**Appendix 1**

**The Pharmacology of Leukotrienes**

The following material summarizes:

* a historical perspective on the pharmacology of leukotrienes and the development of FLAP inhibitors for treating asthma
* a summary of our novel pre-clinical data that delineate a key role of leukotrienes in mediating secondary injury following TBI and the efficacy of FLAP inhibitors in attenuating neuroinflammation that significantly contributes to acute and long-term disabilities following a TBI.

**Kellaway, Bergstrom, Samuelson, Vane and Merck**

In the late 1960s and though the 1970s, studies by Sune Bergstrom, Bengt Samuelsson, and others demonstrated that **leuko**cytes contain 5-lipoxygenase that catalyzes the oxygenation of free arachidonic acid (AA) into several pro-inflammatory mediators that contain a conjugated **triene** as a part of their structure. These lipid mediators were called **“leukotrienes**” (further defined as LTB4, LTC4, LTD4 and LTE4) and shown to mediate potent, potentially dangerous, biological results such as bronchoconstriction and vasoconstriction (3, 4). Related work by John Vane and others showed that another class of lipid mediators called prostaglandins was also derived from the oxidation of AA by the enzymes COX-1 and COX-2 (5). The two inflammatory pathways generated from AA are referred to as the COX pathway and the 5-LO pathway (6). In 1982, Bergstrom, Samuelsson and Vane were awarded the Nobel Prize for their research of lipid inflammatory mediators and their discovery that aspirin and NSAIDs block the COX pathway, thereby, reducing the inflammatory effects of allergens, tissue damage and disease.

Later in the early 1990s, in search of inhibitors of the 5-LO pathway to treat asthma, Merck-Frosst scientists discovered an adaptor protein, later called 5-Lipoxygenase Activating Protein (FLAP), that was required – along with 5-lipoxygenase – for the cellular synthesis of leukotrienes (7, 8). Unlike the COX enzymes that are found in most cells, 5-LO and FLAP are expressed primarily in immune cells and silenced in most non-immune cells.

Merck postulated that the cellular synthesis of leukotrienes could be effectively attenuated or abolished by the use of FLAP inhibitors, and a number of such compounds, including the indole MK-886, and the quinolone-indole MK-591 were developed and tested in Phase 1 and 2 asthma trials (9, 10). Both compounds had excellent clinical safety profiles and showed efficacy in blocking leukotriene synthesis and mitigating asthma symptoms, but were shelved when Merck chose, in the alternate, to market its leukotriene receptor antagonist Montelukast, *Singulair* (11, 12).

**BPP Findings regarding the Biosynthesis of Leukotrienes in the Brain after TBI**

In 2007 Drs. Kim Heidenreich and Robert Murphy discovered that leukotrienes, pro-inflammatory mediators normally absent in the brain, are created within minutes after brain injury by a unique biosynthetic pathway (see Figure 1) involving two separate cell types – immune cells (resident microglia or infiltrating neutrophils) and non-immune cells (neighboring astrocytes or neurons) (13).

Immune cells contain the 2 critical enzymes, 5-lipoxygenase (5-LO) and the 5-lipoxygenase activating protein (FLAP), that work together to convert free arachidonic acid (AA) to the intermediate leukotriene A4 (LTA4). The intermediate LTA4 has 2 fates: 1) it can be converted to leukotriene B4 (LTB4), a potent chemotactic lipid molecule released from immune cells to recruit other endogenous as well as peripheral immune cells to the site of injury, or 2) it is also released from immune cells and taken up by adjacent non-immune brain cells and converted to LTC4, by the action of LTC4 synthase. Metabolism of LTC4 occurs by sequential peptide cleavage reactions involving a *γ* -glutamyl transpeptidase that forms LTD4 and a membrane-bound dipeptidase that converts LTD4 into LTE4. The metabolic transformation of LTA4 to the cysteinyl-leukotrienes (LTC4, LTD4 and LTE4) is critical for determination of their receptor binding specificity and biological activity (14).

