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[^{3}H]BMT-046091 a potent and selective radioligand to determine AAK1 distribution and target engagement

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A B S T R A C T

Adaptor-associated kinase 1 (AAK1), a member of the Ark1/Prk1 family of serine/threonine kinases, plays a role in modulating clatherin coated endocytosis of specific surface receptors. We have demonstrated that AAK1 inhibitors are efficacious in rodent models of neuropathic pain (Kostich et al., 2016). Here we have characterized the binding properties and distribution pattern of the tritiated AAK1 radioligand, [^{3}H]BMT-046091, in rodents and cynomolgus monkeys, and used the radioligand to measure the brain target occupancy following systemic administration of AAK1 inhibitors. We have found that [^{3}H]BMT-046091 is potent and selective AAK1 inhibitor. It inhibits AAK1 phosphorylation of a peptide derived from a physiologic substrate, the μ2 subunit of the adaptor protein complex, with an IC_{50} value of 2.8 nM, and is inactive at >5 μM in a panel of functional or binding assays for receptors, transporters and enzymes. [^{3}H]BMT-046091 binding in the brain is absent in the AAK1 knockout mouse, and is displaceable with a high concentration of AAK1 inhibitors in wild type mice. Specific [^{3}H]BMT-046091 binding is widespread in the brain and spinal cord with the highest density in the cortex, hippocampus, amygdala, striatum and thalamus. In the spinal cord, [^{3}H]BMT-046091 binding appears enriched in the dorsal horn superficial layers. Oral administration of LP-935509, an AAK1 inhibitor, results in a dose-dependent occupation of AAK1 binding sites in the brain and spinal cord. The increase in AAK1 binding site occupancy by LP-935509 correlates with the decrease in antinociceptive responses in the rat chronic constriction injury model of neuropathic pain.

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1. Introduction

Adaptor-associated kinase 1 (AAK1), a member of Ark1/Prk1 family of serine/threonine kinases, is involved in the endocytic pathway (Smythe and Ayscough, 2003). AAK1 is an interacting partner of the adaptor protein (AP2) complex and specifically interacts with the α-adaptin subunit of AP2 and phosphorylates the μ2 subunit of the AP2 complex (Conner and Schmid, 2002). The μ2 subunit binds specific tyrosine motifs of certain membrane receptors and mediates cargo recruitment to clathrin-coated vesicles (Ohno et al., 1995). Phosphorylation of the μ2 subunit by AAK1 promotes a high affinity interaction between the μ2 subunit and cargo with the with the specific tyrosine motif (YXXPhi) thereby playing a role in maximizing the efficiency of receptor internalization (Henderson and Conner, 2007). Clathrin-coated vesicle formation is also needed for recycling of synaptic vesicles particularly after neurotransmission. Although the AP2 complex is important in synaptic vesicle recycling, preventing μ2 phosphorylation through the use of a threonine 156 to alanine replacement does not seem to impact synaptic recycling (Kim and Ryan, 2009).

While AAK1 has mostly been studied in the context of regulation of receptor endocytosis, several studies have suggested AAK1 may be linked in some manner to psychiatric and neurological

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disorders. Analysis of postmortem cerebellum in schizophrenic patients shows that many genes involved in presynaptic vesicular transport including AAK1 are upregulated (Mudge et al., 2008). Single nucleotide polymorphism in an intron of the AAK1 gene has been linked to the onset of Parkinson's disease (Latourelle et al., 2009). AAK1 protein is decreased in the spinal cord of amyotrophic lateral sclerosis (ALS) patients. In rodent models of ALS, AAK1 is found to be mislocated with mutant superoxide dismutase 1 (SOD1) proteins (Shi et al., 2014). Our recent study has revealed an important role of AAK1 in neuropathic pain and anti-neuropathic pain effects of AAK1 inhibition in rodents (Kostich et al., 2016). We have found that AAK1 knockout mice exhibit a reduced response in the persistent pain stage of the formalin model and fail to develop tactile allodynia in a spinal nerve ligation (SNL) model of neuropathic pain. The anti-neuropathic pain effects can be reproduced using small-molecule AAK1 inhibitors (Kostich et al., 2016). Furthermore, AAK1 in the spinal cord appears to be critically important in mediating the antineuropathic pain effects as local administration of AAK1 inhibitors causes reduced nociceptive behavioral responses and inhibits spontaneous neural activity in the spinal cord in the chronic constriction injury (CCI) model of neuropathic pain (Kostich et al., 2016).

Currently there is little information on AAK1 distribution in the brain and spinal cord. There are also no tools to directly relate the binding of selective inhibitors of AAK1 to observed efficacy in animal models. Thus, in the present study we sought to synthesize a tritiated AAK1 inhibitor as a radioligand to map the distribution of AAK1 binding sites in the brain and spinal cord in rodents and cynomolgus monkeys. We then developed an *ex vivo* radioligand binding assay to measure the AAK1 target occupancy in the brain and spinal cord following systemic administration of an AAK1 inhibitor and to correlate the target occupancy with the anti-neuropathic effects of the compound in order to understand the pharmacokinetic-pharmacodynamic relationship.

