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RESVERATROL AS A NEW TYPE OF DNA-CLEAVING AGENT

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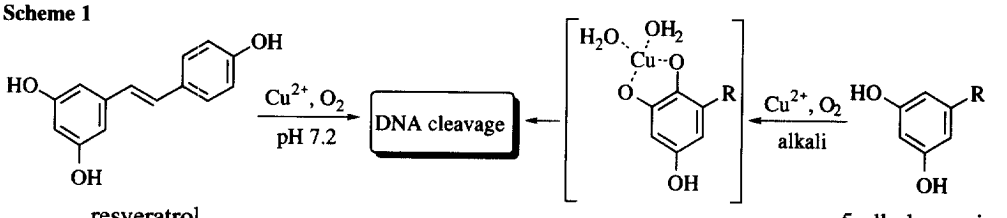
Abstract: The first demonstration of DNA cleavage by resveratrol '3,5,4'-trihydroxy-*trans*-stilbene' is presented. Resveratrol mediated relaxation of pBR322 at micromolar concentrations in the presence of Cu^{2+}. Evidence is provided that resveratrol is capable of binding to DNA, and that the Cu^{2+}-dependent DNA damage is more likely caused by a copper-peroxide complex rather than by a freely diffusible oxygen species.

Recent reports on Cu^{2+}-dependent DNA strand scission by 5-alkyl-1,3-dihydroxybenzene, commonly known as resorcinol, under oxidative conditions pointed to a new structural class of DNA-cleaving molecules as therapeutic agents.[1] This DNA cleavage requires the initial oxygenation of the benzene nucleus, a process that occurs readily at alkaline pH in the presence of Cu^{2+} and O_2. The resulting trihydroxylated benzenes mediate DNA cleavage in a reaction dependent on the presence of Cu^{2+} and O_2.[2] Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), which comprises the same structural elements as resorcinol, is known as an antioxidant[3] and antimutagen[4] found in grapes and other food products. Recently, it has been shown that resveratrol has cancer chemopreventive potential, consistent with the observation of inhibition of tumor initiation, promotion, and progression.[5] Because of the structural similarity of resveratrol with resorcinol, we have focused on its DNA-cleaving activity.

Scheme 1



resveratrol 5-alkylresorcinol

Resveratrol is found to have a greatly potentiated ability to mediate Cu^{2+}-dependent DNA cleavage under aerobic conditions. In a plasmid-based DNA cleavage assay, as shown in Figure 1, low micromolar concentrations of resveratrol efficiently converted supercoiled (form I) DNA to the nicked form (form II),[6] while, in the absence of Cu^{2+}, no cleaving activity was observed even at 100 μM of resveratrol. In the presence of other metal ions, resveratrol did not induce DNA cleavage,[7] consistent with the suggestion that Cu^{2+} is the specific metal ion required for DNA degradation by resveratrol. In this context, it should be noted that Cu^{2+}-dependent DNA strand scission occurred at neutral pH, because it implies that *resveratrol per se has an ability to induce DNA cleavage without oxygenative transformation of he benzene nuclei,*[8] the process required for

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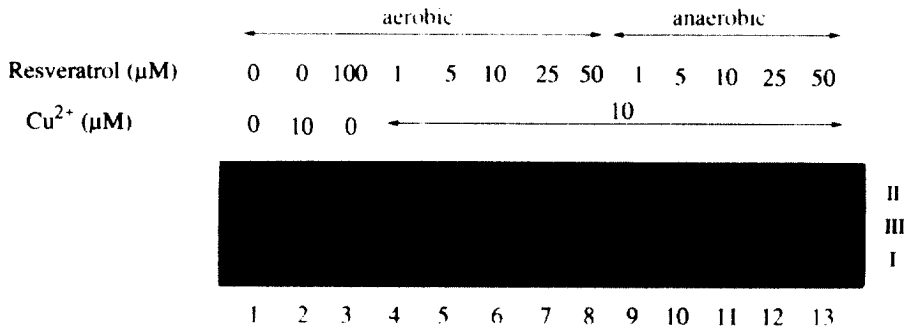


Figure 1. DNA cleavage by resveratrol with Cu^{2+} in the presence and absence of O_2.
Assays were performed in 5 mM sodium cacodylate buffer , pH 7.2, 0.25% DMF, containing 45 μM bp of pBR322 DNA, for 1 h at 37 °C in the presence (lane 1 - 8) and absence (lane 9 - 13) of O_2. Lane 1: pBR322 DNA alone. Lane 2: 10 μM Cu^{2+}. Lane 3: 100 μM resveratrol. Lane 4 - 8: 1, 5, 10, 25, and 50 μM resveratrol, in the presence of Cu^{2+} under aerobic condition. Lane 9 - 13: 1, 5, 10, 25, and 50 μM resveratrol, in the presence of Cu^{2+} under anaerobic condition.

