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MODIFICATION OF DNA BASES BY ANTHRALIN AND RELATED COMPOUNDS*

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Abstract—Modification of bases in calf thymus DNA by treatment with the antipsoriatic drug anthralin was studied. The products of DNA bases were identified and their yields measured by gas chromatography–mass spectrometry with selected ion monitoring. Treatment of calf thymus DNA with anthralin significantly enhanced the amount of modified bases above control levels. Purine bases were modified to products identical with those known to be typical of DNA damage induced by hydroxyl radicals. The yields of Fapy-adenine, 8-hydroxyadenine, Fapy-guanine, and 8-hydroxyguanine were maximally increased at an anthralin concentration of 75 μM. A variety of structural analogues of anthralin were also tested at 75 μM and were either weaker or stronger hydroxylating agents. It is likely that damage to DNA bases induced by anthrones contributes to their antiproliferative activity. The pharmacological implications of these characteristics of the action of anthralin on DNA bases are discussed.

Key words: anthralin; dithranol; psoriasis; DNA; base damage; free radicals

Anthrone derivatives such as anthralin have long been used in the treatment of psoriasis and are still a mainstay in the management of this chronic inflammatory and scaling skin disease [1]. As in most forms of psoriasis therapy the benefits of these drugs are limited by their undesirable irritant effects on the skin. On the basis of *in vitro* studies, three major targets of anthrone action have been identified [2]: enzymes associated with cell proliferation and inflammation, mitochondrial membranes, and DNA.

Among anthrones, anthralin (**1**, dithranol, 1,8-dihydroxy-9(10*H*)-anthracenone) has been particularly well studied. Anthralin is readily oxidized under physiological conditions [3] yielding ESR‡-detectable species [4–7]. Furthermore, one-electron oxidation generates the anthralin radical with concomitant production of superoxide radical [8, 9] and hydroxyl radical [10]. The anthralin anion produces singlet oxygen upon irradiation [11] and is also a quencher of this species [12]. Since active oxygen species are known to have deleterious effects in many biological systems [13], they have been implicated in the mode of action and induction of side effects of antipsoriatic anthrones [2].

The influence of antipsoriatic anthrones on epidermal growth has been extensively studied. Thymidine incorporation into epidermal cells is inhibited [14–16], both replicative and repair modes of DNA synthesis are inhibited [17], and epidermal DNA synthesis is suppressed [18]. A direct interaction between anthralin and DNA was reported [19] but was later disproved [20]. Furthermore, anthralin was reported to induce DNA strand breaks in human leukocytes *in vivo* [21]. Since DNA is among the principal targets for antipsoriatic anthrones it is important to examine the mechanism by which these drugs exert their action on DNA. Active oxygen species have been implicated in the occurrence of cell damage in several pathological circumstances [13, 22]. Therefore, the enhancement of intracellular levels of active oxygen species has often been proposed as one of the mechanisms of cytotoxicity of a variety of drugs [23]. In a recent study we have shown that anthralin causes degradation of deoxyribose to malondialdehyde mediated by hydroxyl radicals [24]. However, we were not able to detect malondialdehyde using intact DNA under similar conditions [24]. Consequently, a preferential attack of anthralin-produced hydroxyl radicals at DNA bases seems likely.

As part of our ongoing research involving anthracenones as potential antipsoriatic agents, we recently described the synthesis and biological evaluation of novel series of 9(10*H*)-anthracenones with modulated redox properties [25, 26]. In the present study, we have characterized the chemical changes produced in DNA by anthralin and a number of these novel compounds (Fig. 1). This characterization and the quantitative measurement of DNA lesions are necessary to gain more insight into the mechanism by which antipsoriatic anthrones produce their biological effects. The base products

* Dedicated to Prof. Dr H. Schönenberger on the occasion of his 70th birthday.

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‡ Abbreviations: ESR, electron spin resonance; GC-MS, gas chromatography–mass spectrometry; SIM, selected ion monitoring; 8-OH-Ade, 6-amino-8-hydroxypurine; Fapy-Ade, 4,6-diamino-5-formamidopyrimidine; Fapy-Gua, 2,4-diamino-6-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 2-amino-6,8-dihydroxypurine; BSTFA, *N,O*-bis(trimethylsilyl) trifluoroacetamide; RMRF, relative molar response factor.

