Eur. J. Med. Chem. **24** (1989) 371–377

Original paper

Metal-complexing, DNA-binding and DNA-cleaving properties of a synthetic molecule AMBIGLU, a simplified model for the study of bleomycin

Abderraouf KENANI[1], Christian BAILLY[1], Nicole HELBECQUE[1], Raymond HOUSSIN[2], Jean-Luc BERNIER[1] and Jean-Pierre HENICHART[1]*

[1]*INSERM U 16, Place de Verdun, 59045 Lille, and*
[2]*Institut de Chimie Pharmaceutique, Université de Lille II, 59045 Lille, France*

(Received June 23, 1988, accepted January 10, 1989)

Summary — On the basis of the previous studies on simple synthetic molecules structurally related to the anti-tumor drug bleomycin-A_2 (BLM-A_2), the roles of the main parts of the parent compound, a metal-chelating peptide, a DNA-binding heterocycle and a protecting activating sugar residue, were delineated. A new synthetic compound, AMBIGLU was designed taking into account these results. The synthesis, the copper-chelating properties, the radical production, the DNA-binding and DNA-cleaving ability are described here and compared to those of BLM-A_2.

Résumé — Complexation des métaux, liaison à l'ADN et coupure de l'ADN par une molécule synthétique, AMBIGLU, un modèle simplifié pour l'étude de la bléomycine. *Trois parties principales dans la structure de la bléomycine-A_2 (BLM-A_2) se sont révélées essentielles pour son activité: une partie peptidique qui complexe les métaux, une partie hétérocyclique qui lie l'ADN et une partie glycannique qui stabilise le complexe formé et active la molécule. Ces trois parties, simplifiées, ont été réunies sur une même molécule, AMBIGLU, dont les propriétés se sont trouvées être tout à fait comparables à celles de BLM-A_2 pour la complexation du cuivre, pour la production de radicaux libres, pour la liaison à la cible ADN et pour la coupure d'ADN.*

bleomycin-like antitumor drug / synthesis / metal-chelating properties / radical production / DNA-binding / DNA-cleaving ability

Introduction

Recent advances in cancer cell biology and in molecular biology allow the identification of new appropriate targets for chemotherapeutics in the treatment of cancer. On the other hand, the discovery of natural toxins with a very high cytotoxicity may reveal compounds with biological effects pertinent to severe human diseases such as cancer. Thus, the rational design of new synthetic drugs with precise action on specific targets can find inspiration from natural products. If the mechanism of action of the parent natural drug is known, by adequate chemical modifications of the initial structure, it is possible to optimize the desired effect and to minimize adverse effects. This approach has been used here starting from the anti-tumor drug bleomycin.

Bleomycin (Fig. 1) is an anti-tumor antibiotic drug isolated from *Streptomyces verticillus* and employed for the treatment of lymphomas, squamous cell carcinomas and testicular carcinomas [1–3]. In fact it is the generic name of closely related natural glycopeptides with a common structure, bleomycinic acid, and which differ only in the substituent on their heterocyclic unit, bithiazole.

The biological activity of bleomycin involves 2 well defined parts of the molecule. A metal ion-chelating fraction is able to form a copper (II) complex which has 2 biological roles: the resistance to the inactivation enzyme and the protection against manifestation of the biological activity [4]. The same part is able to form iron (II) and molecular oxygen an active complex which generates free radicals responsible for the cleavage of DNA [5, 6]. On the other hand, a bithiazole containing moiety contributes to the binding of bleomycin to DNA [7]. DNA-binding and DNA-cleaving are believed to be the 2 essential steps of the cytotoxic process.

By the design of synthetic model molecules [8, 9], we have delineated the exact role of the heterocyclic bithiazole ring of bleomycin in the binding to DNA [10]. The complexing properties of the pseudopeptide chain have also been studied using a synthetic simplified molecule, AMPHIS (11) (Fig. 1). This compound was found to form a copper (II) complex exhibiting ESR parameters substantially similar to those of the copper (II)-bleomycin complex and to produce $OH^{\cdot}$ radicals in the presence of iron (II) and oxygen in the same conditions as bleomycin does

*Author to whom correspondence should be addressed.

[12]. Similar results were obtained with a simple synthetic molecule named AMBI-A_2, related to BLM-A_2 and possessing in its structure both simplified complexing and binding parts of BLM-A_2 [13] (Fig. 1). This compound has been shown to mimic the chelating and binding properties of the parent drug BLM-A_2 but to cleave DNA at higher concentration. At least, to explain the role of the carbohydrate part of bleomycin, we have compared the properties of deglycobleomycin [14] and of bleomycin. It can be stated that the gulose-mannose part undoubtedly plays a role both in the stabilization of iron (II)-oxygen-bleomycin complex and in its activation to produce oxygenated free radicals [15].

