FCY-302, a Novel Small Molecule, Induces Apoptosis in Leukemia and Myeloma Cells by Attenuating Key Antioxidant and Mitochondrial Enzymes

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INTRODUCTION

Leukemia and myeloma are the two forms of cancers that remain as most dreadful conditions in their class. Both the forms of malignancies, once initiated, are characterized by uncontrolled proliferations and are unmet with significant medical need1,2. Current treatment regimens of acute leukemia and myeloma are very much limited to individual or combination chemotherapies with dose-limiting toxicities and drug resistance3. These limitations therefore open a scope for development of safer and efficacious drugs to treat leukemia and myeloma.

Small molecules are focused in the recent past as effective chemotherapeutics to tackle various diseases. Understanding of medicinal chemistry at the molecular level and development of high-throughput screening have resulted in many synthetic chemical compounds to be tested in vitro for bioactivity. These chemical compounds add value to current drug research with emphasis on effective, economical, and less toxic drugs. Chemotherapy considerably improves survivals rates and stops further malignancy but is mostly restricted by its toxic side effects4. To overcome resistance that malignant cells build against anticancer drugs, there is great interest in the search for new small molecule therapeutic agents5.

Arylidene are such small molecules, and their derivatives are widely proven effective for regulation of cancer cell cycle, antimolluscicidal, antidegenerative, hypoglycemic, and hypolipidemic activities6–9. Few derivatives of arylidene have also exhibited excellent cytotoxic potencies10,11. However, there is no literature evidence for arylidene derivatives possessing antiproliferative activity for leukemia and myeloma cells. As it is well proven that relationships exist among antioxidant status and antiproliferative properties in certain cancers4, search of novel compounds targeting oxidant status and therapeutic efficacy against cancer will be largely rewarding. The current investigation was therefore undertaken to screen our lead molecule (2E)-7-methyl-2-(phenylmethylidene)-4-(propan-2-yl)-2,3-dihydro-1H-inden-1-one (internal product code

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FCY-302) for antioxidant and antiproliferative effects in cancer cells. A brief synthesis method of FCY-302 is indicated in Figure 1.

MATERIALS AND METHODS

HL-60, RPMI-8226, Vero, and Jurkat cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Iscove’s modified Dulbecco’s medium, Eagle’s minimum essential medium, RPMI-1640 medium, and fetal calf serum (FCS) were purchased from Hi Media Corp (Mumbai, India). Annexin V and cell cycle analysis kits were from Millipore Corp. (Boston, MA, USA). Bcl-2, Bax, and β-actin antibodies were from Cell Signaling (Danvers, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other biochemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

Vero cells were cultured in Eagle’s minimum essential medium; RPMI-8226 cells were grown in Iscove’s modified Dulbecco’s medium. HL-60 cells and Jurkat cells were grown in RPMI-1640 medium. All complete growth medium was supplemented with 10% FBS, 100 U/ml of penicillin, and 100 U/ml streptomycin. Cells (passages 3–12) were maintained in a humidified atmosphere of 5% CO₂ incubator at 37°C until the confluence stage was attained. The medium was replaced every alternative day, and the maintenance was strictly in accordance with the standard methods.

Cell Proliferation Assay

Proliferation assay was performed as described by Mosmann with modifications; 5,000 cells/well in 100 µl of RPMI media supplemented with 10% FBS and 1% penicillin–streptomycin were plated in triplicates in a 96-well plate. Cells were incubated overnight, and 50 µl of the compound containing the desired final concentration was added along with a DMSO blank. The cells were incubated at 37°C and 5% CO₂ for 72 h. MTT (15 µl of 5 mg/ml) was added and incubated for 3.5 h. Media was aspirated, and MTT was dissolved in 150 µl of DMSO, and absorbance was read at 560 nm with reference at 640 nm. Percent inhibition was calculated after subtracting day 0 MTT read. Results were analyzed using Graphpad Prism 6.0 software (La Jolla, CA, USA).

