Complete Inhibition of P-glycoprotein by Simultaneous Treatment with a Distinct Class of Modulators and the UIC2 Monoclonal Antibody


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ABSTRACT

P-glycoprotein (Pgp) is one of the active efflux pumps that are able to extrude a large variety of chemotherapeutic drugs from the cells, causing multidrug resistance. The conformation-sensitive UIC2 monoclonal antibody potentially inhibits Pgp-mediated substrate transport. However, this inhibition is usually partial, and its extent is variable because UIC2 binds only to 10 to 40% Pgp present in the cell membrane. The rest of the Pgp molecules become recognized by this antibody only in the presence of certain substrates or modulators, including vinblastine, cyclosporine A (CsA), and SDZ PSC 833 (valspodar). Simultaneous application of any of these modulators and UIC2, followed by the removal of the modulator, results in a completely restored steady-state accumulation of various Pgp substrates (calcein-AM, daunorubicin, and 99mTc-hexakis-2-methoxybutylisocyanitile), indicating near 100% inhibition of pump activity. Remarkably, the inhibitory binding of the antibody is brought about by coincubation with concentrations of CsA or SDZ PSC 833 20 times lower than what is necessary for Pgp inhibition when the modulators are applied alone. The feasibility of such a combinative treatment for in vivo multidrug resistance reversal was substantiated by the dramatic increase of daunorubicin accumulation in xenotransplanted Pgp tumors in response to a combined treatment with UIC2 and CsA, both administered at doses ineffective when applied alone. These observations establish the combined application of a class of modulators used at low concentrations and of the UIC2 antibody as a novel, specific, and effective way of blocking Pgp function in vivo.

One of the unresolved problems of cancer, as well as that of AIDS, chemotherapy is the resistance of target tissues against a broad range of compounds applied in the treatment protocols, such as steroids, antineoplastic drugs, immunosuppressive agents, or HIV protease inhibitors (Jones et al., 2001; Borst and Elferink, 2002; Kim, 2003; Glavinas et al., 2004; Sankatsing et al., 2004). This phenomenon is frequently associated with and caused by the overexpression of certain ATP binding cassette (ABC) transporters, including P-glycoprotein (ABCB1; Pgp), multidrug resistance protein 1 (ABCC1), and breast cancer resistance protein (ABCG2), leading to a decreased accumulation of drugs in these tissues (Borst and Elferink, 2002; Glavinas et al., 2004). Multidrug resistance (mdr) mediated by Pgp has been the first to be discovered, and it seems to be the most widely observed mechanism in clinical cases of mdr (Glavinas et al., 2004).

Pgp is composed of two homologous halves, each containing six transmembrane α-helices and an ATP binding site characterized by an “ABC signature” element, in addition to Walker A and B sequence motives. The α-helices form a pore-like structure, allowing the passage of a wide range of hydrophobic substrates against their concentration gradient, and antidrug resistance is associated with the overexpression of Pgp.

ABBREVIATIONS: ABC, ATP binding cassette; Pgp, P-glycoprotein; mdr, multidrug resistance; CsA, cyclosporine A; mAb, monoclonal antibody(ies); ACT, antibody competition test; DMEM, Dulbecco’s modified Eagle’s medium; MIBI, hexakis-2-methoxybutylisocyanitile; PI, propidium iodide; GAMIG, goat anti-mouse IgG; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDZ PSC 833, valspodar; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SCID, severe combined immunodeficient; FK506, tacrolimus; XR9576, tariquidar; SR33557, fanto-
governed by ATP-fueled conformational changes of the protein (Rosenberg et al., 2003).

Numerous studies suggest that the principal physiological role of Pgp is to protect the organism from toxic substances because it is expressed mostly in tissues having barrier functions (e.g., in capillary endothelial cells comprising the blood-brain barrier, placental trophoblasts, and polarized endothelial cells in several organs, such as the gut, liver, or kidneys). Tumors derived from these tissues are intrinsically resistant to chemotherapy, whereas other malignancies may express Pgp or other ABC transporters during later stages of disease progression or in response to chemotherapy (Borst and Elferink, 2002; Leonard et al., 2003; Glavinas et al., 2004).

