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Differential effects of peroxisome proliferator-activated receptor agonists on doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells

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Abstract

P-glycoprotein (*P*-gp)-mediated multidrug resistance (MDR) in tumor cells is still a main obstacle for the chemotherapeutic treatment of cancers. Therefore, identification of safe and effective MDR reversing compounds with minimal adverse side effects is an important approach in the cancer treatment. Studies show that peroxisome proliferator-activated receptor (PPARs) ligands can inhibit cell growth in many cancers. Here, we investigated the effect of different PPAR agonists include fenofibrate, troglitazone and aleglitazar on doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells. The effects of doxorubicin (DOX) following treatment with PPAR agonists on cell viability were evaluated using MTT assay and the reversal fold (RF) values. Rhodamine123 (Rh123) assays were used to determine *P*-gp functioning. *P*-gp mRNA/protein expression was measured by quantitative reverse transcription polymerase chain reaction (*q*RT-PCR) and western blot analysis after incubation with troglitazone and aleglitazar. Our results showed that troglitazone and aleglitazar significantly enhanced the cytotoxicity of DOX and decreased the RF values in K562/DOX cells, however, no such results were found for fenofibrate. Troglitazone and aleglitazar significantly down regulated *P*-gp expression in K562/DOX cells; in addition, the present study revealed that aleglitazar elevated intracellular accumulation of Rh123in K562/DOX cells as short-term effects, which also contribute to the reversal of MDR. These findings show that troglitazone and especially aleglitazar exhibited potent effects in the reversal of *P*-gp-mediated MDR, suggesting that these compounds may be effective for combination therapy strategies and circumventing MDR in K562/DOX cells to other conventional chemotherapeutic drugs.

Key words: PPAR, multidrug resistance, *P*-glycoprotein, chronic myeloid leukemia, doxorubicin, cardiotoxicity.

Introduction

The most important problem in the field of cancer therapy, which is become more sophisticated by increasing researches, is resistance to chemotherapeutic agents. Besides the high costs and labors and more importantly problematic side effects, development of the multi-drug resistance (MDR) is considered as a huge disappointment for patients and physicians(1-4). Therefore, identification of effective MDR reversing compounds with minimal or no adverse effects is an attractive goal in the clinic.

The most common mechanism of MDR is expression of the multi-drug efflux pumps belonging to the ATP-binding cassette (ABC) transporter family(5). *P*-glycoprotein (*P*-gp) that encodes by multiple drug resistance protein 1 (MDR-1; or ABCB1) gene is the most commonly expressed of ABC transporters family in many human cancers and functions as an active transmembrane efflux pump for the different anticancer drugs such as anthracyclines, vinca alkaloids, and taxanes(6, 7). Following binding to *P*-gp, anticancer drugs are constantly pumped outside of the cells, inducing a continuous decline in intracellular drug concentrations. Subsequently, the drug toxicity on tumor cells is slowly weakened, thereby losing efficiency, and lastly, generating MDR in cancerous cells. Hence, because of unavoidable role of *P*-gp in the MDR, many efforts have been concentrated on identifying effective inhibitors of *P*-gp. Some drugs including verapamil and cyclosporin A have been reported as compounds for inhibiting *P*-gp(8, 9). However, these are used as an antiarrhythmic drug and an immunosuppressant, respectively. Consequently, adverse side effects are expected to arise when these drugs are used as MDR-reversing agents with antitumor drugs. Therefore, development of safe and effective MDR reversing agents with minimal adverse side effects is eagerly required.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptors superfamily. PPARs, which are dividing to three main subgroups includingα, β/δ and γ, have exclusive roles in the regulation of lipid/carbohydrate metabolism, growth inhibition and differentiation in normal or tumor cells (10-12). Several preclinical and clinical studies suggest that PPARα and γ agonists were found to increase cytotoxicity effects of chemotherapeutic agents in several types of tumors (11, 13-15). In the present study, we aimed to investigate the effects of fenofibrate (a PPARα agonist), troglitazone (a PPARγ agonist), and aleglitazar (a dual PPARα/γ agonist) on cytotoxicity of doxorubicin, a chemotherapeutic agent that is a substrate for *P*-gp, and P-gp mRNA/protein expression as well as its functioning, on the doxorubicin-resistant

human myelogenous leukemia (K562/DOX) cells.

