AMPLIFICATIONS OF PHLEOMYCIN AND BLEOMYCIN-INDUCED ANTIBIOTIC ACTIVITY IN *ESCHERICHIA COLI* BY AROMATIC CATIONIC COMPOUNDS

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A wide range of aromatic compounds has been shown to amplify phleomycin-induced cell killing in *Escherichia coli*. They include acridines, acridinium chlorides, dihydroanthracenes, anthracenes, dianthracenes, phenanthridinium salts, phenazinium chlorides, phenoxazones, triphenyl methane dyes, benzoquinolizinium chloride, diphenylmethane derivatives, stilbene and diphenyl derivatives. Low concentrations of these amplifiers also amplified the DNA breakage and degradation effects of phleomycin. The minimum structural specification for activity as an amplifying agent is suggested. A representative sample of compounds effective as amplifiers of phleomycin also amplified the antibiotic effects of bleomycins B4 and B6. The amplifiers described are known to vary in their ability to penetrate and accumulate in different organisms or tissues. This suggests the possibility of developing a series of antibiotic regimes using these amplifiers (or the large number of derivative compounds also likely to be active) where the therapeutic index is determined by the properties of the amplifier chosen rather than of the phleomycin or the bleomycin.

The antibiotic phleomycin, a mixture of closely related copper-containing glycopeptides, not only interferes with the ability of cells to replicate, but also causes breakage and degradation of the DNA.[1,2,3,4] Cell killing and DNA degradation are closely related.[5] It has potent antitumor and antimicrobial activity,[1,6,7,8] but it is also nephrotoxic[9]. This failure to demonstrate sufficient selectivity has prevented phleomycin from being used clinically. However, as we have previously reported,[10,11,12] a number of DNA-binding compounds, including a number of purines, coumarin and Pyronin Y are able to potentiate the antibiotic effects of phleomycin. With the exception of Pyronin Y relatively high concentrations of these compounds ($1 \sim 10$ mM) are needed. Nevertheless, the discovery of this amplification phenomenon suggests the possibility of developing a phleomycin-amplifier antibiotic regime with a high therapeutic index, where selectivity of action is determined by the properties of the amplifier rather than of the phleomycin.

The usefulness of this rationale would be greatly enhanced if the range of compounds able to amplify the effects of phleomycin were extended, particularly to classes of substances known to be taken up selectively in different tissues and organisms. In this paper we describe a wide range of conjugated aromatic compounds able to amplify the cell killing effects of phleomycin by up to 50-fold, at concentrations as low as 10^{-8} M and suggest structural features of importance for activity.

Materials and Methods

E. coli strains

Strain *E. coli* B was obtained from the late Dr. RUTH HILL. *E. coli* B *thy* was selected as a spontaneous mutant from strain B by the method of OKADA, YANAGISAWA and RYAN[13].

Materials

Phleomycin (batches A9331-648 and -616) was kindly provided by Dr. W. BRADNER of Bristol Laboratories Inc., Syracuse N.Y. The phleomycin mixture (batch -648) was fractionated by chromatography on CM Sephadex (Pharmacia) using an increasing gradient of ammonium formate.[14] Bleomycins B4 and B6 ("phleomycin" F and G') were isolated from a mixture of phleomycins and tentatively identified from the order of elution, and characterised as bleomycins by their U.V. spectrum. Phleomycin E corresponds in the structure of its terminal amine with bleomycin B4.[15] The sources and names of the other compounds used are listed in Table 1.

Measurement of Cell Killing

The procedures used have been described previously.[12] Stationary-phase cultures of *E. coli* B previously exposed to phleomycin (2 μg/ml) in glucose salts medium for 30 minutes at 37°C were resuspended in the same medium at 37°C, together with the compounds under test for amplifying activity (10^{-1} M to 10^{-2} M). As a control, bacteria were exposed to the same range of concentrations of each compound without phleomycin treatment. To determine viable cell numbers, aliquots of each cell suspension were plated on broth agar medium (Oxoid Blood Agar Base) and incubated overnight at 37°C. All operations with amplifying agents were carried out in dim yellow light to minimise photodynamic effects.

