Anticancer drugs

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This is the first part of a series of three articles which review the chemistry behind a wide range of currently recognised anticancer drugs. Throughout the series the emphasis will be on chemical mechanism of action in this area and its relation to future drug improvement and design. The biochemical targets currently in vogue for anticancer drug design will also be covered – for example, antimetabolites which mimic, in one way or another, crucial metabolic intermediates.

The reviews are written from a distinctly chemical angle, but some coverage of the necessary biochemistry and physiology will be given at a relatively elementary level. However, the purpose of these articles is to show which aspects of a series of drugs are most interesting to chemists in the hope that this will stimulate the application of analogous reasoning to other drug areas. For example, a given drug may be a good example of a transition-state inhibitor, a multi-substrate analogue, an allosteric regulator or a reactive alkylating species. These concepts will be explained as they arise, essentially in situ, *through the medium of an important anticancer drug.*

In this first part, a number of antimetabolites based on the pyrimidine and purine structures are considered. The most outstanding examples in these fields include methotrexate and 5-fluorouracil. This section provides several examples of different ways in which recent advances in our understanding of enzymes in general at a fundamental level have contributed to the understanding and design of effective anticancer drugs.

The major current approaches to cancer treatment are: surgical excision of the tumour, which is frequently limited, not so much by the tumour size, but by its distribution; radiotherapy, which also depends frequently on the degree of tumour dissemination; and chemotherapy. The purpose of chemotherapy is to use chemicals to treat disease without seriously harming the patient. Chemotherapy goes back a long time, from the rather drastic metallic 'remedies' of Paracelsian days, through early Greek and Egyptian medicine, ultimately into the folk medicines which must have existed from time immemorial.

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As with all major diseases, the treatment of cancer is not without its folklore versions of 'cures'. The majority are, sadly, groundless, but increasing numbers of examples of folk medicines are becoming useful after careful re-evaluation – frequently, it must be carefully emphasised, not for the disease for which the folk remedy gained its fame. In the cancer area, good examples are provided by the *Vinca* alkaloids from the periwinkle and possibly also by the famous mandrake root from which epipodophyllotoxin is isolated.

On a rational basis, an obvious goal of cancer chemotherapists is to find a unique biochemical characteristic of the cancerous target tissue, which is absent from normal tissues. Chemical attack can then be focused on this aspect. To date, the number of absolute differences between normal and cancer cells is limited – the differences are frequently reflected as altered levels of a common enzyme activity. An exception is the dependence of some lymphoid malignancies on exogeneous asparagine, which has led to the asparaginase therapy. The recent discoveries of the single base changes in the genetic sequences of some normal and cancerous cells, leading to a small, but highly significant, alteration in a single protein (of, as yet, undefined function) underlines the rather subtle differences one might anticipate between normal and cancerous cells at a biochemical level.

Currently, useful drugs are by-and-large non-selective cytotoxins with severe, dose-limiting (toxic) side-effects. The aim with these compounds is usually an attempt at killing the cancer cell and not at controlling its growth. This contrasts with antibacterial chemotherapy in which it is anticipated that low bacterial levels remaining after treatment will be mopped up reasonably efficiently by phagocytes. Phagocytosis of tumour cells is much less effective, unless it can be maintained indefinitely. There are a number of materials which stimulate macrophage activity – for example, muramyl peptides – so this is an added goal of a more subtle chemotherapy of cancer.

The need to marshal assistance, such as the body's immune system, is clear when one looks at the demands placed on the cytotoxic efficiency of an anticancer cytotoxin. Consider a tumour weighing approximately 100g; it contains approximately 10^{11} cells. By pushing a single course of therapy with a cytotoxic drug to the

