INTERACTIONS BETWEEN BLEOMYCINS AND METALS

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Gel filtration has shown that there are considerable differences between the metal complexes of bleomycin A_2 and B_2 with indium, cobalt or copper. Differences in the rates of formation of the complexes have also been found and it is thought that these effects are due to a difference in co-ordination between the metals and the bleomycin. The co-ordination changes are thought to be the cause of the differences in *in vivo* distribution of metal bleomycin complexes found in the radiodiagnosis of tumours. The ease of formation of the copper or cobalt complexes is suggested as a possible mechanism for the inhibition of the attack of DNA by bleomycins.

The bleomycins are a closely related group of water-soluble glycopeptides (each differing in an amine side chain), and are obtained from the culture of a strain of *Streptomyces verticillus*. Since their discovery by UMEZAWA *et al*[1,2] they have been increasingly used for the chemotherapy of some types of tumours[3] and, when labelled with a radionuclide, as tumour imaging compounds[4~7]. Originally the copper chelate produced by the organism was used for chemotherapy, however, indications were that this form was more toxic and irritating than the copper-free preparation which is now used[8]. Besides the ability to bind copper, bleomycin has the capacity to bind various other metals which has been extensively investigated for tumour imaging by using a complex with metal radionuclides[4~6]. Any differences between the different bleomycin-metal complexes might be expected to effect their biological behaviour. There are indications that the ^{111}In and ^{57}Co complexes at present widely used for tumour imaging exhibit greater differences than the known interactions of the individual metals within the body would suggest[9]. Furthermore, a number of workers have shown that bleomycins cause effects (such as scission of DNA and a reduction in its melting temperature) that are enhanced by agents containing sulfhydryl groups or by hydrogen peroxide, but are inhibited by metal ions such as copper and cobalt, and by EDTA.[10,11] This indicates that metal-bleomycin interactions may have important effects on the attack and inhibition processes.

The chemical formula of bleomycin has been established[12] but the conformation has not yet been elucidated. Gel permeation chromatography has been used to study conformational changes such as those that occur when metals bind transferrin[13] and when organic reagents react with albumin[14]. This paper reports some results of experiments designed to observe the effect on the molecule of binding metals using bleomycin (BLM) as supplied for clinical use (which is a mixture) and separated samples of BLM–A_2 and BLM–B_2 together with radionuclides of indium, cobalt, and copper.

Methods

1. Preparation of compounds

Freeze-dried bleomycin sulphate in 15 mg ampoules for injection was kindly supplied by Lundbeck Ltd., Luton, Bedfordshire, England. They also supplied a quantity of separated BLM–A_2, batch

CM–25 and BLM–B_2, batch F–4. All samples were supplied copper-free.

The radionuclides used were carrier-free and were made in this Unit ^{111}In[15], ^{61}Cu (unpublished work), or supplied by the Radiochemical Centre, Amersham, England(^{111}In, ^{57}Co).

The indium and cobalt bleomycin complexes were made by adding the radionuclides as their chlorides in 0.2 ml of 0.02 M HCl to a solution of 1 mg/ml bleomycin in 0.9% NaCl and after 5 minutes at room temperature, adjusting the pH to 7.4 with 0.1 M $NaHCO_3$ solution.

The copper bleomycin complexes were made by adding ^{61}Cu in 0.2 ml of 0.9% NaCl to a solution of 1 mg/ml bleomycin in 0.9% NaCl at room temperature.

2. Thin-layer chromatography

This was performed using precoated plates of silica gel without binder 0.25 mm thick (Macherey-Nagel). Samples of approximately 10 μl were spotted onto the plates 1 cm from the bottom and dried. The plates were developed in a solvent saturated atmosphere with 10% aqueous ammonium acetate-methanol (1: 1, v/v) until the solvent front had travelled 15 cm from the origin.

After air drying the plates were assayed for radioactivity with a scanning system utilising a 25× 38 mm sodium iodide crystal linked to a 1000 channel analyser in multiscaler mode. After obtaining quantitative data in this way autoradiographs of the plates were made using Ilford Red Seal X-ray film. This gave higher resolution pictures of the radioactive spots on the plate than the scanning system. With the amounts used it was not always possible to visualise the bleomycin spots chemically, however, at all times when labelling occurred the Rf value of the radioactive spots coincided with the known values of stable bleomycin.

