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Review

The molecular targets of resveratrol ☆

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ABSTRACT

Resveratrol has emerged in recent years as a compound conferring strong protection against metabolic, cardiovascular and other age-related complications, including neurodegeneration and cancer. This has generated the notion that resveratrol treatment acts as a calorie-restriction mimetic, based on the many overlapping health benefits observed upon both interventions in diverse organisms, including yeast, worms, flies and rodents. Though studied for over a decade, the molecular mechanisms governing the therapeutic properties of resveratrol still remain elusive. Elucidating how resveratrol exerts its effects would provide not only new insights in its fundamental biological actions but also new avenues for the design and development of more potent drugs to efficiently manage metabolic disorders. In this review we will cover the most recent advances in the field, with special focus on the metabolic actions of resveratrol and the potential role of SIRT1 and AMPK. This article is part of a Special Issue entitled: Resveratrol: Challenges in translating pre-clinical findings to improved patient outcomes.

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene, Rsv) is a small polyphenol that has been intensively studied for a couple of decades in a large spectrum of therapeutic research areas. The natural occurrence of Rsv in a large variety of plant species, in particular, mulberries, peanuts and grapes, has further fostered the attention of the general public and claims around the possibility of using Rsv in the fields of natural medicine and dietary supplementation.

To help understand the impact of Rsv on the general public and the scientific community, two concepts should be introduced: 1) the French Paradox and 2) the Caloric Restriction (CR). The concept of the French Paradox, introduced almost 25 years ago [1], summarizes the apparently paradoxical epidemiological observation that the French population has a relatively low incidence of cardiovascular complications despite having a diet rich in saturated fats. Although the validity of this observation has been a matter of debate, one of the implications derived from it rapidly gained momentum, i.e.: that there was a component in the French diet or lifestyle that could protect against the coronary disease complications associated with high saturated fat consumption. The impact of dietary habits on health is further enhanced by the second concept, CR. CR is defined as a moderate (normally, 20–40%) reduction in caloric intake in the absence of malnutrition or deficits in vitamin or mineral needs. To this date, CR is the most consistent non-pharmacological intervention increasing lifespan and protecting against the deterioration of biological functions in model organisms [2]. Almost 80 years ago, McCay and colleagues identified how CR increased maximal longevity in rats [3]. Multiple lines of evidence indicate that the effects of CR on lifespan extension stretch all along the evolutionary scale and up to a 50% increase in maximum lifespan has been reported in caloric restricted lower eukaryotes (such as yeast and worms), rotifers, insects, fish and mammals [4]. While data on how CR affects primate lifespan is not yet fully consistent, it seems clear that CR prevents age-related physical deterioration at the metabolic, cardiovascular and tissue damage levels, as well as cancer incidence [5,6]. The ability of CR to prevent numerous age-related diseases with apparently disparate etiology suggests that CR affects the fundamental basis of the aging process. However, it is the general view that there would be unwillingness in a large portion of the population to maintain a lifestyle based on CR so as to obtain health benefits. Therefore, as with the French Paradox, it is not surprising that there have been numerous efforts to understand how CR promotes health benefits and whether small molecule compounds could mimic the benefits of CR without the need to commit to major lifestyle changes.

2. Building the links between Rsv, Caloric Restriction and the French Paradox

While previous reports indicated an antioxidant action of Rsv, the initial finding that placed Rsv in the limelight was provided by the Pezzuto lab, which identified Rsv as a cyclooxygenase (COX) inhibitor with antitumoral effects [7]. Rsv efficiently protected against

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carcinogenesis models in the mammary gland and skin from mice [7]. These results fueled the possibility of using this natural compound as a cancer chemopreventive agent. Importantly, CR is an intervention that heavily protects against cancer development in rodents [8]. As discussed later, this was just the first of numerous findings suggesting that Rsv might have some CR-mimicking actions. A second line of evidence that raised the attention of the scientific community was the possibility of Rsv acting as a cardioprotective compound. Rsv effectively protected against plaque development in different animal models of atherogenesis [9,10]. In addition, Rsv enhanced endothelial nitric oxide production [11], potentially improving vasodilatation. The addition of Rsv to a high fat diet ameliorates arterial wall inflammation and other cardiovascular risk markers associated with aging in mouse models [12]. In porcine models, Rsv improved myocardial perfusion, and regional contractility while decreasing oxidative stress [13]. In a recent study using Rhesus macaques fed with high-fat/high-sucrose diet, a two year dietary supplementation of Rsv significantly reduced central arterial wall inflammation and stiffening, which accompanies most age-associated diseases, such as atherosclerosis, hypertension and diabetes [14]. This provided critical evidence indicating that Rsv supplementation could safely reduce many of the negative consequences of excess caloric intake on cardiovascular health. Studies in humans indicate that an acute administration of Rsv is enough to dose-dependently improve endothelium-dependent vasodilatation [15]. Also, a three month administration of Rsv at low doses (10 mg/day) had beneficial effects on left ventricle diastolic function, endothelial function and LDL-cholesterol in patients with coronary artery disease [16]. Other studies in obese humans have also observed that Rsv (150 mg/day) ameliorated plasma lipid profiles and inflammatory markers after one month of treatment [17]. The presence of significant amounts of Rsv in grape skin (20–100 μg of Rsv per gram of dry grape skin, depending on the grape variety) and, consequently, on wine (most notably, red wine, with a concentration around 1.5 to 3 mg/L) [7,18] rapidly forged a popular hypothesis in which Rsv, as a wine component, could explain the French Paradox. Nonetheless, it must be clarified that only a small fraction of the ingested Rsv reaches mammalian tissues. At doses similar to those achievable through normal diet, Rsv levels in plasma are either not detectable or clearly below the micromolar range [19,20], as used in vitro. Even doses up to 5 g would only lead to very low micromolar concentrations in blood (~2–4 μM) [19–21]. However, it must be taken into account that Rsv is a lipophilic molecule that can quickly move across membranes. Therefore, intratissular/cellular Rsv levels might be higher than what plasma levels suggest. Indeed, recent elegant evidence suggests that Rsv might be quickly metabolized in human colorectal cells into stable sulfate-conjugated forms, which would then gradually regenerate into the parental compound, providing the beneficial effects in vivo [21]. Whether Rsv and Rsv-sulfate forms are metabolized similarly in other tissues – e.g.: peripheral tissues, such as muscle or adipose tissue – however, has not yet been explored.

