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Structure-Function Relationship of New Anthralin Derivatives Assayed for Growth Inhibition and Cytotoxicity in Human Keratinocyte Cultures[+)]

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HaCaT keratinocyte cultures were exposed to twelve hydrophilic anthralin derivatives 1 to 12 with substituents at C-1 and C-8 of the anthrone skeleton, of one H at C-10 and of both H's at C-10 by lacton rings (fig. 1). After 3 µM treatment growth was determined by cellular protein content, ^{3}H-thymidine- and ^{14}C-amino-acid-uptake and cytotoxicity by the release of cytoplasmic LDH into the culture medium.- In comparison to acetone control (100%) anthralin suppressed mean protein content, as well as DNA- and protein-synthesis to 33, 28, and 21%, respectively, and the drug revealed an enzyme release of 660%. In relation to the parent drug we found similar cell growth inhibitory effects of compounds 4, 6, 8, 9, 10, and 12. Deriv. 4, 8, and 10 were, however, to some extent less cytotoxic than anthralin, whereas deriv. 6, 9, and 12 were in the same range. An extreme suppression of growth parameters which differed from the anthralin effect by a factor 0.5-0.8 was caused by deriv. 11, showing the same cytotoxicity. Deriv. 1, 2, 3, 5, and 7 did not demonstrate any cytotoxicity. Concerning growth parameters, deriv. 2 induced a slight stimulation, deriv. 3 and 7 were completely ineffective, deriv. 1 and 5 induced slightly to moderately inhibited proliferation but both being much less effective than anthralin. These data indicate that the "minimum structure" concept by *Krebs* and *Schaltegger* - claiming 1-hydroxy-9-anthrone as a precondition for *clinical* antipsoriatic potency - is not valid at least in *cell-biological* tests and point toward possible usefulness of some experimental model compounds as alternative antipsoriatics.

Beurteilung von Struktur-Wirkungs-Beziehungen neuer Anthralin-Derivate aufgrund der Zellwachstumshemmung und Zytotoxizität in Humankeratinozyten-Kulturen

In HaCaT Keratinozyten-Kulturen wurden zwölf hydrophile Dithranol-Derivate 1 bis 12 untersucht, die Substituenten an C-1 und C-8 des Anthrongerüsts, an C-2, an Stelle eines H-Atoms an C-10 und an Stelle beider H an C-10 Lacton-Ringe aufweisen (Fig. 1). Nach der Behandlung mit 3 µM Substanz wurde das Zellwachstum bestimmt anhand des zellulären Proteingehalts, ^{3}H-Thymidin- und ^{14}C-Aminosäuren-Einbaus; die Zytotoxizität war durch die extrazelluläre Freisetzung der zytoplasmatischen Lactatdehydrogenase erfaßbar.- Im Vergleich zu der Aceton-Kontrolle (100%) erniedrigte Dithranol den Proteingehalt, die DNA- und Protein-Synthese im Mittel auf 33, 28 bzw. 21%, dabei kam es zu einer Enzymfreisetzung von 660%. Im Vergleich zu Dithranol fanden wir ähnliche Hemmungen des Zellwachstums für die Derivate 4, 6, 8, 9, 10 und 12. Die Derivate 4, 8 und 10 waren jedoch weniger zytotoxisch als Dithranol, wohingegen die Derivate 6, 9 und 12 diesbezüglich im gleichen Bereich lagen. Eine extreme Hemmung der Wachstumsparameter, wobei diese vom Dithranol-Effekt um einen Faktor 0.5 bis 0.8 abwichen, ergab sich für das Derivat 11, welches gleich stark zytotoxisch wirkte. Die Derivate 1, 2, 3, 5 und 7 waren nicht zytotoxisch. Bezüglich der Wachstumsparameter ließ sich feststellen, daß das Derivat 2 leicht stimulierte, die Derivate 3 und 7 vollständig unwirksam waren und die Derivate 1 und 5 das Zellwachstum leicht bis mäßig hemmten, aber die Hemmung viel geringer ausgeprägt war als die durch Dithranol. Die Ergebnisse zeigen, daß a) das von *Krebs* und *Schaltegger* begründete Konzept der "Minimalstruktur" - demzufolge die 1-Hydroxy-9-anthron-Struktur eine Voraussetzung für *klinische* antipsoriatische Wirksamkeit eines Anthrons darstellt - zumindest für *zellbiologische* Tests nicht allgemein gültig ist und weisen b) auf eine mögliche weitergehende Bedeutung einzelner dieser Modell-Substanzen als antipsoriatische Alternativ-Therapeutika hin.

