DAUNOMYCIN ACCUMULATION IN RESISTANT TUMOR CELLS AS A SCREENING MODEL FOR RESISTANCE MODIFYING DRUGS: ROLE OF PROTEIN BINDING

H.J. BROXTERMAN, C.M. KUIPER, G.J. SCHUURHUIS, J.J.M. van der HOEVEN, H.M. PINEDO and J. LANKELMA

Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (The Netherlands)

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SUMMARY

A potential screening system for drugs that modify anthracycline resistance was investigated. The effect of the calcium antagonistic agents verapamil, diltiazem, bepridil and Ro 11-2933/001 on daunorubicin (DNR) accumulation in the doxorubicin resistant cell line 2780AD was measured. For this purpose cells were suspended in medium with varying concentrations of bovine serum albumin (BSA) and in human plasma to establish the influence of protein binding on DNR accumulation. Increase of protein concentration did not affect DNR accumulation when no calcium antagonists were present but caused an increase in concentrations of calcium antagonists needed to promote DNR accumulation. It appeared that DNR accumulation in suspended cells correlated well with cytotoxicity assays, performed on cells growing in monolayer in 96-well microtiter plates, when accumulation was measured in medium with the same protein constitution as in growth medium (10% fetal calf serum).

It is concluded that protein binding effects should be taken into account in the screening of compounds for the circumvention of multidrug resistance.

INTRODUCTION

Mammalian tumor cell lines, selected in vitro for resistance to doxorubicin, often exhibit cross-resistance to a number of structurally unrelated natural products with different modes of action, a phenomenon which has been termed multidrug resistance (MDR) [2,3]. Such cell lines are extensively used as model systems for studies into the mechanism of MDR and or ways to overcome MDR [15,17].
Most studies identified an increased energy dependent outward transport of drug from resistant cells, associated with an increased expression of a 170,000-dalton plasma membrane glycoprotein [9], enabling the cells to maintain a low intracellular drug concentration [8,15]. Based on these observations, efforts have been made to search for agents which might reverse resistance by increasing drug accumulation in the cells. These studies have particularly focussed on membrane-interacting agents, including the calcium antagonist verapamil and the calmodulin inhibitor trifluoperazine, which appeared highly potent in reversing resistance to a number of cytostatic drugs in vitro [17]. Also increases in life-span of mice inoculated with resistant tumor cells and treated with combinations of verapamil and cytostatic agent compared to treatment with the cytostatic agent alone have been reported [16,18]. A phase I clinical study aiming to achieve plasma levels of verapamil which have been proven to be effective in vitro, failed to be successful [13]. Therefore, in order to detect drugs which are more active in reversing drug resistance in cancer patients, we are in need of more effective preclinical screening models [4,7,10,14].

The measurement of the drug effects on in vitro daunorubicin (DNR) accumulation in resistant cells seems to be a convenient initial screening system. Ideally, for such a system, the effect of potential resistance modifying drugs on DNR accumulation should correlate with effects on DNR cytotoxicity. However, one factor which has not been considered until now [10,12,13], but which may nevertheless obscure the in vitro results and their potential predictive value for in vivo results, is the influence of protein binding of the drugs [11]. In the present study we investigated the effect of a range of protein concentrations on DNR accumulation in a suspended resistant ovarian carcinoma cell line.

MATERIALS AND METHODS

Drugs

[14-14C]Daunorubicin (spec. act. 45 Ci/mol) was obtained from Amersham (Amersham, U.K.). The radiochemical purity was >98% by thin layer chromatography. Daunorubicin-HCl was from Specia (Paris, France). Verapamil-HCl, diltiazem-HCl and bovine serum albumin (charcoal-treated; BSA) were from Sigma Chemical Corp. (St. Louis, MO); Bepridil-HCl (β-[2-methylpropoxy]-methyl]-N-phenyl-N-(phenyl-methyl)-1-pyrrolidine-ethamine · monohydrochloride · monohydrate; ORG 5730) was provided as a sterile solution containing 44.8 mg glucose/ml by Organon Int. (Oss, The Netherlands). Ro 11-2933/001 (N-[3,4-dimethoxyphenyl]-2-[2-naphthyl]-N-methyl-m-dithiane-2-propylamine was a gift from Hoffmann-La Roche (Mijdrecht, The Netherlands) and was dissolved in dimethyl sulfoxide (DMSO).

