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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 overexpression. P-gp inhibition assay indeed confirms that these appropriately modified compounds avert P-gp binding and thus overcome P-gp-mediated drug resistance.

**Keywords:** Daunorubicin; multidrug resistance; P-gp; molecular docking; MsbA

## 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

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(1) Simon, S. M.; Schindler, M. Cell biological mechanisms of multidrug resistance in tumors. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91* (9), 3497−504.



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of biological pathways. Drug efflux, drug inactivation, and prosurvival (antiapoptotic) 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 antineoplastic 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 gene family.

Efflux-mediated MDR is generally characterized by an overexpression 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 bilayer, 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, and 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 overexpress 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-resistant 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 six α-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


(2) Longley, D. B.; Johnston, P. G. Molecular mechanisms of drug resistance. *J. Pathol.* **2005**, *205* (2), 275−92.

(3) Fojo, T.; Bates, S. *Strategies for reversing drug resistance. Oncogene* **2003**, *22* (47), 7512−23.

(4) Dean, M.; Hamon, Y.; Chimini, G. The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid Res.* **2001**, *42* (7), 1007−17.

(5) Gottesman, M. M.; Fojo, T.; Bates, S. E. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat. Rev. Cancer* **2002**, *2* (1), 48−58.

(6) Leslie, E. M.; Deeley, R. G.; Cole, S. P. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* **2005**, *204* (3), 216−37.

(7) Jones, P. M.; George, A. M. Symmetry and structure in P-glycoprotein and ABC transporters what goes around comes around. *Eur. J. Biochem.* **2000**, *267* (17), 5298−305.

(8) Rothnie, A.; et al. The topography of transmembrane segment six is altered during the catalytic cycle of P-glycoprotein. *J. Biol. Chem.* **2004**, *279* (33), 34913−21.

(9) Higgins, C. F.; et al. Structure of the multidrug resistance P-glycoprotein. *Semin. Cancer Biol.* **1997**, *8* (3), 135−42.

(10) Reyes, C. L.; Chang, G. Structure of the ABC transporter MsbA in complex with ADP.vanadate and lipopolysaccharide. *Science* **2005**, *308* (5724), 1028−31.

(11) Chang, G. Structure of MsbA from Vibrio cholera: a multidrug resistance ABC transporter homolog in a closed conformation. *J. Mol. Biol.* **2003**, *330* (2), 419−30.

(12) Chang, G.; Roth, C. B. *Structure of MsbA from E. coli: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science* **2001**, *293* (5536), 1793−800.

(13) Reyes, C. L.; et al. The structures of MsbA: Insight into ABC transporter-mediated multidrug efflux. *FEBS Lett* **2006**, *580* (4), 1042−8.

(14) Peer, M.; et al. Photoaffinity labeling of P-glycoprotein. *Mini-Rev. Med. Chem.* **2005**, *5* (2), 165−72.

(15) Zunino, F.; Capranico, G. DNA topoisomerase II as the primary target of anti-tumor anthracyclines. *Anti-Cancer Drug Des.* **1990**, *5* (4), 307−17.

(16) Wang, J. C. DNA topoisomerases. *Annu. Rev. Biochem.* **1996**, *65*, 635−92.

(17) Gewirtz, D. A. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem. Pharmacol.* **1999**, *57* (7), 727−41.

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 their 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 analogues with sugar modifications as a chemical biology tool. The molecular docking was used to identify the binding configuration of new analogues to MsbA, a homologue 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.

## Materials and Methods

**Synthesis and Structures of 25 Anthracycline Analogues with Various Sugar Modification.** We have previously synthesized 25 daunorubicin (Figure 1) analogues with sugar modifications.[22,23,26,27] The structures of these compounds are listed in Figure 2. To study the uncommon sugar function in anthracyclines, four different groups of anthracyclines with various uncommon sugars as monosaccharides or disaccharides were synthesized (Figure 2).

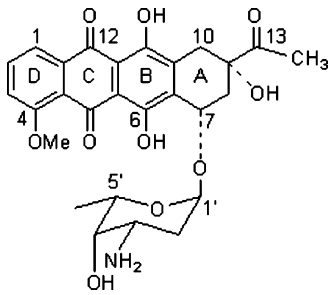


***Figure 1.*** Structure and atom labeling of the parent compound, daunorubicin.

The first group of daunorubicin monosaccharides (compounds DNR-1−DNR-6) were stereoselectively synthesized using TTBP/$AgPF_6$ glycosylation. The second group of daunorubicin analogues were synthesized with disaccharides (compounds DNR-D1−DNR-D8). Based on the molecular docking results as discussed below, we synthesized the third group of daunorubicin analogues with azido sugar or triazole sugars (compounds ADNR, ADNR-T1−ADNR-T4). The fourth group of daunorubicin analogues was an attempt to optimize the lead compound (ADNR). (Figure 2).

**Molecular Docking of Daunorubicin Analogues to MsbA.** Since no crystal structure of mammalian P-gp is available, the crystal structure of MsbA with 3.8 Å resolution, an orthologue 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 nonpolar 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 50°, 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.

**$IC_{50}$ Determination of Daunorubicin Analogues in Drug-Sensitive (K562) and Drug-Resistant 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

(18) Zunino, F.; Pratesi, G.; Perego, P. Role of the sugar moiety in the pharmacological activity of anthracyclines: development of a novel series of disaccharide analogs. *Biochem. Pharmacol.* **2001**, *61* (8), 933−8.

(19) Arcamone, F.; et al. Configurational requirements of the sugar moiety for the pharmacological activity of anthracycline disaccharides. *Biochem. Pharmacol.* **1999**, *57* (10), 1133−9.

(20) Raub, T. J. P-glycoprotein recognition of substrates and circumvention through rational drug design. *Mol. Pharm.* **2006**, *3* (1), 3−25.

