Pergamon

Bioorganic & Medicinal Chemistry 8 (2000) 1065–1073

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis, Hydroxyl Radical Production and Cytotoxicity of Analogues of Bleomycin

Jackie A. Highfield,[a] Lina K. Mehta,[a] John Parrick[a,*] and Peter Wardman[b]

[a]*Chemistry Section, Institute for Physical and Environmental Sciences, Brunel University, Uxbridge, Middlesex, UB8 3PH, UK*
[b]*Gray Laboratory Cancer Research Trust, Mount Vernon Hospital, Northwood, Middlesex, HA6 2JR, UK*

Received 28 September 1999; accepted 30 December 1999

Abstract—Two pyridine analogues of the metal complexing region of the anticancer drug bleomycin and two related but deactivated prodrugs have been linked to a 2,6-diphenylpyridine derivative as a DNA binding unit. The 2,6-diphenylpyridine system is structurally related to known amplifiers of the cytotoxicity of bleomycin. The conjugates were found to bind to DNA more strongly than bleomycin-A_2 and were more cytotoxic than the corresponding compounds lacking the DNA binding unit. On exposure of a mixture of cells and prodrugs to hypoxia and then air, the prodrug containing the nitrohistidine unit was not bioreductively activated but the prodrug having an *N*-oxide group was bioreductively activated. This result represents a novel approach to the improvement of the therapeutic ratio of bleomycin analogues. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

The targeting of a drug to the site of action is desirable because this reduces indiscriminate toxicity and decreases the chance of metabolism before the drug has had chance to act. Generally, if the site of action is DNA, the drug must pass from the blood stream, penetrate the cellular and nuclear membranes and reach the DNA of the target cell without being metabolised and without causing extensive damage to cellular structures or functions. Targeting of anti-cancer drugs is a particularly difficult problem and one approach under active investigation is to try to utilise the hypoxia present in regions of solid tumours by using bioreductive activation of a prodrug in those regions of oxygen deficiency. We have reported our approach to the development of bioreductively activated prodrugs based on the metal complexing region of bleomycin A_2 (BLM) (Figure 1, the asterisks indicate the nitrogen atoms known to be involved in the complexation with iron).[1,2]

The molecular architecture of the bleomycins contains four regions: the metal complexing unit, a disaccharide, a peptide linker unit and a bithiazole DNA binding unit (Fig. 1).[3]

Bleomycin-A_2 binds preferentially in the minor groove of DNA though the method by which certain base sequences in DNA are recognised is still not clear.[4] Several BLM analogues having C-terminal bithiazole units have been prepared and their action on DNA studied.[5] The observation that DNA degradation is enhanced by the addition of a DNA binding unit on to the metal complexing portion of bleomycin has led to the synthesis of many analogues which differ in the DNA binding unit. Distamycin carriers[6] and lexitropsins[7] have been used as the DNA ligand and for these it is known that two or more *N*-methylpyrrole or thiazole nuclei are required for selective binding with DNA.[7,8] Recently, novel acridine derivatives which cleave DNA in a sequence-neutral manner have been reported.[9] In other work, hybrids containing both the metal-binding unit and a DNA ligand were found necessary for DNA degradation.[3] Variation of the linker between the metal complexing region and the DNA-ligand causes changes in the sequence selectivity and the efficiency of DNA degradation.[3]

One method of enchancing the activity of BLM is to use the drug in combination with a compound that alone has no activity or toxicity but which amplifies the action of BLM.[10] The best amplifiers of BLM are composed of at least two fused or unfused but conjugated aromatic nuclei which carry an existing or potential cationic centre.[11] The mechanism of the amplification is not fully understood but it is believed that the cationic side chain of these amplifiers lies in the major groove and that this causes expansion of the minor groove of DNA allowing easier access by BLM.[12] 2,5-Diarylthiophenes, e.g., **1**, and 2,6-

*Corresponding author. Tel.: (44) 1895 256844; fax: (44) 1895 256844; e-mail: john.parrick@brunel.ac.uk

0968-0896/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(00)00042-0

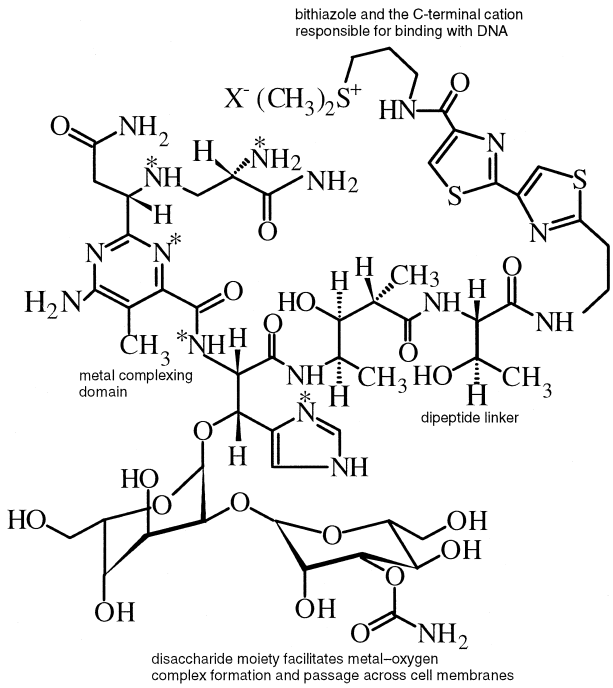


Figure 1. Bleomycin A_2.

diarylpyridines, e.g., **2** (Fig. 2), carrying side-chains which have terminal tertiary amine groups are particularly effective amplifiers for BLM.[11c] The DNA binding constant for **1**[11b] is $13\times10^6\,M^{-1}$ which is greater than that for bleomycin ($10^5\,M^{-1}$).[13] In order that two amino functions in one chain of an aliphatic diamine at pH 7 are protonated the nitrogen atoms must be separated by at least three methylene groups.[11c,14] Thus, these amplifiers have some characteristics which are similar to the bithiazole unit in BLM.

We have described the preparation of analogues (prodrugs) of the metal complexing moiety of bleomycin which do not complex with Fe(II) or Fe(III) because of the presence of deactivating groups (nitro or *N*-oxide groups). It is hoped that these groups would be reduced in the hypoxic cells of solid tumours (bioreductive activation) to form compounds (active drugs) that do bind with iron ions and generate hydroxyl radicals in the presence of oxygen. Here we report the preparation of conjugates where these prodrugs and the corresponding chemically reduced compounds are linked to an analogue of known amplifiers of bleomycin activity. We reasoned that the amplifier would bind with DNA and open the minor groove so allowing the reduced prodrug (formed selectively in hypoxic cells) to bind iron ions and exhibit its cytotoxic action.