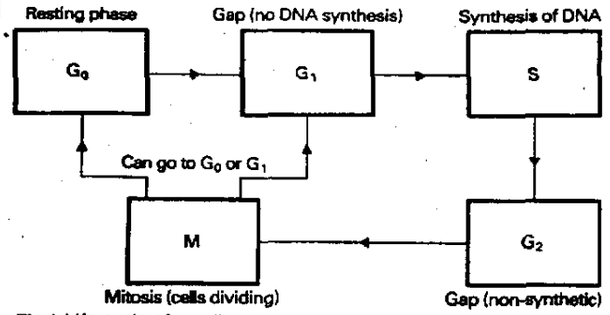


Fig 1 Life cycle of a cell

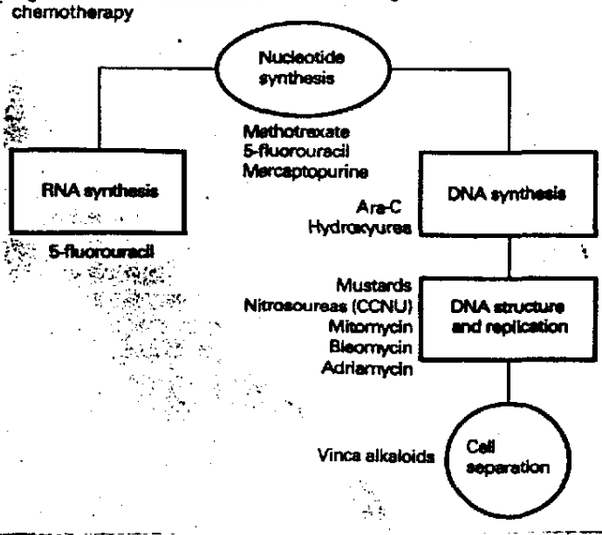
patient's tolerance limit, one may be able to kill 99.9 per cent of the tumour cells. This still leaves 10^9 living tumour cells, which would soon double and eventually regenerate the 100g tumour in a matter of weeks. For a relatively small tumour (10^2-10^3 cells) one can reduce its mass and leave relatively few cancer cells, but therapy must often be maintained. Not surprisingly, a combination of surgery and/or radiotherapy with chemotherapy is often used.

Cell life cycle

Recent use of chemotherapy has shown that appropriately administered combinations of anticancer drugs lead to greater improvements in the patients than anticipated from the properties of any one of the individual drugs. Rational combinations of drugs are often designed with the cell life cycle in mind (see Fig 1). When a new cell is born (mitosis, M phase) it can go either into a resting phase (G_0) or a gap phase (G_1) during which no DNA is synthesised, although many enzymes, for example, are. From G_1, the cell goes to the S phase (synthesis) during which DNA synthesis occurs. Another gap (G_2) then occurs before cell division (M). Each of the main segments of the life cycle has its own characteristics and these must occur in the correct sequence.

In the resting phase, the cell is desensitised towards cytotoxic drugs. Drugs blocking mitosis act only on cells in the M state, while chemotherapeutic attacks on DNA synthesis are best made during the S phase. In such ways

Fig 2 Scheme of successful biochemical targets in anticancer chemotherapy



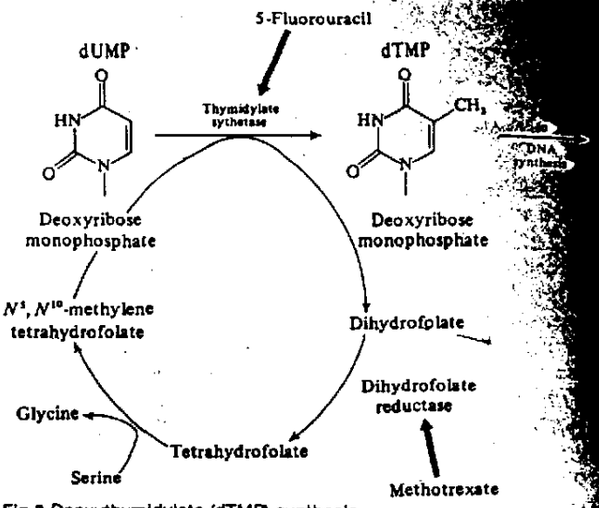


Fig 3 Deoxythymidylate (dTMP) synthesis

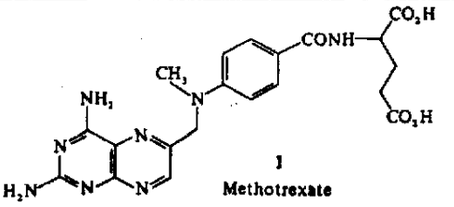
knowledge of the cell cycle and the factors controlling changes from one state to another are crucial. A critical problem with tumour annihilation is that all the cells in the tumour are not at the same phase of the cycle. Combination chemotherapies can be designed to help circumvent problems associated with this – for example, by sequentially attacking at various phases of the life cycle of the tumour cells.

