AMPLIFICATION OF THE ANTITUMOR ACTIVITY OF PHLEOMYCINS IN RATS AND MICE BY HETEROCYCLIC ANALOGUES OF PURINES

T. E. ALLEN, D. J. BROWN[†††], W. B. COWDEN[†††], G. W. GRIGG, N. K. HART[†], J. A. LAMBERTON[†] and A. LANE[††]

The Division of Molecular Biology, The Division of Applied Organic Chemistry[†] and The Division of Food Research[††] of C.S.I.R.O. Australia
The John Curtin School of Medical Research[†††]
Canberra, Australia

(Received for publication November 17, 1983)

Significant and often substantial enhancement of the antitumor properties of several individual phleomycins, by co-administration *via* intraperitoneal injection of a number of purine analogues, is demonstrated in rats and mice having three diverse tumors. It is evident that the dose levels of both the phleomycin and the amplifier are very significant and that optimal levels vary widely with the actual agents used. Constant serum levels of amplifier can be maintained for several days by administration *via* silastic-pellet implantation rather than injection, and this route of administration is an effective alternative for amplifiers of low solubility.

The antibiotic phleomycins[1,2] were found to have marked antitumor activity against Sarcoma 180, Adenocarcinoma 755 and Ehrlich's ascites tumor *in vivo*[3]. However, following the report of an irreversible dose-related nephrotoxicity in dogs and monkeys[4] following intravenous doses of 0.16 mg/kg[5], a planned clinical study was abandoned. It was found subsequently that the presence of two or more guanidine groups in the variable basic group of the phleomycin (PLM) molecule was responsible for the toxicity, and thus that it should be possible to prepare PLMs which do not cause irreversible renal toxicity[6].

Meanwhile, it was noticed[7~9] that the activity of PLM-648, a mixture of PLM-G, PLM-E and PLM-D_1 (see Table 1a; *cf.* ref 10), against the growth of *Escherichia coli* was increased substantially when certain DNA-binding compounds, including caffeine (**1**) and other purines, were used in combination with it. This suggested the possibility of developing a phleomycin-amplifier antibiotic regime having a higher therapeutic index than that of the phleomycin alone. Later, numerous purine analogues were found to amplify the antibiotic properties of PLM-648 and of other PLMs[10]. In the present work several such purine analogues were tested as amplifiers of the activity of phleomycins against

Table 1a. Structure of the variable basic groups and trivial designation of the PLMs used in the experiments.

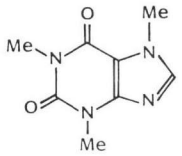
PLM-E	$NH(CH_2)_4NHC(:NH)NH(CH_2)_4NHC(:NH)NH_2$
PLM-G	$NH(CH_2)_4NHC(:NH)NH(CH_2)_4NHC(:NH)NH(CH_2)_4NHC(:NH)NH_2$
PLM-AAGPe	$NH(CH_2)_4NH(CH_2)_4NHC(:NH)NH(CH_2)_2Ph$
PLM-D_1	$NH(CH_2)_4NHC(:NH)NH_2$
PLM-CHP	$NH(CH_2)_8NHC_6H_{11}$[a]
PLM-PEP	$NH(CH_2)_3NHCHMePh$
PLM-648	A natural mixture of PLM-E, PLM-G and PLM-D_1

[a] cyclohexyl

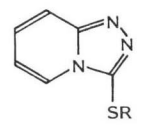
Table 1b. Trivial name, IUPAC* name, formula and source of purine analogues tested as amplifiers of PLMs.

Trivial name	Formula number	IUPAC name	Ref
141/4a	**2**	3-Methylthio-*s*-triazolo[4,3-*a*]pyridine	10
TAP	**3**	2-(*s*-Triazolo[4,3-*a*]pyridin-3′-ylthio)acetamide	11
ETAP	**4**	2-(*s*-Triazolo[4,3-*a*]pyridin-3′-ylthio)propionamide	11
TAPM	**5**	2-(*s*-Triazolo[4,3-*a*]pyrimidin-3′-ylthio)propionamide	11
YI27	**6**	2-(5′,7′-Diethyl-*s*-triazolo[4,3-*a*]pyrimidin-3′-ylthio)acetamide	12
TN37-4	**7**	2-(5′,7′-Dimethyl-*s*-triazolo[1,5-*c*]pyrimidin-2′-ylthio)acetamide	13
LS34	**8**	*N*,*N*-Dimethyl-2-[4′-(thien-2″-yl)pyrimidin-2′-ylthio]ethylamine (HBr)	14
BC55	**9**	*N*,*N*-Dimethyl-2-[4′-(pyridin-4″-yl)thiazol-2′-ylthio]ethylamine (2HBr)	15
BC135	**10**	3-(2-Chlorophenothiazin-10′-yl)-*N*,*N*-dimethylpropylamine (HCl)	Sigma

