Initial Biological Qualification of SBDP-145 as a Biomarker of Compound-Induced Neurodegeneration in the Rat

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ABSTRACT

Detection of compound-related neurodegeneration is currently limited to brain histopathology in veterinary species and functional measurements such as electroencephalography and observation of clinical signs in patients. The objective of these studies was to investigate whether concentrations of spectrin breakdown product 145 (SBDP-145) in cerebrospinal fluid (CSF) correlate with the severity of neurodegeneration in rats administered neurotoxic agents, as part of a longer term objective of developing in vivo biomarkers of neurotoxicity for use in non-clinical and clinical safety studies. Non-erythroid alpha-II spectrin is a cytoskeletal protein cleaved by the protease calpain when this enzyme is activated by dysregulation of calcium in injured cells. Calcium dysregulation is also associated with some toxicological responses in animals, and may be sufficient to activate neuronal calpain and produce SBDPs that can be released into CSF. Neurotoxicants (kainic acid, 2-chloropropionic acid, bromethalin, and pentylenetetrazole) known to affect different portions of the brain were administered to rats in dose-response and time-course studies in which neurodegeneration was measured by histopathology and SBDP-145 concentrations in CSF were measured by ELISA. We consistently observed 3-fold increases in SBDP-145 concentration in rats with minimal to slight neurodegenerative lesions, and 20 to 150-fold increases in animals with more severe lesions. In contrast, compounds that caused non-degenerative changes in central nervous system (CNS) did not increase SBDP-145 in CSF. These data support expanded use of SBDP-145 as a biomarker for monitoring compound-induced neurodegeneration in pre-clinical studies, and support the investigation of clinical applications of this biomarker to promote safe dosing of patients with compounds that have potential to cause neurodegeneration.

Key words: neurodegeneration; SBDP-145; α-2-spectrin; biomarker; calpain

Over-activation of calpain and the resulting proteolysis of dozens of proteins has been linked to a variety of degenerative conditions in the brain (Mansouri et al., 2007; Vanderklish & Bahr, 2000), and may be the unifying mechanistic event in stroke, spinal cord injury, traumatic nerve injury, Parkinson’s disease, amyotrophic lateral sclerosis, Alzheimer’s, and a variety of other diseases (Stracher, 1999). The main trigger of calpain activation appears to be the dysregulation of intracellular Ca2+ levels, which can also be induced through toxic insult from various pharmacologically active compounds. Compounds having pharmacological activity in the brain may cause neurotoxicity at super-pharmacological doses. In the case of glutamate receptor activators such as kainic acid, this phenomenon is referred to as “excitotoxicity” and is related to the elevation of intracellular calcium concentrations through prolonged opening of the glutamate ion channel (Kew & Kemp, 2005). Histomorphological changes in the brain associated with excitotoxicity include neuronal degeneration and necrosis. Excitotoxicity of glutamate re-
ceptor activators can manifest as altered clinical behavior and in severe cases includes seizures. Unfortunately, there are no quantitative biomarkers that can be measured in blood or cerebrospinal fluid (CSF) of humans and veterinary species that correlate with an increased risk for excitotoxicity or indicate that an early stage of excitotoxic injury is in progress. Furthermore, consistent with the target-related mechanism, the margin of safety between efficacy and toxicity of glutamate receptor activators can be narrow.

The pathogenesis of glutamate-related excitotoxicity has been described to present in two phases: a rapid phase in which excessive glutamate receptor activation leads to increased intracellular concentrations of sodium and chloride, resulting in cell swelling; and a more delayed phase in which increased calcium influx and mobilization of calcium from intracellular stores causes activation of Ca-dependant enzymes, including calpains, that are part of necrosis and apoptotic pathways (Slemmer et al., 2005). For example, L-2-chloropropionic acid (CPA), an N-methyl-D-aspartate (NMDA) receptor agonist, causes cerebellar granule cell degeneration approximately 36–48 h after a single oral administration to rats (Widdowson et al., 1997). Immunoblots of tissue homogenates prepared from the cerebellum, but not those from cerebral cortex, revealed CPA-induced increases in concentration of spectrin breakdown products (SB-DPs) at 155–157 kDa compared with the full-length α-2-spectrin (240 kDa) protein. SBDP increases occurred prior to microscopic observation of neurodegeneration, but only in areas in which neurodegeneration became evident microscopically at later time points. Kainic acid-induced neurotoxicity has also been reported to increase SB-DP concentrations in CSF (Glushakova et al., 2012). SB-DPs have been studied in models of traumatic brain injury (Cardali & Maugeri, 2006; Liu et al., 2006; Mondello et al., 2010; Ringer et al., 2004; Zoltewicz et al., 2013) including quantitation in CSF (Pike et al., 1998, 2001). Together, these studies suggested that SB-DP concentrations of SB-DPs could be useful as an accessible biomarker for neuronal injuries that involve calpain activation. Specifically, SB-DP-145 may prove to be a valuable biomarker of biochemical events that precede cytolethal excitotoxicity and could be used in both non-clinical toxicology studies and in clinical trials to help develop glutamate receptor activators for the treatment of human neurological diseases.

