Resveratrol, a remarkable inhibitor of ribonucleotide reductase

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Abstract Resveratrol, a natural phytoalexin found in grapes, is well known for its presumed role in the prevention of heart disease, associated with red wine consumption. We show here that it is a remarkable inhibitor of ribonucleotide reductase and DNA synthesis in mammalian cells, which might have further applications as an antiproliferative or a cancer chemopreventive agent in humans.

Key words: Resveratrol; Ribonucleotide reductase; Tyrosyl radical; Anticancer agent

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a natural phytoalexin found in grapes (Scheme 1). Its physiological function is not well defined but is thought to reside in the protection of the plant from environmental stress and pathogenic attack [1]. On the other hand, it has been suggested to play a role in the prevention of heart disease, associated with red wine consumption, as it inhibits platelet aggregation, alters eicosanoid synthesis and modulates lipid and lipoprotein metabolism [1]. Resveratrol was also recently shown to inhibit cellular events associated with tumor initiation, promotion and progression [2]. In the light of its very weak toxicity, at least in mouse tumor models [2], it was suggested that resveratrol should be investigated as a cancer chemopreventive agent in humans.

Still very little is known about the molecular basis for its biological activities. As a polyphenol molecule, it is a radical scavenger and has been shown to inhibit cyclooxygenase activity [2]. Now we report that it is a remarkable inhibitor of ribonucleotide reductase, the enzyme which provides proliferating cells with deoxyribonucleotides required for DNA synthesis, during the early S-phase of the cell cycle [3]. This is likely to explain its antiproliferative properties.

Ribonucleotide reductases are complex enzymes which catalyze the reduction of ribonucleotides into the corresponding deoxyribonucleotides. Mammalian cells, in common with other eukaryotes, DNA viruses of the Herpesvirus group and some bacteria, notably *Escherichia coli*, have an iron-containing enzyme which consists of two non-identical homodimeric subunits, R1 and R2 [4]. Each protein represents an important target for new antiproliferative drugs [5]. Protein R1 contains the substrate binding sites and can be irreversibly inactivated by certain nucleotide analogs, in particular those having a modification at the 2′ position of the ribose moiety [5–8]. For example, gemcitabine, the 2′-difluoro-2′-deoxycytidine analog, proved to be an excellent ribonucleotide reductase inhibitor and is now used in clinics [7].

Another strategy of inhibition relies on the fact that enzyme activity depends on the presence of a tyrosyl radical on a specific residue of the small R2 protein [9]. Tyrosyl radical scavenging by hydroxyurea [10] or 4-hydroxyanisole [11], both used in clinics as anticancer agents, results in enzyme inactivation and inhibition of DNA synthesis. This strategy was recently revisited in the context of the research for new drugs against the human immunodeficiency virus (HIV), since a combination of hydroxyurea with 3′-azido-3′-deoxythymidine (AZT) or 2′,3′-dideoxyinosine (ddI) proved to have synergistic antiviral effects [12–14]. In view of the secondary effects of hydroxyurea and the development of cell resistance, due to the requirement for rather large doses of the drug, there is a need for new, more efficient inhibitors of ribonucleotide reductase. Resveratrol may be a good candidate.

2. Materials and methods

2.1. Materials

Hydroxyurea and resveratrol were from Sigma; *p*-propoxyphenol was from Aldrich.

Recombinant mouse R2 protein was prepared as previously described [9] from an *E. coli* strain, BL21(DE3)-pETM2, containing the mouse R2 gene, kindly provided by Professor Thelander (University of Umeå, Sweden). After purification, the mouse R2 protein was obtained in the apoprotein form. The radical site was then generated after the anion exchange chromatography step by addition of ferrous iron/ascorbate (molar ratio 1:2) anaerobically followed by addition of oxygen [9]. The protein solution was transferred to ice and the excess iron/ascorbate complex was immediately removed by gel filtration on a Sephadex G25 column. The reconstituted protein was kept in 50 mM Tris pH 7.6, 10% glycerol, frozen in liquid nitrogen and stored at −70°C until used. Its concentration was determined with the Bradford colorimetric assay [15] or from the absorption at 280 nm ($\varepsilon_{280-310} = 62\,000\ M^{-1}\ cm^{-1}$) [9].

2.2. Assay for tyrosyl radical scavenging – EPR spectroscopy

First derivative EPR spectra were recorded at 100 K on a Bruker ESP300E spectrometer. Protein R2 was dissolved in 150 μl of 0.1 M Tris-HCl buffer pH 7.5 in order to have a final protein-bound tyrosyl radical concentration of 10 μM. The solution was transferred to an electron paramagnetic resonance (EPR) tube and a control spectrum was recorded. After addition of a small volume of the radical scavenger solution, incubation was carried out aerobically in the EPR tube at 37°C and was stopped after 5 min by freezing the sample at liquid N_2 temperature. The relative amplitude of the characteristic tyrosyl radical g = 2.00 doublet signal (normalized to 100% for the control) was used to measure the inactivation of R2 at various concentrations of the radical scavengers. Half-maximal inhibition values were used to compare the efficiency of the inhibitors. Recording conditions: microwave power 10 mW; modulation amplitude 3.12 G.

