Cancer Chemother Pharmacol (2005) 56: 379–390
DOI 10.1007/s00280-005-1019-y

ORIGINAL ARTICLE

**Ting-Chao Chou · Yongbiao Guan
Danielle R. Soenen · Samuel J. Danishefsky
Dale L. Boger**

Potent reversal of multidrug resistance by ningalins and its use in drug combinations against human colon carcinoma xenograft in nude mice

Received: 21 October 2004 / Accepted: 12 January 2005 / Published online: 4 May 2005

Abstract *Purpose*: To evaluate the pharmacological properties and the possible therapeutic applications of a series of synthetic marine natural product analogs, ningalins (N1–N6), in terms of cytotoxicity, MDR-reversing activity, and enhancement of drug combinations with antitumor agents in vitro and in vivo. *Methods*: XTT assays, [^{3}H]azidopine binding to P-glycoprotein (Pgp), cellular accumulation and efflux of labeled drugs were carried out in vitro. Drug combinations using combination index, dose-reduction index, and isobologram were performed in vitro and enhancement of efficacy in drug combinations against human colon carcinoma HCT-116 xenografts were conducted with nude mice. *Results*: N3 at sub-IC_{50} cytotoxic concentration (10 μM) was capable of enhancing vinblastine (VBL) cytotoxicity toward human leukemic CCRF-CEM cells about 50,000-fold as measured by the decrease of IC_{50} of VBL. For CCRF-CEM/VBL_{1000} (1,500-fold resistant to VBL and overexpressing Pgp), N3 and N5 enhanced VBL cytotoxicity as much as 6.2 million-fold and 210,000-fold, respectively. Moreover, N3 and N5 collaterally made CCRF-CEM/VBL_{1000} cells 4,000-fold and 130-fold, respectively, more susceptible to VBL than the parent CCRF-CEM cells. In human mammary carcinoma cells MX-1/paclitaxel which were 170-fold resistant to taxol and 38-fold resistant to VBL, N3 was capable of enhancing VBL effect as much as 6,000-fold. Combination therapy on murine P388/doxorubicin (DX) leukemia with DX + N3 or taxol + N3 achieved greater efficacy than the therapy with each drug alone. Impressively, nude mice, bearing human colon carcinoma HCT-116 cells, treated with a suboptimal dosage of taxol in combination with N3, N5 or N6 led to shrinkage of established tumor and achieved total tumor remission, while taxol alone had no tumor disappearance in this xenograft model. Furthermore, the enhancement of antitumor effect by ningalins, at least in parts, are due to inhibiting Pgp which was supported by the observation that the ningalins compete for [^{3}H]azidopine binding to Pgp, increase the cellular accumulation of VBL or taxol, and inhibit drug efflux from the tumor cells. *Conclusion*: The profound enhancement of antitumor cytotoxicity of vinblastine and taxol in vitro by ningalins may have multiple mechanisms including the MDR-reversing effects. The mechanisms for collateral sensitivity by ningalins against sensitive (parent) cells are not yet clear. The marked enhancement of therapeutic effect of taxol by ningalins against xenograft tumors in nude mice suggests potential applications of therapeutic use of ningalins.

Keywords Ningalin · MDR-reversal · Combination index · Therapeutic potentiation

Abbreviations N: Ningalin · MDR: Multidrug resistance · Pgp: P-glycoprotein · CI: Combination index · NAA or 5N-Ac-Ard: 5-*N*-acetyl ardeemin · VRPL: Verapamil · DX: Doxorubicin · VBL: Vinblastine · DMSO: Dimethylsulfoxide · Taxol: Paclitaxel

T.-C. Chou (✉) · Y. Guan · S. J. Danishefsky
Molecular Pharmacology and Chemistry Program,
Sloan-Kettering Institute for Cancer Research,
1275 York Avenue, New York 10021, USA
E-mail: chout@mskcc.org
Tel.: +1-212-6397480
Fax: +1-212-7944342

D. R. Soenen · D. L. Boger
Department of Chemistry, The Scripps Research Institute,
10550 North Torrey Pines Road, Jolla, CA 92037, USA

Introduction

Innate or acquired drug resistance is generally considered a major factor in cancer therapeutic failure. The quest to overcome drug resistance has been a major effort in clinical oncology [1]. Chemotherapeutic agents of natural origins such as vinblastine (VBL), vincristine, doxorubicin (DX), paclitaxel (taxol), actinomycin D, and their analogs are frequently cross resistant, although

they have dissimilar chemical structures and mechanisms of action. Cancer cells with the multiple drug resistance (MDR) phenotypes are known to overexpress P-glycoprotein (Pgp), a membrane protein that mediates the efflux of MDR drugs. Some MDR cells show elevated levels of multidrug resistant-associated protein (MRP), lung resistance protein (LRP) or the increased expression of other members of the ABC transporter gene family [2, 3].

A range of agents has been shown to modify, modulate or reverse the MDR phenotype [4–6]. The classic resistance modifiers include verapamil (VPML), cyclosporines, calmodulin-inhibitors, quinine, progesterone, tamoxiphen, amidorone, and trifluoperazine. The more recent introductions include dexverapamil, SDZ PSC 833, SDZ 280–446, XR9051, GF120918, and ardeemin [7]. Of these, VPML, trifluoperazine, cyclosporine A, and nifedipine have been subjected to phase I/II clinical trials [5, 8]. Many of these compounds already have designated medical indications and are employed in the clinic. Use of these compounds as MDR-reversing agents in combination with cancer chemotherapeutic agents frequently suffers from the lack of desired potency and may expose patients to unacceptable side effects or toxicity at doses required for effectiveness [4, 5, 8].

Herein, we describe detailed studies in a series of marine natural product analogs, ningalin compounds (N1–N6), which we recently discovered to be potent MDR-reversal agents [9–11]. These compounds, which were made readily available through development of an efficient total synthesis entailing a unique tetrazine $\rightarrow$ diazine $\rightarrow$ pyrrole heterocyclic azadiene Diels-Alder strategy, were found to lack intrinsic cytotoxic activity and reverse MDR in vitro. We now show that among them, N3 exhibits enhancement of VBL cytotoxicity against VBL-resistant leukemic cells by as much as several million-fold. Thus, N3 made the VBL-resistant cell line 4,000-fold more susceptible to VBL than the parent, non-MDR leukemic cell line (i.e., collateral sensitivity). To our knowledge, this magnitude of collateral sensitivity and the megafold MDR reversal is unprecedented. In this study, we demonstrate that ningalins compete with [^{3}H]azidopine for the Pgp binding site. At the cellular level, we show that intracellular accumulation and retention of an MDR substrate (e.g., VBL or taxol) are increased by the ningalins. However, the megafold enhancement and the kilofold collateral hypersensitivity suggest that ningalins may have multiple modes of actions in addition to the MDR-reversal. In the present study, we demonstrate that combination doses of N3, plus DX or taxol show very strong synergism in vitro, and enhance therapeutic effects against murine leukemia P388/DX in vivo. Furthermore, in nude mice-xenograft models, combination of suboptimal doses of taxol with N3, N5 or N6 not only led to shrinkage of HCT-116 tumor size but also achieved a complete therapeutic remission, without increasing toxicity toward the host.

Materials and methods

Chemicals

Paclitaxel and vinblastine sulfate (VBL) were purchased from Sigma. The ningalin derivatives N1–N6 were prepared as described [9–11]. All stock solutions of the compounds were prepared using dimethylsulfoxide (DMSO) as solvent (except VBL in saline) and were further diluted to desired concentrations for use. The final concentration of DMSO in the tissue cultures was 0.25% (vol/vol) or less to avoid solvent cytotoxicity. For in vivo studies, the ningalins were dissolved in DMSO for i.p. injection. Taxol in Cremophor/ethanol formulation was commercially obtained as the clinical preparation from Bristol-Myers Squibb. Vinblastine sulfate (Velban; Eli Lilly) and adriamycin (DX or Adr, DX-HCI; Pharmacia & Upjohn Co.) were used as the manufacturer's formulation and diluted with saline.

