4-MU Synergistically Kills Cancer Cells with TRAIL and Suppresses Reversal of Cells from TRAIL-induced Apoptosis

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Declaration

This thesis presentation is based on my original research work. Contributions from additional sources are made clear by due references to corresponding literature and acknowledgement of collaborative research and discussion. This work was carried out under the guidance of Professor FUNG, Ming Chiu at the Chinese University of Hong Kong, Hong Kong SAR.

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Abstract of thesis entitled:

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TRAIL has been widely investigated as an anti-cancer agent due to its high efficacy *in vitro* and its safety to normal cells. However, TRAIL-based agents only showed modest effect in clinical studies because of TRAIL resistance. In addition to apoptosis, TRAIL has also been reported to promote pro-survival signalings, cell migration and metastasis. One of the current strategies in the development of TRAIL-based therapeutics focuses on the search of sensitizing agents that help overcome TRAIL resistance without increasing harm to normal cells.

This study reports a novel combination of TRAIL and 4-methylumbelliferone (4-MU) which can kill HeLa cells and HepG2 cells synergistically without cytotoxicity to Hs68 non-tumorigenic cells. This combination also effectively inhibited cancer cell proliferation and potentiated apoptosis by accumulation of tBid, down-regulation of anti-apoptotic proteins and inhibition of Akt. More importantly, 4-MU could suppress the recovery of HeLa cells from TRAIL-induced apoptosis, a p rocess previously implicated to be associated with cancer relapse and tumor heterogeneity. This study has provided solid evidences substantiating further research on TRAIL-4-MU combination.

摘要

腫瘤壞死因子相關凋亡誘導配體 (TRAIL) 在體外實驗中有良好抗癌作用，且不會傷害正常細胞，使之得到廣泛研究，成為近年熱門的新抗癌分子。然而 TRAIL 在臨床實驗中並沒有顯著抗癌功效，一般認為人體腫瘤細胞對 TRAIL 具有耐藥性。研究文獻亦指出，除了細胞凋亡外，TRAIL 亦會誘發細胞存活機制、促進細胞移行及癌細胞轉移。目前，對於 TRAIL 相關藥品抗癌作用的研究有幾個大方向，其中之一就是尋找良好的增敏分子。良好的增敏分子應能夠增力癌細胞對 TRAIL 的敏感性，對抗癌細胞對 TRAIL 的耐藥性，同時不能殺傷正常細胞。

本研究揭示了一個全新的抗癌藥物聯合。當 TRAIL 聯合 4-甲基傘形酮(4-MU)能產生協同作用，殺傷 HeLa 癌細胞和 HepG2 癌細胞而不會傷害 Hs68 正常細胞。此組合能有效抑制癌細胞生長，並透過增加 tBid 蛋白表達、減少抗凋亡蛋白表達及抑制 Akt 來促進細胞凋亡。更為重要的是，4-MU 能抑制 HeLa 癌細胞自 TRAIL 誘導凋亡的恢復和逆轉。而癌細胞凋亡逆轉一般被視為與癌症復發及腫瘤多樣性有關。本研究提供了實質證據，支持對 TRAIL-4-MU 組合的後續研究。

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List of abbreviations

%	Percent
β	Beta
ºC	Degree Celsius
>	Higher than or more than or larger than
<	Less than or smaller than
±	Plus-minus
4-MU	4-methylumbelliferone
AEs	Adverse effects
Akt	Protein kinase B
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor 1
ATCC	American Type Culture Collection
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
bFGF	Basic fibroblast growth factors
Bid	BH3 interacting-domain death agonist
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
Caspase	Cysteine-dependent aspartate-directed protease
CI	Combination index
CMV	*Cytomegalovirus*
CO_2	Carbon dioxide
Cox-2	Cyclooxygenase-2
Ctrl	Control
Da	Dalton
DcR	Decoy receptor
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DR	Death receptor

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Eq	Equation
EMT	Epithelial–Mesenchymal Transition
ERK	Extracellular regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FADD	Fas-associated protein with death domain
FLICE	FADD-like interleukin-1 beta-converting enzyme
FLIP	Flice-like inhibitory protein
g	Gram
g	Relative centrifugal force, unit of times gravity
GlcA	D-glucoronic acid
Glc-NAc	N-acetylglucosamine
h	Hour
HA	Hyaluronan / Hyaluronic acid
HAS	Hyaluronan synthase
HMWHA	High-molecular-weight hyaluronan
HRP	Horseradish peroxidase
HYAL	Hyaluronidase
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
IκB	Inhibitor of kappa-B
IL	Interleukin
JNK	Jun NH2 terminal kinase
k	kilo
LMWHA	Low-molecular-weight hyaluronan
MAPK	Mitogen-activated protein kinase
MCB	Metaplastic carcinomas of breas
Mcl-1	Myeloid cell leukemia 1
mg	Milli-gram
min	Minute
ml	Milli-litre
mM	Milli-Molar
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilization
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide

NF-kappaB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nano-gram
NK cells	Natural killer cells
nm	Nano-metre
NSCLC	Non-small cell lung cancer
OD	Optical density
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PI3K	Phosphatidylinositide 3-Kinases
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
Rhamm	Hyaluronan-mediated motility receptor
RIP1	Receptor-interacting protein 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNase	Ribonuclease
ROCK1	Rho-associated, coiled-coil-containing protein kinase 1
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SMAC/DIABLO	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
STS	Staurosporine
TAM	Tumor-associated macrophage
tBid	Truncated BH3 interacting-domain death agonist
TBST	Tris-Buffered Saline and Tween 20
TGF	Transforming growth factor
TNFR	Tumor necrosis factor receptor
TRAF2	TNF receptor-associated factor 2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
µg	Micro-gram
µl	Micro-litre
µM	Micro-Molar
uPA	Urokinase-type plasminogen activator
v/v	Volume per volume
w/v	Weight per volume
XIAP	X-linked inhibitor of apoptosis protein

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Chapter 1 Introduction

1.1 TRAIL

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as APO2 ligand (APO2L), belongs to the death receptor (DR) family (Pitti et al, 1996). It is mostly expressed on the surface of immune cells and it also exists in a soluble form (Ehrlich et al, 2003). TRAIL has been widely investigated as an anti-cancer agent as it induces apoptosis in a wide variety of cancer cells, *in vitro* and *in vivo*, without affecting normal cells (Ashkenazi et al, 1999; Walczak et al, 1999).

The physiological role of TRAIL includes immune surveillance and tumor suppression by exerting antiviral and antitumor cytotoxicity. TRAIL can be induced by antigens like lipopolysaccharides and cytokines like interferons (IFN) (LeBlanc & Ashkenazi, 2003; Dimberg et al, 2013). It can selectively induce apoptosis in human CMV-infected fibroblasts without harming uninfected cells. This selective killing can be potentiated by IFN-gamma, which down-regulates the expression of TRAIL-Rs in uninfected cells and lowers the basal level of NF-kappaB activation, an apoptosis-inhibiting mechanism, in infected cells (Sedger et al, 1999). The tumor surveillance role of TRAIL was first shown by Walczak and colleagues, who

demonstrated that administration of soluble recombinant TRAIL reduced the size of human mammary adenocarcinoma xenografts in mice (Walczak et al, 1999). Xenografts of renal carcinoma and mammary carcinoma grew faster and became more metastatic in both TRAIL -/- mice and mice treated with antagonistic anti-TRAIL antibody (Cretney et al, 2002). TRAIL is expressed on liver natural killer cells (NK cells) in mouse. Blocking TRAIL with antagonistic antibody significantly increased liver metastases of several TRAIL-sensitive cell lines. And such anti-metastatic effect is IFN-gamma dependent (Takeda et al, 2001). These findings confirm the contribution of TRAIL to immune surveillance and host defense against tumor formation and metastasis.

At least five TRAIL receptors have been reported in human. TRAIL receptor 1 (TRAIL-R1, also known as death receptor 4, DR4) and TRAIL receptor 2 (TRAIL-R2, also known as death receptor 5, DR5) are transmembrane receptors that carry a cytoplasmic death domain, which can convey apoptotic signals to the cells when TRAIL is bound (Sheridan et al, 1997). The structure of TRAIL-DR5 complex has been elucidated in 1999 as shown in Figure 1. TRAIL receptor 3 and TRAIL receptor 4 (TRAIL-R3 and TRAIL-R4, also known as decoy receptor 1 and 2, DcR1 and DcR2, respectively) lack a functional death domain and do not transmit signals to activate apoptosis (Marsters et al, 1997; Pan et al, 1997; Sheridan et al, 1997). The fifth binding protein is osteoprotegerin (OPG), a soluble TNFR family member, but its affinity to TRAIL is low at physiological temperature (Emery et al, 1998; Truneh et al, 2000).

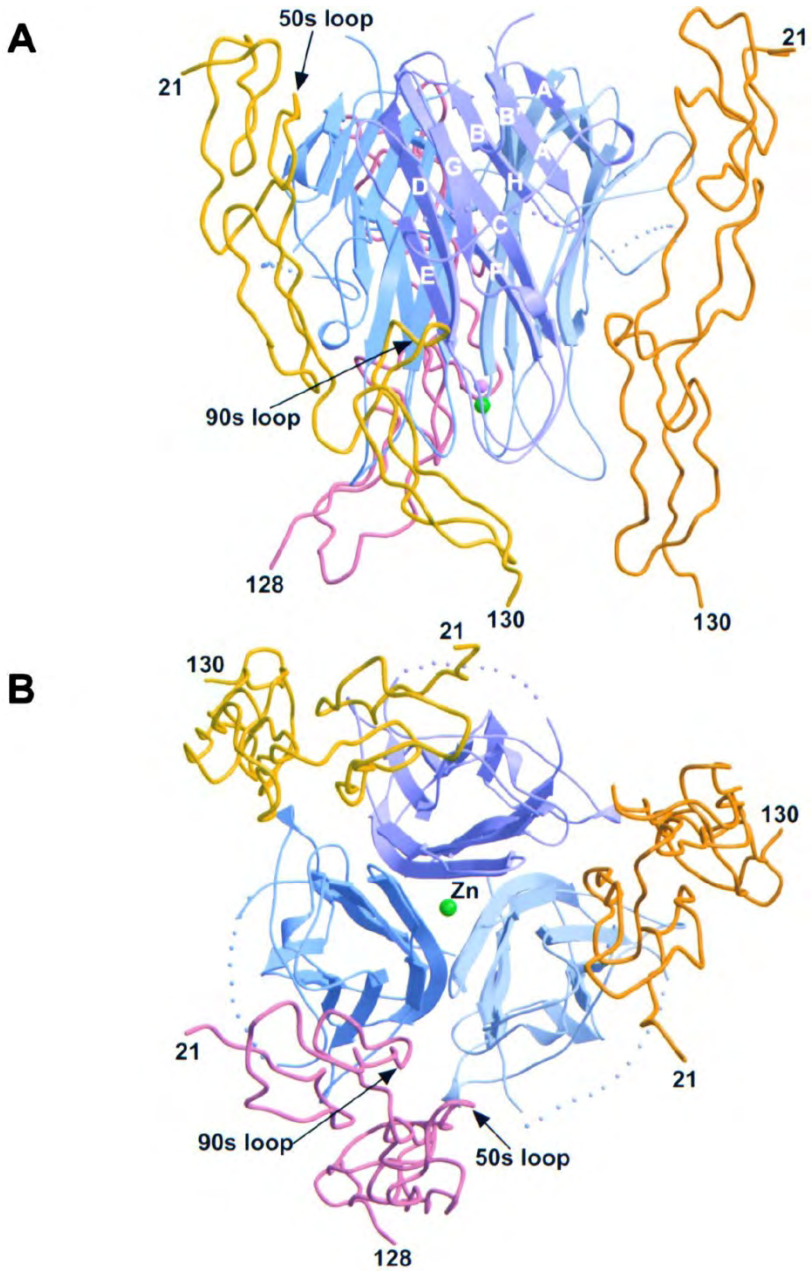


Figure 1. Structure of TRAIL-DR5 complex. The TRAIL trimer is shown as ribbon rendering in gradations of blue. The three DR5 receptors are rendered as tubes in yellow and orange colors. The disordered loop (residues 132–143) in TRAIL is rendered as small spheres. The bound zinc atom is in green, and the bound chloride ion is pink. β strands and relevant loops are labeled. (A) Side view. In this orientation, the membrane of the receptor-containing cell is at the bottom of the figure. (B) View down the 3-fold axis of the complex, perpendicular to (A).

Adapted from Hymowitz et al, 1999.

1.1.1 **TRAIL apoptotic signaling**

TRAIL induces apoptosis mainly via extrinsic pathway as shown in Fig. 1. When ligand (TRAIL or agonistic antibodies) binds to functional TRAIL-R1 or TRAIL-R2, the receptors will trimerize, enabling the recruitment of adaptor protein Fas-associated protein with death domain (FADD) and procaspase-8, an initiator caspase, to form a co mplex named death-inducing signaling complex (DISC). Procaspase-8 will be cleaved and activated, which then further cleave and activate downstream effector caspase-3. Cleavage and activation of caspase-8 can be inhibited by cellular flice-like inhibitory protein (cFLIP), which is a non-functional homolog of procaspase-8 and can compete with procaspase-8 for FADD binding. In some cells (called type I cells), the activated initiator caspases (caspase-8/10) can directly generate sufficient lethal level of effector caspases (caspase-3/7) for cell suicide. In some other cells (called type II cells), the level of activated effector caspase3/7 cleaved by initiator caspase-8/10 is not enough for cell death machinery. A process called mitochondrial outer membrane permeabilization (MOMP) is required to amplify the caspase cascade.

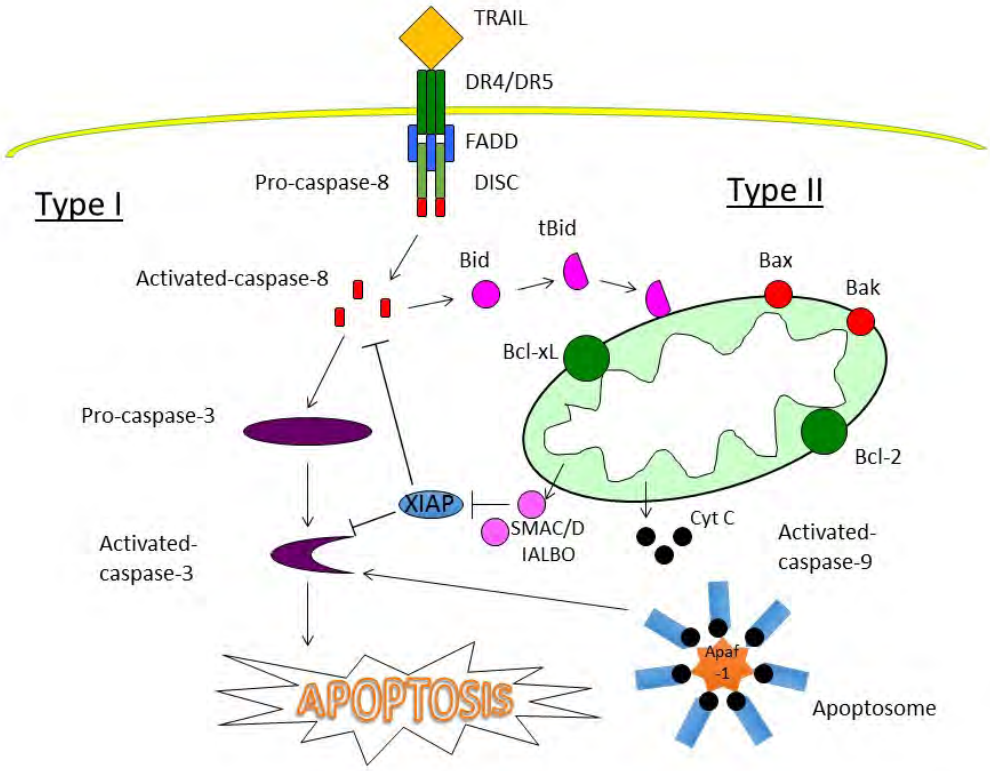


Figure 2. Typical extrinsic apoptotic pathways induced by TRAIL in cells after binding to apoptotic TRAIL receptors. The TRAIL receptors (DR4/DR5) then trimerize and recruit Fas-associated protein with death domain (FADD) and the initiator procaspase-8 to form death-inducing signaling complex (DISC). Procaspase-8 is then cleaved and activated. In some cells (type I cells), the activity of caspase 8 is robust enough to cleave sufficient lethal level of effector procaspase-3 for triggering apoptosis. In other cells (type II cells), the amount of caspase-3 directly cleaved by caspase-8 is not enough for cell suicide. Truncated Bid (tBid) cleaved by caspase-8 to activate pro-apoptotic proteins BAX/BAK to trigger mitochondrial outer membrane permeabilization (MOMP). Cytochrome c is then released from mitochondrial intermembrane space to form apoptosome with Apoptotic protease activating factor 1 (APAF-1) and initiator pro-caspase-9. Activated caspase 9 would further cleave more pro-caspase 3 to yield lethal level of activated effector caspases for apoptosis.

MOMP is the event controlling the mitochondrial apoptotic pathway. The integrity of mitochondrial outer membrane depends on the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. If enough pro-apoptotic Bcl-2 family proteins BAX and BAK are activated, they can self-assemble to form pores on the mitochondrial outer membrane. In type II cells, TRAIL induces caspase-8 activation, which cleaves the pro-apoptotic Bcl-2 family protein BH3 interacting-domain death agonist (BID) to truncated Bid (tBid). tBid can interact with and activate BAX/BAK to trigger MOMP. In intrinsic apoptotic pathway, DNA damage or other cellular stresses could also tip the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins, causing the trigger of MOMP.

Once the outer membrane of mitochondria is permeabilized, pro-apoptotic proteins in the intermembrane space, mainly cytochrome c and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO), are released. Similar to the formation of DISC in extrinsic pathway, another protein complex called apoptosome will be formed in mitochondrial apoptotic pathway. Apoptosome consists of cytochrome c, Apoptotic protease activating factor 1 (APAF-1) and pro-caspase-9, which is cleaved and activated in apoptosome. The activated initiator caspase-9 will then cleave and activate effector caspase-3/7 for cellular demolition.

It was previously thought that difference in the rate of formation of active DISC distinguishes type II cell from type I cells (Peter & Krammer, 1998). Recently,

X-linked inhibitor of apoptosis protein (XIAP), an anti-apoptotic protein that directly inhibits effector caspases and ubiquitinate caspase 3 for proteasome degradation, was found to be the crucial factor in differentiation of type I and type II cells. Type II cells have a high XIAP/caspase 3 ratio that prevents full caspase 3 activation by caspase 8 only (Jost et al, 2009). Deleting the RING domain of XIAP shut down its E3 ligase activity and switched the cells from type II to type I, revealing the role of XIAP in determining caspase 3 turnover and cell fate determination (Aldridge et al, 2011).

1.1.2 **TRAIL-based anticancer therapy**

Selective killing of cancer cells is not the only advantage of TRAIL that put it under the spotlight. Conventional chemotherapy causes DNA damage or cellular stresses to induce intrinsic apoptosis in cancer cells. To trigger intrinsic apoptosis, the DNA/cellular damage has to be recognized, mainly by the p53 tumor suppressor protein, which is also regarded as the guardian of the genome (Lane, 1992). However, more than half of all cancer cells do not have a functional p53 due to inactivating mutaions (Hollstein et al, 1994), causing resistance to conventional chemotherapy in those cancer cells. TRAIL can induce apoptosis in cancer cells with p53 deleted and irrespective of their proliferation status (Ashkenazi et al, 1999), opening a door to the eradication of cancer cells resistant to conventional therapies.

1.1.3 TRAIL-based therapy in clinical trials

In spite of the robust selective cancer-killing effect in preclinical studies, the results of TRAIL-based therapy to date are disappointingly modest (Dimberg et al, 2013). Soluble recombinant human TRAIL ligand (rhTRAIL, also called dulanermin), which targets both DR4 and DR5, and agonistic antibodies targeting the functional receptors DR4 (mapatumumab) and DR5 (conatumumab, lexatumumab, tigatuzumab, drozitumab and LBY-135) have entered clinical trials for treating cancer. More than 35 phase I/II clinical trials of TRAIL-based therapy, both monotherapy and in combination, have been reported. TRAIL-based agents were found to be safe and well-tolerated in all trials (Reviewed in Dimberg et al, 2013; Lemke et al, 2014), reflecting the safety and broad tolerability of all TRAIL-based agents reported. Their efficacies in clinical trials, however, are disappointing. In 22 clinical trials, TRAIL-based agents were reported to have no response or no anticancer activity. A recent meta-analysis screened the trials of TRAIL-based agents conducted between 1995 and 2013 and included 4 randomized controlled trials for further analysis. It was found that TRAIL-based agents did not lead to statistically significant differences regarding objective response rate, progression-free survival, number of patients with any adverse effects (AEs), number of patients with any severe AEs, number of patients with ≥grade 3 AEs and number of fatal AEs (Sun et al, 2014).

Some clinical trials still had positive results though. A combination of dulanermin with paclitaxel, carboplatin, and bevacizumab were reported to give 14 confirmed

responses (1 complete response and 13 partial responses) in a phase 1b trial of non-small-cell lung cancer (Soria et al, 2010). In a phase 1b trial of dulanermin combined with modified FOLFOX6 and bevacizumab in treating metastatic coloreactal cancer, 13 out of 23 patients displayed partial response (Wainberg et al, 2013). In a phase II open-label study of tigatuzumab in combination with gemcitabine in chemotherapy-naïve patients with unresectable metastatic pancreatic cancer, partial response occurred in 8 out of 61 patients (Forero-Torres et al, 2013).

