

Interventions Testing Program Application

Title: Sulindac

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Background

The Msr system and sulindac

I would like to propose that **sulindac** be tested for its effect on longevity. This drug was one of the early NSAIDS marketed by Merck, and can still be obtained by prescription. Sulindac is an NSAID prodrug that must be reduced to sulindac sulfide, a reaction catalyzed by the methionine sulfoxide reductase (Msr) enzymes, MsrA and MsrB (1). As part of the background regarding sulindac, I would like to say a few words about how our laboratory became interested in sulindac and our general approach to the aging problem. We initially identified the Msr system, which protects cells against oxidation of methionine (Met) residues in proteins to methionine sulfoxide (Met(o)), by reducing the Met(o) back to Met. For a review see reference (2). The Msr system is also part of a scavenger system that permits exposed Met residues in proteins to function as catalytic anti-oxidants (3). With regard to longevity we had shown that the Msr system can extend the life span of flies (4), and is involved in life span extension of worms on a caloric restricted diet (5) while others showed that MsrA is involved in extending the life span of worms that have a *daf 2* mutation (6). **These MsrA studies, and other aging studies with SOD and catalase, convinced us that one approach to extend life span is to up-regulate, or activate, one or more of the natural systems that cells use to protect against oxidative damage.** More specifically, our initial goal was to find drugs that will activate the Msr enzymes and we developed a high-throughput screening assay (7), and have screened over 500,000 compounds. We recently have identified in vitro activators of both MsrA and MsrB that are derivatives of the natural product fusaricidin (8), but animal studies have not been done, so these compounds are at too early a stage to test in an aging experiment. However, as mentioned above, the studies on the Msr system led to our interest in sulindac, which is a substrate for the Msr enzymes. **It is now clear that sulindac is a potent pharmacological preconditioning agent that has been shown in vivo to protect the heart, retina and brain against oxidative damage.** This property of sulindac is independent of the Msr system and its NSAID activity

Ischemic and pharmacological preconditioning (IPC) IPC is an important cellular mechanism that is initiated when cells are exposed to a sub-lethal hypoxia, and will protect the cells from a more severe, potentially fatal, ischemia. The mechanism has been studied in great detail, especially in the heart and brain, with regard to protection against ischemia/reperfusion (I/R) damage (9, 10). To summarize, early phase IPC, that occurs within the first few minutes of I/R damage can be triggered by low levels of reactive oxygen species (ROS) and involves, PKC epsilon and activation of an ATP sensitive mitochondrial K⁺ channel, which prevents formation of the mitochondrial permeability

transition pore (MPTP). The MPTP pore, if formed, can lead to cell apoptosis. Late phase IPC, which can occur 12- 18 hours later, involves the expression of a large number of nuclear coded genes that also help to protect the cells against oxidative damage, including stress proteins, phase 2 enzymes that protect against oxidative damage and iNOS. It is known that nitric oxide (NO) can also act as a mitochondrial effector to maintain the IPC response for many additional hours (11). There are known drugs that have been shown to protect cardiac and/or brain cells against I/R damage by what appears to be a preconditioning like mechanism, which is referred to as pharmacological preconditioning. These include sildenafil, rapamycin, metformin, resveratrol and some glitazones (12-16). As discussed below we believe that sulindac has an overall profile that makes it an attractive drug to test in a mammalian aging model.

Studies with sulindac: protection of normal cells by a preconditioning mechanism similar to IPC- Since sulindac was a substrate for the Msr enzymes (7) we thought that it might also function in cells as a catalytic antioxidant, in a similar manner to Met residues in proteins. In our initial experiments we showed that sulindac could protect normal lung cells against oxidative damage. However, it soon became clear that this protective effect of sulindac was independent of the Msr system and its NSAID activity(17). A similar protective effect by sulindac was seen with rat cardiac myocytes exposed to hypoxia and reoxygenation (18), and this initiated a search for the mechanism by which sulindac protected these cells against oxidative damage. Although the initial studies were done using cells in culture, we later moved to studies on the effect of ischemia/reperfusion on the intact rat heart using a Langendorff model of cardiac ischemia (18). For these experiments the sulindac was fed to the 300 gm rats (0.2 mg/ day or about 0.7 mg/kg for two days), before the intact hearts were removed and analyzed in a Langendorff preparation without further drug exposure. The hearts were then subjected to 45 minutes of ischemia followed by 2 hr of reperfusion. Damage to the heart was measured either by LDH released or infarct size using TTC to stain the tissue slices. Sulindac reduced LDH release by close to 60% during the ischemic period and 80% during reperfusion. The size of the infarct was also diminished about 50% in the hearts from animals fed sulindac. In addition, the sulindac effect was significantly inhibited by the drug chelerythrine, a broad spectrum inhibitor of the PKC enzymes, which was also fed to the animals (5 mg/kg) per day for 2 days. PKC epsilon is known to be involved in IPC. In these same studies there was other evidence that the sulindac effect was similar to IPC, since sulindac also increased the expression of both iNOS and HSP 27 in the rat myocardium and the protective effect required ROS (18). The tentative conclusion from these studies was that sulindac was initiating a pharmacological preconditioning response that was similar to what is observed in IPC, but in the absence of hypoxia.

