0026-895X/89/040414-08$02.00/0

MOLECULAR PHARMACOLOGY, 35:414–421

Preparation and Utility of a Radioiodinated Analogue of Daunomycin in the Study of Multidrug Resistance

ROGER BUSCHE, BURKHARD TÜMMLER, JOHN R. RIORDAN, and DANIELLE F. CANO-GAUCI

Zentrum Biochemie II, Abt. Biophysikalische Chemie, Medizinische Hochschule Hannover, Hannover, West Germany (R.B., B.T.) and Research Institute, The Hospital for Sick Children and Departments of Biochemistry and Clinical Biochemistry, University of Toronto, Canada M5G 1X8 (J.R.R., D.F.C.-G.)

Received July 11, 1988; Accepted January 27, 1989

SUMMARY

Anthracyclines are an important class of cytotoxic drugs that are frequently used in cancer chemotherapy, especially in acute leukemia. The pharmacokinetics and disposition of these compounds in whole animals and in cells have been studied employing ^{3}H-labeled forms. However, their usefulness is limited by their low specific activities and the low energy of ^{3}H. Therefore, we have labeled daunomycin using ^{125}I-Bolton-Hunter reagent. The resultant anthracycline analogue, iodomycin, has a specific activity of approximately 2000 Ci/mmol. Although this compound was 10-fold less toxic to normal cells than daunomycin, multidrug-resistant cells were cross-resistant to it. Like other drugs to which these cells are cross-resistant, its accumulation by them was greatly reduced, compared with drug-sensitive cells. We have also utilized this compound in photoaffinity labeling experiments to identify its target in multidrug-resistant cells. We observed the specific binding of iodomycin to P-glycoprotein in membrane vesicles as well as in intact cells, thereby directly demonstrating that this protein specifically binds anthracyclines as well as *Vinca* alkaloids.

Anthracyclines are a widely used class of anticancer drugs (1). Major problems with their use in chemotherapeutic regimens include toxicity to normal tissues, especially cardiac tissue (2), and the outgrowth of drug-resistant tumor cell populations (3–5). The latter phenomenon is frequently associated with the development of MDR, which is characterized by resistance to several unrelated compounds, reduced cellular accumulation of these drugs, and the overexpression of a 170-kDa plasma membrane protein, P-glycoprotein (6). The expression of P-glycoprotein in MDR cells had been correlated with the level of resistance to antineoplastic agents (3). Evidence that P-glycoprotein is causative in MDR was provided by the demonstration that P-glycoprotein cDNA could confer resistance on otherwise drug-sensitive cell lines (7, 8).

Several studies have reported the direct binding of *Vinca* alkaloids, another class of antineoplastic agents, to P-glycoprotein in membranes of MDR cells (9, 10). The binding of anthracyclines to P-glycoprotein has not been reported. Yee *et al.* (11) had previously utilized daunomycin to photolabel mouse Sarcoma 180 cells. The anthracycline target was most likely a plasma membrane protein; it was shown to be on the cell surface, trypsin sensitive, DNase insensitive, and not chloroform/methanol extractable. However, it was not specifically identified. The usefulness of radioactive anthracyclines presently available for such studies is limited by their low specific activities and low energies. For this reason, we attempted to synthesize a higher specific activity analogue of daunomycin. This was accomplished by reacting daunomycin with ^{125}I-labeled Bolton-Hunter reagent (12). This reagent [iodinated 3-(4-hydroxyphenyl) propionic acid *N*-hydroxysuccinimide ester] is an ^{125}I-labeled acylating agent that reacts with free amino acids. Thus, the ^{125}I-labeled ester forms an amide bond via the amino group on the daunosamine moiety of daunomycin, resulting in a new anthracycline that we have called iodomycin. Our aim in synthesizing this reagent was to investigate the potential interaction of anthracyclines with P-glycoprotein of MDR cells. We first established that the compound was still recognized by cells with this phenotype, inasmuch as they were shown to be cross-resistant to it. In addition, iodomycin is maintained at reduced intracellular levels by resistant cells. On photoactivation, it was found to bind specifically to P-glycoprotein in isolated plasma membrane vesicles and also in intact cells. This binding is inhibited by several other drugs known to be specifically recognized by MDR cells.

Experimental Procedures

Materials

Growth medium was obtained from the Ontario Cancer Institute (Toronto, Ontario, Canada), fetal calf serum from Flow Laboratories

---

This work was supported by a Studentship to D.F.C.-G. and a grant from the Medical Research Council of Canada.

ABBREVIATIONS: MDR, multidrug resistance; IC$_{50}$, concentration of drug which is inhibitory to growth of 50% of cells; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

(Mississauga, Ontario, Canada). ^{125}I-Bolton-Hunter reagent (2000 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). [^{3}H-G]Daunomycin (4.9 Ci/mmol) was obtained from NEN (Boston, MA). Unlabeled daunomycin, verapamil hydrochloride, colchicine, and nifedipine were from Sigma Chemical Co. (St. Louis, MO), vinblastine sulfate from Eli Lily (Toronto, Ontario, Canada). Nitrendipine was kindly supplied by Dr. Scriabine of Miles Pharmaceuticals (New Haven, CT), the tiapamil analogue RO 11-2933/001 by Prof. H. Ramuz of F. Hoffmann-La Roche & Co. (Basel, Switzerland). Reagents for SDS-PAGE were obtained from Bio-Rad (Richmond, CA) except for Pyronin Y, which was from Eastman Kodak (Rochester, NY). Fluoram was from Roche Diagnostics (Nutley, NJ). Firefly luciferase-luciferin reagent was obtained from Sigma. ATP used for standards in the ATP assay as well as DNase I were obtained from (Dorval, Quebec, Canada).

