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ORIGINAL ARTICLE

PSC833, cyclosporine analogue, downregulates *MDR1* expression by activating JNK/c-Jun/AP-1 and suppressing NF-κB

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Abstract
Purpose Multidrug resistance (MDR) is one of the major causes of clinical cancer chemotherapy failure. PSC833 is well known as a non-immunosuppressant cyclosporine analogue that functionally inhibits P-glycoprotein (Pgp), a product of the *MDR1* gene. We investigated whether PSC833 could also alter *MDR1* expression and, if so, which mitogen-activated protein kinases (MAPKs) and nuclear factor-kappaB (NF-κB) pathways were involved in this event.
Methods MTT assay and flow cytometry were used for the analysis of cytotoxicity and intracellular drug accumulation, respectively. RT-PCR and Western blot assays for analysis of gene expression and electrophoretic mobility shift assays for determination of DNA-binding activity of transcription factors were used.
Results The doxorubicin-resistant lung cancer cell subline (SK-MES-1/DX1000), selected from SK-MES-1/WT cells, upregulated *MDR1* expression, thereby showing MDR phenotypes. PSC833 sensitized SK-MES-1/DX1000 cells to doxorubicin. PSC833 (5 μM) also decreased the intracellular accumulation of fluorescent Pgp substrates such as rhodamine 123 and daunorubicin in SK-MES-1/DX1000 cells. PSC833 downregulated *MDR1* mRNA and Pgp expression in a time- and concentration-dependent manner. PSC833 activated c-Jun NH2-terminal kinase (JNK)/c-Jun and enhanced AP-1 DNA-binding activity, but suppressed nuclear translocation of NF-κB, all of which were prevented by pretreatment with a JNK inhibitor SP600125.
Conclusions These results indicate that PSC833 not only sensitizes SK-MES-1/DX1000 cells to doxorubicin by enhancing drug accumulation, but also downregulates *MDR1* expression by activating JNK/c-Jun/AP-1 and suppressing NF-κB.

Keywords Multidrug resistance · PSC833 · P-glycoprotein · JNK · C-jun · AP-1 · NF-kB

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Introduction

Multidrug resistance (MDR) is a major problem in anticancer chemotherapy. Overexpression of transporters is the major cause of MDR since these transporters act as efflux pumps for a variety of structurally diverse chemotherapeutic agents, thereby decreasing intracellular drug accumulation. *Multidrug resistance 1* (*MDR1*) P-glycoprotein (Pgp), multidrug resistance-associated protein, and breast cancer resistance protein are members of the ATP-binding cassette superfamily of membrane transporters [5, 20, 26]. In particular, Pgp acts as a representative efflux pump for a number of commonly used cytotoxic agents, e.g., doxorubicin, vincristine, vinblastine, paclitaxel, colchicine, actinomycin D and mitomycin C [7]. Fortunately, MDR cells can be sensitized to anticancer drugs by treatment with a Pgp inhibitor, which is known as a chemosensitizer. Classical first-generation chemosensitizers have been developed from already-existing drugs that are capable of reversing Pgp-mediated MDR include verapamil [22] and cyclosporine A [30]. Second-generation chemosensitizers such as PSC833 [11] were developed to overcome the low efficacy and high toxicity of first-generation

chemosensitizers. PSC833 (Valspodar) is a non-immunosuppressant analogue of cyclosporine A. It is a potent Pgp inhibitor that is at least 10-fold more active than cyclosporine A in vitro [17] and also a very promising chemosensitizer in vivo [3]. Its phase III clinical study was performed in patients with recurring or refractory multiple myeloma [8]. However, it is not known why PSC833 has more potent Pgp-inhibitory activity than cyclosporine A. Our hypothesis was that PSC833 could not only functionally inhibit Pgp, but also transcriptionally downregulate *MDR1* expression. In this study, we found for the first time that PSC833 downregulates *MDR1* expression by activating c-Jun NH2-terminal kinase (JNK)/c-Jun/activator protein 1 (AP-1) and suppressing nuclear factor-kappaB (NF-κB).

