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Modifying the Sugar Moieties of Daunorubicin Overcomes P-gp-Mediated Multidrug Resistance

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Abstract

Anthracyclines are widely used in patients for anticancer activity. However, one of the limitations for their clinical use is P-gp-mediated drug resistance in cancer therapy. We hypothesize that modified anthracyclines will retain their anticancer activity, avert P-gp binding, and thus overcome P-gp-mediated drug resistance. Twenty-five daunorubicin analogues were synthesized with slight structure modifications in sugar moieties. Molecular docking, cytotoxicity, and P-gp inhibition assays in drug-resistant leukemia cells (K562/Dox) were used to identify several candidates that avert binding to multidrug-resistant protein (MsbA) and overcome drug resistance. Molecular docking showed that daunorubicin bound to the cavity between the intracellular domain (ICD) and nucleoside binding domain (NBD) of MsbA, which might be the "entry site" for the transport of its substrate. The molecular docking accurately predicted the substrates of multidrug-resistant protein. Several aspects are important for daunorubicin analogue binding to MsbA: (1) the substitution pattern and stereochemistry of the tetracyclic ring and sugar moiety; (2) the hydrogen bond donor or acceptor capability of the substituent at C`-3 and C`-4. Molecular docking, cytotoxicity, and P-gp inhibition assays identified ADNR, ADNR-1, and ADNR-3 for averting P-gp binding and overcoming drug resistance. The replacement of C`-3-NH2 with azido group in daunorubicin not only abolishes the hydrogen bond between the sugar moiety and MsbA but also completely changes the overall binding conformation, and thus averts the binding to MsbA. Cytotoxicity assays confirmed that these compounds showed high sensitivity against drug-resistant cancer cells (K562/Dox) with P-gp over-expression. P-gp inhibition assay indeed confirms that these appropriately modified compounds avert P-gp binding and thus overcome P-gp-mediated drug resistance.

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Chapter One

Problem Statement

The anthracycline antibiotics daunorubicin, doxorubicin, idarubicin, and epirubicin are potent anti-cancer drugs used in cancer chemotherapy. Unfortunately, their clinical success is limited by two factors: multidrug resistance and cardiotoxicity. This proposal and subsequent experiments will seek to determine the effectiveness of new daunorubicin derivatives in averting multidrug resistance. Additionally, we will ascertain the mechanism of action of new anthracycline derivatives. MDR is one of the major threats to successful anti-neoplastic chemotherapy. MDR is often mediated by P-gp, which exports anthracyclines and other drugs from the cell. An anthracycline which can overcome P-gp related MDR would greatly improve chemotherapy success and enhance the overall and cancer-free survival rates of chemotherapy patients.

Introduction

Multidrug Resistance (MDR) is a mechanism that cancer cells use to evade the cytotoxic effects of chemotherapeutic drugs. MDR can be an inborn property of a cancer cell, or induced by a chemotherapeutic drug. It is important to note that MDR cells show decreased sensitivity not only to the drug which induced the resistance, but to a broad panel of drugs which often show no structural or functional similarity [1]. Currently drug resistance to chemotherapy is believed to cause failure in over 90% of patients with metastatic cancers [2]. Drug resistance to chemotherapy is mediated by a variety of biological pathways. Drug efflux, drug inactivation, and pro-survival (anti-apoptotic) signaling seem to be among the most prominent of the mechanisms by which cancer cells avert death due to chemotherapy. The simplest and most efficient mechanism of drug resistance is drug efflux, therefore, the synthesis of compounds which are effective anti-neoplastic agents which overcome efflux-mediated resistance is of great importance [3].

Although anthracyclines are among the most effective anticancer drugs in use today, either as single agents or in combination therapy, multidrug resistance of anthracyclines is one of the limitations for the clinical use of these drugs. Multidrug resistance of anthracyclines is considered to be mediated by the efflux pump of the products of multidrug resistance genes family.

Efflux-mediated MDR is generally characterized by an over-expression of one or several members of the ATP-Binding Cassette (ABC) transporter family. There are now 48 known ABC transporter genes organized into seven subfamilies (ABCA-ABCG) [4]. ABC transporters efficiently export a broad variety of cytotoxic drugs from the lipid bi-layer, on which they reside [5]. Acting as pumps, these proteins function by exporting a

broad range of lipophilic molecules [4]. These proteins are commonly found in high levels to remove harmful xenobiotics and metabolites in the healthy tissues of the lungs, kidney, digestive tract, liver, as well as the blood-brain, blood-testis and blood-placental barriers [6]. In cancer cells these proteins are overexpressed and function to export anticancer drugs such as anthracyclines from cells. This decreases intracellular drug concentrations, diminishing the chemotherapeutic effectiveness and thus conferring drug resistance.

The multidrug resistance of anthracyclines is mediated by P-glycoprotein (P-gp), the product of the MDR1 (ABCB1) gene. MDR cell lines are often shown to over-express P-gp, which is correlated with anthracycline resistance *in vitro* [2]. P-gp, like other members of the ABC transporter family is predicted to have a 6+6 helical structure. Its tertiary structure appears as a roughly hexagonal toroidal ring [7]. The protein requires hydrolysis of two molecules of ATP to transport one drug molecule (or other molecule) out of the cell.

The mechanism of P-gp transport has been well characterized [8, 9], however the crystal structure of mammalian P-gp still remains to be solved. The lack of crystal structure of P-gp makes it difficult to study the substrate binding and to design the new drugs to overcome P-gp. Recently, Chang *et. al.* have been able to solve the crystal structure of an ABC transporter, MsbA, from bacteria [10-12]. The amino acid sequence of MsbA is 36% identical to that of P-gp. MsbA forms a homodimer as the functional transporter, and each subunit has six transmembrane domains and one ATP binding domain [10-13]. More importantly, MsbA is also in the ABC family of multidrug resistance protein and confers drug resistance in bacteria. Furthermore, MsbA and P-gp

share many common substrates. Thus, the MsbA model may be used to explain the transport activities of the ABC transporter family in general [11], especially because the two transmembrane domains of both contain 6 alpha-helical segments that comprise the substrate transporting channel [8, 11, 14].

Daunorubicin is one of the anthracyclines used clinically against leukemia. Daunorubicin features tetracyclic-ring structure and an amino sugar (daunosamine). The tetracyclic ring functions to intercalate DNA, while the sugar moiety functions as the minor groove binder of DNA. In addition, the sugar moiety may also interact with its molecular target, topoisomerase II [15-17]. Two major side effects are associated with the clinical use of daunorubicin: cardiotoxicity and multidrug resistance.

Previous work [18, 19] shows not only that a structure activity relationship exists between the sugar moiety and drug activity, but that the sugar plays a crucial role in the effectiveness of the drug. However, this relationship is neither clear, nor well understood. Circumventing P-gp-mediated resistance is especially difficult due to the necessity of preserving the cytotoxic activity [20]. It has been shown that modifying the sugar moiety in daunorubicin can change the anticancer efficacy of anthracyclines [21-23]. In addition, the 3'-amino group in the sugar may also play a role for P-gp recognition [24, 25]. Therefore, we hypothesize that modifying the sugar moieties of anthracyclines will retain its anticancer activity, alter their ability for binding to P-glycoprotein (P-gp), and thus overcome P-gp-mediated multidrug resistance (MDR).

We have previously synthesized a series of daunorubicin analogs with sugar modifications as a chemical biology tool. The molecular docking was used to identify the binding configuration of new analogs to MsbA, a homolog of P-gp. The molecular

docking results were confirmed by the anticancer activity in drug-sensitive and drug-resistant leukemia cells. Several candidates were identified to avert P-gp binding and overcome P-gp-mediated drug resistance.

Objectives

To find an effective treatment which overcomes P-gp-mediated MDR we have synthesized and tested 25 daunorubicin analog molecules. Each of these molecules contains a naturally occurring uncommon 2,6-dideoxy sugar conjugated to the ring structure of anthracyclines. There will be two specific aims of this study. The hypothesis pivotal to these aims is that by modifying the sugar moiety of daunorubicin the new analog will no longer be a P-gp substrate, and will therefore reduce drug resistance which will improve chemotherapy success.

Aim One: The primary aim of this study is to examine the effectiveness of these compounds (**Figure 1**) in overcoming P-gp mediated resistance in leukemia and/or solid tumor cells, and thus determine their possible efficacy as the possible next generation of anthracyclines.

Aim Two: Should we find one or more drugs which show promise in overcoming multidrug resistance, we will then study the biological mechanism by which these drugs avert MDR. Specifically, we seek to confirm that the drug overcomes MDR by averting P-gp mediated resistance.

Chapter Two

Methodology/Design

Synthesis and structures of 25 anthracycline analogs with sugar modification

We have previously synthesized 25 daunorubicin analogs with sugar modifications [22, 23, 26, 27]. To study the uncommon sugar function in anthracyclines, four different groups of anthracyclines with various uncommon sugars as monosaccharides or disaccharides were synthesized (**Fig 1**).

The first group of daunorubicin monosaccharides (compound**s** DNR-1 – DNR-6) were stereoselectively synthesized using TTBP/$AgPF_6$ glycosylation. The second group of daunorubicin analogs were synthesized with disaccharides (compounds DNR-D1 – DNR-D8). Based on the molecular docking results as discussed below, we synthesized the third group daunorubicin analogs with azido sugar or triazole sugars (compound**s** ADNR, ADNR-T1 – ADNR-T4). The fourth group of daunorubicin analogs was an attempt to optimize the lead compound (ADNR). (**Fig. 1**)

Molecular Docking of daunorubicin analogs to MsbA

Since no crystal structure of mammalian P-gp is available the crystal structure of MsbA with 3.8 Å resolution, an ortholog in bacteria, was used for docking study [28]. MsbA has 36% homology to human P-gp. MsbA and P-gp also have common substrate specificity and function in the same manner.