(21) Kingma, P. S.; Osheroff, N. Topoisomerase II-mediated DNA cleavage and religation in the absence of base pairing. Abasic lesions as a tool to dissect enzyme mechanism. *J. Biol. Chem.* **1998**, *273* (29), 17999−8002.

(22) Fang, L.; et al. Discovery of a daunorubicin analogue that exhibits potent antitumor activity and overcomes P-gp-mediated drug resistance. *J. Med. Chem.* **2006**, *49* (3), 932−41.

(23) Zhang, G.; et al. Syntheses and biological activities of disaccharide daunorubicins. *J. Med. Chem.* **2005**, *48* (16), 5269−78.

(24) Hortobagyi, G. N. Anthracyclines in the treatment of cancer. An overview. *Drugs* **1997**, *54* (Suppl. 4), 1−7.

(25) Yang, D.; Wang, A. H. *Structure by NMR of antitumor drugs aclacinomycin A and B complexed to d(CGTACG). Biochemistry* **1994**, *33* (21), 6595−604.

(26) Zhu, L.; et al. Syntheses and biological activities of daunorubicin analogs with uncommon sugars. *Bioorg. Med. Chem.* **2005**, *13* (23), 6381−7.

(27) Zhang, G.; et al. Syntheses and biological activities of 3′-azido disaccharide analogues of daunorubicin against drug-resistant leukemia. *J. Med. Chem.* **2006**, *49* (5), 1792−9.

(28) Dong, J.; Yang, G.; McHaourab, H. S. *Structural basis of energy transduction in the transport cycle of MsbA. Science* **2005**, *308* (5724), 1023−8.

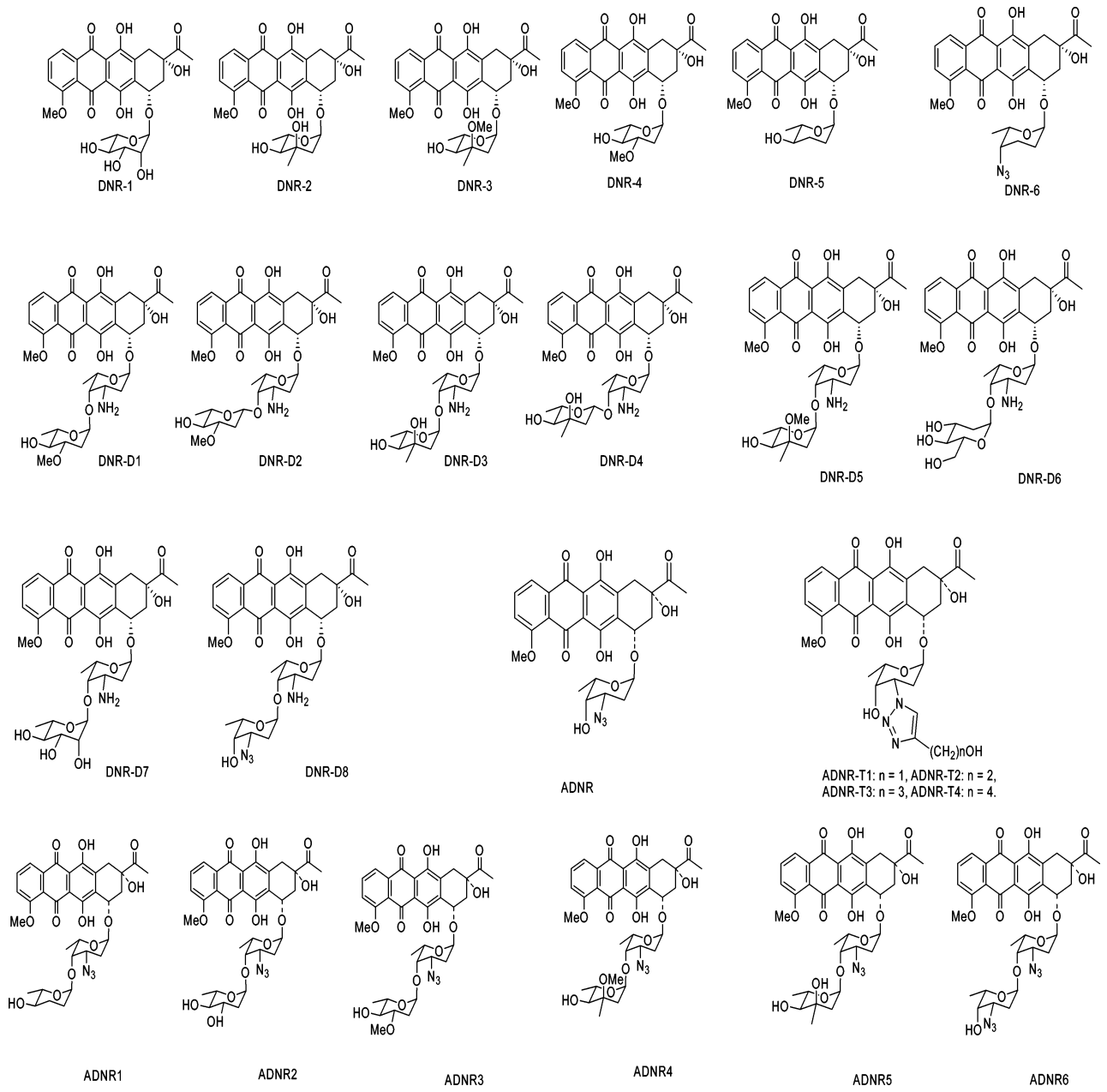


***Figure 2.*** Structures of the 25 daunorubicin analogues with various sugar modifications.

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 $\mu$M doxorubicin. Before each experiment, K562/Dox cells were stimulated with 0.1 $\mu$M doxorubicin at least for 1 week and then cultured for 10 days without doxorubicin stimulation. It was assured that the P-gp expression level was similar in every experiment. The cells were cultured in a 37 °C humidified incubator with an atmosphere of 5% $CO_2$.

