Rational Design of Novel Immunosuppressive Drugs: Analogues of Azathioprine Lacking the 6-Mercaptopurine Substituent Retain or Have Enhanced Immunosuppressive Effects

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Clinical use of the immunosuppressive drug azathioprine is limited by potentially serious toxic effects related to depression of bone marrow function. The immunosuppressive and toxic properties of azathioprine are regarded as being properties of the cytotoxicity of its metabolite, 6-mercaptopurine (6-MP). However, azathioprine has an immunosuppressive effect additional to that attributable to 6-MP alone, and we propose that this is associated with an action of the methylnitroimidazolyl substituent. This suggests a route to the rational design of nontoxic immunosuppressants by replacing the 6-MP component of azathioprine with nontoxic thiols. We have synthesized and tested in vitro 24 such analogues, with two being further tested in vivo. In the human mixed lymphocyte reaction, virtually all compounds showed some degree of activity, 10 compounds being more active than azathioprine. In vivo, two compounds were more effective than azathioprine at prolonging graft survival in mice. In an oral toxicity study in male CD1 mice at doses equivalent to those at which azathioprine caused severe bone marrow depression both analogues had no toxic effects. Our results show that the immunosuppressive effects and bone marrow toxicity of azathioprine are not a consequence of release of 6-MP alone, and with appropriate modification can be separated, an approach which may lead to less toxic immunosuppressive drugs.

Introduction

Immunosuppressants are of use clinically for three main purposes: suppression of the hosts' response to organ allografts, suppression of the response of lymphocytes in the graft to host antigens in bone marrow transplants, and treatment of a variety of conditions when the immune response has been inappropriately stimulated. Drugs used for immunosuppression include the fungal peptide cyclosporin **1**, the structurally dissimilar but mechanistically related macrolide FK-506 **2**, and glucosteroids and cytotoxic agents such as cyclophosphamide **3** and azathioprine **4** (Chart 1). Although FK 506 and cyclosporin are more selective and potent agents than the earlier drugs, they possess severe adverse side effects such as nephrotoxicity, neurotoxicity, and gastric toxicity which limit their clinical utility.[1−3] Consequently, although azathioprine has been available for approximately 30 years, it remains widely used as an immunosuppressive drug to prevent the rejection of organ transplants and in diseases involving the immune system. However, the use of azathioprine itself is limited by potentially serious toxic effects related to depression of bone marrow function.[4,5] There is therefore an ongoing requirement for less toxic and thus safer immunosuppressant drugs for clinical use. These would not only be of use as replacement drugs but also may be of potential use in a number of long term inflammatory conditions, such as psoriasis, rheumatoid arthritis, haemolytic anaemia, ulcerative colitis, Guillain-Barre syndrome, Polyarteritis nodosa, glomerulonephritis, and multiple sclerosis, the treatment of which is currently limited or prevented by the undesirable side effects of currently available therapeutic agents.

Chemically, azathioprine is the 1-methyl-4-nitroimidazyl derivative of 6-mercaptopurine, and it has always been assumed that the immunosuppressive and toxic properties of azathioprine are both a consequence of release of 6-mercaptopurine as the main metabolite in vivo.[6] As azathioprine was originally developed as a prodrug of 6-mercaptopurine, this view point is perhaps not surprising. Physiologically, formation of 6-mercaptopurine is a consequence of the nonenzymatic reaction with the thiol groups of both glutathione and cysteine (Scheme 2) which also gives rise to the alkylation reaction products such as 5-glutathionyl-1-methyl-4-nitroimidazole, 5-cysteinyl-1-methyl-4-nitroimidazole, and 5-mercapto-1-methyl-4-nitroimidazole, which themselves have little or no immunosuppressive activity.[7,8] Although it is tempting to explain both azathioprine's immunosuppression and bone marrow toxicity on the basis of cytotoxicity caused by its nucleotide metabolites, there are several pieces of evidence which suggest that the action of azathioprine cannot be solely explained by release of 6-mercaptopurine.