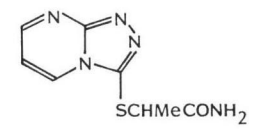
* International Union of Pure and Applied Chemistry.



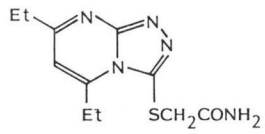
1



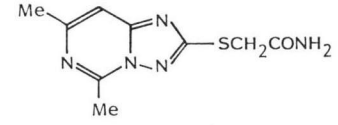
2 R = Me
3 R = CH_2CONH_2
4 R = $CHMeCONH_2$



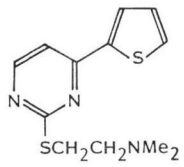
5



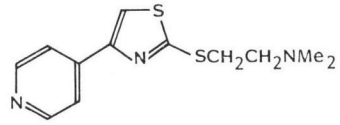
6



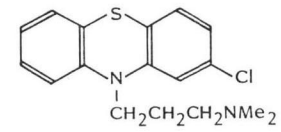
7



8



9



10

diverse animal tumor models *in vivo*.

Materials and Methods

Compounds Used

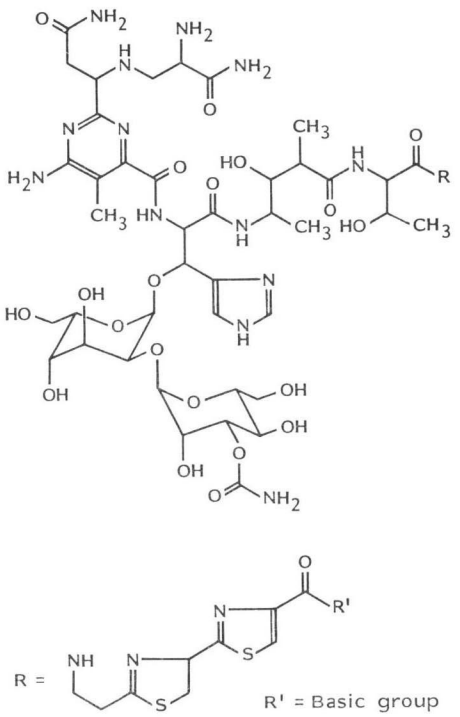
The general structure of the PLMs is shown in Fig. 1. The structure of the variable basic groups and the trivial name for the corresponding PLMs, used in our experiments, are given in Table 1a. Phleomycins were in the copper-containing form in all experiments except those combining PLM-PEP with LS34, PLM-PEP with BC55 and PLM-CHP with BC135. The proper name, trivial name, structure and source of the purine analogues tested as amplifiers are given in Table 1b.

Animal Trials

General Technique: The tests were carried out on 4-week old male Sprague-Dawley rats weighing 100 ± 10 g and on 4-week old male Swiss mice weighing 23 ± 2 g each. Ehrlich tumor (EA) and Sarcoma 180 (S180), both in the ascites form, were used in mice, and Walker 256 carcinosarcoma

