Mechanism of Wogonin-mediated sensitization of HTLV-1-associated ATL cells to TRAIL-induced apoptosis

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Oral examination:……

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ABSTRACT

Targeting apoptosis pathways is one of the key strategies for cancer treatment. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) induces apoptosis in a variety of tumor cells but not in non-malignant cells. Therefore, TRAIL is a promising anti-cancer agent. However, approximately 50 % of cancers are resistant to TRAIL, including the human T-cell leukemia virus type 1 (HTLV-1)-associated adult T-cell leukemia (ATL). So far various treatment strategies are offered to ATL patients, but the mortality of ATL remains very high due to apoptosis resistance.

Our group has previously shown that Wogonin, derived from the Traditional Chinese Medicine (TCM) plant Huang-Qin (*Scutellaria baicalensis Georgi*), can sensitize TRAIL-mediated apoptosis in primary AML (acute myeloid leukemia) cells. Importantly, Wogonin does not affect the viability of peripheral blood T cells derived from healthy donors. However, the mechanisms of Wogonin-mediated sensitization of TRAIL-induced apoptosis are still not known.

In this study, HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 were used to investigate resensitization resistant tumor cells to TRAIL-induced apoptosis. We found that Wogonin sensitized TRAIL-induced apoptosis in HTLV-1-associated ATL cells by down-regulation of caspase-8 inhibitory protein (FLICE)-like inhibitory protein (c-FLIP) expression at the transcriptional level. In addition, Wogonin enhanced the expression of TRAIL receptor 2 (TRAIL-R2) *via* up-regulation of p53. Increased p53 expression was due to inhibition of p53 negative regulator MDM2 at the transcriptional level. We also demonstrated that structurally related natural flavones, *e.g.* Apigenin and Chrysin, inhibited c-FILP at the transcriptional level and increased TRAIL-R2 expression *via* up-regulation of p53 through suppression of p53 negative regulator MDM2 in HTLV-1-associated ATL cells. Our study raises the possibility to develop Wogonin, Apigenin and Chrysin as TRAIL adjuvants for treatment of ATL and other types of tumors.

ZUSAMMENFASSUNG

Einer der Hauptstrategien zur Behandlung von Krebs ist das Auslösen des programmierten Zelltods (Apoptose) in Tumorzellen. Das Protein TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) kann Apoptose in einer Vielzahl von Tumorzelllinien, aber nicht in normalen Körperzellen, induzieren. Allerdings weisen etwa die Hälfte aller Tumore eine Resistenz gegenüber TRAIL auf, unter anderem auch die mit dem humanen T-lymphotropen Virus 1 (HTLV-1) assoziierte adulte T-Zellleukämie (ATL). Aufgrund der Apoptoseresistenz dieser Leukämieart sind alle derzeit verfügbaren Behandlungsstrategien mit geringen Erfolgsaussichten verbunden und die Sterblichkeitsrate bei ATL Patienten ist sehr hoch.

In unserer Arbeitsgruppe konnte gezeigt werden, dass Wogonin, ein Bestandteil der traditionellen chinesischen Heilpflanze Huang-Qin (*Scutellaria baicalensis Georgi*), primäre AML (akute myeloide Leukämie) Zellen gegenüber TRAIL-induzierter Apoptose sensitivieren kann. Die Viablitiät nicht maligner peripherer T-Zellen wird durch Wogonin hingegen nicht beeinflusst. Die molekularen Mechanismen, durch welche Wogonin resistente Tumorzellen gegenüber TRAIL sensitiviert, sind jedoch noch weitgehend unbekannt.

In dieser Studie wurden die HTLV-1-infizierten ATL Zelllinien SP, MT-2 und MT-4 als Modellsystem benutzt, um die Sensitivierung resistenter Tumorzellen gegenüber der TRAIL-induzierten Apoptose zu untersuchen. Wir konnten zeigen, dass Wogonin HTLV-1-infizierte ATL Zelllinien gegenüber TRAIL sensitiviert, indem die Expression des Proteins c-FLIP (caspase-8 (FLICE)-like inhibitory protein) auf transkriptioneller Ebene gehemmt wird.

Darüber hinaus steigerte Wogonin die Expression von TRAIL-Rezeptor 2 (TRAIL-R2) auf der Zelloberfläche durch Stabiliseurng von p53. Ursächlich für die beobachtete p53 Stabilsierung war die von Wogonin vermittelte transkriptionelle Hemmung von MDM2, einem Negativregulator von p53. Außerdem konnten wir nachweisen, dass

die strukturell ähnlichen Flavone Apigenin und Chrysin ebenfalls c-FLIP Expression auf transkriptioneller Ebene inhibierten und die Expression von TRAIL-R2 in HTLV-1-infizierten ATL Zellen verstärkten.

Auf Grundlage dieser Ergebnisse erscheint es vielversprechend, Wogonin, Apigenin und Chrysin in Kombination mit TRAIL zur Behandlung von ATL und anderen Tumoren einzusetzen.

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1. INTRODUCTION

1.1 Apoptosis

Apoptosis is an essential mechanism for development, establishment and maintenance of tissue architecture. Furthermore, apoptosis plays a role in fighting against infections in multicellular organisms. It was in 1842 that Carl Vogt first discovered a new type of cell death occurring in a programmed fashion (Vogt *et al*., 1842). Kerr, Wyllie and Currie used the term "apoptosis" to describe the processes of programmed cell death in 1972 (Kerr *et al*., 1972). "Apoptosis" is a Greek word describing the "dropping off" or "falling off" of petals from flowers or leaves from trees. In 1989, two groups discovered CD95 (APO-1/Fas) by the generation of monoclonal antibodies which induce apoptosis in various cell lines (Trauth *et al*., 1989; Yonehara *et al*., 1989). From then on, apoptosis has attracted great attention and research on apoptosis has dramatically increased.

Apoptosis is characterized by morphological and biochemical traits, including membrane blebbing, cell shrinkage, nuclear DNA fragmentation and chromatin condensation (Wyllie *et al*., 1980; Steller *et al*., 1995). The apoptotic form of cell death is different from necrosis which occurs in response to a major insult as injury by toxins, or ischemia. Necrosis shows cell swelling, the destruction of cellular organelles, rupture of the plasma membranes and finally leakage of the cellular content into the environment. The main physiological difference between apoptosis and necrosis is that necrotic cells can elicit an inflammatory reaction, whereas apoptotic cells are cleared in an inconspicuous fashion, mainly by phagocytosis by neighboring cells or by specialized macrophage-like cells (Okada *et al*., 2004; Krammer *et al.*, 2007).

Apoptosis has to be tightly regulated since dysfunction of apoptosis leads to a variety of diseases. On the one hand, insufficient apoptosis may lead to cancer (Thompson,

1995), certain autoimmune diseases such as Grave's disease (Feldmann *et al.,* 1992) or Hashimoto's thyroiditis (Stassi and De Maria, 2002). On the other hand, an excess of apoptosis has been associated with several other diseases, such as AIDS (acquired immune deficiency syndrome) and neurodegerative disorders (Thompson, 1995; Peter *et al*., 1997; Krammer *et al*., 2007).

The process of apoptosis relies on activation of cysteine proteases which are called caspases (cysteinyl aspartate-specific protease). These caspases are present in the cells as inactive pro-forms (zymogens). Following the induction of apoptosis, these pro-forms can be cleaved to form active enzymes which then cleave substrates on the carboxyl-side of an aspartate residue (Cohen, 1997; Stennicke, 1998). There are two kinds of caspases: initiator caspases including caspase-2, caspase-8, caspase-9 and caspase-10 and effector caspases including caspase-3, caspase-6 and caspase-7. Activation of initiator caspases leads to the proteolytic activation of the effector caspases, which results in the cleavage of specific substrates and execution of cell death (Kumar and Lavin, 1996). In addition, a caspase-independent apoptotic pathway has also been reported which is mediated by AIF (apoptosis inducing factor) (Susin *et al*., 1999; Sevrioukova, 2011).

Apoptotic signaling events can be divided into two pathways depending on the mechanism of initiation. The extrinsic pathway is activated by engagement of extracellular death receptors, while the intrinsic pathway depends on the mitochondria (Fig. 1.1).

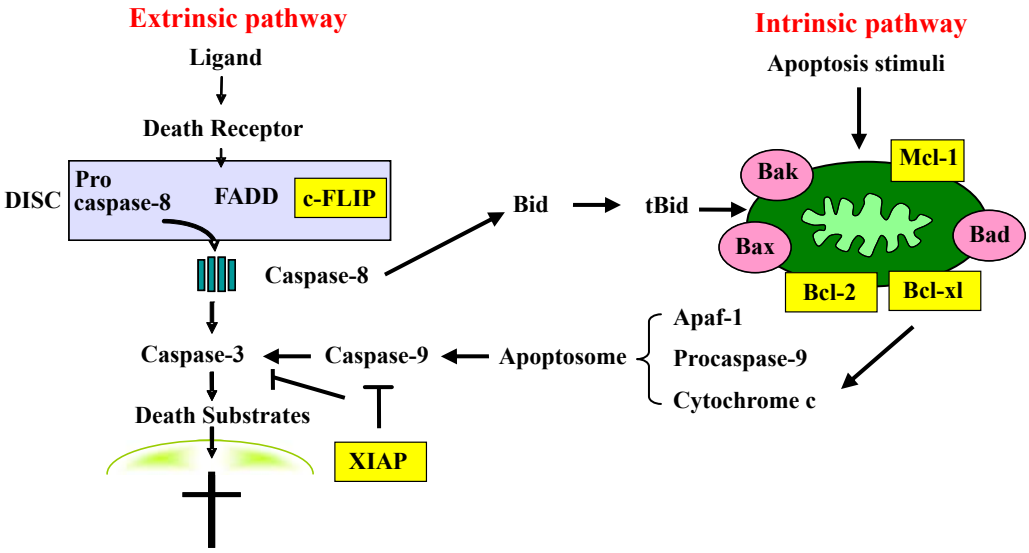

Figure 1.1 Apoptosis signaling pathways. Apoptotic signaling events can be divided into two pathways: the extrinsic pathway which is activated by engagement of extracellular death receptors and the intrinsic pathway which depends on mitochondria. The extrinsic pathway mainly involves binding of ligand to its receptor and activation of the initiator caspase-8 at the DISC level, a process which can be inhibited by c-FLIP. The intrinsic pathway is activated by various stimuli, such as DNA damage, oxidative stress, irradiation or cytotoxic drugs, which lead to release of cytochrome c from mitochondria and activation of caspase-9. This pathway is regulated by the anti-apoptotic and pro-apoptotic members of the Bcl-2 family. Pro-apoptotic factors are shown in red and anti-apoptotic factors in yellow. The extrinsic pathway and the intirnsic pathway are linked by Bid which is cleaved by caspase-8 to a truncated form (tBid). tBid translocates to the mitochondria to induce the intrinsic apoptosis pathway. For details see text.

1.1.1 The receptor-mediated (extrinsic) apoptosis pathway

The extrinsic apoptosis pathway is triggered by the binding of ligands to their corresponding death receptors on the cell surface (Krammer *et al.*, 2007; Lavrik *et al.*, 2005; Schulze-Bergkamen *et al.*, 2004). Six ligands have been reported so far: tumor necrosis factor-α (TNF-α), CD95 ligand (CD95L), TNF-like factor (TL1A), TNF-related apoptosis-inducing ligand (TRAIL), ectodysplasin A (EDA) and nerve

growth factor (NGF). These ligands are type II transmembrane proteins belonging to the TNF/NGF superfamily (Smith *et al.*, 1994). Until now eight death receptors and three decoy receptors have been reported. Death receptors are tumor necrosis factor receptor 1 (TNFR1), CD95, DR3, TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), TRAIL-R2, DR6, ectodysplasin A receptor (EDAR), nerve growth factor receptor (NGFR), and decoy receptors are TRAIL-R3, TRAIL-R4 and osteoprotegerin (OPG) (French and Tschopp, 2003; Wajant, 2003; Lavrik *et al*., 2005; Krammer *et al*., 2007) (Table 1). These death receptors are characterized by a cytoplasmic region of about 80 residues termed the death domain (DD), which is necessary and sufficient for induction of apoptosis (Itoh *et al*., 1993; Tartaglia *et al*., 1993; Lorenzo and Susin, 2004).

CD95 (APO-1/FAS), one of the most studied death receptors, was discovered by the generation of monoclonal antibodies which induce apoptosis in various cell lines (Trauth *et al*., 1989; Yonehara *et al*., 1989). It is a type I transmembrane glycoprotein with a molecular mass of 45 to 52 kDa (Itoh *et al*., 1991; Oehm *et al*., 1992). The ligand for CD95, CD95L, is a 40-kDa type II membrane protein which is only present on few cell types, such as activated T cells (Suda *et al*., 1993) or natural killer (NK) cells (Oshimi *et al*., 1996) as well as cells in immune privileged sites, such as the testis, the placenta, the anterior chamber of the eye and the brain (Lee *et al.*, 1997; Mitsiades *et al.*, 2003).

The CD95/CD95L system plays a major role in maintaining homeostasis in the immune system. In mouse models it has been demonstrated that mice lacking a functional CD95/CD95L system show autoimmune syndromes such as accumulation of peripheral lymphocytes and massive enlargement of lymph nodes (lymphadenopathy) and spleen (splenomegaly) (Takahashi 1994; Watanabe-Fukunaga 1992). In humans, mutations in the CD95/CD95L system are associated with an autoimmune disorder termed ALPS (autoimmune lymphopoliferative syndrome). These patients have accumulation of lymphocytes and an increased risk of developing

lymphomas (Lenardo, 2003; Krammer *et al.*, 2007).

In the extrinsic apoptosis pathway, the DISC (death-inducing signaling complex) is formed within seconds after receptor engagement (Peter and Krammer, 2003). The CD95 DISC consists of oligomerized CD95 receptors, the DD-containing adaptor protein FADD (Fas-associated death domain), the two splice variants pro-caspase-8a and pro-caspase-8b and the cellular FLICE-inhibitory protein (c-$FLIP_{L/S/R}$). FADD plays a crucial role in DISC formation, because it bridges the receptors and caspases by homotypic interactions between the DD and the death effector domains (DEDs). The DD of FADD binds to the DD of the receptor, and *via* its DED, FADD interacts with the DEDs of pro-caspase-8 and -10, which leads to the cleavage process and activation of pro-caspase-8 and -10 (Griffith *et al*., 1999; Chinnaiyan *et al*., 1995; Bouillet and O'Reilly, 2009). Activation of caspase-8 is thought to occur in a two-step mechanism. The initial cleavage gives raise to the p43/p41 and the p12 subunits. In the second step, the active form of caspase-8, a heterotetramer of two small (p10) and two large (p18) subunits, is generated (Micheau *et al*., 2002; Shi, 2002). For execution of apoptosis, it is released into the cytosol to initiate further effector caspase activation or to activate the intrinsic apoptosis pathway *via* cleavage of Bid (Sprick *et al*., 2002; Bouillet and O'Reilly, 2009).

Table 1. Death receptors, decoy receptors and their ligands.

Death receptors		Ligands
Common name	Alternative name	
TNFR1	DR1	TNF-α
	p55	
	p60	
	CD120a	
CD95	DR2	CD95L
	Fas	
	APO-1	
TRAMP	DR3	TL1A
	APO-3	
	WSL-1	
	LARD	
TRAIL-R1	DR4	TRAIL
	TNFRSF10A	
TRAIL-R2	DR5	TRAIL
	APO-2	
	KILLER	
	TRICK2	
	TNFRSF10B	
DR6	TR-7	?
EDAR		EDA1
NGFR		NGF
Decoy receptors		
TRAIL-R3	DcR1	TRAIL
	TRID	
	TNFRSF10C	
TRAIL-R4	DcR2	TRAIL
OPG	TNFRSF10D	TRAIL

1.1.2 c-FLIP, an inhibitor of the extrinsic apoptosis pathway

FLIP (FADD-like interleukin-1β-converting enzyme-like protease-inhibitory protein) is a key regulator of death receptor-mediated apoptotic signaling cascades. The gene for FLIP was first discovered in viruses including Kaposi's-sarcoma-associated human herpesvirus-8 and the tumorigenic human molluscipoxvirus (Thome *et al*., 1997). Viral FLIP inhibits death receptor-induced apoptosis which might delay cytotoxic T lymphocyte-mediated eradication of the infected host cells and prolong viral replication time (Bertin *et al*., 1997; Tschopp *et al*., 1998; Thurau *et al*., 2009). Later mammalian FLIP proteins were also discovered (Goltsev *et al*., 1997; Han *et al*., 1997; Hu *et al*., 1997). Viral FLIP (v-FLIP) and mammalian cellular FLIP (c-FLIP) are structurally similar and characterized by two DED motifs at their amino-terminus (N-terminus). So far, 11 c-FLIP splice variants have been reported but only three are expressed at the protein level: the 26 kDa short form c-FLIP$_S$, the 24 kDa form c-FLIP$_R$ and the 55 kDa long form c-FLIP$_L$ (Bagnoli *et al*., 2010). c-FLIP shares extensive amino acid sequence similarity with pro-caspase-8 and pro-caspase-10 at the N-terminus. Additionally, c-FLIP$_L$ has one pseudo caspase domain at the carboxy-terminus (C-terminus) which is catalytically inactive due to the absence of critical residues required for protease activity, such as the catalytic cysteine (Cohen, 1997). c-FLIP$_S$ and c-FLIP$_R$ have similar structures. c-FLIP$_S$ has been reported habouring an carboxy-terminal (C-terminal) extension of 20 amino acids which is crucial for ubiquitylation and degradation (Chang *et al.*, 2006) (Fig. 1.2).

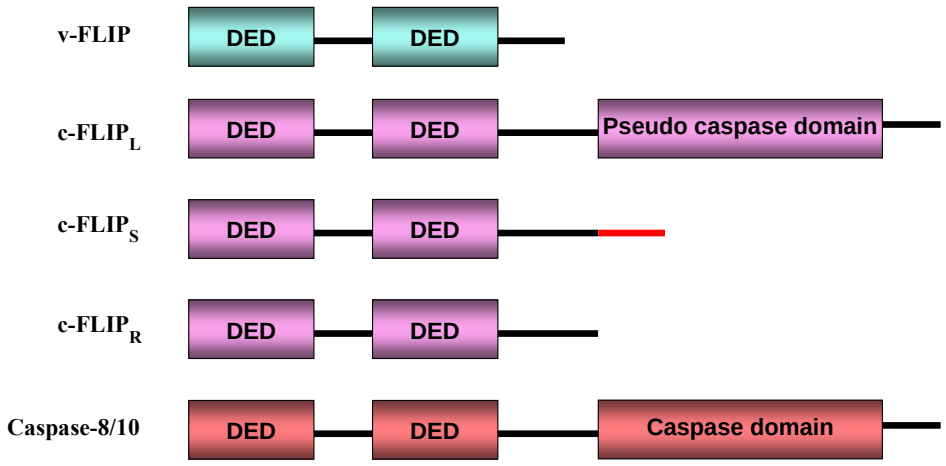

Figure 1.2 Molecular structures of viral and cellular FLIPs. Both v-FLIP and c-FLIP$_S$ are characterized by two DED motifs at their amino terminus, which is similar to pro-caspase-8 and pro-caspase-10. At the carboxy terminus, c-FLIP$_L$ has one pseudo caspase domain which is catalytically inactive. c-FLIP$_S$ and c-FLIP$_R$ have similar structures except that c-FLIP$_S$ has an carboxy-terminal extension of 20 amino acids which is crucial for ubiquitylation and degradation.

c-FLIP expression is positively regulated by the transcription factors NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and NFAT (nuclear factor of activated T-cells), and negatively by c-myc and c-Fos (Shirley and Micheau; 2010). Beyond that, c-FLIP isoforms are heavily regulated at the post-translational level by the E3 ubiquitin ligase Itch *via* the proteasomal pathway (Fukazawa *et al*., 2001). Specifically, the c-FLIP$_S$ protein contains a unique C-terminal extension of 20 amino acids which confers its ubiquitination and degradation, and therefore it has a shorter half-life compared to c-FLIP$_L$ (Poukkula *et al.*, 2005). Generally, c-FLIP isoforms have not only short protein but also short mRNA half-life of about 2 hours. c-FLIP expression can be easily attenuated by using protein or RNA synthesis inhibitors indicating a short half life (Fulda *et al*., 2000; Hernandez *et al*., 2001).

c-FLIP is widely accepted as an anti-apoptotic protein that functions by preventing recruitment and processing of pro-caspase-8 within the DISC. It has been reported that c-FLIP can bind to FADD *via* homotypic DED interactions and partially

processed c-FLIP (p43) is retained at the DISC through binding to the FADD/death receptor complexes which blocks further recruitment of pro-caspase-8 (Goltsev *et al*., 1997; Irmler *et al*., 1997; Krueger *et al*., 2001; Shirley and Micheau; 2010). c-FILP$_S$ can form a caspase/c-FLIP$_S$ heterocomplex, which blocks cleavage of caspase-8 within the DISC. Unlike c-FLIP$_S$, upon heterodimer formation, c-FLIP$_L$ allows a limited cleavage of pro-caspase-8, leading to the generation of p43/41 and p12 caspase-8 subunits. However, no further caspase-8 processing occurs to form an active heterotetramer of caspase-8 (two small p10 subunits and two large p18 subunits) due to the lack of proteolytic activity of c-FLIP$_L$. The cleaved products (p43/41 and p12) remain bound at the DISC, preventing further transduction of the apoptotic signal (Krueger *et al*., 2001; Micheau *et al.*, 2002; Shirley and Micheau; 2010). Furthermore, current studies indicate that the cleavage product of c-FLIP$_L$ interacts with the IKK (IκB kinase) complex to activate the non-apoptotic NF-κB signaling pathway (Neumann *et al*., 2010; Golks *et al*., 2006). Alternatively, c-FLIP$_L$ processed by caspase-8 promotes recruitment of RIP1 (receptor-interacting kinase 1) and TRAF2 (tumor necrosis factor receptor-associated factor 2) and induces activation of the NF-κB signaling pathway (Dohrman *et al*., 2005; Kataoka and Tschopp, 2004).

c-FLIP$_L$ has also been reported to have a pro-apoptotic effect (Lamkanfi *et al*., 2007; Yu and Shi, 2008). It has been shown that c-FLIP$_L$ can function as a pro-apoptotic molecule at low levels of expression, promoting the activation of pro-caspase-8 at the DISC (Chang *et al*., 2002). In addition, Fricker *et al.* demonstrated that c-FLIP$_L$ exerts a pro-apoptotic role only under conditions of moderate expression along with strong receptor stimulation or with high amounts of c-FLIP$_S$ or c-FLIP$_R$ (Fricker *et al*., 2010).

Many tumors have been reported to express abnormally high levels of c-FLIP, such as melanoma (Bullani *et al*., 2001), colon carcinoma (Ryu *et al*., 2001), ATL (adult T-cell leukemia/lymphoma) (Krueger *et al*., 2006) and Hodgkin lymphoma (Thomas *et al*., 2002), which correlates with resistance to apoptosis induced by CD95 or TRAIL.

Overexpression of c-FLIP in tumor cells also results in resistance to apoptosis induced by cytotoxic lymphocytes expressing CD95 ligand (French *et al*., 2002). In addition, high levels of c-FLIP expression in tumor cells were able to protect tumors from eradication by NK (natural killer) cells (Taylor *et al*., 2001). In contrast, c-FLIP knockdown in cells which express abnormaly high c-FLIP levels restores sensitivity to CD95 or TRAIL-mediated apoptosis (Krueger *et al*., 2006; Okamoto *et al*., 2006; Bleumink *et al*., 2010; Shirley *et al.*, 2010). Finally, down-regulation of c-FLIP by various substances, such as doxorubicin, sodium butyrate and cycloheximide can overcome TRAIL resistance in tumor cells (Roth and Reed, 2004). Collectively, these data support the investigation of c-FLIP as a therapeutic target to restore an apoptotic response in cancer cells.

1.1.3 The mitochondrial (intrinsic) apoptosis pathway

The intrinsic apoptosis pathway, also called the mitochondrial pathway, can be initiated by different stress stimuli, such as DNA damage, oxidative stress, irradiation or cytotoxic drugs (Norbury and Zhivotovsky, 2004; Takahashi *et al.*, 2004; Erlacher *et al.*, 2005; Debatin and Krammer, 2004). As a result, the mitochondrial membrane is permeabilized leading to release of cytochrome *c* from mitochondria into the cytosol (Hockenbery *et al*., 1990). Subsequently, a protein complex called apoptosome is assembled which consists of Apaf-1 (apoptosis protease-activating factor 1), cytochrome c and pro-caspase-9 (Zou *et al*., 1999). Apaf-1 serves as an oligomerization platform for cytochrome *c* and caspase-9 in a dATP/ATP-dependent manner (Jiang *et al*., 2000). Apoptosome formation leads to the activation of caspase-9, subsequent activation of effector caspases, and finally to induction of cell death (Zou *et al*., 1999; Acehan *et al*., 2000).

1.1.4 Inhibitors and regulators of the intrinsic apoptosis pathway

The Bcl-2 family:

The most important regulators of the intrinsic apoptosis pathway are proteins of the Bcl-2 family. All members of the Bcl-2 family contain up to four characteristic regions termed the Bcl-2 homology domains (BH1-4). Most of the Bcl-2 members possess a hydrophobic transmembrane domain (TM) at the carboxyl-terminus (Fig. 1.3). These proteins can be divided into two kinds: anti-apoptotic and pro-apoptotic proteins.