Iontropic glutamate receptors are classified into three families based on the small molecules that activate these receptors: kainate, AMPA, and NMDA. Each family of receptors has numerous subtypes expressed in different parts of the brain (Kew & Kemp, 2005). Compounds that modulate each of the three families of ionotropic glutamate receptors were used in the short duration rat toxicology studies reported here to evaluate SB-DP-145 in CSF as a biomarker of acute neuronal degeneration in the rat. Additional compounds with differing mechanisms of neurotoxicity and affecting different neuroanatomic locations were used to help assess the specificity of increased SB-DP-145 in CSF as a biomarker of neurodegeneration. Histopathology was conducted to understand the nature and severity/extent of the brain lesions caused by these compounds, and immunohistochemistry was used to localize the SB-DP-145 in relationship to these microscopic lesions.

**MATERIALS AND METHODS**

**In vivo experiments.** All animal procedures were performed according to protocols approved by the Animal Care and Use Committees of Eli Lilly and Company and/or contract research organizations conducting in vivo studies. Sprague Dawley (SD) rats were used in all studies. Rats were observed predose and several times per day post dose for adverse clinical signs. Kainic acid-treated rats were monitored continuously for 4 h post dose, several times during the remainder of the day, and at least 1x on the day following dose administration for evidence of uncontrolled convulsions. Diazepam (10 mg/kg IP) was administered as needed to depress seizure activity 2 h after onset then rats were hydrated with SQ injection of physiological saline following diazepam administration and again on the following day. Single CSF samples (approximately 30–50 μl) were collected from the cisterna magna of anesthetized rats at necropsy. Rats were euthanized by cervical dislocation under anesthesia or by asphyxiation using CO₂.

**Histopathology.** Brains collected at necropsy were fixed in 10% neutral buffered formalin for up to 24 h then trimmed and transferred to 70% dehydrant prior to processing. After fixation, brains were trimmed into slices of approximately 3-mm thickness as detailed previously (Jordan et al., 2011) to provide four coronal levels. The four slices were processed routinely for embedding into a single paraffin block, and 5-micron thick microtome sections were mounted on slides and stained with hematoxylin and eosin (H&E). The H&E-stained slides were examined with both brightfield illumination and with epifluorescent illumination using a fluorescein isothiocyanate blue excitation filter (Leica L3 filter cube), as previously described to enhance the sensitivity for detection of degenerative neuronal cell bodies and processes (Jordan et al., 2011) (Supplementary online images). Microscopic tissue changes were recorded and assigned a qualitative severity grade on a 5-point scale ranging from minimal to severe. Minimal indicated that only a few individual cells were affected, slight represented changes in a few small clusters of cells, moderate effects indicated a prominent feature, marked indicated an extensive change with some normal tissue remaining within affected areas, and a severe grade indicated an overwhelming effect in a broad area. When neurodegeneration was observed in multiple brain sections for an individual animal, the maximum observed severity was used as the overall severity grade for consideration of associations with CSF SB-DP-145 levels. The study pathologist evaluating tissue changes was blinded to the SB-DP-145 measurements in CSF.

**Immunohistochemistry to label SB-DP-145 in the brain.** Slides-mounted histologic sections were deparaffinized with xylene, rehydrated through graded alcohols to deionized water, and then transferred into Biocare DIVA pretreatment reagent. Antigen retrieval was conducted in a pressure dewaxing chamber by increasing the temperature to 125 °C, then cooling to 90 °C. Slides were rinsed in deionized water then placed on a Leica Bond Rx autostainer (Leica Microsystems Inc., Buffalo Grove, IL) programmed for rabbit antibody protocols. Primary anti-SBDP145 antibody (Banyan Biomarkers, Inc. Alachua, FL) was applied for 15 min at a concentration of 1 μg/ml. The secondary antibody, Leica Bond Polymer (a part of the Polymer Refined Detection Kit, DS9800), was applied for 8 min and then slides were removed from the autostainer, dehydrated through graded alcohols to xylene and coverslipped.

