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REDOX PROPERTIES OF PHENOLS, THEIR RELATIONSHIPS TO SINGLET OXYGEN QUENCHING AND TO THEIR INHIBITORY EFFECTS ON BENZO(a)PYRENE-INDUCED NEOPLASIA

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The inhibitory effects of synthetic phenolic compounds on benzo(a)pyrene-induced neoplasia of the mouse forestomach have been measured by Wattenberg *et al.*[6] The efficiency of this inhibition has been estimated for each phenol, using R, the ratio of the number of tumors per mouse in the protected group over the number of tumours per mouse in the control group. We have observed a linear correlation between the chemoprotection efficiency R and the logarithm of the rate of quenching of singlet oxygen, k, by this family of phenols, log k being itself correlated with the one-electron oxidation potential of the phenols. These correlations suggest a charge transfer mechanism for the inhibition of neoplasia induced by benzo(a)pyrene. The correlations described provide a theoretical basis for scaling the inhibitors of mutagenicity induced by polycyclic aromatic compounds in terms of their oxidation potentials.

KEY WORDS: Oxidation potential of phenols, chemoprotection, benzo(a)pyrene, neoplasia, singlet oxygen.

INTRODUCTION

Cellular prooxidant states, characterized by a high concentration of activated forms of oxygen, including superoxide anion, singlet excited state of oxygen, hydroxyl radical, alkoxyl radical, hydrogen peroxide, organic peroxides and epoxides, appear to play a crucial role in tumor promotion and carcinogenesis.[1] In the particular case of cancer induced by polycyclic hydrocarbons, the mechanism of carcinogenesis involves their metabolic oxidation and their covalent binding to DNA via their epoxides.[2-4] Although a large number of inhibitors of carcinogenesis induced by polycyclic aromatic hydrocarbons have been identified, their protective molecular mechanisms of action have not yet been fully elucidated.[5] The inhibitory effects of phenolic compounds (added to the diet of mice) on benzo(a)pyrene-induced neoplasia of the forestomach have been determined quantitatively by Wattenberg *et al.*[6] The efficiency of this inhibition has been estimated for each phenol, using R, the ratio of the number of tumors per mouse in the treated group over the number of tumors per mouse in the unprotected control group. This ratio is lower than unity when there is an inhibition and above unity when there is an enhancement of the number of tumors.

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Extending a previous study,[7] we have investigated for other compounds the validity of a correlation betwen the ratio R and the rate constant k of their quenching of singlet oxygen, $O_2(^1\Delta_g)$, which is itself correlated with the half-wave oxidation potential of these molecules. Our results emphasize the importance of the oxidation potential of these molecules and suggest a possible molecular mechanism explaining the change from inhibition to enhancement of neoplasia by antioxidants.

MATERIALS AND METHODS

The porphyrins, P, were either hematoporphyrin from Roussel-Uclaf purified by Vever-Bizet *et al.*[8] or tetrakis(p-sulphonatophenyl) porphyrin from Midcentury (Posen, IL., USA); 2,3,5,6-tetrafluorophenol, 2-tert-butylphenol, 4-methoxyphenol, 3,5-di-tertbutylcatechol, trans-cinnamic acid and 2-hydroxycinnamic acid were from Aldrich, phenol from Sigma and the other compounds from Riedel de Haën.

Buffered solutions were prepared via standard procedures[9] using KH_2PO_4, $Na_2B_4O_7$, Na_2HPO_4 and NaOH according to pH. Measurements of pH were made using a Radiometer glass electrode (type B) and the pD was taken as the measured pH + 0.4 as established by Salomaa *et al.*[10]

