# Purine and Pyrimidine Antagonists

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A MAJOR proportion of earlier investigations which dealt with the effects of antimetabolites *in vivo* were concerned with antivitamins. In many of these the symptoms of deprivation of the metabolite had been established by feeding experiments, and when a similar syndrome was produced by the antimetabolite it was reasonable to conclude that the substance possessed the mode of action *in vivo* which had been inferred from structural considerations and *in vitro* studies. The accumulation of a rather large volume of such information supports the view that *in vitro* studies provide presumptive definitions of mechanisms of action which may be extended usefully to other biological systems. However, when one is dealing with metabolites which normally are synthesized by the organism, the deficiency state is not available for comparison with the results of administration of the antimetabolite, and one can only infer what the consequences of deprivation of the metabolite might be from a general knowledge of the biochemical reactions in which it is involved. Consequently, much more in the way of experimental evidence is required to establish the mechanism of action in these instances than in those which involve known signs of deprivation. The reversal study (antagonism between metabolite and antimetabolite) is an important source of such evidence, but has certain pitfalls as, for example, the failure of folic acid to prevent the effects of its 4-amino analogue, aminopterin, in birds and mammals in contrast to the competitive effect in microorganisms. Metabolic studies and, finally, the study of analogues in combination may provide evidence of the mechanisms of action which may be quite convincing. The present paper deals primarily with some examples of the last two types of experiment and their applications to problems of the mechanism of analogue action.

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## EFFECTS OF ANALOGUES

An extensive study of the effects of analogues of the natural purines and pyrimidines on the developing tadpole of *Rana pipiens*[1,2] was designed to glean information both as to the role of nucleic acid biochemistry in embryogenesis and the mechanisms of action of the analogues. A considerable number of inhibitory substances was found. When the dose response relationship was investigated, some tendency for higher concentrations to result in earlier inhibition was noted, but only within definite limitations, and it soon became apparent that whereas certain purine analogues were capable of producing an inhibition of very early development (stage 8, blastula) the antifolic acids were capable of inhibition no earlier than neurula (stage 12–13) at any reasonable concentrations (Table I). These facts suggested that early embryogenesis might occur at the expense of preformed purine-containing nucleic acid fragments which were stored in the ovum and that *de novo* synthesis (involving folic acid) might begin only at neurulation. Several facts support this view. The presence of very large stores of desoxypentose derivatives in the mature oöcyte (sufficient for the chromosomal DNA of about $10^5$ somatic cells) has been confirmed.[3] These stores also are reduced when the female frog is starved during oögenesis (Table II) and

TABLE I

Inhibition of Development

| Substance | Earliest Stage Inhibited |
|---|---|
| 8-Azaadenine | 8 |
| 6-Mercaptopurine | 8 |
| Pyrimethamine | 14 |
| Aminopterin | 13 |

the resultant embryos are hypersensitive to the action of 6-mercaptopurine and other purine analogues.[4] Finally, added confirmation is being obtained from studies of the incorporation of radioactive formate, which is found to begin only at neurulation where

TABLE II

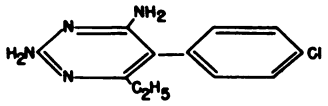
μg Desoxypentose per Oöcyte

| Diameter | Normal Females Cold TCA | Normal Females Hot TCA | Starved Females Cold TCA | Starved Females Hot TCA |
|---|---|---|---|---|
| 1.6 mm | 0.32 | 0.12 | 0.25 | 0.07 |
| 1.8 mm | 1.00 | 0.22 | 0.45 | 0.09 |
| DNA† of Mature Sperm = 3.6 x $10^{-6}$ μg | | | | |

* TCA—trichloracetic acid.
† DNA-desoxyribonucleic acid.

the antagonists of the formylating coenzyme, folic acid, first become active, and to reach a high level at about the point that the stores of preformed nucleic acid fragments are approaching exhaustion. In this example, therefore, the acceptance at face value of the mechanism of action for 6-mercaptopurine which had been assigned on the basis of structural considerations[5,6] and microbiological studies[7,8,9] led, in an *in vivo* system, to a number of metabolic studies, the results of which are entirely consistent with the postulated mechanism of action.

