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Resveratrol

Adding Life to Years, Not Adding Years to Life

Edited by Farid A. Badria

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Meet the editor

Professor Farid Badria obtained his PhD in Microbial Transformation from the University of Mississippi, USA, and two Masters of Science in Pharmacognosy and Cell Biology from Egypt and the USA.
TWAS-ARO- TWAS-ARO in "Public Understanding and Popularization of Science (2013), World Intellectual Property Organization Gold Medal as the Best Inventor in Egypt (2011), Recognition Outstanding Award in Medicine (Egypt, 2001), Outstanding Arab Scholar, Kuwait (2000), and Khawrazmi Award, Iran (2000), are just some of the awards he has received.
Professor Badria has submitted 43 patents, of which 16 have been granted certificates with intellectual protection for 20 years. With over 200 publications, he continues to lead research projects on developing new therapies for liver disorders, arthritis, and skin disorders, and bomarkers for cancer.

Contents

Preface

Biomedical research focuses on trying to cure individual diseases and investigate the consequences of successful treatment by addressing the question: "Do drugs add years to life or add life to years?" Life expectancy has risen in developed countries from about 47 in 1900 to about 80 today, largely due to advances in curing childhood diseases. But those longer lives come with their share of misery. Age-related chronic diseases such as heart disease, cancer, stroke, and Alzheimer's are more prevalent than ever.

The standard medical approach—curing one disease at a time—only makes that worse, says Jay Olshansky, a sociologist at the University of Chicago School of Public Health, who runs a project called the Longevity Dividend Initiative, which makes the case for funding aging research to increase the health span of individuals on health and economic grounds. "I would like to see a cure for heart disease or cancer," he says. "But it would lead to a dramatic escalation in the prevalence of Alzheimer's disease."

Although science has discovered effective drugs for many of the diseases that afflict mankind, many human health problems remain untreatable. The search for novel therapeutic agents is always ongoing.

This book will describe some aspects of resveratrol (3,5,4′-trihydroxy-trans-stilbene), a stilbenoid, a type of natural phenol, and a phytoalexin produced by several plants in response to injury or when the plant is under attack by pathogens such as bacteria or fungi. Even though over 4000 scientific studies have been published on resveratrol and it is used as a dietary supplement, there is no good evidence that consuming resveratrol affects life expectancy or human health.

This book addresses in three sections several controversial issues regarding this interesting natural molecule with fascinating pharmacological and therapeutic effects.

Section 1: Pharmacology and Therapeutic Applications of Resveratrol

This section discusses in detail the pharmacology and effects of resveratrol supplementation in animals.

Section 2: Bioavailability, Metabolism, and Mode of Action of Resveratrol

This section presents a novel drug delivery systems for resveratrol, its protective activity in cardio- and cerebrovascular diseases, and where and how in the mTOR pathway inhibitors fight aging. It also investigates resveratrol as an activator for the treatment of aging and age-related diseases.

Section 3: Resveratrol and Its Role in Chronic and Degenerative Diseases: Does it Add Life to Years or Years to Life?

This section presents new therapeutic approaches: nanotherapy and clinical and therapeutic applications of resveratrol in the management of diabetes and obesity.

We hope this book will be useful to a wide range of people, from students first learning about longevity, to advanced clinicians and researchers who are looking for a review of current treatments and conceptualizations of the condition. It is our hope that this book will motivate readers to approach the evidence on the use of this fascinating molecule with an open mind, and thereby spark an interest in making further contributions to the current scientific debate and treatment development efforts.

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Section 1

Pharmacology and Therapeutic Applications of Resveratrol

Chapter 1

Evaluation of Resveratrol Supplementation on Laboratory Animals, Cats, Pigs, Horses, Dogs, Cattle, and Birds

Mary U. Ememe, Anthony K.B. Sackey and Joseph O. Ayo

Additional information is available at the end of the chapter

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Abstract

This chapter evaluated resveratrol supplementation on laboratory animals, cats, pigs, horses, dogs, cattle and birds. Resveratrol (3, 5, 4′-trihydroxystilbene) is a stilbenoid, a derivate of stilbene. It is found in some plants such as red grape, grape products, cocoa, peanuts, raspberries, mulberries, strawberry and Japanese knotweed roots. The most important dietary source of resveratrol is red wine, and it is often assumed to be an important factor in the French Paradox, a term used to describe the observation that the French population has a very low incidence of cardiovascular disease, despite a diet high in saturated fats. Research has shown some therapeutic effects of resveratrol ranging from antioxidant, anti-inflammatory, cardioprotective, antiatherogenic, antiaging, anti-platelet aggregation, anticancer, antidiabetic, antitumor, and immunomodulatory activities. In laboratory animals, benefits of resveratrol comprise antitumor effects while in cats it has shown to improve hepatic function. In pigs, the antibiotic and antiviral effects of resveratrol have been illustrated. The anti-inflammatory and antioxidative properties of resveratrol in horses and cattle were also reviewed. The supplement was shown to be useful as an antibiotic and an aid in improving alertness in dogs. Resveratrol also showed to increase growth performance in birds. It is therefore concluded that use of resveratrol is a potent aid in improving animal production and health.

Keywords: animals, anti-inflammatory, antioxidant, benefits, resveratrol

1. Introduction

Resveratrol (3, 5, 4′-trihydroxystilbene) is a stilbenoid, a derivate of stilbene. It exists as two geometric isomers: *cis*-(*Z*) and *trans*-(*E*) [1] (**Figure 1**). The *Tran-* and *cis*-resveratrol can be either free or bound to glucose [2].

Resveratrol is a natural polyphenol nonflavonoid compound present in strongly pigmented vegetables and fruits. It is found in more than 70 species of plants such as grapes (*Vitis vinifera*), cranberry (*Vaccinium macrocarpon*), peanut (*Arachis hypogaea*), cocoa, raspberries, mulberries, grapevines, strawberry and Japanese knotweed roots (*Polygonum cuspidatum*) [3] which has the highest concentration of resveratrol (**Figure 2**). Resveratrol is also present in yucca (*Yucca schidigera*) and turmeric (*Curcuma longa*) [4]. The most important dietary source of resveratrol is red wine, and it is often claimed to be an important factor in the French Paradox, a term coined to describe the observation that the French population has a very low incidence of cardiovascular disease, despite a diet high in saturated fats [5]. A new bioconversion system is known to produce resveratrol in the blastospore of *Tremella fuciformis* [6]. *Tremella fuciformis* is a known edible macrofungus that has medicinal value and is widely cultivated in China. Resveratrol has also been produced from tyrosine in metabolically engineered *Saccharomyces cerevisiae* [7].

Resveratrol was first used as a traditional Chinese and Japanese medicine for treatment of human inflammatory, allergic, hypertensive, and lipid diseases [9].

Current research into resveratrol benefits shows that resveratrol has amazing antiaging properties at the cellular level [10, 11]. This effect may be attributed to biochemical impacts of energy restriction [12, 13]. Caloric restriction is an effective means of preventing chronic disease and ultimately increasing lifespan [14]. SIRT1, an NAD + −dependent deacetylase, was identified as one of the molecules through which calorie restriction extends lifespan or delays age-related diseases [15]. This has led to breakthroughs in geriatric and antiaging medicine. Resveratrol is recognized to increases the expression of Sirtuin1 and Peroxisome proliferator-activated receptor co-activator 1 alpha (PGC-1α). Sirtuin1 is a protein encoded by the SIRT1 gene [16] which implies (Silent mating type information regulation 2 homolog 1). SIRT1 is an enzyme that occurs in living organisms and is known to regulate cellular aging, apoptosis and resistance to stress [17]. Sirtuin 1 also aids mitochondria to metabolize glucose more efficiently [14]. The result is increased energy output from cellular metabolic reactions. PGC-1α as well slows the aging process and prevents a number of chronic diseases [18].

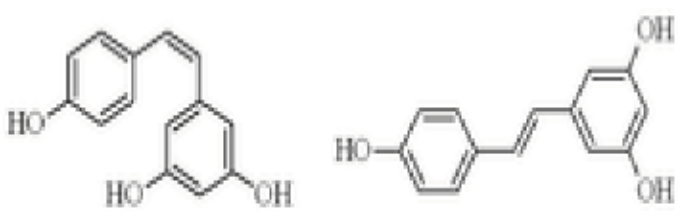

Figure 1. Chemical structures of *cis*-(Z)-resveratrol and *trans*-resveratrol (E)-resveratrol [1].

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Figure 2. Japanese knotweed, a well-known rich source for resveratrol [8].

Khan et al. [19] and Sahin et al. [20], stated that resveratrol increases regulation of antioxidant enzyme like catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). This result in reduction of oxidative stress and attenuation of inflammation, and these mechanisms may account for many of its health benefits. The antioxidant activity of resveratrol may also inhibit oxidation of low-density lipoproteins (LDL), and therefore, decrease endothelial damage associated with cardiovascular disease [21, 22].

Results of cellular and animal studies have indicated that resveratrol inhibits a nuclear cofactor (NF-kappa B) involved in the gene expression of numerous inflammatory compounds, including cyclooxygenases (COX-2), lipoxygenases, peroxidases, nitrous oxide synthases and cytokines such as tumor necrosis factor alpha (TNFα) [23]. Resveratrol has also been shown to suppress apoptosis and inflammatory signaling via its actions on the NF-kappa B pathway in human chondrocytes [24]. It is therefore, important that resveratrol be investigated further for the prophylactic treatment of osteoarthritis in humans and companion animals. Resveratrol regulates neuronal inflammation in various disease models and protects the brain against ischemic injury [25]. This finding supports important benefits of resveratrol in modulation of the excitotoxic cascade postischemia, which are similar with anti-inflammatory effects observed in various pathological models.

Ghanim et al. [26] found that 6 weeks of supplementation with 200 mg of *Polygonum cuspidatum* extract containing 40 mg of resveratrol did not alter fasting plasma concentrations of cholesterol (total, LDL, and HDL), triglycerides, or leptin compared with placebo in 20 healthy individuals. However, mononuclear cells from the resveratrol group demonstrated suppressed nuclear factor kappa B (NFκB) binding, decreased ROS generation, and TNF-α and Interlukin-6. Additionally, plasma TNFα and C-reactive protein (CRP) were significantly reduced. These findings reveal that resveratrol's actions on the cellular level can indeed influence plasma biomarker measurements associated with inflammation and risk for various diseases.

One of the key cardioprotective mechanisms of resveratrol stems from its ability to upregulate endothelial nitric oxide synthase (eNOS), which ultimately increases nitric oxide (NO) mediated vasodilation and increases blood flow [27]. Additionally, human platelets exposed to physiologically attainable concentrations of resveratrol have been shown to increase eNOS activation, leading to greater NO production and decreased platelet activation [28].

Studies have shown that resveratrol has antidiabetic effects [29, 30]. Poulsen et al. [31] observed that resveratrol supplementation as antidiabetic played an important role in improving glucose metabolism and preventing inflammation, metabolic abnormalities, cancer and nonalcoholic fatty liver disease. Furthermore, resveratrol decreased insulin resistance and metabolic disorders, compared to other diets that did not contain resveratrol [32].

Resveratrol or its derivatives also prevent cancer cell proliferation [33]. This is possible because Resveratrol can inhibit the activity of one type of enzyme, matrix metalloproteinase [34] which aids proliferation of cancerous cells as well as angiogenesis which enhances invasive tumors [35, 36].

Das [37] found that resveratrol helps in reduction of thermal stress. Numerous studies have shown that resveratrol can attenuate cellular processes such as protein damage associated with high temperature [20, 37] and UV radiation [38].

Recent advances of resveratrol have shown that it could be used for treatment and prevention of HIV/AIDS, and it has been shown to synergistically enhance the anti-HIV-1 activity [39].

The effects of resveratrol on cellular factors mediating liver damage and regeneration in acute carbon tetrachloride (CCl_4) liver injuries have been investigated [40]. The result showed that resveratrol therapy can be beneficial for acute toxic liver injury.

Resveratrol bioavailability is low or zero and this may be attributed to speed and extensive metabolism or its poor water solubility [41] and the consequent compound of different metabolites such as resveratrol sulfates and resveratrol glucuronides [42]. Encapsulated resveratrol provides a potential approach for improving the solubility of resveratrol, consequently, enhancing its bioavailability. Against this background, the oral resveratrol bioavailability is not related to dose or aqueous solubility [35, 43]. While 70% of orally administered resveratrol is absorbed, its oral bioavailability is approximately 0.5% due to extensive hepatic glucuronidation and sulfation [44]. Microencapsulation of resveratrol product helps to reduce rapid metabolization and excretion of resveratrol when administered to horses [45]. Resveratrol conjugated gold nanoparticles is effectively used as delivery vehicles [46]. Bio-directed synthesis of metal nanoparticles is gaining importance due their biocompatibility, low toxicity and eco-friendly characteristics.

2. Studies of resveratrol in animals

2.1. Laboratory animals

Studies demonstrated that resveratrol supplementation in diet of rats and mice played an important role in protecting heart cells or cardiovascular system from free radical-induced cell death or from damage which occurs through obesity and chronic hypertension [47].

Resveratrol has shown much promise in treating cancer in laboratory animals [48, 49]. A recent study demonstrated resveratrol to decrease liver tumors, while increasing lymphoma and possibly solid tumors. This is consistent with the concern that resveratrol can have pro-oxidant effects, especially in the presence of copper, which is elevated in certain tumors, and that this may exacerbate the effects of cancer [50].

A study on rodent models showed that, oral administration, topical application, and injection of resveratrol inhibited the development of chemically-induced cancer at many sites, including gastrointestinal tract, liver, skin, breast, prostate, and lung [51, 52]. The anticancer effects of resveratrol in rodent models involved the reduction of cell proliferation, the induction of apoptosis, and the inhibition of angiogenesis, tumor growth, and metastasis [53]. Resveratrol has shown promise on skin cancers when used on the body surface of mice [54] and effective against esophageal cancer when ingested orally in rats [55].

The benefits of resveratrol for memory and prevention of neurodegenerative diseases has been documented in laboratory animals [56]. The potential benefits of resveratrol were linked to an increase in the production of a peptide called insulin-like growth factor-I (IGF-I), which is reported to promote the growth of blood vessels and neurons in the hippocampus [57].

Laboratory models have also shown that resveratrol reduces oxidative stress in skeletal muscles during exercise [58] and disuse [59] and suppresses aging-associated decrements in physical performance [60] but does not attenuate sarcopenia [61].

A study revealed that resveratrol does not extend life-span in healthy mice or in a model of premature aging [62] but may delay or attenuate many age-related changes and prevent early mortality in obese animals [63].

An investigation on the effects of resveratrol on the insulin signaling pathway in the liver of obese mice showed that resveratrol restored the phosphorylation levels of proteins involved in the insulin signaling pathway, which were decreased by a high fat diet [64]. Further studies indicated that consumption of red wine containing 20 milligrams of resveratrol per liter improved cognitive function in mice. Japanese researchers, Harada et al. [57] postulated that the average concentration of resveratrol in red wine is 4.7 mg/L.

Resveratrol has been shown to restore spermatogenesis in cryptorchid mice [65]. Mice fed diets supplemented with resveratrol (7 mg/kg/day) for 12 months exhibited a larger follicle pool and number and quality of oocytes than those fed diet without resveratrol [66].

Studies by Hichem et al. [67], on ameliorative effects of resveratrol on lipopolysaccharide (LPS)-induced oxidative stress in rat liver showed that the supplement counteracted LPS-induced lipoperoxidation and depletion of SOD and catalase but slightly reduced that of GPx.

Previous work on suppressive effects of resveratrol on leucocyte count has also been reported in rats [68]. This may be due to the anti-inflammatory property of resveratrol.

2.2. Resveratrol effects in cats

A team of researchers studied the mechanisms of action of resveratrol using a cat model. They induced hepatotoxicity in the experimental cat using arsenic trioxide (As_2O_3). Their findings showed that pretreatment with resveratrol reversed changes in As_2O_3-induced morphological and liver parameters and resulted in a significant improvement in hepatic function. Resveratrol administration also improved the activities of antioxidant enzymes and attenuated As_2O_3-induced increases in reactive oxygen species and malondialdehyde production [69].

2.3. Studies of resveratrol in pigs

Resveratrol showed strong potential as antibiotic alternatives for reversing the adverse effects of weaning stress on growth performance, immunity and digestibility of nutrients and fecal microbial shedding of weaned piglets [70].

Previous work on suppressive effects of resveratrol on leucocyte count has been reported in pigs [71]. This indicates the anti-inflammatory effects of resveratrol.

Will Block [72], reported the inhibitory effect of *Polygonum cuspidatum* and its active components, resveratrol and emodin. They were found to preferentially inhibit the replication of H1N1 swine flu virus.

An *in-vitro* dose-dependent antiviral effect of resveratrol and oxyresveratrol (extracted from mulberry twigs) showed the antiviral activities of these compounds on African swine fever virus (ASFV). Oxyresveratrol differs from resveratrol because it has an extra hydroxyl group, which enhances its antioxidant activity. The antiviral effect of these two compounds achieved a 98–100% reduction in viral titers of ASFV. The compounds allowed early protein synthesis but inhibited viral DNA replication, late viral protein synthesis and factory formation. Resveratrol and oxyresveratrol were therefore postulated to be potential tools for the treatment or prevention of ASFV infection [73].

Fu et al. [74] suggested that resveratrol dry suspension (RDS) could be considered as an adjuvant to enhance immune responses to vaccines and dietary additives for animals to boost humoral and cellular immunity. In their study on immune function in piglets fed different doses of RDS for 2 weeks, they observed significant effects on the development, maturation, proliferation, and transformation of T lymphocytes. The result also showed upregulation and the release of interferon gamma (IFN-γ), downregulation of the release of TNF-α and high resistance to improve total superoxide dismutase (T-SOD) activity. Vaccination of the piglets against classical swine fever virus and foot-and-mouth disease virus as well produced significantly increase in antibody titers after supplementation of RDS.

Cui et al. [75] studied pretreatment with resveratrol dry suspension via basal diet on diarrhea induced rotavirus (RV) infection in piglets for 3 weeks. They observed a decrease in diarrhea, reduction on TNF-α production and elevated IFN-γ level. These results indicated that resveratrol could be used to control RV infection.

A 7 week study to determine effects of red wine and vodka on swine showed that the subjects that were given wine or vodka had significantly increased blood flow to the heart, although the red wine had the larger cardiovascular benefit [76].

2.4. Resveratrol studies in horses

Studies have evaluated the effects of resveratrol in horses [77, 78]. Report by Kohnen et al. [77] showed the inhibitory effect of resveratrol on equine neutrophil myeloperoxidase, while resveratrol treatment (1 g/d) in 20 old horses for 4 weeks decreased equine inflammatory cytokine production both *in vitro* and *in vivo* [45]. The compound has significant potential as a therapeutic agent in the management of acute and chronic inflammatory conditions in horses [45]. Trainers and horse owners have observed an improvement in health, comfort and performance in horses receiving resveratrol therapy. Refs. [79, 80] reported that resveratrol reduces gene expression of inflammatory mediators to allow horses move comfortably during aging, training and competition.

Daily resveratrol administration improves energy metabolism through its effects on mitochondria, the body's cellular power house [45].

Studies by Ememe et al. [82], showed a significant reduction in values of creatine kinase and glucose in the horses administered resveratrol and hyaluronic acid (equithrive joint®) (**Figure 3**) supplement. Elevated levels of these substances have been associated with a reduction in metabolic efficiency in aging animals. Hence, administration of equithrive joint® may help to reduce the harmful effects of these biochemical parameters during aging in horses. Also a study on horses exhibiting hind-limb lameness and poor performance was carried out with equithrive joint®. The researchers injected each horse's lower jock joints with triamcinolone before supplementing with equithrive joint® for 4 months [83]. The result showed higher percentage of riders who reported better performance of their horses. Ememe et al. [84] also reported that administration of equithrive joint to aged and lame horses decreased the serum MDA concentration and modulated the serum content of GPx, catalase, and SOD. The results suggested a potential protective effect of equithrive joint against oxidative stress and aging in

Figure 3. Equithrive®: a horse supplement, containing resveratrol and hyaluronic acid [81].

horses. Siard et al. [85] suggested that polyphenol supplementation such as resveratrol could decrease the amount of nonsteroidal anti-inflammatory drugs given to older horses, thereby reducing the side effects of such drugs.

2.5. Studies in dogs

Japanese Knotweed is the best source of resveratrol for dogs. An is an unproved evidence regarding the use of Japanese knotweed and lyme disease in dogs. Herbalist Stephen Buhner recommends Japanese knotweed in his book, Healing Lyme. He suggested that it is the only herbal treatment that blocks the bacterial phyla, spirochetes, which lead to Lyme disease and other infections like *Bartonella* [86].

Ref. [87] produced Resvantage Canine® (**Figure 4**) which contains resveratrol blended together with a unique combination of nutrients. It is alleged that the supplement maintains longevity by providing powerful support for pet's health needs (**Figure 5**).

A small dosage of resveratrol in the range of five to seven milligrams per 30 pounds of body weight daily has been reported to increase energy levels and alertness in dogs [86].

A research conducted on five known natural chemopreventive agents namely, resveratrol, ellagic acid, curcumin, genistein and quercetin over a period of 3 weeks indicated that the supplementation significantly decreased H_2O_2-inducible DNA damage [88].

2.6. Studies in cattle

The meat of cows that drank a liter of homemade local wine was found to have a wonderful texture and impressive aroma and flavor. Researchers at Thompson Rivers University in British Columbia are studying the effects of wine on cows. The study is to demonstrate the

Figure 4. Resvantage Canine®: a resveratrol supplement used to improve health needs of dogs.

Figure 5. An alert and healthy dog on resveratrol supplementation [86].

effects of wine diet on cow's methane production and possible health benefits of resveratrol in wine [89]. Salzano et al. [90] showed that resveratrol addition to bovine culture medium enhanced the fertility rate, cell numbers, blastocyst development and embryo cryotolerance.

A study on pretreatment of cultured bovine mammary epithelial cells (MAC-T) with resveratrol prevented decrease in cell viability and resulted in lower intracellular reactive oxygen species (ROS) accumulation after H_2O_2 exposure. The study showed that resveratrol could potentially be used as a therapeutic medicine against oxidative stress in lactating animals [91].

2.7. Effects of resveratrol in birds

In a recent study, 42-day-old female blackboned chickens were exposed to heat stress at 37 ± 2°C for 15 days after dietary supplementation of resveratrol at 0, 200, 400, or 600 mg/kg. The performance, immune organ growth index, serum parameters, and expression levels of heat shock protein in the bursa of Fabricius, thymus, and spleen were observed after supplementation. The result showed that administration of resveratrol improved growth performance and reduced oxidative stress biomarkers in the chickens by increasing serum growth hormone concentrations and modulating the expression of heat shock genes in organs of the immune system [38]. Sridhar et al. [92] advised on the use of resveratrol as a feed additive to control aflatoxicosis in poultry farms. In the new study at the National Institute of Animal Nutrition and Physiology in Bangalore, India, 0.5% or 1% resveratrol was administered for 42 days to broilers on aflatoxin-induced toxicity. It was observed that activities of the oxidative enzymes were increased and plasma total antioxidant capacity and total protein improved [93]. They also observed that the severity and degree of the liver lesions were decreased in supplemented birds (**Figure 6**).

Figure 6. Resveratrol administered as a feed additive to control aflatoxicosis in broilers [92].

Xu et al. [94] observed that resveratrol (3.85 μg mL^{-1}) supplementation decreased duck enteritis virus multiplication by 50%. This may be due to the inhibition of viral proliferation in the host cell.

Sahin et al. [95] studied the Effects of dietary resveratrol supplementation on egg production and antioxidant status in quail (*Coturnix coturnix japonica*). They found out that addition of resveratrol at 400 mg/kg into quail diets improved the antioxidant status of birds and eggs.

Supplementation of dried grape pomace to 96 molted 80-week-old *Bovans* laying hens led to the reduction in plasma and egg yolk MDA, and serum glucose levels by 4 and 6% [96]. It was opined that grape pomace supplementation has the potential to extend shelf life. Grape pomace is produced as a by-product during the production of molasses, grape juice, vinegar, dried fruit pulp and wine [97]. The polyphenol content of the grape pomace and seed include some flavonoids such as catechin, epicatechin, procyanidin, and antocyanidin; some phenolic acids such as gallic and ellagic acid, and some stilbenes such as resveratrol and piseid [98].

Supplementation with resveratrol (200, 400 or 800 mg kg^{-1} of diet) to chicks produced the highest values of body weight gain, IgM, thymus weight, cell proliferation index, antibody titers against avian influenza viruses H5 and H9 and Newcastle disease virus. It also enhanced growth hormone receptor gene mRNA expression and insulin-like growth factor-1 than those fed control diet during the study period [99].

3. Conclusion

This review illustrated the useful effects of supplementation of resveratrol in animals. The benefits highlighted consist of protective effects on cardiovascular system, treatment of various cancers, prevention of neurodegenerative diseases, suppression of age related decrements

in physical performance and improvement in cognitive functions. Others included suppressive effects on inflammatory factors, therapeutic medicine against oxidative stress, antibiotic alternative, decrease in viral replication and enhancement of immune responses. In view of the many benefits of resveratrol supplementation in animals, it may be considered as an aid to maintain longevity and increase in production in animals.

4. Recommendation

It is recommended that resveratrol should be included as a feed additive for dogs, cats, horses, pigs and cattle to improve immunity, reduce risk of various diseases and enhance productive performance.

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Section 2

Bioavailability, Metabolism, and Mode of Action of Resveratrol

Chapter 2

Novel Drug Delivery Systems of Resveratrol to Bioavailability and Therapeutic Effects

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Additional information is available at the end of the chapter

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Abstract

Resveratrol is a naturally occurring product used in the prevention and treatment of various diseases by acting as a potent defensive antioxidant. Resveratrol can be used in various fields, but the use is limited due to its poor solubility and hence low bioavailability. For overcoming this limitation, various drug delivery systems of resveratrol were developed. The aim of the novel drug delivery system (NDDS) is to provide a therapeutic amount of drug to the target site to maintain the desired drug concentration. NDDS enhances the duration of therapeutic activity, increases plasma half-life, decreases the immunogenicity, increases the stability of biopharmaceuticals, improves the solubility of low molecular weight drugs so does the bioavailability, and has a potential of targeted drug delivery. However, they have their own advantages as well as limitations. This chapter focuses on: (1) general introduction to resveratrol and its various therapeutic uses, (2) pharmacokinetic- and bioavailability-related problems of resveratrol, and (3) general about various NDDS used in resveratrol formulations.

Keywords: microparticulate systems, cyclodextrin complex, solid lipid nanoparticles (SLNs), vesicular systems and polymeric micelles

1. Introduction

Natural products have been used in the prevention and treatment of diseases throughout history due to their wide acceptability. Among the various groups of natural products and plant metabolites that are available, resveratrol plays an important role in the treatment of various diseases by acting as a potent defensive antioxidant [1]. Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is

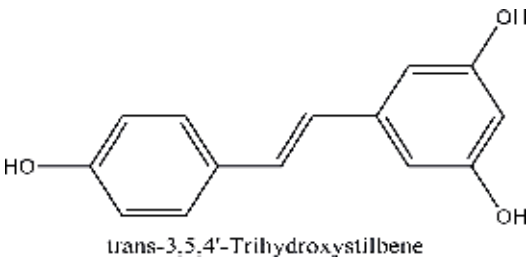

Figure 1. Chemical structure of resveratrol.

a natural phenol and a phytoalexin produced by several plants in response to injury by numerous plants in response to damage. The molecular formula is $C_{14}H_{12}O_3$ and molecular weight is MW 228.25 (**Figure 1**) [2]. Resveratrol is present in plants of families including Gnetaceae, Vitaceae, Cyperaceae, Dipterocarpaceae, and Leguminosae. A variety of food and food products such as grapes, wine, grape juice, cranberries, mulberries, cranberry juice, and peanuts contain resveratrol [3]. Resveratrol is a member of the stilbene family [4], characterized by two benzene rings linked through isopropyl moiety separated by a double bond [5]. Resveratrol plays a role in defense mechanism against various fungal, bacterial infections, viral, and damage from exposure to ultraviolet radiation (UV), which is very well depicted in **Table 1** [6]. Resveratrol also exhibits various therapeutic effects such as antiaging, neuroprotector, antioxidant, cardio protector, etc. The method of drug delivery has significant consequences over the efficacy of the constituent [7]. Drug delivery systems (DDSs) emerged as a new strategy based on interdisciplinary approach combining polymer science, bioconjugate chemistry, pharmaceutics, and molecular biology. DDS increases drug bioavailability and the fraction of

Sr. no	Role of resveratrol	Mechanism of action	Refs
1	Cardioprotective	Inhibition of transforming growth factor (TGF-β1) and atrial natriuretic peptide (ANP), increase in nuclear factor (NF-kB) activity	[13, 14]
2	Antioxidant	Neutralization and inhibition of reactive oxygen species (ROS), lipid peroxidation inhibition, and metal cations chelation	[10, 11]
3	Immunomodulatory	Increase in CD4/CD8 ratio, T lymphocytes proliferation, and B cell-mediated immune response, promotion of humoral immune response and improvement in the formation of antibody cells	[14]
4	Antihypertensive	Inhibition of vascular nicotinamide adenine dinucleotide phosphate (NADPH) oxidase induction and inhibition of COX-2	[20]
5	Anticancer	Suppression of protein kinase and suppression of NF-kB activation, suppression of protein kinase and suppression, induction of apoptosis, cell cycle arrest and suppression of growth factor receptor (GFR)-mediated pathway	[16]
6	Anti-inflammatory	Inhibition of NF-kB and nitric oxide synthases (NOS) expression and also reduction in proinflammatory IL-1β, cytokines, TNF-α, and COX-2	[20–22]

Table 1. Mechanism of action of resveratrol for different activities.

the drug accumulated in the required zone, and minimizes drug degradation, loss and harmful side effects. A variety of DDS are also available for resveratrol for modulating its delivery; for example, cyclodextrin complex; microparticulate systems; nanocarrier systems such as nanosuspension and solid lipid nanoparticles; and vesicular system such as niosomes, liposomes, transferosomes and ethosomes, and nanosponges.

2. Medicinal uses

Plants containing resveratrol have been widely used as a potent antioxidant [8] and also useful in the treatment of various disease [9] (**Figure 2**).

2.1. Antioxidant activity

Oxidative stress caused due to free radicals generated through endogenous and exogenous sources plays a significant role in the pathogenesis of a disease [10]. Resveratrol (RSV) also suppresses the action of the lipopolysaccharide, an inducible form of nitric oxide (NO) synthase, through the vascular endothelium, established by a reduction in nitric oxide production, also inhibiting the oxidation of the low-density lipoprotein (LDL) through the *in vivo* chelation of copper, therefore suggesting a potential role in the prevention of atherosclerosis and coronary heart disease [11]. The first antioxidant activity was reported by Frankel et al. of transresveratrol using different antioxidant assays including lipid peroxidation assay, 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay, total antioxidant activity, hydrogen peroxide scavenging activity assay, and reducing power assay. The results show that resveratrol has 2 μmol higher scavenging activity for diphenylpicrylhydrazyl (DPPH) radical as compared to the control group [12].

2.2. Cardioprotective activity

Various scientific studies reveal that utilization of plant extracts containing polyphenols, especially resveratrol, and red wine decreases mortality of coronary heart disease [13]. Due to its potent antioxidant, resveratrol, showing an overall good cardioprotective effect by inhibiting the oxidation of low-density lipoprotein (LDL), promotes vasodilation, reduces platelet aggregation, and enhances endothelial nitric oxide synthase activity [1].

