Multidrug Resistance-Reversing Agents Increase Vinblastine Distribution in Normal Tissues Expressing the P-Glycoprotein but Do Not Enhance Drug Penetration in Brain and Testis

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Accepted for publication February 11, 1997

ABSTRACT

The aim of this study was to assess whether P-glycoprotein (Pgp) inhibitors altered the blood-brain barrier and enhanced vinblastine (VBL) distribution in brain, testis and other Pgp-expressing tissues. Trifluoperazine, cyclosporin A, amiodarone, quinidine, the nifedipine analog Bay K8644 and verapamil were selected among Pgp inhibitors and were administered intraperitoneally 1 hr before an intravenous dose of 10 mg/kg VBL. Trifluoperazine and cyclosporin A were also administered intraperitoneally for 7 days before VBL. VBL and its metabolite O4-deacetylvinblastine were measured in tissues by high-performance liquid chromatography assay. None of the reversing agents (RA) appreciably raised VBL concentrations in brain and testis, whereas all except quinidine significantly enhanced VBL distribution in liver and kidney; the most effective were trifluoperazine and cyclosporin A. In mice treated with RA and VBL combined, O4-deacetylvinblastine levels in liver and kidney reached either the same or higher levels than in mice treated with VBL alone, indicating that the increase in VBL levels is not due to inhibition of its metabolism. The main conclusions are that (1) inhibitors of Pgp, even at high doses, do not increase the permeability of the blood-brain barrier in mice, suggesting caution in the clinical use of RA combined with antitumor agents for brain tumors; and (2) several RA achieve high enough concentrations to enhance the distribution of VBL in other normal tissues expressing Pgp, thus potentially increasing VBL toxicity.

Pgp was initially identified in cell lines resistant to colchicine (Endicott and Ling, 1989; Gottesman and Pastan, 1993) and found to be expressed in several other cancer cell lines resistant to a variety of anticancer agents, including vinca alkaloids, anthracyclines, epipodophyllotoxins, taxanes and other natural products (Chin et al., 1993; Van der Blik and Borst, 1989). Pgp is made up of two symmetrical halves, each of which contains six membrane-spanning domains and an ATP-binding site. The high homology with a group of bacterial proteins implicated in membrane transport, known as ATP-binding cassette transporters, supports the idea that Pgp is a transport protein (Chen et al., 1986; Devault and Gros, 1990; Gros et al., 1986; Higgins and Gottesman, 1992). In fact, the mechanism by which Pgp confers resistance to anticancer drugs is related to the drug transport out the cells; the efflux of drugs from cells that express Pgp was much faster than that from cells that do not express it (Beck, 1987; Coley et al., 1993; Dano, 1973; Fojo et al., 1985; Inaba et al., 1979).

Mammalian Pgps are encoded by a family of closely related genes, including two members in humans (MDR1 and MDR3) and three in mice (mdr1a, mdr1b and mdr2). Not all these proteins confer drug resistance; transfection experiments have shown that the expression of human MDR1 or rodent mdr1a or mdr1b is sufficient to confer resistance to antitumor agents, whereas MDR3 and mdr2 are involved in the transport of other molecules, such as phospholipids in the bile (Smit et al., 1993). Pgp is expressed not only in tumor cells resistant to anticancer agents but also in several normal organs, such as liver, kidney, adrenals and intestine (Croop et al., 1989; Fojo et al., 1987; Thiebaut et al., 1987).

The localization of the proteins also suggests that the function of Pgp is related to transport mechanisms because there are high levels in the brush border of renal proximal tubules, biliary surface of hepatocytes and apical surface of intestinal mucosal cells. Pgp is also expressed at high levels in capillary endothelial cells of brain and testis (Cordon Cardo et al., 1989, 1990; Thiebaut et al., 1989).

To gain insight into the biological and pharmacological roles of Pgp, Schinkel et al. (1994) recently generated mice homozygous for disruption of the mdr1a. These mice grew

ABBREVIATIONS: Pgp, p-glycoprotein; VBL, vinblastine; TFP, trifluoperazine; CsA, cyclosporin A; AMD, amiodarone; QND, quinidine; Bay K, Bay K8644; VER, verapamil; DVBL, O4-deacetylvinblastine; HPLC, high-performance liquid chromatography; RA, reversing agents; CNS, central nervous system.
normally, were fertile and had an apparently normal phenotype. However, the absence of Pgp was associated with loss of the blood brain-barrier, so they were very sensitive to neurotoxic compounds such as ivermectin or VBL that penetrate their brain but not the brains of mice with a normal expression of the mdr1 gene.

