ABSTRACT

The effect of thaliblastine (TBL, NSC-68075), a plant alkaloid, in overcoming multidrug resistance was investigated in doxorubicin (ADR)-resistant murine leukemic P388/R-84 cells. In the soft agar cloning assay, a non-toxic concentration of TBL (2 μM) reduced the 50% inhibitory dose of ADR (1-h exposure) from 10.8 to 1.4 μM with a dose modification factor of 7.7. Continuous treatment of P388/R-84 cells with ADR and TBL for 24 h further lowered the 50% inhibitory dose from 3.5 to 0.07 μM, the resistance level being decreased from 233-fold in the absence of TBL to 4.7-fold in the presence of TBL as compared to the parental P388 cells. Although ADR or TBL individually had no detectable effects on cell cycle traverse, the combination of the two drugs caused a significant G2 block.

Flow cytometric analysis showed that TBL enhanced ADR retention in P388/R-84 cells in a dose- and time-dependent manner. TBL partially blocked the photolabeling of P glycoprotein with [3H]azidopine, and this blocking effect was further enhanced in combination with ADR. Our results indicate that TBL can reverse multidrug resistance by direct interaction with P-glycoprotein, thereby increasing cellular ADR retention.

INTRODUCTION

MDR is characterized by decreased intracellular drug retention as a result of the increased efflux mediated by the overexpression of the MDR 1,700,000 P-gp (1, 2). P-gp acts as a membrane-bound, ATP-dependent, drug-efflux pump and is believed to transport a number of functionally and structurally unrelated drugs (3-5), resulting in a wide spectrum of cross-resistance. The specific binding of Vinca alkaloids to P-gp was first demonstrated by using a radiolabeled photoactive analogue of vinblastine (6, 7). Since then a number of compounds including verapamil, a typical calcium channel blocker (8), progesterone (9), a quinoline derivate (MS-073) (10), and cyclosporin A (11) have been shown to inhibit this photoaffinity labeling and reverse MDR in several cell lines. Thus, it has been proposed that the photolabeling method may be used for rapid screening of new compounds for MDR reversal (7, 11).

Thalicarpine (TBL) is a dimeric aoporphine benzylisoquinoline alkaloid originally isolated from several species of the genus Thalictrum (family Ranunculaceae) (12). TBL has antitumor activity against several experimental tumors both in vitro and in vivo (13, 14). Recently, TBL was found to have a higher cytotoxic effect in a cisplatin-resistant rat ovarian tumor cell line than in the parental drug-sensitive cell line (15). This effect could be further increased in combination with hyperthermia. Because of its lower toxicity (16) and structural similarity (Fig. 1) with some photoaffinity analogues of P-glycoprotein (11), we have investigated the ability of TBL to reverse MDR in ADR-resistant murine leukemic P388/R-84 cells.

MATERIALS AND METHODS

Chemicals and Cell Culture. Doxorubicin hydrochloride (ADR, NSC-123127) was purchased from Adria Laboratories, Inc., Columbus, OH. A stock solution of ADR (1 mg/ml) prepared in distilled water and kept at -20°C, was diluted in RPMI medium just before use. Thaliblastine hydrochloride (thalicarpine, TBL; Pharmachin, Bulgaria) was obtained from Dr. D. K. Todorov, National Oncological Center, Sofia, Bulgaria, and dissolved in medium before use. Tritium-labeled, photoactive 2,6-dimethyl-[4-(4′-trifluoromethyl)phenyl]-1,4-dihydropropyridine-3,5-dicarboxylic acid, ethyl (N′-4′-azido[3′,5′-3H] benzoyleaminomethyl)diester ([3H]azidopine) (stock solution, 1 μM) was purchased from Amersham Corporation (Arlington Heights, IL).

Mouse leukemic P388 and the doxorubicin-resistant P388/R-84 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10 μM 2-mercaptoethanol.

Cytotoxicity Assay. Soft agar cloning and growth inhibition assays were carried out for determination of long- and short-term cytotoxicity of ADR and TBL, respectively. Following 1-h exposure to ADR in the presence or absence of 2 μM TBL, cells were washed twice with fresh medium and resuspended in RPMI medium containing 15% fetal bovine serum and soft agar at a final concentration of 0.3%. Cells were seeded at a density of 40,000 per well (2 ml) in 6-well plates (Costar, Cambridge, MA) in triplicate. Colonies larger than 50 μm were counted on Day 5; plating efficiency in the control group of both lines was between 45 and 55% (17).

The effect of 2 μM TBL on ADR (0.01 to 10 μM) cytotoxicity was determined by the trypan blue dye exclusion method (18), after incubating 1 × 106 cells with the drug(s) in 1 ml of medium for 24 h at 37°C.

DNA Histogram Analysis. Following drug exposure for 24 h, cells were centrifuged and resuspended in propidium iodide-hypotonic citrate solution for 1 h before flow cytometric analysis for cell cycle distribution (19).