Clinically, observations in patients with kidney transplants have shown a number of unusual differences between the expected and observed mode of action; for example, immunosuppression produced by azathioprine is sufficient to prevent rejection in the absence of neutropenia and is highly dependent on repeatedly taking the drug. Cessation of azathioprine therapy, even for a few days, is frequently associated with transplant rejection.[4,5,9] Moreover, although clonal expansion through rapid cell division occurs in both cellular and humoral immune responses, azathioprine

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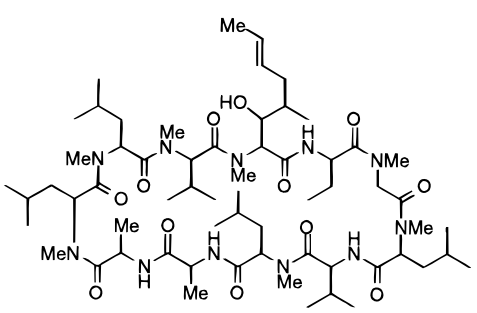
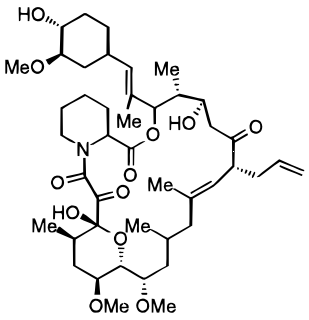
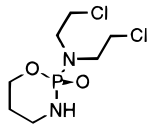
Chart 1. Chemical Structures of Cyclosporin (**1**), FK-506 (**2**), Cyclophosphamide (**3**), and Azathioprine (**4**)



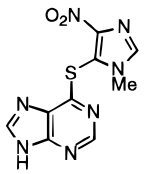
(1)



(2)



(3)



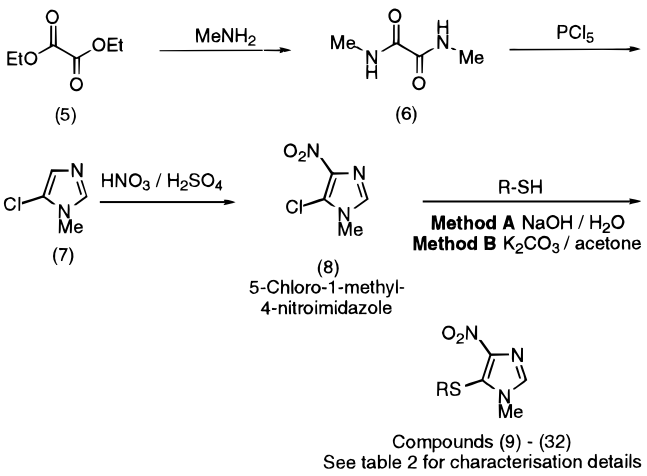
(4)

has a relatively selective effect on the cellular immune system.[10] These phenomena, while somewhat circumstantial, are not consistent with an immunosuppressive mechanism involving inhibition of DNA synthesis, which could be expected to be much slower to respond.

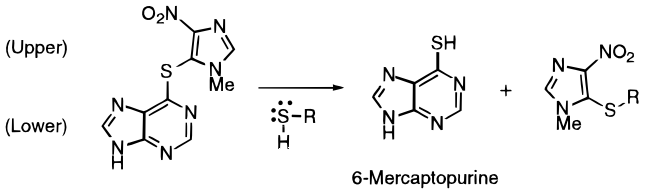
In vitro studies using the human mixed lymphocyte reaction (MLR) as a model of the cellular immune reaction have shown that azathioprine is more active in inhibiting the reaction when added within the first 24 h and is much less effective when added later, when cellular proliferation is maximal.[10,11] Further, 6-mercaptopurine inhibition of the MLR is completely prevented by hypoxanthine and inosine whereas that resulting from azathioprine is unaffected.[12] Additionally, azathioprine inhibits the MLR using lymphocytes from patients with Lesch-Nyhan syndrome that are unable to form active nucleotide metabolites, responsible for cytotoxicity.[13] Taken together, these inconsistencies indicate that azathioprine has an effect subtly different from that which might be expected from the action of 6-MP alone.