Materials and methods

Cell culture

The SK-MES-1 cell line, obtained from the Cancer Research Center in Seoul National University (South Korea), was cultured at 37°C in a 5% (v/v) CO_2 atmosphere using DMEM medium (GibcoBRL, Gland Island, NY, USA) with 10% (v/v) heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). The doxorubicin-resistant lung cancer cell subline SK-MES-1/DX1000 was selected from the parental cell line SK-MES-1/WT after chronic exposure to doxorubicin and were finally cultured in 1,000 ng/ml of doxorubicin.

Cytotoxicity test

The in vitro cytotoxicity of the drugs was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis, MO, USA] assay [25]. Formazan crystals were then dissolved in dimethylsulfoxide (DMSO). The optical density of the wells was measured with a microplate reader at a wavelength of 540 nm. The 50% inhibitory concentration (IC_{50}) of a particular agent was defined as the drug concentration that causes a 50% reduction in cell number versus untreated control. The IC_{50} values were directly determined from semi-logarithmic dose–response curves. All the experiments were carried out at least in triplicate.

Functional drug accumulation assay using flow cytometry

A suspension of log-phase cells was obtained by trypsinization. The cells were washed twice with ice-cold PBS and then incubated in 5 μM rhodamine 123 (Sigma) and 5 μM daunorubicin (Cerubidine®, Rhone-Poulenc Rorer, Canada) with or without 10 μM PSC833 (Novartis Pharmaceuticals, East Hanover, NJ, USA) in PBS for 1 h at 37°C. The cells were subsequently analyzed for cellular drug fluorescence by flow cytometry (FACSCalibur, Becton Dickinson, MA, USA), in which a focused argon laser beam (485 and 480 nm) excited cells in a laminar sheath flow and their fluorescence emissions (546 and 590 nm) for rhodamine 123 and daunorubicin, respectively, were collected to generate a histogram.

Western blot analysis

Proteins were solubilized and then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting analysis was performed by a slight modification of the method first described by Towbin et al. [29]. Polyvinylidene fluoride membranes were incubated with primary antibodies (diluted 1:1,000): Pgp (C219, Signet Laboratories Inc., Dedham, MA, USA), NF-κB p65, IκBα, p-c-Jun and c-Jun (Santa Cruz Biotech, Santa Cruz, CA, USA), p-IκBα (Stressgen, Victoria, BC, Canada), and JNK and p-JNK antibodies (Cell Signaling, Beverly, MA, USA). The membranes were washed, and incubated with HRP-conjugated rabbit anti-mouse IgG (diluted 1:2,000; Sigma) for 1 h. The proteins were then detected using the ECL detection kit (Amersham, Piscataway, NJ, USA).

RNA extraction and reverse transcription-polymerase chain reaction assay

Total RNA was prepared from cells using an RNeasy midi kit (Qiagen, Hilden, Germany); *MDR1* and *β-actin* mRNAs were detected using reverse transcription-polymerase chain reaction (RT-PCR) assays. *MDR1* mRNA expression was detected with 3′- and 5′-primers corresponding to nucleotides 1,179–1,201 and 907–930, respectively, of the published cDNA sequence [4], to produce a 295-bp PCR product. As a control for RNA amount, *β-actin* mRNA expression was detected with 3′- and 5′-primers corresponding to nucleotides 2,392–2,412 and 1,912–1,932, respectively, of the published cDNA sequence [23], to produce a 501-bp PCR product. RNAs from each sample were reverse-transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, USA) and oligo-dT_{18} primer (Invitrogen, Carlsbad, CA, USA) for 1 h at 37°C. The resulting cDNAs were sequentially amplified with 2.5 units of Taq polymerase (Promega, Madison, WI, USA) and 10 pmole of each primer in a GeneAmp PCR9600 (Perkin-Elmer-Cetus, Norwalk, CT, USA) for 32 cycles (21 cycles for *β-actin*) of denaturation (95°C for 30 s), annealing (53°C but 65°C in *MDR1* for 30 s), and

extension (72°C for 30 s). After the last cycle, all PCR products were subjected to a final extension for 5 min at 72°C. PCR products were combined and then electrophoresed on 1.5% agarose gels containing ethidium bromide. Autoradiographic films of the RT-PCR assays were subjected to densitometric analyses using a Kodak Image Station 4000MM (Eastman Kodak, Rochester, NY, USA). The amount of each mRNA transcript was normalized to that of *β-actin* mRNA.