The reversal experiment should not be neglected entirely as a means of demonstration




PYRIMETHAMINE
(DARAPRIM)
COMPOUND 50-63

Figure 1.

of the mechanism of action of an analogue. It may be pertinent here to recall the role of 2,4-diaminopyrimidines as antagonists of folic acid.[10,11] Since these substances are rather more remotely related to folic acid than are 4-amino folic acid (aminopterin) and similar structural analogues, it was of interest to determine whether folic acid antagonism could be demonstrated *in vivo* as well as *in vitro*. For this purpose young rats were fed a purified diet, otherwise adequate but lacking in folic acid, to which 1 g of pyrimethamine[12] (Daraprim; Fig. 1) per kg of diet had been added.

TABLE III

Effects of Metabolites on Toxicity of Pyrimethamine

| Supplement | Amount per kilo diet | Average weight gain | No. of animals | No. of survivors |
|---|---|---|---|---|
| None | — | 15 | 18 | 3 |
| Folic acid | 50 mg | 9 | 6 | 0 |
| Folinic acid | 3.3 mg | 43 | 5 | 4 |
| Folinic acid | 10 mg | 44 | 13 | 10 |
| Folinic acid | 33 mg | 82 | 5 | 5 |
| Folic acid + Ascorbic acid | 10 mg 5 g | 9 | 7 | 3 |
| Liver powder | 50 g | 101 | 24 | 24 |

This diet inhibited growth and was lethal within a few weeks; the animals showed, too, a typical folic acid deficiency syndrome. When the drug-containing diet was supplemented with folic acid, there was no effect; however, Leucovorin (folinic acid) was able completely to prevent death of the animals (Table III). Thus the effects of the diaminopyrimidines resemble closely those of aminopterin and A-methopterin *in vivo*. Perhaps one reservation should be made. It is not possible to titrate the pyrimidine against folinic acid *in vivo* over so wide a range of concentration as is possible with, for example, A-methopterin.[13,14] It is also apparent that liver powder is more effective in the prevention of the toxicity of pyrimethamine than is accounted for on the basis of its content of folinic acid (Table III, 50 g liver powder contain about 1.5 mg of folic acid + folinic acid). Nevertheless, the most probable explanation of these results, at present, would appear to be that the metabolite with which the pyrimidine competes is closely related to but not identical with folinic acid. Final confirmation of this view will have to await the isolation and testing of such a factor. In any case it is quite clear that the mechanism of action of the diaminopyrimidine *in vivo* is closely related to that demonstrated *in vitro*.

## Folic Acid Antagonists

Experience with the folic acid antagonists may serve to illustrate one of the difficulties

in the way of a facile interpretation of the *in vivo* reversal experiment. It will be recalled that the early microbiological studies[15] had shown the action of aminopterin to be preventable by folic acid, although the two were not completely competitive. *In vivo*, however, folic acid had scarcely any effect on the toxicity of aminopterin, and it was only several years later after the discovery and synthesis of folinic acid that the role of aminopterin as a competitor of folinic acid, rather than folic acid, was appreciated. In this instance, therefore, the reversal experiment *in vivo* was at first somewhat misleading, and it seems probable that similar problems will arise with other analogues. Thus, for example, in microbiological experiments, the toxicity of 6-mercaptopurine is prevented by any of the four free purine bases,[7,8,9] but these are not effective *in vivo*.[16,17] However, several of the more complex purine-containing metabolites do effectively block the toxicity.[17] Thus, in a sense, this problem resembles that with aminopterin, in that the antidotal metabolite is more complex than the antimetabolite.

One of the more reassuring lines of evidence regarding the mechanisms of action of antimetabolites *in vitro* has been the ability to make predictions on the basis of *in vitro* studies and biochemical knowledge of the effects of combinations of analogues.

The chief biochemical pathway with which the purines and pyrimidines are concerned may conveniently be diagrammed as shown in Figure 2.

