AMPLIFICATION OF THE ANTIBIOTIC EFFECTS OF THE BLEOMYCINS, PHLEOMYCINS AND TALLYSOMYCINS: ITS DEPENDENCE ON THE NATURE OF THE VARIABLE BASIC GROUPS

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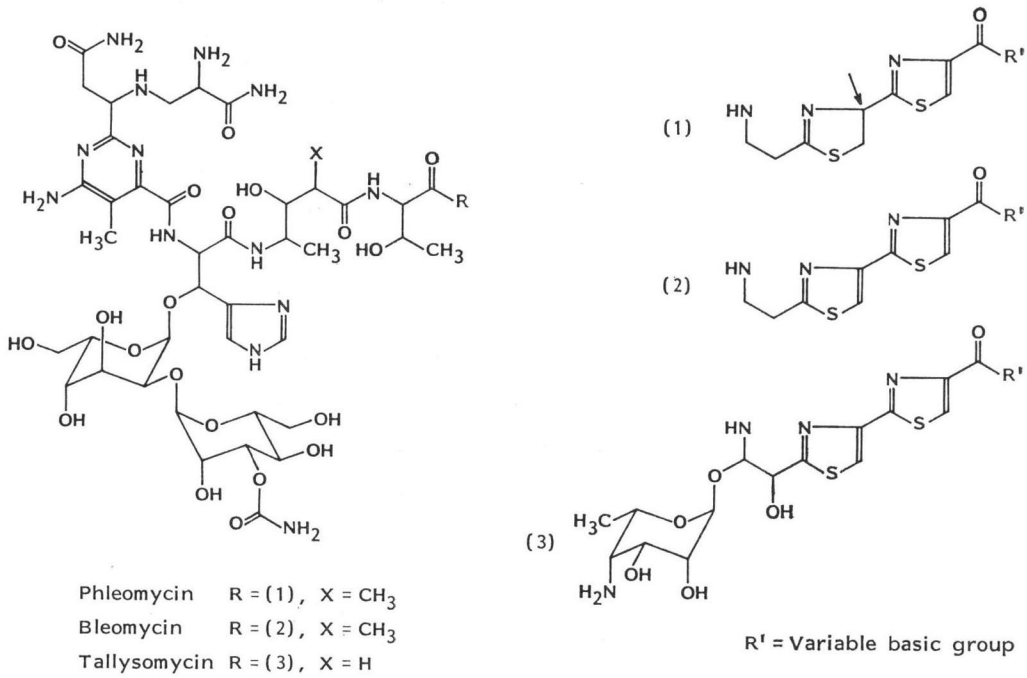
The bleomycins, phleomycins and tallysomycins are structurally similar glycopeptide antibiotics. Within each class, individual members differ only in the structure of a basic group. The antibiotic effect of phleomycin (Bristol batch A9331-648) against *Escherichia coli* is amplified substantially by a number of simple heterocyclic and aromatic compounds. In this paper a sample of 26 such compounds were tested for this property with 25 different phleomycins, bleomycins and tallysomycins. The nature of the variable basic group of the phleomycins, bleomycins and tallysomycins determined the response obtained with all amplifiers, although variation of response was much less marked with caffeine which potentiated the cytotoxic effects of all the phleomycins, bleomycins and tallysomycins tested. Phleomycins and bleomycins having two or three guanidino groups in the variable basic group, or phleomycins having a secondary amino group within a methylene chain and a terminal 2-phenylethyl substituent, were amplified by most compounds, whereas the cytotoxicity of others was enhanced little or not at all. Similar phleomycins, having a secondary amino and a terminal guanidino group and no 2-phenylethyl substituent showed little enhancement, and in these cases the inclusion of a 2-phenylethyl substituent had a major influence in determining amplifiability. Bleomycins and phleomycins having identical basic groups were amplified to similar extents by the sample of 26 amplifying agents used.

