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The interaction of aminoalkylaminoanthraquinones with deoxyribonucleic acid

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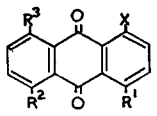
A series of aminoalkylaminoanthraquinones have been prepared as potential intercalating agents. The binding to DNA of these compounds and the known intercalating drugs chloroquine, lucanthone, daunorubicin and doxorubicin has been characterized by spectrophotometric titration. The association constant for the interaction with DNA has been determined for each compound from a Scatchard plot. The compounds synthesized had association constants between 0·5 and 4·2 $\times$ 10^6 compared with 0·93, 0·90, 3·10 and 2·49 $\times$ 10^6 for chloroquine, lucanthone, daunorubicin and doxorubicin respectively.

Many chemotherapeutic agents are thought to exert their cytotoxic effect by intercalation of individual drug molecules between adjacent base pairs of the DNA helix. This model was originally proposed in 1961 by Lerman to explain the interaction between the acridines and DNA and it is now widely accepted that intercalation is the primary mode of this interaction (Peacocke, 1973). Clinically important drugs now also thought to act in this manner include the antimalarials chloroquine (O'Brien, Allison & Hahn, 1966; Waring, 1970) and mepacrine (Lerman, 1963) the schistosomicide lucanthone (Heller, Tu & Maciel, 1974) and its metabolite hycanthone (Waring, 1970), and the antileukaemic agents daunorubicin (daunomycin) (Pigram, Fuller & Hamilton, 1972) and doxorubicin (adriamycin) (Zunino, Gambetta, & others, 1972). The latter is one of the most promising anticancer agents introduced within the last five years (Blum & Carter, 1974) showing an activity not only against leukaemia but also against solid tumours (Carter, Di Marco, & others, 1972). Although these drugs are used clinically they have disadvantages, for example lucanthone, hycanthone, daunorubicin and doxorubicin have a low therapeutic index, and there is widespread resistance to chloroquine (Peters, 1970). Furthermore, for both daunorubicin and doxorubicin there is a 1–2% fatality rate due to the irreversible cardiotoxicity of their aglycone metabolites (Huffman, Benjamin & Bachur, 1972). Since the molecular action of these compounds is thought to be intercalation it may be possible to design an improved intercalating drug. As an initial study, the design of an intercalating nucleus has been attempted.

The essential structural feature for intercalation is an electron-rich planar chromophore. This is interposed between adjacent base pairs causing a local untwisting of the helix (Lerman, 1961). In the compounds considered above, excluding the acridines, this intercalation complex is stabilized by an additional binding force. This secondary force is thought to be an electrostatic interaction between the phosphate groups of the DNA and the aminosubstituted side chain (or amino-sugar) of the drug; this group becoming located in one of the external grooves of the DNA (Newton 1970; Pigram & others, 1972). The model compounds Ia—e incorporate these structural features. These aminoalkylaminoanthraquinones have been synthesized and their binding to DNA has been characterized and compared with the binding of chloroquine, lucanthone, daunorubicin and doxorubicin.



	R^1	R^2	R^3
Ia	H	H	H
b	OH	H	H
c	X	H	H
d	H	X	H
e	H	H	X

X = $-NH-CH(CH_3)[CH_2]_3N(CH_2CH_3)_2$

METHODS

Syntheses

1-(4′-Diethylamino-1′-methylbutylamino)-9,10-anthraquinone hydrochloride (Ia). 1-Chloro-9,10-anthraquinone (5g) was dissolved in 2-amino-5-diethylaminopentane (15 ml) and the solution heated under reflux for 1 h. The solution was allowed to cool and dilute hydrochloric acid (250 ml) added. The acidic solution was extracted first with ether (3 × 200 ml) then with chloroform (3 × 200 ml). The chloroform extracts were evaporated to dryness under vacuum. The residue was dissolved in water; this solution was made alkaline and extracted with ether (3 × 200 ml). The bulked ether extracts were dried with anhydrous magnesium sulphate and evaporated to dryness under vacuum. The residue was dried over phosphorus pentoxide and the hydrochloride salt prepared by bubbling dry hydrogen chloride through a solution of the base in dry ether. The salt was recrystallized from ethyl acetate. Yield after recrystallisation 4·4g (50%). Melting point 175°. Pmr spectrum (in $CDCl_3$) shows 1 H m 0·3τ (exchangeable with D_2O, N–H), 7 H m 1·7–3·0 τ (aromatic protons), 1 H q 6·2 τ ($-CH<$), 6 H m 7·0 τ (3 × $N-CH_2-$), 4 H m 8·1 τ ($-CH_2-CH_2$), 9 H m 8·7τ (3 × CH_3).

