Biochemical Pharmacology 79 (2010) 389–398

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Investigation of anti-leukemia molecular mechanism of ITR-284, a carboxamide analog, in leukemia cells and its effects in WEHI-3 leukemia mice

Yen-Fang Wen [a,1], Jai-Sing Yang [b,1], Sheng-Chu Kuo [c], Chrong-Shiong Hwang [a], Jing-Gung Chung [d], Hsi-Chin Wu [e], Wen-Wen Huang [d], Jia-Hua Jhan [f], Chung-Ming Lin [g,**], Hui-Jye Chen [h,*]

[a] Medicinal Chemistry Laboratory, Biomedical Engineering Research Laboratories, Industrial Technology Research Institute, Hsinchu 300, Taiwan
[b] Department of Pharmacology, China Medical University, Taichung 404, Taiwan
[c] Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 404, Taiwan
[d] Department of Biological Science and Technology, China Medical University, Taichung 404, Taiwan
[e] School of Medicine, China Medical University, Taichung 404, Taiwan
[f] School of Pharmacy, China Medical University, Taichung 404, Taiwan
[g] Department of Biotechnology, Ming Chuan University, Taoyuan 333, Taiwan
[h] Graduate Institute of Molecular Systems Biomedicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 404, Taiwan

ARTICLE INFO

Article history:
Received 30 June 2009
Accepted 10 September 2009

Keywords:
ITR-284
Anti-leukemia activity
Apoptosis
Caspase
Orthotopic mice model of leukemia

ABSTRACT

ITR-284, a potent anti-leukemia agent of carboxamide derivative, has been shown to inhibit the proliferation of leukemia cells. In this study, the underlying molecular mechanisms *in vitro* and anti-leukemia activity *in vivo* of ITR-284 were investigated. ITR-284 reduced the cell viability and induced apoptosis in HL-60 and WEHI-3 leukemia cells. Following exposure of cells to 30 nM of ITR-284, there is a time-dependent decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) and an increase in the reactive oxygen species (ROS). ITR-284 treatment also caused a time-dependent increase of Fas/CD95, cytosolic cytochrome *c*, cytosolic active form of caspase-8/-9/-3, cytosolic Apaf-1 and Bax, and the decrease of Bcl-2. However, the ITR-284-induced caspase-8/-9 and -3 activities can be blocked by pan-caspase inhibitor (Z-VAD-FMK). In addition, the anti-leukemia effects of ITR-284 *in vivo* were further evaluated in BALB/c mice inoculated with WEHI-3 cells. Orally treatment with ITR-284 (2 and 10 mg/kg/alternate day for 7 times) increased the survival rate and prevented the loss of body weight in leukemia mice. The enlargement of spleen and infiltration of immature myeloblastic cells into spleen red pulp were significantly reduced in ITR-284-treated mice compared with control mice. Moreover, ITR-284 application can enhance the anti-leukemia effect of all-trans retinoic acid (ATRA). These results revealed that ITR-284 acted against both HL-60 and WEHI-3 *in vitro via* both intrinsic and extrinsic apoptotic signaling pathways, and exhibited an anti-leukemic effect in a WEHI-3 orthotopic mice model of leukemia.

1. Introduction

Leukemia is one of the commonly diagnosed neoplasm and the major leading causes of human death [1,2]. Bone marrow transplant, radiotherapy and chemotherapy are used in the clinical therapy of leukemia patients [3,4], but all treatments are not yet satisfactory. In clinic, all-trans retinoic acid (ATRA) has been widely used as the differentiation derivation therapy of acute promyelocytic leukemia (APL) [5]. Despite the complete cure of several APL cases by ATRA, severe side effects such as retinoids and ATRA syndrome are still observed [6]. This side effect of ATRA in leukemia is far from acceptable and searching for a novel drug is crucial. So far, the best strategy for killing leukemia cells is through the induction of cancer cell apoptosis [7,8]. The induction of apoptosis in cancer cells has been shown to be the major anti-cancer mechanism for cancer therapy. Apoptosis is characterized by a series of morphological changes including translocation of phosphatidylserine (PS) of the plasma membrane, cell shrinkage, chromatin condensation, DNA fragmentation and the formation of apoptotic bodies [9,10]. Two main apoptotic pathways converge to activate the caspase family proteins when cells undergo apoptosis [11]. The extrinsic pathway or death receptor pathway is triggered by binding of the ligand to its surface receptor such as FasL and CD95/Fas [12,13], sending the death signal from the cell membrane

* Corresponding author at: Graduate Institute of Molecular Systems Biomedicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung 404, Taiwan. Tel.: +886 4 22052121x7722; fax: +886 4 22333641.
** Corresponding author at: Department of Biotechnology, Ming Chuan University, 5 De Ming Rd., Gui Shan District, Taoyuan 333, Taiwan. Tel.: +886 3 3517001x3558; fax: +886 3 3593878.
E-mail addresses: lin.chungming@gmail.com (C.-M. Lin), huijyechen@yahoo.com, huijyechen@mail.cmu.edu.tw (H.-J. Chen).
[1] These two authors contributed equally to this work.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.09.011

to the cytoplasm, activating the caspase-8 and caspase-3 activities [12]. The intrinsic pathway or mitochondria-dependent pathway is triggered by various stimuli including DNA damage, reactive oxygen species (ROS) and chemotherapeutic agents [14,15]. These apoptotic signals converge on mitochondria, causing the loss of mitochondrial membrane potential ($\Delta\Psi_m$), and the release of cytochrome *c*. The released cytochrome *c* assembles with Apaf-1 to form apoptosome and converts pro-caspase-9 into caspase-9. Then pro-caspase-3 is activated by caspase-9, finally leading to apoptosis [16]. This apoptotic signal in mitochondria is also regulated by anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax [17,18].

A variety of cell-based systems are available for the *in vitro* and *in vivo* evaluation of new anti-leukemia agents. Recently, we have designed and synthesized a series of carboxamide derivatives as novel anti-leukemia agents and found that many of these compounds exhibited potent cytotoxicities against leukemia cell lines [19]. Among these compounds, ITR-284 (*N*-(2-dimethylaminoethyl)-4,8-dihydrobenzo [1,2-b,4,5-b′] dithio-phene-2-carboxamide phosphoric acid salt) (Fig. 1A) is the most potent anti-leukemia agent. Our previous report has shown that ITR-284 significantly inhibited the proliferation of human leukemia HL-60 cells, suggesting that ITR-284 represents a promising candidate as an anti-leukemia drug with low toxicity to normal cells [19].

In this study, we further investigated the molecular mechanisms of ITR-284 on HL-60 and WEHI-3 leukemia cells *in vitro* and its anti-leukemia activity in WEHI-3 cells orthotopic model of leukemia *in vivo*. The results demonstrated that ITR-284 can

