Potential Bioreductive Alkylating Agents. 2. Antitumor Effect and Biochemical Studies of Naphthoquinone Derivatives

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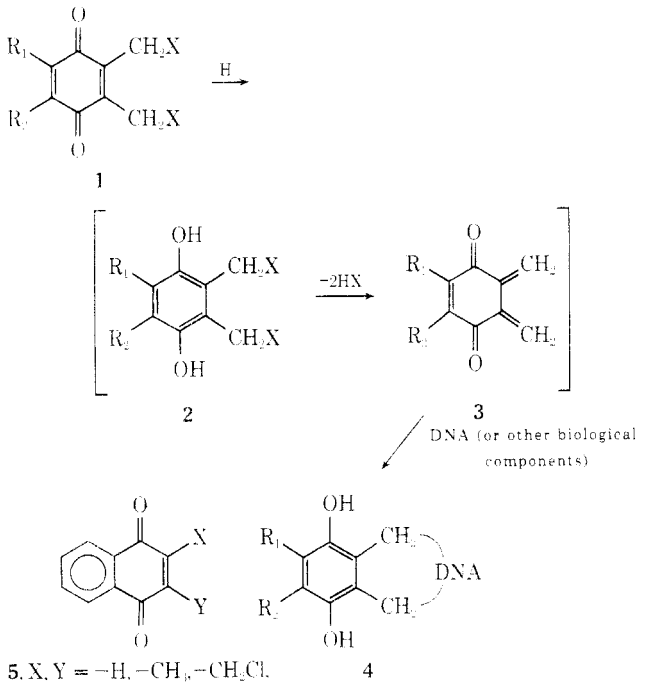
A number of naphthoquinone derivatives with side chains potentially capable of alkylation after reduction were prepared as a new series of potential bioreductive alkylating agents. These agents were found to produce significant prolongation of the survival time of mice bearing either Adenocarcinoma 755 or Sarcoma 180 ascites cells. The synthesis of DNA and RNA in neoplastic cells and the activities of beef heart mitochondrial NADH-oxidase and succinoxidase enzyme systems were inhibited by these compounds. No evidence was obtained to implicate these biochemical processes in the primary mode of action of naphthoquinone derivatives.

The design and synthesis of a series of benzoquinone derivatives as potential bioreductive alkylating agents have been previously reported;[1] these agents were found to be potent inhibitors of (a) the growth of the murine neoplasm Adenocarcinoma 755 and (b) the synthesis of DNA and RNA in these neoplastic cells *in vitro*. The action mechanism for this type of compound has been hypothesized to involve bioreduction *in vivo* of parent quinones **1** to dihydroquinones **2**, which then decompose to generate the reactive species, quinone methides **3**. These reactive intermediates are then visualized to be capable of alkylating DNA or other critical biological components.



1 2 3

5, X, Y = -H, $-CH_3$, $-CH_2Cl$, $-CH_2Br$, or $-CH_2OAc$ 4

Chemical evidence has been obtained to substantiate the existence of quinone methide **3** (R_1, R_2 = $-CH_3$) as the active intermediate of 2,3-dimethyl-5,6-bis(acetoxymethyl)-1,4-benzoquinone [**1**, R_1, R_2 = $-CH_3$; X = $-O-C(O)CH_3$] following sodium borohydride reduction;[2] this evidence was provided in part by the capacity of **3** to alkylate morpholine or aniline at 0-3° *in vitro*. Should quinone methide be generated enzymatically in target cells, a similar capacity to alkylate biological molecules is envisioned. In general, the parent compounds are not good alkylating agents until they are reduced; therefore, if the postulated action mechanism occurs *in vitro*, these agents can be classified as compounds with latent anticancer activity.