The photosensitised production of singlet molecular oxygen $O_2(^1\Delta_g)$ was carried out by energy transfer from triplet porphyrin, $^3P^*$, to molecular oxygen in its ground state $O_2(^3\Sigma_g^-)$. The porphyrin was excited with the frequency doubled line (532 nm) of a Quantel Nd/YAG laser. The unfocused 8 mm diameter laser pulses with energies between 1 and 10 mJ were incident upon solutions contained in a 10 × 10 × 45 mm spectrosil quartz cell. The porphyrin absorbance was in the range 0.4 to 0.8 at the excitation wavelength. The $O_2(^1\Delta_g)$ photoproduction was measured with a system based on that described by Rodgers and Snowden.[11] The characteristic $O_2(^1\Delta_g)$ emission with λ_{max} at 1.27 μm was detected at 90° to the laser beam by a 7 mm^2 Judson J16 germanuim photodiode. The Judson diode photocurrent was passed through a load resistance variable between 1 and 2 kΩ. The resulting voltage signal was then applied to a Judson amplifier whose output was fed to a Tektronix 7912 AD digitizer interfaced with a Hewlett-Packard 9816 microcomputer. A long-pass silicon filter ($\lambda > 1.1\,\mu$m) was inserted between the irradiation cell and the Judson diode to cut off the porphyrin fluorescence and the scattered laser light from the $O_2(^1\Delta_g)$ emission.

The experiments were performed either in CD_3OD (99.8%) manufactured by the Commisariat à l'Energie Atomique (C.E.A., Saclay, France) or D_2O from Janssen Chimica (Beerse, Belgium). The intrinsic lifetimes of $O_2(^1\Delta_g)$ reach approximately 230 μs and 60 μs in CD_3OD and buffered D_2O respectively. The duration of these lifetimes permits an accurate determination of the bimolecular rate constants of reaction with the different phenols added. The addition of the phenolic compounds reduced the $O_2(^1\Delta_g)$ lifetime but did not modify the initial $O_2(^1\Delta_g)$ emission intensity. This indicates that the singlet oxygen formation yield from the sensitizer triplet is unchanged by the addition of the phenols.

RESULTS

The decay of the $O_2(^1\Delta_g)$ phosphorescence was monitored in the absence and presence of added phenolic compounds Q, over a time period of several half-lives. The

simplified scheme of the reactions under our experimental condition is:

$$P + h\nu \rightarrow {}^1P^* \rightarrow {}^3P^* \quad (1)$$

$${}^3P^* + {}^3O_2 \rightarrow P + {}^1O_2 \quad (2)$$

$${}^1O_2 \rightarrow {}^3O_2 \quad (k_d) \quad (3)$$

$${}^1O_2 + Q \rightarrow {}^3O_2 + Q \quad (k_q) \quad (4)$$

$${}^1O_2 + Q \rightarrow \text{oxidation products} \quad (k_r) \quad (5)$$

with k_d the intrinsic first order rate constant of singlet oxygen decay in the solvent and $k = (k_r + k_q)$ the overall bimolecular rate constant for quenching of singlet oxygen by both chemical and physical quenching by Q. After the laser pulse excitation of 6 ns duration, steps 1 and 2 are complete in less then 2 μs in aerated solutions. After that time the singlet oxygen decay can be determined, its rate of decay is given by:

$$-d[{}^1O_2]/dt = \{k_d + (k_r + k_q)[Q]\}[{}^1O_2]$$

In our experimental conditions, the initial concentration of singlet oxygen, $[{}^1O_2]_0$ is $\ll [Q]$, moreover $[Q]$ is not modified as, for phenols, the physical quenching via reaction (4) is usually much greater than the chemical quenching via reaction (5).[13] By integration

$$[{}^1O_2] = [{}^1O_2]_0 \exp[Q](k_{obs}t)$$

with $k_{obs} = k_d + (k_r + k_q)[Q]$. Plotting k_{obs} as a function of $[Q]$ allows values of $k = (k_r + k_q)$ to be determined.

Table 1 reports the k values in CD_3OD obtained for each compound in the range of concentration quoted and indicates the value of R, the quantitative expression of the carcinogenesis chemoprevention estimated by Wattenberg *et al.*[6] Compound 11 of Table 1 is the only non-hydroxy compound; it does not appear to be an inhibitor ($R = 0.92$) and can be compared with its corresponding hydroxy analogue (compound 12), which is an inhibitor with $R = 0.66$.

