0026-895X/95/020241-07$03.00/0

MOLECULAR PHARMACOLOGY, 47:241-247, 1995

Welwitindolinone Analogues that Reverse P-Glycoprotein-Mediated Multiple Drug Resistance

CHARLES D. SMITH, JACK T. ZILFOU, KLEMENS STRATMANN, GREGORY M. L. PATTERSON, and RICHARD E. MOORE

Department of Pharmacology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 (C.D.S., J.T.Z.), and Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822 (K.S., G.M.L.P., R.E.M.)

Received July 5, 1994; Accepted November 15, 1994

SUMMARY

Welwitindolinones are a family of novel alkaloids recently isolated from the blue-green alga *Hapalosiphon welwitschii* as a part of our effort to identify new compounds that overcome multiple drug resistance. The abilities of three structurally similar members of this family to interact with P-glycoprotein have been compared. Similarly to the effects of verapamil, *N*-methylwelwitindolinone C isothiocyanate (compound **1**) attenuated the resistance of MCF-7/ADR cells to natural product anticancer drugs, including vinblastine, taxol, actinomycin D, daunomycin, and colchicine, without affecting the cytotoxicity of cisplatin. These effects of compound **1** were apparent at doses as low as 0.1 μM, indicating that it is considerably more potent than verapamil for reversal of resistance. Welwitindolinone C isothiocyanate (compound **3**) demonstrated weaker reversing activity, whereas an analogue of compound **1** in which the isothiocyanate group is replaced by an isonitrile group (compound **2**) was inactive. The accumulation of [^{3}H]vinblastine in SK-VLB-1 cells was increased by compound **1** > compound **3** > verapamil ≫ compound **2**. Interestingly, only compound **1** and verapamil enhanced [^{3}H]taxol accumulation by these cells. Photoaffinity labeling of P-glycoprotein with [^{3}H]azidopine in membranes from SK-VLB-1 cells was inhibited by compounds **1** and **3**, but not by compound **2**. Therefore, the differences in the size and/or the electronegativity of the isothiocyanate and isonitrile moieties appear to dramatically affect the abilities of the compounds to interact with P-glycoprotein.

The development of resistance to natural product anticancer drugs frequently occurs both in cell culture systems and in tumors (reviewed in Refs. 1 and 2). This protective response of the tumor cells currently limits the efficacy of many first-line chemotherapeutic agents, including *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and many antitumor antibiotics. A primary mechanism of drug resistance *in vitro*, and probably *in vivo*, is through the induction of membrane-associated transport proteins such as P-glycoprotein (1–5). This transporter very effectively reduces the intracellular accumulation of the natural product drugs and so reduces their cytotoxicity. In contrast, P-glycoprotein does not protect against small hydrophilic drugs such as cisplatin, 5-fluorouracil, and mercaptopurine.

In an attempt to overcome MDR, significant effort has been expended in the search for chemical agents that antagonize the effects of P-glycoprotein. The result of this effort has been the identification of a number of compounds that are able to reverse (reviewed in Refs. 6 and 7) or otherwise circumvent (8, 9) P-glycoprotein-mediated MDR *in vitro*. For example, verapamil, cyclosporine A, quinidine, and phenothiazines sensitize MDR cells to P-glycoprotein substrate drugs, promote the intracellular accumulation of these drugs, and compete for drug binding to P-glycoprotein. Although clinical trials of these and other MDR-reversing agents continue, problems with systemic toxicity of these agents have thus far limited their utility (reviewed in Ref. 10). It is therefore desirable that additional reversing agents be discovered and characterized, with the ultimate goal of testing *in vivo*.

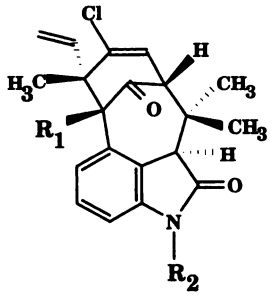
We have surveyed a collection of extracts from approximately 1500 strains of blue-green algae (cyanobacteria) for anti-MDR compounds. The activities of a novel porphyrin from one extract have been described previously (11, 12). In the present report, the abilities of novel alkaloids, termed welwitindolinones, to reverse P-glycoprotein-mediated MDR are described. Interestingly, minor chemical differences among these compounds appear to strongly affect their interactions with P-glycoprotein.

Experimental Procedures

Materials. *N*-Methylwelwitindolinone C isothiocyanate (compound **1** in Fig. 1; molecular weight, 413), *N*-methylwelwitindolinone C iso-

This work was supported by National Institutes of Health Grant CA64983 (C.D.S.), National Science Foundation Grant CHE-9024748 (R.E.M.), and a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (K.S.).

ABBREVIATIONS: MDR, multiple drug resistance; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, concentration that inhibits cell proliferation by 50%.



Compound	Name	R_1	R_2
1	N-Methylwelwitindolinone C isothiocyanate	SCN	CH_3
2	N-Methylwelwitindolinone C nitrile	NC	CH_3
3	Welwitindolinone C isothiocyanate	SCN	H

Fig. 1. Structures of welwitindolinones.

nitrile (compound **2**; molecular weight, 381), and welwitindolinone C isothiocyanate (compound **3**; molecular weight, 399) were isolated from extracts of *Hapalosiphon welwitschii* Elenkin IC-52–3 as described by Stratmann *et al* (13). The compounds were dissolved in dimethylsulfoxide and were stable at 4°. Taxol was obtained from Calbiochem (San Diego, CA), whereas all other drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Vinblastine sulfate and [^{3}H]-taxol were from Moravek Biochemicals (Brea, CA) and [^{3}H]azidopine was obtained from Amersham (Arlington Heights, IL).

