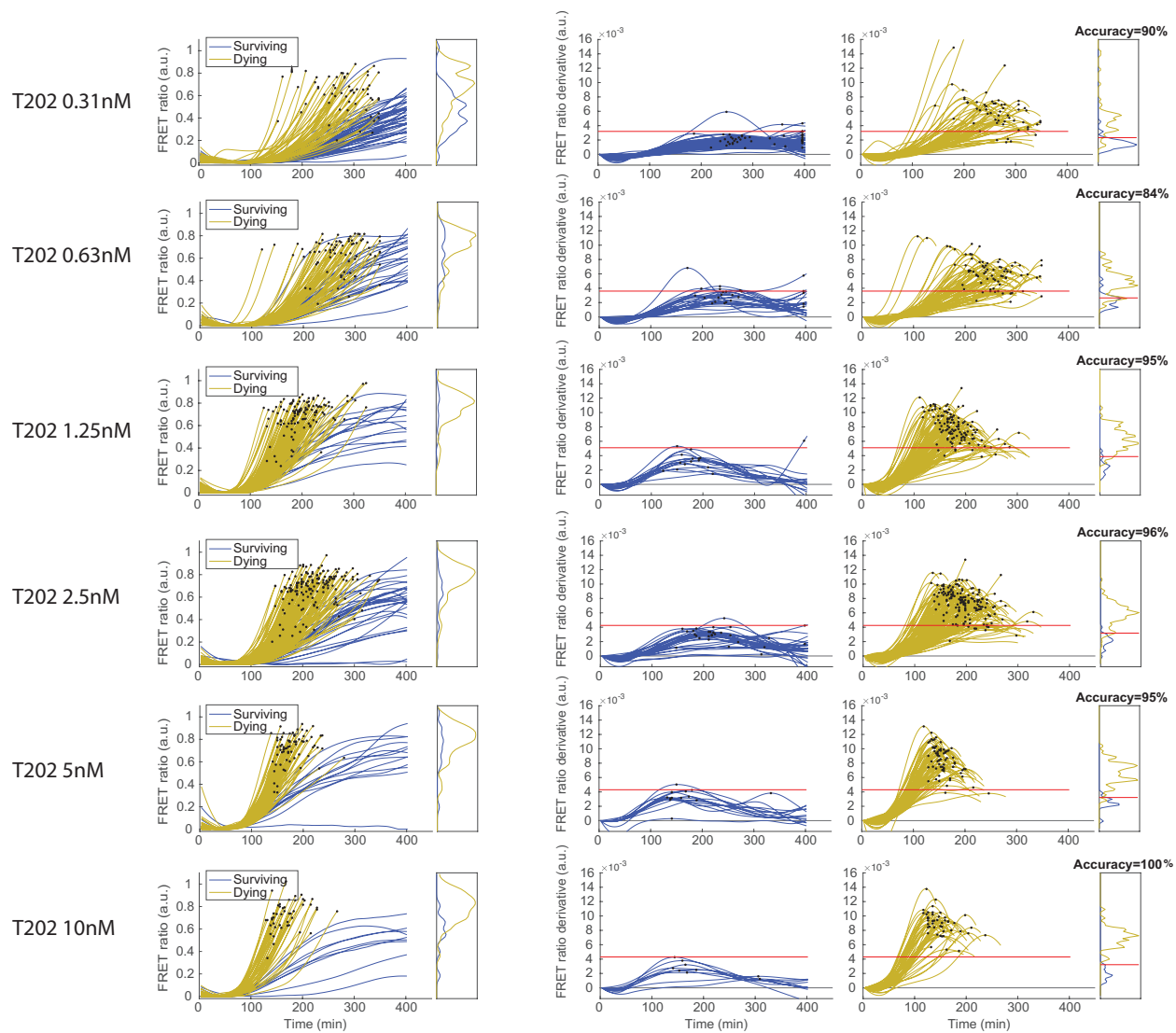


Figure 1 (Continued).

C



6.2 The sensitizing effect of glutamine starvation in 9 additional cancer cell lines

In addition to the 8 cell lines discussed in chapter 3, here I am showing the dose response results of the other 9 cell lines treated with TRAIL and T191 in normal or glutamine-deprived condition.

As shown in Figure 6.2, in the case for TRAIL, HCT116 and H1703 showed synergistic increase in fraction of death after glutamine was depleted from the media as the ‘-gln curves’ were above the expected ‘additive curves’(Figure 6.2A), while in Hs578T and MDA-MB231 cells glutamine starvation has only additive effect as the ‘-gln curves’ overlapped with the expected ‘additive curves’(Figure 6.2B). Glutamine deprivation did not change TRAIL sensitivity of Colo 205, CAL120, MCF7, OVCAR-8, and T47D cells (Figure 6.2B).

