● PERSPECTIVES

Sulindac for stroke treatment: neuroprotective mechanism and therapy

Sulindac, a widely used nonsteroidal anti-inflammatory drug (NSAID) is a prodrug that is reduced by methionine sulfoxide reductase to its active form as an inhibitor of cyclooxygenase 1 and 2. The drug has been shown to elicit tissue protection by processes that may include at least three functions: antioxidant, preconditioning and anti-inflammatory. Sulindac demonstrates neuroprotection that involves inhibition of mitochondrial calcium overload or a decrease in protein oxidation. We have demonstrated the induction by sulindac treatment of pro-survival proteins Hsp27, Akt and Bcl-2 in the ischemic penumbra and core of the central nervous system (CNS) infarct in a rat model of ischemic stroke. Our findings point to sulindac acting on the endoplasmic reticulum (ER) to decrease ATF-6 and on the mitochondrion to increase Bcl-2 as well as decrease pro-apoptotic components BAK and PUMA. The resulting decrease in ER stress and reduction in apoptosis underlies the protective effect of sulindac in reducing infarct size following transient focal brain ischemia. The potent neuroprotective effect of sulindac in the stroke model is obtained with low-dose administration of the drug pointing to the potential of sulindac as a valuable neuroprotective agent against oxidative stress in cerebral ischemia.

Sulindac has been widely used as a NSAID that is capable of inhibiting cyclo-oxygenases (COX) 1 and 2. The molecule is a substituted indeneacetic acid chemically related to indomethacin (Strong et al., 1985). Sulindac is commercially available and known as Clinoril, a prescription NSAID that is approved for use in 34 countries (Martindales Extra Pharmacopeia), including the United States. The U.S. Food and Drug Administration have approved sulindac for treating acute gouty arthritis, acute painful shoulder (bursitis/tendonitis), osteoarthritis, and rheumatoid arthritis (http://www.fda.gov). In addition to its established anti-inflammatory, antipyretic, and analgesic properties, sulindac is thought to provide anticarcinogenic effects based on extensive data from cell culture experiments and animal model systems as well as more limited data from early-phase chemoprevention trials conducted primarily among adenomatous polyposis patients (Reid et al., 2008). Following ingestion, the parent compound (sulindac sulfoxide) is converted into sulindac sulfide, which appears to be the primary COX-inhibiting metabolite. The second major metabolite, sulindac sulfone (exisulind), has also been shown to interrupt carcinogenesis albeit through COX-independent pathways (Brunell et al., 2011) and has undergone more limited clinical development to date in light of some toxicity concerns. The sulfide metabolite is an active moiety and is approximately five times more potent than sulindac, while the sulfone elicits no pharmacological anti-inflammatory response (Strong et al., 1985). Thus sulindac may be regarded as a prodrug that is reduced by the enzyme methionine sulfoxide reductase (Msr) to the active form, with the sulfide as the active species or pharmacophore (Moench et al., 2009). The long half life of the sulfide results in its accumulation during chronic dosing. After a single oral dose of sulindac to man, the peak plasma concentrations of both the sulfide and the sulfone occur about 2 hours after that of the parent compound. This suggests that the tissues rather than the gut flora are the site of both the reduction and oxidation of sulindac (Strong et al., 1985).

In addition to its known anti-inflammatory activity there have been numerous studies in recent years on the ability of sulindac and its metabolites to act as potential anti-cancer agents, based on their ability to slow the progression of colorectal polyps to colon cancer, as well as their ability to kill colon cancer cells and other types of cancer cell (Marchetti et al., 2009). However, the potential value of sulindac for protection in acute cerebral ischemia is still uncharacterized. A growing body of evidence shows that sulindac offers remarkable neuro-protective effects by inhibition of mitochondrial Ca^{2+} overload or reduction of protein oxidation in neurodegenerative disorders (Xing et al., 2012) indicating that sulindac may have potential to exert a beneficial effect in cerebral ischemia.

Recent studies suggest that sulindac protects normal cells against oxidative damage. Previous studies on the heart suggested that sulindac protection against ischemic damage occurs through an ischemic preconditioning mechanism.