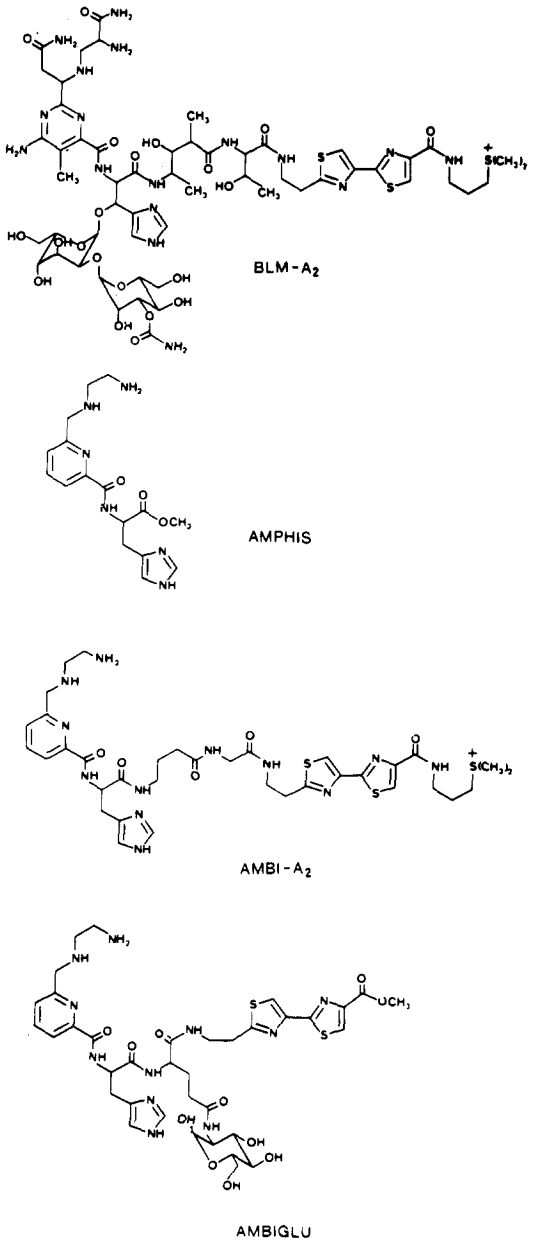
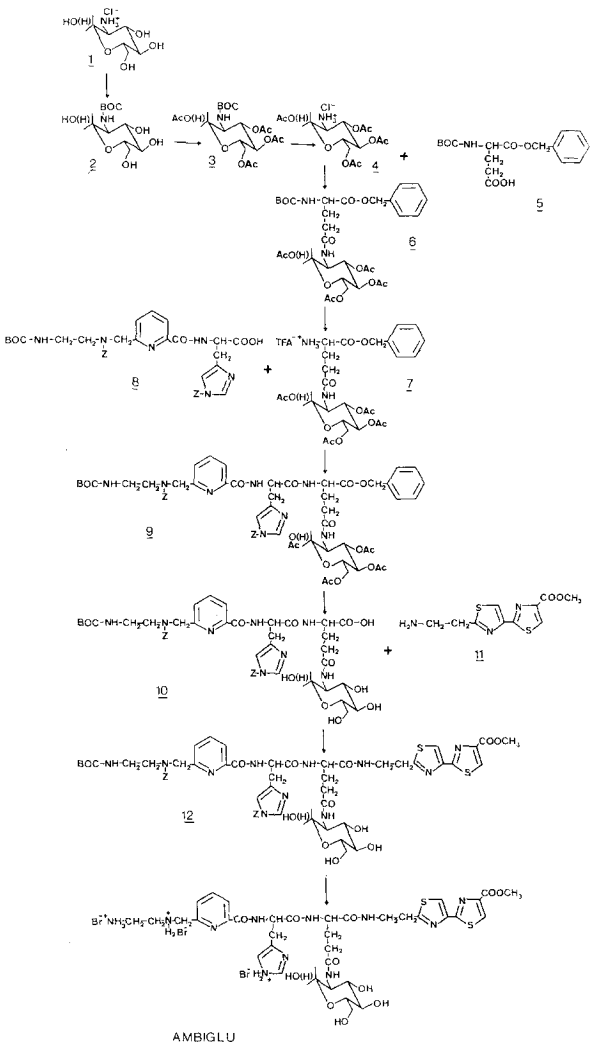


Fig. 1. Structures of BLM-A_2, AMPHIS, AMBI-A_2 and AMBIGLU.

On the basis of these results, a model was designed. Taking into account the previous observations, this molecule named AMBIGLU was built by connecting the simplified chelating part AMPHIS, the DNA-binding part bithiazole and a glucosamine residue to a short spacer, glutamic acid (Fig. 1). We report here the synthesis, the metal-complexing and DNA-binding properties of the synthetic molecule together with the DNA-cleaving ability compared to those of the parent drug bleomycin.

Chemistry

AMBIGLU was synthesized by a multi-step strategy (Scheme 1). The simplified complexing part of BLM (AMPHIS, **8**) [11] was here linked to the bithiazole ring **11** [9] *via* a glycopeptidic spacer: *N*-glutamido glucosamine. This linker part was prepared starting from glucosamine hydrochloride **1**. N-protection of the amine function with di-tertiobutyl dicarbonate led to the tertbuty-



Scheme 1. Synthesis of AMBIGLU.

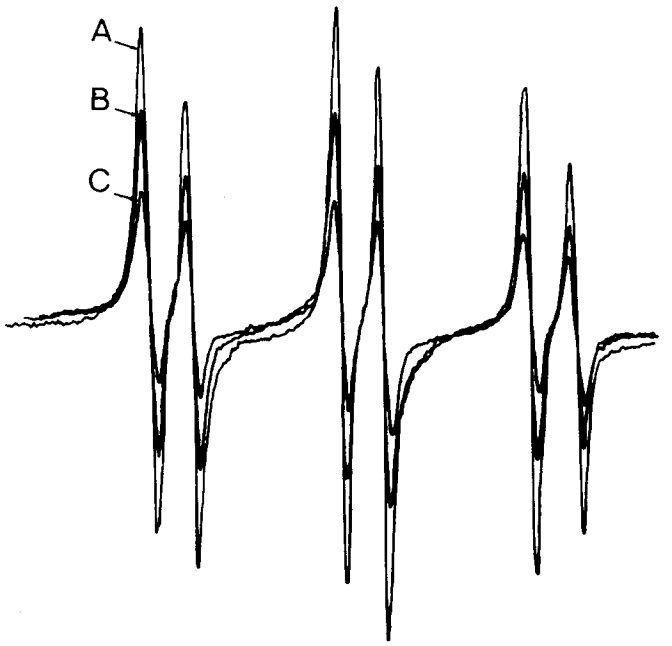


Fig. 2. ESR spin-trapping by phenyl-*N*-t-butyl-nitrone in the presence of BLM-A_2-Fe(II)-O_2 (A), AMBIGLU-Fe(II)-O_2 (B) and AMBI-A_2-Fe(II)-O_2 (C) complexes at 10 mM concentration. ESR spectra consist of triplet of doublet with a g factor of 2.006 and a_N: 15.3 G.