The phleomycins and bleomycins are glycopeptide antibiotics produced as metabolites of *Streptomyces verticillus* and they are isolated as copper chelates. The phleomycins have the general structure shown in Fig. 1, in which R′ represents a basic group that varies from one phleomycin to another[1]. The bleomycins differ from the phleomycins in having a thiazole instead of a dihydrothiazole ring (see arrow, Fig. 1). Because of the large number of variable basic groups in the full range of bleomycins and phleomycins used in this study, all the basic groups are listed in order of increasing complexity in Table 1. For ease of reference, pairs of phleomycins and bleomycins having the same basic group, as shown in Table 1, have been given the same number in order to indicate their similarity. Thus phleomycin G and bleomycin B6, which have the same terminal triguanidine group[2~4] are written as PLM-23 and BLM-23 respectively; and phleomycin E and bleomycin B4, which have the same terminal diguanidine group[5,6] as PLM-22 and BLM-22. Such pairs may be considered as derivatives of the respective phleomycinic and bleomycinic acids. Tallysomycins are similar to bleomycins and phleomycins[7], but in addition to the structural difference shown in Fig. 1, there is another series of tallysomycins, in which a β-lysyl group is interposed between the variable basic group and the rest of the

Fig. 1. Structure of phleomycins and related antibiotics.



molecule.

Bleomycins, as the mixture Blenoxane (Bristol Labs) or as peplomycin, are in clinical use as antitumor agents. The *in vitro* mechanism of action of bleomycin has been intensively studied[8~12] and the primary mechanism of action has been reported to be induction of single and double strand breaks in DNA. Damage to DNA is produced by a reaction involving the Fe^{++} complex of bleomycin and oxygen, and the bithiazole group of bleomycin is thought to bind to DNA by intercalation. The variable basic groups are considered to bind ionically to the phosphate groups of DNA and they appear to influence the selective toxicity of the drug both to microorganisms and to animals[6,13,14] presumably because they modify transport into and within cells, and the distribution within the animal body.

As previously reported by us, each of a large number of low molecular weight aromatic compounds amplifies the antibacterial, antitumor, or DNA-breaking activity of the phleomycin mixture A9331-648 (PLM-648) and of bleomycins BLM-22 and BLM-23[15]. The extent of enhancement of antibacterial activity by these compounds in liquid culture was estimated, in some instances, to be as much as 50-fold, yet they themselves may not exhibit antibiotic activity[15~18]. There are other reports that the mammalian cell toxicity of the bleomycin is enhanced by a number of membrane-interacting drugs[19,20]. If effects similar to those which we obtained in bacteria pertain to mammalian cells it may be possible to develop a bleomycin/amplifier or phleomycin/amplifier regime with a high therapeutic index and reduced side effects, by means of amplifiers which are taken up selectively by tumor cells. The selection of appropriate bleomycins and phleomycins with modified basic groups may enhance this regime.

In this paper we examine the specificity of response to amplifying agents by phleomycins, bleomycins

Table 1. Variable basic groups.

Designation in this paper	Formula of variable basic group	Trivial designation of corresponding antibiotic
– 1	$-HN(CH_2)_3S^+(CH_3)_2X^-$	BLM-A_2
– 2	$-NH(CH_2)_2NH_2$	BLM-EDA
– 3	$-NH(CH_2)_3NH_2$	BLM-A_2'-b
– 4	$-NH(CH_2)_3NHC_6H_{11}$*	BLM-, PLM-, TLM-CHP
– 5	$-NH(CH_2)_3NHCH(CH_3)Ph$	BLM-, PLM-, TLM-PEP
– 6	$-NH(CH_2)_3NH(CH_2)_3OH$	BLM-HPP
– 7	$-NH(CH_2)_3NHC_5H_9$**	BLM-CPP
– 8	$-NH(CH_2)_3NH(CH_2)_4NH_2$	BLM-A_5, TLM-B
– 9	$-NH(CH_2)_3NH(CH_2)_3NHC_6H_{11}$*	BLM-, PLM-CHPP
–10	$-NH(CH_2)_3NH(CH_2)_3NHCH(CH_3)Ph$	BLM-, PLM-PEPP
–11	$-NH(CH_2)_3NH(CH_2)_3NHC_5H_9$**	BLM-CPPP
–12	$-NH(CH_2)_4NHC(=NH)NH_2$	BLM-B_2, PLM-D_1
–13	$-NH(CH_2)_4NHC(=NH)NH(CH_2)_2Ph$	PLM-A_4GPe
–14	$-NH(CH_2)_4NH(CH_2)_3NHC(=NH)NH_2$	PLM-A_4A_3G
–15	$-NH(CH_2)_4NH(CH_2)_4NHC(=NH)NH_2$	PLM-A_4A_4G
–16	$-NH(CH_2)_5NH(CH_2)_3NHC(=NH)NH_2$	PLM-A_5A_3G
–17	$-NH(CH_2)_6NH(CH_2)_3NHC(=NH)NH_2$	PLM-A_6A_3G
–18	$-NH(CH_2)_4NH(CH_2)_3NHC(=NH)NH(CH_2)_2Ph$	PLM-A_4A_3GPe
–19	$-NH(CH_2)_4NH(CH_2)_4NHC(=NH)NH(CH_2)_2Ph$	PLM-A_4A_4GPe
–20	$-NH(CH_2)_5NH(CH_2)_3NHC(=NH)NH(CH_2)_2Ph$	PLM-A_5A_3GPe
–21	$-NH(CH_2)_6NH(CH_2)_3NHC(=NH)NH(CH_2)_2Ph$	PLM-A_6A_3GPe
–22	$-NH(CH_2)_4NHC(=NH)NH(CH_2)_4NHC(=NH)NH_2$	BLM-B_4, PLM-E, TLM-AGG
–23	$-NH(CH_2)_4NHC(=NH)NH(CH_2)_4NHC(=NH)NH(CH_2)_4NHC(=NH)NH_2$	BLM-B_6, PLM-G, TLM-AGGG
–24	$-NH(CH_2)_4NHC(=NH)NH(CH_2)_4NHC(=NH)NH(CH_2)_4NHC(=O)NH_2$	PLM-, TLM-AGGU
–25	$-NH(CH_2)_4NHC(=NH)N[(CH_2)_4NH_2][(CH_2)_4NHC(=NH)NH_2]$	PLM-AG(A)G