3. Gel Permeation Chromatography

This was performed using a BioRad glass column of 7 mm inside diameter filled to 25 cm with G–15 Sephadex (Pharmacia), pre-swollen in the eluant (0.9% NaCl in water). A polythene sinter was placed on the top of the column bed to prevent disturbance of the surface. The eluant head was maintained at 50 cm using a Mariotte flask and gave a flow rate of 0.1 ml/min.

The column eluate was passed through a 2-mm path length, vertical flowthrough optical cell, which was illuminated with UV light of 254 nm wavelength. The absorbance was measured with an ISCO UA–4 ultraviolet absorbance monitor; 0.5-ml fractions were collected using a Gilson MTDC fraction collector coupled to the UA–4 so that fractions were marked on the absorbance trace. Small bore PTFE tubing was used throughout and gave a dead volume between the centre of the flow-through cell and the fraction collector of 0.1 ml. Radioactivity was measured in each tube (after all the samples had been collected) with a well-type scintillation counter. The sample volume was 0.1~0.4 ml.

Results

The bleomycin used clinically is a mixture which consists of approximately 65% BLM–A_2 (Mol. wt. 1,385) and 30% BLM–B_2 (Mol. wt. 1,395). There are some other components, but these do not total more than a few percent collectively. The structure of the two most abundant bleomycins is shown in Fig. 1., the difference between BLM–A_2 and BLM–B_2 is seen to reside in the amine side chain. Fig. 2 shows an autoradiograph of a thin-layer chromatogram of ^{61}Cu-labelled BLM–A_2, BLM–B_2 and mixture. In the samples shown the BLM–A_2 contains 6.6% BLM–B_2 and 5.4% BLM–A_1 and B_1 labelled. The BLM–B_2 is pure and the mixture contains 69.4% BLM–A_2 and 30.6% BLM–B_2 labelled.

The analysis by thin-layer chromatography showed that samples used for gel permeation experiments contained >98% of the radionuclide bound to bleomycins.

The results of the gel permeation experiments are reproduced in Figs. 3 and 4 and are shown as continuous UV absorbance traces with the corresponding radioactivities per fraction collected superimposed. All the absorbance traces were reproducible as regards peak-position over three or more runs, both with samples from the same batch of labelled BLM, and also from batch to batch. There were some minor differences in peak shape that were due to differences in sample application and size.

Fig. 1. The structure of bleomycin (after UMEZAWA)

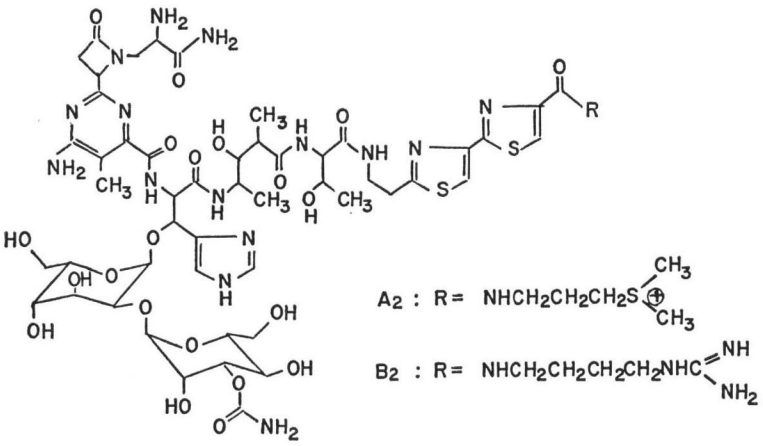
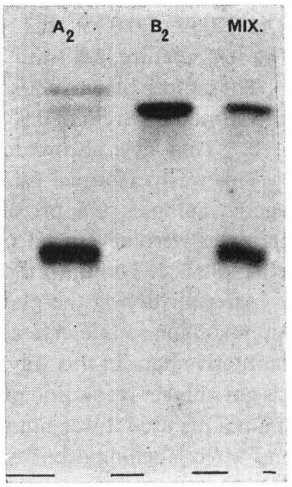
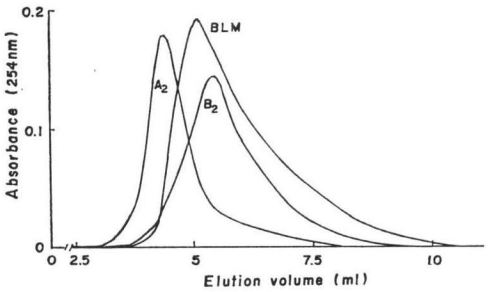