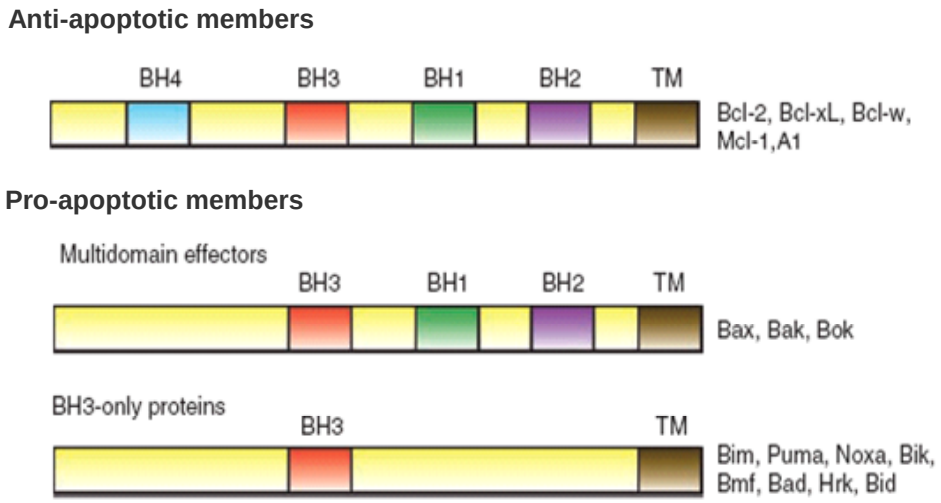

Figure 1.3 The Bcl-2 family. The Bcl-2 family is made up of proteins that contain conserved functional BH (Bcl-2 homology) domains. These proteins can be divided into two kinds: anti-apoptotic and pro-apoptotic proteins. The latter can be further divided into multidomain effectors and BH3-only proteins. Some BH3-only proteins do not possess TM domain (transmembrane domain). Modified from (Giam *et al.*, 2009)

i. The anti-apoptotic proteins of the Bcl-2 family:

The anti-apoptotic proteins of the Bcl-2 family consist of Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1. They contain up to four BH domains and have similar three dimensional structures (Strasser, 2005). Overexpression of any of these anti-apoptotic Bcl-2 family members is able to prevent death induced by apoptotic stimuli, which indicates a functional redundancy between these proteins. However, in physiological conditions, it is unlikely that a single anti-apoptotic protein can ensure the survival of a cell population (Giam *et al.*, 2009). These anti-apoptotic proteins are generally found at the outer membrane of mitochondria (OMM), where they function to inhibit the pro-apoptotic Bcl-2 proteins (Fletcher *et al.*, 2008; Lindsay *et al.*, 2010). It has also been shown that overexpression of anti-apoptotic proteins can block the release of cytochrome *c* to the cytosol, which inhibits apoptosome formation. However, the molecular mechanisms of this are still unclear (Giam *et al.*, 2009).

Mcl-1 (myeloid cell leukemia 1) is an anti-apoptotic member of the Bcl-2 family, which was initially identified as an immediate-early gene expressed in programmed myeloid cell differentiation (Kozopas *et al.*, 1993). Mcl-1 contains three BH domains and its N-terminal region habours a potential regulatory motif which is predicted to regulate its function. Mcl-1 blocks the progression of apoptosis by binding and sequestering Bax and Bak which are capable of forming pores in the mitochondrial membrane, allowing the release of cytochrome c into the cytoplasm. Mcl-1 also binds and sequesters a subset of the BH3-only pro-apoptotic Bcl-2 family members, which act to induce the oligomerization of Bak and Bax (Thomas *et al.*, 2010). Although Mcl-1 is one of the essential anti-apoptotic factors in the development and maintenance of normal cells, such as B and T-lymphocytes (Opferman *et al.*, 2003) and neural development (Arbour et al., 2008), Mcl-1 overexpression has been found in a variety of human hematopoietic (Aichberger *et al.*, 2005; Cho-Vega *et al.*, 2004) and solid tumors (Derenne *et al.*, 2002; Sieghart *et al.*, 2006). Furthermore,

down-regulation of Mcl-1 overcomes Mcl-1-mediated resistance to conventional cancer therapy-induced apoptosis (Nguyen *et al.*, 2007; Hussain *et al.*, 2007; Paoluzzi *et al.*, 2008; Wertz *et al*., 2011). In addition, Mcl-1 has been reported to contain two PEST sequences at the N-terminal region which are the targets for proteolysis by proteasome (Derouet *et al.*, 2006; Akgul, 2009). Therefore, Mcl-1 has a very short half-life and inhibition of its expression will rapidly make Mcl-1-dependent tumor cells more susceptible to apoptosis and provide a therapy option for these cancers.

ii. The pro-apoptotic proteins of the Bcl-2 family:

Multidomain effectors-Bax, Bak:

Bax and Bak are the main effectors of the Bcl-2-regulated pathway. In response to cytotoxic signals, Bax and Bak change their conformation and form membrane-associated homo-oligomers. These oligomers are thought to form pores in the OMM and cytochrome c is released from permeabilized mitochondria. However, the molecular mechanism of pore formation still remains elusive (Newmeyer and Ferguson-Miller, 2003; Green, 2005; Giam *et al.*, 2009). It has also been reported that Bax and Bak can influence the shape of the mitochondria *via* effects on the mitochondrial fission /fusion machinery, but it is still unclear whether this machinery contributes to mitochondria membrane permeabilization (Adams and Cory, 2007; Westermann, 2010). Although Bax and Bak seem to have identical functions, their subcellular localization is different. Bax is largely a cytosolic protein, while Bak is an integral membrane protein at the OMM. When activated, Bax translocates to the mitochondria to form homo-oligomers (Giam *et al.*, 2009; Adams and Cory, 2007).

BH3-only proteins:

The well studied mammalian BH3-only proteins include Bad, Bid, Bim, Noxa and Puma/BBC3. These proteins are all pro-apoptotic and their overexpression can promote apoptotic death in many cell types. However, cell death induced by BH3-only proteins requires presence of Bax and Bak, which indicates that they are upstream of Bax and Bak in the apoptosis pathway (Zong *et al.*, 2001; Giam *et al.*, 2009). All BH3-only proteins bind strongly to at least some anti-apoptotic members of the Bcl-2 family (Chen *et al.*, 2005) (Fig. 1.4). In addition, Bim, Bid and Puma were found to interact with Bax to induce cell death (Giam *et al.*, 2009).

The death receptor pathway and the mitochondrial pathway are linked by the pro-apoptotic BH3-only family member Bid. Upon stimulation, Bid is cleaved by caspase-8 to a truncated form (tBid) which translocates to the mitochondria where it acts together with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the intrinsic apoptosis pathway (Scaffidi *et al.*, 1998).

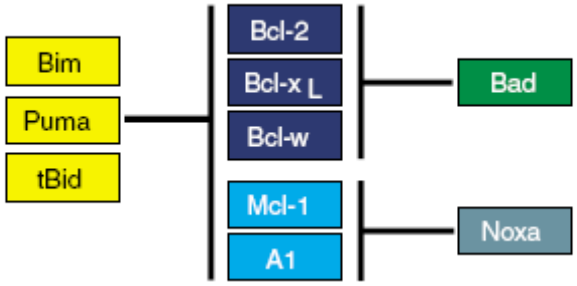

Figure 1.4 Differing binding profiles of BH3-only proteins. Bim, Puma and Bid bind to and inhibit all of the anti-apoptotic proteins of the Bcl-2 family, whereas Bad only inhibits Bcl-2, Bcl-xL and Bcl-w and Noxa suppresses Mcl-1 and A1 (Chen *et al.*, 2005).

XIAP:

In addition to the anti-apoptotic Bcl-2 members, XIAP (X-linked Inhibition of Apoptosis Protein), a 57 kDa protein, is an important inhibitor of the apoptotic pathway. XIAP consists of three major types of structural domains: the BIR (baculoviral IAP repeat) domain, the UBA (ubiquitin-associated) domain and the RING (really interesting new gene) domain (Blankenship *et al.*, 2009). XIAP-mediated inhibition of executioner caspases-3 and -7 is the result of XIAP BIR2 binding to these caspases and blocking their active sites (Stennicke *et al.*, 2002; Chai *et al.*, 2001). XIAP binding to caspase-9 *via* the BIR3 domain prevents caspase-9 dimerization and inhibits its protease activity (Deveraux and Reed, 1999; Gewies, 2003; Mufti *et al.*, 2007). XIAP function can be inhibited by the release of the mitochondrial proteins Smac/DIABLO (second mitochondria derived activator of caspase/direct IAP binding protein with low isoelectric point) into the cytoplasm where they bind to XIAP at precisely the same domains that mediate the interactions of XIAP with the caspases (Chai *et al.*, 2000; Du *et al.*, 2000). So far, several reports have shown that down-regulation of XIAP sensitizes various tumor cells towards TRAIL or CD95-induced apoptosis (Kim *et al.*, 2004; Kim *et al.*, 2005; Loeder *et al.*, 2010).

1.2 TRAIL and cancer therapy

1.2.1 TRAIL and TRAIL receptors

TRAIL (Apo2L, CD253, TNFSF10) is a type II transmembrane protein of about 34kDa, which belongs to the TNF superfamily and was identified by two groups in 1995/1996 (Wiley *et al.*, 1995; Pitti *et al.*, 1996). Similar to most members of the TNF family, TRAIL can be cleaved by metalloproteases at the cell surface to form a soluble molecule (Mariani *et al.*, 1998). Active TRAIL forms trimers and specifically binds to

five TRAIL receptors: two agonistic receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) and three decoy receptors (TRAIL-R3/DcR1, TRAIL-R4/DcR2 and OPG) (Pan *et al.*, 1997; Walczak *et al.*, 1997; Degli-Esposti *et al.*, 1997a; Degli-Esposti *et al.*, 1997b) (Fig. 1.5). TRAIL-R1 and TRAIL-R2 contain two cysteine-rich extracellular ligand-binding domains and a cytoplasmic region (death domain DD), which is important for activation of the extrinsic apoptosis pathway after TRAIL binding. However, TRAIL-R3 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein devoid of DD and TRAIL-R4 is a transmembrane receptor with a truncated DD. Therefore, both of them are thought to play a role in negatively regulating the action of TRAIL. OPG (osteoprotegerin) has the lowest affinity to TRAIL and its role is still not clear (Gonzalvez and Ashkenazi, 2010).

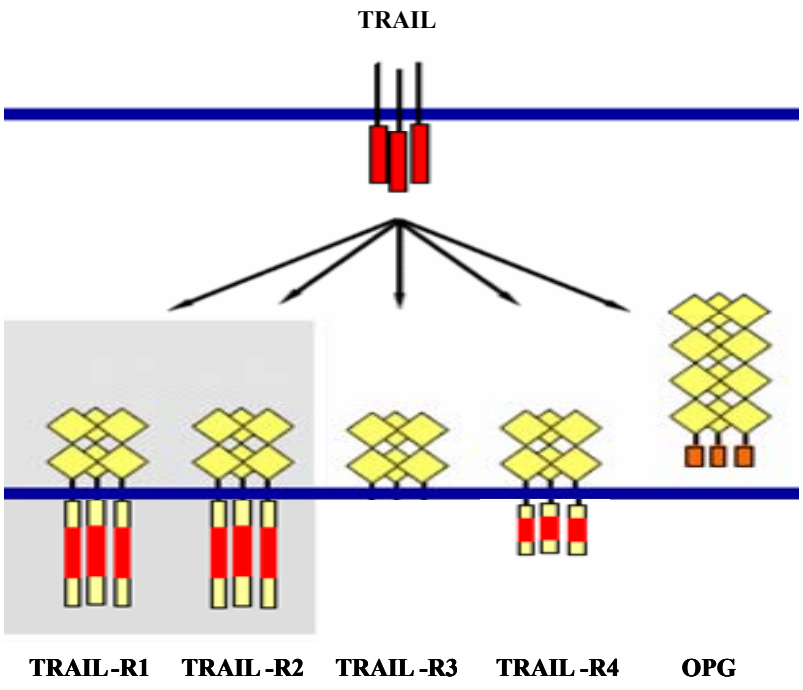

Figure 1.5 TRAIL receptors. In humans five TRAIL receptors have been identified. Two of them, TRAIL-R1 and TRAIL-R2, are transmembrane proteins containing cytosolic death domains that activate the caspase cascade. The others function as decoy receptors, lacking the capacity to activate the cell death pathway. TRAIL-R3 is a GPI-linked protein devoid of death domain. TRAIL-R4 is a transmembrane protein containing a non-functional, truncated death domain. OPG has the lowest affinity to TRAIL and its role is still not clear. Modified from (Lavrik *et al.*, 2005).

1.2.2 Cancer cell resistance to TRAIL

TRAIL has been shown to be an activator of apoptosis in tumor cells while sparing non-malignant cells. Therefore, TRAIL is promising in cancer treatment (Walczak *et al*., 1999; Bruix *et al*., 2004; Abdulghani and El-Deiry, 2010). That TRAIL has cancer cell selectivity has been reported by using stepwise tumorigenic cellular systems. In these systems, normal cells which are resistant to TRAIL were transformed by ectopic expression of oncogenes Ras or c-Myc, and subsequently these cells showed restored sensitivity to TRAIL-induced cell death (Nesterov *et al*., 2004; Wang *et al*., 2005; Nieminen *et al*., 2007). However, various tumor cells show resistance to TRAIL-induced apoptosis (LeBlanc *et al*., 2003; Pan *et al*., 2007), such as prostate cancer cells (Kasman *et al*., 2006), human T-cell leukemia virus 1-infected T cells (Matsuda *et al*., 2005; Bleumink *et al*., 2010) and colon cancer cells (Ishii *et al*., 2007).

Several mechanisms could explain tumor cell resistance to TRAIL-induced apoptosis.

One explanation is that aberrant expression of TRAIL receptors results in resistance to TRAIL-induced apoptosis in tumor cells. Impairment or mutation of TRAIL-R1 or TRAIL-R2 at the critical domains (such as DD or the ligand-binding domain) can hamper TRAIL-induced cell death (Kim *et al*., 2000; Park *et al*., 2001; Fisher *et al*., 2001; McDonald *et al*., 2001). Decoy receptors (DcRs) may compete with death receptors for TRAIL binding (Pan *et al*., 1997; Degli-Esposti MA *et al*., 1997a; Degli-Esposti MA *et al*., 1997b, Mérino *et al*. 2007). Ectopic expression of DcRs in TRAIL-sensitive cells leads to TRAIL resistance, suggesting that DcRs are negative regulators of TRAIL-mediated apoptosis (Marsters *et al*., 1997; Mérino *et al*., 2006). Similarly, TRAIL sensitivity could be restored in resistant cells by using anti-DcRs blocking antibodies or siRNA to decrease DcR expression (Sanlioglu *et al*., 2005; Sanlioglu *et al*., 2007).

Another explaination for tumor cell resistance to TRAIL-induced apoptosis is that

DISC level deficiency in caspase-8 and -10, caused by a loss-of-function mutation or gene promoter methylation, impairs TRAIL-induced apoptosis (Fulda *et al*., 2001; Hopkins-Donaldson *et al*., 2003; Cheng *et al*., 2006). Aberrantly increased expression of c-FLIP is another reason for TRAIL resistance at the DISC level. Various tumors have been reported to express abnormally high levels of c-FLIP, such as melanoma (Bullani *et al*., 2001), colon carcinoma (Ryu *et al*., 2001) and ATL (adult T-cell leukemia/lymphoma) (Bleumink *et al*., 2010), which correlates with resistance to apoptosis induced by TRAIL. c-FLIP knockdown in cells which express abnormal high c-FLIP levels restores sensitivity to TRAIL-mediated apoptosis (Bleumink *et al*., 2010; Shirley *et al*., 2010). Down-regulation of c-FLIP by various substances, such as doxorubicin, sodium butyrate and cycloheximide can also overcome TRAIL resistance in tumor cells (Roth and Reed, 2004). Therefore, c-FLIP is a therapeutic target to restore TRAIL sensitivity in cancer cells.

Furthermore, the Bcl-2 family members have been shown to be major regulators of the intrinsic apoptosis pathway. Impaired expression of the pro-apoptotic members of the Bcl-2 family, such as Bax (LeBlanc *et al*., 2002), or overexpression of anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2 (Fulda *et al*., 2002), can lead to TRAIL resistance. In addition, IAPs which inhibit caspase activities have also been reported to play a role in TRAIL resistance (Hersey *et al*., 2003; Straub, 2010).

1.2.3 Sensitization of cancer cells to TRAIL

Besides the resistance of human tumor cells to TRAIL-induced apoptosis, two other limitations hamper its use in the clinic. First, during the use of TRAIL in a Phase I clinical trial (Genentech and Amgen), it was revealed that TRAIL is rapidly cleared from the circulation, thus a prolonged administration or a sensitizer may be required for its anti-tumor activity (Mérino *et al*., 2007). Second, TRAIL rarely showed a

complete eradication of established tumors in xenografted animal models. These facts strengthen the necessity of combining TRAIL with other compounds or molecules to sensitize tumor cells to TRAIL-induced cell death.

Combination treatment of TRAIL with chemotherapeutic drugs, such as paclitaxel, vincristine, vinblastine and etoposide, could exploit synergistic properties inducing apoptosis and regression in tumors (Singh *et al*., 2003; Buchsbaum *et al*., 2006; Abdulghani and El-Deiry, 2010). *In vivo*, such combination treatment of TRAIL and chemotherapeutics in immunodeficient mice results in the regression of xenografted human tumors, such as breast carcinoma and colon carcinoma (Naka 2002; Singh *et al*., 2003). Chemotherapeutic agents were shown to sensitize tumor cells to TRAIL-induced apoptosis by several mechanisms such as death receptor up-regulation and anti-apoptotic protein down-regulation (Wu *et al*., 2004; Mérino *et al*., 2007; Abdulghani and El-Deiry, 2010).

Another approach is the combination of ionizing radiation and TRAIL, which was shown to sensitize breast cancer (Chinnaiyan *et al*., 2000), leukemic (Gong *et al*., 2000), melanoma (Ivanov *et al*., 2007) and glioma (Ciusani *et al*., 2005) cells to TRAIL-induced cell death. The suggested mechanisms of the synergistic effect through combination of radiation and TRAIL are p53-dependent up-regulation of TRAIL-R2, Bax and Bak induction and suppression of Bcl-2 (Shankar *et al*., 2004; Rahman *et al*., 2009).

Non-toxic compounds derived from natural food products or plants have also been demonstrated to synergize with TRAIL therapy. For example, Rocaglamide, derived from the traditional Chinese medicinal plant *Aglaia*, breaks TRAIL resistance in HTLV-1-associated ATL by translational suppression of c-FLIP expression (Bleumink *et al*., 2010). Resveratrol, present in grapes, was demonstrated to enhance TRAIL anti-tumor activity in colon carcinoma cells through redistribution of death receptors in lipid rafts which increases signaling efficiency (Delmas *et al*., 2004).

Additional molecules are also able to sensitize tumor cells TRAIL-induced apoptosis.

For example, synthetic triterpenoids sensitize AML (acute myeloid leukemia) and breast cancer cells to TRAIL-induced apoptosis by down-regulation of c-FLIP (Suh *et al*., 2003; Hyer *et al*., 2005). Several protein inhibitors have been reported to sensitize resistant tumor cells to TRAIL-induced cell death, such as proteasome inhibitors, Hsp90 inhibitors and anti-apoptotic Bcl-2 family inhibitors (Mérino *et al*., 2007; Kruyt, 2008).

1.2.4 TRAIL-R2, a transcriptional target of p53, Sp1 and CHOP

As mentioned above, up-regulation of TRAIL-R2 can be a mechanism for sensitization of resistant tumor cells to TRAIL-induced apoptosis. It has been shown that several agents, such as potent anti-cancer natural compound triptolide, the antibiotic tunicamycin and histone deacetylase sodium butyrate can sensitize certain cancer cells to TRAIL-mediated apoptosis through p53, CHOP (CCAAT/enhancer-binding protein homologous protein) or Sp1-mediated up-regulation of TRAIL-R2, respectively (Shiraishi *et al*., 2005; Yoshida *et al*., 2005; Carter *et al*., 2008; Kim *et al*., 2004; Kim *et al*., 2010). CHOP, Sp1 and p53 are transcription factors which can bind to the TRAIL-R2 promoter and regulate TRAIL-R2 expression. The CHOP binding site is located at -270 bp of the TRAIL-R2 promoter and the promoter region contains four putative Sp1 binding sites (Yoshida *et al*., 2001). In addition, three p53 DNA-binding sites have been identified in the TRAIL-R2 genomic locus and intron 1 (+ 0.25 Kb downstream of the ATG) is responsible for the p53-dependent transactivation of TRAIL-R2 (Takimoto *et al.*, 2000).

1.2.5 p53 and MDM2

The p53 tumor suppressor protein serves as a genome guardian and has been studied for nearly 30 years (Levine and Oren, 2009; Vousden and Prives, 2009). It functions mainly as a transcription factor by binding to specific DNA sequences to transactivate or repress a large group of target genes (el-Deiry *et al.*, 1992; Laptenko and Prives, 2006). In response to different stimuli, such as DNA damage, hypoxia or deficiency of growth factors or nutrients, p53 binds to its downstream targets to regulate the pathways of cell cycle arrest, apoptosis and DNA repair to maintain a dynamic equilibrium between cell growth and arrest (Mandinova and Lee, 2011).

The p53 activity is strictly controlled by a negative regulator termed MDM2 (mouse double minute 2). MDM2 was originally found in a mouse tumor cell line with enhanced MDM2 expression (Fakharzadeh *et al*., 1991). Later it was proven that MDM2 interacts with p53 and negatively regulates its function. So far, two mechanisms have been reported for MDM2-mediated negative regulation of p53 (Manfredi, 2010). One mechanism is that MDM2 directly binds to the N-terminal end of p53 to inhibit the transactivating function of p53. Another one is that the E3 ubiquitin ligase activity of MDM2 targets p53 for ubiquitination and subsequent degradation by the 26S proteasome. Both p53 and MDM2 are molecules with short half-life. p53 protein usually has a half-life of about 20 min (Weinberg, 2006), and the p53 mRNA half-life is about 4 to 6 h (Zhao *et al.*, 2008; Mazan-Mamczarz *et al.*, 2003). MDM2 mRNA and protein half-life was estimated to be about 20 to 40 min (Wang *et al.*, 2002; Itahana *et al.*, 2007).

MDM2 can function as an oncogene by down-regulation of p53. Additionally, it has been shown that MDM2 acts as an oncogene through p53-independent mechanisms which regulate proliferation, apoptosis and the epithelial-to-mesenchymal transition. As an example, overexpression of MDM2 caused mammary epithelial cells to undergo multiple rounds of S phase without cell division both in p53 wild type and

p53 knockout mice, suggesting that MDM2 regulation of cell cycle progression is independent of p53 (Lundgren *et al*., 1997). Additionally, MDM2 has also been shown to prevent apoptosis by binding to the mRNA of the anti-apoptotic protein XIAP, thereby enhancing XIAP translation and expression (Gu *et al*., 2009). Most notably, MDM2 has been reported to modify E-cadherin for degradation *via* the 26S proteasome, which leads to the epithelial-to-mesenchymal transition, a vital step of metastasis in solid tumors of epithelial origin (Yang *et al*., 2006; Thiery *et al*., 2009). All these findings demonstrate that MDM2 may act as an oncogene independently of p53.

In recent years, a diverse range of p53-targeting drugs have been developed. Remarkably, two drugs named RITA and nutlin have been shown to stabilize wild-type p53 through inhibition of the MDM2-p53 interaction and protection of p53 form being degraded by the ubiquitin-proteasome system. RITA directly binds to p53 and interferes with the interaction of MDM2 and p53, whereas nutlin fits in the p53-binding pocket of MDM2, effectively dislodging p53 from MDM2 (Levine and Oren, 2009).

1.3 Human T-cell leukemia/lymphoma virus-1 (HTLV-1)

1.3.1 Introduction to HTLV-1

HTLV-1, a member of the delta-retrovirus family, is the first retrovirus shown to be directly associated with a human malignancy. It was isolated from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma (Mycosis fungoides) by Robert Gallo's group at the NIH (National Institutes of Health) in 1980 (Poiesz *et al*., 1980). In 1982, Mitsuaki Yoshida and his colleagues in Japan identified the etiologic agent of ATL (adult T-cell leukemia) and named the virus adult T-cell leukemia virus (ATLV) (Yoshida *et al*., 1982). Later HTLV and ATLV were demonstrated to be the

same retrovirus based on homology of the viral genome and viral antigens. The terms were unified to HTLV-1 (Watanabe *et al*., 1984).

Currently, 15 to 20 million people all over the world are infected with HTLV-1 (Matsuoka and Jeang, 2010). HTLV-1 is endemic in south-western Japan, the Caribbean, the Middle-East, inter-tropical Africa and South-America. The highest prevalence rate in the general population is 10% in the south of Japan (Proietti *et al*., 2005). HTLV-1 infects many cell types *in vitro*, but *in vivo* it is detected mainly in CD4+ T cells and to a lesser extent in CD8+ T cells and dendritic cells (Matsuoka and Jeang, 2010). HTLV-1 needs cell to cell contact for transmission, which takes place upon forming of a virological synapse between infected and non-infected cells (Nejmeddine *et al*., 2005). HTLV-1 can be transmitted in three ways: mother-to-child transmission, sexual intercourse, contaminated blood cell transfusion or needle-sharing between intravenous drug users (Proietti *et al*., 2005).