(W256), also in the ascites form, was used in rats. The tumors were passaged by the intraperitoneal (ip) injection of 10^6 cells, prepared by aspirating several ml of ascites fluid from a tumor-bearing rat or mouse. An aliquot (0.1 ml) of mouse ascites fluid was diluted 1,000-fold with Eagle's medium (Minimum Essential Medium for Suspension Cultures, Flow Laboratories Inc., Rockville) and the number of live cells was determined on a haemocytometer after a further 1:1 dilution with Turk's fluid [glacial acetic acid (1 ml), crystalline Gentian Violet (10 mg), water (99 ml)]. The bulk of the ascites fluid was then diluted with Eagle's medium to give a concentration of 10^7 live cells per ml. The mice were injected intraperitoneally with this suspension (0.1 ml each) within one hour of collecting the ascites fluid, all preparations being handled over ice during that period. The rat cells were treated similarly except that they were given two treatments (for 2 minutes each time) in Boyle's fluid [pH 7.2: 0.83% ammonium chloride (9 vol) plus Tris buffer (1 vol)] [composition of the Tris buffer: Tris(hydroxymethyl)aminomethane (1.92 g/liter)+sodium chloride (5.27 g/liter)+disodium hydrogen phosphate dihydrate (0.04 g/liter)+potassium chloride (0.26 g/liter)+potassium dihydrogen phosphate (0.04 g/liter)+calcium chloride (0.11 g/liter)] before an aliquot was counted in the haemocytometer and a dilution to 10^7 live cells per ml was made. In all cases, a Bacto-fluid thioglycollate medium (NIH Thioglycollate Broth, Difco Laboratories, Detroit) was used to test for contamination. In one series of 18 experiments, three purine analogues (TAPM, ETAP and TN37-4) were tested as amplifiers of two PLMs (PLM-E and PLM-AAGPe). Each amplifier was used at two concentrations with each of the two PLMs at 5 concentrations. These tests were carried out on 3 different tumor models (W256 in rats, Ehrlich ascites tumor and Sarcoma 180 in mice), thus making 18 experiments in all. Other single experiments were also carried out with a variety of PLMs at various concentrations and with several other purine analogues being tested as amplifiers. Relevant controls (PLM without amplifier, amplifier without PLM, phosphate buffered saline only) were included, and procedures were based on those outlined by GERAN *et al.*[17]. In the context of amplification, the test group was that treated with the PLM plus amplifier and the control group was that treated with the same dose level of the same PLM without amplifier. All groups were retained until their median survival time had been passed or, in a few instances, until 100 days had elapsed. A T/C (the median survival time of the test group divided by that of the control group, expressed as a percentage) value of 125 or more was taken as a positive result, in keeping with the criterion expressed by the National Cancer Institute of the U.S.A.[17].

Fig. 1. Structure of PLMs.



Implantation of Pelleted Material: Two of the compounds being tested as amplifiers (ETAP and YI27) were given as subcutaneously implanted pellets in some experiments. Serum levels of ETAP after such implantation were estimated.

The pellets were made from equal weights of ETAP and Silastic RTU732 which were mixed and allowed to set on a glass plate. The mixture was then cut into rectangular blocks of 400 mg each. For smaller amounts, as with YI27, the mixture was made similarly and then pressed into a plastic 1 ml tuberculin syringe for setting. Later, the top of the syringe was cut through to allow the cylindrical plug to be expressed and sliced into 100 mg pellets. These were inserted beneath the skin of the flank region of anaesthetised rats. The anaesthetic was made up by diluting 0.25 ml of a stock solu-

tion, consisting of 2,2,2-tribromoethanol (10 g) plus Amyl Alcohol (10 ml), with 9.75 ml of aqueous 0.9% sodium chloride.

Serum Levels of Amplifying Agents

From ETAP Pellets: After implantation of the pellets, blood was collected by tail-bleeding each animal at 2 day intervals; half the animals were bled on even days, the others on odd days. On each occasion the blood was pooled because at least 0.5 ml of serum was required for each assay. Bleeding was more effective when each animal was warmed under infrared lamps for 30 minutes beforehand.

For assay, each sample was treated with Pronase and applied to thin-layer chromatography plates (1 mm silica gel). After drying at 40°C in a fan oven, the sample at the origin was extracted five times with ascending ethanol to remove ETAP from other material which remained at the origin: the ethanol was allowed to rise 2~3 cm above the origin on each occasion before the plate was dried again as above. The ethanol-insoluble material at the origin was finally scrapped off and the ETAP just above was separated from other ethanol-soluble materials in the zone by development with chloroform - ethanol (9: 1): the ETAP band was recognized by its Rf and blue fluorescence in 250~300 nm light. It was subsequently eluted with ethanol and estimated by fluorimetry at the emission wavelength (420 nm) after excitation in short wavelength light (Ito spectrophotometer modified with a Beckman fluorimeter attachment); the blank was 3% transmittance, and full-scale transmittance was set for 10 μg of ETAP.