It must be noted at this point that, using the pulse radiolysis technique, Steenken and Neta[12] have determined the one-electron oxidation potentials E of a group of

TABLE 1

Total singlet oxygen quenching rate constants, ($k_r + k_q$) measured in CD_3OD, and chemoprotection efficiency, (R) for phenols and related compounds.

Compound	Concentration M	$(k_q + k_r)$ M $^{-1}s^{-1}$	R^*
1. Phenol	$7 \times 10^{-2} - 3 \times 10^{-1}$	2.5×10^4	0.88
2. 2,3,5,6-tetrafluorophenol	$5 \times 10^{-3} - 3 \times 10^{-2}$	3.0×10^4	0.73
3. 3,4-di-hydroxycinnamic acid	$2 \times 10^{-3} - 1 \times 10^{-2}$	5.4×10^5	0.62
4. 4-OH-3-methoxycinnamic acid	$1 \times 10^{-3} - 1 \times 10^{-2}$	3.5×10^5	0.60
5. 2-tert-butylphenol	$2 \times 10^{-2} - 6 \times 10^{-2}$	2.5×10^5	0.60
6. 2,6-di-tert-butylphenol	$3 \times 10^{-3} - 2 \times 10^{-2}$	2.6×10^5	0.52
7. 2-tert-butyl-4-hydroxyanisole	$3 \times 10^{-4} - 1 \times 10^{-3}$	2.2×10^7	0.30
8. p-methoxyphenol	$5 \times 10^{-4} - 2 \times 10^{-3}$	5.8×10^6	0.22
9. 2,5-di-tert-butylhydroquinone	$5 \times 10^{-5} - 2 \times 10^{-4}$	1.9×10^8	1.28
10. 3,5-di-tert-butylcatechol	$6 \times 10^{-4} - 2 \times 10^{-3}$	2.5×10^7	0.46
11. trans-cinnamic acid	$5 \times 10^{-2} - 5 \times 10^{-1}$	3.4×10^3	0.92
12. 2-hydroxycinnamic acid	$2 \times 10^{-2} - 2 \times 10^{-1}$	2.1×10^4	0.66

*Chart 1 in ref.[6]

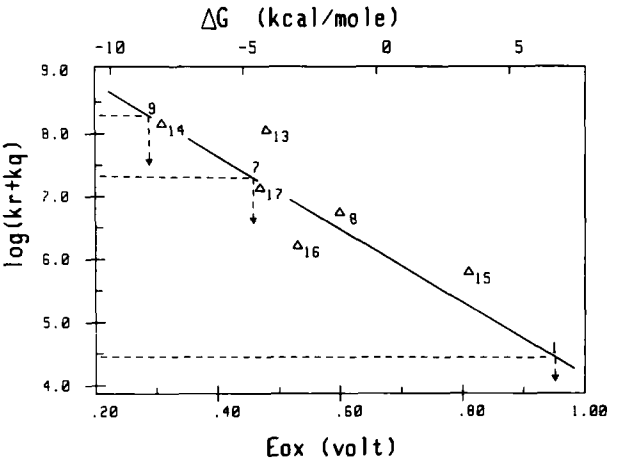

FIGURE 1 Plot of the logarithm of the total $^1O_2$ quenching rate constant in CD_3OD, $\log(k_r + k_q)$ of various phenols (Table 2) versus the one-electron oxidation potential, $E(D^+/D)$ vs NHE in water at 22°C pH = 7(lower scale), and versus free energy of electron transfer ΔG (upper scale). Δ, values for which both the $E(D^+/D)$ and the $\log(k_r + k_q)$ are known and for which the linear least squares fit is calculated, linear correlation coefficient $r = 0.92$. Interpolated $E(D^+/D)$ values using the measured value of $\log(k_r + k_q)$ for compounds 9, 7 and 1 are indicated by dashed arrows.