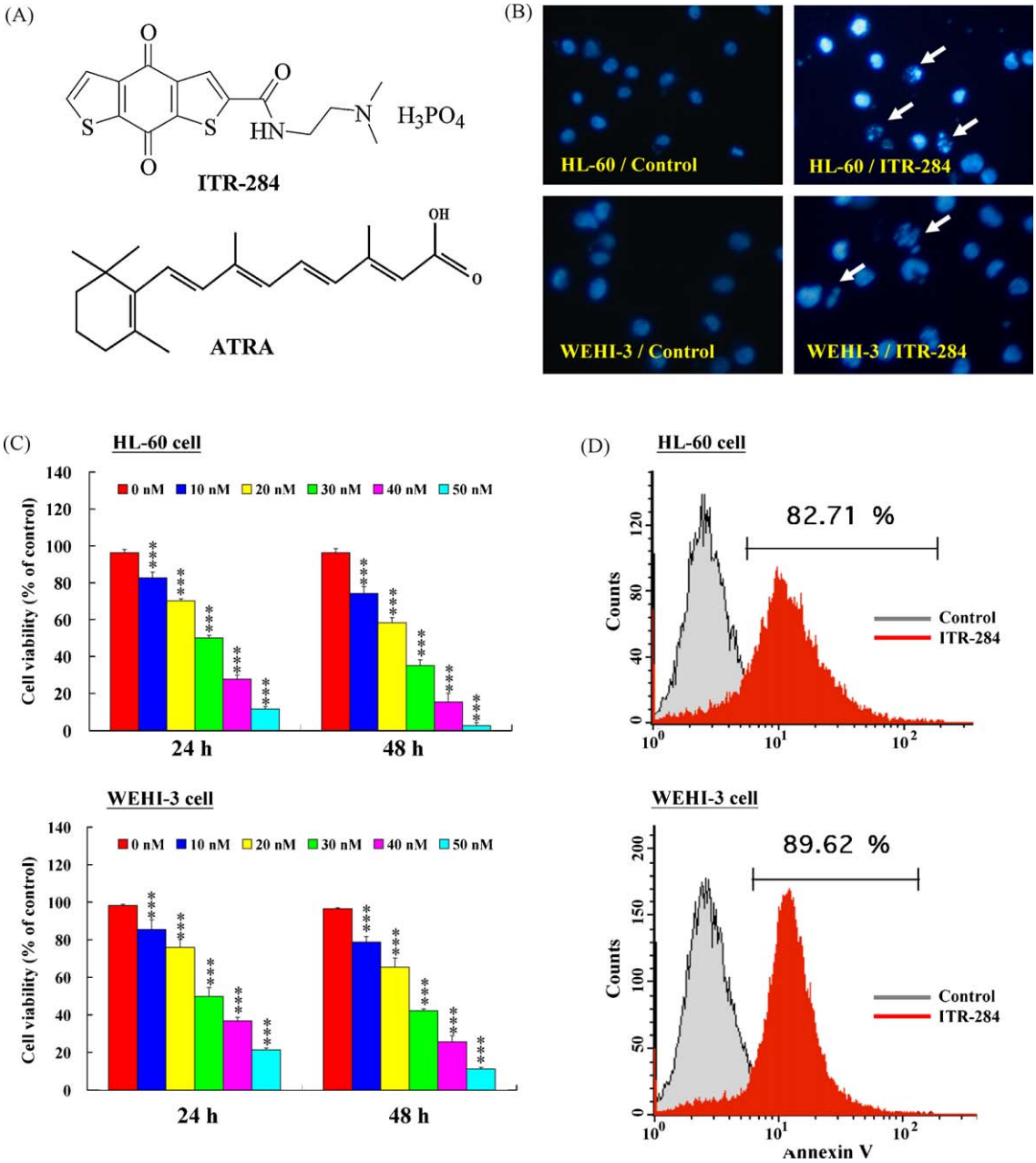

Fig. 1. Effects of ITR-284 on cell viability and apoptosis in HL-60 and WEHI-3 cells. (A) The chemical structures of ITR-284 (upper panel) and ATRA (lower panel). (B) The cell viability of both cells after 24 and 48 h of ITR-284 treatment. Each point is the mean ± S.E.M. of three independent experiments. (C) Cells treated with ITR-284 (30 nM, 24 h) were stained with DAPI and observed by fluorescence microscope (400×). ITR-284 significantly induced chromatin condensation and nuclei fragmentation in treated cells (arrows). (D) ITR-284-treated cells (30 nM, 24 h) were stained for Annexin V and analyzed by flow cytometry. Viable cells were determined by PI exclusion and immediately analyzed by flow cytometry. Gray symbol, control cells. Red symbol, treated cells.

trigger apoptosis through both the intrinsic and extrinsic signaling pathways in HL-60 and WEHI-3 leukemia cells and elicited anti-leukemia effects in the WEHI-3 cells orthotopic model *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Propidium iodide (PI), RNase A, Proteinase K and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pan-caspase inhibitor (Z-VAD-FMK) was from R&D Systems (Minneapolis, MN, USA). 3′-Dihexyloxacarbocyanine iodide ($DiOC_6$) and 4′,6′-diamidino-2-phenylindole (DAPI) was from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture and cell viability assay

The leukemia cell lines HL-60 and WEHI-3 were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine at 37 °C under a humidified 5% CO_2 atmosphere. For cell viability assay, 2.5×10^5 cells were seeded into each well of 24-well plates, then treated with various concentrations of ITR-284 for 24 and 48 h. Percentage of viable cells was determined by PI exclusion method and flow cytometry (FACS Calibur™, Becton-Dickinson, NJ, USA) equipped with a laser at 488 nm wavelength [20]. For treatments with inhibitors, cells (2.5×10^5 cells/mL) were seeded into 24-well plates for 24 h, pretreated with 10 mM *N*-acetylcysteine (NAC, antioxidant) (Sigma–Aldrich), 1 μM cyclosporine A (CSA, $\Delta\Psi_m$ inhibitor) (Sigma–Aldrich) or 500 ng/mL FasL monoclonal antibodies (mAb, Fas inhibitor) (BD Pharmingen Inc., San Diego, CA, USA) for 1 h, followed by treatment with or without 30 nM of ITR-284. The cells were then harvested at 24 h to determine the percentage of viable cells as described previously [21].

2.3. Examination of cell apoptosis by DAPI and Annexin V-FITC staining

For apoptosis assay, cells were cultured in a density of 1×10^4/mL in 6-well plates, then treated with 30 nM of ITR-284 for 24 h. For DAPI staining, cells were harvested, stained with DAPI, and mounted with DABCO (Sigma–Aldrich). Apoptotic cells were determined by the formation of apoptotic bodies under fluorescence microscope. For Annexin V-FITC staining, cells were cultured in a density of 2.5×10^5/mL in 24-well plates, treated with 30 nM of ITR-284 for 24 h, harvested and incubated for 30 min with conjugated Annexin V-FITC (1 μg/mL) at room temperature in the dark according to the manufacturer's instruction (Serotec, MN, USA), and analyzed by flow cytometry [22,23].

2.4. Flow cytometric analysis of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$)

Cells were cultured in a density of 2.5×10^5/mL in 24-well plates, then 30 nM of ITR-284 was added and incubated for 0, 3, 6 and 12 h respectively. After ITR-284 treatment, ROS and $\Delta\Psi_m$ were assessed by cell permeable marker dyes DCFH-DA and $DiOC_6$, and analyzed by flow cytometry [24].

2.5. Western blotting

For the isolation of cytosolic fractions, 1×10^7 cells were cultured in a 75T flask, then 30 nM of ITR-284 was added and incubated for 0, 6, 12 and 24 h. The cells were harvested and re-suspended in 5 volumes of buffer A (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 1 μg/mL aprotinin, 100 μg/mL PMSF, and 250 mM sucrose). The cells were homogenized for 40 strokes, and centrifuged at 1200 rpm for 10 min at 4 °C. The supernatant was collected and further centrifuged at 16 000 rpm for 60 min at 4 °C to obtain the cytosolic fraction for Western blotting analyses. For the isolation of whole cell lysate, the cells were harvested and re-suspended in proteins lysis buffer (20 mM Tris/acetate, pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM orthovanadate, 1 mM sodium glycerophosphate, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 5 mM β-mercaptoethanol, 1 mM benzamidine, 35 μg/mL PMSF, and 5 μg/mL leupeptine), homogenized ultrasonically, and put on ice for 20 min before centrifugation at 13 000 rpm for 15 min. The protein concentration was measured by using a BCA assay kit (Pierce Biotechnology, Rockford, USA). Equal amounts of cell lysate were resolved on 10–12% SDS–PAGE and transferred to PVDF membrane (Amersham Pharmacia Biotech). After blocking, the blots were incubated with individual primary antibodies including Fas/CD95, AIF, Bcl-2 and Bax (Santa Cruz, CA, USA), Apaf-1 (Abcam, Cambridge, UK), caspase-8, caspase-9 and caspase-3 (Cell Signaling Technology, Danvers, USA), at 4 °C for overnight and then incubated for 1 h with HRP-conjugated secondary antibody (Santa Cruz). The signals were detected by ECL kit using a Lumino Image Analyzer (TAITEC) [24,25].

2.6. Quantification of caspase activities

Cells (1×10^6) were cultured in a 10-cm dish, treated with or without 10 μM pan-caspase inhibitor (Z-VAD-FMK) for 3 h before exposure to 30 nM of ITR-284 for 24 h. The cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT). The resulting cell lysates (50 μg protein) were incubated with caspase-3, -9, and -8 specific substrates (Ac-DEVD-pNA, Ac-LEHD-pNA, and Ac-IETD-pNA) (R&D Systems Inc.) for 1 h at 37 °C. The caspase activity was determined by measuring the release of pNA at OD_{405} [24–26].