Although anthralin (syn. dithranol) is a classic topical antipsoriatic, there is still a controversial debate concerning its exact biochemical mode of action. The drug is known to suppress various parameters of cell metabolism, e.g. DNA-replication and -repair[1,2,3,4], protein-synthesis[3], mitochondrial respiration[5,6,7,8], activities of cytosolic glucose-6-phosphate-dehydrogenase[9,10,11], calmodulin[12], thioredoxin-reductase[13,14], plasminogen-activator[15], arachidonic acid lipoxygenase[16], and superoxide anion generation in neutrophils[17]. Comparative dose response studies have favored mitochondrial respiration as the primary cellular target[18,19,20]. With regard to the sequel of subcellular drug effects, to a great extent the experimental difficulties derive from anthralin's instability through oxidation generating a cascade of radicals assumed to be the immediate effectors of cytotoxicity[21,22,23].

Much of the experimental work is impeded by the low degree of solubility of anthralin in aqueous systems[9,24]. This could be overcome by developing and using hydrophilic anthralin derivatives as drug models. Therefore, the present study deals with twelve more aqueous soluble anthralin derivatives (deriv. 1 to 12) with hydrophilic groups

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[+)] Dedicated to Prof. Dr. Dr. h.c. *Gerd Klaus Steigleder* on the occasion of his 65th birthday.
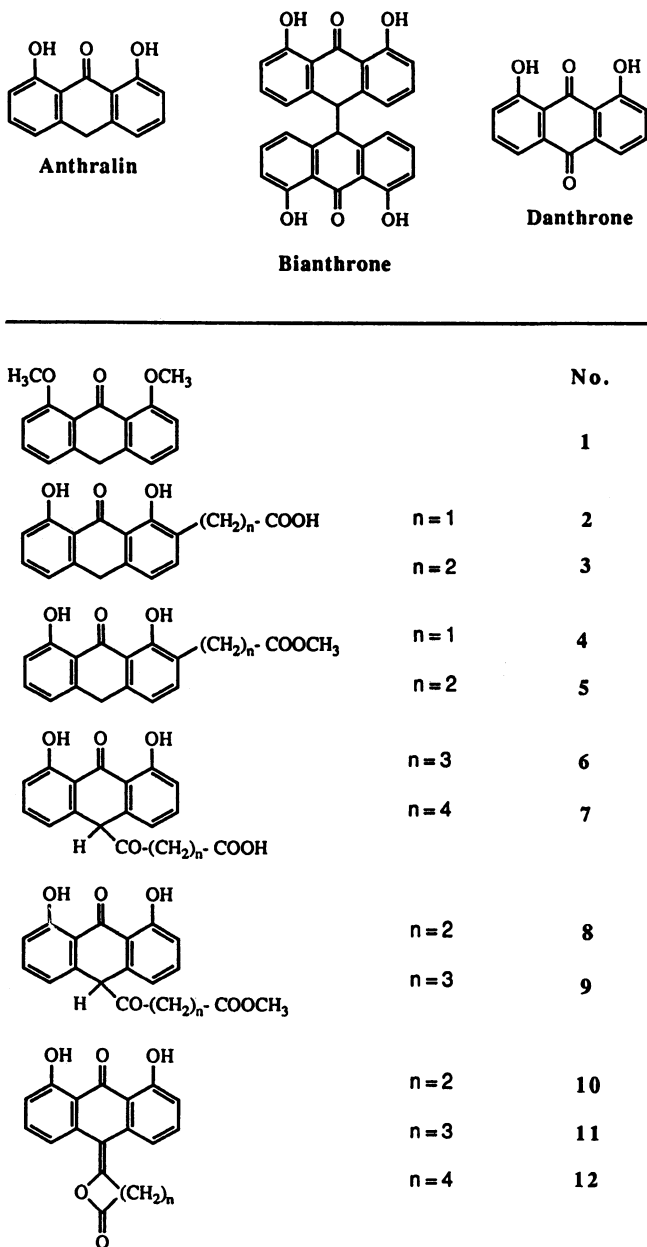
Figure 1: Structural formulas of anthralin, bianthrone, danthrone, and the anthralin derivatives 1 to 12 used in this study.

synthesized recently[24,25]. This panel of derivatives (fig. 1) contains a 1,8-dimethoxy-compound [1] as well as C-2 [2 to 5] and C-10 [6 to 12] substituted anthrones. The reason for *in vitro* screening is the ongoing search for a derivative having antipsoriatic efficacy but lacking skin irritancy or staining of skin (clothes), which both still represent the main disadvantages of clinical anthralin application.

With regard to psoriatic epidermal hyperproliferation we used HaCaT cells, a rapidly multiplying human keratinocyte line[26], as an assay system. This has been described as being extremely sensitive to anthralin as evidenced by a 50% inhibition concentration (IC_{50}) of 0.2 μM for DNA synthesis

and by a significant release of lactate dehydrogenase (indicating cytotoxicity) at a relatively low concentration of 0.3 μM[27].