A general scheme of biochemical targets which have been used successfully (although not often by design) in anticancer chemotherapy is shown in Fig 2, along with some drugs as examples. In this scheme, the major emphasis is on polynucleotides (RNA, DNA), but a number of other targets have been used or are under current investigation. Some of these will be discussed in this series.

Antipyrimidines

The synthesis of DNA is especially important in rapidly dividing cells and it is not surprising that this is the focus of many workers in anticancer drug design. Deoxythymidylate (dTMP) is required for DNA synthesis and the cell synthesises this from deoxyuridylate (dUMP) – a methylation process catalysed by thymidylate synthetase (Fig 3).

The carbon required for the methyl group is supplied by



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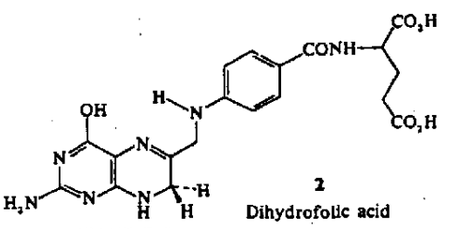
Methotrexate



2
Dihydrofolic acid

N^5, N^{10}-methylenetetrahydrofolate which is converted in the thymidylate synthetase reaction to dihydrofolate. A cycle of reactions reconverts this to N^5, N^{10}-methylenetetrahydrofolate for reuse (see Fig 3). The vital conversion of dUMP to dTMP can be blocked at the thymidylate synthetase stage – for example, by 5-fluorouracil – or in the folate-cycle – for example, at dihydrofolate reductase by methotrexate. Hence there is great activity in these areas of enzyme inhibition.

Methotrexate and dihydrofolate reductase. Blocking regeneration of the cofactor required by thymidylate synthetase is achieved by strong inhibition of dihydrofolate reductase. Methotrexate (1) is a powerful inhibitor, and one of the first effective antimetabolites used in neoplastic diseases. It appears to be a classical antimetabolite, mimicking the structure of the natural substrate (2) rather closely. It is a competitive inhibitor of dihydrofolate reductase and binds extremely tightly.

The X-ray diffraction derived structure of the *L. casei* enzyme complexed with methotrexate and NADPH has been determined at 2.5Å resolution.[1] Consequently, many of the binding interactions can now be assessed in detail. By comparing the structure of this ternary complex and that of the binary complex of methotrexate with the enzyme from *E. coli*,[2,3] it is possible to postulate differences in conformation caused by coenzyme (NADPH) binding (note that the enzymes are from different species). Large movements of NADPH-contacting side chains can be made out in this way. Elegant n.m.r. experiments of bound ligands with dihydrofolate reductase have indicated that there is more than one conformation of enzyme capable of binding NADPH.[4] This view is supported by transient kinetic studies.[5]

There has been a resurgence of interest in methotrexate of late. Current problems with its use include the development of methotrexate resistance by some tumour cells and high toxicity for normal cells, necessitating 'rescue therapy' (by administration of folinic acid) from time to time.

5-fluorouracil and thymidylate synthetase. Fluoropyrimidines, of which the most famous is 5-fluorouracil, act primarily as metabolites and cause cell-kill in one of two ways – inhibition of thymidylate synthetase or by incorporation into RNA, thus altering RNA processing and function.[6,7] I shall concentrate on the chemistry behind the thymidylate synthetase inactivation as it offers a good example of a mechanism-based irreversible enzyme inactivator. Such inhibitors are recognised as substrates and processed in a normal manner by the enzyme, but because of chemical peculiarity designed into them, conversion to products with regeneration of free enzyme does not occur. Rather, the enzyme's own mechanism causes production of a highly reactive intermediate from the pseudosubstrate. This intermediate leads to an inactive enzyme.

