EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 44, No. 11, 653-664, November 2012

Kurarinone promotes TRAIL-induced apoptosis by inhibiting NF-κB-dependent cFLIP expression in HeLa cells

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http://dx.doi.org/10.3858/emm.2012.44.11.074

Accepted 27 August 2012
Available Online 30 August 2012

Abbreviations: Act D, actinomycin D; cFLIP, cellular FLICE-inhibitory protein; DISC, death-inducing signal complex; FADD, Fas-associated death domain; IKK, IκB kinase; Kur, kurarinone; PARP, poly (ADP-ribose)polymerase; tBid, truncated Bid; TRAIL, TNF-related apoptosis inducing ligand; Wit A, withaferin A

Abstract

This study was designed to investigate the effects of the prenylated flavonoid kurarinone on TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis and its underlying mechanism. A low dose of kurarinone had no significant effect on apoptosis, but this compound markedly promoted tumor cell death through elevation of Bid cleavage, cytochrome c release and caspase activation in HeLa cells treated with TRAIL. Caspase inhibitors inhibited kurarinone-mediated cell death, which indicates that the cytotoxic effect of this compound is mediated by caspase-dependent apoptosis. The cytotoxic effect of kurarinone was not associated with expression levels of Bcl-2 and IAP family proteins, such as Bcl-2, Bcl-x_L, Bid, Bad, Bax, XIAP, cIAP-1 and cIAP-2. In addition, this compound did not regulate the death-inducing receptors DR4 and DR5. On the other hand, kurarinone significantly inhibited TRAIL-induced IKK activation, IκB degradation and nuclear translocation of NF-κB, as well as effectively suppressed cellular FLICE-inhibitory protein long form ($cFLIP_L$) expression. The synergistic effects of kurarinone on TRAIL-induced apoptosis were mimicked when kurarinone was replaced by the NF-κB inhibitor withaferin A or following siRNA-mediated knockdown of $cFLIP_L$. Moreover, cFLIP overexpression effectively antagonized kurarinone-mediated TRAIL sensitization. These data suggest that kurarinone sensitizes TRAIL-induced tumor cell apoptosis *via* suppression of NF-κB-dependent cFLIP expression, indicating that this compound can be used as an anti-tumor agent in combination with TRAIL.

Keywords: apoptosis; CASP8 and FADD-Like apoptosis regulating protein; kurarinone; NF-κB; TNF-related apoptosis-inducing ligand

Introduction

Soluble recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is thought to be therapeutic alternative against tumors (Plantivaux *et al*., 2009). TRAIL can induce tumor cell apoptosis with little apoptotic effect to normal cells (Cho *et al*., 2005). Despite this attractive selective tumoricidal activity of TRAIL, many tumor cells show resistance to TRAIL-induced apoptosis and the mechanism of TRAIL resistance is not fully elucidated (Plantivaux *et al*., 2009). Recent studies demonstrated that combination treatment of TRAIL and other drugs or chemicals can synergistically lead to an increase in cell death and apoptosis of various tumor cells by

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suppressing survival signals and gene expression as well as elevating apoptosis-associated gene expression (Neuzil *et al*., 2004; Seol *et al*., 2005; Griffith *et al*., 2011; Gupta *et al*., 2011; Jung *et al*., 2012).

TRAIL induces apoptosis through the trans-membrane type-I receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), but not decoy receptors, DcR1 and DcR2, which do not contain a cytoplasmic domain (DcR1) or contain a truncated death domain (DcR2) (Kang *et al*., 2004). Activated DR4 and/or DR5 by TRAIL recruits Fas-associated death domain (FADD), pro-caspase-8 and/or -10, comprising the death-inducing signal complex (DISC). Recruited pro-caspase-8 can be activated to caspase-8 by its own proteolytic activity. Activated caspase-8 cleaves Bid to truncated Bid (tBid), which sequentially induces mitochondrial cytochrome c release, activation of caspase-9/-3, cleavage of cellular repair proteins and activation of caspase-activated DNase (Cho *et al*., 2005), leading to the promotion of apoptosis. Caspase-8 can also directly promote the proteolytic activation of caspase-3. On the other hand, TRAIL can activate the transcription factor NF-κB, leading to transcription of genes known to antagonize the death signaling pathway.

NF-κB is involved in the regulation of gene expression of various inflammatory and survival genes. Of the NF-κB-mediated cytoprotective genes, the cellular FLICE-inhibitory protein (cFLIP) is a well-known anti-apoptotic gene (Rae *et al*., 2007; Romagnoli *et al*., 2007; Benayoun *et al*., 2008; Plantivaux *et al*., 2009). cFLIP resembles pro-caspase-8 but does not have full caspase activity similar to caspase-8. Between the two major isoforms of cFLIP, $cFLIP_L$ contains an additional caspase-like domain (Matsuda *et al*., 2008) which $cFLIP_S$ do not possess. Since the active-center cysteine, which is required for caspase activity, in the caspase-like domain of $cFLIP_L$ is altered by tyrosine (Matsuda *et al*., 2008), TRAIL-induced apoptosis can be inhibited by $cFLIP_L$ (Murtaza *et al*., 2009). However, TRAIL does not exclusively activate the apoptotic pathway, but also induces NF-κB-dependent up-regulation of anti-apoptotic genes, including cFLIP (Rae *et al*., 2007; Romagnoli *et al*., 2007; Plantivaux *et al*., 2009). Our group and others demonstrated that siRNA-mediated knockdown of cFLIP increases TRAIL-induced apoptosis (Kang *et al*., 2004, 2011). These results suggest that inhibition of the NF-κB pathway promotes TRAIL-induced apoptosis of tumor cells.

Kurarinone, a natural bioactive lavandulyl flavonoid, has several features of bioactivity, including cytotoxic activity in human myeloid leukemia HL-60 cells (Kang *et al*., 2000). This compound has been also shown to inhibit NF-κB-dependent inflammatory gene expression (Han *et al*., 2010) and block nuclear NF-κB translocation and cyclin D expression, leading to the suppression of tumor cell proliferation (Berghe *et al*., 2011). These evidences implicate kurarinone as an attractive inhibitor of the NF-κB signaling pathway, which is responsible for tumor cell proliferation and apoptosis. Thus, we hypothesized that kurarinone could synergistically improve TRAIL-induced tumor cell apoptosis *via* suppression of the NF-κB-mediated survival pathway.

