The Pharmacology of Aging in Drosophila

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Abstract: Recent research indicates that aging is affected by many genes and thus many biochemical pathways. This has led to a failure to find pharmaceuticals that significantly ameliorate the human aging process. Progress in evolutionary and genetic research, however, suggests the possibility of combining experimental evolution, genomic analysis, and mass screening of pharmaceuticals and botanicals to produce effective therapeutics for human aging. The starting point for this strategy is model systems that have outbred populations with substantially increased lifespan. These are easily produced by tuning the force of natural selection in the laboratory. Such biological material is then a good candidate for genomic analysis, leading to the identification of numerous biochemical pathways involved in increased lifespan, in the model system. These biochemical pathways would then be available for pharmaceutical development, first in fruit flies, then in rodents, and eventually in a clinical human population. We include a discussion of the pharmacological methods appropriate to this strategy of drug discovery.

Key Words: Anti-Aging, Lifespan, Drosophila, Aging Pathways, Drug testing, Pharmaceuticals.

INTRODUCTION

Aging is a biological puzzle of long standing, particularly because it manifests itself over a wide range of biological systems, tissues, and functions. For some time, the outstanding task has been to find experimental strategies that make sense of the complexity of aging. There is now reason to hope that such experimental strategies have been found. Pletcher *et al.* profiled the transcripts of *Drosophila* on a genome-wide scale over a range of adult ages, finding that at least 6 % of the 13188 genes on the Affymetrix fly chip show changes in transcription with age [1]. We have argued elsewhere that these results suggest that about 400 loci are involved in aging [2]. Using primitive proteomic technology on a few hundred proteins resolved on a two-dimensional gel, Fleming *et al.* earlier suggested that about 300 loci might be involved in the control of ageing [3]. Though the methodologies of these studies differ substantially, they both yield fairly high estimates of the number of aging loci in common. These findings suggest that aging, even in *Drosophila*, is genetically and biochemically complex. For now, the best conclusion is probably that "*many*" pathways are involved in aging in fruit flies. Vertebrates are unlikely to have fewer genes involved in aging, in view of their larger genomes. This may explain the present absence of effective therapies that postpone or slow the aging process in humans.

DROSOPHILA AS A MODEL SYSTEM

Before 1980, little progress was made in the study of the genetics of aging in *Drosophila*. The problem was that most of the tools of the genetics trade did not give useful results. The large-effect mutants studied by Pearl and his colleagues almost always reduced life span. The *Drosophila* mutants that enhanced lifespan most eliminated the reproductive organs of females [4], making such mutants of doubtful relevance. A new crop of mutants that extend *Drosophila* lifespans have been produced. Mutant *Methuselah* (*mth*) flies have increased resistance to starvation, high temperature, and paraquat and live 35% longer [5]; *mth* appears to be a G Protein Coupled Receptor (GPRC), which is involved in response to stress. Rogina *et al.* [6] reported that *Indy* mutant flies display a state similar to caloric restriction by reducing dicarboxylase acid cotransporter expression. Dicarboxylic acids are the key intermediates in the Krebs cycle. This mutation could mimic caloric restriction. Although, the *Indy* mutants had lifespan extension, their activity and fertility rates were similar to controls [6]. In another study with similar methodology using *Indy* flies, the only factor that resulted in lower fertility in this long-lived *Drosophila* mutant was reduced food quality. Therefore, in a nonstressful environment, *Indy* flies can perform as well as the wild-type flies and can live twice as long as the wild-type [7], but not necessarily otherwise. The *InR* gene is an insulin-like receptor in fruit flies that is homologous to insulin receptors in vertebrate and *daf-2* in worms. The impact of *InR* mutations on life extension depends on allelic combinations. Only a few specific *InR* mutant flies have extended lifespan without compromised fertility. For instance, heteroallelic dwarf mutant *InR* females had as much as 85% increased longevity. Most of the long lived *InR* mutants have decreased fertility [8].

While reproduction exhibits aging also, it substantially complicates the analytical problem to have effects on survival confounded with effects on reproduction. But against this criticism, there is a great deal of evidence that indicates that survival trades-off against reproduction in the genetics of aging, a trade-off that is part of a phenomenon called antagonistic pleiotropy [9].

The evolutionary genetic theory of aging and its subsidiary implications, together with the experiments that support them, reveal that aging is fundamentally harder than many other problems that biologists study. It will not be solved in molecular detail or in medical application as easily. It requires the development of new strategies of experimental research and medical application. Here we propose such a strategy, using *Drosophila* as a model system.

