TITLE:

TRIMERS OF N-ALKYLGLYCINES ARE POTENT MODULATORS OF THE MULTIDRUG RESISTANCE PHENOTYPE

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RUNNING TITLE:

MODULATION OF THE MULTIDRUG RESISTANCE PHENOTYPE

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Abbreviations used are: MDR, multidrug resistance; DNM, daunomycin; P-gp, P-glycoprotein; MRP1, multidrug-related protein 1; VRP, verapamil; 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]glycinamide;

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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 5,120 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 appear to act as P-gp antagonists as evidenced by the augment of DNM accumulation in the cell line L1210P-gp, 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 HL-60R 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 (VRP). 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.
INTRODUCTION

A plethora of cancer chemotherapies are currently in clinical practice. Most of these treatments fail because 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 over-expression of energy-dependent, unidirectional transmembrane drug efflux pumps (Ling, 1997; Gottesman et al, 2002). The P-glycoprotein (P-gp) is a 170 kDa membrane protein that belongs to the ATP-binding cassette family of transporters (Belamy, 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 (Belamy, 1996). P-gp expression in tumors in vivo is associated with poor overall prognosis and response to chemotherapy, in part, 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, over-expression 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 are 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 at al, 1996). For instance, MRPI confers resistance to anthracyclines, vinca alkaloids but not to taxanes (Tan et al., 2000). The MRPI 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 co-expressed with P-gp enhancing the drug resistance of tumors (Legrand et al. 1999). Other proteins such as the breast cancer resistant protein (BCRP) and the lung-resistance-related protein (LRP) 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, dihydropyroquinolines and anthranilic acid derivatives (Robert and Jarry, 2003). Taken together, these discovery efforts highlight the importance of developing inhibitors of proteins involved in MDR as 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 5,120 compounds. We found a set of 20 trimers of N-alkylglycines that antagonized P-gp function expelling out the anthracycline daunomycin (DNM) with an efficacy and potency that rivaled that shown by verapamil (VRP), a well-established P-gp antagonists (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 which, in due turn, may be developed as clinically useful chemosensitizers.
METHODS

Synthesis of trialkylglycines-based combinatorial mixtures and individual compounds.

An optimized library of 5,120 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, 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 the 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 individual oligo N-alkylglycine most active compounds was determined by analytical HPLC, MS and $^1$H and $^{13}$C NMR (see Supplementary Material).

Cell Cultures. DNM-resistant murine L1210R cells and human promyeolocytic (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$^{P-gp}$ 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% CO2 atmosphere (Castro-Galache et al. 2003).

**Daunomycin accumulation assays.** L1210R over expressing the P-gp and HL60R cells over-expressing the MRP1 were washed once with Hepes saline buffer (HBS) and the pellet resuspended in HBS at 1x10^6/mL per sample. Thereafter, cells were incubated with 3 µM DNM (control) and 5 µM VRP (as reference of chemosensitizing effect) or the peptoid mixtures (0.1 mg/ml) for 1 hr at 37 °C. After incubation, steady-state intracellular DNM accumulation was determined by flow cytometry as 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-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, Sigma) assay after plating 2x10^4 cells in a 96-well plate for 48 hrs. 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 PBS 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, mixed thoroughly to dissolve the dark blue crystals, and plates were read on a ELISA Reader using a wavelength of 570 nm. Data were normalized 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\textsubscript{50}).
\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \left( \frac{[\text{DNM}]}{IC_{50}} \right)^{n_H}}
\]

where \(G\) denotes growth at the different DNM concentrations, \(G_{\text{max}}\) indicates growth in the absence of DNM, and \(n_H\) the slope of the isotherm. The degree of resistance to DNM was obtained as the ratio of the IC\(_{50}\) values for the resistant subline and the wild type one.