3. Resveratrol and Sir2/SIRT1 — metabolic health and longevity within our grasp

To understand how Rsv took center stage in the metabolic arena, one needs to jump back to 1999, when Kaeberlein and colleagues discovered that a protein called Sir2 (Silent information regulator 2) was involved in the yeast replicative aging process [22]. In a seminal paper, it was demonstrated that extra copies of Sir2 increased yeast replicative lifespan by 30%, whereas ablation of the *Sir2* gene had the opposite effects, reducing lifespan by 50% [22]. Sir2 was initially characterized as a protein having an unusual NAD^+-dependent enzymatic histone deacetylase activity [23,24]. The deacetylation reaction catalyzed by Sir2 is coordinated with the cleavage of NAD^+ into nicotinamide and 1-O-acetyl-ADP-ribose [23,24]. Later, the deacetylase activity of Sir2 or that of the closest mammalian ortholog, SIRT1, was shown to extend beyond the histone realm into other nuclear and cytosolic targets [25]. Importantly, the K_M of yeast Sir2 (and the mammalian SIRT1) for NAD^+ is over 100 μM, which is in the range of physiological changes in NAD^+ availability [25]. Since NAD^+ acts as a cofactor in various metabolic reactions, the above data suggests that Sir2 could act as a metabolic sensor, capable of fine-tuning gene expression according to the metabolic state of the cell. In line with this, several studies indicated that Sir2 could be a critical mediator of the effects of CR on yeast lifespan [26,27]. In yeast, mimicking CR by reducing glucose concentration in the media from 2 to 0.5% is enough to increase replicative lifespan [27]. This effect, however, was lost in yeast where the gene coding for Sir2 was deleted [27]. Genetic manipulation of Sir2 orthologs was later demonstrated to affect lifespan in higher eukaryotes, such as nematodes [28,29] and insects [30,31], even though the consistency and amplitude of this effect has been a matter of debate [32–34].

In mammals, there are 7 sirtuin enzymes (SIRT1–7). Among them, SIRT1 has been the most extensively studied [25]. A wide range of substrates have already been described for SIRT1, among which are key regulators of mitochondrial respiration, lipid metabolism and the aging process, such as the FOXO family of transcription factors [25]. SIRT1 overexpression does not enhance maximal lifespan in mice under regular food regimes [35]. However, SIRT1 transgenic mice display some features resembling CR. The first reported SIRT1 gain-of function mice were leaner, metabolically more active, and had improved glucose tolerance [36]. A second, independent, SIRT1 transgenic line with a mild overexpression of SIRT1 displayed lower levels of DNA damage, decreased expression of aging-associated markers, a better general health and fewer spontaneous carcinomas and sarcomas upon aging [35]. These effects, however, were not sufficiently potent to affect longevity. One of the major caveats of the gain-of-function approaches is that higher SIRT1 expression does not necessarily result in a proportional increase in SIRT1 activity, particularly in aged rodents, where NAD^+ content declines to the levels that might compromise SIRT1 activity [37,38]. A growing body of evidence derived from in vivo studies suggests that SIRT1 may mediate the effects of CR in mice. The study of SIRT1 knockout mice is complicated, as SIRT1 deletion is lethal at embryonic or early post-natal stages in inbred mice [39,40]. Hence, most studies had to rely on outbred stocks. In general, mice deficient for SIRT1 display developmental and growth defects as well as altered birth rate [39,40]. The few SIRT1 knock-out mice that survive until adulthood, however, are metabolically inefficient and, display a blunted response to CR-induced adaptations, including the effect of CR on longevity [41,42].

Based on the hypothesis that sirtuins are critical mediators of CR and its associated health benefits, the Sinclair lab used an in vitro screening strategy with a fluorescent labeled substrate aimed to identify small molecule activators of SIRT1 [26]. The screen identified a number of polyphenols as possible allosteric SIRT1 activators, Rsv being the most potent hit [26]. Subsequently, Rsv was shown to extend lifespan in lower eukaryotes – including yeast, worms and flies – in a SIRT1 dependent manner [26,43]. This way, Rsv treatment in lower eukaryotes was sufficient to mimic one of the most well-known effects of CR, i.e.: lifespan extension. Chronic treatment of mice with Rsv also had dramatic effects on metabolic health. Mice on a high-fat diet were largely protected against body weight gain when supplemented with Rsv at 400 mg/(kg*day), accompanied by a significant amelioration of impaired whole body glucose and lipid homeostasis [44]. The metabolic benefits of Rsv supplementation culminated in a remarkable protection of treated mice (at ~25 mg/(kg day)) against the lifespan reduction promoted by high-calorie diets [45]. When supplemented at ~25 mg/(kg day) to a regular diet in one year old mice, Rsv induced changes in the transcriptional profile of key metabolic tissues that closely resemble those induced by CR [12]. In the liver and muscle, these changes could also be correlated to the gene expression patterns of younger animals [12]. Rsv supplementation also reduced osteoporosis, cataracts, vascular dysfunction, and the age-related decline in motor coordination [12]. However, Rsv did not increase longevity in mice fed regular, low-fat, diets [12].

