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Substituted 1,4-Naphthoquinones vs. the Ascitic Sarcoma 180 of Mice

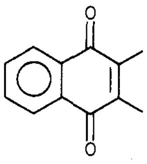
Ernest M. Hodnett,[*,†] Chinda Wongwiechintana,[†] William J. Dunn III,[*,‡] and Pam Marrs[‡]

Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078, and Department of Medicinal Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, Illinois 60680. *Received February 19, 1982*

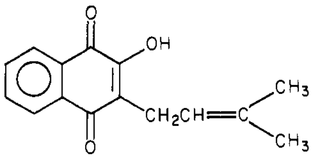
Twelve 1,4-naphthoquinones have been tested against the ascitic form of sarcoma 180 in Swiss mice. Statistical analysis shows that the most important molecular parameter determining their effectiveness in prolonging the life of mice bearing this tumor is their redox potentials. Although the toxicities of the compounds are also related to the redox potentials in the same way, the therapeutic indexes can be increased by adding substituents of greater lipophilicity. The naphthoquinones differ greatly in antitumor activities and may inhibit the growth of malignant cells by different mechanisms.

Quinones occur widely in nature, and some have important roles in the biochemistry of energy production and serve as vital links in the electron–transport system. Some quinones are active in inhibiting the growth of bacteria and fungi.

Several compounds containing the 1,4-naphthoquinone moiety, a, are active against some tumor systems[1-8] in experimental animals. Lapachol, b, obtained from natural sources, has been tested against human cancers,[6,7] since it has activity against some animal cancers. A similar compound, dichloroallyllawsone, c, has also been used against human cancer.[4,9] Some quinones, such as d, that are active against animal tumors appear to function as bioreductive alkylating agents.[7,8] Such quinones are thought to be reduced in vivo to the unstable dihydro forms, which in turn decompose to the reactive *o*-quinone methide, e, which can alkylate important biological materials. The mode of antitumor action of other quinones is unknown but probably involves either addition or substitution on the quinone ring.



a



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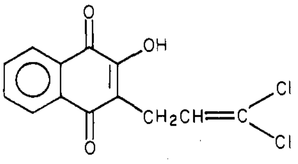

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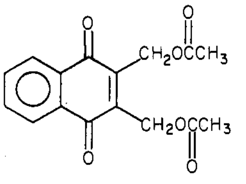

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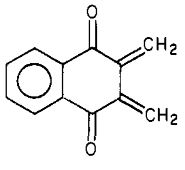

e

We wished to test 1,4-naphthoquinones with substituents having a range of electronic and lipophilic characteristics in order to help elucidate their mechanisms of action as antitumor agents. Twelve 1,4-naphthoquinones were obtained and tested against the ascitic form of the sarcoma 180 tumor of mice. The results were then subjected to regression analysis in order to ascertain the factors most important to their antitumor activity. It was hoped that the information gained might be useful in the design of new antitumor drugs.

Chemistry. Six derivatives of 1,4-naphthoquinone were prepared by known methods, and their structures were verified by elemental analysis and infrared, NMR, and mass spectroscopy. These are listed in Table I as compounds **3**, **5–8**, and **12**. One chlorine atom of 2,3-dichloro-1,4-naphthoquinone (**4**) can be replaced by a variety of nucleophiles under proper conditions. Reaction of **4** with aniline gave **3** [2-chloro-3-(phenylamino)-1,4-naphthoquinone]; with ammonia, **5** (2-amino-3-chloro-1,4-naphthoquinone); with methanol, **6** (2-chloro-3-methoxy-1,4-naphthoquinone); and with ethanol, **7** (2-chloro-3-ethoxy-1,4-naphthoquinone). 2-Chloro-3-propoxy-1,4-naphthoquinone (**12**) was obtained when **4** reacted with 1-propanol. Compound **5** was acetylated to give **8** (2-acetamido-3-chloro-1,4-naphthoquinone).

Since during the study of these compounds large differences in their solubilities were noted, their solubilities in water buffered at pH 7.4 were determined (Table II). Because the transport of drugs in the body is related to their partitioning between water and 1-octanol, we determined the partition coefficients for most of these compounds (Table II). The relationship between the two parameters for this set of compounds was then examined.

