REVIEW

Resveratrol in human cancer chemoprevention – Choosing the 'right' dose

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There is now robust preclinical evidence to suggest that resveratrol possesses cancer chemopreventive properties. A series of clinical pilot studies has provided insights into its pharmacokinetics, and data on its human antineoplastic pharmacodynamics start to emerge. It is likely that resveratrol will be developed further in the clinic as a putative cancer chemopreventive agent. The question that remains unresolved is: What is the most suitable dose of resveratrol for effective cancer preventive intervention? Mechanistic studies in cells in vitro have almost invariably used concentrations of resveratrol in the 10^{-5} to 10^{-4} M range, which is much higher than those which can be achieved in the human biophase after consumption of doses up to 1 g. Many of the preclinical efficacy studies in rodent models of carcinogenesis have employed doses which are dramatically above those which can be ingested with the diet. New experimental paradigms need to be used to obtain information on pharmacological changes elicited by resveratrol when present at very low concentrations or when administered at dietary-relevant doses.

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1 Introduction

It is widely thought that the consumption of ample fruits and vegetables is able to prevent cancer. However, ultimate scientific proof for this contention remains elusive. A recently published, ubiquitously discussed, prospective study of almost 500 000 European subjects suggests that the beneficial impact of fruits and vegetables on cancer incidence, whilst significant, is only small. There was a 4% decrease in the occurrence of all cancers combined accompanying an increment of 200 g of total fruits and vegetables per day [1, 2]. This figure contrasts sharply with a reduction of 30% in the incidence of coronary heart disease or stroke brought about by eating ≥ 5 portions of fruits/vegetables per day, compared with consuming < 1.5 servings per day [3]. It needs to be stressed that in the study by Boffetta et al. [1] the protective effect of fruits and vegetables was computed for all cancers combined. Thus, it is conceivable that the consumption of fruit and vegetables has no retarding effect on the development of certain malignant disease types, e.g. breast cancer, but reduces others, e.g. colorectal cancer, so that the overall effect on the incidence of all cancers is only weak. Another issue that may skew the results of the study by Boffetta et al. [1] is the possibility that specific constituents, or groups of compounds, in fruits and vegetables have a marked favourable effect, but this is masked or counteracted by the whole diet matrix. Therefore, it seems propitious to explore the putative cancer chemopreventive effects of individual diet constituents. The knowledge accrued from such studies may ultimately allow optimisation of diet composition with respect to health-promoting properties and/or the scientifically justified recommendation of certain supplements.

Evidence accumulated over the past 15 years suggests that the naturally occurring polyphenol resveratrol is able to prevent cancer, or delay its onset. This notion is based on studies in a broad variety of in vivo preclinical models of

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Abbreviation: IGF, insulin-like growth factor

carcinogenesis, in which resveratrol has been shown to prevent or delay the development of malignancies [4, 5]. It is, therefore, considered to merit further clinical development as a potential cancer chemopreventive agent in humans [6].

Relatively few diet-derived agents have been subjected to long-term clinical intervention studies aimed at assessing their chemopreventive efficacy. Among these are β-carotene, selenium, vitamin A and folate. The outcome of these studies has been disappointing in that a clear benefit could not be demonstrated [7]. In the case of β-carotene and folate, there was a slight exacerbation of lung and colorectal cancer incidence, respectively, with agent intake. These results have engendered considerable soul-searching in the cancer chemoprevention research community, addressing questions pertaining to the optimal design of such clinical trials. One prominent contentious issue has been the suitability of the doses used in these trials. In general, the doses selected have not been sufficiently optimised, due to either a lack of the required preclinical or early clinical data to make an informed choice or the assumption that higher doses are better, regardless of the available evidence. The use of suboptimal doses may have contributed to the disappointing trial outcomes to date and it is widely appreciated that there is an urgent need to improve the design of future chemoprevention trials of diet-derived agents. The aim of this commentary is to interpret the existing preclinical and clinical data on resveratrol, with a view to identifying the 'right' dose (or doses) to be used in future cancer chemoprevention studies, so as to maximise the chances of efficacy.