From Intraperitoneal TAP: A ^{14}C-labelled sample[12] of TAP was used to determine serum levels. Thus a solution of [^{14}C]TAP (32.31 mg/ml, 1.01 μCi/ml) in phosphate-buffered saline was administered by intraperitoneal injection to 10 male rats (0.2 ml each). These rats were then bled, by decapitation, at 10, 15, 20, 30, 45, 60, 90, 120, 150 and 180 minutes after injection. The serum from each blood sample was spun off and frozen until assayed in a Packard scintillation counter.

Results

A significant and often substantial enhancement of the antitumor effect of several PLMs by a number of purine analogues was detected. The results of a series of trials are shown in Table 2. Amplifiers TAP, TAPM, ETAP, TN37-4, BC55 and BC135 all showed significant enhancement, with a T/C value greater than 200% on at least one testing, indicating that the use of these compounds can increase the life-span to more than twice that of animals receiving PLM alone. With some regimes an increased antitumor effect was produced only at PLM doses which alone had some antitumor properties. With others antitumor activity was observed at PLM doses which, *per se*, had no antitumor effect.

A similar phenomenon has been observed in dose-response curves associated with the amplification of PLM as an antibacterial agent *in vitro*[12,18]. No estimates of reproducibility can be given but the consistency of the enhancement effect can be seen from Table 3 when a series of similar experiments was carried out using five levels of two different PLMs with two levels of three different amplifiers on each of three different tumor models. Of these 18 combinations of antibiotic, amplifier and tumor, only two did not show a T/C of 125% or more, *i.e.* an increase in life-span of 25% due to the effect of the amplifier.

Prior to adopting an ip dose schedule for PLM and amplifier (twice daily for five consecutive days), the serum level of one radioactively labelled amplifier ([^{14}C]TAP) was measured after a single ip administration. Fig. 2 shows that a peak level of 86 μg/ml was attained in the serum after 15 minutes; after 45 minutes the level had decreased to 40 μg/ml, and after 3 hours it was only *ca.* 1 μg/ml. Since the virtually complete elimination of unchanged TAP in the urine is known[12] to take much longer (12~18 hours), a depot effect (possibly through binding to tissue DNA) must be assumed.

Table 2. Enhancement of antitumor properties of PLMs in rats and mice by purine analogues.
Dosage schedule: Twice daily ip injection for first 5 days except where pellet indicated.