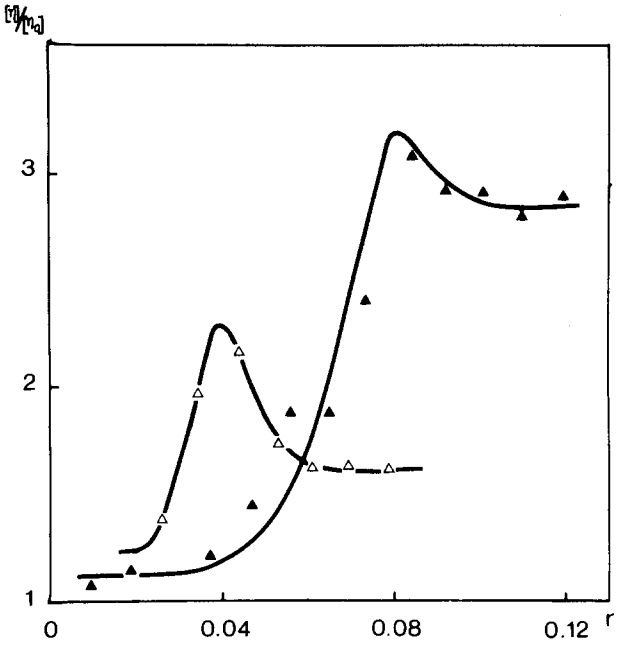


Fig. 3. DNA unwinding produced by AMBIGLU (▲), compared to the one observed for ethidium bromide (△). (DNA: 150 μM, 0.01 SHE buffer).

loxycarbonyl derivative **2**, acetylation of hydroxyl groups with acetic anhydride gave the fully protected sugar **3**. Cleavage of the BOC group with dry hydrogen chloride in acetic acid gave the desired compound **4** with a 86% yield. Coupling of the primary amine **4** with the α-protected glutamic acid derivative **5**, in the presence of dicyclohexylcarbodiimide (DCC) and 1*H*-hydroxybenzotriazole (HOBT), afforded the amide **6** after column chromatography. After cleavage of the BOC-protecting group in trifluoroacetic medium, the amino-glucidic unit **7** was coupled to compound **8** using the mixed anhydride method. The resulting benzyl ester **9** was converted into the acid **10** by saponification. This reaction conducted in MeOH and aqueous sodium hydroxyde during 2 days furnished the deacetylated acid **10** in a 53% yield. This free acid was coupled to the bithiazole moiety **11** using a classical DCC–HOBT procedure. Pure **12** was isolated by careful flash-chromatography.

The last step consisted in removal of the protecting groups by hydrobromic acid in acetic acid medium. The final compound was dissolved in water. After extraction with ethyl acetate, the aqueous layer was lyophilized to give AMBIGLU as a white powder.

Results

Cu(II) complexes

The ESR spectrum of AMBIGLU-Cu(II) looks like that exhibited by BLM-A_2-Cu(II). Parameters values ($g_{//}$:2.20, $g_{\perp}$:2.05, $A_{//}$:177G) are quite similar to those of BLM-A_2-Cu(II) and AMPHIS-Cu(II).

Spin-trapping

The air oxidation of AMBIGLU-Fe(II) gives rise to the formation of an OH· radical adduct with PBN spin-trap, well characterized by the corresponding ESR spectrum: triplet of doublet with a g factor of 2.006 and a_N=15.3G. These values are identical to those found by Harbour *et al.* [22].

Free radical production in the presence of (i) BLM-Fe(II)-O_2 [5, 23], (ii) AMPHIS-Fe(II)-O_2 [12] and (iii) AMBI-A_2-Fe(II)-O_2 [13] has been observed in similar conditions. The present results indicate that the spin density is significantly higher for radicals produced in the presence of AMBIGLU compared to the other analogues. Nevertheless, it is lower than those produced by BLM-A_2 (Fig. 2).

DNA unwinding

The effect of AMBIGLU on the superhelicity of a modified pBR 322 plasmid has been followed by viscometry experiments. It yields the characteristic rise-and-fall response, reflecting removal and reversal of the supercoiling. However, reversal of the supercoiling produced by AMBIGLU is not so pronounced as in the case of ethidium bromide. This can be due to the fact that AMBIGLU binds DNA by different mechanisms including intercalation. Measurement of the length increase of the DNA helix can help in elucidating these mechanisms. At the

maximum of the curve (Fig. 3), when the DNA was completely relaxed, which is hydrodynamically equivalent to nicked circular DNA, the reduced viscosity indicates an apparent unwinding angle of 12.7°. This value is quite similar to those obtained for BLM-A_2 and AMBI-A_2 [13] and can be thought to reflect a similar DNA binding mode.

Helix extension

The change in contour length (from L_o to L) has been related to the change in intrinsic viscosities (from $[\eta_o]$ to $[\eta]$) of the free and complexed DNA [24]. The data are directly transformed from flow times to values for the relative contour length using the expression $L/L_o = [t_c - t_o/t_D - t_o]^{1/3}$ where L is the contour length in the presence of drug, L_o is the contour length of free DNA, t_c is the flow time for the complex, t_D is the flow time for pure DNA, and t_o is the flow time for buffer at a given total volume.

Contrary to BLM-A_2 and AMBI-A_2, which decrease the DNA contour length [10, 13], AMBIGLU induces a linear increase in DNA length (0.82 Å).

DNA cleavage

The extent of mono- and double-strand degradation of pBR 322, induced by BLM-A_2, AMBI-A_2 and AMBIGLU was visualized by agarose gel electrophoresis. As illustrated in Fig. 4**a**, incubation with BLM-A_2 produces much more double-strand degradation of the plasmid than AMBIGLU does.

The DNA break production (forms II and III) was analysed by densitometry of the gel electrophoretic pattern shown in Fig. 4**a** and of 5 other experiments (Fig. 4**b**). The densitometric scanning result shows that with 0.1 μM BLM-Fe(II), $\approx$ 85% of the covalently closed supercoiled (form I) pBR 322 DNA was converted to form II. Then, with increasing concentration of BLM-Fe(II) 1–5 μM, linear double strand DNA (form III) became the major form, leading to a complete degradation of the DNA with 5 μM (Fig. 4**a**, lane **C**). In contrast, both synthetic models AMBI-A_2 and AMBIGLU caused DNA degradation basically by single-strand breakage (Fig. 4**a**, lanes **E**–**F**). As shown in Fig. 4**b**, gradual shift appeared in the production of form II to reach a maximum near 2 μM. The higher DNA cleaving capacity of AMBIGLU compared to AMBI-A_2 was clearly demonstrated when we compared the production of form III. At 10 μM, only 10% form III DNA was obtained with AMBI-A_2-Fe(II), in contrast to AMBIGLU-Fe(II) for which 10 μM produced $\approx$ 45% form III DNA.