**Quantitation of Biomarker Concentration in CSF.** ELISA assays for SB-DP-145 in CSF were conducted at Banyan Biomarkers, Inc. (http://banyanbio.com) using Banyan Biomarker’s proprietary sandwich ELISA (Glushakova et al., 2012).
RESULTS

Sensitivity Studies

The first set of studies was conducted with superpharmacological doses of ionotropic glutamate receptor agonist or potentiator compounds to assess the sensitivity of SBDP-145 in CSF as a signal for compound-induced neurodegeneration in various brain regions (Fig. 1 and Table 1).

Kainic acid. Rats were necropsied at 6, 12, 24, or 48 h following a single 9-mg/kg subcutaneous kainate dose. Neurodegeneration occurred in the hippocampus (Fig. 4B), cerebral cortex, and in many other regions of the rostral brain. Ten of 11 treated rats with minimal or no observed neurodegeneration had less than a 2-fold increase in SBDP-145 compared with vehicle control group mean, whereas all four rats with slight to moderate neurodegeneration had a greater than 2-fold increase (Fig. 4A) indicating a positive association between increased histologic severity grades for neurodegeneration and increased SBDP-145 concentrations. Eight of 15 treated rats had observed convulsions. All rats with convulsions also had minimal to moderate neurodegeneration observed. Generally, rats with convulsions had increased SBDP-145 but three rats with convulsions and minimal neurodegeneration had little to no increase in SBDP-145 compared with vehicle controls (Fig. 4A), weakening the association between convulsions and increased SBDP-145. Only one rat had two observed convulsions and although it had the highest SBDP-145 increase (Fig. 4A), there is insufficient data to associate convulsion incidence with magnitude of SBDP-145 increase. Severity of convulsions was not documented on a grading scale but kainic acid-induced convulsions were more severe and of longer duration than those induced by other compounds that we tested. Kainic acid was the only treatment that required diazepam as humane intervention for more severe and prolonged convulsions (see the Materials and Methods section for details).

LY450108 (AMPA receptor potentiator). Three independent studies were conducted with LY450108 administered to SD rats either in a dose response (from 20–80 mg/kg) or sample time course (4 h to 10 days) design (Table 1). In each study, cerebellar Purkinje cell degeneration/necrosis was recognizable in H&E-stained histologic slides examined with both brightfield (Fig. 2B and online) and epifluorescent (online) illumination. In CSF collected between 4 h and 10 days after a single oral administration of LY450108, concentrations of SBDP-145 increased with increasing histologic severity grade for the 42 treated rats with microscopically confirmed neurodegeneration/necrosis (Fig. 2A). Lesion severity grade was minimal in rats terminated at ≤24 h but included minimal to marked severity at ≥2 days. The highest severity grades for neurodegeneration and the maximum fold-increases of SBDP-145 in CSF both occurred at 2–4 days after dosing. Four of the 52 treated rats with no observed degeneration (ND in Fig. 2A) had CSF concentrations of SBDP-145 that were increased >10-fold. These rats, necropsied at 4–24 h post dose, may have had brain lesions that were not present in the examined histologic slides or subcytolethal neuronal damage sufficient to cause production and accumulation of SBDP-145 in CSF.

2-Chloropropionic acid. Rats were administered two or three daily doses of 2-Chloropropionic acid (2-CPA) and samples collected 24 h after the last dose. Rats receiving three daily doses of 250 or 375 mg/kg survived to study termination but rats receiving 500 mg/kg were euthanized (with CSF and tissue collection) after two doses due to poor clinical condition. Segmental diffuse granule cell degeneration and neuropil vacuolation within the Purkinje cell layer were observed throughout the cerebellum (Figs. 3B and C). SBDP-145 concentration in CSF increased ≥4.5-fold in six of seven rats with cerebellar granule cell degeneration graded slight to marked and the magnitude of increase correlated with the microscopic neurodegeneration severity score (Fig. 3A).