Preparation of ^{125}I-Labeled Daunomycin Derivative

^{125}I-labeling of daunomycin was performed by adding 40 nmol of daunomycin in 1:1 benzene/dimethyl formamide to 0.4 nmol of ^{125}I-Bolton-Hunter reagent [iodinated 3-(4-hydroxyphenyl) propionic acid *N*-hydroxy succinimide ester]. The reaction was allowed to proceed for 24 hr at room temperature in the dark. During this time approximately 80% of the Bolton-Hunter reagent reacted with the daunomycin. The reaction mixture was chromatographed on silica gel 60 using a solvent system of ethyl acetate/diethyl ether (2:1). Bolton-Hunter reagent migrated with R_F values of 0.57 and 0.64 for the mono- and diiodinated compounds; daunomycin remained at the origin. Iodomycin appeared as two separate spots with R_F values of 0.10 and 0.19 for the mono- and diiodinated forms, respectively. These spots were identified by autoradiography (Fig. 1) and scraped from the thin layer plate. Iodomycin was extracted from the silica with a mixture of (1:1) dimethyl sulfoxide/ethanol. The compound was concentrated under a stream of N_2. A mixture of both the mono- and diiodinated forms of iodomycin was used for subsequent experimentation. As determined by γ counting (Beckmann Gamma 8000), the product had a specific activity of 2000 Ci/mmol. Nonradioactive iodomycin was prepared by reacting 5 mmol of daunomycin with 5 mmol of Bolton-Hunter reagent (Sigma) in 100 μM dimethyl formamide. Iodomycin was precipitated by adding 900 μl of 25 mM borate buffer, pH 8.5. The NMR spectra were acquired on a Bruker WH300 Spectograph in dimethyl sulfoxide for iodomycin and *d*-$CHCl_3$ for Bolton-Hunter reagent.

Fig. 1. Autoradiogram of a thin layer plate showing the separation of product from reactants. *Lane A*, unreacted Bolton-Hunter reagent; *lane B*, isolated iodomycin. In each case, two separate spots correspond to the mono- and diiodinated compounds. The solvent system used was ethyl acetate/diethyl ether (2:1).

Tissue Culture

Cell culture. Cell culture of parental, drug-sensitive, Chinese hamster ovary cells (Aux B1) and their colchicine-resistant derivatives (B30) has been described (13, 14). Cells were grown in α-minimum essential medium (15) with added nucleosides and deoxyribonucleosides plus 10% (v/v) fetal calf serum; B30 cells were grown in the presence of 30 μg/ml colchicine.

Drug accumulation. Drug accumulation experiments were carried out on cell monolayers essentially as described (13). [^{125}I]Iodomycin was added from ethanol stock solution to growth medium at a final concentration of 4 μCi/100 ml; [^{3}H]daunomycin was added at 10 μCi/100 ml. To terminate experiments, iodomycin- or daunomycin-containing medium was removed from plates, which were then rinsed three times in ice-cold PBS. Cell samples were lysed with 1 ml of H_2O and counted. To normalize for cell numbers, the DNA content was determined by a modification (16) of the Burton (17) diphenylamine colorimetric assay, as previously described (13).

Growth assays. Cells were seeded at approximately 200, 500, and 1000 per plate in growth medium with the appropriate drug concentration. After colonies of 50 or more cells were observed in the control plates (7–8 days for B1 cells; 8–9 days for B30 cells), colonies in all plates were fixed, stained with 0.5% methylene blue in 50% methanol, and counted.

Preparation of membrane vesicles. Plasma membrane vesicles were prepared as previously described (18) using a Stansted Cell Disruptor (Model A0612) with No. 716 disrupting valve at pressures of 300 and 180 p.s.i. for B1 and B30 cells, respectively. After determination of protein content by the fluorimetric assay of Böhlen *et al.* (19), aliquots were stored at −85°.

Photoaffinity Labeling

Membrane vesicles. Photoaffinity labeling was used to directly measure drug binding. Iodomycin is inherently photoactive (11), obviating the use of an azido or nitrene substitution. Unless otherwise indicated, samples were prepared by adding 1 nM [^{125}I]iodomycin to membrane vesicles (200 μg/ml protein) in 10 mM Tris·HCl, pH 7.5, plus 0.125 M sucrose and 5 mM $MgCl_3$. To test their effects on binding, various other drugs were added as indicated in the figure legends and Table 1. Photoactivation utilized a xenon lamp (SLM Instruments Model XE 450), the beam of which was passed through two 2-cm plastic tissue culture flasks, one containing distilled water and the other, saturated $CuSO_4$, and through a glass filter with cut-off below 377 nm. Optimum time of irradiation was determined by measuring the time course of binding of the drug to the protein. The length of time at which drug binding was almost maximum but protein degradation (as determined by SDS-PAGE) had not occurred was 3 min (at 24°). After irradiation, membrane vesicles were collected by centrifugation in a Beckman Airfuge. Proteins (80 μg/lane) were separated by SDS-PAGE according to the method of Fairbanks *et al.* (20). Gels were stained with Coomassie blue and then scanned using a Joyce Loebl Chromoscan II to compare the amounts of protein per lane. Dried gels containing ^{125}I were autoradiographed. In inhibition experiments, densitometric traces of autoradiograms were obtained in order to quantitate the reduction in binding.

Whole cells. Late log-phase cells were trypsinized and washed once in growth medium and once in PBS plus 10 mM glucose. In order to inhibit cellular energy metabolism, some cells were incubated at 37° in

PBS plus 2 mM KCN for various times before trypsinization. These cells were washed in medium without glucose, followed by washing in PBS plus KCN. Cells were suspended in 2.5 ml of PBS with or without 2 mM KCN to a final concentration of approximately 7×10^6 cells/ml. At this point, some cell suspensions were extracted with 0.4 N perchloric acid in preparation for ATP determinations. Cellular ATP levels were determined by the method of Stanley and Williams (21) using a Beckmann LS3800 scintillation counter. For photoirradiation, cells were irradiated for 5 min, with stirring, in the presence of 4 μCi of iodomycin. To remove unbound iodomycin, cells were pelleted in a bench-top Sorvall centrifuge for 10 min at 1000 rpm. The pellet was suspended in 20 ml of PBS plus broad spectrum protease inhibitors (0.1 mM aprotinin, leupeptin, phenylmethyl sulfonyl fluoride, and EDTA and 10 mM dithiothreitol). After a 10-min incubation at 80 p.s.i. in a Parr cell disruption bomb, the samples were collected and centrifuged as follows: nuclear spin, 300 × *g* for 10 min; mitochondrial spin, 4000 × *g* for 10 min. After incubation for 15 min at 37° in the presence of 8 mg of DNase I, the supernatant was pelleted by spinning at 35,000 × *g* for 30 min. The pellet was resuspended in 10 mM Tris·HCl plus 0.25 M sucrose, layered on 35% sucrose in 10 mM Tris·HCl with 1 mM EDTA, and spun at 16000 × *g* for 30 min. The material banding at the interface was collected, diluted with 10 mM Tris·HCl plus 0.25 M sucrose, and spun at 150000 × *g* for 45 min. The pellet was either stored at −20° or solubilized directly in electrophoresis sample buffer, followed by separation on SDS-PAGE and autoradiography as described above.