In the case for T191, CAL120, H1703 and HCT116 showed synergistic increase in fraction of death after glutamine was depleted from the media (Figure 6.3A), while in Colo 205, Hs578T and MDA-MB231 cells glutamine starvation was only additive to T191’s effect (Figure 6.3B). Similar to TRAIL, glutamine deprivation did not change T191 sensitivity of MCF7, OVCAR-8 and T47D cells (Figure 6.3B).

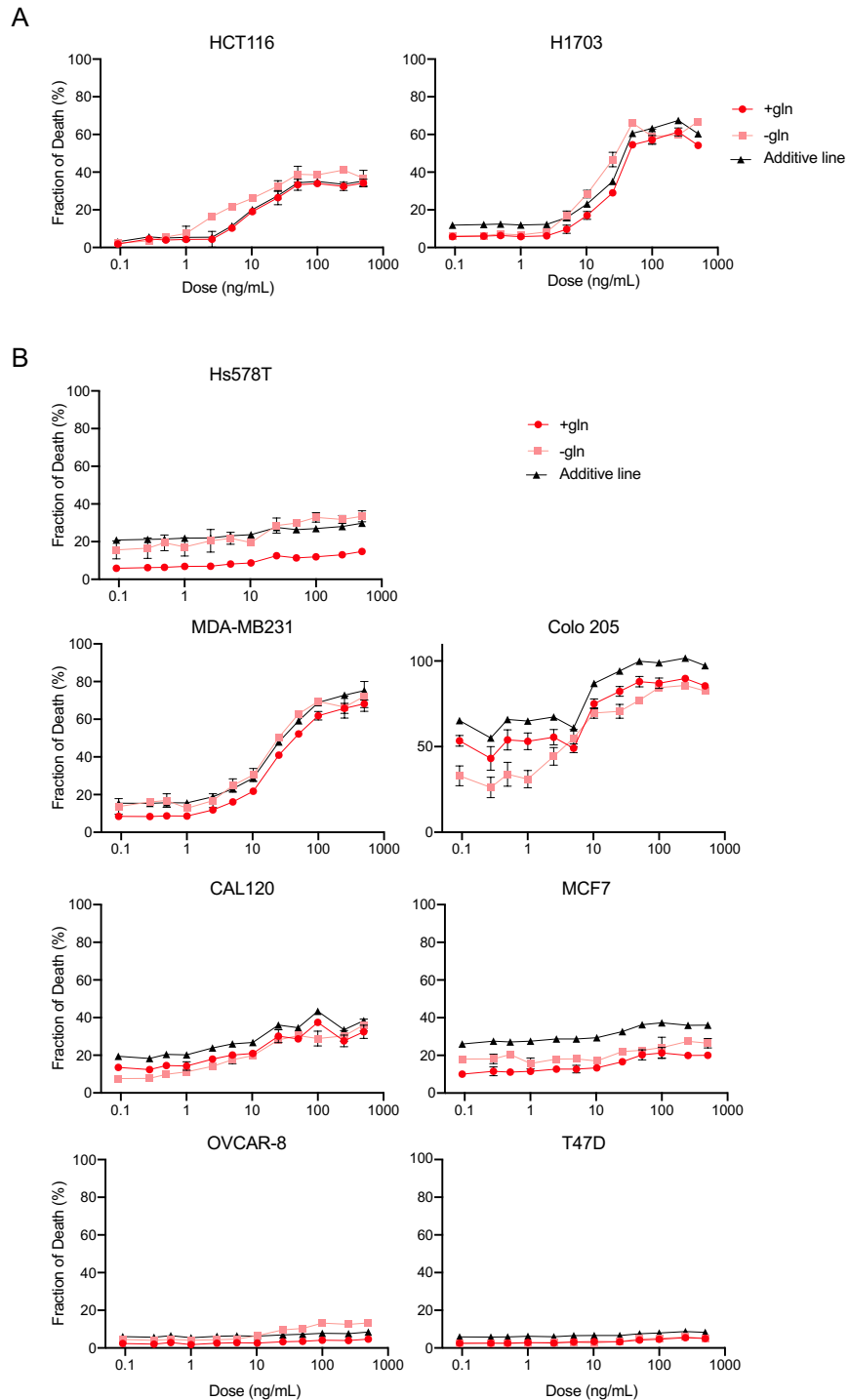
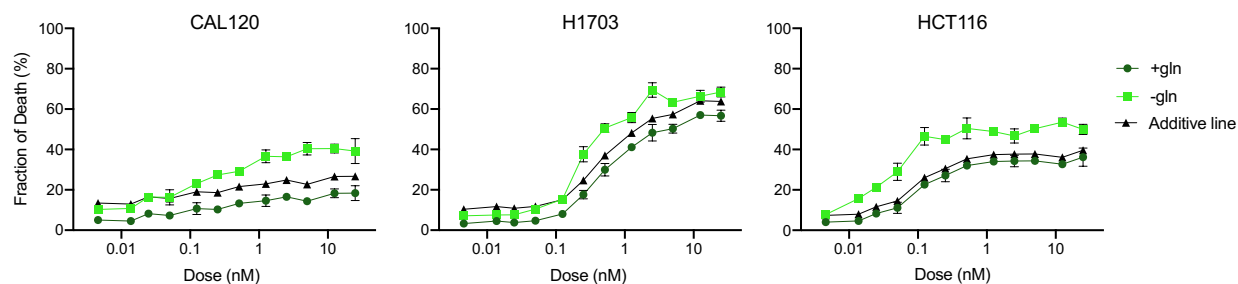