To extend these studies on the mechanism of sulindac protection of normal cells against oxidative damage we decided to use retinal pigmented epithelial cells (RPE), which are known to be sensitive to oxidative stress. These studies initially used the established ARPE19 cell line and the results showed that sulindac could protect the RPE cells against both chemical oxidative damage and UV damage (19). Since there are questions as to whether this established cell line will reflect what occurs in vivo, studies were also performed on early passage human fetal

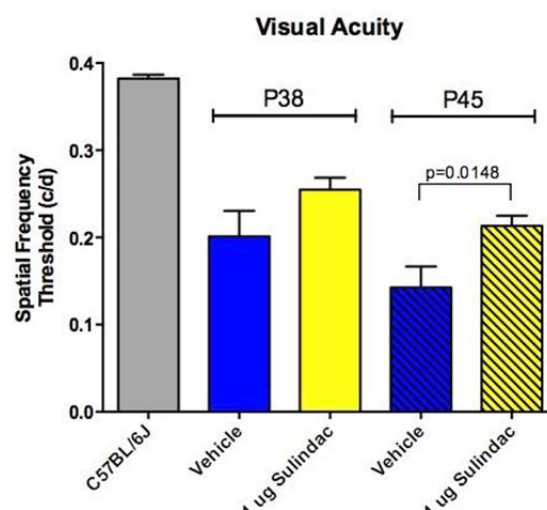


Figure 1: Sulindac treatment rescues scotopic vision in *rd10* mice. OKT measurements were performed at P38 and P45 following intravitreal injection of vehicle or 1 μ g sulindac at P31. Sulindac-treated eyes had significantly improved visual acuity at P45 (unpaired t-test, $p=0.0148$). Data from Dr. R. Farjo at EyeCro.

RPE cells where sulindac again provided excellent protection against chemical oxidation. In this study more evidence was also presented that the mechanism of sulindac protection involved a pharmacological preconditioning response similar to IPC. As example, it was shown that ROS, PKC epsilon, the mitochondrial ATP sensitive K⁺ channel, and PKG were all involved in the protection mechanism, and iNOS and Hsp 70 proteins were induced. It was also shown that PPAR alpha was also involved in the protection of the retina by sulindac [19]. Dr. Rafal Farjo at EyeCro was aware of our retinal results and offered to carry out in vivo experiments. EyeCRO is a CRO that specializes in animal testing of drugs for diseases of the eye. He performed an initial experiment on the ability of sulindac to protect mice carrying a genetic mutation seen in patients with retinitis pigmentosa (RP). RP results from mutations in rhodopsin or proteins involved in the visual pathways in the retina. These unpublished results are shown in Figures 1 and 2. Figure 1 shows that sulindac, 1 ug injected intraocular, will slow the rate of visual loss significantly, and Figure 2 shows that the loss of photoreceptor cells is also diminished in the animals that received either 0.25 ug or 1 ug. Finally, sulindac has also been reported by our colleagues here at FAU to protect rats from I/R damage in an animal stroke model (20).

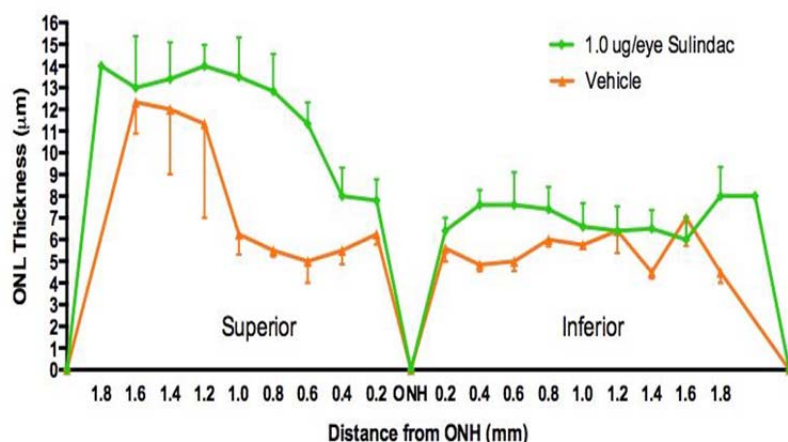


Figure 2. Histological measurements of ONL in rd10 mice treated with sulindac. Quantitative measurements of ONL as thickness are plotted as a function of distance from ONH. Sulindac treatment increased retinal thickness in the ONL and in the INL near the ONH. Data from Dr. R Farjo at EyeCro.