Cell culture and cytotoxicity assay. SK-VLB-1 cells were kindly provided by Dr. Victor Ling of the Ontario Cancer Institute (14). These cells were selected for resistance to vinblastine and overexpress P-glycoprotein at least 100-fold, in comparison with SK-OV-3 cells. Both cell lines were grown in α-minimal essential medium (GIBCO-BRL, Grand Island, NY) containing 10% fetal bovine serum (GIBCO-BRL), 4 mM L-glutamine, and 50 μg/ml gentamycin sulfate. Selection pressure was maintained on SK-VLB-1 cells by the addition of 1 μM vinblastine to stock cultures 24 hr after their passage. MCF-7 breast carcinoma cells and MCF-7/ADR cells, an MDR subline (15), were obtained from the Division of Cancer Treatment, National Cancer Institute, and were grown in RPMI 1640 medium (GIBCO-BRL) containing 10% fetal bovine serum (GIBCO-BRL) and 50 μg/ml gentamycin sulfate.

To test the effects of drugs on growth, cells were seeded in 96-well tissue culture dishes (Corning Glass Works, Corning, NY) at approximately 10% confluency and were allowed to attach and recover for at least 24 hr. Varying concentrations of drugs alone or combined with verapamil or welwitindolinone were then added to each well, and the plates were incubated for an additional 48 hr. The number of surviving cells was then determined by staining with sulforhodamine B, as described by Skehan *et al.* (16). The percentage of cells killed was calculated as the percentage decrease in sulforhodamine B binding, compared with control cultures. Control cultures included equivalent amounts of dimethylsulfoxide, which does not modulate the growth or drug sensitivity of these cells at doses used in these studies. Reversal of MDR is defined as the ability of the compound to potentiate the cytotoxicity of P-glycoprotein-transported drugs.

^{3}H-Labeled drug accumulation assay. SK-OV-3 or SK-VLB-1 cells were plated into 24-well tissue culture dishes and allowed to grow to 90% confluency. The cells were washed with PBS and then incubated for 60 min at 37° in 0.5 ml of α-minimal essential medium containing the test compound and 10–20 nM [^{3}H]vinblastine sulfate (10–15 Ci/mmol) or [^{3}H]taxol (~19 Ci/mmol). The cultures were rapidly washed three times with ice-cold PBS. Intracellular ^{3}H-labeled drug was solubilized with 0.3 ml of 1% sodium dodecyl sulfate in PBS and quantitated by liquid scintillation counting. Parallel cultures were treated with the test compound for an equivalent period of time and then incubated with 0.1% trypan blue for 10 min. The percentage of cells permeable to the dye was determined by microscopic examination.

Photoaffinity labeling of P-glycoprotein. MCF-7 and MCF-7/ADR cells were washed with PBS, detached by gentle scraping, and collected by centrifugation at 500 × *g* for 5 min. The cells were resuspended in cold lysis buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, 5 μM phenylmethylsulfonyl fluoride, and 10 mM HEPES, pH 7.4, and were incubated on ice for 30 min. The cells were then disrupted by gentle homogenization on ice, and the lysate was centrifuged at 5000 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 30 min, and the resulting pelleted membranes were resuspended in lysis buffer and stored at −75° until their use. For photolabeling studies, samples (25 μl) containing approximately 25 μg of membrane protein, 0.75 μM [^{3}H]azidopine (~1 μCi), and the indicated concentrations of verapamil or welwitindolinone in lysis buffer were incubated at room temperature in the dark for 30 min. Samples were then exposed to 200,000 μJ of UV light in a Stratagene UV Stratalinker at room temperature. Reactions were terminated by the addition of electrophoresis sample buffer (containing 1% β-mercaptoethanol), and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.5% gels. The gels were fixed with isopropanol/water/acetic acid (25:65:10, by volume) for 30 min and then soaked in Amplify (Amersham) for 30 min. Gels were subsequently dried and exposed to Hyperfilm-MP (Amersham) at −75° for approximately 7 days. Radiolabeled bands were excised from the gel, minced, rehydrated, and quantitated by liquid scintillation counting. In some experiments, proteins from parallel gels were electrotransferred to nitrocellulose membranes and probed for P-glycoprotein using the mdr(Ab-1) antibody from Oncogene Science (Manhasset, NY) and an amplified alkaline phosphatase Immun-Blot assay kit from Bio-Rad (Melville, NY).

Other methods. Protein concentrations were determined by the method of Bradford (17), using reagents from Bio-Rad and bovine serum albumin as the standard. Molecular modeling of the welwitindolinones and other compounds was conducted by following the Polak-Ribiere minimum energy optimization routines, using the HyperChem for Windows program (release 3; Autodesk, Sausalito, CA).

Results

Reversal of P-glycoprotein-mediated MDR by welwitindolinones. Screening of an extensive collection of extracts from blue-green algae (18) identified *H. welwitschii* Elenkin IC-52–3 as a source of an MDR-reversing agent. The hydrophobic extract from this alga was sequentially fractionated by size exclusion chromatography and silica gel chromatography (13). Fractions containing active compounds were identified by their ability to chemosensitize SK-VLB-1 cells to daunomycin and/or actinomycin D and to promote the accumulation of [^{3}H]-vinblastine in these cells. *N*-Methylwelwitindolinone C isothiocyanate (compound **1**) was isolated by this approach, followed by isolation and identification of welwitindolinone C isothiocyanate (compound **3**). A compound with similar chromatographic properties was subsequently isolated and identified as *N*-methylwelwitindolinone C isonitrile (compound **2**).

