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Interaction of DNA-Intercalating Antitumor Agents with Adrenoceptors

ADRIENNE ADAMS, BEVYN JARROTT, BRYAN C. ELMES, WILLIAM A. DENNY, AND LAURENCE P. G. WAKELIN

Clinical Pharmacology and Therapeutics Unit, University of Melbourne, Austin Hospital, Heidelberg, Victoria 3084 Australia (A.A., B.J.), Commonwealth Scientific and Industrial Research Organization Division of Applied Organic Chemistry, Fisherman's Bend, Victoria 3207 Australia (B.C.E.), Cancer Research Laboratory, School of Medicine, University of Auckland, Private Bag, New Zealand (W.A.D.), and Experimental Chemotherapy Unit, Cancer Institute, Melbourne, Victoria 3000 Australia (L.P.G.W.)

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SUMMARY

The interaction between some examples of mononuclear and binuclear DNA-intercalating antitumor agents and α- and β-adrenoceptors has been studied using radioligand-binding assays. Competition for ^{125}I-BE 2254, [^{3}H]rauwolscine, and (−)-[^{3}H]dihydroalprenolol binding was used to assess affinity for α_1-, α_2-, and β-adrenoceptor-binding sites, respectively. Two homologous series of alkyl-linked diacridines and diquinolines were found to interact poorly with β-adrenoceptors, with only the largest members having appreciable affinity. By contrast, these compounds bind strongly and in a complex manner to α_1- and α_2-adrenoceptors. The affinity of diacridines for both α-adrenoceptor classes has a parabolic dependence on alkyl chain length with the hexyl and pentyl derivatives being the most potent at the α_1- (K_i = 11.5 ± 2.3 nM) and α_2- (K_i = 143 ± 26 nM) binding sites, respectively. The dependence of inhibition constants on linker chain length for the diquinolines is more complicated, with the ethyl- and heptyl-linked dimers having the greatest affinity for each α subclass. There is a nadir in affinity for the pentyl and butyl ligands and an increase in dissociation constant for octyl and longer homologues. Thus, the ethyl diquinoline has K_i values of 6.6 ± 1.2 and 110 ± 14 nM for the α_1- and α_2-adrenoceptors, respectively, and, correspondingly, the heptyl derivative has values of 39 ± 4 and 51 ± 1 nM. These findings are discussed with respect to a model of the α-adrenoceptor in which the radioligand-binding site is situated in a trench or cleft, surrounded by a flat surface bounded by walls. Daunomycin was found to have no affinity for adrenoceptors of any type and mitoxantrone similarly fails to interact with α_2- and β-adrenoceptors, but binds to the α_1 subclass with an inhibition constant (K_i) of 3930 ± 420 nM. Bisantrene also has no affinity for β-adrenoceptors but binds to α_1- and α_2-adrenoceptors with K_i values of 145 ± 24 and 2310 ± 430 nM, respectively. Among the mononuclear acridine drugs studied, only nitracrine shows detectable interaction with β-adrenoceptors (K_i = 760 ± 50 nM). This compound, like bisantrene, has high affinity for the α_1-adrenoceptor (K_i = 131 ± 17 nM) and moderate affinity for the α_2 subclass (K_i = 2180 ± 500 nM). Amsacrine and ethidium have indistinguishable inhibition constants for the α_1-adrenoceptor with K_i values of 1750 ± 230 and 1800 ± 300 nM but amsacrine is more potent at the α_2-adrenoceptor (K_i - 900 ± 150 nM) than ethidium (K_i = 5700 ± 900 nM). Quinacrine and 9-methylaminoacridine have similar affinity for the α_1-adrenoceptor (K_i = 550 ± 130 and 388 ± 27 nM, respectively), but the latter compound is less potent at the α_2 subclass (cf. K_i of 560 ± 90 nM for quinacrine with that of 1053 ± 52 nM for 9-methylaminoacridine). The relationship between the neurologic and cardiovascular toxicities of these agents is discussed with respect to their relative affinities for adrenoceptors and nucleic acids.

INTRODUCTION

Much of the search for new DNA-binding antitumor agents centers upon cationic derivatives of polycyclic aromatic compounds that have structural features which promote intercalative binding to DNA (1–5). However, the resulting requirements of one or more aromatic moieties, frequently accompanied by a positively charged side chain, are structural determinants also shared by many drugs and neurotransmitters active in the cardiovascular and central nervous systems. Consequently, clinically

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useful DNA-binding antitumor agents often have detrimental neurologic and cardiovascular side effects, some of which are so severe as to limit the maximum tolerated dose to values below the chemotherapeutic optimum (6). For example, anthracycline antibiotics like daunomycin and doxorubicin (Adriamycin) have a broad spectrum of antitumor activity and play a major role in the chemotherapy of malignant disease. However, their use has been hampered by conventional toxicities and a unique dose-limiting cardiomyopathy associated with perturbed calcium handling (3, 6). Synthetic analogues of the anthracyclines are being developed to overcome this chronic cardiomyopathy and several examples are undergoing clinical trial. Initial reports for the anthracenediones, mitoxantrone and ametantrone, are encouraging, although electrocardiogram changes, sinus tachycardia, congestive heart failure, hypotension, impairment of cardiac function, and changes in myocardial morphology have been observed (7, 8), and the anthracenedicarboxaldehyde derivative bisantrene has been noted to induce severe phlebitis and to cause serious hypotension (9). Similar toxicities have been found for the acridine antitumor agents amsacrine and nitracrine, the former causing acute electrocardiogram changes and occasionally congestive heart failure in man (10, 11) and a dose-related negative inotropic effect on the isolated rabbit heart (12). Nitracrine has been used extensively in Poland where it was observed to have good antitumor activity and only moderate conventional toxicities. However, it exhibited strong local irritant properties, a characteristic shared by bisantrene, and caused severe hypotension (13). On the basis of the clinical results gathered in Poland, nitracrine was evaluated by the United States National Cancer Institute but was eventually abandoned due to its adverse neurologic and cardiovascular side effects (13).

To enhance the specificity of intercalating agents for DNA, several groups of investigators have synthesized and studied bisanthracyclines, diacridines, diquinolines, bisphenanthridines, and bispyridocarbazoles since these bifunctional ligands have greater affinity for DNA, as well as the potential for improved selective toxicity towards tumor cells (14–18). However, the diacridine NSC 219733, in which two 9-aminoacridine chromophores are linked via their 9-amino positions with a hexamethylene chain (C6-diacridine, see Fig. 1), was rejected when evaluated for clinical trial because of severe neurologic toxicity (13). That mononuclear derivatives of 9-aminoacridine can interact directly with neurotransmitter receptors and related enzymes is well documented. For example, 9-aminoacridine and its tetrahydro derivative tacrine have morphine antagonist activity and inhibit monoamine oxidase (19), and quinacrine binds to the acetylcholine receptor (20) and inhibits both acetylcholinesterase and diamine oxidase activities (19). In this regard, it is noteworthy that the capacity to bind to acetylcholinesterase is also shared by the binuclear derivatives of 9-aminoacridine (21). Evidence that diacridines may bind specifically to plasma membrane components has been presented by Canellakis and colleagues (22), who postulate that the antitumor activity of these compounds is a consequence of disruption of membrane function, rather than the result of inhibition of nucleic acid synthesis.

