Mechanisms under Metformin and Rapamycin as Anti-aging Drugs

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Abstract

As percentages of elderly people rise in many societies, age-related diseases have become more prevalent. Research interests have been shifting to delaying age-related disease by delaying or reversing aging itself. We use metformin and rapamycin, two drugs at the center of anti-aging drug research, as an entry point to talk about important molecular and genetic anti-aging mechanisms that have been extensively studied with them, such as mTOR, AMPK, and epigenetic modifications. We also present a number of observational studies, animal studies, and clinical trials to reflect the potential and actual effects of the mechanisms. At the end, we list remaining concerns that not only apply to researches around metformin and rapamycin but also future researches to explore other anti-aging pathways and therapeutics.

Introduction

The life expectancy in many countries is projected to exceed 85 years by 2030.[1] Globally, one quarter of the population is expected to be in their sixties or older in 2050.[2]Nonetheless, the unprecedented longer life expectancy heralds a staggering number of people living with age-related diseases and considerable burdens on the social, economic, and healthcare systems worldwide. There is a pressing need to combat the challenges posed by age-related diseases and increase the health span of humans. However, while age-related diseases can often coexist, current delivery of health services and research efforts have continued to deal with the diseases ineffectively in an insular fashion.[3] In contrast, mechanisms that account for the phenotypes of old age, such as impaired metabolism, dysregulated immune profile, and abnormal DNA methylome, have been shown to be the underlying determinants of many chronic diseases.[4] Therefore, modifying the mechanisms of aging directly seems to be a more productive approach to fundamentally curb the growth of chronic diseases.

Although aging has been traditionally considered an irreversible process, encouragingly, a growing number of studies have indicated that metformin and rapamycin, two drugs that have been used extensively to treat type 2 diabetes (T2DM) and as an immunosuppressant respectively, have the potential to stall aging and delay the onset of age-related diseases. In this review, we survey a selection of articles about metformin and rapamycin to give a glance of the anti-aging mechanisms that have been studied with these two drugs and their extent of effectiveness. Finally, remaining questions and concerns are highlighted to guide future anti-aging research.

Longevity

Almost all life forms constantly sit on a balance between production and maintenance, and under low nutrient conditions when reproduction is more challenging, in order to ensure reproductive success, increasing somatic

maintenance is necessary to prolong the reproductively competent period and consequently, lifespan.[5] Hence, calorie restriction (CR) without malnutrition is one of the most reliable approaches in extending both lifespan and healthspan in various vertebrate and non-vertebrate species. However, CR is difficult to sustain and implement since individuals must remain in a state of hunger and endure feelings of starvation, fatigue, and irritations. Besides, individuals who practiced CR are more susceptible to viral infections[6] and resistant to wound-healing[7], both of which impede its widespread use. Alternatively, metformin and rapamycin can act as calorie-restriction mimetics (CRM) by triggering the nutrient sensing pathways that sense and respond to the changing intracellular and extracellular energy and nutrient levels without actually restricting calorie intake.[8]

Rapamycin and metformin target respectively the mechanistic target of rapamycin (mTOR) and 5'-AMP-activated protein kinase (AMPK) (Fig.1). The mTOR comprises of two complexes, mTORC1 and mTORC2, and they coordinate a wide range of cellular metabolic processes concerning production, growth, and somatic maintenance, such as protein synthesis, mitochondrial function, and cell proliferation. Activated mTORC1 enhances mRNA translation and protein synthesis in the cell by phosphorylating the p70 ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1);[9] it also suppresses autophagy by phosphorylating ULK1, ULK2, and ATG13 of the ULK complex and the transcription factor EB, both of which essential for the autophagy process.[10] Not surprisingly, mounting studies have shown that deregulated mTOR signaling is implicated in the aging process and the progression of age-related disease such as cancer and diabetes.[10,11] Rapamycin suppresses mTOR signaling by first binding to its immunophilin FK binding protein (FKBP12) and then acting upon mTORC1 and mTORC2.[12] While inhibiting mTORC1 extends life expectancy and confers protection for age-related diseases, inhibiting mTORC2 is associated with unwanted effects such as glucose intolerance and abnormal lipid profiles. Nevertheless, mTORC2 is less sensitive to rapamycin and its inhibition can only be achieved through long-term treatment.[13]