Cell Cycle Analysis

Cancer cells were seeded at a density of 0.5 × 10⁶ cells per well in a six-well plate and incubated with FCY-302 at desired concentrations for 72 h at 37°C in a 5% CO₂ incubator. After incubation, cells were fixed in 70% ethanol and stored at −20°C until analysis. Cells were stained with Guava® Cell Cycle reagent according to the manufacturer’s instructions, and 10,000 events were acquired on a Guava easyCyte™ flow cytometer. Data were analyzed using Express Pro software (Millipore), and percent cell population in different cell cycle stages with respect to control was calculated.

Annexin V Assay

The assay was performed using Annexin-V detection kit from e-Biosciences (Grand Island, NY, USA) as per the manufacturer’s instructions as follows. Cells (0.5 × 10⁶) were grown in six-well plates with complete growth media for 24 h until they are semiconfluent. Cells were treated with respective concentrations of FCY-302 and incubated in a CO₂ incubator for 48 h. Harvested
cells were washed twice with wash buffer and incubated with 0.25 µg/ml annexin V reagent in 1x binding buffer for 15 min. After a couple of washes with wash buffer, cells were resuspended back in binding buffer containing 0.5 µg/ml propidium iodide, and 10,000 events were acquired on a Guava easyCyte™ flow cytometer. Data were analyzed using InCyte software-Millipore. Early and late phase apoptotic cells were segregated with a quadriplot graph, and total percentage of apoptotic cells was represented using Graphpad Prism 6.0 software.

**Western Immunoblotting**

Western blotting procedure for determining the anti- and proapoptotic signaling proteins were carried out as described elsewhere with minor modifications. Briefly, cancer cells were treated with desired concentrations of FCY-302 for 48 h and lysed in buffer containing 50 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 5 µg/ml pepstatin A. Proteins were quantified using Coomassie Plus Protein Assay Reagent kit (Pierce, Rockford, IL, USA), and 20–40 µg of total cellular proteins from cell lysate was separated using 8%–15% SDS polyacrylamide gel electrophoresis. The proteins were further transferred to a nitrocellulose membrane, probed with the respective primary antibodies, and added with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies. Membrane stripping was done by stripping buffer at 50°C for 30 min and quantified. Protein bands were visualized using ECL reagents (Amersham Bioscience, Piscataway, NJ, USA) and exposed to Kodak X-Omat Blue XB-1 films (Rochester, NY, USA). Bands were quantified using ImageJ (Ver: 1.46, NIH) and normalized to actin.

**Mitochondrial Membrane-Bound Enzyme Assays**

HL-60, Jurkat, and RPMI-8226 cells were incubated with respective concentrations of FCY-302 for 48 h. The cells were then pelleted and washed once with phosphate buffer and resuspended in the same buffer and frozen. The suspension was thawed to break the cells and cooled in ice. The cell extract was made in 2.5 mM in MgCl₂ and centrifuged at 6,000×g for 10 min to remove the unbroken cells. The membrane fraction was collected by centrifugation at 50,000×g for 45 min. After washing twice and rehomogenization in the same buffer (ice cold), the membrane fraction was used for the estimation of Na/K+ ATPase, Ca²⁺, ATPase, Mg²⁺.

**Statistical Analysis**

Experiments were carried out at least in triplicate, and results are expressed as the mean±SD. Statistical analyses were performed using Graphpad Prism 6.0. GI₅₀ and IC₅₀ values were calculated using a nonlinear regression fit model with variable slope and plotted accordingly. Differences with a value of p<0.05 were considered statistically significant.

**RESULTS**

**Antiproliferative Efficacy of FCY-302 in Cancer Cell Lines**

We used acute promyelocytic leukemia cell line HL-60, myeloma cell line RPMI-8226, and acute T-cell leukemia cell line Jurkat to assess the antiproliferative/cytotoxic effect of the compound in cancer cells. To check out the nontoxic levels of the compound in normal cells and determine the selectivity toward cancer cells, non-tumorigenic Vero cells were used. The compound inhibited the proliferation of HL-60, Jurkat, and RPMI-8226 cells with GI₅₀ of 395.2 nM, 514.6 nM, and 642.4 nM, respectively (Fig. 2A). GI₅₀ was 4,881 nM for Vero cells (Fig. 2A). FCY-302 was around 8-, 9-, and 13-fold more selective toward RPMI-8226, Jurkat, and HL-60 cells, respectively, in comparison with the normal Vero cells (Fig. 2B). We used the closest GI₅₀ values; 400 nM FCY-302 in HL-60 cells, 520 nM in Jurkat cells, and 650 nM in RPMI-8226 cells for all the following assays.

