THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 313:112–120, 2005

Vol. 313, No. 1
78014/1198227
Printed in U.S.A.

Trimers of *N*-Alkylglycines Are Potent Modulators of the Multidrug Resistance Phenotype[S]

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Received September 16, 2004; accepted January 7, 2005

ABSTRACT

The multidrug resistance (MDR) phenotype is considered a major cause of the failure of cancer chemotherapy. The acquisition of MDR is usually mediated by the overexpression of drug efflux pumps such as glycoprotein P (P-gp) or multidrug resistance-related protein 1 (MRP1). Thus, the identification, validation, and development of compounds that mitigate the MDR phenotype by modulating the activity of these transport proteins is an important yet elusive target. Here, we have addressed this issue and screened an *N*-trialkylglycine-based combinatorial library composed of 5120 compounds to search for modulators of the MDR phenotype. The screening identified 20 trimers of *N*-alkylglycine that increased the intracellular accumulation of daunomycin (DNM) in drug-resistant L1210R tumor cells that overexpressed the P-gp. These compounds seem to act as P-gp antagonists, as evidenced by the augmentation of DNM accumulation in the $L1210^{P\text{-}gp}$ cell line, a drug-sensitive L1210 cell stably expressing the murine P-gp protein. Similarly, several of the active *N*-trialkylglycines also produced an increment in DNM uptake in human HL60R cells, which primarily express the MRP1 protein. Trialkylglycines notably sensitized L1210R and HL60R tumor cells to DNM with a potency that rivaled that of verapamil. These findings provide new molecular scaffolds for the development of effective chemosensitizers against the MDR phenotype that, in due turn, could be used as adjuvant drugs in cancer chemotherapy.

This work was supported by grants from La Fundación La Caixa (to A.F.-M.); Fundació La Marató de TV3 (to A.M.); the Spanish Interministerial Commission of Science and Technology (CICYT) and the European Commission (to A.F.-M. and E.P.-P.). A fellowship to N.C. from the Generalitat de Catalunya is also acknowledged.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.104.078014.

[S] The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

A plethora of cancer chemotherapies are currently in clinical practice. Most of these treatments fail because of the acquisition of the multidrug resistance (MDR) phenotype by tumors (Pastan and Gottesmann, 1987). Although the molecular mechanisms underlying MDR are diverse, a major strategy is through overexpression of energy-dependent, unidirectional transmembrane drug efflux pumps (Ling, 1997; Gottesman et al., 2002). The P-glycoprotein (P-gp) is a 170-

ABBREVIATIONS: MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug-related protein; DNM, daunomycin; VRP, verapamil; N151212C, [*N*-[2-(2′-fluorophenyl)ethyl]glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N161212C, [*N*-[2-(4′-fluorophenyl)ethyl]glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N31212C, [*N*-(Isopentyl)glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N71212C, [*N*-(2-phenylethyl)glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N101212C, [*N*-[2-(4′-methoxyphenyl)ethyl]glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N31412C, [*N*-(isopentyl)glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N71412C, [*N*-(2-phenylethyl)glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N101412C, [*N*-[2-(4′-methoxyphenyl)ethyl]glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N151412C, [*N*-[2-(2′-fluorophenyl)ethyl]glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N161412C, [*N*-[2-(4′-fluorophenyl)ethyl]glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycinamide; N31214C, [*N*-(isopentyl)glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N71214C, [*N*-(2-phenylethyl)glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N101214C, [*N*-[2-(4′-methoxyphenyl)ethyl]glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N151214C, [*N*-[2-(2′-fluorophenyl)ethyl]glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N161214C, [*N*-[2-(4′-fluorophenyl)ethyl]glycyl]-[*N*-[2-(2′,4′-dichlorophenyl)ethyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N31414C, [*N*-(isopentyl)glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N71414C, [*N*-(2-phenylethyl)glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N101414C, [*N*-[2-(4′-methoxyphenyl)ethyl]glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N151414C, [*N*-[2-(2′-fluorophenyl)ethyl]glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide; N161414C, [*N*-[2-(4′-fluorophenyl)ethyl]glycyl]-[*N*-[3,3-(diphenyl)propyl]glycyl]-*N*-[3,3-(diphenyl)propyl]glycinamide.

kDa membrane protein that belongs to the ATP-binding cassette family of transporters (Bellamy, 1996; Ling, 1997; Borst and Elferink, 2002). This protein is proposed to function as an ATP-dependent efflux pump for hydrophobic agents, thus conferring resistance by reducing the intracellular concentration of cytotoxic drugs (Bellamy, 1996). P-gp expression in tumors in vivo is associated with poor overall prognosis and response to chemotherapy partly because of the protein capacity to transport a broad range of compounds, including anthracyclines, vinca alkaloids, and taxanes (Sharom, 1997). Chemotherapeutically-induced expression of P-gp has been well documented in tumors such as acute leukemia and small-lung cancer, breast and ovarian cancer, head and neck tumors, Kaposi sarcoma, and child neuroblastoma (Tan et al., 2000).

In addition to P-gp, overexpression of a series of homologous proteins termed multidrug resistance-related proteins (MRPs) that share many pharmacological properties with P-gp have been discovered more recently (Cole et al., 1992; Borst and Elferink, 2002). The MRP family is made up of 190-kDa membrane proteins encoded by several closely related genes in humans that show low sequence homology to P-gp (Cole and Deeley, 1996). These proteins also contribute to the MDR phenotype (Lautier et al., 1996). For instance, MRP1 confers resistance to anthracyclines and vinca alkaloids but not to taxanes (Tan et al., 2000). The MRP1 protein is overexpressed in most non-P-gp-mediated multidrug-resistant cells (Loe et al., 1996), although its presence is not consistently found in tumors (Lee et al., 2004). Furthermore, MRP1 may be also coexpressed with P-gp, enhancing the drug resistance of tumors (Legrand et al., 1999). Other proteins such as the breast cancer-resistant protein and the lung resistance-related protein have been identified in MDR tumors that do not express P-gp or MRP1 (Tan et al., 2000).

The central role of MDR in clinical oncology has prompted the discovery and development of antagonists of these transport proteins, specifically P-gp. These agents are known as chemosensitizers or revertants of the MDR phenotype. By antagonizing the active efflux of anticancer drugs, chemosensitizers promote an accumulation of these drugs into the tumor cells, thus augmenting the efficacy of the chemotherapeutic treatment (Tan et al., 2000; Robert and Jarry, 2003). The first generation of chemosensitizers consisted of calcium channel blockers, calmodulin inhibitors, antibiotics, cardiovascular drugs, cyclosporins, and other compounds (Robert and Jarry, 2003). Most of these agents exhibit side effects that prevented their clinical development (Robert and Jarry, 2003). Chemical modification of these compounds led to the identification of a new generation of chemosensitizers with improved efficacy and lower, but still unacceptable, toxicity (Robert and Jarry, 2003). The third generation of MDR antagonists included molecules selected on the basis of structural features and then tested in vitro. This strategy led to the identification, among others, of triazine piperidinyl compounds, dihydropyrroquinolines, and anthranilic acid derivatives (Robert and Jarry, 2003). Together, these discovery efforts highlight the importance of developing inhibitors of proteins involved in MDR as a therapeutic strategy to increase the efficacy of current chemotherapy.