Figure 2 Multiple cancer cell lines showed changes in sensitivity of TRAIL with 24-hour pre-culturing in glutamine depleted media.

(A-B) Dose response curves of cell lines sensitive (A), or resistant (B) to the sensitizing effect of 24-hour glutamine starvation before TRAIL treatment; median with glutamine (red), or with glutamine depletion (pink). The black curves ('Additive line') represent the expected fraction of death if glutamine starvation has only additive effect to TRAIL response. Error bars represent the standard deviation of $n = 3$ technical replicates.

A



B

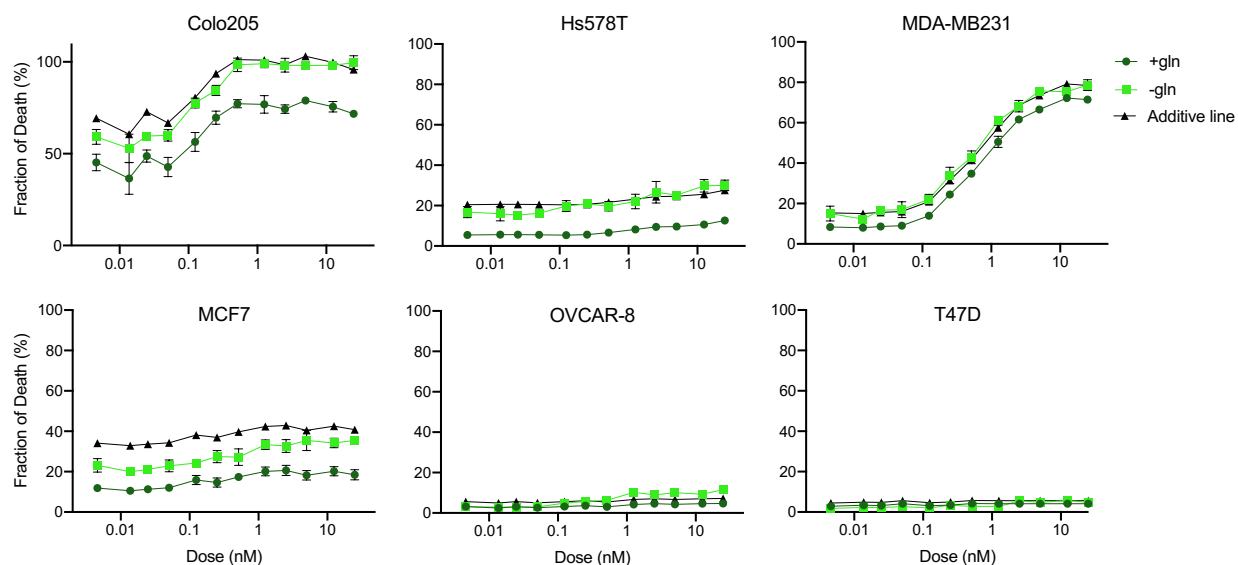


Figure 3 Multiple cancer cell lines showed changes in sensitivity of T191 with 24-hour pre-culturing in glutamine depleted media.