Cellular ADR Content. Log-phase cells (1 × 106/ml) were exposed to 3.6 μM ADR in the absence or presence of TBL (2 or 8 μM) for either 1 or 4 h at 37°C. Cellular ADR fluorescence was monitored after excitation with a 488-nm argon laser line in a FACScan (Becton and Dickinson, San Jose, CA) (17, 20). Fluorescence emission (above 530 nm) from at least 10,000 cells was collected, amplified, and scaled to generate a single-parameter histogram.

Photolabeling of Plasma Membrane. Plasma membranes from P388/R-84 cells were prepared according to the procedures of Gerlach et al. (21) and Yusa and Tsuru (22) with minor modifications. Cells were washed 3 times with ice-cold phosphate-buffered saline [0.15 M NaCl:20 mM sodium phosphate (pH 7.4)] at 200 × g and 4°C for 10 min and incubated at about 5 × 107 cells/ml in the hypotonic lysis buffer [10 mM KCl:1.5 mM MgCl2:2 mM phenethylsulfonfluoride:10 mM Tris (pH 7.4)] on ice for 10 min. The swollen cells were disrupted with a T-line homogenizer (Talboys Engineering Corporation, Emerson, NJ). The homogenate was subjected to centrifugation at 4000 × g for 10 min to remove the nuclei (pellet). The supernatant was centrifuged for 90 min at 4°C and 50,000 × g. The pellet was stored at -70°C until use. The protein content was determined by the Bradford assay (23).

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Thaliblastine, a Plant Alkaloid, Circumvents Multidrug Resistance by Direct Binding to P-Glycoprotein

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The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; TBL, thaliblastine; ADR, Adriamycin; ID50, 50% inhibitory dose; MI, modulator index.

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RESULTS

Reversal of ADR Resistance by TBL. The sensitivity of P388 and P388/R-84 cells to ADR and the potentiating effects of TBL on ADR cytotoxicity in soft agar are illustrated in Fig. 2. The ID_{50} values of ADR (1-h exposure) were 0.028 and 10.8 μM in the sensitive and resistant lines, respectively, indicating an approximate 386-fold resistance (Fig. 2). When TBL was added at a final concentration of 2 μM to P388/R-84 cell cultures, the ID_{50} value for ADR shifted from 10.8 to 1.4 μM with a dose modification factor of 7.7 (Fig. 2B). However, in P388 cells, coincubation with TBL had no detectable effects on ADR cytotoxicity. TBL (2 μM) alone did not have any significant cytotoxicity with the surviving fraction of 94.4 ± 3.1% and 101.7 ± 4.2% (SD, n = 4) in P388 and P388/R-84 cells, respectively. In the soft agar clonogenic assay, even 16 μM TBL was relatively less toxic with a mean surviving fraction of 80.1% and 90.4% in P388 and P388/R-84 cells, respectively, following a 1-h exposure time. Since our goal was to find a nontoxic concentration of TBL which would alter ADR resistance, we have not tested higher TBL concentrations in the clonogenic assay.

In the 24-h growth inhibition assay, P388/R-84 cells were 233-fold more resistant than the sensitive cells (ID_{50}, 0.015 in P388 cells versus 3.5 μM ADR in P388/R-84 cells) (Fig. 3). Simultaneous exposure of the P388/R-84 cells for 24 h to ADR and 2 μM TBL, the latter alone having no significant cytotoxicity (>95% of control), reduced the ID_{50} of ADR from 3.5 to 0.07 μM, resulting in lowered resistance from 233- to 4.7-fold (Fig. 3B). Treatment with 2 μM TBL also slightly enhanced ADR cytotoxicity in the P388 cells (Fig. 3A), which was not observed in the clonogenic assay (Fig. 2A). The ID_{50} values of TBL in the cytotoxicity assay were 8 and 5.8 μM for P388 and P388/R-84 cells, respectively.

Cell Cycle Effects. Following 24-h exposure to ADR and/or TBL, the cellular DNA content (and cell cycle distribution) was analyzed by flow cytometry. DNA distribution histograms in Fig. 4 are of P388 and P388/R-84 cells exposed to different concentrations of ADR and/or TBL. Histograms A (control) and B show that, in P388 cells, TBL (2 μM) had no major effect on cell cycle traverse (G2-M = 9.6 to 11%). In P388 cells (Fig. 4C), ADR (0.01 μM) caused a significant accumulation of cells with the G2-M DNA content (G2-M = 35.9%), and the effect was more pronounced when combined with 2 μM TBL (Fig. 4D, G2-M = 58.6%). In ADR-resistant P388/R-84 cells, either a 100-fold concentration of ADR (1 μM, Histogram E) compared to that in P388 cells or TBL (2 μM, Histogram F) had no effect on cell cycle traverse (G2-M = 11.9%). However, coincubation of cells with ADR (0.1 to 1 μM) and TBL (2 μM, Histograms G and H) caused a concentration-dependent increase in the number of cells with the DNA content of G2-M (20.8, 73.1%) and dead cells (arrow, Fig. 4H). These data confirm the synergistic cytotoxicity data obtained from cytotoxicity and clonogenic assays shown in Figs. 2 and 3.
Increased ADR Retention in P388/R-84 Cells by TBL. Fig. 5 shows cellular ADR fluorescence of P388 (A and B) and P388/R-84 (C and D) cells in the presence and absence of 2 or 8 μM TBL. No significant change in cellular ADR fluorescence of P388 cells was observed upon coincubation with either 2 or 8 μM TBL. In contrast, TBL significantly increased cellular ADR retention of P388/R-84 cells in a dose- and time-dependent manner (Fig. 5D).