Here, we have aimed to identify new molecular scaffolds that define chemosensitizing activity by screening a mixture-based, *N*-trialkylglycine combinatorial library composed of 5120 compounds. We found a set of 20 trimers of *N*-alkylglycines that antagonized P-gp function, expelling the anthracycline daunomycin (DNM) with an efficacy and potency that rivaled that shown by verapamil (VRP), a well established P-gp antagonist (Robert and Jarry, 2003). These compounds also inhibited the MRP1 transporter, as evidenced by their potent revertant activity on human HL60R. Cytotoxicity studies unveiled that several trimers of *N*-alkylglycines chemosensitized the MDR phenotype in DNM-resistant L1210R and HL60R cells. Therefore, these results provide new MDR modulators that, in due turn, may be developed as clinically useful chemosensitizers.

Materials and Methods

Synthesis of Trialkylglycines-Based Combinatorial Mixtures and Individual Compounds. An optimized library of 5120 peptoids in 52 controlled mixtures was synthesized by using the positional scanning format on solid phase. The mixture positions (X, Fig. 1) were incorporated by coupling a mixture of 22 or 16 selected primary amines with the relative ratios adjusted to yield equimolar incorporation (Humet et al., 2003). Briefly, starting from Rink amide resin (0.7 mEq/g; Rapp Polymere, Tuebingen, Germany), the eight-step synthetic pathway involved the initial release of the Fmoc protecting group. Thereafter, the successive steps of acylation with chloroacetyl chloride followed by the corresponding amination of the chloromethyl intermediate, using the selected individual amine (O, Fig. 1) or the mixture of amines (X, Fig. 1), was conducted. Thereafter, the products were released from the resin by using a trifluoroacetic acid/dichloromethane/water cocktail, solvents were evaporated, and the residues were lyophilized and dissolved in 10% dimethyl sulfoxide at a concentration of 5 mg/ml for screening. Individual oligo *N*-alkylglycines were prepared by simultaneous multiple solid-phase synthesis following the same synthetic sequence. The purity and identity of the most active individual oligo *N*-alkylglycine compounds were determined by analytical high-performance liquid chromatography, mass spectometry, and ^{1}H and ^{13}C NMR (see Supplemental Data).

Cell Cultures. DNM-resistant murine L1210R cells and human promyelocytic (HL60R) leukemia cells were obtained by stepwise selection of wild-type L1210 and HL60 with increasing DNM concentrations and maintained in cultures as previously described (Soto et al., 1993; Castro-Galache et al., 2003). $L1210^{P\text{-}gp}$ cells are L1210 tumor cells that stably express the mouse mdr1a cDNA (Castro-Galache et al., 2003). Cultures were maintained at 37°C in humidified 5% CO_2 atmosphere (Castro-Galache et al., 2003).

Daunomycin Accumulation Assays. L1210R overexpressing the P-gp and HL60R cells overexpressing the MRP1 were washed once with Hepes saline buffer, and the pellet was resuspended in Hepes saline buffer at 1×10^6/ml per sample. Thereafter, cells were incubated with 3 μM DNM (control) and 5 μM VRP (as a reference of chemosensitizing effect) or the peptoid mixtures (0.1 mg/ml) for 1 h at 37°C. After incubation, steady-state intracellular DNM accumulation was determined by flow cytometry as previously described (Soto et al., 1993; Martín-Orozco et al., 2004).

In Vitro Proliferation Assays. In vitro proliferation assays compared the growth rate of L1210 and L1210R cells and that of HL60 and HL60R cells by 3-(4,5-dimethylthiazo-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) assay after plating 2×10^4 cells in a 96-well plate for 48 h. Cell plates were incubated with increasing concentrations of DNM (0.01–25 μM) in the absence and presence of 5 μM trialkylglycines or VRP. For the viability assays, cell were washed extensively with phosphate-buffered saline, and the MTT reagent was added to each well and incubated for 4 h at 37°C. Thereafter, 100 μl/well of acid-isopropyl alcohol was added and mixed thoroughly to dissolve the dark blue crystals, and plates were read on an enzyme-linked immunosorbent assay reader using a wavelength of 570 nm. Data were normalized

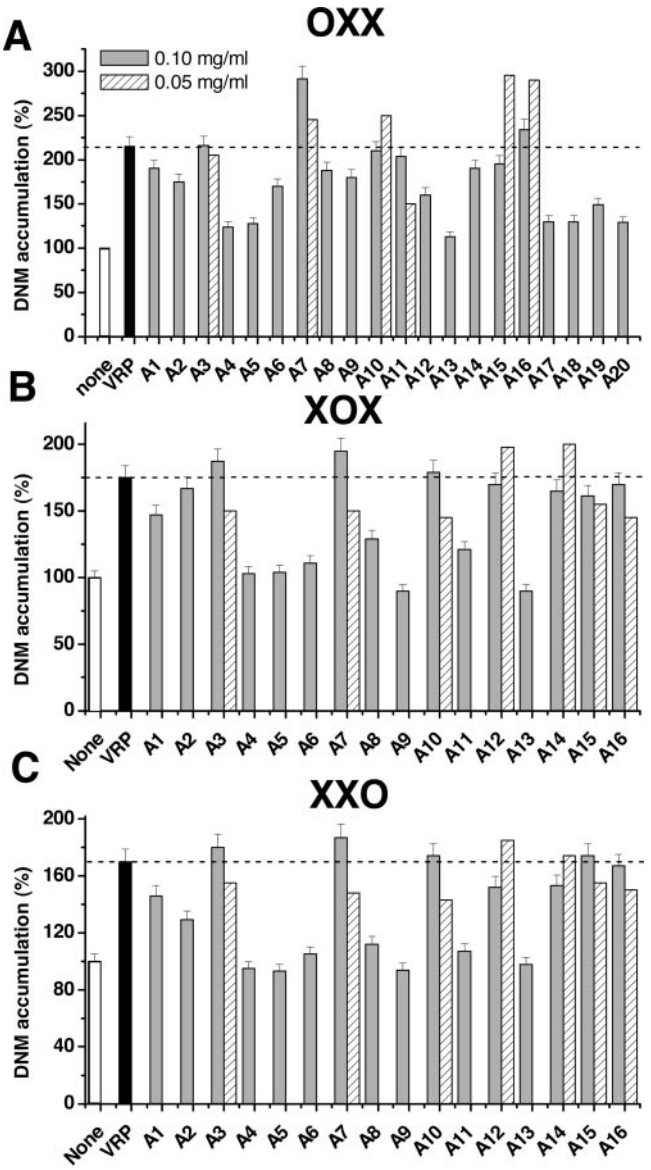


Fig. 1. Screening of an oligo *N*-substituted glycine combinatorial library to identify chemosensitizers of L1210R tumor cells. A–C, DNM intracellular accumulation profile of the 52 library mixtures. Each graph represents the effect on the accumulation of the anthracycline for each of the three positions that compose the library. A1 through A20 refers to the amine number present in Table 1. The bars denote the activity of each mixture as a function of the number of the defined amine used to generate the chemical diversity. Library mixtures were assayed at 100 μg/ml, and those selected positive were assayed at 50 μg/ml. None, intracellular accumulation of anthracycline in L1210R in the absence of chemosensitizers. VRP refers to the DNM accumulation in the presence of 5 μM of compound. Cells were incubated with different mixtures for 1 h at 37°C and thereafter exposed to 3 μM DNM for an additional 1 h at 37°C. Intracellular accumulation of DNM was monitored by flow cytometry. O, defined position in the chemical library; X, combinatorialized position with an equimolar mixture of the amines used.