Preparation of MsbA

MsbA (PDB ID: 1PF4) was chosen for the docking template. The functional binding pocket was selected using Sybyl version 7.1. Polar hydrogens were added and Kollman charges assigned to the selected binding pocket. 3-D affinity grids covering the entire binding pocket were calculated for each of the following atom types: C, A (aromatic C), N, O, H, and e (electrostatic) using Autogrid3.

Preparation of ligands and docking

For the ligands, all hydrogens were added and Gasteiger charges were assigned, after which the non-polar hydrogens were removed. The rotatable bonds were assigned via AutoTors. AutoDock version 3.0.7 was used for the docking simulation. For each compound, the docking parameters were as follows: trials of 50 dockings, random starting position and conformation, rotation step ranges of 50º, and 1 million energy evaluations. Autodock Tools was used to evaluate the binding conformations to select the conformation of best fit. Chimera was used to describe hydrogen bonding and to generate the images.

IC50 determination of daunorubicin analogs in drug-sensitive (K562) and drug-resistance leukemia cells (K562/Dox)

The drug sensitive leukemia cells (K562) were cultured in RPMI-1640 containing 10% fetal bovine serum, 1% Penicillin/Streptomycin, and 1% L-glutamine. The medium was replaced every two to three days. Drug resistant leukemia cells (K562/Dox) were induced for drug resistance by incubation of K562 with doxorubicin for more than 3 months. K562/Dox was cultured in RPMI-1640 medium with 0.5 uM doxorubicin.

Before each experiment, K562/Dox cells were stimulated with 0.1 μM doxorubicin at least for one week and then cultured for 10 days without doxorubicin stimulation. It was assured that P-gp expression level was similar in every experiment. The cells were cultured in a 37 degrees Celsius humidified incubator with an atmosphere of 5% CO_2.

A total of 2,000-5,000 cells (K562 and K562/Dox) were seeded in 96-well plates and incubated for 24 hours. Synthetic compounds were added in a series of dilutions (0.1 nM – 10 μM) and incubated for 3 days. After 3 days, MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium), and phenazine methosulfate (PMS, an electron coupling reagent which allows the metabolization to proceed) were added directly to the cell culture and incubated for 1-4 hours at 37°C in a humidified, 5% CO_2 atmosphere. The MTS/PMS mixture is metabolized by living cells into formazan. The absorbance of formazan (metabolite of MTS in viable cells) is measured at 490 nm. Because the absorbance value of formazan has a linear correlation to the number of viable cells in each well, the absorbance value is used to calculate the surviving cell fraction. The IC_{50} values against drug-sensitive and drug-resistant cells of the tested compounds were calculated with dose-response curves using WinNonlin. The Drug Resistance Index (DRI), which is the ratio of IC_{50} in K562/DOX compared to IC_{50} in K562, was calculated.

P-gp inhibition assay using CsA for daunorubicin analogs

A simple and rapid high throughput P-gp inhibition assay was used to validate P-gp substrates. Co-incubation of P-gp inhibitor (Cyclosporine A, CsA) blocks P-gp exporter function, increases intracellular concentration of daunorubicin analogs, and

therefore increases the cell killing effects of daunorubicin. Therefore, the P-gp inhibition can be quantified by the high throughput MTS assay by comparison of the cell killing effects of daunorubicin analogs in the presence or absence of CsA. Total of 2,000-5,000 cells (K562/Dox) were seeded in 96-well plates and cultured for 24 hrs. The cells were pretreated with 5 μM Cyclosporine A (CsA) for 10 minutes. Then the synthesized compounds (1 μM) were added. After 72 hours, tetrazolium [3-(4,5-dimethythiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, 2 mg/ml) and phenazine methosulfate (PMS, 25 μM) were mixed and added directly to the cells. After incubated for 3 hrs at 37 °C, the absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm.

Expression levels of P-gp in drug-resistant leukemia K562/Dox

Total RNA isolation and cDNA synthesis: Total cellular RNA was extracted and purified using the TRIzol reagent (Gibco). The pellet was resuspended in 30-50 ul of DNase/RNase free-water, and the concentration was measured at 260 nm UV. First-strand cDNA was transcribed from 3μg of total RNA using 50ng random hexamers and SS II reverse transcriptase was used for reverse transcription at 42°C for 50 min. After termination of the reaction, 1μl of RNase H was added to the tube and incubated for 20 min at 37 °C before proceeding to amplification of the target cDNA.

Real-time PCR: SYBR Green PCR Master Mix was purchased from Applied Biosystems. Forward and reverse primers for targeted mRNA were designed using Primer Express version 1.0 software (ABI PRISM, Perkin-Elmer, Branchburg, NJ) based on the human MDR1 or actin sequence. The real-time PCR was completed on the ABI PRISM

Sequence Detector 7000 (Perkin-Elmer) using Sequence Detector version 1.7 software. The PCR was performed at 95 °C for 15 seconds, primer annealing and extension at 60 °C for 1 minute. Fold reduction of siRNA knockdown was calculated as follows: after completion of the PCR, the baselines and thresholds were set for both samples and internal beta-actin controls. Using Ct values (cycle number where sample crosses the threshold value) for samples (sam) and controls, the ΔCt was calculated: $\Delta Ct = Ct_{sam} - Ct_{actin}$. Then the values for each sample (sam) were compared with the negative siRNA control (neg): $\Delta\Delta Ct = \Delta Ct_{sam} - \Delta Ct_{neg}$. The fold change of mRNA level from negative control to sample is $2^{-\Delta\Delta Ct}$. MDR1 level in K562/Dox was compared to K562.

Population and Sample

For the purpose of identifying a compound which is useful against both sensitive and MDR cells we must use cells of each type. For the sensitive leukemia cell line we will use K562 cells. For the resistant leukemia cell line we will use K562/Dox cell line (a gift from J.P. Marie, INSERM, E9912, University of Paris, France) which has been made resistant to anthracyclines. The K562/Dox cells were stimulated with 0.1 μM doxorubicin once a month to maintain their resistance.

Data and Instrumentation

The data collected from the MTS assays will be the absorbance readings, which is directly proportional to the number of cells (gives a linear curve with slope =1). Therefore the absorbance reading will be mathematically converted into a viable cell count (as a percentage of the control group). For measuring the absorbance at 490nm, a Molecular Devices Spectromax Plus^{384} Microplate Spectrophotometer will be used.

The molecular docking simulations were performed by AutoDock version 3.0.7 with ligands and proteins prepared by Sybyl version 7.1. This work was done on a Silicon Graphics SGI02.

Chapter Three

Results

Molecular Docking of Daunorubicin Analogs to MsbA

We hypothesize that the structure modification of daunorubicin will change the binding to P-gp and overcome P-gp-mediated drug resistance. Therefore, we first utilized molecular docking to test our hypothesis with 25 daunorubicin analogs with series modifications on the sugar moiety. However, no crystal structure of mammalian P-gp was available. Fortunately, a distant ortholog (MsbA) in bacteria, which has 36% homology to human P-gp, has been crystallized with 3.8 Å resolution [10-12]. More importantly, MsbA is also in the ABC family of multidrug resistance protein and share common substrate specificity to confer drug resistance in bacteria. Therefore, molecular docking (Autodock version 3.0.7) was performed for daunorubicin analogs against MsbA to test our hypothesis. A summary of each compound's binding energy to MsbA can be found in Table 1. The docking results are shown in Figures 3-8.

Daunorubicin

The molecular docking results showed that daunorubicin (DNR) favorably bound to the cavity between the intracellular domain (ICD) and nucleoside binding domain (NBD) of MsbA (**Fig 2A**). C-6-OH of DNR had hydrogen bonding to the Asn436 in the NBD α2 domain of MsbA. The C'-4-OH of the sugar in DNR formed hydrogen bond with His107 in ICD1 domain, while C'-3-NH2 interacted with His202 in the ICD2 domain and Asp431 in the NBD α2 domain of MsbA (**Fig 2B**). This gave the docked complex a favorable binding energy of -7.92 kcal/mol. The strong hydrogen bonds,

coupled with low energy values showed that DNR is indeed a substrate of this multidrug resistance protein (**Fig 2**).

This binding configuration may have an important indication. It is generally believed that ABC transporters export their substrates via the "funnel" like cavity in the center through a conformation change. It is hypothesized that from here the transporting of the substrates is dynamic as the drug is moved from the cytoplasm, through the membrane into the periplasm [28]. In such a case, the substrate should not bind tightly to the center cavity in order for the transporting to occur. However, the substrate has to bind the transporter protein at an "entry site" in order for the protein to recognize the substrate. The challenge at this point is to find where this "entry site" located. Interestingly, within the cytoplasmic portion of MsbA there exists a cavity or pocket within the tertiary structure of each monomer between the intracellular domain and nucleotide binding domain. The docking results showed that daunorubicin binds to this cavity (**Fig 2B**). This may suggest that daunorubicin comes to the transporting cavity via this "entry site." It appears that the binding to the "entry site" is crucial for determining whether or not a compound is a substrate of MsbA.

DNR monosaccharide analogs

To vary the structure of daunorubicin to overcome P-gp, we synthesized the DNR monosaccharide analogs with monosaccharides linked to the tetracyclic ring structure of anthracycline via a beta glycosidic linkage (DNR-1 to DNR-6). Molecular docking results showed that DNR-1, DNR-2, DNR-3, DNR-4, DNR-5, and DNR-6 all have favorable binding to MsbA with binding energy of -9.05, -8.80, -9.20, -8.13, -8.15, and -

7.50 kcal/mol, respectively. These results indicate that DNR-1 to DNR-6 may still be P-gp substrates (**Fig 3**).