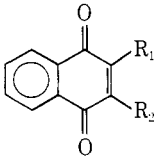
The utilization of benzoquinone or naphthoquinone precursors of quinone methides as potential latent alkylating agents may allow a therapeutic attack to be effectively directed against the more anaerobic cells of solid tumors. Thus, it is visualized that the NADPH-dependent enzyme system that reduces the mitomycins *in vivo*, which apparently is relatively nonspecific, and, therefore, possesses the ability to reduce a variety of mitomycin-like derivatives,[3,4] will also act upon the benzoquinones and naphthoquinones synthesized as bioreductive alkylating agents. Furthermore, it seems reasonable to assume that in the neoplastic cells of solid tumors distal to blood vessels, which traditionally are extremely resistant to chemotherapy, the oxygen tension is decreased, thereby creating conditions conducive to an increase in the ratio of reduced to oxidized pyridine nucleotide coenzymes. Such cells should theoretically be particularly sensitive to quinones which require bioreduction prior to exertion of their growth-inhibitory potential. As a result, compounds of this type may be particularly useful against certain solid tumors.

The potent antitumor activities of benzoquinone derivatives which were reported earlier,[1] as well as the close similarity in chemical reactivity between *o*-benzoquinone methides and *o*-naphthoquinone methides,[5] prompted the synthesis and evaluation of a series of naphthoquinone derivatives **5** which are reported in this paper. The preparation of a number of these compounds has been documented, as referenced in Table I; however, little biological application appears to have been considered.

Chemistry. The reactions involved in the preparation of this series of compounds are relatively uncomplicated. 2-Chloromethyl-1,4-naphthoquinone was synthesized by chromic oxide oxidation of 3-chloromethylnaphthalene by the method of Fieser and coworkers.[6] Although this procedure has been widely used in the preparation of 1,4-naphthoquinones, it has not been employed for the oxidation of benzene analogs to their corresponding 1,4-benzoquinones. 2-Methyl-3-chloromethyl-1,4-naphthoquinone and 2,3-bis(chloromethyl)-1,4-naphthoquinone were prepared by direct chloromethylation of the corresponding 2-methyl-1,4-naphthoquinone and 1,4-naphthoquinone, respectively, according to the procedure of Thomson.[7] This method also has only been applied to naphthoquinones. Treatment of the chloromethyl- or bis(chloromethyl)naphthoquinone with sodium acetate resulted in the corresponding acetoxymethyl- or bis(acetoxymethyl)naphthoquinone. Although the yield of both acetates by this procedure was generally low, it was the shortest accessible route to the preparation of these compounds.

Bromomethylation of 2-methyl-1,4-naphthoquinone using HBr and formaldehyde gave 2-bromomethyl-3-methyl-1,4-naphthoquinone in good yield;[8] however, application of this method to the formation of 2,3-bis(bro-

Table I. 50% Inhibitory Concentrations (ID_{50}) of Quinone Derivatives on the Syntheses of DNA and RNA *in Vitro* in Adenocarcinoma 755 Cells



R_1	R_2	ID_{50}, $M \times 10^{-5}$ DNA	RNA
CH_2Cl[a]	CH_2Cl	0.7	1.0
CH_2Cl[a]	CH_3	0.8	1.2
CH_2Cl	H	1.0	1.0
CH_2Br[b]	CH_2Br	1.0	1.0
CH_2Br[b]	CH_3	1.5	2.5
CH_2Br[c]	H	1.6	1.8
$CH_2OC(O)CH_3$	$CH_2OC(O)CH_3$	0.5	0.5
$CH_2OC(O)CH_3$	CH_3	0.3	0.4
$CH_2OC(O)CH_3$[d]	H	0.7	0.8

[a]Reference 8. [b]Reference 9a. [c]Reference 9b. [d]D. S. Tarbell, D. K. Fukushima, and H. Dam, *J. Amer. Chem. Soc.*, **67**, 197 (1945).

momethyl)-1,4-naphthoquinone by treatment of 1,4-naphthoquinone with HBr and formaldehyde failed. However, the direct bromination of 2,3-dimethyl-1,4-naphthoquinone using *N*-bromosuccinimide as the brominating agent[8] proved successful.

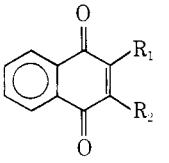
Biological Evaluation. The inhibitory activities of these agents on the synthesis of DNA and RNA were evaluated *in vitro* using Adenocarcinoma 755 ascites cells (Table I). The results indicated that the syntheses of both DNA and RNA were essentially equally susceptible to inhibition by this type of alkylating agent and that the potencies of the various naphthoquinones in this test system approximated those reported earlier for benzoquinone derivatives.[1] No significant difference existed between agents with a single group capable of alkylation and compounds with two reactive arms; however, acetoxy derivatives in general appeared to be better inhibitors of nucleic acid synthesis than chloro analogs which, in turn, were slightly more potent than bromo derivatives.

