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TAMOXIFEN AZIRIDINE, A NOVEL AFFINITY PROBE FOR P-GLYCOPROTEIN IN MULTIDRUG RESISTANT CELLS

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Summary: In this study for the first time we used an electrophilic analog of tamoxifen, [^{3}H]tamoxifen aziridine, and demonstrated that it covalently and specifically binds to P-glycoprotein in multidrug resistant cells. Tamoxifen and its metabolites, N-desmethyltamoxifen and 4-hydroxytamoxifen, were potent inhibitors of [^{3}H]tamoxifen aziridine binding to P-glycoprotein with 4-hydroxytamoxifen > tamoxifen > N-desmethyltamoxifen. The multidrug resistance-related drugs inhibited [^{3}H]tamoxifen aziridine binding with vinblastine > vincristine > doxorubicin > actinomycin D, while colchicine enhanced the binding. Moreover, the multidrug resistance modulators verapamil, nicardipine, diltiazem, prenylamine, cyclosporin A, FK506, dibucaine, reserpine, monensin and progesterone were all potent inhibitors of [^{3}H]tamoxifen aziridine binding to P-glycoprotein. Our data provide the first evidence that [^{3}H]tamoxifen aziridine directly binds to P-glycoprotein and interacts with the binding sites for multidrug resistance-related drugs and modulators.

Cross-resistance of cancer cells to many drugs which show little structural similarity (1, 2) is termed multidrug resistance (MDR). These drugs include Vinca alkaloids, anthracyclines, epipodophyllotoxins, colchicine, taxol, puromycin, actinomycin D, trimetrexate, gramicidin D and some other drugs (1, 2). The distinguishing characteristic of MDR cells is overexpression of a 150-180 kDa membrane phosphoglycoprotein, P-glycoprotein (P-gp) (3). Sensitive cells transfected with the P-gp gene (MDR1) acquire MDR (1). Drugs involved in the MDR phenotype bind specifically to P-gp (2). A wide variety of compounds such as calcium channel blockers, calmodulin antagonists, cyclosporin and its nonimmunosuppressant analogs, progesterone and tamoxifen can enhance the toxicity of MDR-related drugs (2, 4). These agents

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Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; TAM, tamoxifen; NDT, N-desmethyltamoxifen; 4-OH-TAM, 4-hydroxytamoxifen; [^{3}H]TAMA, [^{3}H]tamoxifen aziridine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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bind to P-gp and inhibit the binding and transport of cytotoxic agents out of MDR cells. Previously we have demonstrated that P-gp binds specifically to photoaffinity analogs of vinblastine, colchicine, dihydropyridine calcium channel blocker azidopine, verapamil, the α_1-adrenergic receptor antagonist prazosin (2) and the D_2-dopamine receptor photoaffinity probe iodoazidospiperone (5).

We have previously found that tamoxifen (TAM) and its metabolites, N-desmethyl-tamoxifen (NDT) and 4-hydroxytamoxifen (4-OH-TAM) are promising MDR inhibitors which can reverse MDR at concentrations achievable in vivo (6). TAM and these metabolites stimulate endogenous phosphorylation of P-gp similar to the effects of verapamil and phorbol ester, enhance the accumulation of the cytotoxic agent vinblastine in MDR cells and induce P-gp ATPase activity (6, 7). Since electrophilic agents are generally considered to label receptors efficiently and specifically, in this study, we used [^{3}H]tamoxifen aziridine ([^{3}H]TAMA), an electrophilic analog of tamoxifen (8, 9) and demonstrated that it binds directly and specifically to P-gp. Moreover, we found that the binding of [^{3}H]TAMA is inhibited by TAM, NDT, 4-OH-TAM, some MDR-related drugs and many MDR modulators. Therefore, [^{3}H]TAMA is the first chemical affinity analog of a drug ever shown to specifically bind to P-gp at a site(s) with which many cytotoxic agents and MDR modulators interact.

Materials and Methods

Materials. [^{3}H]TAMA (24 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Protein A-Sepharose was purchased from Pharmacia LKB (Piscataway, NJ). Vinblastine and vincristine were generously supplied by Eli Lilly and Co. (Indianapolis, IN). Cyclosporin A was obtained from Sandoz (Basel, Switzerland). FK506 was obtained from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan).

Monoclonal antibody C219 (3) specific for P-gp was purchased from Signet Laboratories, Inc. (Dedham, CA). Actinomycin D, colchicine, doxorubicin, verapamil, nicardipine, diltiazem, prenylamine, dibucaine, reserpine, progesterone and TAM were purchased from Sigma (St. Louis, MO). Monensin was obtained from Calbiochem (La Jolla, CA). NDT and 4-OH-TAM were obtained from Imperial Chemical Industries (Cheshire, UK).