Cell culture

The human ovarian carcinoma cell line A2780 and its doxorubicin resistant subline 2780AD were obtained from Dr. R.F. Ozols (NCI, Bethesda,
MD) and maintained in culture in bicarbonate buffered Dulbecco's modified essential medium (DMEM) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 10% heat-inactivated fetal calf serum (FCS). 2780AD cells were cultured in the presence of 2 μM doxorubicin for 10−14 days before experiments. All cell culture media and supplements were from Flow Laboratories, (Irvine, U.K.) and plastics were from Greiner (Nürtingen, G.F.R.). Cells were subcultured or used for experiments after detaching in Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (PH 7.4) containing trypsin (0.0025% w/v) and EDTA (0.25% w/v) for 2 min at 37°C.

Growth inhibition assays

IC₅₀-values, i.e. drug concentrations inhibiting cell growth by 50% compared to drug free control cultures, were determined in microtiter plates [6]. A cell suspension (100 μl) (5 × 10⁵ cells/ml) was seeded in microtiter wells. When the cells were attached, 100 μl drug containing medium was added. Each concentration was studied in quadruplicate. The medium was aspirated after 72 h and the wells were washed twice with saline; cells were fixed with 50 μl 70% ethanol for 15 min and stained with 50 μl crystal violet (0.5% w/v in 50% ethanol) for 45 min. Then the wells were washed extensively with water and after drying (at 50°C), the crystal violet was dissolved in 50 μl n-lauryl sarcosine (1% in phosphate buffered saline, PBS). The extinction was measured using a Titertek multiscaner at a wavelength of 596 nm. The method had been validated first by comparing IC₅₀s with IC₅₀s obtained by cell counting. The IC₅₀ was 1.0 (±0.3) × 10⁻⁸ M DNR for A2780 (N = 4) and 2.4 (±0.6) × 10⁻⁸ M DNR (N = 13) for 2780AD.

Daunomycin accumulation experiments

Cells from exponentially growing cultures were suspended with trypsin/EDTA, washed and diluted in medium at densities of 5 × 10⁵−10⁶/ml. The medium consisted of DMEM without bicarbonate and pH-indicator, but with HEPES (pH 7.4) and supplemented with protein as indicated. In one experiment fresh human heparinized plasma (pH 7.4) was used. Incubations were carried out at 37°C in polypropylene vials and initiated with the addition of labeled DNR. Calcium antagonists or vehicle were added 5 min earlier at specified concentrations. Incubations were terminated by dilution with 8 volumes ice-cold PBS and centrifugation. Cell pellets were washed once with 1 ml cold PBS and transferred into vials containing 4 ml. Opti-fluor (Packard, Groningen, The Netherlands). Radioactivity was determined by liquid scintillation counting. Intracellular DNR concentrations are expressed as pmol/10⁶ cells, after correcting radioactivity for immediate binding at 0°C.

RESULTS

The time-course of accumulation of labeled DNR (at an extracellular concentration of 2 μM) by A2780 and 2780AD cells is shown in Fig. 1. Under
the present conditions, i.e. cells suspended in medium + 10% FCS the steady-state accumulation of DNR in resistant cells was 15−20-fold reduced compared to sensitive cells. Moreover when the cells were examined with fluorescence microscopy, apparently all DNR was intranuclear in sensitive cells, while no fluorescence could be observed in 2780AD cells.

For A2780 cells the relationship between DNR accumulation versus extracellular DNR concentration is shown in Fig. 2. A linear relationship was found over the concentration range studied. Thus, differences in extracellular DNR available for cellular uptake will be reflected in steady-state DNR accumulation in A2780 cells.

