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Apoptogenic activity of auraptene of *Zanthoxylum schinifolium* toward human acute leukemia Jurkat T cells is associated with ER stress-mediated caspase-8 activation that stimulates mitochondria-dependent or -independent caspase cascade

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To isolate pharmacologically safe compounds that can induce apoptosis of tumor cells, leaves of an aromatic plant (*Zanthoxylum schinifolium*), which are widely used as a food flavor and herbal medicine in Korea and Japan, were sequentially extracted by organic solvents. An apoptogenic ingredient in the methylene chloride extract was further purified by silica gel column chromatography and identified as auraptene (AUR). The IC_{50} value of AUR against Jurkat T cells was 16.5 μg/ml. After the treatment of Jurkat T cells with AUR, the endoplasmic reticulum (ER) stress-mediated activation of caspase-12 and -8 and subsequent apoptotic events including c-Jun N-terminal kinase (JNK) activation, cleavage of FLICE inhibitory protein and Bid, mitochondrial cytochrome c release, activation of caspase-9 and -3, degradation of poly (ADP-ribose) polymerase and apoptotic DNA fragmentation were induced in a dose-dependent manner. The cytotoxicity of AUR was not blocked by the anti-Fas neutralizing antibody ZB-4. The AUR-induced cytotoxicity and apoptotic events were abrogated by ectopic over-expression of Bcl-xL or addition of the pan-caspase inhibitor z-VAD-fmk. The individual or simultaneous addition of the m-calpain inhibitor (E64d), JNK inhibitor (SP600125) and mitochondrial permeability transition pore inhibitor (CsA) failed to prevent apoptotic events including caspase-8 activation and Bid cleavage, unless the caspase-8 inhibitor (z-IETD-fmk) was combined, whereas AUR-induced caspase-12 activation was sustained even in the concomitant presence of z-IETD-fmk. These results demonstrated that the apoptotic effect of AUR on Jurkat T cells was exerted by the ER stress-mediated activation of caspase-8, and the subsequent induction of mitochondria-dependent or -independent activation of caspase cascade, which could be suppressed by Bcl-xL.

Introduction

Since the induction of apoptosis, a programmed cell death, in tumor cells can lead to their own destruction, apoptosis has been suggested as an efficient mechanism by which malignant tumor cells can be removed when treated with anti-neoplastic drugs. In relation to the apoptogenic activity of chemotherapy toward tumor cells, three different death signaling pathways leading to apoptotic cell death are probably to be implicated; one is the extrinsic death receptor-dependent pathway (1), the second is the intrinsic mitochondria-dependent pathway (2) and the third is the intrinsic endoplasmic reticulum (ER) stress-mediated pathway (3). The initiation of death receptor-dependent apoptotic signaling in tumor cells, following chemotherapy, is provoked by the up-regulation of Fas ligand (FasL) and Fas expressions with the subsequent induction of Fas signaling (4–7), whereas the mitochondria-dependent death signaling begins with the release of cytochrome c from mitochondria into cytoplasm, which together with the apoptotic protease-activating factor-1 activates caspase-9 in the presence of dATP, and then activates an effector caspase, caspase-3, resulting in apoptotic cell death (8,9). The ER stress-mediated apoptotic pathway is known to be initiated by the activation of caspase-12, which can directly activate pro-caspase-9 independently of mitochondrial cytochrome c and apoptotic protease-activating factor-1 (10,11). Along with the caspase-12 activation, ER stress also triggers the activation of caspase-8 and c-Jun N-terminal kinase (JNK), both of which can cause mitochondrial cytochrome c release (12,13). The mechanism in chemotherapeutic drug-induced apoptosis needs to be studied further in order to clarify whether the apoptogenic effect of the drug is confined to tumor cells rather than normal cells.

Recently, we have examined various edible plants to isolate the apoptogenic substance that is pharmacologically safe, based on a simple concept that edible plant-derived cytotoxic components to tumors may be less toxic to normal cells. When we investigated whether *Zanthoxylum schinifolium*, an aromatic plant whose pericarps and leaves are widely used as a pungent condiment and seasoning and herbal medicine in north-eastern Asian countries such as Korea and Japan, possesses apoptogenic activity against human acute leukemia Jurkat T cells, the results indicated the presence of apoptogenic activity in leaves of *Z. schinifolium*. The purification of an apoptogenic ingredient in the leaves, by a serial solvent extraction and subsequent silica gel column chromatography, has led to the identification of auraptene (AUR). AUR, which was initially isolated from citrus fruit, has been reported to possess various biological functions as determined by its dietary administration in animal models. These include anti-inflammatory effect (14,15), antioxidant effect (16), immunomodulatory action on macrophages and lymphocytes (17), promoting effect on the activity of detoxification enzymes (18) and suppressive effect on certain cell proliferation markers (19). In particular, AUR has been shown to possess chemopreventive activity against chemically induced carcinogenesis in rodents (20,21). As a mode of action of AUR, which can be directly associated with such chemopreventive activity, the apoptogenic property of AUR has been shown in human hepatocellular carcinoma and colorectal adenocarcinoma cells *in vitro* (22,23). Little is known, however, about the mechanism responsible for the apoptogenic activity of AUR against tumor cells.

In the present study, we show that AUR, purified from *Z. schinifolium* leaves, possesses potent apoptogenic activity toward human acute leukemia Jurkat T cells. Since AUR can induce apoptosis in Jurkat T cells, the cellular mechanism underlying the induced apoptosis has been further investigated in order to understand the relevancy of apoptogenic activity to the chemopreventive effect of AUR, which was reported previously in rodent models. The results show that although the AUR-induced apoptosis of Jurkat T cells is accompanied by the ER stress-mediated activation of caspase-12 and -8, the caspase-8 activation is a prerequisite for the subsequent mitochondria-dependent or -independent activation of caspase cascade leading to poly (ADP-ribose) polymerase (PARP) degradation, which is negatively regulated by the ectopic over-expression of Bcl-xL. Additional results show that normal human T cells are more refractory to the apoptogenic activity of AUR, as compared with Jurkat T cells.

Abbreviations: AUR, auraptene; ER, endoplasmic reticulum; FasL, Fas ligand; FLIP, FLICE inhibitory protein; IL-2, interleukin-2; JNK, c-Jun N-terminal kinase; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PHA, Phytohemagglutinin A; PI, propidium iodide; tBid, truncated Bid.

Materials and methods

Reagents, antibodies, cells and culture medium

The ECL western blotting kit was purchased from Amersham (Arlington Heights, IL), and Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA). Anti-cytochrome c was purchased from PharMingen (San Diego, CA), and anti-Fas and anti-FasL were obtained from Transduction Laboratories (Lexington, KY). Anti-caspase-3, anti-caspase-12, anti-Bid, anti-FLICE inhibitory protein (FLIP), anti-PARP, anti-Bcl-xL and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-caspase-8 and anti-caspase-9 were from Cell Signaling (Beverly, MA). Phytohemagglutinin A (PHA) was purchased from Sigma (St Louis, MO). All inhibitors including the broad-range caspase inhibitor z-VAD-fmk were obtained from Calbiochem (San Diego, CA). Human acute leukemia Jurkat T cell line E6.1, clone J/Neo transfected with vector and clone J/Bcl-xL transfected with *Bcl-xL* gene were used in this experiment. Jurkat T cells and human peripheral T cells were maintained in RPMI 1640 (Hyclone, Gaithersburg, MD) containing 10% fetal bovine serum, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0), 5×10^5 M β-mercaptoethanol and 100 μg/ml gentamycin. For the culture of both J/Neo and J/Bcl-xL, G418 was added to the RPMI 1640 medium at a concentration of 400 μg/ml.

