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Solid Dispersion of Resveratrol Supported on Magnesium DiHydroxide (Resv@MDH) Microparticles Improves Oral Bioavailability

Roberto Spogli [1], Maria Bastianini [1], Francesco Ragonese [2,3], Rossana Giulietta Iannitti [4], Lorenzo Monarca [2], Federica Bastioli [2], Irina Nakashidze [5], Gabriele Brecchia [6], Laura Menchetti [6], Michela Codini [7], Cataldo Arcuri [3], Loretta Mancinelli [2] and Bernard Fioretti [2,*]

1 Prolabin & Tefarm, Spin-Off Un. of University of Perugia, Via Dell'Acciaio 9, Ponte Felcino, 06134 Perugia, Italy; roberto.spogli@prolabintefarm.com (R.S.); maria.bastianini@prolabintefarm.com (M.B.)
2 Department of Chemistry, Biology and Biotechnologies, University of Perugia, Via Elce di Sotto 8, 06123 Perugia, Italy; francesco.ragonese@studenti.unipg.it (F.R.); lorenzo.monarca@unipg.it (L.M.); federica.bastioli@unipg.it (F.B.); loretta.mancinelli@unipg.it (L.M.)
3 Department of Experimental Medicine, Perugia Medical School, University of Perugia, Piazza Lucio Severi 1, 06132 Perugia, Italy; cataldo.arcuri@unipg.it
4 S&R Farmaceutici S.p.A Bastia Umbra, 08063 Perugia Italy; r.iannitti@srfarmaceutici.com
5 Department of Biology, Faculty of Natural Science and Health Care, Batumi Shota Rustaveli State University, 6010 Batumi, Georgia; irina.nakashidze@bsu.edu.ge
6 Department of Veterinary Science, University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy; gabriele.brecchia@unipg.it (G.B.); laura.menchetti7@gmail.com (L.M.)
7 Department of Pharmaceutical Sciences, University of Perugia, Via A. Fabretti 48, 06123 Perugia, Italy; michela.codini@unipg.it
* Correspondence: bernard.fioretti@unipg.it

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Abstract: Resveratrol, because of its low solubility in water and its high membrane permeability, is collocated in the second class of the biopharmaceutical classification system, with limited bioavailability due to its dissolution rate. Solid dispersion of resveratrol supported on Magnesium DiHydroxide (Resv@MDH) was evaluated to improve solubility and increase bioavailability of resveratrol. Fluorimetric microscopy analysis displays three types of microparticles with similar size: Type 1 that emitted preferably fluorescence at 445 nm with bandwidth of 50 nm, type 2 that emitted preferably fluorescence at 605 nm with bandwidth of 70 nm and type 3 that is non-fluorescent. Micronized pure resveratrol displays only microparticles type 1 whereas type 3 are associated to pure magnesium dihydroxide. Dissolution test in simulated gastric environment resveratrol derived from Resv@MDH in comparison to resveratrol alone displayed better solubility. A 3-fold increase of resveratrol bioavailability was observed after oral administration of 50 mg/kg of resveratrol from Resv@MDH in rabbits. We hypothesize that type 2 microparticles represent magnesium dihydroxide microparticles with a resveratrol shell and that they are responsible for the improved resveratrol solubility and bioavailability of Resv@MDH.

Keywords: resveratrol; magnesium dihydroxide; solubility; bioavailability; dissolution rate; microparticles

1. Introduction

Resveratrol (trans-3,5,4′-tri-hydroxic-stilbene) is a stilbenic structure polyphenol, initially isolated from the root of the white hellebore (*Veratrum Grandiflorum O. Loes*) and later from the root of the

Polygonum cuspidatum, a plant used in traditional Chinese and Japanese medicine. Resveratrol became popular in 1992 when it was suggested that it could be the reason behind red wine's cardio-protective effects (French paradox; [1]), and its popularity increased in 1997 when it was proven that resveratrol was able to prevent colorectal cancer in mice [1]. Resveratrol based compounds present anti-oxidant, anti-inflammatory, anti-viral, cardio-protective, neuro-protective, anti-cancer and anti-angiogenetic activities [1–3]. It has been recently observed in obese human subjects that treatment with trans-resveratrol reduces glucose, triglycerides and inflammatory marker levels with a similar effect to the one induced by caloric restriction [4]. The mechanism of action of resveratrol has not been completely defined yet, and for this reason recently studies have been carried out in order to understand the aspects that are still not clear [4].

Resveratrol is poorly bioavailable because of reduced absorption mainly due to its low solubility and fast metabolism that converts it into glucuronide and sulfates compounds [1,5]. In humans resveratrol can be detected in plasma about 30 min after oral administration, meaning that its absorption already starts at the gastric level and reaches a plasmatic submicromolar concentration peak. Such peak is variable and hardly related to the used dose. For example, by administrating a 25 mg dose of resveratrol a 10 ng/mL plasmatic concentration is obtained, while increasing such dose by 20 times (500 mg/day) its plasma level increases only seven times (72.6 ng/mL) [6]. Differences in resveratrol absorption have been demonstrated by clinical trials based on the oral administration of 150 mg/day of resveratrol for a prolonged period of time. It has been observed that the same dose produces different plasmatic concentrations: 231 ng/mL [4] and 24.8 ng/mL [7]. Several strategies have been performed to increase its bioavailability and improve its potential health properties. A recent revision of the literature highlights how the increased bioavailability of resveratrol is a necessary element in order to evaluate the real pharmaceutical and health potential of this well-known polyphenol [5]. According to the biopharmaceutical classification system (BCS) [8,9], resveratrol belongs to the second class which means that it is characterized by low solubility in water (about 30 mg/L), while it shares a high membrane permeability (log P~3.1) [10]. Among the different strategies, new formulations have been developed that are able to increase its apparent solubility for example by using a lipophilic vehicle or through various processes such as the complexation with cyclodextrins, nanopreparation, or micellar solubilization with biliary acid [10–12]. It has been demonstrated, in in vitro studies, that the increase of apparent resveratrol solubility allows a partial saturation of the mechanisms that are involved in its metabolism (conjugation) with a subsequent increase of resveratrol's bioavailability [13]. This is in accordance with BCS for molecules class II that increasing resveratrol apparent solubility produces a bioavailability improvement [8,9,14], but in a dedicated study the increased solubility with cyclodextrins doesn't modify its bioavailability [12].

In the present study we investigated that the solid dispersion of resveratrol on magnesium dihydroxide increases its solubility and bioavailability indicating that in some instance this approach could be exploited to enhance biological properties of resveratrol. Although resveratrol does not display chelating properties, some studies have shown its ability to interact with heavy metals such as copper, zinc and aluminum [15,16]. In this work we report that resveratrol interacts with magnesium dihydroxide at the microparticle level and that this is able to modify its bioavailability.

2. Material and Methods

2.1. Solid Dispersion of Resveratrol on Magnesium Dihydroxide Preparation

Magnesium dihydroxide and resveratrol (from *Polygomun cuspidatum*, 98% pure) solid dispersion was performed by modified co-precipitation method of Biswicka et al. [17]. Magnesium dihydroxide on resveratrol solid dispersion and pure micronized resveratrol described in this study was obtained by Good Manufacturing Practice (GMP) chain by Prolabin & Tefarm, Ponte Felcino (PG) and distributed by S&R Farmaceutici SpA, Via dei Pioppi 2, 06083 Bastia Umbra (PG), with the trade name, Revifast®(produced by the manufacturer La Sorgente del Benessere, Via Prenestina, 141 -02014

Fiuggi (FR) Italy on behalf of S&R Farmaceutici S.p.A) The resveratrol content in the solid dispersion was evaluated using the HPLC method (see below). The mean value obtained in the three samples was about 30% and 70% of total weight of resveratrol and magnesium dihydroxide, respectively.

2.2. Particle Size Analysis

The size of the particles was determined using a Malvern Mastersizer 2000, a laser diffraction particle size analyzer, for the dried powders.

2.3. Dissolution Assays

A weighed amount of RSV@MDH or resveratrol were placed in series of closed flat-bottomed glass vessels containing 250 mL of Simulated Gastric Fluid (SGF). The composition of SGF was 35 mM of NaCl, pH 1.2 with HCl. The vessels were inserted in shaking water bath (Nuve ST 30) at 37 °C and 110 rpm for 2 h. At appropriate times (1, 3, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min) 2 mL samples were withdrawn and replaced by fresh dissolution medium, then filtered (Spartan 13/02 RC, Whatman GmbH, Dassel, Germany) and analyzed. The drug concentration was determined by HPLC (see below).

2.4. Field Emission Scanning Electron Microscopy

The morphology of the samples was investigated with a FEG LEO 1525 scanning electron microscope (FE-SEM). FE-SEM micrographs were collected by depositing the samples on a stub holder and after sputter coating with chromium for 20 s.

2.5. Fluorescence Microscopy

Microscopic fluorescence analysis of powders was performed using an Axio Esaminer (Zeiss, Jena, Germany) fluorescence microscope with a CCD digital camera Axio Cam 502 Mono. Samples have been observed with DAPI filter (G 365, FT 395, BP 445/50), and with Rhodamine (BP 545/25, FT 570, BP 605/70) using for excitation mercury lamp (HXP 120V). Image acquisition and analysis was performed with Zen 2 software (Zeiss, Jena, Germany).

2.6. In Vivo Absorption Test

The trial was carried out at the experimental farm of the University of Batumi, Georgia. Rabbits were exposed to a continuous photoperiod of 16 h light per day at 40 lx. Room temperature ranged from 18 to 27 °C. Fresh water was always available. Animals were fed with 130 g/day of a standard diet. The experimental protocol was approved by the Local Ethical Committee for Animal Experimentation at the University Batumi, Georgia. All efforts were made to minimize animal distress and to use only the number of animals necessary to produce reliable results. The tests were conducted on New Zealand White hybrid rabbits (4.5–5 kg weight range). Two groups of four animals each were prepared for the comparative treatment of resveratrol (pure resveratrol versus Resv@MDH). The rabbits were fasted for 24 h before administration of a suspension containing 50 mg/kg of pure resveratrol or 50 mg/kg of resveratrol from Resv@MDH according to Jaisamut et al., 2017 [18]. The powders were suspended in 10 mL of a glucose solution and orally administered to a conscious animal by a syringe (0 min). At 0, 5, 15, 30, 45, 90, 120 and 180 min, blood samples were taken (about 2 mL) through the auricular artery and put in heparinized tubes. The samples were centrifuged at 2500 g for 5 min and the plasma was recovered. Acetonitrile was added to the plasma samples (*v:v* 1:1 ratio) and left for 5 min in order to precipitate plasma proteins. After centrifugation the supernatant was recovered for the dosage of resveratrol by HPLC.

2.7. HPLC Analysis

The measurements were performed by an Agilent HPLC 1200 series equipped with an Agilent Zorbax SB C18 4.6 × 250 mm 5-µm Agilent P/N 880975-902 column. Elution was carried out under isocratic conditions using as mobile phase (Water + 0.1% *v*/*v* Trifluoroacetic acid)/(Acetonitrile + 0.1% *v*/*v* Trifluoroacetic acid) = 65/35, with a flow of 1mL/min and a column temperature of 30 °C. A total of 20 µL of samples were injected, after 0.2 µm Nylon membrane filtration, and the analytes were detected by VWD Detector, λ = 306 nm. For the quantification of resveratrol a calibration was performed to detect the polyphenol at a retention time of 5.6 min with a detection limit of 4 ng/mL. All the plasma concentrations were multiplied by 2 to take into account the dilution in acetonitrile during sample preparation and by 3.6 to take into account the yield of extraction of resveratrol from plasma (28%) [19].

2.8. Statistical Analysis

All results are expressed as the mean ± SE. Differences between two related parameters were assessed by Student's *t*-test. Differences were considered significant at $p < 0.05$. The number of animals used in the current experimental trial is based on the work by Jaisamut et al., 2017 [18].

3. Results

3.1. Microscopic Analysis of Solid Dispersion of Resveratrol on Magnesium Dihydroxide

RSV@MDH powder was dispersed in glycerol and was observed by bright-field microscopy. The presence of particles with different scattering profiles in a narrow size range of a few micrometers was observed (Figure 1A). Fluorescence analysis of the samples with DAPI filter showed that about 10–20% of the microparticles emitted fluorescence. These microparticles were defined as type 1 (Figure 1B). The mean size of type 1 microparticles was 1.8 ± 0.1 µm, n = 40 in diameter (given the non-spherical morphology of the particles, the longest diameter has been taken into account). When the sample was analyzed with the rhodamine filter, a comparable population of particles was visualized with a mean size of 2.0 ± 0.2 µm, n = 34 and was named type 2 (Figure 1C). Type 1 microparticles displayed very scant signals when observed with the rhodamine filter similar to the type 2 particles with the DAPI filter. Finally, the majority of the microparticles didn't display any fluorescence in either filter and were defined as type 3 and had medium size similar to others (Figure 1D).

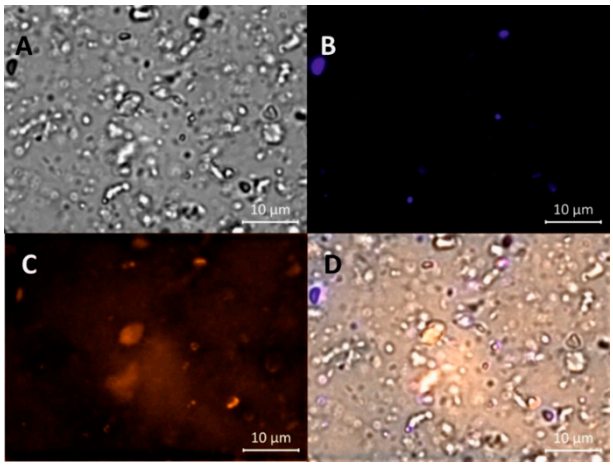

Figure 1. Image of Resv@MDH powder dispersed in glycerol under different excitation sources. (**A**) Bright-field; (**B**) DAPI (4′,6-diamidino-2-phenylindole) fluorescence filter; (**C**) Rhodamine fluorescence filter; (**D**) merging of the Bright-field, DAPI and Rhodamine images.

Thus, the solid dispersion of resveratrol on magnesium dihydroxide was composed by three distinct populations of microparticles based on the fluorescence profile. When we similarly analyzed the dry powder without dispersion in glycerol we observed aggregates of size around 5 µm were present as a possible consequence of the interaction of the three types of microparticles (Figure 2A). In accordance, that the aggregates are based on different types of microparticles, they displayed fluorescence signals from every channel. Granulometric and SEM analysis showed two distinct population sizes, one with size around 1 µm and the second population with size around 6 µm of diameter (Figure 2B,C).

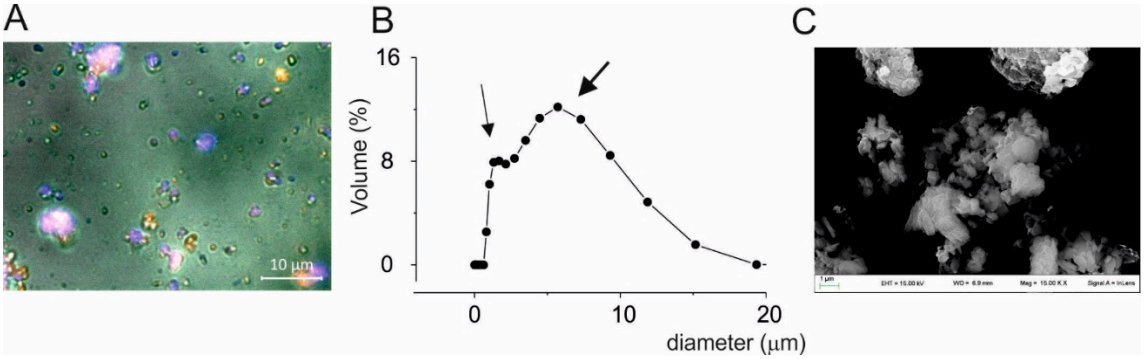

Figure 2. Properties of Resv@MDH dry powder. (**A**) Image created by digital merging of bright-field and DAPI/Rhodamine fluorescence illumination. (**B**) Granulometric analysis of Resv@MDH dry powder. (**C**) SEM image of Resv@MDH dry powder.