2.3. Cell lines

L1210-R2 murine lymphoblastic leukemia cells, in which expression of protein R2 has been amplified 15–20 times with regard to the parental L1210 cell line [16], was used for ribonucleotide reductase assays. K-562 human myelogenous leukemia cells and P-815 murine

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mastocytoma cells were used for assaying [^{3}H]thymidine incorporation into DNA. These cell lines were cultured in RPMI 1640 medium supplemented with antibiotics, 5% heat-inactivated fetal calf serum (Gibco BRL Sarl, Cergy-Pontoise, France), and 25 mM HEPES pH 7.4.

2.4. CDP reductase assay

The L1210-R2-overexpressing cell line [16] was cultured in RPMI 1640 medium supplemented with antibiotics and 5% heat-inactivated fetal calf serum. Cells grown in exponential phase were harvested, washed once in phosphate-buffered saline and resuspended in 100 mM HEPES pH 7.6, 15 mM magnesium acetate and 10 mM dithiothreitol (DTT), at a concentration of 10^8 cells/500 μl. Preparation of cytosolic extracts and assay of ribonucleotide reductase were performed essentially as described in [17], except that CDP concentration was reduced to 80 μM. Inhibitors were added with the CDP substrate, when required.

2.5. Determination of [^{3}H]thymidine incorporation

K-562 human erythroleukemia cells and P-815 murine mastocytoma cells were seeded in 96-well microculture plates (1×10^5 cells/well) in 100 μl culture medium. Inhibitors were added in 100 μl and the cells were labelled with 37 kBq of [^{3}H]thymidine (specific activity 37 GBq/mmol; Amersham, France) for 24 h at 37°C. DNA was then harvested on glass fiber filters and radioactivity determined by scintillation counting [17].

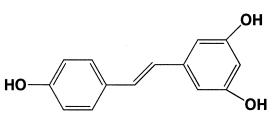
3. Results

3.1. Reaction of the tyrosyl radical in protein R2 with resveratrol

The tyrosyl radical of pure recombinant protein R2 from mouse exhibits an EPR doublet at 100 K at g = 2.0047 and can thus be monitored during reaction with increasing concentrations of radical scavengers [11]. We found that the catalytically essential radical (10 μM) was totally destroyed in the presence of a stoichiometric amount of resveratrol after 5 min incubation at 37°C (Fig. 1). Resveratrol was thus much more active than hydroxyurea since 10 μM hydroxyurea had almost no detectable effect on the tyrosyl radical after 5 min reaction (Fig. 1). Much higher concentrations or much longer reaction times were required for full destruction of the tyrosyl radical, as expected.

3.2. Inhibition of ribonucleotide reductase activity by resveratrol

The ability of resveratrol to destroy the tyrosyl radical was correlated with its strong dose-dependent inhibitory effects on enzyme activity, as assayed in soluble extracts of murine leukemia cells containing high protein R2 expression and high cytidine diphosphate reductase activity suitable for sensitive and reproducible assays (Fig. 2). The IC_{50} value (100 μM) was much smaller than that of hydroxyurea (1 mM). The latter value was in the concentration range previously reported [18]. We also compared it to that of *p*-propoxyphenol, which was recently claimed to be one of the most potent phenol inhibitors of ribonucleotide reductase [11]. The latter was found to be significantly less active with a IC_{50} value of 300 μM, under comparable conditions (Fig. 2). We thus expect an IC_{50} value much below 100 μM in assays where R2 is more limiting, in normal cells.



Scheme 1.

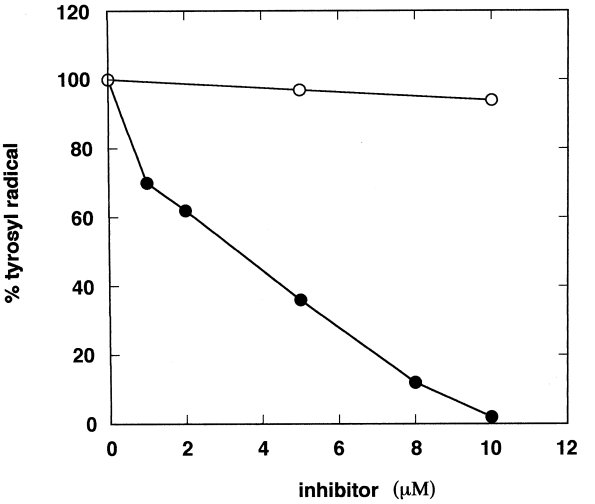


Fig. 1. Inactivation of the R2 protein of mouse ribonucleotide reductase by resveratrol and hydroxyurea. 10 μM of mouse protein R2 tyrosyl radical was incubated with increased concentrations of resveratrol (●) and hydroxyurea (○) at 37°C for 5 min. The sample was then frozen and assayed for its radical content by EPR spectroscopy at 100 K.