We herein examined the synergistic effect between TRAIL and kurarinone on the apoptotic cell death of HeLa cells and investigated its underlying mechanism. We found that kurarinone increased caspase-8/-3 activation, cytochrome c release and apoptotic cell death in TRAIL-treated HeLa cells. Kurarinone suppressed TRAIL-mediated NF-κB activation and $cFLIP_L$ protein expression. These results suggest that kurarinone promotes TRAIL-induced apoptosis through inhibition of NF-κB nuclear translocation and subsequent down-regulation of cFLIP in HeLa cells.

Results

Kurarinone increases TRAIL-induced apoptotic cell death

TRAIL is an interesting protein for cancer therapy, because it has been shown to predominantly kill cancer cells, while having little effect on normal cells. In this study, we first examined whether the prenylated flavonoid kurarinone would increase TRAIL-induced apoptosis in HeLa cells. Microscopic analysis showed that TRAIL (75 ng/ml) alone induced cell death of about 15%, and TRAIL co-treated with 5 μM kurarinone further elevated cell death to 70%, while kurarinone alone did not demonstrate a significant cytotoxic effect (Figures 1A and 1B). Cell death occurs *via* two distinct mechanisms, caspase-dependent apoptosis and -independent necrosis. To differentiate between apoptosis and necrosis, we examined whether caspase inhibitors would regulate kurarinone-mediated synergistic cell death. Cell death was significantly inhibited following treatment with the caspase-8 inhibitor Ac-IETD-cho, the caspase-3 inhibitor Ac-DEVD-cho and was further strongly suppressed by the pan-caspase inhibitor z-VAD-fmk (Figure 1C). These results indicate that kurarinone increases TRAIL-induced caspase-dependent apoptotic cell death in HeLa cells.

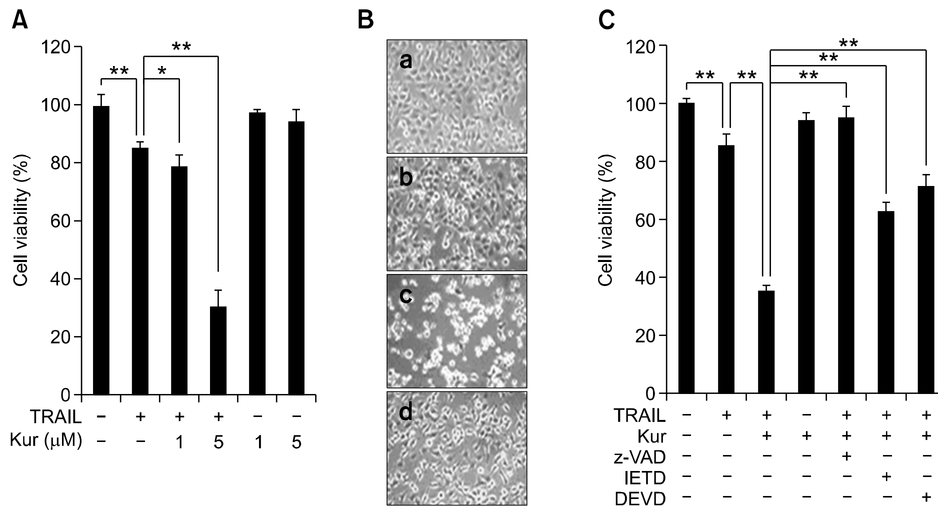

Figure 1. Kurarinone increases TRAIL-induced apoptosis in HeLa cells. HeLa cells were treated with TRAIL (75 ng/ml) for 6 h following pretreatment with the indicated concentrations of kurarinone (Kur) for 1 h. (A) The effect of kurarinone on TRAIL-induced cell death was assessed by the crystal violet staining method. (B) Morphological features of HeLa cells were analyzed with phase contrast microscopy. a, control; b, TRAIL (75 ng/ml); c, TRAIL (75 ng/ml) + kurarinone (5 μM); and d, kurarinone (5 μM). (C) Kurarinone (5 μM)-mediated increase in TRAIL-induced cell death was determined in the presence or absence of 100 μM of caspase inhibitors by crystal violet staining following co-treatment with caspase inhibitors. Data shown in (A) and (C) are the means ± S.D (n = 3). *P < 0.05 and **P < 0.01.

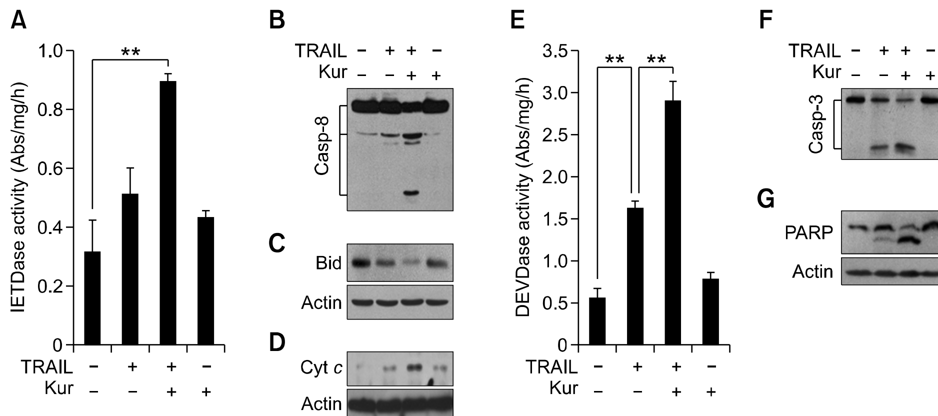

Figure 2. Kurarinone promotes TRAIL-induced apoptotic signal activation. HeLa cells were treated with TRAIL (75 ng/ml) alone or in combination with kurarinone (5 μM) for 4 h. (A) IETDase (caspase-8-like enzyme) and (E) DEVDase (caspase-3-like enzyme) activities were determined in the cell lysates by colorimetric assay. Western blot analyses were performed to determine caspase-8 activation (B), Bid cleavage (C), cytochrome c release (D), caspase-3 activation (F) and PARP cleavage (G) in the cell lysates and cytosolic fractions. Data shown in (A) and (E) are the means ± S.D (n = 3). **P < 0.01.