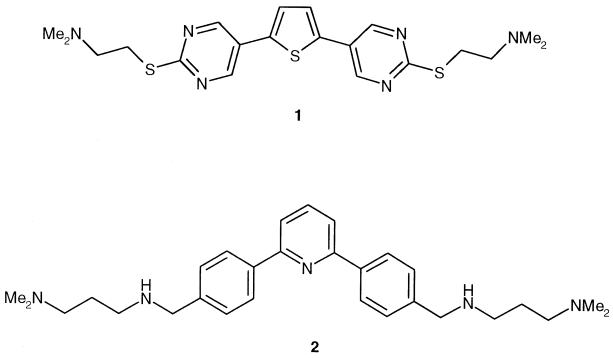


Figure 2. Examples of amplifiers of the cytotoxicity of bleomycin.

Results and Discussion

Chemistry

The synthesis of **3–6** (Fig. 3) was accomplished in a 3-step procedure from the esters **9–11**,[2] and **12**. The first step in the preparation of **12** was the reaction of nitrohistidine methyl ester hydrochloride, obtained by the esterification of nitrohistidine,[15] with the acid (**7**)[2] (Fig. 4) in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBT) as activating agents to give the ester (**12**) in 36% yield. The hydrolysis of the esters (**9–12**) with lithium hydroxide was carried out at 0 °C (Scheme 1). The reaction mixture was neutralised with dilute HCl to avoid removal of the protecting groups as the *tert*-butoxycarbonyl group is labile to strong acids. The acid was isolated by freeze-drying the aqueous solution but was contaminated with a small quantity of lithium chloride. The acids (**13–16**) were used in the next step without purification. Available to us was the novel amplifier (**8**)[16] (Fig. 4) having two different chains: one terminating in a potentially cationic tertiary amine group and the other ending in the primary amine necessary for the envisaged amide linkage to be formed by reaction of the acids (**13–16**) with *N*-(2-aminoethyl)-4-(6-[4-{2-(dimethylaminoethyl)-thiomethyl}phenyl]pyridin-2-yl)benzamide (**8**). Attempted coupling of the acid (**13**) with **8** in presence of diphenylphosphoryl azide[17] to give **17** was unsuccessful because of the difficulty experienced in attempts to isolate the pure product from a complex mixture. However, separate condensation of the acids (**13–16**) with *N*-(2-aminoethyl)-4-(6-[4-{2-(dimethylaminoethyl)thiomethyl}phenyl]-pyridin-2-yl)benzamide (**8**) using EDCI and HOBT as

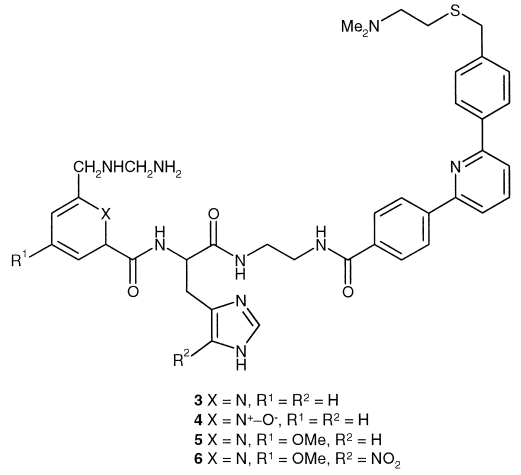


Figure 3. Bleomycin analogues.

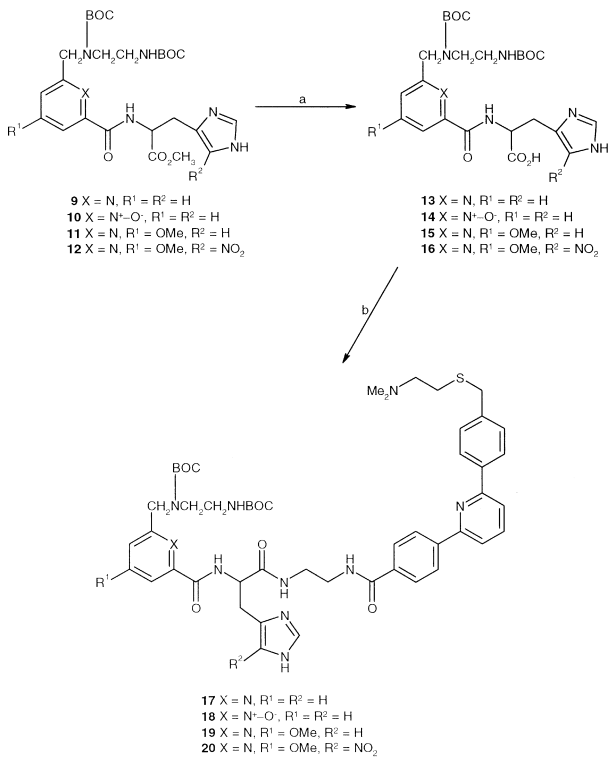
activating agents gave the coupled compounds **17**–**20**, respectively, in 30–36% yield after repeated slow flash chromatography (Scheme 1). The *tert*-butoxycarbonyl groups were removed from **17**–**20** by treatment with trifluoroacetic acid at 0 °C. The corresponding free amines **3**–**6**, respectively, were isolated in high yields by passing an aqueous solution of the peptide trifluoroacetates through an ion exchange resin.

Physico-chemical measurements

The formation of a metal complex and subsequent activation of dioxygen is the means by which bleomycin is believed to exert its cytotoxic effect.[18] The production of hydroxyl radicals with time was measured by observation of the fluorescence produced upon hydroxylation of benzoate to give salicylate. The other isomeric hydroxybenzoates are not fluorescent. The assay was calibrated by ^{60}Co irradiation of an aqueous benzoate solution saturated with nitrous oxide. Under these conditions the formation of salicylate is proportional to the radiation dose.[19]

Iron(II) reacts with hydrogen peroxide to produce hydroxyl radicals (Fenton reaction). The addition of the BLM analogues **3**–**6** to this mixture caused an increase in the hydroxylation of benzoate and **3** and **5** produced a much greater effect than bleomycin (Fig. 5A). An increase in hydroxyl radical formation is presumably due to the chelator facilitating redox cycling of iron(II) and iron(III).

In the presence of iron(III) and hydrogen peroxide, the analogues **3** and **5** and BLM caused some hydroxylation of benzoate but less than that produced by iron(III) and hydrogen peroxide alone (Fig. 5B). The other analogues **4** and **6**, produced a small amount of hydroxyl radicals. As expected, no hydroxylation was produced by iron(III)



Scheme 1. Reagents and conditions: (a) LiOH, 0 °C, 2 h; (b) **8**, EDCI, HOBT, DMF, Et_3N, 24 h.