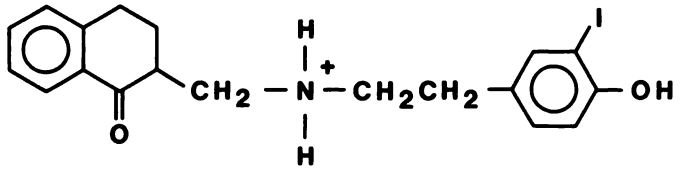
As a first step in investigating the underlying molecular mechanisms responsible for the cardiovascular and neurologic effects of intercalating agents, we have initiated a study of the interaction of diacridines, diquinolines, and mononuclear DNA-binding antitumor agents with adrenoceptors. We have taken advantage of recent progress in the development of radioligands selective for α-adrenoceptor subclasses and measured the affinities of DNA intercalating agents for α_1-, α_2-, and β-adrenoceptors by competition for radioligand binding to membrane preparations from rat cerebral cortex tissue (23, 24). Fig. 1 shows the structural formulae of the diacridines, diquinolines, and α-adrenoceptor radioligands used in this study. We report here that several classes of intercalating drugs, especially the binuclear acridines and quinolines, have high affinity for α-adrenoceptor-binding sites. Moreover, studies of the dependence of inhibition constants on length of the linker chain bridging the diacridines and diquinolines have provided new insights into the topography of adrenoceptors. While it is true that the target receptors for many of the side effects discussed here would appear to be peripheral, rather than central, we have found, at least for α_1-adrenoceptors, that the ligand-binding properties of peripheral receptors, i.e., those in rat kidney cortex membranes, are indistinguishable from the properties of central receptors in guinea pig cerebral cortex membranes (23). Consequently, it is likely that the findings for interaction with central cortical binding sites will have direct bearing on the probable origin of side effects in the periphery.

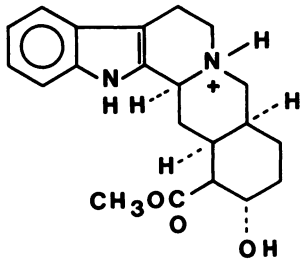
EXPERIMENTAL PROCEDURES

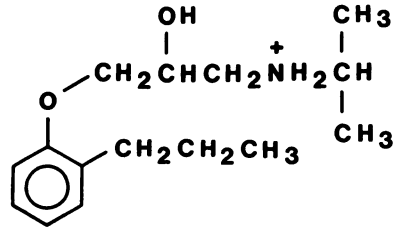
Materials

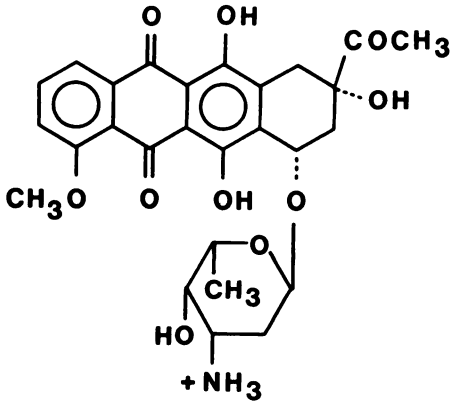
[^{3}H]Rauwolscine (87.4 Ci/mmol) and [^{3}H]DHA[1] (34.5 Ci/mmol) were purchased from New England Nuclear. ^{125}I-BE 2254 was prepared as previously described (23) by using a modification of the chloramine-T method of Maguire *et al.* (25) with Na^{125}I obtained from Amersham International, U.K., and BE 2254 from Beiersdorf, West Germany. Quinacrine, ethidium, daunomycin, isoproterenol, PMSF, spermine, hexamethonium, and (−)norepinephrine were purchased from Sigma Chemical Co. Hexafluorenium was a gift from Wallace Laboratories. Lucigenin was purchased from Aldrich Chemical Co., rauwolscine was from Roth, phentolamine was from Ciba-Geigy Ltd., and mitoxantrone and bisantrene were obtained from the National Cancer Institute, Bethesda, MD. Nitracrine, amsacrine, and mononuclear and binuclear acridines were prepared as described in Refs. 26, 27, and 14, respectively. 4-Aminoquinoline and the diquinolines were synthesized according to the general method described by Deshpande *et al.* (28). Drugs were dissolved, where possible, to give stock solutions of concentration 1 mM in distilled water, or when in their free base form in 10 mM hydrochloric acid, and stored frozen at −20°. Stock solutions were diluted in experimental buffers prior to use. The solvents used were: buffer A, 40 mM potassium phosphate, pH 7.4; buffer B, 50 mM Tris-HCl, 5 mM EDTA, pH 7.6 at 4°; buffer C, 5 mM Tris-HCl, 5 mM EDTA, pH 7.6 at 4°; buffer D, 50 mM Tris-HCl, 5 mM EDTA, pH 7.6 at 25°, containing 0.1% (w/v) ascorbic acid; and buffer E (Krebs phosphate

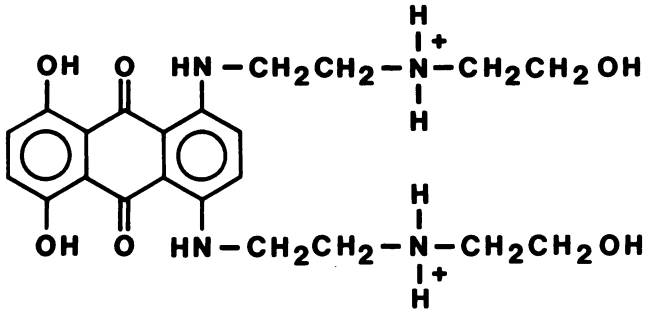
[1] The abbreviations used are DHA, (−)-dihydroalprenolol; BE 2254, 2-β-(4-hydroxyphenyl)ethylaminomethyl)tetralone; PMSF, phenylmethylsulfonyl fluoride.

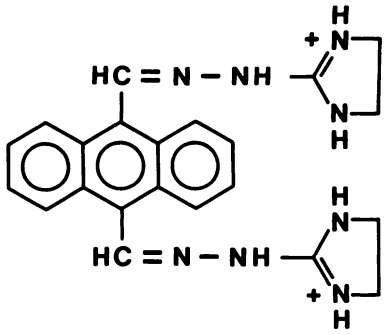












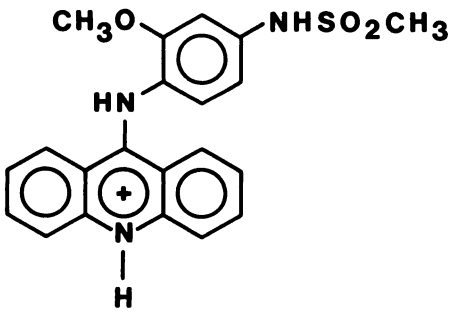
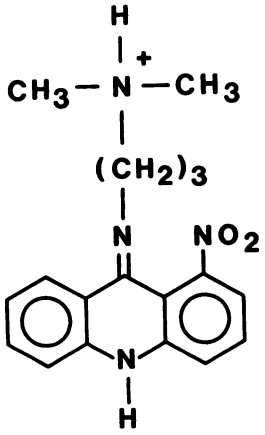
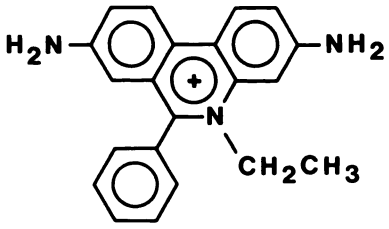
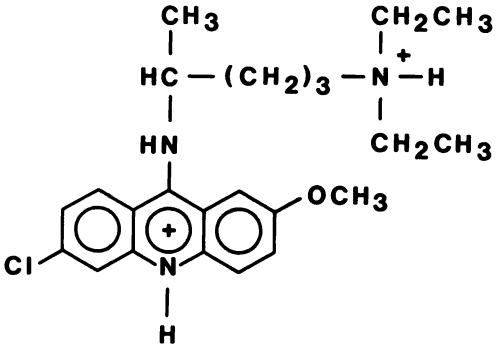