The binding conformation of DNR-1 to DNR-4 was similar to daunorubicin (**Fig. 3A, 3B**). The glycosidic linkage oxygen and the keto group at C-5 of DNR-1 – 4 formed hydrogen bond with Asn436 and Phe429 in the α2 and the hinge region between β4 and α2 regions of the NBD domain of MsbA. Although the C'-3- NH2 group in the sugar was replaced with C'-3-OH, or C'3-OMe, they still have hydrogen bond donating and accepting ability to form hydrogen bonds or favorable electrostatic interactions with His202 in the ICD2 domain. Some subtle differences in hydrogen bonding occurred, for example DNR-2 and DNR-3 did not exhibit true hydrogen bonding, but are probably stabilized by favorable electrostatic interactions between the C'-3 groups and His202. DNR-1 was further stabilized by a hydrogen bond from the C'-3-OH of DNR to Tyr439 of the α2 region of MsbA. These data suggest that the hydrogen donor or acceptor on C'-3 or C'-4 position may be critical to determine the binding of daunorubicin analogs to MsbA.

Therefore, we removed the C3'-OH in the sugar of DNR-5. However, DNR-5 still favorably bound to MsbA with -8.16 kcal/mol binding energy (**Fig 3C**). The favorable binding may come from the hydrogen bond between the keto group and glycosidic oxygen of DNR-5 and Asn436 and Phe429 in the α2 and the hinge region between β4 and α2 regions of the NBD domain of MsbA.

Furthermore, we replaced all polar group from the sugar structure of DNR-6 and added a high electron density group (azido, N_3) on C'-4. Although these modifications significantly changed the binding conformation of DNR-6 to MsbA as compared to DNR,

DNR-6 still favorably bound to MsbA with binding energy of -7.50 kcal/mol. The glycosidic linkage oxygen and the keto group at C-5 still formed hydrogen bond with Asn436 and Phe429 in the α2 and the hinge region between β4 and α2 regions of the NBD domain of MsbA (**Fig 3D**).

These results suggest that both the tetracyclic ring and sugar moiety of daunorubicin contribute the binding to MsbA. In addition, the substitution pattern and stereochemistry of the tetracyclic ring and sugar moiety play an important role for daunorubicin analogs to fit into the binding cavity of MsbA (**Fig 3**).

DNR Disaccharide analogs

In order to introduce more structure variability in the DNR analogs, we synthesized eight daunorubicin analogs with disaccharides (DNR-D1 to DNR-D8), in which the first sugar remained the same as DNR, but various second sugars are linked to the C'-4 position of the first sugar. Both alpha and beta glycosidic linkages were synthesized (**Fig 1**). Surprisingly, all eight analogs favorably bound to MsbA in a similar configuration as daunorubicin (**Fig 4**), and with relatively similar binding energy (ranging from -7.8 kcal/mol to -11.2 kcal/mol).

Each DNR disaccharide analog forms hydrogen bonds from the C'-3-NH2 group to His 202, from the first glycosidic oxygen to Asn436, and from the C-5 keto group to Phe429 (**Fig 4A, 4B, 4C, 4D**). Exceptions are DNR-D1 and DNR-D6 which did not form a hydrogen bond to Asn436. Interestingly, DNR-D4 and DNR-D7 had hydrogen bonding to Cys315 from the C''-3-OH group of the second sugar (**Fig 4C**).

These results strongly suggest that the first sugar moiety of daunorubicin analogs plays a more important role for the binding of MsbA. Addition of the second sugar does not significantly change the binding conformation to MsbA. It appears that it is difficult to avert the binding to multidrug resistant protein without altering the first sugar moiety of daunorubicin (**Fig 4**).

ADNR analogs

Based on the information of the above docking results, it seems that several aspects are important for daunorubicin analogs binding to MsbA: (1) both sugar moieties and tetracyclic ring structure of daunorubicin; (2) C'-3 and C'-4 hydrogen bond donor or acceptor capability; and (3) the overall structure conformation of daunorubicin analogs that determined by the tetracyclic rings and sugar structure.

The binding conformation of DNR-6 to MsbA, in which C'-4-N3 group in the sugar moiety was able to significantly alter the whole binding conformation of DNR-6 to MsbA as compared to DNR. In addition, C'-3-NH2 group has been reported to be critical for P-gp binding [14, 29]. Therefore, we hypothesize that replacement of C'-3-NH2 group with an azido group or large triazole group may be able to change both the binding conformation and/or abolish the hydrogen bond between DNR and MsbA. Thus, ADNR and ADNR-T1 to ADNR-T4 were synthesized to test our hypothesis [22].

Very interestingly, ADNR bound unfavorably to MsbA compared to DNR. Its binding energy (+2.94 kcal/mol) is significantly higher than that of DNR. This modification completely changes the overall conformation of the ADNR binding to MsbA. The tetracyclic rings of DNR and ADNR are roughly perpendicular to each other.

The sugar modification in ADNR completely abolished the hydrogen bond between C'-3 and MsbA. The only hydrogen bonds between ADNR and MsbA are glycosidic oxygen of ADNR to Asn436 and the C'-4-OH group of ADNR to Asp431 of MsbA (**Fig 5A**). These data suggest that ADNR may not favorably bind to multidrug resistant protein and may overcome P-gp mediated drug resistance. Indeed, these findings were confirmed by experimental data as show below.

Similarly, the DNR triazole analogs (ADNR-T1 to ADNR-T4) also unfavorably bind to MsbA with binding energy of + 3.35 to +18.78 kcal/mol (**Fig 5B, 5C, 5D**). Similar to ADNR, ADNR-T1 to ADNR-T4 significantly changed the binding conformation to MsbA, abolished the hydrogen bonds between C'-3 with MsbA. The only existing hydrogen bond is between the C'-4 hydroxyl group and Cys315 of MsbA.

These results indicate that replacement of C'-3-NH2 amino group with azido or triazole group sufficiently avert P-gp binding and overcome drug resistance. Although the tetracyclic rings of azido sugar analogs and triazole analogs remain the same as DNR, it appears that azido or triazole group not only abolishes the hydrogen bond between the sugar moiety and MsbA, but also significantly changes the conformation of whole structure of DNR. These two important changes are critical to avert the binding to MsbA. **(Fig. 5)**

ADNR disaccharide analogs

To further optimize the lead compound ADNR, we synthesized ADNR disaccharide analogs (ADNR-1 to ADNR6) with a second sugar linked to first azido-

sugar via beta-linkage. This was to study the contribution of the second sugar for MsbA binding and anticancer activity.

Although all six analogs were derived from ADNR with azido-sugar, not all six analogs have similar binding to MsbA as compared to ADNR. Similar to ADNR, ADNR-1, ADNR-3, and ADNR-6 showed unfavorably binding to MsbA with binding energy of + 2.38 kcal/mol to +17.25 kcal/mol (**Fig 6**). Very surprisingly, ADNR-2, ADNR-4, and ADNR-5 regained favorable binding to MsbA with binding energy of -8.92, -7.51, and -3.22 kcal/mol, respectively (**Fig 7**). The introduction of second sugar has generated more hydrogen bonding between these analogs and also changed the overall binding conformation.

In fact, the sugar structure of ADNR-1 and ADNR-3 are in completely opposite orientation compared to DNR, while the sugar structure of ADNR-6 is very dissimilar. No H-bond interaction was found between the sugar structure of these three compounds and MsbA. The only hydrogen bonds were found between C6-OH of ADNR-1 and His107 of MsbA, and between C-11-OH, C-12-O of ADNR-3 and Cys315, and between the C-11 hydroxyl group of ADNR-6 with Cys315 of MsbA. Clearly, the overall conformation change and the abolition of critical hydrogen bonding between the sugar and MsbA created an unfavorable binding (**Fig 6**).

In contrast, a second sugar in ADNR-2, ADNR-4, and ADNR-5 introduced the extra hydrogen bond or generated a favorable binding conformation. Although ADNR-2 showed different binding configuration with MsbA, the introduction of second sugar in ADNR-2 re-configured the compound in such a way that the C''-4-OH group of the second sugar interacted with Thr198 in ICD2 domain. Meanwhile the C'-9 hydroxyl

group of ADNR-2 regained H-bond interaction with Arg127 in ICD1 domain. These data suggest that ADNR-2 still weakly binds to multidrug resistant protein which confers drug resistance, which was confirmed by experimental data below. ADNR-4 and ADNR-5 have dissimilar configurations in relation to DNR. ADNR-4 forms hydrogen bonds to Cys315 from both the C-11 and C-12 hydroxyl and keto groups. The tetracyclic rings of the two compounds are nearly perpendicular to each other in docked conformations. Although no extensive hydrogen bonds between the second sugar of ADNR-4 (orADNR-5) and MsbA, the overall conformation of the structure orientated the molecules in such a way that they are fitting very well into the binding pocket (**Fig. 7**).

These data confirm that the C'-3-NH2 in the first sugar of DNR, tetracyclic ring structure, and the overall conformation (determined by tetracyclic and sugar structure) of daunorubicin are critical to determine the binding to MsbA. The replacement of C'-3-NH2 group with an azido group not only abolishes the hydrogen bond between the C'-3 of the sugar moiety and MsbA, but also completely changes the overall conformation of DNR, and thus averts the binding to MsbA. The second sugar moiety in the sugar structure may not be important in overcoming MsbA binding. Rather, the introduction of second sugar sometimes introduces new hydrogen bond or change the overall conformation to fit the binding pocket. However, the introduction of the second sugar may change the anticancer activity and topoisomerase activity (data not shown) (**Fig 6 and 7**).

Expression levels of P-gp in drug-resistant leukemia cells (K562/Dox) compared to drug sensitive cells (K562)

To confirm our hypothesis and molecular docking results, we tested the cytotoxicity in drug-sensitive leukemia cells (K562) and drug-resistant leukemia cells (K562/Dox). First we tested the P-gp expression levels in these two cell lines using real-time PCR. As shown in Figure 9, the mRNA levels of MDR1 gene were more than 500-fold in K562/Dox cells compared to K562. Therefore, K562/Dox will show high drug-resistance for anthracyclines due to the P-gp export function as shown below. That is why K562/Dox was used to test the drug cytotoxicity and drug resistance of our daunorubicin analogs. **(Fig 8)**

Cytotoxicity and drug resistance of daunorubicin analogs in drug-sensitive and drug-resistant leukemia

MTS Assays were utilized to asses the cytotoxicity in both K562 and K562/Dox leukemia cells. These were to confirm if the molecular docking results are in agreement with the anticancer activity in drug-resistant leukemia cells (K562/Dox). The cells were incubated with varying amounts of drug to give a dose-response curve. The IC_{50} of daunorubicin analogs were determined by WinNonlin 4.1. Drug Resistance Index (DRI) was calculated by the ratio of IC50 in the drug-resistant K562 vs. IC50 in the drug sensitive index. These data are summarized in Table 2.

