9:1 $CHCl_3$–MeOH. Pooled TLC-pure fractions were evaporated to a glassy solid, which was triturated with Et_2O, filtered, and dried in vacuo at 60 °C over P_2O_5 to obtain a yellow powder (544 mg, 53%): TLC R_f 0.7 (silica gel, 9:1 $CHCl_3$–MeOH); mp 94–105 °C; IR (KBr) 3440 (br), 3200 (br), 2990, 1740 (ester C=O), 1630 (sh), 1615 cm^{-1}. Anal. ($C_{24}H_{30}N_8O_5 \cdot 0.5H_2O$) C, H, N.

3-[*N*-(4-Amino-4-deoxy-*N*^{10}-methylpteroyl)-*N*-(carboxymethyl)amino]propanoic Acid (7). A solution of **21** (510 mg, 1 mmol) in 50% EtOH (50 mL) was treated with $Ba(OH)_2 \cdot 8H_2O$ (630 mg, 1 mmol) and stirred at room temperature overnight. A solution of NH_4HCO_3 (300 mg, 4 mmol) in a small volume of H_2O was then added, and after 5 min of vigorous stirring, the $BaCO_3$ was removed by filtration. The filtrate was concentrated to remove EtOH and acidified with 10% AcOH. The precipitated solid was collected and dried by lyophilization to obtain a light-yellow powder (362 mg, 76%): TLC R_f 0.9 (cellulose, pH 7.4 phosphate buffer); IR (KBr) 3400 (br), 3200 (sh), 1720 (sh), 1645, 1615 cm^{-1}. Anal. ($C_{20}H_{22}N_8O_5 \cdot 1.75H_2O$) C, H, N.

Biological Assays. The ability of compounds **3–7** to inhibit DHFR from murine leukemic cells, to act as substrates and/or inhibitors of partially purified FPGS from mouse liver, and to inhibit the growth of L1210, L1210/R81, and CEM cells was evaluated by methods described earlier.[15,19,20]

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Registry No. L-**3**, 112113-76-5; L-**4**, 112113-77-6; DL-**5**, 112113-78-7; DL-**6**, 112113-79-8; DL-**6** (R^1 = CHO, R^2 = Me), 112113-89-0; **7**, 112113-80-1; L-**8**, 112138-39-3; L-**9**, 5420-67-7; L-**10**, 112113-81-2; L-**11**, 112113-82-3; L-**12**, 112113-83-4; L-**13**·HCl, 112113-84-5; **14**, 19741-14-1; **15**, 89043-75-4; DL-**16**, 112113-85-6; DL-**17**ηdtHCl, 112113-86-7; **18**, 81167-39-7; DL-**19**, 112113-87-8; **20**, 3783-61-7; **21**, 112113-88-9; DHFR, 9002-03-3; H-L-Glu-OH, 56-86-0; $O_2NC_6H_4$-*m*-COCl, 121-90-4; H-L-Glu(OEt)-OH, 1119-33-1; H-L-Glu(*t*-BuO)-OMe·HCl, 6234-01-1; $O_2NC_6H_4$-*o*-COCl, 610-14-0; H-Gly-OEt·HCl, 623-33-6; 2,4-diamino-6-(bromomethyl)pteridine hydrobromide, 52853-40-4; methyl 4-bromocrotonate, 1117-71-1; ethyl acrylate, 140-88-5.

New Actinomycin D Analogues as Superior Chemotherapeutic Agents against Primary and Advanced Colon Tumors and Colon Xenografts in Nude Mice

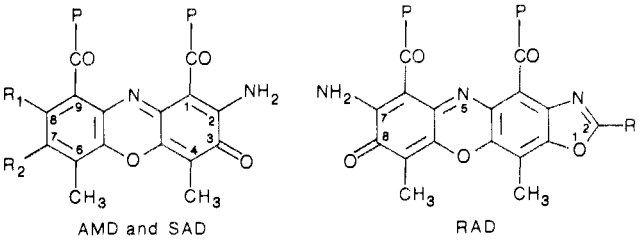
Sisir K. Sengupta,* Yuri Kogan, Christine Kelly, and Josephine Szabo

Departments of Obstetrics and Gynecology, Biochemistry, and Pharmacology, and Experimental Therapeutics, Boston University Medical Center, School of Medicine, Boston, Massachusetts 02118. *Received June 3, 1987*

"Reverse" analogues (RAD's) of actinomycin D (AMD) and their antitumor activity against mouse and human colon tumor cells are reported. RAD's are tetracyclic, and they have an oxazole ring fused on the tricyclic phenoxazine chromophore of AMD. The oxazole ring in RAD is substituted at the C-2 carbon with either a CH_3 (in RAD I), a C_6H_5 (in RAD II), or a $CH_2CONH(CH_2)_4NH_2$ (in RAD III) group. In tumor cells and rat hepatic microsomes, RAD's are metabolized to a tricyclic "symmetrical" analogue of AMD (SAD) with the loss of the oxazole ring and its substituents. RAD and SAD are very active in priming superoxides in the presence of microsomal enzymes as well as in inhibiting the synthesis of DNA and the growth of human colon tumor HT-29 cells in vitro. RAD III and SAD efficiently cleave closed circular plasmid pBR322 DNA like the antitumor agent bleomycin. In addition to their strong inhibitory activity against P388 and B_{16} tumors in vitro and in vivo, RAD III and SAD demonstrate high levels of activity against primary C26 and advanced C38 colon tumors in mice and against a xenograft of human colon adenocarcinoma CX-1 in athymic mice. In all these biological activities, the analogues demonstrate superiority to AMD in several experimental tumors. Also, the analogues, in contrast to AMD, show reduced toxicity in tumor-free mice, which is possibly due to the metabolic deactivation of SAD in host organs.

