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SHORT COMMUNICATIONS

Role of P-glycoprotein in dolastatin 10 resistance

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Abstract—Dolastatin 10, a cytotoxic pentapeptide isolated from the mollusk *Dolabella auricularia*, exhibits potent antitumor activity. The present studies demonstrated that sublines of murine PC4 and human U-937 leukemia cells expressing a multidrug resistance (MDR) phenotype are cross-resistant to this agent. We also demonstrated that such resistance was reversed by verapamil. While these findings suggested the involvement of the P-glycoprotein (P-gp) in dolastatin 10 resistance, we performed similar studies in a CHO cell line transfected with the human *mdr*1 cDNA. Expression of P-gp in the transfected cells was associated with resistance to dolastatin 10 by a verapamil-sensitive mechanism. The demonstration that photoaffinity labeling of P-gp was decreased in the presence of dolastatin 10 further supports the interaction of this cytotoxic peptide with P-gp. Taken together, these findings suggest that resistance to dolastatin 10 is conferred, at least in part, by P-gp and that this cytotoxic peptide is a novel member of the MDR phenotype.

Key words: dolastatin 10; multidrug resistance; P-glycoprotein; cytotoxic peptides; peptide transport; tubulin binding drug

The dolastatins are small peptides isolated from the shell-less marine mollusk *Dolabella auricularia* [1]. Dolastatin 10 (Fig. 1), a member of this family with anticancer activity, is a pentapeptide that in addition to valine contains the unique amino acids dolavaline, dolaisoleucine and dolaproline linked to a dolaphenine amine [2]. This agent has *in vivo* activity against murine B16 melanoma, P388 leukemia and human melanoma xenografts in nude mice [2]. Dolastatin 10 is one of the most potent known anticancer agents *in vitro* with an IC_{50} for P388 cell growth of 5×10^{-11} M [2]. Several studies have suggested that the cytostatic effects of dolastatin 10 are mediated by an interaction with tubulin in the vinca alkaloid binding domain [3, 4]. Binding of dolastatin 10 to this domain has been associated with inhibition of tubulin polymerization and decay [3, 4]. These findings are in concert with the demonstration that this agent inhibits mitosis [3, 4]. The total synthesis of dolastatin 10 has recently made available sufficient quantities for preclinical evaluation of this agent [5]. This agent is now under development at the National Cancer Institute for clinical trials.

The present studies examined mechanisms that confer resistance to dolastatin 10. The results demonstrated that dolastatin 10 resistance is associated with expression of the *mdr*1 gene. We also demonstrated that dolastatin 10 binds to the P-gp*.

Materials and Methods

Cell culture. Parental and multidrug-resistant sublines of the murine PC4 erythroleukemia and human U-937 monoblastic leukemia cells were grown in BME and RPMI 1640 media, respectively, containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine. DUKX/CHO cells deficient in dihydrofolate reductase were transfected with the pED expression construct containing a full-length human *mdr*1 cDNA (designated pED H1). Control DUKX/CHO cells were transfected with the pED vector alone (designated pED) [6]. Both cell lines were grown in Eagle's α-MEM without nucleosides containing 10% dialysed fetal bovine serum and 5 nM methotrexate. Cells were exposed to dolastatin 10 (provided by the Drug Synthesis and Chemistry Branch, DTP, DCT, NCI) after solubilization in DMSO and dilution in culture media.

Cytotoxicity assays. The cytotoxicity of PC4 and U-937 cells grown in suspension was determined using the tetrazolium-based compound MTT [7]. Briefly, 100 mL of diluted drug or drug diluent alone was added to logarithmically growing cells (1×10^4/well) in a 96-well plate. After incubation for 72 hr, 50 mL of 3 mg/mL MTT diluted in PBS was added, and the cells were incubated for an additional 3 hr. Eighty milliliters of 23% SDS/50% dimethylformamide was then added, and the plates were incubated overnight at 37° to allow solubilization of the formazan crystals. The absorbance values for each well were determined in an ELISA reader (model MA310 Automated EIA Plate Reader, Whittaker M.A. Bioproducts, Inc., Walkersville, MD) at 550 nm. The IC_{50} values were obtained by a linear regression analysis of percent absorbance vs log drug concentration.