In view of the great medical importance of overcoming mdr in cancer chemotherapy, as well as combating mdr phenomena decreasing the intracellular concentration of anti-HIV drugs, search for effective and specific reversal strategies continues. These tools include the coadministration of reversing agents (mdr modulators) with the disease-specific drugs to overcome their efflux mediated by the pumps. Concerning Pgp, its antagonists may hinder drug extrusion competitively (e.g., cyclosporine A (CsA), FK506) (Saeki et al., 1993) or allosterically (e.g., XR9576, SR33557, or cis-(Z)-flupentixol) (Martin et al., 1997; Mistry et al., 2001; Maki et al., 2003).

Several monoclonal antibodies (mAb) recognizing discontinuous extracellular epitopes of Pgp have been developed. A few of them (e.g., MRK16, MRK17, MC57, HYB-241, and UIC2 in particular) seem to partially inhibit Pgp-mediated drug export in vitro or in vivo (Chaudhary et al., 1992; Mechetner and Roninson, 1992; Rittmann-Grauer et al., 1992; Jachez et al., 1994), and it was also found that MRK16 potentiates the reversal activity of different cyclosporin analogs in vivo (Naito et al., 1996; Watanabe et al., 1997). Unfortunately, the modulatory effect of the antibodies is partial and extremely variable (Chaudhary et al., 1992; Mechetner and Roninson, 1992; Rittmann-Grauer et al., 1992; Jachez et al., 1994; Naito et al., 1996; Watanabe et al., 1997; and our unpublished observations). Moreover, it depends on the type of the transported substrate (Rittmann-Grauer et al., 1992; Naito et al., 1996; Watanabe et al., 1997); thus, the feasibility of antibody-based mdr reversal strategies in vivo has been highly questionable.

Previously, applying a novel assay [antibody competition test (ACT)] (Nagy et al., 2001, 2004), we have selected a distinct class of Pgp modulators (herein referred to as ACT-positive agents) that elicit a marked increase in UIC2 binding. Here we show that administration of UIC2 in the presence of these modulators (e.g., CsA, SDZ PSC 833, and vinblastine) leads to a near-complete inhibition of Pgp. The inhibitory binding of the antibody is brought about at much lower concentrations of the ACT-positive modulator used than what is required for effective pump inhibition achieved by the modulator alone.

Materials and Methods

Cell Lines. The NIH 3T3 mouse fibroblast cell line and its human mdr1-transfected counterpart (NIH 3T3 MDR1 G185) (Brugemann et al., 1992) obtained from Michael Gottesman’s laboratory (National Institutes of Health, Bethesda, MD) were used in most of the experiments. The cells were grown as monolayer cultures at 37°C in an incubator containing 5% CO2 and maintained by regular passage in Dulbecco’s modified Eagle’s medium (DMEM) (supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, and 25 µg/ml gentamycin). The NIH 3T3 MDR1 cells were cultured in the presence of 670 nM doxorubicin. Cells were trypsinized 2 to 3 days before the experiments and maintained without doxorubicin until use. In some measurements, the A2780 (Pgp )/A2780(CS) (Pgp–) cells were preincubated with 2 µM doxorubicin (Louie et al., 1986) human ovarian carcinoma cell pair was used. The cells were occasionally checked for mycoplasma by the mycoplasma T. C. rapid detection system with a [3H]-labeled DNA probe from Gen-Probe Inc. (San Diego, CA) and were found to be negative.