Results

Characterization of iodomycin. The structure of iodomycin is shown in Fig. 2. NMR spectra at 300 MHz of Bolton-Hunter reagent and iodomycin were obtained to confirm that the product obtained by scraping the spots off the thin layer plate was iodomycin. Although the spectrum was complex, the resonances could be assigned as follows. (a) Daunomycinone moiety: three aromatic protons appear as a complex multiplet at δ7.5–8. H_7 appears at δ4.9. $CH_{2(8)}$ and $CH_{2(10)}$ appear as part of a multiplet at δ1.8 and a triplet at δ3, respectively. A singlet at δ4 represents the —OCH_3 and CO-CH_3 is seen part of a multiplet at δ2.2. The C_9 hydroxyl is seen as a singlet at δ5. These assignments are in agreement with those of Gray and Phillips (22) and Iwamoto *et al.* (23). (b) Daunosamine moiety: a doublet of doublets at δ4.7 represents H-1′. A multiplet at δ1.4 is H-2′. H-3′ appears as part of a multiplet at δ3.8, as do H-4′ and H-5′ at δ2.6 and δ4.2, respectively. A multiplet at δ2.2 is H-6′. The 6′-CH_3 appears as a doublet at δ1.1. These assignments are in agreement with those of Iwamoto *et al.* (23). (c) Bolton-Hunter moiety: aromatic protons corresponding to the mono- and diiodo forms appear as two doublets at δ6.6–6.9 and a singlet at δ7.9. A doublet at δ1.1 is CO-CH_2 and CO-CH_2-CH_2 protons appear as part of the multiplet at δ2.9. These protons were identified by comparison with the spectrum of Bolton-Hunter reagent alone.

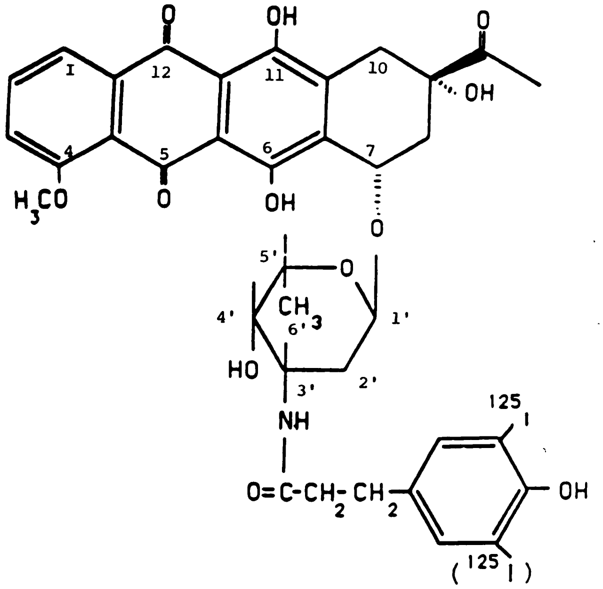

Fig. 2. The structural formula of [^{125}I]iodomycin. The *brackets* indicate that both the mono- and diiodinated compounds were synthesized.

Effect of daunomycin and iodomycin on cell growth. Fig. 3 compares the response of wild type drug-sensitive CHO cells (Fig. 3, A and C) and a highly drug-resistant variant (Fig. 3, B and D) to increasing concentrations of daunomycin or iodomycin. As had been shown previously, whereas 50% of sensitive (B1) cells grow at 0.035 μM daunomycin, the IC_{50} (concentration that inhibits growth of 50% of cells) for resistant (B30) cells is 100-fold higher, at 3.5 μM. The major difference in the response to iodomycin is that the wild type cells (B1) are approximately 10-fold less sensitive to it (IC_{50}, 0.5 μM). On the other hand, the dose response of resistant cells to iodomycin (IC_{50}, 3.3 μM) is almost identical to that of daunomycin. Hence, although the magnitude of the difference in sensitivity of the two cell types is far less for iodomycin than for daunomycin, a substantial difference (10-fold) remains. Thus, B30 cells are cross-resistant to this radioiodinated daunomycin analogue. On this basis, the newly synthesized derivative of daunomycin is recognized by the MDR mechanism.

Cellular accumulation of daunomycin and iodomycin. To determine whether reduced accumulation of iodomycin by MDR cells correlates with cross-resistance to it, its uptake and that of daunomycin by sensitive and resistant cells were compared (Fig. 4). The principle finding is that the accumulation of iodomycin by resistant cells is very greatly reduced, compared with that by sensitive cells. The amount entering the resistant cells over a 2-hr period is extremely low and essentially the same as the quantity of daunomycin that accumulates. In addition, these data also indicate that iodomycin enters sensitive cells less readily than does daunomycin (45 fmol of iodomycin/μg of DNA in 2 hr; 250 fmol of daunomycin). This is entirely consistent with the reduced toxicity of iodomycin to wild type cells relative to that of daunomycin (Fig. 3) and can at least partially account for that difference. Therefore, whereas iodomycin is apparently a useful probe of MDR, the added bulk of the Bolton-Hunter reagent has diminished the ability of this anthracycline derivative to enter and act on normal cells.

