Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport

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Summary The human multidrug transporter MDR1 P-glycoprotein and the multidrug resistance proteins MRP1 and MRP2 transport a range of cytotoxic drugs, resulting in multidrug resistance in tumour cells. To overcome this form of drug resistance in patients, several inhibitors (reversal agents) of these transporters have been isolated. Using polarized cell lines stably expressing human MDR1, MRP1 or MRP2 cDNA, and 2008 ovarian carcinoma cells stably expressing MRP1 cDNA, we have investigated in this study the specificity of the reversal agents V-104 (a pipecolinate derivative), GF120918 (an acridone carboxamide derivative also known as GG918), and Pluronic L61 (a (poly)oxypropylene and (poly)oxypropylene block copolymer). Transport experiments with cytotoxic drugs with polarized cell lines indicate that all three compounds efficiently inhibit MDR1 Pgp. Furthermore, V-104 partially inhibits daunorubicin transport by MRP1 but not vinblastine transport by MRP2. V-104 reverses etoposide resistance of 2008/MRP1 cells, whereas GF120918 does not reverse resistance due to MRP1. V-104 partially inhibits the export of the organic anion dinitrophenyl S-glutathione by MDCKII-MRP1 but not by MDCKII-MRP2 cells. Unexpectedly, export of the organic anion calcein by MDCKII-MRP1 and MDCKII-MRP2 cells is stimulated by Pluronic L61, probably because it relieves the block on entry of calcein AM into the cell by endogenous MDR1 Pgp. © 2000 Cancer Research Campaign

Keywords: multidrug resistance; reversal agent; MRP1; multispecific organic anion transporter; MDR1 Pgp; polarized cell

Simultaneous resistance of tumour cells against a range of cytotoxic drugs is a serious problem in cancer chemotherapy. This phenomenon is called multidrug resistance (MDR). One form of MDR can be caused by members of the ATP-binding cassette (ABC) family of transport proteins (Gottesman et al, 1995; Cole and Deele, 1998). These are large polytopic membrane proteins that actively transport drugs out of the cell, resulting in a decreased intracellular drug concentration. In humans, two ABC transporters have been identified that can cause resistance in tumour cells: MDR1 P-glycoprotein (Pgp) (Juliano and Ling, 1976), and the multidrug resistance protein (MRP1) (Cole et al, 1992). MDR1 Pgp transports drugs in an unmodified form, whereas MRP1 transports drugs either conjugated to the anionic ligands glutathione (GSH), glucuronide, or sulphate (Leier et al, 1994; Müller et al, 1994; Jedlicshky et al, 1997), or transports them in an unmodified form, probably together with GSH (Rappa et al, 1997; Loe et al, 1998).

Another human ABC transporter that might contribute to drug resistance is the liver canalicular multispecific organic anion transporter (cMOAT or MRP2). Studies with mutant rats (TR−/GY or EHBR) that lack the MRP2 protein in the canalicular membrane of the hepatocytes and with cell lines stably transfected with MRP2 cDNA have demonstrated that the substrate specificity of this transporter is similar to that of MRP1, and suggested that MRP2 is able to transport several anti-cancer drugs (e.g. Evers et al, 1998; Masuda et al, 1998; Chen et al, 1999; Cui et al, 1999; Hooijberg et al, 1999). Besides MRP1 and MRP2, at least four other MRP homologs have been identified: MRP3, MRP4, MRP5 and MRP6 (Kool et al, 1997; 1999a). MRP3 has been shown to transport organic anions and confers low-level resistance against etoposide and tenoposide, and high-level resistance against methotrexate (Hirohashi et al, 1999; Kool et al, 1999b). Recently, a correlation was found between the overexpression of MRP4 and resistance against nucleoside-based antiviral drugs (Schuetz et al, 1999).