2 Effective concentrations of resveratrol in vitro

Rational exploration of the efficacious dose of a putative cancer chemopreventive agent requires robust knowledge of its mechanisms of action. Over the past two decades, the molecular pharmacology of resveratrol has been the subject of a vast number of investigations. The plethora of anticarcinogenic mechanisms in which resveratrol can engage has been described in many reviews [see [5, 8] for examples], and only cursory reference is made to them here. In general, the concentrations of resveratrol required to suppress the proliferation of cultured cells in vitro or modulate biochemical processes germane to carcinogenesis tend to be above 5 μM and often close to 100 μM. Among these events are the inhibition of cyclooxygenase-2, decreased nuclear factor κB activity, induction of cell-cycle arrest at the G1 and G1/S phases consequent to the enhancement of the expression of cyclin-dependent kinase inhibitor proteins plus induction of apoptosis and differentiation via modulation of survival genes such as Bax, Bak, Bcl-2, p53 and TRAIL. Resveratrol is also an antioxidant and inhibits certain cytochrome P450 enzymes, reduces angiogenic potential and inhibits cell adhesion, invasion and metastasis. In recent years, resveratrol has received considerable attention for its ability to act as a calorie restriction mimetic.

Most of the studies pertaining to the anti-carcinogenic mechanisms of resveratrol have been conducted using a conventional cell culture paradigm, in which cells are incubated with the test agent at relatively few concentrations for short periods, typically <96 h, after which the phenomenon under scrutiny is assessed. This experimental design is unlikely to reveal the more subtle effects of cancer chemopreventive agents particularly at low, clinically or dietary relevant concentrations, which may only become manifest after long-term exposures. Unpublished experiments in our laboratory support this notion; incubation of HCA7 human colon cancer cells with resveratrol at 10 nM for 3 months caused significant and reproducible alterations in gene expression, but this concentration failed to induce detectable phenotypic changes following a more conventional short-term protocol. Intriguingly, non-conventional or nonlinear dose–response relationships have been described for several biological phenomena elicited by resveratrol in vitro, and the hormetic nature of some of these cellular responses has been the subject of a recent in depth review [9]. 'Hormesis', derived from the Greek word meaning 'rapid motion' or 'eagerness', is used predominantly in toxicology where it characterises stressor stimuli such as toxic agents that exert biological effects at small doses, which are opposite to those caused by large doses. However, the relevance of the concept of hormesis to intact biological organisms is debatable [10]. An example of the biphasic nature of a biochemical effect of resveratrol is its modulation of nitric oxide synthase expression in endothelial progenitor cells in vitro: at 1 μM it increased nitric oxide synthase expression, whereas at 60 nM it decreased expression [11]. This bi-phasicity was confirmed in a murine model of aorta repair in vivo. Resveratrol administered at a dose of 10 mg/kg increased endothelial nitric oxide synthase expression in injured arteries and increased the number of endothelial progenitor cells in the circulation, but a higher dose of 50 mg/kg failed to elicit these responses, illustrating that supra-dietary doses may exert effects very different to those elicited by dietary doses. Such bi-phasicity has hitherto not been described for the effects of resveratrol on oncogenic processes, but it might well exist.

3 Resveratrol doses in vivo

3.1 Preclinical carcinogenesis models

Resveratrol has been shown to prevent cancer in several studies in rodent models of carcinogenesis involving a large range of oral doses, mostly administered with the diet. In the azoxymethane-induced colon cancer model in rats, a daily dose as low as 0.2 mg/kg interfered with tumour development [12]. This dose equates to ~2.5 mg in a human

