Possible Link between the Intrinsic Drug Resistance of Colon Tumors and a Detoxification Mechanism of Intestinal Cells

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ABSTRACT

The insensitivity of colon tumors to various anticancer agents was studied in vitro. The activity of Adriamycin (ADR) in certain colon tumor cell lines was potentiated by the calcium channel blocker verapamil (VER). In the HCT-8 human colon adenocarcinoma cell line, VER potentiation of the activities of ADR and the anthrapyrazole CI-937 appeared to be related to its ability to enhance the net accumulation of both drugs and inhibit their efflux. VER, which potentiated ADR activity in HCT-8 cells by 4-fold, caused a 3.5-fold stimulation of ADR accumulation and a 3.5-fold inhibition of ADR efflux, when compared to non-VER-treated cells. The low level of VER potentiation of CI-937 activity in HCT-8 cells (1.4-fold) was also reflected in CI-937 transport studies which demonstrated a 1.5-fold enhancement of CI-937 accumulation and a 1.4-fold stimulation of its efflux. VER was also found to stimulate ADR activity and accumulation in a normal small intestinal crypt cell line (IEC-6). The mechanism of drug efflux was examined in HCT-8 cells. Agents known to increase the permeability of the plasma membrane did not alter ADR accumulation or its efflux in HCT-8 cells unless these same agents were also capable of interacting with the lysosome. Tween 80 and the lysosomotropic detergent Triton WR-1339 as well as proton ionophores and lysosomotropic amines all stimulated ADR uptake and/or inhibited its efflux from HCT-8 cells. ADR efflux was also partially blocked by cytochalasin B. Based on these observations, we suggest that at least part of the inherent drug resistance of colon tumor cells results from the retention of an enhanced drug efflux mechanism which is found in normal intestinal epithelium where this property may provide protection from plant alkaloids and other xenobiotic agents ingested in the diet.

The mechanism of this drug efflux from HCT-8 cells may involve drug partitioning into acidic vesicles within the cell and their subsequent release from these cells by exocytosis.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer death in the United States (1). It is estimated that 140,000 new cases of colorectal cancer will occur and that 60,000 individuals will die from this disease in 1986. Despite extensive studies on chemotherapeutic approaches to the treatment of this disease, the static survival statistics for colorectal cancer during the past three decades demonstrate the lack of significant progress in the treatment of this disease.

Fojo et al. (2) recently reported that the mdrl gene, which appears to be responsible for MDR in tissue culture systems (3), was also elevated in colon tumor tissue as well. Several other studies have examined the phenomenon of inherent drug resistance in cancer and have observed that VER, which potentiates drug activity in MDR cells, can also increase drug sensitivity in certain intrinsically drug-resistant tumor cells as well (4-6). In the present study, we explored the possibility that VER might also alter anthracycline and anthrapyrazole activity in colon tumor cells. We found that VER potentiates the activity of ADR and the anthrapyrazole, CI-937, in a manner which parallels its effects on drug accumulation and efflux and suggest that this enhanced efflux may be one factor involved in the inherent resistance of certain colon tumors to chemotherapy.

In addition, we present data which lend support to the recent hypotheses proposed by Beck (7) and by Sehested et al. (8) that the endosome-lysosome system is involved in this accelerated drug efflux process.

MATERIALS AND METHODS

Chemicals. The anthrapyrazoles CI-937, CI-941, CI-942, and [14C]-CI-937 (specific activity, 83.1 μCi/mM) were obtained from the Warner-Lambert Co., Ann Arbor, MI (9). RPMI 1640 medium, nutrient F-12 medium, MEM-E, and MEM nonessential amino acids were purchased from KC Biological, Inc., Lenexa, KS. FBS was purchased from HyClone Laboratories, Logan, UT. Adriamycin, daunomycin, Tween 80, Triton WR 1339, amphotericin B, filipin, deoxycholate, verapamil, chloroquine, monensin, nigericin, cytochalasin B, monodansylcadaverine, and CCCP were all purchased from Sigma Chemical Co., St. Louis, MO.

