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Chirally Pure Prodrugs and Their Converting Enzymes Lead to High Supersaturation and Rapid Transcellular Permeation of Benzodiazepines

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ABSTRACT

Water-soluble prodrugs can be rapidly converted by enzymes to hydrophobic drugs, whose aqueous thermodynamic solubilities are low, but are maintained in aqueous solution at supersaturated concentrations due to slow precipitation kinetics. Recently, we investigated avizafone (AVF) in combination with *Aspergillus oryzae* protease as a prodrug/enzyme system intended to produce supersaturated diazepam (DZP). Several fold enhancement of permeation of supersaturated DZP across Madin-Darby canine kidney II-wild type (MDCKII-wt) monolayers was observed, compared to saturated DZP solutions. However, prodrug conversion was incomplete, putatively due to partial racemization of AVF and stereoselectivity of *A oryzae* protease. Here we report synthesis of chirally pure AVF, and demonstrate complete conversion to supersaturated DZP followed by complete DZP permeation at enhanced rates across MDCKII-wt cell monolayers. We also synthesized, for the first time, a chirally pure prodrug of midazolam (MDZ-pro) and carried out the same sequence of studies. *A oryzae* protease was identified as a benign and efficient activating enzyme for MDZ-pro. The MDZ-pro/*A oryzae* protease system showed greater than 25-fold increase in absorption rate of MDZ across MDCKII-wt monolayers, compared to saturated MDZ. Such chirally pure prodrug/enzyme systems are promising candidates for efficient intranasal delivery of benzodiazepine drugs used in the treatment of seizure emergencies.

Introduction

Benzodiazepines (BZDs) such as diazepam (DZP), lorazepam, and midazolam (MDZ) are drugs of first choice for treating seizure emergencies in individuals with epilepsy. Such emergencies require rapid drug delivery to the brain, preferably within 5-10 min.[1] This condition is most frequently treated using intravenous (i.v.) lorazepam or rectal DZP.[2] However, i.v. administration requires admission to an emergency department or the presence of a skilled caregiver, while the rectal route exhibits poor social acceptance and patient compliance.

Because of the difficulties associated with i.v. and rectal administration of BZDs, the intranasal route has attracted recent interest.[3-8] However, the limited volume of the human nasal cavity mandates highly concentrated drug solutions, which are difficult to prepare and store in aqueous media due to the low solubility of BZDs. As a result, several nasal formulations containing organic solvents and surfactants have been developed.[9-12] Because these organic additives may cause irritation and potential toxicity, aqueous formulations would be preferable.

Recently, we reported an aqueous prodrug/enzyme system that may enable rapid intranasal absorption of DZP.[6] Supersaturated drug solutions were prepared *in situ* by co-administering the

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prodrug avizafone (AVF) with a converting enzyme, *Aspergillus oryzae* protease, onto Madin-Darby canine kidney II-wild type (MDCKII-wt) monolayers, a model for the nasal epithelium. These prodrug-enzyme mixtures, which were prepared immediately before administration, enabled several fold faster permeation across the monolayers, without drug precipitation, compared to saturated aqueous DZP. However, prodrug transformation by *A oryzae* protease was incomplete: at most 70%-80% of AVF was converted to DZP, irrespective of prodrug/enzyme ratio and reaction time. Upon further investigation, the AVF used in that study was shown to be a mixture of enantiomers, which we hereafter designate as AVF_m, leading us to speculate that conversion by *A oryzae* protease is stereospecific.

The success of the point-of-administration AVF/*A oryzae* protease technique in accelerating permeation suggests that the water-soluble prodrug/converting enzyme approach might apply to other poorly soluble BZDs. Of particular interest is MDZ, which is increasingly being used for out-of-hospital treatment of seizure emergencies because of its greater potency and lipid solubility.[13]

In this communication we describe experiments in which we synthesized chirally pure AVF (AVF_p) and compared its performance against the enantiomeric mixture with respect to enzymatic conversion and permeation of the DZP product across MDCKII-wt monolayers. We also report the first synthesis of a chirally pure prodrug of MDZ. Remarkably, this prodrug (MDZ-pro) is shown to be a substrate for conversion by *A oryzae* protease. Enzyme kinetics and *in vitro* permeability studies using MDCKII-wt monolayers were also performed for the MDZ-pro/*A oryzae* protease system. This work provides an expanded basis for developing aqueous prodrug/enzyme systems for nasal delivery of poorly soluble BZDs.