We have recently shown in mice that Pgp inhibitors like cyclosporins or the cyclosporin analog SDZ 833 can inhibit Pgp in vivo, thus significantly increasing tissue retention of antitumor agents that are transported by the protein (Colombo et al., 1994; Gonzalez et al., 1995).

The finding that destruction of the mdr1a gene causes an impairment of the blood brain-barrier suggested that Pgp inhibitors might be useful to improve the penetration of drugs that do not normally enter the brain in sufficient amounts. This study therefore investigated whether and to what extent different Pgp inhibitors could circumvent the blood-brain and testicular barriers, raising the concentrations of VBL in brain and testis and, by comparison, in other organs, such as liver and kidney, of mice treated with the antitumor drug.

Materials and Methods

Animals and drugs. All experiments were carried out in male BDF1 mice (20 ± 2 g b.wt.) obtained from Charles River Italia (Caleo, Italy). Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH publication No. 85–23, 1985).

For the tissue disposition studies, multidrug resistance RA were injected intraperitoneally at different doses. CsA was dissolved in olive oil (25 and 200 mg/kg), and TFP (12.5, 25 and 50 mg/kg), AMD (25 mg/kg), QND (10 and 50 mg/kg), Bay K (2 mg/kg) and VER (25 and 50 mg/kg) were dissolved in water. CS A and TFP were also given for 7 days at the dose of 25 and 12.5 mg/kg, respectively. The doses amounted to ≥10% of the LD50. The maximum dose selected was approximately the maximum tolerated dose, as assessed in pilot experiments on male BDF1 mice. After 1 hr, VBL dissolved in ethanol/water (1%) was injected intravenously at the dose of 10 mg/kg. Four mice per time point were exsanguinated while under light ether anesthesia at 4, 8 and 24 hr after VBL, and serum and tissues (brain, testis, liver and kidneys) were removed and frozen at −20°C until use.

Analytical assays. VBL was kindly provided by Eli Lilly (Indianaapolis, IN), and its metabolite (DVBL) was kindly provided by Dr. J. H. Beijnen (Sloterwaart Hospital, Amsterdam, the Netherlands). The concentrations of these compounds were measured by ion-pair reverse-phase HPLC with fluorescence detection according to a method adapted from Debal et al. (1992). After homogenization in water, tissue samples, with 2 mg of added navelbine (kindly provided by Pierre Fabbre, Boulogne, France) as internal standard, were extracted with 8 ml of ethyl-ether. The samples were shaken for 40 min and centrifuged at 3000 rpm for 15 min. The supernatant organic phase was decanted into tubes containing 250 μl of phosphate buffer 0.25 M, pH 2.9, for the second acid extraction. After slow agitation for 30 min, the acidic aqueous extracts were injected into the HPLC system with fluorescence detection at an excitation wavelength of 280 nm and an emission of 320 nm. Separation was achieved with an isocratic solvent system of acetonitrile/0.1 M phosphate buffer (pH 2.9 containing 1 g/liter heptane sulphate/methanol (30:50:20, v/v/v) using a 10-μm, 4.6 × 300 mm, μBondapak C18 column.

Recovery of both VBL and its metabolite DVBL after the addition of a known amount of drug tissue homogenate was 85 ± 5%, and the sensitivity was 20 ng/g for tissue. The drug concentrations in serum and tissues were calculated from a calibration curve in the range of 0.01 to 5 μg/ml. Statistical differences in the serum and tissue levels were assessed by Duncan’s test.

Results

Blood and tissue levels of VBL and DVBL were measured in mice treated with the antitumor agent either alone or in combination with the Pgp inhibitors of different structures CsA, TFP, QND, AMD, Bay K and VER.

Figure 1 shows VBL blood levels at 4, 8 and 24 hr after an intravenous dose of 10 mg/kg VBL. TFP caused the most marked increase in VBL blood levels, with a dose-dependent pattern. The increase at 24 hr was 2- to 5-fold. Repeated TFP doses did not produce a higher effect than single dose. VBL levels also rose significantly in CsA-pretreated mice. Repeated treatments with CsA were as effective as a single dose. The other inhibitors caused no significant changes.