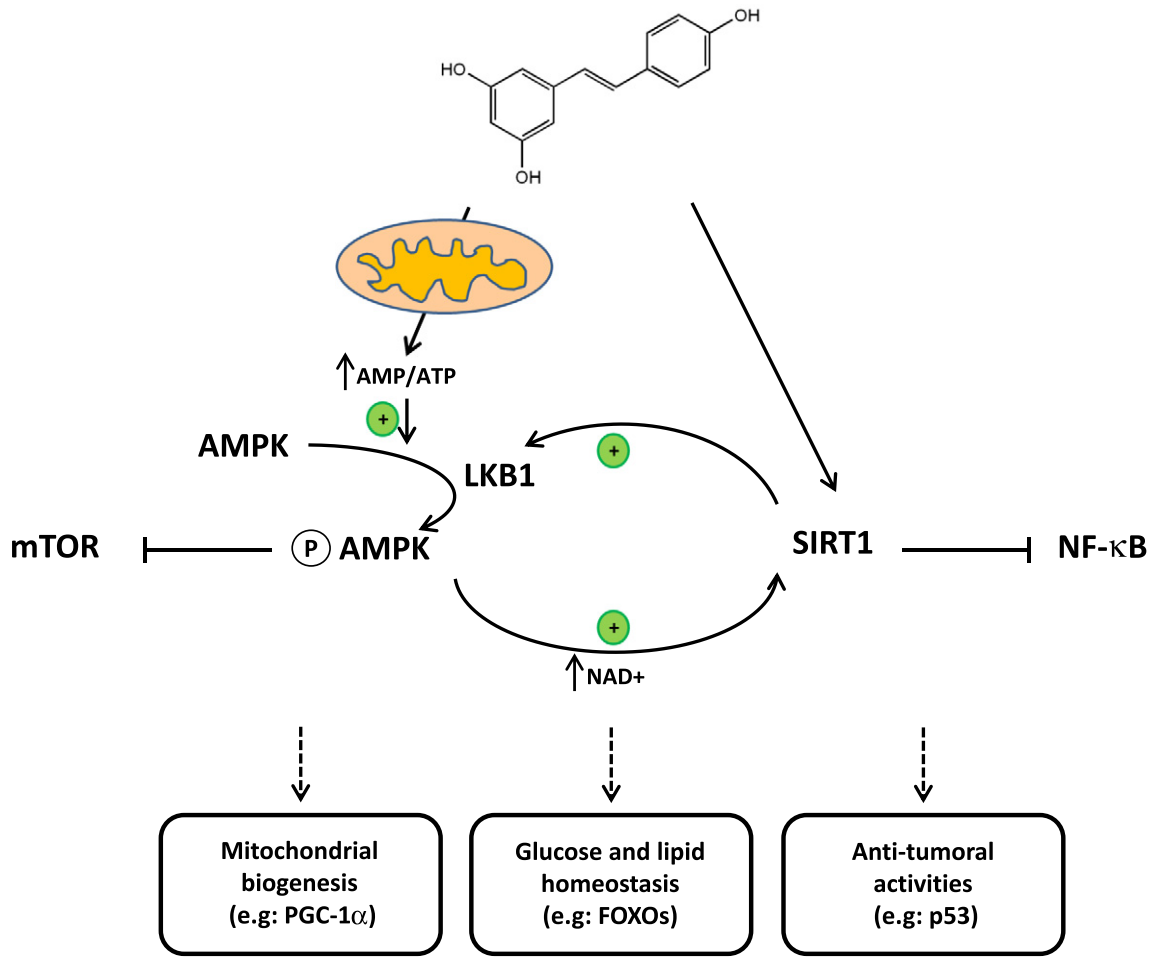

Fig. 1. Resveratrol and the complex relationship between AMPK and SIRT1. AMPK and SIRT1 constitute two key targets of Rsv. As a mild mitochondrial poison, Rsv inhibits mitochondrial ATP production, leading to a higher AMP/ATP ratio and an LKB1-dependent activation of AMPK. Then, AMPK enhances NAD^+ availability, which would overcome the rate-limitation that this cofactor exerts on SIRT1 enzymatic activity. In turn, SIRT1, as a possible direct target of Rsv, can deacetylate LKB1, facilitating the formation of an active kinase complex through the association of LKB1 with STRAD and MO25. This way, SIRT1 could also positively control AMPK activity. Together, AMPK and SIRT1 create a positive feed-forward loop to amplify the adaptation response to nutrient scarcity. Through the modulation of diverse transcriptional regulators, as exemplified, the actions of AMPK and SIRT1 can explain many of the beneficial effects of Rsv against metabolic and age-related complications. In addition, AMPK activation by Rsv would lead to the inhibition of mTOR signaling, while SIRT1 activity would repress NF-κB activity, two key paths by which Rsv modulates cellular growth, autophagy and immune responses.

The health benefits of Rsv in mice have been largely attributed to the ability of Rsv to enhance energy expenditure by boosting mitochondrial respiration and lipid oxidation [44]. Given that defective mitochondrial function has been associated with insulin resistance and other age-related pathophysiological complications, including sarcopenia, cognitive decline and cancer (see [46] for review), improving mitochondrial function might be key to impinge on all these disorders. By acting as a SIRT1 activator, Rsv triggers the deacetylation of diverse metabolic transcriptional regulators in vivo [44]. For example, PGC-1α is one well-established target of SIRT1 that acts as a master orchestrator of mitochondrial biogenesis [47]. Upon deacetylation by SIRT1, PGC-1α increases its activity as a co-activator of numerous transcription factors and nuclear receptors controlling mitochondrial gene expression [48]. Accordingly, Rsv treatment led to large increases in mitochondrial content in key metabolic tissues, such as skeletal muscle or brown adipose tissue [44]. In cultured myotubes, the effect of Rsv on PGC-1α activity was completely blunted when SIRT1 was knocked-down [44], further proving that Rsv action is SIRT1 dependent. Recently, an elegant model has been developed in order to genetically ablate SIRT1 exclusively in adulthood. The deletion of SIRT1 in adult mice did not result in any overt phenotype, but completely impaired the ability of Rsv to increase mitochondrial biogenesis and function [49].

It has been recently proposed that SIRT1 small molecule activators (STACs), such as Rsv, might be acting as direct "assisted allosteric activators" [50]. In this case, STACs would not interact strongly with SIRT1, but rather bind to a steady-state enzyme–substrate complex [50]. By screening different SIRT1 mutations, it was revealed that SIRT1 has an N-terminal domain that is required for STAC binding, which includes E230 (E222 in mice), a critical residue for the activation of SIRT1 by STACs [50]. An E230A or E230K mutation on SIRT1 did not have any major effect on basal SIRT1 activity, but was enough to largely attenuate the ability of Rsv to activate SIRT1 [50]. Accordingly, while Rsv nicely increased mitochondrial mass and ATP levels in SIRT1 KO myoblasts reconstituted with wild type SIRT1, it failed to do so when the reconstitution was made with the E222K mutant form [50]. The elegant work by Hubbard and colleagues additionally pointed out that target deacetylation residues for SIRT1 might need to be surrounded by specific features, such as a hydrophobic residue at the +1 position [50]. In line with this, efforts from the Steegborn lab illustrated how Rsv action only triggers SIRT1-mediated deacetylation in a substrate specific fashion, and even led to a paradoxical inhibition of SIRT1 actions on certain substrates [51]. Altogether, these data indicate that Rsv is not just a mere global SIRT1 activator, but it might only influence the deacetylation of particular groups of substrates. While the work presented above marks a great improvement in our understanding of the mechanism by which Rsv could directly activate SIRT1, a number of findings have strongly contested the possibility of Rsv being a direct SIRT1 activator. First, the assay that initially identified Rsv as a SIRT1 activator has

Table 1
Comparison between Rsv, SIRT1 overexpression and Caloric Restriction effects on diverse metabolic and longevity-related parameters. Unless otherwise specified, the data mentioned refers to mouse models.

