Journal of Cellular Biochemistry 25:15–29 (1984)
Molecular Biology of Host–Parasite Interactions 181–195

The In Vitro Trypanocidal Activity of N-Substituted *p*-Benzoquinone Imines: Assessment of Biochemical Structure-Activity Relationships Using the Hansch Approach

Robert W. Grady, Steven H. Blobstein, Steven R. Meshnick, Peter C. Ulrich, Anthony Cerami, Jafargholi Amirmoazzami, and Ernest M. Hodnett

Department of Medical Biochemistry, The Rockefeller University, New York, New York 10021 (R.W.G., S.H.B., S.R.M., P.C.U., A.C.), Departments of Pediatrics (R.W.G.), Pharmacology (R.W.G.), Dermatology (S.H.B.), and International Medicine (S.R.M.), Cornell University Medical College, New York, New York 10021, and Department of Chemistry, Oklahoma State University, Stillwater, Oklahoma 74078 (J.A., E.M.H.)

It has previously been found that naphthoquinones can potentiate the rate of hydrogen peroxide production by mitochondrial preparations of Trypanosoma brucei brucei and that organisms treated with naphthoquinones are more susceptible to lysis, especially in the presence of compounds such as heme, which promote the homolytic cleavage of hydrogen peroxide. We have evaluated the lytic effect of various N-substituted *p*-benzoquinone imines both in vitro and in vivo and have attempted to correlate their structure with trypanocidal activity using the Hansch approach. While none of the compounds tested proved to be active in vivo, all caused the lysis of trypanosomes in vitro. The parameters that correlated best with trypanocidal activity were the conditional redox potential, the lipophilicity of the substituent attached to the nitrogen atom and the number of active hydrogens on the quinonoid ring. These findings suggest two possible modes of action, which may in fact be related. Conjugate nucleophilic addition and/or oxidative damage could be responsible for lysis of the parasites. These same compounds were previously found to be active against the ascitic sarcoma 180 in mice. The strong correlation between antineoplastic activity in vivo and trypanocidal activity in vitro suggests a similar mode of action in both cases. Further studies aimed at developing a quinonelike compound that will be active against trypanosomes in vivo are now in progress.

Key words: N-substituted *p*-benzoquinone imines, Trypanosoma brucei brucei, trypanocidal drugs, Hansch approach

Trypanosomes cause both human and animal diseases of major importance in Latin America and Africa. Chagas disease, which afflicts more than 7 million Latin

Received March 11, 1983; revised and accepted December 12, 1983.

Americans, is caused by Trypanosoma cruzi [1]. In tropical Africa, trypanosomiasis of cattle due to T congolense, T vivax, and T brucei brucei seriously limits food production [2]. Furthermore, human trypanosomiasis or African sleeping sickness, which is caused by T b gambiense and T b rhodesiense, is currently in a state of controlled endemicity, although health officials fear a major epidemic [3].

There are no drugs that have proven effective against chronic Chagas disease. Moreover, no new drugs to treat African trypanosomiasis have been introduced in more than 30 years [4]. Consequently, those drugs now in use are becoming less effective as resistant strains emerge. Thus, there is an urgent need for new trypanocidal agents.

For several years, we have been studying the biochemistry of T b brucei in order to identify metabolic differences between these organisms and their mammalian hosts. Once such differences are discovered, it may be possible to design drugs that exploit them so as to kill the parasites.

We have been particularly interested in the fact that trypanosomes lack enzymes such as catalase and peroxidase to break down H_2O_2 [5,6]. As a result, these parasites accumulate H_2O_2 and can be readily lysed by heme and heme analogues, presumably because of damage by free radicals formed as a result of homolytic cleavage of hydrogen peroxide [7–9]. Naphthoquinones, such as menadione, also cause lysis of T b brucei in vitro, probably via stimulating overproduction of H_2O_2 [8]. They have been found to lyse T cruzi epimastigotes in a similar manner [10]. We report here the results obtained with 18 N-substituted *p*-benzoquinone imines that have previously been tested for antineoplastic activity [11]. Because these compounds are structurally similar to naphthoquinones, we hypothesized that they might lyse trypanosomes in an analogous manner.

MATERIALS AND METHODS

Chemicals

The preparation of the *p*-benzoquinone imines has been described previously [11]. All other chemicals used were of reagent grade.

Animals

Balb/c and Swiss-Webster mice were obtained from the Charles River Breeding Laboratories, Inc (Wilmington, MA) and the Rockefeller University (New York, NY) respectively. Sprague-Dawley rats were purchased from Taconic Farms, Inc (Germantown, NY).

Organisms

T b brucei (EATRO 110) were obtained from Dr W. Trager (The Rockefeller University) and stored as stabilates in 10% glycerol at −80°C. This strain had been passaged seven times in rodents since it was last transmitted by tsetse flies. Intraperitoneal injection of 2×10^4 motile organisms into Swiss-Webster mice (20-g males) yielded pleomorphic infections, with slender forms predominating 5–7 days after infection.

