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Paper citation:
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Abstract:
A tetrazole ring is often used in drug discovery as a replacement for the carboxylic acid group. Previous work indicates that compounds containing a tetrazole moiety show asymmetric permeability in Caco-2 cells characteristic of an efflux transporter substrate. The aim of this study is to determine which transporters are responsible for polarization of transport of tetrazole containing compounds in Caco-2 cells. Results indicate that only select compounds with tetrazole moieties display asymmetric transport. Three compounds (two commercial drug products and one drug-like structure) were selected for further studies. Losartan appears to be primarily a P-glycoprotein (P-gp) substrate, as previously reported, but MRP inhibitors such as MK-571 and rifampicin also affect the difference between apical to basolateral and basolateral to apical transport. Pemirolast and phenyltetrazole derivative C are sensitive to P-gp inhibition, but transport seems to be mediated by one or more of the MRP family of transporters. Additionally, lowering the pH from 7.4 to 4.0 eliminates the polarization of permeability in Caco-2 cells. These studies indicate that some tetrazole compounds are susceptible to efflux, therefore caution should be used when choosing an appropriate functional group to replace carboxylic acids when synthesizing a drug candidate.

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Text of paper:

Tetrazole Compounds: The Effect of Structure and pH on Caco-2 Cell Permeability

Amber M. Young[1], Kenneth L. Audus[1], John Proudfoot[2] and Mehran Yazdanian[3]

1. The University of Kansas, Department of Pharmaceutical Chemistry, Lawrence, KS
2. Boehringer Ingelheim Pharmaceuticals, Inc., Department of Medicinal Chemistry, Ridgefield, CT
3. Cephalon, Inc., Drug Discovery Support, 145 Brandywine Parkway, West Chester, PA 19380

Introduction:

One major consideration when developing a drug candidate is the potential for interactions with cellular efflux transporters. One such transporter, P-glycoprotein (P-gp) has been shown to significantly decrease the intestinal absorption of numerous compounds.[1] An accurate screening method early in the drug discovery/development process that can detect which candidates are susceptible to P-gp efflux is a highly valuable tool.[1,2]

The Caco-2 assay, using cells derived from a human adenocarcinoma is widely used to assess intestinal permeability, and results from this i*n vitro* system compare favorably with *in vivo* and *in situ* data.[3,4] The apically (luminally) located P-gp is expressed and active in Caco-2 cells, therefore the cell line is employed to evaluate whether a drug candidate is a substrate for this transporter.[5]

Previous studies in our laboratories have shown that drug-like molecules with particular chemical groups often used to improve a drug candidate's physicochemical properties can exhibit polarized transport properties that are characteristic of efflux pump substrates.[6] In particular, benzoic acid, amidine, and tetrazole moieties conferred high basolateral to apical permeability compared to apical to basolateral permeability,

suggesting contributions of polarized transport mechanisms. We have selected tetrazole-containing compounds to study because they showed the highest degree of polarization.[6] Losartan, an angiotensin II antagonist containing a tetrazole ring (Figure 1), has been shown to be a P-gp substrate.[7-9] Its poor oral absorption can at least partially be attributed to its affinity for P-glycoprotein, however it appears that other transporters may

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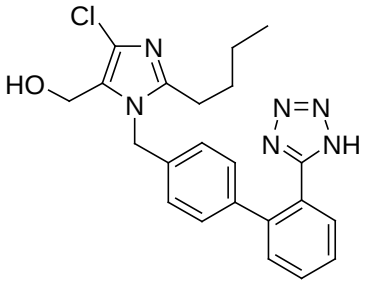

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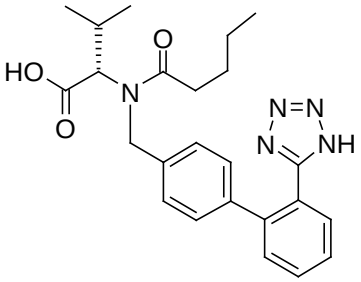

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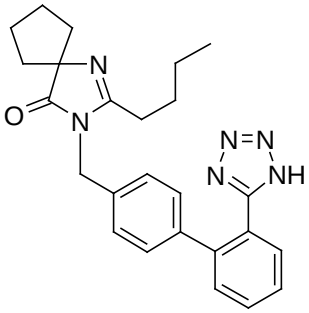