Sulindac was found to induce inducible nitric oxide synthase and Hsp 27 in a PKC dependent manner. It has been widely proposed that compounds that could precondition cells to oxidative stress may have important therapeutic value, since oxidative damage appears to play a major role in age related diseases (Moench et al., 2009).

A number of studies show efficacy of sulindac in models of neurodegenerative disease and importantly the drug is capable of crossing the blood brain barrier (BBB) and therefore may be capable of interacting with therapeutic targets in the CNS. Evidence of the sulindac's ability to cross the BBB is found in studies on guinea pig (Duggan et al., 1980). They detected significant levels of sulindac sulfoxide, sulindac sulfide and sulindac sulfone in brain after single IV injections of C-14 labeled sulindac sulfoxide. In a study, injection with either sulindac epimer (the S-epimer or the R-epimer) in rats resulted in production of both the sulfone and sulfide metabolites in brain (Brunell et al., 2011). Several studies point to the likelihood that sulindac may protect against the progression of Alzheimer's disease and Parkinson's disease either through generalized anti-inflammatory actions or through specific effects on protein aggregate formation. Epidemiological studies have indicated a lower risk of developing Alzheimer's disease or Parkinson's disease associated with use of non-aspirin NSAIDs. NSAIDS have been proposed as agents that modulate Aβ production and sulindac was found to reduce levels of secreted Aβ$_{42}$ in cell culture and to decrease levels of soluble Aβ(1–42) in brains from the Tg2576 mutant mouse. It has been proposed recently that NSAID based γ-secretase modulators may bind directly to the APP/C99 substrate to form a complex that modulates γ-secretase cleavage. This mechanism is controversial however and in contrast it has also been suggested that monomeric γ secretase modulators either do not bind to C99 or Aβ$_{42}$ or only bind non-specifically and weakly. While epidemiological studies on Parkinson's disease point to a decreased incidence with NSAID use, the mechanisms of NSAID protection in Parkinson's remains to be elucidated. The aggregation of α synuclein (αS) in the brain
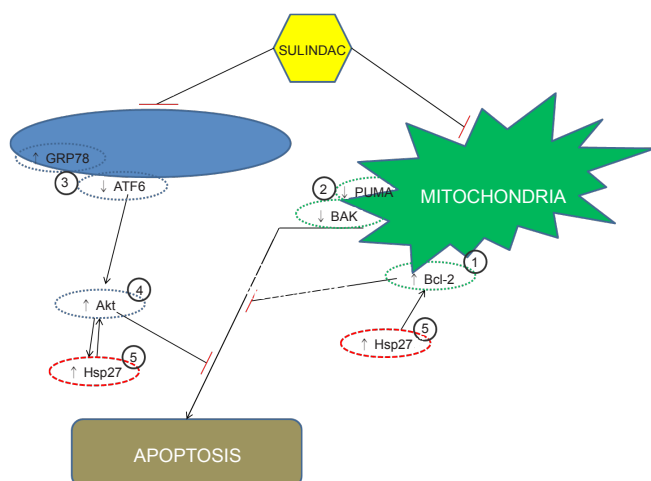
Figure 1 Proposed mechanism of action of sulindac.
The sequence of events leading from the action of sulindac on the mitochondrion and on the ER to neuronal survival is depicted as follows: (1) Increased Bcl-2. (2) Decreased BAK and PUMA. (3) Increased GRP 78 and decreased ATF-6. (4) Increased Akt. (5) Increased Hsp27. Bcl2: B-cell lymphoma 2; BAK: Bcl-2 homologous antagonist killer; PUMA: p53 up-regulated modulator of apoptosis; GRP 78: glucose-regulated protein 78 kDa; ATF-6: activating transcription factor 6; Akt: protein kinase B; Hsp 27: heat shock protein 27.

is a key step in the development of Parkinson's disease. It has been proposed that the precursors of fibrils of αS (f-αS) may be more toxic than the fibrils themselves (Hirohata et al., 2008). Using *in vitro* assays, Hirohata et al. (2008) has shown that sulindac inhibits formation of f-αS and destabilized preformed f-αS.