Abbreviations

DMEM: Dulbecco's modified Eagle's medium G-6-PDH: glucose-6-phosphate dehydrogenase IC_{50}: 50% inhibition concentration LDH: lactate dehydrogenase

Test Compounds

Figure 1 shows the structural formulas of anthralin, its oxidation products bianthrone and danthrone, and twelve anthralin derivatives applied in this study. All test compounds were dissolved in acetone at 3.0 mM under light protection. The solutions were then diluted 1:1000 with Dulbecco's modified Eagle's medium (DMEM, Flow Cat. No. 12-332-54) which was used serum-free in order to avoid binding or inactivation of the test compounds by serum constituents[3]. All solutions were prepared immediately before use.

HPLC-Monitoring of Anthralin Stability

With regard to our special cell culture conditions we prechecked the stability of anthralin which was dissolved at 3 μM in 0.1% acetone and serum-free DMEM at 37°C under 10% CO_2. After adding the drug to the cell-free medium, several samples were taken and analysed by HPLC during the following 240 min[25].
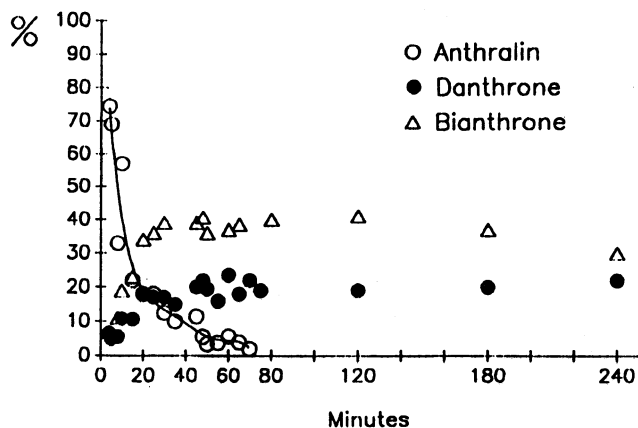
Figure 2: Decay kinetics of anthralin which was dissolved at 3 μM in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.1% acetone (37°C, 10% CO_2). The drug and its main oxidation products bianthrone and danthrone were detected by HPLC[42] and recorded as relative amounts of total substance.

Cell-Culture

HaCaT cells[*] were seeded in Greiner 24-well-multidishes and grown in DMEM, supplemented with 1% antibiotic-antimycotic-solution (Gibco Cat. No. 043-5240H), 4 mM L-glutamine (Gibco Cat. No. 043-5030H) and 10% fetal calf serum (Gibco Cat. No. 011-6290H) at 37°C in a humidified atmosphere of 10% CO_2. Exposure to test compounds was performed on subconfluent cultures at log-growth-phase, which was reached one day after plating of 4 x 10^4 cells per 1.8 cm^2 (series 1) or three days after plating of 1 x 10^4 cells (series 2). The cultures were rinsed two times with DMEM and incubated with 3.0 μM test compound in DMEM (0.1% acetone, as described above) for 30 min; control cultures were exposed to DMEM containing 0.1% acetone. Subsequently, the supernatants were dis-

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[*] HaCaT cells were kindly provided by Dr. *N.E. Fusenig* (German Cancer Research Center, Heidelberg, FRG).

carded and cultivation was continued in the complete growth medium including serum until the 2nd day after the test compound exposure.

For measurement of cytotoxicity by LDH release we employed a protocol which has been described in detail[27].- In brief, 1.8 x 10^{6} HaCaT cells were seeded in a culture flask (75 cm[2], Falcon) and grown to post-confluency for 14 days. The cells were incubated with the test compounds for 30 min (see above) and thereafter kept in 13 ml of fresh DMEM during the following 24 h (see below).

Measurement of Cellular Protein

48 h after exposure with the test compounds, the protein content of attached cells was determined by a modified Bradford method[3,28]. In brief, the cultures were rinsed twice with methanol and dried in the air. The cells were overlayered with 1.5 ml of 0.5 N NaOH per well and kept under occlusion by means of a Parafilm foil in order to prevent evaporation. After the wells had been gently agitated at room temp. for 16 h, the probes were stained with BioRad-reagent (Cat. No. 500-0006) and photometrically measured at 595 nm against bovine serum albumin standard.

