OVERCOMING PGP-RELATED MULTIDRUG RESISTANCE. THE CYCLOSPORINE DERIVATIVE SDZ PSC 833 CAN ABOLISH THE RESISTANCE TO METHOXY-MORPHOLYNIL-DOXORUBICIN

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ABSTRACT

Background. The results obtained so far in studies designed to neutralize P glycoprotein (PGP)-related multidrug resistance (MDR) by using MDR reversal agents, have not yet fulfilled the promise of the experiments which were performed in vitro. In order to improve PGP-related MDR neutralization, we tested in vitro the activity of the cyclosporine derivative SDZ PSC 833 (PSC) together with doxorubicin (DOX) and with two new DOX derivatives named 4' iodo 4' deoxy-doxorubicin (IODODOX) and methoxy-morpholynil-doxorubicin (MMDOX, FCE 23762) using four different human cell lines and their multi-drug resistant variants.

Methods. Anthracycline toxicity was evaluated by using the MTT method after a 7-day culture with continuous exposure to the antitumor drugs with or without the addition of PSC.

Results. PSC significantly downmodulated the toxicity of all three anthracyclines in all the four cell systems. However, despite the great increase caused by PSC in the toxicity of DOX and a more modest effect on the toxicity of the two DOX derivatives, this MDR reversal agent could only completely block the PGP mediated MMDOX resistance whereas DOX refractoriness was only decreased.

Conclusions. The combination of MMDOX or IODODOX with PSC 1.6 µM is more efficient than the combination of DOX plus PSC for the full reversion of PGP-mediated drug resistance. Careful clinical studies are required to evaluate if these associations can also effectively and safely neutralize MDR in vivo.

Keywords: multidrug resistance, P-glycoprotein, reversal agents, anthracyclines

A number of experimental studies showed that the multi drug resistance (MDR) caused by the overexpression of the P-glycoprotein (PGP), can be successfully blocked in vitro by several different methods including the use of a wide range of drugs named MDR reversal agents.1-5 However, the results of the pilot clinical trials designed to overcome MDR in vivo did not fulfill the promise of previous in vitro observations.6-11 While this could reflect a limited clinical relevance of the MDR phenomenon, the scarce results obtained in these clinical trials could also be explained by the use of inappropriate MDR reversal agents or by an inappropriate association with antitumor drugs. In fact, in these first studies drugs as verapamil, quinidine or cyclosporine A were used as MDR reversal agents. It should be noticed that these drugs were not originally designed for the neutralization of the MDR phenomenon. Thus, in the majority of the studies the doses of these modulators were below those required to achieve effective inhibition of PGP function in vitro, because of their dose-limiting toxicity. Moreover, all the studies that were performed up to now tried to downmodulate the resistance to drugs that, like doxorubicin, daunorubicin, etoposide or vincristine, are quickly captured and efficiently pumped out by the PGP.12-16 In the past 20 years, many efforts...
were devoted to select new reversal agents lacking the dose limiting toxicities and to synthesize new antitumor derivatives that are not transported by the P-glycoprotein. With the aim of optimizing the neutralization of PGP-MDR through the use of reversal agents, we tested two lipophilic doxorubicin (DOX) derivatives that are more toxic than the parent compound against MDR cells, namely 4’iodo 4’deoxy DOX (IODODOX) and methoxy-morpholynil DOX (MMDOX) together with the cyclosporine derivative SDZ PSC 833 (PSC). This is one of the most efficient MDR reversal agents developed and tested so far. In comparison with DOX, it was found that the combination of both compounds with PSC was highly efficient and could abolish PGP-related resistance.

Materials and Methods

Drugs

Doxorubicin (DOX) was purchased from Pharmacia S.p.A. Milano, Italy. The two DOX-derivatives 4’iodo4’deoxyDOX (IODODOX) and methoxy-morpholynil-DOX (MMDOX, FCE 23762) were a gift from Pharmacia S.p.A. SDZ PSC 833 was a gift from Sandoz, Basel. DOX and IODODOX were dissolved in water at 100 µg/mL. MMDOX and PSC were dissolved in ethanol at 1 and 5 mg/mL, respectively. All the drugs were stored at –20°C and were immediately thawed and diluted before use.