Effect of sulindac on cancer cells

Another interesting observation was that sulindac does not protect cancer cells against oxidative damage, as seen in normal cells. In contrast sulindac sensitizes the cancer cells to agents that perturb mitochondrial respiration, resulting in enhanced killing. The initial studies used sulindac in combination with TBHP with 3 cancer cell lines, A549 lung cancer cells, RKO colon cancer cells and SCC25, a tongue derived squamous cell carcinoma (17). A later study showed that sulindac could also enhance cancer cell death when used in combination with dichloroacetic acid, an anti-cancer agent that is known to affect mitochondrial respiration (21). We have now evidence that dual drug combinations containing sulindac and other anti-cancer agents such as doxorubicin, arsenic trioxide and cisplatin result in markedly enhanced killing, at much lower concentrations of the anti-cancer drug. In addition, we now have evidence that other agents that have been reported to protect normal cells (cardiac or brain) against ischemia/reperfusion can replace sulindac in these cancer studies. These include sildenafil (viagra) rosiglitazone and pioglitazone (PPAR gamma agonists) and rapamycin. These dual drug combinations, containing a pharmacological preconditioning agent and an anti-cancer agent that affect mitochondrial respiration, show enhanced killing in other cancer cell lines including retinoblastoma, glioblastoma, neuroblastoma and breast cancer. This unique sensitivity of cancer cells to these dual drug combinations appears to be due to a basic metabolic difference in energy metabolism between normal and cancer cells, first described by Warburg (22). With regard to

signaling pathways involved in the enhanced killing of the cancer cells we have strong evidence that sulindac inhibits AKT phosphorylation, which could result in downstream inhibition of mTOR, an important cellular nutritional sensing molecule. It should be noted that the combination of sulindac with difluoro-methylornithine (DFMO) has been reported to reduce the recurrence of colon adenomas in humans by 70-90% (23).

Based on our results with several reported pharmacological preconditioning agents, it is our conclusion that sulindac is an attractive agent to use for an aging study, if one wants to protect normal cells against oxidative damage. We base this on a comparison of the efficacy of sulindac in protecting retinal cells against chemical oxidation, with 4 other agents that have been reported to protect cells against I/R oxidative damage by a preconditioning mechanism, namely, sildenafil, rapamycin, pioglitazone and metformin. The results are shown in Figure 3.

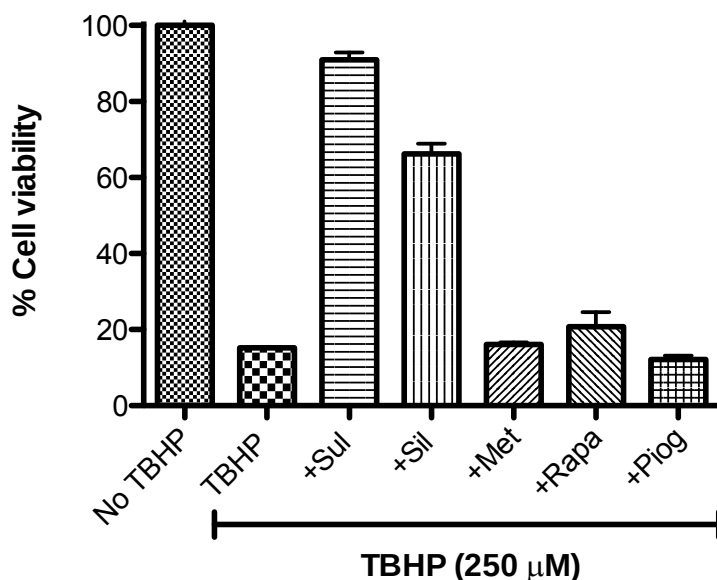


Figure 3. Effect of pharmacological preconditioning agents against oxidative damage in RPE cells. The comparative protective effect of pre-incubating RPE cells with **sulindac** (200 μ M), **sildenafil** (20 μ M), **metformin** (2mM), **rapamycin** (10 nM) and **pioglitazone** (10 μ M) for 24 h prior to exposing them to chemical oxidative stress induced by TBHP (19) .

Of the preconditioning compounds tested only sulindac, under the conditions used, provided almost complete protection of the retinal cells, with sildenafil affording partial protection. Rapamycin, and metformin showed no protection. The concentration of the drugs used in these experiments was close to, or at, their highest non-toxic concentrations. These results suggest that sulindac may be one of the best preconditioning agents against oxidative damage. In considering sulindac for an aging study, one should also take into consideration its low cost, and relatively low toxicity. Regarding the toxicity it should be noted that the preconditioning protective effect on the heart in rodents was obtained at a concentration of sulindac used (0.7mg/kgm) that is <20% of the dose recommended in humans, when used as an anti-inflammatory drug.