3.2. Molecular Nature of Microparticles of Solid Dispersion of Resveratrol on Magnesium Dihydroxide

To define the molecular nature of the different types of microparticles, we studied a powder of pure micronized resveratrol with similar distribution size of solid dispersion. Granulometric analysis confirmed that micronized resveratrol have the size of 1–6 µm in diameter (Figure 3A) similar to the particles size of RSV@MDH (see Figure 1 for comparison). Fluorescence microscopy analysis of the micronized resveratrol displayed all the microparticles emitted fluorescence intensity as type 1 particles (Figure 3B,C), whereas the presence of microparticles that showed fluorescent properties as type 2 and 3 were not observed (Figure 3D,E). No fluorescence was observed (DAPI and Rhodamine filters) during microscopic analysis of pure magnesium dihydroxide, indicating that type 3 microparticles could be constituted by only magnesium dihydroxide. These data suggested that the type 1 microparticles were microparticles of pure resveratrol, whereas the type 3 microparticles represented magnesium dihydroxide. Since the fluorescence properties were due to resveratrol, type 2 macroparticles could be distinguished from type 3 microparticles by presence of resveratrol. The type 2 microparticles were further investigated to define the morphological features. In fact, it was possible to see a shell of fluorescence around a non-fluorescent core and this was due to resveratrol surrounding the core of magnesium hydroxide microparticles (Figure S1). All the features of the microparticles are stated in Table 1.

Table 1. Principle features of microparticles of RSV@MDH.

Characteristic	Type 1 Microparticles	Type 2 Microparticles	Type 3 Microparticles
DAPI filter (G 365, FT 395, BP 445/50)	High intensity	Low intensity	none
Rhodamine (BP 545/25, FT 570, BP 605/70)	Low intensity	High intensity	none
Particles size	~1.8 ± 0.1 µm	~2.0 ± 0.2 µm	~1.7 ± 0.1 µm
Resveratrol contents	High	Low (shell distribution)	none
Dissolution rate	Low	High	n.d.

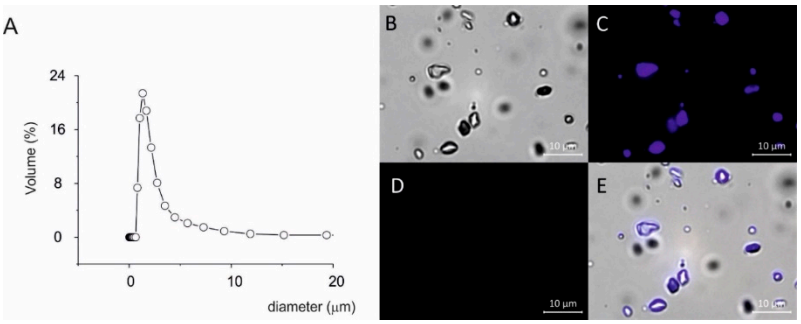

Figure 3. Properties of pure micronized resveratrol. (**A**) Granulometric analysis of microcrystalline resveratrol. (**B**–**E**) Image of crystalline resveratrol powder dispersed in glycerol under different excitation sources. (**B**) Bright-field; (**C**) DAPI fluorescence filter; (**D**) Rhodamine fluorescence filter. (**E**) Merging of the Bright-field, DAPI and Rhodamine images.

3.3. Dissolution of Solid Dispersion of Resveratrol on Magnesium Dihydroxide.

In Figure 4 dissolution profiles of Resv@MDH (red squares) and pure resveratrol (black squares) are presented (mg/L in function of time). The experimental data was fit with exponential equation $C(t) = Cmax\ (1 - \exp(-t/\tau))$, where Cmax = maximum solubility value; t = time; τ = time in which dissolution reaches about 63% of maximum process. The equation represents a form studying the dissolution profiles according to Weibull's models [20]. The best data fit is for Cmax: 40.8 and 13 mg/L for Resv@MDH and resveratrol respectively while τ was 0.4 and 2.2 min for Resv@MDH and resveratrol respectively. These data indicated that Resv@MDH showed a dissolution rate five times higher than resveratrol (compared to τ) and a maximum solubility three times as big (compared to Cmax). To assess the importance of particles size in dissolution rate, we compared the solubility profile of pure micronized resveratrol with similar size particles of Resv@MDH (Figures 3 and 4). It was possible to see (compare black and green squares in Figure 4A) the reduction of particles size modified only dissolution kinetic according to the Noise-Witting law, but did not modify the maximal solubility [21].

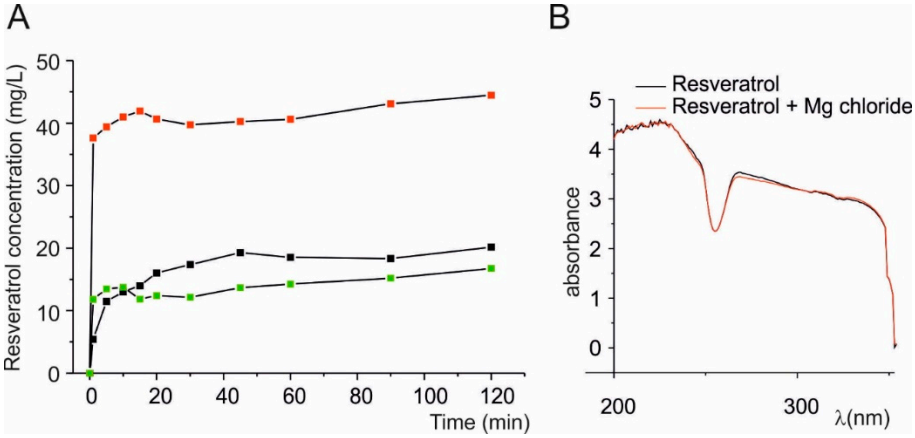

Figure 4. Solubility of resveratrol from Resv@MDH and its interaction with magnesium ion. (**A**) dissolution test of pure resveratrol powder (black MDH square) versus solid dispersion on magnesium dihydroxide (Resv@MDH, red square) and pure micronized resveratrol (green square). (**B**) UV/Vis Absorbance spectroscopy for the study of Resveratrol. Black: 0.008 mM Resveratrol in ethanol:water (75:25, *v*/*v*) in 100 mM HCl; Red: 0.008 mM Resveratrol in ethanol:water (75:25, *v*/*v*) in 100 mM HCl + 0.008 mM of MgCl.

To verify if magnesium ion participates in major solubility (Cmax) of resveratrol by forming a complex, we verified the interaction between them by performing spectrophotometric profile of

resveratrol alone or in presence of magnesium ion in acid environment. It is possible to see in Figure 4B, that the addition of magnesium does not significantly modify the UV absorption spectra, suggesting that the magnesium does not interact with resveratrol and that the major solubility was dependent on other factors.

3.4. Pharmacokinetic Profile of Solid Dispersion of Resveratrol on Magnesium Dihydroxide

The rabbit animal model is excellent to perform pharmacokinetic studies [20] and recently was used to evaluate the bioavailability of a new resveratrol formulation [18]. The mean plasma concentration of resveratrol following oral administration of 50 mg/kg of Resv@MDH and pure resveratrol was investigated in the rabbit animal model. Resveratrol plasma concentration versus time curves from administration is displayed in Figure 5. Pharmacokinetic variables derived from this pharmacokinetic profile are summarized in Table 2. Resveratrol is virtually absent in animal plasma prior to oral administration (0 min) but it seemed to be rapidly absorbed with a peak of maximal concentrations (C_{max}) between 15 and 30 min post-dose. The C_{max} of resveratrol was 76.3 ng/mL and 101.3 ng/mL for resveratrol and Resv@MDH respectively. At 30 up to 90 min from the administration, the resveratrol plasma concentration of Resv@MDH treated animals results statistically greater as compared to resveratrol treated animal, while at 180 min the resveratrol is no longer detectable in the plasma of both groups of animals. The values of Area Under Curve (AUC) of the plasma concentration profile until the 3-h time point was 2698 ng min/mL and 8944 ng min/mL or resveratrol and Resv@MDH respectively. This data demonstrates an enhancement of resveratrols bioaviability by 3.3-fold (ratio of $AUC_{Resv@MDH}/AUC_{resveratrol,}$ Table 2).

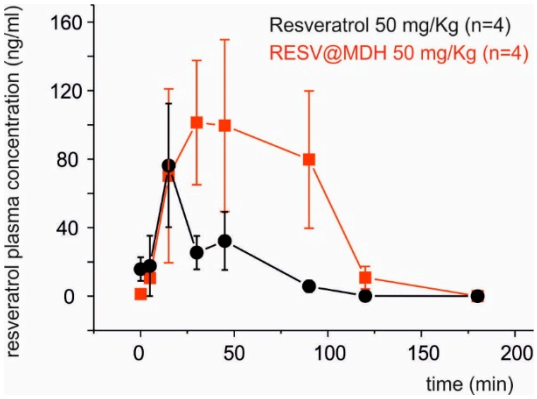

Figure 5. Pharmacokinetic profiles of resveratrol after oral administration in rabbits. Groups of 4 animals each were treated with resveratrol (50 mg/Kg of pure resveratrol versus Resv@MDH). Blood samples taken at 0, 5, 15, 30, 45, 90, 120 and 180 min.

Table 2. Pharmacokinetic parameters of oral administration of 50 mg/Kg of resveratrol from pure resveratrol and from Resv@MDH.

Parameters	Resveratrolo 50 mg/Kg	Resv@MDH (Resveratrol 50 mg/Kg)	Increase %
AUC (Area Under Curve)	2698 ng min/mL	8944 ng min/mL	330
Time to plasmatic peak	15 min	30 min	200
Peak duration	25 min	105 min	420
Cmax	76.3 ng/mL	101.3 ng/mL	130

4. Discussion

Solid dispersion of resveratrol on magnesium dihydroxide (Resv@MDH) represents a new formulation that possesses an increased solubility of resveratrol (spring form). Resv@MDH is able to solubilize itself faster and in greater amounts with respect to resveratrol, with remarkable advantages

in biopharmaceutical terms and therefore of bioavailability. From the physical point of view it is a polydisperse granular material, where the active is supported by inorganic material with a high safety level (magnesium hydroxide). Furthermore, this improves its performance without chemically modifying the natural product's structure. Resv@MDH allows to obtain an apparent solubility much higher with respect to resveratrol as a consequence of an increased dissolution rate and of the establishment of over-saturation phenomena due to different energetic states of resveratrol (Figure 6). This dispersion is formed by three types of microparticles that we define as type 1, 2 and 3. Based on our results we hypothesized that microparticles type 1 and 3 represent resveratrol and magnesium dihydroxide crystals, respectively. The unexpected result is the presence of microparticles type 2 that probably represent the form responsible for enhanced properties of the solid dispersion. Based on the evidence of change of its fluorescence, we suggest that a fraction of resveratrol forms a shell around magnesium dihydroxide microparticles. The better solubility of resveratrol displayed by solid dispersion could be explained by the coexistence of two energetic states of resveratrol related to the two types of microparticles observed (type 1 and 2, Figure 6). It is possible to exclude the involvement of free magnesium ions (Mg^{2+}) in improving the solubility of resveratrol since their absorbance spectrum was not modified by the presence of metals in acidic environment (Figure 4B). The state of over-saturation could lead to the major absorption (increase of the gradient concentration) and therefore increased bioavailability [22].

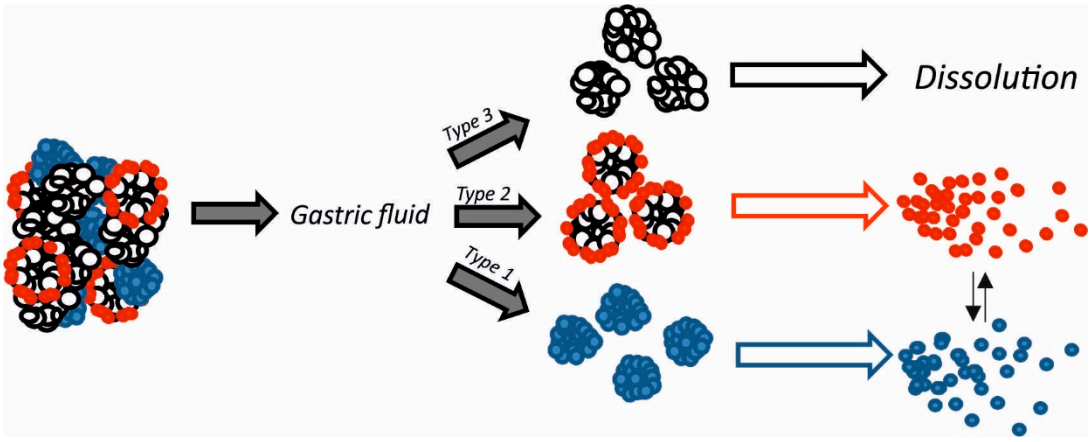

Figure 6. Scheme of hypothetical dissolution events that occur to Resv@MDH powder when it is in contact with simulated stomach fluids. Big powder aggregates divide into three main microparticles named as type 1, 2 and 3. In acidic milieu the type 3 microparticles of magnesium dihydroxide completely dissolves; type 1 microparticles (blue) dissolves in water solution together with type 2 microparticles (red). In this case the limiting step in resveratrol release could be related to acid erosion of dihydroxide core.

The reduced and homogeneous particle size represents a parameter that improves the dissolution rate observed for Resv@MDH is according to Noyes and Whitney law [21]. The comparative dissolution rate of resveratrol displayed in Figure 5 demonstrates that the supersaturating state is not dependent from the particles size of resveratrol. Further studies are needed to clarify the mechanisms of the better solubility of solid dispersion of resveratrol on magnesium dihydroxide. The Resv@MDH represent a new way to uncover the therapeutic potential of resveratrol with possible application as anti-inflammatory, anti-viral, cardio-protective, neuro-protective, anti-cancer and anti-angiogenetic agent [1–3]. As regard the anticancer properties, resveratrol was demonstrated to increase the effect of radio and chemotherapeutic agents [23] in particular against glioblastoma cancer cells [24].

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/12/1925/s1, Figure S1: Image of RESV@MDH powder dispersed in glycerol under different eccitation sources. (**A**) Rohdamine fluorescence filter (**B**) brightfield; Note: white arrow indicates the fluorescent shell of resveratrol around the core of magnesium diihydroxide.

Author Contributions: Conceptualization, B.F. and R.S.; validation, L.M. (Laura Menchetti), F.B. and F.R.; formal analysis, F.R. and L.M. (Laura Menchetti); investigation, R.S., M.B., L.M. (Loretta Mancinelli), F.B, I.N., G.B., M.C., and C.A.; resources, R.S. and R.G.I.; data curation, R.S and B.F.; writing—original draft preparation, L.M. (Loretta Mancinelli) and B.F.; writing—review and editing, L.M. (Loretta Mancinelli), B.F and R.G.I.; visualization, L.M. (Lorenzo Monarca) and F.R.; supervision, B.F.; project administration, B.F.; funding acquisition, B.F.

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Conflicts of Interest: R.S. and B.F. are co-inventors of the patent EPO n EP20130425091; R.G.I is an employee of S&R Farmaceutici S.p.a., whom hold the rights and license of REVIFAST®. All other authors declare no conflict of interest.

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Resveratrol Supported on Magnesium DiHydroxide (Resv@MDH) Represents an Oral Formulation of Resveratrol With Better Gastric Absorption and Bioavailability Respect to Pure Resveratrol

*Rossana Giulietta Iannitti[1], Alessandro Floridi[2], Andrea Lazzarini[2], Alice Tantucci[3], Roberta Russo[3], Francesco Ragonese[4], Lorenzo Monarca[4], Concetta Caglioti[4], Roberto Spogli[5], Lucio Leonardi[1], Massimiliano De Angelis[3], Federico Palazzetti[4] and Bernard Fioretti[4]**

[1] S&R Farmaceutici S.p.A., Perugia, Italy, [2] Forensic Toxicology Laboratory, CRABioN Research Center, Perugia, Italy, [3] Department of Medicine, University of Perugia, Perugia, Italy, [4] Department of Chemistry, Biology, and Biotechnologies, University of Perugia, Perugia, Italy, [5] Prolabin & Tefarm, Spin-Off Un. of University of Perugia, Perugia, Italy

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Sanofi India Limited, India

***Correspondence:**
Bernard Fioretti
bernard.fioretti@unipg.it

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Resveratrol attracts great interest because of the plethora of *in vitro* effects at the micromolar concentration range. Unfortunately, these effects are difficult to establish *in vivo*, due to the low concentration of resveratrol reached. This discrepancy is due to the molecules low solubility in water that favors the propensity for an intestinal absorption rather than in the gastric compartment. To address these challenges, we developed a Solid Dispersion of Resveratrol Supported by Magnesium Di Hydroxide formulation (Resv@MDH). This formulation displays increased water solubility and better bioavailability relative to pure resveratrol in the rabbit animal model. In our study, we evaluated the pharmacokinetics profile of resveratrol in six healthy human subjects following 180 mg of oral resveratrol administration, derived from Resv@MDH or pure resveratrol. Free resveratrol was evaluated in the whole blood sample by using HPLC - MS/MS. In comparison with pure resveratrol that displays an increase of the maximum plasma concentration, Cmax at about 90 min and 2 μM, Resv@MDH displays an earlier peak of resveratrol concentration with an increase of Cmax at about 30 min and 6 μM. The different kinetics suggest a main gastric route for resveratrol absorption from Resv@MDH, where, because of its improved dissolution rate, there seems to be a higher propensity for an acidic environment, as opposed to that with pure resveratrol. This conclusion is also supported by the numerical simulation analysis, which considers the principal steps during the oral route administration. Moreover, there is a 2-fold increase in the amount of free resveratrol with respect to pure resveratrol confirming a better bioavailability observed in the animal model. The characteristic feature of the pharmacokinetic profile of Resv@MDH implies that the beneficial properties of resveratrol in human health should be capitalized on it.

