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Heterocyclic Substituted Anthralin Derivatives as Inhibitors of Keratinocyte Growth and Inducers of Differentiation

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Abstract—Heterocyclic substituted derivatives of the antipsoriatic anthralin were synthesized and evaluated in vitro for their antiproliferative action against keratinocytes and their ability to induce keratinocyte differentiation. The indole-2-carboxylic acid analogue **2e** exhibited the same excellent antiproliferative activity as anthralin and also induced terminal differentiation of keratinocytes. As a benefit of its strongly diminished potential to generate oxygen radicals, **2e** did not induce damage of keratinocyte membranes.

Introduction

Over the past century, a number of therapeutic agents have been employed for the treatment of psoriasis, a skin disorder affecting 2% of the population.[1] Of all these, anthralin (**1**) is the most remarkably consistent and time-honored antipsoriatic drug.[2] It still compares favorably in efficacy with newer treatment methods such as vitamin A and D derivatives.[3] Apart from its therapeutic usefulness, side effects such as inflammation of the healthy skin reduce its patient acceptability.[4] A large body of evidence is consistent with a fundamental role of oxygen radicals in the proinflammatory action of anthralin, which are generated from the deprotonated methylene moiety at the 10-position.[5]

Our strategy to provide agents with diminished intensity of oxygen-radical generation was to modify the critical 10-postion of anthralin,[6–8] while the 1,8-dihydroxy substitution pattern, which is required for potent antiproliferative activity,[9] was retained. Structure–activity relationships of 10-acyl derivatives revealed that the mere presence of an acyl substituent did not necessarily lead to improved activity, and we demonstrated that a terminal phenyl ring was required.[6] These compounds were distinguished by the presence of methylene spacers between the terminal phenyl ring and the carbonyl group at C-10 of anthralin. In our continuing search for anthracenones with an improved benefit/risk ratio, we became interested in analogues lacking the methylene spacers. In that direction, we conducted a preliminary investigation into the potential of several closely related heterocyclic ring systems linked by a carbonyl or a propenone group to the 10-position of anthralin. The rationale behind the choice of these substituents at the 10-position of the anthracenone nucleus was as follows. First, a heterocyclic ring is of particular interest, as this feature suggests a strong impact on anthralin free radical formation, depending on the nature of the heteroatom in the 2-position of the heterocyclic ring. Second, a propenone group would also allow a conjugative stabilization effect by the terminal heterocyclic ring. For the first time, we examined the ability of the novel anthracenones to induce keratinocyte differentiation and we also reported the results of other relevant biological properties.

Chemistry

Acylation of **1** with phenyl terminated acyl chlorides in the presence of pyridine takes place at C-10 via the carbanion, whereas benzoyl chlorides give mixtures of *O*-acylated products and require an alternative route via 1,8-diacetoxy-9(10*H*)-anthracenone.[10] However, by the use of the coupling agent dicyclohexylcarbodiimide (DCC) we were able to directly introduce the heteroarylcarboxylic acids onto the 10-position of anthralin

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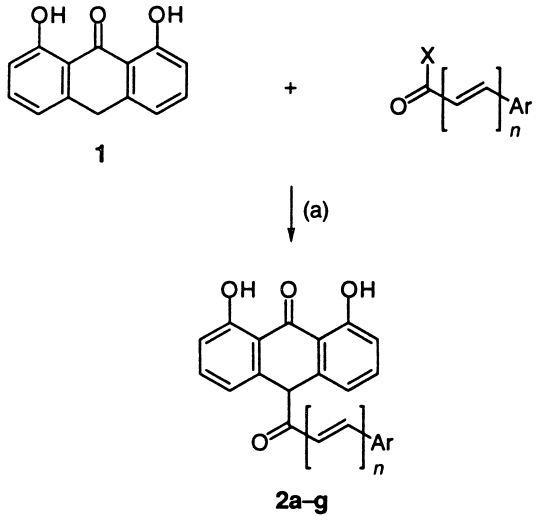

Scheme 1. Reagents: (a) Method A, X = OH, $n = 0$: DCC, pyridine, THF, N_2. Method B, X = Cl, $n = 1$: pyridine, toluene, N_2; Ar is defined in Table 1.