1-(4′-Diethylamino-1′-methylbutylamino)-4-hydroxy-9,10-anthraquinone hydrochloride (Ib). 1,4-Dihydroxy-9,10-anthraquinone(quinizarin) (5g) was dissolved in n-butanol (120 ml) with warming, the solution was heated under reflux and a solution of 2-amino-5-diethylaminopentane (3·2g) in n-butanol (30 ml) added dropwise over ½ h. After further heating under reflux for 4 h the n-butanol was evaporated under vacuum and the residue dissolved in dilute hydrochloric acid (250 ml). The product was extracted and the salt prepared in an identical manner to that for Ia. Yield after recrystallization 2·9g (38%). Melting point 170–3°. Pmr spectrum (in D_2O) shows 6 H m 2·15–3·0τ (aromatic protons), 1 H m 5·9τ ($-CH<$), 6 H q 6·2 τ (3×$N-CH_2-$), 4 H m 7·8 τ ($-CH_2-CH_2-$), 9 H t 8·2 τ (3 × CH_3).

1,4-Di-(4′-diethylamino-1′-methylbutylamino)-9,10-anthraquinone hydrochloride (Ic). Leucoquinizarin (5g), prepared by reduction of quinizarin (Meyer & Sander, 1920), was dissolved in n-butanol with warming. The solution was heated under reflux and a solution of 2-amino-5-diethylaminopentane (3·2g) in n-butanol (30 ml) added dropwise over ½ h. The solution was heated under reflux for a further 4 h. The n-butanol was evaporated under vacuum and the residue dissolved in dilute hydrochloric acid (250 ml). The acidic solution was extracted with chloroform (3 × 200 ml) to remove Ib and the organic layer discarded. The aqueous layer was made alkaline with 20% sodium hydroxide and then extracted with chloroform (3 × 200 ml). The bulked chloroform extracts were evaporated to dryness under vacuum and the oily residue washed exhaustively with hot water, and the aqueous layer discarded. Hot water (250 ml) was added to the residue and evaporated, with heating, under vacuum. The solid residue was dried over phosphorus pentoxide and the hydrochloride salt prepared as previously. The salt is highly deliquescent and was stored over phosphorus pentoxide; it was shown to be pure by t.l.c. in several solvent systems. Yield 3·3g (30%). Pmr spectrum (in D_2O) shows 6 H m 1·7–2·2 τ (aromatic

protons), 2 H m6·2τ (2$\times$ $-CH<$), 12 H m 7·5 τ (6 $\times$ N$-CH_2-$), 8 H m 8·3 τ (2 $\times-CH_2-CH_2-$), 6 H d 8·6 τ (2 $\times$ CH_3), 12 H t 8·9 τ (4 $\times$ CH_3).

1,5-Di-(4′-diethylamino-l′-methylbutylamino)-9,10-anthraquinone hydrochloride (Id). 1,5-Dichloro-9,10-anthraquinone (5g) was dissolved in 2-amino-5-diethylaminopentane (15 ml) and the solution heated under reflux for 3 h. The solution was allowed to cool and dilute hydrochloric acid (250 ml) added. The product was extracted and the salt formed in a manner identical to that for Ic. The salt is deliquescent; it was shown to be pure by t.l.c. in several solvent systems. Yield 5·2g (54%). Pmr spectrum (in D_2O) shows 6 H m 1·7–2·1τ (aromatic protons), 2 H m 5·8τ (2 $\times$ $-CH<$), 12 H m 6·2τ (6 $\times$ N$-CH_2-$), 8 H m 7·7τ (2 $\times-CH_2-CH_2-$), 18 H m 8·15τ (6 $\times$ CH_3).

1,8-Di-(4′-diethylamino-l′-methylbutylamino)-9,10-anthraquinone hydrochloride (Ie). This was prepared from 1,8-dichloroanthraquinone in a manner identical to that for Id. Yield 4·5g (46%). Pmr spectrum (in D_2O) is similar to that for Ic and Id. Spectral data for all the compounds prepared was consistent with the structures proposed.

Estimation of binding to DNA

All solutions were prepared in 0·008 M tris–Cl, 0·01 M sodium chloride buffer, pH 7·0. The DNA solution was prepared by dissolving calf thymus DNA, Sigma Type I, in buffer to give a solution of approximately 1mg ml^{-1}. The solution was dialysed against 4, 3, 2, and 1 M sodium chloride, 3 $\times$ buffer, 0·1 M sodium edetate and 5 $\times$ buffer. The base composition, determined by the method of Ulitzer (1972), was found to be 36% guanine/cytosine. The solution was assayed using the figure $\epsilon\ (P)_{260} = 6\,600$ (Angerer & Moudrianakis, 1972) and found to be $2{\cdot}74 \times 10^{-3}$ M(P).

