Mechanisms of the in Vivo Resistance to Adriamycin and Modulation by Calcium Channel Blockers in Mice

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ABSTRACT

A sensitive fluorometric assay using Hoechst 33258 and a modified alkaline elution procedure were used to quantitate DNA single-strand breaks following an in vivo drug treatment of mice bearing P-388/S and P-388/R cells. After an i.p. treatment of mice with 1 to 20 mg/kg Adriamycin (DOX), the following differences between sensitive and resistant P-388 cells were observed: (a) at 2 h following drug treatment the net intracellular accumulation of Adriamycin in sensitive cells was 2- to 3-fold higher than resistant cells at all doses tested; (b) utilizing a therapeutic dose of DOX (10 mg/kg), the amount of single-strand breaks of DNA in sensitive and resistant cells was significantly different, K × 10^2 = 136.6 ± 1.1 (SD) versus 3.6 ± 0.9, respectively; (c) the 10 and 50% lethal doses for verapamil (VEP) were 10 and 23 mg/kg and for a tiapamil analogue, N-(3,4-dimethoxyphenethyl)-N-methyl-2-(2-naphthyl)-m-dithiane-2-propylamine hydrochloride (DMDP), were 107 and 126 mg/kg, respectively; (d) while the in vivo intracellular accumulation and retention of DOX in sensitive cells were not affected by DMDP or VEP treatment, complete restoration of DOX accumulation and retention was achieved in resistant cells treated with well-tolerated doses of DMDP of 30 and 60 mg/kg. In contrast, utilizing the optimally tolerated dose of VEP (5 mg/kg), only partial restoration of DOX accumulation and retention in resistant cells was achieved; (e) DMDP or VEP did not alter the high level of DNA single-strand breaks induced by DOX in sensitive cells; in resistant cells, however, an increase in single-strand breaks of DNA was observed following treatment with DOX in combination with DMDP and to a lesser extent with VEP; and (f) the rapid DNA repair in resistant cells was inhibited by DMDP but not by VEP. These data demonstrate that DMDP but not VEP can effectively restore the in vivo intracellular accumulation of DOX in resistant cells at achievable nontoxic plasma concentrations.

INTRODUCTION

Acquired and/or intrinsic resistance to DOX is one of the major obstacles to curative therapy with this agent (1–3). Although mechanisms of DOX resistance are multifactorial, major differences in DOX accumulation between sensitive and resistant cell lines have primarily been carried out in in vitro systems, little information is available whether the in vitro and in vivo determinants of response to DOX are identical.

Several investigations have been carried out to evaluate the role of VEP in the restoration of the in vitro sensitivity of resistant cells (14, 17–20). Although conditions utilizing VEP for restoration of the differences existing between sensitive and resistant cells have been identified, the in vitro concentrations of VEP are toxic in vivo (21). Furthermore, simultaneous multifactorial analysis of determinants of resistance to DOX under the same conditions have rarely been carried out. In a search for an alternative to VEP, Kessel and Wilberding (22) identified an agent with greater cytotoxicity than VEP but with effects similar to those of VEP as concerns the restoration of resistance to DOX.

Extensive use of radiolabeled precursors to assess drug-induced DNA damage has been limited to an in vitro situation. Recently the methods of Ducore (23) and Iqbal (24) were modified to quantitate DNA damage induced following an in vivo treatment with DOX alone and in combination with DMDP and VEP.

Studies were carried out to identify in vivo cellular mechanisms of resistance to DOX in P-388 cells and to develop approaches for their modulation by using two calcium channel blockers, VEP and DMDP. Results indicate that DMDP is significantly less toxic in vivo than VEP, more effective in restoring the intracellular accumulation of DOX in resistant cells at achievable nontoxic plasma concentrations.

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drug analysis and DNA damage studies.

DOX Accumulation Assay. At various times following an in vivo treatment with DOX alone and in combination with DMDP and VEP, cells were aspirated from the peritoneal cavity, chilled immediately, and washed at least three times with cold PBS. The cells were then lysed in 0.05% SDS for the determination of DOX accumulation by using the method modified by Finkel et al. (25). The fluorescence of the extracts was detected at 588 nm emission and 480 nm excitation in a Shimadzu RF-540 fluorometer.