Although the therapeutic effects of TRAIL-based agents are just modest in treating a wide range of cancers, a small subset of patients did respond very well to TRAIL-based therapy. The fact that patients were not pre-selected before being admitted to clinical trials may explain the low objective response rate of TRAIL-based agents. It was suggested that identification of biomarkers for TRAIL sensitivity should help the selection of patients who should be benefitted from TRAIL-based therapy (Dimberg et al, 2013).

1.1.4 Drawbacks in TRAIL-based therapy

1.1.4.1 TRAIL resistance

Considering the role of TRAIL in host defense against tumor, it should come as no surprise that a wide range of primary cancer cells which are capable of evading the

surveillance to form tumor are intrinsically TRAIL-resistant. Approximately half of the NSCLC cell lines were found to be intrinsically resistant to apoptosis induction by TRAIL-based agents (Stegehuis et al, 2010). Acquired resistance to TRAIL also occurs in various cancer cell lines after TRAIL treatment *in vitro* (Lane et al, 2006; Wang et al, 2008; van Geelen et al, 2010). Mechanisms of TRAIL resistance are diverse, targeting different steps in TRAIL-induced apoptotic pathway from ligand binding to turnover of effector caspases. A few targets for TRAIL resistance are discussed below.

TRAIL receptors – DR4 and /or DR5 are down-regulated in TRAIL-resistant cancer cells by different means, including silencing by hypermethylation (Horak et al, 2005), deletions or point mutations (Ozoren & El Deiry, 2003; Bin et al, 2007). The effects of soluble TRAIL and agonistic antibodies of DR4 or DR5 vary in different cancers, depending on the expression of functional DR4 and DR5 receptors on the surface of the cells (Dimberg et al, 2013).

Cellular FLICE-like inhibitory protein (cFLIP) – There are three splice variants of cFLIPs, cFLIP-long ($cFLIP_L$), cFLIP-short ($cFLIP_s$) and cFLIP-Raji ($cFLIP_R$). $cFLIP_S$ and $cFLIP_R$ inhibit apoptosis by competing with caspase-8 or -10 to bindng with FADD to prevent DISC formation (Krueger et al, 2001). $cFLIP_L$ has a dual role in apoptosis regulation. $cFLIP_L$ is anti-apoptotic similar to cFLIPs when its expression level is high. At lower level, $cFLIP_L$ is pro-apoptotic by enhancing

recruitment of caspase-8 (Chang et al, 2002). Overexpression of FLIP has been associated with TRAIL resistance in different cancers. Downregulation of FLIP would enhance TRAIL-induced apoptosis (Bagnoli et al, 2010).

Bcl-2 family proteins – Bcl-2 family proteins have three subclasses, (I) the pro-apoptotic MOMP effectors (BAX and BAK), (II) the anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1) and (III) pro-apoptotic BH3-only proteins (Bid, Bim, Puma, Noxa). The balance of pro-apoptotic and anti-apoptotic Bcl-2 family proteins regulate mitochondrial apoptotic pathway of TRAIL in type II cells. Loss of BAX/BAK (Kandasamy et al, 2003) and overexpression of anti-apoptotic Bcl-2, Bcl-xL and Mcl-1 (Fulda et al, 2002; Henson et al, 2003) were reported to confer TRAIL resistance in cancer cells.

Inhibitor of apoptosis proteins (IAPs) – IAPs contribute to TRAIL resistance via direct inhibition of effector caspases (Gyrd-Hansen & Meier, 2010). X-linked inhibitor of apoptosis (XIAP), the most studied IAP members, inhibits TRAIL-induced apoptosis in cancer cells (Cummins et al, 2004). XIAP also possesses E3-ligase activity, which ubiquitinates caspase-3 and -7 and targets the effector caspases for proteasomal degradation (Choi et al, 2009).

1.1.4.2 Non-cell death TRAIL signalings that worsen cancers

Non-canonical TRAIL receptor signalings have been reported in various cancer cells. A secondary signaling complex can be formed after TRAIL receptor activation, subsequent to the assembly of DISC. In addition to FADD and activated caspase-8, this secondary complex consists of receptor-interacting protein 1 (RIP1), TNF receptor-associated factor 2 (TRAF2), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) essential modulator (NEMO/IKK γ) (Varfolomeev et al, 2005). The secondary complex leads to the activation of different kinase pathways, some of which can worsen cancers (Reviewed by Azijli et al, 2013). A few TRAIL-activated pro-survival pathways are discussed below.

NF-κB Survival Signaling – NEMO, part of the secondary complex formed after ligand binding to TRAIL receptors, can recruit IKKα/β. This phosphorylates the inhibitor of κB (IκB) which is subsequently degraded by proteasomal degradation. NF-κB is then released and can translocate to the nucleus, in which it activates the transcription of pro-survival genes, including cFLIP, Bcl-xL, Mcl-1 and cIAPs (Varfolomeev et al, 2005; Henson et al, 2003; Kreuz et al, 2001; Ravi et al, 2001). NF-κB may have dual activity as it can upregulate TRAIL receptors (Rave et al, 2001). It was recently shown that inhibition of NF-κB in glioblastoma cell lines would protect the cells from TRAIL-induced apoptosis (Jennewein et al, 2012).

Mitogen-activated protein kinases (MAPKs) – MAPKs are kinases responsible for cellular signaling and responses to diverse stimuli. They regulate various physiological processes, including gene expression, cell motility, mitosis, proliferation and differentiation (Pearson et al, 2001). There are six groups of MAPKs in mammals, extracellular regulated kinases (ERK1/2), p38 (p38 $\alpha / \beta / \gamma$ /δ), Jun NH2 terminal kinases (JNK1/2/3), ERK3/4, ERK5 and ERK7/8.

JNK can be activated by TRAIL, but its role may be cell type-specific or depending on cellular context. JNK was found to contribute to TRAIL-induced apoptosis in lymphoid cells (Herr et al, 1999) and it took part in TRAIL-induced lysosomal death pathway in cholangiocarcinoma cells (Werneburg et al, 2007). However, JNK inhibition, by RNA interference or with chemical inhibitor, sensitizes TRAIL-induced apoptosis in hepatocellular carcinoma cells but not in normal hepatocytes (Mucha et al, 2009). It was recently found that a recent study has shown that, short JNK1 isoforms (JNK1 α 1 and JNK1 β 1) are anti-apoptotic while the long isoforms (JNK1 α 2 and JNK1 β 2) are pro-apoptotic upon a ctivation of TRAIL in colon cancer cell lines (Mahalingam et al, 2009).

P38 MAPK was found to suppress apoptosis in TRAIL-treated cancer cells. P38 phosphorylation induced by TRAIL protected prostate cancer cells from apoptosis by upregulating the transcription of anti-apoptotic Mcl-1. Inhibition of p38 w ith chemical inhibitor increased TRAIL-induced cell death (Son et al, 2010). P38 inhibition also sensitized breast carcinoma cells for TRAIL (Weldon et al, 2004), suggesting a pro-survival role of p38 in some cells after TRAIL treatment.

ERKs can also be activated by TRAIL. TRAIL phosphorylates and activates ERK1/2 in neuroblastoma cells (Milani et al, 2003). In colon cancer cells, inhibition of ERK 1/2 increased cytotoxicity of TRAIL (Vaculova et al, 2006). In TRAIL-resistant human glioma cells, TRAIL treatment enhanced cell proliferation, which could be abrogated by ERK inhibition (Vilimanovich & Bumbasirevic, 2008).

Phosphatidylinositide 3-Kinases (PI3K)/Akt Signaling – Akt, also known as protein kinase B (PKB), is a critical kinase for cell survival. Activity of PI3K/Akt pathway is enhanced in many malignancies, in which Akt pathway is involved in cell growth stimulation and cell survival (Fresno Vara et al, 2004). TRAIL was found to phosphorylate and activate Akt within 30min in leukemic T Jurkat cells. LY294002, the pharmacological inhibitor of Akt, could sensitize the cells for TRAIL-induced apoptosis (Zauli et al, 2005). Similar Akt activation by TRAIL and sensitization of cells for TRAIL by Akt inhibition were also reported in TRAIL-resistant NSCLC cells, human breast cancer cells, human ovarian cancer cells and human prostate cancer cells (Azijli et al, 2012; Xu et al, 2010; Song et al, 2010).

1.1.4.3 TRAIL-induced Proliferation, Migration and Metastasis

The growth-enhancing and proinvasive property of TRAIL in some cancer cells is also a major concern. TRAIL was reported to induce cell migration and invasion in TRAIL-resistant cholangiocarcinoma cells, which is NF-κB –dependent (Ishimura et

al, 2006). In a xeno-transplantation model with human pancreatic ductal adenocarcinoma cells that overexpressed Bcl-xL, tumor growth and distant metastases were enhanced by administration of aggregated TRAIL (Trauzold et al, 2006). Pro-inflammatory cytokines, including interleukin-8, urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMP)-7 and -9, were stimulated by TRAIL and were associated with the proinvasive property of TRAIL in the pancreatic cancer cells (Trauzold et al, 2006; Zhou et al, 2008). In a recent *in vitro* study on cancer cell lines with dysfunctional in apoptosis, TRAIL led to membrane blebbing, cell detachment and migration in HCT116 Bax-/- cells and Bcl-xL-overexpressing SW480 cells. Cleavage of ROCK1 protein by caspase 3 was essential for these phenotypes (Somasekharan et al, 2013). In TRAIL-resistant NSCLC cells, RIP1/Src/STAT3 pathway was found to mediate TRAIL-dependent migration and invasion (Azijli et al, 2012). DR5 (TRAIL-R2) was shown to be the major mediator for invasion using receptor-specific TRAIL variants. Inhibition of Src/STAT3 could prevent invasion induced by TRAIL without sensitizing the cancer cells for TRAIL-induced cell death.

Considering the drawbacks of therapy with TRAIL, efforts have been made in the following aspects so as to maximize the therapeutic potential of TRAIL-based anti-cancer therapy (Dimberg et al, 2012).

1. Identification of biomarkers that predict patients' response to TRAIL for selecting patients who would be benefitted from TRAIL-based therapy.
2. Development of highly potent TRAIL variants or agonistic antibodies.

3. Identification of effective and cancer-selective sensitizers that sensitize TRAIL-resistant cancer cells, but not normal cells, for TRAIL-induced cell death.

1.2 **Hyaluronan**

Hyaluronan/hyaluronic acid (HA) is a linear anionic, non-sulphated glycosaminoglycan made up of repeating disaccharides of D-glucoronic acid (GlcA) and N-acetylglucosamine (Glc-NAc). The size of HA is variable, from 2000 t o 25,000 disaccharide units (up to 10MDa in molecular mass) (Laurent et al, 1996). HA is a major component of extracellular matrix (ECM). It can be found ubiquitously in human body. More than half of the total HA was found in skin in rat (Reed et al, 1988). The rest occurs in other parts of the body, such as connective tissues, synovial fluid of joints, vitreous body of the eye and intervertebral discs.

1.2.1 Physiochemical properties of HA

At physiological pH, the carboxyl group of D-glucuronic acid is dissociated, making the polymer negatively charged to combine with sodium ions, the most prevalent extracellular cation, to form sodium hyaluronate (Karbownik & Nowak, 2013). The negative charge of HA allows it to form numerous dipolar bonds with water molecules, granting HA its unique hygroscopic property. One gram of HA can retain more than one liter of water (Laurent & Fraser, 1986). This feature is important for

HA as a background material and maintaining tissue hydration and tension. In hydrated state, water around HA molecular is largely immobilized. Large molecules like proteins can then be excluded out of the HA matrix by steric exclusion (Chen & Abatangelo, 1999). HA is also viscoelastic in nature. At low concentration (as low as 0.1%), HA can be entangled resulting a high viscosity. This makes HA a good lubricating material and shock absorbent in synovial fluid of joints. The high viscosity of pericellular HA was also found to impede viral entry to mammalian cells *in vitro* (Clarris et al, 1974).

1.2.2 Biosynthesis and degradation

HA is synthesized at the inner plasma membrane by hyaluronan synthases and the nascent chains are then extruded to the cell surface (Lüke & Prehm, 1999). Hyaluronan synthases are glycosyltransferases, mediating the transglycosylation of UDP-GlcA (UDP: uridine-5'-diphosphate) and UDP-GlcNAc precursors and elongating the HA chain.

There are three HAS genes, *has1*, *has2* and *has3*. HAS-1 synthesizes high-molecular-weight hyaluronan. HAS-2 produces hyaluronan in response to shock, inflammation and wound repair. HAS-3 produces low-molecular-weight hyaluronan (LMWHA) up t o 100kDa (Itano et al, 1999; Karbownik & Nowak, 2013). The expression of HAS genes are regulated temporally and spatially, especially during embryogenesis and pathogenesis, by various cytokines, growth factors and stresses (Itano & Kamata, 2008).

Hyaluronan can be degraded both enzymatically and non-enzymatically. The non-enzymatic breakdown of hyaluronan is mainly by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). The ROS/RNS damage is non-selective and hyaluronan is then broken down into fragments of different lengths. Interestingly, free radicals can at the same time degrade hyaluronan directly and increase the expression of hyaluronidase (HYAL-2), one of the enzymes responsible for hyaluronan degradation (Monzon et al, 2010).

Hyaluronoglucosaminidases, more commonly known as hyaluronidases (HYALs), are the enzymes responsible for enzymatic catabolism of hyaluronan. Six HYAL genes have been identified in human genome: *hyal1*, *hyal2*, *hyal3*, *hyal4*, *ph-20* (or *spam1*) and *phyal1* (pseudogene) (Stern & Jedrzejas, 2006). HYAL-1 degrades HMWHA into tetrasacccharides, mainly in blood plasma in tissues (Csoka et al, 2001). HYAL-2 breaks down HMWHA into LMWHAs of about 20kDa (Lepperdinger et al, 2001). Function of HYAL-3 and HYAL-4 is largely unknown. Knocking out *hyal3* in mouse did not lead to accumulation of hyaluronan (Atmuri et al, 2008), whereas overexpression of hyal3 in Baby Hamster Kidney cells increased HYAL-1 protein and mRNA level (Hemming et al, 2008). PH-20 is involved in fertilization process by digesting the ECM in the *corona radiata* on the surface of the oocyte and to allow sperm to entry (Martin-DeLeon, 2006). The enzymatic breakdown of hyaluronan is in 2 stages. HMWHA bound to CD44, a hyaladherin and a cell surface marker, is broken down by HYAL-2 into LMWHA fragments. These fragments are endocytosed and degraded by lysosomal HYAL-1 or transported to

liver and kidney (Sironen et al, 2011).

Hyaluronan is a dynamic molecule. The biosynthesis and degradation of hyaluronan is continuous with a high turnover rate, depending on location and physiology. The half-life of hyaluronan in blood plasma is only 2-5min but in cartilage it can be about 1-3 weeks. About one-third of total hyaluronan in our body is replaced every day (Karbownik & Nowak, 2013).

1.2.3 Role of hyaluronan in cancer

Not just a supportive material, hyaluronan is also involved in diverse biological processes, such as proliferation, inflammation, recognition, tissue hydration, cell motility, angiogenesis and cell-cell communications. In early stage of mitosis, hyaluronan in pericellular matrix is required for detachment of cells from the substratum and neighboring cells (Evanko et al, 1999). It also helps cell division by filling the space between separating chromosomes (Evando & Wight, 1999).

Level of hyaluronan increases dramatically in many cancer types and high level of hyaluronan is correlated with poor prognosis (Whatcott et al, 2011). In cells or stroma, hyaluronan has a role in malignant transformation and cancer progression in the following aspects.

Invasion and tumor progression – Hyaluronan is found to accumulate in the invasive front of tumors (Bertrand et al, 1992), suggesting its role in cancer invastion and

progression. Ectopic expression of *has2* gene in nontransformed rat 3Y1 cells resulted in the formation of a massive hyaluronan matrix, leading to loss of contact inhibition of growth and enhanced cell migration (Itano et al, 2002). In prostate cancer cells, formation of matrix rich in hyaluronan and vesicant promoted cancer cell motility (Ricciardelli et al, 2007). Matrix metalloproteinases (MMPs) are involved in hyaluronan-mediated invasion. Emmprin (CD147), an inducer of MMP synthesis, promotes anchorage-independent growth and stimulates cell survival pathway signaling in human mammary carcinoma cells by stimulating hyaluronan production (Marieb et al, 2004). Hyaluronan interacts with CD44, a hyaluronan-binding protein and a cell surface marker, which clusters with MMP-9 to promote collagen IV degradation and mediates tumor cell invasion *in vitro* (Yu & Stamenkovic, 1999). The role of HA-CD44 interactions in cancer cell invasion has been demonstrated *in vitro*. Inhibiting HAS expression decreased the invasiveness of SW620 metastatic colon carcinoma cells, which could be restored by addition of exogenous hyaluronan. Interruption of HA-CD44 interactions with anti-CD44 antibody also significantly impaired invasion (Kim et al, 2004). It was later found that the rapid basal motility of invasive mammary cancer cell lines requires Rhamm-CD44-ERK1,2 complexes (Rhamm: CD168, Hyaluronan-mediated motility receptor) and endogenous hyaluronan production. This showed a hyaluronan-dependent autocrine mechanism for sustained ERK signaling for cell motility (Hamilton et al, 2007).

Angiogenesis – A hyaluronan-rich matrix not only increased the motility and invasiveness of cancer cells, it also enhances the recruitment of monocytes and macrophages to the stroma, which is important for angiogenesis. Tumor-associated macrophages (TAMs) can release growth factors promoting angiogenesis and lymphangiogensis (Schoppmann et al, 2002). Co-implantation of hyaluronon-overproducing fibroblasts and MCF-7 human breast carcinoma cells into nude mice resulted in rapid tumor growth together with marked stromal formation and lymphangiogenesis (Koyama et al, 2007). Inoculation of has2-null fibroblasts with tumor cells into nude mice severely retarded macrophage recruitment with concomitant attenuated tumor angiogenesis and lymphangiogenesis, suggesting the role of stromal hyaluronan for TAM recruitment and tumor vascularization (Kobayashi et al, 2010). It has been suggested that hyaluronan oligomers/fragments of 4-25 disaccharides are angiogenic and promotes tumor invasiveness (Lokeshwar et al, 1997). A specific variant of hyaluronidases capable of producing such hyaluronan fragments was discovered in bladder tumors (Lokeshwar et al, 1999). Hyaluronan fragments of similar sizes were also detected in high-grade prostate cancers, squamous cell carcinomas and other cancer types (Sironen et al, 2011).

Epithelial – Mesenchymal Transition (EMT) – EMT takes place in embryonic development as well as metastasis for cell migration. During EMT, epithelial markers like E-cadherin are down-regulated whereas mesenchymal markers like vimentin, fibronectin and smooth muscle actin are up-regulated. MMPs are usually

up-regulated in EMT to enable detachment of cells from each other and penetration of detached cells through the basement membrane by E-cadherin cleavage (Hugo et al, 2007). Hyaluronan is involved in EMT process. *Has2*-null mice failed to undergo EMT resulting in defect in cardiac muscle development (Camenisch et al, 2000). In another study, HAS-2 overexpression increased hyaluronan production, which promoted anchorage-independent growth, stimulated phosphoinositide 3-kinase/Akt pathway and induced EMT in normal epithelial cells (Zoltan-Jones et al, 2003). Stimulation of hyaluronan production with TGF-β/IL-1β in NSCLC cells induced EMT, shown by changes in cellular morphology and E-cadherin/vimentin expression. Overexpression of HAS-3 in epithelial lung adenocarcinoma cell line H358 also led to hyaluronan overproduction associated with EMT and enhanced MMP-2 and MMP-9 activities (Chow et al, 2010). In a recent study, expression of HAS-2 in more than 200 br east carcinomas of different subtypes was examined by immunohistochemistry. HAS-2 was expressed in 30.6% of invasive ductal carcinomas associated with poor overall survival. In metaplastic carcinomas of breast (MCB), a subtype related to EMT, HAS-2 was expressed in 72.7% of all MCB samples. This implicates a correlation between HAS-2 expression and EMT in breast carcinoma (Lien et al, 2014).

Increasing cell proliferation and s urvival by evading apoptosis – HA-CD44 interactions play an important role in regulating cell proliferation and promoting cell survival by regulating growth factor production and activating pro-survival pathway.

In melanoma cells, hyaluronan-mediated release of autocrine growth factors, basic fibroblast growth factors (bFGF) and transforming growth factor-β1 (TGF-β1), is CD44-dependent (Ahrens et al, 2001). CD44 was found to interact with epidermal growth factor receptor (EGFR) and ErbB2 by co-immunoprecipitation in gliomablastoma cell lines whereas hyaluronan treatment led to transient phosphorylation of extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2) (Tsatas et al, 2002). A strong link between HA-CD44 interaction and cyclooxygenase-2 (COX-2) has been established in colon cancer cells via ErbB2/PI3K/Akt/β-catenin signaling axis (Misra et al, 2008). COX-2 mediates the production of prostaglandin E_2 (PGE_2), which promotes cell proliferation and survival. PI3K/Akt is another pro-survival pathway in cells. Akt, also known as protein kinase B, can block apoptosis by phosphorylating and inactivating Bad protein and caspase-9 in apoptotic pathway (Song et al, 2005).

1.2.4 Hyaluronan as therapeutic target in cancer

The increasing understanding in the importance of hyaluronan in cancer progression and survival has inspired the development of different anti-cancer strategies targeting hyaluronan.

Hyaluronan metabolism – Inhibition of hyaluronan biosynthesis with chemical inhibitor of hyaluronan synthase, 4-methylumbelliferone (4-MU), has been adopted in *in vitro* and *in vivo* studies (Further discussed below). Hyaluronidase, the enzyme

responsible for catabolizing hyaluronan, has already been incorporated into chemotherapeutic regimen. This was found to improve chemotherapy in several tumor types including breast, brain, melanoma, and sarcoma (Whatcott et al, 2011).