Figures 2 and 3 show VBL levels in brain and testis of mice receiving the drug alone or with the Pgp inhibitors. None of the inhibitors significantly raised VBL in these organs.

The levels of VBL were also determined in liver and kidney of the same mice. Again, the most marked differences were observed in mice pretreated with single dose of TFP. VBL liver levels (fig. 4) rose significantly when the dose of TFP was escalated from 12.5 to 25 mg/kg, but raising it to 50 mg/kg did not further increase them. Repeated treatments of TFP at the dose of 12.5 mg/kg did not cause any change in liver VBL levels. CsA, at the high dose of 200 mg and after repeated doses of 25 mg/kg, significantly increased VBL liver levels at each time point. AMD, Bay K and VER induced a significant increase in VBL liver levels at 4 hr. Neither drug caused a significant change at 24 hr. QND did not change liver VBL levels.

VBL renal distribution (fig. 5) was markedly enhanced by single doses of TFP and both single and repeated treatments with CsA. VBL kidney levels in mice treated with AMD, Bay K and VER were also increased significantly, whereas QND had no such effect.

The metabolite of VBL, DVBL, was not detectable in blood, testis or brain, but it could be quantified in liver and kidney (tables 1 and 2). In both of these organs, DVBL levels were...
The present study was designed to investigate whether Pgp inhibitors impair the blood-brain barrier; VBL was chosen as a model drug in view of the dramatic increase in its dose, suggesting the possibility that there are other mechanisms of interaction in addition to the inhibition of Pgp.

### Discussion

The efficacy of cancer treatment is often limited by the low penetration of drugs in the tumor. This is a real problem for patients with tumors or metastases in the CNS or testis, where most drugs have negligible penetration. Schinkel et al. (1994) recently reported that in mice that do not express Pgp, the blood-brain and blood-testicular barriers were no longer efficient. Although mice lacking Pgp grew and reproduced normally, they were extremely susceptible to the neurotoxicity of compounds that do not normally cross the blood-brain barrier. In these mice, the brain penetration of several compounds, including ivermectin, VBL (Schinkel et al., 1994), CsA, dexamethasone and digoxin (Schinkel et al., 1995), was much higher than that in mice expressing Pgp. For example, the antitumor agent VBL reached concentrations in the brain 7- to 46-fold those found in mice expressing Pgp (van Asperen et al., 1996). These findings show that Pgp, which is known to be expressed in brain capillaries (Cordon Cardo et al., 1989, 1990; Thiebaut et al., 1989, 1990), plays a major physiological role in preventing potentially toxic compounds from penetrating the CNS.

The availability of several effective Pgp inhibitors (Ford and Hait, 1990) made it feasible to test whether the efficiency of blood-brain barrier was impaired by pharmacological treatments. This possibility is very attractive because the CNS and testis are “pharmacological sanctuaries” where most anticancer drugs cannot reach a sufficiently high concentration to exert their effect (Greig et al., 1990).

The present study was designed to investigate whether Pgp inhibitors impair the blood-brain barrier; VBL was chosen as a model drug in view of the dramatic increase in its

### Tables

**Table 1: Levels of DVBL in liver of mice receiving VBL alone or in combination with different RA**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hr</td>
</tr>
<tr>
<td>Control</td>
<td>0.12 ± 0.03*</td>
</tr>
<tr>
<td>CSA (25 mg)</td>
<td>0.43 ± 0.03**</td>
</tr>
<tr>
<td>TFP (12.5 mg)</td>
<td>0.34 ± 0.05**a</td>
</tr>
<tr>
<td>TFP (25 mg)</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>TFP (50 mg)</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>QND (50 mg)</td>
<td>0.50 ± 0.07**b</td>
</tr>
<tr>
<td>VER (25 mg)</td>
<td>0.93 ± 0.02**b</td>
</tr>
</tbody>
</table>