Data analysis. Data from in vivo studies were initially analyzed by comparing the SBDP-145 concentration in each animal with the mean for the concurrent (intra-study) control group. Because SBDP-145 concentration in CSF of most vehicle control animals was below the quantifiable limit of the assay, fold-increases were calculated from the assay lower limit of quantitation. Prevalence of convulsions and that of overall microscopic neurodegeneration severity score were each plotted against SBDP-145-fold change from control. Unless otherwise specified, graphs include individual animal data across all collection time points. The exploratory studies in this initial biomarker qualification were not designed with the objective of doing statistical analysis but instead to identify positive relationships between increased SBDP-145 and neurodegeneration. Consequently we have graphed each compound that was tested, and consider the data a strong starting point for a more statistics-driven qualification of this biomarker.
### Table 1. Investigative rat biomarker studies to induce injury to various cell types in the rat brain

<table>
<thead>
<tr>
<th>Compound (MOA)</th>
<th>Structure</th>
<th>Pathology and Clinical Signs</th>
<th>Dose(s)</th>
<th>No. of Rats on Study</th>
<th>Sample time (after last dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY450108 (AMPA potentiator)</td>
<td><img src="https://example.com/structure.png" alt="Structure" /></td>
<td>Degeneration–cerebellum, Purkinje cell; vacuolation–cerebellum, molecular layer Convulsion, recumbency, ataxia, rigid stance, labored respiration</td>
<td>Single 0, 80 mg/kg or two daily 70-mg/kg oral doses</td>
<td>20 control, 40 treated</td>
<td>4, 12, 24, 48 h</td>
</tr>
<tr>
<td>2-Chloropropionic acid (NMDA agonist)</td>
<td><img src="https://example.com/structure.png" alt="Structure" /></td>
<td>Degeneration–cerebellum, granular cell &gt; Purkinje cell, cerebrum, hippocampus, brain stem Convulsion, recumbency, ataxia, leg weakness</td>
<td>Daily oral doses of 0, 250, 375 mg/kg (three), or 500 mg/kg (two)</td>
<td>5 control, 15 treated</td>
<td>24 h</td>
</tr>
<tr>
<td>Kainic acid (non-NMDA glutamate receptor agonist)</td>
<td><img src="https://example.com/structure.png" alt="Structure" /></td>
<td>Degeneration–hippocampus, cerebrum &gt; brain stem Vacuolation–hippocampus, Cerebrum Convulsion, recumbency, ataxia</td>
<td>Single oral dose of 0, 9 mg/kg</td>
<td>20 control, 20 treated</td>
<td>6, 12, 24, 48 h</td>
</tr>
<tr>
<td>LY503430 (AMPA potentiator)</td>
<td><img src="https://example.com/structure.png" alt="Structure" /></td>
<td>Degeneration–hippocampus, Cerebrum (No convulsion) increased reactivity to stimulus, increased respiration</td>
<td>7, 10, or 14 daily oral doses of 0, 10 mg/kg, 1, 3, 5, 7, or 10 daily oral doses of 0, 10 mg/kg</td>
<td>12 control, 24 treated</td>
<td>24 h</td>
</tr>
<tr>
<td>Bromethalin (un-coupler of mitochondrial oxidative phosphorylation)</td>
<td><img src="https://example.com/structure.png" alt="Structure" /></td>
<td>Vacuolation–cerebrum, cerebellum, hippocampus, brain stem, spinal cord, white matter (No convulsion) transient hind-limb paralysis (2–3 days)</td>
<td>Single oral dose of 0, 1 mg/kg</td>
<td>12 control, 24 treated</td>
<td>24, 48, 120 h</td>
</tr>
<tr>
<td>Pentylenetetrazole</td>
<td><img src="https://example.com/structure.png" alt="Structure" /></td>
<td>No neurodegeneration (per H&amp;E and autofluorescence) Convulsions (onset 1 min, duration 1 min) in all rats</td>
<td>Single intraperitoneal dose of 0, 50 mg/kg</td>
<td>12 control, 48 treated</td>
<td>2, 10, 48 h</td>
</tr>
</tbody>
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aSimpson et al. (1996); Wyatt et al. (1997); Sigma catalog no. 306797.
bSigma catalog no. K0250.
cMurray et al. (2003).
dBell Labs, CAS No. 63333-35-7.
eSigma catalog No. P6500.
LYS03430 (AMPA receptor potentiator). Rats were necropsied 24 h after 1, 3, 5, 7, 10, or 14 daily doses of LYS03430. Neuronal degeneration, primarily in pyramidal neurons of hippocampal CA1–CA4 (Fig. 5D), was present in some rats after three daily doses and in all treated rats receiving five or more daily doses with histologic severity grades increasing with the number of doses (Fig. 5A). SBDP-145 concentration was slightly increased in CSF of a subset of rats compared with vehicle-treated group mean but there was only a weak association between these increases and the number of daily doses (Fig. 5B) or severity of neurodegeneration (Fig. 5C).