Purification of a cytotoxic component from Z. schinifolium

Zanthoxylum schinifolium leaves were lyophilized to dry, and they (4.4 kg) were extracted with 80% methyl alcohol. The methanol extract (1.0 kg) was dissolved in water (5 l), and extracted with methylene chloride (4 × 3 l) to obtain the methylene chloride fraction (311 g). The aqueous layer was extracted with *n*-butanol (4 × 5 l) to obtain *n*-butanol fraction (385 g). The remaining aqueous layer was concentrated *in vacuo* to obtain aqueous fraction (300 g). The methylene chloride fraction containing the highest level of cytotoxicity was further fractionated through a silica gel column with a gradient elution (100% hexane → 100% ethyl acetate), which afforded 23 fractions (SL-MC-A–SL-MC-W). The recrystallization of SL-MC-I yielded an apoptogenic substance, AUR (100 mg).

Isolation and activation of human peripheral T cells

To prepare human peripheral blood mononuclear cells, heparinized blood obtained from healthy laboratory personnel by venipuncture was centrifuged at 800*g* for 20 min over HISTOPAQUE-1077 (Sigma Chemical) according to the manufacturer's instructions. The T cells were isolated from peripheral blood mononuclear cells using a human T cell enrichment column kit (R&D Systems, Minneapolis, MN). For activation of peripheral T cells, the isolated peripheral T cells at density of 2×10^6 per ml were incubated with PHA at a concentration of 0.2 μg/ml for 60 h. To induce interleukin-2 (IL-2)-dependent proliferation of T cells, the PHA-activated T cells (1×10^5 per well) were cultured with 50 U of recombinant IL-2 in 96-well plates.

Cytotoxicity assay

The cytotoxic effect of the methylene chloride extract of leaves of *Z. schinifolium* or AUR on Jurkat T cells was analyzed by a 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay, reflecting cell viability, as described elsewhere (24). For the MTT assay, Jurkat T cells (5×10^4 per well), human peripheral T cells (2×10^5 per well) or PHA-activated human T cells (1×10^5 per well) were added to a serial dilution of AUR in 96-well plates. After incubation for 20 h, 50 μl of the MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from the MTT was dissolved in 150 μl of dimethyl sulfoxide (DMSO). The optical density (OD) values of the solutions were measured at 540 nm using a plate reader.

DNA fragmentation analysis

The apoptotic DNA fragmentation, induced in the Jurkat T cells following treatment with the methylene chloride extract or AUR, was determined as described elsewhere (24). Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (0.5% Triton X-100, 5 mM ethylenediaminetetraacetic acid, 10 mM Tris–HCl and pH 7.4) for 20 min on ice. After centrifugation, the supernatant was collected and treated for 2 h at 50°C with proteinase K and subsequently with RNase for 4 h at 37°C. After extraction with an equal volume of a buffer-saturated phenol, the DNA fragments were precipitated with 2.5 volumes of ethanol and visualized following electrophoresis on a 1.2% agarose gel.

Flow cytometric analysis

The cell cycle progression of Jurkat T cells was analyzed by flow cytometry as described previously (25). After fixation with 67% ethanol at 4°C for 1 h, the cells (1×10^6) were washed with phosphate-buffered saline and re-suspended with 12.5 μg of RNase in 250 μl of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37°C for 30 min before staining of the cellular DNA with 250 μl of propidium iodide (PI) (50 μg/ml) for 20 min. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content. The extent of necrosis was detected with Annexin V–FITC Apoptosis Kit (Clontech, Takara Bio, Shiga, Japan). The cells (1×10^6) were washed with 1× binding buffer and then incubated with Annexin V–FITC and PI for 15 min before being analyzed by flow cytometry according to the manufacturer's instructions.

Preparation of cell lysates and western blot analysis

The cellular lysates were prepared by suspending 5×10^6 Jurkat T cells in 200 μl lysis buffer [137 mM NaCl, 15 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM 3-(*N*-morpholino)propane sulfonic acid, 2.5 μg/ml proteinase inhibitor E-64 and pH 7.2]. The cells were disrupted by sonication and extracted at 4°C for 30 min. An equivalent amount of protein lysate (20 μg) was subjected to electrophoresis on a 4–12% sodium dodecyl sulfate (SDS) gradient polyacrylamide gel with a 3-(*N*-morpholino)propane sulfonic acid buffer. The proteins were electrotransferred to Immobilon-P membranes and then probed with individual antibodies. The detection of each protein was carried out using an ECL western blotting kit according to the manufacturer's instructions. Densitometry was performed by using ImageQuant TL software (Amersham). Arbitrary densitometric units of the protein of interest were corrected for the densitometric units of β-actin.

Detection of mitochondrial cytochrome c release in cytosolic protein extracts

To assess the mitochondrial cytochrome c release in Jurkat T cells following AUR treatment, cytosolic protein extracts were obtained, as described previously (24). Briefly, $\sim 5 \times 10^6$ cells were washed twice with cold phosphate-buffered saline and then suspended in 0.5 ml of a lysis buffer [250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml E-64, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and pH 7.2]. The cells were allowed to swell on ice for 30 min and homogenized with a Dounce homogenizer (20 strokes). The homogenates were centrifuged at 3500 r.p.m. for 10 min at 4°C, and the supernatants were centrifuged at 13700 r.p.m. for 15 min at 4°C. The supernatants were harvested as cytosolic extracts free of mitochondria, and analyzed for mitochondrial cytochrome c release.

Statistical analysis

Unless otherwise indicated, each result in this paper is representative of at least three separate experiments. Values represent the mean ± standard deviation of these experiments. Statistical significance was calculated with Student's *t*-test. *P* values <0.05 were considered significant.

Results

Identification of AUR as an apoptogenic component in Z. schinifolium

When the substances that were obtained from leaves of *Z. schinifolium*, by 80% methanol extraction, were dissolved in water and then fractionated by a series of solvent extractions using methylene chloride, ethyl acetate and *n*-butanol, the methylene chloride extract appeared to contain the most cytotoxic activity toward Jurkat T cells, whereas the other fractions showed no significant cytotoxic effects. As determined by MTT assay, the methylene chloride extract exerted cytotoxic effect at concentrations ranging from 50 to 150 μg/ml, and its IC_{50} was ~75 μg/ml (Figure 1A). The methylene chloride extract (50–150 μg/ml) was able to induce apoptotic DNA fragmentation (Figure 1B). Under the same conditions, although there were no or barely detectable sub-G_1 cells representing apoptotic cells in the continuously growing Jurkat T cells, the accumulation of sub-G_1 cells increased after treatment with the methylene chloride extract in a dose-dependent manner (Figure 1C). These results demonstrate that the methylene chloride extract of *Z. schinifolium* leaves possesses apoptogenic activity toward Jurkat T cells, and suggest that the cytotoxicity of the methylene chloride extract is due to induced apoptosis.

To purify the apoptogenic component further, the methylene chloride extract was applied to silica gel column chromatography and fractionated into 23 fractions (A to W) by a gradient elution (100% hexane → 100% ethyl acetate). The recrystallization of the I fraction, which corresponded to the ninth fraction among 23 fractions, yielded a substance that was identified as 7-[(3,7-dimethyl-2,6-octadienyl)-oxy]-coumarin (AUR). From 4.4 kg of *Z. schinifolium* leaves, ~100 mg of AUR was recovered. In order to examine the cytotoxicity of

