Abstract. ITR-284 is a carboxamide analog that can inhibit proliferation in human promyelocytic leukemia HL-60 cells. To understand the effects and molecular mechanisms of ITR-284 in human erythromyeloblastoid leukemia, we treated K562 cells with different concentrations of ITR-284 (0, 2, 4, 6, 8 and 10 nM) and all-trans retinoic acid (ATRA) (0, 0.1, 0.5, 1, 5 and 10 µM) for 24 h. The IC₅₀ of ITR-284 was ~10 nM in K562 cells treated for 24 h as determined by MTT assay. May-Grünwald-Giemsa staining and nitro blue tetrazolium (NBT) assays were used to determine cell morphology changes and differentiation after ITR-284 and ATRA treatment. In addition, mRNA expression levels of hematopoietic factors, including GATA‑1, NF‑E2 and GATA‑2, were elevated, while expression levels of BCR‑ABL were downregulated in K562 cells after 24 h of treatment with ITR-284 as determined by quantitative reverse transcription polymerase chain reaction. FOXM1, GLI 1 and c-MYC protein levels were decreased by ITR-284. Taken together, our data show that ITR-284 induced K562 cell differentiation, which led to decreased tumorigenesis. Our findings suggest that ITR‑284 could be a potential candidate for treating chronic myelogenous leukemia.

Introduction

Chronic myelogenous leukemia (CML) is a cancer of the blood cells and bone marrow (1,2). This myeloproliferative disorder is predominantly diagnosed in adults and is characterized by uncontrolled cell growth (3). The exact cause of CML remains elusive. CML is associated with the Philadelphia chromosome, which is a reciprocal chromosome translocation and fusion of the BCR gene on chromosome 22 and the ABL gene on chromosome 9 (4-6). The resulting BCR-ABL fusion protein possesses constitutive kinase activity and promotes the unregulated proliferation of hematopoietic stem cells (5,7).

Properties of differentiation and self-renewal in hematopoietic stem cells are modulated by various lineage-specific transcription factors (8,9). Consensus DNA sequences for the family of GATA transcription factors can be found in the promoters of many hematopoietic linkage-related genes, including β-globin (10,11). GATA binding protein 1 (GATA-1) and nuclear factor, erythroid derived 2 (NF‑E2) are specifically involved in the transcriptional regulation of terminal differentiated erythroid cells (12,13). In contrast, GATA binding protein 2 (GATA-2) and myeloproliferative leukemia virus oncogene (c-MPL) are required for the expansion of multipotent hematopoietic progenitors and the formation of mast cells but not for the terminal differentiation of erythroid cells and macrophages (14,15). c-MYC has been found to be involved in growth, differentiation and apoptosis (16,17). During erythroid and myelomonocytic differentiation, c-MYC plays a critical role. The overexpression of c-MYC leads to the partial inhibition of erythroid differentiation in K562 cells (18-21). In addition, c-MYC suppresses the differentiation induced by imatinib in chronic myeloid leukemia cells (22-24). BCR-ABL kinase inhibitors, such as imatinib and dasatinib, have been used to successfully treat CML (22,23).
ITR-284 (N-(2-dimethylaminoethyl)-4,8-dihydrobenzod (1,2-b:4,5-b') dithio-phene-2-carboxamide phosphoric acid salt), a carboxamide derivative, exhibits potent anticancer effects on various human cancer cell lines (25-27). Our previous study indicated that ITR-284 had anti-proliferative effects on HL60 and WEHI-3 leukemia cells, but it had very low toxicity toward normal cells (26,27). ITR-284 also showed growth inhibitory effects on human hepatocellular cancer cell lines (HepG2, Hep3B, SK-HEP-1 and J5) and colorectal cancer cell lines (HT-29, COLO-205, HCT-116 and SW-620) (26). In this study, we investigated the efficacy of ITR-284 in treating myeloid leukemia and compared its induction of human chronic myelogenous leukemia K562 cell differentiation with that of all-trans retinoic acid (ATRA).

