Reversal of P-glycoprotein-mediated multidrug resistance in vitro by AV200, a new ardeemin derivative

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Abstract

The activity of AV200, a synthetic ardeemin derivative, in reversing the multidrug resistance phenotype has been investigated. At non-toxic doses, AV200 was able to completely restore vincristine and paclitaxel toxicities and partially restore that of doxorubicin in multidrug-resistant cells. The potency of AV200 as a modulator of the resistance to doxorubicin, vincristine and paclitaxel resulted to be seven-, 59 and 12-fold, respectively, higher than that of verapamil. In vitro measurements of rhodamine 123 accumulation in human resistant cells suggest that AV200 reverses multidrug resistance by directly inhibiting the P-glycoprotein-mediated drug efflux. This work underscores the possibility of utilizing ardeemin derivatives as a source of non-toxic modulators of the multidrug resistance phenotype. © 1998 Elsevier Science Ireland Ltd. All rights reserved

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1. Introduction

Resistance to chemotherapy is a common problem in patients with cancer and a major obstacle in the effective treatment of disseminated neoplasms. Although clinical drug resistance in cancer is a multifactorial problem [11], the so-called multidrug resistance (MDR) is one of the most frequent causes of failure of chemotherapy. MDR is characterized by the tumoral cells failing to respond to a variety of chemotherapeutic agents that do not share a common structure or cytotoxic intracellular target. MDR is related to the overexpression of a 170 kDa P-glycoprotein (Pgp) encoded in humans by the MDR1 gene and acting as an efflux transport pump that produces a decrease in intracellular drug accumulation and cytotoxicity [5,6,15].

A goal of MDR research is to improve treatment outcome in patients with cancer by devising strategies that are able to circumvent drug resistance due to Pgp overexpression. A major approach to the circumvention of MDR is the use of resistance modifiers, that is, agents that are able to reduce the degree of drug resistance in multidrug-resistant cells by interfering with the pump’s drug efflux function. These drugs, also referred to as MDR reversal agents, inhibit the efflux of Pgp substrate drugs out of cells in vitro and result in the resensitization of the resistant malignant cell.

Since Tsuruo et al. [23] first reported the pharmacological reversal of MDR in 1981 by showing that non-cytotoxic doses of verapamil could restore the
sensitivity to vincristine in multidrug-resistant cells, a large number of resistance modifier agents (RMAs) has been found that are able to reverse resistance to cytotoxic agents in experimental systems. This has led to the idea that clinical drug resistance in human tumours overexpressing Pgp may be overcome through the administration to patients of an RMA together with chemotherapeutic drugs. Several clinical trials have been tested following this strategy [2,4,22]. Early clinical modulation trials necessarily used first generation modulators, drugs that were originally developed for pharmacological effects other than circumvention of MDR, which had the advantage of being easily available as well as having known toxicity profiles. However, it soon became clear that much higher than standard doses of these agents were required to achieve serum levels sufficient to inhibit Pgp [14,17]. More recently, a new generation of RMAs characterized by a higher potency for reversing Pgp activity in vitro and lower intrinsic toxicity is arising as the product of specific drug discovery programmes. Several agents are much more effective at sensitizing multidrug-resistant cells in vitro than compounds previously examined as modulators have been described, such as the non-immunosuppressive cyclosporin A analogue PSC-833 [24], the cyclopeptolide SDZ 280-446 [12] and lamellarin I, a polyaromatic alkaloid recently isolated from a tunicate [20].

However, in spite of all these efforts, no definitive MDR inhibitor is yet available in the clinic. Poor long-term response in reversal trials indicates the importance of developing more potent but less toxic reversal agents than those presently used in ongoing trials. In the course of screening for compounds able to reverse doxorubicin resistance in a multidrug-resistant cell line, AV200, a synthetic ardeemin derivative, was found to be a potent inhibitor of Pgp function. In the present paper, we compare AV200 MDR-modulating activity in vitro with that of verapamil, which is currently employed as a reference RMA.