Since other workers[11-14] have found correlations between the biological activities of quinones and their redox potentials, we wished to study this relationship for our com-

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[†] Oklahoma State University.

[‡] University of Illinois.

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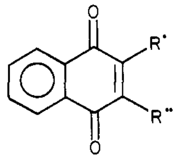
Table I. Antitumor Activities of 1,4-Naphthoquinones against the Ascitic Sarcoma 180 of Mice



no.	R'	R''	ED_{25} mg/kg	 mmol/kg	optimum dose,[b] mg/kg	max T/C,[c] %	LD_{50},[d] mg/kg
1	H	H	1.90	0.012	3	347	5.5
2	OH	C_5H_9	141.0	0.582	100	116	400
3	Cl	NHC_6H_5	94.0	0.331	80	117	1600
4	Cl	Cl	4.89	0.022	6	189	30
5	Cl	NH_2	25.5	0.123	20	112	800
6	Cl	OCH_3	2.49	0.011	4	315	14
7	Cl	OC_2H_5	1.97	0.008	3	230	16
8	Cl	$NHCOCH_3$	2.97	0.010	4	284	11
9	OH	H	33.0	0.190	25	104	100
10	CH_3	H	30.0	0.174	30	126	150
11	OCH_3	H	49.3	0.262	60	154	320
12	Cl	O-*n*-C_3H_7	2.45	0.010	7	271	20

[a] Effective dose, the daily dose of this compound that will give a 25% increase in survival; i.e., T/C = 125%. This value is obtained by interpolation of a semilogarithmic dose-response curve of T/C vs. log dose. Day 0, 10^6 sarcoma 180 cells injected ip; days 1-3, a dose of the compound injected ip. T represents the average survival of the treated mice; C the survival of the untreated mice. [b] The daily dose that will give the maximum T/C according to this protocol. [c] The greatest increase in survival afforded by any dose of the compound using the same protocol. [d] The single dose of the compound given ip that will kill one-half of the mice within 5 days.

Table II. Substituent and Molecular Parameters Used in Statistical Treatment of the Results

no.	π	σ	MR, mL	$-E_{1/2}$,[a] V	mp, °C	solubility in water, mg/L	log P[b]
1	0.00	0.00	0.206	0.164	120	89.4	1.74
2	1.33	−0.52	2.710	0.347	139	9.5	2.69
3	2.08	−0.17	3.607	0.272	209	2.4	(4.04)
4	1.42	0.46	1.206	0.146	190	8.0	(3.16)
5	−0.52	−0.43	1.145	0.382	194	37.7	2.08
6	0.69	−0.04	1.390	0.184	145	39.3	2.15
7	1.09	−0.01	1.850	0.192	79	34.1	(2.61)
8	−0.26	0.23	2.096	0.165	214	106	1.42
9	−0.67	−0.37	0.388	0.330	192	255	(1.07)
10	0.56	−0.17	0.668	0.224	105	10.6	2.25
11	−0.02	−0.27	0.890	0.283	180	31.3	1.59
12	1.55	−0.13	1.496	0.195	37.5		(2.95)

[a] Half-wave potential. Against the saturated calomel electrode. [b] Partition coefficient. Values in parentheses were estimated by the method of Rekker.[10]

pounds. Polarographic half-wave potentials, $E_{1/2}$, have been published by Holmes[15,16] for eight of these 1,4-naphthoquinones. We measured half-wave potentials for the other compounds under the same conditions used by Holmes; these values are reported in Table II.

Biological Evaluation. The acute toxicity (LD_{50}, Table I) of each compound was determined by a protocol described under Experimental Section. For quantitative structure–activity studies, the logarithm of the reciprocal of the LD_{50} [in millimoles per kilogram mouse, log (1/LD_{50})] was used as one of the dependent variables. This parameter plays an important part in the evaluation of antitumor agents, since, in practice, a good drug must inhibit growth of malignant cells without destroying normal cells.