Nuclear protein extraction and electrophoretic mobility shift assays

Nuclear extract was prepared as described previously [14]. Nuclear protein (5 μg) was incubated with 10,000 cpm of a ^{32}P-labeled probe encoding the NF-κB consensus sequence (5′-CAG AGG GGA CTT TCC GAG AG-3′) or AP-1 oligonucleotide (5′-GCA TGA GTC AGA CAC AC-3′). The DNA–protein complexes were incubated for 20 min at room temperature, resolved in a native 5% polyacrylamide gel in 0.5× Tris borate/EDTA at 160 V for 90 min, dried and then autoradiographed.

Protein determination

Protein concentration was determined by the method of Bradford [16] using a Bio-Rad protein assay kit (Hercules, CA, USA) and standardized with bovine serum albumin.

Results

PSC833 not only functionally inhibits Pgp but also transcriptionally downregulates *MDR1* gene expression

Since PSC833 of less than 10 μM showed cytotoxicity of less than 20% in SK-MES-1 cells (data not shown), we used PSC833 of less than 10 μM in this study. As shown in Fig. 1, SK-MES-1/DX1000 cells show a high level of resistance to doxorubicin compared to SK-MES-1/WT cells, due to overexpression of *MDR1* mRNA and Pgp (Fig. 1a, insert). As expected, PSC833 reversed the resistance of SK-MES-1/DX1000 cells to doxorubicin (Fig. 1a). PSC833 (5 μM) also increased the intracellular accumulation of fluorescent Pgp substrates such as rhodamine 123 and daunorubicin in SK-MES-1/DX1000 cells.

Next, we tested whether PSC833 influenced *MDR1* mRNA and Pgp expression using RT-PCR and Western blot analyses. Steady-state level of *MDR1* mRNA was partially decreased by 5 and 10 μM PSC833, and completely by 20 μM PSC833 in 24 h (Fig. 2A). In addition, steady-state levels of *MDR1* mRNA and Pgp began to decrease progressively from 12 to 72 h in a time-dependent manner after treatment with 10 μM PSC833 (Fig. 2b, c).

PSC833 induces phosphorylation of JNK and c-Jun in SK-MES-1/DX1000 cells

The transcription factor c-Jun or the activation of the JNK pathway plays a principal role in downregulation of *MDR1* expression [21, 32]. In this study, we tested whether JNK and c-Jun were involved in PSC833-induced downregulation of *MDR1*. As shown in Fig. 3a, Western blot analyses show that PSC833 increases phosphorylation of JNK in a time-dependent manner, whereas the expression levels of JNK are not changed. For pharmacological inhibition study, SK-MES-1/DX1000 cells were pretreated with the JNK inhibitor SP600125 (2 μM, Calbiochem, La Jolla, CA, USA) for 30 min, and subsequently treated with 10 μM PSC833 for 1 day for RT-PCR assay and 2 days for Western blot analysis. SP600125 inhibited PSC833-induced downregulation of *MDR1* expression in terms of both mRNA and protein (Fig. 3b).

It is well known that c-Jun can be activated through phosphorylation on serines 63 and 73 by JNK [6, 12]. PSC833 (10 μM) increased both phosphorylation and expression of c-Jun in a time-dependent manner (Fig. 4a). Electrophoretic mobility shift assay (EMSA) revealed that the DNA-binding activity of AP-1 was increased by 10 μM PSC833 in a time-dependent manner in SK-MES-1/DX1000 cells (Fig. 4b).

PSC833 inhibits nuclear translocation of NF-κB p65 subunit due to reduced phosphorylation of cytosolic IκBα

The first intron of the human *MDR1* gene has an NF-κB binding site whose activation causes drug resistance through *MDR1* expression in cancer cells [2]. Therefore, we tested whether suppression of NF-κB was involved in PSC833-induced downregulation of *MDR1* expression. Western blot analysis revealed that PSC833 suppressed nuclear translocation of the NF-κB p65 subunit in SK-MES-1/DX1000 cells. PSC833 (10 μM) reduced the phosphorylation of cytosolic IκBα, followed by a concomitant decrease in nuclear NF-κB p65 subunit from 3 h after treatment (Fig. 4c). EMSA showed that PSC833 (10 μM) decreased the DNA-binding activity of NF-κB in a time-dependent manner (Fig. 4d).

