# Transmembrane Inhibitors of P-Glycoprotein, an ABC Transporter

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Drug resistance mediated by ABC transporters such as P-glycoprotein (P-gp) continues to be a major impediment to effective cancer chemotherapy. We have developed a panel of highly specific peptide inhibitors of P-gp based on the structure of the transmembrane domains of the transporter. These peptides are thought to exert their inhibitory action by disrupting the proper assembly of P-gp. A novel 96-well-plate assay based on the efflux of fluorescent P-gp substrate $DiOC_2$ (3-ethyl-2-[3-(3-ethyl-2(3*H*)-benzoxazolylidene)-1-propenyl]benzoxazolium iodide) was developed and used for structure−functional characterization of transporter inhibitors. The studies strongly suggest that potent and selective inhibitors of ABC transporters can now be developed solely on the basis of the primary structures of the target proteins. The inhibition of P-gp with transmembrane peptides was shown to be chirality-independent. A 25-residue long retroinverso D-analogue of transmembrane domain 5 inhibited the efflux of the fluorescent P-gp substrate with an $IC_{50}$ of 500 nM. Transmembrane peptides effectively sensitized resistant cancer cells to doxorubicin in vitro without demonstrating any cell toxicity of their own. The newly synthesized P-gp antagonists appear to be promising nontoxic drug resistance inhibitors that merit further development.

## Introduction

ABC transporters pump a variety of compounds from the cytoplasm and organelles in an ATP-dependent manner, thereby modulating numerous cell functions. Their role in rendering tumors resistant to chemotherapy is well-documented, and significant effort is currently devoted to the development of specific inhibitors of transporters responsible for drug resistance.[1−3] The structural core of ABC transporters consists of two membrane domains composed of six transmembrane helical segments each and two nucleotide binding domains located at the cytoplasmic surface of the membrane. Transmembrane domains (TMs) are involved in substrate binding and are believed to undergo significant structural reorganization during the pumping process.[4,5] While nucleotide binding domains of different ABC transporters show very high homology at both primary and tertiary structure levels, the transmembrane domains display significant variation in amino acid sequences and are arranged differently in the tertiary structures determined to date.[6,7] This diversity suggests that TMs harbor the most unique structural elements of the transporters, and thus, targeting those elements may result in the development of the most specific inhibitors.

We have previously shown that interactions of transmembrane domains of G-protein-coupled receptors (GPCRs) can serve as targets in the design of specific receptor antagonists.[8] Properly constructed synthetic peptide analogues of transmembrane domains of GPCRs were shown to inhibit the function of the target receptors with $IC_{50}$ in the nanomolar range. We have speculated that peptide analogues of membrane helices of ABC transporters may work in a similar way and may be excellent targets for the development of specific inhibitors. P-glycoprotein (P-gp) encoded by the MDR1 gene is a common cause of tumor resistance to chemotherapy.[9]

The synthesis of derivatives of all 12 transmembrane helices of P-gp was carried out, and their activity as inhibitors of P-gp function and their physicochemical properties were determined. Optimization of peptide sequences allowed us to obtain potent P-glycoprotein inhibitors. Extensive structure−activity studies have defined the structural requirements for an active inhibitor.

## Results

**Peptide Synthesis and Solubilization.** P-gp transmembrane peptides are generally very hydrophobic. Consequently, poor solubility and aggregation during the synthesis constitute the major obstacles in the development of transmembrane peptide inhibitors. The application of conductivity monitoring of the Fmoc deprotection together with conditional double coupling protocols on difficult steps helped to increase the yields during the synthesis in many cases. As a result, we succeeded in obtaining pure peptides for all 12 transmembrane domains of P-glycoprotein (Tables 1 and 2). In the antagonist nomenclature used throughout the paper, the first number after the transporter name refers to the transmembrane domain from which the peptide was derived and the second number corresponds to the order in which peptides were synthesized. All

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peptide identities were confirmed using high-resolution electrospray mass spectrometry. We were not able to test inhibitory properties of all peptides because of poor solubility of some in media. Addition of aspartate residues and extension of the sequences to include portions of hydrophilic sequences of the extracellular loops helped to increase the solubility of TM 5, 6, and 12 analogues, but this strategy was not sufficient in making TM 10 derivatives soluble most probably because of the presence of two hydrophobic clusters composed of seven and five aliphatic residues. TM peptides usually readily dissolve in DMSO and DMF. Addition of equimolar amounts of ammonia afforded concentrated DMF solutions of acidic peptides suitable for purification by HPLC. Peptides were purified by reverse-phase chromatography to at least 95% purity as determined by analytical HPLC. DMSO was used for preparing stock solutions of peptides for activity tests. Generally, peptides are more soluble in phosphate-buffered saline (PBS) than in cell culture media. Consequently, shorter testing protocols of fluorescent substrate efflux from the cells could be applied for a larger number of poorly soluble peptides, while precipitation of many TM analogues in cell culture media precluded us from accurately testing their ability to sensitize resistant cancer cells toward the toxic effects of doxorubicin.