Chemicals. All of the Pgp substrates and modulators were from Sigma-Aldrich (Budapest, Hungary), with the exception of SDZ PSC 833 (valspodar), which was from Novartis (Basel, Switzerland), and hexakis-2-methoxybutylisonitrilite (MIBI), which was purchased from F. J. Curie Radiobiological Research Institute (Budapest, Hungary) and labeled with 99mTc according to the manufacturer’s instructions. The working concentrations of the tested Pgp substrates and modulators were determined by measuring the changes in calcein accumulation, as well as in viability by propidium iodide (PI) exclusion, in response to the above agents applied at a wide concentration range. Working concentrations of the tested agents were chosen to bring about maximal inhibition of Pgp-mediated calcein-AM efflux at <10% increase in PI positivity. Cell culture media and supplements were also from Sigma (Budapest, Hungary). Alexa 488- and Alexa 647-conjugated goat anti-mouse IgG (GAMIG) was from Molecular Probes (Eugene, OR). The UIC2 (the hybridoma was from the American Type Culture Collections, Manassas, VA) anti-Pgp mAb preparations were >97% pure by SDS-polyacrylamide gel electrophoresis.

Drug Accumulation Studies. Nearly confluent monolayers of these cells were harvested 2 to 3 min after treatment with [0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS), pH 7.4] and washed twice with PBS before use.

Calcein and daunorubicin accumulation was measured as described in Holló et al. (1994) and Goda et al. (2002). In brief, cells were preincubated with Pgp substrates/modulators for 10 min and then further incubated with UIC2 mAb (10 µg/ml) at 37°C for 30 min. Next, the samples were divided into two parts. Pgp substrates/modulators were removed from one of the aliquots by washing with 1% bovine serum albumin (BSA)-PBS and twice with PBS, whereas the other aliquot was kept at room temperature. Finally, samples were stained with 0.25 µM calcein or 1 µM daunorubicin for 15 or 30 min, respectively. In some experiments, cell-bound UIC2 molecules were labeled with Alexa 647-conjugated GAMIG following the calcein accumulation assay. Washing the samples with 1% BSA-PBS did not affect the extent of UIC2 binding to the cells, even after five consecutive washes.

Calcein accumulation experiments were also carried out in the presence of whole-mouse blood containing 3.8% trinitium citrate. A total of 2.5 × 106 cells were resuspended in 100 µl of whole blood, and the subsequent modulator and UIC2 treatment and calcein assay was carried out as described above.

In 99mTc-MIBI accumulation experiments, 10 µCi/ml 99mTc-MIBI was added to the cells after the modulators and/or UIC2 mAb, and the samples were further incubated for 30 min at 37°C. The uptake was terminated by the addition of ice-cold PBS. The cells were then washed three times with ice-cold PBS and resuspended in 1 ml of PBS, and the radioactivity was measured in a Canberra Industries (Meriden, CT) gamma-well counter. The intracellularly accumulated amount of radiotracer was always less than 5% of the total radioactivity concentration. The displayed data are the mean ± S.E.M. of the results of at least three independent experiments, each carried out with triplicate samples.

In Vitro Cytotoxicity Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (Mosmann, 1983) was used to study the cytotoxic effect of daunorubicin. The cells were seeded in 96-well plates at a cell density of 1 × 104 cells/well. Twenty-four
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Flow Cytometry. Two- or three-color cytofluorimetric analysis was performed by using the Becton Dickinson FACScan or FACS-Calibur flow cytometers (Mountain View, CA), respectively. Dead cells stained with PI were excluded from the analysis. Fluorescence signals were collected in logarithmic mode, and the cytofluorimetric data were analyzed by the BDIS CellQuest (Becton Dickinson) software.