The abilities of these compounds to reverse MDR were initially examined using SK-VLB-1 cells. As demonstrated in Fig. 2, 25 μM daunomycin and 1 μM actinomycin D caused only modest decreases (<5%) in the proliferation of these cells. As expected, verapamil strongly enhanced the ability of both drugs to inhibit cell proliferation (approximately 50% cell kill at 10

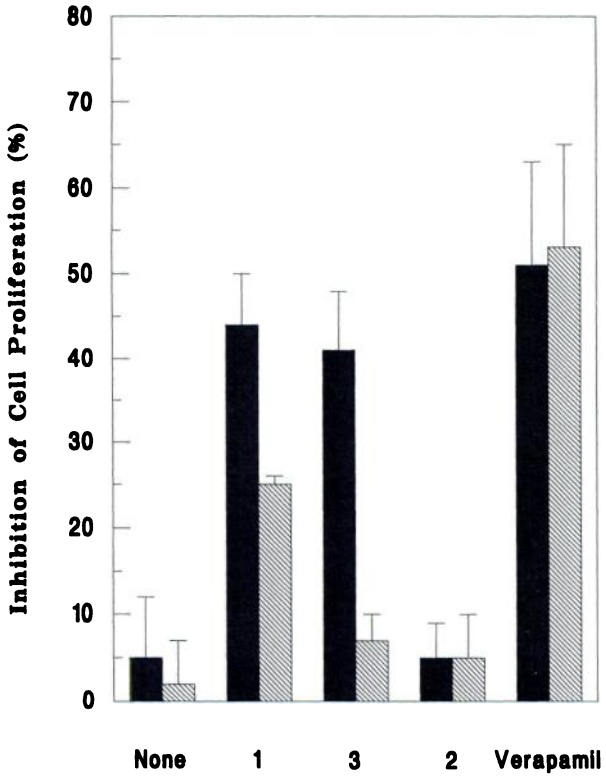


Fig. 2. Chemosensitization of SK-VLB-1 cells by welwitindolinones and verapamil. SK-VLB-1 cells were treated with 1 μM actinomycin D (■) or 25 μM daunomycin (▨) alone or in the presence of 5 μM compound **1**, **2**, or **3** or 20 μM verapamil. The inhibition of cell proliferation was quantitated as indicated in Experimental Procedures. Values represent the mean ± standard error of two experiments, each containing triplicate samples.

μM verapamil). Compounds **1** and **3** (at approximately 5 μM) increased cell killing by actinomycin D to the same extent as did verapamil, whereas compound **2** did not potentiate the cytotoxicity of actinomycin D. Compound **1** also markedly increased cell killing by daunomycin, whereas compounds **2** and **3** did not modulate the toxicity of daunomycin toward SK-VLB-1 cells.

Additional characterization of the abilities of the welwitindolinones to modulate drug resistance used drug-resistant breast carcinoma (MCF-7/ADR) cells. These cells are less resistant than SK-VLB-1 cells (e.g., resistance factors with colchicine of 110 and 990 for MCF-7/ADR and SK-VLB-1 cells, respectively),[1] due to lower levels of P-glycoprotein expression (data not shown), and are more sensitive to the effects of MDR-reversing agents. Compounds **2** and **3** were cytotoxic toward MCF-7 cells, with IC_{50} values of approximately 0.12 μM (Table 1). Interestingly, methylation of the nitrogen atom, i.e., compound **1**, reduced the cytotoxicity of the isothiocyanate compound 25-fold. The cytotoxicities of the three compounds toward MCF-7 and MCF-7/ADR cells were identical, indicating that overexpression of P-glycoprotein does not confer resistance to these welwitindolinones.

To assess the abilities of the welwitindolinones to chemosensitize MDR cells, MCF-7 and MCF-7/ADR cells were treated with combinations of compound **1**, compound **2**, or compound **3** (at their IC_{50} values) and several doses of either vinblastine, taxol, actinomycin D (drugs transported by P-glycoprotein), or

[1] Resistance factor = IC_{50} of resistant cell line/IC_{50} of parental cell line.

TABLE 1

Cytotoxicities of welwitindolinones

MCF-7 and MCF-7/ADR cells were treated with several concentrations of the compounds indicated. The percentage cell survival was determined as indicated in Experimental Procedures and the IC_{50} for each compound was calculated. Values represent the mean ± standard error of at least three experiments.