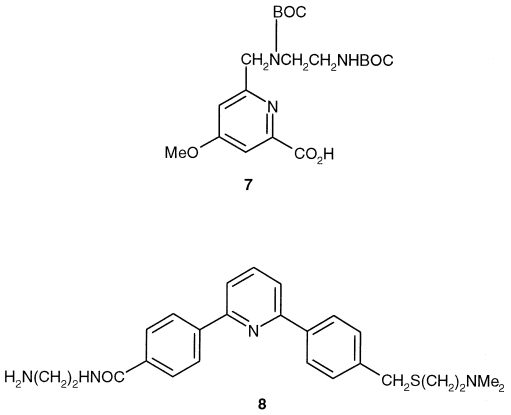


Figure 4. Intermediates in the synthesis of bleomycin analogues.

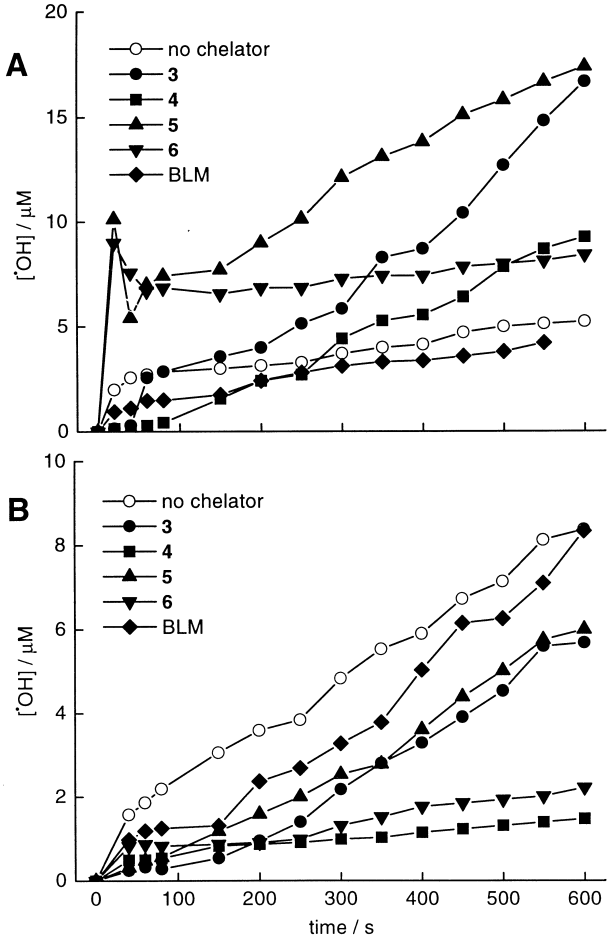


Figure 5. The effect on hydroxyl radical production upon the addition of bleomycin and its analogues to a mixture of hydrogen peroxide and iron(II) **A** and iron(III) **B**.

and the analogues in the absence of hydrogen peroxide because of the lack of a reducing agent.

Hydroxyl radicals are produced when air is passed through a solution of iron(II) and **3** or **5** or BLM. Analogue **5** was more effective than bleomycin (Fig. 6A). No significant hydroxyl radical production was observed with **4** and **6**. In an atmosphere of oxygen BLM was the most effective (Fig. 6B). The *N*-oxide derivative **4** and the nitro compound **6** showed insignificantly small activity as expected.

Surprisingly, the hydroxyl radical production by **3** and **5** was greater than that found for the corresponding metal binding units lacking the DNA binding moiety, AMPHIS (**21**) and **22** (Fig. 7). A similar increased hydroxyl radical production has been reported for radical production when AMPHIS (**21**) metal binding ligand was attached to a bithiazole binding unit.[20]

Caution is prudent in interpreting the results of scavenging oxidizing intermediates with either fluorescent detection of hydroxylated products (Figs 5 and 6) or with spin traps. The formation of salicylate from benzoate was calibrated using 'free' hydroxyl produced radiolytically, and we have therefore labelled Figures 5 and 6 with the equivalent hydroxyl radical concentration. However, many studies point to the participation of

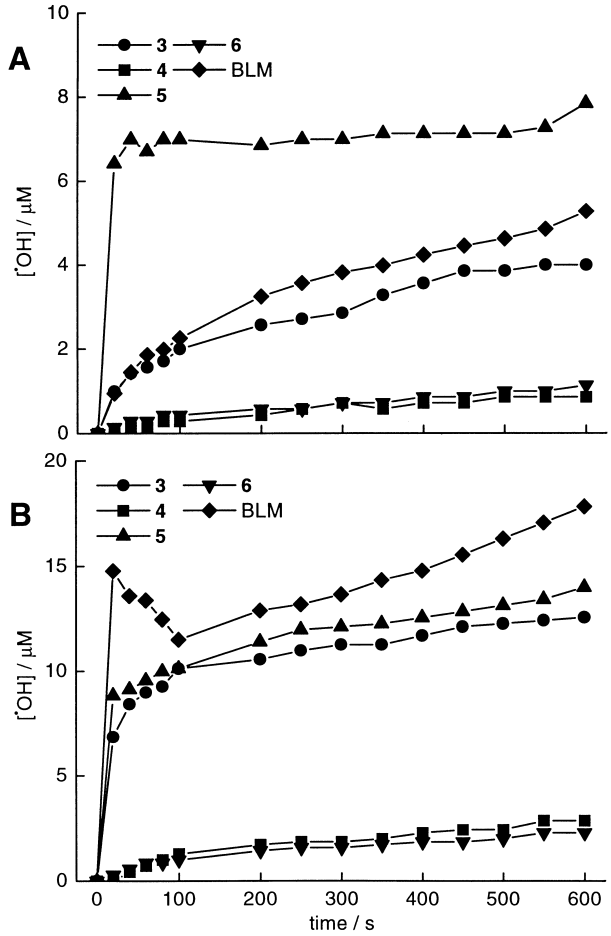
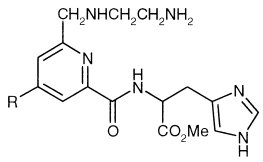


Figure 6. Hydroxyl radical production by bleomycin and its analogues in the presence of iron and air **A** and oxygen **B**.



21 R = H
22 R = OMe

Figure 7. AMPHIS and its methoxy analogue.

iron-oxo intermediates in bleomycin system.[21] Other studies argue for free hydroxyl production, e.g., based on detecting a hydroxylated coumarin.[22] Other factors, in addition to the production of oxidizing intermediates, such as the drug–DNA interaction and the protective effects of histone proteins, must be reflected in the biological efficacy of these compounds. Close parallels between the hydroxylation efficiencies (whether or not reflecting 'free' hydroxyl radicals) and cytotoxicities are not expected.

DNA binding and biological assay

The decrease in ethidium fluorescence with increasing drug concentration was used to measure the binding constant.[23] When a drug which binds to DNA is introduced into a solution of DNA saturated with ethidium, some of the ethidium is displaced into the solution. The amount of ethidium displaced for a given quantity of drug is related to the binding constant of the drug. Ethidium in aqueous solution has little fluorescence compared to ethidium complexed with the hydrophobic region of DNA. The bleomycin analogues **3**–**6** were found to bind to DNA by a factor of ten more strongly than bleomycin itself (Table 1).