Materials and Methods

Materials

Human chronic myelogenous leukemia (K562) cells and doxorubicin-resistant human chronic myelogenous leukemia (K562/DOX) were obtained from Pasteur Institute Cell Culture Collection (Tehran, Iran); doxorubicin (DOX), verapamil, troglitazone and fenofibrate were purchased from Cayman, USA; aleglitazar, Rhodamine123 (Rh123), 3-(4,5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT), RNase, and verapamil (*P*-gp inhibitor) were purchased from Sigma Co, USA; Fetal bovin serum (FBS) and RPMI-1640 medium were purchased from Gibco, USA; *P*-gp and β-actin antibodies and secondary antibody were obtained from Abcam (Cambridge,UK); All other chemicals used in the experiments were commercial products of reagent grade.

Cell culture

K562 cells and K562 /DOX cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37 ^{o}C in a humidified atmosphere of 5% CO_2. K562/DOX cells were cultured in the presence of 0.5 μM DOX and were grown in drug-free medium for 2 week before being used in experiments.

Cell viability assay

Determination of cell viability was accomplished by the MTT assay as described previously(16). This method is based upon the reduction of yellow MTT to the insoluble formazan in the mitochondria of living, viable cells. Breifly, K562 and K562/DOX cells at the logarithmic phase were seeded in a 96-well plate at the density of 2×10^4 cells per well and cultured with RPMI-1640 supplemented with 10% FBS. After 24 h incubation, various concentrations of DOX with or without aleglitazar, troglitazone or fenofibrate were diluted in a RPMI-1640 medium (without FBS) and added into each well. Experiments for each group were performed in triplicate and with a blank control. After 48 h treatment, the medium was removed and 200 μl RPMI-1640 medium supplemented with 10% FBS and 10% MTT (5 mg/ml) was added. After incubation for another 4 h, the reduced intracellular formazan product was dissolved by replacing 100 μlof RPMI-1640 medium with the same volume of dimethylsuloxide (DMSO). Absorbance values were measured at 570 nm with a micro plate reader (State Fax, 2100; Awareness Technology Inc, Palm City, FL, USA).The half-maximal inhibitory concentration (IC_{50}) of each experiment was also calculated. The resistant fold (RF) was calculated according to the following: (IC_{50} value of the resistant experiments) / (IC_{50} of corresponding parental cells).All of these experiments were repeated three times.

Intracellular Rh123 accumulation

K562 and K562/DOX cells at a density of 1×10^5/ml in exponential growth were used for the test. Cells were incubated in the presence or absence of fenofibrate, troglitazone or aleglitazar with medium containing 5 μM Rh123 at 37 ^{o}C for 2 h. The intracellular mean fluorescence intensity (MFI) associated with Rh123 was determined by flow cytometry (Becton Dickinson, New Jersey, USA). Verapamil (10 μM) was used as positive control for *P*-gp function-inhibitory agent.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cellular total RNA was prepared from cells using the AccuZol^{TM} reagent following the manufacturer's instructions (Bioneer, Daedeok-gu, Daejeon, Korea). Total RNA (2μg) was subjected to the RT reaction using MMLV reverse transcriptase (Promega, Madison, WI, USA) and oligo-dT primer. The cDNA products were further subjected to PCR amplification with SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan) using the Rotor-Gene TM 6000 system (Corbett Life Science, Mortlake, NSW, Australia). The sequences of PCR primers were as follows: forward, 5'-CCCATCATTGCAATAGCAGG-3', reverse, 5'-CTACCTCCTGTGGCTAATCTG-3', for *MDR1*, and forward, 5'-TCCCTGGAGAAGAGCTACG-3', and reverse, 5'-GTAGTTTCGTGGATGCCACA-3', for *β-actin*. All experiments were carried out in triplicate, and for each sample assayed, the threshold cycle (Ct) value for target gene or *β-actin* gene was determined. The relative mRNA expression levels of each gene were normalized to the β-actin expression level, which allowed target cDNA calculation as $2^{-(Ct\ target\ gene\ -\ Ct\ \beta\text{-}actin)}$.