* Cyclohexyl.
** Cyclopentyl.

and tallysomycins, and relate it to the nature of their variable basic groups.

Materials and Methods

Antibiotics and Reagents

A list of the variable basic groups of the phleomycins, bleomycins and tallysomycins used is set out in Table 1. Many of the antibiotics studied in this work were prepared previously, and earlier common names or designations used are also shown in the table. Most of the phleomycins and bleomycins were isolated from the culture liquor of *Streptomyces verticillus* strains provided by Dr. T. TAKITA of the Microbial Chemistry Foundation, Tokyo, and Dr. W. BRADNER of Bristol Research Labs., Syracuse, and grown in media to which appropriate precursor amines had been added. Tallysomycins were similarly prepared from cultures of *Streptoalloteichus hindustanus* strain, No. C-801-104-AC10 supplied by Dr. BRADNER. The isolation method, which will be described elsewhere[21], is a modification of published methods[22~24] and involves fractionation by chromatography on CM-Sepharose CL6B (Pharmacia) using an increasing eluent gradient of ammonium formate. We are grateful to Dr. T. TAKITA for advice on isolation and separation methods. A number of bleomycins were the gift of Professor H. UMEZAWA and Dr. T. TAKITA of the Microbial Chemistry Foundation, Tokyo. These were bleomycins EDA (BLM-2), A_2'-b (BLM-3), CHP (BLM-4), PEP (BLM-5), HPP (BLM-6), CPP (BLM-7), CHPP (BLM-9), PEPP (BLM-10), CPPP (BLM-11), B_4 (BLM-22) and B_6 (BLM-23). Phleomycin A9331-648 (PLM-648), a mixture of several phleomycins [principally phleomycins G (PLM-23), E (PLM-22), and D_1 (PLM-12) and small amounts of the corresponding bleomycins], was the gift of Dr. W. BRADNER of Bristol Laboratories. We are greatly indebted to these people and institutions for their assistance.

Bacterial Assay System

Escherichia coli B cells from an overnight culture were grown to stationary phase in GRAY and TATUM's (G.T.) glucose (0.05%) salts medium[25], washed with G.T. buffer (G.T. without glucose) and resuspended in G.T. buffer to provide a cell suspension of 5×10^8 bacteria/ml.

Petri Plate Zone-of-inhibition Method

Amplifying agents were incorporated into molten nutrient agar medium (Blood Agar Base, Oxoid England) held at 45°C prior to pouring as 30 ml aliquots into 9 cm Petri dishes. Concentrations of the amplifying agents were similar to those found effective in liquid cell suspension cultures, but toxicity levels were checked in experiments with spread cells prior to use. Precautions were taken to prevent photodynamic effects whilst bacteria were in contact with potential amplifying agents.