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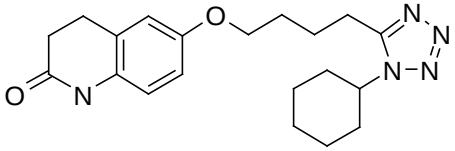

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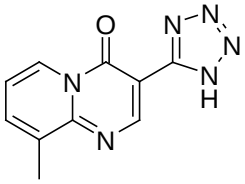

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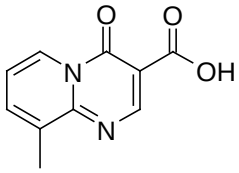

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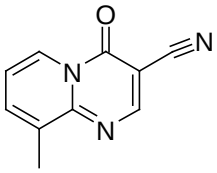

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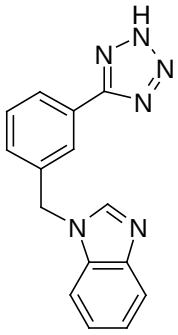

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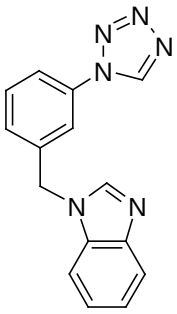

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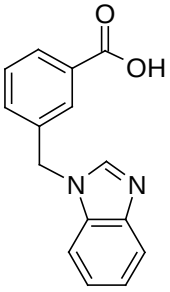

E

Figure 1: Structures of selected compounds

also be responsible for asymmetric transport of losartan in Caco-2 cells.[8] Structurally related angiotensin II antagonists show a wide variability in bioavailability that suggests a difference in efflux transporter affinity among the structures.[9]

In this study, currently marketed drugs as well as a selection of synthesized "drug-like" compounds were used to assess the effect of structure and charge on Caco-2 cell permeability for molecules containing a tetrazole moiety.

Materials:

Dulbecco's Modified Eagle's Medium (DMEM) buffered with HEPES, heat-inactivated fetal bovine serum, non-essential amino acids, and penicillin-streptomycin were obtained from Gibco Invitrogen (Carlsbad, CA). Losartan, valsartan, irbesartan, and cilostazol were purchased from Sequoia Research Products, Ltd. (Oxford, UK). MK-571 was obtained from Biomol Laboratories (Plymouth Meeting, PA). Transwell® plates were from the Costar Corporation (Acton, MA). ^{14}C Mannitol was obtained from Perkin Elmer (Boston, MA). Pemirolast and compounds A through E were synthesized by the Boehringer Ingelheim Pharmaceuticals, Inc. (BIPI) Medicinal Chemistry Department. Caco-2 cells were acquired from ATCC (Manassas, VA). MDCK and MDCK-MDR1 cells were provided by the NIH (Bethesda, MD). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).Cell Culture

Caco-2 cells were grown at 37°C, 90% RH and 5% CO_2. DMEM was buffered with HEPES and supplemented with penicillin-streptomycin (10,000 units/mL), non-essential amino acids (10 mM) and 10% heat-inactivated fetal bovine serum. Cells were grown in Transwell® plates (12 well, 1.12 cm^2 surface area, 0.4 μm pore size) and used for experiments from days 21 to 25. MDCK and MDCK-MDR1 cells were grown using the

same culture conditions listed above. Medium was prepared in the same way as Caco-2, except that colchicine (20 μg/mL) is added for culturing the MDR1 transfected cells. Cells were grown in Transwell® plates for permeability studies. They were used five days post seeding.

Permeability Studies:

Cells were washed with HBSS with or without inhibitor and equilibrated at experimental conditions (4° or 37° C) for 30 minutes. Drug with or without inhibitor was loaded into either the apical or basolateral compartment. Plates were shaken (100 rpm) at experimental conditions, and sampled every thirty minutes for two or three hours depending on the permeability of the compound. Samples were taken from the receiver compartment (300 μL) at each time point and replaced with fresh HBSS with or without inhibitor. For the sodium dependence studies, sodium free salt solution replaced sodium chloride with choline chloride and pH was adjusted with tetramethylammonium hydroxide.[10] The studies at pH=4 were performed in HBSS buffered with 20 mM sodium acetate. The inhibitor concentrations used were as follows: 5 μM for Cyclosporin A, 50 μM for MK-571, and 100 μM for rifampicin, SBP, and CCCP. The concentration of test compounds was either 100 μM or a saturated solution in HBSS (whichever concentration is lower).