All compounds were tested against the ascitic form of sarcoma 180 in mice at several doses by the protocol described under Experimental Section. In order to develop a complete dose–response curve (survival time vs. log dose), at least two dose levels in the effective dose range and at least one dose showing a toxic response were required. From the tests in the effective dose range we were able to interpolate or extrapolate to the dose, ED_{25}, that would give a 25% increase in the survival time compared to that of the controls (Table I). For the quantitative structure–activity studies, the logarithm of the reciprocal in the ED_{25} (in millimoles per kilogram of mouse) was used as the dependent variable. The maximum survival time compared to that of the controls (maximum T/C) and the dose that gave the maximum T/C (optimum dose) were also recorded. The therapeutic index, LD_{50}/ED_{25}, was also used in our statistical evaluations.

Substituent and Molecular Parameters. The substituent parameters π, δ, and NMR represent lipophilic, electronic, and steric factors of substituents. The values shown in Table II are taken from those published by Hansch et al.;[17] the values of MR have been scaled

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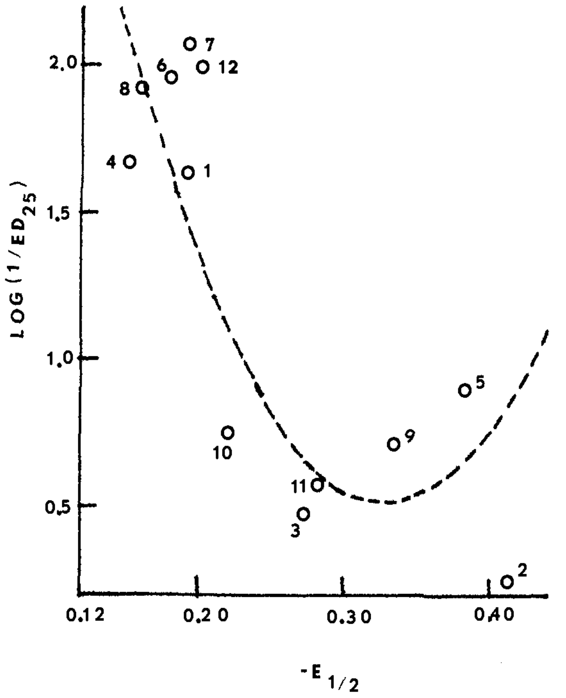

Figure 1. Relationship of antitumor activity and half-wave potentials for 12 1,4-naphthoquinones. The number nearest each circle identifies the compound (Table I). The dashed line is drawn from eq 1.

downward by a factor of 10 to the approximate magnitude of the other factors. Since these naphthoquinones are substituted in the 2- and 3-positions only and since these positions of 1,4-naphthoquinone are identical, we have summed the values for the substituent effects in these two positions. The redox potentials of quinones have been cited as factors in their antitumor activities;[12-14] therefore, the half-wave potential, $E_{1/2}$, has been included as a possible molecular descriptor. The melting point is also included as a possible parameter. Log P, the partition coefficient of the compound for 1-octanol and water, relates to the transport of the drug in the body. The values here are experimentally determined for seven of the compounds. Log P values were estimated for the remaining compounds by using the fragment values published by Rekker.[10] Attempts were made to measure the log P values for compounds **3**, **4**, and **9**. Compound **3** was too insoluble to measure, while compounds **4** and **9** appeared to decompose over the time course of the measurement. Although the log P of **9** has been reported by others, its estimated value was used here for consistency.

Results and Discussion

The most important factor in determining log (1/ED$_{25}$) for all 12 of the naphthoquinones is $E_{1/2}$ ($r = 0.81$). Addition of the $E_{1/2}{}^2$ term is an improvement, giving eq 1 for

$$\log (1/\mathrm{ED}_{25}) = -31.0\ (\pm 13.7)\ E_{1/2} + 45.7\ (\pm 26.1)\ E_{1/2}{}^2 + 5.82\ (\pm 1.65) \qquad (1)$$