In search for actinomycin D (AMD, **1**, Chart I) analogues with a broader antitumor activity and reduced host toxicity, considerable work has been done in our laboratories.[1-6] Recently, we reported the synthesis of two new classes of actinomycin D analogues; one class has a tetracyclic chromophoric structure, which features an oxazole ring attached to the phenoxazinone tricyclic ring of actinomycin D. We termed this class "reverse" analogues (RAD).[7] The other class has the same tricyclic phenoxazinone ring of AMD, but carries two extra groups, an amino and a hydroxyl group. These moieties are substituted at the C-7 and C-8 positions. In this process, the unsymmetrical molecule of AMD is made symmetrical, and

Chart I[a]



[a] P = Thr-D-Val-Pro-Sar-MeVal (cyclic depsipeptide)

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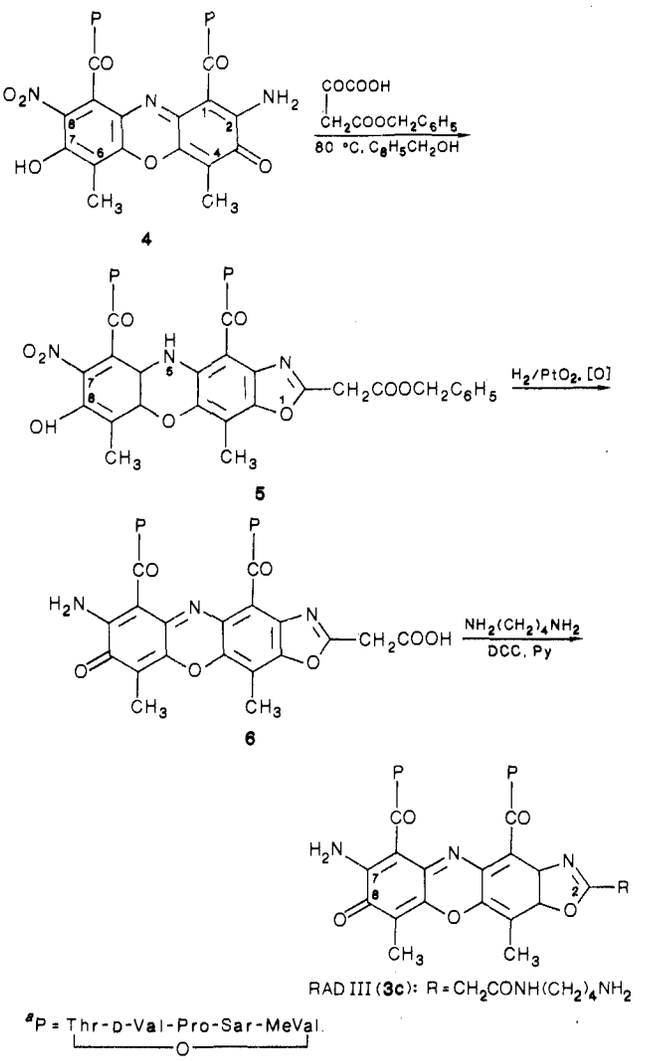
Scheme I[a]



hence it is termed a "symmetrical" analogue or SAD[7] (Chart I).

RAD's usually carry a substituent at the C-2 position; RAD I (**3a**) has a CH_3 substituent, and RAD II (**3b**) has a C_6H_5 substituent. RAD I and II and SAD (**2**) bind to double-stranded DNA strongly, but they do not intercalate. Yet, they are potent inhibitors of nucleic acid synthesis and growth of tumor cells in vitro. RAD's also demonstrate strong antitumor activities against P388 and L1210 leukemias and B_{16} melanoma,[7] and compared to AMD, they exhibit reduced toxicity in tumor-free hosts.[7]

We observed that the tetracyclic RAD II is metabolized to tricyclic SAD, which is further metabolized to several biological conjugates including glucuronide and sulfate; these conjugates are several hundred times less cytotoxic than SAD. In addition, we observed that the rat liver microsomes cause the conversion of RAD and SAD to their free-radical forms, which stimulate the production of superoxides. This process may contribute to their potent cytotoxicity in tumor cells.[7]

More recently, we have synthesized a new analogue of RAD, which has a substituent chain, $CONH(CH_2)_4NH_2$, at the C-2 position on the oxazole ring of RAD. The substituent improves its aqueous solubility, which makes it easy to use for tumor experiments. The new analogue is RAD III (**3c**) (Scheme I).

In this paper we report the biochemical, pharmacological, and new antitumor activity of the RAD's and SAD. We describe the DNA cleavage activity of SAD and RAD on plasmid DNA and also report the superior activity of these analogues in the treatment of both primary and advanced colon carcinoma in mice. We also report the experimental antitumor activity of three analogues, RAD II, RAD III, and SAD, against a xenograft of human colon adenocarcinoma, a representative of a major class of human cancer, heterotransplanted into athymic mice.

Synthesis of "Reverse" III AMD Analogue (RAD III, 3c). The synthesis of RAD III (**3c**) is shown schematically in Scheme I. Compound **4** was reacted with the benzyl ester of oxalacetic acid at 80 °C according to the procedure reported previously for the synthesis of other oxazoles.[6,7] The resulting product **5** with the $CH_2COOCH_2C_6H_5$ substituent at the C-2 of the oxazole ring was debenzylated to **6**, and compound **6** was coupled with excess 1,4-diaminobutane by DCC in pyridine. (see the Experimental Section). The isolated product showed the characteristic UV–vis and NMR characteristics and the specific rotation values of a RAD compound with a $CH_2CO(CH_2)_4NH_2$ substituent. RAD III (**3c**) was polar and more soluble in water than either RAD I (**3a**), RAD II (**3b**), or actinomycin D (**1**) (solubility for RAD III is 2.1 mg/mL at 20 °C; for AMD, 1.0 mg/mL at 20 °C). Like AMD and its other "reverse" analogues, RAD III (**3c**) is 16-fold more soluble in water at 4 °C than at 20 °C.