 **Cysteinyl Leukotrienes**

* Increase vascular permeability
* Cytokine release
* Tissue edema
* Inflammation

**LTB4**

* Chemotaxis
* Chemokinesis
* Neutrophil activation

**FLAP Inhibitor**

**(MK-591)**

**Microglia**

**Figure 1**

The transcellular nature of cysteinyl-leukotriene production contributes to the high level of regulation of the 5-LO pathway. As the cellular substrates and enzymes for making leukotrienes are present and ready for activation without the need of protein synthesis or the assembly of an inflammasome (required for cytokine production), leukotrienes are produced immediately upon tissue damage, and thus represent the “first-responders” of the innate immune response to tissue damage, making them excellent drug targets for blocking inflammation.

**TBI-induced Neuroinflammation**

A traumatic brain injury includes multiple phases of injury, beginning with the **primary** **injury** – caused by mechanical disruption of macro- and microscopic structures within the brain resulting in tissue damage – followed by various **secondary** **injury** responses that include neuroinflammation as a critical and progressive component. Neuroinflammation is an inflammatory response within the brain (depicted in Figure 2) that is mediated by the production of leukotrienes, cytokines, chemokines, reactive oxygen species (ROS), and other second messengers. These mediators are produced by resident glial cells (**microglia and astrocytes**) and peripheral immune cells (**neutrophils and macrophages**) that infiltrate the brain as shown in **Figure 2**.



**Figure 2. Schematic of TBI-induced Neuroinflammation**

As represented in Figure 2, the injured axons or other damaged tissue resulting from a TBI **1** releases signals called damage-associated molecular patterns (DAMPs), ATP, and glutamate which **2** bind to pattern recognition receptors on nearby resting microglia and astrocytes, leading to their activation, migration, and **3** release of pro-inflammatory mediators – leukotrienes, cytokines, chemokines and reactive oxygen species – in the area of the injury.

The pro-inflammatory mediators produced endogenously by the brain **4** increase blood-brain barrier permeability and signal peripheral immune cells to migrate to the site of injury. These infiltrating immune cells (macrophages and neutrophils) **5** secrete additional pro-inflammatory mediators which **6** contribute to excitotoxicity, mitochondrial impairment, and vascular dysfunction.

It is generally believed that following the production and release of pro-inflammatory mediators there is a switch that results in the synthesis and release of anti-inflammatory mediators that promote neurogenesis, plasticity, re-myelination and angiogenesis, each of which promotes regeneration and repair of the brain. These processes allow for injury resolution and recovery usually within 1 to 2 months following a mild TBI like a concussion.

However, **in situations where the pro-inflammatory stimulus persists, due to repetitive or prolonged injury to the brain,** the inflammatory response becomes dysregulated. The now primed microglia release abnormally high amounts of pro-inflammatory mediators thatattack uninjured tissues resulting in **7** further brain damage and a shut-off of regeneration and repair processes. These events lead to a perpetual feed-forward inflammatory cascade **8** that can persist for months and years, and contributes to life-long cognitive and behavioral disabilities and, in some cases, **9** dementia, suicide and death.

There is abundant clinical and experimental data that support the “dysregulated neuroinflammatory scenario” described above where the negative aspects of neuroinflammation outweigh the positive aspects. Following diffuse, penetrating, and/or repetitive head injuries, the pro-inflammatory profile of activated microglia and reactive astrocytes persists for a prolonged period after injury. Furthermore, a substantive fraction (20-30%) of mild TBI patients **do not recover** within the normal time period following injury and suffer a variety of ongoing physical, cognitive, and emotional symptoms seen in Figure 3. **This condition is termed “persistent post-concussive syndrome” (PPCS)**. Although the specific underlying cause of PCS is not fully understood, it is generally believed that PCS involves 1) unresolved neuroinflammation, 2) failure to activate regeneration and repair processes, or 3) a combination of these processes. Further, it is reasonable to propose that a blockade of ongoing neuroinflammatory cascades would be expected to promote recovery of the brain, mitigate post-concussive symptoms, and prevent chronic degenerative processes.



**Figure 3**

**Appendix 9**

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