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PHOTOAFFINITY SUBSTRATES FOR P-GLYCOPROTEIN

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Abstract—A variety of compounds can inhibit the function of P-glycoprotein (Pgp) by binding to it and preventing the efflux of anticancer drug substrates. While the molecular architecture of the drug binding site(s) in Pgp is not known, it is clear that modulators in general appear to conform to some general physical–chemical rules. In this paper, we discuss the basic concepts of drug recognition by Pgp as currently understood. We also examine the compounds used to photoaffinity label this protein and discuss their utility in identifying drug binding sites. Finally, we show that a photoaffinity analog of daunorubicin, [^{3}H]azidobenzoyl-daunorubicin ([^{3}H]AB-DNR), is a good affinity labeling reagent for Pgp. A finding of interest is that vinblastine and verapamil compete more effectively than daunorubicin for [^{3}H]AB-DNR binding to Pgp, suggesting that vinblastine and verapamil have similar structural features not shared by daunorubicin.

We have learned in recent years that the extensively described experimental phenomenon of multidrug resistance (MDR‖) [1] may be a factor in some of the failures of clinical cancer chemotherapy. Different tumor types have been shown to express the *MDR*1 gene or Pgp after chemotherapy, or even before its start [2, 3]. Recent results with pediatric soft tissue sarcomas suggest expression of Pgp may be predictive of clinical response [4]. Although non-Pgp forms of natural product MDR have also been described experimentally [5, 6], they have not yet been demonstrated clinically. Because of the likely role of Pgp in clinical drug resistance, many investigations have focussed on strategies to inhibit the action of this protein [7–9]. By far the largest effort includes screening and rational design of Pgp-inhibitory chemicals. More recent studies are beginning to examine other methods of blocking the action of Pgp and include the use of modulator combinations, inhibitory antibodies, and antisense oligonucleotides [7–15]. The compounds that modulate Pgp-MDR represent a wide range of chemical structures and drug classes and include detergents, hormones, antibiotics, antihypertensives, antimalarials, and immunosuppressives. The structures of many of these agents and descriptions of their Pgp-inhibitory effects are presented in a recent review [8]. To better understand the relationship of drug structure to its action in inhibiting Pgp, we and others have studied various classes of compounds for their modulating activity [16–22]. We have tried to understand the results in terms of the MDR pharmacophore [17], i.e. the basic molecular unit(s) that will inhibit Pgp action. It is possible that there are several pharmacophore classes.

Basic concepts of drug recognition by Pgp: structure–activity studies

Are there common structural determinants for modulators of Pgp-MDR? We showed previously that verapamil and vinblastine shared a *common volume element* and suggested that in three dimensions these compounds could resemble each other [16]. Subsequent analysis by Pearce *et al.* [23] developed the concept of *modulator index* (MI), which is the -fold decrease in anticancer drug IC_{50} produced by the modulator divided by the modulator concentration. Among five modulators examined there was a strong correlation between the modulator index and the *calculated molar refractivity* of the compound suggesting that molecular volume, which is the simplest measure of molecular size, is directly associated with the effectiveness of the modulator.

We originally proposed physical–chemical "rules" for modulators of Pgp-MDR from the study of a series of indole and other alkaloidal compounds [16]. Based on our findings, we established that an ideal modulator of Pgp-MDR would have at least two planar aromatic rings, a tertiary nitrogen that would be charged at physiological pH and would be relatively lipophilic. Despite these observations, it was still not clear whether Pgp recognized specific drug structures or whether it simply formed a "greasy channel" for certain lipophilic molecules of general shape and size. We directly tested these rules by designing a series of analogs based on the reserpine molecule which had proven to be one of the better modulators in our system [16, 24]. Our results revealed that the relative disposition of aromatic rings and a basic nitrogen atom were clearly important for Pgp modulatory action [17] and suggested that there was indeed structural specificity for drug recognition by Pgp. Subsequent work by others has also supported this notion that structural

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‖ Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; NASV, *N*(*p*-azido-3-[^{125}I]salicyl)-*N'*-(β-aminoethyl)vindesine; AB-DNR, azidobenzoyl-daunorubicin; LU49888, ((*S*)-5-[(3-azidophenylethyl)-[*N*-methyl-^{3}H]-methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile; iodo-aryl-azido-prazosin, 2-[4-(4-azido-3-[^{125}I]iodobenzoyl)piperazin-1-yl)-4-amino-6,7-dimethoxyquinazoline.

