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Paxilline enhances TRAIL-mediated apoptosis of glioma cells *via* modulation of c-FLIP, survivin and DR5

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Abbreviations: FADD, fas-associated protein with death domain; TRAIL, tumor necrosis factor-related apoptosis-induced ligand

Abstract

Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) induces apoptosis selectively in cancer cells while sparing normal cells. However, many cancer cells are resistant to TRAIL-induced cell death. Here, we report that paxilline, an indole alkaloid from *Penicillium paxilli*, can sensitize various glioma cells to TRAIL-mediated apoptosis. While treatment with TRAIL alone caused partial processing of caspase-3 to its p20 intermediate in TRAIL-resistant glioma cell lines, co-treatment with TRAIL and subtoxic doses of paxilline caused complete processing of caspase-3 into its active subunits. Paxilline treatment markedly upregulated DR5, a receptor of TRAIL, through a CHOP/GADD153-mediated process. In addition, paxilline treatment markedly downregulated the protein levels of the short form of the cellular FLICE-inhibitory protein (c-FLIP$_S$) and the caspase inhibitor, survivin, through proteasome-mediated degradation. Taken together, these results show that paxilline effectively sensitizes glioma cells to TRAIL-mediated apoptosis by modulating multiple components of the death receptor-mediated apoptotic pathway. Interestingly, paxilline/TRAIL co-treatment did not induce apoptosis in normal astrocytes, nor did it affect the protein levels of CHOP, DR5 or survivin in these cells. Thus, combined treatment regimens involving paxilline and TRAIL may offer an attractive strategy for safely treating resistant gliomas.

Keywords: apoptosis; astrocytes; glioma; paxilline; TNF-related apoptosis-inducing ligand

Introduction

Tumor necrosis factor-related apoptosis-induced ligand (TRAIL), a member of the TNF family, induces apoptosis in diverse tumor cell types but not in normal cells, and is therefore considered a potential anticancer agent (Sheridan *et al*., 1997; Ashkenazi *et al*., 1999). When TRAIL cross-links with its death receptors, DR4 or DR5, the death receptors oligomerize and trigger the recruitment of Fas-Associated protein with Death Domain (FADD), the activation of caspase-8, and the subsequent initiation of apoptosis (Kischkel *et al*., 2000). However, recent studies have shown that many cancer cells, including glioma cells, are resistant to TRAIL-induced apoptosis even though they express TRAIL receptors (Hao *et al*., 2001; Kim *et al*., 2005). Thus, the development of new therapeutic strategies to restore TRAIL-induced apoptosis in cancer cells will be required to improve the efficacy of TRAIL-based cancer therapy.

Malignant gliomas, which are the most common primary brain tumors, are notoriously resistant to traditional therapeutic strategies, such as surgery, radiation therapy, and chemotherapy (Weller, 1986). Increasing evidence suggests that resistance to apoptosis is a fundamental mechanism through which gliomas evade elimination in the face of conventional or targeted treatments (Ziegler *et al*., 2008). Recent studies have shown that inhibition of ion channels or pumps has provided a potential new approach for biochemical therapy of the brain tumors (Yin *et al*., 2007; Lefranc and Kiss, 2008) and inhibition of ion channels or pumps can increase the sensitivity of cancer cells to TRAIL (He *et al*., 2002; Kaddour-Djebbar *et al*., 2006; Monteith *et al*., 2007). Paxilline, an indole alkaloid from *Penicillium paxilli*, inhibits the activity of large-conductance Ca^{2+}-activated K^+ channels (BK

channels) (Sanchez and McManus, 1996) and the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Bilmen *et al*., 2002). Here, we show for the first time that subtoxic doses of paxilline can sensitize TRAIL-resistant glioma cells to TRAIL-induced apoptosis, while sparing normal astrocytes. Furthermore, we provide evidence that CHOP-mediated DR5 upregulation, survivin downregulation and c-FLIP downregulation may all contribute to the glioma-selective killing effects of paxilline/TRAIL co-treatment.

Results

Paxilline recovers TRAIL sensitivity in various glioma cells by inducing caspase-mediated apoptosis

