The novel carboxamide analog ITR-284 induces caspase-dependent apoptotic cell death in human hepatocellular and colorectal cancer cells

YU-REN LIAO[1], CHI-CHENG LU[2], KUANG-CHI LAI[3,4], JAI-SING YANG[5], SHENG-CHU KUO[6], YEN-FANG WEN[7], SHINJI FUSHIYA[8] and TIAN-SHUNG WU[1]

[1]Department of Chemistry, National Cheng Kung University, Tainan 701; [2]Department of Life Sciences, National Chung Hsing University, Taichung 402; [3]School of Medicine, China Medical University, Taichung 404; [4]Department of Surgery, China Medical University Beigang Hospital, Yunlin 651; [5]Department of Pharmacology; [6]Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 404; [7]Medicinal Chemistry Laboratory, Biomedical Engineering Research Laboratories, Industrial Technology Research Institute, Hsinchu 300, Taiwan, R.O.C.; [8]Department of Kampo Pharmaceutical Sciences, Nihon Pharmaceutical University, Saitama 362-0806, Japan

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Abstract. We have previously reported that ITR-284, a potent carboxamide-derived anticancer agent, induced apoptosis in leukemia cells. However, there are no reports showing that ITR-284 inhibits human hepatocellular and colorectal cancer cells. In this study, we investigated the antiproliferative effects and apoptotic induction of ITR-284 on various types of human hepatocellular and colorectal cancer cells *in vitro*. The growth inhibition effect of ITR-284 on cancer cells was evaluated by thiazolyl blue tetrazolium bromide (MTT) assay. Cell morphology was examined under a phase-contrast microscope. The activities of caspase-3, -8 and -9 were determined by caspase colorimetric assay. ITR-284 reduced the cell viability in human hepatocellular cancer cells (Hep G2, Hep 3B, SK-HEP-1 and J5) and colorectal cancer cells (HT 29, COLO 205, HCT 116 and SW 620). ITR-284 had highly selective effects on Hep 3B and COLO 205 cells. ITR-284 stimulated morphological changes of Hep 3B and COLO 205 cells. The activation of caspase-3, -8 and -9 contributed to ITR-284-induced apoptosis. ITR-284-triggered growth inhibition was significantly attenuated by the inhibitors of caspase-3, -8 and -9 in Hep 3B and COLO 205 cells. ITR-284 induced apoptosis in Hep 3B and COLO 205 cells through the caspase cascade-dependent signaling pathway.

Correspondence to: Dr Tian-Shung Wu, Department of Chemistry, National Cheng Kung University, No. 1 Ta-Hsueh Road, Tainan 701, Taiwan, R.O.C.
E-mail: tswu@mail.ncku.edu.tw

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Introduction

Cancer is a major cause of mortality worldwide and in Taiwan (1). Liver cancer is the second most frequent cause of cancer death, and colorectal cancer is the third most frequent cause of cancer death in Taiwan (2,3). Approximately 26.0 per 100,000 individuals succumb to liver cancer and 25.3 per 100,000 individuals succumb to colorectal cancer each year, according to the Department of Health, Executive Yuan, Taiwan in 2010 (www.doh.gov.tw/CHT2006/DM/DM2_2.aspx?now_fod_list_no=12336&class_no=440&level_no=4). Chemotherapy is one of the treatment options in liver and colorectal cancer, but the anticancer effects of chemotherapeutic agents are not fully satisfactory. Thus, the discovery of new antiliver and anticolorectal cancer chemotherapeutic agents is urgently required. The induction of cancer cell apoptosis has been shown to be the major anticancer mechanism for chemotherapeutic agents (4,5). Apoptosis has stimulated interest in caspases as potential therapeutic targets of chemotherapeutic agents (6,7).