LC and LC/MS Detection:

Samples were analyzed using HPLC with an Agilent LC/MSD 1100 instrument. A Zorbax C18 column (4.6 x 150 mm) was used for the LC separation. Some compounds were analyzed using UV detection, while others were analyzed with mass spectrometry (electrospray ionization;single quadrupole).

Liquid Scintillation Counting:

^{14}C Mannitol flux was detected using a Tri-Carb 2700TR liquid scintillation counter.

Permeability Calculations:

Apparent permeability (P_{app}) in cm/sec was calculated using the following equation:

$$\mathbf{P_{app} = dC/dt * V_r/A*C_d}$$

dC/dt is the change in concentration in the receiver compartment over time, V_r is the receiver compartment volume, A is the area of the polycarbonate membrane and C_d is the initial donor compartment concentration. The PDR, or permeability directional ratio, is a measure of polarization of the compound in Caco-2. The following equation determined the ratio:

$$\mathbf{PDR = P_{B\ to\ A} / P_{A\ to\ B}}$$

Statistical Analysis:

Student's t test was used to determine the statistical significance. A p value less than 0.05 was considered statistically significant. All experiments were performed in triplicate.

Results:

Selected Compounds:

Figure 1 shows the structures of drug-like molecules selected for study. Losartan, valsartan, and cilostazol are commercially available compounds that contain a tetrazole ring. Pemirolast, and compounds A-E were synthesized in our laboratories for comparative purposes. To determine the structural effects of the tetrazole ring, a

carboxylic acid (A) and a nitrile (B) analogue of pemirolast were synthesized. Additionally, analogues D and E of the previously studied phenyltetrazole C were evaluated to determine the importance of the tetrazole ring for efflux properties.[6]

Caco-2 Cell Permeabilities:

Apical to basolateral (A to B) and basolateral to apical (B to A) permeability values for each of the chosen molecules are given in Table 1. A PDR that exceeded 2 was considered a significant enough difference to warrant further study. Losartan, pemirolast and compound C all fit this criterion. Additional Caco-2 cell permeability studies were performed to determine whether the presence of inhibitors of various efflux transporters affected the PDR of each drug or drug-like molecule.

Losartan:

Caco-2 cell permeability values were evaluated in the presence of several inhibitors to determine the type of efflux transporters involved (Table 2). Lowering the temperature to 4°C essentially eliminates A to B and B to A transport as compared to the control (0.38×10^{-6} and 3.3×10^{-6} cm/sec, respectively). CCCP (carbonyl cyanide 3-chlorophenyl hydrazone), a metabolic inhibitor, had no significant effect on PDR. Although the ratio remained the same, the A to B and B to A permeability values

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Compound	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)	$P_{B\ to\ A}\ 10^{-6}$ (cm/sec)	PDR
Losartan	0.54 ± 0.07	3.3 ± 0.5	6.0
Valsartan	N/D*	N/D*	N/A
Irbesartan	11.8 ± 1.5	9.7 ± 1.0	0.8
Cilostazol	20.0 ± 0.3	29.2 ± 3.8	1.5
Pemirolast	4.7 ± 0.2	26.4 ± 0.5	5.7
A	19.0 ± 3.5	18.6 ± 2.1	1.0
B	19.2 ± 0.5	20.1 ± 1.4	1.0
C	0.10 ± 0.03	6.6 ± 0.4	66.1
D	18.5 ± 0.1	21.1 ± 1.5	1.1
E	2.0 ± 0.8	4.4 ± 0.03	2.2

Table 1: Permeability in Caco-2 Cells; * indicates that no valsartan transport was detected in the A to B or B to A direction. n=3 for all experiments.