**Figure 2.** Efficacy of FCY-302 in cancer and normal cells. (A) GI₅₀ values of FCY-302 in HL-60, Jurkat, RPMI-8226, and Vero cells showing antiproliferative effect by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B) Fold difference in antiproliferative efficacy by the compound between normal and cancerous cells. Results are average from three individual experiments performed in triplicates. *p<0.05.
Effects of FCY-302 on Cell Cycle Changes in Cancer Cells

To further elucidate the observed antiproliferative effects of the compound, we analyzed the effect of FCY-302 in the cell cycle of cancer cells. Appearance of a hypodiploid peak was observed in all the three types of cancer cells after FCY-302 treatment (Fig. 3). An increase in the percentage of sub-G0 phase of cells with respective controls of 3%–15% to 14.23% in HL-60 cells (Fig. 3A), 3.46% to 11.93% in Jurkat cells (Fig. 3B), and 1.80% to 9.25% in RPMI-8226 cells (Fig. 3C) were observed. FCY-302 Induced Apoptosis in Leukemia and Myeloma Cells

FCY-302 treatment resulted in the apoptotic indicator annexin V+ cells of 32%, 28.87%, and 31.28% in HL-60, Jurkat, and RPMI-8226 cells, respectively (Fig. 4). FCY-302 treatment also decreased the antiapoptotic Bcl-2 levels and increased the proapoptotic Bax protein levels in HL-60, Jurkat, and RPMI-8226 cells (Fig. 5A), thereby significantly decreasing the Bcl-2/Bax ratio in all three cell lines (Fig. 5B).

FCY-302 Attenuated Mitochondrial and Antioxidant Enzyme Activities

In order to augment the observed antiproliferative and apoptosis-inducing effects of FCY-302 in cancer cell lines, we tested the effects of the compound on mitochondrial and antioxidant enzymes for all three cancer cell lines. Significant inhibition of Na+/K+ ATPase activity was observed in HL-60, Jurkat, and RPMI-8226 cells (Fig. 6A). There was a reduction in activities of Ca²⁺ ATPase post-FCY-302 treatment in these cancer cells (Fig. 6B). Mg²⁺ ATPase activities also remained inhibited, suggesting an alteration in the homeostasis of these ions (Fig. 6C). GSH was reduced in all three cancer cells after FCY-302 treatment (Fig. 7A). Activity levels of antioxidant scavenging enzymes like superoxide dismutase (SOD) catalase (CAT), glutathione reductase (Gr), and glutathione-S-transferase

Figure 3. Cell cycle analysis of (A) HL-60, (B) Jurkat, and (C) RPMI-8226 cells after 72 h treatment of FCY-302. Representative cell cycle histograms from three individual experiments showing percentage sub-G0 population in cancer cells after FCY-302 treatments.
Figure 4. Representative histograms from annexin V assay post-FCY-302 treatment in (A) HL-60, (B) Jurkat, and (C) RPMI-8226 cells after 48 h. Results from three independent assays are shown.

Figure 5. (A) Western blot of antiapoptotic and proapoptotic markers after various doses of FCY-302 treatment in HL-60, Jurkat, and RPMI-8226 cells for 48 h. (B) Bcl-2/Bax ratio of HL-60, Jurkat, and RPMI-8226 cells after FCY-302 treatment. Bands were quantified by densitometry using ImageJ (Ver: 1.46, NIH), normalized to actin, results were plotted with GraphPad Prism 6 software. Results are from three individual experiments in duplicates and were statistically significant at *p < 0.5.
(GST) were also found to be decreased with FCY-302 treatment in the three cell lines tested (Fig. 7B–E).