Previously, we designed and synthesized a series of carboxamide derivatives as novel anticancer agents (8). We found that many of these compounds exhibited potent cytotoxicities against various human cancer cell lines (8,9). ITR-284 [N-(2-Dimethylaminoethyl)-4,8-dihydrobenzo (1,2-b;4,5-b') dithio-phene-2- carboxamide phosphoric acid salt] (Fig. 1A) is one of the most potent agents. The previous studies suggested that ITR-284 significantly inhibited the proliferation of HL60 and WEHI-3 leukemia cells, with low toxicity to normal cells (8,9). In the current study, we investigated the antiproliferative effects and apoptotic induction of ITR-284 on human hepatocellular cancer cell lines (Hep G2, Hep 3B, SK-HEP-1 and J5) and colorectal cancer cell lines (HT 29, COLO 205, HCT 116 and SW 620). We demonstrated that ITR-284 has a greater growth inhibition effect than that of other compounds in various cancer cells, with a half maximal effective concen-

tration (EC_{50}) of 50 to 75 nM. We explored the mechanism of apoptotic induction by ITR-284 in Hep 3B and COLO 205 cells. Our results suggest that ITR-284 induced apoptosis in Hep 3B and COLO 205 cells through caspase cascade-mediated pathways. ITR-284 may be selected as the lead compound of an antihepatocellular and colorectal cancer agent to trigger cell apoptosis in the future.

Materials and methods

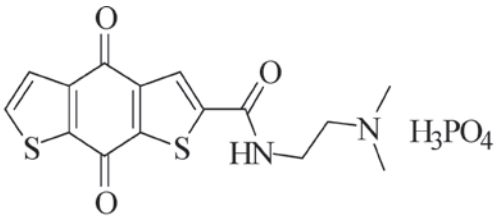
Chemicals and reagents. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, cell culture medium DMEM and trypsin-EDTA were obtained from Gibco/Life Technologies (Carlsbad, CA, USA). Caspase-3 (Z-DEVE-FMK), -8 (Z-IETD-FMK) and -9 (Z-LEHD-FMK) inhibitors were dissolved in DMSO and diluted in cell culture medium prior to use (R&D Systems, Minneapolis, MN, USA).

Cell culture. The human hepatocellular cancer cell lines (Hep G2, Hep 3B, SK-HEP-1 and J5) and human colorectal cancer cell lines (HT 29, COLO 205, HCT 116 and SW 620) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). All cells were cultured with DMEM and plated into a 75-T flask with 2 mM L-glutamine and were adjusted to contain 10% FBS and 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin). All cells were grown at 37˚C in a humidified atmosphere comprised of 95% air and 5% CO_2.

Cell viability assay. Cell viability was assessed using the MTT assay as described previously (10,11). Approximately $2x10^4$ cells/well were plated onto 96-well plates and then were exposed to ITR-284 (0, 20, 40, 60, 80 and 100 nM). DMSO (0.1%) in media served as a vehicle control. Cell viability was also used to examine Hep 3B and COLO 205 cells following pretreatment with or without 10 μM of caspase-3, -8 and -9 inhibitors for 1 h, followed by treatment with 50 nM ITR-284 and 0.1% DMSO as a control. After a 48-h incubation, 100 μl of MTT solution (0.5 mg/ml) was added to each well, and the plate was incubated at 37˚C. Approximately 100 μl of 0.04 M HCl/isopropanol was added and the absorbance at 570 nm was measured for each well. The cell survival ratio was expressed as a percentage of the control. All results were formed of three independent experiments.

Cell morphological examination. A total of $2x10^5$ cells/well of Hep 3B and COLO 205 cells in 24-well plates were exposed to 50 nM ITR-284 for 48 h. The cell morphology was directly examined and images were captured under a contrast-phase microscope (12).

Assays for caspase-3, -8 and -9 activities. The activities of caspase-3, -8 and -9 were determined according to the manufacturer's instructions (Caspase colorimetric kits, R&D Systems). Hep 3B and COLO 205 cells were inoculated into a 75-T flask at a density of $1x10^7$. After being treated with ITR-284 (50 nM) for 48 h, cells were harvested and lysed in lysis buffer (50 μl) for 10 min. After centrifugation, the supernatants containing 100 μg protein were incubated with caspase-3, -8 and -9 substrate (Z-DEVE-pNA, Z-IETD-pNA and Z-LEHD-pNA for caspase-3, -8 and -9, respectively) in reaction buffer. Samples were incubated in a 96-well flat-bottomed microplate at 37˚C for 1 h. The levels of released pNA were measured with an ELISA reader (Anthos Labtec Instruments GmbH, Salzburg, Austria) at a wavelength of 405 nm (13,14).