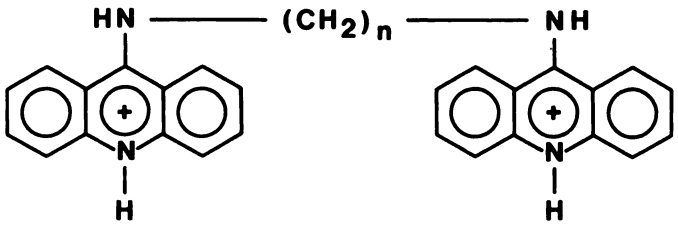
FIG. 1. Ligand structures

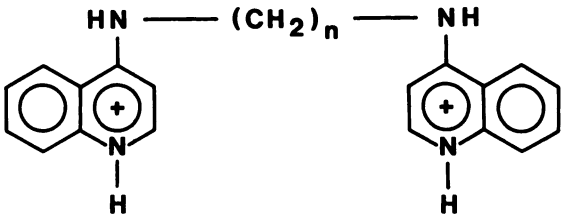
The diacridines and diquinolines are referred to in the text as C*n*-diacridine and C*n*-diquinoline, where C*n* represents the number of methylene groups in the linking chain. This page, left to right and top to bottom: I-BE 2254, rauwolscine, dihydroalprenolol, daunomycin, mitoxantrone, bisantrene, amsacrine. Facing page: nitracrine, ethidium, quinacrine, diacridines, diquinolines, hexafluorenium, lucigenin, 9-methylaminoacridine.

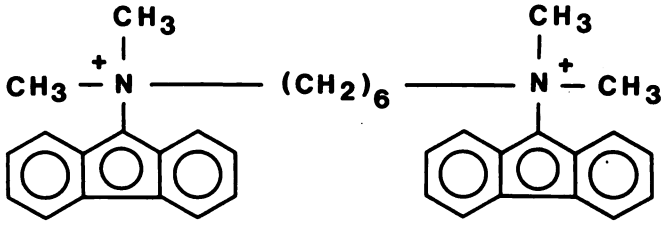


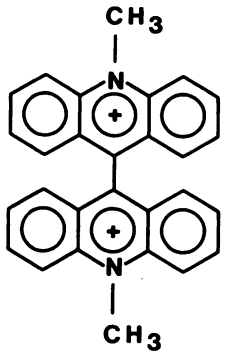












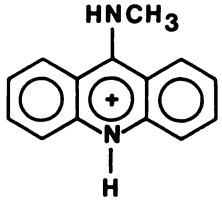


FIG. 1 *continued*

buffer), 119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 10 mM NaH_2PO_4, 1.7 mM $CaCl_2$, pH 7.4.

Methods

Tissue preparation. Cerebral cortices from female Sprague-Dawley rats were homogenized in 20 volumes of ice-cold 0.32 M sucrose in a Kontes all-glass Dounce homogenizer and centrifuged at 1,000 × *g* for 10 min at 4°. The supernatant was centrifuged at 45,000 × *g* for 15 min at 4° and the resultant pellet was washed twice in 50 volumes of buffer (A and C for investigation of α_1- and α_2-adrenoceptors and E for measurements with β-adrenoceptors) containing 10 μM PMSF, with intermediate centrifugation at 45,000 × *g* for 10 min at 4°. This method of tissue preparation ensures minimal contamination by nucleic acids. Final resuspension was in 200 volumes of buffer A for experiments with α_1-adrenoceptors, 50 volumes of buffer D for measurements with α_2-adrenoceptors, and 40 volumes of buffer E for those with β-adrenoceptors. All final membrane suspensions contained 10 μM PMSF.

Receptor-binding assays. Radioligand-binding parameters. Equilibrium dissociation constants were determined for the radioligands ^{125}I-BE 2254, [^{3}H]rauwolscine, and [^{3}H]DHA used to label α_1-, α_2-, and β-adrenoceptors, respectively. The affinity of ^{125}I-BE 2254 was measured essentially as described previously (23) using 1 μM phentolamine to define nonspecific binding at each radioligand concentration. Fifty-μl aliquots of membrane suspension were incubated in polystyrene culture tubes containing eight different concentrations of ^{125}I-BE 2254 between 0.02 and 0.9 nM in a total volume of 200 μl of buffer A. After incubation for 30 min at 37° bound and free radioligand were separated by filtration through 12-mm-diameter Whatman GF/B glass fiber filters. The filters were washed with 3 × 5 ml of ice-cold incubation buffer and then transferred to 10-ml polystyrene tubes and counted in an LKB Multiwell gamma counter. A similar method was used to determine the equilibrium dissociation constant for [^{3}H]DHA in which 0.5 ml of membrane suspension was incubated for 30 min at 37° in the dark with eight different concentrations of radioligand ranging from 0.2 to 30 nM in a total volume of 1 ml of buffer E. Nonspecific binding was determined at each concentration of radioligand by the addition of 200 μM isoproterenol. Bound and free radioligands were separated by filtration through 25-mm-diameter Whatman GF/B glass fiber filters. The filters were washed with 3 × 5 ml of ice-cold buffer E and transferred to plastic scintillation vials; then 3 ml of 2-methoxyethanol and 10 ml of scintillation cocktail [2,5-diphenyloxazole, 0.4%; 1,4-bis[2-(5-phenyloxazolyl)]benzene, 0.01% (w/v) in toluene] were added and the contents were mixed thoroughly. Samples were counted in a Searle Iso-cap liquid scintillation counter with an efficiency of approximately 50%. The dissociation constant for [^{3}H]rauwolscine was measured by the isotope dilution method. Membrane suspensions (0.5 ml) were incubated for 45 min at 25° with approximately 0.5 nM [^{3}H]rauwolscine and eight different concentrations of nonradioactive rauwolscine ranging from 0.2 to 30 nM in a total volume of 1 ml of buffer D. Nonspecific binding was defined using 10 μM phentolamine. Tissue samples were filtered and counted as for [^{3}H]DHA using ice-cold buffer B to wash the filters. Equilibrium dissociation constants for all three radioligands were determined using the computer programs EBDA (29) and LIGAND (30).

Competition binding studies. Studies with ^{125}I-BE 2254 were performed essentially as previously reported (23); 50 μl of membrane suspension was incubated with 50 pM ^{125}I-BE 2254 and six to eight different concentrations of competing ligand in a total volume of 200 μl of buffer A for a period of 30 min at 37°. Nonspecific binding, defined using 1 μM phentolamine, was approximately 10%. Samples were filtered and counted as described for ^{125}I-BE 2254 in the previous section. The experimental procedure for measuring competition with [^{3}H]rauwolscine for binding to membranes was similar to that used for measurement of saturation binding of this radioligand with the modification that the nonradioactive rauwolscine was replaced by six to eight different concentrations of competing ligand. Nonspecific binding, defined using 10 μM phentolamine, was approximately 15%. Competition for [^{3}H]DHA binding was measured by incubating 0.5 ml of membrane suspension for 30 min at 37° in the dark with approximately 1 nM [^{3}H]DHA and six to eight different concentrations of competing ligand in a total volume of 1 ml of buffer E. Nonspecific binding, assessed using 200 μM isoproterenol, was approximately 30%. For competition experiments with [^{3}H]rauwolscine and [^{3}H]DHA, samples were filtered and counted in the manner described for measurement of their equilibrium binding isotherms. Results from the competition binding assays were fitted to a four-parameter logistic function (31) and IC_{50} values were converted to inhibition constants using the Cheng and Prusoff equation for competitive inhibition (32). All assays were carried out in duplicate and each saturation binding or competition binding curve was performed three times using different tissue preparations. The protein content of samples was estimated by the Lowry method (33) using bovine serum albumin as the standard.