Specificity of drug binding to P-glycoprotein. The autoradiogram in Fig. 5A shows the photobinding of 10 nM [^{125}I]iodomycin to P-glycoprotein in MDR membrane vesicles in the presence of increasing concentrations of nonradioactive iodomycin. The identity of P-glycoprotein was confirmed by immunoblots as described previously (6). No proteins were observed to be labeled when membrane vesicles isolated from drug-sensitive cells were used (data not shown). In the presence of unlabeled iodomycin, the photolabeling of P-glycoprotein is reduced in a dose-dependent manner, confirming the specificity of binding of this drug to P-glycoprotein. Densitometric traces of the autoradiogram were obtained to produce the titration curve in Fig. 5B. Iodomycin (25 nM) reduces binding of [^{125}I]iodomycin to P-glycoprotein by 50%. In a similar experiment

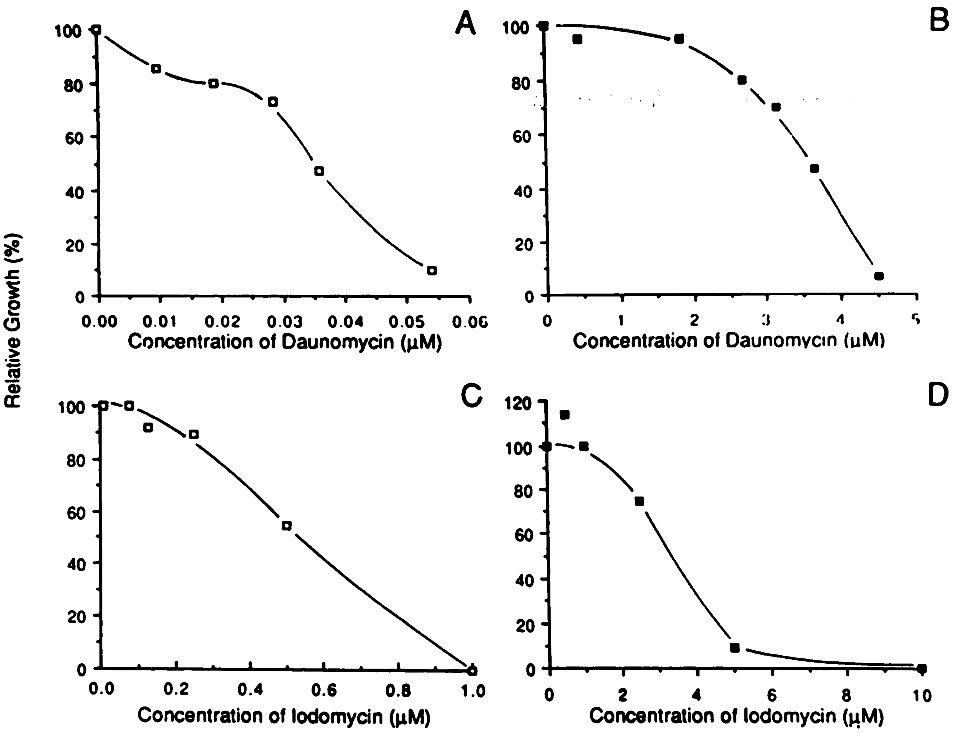

Fig. 3. Effect of daunomycin and iodomycin on growth of drug-sensitive and drug-resistant cells. The influence of increasing concentrations of daunomycin (A, B) and iodomycin (C, D) on the plating efficiencies of sensitive (B1) (□) and resistant (B30) (■) cells is shown. Absolute plating efficiencies of sensitive and resistant cells, respectively, were 61 and 70% for the daunomycin experiment and 62 and 68% for the iodomycin experiment.

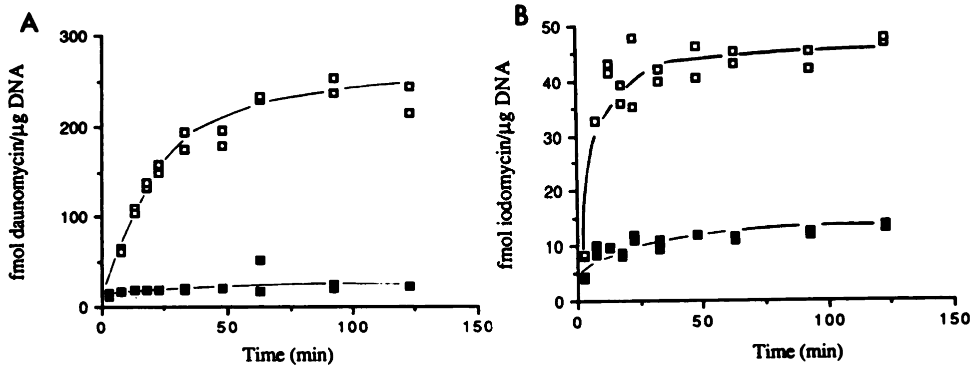

Fig. 4. Accumulation of [^{3}H]daunomycin (A) and [^{125}I]iodomycin (B) by drug-sensitive and drug-resistant cells. The accumulation of 20 nM [^{3}H] daunomycin and 20 nM [^{125}I]iodomycin by sensitive (B1) (□) and resistant (B30) (■) cells was determined at the times indicated.

in which daunomycin instead of iodomycin was used to displace [^{125}I]iodomycin, a considerably higher dose of the unmodified anthracycline was required to bring about a similar reduction in binding. Therefore, although the two compounds are not recognized identically by sites on P-glycoprotein, there is specific high affinity binding of iodomycin. Thus, MDR cells are cross-resistant to this compound, its accumulation by these cells is negligible as compared with that by the parental cells, and the drug binds specifically to P-glycoprotein.