AMPK is the upstream controller of the mTOR signaling pathway (Fig. 1). Numerous studies have indicated that the activating capacity of the AMPK signaling pathway declines with aging, and its decline disturbs autophagy, increases cellular stress, and promotes inflammation, which further provoke many age-associated diseases, such as cardiovascular disease, diabetes, and cancer.[9,14] Correspondingly, increased activation of the AMPK pathway has been shown to extend lifespan in lower organisms in response to CR and pharmaceutical agents, such as metformin.[15] Activated AMPK phosphorylates and activates ULK1 of the ULK complex to promote autophagy as well as activates the FOXO transcription factors that transactivate the genes involved in detoxification, autophagy, tumorigenesis suppression, and energy homeostasis.[5] Furthermore, AMPK activation attenuates the aging process by inhibiting NF-$\varkappa$B, the major regulator of innate and adaptive immunity, and relieves ER stress and oxidative stress by promoting the expression of mitochondrial uncoupling protein (UCP-2).[16]

Metformin and rapamycin are also implicated in DNA methylation, an omnipresent regulatory mechanism for gene expression in our genome. DNA methylation is facilitated by DNA methyltransferases DNMT3A, DNMT3B, and DNMT1 and adds a methyl group to the 5^{th} carbon on cytosine. DNA methylation usually leads to gene silencing by interacting with transcription mechanisms, and global hypomethylation and promoter hypermethylation are often observed in aged people.[17]Since DNA methylation is reversible, it is a promising target for therapeutic interventions.

Rapamycin regulates DNA methylation by inhibiting the mTOR signaling pathway to reduce serine production and glycolytic metabolism, which tune down the serine and 1C metabolism that uses serine to produce SAM, the methyl donor in the methylation reaction.[18]Metformin alters methylomes globally via the H19/SAHH axis. H19, a long noncoding RNA that should be downregulated in adults, causes an aberrant methylation profile by binding to and inhibiting s-adenosylhomocysteine hydrolase (SAHH), which normally hydrolyzes s-adenosylhomocysteine (SAH) and removes its inhibition of DNMT3B. Metformin activates AMPK and upregulates let-7, a family of microRNAs that bind to and degrade H19.[19]

Quite a few studies have corroborated the proposed life-extending effects of metformin and rapamycin. Metformin has been shown to extend healthspan and lifespan in the roundworm *C. elegans* (Table 1).[20]

Furthermore, a low dose of metformin supplemented for middle-aged male mice's diet lead to a 5.83% extension of mean lifespan.[21] A longitudinal study that compares the methylation profiles of the white blood cells from 12 healthy individuals at the beginning, 10 hours, and 7 days after metformin treatment revealed 11 consistently differentially methylated sites. By looking at the associated genes, regions, and networks, the study found several related genes including CAMKK1, a regulator of AMPK and glucose uptake, BACE2, involved in neurodegenerative disorders and insulin production, and ADAM8, which is related to monocyte adhesion and migration and contributes to disorders caused by excessive inflammation such as neurodegenerative disorders, allergy, asthma, and acute lung inflammation. As the cells were from healthy individuals, having ADAM8 in the result shows that metformin's anti-inflammatory effect is independent of diabetic status.[22]

Treating yeast with rapamycin resulted in extended lifespan in a process that has been postulated to mimic CR.[23] Heterogeneous male mice that received a daily dosage of 2.24 mg of rapamycin per kg of body weight beginning at the age of 20 months had 9% extended lifespan while female mice had 14%.[24] In another mice study, the Intervention Testing Program (ITP), genetically outbred mice were used to test the potential of multiple anti-aging manipulations including drugs, diets, and other interventions, and rapamycin was one of the only two drugs that had robust anti-aging effects.[25] Mice treated with rapamycin at 42mg/kg from 4-month to 22-month old had a 6-month decrease on average in epigenetic aging compared with control mice.[26] Moreover, healthy participants in a double blind randomized study aged 65 and older who had taken Everolimus, also a mTOR inhibitor, for 6 weeks then stopped for 2 weeks before a flu shot was given to them had 20% stronger immune responses compared with the control, suggesting that a low dose of rapamycin may delay immunesenescence in the elderly instead of suppressing their immune system.[27]

Cardiovascular Diseases

Cardiovascular diseases (CVDs), marked by lipid-rich plaques accumulating in blood vessels, are the leading cause of death globally, claiming around 17.9 million lives per year.[28] While CVDs arise from a complex combination of hereditary predisposition and environmental factors including lifestyle, aging is the dominant factor.[29] In the US, roughly 70%-75% of people who are 60-79 years old are afflicted with CVDs.[30]