Spectrophotometric titration was carried out by sequential addition of aliquots of the DNA solution (20–100 μl) to 3 ml of a solution (about 5×10^{-5} M) of the compound under test in buffer at room temperature. The absorbance of the solution was monitored at the wavelength in the visible region at which maximum change in extinction occurred. After each addition of DNA (allowing time for incubation if necessary) the extinction was determined, allowing for the change in volume of solution. The volumes of DNA were selected to give at least 20 additions before the maximum reduction in extinction occurred, and further additions were made after this point. The assay was repeated twice giving a minimum of 60 values for each compound. All compounds obeyed Beer-Lambert's Law over the concentration range.

RESULTS AND DISCUSSION

On intercalation of a ligand into DNA there is a red shift in the absorption spectrum of the ligand and a decrease in the extinction at the λ_{max} (Blake & Peacocke, 1968). This is due to a change in the polarity of the medium, the ligand passing from a hydrophilic to a hydrophobic environment (Laurence, 1952). This change in absorption can be used to characterize the binding of a ligand to DNA (Blake & Peacocke, 1968) and has been used here to compare the effects of adding DNA to solutions of compounds Ia-e to those of adding DNA to solutions of the known intercalating agents chloroquine, lucanthone, daunorubicin and doxorubicin. In order to determine the fraction of ligand bound after each addition it is necessary to know not only the observed extinction (ϵ) but also the extinction of the ligand when totally free, or unbound, (ϵ_f) and when totally bound (ϵ_b).

On addition of DNA to a solution of the ligand there is a progressive decrease in the extinction corresponding to an increase in the fraction of ligand bound to DNA until the drug is fully bound. There is therefore a limiting concentration of DNA at which no further decrease occurs. To evaluate ϵ_b a plot of ϵ against Ct/DNAP was constructed where Ct is the molar concentration of total ligand and DNAP is the molar concentration of DNA expressed in terms of phosphate. Addition of DNA was continued after the limiting value was reached and there was then no change in ϵ. The value of ϵ_b was obtained by linear regression analysis of this horizontal region of the curve. All the ligands tested gave this type of curve and the values of ϵb obtained are given in Table 1 along with the limiting values of Ct/DNAP. Since the value of ϵb is taken at low values Ct/DNAP it characterizes the site on the DNA with highest affininity for the ligand.

Table 1. *Binding parameters*

Compound	λ	$\epsilon f \times 10^{-4}$	$\epsilon b \times 10^{-4}$	Ct/DNA	$K \times 10^{-5}$	n
Chloroquine	343	1·8899	0·8365	0·06	9·27	0·10
Lucanthone	448	0·6250	0·4180	0·21	9·03	0·24
Daunorubicin	480	1·0143	0·5975	0·16	31·00	0·19
Doxorubicin	480	0·8513	0·5211	0·22	24·90	0·28
Ia	520	0·7050	0·5227	0·11	5·07	0·19
Ib	560	1·0600	0·7080	0·10	36·0	0·14
Ic	630	2·0200	0·9517	0·08	42·62	0·13
Id	545	1·0487	0·8026	0·16	6·83	0·32
Ie	575	0·8040	0·6730	0·13	12·86	0·29

Determination of association constants

The fraction of ligand bound to DNA (α) after each addition of DNA during the titration was calculated from the equation:

$$\alpha = \frac{\epsilon f - \epsilon}{\epsilon f - \epsilon b} \quad (1)$$

Knowing α, the values c (molar concentration of free ligand) and r (molar concentration of bound ligand/DNAP) were calculated. A plot of r/c against r (a Scatchard plot) was constructed for each ligand.

According to the Scatchard equation (Scatchard 1949):

$$r/c = Kn - Kr \quad (2)$$

Where K is the association constant for formation of the bound ligand /DNA complex, and n is the number of sites/DNAP.

The Scatchard plots obtained for all the compounds were similar; at low values of r the curves are linear corresponding to binding to sites of highest affinity for ligand. At higher values of r there is curvature due to nearest neighbour site exclusion and/or external electrostatic binding, this is typical of drugs which intercalate (Crothers, 1971). Since the slopes of these two regions of the curve vary by several orders of magnitude the effect of the weaker binding can be ignored (Blake & Peacocke, 1968). The points on the linear region of the curve corresponding to the high affinity sites were analysed by linear regression analysis. The slope of the line is –K and the intercept on the ordinate is Kn. The values obtained are given in Table 1.