Figure 2. Medical benefits of resveratrol.

Resveratrol was also showing potential effect on protecting cardiac and vascular tissues by reducing the accumulation of reactive oxygen species (ROS) in renal hypertensive rats [14].

2.3. Immunomodulatory activity

Resveratrol was found to increase B cell-mediated immune response, and also increases CD4/CD8 ratio and T cell proliferation. Li et al. demonstrated immunomodulatory effect on mice with lymphocytic leukemia [15].

2.4. Anticancer activity

Cancer is a chronic disease with a high death rate. Carcinogenesis involves in accumulation of cancer-regulating genes [16]. Various studies suggested that fruit- and vegetable-rich diet leads to reduced incidence of cancer due to presence of polyphenolic compounds [17]. Subramanian et al. showed the antiproliferative, proapoptotic along with antiangiogenic activity of resveratrol by performing different *in vitro* studies on wide tumor cell lines [18]. Similarly, Hu et al. discussed treatment of hepatocellular carcinoma with different compounds such as curcumin, tanshinone II-A, quercetin, berberine, silibinin, resveratrol, and celastrol derived from Chinese herbal medicines [19].

2.5. Antihypertensive activity

Endothelium-dependent vasorelaxation improved using resveratrol. Researchers' findings show that early treatment with resveratrol preserves endothelial function, decreases oxidative stress and superoxide dismutase activity, and gradually lowers the incidence of hypertension with the reduction in hydrogen peroxide levels [20].

2.6. Anti-inflammatory and vasorelaxing activity

Oxidative and inflammatory responses attenuate by polyphenols in various cells [21]. Activatation of microglia causes secreation of neurotoxic and proinfammatory mediators after brain injury. Zhang et al. studied the effect of resveratrol in inhibiting microglia [22]. Resveratrol was also found to decrease the synthesis of prostaglandin (PGE_2) by inhibiting cyclooxygenase (COX-2) enzyme activity, thus useful in inflammation [23]. Anti-inflammatory activity of resveratrol in turbot (a fish species) in vertebrates carried out by Leiro et al. The results showed the drug-dependent inhibition of messenger ribonucleic acid (mRNA) production, and increased tumor necrosis factor (TNF-α) and interleukins (IL-1β) pre-mRNA levels, thus proving its anti-inflammatory activity in vertebrates [24]. A vasorelaxation effect was observed by the activity of nitrous oxide (NO) by Zenebe et al. in red wine polyphenols on rat femoral artery [25]. Resveratrol is a multipurpose compound found in wine polyphenol, that improves the endothelial function with NO and vasorelaxation effect [26].

2.7. Antimicrobial activity

Stilbenes have been studied broadly for their antimicrobial activities [27]. Paolillo et al. studied resveratrol using cells line (U937) and observed time and dose-dependent reduction in

NO level [28]. Another antibacterial study given by Paulo et al. has determined zone of inhibition and minimum inhibitory concentration (MIC) using different strains of gram-negative spiral-shaped bacillus of Helicobacter, *H. pylori*. Most of the strains show similar susceptibility patterns, and MIC was observed in range 25–100 μg/ml. The result shows that action of resveratrol in urease inhibition was found to be higher (90%), as compared to the positive control strains (<75%). Thus, the research confirmed that the antibacterial activity of resveratrol is due to urease inhibition [29].

2.8. Antidiabetic activity

Diabetes mellitus is a complex metabolic disease characterized by high concentration of blood glucose level and increased insulin resistance [30]. Su et al. carried out resveratrol treatment study on streptozotocin-induced diabetic rats, the results decrease in triglyceride concentration by 50.2 ± 3.2%, and plasma glucose level by 25.3 ± 4.2% was obtained on the fourteenth day after treatment. The drug also verifies a stimulatory effect on the glucose absorption by numerous cells including hepatocytes and adipocytes [31].

2.9. Radioprotective activity

Radiation exposure is a major problem of destruction of a living cell, which causes free radical generation and ROS activation. Different polyphenols have been studied for their radioprotective activities [32]. Aziz et al. studied that ultraviolet-B radiation causing skin damage of mouse was prevented using resveratrol. Topical use of 10 μmol solution of resveratrol in 200 μl acetone results in the inhibition of ornithine decarboxylase, increase in cellular proliferation, and protein levels of epidermal COX-2 were observed, thus confirming the radioprotective activity, which was mediated by programmed cell death and elimination of damaged cells [33].

2.10. Neurodegenerative diseases

2.10.1. Parkinson's disease

Neurodegeneration is associated with various factors such as gene alteration, inflammation, oxidative stress, and mitochondrial dysfunction [34]. Okawara et al. observed resveratrol in mid-brain segment culture that shows a protective role of Wistar rats on dopaminergic neurons. Cytotoxicity of 1-methyl-4-phenyl pyridinium (MPP^+) causes neurodegeneration, which was formed from the transformation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Decrease in numbers of viable dopaminergic neurons was observed when segmented culture was applied with 30 μmol of MPP^+, but when applied with resveratrol, dose-dependent protection of neurons, which was due to antioxidative activity [35].

3. Bioavailability and pharmacokinetic studies of resveratrol

The absorption, distribution, metabolism, and excretion (ADME) and other pharmacokinetic parameters of resveratrol are well implicated. Instead of high oral absorption (~75%). The oral

bioavailability is less than 1% because of extensive and fast metabolism in intestine and liver leading into sulfate and glucuronide metabolites [36]. Almeida et al. determined the pharmacokinetic and safety contour of resveratrol. Four groups of five males and five females each were taken. Any two random subjects of each group were administered with placebo, while the remaining eight were administered with 25, 50, 100, and 150 mg of resveratrol, six times daily for 13 doses. Several pharmacokinetic parameters were determined out of which the peak plasma concentration (C_{max}) was found to be 0.8–1.5 h, whereas the mean C_{max} and mean area under the curve (AUC_{0-t}) were found to be dose dependent and found to be increasing with increase in dose from 25 to 150 mg [37].

4. Solubility and bioavailability enhancement of resveratrol

The reported aqueous solubility of resveratrol is <1 mg/ml, which is a key shortcoming of the drug [38]. The metabolic studies of resveratrol showed an increase in bioavailability when aglycone form of resveratrol was administered in solution of hydroxypropyl b-cyclodextrin [39]. Different approaches such as formulation of microparticles to reduce size to micron level [40], complexation with cyclodextrins [41], formulation of various vesicular systems such as liposomes [42], niosomes [43, 52], transfersomes and ethosomes [44], formulation of different nanocarrier systems for example nanostructured lipid carriers (NLCs), solid lipid nanoparticles (SLN) [45], nanosuspension [46], and nanocapsules [47] have been implied to improve different properties of resveratrol such as bioavailability, solubility, photo-sensitivity, and stability.

5. Drug delivery systems

The aim of novel drug delivery system (NDDS) is to provide a therapeutic amount of drug to the target site to maintain the desired drug concentration. NDDS enhances duration of therapeutic activity, increases plasma half-life, decreases the immunogenicity, increases the stability of biopharmaceuticals, improves the solubility of low molecular weight drugs so does the bioavailability, and has a potential of targeted drug delivery. Various NDDS given in **Table 2** can be explained as follows:

5.1. Microparticulate systems

These systems act as the carrier vehicle system for solid or liquid microparticles surrounded by polymeric layer ranging from 0.1 to 200 μm [48] in diameter (**Figure 3**), the type of polymer present in the layer improves bioavailability and controls the release of the drug [49]. Nam et al. improved the stability and antioxidant activity of the resveratrol possessing cyano-groups by formulating its porous polymeric microspheres by dispersion polymerization. Antioxidant property was characterized by DPPH radical scavenging activity [50]. Das et al. formulated microparticles of resveratrol and pectin utilizing glutaraldehyde as hardening agent and zinc ions as crosslinking agent, as resveratrol has potential therapeutic activity

Sr. no.	Drug delivery systems	Method of formulation	Model used	Observations	Refs
1	Microparticulate systems	Microparticles prepared by using resveratrol and pectin, containing glutaraldehyde as hardening agent and zinc ions as cross-linking agent for pectin	*In vitro* and *in vivo* drug release. Plasma appearance (C_p) of drug was delayed for 4–5 h in direct administration into stomach	Result shows enhancement of antioxidant activity and improved stability of resveratrol in microparticulate system as compared to that of pure resveratrol	[54, 56]
2	Microcapsules	Microparticles of resveratrol were prepared by microencapsulation using yeast cells	*In vitro*	Yeast-encapsulated resveratrol formulation shows increased stability, solubility, bioavailability and sustained-release profile	[6]
3	Cyclodextrin complex	Inclusion complexes prepared using combination of resveratrol with β-cyclodextrins and hydroxyl-β-cyclodextrins	*In vitro*	Enhanced solubility, antioxidant activity, and thermal stability	[59, 60]
4	Solid lipid nanoparticles	Lipid nanoparticles of resveratrol were prepared by using modified hot homogenization technique	*In vitro*	Results show increased oral bioavailability of poorly soluble compounds	[42]
5	Nanosuspension	Nanosuspension of resveratrol was prepared by using high-pressure homogenization technique	*In vitro*	High solubility and dissolution velocity, thus increasing the bioavailability	[43]
6	Vesicular systems liposomes	Liposomes was prepared by using Phosphatidylcholine (PC) and resveratrol were dissolved in mixture solvents such as chloroform and methanol using rota evaporator. The liposome suspension was then added dropwise to chitosan solution with stirring and then solution left overnight at 4°C. Finally, chitosan-coated liposomes were harvested using centrifugation	*In vitro*	Increased skin-permeation efficiency with effective transdermal delivery system	[66]
7	Niosomes	Resveratrol-loaded niosomes were prepared using two stage techniques that include mechanical agitation followed by sonication, where two different surfactants, span 80 and span 60, were used	*In vitro*	Results show increased stability and improved the bioavailability	[66]

Sr. no.	Drug delivery systems	Method of formulation	Model used	Observations	Refs
8	Transfersome and ethosomes	Transfersomes and ethanol-containing vesicles were prepared by thin lipid film hydration method (rota evaporator)	*In vitro* and permeation studies (porcine skin carried out on Franz diffusion cells)	Findings show that nanocarriers were found to possess an encapsulation efficiency of >70% with a mean diameter of 83–116 nm	[67]
9	Nanosponges	Nanosponges containing resveratrol were prepared by solubilizing the reagents in dimethyl sulfoxide (DMSO) at 100°C for 4 h	*In vitro* and permeation studies (pigskin)	Results increase the stability, cytotoxicity, and permeation of resveratrol. Decrease in crystallinity of resveratrol was observed after encapsulation	[65]
10	Microspheres	Microspheres of resveratrol (Res) using chitosan were prepared by emulsion chemical crosslinking method, and vanillin was used as the new crosslinker	Higuchi was the most suitable model for the controlled release profile	Microspheres of resveratrol were protected from light and heat as compared to free Res, and increase in stabilization of Res was achieved through crosslinking with vanillin	[67]

Table 2. Novel drug delivery systems for resveratrol (RSV) delivery.

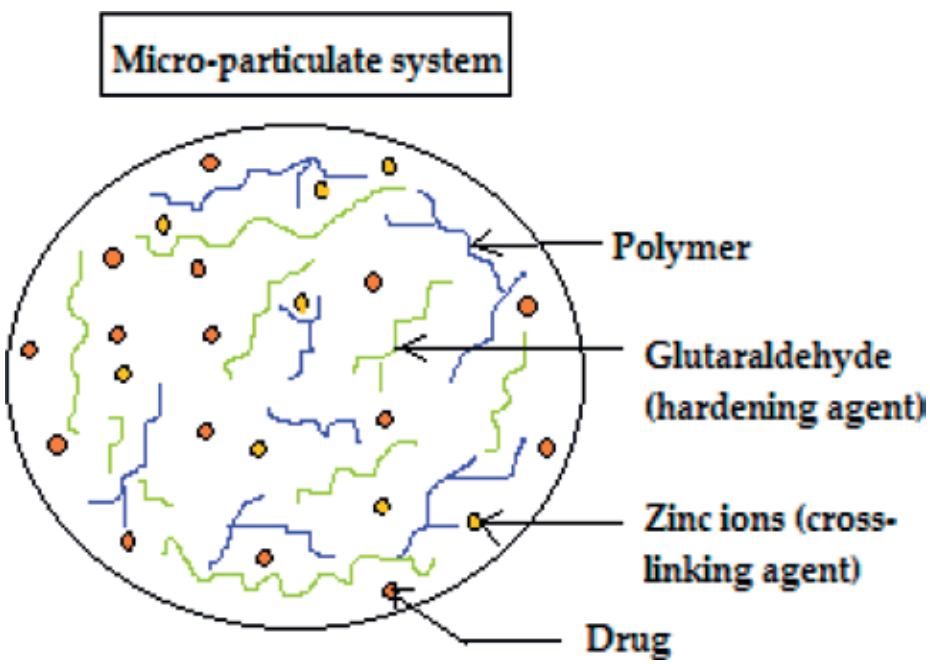

Figure 3. Microparticulate system.

on colitis and colon cancer, and was used as a model drug in the prepared formulation. By fluctuating the formulation variables, a variety of microparticles were prepared, which were investigated on different shape and size of the microparticles, weight loss during drying, moisture content, encapsulation efficiency, drug release pattern from the microparticles, and swelling-erosion ratio. The formed microparticles were spherical with less than 1 mm diameter and with greater than 94% encapsulation efficiencies. Colon-specific *in vivo* and *in vitro* drug release was shown by glutaraldehyde-modified microparticles (GMM) formulated at optimized conditions. GMM delayed the plasma appearance of the drug for 4–5 h after their administration straightaway into stomach, but the AUC was comparable to other control formulations of the experiment; this indicated the potential of the developed microparticle formulation of the resveratrol as a colon-specific drug delivery system [51].

5.2. Microcapsules

Microencapsulation is a technique in which tiny particles are enclosed by a coating to give small capsules (**Figure 4**). Microencapsulation can also be used to enclose solids, liquids, or gases surrounded by a hard or soft soluble film, in order to decrease dosing frequency and avoid the degradation of pharmaceuticals. Encapsulation of resveratrol was done by the yeast cell, which was characterized by FT-IR spectra, fluorescence and confocal micrographs of the microcapsules, yeast cells, and resveratrol. The release of the drug was characterized in simulated gastric fluid (SGF), and storage stability was determined by taking powder sample of the formed microcapsules at 25°C at 75% RH, 25°C at 90% RH, and 60°C under the dark or laboratory fluorescent lighting conditions. It was found that the encapsulated resveratrol possess high scavenging capacity on DPPH radical as compared to nonencapsulated resveratrol. The encapsulation does not cause any chemical change in the active moiety. Besides, the encapsulation enhances the DPPH radical-scavenging activity, stability, sustained-release and also solubility of resveratrol and so it's bioavailability [52].

5.3. Cyclodextrin complex

Cyclodextrins are cyclic oligosaccharides formed during bacterial digestion of cellulose. They are also known as cycloamyloses, cyclomaltoses, and Schrodinger dextrins. These complexes have a lipophilic core with hydrophilic outer surface and hence capable to form inclusion

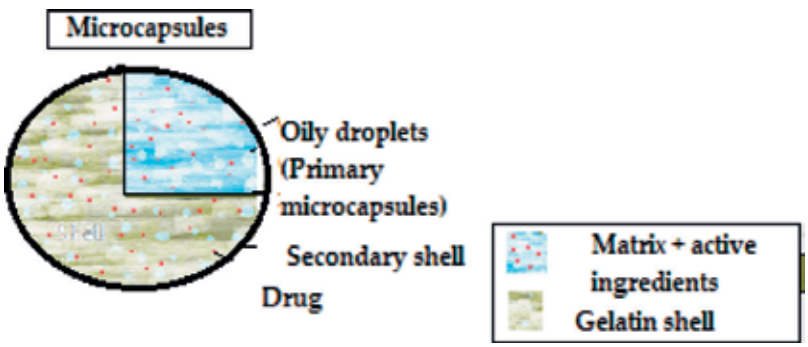

Figure 4. Microcapsules.

complexes and holding lipophilic drugs to their inner cavity (**Figure 5**). Thus, they improve solubility and so does the bioavailability of the poorly soluble drugs. These can be divided into α, β, and γ subtypes on the basis of presence of six, seven, or eight glucopyranose units, respectively. Cyclodextrins protect the drug from oxidation so act as a controlled dosage reservoir [53, 54]. Resveratrol is encapsulated in different native and modified cyclodextrins to form different inclusion complexes to enhance its poor aqueous solubility [55]. Lu et al. observed enhancement in aqueous solubility and antioxidant property of resveratrol by forming inclusion complex of resveratrol with β-cyclodextrins and hydroxyl-β-cyclodextrins. The drug and cyclodextrin interactions were evaluated in the solution for stoichiometry and stability constant by phase solubility analysis. Results proved the increased limited water solubility of resveratrol-cyclodextrins inclusion complexes. Resveratrol/hydroxy-β-cyclodextrin complex also exhibited superior antioxidant potential compared to β-cyclodextrin complex [56].

5.4. Nanocarrier systems

5.4.1. Solid lipid nanoparticles (SLNs)

SLNs are formed by a solid lipid matrix medium, which is stabilized by surfactants in the aqueous media, with potential to enhance drug bioavailability. The diameter ranges between 50 and 1000 nm. SLNs possess various advantages such as target-specific delivery, photo, and acid stability for sensitive drugs (**Figure 6**). SLNs elegantly control drug distribution at cellular, or even at subcellular level [57]. Teskac et al. prepared SLNs of resveratrol by melt-emulsification process and investigated cellular uptake, transport, and internalization of the drug in keratinocytes. Hydrodynamic diameter of loaded SLNs was calculated by photon correlation spectroscopy that was found to be 180 ± 8 nm. This confirmed the upgraded cellular uptake and cellular density of resveratrol by loaded SLNs. As SLNs were observed to concentrate around nuclei, it confirms sustained-release of drug, thus improving the bioavailability and stability [58]. A comparison is made between resveratrol nanostructured lipid carriers (NLCs) and SLNs

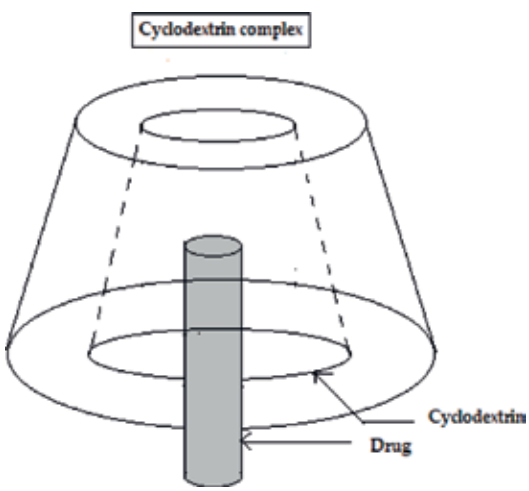

Figure 5. Structure of cyclodextrin complex.

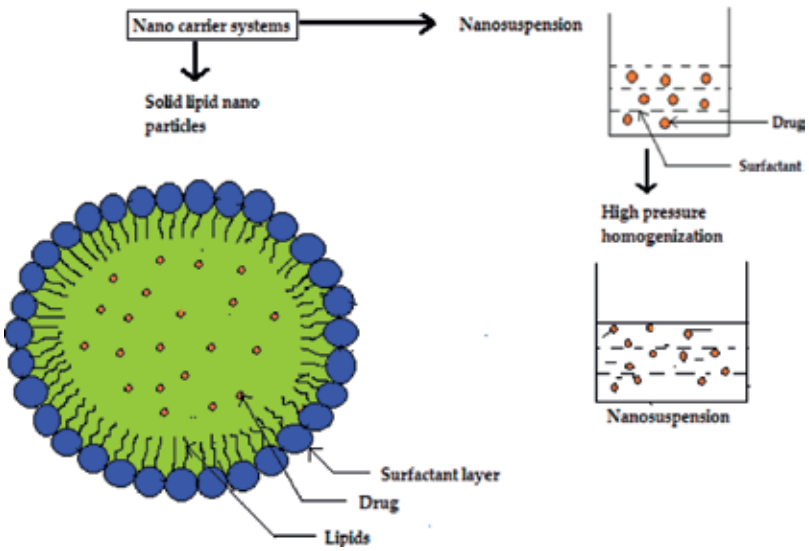

Figure 6. Structure of nanocarrier systems.

prepared by high shear homogenization using compritol 888ATO, myglyol, poloxamer 188, and tween 80. A potent antioxidant activity was observed for both formulations at a concentration of 50 μM, but resveratrol NLCs was observed to penetrate deeper into the skin [59].

Ana et al. developed lipid nanoparticles of resveratrol to increase the oral bioavailability. Modified hot homogenization technique was used to prepare resveratrol-loaded lipid nanoparticles (SLNs and NLCs). The encapsulation efficiency was found to be high for both SLNs and NLCs, with an average around 70%. The existence of solid state of SLNs and NLCs at both room (25°C) and body temperature (37°C) was confirmed by DSC studies. *In vitro* release studies performed in shelf storage conditions showed a negligible release of resveratrol over several hours for both systems, which concluded the high stability of both lipid nanoparticle systems [60].

5.4.2. Nanosuspension

These are the nanorange colloidal dispersions systems stabilized by surfactants, containing poorly water soluble drugs. These systems result in the improvement of bioavailability of the drug by improving its solubility. Kobierski et al. prepared nanosuspension of resveratrol in the range between 150 and 220 nm for dermal application using high-pressure homogenization technique. Nanosuspension showed high solubility and dissolution velocity for resveratrol and thus improved bioavailability [46].

5.5. Vesicular systems

Vesicular systems are the drug delivery systems composed of lipophilic polymeric core, and lipophobic tail or shell. These systems are capable of encapsulating both hydrophilic and

lipophilic drugs (**Figure 7**). They are shown to improve the bioavailability of the drug and thus enhancing duration of therapeutic activity. Examples of these systems are liposomes, niosomes, transfersome, phytosomes, ethosomes, etc. [61].

5.5.1. Liposomes

Liposomes are colloidal vesicular structures prepared from lipids capable of loading both lipophilic and hydrophilic drugs. The bioavailability of resveratrol was found to be improved by incorporating into liposomes [42]. Caddeo et al. formulated liposomes of resveratrol using phosphatidylcholine and oleic acid as penetration enhancers. Deep penetration of the drug into the skin was reported by these systems. The antioxidant potential of the resveratrol was also found to be unchanged by incorporating the drug into these systems [62]. Park et al. investigated chitosan-coated liposomes of resveratrol for improving transdermal delivery of the drug. The stability of the liposome was found to be increasing on chitosan coating, by preventing aggregation. Franz diffusion cells were used to investigate the transdermal delivery of uncoated and 0.1% chitosan-coated liposomes with 0.1% resveratrol. The proportions of resveratrol that permeated through the animal skin were found to be 40.42 and 30.84% for the coated and uncoated liposomes, respectively. This indicated the effective transdermal delivery of chitosan-coated liposomes for delaying skin aging using resveratrol [63].

5.5.2. Niosomes

Niosomes are nonionic surfactant vesicular systems comprised of aqueous inner core enclosed by nonionic surfactant membrane, thus forming a closed bilayer structure. These systems are capable of loading both hydrophilic and hydrophobic drugs. These are prepared by hydration of surfactants. Pando et al. formulated resveratrol encapsulated niosomes using two stage techniques, mechanical agitation followed by sonication by using two different surfactants, span 60 and span 80. The entrapment efficiency and particle size distribution of niosomes prepared from span 80 were found better as comparison to noisome prepared

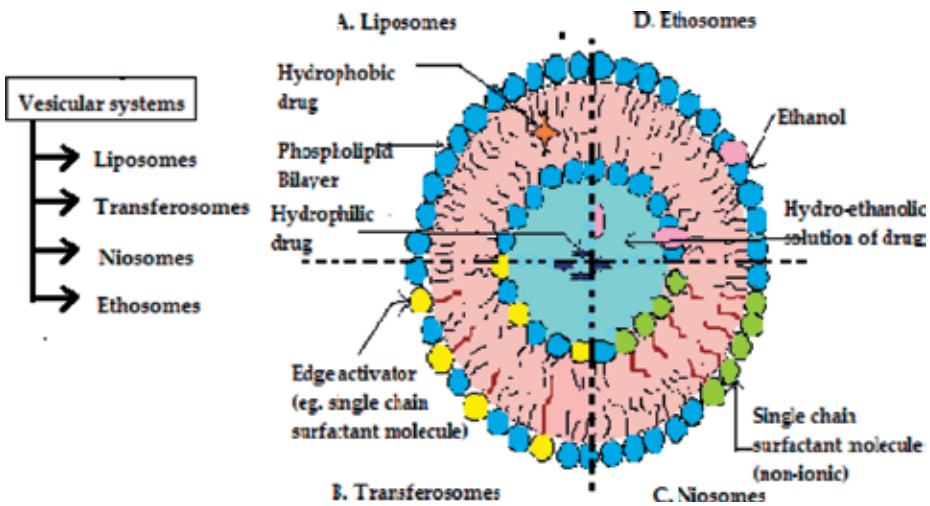

Figure 7. Vesicular systems.

from span 60, hence the bioavailability [43]. Poonam Negi et al. prepared niosome employing span 80 as surfactant by thin film hydration and ether injection methods at three different levels. Selection of best optimized formulation was done on the basis of entrapment efficiency (% EE), sedimentation volume, mean particle size, and microscopy. Optical microscopy and transmission electron microscope (TEM) confirmed the vesicular and spherical nature of the niosomes. Resveratrol entrapped niosomal gel was formulated by gelling in Carbopol 934. Ex vivo permeation studies confirmed better permeation and deposition of resveratrol in skin as compared to plain resveratrol [64].

5.5.3. Transfersomes and ethosomes

Transfersomes are ultraflexible lipid-based elastic vesicles, while ethosomes are vesicles containing phospholipids, ethanol, and water with the potential to penetrate into intact skin and delivering drug into systemic circulation through skin. Topical formulations of resveratrol as a potential drug were prepared as a vesicular system. The diameter of nanocarriers was found to possess a mean diameter of 83–116 nm with more than 70% encapsulation efficiency [44].

5.6. Nanosponges

Nanosponges are nanometric-range size particles with enclosed cavities, capable of encapsulating both lipophilic and hydrophilic drugs (**Figure 8**). These systems improve the solubility of poorly water soluble drugs and thus the bioavailability [65]. Ahmed et al. formulated

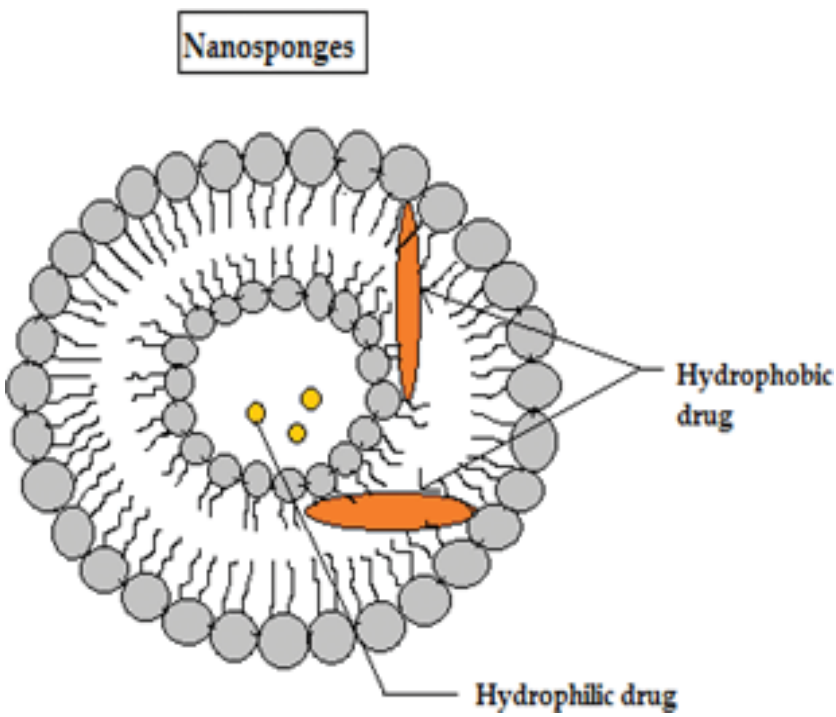

Figure 8. Nanosponges.

nanosponges of resveratrol by dissolving the reagents in dimethyl sulfoxide at 100°C for 4 h. Improvement of the stability and penetration of resveratrol was recorded by this system [66].

5.7. Microspheres

Microspheres are minute spheres comprising of porous/nonporous inner polymeric matrix with an outer, porous and regular to nonporous and irregular, polymeric surface (**Figure 9**). Diameter of these structures ranges from 1 to 500 μm. Peng et al. formulated microspheres by emulsion chemical crosslinking method using vanillin as a crosslinker. Stable chitosan-incorporated resveratrol microspheres were prepared. Encapsulation efficiency of resveratrol within microspheres was found to be 93.68%. Resveratrol encapsulated within microspheres was protected from light and heat compared to the free resveratrol [67].

5.8. Polymeric micelles

This system is made of amphipathic linear polymers formed spontaneously by self-assembly in water and a hydrophobic core in which the drug is encapsulated (**Figure 10**). The size of micelles varies from 20–100 nm, so that they do not cross the normal vessel walls, hence have a low volume of distribution and decrease the chances of side effects of the drugs. They carry various poorly soluble pharmaceutical agents due to their high *in vitro* and *in vivo* stability. They get accumulated in body areas with compromised vasculature and act as carrier systems for drug targeting as they can carry specific ligands on their surface. Katherine E. Washington formulated polymeric micelles of doxorubicin (DOX) and resveratrol (RSV), increasing loading of DOX. Co-loading of DOX and RSV in amphiphilic diblock copolymer micelles of poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG-*b*-PCL) and poly(ethylene glycol)-*b*-poly(γ-benzyl-ε-caprolactone) (PEG-*b*-PBCL) results in improvement of loading efficiency and bioavailability [68].

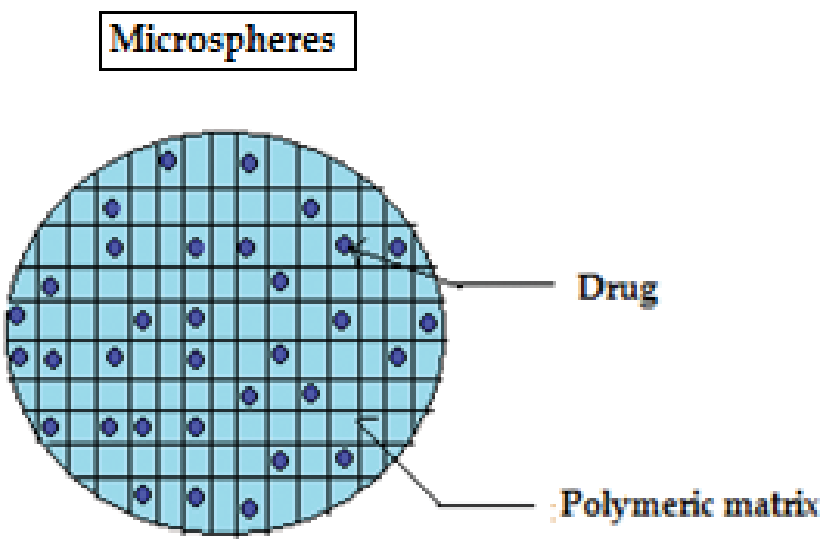

Figure 9. Microspheres.