(method A), as depicted in Scheme 1. The starting materials are commercially available. Anthracenones **2f** and **2g** were obtained from the corresponding acrylic acid chlorides (method B, Scheme 1) which were synthesized following a literature method.[11] NMR spectroscopy indicated the presence of the *E* isomers.

Biological Evaluation

The novel analogues were evaluated in vitro for their potential antipsoriatic activity in complementary assays specifically addressing important aspects of psoriasis and anthralin treatment. First, the immortalized keratinocyte line HaCaT[12] was used as a model for the hyperproliferative epidermis found in psoriasis, as these cells have been described as an extremely sensitive target for the antiproliferative action of anthralin.[13] Second, as HaCaT keratinocytes maintain a highly preserved differentiation capacity,[14] these cells were also used to evaluate the ability of the novel anthracenones to induce keratinocyte differentiation, which is disturbed in psoriasis.[15] Third, in order to confirm that inhibition of keratinocyte growth is not a result of cytotoxic effects against HaCaT cell membranes induced by oxygen radicals generated from anthracenones, plasma membrane integrity was tested by the release of lactate dehydrogenase (LDH) into the culture medium,[7] which is commonly used as indicator in testing cytotoxicity. Finally, the intensity of hydroxyl-radical generation by the novel anthracenones was determined by degradation of deoxyribose and was measured by the release of malondialdehyde.[6]

Results and Discussion

The data in Table 1 show the intensity of analogues **2a**–**g** to generate hydroxyl radicals, their activity against HaCaT cell growth, and their potential to cause membrane damage as a measure of cytotoxicity. The data for anthralin and 10-benzoyl-anthralin (**3**, Fig. 1) are included for comparison purpose. As far as the growth-inhibitory action is concerned, compounds **2b** and **2c** bearing a 2- or 3-thienoyl group were inactive within the concentration range. This is consistent with the role of a thiophene ring as an appropriate bioisosteric replacement of a benzene ring, since the benzoyl analogue **3** was also inactive. However, replacement of the phenyl ring of **3** with other heterocycles possessing a more electronegative heteroatom such as furan (**2a**), pyrrole (**2d**), and indole (**2e**, Fig. 1) resulted in highly potent antiproliferative compounds. Thus, analogues **2d** and **2e** with the more electronegative nitrogen atom were equipotent with anthralin while those with a less negative heteroatom (**2b** and **2c**) or carbon (**3**) were inactive at concentrations up to 5 μM. We also explored the effect of insertion of a *trans*-double bond between the heterocyclic ring and the keto group at C-10 of the anthracenone

Table 1. Deoxyribose degradation, antiproliferative activity and cytotoxicity against HaCaT cells by heterocyclic substituted 1,8-dihydroxy-9(10*H*)-anthracenones

Compound	Ar	*n*	DD (•OH)[a]	AA IC_{50}, μM[b]	LDH[c]
2a	2-Furyl	0	0.41 ± 0.12	1.2	242
2b	2-Thienyl	0	0.65 ± 0.10	> 5	ND[e]
2c	3-Thienyl	0	0.82 ± 0.14	> 5	ND
2d	2-Pyrrolyl	0	1.77 ± 0.04	0.6	202
2e	2-Indolyl	0	0.17 ± 0.10	0.7	167
2f	2-Furyl	1	1.69 ± 0.15	1.5	147
2g	2-Thienyl	1	1.13 ± 0.15	4.2	166
Anthralin			2.89 ± 0.14	0.7	294
3[d]	Phenyl	0	2.31 ± 0.32	> 5	ND

[a]Deoxyribose degradation as a measure of hydroxyl-radical formation. Indicated values are μmol of malondialdehyde per mmol of deoxyribose released by 75 μM test compound (controls < 0.1; values are significantly different with respect to control; $P < 0.01$).
[b]Antiproliferative activity against HaCaT cells. Inhibition of cell growth was significantly different with respect to that of the control, $N = 3$, $p < 0.01$.
[c]Activity of LDH (mU) release in HaCaT cells after treatment with 2 μM test compound (vehicle controls < 140; $N = 3$, SD < 10%).
[d]Ref 10.
[e]ND, not determined.