Incorporation of ^{3}H-thymidine and ^{14}C-amino-acids

48 h after treatment with the test compounds cultures were pulse labeled (2 h) simultaneously with 1.0 μCi methyl-^{3}H-thymidine (specific activity 77.9 Ci/mmol, NEN DuPont, 6380 Bad Homburg, FRG, NET-027Z) and 0.5 μCi ^{14}C-amino-acids (mixture of 15 different amino acids, specific activity 1.85 GBq/milligramatom of carbon, Amersham, 3300 Braunschweig, FRG, CFB.104) in 1 ml DMEM per well of the multidish. Cells were then trypsinized with 0.05% trypsin/0.02% EDTA (Gibco Cat. No. 043-5300M) for 45 min at 37°C and precipitated with 5% trichloroacetic acid. The cell material was harvested by an Inotech apparatus and counted in a Rackbeta scintillation counter (LKB) using the two channel mode. The incorporation of ^{3}H-thymidine and ^{14}C-amino-acids per well was taken as a measure for DNA and protein synthesis of the cultures[3,29].

Lactate Dehydrogenase (LDH) Release Assay

The supernatants of cultures which had been treated with the test compounds 24 h previously were collected for the determination of the extracellular LDH activity. According to the recommendations of the German Society for Clinical Chemistry an optimized standard method of the photometric measurement was performed using a Boehringer Mannheim test kit for BM/Hitachi system 737[27,30,31].

Morphological Monitoring

48 h after incubation, cultures were examined by phase contrast microscopy. The morphological evaluation was performed with special regard to: a) density of attached cells, b) loss of regular "cobble-stone-pattern" with widened intercellular spaces, c) extent of cell detachment, d) alteration of cell shape, e.g. impeded cell-spreading, ballooning, pycnotic shrinking, fragmentation, and e) cytoplasmic changes, e.g. vacuolization or condensation[32,33].

Study Design and Statistical Analysis

The test compounds were studied for their influence on the four mentioned parameters (protein content, thymidine- and amino-acid-incorporation, LDH release) in two series of investigations. Series 1 consisted of a simultaneous testing of bianthrone, danthrone, deriv. 1, 3, 5, 7, 8, 10, and 12; deriv. 2, 4, 6, 9, and 11 were tested in series 2. Both series included a control with 0.1% acetone as well as a standard

with anthralin. Growth studies were conducted in at least six culture wells in three independent experiments (total: 18 cultures) for each compound, and the enzyme release studies were performed in three culture flasks, each in triplicate (total: 9 cultures). For each experiment and parameter the mean value of the acetone control was taken as 100%. Statistical comparisons on the relative data pooled from the three experiments were performed according to Mann and Whitney (U-test[34]). Multiple comparisons required Bonferroni's adjustment[35], with an overall level of significance α < 0.05 corresponding to p < 0.005 (series 1) and p < 0.0083 (series 2).

Results

HPLC monitoring revealed that under cell-free conditions anthralin dissolved with acetone 0.1% in DMEM (37°C, 10% CO_2) decomposed at a rate of 31% at 5 min, 78% at 15 min, 87% at 30 min, and 98% at 70 min. The main oxidation products found were bianthrone and danthrone reaching a plateau of 41% and 24% at 48 and 60 min, respectively. These data (fig. 2) prompted us to restrict the exposure time for all compounds to 30 min.

The data of the protein content, ^{3}H-thymidine- and ^{14}C-amino-acid-incorporation of attached cells are compiled in table 1, 2, and 3. Setting the values for acetone at 100% (control) we found a similar inhibition of cell growth by anthralin in both series 1 and 2, that means a reduced mean cellular protein content of 31 and 35%, DNA-synthesis of 19 and 36% as well as protein-synthesis of 16 and 25%, respectively. Statistical comparison of these data for acetone and anthralin with the growth values of the different test compounds are shown in tables 1, 2, and 3.

In both series the mean LDH release by anthralin exceeded that of the acetone controls by a factor > 6. These results, which represent a measure for cytotoxicity, are calculated on a percentage level in table 4, which also shows statistical comparison of the different compounds with the acetone control and anthralin.

In the phase contrast microscope we found morphological alterations which were strictly correlated with the above mentioned effects on cell growth and toxicity-dependent LDH release of the tested compounds. Thus the state of cell growth and the cytotoxicity were reflected by the density of adherent keratinocytes and the cell degeneration phenomena described in detail under "Material and Methods". Representative examples of the morphological findings after treatment with the rather effective deriv. 10 and the uneffective deriv. 3 are given in fig. 3.