2.7. Anti-leukemia activity in the WEHI-3 orthotopic model of leukemia

The WEHI-3 orthotopic mouse model we used is generated by Chung et al. [25]. For detail, about 60 male BALB/c mice of 22–28 g in weight at the age of 8 weeks were obtained from Laboratory Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan). The mice were divided into 6 groups and each group had 10 animals. WEHI-3 cells were injected i.p. at 1×10^5 cells/100 μL PBS into individual BALB/c mice on day 0; Groups II–VI were treated with either ITR-284 and/or ATRA at day 14th after WEHI-3 cells injection. Group I was only treated with PBS p.o.; Group II were treated with p.o. 2 mg/kg/alternate day of ITR-284 for 7 times; Group III were treated with p.o. 10 mg/kg/alternate day of ITR-284 for 7 times; Group IV were treated with i.p. 30 mg/kg/alternate day of ATRA for 7 times; Group V were treated with i.p. 30 mg/kg/alternate day of ATRA and p.o. 2 mg/kg/alternate day of ITR-284 for 7 times and Group VI were treated with i.p. 30 mg/kg/alternate day of ATRA and p.o. 10 mg/kg/alternate day of ITR-284 for 7 times.

Anti-leukemia activity was assessed as the survival time in the drug-treated and untreated groups. All animals were weighed and sacrificed on day 28th. The spleens were isolated, weighed, and subjected to histopathological analysis. These spleens were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin according to the previous procedure [25,26].

2.8. Cell surface marker analysis of peripheral blood mononuclear cells (PBMC)

Whole blood samples were collected (100 μL) from animals of each group at the end of the experiments, treated immediately with ammonium chloride buffer (Becton-Dickinson, CA, USA) for lysing the red blood cells, then centrifuged for 15 min at 1500 rpm at 4 °C to isolate the PBMC cells. The cells were examined for cell surface markers, including CD3 (T cell), CD19 (B cell), Mac-3 (Macrophage) and CD11b (Monocyte) by staining with anti-CD3-FITC, anti-CD19-PE, anti-Mac-3-FITC and anti-CD11b-PE antibodies (BD Pharmingen Inc.) and then incubated with fluorescently coupled secondary antibody to determine the cell surface marker expression by flow cytometry as previously described [26].

2.9. Statistical analysis

All the statistical results were expressed as the mean ± S.E.M. of triplicate samples, and the difference between groups was analyzed by paired two-tailed Student's *t*-test, and $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was taken as significant.

3. Results

3.1. ITR-284 inhibits cell proliferation and induces apoptosis in leukemia cells

Our previous report [19] mentioned that ITR-284 (Fig. 1A) can inhibit cell proliferation of HL-60 leukemia cells and we wondered whether ITR-284 has the same effect on WEHI-3 leukemia cells. To gain further into the effects of ITR-284, HL-60 and WEHI-3 cells were treated with 10–50 nM of ITR-284 for 24 and 48 h and the number of viable cells was counted by PI exclusion method. ITR-284 inhibited the growth of HL-60 and WEHI-3 cells in a time- and dose-dependent manner. The half maximal (50%) inhibitory concentration (IC_{50}) for 24-h treatment of ITR-284 in HL-60 and WEHI-3 cells were 29.64 ± 1.38 and 32.09 ± 2.54 nM respectively (Fig. 1B). Chromatin condensation and nuclei fragmentation were evident in HL-60 and WEHI-3 cells treated with 30 nM of ITR-284 by DAPI staining and examination by fluorescence microscope (Fig. 1C). Treatment of HL-60 and WEHI-3 cells with 30 nM of ITR-284 also induced the translocation of phosphatidylserine (PS) from inner side of the plasma membrane to the outer layer of the cell membrane by Annexin V analysis (positive cells of ITR-284-treated HL-60 and WEHI-3 cells: 82.71 ± 2.68% and 89.62 ± 3.13%; Fig. 1D). Our results indicated that ITR-284 treatments induced apoptosis in HL-60 and WEHI-3 leukemia cells.

3.2. ITR-284-induced apoptosis in HL-60 and WEHI-3 cells were accompanied by the increase in ROS production and the loss of mitochondrial membrane potential ($\Delta\Psi_m$)

We examined the effects of ITR-284 on the ROS production and loss of $\Delta\Psi_m$ by using the ROS-specific dye DCFH-DA and mitochondria-specific dye $DiOC_6$. A remarkable decrease in the $\Delta\Psi_m$ was observed after 3–12 h of exposure to 30 nM of ITR-284 in HL-60 and WEHI-3 cells (Fig. 2A). Besides, the increase in ROS production was evident after 3 to 12 h of exposure to 30 nM of ITR-284 in HL-60 and WEHI-3 cells (Fig. 2B). Our data showed that ITR-284 triggered apoptosis through the increase of ROS production and the disruption of $\Delta\Psi_m$ in HL-60 and WEHI-3 cells.

3.3. ITR-284-induced apoptosis on HL-60 and WEHI-3 cells involves the activation of caspases-8, -9 and -3

To determine if caspases cascade were involved in ITR-284-induced apoptosis, the caspase-8, -9 and -3 activities were examined by the caspase activity assay. The results showed that caspase-8, -9 and -3 activities were all elevated after 24 h of exposure to 30 nM of ITR-284 in both HL-60 and WEHI-3 both cells (Fig. 3A, dark column). The caspase-8, -9 and -3 activities decreased after ITR-284-treated cells were pre-treated with pan-caspase inhibitor (Z-VAD-FMK) (Fig. 3A, gray column). Therefore, ITR-284-induced apoptosis is through the induction of caspase-8, -9 and -3 activities.

3.4. ITR-284-induced apoptosis is mediated through both death-receptor and mitochondrial apoptotic pathways in HL-60 and WEHI-3 cells

The aforementioned data showed that ITR-284-induced apoptosis is through the activation of caspases-8, -9, and -3 activities. It is well-known that these caspases can be activated in two major apoptotic pathways, the death-receptor- and mitochondria-mediated signaling pathways. We therefore determined whether both of these pathways contribute to ITR-284-induced apoptosis. First, the death receptor relative proteins Fas/CD95, caspase-8 and caspase-3 were examined by Western blotting. The results showed that 30 nM of ITR-284 treatment caused a time-dependent increase in the level of Fas/CD95 protein in both cells (Fig. 4A). Following treatment with ITR-284, the cleavage of pro-caspase-8 and pro-caspase-3 into catalytically active forms were clearly detected in a time-dependent manner (Fig. 4A). Our data suggested that ITR-284 could activate the extrinsic pathway of Fas/caspase-8/caspase-3 cascade. Second, the amount of mitochondrial-regulated relative proteins including cytosolic cytochrome *c*, Apaf-1, AIF, and caspase-9 were determined by Western blotting. As shown in Fig. 4B, 30 nM of ITR-284 treatment caused a time-dependent increase in the cytosolic cytochrome *c*, Apaf-1 and AIF in both HL-60 and WEHI-3 cells. Following treatment with ITR-284, the cleavage of pro-caspase-9 and pro-caspase-3 (Fig. 4A and B) into catalytically active forms were also clearly detected in a time-dependent manner. Our data suggested that ITR-284 could activate the intrinsic pathway (mitochondria-apoptotic pathway) of caspase-9/caspase-3 cascade. Third, we examined whether ITR-284-induced apoptosis is associated with Bcl-2 family proteins, which are the important regulator proteins for the activation of caspase cascade. As shown in Fig. 4C, 30 nM of ITR-284 treatment decreased the amount of anti-apoptotic protein Bcl-2 and increased that of the pro-apoptotic protein Bax, which suggested that both Bcl-2 and Bax proteins are involved in ITR-284-induced apoptosis. Fourth, when cells were pretreated with N-acetylcysteine, cyclosporine A or FasL mAb respectively, and then treated with ITR-284, there is a significant increase in cell viability (Fig. 3B). These data suggested that the death receptor protein Fas/CD95 and the mitochondrial environment are the targets of ITR-284. Thus the effects of ITR-284 on HL-60 and WEHI-3 leukemia cells are through both intrinsic and extrinsic apoptotic pathways.