Measurement of DNA Breakdown

Bacterial cultures were grown overnight to stationary phase in GT[12] (plus 20 μg of thymidine per ml when appropriate). To radioactively label the DNA 20 μg of [^{14}C]thymidine per ml (60 mCi/mmol) was added to the growth medium. Washed cells were resuspended in fresh GT thymidine medium containing phleomycin, and incubated at 37°C for a measured period. Samples were withdrawn, membrane-filtered (Millipore Corp., Bedford, Mass., 0.45 μm pore size) and resuspended in GT-thymidine (20 μg/ml) medium containing various concentrations of compounds under test. Periodically, samples were removed for estimating the proportion of DNA broken down to acid-soluble fragments[12].

Results

Compounds Active as Amplifiers of Phleomycin

Previous studies on amplification of phleomycin by purines showed that when phleomycin-treated bacteria were resuspended at 37°C in growth medium containing an active purine, cell death commenced almost immediately and continued at an exponential rate for 60~90 minutes, after which the rate of killing declined substantially[12]. Other amplifying substances produced similar kinetics of killing, so we have taken the proportion of cells surviving after 120-minutes treatment with the test compound as an appropriate measure of amplification activity. The compounds used are set out in Table 1. Compounds which are designated as active, showed an increase in killing of phleomycin-treated cells over that explained by the effects of the two compounds given separately.

Members of a great many of the classes of tricyclic compounds tested proved to be potent amplifiers of the antibiotic activity of phleomycin. Within these classes compounds which are cations or are capable of forming cations were more likely to be active. Among the diphenylmethanes, only the cationic compounds were active. However, cationic character alone was not sufficient to produce amplification, since simple compounds such as tetramethylammonium chloride and phenyltrimethylammonium chloride were inactive (Table 1).

Table 1. Amplification of phleomycin-induced cell killing and DNA degradation in *E. coli* B cells

Compound (origin)	Concentration of amplifier (M)			Log_{10} (Number of cells killed)[d]	
	Killing[a] starts	Peak of[b] DNA breakdown	Killing[c] levels off	No phleo.	+Phleo.
Acridines (C)					
Acridine (1)	Active 2.8×10^{-4}				
3-Aminoacridine (3)	1.4×10^{-6}	2.6×10^{-5}	5.2×10^{-5}	0.21	3.8
3,6-Diaminoacridine (2)	1.6×10^{-6}	1.1×10^{-5}	1.3×10^{-5}	0.40	3.5
3,6-Dihydroxyacridine (4)	Slightly active $\leq2.4\times10^{-4}$				
Acridinium chlorides (C)					
N-Methylacridinium chloride (5)	Inactive $\leq4.4\times10^{-4}$				
3,6-Bis(dimethylamino)acridine hydrochloride (Acridine orange) (6)	3.3×10^{-6}	—	4.0×10^{-4}	0	4.7
9-Aminoacridine hydrochloride (7)	3.9×10^{-6}	5.2×10^{-5}	8.6×10^{-5}	0.61	3.5
Quinacrine hydrochloride (8)	1.2×10^{-6}	9.8×10^{-6}	1.2×10^{-5}	0	2.9
3,6-Diamino-N-methylacridinium chloride (Acriflavine) (6)	6.0×10^{-7}	3.9×10^{-6}	2.1×10^{-5}	0	3.8
9,10-Dihydroanthracenes (A)					
2,7-Bis(trimethylammonio)-9,10-dihydroanthracene dichloride (10)	1.4×10^{-6}	1.4×10^{-3}	no levelling off	0.25	4.1
2,7-Bis(dimethylamino)-9,10-dihydroanthracene (9)	Inactive $\leq7.5\times10^{-5}$				
Anthracenes (C)					
2,7-Bis(trimethylammonio) anthracene dichloride (10)		3.3×10^{-3}	5.0×10^{-4}	0	3.7
9,9′-Dianthracenes (C)					
2,2′,7,7′-Tetrakis(trimethylammonio)-9,9′-dianthracene tetrachloride (10)	2.0×10^{-5}	—	1.5×10^{-4}	0.32	2.9
2,2′,7,7′-Tetrakis(dimethylamino)-9,9′-dianthracene (10)	Inactive $\leq3.7\times10^{-5}$				
Phenanthridinium salts (E)					
Ethidium Bromide (11)	1.3×10^{-5}	1.3×10^{-4}	5.1×10^{-5}	0.92	4.2
N-Methylphenanthridinium chloride (12)	5.0×10^{-7}	—	1.0×10^{-5}	0	2.5
Phenazines (C)					
Phenazine (18)	Active $\leq4.5\times10^{-4}$				
Phenazinium chlorides (C)					
N-Methylphenazinium chloride(13)	3.3×10^{-7}	—	1.0×10^{-5}		
Neutral Red (14)	3.3×10^{-6}	—	3.0×10^{-5}	0.45	3.1
Safranin T (15)	2.8×10^{-6}	—	1.0×10^{-4}	0	1.1
Phenoxazinium dyes (C), (H)					
Brilliant Cresol Blue (15) (C)	Active 4.0×10^{-4}				
Nile Blue (15) (H)	4.4×10^{-7}	1.4×10^{-5}	1.4×10^{-5}	0.40	3.7
Phenazones (C)					
Phenoxazone	2.5×10^{-6}	No peak	2.5×10^{-5}	0.29	1.4
2-Aminophenoxazone (17)	Active 2.0×10^{-4}				
Phenazathionium dyes (C)					
Methylene Blue (11)	7.0×10^{-7}	1.3×10^{-5}	3.6×10^{-5}	0.37	2.9
Toluidine Blue (15)	2.5×10^{-6}	1.6×10^{-5}	1.7×10^{-5}	0.50	2.6