Hyaladherins – HA-CD44 interaction has drawn much attention as it regulates various signaling pathways accounted for tumor growth and survival, cancer cell migration, invasion and metastasis. Anti-CD44 antibodies have been used to induce apoptosis in cancer cells (Rajasagi et al, 2010) or as a drug vehicle for selectively delivery of cytotoxic drugs to cancer cells (Karbownik & Nowak, 2013). Small hyaluronan oligosaccharides (3-9 disaccharide units) has also been investigated for interrupting HA-CD44 interaction, resulting in inhibited anchorage-independent growth and suppression of PI3K/Akt pathway *in vitro* and reduced tumor growth *in vivo* (Ghatak et al, 2002).

Hyaluronan-based drug delivery – Due to HA-CD44 interaction, HA-cytotoxic drug conjugates can be internalized selectively by cancer cells with a high CD44 level. Hyaluronan is advantageous as a drug delivery system as it is biocompatible, biodegradable and non-immunogenic. HYTAD1-p20, a paclitaxel-HA conjugate was found to be more effective than conventional paclitaxel for its better hydrosolubility and higher anti-cancer activity against human bladder cancer cells (Rosato et al, 2006). An irinotecan-HA conjugate showed better efficacy than irinotecan alone against metastatic colorectal cancer in phase II clinical trial (Gibbs et al, 2011).

1.2.5 4-Methylumbelliferone

4-Methylumbelliferone (4-MU), also known as hymecromone, is a coumerin derivative. Its structure is shown in Figure 3. It is used as a ch oleretic or antispasmodic drug or dietary supplement under different brand names, such as Heparvit, Adesin C and Cantabiline. 4-MU has no know n toxicity and can be consumed at a dose of 1500-2200mg/day (Benitez et al, 2013). 4-MU inhibits hyaluronan biosynthesis by two means, depletion of intracellular UDP-glucuronic acid (UDP-GlcUA) and down-regulation of HAS-2 and HAS-3 (Kultti et al, 2009). Accumulating evidences reveal that 4-MU is a promising anti-cancer agent. 4-MU was shown to induce apoptosis, inhibit proliferation and invasion in various cancer cells, such as liver cancer cells, prostate cancer cells and osteosarcoma cells (Piccioni et la, 2012; Lokeshwar et al, 2010; Arai et al, 2011). It also inhibited tumor microvessel formation of prostate cancer cells (Lokeshwar et al, 2010) and distant metastasis of osteosarcoma cells *in vivo* (Arai et al, 2011). In a mouse model, 4-MU sensitized human pancreatic cancer cells to gemcitabine treatment (Nakazawa et al, 2006). 4-MU also displayed synergistic anti-cancer effect with sorafenib against renal cancer cells (Benitez et al, 2013).

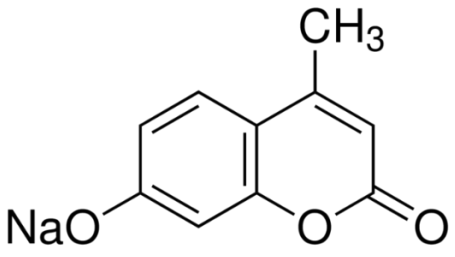


Figure 3. Structure of 4-methylumbelliferone.

1.3 Aims and significance

TRAIL is a promising anti-cancer agent. It can selectively kill cancer cells without harming the normal cells and its killing effect is p53-independent. The apoptotic pathway of TRAIL is already well studied. Unfortunately, clinical efficacy of TRAIL-based therapeutics is only modest. Furthermore, there are several drawbacks of therapeutic treatment with TRAIL, including the development of TRAIL resistance, induction of non-canonical pro-survival and pro-proliferating signaling (eg. PI3K/Akt pathway), and enhanced motility and invasiveness of some of the TRAIL-treated cancer cells. One of the current research directions on TRAIL is to identify anti-cancer agents or other means that can sensitize TRAIL-resistant cancer cells to TRAIL treatment. The combinations should also be low-toxic and non-harmful to normal cells.

Considering the anti-proliferating, anti-motility, anti-invasion effect as well as the reported PI3K/Akt inhibition of 4-MU on different cancer cells, I hypothesize that 4-MU may be a good candidate that can sensitize cancer cells to TRAIL-induced apoptosis. Also, our group has previously discovered the reversal of cancer cells from apoptosis induced by chemical agents (Tang et la, 2009, Tang et al, 2012), which may have implications in cancer relapse and drug resistance. As TRAIL is a typical inducer of extrinsic apoptotic pathway, it is of interest to know if cancer cells can reverse from TRAIL-induced apoptosis and whether 4-MU can inhibit this reversal. The objectives of this study are:

1. To investigate if TRAIL-4-MU combination have any synergistic anti-cancer effect.
2. To investigate the reversibility of TRAIL-induced apoptosis in cancer cells and whether 4-MU can inhibit this cancer cell recovery.
3. To reveal the mechanism of anti-cancer effect of TRAIL-4-MU combination.

This study has revealed a novel synergistic anti-cancer combination, TRAIL-4-MU. This combination should have therapeutic potential as both agents are safe with few adverse side effects and 4-MU is a suitable sensitizing agent that can compensate the drawbacks of TRAIL. This study should have provided a solid evidence for the synergistic anti-cancer effect of this combination without much harm to normal cells *in vitro* and paved the way for further research on this combination.

Chapter 2 Materials and Methods

2.1 Cell culture

HeLa cells (CCL-2, human cervical carcinoma cell line) , HepG2 cells (HB-8065, human hepatocarcinoma cell line) and Hs68 (CRL1635, human foreskin fibroblast line) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultivated in tissue culture flasks with cell culture surface treatment (Nunc^{TM} cell culture treated flask with filter cap, Thermo Fisher Scientific, Waltham, MA, USA) with Dulbecco's Modified Eagle medium:F-12 nutrient mixture (DMEM/F12) culture medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37ºC in a cell culture incubator (Thermo Fisher Scientific, Waltham, MA, USA) under humidified atmosphere of 5% CO_2 as previously described (Tang et al 2009).

Cells were subcultured two times a week when cells reached 70-80% confluence. In brief, culture medium was decanted from culture flask and cell layer was rinsed 2 times with 1X phosphate-buffered saline (PBS) before addition of 0.25% Trypsin-0.5 mM EDTA (Gibco, Carlsbad, CA, USA). Cells were trypsinized at 37 ºC in incubator for a few minutes until the cell layer detached. Trypsinization was stopped by adding double volume of 10% FBS-containing DMEM/F12 culture medium. Cells

were dispersed by agitation and pelleted by centrifugation at 200 g for 5 min. The cell pellets would be resuspended in fresh culture medium with FBS. Around 1:10-1:6 of the dispersed cells were transferred to a new culture flask containing fresh culture medium for further culture.

2.2 MTT Cell Viability Assay

Cells (5000 cells/well for HeLa cells, 10,000 cells/well for HepG2 cells) were plated in 96-well culture plates. After incubation for 24 h, the culture medium would be gently discarded and replaced with 100 µl medium containing various concentrations of *SuperKillerTRAIL*™ (soluble) (human), (recombinant) (Enzo Life Sciences, New York, USA) and/or 4-MU (Sigma, St. Louis, MO, USA) . The cells were further incubated for 24 h or 72 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was prepared by dissolving the MTT powder (Sigma, St. Louis, MO, USA) in 1X PBS and was added to each well at the end of drug incubation. Final concentration of MTT was 0.5 mg/ml. Cells were further incubated at 37 ºC for 3 hour s to allow dehydrogenase enzymes in the cells to metabolize the water soluble MTT compound to form a water insoluble purple formazan crystal. After incubation, culture medium with MTT solution was gently but thoroughly removed and 100 µl DMSO (Dimethyl sulfoxide, (Sigma, St. Louis, MO, USA) was added into each well to dissolve the formazan crystals. The plate was shaken for 5min or until all the crystals dissolved. The absorbance at wavelength 570

nm would be measured by a microplate reader (Molecular Devices Corp, Concord, ON, Canada).

2.3 BrdU cell proliferation assay

Colorimetric BrdU cell proliferation enzyme-linked immunosorbent assay kit (No.1647229, Roche, Basel, Switzerland) was used according to manufacturer's instructions. Cells (5000 cells/well for HeLa cells, 10,000 cells/well for HepG2 cells) were seeded into a 96-well culture plate. After incubation for 24h, t he culture medium would be gently discarded and replaced with 100µl medium containing various concentrations of TRAIL and/or 4-MU. The cells were further incubated for 24 h. BrdU Label was added to cells at 1:1000 dilution from stock without removal of culture medium and the cells were further incubated for 4 h i n incubator. The labeling medium was then removed and the cells were fixed with DNA denatured in fixing solution for 30min. Fixing solution was then removed and the fixed cells were incubated in anti-BrdU–peroxidase conjugate for 1 h at room temperature. After three-time washing, 100 µl peroxidase substrate was added to the cells and allowed to react in dark for 15 min. Absorbance was then measured in a microplate reader at wavelength 450 nm with reference to wavelength 690 nm using a Spectramax 250 plate reader (Molecular Devices Corp, Concord, ON, Canada).

2.4 Colony formation assay

To determine the viability and proliferative ability of TRAIL- and/or 4-MU-treated cells, colony formation assay was performed similar to previously described (Franken et al, 2006). Cells (1000 cells for HeLa cells, 4000 cells for HepG2 cells) were seeded in 6-well culture plates and incubated for 24 h before addition of TRAIL and/or 4-MU. The cells were treated for 12 h followed by gentle washing and replacement with fresh medium. The cells were then further cultured for 10 days to allow colony formation. Afterwards, cell culture medium was removed and wells were carefully rinsed with 1X PBS. Cell colonies were fixed with 3.7% formaldehyde in 1X PBS and stained by 0.5% (w/v) crystal violet in methanol for 15 min at room temperature. Non-specific stain was gently washed off by rinsing twice with tap water until rinse no l onger turn dark. Plates were air dried at room temperature before manual counting. Only colonies with >50 cells were counted.

2.5 Apoptosis detection – DNA Fragmentation and Cell Cycle Analysis

Propidium iodide (PI) can stain the DNA content of single cells to reflect DNA fragmentation cells and cell cycle distribution of cell population (Krishan, 1975). Cells were seeded in 6-well culture plates for 24 h before TRAIL and/or 4-MU treatment for another 24 h. After treatment, adherent cells were trypsinized and pelleted together with floating cells, if any. Cells were fixed in ice-cold 70% ethanol

in PBS at 4 ºC overnight or up to one week. Fixed cells were pelleted and washed with ice-cold PBS before incubation with 0.2mg/ml RNase A (Sigma, St. Louis, MO, USA) in PBS at 37 ºC for 1 h for RNA digestion. PI solution was then added to each sample to achieve at final concentration of 10 µg/ml PI. Cells were kept in dark and analyzed within 15 min on Cytomics FC500 (Beckman Coulter, CA, USA). Cells in Sub-G1 and cell cycle distribution were analyzed with CXP System software (Beckman Coulter, CA, USA) and Multicycle AV software (Phoenix Flow Systems, CA, USA).

2.6 Apoptosis detection – Caspase-3/7 activity

CellEvent™ Caspase-3/7 Green Detection Reagent (CellEvent; Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) was used to determine the proportion of cells with caspase-3/7 activity after apoptotic induction. Cells were pre-incubated in medium with 10 µM CellEvent for 1h before treatment with TRAIL and/or 4-MU similar to published procedures (Miyata et al, 2011). After 24h treatment, all cells, including floating and adherent cells, were collected by trypsinization and resuspended in 500 µl PBS before analysis on Cytomics FC 500 (Beckman Coulter, CA, USA). Cells stained as CellEvent-positive were considered as caspase-3/7 activated.

2.7 Effect on cellular reversibility of apoptosis - Fluorescence-activated cell

sorting (FACS) and Colony formation assay

To determine the effect of TRAIL/4-MU treatment on reversibility of cells from apoptosis, HeLa cells with caspase-3/7 activity were sorted out for recovery from apoptosis. CellEvent^{TM} Caspase-3/7 Green Detection Reagent (CellEvent; Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) was used again. Cells were preincubated with CellEvent for 1h before treatment with TRAIL or staurosporine (STS) for 6 h. FACS was performed on BD FACS Aria I Flow Cytometer (BD Biosciences, CA, USA). During sorting, cells were sorted into CO_2 Independent Medium (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) with 20% FBS. After sorting, sorted cells were pelleted by centrifugation at 200g for 5min and resuspended in DMEM/F12 medium with 10% FBS for further culture. Cells with positive CellEvent signals were sorted out for subsequent colony formation assay or further culture. 4-MU was added together with TRAIL before sorting or after sorting for 24 h in colony formation assay.

Colony formation assay was performed by seeding 5000 sorted cells into one well in 6-well culture plates. Similar to the procedures mentioned above, cells were further cultured for 10 days to allow colony formation. After 10 days, cells were carefully rinsed with 1X PBS and fixed with 3.7% formaldehyde in 1X PBS. Cell colonies were stained by 0.5% (w/v) crystal violet in methanol for 15min at room temperature. Only colonies with >50 cells were counted.

2.8 Measurement of hyaluronan concentration in culture medium

HeLa cells and HepG2 cells were seeded in 96-well plates and incubated overnight before treatment with 50 ng/ml TRAIL and/or 0.5 mM 4-MU. After 24h, conditioned medium was collected by centrifugation at 500 g, 5 min to remove cells or cell debris and stored at -20°C before analysis. Hyaluronan Quantikine ELISA Kit (R&D Systems, Minnesota, USA) was used to measure the HA concentration according to manufacturer's instructions. A standard curve was plotted using the HA standard provided in the kit for quantitation. The conditioned medium collected was diluted ten-fold for the assay. Cell-free medium with FBS was included as a control for the amount of HA naturally present in FBS.

2.9 Western blotting

To study the relative abundance of proteins of interest, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (*SDS-PAGE*) followed by immunoblotting with specific antibodies was performed similar to previously described (Tang et al. 2009; Tang et al. 2012). Trypsinized cells were washed with 1X PBS, pelleted by centrifugation at 300 g for 5 min and lysed with RIPA lysis buffer with protease inhibitor cocktail (Santa Cruz Biotechnology, TX, USA) under brief sonication. Cell lysate samples were centrifuged at 10,000 g for 10 min at 4ºC to pellet cell debris or impurities. Supernatant was collected as protein sample, which was quantified using

2-D Quant Kit (GE Healthcare Life Sciences, PA, USA). Approximately 20μg of proteins were loaded onto an SDS-PAGE gel (10%, 12% or 15% (w/v) depending on the size of targeted protein) and proteins were separated according to their molecular weight. Separated proteins in gel were then transferred onto an Immun-Blot® PVDF membrane (Bio-rad, CA, USA). Non-specific sites on the membrane were blocked by shaking in 5% fat-free milk (w/v) or 5% bovine serum albumin (BSA) in 1X TBST for 1 h at room temperature. The membrane was then incubated with individual primary antibodies (1:1000 in 1X TBST with 5% BSA) overnight at 4ºC. After washing for three times, the membrane was incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 in 1X TBST with 5% fat-free milk) under shaking for one hour at room temperature. The membrane was washed again for three times before applying Amersham™ ECL™ Prime Western Blotting Detection Reagent according to manufacturer's instructions (GE Healthcare Life Sciences, PA, USA). Signals were captured by Lumi-Imager F1 Imager Workstation (Roche, Basel, Switzerland).

2.10 Calculation of combination index

Combination index was calculated based on Chou-Talalay method (Chou et al, 2010). The principle is briefly described here. After a systematic analysis of the classic kinetic models of enzyme-substrate-inhibitor interactions with different number of substrates, different reaction mechanisms, and different types or mechanisms of inhibition, Chou derived two equations about fractional velocity (f_v) and fractional

inhibition (f_i) in the presence of an inhibitor (I) for all cases of enzymatic reactions:

$$f_v = [1 + (I/K_i)(E_x/E_t)]^{-1}$$ ……..Eq 1

and

$$f_i = [1 + (K_i/I)(E_t/E_x)]^{-1}$$…….Eq 2

where K_i is the enzyme-inhibitor dissociation constant, E_t is the total amount of enzyme, and E_x is the amount of the enzyme species with which the inhibitor may combine.

Given that $K_i/I_{50} = E_x/E_t$……Eq 3, I_{50} is the concentration of I required for 50% inhibition, substituting Eq 3 into Eq 1 and 2 will give the following equations:

$$f_v = 1/[1 + (I/I_{50})]$$……Eq 4

and

$$f_i = 1/[1 + (I_{50}/I)]$$……..Eq 5

Combining Eq 4 and 5 will give $f_i/f_v = I/I_{50}$ (Eq 6).

Further analysis of the pharmacological receptor system and the shapes of dose-effect relationship, Chou derived a median-effect equation to describe dose-effect relationship

$$f_a/f_u = (D/D_m)^m$$ ……..Eq 7 (Median-effect equation)

which can be rearranged to

$$D = D_m[f_a/(1 - f_a)]^{1/m}$$ …Eq 8

where D is the dose (or concentration) of a drug, f_a is the fraction affected by D (i.e., percentage inhibition/100), and f_u is the fraction unaffected (i.e., $f_u = 1 - f_a$). D_m is the median-effect dose (e.g., IC_{50}, ED_{50}, or LD_{50}) that inhibits the system under study by 50%, and m is the coefficient signifying the shape of the dose-effect relationship, where m = 1, > 1, and < 1 indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curves, respectively.

The median-effect equation obeys the mass-action law and is called the general theory of dose and effect.

The median effect equation was further extended to multiple drugs.

For two-drug combination in first-order system (m=1), the equation became

$$\frac{(f_a)_{1,2}}{(f_u)_{1,2}} = \frac{(f_a)_1}{(f_u)_1} + \frac{(f_a)_2}{(f_u)_2} = \frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2}$$ ……Eq 9

For higher-order system (m≠1), the equation is

$$\left[\frac{(f_a)_{1,2}}{(f_u)_{1,2}}\right]^{1/m} = \left[\frac{(f_a)_1}{(f_u)_1}\right]^{1/m} + \left[\frac{(f_a)_2}{(f_u)_2}\right]^{1/m} = \frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2}$$ …Eq 10

Based on Eq 9 and 10, the term combination index for quantitation of

synergism/antagonism of 2-drug combination was introduced and can be calculated by

$$\mathrm{CI} = \frac{(\mathrm{D})_1}{(\mathrm{D_x})_1} + \frac{(\mathrm{D})_2}{(\mathrm{D_x})_2} = \frac{(\mathrm{D})_1}{(\mathrm{D_m})_1[f_\mathrm{a}/(1-f_\mathrm{a})]^{1/m_1}} + \frac{(\mathrm{D})_2}{(\mathrm{D_m})_2[f_\mathrm{a}/(1-f_\mathrm{a})]^{1/m_2}}$$

…..Eq 11

where CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively. In the denominator, (D_x) is for D_1 "alone" that inhibits a system x%, and $(D_x)_2$ is for D_2 "alone" that inhibits a system x%.

The general equation for combination of multiple drugs was later derived to be

$$^{n}(\mathrm{CI})_\mathrm{x} = \sum_{j=1}^{n} \frac{(\mathrm{D})_\mathrm{j}}{(\mathrm{D_x})_\mathrm{j}} = \sum_{j=1}^{n} \frac{(\mathrm{D_x})_{1-\mathrm{n}}\{[\mathrm{D}]_\mathrm{j}/\sum_1^n[\mathrm{D}]\}}{(\mathrm{D})_\mathrm{m})_\mathrm{j}\{(f_{\mathrm{a_x}})_\mathrm{j}/[1-(f_{\mathrm{a_x}})_\mathrm{j}\}^{1/m_\mathrm{j}}}$$ Eq 12

Compusyn, a free computer software adopting the above equations developed by Chou and his colleagues, is used in this study for automated data analysis and calculation of CI.

2.11 Statistical analysis

All results were presented as means ± SD. Data were processed with GraphPad Prism 5.00 software. Statistical analysis was performed by using t-test method (for comparison of 2 groups) or one-way analysis of variance (ANOVA) (for comparison

of more than 2 groups) was performed. For one-way ANOVA, Dunnett's posttst or Tukey-posttest was performed for multiple comparisons if necessary. Results were considered as statistically significant when P-value is less than 0.05.

Chapter 3 4-MU synergistically potentiates TRAIL-induced apoptosis

3.1 Introduction

TRAIL can selectively kill cancer cells without affecting normal cells and its killing effect is p53-independent, making it a promising anti-cancer agent. Unfortunately, clinical efficacy of TRAIL-based therapeutics is only modest. Also, there are several shortcomings of TRAIL as anti-cancer agent, including TRAIL resistance, induction of non-canonical, pro-survival and pro-proliferating signaling (eg. PI3K/Akt pathway), and enhanced motility and invasiveness in some of the TRAIL-treated cancer cells. Development of low-toxic, non-harmful combinations that can sensitize TRAIL-resistant cancer cells to TRAIL treatment is one of the current research directions on TRAIL.

4-MU is a safe, anti-proliferating, anti-motility, anti-invasion agent that was reported to inhibit the pro-survival PI3K/Akt pathway in different cancer cells. It inhibits biosynthesis of hyaluronan and thus interrupts HA-CD44 interactions. It was hypothesized that 4-MU may synergistically sensitize cancer cells to TRAIL-induced apoptosis. In this chapter, the cytotoxicity and anti-proliferating effect of

TRAIL-4-MU combinations were investigated. Combination index was calculated by Chou-Talalay method. And the mechanisms for the synergistic cytotoxicity were studied.