**Activity Fluorescence Assay.** The initial tests of transmembrane P-gp inhibitors were conducted utilizing fluorescence-activated cell sorting (FACS) of the cells incubated with rhodamine 123 (data not shown), which indicated that even nonoptimized TM analogues had activity as inhibitors of P-gp. Similarly, laser scanning confocal microscopy of the cells incubated with rhodamine 123 revealed much higher fluorescence of the cells treated with the P-gp substrate in the presence of the peptide inhibitors (Figure 1). However, the expensive and laborious FACS analysis and microscopy techniques were inconvenient for activity characterization of many inhibitors at multiple concentrations. Precipitation of the hydrophobic peptides during the analysis procedure created additional technical difficulties. Consequently, we developed a novel fluorescence-based assay in a 96-well format to measure the ability of the new inhibitors to block the transport of fluorescent P-gp substrates. We compared four known fluorescent P-gp substrates, rhodamine 123,[10] tetramethylrosamine,[11] SYTO 16,[12] and 3-ethyl-2-[3-(3-ethyl-2(3*H*)-benzoxazolylidene)-1-propenyl]benzoxazolium iodide ($DiOC_2$),[13] to determine which one would allow for the most sensitive assay. Cyclosporin A was used as an inhibitor that completely abrogates the transport at 5 $\mu$M. NIH/3T3 cells that were stably transfected with MDR1 were used for the development of the assay. The difference in the fluorescence reading of the cells incubated with the substrate in the presence and absence of the cyclosporin A was used as a measure of the test sensitivity. The fluorescence ratio of the cells treated with the substrate in the presence of the inhibitor to fluorescence of the cells treated with the pure substrate used at 4 $\mu$M was equal to $5.0 \pm 0.2$ for SYTO 16, $4.3 \pm 0.2$ for $DiOC_2$, $2.0 \pm 0.4$ for rhodamine123, and $1.3 \pm 0.3$ for tetramethylrosamine. $DiOC_2$ was chosen over the slightly more sensitive SYTO 16 for economic reasons.

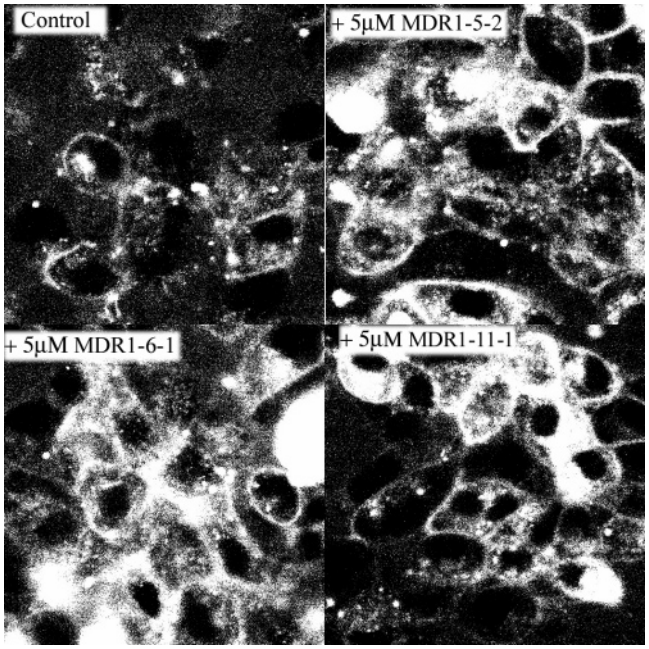


**Figure 1.** Inhibition of rhodamine 123 efflux from NIH/3T3 cells stably expressing MDR1 detected by laser scanning confocal microscopy. The cells were treated with fluorescent P-gp substrate rhodamine 123 in the absence (control) and presence of indicated TM analogues. The images correspond to rhodamine 123 fluorescence of the cells, using 488 nm wavelength excitation and 515−540 nm emission.

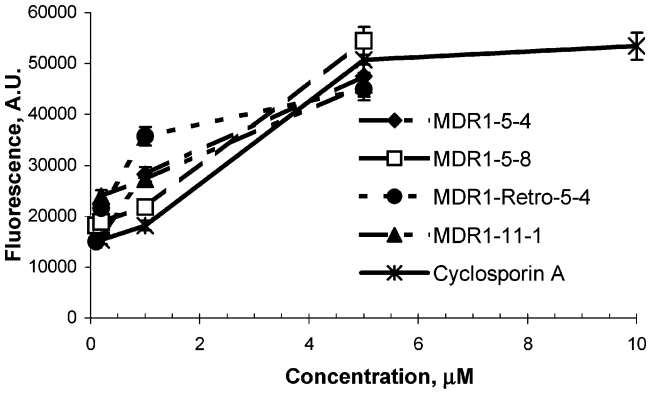


**Figure 2.** Inhibition of fluorescent P-gp substrate efflux from NIH/3T3 cells stably transfected with MDR1 by P-gp inhibitors. $DiOC_2$ was used as a substrate, and the assay was performed in 96-well filtration plates as described in Experimental Section.

All tests were conducted with cells in suspension rather than on attached cells to provide sufficient cell number for detection. The optimal cell number was in the range of 10000−20000 cells per well. In structure−activity studies, the tests were performed with stably transfected NIH/3T3 cells and HCT115 human colon carcinoma cells (Figure 2).