(to be continued)

Table 1 (continued)

Compound (origin)	Concentration of amplifier (M)			Log_{10} (Number of cells killed)[d]	
	Killing[a] starts	Peak of[b] DNA breakdown	Killing[c] levels off	No phleo.	+Phleo.
Xanthene dyes (C)					
Pyronin Y (16)	Active 1.7×10^{-4}				
Rhodamine G (14)	Active 1.0×10^{-4}				
Fluorescein (11)	Inactive $\leq3.0\times10^{-4}$				
Linear tricyclic heterocyclic compounds (C)					
Phenoxazine (18)	Inactive $\leq5.4\times10^{-6}$				
Phenoxathin (19)	Inactive $\leq1.7\times10^{-5}$				
Benzo[b]quinolizinium chloride(20)	5.0×10^{-6}	—	1.0×10^{-4}		
Triphenyl methanes (D)					
Crystal Violet (14)	1.5×10^{-8}	4.9×10^{-7}	3.2×10^{-8}	0.05	4.0
Methyl Green (14)	2.1×10^{-7}	1.3×10^{-5}	3.8×10^{-5}	0.40	4.0
Methyl Violet (14)	4.8×10^{-9}	6.3×10^{-7}	5.4×10^{-8}	0.09	4.3
Diphenyl methanes (B)					
Auramin O (15)	3.3×10^{-6}	—	3.3×10^{-5}	0.15	3.5
4,4′-Bis(trimethylammonio) diphenylmethane dichloride(21)	2.9×10^{-5}	3.0×10^{-4}	2.9×10^{-4}	0.60	4.2
4,4′-Bis(trimethylammonio) benzophenone (22)	Inactive $\leq3.6\times10^{-5}$				
4,4′-Bis(dimethylamino) diphenylmethane (18)	Inactive $\leq4.0\times10^{-6}$				
Other compounds					
Tetramethylammonium chloride (24)	Inactive $\leq9.1\times10^{-5}$				
Phenyltrimethylammonium chloride (25)	Inactive $\leq5.8\times10^{-5}$				
2-Naphthol (6)	Inactive $\leq3.5\times10^{-4}$				
Bromocresol Purple (16)	Inactive $\leq2.0\times10^{-4}$				
4-Dimethylamino-4′-trimethyl ammonio-stilbene chloride(26)(G)	1.4×10^{-6}	—	3.0×10^{-5}(toxic $\geq3.0\times10^{-5}$M)	0	3.0
9,10-Dihydro-8a,10a-diazonia-phenanthrene dichloride (27)	Inactive $\leq4.0\times10^{-4}$				
Benzyl Viologen (11)	Inactive $\leq4.0\times10^{-4}$				
4,4′-Bis(trimethylammonio) diphenyl-dichloride (28) (F)	9.0×10^{-6}	—	9.5×10^{-4}	1.1	5.0
N-Methylquinolinium chloride (27)	Inactive $\leq5.6\times10^{-5}$				
N-Methylisoquinolinium chloride (29)	Inactive $\leq5.6\times10^{-5}$				
N,N,N′,N′-Tetramethyl-1,4-phenylenediamine dihydrochloride (30)	Inactive $\leq4.2\times10^{-5}$				
2-*p*-Dimethylaminostyryl-N-ethylquinolinium chloride(31)(I)	1.5×10^{-8}	—	1.4×10^{-7}	0	2.9

Measurement of amplifying activity was carried out as described for Fig. 1. a – The concentration at which cell killing begins (see Fig. 1). b - Concentration for maximum DNA breakdown from unpublished experiments. c - Concentration for maximum cell killing (see Fig. 1). d - Number of cells killed by amplifier in control and phleomycin-treated cells at concentration to give maximum killing—concentration B in Fig 1. The broad structural class into which each compound or class of compound is grouped in Fig. 3 is indicated by one of the letters (A), (B), (C) . . . (I).