aromatic compounds which includes phenol and p-methoxyphenol, compounds 1 and 8 of Table 1. We have measured for these compounds the total $^1O_2$ quenching rate constant k in CD_3OD and observed a linear correlation between the log of k and the one-electron redox potential E vs. NHE of these molecules (Figure 1 and Table 2). Thomas and Foote[13] and Saito *et al.*[14] have also observed a similar linear correlation between the log of k and the halfpeak potential (vs SCE) $E_{p/2}$ for a number of other aromatic compounds: para substituted 2,6-di-t-butyl phenols, methoxybenzenes and durohydroquinone monoethyl ether. These correlations indicate that the singlet oxygen quenching mechanism can be interpreted[13,14] to be an electron transfer reac-

TABLE 2

Total singlet oxygen quenching rate constants $(k_r + k_q)$ in CD_3OD, concentration range used for the rate constant determination, and one-electron oxidation potentials $E(D^+/D)$ for phenols and related compounds.

Compound	Concentration M	$(k_q + k_r)$ M^{-1} s^{-1}	$E(D^+/D)$	
1. Phenol	$7 \times 10^{-2} - 3 \times 10^{-1}$	2.5×10^4	>0.80[b]	0.95[c]
7. 2-tert-butyl-4-hydroxyanisole	$3 \times 10^{-4} - 1 \times 10^{-3}$	2.2×10^7		0.46[c]
8. p-methoxyphenol	$5 \times 10^{-4} - 2 \times 10^{-3}$	5.8×10^6	0.60[b]	
9. 2,5-di-tert-butylhydroquinone	$5 \times 10^{-5} - 2 \times 10^{-4}$	1.9×10^8		0.29[c]
13. HTCC[a]	$5 \times 10^{-5} - 5 \times 10^{-4}$	1.2×10^8	0.48[b]	
14. ascorbic acid	$1 \times 10^{-5} - 1 \times 10^{-4}$	1.5×10^8	0.30[b]	
15. resorcinol	$6 \times 10^{-4} - 7 \times 10^{-3}$	6.5×10^5	0.81[b]	
16. catechol	$5 \times 10^{-4} - 5 \times 10^{-3}$	1.7×10^6	0.53[b]	
17. hydroquinone	$5 \times 10^{-5} - 7 \times 10^{-4}$	1.4×10^7	0.46[b]	

a. 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid, a water soluble analogue of vitamin E.

b. volts vs. NHE, (Normal Hydrogen Electrode), 22° C, calculated values for pH7 from one-electron redox potential values measured at pH 13.5 by pulse radiolysis (Table V, ref. 12).

c. volts vs NHE, (Normal Hydrogen Electrode) at pH7 and 22°C, values interpolated from Figure 1.

tion on the basis of the following scheme, in which the aromatic compound is an electron donor D:

$$\overset{\Delta G}{}$$

$$^1O_2 + D \leftrightarrow (^1O_2 ... D) \leftrightarrow (O_2^- O_2^- ... D^{+\cdot}) \leftrightarrow {}^3O_2 + D \text{ or reaction products}$$

The free energy change ΔG associated with the electron transfer process is represented by the Rehm and Weller equation:[15]

$$\Delta G = 23.06\,[E\,(D^{+\cdot}/D) - E\,(A/A^{-\cdot})] - e^2/\varepsilon a - E(A^*) \quad (6)$$

where $E\,(D^{+\cdot}/D)$ is the oxidation potential of the electron donor, $E\,(A/A^{-\cdot})$ is the reduction potential of the electron acceptor, $e^2/\varepsilon a$ is the Coulombic attraction term where ε and a are the dielectric constant of the solvent and the radical ion distance, respectively, and $E(A^*)$ is the energy of the excited acceptor molecule. For electron transfer reactions between the members of a homogeneous series of donors D (in our case phenolic compounds) and the same acceptor A (in our case $^1O_2$) a linear relationship between the logarithm of the overall rate constant k (or which is equivalent, the free energy of activation) and ΔG is observed. As all the terms dealing with A are constant in relation (6), log k is linearly dependent on $E(D^{+\cdot}/D)$ as shown by Figure 1. This behaviour is consistent with the Marcus theory[16,17] which predicts that log k will demonstrate a nearly linear dependence on free energy change in the region of low thermodynamic driving force.