1.3.2 HTLV-1 genome and persistence

HTLV-1 contains a relatively small genome (9 kb) but expresses multiple gene products due to transcription of both strands of its pro-viral genome and mRNA alternative splicing. The *gag, pol* (polymerase) and *env* (envolpe protein) genes are flanked by 5' and 3' long terminal repeats (LTRs). In the 3' portion of the genome between the *env* gene and the 3' LTR, is a "pX region" which contains four partially overlapping reading frames (ORFs) encoding the accessory proteins ($p12^{I}$, $p13^{II}$, $p30^{II}$), the post-transcriptional regulator p21REX (ORF III) and the TAX transactivator (ORF IV) (Seiki *et al*., 1983). In addition, HTLV-1 basic leucine zipper factor (HBZ) is encoded from 3' LTR in the complementary strand of the genome (Gaudray *et al*., 2002) (Fig. 1.6).

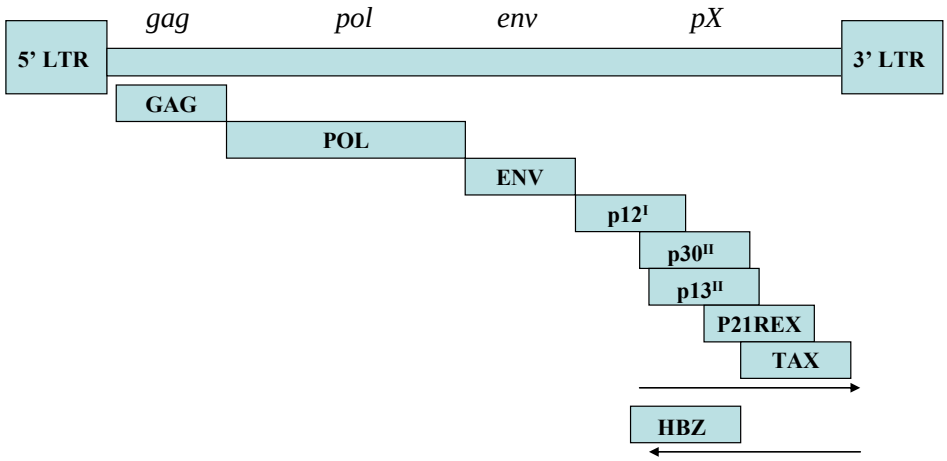

Figure 1.6 HTLV-1 pro-viral genome. HTLV-1 genome contains both a 5'LTR and a 3'LTR, between which the genome encodes the structural proteins, gag, pol, env and pX. In pX region, the viral proteins, $p12^{I}$, $p13^{II}$, p21rex, $p30^{II}$ and Tax are encoded. The HBZ minus strand RNA and protein are synthesized in an antisense fashion from the 3' LTR. Superscript Roman numerals indicate the respective open reading frame used for the translation of the indicated protein.

Homologues of the proteins Gag, Pol and Env are also found in other retroviruses, such as human immunodeficiency virus (HIV), and are required for virus replication and virion formation (Green *et al*., 2005). The regulatory proteins p12, p30, p13 and HBZ are involved in virus infectivity, immune escape and the establishment of a latent state (Nicot *et al*., 2005). Rex is an RNA-binding post-transcriptional regulator which specifically binds to the Rex response element (RRE) located at the 3' region of the viral mRNA to promote the transport of the unspliced and singly spliced viral RNA from nucleus to the cytoplasm to express structural proteins (Kashanchi *et al*., 2005). Among all these regulatory proteins, the most studied viral proteins are Tax and HBZ and they appear to have particularly important roles in viral persistence and pathogenesis.

Tax (p40) is an activator of transcription that binds to triplicate enhancer elements (TRE) located in the 5' LTR, which has been shown to be essential for the replication of HTLV-1 (Chlichlia and Khazaie, 2010). Importantly, this trans-acting factor also

acts as a potent transcriptional regulator of cellular gene expression.

Tax can transcriptionally activate the expression of cellular genes involved in growth and proliferation pathways, such as Akt and NF-κB pathways, to inhibit apoptosis (Taylor *et al*., 2008). Several anti-apoptotic proteins are up-regulated by Tax-mediated NF-κB activation, such as Bcl-xL (Mori *et al*., 2001; Tsukahara *et al*., 1999), IAP family proteins (Kawakami *et al*., 1999; Waldele *et al*., 2006) and c-FLIP (Micheau *et al*., 2001; Kreuz *et al*., 2001). In addition, Tax is able to prevent Bax relocation to mitochondria (Trevisan et al., 2006).

Furthermore, Tax inhibits tumor suppressor proteins, such as retinoblastoma (Rb) protein, which is phosphorylated to promote cell transition from G1 to S phase of the cell cycle (Kehn *et al*., 2005; Chlichlia and Khazaie, 2010). Tax also negatively regulates transcription of the tumor suppressor p53 (Mahieux *et al*., 2000; Pise-Masison *et al*., 2000; Chlichlia and Khazaie, 2010).

The HBZ gene is transcribed as an anti-sense transcript of HTLV-1 from the 3' LTR. It has been reported that the Tax gene transcripts are detected in only about 40% of transformed ATL cells. However, the HBZ gene is ubiquitously expressed in all ATL cells and supports proliferation of adult T cell leukemia cells (Satou *et al*., 2006; Chlichlia and Khazaie, 2010). In addition, HBZ gene expression is well correlated with the amount of integrated provirus in HTLV-1-infected individuals (Matsuoka and Jeang, 2010). Therefore, one hypothesis is that Tax and HBZ may cooperate in the process of leukemogenesis. Tax initiates the transformation process and HBZ supports survival of the infected cells (Chlichlia and Khazaie, 2010; Matsuoka and Jeang, 2010).

1.3.3 HTLV-1 associated diseases

ATL (adult T-cell leukemia/lymphoma) is a peripheral T-cell malignancy caused by HTLV-1 (Poiesz *et al*., 1980; Hinuma *et al*., 1981). The probability of infected people to develop ATL is 2-5 %. There is a long latency period between infection and the appearance of ATL, which is as much as 60 years in Japan and 40 years in Jamaica (Yasunaga *et al*., 2007). A clinical feature of ATL is leukemic cells with flower-like nuclei called 'flower cells'. The immunological phenotype of ATL cells is $CD3^+CD4^+CD8^-CD25^+$ and 59% of ATL cases express the transcription factor forkhead box P3 (FOXP3), suggesting that some ATL T cells originate from virus-infected Treg cells (Karube *et al*., 2004). ATL has four clinical subtypes: acute, lymphoma, chronic and smoldering. The first two types are very aggressive and there is no efficient treatment so far. The chronic and smoldering forms of ATL are indolent, but after a number of years they will develop into the acute forms (Shuh *et al*., 2005). Currently, patients in acute and lymphoma phase are treated with CHOP (cyclophosphamide, hydroxydoxorubicin, vincristine and prednisolone), AMP (doxorubicin, ranimustine and prednisolone) or VECP (vincristine, etoposide, cyclophosphamide and prednisolone) chemotherapy regimens. Other treatment strategies are described in the literature, such as NF-κB pathway blockers and PI3K inhibitors. Although these treatment strategies offer some treatment options, the mortality rate is still very high (Tsukasaki *et al*., 2009; Romanelli *et al.*, 2010).

In addition to ATL, HTLV-1 was also reported to be related to tropical spastic paraparesis (TSP) (Gessain *et al*., 1985) and HTLV-associated myelopathy (HAM) (Osame *et al*., 1986). Later, these two diseases were found to be the same disease (Román and Osame, 1988). More recently, several other diseases such as thyroiditis and Sjögren's syndrome have been correlated with this retrovirus infection (Seguchi *et al*., 2006; Verdonck *et al*., 2007; Cooper *et al*., 2009).

1.3.4 p53 mutation in HTLV-1-associated ATL

p53 mutations are found in 50% of human cancers (Sossi and Wiman, 2007). These mutations occur at variable frequencies in different tumor types. For instance, small cell lung cancer and ovarian carcinoma have the highest occurrence of p53 mutation, while testis cancer rarely shows mutant p53 (Olivier *et al*., 2002). The p53 status in ATL patients has been studied by several groups. These studies have shown that p53 is mutated especially in patients with acute ATL rather than in patients with chronic ATL. In addition, it was suggested that p53 mutations may be involved in the transition from chronic to acute stage of ATL (Nagai *et al*., 1991; Sakashita *et al*., 1992; Yamato *et al*., 1993; Tabakin-Fix *et al*., 2006). In contrast, p53 shows wild type sequence in most of HTLV-1-associated ATL cell lines, but its function is inhibited by the HTLV-1 Tax protein through the activation of the NF-κB pathway (Pise-Masison *et al*., 2000; Jeong *et al*., 2004). The anti-cancer drug 9-aminoacridine (9AA) has been reported to inhibit NF-κB and to induce p53 activity in HTLV-1-transformed cells, leading to a dramatic decrease in cell viability (Jung *et al*., 2008). This suggests a promising strategy for the treatment of HTLV-1 related diseases.

1.4 Wogonin and its analogs

The great potential of natural agents for cancer treatment is becoming more and more evident and consequently much effort is being made to identify promising candidates. In this study, Wogonin and its two analogs Apigenin and Chrysin were tested for their effects on sensitization to TRAIL-induced apoptosis in HTLV-1-associated ATL cell lines. These three compounds belong to a subgroup of flavonoids, named flavones (2-phenyl-chromone), which are composed of two benzene rings (A and B) joined

together by a γ-pyrone ring (C ring) (Li-Weber, 2010; Khoo *et al.*, 2010) (Fig. 1.7).

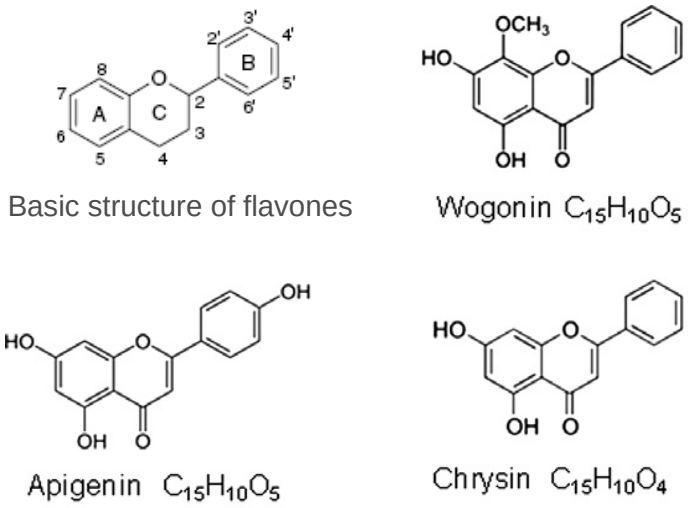

Figure 1.7 Basic structure of flavones and structures of Wogonin, Apigenin and Chrysin.

Wogonin (5, 7-dihydroxy-8-methoxyflavone) is an active compound extracted from Huang-Qin (*Scutellaria baicalensis Georgi*) (Fig. 1.7). Huang-Qin is a herb used in traditional Chinese medicine for treatment of hyperlipemia, hypertension, dysentery, the common cold and inflammatory diseases. Wogonin has been reported to have anti-oxidant, anti-viral, anti-inflammatory and anti-thrombotic activities (Chang *et al.*, 2001; Li-Weber, 2009; Li-Weber, 2010). Wogonin induces cell death in various human tumor cells *in vitro* and inhibits tumor growth *in vivo* in several tumor-bearing mouse models (Li-Weber, 2010). Recently, our group has shown that Wogonin attenuates NF-κB activity by shifting TNFα-induced free radicals $\cdot O_2^-$ to a more reduced nonradical product, H_2O_2, and thereby sensitizes TNFα-resistant leukemia cells to TNFα-induced apoptosis. In addition, Wogonin overcomes TRAIL resistance in primary AML (acute myeloid leukemia) cells. However, the molecular mechanisms need to be further investigated (Fas *et al.*, 2006). Wogonin has also been reported to recover TRAIL sensitivity in resistant prostate cancer cells by reactive oxygen species (ROS) dependent up-regulation of p53 and Puma (Lee *et al.*, 2009). Furthermore, it

has been shown that Wogonin potentiates the anti-tumor effects of several chemotherapeutics and ameliorates their adverse effects, such as etoposide and 5-fluorouracil (Enomoto *et al.*, 2010; Zhao *et al.*, 2010). Recently, our group found that Wogonin blocks transcription in cancer cells by inhibiting CDK9 (cyclin-dependent kinase 9) which plays a major role in regulation of transcription (Polier *et al*., unpublished data).

Importantly, Wogonin does not affect the viability of peripheral blood T cells derived from healthy donors (Fas *et al*., 2006). Moreover, it has been reported that Wogonin offers a wide margin of safety and shows no organ toxicity following long term intravenous administration in Beagle dogs (Peng *et al.*, 2009). Toxicological studies of Wogonin in albino mice or Sprague-Dawley rats suggested that it is a safe and promising anti-cancer agent (Qi *et al.*, 2009).

Apigenin is chemically known as 4' 5, 7, -trihydroxy-flavone (Fig. 1.7). It is abundantly present in common vegetables, such as onions and parsley, fruits, such as oranges and grapefruit and plant derived beverages, such as tea (Birt *et al*., 2001; Surh *et al*., 2003). For centuries Apigenin has been used as a traditional medicine to treat asthma, intransigent insomnia, Parkinson's disease and shingles (Patel *et al*., 2007). So far a variety of studies have shown that apigenin possesses anti-oxidant, anti-carcinogenic and anti-inflammatory activities (Shukla *et al*., 2007; Li-Weber, 2010).

Chrysin (5, 7-dihydroxy-2-phenyl-4H-chromen-4-one) is another natural active compound extracted from honey, propolis and plants (Fig. 1.7). It is an analog of Apigenin (Sanderson *et al*., 2004) and shares the common flavone structure with additional hydroxyls at positions 5 and 7 of the benzene ring A. It has been reported that Chrysin shows anti-inflammatory, anti-viral and anti-oxidant effects as well as anti-cancer activity by induction of apoptosis in a diverse range of human and rat cancer cells (Cho *et al*., 2004; Woodman *et al*., 2004; Khoo *et al.*, 2010; Li-Weber, 2010).

1.5 Aim of the study

Targeting apoptosis pathways is one of the key strategies for cancer treatment. TRAIL, which is considered as a promising anti-cancer agent, triggers apoptosis in a variety of tumor cells but not in non-malignant cells. However, 50 % of tumor types show resistance to TRAIL-induced apoptosis, including HTLV-1-associated ATL. So far various treatment strategies are offered to ATL patients, but because of apoptosis resistance the mortality of ATL is still very high.

Our lab has previously shown that Wogonin, derived from the Traditional Chinese Medicine (TCM) plant Huang-Qin (*Scutellaria baicalensis Georgi*), attenuates NF-κB activity by shifting TNFα-induced free radicals $\cdot O_2^-$ to a more reduced nonradical product, H_2O_2, and thereby sensitizes TNFα-resistant leukemia cells to TNFα-induced apoptosis. In addition, Wogonin overcomes TRAIL resistance in primary AML (acute myeloid leukemia) cells. Importantly, Wogonin does not affect the viability of peripheral blood T cells derived from healthy donors. However, the mechanisms of Wogonin-mediated sensitization of TRAIL-induced apoptosis are still not known.

In this study, HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 are used to investigate resensitization resistant tumor cells to TRAIL-induced apoptosis. These cell lines are found to express a high level of the anti-apoptotic protein c-FLIP which blocks death receptor-mediated apoptosis at the DISC level. Therefore, the natural herbal compound Wogonin is tested for its activity to down-regulate c-FLIP and to sensitize HTLV-1-associated ATL cells to TRAIL-induced cell death. The molecular mechanisms of TRAIL resensitization by Wogonin in HTLV-1 ATL cells are further investigated. The aim of this study is to find a new approach to overcome resistance of HTLV-1-associated ATL cells to TRAIL-mediated apoptosis. The final goal is to develop a strategy for the treatment of ATL and other types of TRAIL resistant tumors.

2. MATERIALS AND METHIODS

2.1 Materials

2.1.1 Chemicals

All chemicals, unless specified, are purchased from Merck (Darmstadt), Roth (Karlsruhe), Fluka (Neu-Ulm), Sigma (München) and Serva (Heidelberg).

2.1.2 Buffers and solutions

Name	**Concentration**	**Reagents**
PBS:	137 mM	NaCl
	8.1 mM	Na_2HPO_4
	2.7 mM	KCl
	1.5 mM	KH_2PO_4, pH 7.4
PBST:		PBS
	0.5 % (v/v)	Tween 20
Nicoletti lysis buffer:	0.1 % (w/v)	Na-citrate
	0.1 % (v/v)	Triton X-100
	50 µg/ml	Propidium iodide
TBE (10 ×):	0.45 M	Tris-Base
	0.45 M	Boric acid
	10 mM	EDTA, pH 8.3
		(Ethylene-diamine-tetra-actetate)

RIPA lysis buffer:	50 mM	Tris-HCl, pH 8.0
	1 %	NP-40
	0.5 %	Na-deoxycholate
	120 mM	NaCl
	2 mM	EDTA
	0.1 %	SDS (Sodium-dodecyl-sulfate)
	1 ×	Protease Inhibitor Cocktail (Roche)
	1 mM	DTT
	200 µM	Na_3VO_4
	25 mM	NaF
	1 mM	PMSF
Blocking buffer:	5 % (w/v)	Non-fat dry milk in PBST
SDS sample buffer (5 ×):	50 mM	Tris-Base, pH 6.8
	10 % (w/v)	SDS
	50 % (v/v)	Glycerol
	0.25 mg/ml	Bromophenol blue
	25 % (v/v)	β-Mercaptoethanol (β-ME)
Running buffer (SDS-PAGE):	0.19 M	Glycin
	0.1 % (w/v)	SDS
	25 mM	Tris-Base, pH 6.8

Transfer buffer (Western blot):	25 mM	Tris-Base
	0.19 M	Glysin
	20 % (v/v)	Methanol
	0.037 % (w/v)	SDS
Laemmli Resolving gel:	37.5 mM	Tris-Base, pH 8.8
	5-15 % (w/v)	Acrylamide/Bisacrylamide 37.5:1
	0.1 % (w/v)	SDS
	0.03 % (w/v)	Ammonium persulfate (APS)
	0.1 % (w/v)	Tetramethylethylendiamine (TEMED)
Laemmli Stacking gel (5%):	24 mM	Tris-Base, pH 6.8
	5 % (w/v)	Acrylamide/Bisacrylamide 37.5:1
	0.1 % (w/v)	SDS
	0.1 % (w/v)	APS
	0.1 % (w/v)	TEMED

2.1.3 Reagents

Name	Supplier
SuperKiller TRAIL	Alexis
Wogonin	BIOTREND
Apigenin	Sigma
Chrysin	Sigma
Uracil-N-Glycolase	Eurogentec
Trypsin-EDTA (1x)	Sigma

2.1.4 Eukaryotic cell lines

Cell line	Description
Jurkat 16 (J16)	Human acute lymphoblastoid T cell line
CEM	Human acute lymphoblastoid T cell line
SP	HTLV-1-associated ATL cell line
MT-2	HTLV-1-associated ATL cell line
MT-4	HTLV-1-associated ATL cell line
Hep3b	Human hepatoma cell line
HepG2	Human hepatoma cell line
PC3	Human prostate cancer cell line
MDA-MB-231	Human breast cancer cell line
HT-29	Human colon cancer cell line
SK-MEL-37	Human melanoma cell line

2.1.5 Culture medium for eukaryotic cell lines

Cell line	Culture media
Jurkat 16 (J16)	RPMI 1640 (Sigma)
CEM	RPMI 1640 (Sigma)
MT-2	RPMI 1640 (Sigma)
MT-4	RPMI 1640 (Sigma)
PC3	RPMI 1640 (Sigma)
MDA-MB-231	RPMI 1640 (Sigma)
HT-29	DMEM (Sigma)
SK-MEL-37	DMEM
SP	RPMI 1640 with 100 U/ml IL-2
Hep3b	MEM (Sigma)
HepG2	DMEM

Media were supplemented with 1 % (v/v) Penicillin-Streptomycin (Sigma), 2 mM L-glutamine (Invitrogen) and 10 % (v/v) FBS (Fetal Bovine Serum). FBS was purchased from GIBCO.

2.1.6 Antibodies for Western blot analysis

Antigen	Origin
α-tubulin	Sigma
Bcl-2	Santa Cruz
Bcl-xl	Cell signaling
Bad	Cell signaling
Bak	Cell signaling
Bax	BD (Biosciences)
Bid	Cell signaling
Caspase-2	Cell signaling
Caspase-3	Cell signaling
Caspase-8 p18 (C15)	Scaffidi *et al*., 1997
Caspase-9	Santa Cruz
c-FLIP (NF-6)	Scaffidi *et al*., 1999
CHOP	Santa Cruz
ERK (MK-12)	BD
FADD	Transduction Laboratories
p53 (1C12)	Cell signaling
TRAIL R1	ProSci
TRAIL R2	Cell signaling
TRAIL R3	Cell signaling
TRAIL R4	Cell signaling
Mcl-1	Santa Cruz
Sp1 (PEP2)	Santa Cruz
XIAP	Cell signaling

2.1.7 Antibodies for flow cytometer analysis

Name	Origin
TRAIL-R1	Alexis, HS101(IgG1)
TRAIL-R2	Alexis, HS201(IgG1)
TRAIL-R3	Alexis, HS301(IgG1)
TRAIL-R4	Alexis, HS401(IgG1)
Mouse IgG1, FITC conjugated	USBio
Goat anti-mouse IgG1, FITC conjugated	Alexis

2.1.8 Primers

Target gene	Sequence
c-FLIP$_L$ forward	5'-GGC TCC CCC TGC ATC AC-3'
c-FLIP$_L$ reverse	5'-TTT GGC TTC CCT GCT AGA TAA GG-3'
c-FLIP$_L$ probe	5'-CAG GAG GAT GTT CAT GGG AGA TTC ATG C-3'
c-FLIP$_S$ forward	5'-ACC CTC ACC TTG TTT CGG ACT AT-3'
c-FLIP$_S$ reverse	5'-TGA GGA CAC ATC AGA TTT ATC CAA A-3'
c-FLIP$_S$ probe	5'-AGA GTG CTG ATG GCA GAG ATT GGT GAG G-3'
p53 forward	5'-GCC CCC AGG GAG CAC TA-3'
p53 reverse	5'-GGG AGA GGA GCT GGT GTT G-3'
p53 probe	5'-TTG GGC AGT GCT CGC T-3'
MDM2 forward	5'-ATG TCT GTA CCT ACT GAT GGT GCT G-3'
MDM2 reverse	5'-TCA AAA GCA ATG GCT TTG GTC T-3'
MDM2 probe	5'-CCA CCT CAC AGA TTC CAG CTT CGG A-3'
18S rRNA forward	5'-GTG ACG GAG AAT TAG GGT TCG A-3'
18S rRNA reverse	5'-CTG CCT TCC TTG GAT GTG GTA-3'
18S rRNA probe	5'-CCG GAG AGG GAG CCT GAG AAA CGG-3'

TRAIL-R2 forward	5'-TGG TTC CAG CAA ATG AAG GTG-3'
TRAIL-R2 reverse	5'-CCG CTG CCT CAG CTT TAG C-3'
GAPDH forward	5’-ACA TCA AGA AGG TGG TGA AGC AGG-3’
GAPDH reverse	5’-CTC TTG CTC TCA GAT CCT TGC TGG-3’

Probes were all conjugated with FAM at the 5’ end and TAMRA at the 3’ end. p53, MDM2, 18S rRNA, TRAIL-R2 and GAPDH primers were described previously (Baumbusch *et al*., 2006; Wilda *et al*., 2004; Osman *et al*., 2008; Sun *et al*., 2008; McCullough *et al*., 2001).

2.1.9 Kits

Kit	**Origin**
RT PCR Kit	Applied Biosystems
PCR-Taqman Kit	Eurogentec
PCR-SYBR Green Kit	Eurogentec
RNeasy Mini Kit	Qiagen
Gel extraction Kit	Qiagen

2.1.10 Instruments

Instrument	**Manufacturer**
Flow cytometer FACScan	Becton Dickinson
Thermocycler	Perkin Elmer
UV transilluminator	Bio-Rad
Agarose gel electrophoresis apparatus	Gibco BRL
Microwave oven HMG 730B	Bosch
Laminar chambers	Bio-Rad
Semi-Dry blotting system	Bio-Rad/PEQLab

Cell culture incubator	Forma Scientific
Cell culture hood	Heraeus
Neubauer Cell-counting chamber	Brand
Light microscope ID 02	Zeiss
pH-Meter Calimatic	LHD Labortechnik
Thermomixer Compact	Eppendorf
Spectrophotometer ND-1000	PEQLab
Thermostated hot-block 5320	Eppendorf
Megafuge 1.0R	Heraeus
Microcentrifuge	Heraeus
Chemiluminescence Chemi Smart	Vilber Lourmat
7500 Real Time PCR system	Applied Biosystems
Water bath	Kotterman

2.2 Cellular biological methods

2.2.1 Cell culture

All cells were cultured in a cell culture incubator at 37 °C with 5 % CO_2. Suspension cell culture, cells were maintained by replacement of medium every two to three days to keep a cell density between 1×10^5 and 4×10^5 cells/ml. Adherent cells were washed once with PBS and suspended with 5 ml trypsin. Then cells were transferred into a new labeled flask containing pre-warmed medium and diluted 1:5 or 1:6. Cells were counted using a Neubauer cell counting chamber. Dead cells were distinguished by trypan blue staining.

2.2.2 Storage of eukaryotic cell lines

Cell freezing:

Cells were centrifuged at 1500 rpm for 5 min at 4 °C and resuspended in fresh medium (1-2 × 10^6 cells/ml) supplemented with 20 % FBS and 10 % DMSO. Next, cells were transferred into cryo-tubes and stored in a -80 °C refrigerator for short term or in liquid nitrogen (-196 °C) for long term storage.