**Potency−Sequence Relations.** The choice of the sequences for transmembrane peptide inhibitors was made on the basis of the transmembrane domain predictions made by available computer programs.[14−17] The overall position of the membrane domains made by different programs were in good agreement for the N-terminal half of the molecule but showed significant disagreements for the C-terminal half. TM 9 predicted by HMMTOP (http://www.enzim.hu/hmmtop/index.html)[14] to span residues 833−852 was not found

**Table 1.** Structures and Activity of Transmembrane Inhibitors of P-Glycoprotein[a]

| Peptide | $IC_{50}$, μM |
|---|---|
| TM1: RYSNWLDKLYMVVGTLAAIIHGAGLPLMMLVFEMTDI▶ | |
| MDR1-1-1 VGTLAAIIHGAGLPLMMLVFGEDDD | 10±3 |
| MDR1-1-2 VGTLAAIIHGAGLPLMMLVFGEMTDD | 11±3 |
| TM2: ◀NLEEDMTRYAYYYSGIGAGVLVAAYIQVSFWCLAAGRQIHKIRKQ | |
| MDR1-2-1: DDYAYYYSGIGAGVLVAAYIQVS | 12±3 |
| MDR1-2-2: DDTRYAYYYSGIGAGVLVAAYIQVS | 4.5±0.6 |
| TM3: KINEGIGDKIGMFFQSMATFFTGFIVGFTRGWKLTLVILA▶ | |
| MDR1-3-1: IGMFFQSMATFFTGFIVGFTRGDD | 15±3 |
| TM4: ◀GWKLTLVILAISPVLGLSAAVWAKILSSFTDKELL | |
| MDR1-4-1: GWKLTLVILAISPVLGLSAAVWAK | >20 |
| MDR1-4-2: DDGWKLTLVILAISPVLGLSAAV | 10±2 |
| TM6: ◀VLSGEYSIGQVLTVFFSVLIGAFSVGQASPSI | |
| MDR1-6-1 DSGEYSIGQVLTVFFSVLIGAFSV | 3.2±0.4 |
| MDR1-6-5 DDGSGEYSIGQVLTVFFSVLIGAFSVG | 0.8±0.3 |
| MDR1-6-6 DDDSGEYSIGQVLTVFFSVLIGAFSVG | 0.75±0.3 |
| TM7: MKLNLTEWPYFVVGVFCAIINGGLQPAFAIIFSKIIGVFT▶ | |
| MDR1-7-1 AIINGGLQPAFAIIFSKIIGDD | >10 |
| MDR1-7-2 VGVFSAIINGGLQPAFAIIFSSDD | 7±0.8 |
| MDR1-7-3 VGVFSAIINGGLQPAFAIIFSSG | >10 |
| TM8: ◀KRQNSNLFSLLFLALGIISFITFFLQGFTFGKAGEILTKRLRY | |
| MDR1-8-1: DDNSNLFSLLFLALGIISFITFF | >10 |
| MDR1-8-3: DQNSNLFSLLFLALGIISFI | 5.4±0.5 |
| MDR1-8-4: DQNSNLFSLLFLALGII | 5.6±0.4 |
| TM9: DAAQVKGAIGSRLAVITQNIANLGTGIIISFIYGWQLTLL▶ | |
| MDR1-9-1: LAVITQNIANLGTGIIISFIYGWDD | 15±2 |
| TM10 ◀GWQLTLLLLAIVPIIAIAGVVEMKML | |
| MDR1-10-1 DDGWQLTLLLLAIVPIIA | >15 |
| TM11: SLRKAHIFGITFSFTQAMMYFSYAGCFRFGAYLVAHKLMS▶ | |
| MDR1-11-1 IFGITFSFTQAMMYFSYAGCFDD | 1±0.2 |
| MDR1-11-2 ITFSFTQAMMYFSYAGCFRFGADSSD | 10±1.5 |
| TM12: ◀AYLVAHKLMSFEDVLLVFSAVVFGAMAVGQVSSFAPDYAK | |
| MDR1-12-4 DSFEDVLLVFSAVVFGAMAVGQV | 3.8±0.4 |
| Cyclosporin | 2.8±0.2 |

[a] Activity was determined in the inhibition of the efflux of the fluorescent substrate $DiOC_2$ in 96-well assay using NIH 3T3 cells transfected with human MDR1. Intramembrane segments predicted by HMMTOP[14] are underlined. The end predicted to be extracellular is marked with a black triangle.

to be sufficiently hydrophobic to constitute a TM helix by TopPred2 (http://www.sbc.su.se/~erikw/toppred2/),[15] Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html),SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html),[16] and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/).[17] Also, other C-terminal helices, while found in the same regions of the sequence, had different orientation with respect to the membrane, depending on the program. We based our initial synthetic efforts on HMMTOP prediction (Table 1) with 12 TMs that appeared to correlate with topology prediction obtained by biochemical methods.[18]

We have previously determined that the peptides do not need to span the entire membrane to be effective as antagonists of G-protein-coupled receptors.[8,19] We assumed that this could be a general rule and limited the synthesized sequences to the length that was easily achievable in one run on a peptide synthesizer (not more than 26 residues). Since we did not aim to span the bilayer, the position of the intracellular end of the peptide could be defined by the inclusion of all residues essential for the interactions for the particular domain. The maximal length that could be synthesized in one run was used as a starting point in a search for active TM analogues (Table 1).