Alkaline Elution Assay. Alkaline elution was performed according to Kohn et al. (26) with the modification as described by Ducore (23) and Iqbal (24). Approximately 5 × 10⁶ ascites cells were layered into 47-mm-diameter 2-µm-pore polycarbonate filters (Nucleopore Corp., Pleasanton, CA) and washed twice with ice-cold PBS. Cells were then lysed with 5 ml of a lysis buffer (2% SDS and 0.025 M tetrasodium EDTA, pH 9.7) containing 0.5 mg/ml of proteinase K and were washed with 10 ml of 0.02 M tetrasodium EDTA solution, pH 10.3. The DNA was eluted in the dark with 10 mM tetrasodium EDTA adjusted to pH 12.3 with 6 N NaOH at a flow rate of 2 ml/h. Fractions were collected every 90 min for 15 h, resulting in a fraction volume of approximately 3.0 ml. DNA retained on the filter was eluted with 3 ml of standard saline citrate containing 1 mg/ml of Pronase, pH 7.2, at 37°C for 1 h and then digested in 3 ml of elution buffer, pH 12.3, at 65°C for 1 h. DNA remaining on the filter holders and barrels was recovered by flushing vigorously twice with 3 ml of the elution buffer.

Fluorometric DNA Assay. The relative DNA concentrations in each eluted fraction, filter digestion, and washing fractions were determined by using the fluorometric Hoechst 33258 assay described by Cesarone et al. (27) and adapted for use with alkaline elution by Stout and Becker (28). Each 1.2-ml sample withdrawn from each fraction was neutralized with approximately 0.8 ml of 0.2 M KH₂PO₄ to pH 7.0-7.2. After flushing vigorously twice with 3 ml of the elution buffer.

RESULTS

DOX Accumulation and Toxicity of DMDP and VEP. Utilizing therapeutic (5–10 mg/kg) and in vivo toxic doses (20 mg/kg) of DOX alone and in combination with DMDP and VEP, DOX accumulation in P-388/S and P-388/R cells were quantitated and the results are shown in Fig. 1. The results indicate that at all doses resistant cells accumulated about 3-fold less drug (Fig. 1A). The effect of DMDP and VEP on DOX accumulation in both sensitive and resistant lines are shown in Fig. 1B. The results indicated that intracellular DOX accumulation in resistant cells was restored almost completely by DMDP (30–60 mg/kg dose) and only partially by VEP even at the highest tolerated dose of 5 mg/kg.

The data in Fig. 2 demonstrate significant differences in the in vivo toxicity between DMDP and VEP. The maximally tolerated doses of DMDP and VEP were 100 and 5 mg/kg, respectively. The data in Fig. 2 also indicate that the plasma concentrations of DMDP following the administration of 100 mg/kg were about 9 µM, significantly higher than the 1–2 µM needed for the in vitro reversal of DOX resistance. Although the plasma concentrations of VEP at the MTD were not determined, it is probably less than 1 µM, which is significantly less than the 10 µM VEP required for the in vitro reversal of resistance to DOX.

DOX-induced DNA Damage. The effects of DOX administered at various doses (1 to 20 mg/kg) on the amount of induced SSBs in DNA were investigated and the results are shown in Fig. 3. The data in Fig. 3A indicate that at all DOX doses, significantly fewer SSBs were obtained in P-388/S and P-388/R. Furthermore, the data in Fig. 3 demonstrate that while SSBs were related to the dose of DOX administered, the amount of SSBs in P-388/R never approached the amount seen in P-388/S, even at the in vivo toxic dose of 20 mg/kg (K × 10² of 14 versus 5).

Effect of DMDP or VEP on DOX-induced DNA SSBs. The effects of DOX alone (10 mg/kg) and in combination with
DMDP or VEP on DNA single-strand breaks in ascites cells from mice bearing P-388/S and P-388/R were investigated and the results are shown in Fig. 4 and Table 1. The data in Fig. 4 indicate that neither DMDP nor VEP has any significant effect on the extent of SSBs induced by DOX in sensitive cells. In contrast, DMDP but not VEP potentiated the extent of SSBs induced by DOX in P-388/R cells. These data demonstrate a clear difference in the effect between DMDP and VEP.