of 70 kg body weight using the animal–human body surface area extrapolation pioneered by Freireich et al. and Reagan-Shaw et al. [13, 14]. In the dimethylhydrazine-induced colon carcinogenesis rat model, resveratrol assuaged cancer formation at 8 mg/kg daily [15], approximating to ~90 mg in humans. In the Apc^{Min} mouse, a model of colorectal cancer caused by an *Apc* mutation, daily doses of resveratrol which inhibited adenoma development have been 15 mg/kg [16] and 240 mg/kg [17], extrapolating to ~95 and 1520 mg, respectively, in humans. Finally, in the TRAMP mouse model of prostate cancer, the reported efficacious dose was 94 mg/kg/day [18], equating to ~600 mg in humans. Only a few of these in vivo studies presented results for more than one dose, so it is conceivable that the doses administered were among the lowest eliciting efficacy. In the case of one study in the Apc^{Min} mouse, the dose of 240 mg/kg was rationalised in terms of the level of resveratrol achieved in the intestinal mucosa, which was 34 nmol/g (or ~34 μM assuming 1 mL has a mass of 1 g) [17], a value comfortably within the concentration range at which resveratrol exhibits effects in vitro consistent with cancer chemoprevention (see Section 2). Even in the absence of extended dose–response relationships, the available data on efficacy of resveratrol in rodents suggest that it is active over a wide dose range, and inhibition of carcinogenic events in different organs may be influenced by the underlying molecular/genetic features of the model as well as the concentrations attained in the particular tissue of interest.

3.2 Clinical pilot studies

There have been a variety of clinical pilot studies of resveratrol, almost exclusively aimed at defining its pharmacokinetics and metabolism. In these studies, resveratrol has been ingested either as a single synthetic agent or as a constituent of a food or drink, at a range of doses and administration schedules [19–30]. In one of the early investigations, a dose equating to a high dietary achievable intake of resveratrol (25 mg/70 kg) was ingested by healthy male subjects in three different matrices, white wine, white grape juice or vegetable juice, to examine the influence on absorption [19]. Free resveratrol accounted for only a small fraction of the total dose in plasma (1.7–1.9%), with glucuronide and sulphate conjugates dominating the profile in both plasma and urine. The low levels of free resveratrol attained (< 40 nmol/L) suggest that circulating concentrations generated by the consumption of dietary sources would be inadequate to elicit biological effects, based on that needed for activity in cultured cells in vitro. In a more comprehensive study, the absorption, bioavailability and metabolic fate of resveratrol was traced through the use of radioisotope labelling [20]. Following oral consumption of a dietary relevant amount (25 mg) of [^{14}C]-resveratrol by six healthy volunteers, at least 70% of the dose was absorbed [21]. Despite relatively efficient absorption, the bioavailability of unchanged resveratrol was very low, due to rapid and extensive metabolism. Peak plasma levels of radioactivity equated to ~2 μM total [^{14}C]-labelled species, and the vast majority of this was due to the presence of phase II metabolites. Only trace amounts of unchanged resveratrol (< 5 ng/mL) could be detected.

Two recent phase I dose escalation studies evaluated the safety and pharmacokinetics of resveratrol administered both on a single and repeat dose schedule (0.5, 1.0, 2.5 or 5.0 g daily) in healthy volunteers [22, 23]. Plasma and urine concentrations of parent and metabolites were determined by HPLC with UV detection, and resveratrol-derived species were structurally identified using LC-tandem mass spectrometry. At the highest single dose, the average peak plasma concentration (C_{max}) of resveratrol was ~2.4 μM. In comparison, the C_{max} levels of two monoglucuronides (resveratrol-3-*O*-glucuronide and resveratrol-4′-*O*-glucuronide) and resveratrol-3-*O*-sulphate were three- to eightfold higher. The area under the plasma concentration versus time curve (AUC) for these metabolites was up to 23-fold greater than for resveratrol, indicating considerably higher systemic exposure to the conjugates. In the follow-up study, resveratrol was administered at the same doses for 29 days to assess the safety and pharmacokinetics following repeated dosing [23]. Resveratrol and metabolite levels were measured in plasma predose, and full pharmacokinetic analysis was performed on a day during the fourth wk of dosing. The mean average plasma concentration (C_{av}) and C_{max} values of parent resveratrol across the four groups increased with dose and ranged from 0.04 to 0.55 μM and 0.19 to 4.24 μM, respectively. Resveratrol-3-*O*-sulphate, resveratrol-4′-*O*-glucuronide and resveratrol-3-*O*-glucuronide were the major plasma metabolites, with C_{max} values between 2.4- and 13-fold greater than resveratrol itself. Consistent with the findings of the single dose trial, AUC values for these metabolites also exceeded those of the parent, in the case of resveratrol-3-*O*-sulphate by up to 20-fold.