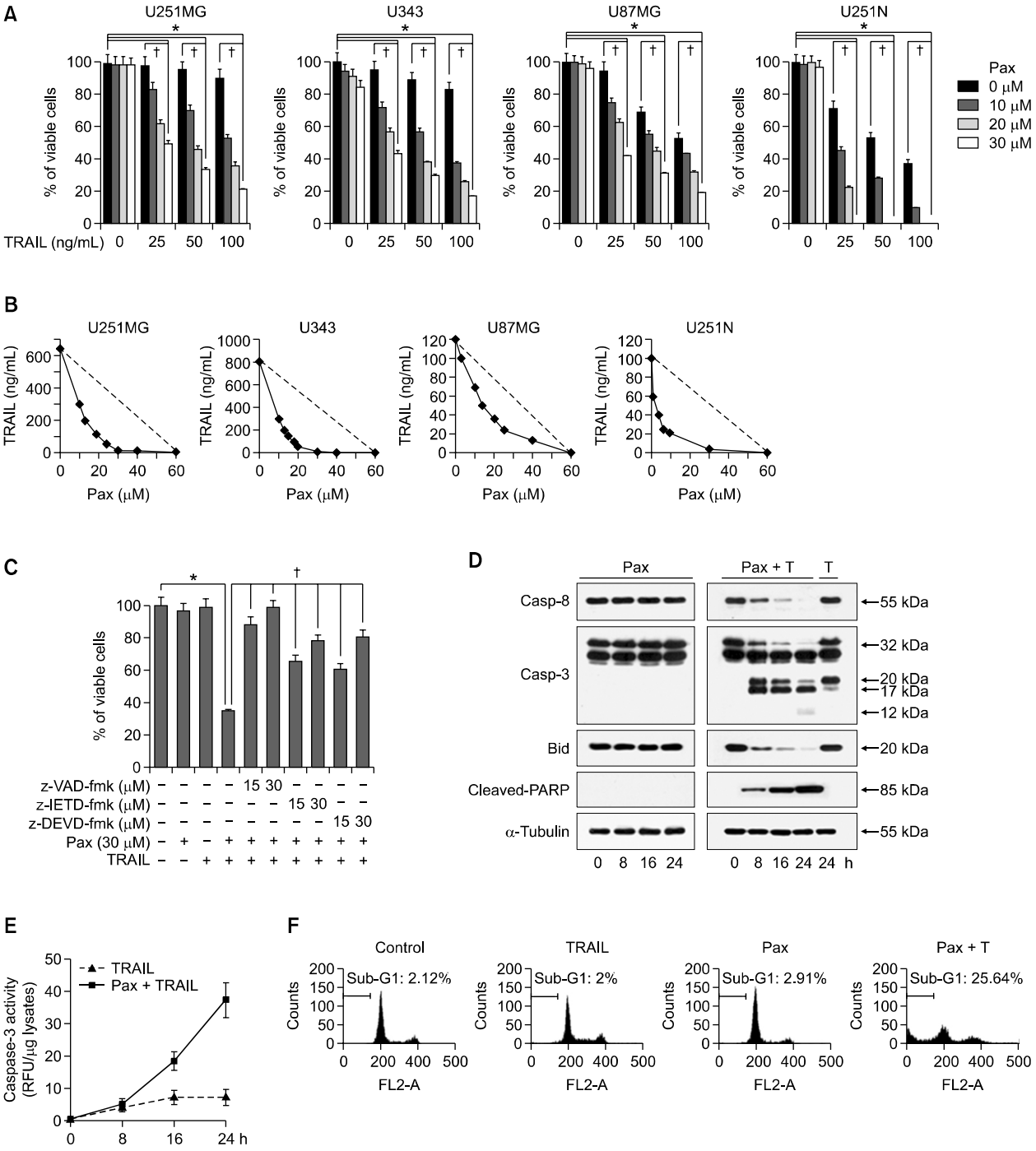
To examine whether paxilline sensitizes various glioma cells to TRAIL-induced cell death, we treated four glioma cell lines (U251MG, U87MG, U343, and U251N) with paxilline alone, TRAIL alone, or both paxilline and TRAIL for 24 h at various concentrations. Assessment of cellular viability using calcein-AM and EthD-1 to detect live and dead cells, respectively, revealed that treatment with TRAIL alone up to 100 ng/mL or paxilline alone up to 30 μmol/L did not induce significant cell death in U251MG or U87MG cells (Figure 1A). However, paxilline/TRAIL co-treatment effectively induced cell death, when we fixed the paxilline concentration and varied the TRAIL concentration, and vice versa. U343 and U251N cells were more sensitive to TRAIL than U251MG and U87MG cells, but co-treatment of U343 and U251N cells with subtoxic doses of paxilline markedly enhanced TRAIL-induced cell death. An isobologram analysis demonstrated that paxilline and TRAIL synergistically induced cell death in all four tested glioma cell lines (Figure 1B). Next, we investigated whether caspases were critically involved in this paxilline/TRAIL-induced cell death. Pretreatment of U251MG cells with z-VAD-fmk (a pancaspase inhibitor), z-IETD-fmk (a caspase-8 inhibitor), or z-DEVD-fmk (a caspase-3 inhibitor) significantly and dose-dependently inhibited cell death in co-treated cells (Figure 1C). Further examination revealed that treatment of U251MG cells with 30 μmol/L paxilline alone did not induce any proteolytic processing of caspase-8 or -3 (Figure 1D). Caspase-8 was not processed in response to TRAIL alone, but it was effectively processed following the combined treatment. Interestingly, treatment with TRAIL alone caused partial processing of caspase-3 to its p20 intermediate, whereas co-treatment with TRAIL and subtoxic doses of paxilline caused complete processing of caspase-3 into its active subunits. Both Bid (a substrate of caspase-8) and PARP (a substrate of caspase-3) were effectively cleaved by paxilline plus TRAIL, but not by either alone. Analysis of caspase-3 activity using the fluorometric substrate, DEVD-AFC (7-amino-4-trifluoromethyl coumarin) showed that caspase-3 activity was very weakly increased by 100 ng/mL TRAIL alone, whereas it was markedly increased by 24 h after co-treatment with paxilline and TRAIL (Figure 1E). These results suggest that the TRAIL-mediated partial priming of pro-caspase-3 into its p20 intermediate form was insufficient to trigger casapse-3 activity. But, co-treatment with paxilline completed this partial processing into the active p17 and p12 subunits, yielding caspase-3 activity. Flow cytometric analysis showed that the hypodiploid sub-G_1 cell population was markedly increased by the combined treatment of U251MG cells with 30 μmol/L paxilline plus 100 ng/mL TRAIL, but not by either alone (Figure 1F). Taken together, these results demonstrate that paxilline enhances the TRAIL sensitivity of glioma cells by inducing caspase-mediated apoptosis.

Upregulation of DR5 and downregulation of c-FLIP and survivin are common responses to paxilline in malignant glioma cells

To explore the underlying mechanisms involved in paxilline-stimulated TRAIL-mediated apoptosis, we examined the expressions of various apoptotic regulators in U251MG cells treated with paxilline alone. Paxilline treatment increased the protein levels of the TRAIL receptor, DR5, but not those of DR4 (Figure 2A). Although paxilline treatment did not alter the protein levels of XIAP and Bcl-2, it triggered the downregulation of survivin (a caspase inhibitor) and c-FLIP (the caspase-8 inhibitor) major isoforms, c-$FLIP_S$ and c-$FLIP_L$, were both strongly downregulated. To confirm that these effects were a common response to paxilline in glioma cells, we tested the effects of various doses of paxilline on the levels of these proteins in other cell lines. Paxilline dose-dependently increased the protein levels of DR5, but decreased those of c-$FLIP_L$, c-$FLIP_S$ and survivin in all tested glioma cells lines, including U251MG, U87MG, U343 and U251N cells (Figure 2B). These results suggest that DR5, c-$FLIP_S$, c-$FLIP_L$ and survivin could be involved in paxilline-mediated sensitization of glioma cells to TRAIL-mediated apoptosis.

