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Effect of D,L-Verapamil, Verapamil Enantiomers and Verapamil Metabolites on the Binding of Vincristine to α_1-Acid Glycoprotein

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Vincristine binding to solutions of α_1-acid glycoprotein (AGP, 2 mg/ml) and the effect of D,L-verapamil, verapamil enantiomers and the verapamil metabolites norverapamil and D617 were investigated *in vitro* using equilibrium dialysis and ^{3}H-labelled vincristine. Vincristine binding to AGP (52.3 ± 3.6%) was concentration independent over the range 0.002–2.0 μg/ml. The displacement of vincristine from AGP varied between 25.1 and 81.3% with D,L-verapamil and verapamil enantiomers added at concentrations in the range 5–50 μg/ml. In contrast, the displacement by D617 (5–100 μg/ml) was weaker and varied between 0 and 47%. The displacement at 20 μg/ml produced by D,L-verapamil, R-verapamil, S-verapamil and norverapamil was 53.1%, 56.8%, 58.9% and 53.9%, respectively, was more than double that for D617 (25%; $P = 0.002$). It is concluded that vincristine, D,L-verapamil and verapamil isomers and metabolites interact at binding sites on AGP. These interactions may be clinically important in multidrug resistance, for example in cancer patients with elevated levels of AGP undergoing treatment with verapamil and vinca alkaloids.
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INTRODUCTION

MULTIDRUG RESISTANCE (MDR) is the commonly used term for crossover-resistance of tumour cells to structurally unrelated cytostatic drugs including vinca alkaloids. One of the mechanisms causing this incurred resistance is the enhanced expression of transmembraneous P-glycoprotein (P-gp) in the tumour cell. P-gp is known to act as an outward flow pump (multidrug-carrier) lowering the intracellular concentration of the cytotoxic drugs [1–3].

Several investigators have reported that verapamil is able to reverse MDR in animals and humans [4–11]. The precise mode of action is not known but it is possible that verapamil binds to P-gp, thereby competitively inhibiting efflux of the cytostatic drug and raising its intracellular concentration. This property is not related to the ability of verapamil to inhibit the transmembraneous flux of $^{2+}$Ca since reversal of MDR can be effected using both R- and S-enantiomers of verapamil and the demethylated verapamil metabolite norverapamil. The dealkylated metabolite of verapamil D617 is inactive in MDR [11].

Chatterjee *et al.* [9, 12] have reported that the acute phase protein α_1-acid glycoprotein (AGP) interferes with verapamil in experimental MDR. At concentrations similar to those in cancer patients, where the concentrations of AGP in plasma is 2- to 3-fold higher than normal [13, 14], AGP completely abolishes the ability of verapamil and toremifene to reverse MDR in Chinese hamster ovary cell lines resistant to doxorubicin. Since AGP strongly binds vinblastine and verapamil [15–17], the possibility exists that binding interactions on AGP modify the pharmacological activity of these agents and that these interactions have clinical relevance in cancer chemotherapy.

The purpose of this study was therefore to investigate the effect of racemic verapamil (D,L-verapamil), verapamil metabolites and verapamil enantiomers on the binding of vincristine to AGP *in vitro* and to assess the clinical importance of interactions with this glycoprotein in patients undergoing chemotherapy.

MATERIALS AND METHODS

AGP was obtained from Fluka GmbH, Neu-Ulm or the Sigma Chemical Co., and vincristine sulphate from Eli Lilly. [^{3}H]Vincristine sulphate, with specific activity 296 MBq/mg, was supplied by Amersham (U.K.). D,L-Verapamil-HCl, R-verapamil-HCl and the verapamil metabolites norverapamil-HCl and D617-HCl [2-methyl-3-cyano-3-(3',4'-dimethoxyphenyl)-6-methylamino-hexane HCl] were supplied by Knoll AG (Ludwigshafen, F.R.G.). S-Verapamil-HCl was obtained from Research Biochemicals Incorporated (U.S.A.).

Stock solutions of labelled vincristine in phosphate buffer were stored at −20°C. Unlabelled vincristine sulphate and solutions of other reagents were prepared on the day of the experiment and used immediately.

Binding studies were carried out at 37°C in phosphate buffer 0.16 mol/l, pH 7.4 using equilibrium dialysis (Dianorm system). The 1-ml chambers were separated by a cellulose semipermeable membrane (Diachema, Munich, F.R.G.) having a cut-off at 5000 Da. The membrane was soaked in 15% ethanol for 10 min, in distilled water overnight and washed with phosphate buffer before use. The dialysis time required to establish equilibrium was 4 h at a rotation speed of 4 rpm [17, 18]. Individual binding experiments were carried out in duplicate. The concentration of AGP was 2 mg/ml. Each experiment contained between 500 and 1000 Bq [^{3}H]vincristine. In the first set of experiments with

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D,L-verapamil the concentration range of vincristine used was 0.002–2.0 μg/ml. In the second set of experiments using verapamil isomers and metabolites the vincristine concentration range used was 0.125–2.0 μg/ml. The concentration of the interacting ligands in the cell was 20 μg/ml. In the third set of experiments, the effect of different concentrations of D,L-verapamil, its R- and S-enantiomers (5–50 μg/ml) and the metabolite D617 (5–100 μg/ml) on the binding of vincristine (1 μg/ml) to AGP (2 mg/ml) was studied.