Some of these differences have been discussed, and the suggestion has been made that azathioprine has an effect related to its chemical reactivity with thiols or amino groups by Elion,[14] but the generally accepted view is that the purine component is essential for immunosuppression and the effect of azathioprine is a consequence of its greater metabolic stability, giving a more controlled release of 6-mercaptopurine. Whereas we acknowledge that release of 6-MP plays a role in the immunosuppressive action of azathioprine, we now propose a mechanism which can explain the different, enhanced effectiveness of azathioprine over 6-mercaptopurine based on an investigation of its chemical mode of action. As shown in Scheme 2, release of mercaptopurine is a consequence of conjugate addition-elimination of a thiol at the 5 position of the imidazole ring. This process can also be regarded as heteroarylation of the attacking thiol. We suggest that this is a secondary effect of azathioprine which also contributes to its immunosuppressive action. As the lymphocyte cell membrane is richly endowed with thiol groups,[15] we postulate that heteroarylation at thiols (or amino groups) on one or more key sites is responsible for the additional immunosuppressive effects seen (Scheme 3). With this working hypothesis as a basis, we set out to explore the possibility that by replacing 6-MP the immunosuppressive and toxic effects of azathioprine may be separable properties. We have therefore synthesized a number of analogues of azathioprine in which the "upper" , proposed arylating portion of the molecule is retained

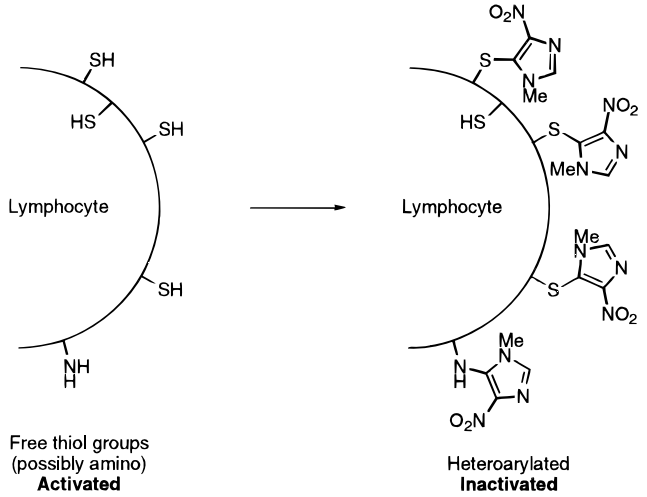
Scheme 1. Synthetic Route to Azathioprine Analogues



Scheme 2. Mechanism of Physiological Release of 6-MP

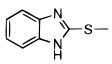
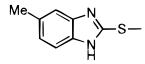
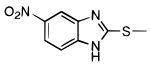
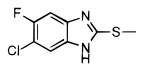
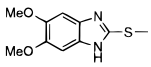
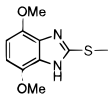
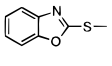
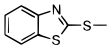
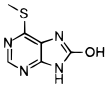
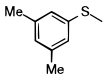
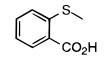
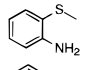
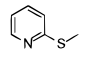
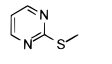
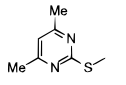
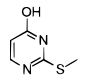
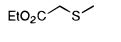
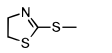
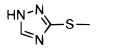
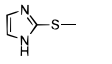
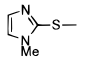
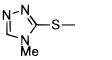
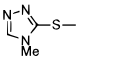
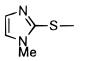


Scheme 3. Proposed Model of Inactivation of Lymphocytes by Heteroarylation



whereas the "lower" part of the molecule (6-mercaptopurine, the toxic component) is replaced with different, nontoxic mercaptans. Development of less toxic immunosuppressants based on a structure−activity-related

