Partial Reversal of Doxorubicin Resistance by Forskolin and 1,9-Dideoxyforskolin in Murine Sarcoma S180 Variants

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ABSTRACT

Acquired resistance to chemotherapeutic agents is an important clinical problem. One preclinical model, termed multidrug resistance (MDR), is characterized by a complex phenotype of cross-resistance to biochemically unrelated antineoplastic agents, the presence of a high-molecular-weight membrane glycoprotein, and impaired accumulation of drug. To determine whether MDR is mediated in part by altered cyclic 3',5'-adenosine monophosphate (cAMP) levels, the effect of incubation with the adenylyl cyclase agonist, forskolin, was investigated in the murine sarcoma S180 cell line and two MDR variants (A5-8, A5-2.5). Basal cAMP levels in sensitive and MDR lines were not significantly different (range, 0.15 ± 0.05 to 0.31 ± 0.09 pmol/mg protein); however, 1-h incubation with forskolin, 10 μM, elevated intracellular cAMP 2-fold in the parent line and 43- and 35-fold in the variants. The adenylyl cyclase agonists, prostaglandin E1 and chola toxin, and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine had no significant effect on cAMP levels. To determine the effect of forskolin on doxorubicin-induced cell lethality, S180 and MDR lines were incubated with doxorubicin plus forskolin for 1 h and closed in soft agar. Coaction with forskolin partially reversed doxorubicin resistance in the MDR lines in a dose-dependent fashion. To determine whether this effect was mediated solely by elevation of intracellular cAMP, the inactive 1,9-dideoxy analogue of forskolin (DF) was used. Incubation with DF resulted in no elevation of cAMP levels in the sensitive or resistant cell lines; however, DF also partially reversed doxorubicin resistance in the MDR variants. Furthermore, coincubation of the A5-2.5 cell line with doxorubicin and 8-bromo cAMP, 1 mM, did not result in reversal of resistance to doxorubicin. To determine whether the reversal of resistance by the diterpenes was associated with alteration of doxorubicin transport, uptake, and efflux of [3H]doxorubicin were measured. Coincubation with both forskolin and DF, 10 μM, enhanced [3H]doxorubicin uptake in the resistant cells, while drug efflux was significantly affected only in the cell line exhibiting intermediate resistance. Since both forskolin and its inactive analogue are effective in partially reversing resistance to doxorubicin and augmenting anthracycline uptake, a mechanism other than elevation of cAMP is most likely responsible.

INTRODUCTION

Acquired resistance to anthracyclines and other classes of cytotoxic drugs limits their clinical utility. One preclinical model designed to study this problem, termed MDR, is characterized by a complex phenotype of cross-resistance to biochemically unrelated natural product antineoplastic agents, gene amplifications, deficient accumulation of drug, partial reversal of resistance in the presence of calcium channel blockers, and the presence of a high-molecular-weight membrane glycoprotein (1–6). Other less-well-defined characteristics of some MDR cell lines have recently been noted including: the presence of a novel, low-molecular-weight, calcium-binding protein (7); increased phosphorylation of a low-molecular-weight phosphoprotein (8); increased glutathione-S-transferase activity (9); enhanced protein kinase C activity (10); and increased levels of intracellular calcium (11). These findings remain to be confirmed in a broad spectrum of MDR cells and to be incorporated into the MDR model. Nevertheless, while the biological significance of these findings remains unclear, they suggest a global diversion of cellular resources allowing the drug-resistant cancer cell to adapt to a toxic environment.

To determine whether cellular regulatory mechanisms are implicated in MDR, investigations of the role of the ubiquitous cell regulatory axis, adenylyl cyclase-cAMP-cAMP-dependent protein kinase, were initiated. In conjunction with the protein kinase C-calmodulin system, the cAMP axis constitutes a synergistic regulatory system over a wide variety of cell functions (12). In addition, cAMP has been shown to modulate the synthesis of a growing list of proteins, and in several cases, the effect on protein synthesis has been demonstrated to occur at the transcriptional level (13).