3.2 Results

3.2.1 Synergistic cytotoxicity of TRAIL and 4-MU combination on HeLa cells and HepG2 cells

Before studying the TRAIL-4-MU combination, dose-response of HeLa cells and HepG2 cells to TRAIL-induced cytotoxicity was studied by MTT assay in order to determine a suitable dose of TRAIL that would have moderate cytotoxicity on the cancer cells (Figure 4). In HeLa cells, treatment with 62.5ng/ml TRAIL for 24h resulted in 50.40%±4.40% viability. But further increase in TRAIL concentration did not result in significant reduction in viability. HeLa cells treated with 500ng/ml TRAIL for 24h still had 41.51%±5.32% viability. HepG2 cells appeared to slightly more resistant to TRAIL in moderate concentration, with 62.03%±2.25% viability at 62.5ng/ml TRAIL. At high TRAIL concentration (500ng/ml), the viability of HepG2 cells was 40.58%±2.00%. In both cell lines, moderate concentration of TRAIL (62.5ng/ml-125ng/ml) could decrease the cell viability to half but higher concentration of TRAIL did not lead to further reduction in viability. In the following cytotoxicity assay for TRAIL-4-MU combinations, two different concentrations of TRAIL, 50ng/ml and 100ng/ml, were used.

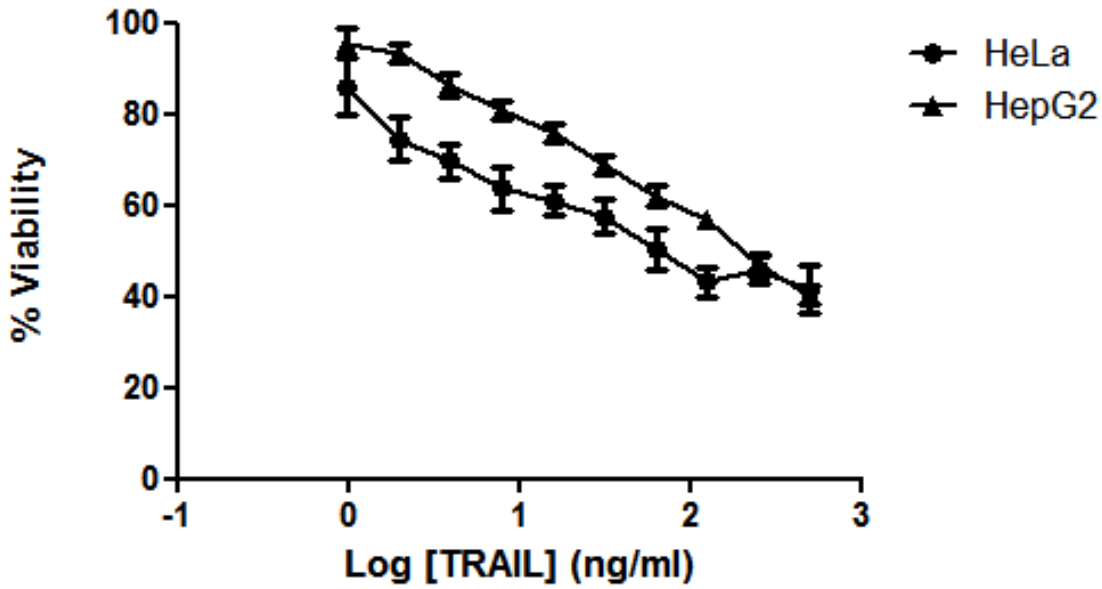


Figure 4. Cytotoxicity of TRAIL on HeLa cells and HepG2 cells. Cells were treated with TRAIL in various concentration for 24h. Cell viability was assessed using MTT assay and is expressed as percentage of untreated control cells. Date represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Dunnett's multiple comparison test to compare all treated samples with untreated control. $P<0.05$ for all TRAIL treatments compared with control in both cell lines.

HeLa cells and HepG2 cells were then treated with TRAIL (50ng/ml or 100ng/ml) and/or various doses of 4-MU (0.1mM, 0.3mM, 0.5mM and 1mM) for 24h. 4-MU enhanced TRAIL-mediated reduction in cell viability in a synergistic manner (Figure 5, Table 1). Combination index (CI) was calculated using software Compusyn (Chou, 2006). All combinations of TRAIL and 4-MU in high or low doses were in synergy according to the CI value (All CI values <1). Combination of 50ng/ml TRAIL and high concentration of 4-MU (0.5mM or 1mM) resulted in strong synergism or very strong synergism in both HeLa cells and HepG2 cells (Table 1). Based on one-way ANOVA analysis followed by Tukey's test, the differences among the percentage of viability of both HeLa cells and HepG2 cells treated with 50ng/ml TRAIL+0.5mM 4-MU, 50ng/ml TRAIL+1mM 4-MU, 100ng/ml TRAIL+0.5mM 4-MU and 100ng/ml TRAIL+1mM 4-MU were not statistically significant.

To evaluate the cytotoxicity of combinations of TRAIL and 4-MU on normal cells, cytotoxicity of the combinations was also tested on Hs68 human foreskin fibroblast cell line. As shown in Figure 6, no statistically significant reduction of viability was detected in Hs68 cells treated with TRAIL and low or moderate concentration (0.1-0.5mM) of 4-MU, suggesting that this combination at low or moderate dose is not harmful to this normal cell line. Considering the toxicity to normal cells of the combination at high concentration of 4-MU (1mM), as well as the insignificant differences in reduction in viability among HeLa cells and HepG2 cells treated with high concentrations of TRAIL (50ng/ml and 100ng/ml) and 4-MU (0.5mM and

1mM), moderate concentration of TRAIL at 50ng/ml and 4-MU at 0.5mM was chosen for further cell death analysis.

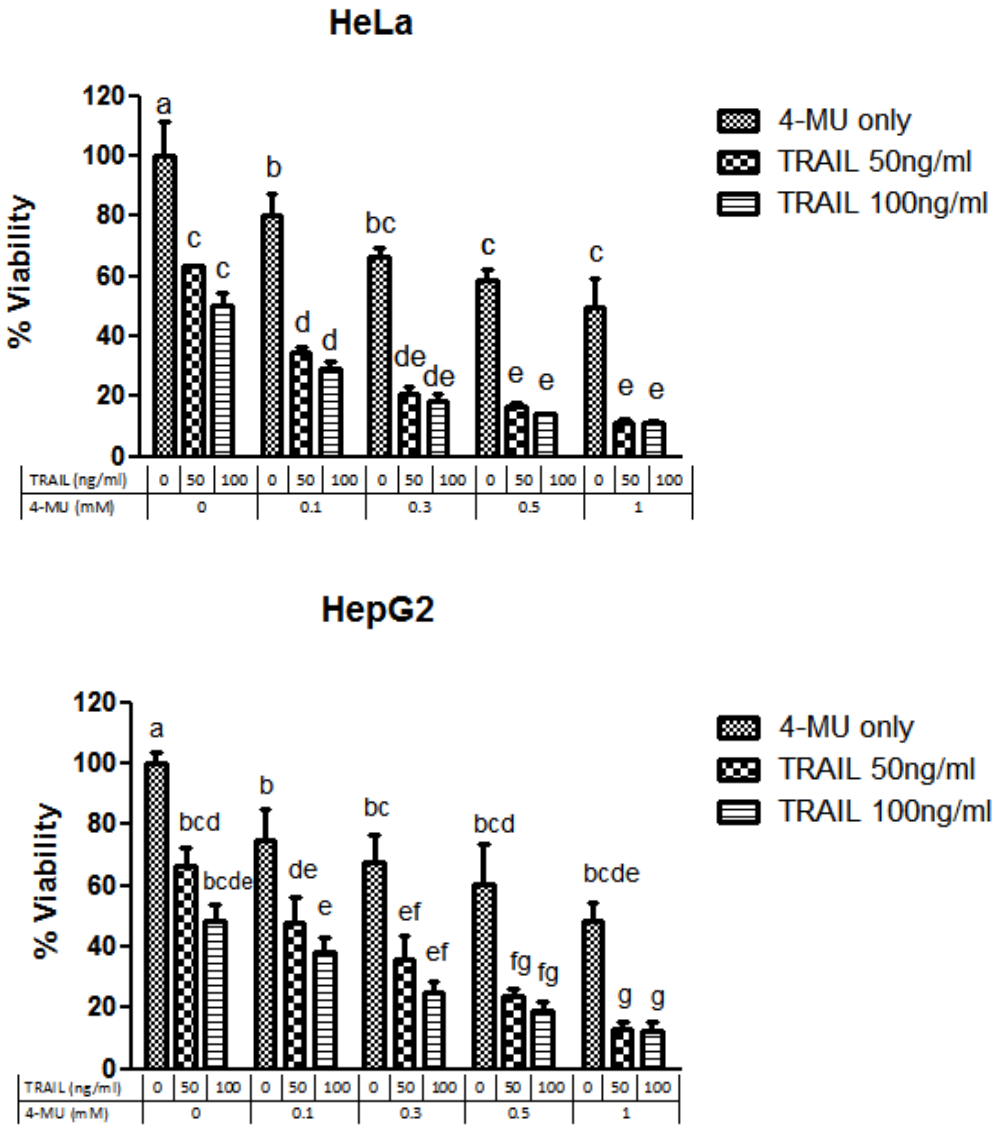


Figure 5. 4-MU enhanced TRAIL-induced cytotoxicity in HeLa cells and HepG2 cells. Cells were treated with indicated concentrations of TRAIL and/or 4-MU for 24h. C ell viability was determined by MTT assay and is expressed as percentage of untreated control cells on the left. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no superscript in common are significantly different from each other ($P<0.05$).

Table 1. Combination index on the cytotoxicity of TRAIL and 4-MU on HeLa cells and HepG2 cells

HeLa cells

TRAIL \ 4-MU	0.1mM	0.3mM	0.5mM	1mM
50ng/ml	0.273^{++++}	0.136^{++++}	0.107^{++++}	0.081^{+++++}
100ng/ml	0.366^{+++}	0.198^{++++}	0.137^{++++}	0.121^{++++}

HepG2 cells

TRAIL \ 4-MU	0.1mM	0.3mM	0.5mM	1mM
50ng/ml	0.566^{+++}	0.402^{+++}	0.236^{++++}	0.117^{++++}
100ng/ml	0.727^{++}	0.423^{+++}	0.314^{+++}	0.197^{++++}

Graded symbols: $^{++}$ moderate synergism (0.7<CI<0.85), $^{+++}$ synergism (0.3<CI<0.7), $^{++++}$ strong synergism (0.1<CI<0.3), $^{+++++}$ very strong synergism (CI<0.1) (Chou, 2006).

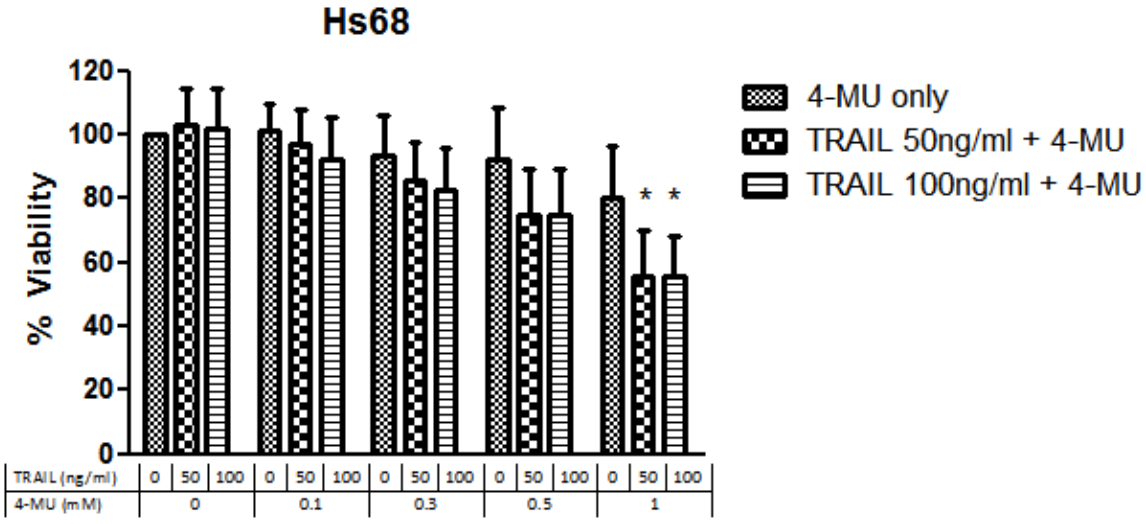


Figure 6. Cytotoxicity of TRAIL and 4-MU combinations on Hs68 human normal fibroblasts was insignificant at low and moderate doses. Cells were treated with indicated concentrations of TRAIL and/or 4-MU for 24h. Cell viability was determined by MTT assay and is expressed as percentage of untreated control cells on the left. Date represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Dunnett's multiple comparison test to compare all treated samples with untreated control. Only 50ng/ml TRAIL+1mM 4-MU and 100ng/ml TRAIL+1mM 4-MU gave statistically significant difference from the control (* P<0.05).

3.2.2 TRAIL-4-MU combinations had significant anti-proliferating effect and inhibited colony formation in HeLa cells and HepG2 cells

The anti-proliferating effect of TRAIL-4MU combinations was evaluated by MTT assay and BrdU colorimeteric assay. The percentages of inhibition of proliferation of different TRAIL-4-MU combinations were significantly higher than TRAIL alone or 4-MU alone at the same concentration in MTT assay (Figure 7). The percentage of inhibition of HeLa cells treated with 50ng/ml TRAIL and 0.5mM 4-MU was 93.12% ±0.29% and that of HepG2 cells treated with the same combination was 82.39%± 2.71%. For HeLa cells, there were no statistically significant differences among combinations of TRAIL and 0.3mM, 0.5mM or 1mM 4-MU. For HepG2 cells, a higher concentration of TRAIL in the combination did not increase the percentage of inhibition. Percentage of inhibition of HepG2 cells treated with 50ng/ml or 100ng/ml TRAIL and the same concentration of 4-MU were not significantly different.

BrdU colorimetric assay measures BrdU incorporation during DNA synthesis. Percentage of BrdU-positive cells significantly decreased with the concentration of 4-MU alone in both HeLa cells and HepG2 cells (Figure 8), which is not surprising given the anti-proliferating effect of 4-MU. Still, the percentages of BrdU-positive cells in cells treated with TRAIL-4-MU combinations were lower than that of cells treated with TRAIL or 4-MU alone at corresponding concentrations. The only exception is HeLa cells treated with TRAIL and 1mM 4-MU, of which the percentage of BrdU-positive cells were not statistically different from that of HeLa cells treated with 1mM 4-MU alone.

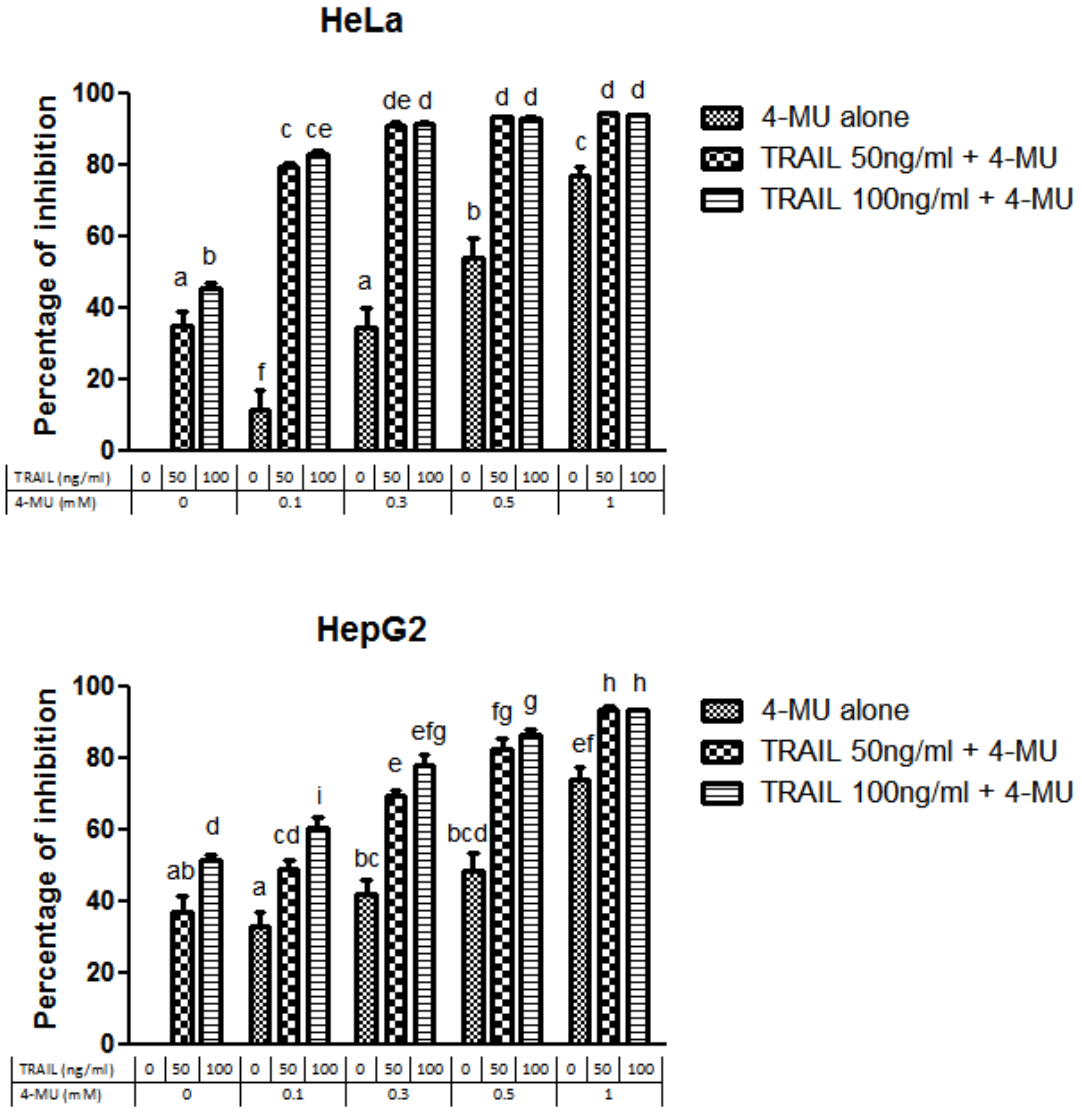


Figure 7. 4-MU enhanced the anti-proliferating ability of TRAIL on HeLa cells and HepG2 cells by MTT assay. Cells were treated with TRAIL and/or 4-MU for 72h before subjected to MTT assay. Percentage of inhibition was calculated as (OD570 ctrl- OD570 treated)/OD570 ctrl*100%. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no superscript in common are significantly different from each other ($P<0.05$).