The negatively charged residues positioned on the end that was intended to be extracellular improved activity in all cases, similar to what had been observed with GPCR inhibitors. Note the 5-fold increase in the potency of MDR1-5-2 upon substitution of C-terminal serine and glycine with two aspartates (MDR1-5-4 in Table 2). Similarly, addition of two aspartates to the C-terminus

**Table 2.** Structure–Activity Relationship in Peptide Inhibitors of P-gp Derived from the Fifth Transmembrane Domain[a]

TM5: KAITANISIGAAFLLIYASYALAFWYGTTLVLSGE▶

| Peptide | | $IC_{50}$, μM |
|---|---|---|
| MDR1-5-2 | LIYASYALAFWYGTTLVLSGEGSSG | 5±0.5 |
| MDR1-5-4 | LIYASYALAFWYGTTLVLSGEGSDD | 1.1±0.3 |
| MDR1-5-5 | ASYALAFWYGTTDD | >10 |
| MDR1-5-6 | YASYALAFWYGTTDD | 5.5±0.5 |
| MDR1-5-7 | IYASYALAFWYGTTDD | 7.5±0.6 |
| MDR1-5-8 | SYALAFWYGTTLVLSDD | 2.5±0.3 |
| MDR1-5-9 | ALAFWYGTTLVLSGDD | 4.3±0.4 |
| MDR1-5-10 | YALAFWYGTTLVLSGDD | 2.8±0.2 |
| MDR1-5-11 | SYALAFWYGTTLVLSGDD | 1.5±0.2 |
| Retro-MDR1-5-4(all D-) | Suc-DDSGEGSLVLTTGYWFALAYSAYIL | 0.5±0.2 |

[a] For experimental details, refer to Table 1. The black triangle indicates the end predicted to be extracellular.

made an active analogue of TM 7 (MDR1-7-3, Table 1), while the same sequence without aspartates had no detectable activity (MDR1-7-2). The peptide that corresponded to the sequence of TM 4 (MDR1-4-1, Table 1) was inactive, while the shorter peptide with negative charges at the extracellular end (MDR1-4-2) displayed detectable activity.

Interestingly, active inhibitors of P-gp could be generated from almost all TMs, although potency varied significantly (Tables 1 and 2). TM 10 was the only exception. The corresponding peptides had a very high tendency to aggregate, which did not allow us to synthesize homologues with more than 13 transmembrane residues. This length may be insufficient for this particular domain to form stable interactions with the rest of the molecule. However, in some cases, longer peptides had lower inhibitory activities than their shorter versions. We attribute this effect to an increased propensity to self-oligomerize (see below), which could interfere with peptide–protein interaction. An increased potency due to shortening of the sequence is evident in the comparison of MDR1-8-1 and MDR1-8-3 (Table 1) and MDR1-5-6 and MDR1-5-7 (Table 2).

However, in the majority of cases, decreased peptide length was accompanied by lower activity, as could be expected from the reduction of the size of the interaction surface. The systematic reduction in the length of the intramembrane portion of the peptide antagonist was performed on TM 5 (Table 2). The minimal length required for detectable activity for the entire peptide was equal to 15 residues (MDR1-5-6). The predicted intramembrane portion necessary for activity appeared to be as short as six residues (MDR1-5-9). However, since the residues that border the membrane-extracellular interface have not been determined experimentally, it is possible that the intramembrane portion of the helix extends further than predicted by the hydrophobicily analysis and that the part of the peptide involved in the intramembrane interaction is actually longer.

The extension of the peptide toward the extracellular loop was found to improve the activity of several GPCR antagonists[8] probably because the initial prediction of the extracellular TM terminus was not quite accurate and several residues that were postulated to be extracellular were indeed parts of the TM helix. For the majority of both GPCR and P-gp TM analogues, addition of a portion of an extracellular loop helped to improve solubility. We were not able to characterize the influence of those additions on activity in many cases because of the poor solubility of truncated analogues.

The retroinverso analogue of MDR1-5-4 was constructed from D-amino acid residues and had a reversed sequence compared to the parent peptide. The retroinverso peptide was as potent as the original MDR1-5-4, provided that the polarity of the peptide chain was preserved (Table 2). An additional positive charge of an amino group is normally gained and a negative charge of the C-terminal carboxy group is lost during inversion of the sequence. In the case of MDR1-5-4, an inverted D-peptide with a nonmodified sequence turned out to be inactive. We have foreseen this problem and synthesized two peptides, with and without N-terminal succinic moiety, in one run of the peptide synthesizer. We divided the resin with protected peptide into two halves after building the sequence. One-half was cleaved from the resin without prior modifications, and the other half was reacted with succinic anhydride before the cleavage from the resin. Thus, the amino terminus was blocked with an addition of a negatively charged carboxy group. Nonsuccinoylated peptide was totally inactive at 10 $\mu$M, while succinoylated peptide inhibited the efflux of the fluorescent P-gp substrate with an $IC_{50}$ of 500 nM (Figure 2, Table 2). It appeared even slightly more active than the original MDR1-5-4 probably because of higher stability in the medium. Thus, inhibition of P-gp with transmembrane peptides was shown to be chirality-independent, and thus, the more metabolically stable all-D peptides could potentially be used as in vivo P-gp inhibitors.