Cell Culture. Sensitive DC-3F Chinese hamster lung cells and MDR variant DC-3F/VCRd-5L cells (2400-fold resistance to vincristine) were grown as previously described (10). DC-3F/VCRd-5L cells were maintained in 50 μg/ml vincristine. Vincristine was removed from the cultures one week before experiments.

Affinity Labeling with [^{3}H]TAMA and Immunoprecipitation. Exponentially growing cells were harvested by rubber blade. Trypan blue viable (>90%) cell suspensions (5 x 10^5 cells/assay) in Ca^{2+}-Mg^{2+}-free Dulbecco's phosphate-buffered saline were incubated in the absence or presence of MDR-related drugs and modulators in a final volume of 0.05 ml with 0.8 μM [^{3}H]TAMA (24 Ci/mmol) for 1 h. For immunoprecipitation with the anti P-gp monoclonal antibody C219 (3), 5 x 10^6 DC-3F/VCRd-5L cells were affinity labeled with [^{3}H]TAMA, detergent solubilized and immunoprecipitated as previously described (11). Samples were analyzed by 5-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 4.5M urea followed by fluorography (11). Quantitation of radioactivity was accomplished by densitometry tracing of fluorograms.

Results and Discussion

Affinity labeling of sensitive DC-3F and MDR variant DC-3F/VCRd cells with 0.8 μM [^{3}H]TAMA (Fig. 1), SDS-PAGE and fluorography revealed that this electrophilic analog of tamoxifen binds to a number of proteins in both sensitive and MDR variant cells (Fig. 2). In the resistant cells a prominently labeled protein with a molecular weight of 150-180 kDa was evident (Fig. 2). The 150-180 kDa protein was immunoprecipitated with the anti-P-gp monoclonal antibody C219; therefore, it is P-gp (Fig. 2). The specificity of [^{3}H]TAMA binding was determined by performing experiments in the presence of excess nonradioactive TAM, 4-OH-TAM and NDT (Fig. 2). While several proteins were labeled with [^{3}H]TAMA, only P-gp was specifically labeled since 10 μM TAM, 4-OH-TAM or NDT inhibited the binding of the probe to P-gp significantly but did not effect the other labeled proteins (Fig. 2). The binding between [^{3}H]TAMA and P-gp appears very stable since the label remains with P-gp following treatment with 2% SDS solution and 1.25% dithiotreitol at 100°C for 5 min. At 10 and 100 μM, TAM inhibited the binding of [^{3}H]TAMA to P-gp by 75 and 100%, respectively. Similarly, NDT at 10 and 100 μM inhibited the binding of the affinity probe to P-gp by 54 and 91%, respectively. 4-OH-TAM was the most effective inhibitor of [^{3}H]TAMA binding to P-gp, and at 10 and 100

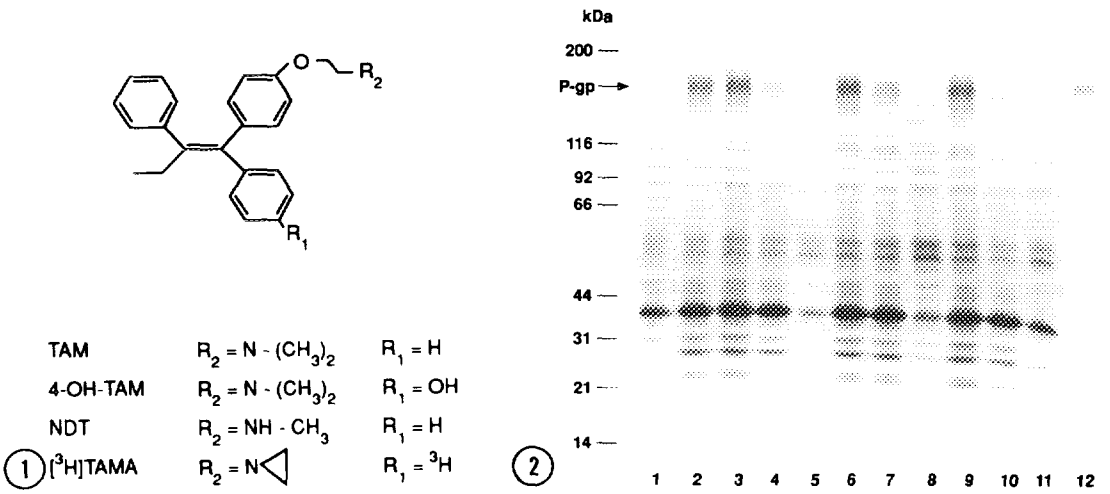

Fig. 1. Chemical structures of TAM, NDT and 4-OH-TAM.