	Resveratrol (Rsv)	SIRT1 overexpression	Caloric Restriction (CR)
Lifespan	Rsv has been shown to enhance lifespan in lower eukaryotes [2]. No effect has been observed in rodents under regular chow diet [12], but mice are prevented against the decreased lifespan induced by high-fat diets [12,45].	Whether SIRT1 extends lifespan in lower eukaryotes, worms and flies, is still under debate [34]. No effect has been observed in rodents [35].	CR has consistently enhanced lifespan in most organisms tested to date. The evidence in primates needs further solidification [5,6,117].
Body weight	Rsv prevents high-fat diet-induced body weight gain at high-doses (400 mg/(kg day)) [44], but not at lower ones (~22 mg/(kg day)) [45]. No effect on chow diet [44].	Lower body weight was observed in one transgenic model [36], but not in 2 other ones, where SIRT1 also failed to prevent high-fat diet-induced body weight gain [56,57]	CR decreases body weight [117]
Energy expenditure	Rsv enhances energy expenditure at doses of 400 mg/(kg day) [44]. However, Rsv decreased resting metabolic rate in humans [17]	More active metabolism has been reported in one model [36], but no differences in energy expenditure were observed in the other two on regular diet [56,57]. A modestly higher energy expenditure on high-fat diet has been reported [56]	CR drives behavioral changes that can affect energy expenditure. In humans, the resting metabolic rate is reduced by CR [17].
Glucose metabolism	Higher glucose tolerance and insulin sensitivity on high-fat fed mice [45]. Upon a hyperinsulinemic–euglycemic clamp, Rsv-treated mice require higher glucose infusion rates [44].	Does not affect peripheral glucose uptake, but decreases hepatic glucose output when mice are on a high-fat diet regime [57].	CR enhances insulin-stimulated peripheral glucose disposal and repression of hepatic glucose output [87].
Mitochondrial biogenesis	Increased mitochondrial content in the liver, skeletal muscle and brown adipose tissues [44,45].	No effects on mitochondrial biogenesis were observed, at least in the skeletal muscle [56].	In most cases, CR does not increase mitochondrial biogenesis, even though this might be regime-dependent [58].
Age-related physiological decline	Reduction of osteoporosis, cataracts, vascular dysfunction, and declines in motor coordination [12]	Protection against age-related glucose intolerance. Improved motor coordination, bone mineral density and wound healing [35]	All around protection against age-related pathologies, even though impairments in immune function and wound healing have been reported [117].
Cancer	Protection against implanted or chemically induced tumors, but not against the incidence of age-related cancer (mostly lymphomas) [7,12]	Lower incidence of malignant tumors. The suppressive effect of SIRT1 on malignant tumors was restricted to carcinomas and sarcomas, but not lymphomas [35]	Reduces tumor incidence in all species tested to date, including prevention against lymphomas [8].

been called into question when it was demonstrated that the nonphysiological "Fluor de Lys" substrate used for screening can lead to artefactual results [52–55]. Several laboratories have demonstrated that Rsv fails to activate SIRT1 with native peptides or full-length protein substrates, whereas they do activate SIRT1 when using fluorphore-containing peptides [52–55]. Second, there are a number of discordances between the in vivo effects of Rsv treatment and SIRT1 genetic gain-of-function models (Table 1). For example, Rsv largely protects against high fat diet-induced body weight gain [44], while SIRT1 transgenic mice gain weight in a similar fashion to control wild-type mice [56,57]. Similarly, Rsv, but not SIRT1 overexpression or CR, leads to a dramatic increase in skeletal muscle mitochondrial content [44,56,58]. In fact, while most data suggests that Rsv increases skeletal muscle glucose uptake [44,59,60], even in an acute fashion [61,62], SIRT1 transgenesis in the muscle does not affect insulin action [63]. More specifically, the positive effects of SIRT1 transgenesis on global insulin sensitivity seem to be largely derived from a protection against hepatic metabolic damage [57]. Importantly, Rsv also ameliorated glucose homeostasis in high-fat fed SIRT1 deficient mice [49], thus additional mechanisms, other than SIRT1 or mitochondrial biogenesis, are required for Rsv to improve metabolic health. Last, but not the least, one has to take into account that there is uncertainty on whether it is Rsv or one of its metabolized forms that accounts for the biological effects upon dietary supplementation. Indeed, it has always been striking how a relatively permeable compound such as Rsv has generally needed long time frames (several hours) in order to drive SIRT1 activation (discussed in [25]). As a whole, while a large body of evidence indicates that Rsv requires SIRT1 for most of its key metabolic actions, the activation of SIRT1 does not necessarily have to be through direct means and SIRT1-independent events definitively exist.

4. Resveratrol as an AMPK activator through affecting mitochondrial metabolism

Could the activation of other pathways drive the metabolic actions of Rsv? Interestingly, the health benefiting properties of SIRT1 activation overlap in many ways with those conferred by AMP-activated protein kinase (AMPK) activation [45]. AMPK has emerged as a key nutrient sensor with the ability to regulate whole-body metabolism. AMPK is an evolutionarily conserved enzyme whose activity is triggered by increases in the AMP/ATP ratio, which reflects the energy status of the cell. A recent report combining structural and biochemical approaches revealed that, apart from AMP, also ADP binding might also crucially contribute to AMPK activation [64], further supporting the exquisite sensitivity of this kinase to be modulated in response to energy needs. Consequently, AMPK is activated under the physiological conditions of energy stress such as hypoxia, fasting and exercise [4,65,66]. Upon activation, AMPK diminishes the rates of most energy consuming metabolic programs such as lipid and protein biosynthesis, and triggers energy producing biochemical processes such as lipid oxidation and glycolysis to counter energy depletion [65]. In addition, AMPK exerts long term changes in energy homeostasis by triggering transcriptional programs related to mitochondrial biogenesis and the use of oxidative metabolism to fuel cellular energy demands [47]. Given the conserved ability of AMPK to sense energy stress and trigger metabolic adaptations, it has also been speculated that AMPK could act as mediator of the beneficial effects of CR (reviewed in [67]). Certainly, while AMPK activation mimics certain aspects of CR and has been shown to be necessary to promote longevity under certain circumstances, whether AMPK is an effector promoting longevity and healthy aging during CR is far from clear [67].