Notes:

(1) Commercial sample, purified by chromatography on alumina and sublimed;

(2) Commercial sample, purified according to Reference 24, p. 112;

(3) Prepared according to ALBERT (Reference 23) p. 101;

(to be continued)

(4) Reference (25);
(5) Reference (24), p. 344, followed by ion exchange on Amberlite I.R.A.–400;
(6) Commercial sample, Drug House of Australia, Tempe, N.S.W.;
(7) Commercial sample, purified according to ALBERT (Ref. 23), p. 292;
(8) Commercial sample, May and Baker Ltd, Dagenham, U.K.;
(9) Reference (25);
(10) Reference (26);
(11) Commercial sample, British Drug Houses Ltd., Poole, U.K.;
(12) Prepared from resublimed phenanthridine *via* the methosulphate, followed by ion exchange and crystallization from methanol-acetone;
(13) Prepared from rechromatographed phenazine *via* the methosulphate, which was subjected to ion exchange;
(14) Commercial sample, G. Gurr, London, purified by crystallization from water;
(15) Commercial sample, purified by crystallization from methanol-benzene;
(16) Commercial sample, G. T. Gurr Ltd. London, U.K.;
(17) Gift from Dr. P. CLEZY, University of N.S.W., Kensington, N.S.W.;
(18) Commercial sample chromatographed on alumina, and recrystallized from ethanol;
(19) Reference (27);
(20) Reference (28);
(21) Commercially available 4,4′-bis(dimethylamino)diphenylmethane was quaternized with dimethyl sulphate, and subjected to ion exchange on Amberlite I.R.A.–400. Repeated crystallization from ethanol gave an analytically pure sample;
(22) Reference (29);
(24) Made from commercially available bromide by ion exchange and crystallization from methanol-acetone;
(25) Made from highly purified N,N′-dimethylaniline and dimethyl sulphate, followed by ion exchange and crystallization from methanol-acetone;
(26) Methylation of 4,4′-bis(dimethylamino) stilbene with methyl iodide in methanol, followed by ion exchange;
(27) From 2,2′-dipyridyl and 1,2-dibromoethane followed by ion exchange;
(28) Reference (30);
(29) Prepared from a highly purified sample of the base and dimethylsulphate, followed by ion exchange and crystallization from methanol-acetone;
(30) Prepared from a commercial sample by repeated crystallization from anhydrous methanol;
(31) Ion exchange on the commercially available iodide.

The relationship between degree of amplification and amplifier concentration was investigated for some of the more interesting compounds. The results of an experiment with Crystal Violet are illustrated in Fig. 1.

The results of similar experiments with other compounds are shown in Fig. 2. The figures have been simplified by including only the data for phleomycin-treated cells. The cell killing effect of amplifying compounds alone can be gauged from the data in Table 1, which compares the toxicity of different compounds and their ability to amplify phleomycin-induced cell killing. The table also includes, for comparison, data on amplification of DNA degradation.

Within classes of compounds tested, there was considerable variation between individual compounds in amplifier activity. In general, the maximum killing due to amplification was con-

Fig. 1. Effect of Crystal Violet on the survival of *E. coli* B cells pretreated with phleomycin.
Stationary phase cells, treated with phleomycin (2 μg/ml) in growth medium for 30 min, were then exposed for 2 hours to various concentrations of Crystal Violet. The top curve shows survival of cells treated with Crystal Violet without previous phleomycin treatment (○).