Physical properties of RAD I (**3a**), RAD II (**3b**), and SAD (**2**) were reported previously.[7] Like its predeccessors, RAD III shows UV–vis double-absorption peaks at 455 and 440 nm in chloroform, characteristic of a phenoxazin chromophore with *o*-aminoquinone substituents. The NMR chemical shifts of the chromophoric and peptide moieties are consistent with that found for the other RAD's.[7] The specific rotation and the NMR values confirm that the peptide conformation in RAD III is practically the same as in AMD.[6,7]

Biophysical Properties. The equilibrium binding constants of RAD III (**3c**), determined as in previously[2,3] reported analogues from the Scatchard plots of binding isotherms with use of calf thymus DNA, are K_{app}, 2.9×10^6 M^{-1}, and B_{app}, 0.040 in 0.01 M phosphate buffer, pH 7.0 at 20 °C. AMD (**1**) shows K_{app}, 2.3×10^7 M^{-1}, and B_{app}, 0.108;[2,7] K_{app} of AMD is 1 log unit higher than RAD III, suggesting a weaker DNA binding for RAD III. In contrast to AMD, RAD III failed to demonstrate any measurable change in the intrinsic viscosity with the increasing ratios of [ligand]/[DNA base pair] in a viscometry experiment using low molecular weight DNA (0.5×10^5 to 1.0×10^5 daltons). The experiments showed that RAD III does not intercalate into DNA.[2]

Biochemical Pharmacology. Previous workers[8,9] reported that the quinone imino chromophore of actinomycin can be subjected to enzyme-catalyzed single-electron reduction to give free-radical species that generate superoxides by reacting with molecular oxygen. The process needs a reductive cofactor NADPH, which is oxidized to $NADP^+$ during the process, giving a change in the absorbance at 340 nm that can be measured spectrophotometrically. Alternatively, the superoxides generated during the process may be allowed to react with epinephrine to produce adrenochrome, a chromophore with strong absorbance at 480 nm that can be measured spectrophotometrically.[7]

We studied the superoxide formation and NADPH oxidation catalyzed by AMD analogues in the presence of rat liver microsomes. The results of these assays demonstrated

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Table I. Stimulation of NADPH Oxidation and Adenochrome Formation by Actinomycin D and Its Analogues by Rat Hepatic Microsomal Incubations

compd	NADPH oxidation,[a] nmol/min per mg of protein	SOD,[b] 5 μg/mL	adrenochrome formation,[a] nmol/min per mg of protein
no drug	9.11 ± 0.30	-	2.11 ± 0.11
AMD (1)	10.01 ± 0.90 (9.9)	-	2.29 ± 0.33 (8.5)
		+	inhibited
SAD (2)	64.3 ± 2.11 (606)	-	19.3 ± 1.04 (815)
		+	inhibited
RAD II (3b)	65.0 ± 5.95 (610)	-	18.8 ± 1.9 (800)
		+	inhibited
RAD III (3c)	87.1 ± 7.1 (812)	-	22.1 ± 2.2 (870)
		+	inhibited

[a] The percentage stimulation = [(drug-stimulated rate - basal rate)/basal rate] × 100 and is shown in parentheses. NADPH oxidation was measured at 340 nm and adrenochrome formation was measured at 480 nm; the values are average ± standard errors of triplicate analyses. [b] SOD, superoxide dismutase.

Table II. Percentage of Closed Circular pBR322 DNA Cleaved by BLM and AMD Analogues[a]

compd	concn, μM	%[b]
AMD (1)	10	10
	100	22
SAD (2)	10	35
	25	50
	50	50
	100	65
RAD III (3c)	10	40
	25	45
	50	50
	100	58
BLM (bleomycin)	10	55
	50	75
	100	<85

[a] Ten microliters of 200 μM pBR322 DNA nucleotides in 10 mM Tris·HCl (pH 7.8) and 50 mM NaCl, containing 4 mM $Na_2S_2O_4$ and 100 μM $FeCl_2$, was incubated with drug concentration at 20 °C for 1 h; the reaction mixture was kept saturated with air throughout the incubation period. [b] Percentage of DNA was calculated from the amounts of open- and closed-circular DNA in the incubation mixture.

that RAD and SAD are extremely efficient agents in stimulating these reactions. The process of adrenochrome formation is inhibited by superoxide dismutase, confirming that superoxide is indeed generated in these systems. (For experimental details, see ref 7 and 10, for data see Table I).

DNA-Cleaving Ability. The cleavage of DNA was followed by monitoring of the conversion of supercoiled closed-circular pBR322 DNA to open-circular DNA (analyzed by agarose gel electrophoresis and quantitated by staining with 0.5 μg/mL of ethidium bromide for 30 min and densitometry;[11] Table II). SAD and RAD show strong DNA-cleaving ability in the presence of sodium hydrosulfite and ferrous chloride. Presumably, sodium hydrosulfite acts as a reducing agent and regenerates Fe(II) from Fe(III) to produce a continuous source of the active metal ion. When the DNA-cleaving reaction with the analogues was performed at 20 °C, the ratio of cleaved DNA (open circular form DNA versus closed circular form DNA) was almost maximum within 1 h. The efficiency of this cleavage is comparable to that of the drug bleomycin (BLM).[12] Cleavage of DNA by the analogues is effected by the actinomycin chromopeptide moiety, which acts as a site-specific binder to DNA, and the aminoquinone function chelated to the ferrous ion is a likely candidate which generates the radical species.

In general, the analogues demonstrate extremely efficient DNA cleaving ability which is approximately 70% as potent as that of BLM. In this respect, actinomycin is only about 30% as efficient as its synthetic analogues, RAD III (3c) and SAD (2c) (Table II).

Metabolic Studies. Metabolism in the Presence of Tumor Cell Homogenates and Rat Liver Microsomes. Details of these studies were reported previously[7] (Table III). The cell homogenates (P388 cells were sonicated) were incubated at 37 °C with analogues for 16 h. Rat microsomes were harvested from phenobarbital-induced rats by standard procedure. Incubations were carried out for 6 h in the presence of a NADPH-generating system, i.e., glucose-6-phosphate dehydrogenase and glucose 6-phosphate.[7] The metabolites and their water-soluble conjugates were isolated and identified following a previously reported procedure[7] (Chart II, supplementary material), with the use of tritiated analogues.