Table 1. Experimental Conditions and Physical Data

No.	R	R^1	R^2	Method	Recryst. solvent	Yield (%)	Formula	mp (°C)
9		Me	H	A	H_2O (ppt)	86	$C_{11}H_9N_5SO_2$	225-228
10		Me	H	A	H_2O (ppt)	58	$C_{12}H_{11}N_5SO_2$	260-270
11		Me	H	A	H_2O (ppt)	76	$C_{11}H_8N_6SO_4$	>240
12		Me	H	A	H_2O (ppt)	51	$C_{11}H_7N_5SO_2F$	>240
13		Me	H	A	H_2O (ppt)	86	$C_{13}H_{13}N_5SO_4.0.5H_2O$	232-235
14		Me	H	A	H_2O (ppt)	68	$C_{13}H_{13}N_5SO_4$	255-256
15		Me	H	B	EtOH	73	$C_{11}H_8N_4SO_3.H_2O$	152-154
16		Me	H	A	EtOH	72	$C_{11}H_8N_4S_2O_2$	127-130
17		Me	H	B[a]	H_2O (ppt)	72	$C_9H_7N_7O_3S$	>280
18		Me	H	B	EtOH	61	$C_{12}H_{13}N_3SO_2$	133-134
19		Me	H	A	Acetone	60	$C_{11}H_9N_3SO_4.H_2O$	215
20		Me	H	B	EtOAc	72	$C_{10}H_{10}N_4SO_2$	130-132
21		Me	H	A	EtOH	85	$C_9H_8N_4SO_2$	119-120
22		Me	H	A	EtOH	63	$C_8H_7N_5SO_2$	165-167
23		Me	H	B	EtOH / EtOAc	75	$C_{12}H_{15}N_5SO_2$	140-141
24		Me	H	A	EtOH	61	$C_8H_7N_5SO_3$	218-220
25		Me	H	B	Ether	75	$C_8H_{11}N_3SO_4$	54-55
26		Me	H	B	EtOH	71	$C_7H_8N_7SO_2$	100-101
27		Me	H	A	EtOH	80	$C_6H_6N_6SO_2$	205-207
28		Me	H	A	EtOH	68	$C_7H_7N_5SO_2$	221-223
29		Me	H	B	Acetone	73	$C_8H_9N_5SO_2$	110-111
30		Me	H	A	Acetone	61	$C_7H_8N_6SO_2$	210-212
31		Et	Me	B	EtOH	71	$C_9H_{12}N_6SO_2$	148-149
32		Et	Me	B	EtOAc	68	$C_{10}H_{13}N_5SO_2$	122-123

[a] DMF used as solvent.

separation of the immunosuppressive and toxic properties of azathioprine has not to our knowledge been undertaken.

Chemistry

Analogues **9**−**32** were synthesized by reacting 5-chloro-1-methyl-4-nitroimidazole **8** with the appropriate thiol using either sodium hydroxide and water (method A) or potassium carbonate in acetone (method B) as reagents (Scheme 1). Key intermediate **8** was synthesized following the methods given by Wallach[16,17] and Sarasin and Wegmann.[18] Briefly, diethyl oxalate **5** is converted to dimethyloxamide **6** on treatment with aqueous methylamine, which on treatment with phosphorus pentachloride gives 5-chloro-1-methylimidazole (**7**). This material can be nitrated using conventional conditions to give **8**, a white, highly crystalline compound in good overall yield. The methylethyl analogues **31** and **32** are made by simply substituting ethylamine for methylamine in the first step. All compounds made were characterized by proton NMR, mass spectroscopy, and microanalysis. (See Table 1 for a full list of compounds made and characterisation details.)

Results and Discussion

As a primary screen, analogues were initially tested in the human mixed lymphocyte reaction. Briefly, antigen responding and stimulating human lymphocytes were incubated together for 5 days at 37 °C in a desiccator flushed with a mixture of 5% CO_2 and 95% air. The response was measured by the incorporation of tritiated thymidine into DNA, with the results being expressed as either the percentage inhibition of the uptake of tritiated thymidine at 10 or 25 μM or the ED_{50} value, unless otherwise indicated, and are presented in Table 2. For comparison at 25 μM azathioprine alone causes 79% inhibition and has an ED_{50} of 7.9 μM.

The results obtained for the first category of compounds, the benzimidazoles **9**−**14**, the benzoxazole **15**, and the benzthiazole **16**, which are somewhat related by the fact that their corresponding thiols are roughly isosteric with 6-MP, show immediately that substitution of the mercaptopurine substituent results in compounds with retained or enhanced ability to inhibit cell activity, with compounds **9**, **10**, **13**, and **15** outscoring azathioprine in this test. Although we were very encouraged by this finding, these compounds were not further

Table 2. Inhibitory Potency of Azathioprine Analogues in the Human Mixed Lymphocyte Reaction

	% inhibition of [^{3}H]thymidine incorporation[a]		
compd no.	10 μM	25 μM	ED50 (μM)[b]
azathioprine	ND[c]	79	7.9
9	ND	90	ND
10	ND	86	ND
11	ND	2	ND
12	ND	59[d]	ND
13	ND	94	ND
14	ND	41	ND
15	ND	87	ND
16	ND	27	ND
17	ND	50	24
18	ND	47	ND
19	ND	34	ND
20	ND	21	ND
21	ND	76	ND
22	ND	47	ND
23	ND	47	ND
24	ND	29	ND
25	ND	65	ND
26	ND	100	ND
27	ND	58	14
28	89	100	2.8
29	82	100	3.15
30	98	100	2.7
31	98	100	2.5
32	98	100	2.9

[a] Values are the average of at least three experiments performed in triplicate. [b] ED50 values determined from a graph with at least four points, each derived from the mean of 3 −10 experiments. [c] ND = not determined. [d] Determined at 50 μM.

investigated at this stage as their solubility was rather low, and 25 μM was about the maximum concentration obtainable in the cell culture medium.