Fig. 2. SDS-PAGE fluorography of [^{3}H]TAMA affinity labeled drug sensitive Chinese hamster lung DC-3F (lane 1) and drug-resistant DC-3F/VCRd-5L cells (2-11) in the absence (lanes 1 and 2) or presence of 1, 10 and 100 μM TAM (lanes 3-5); 1, 10, and 100 μM NDT (lanes 6-8); and 1, 10, and 100 μM 4-OH-TAM (lanes 9-11). Affinity labeling was carried out with 0.8 μM [^{3}H]TAMA and 5 x 10^5 cells/assay. For immunoprecipitation (lane 12), 5 x 10^6 cells were affinity labeled with [^{3}H]TAMA after detergent solubilization (11), P-gp was immunoprecipitated by the anti-P-gp monoclonal antibody C219 (3).

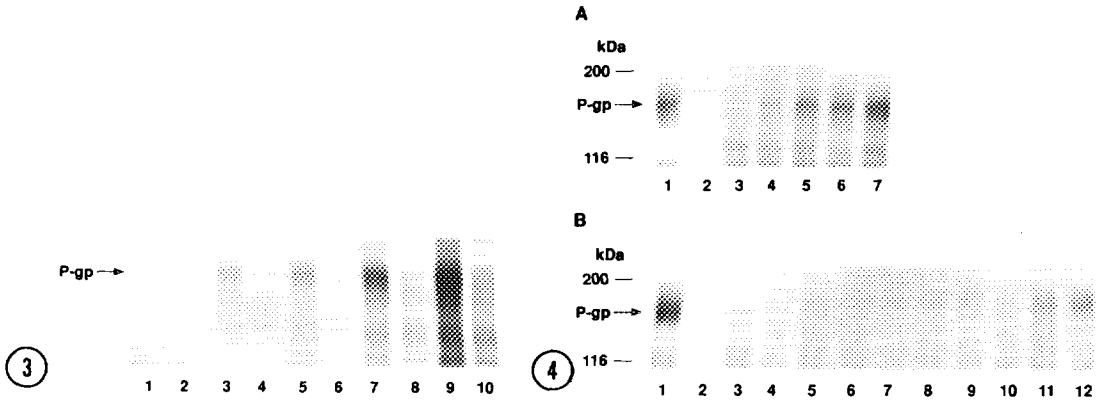

Fig. 3. SDS-PAGE fluorography of [^{3}H]TAMA affinity labeled drug resistant DC-3F/VCRd-5L cells. Affinity labeling was carried out with 5 x 10^5 cells/assay and 0.3, 0.4, 0.5, 1 and 2 μM [^{3}H]TAMA in the absence (lanes 1, 3, 5, 7 and 9) or presence of 100 μM TAM (lanes 2, 4, 6, 8 and 10), respectively.

Fig. 4. (A) SDS-PAGE fluorography of [^{3}H]TAMA affinity labeled DC-3F/VCRd-5L cells (lanes 1-7) in the absence (lane 1) or presence (lanes 2-7) of 100 μM of TAM, vinblastine, vincristine, doxorubicin, actinomycin D and colchicine, respectively. The positions of molecular mass standards in kilodaltons and P-gp are indicated at the left. (B) SDS-PAGE fluorography of [^{3}H]TAMA affinity labeled DC-3F/VCRd5L cells (lanes 1-12) in the absence (lane 1) or presence (lanes 2-12) of 100 μM TAM, verapamil, nicardipine, diltiazem, prenylamine, cyclosporin A, FK506, dibucaine, reserpine, monensin and progesterone, respectively. The positions of molecular mass standards in kilodaltons and P-gp are indicated at the left.

μM it inhibited the binding by 92 and 98%, respectively. [^{3}H]TAMA also labeled P-gp in other MDR cell lines tested including human SHSY-5Y/VCR neuroblastoma cells and KB-V1 epidermoid carcinoma cells, and the labeling was inhibited by TAM, NDT and 4-OH-TAM. These results show that [^{3}H]TAMA is an affinity analog capable of specifically and covalently binding to P-gp. These data and our previous work showing that TAM, 4-OH-TAM and NDT reverse vinblastine resistance by increasing its accumulation in MDR cells (6) suggest that their interaction with P-gp may prevent the efflux of vinblastine from MDR cells.