Upregulation of DR5 and downregulation of c-FLIP and survivin play important roles in the paxilline-triggered enhancement of TRAIL-induced apoptosis

Next, we investigated whether DR5, c-FLIP and survivin are critically involved in the apoptosis induced by paxilline and TRAIL. First, to clarify the functional significance of DR5 upregulation, we employed siRNA against DR5. We found that transfection with the DR5-specific siRNA effectively inhibited the cell death induced by paxilline/TRAIL co-treatment (Figure 3A). Next, to examine whether the cell death induced by paxilline plus TRAIL was affected by the overexpression of c-FLIP$_S$ or



c-FLIP$_L$, we established cell lines that stably overexpressed c-FLIP$_S$ or c-FLIP$_L$ and examined their responses to paxilline/TRAIL co-treatment. We found that both proteins blocked co-treatment-induced caspase-3 activation and cell death, with c-FLIP$_S$ overexpression showing a stronger blocking effect than c-FLIP$_L$ overexpression (Figure 3B). Similarly, overexpression of survivin in U251MG cells also attenuated the caspase-3 activation and cell death induced by paxilline/TRAIL co-treatment (Figure 3C). To further assess whether the downregulation of c-FLIP and/or survivin was critical for paxilline-mediated TRAIL-induced apoptosis, we employed siRNAs against c-FLIP$_S$, c-FLIP$_L$ and survivin. We found that siRNA-mediated suppression of c-FLIP$_S$ enhanced caspase-3 activity and cell death more markedly than suppression of c-FLIP$_L$ in U251MG cells treated with 100 ng/mL TRAIL (Figure 3D). Similarly, siRNA-mediated suppression of survivin expression effectively enhanced caspase-3 activity and cell death in U251MG cells treated with TRAIL (Figure 3E). Taken together, these findings collectively suggest that the upregulation of DR5 and the downregulation of c-FLIP (particularly c-FLIP$_S$) and survivin all contribute to the paxilline-triggered enhancement of TRAIL-mediated cell death.

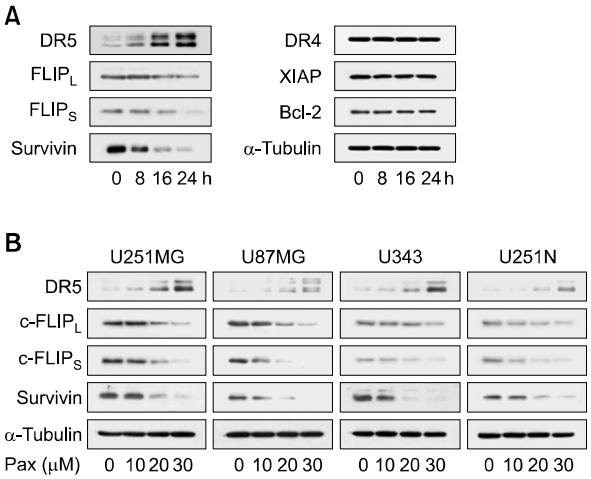


Figure 2. Subtoxic doses of paxilline commonly induce upregulation of DR5 and downregulation of FLIP and survivin in various glioma cells. (A) U251MG cells were treated with 30 μmol/L paxilline for the indicated time points and Western blotting was done to detect the levels of the indicated proteins. Equal loading was confirmed by Western blotting of α-tubulin. (B) Cells were treated with 30 μmol/L paxilline for the indicated time points and Western blotting was used to detect the levels of the indicated proteins.

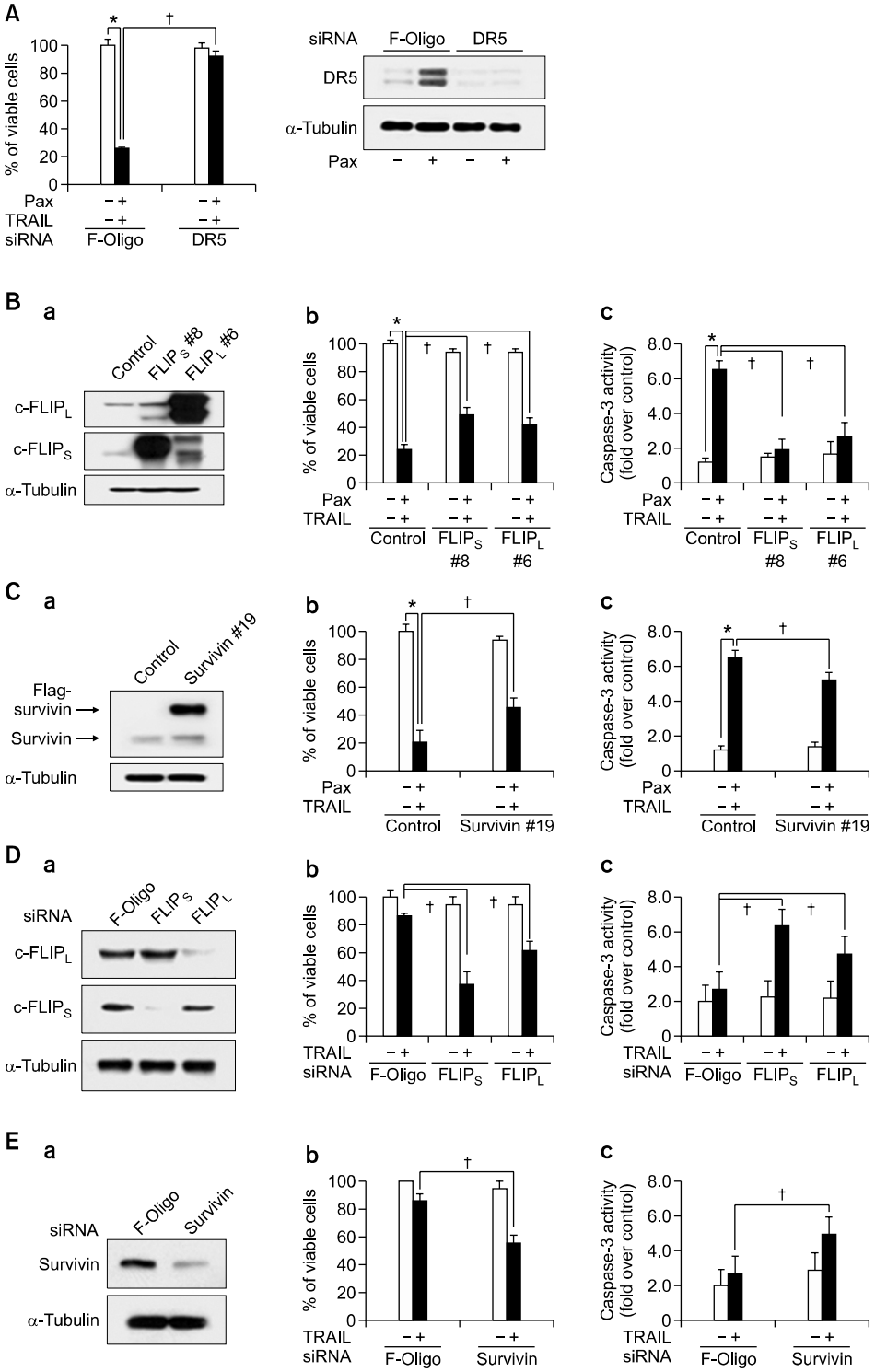
In paxilline-treated cells, CHOP mediates DR5 upregulation, while proteasomes mediate the downregulation of c-FLIP and survivin