3.5. Anti-leukemia activity of ITR-284 in the WEHI-3 orthotopic mice model

In our preliminary *in vivo* test, we demonstrated that the dosage that kills half (50%) of the animals tested (lethal dose; LD_{50}) of ITR-284 for orally fed BALB/c mice was 20.578 mg/kg. All-trans retinoic acid (ATRA) (Fig. 1A) is a well-known anti-leukemia drug [26,27]. To test the effects of ITR-284 and ATRA on the survival rates of WEHI-3 orthotopic model, we administered one tenth and one half LD_{50} dose of ITR-284 (p.o. 2 and 10 mg/kg/alternate day) with or without ATRA (i.p. 30 mg/kg/alternate day) in BALB/c mice after inoculated with WEHI-3 cells. As shown in Fig. 5A and B, ITR-284 treatments significantly prolonged the survival rate on day 28th of WEHI-3/BALB/c leukemia mice. The survival rate of WEHI-3/BALB/

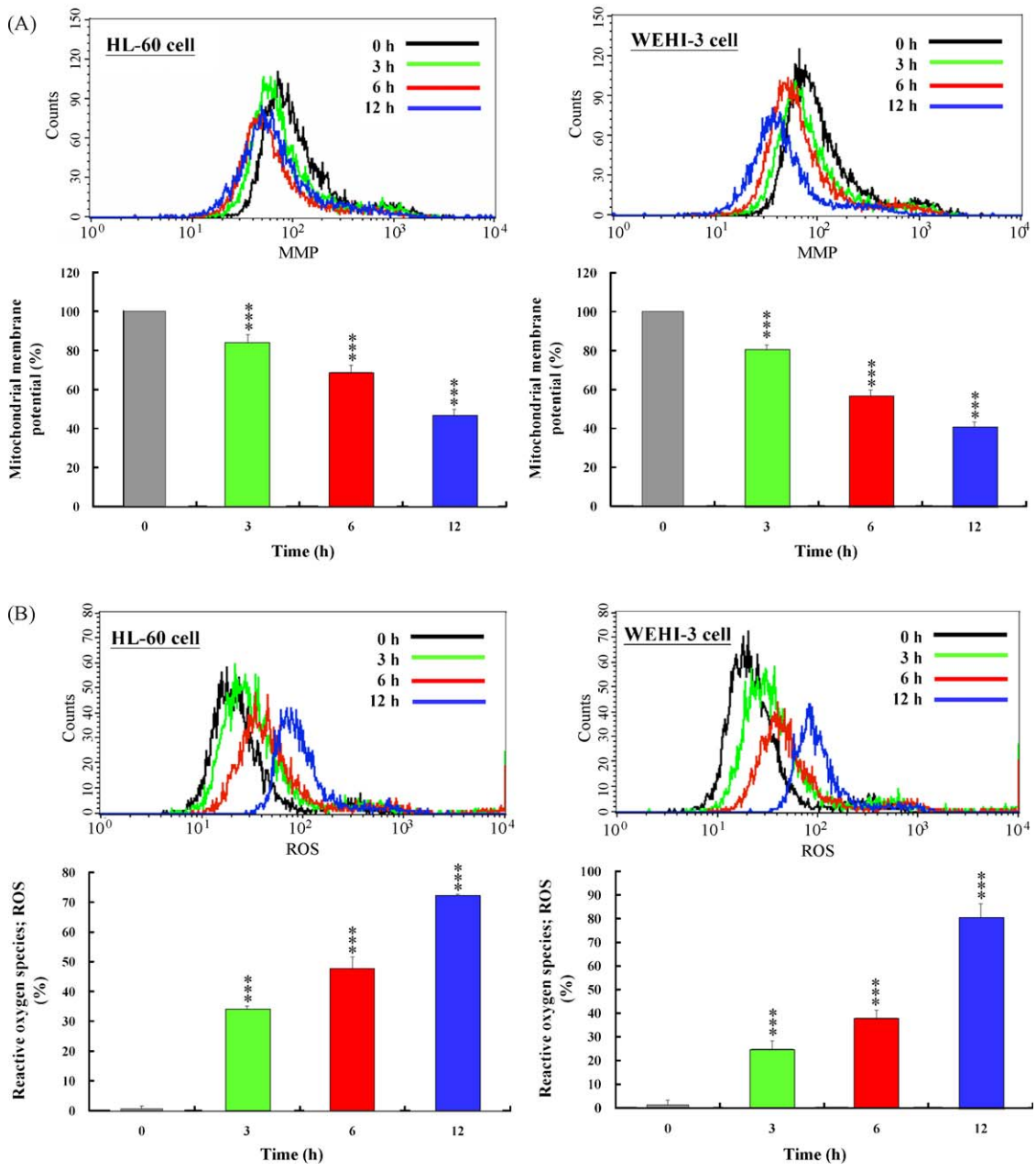

Fig. 2. Effects of ITR-284 on mitochondrial membrane potential ($\Delta\Psi_m$) (A) and ROS production (B) in HL-60 and WEHI-3 cells. The $\Delta\Psi_m$ in ITR-284-treated HL-60 and WEHI-3 cells from each time point (0, 3, 6, or 12 h) is measured by staining with $DiOC_6$. The ROS production in ITR-284-treated cells is measured by staining with DCFH-DA. Data from three independent experiments were presented ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, as compared with control treatments). Color symbols were inserted inside the figures.

c mice was 40%, and the survival rate increased to 50% and 60% when WEHI-3/BALB/c mice were treated with ITR-284 (p.o. 2 and 10 mg/kg/alternate day). The survival rates even increased to 70–80% when WEHI-3/BALB/c mice were treated with ATRA (i.p. 30 mg/kg/alternate day) in combination with ITR-284 (p.o. 2 or 10 mg/kg/alternate day) treatment (Fig. 5B).

Furthermore, we examined the physiology and histopathology of the drug-treated animals at day 28th. As in Fig. 5C, ITR-284 treatments prevented the body weight loss in leukemia mice. The spleens are enlarged in leukemia mice, however, ITR-284 treatments significantly alleviated the enlargement of the spleens (Fig. 5D). Moreover, hematoxylin and eosin (H-E) staining of spleen sections revealed that infiltration of immature myeloblastic cells into splenic red pulp was reduced in ITR-284-treated WEHI-3/BALB/c mice (Fig. 5E). We also examined the cell surface markers of PBMC from BALB/c mice after exposure to ITR-284. As shown in Fig. 5F, ITR-284 treatments increased the levels of CD3- and CD19-positive cell populations but had no effects on the levels of CD11b- and Mac-3-positive cell populations. Combined treatments of ATRA with ITR-284 even enhanced the ATRA effects on the loss of body weight, spleen enlargement, and infiltration of immature myeloblastic cells into splenic red pulp (Fig. 5C–E). Therefore, ITR-284 not only has anti-leukemia activity, but also can augment the anti-leukemia effects of ATRA *in vivo*.