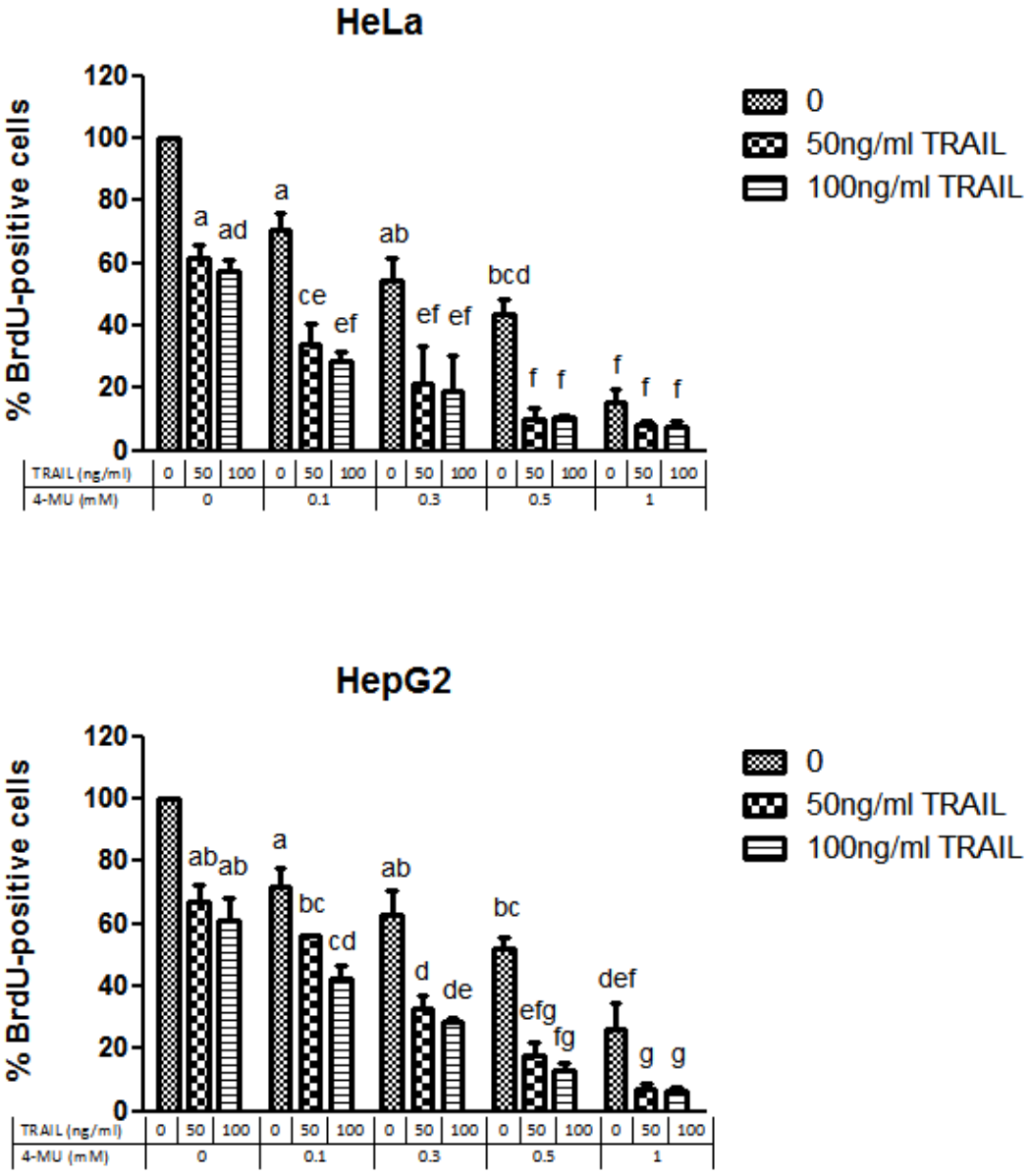
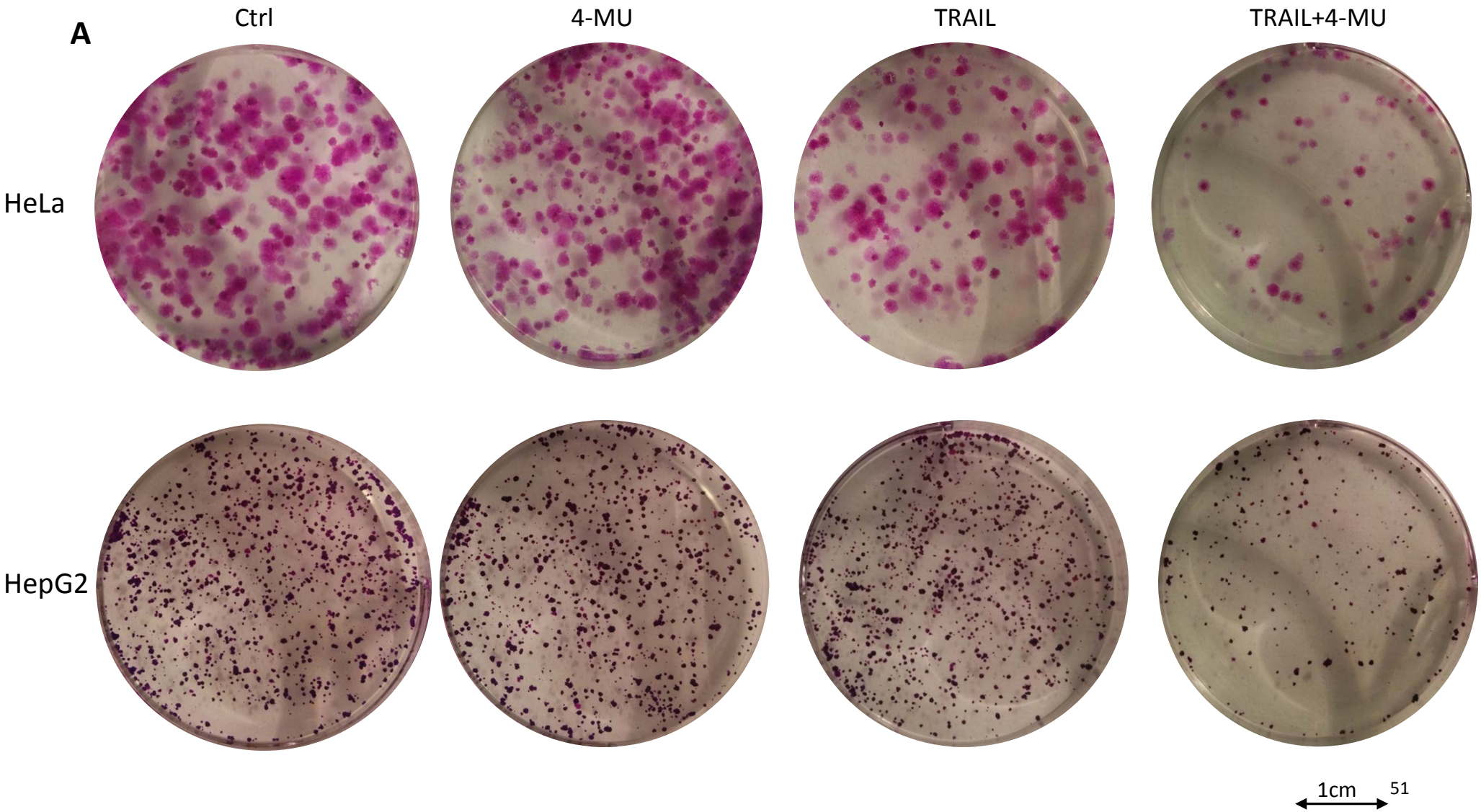


Figure 8. TRAIL and 4-MU combinations significantly inhibited DNA synthesis in HeLa cells and HepG2 cells. Cells were treated with TRAIL and/or 4-MU for 24h before subjected to BrdU colorimeteric assay. Percentage of BrdU-positive cells was expressed as percentage of untreated control cells on the left. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no s uperscript in common are significantly different from each other ($P<0.05$).

To evaluate the effect of short term TRAIL-4-MU treatment on the number of cells with proliferative ability in HeLa cells and HepG2 cells, colony formation assay was performed (Figure 9A). Cells were treated TRAIL and/or 4-MU for 12h and further cultured for 10 days. Each colony should originate from one single cell. Co-treatment of 50ng/ml TRAIL and 0.5mM 4-MU for 12h reduced the percentage of colony formation to 26.77%±4.89% in HeLa cells, and to 8.43%±3.15% in HepG2 cells (Figure 9B). Treatment with 0.5mM 4-MU alone only resulted in modest inhibition in colony formation, with 86.03%±8.50% colony formation in HeLa cells and 80.41%±7.58% colony formation in HepG2 cells. For cells co-treated with 50ng/ml TRAIL and 0.5mM 4-MU, the quantity and size of colonies formed were much smaller, indicating that combinatorial treatment of TRAIL and 4-MU can result in lasting inhibition in cell proliferation, by directly killing the cells and/or arresting the cells.


A
Ctrl
4-MU
TRAIL
TRAIL+4-MU
HeLa
HepG2
1cm
51

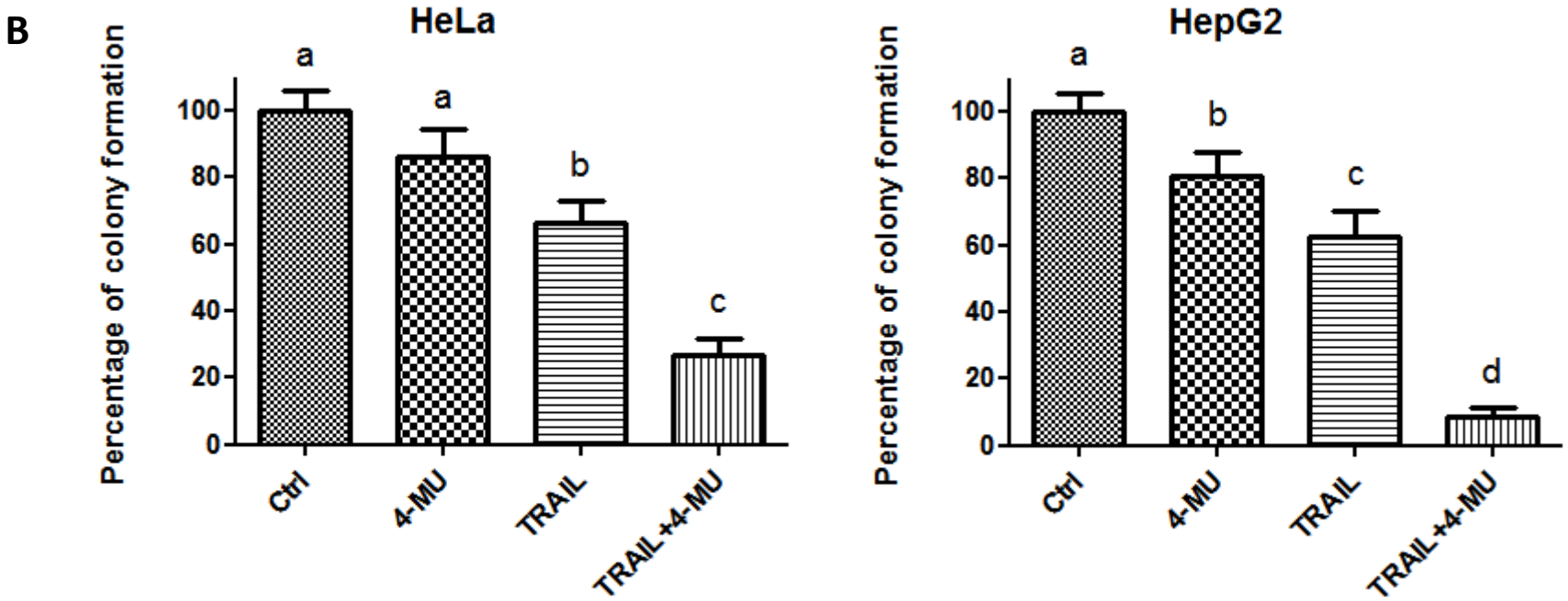


Figure 9. Combination of 50ng/ml TRAIL and 0.5mM 4-MU significantly inhibited colony formation in HeLa cells and HepG2 cells. Cells were treated with 50ng/ml TRAIL and/or 0.5mM 4-MU for 12h before replacement with fresh medium. Cells were further cultured for 10 days to allow colony formation. Colonies were fixed and stained with crystal violet and counted. (A) Images of colonies stained with crystal violet. Scale bar: 1cm. (B) Colonies were counted and expressed as percentage of control. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no superscript in common are significantly different from each other ($P<0.05$).

3.2.3 4-MU enhanced TRAIL-induced apoptosis in HeLa cells and HepG2 cells

TRAIL is the ligand that binds to death receptors on cell surface and triggers apoptosis via the extrinsic pathway. HeLa cells and HepG2 cells treated with TRAIL-4-MU combination showed typical apoptotic morphologies, including cell rounding and shrinkage, loss of adhesion to the growth surface, and membrane blebbing (Figure 10) (Kroemer et al, 2009). But morphological changes are not decisive evidences for apoptotic pathway. To determine if 4-MU augments TRAIL-mediated cell death by enhancing apoptosis, DNA fragmentation was detected by propidium iodide staining and caspase-3/7 activity was measured by commercially available molecular detection probe. CellEvent Caspase-3/7 Green Detection Reagent (CellEvent in short). CellEvent is a probe with a DEVD cleavage target. It would become fluorogenic upon DEVD cleavage by activated caspase-3/7 in cells.

In both HeLa cells and HepG2 cells, 0.5mM 4-MU alone did not lead to DNA fragmentation (Figure 11a and 11b). The percentage of cells in the sub-G1 region of 4-MU-treated cells was not statistically different from that of the untreated control cells. For HeLa cells, co-treatment with TRAIL and 4-MU increased the percentage of sub-G1 cells from 18.70%±4.03% of TRAIL-treated cells to 28.77%±2.26%. The percentage of sub-G1 cells of TRAIL-treated HepG2 cells and TRAIL-4-MU-treated HepG2 cells were 31.50%±0.75% and 67.90%±5.57%, respectively. The significant

increase in percentage of sub-G1 cells in TRAIL-4-MU-treated cells agree with the synergistic cytotoxicity effect shown in Figure 5 and Table 1.

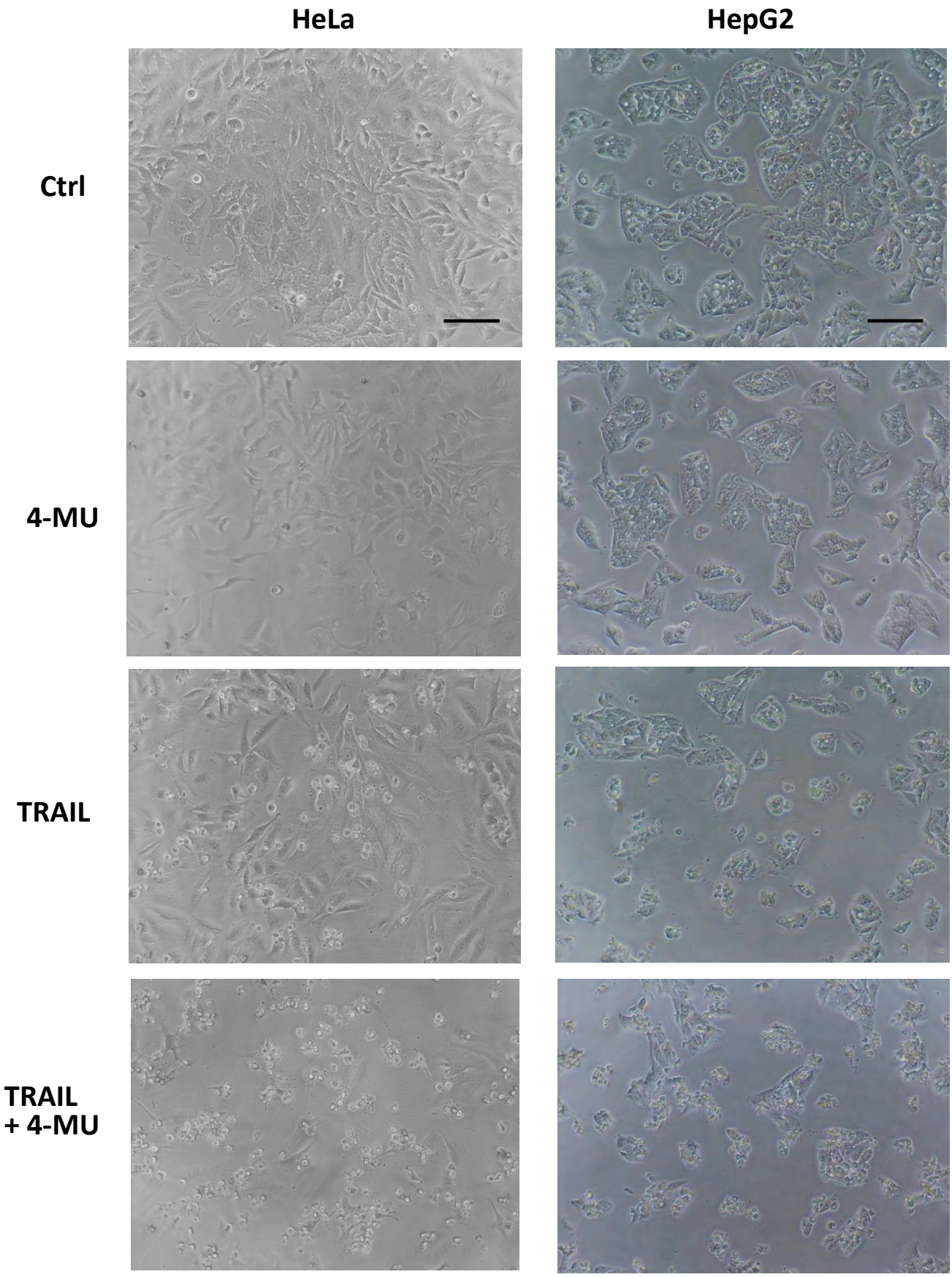
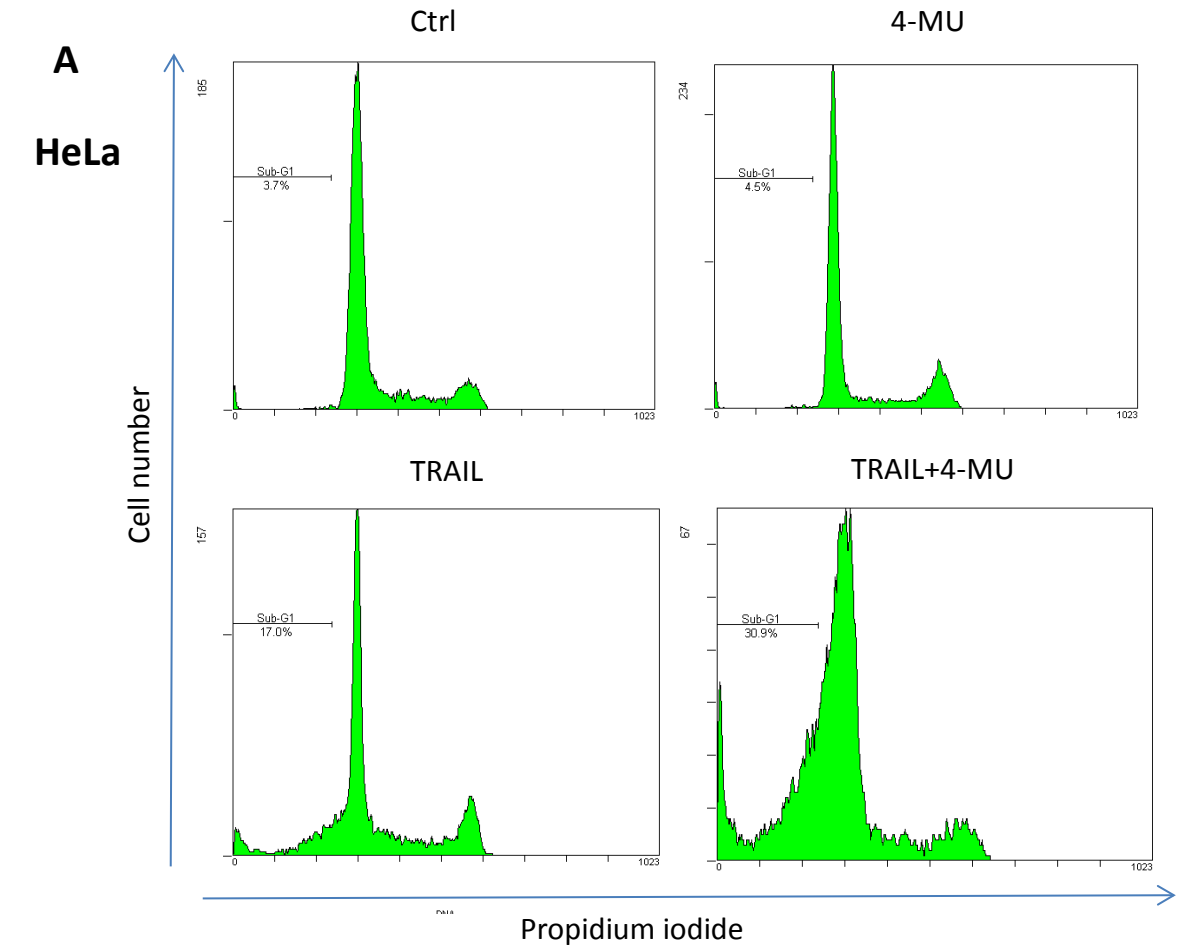
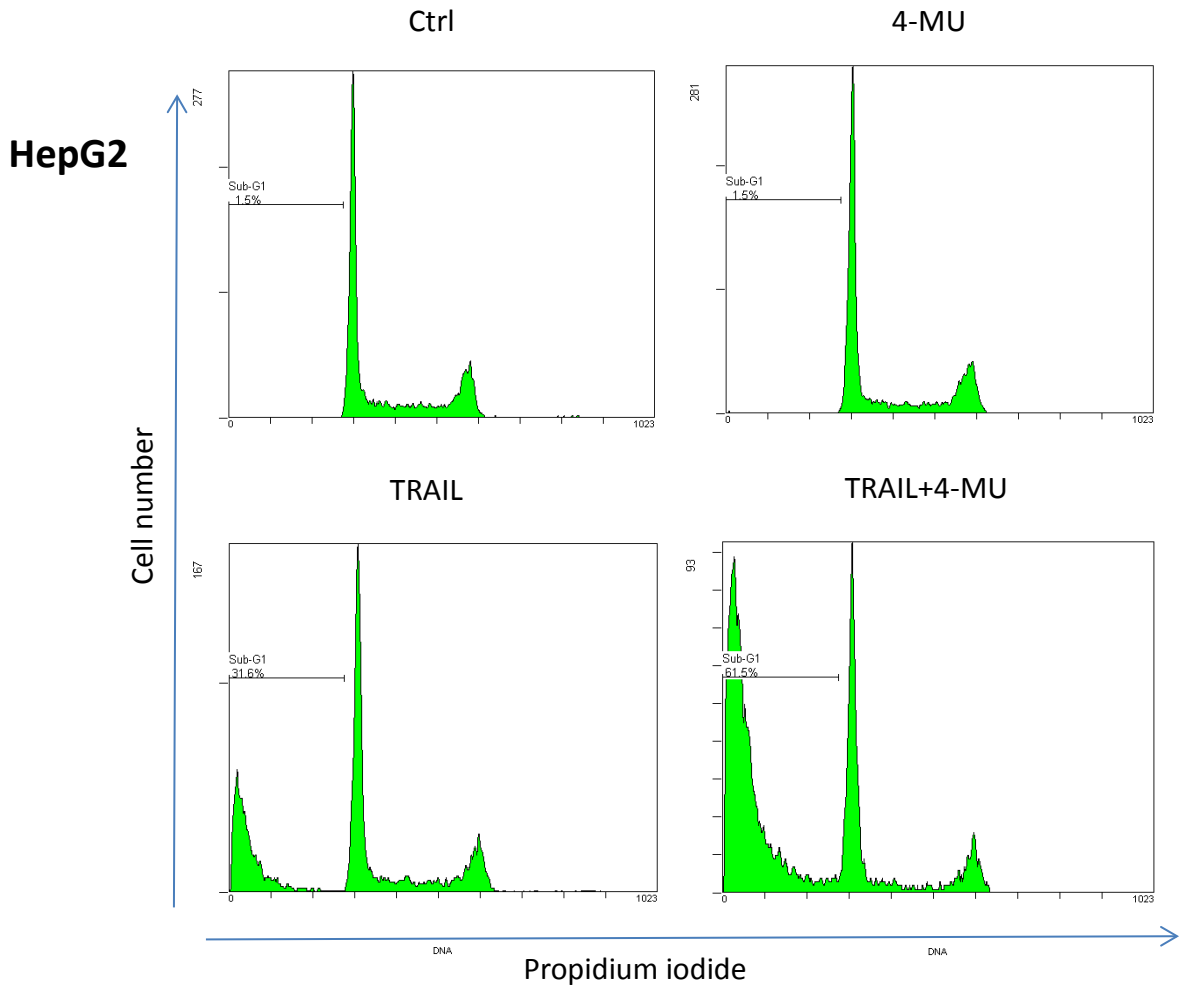


Figure 10. Apoptotic morphologies of (a) HeLa cells and (b) HepG2 cells treated with TRAIL and 4-MU. Cells were treated with 50ng/ml TRAIL and/or 0.5mM 4-MU for 24h. Phase contrast microscopy images were taken. Scale bar = 50μm.


