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Pharmacological effects of a novel isosorbide-based butyrylcholinesterase inhibitor

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ABSTRACT

Isosorbide-2-benzylcarbamate-5-benzoate, a novel butyrylcholinesterase inhibitor, shows interspecies variation in its inhibitory activity (IC_{50} of 4.3 nM for human plasma butyrylcholinesterase, but 1.09 μM for mouse plasma butyrylcholinesterase). Stability studies revealed that this drug is resistant to hydrolysis by human plasma (no degradation in 1 h). However, it was found to undergo rapid degradation when incubated with mouse plasma or mouse liver homogenate, yielding benzyl carbamate and benzoic acid. The addition of the carboxylesterase inhibitor bis-(4-nitrophenyl) phosphate (BNPP) inhibited the degradation of the novel drug, indicating that it may be a substrate for both butyrylcholinesterase and carboxylesterase. The absence of carboxylesterase from human plasma explains the drug's stability in this medium. *In vivo*, pharmacodynamic studies on single doses of 1 mg/kg to naïve male C57BL/6 mice revealed maximal plasma butyrylcholinesterase inhibition 20 min after intraperitoneal administration (~60% inhibition) and 1 h after administration by gavage (~45% inhibition). While this plasma butyrylcholinesterase inhibition was short-lived, the drug also penetrated the blood–brain barrier resulting in a slight (10–15%) but persistent (≥72 h) reduction in brain butyrylcholinesterase activity.

1. Introduction

Butyrylcholinesterase (EC 3.1.1.8, BuChE) is found in neurons and glial cells in the human brain, and peripherally in serum [1]. Although the exact function of BuChE remains unclear, it is responsible for the metabolism of various drugs including the anaesthetic succinylcholine [2], and it efficiently hydrolyses acetylcholine (ACh) at high concentrations [3]. In Alzheimer's disease, BuChE may be increased 40–90% in the brain and high levels of BuChE are found in neuronal plaques [4,5]. Novel and potent inhibitors displaying exceptionally high selectivity for BuChE have been developed in our laboratories, providing a unique pharmacological opportunity to explore the physiological and pathological roles of BuChE and the potential for BuChE inhibition as a therapeutic strategy in Alzheimer's disease [6]. One such compound, isosorbide-2-benzylcarbamate-5-benzoate (compound 51, Fig. 1), which displays >60,000-fold selectivity for BuChE over acetylcholinesterase (AChE), has been tested *in vitro* and *in vivo*. This paper describes studies intended to reconcile the metabolism of compound 51 with its *in vivo* activity.

2. Methods

2.1. In vitro butyrylcholinesterase inhibition

BuChE inhibition was measured *in vitro* using a modification of the Ellman assay [7] employing butyrylthiocholine as substrate [8] and a standardized BuChE concentration (5.88 μmol min^{-1} ml^{-1}). Plasma was removed from fresh blood centrifuged at 3000 rpm for 10 min at 4 °C (Sorvall RT6000B). A stock solution of the drug was prepared in a 1:3 solution of acetonitrile:PBS (phosphate

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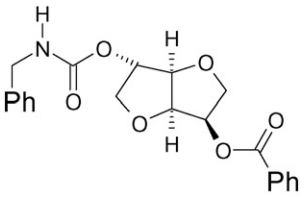

Fig. 1. Compound 51, isosorbide-2-benzylcarbamate-5-benzoate.

buffered saline) pH 8.0 from which serial dilutions (100 μM to 1 nM) were made. The drug and plasma were incubated in 0.1 M PBS (pH 8.0) with 0.3 mM DTNB (5-5′-dithiobis(2-nitrobenzoic) acid) at 37 °C for 30 min. Thereafter 0.5 mM of BTCI (butyrylthiocholine iodide) was added and the absorbance was measured at 405 nm. IC_{50} values were determined from non-linear regression plots using GraphPad Prism 4.0.

2.2. In vivo butyrylcholinesterase inhibition

Healthy naïve male C57BL/6 mice (Harlan, UK) were housed singly with *ad libitum* access to food and water under a 12 h light/dark cycle. A single dose of compound 51 (1 mg/kg) was administered intraperitoneally (ip) or orally (po). At various time points ($n = 3$ per timepoint) submandibular blood samples were collected in lithium heparinized Microvette tubes and centrifuged at 3000 rpm for 10 min at 4 °C. Plasma BuChE activity was determined using the Ellman assay [7] as previously described. Absorbance values were plotted using GraphPad Prism 4.0.