After equilibrium had been established, the radioactivity present in the two chambers of the dialysis cell, representing bound ligand plus free ligand and free ligand alone, was determined by liquid scintillation (Tricarb, Packard) using 4 ml scintillation fluid [Aquasafe 300, Zinsser Analytic, Frankfurt, F.R.G. consisting of 2,5 diphenyloxazol and 1,4-bis-(5-phenyl-2-oxazolyl)-benzol in toluene] which gave a clear solution with 0.25 ml aqueous sample. Quench correction was based on quench curves obtained using tritiated vincristine. Results were expressed as percentage of vincristine bound to AGP after correction for background and non-specific binding. Non-specific binding (on the dialysis membrane and polytetrafluoroethylene cells) did not exceed 10% of added radioactivity. The relationship between vincristine concentration and the percentage displacement was analysed statistically using 2-way analysis of variance (concentration of vincristine vs. displacing ligand).

RESULTS

The binding of vincristine to AGP, 52.3 ± 3.6% (mean ± S.D.; range 46.1–56.2%), was independent of concentration over the range investigated, 0.002–2 μg/ml (Table 1).

In the presence of D,L-verapamil, 20 μg/ml, the mean percentage binding of vincristine was reduced to 22.1 ± 4.1% (range 16.8–28.6%). The magnitude of this reduction, 57.8 ± 6.2%, was also independent of the vincristine concentration (Table 1).

The displacement produced by R- and S-enantiomers of verapamil, norverapamil and D617, each at 20 μg/ml, on the binding of vincristine at concentrations 0.125, 0.25, 0.5, 1.0 and 2.0 μg/ml was concentration independent (Table 2). There was no difference between norverapamil, the R-enantiomer and the S-enantiomer in the ability to displace vincristine from its binding site on AGP but the displacement by D617 was lower than all the other agents tested, $P = 0.002$ (Table 2).

Over the concentration range 5–50 μg/ml, and using a vincristine concentration of 1 μg/ml, D,L-verapamil and its R- and S-enantiomers displaced vincristine by 25.1–81.3% (Fig. 1).

In contrast, the effect of D617 was markedly lower and 100 μg/ml of D617 produced the same displacement as 10 μg/ml of verapamil.

Table 1. Binding of vincristine to AGP in absence and presence of D,L-verapamil and percentage displacement

Vincristine concentration (μg/ml)	Vincristine bound in absence of D,L-verapamil (%)	Vincristine bound in presence of D,L-verapamil (%)	Displacement (%)
0.002	49.8	19.4	61.0
0.009	56.2	16.8	70.1
0.035	51.6	22.0	57.4
0.069	46.6	18.4	60.5
0.125	54.2	25.4	53.1
0.135	46.1	16.9	63.3
0.250	53.6	24.0	55.0
0.500	54.9	28.6	47.9
1.000	55.6	25.4	54.7
2.000	54.2	24.5	54.8
Mean ± S.D.	52.3 ± 3.6	22.1 ± 4.1	57.8 ± 6.2

Table 2. Binding of vincristine to AGP in absence and presence of R-verapamil, S-verapamil, norverapamil and D617. Mean of measurements made using five different vincristine concentrations in the range 0.125–2.0 μg/ml

Compound investigated	Vincristine bound in absence of compound (%)	Vincristine bound in presence of compound (%)	Displacement (%)
R-Verapamil	41.3 ± 2.6	17.7 ± 2.0	56.8 ± 7.6
S-Verapamil	52.2 ± 1.9	21.4 ± 1.0	58.9 ± 3.1
Norverapamil	53.1 ± 2.7	24.5 ± 2.3	53.9 ± 3.4*
D617	48.7 ± 2.0	36.4 ± 1.5	25.2 ± 6.1*

*Data obtained at vincristine concentrations of 0.2 and 2.0 μg/ml only.

