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What Limits the Adsorption of Cyclic Prodrugs of Opioid Peptides into Intestinal Cells (DADLE): Part I. The Function of Efflux Transporters in the Mucosa of the Intestine

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Abstract

In this work, we aimed to understand how P-glycoprotein (P-gp) limits the intestinal mucosal permeability of the opioid peptide DADLE (H-Tyr-D-Ala-Gly- Phe-D-Leu-OH) and its cyclic prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE). By incorporating GF-12098, cyclosporine (CyA), or PSC-833, which are recognized P-gp inhibitors, into the incubation media of AOA-DADLE, CA-DADLE, and OMCA-DADLE (71-117) in the Caco-2 cell model, the high Papp, BL-to-AP/Papp, AP-to-BL ratios were considerably reduced. This indicates that P-gp is limiting the AP-to-BL flow of these cyclic prodrugs. It was shown that AOA-DADLE, CA-DADLE, and OMCA-DADLE had very low mesenteric blood permeation (PB ¼ 0.40, 0.56, and 0.42 × 10—7 cm/s, respectively) in the in situ perfused rat ileum model. All three prodrugs showed a considerable rise in PB values when treated with PSC-833. While PSC-833 significantly increased the PB values of these prodrugs, CyA and GF-12918 had no effect or had a much less effect. These results indicate that P-gp isn't the only factor that limits the permeability of AOA-DADLE, CA-DADLE, and OMCA-DADLE across the intestinal mucosa of rats; other variables, such as substrate activity for other efflux transporters and metabolic enzymes, may also play a role.

Topics covered include: peptide administration, oral absorption, intestinal mucosa, in situ perfused rat ileum, Caco-2 cell efflux transporter, and prodrugs. Glycoprotein P-type

A brief overview

Our lab has developed and manufactured cyclic prodrugs of the opioid peptide DADLE (H-Tyr-D-Ala-Gly-Phe-D-Leu-OH) using a variety of promoiety linkers, including an acyloxyalkoxy (AOA) linker, a coumari-nic acid (CA) linker, and an oxymethyl-modified coumarinic acid (OMCA) linker, in an effort to enhance its oral bioavailability and its ability to pass the blood-brain barrier (BBB) (Fig. 1).The three cyclic prodrugs that resulted—AOA-DADLE, CA-DADLE, and OMCA-DADLE—are characterized by their lack of charges, relative lipophilicity,

and the presence of distinctive structures in solution that facilitate their transcellular route penetration.

Substrate activity for efflux transporters in polarized cells limits the penetration of the three cyclic prodrugs of DADLE, according to in situ rat brain perfusion studies and in vitro cell culture experiments (e.g., Caco-2, MDCK-MDR1, and MDCK-MRP2 cells).4–9 GF-120918 and cyclosporin A (CyA) may suppress the polarized efflux of these cyclic prodrugs in Caco-2, MDCK-MDR1, and MDCK-MRP2 cells.Theseprodrugs have been shown to enhance brain permeability in an in situ perfused rat brain model when administered with 4-9 GF-120918 and Cya. The p-glycoprotein (P-gp), breast cancer resistance protein (BCRP) 11,12, and MRP proteins are all recognized to be inhibited by GF-120918.CyA, which is known to inhibit P-gp and MRP2, is one of thirteen compounds listed.

Oral administration of AOA-DADLE, CA-DADLE, and OMCA-DADLE to rat's results in poor absorption, according to preliminary in vivo pharmacokinetic experiments performed in our lab.

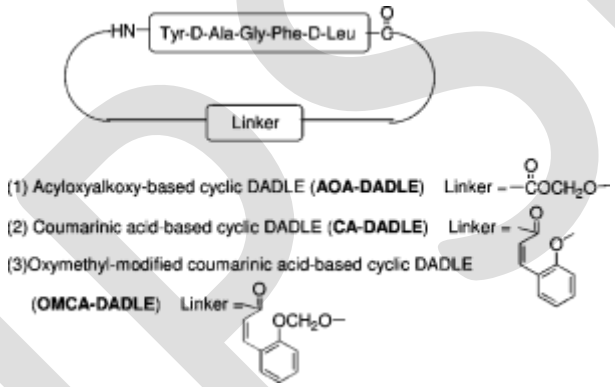

Figure 1. Chemical structures of DADLEanditscyclicprodrugs (AOA-DADLE, CA-DADLE, andOMCA-DADLE).

