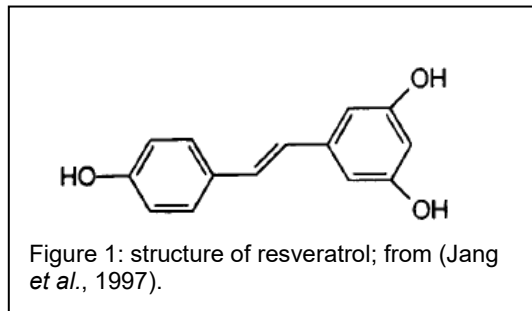


ITP Year 3 proposal: resveratrol

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 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 cyclooxygenase (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 to 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), cyclooxygenase, 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 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-1 α , 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 (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 provide 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-1 α activity as inferred from the percent of total PGC-1 α that is acetylated. Recent studies suggest that the peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) plays a key role in the sirtuin pathway. PGC-1 α 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-1 α at multiple lysine sites, leading to increased PGC-1 α 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-1 α activity. Thus, PGC-1 α activity or decreased acetylation would provide a good measure of resveratrol efficacy. Other possible measures could be to measure the consequences of increased PGC-1 α 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-1 α acetylation and an increase in PGC-1 α 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.

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