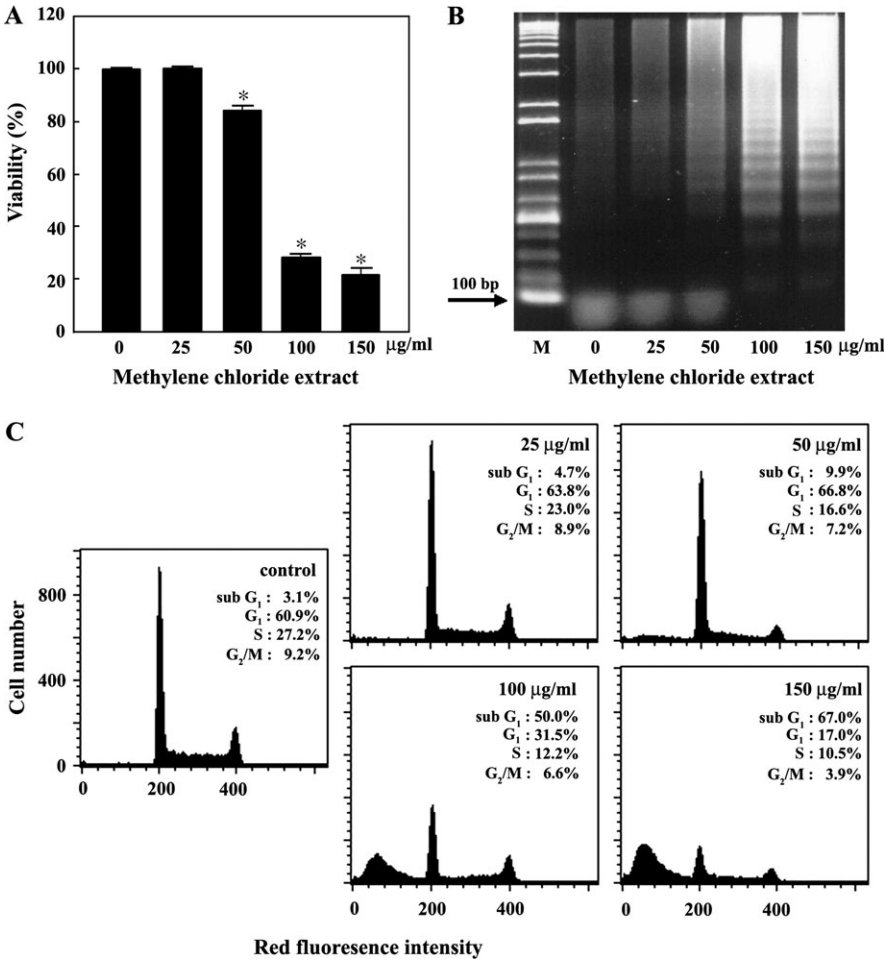

Fig. 1. Effect of the methylene chloride extract of leaves of *Zanthoxylum schinifolium* on cell viability (**A**), apoptotic DNA fragmentation (**B**) and cell cycle distribution (**C**) in Jurkat T cell clone E6.1. Continuously growing Jurkat T cells (5×10^4) were incubated with indicated concentrations of the methylene chloride extract in a 96-well plate for 20 h and the final 4 h were incubated with MTT. The cells were sequentially processed to assess the colored formazan crystal produced from MTT as an index of cell viability. Each value is expressed as mean ± standard deviation ($n = 3$). $^*P < 0.05$ as compared with the control. Equivalent cultures were prepared and the cells were collected to analysis of apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis. To assess cell cycle distribution of Jurkat T cells exposed to the methylene chloride extract for 24 h, the cells were fixed with cold ethanol and then stained with PI for flow cytometric analysis.

AUR toward Jurkat T cells, cell viability following treatment with AUR (5–20 μg/ml) for 20 h was determined by MTT assay. As shown in Figure 2A, after treatment with 5 μg/ml AUR, cell viability remained at the level of control cells untreated. Cell viability, however, declined to the level of 92, 62 and 35% in the presence of 10, 15 and 20 μg/ml, respectively, indicating that the IC_{50} value of AUR was 16.5 μg/ml. Typical apoptotic DNA fragmentation began to be detectable at a concentration of 10 μg/ml and reached a maximum level in the presence of 20 μg/ml AUR, indicating that AUR possesses apoptogenic activity and induces apoptotic DNA fragmentation of Jurkat T cells in a concentration-dependent manner (Figure 2B). Under these conditions, flow cytometric analysis also showed that the accumulation of sub-G_1 cells, following treatment with AUR, was enhanced in a dose-dependent manner (Figure 2C). To examine whether necrosis occurred along with AUR-induced apoptosis, Jurkat T cells treated with AUR at concentrations of 0, 15 or 20 μg/ml for 24 h were stained with Annexin V–FITC and PI and then analyzed by flow cytometry. Although early apoptotic cells stained only with Annexin V–FITC and late apoptotic cells stained with both Annexin V–FITC and PI appeared to significantly increase following AUR treatment dose dependently, necrotic cells stained only with PI were barely detected (Figure 3). These results demonstrate that AUR is an apoptogenic component contained in *Z. schinifolium* leaves.

Involvement of ER stress and mitochondrial cytochrome c release with resultant activation of caspase cascade in AUR-induced apoptosis

To understand the mechanism underlying apoptosis induced by AUR, it was determined whether the apoptogenic activity of AUR could be suppressed by the anti-apoptotic regulatory protein Bcl-xL. When the cytotoxicity of AUR was compared between Jurkat T cells transfected with vector (J/Neo) and Jurkat T cells transfected with *Bcl-xL* gene (J/Bcl-xL) after treatment with AUR at concentrations of 10–20 μg/ml for 20 h, the viability of J/Neo cells, determined by MTT assay, appeared to decrease in a dose-dependent fashion and declined to the level of 32% in the presence of 20 μg/ml (Figure 4A). There was, however, no detectable reduction in the viability of J/Bcl-xL cells in the range of 10–20 μg/ml AUR. DNA fragmentation assay confirmed that AUR at concentrations of 10–20 μg/ml was able to induce apoptotic DNA fragmentation in J/Neo cells in a dose-dependent manner, but it failed to induce DNA fragmentation in J/Bcl-xL cells (Figure 4B). Flow cytometric analysis also showed that the level of sub-G_1 cells was significantly enhanced in J/Neo cells after treatment with AUR, whereas it did not increase in J/Bcl-xL cells (Figure 4C). These results indicate that the AUR-induced apoptosis of Jurkat T cells is negatively regulated by ectopic expression of Bcl-xL and that the cytotoxicity of AUR toward Jurkat T cells is mainly caused by its ability to induce apoptosis.

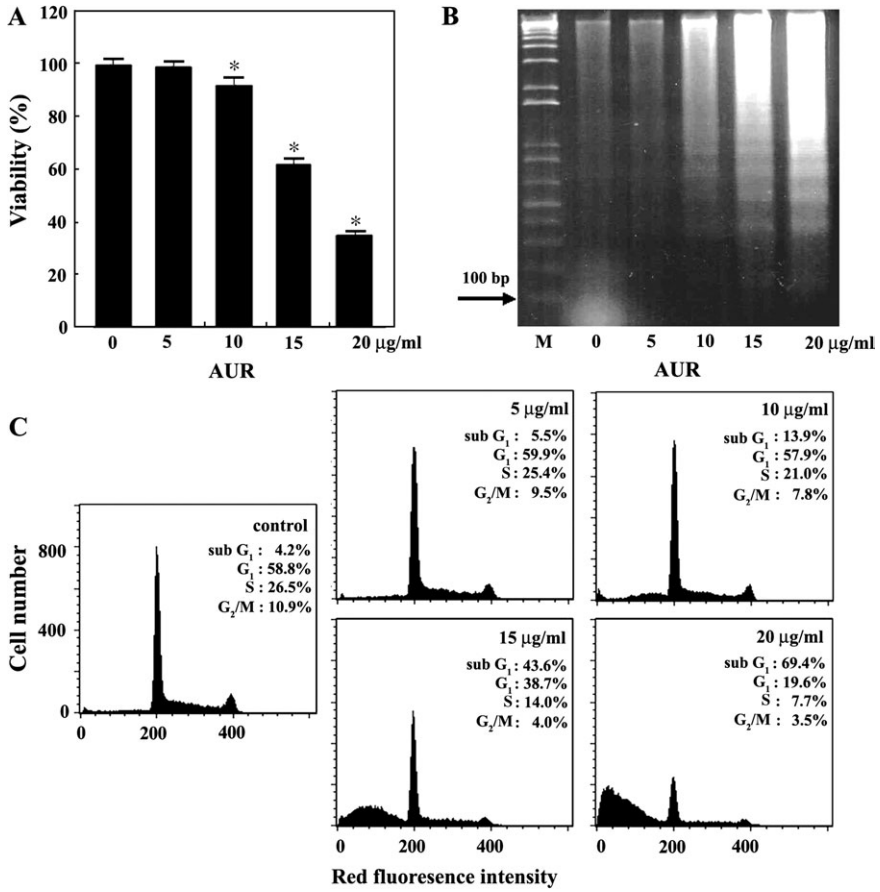

Fig. 2. Effect of AUR on cell viability (**A**), apoptotic DNA fragmentation (**B**) and cell cycle distribution (**C**) in Jurkat T cell clone E6.1. Continuously growing Jurkat T cells (5×10^4) were incubated with indicated concentrations of AUR in a 96-well plate for 20 h and the final 4 h were incubated with MTT. The cells were sequentially processed to assess the colored formazan crystal produced from MTT as an index of cell viability. Each value is expressed as mean ± standard deviation ($n = 3$). $^*P < 0.05$ as compared with the control. Equivalent cultures were prepared and the cells were collected to analysis of apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis. To assess cell cycle distribution of Jurkat T cells exposed to AUR for 24 h, the cells were fixed with cold ethanol and then stained with PI for flow cytometric analysis.