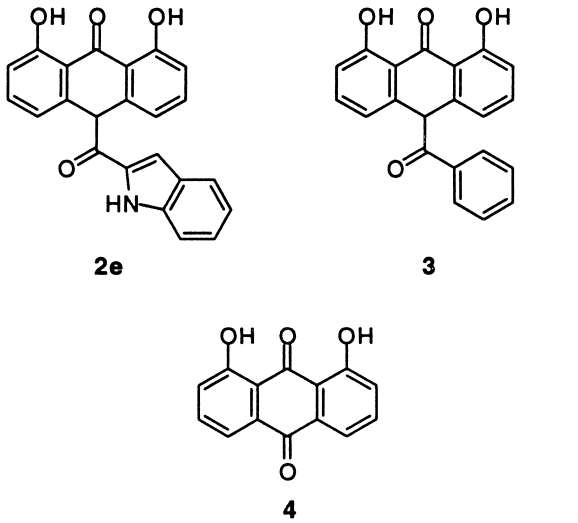

Figure 1. 10-(2-Indoloyl)-anthralin (**2e**), 10-benzoyl-anthralin (**3**) and danthron (**4**).

nucleus. In the case of the furan analogue **2f**, potency did not dramatically change as compared to **2a**, whereas for the thiophene analogue **2g** antiproliferative activity was improved as compared to **2b**.

The data of Table 1 further document that for all novel analogues the intensity of hydroxyl-radical generation was strongly decreased as compared to anthralin. However, as observed with benzoyl analogue **3** there is still a marked amount of hydroxyl radicals produced by the pyrrole analogue **2d** and by the acrylic acid derivatives **2f** and **2g**.

Anthralin is a known inducer of lipid peroxidation in biological membranes, which is mediated by hydroxyl radicals.[16] Also, considerable membrane damage was observed for the pyrrole **2d**, which is also a potent generator of hydroxyl radicals, and surprisingly, for the furan **2a**. For other compounds, the release of LDH activity into the culture medium was not markedly increased as compared to vehicle controls and suggested cytostatic rather than cytotoxic action against keratinocytes.

A further important aspect of psoriasis is the abnormal differentiation of keratinocytes with parakeratosis. Differentiation is an irreversible process, killing aberrant cells which are then lost by sloughing. Accordingly, agents that induce this process can be used in the treatment of hyperproliferative conditions. Even though HaCaT cells are genetically abnormal, they have been documented to exhibit a high differentiation potential under in vitro and in vivo conditions.[12,17] One well-recognized marker of terminal differentiation in keratinocytes is the cornified envelope,[18] which is composed predominantly of an array of cross-linked proteins.[19] Therefore, we have investigated the ability of the novel anthracenones to induce the formation of cross-linked protein envelopes.

The data of Table 2 show that the antipsoriatic anthralin was able to induce cross-linked protein in HaCaT cells in a concentration-dependent manner. By contrast, its metabolite danthron (**4**, Fig. 1), which is therapeutically inactive and does not arrest keratinocyte growth,[9] did not induce terminal differentiation. The results obtained for the novel analogues show that those compounds (**2b** and **2c**) which did not inhibit keratinocyte growth were also not able to induce their differentiation at concentrations up to 5 μM. Reversely, of those compounds that did inhibit cell growth only the 2-furylacrylic acid derivative **2f** was inactive as an inducer of differentiation. This is in agreement with observations that growth arrest does not necessarily result in the onset of differentiation.[20,21] Further, the inactivity of compounds **2b**, **2c** and **2f** indicates that structural features necessary for inducing the differentiation process of keratinocytes do not reside necessarily in the anthralin chromophore. On the other hand, the most potent inhibitors of cell growth were also potent inducers of terminal differentiation. However, induction of significant cross-linking was observed at concentrations slightly above those required to arrest cell growth.

