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HYDROXYL RADICAL DAMAGE TO DNA SUGAR AND MODEL MEMBRANES INDUCED BY ANTHRALIN (DITHRANOL)

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Abstract—The antipsoriatic anthrones anthralin and butantrone caused degradation of the DNA sugar deoxyribose in the presence of ferric salt. The degradation was substantially inhibited by iron-binding hydroxyl radical scavengers, iron chelators, superoxide dismutase (SOD) and catalase, suggesting a mechanism in which antipsoriatic anthrones generate hydroxyl radicals via the Fenton reaction or an iron-catalysed Haber–Weiss reaction. Butantrone was markedly less efficient at generating hydroxyl radicals than anthralin. Using bovine brain phospholipid liposomes as model membranes to study the effects of antipsoriatic anthrones on lipid peroxidation, the peroxidation of liposomal membranes in the presence of ferric salt was maximally enhanced by anthralin and butantrone at 12.5 and 5 μM, respectively. Higher concentrations of the drugs resulted in less peroxidation. Chain-breaking antioxidants and iron chelators strongly decreased anthralin-enhanced lipid peroxidation, suggesting the involvement of hydroxyl, peroxyl or alkoxyl radicals. In contrast to their stimulatory effects on liposomal membrane peroxidation, both anthralin and butantrone diminished Fe^{3+}/ascorbate-induced lipid peroxidation in liposomes. Butantrone was more effective as an inhibitor of lipid peroxidation than was anthralin. The antioxidant properties of antipsoriatic anthrones were determined in terms of their reactivities with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Antioxidant activity of antipsoriatic anthrones requires the presence of free hydroxyl groups at C-1 and C-8 and at least one hydrogen atom at C-10 of the anthrone nucleus. The role of active oxygen species produced by antipsoriatic anthrones and the biological effects on cellular targets are discussed with respect to the mode of action and manifestation of side effects of these drugs.

Anthralin [dithranol, 1,8-dihydroxy-9(10*H*)-anthracenone, Fig. 1] is among the most widely used drugs in the treatment of psoriasis. However, patient compliance is limited by its undesirable irritant effects and staining of the skin [1]. Because of these drawbacks there has been much research into discovering derivatives that retain clinical efficacy with reduced side effects [2]. Initial assessment showed reduced irritation and staining for butantrone (Fig. 1), a C-10-butyryl derivative of anthralin, but there was loss of clinical efficacy [2].

Psoriasis is a scaling skin disease which is characterized by increased cell proliferation of the epidermis and dermal inflammation [3]. Anthralin produces resolution of both features [2]. The biochemical basis for the mode of action of anthralin and induction of side effects is uncertain but there is growing evidence that it is related to its redox activity leading to the production of anthralin free radical [1,8-dihydroxy-9(10*H*)-anthracenon-10-yl radical] and active oxygen species [1]. These latter species, which include singlet oxygen [4, 5], superoxide anion [6, 7], hydroxyl radical [8] and hydrogen peroxide [6], have all been demonstrated to be formed by anthralin under aerobic conditions. Active oxygen species are involved in a variety of oxidative effects [9–12] affecting cellular targets that have been implicated in the mode of action of anthralin: interaction with DNA [13, 14], inhibition of various enzyme systems associated with cell proliferation and inflammation [15–18], and alteration of mitochondrial functions and destruction of membrane lipids [19, 20].

Since no single mechanism is operative, it may be concluded that all these effects represent a common mechanism caused by activation of anthralin and generation of reactive species. In view of the chemical instability of anthralin it is unlikely that the molecule itself is responsible for the biological properties, and the known oxidation products bianthrone and danthron (Fig. 1) are not effective in the treatment of psoriasis. However, little evidence has been presented that the biological effects of anthralin are related to the reactive intermediates formed by its autoxidation. A better understanding of the mechanism by which anthralin exerts its action on DNA and lipid membranes is necessary for future development of new antipsoriatic anthrones.

The initial species formed by electron transfer from anthralin to oxygen is superoxide anion [6]. The production of hydroxyl radical from superoxide anion is dependent on the presence of iron capable of catalysing the reaction [12]. The skin exhibits a certain degree of iron turnover and is a significant site of iron excretion [21]. This may be increased in certain pathologic conditions such as psoriasis [21].

In the present study anthralin and some of its derivatives have been examined for degradation of deoxyribose in the presence of ferric salt under

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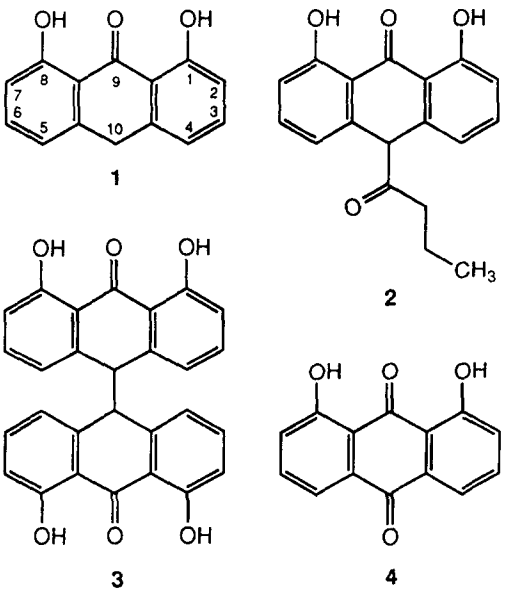

Fig. 1. Structures of anthralin (1), butantrone (2), bianthrone (3) and danthron (4).

aerobic conditions. The simple DNA sugar deoxyribose provides a sensitive and inexpensive detector molecule for establishing free radical damage in cell-free systems [22, 23] and is highly sensitive for detection of hydroxyl radicals [24].