We then investigated the potential mechanisms underlying the paxilline-induced modulation of DR5, c-FLIP and survivin expression. First, we examined whether their expression levels were transcriptionally controlled. RT-PCR analysis demonstrated that paxilline dose-dependently increased the mRNA levels of DR5, but not c-FLIP$_S$, c-FLIP$_L$ or survivin (Figure 4A). Since the CHOP transcription factor has been associated with the transcription of DR5 (Yoshida *et al*., 2005), we tested the possible involvement of CHOP in paxilline-induced DR5 upregulation. We found that paxilline treatment increased CHOP protein levels prior to the upregulation of DR5 (Figure 4B). We then used a luciferase reporter system containing the DR5 promoter to test whether CHOP was directly involved in the paxilline-mediated transcriptional activation of the DR5 promoter. The pDR5-605 plasmid contained 605-bp of the upstream DR5 promoter sequence, while the pDR-605-mCHOP plasmid contained the same sequence harboring mutations in the CHOP-binding site (Figure 4C). Paxilline treatment increased the promoter activity of pDR5-605 but not that of pDR5-605-mCHOP (Figure 4C), suggesting that abrogation of the CHOP-binding site blocked the paxilline-induced activation of the DR5 promoter. The functional significance of CHOP in paxilline-induced DR5 upregulation was further examined using CHOP-specific siRNA. Our results revealed that siRNA-mediated suppression of CHOP significantly inhibited the paxilline-induced upregulation of DR5 (Figure 4D). Moreover, the

Figure 1. Paxilline and TRAIL synergistically induce apoptosis in malignant glioma cells. (A) Cells were treated with paxilline for 30 min and further treated with TRAIL for 24 h at the indicated concentrations. Cellular viability was assessed using calcein-AM and EthD-1. Columns, mean; bars, SD. *, $P < 0.01$ vs. control; †, $P < 0.01$ vs. cell treated with TRAIL alone. (B) Cells were treated for 24 h with various concentrations of paxilline and/or TRAIL, and an isobologram analysis was performed as described in the "Methods" section. (C) U251MG cells were treated with specific caspase inhibitors at the indicated concentrations for 30 min, and further treated with 30 μmol/L paxilline and 100 ng/mL TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. Columns, mean; bars, SD. *, $P < 0.01$ vs. control; †, $P < 0.01$ vs. cells treated with paxilline plus TRAIL. (D) Cell extracts were prepared from U251MG cells treated with 30 μmol/L paxilline and/or 100 ng/mL TRAIL for the indicated time points, and Western blotting was used to detect the levels of the indicated proteins. Equal protein loading was confirmed by Western blotting of α-tubulin. (E) U251MG cells were treated with 100 ng/mL TRAIL with or without 30 μmol/L paxilline for the indicated time points. Caspase-3 activity (RFU, relative fluorescence units) was measured using a fluorometric assay kit, as described in the "Methods" section. The data shown are representative of three independent experiments. Points, mean; bars, SD. (F) U251MG cells were treated with 30 μmol/L paxilline and/or 100 ng/mL TRAIL for 16 h, and the cellular DNA contents were examined by flow cytometric analysis.

siRNA-mediated knockdown of CHOP significantly attenuated the paxilline-induced increase in caspase-3 activity and death of U251MG cells treated with 100 ng/mL TRAIL. Collectively, these results show that CHOP-mediated DR5 upregulation may contribute to the ability of paxilline to recover TRAIL sensitivity in TRAIL-resistant glioma cells.

Since our RT-PCR analysis showed that the mRNA levels of c-FLIP$_L$, c-FLIP$_S$ and survivin were unaffected by paxilline treatment (Figure 4A), we



tested whether the paxilline-induced downregulations of c-FLIP and survivin were associated with proteasome-mediated degradation. We found that pretreatment with the proteasome inhibitor, MG132, dose-dependently recovered the paxilline-induced suppression of c-FLIP$_L$, c-FLIP$_S$ and survivin (Figure 4E). These findings indicate that paxilline downregulates c-FLIP and survivin *via* proteasome-mediated degradation.

Paxilline/TRAIL co-treatment does not induce cell death in normal astrocytes

Finally, we examined the effect of paxilline/TRAIL co-treatment on the viability of normal human astrocytes. We found that astrocytes were resistant to TRAIL or paxilline alone, as well as to paxilline/TRAIL co-treatment (Figure 5A), suggesting that paxilline/TRAIL co-treatment is preferentially toxic to glioma cells over normal astrocytes. While caspase-3 was partially processed into its p20 intermediate form in TRAIL-treated U251MG cells, and further processed into its active subunits in paxilline/TRAIL co-treated cells, no such processing was observed in TRAIL-treated or paxilline/TRAIL co-treated astrocytes (Figure 5B). Paxilline-treated astrocytes showed no evidence of CHOP upregulation, DR5 upregulation, survivin downregulation, or c-FLIP$_L$ downregulation, whereas the levels of c-FLIP$_S$ were slightly reduced in these cells (Figure 5C). Taken together, our results collectively suggest that the TRAIL-mediated partial priming of caspase-3 and the paxilline-mediated modulation of various components in the death receptor-mediated apoptotic signaling pathways (e.g., DR5, c-FLIP and survivin) may allow paxilline/TRAIL co-treatment to selectively kill glioma cells, while sparing normal astrocytes (Figure 5D).