A
HeLa
Ctrl
4-MU
TRAIL
TRAIL+4-MU
Sub-G1
3.7%
Sub-G1
4.5%
Sub-G1
17.0%
Sub-G1
30.9%
Cell number
Propidium iodide


HepG2
Ctrl
4-MU
TRAIL
TRAIL+4-MU
Sub-G1
1.5%
Sub-G1
1.5%
Sub-G1
31.6%
Sub-G1
61.5%
Cell number
DNA
DNA
Propidium iodide

B

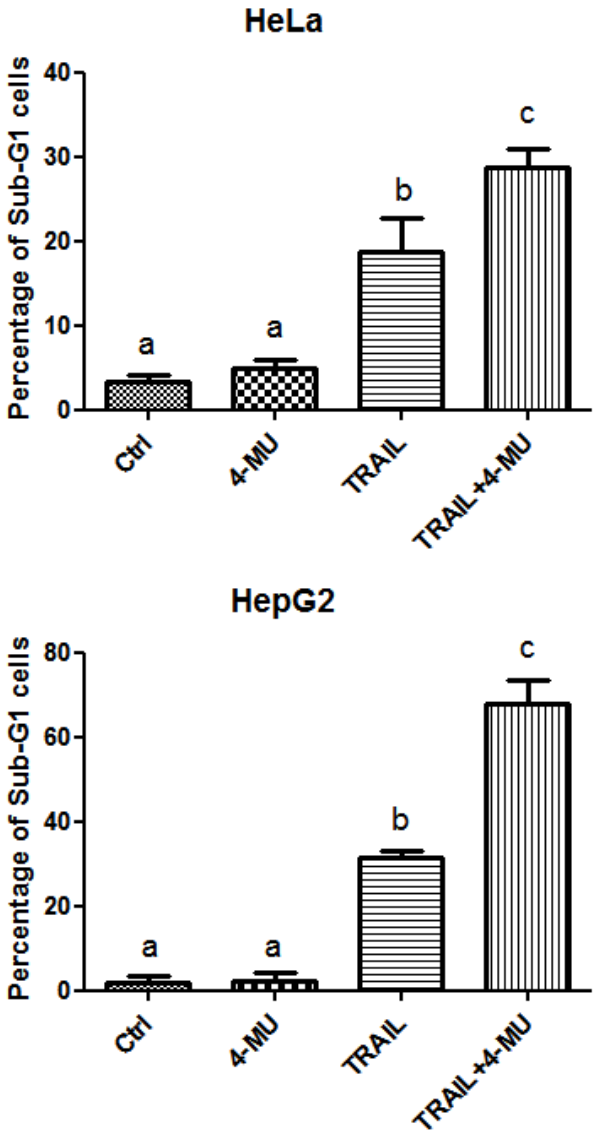


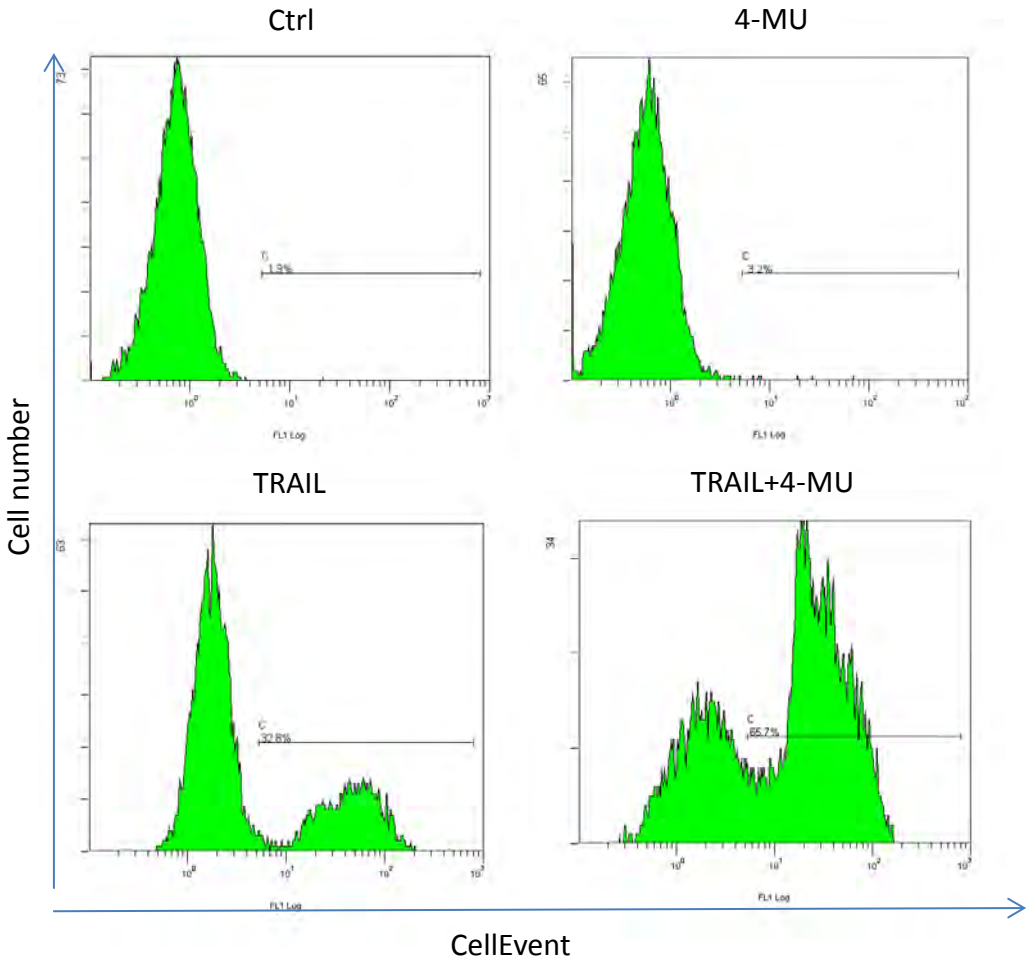
Figure 11. TRAIL-4-MU combination synergistically enhanced DNA fragmentation in HeLa cells and HepG2 cells. Cells were treated with 50ng/ml TRAIL and/or 0.5mM 4-MU for 24h be fore ethanol fixation. Apoptosis was determined by flow cytometry analysis of DNA fragmentation of propidium iodide-stained nuclei. A minimum of 10,000 cells were counted in each group.
(A) Representative results of cell cycle distribution of HeLa cells and HepG2 cells treated with TRAIL and/or 4-MU. (B) Comparison of the proportion of cells with DNA fragmentation, i.e., cells that lie in the sub-G1 region. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no superscript in common are significantly different from each other ($P<0.05$).

From the flow cytometry analysis of caspase-3/7 activity detected by CellEvent molecular marker, it was found that TRAIL-4-MU combinatorial treatment greatly increased the percentage of cells with caspase-3/7 activity than TRAIL treatment alone, in both HeLa cells and HepG2 cells (Figure 12a and 12b). Similar to the results of DNA fragmentation analysis, treatment with 0.5mM 4-MU alone for 24h in HeLa cells and HepG2 cells did not lead to apoptosis, as 4-MU treatment did not result in statistically significant increase in percentage of cells with caspase-3/7 activity. For HeLa cells, co-treatment with TRAIL and 4-MU increased the percentage of cells with caspase-3/7 from 31.80%±0.87% of TRAIL-treated cells to 66.14%±0.59%. The percentage of sub-G1 cells of TRAIL-treated HepG2 cells and TRAIL-4-MU-treated HepG2 cells were 33.60%±0.86% and 81.99%±1.22%, respectively. The synergistic enhancement in caspase-3/7 activity by TRAIL-4-MU combination also agrees with their synergistic cytotoxicity and increase in cells with DNA fragmentation. The percentage of cells with caspase-3/7 activity and the percentage of sub-G1 cells in TRAIL-4-MU-treated HepG2 cells were both high, which were 81.99%±1.22% and 67.90%±5.57%, respectively. However, in HeLa cells, TRAIL-4-MU treatment resulted in 66.14%±0.59% cells with caspase-3/7 activity but only 28.77%±2.26% sub-G1 cells with DNA fragmentation. Activation of effector caspase-3/7 and DNA fragmentation are two different stages in the apoptotic process. During apoptosis, DNA fragmentation is dependent on caspase-3 activity and is, therefore, downstream of caspase-3 activation (Jänicke et al, 1998; Kitazumi et al, 2011). The discrepancy in caspase-3/7 activity and DNA

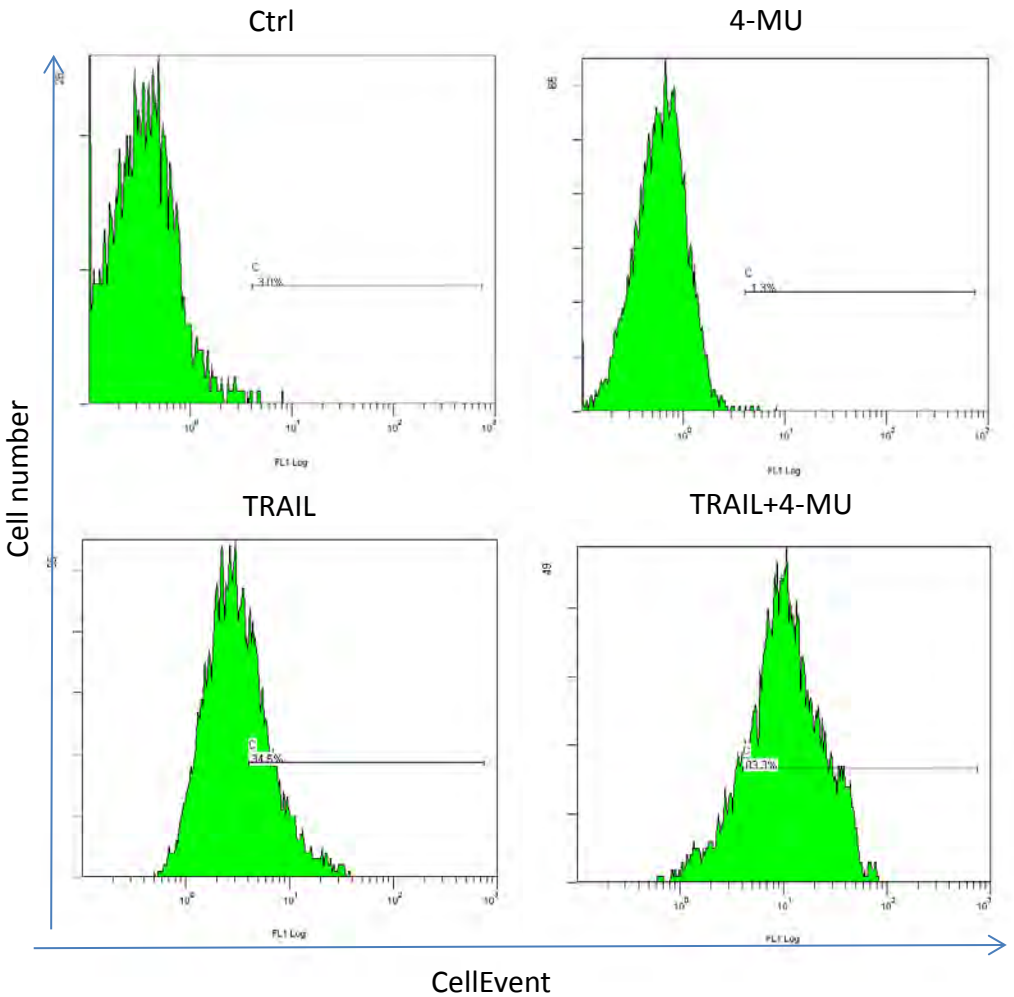
fragmentation in HeLa cells compared with HepG2 cells may indicate the difference in the rate of apoptosis progression after activation of effector caspases between the two cell lines. This difference may be cell type-specific or depending on cellular context.

A

HeLa


Ctrl
4-MU
TRAIL
TRAIL+4-MU
Cell number
CellEvent

HepG2


Ctrl
4-MU
TRAIL
TRAIL+4-MU
Cell number
CellEvent

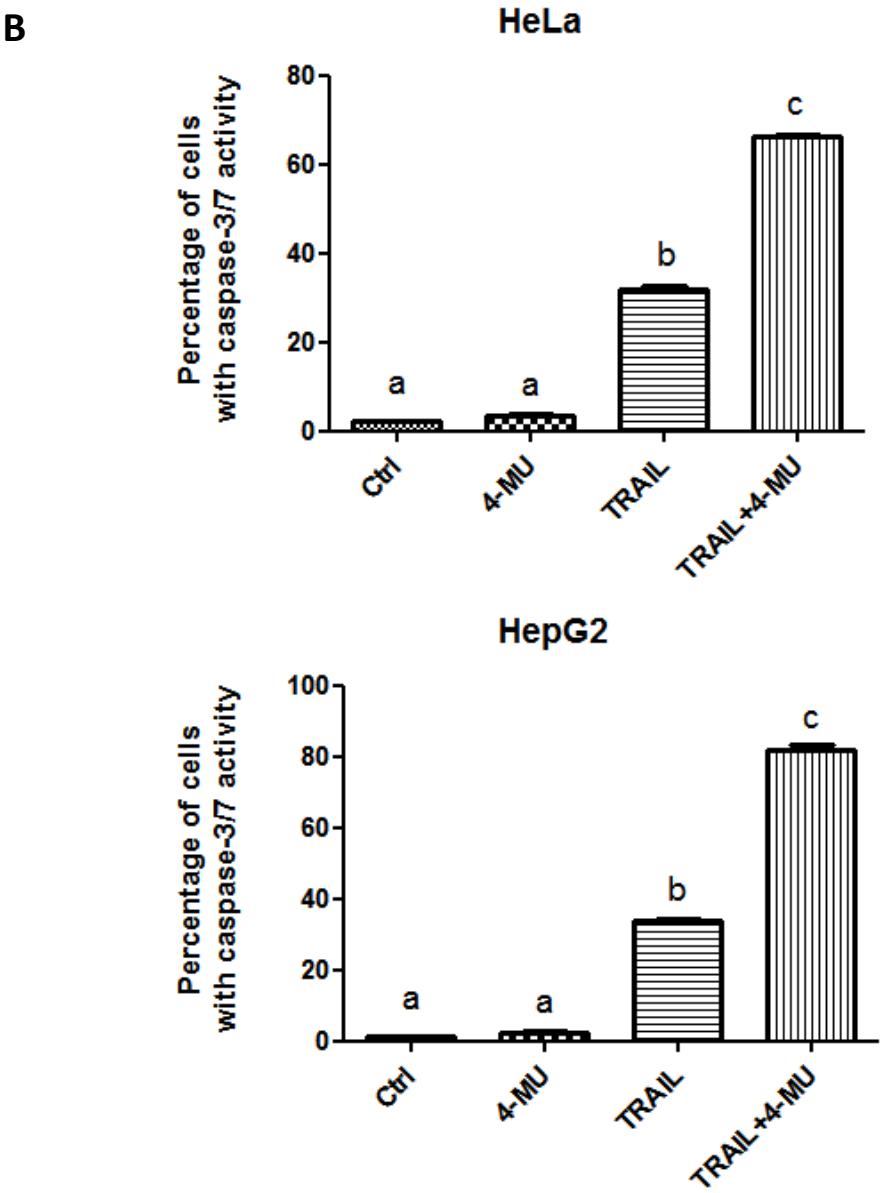


Figure 12. TRAIL-4-MU combination synergistically increased proportion of cells with caspase-3/7 activity in HeLa cells and HepG2 cells. Cells were pre-incubated in 10μM CellEvent molecular marker for 30 minutes before treatment with 50ng/ml TRAIL and/or 0.5mM 4-MU for 24h. Cells were afterwards harvested by trypsinization and apoptosis was determined by flow cytometry analysis of CellEvent signal, which is fluorogenic upon D EVD cleavage by caspase-3/7. A minimum of 10,000 cells were counted in each group. (A) Representative results of CellEvent signal of HeLa cells and HepG2 cells treated with TRAIL and/or 4-MU. (B) Comparison of the percentage of cells with caspase-3/7 activity. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no s uperscript in common are significantly different from each other ($P<0.05$).

3.2.4 4-MU reduces HA production

To investigate whether 4-MU suppresses hyaluronan production, level of hyaluronan in culture medium was detected by commercially available HA Elisa kit (Figure 13). In HeLa cells, 0.5mM 4-MU significantly decreased the HA concentration in the culture medium from 279.30ng/ml±12.48ng/ml of untreated control to 113.62ng/ml± 21.05ng/ml in 4-MU-treated cells and 53.20ng/ml±6.47ng/ml in TRAIL-4-MU-treated cells. In HepG2 cells, 4-MU reduced the HA concentration from 226.53ng/ml±19.20ng/ml of untreated control to 39.92ng/ml±8.59ng/ml in 4-MU-treated cells and to 26.50ng/ml±6.92ng/ml in TRAIL-4-MU-treated cells. The difference in HA concentration between 4-MU-treated cells and TRAIL-4-MU-treated cells was statistically significant in HeLa cells but not in HepG2 cells.

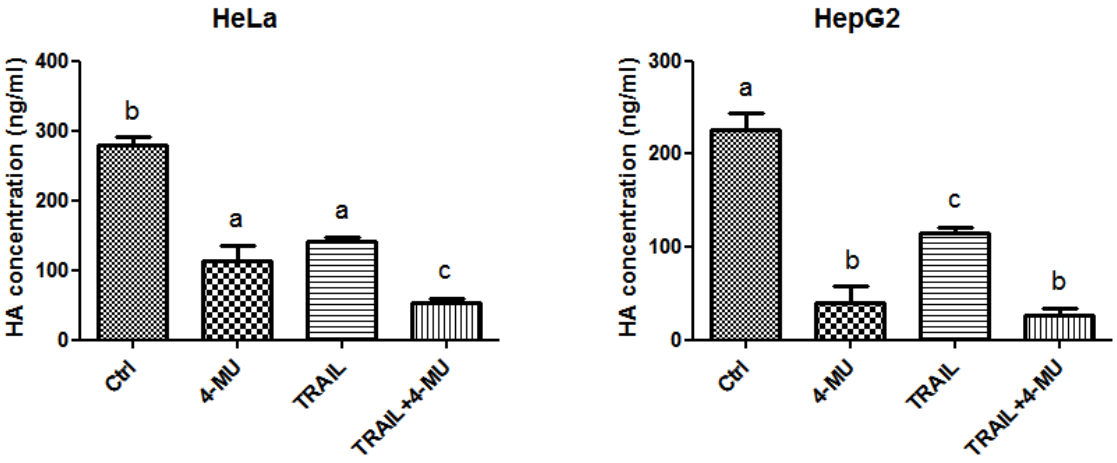


Figure 13. 4-MU suppresses HA production in HeLa cells and HepG2 cells. Cells were treated with 50ng/ml TRAIL and/or 0.5mM 4-MU for 24h. C onditioned medium was collected for measurement of hyaluronan (HA) level by HA Elisa kit. Data represent mean ± SD of three biological replicates. One-way ANOVA was performed followed by Tukey's test. Means that have no superscript in common are significantly different from each other ($P<0.05$).

3.2.5 TRAIL-4-MU enhanced tBid accumulation, down-regulated anti-apoptotic proteins and inhibited Akt phosphorylation

To investigate the mechanisms of 4-MU sensitization to TRAIL-induced apoptosis, expression/abundance of selected proteins related to TRAIL apoptotic pathways were detected by Western Blotting (Figure 14). Increase in cleaved PARP and cleaved caspase-3 again confirmed the enhancement in apoptosis by TRAIL-4-MU combinatorial treatment in HeLa cells and HepG2 cells. The abundance of DR4 (TRAIL-R1) and DR5 (TRAIL-R2) was not changed after treatment, suggesting that the increase in apoptosis after TRAIL-4-MU treatment was not by up-regulating the TRAIL receptors. Abundance of truncated Bid (tBid) has increased to 4.44-fold and 5.08-fold, respectively, in HeLa cells and HepG2 cells, whereas anti-apoptotic protein Bcl-2 was decreased to 0.37-fold and 0.45-fold, respectively. The accumulation of tBid and the down-regulation of the anti-apoptotic Bcl-2 protein on mitochondrial surface suggest that 4-MU sensitized cancer cells to TRAIL-induced apoptosis by promoting MOMP. XIAP was down-regulated to 0.23-fold and 0.20-fold in HeLa cells and HepG2 cells, respectively. P-Akt (S473) was also down-regulated, to 0.34-fold and 0.62-fold, indicating the inhibition of the pro-survival PI3K/Akt pathway upon co-treatment with TRAIL-4-MU. Abundance of CD44 remained unchanged in all treatment conditions.