Effect of other compounds on [^{125}I]iodomycin binding. The influence of several other compounds on [^{125}I]iodomycin photolabeling of P-glycoprotein was determined. The results are presented in Table 1. The inhibition data indicate that these drugs can be classified as interacting with P-glycoprotein with either high, intermediate, or low affinity. Unlabeled iodomycin, vinblastine, and a calcium channel blocker, RO 11-2933/001, are able to inhibit radiolabeled iodomycin binding at the lowest concentrations. In contrast, nifedipine and colchicine are poor inhibitors of this photobinding. Colchicine and nifedipine had previously been reported to be unable to inhibit noncovalent binding of [^{3}H]vinblastine to P-glycoprotein from MDR epidermoid carcinoma cells (24). Nitrendipine, verapamil, and daunomycin all display an intermediate ability to inhibit binding. The diminished ability of daunomycin to inhibit iodomycin binding probably reflects the absence of the iodinated 3-(4-hydroxyphenyl) propionic acid group from the Bolton-Hunter reagent in the former. The various compounds used represent drugs with two distinct actions on MDR cells. Vinblastine, iodomycin, daunomycin, and colchicine are all drugs to which MDR cells are cross-resistant. Conversely, these cells are hypersensitive to verapamil, nitrendipine, nifedipine, and RO 11-2933/001 (13).[1] Because vinblastine is a potent inhibitor of iodomycin photolabeling of P-glycoprotein, these findings suggest either that there are not distinct binding sites for the *Vinca* alkaloids and anthracyclines on this protein or that, if there are distinct sites, they may interact allosterically. Similarly, it may be argued that common or interacting sites are responsible for the binding of the calcium antagonists.

---

[1] Manuscript in preparation.

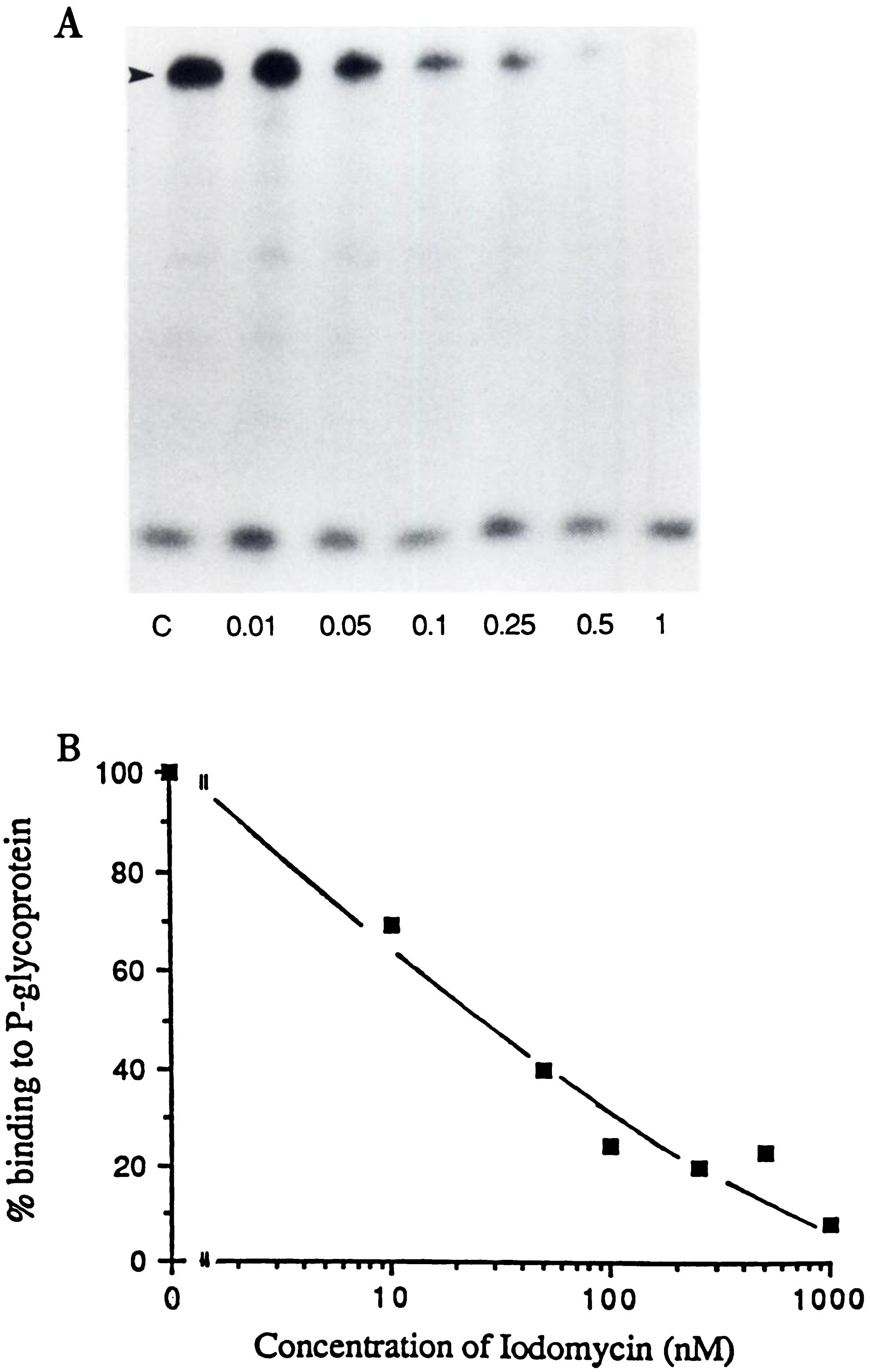

Fig. 5. Specificity of photobinding of [^{125}I]iodomycin to P-glycoprotein. The influence of increasing concentrations of nonradioactive iodomycin on the photobinding of 10 nM [^{125}I]iodomycin is illustrated. A, The autoradiogram obtained from SDS-PAGE of photolabeled membrane vesicles from resistant cells. B, The corresponding plot of relative densitometric intensities of the P-glycoprotein bands (*arrowhead*). The control (C), in which no nonradioactive drug was added, was considered to represent 100% binding.

Overall, the two classes of compounds are clearly not distinguished in terms of their ability to inhibit the photobinding of [^{125}I]iodomycin to P-glycoprotein; different drugs representing either type of compound bind to P-glycoprotein with different affinities.