Cell Cultures. C-38 and C-26 murine colon tumor cell lines, obtained from Southern Research Institute, Birmingham, AL, were grown in MEM-E supplemented with 20% FBS, 16 mM N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid and 8 mM morpholinopropane sulfonic acid. HCT-8 human colon adenocarcinoma cells were grown in RPMI 1640 medium supplemented with 10% FBS. WiDr, LoVo, and LS-174 human adenocarcinoma cell lines and IEC-6 normal rat intestinal epithelial cells were obtained from the American Type Culture Collection, Rockville, MD. IEC-6 cells were cultured in Dulbecco's modified Eagle's medium containing 5% FBS and 0.1 μg/ml of insulin. WiDr cells were grown in MEM-E with 10% FBS, nonessential amino acids, and 50 μg/ml of gentamycin. LoVo cells were grown in nutrient F-12 medium supplemented with 10% FBS and 50 μg/ml of gentamycin. LS-174T cells were grown in MEM-E with 10% FBS.

The effect of anthracyclines and anthrapyrazoles alone or in combination with VER on the growth of the colon tumor cell lines was determined as previously described (10). Cells (2.5 × 10⁴ cells/ml) were added to Linbro 24-multwell tissue culture plates (Flow Laboratories, Inc., McLean, VA) 24 h prior to drug addition. After 72 h of drug exposure, cells were trypsinized and counted with the use of a Model ZC Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Viability was determined by trypan blue exclusion.

Transport Studies. ADR accumulations into HCT-8 and IEC-6 cells were measured in the appropriate culture medium for each cell line at 37°C. Cells (5 × 10⁵) were incubated in 9.2 μM ADR in the presence or absence of 5.0 μg/ml of VER. Incubations were terminated as described by Kessel and Wilberding (11) and washed once in ice-cold saline. ADR was extracted with 50% ethanol-0.3 N hydrochloric acid, and its content was measured spectrofluorometrically using an Aminco SPF-500 spectrophotometer (SLM Instruments, Inc., Urbana, IL) following procedures described by Skogvagard (12).

For studies on the outward transport of ADR from HCT-8 and IEC-6 cells, cells (5 × 10⁵) were preloaded for 4 h at 37°C as described for uptake. Cells were then equilibrated to 4°C and washed twice in ice cold medium. ADR-free medium with or without 5.0 μg/ml of VER was then added to each culture, and drug efflux was determined at 37°C as previously described (12).
INTRINSIC DRUG RESISTANCE

To determine if the effect of VER on ADR efflux was related to an effect on ADR metabolism, both IEC-6 and HCT-8 cells were incubated for 2 h with ADR in the presence or absence of 5 µg/ml of VER. Chloroform:methanol (2:1) extracts of drug-loaded cells were prepared as previously described (13) and chromatographed on silica gel plates in chloroform:methanol:water (80:20:3). Quantitation of ADR and its metabolites was determined according to the method of Cradock et al. (14). As expected, the extracted material was greater than 90% ADR, and this was not altered by the addition of VER.

The uptake and efflux of CI-937 into HCT-8 cells were carried out under similar conditions as described for ADR transport. CI-937 accumulation (final concentration in the medium, 10.3 µM; specific activity, 83.1 µCi/mmol) was measured in the presence or absence of 5 µg/ml of VER at 37°C. Cell pellets were dissolved in N NaOH at 70°C for 1 to 2 h, neutralized, and counted in Beckman Ready-Solv cocktail by liquid scintillation counting.

RESULTS

Verapamil Potentiation of Anthracycline and Anthrapyrazole Activities. ADR activity was determined in the presence or absence of VER in 6 different colon tumor cell lines and the normal rat intestinal crypt cell line, IEC-6 (Table 1). At non-cytotoxic concentrations of VER, ADR activity was potentiated in 5 of the 7 cell lines tested by as much as 17-fold. VER concentrations greater than 5.0 µg/ml were toxic to all of these cell lines. VER potentiated ADR cytotoxicity in LoVo and LS-174T cells by 3-fold, in colon-26 and HCT-8 cells by 4-fold, and in the IEC-6 cells by 17-fold, while it had no effect on ADR activity in colon-38 or WiDr cells. In addition to ADR potentiation, VER also potentiated the activities of daunomycin and 3 anthrapyrazoles in HCT-8 cells (Table 2). Daunomycin activity was increased in these cells by over 4-fold. VER potentiated the activity of the anthrapyrazole, CI-941, by over 4-fold but produced only a 1.5-fold increase in the activities of CI-937 and CI-942 in HCT-8 cells.

