ITP proposal: MIF098 (MIF antagonist)

Co-Sponsors: Rick Bucala (Yale University) and Richard Miller (University of Michigan)

**Rationale.** MIF (macrophage migration inhibition factor) is a widely expressed and pleiotropic cytokine with immunoregulatory, neuroendocrine, and metabolic actions (1). MIF expression is required for optimal pro-inflammatory responses and MIF has a close regulatory interaction with glucocorticoids by virtue of its ability to specifically suppress their anti-inflammatory function (4,5). MIF promotes tumor progression by several mechanisms and is expressed in elevated levels in many invasive and metastatic cancers (6,7). MIF also is a strong inducer of AMP-activated protein kinase (AMPK) and promotes glucose uptake and energy utilization in hypoxic cells (8). It is further notable that MIF is encoded in a functionally polymorphic locus within the human genome (22q11.23). Variant MIF alleles occur commonly in the human population (minor allele frequency >5%) and high expression alleles have been linked to the clinical severity of a number of autoimmune inflammatory, infectious, and oncologic diseases (9), suggesting that pharmacologic reduction of MIF activity could extend healthy, disease-free life.

That MIF expression may influence longevity was suggested initially by studies of caloric restriction (CR), where it was reported that mice maintained on either of two anti-aging diets, CR or a diet low in methionine (Meth-R), had significantly elevated levels of MIF mRNA in the liver (10,11). An influence of MIF on lifespan was addressed directly in a recent study of MIF-KO mice (B6x129 genetic background) fed *ad libitum* (AL) or a CR diet (2). Median survival in the AL control mice was 774 days while median survival in the AL MIF-KO mice was 16% higher (895 days) (Fig. 1). Among CR mice, median survival was 1045 days and was 1064 days in the CR MIF-KO, which is an increase of 35 and 19%, respectively, relative to AL mice. Notably, not only did MIF-KO mice show a lifespan extension in response to CR, they were longer lived than controls under standard AL feeding conditions. Another lifespan experiment, using a different genetic stock with much longer control lifespan, is now in progress but does not so far show a lifespan benefit (R. Miller, *unpublished*). Additional work will be needed to establish whether genetic or pharmacological modulation of MIF action will lead to improved healthspan and lifespan in mice.

![Fig. 1.](image-url) MIF-KO mice are significantly long lived (log-rank, *P*<0.001) relative to (B6×129)F2 control mice, and also exhibit a significant life-span extension in response to CR. Kaplan-Meier survival curves for each genotype and dietary condition; each point represents a single mouse. From (2).

**MIF Antagonism.** Immune neutralization or genetic deletion of MIF reduces disease severity in virtually every pre-clinical model of inflammatory disease that has been studied, prompting the development of pharmacologic MIF inhibitors for clinical application (9,12). An anti-MIF monoclonal antibody developed by the Yale co-sponsor has been humanized for phase I clinical testing for lupus nephritis and for solid tumors (13,14) ([ClinicalTrials.gov Identifier: NCT01765790](https://clinicaltrials.gov/ct2/show/NCT01765790)). Potent and pharmacologically auspicious small molecule MIF antagonists that block MIF interaction with its receptor also have been developed by the Yale laboratory (15). One such antagonist: MIF098 (3-(3-hydroxybenzyl)-5-methylbenzo[d]oxazol-2(3H)-one) is orally bioavailable and well-tolerated in mice, and shows MIF inhibitory activity in mouse models of bronchopulmonary dysplasia, hyperoxic lung injury, and in an OLAW approved collagen-induced model of arthritis (3,16-18) (Figs. 2,3).
Further noteworthy for this proposal are observations that dietary isothiocyanates, such as sulforaphane present in cruciferous vegetables, inactivate MIF by binding covalently to the same domain employed in the structure-based design of MIF098 (19). In a controlled study, subjects ingesting sulforaphane were demonstrated to have reduced MIF activity in urine (20). There is much current interest in the use of sulforaphane to attenuate the severity of oxidant, electrophile, and inflammatory stresses that contribute to the pathogenesis of many chronic diseases including aging (18-20), and it is possible that some of the benefits of sulforaphane or other Nrf2 inducers may be attributable to direct inhibition of MIF function.