Western blotting analyses

Western blot assay was used to analyze the expression of *P*-gp in K562/DOX cells. The protocol was followed as previous study with slight modifications [16]. K562/DOX cells were incubated in the presence or absence of the fenofibrate, troglitazone or aleglitazar at 37 ^{o}C for 24 h, respectively. Cell lysate was collected using a buffer (20 mM Tris-HCl at pH 7.5) containing detergents (0.1% SDS, 1% sodium deoxycholate, and 1%Triton X-100). The protein concentration of the cell lysates were measured by Bradford reagent with bovine serum albumin (BSA) as the standard protein (Bio-Rad, Hercules, CA, USA). Each 50 μg lysate were resolved on corresponding SDS-PAGE gels with different percentages and then transferred onto PVDF membranes (GE Healthcare, Amersham, Buckinghamshire, UK). After milk block, the membranes were incubated with primary antibodies overnight at 4C. Then washed with TBST (Tris-buff ered saline with 0.1% Tween 20) buffer for three times and incubated with HRP-conjugated secondary antibody at room temperature for 2 h. Washed another three times with TBST buffer, the membranes were developed by the ECL reagents(Amersham Pharmacia Biotech, USA).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) followed by Tukey's test was used to determine the significant differences between groups and analyzed using GraphPad Prism software. Values of P less than 0.05 were considered significant.

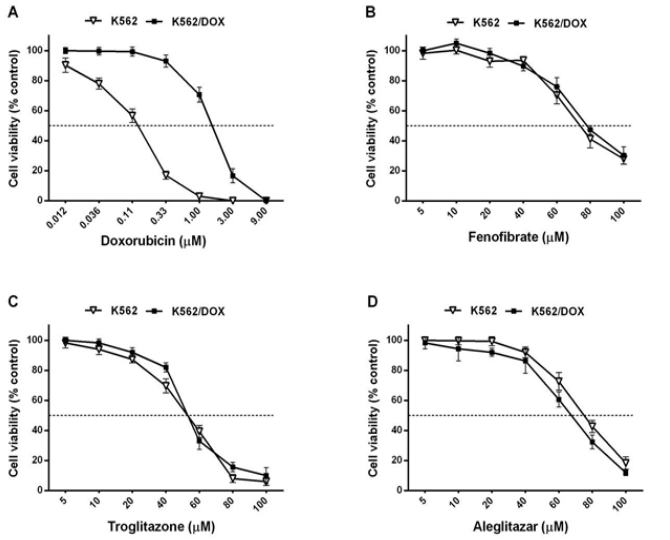

Figure 1. The sensitivity of K562 and K562 /DOX cells toward DOX and PPAR ligands. K562 and K562/DOX cells were treated with various concentrations of DOX (A), fenofibrate (B), troglitazone (C) and aleglitazar (D) for 48 h, the cell viability was determined by the MTT assay. Each point represents the mean ± S.D., n = 3 dependent experiments.

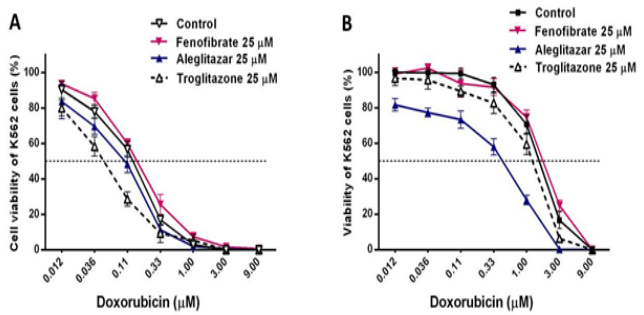

Figure 2. Effects of PPAR ligands on DOX chemotoxicity in K562 and K562/DOX cells toward DOX. K562 cells (A) and K562/DOX cells (B) cells were treated with DOX and DOX plus fenofibrate, troglitazone or aleglitazar 25μM for 48h, and the cell viability was determined by the MTT assay. Each point represents the mean ± S.D., n = 3 dependent experiments.