Although daunorubicin showed very sensitive cell killing effects in K562 cells with IC50 of 16 nM, it indeed exhibited drug resistance in K562/Dox due to the high levels of P-gp expression. The drug resistance index is greater than 320.

DNR monosaccharide analogs

The MTS results showed that all the DNR monosaccharide analogs (DNR-1 to DNR-6) exhibit very high IC50 in K562/Dox and none of them overcame drug resistance against drug-resistant K562/Dox compared to DNR. This data set is in agreement with molecular docking results that none of these analogs averted binding to multidrug resistant protein MsbA. In addition, it is important to note that since sugar modifications in DNR-2, DNR-3, and DNR-6 even resulted in the loss of anticancer activity in drug-sensitive cells, the drug resistance index (DRI) can not be calculated for the confirmation of the P-gp substrate.

DNR disaccharide analogs

DNR disaccharides (DNR-D1, -D2, -D3, -D5, -D8) slightly improved cytotoxicity against drug-resistant K562/Dox compared to DNR. However, the drug resistance index (DRI) is still relatively high (>50). It is also important to note that the cytotoxicity of these compounds may dependent on two factors: drug efflux pump and drug target topoisomerase. Whether these compounds may overcome drug resistance needs to be further confirmed with the experiment below (P-gp inhibition Assays). It is also worth noting that DNR-D4, DNR-D6, and DNR-D7 lost their activity in both drug-sensitive and drug-resistant cells due to the lack of topoisomerase II poisoning (data not shown). Therefore, DRI is not used to confirm the P-gp substrates.

DNR azido sugar (ADNR) and triazole sugar (ADNR-T1 to -T4) analogs

Very interestingly, ADNR with azido sugar significantly reduced the IC50 against drug-resistant cells compared to DNR. The drug resistant index (DRI) significant reduced to less than 13. Together with the molecular docking results that ADNR may avert

binding to MsbA; these data may suggest that ADNR overcome P-gp-mediated drug resistance in cancer cells. In contrast, although ADNR-T1 to T4 also avert MsbA binding by molecular docking, these four compounds also lost their activity against both drug-sensitive and drug-resistant cancer cells. The lost activity is due to the lack of Topoisomerase II poisoning (data not shown). Thus, the DRI is not used to confirm the p-gp substrates for ADNR-T1 to –T4.

ADNR disaccharide analogs

Although the disaccharide reduces the sensitivity of ADNR-1 to ADNR-6 in drug-sensitive cells, these compounds indeed showed high sensitivity against drug-resistant leukemia cells (except for ADNR-2 and ADNR-4). The drug resistance indexes (DRI) were indeed significantly decreased compared to DNR. These results suggest that these compounds (ADNR-1, -3, -5, -6,) may overcome drug resistance. The molecular docking results indeed showed that ADNR-1, ADNR-3, and ADNR-6 indeed averted MsbA binding while ADNR-2 and ADNR-4 showed favorable binding to MsbA. The agreement of these results for compounds (ADNR-1, -2, -3, -4, and -6) and the discrepancy for ADNR-5 need to be further tested in the following experiments.

Molecular docking energy can qualitatively distinguish the P-gp substrate

By analyzing the molecular docking energy of these daunorubicin analogs to MsbA, it is very important to note that there is no simple correlation between associated binding energy and DRI. However, our docking method can accurately predict whether or not a compound is a P-gp substrate. Analyzing tables 1 and 2, it can be shown that our docking model alone was accurate in predicting relative DRI for all but one (ADNR-5) or our 25 compounds. Each favorably docking compound (with binding energy less than 0)

shows a DRI of 12 or more, with the one exception of ADNR-5 (or is inactive), while each unfavorably docking compound (with binding energy great than 0) shows a DRI of 5 or less (or is inactive). This data implies that molecular docking may provide an accurate means of analyzing the ability of compounds to overcome p-gp-mediated MDR.

P-gp inhibition assay

In order to further confirm the molecular docking results and P-gp-mediated drug resistance in drug-resistant cancer cells (K562/Dox), we used a combination of P-gp inhibition and MTS assay to distinguish whether daunorubicin analogs avert P-gp binding and overcome drug resistance. If a daunorubicin analog is a P-gp substrate, it will be exported from the K562/Dox cell by overexpressed P-gp, and thus it will have lower intracellular concentration to confer drug resistance. When a P-gp substrate (such as daunorubicin) is incubated with P-gp inhibitor (cyclosporine, CsA) in K562/Dox, CsA will competitively inhibit P-gp and block the export function. Thus, CsA will significantly increase the intracellular concentration of the P-gp substrates (such as daunorubicin) to exhibit much higher cell killing effects against K562/Dox. In contrast, CsA will not change the cytotoxicity of non-P-gp substrates. However, it is worth noting that if daunorubicin analogs lost their anticancer activity (due to the lack of topoisomerase II poisoning), this experiment can not be used to distinguish the P-gp substrate and can only be used as a negative control.

As shown in Fig 10A, when 1 uM daunorubicin was incubated with 5 uM of CsA in K562/Dox, the cell killing effects increased by 2.25-fold although CsA alone (5 uM) did not show any cytotoxicity. These results indeed confirm the molecular docking results and strongly suggest that daunorubicin is indeed a P-gp substrate.

In DNR monosaccharide analogs, CsA also significantly increased the cell killing effects of DNR-1, DNR-4, and DNR-5 by 1.61 to 3.05-fold. These data also confirm the molecular docking results and suggest that these compounds are P-gp substrates. It is important to note that DNR-2, DNR-3, and DNR-6 lost their anticancer activity in both drug-sensitive (K562) and drug-resistant leukemia (K562/Dox). Even though CsA did not change the cell killing effects of DNR-2, -3, and -6, this experiment can not be used to confirm if they are P-gp substrate or not. These compounds only served as a negative control in this experiment.

In DNR disaccharide analogs, CsA also significantly increased the cell killing effects of DNR-D1, -D2, -D3, -D5, -D8 from 1.85 fold to 5.77-fold, similar to DNR. These data together with molecular docking suggest that these compounds are still P-gp substrates. Since DNR-D4, -D6, and -D7 lost their anticancer activity, we did not test these compounds in this experiment set.

As expected, ADNR, ADNR-1, ADNR-3, and ADNR-6 showed anticancer effects in both drug-sensitive and drug-resistant leukemia; however CsA did not change their cell killing effects in K562/Dox. These results together with molecular docking results strongly suggest that these three compounds avert P-gp binding and overcome drug resistance. In contrast, ADNR-2, ADNR-4, and ADNR-5 did show significantly high cell killing effects when combined with CsA, which indicate that they may still be the substrate of P-gp. These data also confirmed by the molecular docking results. Only some discrepancy was seen for ADNR-4. The molecular docking showed ADNR-4 still binds to MsbA, but CsA did not enhance its cell killing effects. The detailed mechanism for this discrepancy may warrant further study in the future.

Discussion

Several strategies have been explored to overcome P-gp-mediated drug resistance. The first attempts to circumvent the P-gp efflux were inhibition of P-gp's ability to export the drug via competition for binding and transport. Cyclosporine A, a known auto-immune suppressant, and verapamil, a calcium channel blocker, were among the first generation of P-gp inhibitors. By adding these compounds to the regimen of chemotherapeutic drugs they will compete for binding and export via P-gp, leading to higher intracellular drug concentration of other anticancer drugs. Thus the compounds act as competitive inhibitors of P-gp. Both of these were used successfully *in vitro* to overcome P-gp mediated resistance. However, *in vivo* testing for these drugs in combination with anticancer drugs has a variety of detrimental effects. The first generation of P-gp inhibitors required a dose so high that toxic side effects often occurred. Second and third generations of these P-gp inhibiting compounds have since been synthesized and evaluated in pre-clinical and clinical trials, reaching only modest levels of success. The definitive downfall of the use of these compounds is the fact that a variable pharmacokinetic interaction exists in other organs [3]. Therefore, any P-gp inhibitor could potentially pose a threat to the normal, healthy tissues of the kidney, lung, brain, colon, uterus or adrenal glands which depend on the function of P-gp for the efflux of xenobiotics [30]. Given such a long and varied history of problems perhaps small compound P-gp competitive inhibition is not the best strategy for overcoming drug resistance.

A second and more promising strategy for defeating P-gp-mediated MDR is direct modification of the structure of anticancer drugs to maintain anticancer activity and avert

P-gp binding. As reported here, we have modified sugar structure of anthracycline to generate a series of daunorubicin analogs. These analogs were tested to overcome P-gp binding by molecular modeling, cytotoxicity, and P-gp inhibition. Indeed, we have generated several lead compounds that maintain the anticancer activity and avert P-gp binding [23]. In addition, our results have also been confirmed by previous studies. For instance, modification of the structure of anthracycline has demonstrated some success in overcoming drug resistance [17, 31]. The 3'-amino group in the sugar may plays a role for P-gp recognition [24, 25], but it has also been suggested [18] that the amino group may stabilize intercalation of DNA. When the 3'-NH_2 of doxorubicin was replaced with a 3'-OH or 3'-*N*-methylation, the resulting compounds partially reversed drug resistance [32-40], but were slightly less cytotoxic. Furthermore, these daunorubicin analogs in our study are only slightly modified in their structures. These structures are very similar yet several compounds exhibit the capability to overcome P-gp binding and overcome drug resistance. Since P-gp is very difficult to study due to the membrane protein nature, the daunorubicin analogs provide an excellent means for study P-gp functions using chemical biology methods. For instance, since our molecular modeling have identified the amino acid residues in MsbA that interact with daunorubicin analogs and confirmed by the non-P-gp substrates with similar structure (such as ADNR), this information may well be used for further chemical biology study for P-gp for substrate binding sites.