Kurarinone elevates TRAIL-induced caspase-8/-3 activation

TRAIL triggers apoptosis *via* the sequential death signal cascade, which results in the recruitment of FADD to the death domain of receptors and activation of the caspase family proteases (Cho *et al*., 2005). Therefore, we next investigated the regulatory effect of kurarinone on TRAIL-induced apoptotic signal events. HeLa cells treated with TRAIL slightly activated caspase-8-like protease (IETDase) and elevated its catalytic activity as compared with untreated control cells, and these were further increased by co-treatment with kurarinone (Figures 2A and 2B). Activated caspase-8 cleaves Bid to tBid and induces mitochondrial cytochrome c release *via* formation of mitochondrial membrane pore (Cho *et al*., 2005). We next examined whether kurarinone would regulate TRAIL-induced Bid cleavage and cytochrome c release. Treatment with TRAIL alone slightly reduced the level of Bid protein, which was further decreased by co-treatment with kurarinone (Figure 2C). Decreased Bid protein

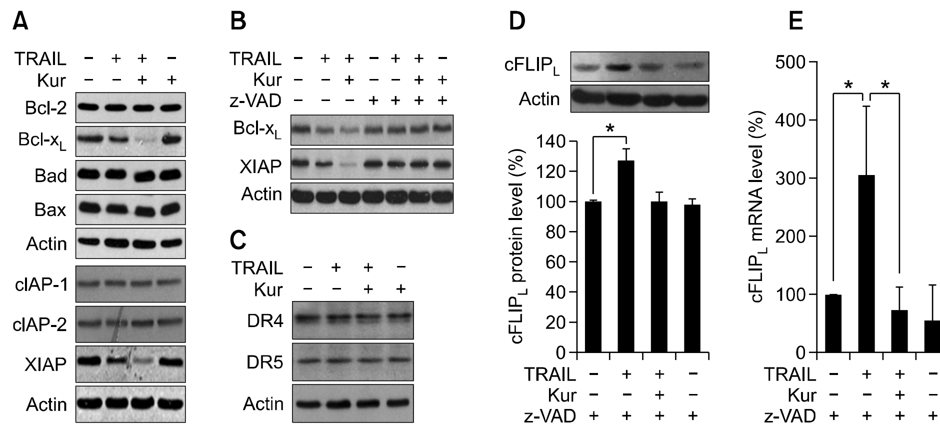

Figure 3. Effects of kurarinone on apoptosis-regulatory proteins. HeLa cells were treated with TRAIL (75 ng/ml) in the presence or absence of kurarinone (5 μM) and z-VAD-fmk (100 μM) for 5 h. (A-D) Cell lysates were separated on SDS-PAGE, and target proteins were determined by Western blot analyses. (E) The mRNA levels of $cFLIP_L$ were determined by quantitative RT-PCR. Data shown in (D) and (E) are the means ± S.D (n = 3). *$P < 0.05$.

levels were completely reversed by the addition of the caspase-8 inhibitor Ac-IETD-cho (data not shown), indicating Bid was fragmented by caspase-8-dependent proteolytic cleavage. Furthermore, kurarinone significantly increased TRAIL-induced mitochondrial cytochrome c release and caspase-3 activation/activity (Figures 2D-2F). We further examined the effect of kurarinone on the cleavage of PARP, an endogenous substrate of caspase-3. Treatment with TRAIL induced PARP cleavage, and this was further increased by co-treatment with kurarinone (Figure 2F). These results suggest that kurarinone promotes TRAIL-induced extrinsic apoptotic signal cascades, such as Bid cleavage, mitochondrial cytochrome *c* release, caspase activation and PARP cleavage, resulting in a synergistic increase in TRAIL-induced tumor cell death.

Kurarinone abrogates TRAIL-induced cFLIP up-regulation

Levels of pro- and anti-apoptotic proteins, to include the Bcl-2 and IAP family proteins, are known to regulate apoptotic cell death. Thus, we examined whether kurarinone regulates the levels of these proteins in the presence or absence of the pan-caspase inhibitor z-VAD-fmk. Kurarinone alone or in combination with TRAIL did not alter the protein levels of Bcl-2, Bad and Bax (Figure 3A). However, treatment with TRAIL alone slightly decreased the $Bcl\text{-}x_L$ protein level, which was further reduced by co-treatment with kurarinone (Figure 3A). Although not shown, TRAIL alone or in combination with kurarinone did not induce Akt activation and Bad phosphorylation at Ser-112/136. We next examined whether kurarinone would regulate the protein levels of the IAP family. Kurarinone alone or in combination with TRAIL did not alter the protein levels of cIAP-1 and cIAP-2 in HeLa cells treated with or without TRAIL. However, TRAIL alone partially decreased the XIAP protein level, and this decrease was further reduced by co-treatment with kurarinone (Figure 3A). However, when z-VAD-fmk was pretreated, the protein levels of $Bcl\text{-}x_L$ and XIAP in HeLa cells were not altered by treatment with TRAIL alone or in combination with kurarinone (Figure 3B). These data suggest that the decreased $Bcl\text{-}x_L$ and XIAP protein levels are due to caspase-dependent cleavage, without changing their protein levels. Since TRAIL-induced apoptosis is regulated by the expression levels of death receptors (Kim *et al.*, 2006; Moon *et al.*, 2011), we next examined the effect of kurarinone on DR4 and DR5 expression. However, we did not find any regulatory effect of kurarinone on DR4 and DR5 expression (Figure 3C). Sensitivity of tumor cells to TRAIL-induced apoptosis is regulated by the expression levels of $cFLIP_L$, a competitive inhibitor of caspase-8 activation (Budd *et al.*, 2006), which can be upregulated by TRAIL-mediated NF-κB activation (Rae *et al.*, 2007; Romagnoli *et al.*, 2007; Plantivaux *et al.*, 2009). Therefore, we investigated whether kurarinone regulates the expression of $cFLIP_L$ in HeLa cells treated with TRAIL. Treatment with TRAIL increased $cFLIP_L$ protein level to about 130%, which was abrogated by co-treatment with kurarinone (Figure 3D). We next examined whether kurarinone regulates the mRNA levels of $cFLIP_L$ by quantitative real-time PCR analysis. Co-treatment with kurarinone inhibited TRAIL-induced increases in $cFLIP_L$ mRNA levels (Figure 3E). Moreover, co-treatment with the transcription inhibitor Act D (actinomycin D, 50 ng/ml) decreased TRAIL-induced increases in $cFLIP_L$ mRNA levels and synergistically increased apoptotic cell death (data not shown). These findings suggest that kurarinone suppresses TRAIL-induced increases in $cFLIP_L$ expression levels at the transcriptional level and sensitizes tumor cells to TRAIL-induced apoptosis.