$$n = 12;\ r = 0.86;\ \mathrm{SD} = 0.406;\ F = 12.73;\ F_{2,9,0.995} = 10.11$$

the best pair of variables. In this and all other equations, the numbers in parentheses are the standard errors, n is the number of compounds, r is the correlation coefficient, SD is the standard deviation, and F is the distribution of variance ratios.[21] A plot of the data for eq 1, given in Figure 1, has the form of a parabola with a minimum in the antitumor activity at about −0.34 V (against the saturated calomel electrode). Antitumor activities of compounds expressed as log (1/ED$_{25}$) increase whether the $E_{1/2}$ of the compound is greater or less than −0.34 V. The antitumor activity increases as the ease of the reduction increases or decreases from the point of minimum activity. Figure 1 also suggests that an $E_{1/2}$ of about −0.20 or less is necessary for antitumor activity in this system. Such thresholds in reduction potential for quinones have been observed previously by Dunn and Wold.[18] These data may explain the variation in relationships between antitumor activity and $E_{1/2}$ shown by naphthoquinones in L1210 leukemia, solid sarcoma 180, and ascitic sarcoma 180 in mice.[14]

The most significant variables determining the acute toxicity for the 12 naphthoquinones are $E_{1/2}$ ($r = 0.81$), $E_{1/2}{}^2$ ($r = 0.77$), and σ ($r = 0.70$). The most significant pairs of variables are $E_{1/2}$ and solubility in water ($r = 0.893$), $E_{1/2}$ and log P ($r = 0.892$), $E_{1/2}$ and π ($r = 0.878$) and $E_{1/2}$ and $E_{1/2}{}^2$ ($r = 0.860$). Equations 2 and 3 show the relationships

$$\log (1/\mathrm{LD}_{50}) = -8.64\ (\pm 1.52)\ E_{1/2} - 0.35\ (\pm 0.16)\ \log P + 3.37\ (\pm 0.56) \qquad (2)$$

$$n = 12;\ r = 0.89;\ \mathrm{SD} = 0.402;\ F = 17.54;\ F_{2,9,0.999} = 16.39$$

$$\log (1/\mathrm{LD}_{50}) = -28.3\ (\pm 15.3)\ E_{1/2} + 38.4\ (\pm 29.2)\ E_{1/2}{}^2 + 4.84\ (\pm 1.84) \qquad (3)$$

$$n = 12;\ r = 0.86;\ \mathrm{SD} = 0.454;\ F = 12.8;\ F_{2,9,0.995} = 10.11$$

for two of these pairs. Equation 3 again represents a parabola with a minimum at about −0.37 V, similar to that for log (1/ED$_{25}$).

Examination of our antitumor data shows that these 12 compounds can be separated into two distinct groups according to their antitumor activities. Compounds **1**, **4**, **6–8**, and **12** increase survival times by 25% at dose levels of 1.90–4.89 mg/kg, whereas the others require 25.5–141 mg/kg to produce a 25% increase in survival. Compounds **1**, **4**, **6–8**, and **12** give survival times of 189–347% that of the controls; the other compounds in this set give survival times of 104–154% of the controls. The latter group of compounds is essentially inactive in this tumor system.

Since log (1/ED$_{25}$) responds to variables such as $E_{1/2}$ in much the same way that log (1/LD$_{50}$) does, it would appear that improving the therapeutic index might not be possible. However, when the therapeutic indexes of the six active compounds are subjected to linear regression analysis, the most significant variables taken one at a time are found to be π ($r = 0.82$), water solubility ($r = 0.78$), and $E_{1/2}$ ($r = 0.63$). Equation 4 shows the relationship of

$$\mathrm{TI} = 2.48\ (\pm 0.87)\ \pi + 4.12\ (\pm 0.88) \qquad (4)$$

$$n = 6;\ r = 0.82;\ \mathrm{SD} = 1.46;\ F = 8.09;\ F_{1,4,0.95} = 7.71$$

therapeutic index (TI) to π for the six active compounds. Equation 4 indicates that for active compounds an increase in lipophilicity leads to an increase in the therapeutic index.