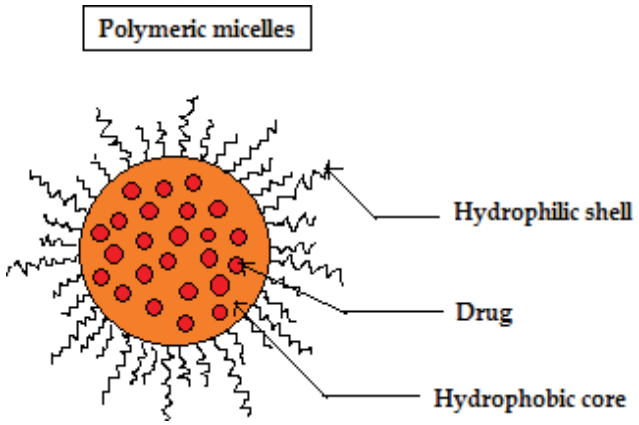

Figure 10. Polymeric micelles.

6. Conclusion

A wide variety of researches use resveratrol as a potential candidate for novel drug delivery. The present chapter provides detailed information about the novel drug delivery systems of resveratrol to bioavailability and therapeutic effects, issues related to drug delivery along with the different approaches utilized to improve the bioavailability of the drug. The novel drug delivery systems of resveratrol show immense benefits in therapeutic terms by various means, for example, by enhancing the solubility and thus the bioavailability, by increasing the half-life of the drug, by reducing the side effects and also focused on the target-specific delivery, etc. Various reports include microparticles formulation that shows colon-specific drug delivery; resveratrol is encapsulated in cyclodextrins to enhance its poor aqueous solubility, SLNs possess various advantages such as target-specific delivery, photo and acid stability, and nanosponges and nanosuspension systems results in the improvement of bioavailability of the drug by improving its solubility. The bioavailability of resveratrol was found to be improved by incorporating into liposomes and also improving transdermal delivery, vesicular systems such as transfersomes and ethosomes containing phospholipids, ethanol, and water with the potential to penetrate into intact skin and delivering drug into systemic circulation through skin; polymeric micelles has the capacity to hold drugs, which are poorly soluble in aqueous solution, results in enhanced bioavailability of resveratrol and also effective to target tumors, and stable chitosan incorporated resveratrol microspheres results controlled release and improved stabilization of resveratrol.

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Chapter 3

Protective Activity of Resveratrol in Cardio- and Cerebrovascular Diseases

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Additional information is available at the end of the chapter

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Abstract

Resveratrol (RSV) is a natural nonflavonoid polyphenol compound containing a stilbene structure similar to that of estrogen diethylstilbestrol. It is a fat-soluble compound existing in cis-, trans-, and piceid isomeric forms, isolated for the first time in 1940 from a plant used in traditional Chinese and Japanese medicine. Although initially used for cancer therapy, it has shown beneficial effects against most cardiovascular and cerebrovascular diseases. Its beneficial effects are mainly related to its antioxidant properties. Here, we review the metabolism and the ability of RSV to modulate redox signaling and to interact with multiple molecular targets of different intracellular pathways exerting protective effects against cardio-cerebrovascular diseases and metabolic disorders such as diabetes, reporting evidence in animal models and its efficacy and toxicity in humans. The aim of this chapter is to highlight the mechanisms, the biology, and the potential use of resveratrol to prevent, protect and aid cardio- and cerebrovascular diseases.

Keywords: resveratrol, cardiovascular diseases, cerebrovascular diseases, molecular mechanisms, clinical effects, nitric oxide, oxidative stress

1. Introduction

Both in the scientific world and in the public opinion, a particular attention is paid to cardiovascular diseases since they represent the first cause of mortality in the Western world. The major risk factors are represented by different factors such as hyperlipidemia, arterial hypertension, diabetes and obesity, and the common anatomopathological basis is atherosclerosis. Since the early 50s of the last century, the pioneering studies of the American scientist

Ancel Keys have highlighted the enormous potential of proper nutrition in the prevention of cardiovascular diseases (CVDs). Since then, the interest in food science has been growing and was corroborated by the discovery of new associations between healthy nutrition and protection against other cardiovascular diseases, such as diabetes, hypertension, atherosclerosis and myocardial infarction. A clear and concise testimony of the work of the American scientist provides it in a recent article that M. Mancini and J. Stamler, certainly two scholars who in their respective countries, Italy and the United States, have contributed most to spread and develop Keys' theorems [1]. The legacy of Keys is fundamentally contained in the seven countries study (SCS), which he initiated and coordinated, in which it has unequivocally demonstrated through the study of different populations that a high intake of saturated fats causes an increase in blood cholesterol and risk mortality for CVDs and that the level of blood cholesterol correlates with the risk of CVDs. Years later, Keys' insights, which were validated by epidemiological population studies, are now validated and investigated at the cellular and molecular level.

In the context of the recognized benefits deriving from "healthy eating," the chemical components that are largely responsible for the protective effects of the diet are gradually being highlighted. The importance of diet in the prevention of vascular diseases and dysfunction is highlighted by the observation that the incidence of certain diseases varies from country to country, where there are different eating habits. In vitro and in vivo studies have shown that some components of the diet, including vitamin E, vitamin C, retinoic acid, carotenoids such as lycopene (a powerful antioxidant in tomato) and polyphenols show a protective effect on the onset of CVDs [2].

On this regard, polyphenols are compounds that own one or more aromatic rings, with one or more hydroxyl groups and are generally classified as phenolic acids, flavonoids, stilbenes, coumarin, and tannins. Polyphenols are products of the secondary metabolism of plants, whose function is to protect the plant from the pathogenic attacks of parasites and also contribute to giving color to the plants [3]. Polyphenols have different structures, but all have aromatic rings, with one or more hydroxyl substituents; due to their structure, they are able to chelate metal ions and have the activity of scavengers of free radicals; they are also able to inhibit inflammation and platelet aggregation, thus exerting a protective action on the vascular system. Due to the acid character of the hydroxyl groups and the nucleophilic properties of the phenolic rings, the flavonoids are highly reactive and appear to have antiviral, antibacterial, immunostimulatory, anti-ischemic, antineoplastic, anti-inflammatory and gastroprotective properties. Flavonoids inhibit the activity of many enzymes including lipoxygenase, cyclooxygenase, monooxygenase, xanthine oxidase, NADPH-oxidase, phospholipase A2, some protein kinases and transcription factors such as NF-kB [4].

Among the flavonoids, resveratrol (3,5,4′-trihydroxystilbene) has attracted considerable attention from the scientific community. It belongs to the stilbenes family. The stilbenes (C6-C2-C6) are low molecular weight phenolic compounds, characterized by the presence of two aromatic rings joined by an ethane or an ethylene bridge. Resveratrol (RSV) (trans-3,4,5′-trihydroxystilbene) is a natural phytoalexin synthesized in response to fungal attacks, or to abiotic agents such as exposure to ultraviolet rays. Present in the peel of grapes and in

red wine, RSV has a wide variety of pharmacological properties [5]. Since its first isolation in 1940 by Takaoka, RSV has been associated with many properties [6]. Above all, the detection of RSV in wine has greatly contributed to finding the cardioprotective effects of this compound. This is testified by the so-called "French paradox," in which despite equal CVD risk factors, French population has a lower mortality rate compared to western countries [7]. This discovery about 25 years ago gave rise to an increasingly compelling urge to research all the mechanisms lying behind RSV and its beneficial effects [8]. This, unfortunately, gave also birth to a "red wine/RSV dogma," hence the concept that red wine benefits are due to its content in highly bioactive RSV. This concept might seem logic at first, as red wine is the main RSV dietary source; however, RSV content in wine is greatly variable and usually low, so its effects are mostly unpredictable and so its biological benefits are rather overestimated [7, 9–11]. Besides this noteworthy mention, consumption of red wine has been associated with beneficial effects on both the healthy and in patients with the previous acute coronary syndrome in terms of reduction in oxidative stress and endothelial function improvement [12, 13]. Other studies have shown that moderate consumption of red wine on man produces a reduction in risk factors for atherosclerosis. In red wine consumers, in fact, a reduction in platelet aggregation leads to an increase in plasma levels of HDL-cholesterol (HDL, high-density lipoprotein, responsible for the disposal of excess cholesterol in the peripheral tissues) and to a more low oxidation of low-density lipoprotein (LDL); these events are associated with a minor formation of atherosclerotic plaques in blood vessels and, therefore, a reduction in cardiovascular events, which makes RSV a cardioprotective agent [14, 15]. RSV, like many phytoalexins, has many biological activities—inhibition of lipid peroxidation and platelet aggregation, and alteration of lipid metabolism—possesses anti-inflammatory activity, is an inhibitor of the damage induced by free radicals and exerts an important vasorelaxant effects in different vascular districts.

2. Resveratrol and cardiovascular diseases: molecular mechanisms

A human takes with the diet small amounts of stilbenes, but one of the most represented is RSV. It is absorbed more in the duodenum; studies conducted on mice, using labeled RSV, have detected, already after 3 h from the administration, the presence of this molecule in the brain, heart, lungs, spleen and testicles, and after 6 h the stay in the liver and kidneys. In plasma, however, its concentration is very low and of short half-life. Trans-RSV has several beneficial effects and can act at different levels such as cellular signals, enzymatic processes, apoptosis and gene expression [16, 17]. Being lipophilic, RSV binds preferentially to HDL, LDL and VLDL lipoproteins, protecting them from oxidation from ionic metals and removing copper ions from both LDL and arterial walls. It is transported in the bloodstream bound mainly to LDL, both as an intact molecule and as its metabolites: trans-RSV-3-O-glucuronide, cis-RSV-3-O-glucuronide and cis-RSV-3-O-glucoside [18]. It has been shown that about 75% of trans-RSV taken with red wine is absorbed by passive diffusion and only <1% is bioavailable in the liver and in the intestine. Despite its very low concentration and of short half-life after assumption, RSV exerts several beneficial effects in different CVDs (**Figure 1**).

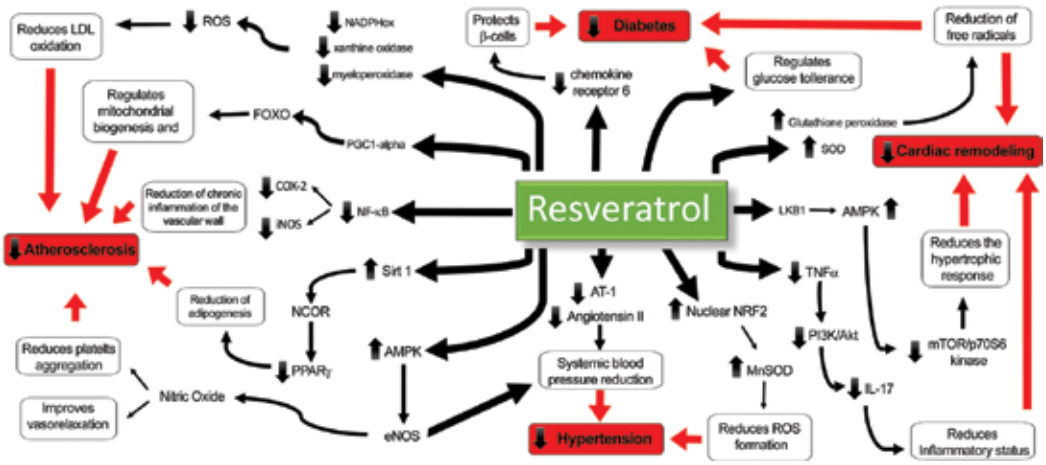

Figure 1. Schematic of molecular mechanisms of resveratrol in cardiovascular diseases.

2.1. Atherosclerosis

Atherosclerosis is a chronic inflammation of the vascular wall that results in the development of plaques and subsequent stenosis of the arteries [19]. A number of cytokines are involved in atherosclerosis-related inflammation; these include tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1). These factors induce the expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin adhesion molecules and lipid homeostasis [20, 21]. Other important cytokines responsible for the cross-talk phenomenon that occurs between inflammatory cells and intrinsic factor wall cells are IL-1β and platelet-derived growth factor cross-reactive material [19]. Inflammation associated with atherosclerosis is mediated via the nuclear factor κB (NF-κB) signaling pathway, implying that substances inhibiting or activating this factor exert an important role in atherogenesis [22].

RSV is able to interfere with the activation of NF-kB, a transcription factor that regulates the expression of various genes involved in inflammation, cell proliferation and carcinogenesis such as COX2 cyclooxygenase and nitric oxide synthase (iNOS). Nitric oxide synthase maintains high concentrations of nitric oxide molecule able to exert vasodilatory effects, to inhibit adhesion and platelet aggregation and to block the cell growth and migration. Recently, it has been shown that RSV is an activator of sirtuins [23]. The sirtuins are a class of NAD-dependent deacetylases, implicated in the transcriptional modulation of the silencing of genes of the aging and cell survival processes. A large number of sirtuins appear to be involved in promoting longevity in mammals. RSV activates human sirtuin 1 (SIRT 1), a homolog of silencing information regulator 2 (SIR 2) of yeast [24]. SIRT 1 is involved in a multiplicity of cellular and metabolic events with a pivotal role in many of them. For example, it mobilizes fats from adipose tissue blocking the activity of peroxisome proliferator activated receptor-gamma (PPAR-gamma) receptors through its interaction with the NCOR nuclear receptor corepressor. Furthermore, it represses the transcriptional activity of the nuclear factor NF-kB by deacetylating the p65 subunit. SIRT 1 also modulates mitochondrial cell metabolism thanks

to the deacetylation performed on the PPAR-gamma receptor coactivator, PGC1 alpha and cell survival in stress conditions thanks to interaction with FOXO proteins. Moreover, it has been demonstrated that RSV is able to induce NOS-3 in direct and indirect manners through the 5′adenosine monophosphate-activated protein kinase (AMPK), SIRT1 and nuclear factor erythroid 2-related factor two pathways, thus modulating the vessels homeostasis [25].

In ApoE −/−/LDLR−/− mice, the lack of apolipoprotein E (ApoE) or LDL receptor (LDLR), and the over-expression of apolipoprotein B (ApoB) gene, leads to an increase in VLDL and LDL, contributing to the promotion of atherosclerosis [26]. In vivo studies in genetically hypercholesterolemic mice (ApoE−/−/LDLR−/−) and treated with oral administration of RSV in association with a high fat diet content have shown that the polyphenol suppresses the formation of atheroma in the aorta and reduces the laser-induced thrombosis in the carotid arteries [27], thus demonstrating the positive effect of RSV. An important antioxidant action of RSV is the inhibition of the oxidation of LDL; this inhibition is protective because the oxidative modification of LDL is considered a primary event in the pathogenesis of atherosclerosis. In fact, several studies have reported that oxidized LDL (ox-LDL) can stimulate platelet aggregation [28] and promote a procoagulant activity on the surface of human monocytes/macrophages, increasing the thromboplastin activity in the tissue [29]. Various enzymatic systems, present in endothelial cells and macrophages, are implicated in the oxidation of LDL. These systems include NADPH-oxidase, hypoxanthine/xanthine oxidase, myeloperoxidase (MPO) and the enzyme nitric oxide synthase (NOS) [30–32]. The products of these enzymes oxidize LDL, which alter endothelial cells, stimulate NADPH-oxidase, release pro-inflammatory cytokines and inhibit the endothelial enzyme nitric oxide synthase (eNOS) involved in the vasorelaxing activity [33]. RSV has been shown to act on these ROS scavenger enzymes by inhibiting the COX-2 cyclooxygenase and thus the expression of the scavenger receptor (SR-A) and inducing the vasorelaxing activity of eNOS [34]. It is well known that vascular smooth muscle cells (VSMCs) contribute to the pathogenesis of atherosclerotic lesions since their migration and proliferation are critical events for the progressive thickening in the intimate and development of atheroma in the vascular wall [35]. Several studies have shown that RSV can inhibit the proliferation of VSMCs [36, 37], induced by different mitogens such as serum, endothelin and PGDF. The antiproliferative effect of RSV is not mediated by the induction of apoptosis, but appears to be produced by the blockade of the G1-S transition of the cell cycle [38, 39] and of the synthesis of DNA [37]. These results suggest that RSV can selectively counteract the pathological proliferation of VSMCs in arterial walls in vivo and thus could exert an important protective effect on the onset of atherosclerosis.

2.2. Hypertension

Hypertension is one of the most important risk factors for cardiovascular diseases, representing the main causes of death in developed countries. It involves from 30 to 45% of the general population, with a tendency to increase incidence from the age of 50 and with an increase in prevalence in the most disadvantaged social classes [40].

The endothelial dysfunction is a hallmark of hypertension and clearly contributes to the onset and progression of the disease. It is important to underline that several risk factors for cardiovascular diseases can be effectively countered by a proper diet and by the intake of nutraceuticals.

On this regard, it has been demonstrated that RSV increases the levels of the vasodilator NO, which protects against the high blood pressure levels and subsequent cardiac hypertrophy and decreases ET-1 and angiotensin II (AngII) concentrations, which are associated with higher hypertension [41, 42]. In several animal models of hypertension, chronic RSV administration reduces systemic blood pressure in different rat models of hypertension [42, 43], suggesting the important beneficial effects evoked by polyphenol. RSV has also been shown to prevent remodeling of the mesenteric artery wall of spontaneously hypertensive rats (SHR), which is also typically observed in hypertensive humans, and to limit the increase in compliance of SHR arteries [44].

In another animal model of metabolic syndrome, the increased systolic blood pressure and reduced aortic eNOS expression were significantly improved by long-term administration of RSV. In the visceral adipose tissue (VAT) of this rat type, RSV treatment lowered tumor necrosis factor-alpha (TNF-α) production and increased the concentration of adiponectin, which improved the inflammatory status [45]. The development of pulmonary hypertension is induced by the proliferation of pulmonary arterial smooth muscle cells, endothelial dysfunction, oxidative stress, and inflammation. In monocrotaline-treated rats, RSV attenuated right ventricular systolic pressure, increased expression of endothelial NO synthase, decreased oxidative stress, and improved endothelial function in small pulmonary arteries. In addition, RSV was able to decrease expression of inflammatory cytokines, such as (TNF-α) and interleukin 6 (IL-6), and to limit leukocyte infiltration in the lung [46]. RSV also inhibited proliferation of pulmonary arterial smooth muscle cells. The increased level of NO induced by RSV is due to the augmentation of eNOS expression and activity [47]. It has been proposed that these effects involve SIRT1, which has been shown to directly deacetylate eNOS [48] leading to the improvement of nitric oxide production. It is well known that endothelial cells are responsible for the synthesis of ET-1, which is a strong vasoconstricting factor. RSV potentially inhibits stress-induced ET-1 gene expression, ET-1 mRNA levels, and ET-1 promoter activity by interfering with the ERK 1/2 pathway improving endothelial function through the decrease of ET-1 levels [49].

2.3. Cardiac remodeling

Chronic cardiovascular disease, such as hypertension, heart failure, or myocardial infarction, induces remodeling of the heart [50]. The remodeling process is characterized by hypertrophy of myocytes, hyperplasia of fibroblasts and vascular smooth muscle cells, excessive collagen deposition, and conduction abnormalities. As described above, RSV is able to prevent increased blood pressure in animal models; thus, it can protect the heart from structural remodeling (i.e., left ventricular hypertrophy, LVH) associated with pressure overload. Another antihypertrophic mechanism of RSV is via AMPK and its upstream kinase LKB1. AMPK not only reduces the hypertrophic response, but also delays the transition from cardiac to heart failure [51]. In hypertensive patients and rats, oxidative stress and lipid peroxidation products, such as 4-hydroxy-2-nonenal (4-HNE), are elevated [52]. 4-HNE produces an inhibitory effect on the LKB1/AMPK signaling pathway, with consequent induction of mTOR/p70S6 kinase-mediated protein synthesis and cardiac myocyte cell growth. RSV prevents the pro-hypertrophic effect of 4-HNE by the activation of AMPK [53]. Thus, RSV inhibits unnecessary protein synthesis and prevents remodeling of the heart [54]. RSV has

also effects on cell proliferation. Block of cell proliferation could also improve cardiac function. In cultured rat cardiac fibroblasts, RSV inhibited their proliferation and differentiation to the hypersecretory myofibroblast phenotype; these are two critical steps in cardiac collagen deposition. Another probable mechanism through which RSV can inhibit the proliferation of cultured rat cardiac fibroblasts is the activation of the NO-cGMP signaling pathway [55]. Since inflammation is a key initiator of fibrosis, the anti-inflammatory properties of RSV could be another contributory mechanism to the changes in cardiac remodeling. In mouse cardiac fibroblasts, RSV inhibited the high expression of PI3K/Akt/ERK-dependent interleukin-17, a pro-inflammatory cytokine, induced by high glucose levels; thus, RSV may decrease high glucose-mediated myocardial inflammation and remodeling [56].

2.4. Diabetes

The term diabetes does not indicate a single pathological entity but rather a clinical syndrome characterized by chronic hyperglycemia with alterations in the metabolism of carbohydrates, fats, and proteins, due to defects in secretion and/or insulin action [57]. The cause of diabetes continues to be unknown, although researchers' attention is increasingly focused on a number of factors: the increase in obesity, the increase in the average age and life expectancy, a style of a more sedentary life, an increase in stress, and, above all, genetics. This disease is called a heterogeneous syndrome because it includes various clinical forms, of which the most frequent are type 1 or insulin-dependent diabetes mellitus (T1DM or IDDM) and type 2 or noninsulin-dependent diabetes mellitus (T2DM or NIDDM). Type 1 diabetes (also called juvenile diabetes because it occurs generally in the first 30 years of life) is determined by an autoimmune destruction of the beta cells of the Isles of Langerhans, which results in a total absence of insulin and represents about 10% of diabetes cases [58, 59]. Type 2 diabetes, which represents approximately 90% of cases, occurs predominantly after 35–40 years of age with reduced insulin secretion associated with a resistance by the tissues to the action of the hormone itself. The onset, difficult to diagnose, is characterized by hyperglycemia and consequent polyuria, polydipsia, and polyphagia. Obesity or overweight is another characteristic of individuals suffering from this pathology [60].

RSV also prevents or delays the onset of chronic age-associated diseases such as type II diabetes, improves insulin sensitivity, reduces blood glucose levels, and reduces high-fat-diet-induced obesity in rodents [56].

In a recent study, it has been reported that the multiple aspects of the action of RSV on the mechanisms control glucose homeostasis [61]. Polyphenol plays a protective role on pancreatic islets by increasing the synthesis of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase, and glutathione-s-transferase, which counteract the action of free radicals [62]. The antiapoptotic power toward β-cells has emerged both in animal models with streptozotocin toxic damage and in the autoimmune insulin of type 1 diabetes mellitus, where the action of RSV manifests itself by reducing the expression of the chemokine receptor 6 and inhibiting the migration of inflammatory cells into the pancreas [63, 64]. Furthermore, RSV modulates glycemic homeostasis at hepatic level, reducing the activity of the enzymes of gluconeogenesis and increasing, on the contrary, that of glycogen synthase [65]. At the muscle level, as well as the hepatic, the optimization of fatty acid metabolism and the reduction of

NF-kB and pro-inflammatory cytokines in the target organs are due to a direct action of the polyphenol, which induces a partial regeneration of β-cells and causes an increase in the plasma concentration of insulin [66]. In type 2 diabetes mellitus, RSV counteracts insulin resistance in rats fed with hyperlipemia diet and high in fructose as well as in those with genetically determined insulin resistance. In obese rats, in fact, the polyphenol contrasts the adipogenesis and reduces the macrophagic infiltrate in the adipose tissue, the main source of adiponectin, coresponsible for the appearance of insulin resistance [67, 68]. Furthermore, the polyphenol determines the reduction of the muscular and hepatic lipid content. These effects are due to the modulation of the action of two important intracellular regulators, a histone deacetylase (SIRT1) and an AMP-dependent kinase (AMPK), which control determinant cellular functions such as intracellular energy metabolism, mitochondrial function, and apoptosis [69]. These data have also been confirmed in primates and obese men where chronic treatment with RSV seems to improve insulin sensitivity [70]. In conclusion, the pleiotropic action of RSV makes this polyphenol a possible additional natural resource in the treatment of the diabetic patient. Its beneficial effect manifests itself through the increase of insulin secretion, the reduction of insulin resistance, and the suppression of hepatic gluconeogenesis. Furthermore, the efficacy of RSV in mitigating the autoimmune destruction of β-pancreatic cells is particularly relevant from the clinical point of view because it could represent a support to the conventional treatment of the patient with type 1 diabetes mellitus.

3. Clinical trials on resveratrol effects in cardiovascular diseases

Why resveratrol and cardiovascular diseases? From a medical standpoint, CVDs are currently the first cause of death according to WHO. So much so, that RSV has been proposed as a possible treatment for prevalent CVD in relation to all the possible cardioprotective effects that have been uprising due to its extensive research [71].

3.1. From preclinical to clinical pharmacokinetics

Most of the extensive research so far has mainly been preclinical, both in vitro and in animal models as seen before. Preclinical studies are certainly a primary fundamental approach to identifying RSV potential direct and indirect molecular targets, mechanisms, and effects. These studies, in fact, give us a preview of the possible pharmacodynamics of RSV. This, however, neglects the main obstacle of all clinical research when introducing in the human body any kind of external element, pharmacokinetics. By this, I mean absorption, distribution, metabolism, and excretion. Research so far is not abundant and is mainly done on a restricted number of individuals. Moreover, studies initially focused on total plasma and urine RSV content due to lack of suitable metabolites standards of identification and quantification. In the last years, the increased knowledge on RSV-derived metabolites in plasma and urine [72, 73], in particular trans-, cis- forms, mono- and di-glucuronides/sulfates and sulfoglucuronides, as well as dihydro-RSV (DHRSV), derived from microbiota metabolism [73, 74] has opened a new frontier on the true difference between these metabolites and their activity level [75]. Let us now take a direct view of the main human studies published on RSV pharmacokinetics:

3.2. Absorption

After oral administration, the RSV plasma peak is reached in 30–60 min. In a clinical study in healthy volunteers, the absorption after oral administration of 25 mg of RSV was 70%, the peak plasma concentration of 491 ng/ml, and the plasma half-life of 9.2 h [74]. Systemic exposure to RSV (concentration-time curve) and peak plasma concentration were decreased (by 46 and 45%, respectively) when RSV (2 g twice daily) was administered with high-fat foods [76].

Interestingly, a six-daily 4-h interval (25, 50, 100, or 150 mg) RSV intake for 13 days on 20 healthy males and 20 healthy females showed that area under curve values was not directly proportional to RSV intake, and that there is a high interindividual variability and that bioavailability was higher after morning administration [77]. Another clinical study showed that bioavailability from wine and grape juice was around six times higher than that from tablets, in 11 healthy males consequent to either a single 250 ml red wine, 10 tablets, or 1 L grape juice intake with a 0.014 mg/kg of average RSV dose. Furthermore, a similar study with 10 healthy men with single ingestion of 375 mL of red wine (6.3 mg total REA content) or 10 capsules containing grape extract (total RSV content of 4.7 mg RSV) showed that grape extract RSV absorption was delayed versus red wine and moreover remained longer in the organism yielding higher RSV-derived metabolites [73].

RSV absorption studies have been performed on animals (mouse, rat) and using human liver cells (hepatocytes) and human tumor cells (colon carcinoma). The RSV contained in red wine is mainly present in the glycosylated trans- and cis-form (The two trans and cis spatial conformations are due to the presence of the double bond between the two phenolic rings.) The glycosidase RSV can be hydrolyzed from intestinal glucosidases to trans- and cis-RSV. In rats, it has been shown that RSV is absorbed in the intestine in a conjugated form with glucuronic acid [78].

It is estimated that in humans, 75% of the RSV administered orally is absorbed by the oral and intestinal mucosa and that the latter occurs mainly by trans-epithelial diffusion [79]. However, bioavailability is very low (<1%) and this could be due to rapid and intense metabolism and/or by the capture of specific tissues (the liver would seem to be able to eliminate most of the RSV from the circulatory stream) [79]. The low bioavailability of RSV is associated with a low solubility in water (<1 mg/ml) [80]. Furthermore, trans-RSV is photosensitive, easily oxidized and with an unfavorable pharmacokinetic profile [81]. With daily feeding, free concentrations of RSV compatible with those that determine its biological actions in vitro (5–100 μmol) are not achieved in the target tissues nor can high doses of pure RSV be administered due to potential undesirable effects and drug interactions.

3.3. Distribution

RSV is distributed in the liver, to which it shows a high affinity, and in the kidneys and, to a lesser extent in the brain, heart, lungs, and testes (pharmacokinetic studies with C14-labeled trans-RSV) [82, 83]. RSV accumulates in conjugated, glucuronide, or sulfated forms in rat liver. Furthermore, a 12-h pharmacokinetic steady-state of RSV has been shown in a 2g RSV twice a day intake with standard breakfast and high-fat breakfast in a sample of five healthy

females and three healthy males [76]. Another interesting clinical trial on nine healthy males with single intake of 500 mL low-fat milk containing RSV (RSV dose 85.5 mg/70 kg) showed a high binding affinity of RSV glucuronide metabolites to plasma proteins [72].

3.4. Metabolism

RSV is metabolized by (1) sulfation (in the bowel/liver: limiting factor), (2) glucuronidation of the phenolic group, and (3) hydrogenation of the aliphatic double bond (intestinal microflora). Metabolic studies have shown that the most common metabolites of RSV are the derivatives conjugated with glucuronic acid (glucuronides) and sulfates, synthesized both in the liver and in the bowel. Moreover, RSV metabolism by human gut microbiota seems to have a pronounced interindividual difference as shown in a study on 12 healthy volunteers with a single oral dose of 0.5 mg RSV/kg body weight in the form of grapevine-shoot supplement [84]. In a study, in healthy volunteers treated with RSV doses of 0.5–2.5 g, the most abundant metabolite was found to be a monosulfate derivative, RSV-3-sulphate. In the blood, the concentration of RSV 3-sulfate and of the two monoglucuronide derivatives is about 20 times that of RSV; the same ratio is present when we compare the concentration-time curve (AUC) of the RSV with that of the RSV-3-sulfate (the AUC of the sulfate is about 18–23 times that of the RSV, while the AUC of the two glucuronides is 4–6 times that of RSV) [85]. Moreover, in another clinical trial, no gender or age-dependent differences were observed in RSV metabolic profile in a small sample healthy subjects distributed in six young and six elderly females and six young and six elderly males [86].

3.5. Excretion

The apparent clearance and mean volume of distribution of RSV are consistent with the low bioavailability of stilbene. The plasma half-life of RSV is between 2.9 and 8.9 h, similar to that of the two glucuronide derivatives (2.9–10.6 h) and the 3-sulfate derivative (3.2–11.5 h) [85].