Inhibition of [3H]Azidopine Photolabeling of P-gp by TBL. The inhibitory effect of TBL on the photolabeling of P-gp in P388/R-84 cells with [3H]azidopine is shown in Fig. 6. The Mr 170,000 polypeptide in P388/R-84 cells was labeled with [3H]azidopine (Fig. 6, Lane 1). TBL at a concentration of 2 μM slightly reduced [3H]azidopine binding of P-gp in the plasma vesicles (Fig. 6, Lane 2). This inhibitory effect was more pronounced at a higher TBL concentration of 8 μM (Fig. 6, Lane 4). When TBL and ADR at a molar 1:1.8 ratio were added together with the isotope, the intensity of labeling decreased significantly (Fig. 6, Lane 5) as compared to either of them alone (Fig. 6, Lanes 2 and 3).

DISCUSSION

The present study clearly shows that TBL at a nontoxic concentration (2 μM) can significantly enhance ADR cytotoxicity in the multidrug-resistant P388/R-84 cells. The dose of TBL needed for this effect (2 μM) is much lower than that of most other doxorubicin efflux blockers, such as chlorpromazine (100 μM)(17) and trifluoperazine (15 μM)(25). If we use the MI (fold decrease in resistance/modulator μM concentration) to represent the effectiveness of an efflux blocker as proposed by Beck and Qian (11), TBL has a MI of 25 in the 24-h continuous exposure schedule. This MI is much higher than that of most other efflux blockers such as verapamil and chlorpromazine in the vinblastine-resistant human leukemic cell line (26, 27). Since TBL is relatively less toxic (50% lethal dose: 480 mg/kg, i.p., in mice; 1500 mg/kg, i.v., in rats) with some manageable side effects such as hypotension, bradycardia, and respiratory depression (16), TBL may be a superior candidate for further in vitro and in vivo studies on reversing MDR in tumor cells.

Flow cytometric determination of intracellular ADR showed that the peak channel of fluorescence in the parental P388 cells (Fig. 5A) was 3-fold higher than the peak channel in the ADR-resistant P388/R-84 cells (Fig. 5C). The low ADR retention in P388/R-84 cells is functionally mediated by the overexpression of the MDR-1 gene at both the mRNA and protein levels and the P-gp-related efflux (17, 28-30). In the present study, TBL (2 to 8 μM) could increase cellular ADR retention in P388/R-84 to a level almost similar to that of the drug-sensitive P388 line (compare Fig. 5A with Fig. 5C, b and c). Although measurement of cellular fluorescence in the present study...
does not distinguish between drug uptake and drug efflux, it does reflect cellular ADR fluorescence after treatment with the identical schedule as in clonogenic assay (1-h exposure). In P388/R-84 cells, coinubcation with TBL for 4 h instead of 2-h exposure further increased ADR retention (compare Fig. 5, C and D). This might in part explain why the longer exposure (24 h) to TBL in the growth inhibition assay results in a higher level of resistance modulation (50-fold).

The photoaffinity labeling experiment with [3H]lazidopine in the presence or absence of TBL (Fig. 6) strongly suggests that TBL enhances cellular ADR accumulation and cytotoxicity in the resistant P388/R-84 cells through competition for closely related binding sites on P-gp. It would be worthwhile to look for the binding sites of TBL on the P-gp molecule, which would improve the selection of compounds to circumvent MDR. Furthermore, TBL alone is an old anti-cancer drug (31), although its mechanism of action is not clearly understood (15). The collateral sensitivity of cisplatin-resistant ovarian tumor cells (15) as well as MDR cells (ID50 8 and 5.8 μM TBL in P388 and P388/R-84 cells, respectively; data not shown) to this compound indicates that, at higher drug concentrations, TBL can affect targets other than P-gp in drug-resistant cells. The development of drug resistance, with or without MDR overexpression (in cisplatin-resistant cells) (15), could increase its cytotoxicity, which deserves further investigation.

It should be pointed out that, even after 24-h continuous exposure, there is still about 5-fold residual ADR resistance in the P388/R-84 cells compared to the parental P388 sensitive cells (Fig. 3). This low level of residual resistance may be related to other mechanisms of resistance in P388/R-84 cells reported earlier from our laboratory (18, 25, 32).

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