	Compound	IC_{50}	
		MCF-7	MCF-7/ADR
		μM	
1	*N*-Methylwelwitindolinone C isothiocyanate	3.03 ± 0.32	2.88 ± 0.20
2	*N*-Methylwelwitindolinone C isonitrile	0.12 ± 0.01	0.14 ± 0.01
3	Welwitindolinone C isothiocyanate	0.13 ± 0.01	0.15 ± 0.01

cisplatin (a nontransported drug). As demonstrated in Fig. 3A, MCF-7 cells were approximately 200-fold more sensitive to vinblastine than were MCF-7/ADR cells. Treatment of the MCF-7/ADR cells with either verapamil or compound **1** increased their sensitivity to vinblastine, such that the IC_{50} was essentially the same as that for MCF-7 cells. Compound **3** decreased the IC_{50} for vinblastine approximately 4-fold, whereas compound **2** had no effect on the cytotoxicity of vinblastine. Similarly, verapamil and compound **1** potentiated the cytotoxicities of taxol (Fig. 3B) and actinomycin D (Fig. 3C) toward MCF-7/ADR cells, whereas compound **3** had only modest effects on responses to actinomycin D and compound **2** was inactive with both drugs. MCF-7 cells were less sensitive to cisplatin than were MCF-7/ADR cells (Fig. 3D), and neither verapamil nor the welwitindolinones significantly modulated the cytotoxicity of this nonsubstrate for P-glycoprotein. Doses of compound **1** as low as 0.1 μM (i.e., noncytotoxic doses) were able to decrease the IC_{50} values of vinblastine, taxol, actinomycin D, colchicine, and daunomycin in MCF-7/ADR cells (Fig. 4). The IC_{50} values for daunomycin and colchicine were reduced approximately 7-fold by compound **1**, whereas the IC_{50} values for vinblastine, taxol, and actinomycin D were reduced 40–90-fold. The sensitivities of MCF-7 cells to these drugs were not modulated by the welwitindolinones (data not shown).

Effects of welwitindolinones on ^{3}H-labeled drug accumulation. The reduction in intracellular accumulation of anticancer drugs due to expression of P-glycoprotein can be at least partially reversed by compounds that interact with the drug transporter. We therefore examined the effects of welwitindolinones on ^{3}H-labeled drug accumulation in SK-OV-3 and SK-VLB-1 cells. SK-OV-3 cells accumulated [^{3}H]vinblastine and [^{3}H]taxol to levels of 14.2 and 12.5 pmol/10^6 cells, respectively. In contrast, the accumulation of [^{3}H]vinblastine and [^{3}H]taxol by SK-VLB-1 cells averaged only 2 and 0.7 pmol/10^6 cells, respectively. As indicated in Fig. 5, verapamil caused dose-dependent increases in the accumulation of [^{3}H]vinblastine and [^{3}H]taxol in SK-VLB-1 cells, reaching 190 and 270% of control, respectively, at 20 μM verapamil. The accumulation of [^{3}H]vinblastine was strongly enhanced by compounds **1** and **3**, reaching 610 and 480% of control, respectively, at 20 μM. In contrast, compound **2** caused only a very modest increase in [^{3}H]vinblastine accumulation. The intracellular accumulation of [^{3}H]taxol was also strongly increased by compound **1**; however, compounds **2** and **3** were virtually ineffective in enhancing [^{3}H]taxol accumulation. None of these compounds increased the accumulation of either [^{3}H]vinblastine or [^{3}H]taxol in SK-