The solidified plates were spread with a 0.1 ml aliquot of the cell suspension, and stainless steel template discs (Lab. line, Constant Ex) were placed on the surface of the spread agar plates. Varying concentrations of the phleomycins and bleomycins (5 μl volume) were pipetted into the six depressions of the steel discs. After the liquid had soaked into the agar medium the stainless steel templates were removed and the assay plates incubated at 37°C overnight. Because the bleomycins and phleomycins used in this study varied in their antibiotic potency (for examples, see Table 2), the range of concentrations used for each was chosen after a preliminary experiment which established those concentrations generating (a) no zone of inhibition, and (b) a zone of inhibition of 20~30 mm. Normally the concentrations used varied from 1 μg/ml to 50 μg/ml.

Effective antibiotic activity was recognized as a cleared area (zone-of-inhibition) in the lawn of bacterial growth. The diameter of the zones of inhibition was measured on assay plates containing nutrient agar alone (control) and nutrient agar supplemented with an amplifying agent (test plates), and the amplifying indices were calculated as the ratios of the antibiotic doses giving equivalent zone diameters on the control and the test plates (*i.e.* an index value of 1.0 indicates zero amplification). The lack of antibiotic activity of each amplifier, *per se,* was evidenced by the background confluent growth on the plates.

Results were expressed as a score ranging from — (a negative or no effect) to ## (a dose enhancement of 27). The relationship between zone-of-inhibition indices and scores is set out below:

Index range	Score	Index range	Score
≤ 1	−	$>3 \sim \leq 9$	++
$>1 \sim \leq 3$	+	$>9 \sim \leq 27$	+++

Stability of the phleomycin solution does not seem to be a factor influencing reproducibility of results; no significant differences were found in a series of tests with six selected amplifiers when solutions of phleomycin A_4A_4GPe (PLM-19) were made up and (i) stored frozen (−20°C) for 3 days before use, (ii) used immediately, (iii) used after storage at 21°C for 3 hours, or (iv) used after storage at 21°C for 6 hours.

Results

Enhancement of Antibiotic Effects by Caffeine

The original discovery[16] of caffeine amplification of phleomycin activity against *E. coli* was made with phleomycin batch A9331-648, and this material has now been shown to consist of phleomycin G (PLM-23) as the major component, with smaller amounts of phleomycins E (PLM-22) and D_1 (PLM-12) and minor amounts of other phleomycins and bleomycins. Subsequently a series of phleomycins and bleomycins which differed in the structure of the variable basic group was prepared[21] and a number of

Table 2. Antibiotic potency of some of the phleomycins** used against *E. coli* B.

Phleomycin	Antibiotic potency*	Phleomycin	Antibiotic potency*
PLM- 4	1.2	PLM-18	1
PLM- 5	0.5	PLM-19	1
PLM- 9	10	PLM-20	2
PLM-10	3	PLM-21	1.5
PLM-12	1.0	PLM-22	3
PLM-13	0.3	PLM-23	1
PLM-14	15	PLM-24	2.5
PLM-15	8	PLM-25	1.5
PLM-16	8		

* Potency is measured as the zone-of-inhibition, expressed relative to that due to PLM-G (PLM-23) at the same concentration.

** The phleomycins were tested as the copper chelate.

Table 3. Amplification of phleomycins, bleomycins and tallysomycins by caffeine.

Variable basic group of PLM/BLM/TLM	PLM	BLM	TLM	Variable basic group of PLM/BLM/TLM	PLM	BLM	TLM
− 1		++		−15	++		
− 3		++		−16	++		
− 4	++	++	++	−18	++		
− 5	++	++	++	−19	++		
− 8		+	+	−20	++		
− 9	++	++		−21	++		
−10	++	++		−22	++	++	+
−12	++	++		−23	+++	++	+
−13	++			−24	++		+
−14	++			−25	++		

For explanation of scoring, see Methods.

Table 4. Dose-enhancement by amplifying agents* of the antibacterial effect of phleomycins and bleomycins.