Cell thawing:

To avoid the toxic effects of high DMSO concentration, cells were transferred into a 15 ml falcon tube with 10 ml fresh pre-warmed medium directly after thawing in 37 °C water bath. After centrifugation at 1500 rpm for 5 min at 4 °C, the supernatant was discarded and cells were resuspended in 10 ml of fresh pre-warmed medium for further culture.

2.2.3 Apoptotic cell death analysis

Apoptotic cell death was analyzed by propidium iodide (PI) staining and flow cytometry according to the protocol of Nicoletti (Nicoletti *et al*., 1991). Cells were centrifuged at 1500 rpm for 5 min, washed once with PBS, resuspended in 200 µl of Nicoletti lysis buffer containing 50 µg/ml PI and stored at 4 °C in dark overnight. Specific DNA fragmentation was calculated by the formula: ((experimental DNA fragmentation – spontaneous DNA fragmentation) / (100 – spontaneous DNA fragmentation)) x 100 %.

2.2.4 Cell surface staining

5 x 10^5 cells were centrifuged at 1500 rpm for 5 min, washed once with PBS (supplemented with 1 % FBS), and incubated with 10 μg/ml of corresponding antibodies for 30 min at room temperature (RT). Then cells were washed once with PBS (supplemented with 1 % FBS), and incubated with 10 μg/ml FITC-conjugated goat anti-mouse antibody for 30 min at RT. Again cells were washed once with PBS (supplemented with 1 % FBS), resuspended in 200 μl PBS and finally measured by flow cytometry in 200 μl PBS. The following antibodies were used: HS101 (TRAIL-R1), HS201 (TRAIL-R2), HS301 (TRAIL-R3), HS402 (TRAIL-R4) (Alexis, San Diego), FITC-conjugated anti-mouse IgG1 (USBio) and FITC-conjugated goat anti-mouse antibody (Alexis).

2.2.5 Cell fixation

For HTLV-1-associated ATL cell lines (SP, MT-2 and MT-4) it is indispensable to fix cells before taking them out of the S3 lab. Infected cells were washed once with PBS, added to freshly thawed or freshly prepared 2-4 % PFA (Paraformaldehyde)/PBS for 90 min at 4 °C. Then cells were washed once with PBS and transferred to a fresh tube for further experiments.

2.3 Molecular biological methods

2.3.1 Total RNA isolation

Total RNA was isolated by using RNeasy Mini Kit (50) (Qiagen). 5 x 10^5 cells were collected by centrifuging at 1500 rpm for 5 min and homogenized with 350 μl lysis buffer RLT (with 10 μl/ml β-ME). 350 μl 70 % Ethanol was added into homogenized

lysates and mixed. After being transferred to RNeasy spin columns, samples were centrifuged 15 s at 10,000 rpm and flow-through was discarded. 700 µl wash buffer RW1 was pipetted onto spin columns and samples were centrifuged as above. Next, spin columns were placed in new collection tubes. Samples were centrifuged twice with 500 µl wash buffer RPE at 10,000 rpm for 15 s and at full speed for 2 min. Finally spin columns were transferred to new 1.5 ml collection tubes and RNA was eluted with 30 µl DEPC-treated water by centrifugation for 1 min at 10,000 rpm. Total RNA concentration was measured by using a spectrophotometer (PEQLab). The RNA samples were stored at -80 °C.

2.3.2 Reverse transcription reaction (RT)

Reverse transcription reaction was carried out by using RT PCR Kit (Applied Biosystems). The reaction system was prepared as follows:

Reagents	**Amount**
RNA	1 µg
50 mM $MgCl_2$	4 µl
10 x PCR buffer	2 µl
100 mM Oligo-dT	1 µl
RNase inhibitor	1 µl
dNTP mixture (10 mM each)	1 µl
DEPC-treated water	Filled up to 19 µl

The mixture was heated at 65 °C for 3 min and cooled at 4 °C for 5 min. After addition of 1 µl of Reverse Transcriptase, the mixture was placed in a Thermocycler (Bio-Rad) for the following steps:

42 °C	45 min
95 °C	5 min
4 °C	5 min

cDNA was stored at -20 °C. For Polymerase Chain Reaction, 4.4 µl of cDNA was added to a total volume of 25 µl PCR mixture.

2.3.3 Polymerase Chain Reaction (PCR)

The purpose of a PCR is to make a huge number of gene copies. The PCR mixture was prepared as follows:

Reagents	**Volume (µl)**
10 x PCR buffer	5 µl
dNTP mixture (10 mM each)	1 µl
Forward primer	1 µl
Reverse primer	1 µl
RT product	20 ng
Taq DNA polymerase	1 µl
H_2O	Filled up to 50 µl

The mixture was placed in a Thermocycler (Bio-rad) for the following steps:

Steps	**Time**	**Temperature**
Start	5 min	95 °C
Denaturation	1 min	95 °C
Annealing	1 min	60 °C
Extension	2 min	72 °C
Termination	10 min	72 °C

Three major steps (denaturation, annealing and extension) were repeated for 25 to 30 cycles. The size of the PCR products was verified by a 1.5 % agarose gel containing ethidium bromide.

2.3.4 Quantitative real-time PCR

TaqMan PCR

Reagents for TaqMan PCR were all purchased from Eurogentec. The reaction system was prepared as follows:

PCR-Mix (1ml):

Reagents	**Volume (µl)**
10 x PCR buffer	200
50 mM $MgCl_2$	400
dNTP mixture (5 mM each with dUTP)	160
HGS Polymerase	10
Uracil-N-Glycolase	20
H_2O	210

Reaction Mixture (µl):

	c-FLIP$_L$/18S rRNA	c-FLIP$_S$	MDM2	p53
Forward Primer	0.88	5.3	5.3	15.8
Reverse Primer	5.3	15.8	0.88	15.8
Probe	3.5	3.5	3.5	3.5
DNA template	4.4	4.4	4.4	4.4
PCR-Mix	43.8	43.8	43.8	43.8
H_2O	29.8	14.9	29.8	4.4

Reaction mixture was pipetted into 96-well plate (Applied Biosystems) with 25 µl each well. Fluorescence signals of samples were measured and analyzed by 7500 Real Time PCR system (Applied Biosystems). Relative mRNA was calculated by the following formula: $2^{-\triangle Ct}$ ($\triangle$Ct= experimental Ct – control Ct) and the standard deviations were calculated from the triplicates.

SYBR Green PCR

Reagents for SYBR Green PCR were all purchased from Eurogentec. The reaction system was prepared as follows:

PCR-Mix (1ml):

Reagents	**Volume (µl)**
10 x PCR buffer	200
SYBR Green 1:2000	60
50 mM $MgCl_2$	120
dNTP mixture (5 mM each with dUTP)	80
HGS Polymerase	10
Uracil-N-Glycolase	20
H_2O	510

Reaction Mixture (µl):

	TRAIL-R2	GAPDH
Forward Primer	5.3	15.8
Reverse Primer	5.3	0.88
DNA template	4.4	4.4
PCR-Mix	43.8	43.8
H_2O	28.9	23.8

Data measurement and analysis are described previously (see TaqMan PCR).

2.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate mixed double stranded DNA fragments according to their differences in length. 1.5 % agarose gels are made by agarose and 0.5 x TBE buffer. After agarose suspension was boiled and cooled down to about 50 °C, ethidium bromide was added to a final concentration of 0.5 µg/ml. 20 µl PCR products with 4 µl 6 x loading buffer were loaded on the agarose gel. The PCR products were electrophoresed at 100 V for 30 min. The ethidium bromide stained DNA fragments were visualized by UV illumination (366 nm).

2.3.6 Extraction and purification of DNA from agarose gels

DNA was extracted and purified by using QIAquick Gel Extraction Kit (Qiagen). All centrifugation steps were carried out at 13000 rpm at RT. DNA fragments were cut from the agarose gel with a clean and sharp scalpel. After weighing in a clean eppendorf tube, the gel slice was added to 3 times volume of Buffer QG (100 µl for

100 mg). When dissolved completely at 50 °C. 1 gel volume of isopropanol was added and mixed. Then the sample was applied to a QIAquick column and centrifuged for 1 min. To remove all traces of agarose, Buffer QG was added to the sample and the column was centrifuged for 1 min. After washing once with 0.75 ml of Buffer PE and centrifugation for 1 min, the column was again centrifuged for 1 min to remove residual ethanol. Then the column was put into a new 1.5 ml microcentrifuge tube and DNA fragments were collected after elution with buffer EB or water by centrifugation for 1 min. DNA was stored at -20 °C for further experiments.

2.3.7 Measurement of RNA and DNA concentration

RNA or DNA concentration was measured by using the spectrophotometer ND-1000 (PEQLab) and the software NanoDrop 1000 3.7.1.

2.3.8 Preparation of protein lysates for Western blot

Cells were collected by centrifuging at 1500 rpm for 5 min at 4 °C. Supernatant was sucked off and pellets were washed once with PBS at 2000 rpm for 5 min at 4 °C. Cells were resuspended with RIPA buffer (200 µl RIPA for 1 x 10^7 cells) and incubated on ice for 20 min. After cells were centrifuged at 13000 rpm for 30 min at 4 °C, supernatants were transferred into new eppendorf tubes. After addition of loading buffer, protein lysates were denatured at 95 °C for 10 min.

2.3.9 SDS PAGE

Bio-Rad 1.5 mm spacer plates, 10-well combs and gel apparatus were cleaned and assembled. Laemmli resolving gel and stacking gel were prepared according to the table below:

Laemmli resolving gel	13 %	10 %	7.5 %
1.5 M Tris-Base + 0.4 % SDS, pH 8.8 (ml)	4	4	4
H_2O (ml)	5.1	6.7	8
30 % Acrylamide mix (ml)	6.9	5.3	4
10 % APS (µl)	100	100	100
TEMED (µl)	10	10	10

Laemmli stacking gel		
1.5 M Tris-Base + 0.4 % SDS, pH 6.8 (ml)	2.5	
H_2O (ml)	5.9	
30 % Acrylamide mix (ml)	1.6	
10 % APS (µl)	100	added before use
TEMED (µl)	10	

The mixed solution for Laemmli resolving gel was loaded and covered with 70 % ethanol. When the gel was polymerized (20 to 30 min), the layer of ethanol was discarded. Then the mixture of Laemmli stacking gel was syringed onto the separating gel and an appropriate comb was inserted immediately. After the stacking gel was polymerized (10 min), denatured protein lysates (95 °C for 10 min) were loaded. SDS PAGE was run about 1 hour at 35 mA per gel until the blue dye front reached the bottom of the gel.

2.3.10 Western blot

When SDS PAGE running was finished, resolving gel was kept for blotting. Each resolving gel was stacked between whatman papers for transfer of proteins onto the nitrocellulose membrane in transfer buffer for 2 hours at a constant current of 60 mA. After transfer, the nitrocellulose membrane was incubated for 1 h in 5 % BSA. After BSA blocking, first antibody was added to the nitrocellulose membrane at 4 °C overnight. Before incubation with second antibody for 1 hour, the membrane was washed three times (5 min each time) with washing buffer. When second antibody incubation was finished, the membrane was washed again three times. For Western blot developing, the ECL kit (PerkinElmer Life Sciences) was used according to the manufacture's introduction. A mixture of solution A and B (1:1) was added to the membrane for 1 min and the membrane signal was detected and analyzed by using Chemiluminescence Chemi Smart (Vilber Lourmat).

3. RESULTS

3.1 Wogonin sensitizes HTLV-1-associated ATL cells to TRAIL-induced apoptosis

3.1.1 HTLV-1-associated ATL cells are resistant to TRAIL-induced apoptosis

TRAIL is a promising candidate for cancer therapy. However, approximately 50 % of tumors show resistance to TRAIL-induced apoptosis including HTLV-1-associated ATL (LeBlanc *et al*., 2003; Matsuda *et al*., 2005; Ishii *et al*., 2007; Bleumink *et al*., 2010). To systematically investigate the mechanism by which HTLV-1-associated ATL cells escape TRAIL-mediated apoptosis, three HTLV-1-associated ATL cell lines, SP, MT-2 and MT-4 were examined. Cells were treated with different concentrations of recombinant TRAIL for 24 h and apoptotic cell death was determined by DNA fragmentation according to the method of Nicoletti ((Nicoletti *et al*., 1991). Apoptotic cell death of the HTLV-1-associated ATL cell lines was compared to a control leukemic cell line, Jurkat 16, which is not HTLV-1-infected and is sensitive towards TRAIL-induced apoptosis. After treatment with TRAIL (10 ng/ml), 80 % of Jurkat 16 cells underwent apoptosis (Fig. 3.1A), whereas even 100 ng/ml of TRAIL had no effect on HTLV-1-associated ATL cells (Fig. 3.1B). This demonstrates that HTLV-1-associated ATL cell lines are resistant towards TRAIL-induced apoptosis.

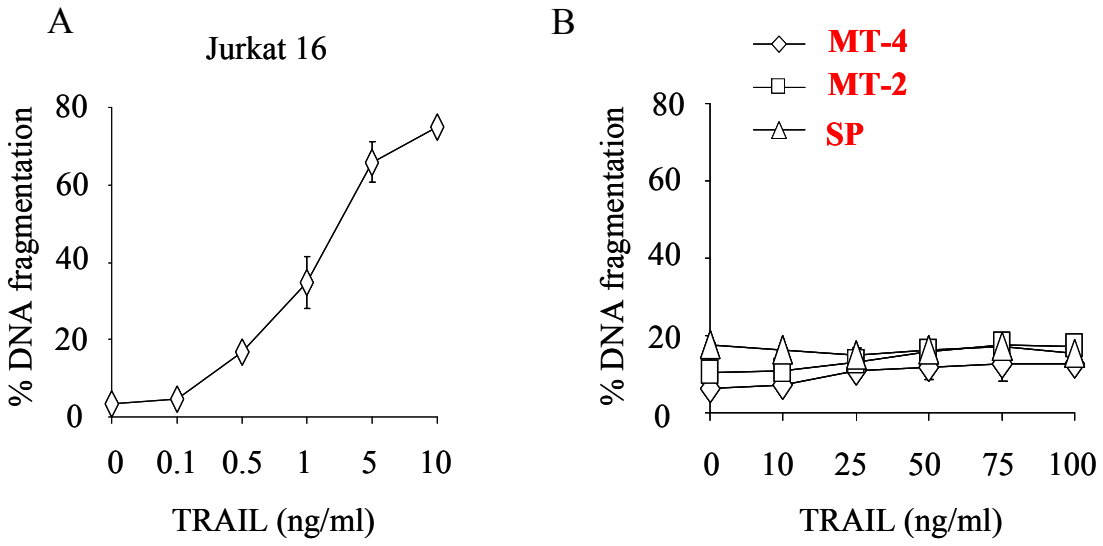

Figure 3.1 HTLV-1-associated ATL cell lines are resistant towards TRAIL-induced apoptosis.

(A) The non-infected leukemic cell line Jurkat 16 and **(B)** the HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 were incubated with different concentrations of TRAIL for 24 h. Apoptotic cell death was determined by the Nicoletti assay. Means ± SD are shown. The results shown are representative of three independent experiments.

3.1.2 Sensitization of HTLV-1-associated ATL cells towards TRAIL-induced apoptosis by Wogonin

We have previously shown that Wogonin, derived from the Traditional Chinese Medicine (TCM) plant Huang-Qin (*Scutellaria baicalensis Georgi*), can sensitize the leukemic cell lines (CEM and Jurkat) and primary acute myeloid leukemia cells towards TRAIL-induced apoptosis. Importantly, Wogonin does not affect the viability of peripheral blood T cells derived from healthy donors (Fas *et al*., 2006). To study whether Wogonin can sensitize HTLV-1-associated ATL cells to TRAIL-induced apoptosis, SP, MT-2 and MT-4 cells were treated with TRAIL alone or in combination with Wogonin (Fig. 3.2). Apoptotic cell death was examined by DNA fragmentation. The experiment showed that about 5 % of the cells underwent apoptosis after

treatment for 24 h with 10-100 ng/ml of TRAIL. Treatment with 50 µM of Wogonin led to apoptosis induction of 5-20 %. However, a combination of Wogonin and TRAIL resulted in 30-80 % of apoptotic cell death. Thus, Wogonin can sensitize HTLV-1-associated ATL cells to TRAIL-induced apoptosis.

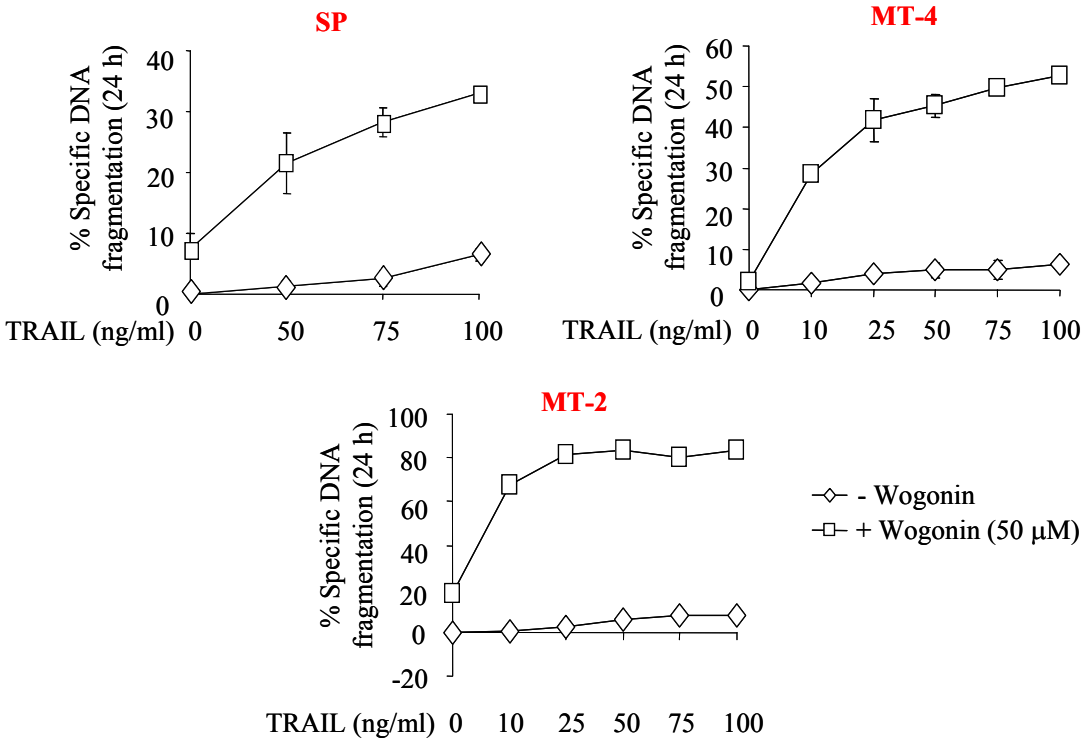

Figure 3.2 Wogonin sensitizes HTLV-1-associated ATL cell lines towards TRAIL-induced apoptosis.

SP, MT-2 and MT-4 cells were treated either with different concentrations of TRAIL or Wogonin (50 µM) alone or in combination for 24 h. Apoptotic cell death was determined by Nicoletti and represented as specific DNA fragmentation. Means ± SD are shown. The results shown are representative of three independent experiments.

3.1.3 Wogonin enhances TRAIL-induced cleavage of caspase, Bid and PARP

Pro-caspase-8 cleavage and activation is the first event after death receptor triggering. Activation of pro-caspase-8 is followed by processing and activation of downstream

effector pro-caspase-3 and, consequently, of PARP. Additionally, cleavage of Bid by caspase-8 leads to crosslinking of the extrinsic with the intrinsic apoptosis pathway. To confirm that sensitization towards TRAIL-mediated apoptosis by Wogonin is triggered through the death receptor, cleavage of pro-caspase-8 and Bid was analyzed. The HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 were treated for 24 h with TRAIL (50 ng/ml) or Wogonin (50 µM) alone or in combination. As shown in Fig. 3.3, Wogonin enhanced TRAIL-induced cleavage of pro-caspase-8 and Bid, leading to enhanced caspase-3 activation in all three HTLV-1-associated ATL cell lines. These findings demonstrate that Wogonin can enhance TRAIL-induced apoptosis by promoting caspase-8 activation and Bid cleavage in HTLV-1- associated ATL cells.

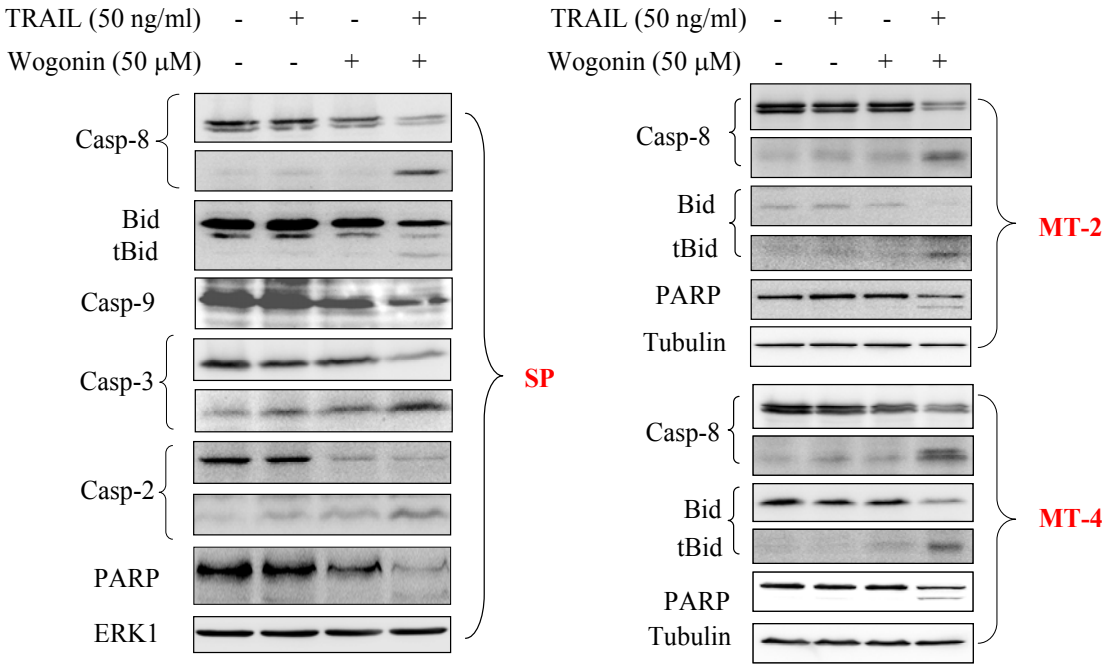

Figure 3.3 Wogonin enhances TRAIL-induced cleavage of caspase, Bid and PARP.
SP, MT-2 and MT-4 cells were treated with TRAIL (50 ng/ml) or Wogonin (50 µM) alone or in combination for 24 h. Processing of pro-caspases, Bid and PARP was analyzed by Western blot. The expression levels of Tubulin and ERK1 served as controls for equal protein loading. The results shown are representative of three independent experiments.

3.1.4 Wogonin does not sensitize TRAIL-induced apoptosis in non-malignant T cells

To test whether Wogonin and TRAIL can induce cell death in non-malignant T cells, the effect of Wogonin and TRAIL on peripheral blood T lymphocytes was analyzed. Freshly isolated peripheral blood T cells from healthy donors were incubated with TRAIL (50 ng/ml) or Wogonin (50 μM) alone or in combination for 24 and 48 h. Cells were harvested and apoptotic cell death was examined by specific DNA fragmentation. As shown in Fig. 3.4, only 0-2% of healthy blood T cells underwent apoptosis when treated with TRAIL or Wogonin alone, or in combination for 24 h. Additionally, less than 10 % of healthy blood T cells underwent apoptosis after treatment of TRAIL and Wogonin in combination for 48 h (Fig. 3.4). These data show that Wogonin has almost no effect on TRAIL-induced apoptosis in non-malignant T cells.

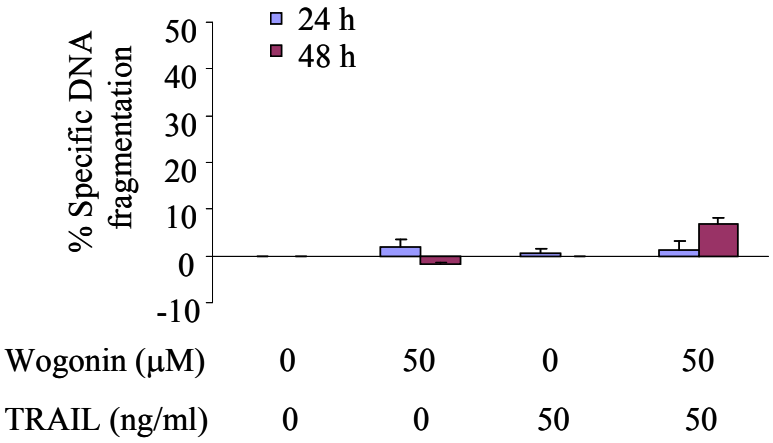

Figure 3.4 Wogonin does not sensitize TRAIL-induced apoptosis in non-malignant T cells
Freshly isolated peripheral blood T cells from healthy donors were treated with TRAIL (50 ng/ml) or Wogonin (50 μM) alone or in combination for 24 or 48 h. Apoptotic cell death was examined by specific DNA fragmentation. The data shown are representative of two independent experiments.

