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Water-soluble benzodiazepine prodrug/enzyme combinations for intranasal rescue therapies

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ABSTRACT

Benzodiazepines (BZDs), including diazepam (DZP) and midazolam (MDZ), are drugs of choice for rapid treatment of seizure emergencies. Current approved use of these drugs involves administration via either intravenous or rectal routes. The former requires trained medical personnel, while the latter is socially unacceptable for many patients and caregivers. In recent years, efforts have been made to formulate BZDs for nasal administration. Because of the low solubility of these molecules, organic vehicles have been used to solubilize the drugs in the nasal products under development. However, organic solvents are irritating, potentially resulting in injury to nasal tissue. Here we report preliminary studies supporting a strategy in which water-soluble BZD prodrugs and a suitable converting enzyme are coadministered in an aqueous vehicle. Diazepam and midazolam prodrugs were synthesized and were readily converted to their active forms by a protease from *Aspergillus oryzae*. Using a permeation assay based on monolayers of Madin–Darby canine kidney II-wild type cells, we found that enzymatically produced BZDs could be maintained at high degrees of supersaturation, enabling faster transport across the membrane than can be achieved using saturated solutions. This strategy not only obviates the need for organic solvents, but it also suggests more rapid absorption and earlier peak concentrations than can be otherwise achieved.

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1. Introduction

Ideally, seizure emergencies should be treated rapidly in order to avoid brain injury, which may occur secondary to prolonged or cluster seizures. Benzodiazepines (BZDs), including diazepam (DZP) and midazolam (MDZ), are the drugs of choice [1,2], but there are practical problems in administering these drugs. Intravenous (iv) administration is optimal from a pharmacokinetic/pharmacodynamic point of view, but it requires trained personnel and may involve long delays due to transport to emergency care facilities. A rectal DZP formulation is also available, but many older children and adults as well as their caregivers find this method of drug administration socially unacceptable. For these reasons, considerable effort has been spent in developing intranasal delivery systems for both DZP and MDZ.

The nasal route poses several challenges. First, the total volume that can be administered via the nose is quite small (~200 μL). Second, BZD aqueous solubilities are very low, which has led to the conclusion that it is impossible to use aqueous vehicles in nasal formulations. In recent years, considerable effort has been spent in developing organic [3,4], mixed aqueous/organic [5,6], and microemulsion-based formulations [7–9] for intranasal BZD administration. While these strategies may meet many of the pharmacokinetic and pharmacodynamic requirements, introduction of organic solvents may lead to irritation and potential risk of side effects.

In this report, we outline and provide evidence in support of an approach for development of aqueous BZD formulations that do not require organic solvents. In this approach, highly water-soluble lysine prodrugs of DZP and MDZ are intranasally coadministered with converting enzymes in an aqueous vehicle. The concentrations of enzymatically released active BZDs can rise far above their maximum solubilities, since precipitation of the active drugs, though thermodynamically favored, is

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kinetically limited. It is postulated and demonstrated, using a cell culture monolayer assay, that the highly supersaturated active drugs are rapidly absorbed across cell membranes. This approach, besides providing an aqueous means to deliver BZDs, may also lead to accelerated absorption. Such formulations would be safer, allow intranasal administration of larger doses in small volumes, and result in earlier termination of seizures.

2. Methods

Details of materials and methods are reported elsewhere [10,11]. Briefly, the HCL forms of the lysine prodrugs, DZP-pro and MDZ-pro, were synthesized and purified. Early studies showed that the selected converting enzyme was stereospecific, so extra steps were taken to ensure enantiopurity of the prodrugs. Each prodrug was tested for conversion by a panel of commercially available proteases, peptidases, aminopeptidases, and esterases, using HPLC. For both prodrugs, *Aspergillus oryzae* (*A.O.*) *protease* was found to be a suitable converting enzyme, and its Michaelis–Menten parameters, K_M and V_{max}, were determined.

As an in vitro model for nasal absorption, Madin–Darby canine kidney II-wild type (MDCKII-wt) cells were cultured as monolayers on polyester membranes in 12-well Transwell plates, as shown in Fig. 1. The basal chamber of the monolayer was filled with a 1.2-mL drug-free assay buffer (cell assay buffer, pH = 7.4). At time zero, proper concentrations of prodrug and enzyme were combined in the assay buffer, with a volume of 0.2 mL, and spiked into the apical chamber. At various time points, samples were taken, with replacement, from the basal and apical chambers, and concentrations of prodrugs and active drugs were determined by HPLC. By this means, the flux of prodrugs and active drugs across the monolayer could be determined, along with the degree of supersaturation on the apical side, as a function of time. The integrity of the monolayer was confirmed by measuring Lucifer yellow permeability and transepithelial electrical resistance. The conversion-transport experiments were carried out at 32 °C, which well approximates the temperature of the intranasal cavity.