The antitumor activity of these compounds was assessed in mice bearing either Adenocarcinoma 755 (Table II) or Sarcoma 180 (Table III) ascites cells. In contrast to benzoquinone derivatives of this class, which showed growth inhibitory activity against Adenocarcinoma 755,[1] but were inactive against Sarcoma 180, the naphthoquinone derivatives possessed good inhibitory activity against both cell lines, with greater potency exhibited in the Adenocarcinoma 755 test system. Except for acetoxymethylnaphthoquinone, which possessed only marginal activity against Sarcoma 180, the differences in maximal activity between compounds in the same tumor system were relatively small. Derivatives with two groups capable of alkylation following bioreduction appeared to produce no greater prolongation of the survival time of mice bearing Sarcoma 180 ascites cells than did the one-armed analogs.

The similarities in the antineoplastic potencies of chloromethyl, bromomethyl, and acetoxymethyl derivatives further implied that the type of leaving group present in the molecule is not critical for tumor inhibitory activity, a finding which agrees with theoretical expectations if these compounds act as bioreductive alkylating agents.

The involvement of coenzyme Q (CoQ) in mitochondrial electron transport[9] has been well recognized in biological systems, including the malarial parasite.[10] Mammalian succinoxidase and NADH-oxidase systems have been extensively studied and may be considered representative of

Table II. Effects of Naphthoquinone Derivatives on the Survival Time of Mice Bearing Adenocarcinoma 755 Ascites Cells

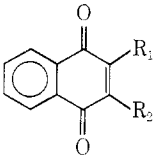


R_1	R_2	Daily dosage, mg/kg[a]	Av Δ wt, %[b]	Av survival, days ± S.E.[c]	T/C[d]
Control			+15.0	13.1 ± 0.7	
CH_2Cl	CH_2Cl	2.5	−4.0	38.6 ± 5.2	2.95
		5.0	−16.1	33.0 ± 5.3	2.52
		10.0	−9.9	35.2 ± 3.3	2.69
CH_2Cl	CH_3	2.5	−11.4	39.8 ± 4.0	3.04
		5.0	−7.7	35.3 ± 3.2	2.69
		10.0	−14.2	28.0 ± 1.4	2.14
CH_2Cl	H	2.5	−1.4	27.0 ± 9.4	2.06
		5.0	−10.2	45.0 ± 3.0	3.44
		10.0	−16.3	47.6 ± 1.5	3.63
CH_2Br	CH_3	2.5	−2.1	42.8 ± 7.2	3.27
		5.0	−7.9	45.4 ± 4.6	3.47
		10.0	−15.7	41.0 ± 4.8	3.13
CH_2Br	H	2.5	+6.0	13.8 ± 1.1	1.05
		5.0	−6.0	39.8 ± 7.6	3.04
		10.0	−16.3	47.6 ± 1.5	3.63
$CH_2OC(O)CH_3$	H	2.5	+12.1	11.6 ± 0.4	0.89
		5.0	−13.2	29.8 ± 3.0	2.27
		10.0	−12.9	25.7 ± 4.1	1.96
		15.0	−16.0	25.4 ± 6.7	1.94
		20.0	−17.6	18.2 ± 4.1	1.39

[a]Administered once daily for 6 consecutive days beginning 24 hr after tumor implantation. [b]Average weight change from onset to termination of drug treatment. [c]Each value represents results from 5 to 20 mice. [d]T/C represents the ratio of the survival time of treated to control animals.