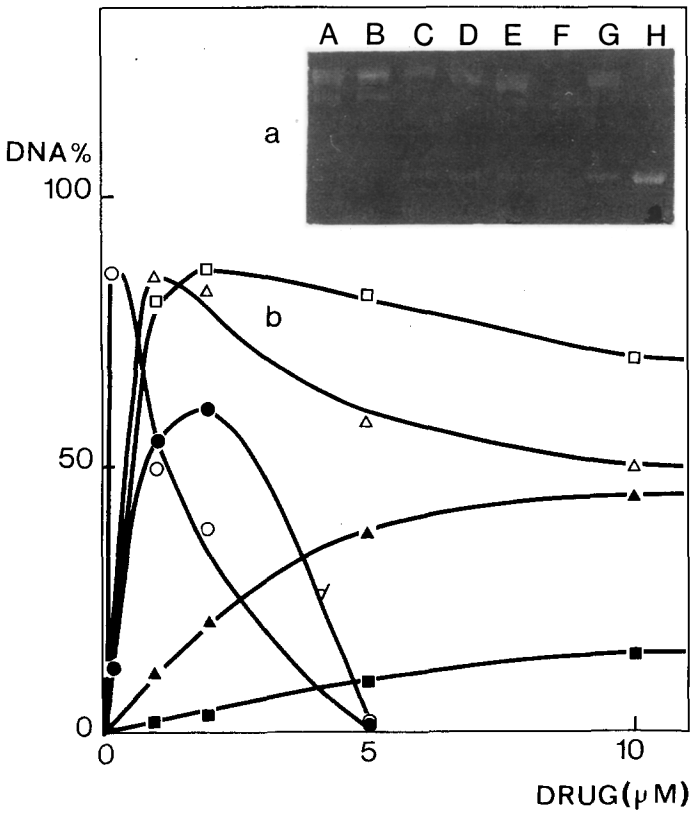


Fig. 4a. Agarose (1%) gel electrophoretic patterns of ethidium bromide-stained pBR322 DNA after treatment with AMBI-A_2-Fe(II) complex (lanes D–E), AMBIGLU-Fe(II) complex (lanes F–H) and control (lanes A–C). DNA migrated from top to bottom in order of decreasing distance of form I, form III and form II DNAs Lane A untreated DNA. Lane B, C: 0.1, 5 μM BLM-Fe(II). Lane D, E: 10, 1 μM AMBI-A_2-Fe(II). Lane F, G, H: 1,5, 10-μM AMBIGLU-Fe(II). **b.** Percentage distribution of DNA pBR322 conformational isomers after treatment with increasing concentrations of BlM (•, ○) AMBI-A_2 (■, □) and AMBIGLU (▲, △). Open and filled symbols represent respectively the forms II and III DNAs. Control experiments with Fe(II) only have been made and are not reported here.

Discussion

On the basis of ESR data, the synthetic model AMBIGLU was found to chelate cupric ions under the same conditions as bleomycin. The ESR parameters similar to those exhibited by the spectra of AMPHIS-Cu(II), AMBI-A_2-Cu(II) and BLM-Cu(II) complexes are in favour of a square–pyramidal coordination geometry (Fig. 5).

The copper ligands are undoubtedly the N^{π} and N^{α} of the histidyl residue, the heterocyclic N atom of the pyridine ring, the N atom of the secondary amine, these 4 atoms forming a square plane, and the primary amine N of the side chain as an apical ligand.

The major structural difference between AMBIGLU and AMBI-A_2 is the presence of a glucosamine residue in the structure of the former. It was reasonable to expect slight differences in the ESR parameters between AMBIGLU-Cu(II) and AMBI-A_2-Cu(II)-complexes spectra as observed between ESR parameters of BLM-A_2 and deglyco-BLM-A_2 [15]. In the case of the parent drug and its aglycon, these differences were analyzed in terms of a Jahn–Teller effect: in the absence of the sugar part, a modification of the Cu(II) complex structure occurs with a displacement of the Cu(II) center out of the plane towards the fifth ligand. Such a modification of the geometry of copper complex was not observed by comparison between AMBIGLU and AMBI-A_2. It can be envisaged that in the parent drug the sugar part gulose–mannose

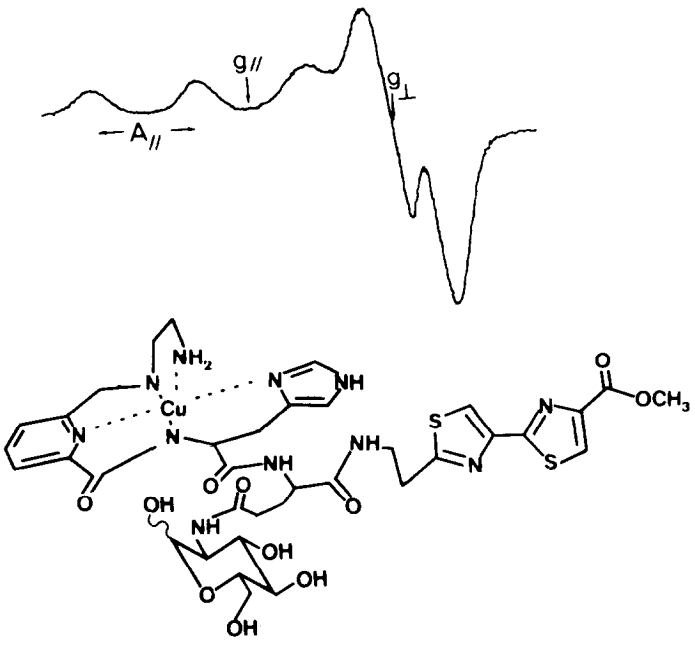


Fig. 5. ESR spectrum and structure of AMBIGLU-Cu(II) complex.