Binding of iodomycin to whole cells. The binding of photoactive drugs to P-glycoprotein in intact cells has not been previously demonstrated. As a further test of its usefulness in the study of MDR, photoaffinity binding of iodomycin to intact MDR cells was carried out. [^{125}I]Iodomycin was observed to bind specifically to P-glycoprotein in cells, as it does in isolated plasma membranes. The extent of binding was augmented by pretreatment of the cells with the metabolic inhibitor KCN. To determine whether this increase was due to the diminished energy charge of the cells, preincubation with 2 mM KCN was performed for varying periods of time and the extent of [^{125}I]iodomycin binding (Fig. 6A) was monitored simultaneously with the resultant decrease in ATP levels (Fig. 6B). ATP was rapidly depleted, from about 2 nmol/10^6 cells (approximately 2 mM) to less than half that level, during the first 10 min. This was followed by a more gradual decrease toward negligible amounts by 1 hr. The autoradiogram shown in Fig. 6A illustrates the relative amounts of [^{125}I]iodomycin that had bound to P-glycoprotein at the surface of cells containing these dimin-

TABLE 1

Effects of cancer drugs and calcium channel blockers on photolabeling of P-glycoprotein with [^{125}I]iodomycin

Reduction in binding to P-glycoprotein relative to controls was determined by obtaining densitometric traces of autoradiographed acrylamide gels, as described in Fig. 5.

Inhibitor	Iodomycin Binding Remaining[a]			
	0.1 μM[b]	1 μM	10 μM	100 μM
		%		
Iodomycin	35	20	14	
Daunomycin	91	65	29	0
Vinblastine	45	15	0	0
Colchicine	97	127	83	22
Nitrendipine		60	4	6
Nifedipine			129	13
RO 11-2933/001	53	11	2	
Verapamil	60	33	11	

[a] Relative to controls in the absence of inhibitors.

[b] Inhibitor concentration.

ished ATP concentrations. By 10 min there was a substantial increase in amount bound and by 1 hr there was a further large elevation. Hence, as cellular ATP levels decrease, the extent of iodomycin binding to P-glycoprotein increases; the presence of the metabolic inhibitor promotes binding to the anthracycline target. At this stage we are unsure whether this simply reflects the increased amount of drug that is known to enter energy-depleted MDR cells (3, 4) or whether the interaction of drug with P-glycoprotein is directly affected. To address the latter possibility, isolated membrane vesicles instead of cells were incubated in the presence of varying ATP concentrations during the photobinding. As shown in Fig. 7, this was without effect on the extent of [^{125}I]iodomycin binding to P-glycoprotein. Hence, the presence of ATP alone is insufficient to reduce iodomycin binding to P-glycoprotein in isolated membranes. This may mean either that the influence on binding to the protein in cells is the indirect result of reduced drug entry or that additional cellular factors are required to bring about a direct effect on the P-glycoprotein-drug interaction. Further reconstitution experiments may help to test the latter possibility.

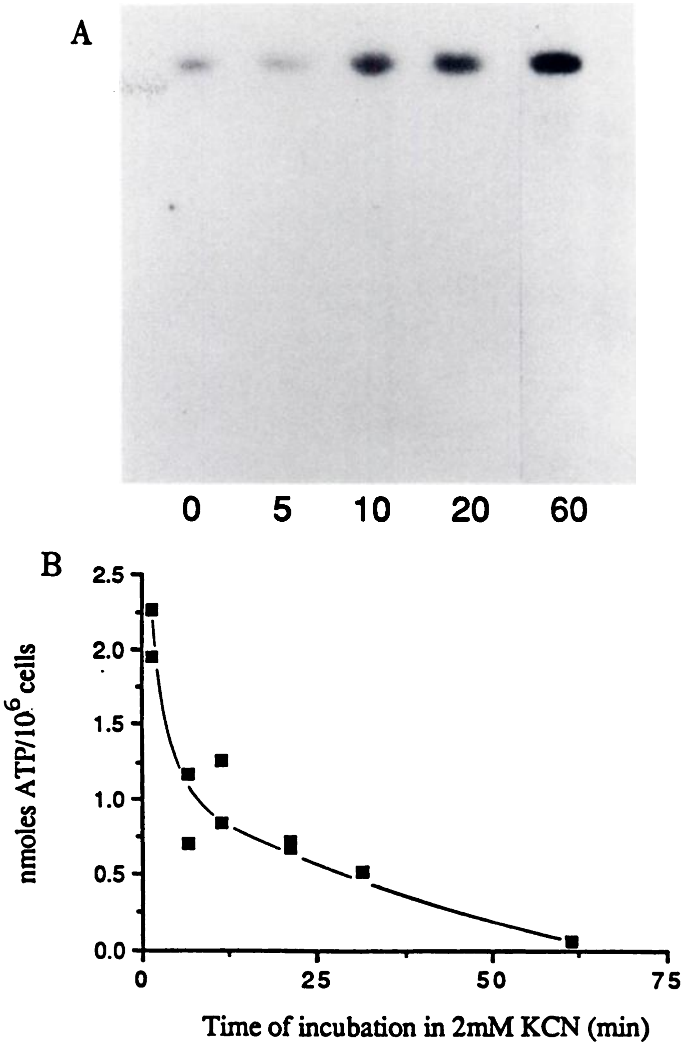

Fig. 6. Binding of iodomycin to intact cells. B30 cells were incubated for the times indicated in 2 mM KCN and either irradiated in the presence of 4 μCi of iodomycin for 5 min to determine binding to P-glycoprotein (A) or extracted with 0.4 N perchloric acid in order to determine cellular ATP levels (B). Cell viability was monitored by trypan blue exclusion and found to be greater than 98% even after 60 min in 2 mM KCN. Membrane vesicles were then isolated from irradiated cells and separated by SDS-PAGE. The autoradiogram of such an experiment is shown here (A). Time of incubation in KCN (in min) is indicated *below* each lane.

Discussion

We report here the synthesis of a new anthracycline analogue that we have called iodomycin. Iodomycin was prepared by allowing the Bolton-Hunter reagent to react with the free amino group of the anthracycline daunomycin. The complete separation of the Bolton-Hunter reagent from iodomycin by thin layer chromatography enabled the isolation of the product in pure form. The 300-MHz NMR spectrum, containing the peaks from the Bolton-Hunter reagent plus the peaks from daunomycin, confirmed that the product had the structure shown in Fig. 2.