Relationship between DOX Accumulation and Induction of DNA Damage. The data in Table 2 summarize the effects of DMDP and VEP on the intracellular accumulation of DOX and on the extent of DNA single-strand breaks following an in vivo administration of DOX alone and in combination with DMDP or VEP to mice bearing P-388/S and P-388/R. These data demonstrate that while DMDP nearly restored the cellular accumulation of DOX in P-388/R, the amount of DNA SSBs in P-388/R were still 35% lower than what was observed in P-388/S. In contrast, VEP was not as effective as DMDP in the restoration of an in vivo drug accumulation and in the potentiation of the extent of DNA SSBs. Data not shown indicate that increasing the DMDP dose to 100 mg/kg (the MTD in DBA/2J mice) did not increase the amount of DNA SSBs. DMDP and VEP did not potentiate further the high level of DNA damage induced by DOX in P-388/S cells.

Repair of DNA SSB. In order to investigate the effect of DMDP and VEP on the repair of DOX-induced single-strand breaks in the ascites cells of mice bearing P-388/S and P-388/R, mice were treated with DOX alone or in combination with DMDP and VEP and at various times thereafter, 2, 4, and 8 h, the amount of SSBs was quantitated. The results are outlined in Fig. 5. The calcium channel blockers have no significant effects on the repair of the induced DNA SSBs of P-388/S cells treated in vivo with DOX. The DNA SSBs in P-388/S were extensive and the repair was slow, independent of DMDP and VEP. In contrast, in P-388/R the repair of DOX-induced DNA SSBs was complete within 4 h after termination of drug treatment where the extent of DNA damage was limited. These data demonstrated that higher DNA SSBs and slower repair was achieved in P-388/R treated with DOX and DMDP but not with DOX and VEP.

DISCUSSION

Rapid emergence of acquired drug resistance following chemotherapy as well as the presence of intrinsic resistant cells in a heterogeneous cell population, are important problems in cancer chemotherapy. Development of in vivo approaches to overcome and/or circumvent drug resistance will depend on better understanding of mechanisms of resistance. The precise mechanism of resistance to anthracyclines, for example, may vary depending on the degree of resistance and the cell type.

Data from the literature (4-8, 15, 16, 29, 30) demonstrated that resistance to Adriamycin at the cellular level was associated with a reduced drug accumulation, retention, and DNA damage. Previously it was shown that although drug accumulation and retention in the 300-fold DOX-resistant P-388 cells can be restored by DMDP, a new calcium channel blocker, the amount

![Fig. 4. Reversal effects of DMDP and VEP on DOX-induced DNA SSBs in the cells of mice bearing P-388/S (G) and P-388/R (W). A, no drug; B, DOX (10 mg/kg); C, DOX (10 mg/kg) plus DMDP (30 mg/kg); D, DOX (10 mg/kg) plus VEP (5 mg/kg).](image)

![Table 1. Effect of different concentrations of DMDP on DOX-induced DNA single-strand breaks in ascites cells of mice bearing P-388/S and P-388/R.](table)

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Drug dose (mg/kg)</th>
<th>No. of animals</th>
<th>K ( 10^7 ) (ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-388/S</td>
<td>0</td>
<td>18</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>DOX (10)</td>
<td>9</td>
<td>13.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (1)</td>
<td>3</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (10)</td>
<td>9</td>
<td>12.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (30)</td>
<td>9</td>
<td>13.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (60)</td>
<td>9</td>
<td>13.5 ± 2.6</td>
</tr>
<tr>
<td>P-388/R</td>
<td>0</td>
<td>18</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>DOX (10)</td>
<td>9</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (1)</td>
<td>3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (10)</td>
<td>9</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (30)</td>
<td>9</td>
<td>8.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>DOX (10) + DMDP (60)</td>
<td>9</td>
<td>8.8 ± 2.0</td>
</tr>
</tbody>
</table>

\( K \) is given by the formula:

\[ K = \frac{-\ln \text{(fraction of DNA retained on filter)}}{V} \]

\( K \) is the elution rate constant (ml^-1) of DNA; \( V \) is the elution volume in ml.

![Table 2. Effect of DMDP and VEP on the intracellular DOX accumulation and DOX-induced DNA single-strand breaks in ascites cells of mice bearing P-388/S and P-388/R at 2 h after the DOX treatment.](table)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Intracellular DOX (ng/10^8 cells ± SD)</th>
<th>DNA SSBs (K ( 10^7 ) ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX (10)</td>
<td>535 ± 138</td>
<td>237 ± 95</td>
</tr>
<tr>
<td>DOX (10) + DMDP (30)</td>
<td>513 ± 123</td>
<td>422 ± 124</td>
</tr>
<tr>
<td>DOX (10) + VEP (5)</td>
<td>523 ± 110</td>
<td>254 ± 112</td>
</tr>
</tbody>
</table>