3.3. Inhibition of DNA synthesis by resveratrol

The antiproliferative properties of resveratrol and its inhibitory effects on DNA synthesis were evaluated from the [^{3}H]thymidine incorporation into DNA assay. For these experiments, we used both murine mastocytoma P-815 cells and human myelogenous leukemia K-562 cells and again compared resveratrol to hydroxyurea. Both cell lines gave comparable results with resveratrol being a much better inhibitor of DNA synthesis with a IC_{50} value of 8–10 μM compared to a IC_{50} value of 250 μM for hydroxyurea (Fig. 3). The IC_{50} values are significantly lower than those obtained in the CDP reductase assay described above. This reflects that the cells used here are not overexpressing R2.

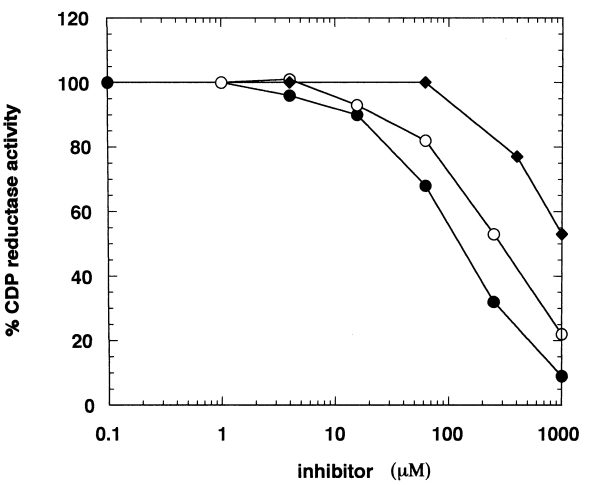


Fig. 2. Inhibition of mouse ribonucleotide reductase activity by resveratrol (●), hydroxyurea (♦) and *p*-propoxyphenol (○). 100% activity corresponded to 100 pmol/min/mg in the experiment with resveratrol and *p*-propoxyphenol and to 22 pmol/min/mg in the experiment with hydroxyurea.

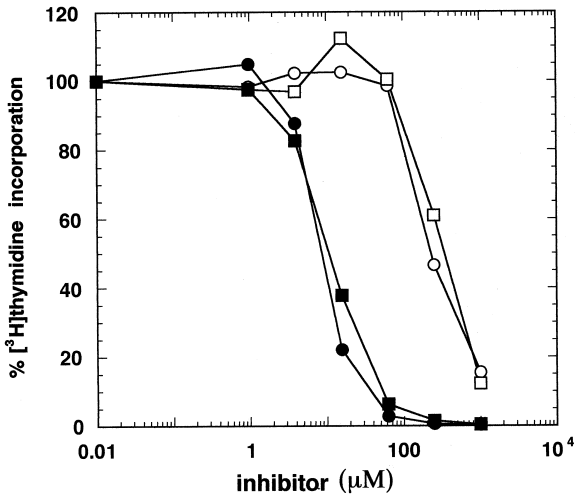


Fig. 3. Inhibition of DNA synthesis by resveratrol and hydroxyurea. K-562 (rounds) and P-815 (squares) tumor cells were incubated with resveratrol (closed symbols) or hydroxyurea (open symbols) and [^{3}H]thymidine at 37°C for 24 h. DNA was then harvested on a glass filter and radioactivity determined by scintillation counting.

4. Discussion

There is still a great interest in the development of inhibitors of ribonucleotide reductase as anticancer, antibacterial and antiviral drugs, including for therapeutic strategies against HIV [12–14,19]. The recent observation that resveratrol, a common constituent of the human diet, had cancer chemopreventive activity [2] and the well-established fact that ribonucleotide reductase is highly sensitive to phenol derivatives led us to investigate whether resveratrol was a good inhibitor of mammalian ribonucleotide reductase.

Our results show for the first time that indeed, at least in vitro, resveratrol is a remarkable inhibitor of ribonucleotide reductase. It is much more effective than hydroxyurea, hydroxyanisole, the only ribonucleotide reductase tyrosyl radical scavengers used in clinics, and the potent *p*-propoxyphenol [11]. The most active radical scavenger described up to now acting on ribonucleotide reductase is 5-amino-1-formyl isoquinoline thiosemicarbazone [20,21]. However, thiosemicarbazones have no application as drugs, because of their very strong toxicity. We suggest that resveratrol should thus be considered as an alternative to the more toxic hydroxyurea, for example in studies of bitherapeutic approaches against HIV.

Furthermore, our results provide new insights into the beneficial biological activities of resveratrol. Resveratrol has in the recent past been a subject of intense research mainly because it may be one of the key components in red wine responsible for the prevention of heart disease [1]. Inhibition of cyclooxygenase might partly explain this effect [2]. Now we confirm the strong antiproliferative properties of resveratrol and show that they are likely to be due to its ability to efficiently scavenge the essential tyrosyl radical of the small protein of ribonucleotide reductase and, consequently, to inhibit deoxyribonucleotide synthesis. Our data thus strongly support the suggestion that, in addition to its cardiovascular beneficial effects, resveratrol merits further investigation as a cancer chemopreventive agent in humans.

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