A monomorphic strain, EATRO 110M, was derived from EATRO 110 via serial passages (> 100) in mice. These organisms were uniformly slender in morphology.

They were routinely harvested from Swiss-Webster mice or Sprague-Dawley rats (250–350-g females) 3–4 days after infection.

Isolation of Trypanosomes

Trypanosomes (EATRO 110M) were isolated from fresh blood according to the procedure of Lanham and Godfrey [12]. Infected rats were anesthetized (ether) and bled by cardiac puncture into heparinized syringes. Whole blood (10 ml) was applied to a column (2.5 × 7 cm) of Whatman DE-52 cellulose and eluted with phosphate-buffered saline containing glucose (PSG 6:4) [12]. The eluant was centrifuged at 1,000g for 10 min to pellet the trypanosomes. The organisms were washed twice with PSG 6:4 and then resuspended in minimal essential medium containing Hank's salts (MEM; GIBCO, Grand Island, NY) to a concentration of 1×10^8 organisms/ml. This suspension of parasites was kept on ice and used within 3 hr. Over this period of time more than 95% of the parasites remained motile and equally susceptible to lysis by heme.

Assessment of Trypanocidal Activity In Vitro

The trypanocidal activity of the quinone imines was assessed in vitro by monitoring their ability to cause lysis of trypanosomes. A suspension of trypanosomes (EATRO 110M) in MEM was diluted 1/10 with PSG 6:4 giving a concentration of approximately 1×10^7 organisms/ml and was then maintained at 37°C. The turbidity of such suspensions was measured at 750 nm in 1-ml cuvettes using a thermostated (37°C) Acta III recording spectrophotometer (Beckman Instruments, Inc, Cedar Grove, NJ). The quinone imines were dissolved in dimethylsulfoxide (DMSO) and added to the suspensions at various concentrations. The values reported are the minimum concentration that caused complete lysis of the trypanosomes in 15 min and the maximum concentration that caused no lysis in the same length of time. In no case was the volume of DMSO added greater than 10 μl/ml of suspension. This amount of DMSO had no effect upon the motility of the organisms or the turbidity of the suspensions. Where lysis was indicated by a decrease in turbidity, it was confirmed by inspection of the suspension by phase contrast microscopy.

Assessment of Trypanocidal Activity In Vivo

Groups of five Balb/c mice were infected with 5×10^4 organisms via an intraperitoneal injection. Three to 4 h after infection and daily for 4 days thereafter, the animals were treated with the quinone imines at a dose equivalent to 1/5 of their LD_{50} as determined by Hodnett et al [11]. The drugs were administered IP as suspensions in 0.9% saline containing 0.25% methylcellulose.

Statistical Assessment of Trypanocidal Activity

The data on the lysis of T b brucei in vitro was analyzed by a mathematical approach developed by Hansch [13,14]. In using this approach one seeks to relate the biological activity of a series of compounds by a linear combination of parameters such as that shown in Equation 1 and thereby to determine structure-activity relationships which can be used to predict the activity of other compounds in the series:

$$\log(BA) = k_1\pi + k_2\sigma + k_3Es + k_4 \ . \qquad (1)$$

In the equation above, BA is a function of the concentration of a compound that will cause a given level of the biological activity being studied, π is a solubility parameter, σ is an electronic parameter, E_s is a steric parameter, and the k's are constants determined for each series of compounds. This method allows one to correlate all variables that may be important in relating the biological activity of a series of compounds under a particular set of conditions and to select that parameter or set of parameters which is most significant in relating structure to biological activity. Having determined the most important parameter (or parameters), one can then predict the biological activity of other compounds in the series provided the correlations are good enough. Sometimes a single variable is sufficient to describe a structure-activity relationship [15], but more often two or more parameters are necessary if the correlation coefficient is to exceed 0.9, 1.0 representing perfect correlation.

The biological activity of a drug generally involves transport to a particular tissue and subsequent reaction with an enzyme or receptor site. Transport of a drug is dependent upon many factors, such as its electronic charge in the biological milieu, water solubility, lipid solubility, etc. The proper distribution of a compound between lipid and aqueous phases is very important if it is to move in biological fluids and penetrate cell membranes. Many workers use 1-octanol as a model of biological fat because it has a similar ability to dissolve a variety of compounds. The partition coefficient, P, is defined as the ratio of the solubility of a compound in 1-octanol to that in water (Equation 2):

$$P = \frac{\text{concentration in 1-octanol}}{\text{concentration in water}} \tag{2}$$

This parameter has been determined for many compounds having biological activity [16]. One can then compare the lipophilicity or hydrophobicity of a substituted member (RX) of a series of compounds to that of the parent compound (RH) using a relationship such as that given in Equation 3.

$$\log \frac{P_{RX}}{P_{RH}} = \log P_{RX} - \log P_{RH} = \pi_X \ . \tag{3}$$

The parameter π_X is often used as a substituent constant expressing the lipophilicity of the group X; values π_X for over 300 groups are listed by Hansch [17].