2. Materials and methods

2.1. Chemicals

Doxorubicin, vincristine and verapamil were purchased from Sigma (St. Louis, MO). Paclitaxel and rhodamine 123 (Rh123) were from Molecular Probes (Eugene, OR). AV200 was kindly provided by Dr C. Avendaño (Universidad Complutense, Madrid, Spain). Its structure is shown in Fig. 1. Cell culture reagents and media were from Gibco (Paisley, UK) and fetal calf serum (FCS) was purchased from Seromed-Biochrom (Berlin, Germany).

2.2. Cell lines and culture

Parental murine leukaemia P388 cells and multidrug-resistant P388/Schabel cells (showing relative resistances of about 100-, 450- and 120-fold to doxorubicin, vincristine and paclitaxel, respectively, compared to those of the P388 cell line) and parental human colon adenocarcinoma LoVo and multidrug-resistant LoVo/Dx cells (showing a relative resistance to doxorubicin of about 30-fold in comparison with its parental cell line) were kindly supplied by Dr M. Grandi (Pharmacia-Farmitalia, Nerviano, Italy). Both resistant cell lines were selected by growth in the presence of doxorubicin for the multidrug-resis-
tant phenotype and overexpress the *mdr1* gene [3,7,8,21]. P388 and P388/Schabel were routinely maintained (37°C, 5% CO₂ in a humid atmosphere) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20 µM β-mercaptoethanol, 100 IU/ml streptomycin–penicillin and 10% FCS. LoVo and LoVo/Dx cells were cultured in Ham’s F12 medium supplemented with 2 mM L-glutamine, 1% vitamins mixture, 100 IU/ml streptomycin–penicillin and 10% FCS. The media for P388/Schabel and LoVo/Dx cells were further supplemented with 300 and 100 ng/ml doxorubicin, respectively, in order to keep their MDR phenotype stable. One day before experimental use, the culture medium of the multidrug-resistant cell lines was removed and the cells were grown in drug-free medium.

### 2.3. Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) dye reduction assay in 96-well microplates was used essentially as described [13]. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenase of viable cell to a blue formazan product which can be measured spectrophotometrically. Cells (2 x 10³ in a total volume of 100 µl of culture medium) were incubated in each well with serial dilutions of the compound to be assayed for its cytotoxicity. After 3 days of incubation (37°C, 5% CO₂ in a humid atmosphere), 10 µl of MTT (5.0 mg/ml in PBS) was added to each well and the plate was incubated for a further 4 h (37°C). The resulting formazan was dissolved in 150 µl of 0.04 N HCl-2 propanol and read at 570 nm. All determinations were carried out in triplicate. The IC₅₀ value was calculated as the concentration of antitumoral drug yielding a 50% rate of cell survival.

### 2.4. Chemosensitization assay

For the chemosensitization assay, a complete antitumoral drug dose–cell growth response curve was constructed as indicated above at each RMA concentration. A whole range of IC₅₀ values were thus obtained in the presence of the different RMA concentrations, the IC₅₀⁺ value being obtained in the absence of RMA. The increase in sensitivity to the antitumoral drug was expressed as a gain of sensitivity [10] and calculated for each RMA concentration from the ratio IC₅₀⁻/IC₅₀⁺.

### 2.5. Rh123 accumulation measurement

LoVo/Dx cells (10⁶ cells/ml) were incubated for 30 min (37°C, 5% CO₂ in a humid atmosphere) with 2.5 µM Rh123 and the indicated RMA concentration. No detectable cytotoxicity was associated with Rh123 under these conditions. Cells were then washed twice with ice-cold PBS and resuspended in PBS just before the cytometric measurement. Untreated cells resuspended in PBS were used as blanks. In order to determine Rh123 accumulation in sensitive cells, LoVo cells were incubated under the same conditions as MDR cells, but in the absence of RMA. Cells were analyzed (10,000 cells per sample) on a FACSsort flow cytometer (Becton & Dickinson, San Jose, CA) essentially as described by Pilarsky and Belch [18]. Excitation was by an argon laser operating at 488 nm and analysis of fluorescence was at 530/530 nm. Cells which had not been exposed to Rh123 were used to determine the background of autofluorescence under these conditions.