As a test of the utility of iodomycin, the nature of its interaction with MDR and drug-sensitive cells was determined. Generally, drugs used in the study of MDR are toxic to resistant cells only at levels much higher than those required for toxicity to drug-sensitive cells lines. In addition, these drugs do not accumulate in MDR cells to the extent that is observed in sensitive cells. Furthermore, some of these drugs, for example *Vinca* alkaloids, are known to bind specifically to P-glycoprotein (9, 10). With iodomycin, we observed that it was not accumulated by MDR CHO cells over a 120-min time period and was toxic to them only at approximately 10-fold higher concentrations than those required for toxicity to drug-sensitive cells. Although this difference is less than that for the parent compound daunomycin, the MDR cells are obviously cross-resistant to iodomycin. The altered cytotoxicity may be due to reduced cellular accumulation of iodomycin (as compared with daunomycin) by B1 (parental) cells. The inherent photoactivity of anthracyclines (11) facilitated the use of iodomycin as a photolabel in testing the potential binding to P-glycoprotein.

We are not aware of previous reports of specific anthracycline binding to P-glycoprotein from MDR cells. Thus, in addition to the previously reported photoaffinity labeling of P-glycoprotein in MDR vesicles with an azido analogue of vinblastine (9, 10) and the calcium channel blockers azidopine (25, 26) and an azido-verapamil derivative (27), the data reported here show that iodomycin also binds specifically to P-glycoprotein. The

Fig. 7. Effect of ATP on photobinding of iodomycin. The indicated concentration was added to MDR membrane vesicle suspensions with iodomycin immediately before irradiation. The autoradiogram of the SDS-PAGE separation of membrane vesicles is shown.

protein, therefore, recognizes anthracyclines as well as other classes of drugs. Data from this and previous studies (14, 25–27) indicate that a wide variety of compounds at variable concentrations considerably in excess of that of the label are able to inhibit binding of these photoactive compounds to their protein target in MDR membrane vesicles. For example, the photobinding of 50 nM azido-vinblastine analogue to P-glycoprotein from KB epidermoid carcinoma cells was blocked by 100 μM verapamil, vinblastine, and vincristine (10). The photolabeling of 3.5 μM azidopine to P-glycoprotein from MDR Chinese hamster lung cells was reduced to varying degrees by several drugs including adriamycin, colchicine, nifedipine, and nitrendipine at 200-fold excess (25). Azidopine photolabeling of P-glycoprotein (at 50 nM) from MDR variants of the murine macrophage-like cell line J774.2 was inhibited by 50 μM vinblastine and, to a lesser extent, colchicine and taxol (26). Photobinding of an azido-verapamil derivative (at 4 nM) to vincristine-resistant Chinese hamster lung cells was inhibited by 50 μM verapamil or 100 μM vinblastine but not by colchicine (27). Hence, several drugs known to be recognized by MDR cells inhibit photobinding to P-glycoprotein. However, in all cases, a large excess of the competing compound was required to reduce binding. In this study, vinblastine, RO 11-2933/001, and nonradiolabeled iodomycin were the most potent inhibitors of iodomycin photobinding; daunomycin, nitrendipine, and verapamil were slightly less effective whereas colchicine and nifedipine were the least inhibitory. Thus, some calcium channel blockers are able to inhibit binding to P-glycoprotein of either calcium channel blockers or cancer drugs wheras some cancer drugs can reduce binding of other cancer drugs or calcium antagonists. The data, therefore, do not indicate that different classes of compounds bind at distinct sites on the protein. In fact, they suggest that a common binding site for several antineoplastic agents and calcium channel blockers may exist. However, the possibility of distinct sites that interact allosterically has not been ruled out. Strict kinetic analyses of the types of inhibition observed as well as localization of the binding sites to specific domains of P-glycoprotein must be carried out before this issue can be resolved with certainty. The identification of such drug-binding domains will be facilitated by the use of the new high specific activity anthracycline.

Iodomycin has also been employed to demonstrate binding of a drug to P-glycoprotein in intact cells for the first time. The extent of this binding was augmented when cellular ATP was depleted. This is probably a reflection of the increased amount of drug that is known to enter energy-depleted MDR cells, rather than an altered drug-protein association, because ATP did not diminish iodomycin photobinding to P-glycoprotein in isolated membrane vesicles. This is in spite of the fact that ATP-dependent drug transport has recently been demonstrated in vesicle preparations from other MDR cell lines (28). Furthermore, although P-glycoprotein does express an ATPase activity (29, 30) and ATP binding (31–33), under the conditions studied here the apparent cellular ATP concentration range in which altered binding is observed is much higher than either the estimated K_m of hydrolysis [0.15 mM (30)] or the concentration at which azido-ATP binds to P-glycoprotein [5 μM (31)]. Therefore, the increased binding may be related to the presence of other ATP-utilizing reactions in intact cells.

Specific binding of vinblastine derivatives to P-glycoprotein from various membrane preparations has been shown to occur in the absence of ATP (9, 10, 34) and could be enhanced by ATP in one case (34). Because it seems likely that binding to P-glycoprotein represents one component of the drug transport process in MDR cells, the lack of a direct effect of ATP on iodomycin photobinding in rodent membranes might suggest that dissociation of drug from the protein may be the ATP-sensitive step detected in an equilibrium binding assay (34).

In summary, we have synthesized a new anthracycline, iodomycin. This compound has a high specific activity and potentially could prove useful in the study of mechanism and targets of actions of anthracyclines in general. However, before it can be used in investigations of anthracyclines with molecules other than P-glycoprotein, its effects on known targets of this class of drug, such as DNA, will have to be characterized. In the present study we utilized it to characterize the nature of the interaction of anthracyclines with P-glycoprotein in MDR cells. Our data showed that these cells are cross-resistant to this drug and capable of maintaining reduced intracellular levels of it, as compared with the drug-sensitive cells. Furthermore, iodomycin binds specifically to P-glycoprotein, directly demonstrating that this protein recognizes anthracyclines as well as calcium channel blockers and *Vinca* alkaloids, as had been previously reported.