It is certainly true that any molecular docking program has limitations. An x-ray crystallography study will always prove to be more definitive and accurate than the best docking simulation. There are many situations, however, where x-ray crystallography is not currently feasible. One of these areas is with membrane proteins such as P-gp. To

validate the accuracy of the molecular docking, cytotoxicity and P-gp inhibition assays were used in P-gp overexpressed leukemia cells (K562/Dox). The cytotoxicity and P-gp inhibition assay are in agreement with the molecular docking study. Although there is no simple correlation between associated binding energy and DRI, our docking method can qualitatively predict whether or not a compound is a P-gp substrate. This data implies that molecular docking may provide an accurate means of analyzing the ability of compounds to overcome p-gp-mediated MDR. Furthermore, while it will be important to support modeling data with biological data, future drug design can progress greatly with computer modeling.

Conclusion

In summary, we have studied previously synthesized 25 daunorubicin analogs with structure modifications in sugar moieties with the aim to overcome P-gp mediated drug resistance. These similar chemical structures provide an excellent probe for chemical biology study of P-gp. Molecular docking, cytotoxicity, and P-gp inhibition assay were used to identify several candidates that successfully avert binding to multidrug resistance protein and overcome drug resistance.

Molecular docking with 25 daunorubicin analogs showed that the cavity between the intracellular domain (ICD) and nucleoside binding domain (NBD) of MsbA is the binding site for daunorubicin, which might be the "entry site" for the transport of the P-gp substrate. The amino acid residues His107, His 202, Asp431, and Asn436 are critical for the substrates to bind to MsbA.

The molecular docking could accurately predict the substrates of the multidrug resistant protein. It appears that several aspects are important for daunorubicin analogs binding to MsbA: (1) The substitution pattern and stereochemistry of the tetracyclic ring and sugar moiety; (2) the hydrogen bond donor or acceptor capability of the substituent at C'-3 and C'-4. The first sugar moiety of daunorubicin analogs plays more important role for the binding of MsbA than the second sugar in the disaccharide analogs.

Molecular docking, cytotoxicity, and P-gp inhibition assay confirmed ADNR, ADNR-1 and ADNR-3 for averting P-gp binding and overcoming drug resistance. The replacement of C'-3-NH2 group with azido group not only abolished the hydrogen bond between the C'-3 of the sugar moiety of daunorubicin and MsbA, but also completely changed the overall conformation of DNR, and thus averted the binding to MsbA.

Cytotoxicity assays confirmed that these compounds showed high sensitivity against drug-resistant cancer cells (K562/Dox) with P-gp over-expression. P-gp inhibition assays indeed confirmed that these compounds averted P-gp binding and overcame P-gp-mediated drug resistance.

Figures and Tables

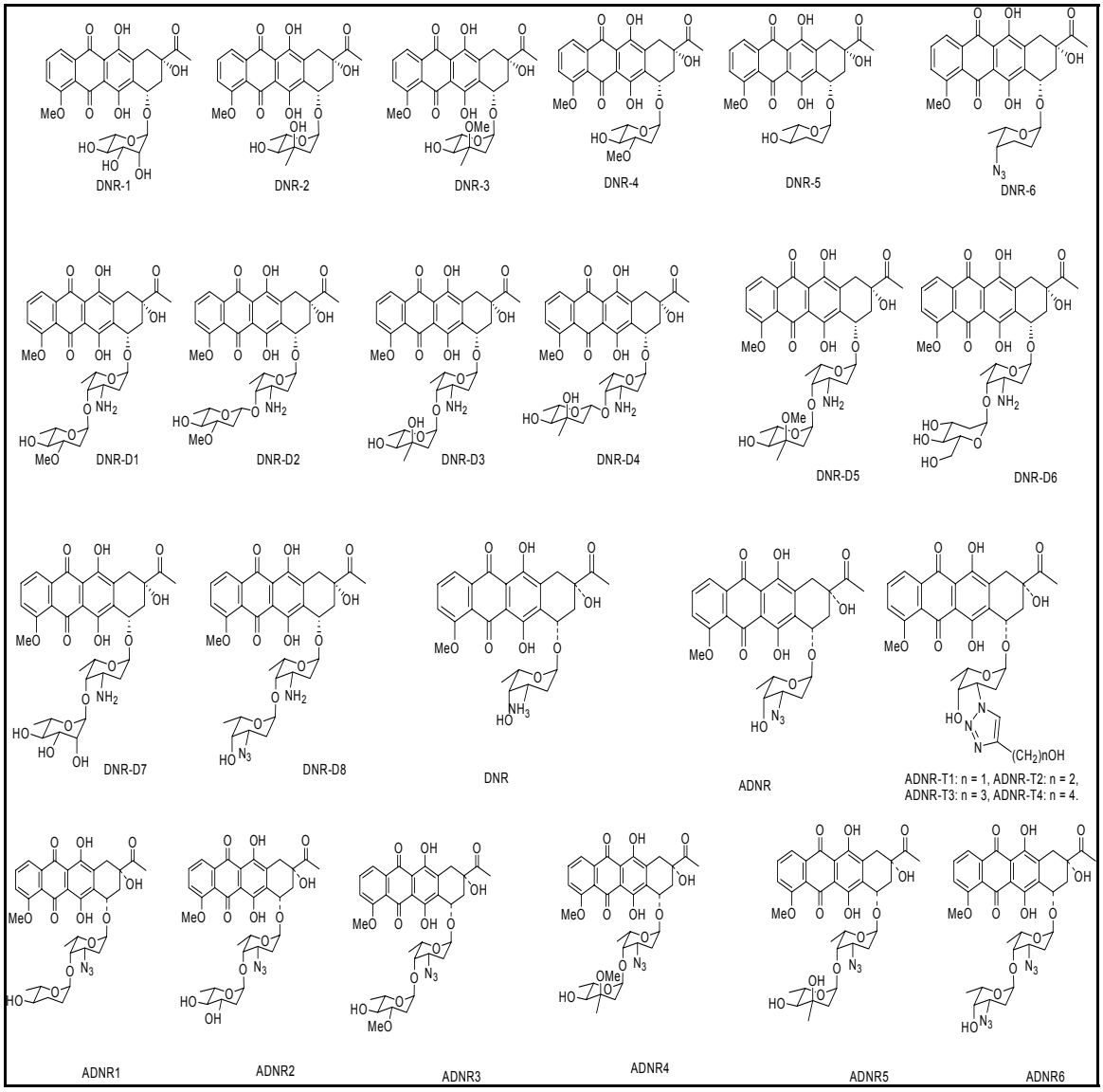

Figure 1

Structures of the 25 daunorubicin analogs with various sugar modifications as well as DNR, the parent compound.

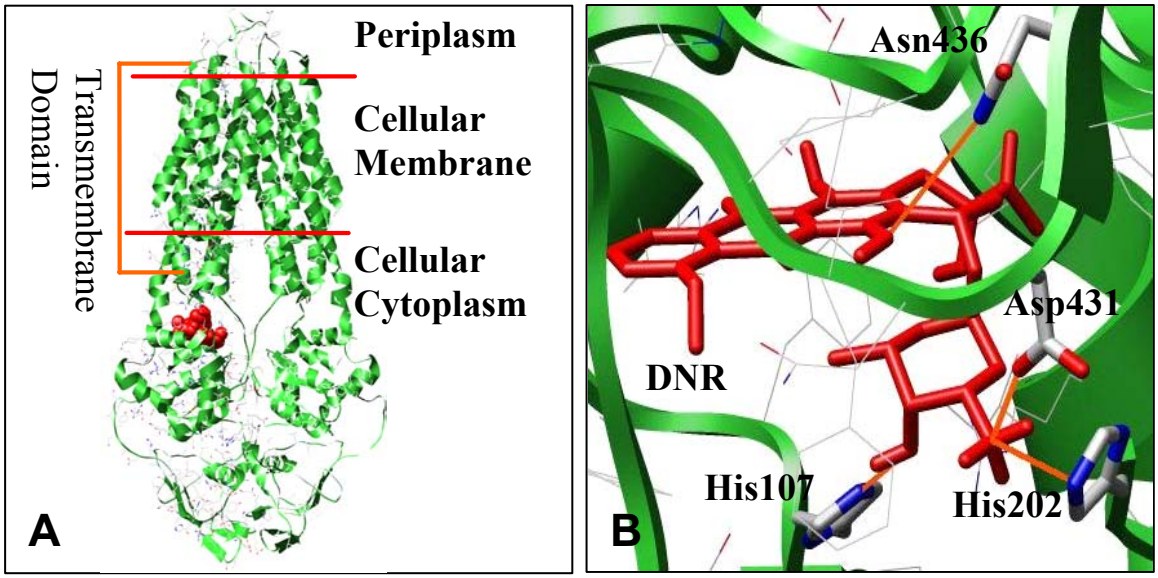

Figure 2

Daunorubicin (DNR) bound to MsbA. DNR binds to the cavity between intracellular domain (ICD) and nucleotide binding domain (NBD) of MsbA. Hydrogen bonds between DNR and MsbA are represented with orange lines. DNR is labeled in red color. Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers.