The effects of anthralin on cellular lipids have been investigated using bovine brain phospholipid liposomes, which provide an ideal model system for lipid peroxidation studies [25].

We also determined the antioxidant properties of antipsoriatic anthrones by use of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH†) [26].

MATERIALS AND METHODS

Reagents. Anthralin, bianthrone (1,8,1′,8′-tetrahydroxy-bis-10,10′-9′(10*H*)-anthracenone) and butantrone (10-butyryl-1,8-dihydroxy-9(10*H*)-anthracenone) were prepared according to known methods [27, 28] and purified by column chromatography (SiO_2/CH_2Cl_2). 2,6-Di-tert-butyl-4-methylphenol and propyl gallate were from Janssen (Brüggen, Germany), danthron (chrysazin, 1,8-dihydroxy-9,10-anthracenedione), 2,2-diphenyl-1-picrylhydrazyl, and 2,2-diphenyl-1-picrylhydrazine were from Aldrich (Steinheim, Germany), 2,2′-azo-bis-(2-amidinopropane) hydrochloride was from Polysciences (Eppelheim, Germany), and doxycycline was a gift from Pfizer (Karlsruhe, Germany). All other reagents were of the highest grades available from the Sigma Chemical Co. (Deisenhofen, Germany) or from Merck (Darmstadt, Germany). Bovine brain was obtained from the local slaughterhouse. HPLC (Kontron 420, Kontron 735 LC UV detector) was performed on a 250 × 4 mm column packed with Nucleosil C_{18} (7 μm particles; Bischoff, Leonberg, Germany). UV spectroscopy was run on a Kontron 810 spectrophotometer. Data were recorded on a MacLab data acquisition system and analysis was performed with the application Peaks on an Apple Macintosh Quadra 700 computer.

Degradation of 2-deoxy-D-ribose. The following reagents were added to glass tubes in the order and at the final concentrations stated: 0.3 mL KH_2PO_4-KOH buffer, pH 7.4 (30 mM), 0.2 mL H_2O bidistilled, 0.2 mL 2-deoxy-D-ribose (2 mM), 0.2 mL $FeCl_3 \cdot 6\,H_2O$ (0.1 mM), 0.1 mL anthralin (75 μM or variable concentrations) in acetonitrile, and where indicated, 0.1 mL of scavengers, enzymes, stimulators or iron chelators (at the concentrations stated). Iron chelators were premixed with $FeCl_3$ before addition. Stock solutions of the compounds were freshly made up before use. Appropriate blanks and controls with the vehicles (acetone and acetonitrile) were conducted. The final reaction volumes were standardized to 1.0 mL (when necessary, the volume of H_2O added was reduced). The reaction mixtures were incubated for 2 hr (unless time dependence was checked) at 37° in a shaking water bath. One mililiter of 2.8% (w/v) trichloroacetic acid and 1.0 mL of 1% (w/v in 0.05 N NaOH) 2-thiobarbituric acid (TBA) were added and the samples were heated at 100° for 15 min and then cooled in an ice bath (5 min). The absorbance at 532 nm was measured against blanks. If the samples were turbid, 2.0 mL of the reaction mixtures were treated with 0.05 mL of 36% (w/v) HCl and 2.0 mL of 1-butanol and vigorously shaken in a vortex mixer (Heidolph) for 15 sec. The organic layer was separated by centrifugation at 3000 rpm (15 min) and the absorbance at 532 nm was measured against 1-butanol. Calibration was performed using a malondialdehyde standard prepared by hydrolysis of 1,1,3,3-tetraethoxypropane [29]. TBA reactive material is expressed in terms of μmoles malondialdehyde (MDA) per mmole deoxyribose (mean values ± SD, N ≥ 3). Interference of test compounds with TBA or MDA was excluded by appropriate control experiments.

Isolation of phospholipids from bovine brain and preparation of liposomes. Phospholipids were prepared essentially as described by Gutteridge [25]. Bovine brain, cooled on ice, was freed from blood vessels and washed repeatedly with 0.15 M NaCl pH 7.4. It was cut into pieces and then macerated with an ultra-turrax, passed through a close-meshed sieve, and extracted four times with four times the volume of acetone. The extraction mixture was filtered by suction to remove the acetone, the residue was dried under vacuum, and then repeatedly extracted with petroleum ether (40–60) using twice the volume of the original brain homogenate. The combined extracts were filtered, dried at 45°, and dissolved in ether (one fifth of the original brain volume). The mixture was treated

† Abbreviations: AAPH, 2,2′-azo-bis-(2-amidinopropane) hydrochloride; BHT, 2,6-di-tert-butyl-4-methylphenol; DETAPAC, diethylenetriaminepentaacetic acid; DMSO, dimethylsulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MDA, malondialdehyde; NDGA, nordihydroguaiaretic acid; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid.