The obvious model systems in which to begin developing therapeutics to manage human aging are the two premier animal genetic systems, *Drosophila melanogaster* and *Caenorhabditis elegans.* Mutants are available that show increased longevity for both species. *Drosophila* have also been bred for postponed aging [10-12], potentially affording hundreds of genetic differences to work with. Recently there has been a controversy concerning the status of the *C. elegans* mutants with increased lifespan. In some laboratories, these mutants show increased longevity according to the degree of reduction in metabolism [13]. If so, then these mutants modulate lifespan by tuning metabolic activity, a discovery well-known in research with poikilotherms since 1917, and of limited interest. By contrast, the *Drosophila* bred for postponed aging are known to have no reduction in metabolic rate, and a substantial increase in their lifelong metabolism [14]. For these reasons, our initial model system recommendation is *Drosophila*. This does not

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1389-4501/06 $50.00+.00

mean that we reject the later use of other model systems, as they are refined for the study of aging.

In addition to the abundant knowledge that has accumulated concerning the evolution and genetics of Drosophila aging, a great deal is known concerning environmental factors that affect fruit fly aging [12 ,15]. Indeed, work on this topic dates back to early in the 20th Century [16,17]. The effect of temperature on fruit fly longevity has been actively studied [18]. Work on the influence of diet on fruit fly lifespan has produced useful results only in the last 20 years, but it is now progressing well [19]. From the standpoint of drug testing, the chief value of this work lies in the background information that it provides concerning the avoidance of experimental artifacts, such as the problem of reduced feeding arising from noxious candidates drugs being detected by flies in their medium.

DRUG TESTING IN FRUIT FLIES

Fruit flies and humans share a vast number of key metabolic pathways such as superoxide metabolism, DNA repair, insulin-like signaling, and so on. Many of these pathways are already considered candidates for aging modulation. Given the successes of developmental genetics in applying the results of homeotic mutation research in *Drosophila* to the cell biology and genetics of development in vertebrates, the ease of transducing information from fruit flies to humans is not controversial.

Unlike mammals, even mice, it is possible to assay aging and mortality in large cohorts of fruit flies, from hundreds to tens of thousands. Complete longevity assays in fruit flies take a few months, but we now know that longevity is a compound of two distinct phases of adult life: aging and late life [20,21]. Therefore, assays of compounds that might manage aging should be confined to the aging phase. In standard short-lived *Drosophila* cultures, the aging phase lasts only a few weeks [22, 23]. Drug screens on large cohorts of fruit flies should therefore take only about a month, with mortality rates during treatment being the primary measured outcome. We now have powerful statistical protocols for the estimation of mortality rates in fruit fly data [24,25]. With large enough cohorts, the impact of a candidate pharmaceutical on aging can be determined very quickly.

It is possible to find drugs that seem to slow aging in fruit flies, the very system in which large gene-number estimates have been obtained [26]. Unfortunately, there is a long history of artifactual responses to toxic or noxious substances by fruit flies. In particular, females that are exposed to toxic substances typically reduce their dietary intake and their reproductive output. Such effects are strongly associated with an increase in longevity [27]. Flies may perceive the pharmaceuticals mixed in their food as toxic substances and therefore reduce their dietary intake. One way to address this problem is to monitor fecundity during drug intake. Drugs that achieve longevity benefits by reducing fecundity as a result of diminished food intake are probably not useful candidates for eventual treatment of aging in humans.