'activation' of 5-alkylresorcinol as a DNA-cleaving agent. In fact, olivetol (5-pentylresorcinol) did not act as a DNA-cleaving agent under the same conditions as employed for resveratrol - Cu^{2+} induced DNA cleavage.[9] It was also found that, under anaerobic conditions, the efficiency of Cu^{2+}- dependent DNA cleavage was not enhanced with increasing the concentration of resveratrol. These results suggest that the DNA cleavage is absolutely dependent on the presence of both Cu^{2+} and O_2.

The effect of bathocuproine, a Cu^{+}-specific chelating agent, was also investigated to examine whether reduction of $Cu^{2+} \rightarrow Cu^{+}$ is essential for the DNA cleavage. As shown in Figure 2, there was a progressive decrease in the production of form II DNA with increasing concentration of bathocuproine, indicating that Cu^{+},

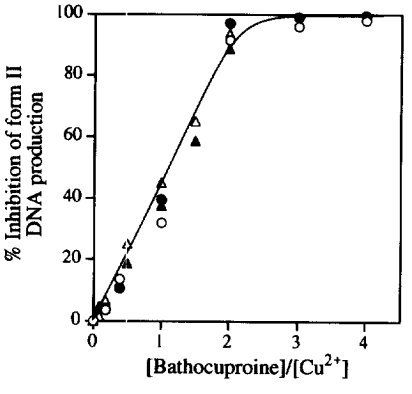


Figure 2. Inhibition of resveratrol-Cu^{2+}-induced DNA strand scission by bathocuproine.
Different concentrations of resveratrol and Cu^{2+} were incubated with supercoiled pBR322 DNA in the presence of bathocuproine, in 50 mM sodium cacodylate (pH 7.2) containing 0.25 % DMF at 37 °C. After 1 h, the amounts of remaining supercoile were determined (○) [resveratrol] / [Cu^{2+}] = 10 μM / 5 μM, (●) 20 μM / 5 μM, (△) 5 μM / 10 μM, and (▲) 10 μM / 10 μM.

which would be formed reductively by resveratrol, is a requisite intermediate in the process that results in DNA strand scission. That is, the reactive species actually responsible for DNA strand scission may be oxygen radicals generated from O_2 and the metal center in combination with resveratrol.[10]

In order to characterize the oxygen radicals leading to DNA cleavage, the effects of several free radical scavengers and inhibitors of DNA breakage were studied. As shown in Table 1, DNA cleavage was strongly suppressed in the presence of catalase, an enzyme that disproportionates H_2O_2 to afford $H_2O + O_2$, suggesting a requirement for H_2O_2 in the resveratrol - Cu^{2+} induced DNA breakage. Thiourea and KI, known as hydroxyl radical scavengers, also inhibited the DNA damage. Meanwhile, no inhibitory effects were observed with several other hydroxyl radial scavengers, such as DMSO, mannitol and ethyl alcohol, suggesting that the participation of the hydroxyl free radical is small. Considering the report by Yamamoto and Kawanishi who found that the DNA damage induced by Cu^{2+} plus H_2O_2 was caused by a copper-peroxide complex,[11] it is most conceivable that the main active species causing resveratrol - Cu^{2+} induced DNA breakage is comparable to that resulting from copper interaction with H_2O_2.

Inhibitors or scavengers	Concentration	% Inhibition of form II DNA production
SOD	100 μg / ml	- 2.0
catalase	25 μg / ml	90.7
tiron	1 mM	96.5
DMSO	50 mM	2.8
ethyl alcohol	50 mM	1.9
mannitol	50 mM	0.6
thiourea	10 mM	93.8
KI	50 mM	61.5
sodium azide	50 mM	1.2
dimethyl furan	1 mM	98.7

Table 1. Effects of inhibitors and scavengers on the DNA strand scission induced by resveratrol and Cu^{2+}.

In a typical assay, 25μM resveratrol with 5 μM Cu^{2+} (mix) was added to each agent with pBR322 DNA (45 μM bp) in 50 mM sodium cacodylate buffer (pH 7.2) containing 0.25 % DMF, and incubated for 1 h at 37 °C. The reaction mixture was analyzed by agarose gel electrophoresis, and the amount of DNA cleavage quantitated by digital imaging. Percent inhibition of the DNA strand scission was calculated as follows: inhibition (%) = 1- [(Sm+a - Sc) / (Sm - Sc)] where Sm+a is % remaining supercoiled after treatment with mix plus agent, Sc is % remaining supercoiled in control untreated plasmid, and Sm is % remaining supercoile with mix without agent.