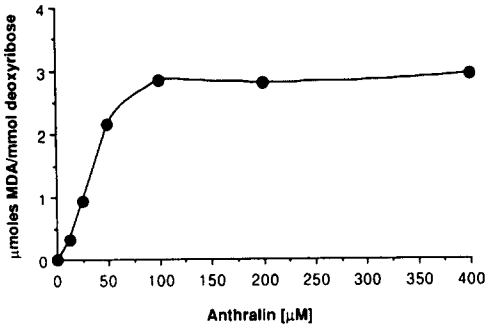

Fig. 2. Concentration-dependent effects of anthralin on deoxyribose (2 mM) degradation in the presence of $FeCl_3$ (0.1 mM) at 37° in KH_2PO_4-KOH buffer/acetonitrile (9 + 1, v/v). Values are expressed as µmoles MDA per mmole deoxyribose per 2 hr (N = 6, SD < 15%), measured by the TBA method.

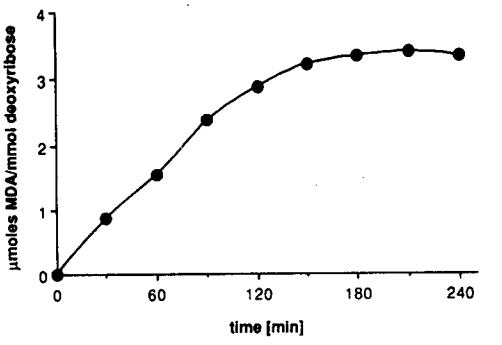

Fig. 3. Deoxyribose (2 mM) degradation induced by anthralin (75 µM) in the presence of $FeCl_3$ (0.1 mM) during a time-course study at 37° in KH_2PO_4-KOH buffer/acetonitrile (9 + 1, v/v). Values are expressed as µmoles MDA formed per mmole deoxyribose (N = 3, SD < 10%), measured by the TBA method.

with five times the volume of acetone, and the resulting precipitate was collected by suction-filtration, dried and stored under N_2 at −20° in the dark.

Bovine brain phospholipids were weighed into glass tubes and shaken in a vortex mixer (Heidolph) in the presence of five small glass beads (diameter 4 mm) for 1 min. The phospholipids were suspended in 0.15 M NaCl, pH 7.4, to a final concentration of 5 mg/mL. The mixture was purged with N_2 for 1 min and vigorously dispersed in a vortex mixer for 5 min. The liposomes were allowed to swell for 1 hr at 4°, and vesicles with a mean size of 1–10 µm were obtained [30].

Assay of lipid peroxidation. The following reagents were added to glass tubes in the order and at the final concentrations stated: 0.3 mL KH_2PO_4-KOH buffer, pH 7.4 (30 mM), 0.29 mL H_2O bidistilled, 0.2 mL liposomes (1 mg/mL), 0.2 mL $FeCl_3 \cdot 6\,H_2O$ (0.1 mM), 0.01 mL anthralin (at the concentrations stated), and where indicated, 0.01 mL of scavengers, 0.1 mL of enzymes or stimulators (at the concentrations stated). Appropriate blanks and controls with the vehicle (acetone) were conducted. The final reaction volumes were standardized to 1.0 mL (when necessary, the volume of H_2O added was reduced). The reaction mixtures were incubated at 37° in a shaking water bath (incubation time as indicated). Ten microliters of 20% (w/v) 2,6-di-tert-butyl-4-methylphenol (BHT), 0.5 mL 25% (w/v) HCl and 0.5 mL of 1% (w/v in 0.05 N NaOH) TBA were added and the samples were heated at 100° for 15 min and then cooled in an ice bath (5 min). 1-Butanol (2.0 mL) was added and the mixtures were vigorously shaken in a vortex mixer (Heidolph) for 15 sec. The organic layer was separated by centrifugation at 3000 rpm (15 min) and the absorbance at 532 nm was measured against 1-butanol. TBA reactive material is expressed in terms of nmoles MDA per mg phospholipid (mean values ± SD, N ≥ 3). IC_{50} values for inhibition of lipid peroxidation were derived by interpolation of a log concentration vs inhibition plot using six concentrations of the compound, spanning the 50% inhibition point. All experiments were run in triplicate.

Determination of the reducing activities against DPPH. One milliliter of test compound solution (0.1 mM) was added to 1 mL of DPPH solution (0.1 mM), each in phosphate-buffered saline/acetone, pH 7.4 (1 + 1, v/v), at room temperature, and the reduction of DPPH was followed spectrophotometrically at 516 nm. A molar absorption coefficient of 12,457 $M^{-1}\,cm^{-1}$ was determined for DPPH. Plots of the reciprocal of DPPH concentrations against time gave straight lines, and the second-order rate constants were obtained from the slopes (mean values ± SD, N = 3–6). The isocratic elution conditions for HPLC analysis were methanol/water/acetic acid (77 + 23 + 0.1, % of volume), adjusted to pH 5.5 with concentrated NH_3, monitored at 256 nm. Retention times of bianthrone and $DPPH_2$ were identical with those of authentic samples.

