6-[(Aminoalkyl)amino]-Substituted 7H-Benzo[e]perimidin-7-ones as Novel Antineoplastic Agents. Synthesis and Biological Evaluation

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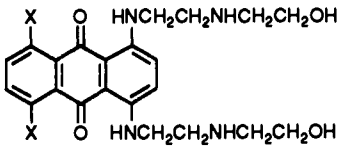
A class of chromophore-modified anthracenediones with an additional pyrimidine ring incorporated into the chromophore system has been obtained in an attempt to provide compounds with diminished peroxidation activity and thus potentially lowered cardiotoxicity. Their synthesis was carried out by the reaction of 6-amino- or 6-hydroxy-7H-benzo[e]perimidin-7-one with a number of alkylamines. Potent activity was demonstrated in vitro against murine L1210 leukemia cells (equipotent with ametantrone) as well as against P388 leukemia in vivo (% T/C = 130–255). We observed that the benzoperimidines did not stimulate free radical formation, perhaps due to their poor substrate properties for NADH dehydrogenase.

Introduction

The anthracycline antibiotics (daunorubicin, DR; adriamycin, ADR) constitute a very important group of antitumor agents.[1] However, their practical use is limited by associated toxicity, especially the risk of cardiotoxicity.[2] Synthetic anthraquinone derivatives, such as mitoxantrone (**1a**) and ametantrone (AME, **1b**), have been shown to have outstanding antineoplastic activity in animals,[3] but cardiotoxicity of **1a** has been reported recently.[4]



1a: X = OH
b: X = H

The mechanism of anthracycline cardiac toxicity remains incompletely understood, but numerous studies have suggested that the cardiotoxicity of this class may be associated in part with the formation of reactive oxygen species and subsequent intracellular lipid peroxidation from enzymatic reduction of the quinone chromophore to a semiquinone radical species.[5]

Anthraquinones can be reduced in cells by flavoprotein enzymes (xanthine oxidase, NADPH-cytochrome P-450 reductase, NADH dehydrogenase).[6] In mitochondria NADH dehydrogenase of the respiratory chain is the site of anthraquinone activation to the reduced form.

Previous studies suggest that the capacity of anthraquinones to produce reactive oxygen species depends on two factors: (1) the redox properties (one electron reduction of oxygen can occur via anthraquinone anion radical[7a] or by direct reduction of quinone–singlet oxygen complex[7b]), and (2) the substrate properties of these compounds for the enzyme catalyzing their one-electron reduction.[8]

Many efforts have been directed toward the design of noncardiotoxic anthraquinone drugs. Among these were the synthesis of 5-iminoadriamycin (Im-ADR) and 5-iminodaunorubicin (Im-DR) with reduced peroxidating activity.[9] The rationale for their synthesis was the expected diminished electron affinity of the derivatives while

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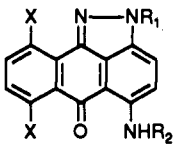
retaining some of the planar, spatial, and electronic characteristics of the parent quinoid structure necessary for molecular recognition and DNA binding. The same approach was used in the synthesis of anthrapyrazoles **2**, the quasi-iminoquinone derivatives of synthetic anthracenediones.[10] Indeed, these iminoanthraquinone derivatives have exhibited diminished cardiotoxicity in preclinical models.[9b,10b]



