Effect of Ring and Side Chain Substituents on the Binding of Naphthothiopheneethanolamines to Deoxyribonucleic Acid. Spectrophotometric Studies

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Some naphthothiopheneethanolamines have been found to have antimalarial activity and a study of their DNA binding was initiated to evaluate this possible mode of antimalarial action. Using solubility increases in the presence of DNA, spectrophotometric change on adding DNA to the compound, and thermal melting analysis, certain of these compounds have been found to bind to DNA. Ring substituents affect binding in a manner that would be predicted for intercalated compounds with the more electronegative substituents increasing binding. The DNA binding of compounds with dibutylamino side chains parallels their antimalarial activity. Compounds with piperidyl side chains also bind strongly to DNA; however, they are devoid of antimalarial activity. Compounds with pyridyl side chains neither bind to DNA nor exhibit antimalarial activity.

Because of the continued high incidence of malaria in the world population and because of the appearance of a variety of the malarial parasite which is resistant to currently used drugs, the need for development of new antimalarial agents has stimulated considerable research.[1] An understanding of the antimalarial action at the molecular level of available drugs is important in determining how these compounds could be modified to improve their activity and in determining what new compounds or classes of compounds should be synthesized. Beginning with the early work of Parker and Irvin[2] there has been increasing evidence that antimalarial quinolines interact with *Plasmodium* DNA. Modern instrumentation has allowed more detailed studies of the interaction of such small molecules with DNA. Lerman's[3] work with quinacrine indicated that antimalarial compounds containing a planar aromatic portion in their structure could intercalate with DNA.

Due to their chemotherapeutic importance and ready availability, there is considerable interest in the mode of action of quinine and chloroquine. A great deal of evidence has been accumulated indicating that these compounds do bind to DNA and that this binding could be involved in their antimalarial action.[4] The mechanism of binding seems to be intercalation according to results from uv spectroscopy,[5,6] flow dichroism,[7] viscosity, and sedimentation[7,8] studies. Some doubt has been cast on this DNA binding model as the primary mechanism of action for chloroquine, quinine, and other similar antimalarial drugs. Van Dyke and coworkers[9] using actual *Plasmodium berghei* cells isolated from parasitized erythrocytes have studied adenosine phosphorylation and polymerization into nucleic acids in the parasite. They concluded that chloroquine and quinine did not exhibit significant inhibition of nucleic acid polymerization.

Recently the synthesis and antimalarial activity have been published for a series of naphthothiopheneethanolamines.[10] These compounds, while structurally similar to the quinoline antimalarial compounds, differ in aromatic ring structure and in solubility at physiological pH from the quinolines. They also do not have a charged ring in contrast with chloroquine. Since several of the naphthothiophenes have shown good activity against *P. berghei* in mice, the question of how their mode of action compares with other antimalarial agents is important. If any of these antimalarial compounds did not bind to DNA, then this mechanism of antimalarial action would be in doubt. On the other hand, if their DNA binding constants parallel their antimalarial activity, then this would be good evidence for the DNA binding mechanism of antimalarial action. The solubility, thermal denaturation, and spectrophotometric binding studies reported here were initiated to help resolve these questions.

Experimental Section

Naphthothiopheneethanolamines. The compounds used in this work were synthesized in our laboratories.[10] Stock solutions of these compounds were prepared in absolute ethanol at concentrations of approximately 10^{-3} *M*.