Estimates of log P can also be determined directly from the structure of a compound using the method of Rekker [18–20]. The skeleton of the molecule is broken down into fragments, each fragment contributing a value f to its lipophilicity. Some fragments have different values depending upon whether they are part of an aliphatic or an aromatic system. Since the quinone imines are not truly aromatic, although having a great deal of resonance energy, it was not clear whether the aliphatic or the aromatic values for the fragments should be used. Therefore, both sets of parameters were considered in making correlations [21], as well as the π_X constants of Hansch. Although the results were nearly identical, the aliphatic f-values gave somewhat better correlations and are those reported (Table III).

In order to simplify the calculations, we have reduced the number of parameters involved based on the intrinsic symmetry of the quinone imines (Fig. 1). Substituents

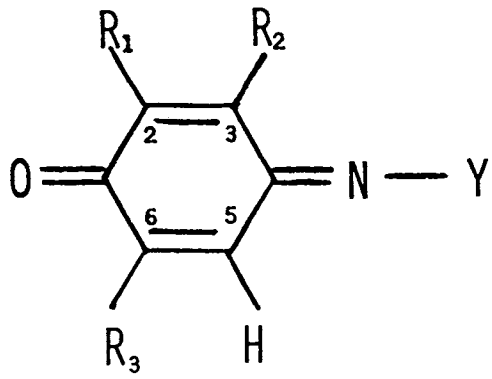

Fig. 1. N-Substituted *p*-benzoquinone imines. The nature of the substituents R_1, R_2, R_3, and Y is given in Table I.

attached to carbon atoms 3 and 5 are identically situated with respect to the carbon attached to the nitrogen. Accordingly, the parameter values of such substituents were summed for use in the correlations, the summations being designated by the subscript 35 (Table III). Likewise, atoms or groups of atoms attached to carbon atoms 2 and 6 are symmetrically arranged about the carbonyl group; their values were also summed (subscript 26, Table III). Values of the parameters for substituents on the nitrogen atom are indicated in Table III by the subscript Y.

As suggested by Equation 1, electronic and steric effects are also important determinants of biological activity. The ability of a given molecule to react with an active site has both an electronic and a steric component. Several sets of substituent constants exist for quantitating the electronic effects of atoms and functional groups, especially those on an aromatic ring. Hammett [22] showed that one could compare chemical equilibria involving a series of aromatic compounds through the use of constants (σ_m and σ_p) that reflect the ability of a meta or para substituent to donate or withdraw electrons from the molecular site undergoing reaction. Electronic effects are transmitted more readily by functional groups para to the reactive site, hence each group has both a meta and a para σ-value. Since the reactive site is unknown in the case of the quinone imines, both sets of σ-values were included in our calculations.

Each substituent also has both a field (inductive) effect, $\mathcal{F}$, and a resonance effect, $\mathcal{R}$, each of which reflects the ability of that substituent to donate or withdraw electrons. These parameters are derived from σ_m and σ_p [23] and have a single value for each atom or functional group. Hansch [17] has published values of $\mathcal{F}$ and $\mathcal{R}$ for more than 300 atoms and groups. In the case of the quinone imines, $\mathcal{F}$ and $\mathcal{R}$ correlated better with the in vitro results than the σ constants; the values of $\mathcal{F}$ and $\mathcal{R}$ used in the calculations are listed in Table III.

Steric effects are not readily quantitated because of a lack of knowledge about the exact dimensions of the active site. Nonetheless, the effect of various substituents can be approximated by a comparison of their molar volumes. The molar refraction, MR, has the dimensions of volume and may be considered a relative volume [17]. Although the molar refraction can be calculated for each structure, we chose to utilize the values of Hansch [17] reduced by a factor of ten to make them comparable in magnitude to the other parameters considered (Table III).

As stated above, any variable that might have an effect on biological activity and for which values are known may be used in the correlations. For example, the number of unsubstituted carbon atoms attached to the ring of the quinone imines may be significant in determining their biological activity as addition is more likely to occur at these carbons than at those already bearing a substituent. Since oxygen is more electronegative than nitrogen, hydrogens attached to carbon atoms 3 and 5 are more reactive than those attached to 2 and 6. Consequently, as one measure of chemical reactivity, we included in our calculations the number of active hydrogens at positions 3 and 5, H_{35}. To the extent that substituents at positions 2 and 6 may alter the reactivity of hydrogens at 3 and 5, one can define modified indexes of chemical reactivity such as that shown in Equation 4:

$$H'_{35} = H_{35} + H_{26} \cdot \mathscr{R}_{26} \ , \tag{4}$$

where H_{26} is the number of active hydrogens at positions 2 and 6, and $\mathscr{R}_{26}$ is the resonance effect of the substituents at the same positions. Such modified assessments of chemical reactivity were also included in our calculations.

The logarithm of the partition coefficient defined above is a measure not only of transport in the biological medium but also of hydrophobic binding to the active site [24,25]. In some cases log P is linearly related to biological activity, while in others the relationship is second order, indicating an optimum value of log P for a given biological effect. Accordingly, both log P and $(\log P)^2$ were included in the calculations, the values of these parameters being estimated from the f-fragments of Rekker [18–20].

