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The potential of 12/15-lipoxygenase inhibitors in stroke therapy

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Stroke is the fourth leading cause of death and the premier cause of disability in the USA. Approximately 800,000 first or recurrent strokes are recorded each year, indicating an enormous need for effective stroke therapies [1]. Nonetheless, only one drug currently has US FDA approval for acute stroke treatment; tissue plasminogen activator (tPA), which helps dissolve the blood clots that cause ischemic strokes. Ischemic strokes represent approximately 85% and hemorrhagic strokes represent approximately 15% of all stroke events; however, since tPA causes and/or exacerbates bleeding in the brain, it can not be administered for hemorrhagic strokes. For this reason, advanced imaging via computed tomography or MRI is necessary to classify the cause of the stroke. The field is, therefore, wide open for novel forms of treatment, which either work as standalone therapeutics or reduce the bleeding complications of tPA. Inhibitors of 12/15-lipoxygenase (12/15-LOX) are in an excellent position to provide such a novel therapeutic approach.

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Lipoxygenases are a family of lipid-oxidizing enzymes, which generate eicosanoids and related compounds from arachidonic acid and other polyunsaturated fatty acids. The 12/15-LOX (aka 15-LOX-1) is special in that it can directly oxidize lipid membranes containing polyunsaturated fatty acids, without the preceding action of a phospholipase, leading to the direct attack on organelles, such as mitochondria. This presumably underlies the cytotoxic activity of 12/15-LOX, which is upregulated in neurons and endothelial cells after stroke and could contribute to both neuronal cell death and blood–brain barrier leakage. The use of 12/15-LOX inhibitors in mice confirmed these hypotheses and provided multifactorial protection, by limiting the expansion of the initial infarct, as well as by reducing edema [2]. In addition, we demonstrated that protection through 12/15-LOX inhibition is both long-lasting and has a suitable time window for treating stroke, with efficient infarct size reduction even when the inhibitor was given 4 h after the onset of ischemia [3]. Strikingly, 12/15-LOX inhibitors also helped to reduce tPA-associated bleeding, greatly expanding the therapeutic potential of these compounds. Finally, we were able to demonstrate that 12/15-LOX is increased not just in rodents, but also in humans following a stroke [3], reinforcing its potential as a human therapeutic.

So what are the challenges to introducing a 12/15-LOX inhibitor into the clinic? For one thing, the climate within the pharmaceutical industry is marked by skepticism towards novel approaches to neuroprotection. Many companies have simply stopped working on stroke and decided to focus on other neurodegenerative diseases because previous clinical trials have failed, and Phase III stroke trials are large and cost millions of dollars. This lack of industry interest is compounded by deficiencies in the design of clinical trials, in which an enormous spectrum of patients, with varying etiologies and severity of strokes, are bundled together, and outcome measures are not appropriate for measuring the efficacy of a specific form of intervention. On the other hand, recent developments have shown that funding agencies such as the US NIH are taking these problems seriously and coming up with new approaches [4]. We, thus, feel that the time is ripe to advance novel 12/15-LOX inhibitors as stroke treatments, and we have come up with a strategy to achieve this goal.

> "…12/15-LOX inhibitors may become a first-line treatment for stroke in the ambulance, and could also be combined with tPA to increase the safety of the only currently available stroke treatment option."

In our view, a successful stroke drug should fulfill several requirements. First, know your target: while a large number of genes are up- or down-regulated following a stroke, there are nonetheless only a specific few that will make good drug targets. These should be the focus of drug development efforts, rather than generalized phenomena such as radical formation. The lack of a well-defined target molecule may have contributed to the failure of the radical scavenger NXY-059 in the SAINT II trial. Second, be selective: many current enzyme inhibitors are promiscuous in their activity against multiple members of the respective enzyme class. While these proteins may be coordinately upregulated, it is still unlikely that they all contribute equally to stroke injury, and, thus, targeting the major contributor is key to achieving high efficacy and avoiding unwanted side effects. Third, time window for administration: early clinical intervention for neuroprotection is clearly important, however, many patients are not available for early treatment because the stroke occurs in their sleep,

the symptoms are not recognized as a stroke and because identifying the nature of the stroke requires advanced imaging by computed tomography or MRI. Fourth, route of administration: stroke patients, especially in the acute phase, often experience problems with swallowing (dysphagia). Therefore, intravenous, rather than oral, administration is seen as preferable.

Our laboratories [5–12] and others [13–15] have identified potent and selective human 12/15-LOX inhibitors over the years, but many of these inhibitors are antioxidants that reduce the active site iron of LOX and/or are promiscuous against multiple LOXs, such as nor-dihydroguaiaretic acid [7], baicalein [11], tocotrienol [16] and curcumin [17]. Bristol-Myers Squibb (NY, USA) [13–15], and Parke-Davis (now Pfizer, Groton, CT, usa) [18] have identified the most drug-like 12/15-LOX inhibitors prior to our investigations, which exhibited low nanomolar potency. However, they had poor physical properties (e.g., solubility and Logp) [13–15] and poor pharmacokinetic properties for use *in vivo*. In addition, these inhibitors were not screened in parallel against mouse 12/15-LOX, so it is unlikely any would be active in the rodent models and, thus, potential drug leads. To remedy this, we therefore developed a comprehensive approach to discovering new inhibitors of 12/15-LOX.

Our pipeline approach to introducing novel 12/15-LOX inhibitors, developed in conjunction with Harvard, UCSC and NCATS/NIH, is intended to identify best candidates by a sequence of *in vitro*, cell culture and *in vivo* tests. Briefly, molecules that inhibit the recombinant human version of 12/15-LOX via high-throughput screening, are classified as initial hits. These hits are then tested for their neuroprotective capacity in neuronal HT22 cells of mouse origin, where a cell death assay dependent on 12/15-LOX activity has been well established. We have adapted this assay to a medium-throughput platform in 96-well plates, allowing us to test up to ten compounds at a time. Compounds that are neuroprotective to HT22 cells are then counter-screened for selectivity against the other lipoxygenases, such as 5-LOX, 12-LOX and 15-LOX-2. The next step is to evaluate their radical scavenging activity, to eliminate molecules with a clear antioxidant mechanism. Finally, we evaluate their structure–function relationship by both medicinal and computational methods and determine if their *in vitro*, *ex vivo* and *in vivo* potency can be improved.

In the course of these studies, we have made a variety of surprising observations. First, a large spectrum of chemotypes have shown activity in our screens. Second, and more importantly, there is significant species-to-species variability in the 12/15-LOX enzyme, meaning we have found compounds that inhibit the human but not the mouse version of 12/15-LOX, and *vice versa*. In our view it is essential that a candidate compound must inhibit both human and rodent versions of 12/15-LOX, because testing in a small animal, typically mouse or rat models of stroke, is required before advancing to human trials. Unfortunately, some compounds that we identified that inhibited the 12/15-LOX in both species, did not always do so with equal potency. Nonetheless, the EC_{50} values determined in the HT22 cell assay can be used as a first approximation of the dosage needed to protect against experimental stroke in mice. We typically start with a mouse model of transient middle cerebral artery occlusion, followed by infarct size determination after 24 h, before additional rodent models of stroke are attempted. This has led to the identification of several

new 12/15-LOX inhibitors, including LOXBlock-1 [3,5,19] and ML351 [20], which reduce infarct size and limit the bleeding side effect of tPA. As such, these 12/15-LOX inhibitors may become a first-line treatment for stroke in the ambulance and could also be combined with tPA to increase the safety of the only currently available stroke treatment option.

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Biographies

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