By early 2007 several labs reported that Rsv had the ability to activate AMPK [45,68,69]. Surprisingly, Rsv led to AMPK activation within minutes in most cell lines tested, including hepatic and cultured skeletal muscle cell models [45,62,69,70] much faster than generally required for SIRT1 activation. Mice fed with Rsv at doses ranging from ~25 to

400 mg/(kg day) displayed enhanced AMPK activation in the liver, skeletal muscle and white adipose tissue [45,71]. The most likely mechanism by which Rsv could activate AMPK relies on the ability of Rsv and related polyphenols (such as piceatannol and quercetin) to directly bind and inhibit the mitochondrial F1F0-ATPase/ATP synthase (Complex V), thus impairing ATP production [72,73]. Elegant experiments using AMPK mutants that are insensitive to changes in AMP/ATP ratio provided further evidence that Rsv activates AMPK as a consequence of decreased ATP production [74], even though the concentration on these assays, 100 μM, was far higher than those generally reported to activate AMPK in cultured cell models, which are generally between 5 and 50 μM.

Many experimental lines indicate that the metabolic effects of Rsv crucially depend on AMPK activation. For example, Rsv required AMPK in order to decrease lipid accumulation in HepG2 cells [75]. Similarly Rsv triggered skeletal muscle glucose transport in an AMPK-dependent manner [62]. Using an AMPKγ_3 knock-out mouse model, it was demonstrated that defective AMPK prevents the activation of SIRT1 upon Rsv treatment [76]. Consequently, PGC-1α was not deacetylated in response to Rsv treatment in AMPKγ_3 knock-out mice [76]. A parallel study by Um and colleagues demonstrated that Rsv required AMPK to promote metabolic benefits in mice, as Rsv failed to improve insulin sensitivity, glucose tolerance, physical endurance and mitochondrial biogenesis in mice deficient for the catalytic AMPKα_1 or AMPKα_2 subunits [71]. Finally, studies in worms indicated that Rsv requires intact AMPK activity to enhance lifespan [77]. Altogether, these observations not only demonstrate that AMPK is a critical mediator of the metabolic actions, but also suggest that AMPK activation could be one of the earliest signals triggered by Rsv.

5. AMPK and SIRT1: partners in crime?

The largely overlapping outcomes of AMPK activation and SIRT1 activation, together with the strong links of Rsv with the activation of both metabolic sensors, quickly fueled the possibility that their actions could be intertwined. From a simplistic perspective, the fact that Rsv requires AMPK and SIRT1 activities for some actions (for example, to trigger mitochondrial biogenesis in vivo [49,71]), suggests that AMPK and SIRT1 might be part of a common signaling pathway or that they might act in concert. However, in what order are they activated? And how are their activities connected?

On the question of whether AMPK or SIRT1 lies upstream of each other, there is evidence to support both directions. As discussed at the end of this section, this is not necessarily contradictory. Intuitively, the very rapid effect of Rsv on AMPK activation would place AMPK in a privileged position to be an upstream driver. Supporting this hypothesis, Rsv has been reported to activate AMPK independently of SIRT1, at least at high doses [49,68,71]. Conversely, defective AMPK activation compromises the ability of Rsv to trigger SIRT1 activity [71,76]. In fact, the activation of AMPK seems to consistently lead to higher SIRT1 activity, irrespective of the nature of the agonist [78]. Furthermore, physiological activation of SIRT1 in response to diverse nutrient and energy stresses, such as glucose restriction, fasting or exercise, seems to depend on AMPK in mammalian cells and tissues [76,79]. Finally, the significant effects of Rsv on mitochondrial biogenesis in the skeletal muscle closely mimic those observed by treatment with AMPK agonists [80] or AMPK gain-of-function models [81], but were not observed in models where SIRT1 was moderately overexpressed [56,82,83]. In all, the above evidence would indicate that, in response to Rsv, AMPK activation is an early event that would lead to a downstream activation of SIRT1. Different mechanisms have been proposed to link AMPK and SIRT1 activities. First, AMPK activation seems to increase NAD^+ availability, both in cultured myocytes or skeletal muscle [78,79]. As discussed in a previous section, NAD^+ can be rate-limiting for SIRT1 activity. Consequently, an increase in NAD^+ availability would favor SIRT1 action. This increase in NAD^+ availability might derive from, at least, two complementary actions: on one hand, the increase in fat oxidation rates induced by AMPK would be permissive for an increase in NAD^+ within hours after treatment [78]. On the other, AMPK triggers nicotinamide phosphoribosyltransferase (Nampt) expression, which would increase NAD^+ salvage from nicotinamide (NAM) [79]. Accordingly, Rsv fails to enhance NAD^+ content in mammalian tissues with defective AMPK activity [10]. The beauty of this model is that the activation of SIRT1 would rely on long term metabolic and/or transcriptional adaptation, explaining why Rsv requires a relatively long time to trigger SIRT1 activity in vivo. A second mechanism by which AMPK could regulate SIRT1 has been proposed recently. According to it, AMPK would directly phosphorylate SIRT1 [84]. This phosphorylation event would disrupt the interaction of SIRT1 with its endogenous negative regulator, Deleted in Breast Cancer 1 (DBC1) [84], therefore rendering SIRT1 active. However, as discussed in a recent review [83], this mechanism might need further clarification given that previous reports failed to detect such phosphorylation event.