and plotted as a function of the DNM concentration and fitted to a Michaelis-Menten isotherm to determine the concentration of DNM that inhibits half-maximal cellular proliferation (IC_{50}):

$$\frac{G}{G_{max}} = \frac{1}{1 + \left(\frac{[DNM]}{IC_{50}}\right)^{n_H}}$$

where G denotes growth at the different DNM concentrations, G_{max} indicates growth in the absence of DNM, and n_H is the slope of the isotherm. The degree of resistance to DNM was obtained as the ratio of the IC_{50} values for the resistant and wild-type subline.

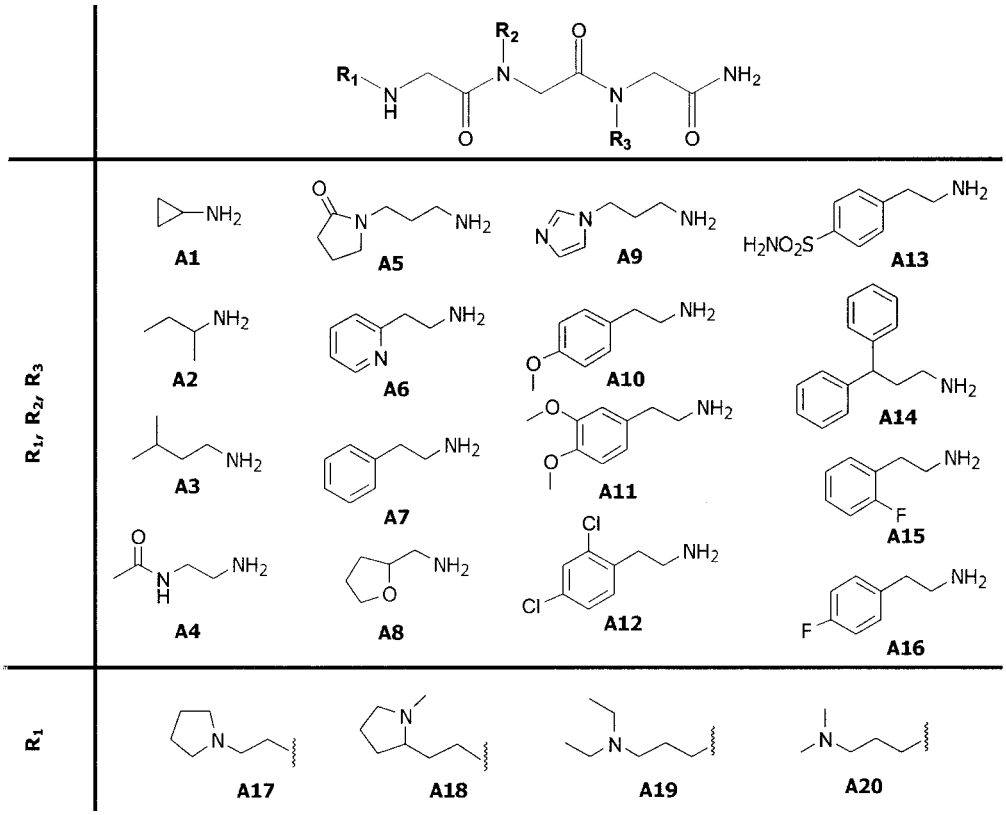
Modeling with Catalyst. The computational molecular modeling studies were carried out using a Silicon Graphics Octane workstation (SGI, Mountain View, CA). The three-dimensional structures of the different ligands of P-gp [(*R*)-verapamil, rhodamine 123, vinblastine, colchicine, and calcein acetoxymethyl ester] were built using Catalyst version 4.7 (Accelrys, San Diego, CA). The number of conformers generated using the "best" feature of the program for each inhibitor was limited to a maximum of 250 with an energy range of 20 kcal/mol. Using the common-features hypotheses function (HipHop) in Catalyst, 10 hypotheses were generated using the above conformers for each of the training set molecules. The eventual protonation of these molecules at the physiological pH and selected features for the inhibitors such as hydrogen bond donor, hydrogen bond acceptor, and hydrophobic and ring aromatic, were considered. After assessing the 10 hypotheses generated, the pharmacophore hypothesis with the lowest energy cost was selected. Selected *N*-trialkylglycines and tariquidar, a potent and specific antagonist of P-gp (Mistry et al., 2001), were fitted into the selected hypothesis. The correct alignment was used with the best-fit procedure to assess how all conformers from both peptoids might orient within the hypothesis, minimizing the distance between the center of the hypothesis features and their mapping to atoms on the molecule.

Results

Screening of a *N*-Trialkylglycine-Based Combinatorial Library to Identify Novel MDR Modulators. A focused, mixture-based combinatorial library made of trimers of *N*-alkylglycines in a positional scanning format was designed and synthesized to identify antagonists of the P-gp protein. The library consisted of three separate positions, each having a single position defined with one of the 20 (OXX) or 16 (XOX, XXO) primary amines used (Table 1), and the remaining two positions had an equimolar mixture of these amines. The rationale of using two different sets of amines was due to side reactions that occur when primary amines bearing an additional tertiary amino moiety were used as diversity sources for the internal or/and C-terminal positions of the trimer. Thus, none of the amines used in the design of the library for the internal and C-end positions had these additional amino groups, whereas four additional amines containing the tertiary amino moiety were added to the set used for the N-terminal position (Humet et al., 2003). The library was organized as an array of 52 separate mixtures. Each mixture contained either 256 (OXX) or 320 molecules (XOX, XXO), and the library chemical diversity comprised 5120 individual trimers. The set of amines included aliphatic and aromatic groups to increase the probability of finding P-gp inhibitors, to enhance membrane permeability, and to improve the bioavailability of the active *N*-trialkylglycines.