DISCUSSION

This investigation addresses the possibility that the chemosensitising action of verapamil in MDR may involve an extracellular effect on vincristine binding in the blood compartment. In a previous study we have shown that verapamil binds to AGP and that AGP contains high affinity receptors for this ligand [17]. If vincristine can also bind to AGP and is displaced by verapamil, the presence of verapamil would increase the free fraction of vincrinstine in plasma and raise tumour cell concentrations of this cytotoxic agent. There is evidence from studies of MDR that vincristine and verapamil interact at intracellular binding sites on P-glycoprotein and, therefore, *in vitro* studies of vincristine/verapamil interaction using AGP may provide information on the mechanism underlying this phenomenon.

The experiments described show that the percentage binding of vincristine to pure solutions of AGP is approximately 50%. The binding is independent of the vincristine concentration up to 2 μg/ml. Binding studies using these two substances have not

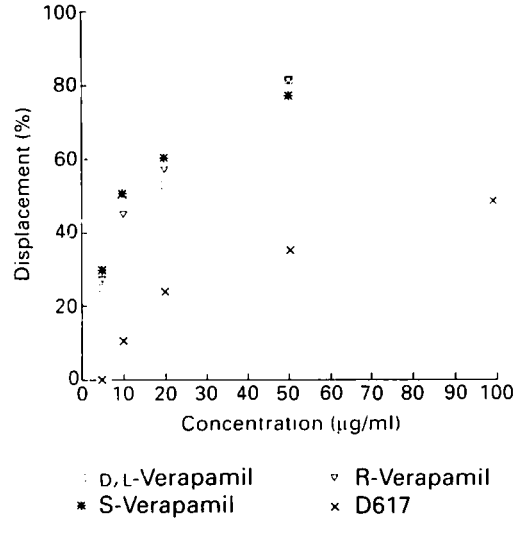

Fig. 1. Percentage displacement of vincristine from binding sites on AGP produced by different concentrations of D,L-verapamil, verapamil enantiomers and the metabolite D617.

been previously reported. The vincristine concentration range used is clinically relevant since the blood concentration attained during chemotherapy is approximately 10 ng/ml and intracellular levels in tissue cultures of neoplastic cells reach 1000 ng/ml [4].

The concentrations of the other binding ligands also correspond to those found *in vivo*. The concentration of AGP, 2 mg/ml, is approximately twice that found in normal human subjects but is similar to that occurring in patients with leukaemia or lymphoma [13, 14, 21]. The lowest concentration of D,L-verapamil and norverapamil used (5 μg/ml) was at least 2 times higher than peak plasma levels (approximately 2 μg/ml) present during verapamil therapy in patients with cardiovascular disease. During verapamil treatment, however, both verapamil and norverapamil are present in plasma in approximately equal amounts and their effects in MDR will be additive [11]. Furthermore, the concentration of verapamil and metabolites in tissues is likely to be much higher than in blood because tissue uptake studies have shown that the concentration of both verapamil and norverapamil in the myocardium is approximately 10 μg/ml [22].

The binding experiments have shown clearly that D,L-verapamil, its metabolites norverapamil and D617 and the individual isomers of verapamil interact with vincristine at binding sites on AGP.

The percentage displacement of vincristine by D,L-verapamil does not change significantly when the vincristine concentration is increased a 1000-fold but the mechanism of the interaction and the nature of the interacting binding sites cannot be fully characterised at present.

Whether the magnitude of the interaction is clinically important is not known. It is generally assumed that drug displacement has clinical significance only when the binding is more than 90% and when the displacement produces large changes in the free-fraction. On the other hand, in assessing the importance of a change in the free concentration of vincristine, it must be kept in mind that vincristine is a highly toxic substance, with a narrow dosage range, where a relatively small increase in concentration may produce a significant increase in the cytotoxic effect. If a doubling of the free-fraction of vincristine, as shown here, produces a corresponding change intracellularly in the tumour, even if only transient, an increase of vincristine cytotoxicity may occur.

The finding that S- and R-verapamil, although having markedly different $^{2+}$Ca-channel blocking properties [19, 20], are equipotent in displacing vincristine, is of considerable interest since both enantiomers are active in reversing MDR and presumably compete with vincristine to the same extent for binding site(s) on P-gp. Thus it might be speculated that the interactions between vincristine, verapamil and AGP resemble those occurring in tumour cells between vincristine, verapamil and P-gp. Previous investigations have shown that D,L-verapamil, the R- and S- enantiomers of verapamil and the verapamil metabolite norverapamil, but not D617, reverse MDR in patients and/or in tissue cultures of human lymphoma [11]. The finding in the present study that D617 is less effective in displacing vincristine from its binding site on AGP offers a possible explanation for the absence of activity of D617 in experimental MDR. These findings also indicate that AGP may have value as a model for interactions involving P-gp, anti-neoplastic drugs and agents capable of reversing MDR. In support of this suggestion, the addition of AGP, within the concentration range used here, completely abolishes the ability of verapamil to reverse MDR in Chinese hamster ovary cell lines resistant to doxorubicin [9].

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