(A-B) Dose response curves of cell lines sensitive (A), or resistant (B) to the sensitizing effect of 24-hour glutamine starvation before T191 treatment; median with glutamine (green), or with glutamine depletion (light green). The black curves ('Additive line') represent the expected fraction of death if glutamine starvation has only additive effect to T191 response. Error bars represent the standard deviation of $n = 3$ technical replicates.

6.3 T191 triggers stronger caspase-8 activity than therapeutic DR antibodies apomab, conatumumab and mapatumumab

Historically, TRAIL is believed to be the only molecule that has a higher potency and efficacy than the therapeutic DR antibodies. When compared with existing therapeutic DR monoclonal antibodies including apomab, conatumumab and mapatumumab, T191, too, triggers significant higher caspase-8 activation in HeLa cells, suggesting T191 is a true competitor molecule to the natural ligand TRAIL (Figure 6.4).

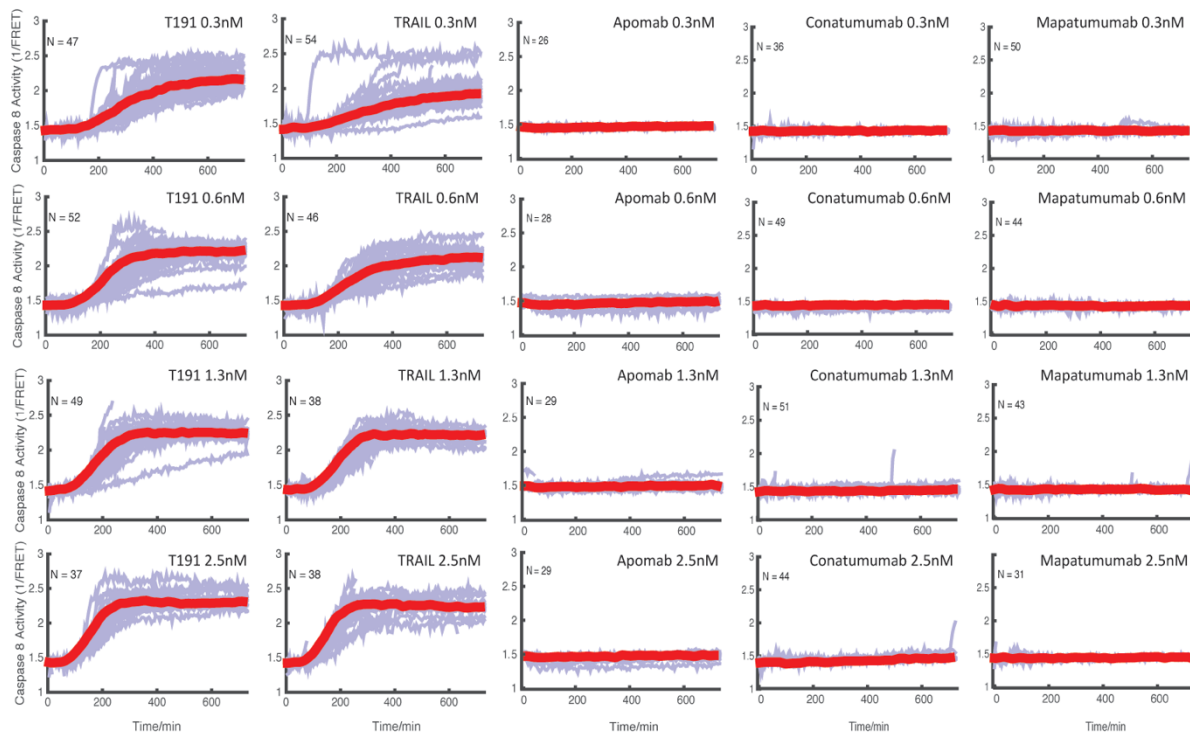


Figure 4 T191 triggers stronger caspase-8 activity than therapeutic DR antibodies apomab, conatumumab and mapatumumab. Caspase-8 activity traces of individual cells (blue traces) and the population average (red traces) with varying doses of treatment.

6.4 A new set of live-cell reporters for better tracking of fast-moving cells

A natural extension of the live-cell caspase-8 activity assay is to measure caspase-8 activity in cell lines other than the four tested (HeLa, DU145, HT29 and HCT116) to assess whether the maximum caspase-8 activity is deterministic of apoptotic response. The major hurdle to apply the same experimental setup to cancer cell lines in general is that the whole-cell tracking mask only works in cells that grow relatively sparsely. For example, the tracking algorithm fails in HeLa cells if the confluency is over 60-70%. In order to solve this problem, I have constructed a new set of live-cell reporters. The system consists of a mCherry nuclear marker for tracking purpose, and a new mTurquoise-mCitrine FRET pair (ICRP2) that is much brighter than the old CFP-YFP pair. As is shown in Figure 6.5A, the new FRET pair ICRP2 is ~10 times brighter than the original ICRP, with comparable FRET traces in HeLa cells treated with 500ng/mL TRAIL (Figure 6.5B). ICRP2 also offers 2-fold wider dynamic range of the FRET ratio comparing with ICRP (Figure 6.5B).