Peptoid mixtures were assayed to find inhibitors of the P-gp transporter. The assay was based on the observation that accumulation of the fluorescent cytostatic drug DNM in L1210R cells, a tumor cell line that overexpresses P-gp (see Supplemental Data), is enhanced in the presence of P-gp antagonists such as verapamil (Robert and Jarry, 2003). Changes in the intracellular accumulation of DNM are readily followed by flow cytometry, thus providing a sensitive and reliable assay for seeking tumor-resistant chemosensitizers (Soto et al., 1993; Nelson et al., 1998; Schwab et al., 2003). Cellular fluorescence was determined after 2 h of

TABLE 1
Set of amines used for the synthesis of the *N*-trialkylglycine-based combinatorial library



incubation in the presence of the peptoid mixtures at a concentration of 100 μg/ml. As illustrated in Fig. 1A, six of the peptoid mixtures that had the first position defined increased the accumulation of DNM to an extent similar to that of 5 μM VRP. The number of positive mixtures was eight for the second position (Fig. 1B) and seven for the third (Fig. 1C). Together, the assay identified a total of 336 peptoids as potential modulators of the MDR phenotype. To isolate the most effective chemistries at the three positions of the trimer, we assayed the positive mixtures at 50 μg/ml for increased DNM cellular accumulation. Figure 1, A through C, shows that amines **A**3, **A**7, **A**10, **A**15, and **A**16 were preferred at R_1, whereas aromatic amines **A**12 and **A**14 were selected for the R_2 and R_3 positions (see Table 1 for structures). The selection of these amines was considered based on both the high activity that restored DNM intracellular accumulation and lack of cytotoxicity at 50 μg/ml.

When used in concert, the data derived from the screening suggest the chemical identity of the bioactive *N*-trialkylglycines in the library (García-Martínez et al., 2002; Humet et al., 2003). Thus, a family of 20 individual *N*-trialkylglycines, resulting from all possible combinations of the active functional groups identified in the deconvolution process, was synthesized. The increase in DNM accumulation in L1210R cells produced by all *N*-alkylglycines (5 μM) is depicted in Fig. 2A. Notice that these peptoids did not affect the accumulation of DNM in L1210 cells (Fig. 2B), consistent with an effect on the MDR phenotype. Several of the compounds displayed a potency in intracellular drug accumulation similar to VRP. In particular, peptoids N151212C and N161212C exhibited a remarkable revertant activity on L1210R cells. The dose-response relationship for these *N*-trialkylglycines shows an IC_{50} of 3.5 $\pm$ 2.1 μM for N151212C (Fig. 2C); for N161212C, a value of 4.3 $\pm$ 1.9 μM was obtained. Both compounds also inhibited the extrusion of the cationic dye rhodamine 123 from L1210R cells (see Supplemental Data). These results indicate that identified *N*-trialkylglycines are chemosensitizers of the MDR phenotype, presumably by inhibiting the P-gp drug transporter.

Active *N*-Trialkylglycines Seem to Be P-gp Antagonists. To investigate whether active peptoids act on the P-gp protein, we used the $L1210^{P\text{-}gp}$ cell line, which is made up of DNM-sensitive L1210 cells that stably express the murine P-gp transporter cDNA (Castro-Galache et al., 2003). $L1210^{P\text{-}gp}$ cells show a reduced intracellular accumulation of DNM compared with untransfected L1210 cells (Fig. 3A). The extent of drug accumulation in $L1210^{P\text{-}gp}$ is akin to that exhibited by DNM-resistant L1210 cells, including the sensitivity to VRP (Fig. 3A). Unlike L1210R cells, however, the $L1210^{P\text{-}gp}$ cell line does not exhibit a drug-induced pleiotropic phenotype, thus allowing a determination of whether newly identified chemosensitizers act as P-gp antagonists. The activity of the 20 identified *N*-trialkylglycines on DNM accumulation in $L1210^{P\text{-}gp}$ cells is displayed in Fig. 3B. It is noteworthy that the most active peptoids in L1210R cells, N151212C and N161212C, were also the most potent in increasing DNM accumulation in $L1210^{P\text{-}gp}$. Dose-response curves indicate that peptoids N151212C and N161212C exhibit a similar inhibitory efficacy (IC_{50}) as that obtained in L1210R cells (data not shown). There-

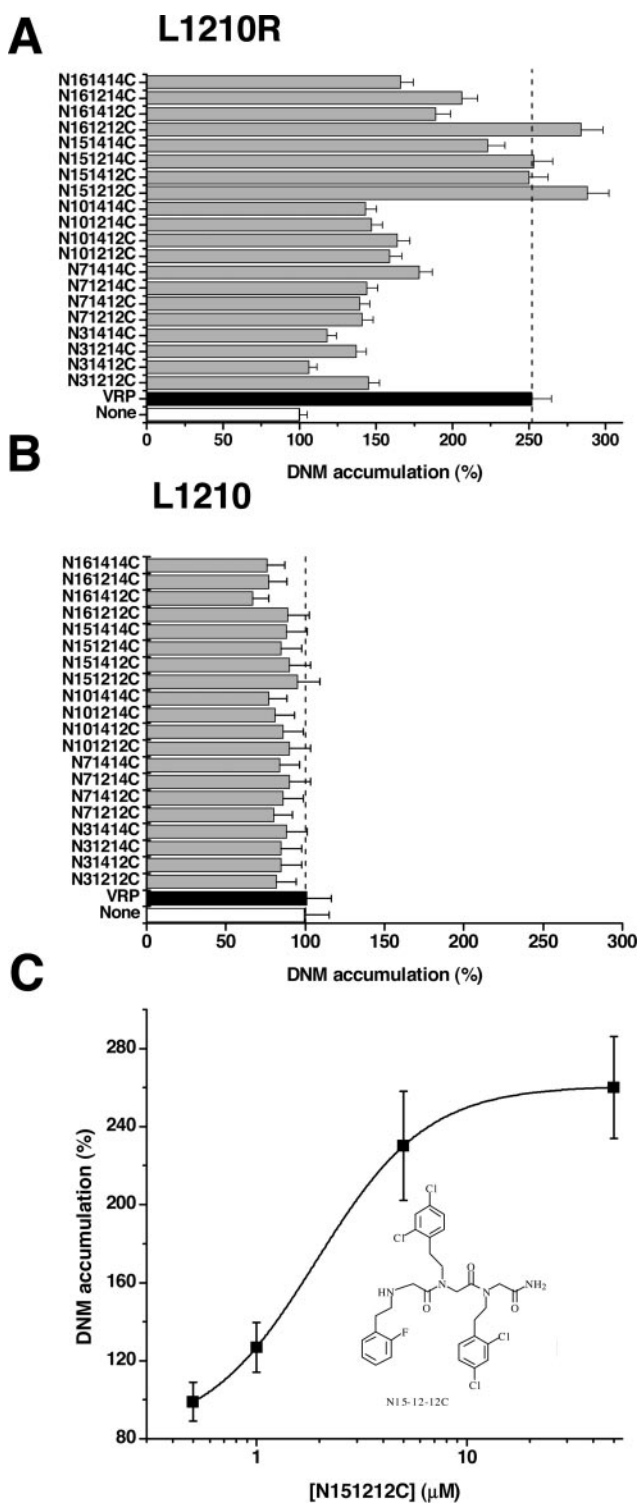


Fig. 2. Identified *N*-trialkylglycines increase the intracellular accumulation of DNM in L1210R cells. Representative activity of *N*-alkylglycine trimers tested at 5 μM that augmented the cytosolic concentration of DNM in L1210R (A) and L1210 (B) tumor cells. None, intracellular accumulation of anthracycline in L1210R in the absence of chemosensitizers. VRP refers to the DNM accumulation in the presence of 5 μM verapamil. C, dose-response relationship for the activity of compound N151212C that augmented the cytosolic accumulation of DNM in L1210R cells. The solid line depicts the best fit to a Michaelis-Menten isotherm. The concentration of DNM that inhibits the half-maximal cellular proliferation (IC_{50}) value was 3.5 $\pm$ 2.1 μM. Data are mean $\pm$ S.E.M., with $n \geq 3$. *N*-trialkylglycines are referred to as the amine number selected from the N-to-C positions. Inset, molecular structure of peptoid N151212C.