4. Trying to make order on CV risk factors in human resveratrol trials

4.1. Hypertension

In the fight against cardiovascular diseases, control of arterial hypertension is what is giving the best results in terms of cost-effectiveness. The large pharmacological intervention studies have shown that the reduction of just 10% of the pressure values resulted in a 40% reduction in mortality from cerebrovascular accidents and 16–20% in mortality from coronary accidents. However, criteria must be followed to set up a rational and adequate treatment to bring the values of blood pressure back to norm or as close as possible to the norm. The first criterion must be based on the degree of hypertension, mild, moderate, or severe which, even if it has a purely indicative value, appears to be extremely useful on the clinical-therapeutic level. In fact, in the patient with mild hypertension, a sufficiently protracted period of controlled clinical observation, up to 4–5 months, is necessary before starting a therapy, since the pressure

could fall within the normal values either spontaneously or with simple hygienic-dietetic measures. Moreover, in mild hypertension, it is advisable to start with a "light" drug therapy, in monotherapy, since the blood pressure control is often easy and the risk of complications is projected far in time and is, however, low. In case of moderate or severe hypertension, on the other hand, there is no longer any doubt as to the appropriateness of immediate pharmacological treatment. In this case, the patient will be initiated to the therapy that must be undertaken gradually and continuously, besides a "step-up," a "step-down" or a "side-step" therapeutical approach. Another criterion used for the purposes of the therapeutic approach is that which is based on the presence or absence of an organ damage, i.e., on the consequences of hypertension. It is evident that the treatment of hypertension that has already caused heart failure, cerebrovascular accidents, or renal failure possesses much more difficult problems than hypertension without obvious complications and requires a considerable commitment on the part of the doctor. A third criterion is that of the presence of concomitant pathologies on which some antihypertensive drugs can negatively interfere or whose treatment can negatively interact with that of hypertension. Fortunately, the vast majority of cases of hypertension are represented as already mentioned by the mild and uncomplicated form so the problem of how to set up the therapy is not so crucial and basically identifies with the problem of choosing the drug or drugs more suitable. The choice of antihypertensive drug is, in fact, still today substantially empirical. In fact, we do not have criteria that allow us to make rational therapeutic choices, that is to say, based on the physiopathological characteristics of the hypertensive state. At most, we can rely on some clinical data, which have some connection with the pathophysiology, but which are not strictly physiopathological. In this hypertension overview, it is important to understand where RSVs place lays. On a pure clinical logical standpoint, RSV could be of great use in two main occasions: first, in borderline hypertension in which drugs have shown to have a degree of adverse effects and so we have this time span in which we, as of today, have no other treatment if not a dietary one and second, it could be a great ally in all degrees of hypertension in association with the standard FDA recommended drugs.

Let us see, however, if this perspective view is backed by clinical trial evidence.

As already specified before, clinical trials today are not sufficient to give a definite all-around answer, but the evidence today at hand is comforting. In fact, a randomized, crossover, double-blind, single-dose, placebo-uncontrolled clinical trial with a single ingestion of 30, 90, 270 mg of synthetic RSV or placebo at weekly intervals, with analyses performed 1 h after consumption on nine healthy over-weighted/obese men or postmenopausal women with mildly elevated blood pressure, showed an acute RSV effect on FMD, which improved by 65% after consumption of 30 or 90 mg RSV and by 88% with 270 mg [87]. Another double-blind, randomized, placebo-controlled, crossover clinical trial on 18 patients with 330 mg of grape seeds and skin, 100 mg of green tea, 60 mg of RSV, 60 mg blend of quercetin and ginkgo biloba and bilberry on a 28 day basis showed a significant reduction in diastolic blood pressure [88]. RSV's main purpose has been studied by this clinical crossover, randomized, double-blind, placebo-controlled, single-center trial, which confirmed that RSV for 4 weeks lowers BP in prehypertensive and stage 1-hypertensive patients [89]. Another interesting study justifies the idea of RSV acting in cohort with standard drugs; in fact, monitoring BP in patients with hypertension (BP prior to and following standard antihypertensive treatment plus RSV,

compared with a control group receiving standard antihypertensive treatment alone) showed that diastolic BP and systolic BP were significantly reduced with the addition of RSV to standard therapy [90] (**Table 1**). Despite great hopes, these studies are still insufficient for RSV recommendation in hypertension as more and better-performed trials must be done in terms of higher sample size and longer follow-ups, without forgetting the pharmacokinetic issue.

4.2. Atherosclerosis

Although atherosclerosis is a slowly progressive disease, at the same time it can be extremely dangerous, considering the risk of its evolution in heart attacks and strokes. Prevention is the best cure: we have seen that obesity is one of the predisposing factors for atherosclerosis; therefore, it is recommended to follow a low-calorie diet, to reduce weight and to practice constant exercise. For the same reason, patients suffering from atherosclerosis or otherwise at risk should stop smoking; hypertensives should constantly monitor blood pressure values to avoid very high peaks, which, as analyzed, can predispose the subject to atherosclerosis and its complications. For similar discourse for patients with high cholesterol, it is recommended to undergo regular blood tests and follow a low-fat diet of lipids, to ensure the body a fair level of cholesterol in the blood. All these risk factors have in common the increased inflammatory state and lipid levels, which lead to mechanical and metabolic damage of the endothelial cells and deposit of lipidic and fibrotic matrix.

According to this, it is clear that an intervention on risk factors and behavioral habits can often block the cascade of events that would inevitably lead to the formation of atheroma.

Sample population	Dose	Duration	Effects	Reference
Hypertension				
19 healthy over-weighted/obese men or postmenopausal women with mildly elevated blood pressure	30, 90, 270 mg of synthetic RSV or placebo at weekly intervals or placebo	Analyses performed 1 h after consumption	FMD improved by 65% after consumption of 30 or 90 mg RSV and by 88% with 270 mg	[87]
18 patients	330 mg of grape seed and skin, 100 mg of green tea, 60 mg of RSV, 60 mg of blend of quercetin and ginkgo biloba and bilberry or placebo	28 days	Significant reduction in diastolic blood pressure	[88]
50 participants with prehypertension and 50 participants with stage 1 hypertension	500 mg of capsules, twice daily or placebo	4 weeks treatment–4 weeks washout–4 weeks treatment	Reduction in blood pressure	[89]
46 stage I hypertension and 51, stage II hypertension patients	Evelor or placebo	6 months	Diastolic BP and systolic BP were significantly reduced with the addition of RSV to the standard therapy	[90]

Table 1. Summary of clinical effects of resveratrol in hypertension.

Atherosclerosis is now a treatable disease: compliance with certain behavioral rules, prevention of risk factors and, possibly, the administration of specific drugs can not only block the degeneration of the disease but also and above all favor its regression.

In this sense, it is interesting to highlight the few clinical trials that invest in RSV and its role on atherosclerosis and on its two main components: oxidative stress and lipid profile.

A randomized, placebo-controlled 40 mg RSV capsule daily for 6 weeks on 20 healthy adults showed that RSV's cellular activity has a direct influence on plasma biomarkers associated with inflammation and risk of various diseases, in terms of reduction in reactive oxygen species generation, p47 and intranuclear nuclear factor-kappaB binding, jun-N-terminal kinase-1, inhibitor of kappaB-kinase-beta, phosphor-tyrosine phosphatase-1B expression, and cytokine signaling-3 suppression in mononuclear cells when compared with the baseline and the placebo. PCE intake also suppressed plasma concentrations of TNF-alpha, IL-6, and C-reactive protein [91]. On the same false line, a crossover, placebo-controlled trial with a high-fat high-carbohydrate meal with placebo or 100 mg of RSV and 75 mg of grape skin polyphenol on a small sample of four healthy male and six healthy women showed an increase in Nrf-2 binding activity following the meal and increased mRNA expression of NQO-1 and GST-π1 genes and attenuated postprandial rise in CD14 and IL-1β mRNA and TLR4 protein in mononuclear cells, and a decreased plasma endotoxin concentration, thus demonstrating an acute reduction in postprandial inflammatory state [92]. A more straightforward study on the possible use of RSV in primary lone prevention of atherosclerosis done on a sample of 44 healthy subjects in a 1 month time period with a double-blind, randomized, placebo-controlled administration of 400 mg of trans-RSV, 400 mg of grape skin extract, and 100 mg of quercetin showed a decreased expression of endothelial cell ICAM, VCAM, and IL-8 as well as a decreased level of plasma IFN-γ and insulin [93]. Other two straightforward studies instead tried to study the possible use of RSV in combination with standard atherosclerosis treatment. In the first, 75 patients on statins treatment at high CVD risk on a three-parallel arm, randomized, triple-blind, placebo-controlled trial on a 6-month daily ingestion of 350 mg placebo (n = 25), RSV containing grape extract (GE-RSV, grape phenolics +8 mg RSV, n = 25) or conventional grape extract lacking RSV (GE) showed a decrease in ApoB (−9.8%) and LDLox (−20%) in RSV-treated patients, beyond their treatment according to standard guidelines for primary prevention of CVD. In the second, with the same cohort as the first but on a 12-month daily ingestion of 350 mg placebo (n = 25), RSV containing grape extract (GE-RSV, grape phenolics +8 mg RSV, n = 25) or conventional grape extract lacking RSV (GE) for 6 months and the double dose for the following 6 months showed a GE-RSV nutraceutical decreased hsCRP (−26%), TNF (−19.8%), PAI-1 (−16.8%), and IL-6/IL-10 ratio (−24%), and increased IL-10 (19.8%). Furthermore, both studies showed no drug interactions or adverse effects [94, 95].

Worth mentioning is a study on healthy smokers, as we said before, smoking is not only an atherosclerosis risk factor but an overall CVD risk factor. In particular, randomized, double-blind, crossover trial on 50 healthy adult smokers, which were allocated to either "RSV-first" group (30 days of 500 mg RSV/day, 30 days washout, 30-day placebo) or to "placebo-first" group (30-day placebo, 30 days washout, 30 days of 500 mg RSV/day) showed a significant CRP and triglyceride concentrations reduction and increased total antioxidant status values [96] (**Table 2**).

Sample population	Dose	Duration	Effects	Reference
Atherosclerosis				
20 healthy adults	40 mg of RSV capsule daily or placebo	6 weeks	Reduction in reactive oxygen species generation, p47 and intranuclear nuclear factor-kappaB binding, jun-N-terminal kinase-1, inhibitor of kappaB-kinase-beta, phosphor-tyrosine phosphatase-1B expression, and cytokine signaling-3 suppression in mononuclear cells when compared with the baseline and the placebo	[91]
Four healthy male and six healthy women	100 mg of RSV and 75 mg of grape skin polyphenol or placebo	1, 3, and 5 hours after meal intake	Acute reduction in postprandial inflammatory state	[92]
44 healthy subjects	400 mg of trans-RSV, 400 mg of grape skin extract and 100 mg of quercetin or placebo	1 month	Decreased expression of endothelial cell ICAM, VCAM and IL-8 as well as a decreased level of plasma IFN-γ and insulin	[93]
75 patients on statins treatment at high CVD risk	RSV-containing grape extract (GE-RSV, grape phenolics +8 mg RSV) or conventional grape extract lacking RSV (GE) or placebo	6 months	Decrease in ApoB (−9.8%) and LDLox (−20%) in RSV-treated patients	[94]
75 patients on statins treatment at high CVD risk	RSV-containing grape extract (GE-RSV, grape phenolics +8 mg RSV) or conventional grape extract lacking RSV (GE) or placebo	6 months + double dose for the following 6 months	Decreased hsCRP (−26%), TNF (−19.8%), PAI-1 (−16.8%) and IL-6/IL-10 ratio (−24%), and increased IL-10 (19.8%)	[95]
50 healthy adult smokers	500 mg of RSV/day or placebo	30 days of 500 mg RSV/day (or placebo), 30 days washout, 30-days of 500 mg RSV/day (or placebo)	Significant CRP and triglyceride concentrations reduction, and increased total antioxidant status values	[96]

Table 2. Summary of clinical effects of resveratrol in atherosclerosis.

These trials took together, although still insufficient as evidence, give us a broad perspective on RSV use in atherosclerosis. RSV supplementation due to its anti-inflammatory, antioxidant, and hypotriglyceridemic effects, it is beneficial to those with increased CVD risk factors, both high and low, such as smokers and as a lone primary prevention therapy as well as in association therapy. Even if repetitive, more and better-performed trials must be done in terms of higher sample size and longer follow-ups in order to recommend RSV as an atherosclerosis therapy.

4.3. Heart diseases

Heart disease is the leading cause of death for men and women of all racial groups and affects all population ages, thus representing the leading cause of death in industrialized countries. Among these, coronary artery disease (CAD) is the most common condition associated with high mortality and morbidity. Clinical manifestations of ischemic heart disease include silent ischemia, stable and unstable angina pectoris, myocardial infarction (MI), heart failure, and sudden death. It has been widely ascertained that acute coronary syndromes in their various forms of presentation share a common pathophysiological mechanism, such as the rupture or erosion of the atherosclerotic plaque (of which we have already mentioned the uses of the RSV), on which phenomena overlap thrombotic and embolism at the distal level, as well as pro-inflammatory state and endothelial dysfunction.

In these terms, we understand the importance of preventing diseases which once again, for the umpteenth time, can only be done by prevention of heart disease that expresses the only winning weapon. Depending on the general health of the patient, it may be necessary to administer drugs for the heart, for obesity, for hypertension, and for hypercholesterolemia, as well as to follow a healthy and balanced diet, without excess exercise constantly. In this context, we try to understand through the various trials available to us how the administration of RSV can also act on the heart component directly without forgetting the already-mentioned beneficial effects associated.

In 40 stable CAD patients, a randomized, two-parallel arm, double-blind, placebo-controlled trial with a 3-month daily ingestion of 10 mg RSV in one of the groups showed that RSV decreased, versus baseline, LDL (8%) and improved endothelial function (50%), left ventricular diastolic function (2%), and protected from unfavorable hemorheologic changes. This highlights the RSV's cardioprotective effects after myocardial infarction [97]. Another interesting trial in 116 patients with stable angina pectoris in a randomized, double-blind, active-controlled, and parallel trial with three groups of subjects who received the test drugs and one control group of subjects who were not randomized with inclusion, 30 and 60 days of oral supplementation with calcium fructoborate (CF) (112 mg/day), RSV (20 mg/day), and their combination showed a significant hs-CRP decrease in all groups at the 30- and 60-day visits: 39.7% at 60 days for the CF group and 30.3% RSV plus CF at 60 days. The N-terminal prohormone of BNP was significantly lowered by RSV (59.7% at 60 days) and by CF (52.6% at 60 days). However, their combination was the most effective and induced a decrease of 65.5%. Lipid markers showed slight changes from baseline in all groups. Overall, this study confirmed the anti-inflammatory and lipid effect of RSV and introduces RSVs effect on left ventricle function enhancement, or more correctly recover, as shown by N-BNP marker [98]. Last but not the least, in 75 patients with stable CAD in a three-parallel arm, randomized, triple-blind, dose-response, placebo-controlled trial with a 12-month daily ingestion of 350 mg placebo (n = 25), RSV-containing grape extract (GE-RSV, grape phenolics +8 mg RSV, n = 25) or conventional grape extract lacking RSV (GE) for 6 months and the double dose for the following 6 months showed a significant increase in adiponectin levels (10%) in GE-RSV

group in addition to a decrease in PAI-1 levels, non-HDL cholesterol decreased significantly in both GE and GE-RSV groups, and downregulation of pro-inflammatory genes expression in PBMCs isolated from GE-RSV group patients. This highlights RSV's possible fibrinolytic effect on such patients confirming its overall anti-inflammatory effect in patients with established disease as did the two trials before [75].

Looking at these three trials and their results, the main suggestion would be to use RSV as an enhancement to the therapies now at our disposal for patients with previous cardiovascular accidents as it has shown not only to be of great importance for prevention of heart diseases through its anti-inflammatory and lipid profile effects but it also seems as it can modify left ventricle function (**Table 3**). Of course, three trials are away from sufficient to make such suggestion an indication, as so much has still to be done.

4.4. Diabetes type 2

Diabetes care is a very complex and articulated. The therapeutic objective is the same for any type of diabetes and consists in taking down high levels of blood glucose within the normal blood glucose values. This objective is more than anything else from a real need since hyperglycemia

Sample population	Dose	Duration	Effects	Reference
Heart diseases				
40 stable CAD patients	Daily ingestion of 10 mg RSV or placebo	3 months	Decreased LDL (8%) and improved endothelial function (50%), left ventricular diastolic function (2%) and protected from unfavorable hemorheologic changes versus baseline	[97]
116 patients with stable angina pectoris	Calcium fructoborate (CF) (112 mg/day), RSV (20 mg/day), and their combination or placebo	30 and 60 days	Significant hs-CRP decrease in all groups at the 30-day and 60-day visits: 39.7% at 60 days for the CF group and 30.3% at 60 days for RSV plus CF. The N-terminal prohormone of BNP was significantly lowered by RSV (59.7% at 60 days) and by CF (52.6% at 60 days). However, their combination was the most effective and induced a decrease of 65.5%. Lipid markers showed slight changes from baseline in all groups. Overall, this study confirmed the anti-inflammatory and lipid effect of RSV and introduces RSVs effect on left ventricle function enhancement, or more correctly recover, as shown by N-BNP marker	[98]
75 patients with stable CAD	RSV-containing grape extract (GE-RSV, grape phenolics +8 mg RSV) or conventional grape extract lacking RSV (GE) or placebo	6 months + double dose for the following 6 months	Increase in adiponectin levels (10%) in GE-RSV group in addition to a decrease in PAI-1 levels, non-HDL cholesterol decreased significantly in both GE and GE-RSV groups and downregulation of pro-inflammatory genes expression in PBMCs isolated from GE-RSV group patients	[75]

Table 3. Summary of clinical effects of resveratrol in heart diseases.

depends not only on the symptoms but also on acute and long-term complications of diabetes mellitus. Therefore, treatments that allow the achievement of the aforementioned objective deserve a quote: the adoption of a healthy and balanced diet, the regular practice of exercise and the intake of specific drugs for the reduction of blood sugar. Finally, concluding this rapid overview of diabetes therapy, it is important to note the importance of the regular monitoring of the effectiveness of the treatments adopted. This aspect is important because it allows the attending physician to understand if the therapy in place is working or not.

In this view, RSV can play an important role, let us see the understandings of few trials at our disposal. In 19 type 2 diabetics, a two-parallel arm, randomized, double-blind, placebo-controlled trial with a daily ingestion of 10 mg RSV for 4 weeks (n = 10 or placebo, n = 9) showed a decrease in insulin resistance possibly due to a decrease in oxidative stress and improvement of insulin signaling via the Akt pathway [99]. In another study, 62 type 2 diabetics in a randomized, two- parallel arm, placebo uncontrolled, unblinded trial with a 3-month daily ingestion of hypoglycemic drugs +250 mg RSV (n = 28) or only hypoglycemic drugs in control group (n = 29) showed that RSV improved systolic and diastolic blood pressures, HbA1c (−5%), total cholesterol, and LDLc concentrations [100]. On the same line, another interesting study on 66 type 2 diabetics in a randomized placebo-controlled, double-blind, parallel clinical trial supplemented with 1 g/day of RSV for 45 days showed significant decrease in systolic blood pressure, fasting blood glucose, hemoglobin A1c, insulin, and insulin resistance, while HDL was significantly increased, when compared to their baseline levels [101].

These studies considered as a whole show that RSV has many important antidiabetic effects as it improves insulin sensitivity, glycemic control, and also acts on associated risk factors such as inflammation and lipid profile (**Table 4**). Overall, RSV seems to have all the main specifics for the perfect antidiabetic drug. Although, as expected, more trials are needed since the observed reductions in HbA1c and HDL with RSV supplementation are so significant, compared to the benefits achieved with frontline antidiabetic drugs; we can hope for the best.

Sample population	Dose	Duration	Effects	Reference
Type 2 diabetes				
19 type 2 diabetics	Daily ingestion of 10 mg of RSV or placebo	4 weeks	Decrease in insulin resistance possibly due to a decrease in oxidative stress and improvement of insulin signaling via the Akt pathway	[99]
62 type 2 diabetics	Hypoglycemic drugs +250 mg RSV(or placebo)	3 months	Improved systolic and diastolic blood pressures, HbA1c (−5%), total cholesterol, and LDLc concentrations	[100]
66 type 2 diabetics	1 g/day of RSV or placebo	45 days	Decrease in systolic blood pressure, fasting blood glucose, hemoglobin A1c, insulin, and insulin resistance, while HDL was significantly increased, when compared to their baseline levels	[101]

Table 4. Summary of clinical effects of resveratrol in type 2 diabetes.

5. Effects of resveratrol in cerebrovascular diseases

The most frequent cause of death in the western world, after heart disease and cancer, is cerebrovascular disease and is the second most common cause of neurological disability, after Alzheimer's disease [102]. It has been found that half of the patients with neurological diseases also present cerebrovascular diseases. The term cerebrovascular disease indicates any cerebral alteration resulting from a pathological process affecting the blood vessels, whether they are arteries, arterioles, capillaries, veins, or venous sinuses (venous sinus). The vascular lesion may have anatomopathological characteristics of an occlusion by a thrombus or an embolus, or a rupture; the consequences at the level of the cerebral parenchyma are of two types: ischemia (with or without infarction) and hemorrhage. An alteration of the vessel wall permeability, hypertension, and increase in blood viscosity or modifications of another rheological characteristic are other pathophysiological mechanisms involved in cerebrovascular pathology.

The most common presentations of cerebrovascular diseases are stroke and transient ischemic attack (TIA). Stroke is caused by cerebral ischemia, that is the interrup-tion of the blood flow to the brain, of the duration enough to determine the appearance of focal signs and symptoms that do not disappear within 24 h, while, TIA consists in a sudden appearance of focal signs and symptoms, attributable to transient cerebral ischemia, which disappear within 24 h.

Most cerebrovascular diseases depend on atherosclerosis and arterial hypertension, and the main forms are characterized by cerebral insufficiency caused by transient disturbances of the blood flow. Moreover, diseases such as diabetes and atherosclerosis can be complicated by hypertension and increase the viscosity of the blood, without forgetting the important role of aging. In fact, an altered vascular permeability is responsible for headache, cerebral edema, and seizures of hypertensive encephalopathy. Signs and symptoms of cerebrovascular diseases reflect the damaged brain areas and not necessarily the affected artery.

The main causes are the formation of atherosclerotic plaques at the level of the carotids, embolisms from the heart, hematological disorders, fibromuscular dysplasia, and vasculitis, i.e., inflammatory processes in the blood vessels that cause the lumen to shrink [102].

5.1. Resveratrol in cerebrovascular diseases

Neurodegenerative diseases share common features such as chronic vascular damage due to oxidative stress and inflammation damage, which underline neural damage and thus neuronal death. Taking into account the mechanisms associated with vascular damage, let us see the role of RSV. As already mentioned, RSV has been shown through great preclinical evidence to have strong anti-inflammatory properties, as well as many antioxidant capacities, apoptosis inhibition, multiple transcriptional signaling pathways, and direct neurological function. As many works show, RSV inflammatory-response inhibition in the nervous system is mainly due to a reduction in TNFα, iNOS, COX-2, IL-1α, IL-1β, MMP-9, and p-p53, all inflammatory markers of great neural importance [103]. Reduction in oxidative stress is mainly mediated by an increase in eNOS, NO, SOD, GPx1, CAT, HO-1 levels accompanied by a decrease in MPO and ROS. Other important oxidative stress mechanisms include an increase in GSH,

VEGF, Trx-2, and mitochondrial biogenesis, while a reduction in XO and MDA. RSV apoptosis inhibition function is associated with a reduction in caspase-3, caspase-7, CYT-c, and Bax with increased Bcl-2. Finally, RSV's direct neuroprotective properties can be identified in the reduction of neurological deficit score and β-amyloid peptide with a contemporary increase in neuron survival, motor function and in TH, dopamine, and AchE levels. Main signaling pathways, protein kinase, and transcriptional factor modulators include Nrf-2, NFκB, p38MAPK, PGC-1 h, PI3K/Akt, mTOR, PPAR, AMPK, CREB, PKC, and SIRT [104–109].

5.2. Clinical trials on resveratrol effects in cerebrovascular diseases

Although RSV's neurological properties are many, the study of the pathologies of the nervous system is still very complex due to the missing knowledge in the exact mechanisms, so we can only depend on biomarkers of the single pathologies.

As far cerebrovascular diseases are considered, two are the main works at our disposal. The first work, on 4 young healthy men and 20 women in a double-blind, placebo-controlled, crossover with a single or once daily on 3 separate days with 250 or 500 mg of RSV, showed that RSV increased cerebral blood flow and hemoglobin but did not enhance cognitive function. Interestingly, the blood flow increase was dose dependent [110]. The second work evaluated if RSV use in coadministration with r-tPA for brain ischemic stroke treatment is more effective than r-tPA alone. These 312 patients were randomly divided according to their onset-to-treatment time (OTT) (early OTT or delayed OTT); afterward they were either treated with the association of r-tPA and placebo or with r-tPA plus RSV. Patients receiving delayed OTT r-tPA plus RSV treatment showed a reduction in matrix metalloproteinase (MMP)-2 and MMP-9 plasma levels; moreover, reductions in both MMPs and patient NIHSS scores were observed [111].

These two trials show a positive effect of RSV in cerebrovascular diseases both in a possible chronic use as seen by the increase in the cerebral blood flow and in an acute state for its positive effects in coadministration with r-tPA. Overall, it is almost impossible to evaluate RSV use in specific cerebrovascular diseases; as of now its use in stroke is the only plausible prospective indication. Of course, the way is still long and both preclinical and clinical trials are needed.

As far as neurodegenerative disease go, at our disposal, there are only two clinical trials both on Alzheimer disease (AD). These two studies were conducted on similar cohorts of 119 patients in a randomized, placebo-controlled, double-blind, multisiter, phase 2 trial manner. In both, 500 mg was administered once daily, with 500 mg dose escalation every 13 weeks, ending with 1000 mg of administration twice daily for 12 months, which, however, found different understandings. The first study showed a reduced CSF MMP9, increased IL-4, and attenuated decline in Aβ42 and Aβ40, while the second showed the same attenuation in Aβ42 and Aβ40 accompanied by increased brain volume loss [112, 113] (**Table 5**).

Overall, these two studies are useful to conclude that RSV metabolites penetrate the blood-brain barrier and have effects on the central nervous system, and these effects are mainly associated with an anti-inflammatory and antineurodegenerative effect. Collectively, we could assess that there is a place for RSV in neurodegenerative disease treatment; however, we are

Sample population	Dose	Duration	Effects	Reference
Cerebrovascular diseases				
Four young healthy men and 20 women	Single or once daily on three separate days 250 or 500 mg of RSV or placebo	After a 45-min resting absorption period	Increased cerebral blood flow and hemoglobin but did not enhance cognitive function	[110]
312 brain ischemic stroke patients	r-tPA + placebo or with r-tPA + resveratrol	24 hours after the treatment, outcomes were assessed	Reduction in matrix metalloproteinase (MMP)-2 and MMP-9 plasma levels; moreover, reductions in both MMPs and patient NIHSS scores were observed	[111]
119 patients	500 mg once daily, with 500 mg dose escalation every 13 weeks, ending with 1000 mg twice daily administration	12 months	Reduced CSF MMP9, increased IL-4, and attenuated decline in Aβ42 and Aβ40	[112]
119 patients	500 mg once daily, with 500 mg dose escalation every 13 weeks, ending with 1000 mg twice daily administration	12 months	Attenuation in Aβ42 and Aβ40 accompanied by increased brain volume loss	[113]

Table 5. Summary of clinical effects of resveratrol in cerebrovascular diseases.

still to understand which viable targets RSV focuses on which could be the biomarkers useful to understand RSV activity and effectiveness. As for as cerebrovascular disease considered, here as well, much is to be done in both preclinical and clinical stages.

6. Conclusion

Prevention policies mainly invest in medical interventions, although it has been known for several years, that lifestyle changes, mainly focused on exercise and nutrition, can produce a significant reduction in cardiovascular risk, with antiatherosclerotic effects, antithrombotic, anti-ischemic, antiarrhythmic with a significant reduction in mortality and incidence of infarction. Dietary and physical activity changes, together with the improvement of body composition, give a sense of general well-being. On the contrary, pharmacological interventions, together with their indispensable curative effect, unfortunately almost always present side effects that worsen the quality of life.

The presented data in this study have demonstrated the role of resveratrol in prevention of cardiovascular abnormalities induced by atherogenic diet. The overall data revealed that resveratrol possesses the cardioprotective effect by improving the serum lipid profile, antioxidant system, improving lipid metabolism, and cardiac tissue damages either in myocardium and aorta.

On the basis of the beneficial properties that resveratrol evoked on human health, the findings support a role for regular consumption of dietary resveratrol by consumption of resveratrol-rich fruits or vegetables to avoid the risk of cardio- and cerebrovascular diseases, in order to add life to the years and not only years to life.

Acronyms and abbreviations

AMPK	adenosine monophosphate-activated protein kinase
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CAT	catalase
COX	cyclooxygenase
CREB	cAMP response element binding
Cyt-c	cytochrome C
eNOS	endothelial nitric oxide synthase
GPx	glutathione peroxidase
GSH	glutathione
HO-1	heme oxygenase-1
IL	interleukin
iNOS	inducible nitric oxide synthase
MAPKs	mitogen-activated protein kinases
MDA	malondialdehyde
MMP	matrix metallopeptidase
MPO	myeloperoxidase
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa B
NO	nitric oxide
Nrf	nuclear factor-E2-related factor
p53	tumor protein 53
PGC	peroxisome proliferator-activated receptor-gamma coactivator
PI3K	phosphoinositide 3-kinase

PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species
RSV	resveratrolo
SIRT1	sirtuin 1
SOD	superoxide dismutase
TH	tyrosine hydroxylase
TNFα	tumor necrosis factor alpha
Trx	thioredoxin
VEGF	vascular endothelial growth factor
XO	xanthine oxidase

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Chapter 4

c-Myc Metabolic Addiction in Cancers Counteracted by Resveratrol and NQO2

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Additional information is available at the end of the chapter

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Abstract

Transcription factor c-myc is frequently amplified/overexpressed in human cancers. One event c-myc controls is metabolic reprogramming or the addiction for glucose and/or glutamine as nutrients. Rewiring of metabolic circuitry provides cancer cells with a gain-of-survival advantage. Accordingly, the aversion of two types of oncogenic-distinct metabolic addictions via c-myc control offers an anti-tumorigenic approach. Resveratrol reportedly inhibits the uptake/transport of glucose or glutamine and reduces c-myc expression in cancer cells. Whether c-myc control by resveratrol involves quinone reductase NQO2 is unknown. NQO2 expressing (shRNA08) and knockdown (shRNA25) CWR22Rv1 prostate cancer cells were generated and used to study the role of NQO2 in growth and cell cycle control. Immunoblot analyses were used to evaluate the changes of cell cycle-associated proteins. NQO2 in mediating degradation of cyclin D1 via AKT/GSK-3β by resveratrol was tested by determining AKT and chymotrypsin-like proteasome activities. Molecular modeling and pull-down/deletion assays were used to evaluate the interaction between NQO2 and AKT. Resveratrol interacts with NQO2, a quinone reductase that plays a key role in resveratrol-induced AKT/GSK3β-mediated degradation of cyclin D1. In this chapter, we unravel control of expression and stability of c-myc by the resveratrol-NQO2 axis as an approach to overcome c-myc-mediated metabolic reprogramming.