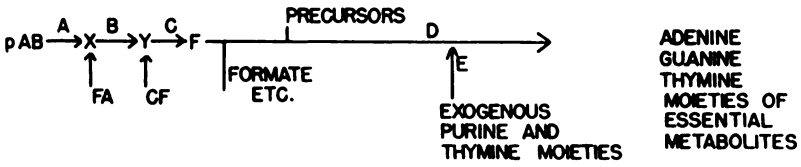



Figure 2.

In this scheme X represents the cellular equivalent of folic acid (FA) which may be formed *de novo* via *para*-aminobenzoic acid or by the incorporation of preformed folic acid, and Y represents the intracellular form of the citrovorum factor. Some member of this series of vitamins (F) is regarded as the formylating coenzyme which is involved in the incorporation of one-carbon fragments into suitable precursors to complete the purine and thymine moieties found in the nucleic acids and various nucleotides. When exogenous purines and thymine are available, they are incorporated (E) and join the main stream of *de novo* synthesis at some as yet unknown points. The availability of sulfonamides, competing in reaction A, diaminopyrimidines and other antifolic acids blocking reaction B, and of end product analogues competing at some point with the products of formylation allows one to test the effects of multiple blockade of a fundamental biochemical mechanism.

Combinations of sulfonamides with diaminopyrimidines provide systems in which the mechanism of action of the latter can be tested. Such combinations provide sequential[18,19] blockade of a biosynthetic pathway and would be expected therefore, to produce potentiative effects. In fact, a wide variety of combinations of sulfonamides with diaminopyrimidines and condensed systems containing the diaminopyrimidine moiety, has been tested *in vitro* and *in vivo* and potentiation has been observed in every instance.[20,21,22,23]

In Figure 3, the data are presented of an *in vitro* experiment using a combination of 2,4-diamino-5-(3′4′-dimethoxybenzyl) pyrimidine (compound 49-210) and sulfadiazine in the growth of *Proteus vulgaris*. In this graph, unity on each axis represents the amount (inhibitory concentration) of the substance required to reduce the growth of the organism to half-maximal. By the use of sub-inhibitory concentrations of one substance and graded amounts of the second, the composition of combinations which produce 50 per cent inhibition are determined graphically and are plotted as decimal fractions of the inhibitory concentrations. Where additive effects occur in combinations, the points fall on the line connecting the inhibitory concentrations of the

individual compounds.[19] Deviation of the locus of points to the left of this line signifies potentiation, and a quantitative expression of the degree of potentiation is found in the sum of the fractional inhibitory concentrations at

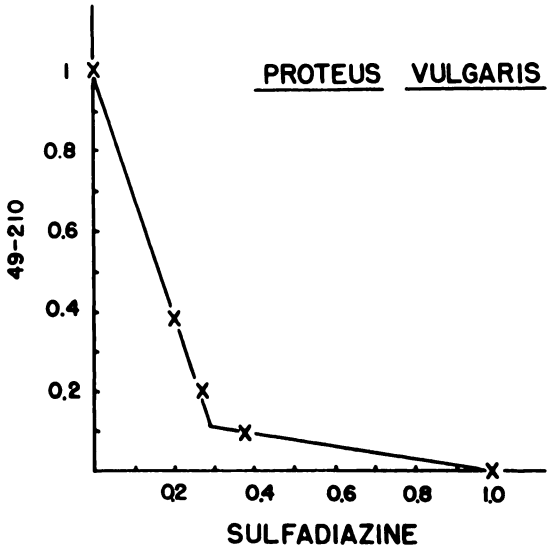



Fig. 3. Effects of combinations of a diaminopyrimidine and sulfadiazine on the growth of *Proteus*. Each point represents the composition of the additive which produces a 50% inhibition of growth. Unity on the ordinate is 30 μg of Compound 49-210 (2,4-diamino-5-(3′,4′-dimethoxybenzyl)pyrimidine). Unity on the abscissa is 20 μg of sulfadiazine.

the intercept of the two lines of the figure. In Figure 3, this amounts to about 0.28 for sulfadiazine, and 0.12 for compound 49-210, a sum of 0.4. Thus the use of two analogues in combination has reduced the required quantity of inhibitor by 60 per cent.