Discussion

We present novel evidence that a potent Pgp inhibitor, PSC833, not only functionally inhibits Pgp, but also

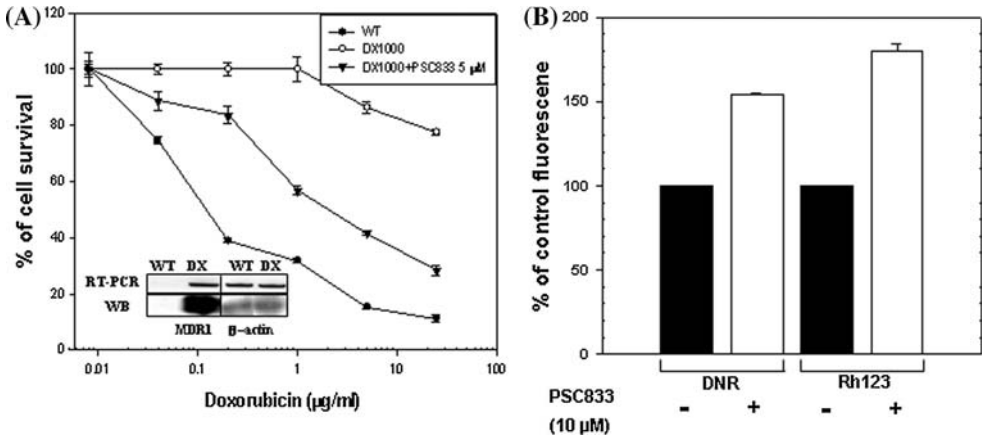

Fig. 1 Effects of PSC833 on chemosensitivity and drug accumulation in SK-MES-1/DX1000 cells. **a** Cytotoxicity was determined by MTT assays (*insert* WT, SK-MES-1/WT; DX, SK-MES-1/DX1000; WB, Western blot). **b** Intracellular drug accumulation was determined by flow cytometry as described in "Materials and methods". *DNR* Daunorubicin, *Rh123* rhodamine 123

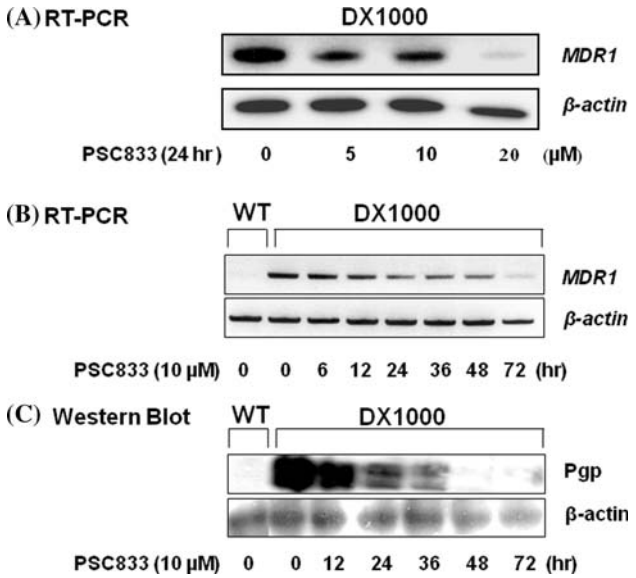

Fig. 2 Effects of PSC833 on *MDR1* expression in SK-MES-1/DX1000 cells. RT-PCR and Western blot analyses were performed to analyze *MDR1* mRNA and protein expression in SK-MES-1/DX1000 cells (1×10^6). Concentration dependency in 24 h (**a**) and time dependency after incubation with 10 μM PSC833 (**b**, **c**) were examined