Tumor and cell lines

The CCRF-CEM human T cell acute lymphoblastic leukemic cells, its teniposide-resistant subline (CCRF-CEM/VM_1), and VBL-resistant subline (CCRF-CEM/VBL_{100}) were obtained from W.T. Beck (University of Illinois, Chicago, IL, USA). These sublines were exposed to increasing sublethal concentrations (IC_{50}–IC_{90}) of VBL for 16 months and taxol for 12 months, respectively (designated CCRF-CEM/VBL_{1000} and CCRF-CEM/taxol, respectively). The drug-containing medium was replenished every 7–14 days. These nascent-resistant cell lines exhibited a 1,500-fold resistance to VBL (IC_{50}: 0.97 μM)and a 206-fold resistance to taxol (IC_{50}: 0.34 μM), respectively, when compared with wild type CCRF-CEM cells. In each case, the drug-exposed cells were resuspended in fresh media for a minimum of 4 days before the cell-growth inhibition experiments were performed. HCT-116 (wild type human colon carcinoma), and the resistant cell line HCT-116/VM46 (MDR) were obtained from D.M. Floyd and C.R. Fairchild (Bristol-Myers Squibb).

The following human cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA): mammary carcinoma (MX-1) and promyelocytic leukemia (HL-60).

Animals

Athymic nude mice, bearing the nu/nu gene, were used for all human tumor xenografts. Outbred, Swiss-background mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Male mice 8 weeks or older, weighing 22 g or heavier, were used for most experiments. The ningalins were administered i.p., and other drugs were given i.v. with an interval of 5 min

between them in drug combinations. Tumor volumes were assessed by measuring length × width × height (or width) using a caliper. All animal studies were conducted in accordance with the guidelines of the National Institutes of Health "Guide for the Care and Use of Animals" and the protocol approved by the Memorial Sloan-Kettering Cancer Center's Institutional Animal Care and Use Committee. In keeping with the policy of this committee for the humane treatment of tumor-bearing animals, mice were euthanized when tumors reached ≥10% of their total body weight.

Cytotoxicity assays

In preparation for the cytotoxicity assays, cells were cultured at an initial density of 2–5 × 10^4 cells per ml. They were maintained in a 5% CO_2-humidified atmosphere at 37°C in RPMI medium 1640 (GIBCO/BRL) containing penicillin (100 U/ml), streptomycin (100 μg/ml, GIBCO/BRL), and 5% heat-inactivated fetal bovine serum. For solid tumor cells growing in a monolayer, cytotoxicity of the drug was determined in 96-well microtiter plates using the sulforhodamine B (SRB) method as described by Skehan et al. [12]. For cells grown in suspension (such as CCRF-CEM and its sublines), cytotoxicity was measured, in duplicate, using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-carboxanilide)-2*H*-terazodium hydroxide (XTT) microculture method [13] in 96-well microtiter plates. For both methods, the absorbance of each well was measured with a microplate reader (EL-340, Bio-Tek, Burlington, VT, USA). Each run entailed six or seven varied concentrations of the tested drugs. Dose–effect relationship data was analyzed with the median-effect plot [14, 15] using a previously described computer program [16, 17].

Development of drug-resistant cell lines

To develop drug resistance in vitro, human breast carcinoma MX-1 cells were repeatedly exposed to doses between the IC_{50} and IC_{90} of various anticancer agents during a 21.4-month period (MX-1/VBL was 50-fold resistant to VBL when compared with IC_{50} in parent cells). Every several weeks, cells were harvested to determine their levels of resistance (using XTT assay) by comparing the IC_{50} value with that of the untreated parent cells. Increasing drug concentrations (1–2-fold of the new IC_{50}) were added to the medium weekly for continued exposure of cells [18].

Intracellular accumulation of [^{3}H]VBL or [^{3}H]taxol

Uptake and intracellular accumulation of [^{3}H]VBL or [^{3}H]taxol were measured by a rapid oil-layer separation technique described by Wohlhueter et al. [19]. A mixture of mineral oil (Sigma Chemical Co., St. Louis, MO, USA) and silicon oil (J.T. Baker Chemical Co., Phillipsburg, NJ, USA), which had a final density of 1.032 g/ml, was used to separate medium and cells following rapid centrifugation (12,000 *g*) using an Eppendorf model 5,415 C microcentrifuge. The [^{3}H]VBL-containing cells rapidly moved to the bottom of the oil-layer whereas the [^{3}H]VBL-containing medium remained atop. The radioactivity in the cell packs was determined by a liquid scintillation counter.

Photoaffinity labeling of Pgp with [^{3}H]azidopine

To measure Pgp in cells, plasma membrane-enriched microsomal fractions were prepared from CCRF-CEM and CCRF-CEM/VBL_{100} cells as described previously [7]. Photoaffinity labeling of Pgp with (52 Ci/mmole) was performed in the absence and presence of various concentrations of ningalins. Electrophoresis on 8% polyacrylamide gel and autoradiography were as described previously [7].

Mathematical analysis of drug combinations

To determine whether synergistic, additive, or antagonistic anticancer effects were achieved in vitro, cell lines were treated with two-drug combinations. The median-effect plot and the combination index-isobologram method of Chou-Talalay [14, 15] and a computer software program [16, 17] were used to analyze the experimental data. The dose-reduction index (DRI) denotes the folds of dose reduction allowed for each drug in the combination when compared with each drug alone, at a given effect level [14, 17].

Xenograft studies

Human tumor cells (5 × 10^6) or tumor tissue (30–50 mg) were implanted s.c. flank in nu/nu mice (Charles River Laboratories, Wilmington, MA, USA). When tumors grew to the specified size, treatment was begun using a schedule as specified. Change in body-weight and lethality were monitored every other day.

Results

Cytotoxicity of ningalins

CCRF-CEM cytotoxicity of the ningalins was evaluated for N1–N6, as shown in Table 1. All the compounds tested are relatively nontoxic with IC_{50} values ranging from 13 μM to 152 μM. Five out of six ningalins (except N5) showed relatively higher IC_{50} values in CCRF-CEM/VBL_{1000} cells than in the parent CCRF-CEM cells.

Table 1 Inhibition of CCRF-CEM subline cell growth by VBL, the ningalin compounds, and their combinations

Compound	CCRF-CEM	CCRF-CEM/VBL_{1000}		Enhancement in CCRF-CEM cells	Enhancement in CCRF-CEM/VBL_{1000}cells	Sensitive cell − N vs Resistant cell + N
		(IC_{50} in μM)[a]	Fold of resistance[c]			
N1	33	47	1.4×			
N2	24	230	9.6×			
N3	13	100	7.7×			
N4	150	210	1.4×			
N5	32	18	0.6×			
N6	42	130	3.1×			
VBL[b]	0.0004 (C)	0.62 (D)	1,500×			
	(A)	(B)	(B)/(A)[c]	[(C)/(A)][d]	[(D)/(B)][e]	[(C)/(B)][f]
+N1	0.00014	0.003	21×	2.9×	210×	0.13×
+N2	0.00015	0.0002	1.3×	2.7×	3,100×	2×
+N3	<0.00001	<0.0000001	0.01×	>40×	6.2×10^6×	>4,000×
+N4	0.00041	0.0009	2.2×	0.98×	690×	0.44×
+N5	<0.000001	0.000003	>3×	400×	210,000×	130×
+N6	0.00005	0.0000058	0.12×	8×	110,000×	69×