Since we have experimental values of the aqueous solubilities and of the partition coefficients for most of these compounds, we wish to correlate these two factors for this set of compounds. The correlation of log S with log P (eq 5) has a form similar to that found by Hansch, Quinlan,

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[18] W. J. Dunn III and S. Wold, *J. Med. Chem.*, **23**, 595 (1980).

$$\log P = -1.19\ (\pm 0.15) \log S + 4.75\ (\pm 0.33) \qquad (5)$$

$$n = 11;\ r = 0.94;\ SD = 0.296;\ F = 64.14;\ F_{1,9,0.999} = 22.86$$

and Lawrence;[19] the slope is very close to those of a large number of compounds listed by these authors. Addition of another term to the relationship, the melting point, does not improve the correlation for this set of compounds; this term is invalid. Yalkowsky and Valvani[20] found that the additional term improved the correlation for their compounds.

Conclusion

The purpose of this work was (1) to increase the effectiveness of these 1,4-naphthoquinones as antitumor agents and (2) to help elucidate their mechanism of antitumor action. Both the antitumor activity and the toxicity of these compounds depend on the same variables, particularly the redox potentials, so that increasing the antitumor activity also increases the toxicity. However, analysis of the therapeutic index of the active compounds indicates that substituents with greater lipophilicity will give larger therapeutic indexes.

The great difference in antitumor activity between the six "good" compounds and the six "poor" compounds must indicate that the two groups differ in some fundamental respect. Dunn and Wold[18] have observed a threshold redox potential for quinones. Since the six "good" quinones have redox potentials more positive than −0.20 V, this may explain the difference between the two groups. The mechanism of antitumor action of the six "good" naphthoquinones may differ from that of the others. Since the chemical reactions of the quinones are complex and varied, involving molecules, ions, and free radicals, it is possible that the two groups of compounds might react differently in a biological medium.

Experimental Section

Of these 12 naphthoquinones, 6 were purchased from Aldrich Chemical Co. or from Eastman Kodak Co. The remaining 6 compounds were prepared in our laboratory by known methods with details given later in this section.

The structures of compounds synthesized were confirmed by elemental analysis, infrared spectrometry, NMR spectrometry, and mass spectrometry. Elemental analyses were performed by M. H. W. Laboratories, Phoenix, AZ, and were within acceptable limits of the theoretical values. The infrared spectra of the compounds were determined in mineral oil mulls (Nujol) on a Beckman IR-5A spectrophotometer. ^{1}H NMR spectra were recorded on a Varian XL-100 analytical nuclear magnetic resonance spectrometer in trifluoroacetic acid, $CDCl_3$, or acetone-d_6 according to their solubilities. Mass spectra of the compounds were determined on a CEC 21-1108 high-resolution mass spectrometer. Melting points were determined with a Fisher-Johns hot stage melting point apparatus and are uncorrected. The solubility studies were made with the help of a Bausch & Lomb Spectronic 20 spectrometer. The partition coefficients were determined with a Cary 118 CX spectrometer. Polarographic studies were made with a Sargent Polarograph Model XV.

Syntheses. 2-Chloro-3-(phenylamino)-1,4-naphthoquinone (**3**) was prepared by refluxing 2,3-dichloro-1,4-naphthoquinone (**4**) and aniline in methanol to give 98% of the theoretical yield. The crystals in the form of red plates melted at 209–110 °C; the recorded melting point is 207–110 °C.[22]

2-Amino-3-chloro-1,4-naphthoquinone (**5**) was prepared by refluxing **4** in 95% ethanol and concentrated ammonium hydroxide while a stream of ammonia was bubbled through the solution. The yield was 78% of theory. The shiny orange crystals melted at 194–195 °C; the reported melting point is 190 °C.[23]

2-Chloro-3-methoxy-1,4-naphthoquinone (**6**) was prepared by refluxing **4** with an equivalent molar quantity of sodium acetate in absolute methanol. The fine yellow needles melted at 145–146 °C; the reported value is 148 °C.[24] The yield was 72% of the theoretical.

2-Chloro-3-ethoxy-1,4-naphthoquinone (**7**) was obtained in a similar fashion from **4**, absolute ethanol, and sodium acetate. The yield of yellow feather needles was 48% of the theoretical; the product melted at 79 °C, although the reported melting point is 97 °C.[25] The structure of the compound was corroborated by elemental analysis, NMR, and infrared spectroscopy. We believe that the melting point of 79 °C is correct.