The cytotoxicity of monolayer DUKX/CHO cells was determined using the SRB colorimetric assay [8]. Briefly, 5×10^4 cells/well were incubated in the presence of drug for 72 hr. The cells were fixed by adding 50 mL of 50% trichloroacetic acid for 1 hr at 4°, washed, and then incubated in 0.4% SRB in 1% acetic acid for 20 min at room temperature. SRB was eluted with 10 mM Tris–HCl, pH 7.4, and absorbance was determined at 550 nm.

Photoaffinity labeling. Whole cell photoaffinity labeling was performed using [^{125}I]AAP (728 mCi/mmol; Dupont-New England Nuclear, Boston, MA). Cells (1×10^6/40 mL) were suspended in 0.15 M NaCl, 10 mM $CaCl_2$, 2 mM K_2PO_4, 4% (v/v) DMSO and 0.75 mCi [^{125}I]AAP for 1 hr at room temperature. The cells were irradiated with a UV 366 nm lamp (model UVL-56, UVP, San Gabriel, CA) for 15 min and then solubilized in SDS with 5% β-mercaptoethanol. Samples were heated at 100° for 2 min, and then separated in a 7.5% SDS-polyacrylamide gel; radioactive bands were visualized by autoradiography.

Results and Discussion

Previous studies have demonstrated that diverse natural products interact with P-gp [9]. In the present work we investigated whether resistance to the cytotoxic peptide

* Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; SRB, SulfonoRhodamine B; AAP, iodoaryl-azidoprazosin; BME, Basal Medium Eagle; MTT, 3-(4,5-dimethylthrazol-2-yl)-2,5-diphenyltetrazolium; MEM, Eagle's Minimum Essential Medium.

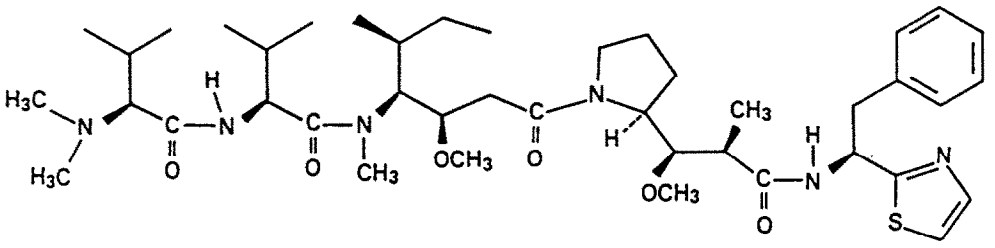

Fig. 1. Structural formula of dolastatin 10.

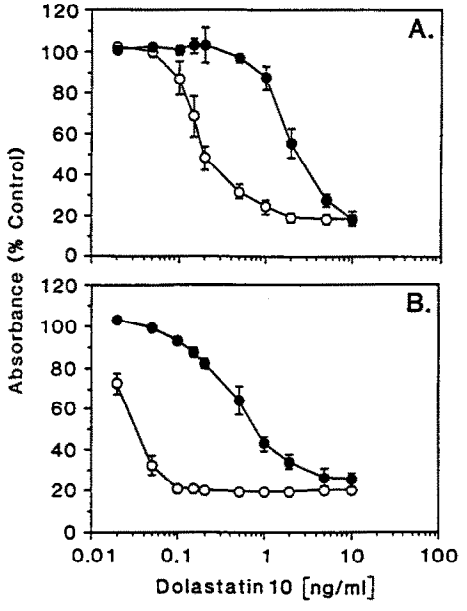

Fig. 2. The IC_{50} determinations of multidrug-resistant PC4 and U-937 cells exposed to dolastatin 10. (A) Wild-type PC4 (○) and vincristine-resistant PC-V160 (●) cells. (B) Wild-type U-937 (○) and vincristine-resistant UV40 (●) cells. Cells were exposed to the indicated concentrations of dolastatin 10 for 72 hr. Viability was determined by the MTT assay. Results are the means ± SEM of six (A) or four (B) separate experiments, each performed in quadruplicate.