3.2 Molecular mechanisms of Wogonin-mediated sensitization of TRAIL-induced apoptosis

3.2.1 Wogonin down-regulates c-FLIP expression

3.2.1.1 Wogonin inhibits c-FLIP protein expression

c-FLIP is a key inhibitor of the death receptor-mediated apoptotic signalling cascade. Our previous study has shown that HTLV-1-associated ATL cells express significantly higher c-FLIP protein levels than non-infected leukemic cells and over-expression of c-FLIP is responsible for resistance to receptor-mediated apoptosis, which was confirmed by knockdown of c-FLIP (Krueger *et al*., 2006). We have also shown that inhibition of c-FLIP expression by the natural compound Rocaglamide breaks TRAIL resistance in HTLV-1-associated ATL cells (Bleumink *et al.*, 2010). Additionally, it was reported that down-regulation of c-FLIP leads to sensitization of TRAIL-induced apoptosis in acute myeloid leukemia (AML) (Suh *et al.*, 2003) or HTLV-1-associated ATL cells (Okamoto et al., 2006). Therefore, the effect of Wogonin on c-FLIP protein expression was analyzed. SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h and c-FLIP protein level was analyzed by Western blot. As shown in Fig. 3.5A, c-$FLIP_L$ and c-$FLIP_S$ protein levels were significantly down-regulated in all three cell lines upon Wogonin treatment. Kinetic analysis of c-FLIP protein levels in SP cells showed that c-$FLIP_L$ and c-$FLIP_S$ protein expression was decreased after 3 h of Wogonin treatment (50 μM) and completely inhibited after 24 h of treatment (Fig. 3.5B). These results demonstrate that the inhibition of c-FLIP expression is one possible mechanism by which Wogonin sensitizes TRAIL-induced apoptosis.

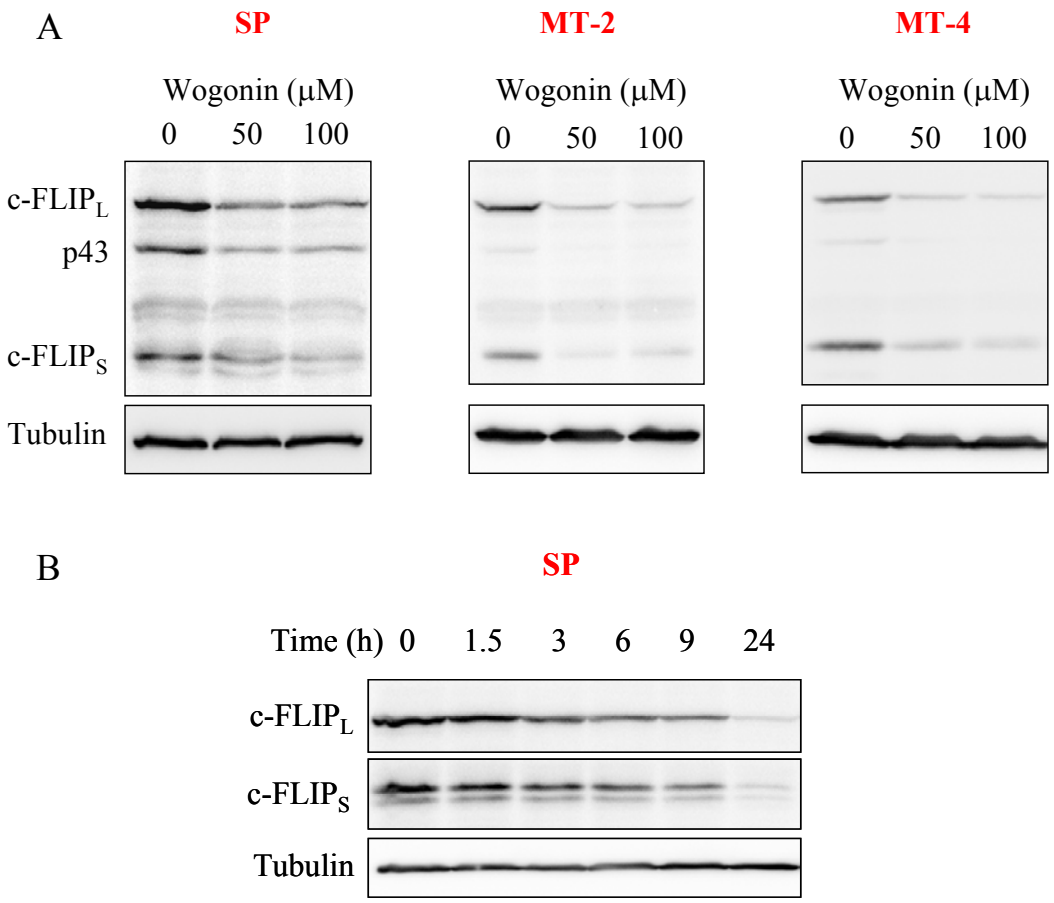

Figure 3.5 Wogonin suppresses the expression of the anti-apoptotic protein c-FLIP
(A) SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h or **(B)** SP cells were treated with Wogonin (50 μM) for indicated time periods (1.5, 3, 6, 9 and 24 h). Cells were lysed and c-FLIP protein levels were analyzed by Western blot. Tubulin expression served as a control for equal protein loading. Data shown are representative of three independent experiments.

3.2.1.2 HTLV-1-associated ATL cells express higher c-FLIP mRNA levels than non-infected leukemic cells

c-FLIP protein levels are increased in HTLV-1-associated ATL cells compared to non-infected leukemic cells (Krueger *et al*., 2006), but it is not clear whether the c-FLIP mRNA levels are also elevated in HTLV-1-associated ATL cells. Therefore, the c-FLIP mRNA levels were measured by TaqMan real time PCR in different HTLV-1-associated ATL cells and control Jurkat 16 cells. As shown in Fig. 3.6, all three HTLV-1-associated ATL cell lines expressed greatly increased mRNA levels of both c-FLIP$_L$ and c-FLIP$_S$ compared to Jurkat 16 cells. These findings show that HTLV-1-associated ATL cell lines express 4-9 fold higher levels of c-FLIP mRNA than non-infected leukemic cell line Jurkat 16.

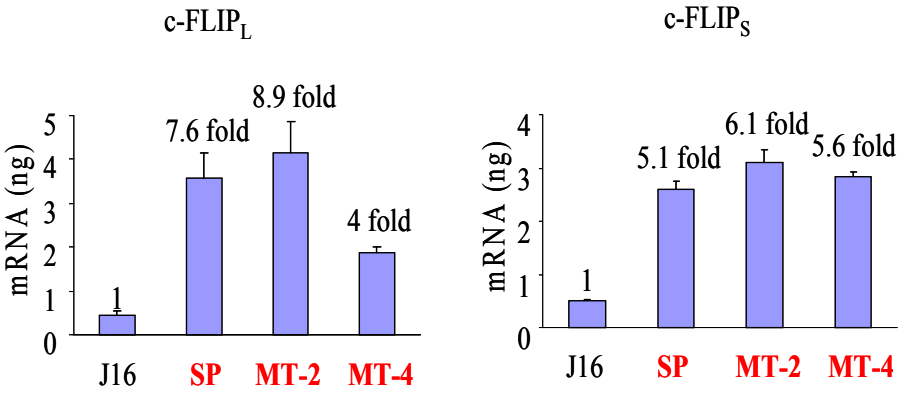

Figure 3.6 HTLV-1-associated ATL cells express increased amounts of c-FLIP mRNA.
c-FLIP absolute mRNA levels in HTLV-1-associated ATL cell lines SP, MT-2, MT-4 and non-infected leukemic cell line Jurkat 16 were analyzed by TaqMan real time PCR described in materials and methods. The data are representative of three independent experiments.

3.2.1.3 Wogonin suppresses c-FLIP expression at the mRNA level

Recently, our group found that Wogonin is a potent inhibitor of CDK9 (cyclin-dependent kinase 9) which plays a major role in regulation of transcription. Additionally, Wogonin induces apoptosis by suppressing transcription of Mcl-1, an anti-apoptotic protein with a short half life (Polier *et al*., unpublished data). It has been previously reported that c-FLIP isoforms are short-lived proteins as well and their expression can be easily attenuated by using protein or RNA synthesis inhibitors (Fulda *et al*., 2000; Hernandez *et al*., 2001). We showed above that HTLV-1-associated ATL cells express elevated c-FLIP mRNA levels (Fig. 3.6). Therefore, we further investigated whether Wogonin down-regulates c-FLIP mRNA levels in HTLV-1-associated ATL cells. Three HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 were treated with Wogonin (50 μM) for different time periods and the relative mRNA expression levels were measured by TaqMan real time PCR. As shown in Fig. 3.7, c-FLIP$_L$ and c-FLIP$_S$ mRNA levels started to decrease after 3 h of treatment with Wogonin (50 μM). These data demonstrate that Wogonin suppresses c-FLIP expression at the transcriptional level.

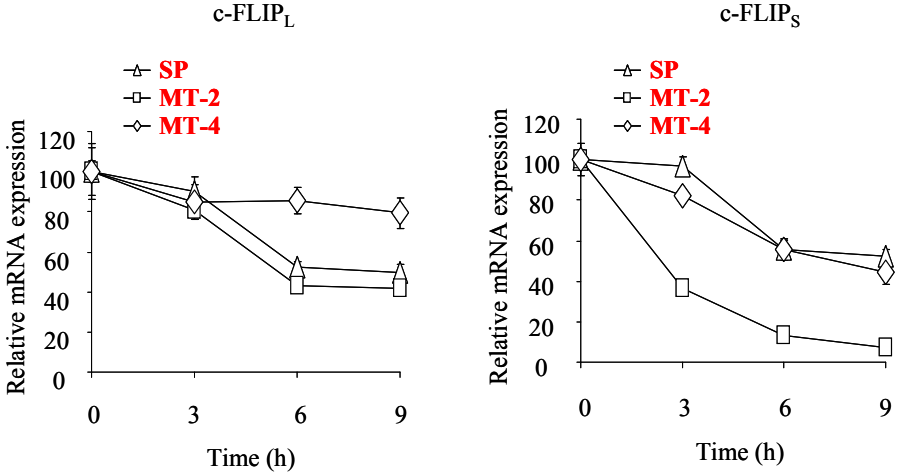

Figure 3.7 Wogonin suppresses c-FLIP mRNA expression

SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM) for 0, 3, 6 and 9 h. The relative c-FLIP mRNA expression level was measured by TaqMan real time PCR using 18S rRNA as a control. The data are representative of two independent experiments.

3.2.2 Wogonin up-regulates TRAIL-R2 expression

3.2.2.1 Wogonin increases TRAIL-R2 protein expression levels

Previous studies have shown that certain drugs can sensitize resistant cancer cells towards TRAIL-mediated apoptosis by up-regulating TRAIL death receptor expression (Kim *et al*., 2004; Shiraishi *et al*., 2005; Yoshida *et al*., 2005; Carter *et al*., 2008; Kim *et al*., 2010). To examine whether Wogonin could affect the expression of TRAIL receptors, SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 16 h and the protein levels of different TRAIL receptors were analyzed by Western blot. Up-regulation of TRAIL-R2 was observed in all three HTLV-1-associated ATL cell lines after treatment with Wogonin (Fig. 3.8A). Kinetic analysis of TRAIL death receptor expression upon treatment with Wogonin (50 μM) for 1.5 h to 24 h showed that TRAIL-R2 protein levels were increased by Wogonin after 9 h of treatment (Fig. 3.8B). The expression of other TRAIL receptors experienced no changes with Wogonin treatment. These experiments demonstrate that Wogonin may sensitize TRAIL-induced apoptosis through up-regulation of TRAIL-R2 expression.

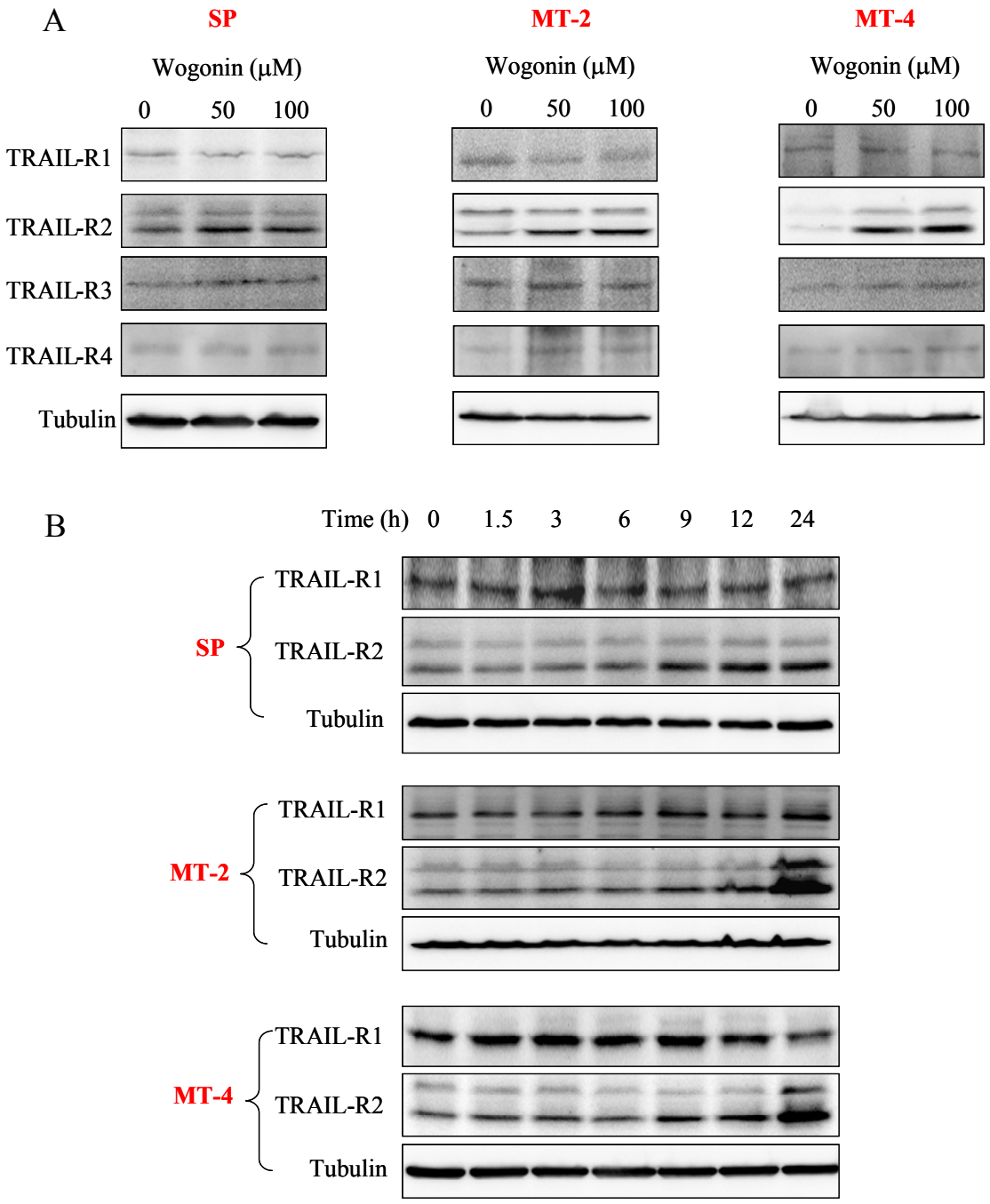

Figure 3.8 Wogonin up-regulates TRAIL-R2 protein expression in HTLV-1-associated ATL cells

(A) HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 16 h or **(B)** with 50 μM of Wogonin for different time periods (1.5, 3, 6, 9, 12 and 24 h). Cells were lysed and TRAIL receptor protein level was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.2.2.2 Wogonin up-regulates TRAIL-R2 surface expression

In addition to increased TRAIL-R2 protein expression level, TRAIL death receptor surface expression was also measured in HTLV-1-associated ATL cells treated with Wogonin (50 μM) for 16 h. As shown in Fig. 3.9, TRAIL-R2 surface expression was increased by Wogonin treatment in all three cell lines, while TRAIL-R1 surface expression was not significantly enhanced. This result is consistent with the total protein expression levels of TRAIL death receptors showing that only TRAIL-R2 but not TRAIL-R1 expression level is elevated by Wogonin treatment (Fig. 3.8). These findings imply that Wogonin may sensitize TRAIL-induced apoptosis through up-regulation of TRAIL-R2 surface expression.

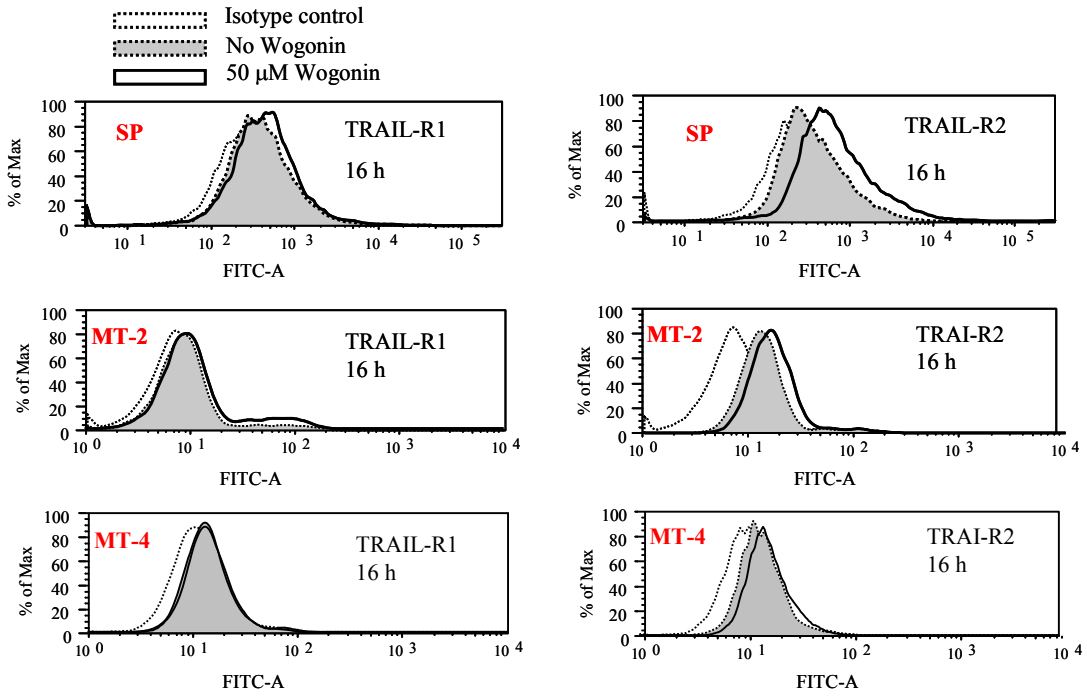

Figure 3.9 Wogonin up-regulates TRAIL-R2 surface expression in HTLV-1-associated ATL cells

SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM) for 16 h. Surface expression of TRAIL death receptor TRAIL-R1 and TRAIL-R2 was analyzed by FACS with HS101 (TRAIL-R1) and HS201 (TRAIL-R2) or an IgG1 isotype control antibody. The data are representative of two independent experiments.

3.2.2.3 Wogonin elevates TRAIL-R2 expression at the mRNA level

To investigate how Wogonin enhances TRAIL-R2 expression, the effect of Wogonin on TRAIL-R2 mRNA expression levels in SP, MT-2 and MT-4 cells was analyzed. All three cell lines were treated with Wogonin (50 μM) for different time periods from 1.5 h to 24 h. Although the TRAIL-R2 mRNA expression level was slightly down-regulated (about 20%-30%) at early time points (0-6 h), it was consistently up-regulated (1.5 to 2.5 fold) after 6 h of treatment (Fig. 3.10). These data demonstrate that Wogonin increases TRAIL-R2 expression at the transcriptional level.

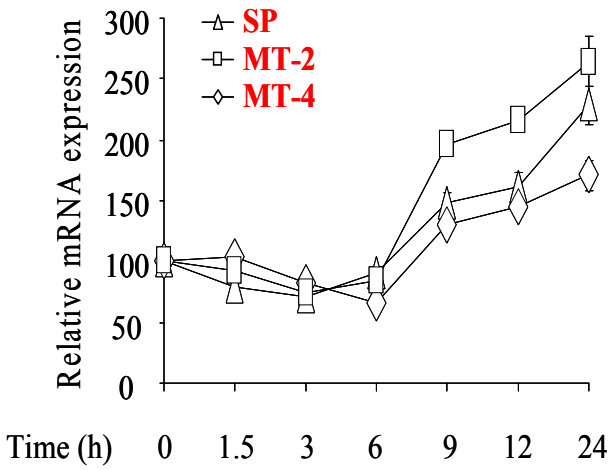

Figure 3.10 Wogonin increases TRAIL-R2 expression at the mRNA level
SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM) for 0, 1.5, 3, 6, 9, 12 and 24 h. The relative TRAIL-R2 mRNA expression level was measured by SYBR Green real time PCR using GAPDH for normalization. Means ± SD are shown. The data are representative of two independent experiments.

3.2.2.4 Increase of TRAIL-R2 mRNA level by Wogonin correlates with increased p53 protein level

TRAIL-R2 mRNA has a short half-life of about 4 h (Kandasamy *et al*., 2008). Since Wogonin can target transcription (Polier *et al*., unpublished data), this may explain the result in Fig. 3.10 that Wogonin suppresses TRAIL-R2 mRNA expression at early treatment. However, the mechanism by which Wogonin increased TRAIL-R2 mRNA level at later points is not clear.

p53 has been shown to transactivate the TRAIL-R2 gene through an intronic sequence-specific DNA-binding site (+ 0.25 Kb downstream of the ATG) (Takimoto *et al*., 2000). It was reported that the natural compound triptolide sensitizes AML cells to TRAIL-induced apoptosis *via* p53-mediated increase of TRAIL-R2 (Carter *et al*., 2008). Therefore, we further investigated whether Wogonin-mediated up-regulation of TRAIL-R2 is also regulated by a p53-dependent mechanism. To investigate this possibility, the effect of Wogonin on p53 protein levels in HTLV-1-associated ATL cells was analyzed. SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) and the protein levels of p53 were analyzed by Western blot. The experiment showed that Wogonin up-regulated p53 expression in a dose- and time-dependent manner (Fig. 3.11). These results suggest that Wogonin may up-regulate TRAIL-R2 protein expression by increasing the p53 protein level.

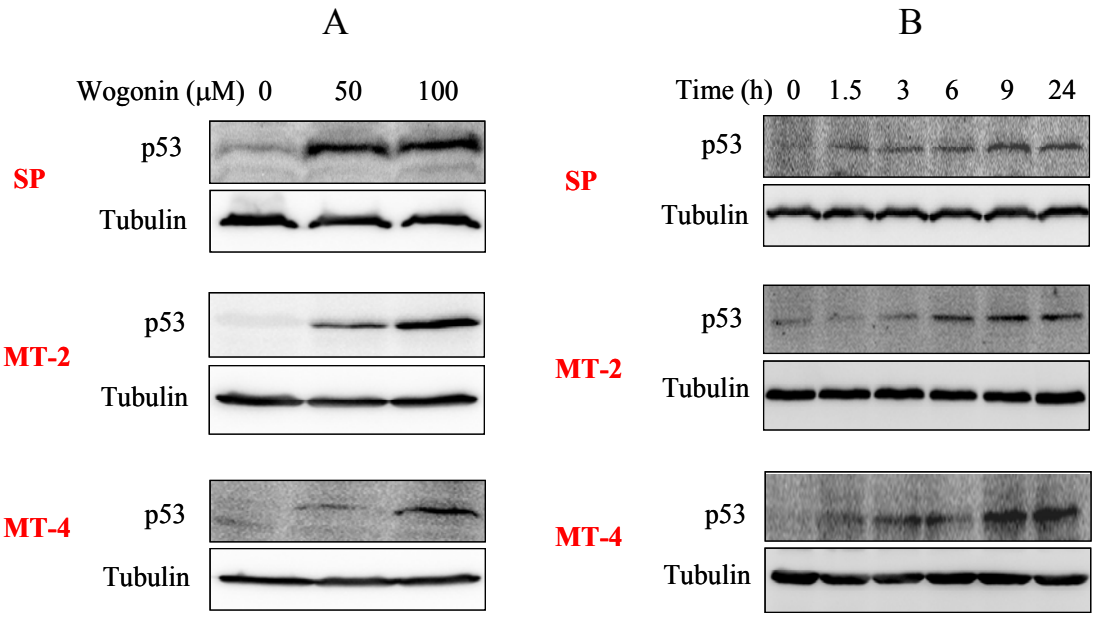

Figure 3.11 Wogonin increases the protein level of the transcription factor p53
(A) SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h or **(B)** with 50 μM of Wogonin for different time periods (1.5, 3, 6, 9 and 24 h). p53 protein level was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The data shown are representative of two independent experiments.

3.2.2.5 The Wogonin-mediated up-regulation of the p53 protein level is due to decreased MDM2 protein expression

The p53 protein level is strictly controlled by the negative regulator MDM2. On the one hand, MDM2 binds directly to the N-terminal end of p53 to inhibit its function (Momand *et al.*, 1992; Oliner *et al.*, 1993). On the other hand, the E3 ubiquitin ligase activity of MDM2 targets p53 for ubiquitination and subsequently degradation by the 26S proteasome (Haupt *et al.*, 1997; Honda *et al.*, 1997). Therefore, the expression levels of MDM2 in Wogonin-treated HTLV-1-associated ATL cells were investigated. SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h or with 50 μM of Wogonin for different time periods (1.5, 3, 6, 9 and 24 h). The MDM2 protein expression levels were analyzed by Western blot. The experiments showed

that Wogonin strongly inhibited MDM2 protein expression (Fig. 3.12A). Kinetic analysis showed that MDM2 was rapidly down-regulated already after 1.5 h of treatment (Fig. 3.12B). These results suggest that Wogonin-mediated increases in p53 protein levels may involve a mechanism of MDM2 suppression.