4. Discussion

Acute promyelocytic leukemia (APL) was found to be specifically sensitive to pharmacological doses of all-trans retinoic acid (ATRA) (Fig. 1A). However, the disease may relapse with resistance

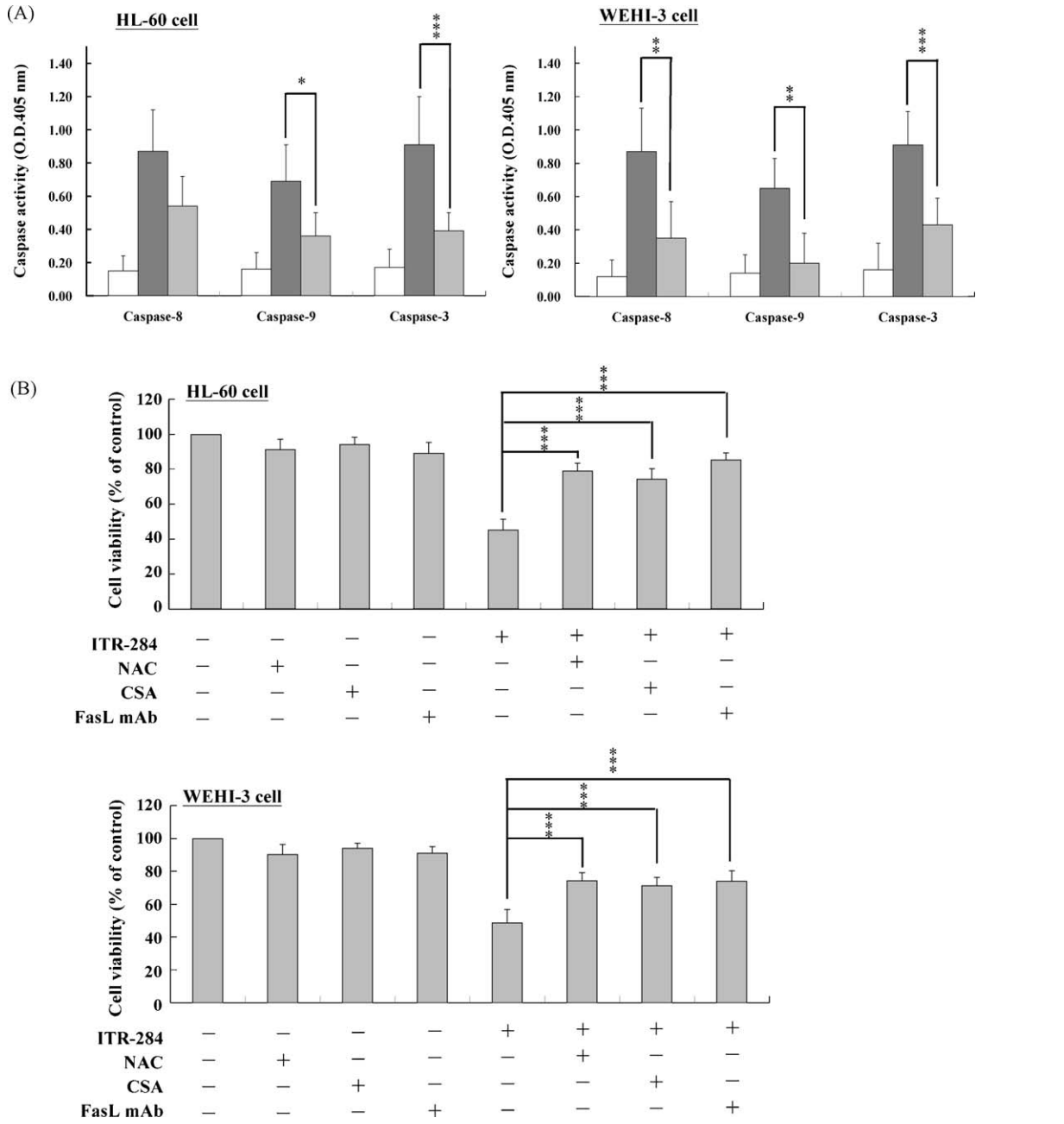

Fig. 3. Effects of ITR-284 and pan-caspase inhibitor on caspase-8/-9/-3 activities (A) and the effects of ITR-284 and specific inhibitors on cell viability (B) in HL-60 and WEHI-3 cells. (A) Cells were treated with 30 nM of ITR-284 or treated with pan-caspase inhibitor (Z-VAD-FMK) for 3 h before ITR-284 treatment, then incubated for 24 h, and the whole-cell lysate were subjected to caspase activity assay. (B) Cells were treated with specific inhibitors, including N-acetylcysteine (NAC), cyclosporine A (CSA) or FasL mAb for 1 h and then treated with ITR-284 for 24 h. Cells were then collected to determine the percentage of viable cells.

to further ATRA and chemotherapy treatments. ITR-284, the promising anti-leukemia compound, was first described and synthesized in our laboratory (Fig. 1A). This drug significantly inhibited the proliferation of HL-60 human promyelocytic leukemia cells [19]. In addition, ITR-284 has much less cytotoxicity on the PBMC than on HL-60 cells, and significantly enhanced ATRA-induced cell differentiation at a lower concentration (25 nM) [19].

In the present study, we examined the molecular mechanisms of anti-leukemia activity of ITR-284 in both HL-60 and WEHI-3 leukemia cells *in vitro* and accessed the effect of ITR-284 and the effects of combined treatment of ITR-284 and ATRA in WEHI-3 orthotopic leukemia mice model *in vivo*. Our results showed that 30 nM of ITR-284 can substantially inhibit the growth of HL-60 and WEHI-3 cells (Fig. 1B). Recently, several studies showed the use of carboxamide derivatives to treat human cancer cell lines, and had revealed some molecular mechanisms including the inhibition of topoisomerases' activities and the induction of apoptosis [28–30]. Our studies showed that ITR-284 treatment decreased the cell viability (Fig. 1B), implicating that ITR-284 may exert its cytotoxicity by the induction of apoptosis in both leukemia cells. As shown in Figs. 1C and D and 2 and 3, ITR-284 indeed induced apoptosis through the activation of caspase cascade in both cells. These results suggested that the anti-leukemia activity of ITR-284 appears to be through the induction of apoptotic cell death.

We further showed that ITR-284 induced the cleavage and activation of the initiator caspases, caspase-8 and caspase-9, and the executor caspase-3 in a time-dependent manner (Fig. 4A and B), suggesting that ITR-284 could trigger both extrinsic and