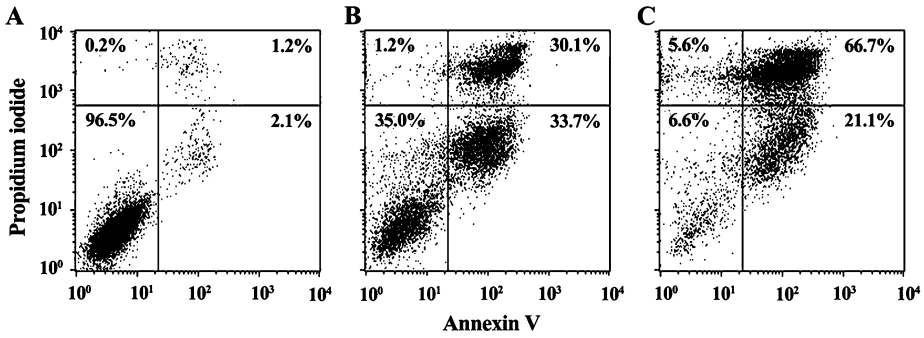

Fig. 3. Annexin V–FITC and PI staining of Jurkat T cell clone E6.1 following treatment with various concentrations of AUR. After Jurkat T cells were incubated with 0 μg/ml (**A**), 15 μg/ml (**B**) or 20 μg/ml (**C**) of AUR for 24 h, the cells were stained and analyzed by flow cytometry to detect Annexin V–FITC-positive and/or PI-positive cells. The unaffected, early apoptotic, late apoptotic and necrotic cells are present in the lower left, lower right, upper right and upper left quadrant, respectively.

The anti-apoptotic role of Bcl-2 and Bcl-xL was initially known to center around their blocking of mitochondrial cytochrome c release into cytosol and the resultant prevention of mitochondrial cytochrome c-dependent activation of caspase cascade (26,27). Recently, it has been reported that stable over-expression of Bcl-xL can protect ER stress-mediated activation of caspase-12 and apoptosis (28). These previous data and current data raised the possibility that mitochondrial cytochrome c release and/or ER stress-mediated apoptotic event, which could be prevented by Bcl-xL, might play an essential role in the AUR-induced apoptosis of Jurkat T cells. To test this prediction, it was investigated in both J/Neo and J/Bcl-xL cells, by western blot analysis, whether the induced apoptosis accompanied not only mitochondrial cytochrome c release that subsequently activated caspase cascade including caspase-9 and caspase-3 but also Fas and FasL up-regulations that could generate Fas signaling to activate caspase-8 prior to the mitochondria-dependent or -independent activation of effector caspases such as caspase-3. As shown in Figure 5A, although there was no detectable cytochrome c in the cytosolic fraction of

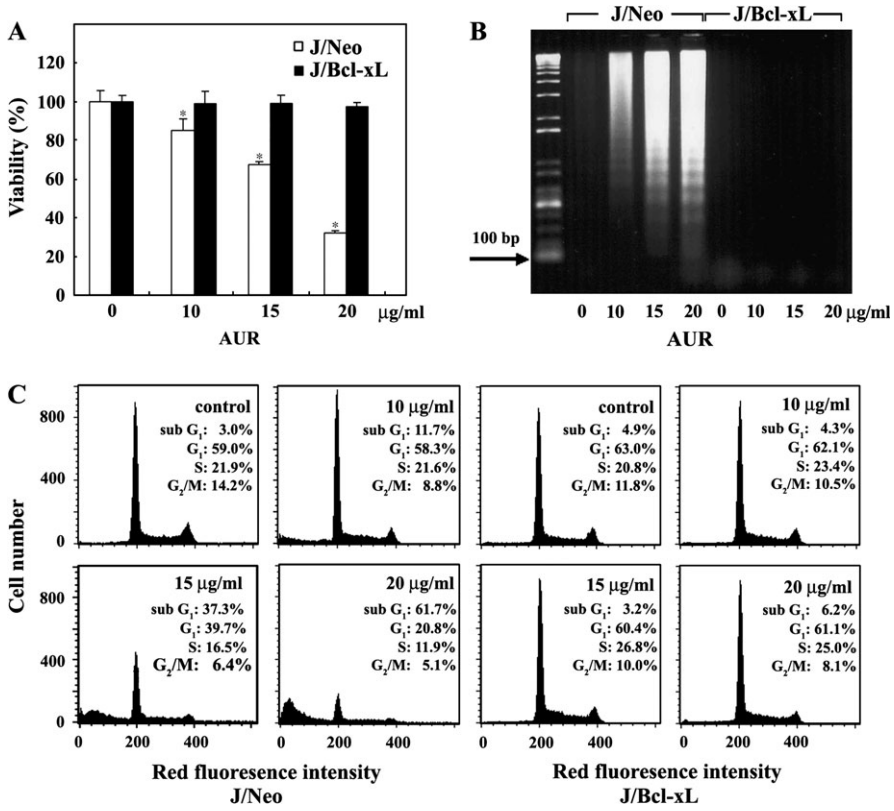

Fig. 4. Effect of AUR on cell viability (**A**), apoptotic fragmentation (**B**) and cell cycle distribution (**C**) in Jurkat T cells transfected with *Bcl-xL* gene (J/Bcl-xL) or vector (J/Neo). J/Bcl-xL cells or control (J/Neo) cells were incubated at a density of 5×10^4 per well with various concentrations of AUR in 96-well plates for 24 h and the final 4 h were incubated with MTT to assess the colored formazan crystals produced from MTT as an index of cell viability. Each value is expressed as mean ± standard deviation ($n = 3$). $^*P < 0.05$ as compared with the control. Equivalent cultures were processed to analyze apoptotic DNA fragmentation. To investigate apoptotic change in cell cycle distribution of J/Bcl-xL or J/Neo cells after exposure to AUR, the cells were fixed with cold ethanol, stained with PI and an equal number of cells (2×10^5) were analyzed by flow cytometry.