Results

Effects of PPAR ligands on the viability of K562 and K562/DOX cells

The multidrug-resistant K562/DOX cells, in which *P*-gp is highly expressed compared with the parental K562 cells, are well-characterized MDR cell model for studying efflux of different anticancer drugs. The effect of fenofibrate, troglitazone and aleglitazar on cell viability was determined with MTT assay. The IC_{50} values against DOX were: K562= 0.121 μM; K562/DOX = 1.95 μM. K562/DOX cells exhibited 16.11 fold resistances (FR) to DOX, compared with the parental K562 cells. As shown in Fig. 1, fenofibrate, troglitazone and aleglitazar (a range concentration from 5 to 100 μM) displayed equal cytotoxicity towards the MDR cells as well as the corresponding parental cells.

Effects of PPAR ligands on MDR in K562/DOX

Data in Fig. 1 showed that PPAR ligands ranging from 5 to 30 μM had no significant inhibitory effects on the growth of K562/DOX and K562 cells, while the anti-proliferative effect was observed at higher concentrations (50–100 μM). To minimize the effect of PPAR ligands itself on the resistant cell growth, we chose lower concentrations than IC_{50} values for PPAR ligands in the reversal experiments. The modulation of fenofibrate, troglitazone and aleglitazar on the sensitivity of DOX against K562 and K562/DOX cells was shown in Fig. 2. Left shifts in the cytotoxicity profiles of DOX upon the addition of aleglitazar and troglitazone were indicative of reversal of MDR in K562/DOX cells. The treatment of aleglitazar and relatively troglitazone at non-toxic concentrations induced a significant decrease of IC_{50} values of DOX against K562/DOX cells. Therefore, significant differences of the FR values were seen in K562/DOX cells for aleglitazar and troglitazone: fenofibrate = 13.83 (p-value > 0.05); troglitazone = 10.02 (p-value < 0.05); aleglitazar = 4.36 (p-value < 0.001); verapamil (10 μM) = 4.07. These findings indicated that troglitazone and aleglitazar could enhance the potency of DOX against K562/DOX cells, supporting the notion that troglitazone and especially aleglitazar could reverse the resistance of K562/DOX cells.

Effects of PPAR ligands on P-gp functionality

To understand whether the PPAR ligands effects were mediated by *P*-gp, the intracellular Rh123-associated MFI, was examined in parental K562 and K562/DOX cells, which representing the function inhibition of *P*-gp. As shown in Fig. 3A, in the uptake study, after K562/DOX cells were incubated with Rh123 in the presence of PPAR ligands, the MFI increased for aleglitazar in a time-dependent manner over 2 h period (p-value < 0.05) and was comparable to that in verapamil-treated