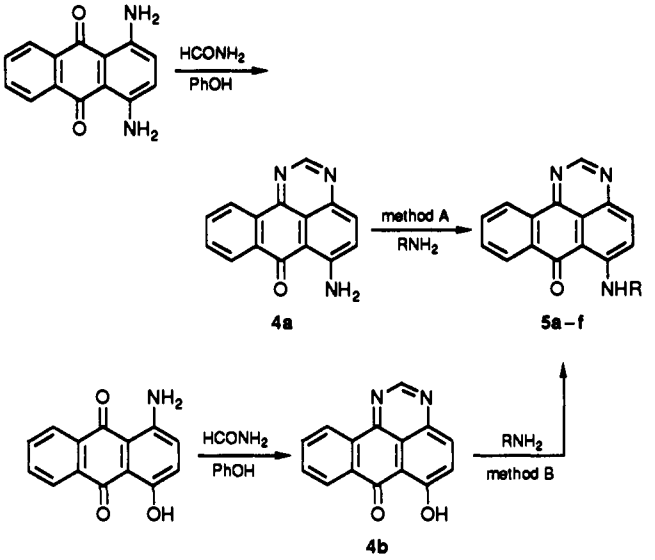
2a: X = H
b: X = OH

R_1 = H, (alkylamino)alkyl
R_2 = H, (alkylamino)alkyl

In our previous studies on the mechanism of peroxidating activity of anthraquinones, we have postulated that it is not the electron affinity but the enzyme substrate properties that are the major factor in stimulating oxygen radical production.[8,11] A number of functional groups essential for the affinity of anthraquinones to the NADH dehydrogenase (the enzyme responsible for the mitochondrial reduction of anthraquinones) have been identified in our investigations with synthetic model compounds.[11] Among them the presence of both quinone carbonyl groups is indispensable. The substitution of one carbonyl by an imino group results in the decrease of substrate affinity.[8,11] The reduced peroxidating activity of Im-ADR, Im-DR, and their derivatives[12] as well as of anthrapyrazoles[10b] corroborates well with these findings.

The favorable properties of the 5-iminoanthracyclines prompted us to attempt the synthesis of the imino analogs of ametantrone (AME). However the synthesis of 10-imino-1,4-bis[[(alkylamino)alkyl]amino]-9-anthracenones failed; instead, we obtained only the slightly biologically active 1-[(aminoalkyl)amino]-4-hydroxy-10-imino-9-anthracenones.[13] In contrast, the anthrapyrazoles in which the central quinone moiety has been modified to a quasi-iminoquinone display very high antitumor activity.[10]

Scheme I



The above findings led us to synthesize the 6-[(aminoalkyl)amino]-substituted 7*H*-benzo[*e*]perimidin-7-ones (hereafter referred as benzoperimidines) in which an additional pyrimidine ring has been incorporated into the anthracenedione chromophore. We hoped that derivatives **5a–f** (see Scheme I) might provide agents with diminished peroxidating properties.

Chemistry

6-[(Aminoalkyl)amino]-substituted benzoperimidines can be obtained by conventional methods[14a,b] using 6-aminobenzoperimidine (method A) or 6-hydroxybenzoperimidine (method B) as starting materials (see Scheme I). 6-Aminobenzoperimidine **4a** was prepared by the reaction of 1,4-diamino-9,10-anthracenedione with formamide in boiling phenol;[14c] under similar conditions, 6-hydroxybenzoperimidine **4b** was synthesized from 1-amino-6-hydroxy-9,10-anthracenedione. The reaction of **4a** or **4b** with appropriate aliphatic amines led to the desired 6-[(aminoalkyl)amino]-7*H*-benzo[*e*]perimidin-7-ones (**5a–f**). It has been noted that the transamination of 6-aminobenzoperimidines with ethylenediamine and N-monosubstituted ethylenediamines may be accompanied by a subsequent cyclization step to form side products as in the reaction of quinizarine with such amines.[2d,15] However, by using 6-hydroxybenzoperimidine (**4b**) as the starting material, we found that this reaction proceeds under milder conditions, thus avoiding cyclization and formation of tar-like byproducts (reaction conditions are indicated in Table I). Unfortunately the synthesis of this substrate is troublesome.

The structures of compounds **4b**, **5a–f** were confirmed and characterized by their spectral data (^{1}H NMR, IR, UV–vis, FDMS) and elemental analysis (see Table I).

Biological Results

The 6-[(aminoalkyl)amino]-substituted benzoperimidines **5a–f** were tested for the growth inhibition of L1210 murine cells in vitro and against P388 murine leukemia

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Table I. Synthesis and Physicochemical Properties of Benzoperimidines 5a,b

no.	R	method	reaction conditions: time (h)	reaction conditions: temp (°C)	yield (%)	mp, °C dec	molecular formula
5a	$CH_2CH_2NHCH_2CH_2OH$	A	4	120	62	268–271	$C_{19}H_{18}N_4O_2 \cdot 1.1HCl$
		B	2	90	75		
5b	$CH_2CH_2N(CH_3)_2$	A	9	110	32	211–214	$C_{19}H_{18}N_4O \cdot HCl \cdot 0.5H_2O$
		B	2.5	100	50		
5c	$CH_2CH_2N(C_2H_5)_2$	A	4	145	84	254–256	$C_{21}H_{22}N_4O \cdot HCl \cdot 0.6H_2O$
5d	$CH_2CH_2NHCH_3$	A	4	120	50	260–265	$C_{18}H_{16}N_4O \cdot HCl \cdot H_2O$
		B	2	90	74		
5e	$CH_2CH_2NH_2$	A	2	120	55	230–238	$C_{17}H_{14}N_4O \cdot HCl \cdot H_2O$
		B	1	100	65		
5f	$CH_2CH_2CH_2NH_2$	A	2	140	65	240–243	$C_{18}H_{16}N_4O \cdot HCl \cdot 1.1H_2O$
		B	1	100	81		