### 3. Results and discussion

#### 3.1. Reversal of doxorubicin, vincristine and paclitaxel resistance by AV200

From primary screening carried out by using a chemosensitization assay previously described [19], AV200 ardeemin derivative was chosen to be further studied for its ability to restore doxorubicin, vincristine and paclitaxel toxicities in P388/Schabel cells. In spite of the wide diversity of chemosensitizer structures, it has been suggested that RMAs are usually hydrophobic and contain two or more planar aromatic rings and a tertiary nitrogen [16,26]. As shown in Fig. 1, the AV200 structure fits this profile. Chemosensitization assays measure the consequences of inhibiting Pgp function for cell growth. They require RMA concentrations which are not inhibitory or toxic by themselves. In the present study, only RMA concentrations yielding less than 10% growth inhibition of P388/Schabel cells, when tested
in the absence of doxorubicin or any other drug, were considered. Fig. 2 shows the effects of AV200 and verapamil on the cytotoxicity of doxorubicin (Fig. 2A), vincristine (Fig. 2B) and paclitaxel (Fig. 2C) on multidrug-resistant cells. As shown in this figure, the vincristine and paclitaxel toxicity curves for P388/Schabel cells resembled that of parental P388 when co-incubated with 2.5 μM AV200. However, no full restoration of doxorubicin toxicity was obtained. Ver-
apam at the same concentration showed a weaker potentiating activity of either of the antitumoral drugs employed on P388/Schabel cells. The potenti-
ing effect of AV200 on doxorubicin, vincristine and paclitaxel toxicities is given in Table 1. As shown in this table, a complete reversion of vincristine and paclitaxel resistance (i.e. the gain of sensitivity equal to the relative resistance between the parental and the multidrug-resistant cell line; see Section 2 for calculation of gain of sensitivity) could be obtained with 2.5 μM AV200, which is within the range of RMA dosages which do not per se cause a substantial inhibition of cell growth. No complete reversion of doxorubicin resistance could be obtained with non-toxic concentrations of AV200. Four-fold higher concentrations of the prototype MDR modulator, verapamil, were required to obtain comparable gains for chemosensitization to doxorubicin, whereas no comparable reversion of the resistance to vincristine and paclitaxel could be reached with non-toxic concentrations of verapamil. If we use the MI (fold decrease in resistance/modulator μM concentration) to represent the effectiveness of an RMA as proposed by Beck and Qian [1], at 2.5 μM AV200 has MI values of 32, 189 and 44 for doxorubicin, vincristine and paclitaxel, respectively. These values are seven-, 59- and 12-fold, respectively, higher than those obtained with 2.5 μM verapamil (4.4, 3.2 and 3.8 for doxorubicin, vincristine and paclitaxel, respectively). The different pattern of chemosensitization by verapamil and

Fig. 2. Dose-dependent effect on the in vitro growth of P388/Schabel cells by doxorubicin (A), vincristine (B) or paclitaxel (C) either in the absence (○) or presence of 2.5 μM verapamil (●) or 2.5 μM AV200 (□). The growth of P388 parental cells is displayed as a reference (■). Cell proliferation is represented as a percentage of control-cell growth in cultures containing no drugs. Each point represents the mean of triplicates; SD values were always lower than 10% and are omitted for clarity.

Fig. 3. Effect of AV200 on the accumulation of Rh123 by multidrug-resistant LoVo/Dx cells in co-treatment conditions. Rh123 accumulation in LoVo parental cells is shown by a dashed line. Each point represents the mean ± SD of three determinations.
AV200 for doxorubicin, vincristine and paclitaxel could be due to the existence of different drug binding-transport sites on the Pgp for different drugs or groups of drugs [9] and it could suggest that verapamil and AV200 have different efficiencies in inhibiting those sites.