With the exception of compound **17**, compounds **17**−**25** represent derivatives made from commercially available mercaptans with no particular attempt to rationalize their design, but to test the generality of the approach. Once again, all compounds tested showed some degree of inhibition of cell stimulation, although none of these is more potent than azathioprine. Compound **17**, 8-hydroxyazathioprine, is worthy of note for its ability to inhibit the mixed lymphocyte reaction, albeit with approximately one-third the activity of azathioprine, as 8-hydroxymercaptopurine is known to lack cytotoxicity in vitro.[7]

The final seven compounds (**26**−**32**) all have in common 5-membered heterocyclic rings attached to the primary imidazole, and these compounds proved to be the most potent. The solubility of these compounds was also optimized so these have been most widely investigated. At 25 μM all except **27** caused complete inhibition of the MLR and for these compounds results obtained at 10 μM and ED_{50} values more accurately express their potency. The imidazoles **28** and **29** showed much better inhibition than azathioprine and were determined to have ED_{50} values of 2.8 and 3.15 μM, respectively, compared to 7.9 μM for azathioprine. The isosteric triazole **30** proved yet more potent, causing 98% inhibition of cell activity at 10 μM and having an ED_{50} value of 2.7 μM. Finally, the methylethyl analogues of **29** and **30**, numerically **32** and **31**, were also tested with **31** giving the lowest ED_{50} value of the whole series.

Two of the most active compounds, the 1-methylimidazole **29** and the 4-methyltriazole **30** were selected

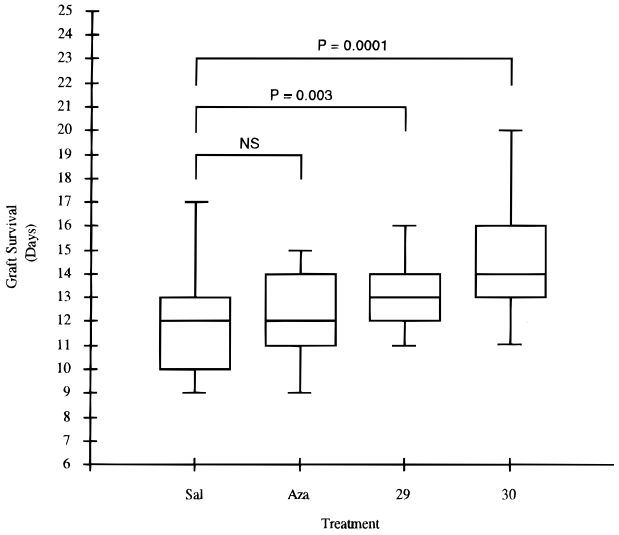


Figure 1. Effect of treatment with immunosuppressive drugs by intraperitoneal injection on BALB/C skin allograft survival in CBA mice. Treatment groups: saline control, 55 mice; azathioprine positive control, 29 mice; compound **29**, 20 mice; compound **30**, 46 mice. The results are shown as a box, representing the median and 25th and 75th centiles, and whiskers representing the lowest and highest values. NS = not significant.

for further testing for their ability to prevent rejection of BALB/C skin allografts on CBA mice. Four groups of mice were tested and treated with saline, azathioprine, and the analogues **29** and **30**, respectively (Figure 1). In this model azathioprine did not prolong skin graft survival when compared to the saline control group with the following results being obtained at equimolar doses: azathioprine median 12, range 9−15 days, n = 29 vs saline median 12, range 9−17 days, n = 55; P = 0.19, 95% CI_{diff} 2, 0 days. However, the two analogues tested were able to prolong skin graft survival, and the results were statistically significant: the 1-methylimidazole analogue **29** median 13, range 11−16 days, n = 20 vs saline median 12, range 9−17 days, n = 55; P = 0.003, 95% CI_{diff} 2, 1 days, and the 4-methyltriazole derivative **30** median 14, range 11−20, n = 46 vs saline median 12, range 9−17 days, n = 55; P = 0.0001, 95% CI_{diff} 2, 3 days. Compound **30** was superior to azathioprine in prolonging graft survival (P = 0.003, 95% CI_{diff} 1, 3 days). Although these results appear relatively modest, this model is a very severe test for immunosuppressive drugs, and the key observation is that the analogues are more effective than the control drug.