Finally, one of the major reasons for evaluating the quinone imines as trypanocidal agents was to determine if they could function as alternate electron acceptors leading to increased H_2O_2 production. Accordingly, we utilized their conditional redox potentials (V) [11] in our correlations. Substituent effects upon redox potentials have not been studied extensively and hence values for given compounds are not readily obtained from published data. Generally one must synthesize the desired compounds and make the measurements or estimate the potentials from those of related compounds.

Having determined the various molecular parameters to be correlated with biological activity, in this case lysis of T b brucei in vitro, we had to assess the best method of expressing the biological data. The trypanocidal activity of a given compound was measured as a function of its concentration. Since the most active compounds were those that caused lysis at low concentrations, we chose to correlate the logarithm of the reciprocal of the concentration and designated this the logarithm of trypanocidal activity, log(TA). Separate computations were made for the lowest concentration of each compound causing lysis of the trypanosomes, the highest concentration failing to cause lysis and the geometric mean of the two. The latter concentration gave somewhat more consistent results. All of the parameters discussed above were evaluated by a computer program to determine which were linearly related to log(TA). The correlation coefficient, r, measures the fit of the data to a straight line, an r of 1.000 indicating a perfect fit. The program RSQUARE of the Statistical Analysis System [26] was used to make a calculation of r^2 for each parameter or variable vs log(TA) and to rank the parameters in decreasing order of

their r^2 values. Beginning with those that fit the best, the program was then used to make r^2 calculations for every combination of two variables and to rank these combinations in decreasing order based on the calculated r^2 values. One can continue this iterative procedure as long as desired or until all combinations of variables are used. Inclusion of additional variables always yields larger values of r^2 but the contribution of each additional variable generally becomes less statistically significant. Once the most significant variables are determined, the program REGRESSION [26] is used for statistical analysis of the series of linear equations so as to determine the best fit of the data.

RESULTS

The 18 N-substituted *p*-benzoquinone imines were evaluated in vitro at concentrations up to 1 mM. All caused complete lysis of the trypanosomes at this concentration. Various dilutions were made until a concentration of imine was reached which failed to cause lysis after incubation at 37°C for 15 min. Reported in Table I is the highest concentration of each compound which failed to cause lysis and the lowest concentration which caused complete lysis. At intermediate concentrations incomplete lysis was observed.

The results suggest that the compounds fall into three groups depending upon the substituent, Y, attached to the nitrogen atom. compounds 1-5, wherein Y is the *p*-dimethylaminophenyl moiety, all caused complete lysis at concentrations ranging from 1 to 5 μM. The five oximes, 6-10 (Y = OH), were approximately 100 times less active, the lowest concentration causing complete lysis ranging from 50 to 750 μM. The N-halogenated imines, compounds 11-16, caused complete lysis over a much broader range of concentrations (0.05–50 μM). In the case of 11 and 12 with hydrogens at positions 2 and 6, the chloroimine, 11, was 1,000 times more active than the bromoimine. The analogous pair of imines with a chlorine atom at positions 2 and 6 (compounds 13 and 14) also differed in activity, however, in this case the chloro derivative was six times less active. A third pair of N-halogenated imines having bromines adjacent to the carbonyl were of equal activity, the lowest concentration causing complete lysis being 2 μM. Lastly, the activities of the two compounds derived from N-*p*-hydroxyphenyl-*p*-benzoquinone imine were similar to those of the *p*-dimethylaminophenyl derivatives. The acetate, 18, was active at 5 μM, while the sodium salt, 17, was ten times less active.

Ten of the 18 quinone imines were evaluated in vivo. At least three compounds, including the most active, were selected for testing from each of the groups referred to above. As seen in Table II, none significantly prolonged the life of T b brucei-infected mice when administered IP for 5 days at a dose equivalent to one-fifth of the LD_{50}. In fact, three of the compounds wherein Y was aromatic (1, 5, and 18) caused a decrease in survival.

Despite these in vivo results, further evaluation of quinones and quinonelike compounds seemed warranted based on the trypanocidal activity of such compounds in vitro. Accordingly, we analyzed our in vitro results using the mathematical ap-

$$\log(TA) = 14.00(\pm 4.00)\ V - 9.17(\pm 2.39) \qquad (5)$$
$$n = 15;\ r = 0.69$$

TABLE I. Lytic Effect of N-Substituted *p*-Benzoquinone Imines on T b brucei in Vitro*