[data that has not been released]. It is possible that the poor oral absorption of AOA-DADLE, CA-DADLE, and OMCA-DADLE is caused by the substrate activity of one or more of the efflux transporters (e.g., P-gp, BCRP, MRP2), which are expressed on the apical membrane of the intestinal epithelial cells.

Using an in situ perfused rat ileum model, we discuss here the findings of permeation experiments that attempted to evaluate the function of efflux transporters in the poor oral absorption of AOA-DADLE, CA-DADLE, and OMCA-DADLE. It is known that the intestinal mucosa expresses several efflux transporters; these investigations aimed to describe the intestinal mucosal permeation of cyclic prodrugs in the presence and absence of inhibitors (GF-120918, CyA, and PSC-833). To assess the efflux transporter's functional activity in this model of the intestinal mucosa, permeability studies were also performed using quinidine, a molecular probe

that is known to be a P-gp substrate. The features of AOA-DADLE, CA-DADLE, OMCA-DADLE, and quini-permeability respectively

to see whether our cell culture model might have anticipated the effects of efflux transporters and inhibitors of these transporters on the intestinal mucosal permeation of these substances. We also tried to determine dine across Caco-2 cell monolayers.

DATA AND PROCEDURE

DADLE, Hank's balanced salts (HBSS) (modified), Dulbecco's phosphate buffered saline, [Leu5]-enkephalin, [14C] We bought CyA and -antipyrine (5.4 mCi/ mmol) from Sigma-Aldrich in St. Louis, MO. Gibco BRL, Life Technologies, Grand Island, NY, supplied the L-glutamine, penicillin, streptomycin, and nonessential amino acids in 85% saline solutions. The concentrations of these substances were 10,000 U/mL, 10,000 g/mol, and 10 mM, respectively. DMEM and a trypsin/EDTA solution [0.25% and 0.02% in Ca2þ- and Mg2þ-free HBSS, respectively] were acquired from JRH Bioscience in Lenexa, KS.

Collaborative Biomedical Products of Bedford, MA provided the rat-tail collagen (type I), while Atlanta Biologicals of Norcross, GA supplied the fetal bovine serum (FBS). It was from Moravek that D-1-[14C]-Mannitol (2.07 Gbq/mmol) was acquired. Brea, California: Biochemicals. All right.We bought quinidine from American Radiolabeled Chemicals in St. Louis, MO, which has a concentration of 1 mCi/mL. A donation from Dr. Kenneth Brouwer of GlaxoSmithKline in Research Triangle Park, NC was GF-120918. Dr. Stephan Ruetz of Novartis in Basel, Switzerland, generously donated PSC-833. The remaining chemicals were of the highest quality and were sourced from either Across Organics distributed by Fisher Scientific in Houston, TX or Aldrich Chemical Co. in Milwaukee, WI. Our laboratory followed the previously published protocols to synthesis AOA-DADLE, CA-DADLE, OMCA-DADLE, and [Leu5]-enkephalin. and

Procedures for In Situ Perfusion of the Rat Ileum: A Statement on Animal Study Compliance

All US Department of Agriculture (USDA) rules pertaining to the Animal Welfare Act (9 CFR, Partsand were followed by this research. Animal Care and Use Committee (AACUC) approval was obtained for all treatments involving animals at The University of Kansas.

Operative Techniques

Preparing the perfused rat ileum for surgery involves methods that are comparable to, but somewhat different from, those already described.20 Test compounds and/or P-gp inhibitors (such as GF-120918, CyA, or PSC-833) were infused into the ileum segment at a flow rate of 0.2 mL/min after surgery. The perfusate, which consisted of 57.9 mM NaH2PO4, 79.5 mM Na2SO4, pH 6.5, and 378C, was collected from the outlet cannula after a single pass perfusion at 5-minute intervals between 30 and 60 minutes. The perfusate and mesenteric blood were drawn at the same time. The plasma fractions were obtained by centrifuging blood samples taken from the mesenteric vein at 1800g for 5 minutes prior to the addition of the internal standards. For

DADLE quantification, the internal standard was [Leu5]-enkephalin, and for DADLE cyclic prodrugs, the standard was the same set of cyclic [Leu5]-enkephalins that include the same linkers as DADLE. After that, the plasma samples were combined with acetonitrile at a ratio of 1:3, volume/volume, and then spun at 21000g for fifteen minutes. The supernatants were centrifuged and then dried using a Centrivap Concentrator (Lab- Company X, located in Kansas City, Missouri. Filtration using Millipore Ultrafree-MC centrifugal filters was performed using 7000g centrifugation for up to three hours after the final residues were dissolved in 0.1 mL of acetonitrile/water (1:1). Injecting 30 microliters of the filtrates allowed for LC-MS/MS verification.