![Fig. 5. Effect of DMDP and VEP on the rate of repair of DOX-induced DNA single-strand breaks at 2, 4, and 8 h posttreatment of mice bearing P-388/S (open symbol) and P-388/R (closed symbol) with 10 mg/kg DOX alone (○, A) and in combination with 30 mg/kg of DMDP (○, ●) or 5 mg/kg of VEP (●, □).](image)
of in vitro DNA single- and double-strand breaks induced by DOX was still significantly less than what was observed in the parent DOX-sensitive cells (15, 16). These resistant cells exhibited an elevation of the P-glycoprotein associated with multidrug resistance (31–35). In addition to the cellular factors mentioned above, additional in vivo factors may also contribute to the mechanism of resistance to DOX. This includes drug delivery to the target site which may be influenced by the degree of vascularity of tumor tissue and the extent of drug distribution and metabolism.

Studies with the calcium channel blockers, VEP and DMDP, demonstrated that these agents altered DOX accumulation and retention only in cells which have exhibited the overexpression of P-glycoprotein (36, 37). The concentrations of VEP required for optimal modulation of DOX effects were relatively high and could not be achieved in vivo without unacceptable toxicity (20, 21). The studies reported herein concentrated on the use of DMDP, with a more favorable effect (Figs. 1, 3–5; Table 1) and less toxicity than VEP (Fig. 2), in an effort to delineate mechanisms of in vivo resistance of P-388/R to DOX and to develop approaches for their modulation.

While in vitro studies demonstrated that 8- to 10-fold less DOX accumulation was observed in resistant cells, under in vivo conditions the differences in DOX accumulation between sensitive and resistant cells were consistently 2- to 3-fold at all the doses tested (Fig. 1A). Administration of a therapeutic dose of DOX to mice bearing sensitive P-388 tumor cells (5 mg/kg) and to mice bearing resistant cells (10 mg/kg) that would achieve comparable cellular accumulation of DOX (280 versus 220 ng/10⁶ cells; Fig. 1) produces significant antitumor activity only against sensitive cells (data not shown). An alternative approach was to use calcium channel blockers to restore the in vivo cellular accumulation and retention of DOX in resistant cells to comparable levels found in sensitive cells treated with a therapeutic dose of 10 mg/kg. The data in Fig. 1B and Fig. 2 demonstrate that: (a) DMDP was more effective than VEP in restoring DOX accumulation in P-388/R to a level comparable with P-388/S; and (b) while DMDP was more toxic in vitro than VEP (1 versus 10 μM), it was less toxic in vivo against DBA/2J mice. The MTD doses were 100 and 5 mg/kg for DMDP and VEP, respectively. A dose of 30 mg/kg DMDP can easily achieve the desired 1 to 2 μM plasma concentration which was found to be sufficient for complete restoration of the in vivo DOX accumulation and retention. In contrast, to achieve the desired 5–10 μM plasma VEP, the doses of VEP were lethal to mice. Even though establishment of favorable pharmacological properties of DOX can be restored in vitro and in vivo by DMDP, augmentation of SSBs of DNA in resistant cells was only partial (Table 2).

The data in Figs. 3 and 4 show that significantly less drug-induced DNA single-strand breaks were observed in resistant cells following treatment with a therapeutic dose of DOX. Although DMDP was more effective than VEP in increasing the extent of DNA single-strand breaks, the amount of DNA damage was significantly less than what was observed in sensitive cells at equimolar intracellular concentrations of DOX in both cell lines. Thus, although equimolar concentrations of DOX in resistant and sensitive cells in the presence of DMDP was achieved and maintained, a significant difference in the extent of DNA SSBs was still evident.

Additionally, the repair of DNA damage in sensitive cells in the presence or absence of DMDP was not evident. In contrast, the induced DNA SSBs in resistant cells by DOX was repaired unless DMDP was continuously infused at a steady-state concentration of 1–2 μM. VEP, however, induces less DNA damage and the repair is more rapid in onset. These data suggest that in order to modulate the therapeutic efficacy of cells resistant to DOX, continuous administration of DMDP must be maintained prior to and for a period of time after the administration of DOX.

These in vivo data collectively demonstrate that at least two distinct mechanisms of resistance are operative in these P-388 highly DOX-resistant cells; a reduced intracellular accumulation of DOX resulted from the overexpression of P-glycoprotein and a rapid onset of DNA repair.

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