Tumor	PLM (see Table 1a)	Amplifier (see Table 1b)	Dose (mg/kg body weight/day)		Best observed dose combination (mg/kg body weight/day)		Medium survival time (days)			T/C (%) Repeats in italics
			PLM concentration range (5 levels)	Amplifier concentration	PLM	Amplifier	PLM alone (C)	PLM+ amplifier (T) (optimal)	Buffered saline alone (C)	
W256	648	TAP	0.004~ 0.008	20; 250	0.008	250	10	38	9	380
EA	AAGPe	141/4a	0.05 ~ 4.05	125; 250	0.45	125	26	38	17	146
W256	AAGPe	TN37-4	0.125~ 2.00	0.294; 2.94	2.00	2.94	27.5	50	8	181
W256	E	TN37-4	0.125~ 2.00	0.369; 3.69	0.50	0.369	24	>100	10	416 (>*266*)
S180	648	TN37-4	0.063~16.00	0.003; 0.31	0.063	0.31	14	21	13	150
S180	CHP	TN37-4	0.125~ 2.00	0.363; 3.63	0.25	0.363	17	24	13.5	141
S180	AAGPe	TN37-4	0.125~ 2.00	0.353; 3.53	2.00	3.53	31.5	45	14	145
EA	AAGPe	TN37-4	0.125~ 2.00	0.365; 3.65	0.25	3.65	17	24	17	141
S180	E	TN37-4	0.125~ 2.00	0.382; 3.82	0.25	0.382	17	24	13	141
W256	AAGPe	ETAP	0.125~ 2.00	25; 250	2.00	25	24	>100	9	416 (>*300*)
W256	E	ETAP	0.125~ 2.00	25; 250	1.00	250	35	59	9	168 (*142*)
EA	E	ETAP	0.125~ 2.00	25; 250	0.50	250	22	30	16	136
S180	E	ETAP	0.125~ 2.00	25; 250	0.25	250	17	24	15	141 (*144*)
S180	AAGPe	ETAP	0.125~ 2.00	25; 250	2.00	25	17	27.5	16	161 (>*176*)
W256	648	ETAP	0.003~ 0.145	50% pellet	0.145	50% pellet	9.5	16.5	10.5 (pellet)	157
W256	E	TAPM	0.125~ 2.00	5.5; 27.5	2.00	5.5	26	42	9	161
W256	AAGPe	TAPM	0.125~ 2.00	5.5; 27.5	0.50	5.5	13.5	29	9	214
W256	648	TAPM	0.004~ 0.008	5.5; 27.5; 55	0.004	27.5	10	37	9	370
EA	E	TAPM	0.125~ 2.00	5.5; 27.5	1.00	5.5	31	>100	13	>322
S180	E	TAPM	0.125~ 2.00	5.5; 27.5	0.50	27.5	17	24	13.5	141
S180	CHP	TAPM	0.125~ 2.00	5.5; 27.5	0.25	27.5	13	17	11	130
W256	E	YI27	0.008~ 0.04	5~50% pellet	0.04	5% pellet	8	12	8 (pellet)	150
EA	D_1	LS34	0.125~ 4.00	3.50; 28.0	1.00	7.00	30	>40	17	>135
EA	PEP	LS34	0.125~ 4.00	10.0; 30.0	2.00	10.0	31.0	>48.5	16	>156
EA	PEP	BC55	0.125~ 4.00	18.75; 150	1.00	75.00	24	>93	17	>387
EA	PEP	BC135	0.118~15.00	2.73; 17.1	0.50	6.8	23	>61	16.5	>265
EA	CHP	BC135	0.500~16.0	4.0; 8.0	2.00	4.0	37	>80	16.0	>216 (>*155*)

Fig. 2. Serum level (μg/ml) of [^{14}C]TAP in Sprague-Dawley rats after ip injection.

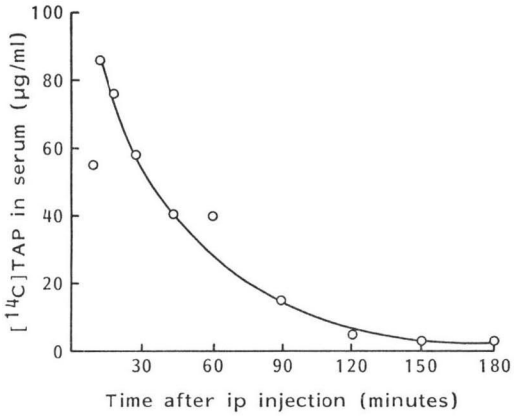


Fig. 3. Serum level (μg/ml) of ETAP in Sprague-Dawley rats following sc implantation of pellets (50% ETAP in Silastic).

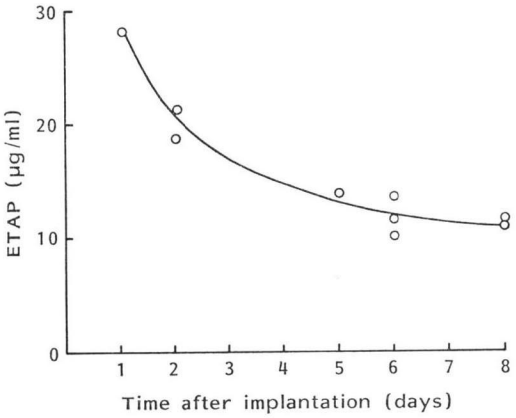


Table 3. Amplification of PLM-E and PLM-AAGPe by ETAP, TAPM and TN37-4.