[a]The IC_{50} values were determined from six to seven concentrations of compound and the dose–effect curves were analyzed with the median-effect plot using a computer software [16, 17]. The concentration for all ningalins was 10 μM which was the sub-IC_{50} concentration
[b]VBL concentrations were varied
[c]Fold of resistance by comparing IC_{50s} of resistant versus sensitive cells
[d]Fold of enhancement of VBL cytotoxicity by the ningalin compound in parent CCRF-CEM cells
[e]Fold of enhancement of VBL cytotoxicity by the ningalin compound in CCRF-CEM/VBL_{1000} MDR cells
[f]Collateral sensitivity for CCRF-CEM/VBL_{1000} cells in the presence of N3 when compared with parent CCRF-CEM cells in the absence of N3

Strong enhancement of VBL cytotoxicity by ningalins

A sub-IC_{50} concentration of ningalins (10 μM each) was found to have profound enhancing effects on the cytotoxicity of VBL especially for CCRF-CEM/VBL_{1000} cells that were 1,500-fold resistant to VBL (Fig. 2). Based on the decrease in IC_{50} values, the enhancement of VBL toxicity was 210-fold, 3,100-fold, and 690-fold, for N1, N2, and N4, respectively, whereas the enhancement for N3, N5, and N6 was 6.2×10^6-fold, 210,000-fold, and 110,000-fold, respectively (Table 1). Notably, the VBL cytotoxicity in the parent CCRF-

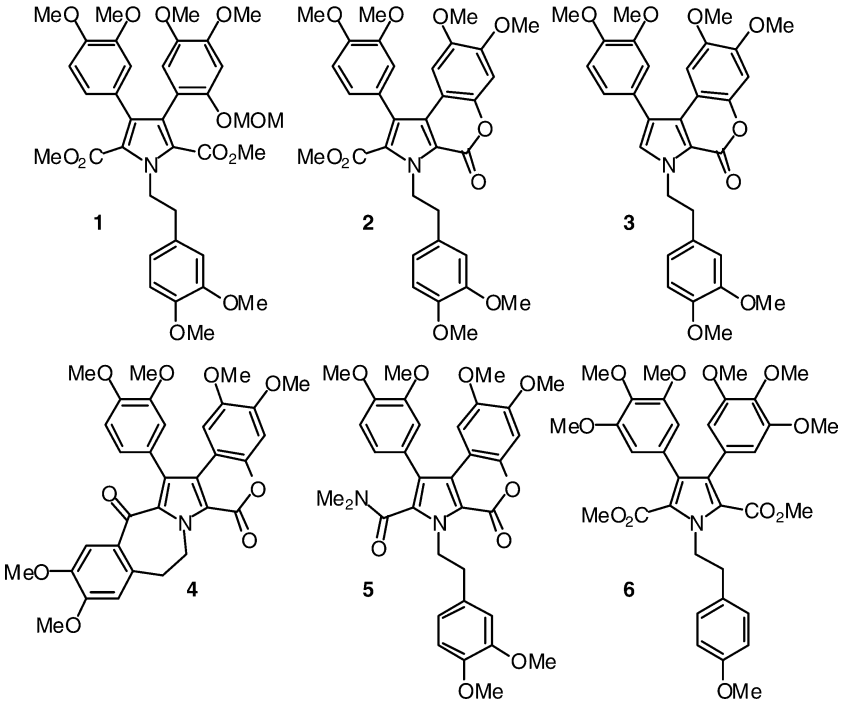

Fig. 1 Structures of ningalin (N) compounds

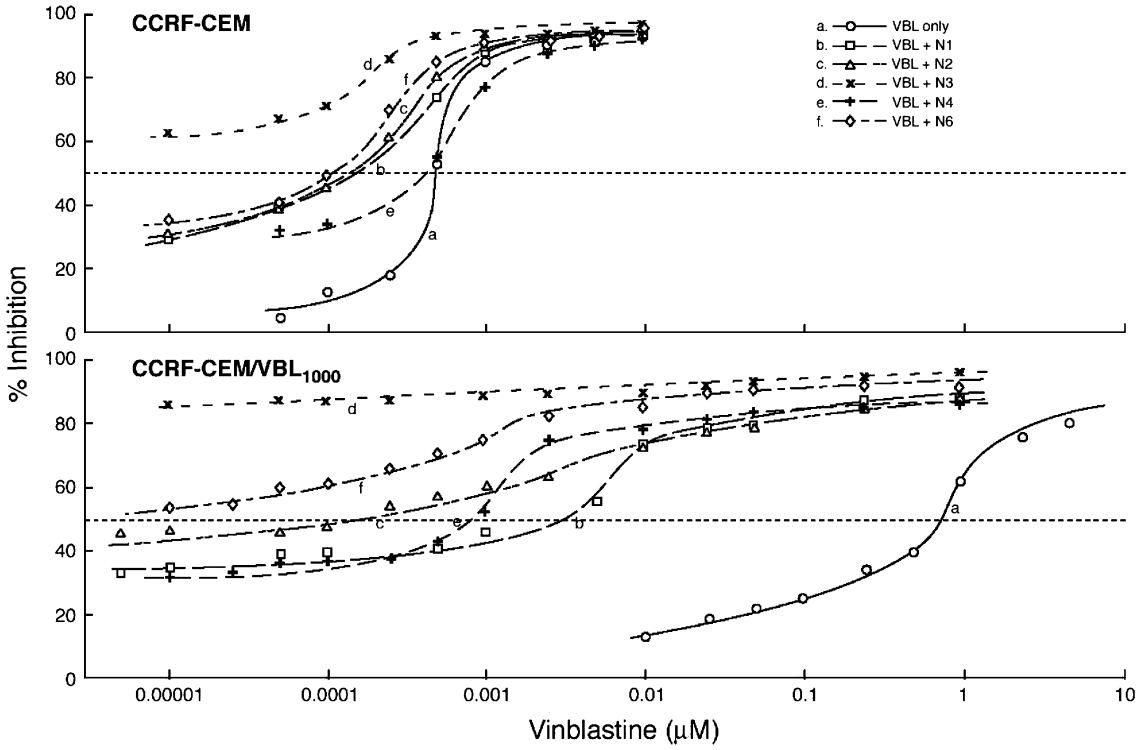

Fig. 2 Dose–effect curves for VBL against cell growth of human leukemia cells in the absence and presence of ningalin agents. **a** CCRF-CEM T-lymphoblastic leukemic cells and **b** CCRF-CEM/VBL_{1000} leukemic cells 1,500-fold resistant to VBL. *Open circle* represents varying concentrations of VBL without ningalin as the control. The others are the controls in the presence of 10 μM of the following ningalins: *open square* N1; *open triangle* N2; *crosses* N3; *plus symbol* N4; and *open diamond* N6. Overall, the concentrations of VBL were varied two million-fold (0.000005–10 μM) and the concentration of ningalins used (10 μM) was less than IC_{50} value toward the CCRF-CEM cells. (see Table 1 for the IC_{50} values of VBL in the presence and absence of ningalins and the folds of shift in IC_{50} values in the presence of 10 μM ningalins)

CEM cells was also enhanced substantially, with enhancements of 40-fold, >400-fold, and 8-fold by N3, N5, and N6, respectively, (see [(C)/(A)] column in Table 1). It is also of interest to note that in the presence of N3 or N6 (10 μM each), the resistant CCRF-CEM/VBL_{1000} cells became 100-fold and 8.6-fold, respectively, collaterally more sensitive to VBL than the parent CCRF-CEM cells (see [(B)/(A)] column in Table 1). This collateral sensitivity (see [(C)/(B)] column in Table 1) was increased to as much as >4,000-fold and 130-fold by 10 μM of N3 and N5, respectively.