Dyslipidemia, insulin resistance, and chronic inflammation that commonly occur in older people make them more susceptible to CVDs. AMPK activation by metformin can suppress fatty-acid desaturase (FADS) genes, reducing the circulating levels of lipid metabolites and LDL cholesterol.[31] Metformin also improves insulin sensitivity, helps losing weight, and reduces perceived hunger and food intake.[32] Recently, growth differentiating factor 15 (GDF15) was found to contribute to the weight loss effect of metformin by interacting with the GFRAL receptor in the central nervous system to suppress appetite. Metformin mediates GDF15 increase by promoting transcription of CHOP and ATF4 most prominently in the liver and gastrointestinal system.[33,34] Although metformin does not directly affect coronary artery disease through the GDF-15 pathway, the GDF-15 dependent weight loss effect may contribute to higher insulin sensitivity.[35] Metformin also inhibits vascular inflammation that can lead to plaque formation by blockading the PI3K-Akt pathway and its downstream NF-ϰB pathway.[36]Furthermore, mitochondria dysfunction and endothelial senescence contribute to higher risks of CVDs, and activated AMPK increases SIRT3 levels and improves mitochondrial biogenesis and function by enhancing trimethylation of H3K79 via the SIRT-DOT1L axis. SIRT3 also delays endothelial senescence by upregulating telomere reverse transcriptase expression.[37] Less is known about the protective effects of rapamycin, but a study found that mTOR inhibition suppressed DNMT1 upregulation caused by disturbed flow in the blood vessels both*in vitro* and *in vivo* .[38]

Metformin's protective effects have been confirmed in both animal and human studies. Chronic low doses of metformin given to ApoE deficient mice that have poor lipid-clearing capabilities and age-related atherosclerosis showed positive effects as well as reduced recruitment of macrophages into subendothelial space of aorta and decreased levels of pro-inflammatory cytokines.[37] Bovine aortic endothelial cells exposed to clinically relevant amounts of metformin have increased activities of nitric oxide synthase (eNOS), endothelium-derived

nitric oxide (NO), and AMPK while no such effect is observed in AMPK knockout mice. NO and eNOS have major roles in maintaining vascular homeostasis and its integrity, suggesting that AMPK activation by metformin exerts vascular-protective effects.[39,40] Treating 32 weeks old mice with metformin at 200 mg/kg per day for 4 weeks also partially reversed left ventricular dilatation caused by δ-sarcoglycan deficiency: the hearts showed less fibrosis, less cardiomyocyte hypertrophy, and fewer degenerative subcellular changes. At the same time, there were also increased autophagy, increased AMPK activity, and suppressed mTOR phosphorylation.[41] Diabetic veterans (mostly white male) who took metformin had lower CVDs and mortality risks compared with those who took sulfonylureas, and similar results were obtained in another clinical trial that compares the CVD risk associated with glipizide and metformin.[42,43] Although in these studies it could not be determined whether the result was caused by the benefits of metformin or damages due to sulfonylureas or both, metformin's protective effects could be more ascertained in the United Kingdom Prospective Diabetes Study (UKPDS), in which metformin treatment conferred a significantly lower incidence of myocardial infraction (33%, P=0.005) compared with dietary therapy for diabetic patients.[44] Furthermore, several clinical trials and meta analyses have found metformin to decrease CVD risk for not just diabetic people, but pre-diabetic and non-diabetic people as well.[45,46]

Tumors

The risk of fatal cancer development increases exponentially with age, and around 60% of cancers are diagnosed in people 65 years or older. Activation of oncogenes and shutdowns of tumor suppressor genes result in reprogrammed energy metabolism and uncontrolled cell growth and division.[47] Hence, it makes sense that over-activation of mTORC1 signaling has been observed in many types of cancer such as lymphoma, endometrial cancer, and renal cell carcinoma.[48–50] Activated mTORC1 promotes aerobic glycolysis by increasing the amount of hypoxia inducible factor (HIF)-1α, a transcription factor that is associated with metastasis by promoting angiogenesis responding to hypoxia.[51] It also indirectly upregulates genes involved in lipogenesis by phosphorylating Lipin-1 and S6K-1, which activates SREBP-1, a lipogenic transcription factor.[52,53] Additionally, phosphorylating S6K1 enhances biosynthesis of purine and pyrimidine, two amino acids required for cancer cell proliferation.[54]As the potent inhibitor of mTORC1, rapamycin can put a brake on the defective tumor metabolism and has been investigated as a promising drug to treat cancer. In 2002, rapamycin was first reported to have antineoplastic properties in mice by suppressing cancer metastasis and angiogenesis.[55] Since then, overwhelming *in vivo* and *in vitro* studies have reported that rapamycin and its derivatives have the potential of ameliorating cancer onset and development, and hundreds of clinical trials have been conducted to test monotherapy or combination therapies of rapamycin.[24,56] For instance, rapamycin treatment decreased both phenotypic progression of tumor and tumor size in mice exposed to the tobacco carcinogen NNK and had lung cancer.[57] Nevertheless, the actual clinical benefits of rapamycin and rapalogs have been mostly modest.[58,59] In a study using transgenic HER-2/neu cancer prone mice, although rapamycin did not extend the lifespan of the mice with established tumor, it effectively delayed spontaneous tumor onset in others and extended their lifespan, suggesting its potential as a measure to prevent cancer.[56]