Laboratory Animals. Twenty adult (10–12-week-old), pathogen-free B-17 severe combined immunodeficiency (SCID) mice were used in this study (Mariani et al., 2003). The Principles of Laboratory Animal Care (National Institutes of Health) was strictly followed, and the experimental protocol was approved by the Laboratory Animal Care and Use Committee of the University of Debrecen. The NIH 3T3 and NIH 3T3 MDR1 cells (4 × 10^5 cells in 300 μl of serum-free DMEM) were injected s.c. into opposite flanks of the mice. The tumors were grown for 10 to 12 days. Animals were pretreated with 10 mg/kg CsA (Sandimmun, Novartis) i.p. and/or UIC2 mAb (5 mg/kg, added i.v.) 4 h before the administration of daunorubicin (5 mg/kg, i.v.). The animals were killed 4 h after the addition of daunorubicin by cervical dislocation, and the tumors were dissected and kept in liquid nitrogen until further use. Eight to 10 pieces of consecutive 6-μm-thick cryosections were prepared from the tumor samples to compare with nearby sections for their daunorubicin accumulation, UIC2 binding, and tissue morphology. The morphology of the tissue sections was routinely checked by conventional H&E staining after paraffin embedding using an Olympus CX31 epifluorescence microscope equipped with a 7.1-megapixel C7070 wide zoom camera (Olympus Hungary, Budapest, Hungary).

Confocal Laser Scanning Microscopy and Laser Scanning Cytometry. The daunorubicin accumulation and UIC2 binding of the cryosections prepared from the tumors were measured by confocal laser scanning microscopy (LSM 510; Zeiss, Jena, Germany) and laser scanning cytometry (iCys; CompuCyte, Cambridge, MA).

Fig. 1. UIC2 reactivity of NIH 3T3 MDR1 cells in the presence of CsA and verapamil. The cells were pretreated with 75 μM verapamil or 10 μM CsA for 10 min. Labeling of the cells with fluorescein-5-isothiocyanate-conjugated UIC2 mAb was detected by flow cytometry. UIC2 binding was expressed as percentage of maximal labeling. Solid lines represent the best hyperbolic fits of the data points (means of three independent measurements ± S.E.M. are shown).

Fig. 2. The effect of Pgp substrates/modulators on the inhibition of Pgp by UIC2 (left). UIC2 binding visualized by indirect immunofluorescence in parallel experiments (right). NIH 3T3 MDR1 cells were pretreated with Pgp substrates/modulators for 10 min and incubated further with UIC2 mAb for an additional 30 min. The Pgp substrates/modulators then were removed by washing one-half of each sample once with 1% BSA-PBS and twice with PBS (left, black bars); the other aliquot was left unwashed (left, gray bars). Finally, all of the samples were stained with 0.25 mg/ml PI. The following Pgp substrates/modulators were used: 10 μM CsA, 75 μM vinblastine, 8 μM SDZ PSC 833, 10 μM valinomycin, 75 μM verapamil, 20 μM quinine, and 125 μM nifedipine. Calcein fluorescence intensities were normalized to the intensity of the modulator and UIC2 untreated control. UIC2 fluorescence intensities were normalized to the intensity of the CsA-treated sample. Means of three independent measurements ± S.E.M. are shown. All of the drugs (in the presence or absence of UIC2 mAb; left, gray bars) significantly increased calcein accumulation compared with the untreated control (P < 0.001). The Pgp inhibitory effect of UIC2 is preserved after the removal of ACT-positive drugs as shown by the significantly higher calcein accumulation levels compared with the UIC2 untreated samples (left, black bars; P < 0.01), except for valinomycin that was not removed from the cells by the washing protocol applied.
The 488-nm line of an argon-ion laser was used in confocal microscopy experiments. Fluorescence intensities were detected through a 505- to 550-nm band-pass filter (for Alexa 488 dye) and a >580 nm long-pass filter (for daunorubicin). Images were collected through a Plan-Apochromat 63× oil-immersion objective (numerical aperture = 1.4). The pinhole was totally open. The images were always recorded at the same laser intensities and detection parameters, making the comparison of the different samples possible.