## Combinations

Since many of the diaminopyrimidines have chemotherapeutic activity, it is not surprising that a number of combinations of these drugs with sulfonamides have found chemotherapeutic applications. One of the more striking of these is the work of Eyles and Coleman[20] on *Toxoplasma gondii*. In previous work these authors had found sulfonamides to have some influence on the course of toxoplasmosis in mice, and pyrimethamine was somewhat more active, but neither of these drugs alone was capable of curing the infection. When they were used in combination, a strong potentiation occurred. In the most effective combination (Fig. 4) one twenty-fifth of an inhibitory dose of pyrimethamine together with one

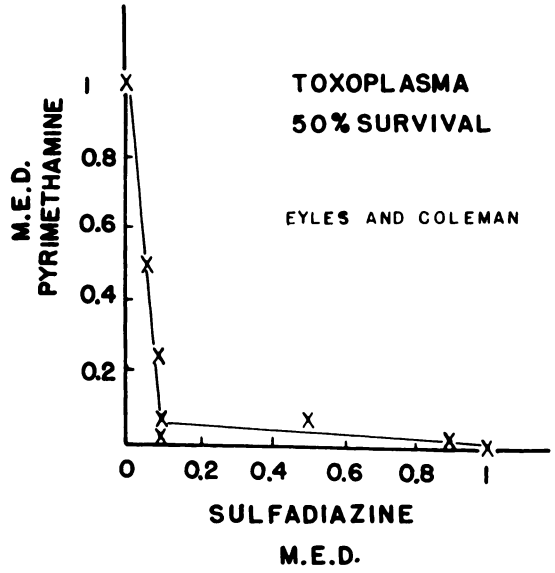



Fig. 4. Effects of combinations of sulfadiazine and pyrimethamine on survival of mice infected with *Toxoplasma gondii*. Drawn from the data of Eyles and Coleman.[20]

twelfth of an inhibitory dose of sulfadiazine was as effective in prolonging the survival of infected mice as a full inhibitory dose of either compound alone. Much more important, however, was the finding that, with combinations of the two drugs, effective clearance of the parasite (cures) could be obtained. Thus the improved potency obtained through the potentiative effect of the sequential blockade resulted in a qualitative improvement in therapy. These observations of Eyles have found a dramatic application in the cure of a case of acute toxoplasmosis which was the result of a laboratory infection in Eyles' own laboratory[24] and have stimulated clinical trials in toxoplasmic chorioretinitis with an apparently favorable outcome.

Finally, the mechanism of action of 6-mercaptopurine may be considered further in relation to its activities against neoplastic disease.[25] The reversal studies of Goldin[17] mentioned above are consistent with the view

that interference with purine metabolism is the primary *modus operandi* of this substance *in vivo* as well as *in vitro*. Potentiative effects with combinations of antifolic acids and 6-mercaptopurine have been observed *in vitro*[19] and *in vivo* with L 1210 leukemia.[26] These

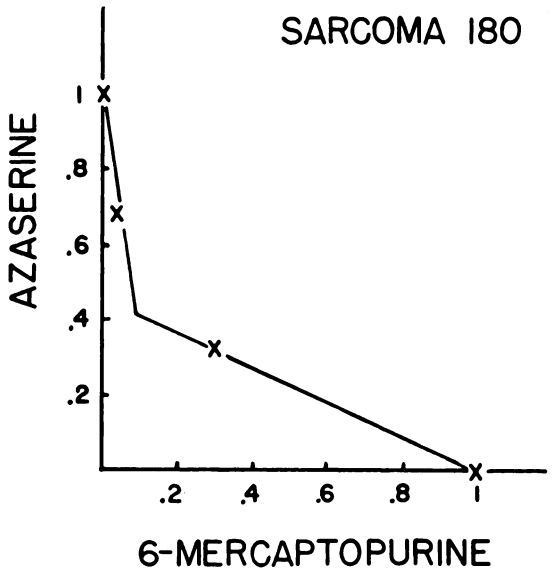



Fig. 5. Effects of combinations of Azaserine and 6-mercaptopurine on the growth of sarcoma 180 in mice. Unity on the ordinate is 7.5 mg/kg of Azaserine; on the abscissa, 155 mg/kg of 6-mercaptopurine.