Another difficulty is that information about administration of drugs to other mammals, including human, will not be directly applicable to fruit flies. Providing drugs to large cohorts of fruit flies depends on their consumption of the drug in their food medium. While the consumption of food by larvae is now fairly well understood, adult feeding is not well characterized. In larvae the rates of feeding are under genetic control [28]. Larval feeding rates affect competitive ability, growth rates, and efficiency of food utilization, all variables that can be easily quantified [29-31]. Yet the majority of pharmacological experiments on aging in fruit flies use adult feeding as the preferred mode of drug delivery. The drug is often dissolved in ethyl acetate or other solvents that do not affect lifespan. The solution is then added and mixed evenly into the cornmeal-sugar-yeast agar diet and the mixture is placed in food plates. However, drug intake may not lead to drug delivery to target tissues beyond the digestive tract. Like many other insects, *Drosophila* has cytochrome P450s in the gut which may metabolize pharmaceuticals. The involvement of various isoforms of P450s in the development of insecticide resistance is well established in the literature [32]. But the number of published studies evaluating the impact of pharmaceuticals on induction and inhibition of CYP families in *Drosophila* is scarce. One study reported that barbiturates may induce CYP4D10 in *Drosophila* [33]. Of note is the fact that, barbiturates are considered a strong inducer of CYP3A3 and CYP3A4 isoforms in humans [34]. To insure adequate drug intake, a variety of methods might be employed: adding food color to the food and drug mixture, administering radiolabled drugs, and measuring drug concentration in fruit flies. Currently there are no standardized methodologies to test drug concentrations in target tissues in adult fruit flies. Although mass spectrometry and enzymatic assays are currently used to test for chemicals in fruit flies, these techniques are not yet validated as tests of drug concentrations. Alternatively, drugs could be injected intraabdominally to fruit flies *via* cell injectors and micromanipulator [35], though this drug delivery system is not ideal for testing numerous drugs using large cohorts. In spite of these uncertainties, the use of food intake for drug delivery in fruit flies is still considered the preferred mode of drug delivery. Possible artifacts could be precluded by use of food color and visual inspection of flies, as well as developing experimental methodologies where different concentration of drugs are tested to evaluate dose response outcomes.

While the organismal physiology of fly aging was little known before 1990, since that date a great deal of progress has been made in unraveling the physiological machinery of aging in fruit flies [27]. This aspect of the problem has gone from a weakness to a strength; the causal controls on human aging and fruit fly aging may now be comparably understood at the level of organismal physiology.

Despite the difficulties that we have adduced here, we nonetheless conclude that testing drugs and botanicals with potential aging treatment properties in fruit flies is one of the best approaches to screening candidate pharmaceuticals and botanicals prior to the testing of vertebrate model systems, like rodents.

WHICH PATHWAYS SHOULD BE TESTED FOR CANDIDATE DRUGS IN FRUIT FLIES?

A number of medications have been proposed as anti-aging pharmaceuticals over the last three decades, without controlled studies. Human growth hormone, Ginkgo biloba, lipoic acid, and resveratrol are among these medications. The majority of these medications are supposed to improve human symptoms of aging such as memory loss, neuronal degeneration, inflammation due to oxidative stress, and the like. None of these medications have supportive data showing that they statistically increase human longevity. All of these medications could be studied, and some have been studied, using large fruit fly cohorts [36-38].

Earlier, we suggested an alternative strategy for the development of aging therapeutics using selection and genomics [23]: the use of genomics to identify candidate biochemical pathways for drug testing from fruit fly stocks that have been bred for delayed and slowed aging. Using modern tools such as gene-expression microarrays would allow the identification of pathways that are up- or down-regulated in stocks with delayed or slowed aging. Pathways conserved in humans and flies that also show changes in gene expression in flies with evolutionarily postponed aging are excellent candidates for drug targeting. Fruit flies, we suggested, could then in turn be used to efficiently screen thousands of candidate drugs specific to these pathways. This approach has the advantage that it does not rely on particular hypotheses concerning the biochemistry of aging. Any genetically-defined biochemical pathway that is associated with postponed aging in fruit flies thereby becomes a target for drug testing.

Genetic pathways associate with postponed aging serve two different purposes. They would provide information concerning the pathophysiology of diseases that contribute to aging and thus could ultimately be used to develop pharmaceuticals to be tested in clinical studies. In addition, these pathways could be used as diagnostics for aging. These diagnostics could potentially shorten the duration of clinical trials of candidate aging pharmaceuticals.

For instance, mutations in *Indy* flies that result in the reduction of dicarboxylic acid transport slowed aging in fruit flies. Dicarboxylate cotransporters control the amount of Krebs cycle intermediates, such as citrate. Citrate or other intermediates of the Krebs cycle may serve as potential biomarkers in clinical trials on pharmaceuticals that inhibit the activation of dicarboxylic acid transport enzymes [6].

Thanks to the sheer quantitative power of the fruit fly as a drug screening system, candidate drugs of any kind can be screened quickly and efficiently using it. In this respect, *D. melanogaster* is similar to *C. elegans* as a testing system for drug screening, which makes the record of pharmaceutical testing in the nematode an important point of comparison. To date, a handful of drugs with potential life extension properties have been tested in *C. elegans*. We will consider these tests in more detail now.