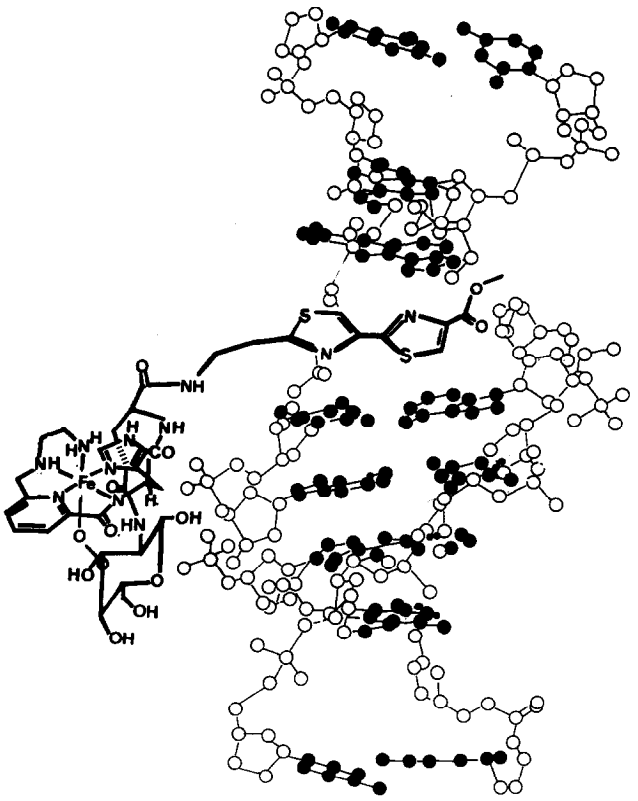


Fig. 6. A proposal for AMBIGLU−DNA interaction.

plays a role which is much more important due to the bulky character of the disaccharide.

However, the glucosaminyl group has been demonstrated to be a key element in the stabilization of the ternary complex AMBIGLU−Fe(II)−O_2 and consequently in the activation of the molecular oxygen. The glucosaminyl residue could form a protecting pocket in the surrounding of the oxygen molecule. The presence on the sugar structure of several hydroxyl groups could explain the establishment of hydrogen bondings with oxygen and its activation. The enhanced production of free radicals by AMBIGLU compared to their formation in the presence of AMBI-A_2 is demonstrative on this point.

Moreover, the DNA strands breakage induced by AMBIGLU was found more effective than by AMBI-A_2. This result confirms the role that the glucosamine residue plays in the stabilization of the complex and in the activation of molecular oxygen.

Concerning the mode of binding of AMBIGLU, a partial intercalation of the bithiazole moiety between the base pairs of DNA can be advanced on the basis of viscometry data (Fig. 6).

These results demonstrate the importance of the terminal amine in the BLM−DNA binding and confirm previous studies indicating that the interaction of BLM-A_2, and probably of AMBI-A_2, with DNA involved primarily the cationic terminus of the drug. AMBIGLU, which lacks the terminal amine cannot establish ionic bonds with the phosphate backbone of the nucleic acid.

Thus, AMBIGLU which possesses in its structure the main parts necessary to a BLM-like activity, *i.e.* a pseudopeptide chelating part and a heterocyclic DNA-binding part, appears to be an acceptable model for the design of further medicinal drugs. This study confirms previous results [13] leading to the conclusion that to have a DNA-cleavage activity comparable to that of BLM, both binding and complexing parts are required on the same molecule. In addition, the presence of a glucosamine residue in AMBIGLU enhances its DNA breakage ability relative to other models which do not contain an osidic part. Therefore the role of the sugar part in the activation of oxygen and production of free radicals can be proposed confirming the hypotheses previously advanced by the comparative study of BLM and deglycoBLM [15].

Experimental protocols

Synthesis

General

The IR spectra were obtained on a Perkin−Elmer 177 spectrophotometer, using KBr pellets. ^{1}H NMR spectra were recorded on a Bruker WP 80 SY or on a Bruker AM 400 WB spectrophotometers. Chemical shifts are reported in ppm from tetramethylsilane as an internal standard and are given in δ units. EI mass spectra were recorded on a Ribermag R10.10 (combined with Riber 400 data system) mass spectrophotometer at 70 eV by using direct insertion. FAB mass spectra were determined on a Kratos MS-50 RF mass spectrometer arranged in an EBE geometry. The sample was bombarded using a beam of xenon with a kinetic energy of 7 keV. The mass spectrometer was operated at 8 kV accelerating voltage with a mass resolution of 3000. Thin layer chromatography (TLC) was carried out using silica gel 60F-254 Merck (0.25-mm thick) precoated UV-sensitive plates, generally in solvent system A ($CHCl_3$-MeOH, 80:20 (v/v) in a saturated NH_3 atmosphere). Spots were visualized by

inspection under UV light at 254 nm and after exposure to vaporized I_2 and/or ninhydrin. Kieselgel 60 (230–400 mesh Merck) was used for chromatography.