Keywords: resveratrol, c-myc, NQO2, metabolic addiction

1. Introduction

The c-myc oncoprotein is a well-studied, multifunctional transcription factor that controls cell proliferation, metabolism, and stress responses [1, 2]. Deregulation (amplification/overexpression) of c-myc occurs in many human cancers and is considered a "transition gatekeeper" for tumorigenesis. c-Myc controls metabolic reprogramming, a key event in tumorigenesis that provides

gain-of-survival adaptive advantage, enabling cancers to thrive in tumor microenvironments with limited nutrient resources [3–5]. This rewiring of metabolic circuitry is characterized by (i) preferred use of glucose to produce ATP by aerobic glycolysis combined with increased lactate production even in oxygen abundance and (ii) dependence on glutamine for conversion to glutamate and subsequent entry into the tricarboxylic acid cycle by anaplerosis [6, 7]. The metabolic calibration by c-myc in cancer cells is orchestrated to adequately meet the demands of energy expenditure, macromolecular syntheses, and disposal of catabolic wastes, in concordance with robust cell growth. For example, the capacity for aerobic glycolysis in highly proliferative cancer cells is controlled by c-myc via an increase in glucose uptake as a result of induced expression of glucose transporter 1 (Glut1) and lactate dehydrogenase (LDH) to stimulate glycolytic flux [8–12]. Similarly, to accommodate the utilization of glutamine, c-myc upregulates the level of expression of sodium-dependent neutral amino acid transporter (SLC1A5), thereby facilitating the transport of glutamate and uptake of glutamine. c-Myc also induces glutaminase GLS which catalyzes the conversion of glutamine to glutamate [8–10]. We hypothesize that the inhibition of c-myc expression and function, in addition to restriction of access to glucose/glutamine are both bona fide anti-carcinogenic approaches.

2. Metabolic addiction in malignant tumors presents new targets for cancer therapy

Cancer biology studies, for the past several decades, have focused on the identification and defining of DNA mutations, the narrowly centered focus being underpinned by the viewpoint that cancer is largely a genetic disease. However, cancer cells are also endowed by the acquisition of several hallmarks that enable them to become tumorigenic; among them is the apparent rewiring of gene:metabolite circuitry coordinating an altered metabolic adaptation with dysregulated cell proliferation in cancer cells [13]. Thus, the ability of cancer cells to preferentially utilize glucose and aerobic glycolysis as a paradoxical coincidence with ample oxygen supply was historically made by Warburg in the 1920s [14]. The so-called Warburg phenomenon amply illustrates the oncogenic addiction for glucose as a rapidly established metabolic adaptation that increases the capacity for ATP generation to meet the energy demands for unrestricted tumor cell proliferation and metastasis [7, 13, 15–17]. A similarly disproportionate reliance on amino acid glutamine is found in various cancer types [3, 4], compared to normal cells [18, 19]. The elevated levels of glutamine metabolism in cancer provide nitrogen for nucleotide and amino acid biosynthesis, and additionally, direct citrate and isocitrate in the TCA cycle for use in lipid synthesis, namely, reprogramming glutamine for energy production and for biosynthetic reactions via anaplerosis [6, 7, 20]. This metabolic rewiring is now recognized as an “Achilles heel” for cancer therapy.

3. c-Myc regulates metabolic addiction, cell growth and tumorigenesis

c-Myc is a transcription factor that controls the expression of a large number of genes including those involved in ribosome and mitochondria biogenesis, glucose and glutamine metabolism,

and lipid biosynthesis [8, 11, 12, 21]. c-Myc controlled gene sets underpin bioenergetics and synthesis of building blocks required for macromolecular assembly, transformation, proliferation and tumorigenesis. As a proto-oncogene, c-myc is frequently amplified/overexpressed in human cancers. Among a myriad of tumorigenic changes, c-myc controls are metabolic reprogramming—preferred use of glucose and glutamine, and sequential regulation of downstream targets/effectors [8, 11, 12, 21, 22]. c-Myc has been shown to induce the expression of glucose transporter Glut1 and glycolytic enzyme LDH to increase glycolytic flux. Similarly, c-myc also activates glutamine transporters SLC1A5 (also known as solute carrier family 1, member 5 and SLC38A5 - solute carrier family 38, member 5) that acts to increase uptake/transport of glutamine [12]. The rewiring of metabolic programming by c-myc provides cancer cells with survival advantage by meeting the demands of energy expenditure, macromolecular syntheses, and catabolic waste disposal. Studies have also shown that upregulation of c-myc is associated with an increase in mitochondrial glutaminolysis, which plays an overarching role on glutamine addiction in cancer cells. c-Myc also induces GLS expression through a dual negative mechanism – suppression of miR-23a/b by c-myc, and inhibition of GLS expression by miR-23a/b [21, 23]. The upregulation of GLS by c-myc facilitates the coordination of metabolic addiction with oncogenesis and the coalescence of metabolic calibration for survival with cellular transformation, proliferation, and cancer-related gene mutations. While metabolic addiction is clinically viewed as favoring carcinogenesis, it is equally plausible that the same nutrient dependence may become a restriction point for designing cancer therapy. Specifically, by limiting access to nutrients within the tumor microenvironments, novel oncogenic targets could become evident and are amenable for rational design of countermeasure strategies. A notable example can be found in the control of c-myc as a "master driver" for cancer; an equally appealing consideration likely involves the downstream targets regulated by c-myc [24, 25].

Resveratrol is a grape polyphenol whose efficacy has been amply supported by tissue culture and animal studies, and in limited clinical trials [26–30]. Resveratrol has been reported to inhibit c-myc in multiple cancer types including medulloblastoma cells, breast cancer cells and osteosarcoma [31–33]. How resveratrol inhibits c-myc via rewiring of metabolite:gene circuitry in tumorigenesis remains largely unknown and will be discussed in the next section. Greater understanding of this will add yet another dimension to the cancer preventive and therapeutic efficacy of resveratrol.

4. Aversion of c-myc metabolic addiction using resveratrol and its target protein NQO2 is an untested anti-tumorigenic approach

Since c-myc is involved in control of metabolic processing and resveratrol can inhibit c-myc expression, we propose that the c-myc-mediated metabolic dysregulation of cancer can be countered using grape-derived resveratrol. It is our hypothesis that resveratrol exerts a dual role in disrupting c-myc-mediated cancer metabolic reprogramming—a **direct effect** impinging on c-myc expression and stability and associated uptake/transport of glucose and/or glutamine, and an **indirect effect** involving NQO2 as the mediator affecting c-myc stability via AKT, its downstream effector GSK3β, and by extension control of the activity and function of the proteasome. This provocative assignment for NQO2 greatly expands its cellular role from a cytosolic flavoprotein discovered in 1961 and classically considered phase II enzyme to a multi-tasking regulator involved in cancer cell metabolic reprogramming. Ample data support this postulation.

NQO2 requires NRH (N-ribosyl dihydronicotinamide) as the cosubstrate for catalysis; the biosynthetic source of NRH in mammalian cells has not been elucidated suggesting that NQO2 may have other novel cellular functions. Mouse keratinocyte studies show that NQO2 controls TNF-induced NF-kB activation; NQO2 deletion potentiates the induction of apoptosis by abolishing TNF-induced cell survival kinases including JNK, AKT, p38, and p44/p42 MAPK [34]. NQO2 stabilizes C/EBPα degradation mediated by 20S proteasomes [35]. We previously showed that NQO2 is a high affinity target protein of resveratrol: NQO2 binds resveratrol with $K_D \leq 50$ nM [36]; X-ray crystal analysis shows that binding to resveratrol occurs in a hydrophobic pocket located between dimeric NQO2, possibly where the cosubstrate NRH binds [37]. Since the plasma concentration of resveratrol in humans can reach from 0.5 μM [38] to as high as 4 μM [39, 40], it may be suggested that *in vivo* levels of resveratrol are sufficient for binding and inhibiting NQO2 enzyme activity and modulation of its other functions. One such novel role may pertain to control of c-myc turnover *via* T58 phosphorylation by AKT/GSK3β [41–43], results which agree with/support/resemble our findings using NQO2-knockdown CWR22Rv1 cells, showing that NQO2: (i) inhibits AKT activity and (ii) controls cyclin D1 stability via AKT/GSK3β mediated threonine T-286 phosphorylation [44]. Moreover, oxidized and reduced NQO2 was recently reported to selectively bind DNA-intercalating agents, including ethidium bromide, acridine orange, and doxorubicin; all three agents functioning as inhibitors at nanomolar levels, thereby raising the provocative tenet that NQO2 is a potential regulator of eukaryotic gene transcription and expression [45]. Accordingly, activators/inhibitors of NQO2 may be developed as drug targets for the management of cancers harboring amplified/overexpressed transcriptional factor c-myc. As hypothesized, inhibitors of NQO2 could modify the interplay between NQO2 and c-myc and disrupt the c-myc-mediated growth advantage in cancer cells. If c-myc control is shown to be connected to and under the rubric of genetic (NQO2) and/or chemical (resveratrol) mediated control in glucose/glutamine addiction cancer, then the control of NQO2-c-myc axis by resveratrol may be a promising cancer preventative and therapeutic lead, providing insights on how to better manage and treat glucose/glutamine addicted diseases.

5. Control of c-myc stability by NQO2

As a powerful factor governing the transcription of large gene sets that encode proteins playing critical roles in numerous cellular processes, both in normal and diseased states, the level of expression of c-myc is under stringent control. Ample data point to c-myc degradation being regulated by sequential phosphorylation of S62 and T58, by two external signal activated kinase cascades, respectively, the RAF/MEK/ERK and PI3K/AKT/GSK3β signaling pathways [46–51]. T58 phosphorylation of c-myc promotes its interaction with the ubiquitin ligases Fbw7 and Skp2, ubiquitination and degradation by the proteasome [52–54]. Additionally, deubiquitinating enzymes, USP28 and USP36, also contribute to c-myc degradation [54–56]. Of note, the reported AKT/GSK3β-mediated c-myc T58 phosphorylation in control of its turnover [41–43] is relevant to our own studies: (i) NQO2 is involved in AKT/GSK3β-mediated cyclin D1 T286 phosphorylation and degradation and (ii) NQO2 knockdown CWR22Rv1-sh25 cells show a 37% decrease in chymotrypsin-like proteasome activity

compared to control CWR22Rv1-sh08 cells [44]. These results suggest a hitherto-never-considered aspect of control of c-myc stability by NQO2. Next, we will discuss a proposed study to test the potential role NQO2 plays as the mediator of control of c-myc stability via AKT/GSK3β-c-myc T-58 phosphorylation, and by regulation of activity and functioning of the proteasome.

Our previous studies showing that resveratrol exerts its effects via its target protein NQO2 provide the impetus for testing that down regulation of c-myc by resveratrol requires the participation of NQO2. Based on our data that NQO2 affects cyclin D1 turnover, we expect that NQO2 will increase c-myc degradation, that is, $\downarrow t_{1/2}$ instead of conferring protection on c-myc stability by competing against proteasome as has been reported for C/EBPα whose degradation by 20S proteasome is attenuated by NQO2 [35]. Since c-myc degradation by proteasomes is known to involve a multitude of mechanisms, there is a possibility resveratrol or NQO2 may directly affect c-myc degradation by exerting control on ubiquitin ligases like Fbw7 and Skp2 or the deubiquitinating enzyme, USP28. As to whether NQO2 interacts with AKT to affect AKT-GSK3β-mediated c-myc degradation, our expectation is that the accumulated c-myc protein in MG132-treated cells will show a higher T58 phosphorylation as compared to nontreated control cells. As a corollary, addition of GSK3β inhibitor LiCl to treated cells should significantly reduce T58 phosphorylated c-myc protein, in parallel with an increase in c-myc protein accumulation. siRNA-knockdown of GSK-3α or -3β in NQO2 expression cells compared to NQO2 knockdown cells will further confirm the role of GSK3β in mediating c-myc degradation. Results of these studies will provide support for the as yet untested hypothesis regarding the indirect role of NQO2 in controlling AKT → GSK3β → c-myc T58 phosphorylation → c-myc degradation by proteasome, and the direct role of resveratrol acting as a metabolic switch to shut off c-myc-mediated metabolic reprogramming in cancer cells.

6. Control of c-myc stability by resveratrol may involve the stimulation of proteasome β5 subunit

Epidemiological studies have shown that moderate intake of red wine is correlated with a reduced incidence of dementia and neurodegenerative disease [57]. Moreover, resveratrol, a tri-hydroxyl stilbene found in abundance in red wine, red grapes, peanuts and a number of other consumed foods in the United States, has been reported to confer protection against oxidative stress in PC-12 cells [58, 59]. It has been determined that the preeminent presence of senile plaques, composed mainly of amyloid-β (Aβ) deposits, is a pathological brain feature in individuals diagnosed with Alzheimer's Disease (AD). The Aβ peptides are derived from cleavage of the amyloid-β precursor protein (APP) and have been shown to destabilize neurons and lead to cell death through the induction of oxidative stress, mediated by the generation of reactive oxygen species (ROS) and elevation in intracellular hydrogen peroxide [60–62]. Compelling evidence supports that Aβ peptides serve as the "primary instigator" of AD [63]. Davies and coworkers[64] used tissue culture studies

combined with biochemical assays and siRNA-silencing approaches to show that resveratrol lowered the levels of secreted and intracellular Aβ-peptides in a concentration and time-dependent manner. Further, this effect occurred not by targeting the Aβ-producing enzymes β and γ-secretases or by affecting the stability of APP or the turnover of its C-terminal fragments; but instead appeared to involve the promotion of intracellular degradation of Aβ via a proteasome subunit β5-dependent mechanism. It is worth noting that resveratrol reportedly also promotes the proteasome-mediated degradation of important regulatory proteins, including cyclin D1 [65], the estrogen receptor-α [66], and the hypoxia-inducible factor-1α [67].

7. c-Myc as an IDP (intrinsically disordered protein) may facilitate its turnover by resveratrol via a ubiquitin-independent, 20S proteasome-mediated mechanism

The notion that a folded three-dimensional structure is required for the biological function of a protein is dispelled by the discovery of intrinsically disordered proteins (IDPs) in the 1990s [68]. IDPs can be viewed as proteins that have minimal structures or are devoid of an overall defined fold, either entirely or in parts and are more likely to exist in dynamic, mosaic states under physiological conditions. The absence of structural orderliness also confers plasticity and "fussiness" to IDPs for diverse protein-protein interactions; however, the very same structural flexibility may also render them difficult and challenging as druggable targets using traditional structure-function drug design approaches.

Eukaryotic transcription factors perform important biological functions in control of gene expression. They play an essential role in identifying the target sequences on DNA located in the vicinity as well as far removed from the transcription start site through direct protein:nucleic acid interaction, and also are required for binding to a large array of co-transcription regulatory proteins via protein:protein interaction. As such, eukaryotic transcription factors have been shown to exhibit a high degree of intrinsic disorderliness; based on bioinformatic model prediction analysis it is estimated that more than 49% of human transcription factors contain intrinsic disorderliness [69]. Studies have also revealed that IDPs, because of their intrinsic destabilized nature circumventing the requirement for unfolding protein substrates for proteolysis by the 26S proteasome, are more likely to be degraded via an ubiquitin-independent mechanism using the 20S proteasomes [70].

The intrinsically unstructured protein (IUP), disorderly theme is also found in transcription factor c-myc; indeed, c-myc only attains an ordered structure after binding to its disordered partner MAX protein (myc-associated factor). Bioinformatics and experimental approaches have estimated that c-myc contains more than 45% of residues which have high probability for disordered structure formation [71]. The possession of intrinsically disordered regions allows c-myc to be degraded independently of ubiquitin, which may account for its observed short half-life at the mRNA [72–74] and protein levels [75–77], and is dynamically

aligned with its switch on/off master transcriptional role independent of collaborative interaction with the pool of cellular ubiquitin that drives 26S proteasome-mediated protein degradation.

It should be noted that the function of the 20S proteasome can also be modulated by interaction with NAD(P)H:quinone oxidoreductase 1 and 2 (NQO1 and NQO2). Previous studies have reported that NQO1 physically binds the 20S proteasome in an NADH-dependent manner [78–80] and to protect IDPs from degradation [81]. A double negative feedback mechanism exists between NQO1 and the 20S proteasome [78]. On the one hand, NQO1 acts to attenuate the proteolytic activity of the proteasome; on the other hand, the proteasome degrades the NQO1 FAD-free apo form which manifests as a partially unfolded structure and a substrate for the 20S proteasome [81]. Studies have shown that NQO2 confers protection against proteolytic degradation by the 20S proteasome [35, 82] albeit by a mechanism independent of NQO1 [83].

8. Conclusion

Resveratrol has been shown to inhibit the uptake/transport of glucose or glutamine, and decrease the expression of c-myc in cancer cells. Rewiring of metabolite:gene circuitry is a key event in tumorigenesis that has been known for decades [7, 15–17], however, with an incompletely understood underlying mechanism. In this chapter, we discuss the aversion of c-myc-mediated reprogrammed cancer cell metabolism by targeting the expression and stability of c-myc using a chemical/genetic disruptive approach focusing on resveratrol and its high affinity target NQO2 we identified [36]. Additionally, the hypothesis we propose broadens the classical function of NQO2 in quinone detoxification to AKT/c-myc-mediated metabolic reprogramming observable in a clinical setting. Taken together, resveratrol/NQO2 in a c-myc controlling role to block metabolic addiction represents a novel diet-based chemoprevention approach in concept, and is transformative in implications warranting further investigation. The results will lay foundation for discovery of drugs able to disrupt AKT/c-myc-mediated reorganized metabolism using NQO2 inhibitors.

In summary, we advance the thesis to avert c-myc-mediated metabolic reprogramming in cancer cells by targeting the control of c-myc and the uptake and metabolism of glucose and glutamine and their downstream effectors using resveratrol. We propose to focus on control of phosphorylation of c-myc T58 by GSK3β, shown to be critical for proteasome mediated c-myc degradation, by the resveratrol target protein NQO2 which we have previously shown to act as a modulator of AKT/GSK3β proteasome mediated degradation of cyclin D1 [44].

The dual role resveratrol plays in disrupting c-myc-mediated metabolic reprogramming in cancer cells—a direct role targeting suppression of c-myc expression and an indirect role involving NQO2-AKT-GSK 3β mediated increase in c-myc T58-phosphorylation for increased degradation by proteasome—is illustrated in **Figure 1**.

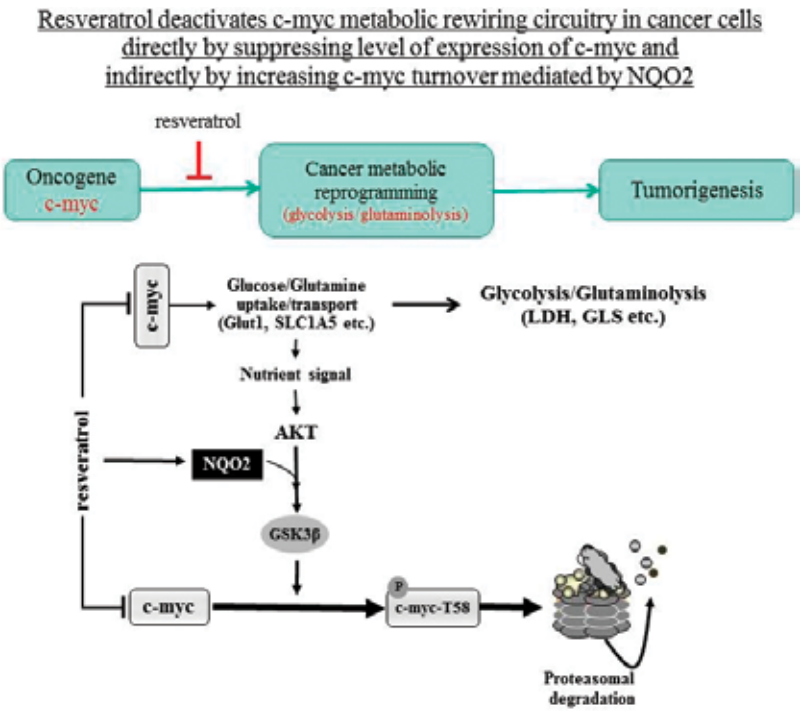

Figure 1. Resveratrol and NQO2 exert dual control of c-myc-mediated glucose/glutamine adaptation in cancer cells by transcription/translation suppression of c-myc expression and by control of proteasome-dependent c-myc stability according to the sequence: NQO2 binds AKT, reducing AKT kinase and increasing GSK 3β activity, resulting in increase in c-myc T58 phosphorylation and facilitating an increase in c-myc degradation by proteasome.

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Chapter 5

Where and How in the mTOR Pathway Inhibitors Fight Aging: Rapamycin, Resveratrol, and Metformin

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Additional information is available at the end of the chapter

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Abstract

The molecular mechanisms underlying the quality and quantity of life extension appear to sometimes be orthogonal. For example, while resveratrol has continued to prove beneficial in reducing obesity, it has had less efficacy in extending lifespan. On the other hand, rapamycin and the chemically similar rapalogs extend lifespan across genera of life from yeast, to nematodes, to mice. Caloric restriction (CR) and bioavailable small molecules, which mimic a fasted state, upregulate autophagy, catabolism of fats over anabolism of carbohydrates, and decrease oxidative stress and inflammation. CR mimics are currently being investigated to elucidate the best dosage, route of administration, timing in life, where best to inhibit in the mTOR pathway, and effects of long-term use on mTORC1 verse mTORC2 complexes. Comparisons between rapamycin, resveratrol, and metformin targets, downstream pathway effects, dosage, and clinical trials will be discussed.

Keywords: rapamycin, rapalogs, resveratrol, metformin, mTOR, senescence, aging, longevity, autophagy, inflammation

1. Introduction

It has been shown across the animal kingdom that caloric restriction (CR) extends lifespan. It is logistically harder to test this in longer living animals due to the length of studies needed, but there are studies in non-human primates [1] and ongoing human test groups who show fewer signs of cardiovascular aging [2]. Two trials calorically constricting macaques began in the 1980s and initially had conflicting results. A study out of University of Wisconsin found a drastic 30% increased survival in the CR group compared to control [3], while a latter study

by the National Institute on aging (NIA) did not find a statistically relevant effect [4]. It was later found that in the NIA study, control monkeys consumed fewer calories than expected, and some in the CR groups began consuming reduced calories as juveniles, which is known to reduce lifespan. A reanalysis of all data by both groups agreed caloric restriction appears to increase macaque longevity by almost 10% (3 years in macaques which would translate to 9 years in humans) [1].

In general results have seemed positive, extending life, but to a lesser degree than in small animal models, such as mice which have seen up to a 50% increase in lifespan from CR [5]. The search for pharmaceuticals to mimic CR life extension will need to continue the long and expensive process of large human studies due to humans' unique interaction with calories in our post-industrial world. Study designs for larger caloric restriction studies are often questioned. Particularly concerning humans, the ability to accurately track caloric consumption in people living outside a clinical setting has often relied on caloric approximations such as food diaries, pictures of food eaten [6–8], or dietary consumption habits at a national level when comparing between countries. The larger percentage life extension effects from CR has been seen across many simpler organisms with budding yeast, fruit flies and worms having their lifespan increased 2–3 fold. However, no mammals have had such large effects. Indeed no one has suggested humans could achieve such great gains with CR which would extend our current upper lifespan from ~100 years to over 200 years. However, mammals such as rats and mice have shown a 20–50% reduction in calories can result in a lifespan increase of up to 50% [9, 10].

Even with these qualifiers in mind, it seems likely that a 30–60% reduction in calories could extend human life 10–20%. This gain of ~1% of lifetime for every ~3% reduction in calories translates to a likely ~10–20 years of extra life for humans, which is similar to the 9-year human equivalent life extension seen in the recent reanalysis of primates undergoing CR [1]. This 1:3 ratio of %lifetimeExtension:%caloricRestriction (LE:CR) may end up being 1:4 or 1:2 in humans, but in either scenario it is most likely caloric restriction will show a statistically significant life extension in humans. However, it is not likely to be a panacea that would give us 50 extra years bringing us past 150 years-of-age, despite that relative effect in mice.

The current dilemma has been elucidating the root cause(s) responsible for life extension which are being targeted as pharmaceutical targets. The "inputs" that one might measure which lead to an increased lifespan in humans (e.g. obesity, cholesterol, cancer, bone density) are numerous and often orthogonal in nature. For example at pharmacological concentrations resveratrol does inhibit obesity but did not inhibit cellular senescence like rapamycin does [11]. While resveratrol and rapamycin were at times thought to act similarly, their mechanistic and pharmacological issues are diverging. While resveratrol had been found to extend life in studies there have been negative results with some labs failing to find life extension in all strains of yeast [12], worms, and flies [13]. Indeed rapamycin but not resveratrol has been shown to extend lifespan in mice [14]. Resveratrol may increase our quality of life while rapamycin (and rapalogs) could increase our quantity of life. In addition, one of resveratrol's main issues is its bioavailability (it's good we just want more), whereas rapamycin may shut down people's immune system too much leading to cancer (it's good but too much is bad). The mechanism of action for rapamycin, resveratrol, and metformin, as well as animal and human studies will be discussed.

The ideal "biological scale" at which aging can be targeted is also still in question (a single gene, pathway, salvaging a cell, or killing unrecoverable cells) (**Figure 1**). Single genes continue to be investigated with inhibition by siRNA, conditional knockouts, or reducing posttranslational modifications such as lipid anchoring [15–21], while activation could be investigated via upregulation of transcription factors or viral therapy such as CRISPR. However, due to overlapping inputs the field often addresses how entire pathways are being affected (such as increased mitochondria biogenesis by caloric restriction). In addition while in vitro studies have often looked at modifying a cells genetic profile to have more of a centenarian profile (i.e. to rescue human cells via an intervention), it has recently been shown many cells become senescent and causing those to undergo apoptosis can save other cells thereby resulting in organism longevity [22–28]. The easiest abnormal aging targets may be the overactive cancerous cells we have become use to targeting via single genetic markers (e.g. targeting estrogen receptor sensitivity in breast cancer). Pathways can be targeted via some important individual targets, for example rescuing p53 deficiency or inhibiting mdm2 over activity to cause apoptosis. However rescuing cells from becoming senescent is the hardest and most distant task, required to truly push human longevity beyond a ~125 year limit (**Figure 1**). An important comparison is the case of the hydra which has been pointed to in the last couple of decades as an immortal multicellular organism [29, 30]. The hydra however, has a structure in which stem cells continually differentiate and move the periphery where they fluff off. There is not a large repository of persistent differentiated cells that can never become senescent for their hydra to continue living. In this regard the hydra can be thought of amputating any problem cells which it can replace [31–33]. Many of humanity's growing diseases involve multi-organ

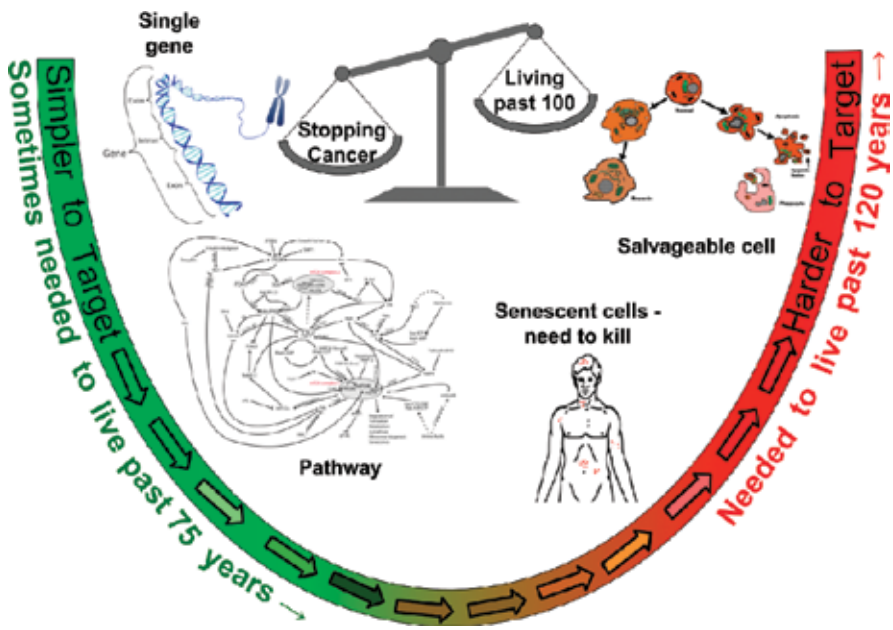

Figure 1. Therapeutics for "aging" will likely having differing levels of complexity in their targets depending on if they are targeting a single gene (easiest), a pathway, cause death of senescent cells, or trying to salvage a cell from becoming senescent (most difficult). Overactive cancerous cells can be targeted simply to kill based on one receptor or gene, while salvaging neurons from death (e.g. various dementias) is a harder therapeutic task.

systems with terminally differentiated cells which cannot be easily replaced. For example, neurodegenerative diseases such as Alzheimer disease (AD) have phenotypic effects when neurons start dying in large numbers. While CR and CR mimics may increase autophagy and delay cell death, as discussed below, there is not evidence that inhibition of the mTOR pathway can perpetually shift humans as an organism to a hydra like state of immortality. Since 1932 the correlation between mass and metabolic rate for mammals has been investigated as a foundation for humans' upper lifespan limit [34, 35]. It could be that the lower molecular activity from CR will shift humans to a longer lifespan following the three-quarters power law (or Kleiber's Law), although more recent studies seem to be elucidating cellular and molecular minutia in a more fine-grained manner than Kleiber's course mass does [36–38].

2. mTOR pathways: rapamycin, resveratrol, and metformin

A wealth of studies has confirmed that rapamycin and rapalogs directly inhibit mTOR, whereas resveratrol's targets are more numerous. Initially resveratrol was thought to act primarily through activation of sirtuins, with sirtuin-1 (SIRT1) known to help reduce obesity [39]. It is now known resveratrol also activates adenylyl cyclase and AMP-activated protein kinase (AMPK), while inhibiting a slew of proteins including lipoxygenase, protein kinase C (PKC), p53, mitogen-activated protein kinase 3 (MAPK3), proto-oncogene tyrosine-protein kinase (Src), signal transducer and activator of transcription 3 (STAT3), and IκB alpha kinase (IKK) [40]. One of the main targets is now AMPK activation which itself activates SIRT1 leading to mTOR inhibition.

2.1. Anabolic vs. catabolic energy production

AMPK is one of the primary metabolic detectors conserved across genera being activated by conditions that cause a low ATP:ADP ratio such as hypoglycemia and hypoxia. Phosphorylation of likely over 1000 targets by AMPK [41] shuts off anabolic pathways (energy-using) and turns on catabolic pathways (energy-generating). One of AMPKs targets for phosphorylation is peroxisome proliferators-activated receptor gamma coactivator-1 alpha (PGC-1α) which becomes active resulting in increased mitochondria biogenesis, membrane potential, and fatty acid oxidation [42], a recurring feature found during caloric restriction [43, 44]. AMPK also activates forkhead transcription factors of the O class (FOXO) which leads to increased autophagy and antioxidants, both leading to increased oxidative metabolism, like PGC-1α does [44].

In the case of life extending interventions dosing becomes very important. Too much of a good thing, can definitely be bad (i.e. cancer), and the molecular mechanism effecting longevity are being elucidated. For example, in *Caenorhabditis elegans*, metformin is found to delay development under well-fed conditions and even reduces life span during starvation [45–47]. The improved mitochondrial function, decreases oxygen consumption needed, which causes a beneficial decrease in reactive oxygen species (ROS) [48, 49]. Mitochondrial biogenesis is controlled differently depending on tissue and disease state. For example, mTOR signaling has been found to increase expression of mitochondrial genes involved in oxidative metabolism,

through PGC-1α and Ying-Yang 1 (YY1). This increased mitochondrial biogenesis in the muscle of healthy individuals, but not in obese individuals perhaps due to decreased insulin sensitivity [50]. Not only is mTORC1 activity cell specific, but it is also concentration dependent being induced and inhibited by low and high levels of ROS respectively [51]. This concentration sensitivity of mTOR is beneficial since it acts as a hub for interdependent pathways, such as mTORs ability to modify both mitochondrial biogenesis and increase autophagy (which helps degrade damaged mitochondria and other organelles). Two models of aging have been established in yeast (*Saccharomyces cerevisiae*): replicative lifespan (RLS) and the chronological lifespan (CLS). RLS measures the number of asymmetric mitotic divisions a cell can undergo before cell cycle arrest and is a valuable model for fibroblasts, lymphocytes, or stem cells in humans [4, 52–54]. CLS in contrast measures how long stationary (G_o) cultures remain viable and is a model for postmitotic cells like neurons or muscle cells [52, 54, 55]. Organ specific analysis of human in vivo studies, while difficult, would help elucidate CR mimics at and upstream of mTOR.