In a 14 day oral dosing study, the toxic effects of the 1-methylimidazole (**29**) and the 4-methyltriazole (**30**) were compared. Daily treatment of male CD1 mice with 100, 200, and 400 mg/kg doses of azathioprine consistently produced marked leucopenia, severe bone marrow depletion, and necrosis, whereas at equimolar doses neither of the analogues produced these effects. That the drugs were sufficiently orally absorbed to cause immunosuppression was shown by T-cell priming of regional lymph nodes.

These results show clearly that the purine moiety of azathioprine is not a prerequisite for its immunosuppressive effects, and furthermore, the imidazole is in fact very tolerant of substitution at this position. Replacement by almost any thiol-containing compound gives rise to molecules that retain the immunosuppres-

sant properties of azathioprine with the overall level of activity being about the same order of magnitude.

Our heteroarylation hypothesis to help explain the mechanism of immunosuppression produced by azathioprine receives considerable support from the results of this work. The wide range of chemical structures of analogues possessing immunosuppressive activity over a fairly narrow concentration range favors the idea of a chemical reaction rather than a specific interaction with a receptor. Their precise locus of action is, however, unknown, and we have made no attempt to investigate it in this study.

Conclusions

We have synthesized 24 analogues of azathioprine lacking a 6-mercaptopurine substituent and found that immunosuppressive effects are retained or even enhanced in these molecules, with 10 more potent than azathioprine in vitro and two (**29** and **30**) being shown to be more potent and less toxic in vivo.

These results indicate that release of 6-MP, and its effects on DNA synthesis, is not the sole explanation for the immunosuppressive effects of azathioprine. Instead it appears that a secondary effect, associated with the nitroimidazole portion of the molecule is very important and with appropriate modification can produce compounds with more potent immunosuppressive properties. It seems possible that rather than release of 6-mercaptopurine being responsible for the immunosuppressive effect of azathioprine, due to its toxicity 6-mercaptopurine may have been an unfortunate choice from among the wide range of thiols which can be substituted on the imidazole ring to produce immunosuppression. These compounds, or others designed using the same principles, offer a lead to immunosuppressive drugs with greatly reduced side effects, which will offer alternative and safer treatment of conditions in which immunosuppressants are already used, both directly and as steroid sparing agents.

Experimental Section

Melting points are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AM-250 (250 MHz) spectrometer supported by an Aspect 3000 data system. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (J) are in hertz. Elemental analyses were within 0.4% of the theoretical values for all compounds. Mass spectra were obtained using either a Kratos MS25 or MS50 spectrometer supported by a DS 55 data system. Thin layer chromatography was run on Merck 7736 60GF silica gel.

5-Chloro-1-methyl-4-nitroimidazole (8) was synthesized using methods previously described in the literature.[16−18] Analogues were prepared from this intermediate by using the appropriate thiol under the conditions described below.

Method A: Typical Example. 3-[(1-Methyl-4-nitro-5-imidazoyl)thio]-4-methyl-4*H*-1,2,4-triazole (30). A solution of sodium hydroxide (1.366 g, 34.1 mmol) in water (100 mL) at room temperature was treated with 3-mercapto-4-methyl-4*H*-1,2,4-triazole (3.571 g, 31.05 mmol) and the resulting suspension stirred until all the solid material was in solution. 5-Chloro-1-methyl-4-nitroimidazole (5 g, 31.05 mmol) was then added and the resulting solution allowed to stir for 1 h at room temperature, after which time TLC showed no starting materials. Neutralization with acetic acid caused the product to precipitate, and this was collected and recrystallized from acetone to give the title compound as fawn crystals (4.53 g, 61%): mp 210−212 °C; NMR (DMSO) δ 8.58 (1H, s, triazole proton), 8.05 (1H, s, imidazole proton), 3.76 (3H, s, methyl protons), 3.69 (3H, s, methyl protons); MS m/z 240 (M^+). Anal. Found: C, 35.19; H, 3.47; N, 34.84; S, 13.12. $C_7H_8N_6SO_2$ requires: C, 34.99; H, 3.35; N, 34.98; S, 13.34%.