We discovered that sulindac at 0.2 mg/kg reduced the brain infarct size when it was administered either before or after middle cerebral artery occlusion (MCAO) surgery. Specifically, animals that were sham operated or MCAO operated with or without sulindac treatment were sacrificed 3 & 11 days after stroke onset, and infarct size in the left hemisphere was measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. Western blotting on tissue from the core and the penumbra of both hemispheres was employed for analysis of the expression of key proteins involved in apoptosis (Bcl-2 family members), and cell protection and survival (two heat shock proteins namely Hsp27 and Hsp70 as well as the signaling kinase Akt). TTC analysis of brain slices indicated a decrease in infarct size in sulindac treated animals at 4 mm, 6 mm and 8 mm from the anterior pole. The western results indicated that sulindac induced Hsp 27 protein expression in ischemic penumbra and core on days 3 & 11. Hsp 27 is a marker of cell stress and plays an important role as a molecular chaperone. There were also significant increases in the protective molecules Akt and Bcl-2 in ischemic penumbra and core (Modi et al., 2014).

Conceptually, the idea of proposing to use sulindac as a novel therapy for stroke is innovative. Despite extensive research to develop drugs for stroke based on the known mechanisms such as glutamate receptor antagonists, calcium channel blockers, enzyme inhibitors, inhibitors of apoptotic pathways and ROS scavengers, the efforts are disappointing. This is partially due to the fact that the underpinning mechanism of stroke-induced brain injury is multi-factorial, and hence it needs a therapeutic intervention that can address
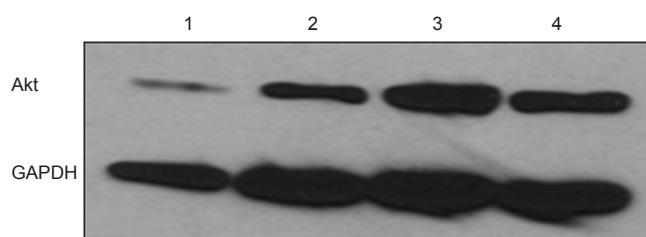

Figure 2 Akt expression in penumbra of ischemic area of stroke on day 11 (After stroke surgery, animals were sacrified on day 11).
1: Sham; 2: penumbra of control with no drug; 3: sulindac treated penumbra; 4: post ischemia sulindac treated penumbra. $n = 3$.

the complicated nature of the disease. Sulindac may act therapeutically in cerebral ischemia through its effects on Hsp 27 and Akt (**Figure 1**). As Hsp 27 and Akt prevent apoptosis it is likely that the elevated levels of the anti-apoptotic molecule Bcl-2 in the current study will contribute by further augmenting the pro-survival effect of sulindac. Hence sulindac exerts its neuroprotective role by inhibiting apoptosis *via* increased Bcl-2 expression and decreased BAK and PUMA expression in the ischemic penumbra (**Figure 1**).

An enhanced expression of GRP78 in the sulindac treated ischemic core and ischemic penumbra implies that sulindac may protect the brain through preconditioning and is consistent with previous studies on ischemic preconditioning showing that an enhanced level of GRP78 expression and protection against slow neuronal death (Modi et al., 2014).

In our study, Akt is activated after ischemia. The sulindac treated groups show greater than 3 fold Akt activation in the penumbra of the ischemic model of stroke compared to the penumbra of the untreated group and approximately a 50% increase in the core at day 3 after vessel occlusion (Modi et al., 2014). At day 11 after vessel occlusion, the sulindac treated group shows a greater than 2 fold increase in Akt in the penumbra compared to the untreated group and an approximately 1.5 fold increase in the penumbra after post ischemia treatment (after 24 hour stroke) with sulindac compared to the untreated group (**Figure 2**).