Keywords: resveratrol, human health, bioavailability, dissolution rate, whole blood concentration

INTRODUCTION

Resveratrol [(E)-5-(p-hydroxystyryl) resorcinol] is a polyphenol with a stilbenic structure and is widely diffused in nature as phytoalexin (1). Biological properties of resveratrol have mostly been related to its antioxidant effects, due to its polyphenolic nature, and mainly to its ability to increase the expression of the intracellular antioxidant mechanism (e.g., SOD, CAT, and GSH). Along with this protective effect on oxidative stress, resveratrol has been shown to promote the mitochondrial metabolism by increasing the number of mitochondria (mitochondrial biogenesis), their activity and production of ATP. Resveratrol exerts its biological effects thought AMPK/SIRT1/Nrf2, ERK/p38 MAPK, and PTEN/Akt- signaling pathways (1–3). Resveratrol has also been shown to have several beneficial properties for human health such as protection from metabolic and cardiovascular diseases, neuroprotection, and both anti-inflammatory and immunomodulation effects (1, 4). Efficacy associated with resveratrol use has been attributed to the activation of the lysine deacetylase, Sirtiun 1 (SIRT1) (5), the cAMP pathway, or the AMP-activated protein kinase (AMPK) (6, 7) and partial agonist in estrogen receptors (8). For this reason, resveratrol has a potential application in the prevention and treatment of chronic diseases, including cancer (9), neurodegenerative diseases such as Alzheimer's (9), and metabolic diseases such as diabetes (10), as well as anti-aging effects. The therapeutic properties of resveratrol are promptly exploited by the pharmaceutical industry and different conventional oral dosage forms have been developed (11).

The efficacy, safety, and pharmacokinetics of resveratrol have been documented in hundreds of clinical trials, with additional trials currently ongoing. A note however is that some of these clinical trials have not yet been published (12). Nevertheless, published trials represent a relatively small portion when one considers the thousands of published reports to date that prove the many *in vitro* effects of resveratrol and its rapid metabolism in the human body, which can limit its clinical use and effectiveness. The bioavailability of oral administration of resveratrol is limited, mainly due to its physical and chemical properties (water solubility) and its biological stability (metabolism). Given resveratrol's low solubility in water and high membrane permeability, it is collocated in the second class of the biopharmaceutical classification system [BCS, (13, 14)]. Due to its chemical and physical profile, after oral administration, resveratrol is slowly absorbed along the gut. The principal site of absorption is at the intestinal level through passive diffusion or active transport via ATP-dependent binding cassette (ABC) transporters (15). Resveratrol can be absorbed through the portal bloodstream to the liver by passive diffusion or receptor-mediated transport, where it is rapidly metabolized in glucuronide and sulfate derivatives. Additionally, resveratrol can efficiently bind in a non-covalent manner, to proteins such as albumin, lipoproteins and, in particular, to a fraction of low-density lipoproteins (LDLs) (16). As a result, plasma-free resveratrol is quite limited due to its short plasma half-life (t1/2) (17) and extensive metabolism in the intestine and liver compartments.

The pharmacokinetics of resveratrol has been investigated in several clinical studies; with various oral doses and regime of administration such as single dose or regime protocols (11). Among single dose studies, Almeida et al. (18) evaluated the pharmacokinetics profile after 25, 50, 100, and 150 mg of resveratrol administration whereas Nunes et al. (19), Vaz-da-Silva et al. (20) and Sergides et al. (15) study the administration of a dose of 200, 400, and 500 mg of resveratrol, respectively. Altogether, the principal results from these studies are that the resveratrol plasma concentration reached *in vivo* is in the submicromolar range with a time peak concentration of about 1 h according to a principal intestinal site of absorption (11). Recently, by using an animal model, a higher distribution of resveratrol in cellular compartment (76%) of blood respect to that in plasma was observed (21). This aspect could be taken into account to estimate the pharmacokinetic properties of single dose of oral resveratrol (15).

Several strategies have been designed to modify resveratrol's pharmacokinetics, thereby increasing its bioavailability and improving its potential health benefits. Recently, we have proposed a solid dispersion of resveratrol on magnesium dihydroxide, Resv@MDH. This strategy increases its solubility and bioavailability, demonstrating its potential use in enhancing the biological properties of resveratrol *in vivo*. Compared with pure resveratrol, Resv@MDH solubilizes itself faster and in greater amounts, has an increased bioavailability demonstrated in the rabbit animal model and thus proving itself advantageous in biopharmaceutical terms (22). The purpose of this study is to assess the pharmacokinetic profile of resveratrol in healthy human subjects by estimating resveratrol from whole blood and comparing it to pure resveratrol from Resv@MDH.

MATERIALS AND METHODS

Materials

Acetonitrile, Ultrapure water, Formic acid and resveratrol used are of analytical grade for LC/MS and purchased from the company SIGMA (Milan, Italy) and/or Carlo Erba (Milan, Italy). The resveratrol used for the Resv@MDH preparation and pure resveratrol were obtained from the Polygonum cuspidatum Siebold & Zucc extract (98% pure). Resv@MDH was obtained by Good Manufacturing Practice (GMP) chain (Prolabin & Tefarm, Perugia, Italy and La Sorgente del Benessere S.p.A, Fiuggi Italy). Resv@MDH is distributed by S&R Farmaceutici S.p.A. Bastia Umbra, Italy, with the trade name of Revifast®. The resveratrol content in Resv@MDH was evaluated using the HPLC method and the mean value obtained in the three samples was about 30 and 70% of total weight of resveratrol and magnesium dihydroxide, respectively.

Dissolution Assays in Sink Condition

Dissolution assay was performed in sink condition to better reproduce the *in vivo* situation. The general procedure used was previously described (22). Briefly, the weighed amount of Resv@MDH or resveratrol was placed in series of closed flat-bottomed glass vessels containing 250 mL of Simulated Gastric Fluid (SGF). The composition of SGF was 35 mM of NaCl, pH

TABLE 1 | Demographic characteristics of the study population.

Patient no	Age	BMI	Systolic BP, mmHg	Diastolic BP, mmHg
1	32	21.4	105	65
2	41	32	121	82
3	32	26	119	74
4	40	26	123	73
5	31	28	117.5	75
6	35	20.4	107	70

1.2 with HCl. The vessels were inserted in shaking water bath (Nuve ST 30) at 37°C and 110 rpm for 2 h. At appropriate times, the reaction was sampled, filtered (Spartan 13/02 RC, Whatman GmbH, Dassel, Germany) and analyzed by HPLC. The measurements were performed by an Agilent HPLC 1200 series equipped with an Agilent Zorbax SB C18 4.6 × 250 mm 5-μm Agilent P/N 880975-902 column and VWD Detector, λ = 306 nm. Elution was carried out under isocratic conditions using as mobile phase (Water + 0.1% v/v Trifluoroacetic acid)/(Acetonitrile + 0.1% v/v Trifluoroacetic acid) = 65/35, with a flow of 1 mL/min and a column temperature of 30°C. For the quantification of resveratrol, a calibration was performed to detect the polyphenol at a retention time of 5.6 min with a detection limit of 4 ng/mL.

Study Design and Treatments

Six healthy adults aged 18–41 years of both sexes with body mass index > 18.5 and <32 kg m^{-2} were eligible for the study if they were willing and able to understand and sign an informed consent (**Table 1**). Inclusion criteria: non-smokers, not on any pharmacological therapy and overall in good health as determined by medical history review, physical examination, and clinical laboratory tests. Key exclusion criteria included pregnancy or lactation, history of hypersensitivity reactions, under drug treatment or dependency or alcohol abuse. Subjects taking dietary supplements containing antioxidants, untreated hypothyroidism were also excluded.

The study was a randomized, single blind, crossover study carried out in a single center in Perugia, Italy. Two single-dose treatments were administered orally under fasting conditions. Phase 1: subjects were given on the first day, a solution of content A or B (see above) obtained by dissolving the contents of the capsule in water. Samples of 2 ml of venous blood were taken in a tube containing EDTA, from the arm vein via ago cannula, at the following times 0 min (fasting before administration), 15, 30, 60, 90, 120, and 180 min. Phase 2: second day, wash-out period: each treatment period was separated by a 1-day washout. Phase 3: during the third day, the person who took solution A in the first day, takes solution B, and *vice versa.* Blood samples were taken as described for Phase 1. See Study design (**Figure 1**).

The treatment sequence for each participant was assigned by a computer-generated randomization list. The treatments were in the form of a suspension obtained by adding the content of capsule containing 180 mg of resveratrol in either the Resv@MDH form or pure resveratrol to water. Subjects fasted overnight for at least 10 h prior to the suspension of resveratrol tablet administration. The study protocol was approved by a regional ethics committee review board (project number 12477/18, title approved on 18/01/2018 by CEAS Umbria, Perugia Italy) and registered in the ClinicalTrials.gov Protocol Registration System (identifier: NCT04258306). The study was conducted in accordance with Good Clinical Practice, and followed the requirements of the Declaration of Helsinki and relevant European regulation and directives. All subjects provided written informed consent.

Pharmacokinetic Profile Quantification

Pharmacokinetic profile quantification was performed using HPLC-MS/MS applied to the whole blood sample to evaluate resveratrol (21, 23). Specifically, the blood samples were diluted with acetonitrile in a ratio of 1: 4, vortexed and centrifuged at 14,500 rpm for 5 min. The supernatant was filtered using a 0.2 μm membrane filter before injecting in HPLC coupled to triple quadrupole linear MS/MS (AB sciex 5500, Shimadzu). Separation was achieved on a C18 column (Agilent Eclipse Plus C18 3.5 μm 4.6 × 100 mm PN 959961-902, SN USUXR18880, LN B14112) at 35°C with a flow rate of 0.9 ml/min. The gradient elution system (A: water and 0.1%; formic acid B: acetonitrile and 0.1%; formic acid) was as follows: 0–2 min (2% B and 98% A), 2–7 min (100% B), 7–10 min (2% B and 98% A). Retention time and the Selected Reaction Monotoring (SRM) transition were developed by using analytical grade resveratrol (Sigma-Aldrich, Milan). In the sample, Resveratrol was quantified by monitoring SRM transition at m/z 229→165. The validation of the analytic methods was performed by adding a known amount of resveratrol in the blood samples. The Lower limit of quantitation (LLOQ) was 5 ng/ml.

Statistical Analysis

All results are expressed as the mean ± SE. Differences between two related parameters were assessed by Student's *t*-test. Differences at $^{*}p < 0.05$ were considered significant.

RESULTS

The Dissolution Rates of Resv@MDH and Pure Resveratrol

In our previous work, we compared the dissolution rate of resveratrol from Resv@MDH with that of pure resveratrol in a condition that predicted the saturation conditions (22). To better investigate the pharmacokinetics of resveratrol following oral administration, we then studied the dissolution rate in sink conditions [(24), no oversaturation condition]. **Figure 2** shows the dissolution profiles of 20 mg of resveratrol in a liter of solution (pure resveratrol vs. Resv@MDH) such an amount is three times lower than the solubility of resveratrol in the used dissolution liquid (max solubility 62 mg/L). The experimental data relevant to the dissolution of pure resveratrol is best described through mono-exponential kinetics, with constant dissolution of about 39 min. In contrast, mono-exponential kinetics (dashed red lines) are required to describe the dissolution data of resveratrol from Resv@MDH. In this case, bi-exponential kinetics are required

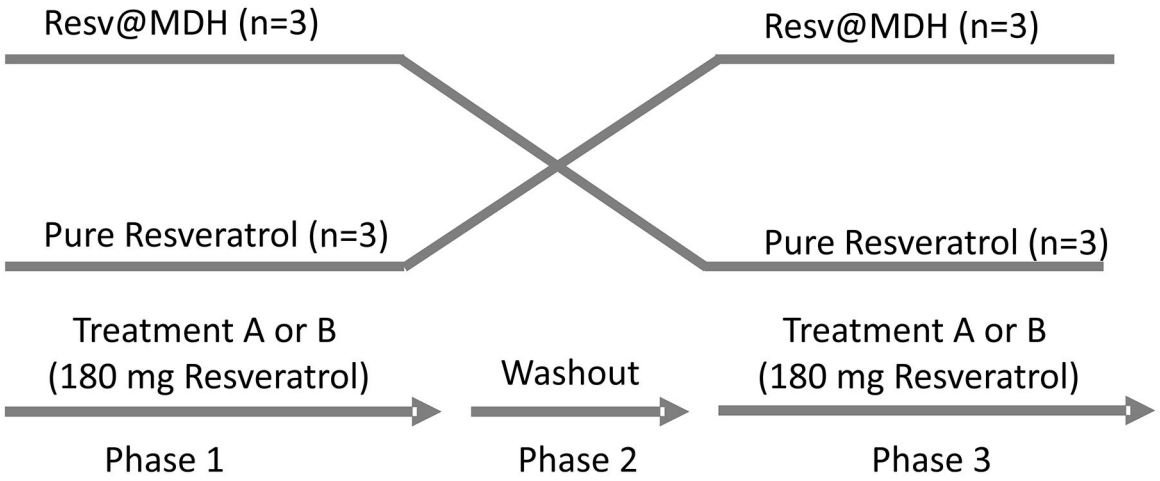

FIGURE 1 | Study design. Randomized, open label, single blind crossover study. Two single-dose treatments of resveratrol were administered orally with a 1-day intermediate washout period. The treatments consist of a solution of either (A) 180 mg pure Resveratrol (Polygonum cuspidatum 98%) or (B) 180 mg Resveratrol from Resv@MDH (600 mg 30% w/w of resveratrol) dispersed in water. After a 1-day washout, subjects who took solution A took solution B and vice versa. No drop out of patients was recording during the trial.

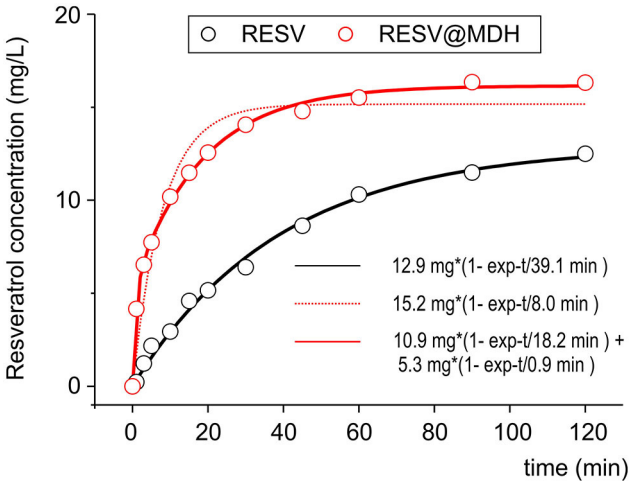

FIGURE 2 | Dissolution profile of resveratrol and Resv@MDH in gastric simulation fluid. The plot reports the solute concentration in mg/L vs. time in minutes. Red dots and black dots indicate the experimental data of Resv@MDH and resveratrol, respectively. The solid red line, black line and red dash line represent the best fits with monoexponential models (black and red dots) and biexponential (red line), respectively, as described in the text.

(R^2 of 0.94 and 0.99 for mono and bi-exponential models, respectively), where there is a fast component of about 1 min (about 5 mg) and a slower one of about 18 min (about 10 mg). The ratio of the fast component over the slower one is 1:2. These dissolution profiles are consistent with the micro-particle composition of Resv@MDH (22).

The dissolution process of the pure resveratrol is given by

$$R_c \rightleftarrows R_{sol} \quad (1)$$

where R_c is the solid crystal resveratrol and R_{sol} is the soluble form (hydrated). It follows a first-order kinetic, given by the equation

$$\frac{d\,[R_{sol}]}{dt} = k_{d1} * ([R_{max}] - [R_{sol}]) \quad (2)$$

where $[R_{sol}]$ is the concentration in water, t is the time, $k_{d1} = 2.56 \cdot 10^{-2}$ min^{-1} is the kinetic constant dissolution 1 and $[R_{max}] = 12.9$ mg/L is the asymptotic concentration of resveratrol in water obtained by fit.