Method B: Typical Example. 2-[(1-Methyl-4-nitro-5-imidazoyl)thio]thiazoline (26). A solution of 5-chloro-1-methyl-4-nitroimidazole (3 g, 18.63 mmol) and 2-mercaptothiazoline (2.21 g, 18.63 mmol) in acetone (60 mL) was treated with potassium carbonate (7.71 g, 55.89 mmol) and the resulting solution allowed to stir at room temperature overnight, after which time TLC showed no starting materials. The solid material was removed by filtration, and the solvent was removed under reduced pressure to give a crude product which was recrystallized from ethanol to give the title compound as light yellow crystals (3.266 g, 71%): mp 100−101 °C; NMR (DMSO) δ 8.19 (1H, s, imidazole proton), 4.16 (2H, t, J = 8 Hz, thiazoline protons), 3.44 (2H, t, J = 8 Hz, thiazoline protons), 3.70 (3H, s, methyl protons); MS m/z 198 (M − 46). Anal. Found: C, 34.33; H, 3.60; N, 22.90; S, 25.96. $C_7H_8N_4S_2O_2$ requires: C, 34.4; H, 3.6; N, 22.95 and S, 26.2%.

Biological Studies. Heparinized venous blood samples were obtained from normal human donors. Lymphocytes were separated from 20 mL blood samples by treatment with dextran (MW 150 000) in normal saline and carbonyl iron powder (type SF, from G. A. F. Ltd., Manchester, U.K.) before isolation on Ficoll-Metrizoate (Lymphoprep, Nygaard AS, Oslo, Norway). Stimulating cells were prepared with mitomycin-C (Sigma Chemical Co. Ltd., Dorset, U.K.) as described by Bach and Voynow.[19] For culture, lymphocytes were suspended in RPMI 1640 (Flow Laboratories, Irvine, U.K.) prepared with glutamine (2 mM), HEPES buffer (20 mM), gentamicin 50 (μg/mL), and amphotericin sodium desoxycholate (Fungizone, 2.5 μg/mL). They were cultured in triplicate in round-bottomed microtitre plates (Flow Laboratories). Each well contained 200 μL, consisting of 50 μL each of antigen responding lymphocytes (10^5), stimulating lymphocytes (10^5), inactivated, pooled human AB serum diluted with culture medium to give a final concentration of 20%, and the test drug dissolved in culture medium. Lymphocytes were incubated for 5 days at 37 °C in a humidified desiccator flushed with a gas mixture of 5% CO_2 and 95% air.

The response was measured by the incorporation of tritiated thymidine ([^{3}H]Tdr) into DNA. Full details have been described previously.[11,12] Results were expressed as the concentration of drug required to inhibit [^{3}H]thymidine incorporation into DNA in the MLR by 50%. The EC_{50} was determined from a graph with at least four points, each derived from the mean of 3−10 experiments.

For skin grafting, the technique of Billingham and Medawar was used.[20] A full thickness piece of Balb/c (white H-2d) mouse tail skin was grafted onto the flank of a CBA (brown skin, H-2) mouse (OLAC, Bicester, U.K.). The graft was protected by a gypsum plaster cast which was removed after 8 days when the condition of the graft was assessed visually by an independent observer, previously blinded to the treatment. Analogues were dissolved in 0.9% sterile saline at 37 °C to give a final concentration of 5 mg/mL. Each was given in a dose of 45 mg/kg/day. Azathioprine (Imuran, Wellcome, U.K.) was prepared in the same way and given in equimolar dosage of 52 mg/kg/day. Drugs were given to groups of 10 mice by intraperitoneal injection 3 h before surgery and then once daily until the graft had been fully rejected. Controls were treated similarly but were given 0.9% saline. Skin graft survival data was analyzed by stem and leaf displays and normal probability plots and found to be not normally distributed. Statistical analysis was performed using the Mann−Whitney U-test. The data is presented as medians, 25th and 75th centiles, and the range. Where comparisons are made, the 95% CIs for the differences are presented (95% CI_{diff}). A P value of <0.05 was considered significant.

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