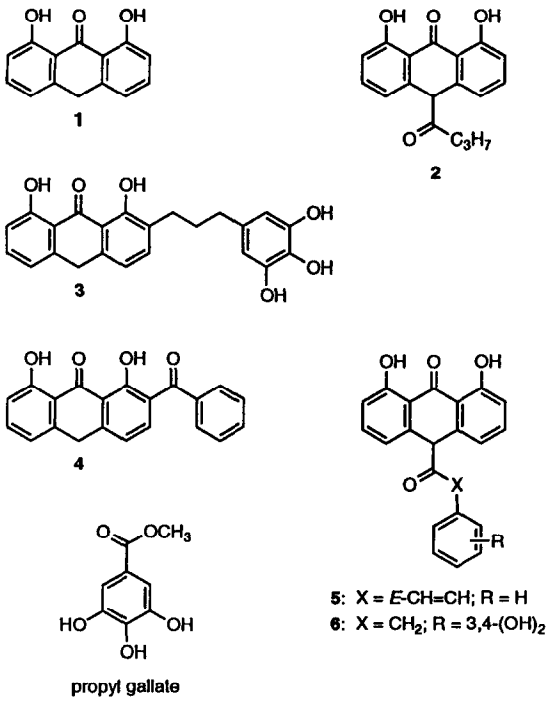

Fig. 1. Structures of anthralin (**1**) and related compounds.

formed as a result of free radical attack on DNA were examined by the use of the highly sensitive technique of GC-MS with SIM, one already shown to be suitable for this purpose [27, 28].

MATERIALS AND METHODS

Reagents. Anthralin (**1**), butantrone [**2**, 10-butyryl-1,8-dihydroxy-9(10*H*)-anthracenone], 1,8-dihydroxy-2-[3-(3,4,5-trihydroxyphenyl)propyl]-9(10*H*)-anthracenone (**3**), 2-benzoyl-1,8-dihydroxy-9(10*H*)-anthracenone (**4**), (*E*)-1,8-dihydroxy-10-(1-oxo-3-phenyl-2-propenyl)-9(10*H*)-anthracenone (**5**), and 1,8-dihydroxy-10-[2-(3,4-dihydroxyphenyl)-1-oxoethyl]-9(10*H*)-anthracenone (**6**) were prepared according to known methods [25, 26]. 8-OH-Ade and Fapy-Gua were prepared from 8-bromoadenine and 2,4,5-triamino-6-hydroxypyrimidine, respectively, as described [29, 30]. Calf thymus DNA, 8-azaadenine, and Fapy-Ade were obtained from Sigma (Deisenhofen, Germany), 8-OH-Gua was from Aldrich (Steinheim, Germany), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane was from Fluka (Buchs, Switzerland), propyl gallate was from Janssen (Brüggen, Germany), acetonitrile was from Baker (Groß-Gerau, Germany), and formic acid (88%, v/v) was from Merck (Darmstadt, Germany). Spectra Por MWCO 3500 dialysis membranes were purchased from Roth (Karlsruhe, Germany).

Determination of the relative molar response factors. Calibration of the mass spectrometer was carried out using mixtures of 8-azaadenine (2 nmol) as the internal standard and authentic samples of Fapy-Ade, 8-OH-Ade Fapy-Gua, and 8-OH-Gua (0.2–2 nmol) [31]. The samples were lyophilized and trimethylated as described below.

Incubation procedure. The following reagents were added to glass tubes at the final concentrations stated: 0.6 mL DNA (0.5 mg/mL), 0.12 mL KH_2PO_4-KOH buffer, adjusted to pH 7.4 (10 mM), and 24 μL of anthrone derivative in acetonitrile (75 μM). The final reaction volumes were standardized to 1.2 mL by adding water (double distilled). Stock solutions of the compounds were made up fresh before use. Appropriate blanks and controls with the vehicle (acetonitrile) were conducted. The reaction mixtures were incubated for 2 hr at 37° in a shaking water bath. Then, the mixtures were extensively dialysed against water at 4° over 3 days. The amount of DNA was measured spectrophotometrically at 260 nm (absorbance of 1 = 50 μg of DNA/mL [31]).