2-Chloro-3-propoxy-1,4-naphthoquinone (**12**) was prepared in a similar fashion from **4**, *n*-propyl alcohol, and sodium acetate. The melting point is 37.5 °C; no literature reference was found.

2-Acetamido-3-chloro-1,4-naphthoquinone (**8**) was prepared from 2-amino-3-chloro-1,4-naphthoquinone (**5**) by treatment with acetic anhydride. The yellow-green crystals (30% yield) melted at 214–215 °C. The reported melting point is 215 °C.[23]

Solubilities in Water. The solubility of each compound in water at pH 7.4 was determined by adding a warm 0.001 M Tris buffer solution to the solid with stirring, allowing the mixture to stand overnight to cool to room temperature, and measuring the absorption of the decanted solution at 340 nm. Comparison of the absorption with a known plot of absorptions vs. concentrations gave the solubility of the compound. Three samples of each compound were used, and the results were averaged to obtain the results given in Table II.

Partition Coefficients between 1-Octanol and Water. Partition coefficients were determined by using the shake–flask method described by Fujita, Iwasa, and Hansch.[26] For each compound, four samples in the range of 1.50–20.0 mg were weighed. Each sample was allowed to equilibrate between known volumes of 1-octanol and water. The equilibrium concentration of solute was determined spectroscopically by analysis of the water phase. The concentration in the 1-octanol phase was determined by difference. The ratio of the concentrations is the partition coefficient. The values so recorded are converted to log *P*. Accuracy is ±0.05 log *P* unit. Results are given in Table II.

Half-Wave Potentials. Half-wave potentials of compounds **2**, **6**, **7**, and **12** were determined under the same conditions used by Currie and Holmes,[16] who published the $E_{1/2}$ for eight of the compounds. The solvent was 75%, v/v, aqueous alcohol containing the reducible substance at 8×10^{-4} M concentration, acetic acid at 0.10 M, and sodium acetate at 0.10 M. The apparent pH of the solution was 6.60. The buffer constituted the carrier electrolyte. A saturated calomel electrode served as the reference electrode. To ensure that our values of $E_{1/2}$ were comparable to published values, we determined $E_{1/2}$ for **10** and **11**. Our values of −0.224 and −0.288 V matched the published values of −0.224 and −0.283 V, respectively. The $E_{1/2}$ of compounds **2**, **6**, **7**, and **12** in Table II were used as determined by us; other values of $E_{1/2}$ were determined by Currie and Holmes.[16]

Acute Toxicities. A group of six Swiss mice each received a single ip injection of a compound suspended in isotonic saline solution. The dose that killed one-half of the mice during 5 days was considered to be the LD_{50}. Results are given in Table I.

Inhibition of Ascitic Sarcoma 180 in Mice. Swiss mice were obtained from Charles River Breeding Laboratories, Wilmington,

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(26) T. Fujita, J. Iwasa, and C. Hansch, *J. Am. Chem. Soc.*, **86**, 5175 (1964).

MA. The ascitic sarcoma 180 cells were originally obtained from Frederic A. French, Chemotherapy Laboratory, Mount Zion Hospital, San Francisco, CA. The details of the protocol are given in Table I. Although each compound was administered at several dose levels, only the dose that gave the maximum T/C value is recorded in Table I. The daily dose that would increase survival times by 25% was obtained by interpolation and extrapolation from the results of other doses in the effective range of the compound.

Statistical Methods. Multiple linear regression studies were performed with the statistical analysis system (SAS)[27] on an IBM 370/168 computer. The RSQUARE procedure was used to determine the independent variable that was most significant for each dependent examined. This procedure determines the correlation coefficient for the dependent variable and each of the independent variables and lists these in order of their correlation coefficients. The REGRESSION procedure may then be used to obtain the equation that relates the index to any variable or variables and other statistical information.

All variables were examined by the CORRELATION procedure of the Statistical Analysis System to determine if any collinearity exits between the variables. A high degree of correlation between variables ($r = 1$) would give a biased result when they are used together.