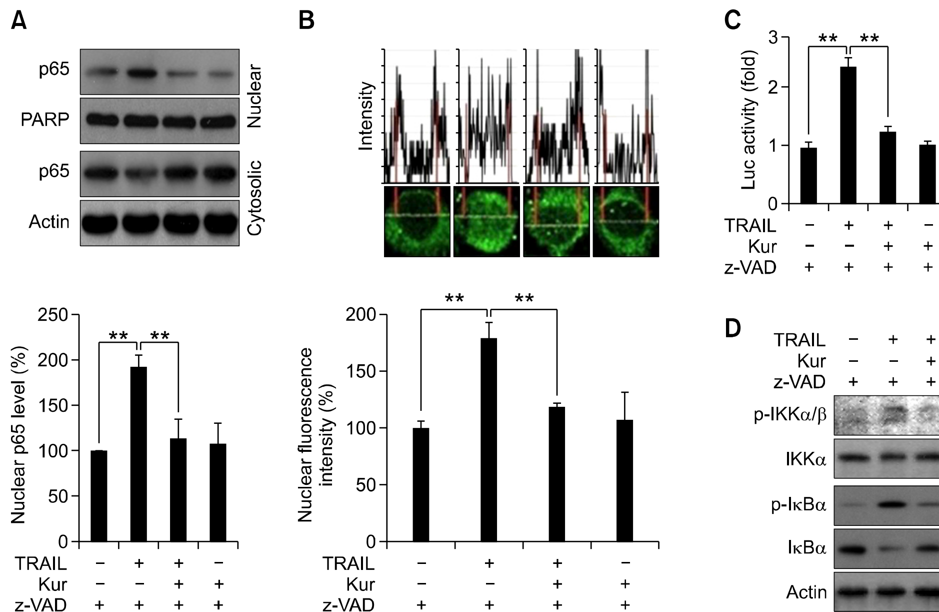

Figure 4. Kurarinone inhibits TRAIL-induced NF-κB activation. HeLa cells were stimulated with TRAIL for 1 h following pretreatment with kurarinone for 1 h in the presence of 100 μM z-VAD-fmk. (A) Nuclear translocation of the NF-κB p65 was determined in the cytosolic and nuclear fractions by Western blotting (upper gels), and protein levels were measured by densitometry (lower graph). (B) The nuclear levels of NF-κB p65 were determined by confocal microscopy (upper photograph), and its level was quantitated by densitometry (lower graph). (C) Cells were transfected with a NF-κB promoter-luciferase construct and pCMV-β-gal plasmid and then treated with TRAIL alone or in combination with kurarinone. Luciferase activity was determined in cell lysates by a luminometer. (D) IKKα/β phosphorylation and IκB phosphorylation/degradation were determined in lysates from HeLa cells treated with TRAIL alone or in combination with kurarinone for 1 h by Western blotting. Graphic data are the means ± S.D (n = 3). **$P < 0.01$.

Kurarinone inhibits TRAIL-induced NF-κB activation

TRAIL has been shown to activate both the apoptotic signal pathway and the survival pathway *via* NF-κB-mediated cFLIP expression (Rae *et al*., 2007; Romagnoli *et al*., 2007; Plantivaux *et al*., 2009). Based on these evidences, we hypothesized that kurarinone could inhibit the expression of $cFLIP_L$ through the inhibition of NF-κB activation. We investigated the effect of kurarinone on TRAIL-induced NF-κB activation in the presence of the pan-caspase inhibitor z-VAD-fmk for blocking proteolytic cleavages of cFLIP and NF-κB p65 by caspases (Ravi *et al*., 1998: Kavuri *et al*., 2011), which can be activated by treatment with TRAIL alone or in combination with kurarinone. Treatment of HeLa cells with TRAIL increased nuclear translocation of the NF-κB subunit p65, resulting in a decrease in cytosolic p65 levels, as determined by Western blotting. Importantly, these cellular events were effectively reversed by co-treatment with kurarinone (Figure 4A). We re-confirmed that kurarinone suppressed TRAIL-induced p65 nuclear translocation by immunohistochemical analysis using confocal microscopy (Figure 4B). We next examined whether kurarinone would regulate TRAIL-induced increase in NF-κB reporter activity. HeLa cells were transiently transfected with the expression vector containing the NF-κB-binding consensus sequence and stimulated with TRAIL in the presence or absence of kurarinone. TRAIL significantly increased NF-κB reporter activity, and this increase was inhibited by co-treatment with kurarinone (Figure 4C). Since the nuclear translocation of NF-κB is preceded by IKK-dependent phosphorylation and proteolytic degradation of IκBα (Traenckner *et al*., 1995; Butcher *et al*., 2001), we determined whether kurarinone regulates IKK activation and IκBα phosphorylation/degradation in HeLa cells treated with TRAIL. Treatment with TRAIL increased phosphorylation-dependent IKKα/β activation and IκBα phosphorylation/degradation, and these events were significantly reversed by co-treatment with kurarinone (Figure 4D). These data suggest that kurarinone inhibits TRAIL-induced NF-κB activation by suppressing IKK activation.

NF-κB inhibitor enhances apoptosis by abrogating TRAIL-induced cFLIPL up-regulation

Since withaferin A (Wit A), a steroidal lactone, inhibits NF-κB activation by preventing IKK activation (Kaileh *et al*., 2007), we investigated whether this inhibitor, similar to kurarinone, regulates NF-κB nuclear translocation, $cFLIP_L$ expression and apoptosis. As