**Modeling with Catalyst\(^ \circledast\).** The computational molecular modeling studies were carried out using a Silicon Graphics Octane workstation. The 3D-structures of the different ligands of P-gp ((R)-verapamil, rhodamine 123, vinblastine, colchicines, and calceine-AM) were built using Catalyst\(^ \circledast\) Version 4.7 (Molecular Simulations, 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 (Hip-Hop) in Catalyst\(^ \circledast\), ten 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, hydrophobic and ring aromatic, were considered. After assessment of the 10 hypotheses generated, the pharmacophore hypothesis with the lowest energy cost was selected. Selected N-trialkylglycines and the novel compound XR-9591, 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, minimising the distance between the centre 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 I), 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 molecules (OXX) or 320 molecules (XOX, XXO), and the library chemical diversity comprised 5,120 individual trimers. The set of amines included aliphatic and aromatic groups to increase the probability of finding P-gp inhibitors and 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 over-expresses P-gp (see supplementary data), that 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 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 for the second position was 8 (Fig. 1B), and for the third was 7 (Fig. 1C). Taken 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 increase DNM cellular accumulation. Figs. 1A-C show that amines A3, A7, A10, A15 and A16 were preferred at R1, whereas aromatic amines A12 and A14 were selected for the R2, and R3 positions (see Table I for structures). The selection of these amines considered both high activity restoring 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 [N-[2-(2’-Fluorophenyl)ethyl]glycyl]-[N-[2-(2’,4’-dichlorophenyl)ethyl]glycyl]-N-N-[2-(2’,4’-dichlorophenyl)ethyl]glycinamide (denoted as N151212C) and [N-[2-(4’-fluorophenyl)ethyl]glycyl]-[N-[2-(2’,4’-
dichlorophenyl)ethyl]glycyl]-N-N-[2-(2’,4’-dichlorophenyl)ethyl]glycinamide (denoted as N161212C) exhibited a remarkable revertant activity on L1210R cells. The dose response relationship for these N-trialkylglycines shows an IC$_{50}$ of 3.5±2.1 µM for N151212C (Fig. 2C). For N161212C, a value of 4.3±1.9 µM was obtained. Both compounds also inhibited the extrusion of the cationic dye Rhodamine 123 from L1210R cells (see supplementary 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 appear to be P-gp antagonists.** To investigate if active peptoids act on the P-gp protein, we used the cell line L1210$^{P\text{-gp}}$ that are 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 as 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). At variance with L1210R cells, however, the cell line L1210$^{P\text{-gp}}$ does not exhibit a drug-induced pleiotropic phenotype, thus allowing to determine 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. Noteworthy, the most active peptoids in L1210R cells were also the most potent increasing DNM accumulation in L1210$^{P\text{-gp}}$, namely N151212C and N161212C. 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). Therefore, these results imply that active peptoids are inhibitors of the P-gp protein that is over-expressed in L1210R cells.
Identified \textit{N-trialkylglycines} chemosensitize L1210R cells to DNM cytotoxicity. We next characterized the chemosensitizing activity of \textit{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 \( \mu \text{M} \) of the two most active peptoids. As positive control, VRP (5 \( \mu \text{M} \))-induced chemosensitization of L1210R cells was evaluated. Dose-response curves of DNM cytotoxicity revealed a concentration of anthracycline that inhibits half-maximal the cellular proliferation (IC\textsubscript{50}) of 0.11±0.03 \( \mu \text{M} \) and 20±2 \( \mu \text{M} \) for L1210 cells and L1210R tumor cells, which represents a degree of DNM resistance (DDR), calculated as the ratio of the IC\textsubscript{50} of L1210R with respect to that exhibited by drug-sensitive L1210 cells, of 180 (Fig. 4A). The presence of 5 \( \mu \text{M} \) VRP reduced ≥3-fold the IC\textsubscript{50} (6.0±0.1 \( \mu \text{M} \)) of resistant cells, leading to a DDR of 54. Similarly, 5 \( \mu \text{M} \) of peptoids N151212C and N161212C significantly decreased the DDR by 3-fold, from 180 to 45 (IC\textsubscript{50}=5.0±0.3 \( \mu \text{M} \)) and 50 (IC\textsubscript{50}=5.5±0.5 \( \mu \text{M} \)), respectively (Fig. 4B). Noteworthy, this concentration of peptoids did not exhibit cytotoxicity, thus implying that chemosensitization of L1210R was due to a direct inhibition of the P-gp pump activity. To further substantiate this notion, RT-PCR analysis of the MDR1 gene shows that the peptoids did not affect the mRNA levels of P-gp in L1210R cells (see supplementary data). Thus, these results indicated that identified peptoids are potent chemosensitizers of the P-gp-mediated MDR phenotype.