Table 2. Induction of keratinocyte differentiation by heterocyclic substituted 1,8-dihydroxy-9(10*H*)-anthracenones

			Cornified envelope assay[a]		
Compound	Ar	*n*	1 μM	5 μM	10 μM
2a	2-Furyl	0	0.5 ± 0.5	3.5 ± 0.2[b]	5.8 ± 0.2[b]
2b	2-Thienyl	0	−0.9 ± 0.4	0.7 ± 0.2	3.7 ± 0.4[b]
2c	3-Thienyl	0	−0.6 ± 0.4	0.6 ± 0.6	3.4 ± 0.4[b]
2d	2-Pyrrolyl	0	2.3 ± 1.3	5.7 ± 1.1[b]	6.5 ± 0.5[b]
2e	2-Indolyl	0	2.0 ± 2.6	4.8 ± 0.2[b]	5.7 ± 0.4[b]
2f	2-Furyl	1	−0.2 ± 1.7	0.6 ± 0.3	1.9 ± 0.5
2g	2-Thienyl	1	2.0 ± 0.9	5.0 ± 0.5[b]	5.6 ± 0.7[b]
Anthralin			3.7 ± 1.8[b]	4.9 ± 0.8[b]	5.8 ± 1.4[b]
4			0.6 ± 1.8	−0.1 ± 0.7	0.0 ± 1.0

[a]Differences of the amounts of cross-linked protein (μg cross-linked protein/mg protein) as a measure of HaCaT keratinocyte differentiation at indicated concentrations of test compounds and vehicle control. Results are the means ± SEM of three independent experiments. [b]Values are significantly different with respect to vehicle control ($p < 0.05$; Student's *t*-test).

In conclusion, within this series of heterocyclic substituted anthralin derivatives the 2-indole analogue **2e** shows potent antiproliferative activity combined with the ability to induce keratinocyte differentiation. Furthermore, the intensity to generate hydroxyl radicals of this compound is dramatically reduced as compared to anthralin. This is also reflected by markedly less damage to keratinocyte membranes and suggests an improved benefit/risk ratio.

Experimental

General procedure for the preparation of heterocyclic substituted anthralin derivatives

Method A. 1,8-Dihydroxy-10-[1-(indole-2-yl-1-oxomethyl)]-9(10*H*)-anthracenone (2e). To a solution of **1** (1.00 g, 4.42 mmol), indole-2-carboxylic acid (0.85 g, 5.30 mmol), and dicyclohexylcarbodiimide (1.10 g, 5.30 mmol) in absolute THF (30 mL) was added dry pyridine (2.0 mL) under N_2. The reaction mixture was stirred at room temperature for 4 h, filtered, and the filtrate was evaporated. The residue was purified by column chromatography (SiO_2; CH_2Cl_2) and recrystallized from benzene to afford **2e** as yellow crystals (28%): mp 265 °C (dec); ^{1}H NMR (DMSO-d_6) δ 11.94 (s, 2H), 11.80 (s, 1H), 8.19–6.96 (m, 11H), 6.62 (s, 1H); FTIR 3320, 1643, 1630 cm^{-1} (CO···HO). Anal. ($C_{23}H_{15}NO_4$) C, H, N.

Method B. *E*-10-[3-(2-Furyl)-1-oxo-2-propenyl]-1,8-dihydroxy-9(10*H*)-anthracenone (2f). To a solution of **1** (1.00 g, 4.42 mmol) in absolute toluene (75 mL) and dry pyridine (0.5 mL) was added dropwise a solution of 3-(2-furyl)propenoyl chloride[11] (0.77 g, 5.50 mmol) in absolute toluene (10 mL) under N_2. The reaction mixture was stirred at room temperature for 4 h, filtered, and the filtrate was evaporated. The residue was purified by column chromatography (SiO_2; CH_2Cl_2:hexane, 9:1) to afford **2f** as a yellow-red powder (36%): mp 169–172 °C; ^{1}H NMR ($CDCl_3$) δ 12.32 (2H), 7.58–6.35 (m, 10H), 6.08 (d, $J = 15$ Hz, 1H), 5.30 (s, 1H); FTIR 1682,

1630 cm^{-1}; MS *m*/*z* 346 (2), 121 (100). Anal. ($C_{21}H_{14}O_5$) C, H.

Biological assay methods. Degradation of 2-deoxy-D-ribose,[7] HaCaT keratinocyte proliferation assay,[21] and LDH release[8] were performed as described previously in full detail.

Cornified envelope assay. The cornified envelope is a well-recognized marker of terminal differentiation in keratinocytes.[19] The assay measures insoluble cross-linked protein envelopes based on a previous report[19] with modifications.[22] HaCaT cells were cultured as described, and protein in envelope preparations were determined by the method of Bradford.[23]

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