Compound no.	R_1	R_2	R_3	Y	Concentration (μM) Maximum causing 0% lysis	Minimum causing 100% lysis	Geometric mean
1	H	H	H	4-$(CH_3)_2NC_6H_4$	2.5	5.0	3.5
2	CH_3	H	H	4-$(CH_3)_2NC_6H_4$	1.0	2.5	1.6
3	OCH_3	H	H	4-$(CH_3)_2NC_6H_4$	2.5	5.0	3.5
4	Cl	H	H	4-$(CH_3)_2NC_6H_4$	2.5	4.0	3.2
5	$NHCOCH_3$	H	H	4-$(CH_3)_2NC_6H_4$	0.4	1.0	0.6
6	H	H	H	OH	500.0	750.0	612.0
7	OCH_3	H	H	OH	50.0	75.0	61.0
8	H	Cl	H	OH	25.0	50.0	35.0
9	H	Br	H	OH	400.0	600.0	490.0
10	H	I	H	OH	100.0	250.0	158.0
11	H	H	H	Cl	0.005	0.050	0.016
12	H	H	H	Br	5.0	50.0	16.0
13	Cl	H	Cl	Cl	5.0	30.0	12.0
14	Cl	H	Cl	Br	0.5	5.0	1.6
15	Br	H	Br	Cl	0.5	2.0	1.0
16	Br	H	Br	Br	0.5	2.0	1.0
17	H	H	H	C_6H_4ONa	5.0	50.0	16.0
18	H	H	H	$C_6H_4OCOCH_3$	0.5	5.0	1.6

*See Figure 1 for diagram.

TABLE II. Effect of Various N-Substituted *p*-Benzoquinone Imines on the Survival of Mice Infected With T b brucei

Compound no.	LD_{50}[a] (mg/kg)	Dose[b] (mg/kg)	Average survival time (days ± SD)	T/C[c] (%)
1	80	16[d]	3.4 ± 0.6	66
2	27	—	—	—
3	25	—	—	—
4	100	20	5.4 ± 0.5	104
5	70	14[d]	3.4 ± 0.9	66
6	260	52	5.6 ± 0.5	108
7	240	—	—	—
8	120	24	5.4 ± 0.5	104
9	380	76	5.2 ± 0.4	100
10	1,090	—	—	—
11	12.5	2.5	5.0 ± 0.0	97
12	40	—	—	—
13	20	—	—	—
14	20	—	—	—
15	63	12.6	5.0 ± 0.0	97
16	25	5	4.8 ± 0.8	93
17	50	—	—	—
18	90	18	4.4 ± 0.9	85

[a]Single IP dose in Swiss mice; from reference [11].
[b]Administered 4 h after infection and once daily thereafter for 4 days.
[c]Ratio of the average survival time of treated animals to that of controls; controls survived an average of 5.2 ± 0.6 days.
[d]Drug administered for only 3 days due to apparent toxicity.

proach of Hansch in an effort to better understand those factors that contribute to trypanocidal activity so as to aid in the design of more effective compounds. The parameter that correlated best with the logarithm of trypanocidal activity was the conditional redox potential (V). The two were related (Equation 5) with a correlation coefficient, r, of 0.69. The numbers in parentheses are the standard errors of the coefficients and n is the number of compounds included in the analysis. A plot of log(TA) versus V is shown in Figure 2, where each compound is represented by a single point and the broken straight line is calculated from Equation 5. The two most active compounds, 5 and 11, are farthest from the straight line, suggesting that factors other than the conditional redox potential contribute to their activity.

Some of the substituent parameters used in the mathematical analysis are given in Table III. The correlation of log(TA) with the lipophilicity of the group attached to the nitrogen atom (f_Y) was also significant, the correlation coefficient being 0.64. Other parameters correlated with log(TA) as follows: H'_{35}, 0.58; $\mathscr{R}_{35}$, 0.55; $\mathscr{F}_{35}$, 0.55; H_{35}, 0.54; MR_{35}, 0.54. Note that neither log P nor $(\log P)^2$ correlated significantly with log(TA). Three variables, $\mathscr{R}_{35}$, $\mathscr{F}_{35}$, and MR_{35}, correlated very strongly with H_{35} as shown by the correlation matrix in Table IV. The strong correlations among these four variables result from the fact that most of the compounds in the study are substituted in position 2 or in positions 2 and 6, and that all substituents in position 3 have positive $\mathscr{F}$-values and negative $\mathscr{R}$-values.

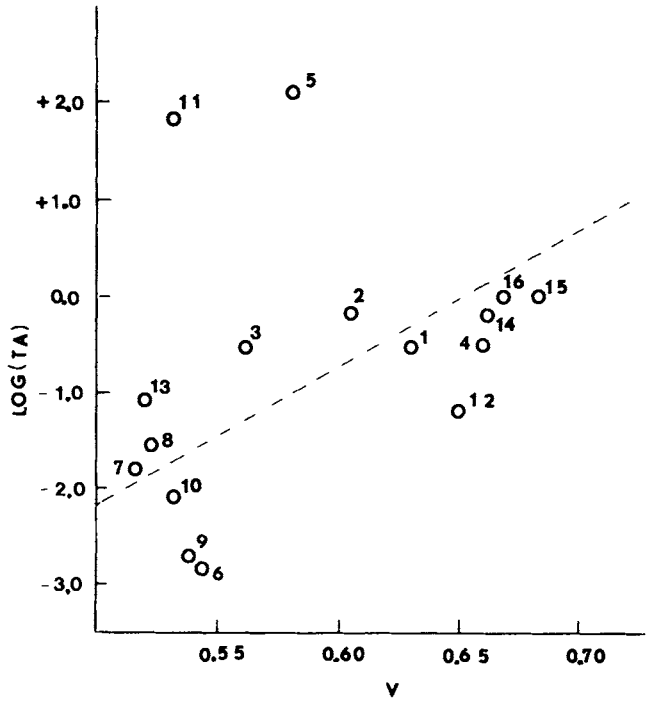

Fig. 2. Trypanocidal activity, log(TA), as a function of the conditional redox potential, V, of 16 N-substituted *p*-benzoquinone imines. The straight line is calculated from Equation 5.