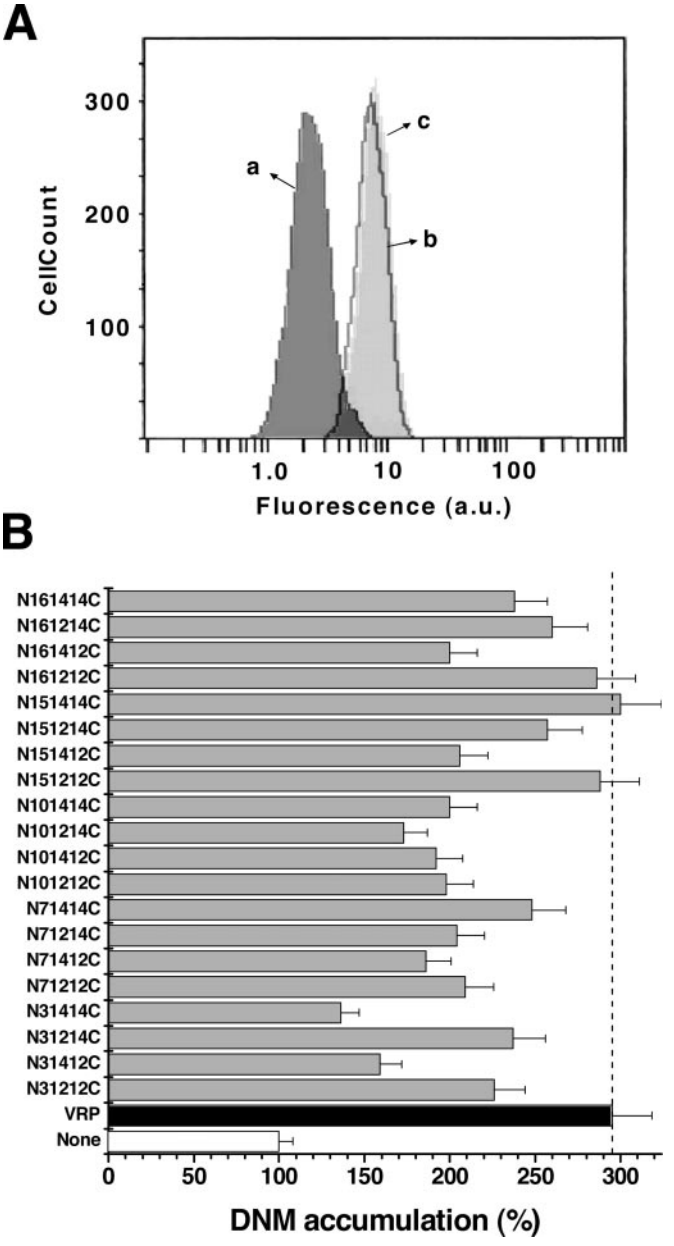


Fig. 3. Identified *N*-trialkylglycines seem to act as antagonists of the P-gp. A, L1210 cells stably expressing the human P-gp protein ($L1210^{P\text{-}gp}$) show a decrease in intracellular accumulation of DNM. The panel shows the flow cytometry distribution peaks of cytosolic DNM in $L1210^{P\text{-}gp}$ (a), $L1210^{P\text{-}gp}$ in the presence of 5 μM VRP (b), and L1210 (c). B, representative profile of the potency exerted by 5 μM *N*-trialkylglycines on the cytosolic uptake of DNM in $L1210^{P\text{-}gp}$. None, accumulation of anthracycline in the absence of any compound or drug. VRP, intracellular concentration of DNM in the presence of 5 μM VRP. Cells were incubated with different compounds for 1 h at 37°C and thereafter exposed to 3 μM DNM for an additional 1 h at 37°C. Intracellular accumulation of DNM was monitored by flow cytometry.

fore, these results imply that active peptoids are inhibitors of the P-gp protein that is overexpressed in L1210R cells.

Identified *N*-Trialkylglycines Chemosensitize L1210R Cells to DNM Cytotoxicity. We next characterized the chemosensitizing activity of the *N*-trialkylglycines N151212C and N161212C in DNM cytotoxicity assays in L1210R tumor cells. For this task, L1210 and L1210R cells were incubated with increasing concentrations of the anthracycline in the absence and presence of 5 μM of the two most active peptoids. As a

positive control, VRP (5 μM)-induced chemosensitization of L1210R cells was evaluated. Dose-response curves of DNM cytotoxicity revealed a concentration of anthracycline that inhibits half-maximally the cellular proliferation (IC_{50}) of 0.11 $\pm$ 0.03 and 20 $\pm$ 2 μM for L1210 cells and L1210R tumor cells, which represents a degree of DNM resistance (DDR), calculated as the ratio of the IC_{50} of L1210R with respect to that exhibited by drug-sensitive L1210 cells, of 180 (Fig. 4A). The presence of 5 μM VRP reduced the IC_{50} (6.0 $\pm$ 0.1 μM) of resistant cells $\geq$3-fold, leading to a DDR of 54. Similarly, 5 μM of peptoids N151212C and N161212C significantly decreased the DDR by 3-fold, from 180 to 45 (IC_{50} = 5.0 $\pm$ 0.3 μM) and 50 (IC_{50} = 5.5 $\pm$ 0.5 μM), respectively (Fig. 4B). It is noteworthy that this concentration of peptoids did not exhibit cytotoxicity, thus implying that the chemosensitization of L1210R was due to a direct inhibition of the P-gp pump activity. To further substantiate this notion, reverse transcriptase-polymerase chain reaction analysis of the MDR1 gene shows that the peptoids did not affect the mRNA levels of P-gp in L1210R cells (see Supplemental Data). Thus, these results indicated that identified peptoids are potent chemosensitizers of the P-gp-mediated MDR phenotype.