The inhibition of P-gp activity with the analogues of the transmembrane domains appears to be very selective. The efflux of the fluorescent substrate of P-gp was not influenced by peptides derived from the TM regions of the chemokine receptor CXCR4[8] or by those derived from another ABC transporter, ABCG2. Moreover, P-gp TM inhibitors had no effect on ABCG2-mediated efflux of fluorescent ABCG2 substrates.

**Oligomerization.** SDS electrophoresis is frequently used to assess the ability of transmembrane peptides to form dimers and oligomers in a membrane-like environment.[20–22] Many P-glycoprotein TM analogues described in this study form stable oligomers easily detectable on SDS gels (Figure 3). The presence of oligomeric structures of various stoichiometries in SDS was evident for peptides derived from TMs 2–4, 6, 8,

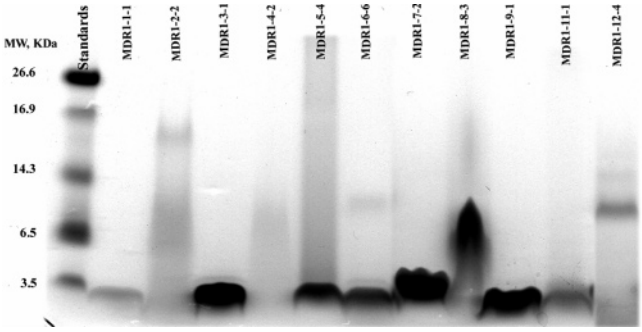


**Figure 3.** Oligomerization of MDR1 transmembrane analogues in SDS micelles visualized by polyacrylamide gel electrophoresis. The first lane corresponds to molecular weight standards, and the rest of the lanes contained the peptides with the sequences indicated in Tables 1 and 2. Samples were separated in a 10−20% gradient of Tris-tricine gel as described in Experimental Section.

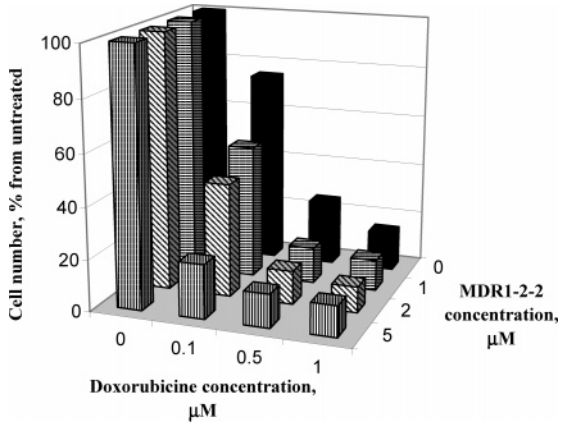


**Figure 4.** Sensitization of HCT15 cells toward doxorubicin by the transmembrane inhibitor MDR1-2-2. The cells were treated with doxorubicin in the presence of varying concentrations of MDR1-2-2 for 96 h, and the cell number was determined using the CyQUANT proliferation kit as described in Experimental Section.

and 12 (Figure 3). TM 2 peptides ran on SDS gels as diffuse bands that corresponded to monomers, trimers, tetramers, and heptamers. TM 3 was mostly monomeric with a faint dimer band consistently present. TM 4 was mostly pentameric. TM 6 was mostly monomeric, but small amounts of dimer and pentamer could also be detected. TM 8 peptides showed a diffuse trimer band along with smaller amounts of a monomer. TM 12 was mostly pentameric with a much less intense band of monomer also present. Most of the P-glycoprotein transmembrane domains contain four or more polar residues. In TMs 1−4, 6−9, and 12, they are spaced by three nonpolar residues and thus are positioned on the same side of the helix. Polar residues are believed to drive interactions of transmembrane helices[23−25] and consequently can cause self-association of the transmembrane peptides. Transmembrane domains 1−3, 7, and 12 contain (A,G)XXX(A,G) motifs (Table 1) that have been implicated in the dimerization of a number of membrane proteins (reviewed in ref 24). TM 2 contains three dimerization motifs, and thus, its ability to form multiple oligomers is not surprising. Similarly, oligomerization can be expected for many other domains. It is unclear at the moment whether this property has any meaning for the function of the transporter. Oligomerization may influence the antagonist properties of the corresponding peptides and thus has to be taken into account during the design of the inhibitors.