ITR-284
(N-(2-Dimethylaminoethyl)-4,8-dihydrobenzo [1,2-b;4,5-b'] dithio-phene-2-carboxamide phosphoric acid salt)

Figure 1. Chemical structure of ITR-284.

Statistical analysis. The statistical results were expressed as the means ± SEM of triplicate samples, and the difference between groups was analyzed using a two-tailed Student's t-test. $P<0.001$ was considered to indicate a statistically significant difference.

Results

ITR-284 inhibits cell growth in human hepatocellular and colorectal cancer cells. Our previous study reported that ITR-284 is capable of inhibiting cell growth of HL-60 and WEHI-3 leukemia cells (8). In the present study, we investigated the growth inhibition effect of ITR-284 on human hepatocellular cancer cells (Hep G2, Hep 3B, SK-HEP-1 and J5) and colorectal cancer cells (HT 29, COLO 205, HCT 116 and SW 620). The anti-proliferative effects of ITR-284 on those cells were evaluated by the MTT assay. As shown in Fig. 2, exposure to various concentrations of ITR-284 (0, 20, 40, 60, 80 and 100 nM) for 48 h resulted in dose-dependent decreases in cell viability of Hep G2 (Fig. 2A), Hep 3B (Fig. 2B), SK-HEP-1 (Fig. 2C) and J5 cells (Fig. 2D). In Fig. 3, we observed that ITR-284 (0, 20, 40, 60, 80 and 100 nM) also dose-dependently reduced cell viability of HT 29 (Fig. 3A), COLO 205 (Fig. 3B), HCT 116 (Fig. 3C) and SW 620 cells (Fig. 3D). The results presented in Table I show the EC_{50} values of ITR-284 in various cancer cell lines. Our results demonstrated that the ITR-284 had highly selective effects on Hep 3B and COLO 205 cells *in vitro*.

ITR-284 induces apoptosis in Hep 3B and COLO 205 cells. ITR-284-induced reduction of cell viability may be due to apoptosis. A 48-h exposure to 50 nM ITR-284 caused the Hep 3B cells (Fig. 4A) and COLO 205 cells (Fig. 4B) to round and shrink morphologically. Treatment of Hep 3B and COLO 205 cells with 50 nM of ITR-284 also induced the