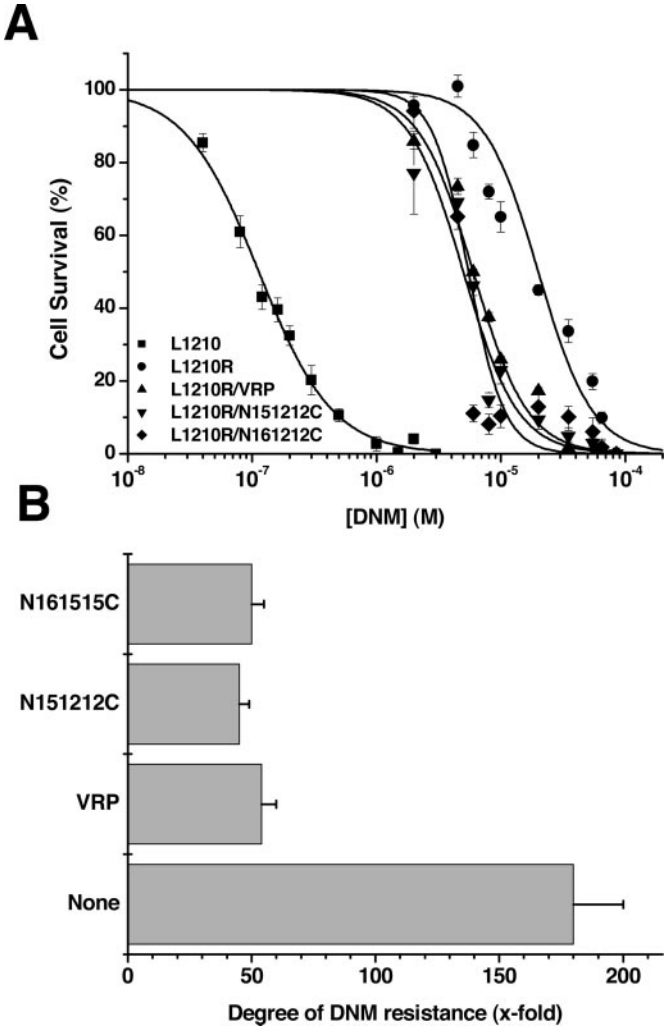


Fig. 4. *N*-trialkylglycines N151212C and N161212C decrease the degree of resistance of L1210R cells. A, dose-response relationships of DNM cytotoxicity on L1210S and L1210R in the absence and presence of chemosensitizers (5 μM). Cell proliferation was determined by the MTT method. Data are normalized with respect to the cellular growth in the absence of cytostatic. Solid lines depict the best fit to a sigmoidal curve. The concentration of DNM that inhibits half-maximal cellular proliferation (IC_{50}) values were 0.11 $\pm$ 0.03 μM for L1012; 20 $\pm$ 2 μM for L1210R; 6.0 $\pm$ 0.1 μM for L1210R in the presence of VRP; 5.0 $\pm$ 0.3 μM for L1210R in the presence of N151212C; and 5.5 $\pm$ 0.5 μM for L1210R in the presence of N161212C. Values are given as mean $\pm$ S.E.M., with $n \geq 3$. C, effect of the compounds on the resistant degree of L1210R. The extent of DNM resistance was calculated as the ratio of DNM IC_{50} of L1210R and the IC_{50} of drug-sensitive L1210 cells in the absence and/or presence of peptoids or VRP.

Identified *N*-Trialkylglycines Also Modulate the MRP1 Transporter. The acquired MDR phenotype may be mediated by overexpression of the P-gp protein or other members of the ATP-binding cassette family of membrane transporters such as the MRP1 protein (Tan et al., 2000). For instance, human HL60 expressed the MRP1 protein upon acquisition of the DNM-induced MDR phenotype (Ross et al., 1996). Hence, we next questioned whether identified active peptoids were also modulators of the resistant phenotype of HL60 cells. To address this question, we determined the potency of the 20 individual peptoids identified that augmented the intracellular accumulation of DNM in HL60R cells. Notably, most of the individual trimers of *N*-alkylglycines increased the uptake of the anthracycline to an extent that competed with that of VRP in HL60R cells (Fig. 5A) but were innocuous to HL60 tumor cells (Fig. 5B). These results indicate that identified peptoids are modulators of the MRP1 protein that is overexpressed in HL60R tumor cells. The activity profile of these compounds in P-gp and MRP1 seems rather similar, suggesting that they may interact with a structurally homologous binding site.

Active Peptoids Chemosensitize HL60R Cells to DNM. Because virtually all 20 trialkylglycines displayed a comparable potency that augmented the intracellular concentration of DNM in HL60R tumor cells, we next evaluated their chemosensitizing activity in cytotoxicity assays. Dose-response relationships of the cellular viability at increasing DNM concentrations reveal an IC_{50} of 0.25 $\pm$ 0.03 μM for HL60 and 7.6 $\pm$ 0.2 μM for HL60R cells, indicating a degree of DNM resistance of 30 (Fig. 6A). This value was decreased up to 3-fold by 5 μM VRP (IC_{50} = 3.1 $\pm$ 0.1 μM). Similarly, the cytotoxicity of DNM to HL60R was notably augmented by the presence of 5 μM of the peptoids (Fig. 6B). All *N*-trialkylglycines sensitized HL60R $\geq$3-fold to DNM cytotoxicity. Note that, as for L1210R, the *N*-trialkylglycine N151212C exhibited the highest chemosensitizing activity on HL60R cells (IC_{50} = 2.1 $\pm$ 0.2 μM). Therefore, identified *N*-trialkylglycines sensitize the MDR phenotype mediated by both P-gp and MRP1 transporter proteins.

Molecular Model of N151212C. We used the Catalyst software to study the fitting of peptoid N151212C into the pharmacophore model generated from a set of different, well established P-gp inhibitors and substrates that presumably bind to the same site on the protein, such as (*R*)-verapamil, rhodamine 123, vinblastine, colchicine, and calcein acetoxymethyl ester. These compounds generated a three-dimensional pharmacophore model featuring the two hydrophobic groups (aromatic or aliphatic chains), one aromatic ring, and two hydrogen bond acceptors, which is consistent by the model reported by Ekins et al. (2002). Peptoid N151212C was fit into the pharmacophoric model to determine whether the compound fulfilled similar properties. As illustrated in Fig. 7A, the lowest energy conformation of peptoid N151212C fit nicely with the pharmacophore. This