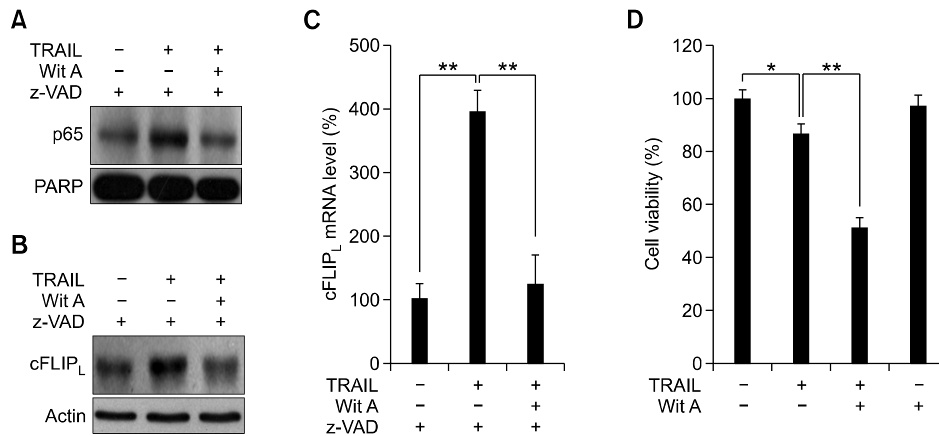

Figure 5. Wit A inhibits NF-κB-dependent cFLIP$_L$ expression and promotes TRAIL-induced apoptosis. HeLa cells were treated with TRAIL in the presence of 100 μM z-VAD-fmk after pretreatment with 10 μM Wit A. (A) After 1 h treatment, nuclear extracts were separated on SDS-PAGE, and nuclear NF-κB p65 levels were determined by Western blotting. After 5 h treatment, cFLIP$_L$ protein (B) and mRNA (C) levels were determined by Western blotting and quantitative RT-PCR, respectively. (D) Cells were treated with TRAIL for 6 h following pretreatment with 10 μM Wit A, and apoptotic cell death was determined by crystal violet staining. Graphic data shown are the means ± S.D (n = 3). *$P < 0.05$ and **$P < 0.01$.

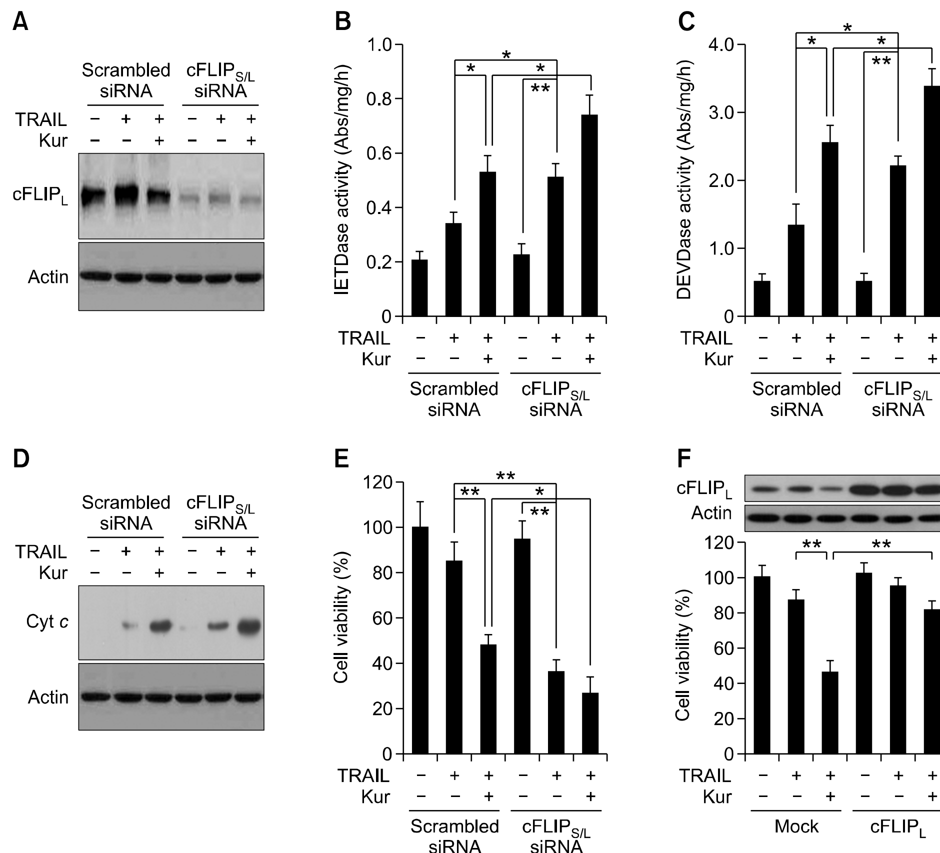

Figure 6. siRNA-mediated knockdown of cFLIP mimics the synergistic effect of kurarinone on TRAIL-induced apoptosis. (A) HeLa cells transfected with scrambled and cFLIP$_{S/L}$ siRNAs by the lipofectamine method. After 48 h, cFLIP$_L$ protein levels were determined by Western blotting. (B, C) Transfected cells were treated with TRAIL alone or in combination with kurarinone for 4 h. IETDase and DEVDase activities were determined in cell lysates by colorimetric assay. (D) Mitochondrial cytochrome c release was determined in cytosolic fractions *via* Western blotting. (E) Apoptotic cell death was measured in siRNA-transfected cells treated with TRAIL alone or in combination with kurarinone by crystal violet staining. (F) cFLIP$_L$ protein levels were determined in HeLa cells transfected with mock or cFLIP$_L$ vector by Western blotting (upper gels). Transfected cells were treated with TRAIL alone or in combination with kurarinone for 6 h, and cell viability was determined by crystal violet staining (lower graph). Graphic data shown are the means ± S.D. (n = 3). *$P < 0.05$ and **$P < 0.01$.

expected, Wit A suppressed TRAIL-induced nuclear translocation of NF-κB p65 (Figure 5A), as well as inhibited TRAIL-induced increases in cFLIP$_L$ protein and mRNA levels (Figures 5B and 5C). Treatment with Wit A synergistically promoted TRAIL-induced apoptosis to about 50%, compared with about 15% and 5% by single treatment with TRAIL and Wit A, respectively (Figure 5D). These results suggest that the NF-κB inhibitor Wit A synergistically increases TRAIL-induced apoptotic cell death by suppressing

expression of the anti-apoptotic protein $cFLIP_L$, similar to kurarinone.

cFLIP knockdown mimics the synergistic effect of kurarinone on TRAIL-induced apoptosis

