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Minireview

Specificity of thymidylate synthase inactivation by 4,5-bisubstituted dUMP analogues*

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Specificity of a drug *vs* its target is an important problem in chemotherapy. Thymidylate synthase (EC 2.1.1.45) catalyzes the $C_{(5)}$ methylation of dUMP in a concerted transfer and reduction of the hydroxymethyl group from $N^{5,10}$-methylenetetrahydrofolate and with concomitant production of dihydrofolate [1, 2]; (Fig. 1). As the sole *de novo* source of thymidylate synthesis in cells, it is a target in anticancer, antiviral, antifungal and antiprotozoan chemotherapy [3 - 7]. A dUMP analogue, 5-fluoro-dUMP (FdUMP), a strong thymidylate synthase inhibitor, is an active form of drugs used in chemotherapy, such as 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FdUrd). The inhibition mechanism is based on the reaction mechanism and involves time-dependent formation of a ternary covalently bound complex of the enzyme with FdUMP and $N^{5,10}$-methylenetetrahydrofolate, resulting in slowly reversible enzyme inactivation. FdUMP inhibition is usually described by the K_i values in the 10^{-9} M range but tumour resistance to FU or FdUrd may be accompanied by the presence of altered thymidylate synthase forms, less sensitive to the inhibitor [8 - 12]. For an experimental tumour system correlation was shown between cell growth inhibition by different 5-substituted 2′-deoxyuridines and both inactivation of cellular thymidylate synthase and inhibition of the isolated enzyme by 5′-monophosphates of respective 5-substituted 2′-deoxyuridines [13, 14].

Recent studies showed that substitution of the pyrimidine ring $C_{(4)}$=O group in FdUMP (Fig. 2) by either $C_{(4)}$=N-OH group (in N^4-hydroxy-5-fluoro-2′-deoxycytidine-5′-monophosphate; N^4-OH-FdCMP) or $C_{(4)}$=S group (in 4-thio-FdUMP) preserves high inhibitory potency of the drug but may alter its specificity for thymidylate synthases from various sources, differing in sensitivity to slow-binding inhibition by FdUMP (Fig. 3). This phenomenon seems to be due to some interplay, of yet unknown nature, between the substituents at $C_{(4)}$ and $C_{(5)}$ in their interaction with the enzyme [15, 16].

Both FdUMP analogues inactivate the enzyme in time- and $N^{5,10}$-methylenetetrahydrofolate-dependent manner. However, although in both cases inactivation mechanisms are apparently based on the reaction mechanism, they are not necessarily identical. While enzyme inactivation by 4-thio-FdUMP seems to be due to the presence of the 5-fluoro substituent, since 4-thio-dUMP behaves as thymidylate synthase substrate [17], with N^4-OH-FdCMP the $C_{(4)}$=N-OH substituent is probably the cause of inactivation and the 5-fluoro substituent potentiates this process [15]. To explain the latter phenomenon, pointing clearly to an interplay between the $C_{(4)}$=N-OH and $C_{(5)}$-F substituents, an intramolecular hydrogen