Amplifier	T/C value					
	PLM-E			PLM-AAGPe		
	W256	EA	S180	W256	EA	S180
ETAP	142	136	144	>300	<125	>176
TAPM	161	>322	141	214	220	<125
TN37-4	>266	136	141	181	141	145

Some of the compounds had too low an aqueous solubility to be administered conveniently by injection and so were implanted subcutaneously as pellets. Serum levels of ETAP were measured following implantation and are shown in Fig. 3: concentrations of 10~20 μg/ml were maintained for at least 8 days. This resulted in no significant increase in T/C value for ETAP over that obtained from ip administration, but the point is made that such implantation does offer a practical alternative administrative route for relatively insoluble amplifiers, such as YI27.

Discussion

The protocols of the National Cancer Institute of the U.S.A. require, in rodent tumor systems using survival as the parameter in evaluation, that a reproducible T/C of 125% is necessary to demonstrate activity[17]. If we use these standards for antitumor activity and apply them to amplification of antitumor activity, the results given in Table 2 show that in all 33 experiments the T/C values were 125% or more; of these, 21 were 150% or more, and 11 were in excess of 200%. Thus there can be no doubt that the compounds tested showed amplification activity. Another criterion of activity can be seen in a patent application[19] where the maximum dose level within the range of longevity ratio (T/C) of 100~200 was considered active. In practice this turned out to be a T/C value approaching 200%. Again it is apparent that the increases in survival time due to amplification obtained in the present study compare favourably with published requirements for activity.

There are few published data describing the enhancement of antitumor effects of one compound by a second compound. However, activity enhancement of 1,3-bis(2-chlorethyl)nitrosourea (BCNU) by vitamin A and of the BCNU and vitamin A combination by the addition of caffeine to the treatment regime has been reported[20]. The results obtained in the present study compare favorably with

those given for the amplification of BCNU by vitamin A and the best of the results in this study (those with T/C values in excess of 200%) also compare favorably with those given for the amplification of the BCNU-vitamin A combination by caffeine, but obtained without the toxicity of the high dosage of caffeine (6.0 mg/mouse) used to amplify the BCNU and vitamin A combination. COHEN[21] also reported that the addition of chlorpromazine (see BC135, Table 1b) to the BCNU/vitamin A combination enhanced the antitumor effect against L1210 leukemia in mice by increasing the cure rate from 5% to 51%. Some of the amplifiers described here have been tested for acute toxicity and have been found to be remarkably non-toxic[12].

The low aqueous solubility of some compounds ruled out the possibility of delivering them by subcutaneously implanted ALZA minipumps (ALZA, Palo Alto, CA, USA) but delivery by subcutaneously-implanted mixtures in Silastic pellets was quite effective with two such compounds (ETAP and YI27). In man, at least some of these insoluble amplifiers could probably be administered in tablet form *per os*, because several of those tested have been found to be stable to the environment of the mouse gut.

The fact that enhancement was found when two amplifiers were given as a subcutaneously-implanted pellet (PLM given by ip injection) indicates that at least in these two cases no initial complexes are formed between PLM and amplifier, and that the enhancement phenomenon observed is not just a result of both compounds acting on the tumor cells by a pseudo *in vitro* effect.