The murine sarcoma S180 cell line and two doxorubicin-resistant MDR variants (14), modified in our laboratory for these investigations, were used to determine the effect of elevations of intracellular cAMP on sensitive and resistant cell properties. The adenylyl cyclase agonist, forskolin, which reproducibly elevates intracellular cAMP in normal human tissues and cultured cell lines, was used as an effector and its inactive 1,9-dideoxy analogue (DF) as a control. Cell lethality as determined by colony-forming assay and drug transport as measured by total cell uptake of radiolabeled doxorubicin were the parameters examined to ascertain drug effect.

MATERIALS AND METHODS

Cell Culture. The S180 cell line, originally isolated from the murine CFW strain in 1955 (15), and its doxorubicin-resistant variant, A5, were the generous gifts of Dr. Thomas Tritton, University of Vermont School of Medicine, Burlington VT. Cells were grown in RPMI 1640 medium with glutamine (GIBCO Laboratories, Grand Island, NY) supplemented with 10% HIHS by the method of Fisher and Sartorelli (16). A5 cells were grown in an identical fashion except that they were cultured in the presence of doxorubicin, 0.8 μg/ml (A5-8), or 2.5 μg/ml (A5-2.5), which was added at the time of cell passage.

Drugs. Doxorubicin (NSC 123127; Farmitalia, Carlo Erba, Milan, Italy) was diluted with sterile 0.15 M NaCl and protected from light. [3H]Doxorubicin ([3H]doxorubicin HCl; 13.4 μCi/mg) was obtained from SRI International (Menlo Park, CA). The radiopurity was greater than 95% as determined by thin-layer chromatographic assay. Forskolin (Sigma Chemical Co., St. Louis, MO) was made up in stock solutions of ethanol at 10 mM. Because ethanol inhibits the effect of forskolin on adenyl cyclase (17), solutions were made such that the volume of ethanol added never exceeded more than 0.1% of the final volume. DF was the generous gift of Dr. Kenneth Seamon of the Food and Drug Administration, Bethesda, MD. DF was handled exactly as forskolin. 8Br cAMP, verapamil, prostaglandin E1, chola toxin, and IBMX were from Sigma. Stock solutions were stored at −85°C.

Clonogenic Assay. The clonogenic assay was performed as previously described (18). Cells in early exponential growth were resuspended in heat-inactivated horse serum; 8Br cAMP, 8-bromocyclic 3',5'-adenosine monophosphate; HIHS, heat-inactivated horse serum; 8Br cAMP, 8-bromocyclic 3',5'-adenosine monophosphate; DF, 1,9-dideoxy forskolin; IBMX, 3-isobutyl-1-methylxanthine.
RPMI 1640 and incubated in the presence or absence of drug (doxorubicin with or without forskolin, DF, 8Br cAMP, or verapamil) in polystyrene tissue culture flasks in the dark at 37°C for 1 h. Cells were then washed 3 times, resuspended in RPMI 1640 with 15% HIHS, twice diluted 1:10, and adjusted to 50–100 cells/ml. Cells were then added to a suspension of RPMI 1640 medium with 15% HIHS and Noble agar (10:1, v/v), incubated at 37°C for 10 to 12 days (S180), 14 to 16 days (A5-8), or 18 to 20 days (A5-2.5), and counted under low-power magnification. Experiments were performed 2 to 5 times.

Cyclic AMP Assay. Cells in exponential growth were adjusted to 2 x 10^6/ml in RPMI 1640, then incubated with or without forskolin, DF, prostataglandin E2, cholera toxin, or IBMX at various concentrations for 1 h. Cells were extracted twice with acidified ethanol. Levels of cAMP were determined by radioimmunoassay by the method of Gilman (19). Protein content was determined by the method of Lowry (20). Values determined were the mean of three experiments. Bovine serum albumin was used as a standard.

Doxorubicin Uptake and Efflux. Uptake and efflux of [14C]doxorubicin in S180, A5-8, and A5-2.5 cells were measured in cells in early exponential growth by a previously described method (21). Cells were adjusted to 2 x 10^6/ml in RPMI 1640 medium with 10% HIHS and allowed to equilibrate in the dark at 37°C for 1 h. Doxorubicin uptake was initiated by the addition of [14C]doxorubicin to the cell suspension at a final concentration of 1.0 µg/ml in the presence or absence of forskolin or DF, 10 µM, which was added simultaneously. At various time points following addition of radiolabeled drug, 0.5-ml aliquots were removed, added directly to microcentrifuge tubes containing 0.6 ml of ice-cold PBS, and centrifuged at 12,000 x g for 1 min to remove cells from the drug-containing medium. Cells were washed with ice-cold PBS, and the medium was aspirated. Aliquots were removed to scintillation fluid for determination of radioactivity.