A total of 2000−5000 cells (K562 and K562/Dox) were seeded in 96-well plates and incubated for 24 h. Synthetic compounds were added in a series of dilutions (0.1 nM to 10 $\mu$M) and incubated for 3 days. After 3 days, MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-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 h 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 Analogues.** A simple and rapid high throughput P-gp

inhibition assay was used to validate P-gp substrates. Coincubation of P-gp inhibitor (cyclosporin A, CsA) blocks P-gp exporter function, increases intracellular concentration of daunorubicin analogues, 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 analogues 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 h. The cells were pretreated with 5 $\mu$M cyclosporin A (CsA) for 10 min. Then the synthesized compounds (1 $\mu$M) were added. After 72 h, tetrazolium [3-(4,5-dimethythiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS, 2 mg/mL), and phenazine methosulfate (PMS, 25 $\mu$M) were mixed and added directly to the cells. After incubationincubated for 3 h 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. (A) 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 $\mu$L of DNase/RNase-free water, and the concentration was measured at 260 nm UV. First-strand cDNA was transcribed from 3 $\mu$g of total RNA using 50 ng of random hexamers, and SS II reverse transcriptase was used for reverse transcription at 42 °C for 50 min. After termination of the reaction, 1 $\mu$L of RNase H was added to the tube and incubated for 20 min at 37 °C before proceeding to amplification of the target cDNA.

**(B) 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 s, primer annealing and extension at 60 °C for 1 min. 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.

## Results

**Molecular Docking of Daunorubicin Analogues 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 analogues with series modifications on the sugar moiety. However, no crystal structure of mammalian P-gp was available. Fortunately, a distant orthologue (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-resistant proteins and shares common substrate specificity to confer drug resistance in bacteria. Therefore, molecular docking (Autodock version 3.0.7) was performed for daunorubicin analogues 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.

***Table 1.*** Associated Binding Energies of Each Compound Binding to MsbA[a]

| compd | MsbA associated binding energy (kcal mol) | compd | MsbA associated binding energy (kcal mol) |
|---|---|---|---|
| DNR | −7.92 | DNR-D7 | −10.3 |
| DNR-1 | −9.05 | DNR-D8 | −10.9 |
| DNR-2 | −8.80 | ADNR | +2.94 |
| DNR-3 | −9.20 | ADNR-T1 | +3.35 |
| DNR-4 | −8.13 | ADNR-T2 | +18.78 |
| DNR-5 | −8.16 | ADNR-T3 | +18.64 |
| DNR-6 | −7.50 | ADNR-T4 | +18.76 |
| DNR-D1 | −11.0 | ADNR-1 | +7.46 |
| DNR-D2 | −7.8 | ADNR-2 | −8.92 |
| DNR-D3 | −11.2 | ADNR-3 | +2.38 |
| DNR-D4 | −8.61 | ADNR-4 | −7.51 |
| DNR-D5 | −11.1 | ADNR-5 | −3.22 |
| DNR-D6 | −11.0 | ADNR-6 | +17.25 |

[a] Energies determined by Autodock.

**(A) 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 (Figure 3A). 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 a hydrogen bond with His107 in the ICD1 domain, while C′-3-NH2 interacted with His202 in the ICD2 domain and Asp431 in the NBD α2 domain of MsbA (Figure 3B). 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-resistant protein (Figure 3).

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

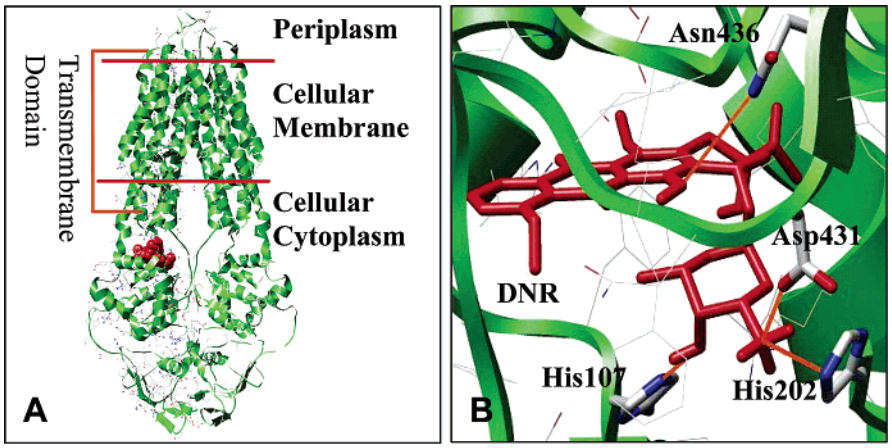


***Figure 3.*** Daunorubicin (DNR) bound to MsbA. DNR binds to the cavity between the intracellular domain (ICD) and the nucleotide binding domain (NBD) of MsbA. Hydrogen bonds between DNR and MsbA are represented with orange lines. DNR is labeled in red. Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers.