Fig. 2. Autoradiograph of a thin-layer chromatogram of ^{61}Cu complexes of bleomycin A_2, bleomycin B_2 and bleomycin mixture



As a result of using "carrier-free" radionuclides each gel filtration experiment had its own internal standard *i.e.* the bleomycin with no radionuclide bound to it. The metal-free bleomycin was detected by its ultraviolet absorption whereas the "carrier-free" metal-bleomycin complexes could only be detected by their radioactivity.

Fig. 3. Gel filtration on G–15 of metal-free bleomycins.



Discussion

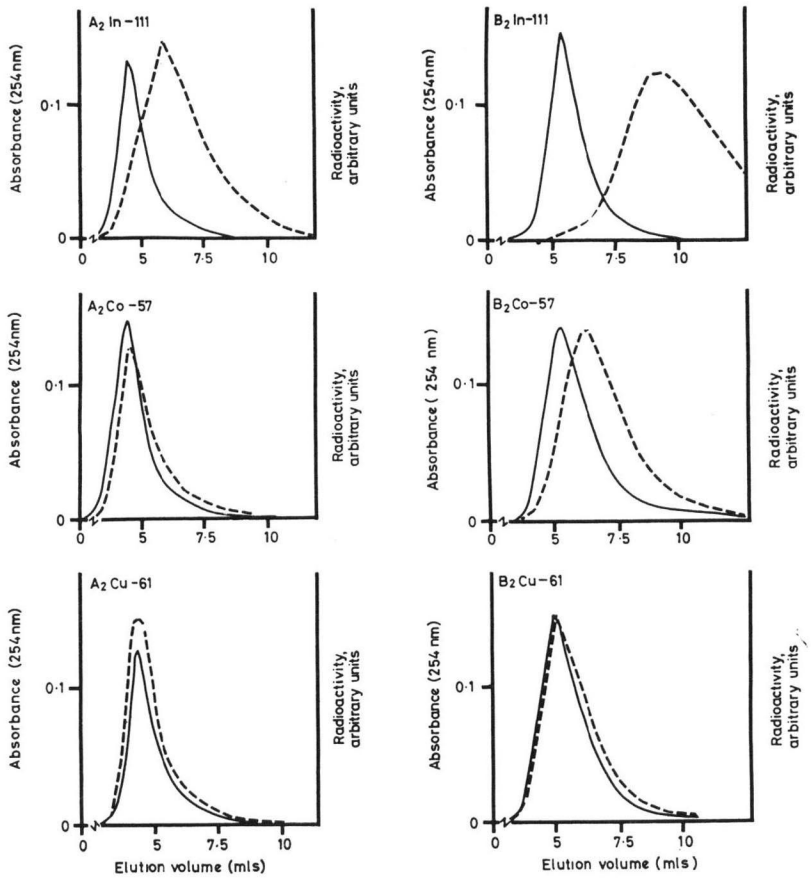
Under optimum conditions the passage of different compounds through a dextran gel such as Sephadex G–15 depends upon the physical size of the molecules. If all the molecules are approximately the same shape, measurement of the elution volumes can lead to a figure for the molecular weight for an unknown compound after suitable calibration procedures[16]. The difference between the molecular weight of BLM–A_2 and BLM–B_2 is $<1\%$ and cannot be resolved by the system used. Any differences in elution volume, therefore, must either be a result of changes in size (*i.e.* STOKES radius of the molecule), or some other means of selectivity such as ion-exchange. It is known that positively charged species may be retarded on dextran gels due to the presence of a small number of –OH groups on the gels, however, this can be minimised if the ionic strength of the eluant is maintained above 0.02[16]. Experiments have shown that in this case the use of isotonic saline (0.9% NaCl, ionic strength 0.15) prevents any obvious ion-exchange effects.

(1) Metal-free Bleomycins

Fig. 3. shows the elution curves for metal-free BLM–A_2, BLM–B_2 and BLM mixture. Because of the smaller elution volume BLM–A_2 apears to be bigger than BLM–B_2. The curve for the mixture is what one would expect from a composite of the curves for BLM–A_2 and BLM–B_2. Curves of solutions containing equal quantities by weight of BLM–A_2 and BLM–B_2 (not shown) exhibit two peaks with the expected elution volumes.