The logarithm of k in CD_3OD, which we have determined for a series of phenolic compounds studied by Wattenberg *et al.*,[6] presents a linear correlation with the parameter R, which expresses their efficiency in cancer chemoprevention (Figure 2; correlation coefficient $r = 0.89$). This correlation is observed for values of R ranging from 0.22 to 1 and values of k ranging from 10^3 to 10^7 $M^{-1}s^{-1}$. A correlation between log k of Wattenberg *et al.*'s compounds (compounds 1–12) and their $E\,(D^{+\cdot}/D)$ can be predicted by the correlation illustrated by Figure 1 and previously noted for other phenols and other aromatic compounds.[13,14] It is interesting to note that compound

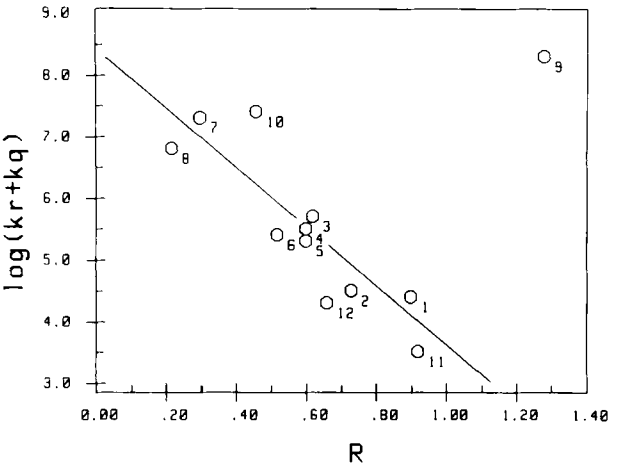

FIGURE 2 Plot of the logarithm of the total $^1O_2$ quenching rate constant in CD_3OD, $\log(k_r + k_q)$ of various phenols (Table 1) versus their chemoprotection efficiency, R (Wattenberg *et.al.*, Chart 1 in ref. [6]), linear correlation coefficient $r = 0.89$, not including data of molecule 9.

9 does not obey the correlation of Figure 1. This particular behaviour could be due to the fact that compound 9 is a hydroquinone and not a phenol. Compound 9 is the best singlet oxygen quencher, with k reaching $1.9 \times 10^8\ M^{-1}s^{-1}$, however in contrast with the other compounds it does not inhibit neoplasia but enhances the number of tumors by approximately one fourth ($R = 1.28$). The oxidation potential of this toxic hydroquinone (compound 9) is 0.29 V while that of the best phenolic inhibitors (compounds 7 and 8) are respectively 0.46 V and 0.60 V vs. NHE at room temperature, at pH 7 (Table 2). It can be assumed from these results that between the oxidation potential of the best inhibitors and the oxidation potential of the toxic compound lies the boundary between protection and adverse effects of hydroxy aromatic compounds. Such an assumption is plausible as it is understandable that xenobiotic molecules with a low oxidation potential will be able to produce electron transfer to biological redox systems upon entering an organism and thus might create a pathway for the generation of free radicals damaging the biological system irreversibly.

In perdeuterated water at the physiological pH, the phenols (ArOD) studied are all in their protonated form as their pK is around 10, thus the inhibitors studied either in the aqueous or in the nonaqueous compartments of the cells will be in the protonated form. As expected, their more easily oxidisable phenolate form (ArO^-) reacts faster with singlet oxygen as shown by Figure 3. The dashed line of Figure 3 represents a calculated quenching rate constant k assuming separate quenching rates for both the phenol k_{ArOD} and the phenolate k_{ArO^-} which gives:

$$k_{obs} = k_d + k_{ArOD}\,[ArOD] + k_{ArO^-}\,[ArO^-]$$

As the equilibrium constant $K_a = [ArO^-][D^+]\,/\,[ArOD]$ and as $[ArOD] + [ArO^-] = C_0$, we get

$$(k_{obs} - k_d)\,/\,C_0 = k_{ArOD} + (k_{ArO^-} - k_{ArOD})\,(1 + 10^{pK_a - pD})^{-1} \qquad (7)$$

