European Journal of Pharmacology 770 (2016) 117–125

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

In vitro bidirectional permeability studies identify pharmacokinetic limitations of NKCC1 inhibitor bumetanide

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ARTICLE INFO

Article history:
Received 6 August 2015
Received in revised form
25 November 2015
Accepted 1 December 2015
Available online 8 December 2015

Keywords:
Bidirectional permeability assay
Efflux transport
Bumetanide
Neonatal seizures
Pharmacokinetics
Blood–brain barrier

Chemical compounds studied in this article:
Bumetanide (PubChem CID: 2471)
Probenecid (PubChem CID: 4911)
Penicillin G Sodium (PubChem CID: 23668834)

ABSTRACT

Recently, it has been suggested that bumetanide, an inhibitor of the Na–K–2Cl co-transporter (NKCC1), may be useful in the treatment of central nervous system (CNS) disorders. However, from a physicochemical perspective, bumetanide may not cross the blood–brain barrier to the extent that is necessary for it to be an effective brain NKCC1 inhibitor *in vivo*. High plasma–protein binding, potentially high brain–tissue binding and putative efflux transporters including organic anion transporter 3 (OAT3) contribute to the poor pharmacokinetic profile of bumetanide. Bidirectional permeability assays are an *in vitro* method to determine the impact of plasma-protein/brain tissue binding, as well as efflux transport, on the permeability of a compound. We established and validated a cell line stably overexpressing human OAT3 using lentiviral cloning techniques for use in *in vitro* bidirectional permeability assays. Using efflux transport studies, we show that bumetanide is a transported substrate of human OAT3, exhibiting a transport ratio of ≥ 1.5, which is attenuated by OAT3 inhibitors. Bidirectional permeability assays were carried out in the presence and absence of either albumin or brain homogenate to elucidate the effect of plasma-protein/brain tissue binding. These tests confirmed the pharmacokinetic limitations for brain delivery of bumetanide. In this experiment, bumetanide is 53% bound to albumin, 77% bound to brain tissue and accumulates in brain cells. Moreover, we conclusively established that bumetanide is a transported substrate of OAT3. Taken together, these bidirectional permeability studies highlight the potential of efflux transporter inhibition as an augmentation strategy for enhanced delivery of bumetanide to the CNS.

1. Introduction

It has been proposed that bumetanide, an inhibitor of the cation chloride Na–K–2Cl cotransporter (NKCC1), may be useful in the treatment of central nervous system (CNS) conditions, including neonatal seizures and autism (Dzhala et al., 2008; Lemonnier et al., 2012). Recently, bumetanide has demonstrated the ability to normalise cortical and hippocampal neuronal activity and prevent structural injury in the hippocampus in a mouse model of benign idiopathic epilepsy syndromes (Marguet et al., 2015). However, in many preclinical studies examining bumetanide in different seizure models, it has shown limited antiepileptic potential, which may be partially due to the fact that it is unclear if bumetanide crosses the blood–brain barrier (BBB) to a sufficient extent to be therapeutically useful *in vivo* (Cleary et al., 2013; Dzhala et al., 2005; Liu et al., 2012; Mares, 2009; Mazarati et al., 2009, Puskarjov et al., 2014).

A dose-finding clinical study of bumetanide in the treatment of neonatal seizures has recently reported an adverse benefit: risk ratio, which may in part be due to the underwhelming efficacy of bumetanide caused by restricted access across the BBB (Pressler

Abbreviations: A→B, apical to basolateral; B→A, basolateral to apical; BBB, blood–brain barrier; CNS, central nervous system; DMEM, Dulbecco's modified Eagle medium; eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FBS, foetal bovine serum; f_u plasma albumin, fraction unbound in plasma albumin; f_u brain, fraction unbound in brain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HEK, human embryonic kidney; hOAT3, human organic anion transporter 3; HPLC, high-performance liquid chromatography; lv, lentiviral; MDCKII, Madin–Darby canine kidney cell line; MDCKII-WT, wildtype MDCKII cells; NKCC1, Na–K–2Cl transporter; NEAA, non-essential amino acids; OAT3, organic anion transporter 3; P, passage; P_{app}, apparent permeability; V_u brain, volume of distribution in brain