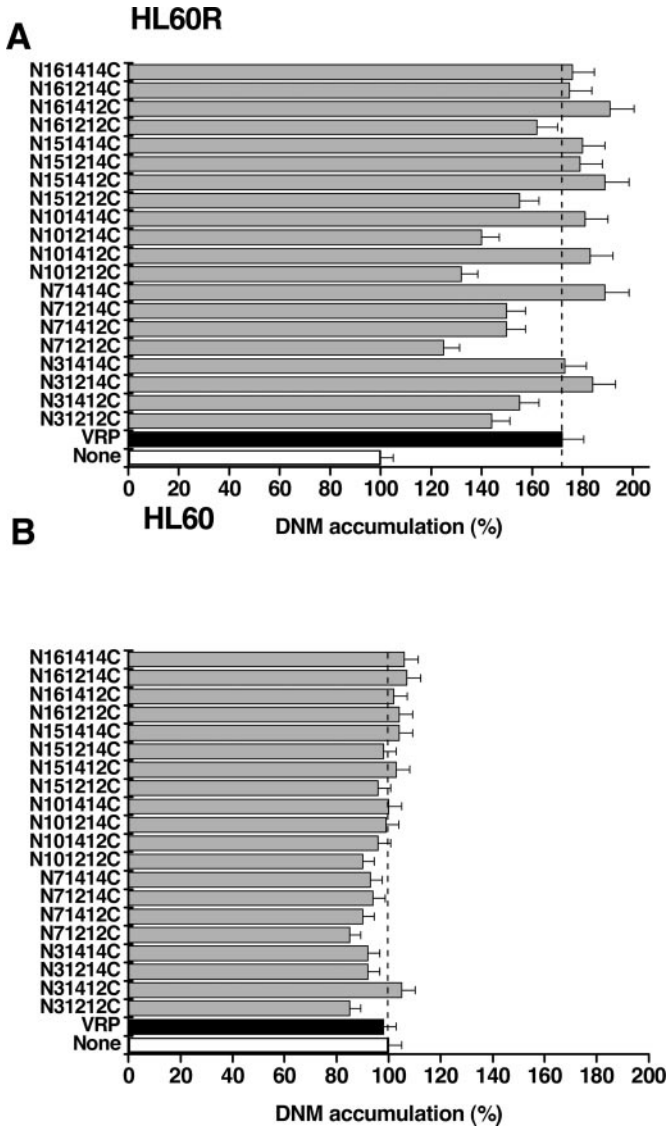


Fig. 5. *N*-trialkylglycines increase the accumulation of DNM in HL60R cells. Profile activity of 5 μM *N*-trialkylglycines on the intracellular accumulation of DNM on HL60R (A) and HL60 tumor cells (B). None, accumulation of anthracycline in the absence of any compound or drug. VRP, intracellular concentration of DNM in the presence of 5 μM VRP. Cells were incubated with different compounds for 1 h at 37°C and thereafter exposed to 3 μM DNM for an additional 1 h at 37°C. Intracellular accumulation of DNM was monitored by flow cytometry.

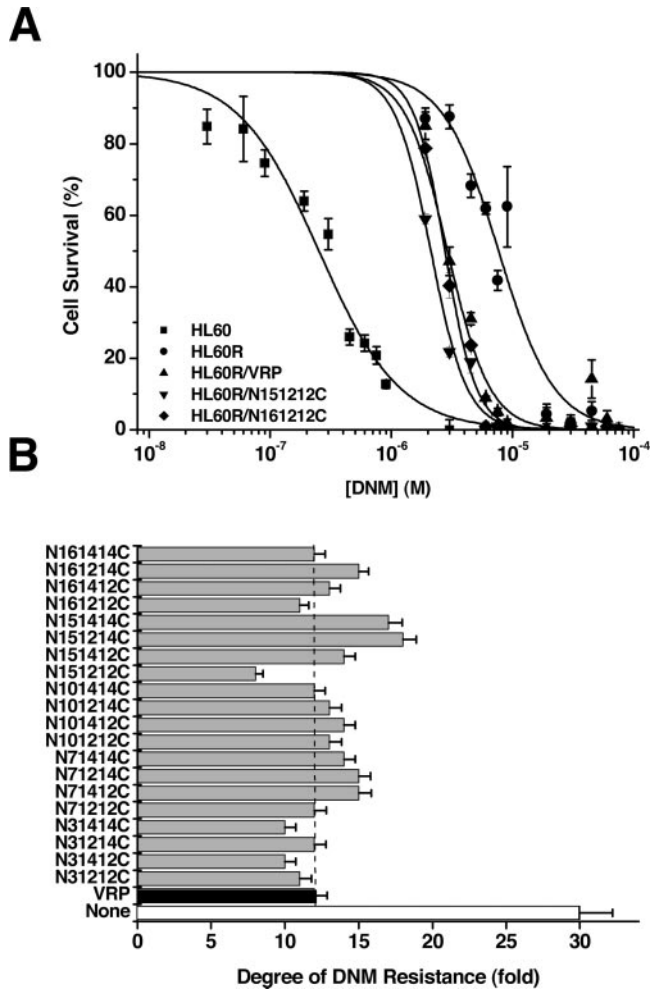


Fig. 6. Trimers of *N*-alkylglycines chemosensitize HL60R cells that express the MRP1 protein. A, dose-response relationships of DNM cytotoxicity on HL60 and HL60R in the absence and presence of 5 μM chemosensitizers. Solid lines depict the best fit to a sigmoidal curve. The IC_{50} values were 0.25 $\pm$ 0.03 μM for HL60; 6.4 $\pm$ 0.5 μM for HL60R; 3.1 $\pm$ 0.2 μM for HL60R in the presence of VRP; and 2.1 $\pm$ 0.4 μM for HL60R in the presence of N151212C. Values are given as mean $\pm$ S.E.M., with $n \geq 3$. B, activity profile of *N*-trialkylglycines on the resistant degree of HL60R. The extent of DNM resistance was calculated as the ratio of DNM IC_{50} of HL60R and the IC_{50} of HL60 cells in the absence and/or presence of peptoids or VRP.

result is similar to that obtained with tariquidar, a potent and specific inhibitor of P-gp currently in clinical trials (Mistry et al., 2001; Robert and Jarry, 2003), which lowest energy conformation also shows an adequate alignment with the model (Fig. 7B) and a good fitting with peptoid N151212C (Fig. 7C). Thus, compound N151212C fulfills the structural properties characteristics of chemosensitizers that antagonize the P-gp and MRP1 pump activities.

Discussion

MDR, the principal mechanism by which many cancers develop resistance to anticancer drugs, is a major factor in the failure of chemotherapy approaches (Pastan and Gottesmann, 1987; Ling, 1997). Resistance to therapy has been correlated to the presence of at least two molecular pumps in tumor cell membranes that actively expel chemotherapy drugs from the cell interior, thus allowing tumors to avoid the toxic effects of cytostatics. The two pumps commonly found to confer chemoresistance to cancer cells are P-gp and MRP1. Because of their importance, they are the targets of several anticancer efforts, as evidenced by the number of chemosensitizers that have been tested in preclinical and clinical trials (Tan et al., 2000; Robert and Jarry, 2003). However, most of these compounds have been discarded because of concerns of toxicity and/or unwanted side effects (Robert and Jarry, 2003). The third generation of revertant drugs may partially attenuate these concerns. These compounds may be used as novel scaffolds that, in due turn, may be developed into clinical chemosensitizers. Nonetheless, the enormous challenge of finding chemosensitizers agents that act as inhibitors of P-gp and MRP1 is still a fundamental endeavor in cancer chemotherapy. Here, we have screened a *N*-trialkylglycine-based library composed of 5120 compounds