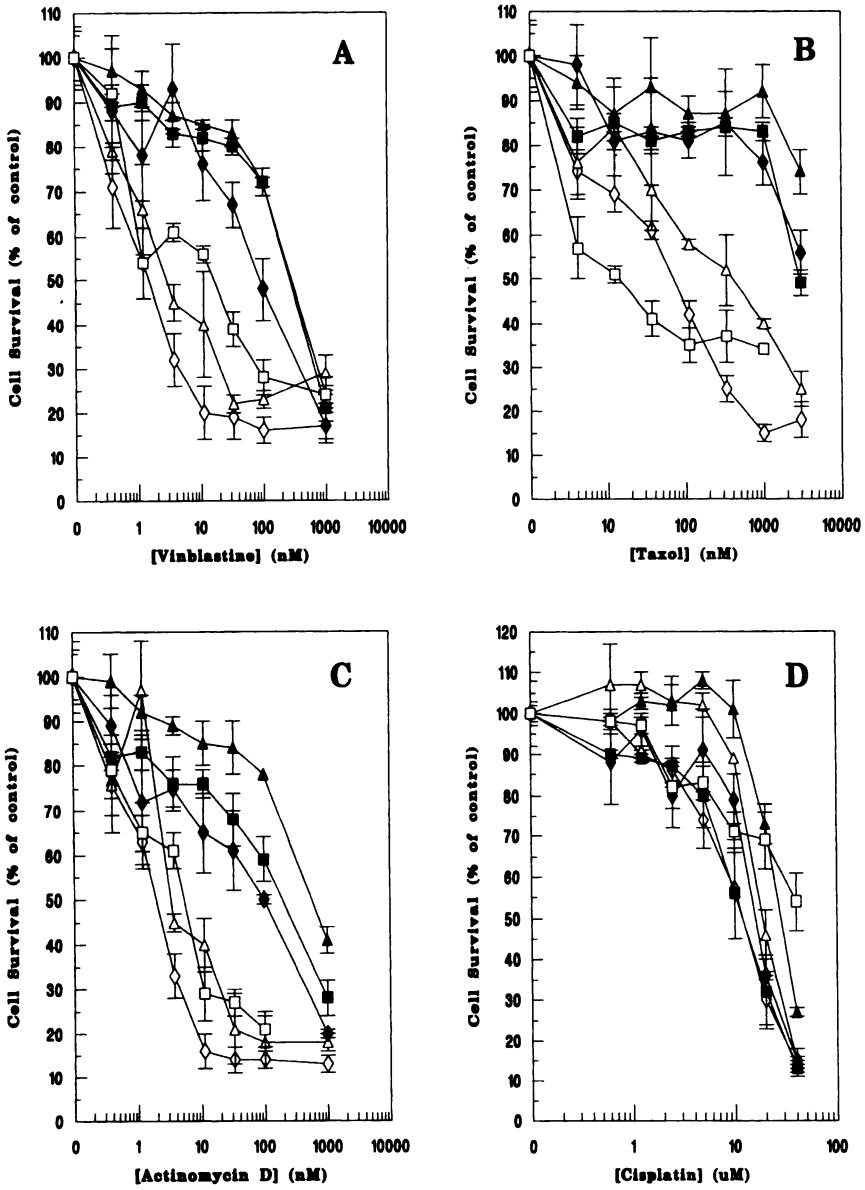


Fig. 3. Chemosensitization of MCF-7/ADR cells by welwitindolinones and verapamil. MCF-7 cells (□) or MCF-7/ADR cells in the presence of dimethylsulfoxide (■), 10 μM verapamil (◇), 2.5 μM compound **1** (△), 0.1 μM compound **2** (▲), or 0.1 μM compound **3** (◆) were incubated with the indicated concentrations of vinblastine (A), taxol (B), actinomycin D (C), or cisplatin (D). Cell survival was assayed as indicated in Experimental Procedures. Values represent the mean ± standard error of two to four experiments, each containing triplicate samples.

OV-3 cells (data not shown). In fact, compounds **2** and **3** (at 20 μM) decreased drug accumulation by 65–90% in SK-OV-3 cells, whereas compound **1** had no effect on drug accumulation by SK-OV-3 cells. These effects are not related to cytotoxicity, because none of the compounds increased the percentages of SK-OV-3 or SK-VLB-1 cells that were permeable to trypan blue after an incubation period equivalent to that used for the drug accumulation studies.

Binding of welwitindolinones to P-glycoprotein. The binding of a compound to P-glycoprotein can be inferred if the compound reduces photoaffinity labeling by substrates such as [^{3}H]azidopine or [^{125}I]iodoarylprazosin (19). To test the effects of welwitindolinones, membranes from MCF-7/ADR cells were incubated with several concentrations of the compounds or verapamil before the addition of [^{3}H]azidopine and exposure to UV light. As demonstrated in Fig. 6, [^{3}H]azidopine was covalently incorporated into a protein that migrated with a molecular mass of approximately 160 kDa and that was not present in membranes from drug-sensitive MCF-7 cells. This protein comigrated with material that was immunoreactive with antibodies against P-glycoprotein (data not shown). Compounds **1** and **3** decreased the binding of [^{3}H]azidopine to P-glycoprotein, such that 10 μM concentrations of either compound inhibited photolabeling by approximately 50%. In contrast, compound **2** at 10 μM did not significantly inhibit [^{3}H]azidopine labeling of P-glycoprotein.

Physical properties of welwitindolinones. The structural characteristics of certain MDR-reversing agents have suggested the need for high hydrophobicity and multiple (usually aromatic) rings for compound binding to P-glycoprotein (6, 7, 20–24). The lowest energy conformation of each welwitindolinone was independently modeled using MM+ molecular mechanics force fields, with the default parameter sets in HyperChem (release 3.0). These models mimicked the X-ray crystallographic structure of compound **1**.[1] The three-dimensional structures of compound **1** and compound **2** were identical except for the presence of the isothiocyanate and isonitrile groups, respectively, indicating that substitution of these moie-

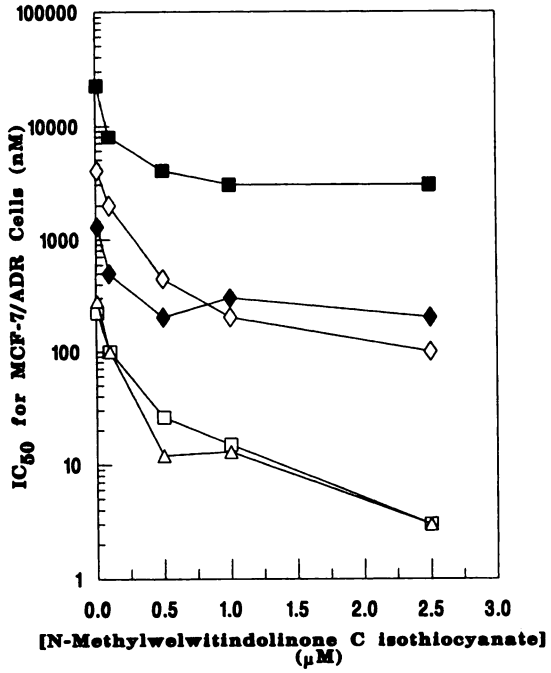


Fig. 4. Dose-response curves for MCF-7/ADR chemosensitization by *N*-methylwelwitindolinone C isothiocyanate (compound **1**). MCF-7/ADR cells were incubated with the indicated concentrations of *N*-methylwelwitindolinone C isothiocyanate and concentrations of daunomycin (■), vinblastine (□), colchicine (♦), taxol (◇), or actinomycin D (△) that inhibited cell proliferation by 0–80%. IC_{50} values were calculated for each drug at each concentration of the compound.

ties does not have an impact on the ring systems of the compounds. The isothiocyanate group of compound **1** projects approximately 1.4 Å beyond the isonitrile group of compound **2**.