dolastatin 10 is also conferred by P-gp. In addressing this issue, we used a multidrug-resistant subline of PC4 cells, designated PC-V160, which has been selected for resistance to vincristine (215-fold) and overexpresses murine *mdr*3 (*mdr*1a) as determined by northern and western analyses [10]. The IC_{50} of dolastatin 10 for parental PC4 cells was 0.2 ng/mL (Fig. 2A). In contrast, the vincristine-resistant PC-V160 cells demonstrated a 10.7-fold increase in resistance to this agent with an IC_{50} of 2.6 ng/mL (Fig. 2A). Similar findings were obtained with a multidrug-resistant derivative of U-937 cells, designated UV40, which was also selected for resistance (2000-fold) to vincristine and expresses the human *mdr*1 gene (data not shown). The IC_{50} of the parental U-937 cells to dolastatin 10 was 36 pg/mL, while that of the UV40 subline was 880 pg/mL (24-fold resistant) (Fig. 2B). These findings suggested that the MDR phenotype is associated with cross-resistance to dolastatin 10.

MDR is characterized by an energy-dependent, verapamil-reversible decrease in the accumulation and retention of certain anticancer agents through efflux mediated by P-gp [9, 11, 12]. Consequently, we asked whether verapamil would modulate resistance to dolastatin 10 in the PC-V160 line. The IC_{50} of dolastatin 10 for PC-V160 cells was decreased to 0.3 ng/mL by verapamil, such that this line was only 2.8-fold resistant compared with verapamil-treated parental PC4 cells (data not shown). The absence of complete reversal of dolastatin 10 resistance by verapamil suggested that in addition to P-gp, other mechanisms, perhaps involving tubulin, contribute to the sensitivity of cells to this cytotoxic peptide. Nonetheless, the demonstration that verapamil reversed dolastatin resistance in these lines provided further support for involvement of P-gp.

Since drug-selected cells can simultaneously express more than one mechanism of acquired drug resistance, we also studied cells that express human *mdr*1 sequences, but have not been exposed to agents of the MDR phenotype. DUKX/CHO cells were transfected with a human *mdr*1 cDNA [13] in the pED expression vector [6] (designated pED H1; Croop J, unpublished data). This construct generates a chimeric transcript with human *mdr*1 and dihydrofolate reductase, permitting selection with methotrexate to produce higher levels of human P-gp (data not shown). In contrast, DUKX/CHO cells transfected with pED vector alone (designated pED cells) were negative for human P-gp expression, whereas hamster P-gp was expressed at low levels (data not shown). The IC_{50} of dolastatin 10 for pED cells was 0.15 ng/mL (Fig. 3A). In contrast, the H1 line transfected with *mdr*1 was 14.1-fold resistant, with an IC_{50} of 2.15 ng/mL (Fig. 3A). Moreover, in the presence of verapamil, sensitivity of the *mdr*1 transfected cells decreased to 0.25 ng/mL or a 10-fold reduction in resistance to dolastatin 10 as compared with pED cells in the presence of verapamil (Fig. 3B). Taken together, these findings indicated that dolastatin 10 resistance is conferred by P-gp.

Photoaffinity labeling of P-gp has been used to demonstrate that agents involved in the MDR phenotype interact with P-gp [11, 14]. The α_1-adrenergic receptor photoaffinity probe [^{125}I]AAP, which binds to P-gp, was used in the present studies. For easier detection of photoaffinity-labeled P-gp, we also used a more resistant U-937 subline (UV1000), which expresses higher levels of P-gp compared with UV40 cells. Radiolabeling of the 170 kDa P-gp was detectable in the UV1000 subline, whereas there was little if any labeling observed with the parental U-937 cells (Fig. 4). Similar studies were performed in the presence of dolastatin 10. The results demonstrated that dolastatin 10 decreases radiolabeling of P-gp (Fig. 4). These findings provide evidence for interaction of dolastatin 10 with P-gp and support the association of P-gp expression with dolastatin 10 resistance.