To explain the case of F-dUMP inhibition, one should consider the normal enzyme mechanism of thymidylate synthetase (see Fig 4). Initially (a), there is a Michael attack of an enzymatic nucleophile, probably a cysteine residue,[8] to give a 5,6-saturated intermediate (3). This enolate ion attacks methylene-THF (N^5,N^{10}-methylenetetrahydrofolate) in the second step (b), followed by a hydride transfer

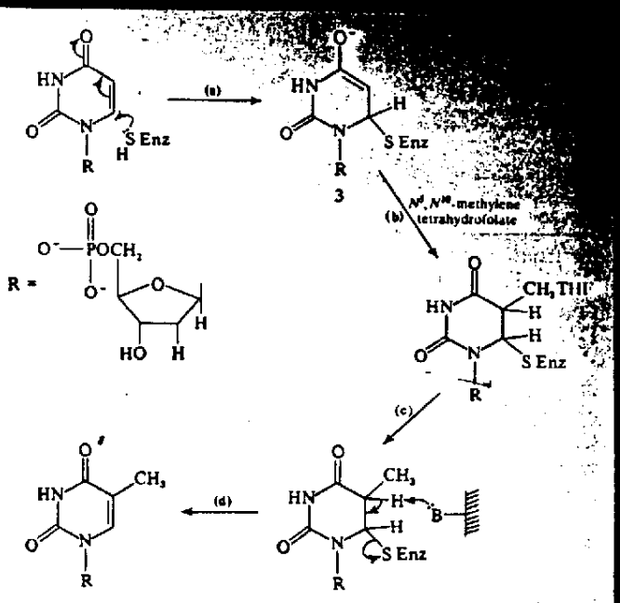


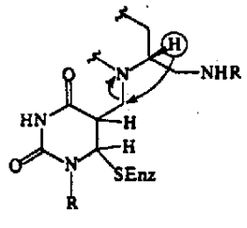
Fig 4 Minimal mechanism of action of thymidylate synthetase

(c), to complete the methylation process at C-5. Enzyme is regenerated by an enzyme-catalysed β-elimination across the 5,6 bond (d).

It is frequently considered likely that the inhibition by FdUMP is caused by the impossibility of the last step (d), which would require an enzyme-catalysed extraction of F^+. An alternative possibility is that reaction with FdUMP is halted by the previous step (c), the hydride transfer.[9] This would probably require an intermediate analogous to that for the normal substrate processing (4) with a 5-fluoro substituent which prevented the 1,3-hydride shift (although it is not clear why).

Ftoraphur (1-(2-tetrahydrofuranyl)-5-fluorouracil) (5) has recently entered clinical trial as it achieves a longer, lower level exposure to FdUMP than direct administration of 5-fluorouracil (to which it is slowly metabolised).[10] Fluorouracil is also incorporated into RNA and then it inhibits RNA methylation and the processing of ribosomal RNA (rRNA) to its functional, lower molecular weight form. These effects may also contribute to cell kill as one cannot completely reverse the toxicity of fluorouracil to normal and malignant cells by administration of thymidine.

Ara-C (1-β-D-arabinofuranosyl cytosine).[11] Ara-C (6) is one of the most effective drugs used in acute myelogenous leukaemia and introduces a new target for design of anticancer drugs. In this molecule, the ribose moiety of cytidine is replaced by the stereoisomeric arabinose. The arabinose



Normal substrate behaviour
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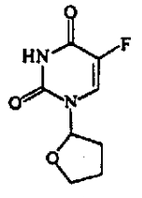
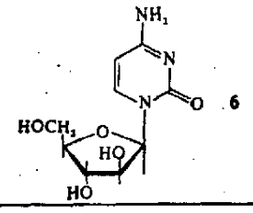


