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CNS uptake of bortezomib is enhanced by P-glycoprotein inhibition: implications for spinal muscular atrophy

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ABSTRACT

The development of therapeutics for neurological disorders is constrained by limited access to the central nervous system (CNS). ATP-binding cassette (ABC) transporters, particularly P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), are expressed on the luminal surface of capillaries in the CNS and transport drugs out of the endothelium back into the blood against the concentration gradient. Survival motor neuron (SMN) protein, which is deficient in spinal muscular atrophy (SMA), is a target of the ubiquitin proteasome system. Inhibiting the proteasome in a rodent model of SMA with bortezomib increases SMN protein levels in peripheral tissues but not the CNS, because bortezomib has poor CNS penetrance. We sought to determine if we could inhibit SMN degradation in the CNS of SMA mice with a combination of bortezomib and the ABC transporter inhibitor tariquidar. In cultured cells we show that bortezomib is a substrate of P-gp. Mass spectrometry analysis demonstrated that intraperitoneal co-administration of tariquidar increased the CNS penetrance of bortezomib, and reduced proteasome activity in the brain and spinal cord. This correlated with increased SMN protein levels and improved survival and motor function of SMA mice. These findings show that CNS penetrance of treatment for this neurological disorder can be improved by inhibiting drug efflux at the blood–brain barrier.

1. Introduction

Pharmacotherapy for neurodegenerative disorders is hampered by the inability of most small molecules to cross the blood–brain barrier (BBB) and the blood spinal cord barrier (BSCB). Three main cell types, the endothelial cells, pericytes, and astrocytes form the BBB and BSCB and protect the central nervous system (CNS) by restricting diffusion of many solutes into the cerebrospinal fluid. This is achieved through a combination of tight junctions formed by the endothelial cells, a lack of pinocytotic activity, and efflux transporters. The predominant efflux transporters, P-glycoprotein (P-gp, *ABCB1*) and breast cancer resistance protein (BCRP, *ABCG2*), are members of the ATP-binding cassette (ABC) class. They are expressed on the apical surface of endothelial cell and function to intercept drugs entering the CNS capillary cells and transport them against their concentration gradient back into the blood (Schinkel et al., 1994, 1996; Rao et al., 1999; Cherry et al., 2013). The BBB and BSCB are fully formed (Saunders et al., 2012) and P-gp and BCRP transporters are correctly localized well before birth (Daood et al., 2008). While there are some differences between the BBB and BSCB including lower protein levels of tight junction markers and increases to permeability there are no significant differences in P-gp levels (Bartanusz et al., 2011).

Spinal muscular atrophy (SMA) is a motor neuron disorder caused by deletions and other mutations of the highly conserved survival of motor neuron-1 gene (*SMN1*) with retention of the nearly identical paralog, *SMN2*. A promising approach to treating SMA is to increase levels of SMN protein by reducing its degradation through the ubiquitin proteasome system (Lefebvre et al., 1995; McAndrew et al., 1997; Kwon et al., 2013). We have previously shown that inhibition of the proteasome by the inhibitor bortezomib increases SMN protein levels in cultured cells and in peripheral tissues of SMA mice. Long term bortezomib treatment resulted in an improvement in motor function in SMA mice compared with vehicle treated animals; however survival was unaffected (Kwon et al., 2011). Pre-clinical studies have shown that bortezomib is unable to penetrate into the CNS, impeding its use for

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treating SMA and other neurological disorders (Nakamura et al., 2007; Rumpold et al., 2007; Lu et al., 2010). To overcome bortezomib's low biodistribution in the CNS, we hypothesized that pre-treatment with an ABC transporter inhibitor would prevent bortezomib's efflux at the blood–brain and –spinal cord barriers.

In this study we used overexpress cell lines, genetic mouse models, and systemic administration of an inhibitor to demonstrate that bortezomib is a substrate of P-gp. Using mass spectrometry we were able to detect bortezomib in the CNS of SMA model mice co-administered with a P-gp inhibitor tariquidar. SMN levels were also increased in the CNS of model mice treated with both drugs. Tariquidar and bortezomib treated mice also lived longer and had improved motor function compared with tariquidar-alone, bortezomib-alone, and vehicle treated counterparts. These data suggest that P-gp inhibition increases the CNS bioavailability of a proteasome inhibitor, which is of particular interest in the development of therapeutics for neurological disorders.

2. Results

2.1. Bortezomib is transported by P-gp

In order to verify that bortezomib is transported by P-gp and not by other transporters (O'Connor et al., 2013) we used MCF-7 and HEK293 cells that express P-gp, BCRP, or no drug efflux transporters and tested bortezomib's transport. MCF-7 cells do not express high levels of P-gp or BCRP at baseline; however selection with flavopiridol increases BCRP expression (Robey et al., 2001) and selection with etoposide increases P-gp levels (Schneider et al., 1994). We therefore generated MCF-7 cells that expressed P-gp or BRCP using these selection processes. Since we were interested in the basal activity of the proteasome in the treated cells we tested several different cell lysate protein concentrations to find an appropriate concentration at which chymotrypsin-like activity of the endogenous proteasome was measurable (Supplemental Fig. 1a). The chymotrypsin-like activity of the endogenous proteasome of 70 μg of cell lysate was well above the residual activity seen with no substrate or no lysate negative controls, but less than the maximal amount seen when excess purified proteasome was tested. The chymotrypsin-like activity of the proteasome of the drug naive cells in the presence of bortezomib and tariquidar was similar to bortezomib alone ($n = 6$, $p > 0.05$) (Fig. 1a). However, MCF-7 cells that had been selected to express P-gp showed further reduction of chymotrypsin-like activity of the proteasome with tariquidar pre-treatment (1 μM, 15 min) before bortezomib treatment (0.5 μM, 30 min) ($n = 6$, $p < 0.05$) (Fig. 1b). However, pre-treatment with tariquidar did not significantly increase the inhibition of the proteasome of the BCRP overexpressing cells when compared with bortezomib alone treatment ($n = 6$, $p > 0.05$) (Fig. 1c).