2. Materials and methods

2.1. Materials

AAK1 inhibitors were synthesized by Neuroscience Discovery Chemistry at Bristol-Myers Squibb (BMS). PK 11195 and gabapentin were purchased from Sigma (St. Louis, Mo). [^{3}H]BMT-046091 (specific activity, 84 Ci/mmol; concentration, 1 mCi/ml; purity, 99%) was synthesized by Radiochemistry Group at BMS. [^{3}H]PK 11195 (NET885) and [^{3}H]Gabapentin (NET1182) were purchased from Perkin Elmer (Waltham, MA).

2.2. Animals and tissue collection

As reported previously (Kostich et al., 2016), AAK1 wild-type and knockout mice were generated on a C57 × 129 hybrid background strain. Sprague-Dawley rats were purchased from Charles River Laboratories. All animals were housed in groups of 2–4 in colony rooms maintained at a constant temperature (21 ± 2 °C) and humidity (50 ± 10%). The rooms were illuminated 12 h per day (lights on at 0600 h). Animals had *ad libitum* access to food (rodent diet; PMI Nutrition International, Brentwood, MO) and water throughout the studies. Animals were maintained in accordance with the guidelines provided by the Animal Care and Use Committee of Bristol-Myers Squibb. Mice and rats were sacrificed by decapitation, and the brain and spinal cord were collected, snap-frozen in dry ice-cold methylpentane citrate and stored at −80 °C until use. Fresh frozen naive cynomolgus monkey brain tissues were purchased from the Analytic Biological Services Company (Wilmington, DE).

2.3. AAK1 kinase assay

Generation of recombinant GST-Xa-hAAK1 (amino acids 30–330) for an AAK1 kinase assay has been described previously (Kostich et al., 2016). Recombinant GST-Xa-hAAK1 (3.5 nM) was then incubated with varying concentrations of AAK1 inhibitors (from 2 μM to 33 pM) in the reaction solution (30 μl in volume) containing 10 mM Tris-HCL pH 7.4, 10 mM MgCl2, 0.01% Tween-20, 1.0 mM DTT, 22 μM ATP, 1.5 μM (5-FAM)-Aha-KEEQSQITSQVTG-QIGWR-NH2, 1.6% DMSO. Kinase reactions were incubated at room temperature for 3 h and terminated by adding 60 μl of 35 mM EDTA buffer to each sample. Reaction solutions were analyzed on the Caliper LabChip 3000 (Caliper, Hopkinton, MA) by electrophoretic separation of the fluorescent substrate and phosphorylated product. IC_{50} values were derived by non-linear regression analysis of the concentration response data.

2.4. In vitro [^{3}H]BMT-046091 binding autoradiography

Frozen brains of mice, rats and monkeys, and frozen spinal cord of rats (naive or CCI) were cut into 20 μm sections on a Cryostat (Thermal Scientific). The sections were mounted on superfrost slides (VWR International, Wilmington, DE) and stored at −80 °C until use. On the day of the experiment, sets of slides were incubated with 10 nM [^{3}H]BMT-046091 in assay solution containing 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.1% BSA for 60 min at 22 °C. Non-specific binding was defined by incubation of adjacent sections with 10 nM [^{3}H]BMT-046091 in the presence of 10 μM BMS-901715 (a potent structurally distinct ligand disclosed in our previous publication that interacts with the same binding site (Kostich et al., 2016)). After incubation, slides were washed in ice cold assay buffer and subsequently dried under a stream of cold air. The slides were then placed in cassettes against [^{3}H]-sensitive storage phosphor-imaging screens (PerkinElmer, Waltham, MA) for 3–7 days, and the screens were then scanned with a Cyclone storage phosphor imaging system (PerkinElmer, Waltham, MA). Captured images were analyzed with OptiQuant Acquisition and Analysis software (PerkinElmer, Waltham, MA). Radioligand binding in a given brain region was measured as digital light units per millimeter squared (DLU/mm^2), and the specific binding density was calculated by subtraction of non-specific from total binding values.

In saturation binding studies, adjacent sections were incubated with varying concentrations of [^{3}H]BMT-046091 from 0.1 nM to 10 nM at 22 °C for 60 min. After washing and drying, the slides plus [^{3}H] standards (American Radiolabeled Chemicals, St. Louis, MO) were placed in cassettes against [^{3}H]-sensitive storage phosphor-imaging screens for 3–7 days, and were then processed as described above for Cyclone storage phosphor imaging acquisition and analysis. Binding signal measured as DLU/mm^2 was converted to μCi/g for K_d and B_{max} calculation.

2.5. In vitro [^{3}H]PK 11195 and [^{3}H]gabapentin binding autoradiography

In vitro [^{3}H]PK 11195 and [^{3}H]gabapentin binding autoradiography procedures were modified from elsewhere (Thurlow et al., 1996; Miller et al., 2013). In brief, for [^{3}H]PK 11195 binding, spinal cord lumbar sections collected from CCI (see below for description of CCI surgery) were incubated with 3 nM [^{3}H]PK11195 in assay solution containing 50 mM Tris-HCl buffer (pH 7.0) at 4 °C for 2 h. The non-specific binding was defined in the adjacent sections incubated in the presence of 10 μM PK11195. For [^{3}H]gabapentin binding, spinal cord lumbar sections collected from CCI or sham-operated rats were incubated with 20 nM [^{3}H]gabapentin in assay solution containing 50 mM Tris-HCl buffer (pH 7.0) at 22 °C

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