Specificity Studies
A second set of rat toxicology studies was conducted to investigate specificity of SBDP-145 as a biomarker of neurodegeneration. Bromethalin is a potent uncoupler of oxidative phosphorylation that causes prominent fluid separation of the layers of myelin sheaths [intramyelinic edema, confirmed by electron microscopy in previous studies reported by van Lier & Cherry (1988)] without evidence of neurodegeneration. Pentylentetrazole (PTZ) has been reported to cause convulsions in laboratory animals without causing neurodegeneration (Mao et al., 2009; Pi et al., 2004). These two compounds were administered (independently) to determine if either intramyelinic edema or convulsions alone would cause increased SBDP-145 in CSF.

Bromethalin. Rats were necropsied 1, 2, or 5 days following a single Bromethalin dose of 1 mg/kg. The transient hind limb weakness/paralysis reported to occur as a result of intramyelinic edema caused by Bromethalin toxicity (van Lier & Cherry, 1988) was observed in this study and served as a live phase indicator that this myelin injury was present. Using light microscopic examination, van Lier & Cherry (1988) observed “a spongy degeneration of the white matter” which, using electron microscopy, was confirmed to be intramyelinic edema. This edema was found to be reversible in rats 8 weeks after a 2-week dosing period. They found no evidence of cell death in brain or nerve tissues. In the current study, microscopic changes typical for white matter edema were present (Fig. 6B) but, consistent with the earlier literature, specific evidence of neuronal
FIG. 3. 2-Chloropropionic acid. (A) SBDP-145-fold change (from vehicle control group mean) in CSF of control and treated rats versus histologic severity grade for degeneration. CSF and tissues were collected 24 h following the last dose. Horizontal lines: mean; + symbol: convulsion observed; ND: treated rats, degeneration not detected; MI: minimal; SL: slight; MO: moderate; MA: marked histologic severity. (B and C) Segmental diffuse cerebellar granule cell degeneration [D] and vacuolation [V] within the Purkinje layer. Morphologically normal granule cell neurons [N].

Degeneration/necrosis was not identified by brightfield or epifluorescent illumination. Due to technical limitations, possibly related to CSF fluid dynamics under conditions of intramyellic edema, CSF samples could not be obtained from several treated rats; therefore, the number of SBDP-145 data points was reduced. In four of five CSF samples obtained at 2 days post dose, SBDP-145 was increased 4–20-fold but was not increased in the few samples obtained at 1 or 5 days post dose (Fig. 6A).

Pentylenetetrazole. Convulsions induced by PTZ occurred in all 40 treated rats beginning less than 5 min after dosing but did not induce morphologic evidence of degeneration in the brain or increase SBDP-145 concentration in CSF at the 2, 10, or 48 h post-treatment termination times (data not shown). PTZ induced relatively mild and short duration convulsions similar to those induced by LY450108 and 2-CPA. By contrast, kainic acid induced frequent or prolonged and more severe convulsions.

SBDP-145 Immunohistochemistry. SBDP-145 immunohistochemical labeling was assessed to verify the localization of the SBDP-145 neoepitope within neurons in brain areas with histologically identifiable neurodegeneration. The brains of vehicle-treated control rats from these studies were not completely devoid of SBDP-145 immunolabeling. However, the labeling pattern in control animals was consistently present in specific regions of the brain levels sampled, which facilitated the ability to distinguish this background labeling from labeling related to compound-induced neurotoxic injury. In the four brain levels sampled in these studies, background labeling of neuronal perikarya and processes in control rats occurred consistently within select nuclei of the lateral thalamus of brain level 2 (Fig. 4E), within the caudal colliculus and select nuclei in the midbrain ventral to the periaqueductal gray matter of level 3, in occasional scattered cerebellar Purkinje cells (Fig. 2D), in neurons of deep cerebellar nuclei and medullary nuclei, and in processes within the deep cerebellar white matter. SBDP-145 immunolabeling did not occur in the hippocampus or cerebral cortex of control brains, areas that were dramatically affected by some of the neurotoxins employed in this study. Positive immunohistochemical labeling in tissues from rats administered neurodegeneration-inducing neurotoxins (Figs. 2C and 4C and D) clearly exceeded this background pattern and correlated with areas of neuronal degeneration/necrosis identified in the H&E-stained sister sections. Examples were also noted in which SBDP-145 immunolabeling was not present in some areas containing recognizably necrotic neuronal cell bodies, though labeling was typically present elsewhere within the same coronal section and sometimes within the neuropil surrounding the unlabeled necrotic cell bodies. Although a specific reason for this apparent variability in labeling was not determined, possible explanations include death of some neuronal populations by pathways not involving calpain activation and subsequent generation of the SBDP-145 neoepitope, leakage of the neoepitope from the confines of the neuronal perikaryon (becoming detectable in extracellular space and CSF), or eventual degradation or masking of the neoepitope within the perikaryon as the necrotic cell deteriorates further.