Discussion

"Minimum structure" concept

Krebs and Schaltegger[36,37] tested about 30 different anthrones for their efficacy in psoriatic patients. They found a potent effect only for anthralin, chrysarobin (1,8-dihydroxy-3-methyl-9-anthrone), 1-hydroxy-9-anthrone, and 1,8,9-
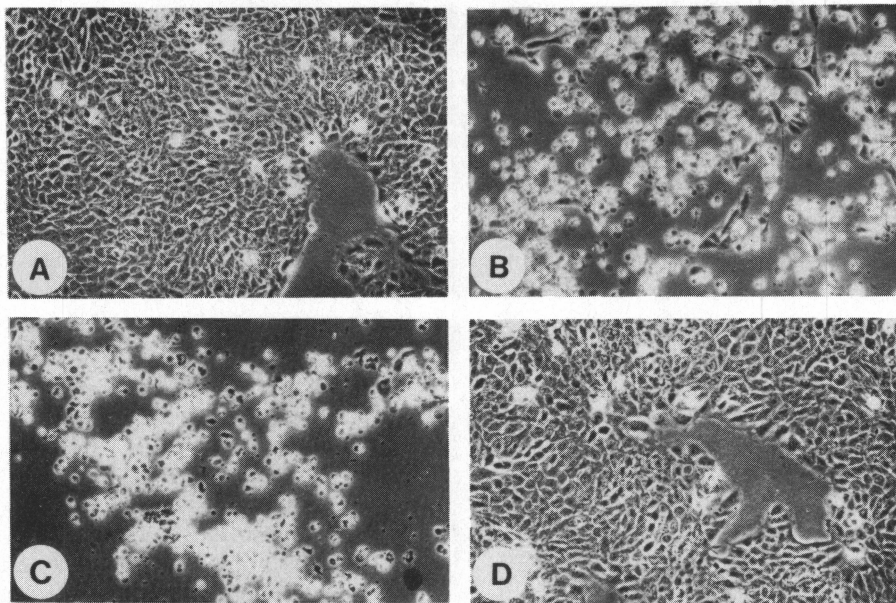
Figure 3: Phase contrast microscopy of cultured HaCaT keratinocytes two days after 30 min treatment with 0.1% acetone (A), with 3.0 μM anthralin (B), and with 3.0 μM of **10** (C) and **3** (D). In comparison to the acetone control, anthralin and deriv. **10** revealed signs of marked inhibition of cell growth and cytotoxicity, i.e. reduced density of attached cells, increased number of detached and deformed cells. With deriv. **3** there was no obvious difference compared to the control (60x).

Table 1: Protein content of attached HaCaT keratinocytes per culture two days after exposure to anthralin derivatives **1** to **12**. Significant differences at an overall level α < 0.05 are indicated by asterisks.

Incubation	Series	Protein content		
Test Compound		Percentage (mean ± s. d.)	Statistical Comparison (p) of the Test compound versus	
			Acetone	Anthralin
Acetone	1	100.0 ± 2.5	-	0.0000*
Anthralin	1	31.0 ± 15.4	0.0000*	-
Bianthrone	1	31.8 ± 12.8	0.0000*	0.4619
Danthrone	1	89.4 ± 12.0	0.0014*	0.0000*
Deriv. 1	1	90.8 ± 8.8	0.0021*	0.0000*
Deriv. 3	1	103.0 ± 5.1	0.0736	0.0000*
Deriv. 5	1	67.0 ± 9.9	0.0000*	0.0000*
Deriv. 7	1	106.3 ± 10.0	0.0290	0.0000*
Deriv. 8	1	28.1 ± 8.5	0.0000*	0.9129
Deriv. 10	1	27.3 ± 7.3	0.0000*	0.9626
Deriv. 12	1	24.5 ± 7.4	0.0000*	0.3888
Acetone	2	100.0 ± 10.8	-	0.0000*
Anthralin	2	34.9 ± 7.9	0.0000*	-
Deriv. 2	2	107.6 ± 16.2	0.0024*	0.0000*
Deriv. 4	2	35.5 ± 14.0	0.0000*	0.5657
Deriv. 6	2	47.9 ± 24.6	0.0000*	0.0120
Deriv. 9	2	32.0 ± 12.2	0.0000*	0.1447
Deriv. 11	2	26.8 ± 10.5	0.0000*	0.0014*

Table 2: DNA-synthesis per culture two days after exposure of HaCaT keratinocytes to various test compounds. Significant differences at an overall level $\alpha < 0.05$ are indicated by asterisks.