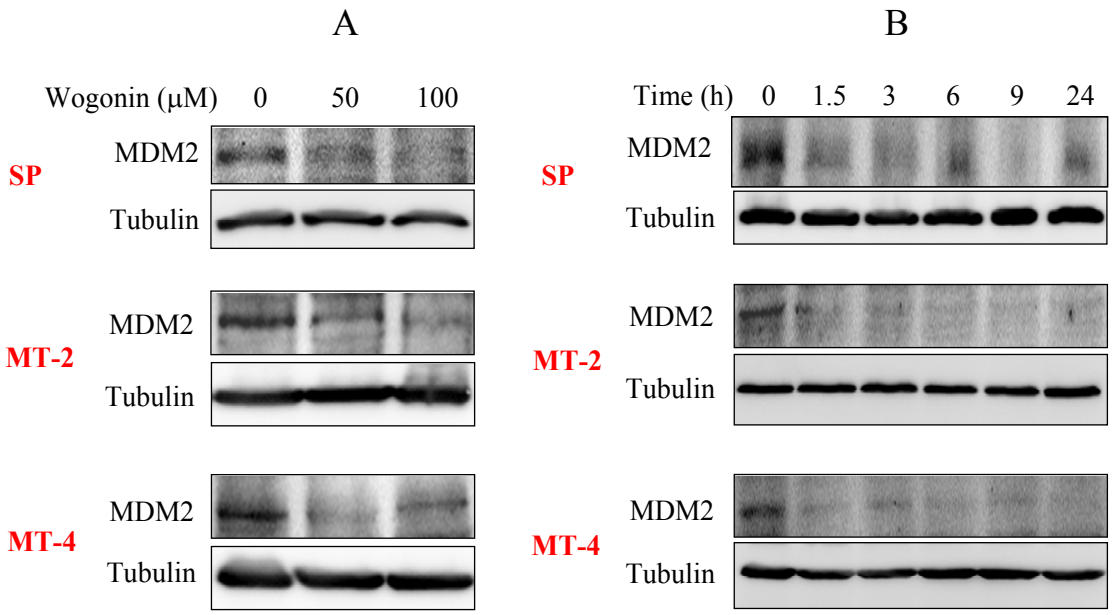

Figure 3.12 Wogonin inhibits MDM2 protein expression

(A) SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h or **(B)** with 50 μM of Wogonin for various time periods (1.5, 3, 6, 9 and 24 h). The MDM2 protein expression level was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.2.2.6 p53 and MDM2 expression is suppressed by Wogonin at the mRNA level

We have recently shown that Wogonin inhibits CDK9 activity and suppresses gene expression at the transcriptional level (Polier *et al*., unpublished data). In order to investigate the effect of Wogonin on the mRNA level of p53 and MDM2, HTLV-1-associated ATL cell lines SP, MT-2 and MT-4 were treated with 50 μM of Wogonin for different time periods (3, 6 and 9 h) and the mRNA expression levels of p53 and MDM2 were measured by TaqMan real time PCR. As shown, Wogonin inhibited both p53 and MDM2 mRNA expression in all three cell lines (Fig. 3.13). These experiments are consistent with our previous finding that Wogonin inhibits mRNA expression.

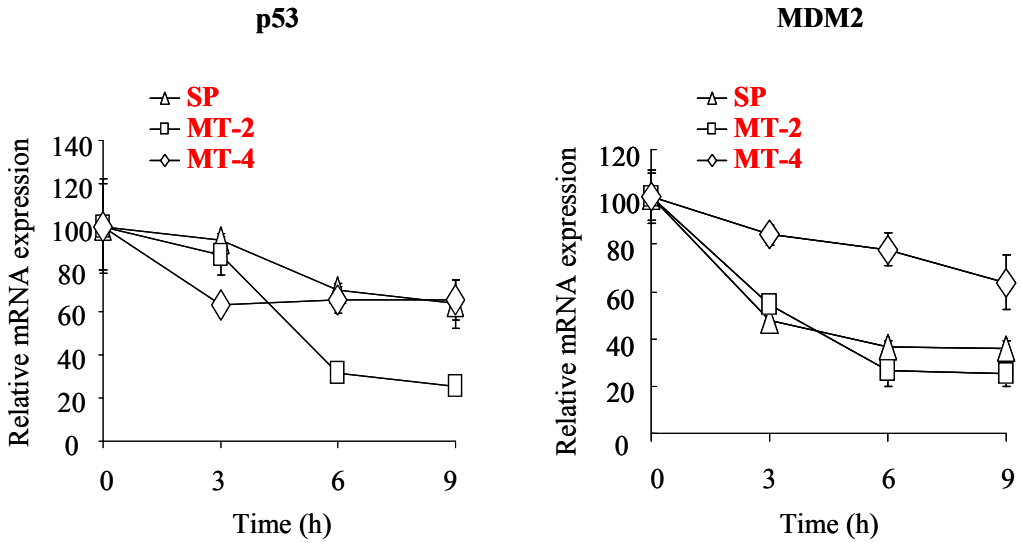

Figure 3.13 Wogonin suppresses p53 and MDM2 mRNA expression
SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM) for 0, 3, 6 and 9 h. The relative p53 and MDM2 mRNA expression level was measured by TaqMan real time PCR, using 18S rRNA as a control. Means ± SD are shown. The data are representative of two independent experiments.

3.2.2.7 TRAIL-R2 protein expression in p53-deleted cell lines is not increased by Wogonin

In order to further investigate the role of p53 in Wogonin-induced TRAIL-R2 expression, the effect of Wogonin on TRAIL-R2 protein expression level was analyzed in two p53-deleted tumor cell lines PC3 (human prostate cancer cell line) and Hep3b (human hepatoma cell line) and in parallel in the control cell line HepG2 (human hepatoma cell line) which harbour wild-type p53. All three cell lines were treated with Wogonin (50 μM) for 8 or 16 h. TRAIL-R1 and TRAIL-R2 protein expression was measured by Western blot. As shown in Fig. 3.14, Wogonin did not increase the TRAIL-R2 expression in the two p53 deficient cell lines, while it significantly enhanced TRAIL-R2 expression levels in the control HepG2 cells. Interestingly, TRAIL-R1 protein level was also increased in HepG2 cells by Wogonin treatment, whereas no enhancement of TRAIL-R1 expression was observed in PC3 and Hep3b cells. This result demonstrates that Wogonin-induced up-regulation of TRAIL-R2 expression is p53 dependent.

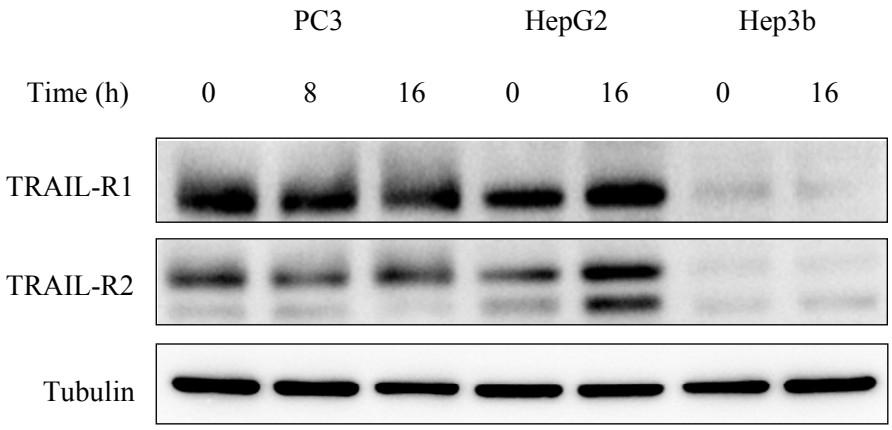

Figure 3.14 Wogonin does not increase TRAIL-R2 protein expression in p53-deleted tumor cell lines

Two p53-deleted tumor cell lines PC3 and Hep3b and a p53 wild type tumor cell line HepG2 were treated with Wogonin (50 μM) for 8 or 16 h. TRAIL-R1 and TRAIL-R2 protein expression level was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.2.2.8 The protein expression of transcription factors CHOP and Sp1 is not influenced by Wogonin

Besides p53, two other transcription factors, CHOP and Sp1, have been implicated in regulating TRAIL-R2 expression. It was shown that the CHOP binding site is localized at -270 bp of the TRAIL-R2 promoter and four putative Sp1 binding sites are located in the region of the TRAIL-R2 promoter spanning nucleotide -605 to +3 (Yoshida *et al*., 2001). Since several publications have reported that certain drugs can sensitize cancer cells to TRAIL-mediated apoptosis through CHOP or Sp1-mediated up-regulation of TRAIL-R2 (Kim *et al*., 2004; Shiraishi *et al*., 2005; Yoshida *et al*., 2005; Kim *et al*., 2010), we asked whether enhancement of TRAIL-R2 expression by Wogonin treatment is also mediated by CHOP or Sp1. To answer this question, SP and MT-4 cells were treated with Wogonin (50 μM) for different time periods and CHOP and Sp1 protein expression were analyzed by Western blot. As seen in Fig. 3.15, CHOP and Sp1 protein expression were not influenced by Wogonin, suggesting that CHOP and Sp1 are not involved in Wogonin-induced TRAIL-R2 up-regulation in HTLV-1-associated ATL cells.

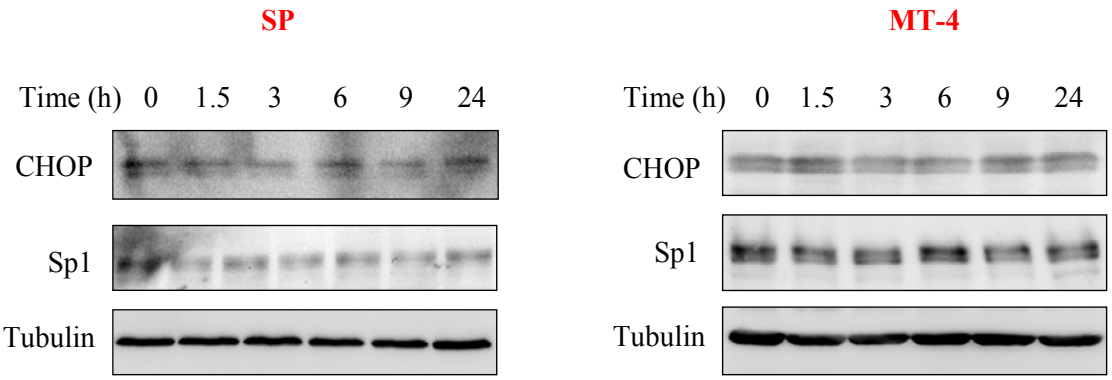

Figure 3.15 Protein expression of the transcription factors CHOP and Sp1 is not influenced by Wogonin

SP and MT-4 cells were treated with Wogonin (50 μM) for different time periods (1.5, 3, 6, 9 and 24 h). CHOP and Sp1 protein expression were analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.2.3 Effects of Wogonin on the expression of pro- and anti-apoptotic proteins

3.2.3.1 Comparison of pro- and anti-apoptotic protein expression levels in HTLV-1-associated ATL cells and non-infected leukemic T cells

Besides c-FLIP, apoptotic resistance in HTLV-1-associated ATL cells has been partly explained by abnormal expression of some other proteins involved in cell death pathways. It has been shown that in HTLV-1-associated ATL cells several anti-apoptotic proteins are up-regulated, such as Bcl-2 (Bleumink *et al.*, 2010), Bcl-xL (Mori *et al*., 2001; Tsukahara *et al*., 1999) and IAP family proteins (Kawakami *et al*., 1999; Waldele *et al*., 2006). In order to obtain an overview of misregulated proteins which may be involved in apoptotic resistance in HTLV-1-associated ATL cells, expression levels of pro- and anti-apoptotic proteins in HTLV-1-associated ATL cells and control Jurkat 16 cells were systematically compared (Fig. 3.16). Consistent with previous reports (Krueger *et al*., 2006; Bleumink *et al.*, 2010), c-FLIP, Bcl-xL and Bcl-2 expression were shown to be increased in HTLV-1-associated ATL cells. In addition, the anti-apoptotic proteins XIAP and Mcl-1 were also found to be up-regulated. However, the expression of pro-apoptotic proteins Bad and Bax was enhanced as well in HTLV-1-associated ATL cells (Fig. 3.16).

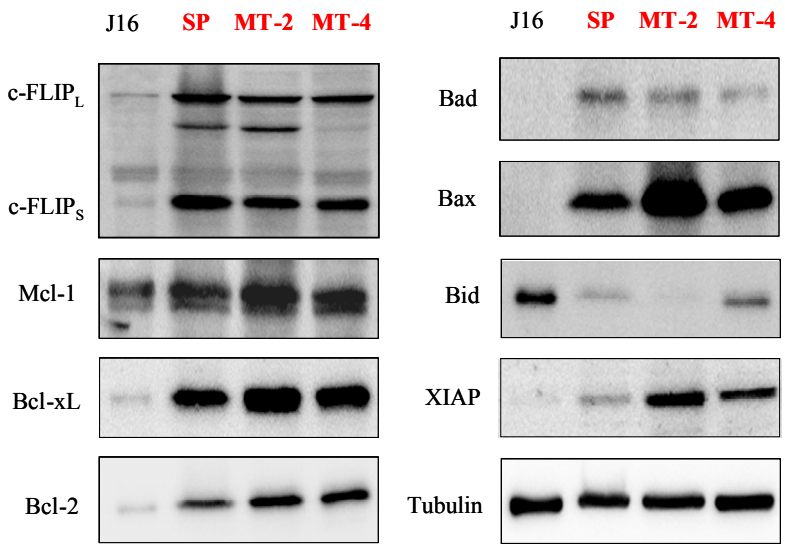

Figure 3.16 Comparison of pro- and anti-apoptotic protein expression in HTLV-1-associated ATL cells and non-infected leukemic T cells

Expression of pro- and anti-apoptotic proteins in HTLV-1-associated ATL cells SP, MT-2, MT-4 and a non-infected leukemic cell line Jurkat 16 cells was analyzed by Western blot. Tubulin levels served as a control for equal loading of proteins. The data are representative of two independent experiments.

3.2.3.2 Wogonin does not influence XIAP protein expression

XIAP is a member of the IAPs family and it suppresses apoptosis by inhibiting the activity of effector caspases, such as caspase-3, -7 and -9. It has been reported that down-regulation of XIAP sensitizes TRAIL- or CD95-induced apoptosis in various tumor cells (Kim *et al*., 2004; Kim *et al*., 2005; Loeder *et al*., 2010). We show that XIAP expression is increased in HTLV-1-associated ATL cells compared to non-infected leukemic Jurkat 16 cells. Therefore, the effect of Wogonin on the protein expression of XIAP in HTLV-1-associated ATL cells was further analyzed. SP, MT-2 and MT-4 cells were treated with Wogonin (50 µM or 100 µM) for 8 h and XIAP protein expression was analyzed by Western blot. As seen in Fig. 3.17A, Wogonin did not influence XIAP protein expression in all three cell lines after 8 h treatment. Kinetic analysis showed that a slightly reduced XIAP protein level was observed only

after 24 h treatment with Wogonin (Fig. 3.17B). These findings suggest that Wogonin-mediated sensitization of TRAIL-induced apoptosis is not mediated through down-regulation of XIAP.

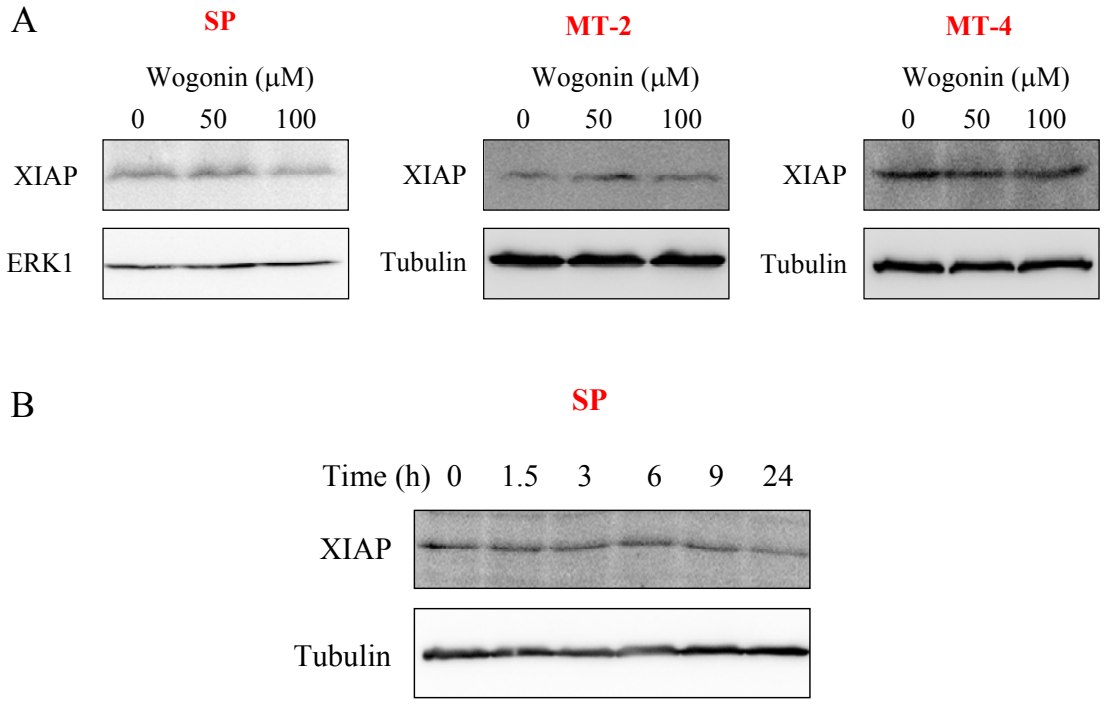

Figure 3.17 Wogonin does not influence XIAP protein expression

(A) SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h and **(B)** SP cells were treated with 50 μM of Wogonin for various time periods (1.5, 3, 6, 9 and 24 h). XIAP protein expression level was analyzed by Western blot. Tubulin and ERK1 expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.2.3.3 Wogonin inhibits protein expression of Mcl-1 but not other Bcl-2 family members

The Bcl-2 family of proteins is the most important regulator of the intrinsic apoptosis pathway. Although both anti- and pro-apoptotic Bcl-2 family proteins are increased in HTLV-1-associated ATL cells compared to non-infected leukemic cells (Fig. 3.16), the effect of Wogonin on expression of these proteins needs to be investigated. Therefore, three HTLV-1-associated ATL cell lines, SP, MT-2 and MT-4, were treated with Wogonin (50 μM or 100 μM) for 8 h or SP cells were treated with Wogonin (50 μM) for different time periods. The expression of Bcl-2 family proteins was analyzed by Western blot. As expected, only the short-lived Mcl-1 protein expression was strongly reduced (Fig. 3.18A), which is consistent with our recent fingding that Wogonin induces apoptosis in cancer cells by suppression of Mcl-1 expression (Polier *et al*., unpublished data). Kinetic analysis showed that Mcl-1 down-regulation occurred as early as after 3 h treatment and persisted until 24 hours (Fig. 3.18B).

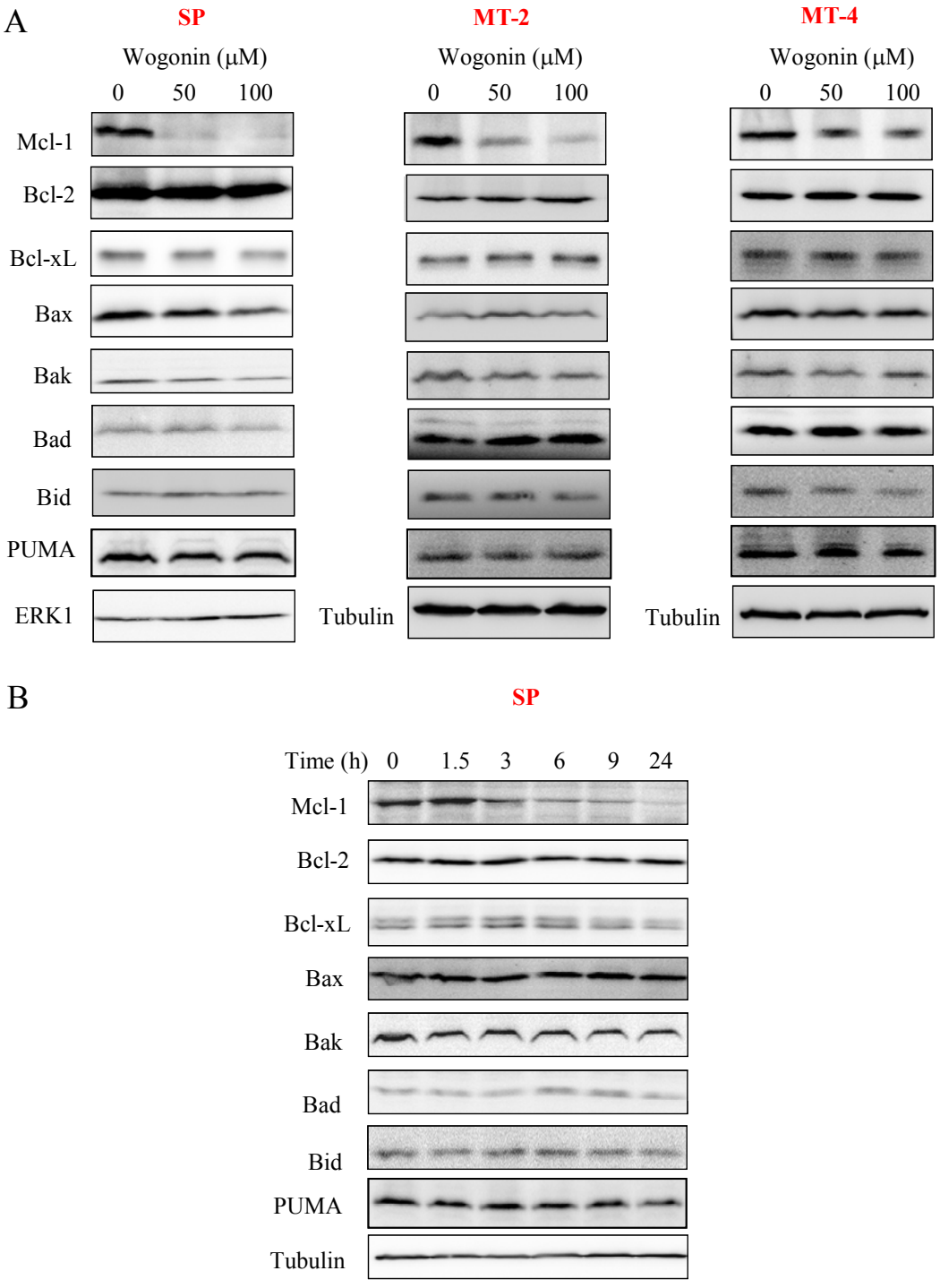

Figure 3.18 Wogonin inhibits Mcl-1 protein expression

(A) SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h or **(B)** SP cells were treated with 50 μM of Wogonin for different time periods (1.5, 3, 6, 9 and 24 h). Bcl-2 family protein expression was analyzed by Western blot. Tubulin and ERK1 expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.2.3.4 Wogonin inhibits mRNA expression of Mcl-1

To investigate the molecular mechanisms by which Wogonin suppresses Mcl-1 protein expression, the mRNA expression level of Mcl-1 was examined. SP cells were incubated with Wogonin for different time periods and the mRNA expression level of Mcl-1 was examined by real-time PCR. As shown in Fig. 3.19, Wogonin suppressed Mcl-1 mRNA expression in a time-dependent manner. This result is consistent with our previous finding that Wogonin inhibits mRNA expression (Polier *et al.*, unpublished data).

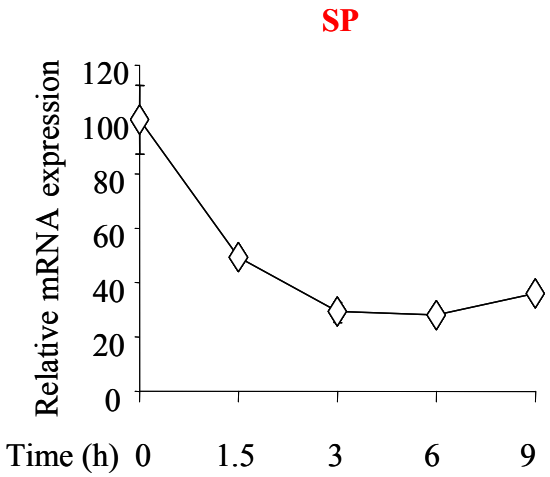

Figure 3.19 Wogonin suppresses Mcl-1 mRNA expression
SP cells were treated with Wogonin (50 μM) for 0, 1.5, 3, 6 and 9 h. The Mcl-1 mRNA expression level was measured by TaqMan real time PCR, using 18S rRNA as a control. Means ± SD are shown. The data are representative of three independent experiments.

3.3 Wogonin sensitizes various cancer cells towards TRAIL-induced apoptosis by down-regulation of c-FLIP and up-regulation of TRAIL-R2 expression

To further investigate whether Wogonin sensitizes TRAIL-induced apoptosis in other cancer cells, apoptotic cell death was measured in four different types of malignant cell lines including the human breast cancer cell line MDA-MB-231, the human melanoma cell line SK-MEL-37, the human hepatocellular carcinoma cell line HepG2 and the human colon cancer cell line HT-29. As shown in Fig. 3.20A, Wogonin sensitized TRAIL-induced apoptosis in all cell lines tested. In addition, Wogonin down-regulated c-FLIP and up-regulated TRAIL-R2 protein expression level in all cell lines tested (Fig. 3.20B). Thus, Wogonin can suppress c-FLIP and enhance TRAIL-R2 expression in tumor cells in general and this may account for one of the mechanisms of Wogonin-mediated sensitization of TRAIL-induced apoptosis in tumor cells.