PLM/BLM	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
BLM-1	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
BLM-2	+	−	−	−	+	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
BLM-3	+	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
BLM-4	+	+	−	−	+	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
PLM-4	+	+	−	+	−	−	+	−	+	−	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−
BLM-5	+	−	+	+	−	−	+	++	+	+	−	+	−	+	−	+	−	+	−	−	−	+	+	−	−
PLM-5	+	+	+	+	+	−	+	++	+	−	−	+	+	++	−	+	−	−	−	−	+	+	+	−	−
BLM-6	+	−	−	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BLM-7	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
BLM-8	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BLM-9	++	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	+	+	−
PLM-9	+	−	−	−	−	+	−	−	−	−	−	−	−	++	−	−	−	−	−	−	−	−	−	−	−
BLM-10	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
PLM-10	+	−	−	−	+	+	+	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−	−	−	−
BLM-11	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
BLM-12	+	−	−	+	−	−	+	+	−	−	−	−	+	−	+	−	−	+	−	−	+	−	−	−	−
PLM-12	+	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
PLM-13	+	+			+	+	+	+	++	+	+	+	+	+	+	+	+	+		+	+				
PLM-14	+	+	−	−	+	−	−		−	+	+	−	+	−	+	−	+	−	+	+	+	−	−	−	−
PLM-15	+	+	−	−	+	−	−	+	−	−	−	−	+	−	+	+	+	−	+	+	+	−	−	−	−
PLM-16	+	+	+	−	+	−	−		−	+	+	−	−	−	+	+	+	+	+	+	+	−	−	−	−
PLM-17	−	−	−	−	−	−	−			−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−
PLM-18	++	++	−	−	++	++	++		++	++	++	++	++	++	++	++	++	++	++	++	+	++	−	−	−
PLM-19	++	+	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	+	−	−	−
PLM-20	+	+	−	−	++	++	+		++	++	++	++	++	+	++	++	++	++	++	++	+	+	+	−	
PLM-21	+	+			+		−		++	++	++	++	++	−	++	++	++		++	++	+		−	−	
BLM-22	+	+		+	+	++	++	++	+	+	+	+	++	++	++	++	++	++	++	+	+	−	−	−	−
PLM-22	+	+		−	++	++	++	++	+	+	+	++	++	++	++	++	++	+	+	+	+	−	+	−	−
BLM-23	+				+	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	+	++	−	−
PLM-23	++	+			++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	+	+		−
PLM-24	++	+			++		++		++	++	++	++	++	++	++	++	++	++		++	++				

* The amplifying agents, B~Z, are listed in Table 5.
For explanation of scores, see Methods.

bleomycins was kindly provided by Professor H. UMEZAWA and Dr. T. TAKITA. We were then able to examine the role of the basic group in determining the extent to which these antibiotics were amplified. The comparative amplifications by caffeine of a range of bleomycins, phleomycins, and tallysomycins that differ in the nature of the basic group are shown in Table 3. The extent of amplification by caffeine appears similar with all bleomycins and phleomycins tested. The effects of caffeine on the cytotoxic effects of the tallysomycins also seemed fairly homogenous but quantitatively smaller. Like caffeine the structurally similar purine theophylline (B in Table 4) enhanced the antibiotic activity of most bleomycins and phleomycins.

Enhancement of Antibiotic Effect by Other Amplifiers

Structural Requirements

A very wide range of compounds has now been found to amplify the antibiotic activity of the phleomycin mixture PLM-648 against *E. coli*[15,18]. A selection of 26 substances, representative of the various classes of compounds, is listed in Table 5 together with the concentrations used in the present study. The results of tests against a range of bleomycins and phleomycins are shown in Table 4. That most of the compounds amplify the cytotoxic effects of phleomycin G (PLM-23) is not surprising

Table 5. Key to amplifiers, concentrations used, and origin.

A	Caffeine (4×10^{-3} M)†
B	Theophylline (8×10^{-3} M)[18]
C	2-(Benzothiazol-2′-ylthio)acetamide (8×10^{-5} M)[18]
D	2-Carbamoylmethylthiobenzoxazole (8×10^{-5} M)[18]
E	9-Aminoacridine hydrochloride hydrate (8×10^{-7} M)[15]
F	3-Aminoacridine (5×10^{-6} M)[15]
G	Quinacrine (1×10^{-4} M)[15]
H	Coumarin (4×10^{-3} M)††
I	2-Carbamoylmethylthio-6,9-dimethylpurine (8×10^{-3} M)[18]
J	2-(5′,7′-Diethyl-*s*-triazolo[4,3-α]pyrimidin-3′-yl)acetamide (8×10^{-3} M)[18]
K	2-(1-Carbamoylethylthio)-*s*-triazolo[4,3-a]pyridine (8×10^{-3} M)[18]
L	3-(1-Carbamoylethylthio)-*s*-triazolo[4,3-a]pyrimidine (8×10^{-3} M)[18]
M	2-(5′,7′-Dimethyl-*s*-triazolo[1,5-c]pyrimidin-2′-ylthio)acetamide (8×10^{-3} M)[18]
N	2-Carbamoylmethylthio-*s*-triazolo[4,3-a]pyridine (1×10^{-2} M)[18]
O	3-Methylthio-*s*-triazolo[4,3-a]pyridine (1×10^{-2} M)[18]
P	Crystal violet (5×10^{-7} M)[15]
Q	1-Ethyl-2-(4′-dimethylaminostyryl)quinolinium chloride (8×10^{-6} M)[15]
R	4-Dimethylamino-4′-trimethylammoniostilbene chloride (3×10^{-5} M)[15]
S	Bindschedler's green (2×10^{-4} M)[35,36]
T	Pyronin Y (1×10^{-4} M)*
U	Nile blue (1×10^{-5} M)[15]
V	Neutral red (1×10^{-5} M)[15]
W	Brilliant cresyl blue (4×10^{-5} M)[15]
X	2-Naphthyltrimethylammonium chloride (8×10^{-5} M)**
Y	Ethidium bromide (5×10^{-7} M)[15]
Z	Toluidine blue (10^{-3} M)[15]