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Inhibitor	Specificity	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)	$P_{B\ to\ A}\ 10^{-6}$ (cm/sec)	PDR
None	-	0.54 ± 0.07	3.3 ± 0.5	6.0
4°C	Active transport	N/D*	N/D*	N/A
CCCP	Active transport	1.6 ± 0.1	8.7 ± 0.4	5.4
Sodium free BSS	Sodium dependent transport	0.28 ± 0.02	7.1 ± 0.5	25.4
CsA	P-gp/MRP	0.56 ± 0.02	0.32 ± 0.12	0.57
MK-571	MRP 1,2	4.6 ± 0.1	3.3 ± 0.3	0.7
Rifampicin	MRP/OATP	2.0 ± 1.9	3.5 ± 0.3	1.7
SBP	OATP	0.53 ± 0.25	4.3 ± 0.4	8.1

Table 2: Losartan permeability in Caco-2 cells * indicates no detectable losartan permeability in the A to B direction. n=3 for all experiments.

increased over two fold. When a sodium free medium was used in place of HBSS, the PDR increased dramatically

from 6.0 to 25.4 (A to B permeability decreased by two fold and B to A permeability increased by two fold). Basolateral to apical permeability decreased approximately ten fold in the presence of cyclosporin A (5 μM), lowering the PDR to 0.6. MRP inhibitor MK-571 and MRP/OATP inhibitor rifampicin also affected the PDR of losartan considerably (6.0 for control and 0.7 with rifampicin). Exposure of Caco-2 cells to 100 μM sulfobromopthalein (SBP), an OATP inhibitor, did not alter permeability values as compared to the control.

Pemirolast:

Permeability values for pemirolast and its carboxylic acid and nitrile analogues, compounds A and B are shown in Table 1. Pemirolast has a PDR of 5.7, and A and B both have PDRs of 1.0. Table 3 includes pemirolast permeability studies performed with the same inhibitors used for losartan. In this case, reducing the temperature to 4°C significantly diminished the A to B and B to A permeabilities. Both were lowered over 20 fold from control values, and the PDR was calculated to be 7.6. Addition of CCCP changed the PDR to 1.0, thereby eliminating any polarization of permeability. A sodium free buffered saline solution reduced the PDR slightly to 3.5, but B to A permeability still exceeded A to B.

P-gp inhibitor cyclosporin A decreased the PDR significantly as did the MRP inhibitors MK-571 and rifampicin (1.5, 1.2 and 1.3, respectively). In addition, SBP decreased the PDR nearly two fold from 5.7 to 3.0.

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Inhibitor	Specificity	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)	$P_{B\ to\ A}\ 10^{-6}$ (cm/sec)	PDR
None	-	4.7 ± 0.2	26.4 ± 0.5	5.7
4°C	Active transport	0.17 ± 0.01	1.3 ± 0.07	7.6
CCCP	Active transport	22.0 ± 1.0	19.1 ± 0.2	0.9
Sodium free BSS	Sodium dependent transport	5.6 ± 0.6	19.8 ± 0.4	3.5
CsA	P-gp/MRP	14.2 ± 0.6	21.6 ± 1.1	1.5
MK-571	MRP 1,2	4.7 ± 0.5	5.7 ± 0.3	1.2
Rifampicin	MRP/OATP	6.0 ± 2.0	7.8 ± 0.3	1.3
SBP	OATP	6.5 ± 0.3	17.9 ± 1.6	3.0

Table 3: Pemirolast permeability in Caco-2 cells; n=3 for all experiments.

Compound C (1-[3-(2H-Tetrazol-5-yl)-benzyl]-1H-benzoimidazole)

One of the drug-like molecules synthesized in our labs showed the highest PDR of any of those screened (Table 1), with a 66.1 fold higher B to A permeability as compared to A to B.[6] In contrast, closely related structural analogues compounds D and E have PDRs of only 1.1 and 2.2, respectively. By reducing the temperature to 4°C, the B to A permeability of compound C is lowered dramatically from 6.0 x 10^{-6} cm/sec to 0.7 x 10^{-6} cm/sec (Table 4). The PDR decreased considerably, but B to A permeability is still seven fold higher than A to B. Permeability of compound C in the presence of CCCP is diminished even further to a PDR of 3.8. The absence of sodium resulted in a large increase in B to A transport and a significantly higher PDR of 7133.

Cyclosporin A caused an increase in B to A permeability and a reduction in A to B permeability that resulted in a modest decrease in the PDR (from 66.1 to 52.2). MK-571 effectively eliminated polarized permeability of benzimidazole compound C by lowering the PDR to 0.8. Rifampicin significantly decreased the PDR to 12.5, while SBP exposure produced an insignificant change in the PDR (from 66.1 to 47).