For studies of drug efflux, cells were loaded for 1 h with [14C]doxorubicin, 1 µg/ml, with or without forskolin or DF, 10 µM, then washed, and resuspended in RPMI 1640 medium with 10% HIHS, with or without forskolin or DF, 10 µM. At various time points 0.5-ml aliquots containing 1 x 10^6 cells were removed to microcentrifuge tubes containing 0.6 ml of ice-cold PBS, washed twice with PBS, and processed for scintillation counting as described above.

Statistical Analysis. Differences in levels of intracellular cAMP were measured by analysis of variance. Differences in cell survival were measured by Student's t test. Differences in uptake of [14C]doxorubicin and rate of efflux of drug were compared by repeated measures analysis of variance and by Student's t test.

RESULTS

Cyclic AMP Assay. Basal cAMP levels and cAMP levels in forskolin-stimulated cells were measured in the parent and doxorubicin-resistant sublines. A 1-h incubation with forskolin was used to ensure that plateau levels were achieved. As shown in Table 1, basal levels of cAMP were not significantly different in the anthracycline-resistant lines as compared to the parent line. Levels were adjusted for the higher protein content of the resistant lines: 146 ng/10^6 cells (S180); 343 ng/10^6 cells (A5-.8); and 357 ng/10^6 cells (A5-2.5). As shown in Table 1, both the parent line and resistant sublines were responsive to forskolin stimulation; however, while 1-h incubation with forskolin, 10 µM, resulted in a 2-fold increase in cAMP in the parent line, in the resistant lines cAMP levels were elevated 43-fold and 35-fold. To determine whether other adenylyl cyclase agonists or phosphodiesterase inhibitors would elevate intracellular cAMP levels in these experimental cell lines, S180 cells and the resistant variants were incubated for 1 h with prostataglandin E2, 10^{-9} and 10^{-8} M, cholera toxin, 1 µg/ml and 10 µg/ml, or IBMX, 0.4 mM. Incubation with these agents resulted in no significant elevation of intracellular cAMP levels. Incubation of S180 cells with the combination of IBMX, 0.4 mM, and forskolin, 10 µM, for 1 h resulted in a 180-fold increase in cAMP levels, to a level comparable to that achieved in the resistant cell lines. Incubation with the inactive analogue of forskolin, DF, 10 µM, resulted in no increase in cAMP levels in any of the cell lines tested.

Clonogenic Assay. To determine whether simultaneous exposure to forskolin alters sensitivity of S180 cells and the anthracycline-resistant variants to doxorubicin, cells were incubated with or without forskolin, 10 µM, and doxorubicin at doses specified in Fig. 1. As shown in Fig. 1A, the 50% inhibitory concentration for the S180 line is 0.2 µg/ml, similar to that previously reported (18). The dose-response curve exhibits a steep exponential decline over 2 logs following 1-h incubation with doxorubicin, 0.6 µg/ml. Simultaneous incubation with either forskolin or DF, 10 µM, produced no alteration in the dose response. When S180 cells were coincubated with IBMX, 0.4 mM, and DF, 10 µM, for 1 h, concentrations which produced 180-fold increase in intracellular cAMP levels, in the presence of doxorubicin, there was no effect on colony-forming efficiency.

As shown in Fig. 1, B and C, the variant sublines are highly resistant to the effects of doxorubicin exhibiting virtually no exponential cell lethality at doses of doxorubicin up to 100 µg/ml. Simultaneous incubation with doxorubicin and forskolin, 10 µM, resulted in partial restoration of anthracycline responsiveness. This effect was dose dependent: increasing concentrations of forskolin markedly enhanced reversal of anthracycline resistance as shown in Table 2.