The above findings elaborate a mechanism in which AMPK is at the apex of the energy sensing machinery and translates the message to SIRT1, which then initiates cellular transcriptional responses aimed to optimize energy production from non-carbohydrate sources. However, several evidences suggest that SIRT1 could also act as an upstream modulator of AMPK. Initial evidence for this was obtained in HepG2 cells, where the use of splitomicin as a SIRT1 inhibitor prevented Rsv-induced AMPK phosphorylation [75]. In a parallel study, the incubation of HepG2 cells with NAM, an end-product inhibitor of SIRT1 activity, also led to a marked decrease in basal AMPK activity [70]. More convincingly, blunting SIRT1 activity using specific shRNAs or through the generation of dominant negative models largely decreased both basal and Rsv-triggered AMPK phosphorylation, either in HepG2 or HEK293 cells [75,85]. These results, however, are seemingly contradictory with other observations showing that the suppression of SIRT1 activity in fibroblasts or muscle C2C12 cells does not lead to decreased AMPK activity [71,78,86]. Similarly, basal AMPK activity in the skeletal muscle seems unaffected by SIRT1 deletion [49,87,88]. This argues that the impact of basal SIRT1 activity on AMPK could be tissue or cell-specific, probably relying on the level of basal SIRT1 activation in the tissue. Interestingly, however, overexpression of SIRT1 has been consistently shown to increase basal AMPK activity in the HepG2 cells, liver and skeletal muscle [49,75]. As a whole, the above results strongly suggest that SIRT1 can also influence AMPK activity. In this sense, a recent study has brought further light into these concepts by demonstrating that the molecular pathway through which Rsv leads to AMPK activation is dose dependent thus emphasizing the dose as a critical factor. This way, low Rsv doses (~25 mg/(kg day) in mice; 25 μM in cultured myotubes) lead to SIRT1-dependent AMPK activation, while high doses (~220 mg/(kg day) in mice; 50 μM in cultured myotubes) can do so in a SIRT1-independent fashion [49]. Therefore, Rsv dosing has to be carefully examined when exploring the mechanistic insights on its action.

As the results above illustrate, SIRT1 can affect the ability of diverse AMPK agonists to trigger AMPK activity. It was, hence, exciting to see that the Liver Kinase B1 (LKB1), the main upstream kinase for AMPK activation [89,90], was a target for SIRT1 deacetylation in cultured cells and rodent tissues [85]. Original experiments in HEK293 cells demonstrated that acetylated LKB1 fails to shuttle from the nucleus to the cytoplasm [85]. Upon deacetylation by SIRT1, LKB1 shuttled more efficiently to the cytoplasm, allowing the binding of STRAD and MO25 and the formation of the final active kinase complex [85]. Consequently, SIRT1 could potentially modulate AMPK activity by influencing the activity of LKB1. The finding that not only Rsv, but also AICAR, the most widely used AMPK agonist, failed to stimulate AMPK when SIRT1 activity was blocked in C2C12 myotubes, primary hepatocytes and primary myoblasts [49], stands fully in line with this possibility. In this sense, Rsv and nutrient deprivation both led to LKB1 deacetylation, potentially triggering AMPK activation [49,85]. The above hypothesis is an

extremely attractive one. However, as with the ones connecting AMPK to SIRT1 activity, it has some weaknesses. First, LKB1 activity in the cell is unlikely to be limiting for energy-stress induced AMPK activation, as even hypomorphic mice with 10-fold lower LKB1 activity retain a significant ability to activate AMPK [90]. Second, and in line with the above comment, AMPK phosphorylation seems to be regulated largely at the phosphatase level in an AMP-dependent fashion, making it difficult for an LKB1-dependent mechanism to explain higher AMPK activity upon SIRT1 overexpression.

While answering some of the caveats of the diverse mechanisms of action proposed will still require further investigation, the weight of evidence indicates that SIRT1 and AMPK are required for Rsv metabolic actions and that both effectors likely act in concert to promote health benefits. For example, blocking either AMPK or SIRT1 is enough to prevent Rsv-induced mitochondrial biogenesis in vivo [49,71]. The common need for both actors might also be explained by a "double hit" hypothesis in which critical mediators might require inputs from both enzymes in order for them to become fully active or inactive. A classic example might be found in PGC-1α, the master transcriptional regulator of mitochondrial biogenesis. AMPK activation promotes mitochondrial biogenesis in a PGC-1α dependent manner [91]. The AMPK–PGC1α axis of action was then mechanistically explained with the discovery that AMPK activation led to direct phosphorylation of PGC1α protein at T177 and S538 and that the phosphorylation of these residues was essential for the induction of PGC1α activity [91]. Thus AMPK-mediated phosphorylation of PGC1α initiated the essential transcriptional circuitry governing mitochondrial metabolism. SIRT1 was also reported to directly control PGC1α activity, in this case by direct deacetylation [48]. Strikingly, treatment of mouse fibroblasts and myotubes with AMPK agonists led to a marked SIRT1-dependent PGC1α deacetylation [78]. So, why should AMPK trigger two independent events stimulating PGC-1α activity (i.e.: phosphorylation and deacetylation). The answer might be provided by experiments demonstrating that PGC-1α forms where both T177 and S538 have been mutated to alanine fail to become deacetylated upon AMPK activation in vivo [78,92]. The above result indicates that the phosphorylation of these residues might be necessary for the PGC-1α protein to be recognized by SIRT1 in vivo, allowing this way its deacetylation and full activation. This, in turn, could constitute an elegant mechanism by which SIRT1 substrates could be specifically targeted for deacetylation upon SIRT1 activation. In addition, it could explain why models of modest SIRT1 overexpression do not necessarily show higher mitochondrial biogenesis [56], as the signal from AMPK might be missing. In line with this observation, AMPK and SIRT1 share a number of downstream targets, including p53 or the Forkhead O box (FOXO) family of transcription factors among others [47], suggesting further interactions of both signaling systems. Finally, the AMPK/SIRT1/PGC-1α path has been proven to be an extremely conserved axis of action through which mitochondrial biogenesis is regulated not only by pharmacological and nutritional cues, but also by diverse key metabolic hormones, including adiponectin [92], leptin [93] and fibroblast growth factor 21 (FGF21) [94]. In conclusion, the above sections demonstrate that AMPK and SIRT1 are core effectors of Rsv action and that their activities closely intertwine (Fig. 1).

6. Other possible effectors for Rsv

While all the above data point to AMPK and SIRT1 as key metabolic effectors of Rsv, it is also true that these two effectors are unlikely to be exclusively responsible for the myriad of effects derived from Rsv treatment. Similarly, the mechanism of activation of AMPK and SIRT1 might be indirect and, therefore, influenced by other direct or indirect Rsv targets. In this section we will briefly discuss some additional relevant targets proposed for Rsv. We will particularly focus on classic and novel possible targets relevant for the metabolic effects of Rsv, and, more specifically, on those where a direct action has been described. For further information on the possible impact of Rsv on other diverse cellular processes, such as anti-oxidant protection or sphingolipid metabolism, the reader is kindly referred to other reviews where the topic has been largely covered [95–98].