continuously growing J/Neo cells, the level of cytochrome c released from mitochondria increased dose dependently in the presence of AUR (10–20 μg/ml). In contrast, the AUR-induced cytochrome c release was abrogated by the ectopic expression of Bcl-xL. Under these conditions, the level of β-actin remained constant, indicating the equivalent loading of the cell lysate in each lane for western analysis. In accordance with mitochondrial cytochrome c release into the cytoplasm of J/Neo cells, the activation of caspase-9, which proceeds through the proteolytic degradation of the inactive pro-enzyme (47 kDa) into the active form (35/37 kDa), was detected (Figure 5B). The activation of caspase-3, through the proteolytic degradation of a 32 kDa pro-enzyme into a 19 kDa activated form, was also detected in a dose-dependent manner in the presence of AUR. As a downstream target of active caspase-3 during the induction of apoptosis, PARP has been reported to be cleaved into two fragments (29). In J/Neo cells after treatment with AUR, the cleavage of PARP was detected along with the activation of caspase-3. There was, however, no alteration in the level of Fas and FasL, excluding the possible involvement of the Fas/FasL system in AUR-mediated death signaling pathway. To understand the contribution of ER stress-mediated apoptotic events as the upstream signals for AUR-induced mitochondrial release of cytochrome c and activation of caspase cascade, the activation of caspase-12, JNK and caspase-8 was also investigated by western blot analysis. In the presence of AUR (10–20 μg/ml), whereas the level of pro-caspase-12 (60 kDa), which appeared to decline dose dependently, became undetectable at 20 μg/ml, the phosphorylated JNK increased by 2-fold. In addition, the activation of caspase-8 via the proteolytic degradation of a 55 kDa pro-enzyme into 41/43 kDa activated form was detected, and the level of 26 kDa Bid protein, which was known to be cleaved by caspase-8 to produce the truncated Bid (tBid) causing the mitochondrial cytochrome c release (30,31), appeared to decline by 83.3-fold in accordance with AUR-induced caspase-8 activation. The level of FLIP, that was reported to inhibit caspase-8 activation (32) and could be cleaved by the active caspase-8 (33), was also down-regulated by 9.2-fold in J/Neo cells. Since caspase-8 as well as pro-caspase-12 was known to be cleaved into the active form in response to ER stress (34) and since the phosphorylated form of JNK, representing the active enzyme that could be translocated to mitochondria to promote cytochrome c release into cytoplasm, was generated by ER stress (35), these results suggest that AUR-induced apoptosis might be initiated by ER stress-mediated apoptotic signals. Under the same conditions, however, these AUR-induced apoptotic events, along with apoptotic DNA fragmentation, were completely prevented in J/Bcl-xL cells. Consequently, these results suggest that AUR-induced apoptosis might be provoked by ER stress-mediated apoptotic signals and the subsequent mitochondria-dependent caspase cascade, which could be negatively regulated by Bcl-xL.

Effect of anti-Fas neutralizing antibody ZB-4 on AUR-mediated apoptosis

Although there was no detectable enhancement in the expression levels of Fas and FasL in Jurkat T cells following treatment with AUR, we examined whether the anti-Fas neutralizing antibody, ZB-4, could block the cytotoxicity of AUR in order to confirm the involvement of the Fas/FasL system in AUR-induced apoptosis. ZB-4 has been shown to prevent the cytotoxic effect of the Fas agonistic antibody, CH-11 (36,37). Pre-treatment using ZB-4 (500 ng/ml), followed by the cytotoxic anti-Fas antibody CH-11 (50 ng/ml), resulted in an almost complete blockage of CH-11-induced cytoxicity in Jurkat

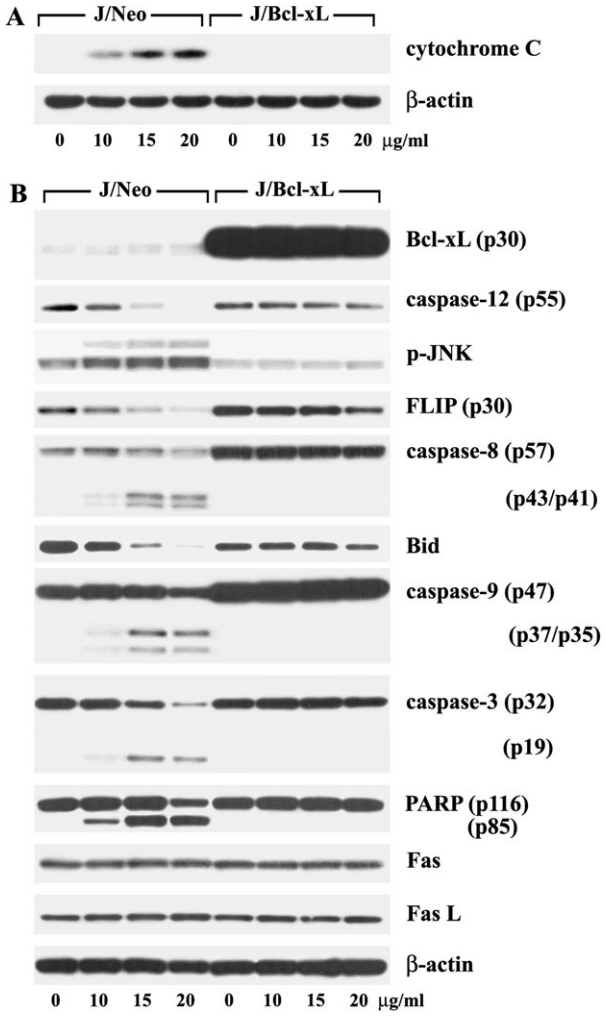

Fig. 5. Western blot analysis of mitochondrial cytochrome c release and β-actin (**A**) and Bcl-xL, caspase-12, phosphorylated JNK, FLIP, caspase-8 activation, Bid, caspase-9 activation, caspase-3 activation, cleavage of PARP, expression levels of Fas and FasL and β-actin (**B**) in Jurkat T cells after treatment with various concentrations of AUR. The cells ($\sim 5 \times 10^6$) were incubated at a concentration of 4×10^5 per ml with indicated concentrations of AUR for 24 h and prepared for cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Materials and methods using ECL western blotting detection system. A representative study is shown and two additional experiments yielded similar results.

T cells (Figure 6A). Under these conditions, the cytotoxic effect of AUR (20 μg/ml) was not reduced by ZB-4 (Figure 6B). These results demonstrate that the AUR-induced apoptosis of Jurkat T cells was not associated with the Fas/FasL system.

Effect of pan-caspase inhibitor z-VAD-fmk on AUR-induced apoptosis

To elucidate further the caspase activation pathway in relation to cytochrome c release for AUR-induced apoptotic cell death in Jurkat T cells, we examined the effect of a caspase inhibitor z-VAD-fmk, which was known to inhibit broad-range caspases (38), on AUR-induced apoptotic events in Jurkat T cells. After Jurkat T cells were pre-treated with z-VAD-fmk at concentrations of 50 or 75 μM for 2 h, the cells were exposed to 20 μg/ml AUR for 20 h. Both cytotoxicity and apoptotic DNA fragmentation caused by AUR were abrogated in the presence of 50 or 75 μM z-VAD-fmk (Figure 7A and 7B). At the same time, AUR-mediated mitochondrial cytochrome c release was prevented in the presence of 50 or 75 μM z-VAD-fmk (Figure 7C). As shown in Figure 7D, all AUR-induced apoptotic events, including the activation of caspase-12, -8, -9 and -3, the down-regulation of FLIP, Bid cleavage, JNK activation and PARP degradation were completely blocked by 50 or 75 μM z-VAD-fmk. These results demonstrate that caspase activation is a prerequisite for AUR-mediated apoptotic DNA fragmentation, and indicate that AUR-mediated caspase activation, which is susceptible to the inhibitory action of z-VAD-fmk, is upstream of JNK activation as well as mitochondrial cytochrome c release during apoptotic signal transduction.

Effect of caspase-8 inhibitor (z-IETD-fmk), calpain inhibitor (E64d), JNK inhibitor (SP600125) or CsA on AUR-induced apoptosis

To elucidate further the death signaling pathway for AUR-induced apoptotic cell death, we investigated the effects of the caspase-8 inhibitor (z-IETD-fmk) (39), calpain inhibitor (E64d) (40), JNK inhibitor (SP600125) (41) or the mitochondrial permeability transition pore inhibitor (CsA) (42) on AUR-induced apoptotic events in Jurkat T cells. After Jurkat T cells were pre-treated with each inhibitor alone or various combinations of inhibitors for 2 h, the cells were exposed to 20 μg/ml AUR for 20 h. As determined by flow cytometry, the apoptotic sub-G_1 peak, induced by AUR without inhibitors, increased to the level of 46.8% (Figure 8A). Whereas the AUR-induced sub-G_1 peak was completely diminished by pre-treatment with z-IETD-fmk, it was not reduced by individual or simultaneous pre-treatment with E64d, SP600125 or CsA unless z-IETD-fmk was concomitantly present. Under these conditions, western blot analysis revealed that AUR-induced apoptotic events, such as the activation of caspase-8, -9 and -3, Bid cleavage and PARP degradation, were abrogated only by z-IETD-fmk, irrespective of the simultaneous presence of E64d, SP600125 and CsA (Figure 8B). The AUR-mediated activation of caspase-12, however, was not abrogated even in the presence of z-IETD-fmk or E64d, indicating that the caspase-12 activation was not downstream of the caspase-8 or m-calpain activation, but was induced independently of the caspase-8 activation by ER stress. In addition, the presence of CsA failed to block not only AUR-mediated caspase-12 activation, caspase-8 activation and Bid cleavage but also the activation of caspase-9 and -3 and PARP degradation. Since the activation of caspase-9 and -3 and the resultant degradation of PARP were known to be mediated by either mitochondrial cytochrome c-dependent or -independent apoptotic pathways (10), these results demonstrated that the AUR-induced activation of caspase-8 and Bid cleavage were upstream of the mitochondrial cytochrome c release, which, in turn, could activate caspase-9 and caspase-3 leading to PARP degradation, and that the AUR-induced activation of caspase-9 and -3 could be also induced, independently of the mitochondrial cytochrome c release, as a downstream event of the activation of caspase-12 and caspase-8. Consequently, these results indicate that the AUR-induced apoptotic signaling pathway, leading to apoptotic DNA fragmentation in Jurkat T cells, is triggered by the ER stress-mediated activation of caspase-12 and -8, and is then relayed by mitochondrial cytochrome c-dependent or -independent activation of caspase-9 and -3.