Clearly there is an appreciable difference in elution of the two bleomycins and since the only

Fig. 4. Gel filtration on G-15 of metal complexes of bleomycin A_2 and bleomycin B_2.
——— absorbance, --------- radioactivity.



chemical difference between the molecules lies in the side chain, this must also be the cause of any elution difference. The terminal guanidine group of B_2 would be protonated at a neutral pH so that there is no charge difference between BLM-A_2 and BLM-B_2. It is possible that a combination of charge and the shorter length of the chain causes the BLM-A_2 amine moiety to project more into the solvent away from the bulk of the molecule, thus increasing its STOKES radius.

(2) Metal Complexes

Fig. 4. shows the effect of binding ^{111}In, ^{57}Co and ^{61}Cu to BLM-A_2 and BLM-B_2.

The curves for indium illustrate different elution characteristics for the complexes compared to the metal-free BLM-A_2 and BLM-B_2. The difference is greater for BLM-B_2 than for BLM-A_2.

The curves for cobalt show that when cobalt binds to BLM-A_2 there is a small change in elution. This is greater for BLM-B_2 than for BLM-A_2, but still smaller than that for indium.

The curves for copper in Fig. 4. show that there is no detectable change of BLM-A_2 or BLM-B_2 when copper is bound.

In all cases the elution profiles for the metal complexes of the bleomycin mixture expected from consideration of the separated bleomycin-metal complexes have been obtained experimentally.

The possible differences in charge between the In^{3+}-BLM complexes and the M^{2+} bleomycin complexes does not appear to be operative in this instance because of the lack of similarity between cobalt and copper complexes and the obvious progression in elution shown by copper, cobalt and indium bleomycins. The elution volume of the ^{111}In in the ^{111}In-B_2 preparation suggests that the STOKES radius of the

complex is approaching that of hydrated metal ions. Dissociation of the complex has been ruled out in this case because analysis of the fractions collected has shown all the indium to be present bound to BLM B_2, and because carrier-free indium is strongly retarded on the Sephadex. It is possible that there are some weak effects peculiar to InBLM-B_2 that are retarding these complexes thus enhancing the apparent natural progression (from copper to indium) of increasing elution volume.

Indium prefers an octahedral configuration, cobalt favours octahedral or tetrahedral configurations depending upon the ligand, and copper favours square planar or distorted tetrahedral configurations. It is reasonable to assume that the site of co-ordination of different metals would be similar for different co-ordination numbers—*i.e.* some of the ligand atoms would be the same in all cases.

The molar ratio for the indium BLM-B_2 complex has been found to be 1 : 1 as it is for the copper complex and one can infer that this is the molar ratio for all metals specifically bound to the bleomycin molecule. The gel filtration data obtained indicate that polymerisation has not occurred.

The changes detected in the elution of the different BLM-metal complexes might be the result of two factors. The first is the different co-ordinatic numbers preferred by the individual metals. The second is the number of co-ordination positions occupied by ligands arising from the bleomycin molecule. Both would produce changes in the conformation of the bleomycin molecule.

Both copper and cobalt bind to bleomycin instantaneously at 20°C in 0.9% NaCl at neutral pH. Indium requires 20 minutes at 80°C before 100% labelling occurs under these conditions. Binding is complete within one minute at pH 2.5 and becomes slower at a lower pH.

Bleomycins have been found to have pKa' values of 2.9, 4.7 and 7.3 which have been assigned to the 4-aminopyrimidine, imidazole and β-aminoalanine groups respectively[12]. If metal binding involves any of these sites as has been suggested for the most basic group[12] proton competition would occur. It follows that if protonation of metal binding sites only is considered, metal binding should be improved at a high pH where protonation is minimised. The pH of the solution does not have any detectable effect on the binding of copper or cobalt to bleomycin within the range investigated.

At pH 2.5 where protonation of the β-aminoalanine and imidazole groups would be essentially complete and where the 4-amino pyrimidine group would be 30% protonated, incorporation of indium is rapid. Thus, these changes in the rate of incorporation of indium into bleomycin cannot be attributed to protonation of the metal binding sites.