Ftoraphur
5



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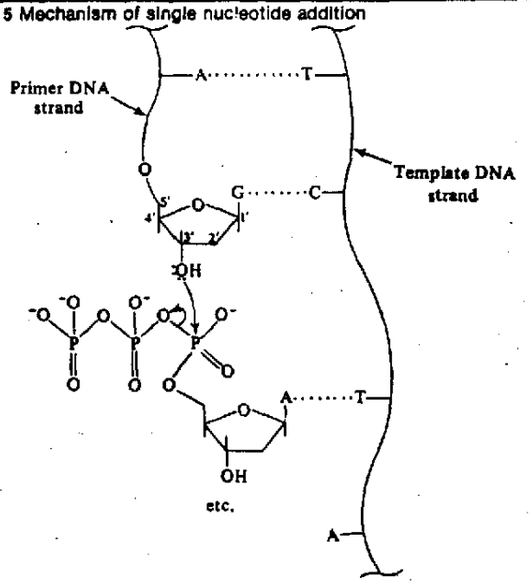
for ribose substitution is a fairly common change made in the search for changed biological activities. At a concentration which has little effect on RNA synthesis, ara-C blocks DNA synthesis.

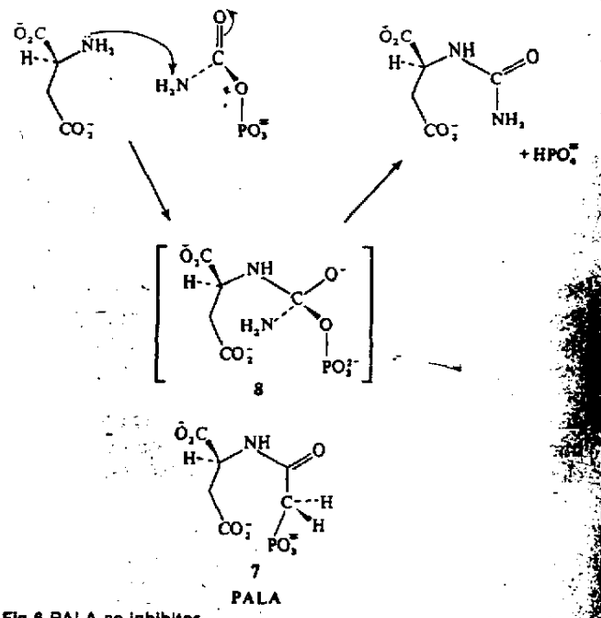
The chemotherapeutic target here is probably DNA-directed DNA polymerase. This catalyses DNA replication by adding deoxyribonucleotide units to a DNA-strand. Consequently, these require a template DNA single strand, which provides the information for the correct sequence of nucleotides in the complementary strand to be synthesised. In addition, these require a short primer strand. A schematised mechanism for the addition of a single nucleotide addition is shown in Fig 5. The 3′-OH group of the bound primer strand nucleophilically attacks the α-phosphorus atom of the next nucleotide triphosphate which is bound appropriately.

The details are a great deal more complex. Ara-C is phosphorylated to the triphosphate form (ara-CTP) which acts as a structural analogue for 2′-deoxycytidine triphosphate. Thus its primary biochemical effect is blocking DNA synthesis by acting as a competitive inhibitor of DNA polymerase. It has other important effects as well – for example, it can be incorporated into DNA, causing chain termination, and it blocks repair of single-strand breaks induced in DNA by UV radiation.

An important feature of any drug design problem is brought out by ara-C – that is, its metabolite fate. Not only must ara-C be metabolised to its final active form (ara-CTP) by the target tissue, but it is also degraded by enzymes of the target. Thus ara-C is destroyed by cytidine deaminase and its monophosphate (ara-CMP) by deoxycytidine deaminase. Therefore, interest has arisen in the use of

Fig 5 Mechanism of single nucleotide addition





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7

PALA

Fig 6 PALA as inhibitor

inhibitors of these metabolic problems in conjunction with ara-C – for example, tetrahydrouridine, a cytidine deaminase inhibitor, is in clinical trial in combination with ara-C.