Another recent study following a similar protocol involved healthy volunteers ingesting 1 g resveratrol daily for 4 wk [24]. An important outcome of this trial was the observation that resveratrol significantly modulated drug and carcinogen-metabolising enzymes. It inhibited the phenotypic indices of plasma cytochrome P450s CYP3A4, 2D6 and 2C9, whilst inducing 1A2. Additionally, in subjects with low baseline values, intervention was associated with an induction of glutathione *S*-transferase GST-π protein expression and UDP glucuronyl transferase UGT1A1 activity.

In the first trial to investigate the tissue levels of resveratrol the question was addressed whether resveratrol can furnish colorectal concentrations commensurate with pharmacological activity [25]. Colorectal cancer patients (ten per group) consumed either one or two 0.5 g resveratrol caplets daily for eight consecutive days prior to resection. During surgery, samples of tumour and adjacent sections of non-malignant colon tissue were obtained for the analysis.

In addition to resveratrol, six metabolic conjugates were identified in the tissue. There was substantial variation in the tissue concentrations measured, both in different samples from the same patient and also amongst individuals. When all ten patients on each dose were considered together, the highest mean concentration of resveratrol was found in benign tissue localised proximal to tumours excised from the right side of the colon, where it reached ~19 and 674 nmol/g, after the 0.5 and 1.0 g interventions, respectively. The corresponding values in tumour tissue were lower, at ~8 and 94 nmol/g (or μM assuming 1 mL has a mass of 1 g). Importantly, the levels of free resveratrol detected in many cases exceeded the concentrations that have been widely reported to have activity in preclinical systems [31].

3.3 High dose versus dietary dose

How can we exploit the information emanating from the results of the preclinical experiments and human pilot studies outlined above to optimise the design of future chemoprevention intervention trials of resveratrol? The data presented above need to be interpreted from two different standpoints: resveratrol has been, and is being, studied on the one hand at low doses relevant to its role as a dietary constituent, and on the other hand at high doses as a synthetic bioactive agent, which just happened to be isolated from fruits originally. It is pertinent to stress that there are not many studies in the literature in which dietary-relevant doses of resveratrol have been studied. Both approaches constitute different paradigms and need to be interpreted separately. Efficacy in rodents has been reported at low [12, 15] and high doses [16–18]. Nevertheless, the lack of extended dose–response relationships encompassing dietary doses in most of the models renders it impossible to ascertain which model responded only to high doses, and whether the efficacy of low-dose resveratrol in the azoxymethane- and dimethylhydrazine-induced rat colon cancer models [12, 15] was also observed at high doses. To enable the identification of optimal doses with maximal efficacy in vivo, more comprehensive analysis of a wide dose range is needed in each preclinical system.