Cytotoxic effect of AUR on human peripheral T cells

Since current data show that AUR possesses cytotoxicity against Jurkat T cells with the IC_{50} value of 16.5 μg/ml, resulting from inducing apoptotic cell death, it was of interest to investigate whether the drug is less toxic to normal cells. In this context, the cytotoxic effects of AUR on the viability of human peripheral T cells or the IL-2-dependent proliferation of activated human T cells, which were obtained by stimulation of human peripheral T cells with 0.2 μg/ml PHA for 60 h, were compared with that on the viability of Jurkat T cells. When the individual cells were incubated with various concentrations of AUR in 96-well plates for 24 h and then cell viability was measured by MTT assay, the viability of resting peripheral T cells was not markedly affected in the presence of 5–30 μg/ml AUR, and remained at the level of 88% at a concentration of 40 μg/ml, whereas the IL-2-dependent proliferation of activated T cells appeared to be more sensitive to the cytotoxic effect of AUR than resting T cells and showed

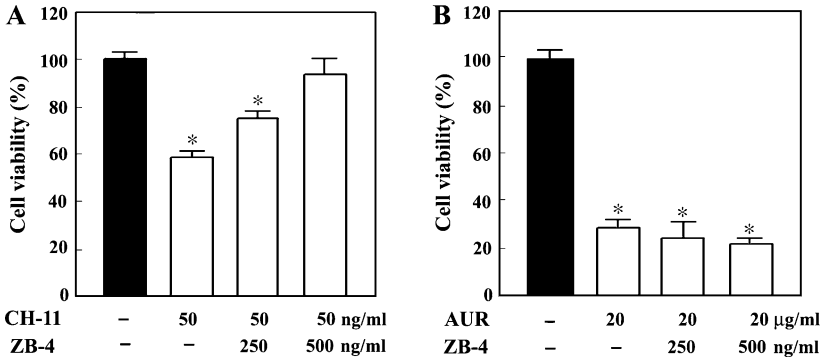

Fig. 6. Effect of anti-Fas neutralizing antibody ZB-4 on anti-Fas agonistic antibody CH-11 (**A**) or AUR-mediated cytotoxicity (**B**) in Jurkat T cell clone E6.1. In 96-well plates, Jurkat T cells were pre-treated for 1 h using 250 and 500 ng/ml of ZB-4 and then challenged with either the anti-Fas agonistic antibody CH-11 (50 ng/ml) or the AUR (20 μg/ml). After 20 h, an MTT assay was performed to determine the cell viability. Each value is expressed as mean ± standard deviation ($n = 3$). $^*P < 0.05$ as compared with the control.

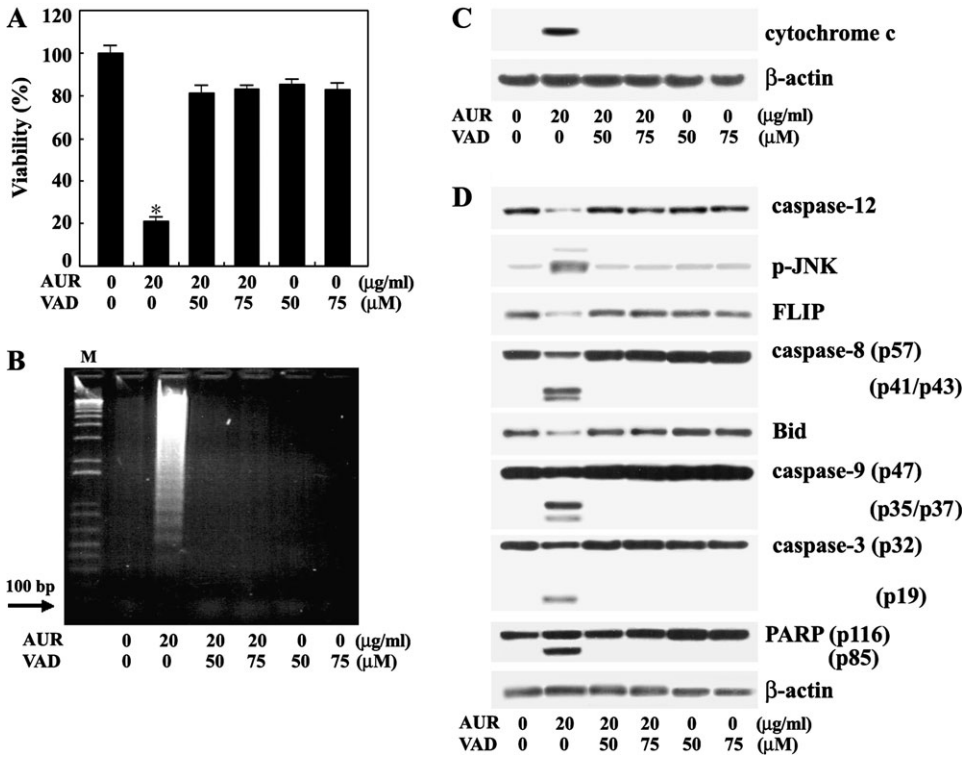

Fig. 7. Suppressive effect of a broad-spectrum caspase inhibitor z-VAD-fmk on the cytotoxicity (**A**), apoptotic DNA fragmentation (**B**), mitochondrial cytochrome c release (**C**) and various apoptotic events including caspase cascade (**D**) induced by AUR in Jurkat T cell clone E6.1. Jurkat T cells were pre-treated with a caspase inhibitor z-VAD-fmk at concentrations of 50 or 75 μM for 2 h, and were then incubated at a density of 5×10^4 per well with 20 μg/ml AUR in 96-well plates. After incubation for 20 h, MTT was added for additional 4 h. The cells were processed to assess the colored formazan crystal produced from MTT as an index of cell viability. Each value is expressed as mean ± standard deviation ($n = 3$). $^*P < 0.05$ as compared with the control. Equivalent cultures were prepared and the cells were processed to analyze apoptotic DNA fragmentation and caspase activation induced by AUR as described in Materials and methods. For western analysis data, a representative study is shown and two additional experiments yielded similar results.

a viability of 85% at a concentration of 20 μg/ml (Figure 9). Under the same conditions, the cell viability of malignant Jurkat T cells began to decrease in the presence of 10 μg/ml, declined to the level of 30% at a concentration of 20 μg/ml and reached a minimum level at a concentration higher than 30 μg/ml. These results indicate that the IC_{50} values for resting human T cells, activated T cells, and Jurkat T cells were >40, 25 and 16.5 μg/ml, respectively, suggesting that leukemia Jurkat T cells are more sensitive to the apoptogenic activity of AUR than are normal human T cells.