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Registry No. 1, 130-15-4; 2, 84-79-7; 3, 1090-16-0; 4, 117-80-6; 5, 2797-51-5; 6, 15707-32-1; 7, 59641-32-6; 8, 5397-78-4; 9, 83-72-7; 10, 58-27-5; 11, 2348-82-5; 12, 84369-93-7; aniline, 62-53-3.

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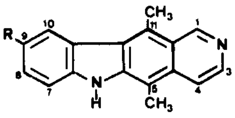
o-Quinone Formation in the Biochemical Oxidation of the Antitumor Drug N^2-Methyl-9-hydroxyellipticinium Acetate

Jean Bernadou,[†] Gérard Meunier,[†] Claude Paoletti,[†] and Bernard Meunier*[,‡]

Laboratoire de Pharmacologie et Toxicologie Fondamentales and Laboratoire de Chimie de Coordination, CNRS, 31400 Toulouse, France. *Received March 23, 1982*

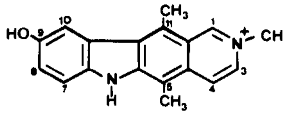
The activation of N^2-methyl-9-hydroxyellipticinium acetate (4) by a peroxidase–H_2O_2 system leads to the formation of an *o*-quinone (**7a**). This *o*-quinone is not directly generated from the starting material but through a quinone imine intermediate (6) which is subsequently oxidized. This reaction is highly dependent on pH values. The *o*-quinone **7a** is easily protonated (**7b**), gives an addition product with methanol (9), and is reduced by cysteine. The *o*-quinone **7b** has a rather low inhibitory effect against L1210 leukemia cell multiplication but acts as an electron carrier and dramatically augments the oxygen consumption in xanthine oxidase–NADH and rat liver microsomes–NADPH systems.



1 R = H

2 R = OCH_3

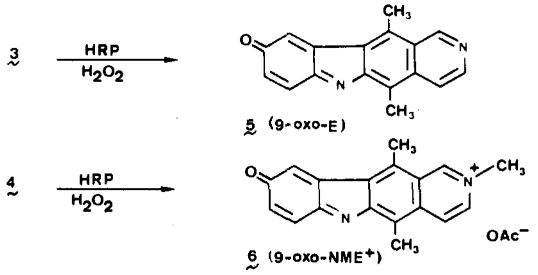
3 R = OH (9-OH E)



4 (9-OH-NME$^+$)

Among the molecules derived from ellipticine (1, 5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole), a cytotoxic alkaloid extracted from plants of the Apocynacae family, those with an alkoxy or an hydroxy group in position 9 have recently been considered for clinical trials.[1] 9-Methoxyellipticine (2), a natural derivative of ellipticine, has been reported to be active in acute myeloblastic leukemia;[1b] 9-hydroxyellipticine (3) and the N^2-methyl-9-hydroxyellipticinium (4, 9-OH-NME$^+$) present a wide range of antitumor activity against experimental tumors.[1c] The latter compound, 4, exhibits an appreciable activity against osteolytic breast cancer metastasis and anaplastic thyroid carcinoma.[1c] The high DNA affinity for these molecules (1–4) by intercalation between base pairs was first suggested to explain the antitumor activity of these drugs.[2] More recently, preliminary investigations on the oxidation of 3 and 4 by a peroxidase/hydrogen peroxide system support the hypothesis of an activation in vivo of the hydroxylated ellipticines.[3]

So the enhanced antitumor activity of 3 and 4 might be related to the DNA intercalation phenomena and the reactivity of oxidized products obtained in vivo from 3 and 4. For example, the two-electron oxidation of 3 and 4 by horseradish peroxidase (HRP) and hydrogen peroxide, respectively, lead to the formation of the two quinone imines 5 and 6.[3] Compound 5 is obtained as a well-defined stable solid, but 6 has not yet been isolated and fully characterized due to its high reactivity in solution.



5 (9-oxo-E)

6 (9-oxo-NME$^+$)

[†] Laboratoire de Pharmacologie et Toxicologie Fondamentales.

[‡] Laboratoire de Chimie de Coordination.

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