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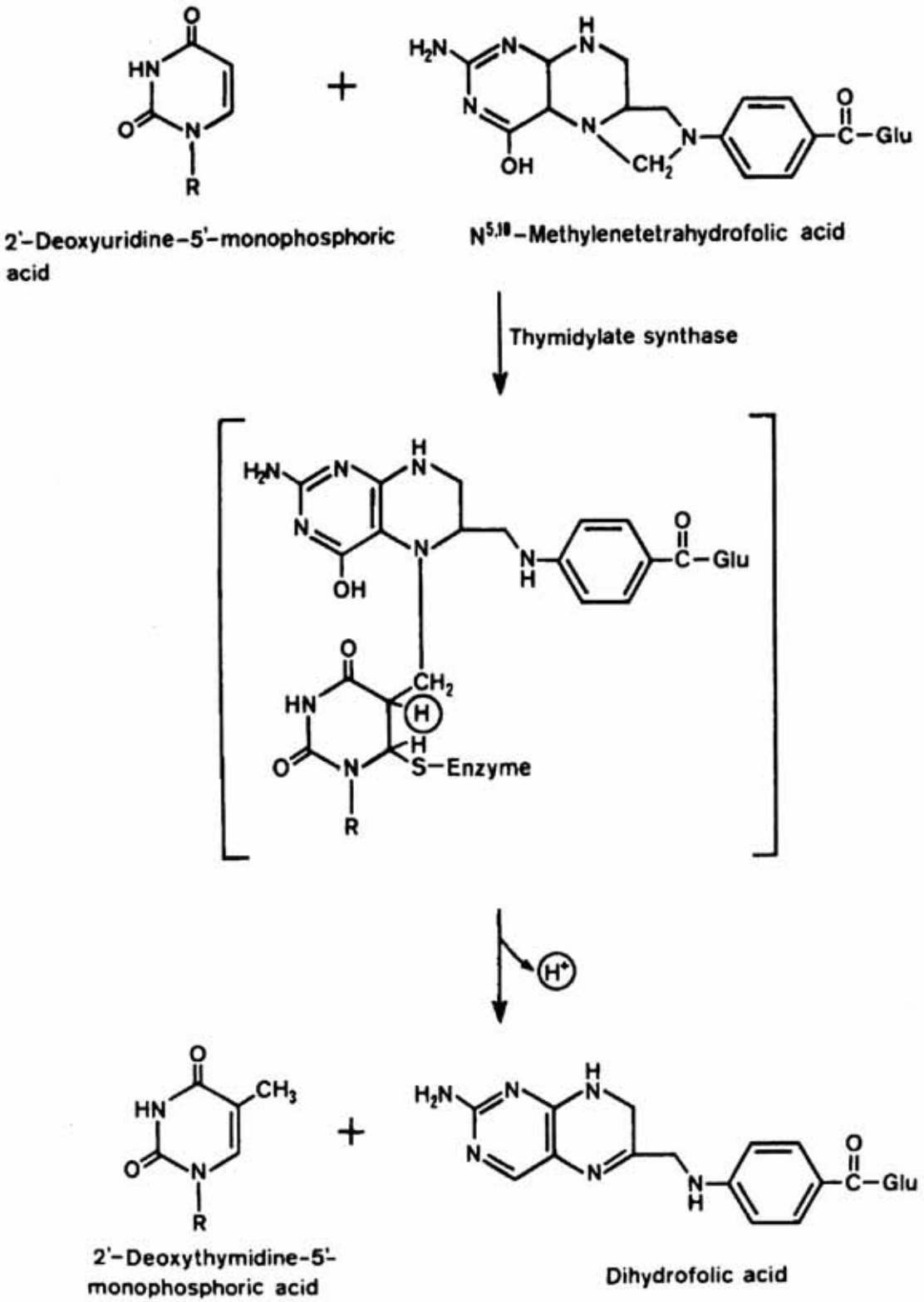

Fig. 1. The reaction catalyzed by thymidylate synthase.

bonding N^4-O-H...F-$C_{(5)}$ was hypothesized, influencing an assumed *syn-anti*, relative to N(3), equilibrium of rotamers around the $C_{(4)}$-N^4 bond (Fig. 4), and resulting in stabilization of the rare species *anti*, found to be the only inhibitory form [15]. However, results of *ab initio* quantum mechanical calculations brought such a mechanism into question [18, 19]. Thus,

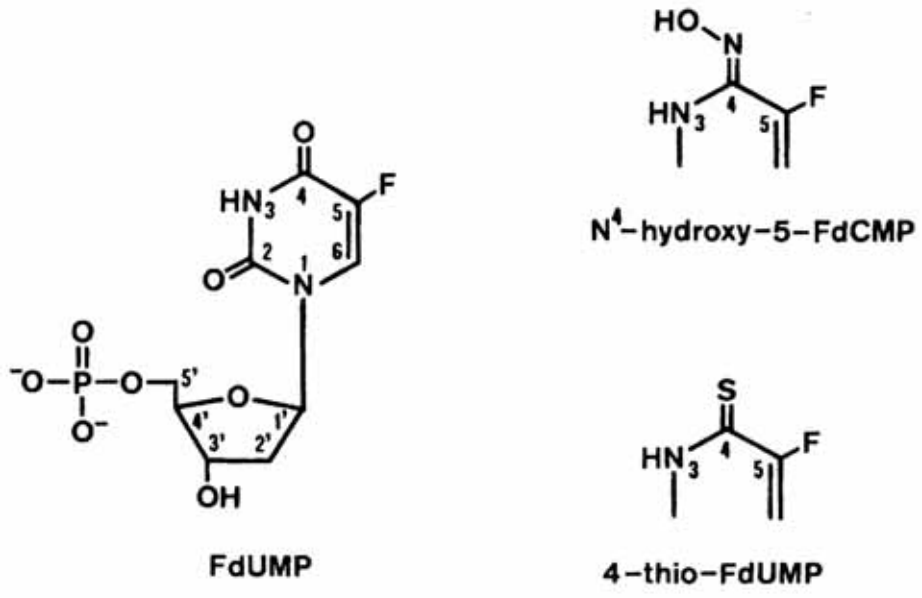

Fig. 2. Structures of FdUMP, N^4-OH-FdCMP and 4-thio-FdUMP.