The tumor specimens were also analyzed with a laser scanning cytometer in one experiment to obtain quantitative results of whole sections. The 488-nm wavelength of the argon-ion laser was used for excitation, and the fluorescence was detected in the green channel (emission: 530 ± 15 nm). For fast setup of scan areas, the “scout” or low-resolution scanning feature of the “Tissue Scan” input module of the iNovator toolkit was applied. The scout scan was used to find the boundaries of tissue samples based on the fluorescence intensity detected in the green channel, and then a high-resolution scan was conducted to analyze only the defined areas. The 20× objective was used for the high-resolution scan, and the phantom contouring feature of the iCyts software was applied to characterize the fluorescence intensity distribution in large areas of the sections (Megyeri et al., 2005). In these experiments, the highest possible numbers of phantom contours were arranged randomly throughout the scan area, with the radius of each contour set to 10 μm and with no overlap allowed between phantom contours. The integral fluorescence (the sum of the pixel intensities inside a contour) of each contour was used to characterize the fluorescence intensities, and contour maps were created with Sigma Plot 8.0 (SPSS Inc., Chicago, IL) for each section, according to the method described previously (Megyeri et al., 2005).

Statistical Analysis. Data have been analyzed using SigmaStat (version 3.1; SPSS Inc.) and are presented as mean ± S.E.M. Comparison of two groups was performed by unpaired t test, whereas in the case of three or more groups statistical significance was assessed using analysis of variance, applying Bonferroni’s multiple comparison test for post hoc pairwise comparison of the results. Differences were considered significant at P < 0.05.

Results

The UIC2 mAb-mediated pump inhibition is partial and variable (Chaudhary et al., 1992; Holló et al., 1994; Raghu et al., 1996; Hochman et al., 2001), most probably because when added at saturating concentrations, UIC2 binds only to 10 to 40% of all of the Pgp present in the cell membrane (Fig. 1) (in accordance with Mechetner et al., 1997; Druley et al., 2001). We have previously shown (Nagy et al., 2001, 2004) that the incubation of cells with certain Pgp substrates and modulators (referred to as ACT-positive agents) increases the reactivity of UIC2 to Pgp to a large extent in a reproducible fashion as opposed to other substrates and modulators. Close to 100% of all of the cell surface Pgp molecules bind UIC2 in the presence of such ACT-positive agents (e.g., CsA, SDZ PSC 833, vinblastine, ivermectin, and valinomycin) compared to untreated cells or the cells incubated with ACT-negative agents, like verapamil, quinine, and nifedipine did not change significantly the number of UIC2-reactive Pgp, whereas CsA and other ACT-positive agents increased UIC2 binding 2 to 3 times, as shown in Fig. 2 (right). Similar results were obtained in different Pgp+ cell lines (high expressor cells: KB-V1 and 2780AD5, 5 × 105 molecules/cell; low expressor cells: KB-8-5, 5–10 × 104 molecules/cell, data not shown).

Figure 3 shows that UIC2 mAb also strongly increases the 99mTc-MIBI and daunorubicin accumulation in NIH 3T3 MDR1 cells when the cells are prelabeled in the presence of CsA, which shows that inhibition of Pgp function is not restricted to calcein-AM pumping.

When Pgp inhibition was measured as a function of the concentration of CsA, a biologically significant (>10 times) increment in intracellular calcein levels occurred at ≥0.2 μM concentration of the modulator when used in combination with UIC2, whereas a CsA concentration ~20 times higher was just as effective when the modulator was applied alone extracted from the cells by this washing protocol, except for valinomycin. At the same time, the ACT-negative verapamil, quinine, and nifedipine did not elicit an inhibitory binding of UIC2 to Pgp molecules as shown by the fact that calcein accumulation decreased to the control level after removal of these agents (Fig. 2, left, black bars). In accordance with the above, verapamil, quinine, and nifedipine did not change significantly the number of UIC2-reactive Pgp, whereas CsA and other ACT-positive agents increased UIC2 binding 2 to 3 times, as shown in Fig. 2 (right). Similar results were obtained in different Pgp+ cell lines (high expressor cells: KB-V1 and 2780AD5, 5 × 105 molecules/cell; low expressor cells: KB-8-5, 5–10 × 104 molecules/cell, data not shown).