We further investigated the functional involvement of cFLIP in kurarinone-mediated synergistic apoptosis in HeLa cells treated with TRAIL, using siRNA. Transfection of HeLa cells with $cFLIP_{S/L}$ siRNA revealed a significant knockdown of cFLIP protein expression, compared with scrambled siRNA-transfected cells (Figure 6A). Transfection with $cFLIP_{S/L}$ siRNA significantly increased caspase-8/-3 activation and cytochrome c release in HeLa cells treated with TRAIL, and these effects were significantly higher than those of scrambled siRNA-transfected cells co-treated with TRAIL and kurarinone (Figures 6B-6D). Furthermore, cFLIP knockdown significantly increased TRAIL-induced HeLa cell apoptosis to about 60%, which was slightly higher than that of scrambled siRNA-transfected cells co-treated with TRAIL and kurarinone (Figure 6E). Moreover, co-treatment with TRAIL and kurarinone significantly increased apoptotic cell death in siRNA-transfected cells, compared with scrambled siRNA-transfected cells (Figure 6E). These results indicate that cFLIP knockdown mimics the synergistic cytotoxic effect of kurarinone on TRAIL-induced apoptotic cell death. Therefore, down-regulation of cFLIP by kurarinone may be critically involved in its synergistic effect on TRAIL-induced apoptotic tumor cell death. In contrast, overexpression of $cFLIP_L$ effectively prevented the synergistic increase in apoptotic cell death in HeLa cells co-treated with TRAIL and kurarinone (Figure 6F), as well as inhibited caspase-8/-3 activities under the same experimental condition (data not shown). Taken together these data suggest that cFLIP is an important modulator for kurarinone-mediated sensitization of TRAIL-induced apoptotic cell death.

Discussion

This study was undertaken to investigate the synergistic effect of kurarinone on TRAIL-induced apoptotic cell death of tumor cells and its underlying mechanism of action. Kurarinone increased TRAIL-induced HeLa cell apoptosis, which was accompanied by caspase-8/-3 activation, Bid cleavage and cytochrome c release. Importantly, kurarinone suppressed TRAIL-induced NF-κB activation and subsequent anti-apoptotic $cFLIP_L$ expression. Furthermore, pharmacological NF-κB inhibitor and $cFLIP_{s/L}$ knockdown enhanced apoptosis in TRAIL-treated HeLa cells, similar to kurarinone. Moreover, ectopic expression of $cFLIP_L$ antagonized kurarinone-mediated TRAIL sensitization. These results suggest that kurarinone modulates TRAIL-induced two distinct signaling pathways, the extrinsic apoptotic cascade and survival pathway *via* NF-κB-mediated cFLIP expression. These evidences indicate that kurarinone in combination with TRAIL is a potent apoptogenic compound and possesses the ability of achieving therapeutic strategy for human tumors.

TRAIL is an attractive protein that is capable of inducing apoptosis in various tumor cells, but elicits minimal cytotoxicity against normal cells. This discrepancy is due to differential expression of death-inducing receptors DR4 and DR5 in tumor cells and decoy receptors DcR1 and DcR2 in normal cells (Pan *et al*., 1997). This suggests that expression levels of DR4 and DR5 play a crucial role in TRAIL-induced apoptosis. Many studies demonstrate that chemotherapeutic agents sensitize tumor cells to TRAIL-induced cytotoxicity by increasing transcriptional expression of DR5 and subsequent activation of the extrinsic apoptotic signal pathway (Kim *et al*., 2006; Kang *et al*., 2011; Moon *et al*., 2011). However, we could not find any evidence that kurarinone regulates the expression levels of DR4 and DR5, indicating that kurarinone-mediated TRAIL sensitization is not directly linked to the regulation of DR4 and DR5 expression. Thus, we focused on exploring other apoptosis-regulated genes.

The expression levels of pro- and anti-apoptotic genes, such as Bcl-2 and IAP family proteins, can regulate apoptotic cell death. The Bcl-2 family is divided into anti- and pro-apoptotic proteins, which are usually involved in the regulation of mitochondrial function. Pro-apoptotic Bcl-2 proteins, such as Bid, Bax, Bak, Bad and Bim, increase mitochondrial cytochrome c release, which triggers the formation of the apoptosome complex by associating with Apaf-1, procaspase-9 and dATP/ATP. Subsequent activation of caspase-9 leads to propagation of down-stream apoptotic cascade. However, anti-apoptotic Bcl-2 proteins, such as Bcl-2, Bcl-x_L, Bcl-w and Mcl-1, prevent cytochrome c release and subsequent apoptosis (Taylor *et al*., 2008). In other words, these two protein types function in opposition, with the overall reaction depending on which protein is activated or up-regulated by an external stimulus (Martinou and Youle, 2011). Tumor cells overexpressing Bcl-2 or Bcl-x_L are resistant to TRAIL-induced mitochondrial permeability, caspase activation and apoptosis (Walczak *et al*., 2000), suggesting that chemicals that regulate Bcl-2 family proteins can be used as chemotherapeutic drugs in combination with TRAIL. In fact, the histone deacetylase inhibitor, suberohydroxamic acid, increases TRAIL-induced

apoptosis by down-regulating Bcl-x_L and subsequently increasing caspase-3 activation (Neuzil *et al*., 2004). Conversely, the natural compound nimbolide up-regulates Bax and sensitizes colon cancer cells to TRAIL-induced apoptosis (Gupta *et al*., 2011). These results indicate that compounds that induce either down-regulation of anti-apoptotic Bcl-2 family proteins or up-regulation of pro-apoptotic Bcl-2 family proteins sensitize tumor cells to TRAIL-induced apoptosis. We herein found that kurarinone does not regulate the protein levels of Bcl-2 family proteins. Although the protein levels of Bcl-x_L and Bid were significantly decreased in HeLa cells co-treated with TRAIL and kurarinone, these protein levels were not altered when the pan-caspase inhibitor was added. These data indicate that the decreased levels of these proteins are due to proteolytic cleavage by caspases and not due to alterations in total protein levels (Clem *et al*., 1998). These results suggest that kurarinone-mediated sensitization of tumor cells to TRAIL-induced apoptosis is not associated with the regulation of Bcl-2 family protein expression.