Identical perfusion protocols were adhered to when investigations using radioactive substances (such as [3H]-quinidine, [14C]-antipyrine, or [14C]-mannitol) were undertaken. For 5 minutes, the blood samples were spun in a centrifuge at 1800g. An LS 6000 IC dual-label scintillation spectrometer from Beckman Coulter, Inc. in Fullerton, CA was used to determine the radioactivity levels in the plasma and perfusate samples.

Examining Rat Ileum Samples Through In Situ Perfusion

An LC-10A gradient system (Shimadzu, Tokyo, Japan) comprised of two LC-10AS pumps, a SCL-10A system controller, and a SIL-10A autoinjector with a sample cooler was used to conduct HPLC analysis with UV detection in order to ascertain the quantities of DADLE and its cyclic prodrugs in the perfusates. A C18 reversed-phase column (300 A˙, 250 4.6-mm i.d.; Vydac, Hesperia, CA) with a C18 guard column was used to separate the samples. Water containing 0.1% v/v trifluoroacetic acid was subjected to gradient elution at a flow rate of 1 mL/min from 26% to 58% acetonitrile (v/v). A UV detector (l 214 nm) was used to detect the eluents. We used the CLASS-VP version 4.2 Chromatography Data System (Shimadzu) to collect and analyze the chromatographic data.

Plasma samples were analyzed using LC/MS/MS on a Micromass Quattro Micro triple quadrupole mass spectrometer (Micromass, Beverly, MA) to determine the amounts of DADLE and its prodrugs. Using a reversed-phase C18 column (50 1.0-mm i.d., 5 mm; Vydac) and a 2690 HPLC System (Waters, Milford, MA), the liquid chromatography was carried out. Two separate mobile phases were prepared: one using water and the other using acetonitrile and 0.1% (v/v) formic acid. A mass spectrometer and an electrospray interface were used to connect the HPLC system. In order to capture samples from different compounds at once, we employed multiple reactions monitoring over multiple channels with a dwell period of 0.1 s and an interchannel latency of 0.03 s. Gathering and analyzing the data were After three to five days, when the cells had grown to about 80% confluence, they were partially digested with a trypsin/EDTA solution. They were then either re-cultured in fresh flasks or seeded at a density of 8.0 104 cells/cm2 on collagen-coated polyester membranes (Transwell, 1.3 mm pore size, 24.5 mm diameter). In order to prepare for transport tests, cells were given culture media every other day for 21 days, with a volume of 1.5 mL at the apex and 2 mL at the basolateral.

Experiments on Transportation

L ~ 2prl ~ Cl

where Q = mL/s, r = intestine luminal radius, l = perfused ileum segment length, and C0 = drug concentration at inlet and Cl = drug concentration at outflow, respectively, in the perfused ileum segment. Following previous descriptions, the concentrations at the exit were adjusted to account for water absorption as measured using nonabsorbable [14C]-PEG 4000.21

Based on the compound's appearance rate in the blood, the apparent mesenteric permeability coefficient (PB) was calculated. In order to get the PB value, the following equation was used:

The equation P = ðDMB=Dt is equal to ð2prl. < C ><C> was the logarithmic mean concentration of the drug inside the segment, which was determined using the following equation: MB was the cumulative quantity of medicines in the blood with time t. The product of C0½1 and ðCl is equal to C0—