Thereafter we examined the effect of a series of calcium antagonists on cellular DNR accumulation in media with different protein concentrations. Bepridil and Ro 11-2933 were compared with the calcium antagonists verapamil and diltiazem which have been studied extensively. The results are summarized in Tables 1−3. Increase of the protein concentration from 1 to 4% had no effect on DNR accumulation in the two cell lines (Table 1), indicating that the fraction of DNR to accumulate in the cells is not significantly dependent on serum albumin concentration, despite considerable albumin binding of the drug [5]. Furthermore, Table 2 shows that no major effects were seen on DNR accumulation in A2780 of any concentration of

![Graph](image_url)

Fig. 1. Time-course of daunorubicin accumulation in A2780 and 2780AD cells, suspended in medium + 10% FCS. Extracellular DNR concentration was 2 μM. S.D.s were <10% for 3 independent experiments.
Fig. 2. Concentration dependence of daunorubicin accumulation (90 min) in A2780 cells, suspended in medium + 10% FCS. S.D.s were <10% for 2 independent experiments.

calcium antagonist tested. However, Table 3 shows that the effect of all calcium antagonists on DNR accumulation in 2780AD cells is smaller in the presence of 4% BSA, compared to medium containing a low protein concentration (1%). Also, it has to be noted that both in A2780 and 2780AD cells, DNR accumulation tended to be lower in the absence of protein supplement in the medium than in the presence of 1% BSA (Tables 1–3). This may not be directly related to calcium antagonist-protein interactions, since factors such as cell morphology or membrane fluidity, but also experimental factors like DNR-plastic interaction may be different in the absence of albumin. In Table 3 also the effects as measured in fresh human plasma are shown.

Following these findings we determined whether the effects of various calcium antagonists on DNR accumulation in suspended cells correlated with

### Table 1

**EFFECTS OF PROTEIN CONCENTRATION ON DAUNORUBICIN ACCUMULATION IN A2780 AND 2780AD CELLS**

Extracellular DNR concentration was 2 µM.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No BSA</th>
<th>1% BSA</th>
<th>4% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>617a</td>
<td>667</td>
<td>637</td>
</tr>
<tr>
<td>2780AD</td>
<td>56</td>
<td>51</td>
<td>52</td>
</tr>
</tbody>
</table>

*a Values are pmol DNR accumulated/10⁶ cells during 60 min accumulation. Results are means from 2 (A2780) or 3 (2780AD) experiments. S.D. <15%.
TABLE 2
EFFECTS OF CALCIUM ANTAGONISTS ON DAUNORUBICIN ACCUMULATION IN A2780 CELLS AT DIFFERENT PROTEIN CONCENTRATIONS

Extracellular DNR concentration was 2 μM.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>(μM)</th>
<th>No BSA</th>
<th>1% BSA</th>
<th>4% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bepridil</td>
<td>(8)</td>
<td>98a</td>
<td>106</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>93</td>
<td>101</td>
<td>97</td>
</tr>
<tr>
<td>Verapamil</td>
<td>(8)</td>
<td>92</td>
<td>120</td>
<td>104*</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>103</td>
<td>126</td>
<td>119</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>(32)</td>
<td>104</td>
<td>119</td>
<td>109</td>
</tr>
<tr>
<td>RO 11-2933</td>
<td>(2)</td>
<td>85</td>
<td>113</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>91</td>
<td>107</td>
<td>95</td>
</tr>
</tbody>
</table>

* Values are expressed as percentage of DNR accumulated without calcium-antagonists. Results are from 2 experiments. S.D. <15%.
* Significant difference (P < 0.05, Student's t-test) between 1 and 4% BSA.

their potentiation of DNR cytotoxicity. Because of the protein related effects, DNR accumulation was measured in medium supplemented with 10% FCS. Figure 3 summarizes the results of 3 independent experiments and shows a good correlation between DMF and the enhancement of DNR accumulation.