CoQ electron transport sequences. A relationship has been documented[11] between antimalarial potency and the degree of inhibition of mitochondrial succinoxidase activity by benzoquinone and naphthoquinone derivatives; however, no comparable evidence is available to indicate a similar relationship between antineoplastic activity of quinone derivatives and inhibition of electron transport. Profound effects of ethyleneiminoquinones, which are relatively potent antineoplastic agents, on the respiration of carcinoma cells have been reported earlier.[12,13] These considerations prompted an investigation of the effects of the compounds synthesized in this report on mitochondrial NADH-oxidase and succinoxidase activities. The results (Table IV) indicated that at concentrations of 1.7–3.3 × 10^{-4} *M*, all quinones tested depressed mitochondrial succinoxidase and NADH-oxidase activities to about 50% or less of the uninhibited controls except for 2-methyl-3-acetoxymethylnaphthoquinone. At a lower concentration (3.3 × 10^{-5} *M*), only bromomethylnaphthoquinone (R_1 = $-CH_2Br$; R_2 = H) strongly inhibited succinoxidase activity (*i.e.*, to below 30% of the uninhibited control). Interestingly, at the same concentration (3.3 × 10^{-5} *M*), bromomethylnaphthoquinone only weakly inhibited NADH-oxidase activity. This finding suggests a site of action by this compound at complex II (*i.e.*, succinate-CoQ reductase). In contrast, the three compounds with a 2-methyl group (R_2 = CH_3) selectively inhibited NADH-oxidase activity at 3.3 × 10^{-5} *M* to at least 50% of control activity. This suggests a preferential site of action at complex I (*i.e.*, NADH-CoQ reductase). Among these three methyl compounds, the chloromethyl (R_1 = CH_2Cl; R_2 = CH_3) and acetoxymethyl (R_1 = CH_2OAc; R_2 = CH_3) ana-

Table III. Effects of Naphthoquinone Derivatives on the Survival Time of Mice Bearing Sarcoma 180 Ascites Cells



R_1	R_2	Daily dosage, mg/kg[a]	Av Δ wt, %[b]	Av survival, days ± S.E.[c]	T/C[d]
Control			+19.0	11.3 ± 0.4	
CH_2Cl	CH_2Cl	2.5	+2.1	15.2 ± 0.8	1.35
		5.0	+1.5	18.2 ± 1.2	1.61
		10.0	−2.9	22.6 ± 2.5	2.00
		15.0	−3.8	18.2 ± 1.2	1.61
		20.0	−6.4	21.8 ± 1.8	1.93
		25.0	−2.2	15.6 ± 3.9	1.38
CH_2Cl	CH_3	2.5	+0.9	15.4 ± 1.2	1.36
		5.0	−0.3	17.0 ± 1.5	1.50
		10.0	+1.5	18.3 ± 0.8	1.62
		15.0	−5.7	19.2 ± 2.9	1.70
		20.0	−5.7	9.2 ± 2.7	0.81
CH_2Cl	H	2.5	+13.3	12.8 ± 0.6	1.13
		5.0	+0.7	20.5 ± 2.7	1.81
		10.0	−0.2	18.5 ± 1.6	1.64
		15.0	−4.1	23.4 ± 3.2	2.07
		20.0	−7.3	19.2 ± 1.7	1.70
		25.0	+3.7	16.4 ± 5.7	1.45
CH_2Br	CH_2Br	2.5	+30.7	10.4 ± 0.4	0.92
		5.0	+10.1	9.2 ± 1.2	0.81
		10.0	−3.0	25.9 ± 3.0	2.29
		20.0	−8.1	19.2 ± 2.2	1.70
		25.0	−11.5	22.8 ± 1.7	2.02
CH_2Br	CH_3	2.5	+8.2	14.4 ± 2.5	1.27
		5.0	−2.2	23.0 ± 0.9	2.04
		10.0	−1.1	22.9 ± 3.0	2.03
		20.0	−7.2	25.0 ± 2.6	2.21
		25.0	−5.0	17.2 ± 2.7	1.52
CH_2Br	H	2.5	+13.0	9.8 ± 0.7	0.87
		5.0	+3.5	19.8 ± 2.8	1.75
		10.0	−2.2	22.8 ± 1.6	2.02
		15.0	−3.2	24.6 ± 2.5	2.18
		20.0	−2.6	21.4 ± 1.6	1.89
		25.0	−10.6	17.0 ± 2.3	1.50
$CH_2OC(O)CH_3$	$CH_2OC(O)CH_3$	2.5	−3.2	21.8 ± 1.7	1.93
		5.0	−7.3	22.8 ± 3.4	2.02
		10.0	−5.3	18.2 ± 1.1	1.61
		15.0	−6.4	13.2 ± 4.1	1.17
		20.0	−1.5	4.4 ± 0.9	0.39
$CH_2OC(O)CH_3$	CH_3	2.5	+9.3	10.6 ± 0.4	0.94
		5.0	−2.4	23.3 ± 3.3	2.06
		10.0	−4.5	16.2 ± 1.7	1.43
		15.0	−6.5	13.2 ± 2.2	1.17
$CH_2OC(O)CH_3$	H	2.5	+17.1	12.4 ± 0.5	1.10
		5.0	+1.5	15.6 ± 1.4	1.38
		10.0	−0.6	16.8 ± 0.7	1.48
		15.0	+1.2	16.5 ± 1.6	1.46
		20.0	−10.8	15.4 ± 2.0	1.36