Concerning the dissolution of Resv@MDH in water, we found that it follows two kinetic laws, arguably given by two different dissolution processes, according to the following scheme:

$$\begin{array}{l} R(\text{type 1}) \\ \qquad\qquad R_{aq} \\ R(\text{type 2}) \end{array} \quad (3)$$

where R (type 1) and R (type 2) are two different forms of resveratrol, namely, R (type 1) is crystalline pure resveratrol, while R (type 2) is formed by resveratrol and brucite agglomeration according to a previous report (22). Both dissolution processes follow a first-order kinetic, given by the following equations:

$$\frac{d\,[R_{sol}]}{dt} = k_{dtype1} * \left(\left[Rtype1_{max}\right] - [R_{sol}]\right) \quad (4)$$

$$\frac{d\left[R_{aqsol}\right]}{dt} = k_{dtype2} * \left(\left[Rtype2_{max}\right] - [R_{sol}]\right) \quad (5)$$

where $k_{dtype1} = 5.48 \cdot 10^{-2}$ min^{-1} and $k_{dtype2} = 1.10$ min^{-1} are, respectively the kinetic constant of dissolution of R (type 1) and

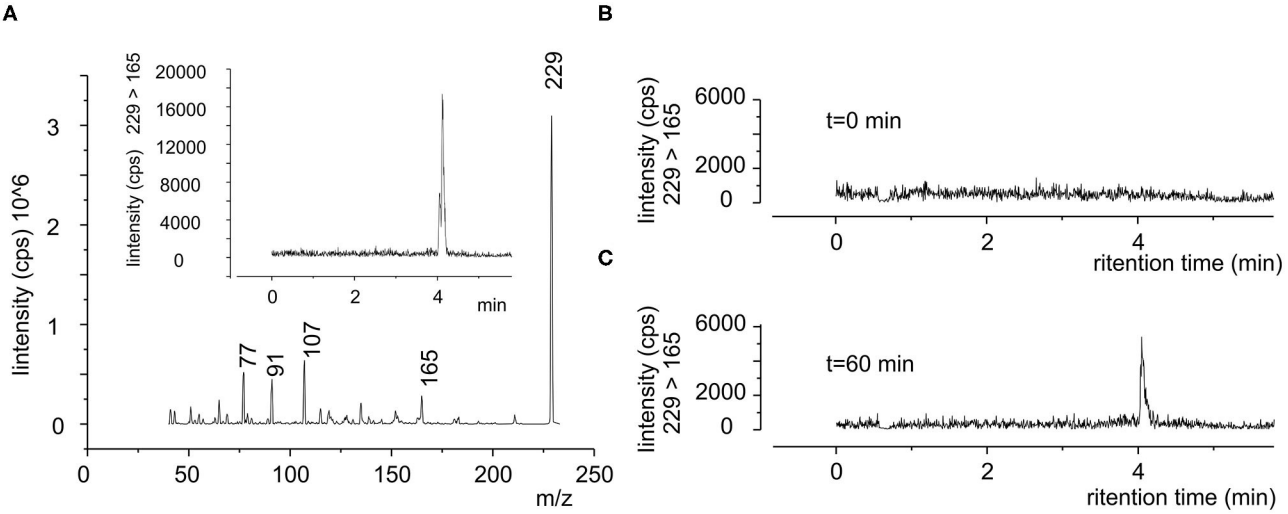

FIGURE 3 | Typical SRM chromatogram of resveratrol in human blood. **(A)** Product ion mass spectra of standard resveratrol (100 ng/ml), where it is possible to see the principal m/z ratio of 229, 165, 107, 91, and 77 peaks. Inset chromatograms of 229-165 SRM in the same sample, shown is the retention time of resveratrol. **(B,C)** Blood sample obtained from same subject (subject 1) before (0 min) and after 60 min after the oral administration of a single dose of pure resveratrol (180 mg), respectively.

R (type 2), while $[\text{Rtype1}_{\max}] = 10.9$ mg/L and $[\text{Rtype2}_{\max}] = 5.3$ mg/L are the asymptotic concentration in water of the two forms of resveratrol. The dissolution curve of Resv@MDH in **Figure 2** is given by the sum of Equations (4) and (5).

The dissolution processes have been indicated as an equilibrium processes; thus, the dissolution kinetics must be intended as a resultant of the direct and -reverse processes. This aspect however, must be investigated in future studies.

Pharmacokinetic Properties of Resv@MDH and Pure Resveratrol After Oral Administration

Since resveratrol cannot be completely recovered from plasma (21), we directly evaluated the polyphenol in the whole blood by using LC/MS-MS. **Figure 3A**, displays the mass spectrum of standard resveratrol solution (100 ng/ml), where the principal fragmentation pattern is apparent. We selected SRM (229-165) to evaluate the retention properties of resveratrol and defined it at about 4 min (inset **Figure 3A**), though a similar result was obtained following other SRM (data not shown). As expected, in the blood sample of fasting subjects no resveratrol trace was observed (**Figure 3B**), whereas it was clearly evident after oral administration of pure resveratrol (**Figure 3C**). The resveratrol concentration in the plasma was obtained from the calibration curve and expressed in μM units (see Methods).

Figures 4A–F and **Supplementary Figure 1** show the single data points of resveratrol in blood samples obtained from the six subjects that participated in the study following oral administration of 180 mg of resveratrol from pure resveratrol (black dots) and Resv@MDH (red dots), respectively. Resveratrol concentration was defined based on a calibration curve by using chromatographic methods described in **Figure 3**. Before the oral administration of either form of resveratrol ($t = 0$), the blood concentration of resveratrol was undetectable indicating also a sufficient wash out period. In all subjects, the blood concentration of resveratrol following Resv@MDH administration was higher than that with pure resveratrol. This observation held true up until the 2-h mark after oral administration, at which point differences between blood concentration of Resv@MDH and pure resveratrol ceased to exist. By using PK solve software (25), we analyzed the pharmacokinetic properties of the plasma profile from each subject. Parameters derived from this analysis are summarized in **Table 2**.

In **Figure 5**, we report the mean concentration of resveratrol in the whole blood as a function of time, combining the results from all subjects. By examining these data, we can specify the differences between the concentration profiles of pure resveratrol and Resv@MDH. A different pharmacokinetic profile was clear between the two sources of resveratrol. Concentration of pure resveratrol increased slowly after oral administration with a steady-state level at around 90 min, whereas with Resv@MDH, a transient profile was observed. Peak concentration occurred at around 30 min after oral administration, with a decay of plasma concentration best described by a mono-exponential model. The dash line in **Figure 5** represent the best fit of experimental data point of resveratrol blood concentration following Resv@MDH administration with the equation $[\text{Resv}]^*\exp((-t)/\tau) + C$ with [Resv] maximal theoretical resveratrol concentration at $t = 0$, τ decay constant and C a constant that describes the resveratrol plasma concentration a infinite time. The parameter obtained is [Resv]= ca. 8 μM, τ = ca. 79 min and C = 130 nM.

The maximum plasma concentration (Cmax) of resveratrol was 2.2 and 6.3 μM for resveratrol and Resv@MDH,

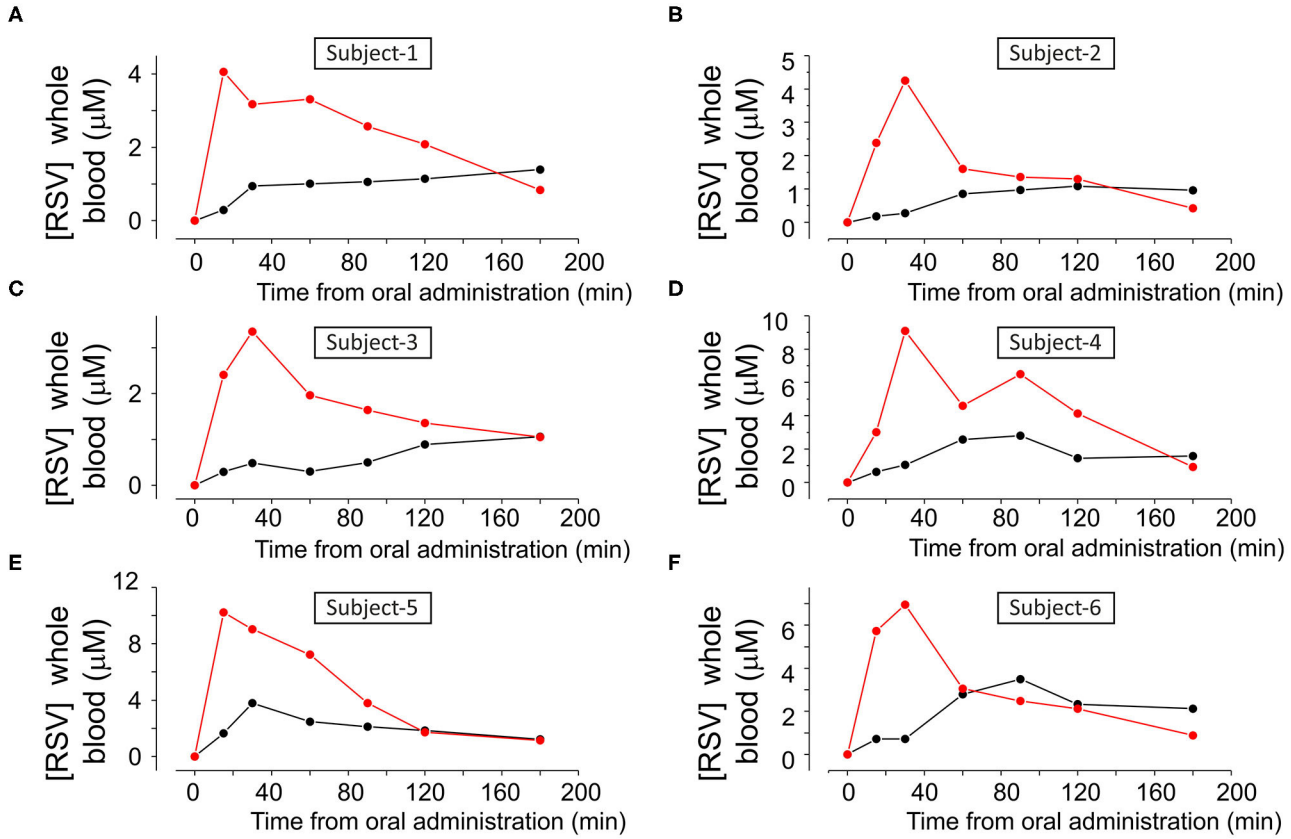

FIGURE 4 | (A–F) Concentration of resveratrol in whole blood as a function of the time, in each subject after treatment with either **(A)** 180 mg pure Resveratrol (Polygonum cuspidatum 98%, (black dots) or **(B)** 180 mg Resveratrol from Resv@MDH after oral administration (red dots).

TABLE 2 | Summary of pharmacokinetic parameters for resveratrol following a single dose of 180 mg resveratrol.

Parameters	Unit	Mean resveratrol	se	*n*	Mean Resv@MDH	se	*n*	*P*
t1/2	Min	128.9	6.5	3	63.6	14.6	6	$p = 0.02$
Tmax	Min	115	23.8	6	25	3.2	6	$p = 0.004$
Cmax	μmol/ml	2.3	0.5	6	6.3	1.2	6	$p = 0.010$
AUC 0–3	νmol/ml*min	248.3	48.7	6	526.0	92.8	6	$p = 0.024$

The parameters are calculated as follows: AUC 0–3, which is the area under the curve that was integrated from the plasma concentrations between time 0 h and the 3 h after oral administration of the resveratrol; Cmax, is the maximum plasma concentration, obtained directly from the data without interpolation; Tmax, is the time to reach the Cmax, obtained directly from the data without interpolation; T1/2, is the plasma half life. All parameter, definitions, and statistical aspects are in accordance to Zhang et al. (25).

respectively ($p = 0.01$). For a time range spanning from 10 to 100 min after oral administration, plasma concentration of Resv@MDH-treated subjects was statistically greater than those treated with pure resveratrol (**Figure 5**). The Area Under Curve (AUC $t = 0$–3) values of the plasma concentration profile up until the 3-h mark were 248 ± 48 nmol/ml*min ($n = 6$) and 526 ± 92 nmol/ml*min ($n = 6$) for resveratrol and Resv@MDH, respectively. The data demonstrates an approximate 2-fold enhancement of resveratrol's bioavailability ($p = 0.024$) in the early phase of absorption (ratio of AUC t = 0–3 Resv@MDH/ AUC $t = 0$–3 resveratrol, **Table 2**). Interestingly, the AUC infinite similarly indicates that the differences between these formulations must be interpreted from a kinetic, not stationary, point of view (data not shown).

Simulation of Pharmacokinetic Properties of Resv@MDH and Pure Resveratrol

To underline the observed pharmacokinetic differences, we developed a simulation process where the unique differences are the dissolution properties described in **Figure 2**. In **Figure 6A**, we report a schematic model of the absorption and elimination/delivery of resveratrol in the human body. The absorption process starts with the dissolution of resveratrol in the stomach (see The dissolution rates of Resv@MDH

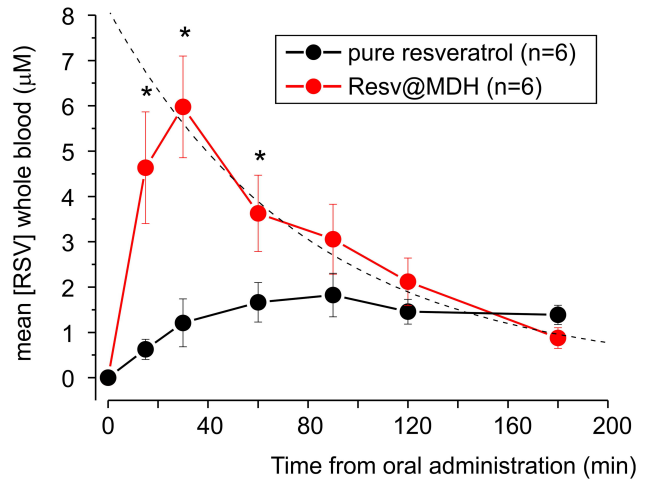

FIGURE 5 | Mean plasma concentration-time curve for free plasmatic resveratrol ($n = 6$) after treatment with of either 180 mg pure resveratrol (Polygonum cuspidatum 98%) or 180 mg resveratrol from Resv@MDH obtained from subjects displayed in **Figure 4**. The dash line represents the best fit of the experimental data point of resveratrol blood concentration following Resv@MDH administration by a mono-exponential model (for details see text in Results section). The black and red dots indicate the resveratrol plasma concentration from the administration of pure resveratrol and Resv@MDH, respectively. *$p < 0.05$.

TABLE 3 | The parameters employed in Equations (6)–(8).

$V_{stomach}$ (dm^3)	$V_{duodenum}$ (dm^3)	V_{plasma} (dm^3)	Dose coefficient	S_S (m^2)	S_D (m^2)
0.25	3	5	3	0.02	0.2
τ_{GE} (s^{-1})	τ_{EI} (s^{-1})	P_S ($m \cdot s^{-1}$)	P_D ($m \cdot s^{-1}$)	r_S	r_D
50	79	$2 \cdot 10^{-5}$	$1 \cdot 10^{-6}$	3.15	3.15

$V_{stomach}$, The volume of the stomach; $V_{duodenum}$, the volume of the duodenum; V_{plasma}, the volume of plasma; S_S and S_D, the dose, the surface of the stomach and duodenum, respectively, τ_{GE}, the gastric empty time; τ_{EI}, the elimination time; P_S, the permeability coefficient of stomach; P_D the permeability coefficient of the duodenum.

and pure resveratrol section) and is, arguably, completed within the first segment of the intestine, the duodenum, where resveratrol is transported after a gastric empty time τ_{GE} (all the parameters employed in the model are reported in **Table 3**).