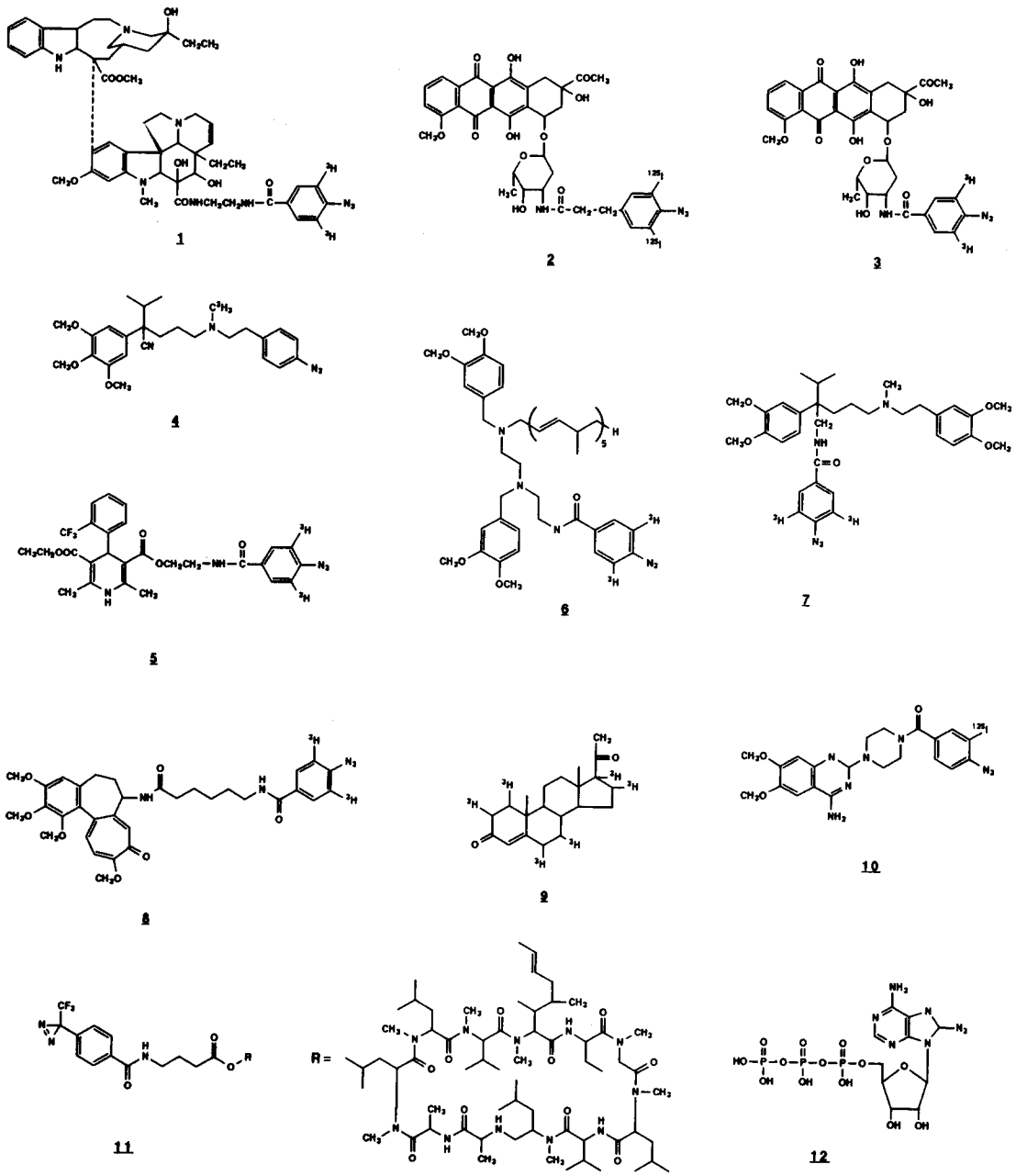

Fig. 1. Structures of photoaffinity analogs shown to covalently label Pgp. (1) NASV [25]; (2) iodomycin [26]; (3) AB-DNR [27, 28]; (4) LU49888 [30]; (5) azidopine [32, 33]; (6) synthetic isoprenoid: *N*-solanesyl-*N*,*N*'-bis(3,4-dimethoxybenzyl)ethylenediamine [35]; (7) *N*-(*p*-azido[3,5-^{3}H]benzoyl)-aminomethyl verapamil [31]; (8) *N*-(*p*-azido[3,5-^{3}H]benzoyl)aminohexanoyldeacetyl colchicine [29]; (9) progesterone [37]; (10) iodo-aryl-azido-prazosin [34]; (11) cyclosporin A [36]; (12) 8-azido ATP [38].

features are important in modulator action [18–22]. It also appears that, in addition to lipophilicity, the cationic charge is critical suggesting that the modulator may recognize specific amino acids or charged domains. Despite these results, however, the common structural determinants for Pgp modulators and binding sites on Pgp for these chemicals remain to be established.