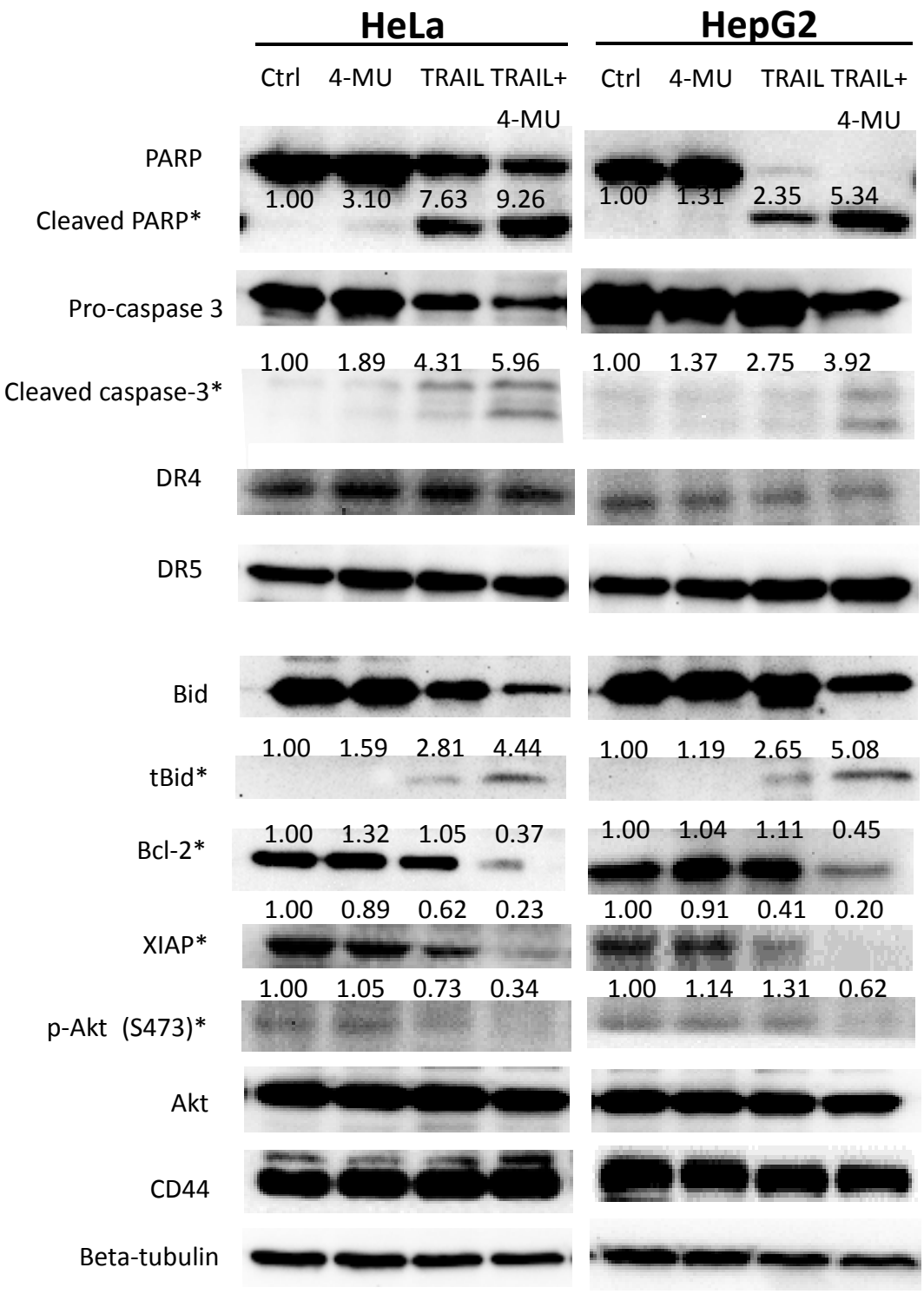


Figure 14. Abundance of selected proteins related to apoptosis or survival pathway detected by Western Blotting. HeLa cells and HepG2 cells were treated with 50ng/ml TRAIL and/or 0.5mM 4-MU for 24h before being harvested. Total cell lysate was prepared for Western Blotting analysis. *Proteins with differential abundance after TRAIL-4-MU treatment. The numbers on top of the protein bands are the mean relative abundance of proteins normalized to beta-tubulin. Two independent experiments were performed for proteins without differential abundance.

3.2.6 Inhibition of Akt sensitize cancer cells to TRAIL-induced apoptosis

To confirm that inhibition of Akt can enhance TRAIL-mediated cell death in HeLa cells and HepG2 cells, MTT cytotoxicity assay was performed on cancer cells co-treated with TRAIL and pharmacological Akt inhibitors. Two Akt inhibitors, LY 294002 and perifosine, were used. Co-treatment of TRAIL and either one of the Akt inhibitors significantly reduced the cell viability in both cell lines (Figure 15). This indicates that Akt inhibition is at least one of the means for the synergistic cytotoxicity of TRAIL-4-MU combinatorial treatment.

Western Blotting revealed enhanced PARP cleavage by TRAIL-LY-294002 co-treatment on HeLa cells and HepG2 cells (Figure 16), suggesting that the cells died of apoptosis. Inhibition of Akt was detected by the lowered level of p-Akt (S473) and unchanged level of total Akt protein.

Taken together, our study has revealed a novel combination of TRAIL and 4-MU, which has synergistic cytotoxicity and anti-proliferating effect. The TRAIL-4-MU combination enhances apoptosis by increasing tBid accumulation, down-regulating anti-apoptotic proteins and inhibition of Akt.

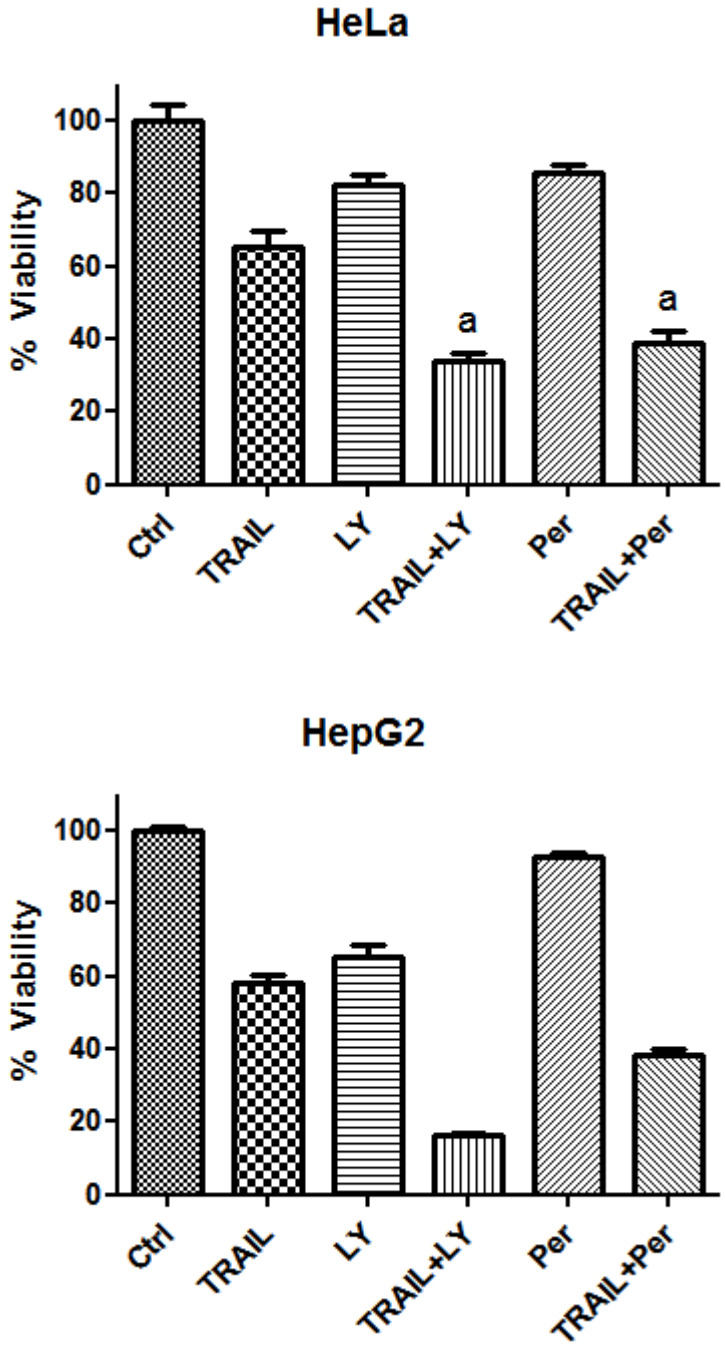


Figure 15. Akt inhibitors sensitized HeLa cells and HepG2 cells to TRAIL-mediated cell death. Cells were treated with 50nM TRAIL and/or 20μM LY-294002/20μM perifosine for 24h before MTT assay. Data represent mean ± SD of three independent experiments. One-way ANOVA was performed followed by Tukey's test. Means that have no superscript in common are significantly different from each other ($P<0.05$). LY: LY-294002, Per: perifosine.

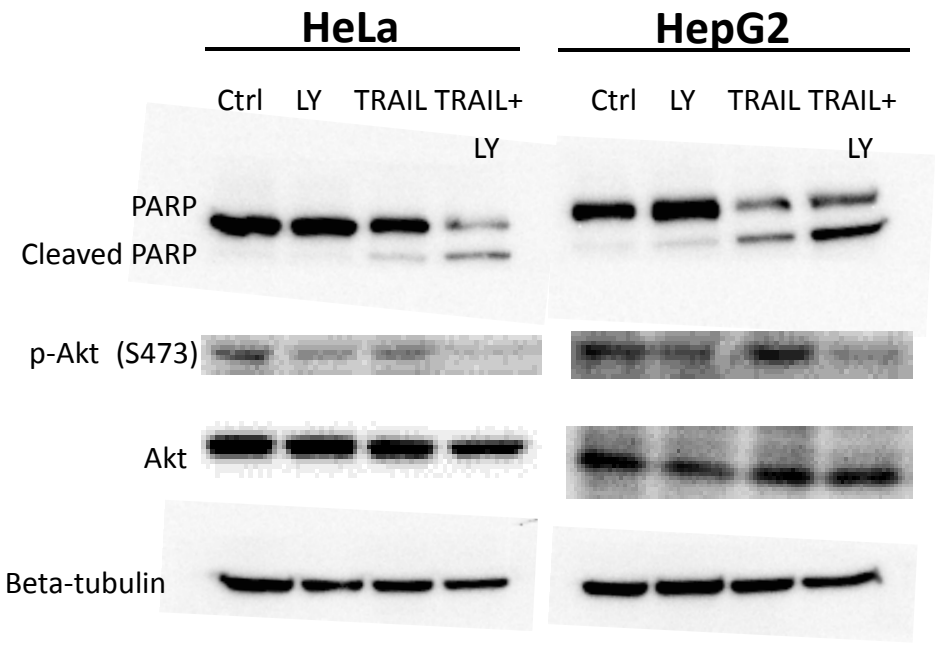


Figure 16. TRAIL-LY-294002 co-treatment increased PARP cleavage and inhibited Akt in HeLa cells and HepG2 cells. HeLa cells and HepG2 cells were treated with 50ng/ml TRAIL and/or 20μM LY-294002 for 24h before being harvested for Western Blotting analysis. Two independent experiments were performed.

3.3 Discussion

In this chapter, a novel anti-cancer combination of TRAIL and 4-MU has been revealed, presenting a promising anti-cancer approach of combining TRAIL-based therapeutics and hyaluronan-targeted strategy. Several pieces of evidences were presented in this work. First, strong synergistic cytotoxicity of TRAIL-4-MU combination was demonstrated in HeLa human cervical carcinoma cell line and HepG2 human hepatocarcinoma cell line, with respective combination index 0.107 and 0.136 for HeLa and HepG2 cells treated with 50ng/ml TRAIL and 0.5mM 4-MU. More importantly, this combination and doses did not have cytotoxicity on Hs68 human foreskin fibroblast cell line in MTT assay, suggesting the existence of a safe therapeutic window with good anti-cancer activity. Second, the effective anti-proliferating activity of TRAIL-4-MU combination was demonstrated by MTT assay, BrdU cell proliferating assay and colony formation assay. Third, 4-MU potentiated TRAIL-mediated apoptosis, supported by the increase in DNA fragmentation and effector caspase-3/7 activity. Fourth, TRAIL-4-MU combination enhanced apoptosis by targeting Akt, an important pro-survival regulator for TRAIL resistance and non-canonical signaling triggered by TRAIL (Azijli et al, 2013).

3.3.1 Potentiation of TRAIL-induced apoptosis with 4-MU

There are various approaches in sensitizing cancer cells to TRAIL-induced apoptosis and counteracting TRAIL resistance, including but not limited to stabilization or increased expression of TRAIL receptors DR4 and DR5, tilting the balance of pro- and anti-apoptotic proteins, promoting degradation of proteins involved in the signaling, switching from type II to type I signaling, and inhibition of pro-survival pathways. Usually, a single sensitizing agent can regulate TRAIL sensitivity of cells at multiple levels (Maksimovic-Ivanic et al, 2012; Kurita et al, 2011).

In this study, TRAIL-4-MU combination displayed synergistic cytotoxicity by enhancing apoptosis. As discussed in Chapter 1, there are many mechanisms for TRAIL resistance in cancer cells. TRAIL resistance may arise theoretically in every step of TRAIL signaling pathways, from death receptor abundance/function to availability of active caspase-3. The expression level of TRAIL receptors, DR4 and DR5 were unchanged under TRAIL and/or 4-MU treatment. But this cannot completely rule out the possibility of regulation at receptor level. The binding of TRAIL to DR4 and DR5 may have been altered as 4-MU reduced hyaluronan production, which may cause changes in the extra-cellular matrix. The trimerization of receptors and formation of DISC may also have been affected. Increase in abundance of anti-apoptotic proteins, such as XIAP, Bcl-2 and Bcl-xL, as well as the engagement in pro-survival, non-apoptotic signaling upon TRAIL binding are also

important means for cancer cell to escape from TRAIL-induced apoptosis. But when 4-MU was added together with TRAIL, abundance of tBid increased significantly. tBid is responsible for the cross-talk between extrinsic and intrinsic apoptotic pathway. Together with the down-regulation of anti-apoptotic Bcl-2 protein, this could amplify the MOMP signal, resulting in the release of cytochrome c and SMAC/Diablo for downstream apoptotic cascade. XIAP was also down-regulated. XIAP is a direct inhibitor to cleaved caspase-3 as well as an E3 ligase ubiquitinating caspase-3 for proteasomal degradation. It is also a determining factor for type I or type II signaling of cells (Aldridge et al, 2011). Its down-regulation would allow the amplification of activated effector caspases.

After TRAIL-4-MU treatment, the level of p-Akt (S473) decreased while Akt level remained unchanged. This indicates an inhibition of Akt either by reduced phosphorylation of Akt or enhanced de-phosphorylation, but not by down-regulating the expression of total Akt. PI3K/Akt signaling is one of the non-apoptotic, non-canonical signalings that can be triggered by TRAIL binding. It is important for cell proliferation and survival. Akt has been reported to stabilize and prevent the degradation of XIAP (Dan et al, 2004). It also prevents Bid cleavage and therefore prevents MOMP and subsequent apoptotic cascade (Majewski et al, 2004). Here, the reduced level of tBid and XIAP agrees with the inhibition of Akt. But whether inhibition of PI3K/Akt is the only cause for lowered tBid and XIAP and the sensitivity of cancer cells to TRAIL-mediated cell death remains elusive.

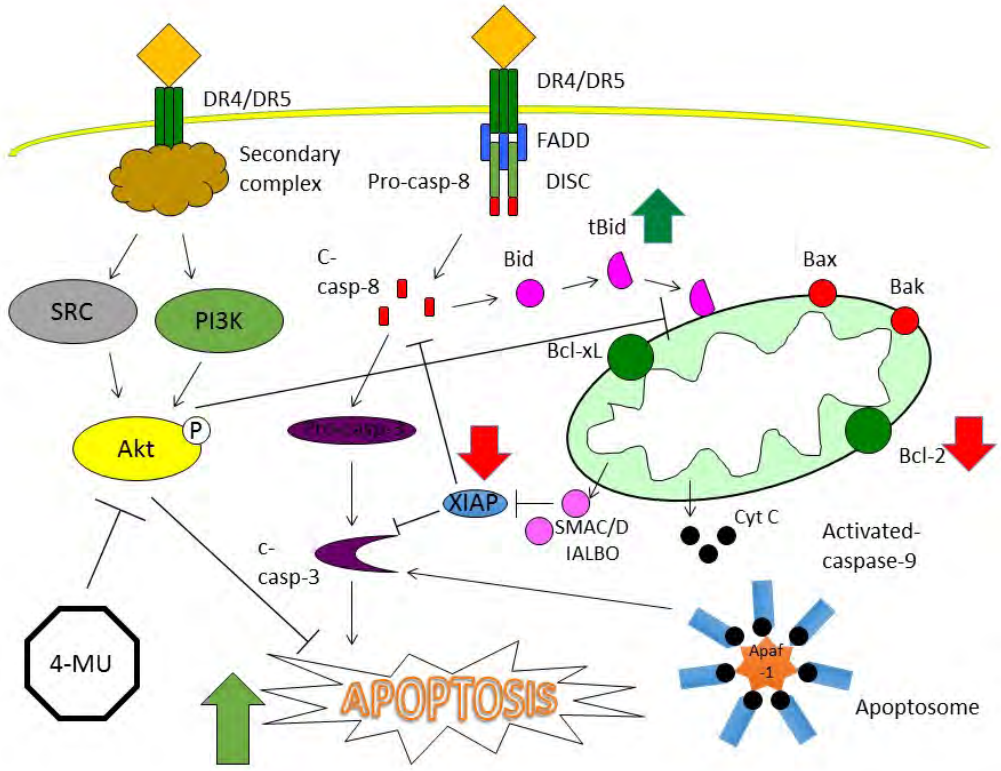


Figure 17. 4-MU potentiates TRAIL-induced apoptosis by inhibiting the pro-survival PI-3K/Akt pathway, up-regulating tBid and down-regulation of anti-apoptotic XIAP and Bcl-2. With the increase in tBid level and reduction in Bcl-2 level, MOMP should have been enhanced. Decrease in XIAP level would leave cleaved caspase-3 un-inhibited and un-ubiquitinated for proteasomal degradation, allowing faster accumulation of active caspase-3 to lethal level for complete apoptosis.

3.3.2 HA-CD44 interactions

Attempts were made to study whether HA-CD44 interactions were disrupted by TRAIL-4-MU combinations. Concentration of hyaluronan in conditioned medium was reduced by 4-MU. However, the level of CD44 remained unchanged in both cell lines in all treatment conditions (4-MU alone, TRAIL alone and TRAIL-4-MU) in Western Blotting. The anti-CD44 antibody used for Western Blotting targets on the cytoplasmic tail of the protein while hyaluronan binds to the CD44 region outside the cells. It cannot be concluded that HA-CD44 interactions were not disrupted.

There are multiple forms of CD44 encoded by one single gene due to alternative splicing. The CD44 variants have different binding affinity to hyaluronan. Some of them seem to be more associated cancer progression and metastasis while others are not. (Misra et al, 2011). It may be worthwhile to investigate the expression level of CD44 variants that are cancer-related after TRAIL-4-MU treatment. On the other way round, it is also interesting to study the combination of TRAIL and antagonistic anti-CD44 antibody that is variant-specific.

Chapter 4 Reversibility of TRAIL-induced apoptosis and its inhibition with 4-MU

4.1 Introduction

Contrary to general perception, activation of apoptotic pathway does not guarantee a commitment to cell death. Evidence for reversibility of apoptosis has been presented more than 2 decades. It was found that cells with externalized phosphatidylserine (PS) labeled by Annexin V can recover and resume growth *in vitro* (Hammill et al, 1999; Geske et al, 2001). Reddien and colleagues later found that in *C. elegans* mutant that failed to externalize PS, the "eat me" signal, during embryogenesis, some cells that should be programed to suicide showed apoptotic morphology but later recovered and survived (Reddien et al. 2001). Since DNA fragmentation is part of the apoptotic process, cells recovered from apoptosis may bear genetic changes as DNA repair is not error-free. It was proposed that cells survive apoptosis should have endured caspase-dependent DNA damages and would potentially carry carcinogenic translocations (Vaughan et al. 2002). Tang and other colleagues in our laboratory later demonstrated the reversibility of cancer cells in real-time by live cell imaging (Tang et al, 2009). We later worked on to show that normal cells could also recover

from apoptosis with the accumulation of genetic alterations and phenotypic transformation (Tang et al, 2012). More recently, our group proved the reversibility of HeLa cells induced by STS after activation of executioner caspases by sorting out cells with caspase-3/7 activity. The reversed cell population displayed altered responses to chemotherapeutic drugs (Fung's lab, unpublished data). All these evidences suggest that reversal of apoptosis, if occurs in tumor *in vivo*, may contribute to tumor heterogeneity, at least in terms of genetic stability and drug resistance.

Since TRAIL does not only induce apoptosis but also other pro-survival or growth-promoting signals in cells (Azijli et al, 2013), it is likely that TRAIL-induced apoptosis is also reversible in cancer cells. In this study, I investigated the reversibility of TRAIL-induced apoptosis in HeLa cells by isolation and further culture of caspase-3-activated HeLa cells induced with TRAIL by fluorescence-activated cell sorting (FACS). As 4-MU can significantly potentiate TRAIL-induced apoptosis, I also investigated if co-treatment of TRAIL and 4-MU or addition of 4-MU to post-sorted cells induced with TRAIL can inhibit the survival of caspase-3-activated cells induced with TRAIL by colony formation assay after FACS.

4.2 Results

To prove the speculation of reversibility of TRAIL-induced apoptosis, HeLa cells were pre-incubated in 10μM CellEvent and treated with TRAIL or STS (as positive control for reversal from apoptosis) with or without 4-MU for 6h. Cells that were positive for CellEvent signal were sorted out for colony formation assay. The CellEvent fluorescent level among HeLa cell populations of different conditions was evaluated before cell sorting. The fluorescent level of un-induced HeLa cells with CellEvent pre-incubation was regarded as the reference for gating CellEvent-positive cells in all samples. Under this gating, there were only 0.4% CellEvent-positive cells in un-induced control. The percentage of CellEvent-positive cells was 56.2% in TRAIL-treated HeLa cells. Co-treatment of TRAIL and 4-MU increased the percentage to 73.8%. For STS treatment, the percentage of CellEvent-positive cells was 50.4% and that for STS-4-MU-treated HeLa cells was 52.1% (Figure 18).