Discussion

Here, we show for the first time that paxilline may be used as an effective TRAIL sensitizer in malignant glioma cells, and further explore the potential mechanisms underlying this effect. Our results revealed that paxilline facilitated the proteolytic processing of caspase-3 in glioma cells exposed to TRAIL *via* downregulation of c-FLIP and survivin. c-FLIP, a homolog of caspase-8, is recruited to the death-inducing signaling complex, where it inhibits the activation of caspase-8 (Irmler *et al*., 1997). We found that the protein levels of c-FLIP$_S$ were more markedly reduced than those of c-FLIP$_L$ in paxilline-treated glioma cells, and experiments involving the overexpression or siRNA-mediated downregulation of c-FLIP$_S$ and c-FLIP$_L$ demonstrated that changes in c-FLIP$_S$ appear to contribute to paxilline-induced TRAIL sensitization more than changes in c-FLIP$_L$. Survivin is expressed more highly in cancer tissues than in normal tissues (Ambrosini *et al*., 1997), and cancer patients with upregulated survivin have been shown to have shortened survival, more unfavorable markers of disease progression, accelerated rates of recurrence (Altieri, 2001) and increase resistance to therapy (Kato *et al*., 2001). Here, we found that paxilline treatment dose-dependently reduced the protein levels of survivin in various glioma cells. Furthermore, siRNA-mediated survivin knockdown increased TRAIL-mediated apoptosis in U251MG cells, whereas survivin overexpression significantly attenuated the cell death induced by paxilline plus TRAIL. To exclude the possibility that these findings could have been affected by clonal variability among the stably transfected cell lines, we transiently overexpressed survivin, c-FLIP$_S$ and c-FLIP$_L$, and examined the cell death induced by paxilline/TRAIL co-treatment. Consistent with the above findings, survivin, c-FLIP$_S$ and c-FLIP$_L$ dose-dependently attenuated

Figure 3. DR5, c-FLIP and survivin are critically involved in the paxilline-stimulated TRAIL-mediated apoptosis of glioma cells. (A) U251MG cells were transfected with 40 nmol/L fluorescent oligonucleotide (F-Oligo; control) or 40 nmol/L siRNA duplexes against the DR5 mRNA, incubated for 24 h, and further treated with or without 30 μmol/L paxilline plus 100 ng/mL TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1 (left). Columns, mean; bars, SD. *, $P < 0.01$ vs. untreated cells; †, $P < 0.01$ vs. cells tansfected with F-Oligo and treated with paxilline plus TRAIL. Cells were transfected with the siRNA, incubated for 24 h, and then subjected to Western blotting of DR5 (right). (B & C) Effect of overexpression of c-FLIP or survivin on the apoptosis induced by paxilline plus TRAIL. (a) Stable cell lines overexpressing pcDNA3, c-FLIP$_S$, c-FLIP$_L$ or survivin were established and the overexpression of each protein was confirmed by Western blotting. (b) The stably overexpressing cells were treated with 30 μmol/L paxilline and 100 ng/mL TRAIL for 24 h, and cellular viability was analyzed using calcein-AM and EthD-1. Columns, mean; bars, SD. *, $P < 0.01$ vs. untreated control; †, $P < 0.01$ vs. control cells treated with paxilline plus TRAIL. (c) Caspase-3 activity was measured as described in the "Methods" section. Columns, mean; bars, SD. *, $P < 0.01$ vs. untreated control; †, $P < 0.01$ vs. control cells treated with paxilline plus TRAIL. (D & E) Effect of siRNA-mediated suppression of c-FLIP or survivin. (a) U251MG cells were transfected with 40 nmol/L fluorescent oligonucleotide (F-Oligo), siRNAs against c-FLIP$_S$, c-FLIP$_L$ or survivin. Twenty-four hours after transfection, knockdown of the relevant proteins was confirmed by Western blotting. (b) To examine the effect of c-FLIP or survivin downregulation on TRAIL-induced cell death, U251MG cells were transfected with each siRNA, incubated for 24 h, and further treated with or without 100 ng/mL TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. Columns, mean; bars, SD. †, $P < 0.01$ vs. control cells transfected with F-Oligo and further treated with TRAIL. (c) Caspase-3 activity was assessed as described in the "Methods" section. Columns, mean; bars, SD. †, $P < 0.01$ vs. control cells transfected with F-Oligo and further treated with TRAIL.