[a] Administered once daily for 6 consecutive days beginning 24 hr after tumor implantation. [b] Average weight change from onset to termination of drug treatment. [c] Each value represents results from 5 to 40 mice. [d] T/C represents the ratio of the survival time of treated to control animals.

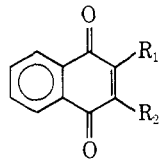
logs showed an equal degree of inhibition of NADH-oxidase activity at the two concentrations tested.

Bioreductive naphthoquinone alkylating agents presumably are reduced to the corresponding dihydronaphthoquinones which then generate reactive intermediates, quinone methides; the involvement of CoQ enzyme systems in the activation process is unknown. Mitomycin C, however, is reduced predominantly by a microsomal NADPH-requiring enzyme system.[14] Nevertheless, it is conceivable that quinone methides may be generated by CoQ-requiring enzyme systems and irreversibly inhibit these enzyme complexes by direct alkylation. The present data do not provide evidence which would permit the unequivocal identification of such a mechanism; however, these data are suggestive of an action other than competition with CoQ, since earlier reports demonstrated structural specificity for a 16- to 17-carbon lipoidal side chain for the competitive inhibition of the CoQ enzyme system, NADH-oxidase.[15,16] The data presented in Table IV demonstrated that relatively potent inhibition of mitochondrial NADH-oxidase activity was obtained with the three methyl derivatives which did not contain a lipoidal side chain.

Experimental Section

Biological Methods. Antineoplastic Activity. Compounds were tested for antineoplastic activity in AKD2F1 mice bearing Adenocarcinoma 755 ascites cells and in CD-1 mice bearing Sar-

Table IV. Inhibition of Mitochondrial Succinoxidase and NADH-oxidase by Naphthoquinone Derivatives



R_1	R_2	NADH-oxidase, %[a,b] 3.3×10^{-4} M	1.7×10^{-4} M	3.3×10^{-5} M	Succinoxidase, %[a,c] 3.3×10^{-4} M	1.7×10^{-4} M	3.3×10^{-5} M
CH_2Cl	CH_2Cl	36.2 ± 8.6		60.3 ± 7.2	10.9 ± 6.1		95.4 ± 9.7
CH_2Cl	CH_3	51.5 ± 9.9		51.7 ± 14.7	16.8 ± 7.7		70.8 ± 19.0
CH_2Cl	H	47.1 ± 7.7		87.6 ± 12.7	24.1 ± 9.8		95.4 ± 5.2
CH_2Br	CH_2Br		23.7 ± 3.8	53.4 ± 16.3		16.0 ± 3.8	55.3 ± 23.4
CH_2Br	CH_3		29.2 ± 3.3	46.1 ± 11.6		24.8 ± 5.0	73.2 ± 8.7
CH_2Br	H		20.0 ± 4.3	81.7 ± 19.8		20.2 ± 3.1	24.1 ± 3.8
$CH_2OC(O)CH_3$	$CH_2OC(O)CH_3$	23.6 ± 8.5		74.5 ± 19.1	24.5 ± 5.8		96.3 ± 17.6
$CH_2OC(O)CH_3$	CH_3	42.9 ± 5.0		37.4 ± 7.1	71.3 ± 10.9		91.2 ± 4.6
$CH_2OC(O)CH_3$	H	31.0 ± 4.4		69.5 ± 15.3	4.3 ± 5.2		95.6 ± 5.4