Discussion

During the past 10 years, it has been widely recognized that acquired drug resistance severely limits the effectiveness of chemotherapy with current anticancer drugs. Biochemical characterization of the mechanisms of acquired drug resistance has placed strong emphasis on P-glycoprotein, because overexpression of this drug transporter appears to be a primary means for MDR both in cell culture and *in vivo*. Pharmacological characterization of agents that bind to P-glycoprotein and modulate drug resistance has been a fruitful area of research, leading to the identification of many compounds with these activities. However, most of these compounds fall into chemical families with similar chemical and physical properties, e.g., dihydropyridines, thioxanthenes, phenothiazines, and other hydrophobic agents. Screening-based approaches have identified a number of compounds with markedly different structures that appear to interact with P-glycoprotein. For example, novel alkaloids (25, 26), naphtho-γ-pyrones (27), a cyclic peptide (28), porphyrins (11, 12), and an acridonecarboxamide (29) have been found to modulate P-glycoprotein-mediated MDR. The identification of new reversing agents is important for two reasons. Firstly, preclinical and, ultimately, clinical testing of these agents may find them to be free of systemic cytotoxicities that preclude the clinical use of current reversing agents (10). Secondly, greater numbers of diverse structures that interact with P-glycoprotein should allow more detailed characterization of the drug binding site(s) of P-glycoprotein. Identification of chemical and physical properties that determine whether a compound can bind to P-glycoprotein should be extremely useful in the rational design of additional MDR-reversing agents. This approach of analogue design and testing has led to the refinement of many current drugs, including *Vinca* alkaloids, podophyllotoxins, anthracyclines, taxanes, and antimetabolites. Therefore, identification of reversing agents with novel structures is likely to be important in anticancer drug development. To contribute to this effort, we have screened a large collection of extracts from blue-green algae for novel compounds with anti-MDR activity.

Using bioactivity-directed fractionation procedures, we have isolated several novel alkaloids from *H. welwitschii* (13). These compounds are structurally very similar but have significantly different cytotoxicities and MDR-reversing activities. Compound **1** is the most interesting as a potential MDR-reversing agent, because it has the lowest intrinsic cytotoxicity and the greatest efficacy in chemosensitization. This compound dem-

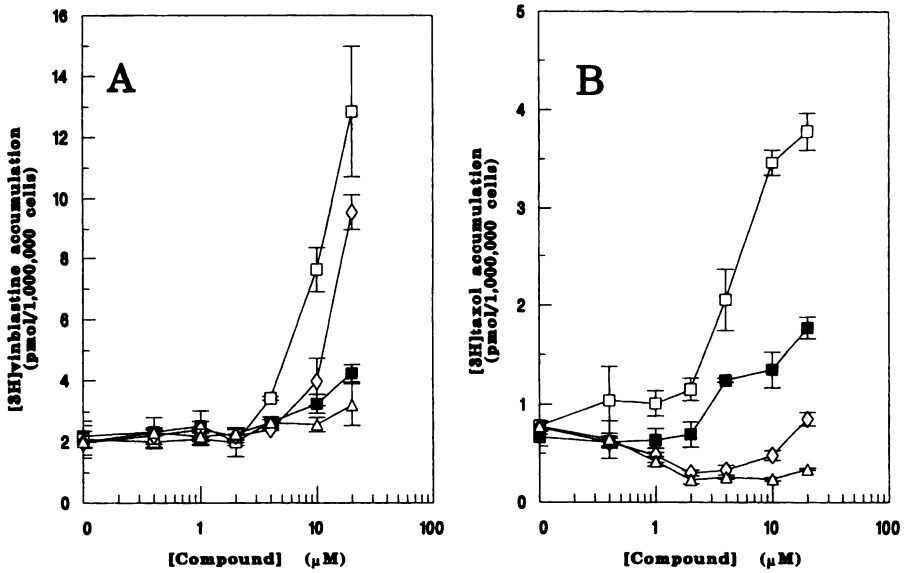


Fig. 5. Effects of welwitindolinones and verapamil on drug accumulation in SK-VLB-1 cells. SK-VLB-1 cells were incubated with the indicated concentrations of verapamil (■), compound **1** (□), compound **2** (△), or compound **3** (◇) for 30 min, as indicated in Experimental Procedures. [^{3}H]Vinblastine (A) or [^{3}H]taxol (B) was then added and its intracellular accumulation after 60 min was determined. Values represent the mean ± standard deviation of accumulation of ^{3}H-labeled drug in one of three similar experiments.

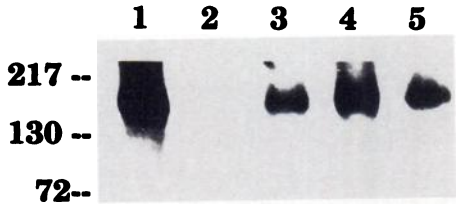


Fig. 6. Photoaffinity labeling of P-glycoprotein. Membranes from MCF-7 cells (*lane 2*) or MCF-7/ADR cells (*lanes 1* and *3-5*) were photolabeled with [^{3}H]azidopine as described in Experimental Procedures. Samples contained buffer (*lanes 1* and *2*), 10 μM compound **1** (*lane 3*), 10 μM compound **2** (*lane 4*), or 10 μM compound **3** (*lane 5*). The positions of the following prestained molecular mass markers are indicated: myosin, 217 kDa; β-galactosidase, 130 kDa; bovine serum albumin, 72 kDa.