The sponsors are currently unaware of any studies planning to address the impact of MIF antagonists on longevity. Because MIF098 emerged from a rational, structure-based design program, shows specificity for the MIF-MIF receptor interaction, and has undergone favorable toxicologic and pharmacokinetic testing in rodents, we propose to utilize it in a study of mouse lifespan extension.

**Fig. 2.** Chemical structure of MIF098, 3-(3-hydroxybenzyl)-5-methylbenzo[d]oxazol-2(3H)-one, identified by structure-based molecular design to inhibit the interaction between MIF and its cell-surface receptor, CD74 (3).

**Activity, Dosage, Bioavailability, and Toxicity.** MIF098 reduces the $K_D$ for MIF binding to its receptor by 5.1 fold (from $K_D = 6.5 \times 10^{-9}$ to $3.3 \times 10^{-8}$ M) and shows superior antagonism of MIF-dependent ERK1/2 phosphorylation when compared to previously described, prototypic MIF inhibitors. In a cell-based signal transduction assays, MIF098 reduces ERK1/2 phosphorylation by 200-fold when compared to the inhibitor ISO-1 (3), a structurally related MIF inhibitor previously shown to protect mice from the severe inflammatory complications of infection or autoimmunity (13,21). MIF098 recapitulates Mif-gene deficiency in established mouse models of broncho-pulmonary dysplasia (17) and hyperoxic lung injury (18). We also have recently completed a study of MIF098 in collagen-induced arthritis, and the drug showed equipotency with prednisolone (dosed at 3 mg/kg qd) and was well-tolerated over the 20 day course of daily therapy (dosed at 20 mg/kg ip bid). A small but significant measure of improved efficacy was observed by dosing MIF098 at 40 mg/kg bid or 80 mg/kg qd. Importantly, equivalent therapeutic efficacy was observed by parenteral (ip) as by oral (gavage) dosing (data available upon request).

**Fig. 3.** Efficacy of MIF098 in the collagen-induced arthritis sensitive DBA1/J mouse strain. Mice were immunized with bovine collagen prior to treatment in either a prophylactic (before disease induction) or therapeutic (after disease onset) protocol.

There is currently no experience with administering MIF098 in food or water, but no special precautions are considered necessary to ensure compound stability. Pilot studies could quickly determine the level of serum MIF098 in UM-HET3 mice given food containing MIF098 at plausible levels, e.g. at 40 mg per kg body weight per day, with dose adjustment if needed prior to the initiation of the longevity study. MIF098 is stable and should maintain full activity in food held at zero degrees for many months.

We note that three of the agents found by the ITP to prolong mouse lifespan have immunosuppressive and/or anti-inflammatory activity, including rapamycin, aspirin, and NDGA. MIF, by opposing the anti-
inflammatory activity of glucocorticoids, is considered pro-inflammatory, and it seems plausible that some of the benefits of MIF inhibitors on illnesses reflect blunted inflammatory responses. The recent report that targeted down-regulation of NFκB pathways in mouse hypothalamus can extend mouse lifespan provides additional justification for testing drugs that blunt inflammatory tone in multiple organ systems (22).

We would propose to start the agent at 4 months of age, and continue lifelong administration. There are no validated biomarkers of quantitative measures of pharmacological efficacy of the MIF098 inhibitor in whole mice, however we will test for MIF’s intrinsic tautomerase activity in treated mice following the protocol that showed the impact of sulphorane ingestion (20).

Cost of a Life-long Intervention Study. Initial quantities of MIF098 (GMP grade) are available from Yale or may be readily synthesized by a third party CRO at reasonable cost ($64 per gm). A study that administers MIF098 at 40 mg/kg qd would require purchase of food at 240 mg of MIF098 per kg of food. (Each mouse would receive 1.2 mg of MIF098 daily). A typical ITP protocol uses 1300 kg of food for the three sites from age 4 months to death. At 240 mg per kg food, this protocol would require a total of 312 grams of MIF098. At a cost of $64 per gram, total cost for the agent would be $19,968 for the entire study, or approximately $7000 per year from 4 months of age onward.