Dose–effect relationships of N3, N5, and N6

The VBL-enhancing dose–effect relationships of N3, N5, and N6 were studied with CCRF-CEM/VBL_{1000} cells. The effects were drastically dose-dependent (Table 2). At 0.1 μM of ningalins, the enhancements were 7–25-fold, and at 1 μM of ningalins, the enhancements were 41–440-fold. At a higher concentration, at 10 μM, the enhancement effects were 620-fold, 210,000-fold, and 6.2×10^6-fold for N6, N5, and N3, respectively (Table 2). Therefore, the enhancement of VBL cytotoxicity by ningalins showed marked concentration dependency. As shown in Table 1, the ningalins by themselves have low cytotoxicity, with IC_{50} values of 42 μM, 32 μM, and 13 μM, for N6, N5, and N3, respectively. Thus, these three ningalins at sub-IC_{50} concentrations exhibited a profound enhancing effect on VBL antileukemic cytotoxicity.

Table 2 Dose–effect relationships for potentiation of cytotoxicity of VBL by N3, N5, and N6 in CCRF-CEM/VBL_{1000} drug-resistant leukemic cells

Compound and concentration	IC_{50} value (in μm) of VBL in CCRF-CEM/VBL_{1000} cells	Fold of potentiation due to the presence of ningalin[b]
VBL[a] alone	0.62	–
VBL[a] + N3		
0.1 μM	0.025	25×
1 μM	0.0014	440×
10 μM	0.0000001	6.2×10^6×
VBL[a] + N5		
0.1 μM	0.03	21×
1 μM	0.009	69×
10 μM	0.000003	210,000×
VBL[a] + N6		
0.1 μM	0.095	7×
1 μM	0.015	41×
10 μM	0.001	620×

[a]Seven serial concentrations of VBL were used to construct each dose–effect curve. The IC_{50} values were determined by the median-effect plot using a computer software [16, 17]

[b]Based on [IC_{50} of VBL in the absence of ningalin/IC_{50} of VBL in the presence of ningalin]

Reversal of VBL and taxol resistance in MX-1/taxol cells by N3

Continuous exposure of human mammary carcinoma MX-1 cells to increasing concentrations of taxol for 27 months led to the development of a cell line that was 167-fold resistant to taxol. These cells were shown to be 38-fold resistant to VBL (Table 3). In the presence of 10 μM N3, the cytotoxicity of taxol and VBL toward MX-1/taxol was enhanced 660-fold and 6,000-fold, respectively. Therefore, 10 μM N3 caused MX-1/taxol cells to be even more sensitive to taxol and VBL when compared with the parent MX-1, i.e., MX-1/taxol cells were 3.9-fold more susceptible to taxol and 160-fold more susceptible to VBL than the parent MX-1 cells.

Synergism between DX and N3 in P388/DX and P388/0 cells

P388/DX cells, 9.1-fold resistant to DX, along with its parent murine leukemic cells, P388/0, were used for in vitro drug combination studies. The combination index (CI) and isobologram method of Chou-Talalay [14, 15] and its computer software [16, 17] were used to determine the degree of synergism or antagonism between DX and N3. By definition, CITable 4, DX + N3 showed strong synergism in P388/0 cells with CI = 0.16–0.066 at IC_{50}–IC_{95}; and showed very strong synergism in P388/DX cells with CI = 0.097–0.0038 at IC_{50}–IC_{95}. As a result of this combination synergism, the IC_{50} values can be reduced 11.5-fold for DX and reduced 13.1-fold for N3 in the P388/0 cells; and the IC_{50} values can be reduced 45.6-fold for DX and 12.8-fold for N3 in P388/DX cells, when compared with the single drug alone. This large reduction in required doses due to synergism should benefit chemotherapy since dose-reduction leads to decreased toxicity toward the host while maintaining therapeutic efficacy.

Table 3 Drug resistance to MX-1/taxol and reversal of drug resistance by N3

Compound	$IC_{50}(\mu)$M		Fold of resistance[a] (B)/(A)
	MX-1/0 (A)	MX-1/taxol (B)	
N3 alone	11.2	60	5.4×
Taxol	0.015	2.5	167×
Taxol + N3	0.0011 (13.6×)[b]	0.0038 (660×)	3.5×
VBL	0.0024	0.09	38×
VBL + N3	0.0003 (8×)	0.000015 (6,000×)	0.05x[c]

N3 concentration was 10 μM
[a]Numbers are fold of drug resistance; (IC_{50} for MX-1/taxol versus IC_{50} for MX-1/0)
[b]Numbers in parentheses are folds of potentiation by N3; (IC_{50} without N3 versus IC_{50} with N3)
[c]Collaterally sensitive due to N3

Combination therapy of N3 with DX or taxol against P388/DX leukemia in vivo

In order to examine the synergism further between DX and N3, $B6D2F_1$ mice bearing P388 leukemia, resistant to DX, (P388/DX) were used as a model for combination chemotherapy. DX alone at 1.5 mg/kg and 3 mg/kg, i.v. QDx3 resulted in 20% and 29% increase in lifespan (ILS). Combination with N3 20 mg/kg, i.p. resulted in 46% ILS (Table 5). These ILS by N3 represent approximately a 1.2-log increase in cell kill. Similarly, taxol alone at 5 and 10 mg/kg, i.v. QDx3 resulted in 4% and 6% ILS, respectively. These ILS were further increased to 12% and 35%, respectively, by combination with N3 (20 mg/kg, i.p. QDx3).

Combination chemotherapy of taxol and ningalins against human colon carcinoma HCT-116 xenograft in nude mice

Cytotoxicity studies with wild type HCT-116 and a MDR cell line HCT-116/VM46 [9, 10] which

Table 4 Synergism between DX and N3 in combination against P388/0 and P388/DX cell growth

Tumor cells	Drug	$IC_{50}\mu$M	CI values[a]				Dose reduction index[b]	Parameters[c]	
			IC_{50}	IC_{75}	IC_{90}	IC_{95}		*m*	*r*
P388/0	DX	0.15					11.5	0.985	0.993
	N3	17					13.1	0.517	0.980
	Combination[d]DX + N3	0.013 + 1.3	0.16	0.100	0.075	0.066		1.105	0.995
P388/DX	DX	0.82					45.6	0.857	0.952
	N3	23					12.8	0.638	0.983
	Combination[d]DX + N3	0.018 + 1.8	0.097	0.028	0.0085	0.0038		2.989	0.982

[a]CI
[b]Folds of dose reduction allowed for each drug caused by synergism at a given effect level [15] (e.g., the DRI values at IC_{50} effect level)
[c]m, the slope of the median-effect plot [16, 17] signifying the shapes of dose–effect curves (i.e., $m = 1$, > 1 and < 1 indicates hyperbolic, sigmoidal, and shallow sigmoidal shapes, respectively). *r*, the linear correlation coefficient of the median-effect plot signifying the conformity of the dose–effect data to the method of data analysis
[d]DX/N3 = 1:100

Table 5 Therapeutic effects of adriamycin, taxol and/or N3 in B6D2F1 mice bearing P388/DX leukemia

Group	Dosage (mg/kg)	Route	Median survival time (days ± SD) (*n*)	% ILS	ILS (in days)	Log cell kill[a] (LCK)
Control	0		9.36 ± 1.53 (7)		0	
N3	20	i.p.	8.54 ± 0.86 (7)	−9	−0.82	
Adriamycin	1.5	i.v.	11.25 ± 1.75 (3)	+20	+1.89	1.42
Adriamycin	3.0	i.v.	12.08 ± 1.81 (3)	+29	+2.72	2.05
Adriamycin	1.5	i.v.	13.67 ± 0.52 (3)	+46	+4.31	3.24
Adriamycin + N3	20	i.p.				
Taxol	5.0	i.v.	9.56 ± 0.62 (4)	+4	+0.37	0.28
Taxol	10.0	i.v.	9.92 ± 0.63 (3)	+6	+0.56	0.42
Taxol	5	i.v.	10.50 ± 1.55 (4)	+12	+1.14	0.86
Taxol + N3	20	i.p.				
Taxol	10	i.v.	12.67 ± 1.63c (4)	+35[b]	+3.31[b]	2.49[b]
Taxol + N3	20	i.p.				