Discussion

Several *in vivo* studies have reported that AUR has valuable chemopreventive activity against the skin, tongue, esophagus and colon carcinogenesis in rodents (18,20,21). Since the AUR-induced apoptosis of tumor cells can be directly associated with its chemopreventive activity, we have investigated apoptogenic activity and underlying mechanism through which AUR causes apoptotic cell death in human acute leukemia Jurkat T cells. This is the first report to demonstrate

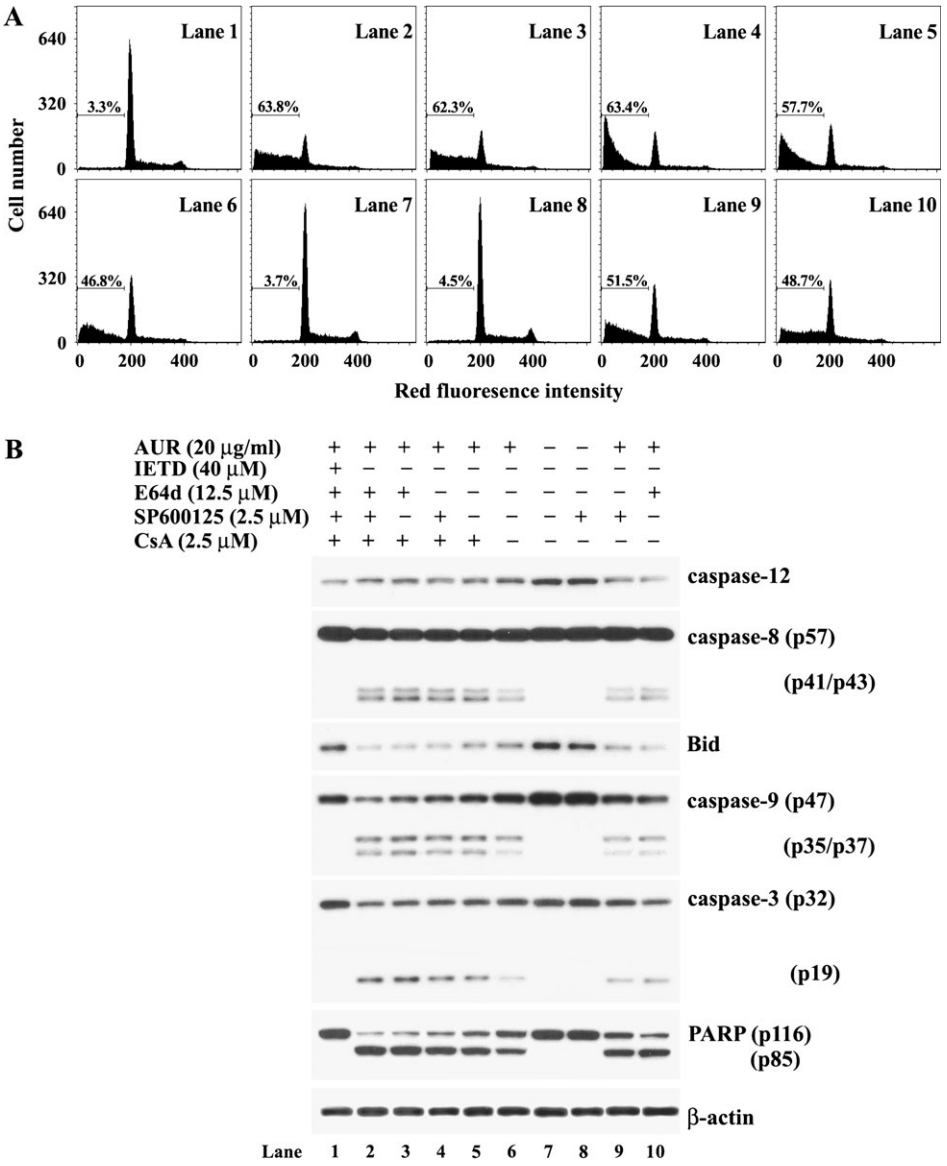

Fig. 8. Western blot analysis of caspase-12, capase-8 activation, Bid, caspase-9 activation, caspase-3 activation and cleavage of PARP (**A**) and apoptotic change in the cell cycle distribution (**B**) in Jurkat T cell clone E6.1 after treatment with 20 μg/ml of AUR in the presence of IETD, SP600125, E64d or CsA (B). Jurkat T cells were pre-incubated at a density of 4×10^5 per ml in the individual or simultaneous presence of z-IETD-fmk, SP600125, E64d or CsA for 2 h and then treated with 20 μg/ml of AUR for 20 h. After the cells were harvested, the analysis of cell cycle distribution was performed on an equal number of cells (5×10^5) by flow cytometry after staining of DNA by PI. Western analysis was performed as described in Materials and methods using ECL western blotting detection system. A representative study is shown and two additional experiments yielded similar results.

that AUR exerts cytotoxic effect on Jurkat T cells via the induction of apoptosis without necrosis. No involvement of necrosis in the cytotoxic effect was evidenced by flow cytometric analysis of Jurkat T cells stained with Annexin V–FITC and PI after AUR treatment. The AUR-induced apoptosis of Jurkat T cells appeared to be initiated by the ER stress-mediated activation of caspase-8, and then mediated by the mitochondria-dependent or -independent activation of caspase cascade, resulting in apoptotic DNA fragmentation. Although it has been shown that chemotherapeutic agents often induced apoptosis of tumor cells by triggering the Fas/FasL pathway (4–7), we could exclude the involvement of Fas/FasL system in the AUR-mediated apoptosis of Jurkat T cells because the expression level of Fas and FasL was not elevated following treatment with AUR, and the cytotoxic effect of AUR was not reduced by the anti-Fas neutralizing antibody ZB-4. The ER stress-mediated pathway, rather than Fas/FasL pathway, through which AUR provokes apoptosis of Jurkat T cells may permit better application of AUR to chemotherapeutic treatment, in that many tumors are refractory to Fas/FasL system-mediated apoptosis by the acquisition of malfunctional Fas (43,44) or its downstream signaling mediator (45).

Since AUR-caused cytotoxicity and apoptotic DNA fragmentation in Jurkat T cells were completely diminished by over-expression of Bcl-xL that was known to prevent induced apoptosis via the blocking of the mitochondrial cytochrome c release and/or ER stress-initiated apoptotic event (26–28), we examined the involvement of mitochondria and ER in the AUR-induced apoptosis. When we investigated cytochrome c release from mitochondria, which was frequently detected in chemotherapeutic agent-induced apoptosis (46–48), and the resultant activation of caspase cascade including caspase-9 and caspase-3, leading to PARP degradation in Jurkat T cells following exposure to AUR, the level of mitochondrial cytochrome c release increased by AUR in a dose-dependent manner. In addition, the activation of both caspase-9 and caspase-3 and the degradation of PARP were enhanced in accordance with the mitochondrial cytochrome c

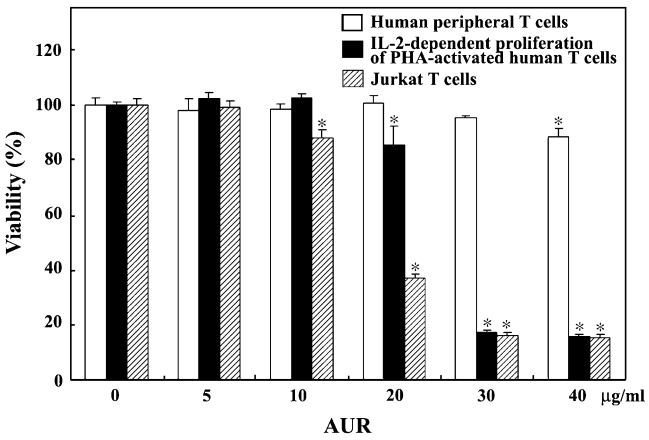

Fig. 9. Cytotoxic effect of AUR on human peripheral T cells unstimulated, IL-2-dependent proliferation of PHA-activated T cells and proliferation of Jurkat T cells. Peripheral T cells (2×10^5 cells per well) were incubated with various concentrations of AUR for 20 h, further incubated with MTT solution for 4 h and then processed to assess the colored formazan crystal produced from MTT as an index of cell viability. To induce IL-2-dependent proliferation of activated T cells, human peripheral mononuclear cells were activated with 0.2 μg/ml of PHA for 60 h, and then the activated T cells were harvested and incubated with various concentrations of AUR at a density of 1×10^5 per well as well as 50 U/ml of recombinant human IL-2 in 96-well plates. For treatment of Jurkat T cells with AUR, the cell density was 5×10^4 per well. Each value is expressed as mean ± standard deviation ($n = 3$). $^*P <$ 0.05 as compared with the control.