Materials and methods

Chemicals and reagents. Fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, and RPMI-1640 medium were obtained from Thermo Fisher Scientific Inc. (Grand Island, NY, USA). All-trans retinoic acid (ATRA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), May-Grünwald stain and nitro blue tetrazolium were purchased from Sigma (Sigma, St. Louis, MO, USA). Primary antibodies against FOXM1, GLI 1, c-MYC, BCL-2, caspase-3 and β-actin were purchased from GenTex (Hsinchu, Taiwan). ITR-284 was synthesized by Dr Yen-Fang Wen at China Medical University.

Cell culture. Human chronic myelogenous leukemia cell line K562 was obtained from the American Type Culture Collection and grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were passaged every two days and maintained in a humidified environment with 95% air and 5% CO₂ at 37˚C for the following experiments (5,28,29).

Cell viability assay. Cell viability was determined by MTT assay. Briefly, K562 cells were seeded at 1x10⁵ cells per well in 96-well plates (Costar, Corning Inc., Corning, NY, USA), allowed to attach overnight, and then exposed to various concentrations of ITR-284 (0, 2, 4, 6, 8 and 10 nM) or ATRA (0, 0.1, 0.5, 1, 5 and 10 µM) for 24 h. The culture media was removed, and 100 µl of 0.5 mg/ml MTT solution was added to each well. After 4 h of incubation at 37°C, the supernatant was removed, and 100 µl of DMSO was added to each well. The absorbance at 595 nm was measured by using an enzyme-linked immunosorbent assay reader, and the control absorbance was normalized to 100%. Six replicate wells were included in each group, and at least three independent experiments were done (23,25,30).

Trypanblue exclusion assay for cell death. Cells were treated with ITR-284 and cell death was evaluated by trypan blue exclusion assay as previously described (31). Briefly, K562, HL60, U937 and WEHI-3 cells were seeded in a 24-well plate (2.5x10⁵ cells per well) and were incubated with ITR-284 as indicated. After 24 h, cells were stained with 0.25% trypan blue solution and the amount of dead cells were determined by Countess Automated Cell Counter (Invitrogen/Life Technologies) (31).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Apoptotic DNA fragmentation was detected using In Situ Cell Death Detection kit, Fluorescein (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, K562 cells (2x10⁵ cells/well) were seeded into 12-well plates and incubated with 0, 5, 10 and 15 µM of ITR-284 for 48 h. Cells were harvested and assayed by In Situ Cell Death Detection kit, Fluorescein (31).

May-Grünwald Giemsa staining. May-Grünwald Giemsa (MGG) staining was used to analyze the morphological features of megakaryocytes. Approximately, 5x10⁵ cells were seeded in each well of a 24-well plate for 24 h and treated with ITR-284 and ATRA at the indicated concentrations for another 24 h. Cells were centrifuged onto a microscope slide at 800 x g for 5 min and fixed with 10% formaldehyde. After air drying, the slides were stained with May-Grünwald solution (Sigma) for 5 min; the solution was removed, and the cells were then stained with Giemsa solution for another 20 min. The stained cells were examined, and images were captured with an inverted microscope (Nikon Eclipse TE2000U) (9).

Cell differentiation. Nitro blue tetrazolium (NBT) reduction assay is used to determine the differentiation of blood leukocytes by measuring the production of alkaline phosphatase. Briefly, 1x10⁶ cells were seeded in each well of a 24-well plate for 24 h and treated with ITR-284 and ATRA at the indicated concentrations for another 24 h. Cells were centrifuged into a 1.5-ml microscope tube at 6000 x g for 5 min and washed with 1 ml of PBS. After centrifugation, the cell pellets were mixed with NBT reagent for 5 min at 37°C and then stained with NBT for 10 min at room temperature. The proportion of blue-stained cells was calculated using a total of 200 cells randomly selected for each sample in triplicate by light microscopy. The mean ± SD of a representative experiment is shown (8,32).

RNA extract. Approximately 1x10⁶ cells from each treatment were harvested. Total RNA was isolated using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, cell pellets were treated with 1 ml of TRIzol at room temperature for 5 min; next, 0.2 ml of chloroform was added to the tubes and mixed well. After centrifugation at 12,000 rpm for 15 min, the upper aqueous layers were transferred to new tubes and mixed with 0.5 ml of isopropanol for 10 min. The tubes were centrifuged at 12,000 rpm for 15 min. The pellets were dried and dissolved in DEPC-treated H₂O. Absorbance was measured at 260 and 280 nm. A ratio of A260:A280 between 1.8 and 2.0 was used to verify the purity of the samples (29,33,34).