MDCK and MDCK-MDR1 permeabilities:

To determine whether any of polarized permeability observed was due to P-gp, the MDR1 transfected MDCK cell line was employed (Table 5). PDRs for all three selected compounds in the wild type MDCK cells were approximately 1. MDCK-MDR1 permeability values differed depending on the particular compound. Losartan permeability could not be detected in the A to B direction, but it was significantly higher from B to A (1 x 10^{-5} cm/sec). Pemirolast permeability in transfected cells is three fold higher from B to A as compared to A to B, a significant increase in

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Inhibitor	Specificity	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)	$P_{B\ to\ A}\ 10^{-6}$ (cm/sec)	PDR
None	-	0.10 ± 0.03	6.6 ± 0.4	66.1
4°C	Active transport	0.1 ± 0.03	0.7 ± 0.1	7.0
CCCP	Active transport	1.9 ± 0.1	7.2 ± 1.4	3.8
Sodium free BSS	Sodium dependent transport	0.03 ± 0.03	214 ± 188	7133
CsA	P-gp/MRP	0.18 ± 0.06	9.4 ± 0.3	52.2
MK-571	MRP 1,2	2.5 ± 0.4	1.9 ± 0.4	0.8
Rifampicin	MRP/OATP	0.64 ± 0.19	8.0 ± 0.8	12.5
SBP	OATP	0.24 ± 0.01	11.3 ± 0.4	47

Table 4: Compound C permeability in Caco-2 cells; n=3 for all experiments.

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Wild type:

Compound	$P_{A\ to\ B}$ 10^{-6} (cm/sec)	$P_{B\ to\ A}$ 10^{-6} (cm/sec)	PDR
Losartan	0.17 ± 0.01	0.23 ± 0.01	1.3
Pemirolast	4.9 ± 0.2	2.1 ± 0.2	0.43
C	0.30 ± 0.06	0.26 ± 0.03	0.9

MDR1 transfected:

Compound	$P_{A\ to\ B}$ 10^{-6} (cm/sec)	$P_{B\ to\ A}$ 10^{-6} (cm/sec)	PDR
Losartan	N/D*	10.1	N/A
Pemirolast	2.2 ± 0.2	6.5 ± 1.4	3.0
C	0.61 ± 0.09	0.88 ± 0.10	1.3

Table 5: MDCK cell permeability. * indicates no losartan permeability detected in the A to B direction. n=3 for all experiments.

PDR as compared to wild type (3.0 and 0.43, respectively). Compound C has a PDR of 1.3 for both wild type and transfected cells.

Permeability at pH=4.0

Caco-2 cell permeability was measured at pH=4.0 to determine whether compound charge had any effect (Table 6). Losartan, Pemirolast, and benzimidazoles C and D all had PDRs of approximately 1.

Monolayer Integrity in the Presence of Inhibitors

Paracellular permeability was measured with radiolabeled mannitol for each of the inhibitors used to confirm that they do not affect the integrity of the monolayer. None of the inhibitors or media used (pH=4.0 and sodium free buffer) caused a significant increase in paracellular permeability when compared to control (Table 7). Additionally, Table 8 shows the permeability of marker compounds caffeine and lucifer yellow in Caco-2 cells (Table 8). There appears to be no significant difference between the values at pH 7.4 and 4.0.

Discussion and Conclusions

The studies presented here show that not all tetrazole-containing compounds are asymmetrically transported in Caco-2 cells (Table 1). Valsartan, irbesartan, cilostazol and benzimidazole D do not show a significant difference between A to B and B to A permeability. Literature results confirm that cilostazol is not subject to significant polarization of transport. Toyobuku and colleagues recently reported that although cilostazol was subject to efflux in cells overexpressing P-gp, the transporter has little

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Compound	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)	$P_{B\ to\ A}\ 10^{-6}$ (cm/sec)	PDR
Losartan	16.0 ± 0.9	20.0 ± 0.7	1.3
Pemirolast	12.6 ± 0.8	16.2 ± 0.5	1.3
C	13.6 ± 1.6	12.1 ± 2.5	0.9
D	18.7 ± 2.4	20.1 ± 1.5	1.1

Table 6: Caco-2 cell permeability at pH=4.0; n=3 for all experiments.