Metformin is a third life extending compound worth contrasting to rapamycin and resveratrol because it inhibits the mitochondrial respiratory chain complex I, leading to decreased ATP:ADP, which activates AMPK [56]. In addition metformin has lots of human data since it is a common oral antidiabetic drug used for overweight people with type 2 diabetes mellitus (T2DM). Metformin inhibits hepatic glucose production, reduces insulin resistance, and has recently been investigated as anti-aging therapeutic. Metformin is currently being investigated for use in various cancers [57, 58]; however, metformin has also been linked to the development of some solid tumors in humans, namely colorectal, breast, and pancreas cancer [59]. Mitochondrial complex I is clearly inhibited by metformin leading to the AMPK dependent activation of TSC2 which inhibits mTOR. AMPK can also directly inactivate mTORC1 complex via phosphorylation of its subunit Raptor. However, it has also been shown that metformin can act in an AMPK independent manner, though that mechanism is less clear but could involve nuclear pore complex (NPC) or late endosome interactions which have been documented [46]. The NPC interaction was found when *C. elegans* ortholog of acyl-CoA dehydrogenase family member 10 (CeACAD10) knockdown was found to have a 3-fold resistance to metformin. CeACAD10 expression was more than doubled by 50 mM metformin, and an unbiased, forward genetic screen found the nuclear pore complex is required for metformin to induce CeACAD10 [47]. That molecular pathway is currently unique to metformin, compared to resveratrol or rapamycin, and while multiple targets have been found for metformin, some pathways overlapping between these three molecules allow a more robust understanding of caloric restrictions possible of life extension mechanisms. Not only mTORC1, but even upstream AMPK, has been shown to be required for the positive effects of all three molecules rapamycin [60], resveratrol [61, 62], and metformin [63, 64]. Metformin's molecular pathway has also been elucidated upstream of AMPK. Metformin interacts with organelle Na1/H1 exchangers (eNHE) and the V-type-ATPase (V-ATPase) which supports the idea of the late endosome/lysosome, which is required by both the AMPK and mTOR pathway, acting as a signaling hub for metabolism [45].

While gross metrics such as weight are often reported in studies and useful to follow, they are not sufficient to investigate the aging phenomenon. For example, mice administered

resveratrol have been found to not lose weight [65, 66]. The degree to which resveratrol mimics caloric restriction (CR) has been shown at a molecular level in mice with changes in gene expression overlapping in the adipose tissue, skeletal muscle, heart, liver, and neocortex. Interestingly, both resveratrol and CR slowed age-related decline in organ function, showing the benefit from resveratrol was not dependent on weight loss [65, 66]. The other side of the caloric coin which is frequently investigated independently of CR is exercise induced caloric deficit. In general CR has more robust life extension properties than an exercise induced caloric deficit. For modern humans it is clear it is extremely difficult to exercise one's way into the same caloric deficit that can be attained through CR. In short, it is harder to run off a fast food meal than to not have the meal in the first place. It has been shown in rodents that increased activity to achieve a 30% relative energy deficit did not extend maximal lifespan but did increase average lifespan [9, 67]. The ability of resveratrol to increase lifespan has varied significantly between studies, but been roughly 40% for yeast, 15% for worms, 30% for fish, and 10% for mice [68].

There are two mTOR multisubunit protein complexes which have been shown to be differentially regulated. mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) share the protein components DEP domain containing mTOR-interacting protein (DEPTOR), mammalian lethal with sec-13 protein 8 (mLST8, also known as GβL), telomere maintenance 2 (telO2), and telO2-Interacting Protein 1 (tti1) (shown as light blue in **Figure 2**). mTORC1 has three core components: mTOR, regulatory-associated protein of mTOR (Raptor), and mammalian lethal with sec-13 protein 8 (mLST8). Whereas mTOR complex 2 (mTORC2) core components share mTOR, mLST8, but also include rapamycin-insensitive companion of TOR (Rictor), and mammalian stress-activated map kinase-interacting protein 1 (mSIN1) (**Figure 2**) [69]. mTORC1 is activated by nutrients and growth factors while being inhibited in low energy cellular states. A known complexity with the mTOR pathway is the difference in response to inhibitors, not only by mTORC1 and mTORC2, but also by tissue. mTORC1 is universally inhibited by rapamycin, whereas mTORC2 needs long term exposure to be inhibited by rapamycin which continues to be investigated. While DEPTOR is known to partially inhibit mTORC1 it may not decrease lipogenesis or inflammation alone, however in conjunction with AKT Serine/Threonine Kinase 1 (AKT) inhibitors can result in both decreases in lipogenesis and inflammation [70]. Combination therapy may be necessary in targeting mTORC1 to attained desired effects.

While multiple targets upstream of mTOR continue to be investigated, well described downstream actions of mTOR help in analysis of in vivo, in vitro, and clinical studies. While the major downstream effect of mTOR activation is anabolic energy production (with inhibitors shifting to catabolic energy production from fat), another significant downstream effect of mTOR activation is increased inflammation. In general people living in the western world live in a state of excess inflammation. Time restricted feeding (TRF) was found to help immune response, reducing systemic low-grade inflammation and age-related chronic diseases linked to immunosenescence, without compromising muscle performance [71]. The reduced inflammation seen in calorically restricted individuals is partially due to an increase in autophagy from CR (see below). The mTOR pathway has been shown to trigger the development of T cells, B cells, and antigen-presenting cells (APC). Indeed resveratrol (found in plants such as

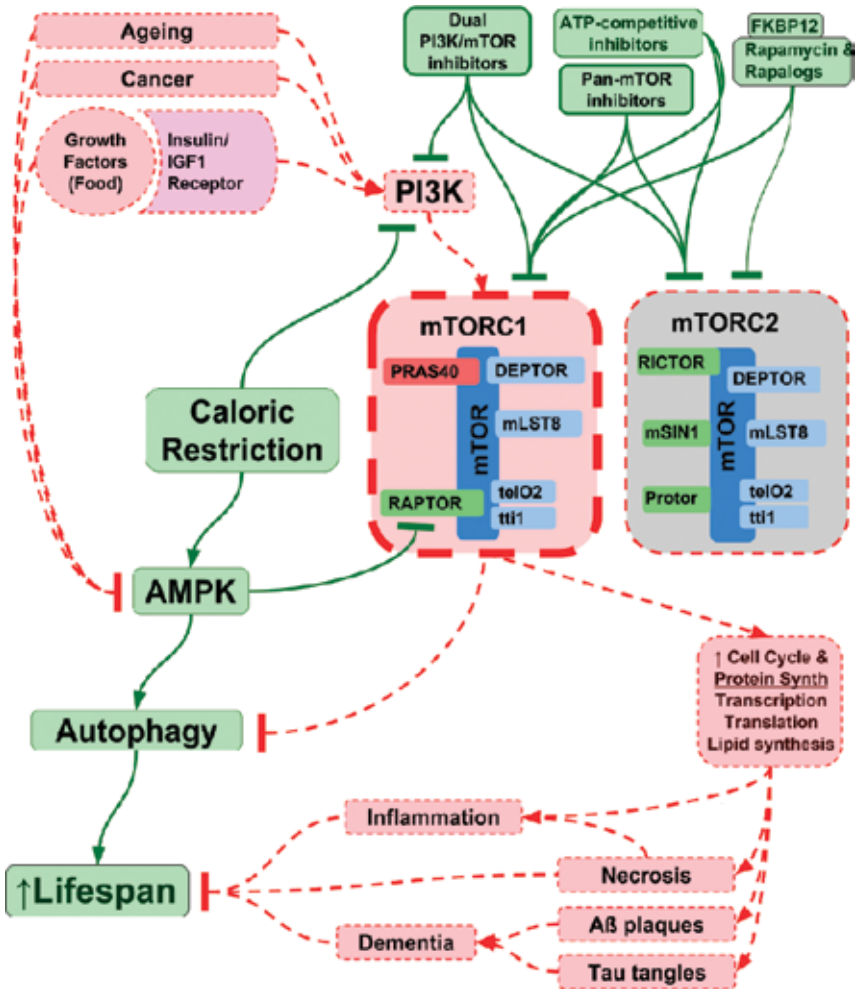

Figure 2. mTORC components, signaling, and inhibitors. Food, old age, and cancer activate PI3K and inactivation of AMPK which cause an increase mTOR activity in both complexes mTORC1 and mTORC2 and decrease the level of cellular autophagy. Autophagy can be restored through mTOR inhibitors (rapalogs, ATP-competitive inhibitors, Pan-mTOR inhibitors, dual PI3K/mTOR inhibitors) or reduced caloric intake (growth signals)-all restore autophagy. Beneficial and deleterious interactions or macromolecules are shown in green and or dashed red respectively. Proteins found in both mTOR1 and mTOR2 are colored blue. *Abbreviations*: AMPK, AMP-activated protein kinase; DEPTOR, DEP domain containing mTOR-interacting protein; mLST8, mammalian lethal with sec-13 protein 8 (also known as GβL); mSin1, mammalian stress-activated map kinase-interacting protein 1; mTOR, mammalian target of rapamycin; mTORC, mammalian target of rapamycin complex; PRAS40, proline-rich Akt substrate 40 kDa; protor1/2, protein observed with rictor 1 and 2; RAPTOR, regulatory-associated protein of mammalian target of rapamycin; RICTOR, rapamycin-insensitive companion of mTOR; telO2, telomere maintenance 2; tti1, telO2-interacting protein 1.

grapes, red wine, mulberries, and peanuts) has been described as a broad spectrum of action anti-inflammatory which attenuates microglial cell overactivation through mTOR inhibition [72]. Resveratrol inhibition of NF-κB causes an increase in superoxide dismutase (SOD) and results in decreased proinflammatory cytokines IL-1β, IL-6, and TNF-α [73–76].

Genome wide analysis will likely be needed to elucidate the beneficial molecular level causes of caloric restriction. For example, Dato et al. recently analyzed pathway-based SNP-SNP interactions of 3 pathways: the insulin/insulin-like growth factor signaling (IIS), DNA repair, and pro/antioxidants. Synergistic effects on longevity were found in the combination of growth hormone secretagogue receptor (GHSR) and double strand break repair nuclease MRE11 homolog (MRE11A) genes which are involved in IIS signaling. TP53 also had synergistic effects with either ERCC Excision Repair 2 (ERCC2) or thioredoxin reductase 1 (TXNRD1). Those results highlighted the central role of TP53 in activating DNA repair and pro-antioxidant pathways [77].

2.2. Autophagy

One pathway difference between rapamycin and resveratrol is the large magnitude with which rapamycin increases autophagy over apoptosis, which helps in regards to life extension but could prove problematic in cancer use. Pharmacological levels of resveratrol on the other hand prevent upregulation of Akt activation and autophagy thereby causing apoptosis. Resveratrol does inhibit obesity at pharmacological concentrations, prevent heightened hyperinsulinemia, or inhibit mTOR in vitro and therefore did not inhibit cellular senescence like rapamycin does [11]. Large levels of resveratrol have recently been shown to induce autophagy when inhibiting mTOR directly through ATP competition [78]. Combination therapy of rapamycin and resveratrol has proven synergistic in treatment of breast cancer cells [79, 80].

Caloric restriction has been shown to increase autophagy through inhibition of mTOR and delay molecular events associated with dementia. The rise in neurodegenerative diseases, which are exacerbated by low autophagy levels, heightens interest in mTOR inhibitors. Caloric restriction achieves mTOR inhibition through two pathways: decreased PI3K activity and increased AMPK activity (**Figure 2**). Cells in low energy states (calorically restricted) have low PI3K activity, lowering Akt activity, which then lowers mTORC1 via inhibition by Tsc1/2 (**Figure 3**). Rapamycin directly inhibits mTOR but metformin and resveratrol inhibit mTOR through upstream pathways, inhibiting the mitochondrial complex I activity and increasing AMPK respectively. In the well fed state mTORC1 inhibits autophagy via inhibition of SIRT1, Unc-51 like autophagy activating kinase (ULK1), transcription factor EB/E3 (TFEB/TFE3). Active mTOR also stimulates eukaryotic translation though phosphorylation and inhibition of 4E-BP1 which in turn releases the bound cap-binding eukaryotic translation initiation factor 4E (eif-4E). When eif-4E is released it can participate in forming the eIF4F complex required for initiation of cap-dependent translation. Ribosomal proteins S6 and S6K are also stimulated by mTOR which leads to increased protein synthesis and lipogenesis. In the fasted state ULK1 starts autophagosome maturation and TFEB/TFE3 increases lysosomal biogenesis and autophagy. Ras-related GTPases (Rags) actually tether mTORC1 to the lysosomal surface and that connection is controlled through amino acid sensing of the vacuolar H^+-adenosine triphosphatase ATPase (v-ATPase) as well as the proton-assisted amino acid transporter 1 (PAT1) (**Figure 3**). SIRT1 is also activated in the fasted state, and by CR mimetics, which increases SOD, p53, and activates FOXO leading to increases in cellular autophagy and mitochondrial biogenesis (**Figure 2**).

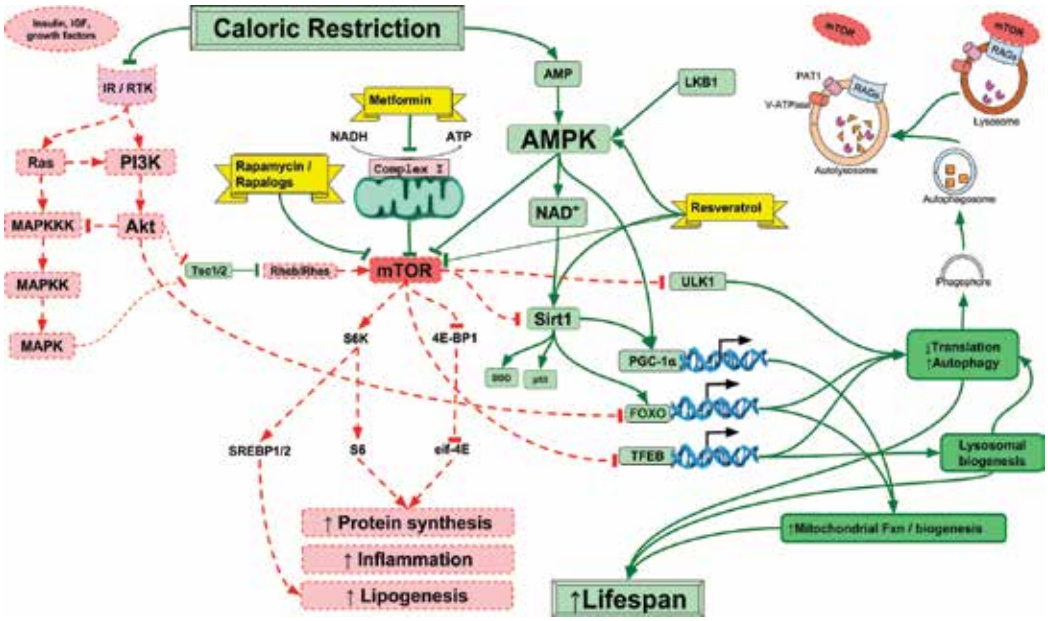

Figure 3. mTOR pathway activation, inhibitors, and downstream effects. The molecular pathways increased by caloric restriction that increase lifespan are also targeted by rapamycin, resveratrol, and metformin. Metformin and resveratrol inhibit mTOR through upstream pathways, inhibiting the mitochondrial complex I activity and increasing AMPK respectively. Rapamycin, and rapalogs, on the other hand inhibit mTOR directly. Beneficial downstream effects of mTOR inhibition include increase mitochondrial function and biogenesis, lysosomal biogenesis, autophagy, and decreased translation. Deleterious effects of an active mTOR pathway include increased lipogenesis and inflammation. Beneficial and deleterious interactions or macromolecules are shown in green and or dashed red respectively. *Abbreviations*: AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; SOD, superoxide dismutase; PGC-1α, peroxisome proliferators-activated receptor gamma coactivator-1 alpha; IR, insulin receptor; RTK, receptor tyrosine kinase; Ras; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; PI3K; Akt = AKT Serine/Threonine Kinase 1; S6, ribosomal protein S6; S6K, ribosomal protein S6 kinase beta-1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; eif-4E, eukaryotic translation initiation factor 4E; SREBP1/2, sterol regulatory element-binding protein; Rheb/Rhes; Tsc1/2, tuberous sclerosis proteins; PAT1, proton-assisted amino acid transporter 1; V-ATPase, vacuolar H^+-adenosine triphosphatase ATPase; RAGs, Ras-related GTPases; ULK1, Unc-51 like autophagy activating kinase; TFEB/TFE3, transcription factor EB/E3. Ras, Ras superfamily (RAt Sarcoma); PI3K, Phosphoinositide 3-Kinase Gamma; Rheb, Ras homologue enriched in brain; Rhes, Ras homolog enriched in striatum.

Therapeutics to help extend human lifespan far past the ~100 year limit will likely need to increase autophagy to avoid dementias, a later life disease state. With various dementias (PD, ALS, HD, and AD) having mitochondrial dysfunction [81–84], and mTOR activation known to increase oxidative stress, antioxidant therapies are being investigated. It has been found conjugating a cation compound to the antioxidant increases uptake into the mitochondria 80-fold and potency up to 800-fold [85] due to its 165 mV negative potential [86]. Low levels of autophagy also result in necrosis instead of apoptosis, with the resulting ramped up immune system increasing inflammation. Intracellular stress acts through Bcl-2 to open the mitochondrial permeability transition pore (mPTP) leading to caspase dependent intrinsic apoptosis [69, 87, 88]. The mPTP is known to exist in 3 states: closed, transiently open in low conductance, and permanently open in high conductance [89–91], the latter resulting in mitochondrial depolarization, loss of ATP production, and caspase independent necrosis since the

Lifestyle	♀	♂
Physical activity (≥30 min/day)	8	7
Not smoking	9	12
Healthy diet	5	4
Low alcohol (15♀, 30♂ g/d = 2♀, 4♂ drinks/d)	3	2
BMI (18–25 kg/m^2)	4	5
Extra years if all 5	14	12

Five healthy lifestyles (exercise, healthy diet, ideal BMI, low alcohol, and not smoking) were found to add 12–14 years of life starting at age 50 when compared to people that did not follow any of the five lifestyles. The healthiest and worst habits within each lifestyle had very different life expectancies as well, with nonsmokers and excessive smokers having the largest lifespan gap (9–12 years). The second greatest gain in lifespan (7–8 years) came from getting more than 30 min of exercise a day compared to never exercising. Data from [94].

Table 1. Five healthy lifestyles that extend lifespan more than 10 years.

controlled apoptotic pathway requires energy [92]. Multiple types of cancer show increased mTOR pathway signaling which is what the first mTOR inhibitors were FDA approved for: sirolimus, everolimus (Afinitor), temsirolimus (Torisel), and ridaforolimus, with sirolimus and everolimus also finding use as immunosuppressants after organ transplants [93].

While caloric mimics will the not be the panacea pushing human life past 200 years it should be pointed out the large effect it could have in humans compared to other currently measurable lifestyle interventions. The effect was recently quantified for the top five frequent lifestyle interventions: smoking cessation, physical activity, healthy diet, healthy BMI, and low alcohol consumption (**Table 1**) [94]. Starting at age 50 women and men were found to be able to add on average 14 and 12 years respectively, if all 5 healthy lifestyles were adopted. Never smoking was the strongest healthy habit of the five, with a close second being engaging in physical activity over 30 min a day (which included brisk walking or anything more strenuous). The healthy diet and BMI (18–25 kg/m^2) can both clearly be linked to a CR lifestyle. It will be interesting to compare the magnitude of CR life extension to the years gained by aspects of a "healthy diet" which is usually cataloged by many more variables than just caloric count (e.g. vitamin/antioxidants, omega-3 vs. omega-6 vs. saturated fat content). In summary, CR alone seems likely to have as big, or slightly larger, of an effect than the 5 healthy lifestyles in concert. If rapamycin, resveratrol, metformin, or a combination thereof, prove capable of reproducing even half the years of life extension that CR extension can, it would be a multi-billion dollar market (USD) and could be among the best therapeutics measured on the Quality Adjusted Life Years (QALY) scale.

3. Preclinical and clinical studies

While there are chemically similar caloric restriction (CR) mimetics such as rapalogs for rapamycin, the main compounds discussed: resveratrol, rapamycin, and metformin are

chemically distinct. Both resveratrol and metformin are hydrogen-donor rich, having hydroxyls and amides, respectively. Rapamycin is a much larger macrocycle molecule (MW = 914) with both hydrogen donor and acceptor moieties compared to the smaller resveratrol (MW = 228) and rapamycin (MW = 129) (**Figure 4**). Future docking, crystallography, and NMR studies would be interesting to determine if other molecules could mimic ATP, directly binding to the ATP pocket on mTOR as it has been suggested resveratrol does [78]. A structural mimic of ATP acting as an antagonist can seem conceptually attractive and likely have broad effects on multiple energy sensing proteins, but would also likely have lower than desired specificity.

All three compounds resveratrol, rapamycin, and metformin have had numerous human clinical trials. Metformin is unique among the three in that it is currently an approved and recommended therapy for a massive population, specifically obese individuals with type II diabetes, and therefore has a much larger dataset of patients to pull safety and efficacy information from. Resveratrol and rapamycin are both natural compounds with a plethora of academic papers in animal models, but rapamycin studies have the added nuance/diversity of involving a host of rapalogs with modified activity. A search of clinical trials including the keywords resveratrol, metformin and rapamycin and grouped by topic is shown in **Table 2** (as of April 11th 2018). Resveratrol has the lowest number of ongoing clinical trials (137), metformin has over 2.5 fold as many (359), and rapamycin has almost fivefold ongoing clinical trials (646). Resveratrol and metformin have largely overlapping pathway targets in clinical trials with the most common being endocrine system diseases, diabetes mellitus, obesity, and insulin resistance. The main topics for rapamycin are neoplasms by histological type, vascular

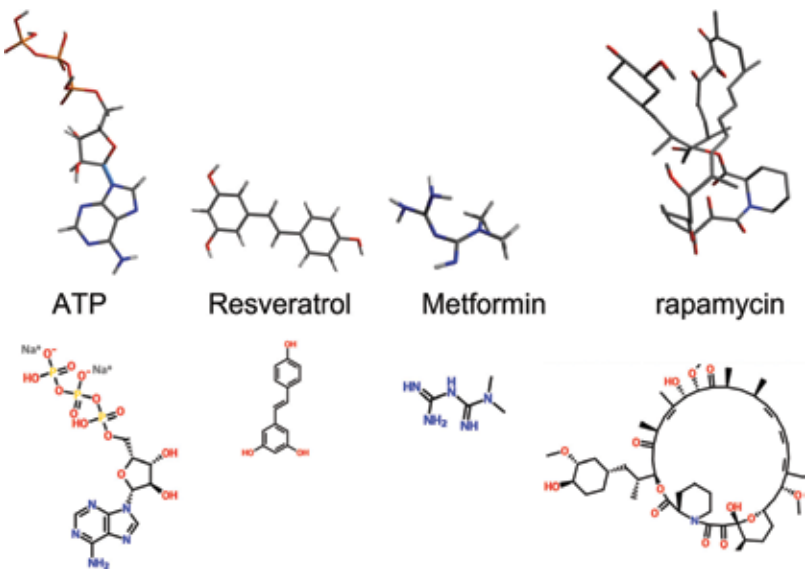

Figure 4. Structures of metabolism modifiers. The structures of compounds discussed: resveratrol, metformin, rapamycin, and ATP. Some have had suggested competitive binding pockets, such as resveratrol and ATP. However on a small molecule scale metformin, resveratrol, and rapamycin have very different complexity and differ by orders of magnitude in molecular weight. All compounds do have significant number of polar groups for hydrogen binding to protein surfaces and pockets.

Compound term search at clinicaltrials.gov	# CT ongoing	Insulin resistance	Diabetes mellitus	Endocrine system diseases	Obesity	Neoplasms by histologic type	Vascular diseases	Myocardial ischemia
Resveratrol	137	28	24	23	19	5	6	1
Metformin	359	34	178	195	34	45	14	5
Rapamycin	646	1	12	42	2	218	172	143

Clinical trials that are ongoing, as of April 11th 2018, were searched for the keywords resveratrol, metformin, and rapamycin. Resveratrol and metformin had overlapping metabolic clinical targets listed, while rapamycin had more numerous trials, which were focused on vascular diseases and cancer.

Table 2. Ongoing clinical trials for resveratrol, rapamycin, or metformin (April 11th 2018 search of clinicaltrials.gov).

disease, and myocardial ischemia. Metformin and rapamycin have some overlap, e.g. metformin has 45 current trials listed under neoplasms by histological type, and rapamycin has 42 trials listed under endocrine system diseases.

4. Conclusions

The use of therapeutics that mimic caloric restriction (CR) is likely to increase and add incremental quality-adjusted life years (QALYs). Natural CR compounds, and analogs based off of them, are fairly cheap with low side effects. Controlled animal studies will likely continue to be the avenue which exposes the degree to which molecular pathways are responsible for the increased quality and quantity of life. Resveratrol and metformin seem robust at increasing molecular pathways linked to quality of health and are useful to combat obesity and type II diabetes; while their ability to increase maximum lifespan remains in question. Data suggests rapamycin and the follow on rapalogs could add years to a human lifespan, although the magnitude of the effect could be enhanced or completely ablated based on accompanying lifestyle choices (diet, exercise, sleep).

Research shedding light on the optimum dosing of caloric mimics should be interesting to follow. Caloric restriction studies in humans fall into three categories: continual modest decrease in calories consumed (~1500 kcal/day), temporary drastic reduction in energy intake (~500 kcal/day), or intermittent fasting (0 kcal/day) in which only water is consumed for 1–3 days. Intermittent fasting has actually slightly outperformed all other methods of dieting methods (atkins, zone, weight watchers, ornish/vegan) in reducing weight in humans, which is partially due to increased compliance [95, 96]. The degree to which molecular pathway changes from intermittent fasting are responsible for reduced weight, such as increased autophagy, remains to be determined. The number of calories that can be consumed above complete fasting, while still increasing autophagy and decreasing inflammation, needs further investigation [1, 10, 44, 97–108].

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5. Recommendations

It would be very useful for clinicians and patients to have a curve in which the x-axis showed calories consumed per day and the y-axis showed %change in these important life extension pathways (e.g. autophagy, inflammation, lipogenesis, lysosomal biogenesis, and protein synthesis). These studies/curves would ideally be done separately for various groups (e.g. males, females, diabetics, elderly predementia, and elderly with early dementia). The interaction of therapeutics that mimic CR in combination with a changing intake of calories from fluctuating diet will require significant large studies and clear simplifications for clinicians and patients to utilize that information and make actionable in daily life. The actionable timeline for CR mimics is still being investigated, but if studies from intermittent fasting apply then administration for months could be useful but lifetime use will be needed to maximize benefits.

Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AD	Alzheimer disease
ALS	amyotrophic lateral sclerosis
AKT	AKT Serine/Threonine Kinase 1
AMPK	AMP-activated protein kinase
APC	antigen-presenting cells
CeACAD10	*C. elegans* ortholog of acyl-CoA dehydrogenase family member 10
CLS	chronological life span
CR	caloric restriction
DEPTOR	DEP domain containing mTOR-interacting protein
eif-4E	eukaryotic translation initiation factor 4E
eNHE	Na1/H1 exchangers
ERCC2	ERCC Excision Repair 2 (ERCC2)
FOXO	forkhead transcription factors of the O class
GHSR	growth hormone secretagogue receptor
HD	Huntington disease
IIS	insulin/insulin-like growth factor signaling

IKK	IκB alpha kinase
MAPK3	mitogen-activated protein kinase 3
mLST8	mammalian lethal with sec-13 protein 8
mPTP	mitochondrial permeability transition pore
MRE11A	double strand break repair nuclease MRE11
mSIN1	mammalian stress-activated map kinase-interacting protein 1
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NIA	National Institute on aging
NPC	nuclear pore complex
PAT1	proton-assisted amino acid transporter 1
PD	Parkinson disease
PGC-1α	proliferators-activated receptor gamma coactivator-1 alpha
PKC	protein kinase C
QALY	Quality Adjusted Life Years
Rags	Ras-related GTPases
Raptor	regulatory-associated protein of mTOR
Rictor	rapamycin-insensitive companion of TOR
RLS	replicative lifespan
ROS	reactive oxygen species
SIRT1	sirtuin-1
SOD	superoxide dismutase
Src	proto-oncogene tyrosine-protein kinase
STAT3	signal transducer and activator of transcription 3
T2DM	type 2 diabetes mellitus
telO2	telomere maintenance 2
TFEB/TFE3	transcription factor EB/E3
tti1	telO2-interacting protein 1
TXNRD1	thioredoxin reductase

ULK1	Unc-51 like autophagy activating kinase
v-ATPase	vacuolar H^+-adenosine triphosphatase ATPase
V-ATPase	vacuolar (H+)-ATPase
YY1	Ying-Yang 1

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Chapter 6

Resveratrol and SIRT1 Activators for the Treatment of Aging and Age-Related Diseases

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Additional information is available at the end of the chapter

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Abstract

Reduced calorie intake is a religious and medical practice known since very old times, but its direct influence on life span in all organisms, included humans, has been demonstrated in the modern era. Not only periodic fasting, but also natural or synthetic compounds that mimic this phenomenon are growing to slow aging and the onset of chronic morbidities. Resveratrol (RSV), a plant polyphenol, is an elixir of longevity for simple organisms and preclinical rodent models even if a beneficial role in humans is still debated. Its main rejuvenating mechanism copes with the activation of specific longevity genes called sirtuins. Among seven known mammalian sirtuins, sirtuin 1 is the most studied. This pleiotropic nicotinamide adenine dinucleotide (NAD)-based deacetylase maintains longevity by removing acetyl group in nuclear histones, transcription factors, and other DNA repairing proteins. Actually, an exciting challenge is to discover and test novel sirtuin 1 activators to extend life span and to treat age-associated disabilities. This chapter updates on the antiaging effect of RSV and sirtuin 1 activators in experimental animals and in humans. Finally, pros and cons on RSV analogues and sirtuin 1 activators tested in preclinical and clinical trials to hamper neurological deficit, cardiovascular complications, diabetes, bone and muscle deterioration, and cancer are discussed.

Keywords: sirtuin 1, resveratrol, aging, neurodegeneration, diabetes, myopathy

1. Introduction

The increase in average life expectancy is a global consequence of sanitary welfare, proper nutrition, and healthy life style [1, 2], but unfortunately, longevity is linked to the onset of chronic irreversible diseases like neurological decline, cardiovascular damage, bone and

muscle frailty, diabetes, and metabolic diseases with high economic and social costs [3–5]. Specific international clinical objectives are to maintain a "healthy aging" defined by World Health Organization (WHO) as the "functional ability that enables wellbeing in old age" by firstly preserving the quality of life together with its duration [6, 7]. Besides unchangeable genetic background, in this context, great credit is due to the quality but also the quantity of dietary nutrients [8–10].