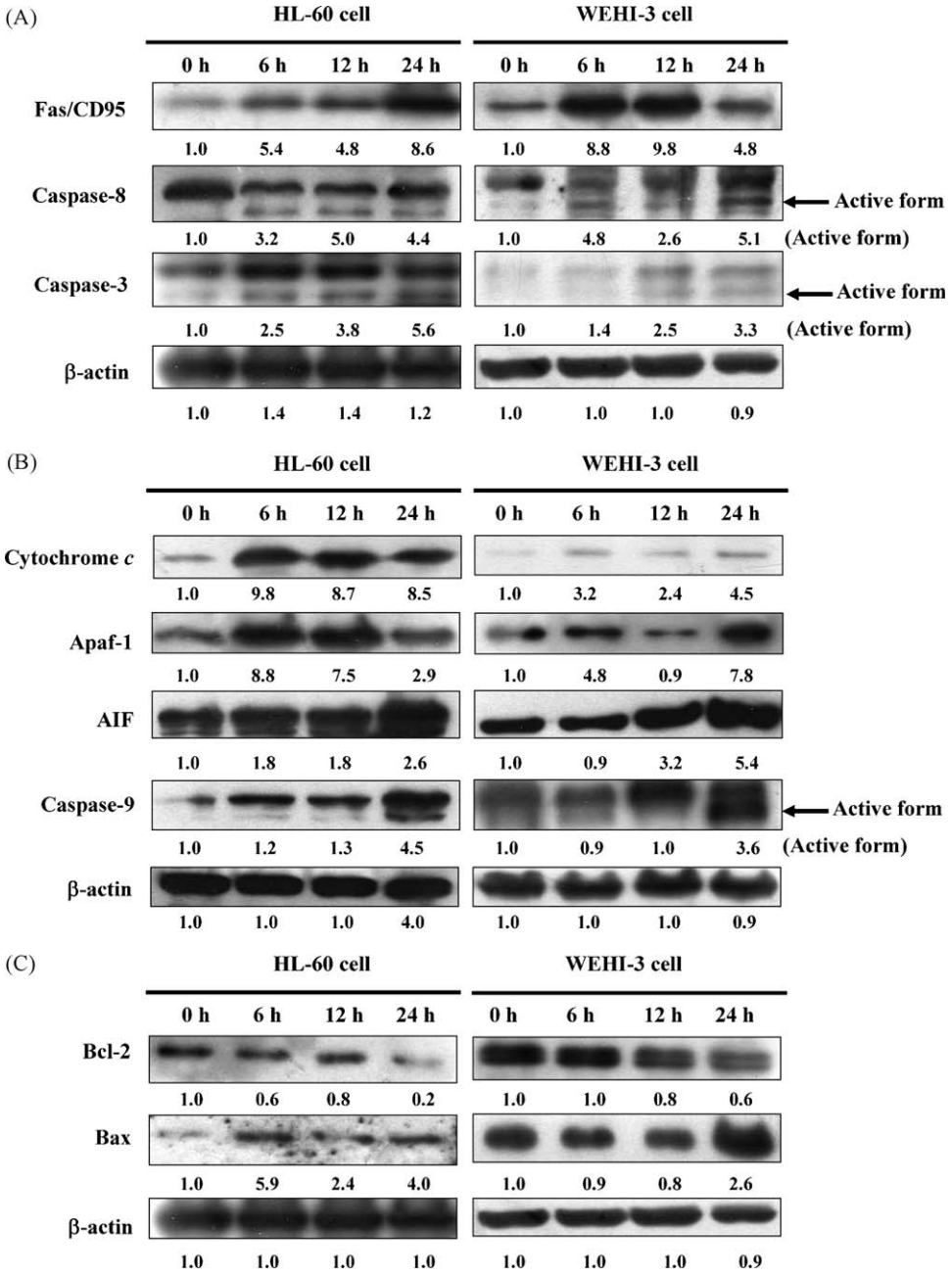

Fig. 4. ITR-284 affected the levels of the associated proteins in both the intrinsic and extrinsic apoptotic pathways in HL-60 and WEHI-3 cells. Cells were treated with 30 nM of ITR-284 for 0, 6, 12 and 24 h, cytosolic proteins or whole cell lysate were prepared, and subjected to Western blotting. The resulting blots were probed for (A) Fas/CD95, caspase-8 and caspase-3 (whole cell lysate), (B) cytochrome *c*, Apaf-1, AIF and caspase-9 (cytosolic proteins), and (C) Bcl-2 and Bax (whole cell lysate). β-Actin served as the loading control.

intrinsic signaling pathways. These findings can be further supported by the following evidences. First, our results demonstrated that ITR-284 induced a time-dependent increase of Fas/CD95 proteins (Fig. 4A). We also observed the activation of both caspase-8 and caspase-3 (Fig. 4A). These data suggested the involvement of ITR-284 in the extrinsic apoptotic pathway in both cells. Second, when mitochondria perceive apoptotic signaling, the outer membrane of mitochondria became permeabilized, then the apoptotic relative proteins including cytochrome *c*, Apaf-1, pro-caspase-9 and AIF were released into the cytosol before the activation of caspase-3 by caspase-9, and the activated caspase-3 was translocated into the nuclei to activate DNase, leading to DNA fragmentation (characteristics of apoptotic cells) [31–33]. Herein, we demonstrated the alteration of $\Delta\Psi_m$ and ROS after ITR-284 treatment for 3 h (Fig. 2A and B) and it is very likely that this alteration led to the release of proteins from mitochondria into the cytosol (e.g. cytochrome *c*, Apaf-1, pro-caspase-9 and AIF, Fig. 4B) and the activation of caspase-9 and caspase-3 cascade (Figs. 3 and 4), as revealed by immunoblotting and caspase activity assays of ITR-284-treated HL-60 and WEHI-3 cells. These results indicated that mitochondria-dependent intrinsic pathway is also involved in ITR-284-induced apoptosis of both cells. These data suggested that ITR-284 can trigger both the intrinsic and extrinsic signaling pathways to induce cell apoptosis. However, a signaling pathway that is involved in the cross-talk between both intrinsic and extrinsic pathways has been substantiated by the observations that the activation of caspase-8 causes the cleavage of Bid that in turn induces a subsequent activation of caspase-9 [34–36]. Our

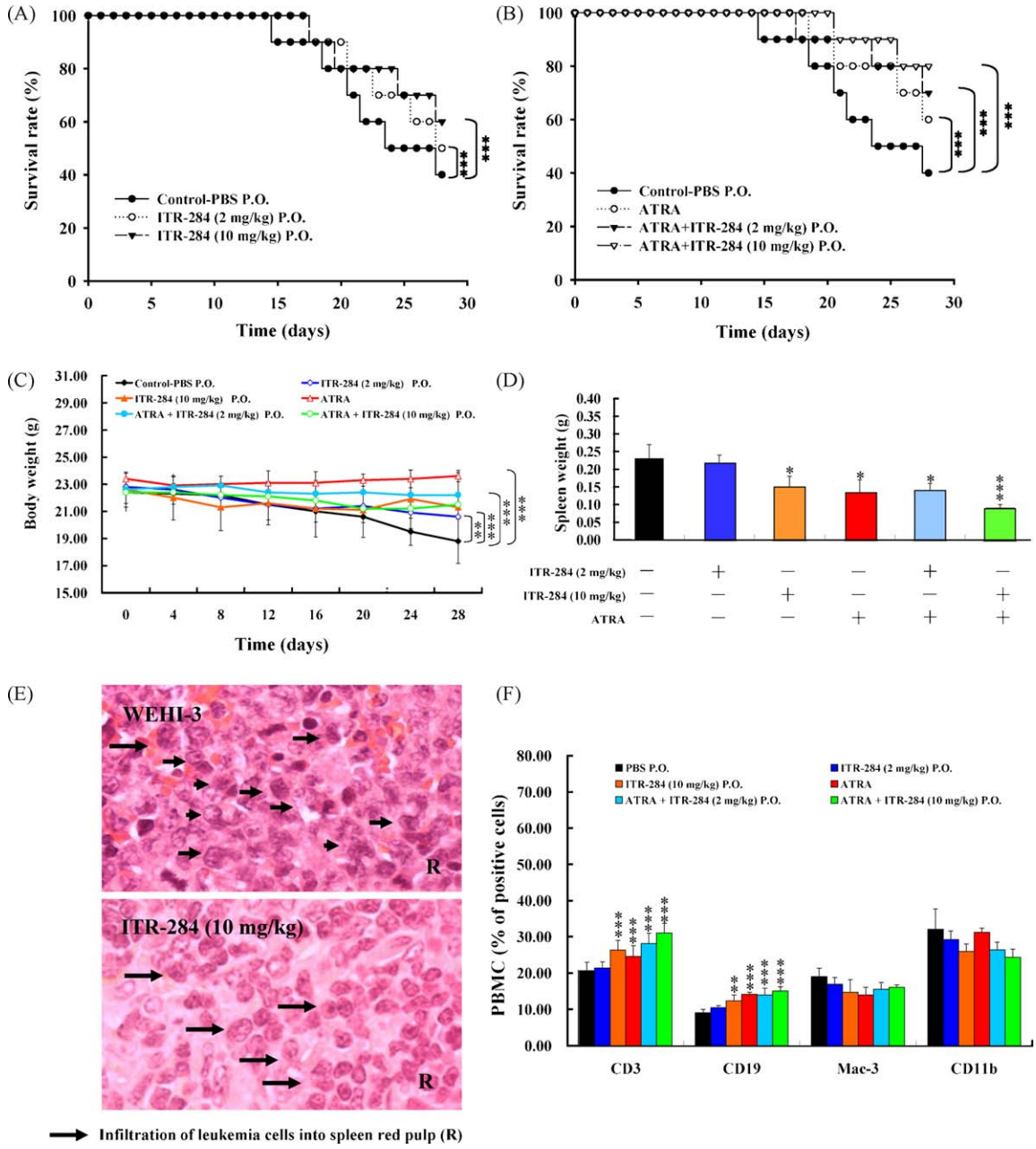

Fig. 5. Effects of ITR-284 and ATRA on WEHI-3 orthotopic model of leukemia *in vivo*. Survival rates of (A) ITR-284 and (B) ATRA and ITR-284-co-treated WEHI-3/BALB/c mice. The mice were inoculated with WEHI-3 cells for 14 days, then either fed orally with ITR-284 (2 and 10 mg/kg alternate day for 7 times) (A) or treated intraperitoneally (i.p.) with ATRA (30 mg/kg/alternate day for 7 times) together with ITR-284 (B) and the number of surviving mice was counted for 28 days. Symbol for each treatment was indicated inside the figure. Alteration of body weight (C) and spleen weight (D) of drugs-treated mice ($n = 4$) were weighed every 4 days for 28 days (C). Symbol for each treatment was shown inside the figure. At day 28th, mice were scarified, and the spleen was taken out and weighed (D). H–E staining of the spleen sections of ITR-284-treated WEHI/BALB/c mice (E) and cell surface marker expression of PBMC (F) of ITR-284 alone and both ATRA and ITR-284-treated WEHI/BALB/c mice. Paraffin sections of spleens from treated animals were prepared and stained with hematoxylin and eosin (E). PBMC cells were collected from control (PBS p.o.) and drug-treated animals and analyzed for cell surface markers (CD3, CD19, Mac-3, and CD11b) by flow cytometry (F). The results of spleen weight were expressed as the mean ± S.E.M. and the difference between the groups were tested by paired two-tailed Student's *t*-test, and $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ was taken as significant.

results indicated that all three caspases (caspase-8, -9, and -3) were activated after ITR-284 treatment. Therefore, it is possible that ITR-284 also exerts its effects through subsequent caspase-8 activation, Bid cleavage, caspase-9 activation, and finally caspase-3 activation, leading to apoptosis in HL-60 and WEHI-3 cells.