The effects of the cAMP analogue, 8Br cAMP, on doxorubicin resistance were also examined. A5-2.5 cells were coincubated with doxorubicin with or without the analogue, 1 mM, for 1 h. There was no alteration in dose response. To confirm that reversal of resistance was not associated with adenylyl cyclase stimulation, A5-.8 and A5-2.5 cells were incubated in the presence of doxorubicin and DF, 10 µM, for 1 h. As shown

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Forskolin (µM)</th>
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<tr>
<td>S180</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.05*</td>
</tr>
<tr>
<td>A5-8</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>A5-2.5</td>
<td>0.31 ± 0.09</td>
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</tbody>
</table>

* Protein, pmol/mg.
* Mean ± SEM of 3 experiments.
* P = 0.0087 by analysis of variance.
* ND, not done.

Fig. 1. Cytotoxic effects of doxorubicin on S180 (A), A5-8 (B), or A5-2.5 (C) cells in the presence of forskolin, 10 µM (□), DF, 10 µM (○, A), or verapamil, 10 µM (C, ○), V, ○, untreated controls. Each point represents the mean of 2 to 5 determinations. In B, differences in cytotoxicity for forskolin-treated cells versus control are significant at the level P < 0.02 (12.8, 40 µg/ml), for DF-treated cells versus control at P < 0.05 (12.8, 40 µg/ml), and for verapamil-treated cells versus control at P < 0.025 by Student's t test. In C, differences in cytotoxicity for forskolin-treated cells versus control are significant at the level P < 0.05 (5, 25, 40 µg/ml) and P < 0.005 (100 µg/ml), for DF-treated cells versus control at P < 0.001 (40 µg/ml) and P < 0.05 (100 µg/ml), and for verapamil-treated cells versus control at P < 0.025 by Student's t test.

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Doxorubicin are significant at the following levels: A. P not significant; B. P < 0.005; C. P = 0.009; D. P = 0.005 by repeated measures analysis of variance.

in Fig. 1, sensitivity to the anthracycline was partially restored, and in the case of the A5-2.5 cells, nearly to levels produced by forskolin. As with forskolin, this effect was dose dependent (Table 2). To compare the effects of the diterpenes with that of the "classical" MDR reversing agent, A5-.8 and A5-2.5 cells were coincubated with doxorubicin and verapamil, 10 μM, for 1 h. As shown in Fig. 1, B and C, anthracycline resistance was partially reversed to a greater extent than that produced by the diterpenes.

Doxorubicin Uptake and Efflux. It has previously been shown that resistant S180 sublines exhibit altered uptake and efflux of doxorubicin (14). To determine whether simultaneous incubation with forskolin or DF results in altered accumulation of doxorubicin in the parent line or further changes in accumulation of doxorubicin in the resistant lines, uptake and efflux of [14C]doxorubicin were measured in the presence and absence of simultaneous exposure to forskolin or DF, 10 μM. As shown in Fig. 2A, the rate of accumulation in the parent line was not influenced by the presence of forskolin. In contrast, as shown in Fig. 2, B and C, uptake of doxorubicin was increased in the forskolin-treated A5-.8 and A5-2.5 sublines as compared with the untreated line at virtually every time point. As shown in Fig. 2D, similar results were seen when A5-2.5 cells were incubated with [14C]doxorubicin in the presence or absence of DF, 10 μM. Rate of efflux of doxorubicin from sensitive and resistant cell lines was reduced by both forskolin and DF as compared with untreated cells, but was only diminished at a statistically significant level in the intermediate-resistant A5-.8 cells (Fig. 3).

DISCUSSION

Several classes of agents have been shown to reverse acquired resistance to anthracyclines in cultured human and animal cell lines, including phenylalkylamines, benzothiazepines, and dihydropyridines, which bind and block the calcium channel at different sites (22); the calmodulin antagonists, trifluoperazine, perhexilene maleate, and N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) (23); the triparanol analogues, tamoxifen and clomiphene (24); and the cardiac antiarrhythmics, quinidine and amiodarone (25, 26). We now report that the plant diterpene, forskolin, an adenylate cyclase agonist, and its 1,9-dideoxy analogue are capable of partially reversing acquired resistance to doxorubicin in resistant variants of the murine sarcoma S180 cell line.