For further exploration of the possible involvement of the copper-peroxide complex in resveratrol - Cu^{2+} mediated DNA degradation, ESR spectroscopy with spin-trapping reagents, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and 2,2,6,6-tetramethyl-4-piperidone (TMPD), was employed. As demonstrated in Figure 3, resveratrol - Cu^{2+} produced a four-line ESR signal ($a^N = a_\beta^H = 14.8$ G) corresponding to the DMPO / ·OH adduct. No superoxide radical adduct of DMPO was detected in this system. The formation of DMPO / ·OH was inhibited completely by 2 mM bathocuproine, 10 mM thiourea and 10 mM tiron, but proved insensitive to

0.2 M DMSO and 0.5 M mannitol. The results strongly indicated that ESR signals did not result from the reaction of a free hydroxyl radical with DMPO. In fact, the effect of scavengers on DMPO / ·OH formation has considerable correlation with their influence on DNA cleavage. Additional evidence, providing a further clue to the nature of the active species, was obtained from the formation of ESR signal for 4-oxo-2,2,6,6,-tetramethyl-1-piperidinyloxy during the reaction of resveratrol with Cu^{2+} in the presence of TMPD. It is reported that a stable nitroxide free radical was formed by the reaction of singlet oxygen with TMPD[12] and the ESR signal corresponding to the nitroxide radical produced from TMPD in the presence of Cu^{2+} plus H_2O_2.[11] Therefore, the production of the ESR signal of the nitroxide radical supports that the radical induced by the reaction of resveratrol with Cu^{2+} has similar reactivity to that generated by the reaction with Cu^{2+} with H_2O_2. That is, the copper-peroxide complex may be considered as the actual oxygen species that induces DNA cleavage by resveratrol and Cu^{2+}.

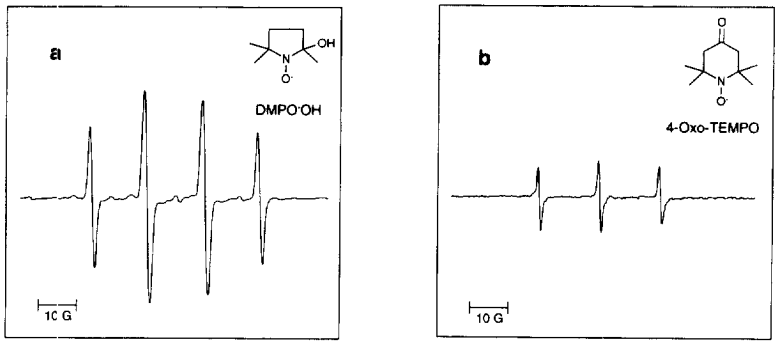


Figure 3. ESR spectra of the spin adducts of DMPO and 2,2,6,6-tetramethyl-4-piperidone during the reaction of Cu^{2+} and resveratrol.

Reactions contained 20 mM sodium phosphate buffer (pH 7.6), 10 mM resveratrol, 1 mM $CuCl_2$. *Spectrum a*, 500 mM DMPO was added; *spectrum b*, 100 mM 2,2,6,6-tetramethyl-4-piperidone was added.

In the study of resveratrol as a DNA-cleaving agent, evaluation of the ability of resveratrol to bind to DNA is of importance, because it is well known that production of a reactive oxygen species by DNA-binding agents affords enhanced DNA-cleaving efficiency relative to that by nonbinding analogues.[13] The binding was examined by NMR spectroscopy in the presence and absence of calf thymus DNA. As is clear from Figure 4, all of the resonances of resveratrol broadened with the decrease in the peak area as the concentration of DNA increased.[14] An analogous observation was also made by fluorescence quenching of resveratrol with calf thymus DNA.[15] These results apparently indicate that resveratrol is capable of binding to DNA, probably by a combination of electrostatic and hydrogen bonding forces.

In conclusion, resveratrol cleaved strongly plasmid DNA in Cu^{2+}-dependent manner. It is characterized that: I) the DNA cleavage is induced without the oxygenation process of the benzene nuclei to the catechol moiety which is a requisite intermediate in DNA cleavage mediated by resorcinol and Cu^{2+}, II) the main active species causing the DNA cleavage are more likely to be copper-peroxide complexes rather than a freely diffusible oxygen species, and III) the ability of resveratrol to generate a copper-peroxide complex and its DNA binding property may facilitate a target delivery of reactive oxygen to the DNA. To date, it still remains unclear