Simultaneously, the water solution is diffused through the membranes of the stomach and duodenum toward the blood compartment. The corresponding flux in the unit time J(t), in mol s^{-1}, is derived by the law of Fick:

$$J(t_i) = \sum_{i=1}^{N} \left\{ P_S S_S r_S \left[\left(mol_R(t_i) - \sum_{l=1}^{i-1} J(t_l) \right) \frac{1}{V_{stomach}} - \sum_{l=1}^{i-1} J(t_l) \frac{1}{V_{plasma}} \right] e^{-\frac{t_i}{\tau_{GE}}} + P_D S_D r_D \left[\left(mol_{R_i} - \sum_{l=1}^{i-1} J(t_l) \right) \frac{1}{V_{duodenum}} - \sum_{l=1}^{i-1} J(t_l) \frac{1}{V_{plasma}} \right] \left(1 - e^{-\frac{t_i}{\tau_{GE}}} \right) \right\} \quad (6)$$

where P_S and P_D are the permeability coefficients in stomach and duodenum, respectively, and are: directly proportional to the diffusion and repartition coefficients and, inversely proportional to the thickness of the membrane, S_S and S_D are the surfaces of the stomach and duodenum wall, respectively; $mol_R(t_i)$ is the number of mol of resveratrol (multiplied by the dose) in stomach at the time i, the summation over $J(t_l)$ is the total number of mol of resveratrol absorbed by the blood through the stomach and duodenum membranes; $V_{stomach}$, $V_{duodenum}$, and V_{plasma} are the volumes of stomach, duodenum and plasma, respectively. The terms in quadratic parenthesis indicate the gradient concentration of: stomach and plasma, and duodenum and plasma; the sign of the gradient indicates the sense of the flux. Finally, the exponential terms $e^{-\frac{t_i}{\tau_{GE}}}$ and $1- e^{-\frac{t_i}{\tau_{GE}}}$ are weight functions which sum is 1 and represent the gastric empty and the duodenum filling, respectively; τ_{GE} indicates the gastric empty time.

The final stage consists in the elimination of resveratrol from the blood compartment, or in the redistribution toward other organs. The kinetics of this process follows an exponential decay

$$[R] = [R_{max}] e^{-\frac{t_i}{\tau_{EI}}} \quad (7)$$

where $[R_{max}]$ is the initial concentration of resveratrol and τ_{EI} is the elimination time from the blood apparatus.

The concentration of resveratrol in plasma as a function of time is therefore given by the following equation:

$$[R]_{plasma}(t_i) = \frac{1}{V_{plasma}} J(t_i) \cdot e^{-\frac{t_i}{\tau_{EI}}} \quad (8)$$

In **Figure 6B**, we report the plasma concentration as a function of time. The plot compares the experimental data of pure resveratrol and Resv@MDH and those calculated by the related model from Equation (8). For each product, we have calculated the gastric and duodenum component. These findings regarding the pecular dissolution of resveratrol could explain the better gastric absorption of Resv@MDH.

DISCUSSION

Resveratrol is a natural molecule that is absorbed quickly along the digestive tract due to its optimal coefficient partition that allows it to maintain a balance between the water compartment and the biological membranes. Several studies have shown that about 80% of an orally administered dose of 25 mg is absorbed systemically and subsequently eliminated through urine (26). In contrast to this favorable absorption, limited bioavailability has been reported for resveratrol as it is rapidly metabolized to sulfates and glucoronidas (11, 26). Specifically, both pre-systemic metabolic activities (gut and liver) were reported through the *in vitro* study of the Caco-2 model (27), as well as systemic metabolism through the pharmacokinetic study of intravenous doses (11, 26).

Due to the metabolism, the plasma half-life of resveratrol following oral administration is about 2–4 h. Additional studies

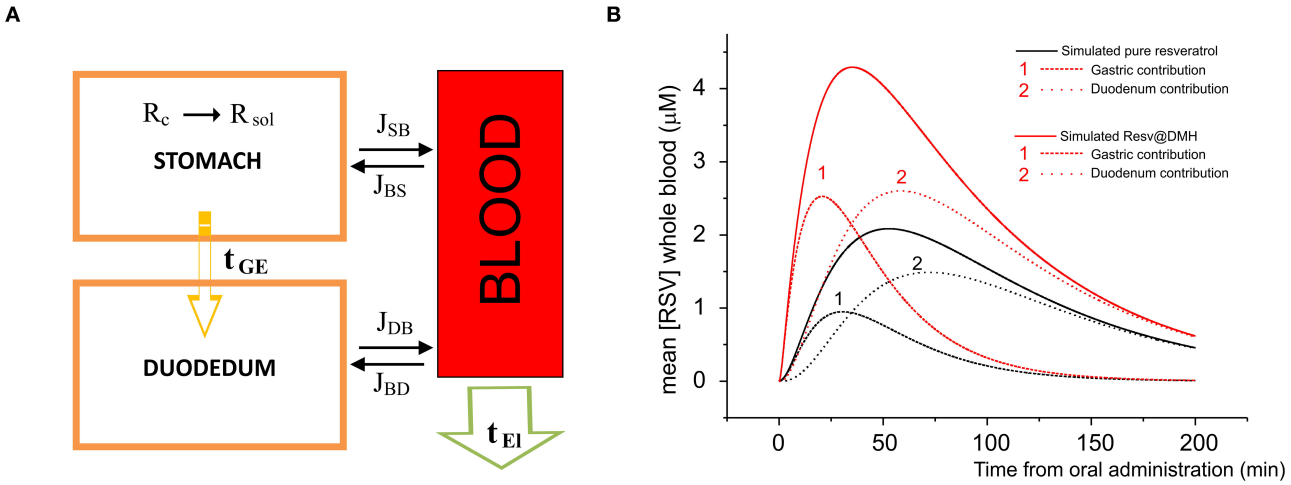

FIGURE 6 | (A) Model of the absorption and elimination of resveratrol occurring in stomach, duodenum, and blood apparatus. Dissolution of resveratrol starts in the stomach and is indicated by R (s) → R (aq); resveratrol is transported toward the duodenum according to a gastric empty time τGE; the water solution of resveratrol is absorbed by the blood apparatus through stomach and duodenum membranes, according to the dissolution time and the gastric empty time; the passage (flux) from the stomach to the blood apparatus and vice versa, and from the duodenum to the blood apparatus and vice versa is indicated by J_{SB}, J_{BS}, J_{DS}, J_{SD}, respectively. Finally, resveratrol is eliminated or redistributed to other parts of the body, according to an elimination time τEl. **(B)** Concentration in μM as a function of the time of pure resveratrol (black line) and Resv@MDH (red line) in plasma. Dots indicate the experimental data, while continuous curves denote the data obtained by our model. For both pure resveratrol and Resv@MDH, the contribution to the plasma concentration due to gastric and duodenum absorption are reported. The number indicates the gastric (1) and duodenal (2) contributions on both simulations.

have shown that plasma concentration largely depends on the administered dose. In a dedicated study using single doses of 0.5, 1, 2.5, and 5 grams of resveratrol, maximum plasma concentrations (Cmax), varying from 73 to 539 ng/ml, were reported [0.3–2.4 μM, (28)]. At lower doses, a similar relationship between dose and plasma peak was also found.

After the first administration of 25, 50, 100, and 150 mg of resveratrol, Almeida et al. (18) observed a Cmax between 1.48 and 24.8 ng/ml with a peak time between 0.8 and 1.3 h. In agreement with Almeida et al. 400 and 500 mg of orally administered resveratrol reach a Cmax of 47 ng/ml (20) (200 nM) and 71 ng/ml (about 300 nM) (15), respectively. In Nunes et. (19), oral administration of 200 mg of resveratrol is comparable to that used in our study (180 mg) and the plasma concentration in elderly and young subjects of both sexes is around 25 ng/ml (about 100 nM), with a peak time ranging from 0.8 to 1.1 h and with a plasmatic half-life ($t/2$) of about 3 h. In our study, we found that the administration of 180 mg of pure resveratrol results in a blood peak concentration of about 2 μM with a latency of 115 min. The main difference between our study and the previous ones, mentioned above, is that we studied the total resveratrol concentration (plasmatic plus cellular) rather than just plasmatic. In fact, recent work on the rat animal model has shown that the amount of absorbed resveratrol is actually underestimated by about 76%, if the considered resveratrol concentration only accounts for that distributed in plasma and does not take into account the cellular component (21).

Although, there are no specific studies conducted in human subjects, we believe that the differences we observed are due to the different mode of blood sampling (whole blood vs. plasma). Dedicated experiments will be needed to discern the apparent differences between our data and those present in literature. Another factor that should be taken into account is the fasting state (our case), which increases the Cmax of resveratrol compared with the presence of food (20). Mode of administration is also important to consider, as done in our study, where both pure resveratrol and Resv@MDH were administered after dispersion of the powder in water.

We previously described the biophysical properties of resveratrol when supported by magnesium hydroxide (MDH). Specifically, we described microscopic properties by observing that, when resveratrol is dispersed into an array of magnesium hydroxide, it is composed of two types of microparticles: one of pure resveratrol and the other in the form of a complex with magnesium (22). Hydroxide magnesium is a safe matrix that dissolves rapidly in acidic settings such as the gastric one, through an acid-based reaction. An important result of this study is that resveratrol, when supported by the magnesium hydroxide matrix (Resv@MDH), shows a completely different plasma profile than that of pure resveratrol. When Resv@MDH is administered, a very early plasma peak (25 min) and a blood concentration of about 6 μM is present. Furthermore, there is greater bioavailability, measured through the AUC calculated on experimental points up to 180 min, by about twice the amount, when compared with that of pure resveratrol. This observation is similar to what has been previously observed in the animal model (22). It is interesting to note that the absolute bioavailability does not change, indicating that the differences are only of kinetic type. Early attainment of the plasma peak allows us to analyse

and estimate the half-life time of resveratrol, which we report as being, on average, 80 min. This half-life component is most likely due to processes of elimination and distribution of resveratrol, and must be analyzed in detail in future works.

To understand whether the different pharmacokinetic behavior was due to the dissolution profile, we developed an absorption model where the only differences were in the dissolution profiles derived from the experiments, shown in **Figure 2**. The model consisted of three compartments: gastric, intestinal and blood. Gastro-enteric emptying was described by estimated mono exponential kinetics in a liquid meal emptying condition, while absorption from the stomach and intestine was described by the law of Fick.

The half-life of the total plasma resveratrol, obtained by the pharmacokinetic data of **Figure 5**, was mono-exponential based. We observe that the different properties of resveratrol based on the dissolution in the gastric environment explain the pharmacokinetic differences between pure resveratrol and Resv@MDH. Specifically, resveratrol with the dissolution properties of the Resv@MDH, in the gastric environment is expected to increase the plasma peak and decrease latency as observed experimentally.

Although the model is a simplification of the *in vivo* condition, it is clear that resveratrol resulting from the administration of Resv@MDH has a mainly gastric absorption. This is in contrast with resveratrol in its pure form, which is predominantly absorbed intestinally (see **Figure 6**). Another aspect we wish to emphasize is that, although the model shows that the main pharmacokinetic differences are due to the peculiar features in the process of dissolution in the gastric environment, other factors must be considered to explain such behaviors. When absorbed mostly at the gastric level, resveratrol could be subject to a different pre-systemic metabolism, compared to that of the model for the intestinal absorption (11). Further studies are required to assess these factors.

In conclusion, resveratrol's increased dissolution rate in the gastric environment, and the factors that lead to such an occurrence, should be leveraged to improve its absorption and to achieve a higher plasmatic peak with less latency time. This improved kinetics could represent the basis for the development of protracted formulations by, for instance, combining resveratrol with different dissolution rates to obtain a higher plasma concentration stability within a given therapeutic window. Considering the numerous health benefits associated with the consumption of resveratrol, our future studies will focus on evaluating possible biological activities of these formulations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional ethics committee review board (project number 12477/18, approved on 18/01/2018 by CEAS Umbria, Perugia Italy). Registered in the ClinicalTrials.gov Protocol Registration System (identifier: NCT04258306). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BF, RI, and FP: conceptualization. BF and FP: formal analysis. AF and AL: analytical analysis. RS: dissolution test. CC, FP, and BF: numerical simulation analysis. AT, RR, MD, and AL: clinical sampling. RI, AF, RS, MD, and BF: data curation. LM, AF, FR, RR, and AL: investigation. BF and RI: writing—original draft preparation. BF and LL: supervision. BF: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.570047/full#supplementary-material

Supplementary Figure 1 | Logarithm of resveratrol concentration in whole blood as a function of the time of the data displayed in **Figure 4**.

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Conflict of Interest: RS and BF are co-inventors of the patent EPO n EP20130425091. RI and LL are employees of S&R Farmaceutici S.p.A., who hold the rights and license of REVIFAST®.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Human Sirtuin Regulators: The "Success" Stories

*Alyson M. Curry[1], Dawanna S. White[1], Dickson Donu[1] and Yana Cen[1,2]**

[1] Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA, United States, [2] Institute for Structural Biology, Drug Discovery and Development, Virginia Commonwealth University, Richmond, VA, United States

The human sirtuins are a group of NAD^+-dependent protein deacylases. They "erase" acyl modifications from lysine residues in various cellular targets including histones, transcription factors, and metabolic enzymes. Through these far-reaching activities, sirtuins regulate a diverse array of biological processes ranging from gene transcription to energy metabolism. Human sirtuins have been intensely pursued by both academia and industry as therapeutic targets for a broad spectrum of diseases such as cancer, neurodegenerative diseases, and metabolic disorders. The last two decades have witnessed a flood of small molecule sirtuin regulators. However, there remain relatively few compounds targeting human sirtuins in clinical development. This reflects the inherent issues concerning the development of isoform-selective and potent molecules with good drug-like properties. In this article, small molecule sirtuin regulators that have advanced into clinical trials will be discussed in details as "successful" examples for future drug development. Special attention is given to the discovery of these compounds, the mechanism of action, pharmacokinetics analysis, formulation, as well as the clinical outcomes observed in the trials.

Keywords: sirtuin, inhibitor, activator, clinical trial, drug development

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***Correspondence:**
Yana Cen
ceny2@vcu.edu

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INTRODUCTION

Initially, sirtuins were classified as epigenetic "eraser" enzymes dedicated for the removal of acetyl groups from histone N-terminal lysine residues (Blander and Guarente, 2004; Trapp and Jung, 2006). The deacetylation of histones causes chromatin condensation, which is closely associated with transcription silencing (Shahbazian and Grunstein, 2007). Unlike the Zn^{2+}-dependent histone

Abbreviations: Aβ, Amyloid beta; AD, Alzheimer's disease; ADME, Absorption, distribution, metabolism, excretion; AE, Adverse effect, AMPK, AMP-activated protein kinase; BBB, Blood-brain barrier; BDNF, Brain-derived neurotrophic factor; C_{max}, Maximum concentration of a drug in the intended organ/tissue after administration; CML, Chronic myeloid leukemia; COPD, Chronic obstructive pulmonary disease; COX, Cyclooxygenase; CRC, Colorectal cancer; $EC_{1.5}$, Concentration at which the enzyme activity is increased by 50%; FOXO, Forkhead box transcription factor; GI, gastrointestinal; HD, Huntington's disease; HDACS, Histone deacetylases; HDL, High density lipoprotein; HDX-MS, Hydrogen deuterium exchange mass spectrometry; HTS, High-throughput screening; IC_{50}, Half-maximal inhibitory concentration; IGFBP, Insulin-like growth factor binding protein; IGF-I, Insulin-like growth factor I; IHD, Ischemic heart disease; ITC, Isothermal colorimetry; K_m, Michaelis-Menten constant; LDL, Low density lipoprotein; m*HTT*, mutant Huntington gene; MI/R- Myocardial/ischemia-reperfusion, MOA, Mechanism of action; NAD, Nicotinamide adenine dinucleotide; NAFLD, Non-alcoholic fatty liver disease; NF-κB, Nuclear factor κB; NFTs, Neurofibrillary tangles; NMR, Nuclear magnetic resonance; PD, Pharmacodynamic; PGC, Peroxisome proliferator-activated receptor-gamma coactivator; PK, Pharmacokinetic; PTEN, Phosphatase and tensin homolog; RSV, Resveratrol; SAR, Structure-activity relationship; SBD, STAC binding domain; SPR, Surface plasmon resonance; STACs, Sirtuin-activating compounds; SULTs, Sulfotransferases; TAMRA, Tetramethylrhodamine; T2D, Type 2 diabetes mellitus; UC, Ulcerative colitis; UGTs, Uuridine-5′-diphospho-glucoronosyltransferases.

deacetylases (HDACs), sirtuins carry out the chemical modifications in an NAD^+-dependent fashion (Imai et al., 2000; Landry et al., 2000). Growing evidence suggests that sirtuins not only act on histone proteins, but also have other cellular targets such as transcription factors and metabolic enzymes (Sauve et al., 2006; Kosciuk et al., 2019). Furthermore, diverse catalytic activities have been uncovered for sirtuins, including, but not limited to, depropionylation, debutyrylation, desuccinylation, and de-fatty acylation (Du et al., 2011; Feldman et al., 2013; Jiang et al., 2013; Anderson et al., 2017). These pleiotropic enzymatic activities give sirtuins their far-reaching functions in maintaining genome integrity, regulating metabolism homeostasis, and promoting organismal longevity (Guarente and Picard, 2005; Cen et al., 2011; Watroba et al., 2017).