Incubation Test Compound	Series	^{3}H-Thymidine Incorporation		
		Percentage (mean ± s. d.)	Statistical Comparison (p) of the Test compound versus	
			Acetone	Anthralin
Acetone	1	100.0 ± 19.1	-	0.0000*
Anthralin	1	19.2 ± 12.3	0.0000*	-
Bianthrone	1	33.2 ± 24.0	0.0000*	0.0186
Danthrone	1	94.1± 25.6	0.3888	0.0000*
Deriv. 1	1	93.9 ± 20.7	0.3550	0.0000*
Deriv. 3	1	101.9 ± 12.8	0.2788	0.0000*
Deriv. 5	1	75.6 ± 17.0	0.0001*	0.0000*
Deriv. 7	1	108.7 ± 10.4	0.0847	0.0000*
Deriv. 8	1	12.3 ± 3.4	0.0000*	0.0320
Deriv. 10	1	11.4 ± 4.3	0.0000*	0.0083
Deriv. 12	1	7.8 ± 4.8	0.0000*	0.0000*
Acetone	2	100.0 ± 26.4	-	0.0000*
Anthralin	2	36.1 ± 8.5	0.0000*	-
Deriv. 2	2	148.4 ± 44.1	0.0000*	0.0000*
Deriv. 4	2	38.1 ± 7.0	0.0000*	0.1710
Deriv. 6	2	48.5 ± 15.8	0.0000*	0.0010*
Deriv. 9	2	21.1 ± 5.9	0.0000*	0.0000*
Deriv. 11	2	22.5 ± 11.5	0.0000*	0.0000*

triacetoxy-anthracene; the last had been introduced by *Hellier* and *Whitefield*[38]. *Krebs* and coworker claimed 1-hydroxy-9-anthrone as the "minimum structure" of an anthrone required for clearing up psoriasis. In detail this "minimum structure" should consist of a) a hydroxy group at C-1, b) a carbonyl group at C-9, and c) a C-10 methylene group (two hydrogens). Determining the activity of isolated glucose-6-phosphate dehydrogenase (G-6-PDH), which as a key enzyme for hexosemonophosphate shunt is known to be significantly elevated in psoriatic tissue[10], *Tanzer* et al.[25] demonstrated that the "minimun structure" compound had an IC$_{50}$-value of 9.4 µM which was very similar to 8 µM for anthralin.

Significance of free hydroxy group at C-1

In accordance with the "minimum structure" concept we found that deriv. 1 characterized by methoxy groups at C-1 and C-8 was not cytotoxic and inhibited the cell growth parameters to only a very low degree. These data are in good agreement with the findings for a very similar compound, namely 1,8,9-trimethoxy-anthracene, which proved to be ineffective against psoriatic lesions[39]. However, contrary to the "minimum structure" concept, an animal study by *Plumier* et al.[40] on 1,8,9-triacetoxy-anthracene revealed that the antiproliferative activity of this derivative, even

though less than that of anthralin, did not depend on degradation to a "minimum structure" compound. Thus, the study denied the pro-drug character of 1,8,9-triacetoxy-anthracene which had initially been suggested[36,37,41]. Also not in favor of the "minimum structure" concept was the finding that the topically applied antiproliferatively active 1,8-diacetoxy-anthracene was not converted to a compound with a free C-1 hydroxy group[40]. In contrast to the results with the HaCaT keratinocytes we recently found nonphenolic anthrones to be more active as inhibitors of arachidonic acid lipoxygenase from bovine polymorphonuclear leucocytes than anthralin, suggesting a different mode of action[42].

Influence of C-2 substitution

Deriv. 2 and 3, which were anthrones substituted at C-2 with acetic and propionic acid increments, respectively, did not reveal any cytotoxicity or inhibition of cell growth at the 3 µM concentration tested. With deriv. 2 we even found a slightly increased cell growth. These data correspond well to G-6-PDH studies demonstrating rather high IC$_{50}$ values for deriv. no. 2 (38 µM) and 3 (18 µM) with regard to anthralin (8 µM[25]).

4 and 5 are methyl esters of 2 and 3. With deriv. 4 we found an identical decrease of cell growth and a reduced cytotoxicity in comparison to anthralin. For deriv. 5 cyto-

Table 3: Protein-synthesis per culture two days after exposure of HaCaT keratinocytes to various test compounds. Significant differences at an overall level $\alpha < 0.05$ are indicated by asterisks.

Incubation	Series	^{14}C-Amino Acid Incorporation		
Test Compound		Percentage (mean ± s. d.)	Statistical Comparison (p) of the Test compound versus	
			Acetone	Anthralin
Acetone	1	100.0 ± 17.3	-	0.0000*
Anthralin	1	16.2 ± 15.5	0.0000*	-
Bianthrone	1	25.0 ± 21.6	0.0000*	0.0449
Danthrone	1	91.3 ± 28.6	0.1916	0.0000*
Deriv. 1	1	94.1 ± 19.4	0.3079	0.0000*
Deriv. 3	1	104.1 ± 11.7	0.1708	0.0000*
Deriv. 5	1	72.4 ± 14.6	0.0000*	0.0000*
Deriv. 7	1	109.4 ± 11.5	0.0847	0.0000*
Deriv. 8	1	8.0 ± 2.0	0.0000*	0.0060
Deriv. 10	1	6.8 ± 1.6	0.0000*	0.0003*
Deriv. 12	1	3.4 ± 1.3	0.0000*	0.0000*
Acetone	2	100.0 ± 24.4	-	0.0000*
Anthralin	2	25.4 ± 8.3	0.0000*	-
Deriv. 2	2	119.1 ± 22.0	0.0004*	0.0000*
Deriv. 4	2	27.1 ± 6.6	0.0000*	0.4345
Deriv. 6	2	35.8 ± 17.3	0.0000*	0.0036*
Deriv. 9	2	12.2 ± 3.2	0.0000*	0.0000*
Deriv. 11	2	13.0 ± 6.1	0.0000*	0.0000*