Table II. Cytotoxic and Antileukemic Activity of Benzoperimidines 5a–f against L1210 Leukemia Cells and P388 Murine Leukemia

compd	L1210 cells: EC_{50} + SEM[a] (nm)	P388 murine leukemia: dose (mg/kg)	% T/C[b]	ToxD/Surv[c]
4a	945 ± 116	100	100	0/7
		200	100	0/7
5a	296 ± 49	50	173	7/7
		100	255	7/7
5b	203 ± 22	50	155	6/6
		100	209	6/6
5c	460 ± 38	50	140	5/7
		100	180	7/7
5d	268 ± 79	50	146	6/6
		100	164	6/6
5e	379 ± 62	25	140	7/7
		50	150	7/7
5f	546 ± 38	50	127	7/7
		100	127	4/7
AME	275 ± 61	3.12	190	6/6
		6.25	210	6/6
		12.50	300	6/6

[a] EC_{50} ± SEM = concentration of compound required to inhibit the cellular protein biosynthesis by 50%. Each experiment was run at least four times and the results are presented as an average value ± standard error of the mean (SEM). [b] % T/C = ratio of medium survival time of the treated to the control mice expressed as a percentage. % T/C values ≥125 are considered indicative of significant activity. [c] ToxD/Surv = ratio of toxic deaths/survivors 7 days after the day 5 injection as a measure of drug toxicity.

in vivo. The results are presented in Table II. All the evaluated compounds (5a–f) exhibited cytotoxicity in vitro comparable to that of ametantrone, but variable antitumor activity in vivo. The optimal derivative 5a has an ametantrone side arm in position 6; a good antileukemic activity has been also shown for compounds 5b–e, while 5f is less active. Compound 4a without a basic side chain is inactive.

The effect of 6-[(aminoalkyl)amino]-substituted benzoperimidines on NADH oxidation, as a measure of potential free radical production, has also been determined. The results are presented in Table III. In contrast to the reference compounds ADR and DR, the benzoperimidines, as well as 5-Im-DR, AME, and anthrapyrazole derivative CI-942, were not effective in stimulating NADH oxidation.

The substrate properties of the tested compounds with regard to NADH dehydrogenase were determined according to our method which is based on the ability of the compounds to decrease the amount of enzymatic cytochrome c reduction at a low concentration of NADH.[16]

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Table III. NADH Oxidation by Benzoperimidines[a]

compd	activity of NADH dehydrogenase (units/mL)	NADH oxidation (μmol/min)
ADR	0.15	30.1 ± 3.5
DR	0.15	33.0 ± 3.7
Im-DR	0.15	7.1 ± 0.7
AME	0.75	8.8 ± 0.6
CI-942[b]	0.75	4.9 ± 0.4
5a	0.75	7.4 ± 0.6
5b	0.75	6.5 ± 0.3
5c	0.75	7.0 ± 7.4
5d	0.75	7.6 ± 0.4
5e	0.75	4.5 ± 0.3
5f	0.75	4.7 ± 0.1

[a] The reaction mixture contained 100 μM test compound, 100 μM NADH, indicatd activity of NADH dehydrogenase, and 0.05 M HEPES (pH 7.4). [b] CI-942 = 5-[(3-aminopropyl)amino]-2-[2-[(2-hydroxyethyl)amino]ethyl]anthra[1,9-*cd*]pyrazol-6(2*H*)-one dihydrochloride.

Table IV. Substrate Properties of Benzoperimidines with Regard to NADH Dehydrogenase[a]

compound	IC_{10} (μM)[b]
benzoperimidines 5a–f	>300
daunorubicin	2
ametantrone	114

[a] The reaction mixture contained 100 mM cytochrome c, 0–200 μM of test compound, 10 μM NADH, 0.125 units/mL NADH dehydrogenase, 50 μg/mL SOD, and 0.50 M HEPES (pH 7.4). [b] IC_{10} = concentration of substrate at which the amount of total cytochrome *c* reduction is decreased by 10%.