Hydrolysis of DNA and derivatization of the products. 8-Azaadenine (2 nmol) was added as an internal standard to aliquots of dialysed DNA samples (170 μg) and the solutions were lyophilized. The samples were then treated with 0.7 mL of formic acid (88%, v/v) in evacuated and sealed reaction vessels at 150° for 40 min. Formic acid was removed and the samples were dried. Subsequently, a mixture of 0.2 mL BSTFA and acetonitrile (2 + 1, v/v) was added and the samples were heated at 130° for 30 min.

Analysis by GC-MS. Gas chromatography (Hewlett-Packard 5890 Series II gas chromatograph) was performed on a Hewlett-Packard fused silica capillary column (12.5 m × 0.20 mm: HP-5 cross-linked 5% phenyl-methyl-silicone, film thickness 0.33 μm) operated in the splitless mode with the septum purge opened after 2 min. Typically 4 μL of the sample were injected with the injection port kept at 250°. After 2 min at 120°, the column temperature was increased at a rate of 8°/min to 250°. Helium was used as the carrier gas (inlet pressure 40 kPa). Selected ion monitoring-mass spectrometry was carried out on a Finnigan MAT 95 mass spectrometer by electron impact ionization (70 eV).

Statistical analysis was carried out by comparing the value of modified purine base product of control and test compound by means of the Student's *t*-test. Each value was obtained from three to six independent measurements and is expressed as mean ± SD. Any value of $P < 0.01$ was accepted as being significant. Standard software on a Macintosh Quadra computer was used for statistical evaluation.

RESULTS

Determination of the relative molar response factors

Quantitative measurement of modified bases in DNA by the GC-MS technique depends on the following prerequisites [31, 32]. Modified bases must be hydrolysed from the sugar–phosphate backbone of the DNA. Formic acid appears to be most suitable for this purpose [32]. Furthermore, modified bases must be converted to sufficiently volatile derivatives so that the GC-MS technique can be applied. The completeness of hydrolysis and the stability of the

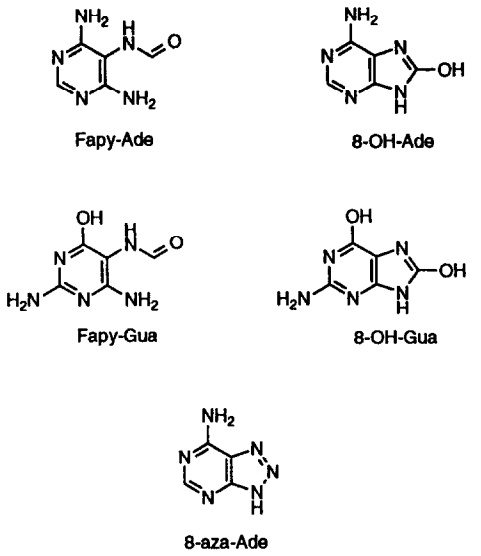

Fig. 2. Structures of the identified products of purine bases and the internal standard 8-azaadenine.

derivatized products have been established [31]. Accordingly, DNA was hydrolysed and converted into volatile derivatives.

Because of the electrophilic nature of the hydroxyl radical, the rate constants for its reaction with the electron-rich imidazole moiety of purine bases are higher compared to those with the electron-deficient pyrimidine bases [33]. Accordingly, purine bases have been shown to be the major products of hydroxyl radical attack on DNA and the best candidates to measure [28, 34]. Of these, 8-OH-Gua has become the species of current interest. The more common tautomeric form is 8-oxo-7-hydroguanine [35], but tautomerism should not affect GC-MS analysis [36]. The chemical structures of the identified products of purine bases are presented in Fig. 2.