Proteins of the inhibitor of apoptosis (IAP) family, such as cIAP-1, cIAP-2 and XIAP, bind to caspases and inhibit their activity (Riedl and Shi, 2004). XIAP directly binds to caspase-9, -7 and -3 and then prevents dimerization and interferes with substrate binding of the corresponding caspase, thereby preventing apoptosis (Fulda and Vucic, 2012). On the other hand, cIAP proteins inhibit the assembly of pro-apoptotic protein signaling complexes and mediate the expression of anti-apoptotic molecules. Therefore, down-regulation of IAP proteins can sensitize tumor cells to TRAIL-induced apoptosis. In fact, it has been demonstrated that combined siRNA-mediated knockdown of cIAP-1, cIAP-2 and XIAP sensitizes PC-3 cells to TRAIL-induced apoptosis (Gill *et al*., 2009). In addition, the combination of a small molecule IAP mimetic Compound A and TRAIL results in highly synergistic apoptosis induction in TRAIL-resistant bladder tumor cells (Griffith *et al*., 2011). The phosphatidylinositide-3-kinase inhibitor Wortmannin and the GlcNAc phosphotransferase inhibitor tunicamycin have been shown to enhance TRAIL-induced tumor cell apoptosis by down-regulating cIAP-2 expression (Seol *et al*., 2005; Jung *et al*., 2012). These evidences indicate that IAP family protein levels play an important role in the resistance of tumor cells to TRAIL-induced apoptosis. In this study, we could not find any evidence that kurarinone regulates the expressional protein levels of cIAP-1, cIAP-2 and XIAP in HeLa cells. These findings indicate that kurarinone-mediated sensitization of HeLa cells to TRAIL-induced apoptosis is not linked to the regulation of IAP family protein expression.

It has been shown that TRAIL induces two different signals, cell death mediated by caspases and gene induction mediated by NF-κB (Falschlehner *et al*., 2007). Thus, TRAIL-induced tumor cell apoptosis can be significantly augmented by inhibitors of the NF-κB activation pathway and the transcriptional inhibitor actinomycin D, leading to attenuation of apoptosis resistance (Jeremias and Debatin, 1998; Wang *et al*., 2007). These evidences suggest that TRAIL-induced apoptosis can be inhibited by the transcriptional activation of cytoprotective or anti-apoptotic genes. Among many anti-apoptotic genes, cFLIP can be up-regulated at the transcriptional level by NF-κB activation (Rae *et al*., 2007; Romagnoli *et al*., 2007). Our group and others have demonstrated that down-regulation of siRNA-mediated knockdown of cFLIP expression increased tumor cell susceptibility to TRAIL-induced apoptosis (Kang *et al*., 2004, 2011). Furthermore, many studies have demonstrated that chemotherapeutic agents sensitize tumor cells to TRAIL- induced cytotoxicity by promoting extrinsic apoptosis pathway *via* down-regulation of cFLIP expression (Lee *et al*., 2009; Kang *et al*., 2011). We here found that kurarinone inhibited TRAIL-induced NF-κB activation and $cFLIP_L$ expression, leading to elevations in TRAIL-induced caspase-8 activation, Bid cleavage, cytochrome c release, caspase-3 activation and apoptosis. Moreover, the NF-κB inhibitor Wit A and siRNA-mediated knockdown of $cFLIP_L$ increased TRAIL-induced cytotoxicity, similar to kurarinone. In contrast, $cFLIP_L$ overexpression antagonized kurarinone-mediated TRAIL sensitization. These results indicate that kurarinone suppresses TRAIL-induced NF-κB activation and cFLIP up-regulation, as well as synergistically increases TRAIL-induced tumor cell apoptosis. Although other NF-κB-dependent anti-apoptotic proteins can be regulated by kurarinone, our results indicate that down-regulation of cFLIP may be regarded as the predominant event of kurarinone-mediated sensitization of HeLa cells to TRAIL.

There have been numerous efforts to improve TRAIL-induced apoptosis through inhibition of NF-κB activation. In fact, several studies have demonstrated that NF-κB inhibitors, such as quinacrine and bortezomib, synergistically increase tumor cell apoptosis *via* inhibition of the NF-κB-mediated survival pathway by combination treatment with TRAIL (Jani *et al*., 2010; Jane *et al*., 2011). Kurarinone has been shown to inhibit NF-κB activation in the macrophage cell line RAW264.7 by lipopolysaccharide (Han *et al*., 2010) and also leads to apoptosis (Han *et al*., 2007). In addition, this compound has been shown to prevent cancer cell

proliferation (Berghe *et al*., 2011). However, the synergistic cytotoxic effect of kurarinone on TRAIL-induced tumor cell apoptosis and its underlying mechanism have not been clearly elucidated. We found that this compound effectively inhibited IKK activation, IκB phosphorylation/degradation and NF-κB nuclear translocation in HeLa cells. Although we did not directly determine how kurarinone inhibits IKK activation, the putative underlying mechanism is likely to involve attenuation of the ERK/RSK signal transduction pathway and scavenging of reactive oxygen species responsible for IKK activation (Han *et al*., 2010; Berghe *et al*., 2011). These events could result in the suppression of NF-κB-dependent transcriptional $cFLIP_L$ expression. Down-regulation of $cFLIP_L$ by kurarinone may effectively promote the recruitment of procaspase-8 to DISC, leading to increases in caspase-8 activation and down-stream signal propagation. Thus, these data suggest that kurarinone sensitizes TRAIL-induced tumor cell apoptosis by suppressing NF-κB-mediated $cFLIP_L$ up-regulation.

In conclusion, the present study demonstrates that kurarinone is capable of modulating NF-κB-dependent survival pathway, specifically $cFLIP_L$ expression. This pathway plays an important role in death receptor-mediated extrinsic apoptotic signaling pathway, thereby sensitizing tumor cells to TRAIL-induced apoptosis. Thus, kurarinone in combination with TRAIL could be used as a potent therapeutic strategy for promoting tumor cell death.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), FBS, penicillin, streptomycin and L-glutamate were purchased from Life Technologies (Gaithersburg, MD). Caspase-8, FADD and Bid antibodies were obtained from PharMingen (San Diego, CA). Polyclonal caspase-3, $cFLIP_L$, Bcl-2, Bad, p65 and cytochrome c antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal $Bcl\text{-}x_L$ and Bax antibodies were purchased from Millipore. Antibody for poly (ADP-ribose) polymerase (PARP) was obtained from Transduction Laboratories (Lexington, KY). Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) and Acetyl-Ile-Glu-Thr-Asp p-nitroaniline (Ac-IETD-pNA) were obtained from Alexis (San Diego, CA). Ac-IETD-cho (IETD, a caspase-8 inhibitor), Ac-DEVD-cho (DEVD, a caspase-3 inhibitor) and z-VAD-fmk (z-VAD, a pan-caspase inhibitor) were purchased from Alexis (San Diego, CA). Kurarinone was provided by Prof. Young Shik Kim (Natural Products Research Institute, Seoul National University), who purified this compound from the roots of *Sophora flavescens* as previously described (De Naeyer *et al*., 2004). Its purity was $>$ 95% as determined by HPLC analysis. Recombinant Human TRAIL was purchased from R&D systems. All other chemicals and reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

Cell culture and drug treatment

The human tumor cell lines, HeLa (cervical carcinoma) were purchased from American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 5 mM Hepes at 37°C in 95% air/5% CO_2. Cells were cultured in 6-well plates (1×10^5 cells/well) or 24-well plates (1×10^4 cells/well). Cells were treated with TRAIL (75 ng/ml) in the presence or absence of various chemicals. If not specifically mentioned, cells were pretreated with kurarinone (5 μM) for 1 h before treatment with 75 ng/ml TRAIL. The pretreatment time for actinomycin D (Act D, 50 ng/ml) or withaferin A (Wit A, 10 mM) before TRAIL treatment was 1 h.