Historically, since fifth century BC, the famous Greek physician Hippocrates declared that fasting was the best practice to block sickness progression, and, on the contrary, too much feeding gave rise to a disease. This old and simple medical concept, i.e., caloric restriction without malnutrition, has maintained for centuries but without a direct impact on life extension. Only in modern times since early 1930s, a pioneering Mc Cay discovery confirmed that undernutrition caused prolonged life span in laboratory rats [11]. By 2000s, Longo and coworkers systematically studied dietary restriction as a therapy for slowing aging and related diseases in human settings [12–15]. Periodic reduction of dietary calorie intake or an intermittent fasting, under medical control, appears alternative tools to pharmacology to delay the onset of aging comorbidities, like metabolic diseases, and to improve health outcomes [16–19]. "Geroscience," a definition coined by Burch et al. [20], identifies the branch of aging biology aimed to reduce human frailty and the impact of associated chronic diseases. However, considering the difference in the duration of life between lower organisms versus humans, to fill this gap, caloric limitation has been used also in nonhuman primates like monkeys. Anyway, significant translational value has been attributed to dietary studies in short-life animals versus nonhuman primates and humans that enhanced their life span under reduced nutrients intake.

This chapter is focused on a natural dietary polyphenol, resveratrol (RSV), able to mimic calorie restriction and to prolong life duration in simple organisms and experimental animal models, but controversial in humans [21, 22]. RSV has been firstly identified as a modulator of conserved survival genes family called sirtuins [23]. Sirtuins, crucial modulators of life span extension and metabolism, have been firstly discovered by Guarente team as product of silent information regulator 2 (Sir 2) gene in yeast, *Saccharomyces cerevisiae* [24], worm, *Caenorhabditis elegans*, and fly fruit, *Drosophila melanogaster* [25]. In mammals, among seven known members, sirtuin 1 (SIRT1) enzyme was most beneficial against aging by mimicking calorie reduction [26, 27]. Moreover, other natural or synthetic compounds, more effective than RSV, like stimulate molecular longevity pathways via SIRT1 or pan-sirtuin activation, are currently under study [28–30]. Therefore, here major attention has been focused on newest preclinical experimental studies on laboratory animals, mainly rodents, and clinical trials based on RSV and other SIRT1 activators published over last 10 years. Due to the impressive wide scientific literature on longevity, we select only more recent articles or reviews and apologize in advance for unintentional omissions. We believe that a primary scope of this chapter is to steer readers, even if not expert in the field, to study in detail if interested, the newest antiaging therapeutic perspectives. Intriguingly, we stressed on the role of SIRT1 activators in cancer but a dichotomy exists based on specific type of cancer and clinical outcome. Indeed, SIRT1 activators (STACs) that will promote benefits in selected tumors may obtain

opposite detrimental effects in others. Therefore, to date, particular caution must be taken in the pharmacological use of SIRT1 modulators in oncology. So best understanding on SIRT1 activation and maintenance by specific modulators is essential to fight age-derived inevitable disorders.

2. Sirtuin 1 history

The history of sirtuin family (silent information regulator 2—Sir 2) as antiaging proteins started in yeast, *Saccharomyces cerevisiae*, where they prolonged life span and regulated the number of replications from a mother cell [31]. Later, it was demonstrated in yeast that Sir 2 was further able to block the formation of extrachromosomal DNA linked to aging and genetic "toxicity." Indeed, an excess of Sir 2 gene ameliorated reproductive cycle and sustained longevity in yeast budding [32]. Moreover, sirtuins were able to influence longevity in other lower organisms like worms, *Caenorhabditis elegans* [33], and dose-dependently in fruit fly, *Drosophila melanogaster* [34, 35]. Guarente team in 2000 demonstrated that the main action of sirtuins was to remove, as deacetylase enzymes, acetyl groups from specific lysine sites in nuclear histones, so allowing DNA silencing and chromosome stability [36]. This peculiar role is basic for an epigenetic regulation of DNA and telomere health and stabilization. Besides on class III DNA histones, sirtuin deacetylation activity has been extended to a plethora of other nonhistone proteins, transcription factors, and cytoplasmic proteins, which, by this posttranslational event, changed their structure and consequently function or signaling [37]. Recently, sirtuins have been considered not only deacetylases but also able to perform other posttranslational changes in their targets and for this reason defined "deacylases" [38]. Moreover, for their enzymatic activity, sirtuins used nicotinamide dinucleotide (NAD+) as a specific substrate, so their role has been related to NAD+ availability in the cell and to the NAD+/NADH ratio. In particular, it is known that during dietary caloric limitation and regular physical exercise, abundant NAD+ is produced and sirtuins are more active. There are two different ways to produce NAD+: one *ex novo* and another by conversion of nicotinamide (NAM) into nicotinamide mononucleotide (NMN) then charged with adenine nucleotide to become NAD. For vertebrates, the limiting enzyme necessary for the final step in the NAD synthesis is nicotinamide phosphoribosyltransferase (NAMPT) that is regulated by circadian rhythm regulator and clock genes (CLOCK and BMALI) [39]. The strict connection between NAD+ availability and sirtuin activity has been recently demonstrated and implied that competition for NAD+ substrate by different enzymes may affect sirtuin level, so contributing to age-associated diseases in mice [40–42]. In particular, NAMPT-mediated NAD synthesis is associated to the transcription of circadian-regulated genes and sirtuin in metabolically active tissues [43]. However, another crucial step in the long sirtuin history was the characterization of mammalian sirtuins, seven different isoforms (sirtuin 1–7) [44]. SIRT1 is the most studied nuclear member, pleiotropic transcriptional factor that drives many cellular activities, like energy metabolism, cell survival, DNA stability, inflammation, and circadian rhythms [45, 46]. SIRT1 is involved not only in deacetylation of a specific histone but in complex chemical reactions for different pathways involved in metabolism, like target of rapamycin (mTOR)

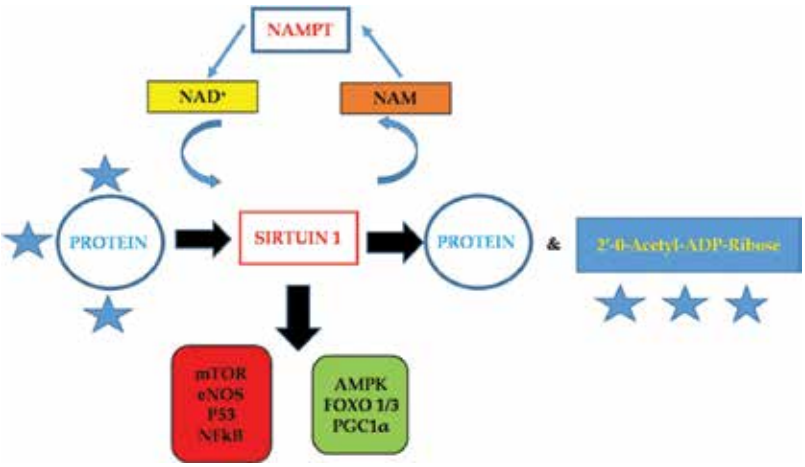

Figure 1. Sirtuin 1 deacetylase activity and its downstream substrates involved in longevity. The red square indicates the inhibited pathways and the green square the activated pathways. Stars indicate acetyl groups; (AMPK): 5′ AMP-activated protein kinase; (eNOS): endothelial nitric oxide synthase; (FOXO1/3): forkhead box protein O 1/3; (NAM): nicotinamide; (NAD^+): nicotinamide dinucleotide; (NAMPT): nicotinamide phosphoribosyltransferase; (NF-kB): nuclear factor k B; (p53): protein 53; (PGC1α): peroxisome proliferatoractivated receptor G coactivator 1α; (mTOR): mammalian target of rapamycin.

[47], insulin signaling [48], and forkhead box O (FOXO) [49] (**Figure 1**). Moreover, different mice overexpressing SIRT1 have been characterized that presented better metabolism, less inflammation, and cancer but a sex-dependent longevity (longer mean life span in males *vs* females) [50]. In these last 5 years, the involvement of SIRT1 in crucial cellular pathways has been demonstrated and the research of new drugs acting as SIRT1 modulators and relative patents exploded [28, 30, 51, 52]. A common intriguing idea is that if SIRT1 acts as a therapeutic target, specific drugs able to activate its signaling might be effective in specific age-related pathologies and in cancer. Herein, we resumed and discuss recent scientific data on SIRT1 modulators (STACs) and their potentiality assessed *in vivo*, in preclinical rodent models or in clinical settings.

3. Resveratrol and its derivatives as sirtuin 1 activators in aging

Since 2006 Sinclair's team in Harvard University reported that RSV, 3,5,4′-trihydroxystilbene, has a therapeutic potential to extend life span by miming caloric restriction to limit metabolic alterations in rodents in more than 140 genic pathways [23, 53]. RSV was added to the rodent diet at concentrations similar to human use (5.2 and 22.4 mg/kg/day) for 6 months. RSV was firstly isolated in late 1930s in leaves of white hellebore *Veratrum grandiflorum* and characterized as a phytoalexin [54], but later in 1990s, it was found in red wine and grapevines [55], in traditional herbs in Asia [56–58] in response to adverse conditions, in over 70 different plants and fruits like blueberry, raspberry, and mulberry [59]. Its first function was to reduce

inflammation and to limit oxidant damage in cardiovascular diseases, the so-called "French paradox." Intriguingly, the use of a glass of red wine reduced the extent of platelet aggregation and cardiovascular side effects in French people despite a high fat diet as reviewed in [60]. However, RSV is a photosensitive molecule, chemically composed by two aromatic rings linked by a methylene bridge, existing in two isomeric configurations, called *trans*-RSV and *cis*-RSV (**Figure 2**). Intriguingly, the most beneficial therapeutic properties are linked to *trans*-RSV even if, when exposed to light and high temperature, more than 80% of *trans*-RSV changes into *cis*-RSV with low solubility and stability [61].

Nevertheless, it has been calculated that more than 110 glasses of wine should be drunk to achieve an anticancer effect in humans [62]. Unfortunately, lipophilicity of RSV conditioned its bioavailability, low intestinal absorption, and rapid clearance from the plasma, making necessary a high dosage in preclinical and clinical trials [63, 64]. In humans, RSV was administered as a dose of 25 mg, then grew in the range from 25 to 1000 mg, up to 5 g daily for almost 1 month still well tolerated [65]. However, when administered in healthy volunteers in the morning, RSV bioavailability and pharmacokinetic ameliorated [66] and tolerability was maintained if associated with other drugs, like quercetin and alcohol [67]. To ameliorate half-life in the plasma, a modified version of RSV was produced, called Longevinex, able to prevent isomerization from *trans* to *cis* and it is rich of vitamin D3 (at a dose 1200 IU) and quercetin very effective in the metabolic syndrome [68] and cardiac health in mice [69]. Interestingly, also in humans, RSV mimicked caloric restriction [70] and attenuated obesogenic changes in the metabolic syndrome but had no effects under normal weight patients [71]. Herein, the RSV ability to sustain SIRT1 expression/activity is effective when the deacetylase is scarce, but unnecessary if SIRT1 level/activity is normal like in postmenopausal women with normal glucose tolerance or in slightly obese men and women (*trans*-RSV was taken at a dose 150 mg daily for 4 weeks) [72]. However, despite extensive data on efficacy of RSV in rodent preclinical trials, actually its beneficial role in humans is still debated, greatly depending

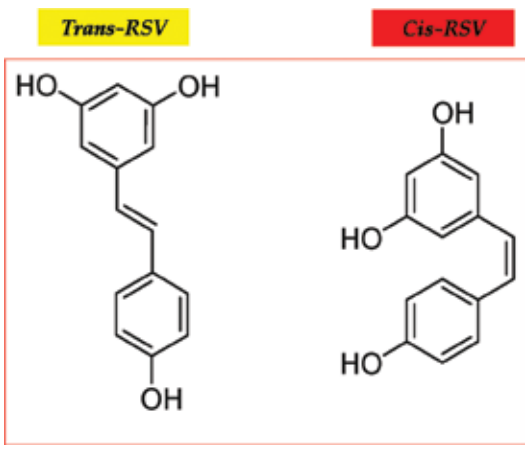

Figure 2. Resveratrol, 3′,5, 4′-trihydroxystilbene, isomers adapted from Nawaz et al. [78].

from doses and time of administration [73]. Antiaging properties of RSV via SIRT1 and mitochondrial health are addressed on amelioration of oxidative metabolism in crucial organs like heart and vessels, muscles, kidney but the mechanism is strictly cell-dependent [74–76]. Remarkably, at high doses, RSV has been reported to induce mild toxicity in humans where somnolence, headache, rash, and myalgia occurred [77]. Herein, to overcome these problems, in the past few years, different RSV derivatives have been synthesized *ex novo* by substituting hydroxyl with methoxy groups, so enhancing lipophilicity, or by adding a 4-hydroxy group in *trans*-RSV or a halogen group to potentiate the therapeutic efficacy [78], although different promising antimicrobial, antioxidant, and cardioprotective effects have been obtained *in vitro*. Moreover, to improve oral bioavailability of RSV-specific complexes with liposomes, lipid or synthetic nanoparticles have been made, even if they resulted with low therapeutic potential [79, 80]. In respiratory diseases reproduced in rodent like pulmonary hypertension, RSV ameliorates asthma and fibrosis via SIRT1 activation [81, 82] and also its derivative, trimethoxystilbene, demonstrated antioxidant and anti-inflammatory properties in rats exposed to hypoxia [83]. Recently, Bastin and Djouadi [84] reviewed RSV potentiality against mitochondrial damage and myopathies, and promising results in an animal model of a fatal genetic disease called Duchenne muscular dystrophy were reported. In dystrophic mice (mdx model), RSV supplementation (0.5% in the diet for 3 weeks) ameliorated muscle atrophy and prevented sciatic denervation signaling [85]. However, caution must be taken to extrapolate successful data obtained in animal models to humans where RSV might modulate different pathways in a dose-dependent manner. Autoimmune diseases represent an emerging type of chronic diseases often associated to aging that affect specific or multiple organs at the same time like rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, and type I diabetes [86]. RSV inhibited transcription factors crucial in autoinflammation, like nuclear factor k B (NF-kB) [87], and curiously in these pathologies, stopped SIRT1 activity. Intriguingly, RSV behaves as a potent drug mainly in animal models, although actually poor patient outcome and reduced number of clinical trials made necessary further studies in humans to exclude side effects or competition with other drugs [88]. RSV has been tested successfully in neuronal inflammation, psychotic mice (up to 30 mg/kg/daily) [89, 90], depressed menopausal women (orally 25 mg/day for 12 weeks) [91], and in patients with minimal hepatic encephalopathy, a frequent complication of cirrhosis [92]. However, the oral dosing of RSV seemed to cross the blood-brain barrier but more potent polyphenols specifically addressed on brain with promising preventive and not only therapeutic properties are under research [93]. Moreover, RSV administered 48 h prior the induction of a subarachnoid hemorrhage activated SIRT1 thereby reducing mortality and improving neurological functions in rats [94]. Pterostilbene is a natural phenolic drug found in sandalwood and some fruits like grapes and blueberries that ameliorated neuronal aging and memory deficit in rats [95]. Chemically, it is a dimethylether analogue of RSV with better pharmacokinetic and oral bioavailability in rats (80% in comparison to 20% RSV) [96]. Recently, pterostilbene demonstrated better efficacy than RSV in the modulation of behavior and functional improving in SAMP8 mouse, a rodent model of accelerated aging validated to study Alzheimer's disease [97]. Moreover, recent evidences indicated that pterostilbene worked well as cardioprotective and anti-inflammatory drug in rodents with ischemia-reperfusion [98]. However, both pterostilbene and RSV efficacy on aging and longevity have been reviewed recently by Li et al. [99], but conclusive data are lacking due to poor water solubility, low bioavailability,

and rapid elimination (half-life only 9 h in humans), the main obstacles to work in clinical trials. However, different strategies have been also undertaken to optimize the delivery of RSV to strengthen its clinical utility: one strategy was to complex it to controlled release devices, another avenue was to use micronized powder (called SRT501) [100]. A crucial year in the history of RSV pharmacology was 1997 when Jang et al. [101] firstly reported an antitumoral effect *in vitro* and *in vivo* in a murine model of skin cancer. However, SIRT1 played a double opposite role in cancer linked to severity grade. Indeed, in early cancer phases, when cellular mutations are scarce, to activate SIRT1 is a good strategy, but in advanced stages with many mutations, SIRT1 may potentiate and accelerate oncogenesis, as demonstrated in breast cancer [102]. Remarkably, Li et al. [103] provided evidences that dietary RSV for 4–5 weeks reduced prostate neoplastic lesions by about 50% in mice through SIRT1-mediated autophagy. More recently in chondrosarcoma cells, xenografted in mice, RSV via SIRT1 stimulated apoptosis and decreased tumor growth [104].

4. Synthetic sirtuin 1 activators (STACs) in aging

RSV and natural polyphenols, like quercetin and butein, have been included into the first generation of SIRT1 activators (STACs). However, to overcome limitations in RSV pharmacology, its aspecific interaction with several sirtuins like human sirtuin 5 and the competition with SIRT1 for the catalytic site [105], Sinclair laboratory since 2000s produced novel synthetic STACs [28] to fight aging-associated disabilities. The screening of potential STACS began *in vitro* looking for more than 18,000 drugs and resulted in 21 compounds able to stimulate the catalytic site in SIRT1 deacetylase enzyme [106]. Using a fluorescence polarization assay verified by mass spectrometry, novel synthetic STACs were obtained but a strong scientific controversy occurred on the efficacy of different drugs on SIRT1 activation. To date, the research on synthetic STACs (came to the fifth generation) has produced more soluble and specific compounds [30] with an *in vitro* 1000-fold greater potency than RSV to mimic calorie restriction. Intriguingly, STACs extended life span in obese mice [107] but also in mice fed a standard diet. [108]. Interestingly, SRT2104 was able to preserve muscle mass, strength, and bone integrity in muscle-atrophy induced by hind limb suspension and fasting [109]. Moreover, also SRT1720 behaved as an effective SIRT1 agonist *in vivo* in two independent rodent models of osteoporosis, where after 1 month or 3 months of oral treatment, femoral bone mass grew about 30% [110]. These studies have great translational implications considering the high frequency of fractures in human osteoporosis. Oral administration of SRT3025 (50 or 100 mg/kg/day) for 6 weeks, starting 6 weeks after ovariectomy, successfully reversed scarcity of bone mass and osteoporosis in mice [111]. Remarkably, SRT1720 intraperitoneally injected daily in female obese mice for 6 weeks improved the vitality of follicles via sustained SIRT1 able to reduce atresia and the abnormal primordial follicles activation [112]. In human plasmacytoma xenografted mice, a model utilized to validate new therapies against multiple myeloma, oral treatment with SRT1720 (200 mg/kg) on five consecutive days/week schedule for 4 weeks reduced tumor growth in combination with other drugs, like bortezomib, potentiated antimyeloma effects [113]. Several STACS have been tested as SIRT1 modulators in preclinical rodent model to fight diabetes, obesity, neurodegeneration, atherosclerosis, bone and muscle mass [114], and most relevant diseases investigated in the preclinical trials have been resumed in **Figure 3**.

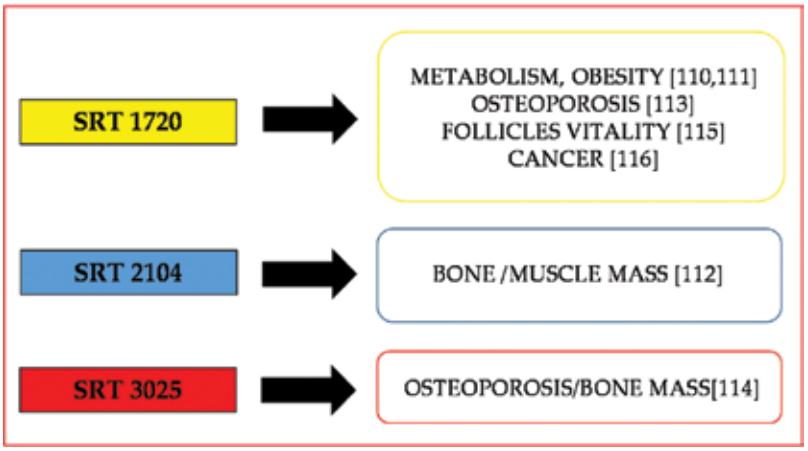

Figure 3. Synthetic SIRT1 activators (STACs) effective in rodent models.

Later, since 2012, an STAC phase I clinical trials started in humans [115]. SRT2104 in doses ranged from 0.03 to 3 g was well tolerated with a bioavailability about 14% in male and female volunteers. Remarkably, the same drug was also tested in elderly volunteers with no side effects [116]. In these last years, various human clinical trials with STACs have been started but to date the only ended with SRT2104 in patients with moderate to severe psoriasis demonstrated a promising efficacy [117]. Despite some encouraging evidences, in other clinical trials, SRT2104 administration for 28 days to diabetic patients (n = 15) was ineffective on insulin resistance and endothelial function but induced a striking weight reduction not observed in placebo [118]. Moreover, patients with mild to moderate ulcerative colitis were treated with SRT2104 at 50–500 mg daily for 8 weeks, and they not presented any clinical remission so the clinical trials stopped [119]. However, the times are ripe to start further clinical trials and to test novel STACs for longer times, hoping in new exciting results.

5. Recommendation

RSV, the main member of the first generation STACs, is not found in meat or dairy but is present in vegetables and herbs. Renisalo et al. [120] have indicated main sources of RSV in vegetables and seeds like cocoa, grape, hop, peanuts, pistachios, tomato, and berries. However, RSV is also present in common Asian herbs like *Polygonum cuspidatum*, known as Japanese knotweed. Its dried roots have been infused to produce "Itadori" tea, which means "well-being" in Japanese, a folk beverage largely used for the treatment of heart disease and stroke [59].

To date, pleiotropic effects of *trans*-RSV in degenerative and metabolic diseases in elderly are recognized, but despite a lot of evidences *in vitro* and in rodent model, their therapeutic role in humans is still debated. Firstly, its beneficial effect is strictly dose-dependent, and to potentiate mitochondria health, it is required at least an oral dose of RSV of 1 g/day in Alzheimer's patients and generally from 0.5 g up to 5 g/day to reach a therapeutic level in plasma (5 μM) [121]. Furthermore, RSV efficacy is also time-dependent, and an oral daily intake for 2 months is effective in patients with angina pectoris only if *trans*-RSV (at 20 mg) is

associated with calcium fructoborate [122]. Moreover, controversial RSV effects reported on nonalcoholic fatty liver (NAFLD) are probably linked to different oral doses (from 500 mg/day up to 3000 mg/day), time of administration (from 56 days up to 6 months), and number of patients considered in clinical trials [123]. On the contrary, promising results have been recently obtained on 119 patients with mild to moderate Alzheimer's disease orally supplemented with RSV at 1 g twice a day for 52 weeks, which presented reduced inflammatory markers in plasma and cerebrospinal fluid [124]. Herein, there is still a lot of research to do on the RSV and other STACs drug and caution must be taken to sustain their definitive clinical therapeutic effects in humans.

6. Conclusions

A constant effort has been made to synthesize novel drugs to extend life span, to prolong the quality of the life in elderly, and to limit age-associated diseases. Unfortunately, to date, there is no drug that could be an elixir of longevity for humans even if more than a hundred clinical trials on natural or synthetic SIRT1 activators are ongoing worldwide. However, there are still many challenges to discover and test the efficacy of antiaging drugs in clinics together with the necessity to get funds for a long time. The main final message from this chapter may be that a big scientific awareness is placed in the aging research, as indicated by the tremendous number of published articles on this topic. Indeed, all actors in the biogerontology scenario agree that not only the extension of the life is important in elderly, but it is fundamental its quality, maintaining a good health. To reach this aim, dietary intervention with caloric restriction mimetics is crucial, but remember to start early during adult age to best adapt your metabolism to counteract aging.

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Conflict of interest

Authors declare no conflict of interest.

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Section 3

Resveratrol and its Role in Chronic and Degenerative Diseases: Adding Life to Years

Chapter 7

Resveratrol in Management of Diabetes and Obesity: Clinical Applications, Bioavailability, and Nanotherapy

Vinitha M. Thadhani

Additional information is available at the end of the chapter

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Abstract

Diabetes is the most common serious metabolic disorder and one of the five leading causes of death worldwide. It is characterized by persistent hyperglycemia coincident with the induction of oxidative stress and alterations in glucose and lipid metabolism–regulating enzymes. Resveratrol has immerged as one of the leading natural ingredients to combat diabetic and its complications. Despite an abundance of laboratory and animal research, there is little clinical evidence to establish resveratrol effectiveness as a therapeutic against diabetes. Further, the poor bioavailability and stability of resveratrol in humans have been a major concern for translating basic science findings into clinical utility. In this review, we embark on large, well-controlled clinical studies to confirm the efficacy of resveratrol in the management of diabetes mellitus and gain a better insight into its biological effects in humans. Further possible methods of increasing the stability and bioavailability for such trials are also discussed.

Keywords: resveratrol, antidiabetic, antioxidant, bioavailability, clinical trials

1. Introduction

Diabetes mellitus (DM) has been an ever-increasing global epidemic and one of the most challenging health problems of twenty-first century. In 2010, more than 285 million people about the world were afflicted with diabetes, and it was then calculated that the number of people with diabetes will increase to 439 million by 2030. Interestingly, the reports of 2015 show that globally 415 million (215.2 million men and 199.5 million women) had DM with a prevalence of 8.8%, and projections indicate that approximately 600 million people would be suffering from diabetic in 2030. In other words, one in eleven people has DM. The economic impact

of diabetes is extensive. A significant component of health care expenditures is attributed to diabetes, and its complications and global spending for treating it in 2015 alone were US$ 673 billion (12% of health expenditure) [1].

Two primary groups of DM are distinguished: (1) autoimmune T1DM or insulin-dependent DM or juvenile DM and (2) T2DM or noninsulin-dependent DM or maturity onset DM. Close to 90% of people with DM around the world have type 2 DM (T2DM) [2].

The treatment of T1DM requires insulin replacement via injections as the pancreatic β-cells are destroyed and do not secrete adequate insulin. On the other hand, T2DM is characterized by insulin resistance and a decreased capacity of insulin secretion by β-cells. Natural/herbal medicines that have claimed to be effectual in the treatment of DM are thus more efficient in the treatment of T2DM [3]. They act either as insulin sensitizers or as substances that reduce the plasma glucose levels.

Further recent inventions on natural products have established a new understanding into the use of antioxidants to combat diabetic complications [4]. Oxidative stress leads to the dysfunction of β-cells and thus plays a crucial role in the pathogenesis of diabetes and its associated complications. In fact, increasing morbidity and mortality rates of T2DM patients are mainly due to the high occurrence and severity of diabetic complications.

Thus, β-cells apoptosis can be protected, and their functions can be preserved by the use of antioxidants [5]. Thus, a potent antioxidant compound is expected to show greater effects on diabetes and its associated complications. Therefore, antioxidant therapy is, a different, innovative but, a fundamental approach for treating diabetic complications [6, 7].

The antioxidant activity and its related health benefits of dietary plant polyphenols are well documented. In recent years, there is growing evidence on the effectiveness of plant polyphenols against the treatment of type 2 diabetes mellitus and its ramifications. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include flavonoids, phenolic acids, β-orcinols, stilbenes, and lignans. **Figure 1** illustrates the different groups of polyphenols and their chemical structures reported as antioxidant and antidiabetic activities.

The hypoglycemic effects of polyphenols are mainly ascribed for reducing the intestinal absorption of dietary carbohydrate, for the modulation of carbohydrate and lipid metabolism enzymes, and they stimulate insulin secretion and insulin action and improve β-cell functions by reducing oxidative stress, stress-sensitive signaling pathways, and inflammatory processes [8].

We have already reviewed the antidiabetic effect of least-studied β-orcinol compounds of lichen origin and found in accordance of its antioxidant and antidiabetic effect [9]. The current chapter focuses on the reported antidiabetic effect of stilbenoid type polyphenols. Stilbenoids are phylotaxins and are mainly found in *Vitis vinifera* L., the wine producing grape fruits, together with other plant families, such as Dipterocarpaceae, Gnetaceae, and Fabaceae.

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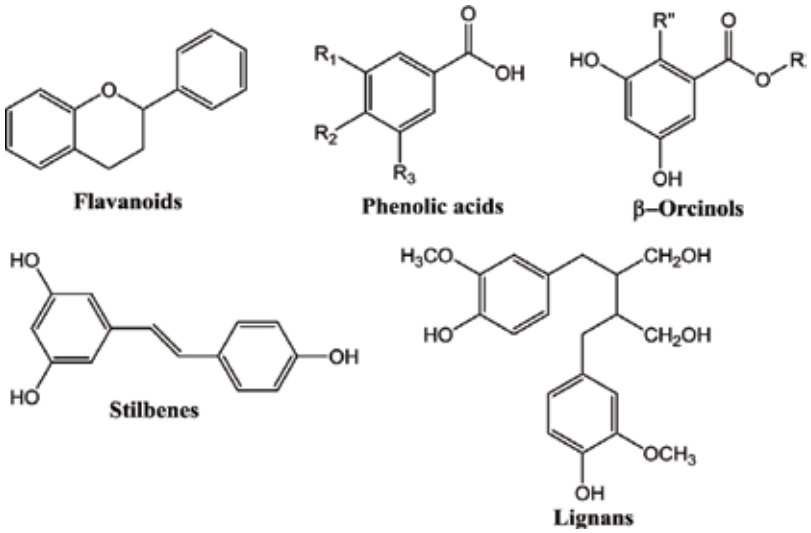

Figure 1. Chemical structure of major classes of polyphenols reported for antioxidant and antidiabetic effects.

The backbone structure of stilbene which is 1,2-diphenylethylene is common, but the type and position of substituents on the rings differ. The hydroxylated derivatives of stilbenes provide the class with a wide variety of polymerization and oligomeric construction. However, the most widely studied hydroxylated stilbenoid is resveratrol (3,5,4′-trihydroxystilbene), which is considered as one of most potent natural biological active compound. The other structural analogs with potentially beneficial medicinal properties include pterostilbene (methylated derivatives), viniferin (glycone derivatives), and hopeaphenol (oligomeric forms—tetramer). Some of the common stilbenoid structures are illustrated in **Figure 2**.

Biological activities of resveratrol have been well examined by a great variety of test systems. Its beneficial properties on humans include neuroprotective, antiviral, antiatherogenic, and estrogen-like growth-promoting effect. Further, its effects on promotion of vasodilatation and prevention of platelet aggregation and its positive effect on the circulatory system especially by increasing production of high-density lipoprotein cholesterol and preventing the development of arteriosclerosis are reported. Furthermore, it was shown that resveratrol is a chemopreventive agent [10]. Due to the wide variety of biological activities shown by this marvel compound, resveratrol-based medicinal chemistry has become rapidly evolving and increasingly active topics in the past decade, covering almost the whole range of therapeutic fields. There are several reports composing the antioxidant, antiinflammatory, and antidiabetic effect of resveratrol. Due to its antiinflammatory and antioxidant effects, resveratrol can mitigate the development of diabetic complications associated with inflammation and oxidative stress. Beneficial effects of resveratrol on the management of blood glucose in diabetes are summarized in **Figure 3**. The aim of this chapter is to highlight the importance of resveratrol along with other stilbenes as an antidiabetic compound with antioxidant properties.