The cytotoxicity of the compounds against Chinese hamster V79 cells was measured after mammalian cells (V79 hamster fibroblast like) were exposed to a solution of the BLM analogues in an atmosphere of either air for 1 h or nitrogen (30 min) and then air (30 min). As expected, in air alone, only the analogues **3** and **5** produced any cell kill, but at a higher concentration of these compounds than was required for bleomycin (Fig. 8). The compound **5** carrying a 4-methoxy group on the pyridine ring of the metal-binding unit was somewhat more cytotoxic than the AMPHIS derivative **3**. In addition, compounds **3** and **5** were approximately one order of magnitude more cytotoxic than the corresponding compounds **21** and **22** without the DNA-binding moiety.[2] The prodrugs **4** or **6** and cells were treated differently. They were exposed to hypoxia for

Table 1. Binding constants of bleomycin analogues to DNA

BLM analogue	DNA binding constant at pH 7.5/M^{-1}
BLM-A_2	1.2×10^{5},[a]
3	7.5×10^{6}
4	3.0×10^{6}
5	8.2×10^{6}
6	6.2×10^{6}

[a]At pH 8.4.

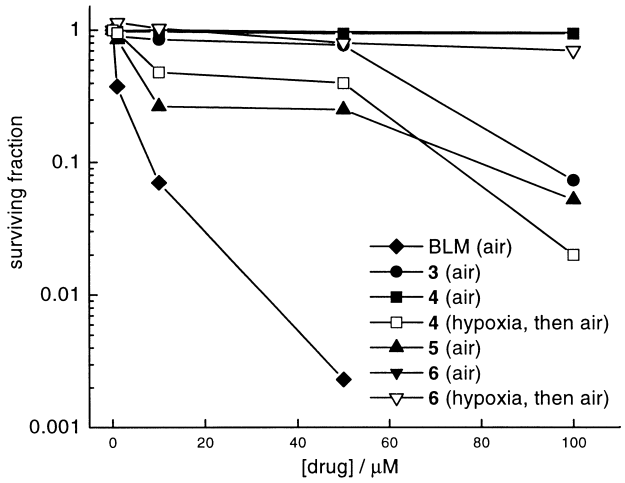


Figure 8. Cytotoxicity to Chinese hamster V79 cells. Solid symbols refer to exposure to air (1 h) and open symbols refer to incubation in hypoxia (30 min) followed by exposure in air (30 min). The data set for compound **6** in air is indistinguishable from that for **4** in air.

30 min and then allowed to stand in air for 30 min. The cytotoxicity of **6** showed little change compared to that found in air alone. However, the *N*-oxide prodrug **4** showed a marked increase in cytotoxicity due to it being bioreductively activated in hypoxia.

Two important points concerning the mechanism of activation merit mention. Firstly, reductive activation in hypoxia followed by reoxygenation of the same region in tumours is possible.[24] Thus a diffusible cytotoxin is not essential. However, agents which bind strongly to DNA are unlikely to diffuse far and exhibit a 'bystander effect', i.e., kills cells distant from the site of reduction. A second factor in comparing the toxicities of nitroarenes and the corresponding amines is that while the amines are easiest to synthesize and handle experimentally, intermediate bioreduction products, particularly the hydroxylamine, are likely to be more important, especially with nitroimidazoles.[25] However, the major shift in metal binding properties which accompanies nitroreduction is likely to be broadly similar regardless of whether nitroreduction is to the amine or the hydroxylamine.

The lack of significant toxicity of the nitroarene **6** after hypoxia/reoxygenation could be due to the quite short period of hypoxic incubation and/or the likely rather low reduction potential of this 4(5)-nitroimidazole, which would result in a low extent or rate of nitroreduction. Further studies are needed to assess the potential value of this approach using nitroarenes.

In conclusion, the differential behaviour found for the prodrug **4** represents a novel approach to improving the therapeutic ratio of bleomycin analogues.

Experimental

Melting points (mp) are determined on an Electrothermal digital apparatus and are uncorrected. ^{1}H NMR spectra were obtained on Jeol FX200 (200 MHz) or Bruker AM360 (360 MHz) spectrometer using TMS as internal standard. *J* values are given in Hertz (Hz) for solutions in DMSO-d_6. Accurate mass measurements were carried out at the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea, UK. Fluorescence spectra were measured using a Perkin–Elmer LS50B luminescence spectrometer.

Thin layer chromatography was performed on silica gel plates (0.25 mM with fluorescent indicator UV_{254}) obtained from Camlab, Germany. Column chromatography was carried out using silica gel MPD 60Å (40–60 microns) and developed under slight positive pressure. Petroleum ether refers to light petroleum (bp 40–60 °C) and ether refers to diethyl ether. All solvents were redistilled before use.

Eagle's minimum essential medium, Earle's salt and Hank's saline were obtained from Sigma Chemical Company, Poole, UK.

2-(*N*-[*tert*-Butoxycarbonyl]-*N*-[2-*tert*-butoxycarbonylaminoethyl]aminomethyl)-4-methoxy-*N*-(5-nitro-2-imidazol-4-yl]-1-[methoxycarbonyl]ethyl)pyridine-6-carboxamide (12). A mixture of 2-([*N*-{2-([*tert*-butoxycarbonyl]amino)ethyl} - *N* - (*tert* - butoxycarbonyl)amino]-methyl)-4-methoxypyridine-6-carboxylic acid (**7**) (0.13 g, 0.31 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.1 g, 0.52 mmol) and 1-hydroxybenzotriazole (0.07 g, 0.52 mmol) in anhydrous DMF (5 mL) was stirred under nitrogen at room temperature for 30 min. To this solution was added dropwise triethylamine (1.5 mL) and subsequently the nitrohistidine methyl ester hydrochloride (0.12 g, 0.48 mmol). The solution was stirred overnight, solvent removed in vacuo and the residue dissolved in ethyl acetate. The solution was washed with saturated aqueous sodium bicarbonate, dried (Na_2SO_4) and the solvent evaporated. The residue was purified by silica-gel column chromatography (ethyl acetate:petroleum ether, 8:2) to give **10** (0.09 g, 50%), mp 90–92 °C. IR (KBr) ν 3370 (NH), 1698 (C=O), 1514 (NO_2), 1388 (NO_2) cm^{-1}; ^{1}H NMR (360 MHz, $(CD_3)_2SO$) δ 1.35 (18H, s, 2×COOC $(CH_3)_3$), 3.11 (2H, m, CH_2), 3.35 (2H, m, CH_2), 3.53 (2H, m, CH_2), 3.65 (3H, s, CH_3), 3.87 (3H, s, OCH_3), 4.44 (2H, s, CH_2), 4.87 (1H, m, CH), 6.80 (1H, brs, NH exchanged with D_2O), 6.85 (1H, s, 3-H), 7.38 (1H, s, 5-H), 7.70 (1H, s, 2-H of imidazole), 8.89 (1H, brs, NH exchanged with D_2O), 13.14 (1H, brs, NH exchanged with D_2O); FAB–HRMS calcd for $C_{27}H_{40}N_7O_{10}$ (M + H) 622.2845. Found: 622.2836.