Multiple studies have also supported that the growth inhibition caused by metformin's interaction with the AMPK/mTOR pathway to be effective against various cancers including lung cancer, breast cancer, and colorectal cancer.[60] Metformin delayed the first tumor onset by 22% and 25% respectively in female mice at the age of 3 months and 9 months.[61] Furthermore, metformin inhibited NNK-induced lung cancer cell proliferation in mice by decreasing the levels of circulating insulin and IGF-1, which suppressed the IIS pathway and downregulated the downstream PI3K-Akt and mTOR signaling pathway (Fig. 1).[62] In endometrial cancer cells, metformin significantly reduced the levels of Ki-67, an indicator of tumor progression, topoisomerase IIα, associated with DNA instability, and phospho-ribosomal protein S6 and phospho-ERK 1/2, both of which activated by mTOR. Significantly increased AMPK and p27 levels and subsequent cell cycle inhibition were also observed.[63] H19 is found in almost all cancer cells. Genome-scale DNA methylation profiling showed that tumor promoting pathway genes became repressed and genes involved in neuronal development, cell morphology, and intracellular communication were activated after metformin treatment. Interestingly,

the H19 gene was also inactivated, suggesting a feed-forward response to continuously suppress H19 can be established by metformin.[19] In addition, the 11 metformin-induced differentially methylated CpG sites mentioned earlier were related to multiple tumor-related genes: SIX3 is downregulated in lung cancer due to promoter methylation, which was rescued by metformin. POFUT2 is linked to glioblastoma and adenocarcinoma. MUC4 is implicated in pancreatic cancer. KIAA1614 is related to colon cancer. Lastly, UPF1 is associated with genome stability. The differentially methylated regions included the gene EPHB1, whose underexpression leads to gastric carcinoma and invasion of colorectal cancer cells, and SERP2, which is positively correlated with BMI and abnormal glucose tolerance as well as colorectal cancer. Pathway enrichment analysis found association between the CpG sites and the unfolded protein response, which is involved in metformin-induced apoptosis in acute lymphoblastic leukemia.[22] In 2005, a case control study first discovered reduced risk of cancer associated with metformin in diabetic patients.[64] Compared with people who took sulfonylureas, insulin, and other anti-diabetic drugs, metformin users had a significantly lower risk of cancer (Hazard Ratio [HR] 0.63, 95% Confidence Interval [CI] [0.53-0.75]).[65] Diabetic patients who took metformin also had 7% less chance of getting hepatocellular cancer for each incremental year they took metformin, and it was attributed to inhibited proliferation and cell cycle arrest induced by metformin in the hepatocytes.[66] Nevertheless, there are studies that do not support metformin's beneficial role in cancer. Evidence from randomized control trials has been large inconclusive.[67,68] Additionally, in a study that compares metformin with rosiglitazone and sulfonylureas, metformin users did not show lower malignancy rates.[69] Multiple meta-analyses also did not find any evidence showing metformin reduces cancer incidence.[70,71] Work is still needed to resolve these inconsistencies.

Neurodegeneration

Alzheimer's Disease (AD) is a degenerative neurological disorder, accounting for 60-80% of dementia.[72] AD affects up to one third of the population aged > 65 years, making it the fifth leading cause of death globally.[73] Currently no drug exists to treat or slow down the development of AD. Instead, patients use drugs such as cholinesterase inhibitors and glutamate inhibitors to prevent the breakdown of acetylcholine and the overexcitation of neurons to amend their impaired cognitive functions.[74–77]

Applying metformin to treat AD stems from the widely observed association between AD and type 2 diabetes mellitus (T2DM). In 20 non-diabetic AD patients, treatment with metformin for 8 weeks resulted in improved cognitive functions (Koenig, Mechanic-Hamilton et al.).[78] Nonetheless, a larger study that analyzed data from 7,086 dementia patients and matching number of healthy controls from the United Kingdom-based General Practice Research Database (GPRD) concluded otherwise; Individuals who did not receive any drug for diabetes (AOR=0.88, 95% CI=0.71–1.10) or those who took antidiabetic drugs (AOR=1.03, 95% CI=0.90–1.19) had a similar risk of developing AD as individuals without diabetes (AOR=1). Furthermore, patients who had been exposed to various anti-diabetic drugs and had received more than 60 prescriptions of metformin had increased the risk of developing AD (AOR=1.71, 95% CI=1.12-2.60), which was attributed to the production of A-β peptides, a hallmark for AD. However, the increased risk was not confirmed in patients who had taken metformin exclusively (AOR=1.00, 95% CI=0.55-1.81, >30 prescriptions), and there was no trend of increasing risk of AD with increasing number of metformin prescriptions.[79] The increased production of A-β peptides caused by metformin was explained on cell cultures of primary cortical neurons and N2a neuroblastoma cells expressing human amyloid precursor protein (APP). Metformin upregulates the transcription of β-secretase, which cleaves APP into A-β peptides. Intriguingly, metformin combined with insulin reduced A-β peptide levels.[80] In another study which diabetes model mice were used to evaluate AD-like brain changes and the effect of metformin on those changes, metformin attenuated the increase of total tau, phospho-tau, and activated JNK, a tau kinase, in the mice. Metformin also attenuated the decrease of synaptophysin and preserved the neural structures, but did not improve spatial learning and memory abilities.[81] These studies together suggest that having taken metformin in the past does not reduce the risk of AD, but metformin used with insulin may be an effective short-term treatment.