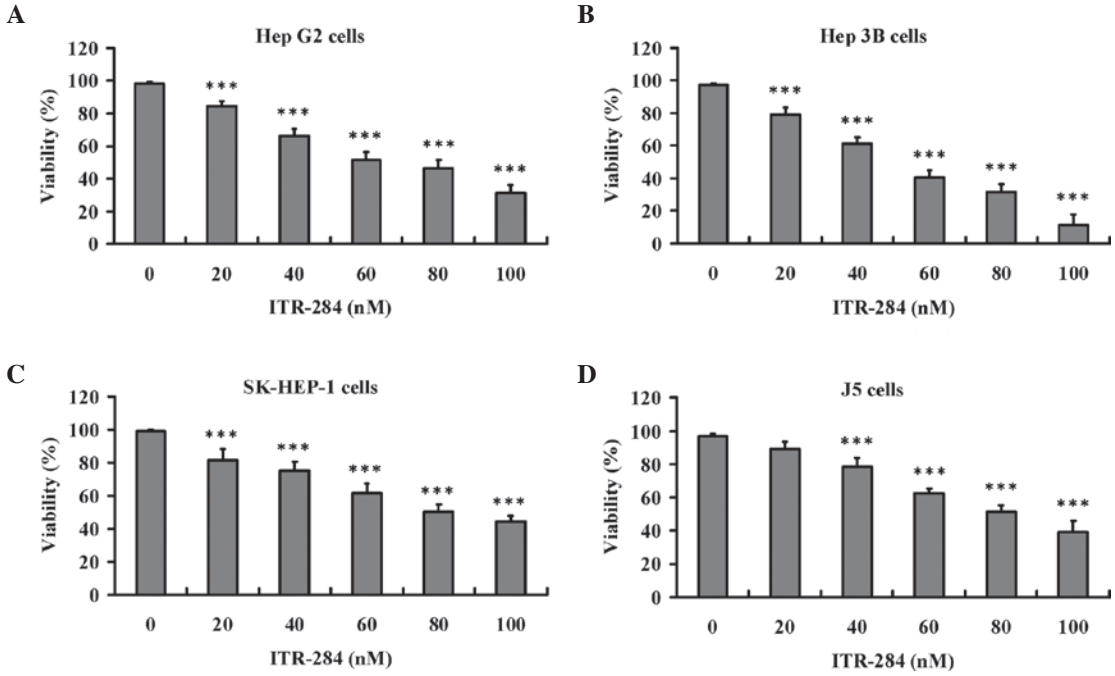


Figure 2. Effects of ITR-284 on cell viability in human hepatocellular cancer cell lines. Approximately $2x10^4$ cells/well were plated onto 96-well plates and then exposed to ITR-284 (0, 20, 40, 60, 80 and 100 nM) for 48 h, and 0.1% DMSO in media served as a vehicle control. The cell viability of (A) Hep G2, (B) Hep 3B, (C) SK-HEP-1 and (D) J5 cells after 24 and 48 h of ITR-284 treatment were determined using the thiazolyl blue tetrazolium bromide (MTT) assay as described in Materials and methods. Each point is the mean ± SEM of three independent experiments. ***P<0.001 compared with the control (0 nM ITR-284).

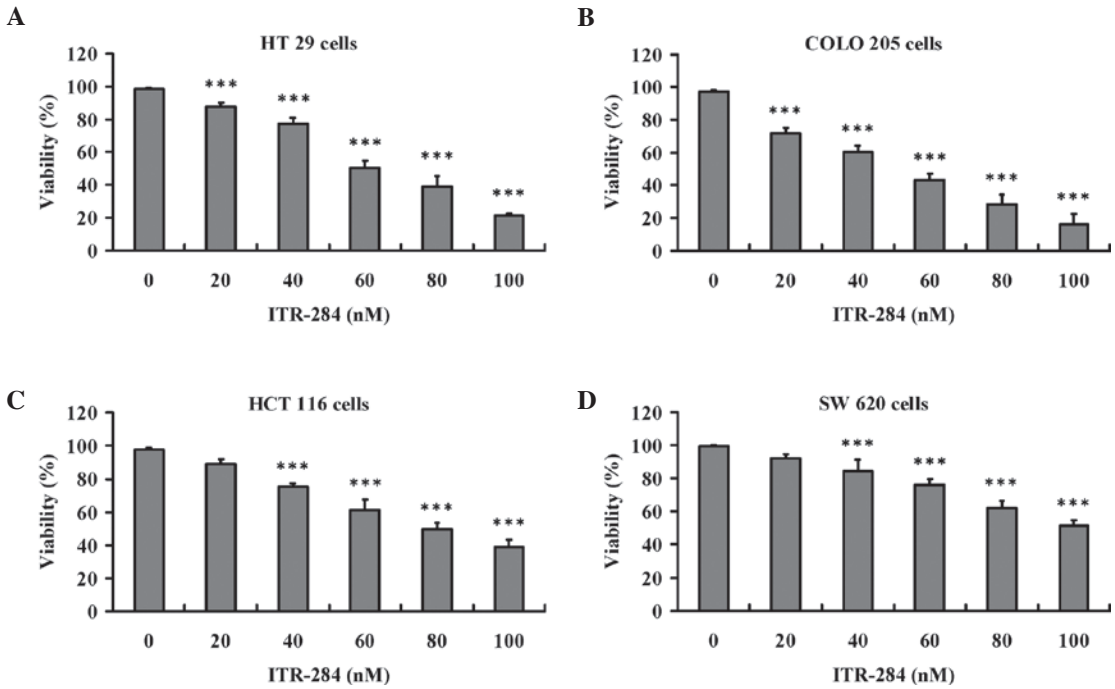


Figure 3. Effects of ITR-284 on cell viability in human colorectal cancer cell lines. Cells ($2x10^4$ cells/well) were incubated in the absence or presence of 0, 20, 40, 60, 80 and 100 nM of ITR-284 for 48 h. Cytotoxic effects on ITR-284-treated (A) HT 29, (B) COLO 205, (C) HCT 116 and (D) SW 620 cells were determined using the thiazolyl blue tetrazolium bromide (MTT) assay as described in Materials and methods. The data are presented as the means ± SEM in triplicate by comparing the treated and untreated control cells. ***P<0.001 compared with the control (0 nM ITR-284).

translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer of the cell membrane by Annexin V analysis (data not shown). Our results indicated that ITR-284 treatments provoked apoptosis in human hepatocellular cancer Hep 3B and colorectal cancer COLO 205 cells.

ITR-284-triggered apoptosis involves the activation of caspase-3, -8 and -9. To determine whether caspases are majorly involved in ITR-284-induced apoptotic cell death, the caspase-3, -8 and -9 activities were examined using the caspase colorimetric activity assay. Our results demonstrated that

Table I. *In vitro* cytotoxicity of ITR-284.

Cell line	Cell type	EC_{50}[a] (nM)
Hep G2	Human hepatoblastoma	86.39±4.18
Hep 3B	Human hepatocellular carcinoma	51.23±2.98
SK-HEP-1	Human hepatocarcinoma cells	95.69±3.25
J5	Human hepatocellular carcinoma	106.25±4.40
HT 29	Human colorectal adenocarcinoma	76.58±6.25
COLO 205	Human colon adenocarcinoma	47.56±3.69
HCT 116	Human colorectal carcinoma	96.25±5.58
SW 620	Human colorectal adenocarcinoma	126.32±4.01

[a]Concentration of compound which afforded 50% reduction in cell number for a 48-h treatment.

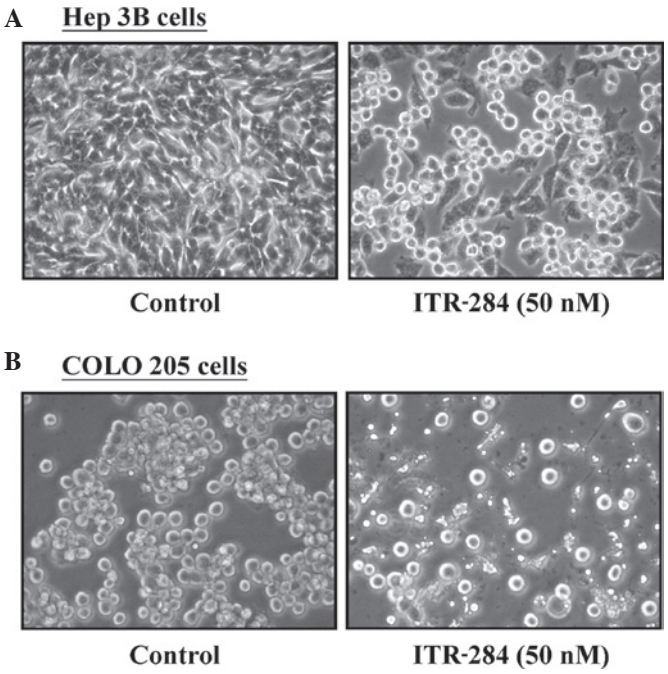


Figure 4. ITR-284 caused apoptotic morphological changes in Hep 3B and COLO 205 cells. (A) Hep 3B and (B) COLO 205 cells treated with 50 nM ITR-284 for 48 h were consequently photographed under a phase-contrast microscope (magnification, x200) to determine apoptotic morphological changes. Data were obtained from three independent experiments with similar results.

caspase-3, -8 and -9 activities were all elevated following 48 h of exposure to 50 nM ITR-284 in both Hep 3B (Fig. 5A) and COLO 205 cells (Fig. 5B). We suggested that ITR-284-induced apoptosis occurs through the induction of caspase-3, -8 and -9 activities.