In an early report,[7] we observed that the RAD analogues are metabolized to SAD (2) with the loss of oxazole ring. By use of both microsomal and tumor systems, the metabolic conversion of RAD III (3c) was found to generate several biological conjugates. In tumor cells, a larger fraction of SAD (2) (30–50%) remains unmetabolized. SAD is found to form glucuronide and sulfate conjugates only in tumor cells. Fractions of large molecular protein adducts are also isolated from SAD (2) and RAD III (3c) in both tumor cell and microsomes, suggesting that other active metabolite(s), e.g., radical species, might have been formed (Table III). Isolation of SAD from RAD analogues is a very important step, because SAD is found to be a potent and effective antitumor agent in several murine tumor systems (Tables V–XII).

In Vitro Inhibition of Tumor Cell Growth. These experiments were performed according to the methods reported by us previously.[5,7]

The comparative cell growth inhibitory activity of RAD's and SAD are reported (data in Table IV, supplementary material). The cell lines used are human lymphoblastic leukemia (CCRF-CEM), murine lymphocytic leukemia (P388) and lymphoid leukemia (L1210), and mouse melanoma (B_{16}). Compared to AMD, the analogues RAD and SAD demonstrate uniformly superior activity in all the cell lines; the best activities are found to be against the solid melanoma line B_{16}. The analogues SAD and RAD III are two- to fourfold more potent than AMD in this cell line. In general, the cytotoxic potencies of the analogues are in the order: SAD = RAD III > RAD II = RAD I > AMD.

Furthermore, the activity of the analogues against a human colon[13] carcinoma HT-29 cell line in culture was evaluated. After the cells were treated for 2 h with 0.01–10.0 μM of the drugs at 37 °C, the cells were washed with ice-cold PBS (phosphate buffered saline), and the cell viability was assessed by soft agar cloning.[13] Cells viability was expressed as the percentage of colonies from the drug-treated cells relative to control cells after correcting for the cloning efficiency, which ranged from 60% to 80%. The values of the drug concentration producing 90% reduction (LD_{90}) in cell viability extrapolated from the plot of concentration of drug versus ability of colony formation again establish SAD (2) as the most active agent (LD_{90} =

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Table III. Percent Metabolite Isolated from Incubation Mixture Containing Tumor Cell Homogenate (T) or Rat Microsomes (M)

	drugs examined		
	AMD (1), %	SAD (2), %	RAD III (3c), %
uncoverted	100 (T, M)	30.0 ± 3.0 (T), 50.0 ± 10.5 (M)	20.0 ± 2.5 (T), 10.0 ± 1.0 (M)
SAD, 7.7 min (I), 20.0 min (II)[a]			20.0 ± 2.0 (T), 65.0 ± 5.5 (M)
SAD-glucuronide and/or sulfate, 8.0, 10.5 min (II); 16.0, 21.0 min (III)[a]		47.0 ± 6.5 (T), nil (M)	40.0 ± 5.5 (T), nil (M)
protein conjugates		5.5 ± 1.5 (T), 20.0 ± 5.0 (M)	8.0 ± 2.0 (T), 10.0 ± 10 (M)
tissue bound		7.5 ± 1.0 (T), 15.0 ± 3.0 (M)	5.0 ± 1.0 (T), 5.0 ± 0.5 (M)
unidentified metabolite		10.0 ± 1.0 (T), 15.0 ± 4.0 (M)	5.0 ± 2.0 (T), 8.0 ± 1.0 (M)

[a] HPLC t_R in minutes (systems used were I, II, and III). For details, see ref 17 and Chart II (in supplementary material).

Table V. Synthesis by AMD and Analogues in Tumor Cells in Vitro

	IC_{50} values, nM			
	P388		HT-29[a]	
compd	DNA synth	RNA synth	DNA synth	RNA synth
SAD (2)	12 ± 4	15 ± 2	70 ± 11	55 ± 6
RAD III (3c)	430 ± 38	55 ± 7	365 ± 45	65 ± 8
RAD II (3b)	780 ± 66	70 ± 5	580 ± 55	68 ± 10
AMD (1)	1100 ± 90	50 ± 5	900 ± 80	100 ± 11

[a] Human colon carcinoma in culture. IC_{50}, drug concentration producing a 50% inhibition of incorporation of percursor [^{3}H]thymidine into DNA and [^{14}C]uridine into RNA in the cell lines, isolated as acid precipitable materials. Data are average of replicate experiments.

0.1 μM) followed closely by RAD II (**3b**) (LD_{90} = 0.2 μM) and RAD III (**3c**) (LD_{90} = 0.5 μM). Comparably, AMD showed an LD_{90} value at 5.0 μM concentration.

In Vitro Inhibition of Nucleic Acid Synthesis in Tumor Cells. AMD is known to inhibit RNA synthesis more efficiently than DNA synthesis in P388 cells in culture;[5,7,9] compared to AMD, the analogue SAD shows a very high potency and superior activity in inhibiting DNA synthesis in P388 cells.

In addition to the P388 cells, we have also examined this inhibitory activity in human carcinoma HT-29 grown in tissue culture. The values of the drug concentration producing 50% inhibition of the incorporation of [^{3}H]-thymidine and [2-^{14}C]uridine into nucleic acid macromolecules are presented in Table V. These values are taken as estimates of the inhibition of DNA and RNA synthesis. Following 2-h exposure, SAD and RAD preferentially inhibit the synthesis of DNA; AMD inhibits RNA synthesis preferentially. Furthermore, the inhibitory activities of these analogues are three- to fivefold higher than AMD; the order of potency of the analogues for their inhibition of DNA synthesis is: SAD (**2**) > RAD III (**3c**) > RAD II (**3b**) ≫ AMD (Table V).

Antitumor Activity in Vivo. The analogues were tested for their antitumor activities in P388 lymphocytic leukemia in male BDF_1 mice. In this system, in which AMD is known to be very effective, RAD II, RAD III, and SAD are found to be two- to threefold more effective with respect to %ILS and also in producing long-time survivors (Table VI).