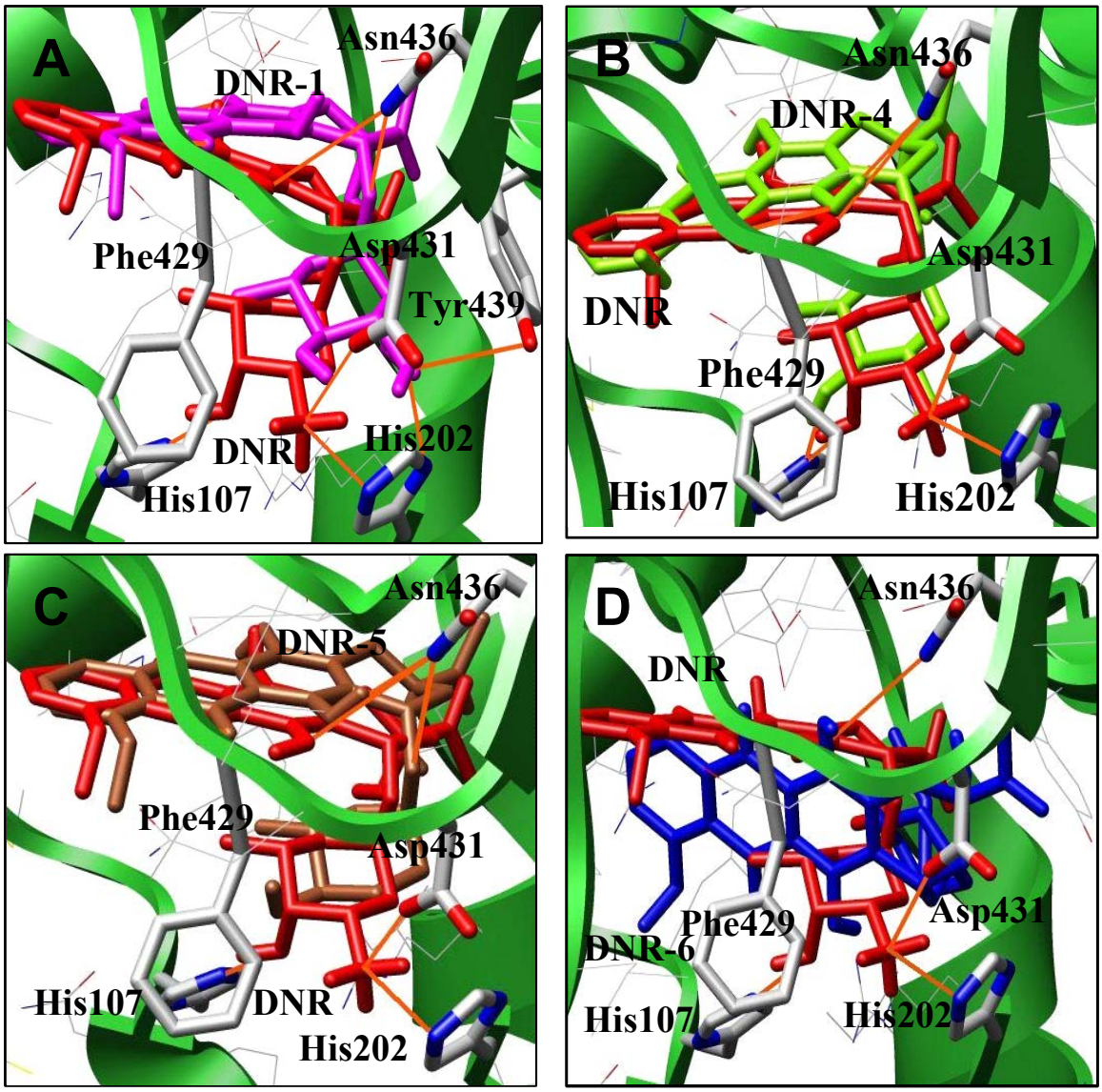

Figure 3

DNR monosaccharide analogs bound to MsbA as compared to the docked structure of DNR (red color). A. DNR-1 (purple color); B. DNR-4 (green color); C. DNR-5 (sienna color); and D. DNR-6 (blue color). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

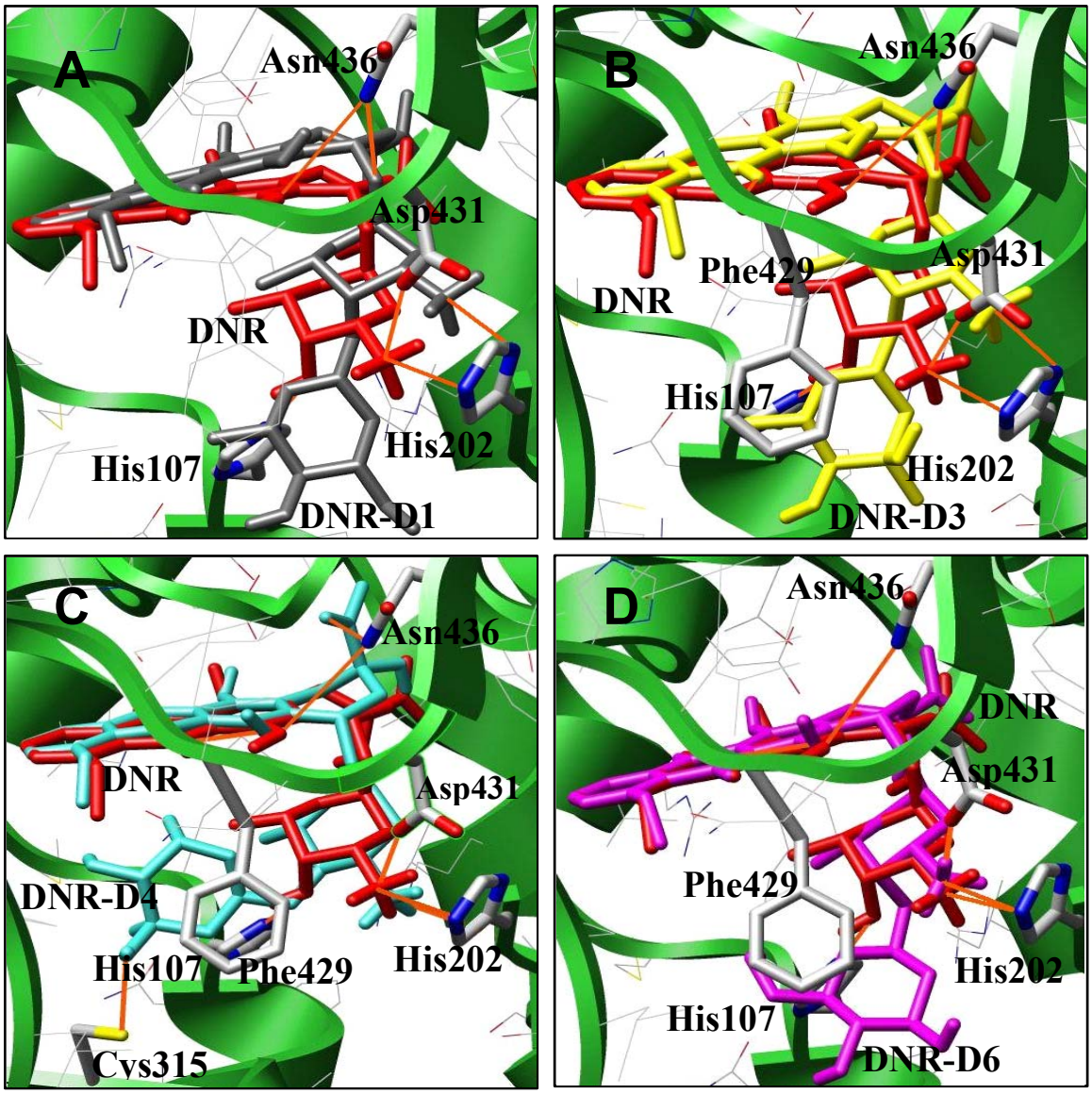

Figure 4

DNR disaccharide analogs bound to MsbA as compared to the docked structure of DNR (red color). A. DNR-D1 (silver color); B. DNR-D3 (yellow color); C. DNR-D4 (light blue color); and D. DNR-D6 (purple color). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