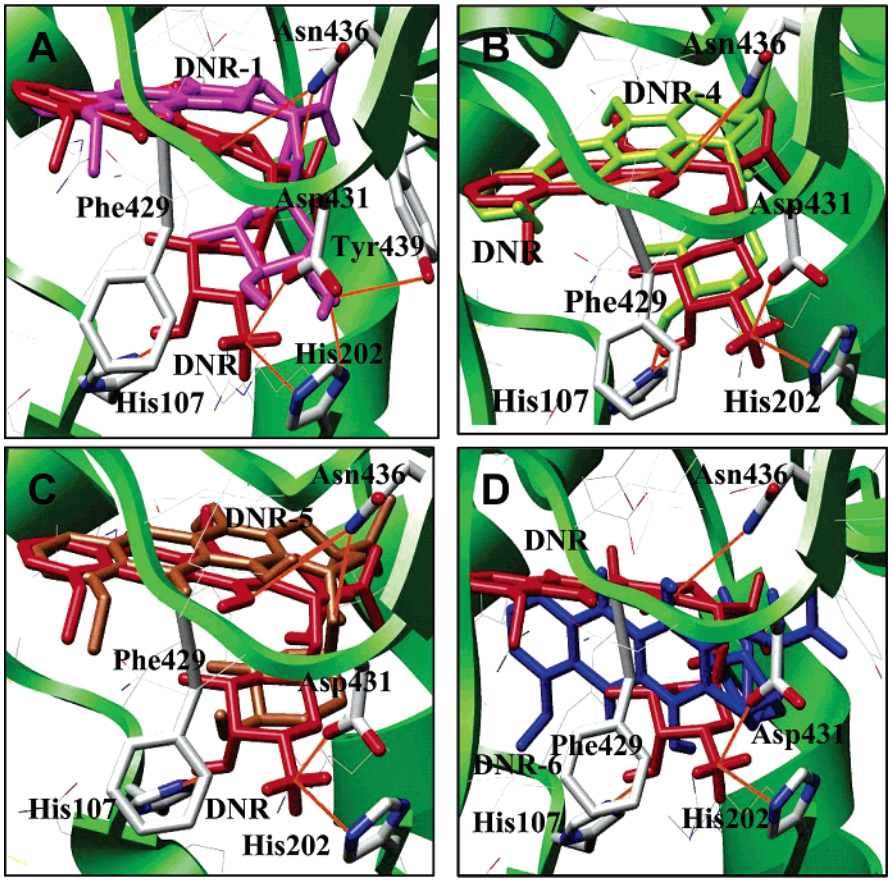


***Figure 4.*** DNR monosaccharide analogues bound to MsbA as compared to the docked structure of DNR (red): (A) DNR-1 (purple); (B) DNR-4 (green); (C) DNR-5 (sienna); and (D) DNR-6 (blue). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines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

tertiary structure of each monomer between the intracellular domain and nucleotide binding domain. The docking results showed that daunorubicin binds to this cavity (Figure 3B). 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.

**(B) DNR Monosaccharide Analogues.** To vary the structure of daunorubicin to overcome P-gp, we synthesized the DNR monosaccharide analogues 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

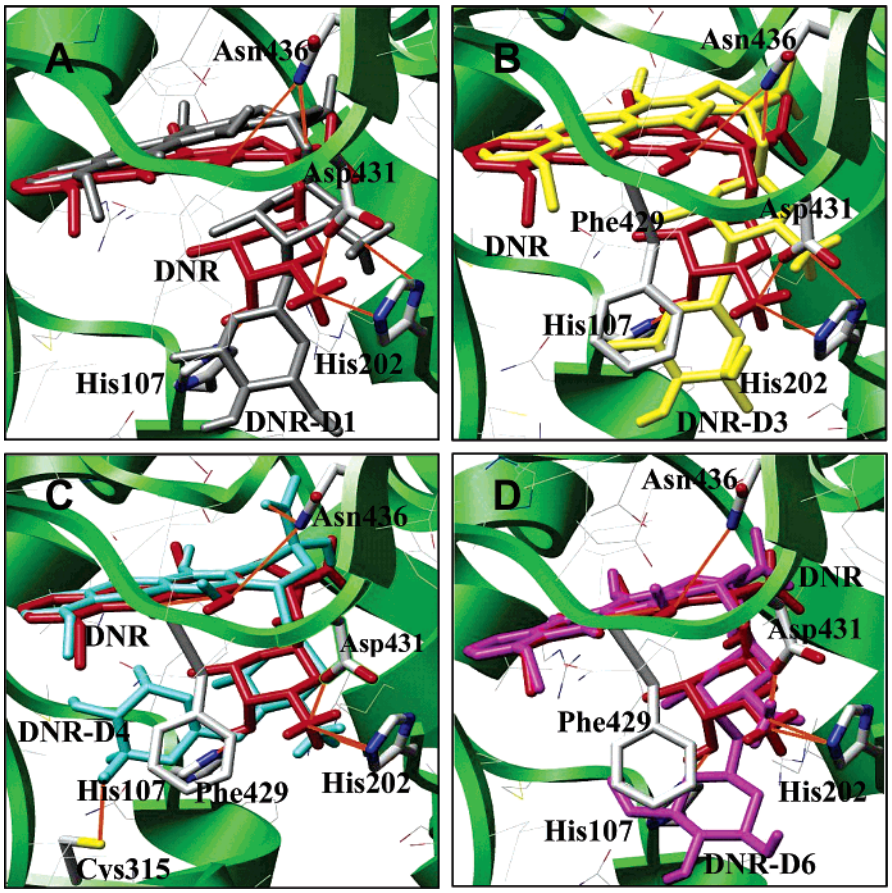


***Figure 5.*** DNR disaccharide analogues bound to MsbA as compared to the docked structure of DNR (red): (A) DNR-D1 (silver); (B) DNR-D3 (yellow); (C) DNR-D4 (light blue); and (D) DNR-D6 (purple). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

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 (Figure 4).

The binding conformation of DNR-1 to DNR-4 was similar to that of daunorubicin (Figure 4A,B). 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 $\beta$4 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 the C′-3 or C′-4 position may be critical to determine the binding of daunorubicin analogues 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 (Figure 4C). 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 $\beta$4 and α2 regions of the NBD domain of MsbA.

Furthermore, we replaced all polar groups 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 a 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 $\beta$4 and α2 regions of the NBD domain of MsbA (Figure 4D).

These results suggest that both the tetracyclic ring and the 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 analogues to fit into the binding cavity of MsbA (Figure 4).