† A commercial sample from British Drug Houses Ltd.

†† A commercial sample from Merck AG

* A commercial sample from Serva Feinbiochemica, Heidelberg, was chromatographed on Sephadex LH-20 and precipitated from methanol - benzene.

** Prepared from purified 2-*N*,*N*-dimethylaminonaphthalene and methyl iodide, followed by ion exchange and crystallization from methanol - acetone.

because they were chosen — albeit in a different test system — on the basis of their ability to amplify the phleomycin mixture PLM-648 of which phleomycin G is the major component.

There were however wide differences in the degree of amplification found, and for most of the selected amplifiers there was a striking dependence of activity on the structure of the variable basic group of the phleomycin or bleomycin. The biological properties of those phleomycins or bleomycins having more than one guanidino group within the variable basic group, *viz.* PLM/BLM-22, PLM/BLM-23 and PLM-24 were amplified by a wide variety of compounds. The strong amplification of the ureido compound PLM-24 shows that a terminal guanidino group is not essential for amplification, and the requirement for an extended system which includes strongly basic centres is shown by the poor amplification of the monoguanidines PLM/BLM-12.

A similar situation is found with PLM-13 which has a terminal 2-phenylethylguanidino group as the only basic centre within the variable basic group (a).

(a) $-NH(CH_2)_4NHC(=NH)NH(CH_2)_2Ph$

The cytotoxic effects of PLM-13 were amplified only moderately by 9-aminoacridine, coumarin, crystal violet *etc.*, as compared to the phleomycins PLM-18, PLM-19, PLM-20 and PLM-21. These compounds have variable basic groups extended by an additional secondary amino group and additional methylene groups and range in structure from (b) to (c).

(b) $-NH(CH_2)_4NH(CH_2)_3NHC(=NH)NH(CH_2)_2Ph$

(c) $-NH(CH_2)_6NH(CH_2)_3NHC(=NH)NH(CH_2)_2Ph$

Within this set of compounds the 2-phenylethyl substituent is an essential requirement for high amplifiability, and the corresponding phleomycins which lack this substituent show little amplification (*cf.* PLM-14 and PLM-18, PLM-15 and PLM-19, PLM-16 and PLM-20, PLM-17 and PLM-21 in Table 4).

Many bleomycins and phleomycins, *e.g.* BLM-A_2 (BLM-1), BLM-EDA (BLM-2), BLM-A_2'-b (BLM-3), BLM-HPP (BLM-6), BLM-A_5 (BLM-8), BLM-CPPP (BLM-11), BLM-CPP (BLM-7) and PLM-CHPP (PLM-9) showed little indication of being amplified by most of the compounds tested excepting caffeine and theophylline.

This dependence of amplifiability on the structure of the variable basic group was not restricted to cationic-type amplifiers but extended also to other compounds, including for example, triazolopyrimidine and triazolopyridine derivatives (amplifiers J ~ O inclusive) all of which might be regarded as purine analogues and therefore more likely in their action to show a resemblance to caffeine than to the cationic amplifiers. In some instances compounds from this group of amplifiers showed relatively weak amplification of other phleomycins, but strong amplification was restricted to the same group of guanidino phleomycins that was amplified by the cationic amplifiers.