In summary, this proposal is based on the assumption that oxidative damage is a significant, if not the most important, factor in the aging process, and that increasing the activity of one or more of the cellular protective systems will slow the aging process. This hypothesis is supported by previous genetic results that have demonstrated up-regulation of oxidative damage protective mechanisms, such as the Msr system, SOD and catalase can extend the life span of lower animals. It seems

reasonable that a drug that induces a protective preconditioning mechanism should also be tested for its effect on life span.

SUGGESTED TREATMENT PROTOCOLS

1.Sulindac is readily soluble in aqueous solution around neutral or slightly basic pH. In our previous experiments on ischemic/reperfusion (I/R) damage to the rat heart the sulindac was prepared in the food pellets (18). The dose that was effective to prevent cardiac I/R damage in the rat was about 0.7 mg/kg. For a 25-30 gm mouse that would be about 20 µg per mouse per day or about 7.3 mg per year. For 3 years the amount of sulindac needed would be about 22 mg. For 100 mice 2.2 gms of sulindac will be needed for the 3 year study.

2.Sulindac can be purchased from Sigma for about \$12 per gm. Since the total amount of sulindac needed for the 3 year study with 100 mice is 2.2 gm, the total cost will be about \$27 for the sulindac. The amount of standard mouse diet for 1 year will be 1.8 kg, or 5.5 kg for 3 years. The estimated cost for the mouse diet (2014- breeder) with sulindac added would be \$22/ kg (Tekland diets, Envigo). Based on our estimates above, the total cost of a standard mice diet with sulindac added, we estimate to be around \$13,183) (Table 1).

Stability- Sulindac is stable in solution but may decompose if exposed to bright light for long periods. Any sulindac powder should be kept in a dark bottle.

Table 1: Estimated costs for sulindac

Cost of sulindac (S8139, Sigma)	\$12/g
Amount of sulindac/mouse/yr	7.3 mg
Amount of sulindac/mouse/ 3yr	22mg
Amount needed for 100 mice/3 yr	2.2 gm
Total sulindac cost/100mice/3 yr	\$27
Standard mice diet [2014(breeder)] with sulindac	\$22/kg
Cost of standard mice diet with sulindac/100 mice/3 yr	\$13,156
Total cost	\$13,183

3. We have developed assays, based on HPLC separation, to measure sulindac and its two major metabolites sulindac sulfide and sulindac sulfone [described in reference 1], so that blood levels can be followed. In these experiments larger doses (2mg, delivered IP, per rat) were used, and plasma levels were easily detected after 4 hrs (1). Our recommendation for the mouse aging studies, based on the rat cardiac protection results, is for about 0.02 mg of sulindac per animal per day, administered

in the diet. If this dosage turns out to be too low for detection in the plasma, it may be necessary to increase the daily dose of sulindac in the aging experiments or develop a fluorescent assay. But based on our experience, even at lower doses of sulindac, we should be able to detect sulindac and its metabolites if sufficient blood is available. The best biochemical markers for late stage aging with this drug would be evidence of decreased oxidative damage to macromolecules in the tissues. This could include evidence of protein oxidation (lysine carbonylation using dinitrophenylhydrazine (DNPH) (24) or lipid oxidation using thiobarbituric acid (25). The DNPH test requires a centrifuge, some standard reagents and a spectrophotometer.

4. I think 4 months of age would be a reasonable time to start the experiment.

Animal safety information

Merck had done all of the initial required animal testing on sulindac more than 40 years ago, and these data are, we believe, still accepted by the FDA. As with all NSAIDs, in humans there is a danger of gastric bleeding and kidney damage with long-term usage. However, at the low dose that is suggested for the aging studies the toxicity should be minimal.

Statement of Understanding

I agree to the following:

- I understand all information presented in the proposal can be freely shared with members of the ITP Steering Committee and Access Panel during their evaluation of proposals, but will otherwise be considered confidential.
- If my proposal, or a modification of it (such as altered dosage or frequency of administration), is accepted for inclusion in a research protocol, I will be asked to help evaluate the data and to prepare the data for written and oral publications, on each of which I will be offered coauthorship. I understand the ITP intends to submit the results of all ITP-supported studies—regardless if they produce data showing positive or negative effects on health status in mice—for publication.
- I understand data generated by ITP-supported experiments using the compound/diet proposed will be made publicly available and can be used in applications for further research support by anyone. I also will be free to use ITP-generated data in the context of applications for research support or for any other purpose.

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