Toward identification of drug binding sites on Pgp: photoaffinity labeling studies

Knowledge of the drug and modulator binding sites on Pgp will be invaluable not only to an understanding of how drugs interact with Pgp but also to the design of better and more specific inhibitors of this protein. One way to identify drug binding sites on Pgp is to use photoaffinity analogs

of compounds that are substrates for or inhibitors of this protein. Since the original observation by Safa *et al.* [25], who synthesized a photoaffinity analog of vinblastine NASV to label Pgp [25], a number of other compounds have been used to affinity label Pgp. We thought that it would be useful to show these structures together, which has not been done before, in order to get a better sense of the variety of reagents that have been used. Most of the reported Pgp-labeling agents are shown in Fig. 1. Inspection of the Figure reveals similarities as well as marked differences among the 12 compounds. Compounds 1–11 represent a variety of different structures and drug classes while compound 12 is an ATP analog. Among the anticancer drugs, photoaffinity analogs of vinblastine (1 [25]), daunorubicin (2 [26] and 3 [27, 28]), and colchicine (8 [29]) have been shown to label Pgp. Of the modulators, photoaffinity analogs of verapamil (4 [30] and 7 [31]) and other calcium channel blockers (5 [32, 33] and 10 [34]) do the same. Furthermore, a photoaffinity analog of a synthetic isoprenoid resembling verapamil (6 [35]) and a photoaffinity analog of cyclosporin A (11 [36]) likewise label Pgp. Progesterone (9), which is inherently photoactivatable, also covalently binds to Pgp when activated by UV light [37]. It is likely that most of these compounds label similar or closely related sites on Pgp since other modulators and anticancer drugs can generally compete with them for binding to this protein. Compound 12, 8-azido-ATP, also affinity-labels Pgp [38] but this labeling is not competed by these other agents, presumably because binding is to the nucleotide binding site(s) on Pgp which clearly differ from the aforementioned putative drug binding sites.

A few laboratories have used azidopine (5, Fig. 1) or iodo-aryl-azido-prazosin (10, Fig. 1) to photolabel Pgp and identify those peptide(s) in the protein to which these agents bind. Using antibodies that recognize distinct cytoplasmic peptides in Pgp, Yoshimura *et al.* [39] showed that azidopine photolabeled a peptide of ≤55 kDa in the carboxy terminus of Pgp. Greenberger *et al.* [40] recently reported preliminary results localizing the binding site of iodo-aryl-azido-prazosin to a similar region of Pgp. In neither case were the amino acids bound by these affinity labels identified. Using photoaffinity analog of verapamil, LU49888 (4, Fig. 1), Striessnig *et al.* [41] localized the verapamil binding site on the α1 subunit of the calcium channel to domain IV containing membrane-spanning segment 6 and the beginning of the long cytoplasmic carboxy terminus, a region apparently homologous to the modulator binding sites of Pgp [39, 40]. We are using the same compound, LU49888, to attempt to identify the verapamil binding site(s) in Pgp. Our preliminary results (Qian and Beck, unpublished) suggest that this compound also labels at least one peptide in the carboxy-terminal portion of the protein. Thus, it appears that results from photoaffinity labeling studies suggest that calcium channels and Pgp have domains or structures in common and also suggest a basis for the effectiveness of calcium channel blocking agents in inhibiting Pgp. Still to be determined are the amino acids in Pgp that are labeled by these reagents.