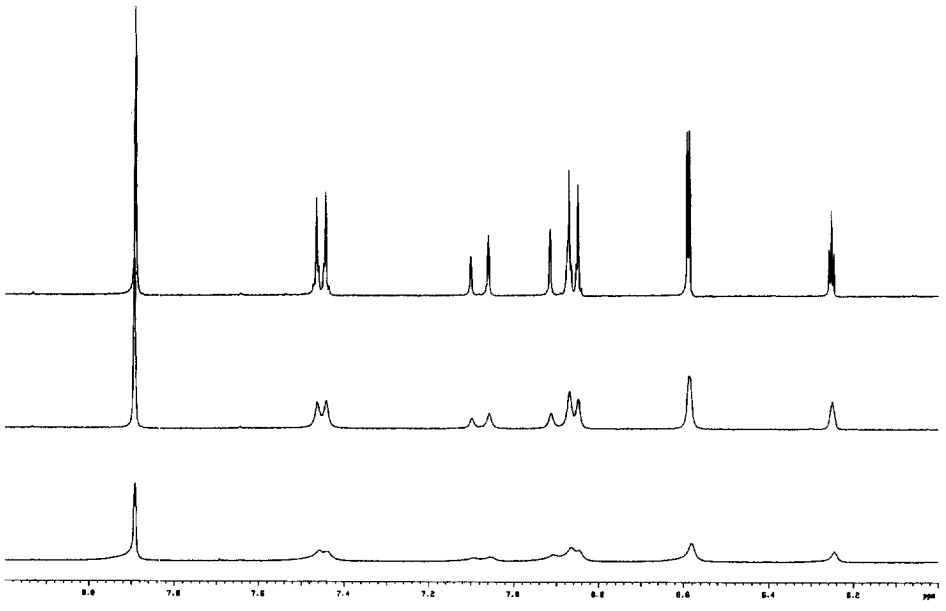
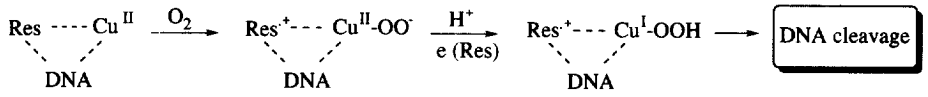


Figure 4. ^{1}H-NMR spectra of resveratrol; effect of DNA.

Top, 1 mM resveratrol in 10 mM phosphate and 90 mM NaCl, contained 10 % DMF-d_7 at pH 7.6 and 25 °C. *Middle and Bottom*, 1.0 and 2.0 base pairs of calf thymus DNA per drug molecule were added, respectively.

whether a series of polyphenol generates an oxygen radical, although it is well known that *o*- and *p*-quinone and the corresponding reduced forms mediate oxygenation of DNA bases and/or DNA strand scission by generation of oxygen radicals,[16] in a process that results in the occurrence of both antitumor activity and carcinogenicity. Recently, Belguendouz et al. have shown that resveratrol has a property to bind Cu^{2+}.[17] This may be related to the ability of resveratrol to induce DNA cleavage without oxygenative transformation of benzene nuclei. That is, as shown in Scheme 2, the binding may facilitate the generation of copper-peroxide complex with concomitant electron transfer of resveratrol to O_2. Originally, resveratrol has the potential to

Scheme 2



Res: resveratrol

to quench free hydroxyl radicals through the Fenton reaction and this may reflect upon the ability of resveratrol as an antioxidant.[18] However, the finding that resveratrol is a Cu^{2+}-dependent DNA damaging agent is of importance from both biomedical and toxicological viewpoints and may be considered in evaluation of its various biological effects, particularly with regard to its application as an antileukemic agent,[19] a cancer chemopreventive agent,[5] or an inhibitor of tyrosine kinase.[20] Further studies on the structure activity relationship of resveratrol and its synthetic analogues are in progress and the results will be reported in due course.

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6. When comparisons of DNA cleavage induced by resveratrol were made for times of 0 - 4 h, the cleavage reaction began immediately and continued with time.
7. Resveratrol (50 μM) did not induce DNA cleavage in the presence of Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} or Fe^{3+} (50 μM or 250 μM).
8. No chemical transformation of resveratrol to catechol in the presence of Cu^{2+} at neutral pH was observed by UV spectroscopy.
9. DNA strand scission by olivetol with Cu^{2+} was not observed, when 100 μM olivetol and 200 μM Cu^{2+} was incubated with pBR322 DNA in 50 mM sodium cacodylate buffer pH7.2 for 1h at 37 °C.
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14. ^{1}H-NMR data for resveratrol are as follows, (400 MHz, D_2O/DMF-d_7) δ: 6.25 (1H, t, *J*=2.0 Hz), 6.58 (2H, d, *J*=2.0Hz), 6.86 (2H, d, *J*=8.6 Hz), 6.90 (1H, d, *J*=16.7 Hz), 7.08 (1H, d, *J*=16.7 Hz), 7.45 (2H, d, *J*=8.6 Hz), 7.89 (s).
15. The addition of calf thymus DNA to resveratrol caused a decrease in fluorescence emission (excitation wavelength 260 nm), and a decrease in the degree of fluorescence was observed with increase in the DNA concentration.
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