The human sirtuins, SIRT1-SIRT7, have been intensively investigated for their enzymatic activities and biological functions. There are numerous wonderful review articles highlighting the significance of these enzymes in regulating normal physiology and pathophysiology (Chang and Guarente, 2014; Herskovits and Guarente, 2014; Kumar and Lombard, 2018; Chang et al., 2020; Jaiswal et al., 2021). For example, overexpression of SIRT1 has been observed to increase carcinogenesis in prostate and thyroid tumors in mice with a deficiency of tumor suppressor PTEN (phosphatase and tensin homolog) (Herranz et al., 2013). SIRT1 also plays an important role in the development of drug resistance in chronic myeloid leukemia (CML) cells (Wang et al., 2013). It has been shown to activate error-prone DNA repair which can lead to increased incidence of genetic mutations (Wang et al., 2013). Thus, a SIRT1 inhibitor would be beneficial in combination with established chemotherapeutics to reduce drug resistance. Sirtuin activators can also play an important role in disease modulation. SIRT1 is considered to have a neuroprotective role in the brain, as it helps to regulate apoptosis and BDNF (brain-derived neurotrophic factor) expression (Luo et al., 2001; Zocchi and Sassone-Corsi, 2012). Activation of SIRT1 may be beneficial for the treatment of Alzheimer's disease (AD), in which SIRT1 levels are typically reduced (Lutz et al., 2014). AD is characterized by the presence of amyloid plaques containing amyloid beta (Aβ) and neurofibrillary tangles (NFTs) containing hyperphosphorylated tau (Ittner and Götz, 2011). SIRT1 overexpression was shown to increase α-secretase activity, thus reducing the formation of Aβ (Endres and Fahrenholz, 2012). SIRT1 also prevents AD pathology through the deacetylation of tau (Min et al., 2010). Tau acetylation inhibits the degradation of tau and is detected in early stages of diseases with abnormal tau accumulation (Min et al., 2010). Therefore, SIRT1 serves as a possible therapeutic target for the treatment of AD.

Naturally, the development of small molecule regulators targeting human sirtuins has become a hot topic of research. Despite all the efforts over the last few decades, the success stories were scarce. Many small molecule sirtuin inhibitors and activators can only be called "chemical probes" at the present time due to the lack of isoform selectivity, moderate potency, limited bioavailability, and poor pharmacokinetic (PK) and pharmacodynamic (PD) profiles. There is a clear gap between the pre-clinical probe discovery and clinical drug candidate development. There is also a gap in the amount of research effort put into studying the various sirtuin isoforms. SIRT1 is by far the most studied isoform, with over 11,000 articles indexed in PubMed. In comparison, the other two most studied isoforms, SIRT2 and SIRT3, together have only around 3,700 articles. This disparity in research translates to fewer small molecule modulators targeting the other isoforms. Thus, the modulators that have entered clinical trials are primarily focused on SIRT1.

In this review, we will "tell the tales" of several human sirtuin regulators that have advanced into clinical investigation for the treatment of various diseases. The focus of the discussions will be the discovery of these compounds, their mechanism of action (MOA), and the rationale and outcome of the clinical trials. Although we brand these compounds as the "success" stories, they are not without controversy or limitation. On the flip side, the lessons we learn from these examples may help guide the design and development of the next generation of sirtuin regulators as therapeutic candidates.

For the benefit of the general audience, we would like to briefly discuss the basic theories behind the development of small molecule drug candidates toward clinical trials. The candidate compounds are normally small molecules, either natural products or synthetic compounds, with desired biological activity toward target proteins or enzymes in the *in vitro* setting. These candidates are the results of rounds of optimization for improved potency, selectivity, and solubility. For example, Lipinski's rule of five (Ro5) has been the golden standard to prioritize the drug-like properties of orally active compounds (Lipinski et al., 2001). The "druggability" of the candidates will then be analyzed through ADME (absorption, distribution, metabolism and excretion) studies. These studies will assess the bioavailability, distribution, stability, and elimination of the candidate compounds. In the following sections, C_{max} of certain sirtuin modulators will be discussed. This critical parameter in ADME analysis describes the maximum concentration of a candidate compound in targeted tissue/organ after administration. The results from ADME studies will guide the further optimization of the candidate molecules. The best dosage, administration route, and formulation need to be evaluated as well. The formulations of resveratrol (RSV) will be discussed in detail in the next section. The active ingredient, RSV, has been combined with variety of substances to improve its bioavailability.

RESVERATROL AND RELATED SRT COMPOUNDS

Resveratrol

In the 1980s, epidemiologists observed that developed countries with increased wine consumption had decreased deaths due to ischemic heart disease (IHD) (St Leger et al., 1979). This later came to be known as the "French Paradox" because of the lowered IHD mortality rates in France despite having no difference in saturated fat intake or blood cholesterol levels (Burr, 1995). In Bertelli et al. (1995) implicated resveratrol (RSV, **Figure 1**) as the mediator of the cardioprotective effects of wine, thus spurring interest in the molecule as a potential therapeutic. The

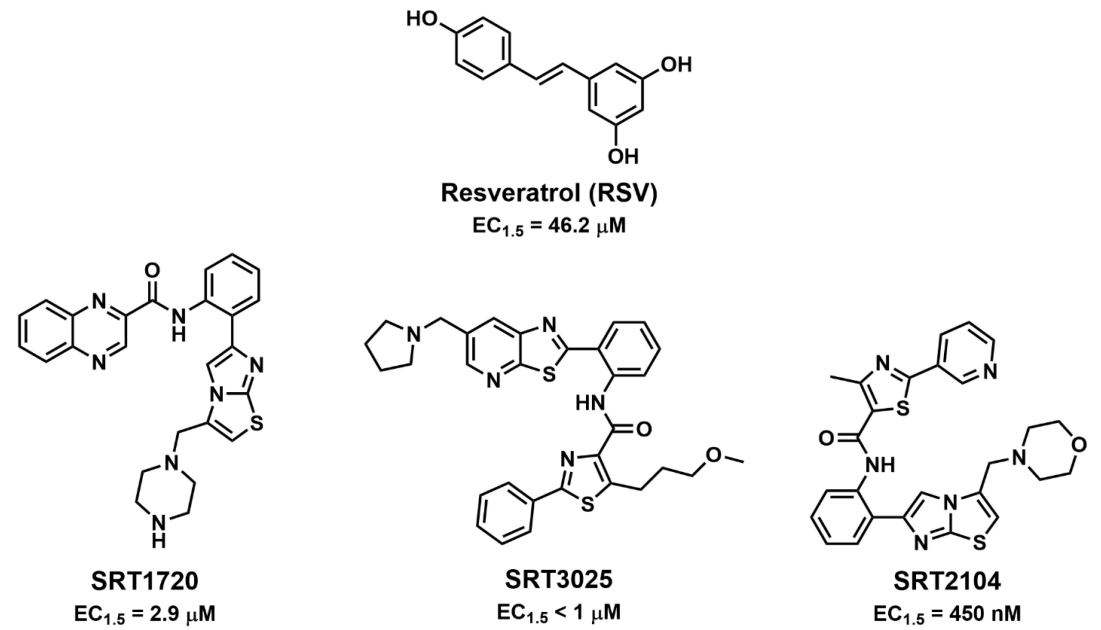

FIGURE 1 | Structures of resveratrol and the SRT family of SIRT1 activators.

association between RSV and SIRT1 was discovered in 2003 by high-throughput screening (HTS) utilizing a Fluor de Lys deacetylation assay on a library of plant-derived polyphenols (Howitz et al., 2003). Howitz et al. (2003) found that RSV activated SIRT1 deacetylase activity by decreasing the K_M for both NAD^+ and the acetylated peptide. In addition to its effects on SIRT1, RSV was also shown to act on a wide range of enzymes, including COX-1, cAMP degrading phosphodiesterases, and nuclear factor-κB (NF-κB) (Jang et al., 1997; Manna et al., 2000; Pezzuto, 2011; Park et al., 2012). Consequently, there is debate whether the observed effects of RSV treatment are due to SIRT1 activation. For example, RSV has been shown to promote autophagy and many have attributed this effect to SIRT1 activation (Morselli et al., 2010; Wu et al., 2011). But this assertion was disputed by a study that observed the direct inhibition of mTOR, an inhibitor of autophagy, by RSV (Park et al., 2016). Despite the promiscuous nature of RSV, a study by Price et al. (2012) found that the presence of SIRT1 was necessary for RSV-mediated mitochondrial biogenesis and AMPK activation.

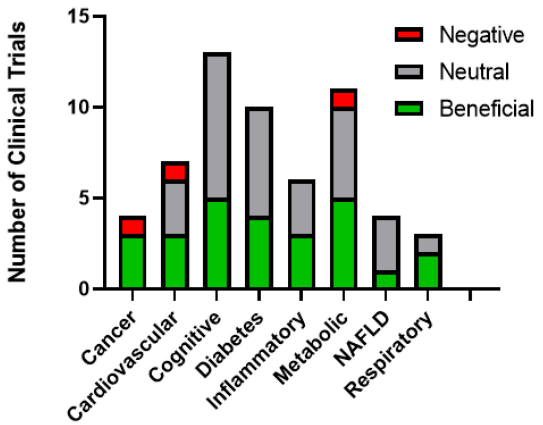

FIGURE 2 | Summary of resveratrol clinical trial outcomes.

After the discovery of RSV as a SIRT1 activator, the validity of the study and its use of the Fluor de Lys assay was disputed. As the assay uses a synthetic peptide substrate that contains a fluorophore, some argued that it was not physiologically relevant and could produce false positives. Kaeberlein et al. (2005) were able to replicate the activation of SIRT1 by RSV, but only when the fluorescent moiety was present on the peptide substrates. Other studies confirmed that the fluorophore was necessary for RSV-promoted activation of SIRT1 (Borra et al., 2005; Beher et al., 2009; Pacholec et al., 2010). In one such study, Pacholec et al. (2010) used NMR, SPR, and ITC to prove that the sirtuin-activating compounds (STACs) were directly interacting with the fluorophore attached to the peptide, even in the absence of SIRT1. Altogether, the contradictory results called into question the reliability of the fluorometric assay and raised serious doubts concerning RSV's mechanism of action and its ability to bind to and activate SIRT1 (Schmidt, 2010).

In response to the controversy, scientists at Sirtris, a company established after the initial discovery of SIRT1 activation by RSV, provided an explanation for the importance of the fluorescent moiety. Dai et al. (2010) observed the formation of STAC-substrate complexes, but found no correlation between the potency of a STAC and its affinity for the fluorescent TAMRA group. They further found that the ability of a STAC to activate SIRT1 was dependent on the substrate structure and were able to induce RSV-activation of SIRT1 using substrates composed of

natural amino acids (Dai et al., 2010). This finding was also seen by Lakshminarasimhan et al. (2013) in which they observed RSV-activation of SIRT1 after replacing the fluorophore with a large, hydrophobic residue. Hubbard et al. (2013) further found that the fluorophore or its hydrophobic replacement has a positional requirement and were able to show that hydrophobic motifs within PGC-1α and FOXO3a could facilitate SIRT1 activation. Additionally, they also determined that the conserved residue Glu230 was critical for RSV activation and found that the benefits of RSV were attenuated when SIRT1 Glu230 mutants were expressed in myoblasts (Hubbard et al., 2013).

After enough evidence was presented confirming an interaction between RSV and SIRT1, the focus shifted to identifying how RSV interacts with SIRT1. When the association between RSV and SIRT1 was first reported, RSV was categorized as an allosteric modulator of the "K system" type (Monod et al., 1965; Howitz et al., 2003). For "K system" modulators, the K_M value is affected while the V_{max} remains the same. Identifying the region of SIRT1 where RSV binds was the first step in characterizing the interaction. SIRT1 is comprised of three major structured regions: an N-terminal domain (183–229), a catalytic domain (229–516), and a C-terminal regulatory region (641–665) (Davenport et al., 2014). The *apo* enzyme undergoes a conformational change following the binding of NAD^+ into a "closed" form that traps the substrate (Yuan and Marmorstein, 2012; Davenport et al., 2014). Using hydrogen deuterium exchange mass spectrometry (HDX-MS), Dai et al. (2015) were able to investigate the STAC binding domain (SBD) and determine that residues 183–229 were necessary for STAC activation of SIRT1. A crystal structure of a SIRT1/FdL peptide/RSV complex by Cao et al. (2015) indicated the binding of 3 RSV molecules for each SIRT1. Two of the RSV molecules form hydrogen bonds with the peptide and the SBD, thus bringing the two domains together in a "closed" conformation with improved substrate binding (Cao et al., 2015; Dai et al., 2018). The perspective of RSV acting as a stabilizing force between the substrate and SIRT1 has also been observed in a computational study in which RSV restored binding of "loose-binding" substrates (Hou et al., 2016).

Bioavailability is the primary obstacle in the development of RSV as a therapy. In human trials, Walle et al. (2004) observed that RSV had an absorption of ∼70%, but were unable to detect unmodified RSV 30 min after administration. After absorption, RSV is rapidly metabolized in the liver where it is conjugated to either sulfate or glucuronate (Springer and Moco, 2019). The RSV conjugates are produced by sulfotransferases (SULTs) and uridine-5′-diphospho-glucoronosyltransferases (UGTs) (Springer and Moco, 2019). Both SULTs and UGTs have genetic polymorphisms that can affect their ability to metabolize drugs, and thus can lead to high variability in levels of unmodified RSV between individuals (Ung and Nagar, 2007; Mehboob et al., 2017). The sulfate-conjugated metabolites, which are the primary conjugated form of RSV, were shown to have similar actions to RSV. Calamini et al. (2010) found that the sulfate conjugate of RSV was able to inhibit COX-1 and COX-2, and could activate SIRT1 only in the presence of a Fluor de Lys substrate. As the level of the conjugated forms of RSV surpass that of free RSV, it remains unclear which molecule is responsible for the experimentally observed effects of RSV supplementation.

Development of novel formulations of RSV emerged as its bioavailability became a prominent issue. One approach was to inhibit the enzymes responsible for conjugating RSV. Co-administration of RSV with quercetin was shown to decrease the formation of RSV-sulfate conjugates through the inhibition of SULT1A1, the major SULT isoform expressed in the liver and kidneys (De Santi et al., 2000). Multiple studies have observed the synergistic effect between RSV and quercetin from reducing adipose tissue weight following a high fat diet (Arias et al., 2016) to inhibiting the development of prostate cancer in a mouse model (Singh and Ahmad, 2015). In a similar fashion, Reen et al. (1993) combined RSV with piperine, an alkaloid previously shown to diminish the activity of UGTs in the intestines of rats. The study found that the addition of piperine to RSV resulted in a more than 1000% increase in the maximum serum concentration of RSV (Johnson et al., 2011). Despite the promising results seen in animal models, outcomes from human trials in healthy subjects were ambiguous (**Table 1**). Two trials co-administering RSV and quercetin found no improvement in RSV pharmacokinetics (la Porte et al., 2010; Huhn et al., 2018). Piperine supplementation was found to be useful in improving RSV bioavailability in one study examining cerebral blood flow (Wightman et al., 2014), but had no beneficial effect on serum levels of RSV in another study (Bailey et al., 2021).

Other groups tried to solve the bioavailability issue by focusing on the drug delivery method. The Fioretti lab developed a novel formulation of RSV as a solid dispersion on Magnesium dihydroxide microparticles. In their early studies, they found that the new formulation (later termed Revifast) was three times more soluble than the unmodified RSV and had enhanced bioavailability in rabbits (Spogli et al., 2018). In human trials, Revifast displayed an earlier peak in RSV as well as a twofold increase in free-RSV levels in plasma (Iannitti et al., 2020). Another method used to increase bioavailability of RSV is micronization. Sirtris developed SRT501, in which they reduced the particle size below 5 μm in order to improve solubility by increasing the surface area of RSV. In an early clinical trial, SRT501 was well tolerated in colorectal cancer (CRC) patients and had an improved C_{max} compared to conventional RSV (Howells et al., 2011). Following that success, SRT501 was used as a treatment for patients with refractory or relapsed multiple myeloma. The clinical trial was terminated early due to severe side effects including nephrotoxicity (Popat et al., 2013). SRT501 was later discontinued for development as the company focused on other SIRT1-activating drug candidates.

RSV has been extensively studied in clinical trials. In the NIH clinical trial registry, over 150 trials using RSV are in various stages of completion. The trials concerned with sirtuin activation and the resulting therapeutic effects have been compiled in **Tables 2–9**. When summarizing the clinical outcomes of RSV (**Figure 2**), the issues with RSV are apparent. For most disease states RSV had a neutral effect, suggesting that the bioavailability issues are a major obstacle. Most of the trials in which RSV had a positive effect had higher dosages, typically over 500 mg/day, and the more recent trials are trending toward higher dosages as

TABLE 1 | Clinical trials examining pharmacokinetics of novel resveratrol formulations and administrations.