The analogues were also tested against P388/ADR murine leukemia following the standard protocol[14] (Table VI). This tumor line is known to be resistant to intercalating agents including actinomycin D and adriamycin; however, the tumor is highly sensitive to mitomycin C, which is believed to act via activation of its quinone chromophore to a free radical form.[15] Again, RAD III and SAD show the highest levels of activity in these tumor lines, and we have observed that these analogues do not intercalate into DNA. The activities of these analogues were found to be fairly high over a broader dose range compared to AMD in this and also in the AMD-sensitive P388/S lines.

Following a standard protocol[16,19] (Table VII), the analogues were tested in vivo in B_{16} melanoma; again, compared to AMD, RAD and SAD were found to be superior agents.

The analogues were moderately active against ip inoculated L1210 leukemia when the drugs were administered, also ip on a multidose treatment schedule (Table VIII, supplementary material); increases in survival time of 71–100% were observed, but there were no 60-day survivors. The activities of the analogues were better than the activity demonstrated by AMD in this tumor line.

Both RAD III (**3c**) and SAD (**2**) displayed significant activity against colon tumor 26 implanted in CDF_1 mice. Drugs were administered ip in three doses over 9-day period and produced a 50–150% increase in %ILS (by RAD III) and a 57–118% increase in ILS (by SAD) over controls, and the survival rate increased 10–40% at 60 days; these activities are superior to that demonstrated by adriamycin, which is known to be effective in this tumor line (data in Table IX, supplementary material).

In view of favorable activity against colon 26 carcinoma, further experiments using the active analogues were carried out on the advanced colon adenocarcinomas of mouse, colon 38.[17] The results obtained on the colon 38 tumor are shown in Table X. Administered every 4 days, three times, starting on day 1 after tumor implant, RAD III (**3c**) and SAD (**2**) are about 150% as active as AMD at equivalent doses; also, their optimum dose activities are either equivalent or better than a highly active agent, adriamycin. The superiority of RAD III and SAD over AMD (**1**) in inhibiting colon 38 carcinoma is evident from the data of both tumor weight inhibition and increases in survival time of the treated over nontreated mice and the larger number of long-term survivors produced by these analogues.

Tables XI and XII show the effectiveness of SAD (**2**) and RAD (**3b** and **3c**) against CX-1 human colon adenocarcinoma heterotransplanted into athymic mice, with three iv treatments with drugs on a weekly schedule starting on the 15th day of tumor transplant.[18] AMD and the analogues were tested at doses of 45 and 60 μg/kg. The

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Table VI. Activity of Actinomycin D (AMD) and "Reverse" (RAD) and "Symmetrical" (SAD) Analogues in P388 in Vivo. AMD-Sensitive and Adriamycin-Resistant Leukemia[a]

drug	dose range, mg/kg per inj, ip	P388/S[b] optimal dose, mg/kg, %ILS	P388/ADR[b] optimal dose, mg/kg, %ILS
adriamycin	2.5–4.5	3.0, 97 (1/8)	3.5, 33
actinomycin D (AMD)	0.075–2.5	0.25, 132 (1/8)	0.25, 22
RAD II (**3b**)	0.10–0.8	0.35, 382 (4/8)[c]	0.6, 132 (1/8)
RAD III (**3c**)	0.10–1.0	1.0, 386 (6/8)	1.0, 188 (2/8)
SAD (**2**)	0.3–1.8	0.6, 382 (4/8)[c]	1.2, 176 (3/8)
mitomycin C	1.0–5.0	3.0, 136 (1/8)	3.0, 400 (5/8)

[a] Male CDF_1 mice inoculated ip: 10^6 cells inoculum, day 0. Drugs administered in 10% dimethyl sulfoxide–saline on days 1, 5, and 9. %ILS = percent increase in life span. Fractions in parentheses = tumor free survivors/total on day 60. [b] P388/S (adriamycin sensitive) and P388/ADR (adriamycin resistant) tumors were evaluated in the same experiment with agent listed. [c] Data reported previously (see ref 17).

Table VII. In Vivo Antitumor Activity of AMD, RAD, and SAD against B_{16} Melanoma[a] Treatment: Days 1, 5, 9

compd	optimal dose, μg/kg per inj	MST[b] (range)	%ILS (surv)[c]
no drug (control)		29 (20–48)	
actinomycin D (AMD, 1)	250	43 (36–54)	48 (2/9)
RAD I (**3a**)	300	65 (55–62)	124 (6/9)
RAD II (**3b**)	300	59 (53–59)	103 (5/9)
RAD III (**3c**)	300	62 (53–61)	114 (6/9)
SAD (**2**)	300	57 (33–61)	96 (4/9)

[a] 0.2 mL of 1:5 (weight/volume) brei of B_{16} melanoma implanted ip on day 0 in groups of nine BDF_1 mice. Drugs administered ip. [b] MST (range), median survival time in days (range of days for death of individual animal). [c] %ILS (surv), percent increase in life span (survivors on day 65/total injected).

tumor responded remarkably well to the treatment of RAD III at doses of 45 and 60 μg/kg. The tumor also responded fairly well to SAD treatment but at 60 μg/kg only. RAD II (**3b**) showed marginal but positive activity, and AMD (1) and adriamycin were ineffective in this tumor at the doses tested.[18]

The result of treatment of mammary MX-1 and lung LX-1 are shown in Table XIII (supplementary material), and the data show that at the doses tested AMD and the analogues RAD III and SAD were practically ineffective. Only adriamycin showed some positive response at 60 mg/kg.[18]

The most significant results of these investigations are findings that analogues RAD III (**3c**) and SAD (**2**) are active against murine leukemias P388/S and P388/ADR, B_{16} melanoma, and especially murine and advanced carcinomas[17] of the colon tumors C26 and C38. The data are in good agreement with the superiority of RAD III (**3c**) and SAD (**2**) against human colon adenocarcinomas transplanted in athymic mice. The good agreement between the results obtained in the antitumor activity test, carried out in such different systems as in conventional mice transplanted with syngenetic tumors and in nude mice transplanted with human tumors, makes us confident about the reliability of the data and the prospects of real benefit from these analogues in the treatment of a major cancer, e.g., colon carcinoma in humans.