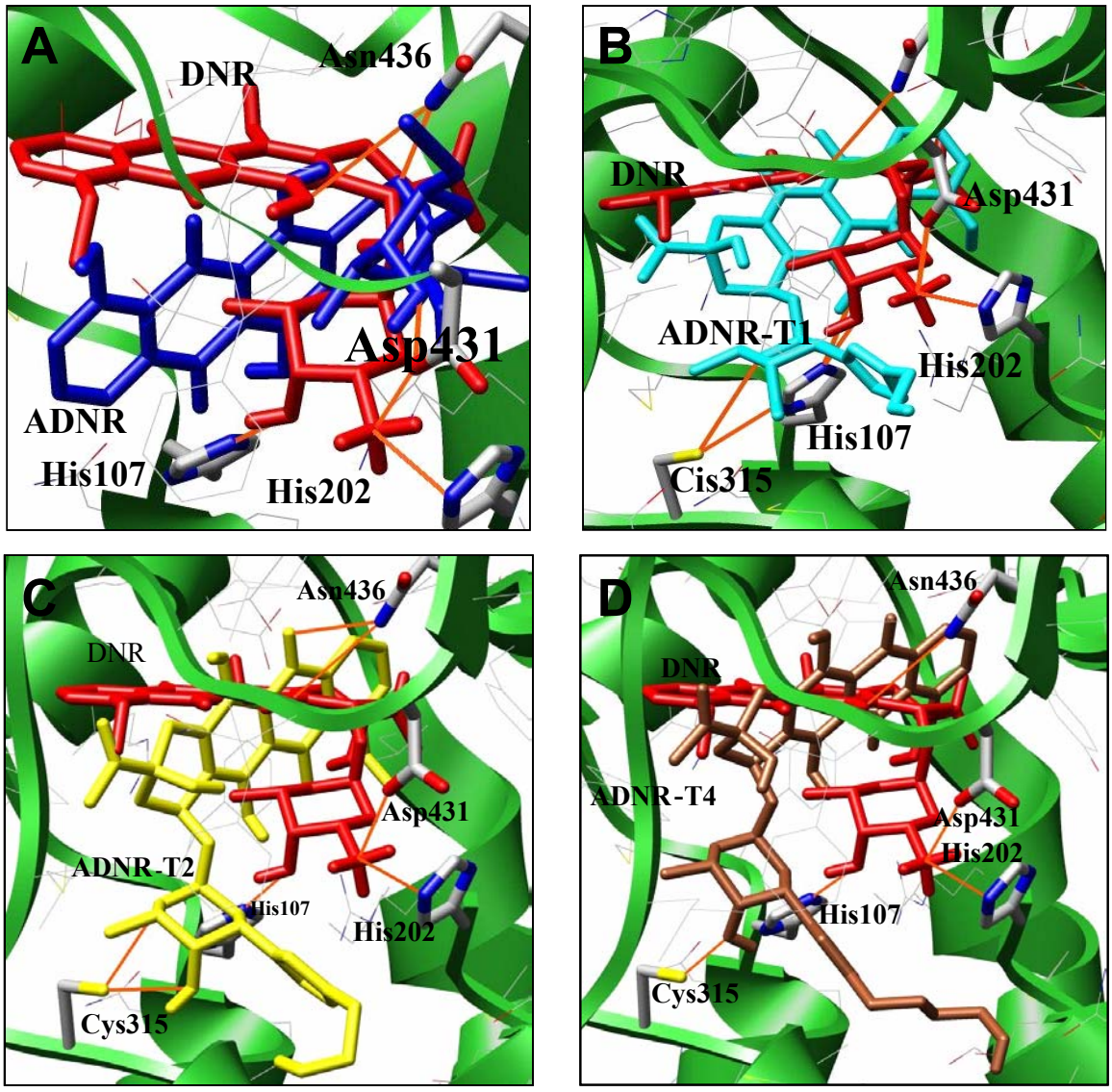

Figure 5

ADNR and ADNR-T1 bound to MsbA as compared to the docked structure of DNR (red color). A. ADNR (dark blue color); B. DNR-T1 (light blue color); C. DNR-T2 (yellow color); and D. DNR-T4 (sienna color). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

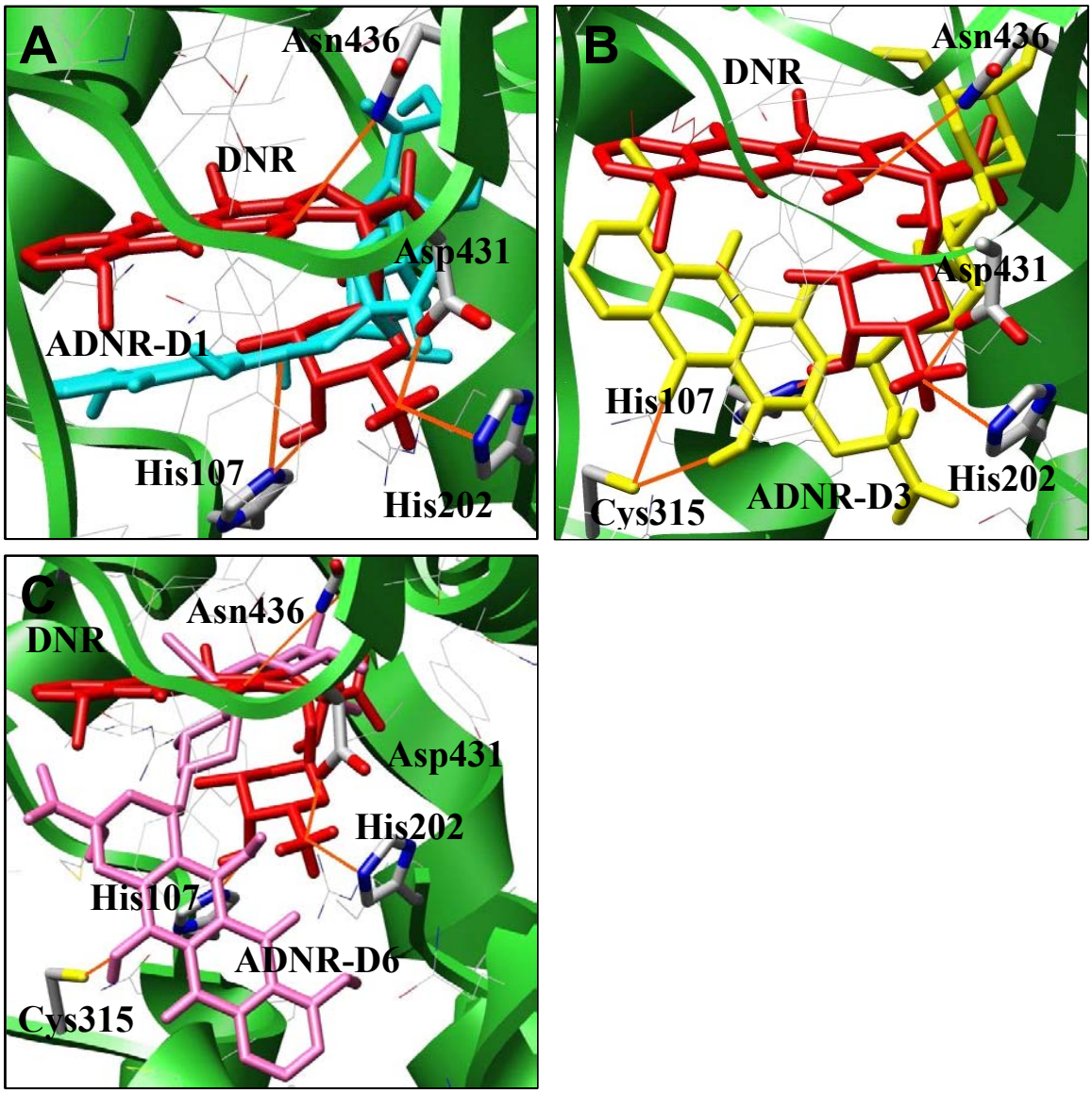

Figure 6

ADNR disaccharide analogs (non-P-gp substrates) bound to MsbA as compared to the docked structure of DNR (red color). A. ADNR-1 (light blue color); B. ADNR-3 (yellow color); C. ADNR-6 (purple color). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

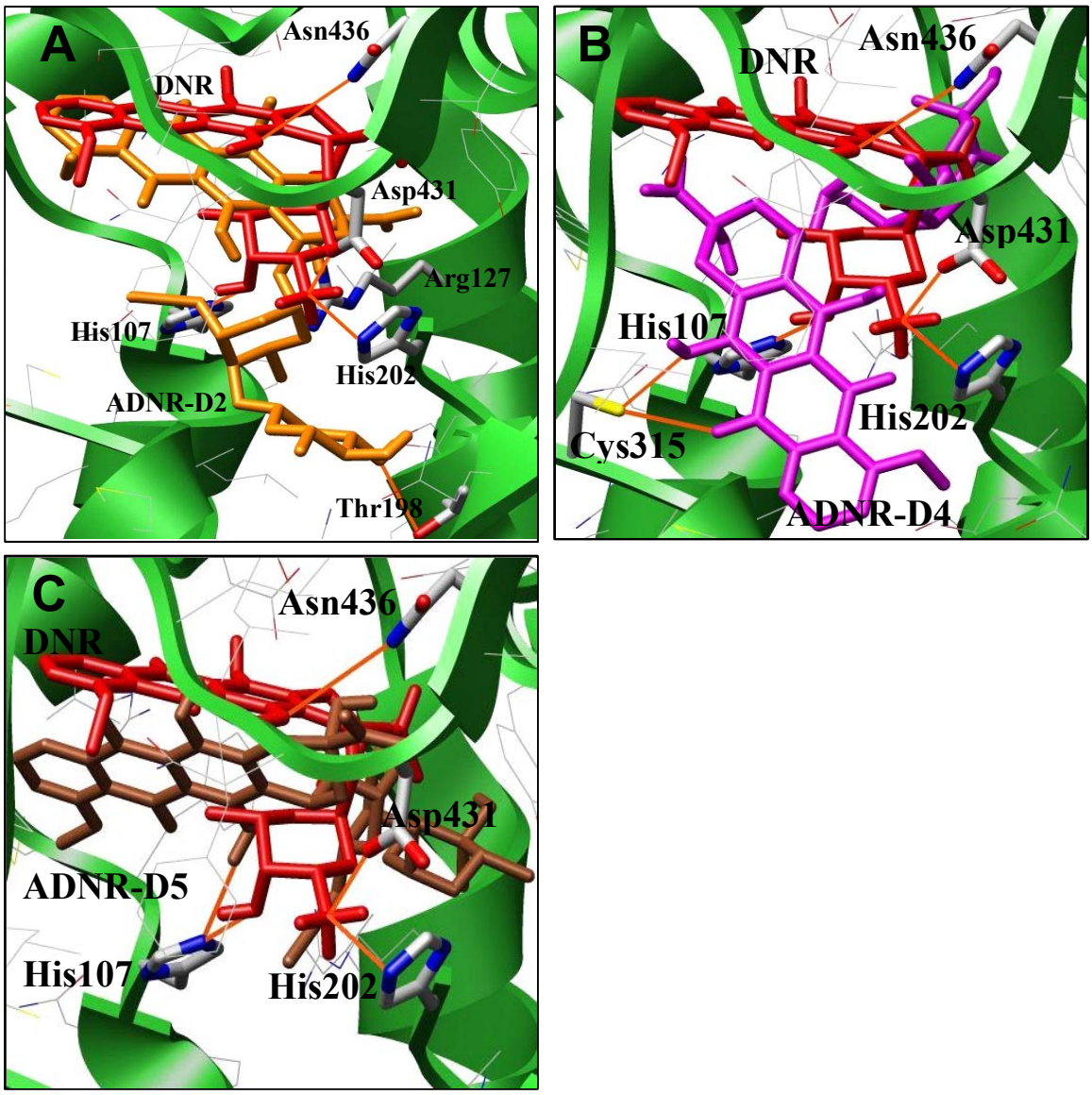

Figure 7

ADNR disaccharide analogs (P-gp substrates) bind to MsbA as compared to the docked structure of DNR (red color). A. ADNR-2 (gold color); B. ADNR-4 (purple color); C. ADNR-5 (brown color). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