DNA. Calf thymus DNA was purchased from Miles Laboratories Inc. (lot no. 36-155, RNA less than 1%, protein 0.6%, relative viscosity of 1.83 at 0.5 mg/ml). The T_m in Tris-saline-EDTA buffer, pH 7.6, was 61.5°, in good agreement with the value of 61.3° obtained by Mahler, *et al.*,[11] in this buffer. DNA stock solutions, for the experiments described below, were prepared in 7.5×10^{-3} *M* Na_2HPO_4 and 1×10^{-3} *M* EDTA (now called standard buffer) adjusted to pH 6.0 with NaOH. An extinction coefficient of 6600/DNA-P at 260 nm was used.[12]

Thermal Denaturation. Solutions were prepared by adding the naphthothiophene in ethanol to a DNA solution (7.56×10^{-5} *M* DNA phosphate final concentration) in a 10-ml volumetric flask. Absolute ethanol was added to obtain a final ethanol concentration of 5% by volume. A pH 6.0 buffer containing 15.0×10^{-3} *M* Na_2HPO_4 and 2×10^{-3} *M* EDTA was added in equal amount to the total ethanol volume to maintain constant ionic strength. The solution was then diluted to 10 ml with standard buffer.

Denaturation experiments were performed on a Beckman Acta V spectrophotometer equipped with an electrically controlled and heated cell block. Absorbances were measured at varying temperatures after allowing time for thermal equilibration as indicated by a lack of change in absorbance of the DNA sample with time.

Spectrophotometric Binding Experiments. Solutions for binding experiments were prepared by adding naphthothiophenes in ethanol to a 7.5×10^{-3} *M* phosphate and 1×10^{-3} *M* EDTA buffer (final concentration), pH 5.8. The solutions were allowed to equilibrate for several hours, precipitate was removed by centrifugation, and the solution was diluted (to prevent precipitation) by the addition of buffer or DNA and buffer (8 ml of naphthothiophene solution diluted to 10 ml). The naphthothiophene concentration is constant in each sample and determined using the molar extinction coefficient. All samples contained ethanol at a final concentration of 5% by volume. Spectra of the compound with graded amounts of DNA were then determined on a Beckman Acta V spectrophotometer. Results from these experiments were analyzed using the Scatchard equation[13]

$$\frac{r}{[\mathrm{C}]} = K_{ap}(N_{ap} - r)$$

where r is the moles of naphthothiophene bound per mole of DNA phosphate, [C] is the molar concentration of free naphthothiophene, and K_{ap} and N_{ap} are the apparent binding equilibrium constant and number of binding sites, respectively. By experimentally measuring the spectra of naphthothiophenes not bound and completely bound to DNA, r and [C] can be calculated from the initial naphthothiophene and DNA concentrations. K_{ap} and N_{ap} can then be determined from a plot of r/[C] as a function of r. This treatment assumes a linear change in naphthothiophene spectra between unbound and completely bound states.

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Solubility Studies. Naphthothiophenes in ethanol were added to buffer or buffer and DNA, equilibrated, and precipitate was removed by centrifugation. Spectra of the solutions were then determined on an Acta V spectrophotometer. All solutions had a final ethanol concentration of 5% by volume and ionic strength was held constant by the addition of double strength buffer as in the experiments described above. Concentrations were determined spectrophotometrically from the sample with no DNA.

Miscellaneous. Measurements of pH were done on a Fisher Accumet Model 320 expanded scale pH meter equipped with a Fisher micro-combination electrode (no. 13-639-92). All chemicals not described above were of highest purity commercially available. Water was glass redistilled.

Results

Solubility Studies. One of the difficulties encountered in this work was the very low solubility of most of the naphthothiopheneethanolamines at physiological pH. Plots of solubility as a function of pH for a naphthothiophene with a dibutyl side chain and a piperidyl side chain are shown in Figure 1 (structures and antimalarial data for compounds used in these experiments are given in Table I). As can be seen, the piperidyl compound 8 has greater solubility than the dibutyl compound 4. This was found to be a general rule for compounds containing these side chains. An indication that these compounds bind to DNA is also illustrated in Figure 1 by the greatly increased solubility of the naphthothiophenes in the presence of DNA.