Formulation/ administration	Description	Outcomes	References
Revifast	Solid dispersion of RSV on Magnesium dihydroxide	Revifast had C_{max} threefold higher than RSV and an earlier peak in RSV	Iannitti et al., 2020
SRT501	Micronized RSV; 5,000 mg/day	SRT501 had a 3.6-fold increase in plasma RSV levels compared to non-micronized RSV	Howells et al., 2011
JOTROL	Micellar emulsion	Recruiting	NCT04668274
With food	High fat meal	RSV taken with food had delayed absorption, but the amount of absorption was not affected	Vaz-da-Silva et al., 2008
RSV/Piperine	2,500 mg RSV + 0/5/25 mg Piperine	No significant change in pharmacokinetics was seen	Bailey et al., 2021
RSV/Quercetin	2,000 mg RSV + 500 mg Quercetin	No significant change in RSV exposure	la Porte et al., 2010

TABLE 2 | Resveratrol clinical trials for cancer.

Condition	Phase	Subjects	Dose	Outcome	References
Cancer prevention	I	40	500, 1,000, 2,500, 5,000 mg/day 29 day	RSV safe, but higher doses had GI side effects; RSV treatment caused decrease in IGF-I and IGFBP-3 levels	Brown et al., 2010
CRC	I	20	5,000, 1,000 mg/day 8 day	RSV treatment reduced tumor cell proliferation by 5%	Patel et al., 2010
Cancer prevention	I	42	1,000 mg/day	RSV affected enzymes involved in carcinogen activation and detoxification (CYP3A4, CYP2D6, CYP2C9)	Chow et al., 2010
Multiple Myeloma	II	24	5,000 mg	Terminated early-severe renal side effects	Popat et al., 2013

TABLE 3 | Resveratrol clinical trials for cardiovascular diseases.

Condition	Phase	Subjects	Dose	Outcome	References
Vascular function	I/II	64	90 mg ResArg	ResArg had improved benefits for vascular function and platelet reactivity compared to RSV	Djurica et al., 2016
Cardiovascular disease	NA	27	300 or 1,000 mg RSV	Higher RSV dosage increased the cardiovascular disease biomarkers, lower RSV dose had no change	Mankowski et al., 2020
Exercise-induced cardiovascular benefits	NA	27	250 mg/day RSV 8 week	RSV diminished positive cardiovascular effects of exercise and had no effect on SIRT1 protein levels	Gliemann et al., 2013
Congestive heart failure	II	40	1,000 mg/day 8 week	Recruiting	NCT03525379
Diabetic coronary artery disease	II	56	500 mg/day 4 week	RSV increased HDL, had beneficial effects on insulin resistance, and upregulated SIRT1	Hoseini et al., 2019
Peripheral artery disease	III	90	125 mg RSV + 1,000 mg NR 6 m	Recruiting	NCT03743636
Peripheral artery disease	NA	66	125 or 500 mg/day	RSV had no consistent effect on walking performance in patients with peripheral artery disease	McDermott et al., 2017
Hypertension	I/II	300	150 or 300 mg/day 12 m	Recruiting	NCT01842399
Endothelial dysfunction	I	24	300 mg	RSV treatment improved endothelial function, but had no effect on blood pressure	Marques et al., 2018
Endothelial dysfunction	NA	25	250 mg	RSV had small beneficial effect on endothelial function, but no additional benefit was seen with exercise	Ozemek et al., 2020

well. Although it is unclear if RSV can directly activate sirtuins when taken orally, it is likely that work with RSV will continue, as it is a readily available natural product with limited adverse reactions. Despite the early promise seen in the lab, the current reality of RSV has shown the need for developing better, more selective activators of sirtuins.

Resveratrol-Related Activators

Following the discovery of RSV, Sirtris began to develop new small molecule activators of SIRT1. From HTS, they discovered SIRT1 activators that were structurally distinct from RSV and had improved SIRT1 activating abilities (Milne et al., 2007). One of the compounds, SRT1720 (**Figure 1**), activated SIRT1 with an $EC_{1.5}$ = 2.9 μM and a maximum activation of approximately 4.5-fold, whereas RSV had an $EC_{1.5}$ = 46.2 μM and a maximum activation of 2-fold (Milne et al., 2007). Using ITC, it was determined that the SRT STACs could only bind SIRT1 in the presence of the peptide substrate and they had a mechanism of action similar to RSV. It was further found that they used the same binding site as RSV. In a rodent model of insulin resistance, SRT1720 treatment resulted in a decrease in the blood glucose level and an increase in insulin sensitivity (Milne et al., 2007).

Thus far, clinical trials of three compounds related to SRT1720 have been completed. SRT2104 (**Figure 1**) has the

TABLE 4 | Clinical trials of resveratrol for cognition and neurological disorders.

Condition	Phase	Subjects	Dose	Outcome	References
Alzheimer's disease	III	27	10 g Dextrose, 10 g Malate, 10 mg RSV/day 12 m	RSV had small, but insignificant effects on mental deterioration	Zhu et al., 2018
Alzheimer's disease	II	119	500–2,000 mg/day 12 m	RSV and metabolites crossed the BBB; RSV decreased MMP9, neuroinflammation, and induced adaptive immunity	Turner et al., 2015; Moussa et al., 2017
Brain function/structure	NA	60	200 mg RSV, 320 mg Quercetin/day 18 week	No improvement in verbal memory after RSV treatment	Huhn et al., 2018
Cognition and cerebral blood flow	NA	22	250, 500 mg	RSV increased cerebral blood flow, but no change in cognitive function was observed	Kennedy et al., 2010
Cognition	NA	27	500 mg	No cognitive changes seen in healthy patients ages 18–35	Wightman et al., 2019
Cognitive impairment	II/III	40	200 mg/day 26 week	Beneficial, but non-significant changes in markers of diabetes and resting-state functional connectivity	Köbe et al., 2017
Depression	IIII	22	500 mg/day 28 day	RSV did not have a significant antidepressant effect	Aftanas et al., 2020
Friedreich ataxia	I/II	27	1,000 or 5,000 mg/day 12 week	Improvement in oxidative stress markers and ataxia seen only in higher dosage group	Yiu et al., 2013
Friedreich ataxia	II	40	1,000 mg/day Micronized RSV	Recruiting	NCT03933163
Gulf war illness	II	68	2,000 mg/day	Recruiting	NCT03665740
Gulf war illness	NA	64	200–600 mg/day 4 week	RSV reduced Gulf War Illness symptoms	Hodgin et al., 2021
Cognition	I	60	500 mg/day 28 day	RSV treatment reduced fatigue, but had no effect on sleep, health, or cerebral blood flow	Novelle et al., 2015
Cognition	NA	24	500 mg	RSV treatment group had fewer errors in serial subtraction test	Wightman et al., 2019
Schizophrenia	II	19	200 mg/day 4 week	RSV treatment did not improve cognition in patients with schizophrenia	Zortea et al., 2016
Sports concussion	I/II	12	500 mg/day 30 day	No significant effects seen with RSV treatment	NCT01321151

TABLE 5 | Clinical trials of resveratrol for diabetes.

Condition	Phase	Subjects	Dose	Outcome	References
Dyslipidemia	NA	50	150 mg/day 4 week	RSV treatment did not change cardiovascular or metabolic risk markers	van der Made et al., 2015
Dyslipidemia	II	8	1,000 mg/day, then 2,000 mg/day 2 week	RSV treatment reduced lipoprotein production	Dash et al., 2013
Type 2 diabetes	I	10	3,000 mg/day 12 week	RSV treatment increased SIRT1 and AMPK expression	Goh et al., 2014
Type 2 diabetes	NA	30	2,000–3,000 mg/day 6 week	No changes in T2D markers, but changes in expression of genes involved in mitochondrial activity	Pollack et al., 2017
Type 2 diabetes	NA	17	150 mg/day 30 day	RSV treatment did not improve insulin sensitivity	Timmers et al., 2016
Insulin resistance	NA	112	150 mg/day 12 week	RSV treatment did not impact liver fat content or cardiovascular risk factors	Kantartzis et al., 2018
Type 2 diabetes	NA	54	100 mg/day 2 week then 300 mg/day 2 week	RSV treatment decreased arterial stiffness and had a positive, but insignificant effect on SIRT1 activity	Zhang et al., 2017
Type 2 diabetes	III	192	40 or 500 mg/day 6 m	Higher RSV dosage group had increased SIRT1 levels and antioxidant markers, and decreased H3K56Ac and body fat percentage	Bo et al., 2018
Pre-diabetes	NA	15	150 mg/day 30 day	RSV increased muscle mitochondrial function, but no other metabolic benefits were observed	de Ligt et al., 2018
Pre-diabetes	I	48		Recruiting	NCT02502253
Pre-diabetes	NA	42	150 mg/day 6 m	RSV had no effect on pre-diabetes markers	de Ligt et al., 2020
Insulin resistance	NA	270	RSV + Vitamin C	Recruiting	NCT03090997
Type 2 diabetes	NA	40		Recruiting	NCT03762096
Type 1 diabetes	NA	198		Recruiting	NCT03436992
Type 1 diabetes	Early I	24		Recruiting	NCT04449198

greatest number of registered clinical trials (**Table 10**), but SRT2379 and SRT3025 (**Figure 1**) have also made it into the clinic (**Table 11**). The single clinical trial to assess the safety and pharmacokinetics of SRT3025 was interrupted after the researchers found a prolongation effect of SRT3025 on the corrected QT interval, a warning that continuation could lead to

TABLE 6 | Clinical trials of resveratrol for inflammatory diseases.

Condition	Phase	Subjects	Dose	Outcomes	References
Chronic kidney disease	III	20	500 mg/day 4 week	RSV treatment had no antioxidant or anti-inflammatory effects	Saldanha et al., 2016
Inflammatory markers	NA	44	400 mg RSV + 100 mg Quercetin/day 30 day	RSV treatment had beneficial effect on some inflammatory markers and reduced fasting insulin concentration	Agarwal et al., 2013
Inflammatory markers in smokers	III	40	500 mg/day 30 day	RSV treatment had beneficial effects on some inflammatory markers and the antioxidant level	Bo et al., 2013
Polycystic ovary syndrome	NA	40	1,500 mg/day 3 m	RSV treatment reduced ovarian and adrenal androgens	Banaszewska et al., 2016
Inflammatory markers	NA	22	200 mg RSV + 100 mg Curcumin	RSV/Curcumin treatment had no effect on inflammation after consumption of a high-fat meal	Vors et al., 2018
Endometriosis	IIII	44	40 mg/day 42 day	RSV treatment had no effect on endometriosis pain	Mendes da Silva et al., 2017
Osteoarthritis	III	164		Recruiting	NCT02905799
Chronic kidney disease	NA	25		Recruiting	NCT03597568

TABLE 7 | Clinical trials of resveratrol for obesity and metabolic disorders.

Condition	Phase	Subjects	Dose	Outcomes	References
Aging	II	60	500 or 1,000 mg/day 12 week	RSV treatment coupled with exercise had beneficial effects on physical and mitochondrial function	Harper et al., 2021
Metabolism	I	32	300 or 1,000 mg/day 90 day	RSV treatment reduced glucose levels in overweight adults	Anton et al., 2014
Metabolic syndrome	NA	25	250 mg/day 3 m	RSV treatment improved many metabolic markers, including total cholesterol, urea, and creatinine	Batista-Jorge et al., 2020
Metabolic syndrome	II	24	1,500 mg/day 90 day	RSV treatment reduced weight, BMI, and total insulin secretion	Méndez-del Villar et al., 2014
Obesity	NA	24	1,500 mg/day 4 week	RSV treatment had no effect	Poulsen et al., 2013
Mitochondrial myopathy	NA	20	1,000 mg/day 8 week	RSV treatment did not improve exercise capacity in subjects with mitochondrial myopathy	Løkken et al., 2019
Obesity	NA	18	150 mg/day 30 day	RSV treatment had no effect on incretin levels, but reduced glucagon levels after eating in obese subjects	Knop et al., 2013
Metabolic syndrome	NA	76	150 or 1,000 mg/day 16 week	RSV treatment did not improve inflammation and increased total cholesterol and LDL cholesterol in subjects with metabolic syndrome	Kjaer et al., 2017
Metabolic syndrome	NA	28	2,000 mg/day 30 day	RSV treatment improvement insulin sensitivity for Caucasian subjects, but non-Caucasian subjects had no difference	Walker et al., 2018
Metabolism	NA	58	75 mg/day 12 week	RSV treatment had no effect on metabolic markers or SIRT1 expression	Yoshino et al., 2012
Obesity	NA	48	500 mg/day 30 day	RSV treatment increased serum levels of SIRT1	Roggerio et al., 2018

TABLE 8 | Clinical trials of resveratrol for NAFLD.

Condition	Phase	Subjects	Dose	Outcome	References
NAFLD	II/III	50	500 mg/day 12 week	RSV treatment improved inflammatory markers	Faghihzadeh et al., 2014
NAFLD	NA	28	1,500 mg/day 6 m	RSV had no consistent beneficial effect for NAFLD	Heebøll et al., 2016
NAFLD	NA	90	600 mg/day 12 week	RSV treatment led to weight loss, but did not change SIRT1 level or induce other beneficial effects of CR	Asghari et al., 2018
NAFLD	NA	26	1,500 mg/day 6 m	RSV treatment had no effect on metabolic markers for subjects with NAFLD	Poulsen et al., 2018

a potentially fatal proarrhythmia in the subjects (NCT01340911, GSK Study Register). Further development of SRT2379 was also terminated due to preclinical toxicities observed by the researchers (NCT01416376, GSK Study Register).

For the lead compound, SRT2104, 5 out of the 8 clinical trials that focused on clinical outcomes had neutral or statistically insignificant results. A pharmacokinetic study found that the bioavailability of SRT2104 was 14% and exposure was improved when administered with food (Hoffmann et al., 2013). Most trials observed highly variable pharmacokinetics, leading some to have inconsistent clinical outcomes. A few trials observed beneficial effects of SRT2104 treatment on lipid profiles (Baksi et al., 2014),

TABLE 9 | Clinical trials of resveratrol for respiratory conditions.

Condition	Phase	Subjects	Dose	Outcome	References
Common cold	III	89	Nasal solution of RSV/carboxymethyl-β-glucan	c β G/RSV treatment provided minor benefit for nasal symptoms in infants	Baldassarre et al., 2020
COPD	NA	21	150 mg/day 4 week	RSV treatment did not improve mitochondrial function in subjects with COPD	Beijers et al., 2020
Seasonal allergies	III	76	Nasal solution of RSV/carboxymethyl-β-glucan	c β G/RSV treatment reduced nasal symptoms	Miraglia Del Giudice et al., 2014
COPD	NA	48		Recruiting	NCT03819517
Cystic fibrosis	NA	36		Active, not recruiting	NCT04166396
COVID-19	II	100		Active, not recruiting	NCT04400890
COVID-19	II	60		Active, not recruiting	NCT04542993
COVID-19	NA	30		Recruiting	NCT04799743

TABLE 10 | Clinical trials on SRT2104.

Condition	Phase	Subjects	Dose	Outcome	References
Pharmacokinetics	I	20	500 mg	SRT2104 had increased absorption with ingested with food; Headache was most common AE	Hoffmann et al., 2013
Type 2 Diabetes	I	10	2,000 mg/day 7 day	SRT2104 increased after multiple doses; Headache was the most common AE (affecting 50% of the treatment group)	Hoffmann et al., 2013
COPD	I	20	250–2,000 mg	SRT2104 had no effect on inflammatory markers; variable pharmacokinetic parameters	NCT00920660, GSK Study Register
Muscular atrophy	I	24	500 or 2,000 mg/day 28 day	SRT2104 treatment decreased cholesterol and LDL levels, but had variable pharmacokinetics.	Libri et al., 2012
Sepsis/Inflammation	I	24	2,000 mg/day 7 day	SRT2104 treatment had anti-inflammatory and anticoagulant effects	van der Meer et al., 2015
Type 2 diabetes	I	38	2,000 mg/day 28 day	SRT2104 treatment had a beneficial metabolic effect and improved lipid profiles and arterial stiffness. It had inconsistent effects on endothelial function	Venkatasubramanian et al., 2013, 2016; Noh et al., 2017
Psoriasis	II	40	250, 500, 1,000 mg/day 84 day	35% of SRT2104 treatment group had improvement in psoriasis; 69% had AEs; SRT2104 exposure was highly variable	Krueger et al., 2015
Type 2 diabetes	II	86	2,000 mg/day 28 day	SRT2104 had no consistent effects on insulin sensitivity	NCT01018017, GSK Study Register
Type 2 diabetes	I	227	250–2,000 mg/day 28 day	SRT2104 did not improve glucose or insulin control; Exposure was highly variable	Baksi et al., 2014
Ulcerative colitis	II	17	50,500 mg/day 8 week	SRT2104 did not improve UC	Sands et al., 2016
Pharmacokinetics	I	65	30–3,000 mg/day 7 day	SRT2104 bioavailability was 14%; Administration with food increased drug exposure	Hoffmann et al., 2013

TABLE 11 | Clinical trials of SRT2379 and SRT3025.

