

# A triple drug combination targeting components of the nutrient-sensing network maximizes longevity

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Increasing life expectancy is causing the prevalence of age-related diseases to rise, and there is an urgent need for new strategies to improve health at older ages. Reduced activity of insulin/insulin-like growth factor signaling (IIS) and mechanistic target of rapamycin (mTOR) nutrient-sensing signaling network can extend lifespan and improve health during aging in diverse organisms. However, the extensive feedback in this network and adverse side-effects of inhibition imply that simultaneous targeting of specific effectors in the network may most effectively combat the effects of aging. We show that the mitogen-activated protein kinase kinase (MEK) inhibitor trametinib, the mTOR complex 1 (mTORC1) inhibitor rapamycin, and the glycogen synthase kinase-3 (GSK-3) inhibitor lithium act additively to increase longevity in *Drosophila*. Remarkably, the triple drug combination increased lifespan by 48%. Furthermore, the combination of lithium with rapamycin cancelled the latter's effects on lipid metabolism. In conclusion, a polypharmacology approach of combining established, pro-longevity drug inhibitors of specific nodes may be the most effective way to target the nutrient-sensing network to improve late-life health.

**Keywords:** aging, lithium, poly-pharmacology, rapamycin, trametinib, triglycerides.

Aging is a complex process of progressive cell, tissue and systemic dysfunction that is involved in the etiology of age-related diseases (1). Genetic, dietary and pharmacological interventions can ameliorate the effects of aging in laboratory animals and may lead to therapies against age-related diseases in humans (2–4).

In organisms ranging from invertebrates to mammals, reducing the activity of the nutrient-sensing mTOR and insulin/IGF signaling (IIS) network can promote longevity and health during aging (2, 3). Lowering network activity can also protect against the pathology associated with genetic models of age-related diseases (1, 2). The network contains many drug targets, including mTOR, MEK and GSK-3 (**Fig. 1A**). Down-regulation of mTOR activity by rapamycin, GSK-3 by lithium, or MEK by trametinib can each individually extend lifespan in laboratory organisms (5–11), and brief inhibition of mTOR has recently been shown to increase the response of elderly people to immunization against influenza (12). **In addition, both mTOR and MEK inhibitors have shown to reduce senescent phenotypes in human cells (13), while lithium levels in drinking water correlate with reduced all-cause mortality in a Japanese population (10).** An advantage of pharmacological interventions is that the timing and dose of drug administration are relatively simple to optimize, and drugs can be easily combined (4, 14–16). Combination drug treatments also have the potential to counter resistance from feedback and to reduce each other's side-effects (17). Rapamycin, trametinib and lithium each target different kinases and transcription factors to extend lifespan (5, 8, 11), and therefore their effector mechanisms are therefore at least partially different from each other. Simultaneous inhibition of multiple targets within the nutrient-sensing network may hence be needed to optimize effector outputs and health benefits. Here, we measure the effects of combination treatments of rapamycin, lithium and trametinib on lifespan and other traits, using *Drosophila* as a model organism.

## RESULTS AND DISCUSSION

Rapamycin treatment, from *C. elegans* to humans, is associated with altered metabolism, including hypertriglyceridemia and obesity (5, 18). Alone, a lifespan-extending dose of lithium (11) did not alter triglyceride levels, but simultaneous treatment with both lithium and rapamycin reversed the dyslipidemia caused by rapamycin (**Fig. 1B**). To confirm that this change in lipid levels was physiologically relevant, we pre-treated (14d) flies with lithium, rapamycin or a combination, and assessed their survival under starvation. Lithium did not alter survival under starvation conditions, while rapamycin increased it (**Fig. 1C**). Consistent with their effects on lipid levels, combining lithium and rapamycin treatment resulted in control levels of starvation resistance (**Fig. 1C**). Lithium can therefore reverse metabolic storage alterations associated with mTOR inhibition.