None of the parameters considered is solely responsible for the trypanocidal activity of a given compound; all contribute to varying degrees. One of the most significant pairs of variables was f_Y and H'_{35}, the lipophilicity of the substituent attached to the nitrogen atom and the reactivity index of the hydrogens attached to carbon atoms 3 and 5. The correlation coefficient between this pair of parameters and log(TA) was 0.73, a value significantly greater than that when either parameter was evaluated alone (0.64 and 0.58 respectively). The relationship between these parameters and log(TA) is given by Equation 6,

$$\underset{\substack{13.30 \\ n = 18;\ r = 0.73,}}{\log(TA) = 0.52(\pm 0.21) f_Y + \underset{3.73}{0.46(\pm 0.24)\, H'_{35}} - 1.29(\pm 0.29)} \quad (6)$$

where the numbers in parentheses are the standard errors of the coefficients of the polynomial and the numbers below the coefficients are the partial *F*-values used in statistical analysis [27]. The *F*-values are indicative of the significance of each term in the polynomial, larger values indicating greater significance. The significance of each term is also indicated qualitatively by the size of its coefficient relative to that of its standard error.

TABLE III. Substituent Parameters Used in Analysis

Compound no.	f_{26}	$\mathscr{F}_{26}$	$\mathscr{R}_{26}$	MR_{26}	f_{35}	$\mathscr{F}_{35}$	$\mathscr{R}_{35}$	MR_{35}	f_Y	$\mathscr{F}_Y$	$\mathscr{R}_Y$	MR_Y	H_{26}	H_{35}
1	0.38	0.00	0.00	0.02	0.38	0.00	0.00	0.02	1.00	0.02	−1.00	4.09	2	2
2	0.89	−0.04	−0.13	0.67	0.38	0.00	0.00	0.02	1.00	0.02	−1.00	4.09	1	2
3	0.43	0.26	−0.51	0.89	0.38	0.00	0.00	0.02	1.00	0.02	−1.00	4.09	1	2
4	1.12	0.41	−0.15	0.60	0.38	0.00	0.00	0.02	1.00	0.02	−1.00	4.09	1	2
5	−1.00	0.28	−0.26	1.60	0.38	0.00	0.00	0.02	1.00	0.02	−1.00	4.09	1	2
6	0.38	0.00	0.00	0.02	0.38	0.00	0.00	0.02	−1.40	0.29	−0.64	0.28	2	2
7	0.43	0.26	−0.51	0.89	0.38	0.00	0.00	0.02	−1.40	0.29	−0.64	0.28	1	2
8	0.38	0.00	0.00	0.02	0.71	0.41	−0.15	0.60	−1.40	0.29	−0.64	0.28	2	1
9	0.38	0.00	0.00	0.02	0.86	0.44	−0.17	0.99	−1.40	0.29	−0.64	0.28	2	1
10	0.38	0.00	0.00	0.02	1.12	0.40	−0.19	1.50	−1.40	0.29	−0.64	0.28	2	1
11	0.38	0.00	0.00	0.02	0.38	0.00	0.00	0.02	0.06	0.41	−0.15	0.60	2	2
12	0.38	0.00	0.00	0.02	0.38	0.00	0.00	0.02	0.24	0.44	−0.17	0.89	2	2
13	1.86	0.82	−0.30	1.21	0.38	0.00	0.00	0.02	0.06	0.41	−0.15	0.60	0	2
14	1.86	0.82	−0.30	1.21	0.38	0.00	0.00	0.02	0.24	0.44	−0.17	0.89	0	2
15	2.34	0.46	0.88	−0.34	0.38	0.00	0.00	0.02	0.06	0.41	−0.15	0.60	0	2
16	2.34	0.46	0.88	−0.34	0.38	0.00	0.00	0.02	0.24	0.44	−0.17	0.89	0	2
17	0.38	0.00	0.00	0.02	0.38	0.00	0.00	0.02	1.36	0.27	−0.57	2.82	2	2
18	0.38	0.00	0.00	0.02	0.38	0.00	0.00	0.02	1.44	0.49	−0.15	3.78	2	2

TABLE IV. Correlation Matrix of the Parameters Used in Analysis

	f_Y	H_{35}	H'_{35}	$\mathscr{R}_{35}$	$\mathscr{F}_{35}$	MR_{35}	MR_Y	$\mathscr{R}_Y$
f	1.000							
H_{35}	0.663	1.000						
H'_{35}	0.416	0.768	1.000					
$\mathscr{R}_{35}$	0.659	0.994	0.765	1.000				
$\mathscr{F}_{35}$	−0.662	−0.999	−0.767	−0.992	1.000			
MR_{35}	−0.615	−0.928	−0.718	−0.962	0.921	1.000		
MR_Y	0.819	0.394	−0.106	0.392	−0.394	−0.365	1.000	
$\mathscr{R}_Y$	−0.240	0.092	0.657	0.092	−0.093	−0.086	−0.753	1.000