The mechanism of arsenic trioxide (As_2O_3)-induced leukemia cell apoptosis is through activation of caspase cascade. As_2O_3 can cause the loss of mitochondria membrane potential ($\Delta\Psi_m$) and the increase in mitochondrial membrane permeability, leading to the degradation phase of apoptosis [37]. Previous reports showed the caspase-9 activation was observed in leukemia cells treated with 1 μM ATRA and 4 μM As_2O_3, but As_2O_3-induced caspase-8 activation was only observed in 4-5 μM As_2O_3, suggesting that signaling through death receptors extrinsic pathway or mitochondria-dependent intrinsic pathway is dependent on drug concentration.

ITR-284 could have the potential to be a novel anti-leukemia drug because it enhanced all-trans retinoic acid (ATRA)-induced cell differentiation *in vitro* [19]. WEHI-3 leukemia cells, a monomyelocytic leukemia cell line originally derived from the BALB/c mouse, are proved to be an ideal system for the studies of potential therapeutic agents that can induce differentiation and

apoptosis *in vivo* [25,26]. In the WEHI-3 cells orthotopic mice model, the BALB/c mice were injected i.p. with WEHI-3 cells for 14 days before treatment with ITR-284, and then the animals were sacrificed at day 28th. ITR-284 treatments significantly prolonged the survival rate (Fig. 5A), prevented the loss of body weight (Fig. 5C), inhibited the spleen enlargement (Fig. 5D), and reduced the infiltration of immature myeloblastic cells into splenic red pulp (Fig. 5E). We also demonstrated that ITR-284 could increase the amount of T and B cells in peripheral blood from BALB/c mice inoculated with WEHI-3 cells (Fig. 5F). ATRA is a naturally derivative of vitamin A and has been shown to act therapeutically by the induction of cell differentiation in HL-60 and WEHI-3 leukemia cell lines [26,27]. It has been successfully used in the therapy of hemopoietic malignancies *in vivo* [27,38]. The combined treatment of ITR-284 with ATRA increased the survival rate, prevented the loss of body weight, and lessened the enlargement of the spleen than that of either ITR-284 or ATRA alone (Fig. 5) suggests that combined treatment of ITR-284 with ATRA is more effective for the differentiation therapy of leukemia. Our results indicated that ITR-284 represents a promising candidate as an anti-leukemia drug with low toxicity to normal cells.

Promyelocytic leukemia is a different subtype of acute myeloid leukemia. Chemotherapy agents such as all-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) were the first-line treatment with a complete remission rate of 75–95% in patients [39]. Many studies demonstrated that ATRA (at concentrations of 0.5–1 μM) strongly induces terminal differentiation of HL-60 and promyelocytic leukemia cells [39]. However, the retinoic acid syndrome (RAS) in some patients is recognized as a distinct complication and a significant percentage of patients still develop resistance to this treatment [40]. Arsenic trioxide (As_2O_3) has been reported to cause the remissions in APL patients. *In vitro* studies showed that HL-60 cells underwent differentiation with As_2O_3 at low concentrations (0.1–0.5 μM) but were induced into apoptosis at high concentrations (0.5–2 μM). The As_2O_3 exerts its cardiac toxicity in human patients even at 0.16 mg/kg and repeated injection of As_2O_3 produces cardiac toxicities in mice [41]. Oral administration of As_2O_3 to mice at doses of 3 or 6 mg/kg for 30 days results in significant reductions in the body weight, protein and glycogen content, as well as the reduction in the ascorbic acid content of livers and kidneys [41]. On the other hand, low doses of As_2O_3 were shown to stimulate angiogenesis and tumorigenesis [42]. Therefore the therapeutic dosage of As_2O_3 should be carefully investigated before it is applied to treat solid tumors in patients. ITR-284 has a better activity than other clinical drugs, such as ATRA and arsenic trioxide (As_2O_3) because (i) it exhibits low cytotoxicity in both leukemia cells and induces apoptotic cell death *in vitro* at low concentration (10–50 nM); (ii) it possesses better anti-cancer activity in orthotopic WEHI-3 model of leukemia than ATRA *in vivo*; (iii) the combined treatment of ITR-284 with ATRA has synergic effect and thus can decrease the concentration of ATRA in clinic application.

In conclusion, ITR-284 has growth inhibition effects on HL-60 and WEHI-3 leukemia cells by the induction of cell apoptosis. In Fig. 6, we have outlined the molecular mechanism and the overall possible signaling pathways for ITR-284-induced apoptosis in HL-60 and WEHI-3 leukemia cells. Our studies have clearly pointed out that the death receptor protein Fas/CD95 and the mitochondrial environment are the targets of ITR-284 and the effects of ITR-284 on HL-60 and WEHI-3 cells are through intrinsic and extrinsic apoptotic pathways. In addition, our *in vivo* studies in WEHI-3 orthotopic leukemia mice model indicated that ITR-284 has anti-leukemia activity and combined treatments with ATRA has synergistic effect. Therefore, ITR-284 has the potential to be one of the best leading compounds for the development of a novel anti-leukemia agent or synergistic therapy agent in the future.

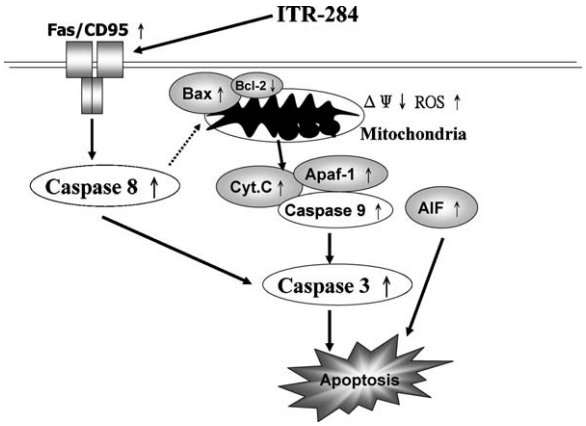

Fig. 6. A proposed model illustrates the molecular mechanism and the overall possible signaling pathways for ITR-284-induced apoptosis in HL-60 and WEHI-3 leukemia cells.

Acknowledgements

The authors thank the Ministry of economic affairs of the Republic of China for the financial support. The investigation was supported by research grant from the National Science Council of the Republic of China (NSC 95-2320-B-039-049-MY2) and grant from the China Medical University (CMU 96-221), Taiwan.