**(C) DNR Disaccharide Analogues.** In order to introduce more structure variability in the DNR analogues, we synthesized eight daunorubicin analogues 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 α and $\beta$ glycosidic

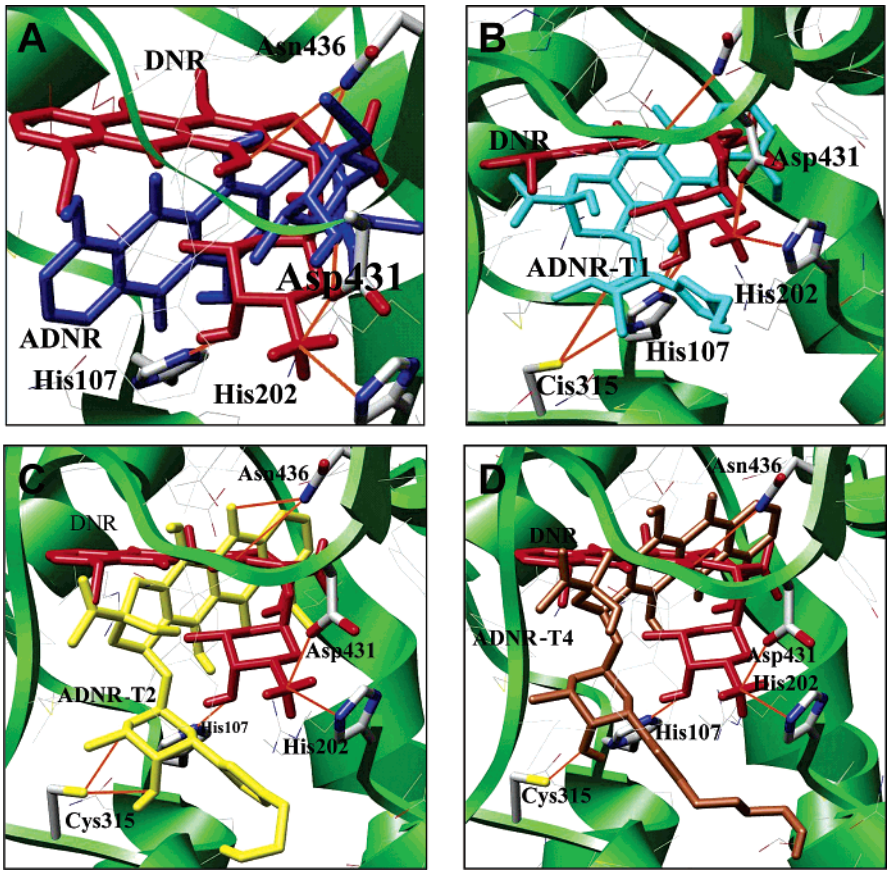


***Figure 6.*** ADNR and ADNR-T1 bound to MsbA as compared to the docked structure of DNR (red): (A) ADNR (dark blue); (B) DNR-T1 (light blue); (C) DNR-T2 (yellow); and (D) DNR-T4 (sienna). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

linkages were synthesized (Figure 2). Surprisingly, all eight analogues favorably bound to MsbA in a similar configuration as daunorubicin (Figure 5), and with relatively similar binding energy (ranging from −7.8 kcal/mol to −11.2 kcal/mol).

Each DNR disaccharide analogue 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 (Figure 5A,B,C,D). 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 (Figure 5C).

These results strongly suggest that the first sugar moiety of daunorubicin analogues 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 (Figure 5).

**(D) ADNR Analogues.** Based on the information of the above docking results, it seems that several aspects are important for daunorubicin analogues 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 analogues determined by the tetracyclic rings and sugar structure.

The MsbA binding conformation of DNR-6, which contained the C′-4-N3 group, was significantly altered from that of DNR. In addition, the C′-3-NH2 group has been reported to be critical for P-gp binding.[14,29] Therefore, we hypothesize that replacement of the C′-3-NH2 group with an azido group or large triazole group may be able to change 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 (Figure 6A). These data suggest that ADNR may not

(29) Priebe, W.; Perez-Soler, R. Design and tumor targeting of anthracyclines able to overcome multidrug resistance: a double-advantage approach. *Pharmacol. Ther.* **1993**, *60* (2), 215−34.