Cell lines

Four systems of human cell lines were used. Each system included a parental, drug sensitive, line without the amplification of the mdr-1 gene and PGP overexpression, and one drug-selected MDR variant with mdr-1 gene amplification and PGP overexpression. The first cell system included the acute myeloid leukemia HL60 cell line and its daunorubicin-selected variant HL60DNR. The second system included the acute lymphocytic leukemia cell line CCRFCEM and its vinblastine-selected variant CEMVLB300. The third system included the colon adenocarcinoma cell line LOVO109 and its DOX-selected variant LOVODOX. The fourth system included the breast cancer cell line MCF7 and its DOX-selected variant MCF7DOX. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂ and maintained in an exponential growth in RPMI 1640 (Biochem KG Seromed) with 10% heat-inactivated foetal calf serum (Biochem KG Seromed), 2 mM glutamine solution, 100 U/mL penicillin and 100 µg/mL streptomycin (Biochem KG Seromed). A selected pressure of the appropriate antitumoric drug (HL60DNR, daunorubicin 0.4 µg/mL; CEMVLB300, vinblastine 0.3 µg/mL; LOVODOX, DOX 0.2 µg/mL; MCF7DOX, DOX 0.2 µg/mL) was constantly maintained just for the MDR cell lines. Before each drug sensitivity assay the PGP expression and the expression of other MDR-associated proteins (lung resistance protein or LRP, multidrug resistance-associated protein or MRP) and of the GSTπ enzyme were evaluated by a flow cytometry assay by using the MRK-16 (Kamiya), the LRP56 (Kamiya), the MRPm6 (Kamiya) and the GSTπ (Dako) monoclonal antibodies. PGP was evaluated as described further on. The LRP56, the MRPm6 and the GSTπ monoclonal antibodies were used following the company guidelines. To quantitate the expression of these MDR associated proteins, the number of the MoAbs bound sites per cell was evaluated by using the Quantum Simply Cellular Kit (Sigma). The results were expressed in units of antibody binding capacity (ABC) as required for this method. Before the experiments the non MDR parental cell lines had an ABC of 177×10^3 (HL60), 98×10^3 (CCRFCEM), 65×10^3 (LOVO109) and 38×10^3 (MCF7). The respective MDR cell lines had an ABC of 3650×10^3 (HL60DNR), 1477×10^3 (CEMVLB300), 6903×10^3 (LOVODOX), and 7517×10^3 (MCF7DOX). No drug selected cell line variants overexpressed LRP, MRP or GSTπ in comparison with the respective parental sensitive cell lines, except LOVODOX, whose reactivity to LRP56 was about three times more than in LOVO109.

Drug sensitivity assay

Cell growth in presence or absence of drugs was determined by using the MTT-microcultured tetrazolium colorimetric assay, as described elsewhere. Briefly, exponential
growing cells were harvested, washed twice in RPMI 1640 (Biochrom KG Seromed), checked for their vitality through the tripan blue exclusion test, and plated into 96 well microtiter plates at the required concentration in 200 µL of a complete culture medium (RPMI 1640 plus 10% fetal calf serum, 2 mM glutamine solution, penicillin and streptomycin). After a 24-hour incubation, ever increasing doses of anthracyclines (0.5–3000 ng/mL) with or without PSC 1.6 µM were added. Cell growth was evaluated after a 7-day incubation at 37°C and 5% CO₂, by using 50 µL per well of the MTT solution (5 mg/mL). Formazan crystals were dissolved in DMSO and their optical density (OD) was read at 540 nm. As required, wells containing no drugs or containing PSC were used to control cell viability. Wells containing no cells were used to blank the spectrophotometer (Novapath Microplate Reader, BioRad). The inhibition dose (ID) was calculated according to Pieters et al. by the following equation, where ID=(mean OD treated wells/mean OD control wells) ×100. Every point of the dose-response curves was the mean of three tests at least. The ID₅₀ was defined as the drug dose that inhibited the cell growth to 50% of the control. The resistance index (RI) was calculated by dividing the ID₅₀ of the MDR cell line with the ID₅₀ of the respective non MDR cell line.

**Results**

For all the MDR and non MDR cell lines the dose response curves obtained by testing the toxicity of all the anthracyclines in presence or in absence of PSC, were drawn to calculate the inhibition dose 50 (ID₅₀) and the resistance index (RI). Table 1 summarizes all the ID₅₀ obtained testing DOX. As expected DOX was several times less toxic in the MDR cell lines than in the parental ones. In the parental cell lines PSC was inactive, whereas in resistant cells PSC the resistance to DOX was substantially decreased. In fact, in the MDR cell lines the ID₅₀ fell from 433 to 27 ng/mL (CEMVLB300), from 1477 to 30 ng/mL (HL60DNR), from 718 to 15 ng/mL (LOVODOX) and from 1230 to 48 ng/mL (MCF7DOX) (Table 1). However, despite the impressive reductions of the ID₅₀ caused by PSC, in the MDR cell lines the sensitivity to DOX never reached the level of the respective parental lines. A possible exception was LOVODOX. Table 2 reports the toxicity of IODODOX alone or in presence of PSC in the MDR and non MDR cell lines. In the MDR cell lines, IODODOX was by itself more toxic than the parental agent DOX. In fact, as shown in Table 2, the RI was 9.4 for the CEM system (CEMVLB300/CCRFCEM), 17.7 for the HL60 system (HL60DNR/HL60), 4 for the LOVO system (LOVODOX/LOVO109) and 3 for the MCF7 system (MCF7DOX/MCF7). Once again the addition of PSC increased the anthracycline toxicity only in the MDR cell lines. Thus, the ID₅₀ of IODODOX fell from 18 to 7.7 ng/mL in CEMVLB300, from 55 to 6.8 ng/mL in HL60DNR, from 26 to 9.9 in LOVODOX and from 57 to 26 ng/mL in MCF7DOX. Therefore, for IODODOX an almost complete neutraliza-
tion of PGP activity, that means a RI close to 1, was obtained in LOVODOX/LOVO109 (RI = 1.6) and in MCF7DOX/MCF7 (RI = 1.4) cell lines. Table 3 summarizes the results obtained with MMDOX. In the drug resistant cell lines MMDOX itself was highly toxic. Its power was particularly evident in MCF7DOX and in CEMVLB300 where the dose response curves of MMDOX alone were very close to the curves obtained in the respective parental cell lines (Figure 1). The coincubation of this anthracycline derivative with PSC further increased MMDOX toxicity in the MDR cell lines so that the addition of PSC could almost completely neutralize the residual resistance to MMDOX (Table 3 and Figure 1).