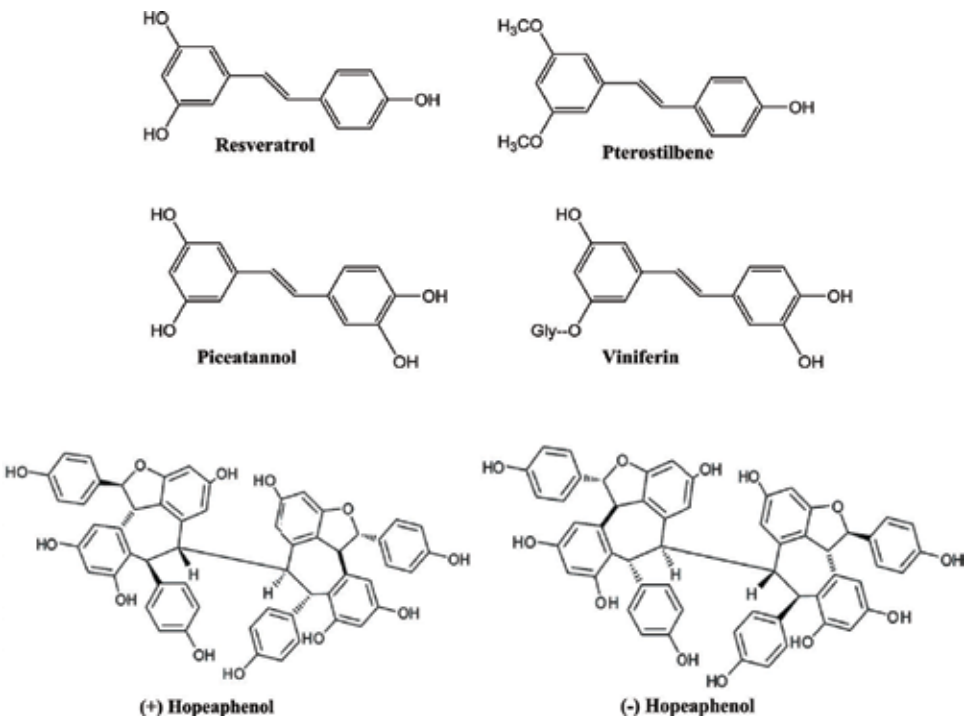

Figure 2. Most common stilbene derivatives reported for antidiabetic activities.

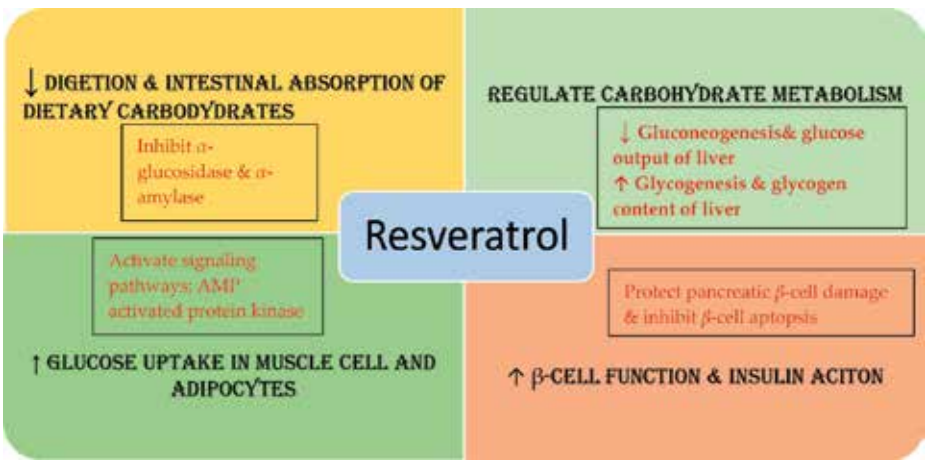

Figure 3. Beneficial effects of resveratrol on the management of blood glucose in diabetes.

2. The role RSV in obesity and diabetes and its molecular mechanism

The appreciable magnitude of scientific evidence is available, which ascribes antidiabetic properties of resveratrol and fights against obesity. There are over 800 publications ascribing

the hypoglycemic action of resveratrol, through both *in-vivo* and *in-vitro* studies. Multiple modes of action, and diversity of molecular targets, keep resveratrol well ahead of its other natural analogs. Modes of action include inhibition of carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase), through their role as an effective antioxidants and through their effect on amelioration of insulin sensitivity. The resveratrol improves defective insulin signaling, prevents pancreatic β-cell apotopsis and dysfunction, inhibits abnormal glucose uptake and storage, mitigates hyperlipidemia and dyslipidemia, and thus shows high pharmokinetic potential as antidiabetic agent [11]. The complex physiological action of resveratrol as an antidiabetic agent could be attributed to its capacity to modulate different pathways and to its diversity of molecular targets including phospodiesterases, adenylyl cyclase, kinases, sirtuins, transcription factors, cytokines, and others, some of which are described below.

Pancreatic β-cells are key players in the development of T2DM, as they are required to secrete increasing amounts of insulin so as to compensate for increasing insulin resistance. Consequently, the β-cells come under increasing metabolic stress and finally their function deteriorates. Thus, it is important to find a mean to preserve the health of β-cells.

Cyclic nucleotide phosphodiesterases (PDEs) belong to a class of enzymes that hydrolyze the phosphodiester bonds of cAMP and cGMP to their biologically inactive 5′ derivatives. Cyclic AMP is known as a key mediator of metabolic regulation. Resveratrol acts as PDE inhibitor, leading to increased cAMP levels, which amplifies glucose-induced insulin secretion [12].

Resveratrol triggers cascade of biological pathways that are induced during calorie restriction. Primarily increased cAMP levels activate PKA (protein kinase A), which directly phosphorylates and activates histone deacetylase Sirtuin1 (SIRT1), which increases insulin sensitivity and protects against metabolic damage resulting from a high-fat diet. In detail, SIRT1 catalyzes NAD^+-dependent protein deacetylation, yielding nicotinamide and *O*-acetyl-ADP-ribose. SIRT1 facilitates the conversion of changes in the nutritional status, which it senses via NAD^+ levels, mediates the metabolic stress situations, such as high-fat-diet-induced obesity, and plays a context-dependent role in health span regulation. In addition to the c-AMP mediated pathway, resveratrol also increases SIRT1 activity through an allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD^+ and the acetylated substrate. SIRT1 promotes many beneficial metabolic changes, such as an increase in fatty acid oxidation, gluconeogenesis, and mitochondrial respiration and a decrease in triglyceride synthesis, glycolysis, ROS production, and inflammation. In light of the rising number of patients suffering from metabolic diseases, compounds that activate SIRT1 directly or indirectly offer protection against the onset of metabolic damage and encourage healthy aging [13].

The regulation of glucose uptake and its subsequent utilization is critical for the maintenance of glucose homeostasis. Homeostasis of blood glucose by insulin involves stimulation of glucose uptake by translocation of glucose transporter Glut-4 from intracellular pool to the caveolar membrane system. Resveratrol increases the expression of this glucose transporter Glu-4 and excites the glucose uptake.

Skeletal muscle is the largest organ in the body and contributes to immeasurable features of organismal biology, and its dysfunction stimulates numerous diseases, including diabetes. Skeletal muscle is the main site of glucose disposal after glucose ingestion. Insulin resistance in skeletal muscle is thus the main driver of postprandial hyperglycemia. The transcriptional

coactivator PGC-1α has emerged as a key driver of metabolic programming in skeletal muscle, both in muscle health and disease. PGC-1α has different roles in different tissues, but in nearly every context, PGC-1α stimulates the transcriptional program of mitochondrial biogenesis. PGC-1α dysfunction, and thus mitochondrial insufficiency, contributes to insulin resistance in skeletal muscle. Resveratrol also has proven to enhance the PGC-1α–skeletal muscle protein levels.

Further resveratrol also activates Akt expression, a modulator of insulin-signaling pathway. Akt is the major effector of the IR-IRS-1-PI3K pathway and is activated by phosphorylation. Resveratrol treatment increases the phosphorylation level of Akt, particularly of its Thr308 and Ser473 residues which is essential for its basal and full activation.

Several studies have found that resveratrol has positive effects on inhibiting the insulin secretion from pancreatic β-cells and prevents it from chronic overstimulation, decreases the plasm insulin concentration, and increases the insulin sensitivity. Possible explanations include resveratrol-mediated suppression of cytokine action through decreased DNA binding of nuclear transcription factor κB, production of nitric oxide, and expression of inducible nitric oxide synthesis [14].

2.1. Antioxidant effect of RSV

The advancement in the knowledge of potent antioxidants has uncovered the way for greater insight in the treatment of diabetic complications. The antioxidant activity of resveratrol is well proven, and there is a good accordance between antioxidant and antidiabetic activity of resveratrol. Resveratrol maintains the concentration of intracellular antioxidants in biological systems by dual methods, that is, by acting as scavenger of free radicals and by increasing the activity of antioxidant enzymes such as catalase, glutathione peroxidase, glutathione S-transferase, and glutathione reductase [14]. In a study on isolated liver mitochondria, addition of resveratrol to the incubation medium significantly increased the activity of manganese-containing superoxide dismutase and diminishes ROS generation. Resveratrol acts as a free radical scavenger of ROS and reactive nitrogen species such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide, thus, prevent DNA lesions and lipid peroxidation in cell membranes. It has been shown that resveratrol significantly reduced the oxidation of thiol groups in proteins of human platelets [15].

2.2. Effect of RSV on carbohydrate metabolism, through its α-amylase and α-glucosidase inhibitory activities

α-Amylase and α-glucosidase are key enzymes involved in carbohydrate digestion. α-Amylase hydrolyzes starch and glycogen into maltose and ultimately increases the blood sugar. α-Glucosidase hydrolyzes oligosaccharides and disaccharides into glucose, which is absorbed through the gut wall to become blood glucose. Thus, inhibition of the activity of these enzymes is viewed as one of the most effective therapeutic approaches in the reduction of glucose levels in plasma, as a consequence, the suppression of postprandial hyperglycemia.

Various plant extracts containing resveratrol have been evaluated for α-amylase inhibitory activity and have shown beneficial effects in bringing down the pace of digestion and assimilation of sugars and thereby leading to the effective management of type 2 diabetes by decreasing the postprandial hyperglycemia, some of which are highlighted below.

Antioxidant and α-glucosidase inhibitory potential of reservatrol isolated from *Rumex bucephalophorus* have been reported, which revealed that reservatrol was at least five times more potent α-glucosidase inhibitory activity as compared to standard drug acarbose [16]. A study on peanut extracts correlated the reservatrol content with the α-amylase and α-glucosidase inhibitory activity. The EtOAc extracts of peanuts with higher resveratrol content (3 μg/ml) showed higher α-amylase and α-glucosidase inhibitory activity (4.32 and 5.93%, respectively) as compared to MeOH extract (3.9 and 4.9%) with resveratrol content of (0.5 μg/ml). The standard resveratrol sample showed α-amylase and α-glucosidase inhibitory activity (5.18 and 5.94%) [17].

In another study, resveratrol had shown potent α-glucosidase inhibitory activity against both yeast and mammal α-glucosidase with (IC_{50}, 0.091 mg/ml) and (IC_{50}, 0.12 mg/ml), respectively. The standard drug acarbose showed IC_{50} = 0.247 mg/ml (yeast α-glucosidase) and IC_{50} = 0.013 μg/ml (mammal α-glucosidase) [15]. Piceatannol, with an additional OH group as compared to resveratrol, showed higher α-glucosidase inhibitory activity as compared to resveratrol [18].

In a study, wistar rats when administered with 30 mg/kg BW resveratrol 60 min prior to sucrose- or starch-loading had a delayed absorption of carbohydrates, resulting in significant lowering of postprandial blood glucose concentrations [18].

The structure activity relationship of polyphenols isolated from other plant sources has been extensively reviewed as inhibitors of α-amylase and α-glucosidase. Detailed SAR has revealed that both α-amylase and α-glucosidase share the same properties in terms of structural requirements for inhibition [19]. Studies reveal that inhibitory activity is influenced by a number of hydroxyl groups and their positions, methylation, methoxylation, glycosylation, etc. Broadly, it is considered that hydroxylation of phenols increases the α-amylase inhibitory activity and methoxylation, which blocks the free hydroxyl groups and reduces the inhibitory activity [19]. Apparently, the activity is increased by more phenolic substitutions. Piceatannol with four OH groups showed higher activity than resveratrol with three free hydroxyl groups as for the study of Zhang et al. [18]. This is further evident by the study of Lam et al. [26], where several stilbenoids isolated from seeds of *Syagrus romanzoffiana* were evaluated for inhibitory activity against α-glucosidase *Bacillus stearothermophilus*. Pentahydroxystilbene (5 OH groups) showed higher inhibitory activity (IC_{50} 19.23 μM) as compared to piceatannol with 4 OH groups (IC_{50} 23.24 μM).

Molecular docking studies have revealed that, overall, the inhibitory activity of phenols depends on two parameters: (i) hydrogen bonding capacity of the OH groups of the phenols with the side chains of amino acids such as Asp197 and Glu233, and (ii) planarity of aromatic rings to form an efficient conjugated π-π system with the indole Trp59 of the active site [20].

(+) Hopeaphenol and (−) hopeaphenol oligomer (tetramer) of resveratrol isolated from *Ampelocissus indica* (L.) and *Vateria indica* Linn., respectively, displayed IC_{50} values of 21.21 ± 0.987 and 9.47 ± 0.967 mM in an α-glucosidase inhibitory assay [21], which were higher than the standard acarbose (IC_{50} 81.3 ± 1.10). The compounds showed a concentration-dependent inhibition of both α-glucosidase and α-amylase enzymes. Further, the study also indicated the positive effect of hopeaphenols as antiglycating agents, with IC_{50} values of 81.9 ± 1.176 and 50.96 ± 0.897, respectively, for (+) and (−) isomers which again were less than the ascorbic acid standard (IC_{50} 158.23 ± 0.718). The results indicated that the hopeaphenols can be a promising natural compound in diabetic management [21]. The effect of glucose uptake performed by 2-NBDG in L6 rat skeletal muscle cells using flow cytometry (BD FACS Aria II, USA) showed potent glucose uptake by (+) and (−) hopeaphenol of 31 and 26.4%, respectively [21].

Few reports exist on the *in-vivo* studies of stilbenoids other than resveratrol. Among them is the effect of pterostilbene, which improves glycemic control in insulin-resistant obese rats by increasing hepatic glucokinase activity and increasing skeletal muscle glucose uptake [22]. *In vitro* studies also indicate that pterostilbene protected pancreatic beta cells against oxidative stress and apoptosis [23]. Antihyperglycemic properties of pterostilbene along with other phenolic constituents of *Pterocarpus marsupium* have been reported [24, 25], whereas pterostilbene has been shown to be beneficial in animal models of diabetes and metabolic disorders. Further, the study by Lam et al. also revealed that pentahydroxystilbene (3,3′,4,4′,5′-pentahydroxy-trans-stilbene) possesses significant effect in reducing the postprandial blood glucose level of sucrose-challenged normal wistar rats [26].

2.3. Clinical studies on RSV on diabetic

Although numerous data exist on the beneficial outcomes of resveratrol in diabetic animals and *in vitro*, there are limited studies that have specifically investigated the antidiabetic effects of resveratrol in humans. Further, because of not only a limited number of clinical surveys, but also limited sample size and conflicting data, the use of resveratrol as an effective antidiabetic agent has been delayed [27]. Few of the reported clinical trial data are discussed below.

Glycated hemoglobin (HbA1c) levels reflect glycemic control and can, consequently, be employed as a predictor of the microvascular and macrovascular complications associated with type 2 diabetes. HbA1c levels seem to be determined by postprandial hyperglycemia. Bhatt and colleagues demonstrated that resveratrol (250 mg/day for 3 months) administered along with glibenclamide and/or metformin demonstrated improvement in glycaemic parameters in diabetic patients as compared to metformin or glibenclamide alone [28]. The study reported improvement in HbA1c, systolic blood pressure, and total cholesterol in patients with type 2 DM treated with resveratrol combined with the oral hypoglycemic agents. Recently, Movahed and colleagues also reported [29] that 1 g/day of resveratrol supplementation for 45 days notably reduced fasting blood glucose, HbA1c, insulin, and systolic blood pressure. Brasnyó and colleagues [30] reported an improvement in insulin sensitivity in type 2 diabetic patients after treatment with a much lower dose of resveratrol (5 mg twice daily) for 4 weeks. The study showed that resveratrol did not cause any changes in a glucose-dependent

insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) levels in diabetes patients. However, they did show that resveratrol significantly decreased insulin resistance and blood glucose and delayed glucose peaks after meals. In the study, resveratrol treatment was shown to significantly decrease HbA1c, systolic blood pressure, and total cholesterol. A decrease in oxidative stress assessed by measuring urinary ortho-tyrosine excretion, a bio marker of oxidative stress, was also reported. Nevertheless, the authors found no evidence that resveratrol influenced homeostasis model of assessment of β-cell function (HOMA-β) and therefore suggested that the mechanism of antidiabetic effects might be referable to a reduction in oxidative stress and a more-efficient insulin signaling. Resveratrol activated the Akt insulin signaling pathway by increasing the phosphoAkt:Akt ratio in platelets.

Most significant notice from the above two studies is the extra security of resveratrol as compared to available standard antidiabetic medication [31].

In contrast, in a randomized control trial by Thazhath et al., 500 mg of resveratrol was administration twice daily for 5 weeks in diet-controlled type 2 diabetes. The study revealed no significant improvement in glycemic control [32]. They studied two incretin hormones that affect postprandial hyperglycemia: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) from the bowel. In healthy people, both hormones stimulate insulin, but in type 2 patients, only GLP-1 can act to stimulate insulin. GLP-1 can also suppress glucagon secretion and energy intake and slow gastric emptying, thereby targeting postprandial hyperglycemia. In rodent models, resveratrol has been shown to upregulate GLP-1 and lower glycemia, but Thazhath et al. found that in human patients, there was no difference between the GLP-1 secretion, fasting glucose level, postprandial glucose level, HbA1c, gastric emptying, body weight, or energy intake in the resveratrol-treated versus the placebo group. As such, resveratrol's efficacy in improving glycemic control is indeterminate. Similarly, resveratrol treatment for 6 months did not improve metabolic parameters in type 2 diabetic patients [33].

Crandall et al. studied older adults with impaired glucose tolerance (IGT), a major risk factor for diabetes as well as cardiovascular disease. They establish that although fasting plasma glucose was unchanged with low dose of resveratrol treatment, peak postmeal glucose and 3-h glucose declined. Further postmeal insulin decreased and insulin sensitivity imporved. Thus, established resveratrol as a promising therapy for insulin resistance [34].

A meta-analysis was carried out by Liu et al. in 2014 [35] and more recently by Zhu et al. in 2017 [36] with the aim of qualitatively comparing the published data on effect of resveratrol on plasma glucose levels, glycated hemoglobin (HbA1c), and insulin sensitivity. A fixed-effect model analysis was carried out to pool the data, nine studies with 283 participants in case by study of Zhu et al. and 388 participants of 11 eligible studies in case of Liu et al. The sample size of studies varied from 8 to 66 participants, with resveratrol dose ranging from 8 mg/d to 3000 mg/d and with duration of intervention differing from 2 weeks to 12 months. Both meta-analysis studies revealed that resveratrol was able to reduce the fasting plasma glucose levels only at high concentrations (1 g/day) as compared with placebo/control in patients with T2DM. Resveratrol was unable to reduce the plasma glucose levels at low concentrations. These studies also revealed that compared to placebo group, the patients who received

resveratrol supplement also showed low insulin levels. Further resveratrol was also effective in reducing the systolic and diastolic blood pressures. However, no significant difference was observed in LDL and HDL levels.

On the other hand, effect of pterostilbene on human type 2 diabetes is yet to be researched. Administration of blueberry (*Vaccinium myrtillus*) and sea buckthorn (*Hippophae rhamnoides*) extract for children with type 1 diabetes for 2 months elicited a reduction in HBA1c levels and an increase in SOD and glutathione peroxidase levels [37]. Since pterostilbene has been isolated from *Vaccinium myrtillus* [38], this effect may be ascribable to the presence of pterostilbene alongside other bioactive compounds in the excerpt.

Some beneficial effects have also been reported in resveratrol treatment in nondiabetic humans. In obese subjects, Timmers and colleagues [39] reported significant improvement in the metabolic profile and general health after resveratrol supplementation for 30 days, thereby describing resveratrol as a calorie restriction mimetic. Resveratrol showed beneficial effects on glucose homeostasis and insulin sensitivity, reduced intrahepatic lipid (IHL) content and expression of inflammatory genes and improved mitochondrial efficiency. These effects may be linked with the activation of AMPK and increased SIRT1 and PGC-1α protein content in the muscle [39].

3. Way forward in clinical utility of resveratrol

One of the major challenges surrounding the clinical utility of resveratrol is achieving its stability and adequate bioavailability at tolerable doses—a common issue in translating promising findings from cell culture and animal models into clinical efficacious drugs. Recent clinical trials proved that resveratrol is well tolerated and pharmacologically safe at doses up to 5 g/day. However, the data on toxicity of resveratrol in long-term experiments are scarce.

Low solubility of resveratrol in water (<0.05 mg/ml), caused by its chemical structure, affects its absorption. Its reported oral bioavailability values range from 20 to 29.8%. After intravenous administration, resveratrol exhibited a very short half-life of 14 min due to rapid metabolism. This poor bioavailability can be ascribed to the rapid conjugation of trans-resveratrol to glucuronic acid and sulfates, producing glucuronides and sulfate conjugates that accumulate in plasma and urine.

In detail, resveratrol is absorbed in a relatively high rate through the small intestine either via passive diffusion due to its nonpolar character or through active diffusion across the intestinal epithelium via cell ATP-dependent binding cassette transporters. Inside the enterocytes of the small intestine and hepatocytes of the liver, the glucuronide and sulfate conjugation of trans-resveratrol to the major metabolites are extensive. This conjugation to sulfates and glucuronides increases resveratrol's aqueous solubility, reduces flux across membranes, preventing nonpolar molecules from interacting with essential macromolecules, and allows excretion by the kidneys via urine. The extensive metabolism to glucuronide and sulfate conjugates during absorption is well described and decreases circulating levels of free trans-resveratrol.

Thus, metabolism of resveratrol ultimately results in relatively small amounts of free trans-resveratrol in the plasma to be delivered to other tissues. Strategies to increase bioavailability from oral delivery of resveratrol are generally focused on increasing the rate of resveratrol absorption into the enterocytes and decreasing intracellular metabolism [40]. Further, the photostability of the resveratrol itself must also be considered when developing formulations, as resveratrol is sensitive to both heat and UV light. New approaches to increase the bioavailability of resveratrol can help to actualize its potentials as a therapeutic agent in DM and related complications.

Different approaches have been utilized by various researchers to increase the stability and bioavailability, some of which are discussed below.

3.1. Co-administration of resveratrol with other phenolic compounds

One simple approach to enhance bioavailability has been the consumption of resveratrol in combination with other phenolic compounds that play as the substrate for enzymes involved in resveratrol metabolism; such compounds which have demonstrated the positive effects are piperine, quercetin, etc. [41]. Combined effect of resveratrol along with curcumins was evaluated by Rouse et al. on animal models and human islet cell lines. Beneficial effects were demonstrated on insulin secretion by these naturally occurring polyphenols. However, the study revealed that the combination of resveratrol along with curcuminoids either did not yield any additional benefits or reduced the beneficial effects observed with the individual treatments. It would be noteworthy to test the combined effect of these two well-studied compounds on human models along with cinnamon and another known natural compound effective for diabetics. Further, clinical data are available on co-administration of resveratrol with various food and beverage, which contain subsequent amounts of other polyphenols such as grape juice, etc.; unfortunately, neither of these studies included a control condition to determine whether food or beverages enhanced or impaired bioavailability compared to resveratrol itself [41]. Due to 3-hydroxyl groups, resveratrol rapidly undergoes glucuronidation or sulphation. The presence of two methoxy groups in the pterostilbene structure makes it more lipophilic and thus more bioavailable and also more metabolically stable because it has only one free hydroxyl group available for glucuronidation or sulphation. However, the data also reveal that more the free hydrozyl groups, it shows better activity in *in-vitro* assays. Furthermore, administration of pterostilbene in a clinical trial at a dose of 125 mg twice daily for 6–8 weeks was found to be safe and did not evoke any remarkable adverse reactions. Still, there are no clinical studies on the antidiabetic effect of pterostilbene on diabetic patients and its co-treatment with resveratrol.

3.2. Prodrugs and resveratrol formulations to increase the stability and bioavailability

Another approach to increase the absorption of resveratrol in the gastrointestinal tract is improving the material properties of resveratrol used in the oral dosage, given the rapid metabolism of resveratrol. This is the basis for SRT501, the patented formulation of micronized oral version of resveratrol that may have higher bioavailability. In this process, resveratrol is

microionized to particle sizes <5 μm, mixed with flavorings, colorings, and emulsifying agents such as docusate sodium and mixed with water for ingestion. The small particle size with the emulsifiers in solution theoretically increases surface area for intestinal absorption while also improving suspension properties [42].

Another approach to maximize the bioavailability of free trans-resveratrol is to develop resveratrol prodrugs, which could be used to improve the anti-diabetic efficacy of resveratrol. Assuming that maximizing free trans-resveratrol is the primary goal, resveratrol prodrug generates *in vivo* resveratrol through enzymatic reactions. Some of these technologies have been investigated in animal studies with no report in humans. Metabolism of prodrugs into resveratrol in tissues of interest can maximize tissue concentration and can be beneficial in the treatment of tissue specific complications in diabetic patients. Targeted delivery of resveratrol prodrugs into tissues of interest via delivery systems such as liposome-mediated delivery or nanotechnological approaches may result in the improved therapeutic effect. Also, intravenous injection as an option to the traditional oral route of administration of resveratrol may bypass gastrointestinal absorption, conjugation, and hepatic metabolism, therefore resulting in increased bioavailability and improved results in diabetic patients.

3.3. Nanotechnological approaches to enhance the stability and bioavailability of resveratrol

A routine of recent surveys have concentrated on applying nanotechnology to improve the bioavailability of resveratrol and have generally demonstrated improved stability and bioavailability with minimal side effects compared to oral dosing. Nanoformulations can improve resveratrol's solubility and transport across the plasma membrane and therefore enhance its effects within cells.

The nanoencapsulation methods include polylactic coglycolic acid nanoparticles [43, 44], carboxymethyl chitosan nanoparticles [45], solid lipid nanoparticles [46], and cyclodextrin nanoparticles [47]. Studies revealed sustained release profiles, which enhanced plasma bioavailability compared to free resveratrol. Nanoencapsulation was also effective in improving the solubility and stability of resveratrol. All the same, no clinical or paraclinical studies have been done to determine the efficacy of resveratrol nanovectors against antidiabetic potential.

Nanovectors delivering resveratrol have been described by Singh and Pai that drew a sustained release of trans-resveratrol from orally administered polylactic-co-glycolic acid nanoparticles (drug encapsulation efficiency more than 78%, with a molecule size of about 170 NM) [43]. The same authors encapsulated resveratrol in Eudragit RL 100 nanoparticles with a drug incorporation efficiency of 84% and the average size of 180 nm. *In vivo* studies in a rat model showed prolonged plasma levels up to 16 h, in comparison with the free drug being cleared within 6 h [44]. Zu et al. developed carboxymethyl chitosan nanoparticles as a carrier for resveratrol [45]. These nanoparticles (155 nm-sized, with an encapsulation efficiency of 44%) improved the solubility of resveratrol, thereby greatly affecting the antioxidant activity of the drug. Additionally, resveratrol-loaded solid lipid nanoparticles were synthesized with a controlled release profile, due to an initial burst release of 40% caused by the active

principle associated with the particle shell and a subsequent prolonged release of the drug located in the lipid matrix. In this system, the efficiency of the cellular uptake depended on the molecular interactions with the biological membrane organization, lipid rafts, and the actin cytoskeleton invaginations for the receptor-mediated entrance [48]. Resveratrol-loaded solid lipid nanoparticles have been also prepared by Pandita et al. with a drug incorporation efficiency of 89% and an average diameter of 134 nm [46]. This drug delivery system showed prolonged release *in vitro* up to 120 h in a Wistar rat model, enhancing plasma bioavailability compared to a free drug suspension. Finally, cyclodextrins-resveratrol complexes have been used to increase the concentration of polyphenol in aqueous solution while maintaining its biological activity. For example, spherical cyclodextrin-based nanosponges showed increasing solubility and stability, together with good drug encapsulation efficiency, compared to free resveratrol [48].

4. Conclusion

There is a large body of evidence indicating resveratrol as an antidiabetic agent. Numerous studies have demonstrated that resveratrol can prevent, attenuate, or reverse diabetic dysfunction through diverse mechanisms and multiple molecular targets, which lead to pleiotropic therapeutic action in the whole organism. The exerted effects include inhibition of carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase) resulting in improved glycemic control, antioxidant properties, and antiinflammatory properties, which ultimately ameliorates diabetes and its complications. Resveratrol enhances insulin sensitivity and decreases insulin resistance, by changes in expression and activity of phosphodiesterases, kinases, AMPK, and SIRT1 in different tissues, which ultimately leads to protection of pancreatic β-cells from deterioration.

Despite widespread use of resveratrol as a nutritional supplement and the fact that animal models have provided a strong case for resveratrol as an antidiabetic agent, however, due to limited number of well-designed human clinical trials and various other limitations, this compound is still under investigation as an antidiabetic drug. The poor stability and bioavailability of resveratrol in humans have been a major concern for translating basic science findings into clinical utility [49].

From the 11 human clinical trial data available on effect of resveratrol as antidiabetic agent, all studies have shown positive effect of resveratrol in reducing the fasting plasma glucose level at higher concentration. But still there remain many discrepancies such as on the Hb1AC levels. The origins of these discrepancies are not definitively known but may be due to different quantification techniques (e.g., HPLC vs. MS/MS, etc.), different formulations and dosing protocols, and differing sample size, dosage duration, and effects of other drugs/materials used in combination with resveratrol. In addition, inter-bioavailability of resveratrol can vary from person to person, which may cause inconsistent physiological responses between individuals and limited clinical applicability. Thus, further well-defined clinical trials should exploit the efficacy of resveratrol itself or when used in combination with other antidiabetic

drugs (e.g., metformin, etc.) or with other known antidiabetic natural products (curcumins, cinamaldedhye, etc.) as a potential pharmaceutical intervention.

A number of approaches have been developed to improve the stability and bioavailability of resveratrol, including consumption with various foods containing multiple polyphenols and micronized powders, combining it with additional phytochemicals, controlled release devices, and nanotechnological formulations. Animal studies demonstrate that these advanced formulations could improve tolerability in humans while also increasing its bioavailability; nonetheless, these nanotechnological and other advanced approaches are yet to be attempted in humans [41]. A combinational approach, as well as improved formulations of resveratrol, may help to overcome the challenge of maintaining an effective concentration at the site of action for an appropriate period, which needs to be confirmed by human studies.

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This book intends to strike a balance between developments in research and the fact that researchers must absorb and link to scientific advances with clinical practice so that the management of diseases can be based on sound physiological concepts.

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It is our hope that this book will motivate readers to approach the evidence on the use resveratrol and thereby spark an interest in making further contributions to the current scientific debate and treatment development efforts.

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