\textbf{Identified \textit{N-trialkylglycines also modulate the MRPI transporter}.} The acquired MDR phenotype may be mediated by over-expression of the P-gp protein or other members of the
ABC family of membrane transporters such as the MRP1 protein (Tan et al., 2000). For instance, human HL-60 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 augmenting 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 over-expressed in HL60R tumor cells. The activity profile of these compounds in P-gp and MRP1 appears rather similar, suggesting that they may interact with an structurally homologous binding site.

**Active peptoids chemosensitize HL60R cells to DNM.** Because virtually all 20 trialkylglycines displayed a comparable potency augmenting 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 \(\mu\)M for HL60 and 7.6\(\pm\)0.2 \(\mu\)M HL60R cells, indicating a degree of DNM resistance of 30 (Fig. 6A). This value was decreased up to 3-fold by 5 \(\mu\)M VRP (IC\(_{50}\)=3.1\(\pm\)0.1 \(\mu\)M). Similarly, the cytotoxicity of DNM to HL60R was notably augmented by the presence of 5 \(\mu\)M 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±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 AM. 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 if the compound fulfilled similar properties. As illustrated in Fig. 7A, the lowest energy conformation of peptoid N151212C nicely fit with the pharmacophore. This result is similar to that obtained with the compound XR9501, 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), as well as 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 anti-cancer 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 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 which, 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 5,120 compounds to find antagonists of P-gp and MRP1. Trimers of N-alkylglycines are a family of non-natural molecules attractive for the drug discovery process due to 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 \textit{in vivo} activity (García-Martínez et al., 2002; Montoliu et al. 2002, Planells-Cases et al., 2002). Thus, the
diverse activities associated to N-trialkylglycines, along with their proteolytic resistance and in vivo stability and low-to-moderate toxicity, makes 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 appears associated with 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. Noteworthy, the two most active compounds antagonizing the activity of P-gp, N151212C and N161212C, reduced the degree of resistance of L1210R cells by ≥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 over-express the MRP1 pump. Notice that virtually all of the N-trialkylglycines identified in the screening modulated MRP1 activity to an analogous or a 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 ≥3-fold, which is comparable to that of P-gp expressing cells. Taken 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 R2 and R3 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 non-toxic 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 knock out of P-gp may lead to increased accumulation of cytostatics in sensitive tissues such as brain, as concluded from studies from the mdr1-/- knock out mice (Schinkel et al., 1997). 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 co-express 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.). Advantageously, the structural
simplicity of $N$-trialkylglycines makes these peptidomimetics susceptible of broad structural manipulation and, therefore, of lead-like property optimization.

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REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1. Screening of an oligo N-substituted glycine combinatorial library to identify chemosensitizers of L1210R tumor cells. (A), (B) and (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-A20 refers to the amine number present in Table I. 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 at 50 µg/ml. None, denotes the 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 37ºC and, thereafter exposed to 3 µM DNM for an additional 1h at 37ºC. Intracellular accumulation of DNM was monitored by flow cytometry. O, refers to the defined position in the chemical library, whereas X, refers to the combinatorialized position with an equimolar mixtures of the amines used.