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http://dx.doi.org/10.1016/j.ejphar.2015.12.001
0014-2999/

et al., 2015; Puskarjov et al., 2014). Based on physicochemical properties such as plasma protein binding and ionisation, bumetanide permeability across the BBB is expected to be very low, and this has been shown *in vivo* in adult and neonatal rats (Cleary et al., 2013; Donovan et al., 2015). Moreover, it has been reported that bumetanide interacts with efflux transporters, including rat organic anion transporter 3 (Oat3), an efflux transporter that is expressed on the basolateral membrane of both the proximal tubule cells in the kidney and at the BBB (Harati et al., 2013; Hasannejad et al., 2004; Kusuhara et al., 1999; Uchida et al., 2007). Previous studies investigating the possible augmentation of bumetanide concentrations in the brain using Oat inhibitor probenecid have demonstrated that the increased bumetanide levels are predominantly due to inhibition of Oat3 in the kidney, which results in reduced clearance and increased plasma concentrations of bumetanide (Donovan et al., 2015; Tollner et al., 2015). There are species differences in substrate specificities of OATs, including for OAT3 (Chu et al., 2013; Tahara et al., 2005). Thus, it is imperative to examine the interaction of bumetanide with human OAT3 (hOAT3) to establish if hOAT3 inhibition is a feasible therapeutic augmentation strategy for bumetanide treatment of CNS disorders in a clinical setting.

Bidirectional permeability assays are reported to be the most direct method of measuring efflux (Polli et al., 2001). We established a hOAT3-overexpressing Madin–Darby canine kidney (MDCKII) cell line for use in bidirectional permeability assays to measure the interaction of hOAT3 and bumetanide. MDCKII cells were chosen because they have shown promise to distinguish between compounds that enter the brain by passive diffusion versus active influx/efflux, as well as being of kidney origin which is the proposed main site of OAT3 inhibition (Donovan et al., 2015; Garberg et al., 2005; Tollner et al., 2015). Inhibitors of hOAT3, probenecid and penicillin G, were employed to ascertain if hOAT3 was responsible for efflux. Probenecid has previously demonstrated an ability to reduce efflux of compounds via Oat3, and is safe for clinical use (Maeda et al., 2014; Minematsu et al., 2008; Miyajima et al., 2011; Robbins et al., 2012). In addition, penicillin G has shown specificity for hOAT3 over human OAT1, while lacking affinity for organic anion-transporting polypeptide 2, which can be found endogenously in MDCKII cells (Nozaki et al., 2007; Ohtsuki et al., 2007; Sasaki et al., 2002; Tollner et al., 2015).

Furthermore, it has been reported that a variation of the bidirectional permeability assay can be employed to determine pharmacokinetic parameters that predict the rate and extent of transport of bumetanide across the BBB, including the fraction unbound in plasma albumin (f_u plasma albumin), fraction unbound in brain (f_u brain) and volume of distribution in brain (V_u brain) (Mangas-Sanjuan et al., 2013). These parameters are useful for future *in silico* predictions of bumetanide concentration–time profiles.

2. Materials and methods

All chemicals were purchased from Sigma Aldrich (Arklow, Ireland) unless otherwise stated.

2.1. Establishing a hOAT3-overexpressing cell line

A hOAT3-overexpressing MDCKII cell line was established using lentiviral (lv) cloning techniques. Briefly, hOAT3 lentiviral particles were produced using a lentiviral expression plasmid containing an open reading frame of hOAT3 (accession code NM_001184732.1) linked by an internal ribosome entry site to an enhanced green fluorescent protein (eGFP) tag (Ex-Z0349-Lv205, Source BioScience UK Ltd, Nottingham) and a third generation packaging kit (Genecopoeia Lenti-Pac HIV Expression Packaging Kit, Source BioScience UK Ltd, Nottingham).