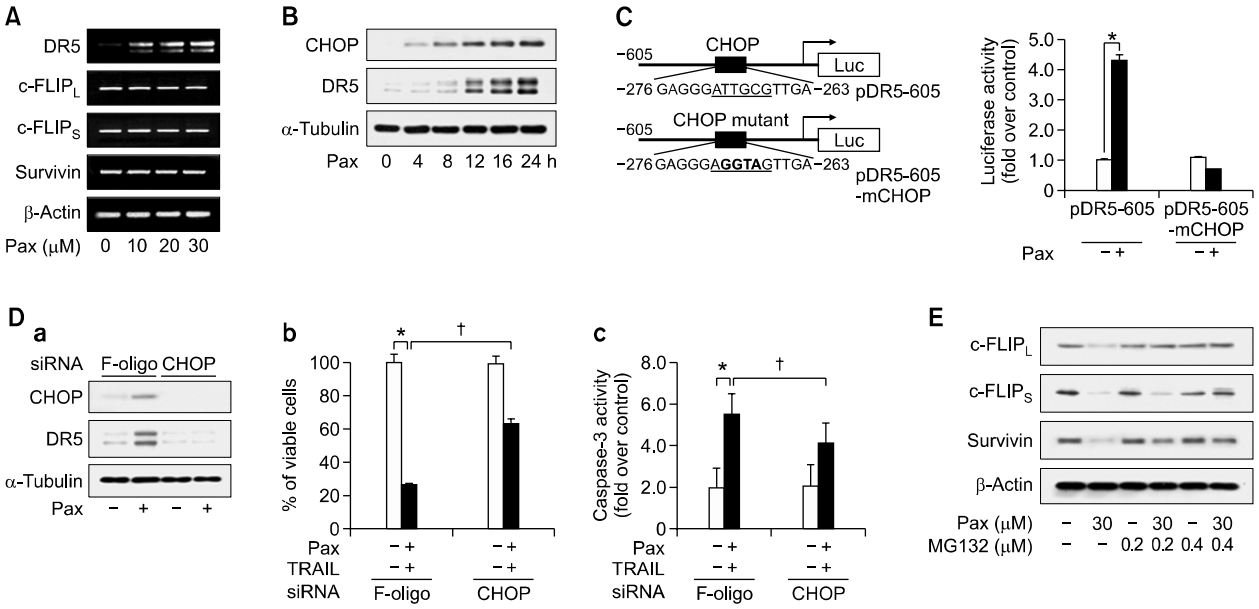


Figure 4. Paxilline induces CHOP-mediated DR5 upregulation and proteasome-mediated downregulation of c-FLIP and survivin. (A) U251MG cells were treated with the indicated concentrations of paxilline and total RNA was prepared for RT-PCR of the indicated gene products, with β-actin assessed as an internal control. (B) U251MG cells were treated with 30 μmol/L paxilline for the indicated time points and the indicated proteins were detected by Western blotting. (C) Schematic diagram of the DR5 promoter constructs used for the luciferase activity assays (top). U251MG cells were transfected with pDR5-605 or pDR5-605-mCHOP, treated with 30 μmol/L paxilline, lysed, and assayed for luciferase activity (bottom). Data are expressed as fold-change relative to the untreated cells transfected with pDR5-605. Columns, mean; bars, SD. *, $P < 0.01$ vs. cells transfected with pDR5-605 and untreated. (D) U251MG cells were transfected with 40 nmol/L F-Oligo or siRNA duplexes against the CHOP mRNA, and incubated for 24 h. (a) Western blotting was used to confirm CHOP knockdown and examine its effect on paxilline-induced DR5 upregulation. Equal loading of protein samples was confirmed by Western blotting of α-tubulin. (b) Transfected cells were treated with or without or 30 μmol/L paxilline plus 100 ng/mL TRAIL for 24 h, and cellular viability was assessed using calcein-AM and EthD-1. Columns, mean; bars, SD. *, $P < 0.01$ vs. cells transfected with F-Oligo and untreated; †, $P < 0.01$ vs. cells transfected with F-Oligo and treated with paxilline plus TRAIL. (c) Caspase-3 activity was assessed as described in the "Methods" section. Columns, mean; bars, SD. *, $P < 0.01$ vs. cells transfected with F-Oligo and untreated; †, $P < 0.01$ vs. control cells transfected with F-Oligo and treated with paxilline plus TRAIL. (E) U251MG cells were treated with 30 μmol/L paxilline with or without the indicated concentrations of MG132 for 12 h. Western blotting was used to assess changes in the protein levels of c-FLIP$_L$, c-FLIP$_S$ and survivin.

paxilline/TRAIL-induced cell death (Supplemental Data Figures S1 and S2) and the death-blocking effect of c-FLIP$_S$ was stronger than that of c-FLIP$_L$ (Supplemental Data Figure S2). Furthermore, pretreatment of U251MG cells with the proteasome inhibitor, MG132, effectively blocked the paxilline-induced downregulation of c-FLIP$_S$, c-FLIP$_L$ and survivin (Figure 4E). These findings collectively indicate that paxilline stimulates TRAIL-mediated apoptosis in glioma cells *via* the proteasome-dependent degradation of c-FLIP and survivin. Interestingly, treatment with TRAIL alone induced the partial proteolytic processing of caspase-3 to its p20 intermediate form and weakly increased the caspase-3 activity in U251MG cells, demonstrating that caspase-3 activation was partially primed in TRAIL-treated glioma cells (Figures 1D, 1E, and 5B). Co-treatment with paxilline and TRAIL led to the further processing of caspase-3 into its p17 and p12 subunits. Based on this, we speculate that the paxilline-triggered proteasome-mediated degradation of c-FLIP may reduce the barriers limiting the efficient formation of the death-inducing signaling complex (DISC), thereby enhancing the activation of caspase-8. In addition, the paxilline-induced degradation of survivin may further facilitate the complete proteolytic processing of caspase-3, which is partially processed by TRAIL treatment.

Paxilline is known to inhibit BK channels (Sanchez and McManus, 1996) and various isoforms of SERCA, with IC_{50} values of 5 to 50 μmol/L (Bilmen *et al*., 2002). Recent studies have shown that BK channel activation is involved in the proliferation of diverse cancer cells, including glioma cells (Bloch *et al*., 2007; Coiret *et al*., 2007), and the expression levels of BK channels have been closely correlated with the grades of glioma malignancy (Weaver *et al*., 2004). Thapsigargin, a well-known SERCA inhibitor (Lytton *et al*., 1991), was previously shown to potentiate TRAIL-induced apoptosis in giant cell bone tumor, perhaps by perturbing intracellular Ca^{2+} homeostasis (Huang *et al*., 2004). In addition, SERCA inhibition increased

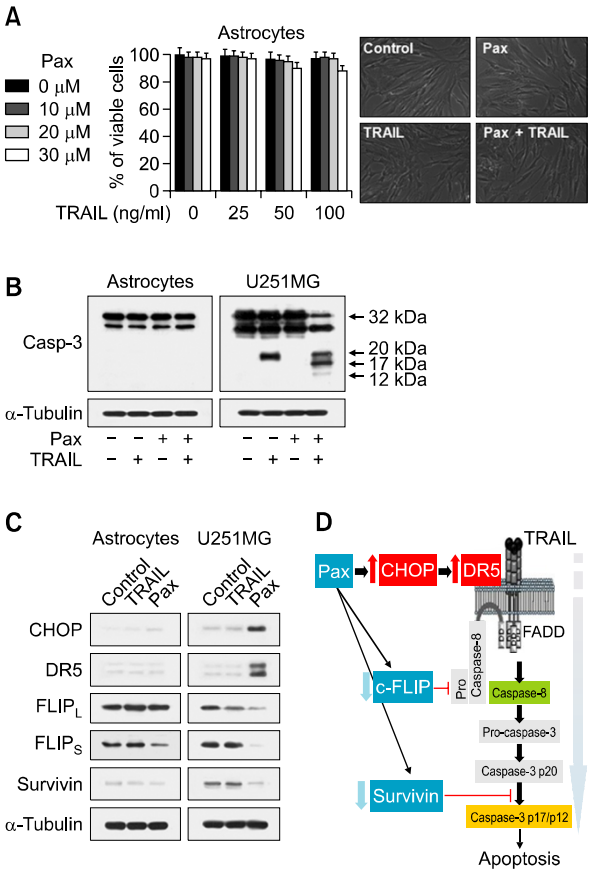


Figure 5. Combined treatment with paxilline and TRAIL does not induce cell death in normal astrocytes. (A) Human astrocytes were treated with paxilline for 30 min and further treated with the indicated concentrations of TRAIL for 24 h. Cellular viability was assessed using calcein-AM and EthD-1. Columns mean; bars, SD (left). Astrocytes were treated with 30 μmol/L paxilline and/or 100 ng/mL TRAIL for 24 h. Representative pictures are shown (right). (B & C) Astrocytes or U251MG cells were treated with 30 μmol/L paxilline and/or 100 ng/mL TRAIL for 24 h, and cell extracts were subjected to Western blotting of the indicated proteins. (D) Schematic diagram of the apoptotic pathway that appears to be induced by paxilline and TRAIL co-treatment of glioma cells.