In all instances in which bleomycins and phleomycins having the same variable basic group were available for comparison, the amplification of antibiotic activity was similar. Thus bleomycin BLM-B_6 (BLM-23) was amplified by virtually all the compounds tested, and the degree of amplification was similar to that observed with the corresponding phleomycin G (PLM-23); while other bleomycins such as BLM-CHPP (BLM-9) and bleomycin PEPP (BLM-10) were amplified by few substances, as were

Table 6. Amplification and antibiotic potency of phleomycins.

Trivial designation		Potency	Amplification by compound*								
			F	G	H	J	K	L	P	Q	T
PLM-14	PLM-A_4A_3G	15	+	−	−	−	+	+	+	−	+
PLM-18	PLM-A_4A_3GPe	1	++	++	++	+++	+++	+++	+++	+++	++
PLM-15	PLM-A_4A_4G	8	+	−	−	−	−	−	+	+	+
PLM-19	PLM-A_4A_4GPe	1	++	++	++	+++	+++	+++	+++	+++	+++
PLM-16	PLM-A_5A_3G	8	+	−	−	−	+	+	+	+	+
PLM-20	PLM-A_5A_3GPe	2	++	++	+	++	+++	+++	+++	++	+++
PLM- 4	PLM-CHP	1.2	−	−	+	+	−	+	−	+	−
PLM- 5	PLM-PEP	0.5	+	−	+	+	−	−	−	+	−
PLM- 9	PLM-CHPP	10	−	−	−	−	−	−	−	−	−
PLM-10	PLM-PEPP	3	+	+	+	−	−	−	−	+	+
PLM-12	PLM-A_4G	1	−	−	−	+	−	−	−	−	+
PLM-13	PLM-A_4GPe	0.3	+	+	+	++	+	+	+	+	
PLM-22	PLM-AGG	3	++	++	++	+	+	+	++	+++	+
PLM-23	PLM-AGGG	1	++	++	++	+++	++	+++	+++	+++	+++

* See Table 5 for key to compound code.

the corresponding phleomycins.

A few Cu-free bleomycins were included in the trial although the majority were Cu-chelates. Copper salts were present in the incubation medium in amounts sufficient to saturate the bleomycins present, and no differences were observed in the pattern of amplification of bleomycin CHPP (BLM-9) or bleomycin CPPP (BLM-11) either as the copper-free or copper-containing compounds, but since the cytotoxic effects of these antibiotics were poorly amplified by most compounds other than caffeine, this result is of limited usefulness.

Is There an Association between Antibiotic Potency and Ability to be Amplified?

Taking the phleomycins and bleomycins together as one population of antibiotics, clearly there is no correlation between cytotoxic potency and enhancement of that cytotoxicity by a variety of amplifying compounds (Tables 2, 4 and 6). If, however, we consider only the subgroup of phleomycins with basic groups of the general form $-NH(CH_2)_nNH(CH_2)_mNHC(=NH)NH_2$ without a terminal 2-phenylethyl group (abbreviated -A_nA_mG) or with a terminal 2-phenylethyl group (abbreviated to -A_nA_mGPe), a constant difference seems apparent (Table 6). Those phleomycins having a terminal 2-phenylethyl group were amplified well by a variety of compounds whereas those phleomycins lacking this group were not; and, whereas the latter had a relatively high cytotoxicity when administered alone to bacteria, the corresponding phleomycin having a terminal 2-phenylethyl group had a relatively low cytotoxicity.

Some compounds, *e.g.*, ethidium bromide, at the concentration tested either had no effect or they actually inhibited the cytotoxic effect of particular phleomycins or bleomycins when presented simultaneously to the target bacteria although they have been reported to amplify cytotoxic effects at other concentrations in other treatment regimes[15] where the phleomycin/bleomycin and the compound were administered sequentially to the bacteria rather than simultaneously as in this study.

Bleomycins *versus* Phleomycins

In vitro studies with DNA[26,27] demonstrate little difference between the mode of action of bleomycins and phleomycins. Our results on amplification *in vivo* also exhibit a close similarity between corresponding phleomycins and bleomycins. Although tallysomycins displayed similar effects to bleomycins and phleomycins the extent of amplification was not as pronounced.