Melov *et al.* reported a 44% increase in the life span of wild-type *C. elegans* with two superoxide dismutase/catalase mimetics, EUK-8 and EUK-134, which are now referred to an Eukarian antioxidant compounds [39]. Later they tested these synthetic mimetics in mice and reported that these compounds extend the lifespan of SOD2 (the mitochondrial form of SOD) nullizygous mice threefold. These nullizygous mice normally die during the first week of their life due to a variety of organ failures and mitochondrial enzymatic defects [40]. The life-extension mechanism of action of these compounds was proposed to be an amelioration of the oxidative damage caused by reactive oxygen species. It is notable that these synthetic mimetics did not result in detectable pleiotropic effects on reproduction and metabolic rate [39] (Table **1**).

Evason *et al.* tested anticonvulsants in *C. elegans* and reported life-span-extension with two FDA approved anticonvulsants, ethosuximide and trimethadione. Three different doses of each drug were tested. The human therapeutic concentration of ethosuximide gave the greatest increase in lifespan. These compounds affect neuronal activity. This is especially significant because the well-established insulin-like signaling pathway that controls life span in *C. elegans* is dependent on the function of sensory neurons [41] (Table **1**).

Anticonvulsants and SOD/catalse mimetics can be readily tested in fruit flies too. The life-extension properties of popular antioxidants such as vitamin E, lipoic acid, and resveratrol have been evaluated in fruit flies. Driver *et al.* reported a concentration-dependent increase in longevity with Vitamin E, though doses above 20 μ/ml resulted in toxicity and doses much below this concentration had no impact on longevity, suggesting a very narrow window for efficacy. The investigators attributed these variable results to a narrow therapeutic window of Vitamin E as a scavenger of reactive oxygen species [42] (Table **1**), a pattern that arises in clinical trials as well [43]. The positive impact of antioxidants on the life span of fruit flies might not be extrapolable to human longevity yet. Until recently, the medical literature was saturated with studies praising the health benefits of Vitamin E. But recently two independent meta analyses on the impact of Vitamin E on cardiovascular disease and all cause mortality reported that vitamin E supplementation may increase all-cause mortality and the incidence of cardiovascular diseases [44,45]. A similar controversy exists concerning the efficacy and mechanism of anti-aging activity of resveratrol.

PHARMACEUTICAL TRIALS PERTAINING TO DROSOPHILA AGING

There are some pioneering studies of 'anti-agathic' pharmaceuticals in *Drosophila*. In a study of lifespan-extending interventions in *Drosophila*, Bauer *et al.* examined the effect of the popular antioxidant, lipoic acid. Since lipoic acid increased the life-span of female *Drosophila* only, the investigators reported that future studies are needed to clarify their results and to explain the lack of life-extension in the male [38]. Resveratrol increased life span in two independent studies [36,38]. In Bauer's study, 200 μM of resveratrol resulted in an average 16% and 10% increase in life span in female and male flies, respectively. Wood *et al.* reported 20% and 16% increase in life span of female and male *Drosophila* using 100 μM of resveratrol. Although a gut-filling assay and food color uptake were used to evaluate feeding rate in Wood's study, none of the investigators reported the drug concentrations achieved in the tissues of the experimental fruit flies. Resveratrol is thought to be a sirtuin2-activating antioxidant compound [36,38] (Table **1**). Sirtuins are proteins that are involved in several cellular processes, such as DNA repair, with direct and indirect effects on aging. The authors of these studies suggested that these compounds act as calorie restriction mimetics due to sirtuin activation. But recently Kaeberlein *et al.* reported that resveratrol had no effect on SIRT2 activity in three yeast strain background [46]. As a result, further *in vivo* as-

Table 1. Examples of Anti-Aging Animal Studies in *C. elegans* and *Drosophila*

Compound Class	Compound	Animal Model	Possible Pathway	Reference
Anticonvulsants	Ethosuximide Trimethadione	*C. elegans*	Daf-2/Daf-16 (Insulin)	Evason K 2005 [41]
Antioxidants (sirtuin activating)	Resveratrol	*C. elegans* *Drosophila*	Sir2 Activation Caloric Restriction	Bauer JH 2004 [38] Wood JG 2004 [36]
Antioxidants (superoxide dismutase/catalase mimetics)	EUK-8 and EUK-134	*C. elegans*	Reactive Oxygen Species	Melov S 2000 [39]
Antioxidants	Lipoic Acid	*Drosophila*	Reactive Oxygen Species	Bauer JH 2004 [38]
Vitamins	Vitamin E	*Drosophila*	Reactive Oxygen Species	Driver C and Georgeou A 2003 [42]
Histone Deacetylase Inhibitors	Phenylbutyrate (PBA)	*Drosophila*	Histone acetylation and transcriptional regulation	Kang JL 2002 [37]

says are needed to evaluate the mechanism of the potential longevity effects of resveratrol.