The potential involvement of the overproduction of drug pumps in clinical drug resistance in tumour cells has led to a search for compounds that can be used to inhibit these transporters in cancer patients. These so-called reversal agents or modulators should preferably be: i) Selective for one or more transporters and bind to these transporters with a high affinity, ii) non-toxic, and iii) stable in human plasma (Sarkadi and Müller, 1997). Several compounds have been described that effectively block MDRI Pgp-mediated drug resistance, some of which are currently being tested in the clinic (e.g. Fisher and Sikic, 1995; Rowinsky et al, 1998). Examples of such inhibitors are the non-immunosuppressive cyclosporin A analogue PSC833 (Twentyman and Bleehen, 1991), the pipecolinic derivative VX710 (Germann et al, 1997a; Yanigazawa et al, 1999), the acridone carboxamide derivative GF120918 (Hyafil et al, 1993), and the (poly)oxypropylene and (poly)oxypropylene block copolymers Pluronic L61 and P85 (Venne et al, 1996; Müller et al, 1997; Butarakova et al, 1998).
Recently, we established two model systems suitable for testing inhibitors of MDR1 Pgp, MRPI and MRP2: i) A set of polarized cell lines stably expressing MDR1, MRPI, or MRP2 cDNA (Schinkel et al, 1995; Evers et al, 1996; 1998). In these cells, MDR1 Pgp and MRPI are localized in the apical plasma membrane, whereas MRPI is localized in the lateral plasma membrane. The ability of these cell lines to grow in a monolayer makes them suitable for drug-transport studies and for determining the specificity and efficiency of inhibitors (Evers et al, 1996; 1998; Smith et al, 1998); ii) 2008 ovarian carcinoma cells stably expressing various MRPs (Hooijberg et al, 1999; Kool et al, 1999b).

To further explore the suitability of transfected (polarized) cell lines for the screening of reversal agents and to investigate the specificity of previously identified reversals, we describe in this report the effect of the inhibitors Pluronic L61, V-104 (an analogue specific for Pgp), and VRT-010367) from Merck (Rahway, NJ, USA) and cultured for 3 days with a daily medium replacement.

Transport assays

\[^{3}H\]Vinblastine and \[^{14}C\]Daunorubicin transport assays were carried out essentially as described (Evers et al, 1996). Cells were seeded on microporous polycarbonate filters (3 μm pore size, 24.5 mm diameter, Transwell™ 3414; Costar Corp., Cambridge, MA, USA) and cultured for 3 days with a daily medium replacement. The experiment was started \((t = 0)\) by replacing the medium at either the apical or the basal side of the cell layer with 2 ml of complete medium containing 2 μM of drug (at 0.25 μCi ml\(^{-1}\)). \[^{3}H\]-labelled vinblastine (0.025 μCi ml\(^{-1}\)), and the amount of inhibitor indicated. The cells were incubated at 37°C, 5% CO\(_2\), 50 μl aliquots were taken from each compartment at various time-points. Radioactivity was measured as the fraction of radioactivity added at the beginning of the experiment. The paracellular flux was monitored by the appearance of inulin \[^{14}C\]carboxylic acid in the opposite compartment and was typically below 1.5% per h. Inhibition of transport by inhibitors was calculated by dividing the net flux of drug in the control experiment \((t = 4\ h)\) by the net flux determined in the presence of inhibitor. Export of \[^{14}C\]DNP-GS was determined by incubating cells with 2 μM of \[^{14}C\]DNP (15 nCi ml\(^{-1}\)) at room temperature as described previously (Evers et al, 1996; 1998), with the modification that the experiments were performed in Hanks Balanced Salt Solution (containing 1.3 mM CaCl\(_2\)). If inhibitors were used in the experiment, cells were first incubated with HBSS containing inhibitor for 10 min before adding the substrate. Export of calcein was determined by incubating monolayers in HBSS containing calcein AM (1 μM) at room temperature. Samples (200 μl) were taken at the time-points indicated. 800 μl HBSS was added and fluorescence was determined in a fluorimeter (Perkin-Elmer). The amount of calcein exported was calculated using a calibration curve made with free calcein.

All transport experiments were performed in duplicate and repeated at least twice.