Fig. 3. The effect of CsA and UIC2 on the accumulation of 99mTc-MIBI and daunorubicin into NIH 3T3 MDR1 cells. Samples were preincubated with 10 μM CsA for 10 min and then further incubated with UIC2 for an additional 30 min. CsA was removed by washing the cells with 1% BSA-PBS/PBS, and all of the samples were subsequently incubated with 10 μCi/ml 99mTc-MIBI (A) or 1 μM daunorubicin (B). 99mTc-MIBI uptake is expressed as the percentage of the initial radioactivity of the incubation medium. Mean ± S.E.M. of three independent experiments is shown. UIC2 mAb significantly increased 99mTc-MIBI (P < 0.001) and daunorubicin (P < 0.02) accumulation in NIH 3T3 MDR1 cells when the cells were prelabeled in the presence of CsA.
(Fig. 4, top). Thus, an inhibitory binding of UIC2 is brought about at much lower concentrations of CsA than what is necessary for blocking transport by the drug acting as a competitive inhibitor. At \(0.2 \mu M\) CsA concentration, UIC2 labeled approximately \(>70\%\) of all of the cell surface Pgp, as detected by indirect immunofluorescence in parallel samples (Fig. 4, top, insert). A large (25 times) increase of calcine accumulation was achieved also in response to a combined treatment of NIH 3T3 MDR1 cells with UIC2 and 10 nM SDZ PSC833; when applied alone, the modulator was completely ineffective at this concentration, and the same increment was achieved only at a concentration 10 times higher (Fig. 4, bottom). The combined addition of UIC2 and low concentrations of SDZ PSC 833 significantly increased the cytotoxic effects of daunorubicin compared with treatments with UIC2 or the modulator alone (Fig. 5).

The presence of blood plasma greatly reduces the effect of Pgp modulators (Ludescher et al., 1995; Ayesh et al., 1996). Therefore, as a prelude to an in vivo attempt of mdr reversal based on the combined application of CsA and UIC2, inhibition of Pgp function by CsA and antibody was shown in the presence of whole-mouse blood using modulator concentrations that were not effective when applied alone. When added together with UIC2, a concentration of CsA \(\approx 5\) times smaller (2 \(\mu M\)) was sufficient to achieve a complete block in Pgp function compared with what was required in the absence of the antibody (similar results were obtained with ivermectin, another ACT-positive modulator; data not shown).

Based on these results, we have designed in vivo experiments to test the above strategy. SCID mice were injected in their two opposite flanks with NIH 3T3 MDR1 Pgp\(^\text{+}\) and NIH 3T3 Pgp\(^{-}\) cells, respectively. Palpable s.c. tumors developed in 10 to 12 days. These mice then were treated with CsA and/or UIC2 mAb, followed by the administration of daunorubicin, and the accumulation of this chemotherapeutic agent, as well as UIC2 binding, was measured in cryosections of the tumors, both by confocal microscopy and laser scanning cytometry. Nuclear accumulation of daunorubicin was observed (Figs. 6, A, C, D, and F) deep inside the tumor tissue (also see Fig. 7, left). The combined application of 10 mg/kg CsA and UIC2 increased daunorubicin accumulation of the Pgp\(^{-}\) tumor approximately to the level of the Pgp\(^{+}\) tumor in the same animal (Figs. 6, A and C, and 7, histograms A and C). At the same time, daunorubicin accumulation did not increase significantly in the Pgp\(^{+}\) tumors treated with the antibody or 10 mg/kg CsA alone compared with the untreated mice (Fig. 6, B and E); a CsA concentration 5 times higher was required to reach effective pump inhibition without codistribution of the antibody (compare Fig. 6, A and D).