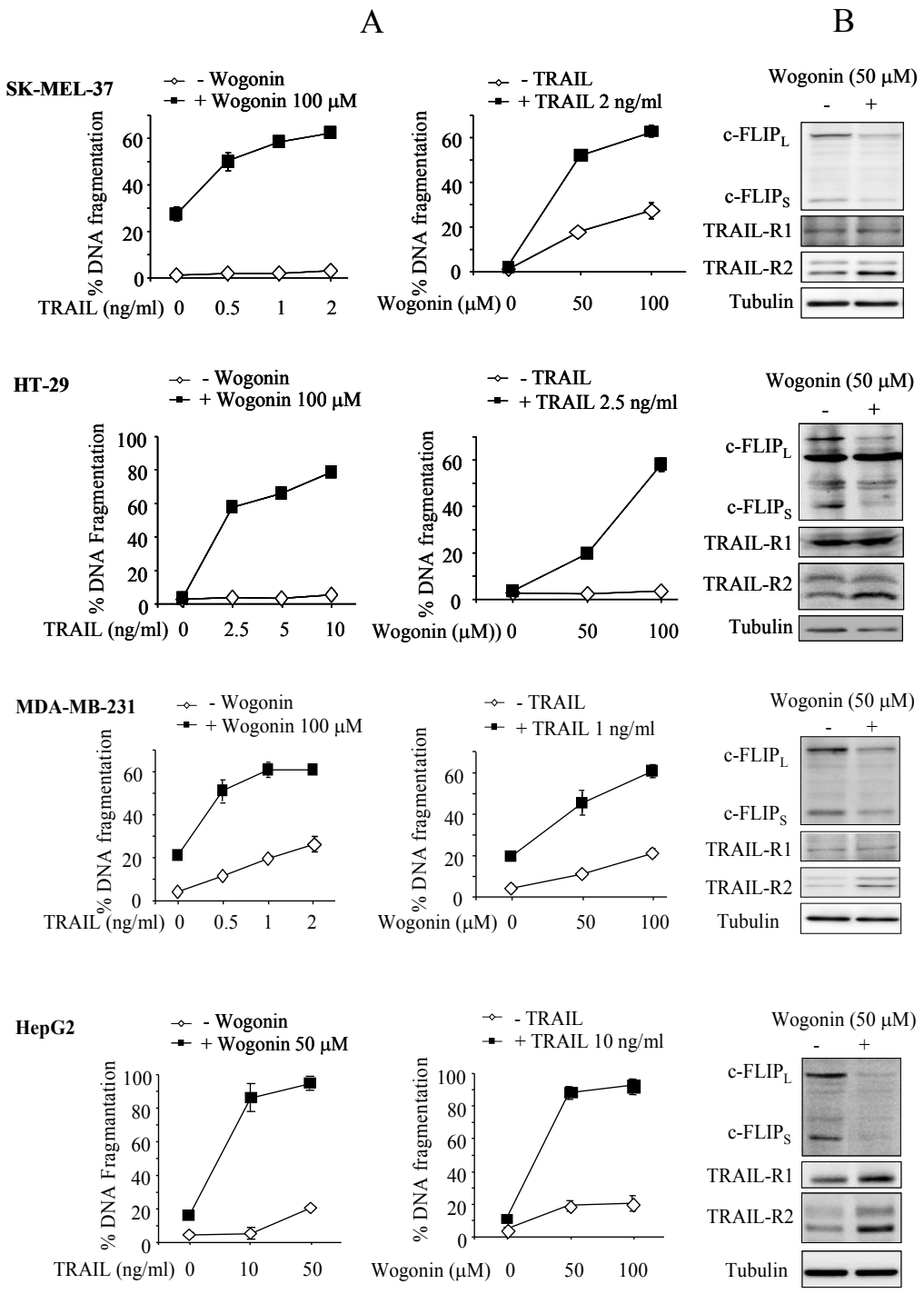

Figure 3.20 Wogonin sensitizes different cancer cell lines towards TRAIL-induced apoptosis by down-regulation of c-FLIP but up-regulation of TRAIL-R2 protein expression

(A) Four different types of cancer cell lines MDA-MB-231, SK-MEL-37, HepG2 and HT-29 were treated either with different concentration of TRAIL or Wogonin (50 µM) alone or in combination for 24 h. Apoptotic cell death was determined by DNA fragmentation. Means ± SD are shown. **(B)** Four indicated cell lines were treated with Wogonin (50 µM) for 16 h. Cell lysates were analyzed by Western blot.

3.4 Apigenin and Chrysin sensitize HTLV-1-associated ATL cells towards TRAIL-induced apoptosis

3.4.1 Sensitization of HTLV-1-associated ATL cells towards TRAIL-induced apoptosis by Apigenin and Chrysin

So far, several natural flavones have been reported to have anti-carcinogenic activities. However, their modes of function as anti-cancer agents are still largely unknown. To investigate whether other flavones besides Wogonin could also sensitize TRAIL-induced apoptosis, two naturally occurring anti-tumor flavones, Apigenin and Chrysin, were tested. The HTLV-1-associated ATL cell line MT-2 was treated with 50 μM of Apigenin or Chrysin alone or in combination with different concentrations of TRAIL for 24 h (Fig. 3.21A). Both Apigenin and Chrysin were shown to sensitize TRAIL-induced apoptosis. The experiments showed that at maximum about 20% of the cells underwent apoptosis after treatment with 10-100 ng/ml of TRAIL for 24 h and approximately 30-40% of cell death was detected after treatment with 50 μM of Apigenin or Chrysin. However, a combination of Apigenin or Chrysin and TRAIL resulted in a synergistic increase in apoptosis (about 80%). As shown in Fig. 3.21B, treatment with 12.5, 25 or 50 μM of Apigenin or Chrysin restored TRAIL sensitivity in a dose-dependent manner. These results demonstrate that Apigenin or Chrysin can sensitize HTLV-1-associated ATL cells to TRAIL-induced apoptosis.

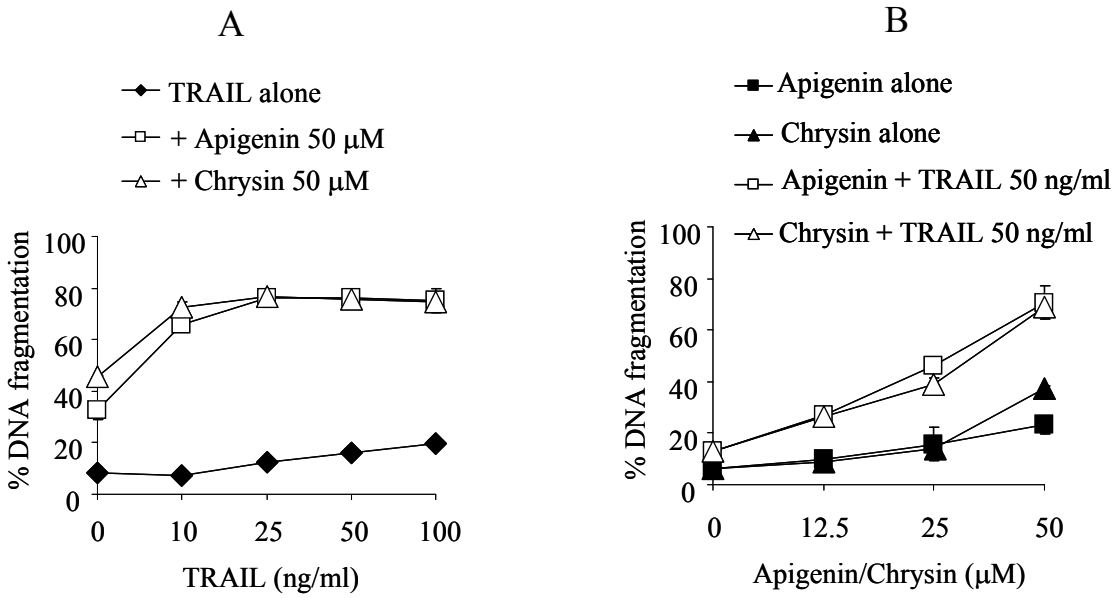

Figure 3.21 Apigenin and Chrysin sensitize HTLV-1-associated ATL cells towards TRAIL-induced apoptosis

(A) The HTLV-1-associated ATL cell line MT-2 was treated either with different concentration of TRAIL or 50 μM of Apigenin/Chrysin alone or in combination for 24 h. **(B)** MT-2 cells were treated with 12.5-50 μM of Apigenin or Chrysin alone, or 50 ng/ml of TRAIL alone or in combination for 24 h. Apoptotic cell death was determined by DNA fragmentation. Means ± SD are shown. The results shown are representative of two independent experiments.

3.4.2 Apigenin and Chrysin suppress c-FLIP protein expression at the transcriptional level

Further, the mechanisms by which Apigenin and Chrysin sensitize HTLV-1-associated ATL cells to TRAIL-induced apoptosis were investigated. As shown previously (Fig. 3.5 and Fig. 3.7), Wogonin sensitizes TRAIL-induced apoptosis in HTLV-1-associated ATL cells by down-regulation of c-FLIP expression at both mRNA and protein levels. Therefore, the effects of Apigenin and Chrysin on expression of c-FLIP in HTLV-1-associated ATL cells were tested. MT-2 cells were treated with Apigenin or Chrysin (50 μM or 100 μM) for 8 h and c-FLIP protein expression was analyzed by Western blot. The results showed that 50 μM of Apigenin

and Chrysin strongly suppressed c-FLIP$_L$ and c-FLIP$_S$ protein expression (Fig. 3.22A). In addition, the c-FLIP mRNA level was also remarkably inhibited by Apigenin and Chrysin in a time-dependent manner (Fig. 3.22B).

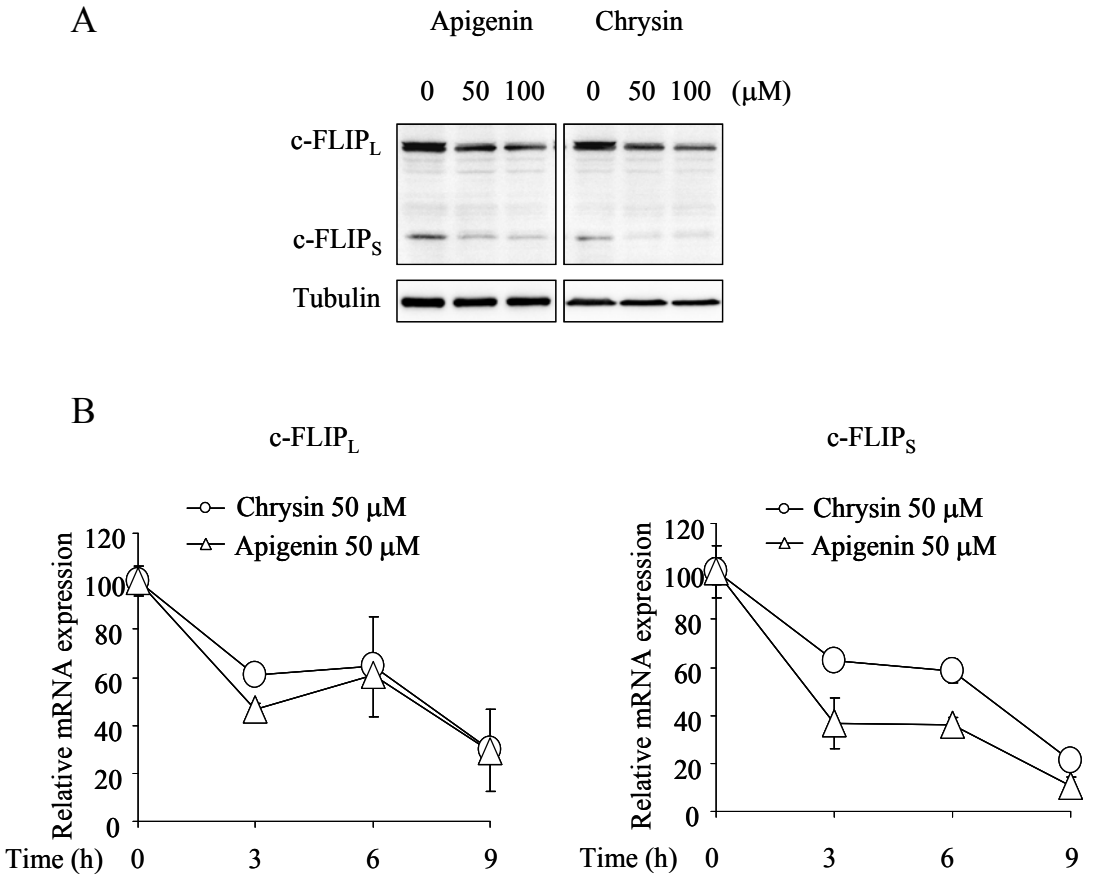

Figure 3.22 Apigenin and Chrysin suppress c-FLIP expression at both mRNA and protein level

(A) The HTLV-1-associated cell line MT-2 was treated with Apigenin or Chrysin (50 μM or 100 μM) for 8 h. c-FLIP protein expression was measured by Western blot. **(B)** MT-2 cells were treated with Apigenin or Chrysin (50 μM) for 3, 6 and 9 hours. The c-FLIP mRNA expression levels were measured by TaqMan real time PCR, using 18S rRNA as a control. Means ± SD are shown. The data are representative of two independent experiments.

3.4.3 Apigenin and Chrysin up-regulate TRAIL-R2 expression

As shown previously, Wogonin not only down-regulates c-FLIP expression, but also enhances TRAIL-R2 expression, which may be another mechanism by which Wogonin sensitizes TRAIL-induced apoptosis. To investigate whether Apigenin or Chrysin also influence TRAIL receptor protein expression, MT-2 cells were treated with 50 μM of Apigenin or Chrysin for 16 h and the expression level of TRAIL was analyzed by Western blot (Fig. 3.23). Similar to Wogonin, both Apigenin and Chrysin increased TRAIL-R2 protein expression but not TRAIL-R1 and TRAIL-R4 in MT-2 cells. However, in contrast to Wogonin, down-regulation of TRAIL-R3 by Apigenin (100 μM) and Chrysin (50 μM and 100 μM) was observed.

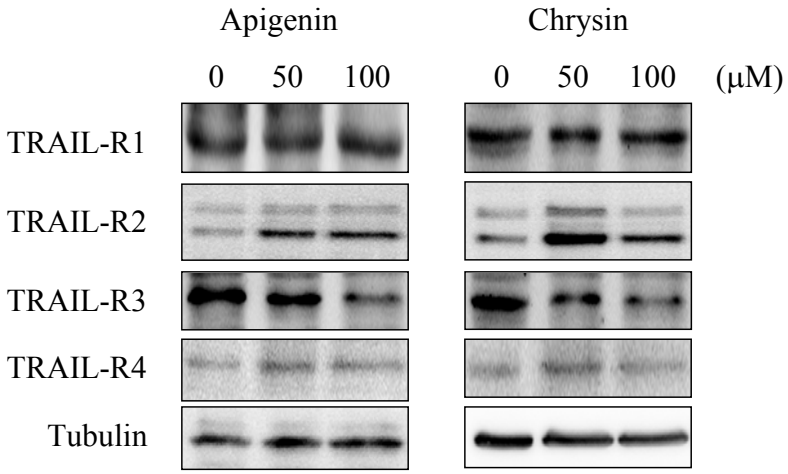

Figure 3.23 The effect of Apigenin and Chrysin on expression of TRAIL receptors
MT-2 cells were treated with Apigenin or Chrysin (50 μM or 100 μM) for 16 h. Protein expression of TRAIL death receptors (R1 and R2) and decoy receptors (R3 and R4) was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.4.3.1 The p53 protein level is elevated by Apigenin and Chrysin

As shown previously in Fig. 3.10 and 3.11, increased TRAIL-R2 expression by Wogonin is mediated by up-regulation of p53. Therefore, the effects of Apigenin and Chrysin on p53 protein expression levels were investigated. As seen in Fig. 3.24, treatment of MT-2 cells with Apigenin or Chrysin (50 μM or 100 μM) for 8 h led to significantly elevated p53 protein levels. This experiment demonstrates that the Wogonin analogs Apigenin and Chrysin also up-regulate TRAIL-R2 expression by enhancing the level of p53.

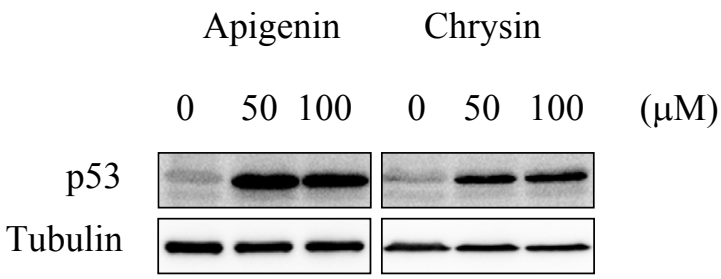

Figure 3.24 The effect of Apigenin and Chrysin on expression of p53
MT-2 cells were treated with Apigenin or Chrysin (50 μM or 100 μM) for 8 h. p53 protein level was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.4.3.2 MDM2 protein expression level is reduced by Apigenin and Chrysin

Since MDM2 is a negative regulator of p53 and its expression can be down-regulated by Wogonin, we hypothesized that Apigenin and Chrysin act like Wogonin to increase p53 protein level *via* inhibiting MDM2. Indeed, after treatment of the HTLV-1-associated ATL cell line MT-2 with Apigenin or Chrysin (50 μM or 100 μM) for 8 h, MDM2 expression was completely suppressed (Fig. 3.25).

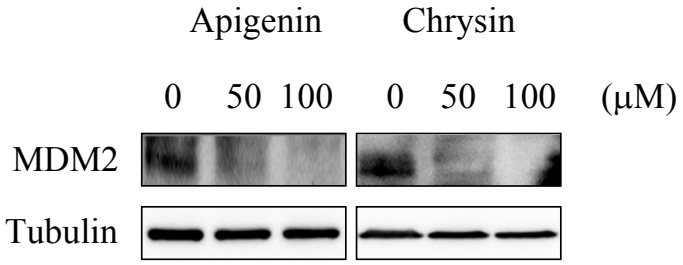

Figure 3.25 The effect of Apigenin and Chrysin on the expression of MDM2
MT-2 cells were treated with Apigenin or Chrysin (50 μM or 100 μM) for 8 h. MDM2 protein expression was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.4.4 Apigenin and Chrysin inhibit Mcl-1 expression in HTLV-1-associated ATL cells

Recently our group has found that Wogonin, Apigenin and Chrysin induce apoptosis in cancer cells by suppression of Mcl-1 expression (Polier *et al*., unpublished data). Therefore, the effect of Apigenin and Chrysin on Mcl-1 expression in HTLV-1-associated ATL cells was investigated. MT-2 cells were treated with Apigenin and Chrysin for 8 h and the expression levels of the major anti- and pro-apoptotic proteins involved in the mitochondrial pathway were analyzed by Western blot. Consistent with our findings for Wogonin, only Mcl-1 but not other proteins were down-regulated by Apigenin and Chrysin. Even 50 μM of each compound remarkably inhibited Mcl-1 protein expression (Fig. 3.26).

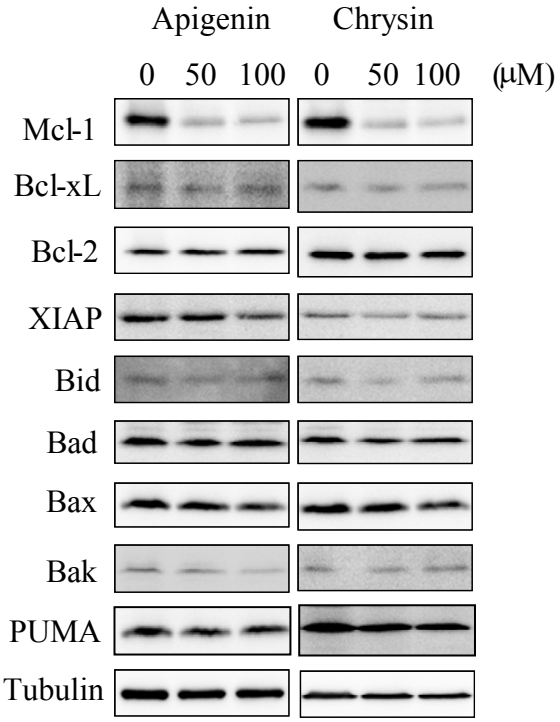

Figure 3.26 Apigenin and Chrysin inhibit Mcl-1 expression
MT-2 cells were treated with Apigenin or Chrysin (50 μM or 100 μM) for 8 h. Protein expression of anti- and pro-apoptotic proteins of the mitochondrial pathway was analyzed by Western blot. Tubulin expression served as a control for equal protein loading. The results shown are representative of two independent experiments.

3.5 Wogonin, Apigenin and Chrysin do not significantly suppress Tax protein expression

Several anti-apoptotic proteins are up-regulated by Tax-mediated NF-κB activation, such as Bcl-xL (Mori *et al*., 2001; Tsukahara *et al*., 1999), IAP family proteins (Kawakami *et al*., 1999; Waldele *et al*., 2006) and c-FLIP (Micheau *et al*., 2001; Kreuz *et al*., 2001). In addition, Tax is able to prevent Bax relocation to mitochondria (Trevisan *et al*., 2006). Therefore, we asked whether Wogonin, Apigenin and Chrysin can suppress Tax protein expression leading to down-regulation of anti-apoptotic protein expression and sensitization of TRAIL-induced apoptosis in HTLV-1-associated ATL cells. Thus, the effects of Wogonin, Apigenin and Chrysin on Tax expression were analyzed. As seen in Fig. 3.27A, SP, MT-2 and MT-4 cells were treated with Wogonin for 8 h. The protein expression of Tax was not influenced in these three cell lines except that the Tax protein level was marginally reduced in SP cells with 100 μM of Wogonin treatment for 8 h. Kinetic analysis of Tax protein expression in SP cells showed that Tax protein expression was slightly decreased only after 24 h treatment with Wogonin (50 μM) (Fig. 3.27B). Moreover, Tax expression was analyzed after Apigenin and Chrysin treatment for 10 h in MT-2 cells. As shown in Fig. 3.27C, Apigenin and Chrysin did not influence Tax protein expression in MT-2 cells. These results demonstrate that Wogonin, Apigenin and Chrysin do not significantly suppress Tax protein expression in HTLV-1-associated ATL cells.

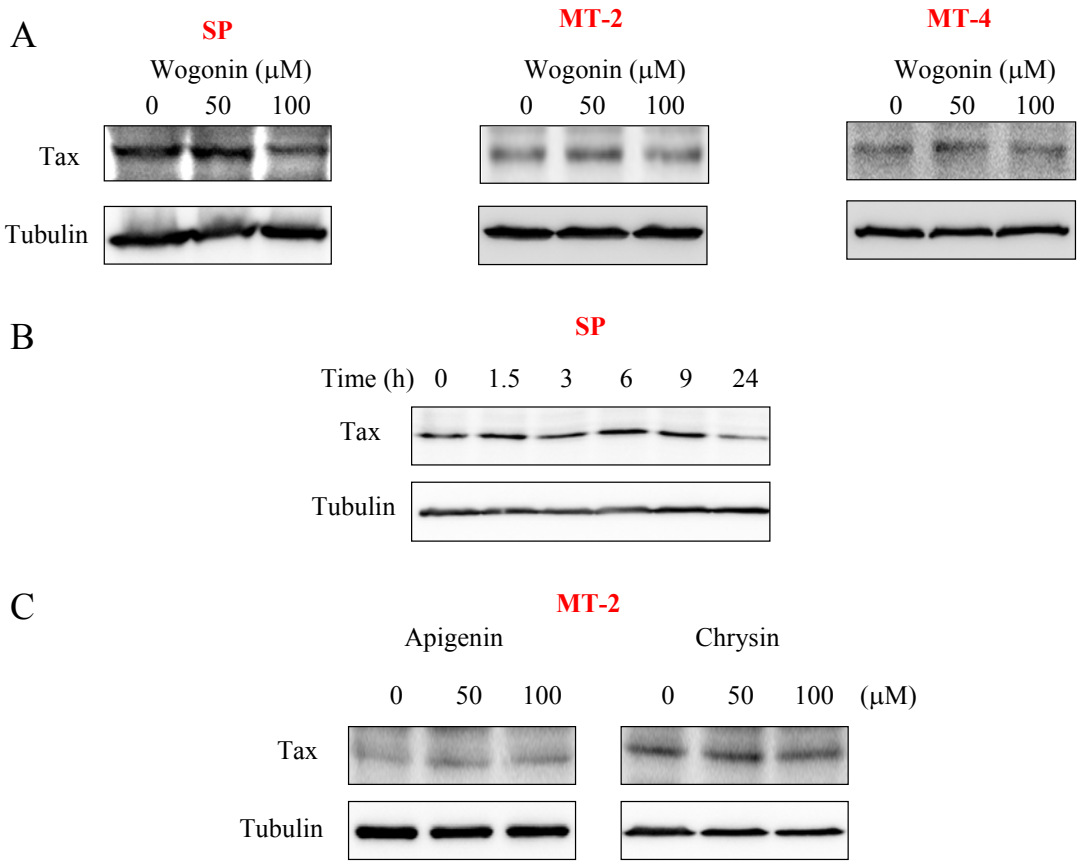

Figure 3.27 Wogonin, Apigenin and Chrysin do not significantly suppress Tax protein expression

(A) SP, MT-2 and MT-4 cells were treated with Wogonin (50 μM or 100 μM) for 8 h, **(B)** SP cells were treated with Wogonin (50 μM) for different time periods (1.5, 3, 6, 9 and 24 h), or **(C)** MT-2 cells were treated with Apigenin or Chrysin (50 μM or 100 μM) for 10 h. Cells were lysed and Tax protein levels were analyzed by Western blot with antibodies against Tax. Tubulin expression served as a control for equal protein loading. Data shown are representative of two independent experiments.