Quantitation of gene expression by real-time RT-PCR. Quantitative RT-PCR was performed according to the Takara One Step SYBR Ex Taq™ qRT-PCR kit. Briefly, one-step RT-PCR was performed in a 20-µl reaction volume containing 100 ng of total RNA, 8 pmol of each forward and reverse primer, Prime Script 1 Step Enzyme Mix, 2X one-step SYBR RT-PCR buffer and RNase-free dH₂O. Quantitative RT-PCR was performed on an ABI 7900HT system (Applied Biosystems). Quantitative RT-PCR conditions were as follows:
stage 1 was cDNA synthesis at 42°C for 5 min and then denaturation at 95°C for 10 min. Stage 2 was RT-PCR amplification for 40 cycles, denaturation at 94°C for 30 sec and annealing and elongation at 60°C for 15 sec. The relative expression level of target genes was determined by normalizing the RNA concentration to that of the β-actin internal control. The relative expression levels of mRNA represent the mean ± SD of duplicates. The primer sequences used for quantitative RT-PCR were as follows: i) actin, forward: 5'-CCAACCGCGAGAAGATGA3' and reverse: 5'-TCCATCACGATGCCAGTG-3'; ii) GATA-1, forward: 5'CTGAGGGCTTGGATGCAG-3' and reverse: 5'-TGGGTACACCTGAAAGACTGG-3'; iii) GATA-2, forward: 5'-TGGCGCACAACTACATGG-3' and reverse: 5'-GCGAGTCGAGGTGATTGAAG-3'; iv) NF-E2, forward: 5'-GCTGTCCACTTCAGAGCTAGG-3' and reverse: 5'-GCTCACTTGAGACATTCAGA-3'; v) c-mpl, forward: 5'-CCTCTTCTGGTCACCTCCTCCTC3' and reverse: 5'-AGGAGACATCTTGGCTGCTG-3'; vi) c-myc, forward: 5'-CGGTGCAGCCGTATTCTAC-3' and reverse: 5'-CAGCAGCTCGAATTTCTTCC-3'; and vii) BCR-ABL, forward: 5'-CACAGCATTCCGCTGACCATCA-3' and reverse: 5'-GCTTCACACCATTCCCCATTGT-3' (29,33,34).

Statistical analysis. All data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with a one-tailed Student's t-test was used for multiple comparisons among groups. A value of p<0.05 was considered statistically significant (5,33,35).

Results

ITR-284 and ATRA inhibit cell viability of K562 cells. The trypan blue exclusion assays were performed to investigate the cytotoxic effects of ITR-284 on several leukemic cell lines including K562, HL60, U937 and WEHI-3 (Fig. 1A). K562 cells were more sensitive than other cells. We investigated the cytotoxic effects of ITR-284 and ATRA on the proliferation of K562 cells by the MTT assays. ITR-284 (Fig. 1B) and ATRA (Fig. 1C) inhibited the viability of K562 cells in a concentration-dependent manner. The IC50 values of ITR-284 and ATRA were ~9 nM and 8.5 µM, respectively (**p<0.005). (D) K562 cells were treated with higher concentrations of ITR-284 for 24 h. Apoptosis was determined by TUNEL assay.
and showed the nuclei characteristics of white blood cells, not those of red blood cells (Fig. 2A). Likewise, ATRA-284 treated cells had morphological changes at concentrations >0.5 µM. More nuclei were observed in ATRA-284-treated cells than in ITR-284-treated cells (Fig. 2B). Furthermore, to examine whether ITR-284 and ATRA promote cell differentiation, we used the nitro blue tetrazolium (NBT) assay to detect the production of alkaline phosphatase. NBT was used as a substrate for alkaline phosphatase, which converted the yellow NBT dye to purple-blue formazan in cells. After treatment with either ITR-284 or ATRA for 24 h, the K562 cells had differentiated significantly (Fig. 3).
ITR-284 and ATRA regulated GATA-1 and NF-E2 mRNA expression. To determine the relationship of GATA transcription factors and EF-E2 in ITR-284- and ATRA-induced cell differentiation, we examined the mRNA expression levels of GATA-1 and NF-E2 by quantitative RT-PCR. GATA-1 (Fig. 4A) and NF-E2 (Fig. 5A) mRNA levels increased in the ITR-284-treated K562 cells, while GATA-1 (Fig. 4B) and NF-E2 (Fig. 5B) mRNA levels decreased in the ATRA-treated K562 cells compared to those in the untreated cells.