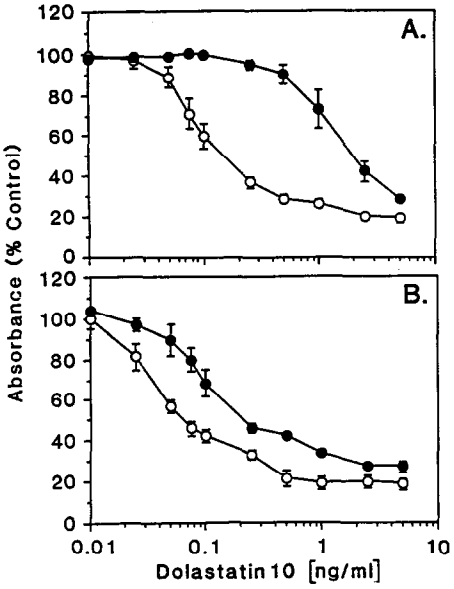

Fig. 3. Sensitivity of CHO cells expressing a human *mdr*1 cDNA to dolastatin 10 ± verapamil. (A) DUKX/CHO pED cells (○) and the pED H1 subline transfected with a human *mdr*1 cDNA (●) were exposed to the indicated concentrations of dolastatin 10 for 72 hr. (B) DUKX/CHO pED (○) and pED H1 (●) cells were exposed to 10 mg/mL verapamil and dolastatin 10 for 72 hr. Viability was determined by the SRB assay. Results are the means ± SEM of three separate experiments, each performed in quadruplicate.

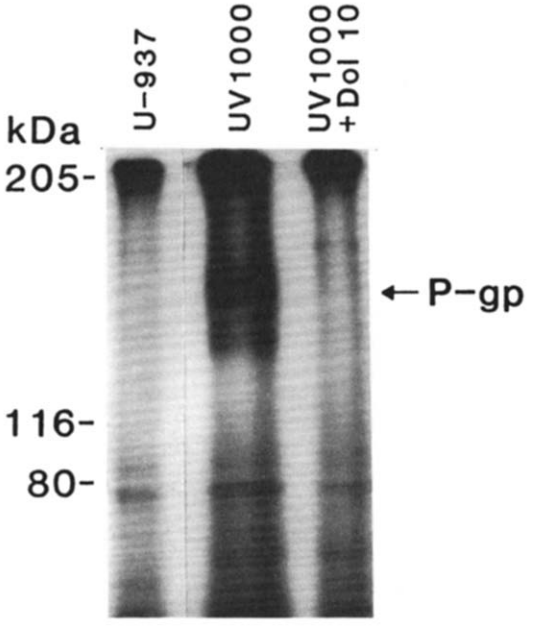

Fig. 4. Effect of dolastatin 10 on photoaffinity labeling of P-gp. UV1000 cells were incubated with [^{125}I]AAP in the absence and presence of 1.3×10^{-4} M dolastatin 10. The cells were subjected to UV irradiation at 366 nm. Dolastatin 10 has no detectable absorption at this wavelength (data not shown). The proteins were separated in a 7.5% SDS-polyacrylamide gel. Radioactive bands were visualized by autoradiography.

Previous work has demonstrated amino acid homology between P-gp and bacterial proteins, such as oppD and *hly*B, involved in peptide transport [15, 16]. The oppD locus encodes a membrane component for an oligopeptide permease in *Salmonella typhimurium* [17], whereas the *hly*B gene product from *Escherichia coli* transports the active component of hemolysin [15, 18]. Other peptide transporters related to P-gp have been identified in eukaryotes. For example, the STE6 gene of *Saccharomyces cerevisiae* encodes a P-gp homolog involved in the secretion of a 12 amino acid pheromone [19, 20]. Moreover, MHC class II region encoding proteins have been identified that are related to P-gp and are capable of transporting short peptides from the cytosol to the endoplasmic reticulum [21–23]. Taken together, these findings suggested that P-gp might also function as a peptide transporter. Although many agents such as valinomycin isolated from Streptomyces [24] and the antibacterial gramacidin D [25] may be substrates or modulators of P-gp, direct evidence for the role of P-gp as a peptide transporter has been demonstrated only for the synthetic, cytotoxic tripeptide, *N*-acetyl-leucyl-leucyl-norleucinol [26]. The present results with dolastatin 10 further demonstrate the involvement of P-gp in the secretion of peptides and support the possibility that a physiologic role of the *mdr*1 gene product is the transport of peptides or cellular proteins.

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