Cytotoxicity assays

The drug sensitivity of cells was determined as described (Kool et al, 1999b) in growth inhibition assays with continuous exposure to drug.

RESULTS

Inhibition of drug transport by Pluronic L61

To examine whether Pluronic L61 inhibits transport mediated by MDR1 Pgp, vectorial transport of vinblastine was measured in LLC-MDR1 cells in the presence of various Pluronic L61 concentrations. Figure 1 shows that transport was blocked by this compound; total inhibition of vinblastine transport was already obtained at a Pluronic L61 concentration of 0.04% (w/v). No effect on the paracellular \[^{14}C\]inulin flux was observed at concentrations up to 0.1% (w/v) Pluronic L61 (results not shown). To investigate whether Pluronic L61 could inhibit vinblastine transport by MRP2, we transfected MDCKII cells (Evers et al, 1998), as MRP2-mediated transport was very low in transfected LLC-PK1 cells (RE and PB, unpublished data). In these experiments a low concentration of the Pgp inhibitor PSC833 (0.1 μM) was present to inhibit the endogenous Pgp in these cells. This concentration of PSC833 only partially inhibits MDR1 Pgp in MDCKII-MDR1 cells and has no effect on vinblastine transport by MDCKII-MRP2 cells. At the highest concentration Pluronic L61 (0.1% (w/v)) tested, some inhibition (1.5-fold) of net vinblastine transport by MDCKII-MRP2 cells was observed, whereas transport mediated by MDR1 Pgp was completely blocked under these conditions (Figure 2). At lower concentration of Pluronic L61 (0.04% (w/v)), transport by MRP2 was not affected at all, whereas transport by MDR1 Pgp was totally blocked (data not shown).

The effect of Pluronic L61 on MRP1 is summarized in Table 1. In polarized cells MRP1 routes to the basolateral membrane (Evers et al, 1996) and not to the apical membrane, like Pgp and MRP2.
Calcein acetoxy-methyl ester (calcein AM) is a lipophilic compound that enters the cell by passive diffusion over the plasma membrane. Cleavage of the ester bonds by intracellular esterases converts calcein AM into the highly fluorescent organon ion calcein. Calcein AM itself is efficiently transported by MDR1 Pgp (Homolya et al, 1996), and MRP1 transports both calcein AM and calcein (Feller et al, 1995; Holló et al, 1996). As transport of fluorescent substrates can be conveniently measured using a fluorimeter, we investigated whether calcein could be used as a substrate to study the function of MRP1 and MRP2 in MDCKII cells. Cells were incubated in the presence of calcein AM and the amount of calcein appearing in the medium was determined. Figure 3A shows that in MDCKII wild-type cells calcein export to the apical and basolateral side was similar. Hardly any calcein export was detected in MDCKII-MDR1 cells (Figure 3B), some increased (2-fold) basolateral export was observed in MDCKII-MRP1 cells (Figure 3C), and a 3-fold increased apical export was measured in MDCKII-MRP2 cells (Figure 3D). Unexpectedly, in the presence of Pluronic L-61 calcein export to the basolateral side by the MDCKII-MRP1 cells was increased 2-fold (t = 2 h), and export to the apical side by the MDCKII-MRP2 cells was increased 4-fold. Similar data were obtained in the presence of higher Pluronic L61 concentrations (data not shown). An obvious explanation for this paradoxical stimulation is that Pluronic L61 inhibits the endogenous MDR1 Pgp present in MDCKII cells and that this Pgp limits the entry of calcein AM in the absence of Pluronic L61. To test this hypothesis, calcein transport was measured in the presence of PSC833 (0.1 μM). This concentration of PSC833 had some stimulatory effect on export by MDCKII-MRP2 cells, but no effect on export by MDCKII-MRP1 cells (Figure 3C and 3D).