Dissociation kinetics. The rates of dissociation of bound ^{125}I-BE 2254 and [^{3}H]rauwolscine induced by challenge with excess C2- and C7-diquinoline, C6-diacridine, phentolamine, and norepinephrine (the latter for [^{3}H]rauwolscine only) were measured at 25°. For ^{125}I-BE 2254, tissue homogenate (50 μl) was incubated to apparent equilibrium (1 hr at 25°) with 50 pM radioligand in a total volume of 190 μl of buffer A. A 10,000-fold molar excess of ligand was added in 10 μl of buffer and samples were filtered at various time intervals. The dissociation rate of [^{3}H]rauwolscine in the presence of a 10,000-fold excess of ligand was similarly measured by incubating 0.5 ml of membrane suspension to equilibrium with approximately 0.5 nM [^{3}H]rauwolscine in a total volume of 1 ml of buffer D for 30 min at 25°. Competing ligands were again added in a volume of 10 μl and samples were filtered periodically. The dissociation rate constant (k_{-1}) was calculated as the negative value of the gradient of a plot of ln (concentration of specifically bound radioligand) versus time. In the case of [^{3}H]rauwolscine, this plot was found to be biphasic and the two exponential components were sufficiently well separated in time to be resolved by standard graphic curve-stripping procedures.

RESULTS

Radioligand-binding parameters. Linear Scatchard plots (data not shown) were obtained for the interaction of all three radioligands with rat cerebral cortex membranes. The dissociation constant found for ^{125}I-BE 2254 of 85 ± 8 pM is indistinguishable from that previously reported for binding to the α_1-adrenoceptor in guinea pig and rat brain membranes (34, 35). The binding data yield a value of 127 ± 14 pmol of radioligand/μg of protein for the number of ^{125}I-BE 2254-binding sites, with a Hill coefficient of 1.06. The binding isotherm for [^{3}H]rauwolscine is characterized by a dissociation constant of 5.0 ± 0.4 nM and a Hill coefficient of 1.01 with a tissue binding capacity of 238 ± 20 pmol of radioligand/μg of protein. These values are in satisfactory agreement with those found by other investigators for binding of [^{3}H]rauwolscine to rat (36) and bovine (24) cerebral cortex membranes. Similarly, [^{3}H]DHA was also found to interact with a single class of high affinity binding sites (Hill coefficient, 0.98), its dissociation constant of 2.15 ± 0.14 nM comparing favorably with the value determined by others in rat (37) and bovine brain membranes (38). The number of binding sites for [^{3}H]DHA was found to be equivalent to 148 ± 13 pmol of radioligand/μg of protein.

Interaction of diacridines with adrenoceptors. To investigate the structural limitations for diacridine binding to adrenoceptors, affinities to α_1-, α_2-, and β-adrenoceptors were measured as a function of linker chain length. Comparison of Figs. 2, 3, and 4 (open symbols) reveals that C6-diacridine has the highest affinity for α_1-adre-

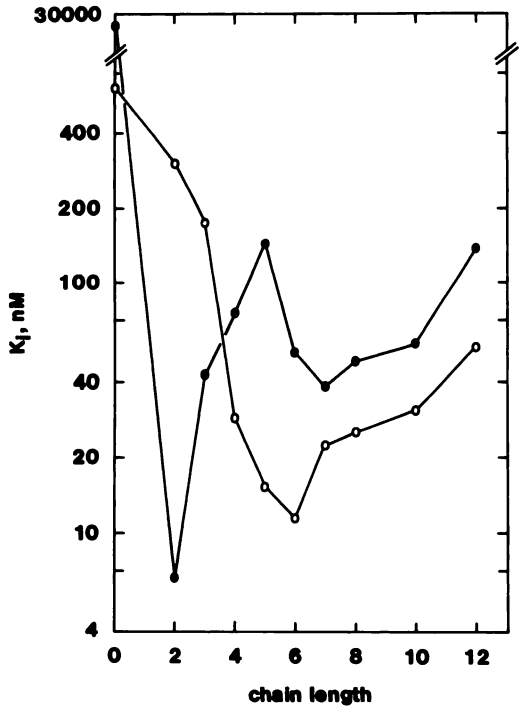


FIG. 2. *Chain length dependence of affinity of diacridines (○) and diquinolines (●) for the α_1-adrenoceptor-binding site*

Inhibition constants are the mean of three measurements whose average standard error is ±13 and 16% for the diacridines and diquinolines, respectively. Results shown for $n = 0$ are those of 9-aminoacridine and 4-aminoquinoline. Slope factors did not differ significantly from unity except for the C2-diacridine (slope factor, 0.6) and the C10- and C12-diquinolines (slope factor, 1.8). The unlabeled scale marks on the ordinate represent the 7th decile of the relevant decade.

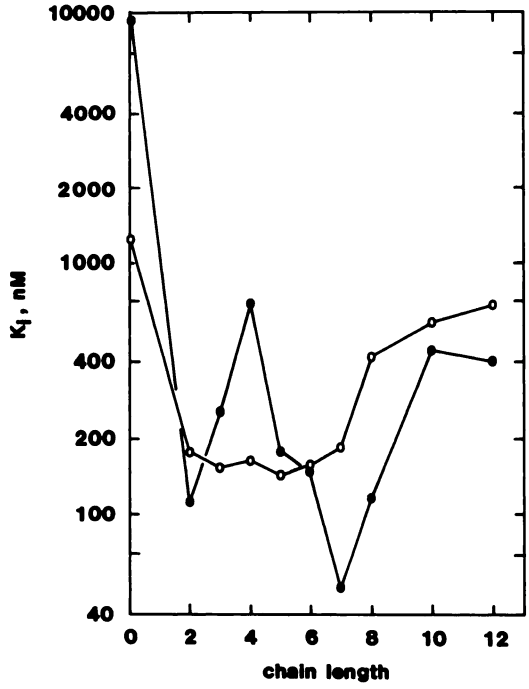


FIG. 3. *Chain length dependence of affinity of diacridines (○) and diquinolines (●) for the α_2-adrenoceptor-binding site*

Inhibition constants are the mean of three measurements whose average standard error is ±13% for both classes of ligand. Results shown for $n = 0$ are those of 9-aminoacridine and 4-aminoquinoline. Slope factors did not differ significantly from unity except for the C2-diacridine (slope factor, 0.5). The unlabeled scale marks on the ordinate represent the 7th decile of the relevant decade.

