Purines as Amplifiers of the Antibiotic Activity of Phleomycin against *Escherichia coli* B

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Phleomycin is a copper-containing compound isolated from *Streptomyces verticillus* (Maeda, Kosaka, Yagishita & Umezawa, 1956; Takita *et al.* 1972) with a broad range of antibiotic and antitumour activity (Smale, Montgillion & Pridham, 1961; Bradner & Pindell, 1962; Tanaka, Yamaguchi & Umezawa, 1963; Kihlman, Odmark & Hartley, 1967; Iwata & Consigli, 1971; Reiter, Milewski & Kelley, 1972). At low concentrations it induces DNA breakdown and death in growing cultures of *Escherichia coli* B but has little effect on stationary-phase bacteria (Grigg, 1969). At higher concentrations this difference disappears. Exposure of phleomycin-treated bacteria to caffeine, pyronin Y, coumarin or certain thio-purines initiates DNA degradation and death. The effects are similar to those caused by increasing the phleomycin concentration up to 100-fold (Grigg, 1970; Grigg, Edwards & Brown, 1971).

Phleomycin binds at the adenine–thymine rich regions in DNA, probably to the thymine bases (Falaschi & Kornberg, 1964; Pietsch & Corbett, 1968), and distorts its secondary structure, inducing localized denaturation (Sleigh & Grigg, 1974). These changes are thought to sensitize the DNA *in vivo* to enzymic attack, resulting in DNA breakdown and death (Grigg *et al.* 1971). Caffeine interacts with phleomycin-treated DNA to increase the frequency of locally denatured regions, and we have proposed that the ability of caffeine and other ligands such as coumarin and the thiopurines to bind preferentially to single-stranded DNA (Grigg *et al.* 1971) enables these compounds to enhance local denaturation around phleomycin–thymine complexes, resulting in the observed nuclease-induced DNA breakage, degradation and death.

In the structure–activity relationships of biologically active compounds, the relative lipophilicity indicated by the partition coefficient (p.c.) between octanol and water, may or may not be important (Albert, 1973). If the p.c. is a factor (as in antibacterial oxine derivatives), transport across a fatty cell membrane may be inferred; if not (as in the local anaesthetics), it may be assumed that no such process is involved. Preliminary observations (Brown, Jones, Angyal & Grigg, 1972) suggest that the lipophilicity conferred by substituents could be important to the potentiating activity of purines towards phleomycin.

We report the potentiating activities and p.c. values for 63 purines, and some tentative correlations between structure and biological activity.

Table 1. *Phleomycin: amplifying activities, partition coefficients and sources of purines*