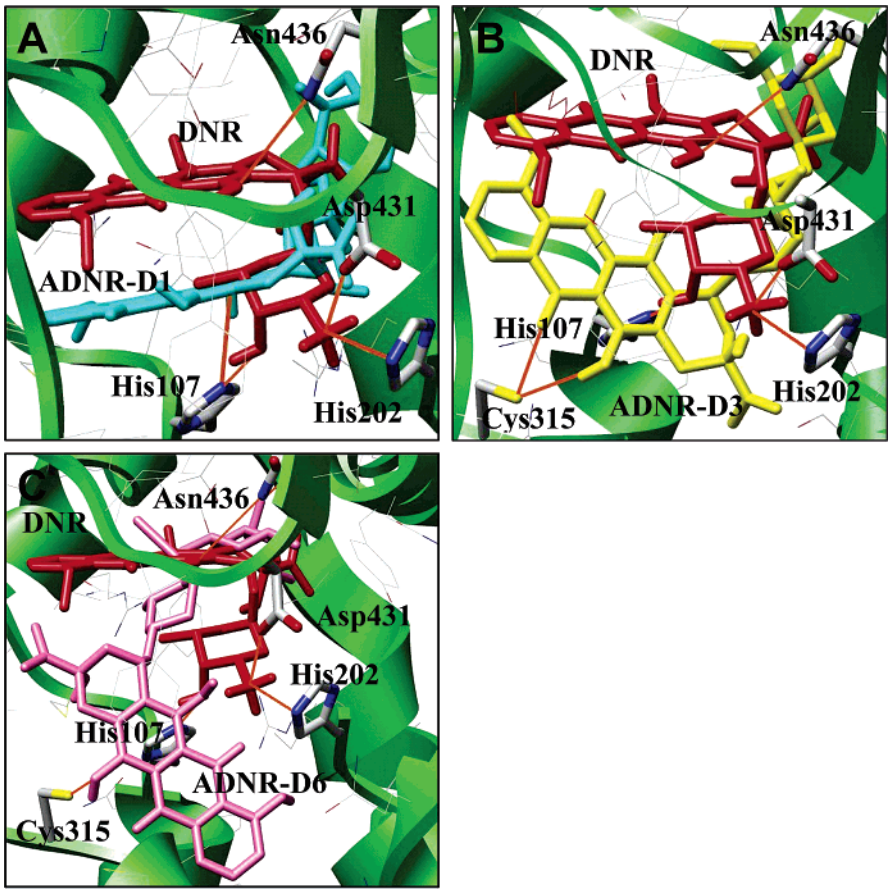


***Figure 7.*** ADNR disaccharide analogues (non-P-gp substrates) bound to MsbA as compared to the docked structure of DNR (red): (A) ADNR-1 (light blue); (B) ADNR-3 (yellow); (C) ADNR-6 (purple). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

favorably bind to multidrug-resistant protein and may overcome P-gp-mediated drug resistance. Indeed, these findings were confirmed by experimental data as shown below.

Similarly, the DNR triazole analogues (ADNR-T1 to ADNR-T4) also unfavorably bind to MsbA with binding energy of +3.35 to +18.78 kcal/mol (Figure 6B,C,D). Similar to ADNR, ADNR-T1 to ADNR-T4 significantly changed the binding conformation to MsbA and abolished the hydrogen bonds between C′-3 group and MsbA. The only existing hydrogen bond is between the C′-4 hydroxyl group and Cys315 of MsbA.

These results indicate that replacement of the C′-3-NH2 amino group with an azido or triazole group sufficiently averts P-gp binding and overcomes drug resistance. Although the tetracyclic rings of azido sugar analogues and triazole analogues remain the same as DNR, it appears that an azido or triazole group not only abolishes the hydrogen bond between the sugar moiety and MsbA but also significantly changes the conformation of the whole structure of DNR. These two important changes are critical to avert the binding to MsbA **(**Figure 6).

**(E) ADNR Disaccharide Analogues.** To further optimize the lead compound ADNR, we synthesized ADNR disaccharide analogues (ADNR-1 to ADNR-6) with a second sugar linked to the first azido sugar via a $\beta$-linkage. This was to study the contribution of the second sugar for MsbA binding and anticancer activity.

Although all six analogues were derived from ADNR with an azido sugar, not all six analogues 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 (Figure 7). 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 (Figure 8). The introduction of a second sugar has generated more hydrogen bonding between these analogues and also changed the overall binding conformation.

In fact, the sugar structures 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 (Figure 7).

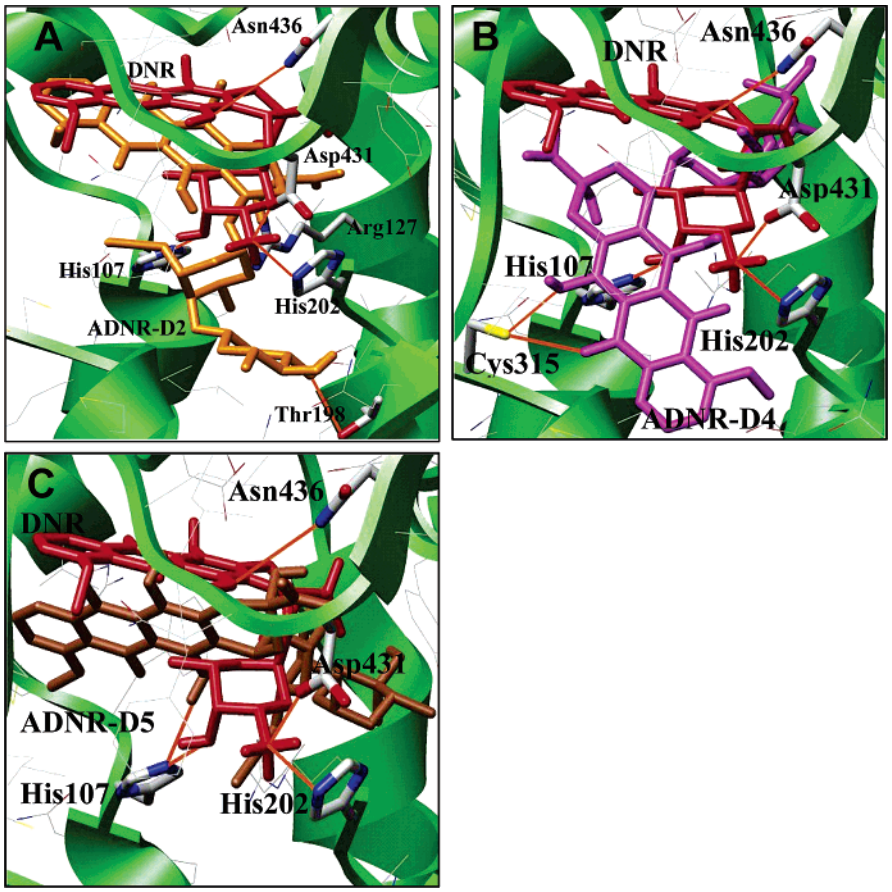


***Figure 8.*** ADNR disaccharide analogues (P-gp substrates) bound to MsbA as compared to the docked structure of DNR (red): (A) ADNR-2 (orange); (B) ADNR-4 (purple); (C) ADNR-5 (brown). Green ribbon represents MsbA protein. Critical amino acids in MsbA are labeled with numbers. The thin orange lines represent hydrogen bonds.

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 a second sugar in ADNR-2 reconfigured the compound in such a way that the C″-4-OH group of the second sugar interacted with Thr198 in the 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 there are 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 (Figure 8).

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 the 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 a second sugar sometimes introduces a new hydrogen bond or changes 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) (Figures 7 and 8).