References

[1] Krueger H, McLean D, Williams D, Leukemias. Progress in Experimental Tumor Research Fortschritte der Experimentellen Tumorforschung 2008;40:45–50.
[2] Batsi C, Markopoulou S, Kontargiris E, Charalambous C, Thomas C, Christoforidis S, et al. Bcl-2 blocks 2-methoxyestradiol induced leukemia cell apoptosis by a p27(Kip1)-dependent G1/S cell cycle arrest in conjunction with NF-kappaB activation. Biochemical Pharmacology 2009;78:33–44.
[3] Nau KC, Lewis WD. Multiple myeloma: diagnosis and treatment. American Family Physician 2008;78:853–9.
[4] Fotoohi AK, Assaraf YG, Moshfegh A, Hashemi J, Jansen G, Peters GJ, et al. Gene expression profiling of leukemia T-cells resistant to methotrexate and 7-hydroxymethotrexate reveals alterations that preserve intracellular levels of folate and nucleotide biosynthesis. Biochemical Pharmacology 2009; 77:1410–7.
[5] Kim SH, Yoo JC, Kim TS. Nargenicin enhances 1,25-dihydroxyvitamin D(3)- and all-trans retinoic acid-induced leukemia cell differentiation via PKCbetaI/MAPK pathways. Biochemical Pharmacology 2009;77:1694–701.
[6] Tallman MS. Differentiating therapy in acute myeloid leukemia. Leukemia 1996;10(Suppl. 2):s33–8.
[7] Schimmer AD. Novel therapies targeting the apoptosis pathway for the treatment of acute myeloid leukemia. Current Treatment Options in Oncology 2007;8:277–86.
[8] Lee MO, Han SY, Jiang S, Park JH, Kim SJ. Differential effects of retinoic acid on growth and apoptosis in human colon cancer cell lines associated with the induction of retinoic acid receptor beta. Biochemical Pharmacology 2000; 59:485–96.
[9] Floryk D, Thompson TC. Perifosine induces differentiation and cell death in prostate cancer cells. Cancer Letters 2008;266:216–26.
[10] Kang MR, Kang JS, Han SB, Kim JH, Kim DM, Lee K, et al. A novel delta-lactam-based histone deacetylase inhibitor, KBH-A42, induces cell cycle arrest and apoptosis in colon cancer cells. Biochemical Pharmacology 2009;78:486–94.
[11] Khan N, Mukhtar H. Multitargeted therapy of cancer by green tea polyphenols. Cancer Letters 2008;269:269–80.
[12] Ma E, Wang X, Li Y, Sun X, Tai W, Li T, et al. Induction of apoptosis by furanodiene in HL60 leukemia cells through activation of TNFR1 signaling pathway. Cancer Letters 2008;271:158–66.
[13] Trincavelli ML, Falleni A, Chelli B, Tuscano D, Costa B, Gremigni V, et al. A(2A) adenosine receptor ligands and proinflammatory cytokines induce PC 12 cell death through apoptosis. Biochemical Pharmacology 2003;66:1953–62.
[14] Rustin P, Kroemer G. Mitochondria and cancer. Ernst Schering Foundation Symposium Proceedings 2007;1–21.
[15] Saitoh M, Nagai K, Nakagawa K, Yamamura T, Yamamoto S, Nishizaki T. Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase. Biochemical Pharmacology 2004;67:2005–11.

[16] Ashkenazi A. Targeting the extrinsic apoptosis pathway in cancer. Cytokine & Growth Factor Reviews 2008;19:325–31.
[17] Ow YP, Green DR, Hao Z, Mak TW. Cytochrome *c*: functions beyond respiration. Nature Reviews Molecular Cell Biology 2008;9:532–42.
[18] Marzo I, Naval J. Bcl-2 family members as molecular targets in cancer therapy. Biochemical Pharmacology 2008;76:939–46.
[19] Wen YF, Lee KH, Huang PT, Chen MH, Shin WC, Huang LJ, et al. Cell differentiation enhancement by hydrophilic derivatives of 4,8-dihydrobenzo[1,2-b:5,4-b′]dithiophene-4,8-diones in HL-60 leukemia cells. Bioorganic & Medicinal Chemistry Letters 2007;17:2908–12.
[20] Kemeny-Beke A, Aradi J, Damjanovich J, Beck Z, Facsko A, Berta A, et al. Apoptotic response of uveal melanoma cells upon treatment with chelidonine, sanguinarine and chelerythrine. Cancer Letters 2006;237:67–75.
[21] Kuo TC, Yang JS, Lin MW, Hsu SC, Lin JJ, Lin HJ, et al. Emodin has cytotoxic and protective effects in rat C6 glioma cells: roles of Mdr1a and nuclear factor kappaB in cell survival. The Journal of Pharmacology and Experimental Therapeutics 2009;330:736–44.
[22] Park WH, Han YW, Kim SW, Kim SH, Cho KW, Kim SZ. Antimycin A induces apoptosis in As4.1 juxtaglomerular cells. Cancer Letters 2007;251:68–77.
[23] Liu B, Wang T, Qian X, Liu G, Yu L, Ding Y. Anticancer effect of tetrandrine on primary cancer cells isolated from ascites and pleural fluids. Cancer Letters 2008;268:166–75.
[24] Lin SS, Huang HP, Yang JS, Wu JY, Hsia TC, Lin CC, et al. DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. Cancer Letters 2008;272:77–90.
[25] Chung JG, Yang JS, Huang LJ, Lee FY, Teng CM, Tsai SC, et al. Proteomic approach to studying the cytotoxicity of YC-1 on U937 leukemia cells and antileukemia activity in orthotopic model of leukemia mice. Proteomics 2007;7:3305–17.
[26] Lin JP, Yang JS, Lu CC, Chiang JH, Wu CL, Lin JJ, et al. Rutin inhibits the proliferation of murine leukemia WEHI-3 cells in vivo and promotes immune response in vivo. Leukemia Research 2009;33:823–8.
[27] Dawson MI, Elstner E, Kizaki M, Chen DL, Pakkala S, Kerner B, et al. Myeloid differentiation mediated through retinoic acid receptor/retinoic X receptor (RXR) not RXR/RXR pathway. Blood 1994;84:446–52.
[28] Lukka PB, Kestell P, Paxton JW, Baguley BC. Development and validation of a liquid chromatography–mass spectrometry (LC–MS) assay for the determination of the anti-cancer agent N-[2-(dimethylamino)ethyl]-2,6-dimethyl-1-oxo-1,2-dihydrobenzo[b]-1,6-naph thyridine-4-carboxamide (SN 28049). Journal of Chromatography B 2008;875:368–72.
[29] Feng W, Satyanarayana M, Tsai YC, Liu AA, Liu LF, LaVoie EJ. 11-Substituted 2,3-dimethoxy-8,9-methylenedioxybenzo[i]phenanthridine derivatives as novel topoisomerase I-targeting agents. Bioorganic & Medicinal Chemistry 2008; 16:8598–606.
[30] Creighton-Gutteridge M, Cardellina 2nd JH, Stephen AG, Rapisarda A, Uranchimeg B, Hite K, et al. Cell type-specific, topoisomerase II-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation by NSC 644221. Clinical Cancer Research 2007;13:1010–8.
[31] Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. Oncogene 2008; 27:6245–51.
[32] Wojtczak L, Zablocki K. Mitochondria in cell life, death and disease. Postepy Biochemii 2008;54:129–41.
[33] Higuchi Y. Chromosomal DNA fragmentation in apoptosis and necrosis induced by oxidative stress. Biochemical Pharmacology 2003;66:1527–35.
[34] Srivastava RK. TRAIL/Apo-2L: mechanisms and clinical applications in cancer. Neoplasia (New York NY) 2001;3:535–46.
[35] Barnhart BC, Alappat EC, Peter ME. The CD95 type I/type II model. Seminars in Immunology 2003;15:185–93.
[36] Clarke P, Tyler KL. Reovirus-induced apoptosis: a minireview. Apoptosis 2003;8:141–50.
[37] Evens AM, Tallman MS, Gartenhaus RB. The potential of arsenic trioxide in the treatment of malignant disease: past, present, and future. Leukemia Research 2004;28:891–900.
[38] He LZ, Merghoub T, Pandolfi PP. In vivo analysis of the molecular pathogenesis of acute promyelocytic leukemia in the mouse and its therapeutic implications. Oncogene 1999;18:5278–92.
[39] Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood 2008;111:2505–15.
[40] Patatanian E, Thompson DF. Retinoic acid syndrome: a review. Journal of Clinical Pharmacy and Therapeutics 2008;33:331–8.
[41] Liu B, Pan S, Dong X, Qiao H, Jiang H, Krissansen GW, et al. Opposing effects of arsenic trioxide on hepatocellular carcinomas in mice. Cancer Science 2006;97:675–81.
[42] Soucy NV, Ihnat MA, Kamat CD, Hess L, Post MJ, Klei LR, et al. Arsenic stimulates angiogenesis and tumorigenesis in vivo. Toxicological Sciences 2003;76:271–9.