**Sensitization of Cancer Cells.** The most soluble among TM inhibitors, MDR1-2-2, was tested for its ability to sensitize highly resistant human colon cancer cells HCT15, which express a high level of MDR1,[26] to the action of doxorubicine (Figure 4). Unfortunately, other more potent peptide P-gp antagonists could not be tested for their ability to reverse resistance to chemotherapeutic agents because of poor solubility in cell media. We observed a several-fold increase in toxicity of doxorubicin in the presence of MDR1-5-4, MDR1-11-2, and MDR1-6-6 (data not shown). However, most of the peptides that were added to the media at 1−5 $\mu$M concentrations precipitated. This precluded a correct determination of inhibitor concentration. MDR1-2-2 had sufficient solubility in cell media for testing at 1 and 2 $\mu$M with traces of precipitate observed at 5 $\mu$M. The relative cell number after exposure to the agents was determined with the use of the fluorogenic dye CyQUANT. The more commonly used MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) toxicity assay was found to be inappropriate because MTT appeared to be a substrate of P-gp and addition of inhibitors resulted in seemingly increased cell proliferation. Staining of the MDR1-expressing cells with CyQUANT did not depend on the presence of transporter inhibitors and could be reliably applied for cell proliferation monitoring. At 1 $\mu$M, MDR1-2-2 reduced the $IC_{50}$ for doxorubicin 5-fold (Figure 4), which is comparable to the effects of other "third-generation" P-gp inhibitors currently in clinical trials, such as LY335979[27] or XR9576.[28] Increasing the concentration of the peptide to 2 $\mu$M caused little additional gain in doxorubicin toxicity (Figure 4). Reduced gain in activity with increasing concentrations of MDR1-2-2 may be caused by an increased degree of oligomerization of the peptide that was shown to form trimers, tetramers, and heptamers (Figure 3). Similar sensitization effects were observed in the DLD-1 colon cell line that also has a high level of MDR1 expression[26] (data not shown). Peptides did not influence the growth of the cells on their own, and no toxicity could be detected.

## Discussion

Analogues of transmembrane domains have been successfully used for the inhibition of G-protein-coupled receptors both in vitro[8,29,30] and in vivo.[31] Diacylglycerol kinase from *E. coli* was the only other polytopic membrane protein that was specifically inhibited with a peptide mimic of its transmembrane domain.[32] We examined the use of transmembrane domain analogues for inhibition of the ABC transporter P-glycoprotein with the intent to eventually develop specific inhibitors for future clinical applications. However, we also wanted to test the general applicability of the approach to this class of membrane proteins. The major advantage of the transmembrane inhibitors is in the highly rational nature of their design. A selective inhibitor can be developed on the basis of no more than a primary structure of the target protein. The major challenges evolve from poor solubility of the highly hydrophobic peptides and their high tendency for aggregation during the synthesis and subsequent biological applications. All

drawbacks intrinsic to peptides such as instability in plasma and poor bioavailability also apply. However, stability in plasma varies significantly depending on peptide sequence, and preliminary studies in the animals have been quite successful.[31]

The present data have demonstrated that specific ABC transporter inhibitors can be obtained using the approach, and many transmembrane domains can serve as templates for inhibitor design. Choosing the least hydrophobic domain appears to be the most practical approach for two reasons. First, it results in a more soluble compound. Second, since polar residues are believed to stabilize interhelical interactions within the membrane bilayer, the helix with a larger number of polar residues, especially when they are conserved, is likely to be involved in extensive interactions essential for tertiary structure stabilization.

The data on structure−activity relationships of the P-gp TM inhibitors (Tables 1 and 2) are in a good agreement with the previously obtained results for GPCRs.[8,19] It confirmed that addition of negative charges to the extracellular end of the peptide significantly improves the activity. We assumed earlier that those negative charges facilitate the correct orientation of the peptide in the bilayer.[19] Collins[33] has proposed that anions are more efficient in water ordering than cations because of the asymmetry of the charge in a water molecule. That is why the transfer of a negatively charged carboxyl group from water into the lipid bilayer requires much more energy than the transfer of positively charged groups.[33] Consequently, anionic groups function as stop signals for peptide insertion into lipids while positive charges travel through the bilayer relatively easily. Correct orientation of the transmembrane segment analogue may serve to decrease the entropy of inhibitor binding to other TMs of the protein. We have observed significant gains in activity of antagonists upon addition of the negative charges at the termini that are intended to be extracellular in all cases examined so far without exception (Tables 1 and 2). The improvement in the inhibitor potency upon addition of the negative charges also confirms the predicted orientation of the transmembrane segments, proving that the assumed secondary structure of the P-glycoprotein with 12 transmembrane helixes is very likely to be correct.

Synergistic inhibitory effects have been observed in mixtures of GPCR TM analogues.[8] We have not been able to detect synergism in MDR1 peptide action when using mixtures of MDR1-11-1 + MDR1-12-4 or MDR1-5-4 + MDR1-6-6. However, the repulsion of two negatively charged peptides may have prevented simultaneous binding. Further studies with at least one peptide lacking the charge need to be conducted to explore possible synergistic effects of MDR1 TM inhibitors.