RESULTS

Deoxyribose degradation

It should be noted that antipsoriatic anthrones are poorly soluble in aqueous solution. It is often stated that anthralin possesses amphiphilic characteristics, with one part of the molecule being hydrophilic due to the presence of the oxygen functions [1, 2]. However, the strong intramolecular hydrogen bonding between both hydroxyl functions at C-1 and C-8 and the C-9 keto group [31] makes the molecule even more lipophilic than the unsubstituted 9-anthrone. Log P values (partition coefficients) as a measure of lipophilicity are 4.23 for anthralin and 3.46 for 9-anthrone, respectively, as determined by a reversed-phase HPLC method [32].

Dissolving the compounds in alkaline solutions and then readjusting the pH to 7.4 was not practicable because anthrones are oxidized to the corresponding anthraquinones under alkaline conditions. Addition of the compounds to the reaction tubes in an organic

Table 1. Effects of different stimulators on deoxyribose degradation

Stimulator	μmol MDA/mmol deoxyribose
Control (Fe^{3+})	0
Fe^{3+}/H_2O_2 (1 mM)	0.93 ± 0.03
Fe^{3+}/H_2O_2 (1 mM)/ascorbic acid (0.1 mM)	3.90 ± 0.06
Anthralin (75 μM)	2.89 ± 0.14
Butantrone (75 μM)	0.24 ± 0.03
Doxycycline (75 μM)	0.66 ± 0.13
Doxycycline (75 μM, without acetonitrile)	3.01 ± 0.30*

Incubation was performed for 2 hr at 37° in KH_2PO_4-KOH buffer/acetonitrile (9 + 1, v/v). The incubation mixtures contained deoxyribose (2 mM), $FeCl_3$ (0.1 mM) and indicated concentrations of stimulators.

Indicated values are μmol MDA/mmol deoxyribose (mean values ± SD, $N \geq 3$), measured by the TBA method.

* Indicates significant difference with respect to the corresponding sample containing acetonitrile ($P < 0.001$).

solvent followed by evaporation of the organic solvent under a N_2 stream did not lead to reproducible results. Therefore, an organic solvent had to be employed as vehicle. However, many organic solvents by themselves caused significant inhibition of deoxyribose degradation at concentrations of 10% and above. Because deoxyribose degradation was least affected by acetonitrile all experiments were run under the same conditions, with addition of equal amounts of acetonitrile to obtain comparable values. For comparison, the known hydroxyl radical generator doxycycline [33] and the system $FeCl_3/H_2O_2$/ascorbic acid [34] were tested under identical conditions.

Figure 2 shows that anthralin led to deoxyribose degradation in the presence of ferric salt at pH 7.4. The effect increased with concentration up to a maximum. Solubility limited assay concentration of anthralin to 100 μM. With an anthralin concentration of 75 μM degradation of deoxyribose increased linearly within 2 hr and then gradually stagnated, with no further degradation after 3 hr of incubation (Fig. 3). Table 1 demonstrates the effects of different stimulators on deoxyribose degradation. The 10-substituted derivative of anthralin, butantrone, was markedly less active than anthralin, the same holds true for the antibiotic doxycycline. Deoxyribose degradation induced by doxycycline was significantly increased when incubation was performed without acetonitrile, demonstrating the inhibitory action of this solvent. Ferric iron alone (control) was without effect. The standard system $FeCl_3/H_2O_2$/ascorbic acid for the generation of hydroxyl radicals was the most effective stimulator of deoxyribose degradation under our test conditions (Table 1).

Table 2 shows the influence of hydroxyl radical scavengers, ferric iron chelators and antioxidant enzymes on deoxyribose degradation stimulated by anthralin in the presence of ferric salt. Urea which scavenges hydroxyl radicals only weakly and non-iron-binding hydroxyl scavengers such as ethanol, dimethylsulfoxide (DMSO) and sodium benzoate did not effect anthralin-induced deoxyribose degradation. On the other hand, iron-binding scavengers such as sodium salicylate, mannitol and glucose as well as the ferric iron chelators EDTA, diethylenetriaminepentaacetic acid (DETAPAC) and desferrioxamine significantly inhibited deoxyribose degradation. Propyl gallate and rutin even stimulated deoxyribose degradation. Catalase was most effective in protecting deoxyribose from being degraded by anthralin whereas superoxide dismutase (SOD) was less effective. The inhibitory activity of catalase was substantially decreased by heat denaturation whereas that of SOD was slightly stimulated.

Enhancement of lipid peroxidation in model membranes

The effect of anthralin on the extent of lipid peroxidation in bovine brain phospholipid liposomes is shown in Fig. 4. Peroxidation slightly, but significantly, increased with anthralin concentration in the presence of ferric salt and was maximally stimulated by anthralin at 12.5 μM, but then became smaller and finally reached its initial value. Concentration-dependent stimulation of lipid peroxidation by butantrone was similar to that of anthralin, but the maximum of MDA release was observed at 5 μM, although the extent of lipid peroxidation was smaller (data not shown). Figure 5 presents the effect of varying the time of incubation on lipid peroxidation in the absence and presence of anthralin (12.5 μM). During a time course of 3.5 hr peroxidation was roughly linear.

Addition of chain-breaking antioxidants, i.e. substances that are able to scavenge intermediate hydroxyl, peroxyl or alkoxyl radicals [12], such as BHT, vitamin E and propyl gallate strongly decreased anthralin-enhanced lipid peroxidation (Fig. 6). The so-called preventative antioxidants [12] sodium salicylate and mannitol, which prevent first-chain initiation by scavenging hydroxyl radicals, were ineffective (Fig. 6). The antioxidant enzyme SOD only slightly inhibited peroxidation whereas catalase slightly stimulated (Fig. 6). Moreover, iron-chelating agents such as desferrioxamine and EDTA were highly effective in preventing lipid peroxidation (Fig. 6), suggesting an important role for ferric ions.