General procedure for the hydrolysis of esters

A solution of the appropriate ester (0.7 mmol) in THF:MeOH:H_2O (3:1:1, 20 mL), was treated with aqueous 1 N lithium hydroxide (1 mL) and the mixture stirred at 0 °C for 2 h. After most of the THF and methanol had been evaporated, the aqueous phase was extracted with chloroform (50 mL). The aqueous phase was acidified with 1.2 N HCl to pH 7 and the mixture was freeze–dried to give a solid which was used in the next step without further purification.

2-(*N*-[*tert*-Butoxycarbonyl]-*N*-[2-*tert*-butoxycarbonylaminoethyl]aminomethyl)-*N*-(1-carboxy-2-[imidazol-4-yl]ethyl)pyridine-6-carboxamide (13). (IR) (KBr) ν 3424 (OH), 1670 (C=O) cm^{-1}; ^1H NMR (360 MHz, $(CD_3)_2SO$) δ 1.36 (18H, s, 2×$COOC(CH_3)_3$), 3.06 (2H, m, CH_2), 3.12 (2H, m, CH_2), 3.38 (2H, m, CH_2), 4.34 (1H, m, CH), 4.49 (2H, s, CH_2), 6.73 (1H, s, 5-H of imidazole), 6.88 (1H, brs, NH exchanged with D_2O), 7.35 (1H, t, $J=8$ Hz, 4-H), 7.50 (1H, s, 2-H of imidazole), 7.90 (1H, d, $J=7$ Hz, 3-H), 7.95 (1H, m, 5-H), 9.85 (1H, m, NH exchanged with D_2O).

2-(*N*-[*tert*-Butoxycarbonyl]-*N*-[2-*tert*-butoxycarbonylaminoethyl]aminomethyl)-*N*-(1-carboxy-2-[imidazol-4-yl]ethyl)-1-oxo-pyridine-6-carboxamide (14). (IR) (KBr) ν 3444 (OH), 1654 (C=O) cm^{-1}; ^1H NMR (360 MHz, $(CD_3)_2SO$) δ 1.36 (18H, s, 2×$COOC(CH_3)_3$), 3.09 (2H, m, CH_2), 3.32 (2H, m, CH_2), 4.49 (2H, s, CH_2), 4.70 (1H, m, CH), 6.89 (1H, s, 5-H of imidazole), 7.35 (1H, t, $J=8$ Hz, 4-H), 7.60 (1H, d, $J=8$ Hz, 3-H), 7.68 (1H, s, 2-H of imidazole), 8.18 (1H, d, $J=6$ Hz, 5-H), 11.52 (1H, m, NH exchanged with D_2O).

2-(*N*-[*tert*-Butoxycarbonyl]-*N*-[2-*tert*-butoxycarbonylaminoethyl]aminomethyl)-*N*-(1-carboxy-2-[imidazol-4-yl]ethyl)-4-methoxypyridine-6-carboxamide (15). (IR) (KBr) ν 3320 (OH), 1708 (C=O), 1664 (C=O) cm^{-1}; ^1H NMR (360 MHz, $(CD_3)_2SO$) δ 1.36 (18H, s, 2×$COOC(CH_3)_3$), 3.04 (2H, m, CH_2), 3.12 (2H, m, CH_2), 3.37 (2H, m, CH_2), 3.88 (3H, s, OCH_3), 4.44 (2H, s, CH_2), 4.61 (1H, m, CH), 6.50 (1H, brs, NH exchanged with D_2O), 6.86 (2H, s, 3-H and 5-H of imidazole), 7.43 (1H, s, 5-H), 7.62 (1H, s, 2-H of imidazole), 8.97 (1H, brs, NH exchanged with D_2O); FAB–HRMS calcd for $C_{26}H_{39}N_6O_8$ (M + H) 563.2829. Found: 563.2855.

2-(*N*-[*tert*-Butoxycarbonyl]-*N*-[2-*tert*-butoxycarbonylaminoethyl]aminomethyl)-*N*-(1-carboxy-2-[5-nitroimidazol-4-yl]-ethyl)-4-methoxypyridine-6-carboxamide (16). (IR) (KBr) ν 3442 (OH), 1664 (C=O) cm^{-1}; ^1H NMR (360 MHz, $(CD_3)_2SO$) δ 1.36 (18H, s, 2×$COOC(CH_3)_3$), 3.11–3.59 (6H, m, 3×CH_2), 3.88 (3H, s, OCH_3), 4.25 (1H, m, CH), 4.45 (2H, s, CH_2), 6.50 (1H, brs, NH exchanged with D_2O), 6.81 (1H, s, 5-H of imidazole), 6.86 (1H, s, 3-H), 7.40 (1H, s, 5-H), 7.59 (1H, s, 2-H of imidazole), 8.96 (1H, m, NH exchanged with D_2O).

General procedure for the preparation of the histaminamides (17–20)

A mixture of the appropriate acid (0.18 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (0.21 mmol) and 1-hydroxybenzotriazole (0.22 mmol) in anhydrous DMF (10 mL) was stirred under nitrogen at room temperature for 30 min. To this solution was added dropwise triethylamine (0.7 mL) and subsequently *N*-(2-aminoethyl)-4-(6-[4-{(2-dimethylaminoethyl)thiomethyl}-phenyl]pyridin-2-yl)benzamide (**8**) (0.23 mmol). The solution was stirred overnight, solvent removed in vacuo and the residue dissolved in ethyl acetate. The solution was washed with saturated aqueous sodium bicarbonate, dried (Na_2SO_4) and the solvent evaporated. The residue was purified by column chromatography.

***N*-(6-[*N*-{*tert*-Butoxycarbonyl}-*N*-{2-*tert*-butoxycarbonylaminoethyl}aminomethyl]-pyridine-2-carbonyl)-*N'*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]phenyl)-pyridin-2-yl}benzamido]ethyl)histaminamide (17).** Yield, 30% (silica-gel eluted with methanol:dichloromethane, 1:19) as a colourless amorphous solid; ^1H NMR (200 MHz, $(CD_3)_2SO$) δ 1.35 (18H, s, 2×$COOC(CH_3)_3$), 2.19 (6H, s, $N(CH_3)_2$), 2.53 (2H, m, CH_2), 3.09 (2H, m, CH_2), 3.15 (2H, m, CH_2), 3.43 (8H, m, 4×CH_2), 3.83 (2H, s, CH_2S), 4.52 (2H, s, CH_2N), 4.65 (1H, m, CH), 6.78 (1H, brs, NH exchanged with D_2O), 6.83 (1H, s, 5-H of imidazole), 7.25 (2H, m, 3- and 4-H), 7.50 (3H, m, 2-H of imidazole and ArH), 7.96 (5H, m, ArH), 8.20 (5H, m, 5-H and ArH), 8.72 (1H, brs, NH exchanged with D_2O); FAB–HRMS calcd for $C_{50}H_{64}N_{10}O_7SNa$ (M + Na): 971.4578. Found: 971.4587.