In submitting this proposal, we agree to the following:

We 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 our proposal, or a modification of it (such as altered dosage or frequency of administration), is accepted for inclusion in a research protocol, we will be asked to help evaluate the data and to prepare the data for written and oral publications, on each of which we will be offered coauthorship. We 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.

We 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. We also will be free to use ITP-generated data in the context of applications for research support or for any other purpose.

The compound/diet proposed makes use of materials that are not yet freely available and whose production depends on proprietary or unpublished methods. If our application is approved for incorporation in the ITP, a mutually acceptable Materials Transfer Agreement that would permit us to provide the ITP with the compound(s) needed for the experimentation will be developed with the institutions involved in this program.

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References


ITP Year 3 proposal: resveratrol

Summary of rationale: there has been great popular interest in the possibility that resveratrol (3,5,4-trihydroxy-trans-stilbene) may retard aspects of the aging process in mice. Figure 1 shows the structure of this compound. Initial work was stimulated by genetic studies, in yeast and then in nematodes, suggesting that longevity could be increased by stimulation of molecules of the sirtuin family of protein deacetylases. Resveratrol earned special attention when it emerged as one of the most potent sirtuin stimulators in a screen of natural products. Resveratrol is present at relatively high concentrations in some varieties of red wine, and initial speculation suggested that it might be one of the factors that improved longevity and postponed cardiovascular illnesses in population groups that consumed large amounts of red wine, although this idea now seems unlikely. Recent publications have suggested that resveratrol can extend life span in a short-lived vertebrate, the fish *Nothobranchius furzeri* (Valenzano et al., 2006), and can extend at least median life span in mice given a diet sufficient high in fat that it induces obesity (Baur et al., 2006a). There are no published studies addressing the effects of resveratrol on maximal longevity, or which employ genetically heterogeneous mice, and thus a good rationale for including it in the ITP experiments.

Background information:

Baur and Sinclair have published an excellent summary of current knowledge of the physiological and pharmacological effects of resveratrol (Baur and Sinclair, 2006b), and I have relied heavily on this information in compiling parts of this background section.

(a) Effects on lifespan: invertebrates. Resveratrol extends life span of the yeast *Saccharomyces cerevisiae* by 70% (Howitz et al., 2003); extends life span in *C. elegans* by an average of 10% in a series of replicate experiments, and also extends life span in *Drosophila melanogaster* by approximately 15% to 20%, depending on gender and media composition (Wood et al., 2004).

(b) Effects on lifespan: vertebrates. The only complete survival experiment reported in vertebrates to date (Valenzano et al., 2006) employed the short-lived killifish *Nothobranchius furzeri*, whose life-cycle, adapted to short-lived ephemeral ponds, has selected for rapid maturation. In laboratory conditions, these fish reach sexual maturity at 4 weeks of age, and have a maximal recorded life span of 13 weeks. When these fish were given resveratrol at a dose of 600 microgram per gram of food, they showed a 56% increase in median longevity and a 59% increase in maximal longevity compared to fish receiving the same amount of unsupplemented food (p < 0.001). An intermediate dose (120 micrograms/gm food) extended maximal longevity by 27%, significant compared to controls. Longevity was increased in males and females, and fish of both sexes remained reproductively competent at ages at which all controls had died. Analysis of the mortality risk curve suggested that treated fish showed a decline in the rate of increase in mortality with age, but an increase in mortality risk in the first few weeks after administration of resveratrol. Age-dependent decreases in locomotor activity and cognitive performance were also delayed in the resveratrol-treated fish.