Table 2. Effects of hydroxyl radical scavengers, ferric iron chelators and antioxidant enzymes on deoxyribose degradation by anthralin and $FeCl_3$

Scavenger	μmol MDA/mmol deoxyribose
Control (75 μM anthralin)	2.89 ± 0.14
Ethanol (5 mM)	2.74 ± 0.25
DMSO (5 mM)	2.84 ± 0.25
Urea (5 mM)	2.80 ± 0.28
Sodium benzoate (5 mM)	2.66 ± 0.18
Sodium salicylate (5 mM)	0.85 ± 0.24*
Mannitol (5 mM)	0.18 ± 0.04*
Glucose (5 mM)	0.63 ± 0.02*
Propyl gallate (50 μM)	3.48 ± 0.89
Rutin (5 μM)	3.65 ± 0.11
EDTA (0.2 mM)	0.40 ± 0.02*
DETAPAC (0.2 mM)	0.23 ± 0.03*
Desferrioxamine (0.2 mM)	0.13 ± 0.02*
Catalase (300 U/mL)	0.07 ± 0.03*
Catalase (heat denatured)	2.07 ± 0.21*
SOD (175 U/mL)	1.77 ± 0.15*
SOD (heat denatured)	1.22 ± 0.06*

Incubation was performed for 2 hr at 37° in KH_2PO_4-KOH buffer/acetonitrile (9 + 1, v/v). The incubation mixtures contained deoxyribose (2 mM), anthralin (75 μM), $FeCl_3$ (0.1 mM) and indicated concentrations of scavengers.

Indicated values are μmol MDA/mmol deoxyribose (mean values ± SD, $N \geq 3$), measured by the TBA method.

* Scavenger/chelator/enzyme treatments all inhibited deoxyribose degradation significantly ($P < 0.01$).

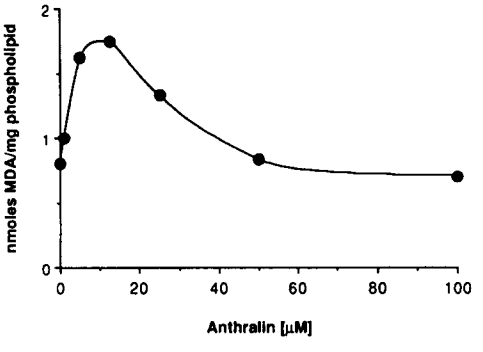

Fig. 4. Concentration-dependent effects of anthralin on lipid peroxidation in bovine brain phospholipid liposomes (1 mg/mL) in the presence of $FeCl_3$ (0.1 mM) at 37° in KH_2PO_4-KOH buffer, pH 7.4. Values are expressed as nmoles MDA formed per mg phospholipid per 60 min (N = 6, SD ≤ 10%), measured by the TBA method. Values are significantly different from controls which were performed in the absence and presence of $FeCl_3$ (0.1 mM), $P < 0.001$.

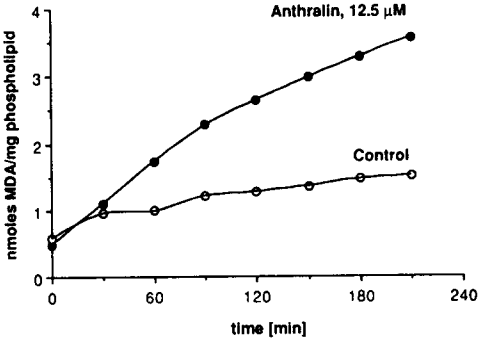

Fig. 5. Time course of lipid peroxidation in bovine brain phospholipid liposomes (1 mg/mL) stimulated in the presence (●) and absence (○) of anthralin. Incubation was performed in the presence of $FeCl_3$ (0.1 mM) at 37° in KH_2PO_4-KOH buffer, pH 7.4. Values are expressed as nmoles MDA formed per mg phospholipid (N = 6, SD ≤ 10%), measured by the TBA method.

Inhibition of Fe^{3+}/ascorbate-induced lipid peroxidation in model membranes by antipsoriatic anthrones

Although ascorbate is an antioxidant it has also been reported to possess pro-oxidant properties at low concentrations [35]. This effect results from the reduction of ferric iron to ferrous iron which may then react with hydrogen peroxide (Fenton reaction, see Discussion Eqn 5). Incubation of bovine brain phospholipid liposomes with equimolar amounts of Fe^{3+}/ascorbate (0.1 mM) at pH 7.4 for 1 hr caused extensive peroxidation, as detected by the release of MDA. This system increased lipid peroxidation more than 7-fold as compared to the azo initiator 2,2'-azo-bis-(2-amidinopropane)-hydrochloride (AAPH). Therefore, we used Fe^{3+}/ascorbate to examine the effects of antipsoriatic anthrones on lipid peroxidation. Interestingly, both anthralin and butantrone diminished lipid