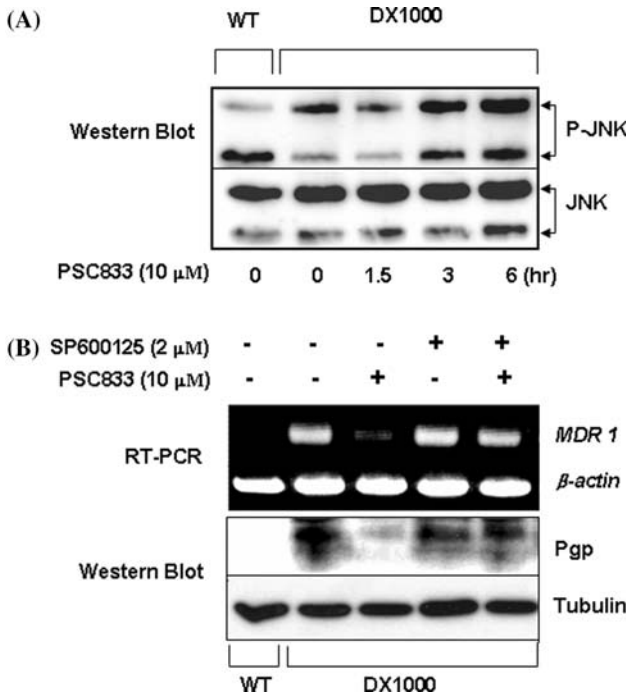

Fig. 3 Involvement of JNK in PSC833-induced downregulation of *MDR1* expression. **a** Effects of PSC833 on p-JNK and JNK. **b** Effects of the JNK inhibitor SP600125 (2 μM) on PSC833-induced downregulation of *MDR1* expression

downregulates Pgp expression by activating JNK/c-Jun/AP-1 and suppressing NF-κB. This effect of PSC833 on *MDR1* expression is quite different from those of other Pgp inhibitors such as verapamil, nifedipine, and cyclosporine A which have been reported to post-transcriptionally increase the levels of *MDR1* mRNA and Pgp in the human colon cancer cell line and its resistant sublines [10]. We wondered if PSC833 could downregulate *MDR1* expression in another Pgp-expressing cell Colo320. PSC833 also downregulated *MDR1* mRNA expression in Colo320 cells overexpressing Pgp (data not shown). Thus, it appears that these dual effects of PSC833 on the function and expression of Pgp may represent, at least in part, a potent Pgp-inhibitory activity compared with other Pgp inhibitors such as cyclosporine A.

What signaling pathways are involved in PSC833-induced downregulation of *MDR1* expression? Since active efflux of xenobiotics is a major defense mechanism of cells against stress, *MDR1* expression is regulated by mechanisms that are generally involved in the stress response [27]. In general, NF-κB and JNK are known to be activated simultaneously under a variety of stress conditions. Since activation of NF-κB inhibits JNK activation, inhibition of NF-κB sensitizes stress responses through enhanced or prolonged JNK activation [31]. In addition, activation of