The two reporters were constructed as following: 1) ICRP2 was constructed by replacing the CFP and YFP in ICRP by mTurquoise and mCitrine; 2) new tracking and MOMP reporter (NLS-MC-BerFP-MO2) was constructed by inserting Kozak sequence (GGACCCAGATCTCGAGCACCATG), NLS sequence (AAGAAGCGTAAGGTA), BerFP (ATGGTGTCTAAGGGCGAAGAGCTGATTAAGGAG), P2A sequence (GCTGAAGCAGGCCGGCGACGTGGAGGAGAACCCCGGCCCC) and SMAC (TGGCGGCTCTGAAGAGTTG) after the multi-cloning site in N1 vector.

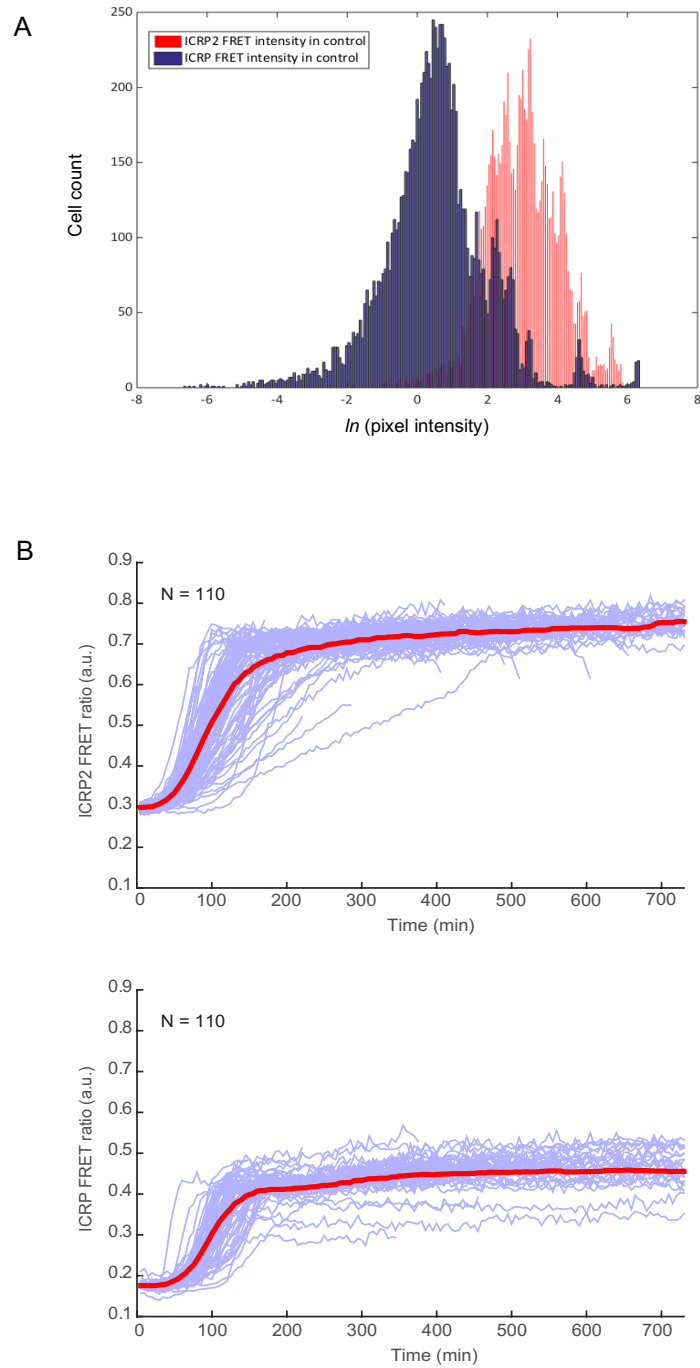


Figure 5 A new set of live-cell reporters for better tracking of fast-moving cells
A) New FRET pair ICRP2 is ~10 fold brighter than old ICRP. B) ICRP2's trace is comparable in HeLa cells treated with 500 ng/ml.

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