Transport of Adriamycin and CI-937. The transport of ADR in the presence or absence of VER was examined in HCT-8 human colon adenocarcinoma cells and in the IEC-6 cell line (Figs. 1 and 2). After 6 h of incubation in medium containing ADR, VER stimulated the net accumulation of ADR in both HCT-8 and IEC-6 cells by greater than 3-fold (Fig. 1). The

Table 1 Effect of verapamil on Adriamycin cytotoxicity in IEC-6 and colon tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>± Verapamil (5.0 µg/ml)</th>
<th>ID₅₀ (nM)*</th>
<th>Degree of potentiation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEC-6</td>
<td>-</td>
<td>87.5 ± 4.8</td>
<td>17.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Colon-38</td>
<td>-</td>
<td>125 ± 9.6</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>132 ± 12.1</td>
<td></td>
</tr>
<tr>
<td>WiDr</td>
<td>-</td>
<td>94 ± 4.0</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>97 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>HCT-8</td>
<td>-</td>
<td>210 ± 22.1</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>53 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Colon-26</td>
<td>-</td>
<td>237 ± 10.1</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>60 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>LoVo</td>
<td>-</td>
<td>57 ± 3.3</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>LS-174T</td>
<td>-</td>
<td>63 ± 4.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were grown for 72 h at 37 °C in the presence of Adriamycin ± 5.0 µg/ml of verapamil.

* Degree of potentiation = \frac{ID₅₀ - verapamil}{ID₅₀ + verapamil}.

Average ± SD of two experiments, each performed in triplicate.

Table 2 Effect of verapamil on anthrapyrazole and anthracycline activity in HCT-8 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>± Verapamil (5 µg/ml)</th>
<th>ID₅₀ (nM)*</th>
<th>Degree of potentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>-</td>
<td>210 ± 22.1</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>53 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>Daunomycin</td>
<td>-</td>
<td>129 ± 4.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>39 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>CI-937</td>
<td>-</td>
<td>304 ± 12.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>221 ± 29.0</td>
<td></td>
</tr>
<tr>
<td>CI-941</td>
<td>-</td>
<td>87 ± 5.4</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>CI-942</td>
<td>-</td>
<td>1003 ± 88.5</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>626 ± 32.5</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were grown as described in Table 1 in the presence or absence of the indicated compound.

* Average ± SD of two experiments, each performed in triplicate.
efflux of ADR was examined in cells preloaded for 4 h with ADR. In concert with its effect on ADR accumulation, VER inhibited the high level of ADR efflux in both HCT-8 cells and IEC-6 cells by more than 3-fold (Fig. 2).

The transport of the anthrapyrazole CI-937 was also determined in HCT-8 cells (Figs. 3 and 4). In accord with VER’s potentiation of CI-937 cytotoxicity in HCT-8 cells (see Table 2), VER increased the accumulation of CI-937 (Fig. 3) and inhibited its efflux (Fig. 4) by approximately 1.5-fold.

Studies on the Mechanism of Drug Efflux. Several membrane active agents were compared with VER for their effects on ADR accumulation and efflux in HCT-8 cells (Figs. 5 and 6). At concentrations that were nontoxic in growth inhibition assays (data not shown), the nonionic detergents Triton WR-1339 and Tween 80 stimulated ADR uptake and inhibited its efflux to the same extent as observed for VER. However, at concentrations that caused an approximately 20% inhibition of cell growth over 72 h (data not shown), the polyene antibiotics, amphotericin B and filipin, and the ionic detergent, deoxycholate, had no effect on ADR accumulation and/or efflux (Figs. 5 and 6). Lower nontoxic concentrations of these 3 compounds were similarly without effect on ADR transport (data not shown).