Condition	Phase	Subjects	Dose	Outcome	References
Type 2 diabetes	I	64	25–3,000 mg SRT2379	SRT2379 exposure increased in a dose-dependent manner	NCT01018628, GSK Study Register
Inflammation	I	17	1,000 mg SRT2379	SRT2379 treatment had a trend of anti-inflammatory effects, but was not statistically significant	NCT01262911, GSK Study Register
Inflammation	I	39	50–1,000 mg SRT2379	SRT2379 treatment did have a significant anti-inflammatory effect	Wiewel et al., 2013
Type 2 diabetes	I	78	50–3,000 mg SRT3025	SRT3025 treatment stopped due to potential adverse cardiovascular side effects	NCT01340911, GSK Study Register

histological examinations of subjects with psoriasis (Krueger et al., 2015), and inflammation (van der Meer et al., 2015). McCallum et al. (2014) tried to improve the pharmacokinetics of SRT2104 by using different release formulations, but were unsuccessful. As of current, it appears that SRT2104 is no longer in development. Despite issues observed in clinical trials, SRT2104 continues to be used in studies as a SIRT1 activator (Miller et al., 2021).

TABLE 12 | Clinical trials of EX-527 for Huntington's disease.

Condition	Phase	Subjects	Dose	Outcome	References
Huntington's disease	I	55	10, 100 mg/day 14 day	EX-527 was well tolerated in early stage HD patients at 10 and 100 mg/day, baseline to day 1 improvement	Sussmuth et al., 2015
Huntington's disease	I	26	100 mg/day 14 day	N.R.P[a]	NCT01485965
Healthy subjects	I	88	5, 25, 75, 150, 300, 600 mg/day 100, 300 mg/day	EX-527 was well tolerated at a max single dose of 600 mg and max multiple doses of 300 mg/day	Westerberg et al., 2015
Huntington's disease	II	144	50, 200 mg/day	N.R.P	NCT01521585

[a]*N.R.P, no results posted.*

EX-527 (SELISISTAT)

Before 2005, many small molecule regulators targeting sirtuins showed modest potency, isoform selectivity, and solubility (Bedalov et al., 2001; Grozinger et al., 2001; Bitterman et al., 2002; Mai et al., 2005; Olaharski et al., 2005; Porcu and Chiarugi, 2005). Isoform-selective small molecule probes were highly sought after for a better understanding of the biological functions of these enzymes (Porcu and Chiarugi, 2005). Napper et al. (2005) performed an HTS of a library of 280,000 compounds against human recombinant SIRT1 using a fluorometric assay in order to expand available SIRT1 small-molecule probes. The initial screening identified indole as a viable scaffold for SIRT1 targeting. The subsequent SAR study led to the discovery of EX-527 (or Selisistat) and the structurally related compound CHIC-35 (**Figure 3**) as selective SIRT1 inhibitors with IC_{50} values of 0.098 and 0.063 μM, respectively (Napper et al., 2005). EX-527 exhibited 100-fold selectivity for SIRT1 over SIRT2/3, Class I/II HDACs, and NAD^+ glycohydrolase (Napper et al., 2005). Kinetic studies indicated that EX-527 is an uncompetitive inhibitor of SIRT1 regarding NAD^+; thus, inhibition depends on the concentration of NAD^+ (Napper et al., 2005; Gertz et al., 2013). Additionally, EX-527 exhibits exceptional ADME properties such as oral bioavailability, metabolic stability, and membrane penetrability, thus elevating EX-527 from a small-molecule probe to a therapeutic candidate (Napper et al., 2005). In contrast, the study of CHIC-35 has been limited to pre-clinical investigations, primarily on its anti-inflammatory effects (Lugrin et al., 2013; Asad and Sachidanandan, 2020).

SAR studies of EX-527 revealed that the primary carboxamide was necessary for an effective inhibition and modifications to the lead compound were generally not well tolerated (Napper et al., 2005). The isoform selectivity of EX-527 was initially attributed to possible differences among the sirtuin active sites (Gertz et al., 2013; Broussy et al., 2020). A recent structural biology study suggested that EX-527 occupies the C-pocket (nicotinamide binding site) and a neighboring hydrophobic pocket which are highly conserved in the sirtuin family (Gertz et al., 2013). Further kinetic analysis indicated that the isoform selectivity of EX-527 stemmed from the differences in the kinetics of catalysis rather than any significant structural variation (Gertz et al., 2013; Broussy et al., 2020).

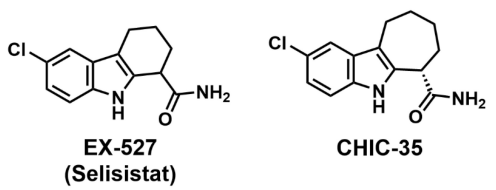

FIGURE 3 | Chemical structures of EX-527 and CHIC-35.

EX-527 has been explored as a potential therapeutic for Huntington's disease (HD). HD is a neurodegenerative disease characterized by abnormal movements, personality changes, and cognitive decline (Bates et al., 2015). A hallmark of HD is the expression of the mutant Huntington gene (m*HTT*), which has an expansion of a cytosine-adenine-guanine (CAG) repeat (Bates et al., 2015; Tabrizi et al., 2020). This extension leads to the protein misfolding and the formation of protein aggregates in HD patients (Bates et al., 2015; Tabrizi et al., 2020). As such, a potential therapeutic approach has focused on the degradation and removal of these aggregates. Acetylation of mHTT can direct the protein to autophagosomes for degradation (Jeong et al., 2009), thus facilitating the removal of the mutant protein. SIRT1 has been pursued as a therapeutic target for the treatment of HD because it has been shown to deacetylate mHTT to prevent its degradation. Genetic loss or pharmacological inhibition of Sir2 (the *Drosophila melanogaster* homolog of human SIRT1) was found to be neuroprotective for m*HTT*-challenged fruit flies (Pallos et al., 2008). Indeed, Smith et al. (2014) demonstrated that in a *Drosophila* model of HD, 10 μM of EX-527 could rescue neurodegeneration at a comparable level as the genetic elimination of Sir2. Additionally, in the R6/2 mouse model of HD, EX-527 can restore neural functions at 5 and 20 mg/kg dosages (Smith et al., 2014). It is important to note that the role of SIRT1 in HD remains controversial, as some view it as protective in HD modulation (Duan, 2013).

The aforementioned preclinical animal studies were essential for clinical trials involving EX-527 as an HD treatment option (**Table 12**). In a Phase 1 trial (NCT01521832), EX-527 was assessed for its safety in healthy human subjects. The study employed healthy men and women with two separate dosage regimens: a single dosage of 5, 25, 75, 150, 300, or 600 mg, and multiple dosages of 100, 200, or 300 mg/day (Westerberg et al., 2015). In this study, EX-527 was found to be well-tolerated and safe after multiple doses of 300 mg/day and at a single dose of 600 mg. Based on the promising safety profile and dosing information, another clinical trial (NCT01485952)

sought to investigate the feasibility of targeting SIRT1 as a potential treatment for HD (Sussmuth et al., 2015). In this study, human subjects with HD were treated with either 0, 10, or 100 mg/day of EX-527. There were improvements across the clinical, cognitive, and neuropsychiatric assessments from the baseline (day -1) to day 1 with no additional improvement at day 14 (Sussmuth et al., 2015).

A relatively new clinical application of EX-527 is improving *in vitro* fertilization (IVF) outcomes through the treatment of endometriosis (NCT04184323). Endometriosis is a chronic inflammatory reproductive disorder involving the growth of uterine endometrial cells outside of the uterine cavity (Zondervan et al., 2020). These delocalized endometrial growths can become lesions that lead to chronic localized pelvic pain and cramping with accompanying fertility issues. Standard treatment options for endometriosis involve mechanical lesion removal, hormonal therapy, or a combination of the two (Budinetz and Sanfilippo, 2010). However, reemergence of the lesions and complications associated with the hormonal therapy remain limitations. Thus, alternative treatment options are needed. A recent study demonstrated a KRAS activation-triggered SIRT1 overexpression in women with endometriosis, which has been suggested to contribute to infertility and the pathogenesis of endometriosis (Yoo et al., 2017). Targeting SIRT1 with small molecule inhibitors thus serves as a potential therapeutic treatment for endometriosis-mediated IVF failure. A planned clinical trial (NCT04184323) will seek to explore inhibition of SIRT1 by EX-527 as a possible treatment for the inflammation associated with endometriosis.

QUERCETIN

Quercetin (**Figure 4**) is a flavonoid phytoestrogen which has demonstrated activity in the management of brain, blood, salivary gland and uterine cancers (Dolatabadi, 2011; Sak, 2014; Sinaga et al., 2017), as well as viral infections such as HCV (Rojas et al., 2016) and Zika virus (Wong et al., 2017) and bacterial infections (Wang et al., 2018; Zeng et al., 2019; Oktyabrsky et al., 2020) in both *in vivo* and *in vitro* studies. The structure of quercetin consists of three rings and five hydroxyl groups at the 3, 5, 7, 3′ and 4′-positions of the basic flavanol skeleton (**Figure 4**). The name "quercetin" was derived from the Latin word "Quercetum" which means oak forest. It is one of the most abundant flavonoids found in fruits and vegetables, and was discovered alongside other bioflavonoids by Albert Szent Gyorgyi in 1936 (Moskaug, Carlsen et al., 2004).

In the same HTS that identified RSV, quercetin was observed to increase SIRT1 activity by fivefold (Howitz et al., 2003). Studies have shown the inhibitory role of quercetin on the progression of breast, colon, prostate, and lung cancers (Baghel et al., 2012; Smith et al., 2016). Quercetin has been found to alleviate kidney fibrosis, intervertebral disc degeneration, and diabetic encephalopathy *via* activation of SIRT1-mediated pathways (Dong et al., 2014; Hu et al., 2020; Liu et al., 2020). Quercetin treatment of Herpes simplex virus-1 infected neuronal cell lines increased the survival of the cells by inhibiting viral production and improved neurodegenerative markers *via* SIRT1 activation (Leyton et al., 2015). It was also found that quercetin inhibits oxidative injury in human endothelial cells through SIRT1 activation, leading to the upregulation of the SIRT1/AMPK pathway (Chen et al., 2013). In addition, quercetin regulates oxidative stress in the body by directly reducing the level of reactive oxygen species (Oboh et al., 2016). In myocardial/ischemia-reperfusion (MI/R) injury in rats, quercetin supplementation increased the expression levels of SIRT1 and PGC-1α, leading to the activation of the SIRT1/PGC-1α pathway and subsequent reduction in MI/R-induced myocardial damage (Tang et al., 2019). In addition to SIRT1, quercetin has a mild stimulating effect on SIRT6 (You et al., 2019). When modified with a bulky trihydroxy benzoyl group at the 3-OH group, as in catechin gallate, it inhibits SIRT6 activity (Rahnasto-Rilla et al., 2018; You et al., 2019).

Quercetin exists as a glycone or an aglycone in plants. When ingested, the glycone form can be hydrolyzed to the aglycone form that can be easily absorbed in the small intestine due to its hydrophobic nature (Massi et al., 2017). In human plasma, ingested quercetin glycosides are predominantly metabolized into quercetin 3-*O*-β-D-glucuronide and quercetin 3′-*O*-sulfate (**Figure 4**; D'Andrea, 2015; Moodi et al., 2021). Modifications such as glycosylation and methylation of the quercetin scaffold result in derivatives with distinct biological activities (Lesjak et al., 2018). For example, isoquercetin, the 3-*O*-glucoside of quercetin (Magar and Sohng, 2020), demonstrates

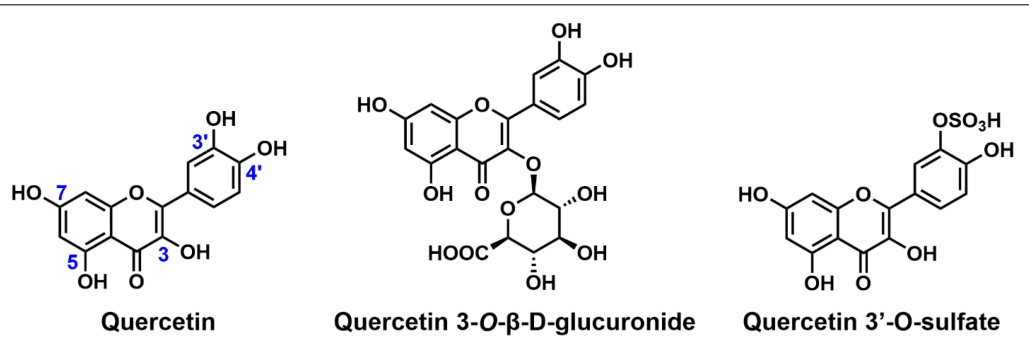

FIGURE 4 | Chemical structures of quercetin and its derivatives.

SIRT6 stimulation activity with no influence on SIRT1 activity (You et al., 2019).

Quercetin's therapeutic applications have been limited by its low bioavailability, poor solubility, and short half-life (Gugler et al., 1975; Ferry et al., 1996; KaŞıkcı and Bağdatlıoğlu, 2016). Modifications have been made to improve these properties (Massi et al., 2017). In one study, the bioavailability of quercetin was increased by about 20 times through a phytosome delivery system (Riva et al., 2019). Several other studies have utilized conjugation to various amino acids and nanoparticle delivery systems to improve the bioavailability of quercetin. Acylated quercetin analogs synthesized by Duan et al. (2017) were about 10-fold more soluble in water than quercetin. Of the several quercetin human clinical trials targeting various disease states, only one ongoing study (NCT03943459) is aimed at investigating the activation of SIRT1 by quercetin in coronary disease.

CONCLUSION AND PERSPECTIVES

A simple search in PubMed provides hundreds of publications related to sirtuin inhibitor/activator development, demonstrating the critical roles these enzymes play in regulating diverse cellular events and the intense interest in pursuing them as therapeutic targets. Unfortunately, tremendous efforts have only resulted in a handful of small molecules in clinical studies as described in this review article. Translating sirtuin regulators from the bench to the clinics has been hampered by the lack of isoform-selective candidate compounds with favorable pharmacological profiles. The catalytic domain is highly conserved between sirtuins and therefore represents a promiscuous target for NAD^+ or peptide-competitive inhibitors (Dai et al., 2018). In the case of activators, the binding sites are often not readily apparent by the inspection of a crystal structure, and there is no general and systematic strategy to identify and target these sites. Furthermore, for several human sirtuin isoforms, novel enzymatic activities were discovered recently (Du et al., 2011; Feldman et al., 2013; Jiang et al., 2013; Anderson et al., 2017). Potent regulators targeting specifically these new activities are still in the making. Pre-clinical investigations using animal models may differ in the genetic background or the assessment methods which have caused controversies and ambiguities that still need to be reconciled. In spite of numerous reports on the endogenous substrates of sirtuins and the pathways they regulate, our understanding of the biological functions of sirtuins is still in its infancy. For example, SIRT1 has been closely associated with cancer pathology, and has been suggested as either a tumor promoter or a suppressor in a context-dependent manner (Deng, 2009). All the research effort has only scratched the surface of sirtuin biology. A comprehensive and thorough picture of these intriguing enzymes still awaits description.

Of course, sirtuin-targeting drugs still hold great therapeutic potential, and progress in the field will accelerate the development of small molecule drug candidates. Apart from their highly conserved catalytic core, sirtuins harbor structurally unique N- or C-terminal extensions that can be targeted for selectivity or even specificity. The conformational plasticity of the active site that explains the isoform selectivity of EX-527 (Gertz et al., 2013; Broussy et al., 2020) has also been suggested as a novel target for inhibitor development. The clinical success of sirtuin-targeting medications requires a clear understanding of the "sirtuin-dependency" of the disease, robust lead compounds that are potent and selective with ideal drug-like properties, PK/PD profiling and improvement, as well as advances in formulation. The combined efforts from all these aspects will bring more sirtuin regulators into the clinic for treating diseases with considerable unmet medical needs.

AUTHOR CONTRIBUTIONS

AC, DW, and YC: conceptualization. AC, DW, DD, and YC: writing. YC: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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