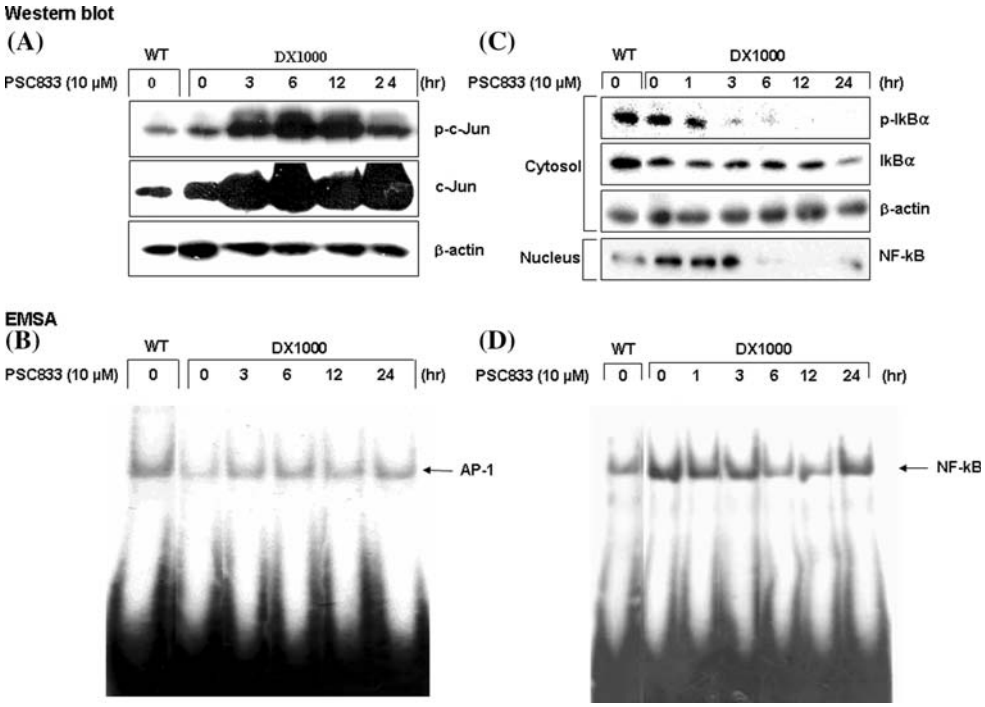

Fig. 4 Effects of PSC833 on c-Jun/AP-1 and NF-κB pathways. Western blot analysis of c-Jun and phosphorylated c-Jun (p-c-Jun) (**a**), and cytosolic phosphorylated IκB (p-IκB) and NF-κB and nuclear NF-κB (**c**). EMSA of AP-1 (**b**) and NF-κB (**d**)

the JNK pathway or the transcription factor c-Jun plays an important role in downregulation of *MDR1* expression [21, 32] although the JNK pathway activates *MDR1* expression in hypoxia which is dependent on hypoxia-inducible factor 1 rather than AP1 [18]. On the other hand, activation of NF-κB induces *MDR1* expression [2]. Recently, it has been reported that NF-κB and JNK signaling pathways are functionally interconnected. The anti-apoptotic function of NF-κB is mediated in part through its ability to downregulate JNK activation [24]. Thus, it is not surprising that JNK and NF-κB are involved as rivals in PSC833-induced downregulation of *MDR1* expression. What is the mechanism by which PSC833 suppresses NF-κB activation? Reactive oxygen species (ROS) increase *MDR1* expression through a process involving NF-κB activation [28]. First, we examined whether PSC833 had an antioxidant effect like the NF-κB inhibitors, *N*-acetylcysteine and pyrrolidine dithiocarbamate. However, PSC833 had no antioxidant effect against ROS such as hydrogen peroxide and superoxide (data not shown). NF-κB is a dimeric transcription factor that regulates the expression of various target genes including inflammatory cytokines such as iNOS and COX2 and anti-apoptotic molecules such as XIAP, C-FLIP and BCL-X_L [1, 9, 13]. In its inactive state, NF-κB is present in the cytosol as a heterodimer composed of p65, p50, and IκB subunits. In response to various stimuli including inflammatory cytokines, such as tumor necrosis factor-α, and cellular stresses, such as irradiation, the IκBα subunit is phosphorylated by an upstream IKKa, triggering ubiquitination and proteosomal degradation of the inhibitory NF-κB subunit, IκBα. This degradation of IκBα facilitates the translocation of the p50–p65 heterodimer into the nucleus [9, 19]. Thus, the IκBα kinase inhibitor Bay 11-7082 prevented both *MDR1* overexpression and degradation of IκBα induced by cadmium [28]. In this study, PSC833 inhibited degradation of IκBα and thereby suppressed nuclear translocation of NF-κB. This effect of PSC833 was supported by the finding that it inhibited lipopolysaccharide-induced upregulation of iNOS expression and NO production in J774A.1 cells (data not shown). This result was also consistent with a recent report that cyclosporine A or PSC833 reduced the expression level or nuclear translocation of the NF-κB p65 subunit in HL60 cells [8] and that JNK/AP-1 activity is under negative feedback control of NF-κB [15].

These results for the first time demonstrate that PSC833 downregulates *MDR1* expression by activating JNK/c-Jun/AP-1 and suppressing NF-κB.

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