We hypothesize that the analogues of the transmembrane domains interfere with the proper assembly and folding of the target protein. However, the direct structural proof for this mechanism of inhibition by TM analogues is still missing because of the difficulties in the structural studies of membrane proteins. Several hydrophobic peptides have been shown to be the substrates of P-gp.[34] The possibility that transmembrane domain mimetics inhibit the pump by competing for binding with the fluorescent ligand has been excluded by the observation that the inhibition was highly sequence-specific and that even minor changes in the structure could abrogate the inhibitory activity of the peptides. In conclusion, the function of P-gp and likely of other ABC transporters can be disrupted by externally added molecules that compete for interactions between transmembrane domains of the target protein. Potent selective inhibitors can be designed solely on the basis of the primary structures of the proteins.

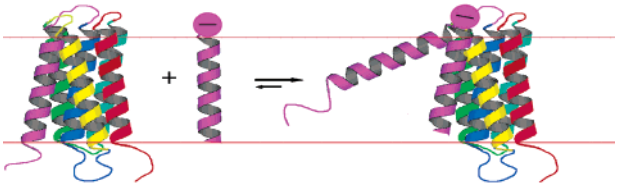

**Figure 5.** Hypothetical mechanism of ABC transporters inhibition with TM analogues. Only the transmembrane part of a half-transporter is depicted.

The following are the general rules for the construction of potent inhibitors:

(1) The extracellular terminus of the peptide must have a net negative charge under physiological conditions. The charge of the intramembrane terminus of the peptide (or the one that faces the cytoplasm) can be positive or neutral.

(2) The peptides do not need to span the entire bilayer, although the minimal length varies for different transmembrane domains.

(3) Retro-inverso peptides can be used as inhibitors; however, additional positive charge acquired during inversion needs to be compensated.

The newly synthesized P-gp antagonists appear to be promising nontoxic drug resistance inhibitors that merit further development.

## Experimental Section

**Peptide Synthesis.** The peptides were synthesized by solid-phase synthesis on a model 433A peptide synthesizer (Applied Biosystems, Foster City, CA) equipped with a conductivity monitoring unit utilizing 9-fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives (AnaSpec, San Jose, CA). Preloaded Fmoc-Asp-HMP resin was used for the synthesis of peptides with C-terminal aspartates, and Fmoc-amide (Rink) resin (Applied Biosystems, Foster City, CA) was used for the systhesis of peptide amides. The synthesis was performed with conditional blocking of unreacted amino groups with acetic anhydride for easier purification of the resulting peptides. Standard coupling procedures utilizing the HBTU/HOBt mixture as activating agent were applied in the FastFmoc protocol of the synthesizer. The synthesis was run in 0.1 mmol scale with a 10-fold excess of reacting amino acids. Conditional double coupling was used for difficult coupling steps. Peptides were cleaved from the resin with 87.5% trifluoroacetic acid containing 5% water, 5% thioanisol, and 2.5% ethandithiol (1 mL of mixture for every 100 mg of resin). The cleavage mixture was added to resin at −5 °C. After 15 min at −5 °C, the stirring was continued for 2 h at room temperature. The resin was filtered off, and the peptides were precipitated with a 15-fold volume of cold diethyl ether, washed five times with ether, and dried in a vacuum overnight. Peptides dissolved in dimethylformamide were purified by HPLC on a semipreparative (9.4 mm × 250 mm) Zorbax CN reverse-phase column (Agilent, Palo Alto, CA) in a gradient of 0.05% trifluoroacetic acid in water and acetonitrile containing 0.05% trifluoroacetic acid. The fractions were analyzed by electrospray LC/MS on an Agilent series 1100 instrument (Agilent Technologies, Palo Alto, CA) with the use of a Zorbax 300SB-C3 Poroshell column

**Table 3.** Analytical Data for the P-gp Transmembrane Peptides

| peptide | mass (calculated) | mass (found) |
|---|---|---|
| MDR1-1-1 | 2440.9 | 2441.0 |
| MDR1-1-2 | 2619.1 | 2618.9 |
| MDR1-2-1 | 2457.7 | 2457.8 |
| MDR1-2-2 | 2715.0 | 2715.1 |
| MDR1-3-1 | 2657.0 | 2657.0 |
| MDR1-4-1 | 2506.1 | 2506.0 |
| MDR1-4-2 | 2350.8 | 2350.4 |
| MDR1-5-2 | 2463.8 | 2463.9 |
| MDR1-5-4 | 2713.0 | 2713.3 |
| MDR1-5-5 | 1580.6 | 1580.2 |
| MDR1-5-6 | 1743.8 | 1744.3 |
| MDR1-5-7 | 1857.0 | 1857.8 |
| MDR1-5-8 | 1922.1 | 1922.4 |
| MDR1-5-9 | 1728.9 | 1728.9 |
| MDR1-5-10 | 1892.1 | 1892.0 |
| MDR1-5-11 | 1979.2 | 1978.7 |
| retro-MDR1-5-4 | 2812.0 | 2812.0 |
| MDR1-6-1 | 2534.9 | 2534.7 |
| MDR1-6-5 | 2764.1 | 2763.8 |
| MDR1-6-6 | 2822.1 | 2822.0 |
| MDR1-7-1 | 2273.7 | 2272.7 |
| MDR1-7-2 | 2438.8 | 2439.0 |
| MDR1-7-3 | 2265.6 | 2266.3 |
| MDR1-8-1 | 2607.0 | 2607.0 |
| MDR1-8-3 | 2224.6 | 2224.9 |
| MDR1-8-4 | 1877.2 | 1876.9 |
| MDR1-9-1 | 2708.1 | 2708.1 |
| MDR1-10-1 | 1963.4 | 1963.6 |
| MDR1-11-1 | 2663.0 | 2662.9 |
| MDR1-11-2 | 2915.2 | 2914.8 |
| MDR1-12-4 | 2381.7 | 2381.2 |