References

1) MAEDA, K.; H. KOSAKA, K. YAGISHITA & H. UMEZAWA: A new antibiotic, phleomycin. J. Antibiotics, Ser. A 9: 82~85, 1956
2) TAKITA, T.; Y. MURAOKA, T. NAKATANI, A. FUJII, Y. UMEZAWA, H. NAGANAWA & H. UMEZAWA: Chemistry of bleomycin. XIX. Revised structures of bleomycin and phleomycin. J. Antibiotics 31: 801~804, 1978
3) BRADNER, W. T. & M. H. PINDELL: Antitumor properties of phleomycin. Nature 196: 682~683, 1962
4) ISHIZUKA, M.; H. TAKAYAMA, T. TAKEUCHI & H. UMEZAWA: Studies on antitumor activity, antimicrobial activity and toxicity of phleomycin. J. Antibiotics, Ser. A 19: 260~271, 1966
5) RAKIETEN, N.; M. V. NADKARNI, M. L. RADKIETEN & B. S. GORDON: Toxicologic and pharmacologic evaluation of phleomycin including special studies on its nephrotoxicity. Toxicol. Appl. Pharmacol. 14: 590~602, 1969
6) UMEZAWA, H.: Structure and action of bleomycin. Progr. Biochem. Pharmacol. 11: 18~27, 1976
7) GRIGG, G. W.; M. J. EDWARDS & D. J. BROWN: Effects of coumarin, thiopurines and Pyronin Y on amplification of phleomycin-induced death and deoxyribonucleic acid breakdown in *Escherichia coli*. J. Bacteriol. 107: 599~609, 1971
8) BROWN, D. J.; R. L. JONES, A. M. ANGYAL & G. W. GRIGG: Purine studies. VII. The synthesis of purines as amplifiers of phleomycin against *E. coli*. J. Chem. Soc., Perkin Trans I 1972: 1819~1825, 1972
9) GRIGG, G. W.; A. M. GERO, J. M. HUGHES, W. H. F. SASSE, M. BLIESE, N. K. HART, O. JOHANSEN & P. KISSANE: Amplification of phleomycin and bleomycin-induced antibiotic activity in *Escherichia coli* by aromatic cationic compounds. J. Antibiotics 30: 870~878, 1977
10) BROWN, D. J. & G. W. GRIGG: Heterocyclic amplifiers of phleomycin. Med. Res. Rev. 2: 193~210, 1982
11) POTTS, K. T.; H. R. BURTON & S. K. ROY: 1,2,4-Triazoles. XIV. Reactions of the *s*-triazolo 4,3-*a* pyridine ring system. J. Org. Chem. 31: 265~273, 1966
12) BROWN, D. J.; W. C. DUNLAP, G. W. GRIGG, L. DANCKWERTS & T. NAGAMATSU: Purine analogues as amplifiers of phleomycin. II. Some 3-triazolo 4,3-*a* pyridines, *s*-triazolo 4,3- or 1,5-*a* pyrimidines and *s*-triazolo 3,4-*b* benzothiazoles. Aust. J. Chem. 31: 397~404, 1978
13) BROWN, D. J. & Y. IWAI: Purine analogues as amplifiers of phleomycin. VI. The synthesis and metabolism of some benzothiazole, benzoxazole *s*-triazolopyrimidine amplifiers. Aust. J. Chem. 32: 2727~2733, 1979
14) BROWN, D. J.; G. W. GRIGG, Y. IWAI, K. N. MCANDREW, T. NAGAMATSU & R. VAN HEESWYCK: Purine analogues as amplifiers of phleomycin. V. Thioethers of several heterocyclic systems with one or two rings. Aust. J. Chem. 32: 2713~2726, 1979

15) BROWN, D. J.; W. B. COWDEN & L. STREKOWSKI: Unfused heterobicycles as amplifiers of phleomycin. VI. Some thienyl- and thiazolyl-pyrimidines with strongly basic side chains. Aust. J. Chem. 35: 1209~1214, 1982
16) BROWN, D. J.; B. B. BUTTLER, W. B. COWDEN, G. W. GRIGG, D. KAVULAK & D. M. PODGER: Unfused heterobicycles as amplifiers of phleomycin. III. Thiazolopyridines and bipyrimidines with strongly basic side chains. Aust. J. Chem. 34: 2423~2429, 1981
17) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against animal tumors and other biological systems. 3rd Ed., Cancer Chemother. Rep. 3: 1~103, 1972
18) BROWN, D. J.; W. C. DUNLAP, G. W. GRIGG & L. DANCKWERTS: Purine analogues as amplifiers of phleomycin. III. Some 2-alkylthio derivatives of imidazole, benzimidazole, benzoxazole and benzothiazole. Aust. J. Chem. 31: 447~450, 1978
19) UMEZAWA, H.; A. FUJII, T. TAKITA & A. N. SHIMADA: Novel process for producing phleomycin group antibiotics. US Patent 3,984,390, 1976
20) COHEN, M. H.: Enhancement of the antitumor effect of 1,3-bis(2-chloroethyl)-1-nitrosourea by vitamin A and caffeine. Natl. Cancer Inst. J. 48: 927~932, 1972
21) COHEN, M. H.: Enhancement of the antitumor effect of 1,3-bis(2-chloroethyl)-1-nitrosourea by various psychotropic drugs in combination with caffeine. J. Pharmacol. Exp. Ther. 194: 475~479, 1975