Solubility studies were also used to determine the effect ethanol might exert on the naphthothiophene binding to DNA. At pH 6.0 the increase in solubility of **3** in the presence of DNA was determined in the presence of 2 and 5% ethanol by volume (ionic strength held constant). Slightly less of the drug was bound to DNA in 2% ethanol, but since the free solution solubility of the drug decreased by approximately 20%, this was expected. It was found that in 40% by volume 2-propanol the naphthothiophenes could be dissolved to a concentration of at least 10^{-4} *M*, even above pH 7. However, no binding of the naphthothiophenes to DNA could be detected in this solvent. This cannot be explained by DNA denaturation since the DNA gave a T_m of 59.0° and had a hypochromisity of 40% in this solvent. It seems that low concentrations of ethanol do not drastically affect naphthothiophene binding to DNA, but high concentrations of organic solvents can reduce the binding energy.

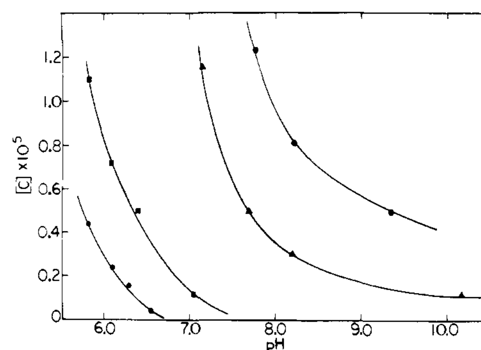

Figure 1. Solubility of naphthothiophenes with and without DNA (7.56×10^{-5} *M*) as a function of pH: compound 4 without DNA (-●-) and with DNA (-■-); compound 8 without DNA (-▲-) and with DNA (-●-). The concentration, [C], is in moles per liter.

Thermal Melting of DNA. Although increased solubility provided a qualitative indication of binding, thermal melting of DNA-naphthothiophene complexes seemed to offer a better way to quantitatively compare interactions of these compounds with DNA. The low solubility of the dibutyl compounds created a problem, however. As the DNA denatured, the dibutylnaphthothiophenes precipitated so that actually two equilibria were being studied: DNA denaturation and naphthothiophene aggregation and precipitation. This problem was compounded by the fact that the solubility of the naphthothiophenes decreased with increased temperature. For these reasons no quantitative data could be obtained from T_m studies on compounds with dibutyl side chains.

As indicated in Figure 1, compounds with piperidyl side chains have higher solubility and at pH 6.0 they are in a solubility range that allows T_m evaluations. Four of these compounds have been synthesized and were studied at pH 6.0. The T_m results are shown in Figure 2. Naphthothiophenes with pyridyl side chains (**9** and **10**) were intermediate in solubility between the piperidyl and dibutyl compounds. T_m studies on **9** and **10** indicated negligible increases on their addition to DNA solutions.

Binding Studies. The compounds which exhibit the highest antimalarial activity in the naphthothiophene-

Table I. α-(Alkylaminomethyl)-4-naphtho[2,1-*b*]thiophenemethanols. Structure and Antimalarial Data

Compd[a]	R_1	R_2	Y[c]	Antimalarial activity,[a] IMST (days) after a single dose (mg/kg) 320	640
1	H	H	$CHOHCH_2N(n\text{-}Bu)_2 \cdot HCl$	2.7	5.9
2	Br	H	$CHOHCH_2N(n\text{-}Bu)_2 \cdot HCl$	10.5	12.7
3	CF_3	H	$CHOHCH_2N(n\text{-}Bu)_2 \cdot HCl$	15.1	2 cures
4	Cl	Cl	$CHOHCl_2N(n\text{-}Bu)_2 \cdot HCl$	3 cures	5 cures
5	H	H	$CHOHCH_2$-1-pip·HCl	0.3	0.3
6	Br	H	$CHOHCH_2$-1-pip·HCl	0.3	0.3
7	CF_3	H	$CHOHCH_2$-1-pip·HCl	0.3	0.5
8	Cl	Cl	$CHOHCH_2$-1-pip·HCl	0.3	0.6
9[b]	H	H	CHOH-2-pyr·HCl	0.3	0.5
10[b]	CF_3	H	CHOH-2-pyr·HCl	0.7	0.9

[a]Test data on compounds 1–8 were taken from Das, *et al.*[10] Increase in mean survival time (IMST) in days of the test group at dosages of 320 and 640 mg/kg are shown. The mean survival time of untreated mice is 6.1 days. A compound is active if IMST exceeds 6.1 days and curative if one or more of the five tested mice live 60 days postinfection. [b]Unpublished results, B. P. Das and D. W. Boykin, Jr. [c] pyr = pyridyl; pip = piperidinyl.