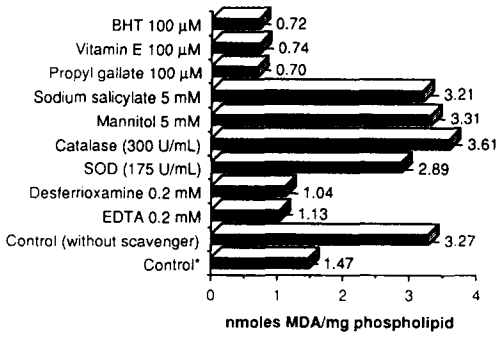

Fig. 6. Effects of antioxidants and iron chelators on lipid peroxidation in bovine brain phospholipid liposomes (1 mg/mL) stimulated by anthralin (12.5 μM) in the presence of $FeCl_3$ (0.1 mM). Incubation was performed for 3 hr at 37° in KH_2PO_4-KOH buffer, pH 7.4. Values are expressed as nmoles MDA formed per mg phospholipid (N = 3, SD ≤ 10%), measured by the TBA method. *Control: 0.1 mM $FeCl_3$ (without anthralin).

Table 3. Inhibition of lipid peroxidation by antipsoriatic anthrones

	IC_{50} (μM)
Anthralin*	12
Anthralin†	33
Butantrone*	4
NDGA*	1

Lipid peroxidation in bovine brain phospholipid liposomes (1 mg/mL) was stimulated by *$FeCl_3$/ascorbate (0.1 mM each) and resulted in the formation of 25.2 ± 1.1 nmol MDA/mg phospholipid; †AAPH (10 mM) as stimulator resulted in the formation of 3.52 ± 0.20 nmol MDA/mg phospholipid.

Incubation was performed for 1 hr at 37° in KH_2PO_4-KOH buffer, pH 7.4.

IC_{50} is the concentration causing 50% inhibition of peroxidation based on interpolation of a log concentration vs inhibition plot (six different concentrations of test compound, spanning the 50% inhibition point, N = 3).

peroxidation with IC_{50} values of 12 and 4 μM, respectively (Table 3). For comparison, the antioxidant nordihydroguaiaretic acid (NDGA) gave an IC_{50} of 1 μM under identical conditions. When AAPH (10 mM) was used instead of Fe^{3+}/ascorbate for initiating lipid peroxidation, anthralin inhibited with an IC_{50} of 33 μM.

Antioxidant determination

Because a radical scavenging antioxidant reacts rapidly with the stable free radical DPPH [26], we determined the reactivities of anthralin and its analogs with DPPH by decrease in absorbance at 516 nm in a mixture of phosphate buffer/acetone, pH 7.4. There is general agreement that the reaction of DPPH with phenols is a second-order reaction [36, 37], and the reaction was also found to be of

Table 4. Reducing activity of anthralin and related compounds against DPPH

	k ($M^{-1}sec^{-1}$)
Anthralin	24.2 ± 4.2
Butantrone	7.0 ± 0.6*
Bianthrone	<1
Danthron	<1
1,8-Diacetoxy-9(10*H*)-anthracenone	<1
NDGA	>100

The reaction mixtures contained test compounds (0.1 mM) and DPPH (0.1 mM) in phosphate buffer/acetone, pH 7.4 (1 + 1, v/v), at room temperature.

Second-order rate constants are expressed as mean values ± SD (N = 3–6).

* Indicates significant difference with respect to anthralin ($P < 0.01$).

second order with respect to anthralin and gave a value of 24.2 ± 4.2 $M^{-1}sec^{-1}$ (Table 4). HPLC analysis of the reaction products arising from equimolar amounts of anthralin and DPPH demonstrated that DPPH was reduced to the corresponding hydrazine ($DPPH_2$), anthralin was oxidized to its dimer bianthrone, presumably by dimerization of the initially produced 1,8-dihydroxy-9(10*H*)-anthracenon-10-yl radical. The known oxidation product of anthralin, danthron [38], was not detected. Both bianthrone and danthron did not react with DPPH at an appreciable rate. Introduction of an acyl function at C-10 of anthralin (butantrone) significantly decreases the reaction rate, whereas substitution of both 1- and 8-hydroxyl groups results in loss of antioxidant activity. For comparison, the antioxidant NDGA was far more reactive than anthralin (Table 4).

DISCUSSION

A variety of data derived from chemical reactions and biochemical results has demonstrated that anthralin, in particular its deprotonated form [6, 39, 40] (Eqn 1), can produce activated forms of oxygen during its autoxidation process [4, 6, 8]. Active oxygen species are known to react with DNA either at the sugar backbone or at a base [41, 42]. Firstly, we used the deoxyribose assay to establish the formation of hydroxyl radicals by anthralin. Neither superoxide anion nor H_2O_2 undergoes any chemical reaction with DNA as measured by chemical changes in the deoxyribose [42]. Thus, the release of TBA reactive material from deoxyribose clearly demonstrates the formation of the highly reactive hydroxyl radical by anthralin.

Secondly, we intended to provide evidence that the biological effects of this drug on DNA are mediated by oxygen radicals. Anthralin has been shown to suppress epidermal DNA synthesis [14] and to inhibit the [^{3}H]thymidine incorporation into mouse epidermis [43]. DNA replication and repair are inhibited by anthralin [13]. A direct interaction with DNA was suggested [44] but was disproved later [45].