Baur, Sinclair, and their colleagues have recently published an initial report (Baur et al., 2006a) of an experiment in which C57BL/6Nia mice were treated with resveratrol while also exposed to...
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a diet containing 60% of its calories in the form of fat ("HC diet"), starting when the mice were one year old. Some of the mice on the HC diet also received resveratrol at 22.4 mg per kg body weight per day, and the study included control mice on a "standard diet" (SD group). Mice placed on the HC diet showed a dramatic increase in body weight, from 34 grams at the outset to a peak of about 52 grams at 75 weeks of age (i.e. 23 weeks after initiation of the HC diet). Resveratrol inhibited this weight gain only slightly, but did not alter body temperature, food consumption, or post-mortem body fat distribution. The HC mice reached median survival at 108 weeks of age. An analysis was done at 114 weeks, at which point 58% of the HC mice had died, significantly higher (p = 0.02 by Cox regression) than HC mice receiving resveratrol (42%) or mice on the standard diet (also 42%). This study did not include information useful for assessing maximal lifespan, nor information on resveratrol effects on mice receiving a diet that does not induce unusual levels of obesity.

(c) Anti-cancer effects in rodents. A report by Jang et al. (Jang et al., 1997) provided a good rationale to suggest that resveratrol might be a useful agent for tumor chemoprevention in mammals. Resveratrol was originally isolated from the small evergreen tree Cassia quinquangulata (now Senna quinquangulata) in the context of a search for inhibitors of cycloxygenase (COX). COX-1 is thought to promote tumor initiation and growth both by catalyzing synthesis of pro-inflammatory prostaglandins, and by activating carcinogens to mutagens. Resveratrol (given at 3 or 8 mg per kg body weight, given daily for 7 days) was found in inhibit both acute and chronic inflammation in a rat pedal edema model with an effectiveness similar to that shown by indomethacin, a potent anti-inflammatory agent. In vitro studies showed that resveratrol inhibited free radical formation in HL-60 promyelocytic cells exposed to TPA, and that resveratrol had anti-mutation activity in a DMBA-exposed Salmonella. The agent was also shown to induce, in cultured hepatoma cells, an enzyme, quinone reductase, thought to be important in the detoxification of carcinogens. Resveratrol was also able, in vitro, to induce differentiation of HL-60 cells to a terminal (non-proliferative) state. Using an organ culture model for DMBA-stimulated mammary carcinogenesis, resveratrol was shown to inhibit the development of preneoplastic nodules with an ED50 of 3.1 microM. Lastly, this group evaluated resveratrol in the classical two-stage skin cancer system, using DMBA as initiator and TPA as promoter in CD-1 mice. In this study resveratrol was administered to the skin site at doses of 1 to 25 microM, twice weekly for the 18 weeks of the study. The number of skin tumors seen was diminished by 68% to 98% in a dose dependent fashion, with no signs of systemic toxicity or weight loss.

In a separate study (Chen et al., 2004), resveratrol was given at a dose of 40 mg/kg body weight by daily intraperitoneal injection to mice that had been inoculated with subcutaneous neuroblastoma cells. By 40 days, all controls had died of tumor, but none of the resveratrol-treated mice had died; 70% of the treated mice survived to 70 days, at which point the experiment ended (p < 0.05; 10 mice per group). Survival after subcutaneous injection of glioma cells was also improved by resveratrol at this dose, though not at a lower dose (10 mg/kg/day ip), perhaps because of diminished neovascularization of the tumor site (Tseng et al., 2004).

Mechanism of action

The mechanism by which resveratrol leads to physiological effects in mammals is not known, and it is possible that multiple mechanisms are involved, and possible, too, that pathways implicated in invertebrates may be different from those important in rodents and (perhaps) primates. Resveratrol appears to have effects on sirtuins, the estrogen receptor (Gehm et al., 1997), cycloxygenase, toll receptors (Youn et al., 2005), and cytochrome P450 enzymes (Chang et al., 2001). It is also plausible that some of the effects seen in rodents may represent the actions of conjugation products with longer half-life or difference activity spectrum, rather than native resveratrol itself. A recent study (Lagouge et al., 2006) in which resveratrol was
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administered to C57BL/6 mice at 400 mg/kg/day for up to 15 weeks suggested that its primary effect may involve increase in the activity of PGC-1α, whose deacetylation can be brought about by SIRT1, and which has been implicated previously as a mediator of the anti-aging effects of low calorie diets. Activation of AMPK, an enzyme that mediates stress resistance and cell proliferation in response to fluctuations in nutrient levels, may also be critical to some of the physiological effects in rodents (Koo and Montminy, 2006; Baur et al., 2006a). Although in some invertebrate systems the effects of resveratrol seem to involve sirtuin-induced activation of the FoxO family proteins (like daf-16 in *C. elegans*), there is also evidence for daf-16 independent pathways for resveratrol-mediated lifespan increases in worms, and parallel evidence that this effect involves induction of proteins that protect cells from death after activation of the unfolded protein response (Viswanathan et al., 2005; Tatar, 2005).