Kang *et al*. evaluated the effect of 4-phenylbutyrate (PBA) on *Drosophila* lifespan. PBA is an FDA-approved medication that is indicated for the treatment of sickle cell anemia and cystic fibrosis.

Various concentrations of PBA were tested and 10 mM of PBA resulted in lifespan extension in a white mutant strain. At higher concentrations, PBA was reported to be toxic. The lifespan extension property of PBA was also dependent on the strain of *Drosophila*. The wild type strain Canton-C required only 5 mM of PBA for lifespan extention. Thus the genetic background affected the required PBA concentration. PBA inhibits the activity of histone deacetylase and thereby regulates gene transcription *via* hyperacetylation of histones. PBA induces a number of genes, such as superoxide dismutase (SOD), and represses a number of genes, such as fatty acid synthetase, that are involved in longevity [37]. Phenylbutyrate is a ligand for peroxisome proliferator activator receptor γ (PPARγ). Peroxisome proliferator activator receptor ligands play an important role in lipid and glucose metabolism. For instance, SIRT1, the mammalian SIRT2 ortholog, represses PPAR γ transactivation and results in a decrease in lipid accumulation in adipocytes [47]. Life span extension due to calorie restriction appears to be due to a reduction in white adipose tissue [48]. Therefore, PBA could have an effect on longevity through a number of anti-aging mechanisms.

Regardless of their anti-aging mechanism in *C. elegans* and *Drosophila*, the promising results of these drug studies on aging direct us to high-throughput screening of additional pharmaceuticals and botanicals that might have an impact on aging. Pharmaceutical longevity studies may take a few days in *C. elegans*, a few weeks in *Drosophila*, a few years in mice, but many years in human. Genomic research on populations with postponed aging in model systems could also play a crucial role in identifying diagnostics that could expedite clinical trials in human.

For instance, one of the most corroborated mechanisms of aging across species, from yeast to humans, is the oxidative stress hypothesis, the idea that reactive oxygen species (ROS) contribute to and accelerate aging [49] and that aging results in increased levels of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals. Due to recent advances in *Drosophila* genomics, the oxidative stress pathway and SOD forms are well characterized in this organism. Therefore, *Drosophila* could be particularly a particularly attractive model to study the role of this pathway in aging. [50]. It has been shown that, decreased levels of catalase and superoxide dismutase (SOD) may shorten *Drosophila* lifespan. Parkes *et al.* showed that overexpression of human SOD1 in adult motorneurons resulted in a 40% increase in the lifespan of *Drosophila* [51].

CONCLUSION

The study of aging in the two major invertebrate model systems, *C. elegans* and *D. melanogaster*, is turning a corner. For the last twenty years, research on these model systems has focused on the evolutionary [9, 27] and genetic [52,53] foundations of aging. These foundations are now relatively well-understood, especially relative to the level of understanding of the mechanisms of organismal aging that had been achieved prior to 1980. Now research is turning to pharmaceutical intervention , an area of research that was of marginal importance in these model systems before 2000 [26]. Yet thanks to the extensive work on the evolutionary and genetic foundations of aging in *Drosophila*, we can do much better than making guesses when choosing candidate pharmaceuticals and botanical for screening "anti-aging drugs." We can instead base our choices on detailed physiological, genetic, and indeed genomic research. This will give a considerable advantage to the initial screening of aging pharmaceuticals with fruit flies.

Ultimately, as our understanding of the pharmacology of aging in *Drosophila* grows, we will be able to proceed to the testing of the most promising drug candidates in rodents, as a preliminary to clinical trials. Genomic research will also allow us to select candidate diagnostics to accelerate rodent screening for drug efficacy. Once rodent aging pharmaceuticals are identified, the next step is to design clinical trials in humans. The use of diagnostics and mortality rates, in lieu of total longevity, could potentially shorten the duration of these studies. The question is not whether drug testing in fruit fly research will yield fly anti-aging compounds. The question is when such compounds will be available for human use.

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