The analogues were also tested in tumor-free male mice for their LD_{10} values[5,7] (Table XIV). The animals were given a broad range of doses (ip) on days 1, 5, and 9. The maximum tolerated doses of AMD and the analogues that caused death in only 10% of the tested animals in 21 days (i.e., LD_{10}) were recorded as MTD. The data in Table XIV show that the MTD values of all the analogues are 7–14-fold higher than corresponding dose value of AMD; however, the MED values are about half the MED value of AMD. On the basis of these values, the calculated therapeutic index values of SAD is 96, and those of RAD I, II, and III are 60, 67, and 70, respectively.

The above data indicate that RAD and SAD show significant reduction in their toxicity in vivo compared to their very high levels of cytotoxicity in cells in vitro. This may derive from several processes, e.g., the microsomal activation in cells, i.e., conversion from RAD to SAD and/or to the other bioactive forms (free radicals, superoxides) necessary for activation, followed by deactivation by processes of bioconjugation to generate inactive forms in the organs of host. A detailed examination of their in vivo metabolism in liver, in tumors, and in the whole animal and the pharmacokinetic distribution of the drug and the active and inactive metabolites in animal organs and tissues is important for understanding the mechanism of action and the reasons for the high therapeutic index values in tumor-bearing host animals exhibited by the new analogue of actinomycin D.

Table X. Activity of Actinomycin D and Its Analogues against Advanced Colon Carcinoma C38 Tumor in CDF_1 Mice[a]

compd	dose, mg/kg	%[b] inhibn of tumor	% T/C[c]	toxic deaths[d]
AMD (1)	0.075	30	100	0/9
	0.15	30	107	1/9
	0.30	67	134	0/9
	0.45	83	170	1/9
	0.60	89	103	4/9
adriamycin	2.0	29	103	0/9
	3.0	70	98	0/9
	4.0	93	250 (5/9)	3/9
	5.0			8/9
RAD II (**3b**)	0.15	29	103 (1/9)	1/9
	0.30	70	137	0/9
	0.60	83	170 (3/9)	0/9
	1.20	88	103 (2/9)	4/9
RAD III (**3c**)	0.30	80	170 (1/9)	0/9
	0.60	93	250 (6/9)	1/9
	1.20	94	231 (3/9)	2/9
SAD (**2**)	0.30	95	278 (5/9)	0/9
	0.60	97	278 (6/9)	0/9
	1.20	99	211 (2/9)	4/9

[a] CDF_1 mice were injected sc with colon 38 adenocarcinoma fragments (70 mg) and treated with drugs with iv injections on day 1, 5, and 9 following tumor implantation on day 0. [b] [100 – (tumor weight of treated mice/tumor weight of untreated mice)] × 100; data pooled from two experiments, tumors measured on day 21 after implantation. [c] (Median survival time of treated mice/median survival of untreated mice) × 100; in parentheses, number of over 100-day survivors/number of total mice in the group. [d] Number of toxic deaths/number of mice in a group of nontumored mice treated in parallel with the tumor-bearing mice and observed for 100 days.

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus at a heating rate of 2 °C/min. Thin-layer chromatography was performed on silica gel plates (Brinkmann Instrument Inc.). Solvent systems were (A) EtOAc–acetone (4:1), (B) $CHCl_3$–MeOH (9:1), and (C) cifferri, the organic phase of the mixture EtOAc–MeOH–H_2O (20:1:20). High-performance liquid chromatography was carried out on a Varian Model 5020 gradient liquid chromatograph equipped with a CD-111L chromatography data system and fitted with a Varian reversed-phase C_{18} column

Table XI. Experimental Chemotherapy of Human Colon CX-1 Tumor Heterotransplanted in Nude Mice

	tumor regression[a]												
	adriamycin, mg/kg				AMD (1), μg/kg			RAD II (3b), μg/kg		RAD III (3c), μg/kg		SAD (2), μg/kg	
expt	4.4	6.0	6.6	10.0	30	45	60	45	60	45	60	45	60
1	-	+	+	+[b]	±	+	+[b]	+	++[b]	++++[b]	++++[b]	+++[b]	+++[b]
2	-	+	ND[c]	+	-	±	+	±	+	++	++[b]	+	++[b]
3	-	±	+	+[b]	±	+	+[b]	+	++	+++[b]	++++[b]	++[b]	+++[b]

[a] -, % T/C higher than 50%; ±, % T/C close to 50%; +, % T/C from 50% to 35%; ++, % T/C from 35% to 20%; +++, % T/C lower than 20%; ++++, regression of the tumor to a volume lower than 10% of the volume at the start of treatment. [b] Statistically significant as evaluated by the Student's *t* test. [c] ND, not done.

Table XII. Activity of Actinomycin D and Its Analogues RAD and SAD against Human Colon Carcinoma CX-1 Tumor Heterotransplanted in Nude Mice

compd	dose,[a] μg/kg	TST[b]	%T/C, optimal
adriamycin	6×10^3	15	38 (22)[c]
	10×10^3	15	39 (22)
AMD (1)	45	15	34 (22)
	60	15	36 (22)
RAD II (3b)	45	15	48 (22)
	60	15	24 (22)
RAD III (3c)	45	15	5 (22)
	60	15	3 (22)
	75	18	13 (27)
SAD (2)	40	15	18 (22)
	55	15	19 (22)
	70	15	5 (32)

[a] Intravenous treatment, once a week for three weeks; eight mice per group. [b] TST: number of days between tumor transplant and starting of treatment. [c] Numbers in parentheses: day of observation after the start of treatment.