Tumor implanted i.p. (106 cells) on day 0; Treatment i.v. (adriamycin, taxol) and/or i.p. (N13) started 6 h after implantation; All treatments were daily for 3 days

[a]Calculated assuming doubling time is 0.4 day (9.6 h); LCK = $\log 2^{(ILS/0.4)}$

[b]One out of four mice died of toxicity on day 4

overexpresses Pgp established that N3, N5, and N6 were relatively nontoxic and equipotent in the two cell lines (data not shown). Moreover, all three have been found to reverse MDR resensitizing HCT-116/VM46 to both VBL and DX. This resensitization was observed at concentrations as low as 1 μM with N3 and N5 reestablishing the HCT-116 wild type sensitivity to HCT-116/VM46 for both VBL and DX (Table 6) and the remarkable hypersensitivity was observed at 7.5 μM (N5 > N6 > N3), the only higher dose examined. Athymic nude mice bearing human colon carcinoma HCT-116 xenografts were used for combination therapy with two doses of taxol (5 and 10 mg/kg, i.v. Q2D) plus N3 (20 mg/kg, i.p. Q2D). The 5 and 10 mg/kg doses selected for taxol were submaximal tolerated dose (sub-MTD) since our recent studies [18] in nude mice with the same xenograft, same schedule and route of treatments have indicated that the MTD for taxol was 20–24 mg/kg, i.v. Q2D. The selection of sub-MTD for taxol not only produced less toxicity toward the host but also provided room to demonstrate the enhanced therapeutic efficacy by N3. Following treatment with taxol at 5 mg/kg and 10 mg/kg on day 16, 18, 20, 26, 28, 30, 32, 40, and 42 tumor size was reduced 26.3% and 92.8%, respectively, when compared with the solvent treated control group, while tumor size was reduced 54.8% and 96.6%, respectively, in combination with N3 (Fig. 3). Despite the last treatment for taxol 10 mg/kg and N3 20 mg/kg on day 42, two out of four mice were tumor free on day 44 and on day 48. The tumors in the remaining two mice continued to decrease in size and on day 58, tumor size was less than half of the size on day 42. By contrast, the group treated with taxol (10 mg/kg) alone showed no tumor disappearance, instead slow tumor regrowth was observed. On day 56, the tumor grew to more than double the size of that on day 42 in the taxol-treated group (Fig. 3a). It should be noted that the group treated with N3 alone (20 mg/kg, i.p.) showed little therapeutic effect as evidenced on day 42 with only 19.1% tumor reduction. Therefore, N3 modulated taxol to achieve curative effects that taxol alone was unable to achieve. A special property of HCT-116 xenograft is that as tumor grows, the body weight decreases. Therefore, drug-treated and tumor-bearing mice may have higher body weight than the untreated control (Fig. 3b).

Subsequent studies of the combination of the suboptimal dose of taxol (10 mg/kg, i.v.) with N5 (15 mg/kg, i.p.) or N6 (20 mg/kg, i.p.) with a Q4D schedule is shown in Fig. 4. For the carrier treated control group, the tumor size reached 3 g in 32 days after tumor implantation. N5 or N6 as a single agent yielded no significant therapeutic effects. Taxol (10 mg/kg) alone had partial therapeutic effects, which suppressed tumor growth but had insufficient efficacy to shrink the tumor. Impressively, the combination of taxol with N5 or N6

Table 6 MDR-reversing activity of ningalins in HCT-116/VM46 cells

Ningalin compound	VBL IC_{50} (μM)[a]	Gain in sensitivity [b] (% reversion)	DX IC_{50} (μM) [a]	Gain in sensitivity [b] (% reversion)
N3				
1 μM	0.002	35 (100)[c]	0.02	4 (50)[c]
7.5 μM	0.001	70 (200)[c]	0.01	7 (100)[c]
N5				
1 μM	0.001	83 (100)[d]	0.1	67 (100)[d]
7.5 μM	0.0001	830 (1000)[d]	0.009	744 (1110)[d]
N6				
1 μM	0.009	22 (33)[e]	0.6	3.6 (40)[e]
7.5 μM	0.0008	250(370)[e]	0.08	31(280)[e]

[a]Each compound was tested at different dates, control IC_{50}'s are provided on [c–e]

[b]See references [9] and [10]

[c]HCT-116/VM46 IC_{50} = 0.07 μM (VBL) and 0.07 μM (DX). HCT-116 IC_{50} = 0.002 μM (VBL) and 0.01 μM (DX)

[d]HCT-116/VM46 IC_{50} = 0.08 μM (VBL) and 6.7 μM (DX). HCT-116 IC_{50} = 0.001 μM (VBL) and 0.1 μM (DX)

[e]HCT-116/VM46 IC_{50} = 0.2 μM (VBL) and 2.2 μM (DX). HCT-116 IC_{50} = 0.003 μM (VBL) and 0.2 μM (DX)

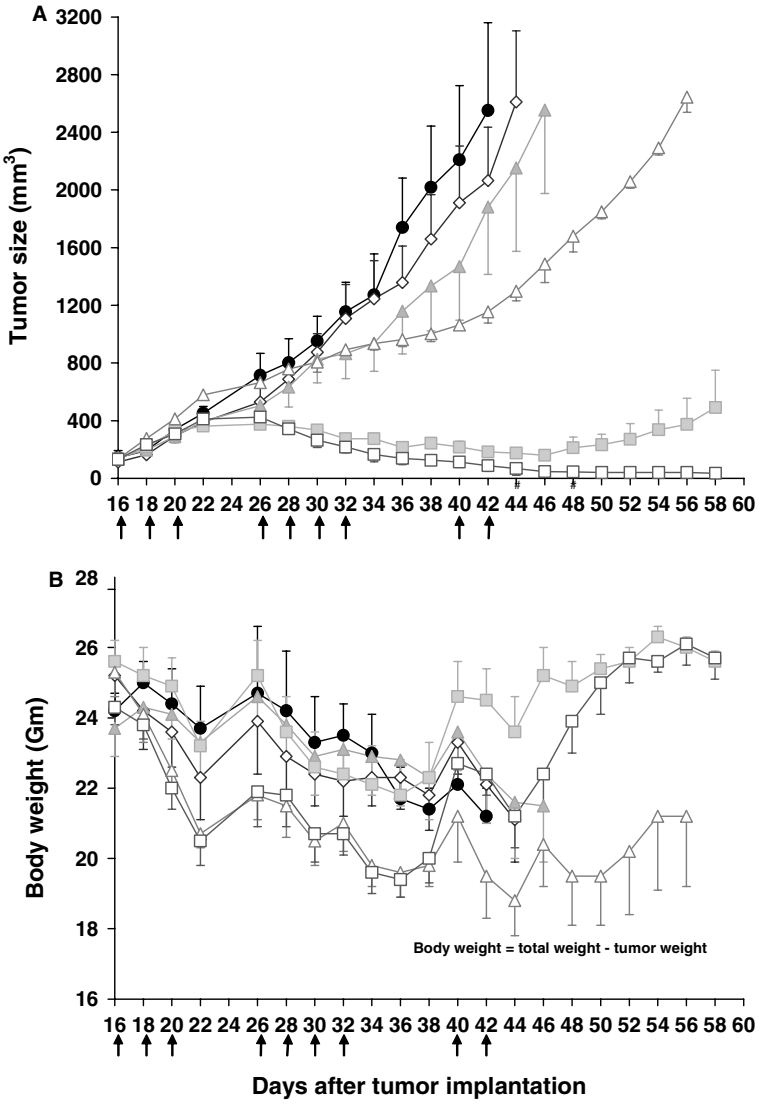

Fig. 3 Therapeutic effects of taxol alone and in combination with N3 in nude mice bearing human colon carcinoma HCT-116 xenograft. HCT-116 tumor tissue 50 mg/mouse was implanted s.c. on day 0. Treatments on days 16, 18, 20, 26, 28, 30, 32, 40, and 42 as indicated by *arrows* with 5 mg/kg taxol (*filled triangles*), 10 mg/kg taxol (*filled square*), 20 mg/kg N3 (*open diamond*), 5 mg/kg taxol + 20 mg/kg N3 (*open triangle*) and 10 mg/kg taxol + 20 mg/kg N3 (*open square*). Taxol was given i.v. and N3 was given i.p. All groups consisted of four mice. The controls (*filled circle*) received DMSO vehicle 75 µl i.p. (two mice) and cremophor-ethanol (1:1) 75 µl i.v. (two mice). The *vertical bars* indicate **a** mean tumor size in mm^3 ± SE, and **b** mean body weight ± SE. Body weight was measured by the total body weight minus tumor weight. In the 10 mg/kg taxol and 20 mg/kg N3 group (*open square*), two out of four mice achieved tumor disappearance on day 44 and day 48

resulted in total tumor disappearance in all mice without any tumor relapse even after 52 days following tumor transplantation (Fig. 4).