and a gradient of 5% acetic acid in water and acetonitrile. Only fractions containing more than 95% pure product were combined and freeze-dried. The purity and structure were further confirmed by LC/MS with separation on a Zorbax 300SB-C3 analytical column (Table 3).

**Activity Determination in the Efflux of Fluorescent Substrates.** The fluorescence-activated sorting of the cells incubated with rhodamine 123 was performed as previously described.[35]

For the measurement of drug efflux in a 96-well format, we have used Unifilter 96-well plates with GF/D grade filters (Whatman, Clifton, NJ). The cells expressing MDR1 (transfected NIH 3T3 cells[36] or HCT15 cells (ATCC) were grown in 75 mL tissue culture flasks in phenol red free RPMI1640 medium containing 10% fetal bovine serum to near confluency, rinsed with PBS, trypsinized with 0.25% trypsin (Invitrogen, San Diego, CA) for 10 min, and suspended in 5.5 mL of media. Incubation mixtures consisted of 50 $\mu$L of cell suspension, 100 $\mu$L of 2X inhibitor solution in PBS, and 50 $\mu$L of 4X fluorescent substrate solution. The 5 mM stock solutions of inhibitors and substrates were prepared in DMSO and further diluted to 0.2−20 $\mu$M solutions in PBS. After incubation for 1 h in a $CO_2$ incubator, the plates were placed in MultiScreen vacuum manifold (Millipore, Billerica, MA) and the medium was removed by slow filtration. The cells were washed four times with 250 $\mu$L of PBS with filtration and resuspended in 200 $\mu$L of PBS, and the fluorescence of the cells was read in a FluoStar plate reader (BMG, Durham, NC) from the top. A 485 nm filter was used for excitation and a 520 nm filter was used for the emission of SYTO 16, $DiOC_2$, and rhodamine 123. Tetramethylrodamine was excited using a 544 nm filter, and the fluorescence was measured using a 590 nm filter. $IC_{50}$ calculations were performed using the Solver tool of Microsoft 2000 Excel (Microsoft Corp., Redmond, WA). Cyclosporin A was included as a positive control in each plate. Fluorescence in the presence of 5 $\mu$M cyclosporin was assumed to correspond to complete transport inhibition and was used to calculate the $IC_{50}$ for inhibitors for which insufficient solubility did not allow reaching of the plateau in the dose−response curve. Six wells on the plate were used for computing an average fluorescence and standard deviations for each concentration point. An $IC_{50}$ value for each compound is the average of at least two independent experiments.

**Confocal Microscopy.** NIH/3T3 cells stably expressing MDR1 were grown in coverslip-bottom chambers in phenol red free RPMI medium to about 75% confluency. Rhodamine 123 in PBS was added to the cells at 0.5 $\mu$g/mL, and the cells were incubated at 37 °C in the absence or presence of 5 $\mu$M TM inhibitors. After 40 min of incubation, the cells were rinsed once with PBS and observed with a Zeiss LSM 410 Micro System in the confocal mode. For rhodamine 123 excitation, an argon/krypton laser at 488 nm wavelength was used and the emitted fluorescence was detected with a 515−540 nm band-pass filter. Data processing was performed using the Zeiss LSM software.

**Gel Electrophoresis.** The peptides were dissolved in SDS−PAGE tricine sample buffer, heated at 95 °C for 5 min, and run on Novex 10−20% gradient Tris-tricine gels (Invitrogen, San Diego, CA) for 1.5 h at 100 V. The gels were fixed in an acetic acid/methanol/water (1:2:2) mixture for 30 min and stained with Bio-Safe Coomassie stain (BioRad, Hercules, CA).

**Drug Toxicity Assay.** HCT 15 or DLD-1 cells (American Type Culture Collection, Manassas, VA) were inoculated in 96-well plates at 200−400 cells/well in RPMI1640 medium containing 10% fetal bovine serum and allowed to attach for 24 h. An amount of 100 $\mu$L of cell suspension was used for each well. A total of 100 $\mu$L of doxorubicin/P-gp inhibitor mixture at 2× concentration was added the next day, and the sample was kept in the $CO_2$ incubator for 96 h. The medium was removed by gentle turning-over and blotting onto filter paper. The cells were frozen and processed for staining with a CyQUANT proliferation kit (Molecular Probes, Eugene, OR) according to the manufacturer's manual. The fluorescence of the dye was detected in a FluoStar plate reader (BMG, Durham, NC) using a 485 nm filter for fluorescence excitation and a 520 nm filter for emission. An average of six well readings was used for each drug concentration point.

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