No.	Purine	Adjusted activity*	Partition coefficient†	Source
1	Unsubstituted	2 +	0.4	Albert & Brown, 1954
2	2-SMe	20 ++	10	Albert & Brown, 1954
3	2,8-(SMe) ₂	70 +++	85	Albert, 1966
4	2-SMe-6-OH	30 ++	—	Elion, Lange & Hitchings, 1956
5	2-SMe-8-OH	20 ++	—	Noell & Robins, 1959
6	2-SEt	70 ++	36	Badger, Brown & Lister, 1973
7	2-SEt-6-OH	7 ++	—	Gouch & Maguire, 1967
8	6-SEt	60 ++	30	Elion, Goodman, Lange & Hitchings, 1959
9	8-SEt	70 ++	13	Badger <i>et al.</i> 1973
Monoalkyl				
10	6-Me-2-SEt	280 ++++	62	Badger, Brown & Lister, 1974
11	6-Me-2,8-(SMe) ₂	70 +++	153	Brown <i>et al.</i> 1972
12	6-Me-2-NMe ₂	220 ++++	—	Badger <i>et al.</i> 1974
13	6-Et-2-SMe	2 +	65	Badger <i>et al.</i> 1974
14	6-Et-2-NMe ₂	95 +++	96	Badger <i>et al.</i> 1974
15	8-Me-2-SMe	120 ++++	17	Brown <i>et al.</i> 1972
16	8-Me-2-SEt	450 ++++	55	Badger <i>et al.</i> 1973
17	8-Et-2-SMe	200 ++++	42	Badger <i>et al.</i> 1973
18	8-Et-2-SEt	580 ++++	151	Badger <i>et al.</i> 1973
19	8-Ph-2-SEt	2 +	< 750	Badger <i>et al.</i> 1974
20	8-CF ₃ -2-OH	< 1 +	0.2	Albert, 1966
21	9-Me-2-SMe	60 ++	10	Brown & Ford, 1969
22	9-Me-2-SEt	110 +++	—	Badger <i>et al.</i> 1974
23	9-Me-6-SMe	30 ++	7	Brown & Ford, 1969
24	9-Me-8-SMe	8 ++	—	Brown & Ford, 1969
25	9-CH ₂ Ph-8-SMe	4 ++	263	Brown, Ford & Tratt, 1967
Dialkyl				
26	2,9-Me ₂ -6-SMe	25 ++	25	Brown <i>et al.</i> 1967
27	2,9-Me ₂ -8-SMe	310 ++++	10	Brown <i>et al.</i> 1967
28	6,7-Me ₂ -2-SMe	500 ++++	4	Brown <i>et al.</i> 1967
29	6,8-Me ₂ -2-SMe	80 +++	—	Brown <i>et al.</i> 1972
30	6,8-Me ₂ -2-NMe ₂ (H ₂ O)	230 +++	31	Badger <i>et al.</i> 1974
31	6-Et-8-Me-2-SMe	30 ++	85	Badger <i>et al.</i> 1974
32	6-Et-8-Me-2-NMe ₂	60 +++	110	Badger <i>et al.</i> 1974
33	8-Et-6-Me-2-SMe	0 ++	69	Badger <i>et al.</i> 1973
34	8-Et-6-Me-2-NMe ₂	45 +++	69	Badger <i>et al.</i> 1974
35	6,9-Me ₂ -2-SMe	750 ++++	21	Brown <i>et al.</i> 1967
36	6,9-Me ₂ -2-SEt	80 ++	75	Brown <i>et al.</i> 1972
37	6,9-Me ₂ -2-SH	35 ++	7	Brown <i>et al.</i> 1967
38	6,9-Me ₂ -2-SOMe	3 +	0.1	Brown <i>et al.</i> 1972
39	6,9-Me ₂ -2-SO ₂ Me	20 ++	0.1	Badger <i>et al.</i> 1974
40	6,9-Me ₂ -2-SCH ₂ CO ₂ ⁻	< 1 +	< 0.1	Grigg <i>et al.</i> 1971
41	6,9-Me ₂ -2-SCH ₂ CONH ₂	510 ++++	0.4	Badger <i>et al.</i> 1974
42	6,9-Me ₂ -2-NMe ₂	80 ++	40	Brown <i>et al.</i> 1972
43	6,9-Me ₂ -2-OMe	55 ++	4	Brown <i>et al.</i> 1972
44	6,9-Me ₂ -2-SMe-8-OH	2 +	30	Brown <i>et al.</i> 1972
45	6,9-Me ₂ -2,8-(SMe) ₂	4 ++	265	Brown <i>et al.</i> 1972
46	6-Et-9-Me-2-SMe	155 +++	71	Badger <i>et al.</i> 1974
47	6-Et-9-Me-2-NHMe	430 ++++	—	Badger <i>et al.</i> 1974
48	6-Et-9-Me-2-NMe ₂ (HCl)	330 ++++	183	Badger <i>et al.</i> 1974
49	9-Et-6-Me-2-SMe	40 ++	68	Badger <i>et al.</i> 1974
50	9-Et-6-Me-2-NMe ₂ (HCl)	25 ++	142	Badger <i>et al.</i> 1974

Table 1 (*cont.*)

No.	Purine	Adjusted activity*	Partition coefficient†	Source
	Trialkyl			
51	Caffeine	11, 30 ++	0.9	—
52	2,6,8-Me ₃	15 ++	216	Brown <i>et al.</i> 1972
53	2,6,9-Me ₃	< 1 +	1	Brown <i>et al.</i> 1972
54	6,8,9-Me ₂ -2-SMe	30 ++	32	Brown <i>et al.</i> 1972
55	6,8,9-Me ₃ -2-NMe ₂	20 ++	46	Brown <i>et al.</i> 1972
56	6,8,9-Me ₃ -2-OMe	80 ++	5	Brown <i>et al.</i> 1972
57	6-Et-8,9-Me ₂ -2-SMe	8 +	80	Badger <i>et al.</i> 1974
58	6-Et-8,9-Me ₂ -2-NHMe	430 + + + +	21	Badger <i>et al.</i> 1974
59	6-Et-8,9-Me ₂ -2-NMe ₂ (HCl.H ₂ O)	350 + + + +	—	Badger <i>et al.</i> 1974
60	8-Et-6,9-Me ₂ -2-SMe	110 + + +	61	Badger <i>et al.</i> 1974
61	8-Et-6,9-Me ₂ -2-NMe ₂	25 ++	122	Badger <i>et al.</i> 1974
62	9-Et-6,8-Me ₂ -2-SMe	90 + + +	76	Badger <i>et al.</i> 1974
63	9-Et-6,8-Me ₂ -2-NMe ₂ (H ₂ O)	15 ++	—	Badger <i>et al.</i> 1974
	Tetra-alkyl			
64	2,6,8,9-Me ₄	6 +	2.5	Brown <i>et al.</i> 1972

* Values at 8 mM or 2 mM (italicized) concentration relative to 8 mM-caffeine (30). Very highly active (+ + + +) = > 10 times the activity of caffeine at the same concentration; highly active (+ + +) = 3 to 10 times caffeine; moderately active (+ +) = 0.3 to 3.0 times caffeine; and slightly active (+) = < 0.3 times caffeine

† *n*-Octanol:water at 20 °C; aqueous buffer (pH 7) used for hydrochlorides.

METHODS

A culture of *E. coli* B was obtained from Ruth Hill. Phleomycin (batches A 9331-648 and 616) was a gift of Bristol Laboratories Inc., Ithaca, New York, U.S.A. and the *n*-octanol and caffeine were commercial samples (Evans Medical Ltd and BDH, respectively). The sources of other purines are indicated in Table 1.