In this experiment, the body weights of the mice in all groups were monitored every other day (Fig. 3b). One of the unique features of the HCT-116 xenografts in nude mice is the observation that body weight of the mice decreases when tumor size increases (Figs. 3 and 4). It is remarkable that in the taxol + N3 (Fig. 3) and taxol + N5 or N6 (Fig. 4) treated groups, HCT-116 tumor disappeared and the body weight of the nude mice recovered to levels far above the other groups, suggesting that these ningalins combined are nontoxic in curative treatments.

Inhibition of azidopine photo-affinity labeling for Pgp

Evidence that ningalins interact directly with the MDR drug transporter, Pgp, was obtained by examining their effect in the inhibition of photo-affinity labeling of [^{3}H]azidopine, a photo-activatable substrate of Pgp. As shown in Fig. 5, N5 and N6 inhibited [^{3}H]azidopine binding to Pgp in a dose-dependent manner.

Intracellular accumulation of [^{3}H]taxol and the effect of N3

Intracellular accumulation of [^{3}H]taxol in MX-1/0 and MX-1/taxol were measured by an oil-layer rapid microcentrifuge method [18]. This method allows quick separation of the labeled cells from the radioactive medium. With the parent MX-1/0 cells, (Fig. 6a), intracellular concentrations of [^{3}H]taxol were increased by N3. The presence of DMSO (the vehicle control) and VBL showed similar accumulation of [^{3}H]taxol. Addition of unlabeled taxol decreased [^{3}H]taxol intracellular concentration. With the taxol resistant MX-1/taxol cells,

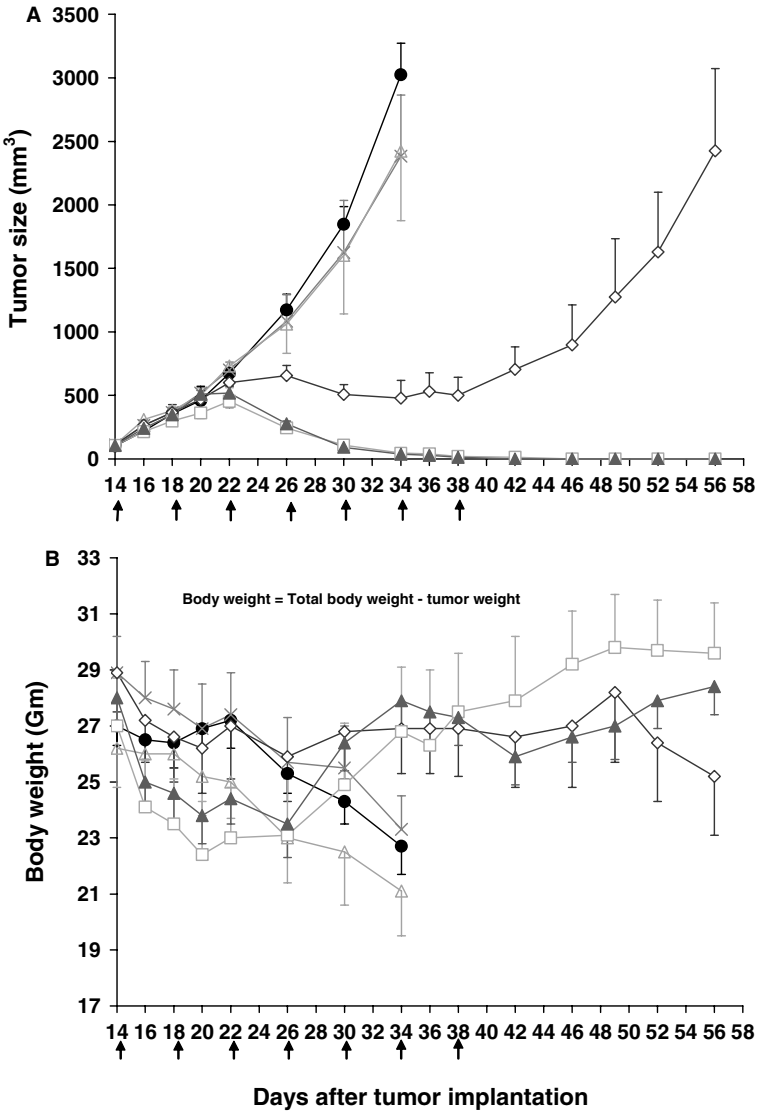

Fig. 4 Therapeutic effects of taxol alone and in combination with N5 and N6 in nude mice bearing human colon carcinoma HCT-116 xenograft. HCT-116 tumor tissue 50 mg/mouse was implanted s.c. on day 0. Treatment on day 14, 18, 22, 26, 30, 34, and 38 as indicated by *arrows* with 10 mg/kg taxol (*open diamond*), 15 mg/kg N5 (*crosses*), 20 mg/kg N6 (*open triangle*), 10 mg/kg taxol + 15 mg/kg N5 (*open square*), and 10 mg/kg taxol + 20 mg/kg N6 (*filled triangle*). Taxol was given i.v. and N5 or N6 was given i.p. The controls (*filled circle*) received DMSO vehicle 75 µl i.p. (two mice) and cremophor-ethanol (1:1) 75 µl i.v. (two mice). All groups consisted of four mice except for the N5 and N6 groups which consisted of three mice per group. The *vertical bars* indicate **a** mean tumor size in mm^3 ± SE, and **b** mean body weight ± SE. Body weight was measured by total body weight minus tumor weight. In the 10 mg/kg taxol + 15 mg/kg N5 group (*open square*), all four mice achieved tumor disappearance on day 42, 42, 46, and 46. In the 10 mg/kg taxol + 20 mg/kg N6 group (*filled triangle*). All four mice achieved tumor disappearance on day 42

(Fig. 6b), [^{3}H]taxol accumulation was increased by N3 and decreased by unlabeled taxol. The presence of VBL did not affect [^{3}H]taxol accumulation.

Effect of intracellular [^{3}H]VBL and the effect of N3

Human T cell acute lymphoblastic leukemic cells, CCRF-CEM, and its VBL-resistant subline, CCRF-CEM/VBL_{100}, were preloaded with [^{3}H]VBL. The efflux of radioactivity into fresh medium was measured by the decrease in extracellular radioactive drugs using the oil-layer microcentrifuge method [7, 19]. For the parent CCRF-CEM cells, Fig. 7a, the efflux of [^{3}H]VBL was partially blocked by the MDR reversing agents: N3 (10 µM), VPML (50 µM), and 5-*N*-acetyl ardeemin (5NAc-Ard or NAA, 30 µM). For the drug resistant CCRF-CEM/VBL_{100} cells, Fig. 7b, the efflux of [^{3}H]VBL was partially blocked by VPML (50 µM) and 5NAc-Ard (30 µM) and strongly blocked by N3 (10 µM). Thus, N3 not only increased intracellular accumulation but also decreased the efflux of the drug.