Discussion

Reliability of the Test Method

The zone-of-inhibition plate method used to produce the results in Tables 2, 3, 4 and 6 has been found to give sufficiently reproducible results to enable most combinations of antibiotic and amplifier to be assigned consistently to one of the designated categories of amplification score (−, +, ++ or +++). As the method is not sensitive to small differences in amplifiability, this assignment will necessarily be less reliable when the determined index value lies close to a boundary between categories. For example, the category + will include all combinations experimentally given an index $>1 \sim \leq 3$, and this will necessarily include a number of doubtfully amplified combinations. Greater reliability may be attached to those values that are consistent with a general pattern of amplification.

There are several factors inherent in the test procedure that limit quantitative comparison of amplification indices for different antibiotics. Because different phleomycins differ in their antibiotic activity against *E. coli* (Table 2) and the estimate of amplifiability is made at approximately equal activity, it is made at different concentrations. Neither the effect of this difference on the diffusion gradient, nor the effect of variable basic group structure on the rate of diffusion is known.

Mechanism of Amplification

Caffeine is a well known DNA repair inhibitor in *E. coli* and inhibition of repair of PLM-damaged DNA could explain the observed enhancement of the biological effects of the phleomycins and bleomycins. In *E. coli* B, and at concentrations greater than 1 mM and less than 8 mM, caffeine is an efficient inhibitor of UV-excision repair mediated by the *uvr* repair pathway whilst the other major repair pathway mediated by the *recA* pathway is unaffected[28]. In addition the repair of phleomycin-induced breakage in buffer is unaffected by caffeine (SLEIGH and GRIGG, unpublished observations). Moreover *E. coli uvrA* and *uvrB* mutants defective in excision-repair capability are no more sensitive to phleomycin than are the corresponding *uvr*$^+$ strains. Indeed in strain *E. coli* B, *uvrA* and *uvrB* mutants are significantly less sensitive[25]. Thus, the excision-repair pathway appears to be unimportant in the repair of phleomycin-induced damage to DNA in *E. coli*. It also seems unlikely that inhibition of DNA repair, in general, is the sole or principal explanation of the amplification of the biological effects of phleomycin by caffeine or by a representative collection of other amplifying agents such as crystal violet, methylene blue, 3-aminoacridine and ethidium bromide (GRIGG, unpublished observations).

Modification of membrane permeability of *E. coli, e.g.* with lysozyme and EDTA treatment, enhances sensitivity to lower concentrations of phleomycin[29], and MIZUNO and ISHIDA[19] demonstrated that a number of membrane-modifying compounds may enhance the cytosomic effects of bleomycins. The effects of caffeine and indeed of the other substances listed in ref 15 and used in this study cannot be explained on this basis. Pretreatment of *E. coli* with caffeine does not enhance the biological effects of phleomycins whereas post-treatment does[16]. In general, post-treatment with an amplifying compound of bacteria previously exposed to phleomycins is at least as effective as and often more effective than simultaneous exposure[15].

Whether some of the other amplifiers listed in Table 5 influence cell-uptake of phleomycins, or rate of reaction with DNA, or both, is not known but it is remarkable that such a diverse group of substances should have similar effects, and particularly that there should be in most instances a similar dependence of amplifier activity on the structure of the variable basic group. The list of amplifiers (Table 5) used in this study includes substances which are known DNA intercalating agents (*e.g.* the amino acridines) and others such as crystal violet, which are known to bind to (AT-rich regions of)

DNA in a non-intercalator fashion and to bring about unwinding of supercoiled DNA[30].

In the absence of degradative DNA nucleases DNA breakage by bleomycins and phleomycins proceeds by a free radical-mediated pathway which requires as cofactors a metal chelate (usually Fe), O_2, and an electron exchange agent such as a thiol[8~12]. The reaction is efficiently and completely inhibited by potassium iodide[31]. Unlike the *in vitro* results, in bacteria this potassium iodide-mediated inhibition is complete only at low (<5 μg/ml) phleomycin concentrations[32]. The requirement for a thiol cofactor at low phleomycin or bleomycin concentrations disappears at higher concentrations[33]. Taken together, these observations tend to suggest that an additional DNA breakage mechanism can operate *in vivo* in addition to the active radical-medated process. Recently we have detected an endonuclease which has a role in modifying sensitivity to phleomycins and bleomycins (HALL, unpublished observations).

Amplification of the biological effects of phleomycins and bleomycins, described here, extends also to mammalian tumor cells and hence may have possible practical significance[34].

Recent studies using phleomycin/bleomycin-resistant mutants of *E. coli* suggest that the mechanism is associated with DNA structural changes. This will be the subject of a future communication.

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