Dysregulated mTOR activity and autophagy have been observed in patients with early Alzheimer's disease.[82] AD and mTOR's connection has also been shown in a genome wide association study (GWAS), in which it identified 5 significant SNPs (rs6723868, rs30986, rs27709, rs26840, rs27648) that had to do with cerebellar age acceleration, defined to be negative when the epigenetic age is less than the chronological age (Table 2). Correlating the SNPs with mRNA levels of neighboring genes found genes that overlapped with those related to AD, age related macular degeneration, and Parkinson's disease (PD). Among them, the gene MLST8 was significantly correlated with cerebellar age acceleration, and MLST8 is an integral part of the mTOR complexes.[83] Hence, the mTOR inhibitor rapamycin has also been investigated to treat AD. Mice with increased mTOR activity had higher levels of tau and Aβlevels.[84]Administrating rapamycin to young 3xTg-AD mice induced mTOR-mediated autophagy and reduced Aβ and tau levels.[85]Nonetheless, rapamycin could only accelerate autophagy of tau and plaques before their formation: a significant reduction of tau and plaques was observed when treating AD mice at 2 months with rapamycin while at 15 months the treatment had no such effect.[86]Moreover, administering rapamycin to APOE4 mutant carrier mice, a transgenic AD mice model, resulted in restored cerebral blood flow and maintenance of blood-brain barrier integrity, pointing to rapamycin's beneficial role in reducing vascular progression in AD.[87]

PD is known for tremors, difficulty in walking, and muscle rigidity in its patients due to dysfunction of the motor system.[88]PD affects around 1% of people aged > 60 years.[89] Loss of dopaminergic neurons is characteristic of PD and leads to a decreased amount of dopamine as well as imbalance between dopamine and acetylcholine. According to the acetylcholine-dopamine balance hypothesis, over-activation of cholinergic system activity causes motor and cognitive disturbances. Hence, the current PD drugs either provide more dopamine or reduce the amount of acetylcholine to restore the balance, working as a remedy instead of neuroprotective agents.[90–92]

PD, diabetes, and dementia share the disorder of mitochondrial bioenergetics and abnormal protein folding in their pathogenesis, and several studies have found metformin to alleviate PD. An analysis of a cohort of 800,000 people from the Taiwan National Health Insurance database showed that having T2DM increased the risk of PD 2.2-fold, and metformin-inclusive sulfonylurea therapy reduced the risk (HR=0.78 relative to diabetes-free, 95% CI=0.61-1.01).[93] The reason has to do with metformin's ability to reduce α-synuclein release, a component of the Lewy bodies and Lewy neurites that are characteristic of PD. MPTP, a prodrug to the neurotoxin MPP+, was used to damage the mice dopaminergic neurons, leading to astroglial activation, which increased release of α-synuclein. Then metformin mitigated astroglial activation and promoted methylation of protein phosphatase 2A (PP2A), helping α-synuclein dephosphorylation. AMPK activation by metformin also increased ATP production in mitochondria and restored mitochondria function. However, the timing and dosage of metformin was also critical. When MPTP and metformin were given in the same day, 75% lethality ensued in the mice. Although metformin increased the levels of two neurotrophic factors BDNF and GDNF, high dosage (400 mg/kg) killed all the mice.[94,95] In another study, metformin rescued tumor necrosis factor type 1 receptor associated protein (TRAP1) mutation associated changes in mitochondrial protein balance. TRAP1 is a protein associated with stress sensing in mitochondria, and its absence due to mutation has been identified to increase the risk for PD. Metformin reversed elevated mitochondrial respiration, reduced mitochondrial membrane potential, and imbalance of nuclear and mitochondrial protein production caused by the loss of TRAP1.[96] In summary, metformin intervenes the pathogenesis of PD by preserving neurons, reducing inflammation, and protecting mitochondria functions. It is a promising new help for PD patients, but further studies are still needed to understand the influence of dosage and timing.