Figure 2. Identified N-trialkylglycines increase the intracellular accumulation of DNM in L1210R cells. Representative activity of N-alkylglycine trimers tested at 5 µM augmenting the cytosolic concentration of DNM in L1210R (A) and L1210 (B) tumor cells. None, denotes the intracellular accumulation of anthracycline in L1210R in the absence of chemosensitizers. VRP refers to the DNM accumulation in the presence of 5 µM of verapamil. (C) Dose-response relationship for the activity of N151212C compound incrementing the cytosolic accumulation of DNM in L1210R cells. Solid line depicts the
best fit to a Michaelis-Menten isotherm. The concentration of DNM that inhibits half-maximal cellular proliferation (IC$_{50}$) value was 3.5±2.1 µM. Data are mean±sem, with n≥3. 

N-trialkylglycines are referred to as the amine number selected form the N-to-C positions. 

(Inset) Molecular structure of peptoid N151212C.

**Figure 3.** Identified N-trialkylglycines appear to act as antagonists of the P-gp. (A) L1210 cells stably expressing the human P-gp protein (L1210$^{P-gp}$) show a decrease intracellular accumulation of DNM. The panel shows the flow cytometry distribution peaks of cytosolic DNM in L1210$^{P-gp}$ (a), and L1210$^{P-gp}$ in the presence of 5 µM VRP (b) and, in L1210 (c). (B) Representative profile of the potency exerted by 5 µM N-trialkylglycines on the cytosolic uptake of DNM in L1210$^{P-gp}$. None, denotes the accumulation of anthracycline in absence of any compound or drug. VRP, refers to the 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 1h at 37°C. Intracellular accumulation of DNM was monitored by flow cytometry.

**Figure 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 5 µM chemosensitizers. 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±0.03 µM for L1012, 20 ± 2 µM L1210R, 6.0±0.1 L1012R in the
presence of VRP, 5.0±0.3 μM L1210R in the presence of N151212C, and 5.5±0.5 μM L1210R in the presence of N161212C. Values are given as mean ± sem, with n≥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 \textsubscript{50} of L1210R and the IC \textsubscript{50} of drug sensitive L1210 cells in the absence and/or presence of peptoids or VRP.

**Figure 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, denotes the accumulation of anthracycline in absence of any compound or drug. VRP, refers to the intracellular concentration of DNM in the presence of 5 μM VRP. Cells were incubated with different compound for 1 h at 37ºC and, thereafter exposed to 3 μM DNM for an additional 1h at 37ºC. Intracellular accumulation of DNM was monitored by flow cytometry.

**Figure 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 \textsubscript{50} values were: 0.25±0.03 μM for HL60, 6.4±0.5 μM for HL60R, 3.1±0.2 μM for HL60R in the presence of VRP, and 2.1±0.4 μM for HL60R in the presence of N151212C. Values are given as mean ± sem, with n≥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 \textsubscript{50} of HL60R and the IC \textsubscript{50} of HL60 cells in the absence and/or presence of peptoids or VRP.
Figure 7. Identified $N$-trialkylglycines fit onto the pharmacophore model characteristic of antagonists and substrates of P-gp. N151212C (A) and compound XR-9501 (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 acceptors features (green). For the sake of clarity, one of the hydrogen bond acceptors showing no interactions with N151212C has been removed. (C) N151212C aligned with compound XR-9501 in the pharmacophore model generated by Catalyst©. For clarity, one of the hydrogen bond acceptors showing no interactions with N151212C has been removed. The lowest energy conformation of compounds N151212C and XR-9501 are displayed.
Table 1. Set of amines used for the synthesis of the $N$-trialkylglycine-based combinatorial library.

| $R_1$, $R_2$, $R_3$ | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 | A9 | A10 | A11 | A12 | A13 | A14 | A15 | A16 | A17 | A18 | A19 | A20 |
|---------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6