Suppression of P-Glycoprotein Expression and Multidrug Resistance by DNA Cross-Linking Agents

Michael A. Ihnat, Jean P. Lariviere, Amy J. Warren, Nicole La Ronde, Johanna R. N. Blaxall, Karana M. Pierre, Bruce W. Turpie, and Joshua W. Hamilton

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755-3835, and Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire 03755-0001

ABSTRACT

Overexpression of the trans-membrane drug efflux pump P-glycoprotein is one of the major mechanisms by which cancer cells develop multidrug resistance. We demonstrated previously that noncytotoxic doses of various genotoxic chemicals, particularly DNA cross-linking agents, preferentially altered expression of inducible genes. These effects occurred principally at the transcriptional level and were closely correlated temporally with DNA damage. Because the mdrl gene coding for P-glycoprotein has been reported to be highly inducible, we were interested in the effects of genotoxic cancer chemotherapy agents on its expression. We report that the DNA cross-linking agent mitomycin C significantly suppressed mRNA and protein expression of P-glycoprotein and decreased the rate of drug efflux. Mitomycin C pretreatment also significantly increased the sensitivity of cancer cells to subsequent killing by the P-glycoprotein substrate doxorubicin, decreasing the ED₅₀ by 5- to 10-fold. Suppression of P-glycoprotein expression was also observed with subtoxic doses of the DNA cross-linking agents cisplatin, BMS181174, and chromium(VI). These effects occurred in both human and rodent cell lines; in cell lines derived from colon, breast, leukemia, neuroblastoma, and hepatoma tumors; and under both monolayer and “spheroid” culture conditions. These results suggest the basis for novel clinical cancer chemotherapy regimens aimed at drug-resistant tumors, in which a subchemotherapeutic dose of a DNA cross-linking agent is used to modulate the multidrug resistance phenotype prior to treatment with a second cytotoxic agent.

INTRODUCTION

One of the major obstacles to the successful treatment of cancer is the development of MDR by the tumor (1). The MDR phenotype is characterized by the ability of the tumor cells to be cross-resistant to the actions of a wide variety of structurally and functionally unrelated drugs and other xenobiotics. A number of different mechanisms for MDR have been identified or proposed, including alterations in drug uptake, drug efflux, drug metabolism, DNA repair, and apoptotic pathways in response to drug-induced DNA damage (2). One of the principal mechanisms by which human tumors develop the MDR phenotype is overexpression of Pgp (1-3). Pgp is an ATP-dependent trans-membrane drug efflux pump, the action of which can significantly lower the intracellular concentration of cytotoxic agents (1-3). Pgp is expressed at lower levels in a variety of normal tissues, including liver, kidney, and colon, and is overexpressed in tumors from those tissues as well as those that do not normally express Pgp, such as breast and lymphoid tissues (1, 4, 5). This overexpression of Pgp can occur spontaneously, as in the case of liver, kidney, and many liquid tumors (1, 4-6), or can arise after multiple rounds of chemotherapy in a previously Pgp-negative tumor, as in the case of breast cancer (5, 7). Substrates for the Pgp pump include many of the most clinically used anticancer agents, including the epipodophyllotoxins, Vinca alkaloids, anthracyclines, and Taxol and its derivatives (3).

Interestingly, there appears to be a striking difference in the mechanism for Pgp overexpression in human and animal tumors in vivo as compared to tumor cells that have been selected for drug resistance in cell culture. In culture, Pgp overexpression has been reported to occur primarily as a result of amplification and/or mutation of the mdrl gene, which codes for Pgp, whereas amplification or mutation of the mdr gene occurs rarely in human tumors in vivo (8). The mechanism for up-regulation of Pgp expression in tumors in vivo is still unknown. Current efforts to reverse the Pgp-based MDR phenotype have focused principally on competitive inhibitors of the Pgp pump, using agents such as the Food and Drug Administration-approved drugs verapamil and nifedipine; cyclosporin; and analogues such as PSC833, tamoxifen, trifluoperazine, and erythromycin (9). However, effective inhibition of Pgp requires relatively constant, stoichiometric concentrations of these drugs, typically at doses that exceed their pharmacological doses, and these drugs each have significant toxicity profiles (9). Clinical trials...