8-Azaadenine was used as an internal standard [32]. Following the method described by Dizdaroglu and Gajewski [32], a calibration plot for the response of the mass spectrometer to known amounts of bases and the internal standard was obtained by measuring an intense and characteristic ion of each compound in the SIM mode. Figure 3 shows the ion-current ratio of the ions as a function of the ratio of the molar amounts of the compound and the internal standard. The slopes of these plots give the RMRF [32] which is defined as follows:

$$\mathrm{RMRF} = \frac{\dfrac{\text{amount of standard}}{\text{amount of analyte}}}{\dfrac{\text{peak area of analyte}}{\text{peak area of standard}}}.$$

Damage to DNA bases induced by anthralin

The poor solubility of anthralin in aqueous solution and the problems resulting from the addition of organic solvents have recently been discussed [24]. Therefore, the amount of acetonitrile added to each reaction mixture was limited to 2% (v/v) to minimize hydroxyl radical-scavenging of the solvent [24].

The maximum concentration for the formation of 8-OH-Gua by anthralin was observed at 75 μM, in excellent agreement with the maximum concentration of anthralin for deoxyribose degradation [24]. Higher concentrations of anthralin did not yield higher amounts of 8-OH-Gua (data not shown). This may be related to the poor aqueous solubility of anthralin. A time-dependent study revealed that with an anthralin concentration of 75 μM, formation of 8-OH-Gua slowly but significantly increased within 2 hr but subsequently dropped to its initial level (Fig. 4).

Table 1 presents the yields of the purine base products measured in DNA samples by treatment with anthralin at 75 μM. The calf thymus DNA used in these experiments already contained some hydroxylated purine bases, with 8-OH-Gua being the major product measured in untreated DNA. This is consistent with observations of earlier studies [28, 34, 37]. Incubation of DNA with 25 μM or 100 μM anthralin did not produce an increase in modified bases over the background levels (data not shown), whereas 75 μM anthralin significantly increased the yields of Fapy-Ade, 8-OH-Ade, Fapy-

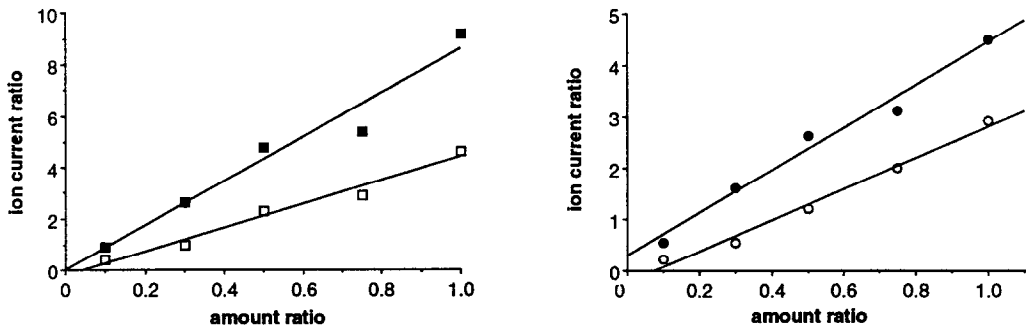

Fig. 3. Calibration plots for the Me_3Si derivatives of modified purine bases relative to 8-azaadenine (m/z 265). The slope of the lines gives the relative molar response factors for (■) Fapy-Ade (m/z 354), (□) 8-OH-Ade (m/z 352), (○) Fapy-Gua (m/z 442), (●) 8-OH-Gua (m/z 440). Data points are mean values (N = 3, SD ≤ 15%).

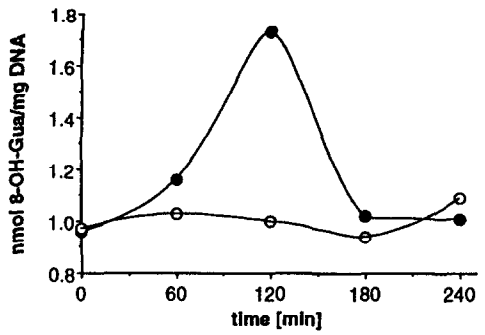

Fig. 4. Time-course of formation of 8-OH-Gua in DNA in the presence (●) and absence (○) of anthralin (75 μM) at 37° in KH_2PO_4-KOH buffer, pH 7.4. Values are expressed as nmols 8-OH-Gua per mg DNA per 2 hr (N = 6, SD < 15%), measured by GC-MS/SIM. Values at 1 and 2 hr are significantly different from controls, $P < 0.01$.