***N*-(6-[*N*-{*tert*-Butoxycarbonyl}-*N*-{2-*tert*-butoxycarbonylaminoethyl}aminomethyl]-1-oxopyridine-2-carbonyl)-*N'*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]phenyl)-pyridin-2-yl}benzamido]ethyl)histaminamide (18).** Yield, 36% (silica-gel eluted with methanol:dichloromethane, 1:19) as a colourless amorphous solid; ^1H NMR (200 MHz, $(CD_3)_2SO$) δ 1.36 (18H, s, 2×$COOC(CH_3)_3$), 2.19 (6H, s, $N(CH_3)_2$), 2.53 (4H, m, 2×CH_2), 3.26–3.83 (10H, m, 5×CH_2), 3.82 (2H, s, CH_2), 4.52 (2H, s, CH_2N), 4.71 (1H, m, CH), 6.70 (1H, brs, NH exchanged with D_2O), 6.83 (1H, s, 5-H of imidazole), 7.50 (5H, m, 2-H of imidazole, 3- and 4-H and ArH), 7.97 (5H, m, ArH), 8.13–8.27 (5H, m, 5-H and ArH), 8.67 (1H, brs, NH exchanged with D_2O); FAB–HRMS calcd for $C_{50}H_{64}N_{10}O_8SNa$ (M + Na): 987.4527. Found: 987.4546.

***N*-(6-[*N*-{*tert*-Butoxycarbonyl}-*N*-{2-*tert*-butoxycarbonylaminoethyl}aminomethyl]-4-methoxypyridine-2-carbonyl)-*N'*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]-phenyl)-pyridin-2-yl}benzamido]ethyl)histaminamide (19).** Yield, 36% (silica-gel eluted with methanol:dichloromethane, 1:9) as a colourless amorphous solid; ^1H NMR (360 MHz, $(CD_3)_2SO$) δ 1.34 (18H, s, 2×$COOC(CH_3)_3$), 2.11 (6H, s, $N(CH_3)_2$), 2.40 (2H, m, CH_2), 2.19–3.19 (10H, m, 5×CH_2), 3.82 (2H, s, CH_2S), 3.95 (3H, s, OCH_3), 4.45 (2H, s, CH_2N), 4.57 (1H, m, CH), 6.84 (1H, s 5-H of imidazole), 7.07 (1H, s, 3-H), 7.42 (1H, s, 5-H), 7.47 (2H, d, $J=8$ Hz, ArH), 7.64 (1H, s, 2-H of imidazole), 7.98 (5H, m, ArH), 8.17 (2H, m, ArH), 8.27 (2H, m, ArH), 8.85 (1H, m, NH exchanged with D_2O); FAB-HRMS calcd for $C_{51}H_{67}N_{10}O_8S$ (M + H): 979.4864. Found: 979.4901.

***N*-(6-[*N*-{*tert*-Butoxycarbonyl}-*N*-{2-*tert*-butoxycarbonylaminoethyl]aminomethyl]-4-methoxypyridine-2-carbonyl)-*N'*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]phenyl)-pyridin-2-yl}benzamido]ethyl)-4(5)-nitrohistaminamide (20).** Yield, 36% (silica-gel eluted with methanol:dichloromethane, 6:94) as a yellow amorphous solid; ^1H NMR (360 MHz, $(CD_3)_2SO$) δ 1.35 (18H, s, 2×$COOC(CH_3)_3$), 2.21 (6H, s, $N(CH_3)_2$), 2.55 (2H, m, CH_2), 3.13 (2H, m, CH_2), 3.36–3.46 (10H, m, 5×CH_2), 3.83 (2H, s, CH_2S), 3.89 (3H, s, OCH_3), 4.44 (2H, s, CH_2N), 4.83 (1H, m, CH), 6.82 (1H, s, 3-H), 7.36 (1H, s, 5-H), 7.49 (2H, d, $J=8$ Hz, ArH), 7.65 (1H, s, 2-H of imidazole), 7.97 (5H, m, ArH), 8.18 (2H, d, $J=8$ Hz,

ArH), 8.26 (2H, d, $J=9$ Hz, ArH), 8.58 (1H, brs, NH exchanged with D_2O); FAB–HRMS calcd for $C_{51}H_{66}N_{11}O_{10}S$ (M + H): 1024.4714. Found: 1024.4646.

General method for the removal of tert-butoxycarbonyl groups

To the appropriate protected compound (0.1 g), trifluoroacetic acid (5 mL) was added and the mixture stirred for 1 h at 0 °C. Trifluoroacetic acid was then removed in vacuo and the residue dissolved in water. The aqueous solution was charged on to a column of Amberlite IRA 93 (as a free base, 7 cm height of the ion exchange resin in the column) and the column was eluted with water. The first five fractions were combined and freeze-dried to give the target compounds as hygroscopic foams.

***N*-(6-[*N*-{Aminoethyl}aminomethyl]pyridine-2-carbonyl)-*N′*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]phenyl)-pyridin-2-yl}benzamido]ethyl)histaminamide (3).** Yield, 94%; ^{1}H NMR (200 MHz, $(CD_3)_2SO$) δ 2.22 (6H, s, $N(CH_3)_2$), 2.57 (2H, m, CH_2), 2.81–3.34 (12H, m, 6×CH_2), 3.83 (2H, s, CH_2S), 3.94 (2H, s, CH_2N), 4.64 (1H, m, CH), 6.83 (1H, s, 5-H of imidazole), 7.55 (5H, m, 2-H of imidazole, 3- and 4-H, ArH), 7.96 (5H, m, ArH), 8.21 (5H, m, ArH), 8.66 (1H, brs, NH exchanged with D_2O); FAB–-HRMS calcd for $C_{40}H_{49}N_{10}O_8S$ (M + H): 749.3709. Found: 749.3652.

***N*-(6-[*N*-{Aminoethyl}aminomethyl]-1-oxopyridine-2-carbonyl)-*N′*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]-phenyl)pyridin-2-yl}benzamido]ethyl)histaminamide (4).** Yield, 92%; ^{1}H NMR (200 MHz, $(CD_3)_2SO$) δ 2.12 (6H, s, $N(CH_3)_2$), 2.56 (2H, m, CH_2), 2.82–3.49 (12H, m, 6×CH_2), 3.82 (2H, s, CH_2S), 3.85 (2H, s, CH_2N), 4.70 (1H, m, CH), 6.82 (1H, s, 5-H of imidazole), 7.52 (5H, m, 2-H of imidazole, 3- and 4-H, ArH), 7.96 (5H, m, ArH), 8.13–8.26 (5H, m, 5-H and ArH), 11.47 (1H, brs, NH exchanged with D_2O).