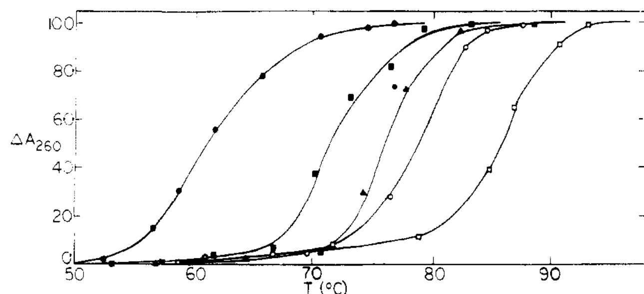

Figure 2. The per cent change in absorbance at 260 nm as a function of temperature for DNA and DNA complexes with piperidyl side chain naphthothiophenes: control (-●-, T_m = 61.1°); compound **5** (-■-, T_m = 72.6°); compound **6** (-▲-, T_m = 76.0°); compound **7** (-○-, T_m = 78.7°); compound **8** (-□-, T_m = 85.7°).

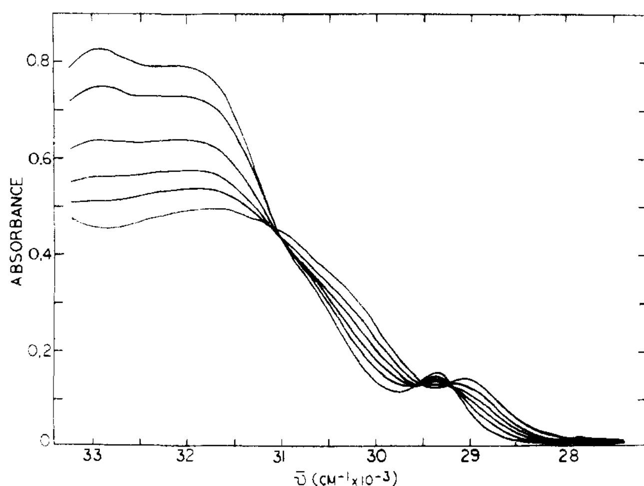

Figure 3. The absorption spectra of compound **8** as a function of added DNA. The DNA concentrations for the six curves from top to bottom are (1) zero, (2) 1.68 × 10^{-5} *M*, (3) 7.56 × 10^{-5} *M*, (4) 11.35 × 10^{-5} *M*, (5) 15.12 × 10^{-5} *M*, and (6) 26.50 × 10^{-5} *M*. In this experiment the naphthothiophene concentration was 4.11 × 10^{-5} *M*.

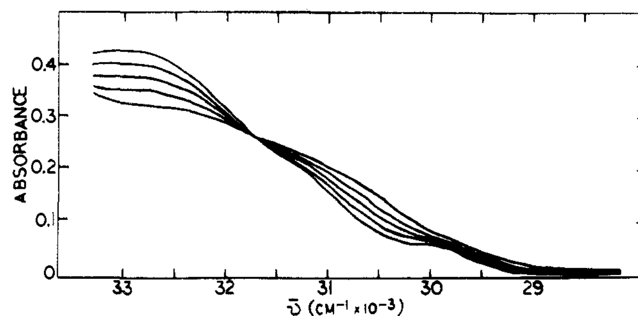

Figure 4. The absorption spectra of compound **3** as a function of added DNA. The DNA concentrations for the five curves from top to bottom are (1) zero, (2) 3.36 × 10^{-5} *M*, (3) 7.56 × 10^{-5} *M*, (4) 11.35 × 10^{-5} *M*, and (5) 18.91 × 10^{-5} *M*. In this experiment the naphthothiophene concentration was 2.14 × 10^{-5} *M*.