None of the currently available pharmacological interventions can provide an effective treatment for stroke. This is partially due to the fact that the underpinning mechanism of stroke-induced brain injury is multi-factorial, and hence it needs a therapeutic intervention that can address the complicated nature of the disease. Sulindac is a substrate for the enzyme methionine sulfoxide reductase which reduces sulindac to sulindac sulfide and elicits the anti-inflammatory action of the drug. A second mechanism of action of sulindac is through acting in conjunction with the catalytic antioxidant action of Msr to decrease cellular oxidant levels (Weissbach et al., 2005). A further mode of action by sulindac is as a preconditioning agent switching on key protective pathways as was previously reported in studies on myocardial ischemia. Hence, sulindac can combat types of brain injury in stroke that are either related to inflammation or to oxidative stress both of which are believed to be important mechanisms underlying tissue damage in stroke. Based on its mode of action as described above, the use of sulindac, a potent anti-oxidant and anti-inflammatory agent, in stroke interventions is both innovative and novel. Another innovative feature of the current application of sulindac is its safety and efficacy since sulindac is a U.S. Food and Drug Administra-

tion approved drug and it has been widely used clinically for decades. Furthermore, in this invention we have shown that sulindac at low concentration is effective in reducing the size of brain infarction by increasing the level of molecules that are important for cell protection and survival.

In future experiments, we plan to specifically address the mechanism of protection by sulindac, and also test whether sulindac is functioning as a preconditioning agent in stroke. To understand this protective mechanism we will evaluate changes in preconditioning markers, the role of reducing enzymes and mitochondrial function. In conclusion, we believe that sulindac may represent a novel therapeutic agent for oxidative stress induced ischemic diseases. Sulindac and its metabolites, sulindac sulfide and sulindac sulfone, inhibit the activation of the nuclear factor-kappaB (NF-κB) pathway by inhibiting IKKb kinase activity in colon cancer (Yamamoto et al., 1999). This result suggests that inhibition of components of the NF-κB pathway may at least in part be involved in the anti-inflammatory properties of sulindac. Investigation of the NF-κB pathway in ischemic brain represents an important future direction for elucidating the mechanisms of protection of sulindac in stroke.

The reported side effects of sulindac are important to consider and are dependent on dose and timing of administration. Sulindac has been reported to elicit gastrointestinal damage because of inhibition of PGE synthesis through COX inhibition. In circumstances where potential gastrointestinal deficits are a concern clinically, administration of the antacid lansoprazole has offered an effective route for abolishing such adverse effects. Many NSAIDs including sulindac are reported clinically to increase the risk of heart attacks (Shau et al., 2012). As a possible solution to this problem, the low dose administration of sulindac employed for tissue protection in a stroke model by Modi et al. (2014) and in a model of myocardial ischemia by Moench et al. (2009) may not elicit COX inhibition and could avoid potential cardiovascular side effects. Hypertensive side-effects may arise from the use of NSAIDs and these are likely to depend on the specific NSAID used as well as the type of antihypertensive agent used if they are taken concurrently. Two meta-analyses based on data from younger adults demonstrated that NSAID use results in an increase in mean blood pressure of 5.0 mm Hg. Comparison of different NSAIDs demonstrated that piroxicam and indomethacin showed the largest and sulindac the smallest pressor effect.

The introduction of sulindac as a pharmacological intervention will greatly improve the effectiveness of stroke treatment over traditional drug treatments since it will combat two of the most likely mechanisms involved in the stroke-induced brain injury. The future clinical impact of sulindac treatment to the field of therapeutic intervention for brain diseases including stroke, Parkinson's disease and Alzheimer's disease will be highly significant. The novel application of this drug has already been demonstrated as a "proof of concept" for treatment of stroke and other brain diseases.

The protective effect we have observed with sulindac in cerebral ischemia is contrary to several studies that NSAIDs cause increased risk of stroke and heart attack. It should be noted that in the rat feeding experiments described in this study the daily dose of sulindac (0.2 mg/d) was only 10–15%, on a weight basis, compared to the doses taken clinically as an anti-inflammatory agent. Hence sulindac at the low dose administered in this study is likely to be very valuable as a neuro-protective agent against oxidative stress in cerebral ischemia (Modi et al., 2014).

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