The equation lnðC0=ClÞ.Experimental Setups Using Caco-2 Cells

Settings for Cell Culture

The American Type Culture Collection in Rockville, MD, provided the Caco-2 cells. The 7 cells were cultured in a 150 cm2 flask with a medium that included DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, 100 mg/mL streptomycin, 100 U/mL penicillin, and 1% L-glutamine, as previously described. The controlled atmosphere included 5% CO2 and 95% relative humidity. The temperature was maintained at 378C. Filters made of polyester and coated with collagen (Transwell1) were used for the growth of Caco-2 cell monolayers. A shaking water bath was used to conduct all experiments in triplicate at a temperature of 378C. The stability of the monolayers of cells

was ascertained by taking frequent readings of the [14C]-mannitol flow, a paracellular marker. After three washes with prewarmed HBSS (pH 7.4), cell monolayers were treated for 20 minutes with either blank HBSS or HBSS containing the test chemical, with or without different doses of efflux transporter inhibitors (e.g., GF-120918, CyA, or PSC-833). For instance, the donor compartment (AP: 1.5 mL) was treated with [3H]-quinidine in HBSS containing different doses of P-gp inhibitors. At 2.5 mL BL, P-gp inhibitors or not were introduced to the receiver compartment. Samples were obtained at 10 mL donor intervals and 100 mL receiver intervals for up to 90 minutes. The radioactivity of the samples was then measured using a dual-label scintillation spectrometer (LS 6000 IC; Beckman Coulter, Inc.). The compounds' permeability coefficients (Papp) were determined using:

This equation states that the product of the linear appearance rate of mass in the receiver solution (DQ/Dt), the cross-section area (A), and the starting concentration of the donor side (C0) at time zero (t ¼ 0 min) is equal to P þ DQ= DtÐ ð D × C0Ð.

Finding the Half-Life of GF-120918, CyA, and PSC-833 as They Relate to Quinidine Efflux Inhibition [3H]- AAs a marker to track the functioning P-gp, quinidine was utilized.18 Quinidine is a recognized P-gp substrate.

The Intestinal Absorption of Cyclic Prodrugs is Limited by Efflux Transporters

The small intestine mucosa expresses a large number of efflux transporters. Limiting xenobiotic absorption into mesenteric blood flow is a critical function of certain of these transporters. On the apical side of intestinal epithelial cells, for instance, you'll find P-gp and MRP2, two proteins that help with the efflux of cationic and anionic medicines, respectively. Also, certain medications, including topotecan, have their absorption reduced because BCRP is located on the apical side of intestinal epithelial cells. Intestinal expression of other membrane-associated efflux transporters, such as lung resistance protein (LRP) and a transporter associated with antigen processing (TAP) has not yet led to a complete characterization of their physiological functions.

The function of P-gp in limiting the oral absorption of medicines has been studied to a greater extent than any of the other efflux transporters expressed in the intestinal mucosa. Thus, three P-gp inhibitors (GF120918, CyA, and PSC833) were tested for their capacity to increase the oral absorption of cyclic prodrugs, and the function of P-gp in limiting their intestinal absorption was the primary focus of this investigation.

The mesenteric permeability coefficients of the cyclic prodrugs were lower (PB values ¼ 0.40 to 0.52 × 10—7 cm/s) compared to DADLE (PB value ¼ 2.46 0.64 × 10—7 cm/s), as shown in Table 3. Cyclic prodrugs have PB values that don't match up with their lipophilic properties.4–7 in The low AP-to-BL Papp values for the cyclic prodrugs and DADLE in the Caco-2 cell model were in good agreement with the low PB values in this in situ perfused rat ileum model (Tab. 2). These results suggest that the cyclic prodrugs' substrate activity for efflux transporters (e.g., P-gp) is limiting their passive permeation across cell membranes in both models of the intestinal mucosa, which is surprising given their higher lipophilicities compared to DADLE. How GF-120918, CyA, and PSC-833, which are P-gp inhibitors, worked on The Differential Effects of GF120918, CyA, and PSC833 in Improving Cyclic Prodrug Permeability in Intestinal Mucosa Models

Using the in situ perfused rat ileum model, we determined how these P-gp inhibitors affected the cyclic prodrug penetration. According to the results of these tests, PSC-833 considerably increased the mesenteric permeability (PB) of the three cyclic prodrugs tested: AOA-DADLE by 7.6-fold, CA-DADLE by 13.4-fold, and OMCA-DADLE by 15.7-fold (Tab. 3). To the contrary, PSC-833 was determined to be much more successful than GF-120918 and CyA (i.e., CyA only raised the PB value for CA-DADLE, while GF-120918 only raised it for OMCA-DADLE) (Tab. 3). The findings obtained here contrast significantly from those shown in the Caco-2 cell assays (Tab. 2), where the polarized efflux of these cyclic prodrugs was completely blocked at 10 mM doses by GF-120918, CyA, and PSC-833. To shed light on these variations, four possibilities were considered: I. The substrates used by the human P-gp (MDR1) and its rat homologue, the AOA-DADLE, CA-DADLE, and OMCA-DADLE, are distinct.