6.1. Cyclooxygenases (COX)

The initial cancer chemopreventive activity of Rsv was attributed to the inhibition of cyclooxygenases (COXs) [7]. COX activity catalyzes the conversion of arachidonic acid to pro-inflammatory substances, such as prostaglandins, which act as critical second-messengers for immune processes and stimulate tumor growth [7]. Jang et al. performed a fractionation study on an extract from a leguminous plant, *Cassia quinquangulata*, known to inhibit COX activity. The authors identified Rsv as the active component, inhibiting COX-1 at a median effective dose of 15 μM [7]. Rsv could also inhibit COX-2, but at ED50 ~5 times higher than those described for COX-1 [7]. It was, hence, not surprising that Rsv does not only display anti-inflammatory actions, but it has even been also proposed to display analgesic actions [99]. However, despite strong data indicating that Rsv can directly inhibit COX activity, there is also a large body of evidence indicating that, in vivo, Rsv reduces COX activity by transcriptional means. This way, Rsv has been shown to dramatically reduce COX-2 transcription by the combined downregulation of the Akt, MAPK and NF-κB pathways [100,101]. Irrespective of mechanism, COX activity seems a clear mode of action by which Rsv reduces inflammation and tumorigenesis. In addition, Rsv has also been described to inhibit lipooxygenases (LOX) activity [102,103], which is also involved in leukotriene synthesis, further leading to the production of other inflammatory and carcinogenic-related signals [104]. This further solidifies the key action of Rsv in the regulation of eicosanoid metabolism and their action as second messengers.

6.2. Phosphodiesterases

A recent study by Park and colleagues provided evidence suggesting that the direct inhibition of phosphodiesterases (PDEs) could be at the root of the metabolic benefits exerted by Rsv [105]. The direct inhibition of PDE1, 3 and 4 by Rsv, at IC_{50} between 6 and 14 μM, would lead to PKA activation and to the phosphorylation of AMPK in a Ca^{2+}-dependent manner, increased NAD^+ availability and, finally higher SIRT1 activity [105]. Accordingly, the treatment of mice with rolipram as a PDE4 inhibitor led to very similar metabolic effects to those described for Rsv, such as prevention against diet-induced obesity and an increase in mitochondrial function [105]. While attractive, this idea will need further work to gain solidity and explain some inconsistencies. For example, Price and colleagues have reported that Rsv can increase mitochondrial biogenesis without affecting cellular NAD^+ levels [49]. Further, the activation of PKA can directly lead to SIRT1 activation via phosphorylation [82]. While some reports support that cAMP signaling might trigger AMPK activation [106–108], PKA has also been reported to directly phosphorylate and negatively influence AMPK activity [109]. Also, the outcomes of enhancing cAMP levels can have diametrically opposite effects to AMPK activation in some Rsv target tissues, such as the liver, where cAMP signaling is generally associated with increased hepatic glucose production. In this sense, however, it is important to note that Rsv has a strong protective effect against hepatic steatosis upon high-fat feeding [45], which might override possible detrimental effects of cAMP signaling on gluconeogenesis. Finally, Rsv failed to activate mutant AMPK forms insensitive to changes in AMP/ATP ratio [74], which refutes a significant role for the Ca^{2+}–CAMKKβ pathway.

6.3. NF-κB

A wealth of data has been gathered indicating how Rsv might interfere with the nuclear factor-kappaB (NF-κB) family of transcription factors. This family includes RelA (p65), NF-κB1 (p50 and p105), NF-κB2

(p52 and p100), c-Rel and RelB [110]. These factors are generally retained in the cytoplasm through their interaction with IκB, which prevents NF-κB and nuclear translocation [110]. By enhancing the degradation of IκB, NF-κB can then migrate into the nuclear compartment, where these transcription factors act as heterodimers that bind the DNA promoters of many inflammatory and immune response genes [110]. Rsv has been largely reported to interfere with NF-κB activity. Initial hypothesis suggested that Rsv might either reduce NF-κB nuclear presence [111] or interfere with the transcriptional activity [112]. The mechanism underlying such actions, however, remains largely obscure. In fact, no evidence exists for a direct effect of Rsv on NF-κB. To date, most hypotheses converge on the possibility that Rsv reduces the ability of several pro-inflammatory stimuli, such as tumor necrosis factor α (TNF-α) lipopolysaccharide (LPS) or H_2O_2 to trigger IκB phosphorylation and degradation [113], hence hampering NF-κB translocation. To do so, it has been suggested that Rsv might lead to the suppression of IκB kinase (IKK) activity [114,115], therefore preventing IκB degradation and, consequently, NF-κB nuclear translocation. Interestingly, SIRT1 physically interacts with the RelA/p65 subunit of NF-κB deacetylates it at K310, inhibiting its transactivation potential [116]. It is likely that Rsv might also negatively influence NF-κB indirectly through this path. In agreement, Rsv reduced RelA/p65 acetylation levels [116].

6.4. PI3K/Akt signaling

Given that a modest inhibition of the insulin signaling pathway can enhance lifespan in virtually all species tested to date [117], it could be hypothesized that Rsv might enhance lifespan by reducing insulin action. While evaluating the impact of Rsv on insulin signaling, Frojdo and colleagues unveiled that Rsv could inhibit PI3K signaling independently of SIRT1 [118]. Rather, this was due to a direct inhibitory action of Rsv on class IA PI3K catalytic subunits p110α and p110β [118]. Their results indicated that Rsv targeted the ATP binding site in a non-covalent fashion at an IC_{50} of 25 and 50 μM (for α and β isoforms, respectively) [118]. Consequently, Rsv prevented the activation of Akt by a number of different stimuli [118]. Indeed, such mechanism would explain the multiple lines of evidence indicating decreased Akt signaling in a number of cell lines following Rsv treatment (see [95] and [98]), also supporting the Rsv chemopreventive activity. That said, some authors have also suggested that Rsv might be inhibiting insulin-stimulated interaction between IRS1 and PI3K, as well as between IRS-1 and Grb2, which drives MAPK activation [119], although the genuine molecular mechanism was not identified in that case. It must be noted, however, that chronic treatment with Rsv in vivo can actually lead to insulin sensitivity [44,45], probably through transcriptional mechanisms leading to a SIRT1-driven downregulation of Protein Tyrosine Phosphatase 1B (PTP1B) [120], which downregulates insulin signaling, or by enhancing mitochondrial lipid catabolism [44,45] and preventing the subsequent accumulation of potential lipid intermediates that could interfere with insulin action, such as ceramides or diacylglycerols.