noceptors (K_i = 11.5 ± 2.3 nM) and that it is 14- and 105-fold less potent at α_2- and β-adrenoceptors, respectively. This degree of avidity for α-adrenoceptors is in the range typically observed for nonselective α-adrenoceptor ligands, e.g., phentolamine and dihydroergocryptine (23), but falls short of that of the most potent α_1-selective ligands, e.g., prazosin and BE 2254 (23). Fig. 2 shows that affinity for the α_1-adrenoceptor increases as the chain length is extended from 2 to 4 methylene groups, reaches a zenith at 6 carbon atoms, and thereafter declines as the linker is further lengthened. A different pattern is observed at the α_2-adrenoceptor (Fig. 3) where the region of highest affinity encompasses 2 to 7 methylene groups and the potency rapidly falls when the linkage contains 8 or more carbon atoms. With the exceptions of the two shortest-linked dimers, the diacridines have greater affinity for the α_1- than for the α_2-adrenoceptor but differences in structure-activity relationships at the two binding sites result in variable α_1/α_2-selectivity ratios. The diacridines bind less tightly to β- than to α-adrenoceptors and, in contrast to interaction with the latter, binding to β-adrenoceptors does not show a parabolic dependence of affinity on chain length, but inhibition constants steadily diminish as the linkage becomes longer (Fig. 4). Modifying the bridging chain so that it contains one or more positively charged amino groups (i.e., so that it becomes —$(CH_2)_3$-$^+NH_2$-$(CH_2)_3$-

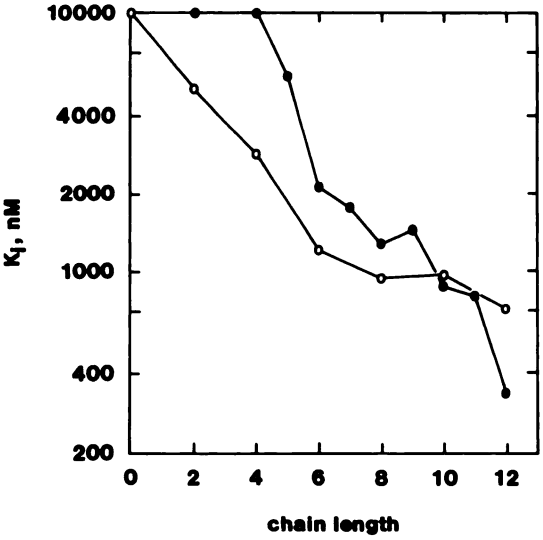


FIG. 4. *Chain length dependence of affinity for diacridines (○) and diquinolines (●) for the β-adrenoceptor-binding site*

Inhibition constants are the mean of three measurements whose average standard error is ±7 and 18% for the diacridines and diquinolines, respectively. Ligands whose inhibition constants were found to be >10,000 nM are shown as having K_i = 10,000 nM. Results shown for $n = 0$ are those of 9-aminoacridine and 4-aminoquinoline, both of which have K_i > 10,000 nM. Slope factors did not differ significantly from unity. The unlabeled scale marks on the ordinate represent the 7th decile of the relevant decade.

and $(CH_2)_3$-$^+NH_2$-$(CH_2)_4$-$^+NH_2$-$(CH_2)_3$-) reduces affinity to all classes of adrenoceptor compared to the alkyl analogue, effectively abolishing reaction with the β-subclass (Table 1). Activity is also completely lost when the acridine moieties are directly joined together so that their aromatic rings are at right angles to each other (see lucigenin, Table 1).

Interaction of diquinolines with adrenoceptors. The importance of size of the aromatic moiety as a structural determinant of binding was investigated by studying the interaction of a series of diquinolines with adrenoceptors. The diquinolines bind poorly to β-adrenoceptors but, like the diacridines, the higher homologues become progressively more potent (Fig. 4, filled symbols). In contrast to the diacridines, however, their interaction with α-adrenoceptors is more complex. In Figs. 2 and 3 (filled symbols), it can be seen that at both α_1- and α_2-adrenoceptors there are two domains of high affinity for diquinolines occurring when the linkage comprises 2 and 7 methylene groups. The barrier between these domains is well defined with 5 and 4 methylene groups marking the nadir in affinity at α_1- and α_2-adrenoceptors, respectively. The inhibition constant of 6.6 ± 1.2 nM for C2-diquinoline at the α_1-adrenoceptor shows this compound to have twice the affinity of C6-diacridine and firmly places it among the family of high affinity α-selective adrenoceptor ligands.

Importance of the aromatic moiety. That the interaction of binuclear aromatic ligands with α-adrenoceptors is not confined to heteroaromatic dimers (i.e., those containing nitrogen atoms in their rings) is made clear by the finding that hexafluorenium, having two carbocyclic fluorene moieties joined by a hexamethylene linker, has very similar affinities at α-adrenoceptors to those of the corresponding diacridines and diquinolines (Table 1). However, the importance of the aromatic moiety per se is dramatically revealed by the total loss of affinity when the fluorene residue is replaced by a methyl group to give the bisquaternary ammonium cation hexamethonium (see Table 1). This phenomenon is also observed in the diacridine series where the polyamine spermine has no affinity for adrenoceptors in contrast to its diacridine derivative (see Table 1).

TABLE 1

Inhibition constants for ligand binding to the α_1-, α_2-, and β-adrenoceptor

Measurements were made as described in Methods, with the α_1-, α_2-, and β-adrenoceptor-binding sites being labeled with ^{125}I-BE 2254, [^{3}H]rauwolscine, and [^{3}H]DHA, respectively. Slope factors are all approximately 1 except for those characterizing the binding of spermine diacridine, which have values of 1.5 at both the α_1- and α_2-adrenoceptor-binding sites. Values for C2- and C7-diquinoline and C6-diacridine are included for the purpose of comparison. Values are ± SE.

Compound	Adrenoceptor subclass K_i		
	α_1	α_2	β
		nM	
Daunomycin	>100,000	>10,000	>10,000
Mitoxantrone	3,930 ± 420	>10,000	>10,000
Bisantrene	145 ± 24	2,310 ± 430	>10,000
Amsacrine	1,750 ± 230	900 ± 150	>10,000
Nitracrine	131 ± 17	2,180 ± 500	760 ± 50
9-Methylaminoacridine	388 ± 27	1,053 ± 52	>10,000
Quinacrine	550 ± 130	560 ± 90	>10,000
Ethidium	1,800 ± 300	5,700 ± 900	>10,000
Lucigenin	31,000 ± 4,000	>10,000	>10,000
C3-N-C3 diacridine[a]	66 ± 12	1,550 ± 140	>10,000
Spermine diacridine[b]	105 ± 14	1,650 ± 380	>10,000
Hexafluorenium	38 ± 6	122 ± 12	>10,000
Hexamethonium	>100,000	>10,000	>10,000
Spermine	>100,000	>10,000	>10,000
C2-diquinoline	6.2 ± 1.2	110 ± 14	>10,000
C7-diquinoline	39 ± 4	51 ± 1	1,780 ± 150
C6-diacridine	11.5 ± 2.3	156 ± 30	1,210 ± 70

[a] C3-N-C3 diacridine is 9-aminoacridine-$(CH_2)_3$-$^+NH_2$-$(CH_2)_3$-9-aminoacridine.

[b] Spermine diacridine is 9-aminoacridine-$(CH_2)_3$-$^+NH_2$-$(CH_2)_4$-$^+NH_2$-$(CH_2)_3$-9-aminoacridine.