Table XIV. Comparison of AMD with Chromophore-Substituted and Tetracyclic Chromophoric Analogues of AMD vs P388 Leukemia[a]

drug	MED[b]	MTD[c]	therapeutic index, MTD/MED
AMD (1)	0.0625	0.25	4[d]
RAD I (3a)	0.03	1.80	60
RAD II (3b)	0.03	2.00	67[d]
RAD III (3c)	0.05	3.50	70
SAD (2)	0.025	2.40	96[d]

[a] Drugs administered ip once on days 1, 5, and 9 starting 1 day after tumor implantation. Determinations were made from analysis of plotted log-dose response data. [b] MED (minimum effective dose) is the dose (milligram/kilogram) providing an increase in life span of 45% over control in P388 tumor bearing mice. [c] MTD (maximum tolerated dose) is the lethal dose (milligram/kilogram) for 10% normal BDF_1 male mice (18–22 g); animals observed for deaths during 21 days (LD_{10} = 21 days). Values were calculated from a plot of log dose vs percent mortality. [d] Data reported previously are incorporated for comparison; for details, please refer to ref 17.

with the following solvent systems: I, CH_3CN–5 mM NH_4OAc buffer, pH 6.4 (68:32 isocratic), flow rate 1.5 mL/min; II, 65–95% water–methanol gradient for 60 min, 1 mL/min; III, 10–90% H_2O–MeOH gradient for 120 min, 1 mL/min, with UV–vis variable- and fixed-wavelength dual detectors at 254, 440, 470, and 520 nm. IR spectra were obtained on a Perkin-Elmer Model 237 Infracord with KBr micropellets or in chloroform solution. UV–vis spectra were obtained on a Gilford 250 spectrophotometer. Specific rotation values were determined in chloroform solutions with a Cary 60 spectropolarimeter. NMR spectra were obtained in a JEOL FQ 90-MHz spectrometer equipped with Fourier transform. All elemental analyses were within +0.4%. The α-keto acid ester was purchased from Aldrich Chemical Co. [^{3}H]Actinomycin D was purchased from Amersham. Other labeled compounds were from New England Nuclear. Actinomycin D (NSC 3053, lot 49300) was purchased from Calbiochem. Calf-thymus DNA, glucarase (bovine liver), superoxide dismutase (E.C. 1.15.1.1), mitomycin C, and bleomycin sulfate were purchased from Sigma Chemical Co. Sephacryl S-200 superfine gel was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. NADPH, L-epinephrine, and plasmid DNA pBR322 were obtained from Boehringer Mannheim and from Calbiochem-Behring, San Diego, CA. A sample of adriamycin was obtained through the courtesy of Dr. Mervyn Israel of Dana Farber Cancer Center, Boston, MA.

Synthesis of Analogue RAD III (3c). Compound 4[17] (200 mg, 0.15 mmol) in benzyl alcohol was allowed to react with 100 mg of benzyl ester of oxalacetic acid at 80 °C (Chart I) according to the procedure reported in ref 4 and 7. Benzyl alcohol was removed at 60 °C (4 mm), and the residue was purified on a TLC plate (system A) to yield compound **5**, R_f 0.88, and further crystallized from heptane-CH_2Cl_2 (1:3) to a pure red solid (175 mg, 75% yield): λ_{max} ($\epsilon \times 10^{-3}$, $CHCl_3$) 290 nm (10.00), 303 (10.20), 320 (7.70), 392 (7.70), and 498 (8.90); IR (KBr) 6.5 μm (nitro peak); NMR ($CDCl_3$) δ 8.4–8.6 (m, 2 H) and 7.75–7.70 (m, 5 H) all for C_6H_5, 4.07 (d, 2 H, $C_6H_5CH_2$), 7.03 (s, 1 H, 8-O*H*), 3.07 (d, 2 H, 2-CH_2), 2.20 (s, 3 H, 9-CH_3), and 2.50 (s, 3 H, 11-CH_3). Anal. ($C_{72}H_{93}N_{13}O_{21}\cdot H_2O$) C, H, N. Compound **5** (150 mg, 0.10 mmol) in methanol (20 mL) was hydrogenated in the presence of PtO_2. The initial purple color of the solution was discharged, and as the resulting yellow solution was being filtered, the filtrate started turning deep yellow in air. After filtration and evaporation of methanol, the residue was chromatographed on Woelm alumina I. Washing the column with CH_2Cl_2 and eluting with methanol gave a pure red solid (125 mg of **6**, 90%): λ_{max} ($CHCl_3$, $\epsilon \times 10^{-3}$), 247 nm (12.1), 435 (12.9), and 450 (14.0); NMR ($CDCl_3$) δ 5.49 (d, 2 H, 7-N*H*$_2$), no peak for $C_6H_5CH_2$ (at 7.70–8.40) or at 11.45 for 8-O*H*; R_f 0.35 (system A). Anal. ($C_{65}H_{87}N_{13}O_{19}\cdot H_2O$) C, H, N.

Compound **6** (100 mg, 0.07 mmol) was dissolved in dry pyridine (3 mL) at 60 °C, and 50 mg of dicyclohexylcarbodimide (DCC) was added followed by 15 mg of 1,4-diaminobutane, $H_2N(CH_2)_4NH_2$ (DAB); the mixture was stirred at 60 °C for 5 h. Excess DCC was decomposed with ice-cold H_2O, the precipitated dicyclohexylurea was filtered, and unreacted DAB and pyridine were removed at 60 °C (4 mm). The residue was dissolved in 1.0 N HCl and extracted in CH_2Cl_2, and acidic layer was lyophilized and dissolved in 0.1 M $NaHCO_3$ (pH 8.9) and extracted in CH_2Cl_2.

The product, RAD III (**3c**), crystallized from THF gave a red solid (85 mg, 80%): R_f 0.51 (system A); λ_{max} ($CHCl_3$, $\epsilon \times 10^{-3}$) 249 nm (15.1), 440 (14.1), and 455 (15.7); IR (KBr), no COOH peak; NMR ($CDCl_3$) δ 6.3 (m, 2 H, ω-N*H*$_2$), 5.45 (d, 2 H, 7-N*H*$_2$), 3.77 (d, 2 H, 2-CH_2), 3.90–3.00 (m, 8 H, $(CH_2)_4$), 2.81 (s, 3 H, 11-CH_3), and 2.66 (s, 3 H, 9-CH_3); $[\alpha]^{20}_{644}$ −288 ± 18° (*c* 0.1, $CHCl_3$); HPLC t_R 3.9 min (system I) and 17.9 min (system II). Anal. ($C_{69}H_{97}N_{15}O_{18}\cdot 2H_2O$) C, H, N.