Cell viability assay

Cell viability was determined by the crystal violet staining method. In brief, cells plated in 24-well dishes at a density of 1×10^5 cells/ml were cultured for 8 h in DMEM containing 10% FBS and treated with TRAIL (75 ng/ml) for 6 h following pretreatment with the indicated concentrations of kurarinone. Cells were stained with 0.5% crystal violet in 99% ethanol and 3% formaldehyde for 10 min at room temperature. Plates were then washed four times with tap water. After drying, cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using a 96-well plate reader. Cell viability was calculated from relative dye intensity compared with those of untreated samples.

Western blotting

Cells were treated with TRAIL (75 ng/ml) in the absence or presence of kurarinone for the indicated times and harvested. Cells were resuspended in RIPA buffer (25 mM, pH 7.4) and lysed by three cycles of freeze and thaw (Cho *et al*., 2005). Cell extracts were obtained by centrifugation at 12,000 *g* for 10 min at 4°C. Cells were homogenized using a Dounce homogenizer in ice-cold mitochondrial fractionation buffer (20 mM Hepes, 1 mM EDTA, 10 mM KCl, 1 mM DTT, 10 μM aprotinin, 10 mM PMSF and 250 mM sucrose), and cytosolic fractions were also obtained by centrifugation at 12,000 *g* for 20 min at 4°C. Nuclear extracts were prepared as described previously (Kim *et al*., 1997). Proteins (40 μg protein) were separated on an SDS-PAGE and subjected to Western blot analysis using the indicated antibodies. The levels of target proteins were measured by densitometry.

Determination of caspase activity

Cells resuspended in 100 mM Hepes buffer, pH 7.4, containing a protease inhibitor mixture (5 mg/ml aprotinin and pepstatin, 10 mg/ml leupeptin and 0.5 mM PMSF), were lysed by three freeze-thaw cycles, and the cytosolic fraction was obtained by centrifugation at 12,000 *g* for 20 min at 4°C. Caspase activity was determined by measuring

proteolytic cleavage of the chromogenic substrate Ac-DEVD-pNA (caspase-3-like activity) or Ac-IETD-pNA (caspase-8-like activity) at 405 nm every 20 min as described previously (Kim *et al*., 2000).

Quantitative real-time RT-PCR

Total RNA was obtained from HeLa cells using the Trizol reagent kit (Life Technology Inc.). Quantitative real-time PCR was performed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems) to analyze induction or suppression of $cFLIP_L$ expression, according to the manufacturer's instruction. The 25 μl reaction mixture consisted of 5.5 μl RNase/DNase-free water, 5.5 μl cDNA, 1 μl of primer (100 pmole/μl), 12.5 μl iTaq SYBR Green Supermix with ROX (Bio-Rad Lab). An ABI PRISM 7000 detector sequence was programmed for the initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative quantitation of gene expression was determined using comparative threshold multiplex PCR in the same tube after validating that the efficiencies of the target genes, and GAPDH PCR was approximately equal. All calculations followed procedures outlined in the ABI PRISM 7000 sequence detective system.

Transfection and promoter assay

A 1.5 ml tube was used to mix 1 μg of NF-κB promoter-luciferase construct and 3 μl of Fugene® HD Transfection Reagent in 100 μl of DMEM. The tube was incubated at room temperature for 30 min, and cells were washed once with DMEM during the incubation. Next, the prepared mixture was added to the plates of the cells, and the cells were cultured for 24 h. Cells were pretreated with 5 μM kurarinone and 100 μM z-VAD-fmk for 1 h and further treated with TRAIL for 6 h. Cells were lysed with Reporter lysis buffer (Promega, Madison, WI) or buffer containing 1% Triton X-100, 5 mM dithiothreitol, 50% glycerol, 10 mM EDTA, and 125 mM Tris-HCl (pH 7.8). Luciferase activity was measured by a luminometer.

Confocal microscopy

To monitor nuclear translocation of the NF-κB subunit p65, HeLa cells were grown on cover slips and incubated with TRAIL in presence or absence of kurarinone for 1 h in culture medium. The cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 30 min, washed with PBS and permeabilized with 0.2% Triton X-100 for 30 min. Cells were incubated with blocking solution in PBS for 1 h, incubated with primary antibody in PBS for 2 h, washed with PBS, incubated with secondary antibody (conjugated with FITC) in PBS for 1 h, washed with PBS and observed with a confocal microscope (FV 300; Olympus, Tokyo, Japan). Approximately 20 cells were randomly selected from three independent experiments, and fluorescence intensities were measured at a single cell level.

Knockdown of cFLIP expression and overexpression of cFLIP

Small interfering RNA (siRNA) against $cFLIP_{S/L}$ and scrambled RNA for scrambled control were purchased from Santacruz. For $cFLIP_{S/L}$ siRNA experiments, HeLa cells were plated at a concentration of 1×10^5 cells/well in 6-well plates and incubated overnight. The cells were transfected with 50 nM siRNA using Lipofectamine 2000 (Invitrogen). After 6 h of initial incubation, the medium was replaced with fresh medium and incubated for 24 h. For ectopic expression of cFLIP, HeLa cells were transfected with mock vector (pcDNA3) or vector containing cFLIP using lipofectamine. Clonal selection was conducted by culturing with G418 (800 mg/ml), followed by serial dilution of the cells in 96-well plates. Stable transfectant clones with high expression of the target genes were identified by Western blot analysis.

Data analysis and statistics

The data are presented as means ± standard deviation (S.D.) of at least three separate experiments. Comparisons between two groups were analyzed using Student's *t* test. P values less than 0.05 were considered statistically significant.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2011-0028790) (YMK) and the Kangwon National University (No.120080349) (JHK).

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