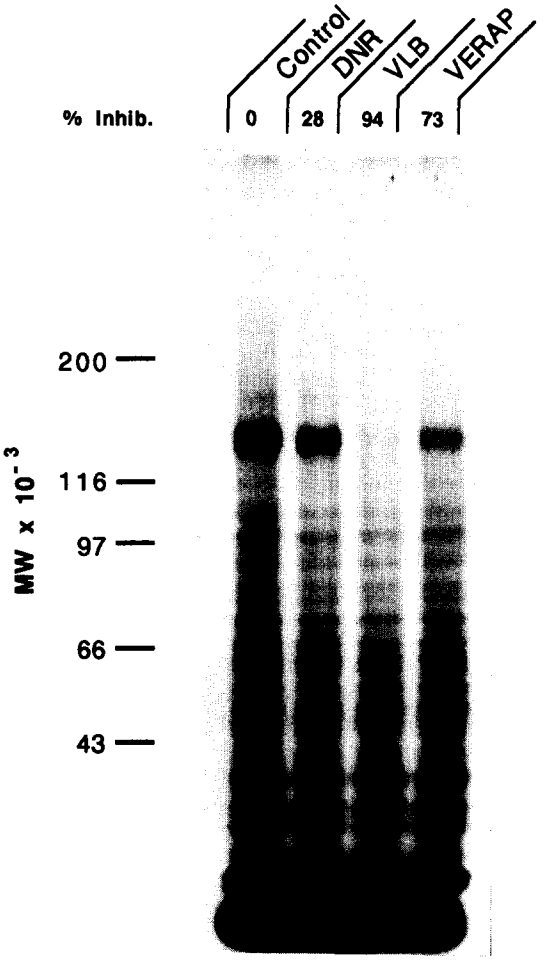

Fig. 2. Photoaffinity labeling of plasma membranes from CEM/VLB$_{5K}$ cells by [^{3}H]AB-DNR. AB-DNR was synthesized as described in the text. Cell membrane fractions (200 μg protein were prepared from CEM/VLB$_{5K}$ cells as described [30]). Photoaffinity labeling was as described previously [30], using increasing concentrations of AB-DNR. Labeled proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detected by fluorography [30]. Identity of Pgp was confirmed by immunoprecipitation and quantitation of labeled Pgp was by densitometry [30].

Photoaffinity labeling of Pgp with [^{3}H]*AB-DNR*

We have recently taken a fresh look at an "old" photoaffinity labeling reagent, [^{3}H]AB-DNR (compound 4, Fig. 1). This compound, originally synthesized by Felsted *et al.* [27], had been shown to label an 18 kDa mitochondrial protein, but its ability to label Pgp had not been described. Furthermore, using the vinblastine analog NASV to label Pgp, daunorubicin was shown to be a poor competitor compared to other Pgp substrates [42], suggesting that these drugs either had different binding affinities or binding sites on Pgp. We

synthesized AB-DNR as follows: 50 μL of daunorubicin (1 mg/mL) in dimethyl sulfoxide/chloroform was mixed with 50 μL (16 μM) of [^{3}H]-*N*-hydroxysuccinimidyl azidobenzoate. After keeping the reaction mixture in the dark at room temperature overnight, it was spotted on a silica gel TLC plate which was developed in ethyl acetate/hexane (3:1). The product of the reaction, [^{3}H]AB-DNR, was visualized by autoradiography and purified by scraping the authentic band from the plate. Our preliminary results reveal that this drug does indeed label Pgp, as seen in Fig. 2, suggesting that it will be a useful Pgp-labeling reagent.

In our earlier studies [30, 37], we showed that LU49888 (the verapamil analog; 4, Fig. 1) and progesterone (9, Fig. 1) could readily photoaffinity label Pgp. Of interest and consistent with earlier studies using NASV [24], two of the most potent drugs that inhibited labeling of Pgp by these agents were verapamil and vinblastine. We now report that this is also true for AB-DNR. As seen in Fig. 2, vinblastine and verapamil inhibited strongly AB-DNR labeling of Pgp by 94 and 73%, respectively. By contrast, the parent compound daunorubicin had minimal inhibitory activity (28%). These results prompt two conclusions: (1) the binding affinity of daunorubicin to Pgp is probably lower than that of vinblastine and verapamil, supporting the earlier findings of Cornwell *et al.* [42]; and (2) vinblastine and verapamil probably have similar three dimensional structures and bind tightly to a common site(s) in Pgp. This would be consistent with our earlier observation that vinblastine and verapamil share a common volume element [16], as discussed above.

Conclusions

It is clear that a variety of compounds can inhibit Pgp function and many appear to do this by binding to the protein. The molecular architecture of the drug binding site(s) in Pgp and the drugs themselves are not yet known, although modulators in general appear to conform to some general physical–chemical rules. Photoaffinity analogs of anticancer drugs that are substrates for Pgp and others that block Pgp function all appear to bind to Pgp but it is not known if they bind to the same site(s) on the protein. These affinity analogs have proven to be useful in identifying binding peptides in Pgp and should help to locate the amino acids in Pgp involved in drug binding.

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