ITR-284 and ATRA regulated GATA-2 and c-MPL mRNA expression. Myeloproliferative leukemia virus oncogene (c-MPL, TPO receptor) plays a crucial role in the differentiation of megakaryocytes and platelets (36-38). To determine whether ITR-284 and ATRA are involved in megakaryocyte differentiation, we treated K562 cells with either ITR-284 or ATRA for 24 h and examined the mRNA expression levels of GATA-2 and c-MPL by quantitative RT-PCR. GATA-2 mRNA expression levels increased in the ITR-284-treated K562 cells but decreased in the ATRA-treated K562 cells ("p<0.005).
YANG et al: ITR-284-INDUCED DIFFERENTIATION IN K562 CELLS

while GATA-2 mRNA levels decreased in the ATRA-treated K562 cells compared to those in untreated cells (Fig. 6B). The mRNA expression levels of c-MPL increased in the ITR-284-treated K562 cells (Fig. 7A), while the c-MPL mRNA levels did not change in the ATRA-treated K562 cells (Fig. 7B).

**ITR-284 inhibits BCR-ABL mRNA expression in K562 cells.** To examine whether ITR-284 and ATRA regulate BCR-ABL mRNA expression, we treated K562 cells with either ITR-284 or ATRA for 24 h and measured BCR-ABL mRNA expression by quantitative RT-PCR. The BCR-ABL mRNA levels decreased in the ITR-284-treated K562 cells (Fig. 8A), while there was no significant change in the ATRA-treated K562 cells compared to untreated cells (Fig. 8B). Our results suggest that ITR-284 inhibits BCR-ABL oncogene expression.

**ITR-284 regulates cell differentiation- and apoptosis-related protein expression in K562 cells.** Cell differentiation- and apoptosis-related protein expression levels were analyzed. We treated K562 cells with ITR-284 for 24 h and measured the protein levels of forhead box (Fox) M1 (FOXM1), GLI 1, c-MYC, BCL-2 and caspase-3 by western blotting. As shown in Fig. 9, the protein levels of FOXM1, GLI 1, c-MYC and BCL-2 were downregulated in ITR-284-treated K562 cells compared to those in untreated cells.

**Discussion**

Human chronic myelogenous leukemia (CML) is characterized by the presence of the Philadelphia chromosome, BCR-ABL...
gene fusion with the constitutively active tyrosine kinase, and increased myeloid cells in the bone marrow and the peripheral blood (3). K562 cells contain a Philadelphia chromosome with the chimeric *BCR-ABL* gene transcript. The BCR-ABL fusion protein acts a constitutively active tyrosine kinase in malignant transformation. In recent years, target therapeutic agents have been successfully used to improve the efficacy and adverse effects observed for traditional chemotherapeutic agents (39,40). Many studies have demonstrated that the novel compounds exhibit significant effects in leukemia treatment by inducing cell differentiation and apoptosis (39). The K562 cell line is a good model to investigate hematological cell differentiation. K562 cells can be differentiated to erythroid cells, macrophages and megakaryocytes by phorbol 12-myristate 13-acetate (PMA) (41,42), arsenic trioxide (As2O3) (43,44) and ATRA (45,46). In this study, we evaluated the anti-leukemia effects of ITR-284 on cell differentiation and investigated its mechanism of action in K562 cells. We found that ITR-284 and ATAR simultaneously induced anti-proliferation (Fig. 1), morphological changes (Fig. 2) and megakaryocytic differentiation (Fig. 3) in K562 cells according to the results of the MTT assay, May-Grünwald-Giemsa staining and nitro blue tetrazolium (NBT) assay.