Dissociation kinetics of α-adrenoceptor radioligands. The implicit assumption in the data analysis that the diacridines and diquinolines bind reversibly to adrenoceptors is in agreement with their known chemistry, which shows these compounds to be stable unreactive molecules in neutral aqueous solution at physiologic temperatures (39). Moreover, there is no evidence for the involvement of any covalent interactions in their complexes with DNA (14, 15, 39). However, to address the possibility that the diacridines and diquinolines may prevent radioligand binding at adrenoceptors by an allosteric mechanism rather than by direct competitive inhibition, we have measured the rates of dissociation of membrane-bound ^{125}I-BE 2254 and [^{3}H]rauwolscine when challenged with a large molar excess of selected members of these series. The kinetics of dissociation of ^{125}I-BE 2254 was a first order process for all unlabeled ligands tested (data not shown), with rate constants comparable to those for phentolamine (see Table 2). Furthermore, the range of k_{-1} values observed in the present studies, 2.3–3.1 × 10^{-3} sec^{-1} at 25° may be compared with the values of 1.5 (35), 2.3 (40), and 6.1 × 10^{-3} sec^{-1} (23) reported for phentolamine-induced dissociation of ^{125}I-BE 2254 from rat and guinea pig brain membranes at 37°.

In contrast to the simple dissociation process observed for ^{125}I-BE 2254, [^{3}H]rauwolscine was found to dissociate by a complex mechanism involving at least two transiently distinguishable bound forms of the radioligand. Fig. 5 illustrates representative plots for the dissociation of [^{3}H]rauwolscine in the presence of phentolamine, C2-diquinoline, C7-diquinoline, or C6-diacridine. In all cases, the biphasic nature of the curves is clearly visible. The data can be resolved into two exponential components whose rate constants, recorded in Table 2, are separated by approximately 1 order of magnitude. Each rate constant characterizes dissociation of about half the total amount of bound [^{3}H]rauwolscine. Having regard for the large errors inherent in the analysis of multicomponent exponentials (estimated here to be about ±20%), there are no discernable differences in the rates of dissociation. This is made more evident by comparison of the weighted mean dissociation rates (a single parameter that may be used to define an average rate of dissociation for complex systems) shown in Table 2. It is interesting to note that the kinetic profile for the dissociation of

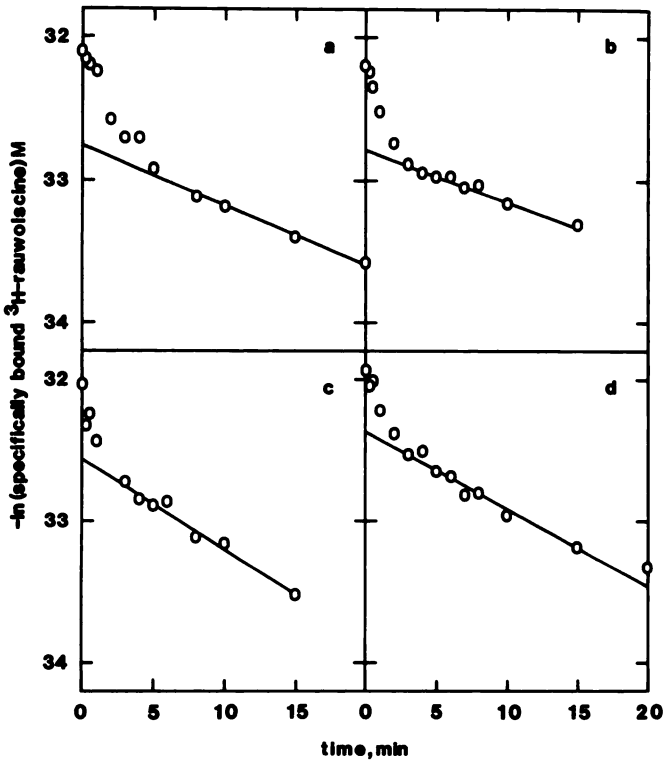


FIG. 5. *Kinetics of dissociation of membrane-bound [^{3}H]rauwolscine* Measurements were made in buffer D at 25°. Panel a, dissociation induced by phentolamine. Panel b, dissociation induced by C2-diquinoline. Panel c, dissociation induced by C7-diquinoline. Panel d, dissociation induced by C6-diacridine. The straight line shows the fit to the slowest step of the dissociation process, the faster component being determined by a curve-stripping procedure.

[^{3}H]rauwolscine is the same whether radioligand dissociation is assessed in the presence of the agonist norepinephrine or the antagonist phentolamine (Table 2).

Little information on the dissociation kinetics of [^{3}H] rauwolscine is available in the literature for the purposes of comparison. Lafontan *et al.* (41) report that, in the presence of excess phentolamine, the ligand dissociates with k_{-1} equal to 8.2×10^{-4} sec^{-1} at 25° from α_2-adrenoceptors in human fat cell membranes, a value equivalent to the slow component measured here. However, it is clear from these authors' data (Fig. 1c in Ref. 41) that they did not take measurements at early time intervals and the extrapolation of their linear first order plot to zero time reveals that they may have missed a faster dissociating component. Perry and U'Prichard (24) found that dissociation of [^{3}H]rauwolscine from rat brain membranes in the presence of excess (−)norepinephrine was monophasic at 4° with a half-life of 50 min, equivalent to a dissociation rate constant of 2.3×10^{-4} sec^{-1}. Our finding that [^{3}H]rauwolscine dissociates from rat brain membranes in a biphasic manner is consistent with the notion that rauwolscine binds to two sites, whether they are α_{2H}- and α_{2L}-receptor states (24) or two distinct high and low affinity α_2-adrenoceptors (42). However, the data are also in accord with a scheme in which the radioligand dissociates in two sequential steps, the slower of which is a monomolecular rearrangement, from a single class of binding sites, and the available information does not permit distinction between this mechanism and the above possibilities. It should be noted that the existence of a complex pathway for dissociation of [^{3}H] rauwolscine does not necessarily imply that the ligand binds with more than one discernable equilibrium constant.

Interaction of antitumor agents with adrenoceptors. To determine to what extent the cardiovascular side effects associated with the use of DNA-intercalating antitumor agents can be related to direct antagonism of sympathetic transmission, we have investigated the affinities of these antitumor drugs for α- and β-adrenoceptors. The results, shown in Table 1, indicate that the anthracycline daunomycin has no affinity for either class of adrenoceptor. By contrast, the anthracenedione and anthracenedicarboxaldehyde derivatives mitoxantrone and bisantrene, which have moderate and high affinity for the α_1-adrenoceptor, respectively, both fail to bind to β-adrenoceptors. Bisantrene interacts moderately well with α_2-adrenoceptors. Further inspection of the table reveals that amsacrine fails to bind to β-adrenoceptors, but has moderate affinity for α_1- and α_2-adrenoceptors, being twice as potent as the latter. Nitracrine, on the other hand, interacts with all three adrenoceptor classes: it has high affinity for the α_1-adrenoceptor and binds reasonably tightly to α_2- and β-adrenoceptors. While not clinically useful antitumor agents, the DNA intercalators ethid-

TABLE 2

Kinetics of dissociation of membrane-bound ^{125}I-BE 2254 and [^{3}H]rauwolscine

Measurements were made at 25° in buffer A for ^{125}I-BE 2254 and buffer D for [^{3}H]rauwolscine. For dissociation of [^{3}H]rauwolscine, fast, k_{-1F}, and slow, k_{-1S}, processes were resolved. a_{-1F} and a_{-1S} represent the proportion of the reaction (expressed as percentage of total reaction) characterized by the rate constants k_{-1F} and k_{-1S}, respectively. k_{-1A} is the weighted average of the dissociation rate constants, i.e., $k_{-1A} = a_{-1F}/100 \times k_{-1F} + a_{-1S}/100 \times k_{-1S}$. For dissociation of ^{125}I-BE 2254, k_{-1} is given as the mean of three determinations ± standard error, whereas the errors in the parameters of dissociation of [^{3}H]rauwolscine are estimated to be ±20%.