There are other similar approaches being taken to DNA polymerase inhibition, using ara-A(1-β-D-arabinofuranosyl adenine), F_3dThd(5-trifluoromethyl-2′-deoxyuridine) and its triphosphate. Suicide inactivators (mechanism-based irreversible inhibitors) of DNA polymerase I from *E. coli* are also under investigation (for example, an epoxy ATP) but are not at the chemotherapeutic stage yet.

With an enzyme as complex as a DNA polymerase, inhibitors can act in a number of ways. One is certainly not restricted to competitive inhibitors and suicide inactivators along the lines of common enzyme inhibition. Template disruptors such as bleomycin, intercalators and template analogues can also be considered.

PALA (Phosphonoacetyl-L-aspartate).[11] Another approach to blockade of DNA biosynthesis has been to attack the enzyme involved in biosynthesis of the pyrimidine ring, aspartate transcarbamylase. This important regulatory enzyme catalyses the reaction of carbamyl phosphate with L-aspartate (Fig 6), the first committed step in pyrimidine biosynthesis. Collins and Stark synthesised *N*-phosphonoacetyl-L-aspartate (PALA) (7) as a tightly binding inhibitor of aspartate transcarbamylase.[12] Promising results have been obtained with it in Phase I anticancer trials.

The reaction catalysed involves nucleophilic attack by the aspartate amino-group at the carbonyl site of carbamyl phosphate, presumably giving an enzyme-bound tetracoordinate species (8) which cleaves to give the products. PALA incorporates most of the structural features of the two cosubstrates which contribute to this species (8). However, it is sp^2-hybridised at the carbon atom which is the seat of action, as opposed to sp^3 in the natural substrate reaction. PALA (with K_i (inhibition constant) equal to 2.7×10^{-8}M) binds about 1000-fold more tightly than

carbamyl phosphate, the more tightly bound of the cosubstrates, and about 40-fold more tightly than given by the product of the K_m (Michaelis constant) values of the substrates (that is, 2.7×10^{-3}M (carbamyl phosphate) × 0.017M (L-aspartate) = 5×10^{-7}M).

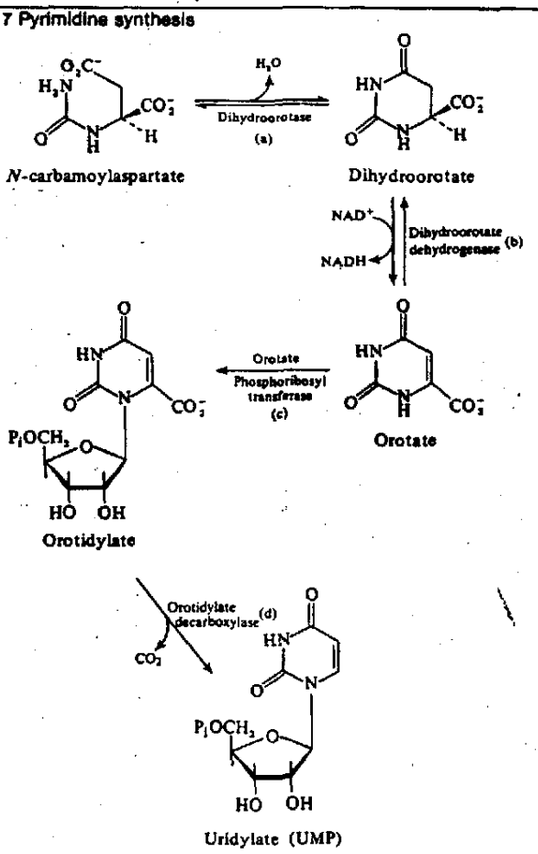
The transition state of the aspartate transcarbamyl reaction would be anticipated to resemble 8 energetically and, hence, structurally. For some time it was considered possible that the PALA-enzyme complex was mimicking the binding interactions present in the transition-state of the enzyme-catalysed reaction – that is, it was a so-called transition-state analogue. However, a transition-state analogue would be expected to bind much more tightly. The figures given indicate that PALA binds about as tightly as expected from the product of the K_m values of the two substrates of this enzyme. It is likely that PALA is merely an effective multisubstrate analogue.[13]