3.2. Effect of lamellarins on Rh123 accumulation in multidrug-resistant cells

One simple assay to quantify the potency of Pgp inhibitors measures the increase in the amount of intracellular cytotoxic drug substrate due to Pgp inhibitors, first reported for verapamil in P388 cells [23]. A modification of this approach uses the fluorescent probe Rh123 in a method that can be automated to allow large scale screening [19]. Rh123, which selectively localizes in mitochondria, is effluxed more efficiently by MDR cells and this efflux can be inhibited by verapamil and other RMAs [25].

Fig. 3 shows the effects of increasing concentrations of AV200 on the intracellular accumulation of Rh123 in human LoVo/Dx cells after 30 min of incubation with 2.5 μM Rh123. As shown in this figure, AV200 increased the intracellular concentration of Rh123 in multidrug-resistant cells in a dose-dependent manner, raising it to the level observed in the sensitive cells. The maximal enhancement in accumulation in resistant cells was obtained using 10 μM of AV200. AV200 caused an increase in Rh123 accumulation in P388/Schabel cells, but did not modulate Rh123 accumulation in the LoVo or the P388 sensitive cell lines (data not shown). Measurement of Rh123 accumulation yields a direct measurement of the inhibition of Pgp function. The increase in accumulation of Rh123 in multidrug-resistant cells after the addition of AV200 supports the hypothesis that this compound causes a modulation of resistance by inhibiting the pump function of Pgp.

In conclusion, the results reported here show that AV200 is able to completely restore vincristine and paclitaxel toxicities and partially restore that of doxorubicin in a multidrug-resistant cell line. AV200 was shown to be seven- to 59-fold more active than the reference compound, verapamil. The fact that AV200 is able to increase Rh123 accumulation in MDR cells suggests that this compound may act by inhibiting the Pgp transport activity.

Table 1

Gains of sensitivity to doxorubicin, vincristine and paclitaxel for multidrug-resistant P388/Schabel cells obtained with different concentrations of verapamil and AV200

<table>
<thead>
<tr>
<th>Antitumoral drug</th>
<th>RMA</th>
<th>RMA (μM)</th>
<th>0.3</th>
<th>0.6</th>
<th>1.2</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>Verapamil</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.5 ± 0.7</td>
<td>11.0 ± 3.5</td>
<td>23.0 ± 9.7</td>
<td>70.0 ± 15.0</td>
</tr>
<tr>
<td></td>
<td>AV200</td>
<td>7.0 ± 3.6</td>
<td>8.9 ± 0.8</td>
<td>27.0 ± 11.0</td>
<td>79.0 ± 12.0</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>Verapamil</td>
<td>ND</td>
<td>ND</td>
<td>4.8 ± 1.3</td>
<td>8.1 ± 2.1</td>
<td>35.0 ± 5.9</td>
<td>75.0 ± 15.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AV200</td>
<td>16.0 ± 3.0</td>
<td>27.0 ± 1.4</td>
<td>77.0 ± 4.9</td>
<td>472.0 ± 57.0</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Verapamil</td>
<td>ND</td>
<td>ND</td>
<td>4.2 ± 0.8</td>
<td>9.4 ± 3.2</td>
<td>21.0 ± 4.9</td>
<td>54.0 ± 16.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AV200</td>
<td>12.0 ± 1.5</td>
<td>21.0 ± 0.4</td>
<td>49.0 ± 11.0</td>
<td>110.0 ± 21.0</td>
<td>*</td>
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</tr>
</tbody>
</table>

ND, not determined; RMA, resistance modifier agent.
Values represent the mean ± SD gains of sensitivity of three to four determinations in triplicate. See Section 2 for calculation of gains of sensitivity.

* >50% Growth inhibition by RMA alone.

References