In view of the similarity of results between VER and the lysosomotropic detergent, Triton WR-1339, other agents known to interact with the lysosome were also tested for their effect on ADR accumulation and efflux in HCT-8 cells (Figs. 7 and 8). The proton ionophores nigericin and monensin were as effective as VER in both increasing ADR uptake (Fig. 7) and inhibiting its efflux (Fig. 8). The other proton ionophore, CCCP, was somewhat less effective in potentiating ADR uptake (Fig. 7). The lysosomotropic amines chloroquine and monodansylcadaverine also potentiated cellular accumulation of ADR and/or blocked its exodus (Figs. 7 and 8). Cytochalasin B, which can disrupt the microfilamentous component of the cytoskeleton, also inhibited ADR efflux from HCT-8 cells (Fig. 8). Neither methylamine or ammonium chloride, however, affected ADR accumulation or efflux in these cells (Figs. 7 and 8). Reducing the concentration of ADR in the incubation me-
Several recent studies have observed that VER, which potentiates drug activity in acquired or pleiotropically drug-resistant tumors, can also increase drug sensitivity in inherently drug-resistant colon tumor cells (9-11). As in MDR cells, fluorescent studies by Chauffert et al. (4) suggested that VER as well as other pharmacological drugs could potentiate ADR activity in a rat colon tumor cell line by inhibiting its exodus from these cells. In the present study, we demonstrated that VER could potentiate ADR activity in a number of colon tumor cell lines and that, at least for the HCT-8 human colon tumor line, this drug-potentiating activity was related to the ability of VER to inhibit drug efflux. ADR efflux from HCT-8 cells appeared to be significantly enhanced over that found for other drug-sensitive cell lines such as P388S cells (10). The degree of potentiation of ADR and CI-937 activities by VER in HCT-8 cells was the same or similar to VER’s effect in blocking either drug from being transported out of these cells (Table 2; Figs. 2 and 4). While there are undoubtedly numerous factors which contribute to the overall intrinsic drug resistance of colon tumors, our data along with previous observations (4-6) suggest that this intrinsic resistance phenomenon, like acquired MDR, may, in part, be related to the ability of the tumor to transport drug out of the cell. This enhancement of drug efflux in HCT-8 may, in fact, be a property of normal intestinal epithelial cells as well. In a carefully characterized normal IEC-6 rat intestinal epithelial cell line (15), we demonstrated that VER potentiated ADR activity in these cells by increasing ADR uptake and inhibiting its efflux (Table 1; Figs. 1 and 2). The enhanced outward transport of agents such as ADR in normal intestinal cells might be one of the mechanisms involved in the protection of intestinal epithelium from plant alkaloids and other xenobiotic agents ingested in the diet. Perhaps many colon tumors retain this property.

Fojo et al. (2) observed that the mdrl gene was overexpressed in some but not all human colon tumors and adjacent normal colon tissue. Even in the limited number of colon cell lines we studied, we observed that VER did not alter ADR activity in two of six cell lines (Table 1), indirectly indicating that these two cell lines did not efflux appreciable amounts of ADR. Presumably those colon tumors which lack this efflux “pump” have some other mechanism to afford protection against xenobiotics. Labeling surface glycoproteins by treating cells with neuraminidase and galactose oxidase-[3H]borohydride as described by Beck et al. (16), we could not detect the presence of a glycoprotein with a molecular weight of 140,000 to 170,000 in these cell lines (data not shown), but this procedure may be too insensitive to detect P-gp in these cells, and more sensitive complementary DNA probes may be necessary.

Since we could not detect P-gp by surface labeling and polyacrylamide gel electrophoresis in any of the cell lines studied including HCT-8 cells, we cannot be certain that the drug efflux demonstrated in HCT-8 cells is due to the same mechanism as that observed in MDR cells. However, the evidence from other studies (4-6) and from the present study for VER potentiation of drug activity and blocking of drug efflux in colon tumor cells as well as the finding that the mdrl mRNA is amplified in many colon tumors (1) suggests that P-gp is probably responsible for the efflux of ADR from HCT-8 cells. What is still not clear, however, is the mechanism by which P-gp removes drugs from these cells. Several groups have proposed that the decreased ability of MDR cells to retain certain drugs was due to an active efflux “pump” (13, 17, 18), and based on its primary sequence, its apparent transmembrane location, its potential nucleotide binding sites, and its homology with bacterial transport proteins (19), Chen et al. (19) also concluded that P-gp functioned as an energy-dependent drug efflux pump. Cornwall et al. (20) recently reported that vesicles from MDR cells bound considerably more radio-labeled vinblastine than did vesicles from drug-sensitive cells. Moreover, they also demonstrated that VER inhibited vinblastine photoaffinity labeling of P-gp (21), and from these and other observations concluded that VER and other modifiers of drug resistance act by inhibiting the binding of drugs such as vinblastine to P-gp which is somehow involved in their efflux by an energy-dependent mechanism.

Our data, however, lend additional support not to the active
pump theory, but rather to a new hypothesis independently described by Sehested et al. (8) and by Beck (7). They suggest that the lysosome or an associated acidic compartment in the MDR cell is involved in drug exodus and that drugs such as ADR or vinblastine, which are weak bases, can become entrapped in these acidic compartments by protonation. These acidic drug-containing vesicles then migrate to the plasma membrane where they fuse and extrude their contents to the outside. Beck (7) suggests that, rather than serving as an efflux pump, P-gp may function at the plasma membrane to alter membrane turnover or vesicular trafficking, or alternatively, to serve to transport certain drugs across the plasma membrane or into acidic vesicles.