6.5. mTOR signaling

The mammalian target of rapamycin (mTOR) is a central controller of cell growth, proliferation and metabolism [121]. As part of the mTOR complexes 1 and 2 (mTORC1 and mTORC2), the mTOR kinase plays a key role in several pathways involved in cancer and metabolic diseases [121]. Genetic or pharmacological strategies aimed to decrease mTOR signaling have been shown to enhance lifespan across a broad range of species [121]. Therefore, inhibition of mTOR signaling could also be a mechanism by which Rsv promoted health benefits. Recent studies have also demonstrated that Rsv inhibits mTOR signaling via a SIRT1-independent mechanism [122] and that it represses protein synthesis [123]. Intriguingly, the disruption of upstream regulators of the mTOR pathway, such as Akt signaling and tuberous sclerosis 1 and 2 (TSC1/2) expressions has no significant effect on the inhibitory effect of Rsv on leucine-stimulated mTOR activation [122]. AMPK activation can trigger the phosphorylation of Raptor, a component of the mTORC1 complex, thereby repressing mTORC1 activity [124]. Hence, the activation of AMPK by Rsv could explain such an effect. Nonetheless, Liu and colleagues have proposed that Rsv could promote the association between mTOR and DEPTOR, an inhibitor of mTOR [125], thus uncovering a novel mechanism by which Rsv inhibits mTOR signaling [122]. In this study, the ability of Rsv to inhibit mTORC1 activity was maintained in C2C12 cells where $AMPK\alpha_2$ was knocked-down [122], arguing in favor of a direct inhibition mechanism. Further evidence, however, will need to be collected to prove that AMPK activity was truly impaired in this cell model and to more finely establish how Rsv promotes the binding of DEPTOR to mTOR and whether the concentrations at which this event takes place are biologically relevant. Interestingly, Rsv has also been found through an in vitro screen to act as a direct inhibitor of p70S6K [126], a key downstream target of the mTOR pathway regulating autophagy and protein translation rates, albeit at an IC_{50} of 25 μM [126], which is higher than the low micromolar concentrations at which Rsv enhances AMPK activity [45,127] and its subsequent induction of autophagy [128,129].

6.6. Estrogen receptors

Based on the structural similarities between Rsv and diethylstilbestrol, a synthetic estrogen, Gehm and colleagues examined whether resveratrol might be a phytoestrogen. Rsv inhibited the binding of 17-β-estradiol (E2) to the estrogen receptor (ER) in a competitive manner at an IC_{50} of 10 μM [130], indicating that it was a relatively weak ligand for the receptor. Rsv binding acted as an agonist of ER and increased the transcription of estrogen-responsive reporter gene [130]. The amplitude of the effect, however, was cell type-dependent. This way, in the MCF-7 human breast cancer cell line, Rsv produced a greater maximal transcriptional response than estradiol whereas in BG-1 (a human ovarian carcinoma line) it was weaker [130]. These results demonstrate that Rsv can act as a phytoestrogen. It was later reported that Rsv interacted directly with ERα and ERβ with Ki values of 8 μM and 25 μM respectively, though it appears to exert particularly strong transcriptional effects via ERβ [131]. Rsv appears to have a biphasic effect on cell proliferation, stimulating growth at low concentrations and suppressing growth at high concentrations [131,132]. Interestingly, Rsv inhibited cell proliferation at all concentrations in ER-negative cancer cell lines [131,132]. This further demonstrates how Rsv exerts its biological effects by various mechanisms. While the phytoestrogenic action of Rsv could support a chemoprotective role in breast cancer, there is also evidence suggesting possible adverse effects [132], likely depending on the distinctive patterns of ERα and ERβ expressions in different cell types.

6.7. MAPK signaling

Some activities of Rsv have been linked to the activity of the mitogen-activated protein kinases (MAPKs), including the extracellular signal regulated kinases 1 and 2 (ERK1/2; also known as p44/42 MAPK) pathway, the p38MAPK and the c-Jun N-terminal protein kinase (JNK). MAPKs critically influence cellular proliferation, survival and differentiation. As with other effectors, it seems that the dosage of Rsv is key to determine MAPK activity. For example, doses up to the low micromolar range might increase ERK signaling, whereas Rsv is inhibitory at the most commonly used, higher, doses (>50 μM) [133]. This clearly suggests that the impact on this path might be indirect, where MAPKs might act as integrators of diverse signaling paths triggered by Rsv. In this sense, while the literature is abundant on identifying a MAPK/p53-dependent path for Rsv to promote cell death and senescence [95,

98], no particular mechanisms for direct activation/repression of a particular kinase in the path have been described.

7. Conclusions

Rsv has gathered >6500 references on PubMed since the landmark finding of its chemoprotective activity by Jang and colleagues. Meanwhile, the benefits of Rsv in a constellation of age-related complications gained a strong momentum due to its finding as a possible direct SIRT1 activator, capable of mimicking the benefits of CR in multiple organisms. The evidence described above supports that Rsv attenuates many age-related chronic diseases and improves overall health status in mammals, including humans. The exact mechanism by which Rsv promotes such a wide range of beneficial effects is, still to this date, unclear. The intrinsic anti-oxidant capacity of the Rsv molecule and its ability to trigger the activation/repression of a wide range of membrane receptors, kinases and other enzymes have turned the quest for a molecular mechanism of action into an epic task. It is always difficult to judge how much detail is needed on the mechanism of action of a dietary component in order to make clinical use. However, the further we understand Rsv actions, the more we can decrease the risk for adverse effects and define novel therapies or agents to obtain health benefits. It might also be true that it is the combination of the many different actions triggered by Rsv that ultimately lead to health benefits. Transgenic mouse models provide information about requirements, but rarely about mechanisms. In this sense, transgenic mouse models clearly illustrate that AMPK and SIRT1 are key mediators of the metabolic health actions prompted by Rsv. However, it seems likely that new analytical methods and paradigms might be needed to understand the fascinating nature of this compound that makes it different from the thousands of other polyphenols found in the western diets.

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