4. DISCUSSION

4.1 Sensitization of HTLV-1-associated ATL cells to TRAIL-induced apoptosis by Wogonin

TRAIL, considered to be a promising anti-cancer agent, is selectively cytotoxic to tumor cells but not to non-malignant cells (Walczak *et al.*, 1999; Bruix *et al*., 2004; Abdulghani and El-Deiry; 2010). However, various tumors show resistance to TRAIL-induced apoptosis (LeBlanc *et al*., 2003; Pan *et al*., 2007), *e.g.*, prostate cancer (Kasman *et al*., 2006), colon cancer (Ishii *et al*., 2007) and HTLV-1-associated ATL (Matsuda *et al*., 2005; Bleumink *et al*., 2010). Here it has been demonstrated that HTLV-1-associated ATL cells are resistant towards TRAIL-induced apoptosis (Fig. 3.1).

Wogonin, an active compound extracted from Huang-Qin (*Scutellaria baicalensis Georgi*), has been reported to have anti-oxidant, anti-viral, anti-inflammatory and anti-thrombotic activities (Chang *et al*., 2001; Li-Weber, 2009; Li-Weber, 2010). In addition, Wogonin induces apoptosis in various human tumor cells *in vitro* and inhibits tumor growth *in vivo* in several tumor-bearing mouse models (Li-Weber, 2010). Our group has reported that Wogonin attenuates NF-κB activity by shifting TNFα-induced $\cdot O_2^-$ radicals to a more reduced nonradical product, H_2O_2, and thereby sensitizes TNFα-resistant leukemia cells to TNFα-induced apoptosis. In addition, Wogonin overcomes TRAIL resistance in primary AML (acute myeloid leukemia) cells (Fas *et al*., 2006). However, the molecular mechanisms need to be further investigated.

In this study, we showed that Wogonin restored TRAIL sensitivity in HTLV-1-associated ATL cells and the effect of Wogonin in combination of TRAIL was rather synergistic than additive (Fig. 3.2). Importantly, Wogonin does not affect the viability of peripheral blood T cells derived from healthy donors. Consistent with

our previous finding (Fas *et al*., 2006), Wogonin does not sensitize TRAIL-induced apoptosis in non-malignant T cells (Fig. 3.4). Moreover, it has been reported that Wogonin offers a wide margin of safety and had no organ toxicity following long term intravenous administration in dogs (Peng *et al.*, 2009). Thus, Wogonin is a promising agent to overcome TRAIL resistance.

4.2 Mechanisms of Wogonin-mediated sensitization of TRAIL-induced apoptosis

4.2.1 Wogonin suppresses expression of c-FLIP

Elevated c-FLIP expression can confer resistance to TRAIL-induced apoptosis in various tumor cells (Roth and Reed, 2004; Shirley and Micheau, 2010). Previously, our group has shown that high expression of c-FLIP protein in HTLV-1-associated ATL cells suppresses CD95-mediated apoptosis (Krueger *et al*., 2006). c-FLIP knockdown in cells which express abnormal high c-FLIP levels restores sensitivity to CD95 or TRAIL-mediated apoptosis (Krueger *et al*., 2006; Okamoto *et al*., 2006; Bleumink *et al*., 2010). In this study, c-FLIP protein expression is inhibited by Wogonin (Fig. 3.5), suggesting that the suppression of c-FLIP expression is one possible mechanism by which Wogonin recovers TRAIL sensitivity in HTLV-1-associated ATL cells.

c-FLIP mRNA expression in ATL cells is elevated compared to Jurkat 16 cells (Fig. 3.6) and can be rapidly reduced by Wogonin treatment (Fig. 3.7). This reveals that Wogonin is modulating c-FLIP expression on the level of mRNA. Recently, our group has demonstrated that Wogonin is a potent inhibitor of CDK9 which plays a major role in regulation of transcription. The expression of proteins is suppressed at the transcriptional level by Wogonin treatment (Polier *et al*., unpublished data). It has been reported that the mRNA of c-FLIP isoforms have a short half-life of about 2

hours (Fulda *et al*., 2000; Hernandez *et al*., 2001). This is possibly the mechanism by which Wogonin inhibits c-FLIP mRNA expression at the early time periods.

c-FLIP is reported to be a transcriptional target of NF-κB (Shirley and Micheau; 2010). The Tax protein plays a role in NF-κB activation by constitutively activating IKK through physical interaction with it (Sun and Yamaoka, 2005; Mori *et al*., 2001; Tsukahara *et al*., 1999). Therefore, the effects of Wogonin on Tax expression were analyzed to investigate whether Wogonin inhibits c-FLIP expression by suppression of Tax. However, we found that Wogonin does not inhibit Tax expression (Fig. 3.27). Thus, down-regulation of c-FLIP expression by Wogonin is not through inhibition of Tax.

Our group has shown previously that Wogonin attenuates NF-κB activity by shifting TNFα-induced free radical $\cdot O_2^-$ to a more reduced nonradical product, H_2O_2, and thereby sensitizes TNFα-resistant leukemic cells to TNFα-induced apoptosis (Fas *et al*., 2006). Several other publications also reported that Wogonin suppresses NF-κB activity by inhibiting the NF-κB nuclear translocation and IκB phosphorylation (Zhao *et al.*, 2010; Piao *et al.*, 2008; Li-Weber, 2010). Therefore, inhibition of NF-κB activity by Wogonin may contribute to the down-regulation of c-FLIP expression and the recovery of TRAIL sensitivity.

c-FLIP is widely accepted as an anti-apoptotic protein by preventing further recruitment and processing of pro-caspase-8 at the DISC (Shirley and Micheau; 2010). However, it was also reported that c-$FLIP_L$ has pro-apoptotic effects (Lamkanfi *et al*., 2007; Yu and Shi, 2008) and it can function as a pro-apoptotic molecule at low expression, promoting the activation of pro-caspase-8 at the DISC (Chang *et al*., 2002). c-$FLIP_L$ expression was decreased after 3 h of Wogonin treatment and dramatically inhibited after 24 h in ATL cells (Fig. 3.5B). These results suggest that Wogonin may down-regulate c-$FLIP_L$ expression to a low level as a pro-apoptotic protein to sensitize towards TRAIL-induced apoptosis. More recently, our laboratory has defined the exact quantities of c-$FLIP_L$ which lead to pro-apoptotic effects.

c-FLIP$_L$ has a pro-apoptotic role only at moderate expression of c-FLIP$_L$ with strong receptor stimulation or with high amounts of c-FLIP$_S$ or c-FLIP$_R$ (Fricker *et al*., 2010). As seen in Fig.3.16, c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$ are all aberrantly elevated in ATL cells compared to Jurkat 16 cells. According to the finding of Fricker and his colleagues, high c-FLIP isoform expression levels in ATL cells do not fit the condition for c-FLIP$_L$ acting as a pro-apoptotic protein. In this case, c-FLIP$_L$ functions as an anti-apoptotic protein and down-regulation of it by Wogonin contributes to TRAIL sensitization.

Taken together, down-regulation of c-FLIP expression by Wogonin is one of the mechanisms to overcome TRAIL resistance in HTLV-1-associated ATL cells. The inhibition of c-FLIP expression at the transcriptional level is possibly due to CDK9 suppression and/or NF-κB inactivation by Wogonin treatment.

4.2.2 Wogonin up-regulates TRAIL-R2 expression

Aberrant expression of TRAIL receptors can result in tumor cell resistance to TRAIL-induced apoptosis. Low or no expression of TRAIL death receptors, or increased expression of TRAIL decoy receptors, may temper TRAIL-induced cell death (Fisher *et al*., 2001; McDonald *et al*., 2001; Mérino *et al*. 2007). Up-regulation of TRAIL death receptor expression by several agents has been reported as a mechanism to overcome TRAIL resistance (Shankar *et al*., 2004; Debatin and Krammer, 2004). In this study, we found that Wogonin enhances TRAIL-R2 protein and surface expression without influencing the expression of the other TRAIL receptors (Fig. 3.8 and Fig. 3.9). Although surface expression of TRAIL death receptors in HTLV-1-associated ATL cells are comparable with those in control CEM and Jurkat cells (Bleumink *et al*., 2010), the increased expression of TRAIL death receptors may still be a mechanism of sensitizing tumor cells to TRAIL-induced

apoptosis.

Studying the mechanism by which Wogonin up-regulates TRAIL-R2 expression revealed that Wogonin suppressed TRAIL-R2 mRNA expression level at early time points (0-6 h) but increased its mRNA expression after 6 h of treatment (Fig. 3.10). Since TRAIL-R2 transcripts have a half-life of about 4 h (Kandasamy *et al*., 2008), this is consistent with our finding that Wogonin suppressed TRAIL-R2 mRNA expression after 0-6 h of treatment. Additionally, the observation that TRAIL mRNA expression was increased by Wogonin at later time points (9 to 24 h) could be explained by up-regulation of p53 (Fig. 3.10 and Fig. 3.11). Three p53 DNA-binding sites have been identified in the TRAIL-R2 genomic locus located upstream (-0.82 Kb) of the ATG site, within Intron 1 (+0.25 Kb downstream of the ATG) and within Intron 2 (+1.25 Kb downstream of the ATG). p53 transactivates the TRAIL-R2 gene through the intronic sequence-specific DNA-binding site (+ 0.25 Kb downstream of the ATG) (Takimoto *et al.*, 2000). Therefore, up-regulation of p53 by Wogonin could increase TRAIL-R2 expression in HTLV-1-associated ATL cell lines. Several agents, such as the natural compound triptolide and MDM2 inhibitor nutlin-3 have also been shown to sensitize AML or human melanoma cells to TRAIL-induced apoptosis *via* a p53-mediated increase of TRAIL-R2 (Carter *et al*., 2008; Tseng *et al.*, 2010).

The mechanism by which Wogonin increases p53 protein expression was further analyzed. p53 protein expression is strictly controlled by its negative regulator MDM2 which targets p53 for ubiquitination and subsequently degradation by the 26S proteasome (Manfredi, 2010). We showed that Wogonin rapidly suppressed MDM2 protein expression, which explains the increased p53 protein levels after Wogonin treatment (Fig. 3.11 and Fig. 3.12). In addition, Wogonin can suppress CDK9 activity and thus inhibits gene transcription (Polier *et al*., unpublished data). p53 mRNA half-life is about 4 to 6 h (Mazan-Mamczarz *et al.*, 2003; Zhao *et al.*, 2008) and MDM2 mRNA is about 20 to 40 min (Wang *et al.*, 2002; Itahana *et al.*, 2007). Therefore, this explains that MDM2 is down-regulated faster than p53 at the

transcriptional level upon Wogonin treatment (Fig. 3.13).

It has been reported that MDM2 can act as an oncogene through p53-independent activities which regulate proliferation, apoptosis and epithelial-to-mesenchymal transitions. As an example, overexpression of MDM2 caused mammary epithelial cells to undergo multiple rounds of S phase without cell division both in p53 wild type and p53 knockout mice, suggesting that MDM2 regulation of cell cycle progression is independent of p53 (Lundgren *et al*., 1997). Additionally, MDM2 has been shown to prevent apoptosis by physically binding to the mRNA that encodes XIAP and enhancing its expression (Gu *et al*., 2009). Most notably, MDM2 has been reported to modify E-cadherin for degradation *via* the 26S proteasome, which leads to an epithelial-to-mesenchymal transition, a vital step of metastasis in solid tumors of epithelial origin (Yang *et al*., 2006; Thiery *et al*., 2009). In this study, Wogonin suppresses MDM2 expression suggesting that even in p53 mutated tumors Wogonin may still play an anti-cancer role by reducing the expression of the oncogene MDM2.

Except for p53, CHOP and Sp1 have also been reported to be transcriptional regulators of TRAIL-R2 (Yoshida *et al*., 2001). Several articles have shown that certain drugs, such as roscovitine and beta-Ionone, sensitize cancer cells to TRAIL-mediated apoptosis through CHOP or Sp1-mediated up-regulation of TRAIL-R2 (Kim *et al*., 2004; Shiraishi *et al*., 2005; Yoshida *et al*., 2005; Kim *et al*., 2010). However, Wogonin influences neither CHOP nor Sp1 protein expression in SP and MT-4 cells (Fig. 3.15). Thus, in HTLV-1-associated ATL cells up-regulation of TRAIL-R2 by Wogonin is unlikely to be mediated through the transcription factors CHOP and Sp1.

Recently, one publication has reported that Wogonin enhances TRAIL-induced apoptosis through the up-regulation of p53 and Puma, mediated by DNA damage and ROS, in the human prostate cancer cell line LNCaP which is resistant to TRAIL (Lee *et al*., 2009). However, Puma elevation upon Wogonin treatment was not observed in HTLV-1-associated ATL cells and CEM cells (Fig. 3.18; Polier *et al.*, unpublished

data). In addition, our group found that Wogonin-mediated apoptosis can not be blocked by the ROS scavenger NAC in various cancer cell lines (Fas *et al*., 2006; data not shown). Moreover, Wogonin and flavones are compounds with anti-oxidant activities (Li-Weber, 2009; Li-Weber, 2010). Therefore, it is unlikely that ROS play a role in Wogonin-mediated sensitization of TRAIL-induced apoptosis in HTLV-1-associated ATL cells.

In this study, Wogonin up-regulates TRAIL-R2 *via* elevated p53 expression in HTLV-1-associated ATL cell lines. p53 is wild type in sequence in most of HTLV-1-associated ATL cell lines, including the ones used in this study. However, it has been reported that p53 function is inhibited by the Tax-mediated NF-κB activation in ATL cell lines (Pise-Masison *et al*., 2000; Jeong *et al*., 2004). Since Wogonin can inhibit NF-κB activity by shifting the free radical $\cdot O_2^-$ to a more reduced nonradical product, H_2O_2, or by inhibiting the NF-κB nuclear translocation and IκB phosphorylation (Fas *et al*., 2006; Zhao *et al.*, 2010; Piao *et al.*, 2008; Li-Weber, 2010), this suggested that suppression of NF-κB activity by Wogonin may contribute to the enhanced expression of p53 in HTLV-1-associated ATL cell cells. However, it has to be further investigated whether Wogonin can increase the activity of p53.

p53 status in ATL patients has been studied by several groups. They have shown that p53 is often mutated in patients with acute ATL rather than in patients with chronic ATL. In addition, it was suggested that p53 mutations may be involved in the transition from the chronic to the acute stage of ATL (Nagai *et al*., 1991; Sakashita *et al*., 1992; Yamato *et al*., 1993; Tabakin-Fix *et al*., 2006). These reports imply that for the best therapeutic effect, Wogonin treatment should be performed in ATL patients as early as possible at the chronic stage.

4.2.3 Effect of Wogonin on the expression of pro- and anti-apoptotic proteins

In this study, we demonstrate that Wogonin overcomes TRAIL resistance in HTLV-1-associated ATL cells *via* suppression of c-FLIP expression and enhancement of TRAIL-R2 expression. It has been reported that impaired expression of pro-apoptotic proteins, such as Bax (LeBlanc *et al*., 2002), or over-expression of anti-apoptotic proteins, *e.g.*, XIAP (Hersey *et al*., 2003), Mc-1 (Kim *et al.*, 2008) and Bcl-2 (Fulda *et al*., 2002) may also confer resistance to TRAIL in cancer cells. Therefore, expression of pro- and anti-apoptotic proteins in the intrinsic apoptosis pathways were compared in HTLV-1-associated ATL cells and control Jurkat 16 cells. This analysis showed that consistent with other studies (Krueger *et al*., 2006; Bleumink *et al.*, 2010) that the anti-apoptotic proteins, Bcl-xL, Bcl-2 and Mcl-1 are increased in HTLV-1-associated ATL cell lines. However, the pro-apoptotic proteins Bad and Bax are also elevated when compared to Jurkat 16 cells (Fig. 3.16). It has been reported that the fate of a cell is largely determined by the amounts of pro- and anti-apoptotic proteins of the Bcl-2 family (Fletcher and Huang, 2008). Since the expression of both anti- and pro-apoptotic proteins is elevated in HTLV-1-associated ATL cells, it is hard to conclude whether these proteins play a role in TRAIL resistance in ATL cells.

In addition, treatment of Wogonin in HTLV-1-associated ATL cells inhibits Mcl-1 expression (Fig. 3.18), which explains that Wogonin alone can induce about 10 % to 20 % of cell death (Fig. 3.2). We also found that Wogonin inhibits Mcl-1 expression at the transcriptional level (Fig. 3.19). Since Wogonin suppresses CDK9 activity and blocks gene transcription elongation (Polier *et al.*, unpublished data) and Mcl-1 has a short mRNA half life of about 2 h (Derouet *et al.*, 2006; Akgul, 2009), this explains that Wogonin inhibits Mcl-1 transcription at early time periods.

4.3 Apigenin and Chrysin overcome TRAIL resistance

Wogonin, Apigenin and Chrysin belong to the chemical class of flavones and share a common chemical structure, consisting of fused A and C rings, and a phenyl B ring attached to position 2 of the C ring (Fig. 1.5). Both Apigenin and Chrysin are reported to have anti-inflammatory, anti-viral, anti-oxidant and anti-cancer activities (Shukla *et al*., 2007; Li-Weber, 2010; Khoo *et al.*, 2010). Additionally, Apigenin has also been shown to inhibit tumor invasion and metastasis by suppressing the expression of HIF-1α and VEGF in human ovarian cancer cells (Fang *et al*., 2005) and by inhibiting intracellular adhesion molecule 1 (ICAM1) in human endothelial cells (Panés *et al*., 1996).

Our group has recently shown that these compounds inhibit CDK9 activity and thus suppress transcription elongation (Polier *et al*., unpublished data). In this study, Apigenin and Chrysin act similar to Wogonin to sensitize HTLV-1-associated ATL cells to TRAIL-induced apoptosis. Apigenin and Chrysin both down-regulate c-FLIP expression at the transcriptional level and increase TRAIL-R2 *via* up-regulation of p53 through inhibition of the p53 negative regulator MDM2 (Fig. 3.22 - Fig. 3.25). Additionally, suppression of TRAIL-R3 was observed in MT-2 cells after treatment with Apigenin and Chrysin (Fig. 3.23). It has been reported that decoy receptors may compete with death receptors for TRAIL binding (Pan *et al*., 1997; Degli-Esposti MA *et al*., 1997a; Degli-Esposti MA *et al*., 1997b; Mérino *et al*. 2007). Ectopic over-expression of decoy receptors in TRAIL-sensitive cells leads to TRAIL resistance (Mérino *et al*., 2006; Marsters *et al*., 1997). Similarly, TRAIL sensitivity could be restored in resistant cells by using anti-DcRs blocking antibodies or siRNA to decrease DcR expression (Sanlioglu *et al*., 2005; Sanlioglu *et al*., 2007). Therefore, inhibition of TRAIL-R3 expression might be another mechanism by which Apigenin and Chrysin overcome TRAIL resistance. It needs to be further investigated whether this is the case in HTLV-1-associated ATL cells.

4.4 Schematic representation of the mechanism by which Wogonin, Apigenin and Chrysin sensitize TRAIL-induced apoptosis

In summary, our group found previously that Wogonin, Apigenin and Chrysin inhibit CDK9 activity and suppress gene expression at the transcriptional level (Polier *et al.*, unpublished data). In this study we show that Wogonin, Apigenin and Chrysin can sensitize TRAIL-mediated apoptosis by down-regulating c-FLIP expression at the transcriptional level and by increasing TRAIL-R2 expression *via* up-regulation of p53 through inhibition of the p53 negative regulator MDM2 at the transcriptional level (Fig. 4.1). Thus, this study raises the possibility to develop Wogonin, Apigenin and Chrysin as TRAIL adjuvant for treatment of ATL and other types of tumors.

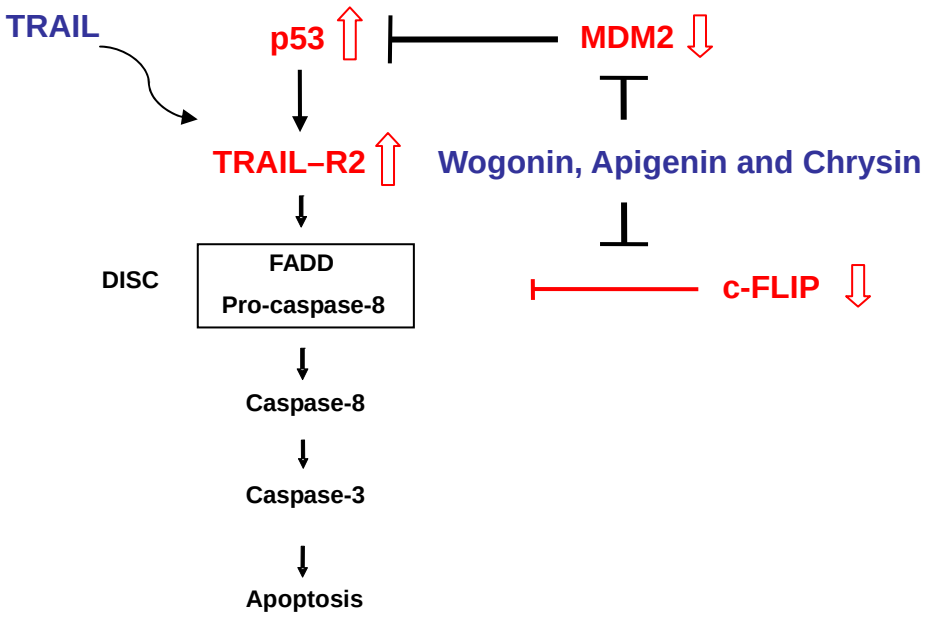

Figure 4.1 Schematic representation of the mechanism by which Wogonin, Apigenin and Chrysin sensitize TRAIL-induced apoptosis

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ABBREVIATIONS

Ab – antibody

AIF – apoptosis inducing factor

AIDS – acquired immune deficiency syndrome

AML – acute myeloid leukemia

AP-1 – activating protein 1

APS – ammonium persulphate

Apaf-1 – apoptosis activating factor-1

ATL – adult T-cell leukemia/lymphoma

Bcl-2 – B-cell lymphoma-2

BH3 – Bcl-2 homolgy 3 domain

BIR – baculoviral IAP repeat

bp – base pair

caspase – cysteine aspartate-specific protease

CD – (CD4 / CD95) - cluster of differentiation

CD95L – CD95 ligand

CDK – cyclin-dependent kinase

c-FLIP – cellular FLICE inhibitory protein

CTL – cytotoxic T cell

Cyt *c* – cytochrome *c*

DcR – decoy receptor

DD – death domain

DED – death-effector domain

DISC – death-inducing signaling complex

DMEM – Dulbecco'smodified Eagles Medium

DMSO – dimethyl sulphoxide

DR – death receptor

DTT – dithiothreitol

ECL – enhanced chemiluminescense

EGR1 – early growth response 1

EDTA – ethylene-diamine-tetra-actetate

ERK – extracellular signal-regulated kinase

FACS – fluorescence-activated cell sorter

FADD – Fas-associated death domain protein

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

FL – fluorescence

FLIP (FLIPS / FLIPL) – FADD-like interleukin-1β-converting enzyme-like protease [FLICE/caspase-8] - inhibitory protein (short form / long form)

GLUT1 – glucose transporter 1

GRP – guanyl nucleotide-releasing protein

HAM – HTLV associated myelopathy

HDAC – histone deacetylases

h – hour

HRP – horseradish peroxidase

HSPGs – heparin sulfate proteoglycans

HTLV – human T-cell leukemia/lymphoma virus

IAP – inhibitor of apoptosis

Ig – immunoglobulin

IκB – inhibitor of NF-κB

IKK – IκB kinase

kD – kilo Dalton

LTR – long terminal repeat

MAPK (MAP kinase) – mitogen-activated protein kinase

Mcl-1 – myeloid cell leukemia sequence 1

MDM2 – mouse double minute 2

mTOR – mammalian target of rpamycin

NFAT – nuclear factor of activated T cells

NF-κB – nuclear factor – κB

NK – natural killer

NRP1 – neuropilin 1

ORF – overlapping reading frame

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffer saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

PI – propidium iodide

PI3K – phosphoinositide 3 kinase

PMSF – phenyl-methanesulphonylfluoride

RIPA (lysis buffer) – radioimmunoprecipitation (lysis buffer)

ROS – reactive oxygen species

rpm – rotations per minute

RPMI medium – Roswell Park Memorial Institute medium

RRE – Rex response element

RT– reverse transcriptase

RT – room temperature

SDS – sodium dodecyl sulfate

Smac/DIABLO – second mitochondria-derived activator of caspase/directed IAP binding protein with low pI

TBE – Tris-Borate-EDTA

TEMED – N,N,N',N' – tetramethyl-ethylenediamine

TM – transmembrane domain

TNF – tumor necrosis factor

TRE – triplicate enhancer element

TSP – tropical spastic paraparesis

TRAF – tumor necrosis factor receptor-associated factor

TRAIL – TNF-α-related apoptosis-inducing ligand

TRAIL-R – TRAIL receptor

Tris – Tris-[hydroxymethyl]amino-methane

U – unit

UV – ultraviolet

XIAP – X-linked Inhibition of Apoptosis Protein

DECLARATION

Herewith, I declare that I wrote this thesis independently under supervision and no other sources and aids than those indicated in the manuscript were used.

Heidelberg, …… ……..

Jie Ding