onstrates reversing efficacy at least as great as that of verapamil in two different MDR cell lines. In the extremely resistant SK-VLB-1 cell line, compound **1**, like verapamil, markedly potentiated cell killing by actinomycin D and daunomycin. Studies with the moderately resistant MCF-7/ADR cell line demonstrated that compound **1** is able to fully reverse resistance to vinblastine and actinomycin D and is as efficacious as verapamil in chemosensitizing the cells to taxol. In contrast, neither verapamil nor compound **1** significantly modulated the sensitivity of these cells to cisplatin, indicating that the effects of compound **1** are probably due to antagonism of P-glycoprotein. Compound **1** appears to have approximately the same potency for chemosensitization of MCF-7/ADR cells to daunomycin, vinblastine, colchicine, taxol, and actinomycin D. This strongly suggests that its action is due to interaction with a common factor for these drugs, i.e., P-glycoprotein, rather than to a particular drug target such as microtubules or DNA metabolism. The ability of compound **1** to chemosensitize the cells at doses as low as 0.1 μM demonstrates that this agent is 20–100-fold more potent than verapamil. This dose is approximately 30-fold lower than its IC_{50}, suggesting that the potential therapeutic index for this compound is reasonably large. Interestingly, the lack of resistance of MCF-7/ADR (and SK-VLB-1) cells to the cytotoxicity of compound **1**, as well as compounds **2** and **3**, suggests that P-glycoprotein may not effectively transport these agents. The mechanism of cytotoxicity induced by these welwitindolinones is presently unknown; however, the specificity of the target is clearly different from the specificity of P-glycoprotein, because compound **2** is much more cytotoxic than compound **1**.

All available information points to direct binding to P-glycoprotein as the mechanism of chemosensitization by compound **1**. Specifically, the generality of its effects on P-glycoprotein substrate drugs, its ability to promote the accumulation of [^{3}H]vinblastine and [^{3}H]taxol in MDR cells, and its ability to reduce P-glycoprotein photoaffinity labeling by [^{3}H]azidopine provide experimentally independent and mutually supportive evidence for this mechanism of action. We therefore believe that *N*-methylwelwitindolinone C isothiocyanate represents the lead compound for a new family of P-glycoprotein antagonists. Some interesting structure-function relationships are apparent even with the small number of compounds currently available. Absence of the methylated nitrogen, i.e., compound **3**, markedly increases the cytotoxicity but does not preclude interaction of the agent with P-glycoprotein, as indicated by the retention of its ability to promote [^{3}H]vinblastine accumulation and to reduce P-glycoprotein photolabeling. However, the consequent reduction in the ratio of the EC_{50} for chemosensitization to the IC_{50} for cell proliferation makes compound **3** less attractive than compound **1** as a potential MDR-reversing agent.

Consideration of compound **2** also reveals some interesting structural relationships. Firstly, replacement of the isothiocyanate group of compound **1** with a nitrile group to yield compound **2** also strongly increases cytotoxicity. However, without knowledge of the molecular target, the details and significance of this difference are unclear. More pertinent to the present study is the observation that compound **2** is much less effective at interfering with drug binding to P-glycoprotein than is compound **1**. This is demonstrated by its inability to chemosensitize either SK-VLB-1 or MCF-7/ADR cells, its lack of ability to enhance ^{3}H-labeled drug accumulation by SK-VLB-1 cells, and its poor competition with [^{3}H]azidopine binding to P-glycoprotein. Therefore, the seemingly small chemical difference between compounds **1** and **2** has a major impact on binding to both their molecular target and P-glycoprotein. Computer modeling studies indicate that the overall structures of compounds **1** and **2** are very similar. The primary differences between compounds **1** and **2** are the extension heights and the electronegativities of the R_1 functional groups. These differences appear to be crucial for determining interaction with the drug binding site of P-glycoprotein.

Acknowledgments

We thank Dr. Victor Ling of the Ontario Cancer Institute for providing the SK-OV-3 and SK-VLB-1 cell lines used in these studies, L. K. Larsen for growing *H. welwitschii*, and Xinqun Zhang for assistance with some of these studies.