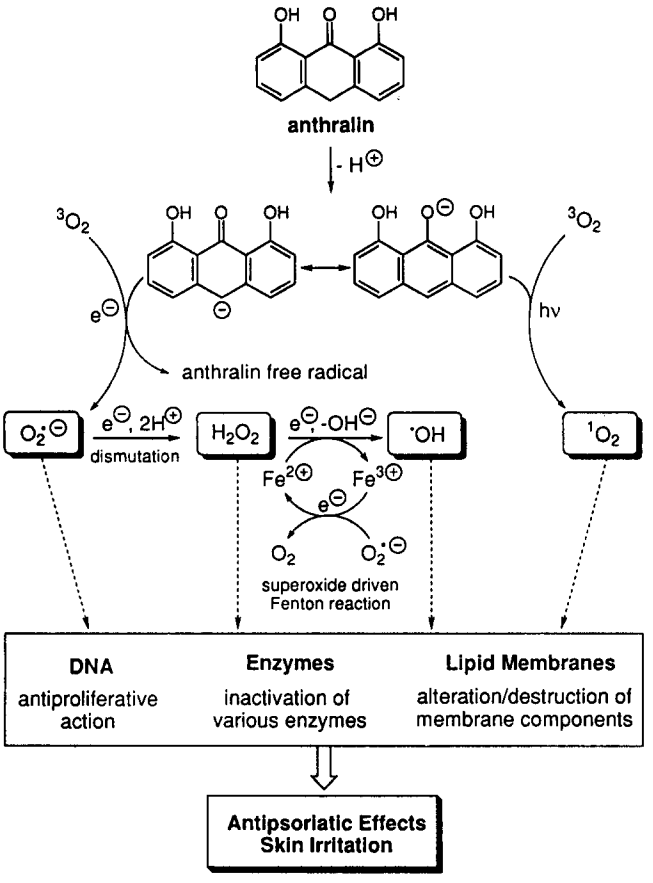

Fig. 7. Generation of active oxygen species and proposed mechanism for the mode of action of anthralin. Energy transfer from the excited anthralin anion to triplet oxygen ($^3O_2$) yields singlet oxygen ($^1O_2$), whereas electron transfer results in the formation of superoxide anion ($O_2^{\cdot-}$). Further steps of one-electron reduction lead to hydrogen peroxide (by dismutation of superoxide anion) and hydroxyl radical ($^{\cdot}OH$, by a superoxide driven Fenton reaction). These activated forms of oxygen can attack several biological targets, in particular DNA, enzymes and lipid membranes and strongly suggest an important role in the mode of action of anthralin and anthralin-caused skin irritation.

Our results clearly demonstrate that anthralin is capable of degrading DNA sugar mediated by hydroxyl radicals. On the other hand, DNA itself was not appreciably affected under identical conditions (data not shown). Reasons may be the preferential attack of hydroxyl radicals at DNA bases with rate constants that are near the diffusion-controlled limit [46].

The ferric iron chelators substantially reduced deoxyribose damage by anthralin suggesting a key role for iron salts in the formation of hydroxyl radical by this drug. This is supported by the inhibitory activity of the iron-binding hydroxyl scavengers [47] sodium salicylate, mannitol and glucose. The ineffectiveness of the hydroxyl radical scavengers ethanol, DMSO and sodium benzoate, which do not bind iron, is in line with the hypothesis that iron is bound to deoxyribose and catalyses a site-specific production of hydroxyl radicals resulting in site-specific damage [47]. Thus, iron-dependent damage to deoxyribose by anthralin cannot be protected against by these hydroxyl radical scavengers that do not effectively compete for hydroxyl radical produced at a specific site where iron is bound. Addition of the antioxidants propyl gallate and rutin led to an increase in sugar damage. This observation is in agreement with other studies that have documented pro-oxidant properties of propyl gallate and catechols [34, 35]. Catalase is a powerful inhibitor of anthralin-stimulated deoxyribose degradation suggesting the involvement of H_2O_2 in the production of hydroxyl radical [8]. This antioxidant enzyme probably inhibits deoxyribose damage in the presence of anthralin and ferric salt by preventing the production of hydroxyl radicals via the Fenton reaction (Eqn 5). SOD, which removes superoxide anion by dismutation to H_2O_2 (Eqn 3), may be effective by preventing the reduction of ferric iron by superoxide anion (Eqn 4), thus interfering with the iron-catalysed Haber–Weiss reaction (Eqn 6). Superoxide anion is initially produced by electron transfer from anthralin anion (AN^-) to molecular oxygen to produce anthralin radical (Eqn 2), which then dimerizes to bianthrone [6]. The observation that

SOD is only slightly effective suggests that ferric iron may still be reduced by another compound, e.g., anthralin. Possible reactions for the generation of hydroxyl radicals by anthralin (ANH) are summarized in Eqns 1–6.

$$ANH + H_2O \rightarrow AN^- + H_3O^+ \quad (1)$$

$$AN^- + O_2 \rightarrow AN^{\cdot} + O_2^{\cdot -} \quad (2)$$

$$2\,O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (4)$$

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow {}^{\cdot}OH + Fe^{3+} + H_2O \quad (5)$$

$$H_2O_2 + O_2^{\cdot -} + H^+ \rightarrow {}^{\cdot}OH + O_2 + H_2O \quad (6)$$

A prevalence of unsaturated fatty acids makes the phospholipids of biological membranes highly susceptible to peroxidation [12]. Hydroxyl radicals can readily abstract allylic hydrogens and initiate the process [12]. Moreover, singlet oxygen can accelerate lipid peroxidation by direct reaction with unsaturated fatty acyl moieties to give hydroperoxides with double bonds shifted to the allylic position [48]. This type of reaction has already been demonstrated for anthralin [4]. Furthermore, many active oxygen species are more soluble in a lipid environment than in aqueous systems and can readily cross biological membranes [11, 12]. Therefore, it may be expected that release of active oxygen species from anthralin could initiate lipid peroxidation in cellular membranes.