**Inhibition of drug transport by V-104**

VX710 is a potent inhibitor of MDR1 Pgp in several drug-selected cell lines that overexpress MDR1 (Germann et al, 1997a). In addition, VX710 partially reverses the MDR phenotype of MRP1 overexpressing cells (Germann et al, 1997b; Yanagisawa et al, 1999). An analogue of VX710 has been developed called V-104. At 15 μM, V-104 effectively inhibited daunorubicin and vinblastine transport by MDCKII-MDR1 cells (Figures 4B and 5B). Hardly any inhibition of MDR1 was observed at lower concentrations of V-104 (5 μM) (data not shown). V-104 at 15 μM had a modest inhibitory effect on MRP1. The low daunorubicin transport by MDCKII-MRP1 shown in Figure 4C was somewhat decreased by the drug. DNP-GS export (Table 1) was decreased as well. Note that the experiments with MDCKII-MRP1 cells in Table 1 show that V-104 decreases basolateral DNP-GS transport by nearly 30%, whereas endogenous DNP-GS goes up 2-fold. As substrate (DNP-GS) production could be limiting to some extent in these cells, the 30% inhibition is a minimal value. In contrast, vinblastine transport by MDCKII-MRP2 cells was not affected by V-104 (Figure 5C). Table 1 shows that export of DNP-GS from MDCKII-MRP2 cells was not significantly affected by V-104 either.

**Inhibition of drug transport by GF120918**

GF120918 is an efficient inhibitor of MDR1 Pgp (Hyafil et al, 1993; Wallstab et al, 1999). Figures 4B and 5B show that GF120918 completely inhibited daunorubicin and vinblastine...
transport by MDCKII-MDR1 cells, whereas transport of daunorubicin by MDCKII-MRP1 cells (Figure 4C) and vinblastine transport by MDCKII-MRP2 cells (Figure 5C) was not affected. Table 1 shows that export of DNP-GS by MDCKII-MRP1 and MDCKII-MRP2 cells was not inhibited by GF120918 either.

**Reversal of drug resistance by V-104 and GF120918**

To test whether the reversal agents GF120918 and V-104 were able to reverse the drug resistance of 2008/MRP1 cells, we performed growth inhibition assays with continuous drug exposure with or without inhibitor. The maximum concentrations of the inhibitors that were not cytotoxic during a continuous exposure were 10 μM and 5 μM for GF120918 and V-104, respectively. We examined reversal of resistance to etoposide, as 2008/MRP1 cells are highly resistant (20-fold) against this drug. V-104 reversed etoposide resistance of the 2008/MRP1 cells, but reversal was not complete, even at 5 μM (Table 2). Sensitivity of mock-transfected cells to etoposide also increased somewhat in the presence of V-104, presumably because of the inhibition of endogenous transporters. Inhibition of endogenous Pgp may also explain the effect found for GF120918 on etoposide resistance, as this effect did not clearly increase with GF120918 concentration.

**DISCUSSION**

Specific inhibitors of drug transporters are potentially useful tools for the dissection of complex drug-resistance phenotypes and, eventually, for the treatment of patients with drug-resistant cancer. We have tested the specificity of three novel compounds, Pluronic L61, V-104 and GF120918, on three drug transporters, MDR1 Pgp, MRP1 and MRP2, in intact (transfected) cells. The transfected LLC-MDR1 cell line used in this study was selected with vincristine, whereas the MDCKII-derived cell lines were selected with geneticin (G-418). We therefore cannot formally exclude that
these lines have developed some unknown mechanisms of drug resistance. We consider this, however, an unlikely possibility as untransfected cells never formed colonies after selection with vincristine or G-418, respectively. All three compounds completely inhibit MDR1 Pgp-mediated transport of vinblastine and daunorubicin at non-cytotoxic concentrations in our assays. GF120918 and V-104 have no effect on transport by MRP2. V-104 is a weak inhibitor of MRP1, but we only observed clear inhibition at drug concentrations close to cytotoxic levels. It is therefore unlikely that this drug will be useful for inhibiting MRP1 in human cancer. A high concentration (0.1%) of Pluronic L61 partially inhibited vinblastine transport by MRP2 (Figure 2), but had only a small effect on DNP-GS transport by MRP2. Our data also show that the interpretation of inhibitor studies can be complicated by the presence in the cells of other endogenous transporters with a high affinity for the inhibitor, the transport substrate used, or both. In the screening for inhibitors of MRP1 and MRP2, it is obviously important to use both organic anions and cytotoxic drugs as substrates, as inhibitors may have a differential effect on the transport of different classes of drugs.