$[Ca^{2+}]_i$ more quickly and to a higher degree in glioma cells compared to normal astrocytes (Kovacs *et al*., 2005). Therefore, we examined whether the sensitizing effect of paxilline on TRAIL-mediated apoptosis in glioma cells was associated with the inhibition of BK channels and/or SERCA. We found that subtoxic doses of two SERCA inhibitors, thapsigargin (Huang *et al*., 2004) and cyclopiazonic acid (Goeger *et al*., 1988), effectively sensitized U251MG cells to TRAIL-induced cell death, whereas two BK channel inhibitors, iberiotoxin (Galvez *et al*., 1990) and penitrem A (Knaus *et al*., 1994), did not (Supplemental Data Figure S3A). Furthermore, treatment with thapsigargin or cyclopiazonic acid dose-dependently downregulated c-FLIP and survivin, but upregulated CHOP and DR5 (Supplemental Data Figure S3B), in a manner similar to that seen for paxilline (Figure 2B). In previous studies, thapsigargin-induced downregulation of survivin (Sohn *et al*., 2003) and alpha-TEA (RRR-alpha-tocopherol ether-linked acetic acid analog)-induced downregulation of c-FLIP$_L$ (Tiwary *et al*., 2010) were shown to involve endoplasmic reticulum (ER) stress. Since inhibition of SERCA activity has been shown to induce ER stress by causing intracellular Ca^{2+} imbalances (Lytton *et al*., 1991), we examined whether paxilline could induce the unfolded protein response (an indicator of ER stress) in our system. We found that paxilline treatment increased KDEL protein levels and eIF2α phosphorylation (Supplemental Data Figure S4), in addition to increasing CHOP levels (Figure 4B). Taken together, our findings collectively show that the sensitizing effect of paxilline on TRAIL-mediated apoptosis may be closely associated with SERCA inhibition, which may cause ER stress and subsequent CHOP upregulation. This CHOP-dependent DR5 upregulation may amplify the death receptor-mediated apoptotic signaling in glioma cells exposed to both paxilline and TRAIL. However, future work will be required to clarify the potential relationships among SERCA inhibition, proteasome-mediated degradation, and the downregulation of c-FLIP and survivin.

In cancer therapy, success often depends on both effectively killing cancer cells and maintaining the safety of normal cells. Here, we found that normal astrocytes were very resistant to treatment with paxilline and TRAIL, both alone and in combination (Figure 5A). Furthermore, astrocytes did not show evidence of the observed TRAIL-induced partial processing of caspase-3 (Figure 5B), paxilline-induced downregulation of c-FLIP and survivin, or paxilline-induced upregulation of DR5 (Figure 5C). These findings suggest that astrocytes are doubly protected from the death-inducing activities of paxilline/TRAIL co-treatment.

In summary, the present study shows that paxilline is capable of modulating multiple targets in the death receptor-mediated apoptotic signaling pathway, including DR5, c-FLIP and survivin, thereby selectively sensitizing glioma cells to TRAIL-induced apoptosis. Thus, paxilline and TRAIL co-treatment may offer a safe and effective strategy for selectively killing malignant glioma cells.

Methods

Chemicals and antibodies

Paxilline was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Recombinant human TRAIL (the non-tagged 19 kDa protein, amino acids 114-281) was from KOMA Biotech (Seoul, South Korea). Calcein acetoxymethyl ester (calcein-AM) and ethidium homodimer-1 (EthD-1) were from Invitrogen (Carlsbad, CA). Caspase inhibitor benzyloxy-carbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone (z-VAD-fmk), benzyloxy-carbonyl-Asp-(OMe)-Glu-(OMe)-fluoromethyl ketone (z-DEVD-fmk), and benzyloxy-carbonyl-Ile-Glu-(OMe)-Tyr-Asp-(OMe)-fluoromethyl ketone (z-IETD-fmk) were from R&D system (Minneapolis, MN). The following antibodies were used: anti-caspase-3, -survivin, and -XIAP (Stressgen, British Columbia, Canada); anti-DR4, -CHOP/growth arrest and damage-inducible gene 153 (GADD153), and -Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-c-FLIP (Alexis, San Diego, CA); anti-cleaved PARP (Poly (ADP-ribose) polymerase) (Epitomics, Burlingame, CA); anti-Bid (Cell Signaling, Beverly, MA); anti-α-tubulin and DR5 (KOMA Biotech); horseradish peroxidase-conjugated anti-rabbit IgG and horseradish peroxidase-conjugated mouse IgG (Invitrogen).

Culture of glioma cell lines and normal human astrocytes

The human malignant glioma cell lines U251MG, U87MG, U343, and U251N were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (GIBCO-BRL, Grand Island, NY). Primary cultures of normal human astrocytes were prepared from 14-week fetal cerebrum tissues, as described previously (Kim *et al*., 1986). Human astrocytes were cultured in high-glucose DMEM supplemented with 10% FBS and 20 mg/mL gentamicin. The cells were subcultured every two weeks, and cell cultures of five or fewer passage were used.

Measurement of cellular viability

Cells (3.5×10^4 cells) were cultured in 24-well plates and treated as indicated. The test drugs were then removed and the cells were washed twice with PBS. For measurement of cellular viability, 200 μL of 2 μmol/L calcein-AM (which indicates intracellular esterase activity with a green fluorescence that was taken as a representing living cells) and 4 μmol/L EthD-1 (which indicates membrane-damaged cells with a red fluorescence that was taken as indicating dead cells) were added to each well, and the plates were incubated for 30 min in a moist chamber at room temperature. The cells were then viewed under a fluorescence microscope (Axiovert 200M, Carl Zeiss, Germany) equipped with a Zeiss filter set #10 (excitation/emission wavelengths of 450-490/515-565 nm) and #34 (excitation/emission wavelengths of 390/460 nm). The numbers of green, red, and bicolored cells were counted from five fields per well at 400 $\times$ magnification. The percentage of cells with exclusively green fluorescence (viable cells) was calculated.

Flow cytometry to measure apoptosis

DNA content was assessed using Cell Cycle TEST PLUS DNA^{TM} reagent kit (Becton Dikinson and Co., San Jose, CA), according to the manufacturer's instruction, and analyzed in a FACS sorter (Becton Dikinson and Co.).