***N*-(6-[*N*-{Aminoethyl}aminomethyl]-4-methoxypyridine-2-carbonyl)-*N′*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]phenyl)pyridin-2-yl}benzamido]ethyl)histaminamide (5).** Yield, 90%; ^{1}H NMR (360 MHz, $(CD_3)_2SO$) δ 2.18 (6H, s, $N(CH_3)_2$), 2.69 (2H, m, CH_2), 2.77 (2H, m, CH_2), 2.90 (2H, m, CH_2), 3.16–3.35 (8H, m, 4×CH_2), 3.83 (3H, s, CH_2S), 3.87 (5H, s, OCH_3 and CH_2N), 4.61 (1H, m, CH), 6.85 (1H, s, 5-H of imidazole), 7.22 (1H, d, $J=1$ Hz, 3-H), 7.39 (1H, d, $J=1$ Hz, 5-H), 7.49 (2H, d, $J=8$ Hz, ArH), 7.59 (1H, s, 2-H of imidazole), 7.99 (5H, m, ArH), 8.18 (2H, d, $J=8$ Hz, ArH), 8.26 (2H, d, $J=6$ Hz, ArH), 8.95 (1H, brs, NH exchanged with D_2O); FAB–MS m/z 779 (M + H).

***N*-(6-[*N*-{Aminoethyl}aminomethyl]-4-methoxypyridine-2-carbonyl)-*N′*-(2-[4-{6-(4-[{2-dimethylaminoethyl}thiomethyl]phenyl)pyridin-2-yl}benzamido]ethyl)-4(5)-nitrohistaminamide (6).** Yield, 90%; ^{1}H NMR (360 MHz, $(CD_3)_2SO$) δ 2.16 (6H, s, $N(CH_3)_2$), 2.62 (2H, m, CH_2), 2.86–3.21 (12H, m, 6×CH_2), 3.75 (2H, s, CH_2S), 3.76 (3H, s, OCH_3), 3.83 (2H, s, CH_2N), 4.61 (1H, m, CH), 7.25 (1H, s, 3-H), 7.40 (1H, s, 5-H), 7.37 (2H, d, $J=8$ Hz, ArH), 7.82 (5H, m, ArH), 8.05 (2H, d, $J=8$ Hz, ArH), 8.12 (2H, d, $J=8$ Hz, ArH), 8.50 (1H, brs, NH exchanged with D_2O); FAB–HRMS calcd. for $C_{41}H_{50}N_{11}O_6S$ (M + H): 824.3666. Found: 824.3643.

Physico-chemical measurements

Benzoate hydroxylation assay. The formation of oxidising species was detected by the addition of ammonium ferrous sulfate (100 mmol in buffer) to a mixture of sodium benzoate (5 mmol), the BLM analogue (110 μmol) and phosphate buffer (10 mmol, pH 5) and noting the change in fluorescence intensity with time. Hydroxylation of benzoate gives the fluorescent, 2-hydroxybenzoate, together with the other non-fluorescent isomers. The assay was calibrated using steady-state γ-irradiation (from a ^{60}Co source, dose rate of 0.0941 Gys^{-1}, determined by Fricke dosimetry) of aqueous benzoate (5 mmol in 10 mL phosphate buffer at pH 5.0) saturated with N_2O, when the formation of salicylate is known to be proportional to the radiation dose.[19b] The effect of oxygen or air on the system was investigated by passing the gas through the reaction mixture before the addition of ammonium ferrous sulfate.

Effect of 3–6 and bleomycin on the Fenton reaction. Ammonium ferrous sulfate (100 μM in buffer) or ammonium ferric sulfate (100 μM in buffer) was added to a solution of hydrogen peroxide (1 mM) and sodium benzoate (5 mM) in phosphate buffer (10 mM, pH 5), and the change in fluorescence intensity with time was measured.

A similar study of fluorescence obtained from solutions containing additionally the compounds **3–6** or bleomycin (110 μM) was made.

Production of hydroxyl radicals in the presence of air or oxygen. Either air or oxygen was bubbled through a solution of sodium benzoate (5 mM) and **3–6** or bleomycin (110 μM) in phosphate buffer (10 μM, pH 5) for 10 min. Ammonium ferrous sulfate solution was then added to give a final concentration of 100 μM. The change in fluorescence with time was measured.

DNA binding assay. The binding of the BLM analogues to DNA was measured by recording the loss of fluorescence of the ethidium bromide–DNA complex (λ_{exc} 525 nM, λ_{em} 600 nM) as the concentration of the analogue increases.

A solution of ethidium bromide (8 μmol) in TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, EDTA buffer (0.5 mmol) and DNA (9.92 μmol) at pH 7.5 or 6 (adjusted with aq HCl) was prepared. The quenching of the fluorescence of this solution by the consecutive addition (μL) at 5 min intervals of the solution of the analogue was recorded. All fluorescence readings were corrected by the blank value obtained for a solution of ethidium bromide in buffer alone. The process was continued until the fluorescence was quenched by more than 50%. These data allow the binding constants to be calculated.[21]

Biological assays

Cytotoxicity studies in air. The Chinese hamster cells used had a doubling time of 12 h in Eagle's minimum essential medium supplement with Earle's salts and 10% fetal calf serum. The pH of the medium during subculture and colony growth was controlled by the addition of sodium bicarbonate ($2.2\,gL^{-1}$) and the maintenance of 5% carbon dioxide in the incubator at 37 °C.

Cells were plated to yield 2000 and 200 cells per dish and were incubated for 1 h prior to exposure. The medium was then removed and replaced by 2 mL of the drug solution in the medium and the control treated with the medium alone. The cells were exposed for 4 h, except for those treated with **3**, **4**, **5** and **6** which were exposed for 1 h, while still attached and in the incubator.

At the end of the treatment, the medium containing drug was removed and each culture dish washed once with Hank's saline (2 mL). The cells were then incubated for 7 days in culture medium (4 mL). After 7 days, the cell colonies were stained with crystal violet and the number of surviving colonies counted.

Cytotoxicity studies in hypoxia followed by air. The cell suspension in Eagle's spinner medium (SMEM) was diluted to a concentration of 2×10^5 cells/mL. The suspension was then split into 4 mL portions and spun down at 2000 rpm for 5 min. The pellets were resuspended in solutions of compound **3** and **5** (4 mL) of a range of concentrations in SMEM and in SMEM alone as a control. The cell samples were then incubated for 2 h under flowing atmosphere of nitrogen and carbon dioxide (95:5%, respectively) at 37 °C. The cell samples were then incubated under a stationary atmosphere of nitrogen and carbon doxide (95:5%). A sample (0.5 mL) was then removed from each cell sample and spun down at 2000 rpm. The pellet was washed with Hank's saline (2 mL) and resuspended in SMEM (5 mL) to give a cell concentration of about 2×10^4 cells/mL. The cell suspension (1 mL) was diluted with SMEM (9 mL). The diluted suspension (1 mL) was plated in culture medium (3 mL) to yield 2000 cells per dish and additionally the diluted suspension (100 μL) was plated in culture medium (4 mL) to yield 200 cells per dish. The cells were incubated for 7 days and then counted.