1 The abbreviations used are: MDR, multidrug resistance; MMC, mitomycin C; Pgp, P-glycoprotein; FBS, fetal bovine serum; TBS, Tris-buffered saline.
Fig. 1 Cytotoxicity of MMC in H4IIE cells. Cells were split and seeded to 25% confluency, and upon reaching 30% confluency, they were treated with MMC for 4 h in serum-free medium. Cytotoxicity was measured by a colony-forming assay as described in “Materials and Methods.” Data points, means (bars, SD) of measurements from independent samples (n = 3–4). The highest dose that produced no cytotoxicity was determined to be 0.1 μM MMC, and this dose was used in all subsequent experiments. Bars are shown where they are larger than the symbol.

with several of these agents have been largely unsuccessful to date (9).

We have taken a unique approach to the problem of Pgp overexpression. Our laboratory has demonstrated previously that DNA-damaging agents, including simple alkylating agents such as the alkyl methanesulfonates and alkylisoureas; agents that induce bulky DNA lesions such as benzo(a)pyrene and aflatoxin B; and DNA cross-linking agents such as cisplatin, chromium(VI), and MMC, all exhibit strong preferential effects on the expression of inducible genes as compared to constitutively expressed genes (10–15). These effects occur after a single, nonovertly toxic dose of carcinogen, are principally a result of changes in the transcriptional levels of the targeted genes, and are strongly correlated both with the specific type and duration of DNA damage (10, 12). The promoter of the affected gene appears to represent the target for these effects, and chromatin structure within the promoter is also likely to be important for the response (11, 14, 16, 17). These effects occur both in vivo and in cell culture and with a wide variety of inducible genes, the regulatory mechanisms of which differ widely (12, 18–21).

Our initial impetus for these studies was to determine whether chemical carcinogens "target" their genotoxic effects to specific genes as a component in the initiation process of carcinogenesis. However, we were also interested in whether this phenomenon could be used to improve cancer chemotherapy regimens using specific DNA-damaging agents. Pgp is coded for by the mdr1 gene in humans (4) and rats (Ref. 22; the rat gene is also referred to as mdr1b; Ref. 23), which is an inducible gene, the expression of which can be modulated by drugs, hormones, and other stimuli (23–25). Thus, we postulated that the mdr1 gene would respond at the transcriptional level to carcinogen-induced DNA damage as predicted in our targeting model. We first tested this idea using the chemotherapeutic drug and DNA cross-linking agent, MMC. We demonstrated previously that MMC exhibited the strongest preferential effects on the expression of a variety of inducible genes to date (17, 26). In addition, MMC is used clinically as a first-line chemotherapy agent for anal and certain lung cancers and as a secondary agent in end-stage breast, colon, gastric, and pancreatic cancers as well as chronic myeloid leukemia. As with cisplatin, the preferential killing of cancer cells by MMC (and its cytotoxicity) is thought to be due to its ability to damage DNA, specifically by forming DNA interstrand cross-links (27–29). We examined whether MMC would preferentially modulate Pgp expression in our single, low-dose model. We report that MMC and other DNA cross-linking agents are potent down-regulators of Pgp expression, leading to reversal of the MDR phenotype in a variety of human and rat tumor cell lines.

MATERIALS AND METHODS

Measurement of mdr1 Steady-State mRNA Expression.

All cell lines were maintained at 37°C in 5% CO2 using α-MEM (H4IIE, SK-HEP-1, and U87), Iscove’s MEM (MDA-MB-435), or RPMI 1640 (K562) with 12% serum (H4IIE; horse serum; FBS, 1:1) or 10% FBS (all other lines) with 50 units/ml peni-
cillin and 50 μg/ml streptomycin. Total RNA was isolated, and mdr1 mRNA levels were quantified essentially as described previously (18). Briefly, at each time point, the medium was removed; the cells were washed in PBS; 4 ml of guanidine isothiocyanate-2-mercaptoethanol buffer were added; and the cell lysate was scraped, collected, and frozen at −20°C. Lysate was centrifuged on a cesium chloride-EDTA gradient, and the resulting RNA pellet was redissolved in water, quantified by spectrophotometry, and frozen at −75°C. Steady-state mRNA levels were measured with a 32P-end-labeled gene-specific mdr1 synthetic oligonucleotide cDNA probe by solution hybridization as described (18). The rat mdr1 probe is a unique and specific 24-mer with the sequence 5'-AAATAGAGAAGAAGACGGTAAGCA-3', which is complementary to nucleotides 1091-1114 of the rat mdr1/mdrlb mRNA sequence, as published previously (22).