[a] Per cent of uninhibited controls ± standard deviation. Mitochondrial protein was from 0.55 to 0.85 mg/assay flask. [b] The uninhibited NADH-oxidase specific activity ranged from 0.500 to 1.00 μatoms of oxygen consumed/min/mg of protein. [c] The uninhibited succinoxidase specific activity ranged from 0.350 to 1.360 μatoms of oxygen consumed/min/mg of protein.

coma 180 ascites cells. Complete details of the biological methods have been described earlier.[17]

Measurement of the Synthesis of DNA and RNA. Six-day-old Adenocarcinoma 755 ascites cells were incubated in the presence of either [^{3}H]thymidine or [^{3}H]uridine to measure the synthesis of DNA and RNA, respectively, and were processed as reported previously.[1]

Determination of NADH-Oxidase and Succinoxidase Activities. Heavy beef heart mitochondria were isolated by differential centrifugation as described by Smith.[18] The activities of the succinoxidase and NADH-oxidase enzyme systems were determined manometrically in the absence or presence of the various inhibitors according to procedures reported earlier.[19] The various quinone test compounds were added in ethanol which was maintained at a constant level in all of the assay flasks (0.1 ml of ethanol in 3 ml of buffer). Mitochondrial protein was determined by the method of Layne.[20]

Chemical Methods. All melting points were measured on a calibrated Thomas-Hoover capillary melting point apparatus. Analyses were performed by the Baron Consulting Co., Orange, Conn. Spectral data were obtained using a Perkin-Elmer 257 grating ir spectrophotometer and Varian A-60 and A-60A spectrometers. The latter instrument used Me_4Si as an internal standard. Where analyses are indicated only by symbols of elements, analytical results obtained for those elements are within ±0.4% of the theoretical values.

2-Chloromethyl-1,4-naphthoquinone. 2-Chloromethylnaphthalene (5 g, 0.028 mol) in 40 ml of AcOH was added dropwise over 2 hr to a well-stirred solution of 20 g of CrO_3 in 50 ml of 80% AcOH. The solution was cooled in an ice bath during the addition to keep the temperature from rising above 20°. The solution was stirred at room temperature for 24 hr and then poured into 500 ml of ice-H_2O and recrystallized from MeOH to give 2 g (35%) of yellow crystals: mp 86–88°. *Anal.* ($C_{11}H_7O_2Cl$) C, H, Cl.

2-Methyl-3-acetoxymethyl-1,4-naphthoquinone. 2-Methyl-3-chloromethyl-1,4-naphthoquinone[7] (1 g, 5 mmol) and NaOAc (0.5 g, 5 mmol) were refluxed in 10 ml of AcOH for 2.5 hr. AcOH was evaporated to dryness under reduced pressure. H_2O (20 ml) was added and the mixture was extracted twice with $CHCl_3$. The $CHCl_3$ extracts were combined, dried over Na_2SO_4, and evaporated to dryness to give a yellow powder. The yellow powder was chromatographed on a silica gel column (4 × 48 mm), using as eluent EtOAc–petroleum ether (1:4 v/v), to give 0.2 g (17%) of yellow crystals after recrystallization from EtOAc and ligroine (bp 65–90°): mp 58–60°. *Anal.* ($C_{14}H_{12}O_4$) C, H.

2,3-Bis(acetoxymethyl)-1,4-naphthoquinone. 2,3-Bis(chloromethyl)-1,4-naphthoquinone[7] (2 g, 8 mmol) and KOAc (2 g, 20 mmol) were refluxed in AcOH (20 ml) for 3 hr. After evaporation to dryness under reduced pressure, the residue was extracted with hot EtOH repeatedly. The EtOH extracts were combined and cooled to room temperature. A brown amorphous solid was removed by filtration and the filtrate was concentrated to 10 ml. On standing pale yellow crystals (0.25 g, 11%) were obtained which were recrystallized from EtOH to give fine yellow crystals: mp 129–131°. *Anal.* ($C_{16}H_{14}O_6$) C, H.

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