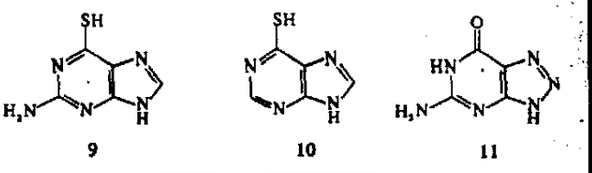
In a two-substrate reaction, if the two substrates bind essentially independently of each other, the free energy of interaction for the system (comparing $E + S_1 + S_2$ with ES_1S_2, where E is the enzyme, S_1 and S_2 are first and second substrates respectively and ES_1S_2 the ternary enzyme-substrates complex) will be given by:

$$\Delta G^\circ = \Delta G^\circ_{s_1} + \Delta G^\circ_{s_2}$$

Clearly, as $\Delta G^\circ = -RT\ln(K)$, the binding constant (K) will be related to the product of the binding constants for S_1 and S_2. Combining the structural (binding) elements of S_1 and S_2 into a single molecule can thus lead to extremely tightly binding inhibitors – even without the luxury of transition-state mimicry.

Fig 7 Pyrimidine synthesis



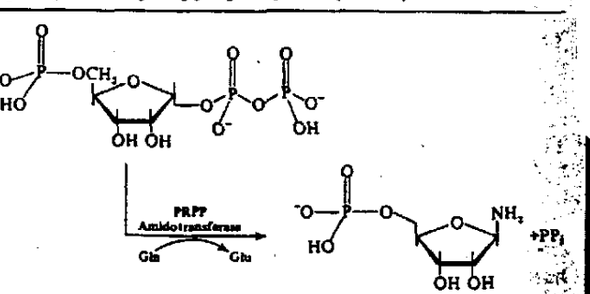


9 10 11

Other pyrimidine biosynthetic blocks. The biosynthetic steps subsequent to the formation of *N*-carbamylaspartate which lead to pyrimidines are outlined in Fig 7. The third step (c) is essentially irreversible as the pyrophosphate formed is cleaved by pyrophosphatase; the decarboxylation (d) is irreversible. These steps would appear to be good targets for lethal enzyme inhibitors, and there is now interest in this area as a hopeful route to new anticancer structures.

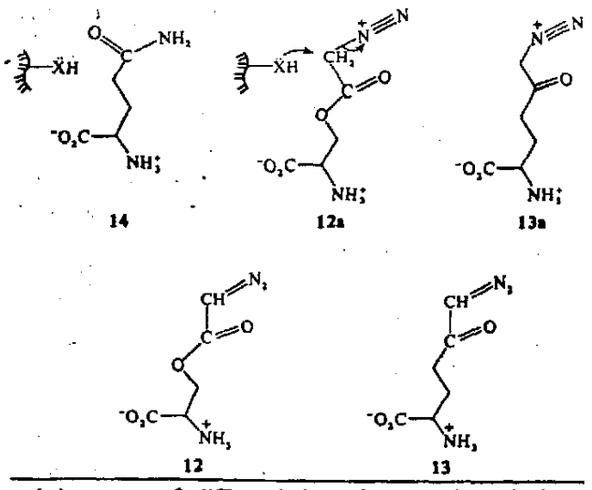
Antipurines

6-thioguanine and 6-mercaptopurine.[11] Some commonly used purine antagonists are 6-thioguanine (9) and 6-mercaptopurine (10). These are believed to act by incorporation of their nucleotide metabolites into DNA. The cytotoxicity of 6-thioguanine probably results from its mistaken incorporation during DNA synthesis giving functionally changed polynucleotides. In contrast, 6-mercaptopurine probably affects a number of enzyme activities, the most important of which appears to be its blockade of phosphoribosyl-pyrophosphate amidotransferase. This suppresses *de novo* purine synthesis as ribosylamine 5-phosphate can no longer be synthesised from glutamine and 5-phosphoribosyl-1-pyrophosphate (PRPP).