Another pair of variables that correlated almost as well was the lipophilicity and resonance effect of the substituent attached to the nitrogen atom. This is shown by Equation 7:

$$\log(TA) = \underset{12.40}{0.73(\pm 0.20)}\, f_Y + \underset{2.40}{0.92(\pm 0.59)}\, \mathscr{R}_Y - 0.38(\pm 0.38) \quad (7)$$
$$n = 18;\ r = 0.70\ .$$

Of the two parameters, f_Y made the greater contribution, as evidenced by the size of the *F*-value. When the conditional redox potential was paired with the other variables evaluated, the correlation with log(TA) was not significantly improved. For example, when V and log *P* were evaluated simultaneously, a correlation coefficient of 0.71 was obtained, not significantly larger than that when V alone was considered (r = 0.69). All other combinations of V and another parameter correlated less well.

DISCUSSION

Although the structure-activity relationships shown by these compounds in their ability to cause lysis of trypanosomes in vitro are not perfect, they do offer some insight into the design of more efficacious drugs. The various structural relationships are compatible with two modes of action that have been proposed for the biological activity of these compounds. First, the strong correlation of the conditional redox potential with log(TA) suggests that these compounds may be causing oxidative damage to cellular membranes. This is supported by the fact that electron-withdrawing groups that increase the redox potential also increase trypanocidal activity. This mode of action is also supported by the fact that the lipophilicity of the substituent attached to the nitrogen atom correlates with trypanocidal activity because lipophilic compounds tend to localize in membranes. One would expect that increasing the concentration of an oxidant in membranes would lead to membrane damage and hence to parasite destruction.

Secondly, the statistical significance of H'_{35}, a measure of the number of active hydrogens on the *p*-benzoquinone imine nucleus, suggests that conjugate nucleophilic addition to the molecule may be of mechanistic importance. That quinonoid systems are susceptible to such addition by a variety of biological nucleophiles has been well documented [28–34]. The enhanced correlation observed when H'_{35} was evaluated in combination with either the resonance effect of Y (Eq. 7) or its lipophilicity (Eq. 6) is compatible with such a mode of action. An increase in the resonance effect of the group attached to nitrogen would be expected to make a given compound more susceptible to 1,4-addition. If, in fact, these compounds kill trypanosomes as a result of such addition reactions, this should lead to increased trypanocidal activity. Indeed, this is the effect seen. While we did not initially consider this second mode of action, its significance cannot be overlooked. Perhaps both mechanisms are operative, either alone or in sequence. For instance, 1,4-addition may serve to covalently bind a quinone or quinonelike compound to a particular site whereupon destruction of the parasite results from subsequent oxidative damage.

Unfortunately, none of the compounds tested were trypanocidal in vivo. This might have been due to a number of factors including metabolism and failure of the

compounds to reach the parasites. With respect to the latter, it is of interest that most of the compounds prolonged the survival of Swiss mice bearing the ascitic sarcoma 180, in several cases by more than 200% [11]. More than likely the fact that the drug was given IP is responsible for the latter effect. While the body burden of trypanosomes is confined mainly to the bloodstream, the tumor cells tend to proliferate in the peritoneal cavity. Thus, the tumor cells were bathed directly with drug, while binding of the drug to serum proteins [28,32] may have prevented it from reaching the trypanosomes. Likewise, reduction and subsequent glucuronidation [35,36] is much more likely to be a factor with respect to parasites present in the bloodstream.

When the activity of the compounds against the ascitic tumor was compared to their trypanocidal activity in vitro, the correlation was highly significant. It was found that the dose (mg/kg) that gives the most prolonged survival time is strongly correlated with trypanocidal activity, the correlation coefficient being 0.89. Unfortunately, the mechanism of action of these quinonelike compounds in inhibiting the growth of malignant cells is not clearly understood. While quinones may cause an increase in the intracellular concentration of H_2O_2, some appear to act as alkylating agents [37]. Both mechanisms may be operational at the same time. Thus, quinones might add to thiols or amines via 1,4-nucleophilic addition and still be free to undergo oxidation and/or reduction yielding free radicals. Bachur et al [38] have proposed that antineoplastic quinones may be activated to site-specific free radicals which bind to RNA and DNA, causing damage either directly or through the production of superoxide (O_2^-) or hydroxyl radicals ($HO\cdot$). Tumor cells may have enhanced susceptibility to these drugs because they contain decreased amounts of superoxide dismutase, even though they contain adequate catalase [39]. In contrast, trypanosomes may be susceptible due to their lack of catalase and peroxidase.