2-Deoxy-2[[(1,1-dimethylethoxy)carbonyl]amino]-D-glucopyranose **2**
A solution of glucosamine hydrochloride **1** (6 g, 27.8 mmol, Aldrich) in 1,4 dioxane-H_2O (2/1, v:v, 200 ml), adjusted to pH 9 with dilute NaOH, was treated with di-*tert*-butyldicarbonate (5.73 g, 27.8 mmol) for 4 h. After removal of dioxane and acidification to pH 3 with dilute HCl, the N-protected sugar **2** precipitated as a white powder with a 87% yield. mp: 186°C, litt. 191°C (16); R_f(pyridine/ethyl acetate/acetic acid/H_2O, 5/5/1/3, v:v:v:v):0.82; IR ν 1690 (BOC) cm^{-1}; ^1H NMR 80 MHz(Me_2SO-d_6) δ 1.45 (s, 9H, $(CH_3)_3$), 3.15–3.45 (m, H_2, H_4, H_5, H_6), 4.30 (m, H_3), 4.95 (d, 1H, H_1), 6.0 (m, 1H, NH); $[\alpha]_D^{18}$: +20.3° (c, 0.1, CH_3OH), litt. $[\alpha]_D^{20}$: +65.5 (C_1, CH_3OH) [16]; MS–*m/e* 279 (M^+); Anal. calcd. for $C_{14}H_{21}NO_7$: C, 47.3; H, 7.5; N, 5.0; found: C, 46.5; H, 7.5; N, 4.5.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2[[(1,1-dimethylethoxy)carbonyl]amino]-D-glucopyranose **3**
50 ml of acetic anhydride were slowly added to a solution of **2** (6.8 g, 24 mmol) in dry pyridine (50 ml). After stirring for 18 h at room temperature, the solution was evaporated *in vacuo*, the slurry residue was then dissolved in ethyl acetate and the starting material was extracted with water. The organic layer was dried, and evaporated. Compound **3** was obtained as a white powder with a 97% yield. mp: 59°C; R_f (A): 0.87; IR ν 2990 (NH), 1760 (CO), 1700 (O–CO), 1240 (acetyl CH_3) cm^{-1}; ^1H NMR 80 MHz(Me_2SO-d_6) δ 1.36 (s, 9H, $(CH_3)_3$), 1.93, 1.98, 2.02, 2.14 (4s, 12H, 4CO-CH_3), 3.95 (m, 2H, H_2-H_5, 4.05 (m, 2H, H_6), 5.05 (m, 2H, H_3, H_4), 5.98 (d, 1H, H_1, *J*: 5.45 Hz), 6.95 (d, 1H, NH, *J*: 9.1 Hz); $[\alpha]_D^{17.5}$: +18.1° (c,0.1, CH_3OH); MS–*m/e* 447 (M^+); Anal. calcd. for $C_{19}H_{29}NO_{11}$: C, 51.0; H, 6.5; N, 3.1; found: C, 51.0; H, 6.6; N, 2.9.

2-Amino-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-glucopyranose hydrochloride **4**
A solution of **3** in 1,4 dioxane was flushed with dry HCl. After 30 min stirring, removal of the solvent left the crude amine **4**. This compound was recrystallized from ethanol to give a white powder; mp: 170°C; 86% yield; R_f (A): 0.66; IRν2800–3000 (broad, NH_3^+), 1740–1770 (CO), 1240 (acetyl CH_3) cm^{-1}; ^1H NMR 80 MHz(Me_2SO-d_6) δ 1.94, 1.97, 2.04, 2.19 (4s, 12H, 4CO–CH_3), 3.77 (m, 2H, H_2-H_5), 4.08 (m, 2H, H_6), 5.19 (m, 2H, H_3, H_4), 6.29 (d, 1H, H_1, *J*: 5.45 Hz), 8.94 (m, 3H, NH_3^+); $[\alpha]_D^{17.5}$: +10.1° (c,0.1, CH_3OH), litt. $[\alpha]_D^{21}$: 29.7° (water) [17]; MS–*m/e* 347 (M^+); Anal. calcd. for $C_{14}H_{22}NO_9Cl$: C, 43.8; H, 5.7; N, 3.6; found: C, 44.1; H, 5.7; N, 3.5.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2[(N-tert-butyloxycarbonyl)glutamamido α-benzylester]-D-glucopyranose **6**
A solution of α benzyl *tert*-butyloxycarbonylglutamate **5** (2.72 g, 8.06 mmol, Serva) in anhydrous CH_2Cl_2 (100 ml) was cooled to 0°C and dicyclohexylcarbodiimide (1.82 g, 8.87 mmol) and 1*H*-hydroxy-1, 2, 3-benzotriazole hemihydrate (1.35 g, 8.87 mmol) in 10 ml of CH_2Cl_2 were added. After 1 h 30, a cooled (0°C) solution of **4** (3.09 g, 8.06 mmol) in 30 ml of CH_2Cl_2 containing triethylamine (1.12 ml, 8.06 mmol) was added. The mixture was stirred at 0°C for 2 h and allowed to rise to room temperature; stirring was continued for 10 h. The precipitated dicyclohexylurea was collected and the CH_2Cl_2 solution was washed successively with 30 ml of 1 N HCl, H_2O and 1 M $NaHCO_3$. After drying over Na_2SO_4, the solvent was removed *in vacuo*. The remaining dicyclohexylurea was discarded by precipitation with acetone. The residue obtained after evaporation of the solvent was thoroughly triturated with diethylether. The yield of crude, chromatographically pure product obtained as a white powder was 3.65 g (68% yield). mp: 184°C; R_f (A): 0.91; IRν2980 (NH), 1740 (CO), 1690 (OCO), 1670 (CONH), 1220 (acetyl CH_3) cm^{-1}; ^1H NMR 400 MHz(Me_2SO-d_6) δ 1.38 (s, 9H, $(CH_3)_3$), 1.91, 1.97, 2.01, 2.15 (4s, 12H, 4CO-CH_3), 3.95 (m, 2H, H_5-H_2), 4.16 (m, 2H, H_6), 4.25 (m, 1H, αCH), 4.98 (m, 1H, H_3), 5.12 (m, 2H, CH_2Bzl), 5.15 (m, 1H, H_4), 5.93 (d, 1H, H_1), 7.25 (1H, NH–CO), 7.34 (s, 5H, aromatic), 7.98 (m, 1H, NHCO); $[\alpha]_D^{17.8}$: +4.4° (c,0.1, $CHCl_3$); Anal. calcd. for $C_{31}H_{42}N_2O_{14}$: C, 55.8; H, 6.3; N, 4.2; found: C, 55.0; H, 6.5; H, 4.5.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2(glutamamido α-benzyl ester)-D-glucopyranose **7**
The *t*-BOC protected amine **6** (1 g, 1.5 mmol) was deprotected with pure TFA (10 ml) to give the corresponding free amine **7** in a good yield (>85%). After 1 h stirring, the excess of TFA was evaporated and the residue was diluted with absolute ethanol (30 ml) before evaporation of the solvent. This procedure was repeated 3 times and resulted in the complete elimination of TFA. After thorough drying which is necessary to eliminate the retained ethanol, the resulting **7** (hygroscopic powder, 0.95 g, 92%) is quite suitable for the next reaction. mp: 53°C; R_f(A): 0.82; IR ν 3200–3600 (broad, NH), 1750 (CO), 1660 (CONH), 1220 (CH_3) cm^{-1}; FAB-MS: 567 (M^++1); Anal. calcd. for $C_{28}H_{35}N_2O_{14}F_3$: C, 49.4; H, 5.2; N, 4.1; found: C, 48.2; H, 5.4; H, 4.1.