Enhancement of lipid peroxidation in bovine brain phospholipid liposomes by anthralin at 12.5 μM was significantly different with respect to controls, although higher concentrations of the drug resulted in less peroxidation. Similar effects were observed for butantrone, but the extent of peroxidation was less pronounced. The enhancement of MDA release in the presence of catalytic amounts of Fe^{3+} documents the catalytic function of transition metals in the formation of active oxygen species by anthralin. This is of particular interest because it is known that skin is a significant site of iron excretion and increased iron content has been reported in the epidermis of untreated psoriatic patients [21]. The involvement of ferric iron is further supported by the fact that the chelators desferrioxamine and EDTA substantially diminished the extent of peroxidation. On the other hand, removal of hydrogen peroxide or superoxide anion by catalase or SOD did not inhibit lipid peroxidation, suggesting that these active oxygen species are not involved. However, the participation of hydroxyl radical, peroxyl radicals or alkoxyl radicals is indicated by the fact that BHT, vitamin E and propyl gallate prevented MDA formation.

The production of hydroxyl radicals and enhancement of lipid peroxidation in model membranes by anthralin may be related to the therapeutic efficiency as well as irritancy. In particular mitochondrial membranes are sensitive to damage resulting from the peroxidation of membrane phospholipids [49]. Both mitochondrial structure and mitochondrial function can be damaged [49]. Anthralin is an inhibitor of mitochondrial membrane functions [20], causes dramatic structural changes in mitochondria [40] and influences redox properties of energy transducing membranes [19], which all may contribute to its antiproliferative activity. Thus, some of the effects of anthralin on mitochondrial structure and biochemical function may be mediated by anthralin-enhanced lipid peroxidation.

Cellular protective mechanisms have evolved against oxygen toxicity and under normal conditions the antioxidant systems present in the cell are able to prevent or revert most of the adverse effects [11]. However, local concentrations of active oxygen species produced by anthralin may overwhelm the protective enzymes. In addition, the activity of SOD is decreased in psoriasis [50, 51]. Consequently, the removal of the superoxide anion as a precursor of the hydroxyl radical is diminished in psoriasis and this factor may promote the susceptibility of psoriatic epidermis to oxidative injuries. Furthermore, selective cytotoxicity of anthralin-derived oxygen radicals to psoriatic tissue may be expected from the fact that in contrast to healthy skin psoriatic skin is deficient in SOD.

On the other hand, an antioxidant effect could be observed for anthralin, although small as compared to that of the antioxidant NDGA. Butantrone was three times more effective as an inhibitor of lipid peroxidation than anthralin. Also, inhibition by anthralin was lower in the AAPH system than in the Fe^{3+}/ascorbate system. Anthralin and butantrone may exert their antioxidant effect by donating a hydrogen atom to a radical intermediate with formation of a lower energy anthracenon-10-yl radical thus interfering with the intended pathway of the peroxidation reaction. The results from our studies with DPPH show that reactivity of anthrones with DPPH depends on the presence of at least one hydrogen at C-10 and of the ability of the resulting anthracenon-10-yl radical to be stabilized by additional delocalization as a consequence of intramolecular hydrogen bond. The 10-butyryl derivative butantrone was less reactive than anthralin, probably on account of impaired radical formation at C-10. Substitution of the two 1,8-hydroxyl groups [1,8-diacetoxy-9(10*H*)-anthracenone] resulted in loss of reactivity, which was also observed for the oxidation products of anthralin, bianthrone and danthron. The latter compound demonstrates that the presence of free hydroxyl groups at C-1 and C-8 is not sufficient for antioxidant activity. In addition, at least one hydrogen atom at C-10 must be present. Whether these antioxidant properties of antipsoriatic anthrones do play an important role *in vivo*, remains to be determined. It seems more likely, however, that possible antioxidant effects may be surpassed by the strong pro-oxidant actions mediated by active oxygen species.

We have reported previously that superoxide anion generated by anthralin may inactivate glucose-6-phosphate dehydrogenase [16], an enzyme associated with cell proliferation. The present study extends this observation on other cellular targets that have been implicated in the mode of action of anthralin. The results demonstrate that the mechanism of anthralin-induced DNA sugar damage and membrane lipid peroxidation involves several

forms of activated oxygen. Figure 7 is a comprehensive presentation of the generation of active oxygen species by anthralin and their implication in the mode of action, which is consistent with the results we have obtained from this and previous studies.

In summary, the present study shows the production of hydroxyl radicals by anthralin. This and other active oxygen species cause damage to DNA sugar and enhance membrane lipid peroxidation *in vitro* and suggest an important role of these species in the antipsoriatic action and manifestation of side effects caused by anthralin treatment *in vivo*.

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