Displacing ligand	^{125}I-BE 2254 k_{-1}	[^{3}H]Rauwolscine				
		a_{-1F}	k_{-1F}	a_{-1S}	k_{-1S}	k_{-1A}
	sec^{-1}		sec^{-1}		sec^{-1}	sec^{-1}
Phentolamine	$2.4 \pm 0.1 \times 10^{-3}$	58	6.5×10^{-3}	42	7.5×10^{-4}	4.1×10^{-3}
(−)-Norepinephrine		36	7.7×10^{-3}	64	7.9×10^{-4}	3.4×10^{-3}
C2-diquinoline	$2.3 \pm 0.2 \times 10^{-3}$	45	9.4×10^{-3}	55	5.4×10^{-4}	4.5×10^{-3}
C7-diquinoline	$3.1 \pm 0.1 \times 10^{-3}$	43	1.5×10^{-2}	57	1.0×10^{-3}	7.0×10^{-3}
C6-diacridine	$3.1 \pm 0.1 \times 10^{-3}$	30	9.2×10^{-3}	70	1.1×10^{-3}	3.5×10^{-3}

ium, 9-methylaminoacridine, and quinacrine have also been studied because of their structural similarities to amsacrine and nitracrine. We find that ethidium has the same affinity as amsacrine for the α_1-adrenoceptor and that it similarly fails to bind to β-adrenoceptors. Unlike amsacrine, however, ethidium binds more poorly to α_2- than to α_1-adrenoceptors. 9-Methylaminoacridine is equipotent with amsacrine at the α_2-adrenoceptor but is 5-fold more active at the α_1-adrenoceptor. This agent also fails to bind to β-adrenoceptors. Quinacrine interacts strongly, and with equal affinity, with both α_1- and α_2-adrenoceptors and again fails to bind to β-adrenoceptors.

DISCUSSION

Diacridine and diquinoline binding to α-adrenoceptors. The division of the diquinolines into two high affinity groups at both classes of α-adrenoceptor speaks strongly for the existence of two different binding sites for these ligands at each receptor. Examination of space-filling molecular models reveals close spatial resemblances between the C2-diquinoline and the radioligand ^{125}I-BE 2254 and [^{3}H]rauwolscine (Fig. 6, C and D), which are not immediately apparent from their structural formulae (see Fig. 1). The ability to superpose their three-dimensional structures rapidly diminishes as the linker is lengthened, and is abolished if the quinoline moieties are replaced by acridines. In addition, comparison of space-filling molecular models of C2-diquinoline and the β-adrenoceptor radioligand [^{3}H]DHA shows little similarity. Our experimental findings together with these observations suggest a model for the surface features of α-adrenoceptors in which the radioligand-binding sites are situated in a trench or cleft. Surrounding the trench is a flat surface bounded by walls which are more restrictive in the case of α_2-adrenoceptors (cf., the shapes of the curves in Figs. 2 and 3). The experimental data are consistent with two possible variations of this model which are illustrated in Fig. 6. In the first scheme (Fig. 6A), the flat surface completely circumscribes the radioligand-binding site and the shorter linked diquinolines are small enough to fit in the trench (witness the high affinity of 6.6 ± 1.2 nM for the ethyl derivative at the α_1-adrenoceptor), whereas the corresponding diacridines and the longer homologues in both series cannot, for they are too large. However, the diacridines and longer diquinolines bind on the flat surface above the trench with their bridging chains spanning and occluding the radioligand-binding site. The dependence of affinity on chain length then reflects specific intermolecular interactions and the topography of this part of α-adrenoceptors, for example, the width or length of the trench, the chemical nature and flatness of the surface, and the disposition and character of the walls. In the second variation (Fig. 6B), the flat surface extends only partly around the radioligand-binding site and the longer bi-

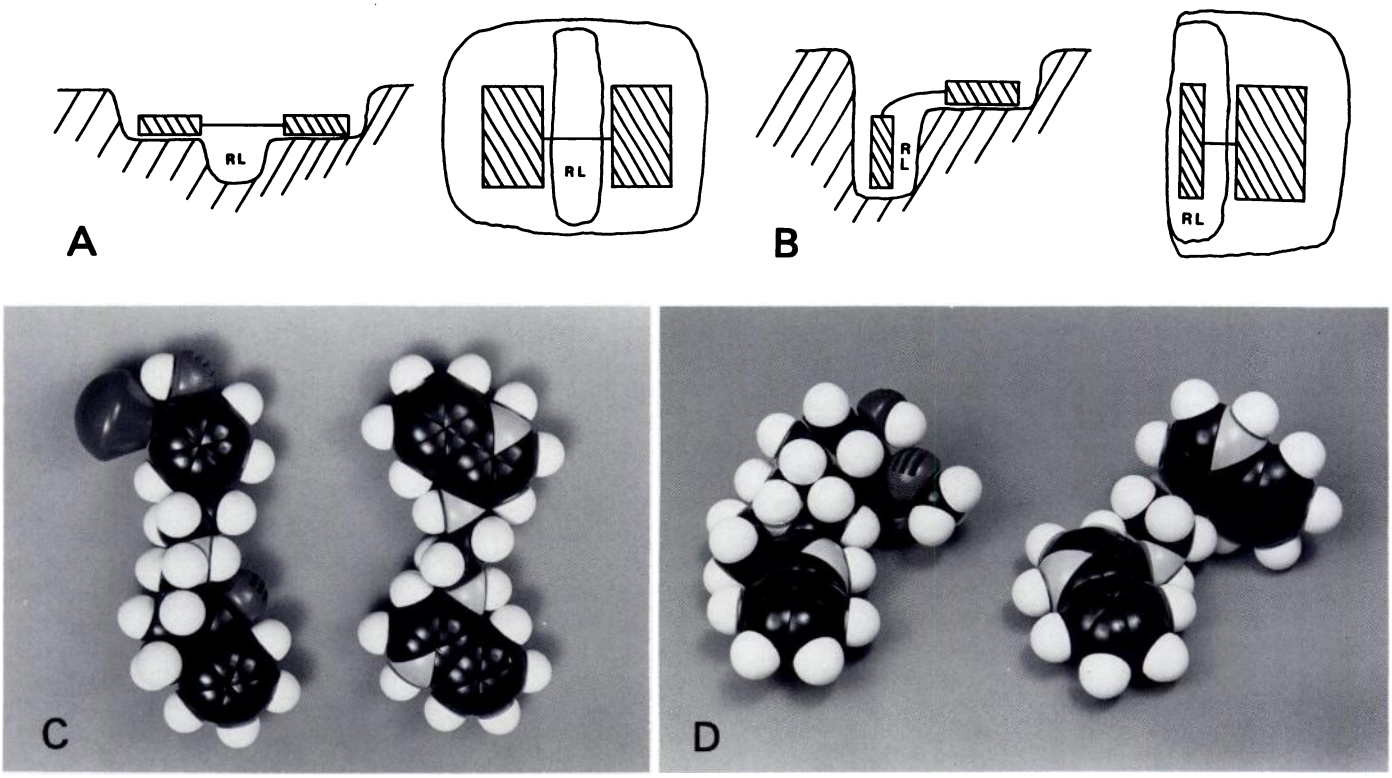


FIG. 6. *Schematic representation of binding of diacridines and diquinolines to α-adrenoceptors*

A, model in which flat surface circumscribes the radioligand-binding site (*RL*), viewed in cross-section (left) and plan (right). B, model in which flat surface lies to one side of the radioligand-binding site (*RL*), viewed in cross-section (left) and plan (right). C, space-filling molecular models of ^{125}I-BE 2254 (left) and ethyl-linked diquinoline (right) in extended conformations. D, space-filling molecular models of rauwolscine (left) and ethyl-linked diquinoline (right) in folded conformations.

nuclear ligands are envisaged as having one chromophore bound in the trench and the other on the flat surface. Highest affinity, with the exception of the shorter linked diquinolines which bind wholly in the trench, is obtained when the chain length permits optimal interaction of each chromophore with the two binding areas.

The above overlapping site model for the interaction of diacridines and diquinolines with α-adrenoceptors is the simplest scheme consistent with the available data. McGrath (43) has described a model for the norepinephrine-binding site(s) of α-adrenoceptors in which he gives molecular details of an area where agonists and small antagonists bind. However, large antagonists such as prazosin are too bulky to be accommodated therein and he proposes that prazosin occupies only part of the norepinephrine site, the rest of the molecule binding to an adjacent feature on the receptor surface. Our findings, and their interpretation, are consistent with this model insofar as the C2-diquinoline-binding sites appear to be equivalent to McGrath's norepinephrine site(s) and that the longer chain length diquinolines and diacridines may be interacting with the additional site hypothesized for prazosin (43). The relevance of the topological model of the α-adrenoceptor surface described by Melchiorre (44), which derives from studies of irreversible binding of the very large tetramine disulfides with α-adrenoceptors, to diquinoline and diacridine binding remains to be established. For example, it is not clear whether or not these two very dissimilar ligand types interact with coincident areas on the receptor surface. It is unlikely that the binuclear acridines and quinolines interact bifunctionally in the manner that each aromatic moiety binds simultaneously to two different α-adrenoceptors juxtaposed in the plasma membrane (see Ref. 45 for this mode of binding of bispharmacophores to opioid receptors). The internuclear distances in the maximally extended conformation of those homologues with the greatest affinity would necessitate extreme proximity of the two receptors' binding sites; this is especially so for the ethyl-linked diquinoline. Moreover, 4-aminoquinoline has little, if any, affinity for α-adrenoceptors, implying that binding of a single quinoline moiety to each receptor would be very weak.

Other explanations for diacridine and diquinoline binding may encompass notions of different sites for agonists and antagonists (46) and/or allosteric regulatory sites distant from the radioligand-binding site (47), or they may invoke further subdivision of α_1- and α_2-adrenoceptors (42, 46, 47). However, our results indicate that both α_1- and α_2-adrenoceptors provide, at least qualitatively, similar environments for binding diacridines and diquinolines, and the experimental evidence is not compelling that any of these alternative explanations could satisfactorily account for interaction with both classes of α-adrenoceptor. In particular, it seems unlikely that the diacridines and diquinolines bind to allosteric sites on either α_1- or α_2-adrenoceptors since the rates of dissociation of bound ^{125}I-BE 2254 or [^{3}H]rauwolscine in the presence of these compounds are indistinguishable from the rates of dissociation observed in the presence of the classical α-adrenoceptor antagonist, phentolamine. While there is some evidence that rauwolscine may bind to two types of α_2-adrenoceptor (42), and we observed a complex dissociation mechanism for this radioligand, we found no signs of biphasic competition at equilibrium by the diacridines or diquinolines. Similarly, there was no evidence for biphasic competition profiles for ^{125}I-BE 2254 binding to the α_1-adrenoceptor by these ligands. The only exception to this observation is with C2-diacridine, which has a slope factor of 0.5 in competing for [^{3}H]rauwolscine and 0.6 in competing for ^{125}I-BE 2254.

To summarize, the combined use of these homologous series of diquinolines and diacridines has provided a novel probe of the topography of adrenoceptors and allowed characterization of regions of the receptor in and adjacent to the norepinephrine-binding site. The results show that α_1- and α_2-adrenoceptors are topologically similar in the region surrounding their radioligand-binding sites, though they clearly differ in detail, whereas this area is structurally quite different in the β subclass. The binding behavior of the diquinoline series at each receptor leads to similar conclusions about the relative topographies of the radioligand-binding sites themselves, as has been noted by other investigators (46). It is a general finding that the α_2-adrenoceptor has more stringent requirements for binding both diacridines and diquinolines at its two sites. This is revealed by the more precipitous loss in affinity at the α_2-adrenoceptor, compared to that at the α_1, as the linker chain length deviates from optimal values. It is probable, therefore, that the C2-diquinoline- and diacridine-binding sites are smaller in α_2- than in α_1-adrenoceptors.

Implications for DNA-binding antitumor agents. It is clear that the cardiotoxicity of daunomycin, which is thought to result from perturbed Ca^{2+} handling by cardiac tissues, cannot be related to direct blockade of adrenoceptors, notwithstanding their involvement in the electrophysiology of Ca^{2+} ions. By contrast, the antitumor agents showing the greatest degree of hypotensive, local irritant, and neurologic side effects, bisantrene, nitracrine, and C6-diacridine (NSC 219733), all bind strongly to α_1-adrenoceptors. Mitoxantrone and amsacrine interact only weakly with the α_1-adrenoceptor although amsacrine has higher affinity for the α_2-adrenoceptor. With the exceptions of daunomycin, mitoxantrone, and the polyamine-linked diacridines, it is a general finding that the affinity for α_1-adrenoceptors of all the compounds studied is comparable to, or greater than, their DNA-binding constants where these are known (the latter are typically in the range 1–100 μM). Further structure-activity relationships for binding to adrenoceptors are revealed by these agents, since it is apparent that where two aromatic rings are fixed orthogonally to one another (lucigenin, amsacrine, ethidium) the binding constant is reduced, in the case of lucigenin quite dramatically. The mononuclear compounds with high affinity for α_1-adrenoceptors (nitracrine, bisantrene, 9-methylaminoacridine, and quinacrine) have side chains attached to the middle ring of their fused aromatic systems, whereas those which bind poorly, if at all (mitoxantrone and daunomycin), are substituted on a terminal ring. It is noteworthy that the structure-activity relationships

for diacridine binding to α-adrenoceptors differ from those found for interaction with DNA. For example, 9-aminoacridine and C2-, C3-, C4-, and C5-diacridine have indistinguishable DNA-binding constants whereas the C6- diacridine binds 10 times more tightly, attributable to the transition from mono- to bifunctional intercalation, and thereafter the binding constant continues to rise as the linker chain length increases (48). Furthermore, the polyamine-linked diacridines have a much higher affinity for DNA compared with their alkyl homologues as a consequence of their increased cationic charge, whereas this feature results in lower affinity at α-adrenoceptors. Nevertheless, it should be noted that many members of the diacridine and diquinoline series have affinities for the α_1-adrenoceptor that fall in the range normally characteristic of ligands specifically designed to interact with adrenoceptors. These findings strongly suggest that in anticancer drug development programs based on DNA-intercalating agents it may be advisable to investigate neurochemical and cardiovascular activity at an early stage. Since the radioligand-binding assay efficiently provides quantitative information about affinity and specificity of drug-receptor interactions, it may prove to be the method of choice for assessing potential neurologic and cardiovascular effects.

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Send reprint requests to: Adrienne Adams, Clinical Pharmacology and Therapeutics Unit, University of Melbourne, Austin Hospital, Heidelberg, Victoria 3084, Australia.