Our data demonstrating inhibition of ADR efflux from HCT-8 cells by lysosomotropic amines, proton ionophores, and cytochalasin B (Fig. 8) support this notion of a lysosomal involvement in drug efflux. Our findings for HCT-8 cells in the present study are, indeed, virtually identical with those we have observed in our P388 MDR model.3 These findings are also in agreement with those recent observations by Zamora and Beck (22) and by Shiraishi et al. (23) demonstrating that lysosomotropic amines, such as chloroquine and other agents known to interact with the lysosome, could reverse drug resistance to anthracyclines and Vinca alkaloids in MDR cells. In addition, in accord with the findings of Tsuruo and Iida for MDR cells (24), we observed that cytochalasin B, an agent that has several cellular effects including inhibition of microfilament function and secretion (25, 26), also partially blocked the outward transport of ADR from HCT-8 cells (Fig. 8).

Beck (7) suggests that cytochalasin B might inhibit drug efflux by impairment of membrane flow, thereby inhibiting the movement of drug-containing vesicles to the plasma membrane. It should be pointed out, however, that cytochalasin B as well as the proton ionophores and lysosomotropic agents can all have multiple effects on cellular functions including alterations in plasma membrane functions. Several previous studies on MDR, in fact, have demonstrated that agents that interact with the plasma membrane can also act as potentiators of drug activity in MDR cells (27–29), and we have recently found that at least for the detergent, Tween 80, this potentiation was due to blocking of the enhanced drug efflux in resistant cells (10). In the present study, Tween 80 and lysosomotropic detergent, Triton WR-1339, also increased the net accumulation of ADR in HCT-8 cells and inhibited its efflux to a level comparable with VER (Figs. 5 and 6). At slightly toxic (approximately 20% reduction in cell growth in growth inhibition assays) as well as nontoxic concentrations (data not shown), neither amphotericin B, filipin, nor the ionic detergent, deoxycholate, altered ADR uptake and/or efflux from HCT-8 cells (Figs. 5 and 6), yet both of the polyene antibiotics have been demonstrated to cause pore formation in cell membranes at concentrations used in the present study (30). It appears, therefore, that the ability of compounds to interact with plasma membrane lipids may not alone be a sufficient criterion for inhibiting drug exodus. Perhaps another prerequisite for inhibiting drug efflux involves the lysosome. The nonionic detergent Triton WR-1339 has been found to preferentially accumulate in the lysosome of many cells (31), and it is possible that the other nonionic detergent, Tween 80, may also be sequestered there as well. If the hypothesis of Beck (7) and Sehested et al. (8) is correct, then the primary effect of these detergents may not be at the plasma membrane of drug-resistant cells, but rather in the lysosome where their concentration results in alterations in lysosomal membrane phospholipids and thereby modifies lysosomal function. This possibility is supported by our observations for ADR transport in HCT-8 cells in the presence of proton ionophores and lysosomotropic amines (Figs. 7 and 8), both of which are known to alkalize acidic vesicles and alter lysosomal function (32, 33). However, if the lysosomal system is somehow involved in drug efflux from certain inherently resistant tumor cells, it is unclear why primary amines such as ammonium chloride and methylamine have no effect on ADR accumulation or exodus from HCT-8 cells (Figs. 7 and 8) or altered vincristine activity in MDR cell lines (34). Primary amines, like proton ionophores and agents such as chloroquine, have been shown to alkalize lysosomal vesicles (Ref. 21; Footnote 3), although several recent studies have noted different cellular responses to primary amines versus proton ionophores or chloroquine (22–24), perhaps because of the varying degrees of efficiency by which charged primary amines are transported into certain cells or cellular compartments.

In conclusion, our data suggest that an enhanced drug efflux may be an important factor in the overall level of inherent drug resistance in certain colon tumors. This elevated efflux may be a property of normal intestinal epithelium where it provides some protection from toxins and carcinogens ingested in the diet. Our data for modifiers of ADR exodus from HCT-8 cells lend considerable support to the hypothesis for MDR cells that endosomal/lysosomal vesicles may be involved in drug exodus and, therefore, drug resistance.

**REFERENCES**


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