Reaction of pBR322 with SAD and with BLM. Plasmid DNA pBR322 (0.5 μg) was dissolved in 8 μL of a reaction buffer (5 mM $Na_2S_2O_4$, 10 mM Tris·HCl). To this solution, 1 μL of the 10 mM aqueous solution of the agent containing 1 mM $FeCl_2$ was added. The mixture was incubated for 0.5–2 h at 20 or 37 °C. Then, the incubation mixture was mixed with 10 μL of sample loading buffer (50% glycerin in 2 × Boyer's buffer containing 0.01% bromophenol blue).[20] Finally, 5 μL of the mixture was analyzed by 0.7% agarose gel electrophoresis (120 V, Boyer's buffer;[20] the migration distance of bromophenol blue was 5.0 cm). The gel was stained with 0.5 μg/L of ethidium bromide and then quantitated by densitometry.

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Inhibition of Nucleic Acid Synthesis in Tumor Cells in Vitro. DNA and RNA synthesis in P388 and HT-29 cell lines was monitored in the presence of drugs, and the rates of inhibition of their synthesis were estimated from the amounts of [2-^{14}C]-uridine and [^{3}H]thymidine into RNA and DNA, respectively, according to the methods described previously.[4]

In brief, for HT-29 colon carcinoma monolayer cells, 10^4 cells in each of five wells per concentration, were incubated with radiolabeled nucleosides (0.5 μCi each) for the last hour of a 2-h drug exposure, following which the cells were aspirated and washed with buffered medium. Cells were trypsinized and counted in the Coulter counter. The cell suspensions were then precipitated onto glass fiber filter discs (Whatman 934H), with 10% trichloroacetic acid. Filters were washed twice with ice-cold 10% trichloroacetic acid and twice with 95% ethanol and dried, and the incorporation of radionucleotides was estimated.

In addition, to investigate the cross contamination of radioactivity from [2-^{14}C]uridine into DNA in these cells, two sets of experiments were set up using a large number of cells (10^6–10^7 cells). After incubation with [2-^{14}C]uridine, the cells were lysed with ice-cold 1% Triton X for 2.5 min. Macromolecules were precipitated with the addition of 10% perchloric acid (5% in the medium) and centrifuged; the pellet in 1% NaCl was treated with an equal volume of phenol reagent, the emulsion was centrifuged at 10000g for 30 min at 4 °C, and the separated layer was adjusted to 1% NaCl and treated with an equal volume of 2-ethoxyethanol to precipitate the nucleic acids. The precipitate was washed with 75% ethanol and dissolved in an aqueous solution containing 2% sodium acetate and 1.5% NaCl. An aliquot was used for estimation of the total radionucleotide incorporation. Ribonuclease, previously heated to 80 °C for 10 min to destroy deoxyribonuclease activity, was added, and the mixture was incubated at 37 °C for 30 min. DNA was thus freed from RNA with the addition of 2-ethoxyethanol as in the above and was found to have minimal radioactivity (8–12% of the total incorporated); the supernatant, which contained all the RNA digest, accounted for the remainder, 87–92%, of the radioactivity found in the above mixture of nucleic acids, demonstrating only a marginal contamination in the DNA from the added [2-^{14}C]uridine.

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Supplementary Material Available: The metabolism flow sheet (Chart II), in vitro data (Table IV), in vivo data (Tables VIII, IX, and XIII), and figures depicting the electrophoretic analysis of 3′- and 5′-end ^{32}P-labeled DNA fragments are presented (7 pages). Ordering information is given on any current masthead page.

Potential Antitumor Agents. 55. 6-Phenylphenanthridine-4-carboxamides: A New Class of DNA-Intercalating Antitumor Agents

Graham J. Atwell, Bruce C. Baguley, and William A. Denny*

Cancer Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand.
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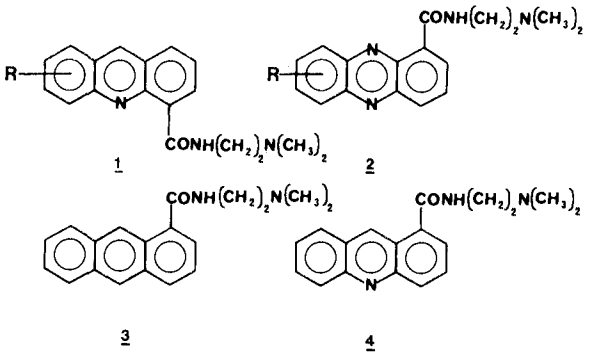
Derivatives of the DNA-intercalating agent *N*-[2-(dimethylamino)ethyl]phenanthridine-4-carboxamide (**7**) have been prepared and shown to have moderate in vivo antitumor activity against both the P388 leukemia and Lewis lung carcinoma. This demonstrates that the effective pharmacophore in the broad class of tricyclic carboxamides is not limited to linear tricyclic chromophores. Both **7** and the 6-phenyl derivative **10** have identical DNA binding properties, suggesting that the phenyl ring of **10** is not involved in the DNA intercalation site. A series of phenyl-substituted derivatives of **10** was evaluated. Aza substituents led to compounds with the highest in vivo cytotoxicity and in vivo P388 activity, but the in vivo solid tumor activity of the substituted 6-phenylphenanthridine-4-carboxamides was in general low.

Recent studies[1–4] have identified a number of linear, tricyclic carboxamides as DNA-intercalating antitumor agents. In particular, the weakly basic acridine-4-carboxamides (**1**) and phenazine-1-carboxamides (**2**) have broad-spectrum in vivo activity, with members of both series showing curative effects against both the P388 leukemia and the Lewis lung (LL) carcinoma.[2,3] In such compounds, the constitution and disposition of the carboxamide side chain has been shown to be critical.[2,5] Other work[4] has shown the necessity of having an aromatic nitrogen atom in the chromophore, peri to the carboxamide side chain; the linear, coplanar tricyclic anthracene-1-carboxamide (**3**), acridine-1-carboxamide (**4**), 9-oxoacridan-4-carboxamide (**5**), and phenoxazine-4-carboxamide (**6**) compounds are inactive,[4] although they all bind to DNA by intercalation with very similar binding constants to those of the active compounds **1** and **2**. Thus the



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