The results are expressed by an IC_{10} parameter which is defined as the concentration of substrate at which the amount of total cytochrome *c* reduction is decreased by 10%. The IC_{10} for the tested benzoperimidines were above 300 μM (Table IV). For comparison, the IC_{10} for daunorubicin and ametantrone is 2 and 114 μM, respectively. Unfortunately the determination of IC_{10} for Im-ADR and Im-DR was not possible, because the compounds exhibit a peak absorption at 550 nm resulting in artifactual readings; neither was the IC_{10} measured for anthrapyrazoles as they reduce the cytochrome c without enzyme participation (unpublished data).

The results in Table IV indicate that the very low peroxidizing activity of benzoperimidine derivatives is related to the very poor enzyme substrate properties of these compounds.

Experimental Section

Melting points, determined with a Boeticus PHMK05 apparatus, are uncorrected. Analyses are within ±0.4% of the theoretical values and were carried out by the Laboratory of Elemental Microanalysis, University of Camerino. A Beckman 3600 spectrophotometer was used for UV spectral determination and for measurements of NADH oxidation. IR spectra were

recorded on a UR 10 Zeiss spectrometer in KBr pellets; ^{1}H NMR spectra were taken on a Varian 300 MHz spectrometer in Me_2SO-d_6 using tetramethylsilane as internal standard. Molecular weights were determined by mass spectrometry (FD) on a Varian MAT 711 instrument. The instrumental conditions were the following: wire heating current 5–20 mA, ion source temperature 70–100 °C, accelerating voltage 4–6 kV. Column chromatography was performed on silica gel 60 Merck (70–230 mesh) and on Sephadex LH-20 (Pharmacia).

Cytochrome c (type VI from horse heart), NADH (grade III), and cardiac NADH dehydrogenase were obtained from Sigma Chemical Corp., St. Louis, MO. Stock solutions were prepared just prior to use.

6-Amino-7*H*-benzo[*e*]perimidin-7-one (4a). A mixture of 2.4 g (10 mmol) of 1,4-diaminoquinizarin and 10 mL (250 mmol) of formamide was heated at reflux in 50 g of phenol for 5 h. The course of reaction was monitored by TLC in the solvent system toluene/acetone 5:1 or $CHCl_3$/MeOH/25% aqueous NH_4OH 5:1:1. The reaction mixture was diluted with $CHCl_3$ and washed several times with dilute NaOH and then with water. The $CHCl_3$ layer was evaporated in vacuo, and the crude product was triturated with hexane and then crystallized from 10:1 MeOH/$CHCl_3$ or pyridine to afford 1.8 g (75%) of **4a**: mp 270–275 °C (lit.[14c] mp 277 °C); ^{1}H NMR (d_6-DMSO) δ 7.7 (d, 1, J = 9 Hz), 7.9 (m, 2, J = 8.5 Hz), 8.05 (d, 1, J = 8 Hz), 8.45 (dd, 1, J = 9 Hz), 8.9 (dd, 1, J = 7.5 Hz), 9.3 (s, 1), 10.2 (br s, 2).

6-Hydroxy-7*H*-benzo[*e*]perimidin-7-one (4b). A mixture of 2.4 g (10 mmol) of 1-amino-4-hydroxyquinizarin and 10 mL (250 mmol) of formamide was heated at reflux in 50 g of phenol for 3 h. The course of reaction was monitored by TLC as described above. The reaction mixture was diluted with $CHCl_3$ and extracted several times with 25% H_2SO_4. NaOH (1 N) was added to the cold acidic layer to pH 3–4, which was then extracted several times with $CHCl_3$. The $CHCl_3$ layer was washed with water and evaporated to afford 1.2 g of the crude **4b**, which was used for further reaction without purification. For analytical data a sample of **4b** was purified using silica gel column chromatography ($CHCl_3$/MeOH, 80:1) and then crystallized from pyridine: mp >300 °C dec; ^{1}H NMR (d_6-DMSO) δ 7.3 (d, 1, J = 9 Hz), 7.75 (d, 1, J = 8.5 Hz), 7.9 (m, 2), 8.5 (d, 1, J = 9 Hz), 8.95 (d, 1, J = 9 Hz), 9.3 (s, 1); IR (KBr, major peaks, cm^{-1}) 1575, 1625; FDMS m/z (relative intensity, %) 248 ([M]$^+$, 100). Anal. ($C_{15}H_8N_2O_2$·0.05H_2O) C, H, N.