ethanolamine series have dibutyl side chains (Table I). For this reason a quantitative comparison of these compounds was of particular interest, and, due to their low solubilities, spectrophotometric binding studies seemed to be the best method for obtaining these data. A stock solution of a compound was prepared (see Experimental Section) and spectra of the compound after addition of graded amounts of DNA and buffer were then determined. Figures 3 and 4 are tracings from the Acta V spectrophotometer of experimental data for a piperidyl and a dibutyl side chain compound, **8** and **3**, respectively. Several experiments of this type were conducted using different initial drug concentrations so that binding data over a broad range could be obtained. The results were analyzed by the method of Scatchard[13] and are plotted in Figure 5. The best lines through the points at low *r* values, which allow determination of the equilibrium constant and number for strong binding sites, are shown. Several dibutyl compounds were analyzed by this method and the equilibrium constants and number of sites for strong binding are collected in Table II. Compound **4** was of such low solubility even at pH 5.8 that it could not be studied.

Discussion

A postulated mode of antimalarial action for quinoline-type compounds has emerged from many different studies over the past decade. In brief, the model has the planar aromatic portion of the quinoline intercalated into the DNA helix and the positively charged side chain interacting with the phosphate groups of the DNA chain.[4] Because of the selective concentration of these drugs in parasitized red blood cells[14] and the inhibition of DNA and RNA polymerases from certain bacteria in their presence,[6] it has been proposed that at least part of their mode of action involves complexing with parasite DNA. Other experiments involving isolated parasites have suggested that the drugs do not act primarily at the nucleic acid level *in vivo*.[9]

Some naphthothiopheneethanolamines with antimalarial activity have been synthesized, and a determination of their DNA binding characteristics is of importance in evaluating their mechanism of antimalarial action. Solubility studies (Figure 1), although indicating that the naphthothiophenes bind to DNA, provide no results that can be used for quantitative comparisons of naphthothiophene–DNA interactions. Binding studies (Figures 3–5 and Table II) are less accurate and more difficult to experimentally perform than solubility or T_m measurements but they do offer a method for quantitatively comparing compounds of lower solubility. It should be mentioned that data analysis of these binding experiments is subject to some error when multiple sites and electrostatic interactions are involved.[15,16] Comparisons of the type presented in Figure 5 and Table II should be valid, however, even though the actual numbers obtained could represent equilibrium constants averaged over several similar classes of strong binding sites. The fact that rather large differences in binding equilibrium constants were found as a function of structure also aided in making comparisons. The dibutyl compounds that could be studied (because of solubility) gave binding data in the same order as their efficacy as antimalarial agents (Tables I and II), indicating that DNA binding could play a role in their mode of action.

Although the experiments reported here are not sufficient to establish a binding mechanism for the naphthothiophenes, the spectral shifts obtained on DNA binding (Figures 4 and 5), the fact that increasingly electronegative ring substituents increase binding (Figure 2 and Table II), and the structure of these compounds (planar aromatic portion and positively charged side chain) all support intercalation as their mode of binding. It should also be pointed out that there are at least two classes of binding sites as indicated by the curved Scatchard plots in Figure 5. The results in Table II are for the strong binding sites. Waring[17] has postulated for ethidium bromide binding to DNA that strong binding is a result of intercalation while the weaker binding results primarily from an ionic attraction of the positive charge on the dye to the negatively charged DNA phosphate groups. Wheth-