The specificity of P-gp labeling with [^{3}H]TAMA was characterized in more detail by affinity labeling in intact cells using increasing concentrations of [^{3}H]TAMA (0.2-2 μM) in the presence of 100 μM TAM (Fig. 3). Under these conditions, P-gp radiolabeling increased with increasing concentrations of [^{3}H]TAMA and 100 μM TAM inhibited the labeling, suggesting that at the concentration range of 0.2-2 μM [^{3}H]TAMA binds to P-gp specifically. When 2 μM [^{3}H]TAMA was used in the affinity labeling experiments, 0.3 pmol/10^6 cells were specifically bound to P-gp.

To examine whether the [^{3}H]TAMA affinity labeling is inhibited by the MDR-related drugs, affinity labeling in the presence of 100 μM of vinblastine, vincristine, doxorubicin, actinomycin D, and colchicine was performed (Fig. 4A and Table 1). [^{3}H]TAMA affinity

Table 1: Effect of MDR-Related Drugs and MDR modulators on [^{3}H]TAMA Affinity Labeling of P-gp

Drugs	[^{3}H]TAMA (% Control)
Control	100
Vinblastine	12
Vincristine	30
Doxorubicin	87
Actinomycin D	95
Colchicine	180
Verapamil	9
Nicardipine	4
Diltiazem	9
Prenylamine	3
Cyclosporin A	4
FK506	7
Dibucaine	11
Reserpine	6
Monensin	20
Progesterone	21

Affinity labeling was carried out using 0.8 μM [^{3}H]TAMA in the absence or presence of 100 μM of drugs as described in Materials and Methods. Quantitation was carried out by densitometric analysis of SDS-PAGE autoradiograms.

labeling of P-gp was inhibited by vinblastine > vincristine > doxorubicin > actinomycin D. However, colchicine enhanced the binding of [^{3}H]TAMA to P-gp (Table 1), suggesting that colchicine may bind to a separate site and allosterically enhance the binding of the affinity probe. Consistent with these results, we have previously demonstrated that Vinca alkaloids are the most effective inhibitors of photoaffinity drug analogs that interact with P-gp (2).

Many lipophilic, cationic agents can reverse MDR by competitive and noncompetitive interaction with the cytotoxic drug binding sites of P-gp (2, 12-14). Furthermore, we recently demonstrated that P-gp may interact with some of its substrates stereoselectively (5). To examine whether MDR modulators inhibit the binding of [^{3}H]TAMA to P-gp, we used several agents known to reverse MDR in affinity labeling experiments (Fig. 4B). At 100 μM, verapamil, nicardipine, diltiazem, prenylamine, cyclosporin A, FK506 and reserpine inhibited [^{3}H]TAMA affinity labeling of P-gp by greater than 90% (Table 1). Dibucaine, monensin, and progesterone inhibited the [^{3}H]TAMA affinity labeling by 89, 80, and 79%, respectively (Table 1). These data suggest that [^{3}H]TAMA labels a site on P-gp with which many MDR modulators can also interact. However, whether TAM is effluxed by P-gp or whether it simply binds to P-gp and prevents efflux of the cytotoxic agent remains to be determined. Drug binding and transport studies have identified two classes of MDR modulators: (a) those which are transported by P-gp

and block both the initial binding and transport of anticancer agents out of the cells (e.g., cyclosporin A, verapamil, azidopine and iodoazidospiperone) (5, 12-15), and (b) those which are not transported by P-gp but circumvent MDR by only blocking the binding of the cytotoxic agent to P-gp (e.g., progesterone and CP-1100356) (16-18).

The specific amino acid residues involved in the drug binding sites of P-gp have not been identified. However, we recently reported that modulators of P-gp may interact with separate or overlapping sites on P-gp (5). We do not have any information as to the nature of the site on the P-gp molecule to which [^{3}H]TAMA covalently binds. However, as proposed for [^{3}H]TAMA binding to the estrogen receptor (8), the functional group aziridine can be selectively activated in the tamoxifen binding site of P-gp, presumably by accepting a proton to form a highly reactive aziridium ion, which in turn can alkylate a nearby nucleophilic group. This covalent labeling process resembles the selective activation of enzyme suicide inactivators in the active site of enzyme. [^{3}H]TAMA has been shown to covalently and specifically label cysteine 530 of the human estrogen receptor (9) suggesting that it may also label a cysteine residue in the TAM binding site of P-gp.

Our data provide the first direct evidence that [^{3}H]TAMA is a specific affinity-labeling agent for P-gp. This is the first affinity analog of P-gp substrates capable of covalently and specifically interacting with P-gp without photoactivation. This affinity agent can be used in characterizing the TAM binding site of P-gp. Moreover, this work offers insight into the synthesis and development of novel electrophilic analogs of cytotoxic agents and MDR modulators for unraveling the architecture of the drug binding sites of P-gp and its inactivation.

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