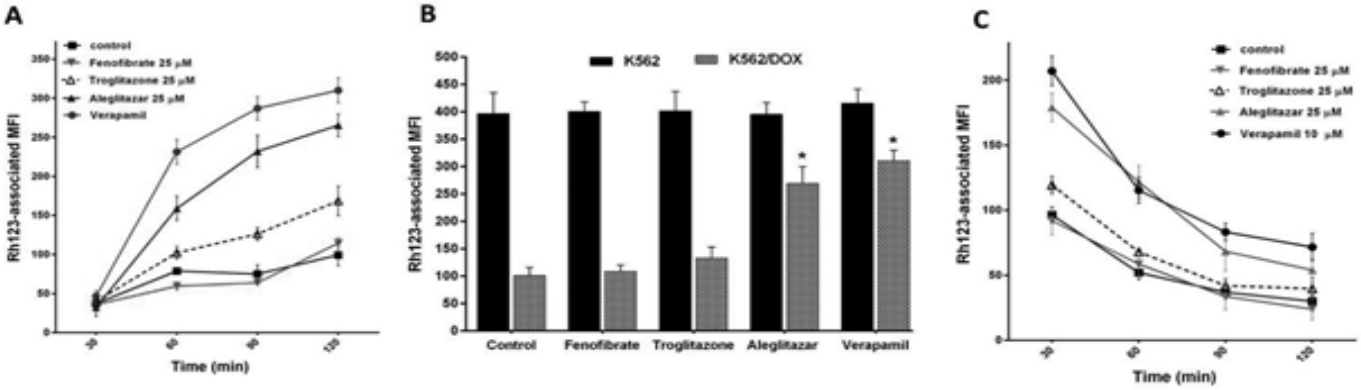

Figure 3. Effect of PPAR ligands on the uptake, intracellular accumulation and efflux of Rh123 in K562 and K562/DOX cells. Each bar represents the mean ± SD (n = 5) and *p < 0.05 vs control.

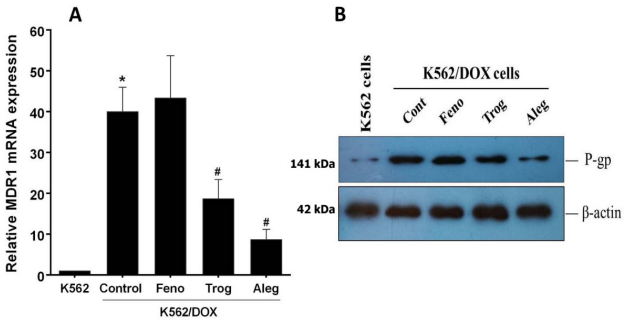

Figure 4. Effects of PPAR ligands on *P*-gp expression. K562/DOX Cells cultured in absence or presence of PPAR ligands for 48. *MDR1* mRNA levels were determined by qRT-PCR and relative mRNA expression were quantified by the 2 $^{(-\Delta\Delta Ct)}$ method (A); and *P*-gp and *β-actin* proteins expression were examined by western blotting analyses (B). The data represent mean±SD (n=3); *p<0.01 vs K562 cells, #p<0.05 vs to untreated K562/DOX cells.

K562/DOX cells (Verapamil as used positive control); however, in other groups did not accumulate the substrate over this same time period. Fig. 4B illustrated clearly that aleglitazar not troglitazone and fenofibrate, enhanced Rh123 accumulation in K562/DOX cells after 2 h; in contrast, no such increase in MFI was observed in aleglitazar-treated K562 cells. Also in the efflux study, result showed that aleglitazar inhibited the efflux of Rh123 from K562/DOX cells in a time-dependent manner (Fig. 3C). All these findings suggested that aleglitazar may inhibit the activity of *P*-gp in K562/DOX cells.

Effects of PPAR ligands on MDR1/P-gp expression in K562/DOX cells

We first evaluated the mRNA and protein levels of *P*-gp in K562 and K562/DOX cells. The findings showed that parental K562 cells expressed a low basal level of the *P*-gp transporter (Fig. 4). We also found troglitazone and aleglitazar down regulated *P*-gp mRNA and protein expression level in K562/DOX cells, which can explain the enhanced DOX intracellular accumulation and cytotoxicity in the troglitazone or aleglitazar -treated K562/DOX cells.