If the dose chosen for clinical intervention studies is to be based on the results of the published in vitro experiments using cultured cells, it appears that doses capable of generating tissue levels exceeding ~5 μM would be required, since this is arguably the lowest concentration at which resveratrol precipitates biochemical changes consistent with anti-cancer activity. Thus far, the highest well-tolerated dose reported in clinical trials in which resveratrol was administered repeatedly is 1 g [22, 23]. This regimen afforded peak plasma levels of parent compound below 5 μM; however, peak plasma concentrations of resveratrol conjugates were close to, or above, this value (Table 1). In the mammalian organism, resveratrol undergoes avid metabolism by conjugation with sulphate and glucuronic acid, and the levels of resveratrol sulphates and glucuronides in the blood of individuals who consumed resveratrol dramatically exceed those of parent agent. Little is known about the pharmacological properties of resveratrol conjugates, although recent in vitro studies hint at the possibility that resveratrol-3-*O*-sulphate and resveratrol-4′-*O*-sulphate may contribute to some of the pharmacological properties elicited by their parent [32, 33]. If the sulphate and/or glucuronide metabolites will be proven to contribute to activity, a dose of 1 g resveratrol may potentially yield efficacious plasma levels of total resveratrol species. Furthermore, the levels of resveratrol measured in human colorectal tissue after repeated oral ingestion of 1 g [25] were consistent with concentrations at which resveratrol elicits anti-carcinogenic effects in cells in vitro and those achieved in the gastrointestinal tract of Apc^{Min} mice after a dose which interfered with adenomagenesis [17] (Table 1). The above consideration suggests that 1 g might be a potentially suitable dose for colorectal cancer chemopreventive activity. Nevertheless, one needs to be extremely mindful that this dose of resveratrol can modulate drug metabolising enzyme activity [24] and may thus cause untoward drug interactions, so lower doses may be necessary in certain populations.

Furthermore, this dose is irrelevant to the potential efficacy of resveratrol as a diet constituent. The concentration of resveratrol in red wine is between 0.2 and 5.8 mg/L [34]. Assuming a resveratrol content of 5 mg/L, the intake of resveratrol with red wine in humans after moderate consumption (250 mL in a 70 kg person) would be 1.25

Table 1. Doses and concentrations of resveratrol (Res) in humans as discussed in the text

Doses	
Highest tolerated dose (daily for 29 days)[a)]	1 g
Pharmacodynamically active dose (IGF-1/IGFBP-3 reduction)[a)]	2.5 g
Typical dose with moderate red wine intake[b)]	~1.25 mg
Dietary dose in Apc^{Min} mice which reduced adenomagenesis[c)]	240 mg/kg
Concentrations	
Res plasma peak after 1 g (daily ~21 days)[a)]	0.6 μM
Res-3-*O*-sulphate plasma peak (same dose)[a)]	7.4 μM
Res-4′-*O*-glucuronide plasma peak (same dose)[a)]	3.1 μM
Res in gut tissue after 1 g (daily 8 days)[d)]	up to 674 nmol/g
Res in Apc^{Min} mouse plasma after 'active' dietary dose[c)]	<0.1 μM
Res in Apc^{Min} mouse gut mucosa after 'active' dietary dose[c)]	~36 nmol/g

a) Ref. [23].
b) Ref. [34].
c) Ref. [17].
d) Ref. [25].

mg/day. It is conceivable that resveratrol exerts its optimal cancer chemopreventive activity at doses of this low order of magnitude, although this idea has been little explored to date. If the aim of an intervention study is to examine the potential role of resveratrol in mediating the chemopreventive activity of a resveratrol-containing diet, trials using such low doses should be conducted. These types of studies would require experimental approaches different from those performed with high resveratrol doses. For example, standard analytical techniques lack the necessary sensitivity to detect resveratrol or its metabolites in humans after consumption of a dietary relevant dose. Therefore, in unpublished studies we have employed [^{14}C]-labelled resveratrol and accelerator mass spectrometry [35] to elucidate the full plasma pharmacokinetic profile of a dietary attainable dose of resveratrol in healthy volunteers as well as measuring prostate and colorectal tissue concentrations in patients undergoing surgery.

It seems worth mentioning that these considerations do not take any effect of dietary factors on resveratrol metabolism and bioavailability into account. Some evidence suggests that the food matrix that accompanies resveratrol in the human diet can affect resveratrol bioavailability [29], perhaps via modulation of phenol sulphate and glucuronide formation.