release. These AUR-induced events, however, were completely inhibited by ectopic over-expression of Bcl-xL. These results indicated that the AUR-mediated activation of mitochondria-dependent caspase cascade was negatively regulated by Bcl-xL and was required for the induced apoptosis. As an upstream event of mitochondrial cytochrome c release in the chemotherapeutic agent-induced apoptosis of tumor cells, ER stress-mediated death signaling has been implicated (12,13). To examine the involvement of ER stress in AUR-induced mitochondrial cytochrome c release, we investigated whether AUR-induced apoptosis in Jurkat T cells accompanied the activation of three pro-apoptotic regulators such as caspase-12, caspase-8 and JNK, which could be mediated by ER stress (34,35). Although the treatment of Jurkat T cells with AUR resulted in the activation of caspase-12, caspase-8 and JNK, the active caspase-8 turned out to play a central role in the mitochondrial cytochrome c release and the activation of caspase-9 and caspase-3 in the AUR-induced apoptosis. This role of caspase-8 appeared to be executed by the Bid protein (26 kDa), which could be cleaved by the caspase-8 into a tBid (15 kDa). Previously, it was reported that the tBid, generated from the Bid protein by the active caspase-8, was rapidly translocated to mitochondria in order to allow mitochondrial membrane transition pore opening and subsequent release of cytochrome c (31,49). Although we failed to observe the generation of tBid, by western blot analysis, in Jurkat T cells following exposure to AUR, presumably due to the short half-life of tBid, we clearly detected that a significant reduction in the level of Bid protein (26 kDa), in accordance with the AUR-induced caspase-8 activation as well as mitochondrial cytochrome c release, reflecting that AUR-induced cytochorome c release was mediated through Bid cleavage by caspase-8. Like the Bid protein, the FLIP was known to be the substrate of caspase-8 (33). Along with the AUR-induced activation of caspase-8 as well as the resultant reduction in the level of the Bid protein, the FLIP level was also reduced. This supported the fact that the active form of caspase-8, which was detectable by western analysis in Jurkat T cells following exposure to AUR, was enzymatically active enough to cleave the substrates, Bid and FLIP. Again, these AUR-mediated apoptotic events were completely blocked by ectopic over-expression of Bcl-xL. Consequently, these results indicated that AUR-induced apoptosis was provoked by ER stress-mediated apoptotic signals and the subsequent activation of mitochondria-dependent caspase cascade, which could be negatively regulated by Bcl-xL.

In our studies, AUR-induced caspase-8 activation and the resultant Bid cleavage, as the upstream event of the cytochrome c release, became more evident by examining the AUR-induced apoptotic events in the individual or simultaneous presence of inhibitory chemicals such as a pan-caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), m-calpain inhibitor (E64d), JNK inhibitor (SP600125) and mitochondrial permeability transition pore inhibitor (CsA). AUR-induced caspase-8 activation and Bid cleavage were abrogated in the presence of z-VAD-fmk, which appeared to prevent AUR-induced mitochondrial cytochrome c release as well as apoptosis. These events, however, were not abrogated in the individual or simultaneous presence of E64d, SP600125 and CsA, unless z-IETD-fmk was combined. In response to ER stress, the m-calpain, a family member of cysteine proteases, was also known to cleave the Bid protein, which resulted in mitochondrial cytochrome c release (50). In our studies, however, the contribution of m-calpain to Bid cleavage could be excluded because the presence of the m-calpain inhibitor E64d was unable to block AUR-induced Bid cleavage.

Besides cleaving the Bid protein, m-calpain was known to be involved in the ER stress-mediated activation of caspase-12 (51). In this process, a proposed mechanism of the caspase-12 activation is that Ca^{2+} released from the ER in response to ER stress activates the m-calpain, which is then translocated from cytosol to ER to cleave off the CARD pro-domain of caspase-12, resulting in caspase-12 activation. Alternatively, caspase-7 has been reported to be translocated to ER and cleaves pro-caspase-12 to generate active caspase-12 (34). Whereas the AUR-mediated activation of caspase-12 was abrogated in the presence of z-VAD-fmk, it was not affected by other inhibitors including z-IETD-fmk, E64d and CsA, demonstrating that such caspase-12 was not downstream of the activation of caspase-8 and m-calpain or mitochondrial cytochrome c release. These results have suggested that a caspase such as caspase-7, rather than the calpain, was most probably involved in the AUR-mediated activation of caspase-12. In addition, although caspase-12 has been reported to directly activate pro-caspase-9, independently of both the mitochondrial cytochrome c and apoptotic protease-activating factor-1, in murine cells (10,11), it was not the case for AUR-induced apoptosis in human Jurkat T cells. AUR-mediated caspase-12 activation, which was detected regardless of the individual or simultaneous presence of z-IETD-fmk, E64d, SP600125 and CsA, did not accompany the activation of caspase-9 and -3 and PARP cleavage only when the caspase-8 activation was prevented by z-IETD-fmk. Thus, it seemed likely that caspase-8 activation, rather than caspase-12 activation, resulting from AUR-caused ER stress played an essential role in the mitochondria-independent activation of caspase-9 and -3, leading to PARP degradation. It is noteworthy that in contrast to murine cells, a functional caspase-12 protein may not be expressed in human cells because the human caspase-12 gene possesses deleterious mutations that prevent the expression of a full-length protein (52). Thus, human caspase-4 and caspase-5, both of which have a CARD pro-domain at the N-terminal and show a high similarity to mouse caspase-12, have been proposed to play roles in the ER stress-mediated apoptosis of human cells (53). Further studies, however, are required to clarify the involvement of caspase-4 and caspase-5, instead of a human isoform of caspase-12, in the mitochondria-independent activation of caspase-9 and -3 during the AUR-induced apoptosis of Jurkat T cells.

In addition to activating caspase-8, ER stress was known to activate JNK, which was then translocated to the mitochondrial membrane in order to stimulate the phosphorylation of Bim, causing mitochondrial cytochrome c release (12,13). For the ER stress-mediated activation of JNK, the IRE1α localized to the ER membrane was known to recruit the cytosolic adaptor protein, tumor necrosis factor receptor-associated factor 2, which then recruited and activated ASK, the proximal component of the JNK pathway (13,54). Since the JNK activation, which was detected in Jurkat T cells following exposure to AUR, was completely prevented by the addition of z-VAD-fmk, it

was likely that AUR-induced activation of JNK might not be dictated by ER stress but by the caspase-dependent process.

To examine if there is a difference in the apoptogenic effect of AUR on tumor cells and normal cells, we have compared the cytotoxicity of AUR against Jurkat T cells with that against normal human T cells. The IC_{50} value for resting human T cells, activated T cells and Jurkat T cells appeared to be >40, 25, 16.5 μg/ml, respectively. This indicated that normal human T cells were more refractory to the cytotoxicity of AUR as compared with Jurkat T cells, which might permit the better application of AUR to chemotherapeutic treatments. Since the cytotoxicity of AUR in T cells was due to apoptosis initiated by the ER stress-mediated activation of caspase-8 and the subsequent mitochondria-dependent or -independent activation of caspase cascade, it seemed likely that the best resistance of unstimulated peripheral T cells, as compared with other cells tested against AUR, might result from a poorly developed ER and mitochondria and a low level of death signaling mediators in unstimulated peripheral T cells.

In conclusion, these results demonstrated that AUR, a purified apoptogenic substance from the leaves of *Z. schinifolium*, induced the apoptotic cell death of human acute leukemia Jurkat T cells via the ER stress-mediated activation of caspase-8 and the subsequent induction of mitochondria-dependent or -independent activation of caspase-9 and caspase-3, leading to PARP degradation. These findings will be useful for evaluating the potency of AUR as an antitumor agent.

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