Discussion

In the present study, we have evaluated the efficacy of PPAR ligands to reverse MDR in the DOX resistant K562 cells. Our findings showed that treating resistant cells with fenofibrate (a PPARα agonist), troglitazone (a PPARγ agonist) and aleglitazar (a dual PPARα/γ agonist) cause equal cytotoxity in MDR cells and their corresponding parental cells. In addition, troglitazone and aleglitazar, but not fenofibrate increased the therapeutic potential of DOX on the K562/DOX cells, which means these agonists can help cells to overcome the MDR. Aleglitazar and troglitazone suppressed the expression of *P*-gp in both mRNA and protein levels, and hence decreased the availability of *P*-gp to pump DOX to the extracellular environment. Therefore, the MDR reversing effects of aleglitazar is mediated by the inhibition of *P*-gp activity and increase in the accumulation of *P*-gp substrate in K562/DOX cells.

As a result of many efforts toward overcoming MDR, it is indicated that there are at least two possible approaches to reverse MDR. One of them is to design transport inhibitors which interfere with the drug efflux mediated by pump(9). In this level some problems such as expression of *P*-gp in other non-cancerous tissues, non- selectivity and non- safety features of the inhibitors decrease the efficacy of the approach. The second one is the use of a pharmacological approach to down regulate the *P*-gp expression as a combination agent(17). *P*-gp a 170kDa transmembrane protein, which causes the efflux of therapeutic agents from tumor cells through an ATP-dependent mechanism, is a significant target for such studies(18). Previous accumulating studies showed that PPAR ligands are more effective at cancer therapy by inhibiting cell proliferation and inducing apoptosis. For example, in a study by Valentiner *et al*., it was found that rosiglitazone as a PPARγ selective agonist; inhibits the growth and viability of the human neuroblastoma cell lines (19). The similar findings were reported in human adenocortical cancer cell lines (20). DOX is an effective anti-cancer agent with congested dose dependent cardiotoxicity, which limits its long-term use (21, 22). Therefore, decrease the therapeutic dose of DOX without the elimination of its anti-cancer efficacy is achieved by using another alternative agent, such as PPARs in the case of our study. The most important feature of PPAR ligands, which makes them more popular in the cancer research, is their safety. It is well established that PPAR ligands, in particular aleglitazar and troglitazone are not only non-cardiotoxic, but they are also useful in the treatment of cardiovascular disorders (23). More interestingly, combination of DOX and aleglitazar or troglitazone at non-toxic levels in our study, decreased the IC_{50} values of DOX against K562/DOX cells. In another words, using these agonists caused a significant reduction in the effective dose of DOX in cancer cells treatment and possible elimination of its side effects. It is postulated that the increase in the efficacy of PPAR ligands in killing K562/DOX cells is caused by various mechanisms. For instance, Davies *et al*., showed that troglitazone induce DNA damage and activate apoptotic pathways. Moreover, epigenetic changes such as inhibition of the activity of histone acetyltransferase were shown to be induced by troglitazone. Therefore, troglitazone is capable of binding to acetyltransferase and suppress its function(24).

Zhang *et al*., in another study showed that rosiglitazone, a PPARγ agonist, down-regulated the expression of *P*-gp and reversed MDR by inhibiting Wnt/β-catenine pathway in a MDR ovarian cancer cell line, A2780/Taxol, in a dose-dependent manner (25, 26). A recent study which was carried out by Prost and colleagues indicated that pioglitazone, another PPARγ agonist, combined with imatinib eradicated CML stem cell pool by downregulating the STAT5 and its downstream targets hypoxia-inducible factor 2α (HIF2α) and CITED2, key genes in regulating the stemness of CML stem cells(27). The results of our study showed that aleglitazar but not troglitazone and fenofibrate, increased the intracellular accumulation of Rh123 in K562/ DOX cells in a time-dependent manner. Given this, intracellular contents of DOX was also elevated by the aleglitazar treatment, which caused potentiated DOX cytotoxicity in K562/DOX cells in addition to decreased IC_{50} values of DOX.

In conclusion, our results showed that troglitazone and in special aleglitazar reverse MDR in K562/DOX

cells by suppressing *P*-gp expression or function, leading to an increased intracellular content of DOX. These findings suggest troglitazone and aleglitazar may be effective for combination therapy strategies and circumventing MDR in leukemia to other chemotherapeutic drugs.

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