References

1. Endicott, J. A., and V. Ling. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* **58**:137–171 (1989).
2. Gottesman, M. M., and I. Pastan. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**:385–427 (1993).
3. Moscow, J. A., and K. H. Cowan. Multidrug resistance. *J. Natl. Cancer Inst.* **80**:14–20 (1988).
4. Goldstein, L. J., H. Galski, A. Fojo, M. Willingham, S.-L. Lai, A. Gazdar, R. Pirker, A. Greene, W. Crist, G. M. Brodeur, M. Lieber, J. Cossman, M. M. Gottesman, and I. Pastan. Expression of a multidrug resistance gene in human cancers. *J. Natl. Cancer Inst.* **81**:116–124 (1989).
5. Fojo, A., K. Ueda, D. J. Salman, D. G. Poplack, M. M. Gottesman, and I. Pastan. Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. USA* **84**:265–269 (1987).
6. Ford, J. M., and W. N. Hait. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol. Rev.* **42**:155–199 (1990).
7. Pearce, H. L., M. A. Winter, and W. T. Beck. Structural characteristics of compounds that modulate P-glycoprotein-associated multidrug resistance. *Adv. Enzyme Regul.* **30**:357–373 (1990).
8. Smith, C. D., S. Carmeli, R. E. Moore, and G. M. L. Patterson. Scytophycins: novel microfilament-depolymerizing agents which circumvent P-glycoprotein-mediated multidrug resistance. *Cancer Res.* **53**:1343–1347 (1993).
9. Smith, C. D., X. Zhang, S. L. Mooberry, G. M. L. Patterson, and R. E. Moore. Cryptophycin: a new antimicrotubule agent active against drug-resistant cells. *Cancer Res.* **54**:3779–3784 (1994).
10. Raderer, M., and W. Scheithauer. Clinical trials of agents that reverse multidrug resistance: a literature review. *Cancer (Phila.)* **72**:3553–3563 (1993).
11. Prinsep, M. R., F. R. Caplan, R. E. Moore, G. M. L. Patterson, and C. D. Smith. Tolyporphin, a novel multidrug resistance reversing agent from the blue-green alga *Tolypothrix nodosa*. *J. Am. Chem. Soc.* **114**:385–386 (1992).
12. Smith, C. D., M. R. Prinsep, F. R. Caplan, R. E. Moore, and G. M. L. Patterson. Reversal of multiple drug resistance by tolyporphin, a novel cyanobacterial natural product. *Oncol. Res.* **6**:211–218 (1994).
13. Stratmann, K., R. Bonjouklian, J. B. Deeter, R. E. Moore, G. M. L. Patterson, C. D. Smith, T. A. Smitka, and S. Shaffer. Welwitindolinones, unusual alkaloids from the blue-green algae *Hapalosiphon welwitschii* and *Westiella intricata*: relationship to fischerindoles and hapalindoles. *J. Am. Chem. Soc.* **116**:9935–9942 (1994).
14. Bradley, G., M. Naik, and V. Ling. P-glycoprotein expression in multidrug resistant human ovarian carcinoma cell lines. *Cancer Res.* **49**:2790–2796 (1989).
15. Fairchild, C. R., S. P. Ivy, C.-S. Kao-Shan, J. Whang-Peng, N. Rosen, M. A. Isreal, P. W. Melera, K. H. Cowan, and M. E. Goldsmith. Isolation of

amplified and overexpressed DNA sequences from Adriamycin-resistant human breast cancer cells. *Cancer Res.* **47**:5141–5148 (1987).
16. Skehan, P., R. Stoneng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**:1107–1112 (1990).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of proteins using the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
18. Patterson, G. M. L., C. L. Baldwin, C. M. Bolis, F. R. Caplan, H. Karuso, L. K. Larsen, I. A. Levine, R. E. Moore, C. S. Nelson, K. D. Tschappat, and G. D. Tuang. Antineoplastic activity of cultured blue-green algae (cyanobacteria). *J. Phycol.* **27**:530–536 (1991).
19. Beck, W. T., and X.-D. Qian. Photoaffinity substrates for P-glycoprotein. *Biochem. Pharmacol.* **43**:89–93 (1992).
20. Pearce, H. L., A. R. Safa, N. J. Bach, M. A. Winter, M. C. Cirtain, and W. T. Beck. Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance. *Proc. Natl. Acad. Sci. USA* **86**:5128–5132 (1989).
21. Ford, J. M., W. C. Prozialeck, and W. N. Hait. Structural features determining activity of phenothiazines and related drugs for inhibition of cell growth and reversal of multidrug resistance. *Mol. Pharmacol.* **35**:105–115 (1989).
22. Ford, J. M., E. P. Bruggemann, I. Pastan, M. M. Gottesman, and W. N. Hait. Cellular and biochemical characterization of thioxanthenes for reversal of multidrug resistance in human and murine cell lines. *Cancer Res.* **50**:1748–1756 (1990).
23. Nogae, I., K. Kohno, J. Kikuchi, M. Kuwano, S.-I. Akiyama, A. Kiue, K.-I. Suzuki, Y. Yoshida, M. M. Cornwell, I. Pastan, and M. M. Gottesman. Analysis of structural features of dihydropyridine analogs needed to reverse multidrug resistance and to inhibit photolabeling of P-glycoprotein. *Biochem. Pharmacol.* **38**:519–527 (1989).
24. Ramu, N., and A. Ramu. Circumvention of Adriamycin resistance by dipyridamole analogues: a structure-activity relationship study. *Int. J. Cancer* **43**:487–491 (1989).
25. Chen, G., C. Ramachandran, and A. Krishan. Thaliblastine, a plant alkaloid, circumvents multidrug resistance by direct binding to P-glycoprotein. *Cancer Res.* **53**:2544–2547 (1993).
26. Kadam, S., M. Maus, J. Poddig, S. Schmidt, R. Rasmussen, E. Novosad, J. Plattner, and J. McAlpine. Reversal of multidrug resistance by two novel indole derivatives. *Cancer Res.* **52**:4735–4740 (1992).
27. Ikeda, S., M. Sugita, A. Yoshimura, T. Sumizawa, H. Douzono, Y. Nagata, and S. Akiyama. *Aspergillus* species strain M39 produces two naptho-γ-pyrones that reverse drug resistance in human KB cells. *Int. J. Cancer* **45**:508–513 (1990).
28. Williams, A. B., and R. S. Jacobs. A marine natural product, patellamide D, reverses multidrug resistance in a human leukemic cell line. *Cancer Lett.* **71**:97–102 (1993).
29. Hyafil, F., C. Vergely, P. Du Vignaud, and T. Grand-Perret. *In vitro* and *in vivo* reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. *Cancer Res.* **53**:4595–4602 (1993).

Send reprint requests to: Charles D. Smith, Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.