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Inhibitor	Specificity	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)
None	-	1.3 ± 0.2
4°C	Active transport	$0.13 + 0.07$
CCCP	Active transport	1.3 ± 0.3
Sodium free BSS	Sodium dependent transport	0.95 ± 0.05
CsA	P-gp/MRP	1.0 ± 0.1
MK-571	MRP 1,2	1.0 ± 0.2
Rifampicin	MRP/OATP	1.4 ± 0.3
SBP	OATP	3.8 ± 2.8
pH = 4.0	N/A	0.27 ± 0.12

Table 7: Mannitol permeability values, n=3 for all experiments

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Compound	pH	$P_{A\ to\ B}\ 10^{-6}$ (cm/sec)	$P_{B\ to\ A}\ 10^{-6}$ (cm/sec)
Caffeine	7.4	22.9 ± 1.1	26.7 ± 0.7
Caffeine	4.0	25.0 ± 2.0	29.1 ± 1.0
Lucifer Yellow	7.4	0.37 ± 0.09	0.31 ± 0.03
Lucifer Yellow	4.0	0.28 ± 0.03	0.34 ± 0.05

Table 8: Caco-2 cell permeability at pH=4.0 for high (caffeine) and low (lucifer yellow) permeability markers; n=3 for all experiments.

effect on *in situ* intestinal absorption because of a countering apical uptake mechanism.[11] Three of the compounds studied (losartan, pemirolast and benzimidazole C) had PDRs greater than 2 that warranted further investigation in to what efflux transporters are involved in their asymmetric transport.

Losartan is well established as a P-gp substrate in the literature.[8-10] Rose and Audus reported that losartan and other angiotensin II antagonists act as P-gp substrates in the blood-brain endothelial barrier.[7] Soldner and colleagues assert that most of the polarization of losartan in Caco-2 is due to P-gp, however another transporter must also be involved.[8]

Our work confirms that losartan is a substrate of P-gp. The PDR is reduced substantially in the presence of cyclosporin A (Table 2), and the MDR1 transfected cell line shows significant B to A permeability and no detectable permeability in the A to B direction. There was likely some permeability from the apical to basolateral compartments, but it fell below the limits of UV detection for the chosen HPLC method. Cyclosporin A has been shown to inhibit transporters in addition to P-gp (e.g., MRP7), therefore the resulting PDR maybe not be entirely due to P-gp inhibition.[12]

Additionally, the MRP inhibitors MK-571 and rifampicin were able to inhibit efflux pump activity in Caco-2 cells. The MRP family of transporters is not fully characterized, and little is known regarding the substrate specificity of each isoform. Both inhibitors are known to affect MRP1 and MRP2, but it is likely that other MRPs could be affected by these compounds. Soldner et al. used MRP1 and MRP2 transfected MDCK cells to demonstrate that losartan efflux was not mediated by these transporters.[8] Our work does not directly contradict these findings. Rather, we assert that other MRP transporters may be responsible for asymmetric losartan transport in the Caco-2 cell line.

Recent work by Prime-Chapman and colleagues shows the expression of MRP 1 through 6 in Caco-2 cells. The differences in functional activity between isoforms has not yet been clarified.[13]

Pemirolast, another currently marketed pharmaceutical, shows a polarization of permeability in Caco-2 cells. The low PDR values for compounds A and B (structural analogues of pemirolast) indicate a clear structure-activity relationship. Both carboxyl (A) and nitrile (B) analogues have equal A to B and B to A permeability values. It seems that the tetrazole moiety is responsible for the polarization of permeability in the drug molecule.

Pemirolast also appears to be a substrate for P-glycoprotein and at least one of the MRP isoforms. As described earlier, the specificity of the MRP inhibitors is unknown, therefore the particular isoform cannot be directly identified. Asymmetric transport in the presence of cyclosporin A was decreased significantly, although not completely (Table 3). The MDCK studies show a threefold greater B to A permeability as compared to A to B in the MDR1 transfected cell line. It is important to note that the PDR for the wild type MDCK is 0.43, indicating that A to B transport exceeds B to A by more than twofold (Table 5). Although untransfected MDCK cells are generally believed to lack most efflux transporters, Ng and colleagues have found MRP2-like and OATP2-like transport activity of organic anions in wild type MDCK cells.[14] It is possible that pemirolast is a substrate of a transporter not found in Caco-2 cells.