Cancer chemoprevention efficacy studies take many years until the clinical endpoint, cancer occurrence, is reached. This fact renders intervention trials immensely costly, and these investigations are only feasible if surrogate endpoints can be employed that indicate efficacy long before cancer occurs. It is now appreciated that the only way to detect potential efficacy at an early stage of clinical intervention is through the use of biomarkers, i.e. biochemical changes intrinsically related to the anti-carcinogenic effects of test agents. There is a paucity of published information on candidate biomarkers that are modulated by resveratrol in humans. One recent clinical trial in healthy volunteers provided evidence that repeated administration of resveratrol can attenuate components of the insulin-like growth factor (IGF) signalling system, which influences malignant development; circulating levels of IGF-1 and IGF-binding protein 3 were both lowered by intervention [23]. This effect was maximal at 2.5 g but absent at the highest dose of 5.0 g. The potential of resveratrol to affect these proteins needs to be investigated over a wider range, encompassing dietary doses.

4 Towards a rational dose for intervention studies

Figure 1 outlines an experimental strategy emanating from the insights gained thus far, which may help define the optimum dose of resveratrol to be used in definitive chemoprevention intervention studies. Resveratrol should be subjected to extended dose-finding efficacy studies in a suitable rodent model of carcinogenesis, exemplified by the above-mentioned murine *Apc*^{Min} and TRAMP models of colorectal and prostate carcinogenesis, respectively. The levels of resveratrol and its metabolites accompanying efficacy should be measured in the circulation and especially the target organs of these animals. The concentrations of resveratrol detected in the target organ should then be used to elucidate potential mechanisms engaged in cell culture studies in vitro, using long-term exposure which mimics the chemoprevention setting more faithfully than conventional cell culture experiments (see above). Based on the mechanisms identified, evidence of appropriate biomarker changes should be sought in the blood and tissues of rodents, which received resveratrol at efficacious doses.

Armed with all the information accrued in these preclinical studies, pilot trials of resveratrol should be undertaken in healthy volunteers and/or cancer patients. Dosing might commence at the level equivalent to that which showed activity in the relevant rodent model. Concentrations of resveratrol and its metabolites in the blood and, if possible, in target tissue should be measured and compared with the levels achieved in the preclinical model in which resveratrol was efficacious. Subsequently, dosing could be adjusted depending on the concentrations attained and how they relate to efficacious levels established preclinically, although this will also be dictated by clinical safety and tolerability data. Then, the hypothesis should be tested that the biomarkers of resveratrol efficacy identified in rodents are also affected by resveratrol in humans. If this is found to be the case, the biomarkers would then be subject to full validation.

Many recent clinical cancer chemoprevention efficacy studies of diet-derived agents such as β-carotene, folate or selenium have been disappointing, and sub-optimal dosing may well have contributed to this outcome. To avoid similar failures with the clinical evaluation of other diet constituents such as resveratrol, the trial design needs to be carefully optimised. We believe that the approach outlined here will help establish the right dose for definitive intervention

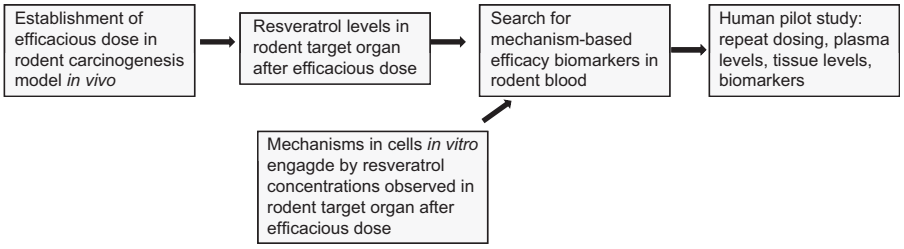

Figure 1. Rational strategy to establish the 'right' dose of resveratrol to be used in efficacy intervention studies.

studies of resveratrol and is especially mindful of the possibility that very low doses may be the optimal ones.

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