Materials and Methods

Materials

DZP, MDZ, phenytoin (internal standard or I.S.), *A oryzae* protease (1474.47 U/mL, cat# P6110), and chemicals used for "cell assay buffer" pH 7.4 (122 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3 mM KCl, 1.2 mM $MgSO_4$, 1.4 mM $CaCl_2$, and 0.4 mM K_2HPO_4) were purchased from Sigma. Lucifer yellow, analytical grade methanol, acetonitrile, and water were purchased from Fisher Scientific. Dulbecco's modified Eagle's medium, antibiotics, and fetal bovine serum were purchased from Invitrogen. MDCKII-wt cells were generously provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam).

Synthesis and Characterization of Prodrugs

AVF_m was synthesized as previously described.[6] AVF_p (*S*-enantiomer of AVF) was synthesized by reacting (5-chloro-2-(methylamino) phenyl) (phenyl)methanone, first with a glycine derivative and then with a lysine derivative to form a lysine-glycine dipeptide side chain. MDZ-pro was produced from MDZ by opening the diazepine ring and adding a lysine group. Details of synthesis, purification, and characterization of AVF_p and the MDZ-pro are presented in Supporting Information.

Enzyme Kinetics

To determine the enzyme kinetic parameters K_M and V_{max} for a prodrug/enzyme system, various concentrations of prodrug were incubated in assay buffer, pH 7.4 containing *A oryzae* protease, at 32°C, for a fixed time period *t*, at which point the reaction was stopped using methanol as an enzyme denaturant. Conversion from prodrug to active drug for each initial prodrug concentration was determined by high performance liquid chromatography (HPLC). The reactions were carried out to times when there was already substantial conversion. Concentrations of product, $C_d(t)$, were fit against initial prodrug concentrations, $C_p(0)$, using the integrated Michaelis-Menten equation:

$$C_d(t) + K_M \ln\left[1 - C_d(t)/C_p(0)\right] = V_{max} t \quad (1)$$

Fitting was carried out using the MATLAB nlinfit function (Mathworks).

Cell Monolayers

Following previously published procedures,[6,7] MDCKII-wt (passages between 10 and 20) cells were cultured and grown as monolayers in 12-well transwell plates (0.4 mm pore size, polyester; Corning). Transepithelial electrical resistance (TEER) was measured to assure membrane integrity just prior to conversion/permeation experiments. TEER and a Lucifer yellow dye permeability assay were used to reassess monolayer integrity at the conclusion of each experiment.

HPLC

The HPLC method for analyzing AVF and DZP is published elsewhere.[6] MDZ-pro and MDZ concentrations were analyzed using HPLC (Beckman Coulter SYSTEM GOLD: solvent module 126, autosampler 508, and UV detector 166 with 32.0 Karat software v5). The mobile phase was 50 mM monobasic potassium phosphate (containing 0.2% diethylamine) and acetonitrile (30:70 vol/vol), and the flow rate was 2 mL/min. For analysis, a 50 μL sample (containing 4 μg/mL I.S.) was injected onto a SUPELCOSIL LC-18 column (250 × 4.6 mm, 5 μm particle size) and chromatograms were obtained at 220 nm. The drug peak was normalized by an I.S. (phenytoin) peak area and converted to drug concentration using a calibration curve. A typical HPLC chromatogram for MDZ-pro, MDZ, and phenytoin is shown in Figure S1 (Supporting Information), with validation parameters (Table S1 in Supporting Information) as per FDA guidelines.[14]

Supersaturated MDZ Solutions

Appropriate molar concentrations of MDZ-pro equivalent to supersaturated MDZ solution were incubated with the converting enzyme in assay buffer, pH 7.4 at 32°C (nasal temperature). Supersaturation potential (SP) was calculated as the ratio between the molar concentration of administered MDZ-pro and the molar concentration of saturated MDZ:

$$SP = \frac{\text{Initial}[\text{MDZ} - \text{pro}]}{\text{Saturated}[\text{MDZ}]}$$

This is the degree of supersaturation that would be attained if enzymatic conversion on MDZ-pro to MDZ was immediate and without precipitation.

Statistics

For multiple comparisons, one-way analysis of variance with Dunnett's *post hoc* test was used.