Although indium is known to precipitate above pH 3.5[17] labelling still occurs, at least upto pH 7.5. Any carrier-free hydrolysed indium species present do not prevent eventual binding to bleomycin. Olation would not be expected to occur as the indium is "carrier free". Trace metal impurities such as iron which may be capable of forming polynuclear species with the indium, but which do not bind to the bleomycin themselves, do not effect binding of indium to bleomycin under the conditions used.

The aqueous chemistry of indium has been investigated[18] and it has been shown that with a large excess of chloride present the $[In(H_2O)_5Cl]^{2+}$ species is the predominant one. One can deduce, therefore, that the changes of binding rate of indium to bleomycin induced by changes in pH cannot be the result of changes in the indium species present.

It is possible that changes in pH would effect the conformation of the bleomycin molecule. As copper and cobalt bind immediately to bleomycin at a neutral pH one can assume that the conformation of the bleomycin is already adequate for these metals to bind.

The increase in temperature at neutral pH, or the lowering of pH required for indium to bind rapidly could be due to a necessary change in conformation of the bleomycin which results in an increase in STOKES radius. If this is so, it would be correct to attribute the differences between cobalt and copper to be due to difference in co-ordination type - tetrahedral v/s square planar, (both having co-ordination numbers of four). Furthermore, one may explain the difference between indium and the other two metals to be primarily due to co-ordination number (six), but also influenced by co-ordination type.

As yet the exact binding site of metals has not been elucidated. The d–d spectrum of Cu BLM shows strong absorption at 595 nM which is characteristic of copper bound to four nitrogen atoms. The molar extinction ~ 300 is in agreement with other values obtained when copper is chelated to a large molecule. There is no spectral evidence of copper-sulphur bonding.

UMEZAWA[12] has suggested that the α-amino group of the β-aminoalanine moiety is a site of copper binding because of the disappearance of the pKa' of this group when copper is bound. The carboxamide group of this area of the molecule appears to be important in the attack of DNA by bleomycins as an inactivating enzyme has been shown to attack this group[19]. Due to the close proximity of a proposed metal binding group and a group that is necessary for DNA attack it is feasible that the way in which copper prevents the attack of DNA by bleomycin is to alter the conformation of the bleomycin molecule and to introduce steric hindrance between the reacting groups.

The 3-*O*-carbamoyl group is known to undergo rearrangement to the 2-position[20]. This rearrangement is prevented by copper chelation but each isomer can still bind copper. Thus, this group is indicated as providing a ligand atom. There are no other strong indications for ligand providing groups apart from the possible involvement of the amine side chains with some metals.

The strength of the bond between the metal and the bleomycin is quite high. It has been shown that bleomycin will remove 74% of cobalt from a CoEDTA complex over a period of 72 hours[21], but the reverse reaction does not occur.

I have found that copper in ten-fold excess with respect to bleomycin rapidly substitutes ^{111}In in ^{111}In-BLM and that cobalt removes ^{111}In at a slower rate[21]. The indium complex is thus not stable in the presence of Cu^{2+} or Co^{2+}. It follows that if ^{111}In–BLM encounters available copper *in vivo* it is likely that the copper complex will be formed and the ^{111}In released. Metals found to inhibit attack of DNA by bleomycins react with sulfhydryl groups. Work is at present in progress to determine the interactions between Cu^{2+}, Co^{2+}, Zn^{2+}, 2-mercaptoethanol, and bleomycin to try and elucidate mechanisms for the changes in attack of DNA by bleomycins. Preliminary results suggest that when there is a large excess of 2-mercaptoethanol compared to Cu^{2+}, Co^{2+} or Zn^{2+} the competition between 2-mercaptoethanol and the bleomycin for the metals favours the 2-mercaptoethanol. However, when there is an increased amount of these metals present the bleomycin is all complex with them so that inhibition of attack of DNA occurs.

Conclusions

As yet, no crystallographic data on the complete bleomycin-metal complex system have been published. The results of experiments reported in this paper show that there are differences in the Gel Permeation elution characteristics of BLM-metal complexes which show some progression from copper through cobalt to indium. This is thought to be mainly due to differences in STOKES radius caused by the different co-ordination characteristics of the metals, however, the elution characteristics of the ^{111}In–B_2 complex suggests that there may be in addition other selectivity effects. These effects may be operative *in vivo* and could contribute to the differences in character of the various BLM-metal complexes which are thought to alter their *in vivo* chemistry and thus their usefulness in medicine.

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