Discussion

Although current research foreshadows a promising perspective for using metformin and rapamycin as anti-aging drugs, there are still some concerns that need to be highlighted, and they apply not only to the researches of metformin and rapamycin, but to other anti-aging mechanism and anti-aging drug researches as well.

First, despite the positive outcomes from many studies, it is not uncommon to find a change in dosage turning the result from life-extending to life-ending. When a low dose of metformin (0.1%) was given to middle-aged male mice with their diet, their lifespans were extended by 5.83% on average, but a higher concentration (1%) became toxic.[21] In another study, although metformin activated AMPK and suppressed lipid storage in fruit flies, their lifespan did not increase. At higher doses (25mM and 50mM), metformin reduced the survival rates. The authors reasoned the causes to be excessive starvation, disrupted intestinal fluid homeostasis, or metformin toxicity.[97] In the PD study with mice models created with MPTP, when MPTP and metformin were given in the same day, 75% lethality ensued in the mice. Furthermore, although metformin increased the levels of BDNF and GDNF, two neurotrophic factors, the high dosage (400 mg/kg) killed all the mice.[95] The issues with dosage along with physical and genetic differences between humans and animals make scaling the positive lab results for human use a tricky matter. In the study that showed metformin's beneficial effects for treating CVDs in mice, the dosage was 200mg/kg, a number that can no way be applicable to humans.[41] Hence, conducting human clinical trials may be a more efficient approach to find a safe and effective dosage for human use. Encouragingly, when anti-diabetic doses of metformin were given to 12 pre-operative endothelial cancer patients and comparison of their tissue samples before and after the operation were made, the same effects observed in vitro were found – increased AMPK phosphorylation, decreased tumor cell proliferation, and decreased H19 levels.[19]

Another issue that stands in the way is the side effects associated with chronic use of drugs. About 25% of patients treated with metformin have gastrointestinal side effects associated with the phenotype of organic cation transporter 1 (OCT1).[98] Besides, chronic use of metformin can cause dose-dependent vitamin B12 deficiency, increasing the risk for anemia and neuropathy.[99,100] Lactic acidosis has been reported as a side effect of metformin, but there has been controversies, and in the study using diabetes model mice to study AD-like brain changes, metformin did not further increase the serum lactate concentrations.[81] Whether this holds true in healthy mice or humans is yet to be seen. As for rapamycin, over a third of users have reported diarrhea and nausea, accounting for around 5% of treatment discontinuation.[101] The issues with side effects can be addressed in four ways: the first is to selectively take supplements, such as vitamin B12, to make up for the loss. The second is to reduce the dose and increase the interval between every dose. A small RCT suggests short-term use of rapamycin to be relatively safe approach.[102] Intermittently administering 2mg/kg of rapamycin every 5 days has also reduced incidence of side effects in mice and extended their lifespan.[103] More clinical trials are needed to calibrate the balance between safety and anti-aging effectiveness. The third way is taking a variety of anti-aging drugs (also known as drug cocktail therapy), each with a very low dose, instead of taking only metformin or rapamycin since the side effects are dose-dependent. Although cultured cells and a mice study both showed metformin combined with insulin reduced A-β peptide levels,[80,81] the GPRD study showed long-time combined exposure to metformin and other anti-diabetic drugs increased the risk of AD while using only metformin did not show any difference.[104] Therefore, this method requires further validation as well. The fourth way is to find analogs with fewer side effects. Recent study found DL001, an effective mTORC1 specific rapalog, does not induce metabolic disruption and immunesuppression.[105] Furthermore, a low dose of rapalog (RAD001) combined with the catalyst BEZ235 reduced infection by 40% and improved response to influenza vaccination in healthy elderly (aged 65 and older) subjects. More importantly, this combination therapy was well-tolerated in majority of the subjects.[106]Rapalogs have been approved for treating multiple cancers, including renal cell carcinoma, hepatocellular carcinoma and mantle cell lymphoma, making it by far the most promising way to circumvent the side effects of rapamycin.[107,108] Nevertheless, as reported clinical benefits have been modest,[58,59] the quest for more effective rapalogs is still ongoing. On the other hand, metformin had fewer available analogs with well-studied side effects. Phenformin and buformin, which are biguanide drugs like metformin, were withdrawn from the market due to fatal lactic acidosis.[109]Mito-metformin, synthesized by adding a positively charged triphenylphosphonium group to metformin, showed 100-fold to 1000-fold more anti-proliferative effects depending on alkyl chain lengths, but how the drastically improved potency will impact healthy cells is poorly understood at the moment.[110]

Future research should also work to elucidate how gender influences drug effectiveness. Metformin increased

mean lifespan of female mice by 4.4% while decreased that of male mice by 13.4%.[111] Male pre-diabetic patients who received metformin had a significantly lower coronary calcium score compared with control while the female group did not.[46] Such sexual dimorphism also affects the lifespan of mice that took rapamycin – female mice had greater lifespan increase than male mice did.[24,112] These studies have showed varied amount by which gender influences drug effectiveness, and studying drug-hormone interactions could help finding the reason.