are regarded as the result of concurrent blockade, in the scheme above, of the main *de novo* synthetic pathway at B, and the by-pass mechanism at E. The observations of Clarke[27] dealing with the potentiation of 6-mercaptopurine by Azaserine in the inhibition of sarcoma 180 are of considerable interest. In Figure 5 the data of Clarke have been plotted in terms of fractional inhibitory concentrations.* It is apparent that potentiation does occur and that a combination of 0.4 of an inhibitory dose of Azaserine and 0.1 of an inhibitory dose of 6-mercaptopurine has the effect of a full inhibitory dose of either drug alone. Moreover, as in other instances, it was possible to attain greater inhibitory effects and a greater percentage of tumor regressions with combination therapy than with either substance given singly (Table IV).

When this was first discovered, the biochemical interrelationship between the two inhibitors was not apparent, but this has been

TABLE IV**

Effects of Combinations of Azaserine and 6-Mercaptopurine on Recoveries from Sarcoma 180

| Exp. No. | Compound | Dose mg/kg | Recoveries* |
|---|---|---|---|
| 1 | 6-Mercaptopurine | 50 | 2 |
| 2 | Azaserine | 10 | 3 |
| 3 | 6-Mercaptopurine + | 25 | 8 |
| | Azaserine | 5 | |
| 4 | 6-Mercaptopurine + | 12.5 | 9 |
| | Azaserine | 2.5 | |
| 5 | Controls | — | 0 |

* 19 mice in Exp. No. 2, 20 mice in each of the others.

** Abstracted from the data of Clarke *et al.*[27] (To be published in full).

clarified by recent observations of Skipper[28] and Buchanan,[29] who have shown that Azaserine interferes with the formylation specifically of 4-amino-imidazole-5-carboxamide ribotide, a precursor of inosinic acid (hypoxanthine-ribosephosphoric acid). 6-Mercaptopurine also appears to become involved rather specifically with the metabolism of a hypoxanthine-containing metabolite.[8] Figure 6 may be regarded as a schematic representa-

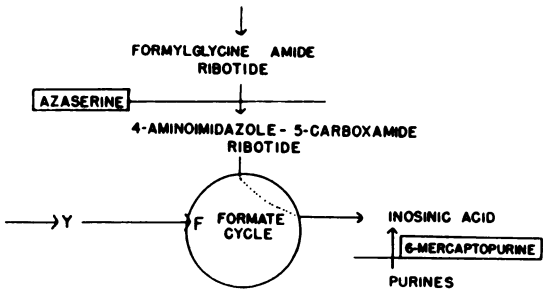



Fig. 6. Diagrammatic representation of biochemical reactions which involve Azaserine and 6-mercaptopurine.

tion of the interrelationship between the two blocking agents, and an enlargement of a segment of the general scheme which was diagrammed above. The synergism between Azaserine and 6-mercaptopurine would thus be

* These data were drawn from experiments not primarily designed for this purpose, and some extrapolation of the dose-response curves for the individual drugs was necessary. However, the validity of this procedure is supported by collateral experiments with the same compounds.

viewed as an additional example of the concurrent blockade of two pathways to the same essential metabolite.

## Comment

Studies *in vivo* of a considerable number and variety of antimetabolites have, with reassuring frequency, given results which are consistent with the mechanisms of action which have been assigned on the basis of structural considerations and *in vitro* studies. Such assurance is of particular importance in the application of antimetabolites to practical problems of chemotherapy. It has led, with increasing frequency, to the use of combinations of antimetabolites which have resulted in enhanced therapeutic effects. At the same time, potentiation in drug combinations can be viewed as an integration of the antimetabolic activities of the individual drugs, and can contribute supporting evidence regarding the mechanisms of action of the substances involved. That potentiative effects have become in a measure predictable offers considerable encouragement to the biochemical approach to chemotherapy.

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