**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

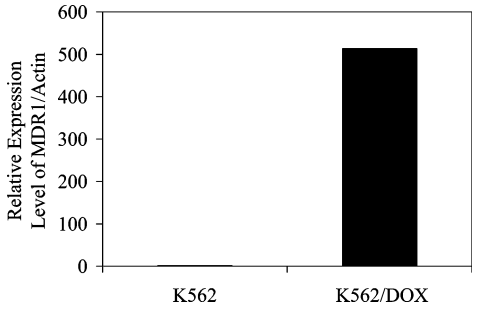


***Figure 9.*** Relative expression levels of MDR-1 mRNA in K562 and K562/DOX as normalized by the expression of $\beta$-actin.

used to test the drug cytotoxicity and drug resistance of our daunorubicin analogues **(**Figure 9).

**Cytotoxicity and Drug Resistance of Daunorubicin Analogues in Drug-Sensitive and Drug-Resistant Leukemia.** MTS assays were utilized to assess 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 analogues were determined by WinNonlin 4.1. The drug resistance index (DRI) was calculated by the ratio of $IC_{50}$ in the drug-resistant K562 vs $IC_{50}$ in the drug-sensitive index. These $IC_{50}$ are summarized in Table 1.

Although daunorubicin showed very sensitive cell killing effects in K562 cells with $IC_{50}$ 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.

**(A) DNR Monosaccharide Analogues.** The MTS results showed that all the DNR monosaccharide analogues (DNR-1 to DNR-6) exhibit very high $IC_{50}$ 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 analogues 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) cannot be calculated for the confirmation of the P-gp substrate.

**(B) DNR Disaccharide Analogues.** 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 depend 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.

**(C) DNR Azido Sugar (ADNR) and Triazole Sugar (ADNR-T1 to ADNR-T4) Analogues.** Very interestingly, ADNR with azido sugar significantly reduced the $IC_{50}$ against drug-resistant cells compared to DNR. The drug resistance index (DRI) significantly reduced to less than 13. Together with the molecular docking results that ADNR may avert binding to MsbA, these data may suggest that ADNR overcomes P-gp-mediated drug resistance in cancer cells. In contrast, although ADNR-T1 to T4 also averts 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 ADNR-T4.

**(D) ADNR Disaccharide Analogues.** 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 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.

**(E) Molecular Docking Energy Can Qualitatively Distinguish the P-gp Substrate.** By analyzing the molecular docking energy of these daunorubicin analogues 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) of our 25 compounds. Each favorably docking compound (with binding energy less than 0) shows a DRI of 12 or more (or is inactive), with the one exception of ADNR-5, 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 analogues avert P-gp binding and overcome drug resistance. If a daunorubicin analogue 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,

***Table 2.*** $IC_{50}$ and Drug Resistance Index (DRI) of 25 Daunorubicin Analogues in Drug-Sensitive (K562) and Drug-Resistant Leukemia Cells (K562/Dox)

| | $IC_{50}$ | | | | $IC_{50}$ | | |
|---|---|---|---|---|---|---|---|
| compd | in K562 (nM) | in K562/Dox ($\mu$M) | DRI | compd | in K562 (nM) | in K562/Dox ($\mu$M) | DRI |
| DNR | 16 | >5 | >320 | DNR-D7 | >5000 | >5 | n/a |
| DNR-1 | 265 | >5 | >19 | DNR-D8 | 31 | 2.4 | 79 |
| DNR-2 | >5000 | >5 | n/a[a] | ADNR | 75 | 0.37 | 5 |
| DNR-3 | >5000 | >5 | n/a | ADNR-T1 | >5000 | >5 | n/a |
| DNR-4 | 104 | >5 | >48 | ADNR-T2 | >5000 | >5 | n/a |
| DNR-5 | 350 | 4 | 12 | ADNR-T3 | >5000 | >5 | n/a |
| DNR-6 | >5000 | >5 | n/a | ADNR-T4 | >5000 | >5 | n/a |
| DNR-D1 | 40 | 1.4 | 34 | ADNR-1 | 788 | 1.9 | 2 |
| DNR-D2 | 46 | 3.2 | 69 | ADNR-2 | 287 | >5 | >17 |
| DNR-D3 | 44 | 2.3 | 51 | ADNR-3 | 278 | 0.28 | 1 |
| DNR-D4 | >5000 | >5 | n/a | ADNR-4 | 225 | >5 | >22 |
| DNR-D5 | 21 | 1.1 | 52 | ADNR-5 | 448 | 1.6 | 4 |
| DNR-D6 | >5000 | >5 | n/a | ADNR-6 | 650 | 1.4 | 2 |

[a] Not applicable.

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 analogues lost their anticancer activity (due to the lack of topoisomerase II poisoning), this experiment could not be used to distinguish the P-gp substrate and could only be used as a negative control.

As shown in Figure 10A, when 1 $\mu$M daunorubicin was incubated with 5 $\mu$M CsA in K562/Dox, the cell killing effects increased by 2.25-fold although CsA alone (5 $\mu$M) 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 analogues, 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 cannot 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 analogues, CsA also significantly increased the cell killing effects of DNR-D1, -D2, -D3, -D5, and -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 indicates that they may still be substrates of P-gp. These data also confirmed by the molecular docking results. Only some discrepancy was seen for ADNR-4. The molecular docking showed that 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. Cyclosporin A, a known autoimmune 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 preclinical 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