Figure 3  Export of calcein by MDCKII cell monolayers. (A, left graph) MDCKII wild-type cells. At $t = 0$ calcein AM ($1 \mu M$) was added to both the apical and basolateral medium, and the amount of calcein appearing in the medium was measured using a fluorimeter. Samples were taken at $t = 1$ and $t = 2$ h. (Middle and right graph) Same as left graph, but the assay was performed in the presence of PSC833 (0.1 $\mu M$) or Pluronic L61 (0.04%), respectively. (B–D) Same as (A), but MDCKII-MDR1, MDCKII-MRP1 and MDCKII-MRP2 cells were tested, respectively. Squares: export to apical compartment. Circles: export to basolateral compartment.
Previous work has shown that P85, an analog of Pluronic L61, inhibits an apically localized drug transporter in CaCo-2 cells, most likely MDR1 Pgp (Batrakova et al, 1998). With transfected cell lines we show here that MDR1 Pgp is indeed inhibited by this class of compounds. The mechanism of action of the copolymer Pluronic L61 is not clear. It is not known whether it is a substrate for MDR1 Pgp (and therefore acts as a competitive inhibitor) or blocks MDR1 Pgp by another mechanism. Amphiphilic block copolymers have a low critical micelle concentration (cmc); the cmc for Pluronic L61 is 0.02% (w/v) (Batrakova et al, 1996). As we see a major inhibition of MDR1 Pgp below 0.02% (w/v) (Figure 1), we conclude that the monomeric form of Pluronic L61 is the effective species in inhibiting transport, in agreement with earlier suggestions by Miller et al (1997) and Batrakova et al (1998) for Pluronic analogues, and Nerurkar et al (1997) for other neutral surfactants. Our results show that the inhibitory effect of Pluronic L61 on Pgp is highly specific. We clearly show that Pluronic L61 does not increase the rate of passive diffusion of drug through the monolayer and that it has no clear effect on MRP1 or MRP2. It is therefore likely that Pluronic L61 directly binds to Pgp, like other inhibitors, but we have not verified this in a simple vesicular transport system, e.g. by vanadate-mediated nucleotide trapping (Szabo et al, 1998). Very recently, Miller et al (1999) suggested that Pluronic L61 and P85 do inhibit MRP1 in a human pancreatic adenocarcinoma cell line. These authors studied the accumulation of fluorescein and observed some increased intracellular accumulation in the presence of various Pluronic analogues. The discrepancy with our data may be explained by the presence of other organic anion transporters than MRP1 in a human pancreatic adenocarcinoma cell line. These authors studied the accumulation of fluorescein and observed some increased intra- cellular accumulation in the presence of various Pluronic analogues. The discrepancy with our data may be explained by the presence of other organic anion transporters than MRP1 in pancreatic adenocarcinoma cells; one of these might be sensitive towards Pluronic analogues. Our data clearly indicate that in MDCKII cells, neither MRP1 nor MRP2 are inhibited significantly by Pluronic L61. Firstly, DNP-GS export by MRP1 or MRP2 was only slightly impaired (Table 1) and, secondly, vinblastine

Figure 4 Effect of V-104 and GF120918 on daunorubicin transport through polarized MDCKII cell monolayers. Transport was measured as described in Figure 2, but with [3H]daunorubicin as substrate. (A, left graph) MDCKII cells. Transport in the presence of PSC833 (0.1 µM) alone. (Middle and right graph) Same as left graph, but in addition to PSC833, V-104 (15 µM) and GF120918 (1 µM) were added at t = 0. (B,C) Same as A, but MDCKII-MDR1 and MDCKII-MRP1 cells were tested, respectively. Squares: translocation from basolateral to apical. Circles: translocation from apical to basolateral.
Figure 5  Effect of V-104 and GF120918 on vinblastine transport through polarized MDCKII monolayers. Transport was measured as in Figure 4, but with $[^3]$Hvinblastine as substrate.