Lithium inhibits GSK-3 activity to extend lifespan (11), implying that activation of GSK3 is likely, if anything, to shorten lifespan. Inhibition of IIS in the canonical PI3K pathway can extend lifespan and healthspan, but reduces inhibitory phosphorylation of GSK3 by Akt (**Fig. 1A**), and hence activates GSK3 (4), a potentially deleterious side-effect of lowered IIS (19). We therefore tested whether lithium could have additive effects in combination with genetic inhibition of IIS upstream of Akt. Lithium was able to further extend the lifespan of flies lacking the insulin-like peptides 2, 3 and 5 (*dilp2-3,5*) (**Fig. 1D**) (20). In contrast, rapamycin or trametinib, neither of which inhibit GSK3, were not able to extend the lifespan of *dilp2-3,5* flies (**Fig. 1E-F**). Lithium thus reverses an adverse side-effect of inhibition of the canonical IIS pathway.

Because rapamycin, lithium and trametinib extend lifespan by at least partially independent mechanisms, we investigated the effects on lifespan of their double and triple combinations. Double combinations of lithium and rapamycin, lithium and trametinib, or rapamycin and trametinib produced a reproducibly greater lifespan extension than controls, on average 30%, compared to each compound alone, which extended lifespan by an average of 11% (**Fig. 2A-B**). Importantly, the triple combination of rapamycin, trametinib and lithium promoted longevity beyond that of the double combinations, extending median lifespan by 48% (**Fig. 2A-B**). Thus, each compound independently displayed an additive effect on lifespan. **The additive effect of rapamycin, trametinib and lithium on lifespan is unlikely to have been due to changes in feeding behavior, because feeding frequency, food intake, and drug uptake were unaltered by the treatment regimens (Fig. 2C-D).** Fecundity is often reduced in interventions that promote lifespan extension (21), and this could provide a potential explanation for the greater longevity with drug combinations. However, at the concentrations used, only trametinib and combinations containing trametinib significantly reduced fecundity (**Fig. 2E**). Importantly, the triple drug combination did not reduce egg-laying below that achieved with double trametinib-containing combinations, or trametinib treatment alone (**Fig. 2E**). Thus, a trade-off with fecundity is unlikely to explain the greater longevity observed with the triple drug combination.

Given the complex nature of the aging process, it is unlikely that the most effective preventative anti-aging therapy could be achieved by a single compound with a single target. We have shown that simultaneous inhibition by three components of different nodes in the nutrient-sensing network using a combination of drugs already approved for human use is a viable strategy to maximize animal longevity and to reduce a side-effect. Rapamycin treatment results in insulin-resistance and dyslipidemia in patients and mice (4, 18, 22), and this disturbance manifests as hypertriglyceridemia in *Drosophila* (5). Lithium reversed this and the starvation resistance associated with rapamycin treatment. Taken together, our results highlight a potential therapeutic avenue to promote longevity,

co-administrating compounds that act on different nodes of the nutrient-sensing network, to maximize their beneficial effects while minimizing negative side-effects.

## METHODS

**Fly stocks, husbandry, and lifespan analysis:** For all experiments, a wild type white *Dahomey* ( $w^{Dah}$ ) stock, or when noted *dilp2-3,5* mutant flies ( $w^{Dah}$  backcrossed) were used, and raised as previously described (20). LiCl (Sigma) in ddH<sub>2</sub>O, Trametinib (LC laboratories) in DMSO, and Rapamycin (LC laboratories) in 100% ethanol, were added to SYA medium to a final concentration of 1mM, 15.6μM, and 50μM, respectively (5, 8, 11). Equivalent volumes and concentrations of vehicle were added to SYA medium for control treatments. Drug treatments were started 2 days post-eclosion. Lifespan analysis: Female flies ( $n=130-200$ , 15-20/vial) were sorted on to SYA medium that was replaced every 2-3 days throughout life. Lifespan data are available under the doi #####. Starvation assay was performed as previously described (11).