Quantitative evaluation of the changes was performed in one of the experiments by laser scanning cytometry (Fig. 7). The mean daunorubicin fluorescence intensity in a section of the Pgp\(^{-}\) tumor of the mouse treated with 10 mg/kg CsA and the antibody was \(3.35 \times 10^6\) (CV = 26.87\%); the mean daunorubicin fluorescence of the Pgp\(^{+}\) tumor of the same mouse was \(2.98 \times 10^6\) (CV = 20.43\%) compared with the \(1.64 \times 10^6\) (CV = 18.12\%) mean value measured in the Pgp\(^{+}\) tumor of the mouse treated only with UIC2 in the same experiment.

UIC2 applied together with CsA could readily penetrate into the compact solid tumors, intensively staining cell surface Pgp (Fig. 8, D and E). The antibody binding was specific.
as no significant labeling of the Pgp− tumors was detected (Fig. 9, D–F). Quantitative tissue section analysis showed that the whole tumor section was labeled by UIC2 in the presence of CsA, whereas it barely labeled the cells when added without CsA, as shown in Fig. 9, A and B. The localization of strong UIC2 binding correlates with the distribution pattern of the daunorubicin fluorescence intensity in the specimen (compare Figs. 9A and 7, right). H&E staining of the adjacent sections showed typical tumor tissue histology. In the central tumor areas of lower cell density, we also observed mostly intact cells with strong cell surface Pgp staining at higher magnification, excluding the possibility that nonspecific binding of the antibody to necrotizing regions has been observed (Fig. 8, A–E).

Discussion

When the cells are incubated with the UIC2 mAb in the presence of ACT-positive modulators/substrates, an enhanced and inhibitory binding of the antibody is observed, and this inhibited state of Pgp is preserved after the removal of the modulators. At the same time, ACT-negative agents do not induce an inhibitory binding of UIC2 (Fig. 2), even if they elicit a mild increase in UIC2 reactivity (Nagy et al., 2001, 2004). As calcein accumulation was increased significantly only at ≥60 to 70% ligation of cell surface Pgp with UIC2 (Fig. 4), a prominent inhibition of drug transport is not expected when UIC2 binds to only 20 to 50% of all of the cell surface Pgp in the absence of modulators or in the presence of ACT-negative drugs. These data are best interpreted in terms of the conformational/topological changes of the transporter elicited by the ACT-positive drugs that make all of the cell surface Pgp UIC2-reactive.

Coincubation of Pgp− cells with CsA or SDZ PSC 833 and UIC2 leads to the inhibitory binding of the antibody to most cell surface Pgp at ~20 times lower modulator concentration than what is necessary for the complete blocking of transport by the modulator acting merely as a competitive inhibitor. We propose that a rather low concentration of ACT-positive agents (under the KM of their transport) is sufficient to initiate the catalytic cycle so that all of the cell surface Pgp

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**Fig. 6.** Confocal laser scanning microscopic images of daunorubicin accumulation in cryosections of NIH 3T3 MDR1 and NIH 3T3 tumors developed in SCID mice (A, B, D, and E, and C and F, respectively). Tumors were palpable when mice were injected i.v. with various amounts of UIC2 antibody and/or CsA, followed by daunorubicin according to the schedule described under Materials and Methods. The panels represent treatment with 10 mg/kg CsA + 5 mg/kg UIC2 (A); 5 mg/kg UIC2 (B and C); 50 mg/kg CsA (D); and 10 mg/kg CsA (E and F). Representative viewing fields of the 6-μm cryosections are presented. Bars indicate 10 μm.