Fluorometric assay of caspase-3 activity

Caspase-3 activity was assayed with a caspase-3/CPP32 fluorometric assay kit (Biovision, CA). Cells (2×10^5 cells) were cultured in 6-well plates and treated. The cells were then harvested, washed twice with cold PBS, and re-suspended with cell lysis buffer following the instructions provided with the assay kit. The protein concentrations of the cell lysates were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Berkeley, CA), and 80 μg of total protein from each sample was mixed with a reaction buffer containing the caspase-3 fluorogenic substrate, DEVD-AFC (7-amino-4-trifluoromethyl coumarin). The samples were incubated for 1 h at 37^{o}C, and the results were obtained using a fluorescence microplate reader (Molecular Devices, CA) at excitation/emission wavelengths of 400/505 nm. To compare the caspase-3 activity levels among the different treatment groups, the fold increases in caspase-3 activity were determined by comparing the absorbance from each apoptotic sample with that from the corresponding untreated control.

Reverse transcription-PCR analysis

Total RNA was extracted from U251MG cells using the TRIzol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) was done, following the manufacturer's protocol (TaKaRa Shuzo Co., Otsu, Shiga, Japan). The cDNAs were amplified by PCR (30 s at 94^{o}C, 30 s at 60^{o}C, and 1 min 74^{o}C) with *Taq* DNA polymerase. Conditions for final analysis were chosen when amplification of mRNA was in the middle of the exponential amplification phase for 30 μmol/L paxilline tested. Human DR5 mRNA was amplified using the sense primer 5'-GTCTGCTCTGATCACCCAAC-3' and the antisense primer 5'-CTGCAAACTGTGACTCCT-ATG-3' (corresponding to a 424-bp region of DR5). For c-FLIP$_L$, the sense primer 5'-CGGACTATAGAGTGCTGA-TGG-3' and the antisense primer 5'-GATTATCAGGCA-GATTCCTAG-3' (corresponding to a 655-bp region of c-FLIP$_L$), and for c-FLIP$_S$, the sense primer 5'-CGGACT-ATAGAGTGCTGATGG-3' and the antisense primer 5'-AGATCAGGACAATGGGCATAG-3' (corresponding to a 561-bp region of c-FLIP$_S$) were used. For survivin, the sense primer 5'-CAGATTTGAATCGCGGGACCC-3' and the antisense primer 5'-CCAGAGTCTGGCTCGTTCTCAG- 3' (corresponding to a 206-bp region of survivin). For β-actin, the sense primer 5'-CAGGTCATCACCATTGGCAATGAGC-3' and the antisense primer 5'-GATGTCCACGTCACACT-TCATGA-3' (corresponding to a 132-bp region of β-actin) were used. The PCR cycling conditions (30 cycles) chosen were as follows: (a) 30 s at 94^{o}C; (b) 45 s at 52^{o}C for CHOP, 30 s at 68^{o}C for DR5, 30 s at 55^{o}C for c-FLIP$_S$, 30 s at 56^{o}C for c-FLIP$_L$, and 1 min at 60^{o}C for survivin, 30 s at

60°C for β-actin; (c) 1 min 30 s at 72°C, with a subsequent 10 min extension at 72°C. Reaction products were analyzed on 2% agarose gels. The bands were visualized by ethidium bromide.

Small interfering RNA

25-nucleotide small interfering RNA (siRNA) duplexes used in this study were purchased from Invitrogen and have the following sequences: DR5 (E11), AUCAGCAUCGUGUACAAGGUGUCCC; CHOP, UUCACCAUUCGGUCAACAGAGCUC; c-FLIP$_S$, AACAUGGAAC UGCCUCUACUU; c-FLIP$_L$, AAGGAACAGCUUGGCGCUCAA; survivin, UUUAAGGCUGGGAGCCAGAUGACGC. BLOCK-IT Fluorescent Oligo (Invitrogen) was used as the control. Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Plasmids, transfection, and luciferase assay

The pDR5/-605 plasmid [containing DR5 promoter sequence (-605/+3)] was a gift from Dr. T. Sakai (Kyoto Prefectural University of Medicine). CHOP-binding sites in the DR5/-605 promoter were point mutated using a two-step PCR method and mCHOP primers (5'-CTTGCGGAGGAGGTAGTTGACGA and 5'-TCGTCAACTACCTCCTCCGCAAG). The resulting mutant was confirmed by sequencing. For transfection, cells (3×10^5 cells) were plated onto 60-mm culture dishes, grown overnight, and then transfected with 1 μg of the indicated luciferase reporter construct using the Lipofectamine Plus reagent (Invitrogen). After incubation for 24 h, the transfected cells were further treated with or without 30 μmol/L paxilline, and their luciferase and β-galactosidase activities were assayed following the manufacturer's protocol (Promega, Madison, WI). The activity of luciferase was normalized with regard to that of β-galactosidase, and the results are given as the average of three independent experiments.

Overexpression of c-FLIP$_S$, c-FLIP$_L$, or survivin

U87MG cells were transfected with a vector containing Flag-tagged c-FLIP$_S$, c-FLIP$_L$ (kindly provided by Dr. Park SI, Korea Centers for Disease Control and Prevention, Korea), or survivin (kindly provided by Dr. JS Lee, Ajou University, Korea). Overexpression of c-FLIP or survivin was analyzed by Western blotting using anti-c-FLIP (Alexis) and anti-survivin (Stressgen) antibody.

Statistical analysis

All data are presented as means ± SD from at least three independent experiments. The statistical significance of differences was assessed using an analysis of variance with Bonferroni correction or a repeated-measures analysis of variance followed by Greenhouse-Geisser adjustment. Values of $P < 0.05$ were considered significant. The synergy of paxilline and TRAIL was evaluated by the isobologram method (Berenbaum, 1981). In brief, the cells were treated with different concentrations of each agent (paxilline or TRAIL) alone or in combination. The relative survival was assessed, and the IC_{50} (the half maximal inhibitory concentration) values for each drug alone or in combination with a fixed concentration of the second agent were established from the concentration-effect curves. In a graphical presentation, the straight line connecting the IC_{50} values of the two agents when applied alone corresponds to the additive effects of both agents. Values below this line indicate synergy, while values above this line indicate antagonism.

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Supplemental data

Supplemental Data include four Figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-1-04.pdf.

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