Impact of blood contamination on SBDP-145 in CSF. We visually evaluated all CSF samples for pink discoloration as an indicator of blood contamination. Eight of 10 control rat CSF samples with visual evidence of blood contamination had SBDP-145 increased >2× compared with the concurrent study control group mean but only one of 76 clear control samples had SBDP-145
FIG. 4. Kainic acid. (A) SBDP-145-fold change (from vehicle control group mean) in CSF of control and treated rats versus histologic severity grade for degeneration. CSF and tissue collection times ranged from 6 to 48 h after dosing. Horizontal lines: mean; + symbol: convulsion observed; ND: treated rats, degeneration not detected; MI: minimal; SL: slight; MO: moderate, histologic severity. (B) Neuronal necrosis (arrow) within the entorhinal cortex 48 h following a single dose of kainic acid. (C and D) SBDP-145 immunolabeling of neuronal perikarya and neuropil was extensive in regions of injury identified in H&E stains. A similar brain region from an uninjured control rat (E) depicting typical background SBDP-145 immunolabeling within the lateral thalamus.
FIG. 5. LY503430. (A) Maximum histologic severity grade among evaluated histologic sections for neurodegeneration for each individual versus number of daily doses. (B and C) SBDP-145-fold change (from vehicle control group mean) in CSF of control (C only) and treated rats versus number of daily doses (B) or histologic severity grade for degeneration (C). CSF and tissues were collected 24 h following the last dose. Horizontal lines: mean; o symbol: convulsion not observed; ND: treated rats, degeneration not detected; MI: minimal; SL: slight; MO: moderate; MA: marked histologic severity. (D) Marked pyramidal cell degeneration and loss in hippocampal CA4, here at 24 h after the 10th daily dose. (E) Normal hippocampal CA4 from a control rat.
**FIG. 6.** Bromethalin. (A) SBDP-145-fold change (from vehicle control group mean) in CSF of control and treated rats versus histologic severity grade for vacuolation. CSF and tissue collection times ranged from 1 to 5 days after dosing. Horizontal lines: mean; sample days post dose indicated by o symbol (1 day), △ (2 days), □ (5 days); MI: minimal; SL: slight; MO: moderate histologic severity. (B) Intramyelinic edema [E], here at 5 days post-treatment, indicated by pallor of the cerebellar white matter. Normal myelin density [N] in cerebellum from control rat (inset).

**FIG. 7.** Blood contamination in CSF. We observed that eight of the 10 vehicle control rat CSF samples with visual evidence of blood contamination (pink color) had SBDP-145 increased ≥2×. Only one of the 76 clear control CSF samples had SBDP-145 increased ≥2×. Horizontal lines: mean.

Increased ≥2× (Fig. 7). Based on this empirical observation and on reported evidence that platelets contain non-erythroid alpha spectrin (Fox et al., 1987), a potential source of SBDPs, we excluded blood-contaminated samples from analysis. Note: Table 1 lists the number of animals on study regardless of exclusion for blood contamination.

**DISCUSSION**

Previous investigations have shown increased CSF concentrations of SBDP-145 following traumatic (physical) brain injury to rats (Pike et al., 1998, 2001) and to humans (Pineda et al., 2007). Those studies have supported the use of SBDP-145 to assess the severity of neuronal injury in people involved in vehicle accidents and in combat. The data presented here show that this biomarker may also be useful for detecting compound-related excitotoxic neuronal injury. It is reasonable to hypothesize that this biomarker may be a useful sentinel of acute or ongoing neuronal injury induced by a variety of pathogenic mechanisms and that it can provide a useful monitoring tool for preclinical and clinical drug development of compounds with the potential to cause neurodegeneration.