It is very likely that much more will be learned, in the coming five years, about the cellular pathways that are triggered by resveratrol and its metabolites to affect rodent physiology, metabolism, and tumor biology. Untangling of this complex network of pathways is not a prerequisite for initiation of a study of resveratrol within the ITP program, because the rationale for the resveratrol proposal rests on reports of its effects on life span and risk factors of age-sensitive diseases.

**Dosage, bioavailability, and toxicity**

Resveratrol is rapidly metabolized after administration to mammals. Rabbits injected with a dose of 20 mg/kg body weight had serum levels of 42 µM 5 min after injection, which decreased to 0.9 µM by 60 minutes (Asensi et al., 2002). Oral administration of the same dose to mice produces a peak level of 2 to 3 µM, which decreases to less than 0.1 µM within 60 minutes. The calculated half-life in rabbits is 14.4 minutes whether measured in plasma or whole blood. When mice were allowed to drink water containing resveratrol at 23 mg/liter (the highest soluble concentration) for 10 days, plasma levels were 0.08 µM. After oral administration, levels of resveratrol in rat and mouse brain, lung, liver, and kidney were highest 10 minutes after administration, but never exceeded 1 nM, and fell to lower levels within 60 minutes. Isolated rat hepatocytes were found to metabolize resveratrol rapidly in vitro, suggesting the hepatic conversion to other metabolites may also occur in intact animals.

A review of the literature by Baur and Sinclair (Baur et al., 2006b) lists studies reporting in vivo effects of resveratrol at doses ranging from 100 ng to 1500 mg per kg body weight per day. Increase in median longevity of mice on a high fat diet (discussed above) was seen at 22.4 mg/kg/day. Positive effects on cardiovascular function have been noted in studies using doses of 1 to 16 mg/kg/day given in the drinking water. The neuroblastoma survival study noted above found effects in mice given 40 mg/kg/day by ip injection.

A study (Juan et al., 2002) in which adult rats were given resveratrol orally at 20 mg/kg body weight per day for 28 days found no abnormalities in body weight, food or water consumption, numbers of platelets, erythrocytes, or leukocytes, proportions of various leukocyte classes, or levels of glucose, triglycerides, HDL, LDL, serum ion concentrations, serum protein, bilirubin, or creatinine. Histopathology revealed no sign of abnormalities in any of the organs evaluated, except that both brain weight and testicular weight were higher in the resveratrol-treated rats (normalized for body weight). A second study (Crowell et al., 2004) evaluated rats given doses of resveratrol between 300 and 3000 mg/kg body weight per day, by oral gavage, for a 28 day period. Rats receiving the highest dose had lower food consumption and signs of renal toxicity (elevated BUN and creatinine), as well as lower hematocrit, increased white blood cell counts, and elevated serum ALT levels; they also showed histopathological signs of nephropathy. Rats given 1000 mg/kg/day showed only diminished weight gain (females) or elevated WBC count.
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(males), and rats given 300 mg/kg showed no abnormalities. A third report (Lagouge et al., 2006) found beneficial effects on insulin sensitivity and muscle function in mice given doses of 400 mg/kg/day for a period of up to 15 weeks. The Baur study (Baur et al., 2006a) of resveratrol effects on C57BL/6 mice given a high calorie diet used 22.4 mg/kg/day for a period of over one year, starting at one year of age, and noted no ill effects.

Therefore it seems likely that long-term administration of resveratrol at doses of 25 mg/kg/day is likely to be safe for mice, and there is no evidence of acute toxicity even at doses as high as 400 mg/kg/day over a 15 week test period.