Measurement of Pgp Protein Expression. Following drug treatment, all cells were washed with cold PBS, and then a modified Garrison’s buffer (20 mM Tris-HCl, 2 mM EDTA, 1 mM EGTA, and 0.1% digitonin) was added to remove the cytosolic fraction. A 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid-based buffer consisting of 10 mM Tris, 2.5% glycerol, 0.5% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, 0.5% SDS, and 0.1% bromphenol blue was then added to the cells to remove and collect the plasma membrane and nuclear fraction (30). Protein concentrations were determined by a standard absorbance assay (bicinchoninic acid assay; Pierce Chemical Co.). Western (immunoblot) analysis of Pgp protein levels was performed essentially as described previously (31). Five or 10 μg of the plasma membrane fraction were electrophoresed using a 7.5% SDS-polyacrylamide gel in a minigel apparatus (Bio-Rad). Gels were then transferred to 0.2-μm nitrocellulose membranes using a semidry transfer apparatus (Bio-Rad) and blocked with 5% Signam instant nonfat dry milk in TBS at 4°C overnight. A primary antibody specific for Pgp, monoclonal C219 from Signet Biotech (diluted to 1 μg/ml in TBS), was then added for 1 h at 4°C. The blot was then washed three times in 1% Carnation instant nonfat dry milk-TBS-0.5% Tween. The secondary antibody, a goat antimouse IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology), was added at a 1:2000 dilution in TBS for 30 min at room temperature. The membrane was again rinsed three times in TBS-Tween and then once with TBS. The membrane was then exposed to a chemiluminescent horseradish peroxidase substrate (ECL; Amersham Corp.). Autoradiographs were digitally scanned, and the densities of specific bands were quantified using IP Lab Gel densitometry software (Molecular Dynamics).

Measurement of Drug Efflux. Following MMC treatment, H4IIE cells were incubated with 1 μM rhodamine 123 in PBS for 1 h at 37°C. Drug-loaded cells were then pelleted and resuspended in either warm PBS or warm PBS containing 16.7 μM verapamil. An aliquot of cells was then taken every 4 min up to 48 min after the wash, and flow cytometric analysis was performed using a FACScan II flow cytometer (Becton Dickinson). Ten thousand cells were gated with respect to forward and reverse scatter, and the mean fluorescence at 530 nm was measured. Rate constants were calculated from the linear portion of the initial decay curve, which measured the decrease in arbitrary fluorescence units of rhodamine over time.

Cytotoxicity Studies. Monolayer cells were plated at a density of 2500 cells/96-well plate. Upon attachment, cells were treated with MMC, and 24 h later they were treated with various doses of doxorubicin (Adria-Pharmacia) for 1 h in incomplete medium followed by replacement with complete medium. At 72 h after initiation of doxorubicin treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-phenazine methosulfate (Cell-Titer assay, Promega Biotech) were added to each well. Absorbance (490–650 nm) was taken at hourly time points after substrate addition using a Thermo-max reader (Molecular Devices). Three
Mitomycin C Suppression of Multidrug Resistance

**Table 1** Summary of effects of MMC on Pgp expression in various human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin and tumor type</th>
<th>Pgp level</th>
<th>Maximal effect</th>
<th>% Pgp expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-435</td>
<td>Human breast carcinoma</td>
<td>++</td>
<td>96 h</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>HT-29</td>
<td>Human colon carcinoma</td>
<td>++</td>
<td>72 h</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>SK-HEP-1</td>
<td>Human hepatoma</td>
<td>+</td>
<td>24 h</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>U87</td>
<td>Human neuroblastoma</td>
<td>++ +</td>
<td>24 h</td>
<td>55 ± 1</td>
</tr>
<tr>
<td>K562</td>
<td>Human chronic myeloid leukemia</td>
<td>+</td>
<td>24 h</td>
<td>74 ± 1</td>
</tr>
</tbody>
</table>

- Relative Pgp expression of untreated cells on a scale of 0 (no detectable expression); ± (barely detectable); or +, + +, or + + +, indicative of moderate, high, and very high expression of Pgp, respectively, using H4IIE control expression as a standard (“Materials and Methods” and in Figs. 1 and 2).

- Time point of maximal suppression of Pgp expression (h after MMC treatment) as determined in a time course experiment similar to that shown in Fig. 2.

- Pgp expression at the point of maximal suppression, expressed as a percentage of the control (time “0” h) value for each experiment, as described in “Materials and Methods” and in Fig. 2.

RESULTS

H4IIE rat hepatoma cells were chosen initially as a useful experimental system, because they express Pgp constitutively at relatively high levels. The *mdrl* gene is also inducible in these cells, and they have not been selected in culture for drug resistance. H4IIE cells were maintained between 25 and 80% confluence for all experiments because we had previously observed significant changes in Pgp expression when these cells were plated below 25% or were allowed to grow to greater than 80% confluence. It had also previously been reported that several other phenotypic changes occur in these cells, including changes in cytochrome P-450 and PEPCK expression, when they are grown outside these parameters (32, 33). Cells were treated with 0.1 μM MMC for 4 h, a treatment that caused no overt cytotoxicity and did not significantly decrease cell survival, as determined by a clonogenic assay (Fig. 1). The drug was removed after 4 h to mimic the first-pass kinetics and rapid clearance of MMC in vivo (34). This treatment was used in all subsequent experiments. MMC treatment of H4IIE cells caused a significant decrease in *mdrl* steady-state mRNA expression (Fig. 2). Levels decreased to less than half of the control value between 12 and 24 h after treatment with the drug. This is similar to the effects of MMC on other inducible genes, particularly the hormone-inducible gene, PEPCK, which is also suppressed in a similar fashion both in H4IIE cells and in chicken embryo liver in vivo (16, 17). In contrast, this treatment had no effect on the constitutive expression of β-actin mRNA, similar to what had been observed previously (10, 11, 13).

MMC treatment also caused a significant suppression in Pgp protein levels over a 96-h time course, as measured by Western blot analysis (Fig. 3B). After a single 4-h MMC treatment, there was a rapid decrease in Pgp levels with an apparent decay very similar to the reported half-life of 18 h for Pgp protein (35). Maximal suppression occurred between 72 and 84 h after the first drug treatment, and Pgp levels appeared to be returning to control levels at 96 h (Fig. 3B). This initial time course was not extended further, because these cells had reached confluence by the 96-h time point. To examine the longer-term effects of MMC on Pgp levels, the MMC-treated cells were split at 80% confluence (96 h), and after obtaining stable cultures at...
Table 2 Summary of effects of various DNA-alkylating agents on Pgp expression in the human MDA-MB-435 breast carcinoma and rat H4IIE hepatoma cell lines

Cells grown in monolayer were treated with a single administration of each drug for 4 h and analyzed for Pgp expression as described in “Materials and Methods” and in Figs. 1 and 2. The rate of rhodamine 123 efflux following treatment was measured by flow cytometry as described in “Materials and Methods” and compared to untreated cells and untreated cells loaded with verapamil during efflux.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Maximal effect</th>
<th>% Pgp expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat H4IIE</td>
<td>0.1 μM MMC</td>
<td>72 h</td>
<td>24 ± 8%</td>
</tr>
<tr>
<td></td>
<td>0.2 μM cisplatin</td>
<td>72 h</td>
<td>25 ± 5%</td>
</tr>
<tr>
<td></td>
<td>1 μM chromium (VI)</td>
<td>72 h</td>
<td>27 ± 14%</td>
</tr>
<tr>
<td></td>
<td>0.3 μM doxorubicin</td>
<td>36 h</td>
<td>519 ± 217%</td>
</tr>
<tr>
<td></td>
<td>10 μM streptozotocin</td>
<td>(60 h)</td>
<td>(140 ± 15)</td>
</tr>
<tr>
<td>Human MDA-MB-435</td>
<td>0.1 μM MMC</td>
<td>72 h</td>
<td>7 ± 4%</td>
</tr>
<tr>
<td></td>
<td>0.2 μM cisplatin</td>
<td>72 h</td>
<td>16 ± 2%</td>
</tr>
<tr>
<td></td>
<td>0.001 μM BMS181174</td>
<td>72 h</td>
<td>26 ± 3%</td>
</tr>
</tbody>
</table>

* Time point of maximal change in Pgp expression (h after drug treatment) as determined in a time course experiment similar to that shown in Fig. 2. Values in parentheses represent point of maximal effect, which was not statistically different from controls.

** Pgp expression at the point of maximal change from control, expressed as a percentage of the control (time “0” h) value for each experiment, as described in “Materials and Methods” and in Fig. 2.

* Significantly different from controls at P < 0.05 using Student's t test.

Table 3 Effects of MMC on the rate of Pgp substrate drug efflux in H4IIE cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of efflux (% control)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(100)</td>
</tr>
<tr>
<td>16.7 μM verapamil</td>
<td>86 (56 and 11)</td>
</tr>
<tr>
<td>66 h MMC pretreatment</td>
<td>67 (75 and 57)</td>
</tr>
<tr>
<td>90 h MMC pretreatment</td>
<td>56 (66 and 42)</td>
</tr>
</tbody>
</table>

* Data represent the average rate of efflux expressed as a percentage of the control rate in untreated cells (replicate values from two separate experiments are shown in parentheses).

Fig. 5 Effects of MMC on cytotoxicity of doxorubicin in H4IIE cells. Cells were grown and treated with MMC (●), as described in Fig. 2, or were treated with solvent alone (■), and then they were treated with various concentrations of doxorubicin for 72 h. Cytotoxicity was measured by a colorimetric substrate-based assay and is expressed as a percentage of control values. Data points, means ± SD of measurements from independent samples (n = 5–6). The ED₅₀ for doxorubicin alone was estimated to be approximately 10 μM, and the ED₅₀ for doxorubicin following MMC pretreatment was estimated to be approximately 2 μM using probit analysis. Bars are shown where they are larger than the symbol.

25–30% confluence, the cells were treated a second time with MMC, and the Pgp levels were again measured over time (see scheme in Fig. 3A). Interestingly, at the beginning of the second time course, the Pgp levels had returned to that of the original control cultures, suggesting that the effects of MMC on Pgp expression are transient. In addition, cells that were not treated the second time with MMC had constant Pgp levels throughout the second time course experiment, indicating that these cells do not up-regulate Pgp expression in response to the first MMC treatment. The second MMC treatment suppressed Pgp protein expression to a similar extent and over a similar time course as the first treatment, demonstrating that these cells had also not altered their response to the drug as a result of the first treatment (Fig. 3B).

Effects of MMC on Pgp protein expression were also examined in various human tumor cell lines. Similar time courses and magnitudes of suppression of Pgp protein levels were seen in human breast carcinoma MDA-MB-435 and colon cancer HT-29 cells grown in monolayer following a single 4-h 0.1 μM MMC treatment and analysis, as in Fig. 3B (Table 1). Suppression of Pgp by MMC was also seen in the human SK-HEP-1 (hepatoma), U87 (neuroblastoma), and K562 (chronic myeloid leukemia) cell lines, although the magnitude of suppression was less, and the maximal suppression was seen at 24 h after MMC in these lines (Table 1). Three other DNA cross-linking agents, the cancer chemotherapy drugs, cisplatin and BMS181174 (a MMC analogue), and the human lung carcinogen, chromium(VI), were also examined for their effects on Pgp protein expression in H4IIE cells. These agents suppressed Pgp levels to a similar extent and over a similar time course as MMC when administered at their respective highest noncytotoxic doses (Fig. 4; Table 2). These four agents have different chemical structures and undergo different metabolic activation. However, all induce significant levels of DNA damage at low doses, including high levels of both monoadducts and DNA
interstrand cross-links. Interestingly, the chemotherapeutic agent doxorubicin, a Pgp substrate, caused a significant increase in Pgp expression when administered to H4IIE cells at the highest nontoxic dose (Table 2). In contrast, melphalan, a bifunctional alkylating chemotherapeutic agent, and streptozotocin, a monofunctional alkylating agent, had no significant effect on Pgp expression over a 96-h period (Table 2). These results suggest that suppression of Pgp expression by MMC and the other DNA cross-linking agents is specifically a direct or indirect result of formation of DNA cross-links rather than DNA damage per se, and that different chemotherapeutic drugs have different effects on Pgp expression.