Effects of caspase-3, -8 and -9 inhibition on apoptosis in ITR-284-treated cells. The aforementioned results showed that ITR-284-induced apoptosis occurs through the activation of caspase-3, -8 and -9 activities. In the present study, Hep 3B and COLO 205 cells were pre-treated with 10 μM caspase-3, -8 and -9 inhibitors for 1 h, and then exposed to 50 nM ITR-284. Subsequently, cells were harvested for

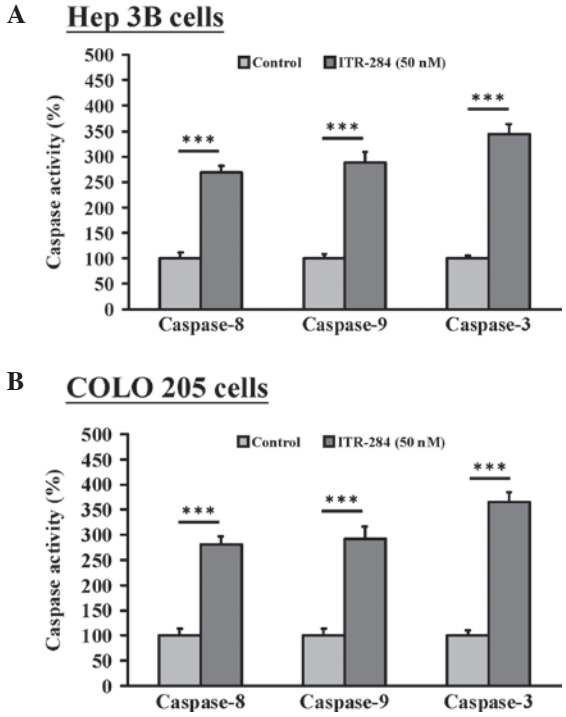


Figure 5. Effects of ITR-284 on caspase-3, -8 and -9 activities of Hep 3B and COLO 205 cells. (A) Hep 3B and (B) COLO 205 cells were treated with 50 nM ITR-284 and then incubated for 48 h, and the whole-cell lysate was subjected to caspase activity assay. Each result is shown as the mean ± SEM in triplicate by comparing the treated and untreated control cells. ***P<0.001 compared with the control.

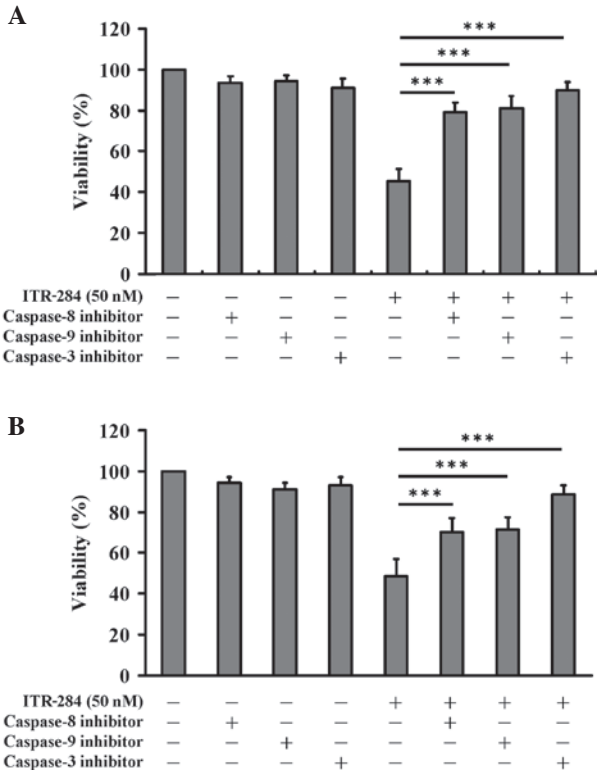


Figure 6. Caspase-3, -8 and -9 are required for ITR-284-triggered apoptosis of Hep 3B and COLO 205 cells. (A) Hep 3B and (B) COLO 205 cells were exposed to 50 nM ITR-284 for 48 h prior to pretreatment with or without 10 μM of caspase-3, -8 and -9 inhibitors, respectively, as described in Materials and methods. All results are expressed as the means ± SEM in triplicate by comparing the treated and untreated control cells. ***P<0.001 compared with the control.

measuring the cell viability by MTT assay. Pre-incubation with caspase-3, -8 and -9 specific inhibitors significantly reduced ITR-284-induced viability in Hep 3B (Fig. 6A) and COLO 205 cells (Fig. 6B). Our results suggest that caspase-3, -8 and -9 activation may be involved in ITR-284-induced apoptotic cell death.