DISCUSSION

Novel chemotherapeutics for leukemia and myeloma are needs of the hour, and biologically active small molecule has been the key research focus in recent past. FCY-302 is shown to inhibit the proliferation of three different cancer cells in the current investigation. Observation of 8- to 13-fold potency in the tested cancer cells over non-cancerous Vero cells clearly indicates the selectivity of FCY-302 to inhibit cancer cell proliferation with a reasonable therapeutic window in vitro. Appearance of the hypodiploid peak in cell cycle analysis post-FCY-302
treatment indicates DNA damage induced by the compound in cancer cells\(^2\).

Annexin V binds with high affinity to phosphatidyl serine of the mitochondrial membrane when exposed to apoptotic cell surface and differentiates healthy cells from apoptotic cells by considering the cellular membrane integrity\(^3\). The occurrence of annexin V cells after FCY-302 treatment indicated apoptotic induction by the compound in leukemia and myeloma cells. It is reported that apoptotic events in cancer cells are closely regulated by the antiapoptotic Bcl-2 protein and the proapoptotic Bax protein\(^4\). Overexpression of the Bcl-2 proteins is believed to provoke proliferation and confer resistance in many cancer cell types\(^5\). On the other hand, studies also indicate that upregulation of the Bax protein is responsible for apoptotic induction\(^6\). The current investigation supports these claims with downregulation of the Bcl-2 and upregulation of Bax proteins, thereby favoring apoptosis in the cancer cells.

Studies indicate a tight correlation between the Bcl-2, Bax proteins, and mitochondrial membrane integrity\(^7\). The susceptibility of the cells to undergo apoptosis is controlled by Bcl-2 protein by regulating the membrane integrity of the mitochondria, while Bax helps in permeabilization of the mitochondrial membrane favoring apoptosis\(^8\). Bcl-2/Bax ratio is therefore a key event for apoptotic induction mediated by mitochondria. Our observations of decrease in the Bcl-2/Bax ratio by FCY-302 treatment can therefore be attributed to mitochondria-mediated apoptosis in leukemia and myeloma cells. Therefore, it could be inferred that the increased levels of Bax and decreased levels of Bcl-2 would have led to loss of mitochondrial potential leading to cell suicide and making the cells bioenergetically deficient\(^9\). These claims were further confirmed by the decrease in the mitochondrial membrane-bound enzyme activities upon FXY-302 treatment, which was in line with previously published research\(^10,11\).

To further augment our observations, we checked the status of key antioxidant enzymes after FCY-302 treatment in cancer cells. In aerobic organisms, reactive oxygen species (ROS) including \(O_2^-\), \(OH^-\), and \(H_2O_2\) are continuously generated by mitochondrial action, which remains a potent threat to cell survival by inducing oxidative stress. To detoxify ROS, the mitochondria have developed several defense mechanisms by combined action of antioxidant enzymes\(^12\) like SOD, CAT, GST, and \(GSH\). Out of these enzymes, SOD catalyzes the dismutation of the superoxide anion to \(H_2O_2\), while catalase favors the conversion of \(H_2O_2\) to water and oxygen\(^13\). Though appearing hostile, oxidative stress could be beneficial by inducing apoptosis in cancer cells\(^14,15\). Studies indicate oxidative stress and free radicals that are created by chemotherapeutic agents and could be helpful in treatments of leukemia and myeloma\(^16,17\). The activity levels of SOD, CAT, GST, and \(GSH\) were markedly reduced in all the tested cells following FCY-302 treatment. These overall observations are in par with the report of Chang et al., who demonstrated the anticancer effect of a traditional medicine by induction of oxidative stress leading to the loss of mitochondrial membrane potential, down-regulation of Bcl-2, and upregulation of Bax protein\(^18\).

**CONCLUSION**

Outcomes of this study indicate excellent antiproliferative activity of FCY-302 in models of leukemia and myeloma cells. The activity was supported by attenuation of key mitochondrial and antioxidant enzymes to favor apoptosis. These findings drive more attention toward this compound for further investigation and development of analogs to test biological efficacy against cancer types.

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