References

1. Aubel-Sadron, G., and D. Londos-Gagliardi. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. *Biochimie (Paris)* **66**:333–352 (1984).

2. Bossa, R., I. Galatulas, and E. Mantovani. Cardiotoxicity of daunomycin and adriamycin. *Neoplasma (Bratisl.)* **24:**405–410 (1977).
3. Riordan, J. R., and V. Ling. Genetic and biochemical characterization of multidrug resistance. *Pharmacol. Ther.* **28:**51–76 (1985).
4. Gerlach, J. H., N. Kartner, D. R. Bell, and V. Ling. Multidrug resistance. *Cancer Surv.* **5:**25–46 (1986).
5. Kaye, S., and S. Merry. Tumour cell resistance to anthracyclines: a review. *Cancer Chemother. Pharmacol.* **14:**96–103 (1985).
6. Kartner, N., J. R. Riordan, and V. Ling. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science (Wash. D. C.)* **221:**1285–1288 (1983).
7. Gros, P., V. B. Neriah, J. M. Croop, and D. E. Housman. Isolation and expression of a cDNA (mdr) that confers multidrug resistance. *Nature (Lond.)* **323:**728–731 (1986).
8. Ueda, K., C. Cardarelli, M. M. Gottesman, and I. Pastan. Expression of a full length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin and vinblastine. *Proc. Natl. Acad. Sci. USA* **84:**3004–3008 (1987).
9. Safa, A. R., C. J. Glover, M. B. Meyers, J. L. Beidler, and R. L. Felsted. Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. *J. Biol. Chem.* **261:**6137–6140 (1986).
10. Cornwell, M. M., A. R. Safa, R. L. Felsted, M. M. Gottesman, and I. Pastan. Membrane vesicles from multidrug resistant human cancer cells contain a specific 150–170 KDa protein detected by photoaffinity labelling. *Proc. Natl. Acad. Sci. USA* **83:**3847–3850 (1986).
11. Yee, G., M. Carey, and T. R. Tritton. Photoaffinity labelling of the sarcoma 180 cell surface by daunomycin. *Cancer Res.* **44:**1898–1903 (1984).
12. Bolton, A. E., and W. M. Hunter. The labelling of proteins to high specific radioactivity by conjugation to a ^{125}I-containing acylating agent. *Biochem. J.* **133:**529–539 (1973).
13. Cano-Gauci, D. F., and J. R. Riordan. Action of calcium antagonists on multidrug resistant cells. *Biochem. Pharmacol.* **34:**2115–2123 (1987).
14. Ling, V., and L. H. Thompson. Reduced permeability in CHO cells as a mechanism of resistance to colchicine. *J. Cell. Physiol.* **83:**103–116 (1974).
15. Stanners, C. F., G. L. Eliceiri, and H. Green. Two types of ribosomes in mouse-hamster hybrid cells. *Nature New Biol.* **230:**52–54 (1971).
16. Leyva, J. R., and W. N. Kelley. Measurement of DNA in cultured human cells. *Anal. Biochem.* **62:**173–179 (1974).
17. Burton, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62:**315–323 (1956).
18. Riordan, J. R., and V. Ling. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J. Biol. Chem.* **254:**12701–12705 (1979).
19. Böhlen, P., S. Stein, D. Dairman, and S. Udenfriend. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* **55:**213–220 (1973).
20. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10:**2606–2617 (1971).
21. Stanley, P. E., and S. G. Williams. Use of liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.* **29:**381–392 (1969).
22. Gray, P. J., and D. R. Phillips. Ultraviolet photoirradiation of daunomycin and DNA-iodomycin complexes. *Photochem. Photobiol.* **33:**297–303 (1981).
23. Iwamoto, R. H., P. Lim, and N. S. Bhacca. The structure of daunomycin. *Tetrahedron. Lett.* **36:**3891–3894 (1968).
24. Cornwell, M. M., I. Pastan, and M. M. Gottesman. Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J. Biol. Chem.* **262:**2166–2170 (1987).
25. Safa, A. R., C. J. Glover, J. L. Sewell, M. B. Meyers, J. L. Beidler, and R. L. Felsted. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. *J. Biol. Chem.* **262:**7884–7888 (1987).
26. Yang, C.-P. H., W. Mellado, and S. B. Horwitz. Azidopine photoaffinity labeling of multidrug resistance-associated glycoproteins. *Biochem. Pharmacol.* **37:**1417–1421 (1988).
27. Safa, A. R. Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. *Proc. Natl. Acad. Sci. USA* **85:**7187–7191 (1988).
28. Horio, M., M. M. Gottesman, and I. Pastan. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc. Natl. Acad. Sci. USA* **85:**3580–3584 (1988).
29. Hamada, H., and T. Tsuruo. Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance. *J. Biol. Chem.* **263:**1454–1458 (1988).
30. Hamada, H., and T. Tsuruo. Characterization of the ATPase activity of the Mr 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM Cells. *Cancer Res.* **48:**4926–4932 (1988).
31. Cornwell, M. M., T. Tsuruo, M. M. Gottesman, and I. Pastan. ATP-binding properties of P-glycoprotein from multidrug-resistant KB cells. *FASEB J.* **1:**51–54 (1987).
32. Gros, P., J. Croop, and D. Housman. Mammalian multidrug resistance gene: complete cDNA sequence indicates strong homology to bacterial transport proteins. *Cell* **47:**371–380 (1987).
33. Chen, C.-J., J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson. Internal duplication and homology with bacterial transport proteins in the MDR1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* **47:**381–389 (1987).
34. Naito, M., H. Hamada, and T. Tsuruo. ATP/Mg^{2+} dependent binding of vincristine to the plasma membrane of multidrug-resistant K562 Cells. *J. Biol. Chem.* **263:**11887–11891 (1988).

Send reprint requests to: John R. Riordan, Research Institute, The Hospital for Sick Children, 555 University Ave., Toronto, Canada M5G 1X8.