Discussion

Our current studies indicate that ningalins N3, N5, and N6 could be particularly promising candidates for combination chemotherapy of neoplastic diseases. Firstly, they have enormous potency in MDR-reversal and collateral efficacy enhancements (Tables 1 and 2). To our knowledge, these effects have surpassed those of previously published MDR-reversing agents. Secondly, they are relatively nontoxic with IC_{50}'s of 13 µM, 32 µM, and 42 µM for N3, N5, and N6, respectively (Table 1). Thirdly, the combination of N3 with DX and N3 with taxol showed markedly increased efficacy against P388/DX murine leukemia when compared with

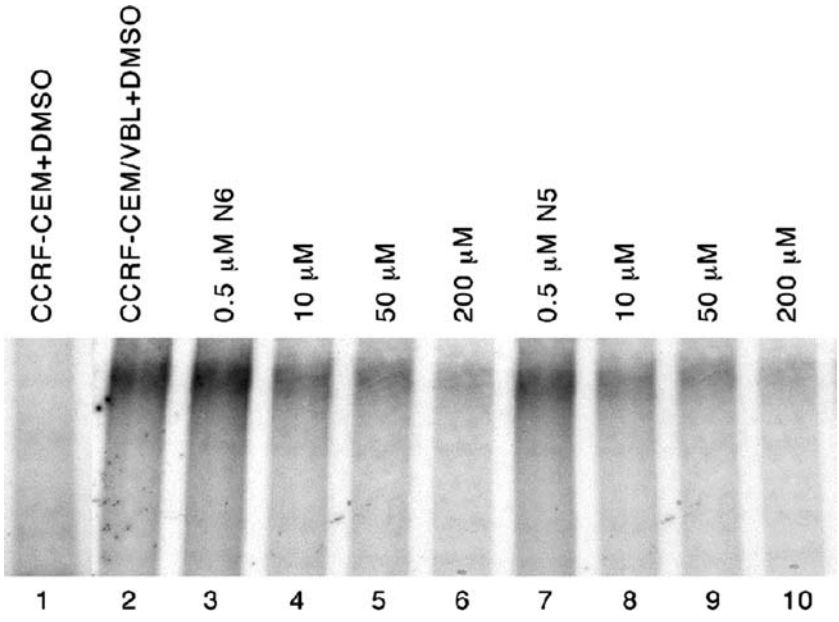

Fig. 5 Effect of N6 and N5 on [^{3}H]azidopine photo-affinity labeling of Pgp. DMSO control with CCRF-CEM Pgp only (lane 1) and with CCRF-CEM/VBL$_{100}$ Pgp only (lane 2). Lanes 3–6, plus N6, 0.5 μM, 10 μM, 50 μM and 200 μM ; and lanes 7–10, plus N5, 0.5 μM, 10 μM, 50 μM and 200 μM, respectively

single drug treatment alone (Table 5). Impressively, the synergistic combination of a suboptimal dose of taxol with N3, N5 or N6 against xenografts of human colon carcinoma HCT-116 in nude mice achieved curative effects whereas single drug treatment alone could not achieve tumor disappearance (Figs. 3 and 4).

Chou et al. [7] previously reported that NAA reversed drug resistance to VBL or taxol as much as 700-fold at relatively noncytotoxic concentrations in CCRF-CEM/VBL$_{100}$ cells that were 760-fold resistant to VBL. In many aspects, the ningalins and ardeemins share similar pharmacological properties, although the chemical structures are very different. The pharmacological similarities of ningalin and ardeemin include: (1) strong reversal of MDR to VBL or taxol; (2) collateral sensitivity in drug-resistant cells; (3) low cytotoxicity by themselves as shown by the IC$_{50}$'s above 10 μM in CCRF-CEM cells; (4) synergistic effects in DX in P388/0 and especially strong synergism in P388/DX or HCT-116/VM46 cells in vitro (Tables 5 and 6); (5) increase intracellular accumulation of VBL (Fig. 5) and retard VBL efflux (Fig. 6) in CCRF-CEM and CCRF-CEM/VBL cells; and (6) improve cancer chemotherapeutic efficacy of DX or taxol in nude mice bearing human tumor xenografts of MX-1 [7] or HCT-116 (Figs. 3 and 4). The in vitro results show that the ningalins are more potent than the ardeemins even though the present studies used CCRF-CEM/VBL$_{1000}$ cells (1,500-fold resistant) while CCRF-CEM/VBL$_{100}$ cells (750-fold resistant) were used previously for the ardeemin studies [7].

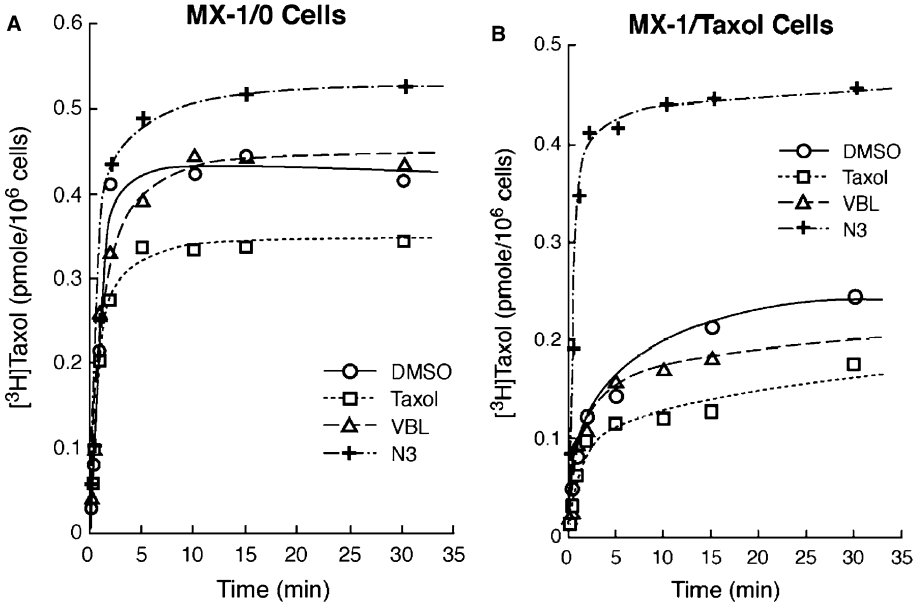

Fig. 6 Intracellular accumulation of [^{3}H]taxol in **a** MX-1/0 cells and **b** MX-1/taxol cells, with DMSO control (*open circle*); 2 μM taxol (*open square*); 0.1 μM VBL (*open triangle*) and 10 μM N3 (*crosses*). Cells were exposed to [^{3}H]taxol and other drugs at 37°C for various times. At each time point, intracellular [^{3}H]taxol concentrations were determined by the oil-layer microfuge method and with scintillation counting [7, 19]