toxicity was lacking and slowing down of cell growth was much less pronounced than with anthralin.

Significance of ligands replacing one hydrogen atom at C-10

The "minimum structure" concept - claiming free hydrogens at C-10 for *clinical* antipsoriatic activity - was developed from the observation that capaloin, an anthralin derivative glucosylated at C-10, did not clear up psoriasis[36,37]. Our data demonstrate that this postulation does not have general validity. Thus, the acid 6 effected an equal cytotoxicity and an only slightly reduced inhibition of cell growth as compared with anthralin. The homologous acid 7 was, however, completely inefficacious. Deriv. 8 and 9 are esters. In the case of 8 we found a decreased cytotoxicity with an equal inhibition of cell growth, and with 9 an equal cytotoxicity with a pronounced inhibition of cell growth, in comparison with anthralin. The ranking of drug potency by means of the HaCaT keratinocyte culture system was rather similar to that by means of the G-6-PDH assay[25] for deriv. 6, 8, and 9. In the case of 7 there was, however, a marked discrepancy between complete ineffectiveness in cell culture and an extremely low IC$_{50}$-value of 0.9 µM in the G-6-PDH assay, which was more than eightfold lower than the reference value of anthralin.

Deriv. 8 and 9 also show activity similar to that of anthralin in the inhibition of leucotriene production; the acids 6 and 7, however, were more potent than anthralin. Their IC$_{50}$-values for 5-lipoxygenase-inhibition were found to be between 5 and 10 µM (anthralin: IC$_{50}$ ≈ 40 µM)[42].

Concerning anthralin derivatives bearing acyl chains of different length at C-10 Mustakallio[43,44] has already stated that at this position substituents less bulky than glucose do not hamper antiproliferative activity. Thus, in short contact therapy butantrone (syn. 10-butyryl-anthralin) was beneficial in the treatment of psoriasis. Its efficacy as well as irritancy and staining were, however, less than with anthralin. When conventionally applied for several h per day over several weeks butantrone caused increased irritation as compared to anthralin. This was explained by a drug accumulation because of retarded degradation. The compound lacked any pro-drug character since anthralin was not produced as evidenced by HPLC analysis[45]. In HeLa cell cultures the antiproliferative activity of butantrone was confirmed[46], as was the case for the so-called 10-acetyl-anthralin, another C-10-derivative, which inhibited thymidine incorporation in human skin fibroblasts[47].

As a special example of C-10-liganding we studied bianthrone which - on an equimolar base - has an identical degree of cytotoxicity and growth inhibitory potency as anthralin. Conversely, in psoriasis *Krebs* and *Schaltegger* did

Table 4: Activity of lactate dehydrogenase in the supernatants of keratinocyte cultures one day after treatment with anthralin, bianthrone, danthrone or anthralin derivatives 1 to 12 for 30 min. The enzyme release from cytoplasm was taken as measure for cytotoxicity implying substantial plasma membrane damage. Significant differences at an overall level $\alpha < 0.05$ are indicated by asterisks.

Incubation	Series	Lactate Dehydrogenase release		
Test Compound		Percentage (mean ± s. d.)	Statistical Comparison (p) of the Test compound versus	
			Acetone	Anthralin
Acetone	1	100.0 ± 36.7	-	0.0000*
Anthralin	1	641.1 ± 195.3	0.0000*	-
Bianthrone	1	659.7 ± 286.2	0.0000*	1.0000
Danthrone	1	150.1 ± 65.8	0.0625	0.0000*
Deriv. 1	1	129.5 ± 44.2	0.2973	0.0000*
Deriv. 3	1	75.9 ± 32.4	0.1135	0.0000*
Deriv. 5	1	126.5 ± 42.6	0.3865	0.0000*
Deriv. 7	1	161.7 ± 51.3	0.0188	0.0000*
Deriv. 8	1	392.8 ± 74.3	0.0000*	0.0008*
Deriv. 10	1	228.2 ± 73.7	0.0008*	0.0000*
Deriv. 12	1	434.7 ± 156.4	0.0000*	0.0315
Acetone	2	100.0 ± 34.7	-	0.0000*
Anthralin	2	685.0 ± 97.8	0.0000*	-
Deriv. 2	2	101.8 ± 29.6	0.6665	0.0000*
Deriv. 4	2	416.0 ± 170.5	0.0002*	0.0012*
Deriv. 6	2	640.1 ± 147.8	0.0000*	0.6048
Deriv. 9	2	803.6 ± 207.5	0.0000*	0.1359
Deriv. 11	2	712.1 ± 177.3	0.0000*	0.3401