In the form of its monophosphate, 8-azaguanine (11) is a powerful negative allosteric effector of PRPP-amidotransferase.[14] Allosteric inhibitors offer a more subtle form of enzyme control than do inhibitors which bind directly to the active site of the enzyme. Allosteric effectors affect enzyme activity (catalytic reactivity and/or substrate specificity) by binding at a locus outside the immediate region of the catalytic groups of the enzyme. This may be on a different sub-unit of the (oligomeric) enzyme, for example.

These regulatory sites on enzymes located at crucial control points of vital metabolic sequences offer an exciting prospect for future development of new drugs. Not surprisingly, cancer and normal cells have not offered many major enzymatic differences on which to hang chemotherapeutic attacks. However, one could reasonably expect that the detailed manner in which cells regulate their own biosynthetic and other activities depends on criteria such as the nature of the cell, whether it is a rapidly-dividing type



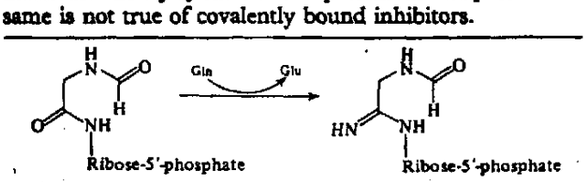
14 12a 13a

12 13

and its stage of differentiation. At least in principle, chemotherapeutic agents based on subtle molecular differences in the sites for regulatory effects on these enzymes should be possible. The example of PRPP amido-transferase is one probable example. Other suitable targets include ribonucleotide reductase (*vide infra*), but detailed coverage of allosteric targets for chemotherapeutic design will be left until later.

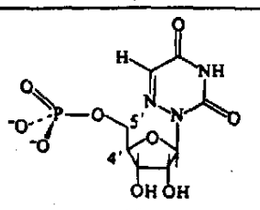
Azaserine and 6-diaza-5-oxo-norleucine. At this point, an additional approach in enzyme inhibition useful for producing drugs can be introduced – that is, irreversible inhibition by active-site alkylation. Two examples are azaserine (12),[15] and 6-diazo-5-oxo-norleucine (DON) (13).[14] These block three reactions in purine biosynthesis, but their main activity appears to be at the level of formylglycinamideribotide amidotransferase, which catalyses an early step.

The amidino-nitrogen of the product is supplied by L-glutamine (14). Azaserine and DON mimic this and are bound at the glutamine site. A schematic view of the alkylation is given in 12a and 13a. This appears to be a classical example of affinity labelling. While affinity labels have the apparent advantage of 'infinitely' tight binding, these suffer drawbacks from a pharmaceutical point of view. The first problem is their inherent chemical reactivity, which means that these will probably react with cellular components other than their primary (selected) target, with unpredictable consequences. It further means that they will be destroyed by hydrolysis. Finally, their effective lifetimes can become limited by the rate of turnover of the target enzyme, as new, non-labelled enzyme will be synthesised. A noncovalently-bound drug will be released from its enzyme complex on proteolytic degradation of the enzyme and so become available to inactivate newly-synthesised replacement enzyme. The same is not true of covalently bound inhibitors.



Formylglycinamide ribotide

6-azauridine.[16-18] This uridine analogue has been used successfully in the treatment of leukaemia. It is metabolised by uridine kinase to 6-azauridine-5'-phosphate (15), which strongly inhibits orotidylate decarboxylase (see Fig 7). An interesting explanation of this inhibition has been offered. Orotidylate will occur in a *syn*-conformation (because the



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$C_6 - CO^-_2$ group of the uracil will not readily lie over the ribose in the *anti*-form), leading to a $C_4 - C_5$ bound orientation different from that usually preferred by all normal 5'-ribonucleotides. As a crystal, 6-azauridine-5'-phosphate adopts a similar and unusual $C_4 - C_5$ orientation. In agreement with this is the inhibition observed of this enzyme by other 5'-ribonucleotides with locked *syn*-conformations.

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