General Procedure for the Synthesis of Compounds 5a–f. Method A. 6-[[2-[(2-Hydroxyethyl)amino]ethyl]amino]-7*H*-benzo[*e*]perimidin-7-one (5a). A sample of **4a** (0.25 g, 1 mmol) was refluxed with 2 mL (18 mmol) of 2-(2-aminoethyl)-ethylamine in 3 mL of *N,N,N′,N′*-tetramethylenediamine under nitrogen for 4 h. The course of reaction was monitored by TLC in the solvent system $CHCl_3$/MeOH/25% NH_4OH (5:1:0.1). The reaction mixture was diluted with 100 mL of $CHCl_3$, washed several times with H_2O, and then extracted with 0.01 N HCl. The acidic water layer was made alkaline and extracted several times with $CHCl_3$. The crude product, after evaporation in vacuo, was crystallized from MeOH/$CHCl_3$ and then converted into the hydrochloride salt by addition of HCl/ethyl ether to the cold chloroform solution; the separated salt of **5a** was purified on Sephadex LH-20 column with the solvent system MeOH/H_2O (10:1): IR (KBr, major peaks, cm^{-1}) 1575, 1625; UV–vis (MeOH) λ_{max} nm (ϵ) 490 (22.480), 455 (17.830), 255 (51.930), 215 (44.180); ^{1}H NMR (d_6-DMSO) δ 3.03 (m, 2), 3.48 (m, 2), 3.68 (t, 2, J = 6 Hz), 4.08 (q, 2, J = 6.6 Hz), 5.3 (br s, 1, exchangeable with D_2O), 7.9 (m, 2), 8.03 (d, 1, J = 9 Hz), 8.17 (d, 1, J = 9 Hz), 8.45 (dd, 1, J = 7 Hz, 1.2 Hz), 8.92 (dd, 1, J = 8.5 Hz, 1.5 Hz), 9.02 (br s, 1, exchangeable with D_2O), 9.33 (s, 1), 11.4 (t, 1, exchangeable with D_2O); FDMS m/z (relative intensity, %) 334 ([M]$^+$, 100). Anal. ($C_{19}H_{18}N_4O_2$·1.1HCl) C, H, N, Cl.

Method B. A sample of **4b** (0.25 g, 1 mmol) was heated with a large excess of the appropriate aliphatic amine except in the case of the synthesis of **5a**, where *N,N,N′,N′*-tetramethylethylenediamine was used as the solvent and in the case of the synthesis of **5b** and **5e** where water was added to the reaction mixture (aliphatic amine/water 1:1). The isolation and purification procedures of the crude products were the same as described in the method A.

Biological Tests. In Vitro Cytotoxicity Evaluation. Tissue Culture. The murine L1210 leukemia cells (RPMI) were grown in RPMI 1640 medium supplemented with 5% fetal calf serum and penicillin (100 000 units/L) plus streptomycin (100 mg/L) in a controlled (air/5% CO_2), humidified atmosphere at 37 °C. The cells were transplanted every 2–3 days.

Cytotoxicity Evaluation. The L1210 cells in logarithmic growth were suspended in the growth medium to give the final density of 0.05 × 10^6 cells/mL. The evaluation was performed as described previously.[17] The cytotoxic activities (EC_{50} values) of the compounds were defined as their in vitro concentrations causing 50% inhibition of cell growth after 48 h as measured by the protein content of the cells.

In Vivo Antileukemic Evaluation. Murine P388 leukemia was obtained from the Institute of Immunology and Experimental Therapy of Polish Academy of Science and injected ip in DBA/2 mice according to standard protocols from National Cancer Institute.[18] For test purposes mice [first generation hybrid (BALB/c × DBA/2)F_1] were given 10^6 P388 cells ip on day zero. Twenty-four hours after tumor implantation, solutions of compounds in physiological saline were administered ip daily for 5 consecutive days. The treated group consisted of seven animals and the control group of eighteen animals. The medium survival time (MST) of the treated (T) and control (C) groups was determined and the percent of T/C was calculated by using the following formula: % T/C = [(MST treated)/(MST control)] × 100.

Determination of NADH Oxidation and Cytochrome *c* Reduction. NADH dehydrogenase activity was determined with cytochrome c as the electron acceptor.[16] It was examined by following cytochrome c reduction at 550 nm with an extinction coefficient for cytochrome c (reduced minus oxidized) of 19 600 M^{-1} cm^{-1}. NADH oxidation was determined at 340 nm with the excitation coefficient 6220 M^{-1} cm^{-1}. All reactions were initiated by the addition of NADH dehydrogenase.

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