2-[N-[2-(Tert-butyloxycarbonylamino)-ethyl]-N-(benzyloxycarbonyl)-aminomethyl]-pyridine-6-carboxyl-N-π-(benzyloxycarbonyl)-histidyl-γ-[(tetra-O-acetyl)-glucosaminyl]-glutamic acid α-benzyl ester **9**
To 0.44 g (0.55 mmol) of **8** (*N*-BOC, *N'*-Z, *N''*-AMPHIS; see ref. [11]) dissolved in 10 ml of dimethylformamide at −15°C were added 73 ml of isobutylchloroformate (0.55 mM) and 61 μl of *N*-methylmorphine (0.55 mM). The solution was vigorously stirred for 30 min at −15°C before adding 374 mg (0.55 mM) of **7** and 61 μl of *N*-methylmorpholine (0.55 mM) in 10 ml of dimethylformamide. The solution was stirred for 15 min at −15°C and overnight at room temperature. Solvent was then evaporated *in vacuo* (< 50°C). The residue was dissolved in CH_2Cl_2 (100 ml) and the solution washed successively with 1 N HCl, H_2O, 1 M $NaHCO_3$ and satured aqueous NaCl and dried over Na_2SO_4. The solvent was then evaporated to dryness and the desired product obtained as a grey powder. 67% yield, R_f(A): 0.95; IR ν2980 (NH), 1760 (CO), 1680 (OCO), 1220–1250 (CH_3 acetyl)cm^{-1}; FAB–MS: 1108 (M^+-Z).

2-[N-[2-(tert-butyloxycarbonylamino)-ethyl]-N-(benzyloxycarbonyl)-aminomethyl]-pyridine-6-carboxyl-N-π-(benzyloxycarbonyl)-histidyl-γ-[(tetra-O-acetyl)-glucosaminyl]-glutamic acid **10**
The benzyl ester **9** (0.2 g, 0.16 mmol) was saponified in the presence of aqueous sodium hydroxide in methanol for 48 h at room temperature. After acidification, evaporation of the solvent, trituration in hot absolute ethanol, elimination of sodium chloride by filtration and evaporation of the solvent, the resulting residue was identified as the deacetylated acid **10**: 53% yield, R_f (A): 0; ^1H NMR (Me_2SO-d_6) δ 1.4 (CH_3), 2.9–4.1 (m, CH_2), 4.25–5.3 (m, αCH, CH-carbohydrate), 6.5 (m CH_2-histidyl), 7.2 (m, NH), 7.35 (s, CH-benzyl), 7.5–8.4 (m, CH-pyridyl, CH-imidazole, NH); FAB-MS: 991 (M^++1, FAB^+), 989 (M^+−1, FAB^-).

Methyl 2'-[2-[N-[2-(tert-butyloxycarbonylamino)-ethyl-N-(benzyloxycarbonyl)-aminoethyl]-pyridine-6-carboxyl-N-π-(benzyloxycarbonyl)-histidyl-(glucosaminyl)-glutamyl-(2-aminoethyl)]-2,4'-bithiazole-4-carboxylate **12**
The acid **10** (50 mg, 0.05 mmol) was coupled to methyl 2'-aminoethyl-2,4' bithiazole-4-carboxylate **11** (18 mg, 0.05 mmol, see ref. [9]) using DCC and HOBt as described for **6**. The protected compound **12** was purified by flash-chromatography in the system solvent CH_2Cl_2/methanol, 95:5 (v/v). White powder, 46% yield, R_f(A): 0.71; FAB-MS: 1242 (M^++1).

Methyl [2'-[2-(2-aminoethyl)-aminomethyl]-pyridine-6-carboxyl-hystidyl-γ-(glucosaminyl)-glutamyl-2-(aminoethyl))]2,4'-bithiazole-4-carboxylate trihydrobromide (AMBIGLU)
A solution of **12** (30 mg, 0.024 mmol) in acetic acid (5 ml) saturated by bromhydric acid was stirred for 15 min and evaporated to dryness. The crude residue is dissolved several times in ethanol and evaporated to assume complete elimination of the HBr. The final product was then dissolved in water, extracted twice with ethyl acetate and CH_2Cl_2. Final lyophilisation of the aqueous layer afforded AMBIGLU as a white powder. 73% yield, R_f(A): 0; ^1H NMR (Me_2SO-d_6) δ 2.85–4.1 (m, CH_2), 4.25–5.5 (m, αCH, CH-carbohydrate), 6.5 (m, CH_2-histidyl), 7.2–8.8 (m, CH-pyridyl, CH-imidazole, NH); FAB-MS: 908 (M^++1).

ESR measurements
ESR measurements were recorded on a Varian E-109 X-band spectrometer with a dual cavity operating in the TE 104 mode. A 100 kHz high frequency modulation with a maximum amplitude of 8 gauss was used with a 10 mW microwave power and g values were determined from α,α'-diphenyl-β-picrylhydrazyl (g = 2.0036). For the spin-trapping experiments, we used a cavity operating in the TM 110 mode with a maximum modulation amplitude of one gauss.

Cu(II) complexes
Bleomycin-A_2-Cu(II) and AMBIGLU-Cu(II) complexes were prepared by adding cupric perchlorate 10^{-3} M to a pH 6.9 phosphate buffer containing the drugs in a 1:1 ratio, or by adding NaOH to aqueous solutions of the drugs and the cupric ion. The samples were disposed into a 3 millimeters diameter cylindrical quartz tube. ESR analyses were conducted at 77K on glycerol glasses.