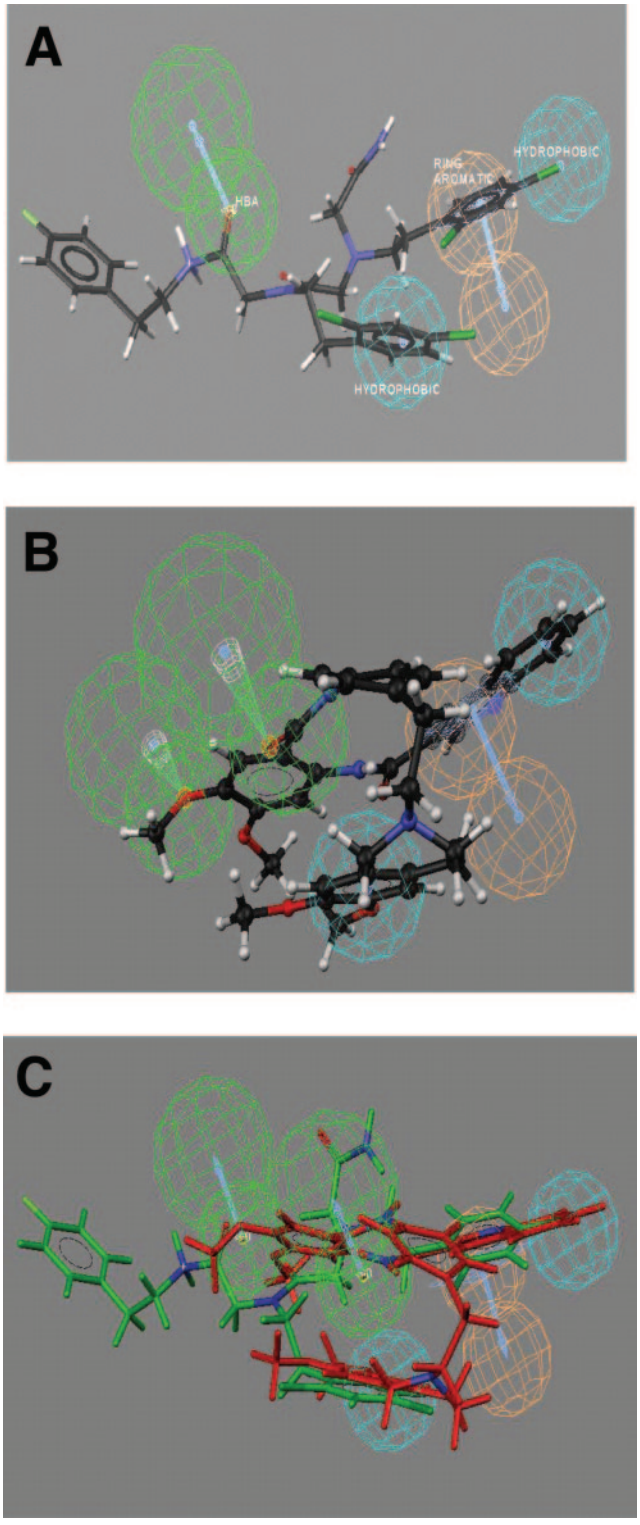


Fig. 7. Identified *N*-trialkylglycines fit onto the pharmacophore model characteristic of antagonists and substrates of P-gp. N151212C (A) and tariquidar (B), a potent chemosensitizer of the MDR phenotype (Mistry et al., 2001), fitted into the pharmacophore hypothesis generated by Catalyst. Pharmacophore contains two hydrophobic regions (cyan), one ring aromatic (orange), and two hydrogen bond acceptor features (green). For the sake of clarity, one of the hydrogen bond acceptors that showed no interactions with N151212C has been removed. C, N151212C aligned with tariquidar in the pharmacophore model generated by Catalyst. For clarity, one of the hydrogen bond acceptors that showed no interactions with N151212C has been removed. The lowest energy conformation of compounds N151212C and tariquidar are displayed.

to find antagonists of P-gp and MRP1. Trimers of *N*-alkylglycines are a family of non-natural molecules that are attractive for the drug discovery process because of their broad variety of biological activities. They have been successfully used for the identification of diverse activity, ranging from high-affinity ligands for membrane receptors to disruptors of macromolecular complexes (Heizmann et al., 1999; Humet et al., 2003). Furthermore, *N*-trialkylglycines have been used to identify anti-inflammatory, analgesic, and neuroprotectant drugs that exhibit in vivo activity (García-Martínez et al., 2002; Montoliu et al., 2002; Planells-Cases et al., 2002). Thus, the diverse activities associated with *N*-trialkylglycines, along with their proteolytic resistance and in vivo stability and low-to-moderate toxicity, make them good chemosensitizer candidates. The most salient contribution of this study is the identification of a set of 20 trimers of *N*-alkylglycines that selectively increase the intracellular accumulation of DNM in cancer resistant cells. This activity seems to be associated with the inhibition of the P-gp pump, as indicated by the chemosensitizing activity of these molecules in a cancer-sensitive line that stably expresses the murine P-gp. It is noteworthy that the two most active compounds antagonizing the activity of P-gp, N151212C and N161212C, reduced the degree of resistance of L1210R cells by $\geq$3-fold at a concentration that rivaled that of VRP. This inhibition was partially reversible, as evidenced by the modest effect on the intracellular accumulation of DNM of washing out the peptoids before incubation of the cytostatic (data not shown). Furthermore, these 20 compounds also augmented the intracellular accumulation of anthracycline in human HL60R tumor cells that overexpress the MRP1 pump. Notice that virtually all of the *N*-trialkylglycines identified in the screening modulated MRP1 activity to an analogous or higher extent than VRP. This observation is further substantiated in the DNM cytotoxicity assays where all the peptoids decrease the degree of resistance of HL60R cells by $\geq$3-fold, which is comparable to that of P-gp-expressing cells. Together, these results indicate that the identified peptoids are potent chemosensitizers with similar efficacy for P-gp and MRP1.

All selected peptoids contain the chemical groups 2,4-dichlorophenylethyl and 3,3-diphenylpropyl at the R_2 and R_3 positions, suggesting a key role for hydrophobic planar groups at these positions of the oligomer. The first diversity position, however, allowed slightly higher chemical diversity, although fluorophenylethyl groups were preferred. The abundant presence of hydrophobic planar groups, along with hydrogen bond acceptors, is consistent with the pharmacophoric properties proposed for P-gp antagonists. This tenet was further substantiated by the alignment of peptoid N151212C with the pharmacophoric model generated by Catalyst. However, one hydrogen acceptor was not properly fitted to the pharmacophore, suggesting that compound N151212C requires structural refinement for improved binding to P-gp.

A major effort is being devoted to develop nontoxic chemosensitizer drugs that could be used as adjuvants of current chemotherapy. The unwanted toxicity and side effects associated with current chemosensitizers have been attributed to interference with other cellular mechanisms or high-affinity blockade of P-gp. Complete pharmacological knockout of P-gp may lead to increased accumulation of cytostatics in sensitive

tissues such as brain, as concluded from studies from the mdr1−/− knockout mice (Schinkel et al., 1997; Zhang et al., 2000). These observations imply that moderate affinity antagonists that partially block the activity of these pumps may play a relevant therapeutic role by attenuating the side effects emerging from full blockade of these extrusion pumps. In addition, moderate-affinity compounds that act with similar efficacy on P-gp and MRP1 may have an additional benefit by tuning down the activity of the major proteins involved in MDR. Furthermore, this class of compounds may be valuable for those tumors that coexpress these two drug efflux pumps. Therefore, the peptoids that we have identified provide a new pharmacophore scaffold that could be considered as a pillar for the direct evolution of more active and safer chemosensitizing drugs. In support of this notion, the acute, single-dose toxicity of compounds N151212C and N161212C is ≥300 mg/kg (i.p.). The structural simplicity of *N*-trialkylglycines advantageously makes these peptidomimetics susceptible to broad structural manipulation and, therefore, lead-like property optimization.

Acknowledgments

We thank Adelina Calviño for technical assistance in the purification of peptoids.

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