Human embryonic kidney (HEK) 293T/17 cells were seeded in a 10 cm dish at a density of 1.4 million cells/10 ml two days prior to transfection, to ensure that cells were 70–80% confluent at the time of transfection. DNA–endofectin lenti complexes were formed as per manufacturer's instructions with both hOAT3-eGFP expression plasmid and eGFP control plasmid (Invitrogen, Dun Laoghaire, Co. Dublin). These complexes were added dropwise to plated HEK 293T/17 cells, and incubated overnight. The culture medium was replaced with fresh media consisting of Dulbecco's modified Eagle medium (DMEM), 2% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 1% penicillin–streptomycin and 0.2% TiterBoost within 14 h of transfection. The virus-containing medium was collected 48 h post transfection and filtered through a 0.45 μm low protein-binding filter.

Transductions were carried out by seeding wild-type cells (MDCKII-WT) in 24-well plates at a density of 100,000 cells per well. After overnight growth, each well was incubated with a dilution of virus in transduction media (DMEM, 2% FBS, 8 μg/ml polybrene) at 4 °C for two hours before being transferred to a 37 °C incubator. Following another overnight incubation, transduction media was replaced with regular media containing puromycin 4 mcg/ml to select for transduced cells. After 24–48 hours, fluorescence was visualised using an inverted microscope (CKX41, Olympus, Hamburg) set-up with a sensitive XM10 camera (C-BUN-F-XM10-BUNDLE) with an infrared filter, mercury burner (USH-103OL) and fluorescence condenser (CKX-RFA, Olympus). The percentage of successfully transduced cells was determined using flow cytometry (BD Accuri C6 Flow Cytometer).

Polyclonal populations of > 90% fluorescent MDCKII-lv-eGFP and MDCKII-lv-hOAT3-eGFP were selected using a BD fluorescence-activated cell sorter (FACS) Aria II 7-colour. 7-aminoactinomycin viability stain (5 μl) (eBioscience Ltd., Hatfield, United Kingdom) was added to the cells prior to FACS in order to exclude any non-viable cells. Cell populations were propagated and puromycin 4 mcg/mL was added with each media change to select for transduced cells, resulting in populations that were 60–80% fluorescent.

2.2. Western blot

Western blot was carried out on cell pellets of MDCK-lv-hOAT3-eGFP and MDCK-lv-eGFP to confirm protein expression of OAT3 following transduction. Cell pellets were collected at each passage between passages 8 and 11 following trypsin treatment. These were stored at −20 °C until analysis. Cell pellets (containing approximately 6 million cells) were lysed in 300 μl lysis buffer (T-Per lysis buffer containing Roche cOmplete Ultra protease and PhosSTOP phosphatase inhibitors). Cell protein was quantified using Pierce BCA protein quantification kit (Life Technologies, Dun Laoghaire, Co., Dublin) and cell protein samples were made up with sample buffer and deionised water to a final concentration of 1 mg/ml. Following denaturation at 95 °C for 5min, 30 mcg of cell protein was loaded into each lane of a 4–20% ExpressPlus PAGE Gel (Genscript), and run at 60–100 V for 2 h. After transfer onto a 0.2 μm polyvinylidene difluoride membrane and blocking with 5% skimmed milk and 0.1% Tween in Tris-buffered saline, the membrane was cut at the 45 kDa mark and the section from 45 to 100 kDa was incubated overnight at 4 °C with the OAT3 primary polyclonal antibody (1:2000 dilution in 0.5% skimmed milk) (antibodies-online, Aachen, Germany), while the section from 20 to 45 kDa was probed under the same conditions with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary monoclonal antibody (1:20,000 dilution in 0.5% skimmed milk). Both membranes were incubated with a secondary antibody for 2 h at