Spin-trapping technique
The technique of spin-trapping makes use of diamagnetic spin traps which react with free radicals giving rise to relatively more stable ESR-observable free radicals. Phenyl *N-t*-butyl-nitrone (PBN) [18] was used to detect the production of OH· radicals. The reaction mixture for spin-trapping experiments consisted of 1:1 bleomycin-A_2-Fe(II), or 1:1 AMBIGLU-Fe(II) complexes (10 mM in aqueous solution) and PBN (80 mM ethanolic solution) in buffered solution at pH 6.9. Oxygen was bubbled through the mixture; an aliquot for the sample solution was rapidly transferred to the quartz flat cell and the spectrum recorded.

Control experiments were made to be sure that ESR spectra neither resulted from nitrone spin trap alone, nor from the separate addition of Fe(II) or drugs.

Viscometry
Unwinding studies using closed-circular DNA (plasmid pBR322 containing fragments of adenovirus) were performed essentially as previously described [19, 20] using a Ubbelohde semi micro dilution viscometer. Temperature was maintained at 25 ± 0.01°C in a thermostatically controlled water bath. Flow times were electronically measur-ed to an accuracy of 0.1 s (Schott ABS / G type detector). The viscometer contained 2.0 ml of a 150 μM solution of DNA. AMBIGLU was added in increments of 5–10 μl from a stock solution (c = 150 μM). Ethidium bromide was used as reference, inducing an unwinding angle of 26°C [21].

For the helical lengthening measurements, calf thymus DNA was reduced to rod-like species with a French press and experiments were carried out in 0.01 SHE buffer (9.4 mM NaCl / 2 mM HEPES / 10 mM EDTA buffer, pH 7.0). Solutions were filtered through 0.45 μM Millipore filters before measurements. The viscometer contained 2.0 ml of a 500 μM solution of DNA. AMBIGLU was added in increments of 5–10 μl from a stock solution (c: 3 mM).

DNA degradation
Single-strand and / or double-strand DNA breaks were visualized by the use of supercoiled DNA (form I). The appearence of relaxed circular DNA (form II) and open linear DNA (form III), with either BLM-A_2, AMBI-A_2 or AMBIGLU was observed on a 1% agarose gel, containing 0.5 μg / ml of ethidium bromide.

Plasmid pBR 322 was incubated in 50 mM Tris–HCl, pH 8.0 buffer containing 10 mM 2-mercaptoethanol. $Fe(NH_4)_2(SO_4)_2$, $6H_2O$ was added in the same final concentration as the product. After 20 min at room temperature, the reaction was terminated with the addition of EDTA 2.5 mM; 5 μl of 0.01% bromophenol blue were added to the reaction mixture (50 μl). Agarose electrophoresis was performed in TBE buffer at 5V / cm for 6 h and examined under a 254 nm UV light. The negative films of gels were used for densitometric scannings.

Acknowledgments

The authors are indebted to the Fédération des Centres de Lutte contre le Cancer and the Institut National de la Santé et de la Recherche Médicale for financial support.

References

1 Crooke S.T. (1978) *in: Bleomycin: Current Status and New Developments* (Carter S.K., Crooke S.T. & Umezawa H., eds.), Academic Press, New York, pp. 1–8
2 Carter S.K. (1978) *in: Bleomycin: Current Status and New Developments* (Carter S.K., Crooke S.T. & Umezawa H., eds.), Academic Press, New York, pp. 9–14
3 Umezawa H. (1979) *in: Bleomycin: Chemical, Biochemical and Biological Aspects* (Hecht S.M., ed.), Springer-Verlag, New York, pp. 24–36
4 Sugiura Y., Muraoka Y., Fujii A., Takita T. & Umezawa H. (1979) *J. Antibiot.* 32, 756–758
5 Oberley L.W. & Buettner G.R. (1979) *FEBS Lett.* 97, 47–49
6 Burger R.M., Peisach J. & Horwitz S.B. (1981) *J. Biol. Chem.* 256, 11636–11644
7 Chien M., Grollman A.P. & Horwitz S.B. (1977) *Biochemistry* 16, 3641–3647
8 Houssin R., Bernier J.L. & Hénichart J.P. (1984) *J. Heterocyclic Chem.* 21, 465–469
9 Houssin R., Bernier J.L. & Hénichart J.P. (1984) *J. Heterocyclic Chem.* 21, 681–683
10 Hénichart J.P., Bernier J.L., Helbecque N. & Houssin R. (1985) *Nucl. Acids Res.* 13, 6703–6717
11 Hénichart J.P., Houssin R., Bernier J.L. & Catteau J.P. (1982) *J. Chem. Soc. Chem. Commun*,. 1295–1297
12 Hénichart J.P., Bernier J.L., Houssin R., Lohez M., Kenani A. & Catteau J.P. (1985) *Biochem. Biophys. Res. Commun.* 126, 1036–1041
13 Kenani A., Lohez M., Houssin R., Helbecque N., Bernier J.L., Lemay P. & Hénichart J.P. (1987) *Anti-Cancer Drug Design* 2, 47–59
14 Kenani A., Lamblin G. & Hénichart J.P. (1988) *Carbohydr. Res.* 177, 81–89
15 Kenani A., Bailly C., Helbecque N., Catteau J.P., Houssin R., Bernier J.L. & Hénichart J.P. (1988) *Biochem. J.* 253, 497–504
16 Pozbnev V.F. (1980) *Khim. Prir. Soedin.* 3, 408–409
17 Bergmann F. & Zervas L. (1931) *Chem. Ber.* 64B, 975–980
18 Janzen E.G. & Blackburn B.J. (1968) *J. Am. Chem. Soc.* 90, 5909–5910
19 Saucier J.M., Festy B. & Le Pecq J.B. (1971) *Biochimie* 53, 973–980
20 Revet B.M.J., Schmir M. & Vinograd J. (1971) *Nature New Biol.* 229, 10–13
21 Wang J.C. (1974) *J. Mol. Biol.* 89, 783–801
22 Harbour J.R., Chow V. & Bolton J.R. (1974) *Can. J. Chem.* 52, 3549–3561
23 Sugiura Y. & Kikuchi T. (1978) *J. Antibiotics* 31, 1310–1315
24 Cohen G. & Eisenberg H. (1966) *Biopolymers* 4, 429–440