specificities; (ii) the human form of P-gp (MDR1) and the rat equivalent of this efflux transporter have different inhibitor (e.g., GF-120918, CyA, PSC-833) specificities; (iii) AOA-DADLE, CA-DADLE, and OMCA-DADLE are substrates of other efflux transporters (e.g., MRP2, BCRP) that play important roles in limiting the intestinal mucosal absorption of these cyclic prodrugs and these transporters are differently expressed in rat intestine and the applied Caco-2 cell model and thus apparent permeability in the two models are differentially inhibited by PSC-833, CyA, and GF-120918; and (iv) AOA-DADLE, CA-DADLE, and OMCA-DADLE are substrates for metabolic enzymes (e.g., cytochrome P-450s, esterase's, phenol sulfotransferases, glucanosyltransferases) that play important roles in limiting the

There are a number of reasons why the first hypothesis—that P-gp substrate specificity varies among species—is very improbable. Rat P-gp sub-segmentation of the three cyclic prodrugs There is still a lack of information on the substrate activity of AOA-DADLE, CA-DADLE, and OMCA-DADLE for certain efflux transporters. The action of these cyclic prodrugs on MDR1 and MRP2 substrates was recently studied in our lab using MDCK-MDR1 and MDCK-MRP2 cells, respectively. Research has shown that AOA-DADLE mostly binds to MDR18, while CA-DADLE and OMCA-DADLE bind to MDR1 and MRP2, respectively.We don't know anything about these cyclic prodrugs' substrate activity for BCRP at the moment.

The observation that AOA-DADLE, CA-DADLE, and OMCA-DADLE all displayed significant polarized efflux in this cell line and that this efflux could be inhibited by GF-120918, CyA, and PSC-833 (Tab. 2) is not surprising given that our laboratory's stock of Caco-2 cells expresses high levels of MDR. The expression level of MRP2, an efflux transporter, is very low in our Caco-2 cells; hence, it is highly improbable that compounds with cosubstrate activity for MDR1/MRP2 could be distinguished. Also, according to our unpublished findings, our Caco-2 cell stock does not contain any BCRP. Consequently, distinguishing between substances that serve as cosubstrates for MDR1/BCRP, MRP2/BCRC, or MDR1/BCRP/

MRP2 is very improbable.

It was not surprising that we found no correlation between the permeation data of Caco-2 cells and the intestinal mucosal permeation data of rats for certain compounds, especially those that show substrate activity for multiple efflux transporters, given the information given about the "efflux transporter profile" of our Caco-2 cell stock. Species variations in P-gp substrate/inhibitor specificity cannot account for the observed lack of association, as shown before. It seems that the cyclic prodrugs may be substrates for unknown efflux transporters expressed in rat intestinal mucosa but not in Caco-2 cells, which could explain why the two models show different levels of cyclic prodrug permeation when efflux inhibitors are present (Hypothesis #3). Curiously, when efflux inhibitors are not present, these variations do not become apparent. They appear only themselves when efflux transporter inhibitors are present. This might point to a different interpretation, namely that GF-120918, CyA, and PSC-833 block efflux transporters with larger specificities than what has been previously reported.

Conclusion

Lastly, considering the information given in this article, it is not possible to dismiss hypothesis #4, which states that AOA-DADLE, CA-DADLE, and OMCA-DADLE are substances that are broken down by specific metabolic enzymes. These enzymes, such as cytochrome P-450s, esterases, phenol sulfo-transferases, and glucuronyltransferases, are crucial in preventing the intestinal mucosal absorption of these cyclic prodrugs. It is hypothesized that PSC-833, CyA, and GF-120918 differentially inhibit these enzymes. While AOA-DADLE, CA-DADLE, and OMCA-DADLE are great substrates for cytochrome P-450 enzymes, this metabolic route is not a major factor in the poor oral absorption of these drugs, as we demonstrate in an ancillary study. Rather, the substrate activity for efflux transporters in the rat intestinal mucosa seems to be the primary factor limiting the oral absorption of AOA-DADLE, CA-DADLE, and OMCA-DADLE.

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Conflict of Interest

The Author declares that there is no conflict of interest.

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