3. Results

The structures of DZP-pro and MDZ-pro are shown in Scheme I. The DZP prodrug was prepared by reacting (5-chloro-2-(methylamino) phenyl)(phenyl)methanone sequentially with a glycine and a lysine derivative to form a lysine–glycine dipeptide [10]. The MDZ prodrug was produced by opening the diazepine ring of MDZ and attaching a lysine endgroup [11]. There are two ionizable amines on the dipeptide, which are protonated at physiological pH, leading to high solubility. When the terminal lysine is cleaved by the enzyme, the glycine rapidly reforms the diazepine ring, and the ionization sites are lost. Midazolam and MDZ-pro also possess another protonation site with a very low pKa, which is not relevant to our formulation.

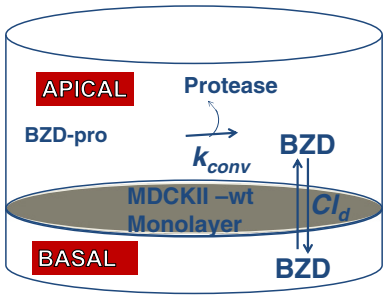

Fig. 1. Schematic of the Transwell assay used to assess BZD prodrug concentration to active BZD, followed by reversible transport across the MDCKII-wt monolayer membrane. The parameters k_{conv} and Cl_d refer to rate constants for enzymatic conversion of BZD-pro to DZP and the clearance (permeability × area) of drug across the monolayer, respectively. Adapted from Reference [13] with permission.

The DZP prodrug has been known for several decades as avizafone (AVF). It was originally developed for battlefield anticonvulsant applications [12]. Aqueous AVF is administered by intramuscular injection and is converted to DZP by endogenous enzymes. To our knowledge, these enzymes have not been identified. We conducted preliminary studies (unpublished) in dogs, which showed that only a fraction of AVF is converted to DZP endogenously and absorbed. We therefore screened a panel of commercially available enzymes that were known to cleave lysine–glycine peptide bonds, and *A.O. protease* was found to convert both prodrugs to their corresponding active forms. The Michaelis–Menten parameters ± s.e.m. are as follows:

$$\begin{array}{ll} \text{DZP-pro}: & K_M = 1.19 \pm 0.19\,\mu\text{M}; \quad V_{max} = 0.108 \pm 0.007\,\mu\text{M/min (at 0.25 U/mL)} \\ \text{MDZ-pro}: & K_M = 5.86 \pm 0.83\,\mu\text{M}; \quad V_{max} = 0.223 \pm 0.022\,\mu\text{M/min (at 4 U/mL)}. \end{array}$$

Having successfully identified an enzyme, we moved to the Transwell assay (Fig. 1) to assess the ability of the BZD-pro$_s$ and *A.O. protease* to create supersaturated BZD solutions, which would then cross model cell membranes. Figs. 2a and b present the time course of appearance of DZP in the apical and basal compartments of the Transwell, respectively, following coadministration of DZP-pro and *A.O. protease* at various prodrug and enzyme concentrations. According to Fig. 2a, DZP-pro was converted rapidly to DZP in the apical chamber, achieving peak concentration in 5–10 min and with conversion more rapid at higher enzyme concentration. Accumulation of DZP in the apical chamber was also proportional to initial DZP-pro concentration. Following the peak, the concentration of DZP in the apical chamber decayed exponentially. The blue horizontal line in Fig. 2a corresponds to the aqueous solubility of DZP, $C_{d,sat}$. It can be concluded, based on this figure, that DZP was present in the apical chamber at highly supersaturated concentrations following conversion. The absence of precipitates was confirmed under an optical microscope, and membrane integrity remained satisfactory.

Fig. 2b charts the appearance of DZP in the basal compartment as a function of time. The concentration rose and eventually reached a final value, with initial rate and final concentration proportional to the concentration of DZP-pro administered in the apical compartment. A convenient parameter for identifying these curves is the *supersaturation potential*, *S*, which is the molar ratio of administered BZD-pro (here DZP-pro) to $C_{d,sat}$. This ratio can be interpreted as the degree of supersaturation of active BZD that would be achieved if all prodrug was converted instantly upon administration. Curves in Fig. 2b are labeled with *S*. In Fig. 2c, the initial rate of flux across the membrane is plotted versus *S*. The linearity of this plot, all the way to the highest value of *S*, provides further confidence that there was no precipitation of supersaturated drug in the apical chamber, since such a process would cause the graph in Fig. 2c to reach a plateau.

An analogous set of plots for MDZ-pro and MDZ is presented in Fig. 3. Similar behaviors are observed as in Fig. 2. Together, these studies establish that rapid enzymatic conversion of BZD prodrugs leads to highly supersaturated active BZD, which can be rapidly absorbed across cell membranes without precipitation.

4. Discussion and conclusions

The enzyme *A.O. protease* is derived from a fungal source and may be allergenic. While human converting enzymes have not yet been identified, it is probable that they exist since AVF (DZP-pro) is converted to DZP following intramuscular injection. Also, *A.O. protease* was identified following a very limited search. A directed, homology-based search of