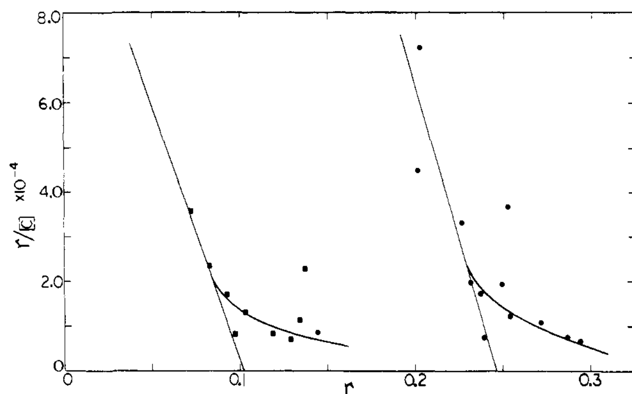

Figure 5. Scatchard plot using data from Figures 3 and 4 and other similar experiments: compound **3** (-■-) and compound **8** (-●-).

er this is true for the naphthothiophenes cannot be determined from our data, but the fact that there are two major groups of sites with very different equilibrium constants is certain from analysis of Figure 5.

Ring substituents on the naphthothiophenes affect DNA binding in a manner that would be predicted for intercalated compounds with the more electronegative substituents increasing binding.[18] An unexpected result from this study, however, was the dramatic effect which side chains have on binding. Compounds with pyridyl side chains gave negligible increases in DNA T_m values, in agreement with their lack of antimalarial activity.‡ As can be seen from Figures 2, 5, and Table II naphthothiophenes with piperidyl side chains have strong interactions with DNA. None of the piperidyl compounds give any appreciable antimalarial activity, however, while dibutyl compounds with lower binding constants give much better activity against *P. berghei*. The piperidyl-substituted compounds must have a molecular configuration which makes their interaction with DNA especially favorable. Their lack of effect as antimalarial agents cannot now be explained from our studies. They do differ in solubility at physiological pH from the dibutyl compounds and it seems reasonable that this could change their membrane permeability making it more difficult to reach the parasite DNA. Chloroquine resistance in *P. berghei* seems to arise due to failure of the parasite to concentrate the drug.[14] The failure of piperidyl compounds to act as antimalarial agents could then be due to a molecular change which essentially gives the same final effect. Solubility alone, however, cannot explain the difference between dibutyl and piperidyl compounds. Compounds **1** and **8** have similar solubilities, but compound **1** has greater antimalarial activity. DNA binding does not explain this effect since compound **8** has a much higher binding constant than compound **1** (Table II).

All of the compounds we have studied that have antimalarial activity bind to DNA. The binding data from Table II for the dibutyl compounds parallel the antimalarial activity from Table I.§ Extrapolation of this data to the dichloro-substituted dibutyl compound **4**, which had

‡Cheng[18] has presented evidence, based on molecular model building, that compounds with structures similar to the naphthothiophenes could not bind to DNA by intercalation if they contained a pyridyl side chain.

§Figure 2 and Table II indicate that dibutyl and piperidyl side chain compounds bind to DNA in the same order with regard to ring substitution (CF_3 substituted > Br substituted > unsubstituted).

Table II. Data for Strong Binding of Naphthothiophenes to DNA

Compd	K_{ap} ($\times 10^5$)	N_{ap}
8	12.1	0.25
3	11.6	0.10
2	4.0	0.11
1[a]	<1.0	<0.1

[a]Compound **1** bound too weakly to DNA to obtain accurate binding data by this method.

solubility too low for study, would suggest that its binding constant should be the highest in the dibutyl series which would also agree with antimalarial data. The extrapolation is based on the fact that the dichloro compound **8** binds more strongly than the CF_3 compound **7** in the piperidyl side chain series (Figure 2). These results are in agreement with the DNA binding model of antimalarial action.[4] This model, however, does not explain why the piperidyl compounds are not good antimalarial agents. Whether this arises due to failure of the parasite to concentrate the drug in the erythrocyte or whether a different mechanism of drug action is involved will require *in vivo* studies on these compounds. It is obvious that other pharmacological phenomena besides DNA binding are playing an important role in the lack of antimalarial action of the piperidyl compounds. These additional phenomena must be determined and taken into consideration before a true molecular understanding of the antimalarial action of these compounds can be proposed.

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