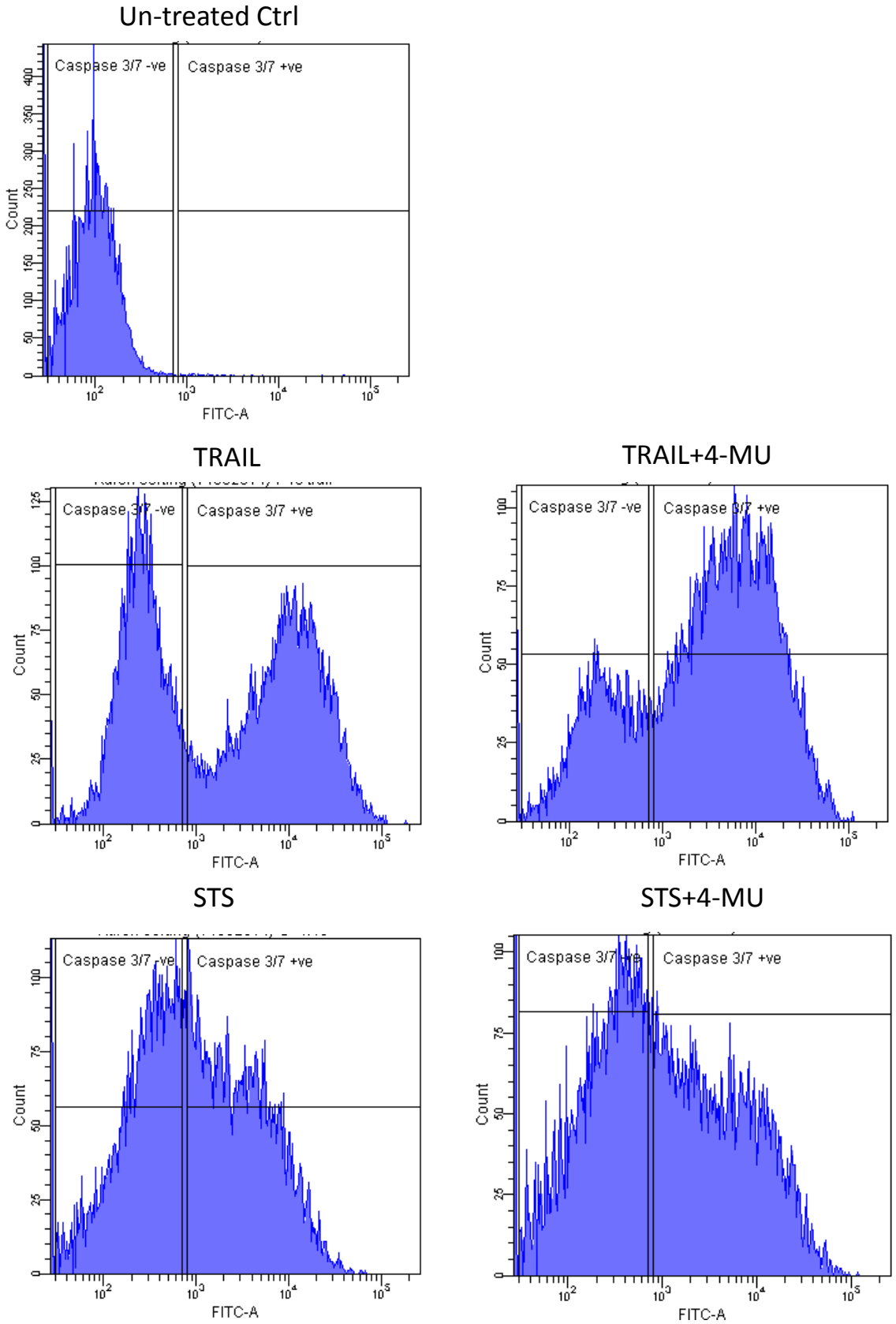


Figure 18. Caspase-3/7 activity in HeLa cells to be isolated by FACS. HeLa cells were pre-incubated with 10µM CellEvent probe for 30min and treated with 50ng/ml TRAIL or 2µM STS with or without 0.5mM 4-MU for 6h before FACS analysis and sorting.

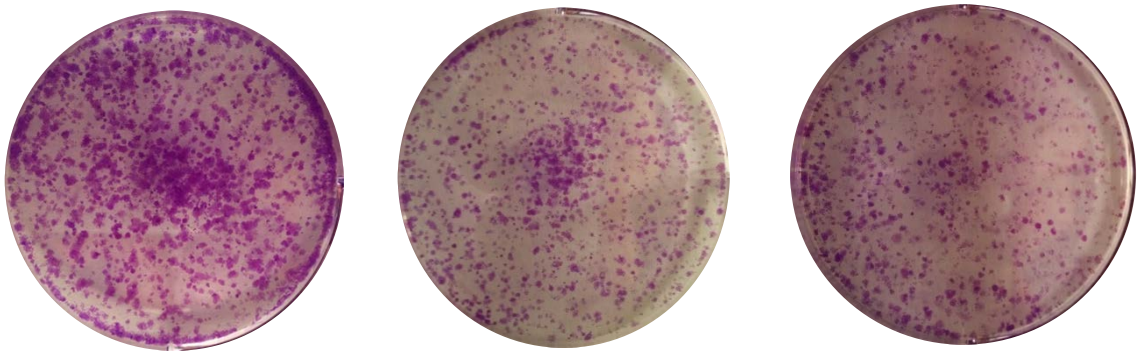
After analysis, CellEvent-negative cells were sorted out for untreated control and CellEvent-positive cells were sorted out for TRAIL- or STS-treated HeLa cells with or without 4-MU. Sorted cells were seeded in 6-well plates (5,000 cells per well) and incubated in fresh medium or medium with 4-MU or genistein for 72h before medium replacement for further culture for another 7 days. The colonies were then fixed and stained with crystal violet for counting. Representative images of colonies were shown in Figure 19. The number of colonies was counted and expressed as percentage of untreated cells in Table 2. Genistein was previously shown to inhibit recovery of HeLa cells from ethanol-induced apoptosis and was included here as positive control for inhibition of reversal of apoptosis (Xie et al, 2013).

From the results of colony formation assay, a small proportion of TRAIL- or STS-induced HeLa cells could recover from apoptosis and further proliferate (7.19%±0.43% for TRAIL-induced cells and 4.74%±0.49% for STS-induced cells). Addition of genistein or 4-MU to TRAIL-/STS-induced HeLa cells after sorting significantly reduced the percentage of colony formed to 1.34%±0.48%-2.61%±0.18%, suggesting that 4-MU could inhibit reversal of cells from TRAIL- or STS-induced apoptosis. But the inhibitory effect was even higher when 4-MU was added to the cells together with the apoptotic inducer. The percentage of colony formed for TRAIL-4-MU-treated HeLa cells was only 0.63%±0.27% and that for STS-4-MU-treated HeLa cells was only 1.91%±0.36%. This suggests that 4-MU could effectively sensitize HeLa cells to TRAIL-induced

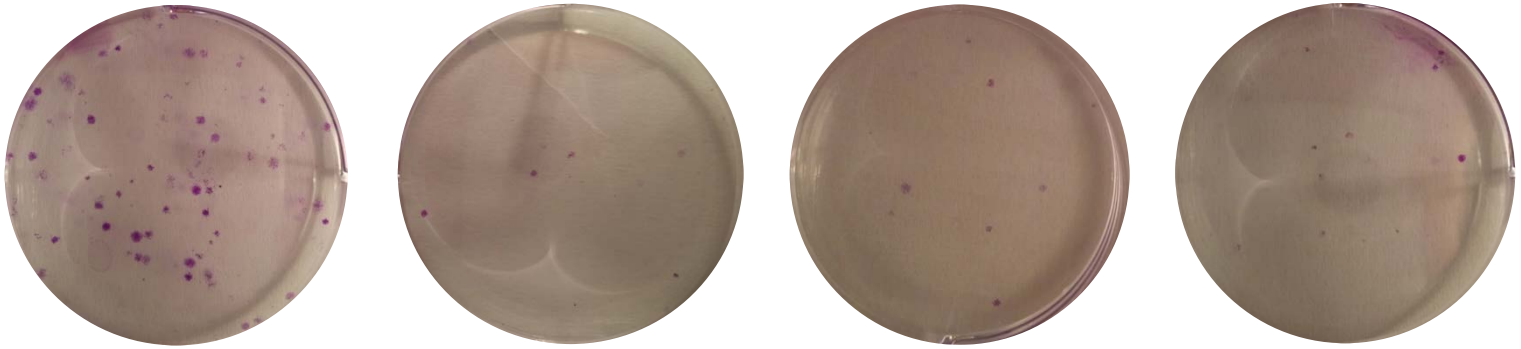
apoptosis and ensure the cells commit to apoptotic cell death.

Table 2. Colonies formed from TRAIL- or STS-induced HeLa cells isolated by FACS

Before sorting	After sorting	Percentage of colony formed	Before sorting	After sorting	Percentage of colony formed	Before sorting	After sorting	Percentage of colony formed
Untreated	/	100%±5.14%	TRAIL	/	7.19%±0.43%	STS	/	4.74%±0.49%
Untreated	Genistein	65.68%±8.19%	TRAIL	Genistein	1.49%±0.18%	STS	Genistein	2.20%±0.30%
Untreated	4-MU	84.51%±9.11%	TRAIL	4-MU	1.34%±0.48%	STS	4-MU	2.61%±0.18%
			TRAIL +4-MU	/	0.63%±0.27%	STS+ 4-MU	/	1.91%±0.36%



Before sorting (6h)	Untreated	Untreated	Untreated	
After sorting (72h)	Untreated	0.5mM 4-MU	5µg/ml Genistein	



Before sorting (6h)	50ng/ml TRAIL	50ng/ml TRAIL	50ng/ml TRAIL+0.5mM 4-MU	50ng/ml TRAIL
After sorting (72h)	Untreated	0.5mM 4-MU	Untreated	5µg/ml Genistein

1cm

(Con't)

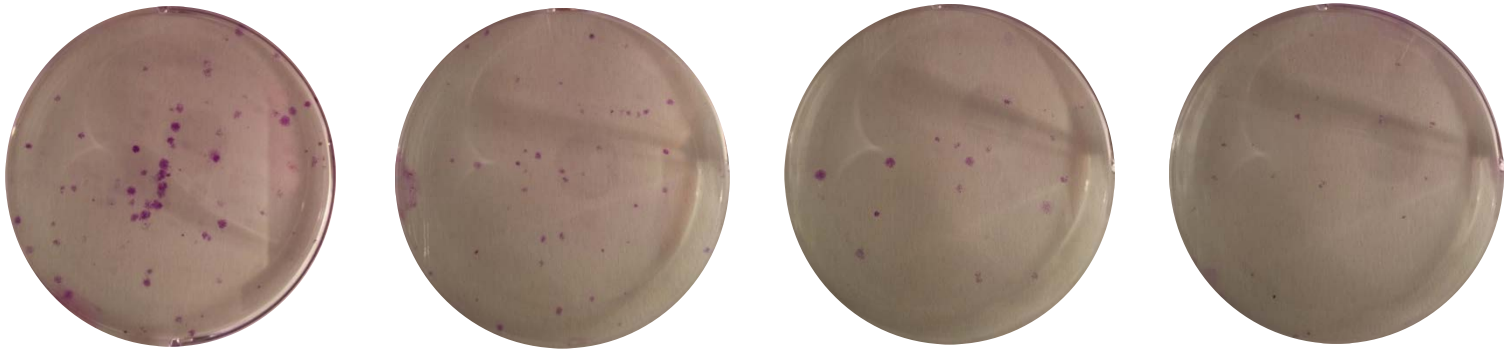


Figure 19. Recovery and proliferation of apoptotic HeLa cells induced by TRAIL or STS shown by colony formation assay. HeLa cells analyzed in Figure 18 were isolated by FACS. For untreated control cells, CellEvent-negative population was isolated. For cells treated with TRAIL or STS with or without 4-MU before sorting, CellEvent-positive cells were isolated. Sorted cells were counted and 5,000 cells were plated in triplicates in 6-well plates for each condition, in fresh medium or medium with 4-MU/genistein for 72h. Medium was then replaced and the cells were cultured for another 7 days to allow colony formation before fixation and counting. Scale bar:1cm.

4.3 Discussion

Apoptosis is an evolutionarily conserved mode of cell death that involves a controlled phase of demolition of cellular architecture to minimize disruption to neighboring cells and to avoid triggering immune responses (Taylor et al, 2008). The apoptotic signalings are carefully controlled and the fate of a cell, survival or death, is orchestrated by the coordination and balance of various pro-apoptotic proteins and anti-apoptotic proteins. The commitment of individual cells to apoptosis varied even within the same population.

It has long been known that individual cells in a clonal cell population can respond to stimuli differently. Goldstein et al observed that the time between stimuli exposure and MOMP varied depending on the type and strength of stimuli, whereas the rate and extent of cytochrome c release was constant, taking ~5min to reach completion (Goldstein et al, 2000). Rehm et al observed similar cell-to-cell variable delay between proapoptotic insult and effector caspase activity with a reporter of effector caspase activity. Kinetics of effector caspase cleavage is also dose invariant (Rehm et al, 2002). The variable delay in time-to-death and the sudden and rapid progression downstream MOMP constitutes a variable delay, snap-action switch in regulation of apoptosis. While the time delay between exposure to stimuli and activation of effector caspase is variable, execution of effector caspase cleavage is fast and efficient in almost all cells, taking only ~20min for complete PARP cleavage (Albeck et al, 2008a). Using

a mathematical simulation model combined with large-scale single cell analysis, Albeck et al further showed that stochastic variation in level of X-linked inhibitor of apoptosis protein (XIAP), a caspase inhibitory protein, and the difference in proteasome-mediated destruction of activated effector caspases are the causes for the variable delay between death receptor stimulation and MOMP (Albeck et al, 2008b).

As caspase 3 can cleave and activate several proteins such as ICAD (inhibitor of caspase-activated DNase), p53 and DNA-PK (DNA-dependent protein kinase) (Taylor et al, 2008; Luthi et al, 2007), cells surviving partial activation of caspase 3 are expected to harbor DNA damages (Vaughan et al. 2002; Albeck et al, 2008b). The accumulation of chromosomal aberrations may be oncogenic and may promote tumor heterogeneity, drug resistance and metastasis. In this study, the growth and proliferation of live HeLa cells with caspase-3/7 activity induced by TRAIL proved that TRAIL-induced apoptosis in cancer cells can be reversible *in vitro*. This fact can be devastating to the development of TRAIL-based anti-cancer therapy, considering that TRAIL has also been reported to promote cancer cell migration and metastasis. The possibility that cancer cells treated with TRAIL may survive with genetic alterations, increased drug resistance and the ability to metastasize is definitely deterring. Means that can inhibit the reversal should be undertaken to suppress cancer cell recovery from TRAIL-induced apoptosis.

For reversal of apoptosis to occur *in vivo*, four conditions must be achieved. The first

one is to limit effector caspase activity and even prolong execution time so that instant demolition of cellular structures would be prevented. XIAP and other inhibitor of apoptosis proteins (IAPs) should play a role here as they can act as direct caspase inhibitors as well as regulators for pro-survival NF-κB signal transduction (Albeck et al, 2008b; Gyrd-Hansen et al, 2010). The second one is the restoration of cellular environment and removal of cleaved proteins and damaged organelles by proteasome degradation. The third one is the maintenance of bioenergetic function. MOMP, besides cleavage of Bid, can also lead to decline of bioenergetic function due to loss of mitochondrial membrane potential (Kushnareva et al, 2010). A fast bioenergetic collapse would divert the cells toward necrotic demise. The fourth one is the evasion from phagocytosis *in vivo*. Given that cancer cells may escape from immune surveillance by evasion from phagocytosis, escape and survival of apoptotic cancer cells is not impossible. These four conditions can be useful therapeutic targets to prevent reversal of cancer cells from apoptosis after TRAIL therapy.

Here, we show that 4-MU can inhibit recovery and proliferation of cells from TRAIL-mediated apoptosis, both in co-treatment (TRAIL and 4-MU added together) or in sequential treatment (4-MU added after TRAIL treatment). There are two means to suppress cellular recovery, enhancing the force of cellular demolition and eradicating the pro-survival/repair mechanics of the cells. Based on the results from Fig. 13, I speculate that TRAIL-4MU combination has adopted both of them. Accumulation of tBid and down-regulation of Bcl-2 and XIAP caused by

TRAIL-4-MU combination should have promoted MOMP and augmented the apoptotic cascade, which was reflected by the increase in caspase-3/7 activity. The inhibition of Akt by TRAIL-4-MU combination suppressed the important PI3K/Akt pro-survival pathway.

Xie and other colleagues in our group have previously shown that genistein, an isoflavone, inhibits recovery of HeLa cells from ethanol-induced apoptosis by down-regulating XIAP (Xie et al, 2013). This study provides another piece of evidence supporting the role of XIAP in reversal of apoptosis, which should not be surprising given the caspase-inhibitory ability and its role in targeting caspase for degradation.

Chapter 5 Summary and Future studies

5.1 Summary

A novel synergistic anti-cancer combination of TRAIL and 4-MU has been reported in this study. Both TRAIL and 4-MU are not safe and not harmful to normal cells. The doses chosen here effectively kill HeLa and HepG2 cancer cells but not Hs68 human foreskin fibroblast, suggesting the presence of a therapeutic window that kills cancer cells without harming normal cells.

TRAIL-4-MU combination had synergistic cytotoxicity and anti-proliferating activity on HeLa cells and HepG2 cells. The enhanced cytotoxicity is caused by the potentiation of apoptosis by this combination. DNA fragmentation and caspase-3/76 activity were increased after the combinatorial treatment. TRAIL-induced apoptosis in HeLa cells was reversible, which was proven by the survival and proliferation of TRAIL-treated HeLa cells labeled for caspase-3/7 activity from FACS. Western Blotting results showed that TRAIL-4-MU combination inhibited Akt. Abundance of tBid has increased while anti-apoptotic protein Bcl-2 and were down-regulated. This suggested that TRAIL-4-MU combination enhanced apoptosis via mitochondria-dependent pathway (or the intrinsic pathway). Inhibition of Akt by

pharmacological inhibitors LY-294002 and perifosine also sensitized HeLa cells and HepG2 cells to TRAIL-mediated cell death with decreased p-Akt (S473) abundance, confirming the role of PI3K/Akt pathway in TRAIL-4-MU synergy.

This study has provided solid evidences substantiating further research on combination of TRAIL-based therapy and 4-MU or other hyaluronan-targeting therapy.

5.2 Future studies

5.2.1 TRAIL-4-MU synergy - cell migration, invasion and *in vivo* model

As discussed in Chapter 1, TRAIL may promote cell migration, invasion or even metastasis in part of the treated cells when it kills the rest of the population. Hyaluronan in the extra-cellular matrix can facilitate cell migration and invasion. 4-MU has also been reported to inhibit cancer cell invasion *in vitro* and lung metastasis *in vivo* (Arai et al, 2011; Kuwabara et al, 2011). Our next step should work on whether TRAIL-4-MU combination can inhibit cell migration and invasion *in vitro*. The combination should also be tested in xenograft tumor model in mice in order to prove the general safety, anti-cancer cytotoxicity and anti-proliferating activity of TRAIL-4-MU combination *in vivo*. After that, *in vivo* model can also be adopted to test whether TRAIL-4-MU combination suppresses metastasis.

5.2.2 Cancer stem cells, reversal of apoptosis and 4-MU

Cancer stem cells, also called tumor initiating cells, have become a very popular topic in cancer research. Cancer stem cells usually only take up a low percentage of the entire population in tumor and they often have different metabolic or proliferative behavior than the rest of the cancer cells. These differences are generally believed to make cancer stem cells more resistant to chemotherapy. Cancer stem cells are now regarded as one of the potential causes for cancer relapse and poor prognosis (Hanahan & Weinberg, 2011).

It was recently found that apoptotic cancer stem cells can form a “shield”-like structure from apoptotic blebs, termed blebbishield, to survive in the apoptotic process (Jinesh et al, 2013). Formation of blebbishield requires caspase activity, suggesting that the cancer stem cells survived may bear genetic changes. It would be of interest to characterize the population of cells reversed from TRAIL-induced apoptosis, to see if cancer stem cells are more likely to reverse from apoptosis than their cancer cell counterparts. A deeper understanding in the mechanism of reversal of apoptosis may help develop therapeutic strategies targeting cancer stem cells. TRAIL-4-MU combination was shown to inhibit reversal of HeLa cells from TRAIL-induced apoptosis in this study. It would be of therapeutic interest to investigate whether TRAIL-4-MU can eradicate cancer stem cells. Also, CD44 is a common cancer stem cell marker in various cancer types. 4-MU or other therapies

targeting HA-CD44 interactions may help killing cancer stem cells by suppressing reversal of apoptosis.

5.2.3 *In vivo* model for reversibility of TRAIL-induced apoptosis in cancer cells and potential consequences

In this study, reversibility of TRAIL-induced, apoptotic HeLa cells *in vitro* was shown by isolation and further culture of CellEvent-labeled cells by FACS. Further experiments will be needed to prove the reversibility of cancer cells from apoptosis *in vivo* and to investigate whether chromosomal aberrations and consequential phenotypic changes occur *in vivo*. CellEvent, which binds to DNA after caspase cleavage, is not an ideal choice for labeling caspase-3-activated cells for study of chromosomal or other phenotypic changes. A biomarker that can label the reversed cells and their progeny outside the nucleus would be good for studying the consequential changes of reversed cells *in vivo*.

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