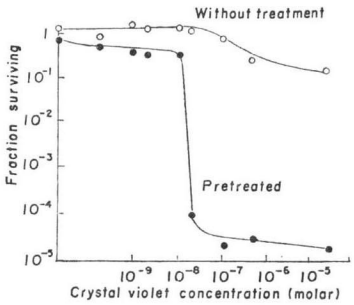
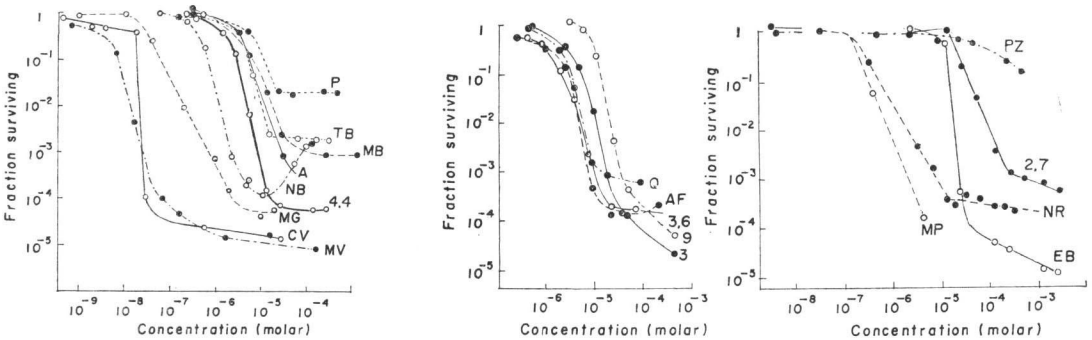


Fig. 2. Relation between fraction of phleomycin-treated bacteria surviving and concentrations of various amplifying compounds.

The procedure was exactly as described for Fig. 1, but only results for phleomycin-treated cells are shown.



(a) 4,4: 4,4-bis(trimethylammonio)diphenylmethane;
A: Auramin; CV: Crystal Violet; MB: Methylene Blue; MG: Methyl Green; MV: Methyl Violet; NB: Nile Blue; P: Phenoxazine; TB: Toluidine Blue.

(b) 3: 3-aminoacridine;
3,6: 3,6-diaminoacridine;
9: 9-aminoacridine HCl;
AF: acriflavine;
Q: Quinacrine.

(c) 2,7: 2,7-bis(trimethylammonio)-anthracene dichloride;
EB: ethidium bromide;
MP: N-methylphenanthridinium chloride;
NR: Neutral Red;
PZ: phenazine.

stant — a decrease in survival of $10^{-3} \sim 10^{-4}$ compared with controls — but compounds such as phenazine and Methylene Blue produced a smaller effect. For each compound there appeared to be a threshold concentration below which no amplification was observed, but at higher concentrations, the survival of phleomycin-treated cells decreased rapidly (Figs. 1 and 2). This threshold concentration generally coincided with the concentration necessary to initiate DNA strand breakage (unpublished observations).

The toxicity of the amplifiers in the absence of phleomycin varied from the trivial (Auramin, 2,7-bis(trimethylammonio)-9,10-dihydroanthracene and N-methylphenanthridinium chloride), to the severe (ethidium bromide, benzo[b]quinolizinium chloride). Intrinsic toxicity was not related to amplifying activity.

Similar trials using identical concentrations and conditions were conducted with bleomycins B4

Table 2. Inhibition of cell killing by amplifiers present during phleomycin treatment

Amplifier	Concentration (M)	Percentage of cells surviving	
		Phleomycin, then amplifier	Phleomycin+amplifier, then amplifier
Acridine	2.8×10^{-4}	0.44	7.6
6,9-Dimethyl-methylthiopurine	8.0×10^{-3}	7.6	6.4
Neutral Red	1.7×10^{-4}	0.66	15
Brilliant Cresol Blue	3.2×10^{-4}	21	100
Pyronin Y	1.7×10^{-4}	1.4	60
Ethidium bromide	1.3×10^{-5}	3.6	2.8

Stationary phase *E. coli* B was treated in growth medium for 30 minutes with phleomycin (1 μg/ml) with or without an amplifying compound present. Cells were then transferred to growth medium containing the amplifier above, and the percentage of cells surviving after 120 minutes was assayed.

and B6 ("phleomycins" F and G′). The antibacterial effects of the latter were amplified by a representative sample of compounds effective as amplifiers of phleomycin consisting of caffeine, 3-aminoacridine, Neutral Red, Nile Blue, Crystal Violet, Auromin O, benzo[b]quinolizinium chloride, 4-dimethylamino-4′-trimethylammonio-stilbene chloride and 2-*p*-dimethylaminostyryl-N-ethylquinolinium chloride. The extent of the amplification was similar to that observed with phleomycins E and G′ which are thought to have identical side chains to bleomycins B4 and B6.