Functional studies were performed to assess whether suppression of Pgp protein levels could lead to a change in the rate of drug efflux in these cells. A flow cytometric assay was developed for estimating drug efflux using the change over time in intracellular fluorescence of the fluorescent dye and Pgp substrate, rhodamine 123. MMC-pretreated H4IIE cells demonstrated a decrease in the rate of rhodamine efflux relative to control cells at 66 h after MMC and a further decrease in the rate of efflux at 90 h after MMC (Table 3), consistent with the Western blot data in Fig. 3B. To assess whether MMC suppression of Pgp would lead to changes in the sensitivity of these cells to subsequent killing by other cytotoxic agents, cytotoxicity experiments were then performed. H4IIE cells pretreated with MMC exhibited a marked decrease in the ED50 for doxorubicin-induced cell killing (approximately 5-fold) as compared to non-MMC-treated cells (Fig. 5). This was not simply due to a combined toxicity of the two drugs, because cytotoxicity from treatment with MMC administered simultaneously with doxorubicin was no different from that of doxorubicin alone (data not shown).

The effects of MMC on Pgp levels and doxorubicin cytotoxicity were also examined in the MDA-MB-435 human breast carcinoma cell line. Recent work has suggested that the MDR phenotype of tumor cells in culture can be modulated by their culture environment, particularly in regard to whether the cells are grown as monolayer or as three-dimensional cultures (36). Human 435 cells were grown either as monolayers or as “spheroids,” which are individual aggregates of approximately 5,000–10,000 cells, each growing in suspension over agar. Interestingly, the apparent ED50 values for cytotoxicity of both MMC and doxorubicin increased approximately 10- to 20-fold in three-dimensional spheroid cultures as compared to monolayers (Fig. 6), suggesting that three-dimensional culture conditions provided some measure of increased drug resistance, as has been reported previously (36). However, at the appropriate dose, MMC pretreatment also caused a decrease in the ED50 for doxorubicin cytotoxicity in the 435 cell line spheroid cultures (Fig. 6). There was no change in basal Pgp expression in these cultures over the course of these experiments (data not shown). These results suggest that the suppressive effects of MMC may occur in both solid as well as liquid tumors in vivo. In addition, the similarity in results in the rat hepatoma H4IIE and human colon HT-29 and breast 435 cells indicates that this phenomenon is not restricted to a particular species or tumor cell type. This is encouraging if this approach is to be translated into clinical usefulness with other tumor types. In contrast to the results in Figs. 5 and 6, MMC pretreatment of H4IIE cells had no effect on subsequent cytotoxicity of cisplatin, which is not a substrate for Pgp efflux (Fig. 7).

