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

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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 clonogenic 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 47-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 M, 170,000 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).

Thalibarine (TBL) is a dimeric aporphine benzyliquinoline 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-gp-inhibitory chemicals (11), we have investigated the ability of TBL to reverse MDR in ADR-resistant murine leukemic P388/R-84 cells.

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4 The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; TBL, thaliblastine; ADR, adriamycin; IDso, 50% inhibitory dose; MI, modulator index.
5 G. Chen et al., unpublished data.

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-[4-2′-trifluoromethyl]phenyl-1,4-dihydropyridine-3,5-dicarboxylic acid, ethyl, (N-4′-azido[3′,5′-3H] benzozyaminomethyl)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 μg/ml of streptomycin, and 10 μg mercury. ADR-resistant mouse leukemia P388 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μg/ml of streptomycin, and 10 μg/2-mercaptoethanol.

Cytotoxicity Assay. Soft agar clonogenic 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 trypsin 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 50,000 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 Tsuruo (22) with minor modifications. Cells were washed 3 times with ice-cold phosphate-buffered saline [0.15 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 [0.15 NaCl:1.5 mM MgCl2:2 mM phenylmethylsulfonfluoride: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).

Photolabeling of Plasma Membrane. Plasma membranes from P388/R-84 cells were prepared according to the method described by Safa et al. (24). The membrane vesicles (50 μg of protein) were photolabeled in 40 mM potassium phosphate buffer (pH 7.0) containing 10 μM CaCl2, 4% dimethyl sulfoxide, and 0.2 μM [3H]azidopine (10 μCi) in a final volume of 50 μl. This mixture preincubated for 1 h at room temperature in the dark (in the presence or absence of 3.6 μM ADR and/or 2 or 8 μM TBL) was irradiated for 20 min using a UV cross-linker (UV Stratalinker 1800, Stratagene, La Jolla, CA). The photolabeled membrane protein preparations were separated on a 7.5% sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. Covalent incorporation of [3H]azidopine was detected by fluorography using Enhance (Du Pont, MA).

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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 ID50 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 ID50 value for ADR shifted from 10.8 to 1.4 μM with a dose modification factor of 7.7 (Fig. 2B). In P388 cells (Fig. 2C), 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. 2D, 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.

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.
REVERSAL OF MDR BY THALI Blastine

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

Fig. 4. DNA distribution histograms of P388 (A to D) and P388/R-84 (E to H) cells exposed to ADR (C and E), TBL (B and F), or a combination of ADR and TBL (D, G, and H). Note that, in P388 cells, ADR-caused G2 block (C) was further enhanced by coincubation with TBL (D). In P388/R-84 cells, ADR (1 μM, E) or TBL (2 μM, F) had no effect on cell cycle traverse but a combination of the two caused a pronounced increase in G2-M (G and H) and dead cells (H, arrow).

Fig. 5. Flow cytometric histograms of ADR cellular fluorescence in P388 (A and B) or P388/R-84 (C and D) cells. Solid (shaded) histograms are of cells incubated with ADR alone. In P388 cells, coincubation with 2 (b) or 8 (μM) TBL for 1 to 4 h did not enhance cellular ADR retention (Fig. 4, A and B). In P388/R-84 cells, coincubation with TBL, 2 to 8 μM (b and c), significantly increased ADR fluorescence (Fig. 4, C and D).

Fig. 6. Blocking of [3H]azidopine photoaffinity labeling of P-glycoprotein by TBL and/or ADR. Lane 1, positive control ([3H]azidopine alone); Lane 2, 2 μM TBL; Lane 3, 3.6 μM ADR; Lane 4, 8 μM TBL; Lane 5, 2 μM TBL + 3.6 μM ADR.

Fig. 4. DNA distribution histograms of P388 (A to D) and P388/R-84 (E to H) cells exposed to ADR (C and E), TBL (B and F), or a combination of ADR and TBL (D, G, and H). Note that, in P388 cells, ADR-caused G2 block (C) was further enhanced by coincubation with TBL (D). In P388/R-84 cells, ADR (1 μM, E) or TBL (2 μM, F) had no effect on cell cycle traverse but a combination of the two caused a pronounced increase in G2-M (G and H) and dead cells (H, arrow).
does not distinguish between drug uptake and drug efflux, it does reflect cellular ADR fluorescence after treatment with the identical cultures, A. Sauerteig, and G. McPhee for help in flow cytometric analysis and preparation of the manuscript.

REFERENCES

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