Interference of Amplifying Compounds with Phleomycin Activity

Studies with caffeine indicated that the extent of the amplification effect was the same, whether or not caffeine was present during the phleomycin treatment period or only afterwards.[12] Similar results were obtained for amplification by other purines and ethidium bromide, but the tricyclic compounds Neutral Red, Brilliant Cresol Blue, Pyronin Y and acridine behaved differently. The presence of these compounds in the medium during phleomycin treatment considerably reduced the cell death normally observed after subsequent treatment with amplifier (Table 2).

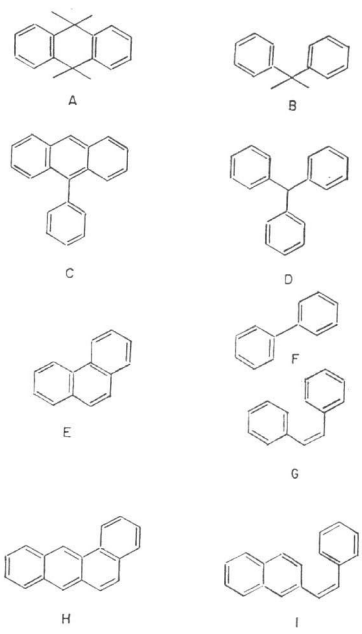
Discussion

Ability to potentiate the antibacterial effects of phleomycin appears to be a widespread characteristic of many classes of organic molecules. Our results show that at least three structural features affect amplifying activity and that none of these alone is sufficient to produce an active compound. All amplifiers reported here contain six-membered carbocyclic or heterocyclic aromatic rings; all active compounds are cationic or can acquire a positive charge by protonation of a nitrogen; and activity is associated only with molecules whose areas can exceed those of simple monocyclic and bicyclic cations.

In particular, the phenyltrimethylammonium, N-methylquinolinium and N-methylisoquinolinium cations were inactive but all of the tricyclic and one tetracyclic annelated cations tested were active. Moreover, cations in which single six-membered rings were linked to each other directly, or by one or two carbon atoms, were active and we note that the active compounds in this structurally divergent group can adopt, or approach, conformations that are similar in area to those active cations with relatively rigid annelated structures (Fig. 3). In the examples shown heteroatoms have been omitted.

Of the active compounds, many are known to form active radicals which are stabilised in the presence of DNA[16,17], a property which apparently explains their ability to sensitise DNA to breakage in the presence of visible light and oxygen[16,18,19]. However, the experiments described here were carried out in dark condition to minimise these effects. Since there was ap-

Fig. 3. Basic general structural conformations which fit all compounds shown in this study to amplify phleomycin activity.
Heteroatoms and possible substituent groups have been omitted. For representatives of each structural class A . . . I, see Table 1.



parently no increase in the permeability of cells to this type of compound as a result of phleomycin treatment[20], the intrinsic ability to break DNA cannot explain their phleomycin amplifying activity. Some compounds, such as Neutral Red and Pyronin Y, were less effective in potentiating the antibiotic effects of phleomycin if the bacteria were exposed simultaneously to phleomycin and compound rather than sequentially to phleomycin followed by compound. This is explicable if the amplifier competes with phleomycin for binding sites in or on the cell *e.g.* on the DNA. Perhaps those amplifiers which bind to A-T base pairs of A-T sequences in the DNA[21] might be expected to interfere with phleomycin binding, which is thought to occur at thymine residues[22].

Two of the amplifiers, proflavine and ethidium bromide, have been reported to stimulate DNA breakage *in vitro* by bleomycin, an antibiotic related to phleomycin both in structure and mechanism of action[23]. However, the concentrations of bleomycin used in these experiments were 1,000 times greater than those used to produce DNA breakage *in vivo* and $10^4 \sim 10^5$ times greater than were required in the amplification experiments reported here.

The list of substances which we have described as potentiators of phleomycin is now quite large, including more than 30 purines, coumarin,[10,12] and the cationic aromatic compounds listed here. The classes of compounds effective as potentiators of phleomycin exhibit considerable diversity in the ability to penetrate and interact with different organisms[24]. We believe the possibility now exists for developing a series of selective antibiotic regimes based on phleomycin amplification, where the selectivity of antibiotic effect is determined by the properties of the particular amplifier chosen from among the many phleomycin-amplifier regimes possible.

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