Forskolin activates adenylate cyclase in a dose-dependent fashion in a variety of cell types. While the exact mechanism of action is unclear, activation of the enzyme appears to occur at the level of the catalytic subunit or regulatory protein, bypassing the receptor (27). In addition to the well-characterized membrane and genetic abnormalities in cells exhibiting MDR, alterations in protein phosphorylation (9), increased basal levels of protein kinase C (11), and increased glutathione S-transferase levels (10) have been detected in resistant MCF-7 cells. We now report an additional alteration in enzyme activity in the anthracycline-resistant lines described in this paper, specifically a markedly augmented effect of the adenylate cyclase agonist, forskolin, on intracellular cAMP levels in the resistant variant lines as compared with the parent line. Whether this results from the presence of an altered adenylate cyclase or increased amounts of the enzyme in the cell membrane of the resistant cells cannot be determined from the data presented. Receptor-mediated adenylate cyclase stimulating agents, prostaglandin E2 and cholera toxin, and the phosphodiesterase inhibitor, IBMX, had no effect on intracellular levels of cAMP.

Our findings suggest that the ability of forskolin to reverse doxorubicin resistance in a dose-dependent fashion is not solely a function of elevation of intracellular cAMP levels. While forskolin produced partial reversal of doxorubicin resistance in

<table>
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<tr>
<th>Cell line</th>
<th>Drug treatment</th>
<th>Dose (μM)</th>
<th>( 1 )</th>
<th>( 5 )</th>
<th>( 10 )</th>
<th>( 50 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5-8</td>
<td>Forskolin</td>
<td>31 ± 4.3*</td>
<td>3.0 ± 0.5</td>
<td>1.7 ± 0.4</td>
<td>1.0 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>59 ± 4.3</td>
<td>ND</td>
<td>32.3 ± 9.5</td>
<td>1.1 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>A5-2.5</td>
<td>Forskolin</td>
<td>96 ± 3.2</td>
<td>97 ± 1.0</td>
<td>67 ± 2.0</td>
<td>14 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DF</td>
<td>85 ± 0.7</td>
<td>ND</td>
<td>29 ± 8.0</td>
<td>21 ± 2.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

\* Percentage.
\* Mean ± SEM.
\* ND, not done.
the resistant cell lines studied, the inactive analogue, DF, also produced partial reversal of resistance, and in the case of the A5-2.5 line, nearly to those produced by forskolin. Furthermore, the 8-bromo cAMP analogue failed to reverse doxorubicin resistance following simultaneous incubation.

Thus, other possible mechanisms to account for the action of the diterpenes may be invoked. Forskolin, for example, produces cAMP-independent inhibition of glucose transport in human erythrocytes (28). Forskolin also alters membrane fluidity in rat liver plasma membranes, but only at concentrations 10-fold higher than those used in these experiments (29); therefore, it is unlikely that the effects noted in the S180 variants are due to a direct effect on membrane fluidity. Forskolin and DF may enter the membrane bilayer, disrupting lipid structure at levels that do not produce measurable alteration in membrane fluidity, but which do enhance permeability of the membrane to doxorubicin. Such a mechanism has been proposed to account for forskolin- and DF-mediated inhibition of carbachol-stimulated uptake of 86Rb+ via nicotinic receptors in the PC 12 rat pheochromocytoma cell (30). Alternatively, forskolin may work as a channel blocker, similar to verapamil, which reverses resistance in a variety of MDR cells; however, the fact that most channel blockers have a positive charge and are not as lipophilic as forskolin makes this somewhat less likely.

As with other agents that reverse resistance to doxorubicin, both forskolin and DF enhanced accumulation of the anthracycline in the resistant variants. The predominant effect in the highly resistant variant appeared to be increased influx of drug with augmentation at virtually every time point from 7 to 60 min. In light of data implicating increased activity of a membrane-associated pump in acquired drug resistance, particular attention was given to drug efflux in our system. There was virtually no effect on rate of drug efflux by forskolin or DF in the sensitive or the highly resistant cell lines. In the intermediate resistant cell line, both influx and efflux were significantly affected. This contrasts with the effects noted with the calcium channel blocking agents and calmodulin inhibitors which predominantly inhibit drug efflux (31, 32). We conclude that further studies are warranted to determine the mechanisms by which the plant diterpenes enhance accumulation of doxorubicin in resistant cells and partially reverse resistance to this drug.

REFERENCES

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