DISCUSSION

We have demonstrated in cell culture that MMC and other DNA cross-linking agents can transiently but significantly suppress Pgp mRNA and protein expression and drug efflux. Pre-
treatment with MMC also led to a significant increase in sensitivity to subsequent killing by a Pgp substrate drug, doxorubicin. It is likely that the suppression of Pgp levels is the principal contributor to this change in cytotoxic response, although MMC effects on other inducible genes and gene products could also be contributing to the overall response of the cells to the second agent. Suppression of Pgp occurred in cell lines of hepatoma, breast carcinoma, colon carcinoma, neuroblastoma and leukemia origin that were derived from both rats and humans; in cells grown under both monolayer and spheroid culture conditions; and with four different DNA cross-linking agents, i.e., MMC, cisplatin, BMS181174, and chromium(VI). This, thus, appears to be a general phenomenon. On the basis of previous work from our laboratory (10–15), we postulate that this is most likely a result of preferential suppression of inducible mdr1 gene expression. In contrast, a Pgp substrate and chemotherapeutic agent that forms DNA monoadducts, doxorubicin, significantly increased Pgp expression, whereas two other chemotherapeutic drugs, mechlorethamine and streptozotocin, had no significant effect on Pgp expression. Increases in mdr1 gene expression had previously been reported for other monoadducting agents, including aflatoxin B1, 3-methylcholanthrene, and 2-acetylaminoﬂuorene (23, 32). Thus, suppression of Pgp expression appears to be specific effect of DNA cross-linking agents. Suppression of Pgp protein levels by MMC resulted in a decrease in the rate of Pgp-mediated drug efflux and sensitization of rat hepatoma H4IIIE and human breast MDA-MB-435 cells to killing by doxorubicin. The effects on Pgp expression appear to be transient, consistent with a model based on chemically induced DNA damage and repair (10–12, 14). This pretreatment provides a “window” of Pgp suppression during which there is an increase in the efficacy of cell killing by a second agent. This suggests that, if a similar phenomenon occurs in vivo, this pretreatment might increase the efficacy of cancer chemotherapy in drug-resistant tumors.

This window of opportunity would have to be clearly defined temporally for maximum clinical benefit. However, there are several potential advantages to such a chemotherapeutic approach: (a) the doses of MMC and cisplatin needed to suppress the MDR phenotype in culture are noncytotoxic, and presumably this would also occur at subchemotherapeutic doses in vivo; (b) because the suppressive effect on Pgp is predicted to lag by at least 24–48 h, second agents could be administered a day or more after the first agent, which would lessen any potential combined toxicities and allow high-dose second-agent chemotherapy; (c) this treatment does not appear to elicit a subsequent up-regulation of Pgp expression in these cells and has similar suppressive effects when administered a second time, indicating that multiple rounds of such therapy may be possible in vivo without inducing further drug resistance. This is important if such an approach is to be used clinically because, it would be desirable to be able to perform multiple cycles of this treatment in patients with highly refractory tumors; and (d) because MMC and cisplatin are approved drugs and have already been used singly and in combined chemotherapy settings, there are considerable data on their use in various cancers, providing an opportunity to rapidly translate these results to the clinic.

There are also many new and promising analogues of MMC and cisplatin being developed for clinical use. The similar suppression of Pgp obtained with all four DNA cross-linking agents suggests that many other drug combinations may also be possible with this approach. In addition, it is likely that other inducible genes, the increased expressions of which have also been associated with the MDR phenotype, may be suppressed in a fashion similar to that of Pgp. Candidates would include the MDR-related drug pump, MRP (37); the LRP protein (38), and Phase I and II metabolic enzymes, such as members of the cytochrome P-450 and glutathione S-transferase families of isozymes, respectively (39). Such a pleiotropic effect would be beneficial, particularly because individual tumors display different subsets of these MDR-associated proteins, which appear to vary considerably among tumor types and even among individuals with the same tumor type. Clearly, it will be important to determine the effectiveness of this treatment to suppress Pgp and other proteins associated with the MDR phenotype in human patients in vivo. Both animal studies and human trials are currently under way to assess this treatment in an in vivo setting. However, preliminary results from a human clinical trial in late-stage breast cancer patients (DMS-9503) indicate that a single MMC treatment simultaneously suppresses expression of Pgp, MRP, LRP, and glutathione S-transferase α in these patients over a similar time course and to a similar extent as in these cell culture experiments (40). These results suggest the basis for development of a novel chemotherapeutic approach aimed at refractory tumors, in which a subchemotherapeutic dose of a DNA cross-linking agent is used to modulate the MDR phenotype prior to treatment with a second cytotoxic agent.

ACKNOWLEDGMENTS

We gratefully acknowledge the advice, technical assistance, and/or use of methods and reagents from Karen Wetterhahn, Frank Valone, Jeff Silverman, Aaron Barchowsky, Melinda Treadwell, Stephen Anthony, Alice Givan, Gary Ward, and Robert Kerbel.

REFERENCES

Suppression of P-glycoprotein expression and multidrug resistance by DNA cross-linking agents.