We investigated SBDP-145 as a CSF-based biomarker of acute neuronal injury in the rat. Our primary focus was on compounds, including kainic acid and AMPA potentiators, which cause excitotoxicity by activation of glutamatergic receptors. These studies demonstrated that the concentration of SBDP-145 was increased in CSF of rats following administration of compounds that caused acute neurodegeneration in a variety of brain locations and cell types, including cerebellar granule cells (2-CPA), cerebellar Purkinje cells (LY450108), hippocampal pyramidal cells (LY503430), and more broadly in the hippocampus, cerebral cortex, and in many parts of the rostral brain (kainic acid). Furthermore, increased CSF SBDP-145 correlated in most cases with presence and severity of microscopically evident acute neuronal degeneration/necrosis.

Immunolabeling for SBDP-145 was done to better understand the relationship between neurodegeneration and increased SBDP-145 in the CSF, not to evaluate SBDP-145 immunohistochemistry as an independent diagnostic tool. Cell bodies and axons of injured neurons preferentially immunolabeled with SBDP-145 polyclonal antibody compared with uninjured (morphologically normal) neurons of control and treated animals (Figs. 2 and 4). Labeling in areas of neurodegeneration in treated animals was often intense and frequently colocalized with degenerating neuronal cell bodies that were identified by light microscopic examination, providing evidence that increased SBDP-145 measured in CSF originated in degenerating neurons. However, there was also a reproducible “background” immunolabeling for SBDP-145 in vehicle control animals with no detectable neurodegeneration using H&E and autofluorescence and without increased SBDP-145 in CSF. This background labeling had two distinct features: bilateral labeling of nuclei in the lateral thalamus and in the midbrain and a more scattered labeling of individual perikarya/processes that was generally faint but moderately strong in a few individuals. The significance of and specific reason for this consistent background labeling pattern in apparently healthy neurons was not determined in this...
study, but suggests that the mere presence of the neoepitope in situ may not always signal neuronal injury. Evidence for a low level of calpain processing of α-spectrin was reported by Huh et al. (2001) and suggested to be a constitutive process in the adult human brain. It is therefore possible that the background staining we have observed is due to an analogous process in the rat brain. The sensitivity of increased SBDP-145 as a biomarker of compound-induced neurodegeneration was evaluated over several in vivo experiments. These were designed with dose levels to induce a range of neurodegeneration severity grades and with sample collection times aimed at understanding SBDP-145 increases in relation to histopathologic injury progression. Neuronal degeneration/necrosis was identified by careful examination of H&E-stained histologic sections under brightfield illumination; additional sensitivity for recognition of rare and/or widely scattered degenerate neurons was gained by examining the same slides under epifluorescent illumination using filters appropriate for detecting the characteristic autofluorescence of degenerating neuronal cell bodies and processes under these conditions (Jordan et al. 2011) (Supplementary online images).

The magnitude of SBDP-145 increase in rat CSF differed significantly between treatments. Degeneration of Purkinje cells in the cerebellum of rats treated with AMPA potentiator LY450108 was particularly well detected: biomarker concentrations increased up to 150-fold at 96 h following a single administration. Degeneration of cerebellar granule cells (2-CPA) and of hippocampus, cerebrum, and brain stem (kainic acid) was generally less severe compared with LY450108-induced injury and resulted in lower magnitude SBDP-145 increases compared with LY450108-treated rats. However, fold changes in SBDP-145 still correlated well with histopathologic severity grade induced by each of these three neurotoxins.

In contrast, LY503430-induced hippocampal degeneration was of comparable histopathologic severity grade as LY450108-induced cerebellar degeneration but LY503430 caused much smaller (10-fold) and less prevalent (only four of 74 rats with >3-fold) increases in SBDP-145 (Fig. 5C). Furthermore, the magnitude of LY503430-induced SBDP-145 increases did not correlate well with either the severity of neurodegeneration (Fig. 5C) or the number of daily doses (Fig. 5B).

To accurately apply SBDP-145 as a diagnostic biomarker of neurodegeneration, better understanding is needed of additional variables related to treatment and/or injury mechanism that impact measurable SBDP-145. For example, it is possible that some injury mechanisms differentially impact the kinetics/magnitude of calpain activation to result in different influences on SBDP production and detection. Some injury mechanisms may involve different proteases that do not lead to the production of SBDP’s, even though they may result in significant levels of neurodegeneration. The SBDPs produced in certain brain regions or cell types may be less likely to accumulate in high concentrations in CSF.