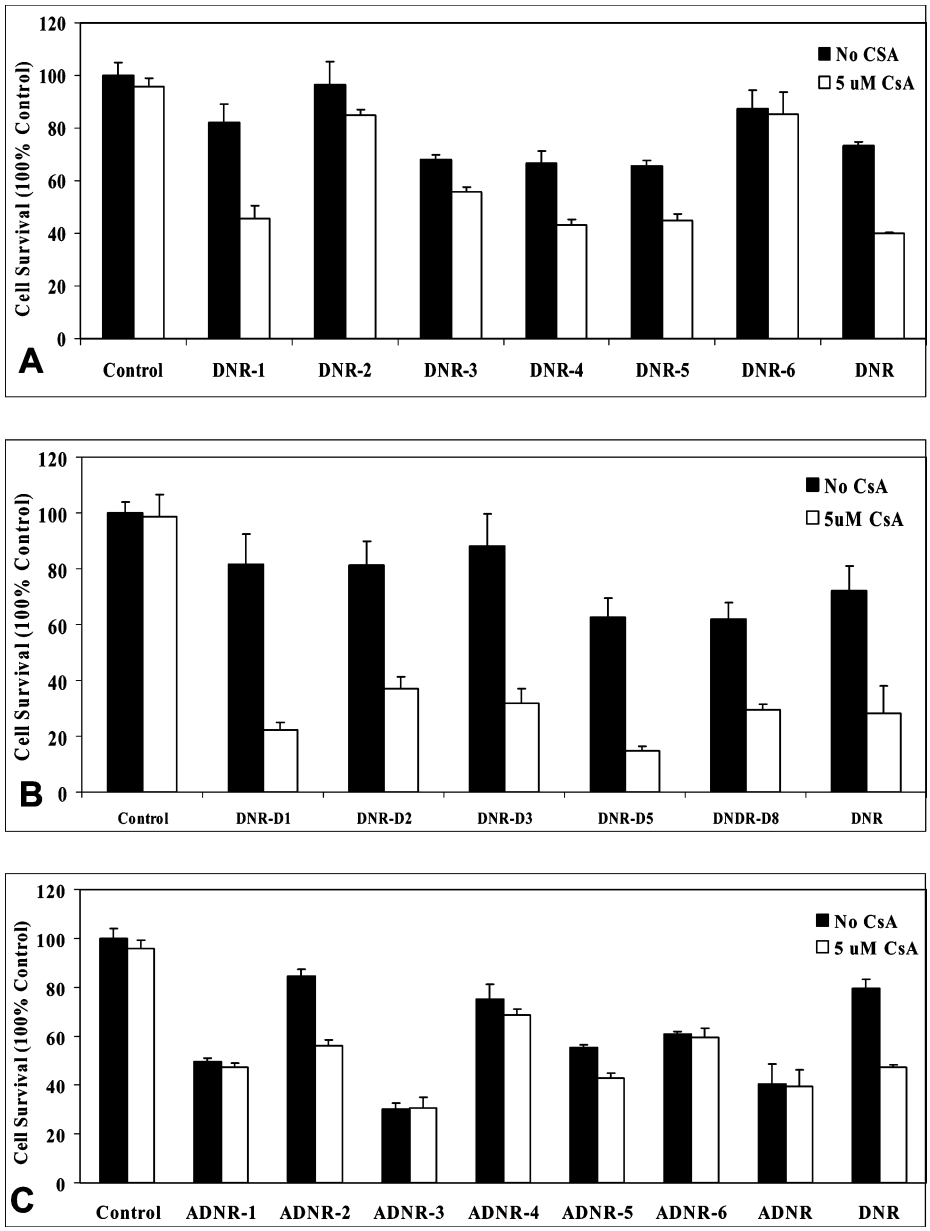


***Figure 10.*** P-gp inhibition assay by P-gp inhibitor (cyclosporine, CsA). 1 $\mu$M concentrations of the daunorubicin analogues was used in the presence (black bar) or absence (blank bar) of $\mu$M concentration of CsA: (A) DNR monosaccharide analogues; (B) DNR disaccharide analogues; and (C) ADNR and ADNR disaccharide analogues.

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 the sugar structure of anthracycline to generate a series of daunorubicin analogues. These analogues 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

(30) Tsuruo, T.; et al. Molecular targeting therapy of cancer: drug resistance, apoptosis and survival signal. *Cancer Sci.* **2003**, *94* (1), 15−21.

(31) Minotti, G.; et al. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* **2004**, *56* (2), 185−229.

(32) Lepage, P.; Gros, P. Structural and functional aspects of P-glycoproteins and related transport proteins. *Curr. Opin. Nephrol. Hypertens.* **1993**, *2* (5), 735−43.

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 analogues 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 analogues provide an excellent means for studying P-gp functions using chemical biology methods. For instance, since our molecular modeling has identified the amino acid residues in MsbA that interact with daunorubicin analogues and been 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 25 previously synthesized daunorubicin analogues 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-resistant protein and overcome drug resistance.

Molecular docking with 25 daunorubicin analogues 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 analogues 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 analogues plays a more important role for the binding of MsbA than the second sugar in the disaccharide analogues.

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 overexpression. P-gp inhibition assays indeed confirmed that these compounds averted P-gp binding and overcame P-gp-mediated drug resistance.

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(33) Lee, V. H. Membrane transporters. *Eur. J. Pharm. Sci.* **2000**, *11* (Suppl. 2), S41−50.
(34) Kaye, S. B. The multidrug resistance phenotype. *Br. J. Cancer* **1988**, *58* (6), 691−4.
(35) Hosoya, K. I.; Kim, K. J.; Lee, V. H. Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers. *Pharm. Res.* **1996**, *13* (6), 885−90.
(36) Honjo, Y.; et al. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res.* **2001**, *61* (18), 6635−9.
(37) Gottesman, M. M. How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res.* **1993**, *53* (4), 747−54.
(38) Germann, U. A. Molecular analysis of the multidrug transporter. *Cytotechnology* **1993**, *12* (1−3), 33−62.
(39) Efferth, T. The human ATP-binding cassette transporter genes: from the bench to the bedside. *Curr. Mol. Med.* **2001**, *1* (1), 45−65.
(40) Doyle, L. A.; Ross, D. D. *Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene* **2003**, *22* (47), 7340−58.