not observe a clearing efficacy of this dimeric compound[36,37], and it did not influence cell cycle kinetics in hairless mouse epidermis[48]. With regard to former in vitro studies the findings for bianthrone are also conflicting. It thus inhibited in comparison to anthralin a) the thymidine incorporation, glutamine respiration and CO_2 production from glucose of SV-40 transformed human keratinocytes[3] as well as mitochondrial membrane functions[5] each to a lower degree and b) the thymidine incorporation and growth in human skin fibroblasts to a higher degree[47,49]. Bianthrone did not alter the migration and superoxide anion production from human neutrophils in comparison to a solvent control[17]. In vitro bianthrone produces the same radical species and decomposition products as anthralin[50].

Significance of ligands replacing both hydrogens at C-10

Deriv. 10, 11, and 12 bear lacton rings at C-10 consisting of four, five and six C-atoms, respectively. The keratinocyte growth inhibitory efficacy of all three derivatives exceeded that of anthralin, and deriv. 11 exerted the maximum effect ever recorded in our experiments. Therefore, it seems to be excluded that these anthralin derivatives act as pro-drugs by degradation to anthralin. In good agreement with these re-

sults is the finding that deriv. 10 to 12 are potent lipoxygenase inhibitors, as well[42]. Their IC$_{50}$-values were found to be about 2 μM in our test system for derivatives 11 and 12; deriv. 10 was slightly less active (IC$_{50}$ ≈ 3 μM). We found an identical cytotoxicity for deriv. 11 and 12 as compared to anthralin; a to some extent lower cytotoxic effect was only found in the case of deriv. 10.

With regard to substitution of both hydrogens at C-10, danthrone, clinically lacking any antipsoriatic quality[36,37], may be regarded as a special case. Under our assay conditions this degradation product did not reveal any cytotoxicity. We found only a very minor inhibition of cell growth. In the micromolar range of concentration danthrone was, however, completely inactive concerning the proliferation as well as glucose- and glutamine-metabolism in keratinocyte cultures[3], the thymidine incorporation in fibroblasts[47] and the motility and superoxide anion generation of neutrophils[17]. Concerning a multifunctional analysis of the interaction with the inner mitochondrial membrane danthrone is found to be almost inactive[5]. The slight growth inhibition effects observed in the HaCaT cells under danthrone might be explained by their especially high sensitivity due to a relatively short population doubling time of 21 h under the subconfluent growth conditions[26].

Conclusion

There is good evidence by clinical and *in vitro* studies on different compounds (i.e. anthralin, danthrone, butantrone) that the antipsoriatic efficacy of a drug may be tested by its antiproliferative and cytotoxic effects in cell cultures[1,3,29,46]. Employing such a keratinocyte assay the present results on anthralin derivatives mostly reveal a good correlation with the results gained by evaluating compounds' effects on G-6-PDH activity[25]. There was only one compound [7] with a striking discrepancy between the cell culture and the enzyme assays. In the case of anthralin the sensitivity of the enzyme system ($IC_{50} = 8\mu M$[25]) is much lower as compared to the more complex HaCaT keratinocyte system ($IC_{50} = 0.2 \mu M$ for ^3H-thymidine-incorporation[27]). This obviously excludes G-6-PDH as a primary target for the drug's action. This conclusion is corroborated by studies concerning the intracellular distribution of ^{14}C-anthralin, revealing that the cytosol remained essentially unlabeled[3].

Substitution not only at C-1 (deriv. **1**) and C-10 (deriv. **7**) of the anthrone skeleton but also at C-2 (deriv. **3**) may cause loss of efficacy, and, paradoxically, with an acetic acid ligand at C-2 (deriv. **2**) we even observed a slight stimulation of cell growth. Among the anthralin derivatives tested we found seven compounds (deriv. **4, 6, 8, 9, 10, 11, 12**) exerting cell growth inhibitory and cytotoxic effects approximating or exceeding those of anthralin. For future investigations these substances would be of special interest as hydrophilic anthralin model compounds and antipsoriatic drugs.

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