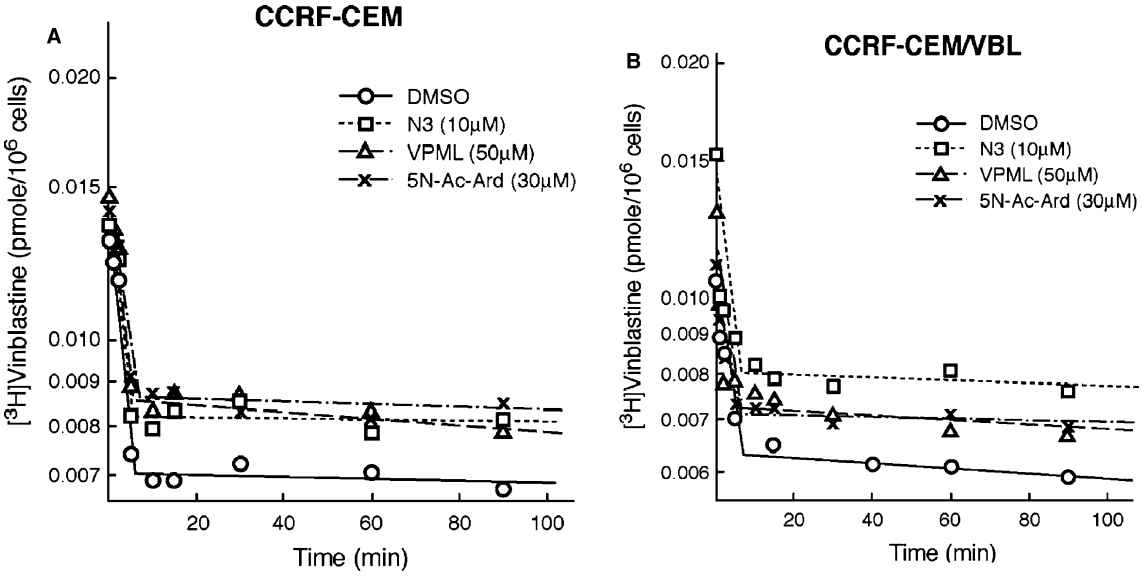

Fig. 7 Efflux kinetics for preloaded [^{3}H]VBL in **a** CCRF-CEM cells and **b** CCRF-CEM/VBL$_{1000}$ cells in the presence and absence of N3, VPML and 5N-Ac-Ard. Control (DMSO vehicle, 0.2% vol/vol) (*open circle*); 10 μM N3 (*open square*); 50 μM VPML (*open triangle*), and 30 μM 5NAc-Ard (*crosses*), was added at 0 min and incubated at 37°C for various time periods. [^{3}H]VBL was preloaded for 30 min at 37°C

Increased intracellular accumulation and retarded efflux of the anticancer drug from cells undoubtedly contributed to the therapeutic resensitization effected by the ningalins. These observations are consistent with the inhibition of Pgp described earlier [9, 10]. However, the megafold enhancement of VBL cytotoxicity suggest that ningalins might have additional mechanisms contributing to the properties such as changes in intracellular localization of the anticancer drugs or induction, modulation or modification in cellular regulatory pathways including those pathways that lead to programmed cell death (e.g., apoptosis).

It is of interest to note that slight modification of the ningalin structure by addition of substituents, lactone ring opening or further ring closures as shown in Fig. 1, substantially affect their pharmacological potency as shown in Table 1. Further studies of the impact of structural features on the MDR-reversal properties of the ningalins are in progress and will be reported in due course.

The three major criteria for successful application of ningalins in cancer chemotherapy would be: (1) potent pharmacological efficacy in drug combination, (2) low toxicity by themselves and devoid of severe toxicity in combination; and (3) selective synergy or enhancement against tumors. The experimental results presented in this paper fulfill these criteria. In a practical sense, it is particularly important that the ningalins permit a reduction in the dosage of anticancer drugs while enhancing or achieving a curative effect as shown in Figs. 3 and 4. It is particularly encouraging that in ningalin- and taxol-treated groups, the tumors disappeared and the cured mice regained body weight at levels above all the other groups. Further exploration of the ningalins as adjuvants of combined cancer chemotherapies is warranted.

In a separate study using human mammary carcinoma MX-1/taxol cells that were 77-fold resistant to taxol and 41-fold resistant to VBL, addition of 10 μM of N3 resulted in 300-fold enhancement for taxol and 6,000-fold enhancement for VBL (data not shown). Interestingly, for the non-Pgp substrate compound, 12,13-desoxyepothilone B (dEpoB) that are not cross-resistant to taxol nor VBL [18], addition of 10 μM of N3 only increased cytotoxicity to fourfold. These data suggest that N3 target is predominately Pgp in which VBL is its well-known substrate.

The observations of the megafold reversal of MDR of VBL by N3 and the kilofold collateral hypersensitivity of VBL by N3 in CCRF-CEM/VBL$_{1,000}$ cells are indeed amazing. The present experimental attempts can only partially explain the MDR-reversing phenomena. There is no explanation as yet for the collateral sensitivity. New avenues are required to explore this interesting new field of investigations. These will pose challenges for years to come.

Acknowledgments We thank Quen-Hui Tang and Luan-Ing Chen for technical assistance. This research was supported by National Institute of Health Grant CA-28824 (S.J.D.) and Core Grant CA-08748 (T.-C. C.)

References

1. Laing NM, Tew KD (1997) Drug resistance to chemotherapy: mechanisms. In: Bertino JR (ed) Encyclopedia of cancer. San Diego, vol 1, pp 560–570
2. Anthony A, Kayne SB (1999) Drug resistance: the clinical perspective. In: Brow R, Bouger-Brown U (eds) Cytotoxic drug resistance mechanisms. Humana Press, Totowa, pp 1–16
3. Kuska B (1999) As easy as ABC: scientists fish out another drug resistance gene. J Natl Cancer Inst 91:402–404

4. Kellen JA (1994) Multidrug resistance. In: Kellen JA (ed) Reversal of multidrug resistance in cancer. CRC Press, Boca Raton, pp 1–19
5. Robert J (1999) Multidrug resistance in oncology: diagnostic and therapeutic approaches. Eur J Clin Invest 29:535–545
6. Chou T-C, Chang T-T (2001) Models for drug development and drug resistance. In: Nicolson GL, Haier J (eds) Cancer handbook, chap 76. MacMillan Publishers, Ltd, London
7. Chou T-C, Depew KM, Zheng Y-H, Safer ML, Chan D, Helfrich B, Zatorska D, Zatorski A, Bornmann W, Danishefsky SJ (1998) Reversal of anticancer multidrug resistance by the ardeemins. Proc Natl Acad Sci USA 95:8369–8374
8. Volm M (1998) Multidrug resistance and its reversal. Anticancer Res 18:2905–2917
9. Boger DL, Boyce CW, Labroli MA, Sehon CA, Jin Q (1999) Total syntheses of ningalin A, lamellarin O, lukianol A, and permethyl storniamide A utilizing heterocyclic azadiene diels-alder reactions. J Am Chem Soc 121:54–62
10. Boger DL, Soenen DR, Boyce CW, Hedrick MP, Jin Q (2000) Total synthesis of ningalin B utilizing a heterocyclic azadiene diels-alder reaction and discovery of a new class of potent multidrug resistant (MDR) reversal agents. J Org Chem 65:2479–2483
11. Soenen DR, Hwang I, Hedrick MP, Boger DL (2003) Multidrug resistance reversal activity of key ningalin analogs. Bioorg Med Chem Lett 13:1777–1781
12. Skehan P, Storeng RH, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenny S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107–1112
13. Scudiero DA, Shoemaker RH, Pauli KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res 48:4827–4833
14. Chou T-C, Talalay PT (1984) Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
15. Chou T-C (1991) In: Chou T-C, Rideout DC (eds) Synergism and antagonism in chemotherapy. Academic, San Diego, pp 61–102
16. Chou J, Chou T-C (1987) Dose-effect with microcomputers quantitation of ED 50, synergism, antagonism, low-dose risk, reception-ligand binding and enzyme kinetics. Biosoft, Cambridge
17. Chou T-C, Hayball M (1996) CalcuSyn for Windows 3.1 and Windows 95: multiple drug dose-effect analyzer and manual for IBM-PC. Biosoft, Cambridge
18. Chou T-C, O'Connor OA, Tong WP, Guan Y, Zhang X-G, Stachel SJ, Lee C, Danishefsky SJ (2001) The synthesis, discovery, and development of a highly promising class of microtubule stabilization agents: curative effects of desoxyepothilones B and F against human tumor xenografts in nude mice. Proc Natl Acad Sci USA 98:8113–8118
19. Wohlhueter RM, Marz R, Graff JC, Plagemann RGW (1978) A rapid-mixing technique to measure transport in suspended animal cells: applications to nucleoside transport in Novikoff rat hepatoma cells. Methods Cell Biol 20:211–236