Equation 1 can only be applied after certain conditions have been verified:

1. The free and bound forms of the ligand must obey Beer-Lambert's Law over the concentration range used in the assay. This was proved for all the ligands tested by

construction of a calibration curve for the ligand and by dilution of the final solution (containing excess DNA) at the end of the spectrophotometric titration. A linear relation was found in all cases.

2. The extinction of the bound drug must not vary with r. The presence of a clear isosbestic point is usually taken to mean that only two species of ligand (bound and free) are present and therefore that equation 1 can be applied (Blake & Peacocke, 1968). However an isosbestic point is only shown when there is a shift in the position of λ_{max} and even then the presence of an isosbestic point does not conclusively prove that only two species of ligand are present (Cohen & Fischer, 1962). The test compounds Ia-e gave indistinct isosbestic points since they give only a small shift in λ_{max} on transfer from a hydrophilic to a hydrophobic environment. Further confirmation that ϵb is linear with r for these compounds came from the finding that ϵb does not vary on increasing the DNA concentration above the limiting concentration and that the Scatchard plots are linear over the range of values of r for which the plots are linear for the standard ligands.

The known intercalating agents chloroquine, lucanthone, daunorubicin and doxorubicin were used as standards under identical experimental conditions since the association constant can be dependent upon the conditions used. For example the association constant for chloroquine is known to decrease with increasing ionic strength (Cohen & Yielding, 1965; Washington, White & Holbrook, 1973). The values obtained by Cohen & Yielding (1965) by spectrophotometric titration ($K = 9{\cdot}8 \times 10^3\ M^{-1}$) and by Yielding & Blodgett (1968) by ultra-centrifugation ($K = 1{\cdot}84 \times 10^4\ M^{-1}$) are much lower than that obtained in this work. However the ionic strength of their buffer was 0·18 compared to 0·018 for the buffer used in this work. Washington & others (1973) quote a value of $K = 5{\cdot}26 \times 10^4$ using equilibrium dialysis in a buffer of similar ionic strength to that used in this work but the results are again not directly comparable as their studies were carried out at 5°. Considering daunorubicin and doxorubicin, the value obtained ($K = 31{\cdot}00 \times 10^5\ M^{-1}$ and $24{\cdot}90 \times 10^5\ M^{-1}$ respectively) are comparable to those obtained by Zunino & others (1972) ($K = 33 \times 10^5\ M^{-1}$ and $23 \times 10^5\ M^{-1}$ respectively). Indeed, variation in ionic strength is known to have no effect on the association constants for these two compounds. Considering now lucanthone, this drug is thought to intercalate. Its metabolite hycanthone is known to intercalate (Waring, 1970) and lucanthone itself has been found to form a complex with isolated hydrogen-bonded base pairs in an analogous manner to actinomycin (Heller & others, 1974). By inference, lucanthone should bind to DNA by intercalation and the results obtained here support this supposition in that the binding and association constant are typical of those for the proved intercalating agents. All the synthetic alkylaminoalkylanthraquinones were found to give a decrease in absorbance (similar to that for the standards) on addition of DNA. The Scatchard plots resemble the plots for the known drugs and the association constants (Table 1) fall within the range of those determined for the standards. An increase in the electron density of the chromophore by introduction of a hydroxyl group into the basic structure (Ia) to give compound Ib gives a marked increase in the association constant. (Table 1). Increasing the capacity for electrostatic binding by incorporation of a second aminoalkyl chain (Ic–e) also gives an increase in activity, the most potent compound being Ic in which the anthraquinone nucleus is substituted at the 1 and 4 positions. This is consistent with intercalation as

the mode of interaction. If this is the case then the individual ligand molecules could be orientated with the long axis of the ligand parallel to the long axis of the base pairs analogous to what is thought to be the orientation of the acridines on intercalation (Galley & Purkey, 1972). This type of interaction is not possible for daunorubicin (and by analogy doxorubicin also) as the presence of the amino-sugar sterically prohibits formation of a complex of this nature. Data from X-ray diffraction studies on daunorubicin—DNA complexes suggests the aminosugar lies in the major groove of the DNA with the chromophore intercalated with considerable but not complete overlap with the adjacent base pairs (Pigram & others, 1972). Compounds Ia-e were designed to incorporate the structural features of daunorubicin and doxorubicin thought to be essential for the binding to DNA, namely the anthraquinone chromophore with an aminosubstituted side chain. They could be accommodated in the DNA in a similar manner to daunorubicin: considering compounds Ic-e then Ic would probably have the highest association constant and this is found to be the case. Although compounds Ia-e are less complex structurally than daunorubicin and doxorubicin they bind avidly to DNA and the evidence is consistent with a similar mode of interaction. Compounds of this type are more amenable to structural variation than daunorubicin or doxorubicin themselves thereby providing a possible lead to drugs with higher activity and reduced toxity.

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