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Immunosuppression by 9-alkyl-6-thiopurines

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AZATHIOPRINE [6-(1-methyl-4-nitro-5-imidazolyl)thiopurine] is widely used as an immunosuppressive drug in the treatment of autoimmune diseases and in the prolongation of kidney transplants.[1,2] Almost certainly the biological activity of this drug involves its conversion into 6-mercaptopurine by non-enzymic thiolysis[3] followed by the formation of the nucleotide analogue, 6-thioinosine monophosphate. This analogue and related nucleotides formed from it (particularly the *S*-methylated derivative and the corresponding thio-analogues of GMP) interfere with cellular processes by inhibiting purine nucleotide synthesis[4] and possibly by direct incorporation into nucleic acids.[5] Similar formation of toxic nucleotides occurs from the thioguanine analogues.[6]

Robins has shown that various 9-alkyl derivatives of 6-mercaptopurine and 6-thioguanine exhibit potent anti-tumour activity.[7,8] In many cases the chemotherapeutic indices of the 9-alkyl analogues were superior to their 9-unsubstituted counterparts. It is likely that the 9-alkyl derivatives have a different mode of action to the unsubstituted thiopurines as 9-substituted thiopurines have been shown to inhibit the growth of tumours resistant to 6-thioguanine[9] and 6-mercaptopurine.[10] Although the metabolic stabilities of many of the 9-substituents have not been established, *in vivo* dealkylation of the 9-butyl compounds does not occur in man, rat and mouse;[11–13] consequently the formation of active nucleotide analogues is blocked.

Recently, immunosuppressive effects have been obtained with 9-butylazathioprine;[14,15] at doseages well tolerated by the test animals, this drug inhibited the rejection of skin grafts across a H-histocompatibility barrier in mice, prolonged the survival of dogs after renal transplantation and inhibited the antibody response of mice to sheep red blood cells (SRBC). In this paper we report the relative abilities of various thiopurines and their 9-substituted derivatives to suppress the antibody response of mice to SRBC.

SRBC (0·25 ml of a 30 per cent suspension in normal saline) was administered interperitoneally on day 0 to Balb/c mice, and drugs were administered on days 0, 1 and 2; there were five animals in each of the test and control groups. Mice were killed on day 4 and numbers of spleen cells producing antibody against SRBC estimated by the Jerne-plaque technique.[16] The average number of plaque-forming cells/10^6 spleen cells in control animals ($n = 13$) was 267 ± 47 (standard error of the mean, S.E.M.). Student's t test, as adapted for small samples, was used to assess the significance or otherwise of test groups when compared with the control groups.

The analogues used were obtained as follows; 6-mercaptopurine, 6-thioguanosine and 6-methylmercaptopurine riboside were obtained from the Sigma Chemical Co. Azathioprine and guaneran (2-amino-6-(1-methyl-4-nitro-5-imidazolyl)thiopurine), were gifts from Burroughs–Wellcome, Australia and 9-isobutylguaneran was a gift from Dr. R. K. Robins. 9-Butylazathioprine,[11] 9-butyl-6-mercaptopurine,[11] 9-butyl-6-thioguanine,[17] 9-butylguaneran,[18] 6-methylthioguanosine,[18] 6-thioguanine[18] and 5-chloro-1-methyl-4-nitroimidazole[19–21] were synthesized by known methods.

A summary of the results obtained is presented in Tables 1 and 2. It is clear that the 6-thioguanine derivatives are much more potent immunosuppressive agents than the 6-mercaptopurine derivatives and this is reflected in their reported general highly cytotoxic effects.[17,22] It should be remembered that toxicity to thioguanine derivatives may vary between species. 6-Thioguanine is less toxic in man than in mouse because of detoxification to 6-methylthioguanine in man which is then excreted in the urine; little methylation occurs in the mouse.[23] However the analogue 6-methylthioguanosine is relatively non-toxic and is well tolerated by mice at doses up to 225 mg/kg.[18] The mechanism of action of this methylated analogue is unknown (indirect evidence suggests that nucleotide derivatives are not formed from this 6-thioribonucleoside[24,25]) and further investigation is warranted. The 9-butylthioguanine derivatives were highly active compounds.