Table 2  Growth inhibition of 2008/MRP1 and mock transfected 2008/pCMV-neo cells by etoposide

|                      | 2008/pCMV-neo IC$_{50}$ (nmol) | 2008/MRP1 IC$_{50}$ (nmol) |
|----------------------|-------------------------------|-----------------------------|
| Etoposide            | 0.42 ± 0.06 (1.0)             | 8.84 ± 1.44 (20)            |
| Etoposide + 1.0 µM GF120918 | 0.36 ± 0.00 (0.9)       | 9.77 ± 5.24 (27)            |
| Etoposide + 2.5 µM GF120918 | 0.31 ± 0.03 (0.7)       | 4.27 ± 0.80 (14)            |
| Etoposide + 5.0 µM GF120918 | 0.31 ± 0.01 (0.7)       | 6.98 ± 1.86 (23)            |
| Etoposide + 1.0 µM V-104 | 0.34 ± 0.02 (0.8)       | 5.00 ± 2.17 (15)            |
| Etoposide + 2.5 µM V-104 | 0.23 ± 0.00 (0.5)       | 2.95 ± 0.87 (13)            |
| Etoposide + 5.0 µM V-104 | 0.30 ± 0.11 (0.7)       | 1.46 ± 0.17 (5)             |

IC$_{50}$s are means of an experiment performed in triplicate. Numbers in parentheses represent resistance factors (relative to 2008/pCMV-neo incubated with etoposide alone for 2008/pCMV-neo cells; relative to the identically treated 2008/pCMV-neo cells for 2008/MRP1 cells).
transport by MRP2 was only weakly affected at the highest concentration of Pluronic L61 tested (Figure 2). We observed stimulation of calcein export by MRP1 and MRP2 in the presence of Pluronic L61 (Figure 3), probably caused by the efficient inhibition of canine Pgp by this compound. As PSC833 could only partially mimic this effect, PSC833 cannot block calcein AM transport by Pgp completely, whereas Pluronic L61 can, or there is another endogenous transporter of calcein AM inhibited by Pluronic L61 but not by PSC833.

It is remarkable that many of the reversal agents that efficiently block MDR1 Pgp, like PSC833, GF120918 and Pluronic L61, have little or no effect on MRP1 and MRP2 (e.g. Böhme et al., 1993; Wallstab et al., 1999). It has been shown that both GF120918 and PSC833 are competitive inhibitors that probably bind with high affinity to the substrate binding site(s) of MDR1 Pgp (Smith et al., 1998; Wallstab et al., 1999). Obviously the drug binding sites of MDR1 Pgp have a higher affinity for hydrophobic inhibitors than the drug binding sites of MRPs. This could be due to a difference in transport mechanisms. Whereas MDR1 Pgp does not require any co-factor for the transport of hydrophobic drugs, transport by MRP1 and MRP2 of these drugs is associated with the export of reduced glutathione (Rappa et al., 1997; Loe et al., 1998; Evers et al., 2000). The compounds that we used here are all relatively hydrophobic and not negatively charged. They may therefore require GSH for binding to MRPs, and this may result in a relatively low affinity for the transporter.

Most high-affinity substrates for MRP1 and MRP2 found thus far are organic anions with a substantial hydrophobic moiety and one, but preferably two, negative charge(s). Examples are leukotriene C4, S-decylglutathione and the leukotriene D4 antagonist MK571 (Loe et al., 1996; Keppeler et al., 1998). These substrates do not readily enter cells and they therefore do not provide obvious lead compounds for drug development. As it seems unlikely that more membrane-permeable compounds can be found with high affinity for MRP, good inhibitors will have to be made as prodrugs in which the charged moiety is shielded and can be converted into active drug in the cell, like CDNB or calcein AM.

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