Besides these issues, much more can be found about the genetic mechanisms that regulate lifespan. Although many positive outcomes have come out of attempts to control DNA methylation with metformin and rapamycin, the full picture of epigenetic modifications have not been understood. Metformin treatment led to a combination of hyper, hypo, and non-differentially methylated CpG sites, and this was due to a combination of direct and indirect effects. For example, hypermethylation of one site can lead to reduced expression of a protein, and this can have downstream effects that alter methylation status of other sites.[19] Understanding this complex network of interactions will not only promote further understanding of metformin and rapamycin, but also help developing more anti-aging measures. APOE, a locus on chromosome 5q33.3, and FOXO3A are all known to correlate with longevity. It has also been mentioned above that a GWAS identified 5 SNPs related to cerebellum aging.[83]In addition, SNPs in the human genome also affects the efficacy of drugs. For example, rs2740574, located in CYP3A4 changes breast cancer cells' response to rapamycin by altering drug metabolisms in liver, and rs2282143, located in SLC22A1, changes breast cancer cells' response to metformin by affecting the rate of drug entering cells.[113] A locus on chromosome 11 (rs11212617) is associated with the glycemic response to metformin.[114]Three SNPs (rs8111699, rs11212617, rs9803799), which are located in the LKB1, ATM and PRKAA2, have been identified as significant influencers on metformin therapy by affecting the AMPK pathway.[115–117] As DNA sequencing becomes more convenient and accessible, it is reasonable to assume that increasing amount of genetic data and research efforts will reveal many more such connections, and they can point to novel genes and drug targets or be used for precision medicine to improve current treatments.

The issues of dosage, side effects, sexual dimorphism, and genetic regulatory mechanisms all point to the need for a large-scale clinical trial. The Targeting Aging with Metformin (TAME) trial is a large placebo-controlled trial that has been designed to enroll 3000 subjects to test whether metformin delays age-related diseases.[118] The TAME trial received FDA approval in 2015, and after receiving all the required budget in 2019, it was set to start at the end of the same year. The TAME trial may make metformin the first approved drug for anti-aging, but more importantly, since it is not testing metformin against a single disease but a collection of age-related ones, it establishes aging as a medical condition that can be intervened or treated instead of an irreversible process outside human control. The shift in the notion of aging will make future anti-aging clinical trials proceed with much more ease.[119]

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Table 1. Summary of major animal studies and clinical trials about the effects of metformin and rapamycin

Study	Organisms	Application scheme	Effect
Metformin	Metformin	Metformin	Metformin
Life extension effect	Life extension effect	Life extension effect	Life extension effect
Slack 2012[97]	Fruit flies	1,10,100 mM, every day	1-10 mM, no effect on survival; >10mM, lifespan decrease Increase mean lifespan by 18%, 36%, 3%
Cabreiro 2013[20]	C. elegans	25, 50, 100 mM, every day	Increase mean lifespan by 14%, 6%, 0%
Martin-Montalvo 2013[21]	Mice	100 mg/kg, every day, started at 3, 9 or 15 months	0.1%, lifespan increase by 5.83%; 1%, lifespan decrease by 14.4%

Study	Organisms	Application scheme	Effect
Zhong 2017[19]	Human	Initial dose: 750 mg/day; increased weekly up to 1500 or 2250 mg/day for 3-12 weeks	Increase in AMPK phosphorylation; Decreased tumor cell proliferation; Decreased H19 levels; Increased methylation in genes H19, DMRTA2, KCNG2, PSMD10, TRA2A
Anticancer effect	Anticancer effect	Anticancer effect	Anticancer effect
Mitsuhashi 2014[63]	Human	1500-2250 mg/day, for 4 to 6 weeks	Inhibited endometrial cancer cells grow in vivo
Anisimov 2011[61]	Mice	100 mg/kg-bodyweight starting at the age of 3 months, 9 months, and 15 months	Delayed tumor onset by 22% and 25% respectively at the age of 3 months and 9 months
H. P. Chen 2013[66]	Human	Based on the condition of patients with type 2 diabetes mellitus	Hepatocellular carcinoma risk decreases
Reduce cardiovascular disease risk	Reduce cardiovascular disease risk	Reduce cardiovascular disease risk	Reduce cardiovascular disease risk
Lexis 2015[45]	Human	ST-segment elevation myocardial infarction (STEMI) patients, 500 mg twice daily, for 4 months	Cardiovascular risk decreases
Goldberg 2017[46]	Human	850 mg twice daily, for over 3.2 years	Coronary atherosclerosis risk decreases
Karnewar 2018[37]	Mice	50 mg/kg-bodyweight for 14 months	Ameliorated age-related atherosclerosis; Significantly reduced macrophage recruitment and inflammatory cytokines
Kanamori 2019[41]	Mice	200 mg/kg-bodyweight per day for 4 weeks	Partially reversed left ventricular dilatation caused by δ-sarcoglycan deficiency
Anti-Alzheimer's disease effect	Anti-Alzheimer's disease effect	Anti-Alzheimer's disease effect	Anti-Alzheimer's disease effect
Chen 2009[80]	Mice	2–5 mg/mL for 6 days	Both intracellular and extracellular Aβ species increases
Li 2012[81]	Mice	200 mg kg^{-1} d^{-1} for 18 weeks	AD-like biochemical changes decrease