The molecular mechanism of the chemoprotection by phenols *in vivo* is still incom-

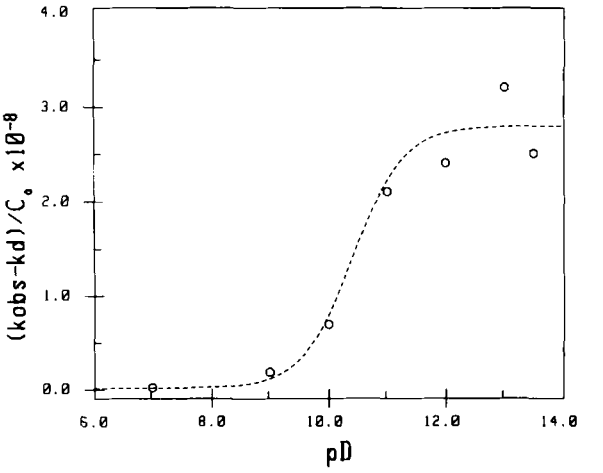

FIGURE 3 Plot of $(k_{obs} - k_d)\,/\,C_0$ in $M^{-1}s^{-1}$ for phenol in buffered D_2O versus pD. The calculated curve (---), equation (7), was obtained using $k_{ArOD} = 1.3 \times 10^6\ M^{-1}s^{-1}$ and $k_{ArO^-} = 2.8 \times 10^8\ M^{-1}s^{-1}$, and pK_a (phenol) = 10.4 to give the best fit to the experimental data.

pletely understood. However, this mechanism might be related to the ability of phenolic antioxidants and other chemoprotective agents to induce the activities of enzymes that inactivate the reactive electrophilic forms of carcinogens, as observed by Sparnins and Wattenberg[18]and by Talalay *et al.*[19] In the special case of light irradiation where benzo(a)pyrene B(a)P shows enhanced carcinogenicity probably involving singlet oxygen,[20,21] phenolic compounds might inhibit the production of tumors by quenching $^1O_2$. In the case of ellagic acid, a polyphenol, it has been suggested from studies *in vitro* that the epoxide ring of the ultimate carcinogenic metabolite of B(a)P might be opened via hydrolysis by interaction with an OH of the ellagic acid.[20] However, as the pK_a values for both 4-methoxyphenol (an efficient inhibitor) and for phenol (a poor inhibitor) are approximately[23] $pK_a = 10$, this similarity would rather favor another mechanism.

The results described above (Figure 1 and 2) suggest that the parameter which rules either the chemoprotection efficiency or the adverse effects of the phenolic compounds studied is their oxidation potential. This property should allow them to modify the course of the metabolic activation of benzo(a)pyrene. The fact that metabolisation of polycyclic aromatic hydrocarbons producing their ultimate carcinogenic forms occurs via activating steps involving one or more electrons[24] supports our hypothesis, This hypothesis is also in agreement with the idea that the major parameter unifying the broad variety of chemical carcinogens is the electrophilicity of their critical intermediates.[24]

In conclusion, the correlations observed emphasize the interest in scaling the following compounds with respect to their oxidation potentials: (i) inhibitors of chemical carcinogenesis, usually antioxidants, which increase the reducing cell environment, (ii) chemical compounds inducing or enhancing carcinogenesis, usually prooxidants, which increase the oxidizing cell environment and (iii) cellular biomolecules involved in the damage, which are usually nucleophilic molecules. This scaling might provide a sorting device for the toxicity of xenobiotics as already suggested by Eberson.[17] It should help to differentiate between electron transfer and other processes involved in chemically-induced neoplasia and in chemoprotection.

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