At the study endpoints, 24, 48 and 72 h post administration of the compound, the level of BuChE inhibition in the brain was assessed. The animals were euthanized by carbon dioxide asphyxiation and the brains were carefully removed and placed on ice until processed. The brain samples were homogenized in physiological saline on ice in three 5-s bursts using an Ultra-turrax T10. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. BuChE activity was analysed using the Ellman assay [7].

2.3. Hydrolysis studies

The rate of hydrolysis of compound 51 was determined *in vitro* in human plasma, mouse plasma and mouse liver homogenate using a gradient HPLC method.

2.3.1. Preparation of plasma samples

Fresh plasma samples were prepared by centrifugation of heparinized venous human or mouse blood for 10 min at 3000 rpm at 4 °C. One millilitre of plasma was added to 2 ml of PBS pH 7.4 (33% solution).

2.3.2. Preparation of liver samples

Mouse liver was homogenized on ice in 10 volumes of PBS pH 7.4 in three 5-s bursts using an Ultra-turrax T-10 (IKA-Werke). The homogenate was then centrifuged for 10 min at 10,000 rpm and 4 °C. Liver homogenate (20 μl) was added to 3 ml of PBS pH 7.4.

2.3.3. Hydrolysis

One hundred microlitres of compound 51 (8 μM) in acetonitrile was added to the plasma/buffer or liver/buffer solution and incubated at 37 °C. Aliquots (250 μl) were taken at various time points and transferred to Eppendorfs containing 500 μl of a 2% zinc sulfate solution in acetonitrile (1:1). The Eppendorfs were then centrifuged at 10,000 rpm for 7 min. A 20-μl aliquot of the clear supernatant was analysed by HPLC.

Hydrolysis studies using the carboxylesterase inhibitor, bis-(4-nitrophenyl) phosphate (BNPP), were also carried out to determine if carboxylesterase played a role in the hydrolysis of compound 51. BNPP (100 μM) was incubated at 37 °C with the mouse plasma or liver homogenate for 10 min prior to drug addition.

2.3.4. Chromatography

High-performance liquid chromatography was performed using a system consisting of a Waters 600 pump and controller, Waters 717 autosampler and a Waters 2996 photodiode-array (PDA) detector controlled by Empower 2 software (Waters). A Hichrom Nucleosil C18 4.0 mm × 250 mm column was used. The plasma and liver samples were both analysed using a gradient method consisting of a 16 mM phosphate buffer (pH 2.5): acetonitrile 80:20 for the first 10 min, grading to 20:80 for the next 5 min, then 60:40 for the next 2 min and finally returning to 80:20 for the last 3 min of the 20 min run. The flow rate was 1 ml/min and the buffer and acetonitrile were helium sparged at a rate of 20 ml/min. The retention times in this system were 9.5 min for benzyl carbamate, 10.0 for benzoic acid and 15.2 min for compound 51. Peak areas were compared quantitatively with external standards of approximately the same concentration. The gradient method was validated for specificity, sensitivity and precision.

2.4. Statistical analysis

BuChE activity data were entered into SPSS v.14 (SPSS Inc., Chicago) for analysis. Standard descriptive parameters were calculated and significant differences were determined using Student's *t*-test where appropriate, taking $p < 0.05$ to be significant.

3. Results and discussion

Compound 51 (isosorbide-2-benzylcarbamate-5-benzoate) was synthesised in house as part of an ongoing programme of research into isosorbide-based cholinesterase inhibitors, and has proved to be a potent inhibitor of human plasma BuChE *in vitro*. However, interspecies differences were apparent, its IC_{50} of 4.3 nM in human plasma contrasting with an IC_{50} of 1.09 μM in mouse plasma.

In vivo, a single dose of 1 mg/kg given to male C57BL/6 mice displayed maximal plasma BuChE inhibition 20 min after ip administration (~60% inhibition), and 1 h after administration po (~45% inhibition). However, the duration of action was short-lived by both routes, restoration of BuChE activity being substantially complete