Biological activity. The *E. coli* B was grown overnight to stationary phase in a glucose + salts medium (GT) (Grigg, 1969). Washed bacteria at a concentration of 10⁸/ml were suspended in fresh GT containing phleomycin (1 to 2 µg/ml; dissolved immediately before the experiment) and incubated at 37 °C for 30 min. The bacteria were membrane-filtered (0.45 µm pore size) and resuspended in GT containing the purine under test (8 or 2 mM, according to solubility). Samples were removed at the outset and at intervals during 120 min for estimation of the number of viable bacteria present (Grigg, 1970) using a commercial broth agar, Oxoid blood agar base, for counts. In each series of assays, 8 mM-caffeine was included as a standard (Grigg *et al.* 1971).

The amplifying activity of each purine is expressed in Table 1 as an adjusted activity relative to the mean activity of 8 mM-caffeine set at 30, and hence independent of variations in conditions or in the amount or intrinsic activity of the phleomycin. Thus,

$$A_m = N_0/N_{120} \quad \text{and} \quad A_{ad} = 30 A_m/A_{caf},$$

where A_m is the measured activity of the purine, A_{ad} is the adjusted activity, N_0 and N_{120} are the numbers of viable bacteria initially and after 120 min, respectively, and A_{caf} is the measured activity of 8 mM-caffeine. Since the dose response curve is not linear over the range 2 to 8 mM for many compounds, activities for purines measured at 2 mM concentration (for solubility reasons) are not strictly comparable with those at 8 mM.

Partition coefficients. A solution of each purine in octanol-saturated water at 20 °C was prepared at a concentration measurable in a spectrophotometer. A sample was filtered using

a syringe and Swinney adaptor containing a membrane filter (pore size 0.2 to 0.45 μm). The extinction (E_1) of the clarified solution was measured at a suitable wavelength. A measured volume of the unfiltered solution was shaken for 10 min with a volume of octanol appropriate to the expected partition coefficient. After settling, the aqueous layer was withdrawn, filtered twice, and the extinction (E_2) measured at the above wavelength. The whole procedure was repeated twice, with extended shaking times of 20 and 30 min, respectively, to ensure that equilibrium had been attained. The p.c. was calculated for each sample from the equation:

$$\text{p.c.} = \frac{V_w(1-E_2/E_1)}{V_o(E_2/E_1)},$$

where V_w was the volume of aqueous solution and V_o the volume of octanol. A spread of 10 % in p.c. values was accepted. It proved essential to prewash filter membranes free of residual detergent which otherwise interfered with extinction measurements.

RESULTS AND DISCUSSION

It is evident from the results in Table 1 that a high level of potentiation is achieved in the monoalkylpurines with a 2-alkylthio or 2-dimethylamino substituent (nos. 10 to 12, 14 to 18, 22; exception: 13); in the dialkylpurines with a 2- or 8-methylthio group or a 2-mono- or 2-di-methylamino group (nos. 27 to 30, 32, 34, 35, 46 to 48; exceptions: 31, 45, 50); and in a few trialkylpurines with similar substituents (nos. 54, 58 to 60, 62; exceptions: 55, 57, 61, 63). The dialkylated purineamide (no. 41) is also highly potentiating. Moderate potentiation, of the same order as caffeine, is shown by the unalkylated purines (nos. 2 to 9); by the 6-methylthiopurines (nos. 23, 26); and by the hydroxy-, benzyl-, mercapto-, methylsulphonyl- and methoxypurines (nos. 4, 5, 7, 25, 37, 39, 43, 56). Slight potentiation occurs from purine itself and its derivatives bearing trifluoromethyl, hydroxy, methylsulphiny, carboxymethylthio, and (only) alkyl groups (nos. 1, 20, 38, 40, 44, 53, 64; exception: 52). The connexion (Table 1) between p.c. and potentiating activity is unclear at present: purines within the very highly active group (+++), range in p.c. from 0.4 to 183, and the spread within each of the other activity groups is similar. It follows that negotiation of a fatty barrier is not a critical factor in activity.

The use of a two-component antibiotic regime, namely phleomycin plus a purine – the two having an antibiotic effect equal to that generated by a substantially higher phleomycin level, increases the possibility of introducing target cell specificity. Phleomycin is taken up by most cellular species, whereas purines, though capable of permeating most cells, bind weakly and, given to an animal, are rapidly eliminated through the kidney to the bladder. They or their corresponding metabolites can be recovered quantitatively in the urine. In principle an appropriate regime of phleomycin plus purine may therefore be useful in selectively treating pathological conditions of the bladder. 6,9-Dimethyl-2-methylthiopurine (No. 35), although of low toxicity to mice (Angyal, unpublished data), is metabolically modified by oxidation to a sulfoxide (No. 38) (Brown *et al.* 1972) which is ineffective as a potentiator of phleomycin. The addition of a carbamoyl group to the methylthio group (no. 41) prevents such enzymic modification (Brown & Stephanson, 1974) without appreciably affecting its biological activity. Accordingly, its utility with phleomycin as a specific bladder antibiotic is being explored.

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