The task then, with respect to trypanosomiasis, is to design a quinonelike drug that will reach the bloodstream, possess sufficient lipophilicity to enter the parasite, be hydrophilic enough to avoid being strongly bound to serum proteins, and finally, a redox potential such that radical formation and/or peroxide production is facilitated. To achieve this we are utilizing the Hansch approach to predict the effect of various substituents so that our synthetic effort can be directed towards those compounds the calculated trypanocidal activity of which is significantly enhanced. Additional parameters are also being considered in an effort to refine our understanding of the molecular mechanisms involved in the trypanocidal activity of these compounds.

ACKNOWLEDGMENTS

We wish to thank P.J. McGrath and K.K. Wong for their technical assistance. Financial support was received from the Rockefeller Foundation (RF-77088) and WHO/UNDP/World Bank Special Programme for Research and Training in Tropical Disease.

REFERENCES

1. Zeledon R: In: "Trypanosomiasis and Leishmaniasis." Ciba Foundation Symposium No. 20, New York: Elsevier, 1974, pp 51–85
2. Wilson SG, Morris KRS, Lewis IJ, Krog E: Bull WHO 28:595, 1963.
3. DeRaadt P: Tran R Soc Trop Med Hyg 70:114, 1976.

4. Williamson J: In Mulligan HN (ed): "The African Trypanosomiasis." New York: Wiley-Interscience, 1970, pp 125–221.
5. Chang KP, Chang CS, Sassa S: Proc Natl Acad Sci, USA 72:2979, 1975.
6. Krassner SM: J Protozool 13:286, 1966.
7. Meshnick SR, Chang KP, Cerami A: Biochem Pharmacol 26:1923, 1977.
8. Meshnick SR, Blobstein SH, Grady RW, Cerami A: J Exp Med 148:569, 1978.
9. Meshnick SR, Grady RW, Blobstein SH, Cerami A: J Pharmacol Exp Ther 207:1041, 1978.
10. Docampo R, Cruz FS, Boveris A, Muniz RPA, Esquivel DMS: Arch Biochem Biophys 186:292, 1978.
11. Hodnett EM, Prakash G, Amirmoazzami J: J Med Chem 21:11, 1978.
12. Lanham SM, Godfrey DG: Exp Parasitol 28:521, 1970.
13. Hansch C, Fujita T: J Am Chem Soc 86:1616, 1964.
14. Hansch C: Acct Chem Res 2:232, 1969.
15. Hansch C, Dunn WJ: J Pharm Sci 61:1, 1972.
16. Leo A, Hansch C, Elkins D; Chem Rev 71:525, 1971.
17. Hansch C, Leo A, Unger SH, Kim KH, Nikaitani D, Lien EJ: J Med Chem 16:1207, 1973.
18. Nys GG, Rekker RF: Chim Ther 8:521, 1973.
19. Nys GG, Rekker RF: Chim Ther 9:361, 1974.
20. Rekker RF: In Nauta WTh, Rekker RF (eds): "The Hydrophobic Fragmental Constant: Its Derivation and Application, A Means of Characterizing Membrane Systems." Amsterdam: Elsevier Scientific Publishing Company, 1977.
21. Dunn WJ: In Roche EB (ed): "Design of Biopharmaceutical Properties Through Prodrugs and Analogs." Washington, DC: Am Pharm Assoc Acad Pharm Sci, 1972, pp 45–56.
22. Hammett LP: "Physical Organic Chemistry: Reaction Rates, Equilibria, and Mechanisms," 2nd Ed. New York: McGraw-Hill, 1970.
23. Swain CG, Lupton EC: J Am Chem Soc 90:4328, 1968.
24. Dunn WJ: J Med Chem 16:484, 1973.
25. Rekker RF: Il Farmaco-Ed Sci 34:346, 1978.
26. Service J: "A User's Guide to the Statistical Analysis System." Raleigh: North Carolina State University, 1972.
27. Draper N, Smith H: "Applied Regression Analysis." New York: John Wiley, 1966, pp 24.
28. Lorentz K: Experentia 32:1502, 1976.
29. Foster R, Kulevsky N, Wanigasikera DS: J Chem Soc [Perkin Transactions I]1318, 1973.
30. Moxon GH, Slifkin MA: J Chem Soc [Perkin Transactions II]1159, 1972.
31. Liberato DJ, Byers VS, Dennick RG, Castagnoli N Jr: J Med Chem 24:28, 1981.
32. Pierpoint WS: Biochem J 112:619, 1969.
33. Mason HS, Peterson EW: Biochim Biophys Acta 111:134, 1965.
34. Young JM: Biochem Pharmacol 20:163, 1971.
35. Williams RT: In: "Detoxification Mechanisms." New York: John Wiley and Sons Inc, 1959, pp 432–344.
36. Thompson RM, Gerber N, Seibert RA, Desiderio DM: Res Commun Chem Pathol Pharmacol 4:543, 1972.
37. Lin AJ, Cosby LA, Sartorelli AC: Cancer Chemo Rep (Part 2)4:23, 1974.
38. Bachur NR, Gordon SL, Gee MV: Cancer Res 38:1745, 1978.
39. Oberly LS, Buettner GR: Cancer Res 39:1141, 1979.