Discussion

ITR-284 is a prospective anticancer compound and was first described and synthesized in cooperation with the laboratory of Dr. Yen-Fang Wen. An earlier study has verified that ITR-284 significantly inhibited the cell proliferation of human leukemia cells (8). Furthermore, ITR-284 has much less cytotoxicity in normal peripheral blood mononuclear cells (PBMCs) than in leukemia cells (8). The previous study has demonstrated that ITR-284 (30 nM) substantially inhibits the growth of HL60 and WEHI-3 leukemia cells *in vitro*. In a leukemia orthotopic model, ITR-284 significantly prolonged the survival rate, prevented body weight loss, inhibited spleen enlargement and reduced infiltration of immature myeloblastic cells into splenic red pulp in an *in vivo* experiment (8). However, combined treatment of ITR-284 with ATRA is more effective for differentiation therapy of leukemia. Our data indicated that ITR-284 represents a promising candidate as an anticancer drug with low toxicity to normal cells (8). The purpose of this study was to determine whether ITR-284 affects cell growth, and we investigated cell death signaling pathways and induction of apoptosis in human hepatocellular and colorectal cancer cells.

A number of studies have showed that the carboxamide derivatives function via certain molecular mechanisms, including the inhibition of topoisomerase activities and the induction of apoptosis (15-17). In the present study, our results demonstrate that ITR-284 treatment decreased the viability (Figs. 2 and 3) of human hepatocellular cancer cells (Hep G2, Hep 3B, SK-HEP-1 and J5) and colorectal cancer cells (HT 29, COLO 205, HCT 116 and SW 620). ITR-284 may cause cytotoxicity by inducing cell death. Notably, the EC_{50} for 48-h treatment of ITR-284 in hepatocellular and colorectal cancer cell lines was different (Table I); one of the reasons for the differences in sensitivities of different cell lines may be the inherent different doubling time in various cell lines, and another reason may be the differential gene expression in various cell types. It is well known that Hep G2, J5 and SK-HEP-1 cell lines are p53-positive, but Hep 3B cells are p53-negative. HT 29, COLO 205 and SW 620 lines have p53 mutation, but the HCT 116 cell line has wild-type p53.

This is the first study to investigate the anticancer effects of ITR-284 on human hepatocellular and colorectal cancer cells, and the results suggest that ITR-284 induced apoptotic cell death and inhibited the growth of cancer cells in a concentration-dependent manner. This observation is similar to our earlier study addressing ITR-284, which showed that ITR-284 initially affected the induction of apoptosis in HL60 and WEHI-3 leukemia cell lines. As shown in Fig. 5, ITR-284 induced apoptosis through the activation of caspases-3, -8 and -9 in Hep 3B and COLO 205 cells. These results suggest that the anticancer activity of ITR-284 occurs through the induction of apoptotic cell death. Hep 3B and COLO 205 cells were pretreated with caspase-3, -8 and -9 inhibitors and then exposed to ITR-284, leading to increases in the percentage of viable cells when compared with the ITR-284-treated only cells (Fig. 6). Our data indicated that these three caspases (-3, -8 and -9) were activated following ITR-284 treatment. Thus, we proposed that ITR-284-induced apoptosis may be carried out through the extrinsic and intrinsic signaling pathways.

In conclusion, ITR-284 has growth inhibition effects on human hepatocellular cancer cells (Hep G2, Hep 3B, SK-HEP-1 and J5) and colorectal cancer cells (HT 29, COLO 205, HCT 116 and SW 620) by inducing cell apoptosis. Our study has clearly revealed that the activation of caspase-3, -8 and -9 is the major pharmacological action of ITR-284. Based on our results, ITR-284 has the potential to become one of the leading compounds for the development of a novel antihepatocellular and colorectal cancer agent in the future.

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