Study	Organisms	Application scheme	Effect
Koenig 2017[78]	Human	Metformin or placebo for 8 weeks	Executive functioning improves
Anti-Parkinson's disease effect	Anti-Parkinson's disease effect	Anti-Parkinson's disease effect	Anti-Parkinson's disease effect
Katila 2017[95]	Mice	200 mg kg-¹ d-¹ for 7 days	Metformin provides neuroprotection against MPTP neurotoxicity
Rapamycin			
Life extension effect	Life extension effect	Life extension effect	Life extension effect
Aliper 2017[23]	Yeasts	100, 300, 600, 1000pg/mL in the culture medium	Lifespan increase in a dose-responsive manner
Harrison 2009[24]	Mice	Begins at 600 days, 14.7 mg/kg	females' lifespan increases by 14%; males' lifespan increases by 9%
Miller 2014[112]	Mice	Begins at 3 months, everyday	females, lifespan increase by 26%; males, lifespan increase by 23%
Mannick 2014[27]	Human	0.5mg daily, or 5 mg weekly, or 20 mg weekly for 6 weeks	20% improved response to influenza vaccine
Wang 2017[26]	Mice	42mg/kg-bodyweight dietary rapamycin treatment from 4 to 22 months	Had 6-month decrease in epigenetic age compared to control
Anticancer effect	Anticancer effect	Anticancer effect	Anticancer effect
Guba 2002[55]	Mice	1.5 mg/kg/d, begins on day 0 or 7 relative to tumor implantation	Inhibited liver tumors grow
Granville 2007[57]	Mice	1 weeks after NNK administration	Tumors show decreased phenotypic progression and a 74% decrease in size
Anisimov 2011[56]	Mice	Begins at 2 months ,3 times a week, for 2 weeks, followed by a 2 weeks break, for 2 years	Shifted the tumor-yield curve to the right and prolonged mean lifespan
Anti-Alzheimer's Disease effect	Anti-Alzheimer's Disease effect	Anti-Alzheimer's Disease effect	Anti-Alzheimer's Disease effect
Caccamo 2010[85]	Mice	2.24 mg/kg, every day	RAPA improves learning and memory and reduces Aβ and tau pathology.
Lin 2017[87]	Mice	14mg/kg, every day	Block progression of early cognitive deficits

Table 2. A summary of major studies that have shown the effects of SNPs on metformin/rapamycin therapy

Study	SNP	Gene	Effect
Metformin	Metformin	Metformin	Metformin
Trilla-Fuertes L 2018[113]	rs2282143	SLC22A1	Affecting the efficiency of metformin entering cells
Lopez-Bermejo A 2010[115]	rs8111699	LKB1	Influencing both insulin sensitivity and metformin efficacy in hyperinsulinemic girls with androgen excess
Jablonski KA 2010[116]	rs11212617	ATM	Affecting glycemic response to metformin
Zhou 2011[117]	rs9803799	PRKAA2	Affecting generation of AMPK
Rapamycin	Rapamycin	Rapamycin	Rapamycin
Trilla-Fuertes L 2018[113]	rs2740574	CYP3A4	Affecting the metabolism of rapamycin in the liver
Lu 2016[83]	rs6723868	DHX57	Correlated with chronological age
Lu 2016[83]	rs30986 rs27709 rs26840 rs27648	MLST8 MLST8 MLST8 MLST8	Correlated with smaller epigenetic age than chronological age in the cerebellum, a part of mTOR complexes

Figure Legends

Figure 1. Metformin and Rapamycin could decrease the incidence of age-related diseases.

In terms of mechanism, metformin and rapamycin achieve similar calorie restriction effect in different ways. Metformin can activate AMPK like calorie restriction and further cause a series of pathways changes, which could arouse anti-aging effects such as Inhibition of pro-inflammatory effect and ROS detoxification. Rapamycin can inhibit mTORC1, which relate to Autophagy and Protein Synthesis pathway.