HEK293 cells do not typically express P-gp (Robey et al., 2011); bortezomib (0.5 μM, 30 min) inhibited the chymotrypsin-like activity of the proteasome ($n = 6$, $p < 0.01$) and there was no additive effect when the cells were pretreated with tariquidar ($n = 6$, $p > 0.05$) (Supplemental Fig. 2a). Cells that stably express P-gp showed increased inhibition to the chymotrypsin-like activity of the proteasome when treated with tariquidar (15 min, 1 μM) before bortezomib treatment (0.5 μM, 30 min) ($n = 6$, $p < 0.05$) (Supplemental Fig. 2b). Tariquidar treatment alone does not inhibit the chymotrypsin-like activity of the proteasome in either the MCF-7 cells (Fig. 1a, b, c) or the HEK cells (Supplemental Fig. 2c). Together, these data indicated that bortezomib was a substrate of P-gp, but not of BRCP.

2.2. Bortezomib levels and proteasome inhibition are enhanced in spinal cord of P-gp1a/b BCRP knockout mice compared with control mice

We verified that bortezomib was transported by P-gp *in vivo* by treating P-gp1a/b/BCRP triple knockout mice (FVB.129P2-*Abcb1a*^{tm1Bor}:*Acb1b*^{tm1Bor}:*Abcg2*^{tm1Ahs} N7) or the parental strain (FVB) mice with a single dose of bortezomib (Velcade) by intraperitoneal (i.p.) injection (0.15 mg/kg). Mice carry two P-gp genes, unlike humans, and both genes must be knocked down to prevent drug extrusion from the cytoplasmic membrane (Croop et al., 1989). Tariquidar is a known inhibitor of both P-gp and BCRP (Bauer et al., 2013) and these triple knockout mice most closely mimic the anticipated effects of tariquidar treatment.

We measured the levels of bortezomib in the CNS by liquid chromatography–tandem mass spectrometry (LCMS/MS) and observed that bortezomib in the spinal cord was 3.2 fold higher 1 h post injection in the P-gp1a/b:BCRP knockout mouse spinal cords (257.9 ± 79.1 ng/mg tissue) compared with the spinal cords of the parental strain mice (38.7 ± 0.9 ng/mg tissue) ($p < 0.01$) (Fig. 2a). Additionally, the chymotrypsin-like activity of the proteasome was decreased in the spinal cord lysates 1 h post-injection of the bortezomib- treated P-gp1a/b/BCRP knockout mice compared with the bortezomib-treated parental strain mice ($p < 0.05$) (Fig. 2b). A single dose of bortezomib (0.15 mg/kg) did

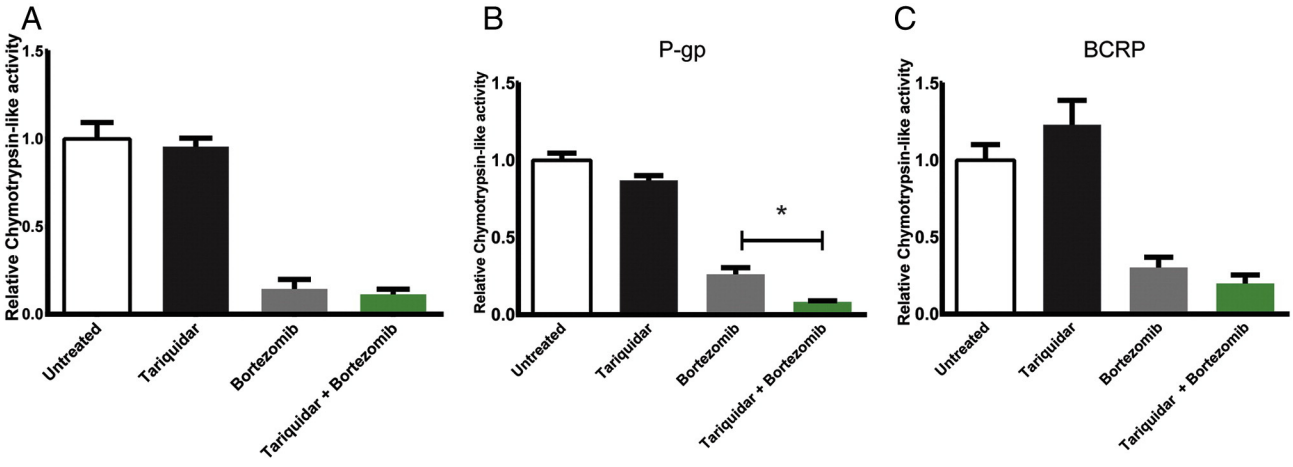

Fig. 1. Bortezomib is transported by P-gp1, but not by BCRP in cultured MCF-7 cells. Cells drug selected for P-gp, BCRP, or no drug treatment were treated with tariquidar (1 μM) or DMSO (1 μL per 1 mL) for 15 min then with bortezomib (0.5 μM) or PBS (1 μL per 1 mL) for 30 min. The cells were lysed and the chymotrypsin-like activity of the proteasome was assessed by examining the cleavage of the fluorogenic peptide Suc-LLVY-AMC (a,b,c) when it was added to the cell lysates. Values represent chymotrypsin-like activity relative to vehicle control ± SEM,*$p < 0.05$, $n = 6$.