Cost of a life-long intervention study

Dr. Sinclair, in a personal communication, has recommended the use of resveratrol purchased from Michel Baudet at Lalilab, who can apparently provided synthetic purified resveratrol, derived from Orchid Pharmaceuticals in India, at a cost of approximately $3,000/kg. Dr. de Cabo, in a personal communication, notes that future studies of resveratrol at NIH will use material purchased from a supplier called Royal Mount Pharma; no price estimate was provided.

A study that administers resveratrol at 25 mg/kg/day would require purchase of food at 150 mg resveratrol per kg of food. (Each mouse would receive 750 micrograms/day). Our protocol uses approximately 50 kg of food, per month, split among 3 test sites, for each test compound. This requires purchase of 600 kg food per year for each test substance. At 150 mg resveratrol per kg food, 600 kg food would require 90 grams/year of resveratrol. Thus cost for the agent at this dose is likely to be less than $1000/year.

A study that administers resveratrol at 100 mg/kg/day would require 360 gm resveratrol per year (for all three sites combined), at a projected cost of about $1000/year.

Plans for assessment of physiological effects of resveratrol in treated monitor mice

There are several possible ways to measure the biological efficacy of resveratrol in treated monitor mice. The most direct way would be to measure PGC-1α activity as inferred from the percent of total PGC-1α that is acetylated. Recent studies suggest that the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) plays a key role in the sirtuin pathway. PGC-1α is involved in a wide range of biological responses, e.g. mitochondrial biogenesis, energy metabolism, thermal regulation, glucose metabolism, fat metabolism and muscle fiber type switching (Puigserver and Spiegelman, 2003). SIRT1 has been shown to physically interact with and deacetylate PGC-1α at multiple lysine sites, leading to increased PGC-1α activity (Nemoto et al., 2005; Rodgers et al., 2005). Resveratrol has been shown to significantly increase SIRT1 activity through an allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD and the acetylated substrates (Howitz et al., 2003). Baur et al. (Baur et al., 2006a) also observed that feeding resveratrol to mice induced PGC-1α activity. Thus, PGC-1α activity or decreased acetylation would provide a good measure of resveratrol efficacy. Other possible measures could be to measure the consequences of increased PGC-1α activity. Lagouge et al. (Lagouge et al., 2006) showed that mice fed resveratrol had increased aerobic capacity, as evidenced by their increased running time and consumption of oxygen in muscle fibers, and an induction of genes for oxidative phosphorylation and mitochondrial biogenesis. These changes were largely explained by a decrease in PGC-1α acetylation and an increase in PGC-1α activity, which is consistent with resveratrol activating SIRT1. Thus, additional measures indicative of the efficacy of resveratrol could include assessing increases mitochondrial number, increased insulin sensitivity, increases in IGF-I levels, and improvement or maintenance of motor function into old age. The latter is already included in the current SOP.
Proposal

Subject to discussion and approval by the ITP steering group, I propose that resveratrol be administered to mice in the Year 3 ITP cohort at each of two doses: 25 mg/kg/day and 100 mg/kg/day. Dosing would be initiated at whatever age has been reached by the oldest group of Cohort 3 mice at the time resveratrol-containing food becomes available to us, but presumably not later than 12 months of age.

I propose that initiation of this study NOT be delayed pending outcome of studies of blood levels or physiological effects. The rationale is that doses in the range used have been suggested to have effects, in intact rodents, on endpoints of interest including tumor growth, insulin sensitivity, and survival curves (at least for obese mice). Blood levels of resveratrol are reportedly very low and difficult to measure, and developing clear and validated tests physiological effects may also take many months of effort. I propose that tests of physiological effect be developed promptly so that monitor mice can be evaluated once they have been on the resveratrol diets for several months prior to assessment.

REFERENCES


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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 (11). More recently, late phase IPC has been shown to be linked to the activation of a cAMP-dependent pathway (12). Research is under way to identify targets for IPC and to develop drugs that will allow IPC to be activated in vivo. The Msr system is thought to play a role in IPC (13), but it is likely that the role of IPC in aging will be more complex than that of the Msr system.
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 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).

**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.](image)

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

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

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.
References:


