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STRUCTURE ACTIVITY RELATIONSHIPS IN A SERIES OF ANTI-NEOPLASTIC DINITROPHENYL AZIRIDINES—PROTEIN BINDING STUDIES

JOHN A. HICKMAN

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W.3. England

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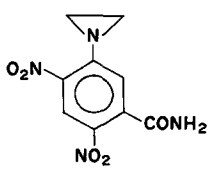
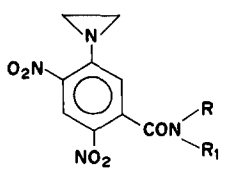
Abstract—The reversible binding of a series of antineoplastic 2,4-dinitrophenylaziridines to bovine serum albumin has been studied. The methods of gel filtration and continuous diafiltration were used to estimate the amount bound and the reversibility of binding. The compounds were shown to be weakly but reversibly bound and the extent of binding correlated well with biological activity and molecular structure.

THE ANTI-TUMOUR activity of alkylating agents is normally associated with two or more alkylating functions within the molecule. The compound CB 1954 (I) is of exceptional interest since it is formally a monofunctional agent but exhibits a very high chemotherapeutic index against the Walker Carcinoma 256 in rats.[1] Studies on its mechanism of action have shown it to have certain properties in common with difunctional agents such as melphalan, and with antimetabolites, the latter property suggesting the existence of a specific receptor site in the Walker tumour.[2] This site was also suggested by the finding that a number of agents could reverse the cytotoxicity.[3]



(I)



(II)

The synthesis of an extended series of compounds showed that the minimal requirements for activity were an aziridine ring, implying a requirement for an alkylating centre, and a 2,4-dinitrophenyl moiety.[4] The optimal structure was the 5-carboxyamido-2,4-dinitrophenylaziridine (I) several *N*-substituted amido derivatives (II, R = alkyl, aryl, R_1 = H) were the only compounds to retain high chemotherapeutic indices.[5] A complete loss of activity was found when both hydrogens of the amide were substituted (II, R = R_1 = alkyl). A study of structure–activity relationships of this series[5] showed no correlation between the rates of alkylation and anti-tumour activity, and no overall correlation between partition coefficient and activity.

This study of the reversible binding to bovine serum albumin of a number of compounds has been undertaken in order to provide a model for a possible drug–receptor interaction and to correlate biological activity with the ability of compounds to bind to a specific receptor site.

MATERIAL AND METHODS

Bovine serum albumin (BSA), fraction V, was purchased from Armour Pharmaceuticals and used without further purification. All solutions were in 0·1 M potassium phosphate buffer pH 7·4. Dimethylsulphoxide, 2 per cent, was used as cosolvent for drug solutions. The diafiltration apparatus was purchased from Amicon Ltd. The drugs were synthesized by Mr. A. M. Mirza of this Institute.

(1) *Comparisons of binding of* 0·3 *mM solutions of dinitrophenylaziridines to* 6% *BSA by gel filtration method*[6]

A Pharmacia water jacketed column (K16/20) packed with Sephadex G25 (medium), was maintained at 20° and equilibrated with a 0·3 mM solution of the drug. (Three times the void volume of the column was sufficient to achieve equilibrium). To 2 cm^3 of this drug solution was added 120 mg of BSA and this mixture stirred at 20° for 0·5 hr. One cm^3 of this solution was applied to the equilibrated gel and eluted from it with the drug solution at a flow rate of 15 $cm^3\ hr^{-1}$. The eluate was passed directly through a Unicam "flow-through" cell mounted in a Unicam SP800 u.v. spectrophotometer and changes in absorbance were monitored at the λ_{max} for the drug (Table 1). A continuous record was made using a scale expansion unit coupled to a Unicam SP21 slave recorder.

(2) *Comparisons of binding isotherms of* 1·0 *mM solutions of dinitrophenylaziridines to* 6% *BSA by a continuous diafiltration method*[7]

An Amicon Model 202 stirred ultrafiltration cell was fitted with an Amicon PM 10 filter and used with an RS 24 stainless steel reservoir and a concentration/dialysis selector. The ultrafiltrate exit port was connected to a Unicam "flow-through" cell in a Unicam SP 800 u.v. spectrophotometer and changes in absorbance monitored as described above. Flow rates were approximately 250 $cm^3\ hr^{-1}$ in the absence of protein and about 90 $cm^3\ hr^{-1}$ with 6% BSA in the cell. All determinations were made using a nitrogen pressure of 3 $kg\ cm^{-2}$ and the apparatus was maintained at 25°.

TABLE 1. COMPARISON OF THE BINDING OF CB1954 AND ANALOGUES, BY GEL FILTRATION, WITH BIOLOGICAL ACTIVITY

Compound	λ_{max}nm	Chemotherapeutic index (LD_{50}/ED_{90})[2]	Drug–protein ratio (mole–mole) bound
$R = R_1 = H$ (CB1954)	320	70	0·173
$R = H, R_1 = Me$	317	30	0·150
$R = H, R_1 = Et$	320	30	0·150
$R = R_1 = Me$	315	0	0·046
$R = R_1 = Et$	315	0	0·081

(3) *Confirmation of reversible binding*

By gel filtration. Comparisons of the amount of 0·3 mM CB 1954 (I) bound to 6% BSA were made, by following the method described above (1), after intervals of 0·25, 0·5, 1·0, 2·0, 4·0 and 8·0 hr incubation of drug and protein at 20°. These incubates were also applied to a column of the gel which had not been equilibrated with drug but with buffer alone. The recovery of total drug from this procedure was calculated from a u.v. monitor of eluate. The λ_{max} of the drug was used throughout for this purpose (Table 1).

A binding isotherm for 1·0 mM CB 1954 (I) to 6% BSA was determined as described above (2). When the concentration of drug in the ultrafiltrate from the cell had reached its maximal value the procedure was stopped and buffer placed in the reservoir instead of the drug solution. The experimental procedure was then repeated, this time adding buffer to the protein plus drug in the cell and a "de-binding" isotherm determined.

RESULTS

(1) *Comparison of the binding of 0·3 mM drug by gel filtration method*

This method permitted the comparison of binding at the low concentrations necessary because of poor solubility. The results represent the mean values of at least three determinations at 20°. The amount bound was calculated by the method of Fairclough and Fruton,[8] and the results are shown in Table 1.

(2) *Comparison of binding isotherms by continuous diafiltration*

A typical ultrafiltration profile is shown in Fig. 1. Curve A represents the profile for the drug added to buffer alone in the ultrafiltration cell. Curve B represents the profile for the drug added when a protein solution is in the cell. The area between the two curves represents the total amount of ligand bound at a drug concentration C and ultrafiltrate volume V. The amount bound at a concentration of ligand C_n at an ultrafiltrate volume V_n is given by calculating the area within the two curves. The amount of drug bound with changing drug concentration is shown in Fig. 2 for four of the amides. The monoethylamide was insufficiently soluble for a determination by this method.

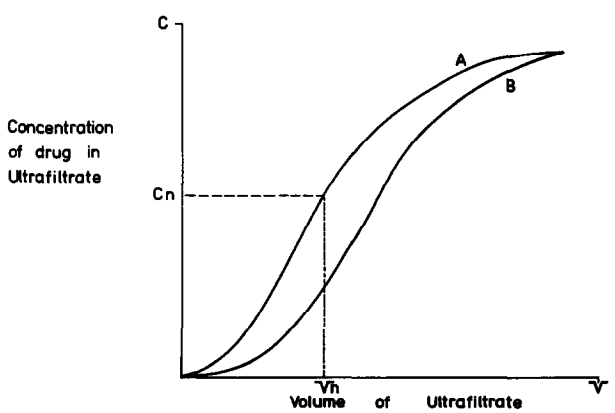


FIG. 1. Ultrafiltration profiles for addition of drug to ultrafiltration cell under conditions of diafiltration. For description see text.

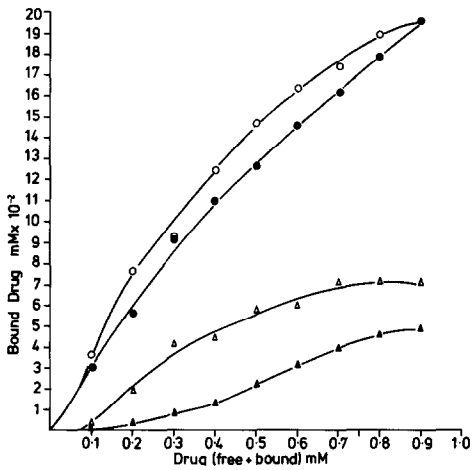


FIG. 2. The 25° isotherms of bound drug vs total drug concentration. (●) CB1954(I); (○) monomethylamide(II), R = H, R_1 = Me; (△) diethylamide, (II), R = R_1 = Et; (▲) dimethylamide, (II), R = R_1 = Me.

(3) *Confirmation of reversibility of binding*

By gel filtration. The extent of binding of a 0·3 mM solution of CB 1954 did not increase over an incubation period of 8 hr at 20°. Recovery of CB 1954 from 6% BSA incubated for this time was 96–100 per cent when the mixture was eluted with buffer from Sephadex G25. This process was sufficient to remove all of the drug from the protein and shows the strength of binding to be low.

By continuous diafiltration. The ultrafiltration profile of the binding and "debinding" experiment are shown in Fig. 3. Not all the CB 1954 was dissociated from the protein according to this method. The concentration of CB 1954 remaining on the protein was 0·02 mM. The duration of this experiment was 15 hr. A 15 hr incubate of CB 1954 (1·0 mM) showed no detectable signs of alkylation of the protein, as measured by the u.v. absorbance at 360 nm (the wavelength corresponding to the ring-opened compound[9]).

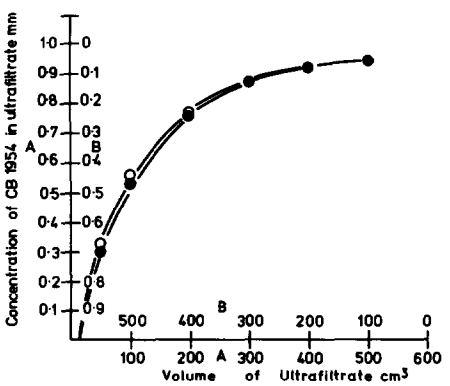


FIG. 3. Ultrafiltrate profiles for binding and 'debinding' isotherms of CB1954 (1·0 mM) measured by continuous diafiltration on A, and ● = binding curve; B, and ○ = debinding curve.

DISCUSSION

The attempt to correlate certain physical parameters with the biological activity of drugs provides information about the site and mechanism of action. A previous study of the reversible binding of dinitrophenyl compounds to BSA suggested that a specific site for the binding of this ligand might exist,[10] and a correlation between protein binding and the ability of isomeric dinitrophenols to uncouple oxidative phosphorylation has been shown.[11] That a similar correlation might exist for the anti-tumour activity of the dinitrophenylaziridine amides was suggested by an examination of Courtauld molecular models of the compounds. The amide, CB 1954 (I), and the monosubstituted amides were seen to be able to maintain the presentation of a planar face of the molecule to a hypothetical receptor site. This was not the case with the disubstituted amides and in addition some steric hindrance of the *p*-nitro group was apparent.

The choice of methods for the study of binding of these alkylating agents was limited by their very low water solubility,[4,5] and by the fact that they are potentially capable of irreversible reaction with the protein. For these reasons two relatively rapid methods of determining binding have been used on a limited but representative number of compounds.

The results indicate that very little irreversible binding took place during the time of each determination, so that measurements represent reversible binding. The extent of binding of these compounds is low when compared to organic ligands with a polar, potentially ionic group and this was expected from the results of previous work.[10] Despite this low level of binding, a comparison of these amides shows that disubstitution lowers the affinity of the compounds for the protein and this is in accord with their activity as anti-tumour compounds (Table 1). A comparison of the two methods used here shows the continuous diafiltration technique to give a lower estimate for the amount bound. Compared to the gel filtration method, where time is available for drug and protein to equilibrate, this method allows negligible time for equilibration at any one concentration. The higher temperature of these determinations may also have had an effect.

The correlation of biological activity and molecular structure with the degree of binding to a model receptor appears to be valid here for the limited number of compounds studied. A single parameter cannot generally be used to account for overall biological activity and so although a clear difference was seen between the active and inactive compounds there is little difference between the active compounds. Overall activity is likely to depend upon a number of other parameters.

These results suggest, in common with previous work,[2] that a "receptor site" may exist for CB 1954 and that ability to bind to such a site is essential for anti-tumour activity. Work to characterize this site is in progress.

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