*P < .05 vs. controls, Duncan’s test.
**P < .01 vs controls, Duncan’s test.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time after treatment</th>
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<tbody>
<tr>
<td></td>
<td>4 hr</td>
</tr>
<tr>
<td>Control</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>CSA (25 mg)</td>
<td>0.23 ± 0.001**b</td>
</tr>
<tr>
<td>TFP (12.5 mg)</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>TFP (25 mg)</td>
<td>0.14 ± 0.001</td>
</tr>
<tr>
<td>TFP (50 mg)</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>QND (50 mg)</td>
<td>0.23 ± 0.02**b</td>
</tr>
<tr>
<td>VER (25 mg)</td>
<td>0.29 ± 0.02**b</td>
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*P < .05 vs. controls, Duncan’s test.
**P < .01 vs controls, Duncan’s test.
levels in mice not expressing Pgp. Because it was proposed that Pgp might be implicated in the blood-testicular barrier (Cordon Cardo et al., 1989, 1990) and a comparison of testicular levels of drugs in mice expressing and not expressing Pgp supported this view (Schinkel et al., 1994), we also investigated the penetration of VBL in testicular tissue. We included various Pgp inhibitors with different structures (Ford and Hait, 1990; Lum et al., 1993) belonging to different pharmacological classes, such as calcium channel blockers (nifedipine analog Bay K and VER), calmodulin inhibitors (TFP), cardiovascular drugs (AMD and QND) and cyclosporins. These compounds possess either a direct or an indirect mechanism of inhibition of Pgp (Ford and Hait, 1990), different penetration into the brain and different patterns of toxicity (Dollery, 1991).

None of the Pgp inhibitors enhanced VBL penetration into either brain or testis. The reason for the different findings in mice not expressing Pgp and in mice treated with Pgp inhibitors might be due to the fact that despite the relatively high doses used, the pharmacological treatment was still insufficient to completely block the Pgp in brain and testicular capillaries.

Another possible explanation involves the mice with disruption of the mdr1 gene. The data on functional impairment of the blood-brain barrier in mice lacking expression of Pgp are very convincing, but can this impairment be attributed totally to the lack of Pgp? The lack of expression of Pgp may induce important changes in the cell membranes that are then responsible for the altered permeability. Pharmacological inhibition may not cause a similar change in the membrane structure, thus explaining why Pgp inhibitors did not impair the blood-brain barrier.

The results of the present study suggest that there is no real advantage in combining an anticancer drug with a RA for CNS tumors or meningeval or testicular leukemia. In view of the results of the present study, it seems mandatory to obtain pharmacokinetic data (e.g., by investigating whether RA do increase the passage of VBL or other anticancer drugs into the CNS) before organizing clinical trials with therapies designed to overcome the low penetration.

Previous studies conducted in this laboratory had shown that cyclosporins (Colombo et al., 1994; Gonzalez et al., 1995) significantly modify the distribution of anthracyclines in tissues expressing Pgp, such as liver or kidney. The present study shows that CsA and other RA such as TFP, AMD, the nifedipine analog Bay K or VER increase the concentrations of VBL in these tissues. This effect is probably due to the fact that these compounds inhibit Pgp or other proteins involved in drug efflux from tissues (i.e., MRP) that express these proteins (Cole et al., 1994; Brock et al., 1995; Eijgenda et al., 1995; Zaman et al., 1994). The increased concentrations of VBL in liver and kidney do not appear to be due to inhibition of the drug metabolism. In fact, the tissue concentration of DVBL was actually increased, suggesting that this metabolite may be a substrate for Pgp and that its tissue retention is enhanced by the RA.

VBL distribution was not affected to the same extent by the RA tested. The order of effectiveness for liver was TFP > CsA > VER > AMD = Bay K > QND, and the order for kidney TFP > CsA > VER > AMD = Bay K > QND. The doses of RA were based on toxicity data in rodents (Dollery, 1991) and on pilot experiments in the mouse strain that was then used for VBL pharmacokinetic studies. For all RA, at least one dose was close to the maximum tolerated dose; however, because the mechanism of toxicity is likely to be different from that causing inhibition of Pgp, the levels of these compounds achieved in plasma and tissues, although close to the toxic ones, may not necessarily be equally effective in inhibiting Pgp. For example, QND did not enhance VBL distribution in liver and kidney, even though the largest dose used (50 mg/kg) was ~40% of the LD50.

In conclusion, the present study indicates that RA do not increase the penetration of VBL in the mouse CNS, a finding that does not support the clinical use of RA in combination with antitumor agents for the therapy of brain tumors. The study found that RA can enhance the penetration of antitumor drugs in normal tissues such as liver and kidney, thus suggesting the need to reduce anticancer drug doses to avoid toxicity.

Acknowledgments

The generous contribution of the Italian Association for Cancer Research (Milan, Italy) is gratefully acknowledged.

References


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