**Discussion**

SDZ PSC 833 (PSC) is a novel non immuno-suppressive analog of cyclosporine. It was recently completed phase-1 trials which show that at the maximum tolerated dose plasma levels ranged between 2 and 4 µg/mL (1.6-3.2 µM). Laboratory studies showed that at these concentration ranges PSC can provide highly efficient chemosensitization, but also that a complete MDR elimination may be compromised by a high overexpression of the mdr-1 gene. The new anthracyclines IODODOX and MMDOX where shown to be very powerful DOX derivatives. Compared to their drug parent, they are characterized by a higher lipophilicity which seems to be the basis of their rapid spreading through the cell membrane and also by their higher intracellular accumulation in MDR cells. With the aim of optimizing the neutralization of PGP-related MDR through the use of reversal agents we selected the two best available DOX derivatives (IODODOX and MMDOX). We tested their toxicity in comparison with DOX, with or without the addition of PSC in an experimental model of four different human cell line systems, including a sensitive line and a PGP-overexpressing MDR variant. Our findings confirmed prior reports, showing that both DOX derivatives alone were more toxic than DOX in the MDR cell lines. However,
to completely remove the anthracycline resistance caused by PGP in an experimental model based on MDR positive and negative cells, it is necessary that in the presence of the reversal agent, the ID$_{50}$ of the MDR positive cell line variant must equal the ID$_{50}$ of the parental line. In other words, the RI must fall close to 1. In our example, PSC at 1.6 µM which was shown to be a safely achievable concentration also in vivo, was inactive in the non MDR cell lines, while it influenced the toxicity of all the anthracyclines in the MDR cell lines. The power of PSC in blocking PGP was particularly evident when it was used in combination with DOX. In fact this association could reduce DOX ID$_{50}$ more than 15 fold, while the ID$_{50}$ of IODODOX and MMDOX was only reduced by 2-5 folds. However, despite the great effect in sensitizing DOX and the apparently modest effect in sensitizing DOX derivatives, when the toxicity of the anthracyclines plus PSC in the MDR cell lines was compared with the toxicity in the non MDR cell lines, it emerged that only MMDOX resistance could be almost completely abolished. On the contrary, resistance to DOX only decreased.

A possible exception was LOVODOX; in this cell line, PSC was highly efficient in blocking PGP, and in the case of DOX it could reduce the RI to almost 1. The discrepant effect of reversal agents in counteracting the drug resistance to different drugs in different cell lines has already been described. A possible explanation could be that cell lines selected with a positive drug pressure can simultaneously develop different mechanisms of resistance. In our cell lines, an amplification of the mdr-1 gene has already been described and a high PGP overexpression was confirmed in our laboratory by using the MRK-16 MoAb and functional assays. To better define the mechanisms of MDR in our cell lines, other possible MDR factors were investigated. This screening included the evaluation of the
expression of two other drug transporter proteins which belong to the ATP binding cassette superfamily, called the lung resistance protein (LRP) and the multidrug resistance-associated protein (MRP), as well as the study of the expression of the enzyme GSTπ. We found that only LOVODOX displayed a modest increase of the LRP. Therefore, the results that were obtained in this study can apply specifically only to PGP-related MDR. Our observations show that PGP-related MDR can be almost completely removed by using PSC, even when tested cells have a very high PGP expression, if the parent drug DOX is substituted by its more lipophilic derivatives. This suggests that PSC is more efficient when it is used in association with drugs which by themselves can partially avoid the P-glycoprotein transport. Concerning the possible clinical application of these data, preliminary studies have already described the toxic, metabolic and pharmacokinetic properties of IODODOX, MMDOX and PSC respectively. However, it must be pointed out that in vitro studies can describe and clarify the antitumor effect of a single drug or of a combination of drugs, but they can not predict therapeutic results and in vivo side-effects. Moreover, from the preclinical studies with reversal agents, including PSC, performed until now, we have learnt that the introduction of agents blocking the PGP-mediated drug efflux can also alter the antitumor drug metabolism, its delivery to the tumor and the pharmacokinetic.

We conclude that the association of PSC and lipophilic anthracyclines like IODODOX and MMDOX is worth evaluating for antitumor effect and for toxicity in experimental models that is in high risk, primary chemoresistant human tumors.

References
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