Gua, and 8-OH-Gua. In terms of increase in yields of modified purine bases over background levels, anthralin-induced free radical attack on DNA gave the greatest change in the yields of Fapy-Ade and 8-OH-Gua.

Damage to DNA bases induced by anthralin derivatives

In addition to anthralin, the following compounds were evaluated for their ability to induce damage to DNA bases (Table 1). Butantrone (**2**) has shown clinical efficacy against psoriasis [38], but is more irritating than anthralin itself [39]. The 2-substituted anthrones **3** and **4** as well as the 10-substituted derivatives **5** and **6** are strong producers of hydroxyl radical, which was confirmed by using the deoxyribose assay [25, 26]. Furthermore, even though propyl gallate is an antioxidant, this compound enhanced anthralin-induced deoxyribose degradation [24]. The yields of the purine base products obtained by treatment of DNA with anthralin and related compounds are given in Table 1. Incubation of DNA with propyl gallate and derivative **4** did not lead to significantly increased amounts of modified bases. All other derivatives produced significantly increased yields of at least 8-OH-Gua. In particular, the pyrogallol derivative **3** produced by far the highest yields of the modified purine bases.

DISCUSSION

Formation of oxygen radicals upon anthralin autoxidation has been documented extensively [8–10, 24]. However, neither superoxide radical nor hydrogen peroxide causes any strand breakage or chemical modification of DNA [28, 34, 40]. Their biological effects are thought to result from their iron-dependent conversion to hydroxyl radical, so reactive that it can attack all components of the DNA [40]. Thus, generation of hydroxyl radical by an iron-catalysed Haber–Weiss reaction (Fenton-type reaction) has been demonstrated, leading to degradation of deoxyribose [24]. This has also been confirmed by ESR spectroscopy [41]. Nonetheless, the mechanism by which anthralin exerts its action on DNA is not clear at present. To investigate further the role of oxygen-derived species in anthralin action, we evaluated the ability of anthralin and related analogues to hydroxylate DNA bases.

For this purpose, both HPLC separation coupled with electrochemical detection [42] and GC-MS have been applied [29]. Contrary to the HPLC technique, GC-MS allows the determination of a wide variety of chemical changes in DNA bases [27]. Both techniques have comparable sensitivity, although they do not always give similar results [36]. In particular, HPLC may underestimate the real amount of 8-OH-Gua, while GC-MS may overestimate it. Potential reasons for this have been discussed in detail [36]. However, the background levels of purine modifications, including the level of 8-OH-Gua of our study, are in good agreement with the values observed previously under similar conditions [28, 34, 37].

The data of our present study demonstrate that

Table 1. Yields of purine base products in DNA by treatment with anthralin and related compounds

Compound	Fapy-Ade	8-OH-Ade	Fapy-Gua	8-OH-Gua
Untreated DNA	0.010 ± 0.003	0.26 ± 0.02	0.11 ± 0.01	1.00 ± 0.07
Anthralin (**1**)	0.030 ± 0.003*	0.33 ± 0.01*	0.14 ± 0.01*	1.73 ± 0.05*
Butantrone (**2**)	0.014 ± 0.001	0.29 ± 0.05	0.13 ± 0.01*	1.66 ± 0.09*
3	0.036 ± 0.007*	0.65 ± 0.11*	0.16 ± 0.03*	2.94 ± 0.43*
4	0.014 ± 0.003	0.18 ± 0.03	0.10 ± 0.01	1.02 ± 0.15
5	0.021 ± 0.004	0.18 ± 0.02	0.14 ± 0.02	1.28 ± 0.08*
6	0.011 ± 0.002	0.24 ± 0.04	0.19 ± 0.01*	1.47 ± 0.19*
Propyl gallate	0.013 ± 0.001	0.18 ± 0.01	0.09 ± 0.01	0.98 ± 0.09

Incubation was performed for 2 hr at 37° in KH_2PO_4-KOH buffer, pH 7.4. The reaction mixtures contained DNA (0.5 mg/mL) and 75 μM anthrone derivative. The structures of the compounds are given in Fig. 1. Indicated values are nmols modified base per mg DNA (mean values ± SD, $N \geq 3$), measured by GC-MS/SIM.