The specificity of increased SBDP-145 in CSF as a marker for neurodegeneration was also explored. We considered the possibility that convulsions might contribute to increases in SBDP-145 concentrations, such as in rats treated with kainic acid, which caused both convulsions and neurodegeneration and resulted in increased concentrations of SBDP-145 in many treated rats. PTZ administration caused relatively mild and intermittent convulsions in all rats, similar in intensity to those induced by AMPA potentiators and 2-CPA, but caused no histologically detectable neurodegeneration and no detectable change in the concentration of SBDP-145 in CSF. These data indicate that convulsions alone, at least when relatively mild, do not cause increased SBDP-145 in CSF. The three kainic acid-treated rats with convulsions but only minimal neurodegeneration and SBDP-145 increases (Fig. 4A) indicate that even fairly severe and longer duration convulsions may have less impact on SBDP-145 than severity of neurodegeneration.

We also investigated the possibility that intramyelinic edema without neurodegeneration would increase SBDP-145 concentrations. Bromethalin administered to rats produced the expected intramyelinic edema and associated transient hind limb paralysis with no microscopically evident neurodegeneration. Surprisingly, several Bromethalin-treated rats did have increased concentrations of SBDP-145 in CSF (Fig. 6A). Based on the lack of Bromethalin-induced neurodegeneration detected by van Lier & Cherry (1988) and by our laboratory, we do not believe that sparse neurodegeneration was the cause for SBDP-145 increases in these three rats.

As the LY503430 neurodegeneration mechanism appears to involve variables that do not lead to significant SBDP-145 increases in CSF, the Bromethalin injury mechanism, based on our limited data set, appears to involve variables that cause SBDP-145 production to be more closely associated with timing (possibly the degree of calpain activation at time of CSF collection) than simply associated with the severity of the edema. Looking more broadly across our experiments, a relatively small number of rats had no observed neurodegeneration yet had SBDP-145 increased more than 4-fold compared with concurrent control groups (four LY450108-treated, Fig. 2A, ND group; five Bromethalin-treated, Fig. 6A, SL-MO vacuolation; two 2-CPA-treated, Fig. 3A, ND group). A potential explanation for the apparent disconnect between increased SBDP-145 and lack of observed neurodegeneration in these rats could be that brain lesions were present in unexamined brain areas and led to increased SBDP-145. However, we include an alternate hypothesis that should be considered as qualification and interpretation of this biomarker proceeds: We would expect that in a gradient of compound-induced in vivo toxicity, a certain degree of subcytotoxic neuronal damage occurs in some subjects that is not sufficient to cause detectable histopathologic lesions but is sufficient to cause production of, and possibly a detectable accumulation of, SBDP-145 in CSF. Di Stasi et al. (1991) observed that calpain-dependent cleavage of αII-spectrin alone (based on increased concentration of SBPD-150) without cleavage of βII-spectrin did not cause cell death in cultured cerebellar neurons and suggested that limited αII-spectrin cleavage may push cells to a “pre-toxic” but reversible injury state. In our Bromethalin experiment, increased SBDP-145 aligned more with sample day (Fig. 6A, day 2 but not days 0 or 5) than with severity of vacuolation. The alignment of this SBDP timeline with the timing and transient nature of Bromethalin-induced hind limb paralysis as well as the reversible nature of the edema reported by van Lier & Cherry (1988) supports the association of increased SBDP-145 in this experiment with Di Staci’s observation of a “pre-toxic” but reversible injury state. More data are needed to understand the tipping point between “pre-toxic” and cytotoxic (degenerative) injury mechanisms and to more precisely characterize the lower limits of SBDP-145 sensitivity for various neuronal injuries.

A small number of CSF samples collected from untreated rats became visually contaminated with blood during CSF collection. Most of these samples contained increased concentrations of SBDP-145 (Fig. 7). Though the number of data points is small, the association between blood contamination and increased SBDP-145 is compelling enough to warrant discussion. Fox et al. (1987) reported immunodetection of α-spectrin in platelets using anti-
bodies against brain spectrin. Based on this report and our data, it is possible that blood contamination, not neuronal injury, was the source for increased SBDP-145 in these blood-contaminated samples. Caution should therefore be taken to avoid blood contamination when collecting CSF for analysis of SBDP-145.

In summary, SBDP-145 is a promising CSF biomarker of acute compound-induced neurodegeneration based on strong associations between increased concentrations in CSF and the presence/severity of neurodegeneration in the rat. Further biological qualification is needed to understand situations where a lack of correlation occurs in order to more effectively apply SBDP-145 as a biomarker in preclinical and clinical studies.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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