**Fig. 7.** Daunorubicin accumulation determined by laser scanning cytometry in tissue cryosections. Mice bearing xenotransplants were treated with UIC2 and/or CsA followed by the i.v. injection of 8 mg/kg daunorubicin. Left, fluorescence intensity distribution histograms of sections corresponding to the upper row images of Fig. 6; A, NIH 3T3 MDR1 xenotransplant treated with 5 mg/kg UIC2 monoclonal antibody and 10 mg/kg CsA. B and C, treatment with 5 mg/kg UIC2 of NIH 3T3 MDR1 and NIH 3T3 tumor grafts, respectively. Right, topographic image of daunorubicin fluorescence intensity in the whole cryosection from which histogram A was constructed. The fluorescence intensity distribution of the phantom contours was evaluated on x-y scattergrams (see under Materials and Methods). Only the evenly fluorescent middle region of the selected sections was evaluated, leaving out the intensively fluorescent edges arising as a result of the folding back tissue elements.
gradually enter and become trapped in a UIC2-reactive conformational state. The effect of the ACT-positive drugs on Pgp conformation was shown to be indistinguishable from that of ATP depletion, suggesting that in the presence of these agents the pump assumes a conformational state that is part of the catalytic cycle (Goda et al., 2002; Nagy et al., 2004). This interpretation is also in line with the fact that inhibition of drug transport is not specific for a particular substrate or a class of substrates (Figs. 2 and 3).

To our knowledge, these data represent the first successful attempt to achieve a near-complete antibody-mediated Pgp inhibition in in vivo conditions. We have shown in the SCID mouse model that 1) the UIC2 mAb reaches its cell surface Pgp targets deeply buried in solid tumors; 2) daunorubicin, a fluorescent anthracycline anticancer agent, readily enters Pgp-expressing cells when inhibited by UIC2; 3) there is a positive correlation between the extent of UIC2 binding and the increment in daunorubicin uptake; and 4) CsA augments the inhibitory UIC2 binding at a ≈5 times lower concentration than its effective concentration for the competitive inhibition of Pgp function.

A concern in the clinical applications of an antibody-based Pgp reversal strategy would be posed by the possible side effects caused by the expression of Pgp molecules in certain normal tissues. For example, inhibition of Pgp molecules expressed in the blood-brain barrier might lead to the increased accumulation of Pgp substrates. Indeed, neurotoxicity in the central nervous system was experienced in mdr1a/b knockout mice (Schinkel et al., 1994, 1997). However, in several clinical trials, administration of relatively Pgp-selective modulators did not cause toxicity to the central nervous system (Sikic et al., 1997), perhaps because of the presence of other ABC transporters (e.g., ABCC1, ABCG2) with overlapping substrate spectra (Loesch and Potschka, 2005). Alternatively, or in addition, the reversal agents used in these studies (Sikic et al., 1997) might not have caused complete Pgp inhibition at the doses applied. Thus, Pgp inhibition is probably tolerated, and the absolute Pgp specificity of antibody-mediated inhibition may further mitigate possible damage to normal tissues. In addition, direct injection of the antibody into the tumor tissue may decrease the possible systemic side effects because the antibody is expected to stay bound within the injected tissues, allowing a reduction of the dose to be applied. Because the UIC2 mAb is specific for the human and primate Pgp (Mechetner et al., 1992), a primate animal model will be needed for toxicological studies related to the use of UIC2 in mdr reversal strategies.

The experimental system described herein offers a conve-
noid model to determine the minimal effective doses of the cytotoxic drug and antibody. Because 5 mg/kg UIC2 mAb in combination with CsA completely restored daunorubicin accumulation in Pgp− tumors, we expect significant reduction of tumor size at this concentration. The Kp of Herceptin binding (trastuzumab; a humanized antibody used in the immunotherapy of ErbB2-positive cancers) is similar to that of UIC2 (1 µg/ml) (Raghu et al., 1996), the inhibitory character of the antibody would hopefully be retained after humanization. This effort may be rendered worthwhile in view of the fact that mdr is a central problem in cancer chemotherapy and Pgp is among the ABC transporters most frequently held responsible for the mdr phenotype of tumor cells. The Pgp-mediated active efflux can also limit the brain access of HIV protease inhibitors, diminishing the efficiency of virus eradication from the brain. Effective treatment of mental illnesses and tumors of the central nervous system may also be hindered by the gatekeeper function of Pgp. Therefore, potential human applications of our protocol include several pathological scenarios in which specific inhibition of Pgp could be useful: multidrug resistance, need for compromising blood-brain barrier function, overcoming anti-HIV drug resistance, among others.

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