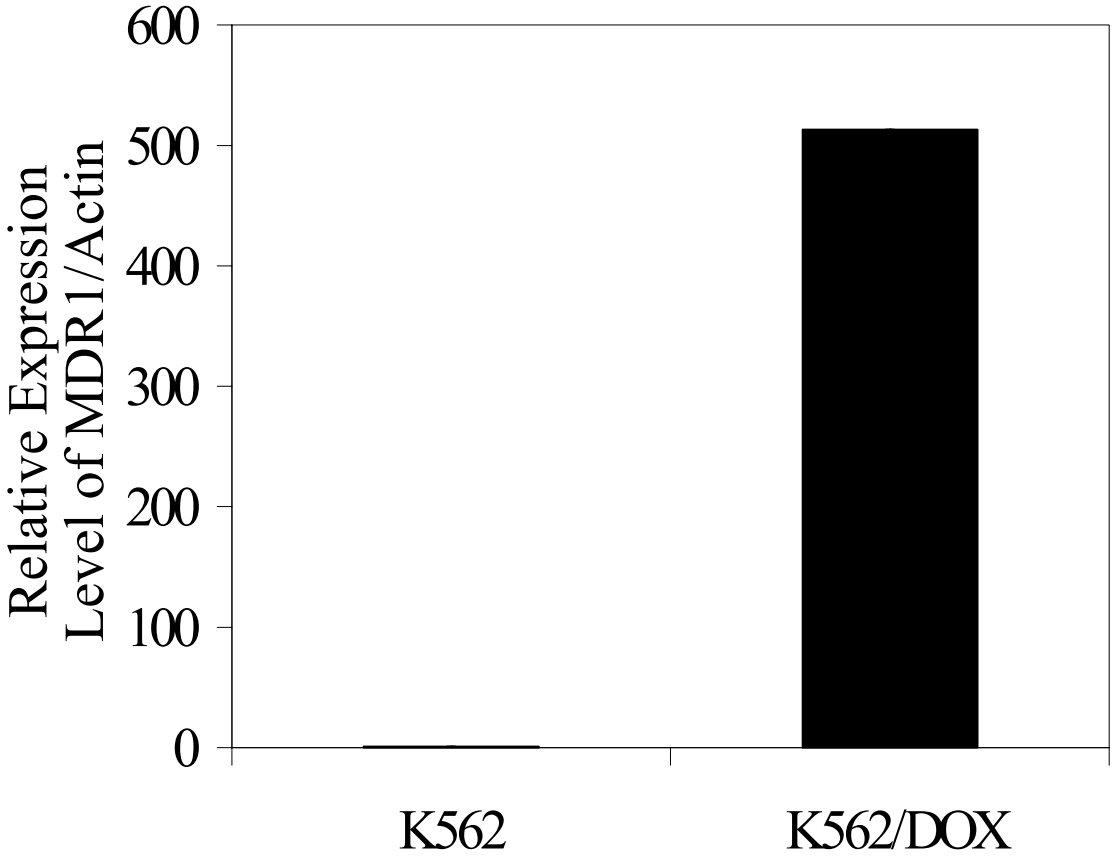

Figure 8
Relative expression levels of MDR-1 mRNA in K562 and K562/DOX as normalized by the expression of beta-actin.

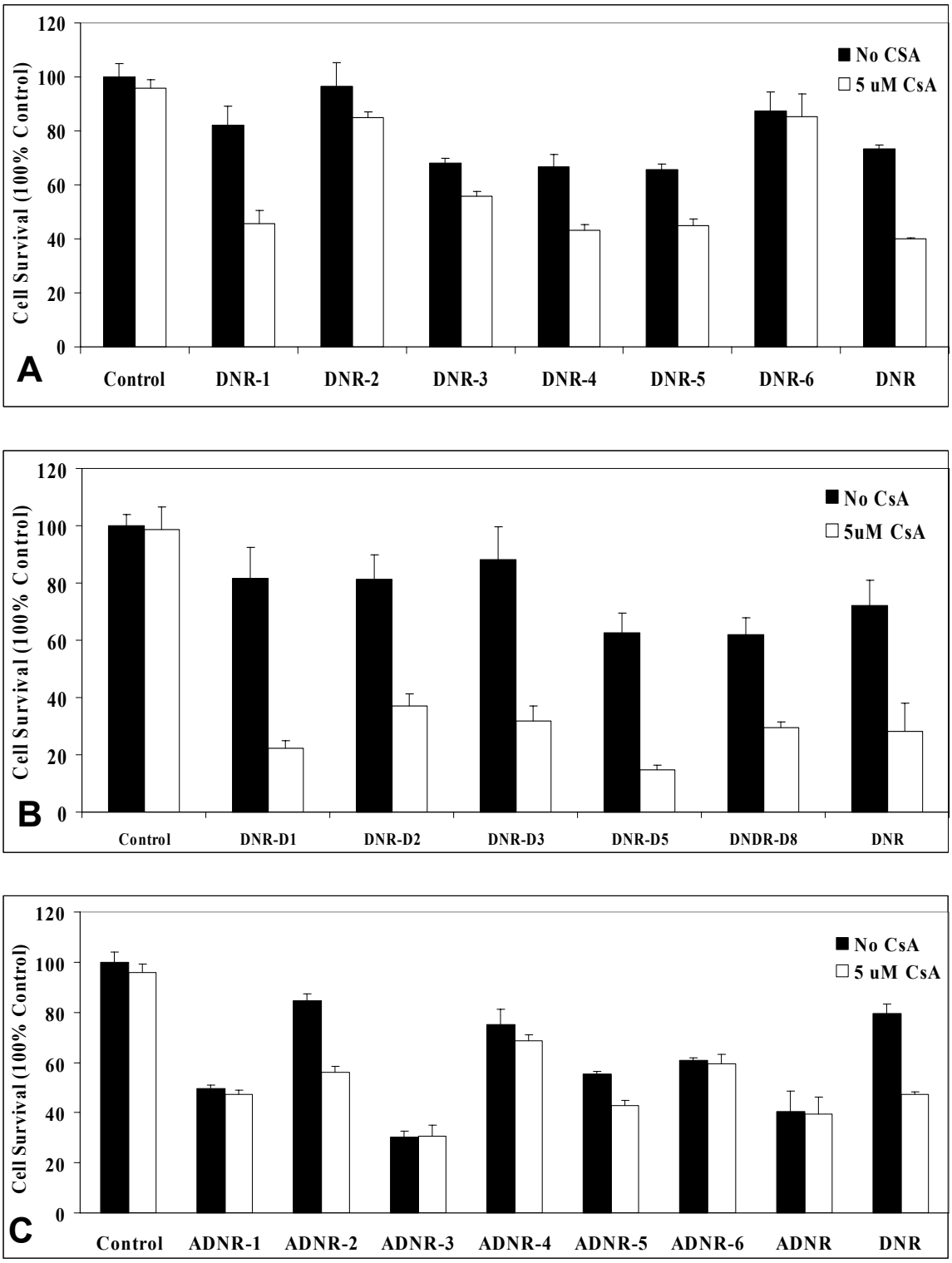

Figure 9

P-gp inhibition assay by P-gp inhibitor (cyclosporine, CsA). 1 uM concentrations of the daunorubicin analogs were used in the presence (black bar) or absence (blank bar) of 5 uM concentration of CsA. A. DNR monosaccharide analogs; B. DNR disaccharide analogs; and C. ADNR and ADNR disaccharide analogs.

Table 1
Associated Binding Energies of Each Compound Binding to MsbA
Energies Determined by Autodock

Compound	**MsbA Associated Binding Energy**
DNR	-7.92 kcal/mol
DNR-1	-9.05 kcal/mol
DNR-2	-8.80 kcal/mol
DNR-3	-9.20 kcal/mol
DNR-4	-8.13 kcal/mol
DNR-5	-8.16 kcal/mol
DNR-6	-7.50 kcal/mol
DNR-D1	-11.0 kcal/mol
DNR-D2	-7.8 kcal/mol
DNR-D3	-11.2 kcal/mol
DNR-D4	-8.61 kcal/mol
DNR-D5	-11.1 kcal/mol
DNR-D6	-11.0 kcal/mol
DNR-D7	-10.3 kcal/mol
DNR-D8	-10.9 kcal/mol
ADNR	+2.94 kcal/mol
ADNR-T1	+3.35 kcal/mol
ADNR-T2	+18.78 kcal/mol
ADNR-T3	+18.64 kcal/mol
ADNR-T4	+18.76 kcal/mol
ADNR-1	+7.46 kcal/mol
ADNR-2	-8.92 kcal/mol
ADNR-3	+2.38 kcal/mol
ADNR-4	-7.51 kcal/mol
ADNR-5	-3.22 kcal/mol
ADNR-6	+17.25 kcal/mol

Table 2. IC_{50} and drug resistance index (DRI) of 25 daunorubicin analogs in drug-sensitive (K562) and drug-resistant leukemia cells (K562/Dox).

Compound	**IC_{50} in K562 (nM)**	**IC_{50} in K562/Dox (μM)**	**Drug Resistance Index (DRI)**
DNR	16	>5	>320
DNR-1	265	>5	>19
DNR-2	>5000	>5	N/A
DNR-3	>5000	>5	N/A
DNR-4	104	>5	>48
DNR-5	350	4	12
DNR-6	>5000	>5	N/A
DNR-D1	40	1.4	34
DNR-D2	46	3.2	69
DNR-D3	44	2.3	51
DNR-D4	>5000	>5	N/A
DNR-D5	21	1.1	52
DNR-D6	>5000	>5	N/A
DNR-D7	>5000	>5	N/A
DNR-D8	31	2.4	79
ADNR	75	0.37	5
ADNR-T1	>5000	>5	N/A
ADNR-T2	>5000	>5	N/A
ADNR-T3	>5000	>5	N/A
ADNR-T4	>5000	>5	N/A
ADNR-1	788	1.9	2
ADNR-2	287	>5	>17
ADNR-3	278	0.28	1
ADNR-4	225	>5	>22
ADNR-5	448	1.6	4
ADNR-6	650	1.4	2

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