TABLE 3
EFFECTS OF CALCIUM ANTAGONISTS ON DAUNORUBICIN ACCUMULATION IN 2780AD CELLS AT DIFFERENT PROTEIN CONCENTRATIONS

Extracellular DNR concentration was 2 μM.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>(μM)</th>
<th>No BSA</th>
<th>1% BSA</th>
<th>4% BSA</th>
<th>Human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bepridil</td>
<td>(4)</td>
<td>186a</td>
<td>208</td>
<td>115</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>229</td>
<td>250</td>
<td>131</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>334</td>
<td>436</td>
<td>251</td>
<td>140</td>
</tr>
<tr>
<td>Verapamil</td>
<td>(4)</td>
<td>188</td>
<td>214</td>
<td>138</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>235</td>
<td>321</td>
<td>196</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>351</td>
<td>447</td>
<td>334</td>
<td>214</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>(32)</td>
<td>324</td>
<td>338</td>
<td>263</td>
<td>221</td>
</tr>
<tr>
<td>RO 11-2933</td>
<td>(2)</td>
<td>566</td>
<td>490</td>
<td>212</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>732</td>
<td>716</td>
<td>527</td>
<td>124</td>
</tr>
</tbody>
</table>

* Values are expressed as percentage of DNR accumulation without calcium-antagonist. Results are from 2—4 experiments. S.D. <15%. All values between 1 and 4% BSA were significantly different (P < 0.05, Student's t-test).
DISCUSSION

In recent years a series of papers have appeared showing the potency of verapamil and other calcium-antagonists to increase cytotoxicity of anthracyclines and vinca-alkaloids in resistant cell lines (for reviews see 7, 15). The potential clinical usefulness of such combinations will highly depend on the feasibility to attain plasma levels of the agent to modify resistance and finally to enhance anthracycline responsiveness in cancer patients with drug resistant tumors. For this reason the establishment of in vivo toxicity of potential agents at resistance modifying concentrations is required. In this regard verapamil is considered to be too toxic to achieve high enough dose levels [13] requiring more selective agents.

In vitro testing is the method of choice for a first screening of drugs, since a great number of compounds can be tested at different concentrations. In the present study we investigated in an in vitro model to what extent protein binding influences the effect of calcium-antagonists on DNR accumulation in resistant tumor cells. This factor, which is usually not taken into account, will give useful information for in vivo use of these compounds. We showed that DNR accumulation in suspensions derived from resistant anchorage-dependent growing 2780AD cells correlated well with cytotoxicity assays performed on monolayers in 96-well plates in the presence of calcium antagonists [15]. Thus in our model which is easy to handle drug
accumulation appears to predict for cytotoxicity. This may be related to the fact that the difference in DNR accumulation between A2780 and 2780AD is relatively large enabling reliable experimental measurements. Also, in the presence of calcium antagonist DNR accumulates for the great part in the cell nuclei, the site where it exerts its cytotoxic action.

The accumulation of DNR in the absence of calcium antagonists was not influenced by the protein concentration in the incubation medium, neither in sensitive nor in resistant cells. Moreover, calcium antagonists did not significantly enhance DNR accumulation in sensitive cells, indicating that there is no interference with the accumulation assay by displacement of DNR from protein binding sites. Therefore, the influence of protein on the effect of calcium-antagonists in resistant cells may be considered to be the result of differences in availability of calcium antagonist itself.

An increasing BSA concentration clearly lowered the effect of all calcium antagonists on DNR accumulation in 2780AD cells. The effects of calcium antagonists in human plasma were also less pronounced than in medium with low protein. As a result a higher concentration of calcium antagonist is required under such circumstances in order to produce an increase in DNR accumulation. The influence of protein concentration seemed to be somewhat higher for bepridil, which has a higher reported plasma protein binding (>99%) [1], compared to verapamil (90%). Diltiazem effects were relatively little affected by protein, which may correlate with its lower protein binding (78%) [1]. Since other factors also influence in vivo accumulation in tumor cells, such as lipid solubility, distribution volume and bioavailability, which are all greater for bepridil than for verapamil [1], a direct comparison of in vivo availability of the drugs at the site of the tumor cannot be made from these in vitro accumulation data. However, knowledge of how protein affects calcium antagonist induced DNR accumulation in tumor cells should help to evaluate in vivo data. Especially, for the prediction of clinical usefulness in hematologic malignancies, the measurement of protein effects seems warranted.

In conclusion our results suggest that measuring protein effects on DNR accumulation in resistant cells should be included in the screening for more effective agents that might reverse drug uptake related resistance in tumor cells. This approach may help to predict or explain in vivo effects.

ACKNOWLEDGEMENT

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REFERENCES