The cytotoxicity of compounds **4** and **6** was assessed by the same procedure except that the times allowed under hypoxic conditions and in air were for 30 min in each case.

Acknowledgements

We are grateful to the EPSRC and the Gray Laboratory Cancer Research Trust (GLCRT) for financial support (to J.A.H.) and to Lisa Folkes (GLCRT) for help and advice concerning the biological assays. We thank the EPSRC National Mass Spectrometry Service Centre, Swansea, for the FAB spectra and accurate mass determinations. P.W. is supported by the Cancer Research Campaign.

References and Notes

1. Highfield, J. A.; Mehta, L. K.; Parrick, J.; Candeias, L. P.; Wardman, P. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2609.
2. Highfield, J. A.; Mehta, L. K.; Parrick, J.; Candeias, L. P.; Wardman, P. *J. Chem. Soc., Perkin Trans 1* **1999**, 2343
3. Huang, L.; Quada, J. C.; Lown, J. W. *Curr. Med. Chem.* **1995**, *2*, 543.
4. Kane, S. A.; Natrajan, A.; Hecht, S. M. *J. Biol. Chem.* **1994**, *269*, 10899.
5. (a) Kross, J.; Henner, W. D.; Heseltine, W. A.; Rodriguez, L.; Levin, W. D.; Hecht, S. M. *Biochemisry* **1982**, *21*, 3711. (b) Fisher, L. M.; Kuroda, R.; Sakai, T. T. *Biochemistry* **1985**, *24*, 3199. (c) Kennani, A.; Lohez, M.; Houssin, R.; Helbecque, N.; Bernier, J. L.; Lemay, P.; Henichart, J. P. *Anti-Cancer Drug Design* **1987**, *2*, 47. (d) Vloon, W. J.; Kruk, C.; Pandit, U. K.; Hofs, H. P.; McVie, J. G. *J. Med. Chem.* **1987**, *30*, 20. (e) Carter, B. J.; Murty, V. S.; Reddy, K. S.; Wang, S.-N.; Hecht, S. M. *J. Biol. Chem.* **1990**, *265*, 4193. (f) Boger, D. L.; Colletti, S. L.; Honda, T.; Menezes, R. F. *J. Am. Chem. Soc.* **1994**, *116*, 5607.
6. (a) Otsuka, M.; Masuda, T.; Haupt, A.; Ohno, M.; Shiraki, T.; Sugiura, Y.; Maeda, K. *J. Am. Chem. Soc.* **1990**, *112*, 838. (b) Owa, T.; Haupt, A.; Otsuka, M.; Kobayashi, S.; Tomioka, S.; Itai, A.; Ohno, M.; Shiraki, T.; Uesugi, M.; Maeda, K. *Tetrahedron* **1992**, *48*, 1193.
7. Huang, L.; Quada, J. C.; Lown, J. W. *Bioconjugate Chem.* **1995**, *6*, 21.
8. Hamamichi, N.; Natrajan, A.; Hecht, S. M. *J. Am. Chem. Soc.* **1992**, *114*, 6278.
9. (a) Searcey, M.; McClean, S.; Madden, B.; Wakelin, L. P. G. *J. Chem. Soc., Perkin Trans. 2* **1997**, 523. (b) Searcey, M.; McClean, S.; Madden, B.; McGrown, A. T.; Wakelin, L. P. G. *Anti-Cancer Drug Design* **1998**, *13*, 837.
10. Strekowski, L.; Chandrasekaran, S.; Wang, Y.-H.; Edwards, W. D.; Wilson, W. D. *J. Med. Chem.* **1986**, *29*, 1311.
11. (a) Strekowski, L.; Strekowska, A.; Watson, R. A.; Tanious, F. A.; Nguyen L. T.; Wilson, W. D. *J. Med. Chem.* **1987**, *30*, 1415. (b) Strekowski, L.; Makrosz, J. L.; Tanious, F. A.; Watson, R. A.; Harden, D.; Mokrosz, M.; Edwards, W. D.; Wilson, W. D. *J. Med. Chem.* **1988**, *31*, 1231.
12. Wilson, W. D.; Tanious, F. A.; Barton, H. J.; Wydra, R. L.; Jones, R. L.; Boykin, D. W.; Strekowski, L. *Anti-Cancer Drug Design* **1990**, *5*, 31.
13. Chien, M.; Grollma, A. P.; Horwitz, S. B. *Biochemistry* **1977**, *16*, 364.
14. Strekowski, L.; Makrosz, M.; Makrosz, J. L.; Strekowska, A.; Allison, S. A.; Wilson, W. D. *Anti-Cancer Drug Design* **1988**, *3*, 79.
15. Trout, G. E. *J. Med. Chem.* **1972**, *15*, 1259.
16. The synthesis and properties of **8** will be reported elsewhere.
17. Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203.
18. Stubbe, J.; Kozarich, J. W.; Wu, W.; Vanderwall, D. E. *Acc. Chem. Res.* **1996**, *29*, 322.
19. (a) Sandstom, B. E.; Svoboda, P.; Granstrom, M.; Harms-Ringdahl, N.; Candeias, L. P. *Free Rad. Biol. Med.* **1997**, *23*, 744. (b) Candeias, L. P.; Patel, K. B.; Stratford, M. R. L.; Wardman, P. *FEBS Lett.* **1993**, *333*, 151.
20. Kennani, A.; Bailly, C.; Helbecque, N.; Houssin, R.; Bernier, J.-L.; Henichart, J. P. *Eur. J. Med. Chem.* **1989**, *24*, 371.
21. Loeb, K. E.; Zaleski, J. M.; Hess, C. D.; Hecht, S. M.; Solomon, E. I. *J. Am. Chem. Soc.* **1998**, *120*, 1249.

22. Chakrabarti, S.; Makrigiorgos, M. G.; O'Brien, K.; Bump, E.; Kassis, A. I. *Free Rad. Biol. & Med.* **1996**, *20*, 777.
23. Morgan, A. R.; Lee, J. S.; Pulleybank, D. E.; Murray, N. L.; Evans, D. H. *Nucleic Acids Res.* **1979**, *7*, 547.
24. Reviews in *Blood Perfusion and Microenvironment of Human Tumours, Implications for Clinical Radiobiology*; Molls, M.; Vaupel, P., Eds.; Springer, Berlin, 1998.
25. McClelland, R. A.; Panicucci, R.; Rauth, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 4308.