MRP inhibitors effectively decreased the permirolast PDR to 1 in Caco-2 cells. The addition of sulfobromopthalein (SBP), an OATP inhibitor, reduced the PDR from 5.7 to 3.0, though not to a significant extent.

Benzimidazole compound C showed the most dramatic difference between A to B and B to A permeability (PDR = 66.1). Like pemirolast, it seems that the tetrazole ring is the portion of the structure primarily responsible for the differences in directional permeability. Compound E, the carboxylic acid analogue of C, has a PDR of 2.2. This is consistent with previous findings in our lab, showing asymmetric transport with tetrazole and carboxyl containing compounds.[6] Inhibition of active transport (4°C and CCCP) reduced the PDR to 7.0 and 3.8, respectively, suggesting both active and passive/facilitative transport mechanisms. Sodium free medium actually increased the PDR quite significantly, thereby removing facilitative transporters such as OATP from consideration.

Inhibition of P-gp via cyclosporin A results in a small decrease in polarization of compound C, indicating that this transporter is not the primary cause of the asymmetry of transport. MK-571 effectively eliminates any differences between A to B and B to A permeability, while the less-specific rifampicin lowers the PDR from 66.1 to 12.5. SBP exposure produces little change in the polarization of transport (Table 4).

Aside from efflux transporters, another main consideration is the effect of pH and ionization of the compounds on permeability. The pK_a of tetrazoles is in the range of 4-5, similar to that of carboxylic acid groups.[9] Therefore, the structures should be considered negatively charged at experimental conditions (pH 7.4). One notable exception is benzoimidazole compound D, which is attached to the rest of the molecule via a nitrogen as compared to a carbon. As a result, the tetrazole ring is uncharged at pH values in the physiological range. Comparing the PDRs of the two compounds in Caco-2 cells show the startling difference between the two (66.1 for C and 1.1 for D).

Caco-2 cell permeability studies were performed at pH 4.0 to determine the effect of charge. Surprisingly, all of the compounds showing asymmetric permeability values at 7.4 showed no differences in permeability at acidic conditions. Compounds C and D both had PDRs of 1. At pH 4.0, the permeability in both directions increased significantly (Table 6) for all compounds except benzimidazole D. This is reasonable because all the compounds studied with the exception of D go from negatively charged to neutral, therefore passive diffusion across the plasma membrane is much more favorable. Compound D is neutral under all the experimental conditions, so the passive permeability should not be altered. Literature results have demonstrated that a higher fraction of charged species reduces Caco-2 cell permeability.[15] Our mannitol studies (Table 7) confirm that pH= 4.0 media does not increase the paracellular permeability and thereby artificially increase the numbers during the course of the experiment. As an additional control, the high and low permeability markers (caffeine and lucifer yellow, respectively; Table 8) showed no difference in permeability when the media was pH 7.4 or 4.0.

Little is known about the effect of pH on the efflux transporters themselves. One explanation of the data at pH = 4.0 is that the functional activity of the transporters is impaired at the experimental conditions. Additional studies at pH = 4.0 with known transporter substrates would help to clarify the matter. The pH of the gastrointestinal tract can vary depending on diet and disease state (typically from 5 to 8), therefore is important to recognize the possibility of a pH dependent variability of absorption. A more realistic representation of the *in vivo* situation would be to study drug transport with a range of pH values in the apical compartment, while keeping the basolateral compartment value a constant pH 7.4.[16]

This work demonstrates that certain tetrazole compounds are efflux transporter substrates. All the selected compounds screened in this work seem to have an interaction with a member of the MRP family. The particular isoform is not known due to substrate overlap and prior work that indicates that losartan efflux is not MRP1 or MRP2 mediated.[8] To an extent, losartan and pemirolast also show some interaction with P-gp. In this case, interaction with the relevant transporter(s) seems to be based on the tetrazole structure recognition as well as the negative charge at physiological pH.

This paper also raises some concerns about the use of tetrazole groups as a replacement for carboxylate groups during the lead optimization process. Data presented here suggest that this substitution could significantly affect the efflux transporter affinity, and therefore the absorption and bioavailability of the candidate.

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