* Indicates significant difference with respect to untreated DNA, $P < 0.01$.

biologically relevant concentrations of anthralin significantly generate modified bases in calf thymus DNA similar to those induced by hydroxyl radicals [28, 34, 37]. Hydroxyl radical attack on DNA induced by anthralin or related anthrone derivatives produced the greatest change in the yield of 8-OH-Gua.

Among the anthrone derivatives examined, the pyrogallol compound **3** produced the highest yield of modified purine bases in DNA. Even though the 2-benzoyl derivative **4** was as potent in degrading deoxyribose as **3** [26], this compound produced no significant increase in base damage. The 10-substituted anthrone **6**, which generates twice as many hydroxyl radicals as anthralin [25], produced significant increases only in the yields of Fapy-Gua and 8-OH-Gua, but the ratios of increase over background levels were substantially less than those measured for anthralin and **3**. This may be rationalized on the basis of the association of the molecules with DNA, followed by hydroxyl radical generation at that particular site. The results of this study suggest that the nature of the interaction of the molecules with DNA was much better for compound **3** than for the other anthrones. Indeed, site-specific generation of hydroxyl radicals followed by DNA damage is often referred to as site-specific damage to DNA [43]. Surprisingly, butantrone, which was only weakly effective in damaging deoxyribose [24], significantly increased the yields of Fapy-Gua and 8-OH-Gua, suggesting a production of hydroxyl radicals in the vicinity of DNA. The cinnamoyl derivative **5** led to significant increases only in the yield of 8-OH-Gua, but these were markedly less pronounced than those of anthralin and **3**.

Although the antioxidant propyl gallate can enhance oxidative damage to deoxyribose [24] and DNA [44, 45], it is not necessarily a prooxidant with respect to DNA bases. This is documented by the fact that the yields of base products were not altered as compared to those of untreated DNA. Taken together, the hydroxyl radical-generating anthrones [24–26] anthralin, butantrone, and compounds **3** and **6** are all able to cause damage to DNA bases, which is probably related to the generation of hydroxyl radicals at a specific site on the DNA helix.

As the cell is particularly sensitive to DNA damage [46], the effects of oxidative stress upon DNA may be a major contribution to the mechanism of the antiproliferative activity of anthrones. Hydroxyl radicals can attack DNA either at the sugar moiety or at the bases [40, 46]. The former ultimately leads to sugar fragmentation, base loss, and strand breaks, the latter results in damaged bases. Since induction of DNA strand breaks [21] and inhibition of DNA replication [17] by anthralin have been demonstrated, the formation of modified DNA bases may be responsible for these effects.

Formation of 8-OH-Gua can be used as a marker for monitoring oxidative damage in evaluating the carcinogenic potential of various oxygen radical-generating agents [47]. 8-Hydroxyguanine might lead to mutations by inducing misreading [40]. It has been reported that antioxidants were effective inhibitors of anthrone tumour promotion, indicating an important role of oxygen radicals in the mechanism of action of this class of tumour promoters [48]. Anthralin seems to be a tumour promoter in mouse skin [49–51]. However, these results should not be extrapolated to its use in psoriasis therapy [52]. In spite of its clinical use for more than 70 years, an increased instance of any type of tumour has not been observed [52].

DNA damage resulting from oxygen radical attack by anthralin need not necessarily lead to tumour promotion. Although the yields of modified bases in DNA produced by anthralin were significantly enhanced, the ratio of increase over the background levels was comparatively low with respect to well-known hydroxyl radical-generating systems [28, 34]. For example, base modification in DNA by Fe^{3+}-nitrilotriacetic acid/H_2O_2 may account for the carcinogenicity associated with this system [34]. Low levels of damage induced by anthralin may be repaired by enzymes that mediate the removal of base and sugar damages from DNA and help counteract the potential cytotoxic, mutagenic and carcinogenic effects [53]. On the other hand, high levels of damage cause cell death. As a consequence, initiated cells do not remain in the organism [40].

In summary, we have identified and quantitatively determined the modification of purine bases in DNA induced by anthralin and some of its derivatives. Purine bases were modified to the same products known to be typical of DNA damage induced by hydroxyl radicals. This may contribute to the mechanism of the antiproliferative activity of the anthrones.

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