**Figure 10. Possible mechanisms of action of ITR-284 in K562 cells.** The diagram shows that after treatment with ITR-284, K562 cells differentiate into the erythroid lineage or the myelomonocytic lineage according to qRT-PCR and western blot analyses. Upon differentiation, the cells might trigger apoptosis.

Many lineage-specific transcription factors are involved in hematopoietic stem cell differentiation (47-49). The GATA transcription factor is one of the regulators of hematopoietic stem cell differentiation. GATA-1 and GATA2 bind to the consensus DNA sequence (A/T)GATA(A/G) and regulate lineage-restricted gene expression during hematopoietic stem and progenitor cell differentiation (47,49). GATA-1 is required for the differentiation of erythroid cells, granulocytic cells, mast cells, megakaryocytes, and eosinophils, whereas GATA2 is indispensable for the maintenance of hematopoietic stem and progenitor cells (49). Our results showed that *GATA-1* (Fig. 4A) and *GATA-2* (Fig. 6A) mRNA levels increased in the ITR-284-treated K562 cells, but *GATA-2* (Fig. 6B) mRNA levels decreased in the ATRA-treated K562 cells compared to untreated cells. No report has demonstrated the exact roles of GATA-1 and GATA2 in K562 cell differentiation. Ryningen et al demonstrated that ATRA decreased the levels of GATA-2 in acute promyelocytic leukemia (APL) (50). The transcription factor nuclear factor-erythroid 2 (NF-E2) is another specific transcription factor that is functionally linked to the megakaryocytic lineage (51). It has been reported that NF-E2 was significantly reduced in malignant megakaryocytes from essential thrombocytopenia (ET) patients and in K562 cells (51). The *NF-E2* mRNA levels were increased in the ITR-284-treated K562 cells (Fig. 5A), suggesting that NF-E2 plays a crucial role in the ITR-284-induced differentiation of K562 cells.

Myeloproliferative leukemia virus oncogene (c-MPL, hematopoietic cytokine receptor) mainly controls the megakaryocytic lineage of cell differentiation. When K562 cells were treated with either ITR-284 or ATRA, *c-MPL* mRNA was increased (Fig. 7A). Targeting the BCR-ABL kinase is an attractive approach for CML treatment. Elevated BCR-ABL kinase activity causes cell proliferation, while the reduction of BCR-ABL kinase activity induces hematopoietic stem cell to undergo differentiation, which leads to apoptosis (52,53). Our results showed that the BCR-ABL mRNA levels decreased in the ITR-284-treated K562 cells (Fig. 8A). Therefore, BCR-ABL may be a target of ITR-284.

Forkhead box (Fox) M1 (FOXM1) and c-MYC have been characterized as human proto-oncogenes and play an important role in human malignancies (54,55). The FOXM1 protein, a transcriptional regulator, is involved in leukemia cell survival, proliferation and chemotherapy resistance (56-58). The c-MYC protein encodes a transcription factor that regulates the expression of various genes in cell proliferation, cell death and differentiation (59,60). In this study, our results demonstrate the downregulation of the cell survival proteins FOXM1, GLI 1, c-MYC and Bcl-2. In contrast, the cleaved caspase-3 protein levels were increased in the ITR-284-treated K562 cells. These findings suggest that ITR-284 can inhibit K562 cell proliferation by inducing cell differentiation and apoptotic cell death.

In conclusion, our data showed that ITR-284 inhibits growth and cell differentiation in leukemia cells. The possible mechanisms of action of ITR-284 in K562 cells are summarized in Fig. 10. ITR-284 induces cell differentiation by upregulating...
Acknowledgements

This study was supported by a grant to Dr Jai-Sing Yang from China Medical University Hospital, Taichung, Taiwan (DMR-106-122). This study was supported by research grants (no. CMU100-TC-08 and no. CMU106-S-26) to Dr Shih-Chang Tsai from China Medical University, Taichung, Taiwan.

References


GATA-1, NF-E2 and GATA-2, as well as c-MPL, and down-regulating BCR-ABL mRNA expression in K562 cells. Taken together, our findings provide important new insight into the possible molecular mechanism of the anti-chronic myelogenous leukemia activity of ITR-284.