**Food intake, fecundity, and triglyceride measurements:** Feeding behavior (proboscis extension at 1 and 15 days of treatment) and food intake (quantified by dye-calibrated feeding) (4-5 flies/replicate,  $n=8-10$ ) were measured as previously described (23). Fecundity was quantified as # of eggs laid within 24h (15d) and triglyceride measurements (5 flies/replicate,  $n=8$ ) were performed as previously described (5, 11).

**Mass spectrometry:** Flies ( $n=5$ , 15 flies) were treated with drugs (15d), their digestive system allowed to void (1h), snap frozen, drugs extracted as previously described (5), and resuspended in 100μl acetonitrile/isopropanol 70:30 for measurement with an Acquity UPLC™ I-class System/Xevo TQ-S (Waters) with MassLynx and absolute quantification.

## Figure 1. Lithium blocks negative side-effects of mTORC1 and IIS inhibition.

A simplified diagram of the *Drosophila* nutrient-sensing network showing the target kinases of rapamycin, trametinib and lithium (A). Lithium reversed the (B) hypertriglyceridemia ( $n=6$  replicas of 5 flies per condition, 1-way ANOVA) and starvation resistance (C) induced by rapamycin (50 $\mu$ M) ( $n=75$ ). Lithium treatment significantly extended lifespan of both  $w^{Dah}$  and  $dilp2-3,5$  mutant flies (D). Neither rapamycin (E;  $p=0.58$ ) nor trametinib (F;  $p=0.14$ ) further extended lifespan of  $dilp2-3,5$  mutant flies (log-rank test ( $n=150$ )). Cox Proportional Hazard analysis showed a significant genotype by treatment interaction for rapamycin ( $p=0.002$ ) and trametinib ( $p=0.0018$ ). Error bars represent SEM. \*\*\*  $p<0.001$  (1-way ANOVA or log-rank test).

## Figure 2. A triple drug combination maximizes longevity.

(A) Representative survival curve and associated pairwise log-rank tests. (B) Replicated median/max. lifespans plotted for all single ( $n=4$ ), double ( $n=3$ ), and triple ( $n=2$ ) combinations of rapamycin, trametinib and lithium treatments. Each lifespan contained 130-200 flies per treatment. Numbers in parentheses show (total number of flies/number of censors). (C) Proboscis extension feeding behavior assay (1 and 15 days of treatment) and quantification of ingested non-absorbable (bottom) blue dye ( $n=8$  replicas of 4-5 flies 15d old, 1-way ANOVA with Dunnett's test). Mass spectrometry of systemic trametinib (top) or rapamycin (bottom) levels when other drugs were co-administered (D) ( $n=5$ , 1-way ANOVA). Fecundity of treated (15d) flies within a 24h period ( $n=8$  replicas of 4-5 flies) (E). Error bars show Tukey whiskers and outlying data points are shown as dots. \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$  (Kruskal-Wallis test and Dunn's pairwise tests).

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## **AUTHOR CONTRIBUTIONS**

JICQ and LP conceived experiments. JICQ, KJK, LL, SG, YH and IB performed experiments. JICQ, LST and LP analyzed and interpreted data. LP and TKB supervised experiments. JICQ, LST, and LP wrote the manuscript.

## **ACKNOWLEDGEMENTS**

We are grateful to Prof David Gems and Drs. Helena Cochemé, Natalie Moroz and Filipe Cabreiro for advice and comments and Rachel Beltzhoover for proof-reading. We thank Drs Fiona Kerr, Anna Tillmann, and Giovanna Vinti for technical advice and assistance. We acknowledge funding from UCL Scholarships (JICQ), AFAR/Glenn Foundation for Medical Research Postdoctoral Fellowship (JICQ), Max Planck Society (JICQ, LST, SG, YH and LP), National Institutes of Health (AG54215 and GM122610 to TKB), this project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No 741989), Research Into Ageing (IB, LP), Parkinson's UK (LL, LP), Wellcome Trust (KJK, LP) and Academy of Medical Sciences (KJK).

## **COMPETING FINANCIAL INTERESTS**

All authors declare the absence of financial competing interests.