It has previously been shown that on a molar basis, azathioprine is a more potent immunosuppressive agent than the parent compound, 6-mercaptopurine.[23] Like azathioprine, the alkylating agent 5-chloro-1-methyl-4-nitroimidazole (chloroimidazole) can react with sulphydryl compounds. An example is its reaction with glutathione to yield the corresponding 5-glutathionyl-1-methyl-4-nitroimidazole adduct (GSIM; A. H. Chalmers, unpublished result). Chloroimidazole did have some immunosuppressive activity (Table 1) and GSIM was inactive, indicating that the alkylating function of the imidazole ring is essential to its action as an immunosuppressive.

Thus two classes of 6-thiopurines have been shown to have immunosuppressive activity; 9-unsubstituted derivatives which form nucleotide analogues inside cells, and stable 9-alkyl derivatives

TABLE 1. EFFECT OF DERIVATIVES OF 6-MERCAPTOPURINE ON THE ANTIBODY RESPONSE OF MICE TO SHEEP RED BLOOD CELLS

Drug	Dose (mg/kg)	Control % (± S.E.M.)	Significance (P)
6-Mercaptopurine	28	14 (± 5)	< 0·01
	57	4 (± 2)	< 0·01
9-Butyl-6-mercaptopurine	57	94 (± 10)	N.S.*
	110	47 (± 13)	< 0·01
Azathioprine	50	19 (± 6)	< 0·001
	100	< 1	< 0·001
9-Butyl-azathioprine	100	35 (± 9)	< 0·02
	200	31 (± 12)	< 0·01
6-Methylthioinosine	11	27 (± 16)	< 0·01
	23	2 (± 0·7)	< 0·001
Chloroimidazole†	50	47 (± 8)	< 0·001
	100	57 (± 11)	< 0·01
GSIM‡	80	150 (± 23)	N.S.*
	160	118 (± 30)	N.S.*

* Not significant; $p > 0·1$.
† 5-Chloro-1-methyl-4-nitroimidazole.
‡ 5-Glutathionyl-1-methyl-4-nitroimidazole. This compound was prepared by reacting azathioprine with glutathione at pH 8–9; details are in preparation.

TABLE 2. EFFECT OF DERIVATIVES OF 6-THIOGUANINE ON THE ANTIBODY RESPONSE OF MICE TO SHEEP RED BLOOD CELLS

Drug	Dose (mg/kg)	Control % (± S.E.M.)	Significance (P)
6-Thioguanine	4·5	15 (± 5)	< 0·001
	9·0	< 1	< 0·001
9-Butyl-6-thioguanine	5·4	27 (± 16)	< 0·01
	10·6	6 (± 3)	< 0·001
Guaneran	3·7	136 (± 16)	N.S.
	7·4	4 (± 0·7)	< 0·001
9-Butylguaneran	5·5	39 (± 12)	< 0·05
	11·2	7 (± 2)	< 0·001
9-Isobutylguaneran	6·3	104 (± 15)	N.S.
6-Thioguanosine	10·0	< 1	< 0·001
6-Methylthioguanosine	10·0	62 (± 14)	< 0·05
	20·0	37 (± 6)	< 0·01

which have a mechanism of action unrelated to nucleotide formation. Further work is required to determine the sites of action of 9-alkyl thiopurines, and to establish whether these compounds have clinical potential, either alone or in combination with unsubstituted thiopurines.

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Effect of calcium, sodium and potassium on adrenal tyrosine hydroxylase activity *in vitro*

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THE REGULATION of synthesis of catecholamines (CA) is currently thought to be mainly through the inhibitory effect of CA on the rate limiting step, i.e. tyrosine hydroxylase (TH).[1] The regulatory function is ascribed to a specific "pool" of CA which presumably interacts with this enzyme, although such a pool has not been clearly defined.[2] However, inhibition of TH by CA can be demonstrated *in vitro*.[3]

A different type of experiment has shown a mechanism regulating CA synthesis *in vivo* through increased sympathetic stimulation.[4,5] This has been coined as "trans-synaptic induction" and involves increased synthesis of TH by prolonged presynaptic stimulation.[5] In addition to the gradual increase of TH following presynaptic stimulation, which is due to synthesis of the enzyme, an immediate increase in conversion of tyrosine to noradrenaline (NA) has been demonstrated *in vivo* even with a short stimulation, when new synthesis of TH cannot account for the enhanced NA synthesis.[6] This immediate increase in NA synthesis may be ascribed to depletion of the specific NA pool mentioned above.[2] However, several other phenonena accompany nerve stimulation to the adrenal medulla.