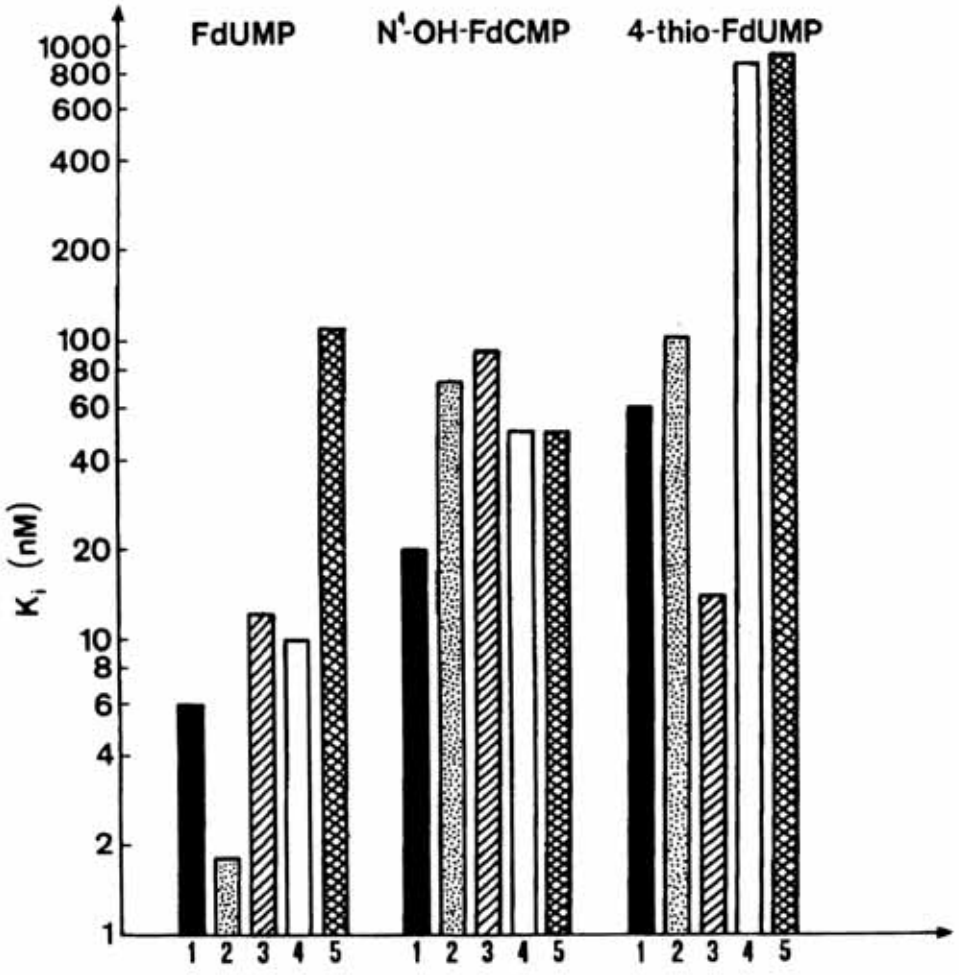

Fig. 3. K_i values describing inactivation by FdUMP, N^4-OH-FdCMP and 4-thio-FdUMP of thymidylate synthases from: Ehrlich carcinoma (1), L1210 parental (2) and FdUrd-resistant (3) cells, regenerating rat liver (4), and the tapeworm, Hymenolepis diminuta (5) [15, 16, 23].

mechanisms of the interplay between the $C_{(4)}$=N-OH and $C_{(5)}$-F, and between the $C_{(4)}$=S and $C_{(5)}$-F groups, influencing enzyme inactivation in such a way that it becomes sensitive to some non-conservative amino-acid residue(s) undergoing variations in active centres of different thymidylate synthases, do not have to be different.

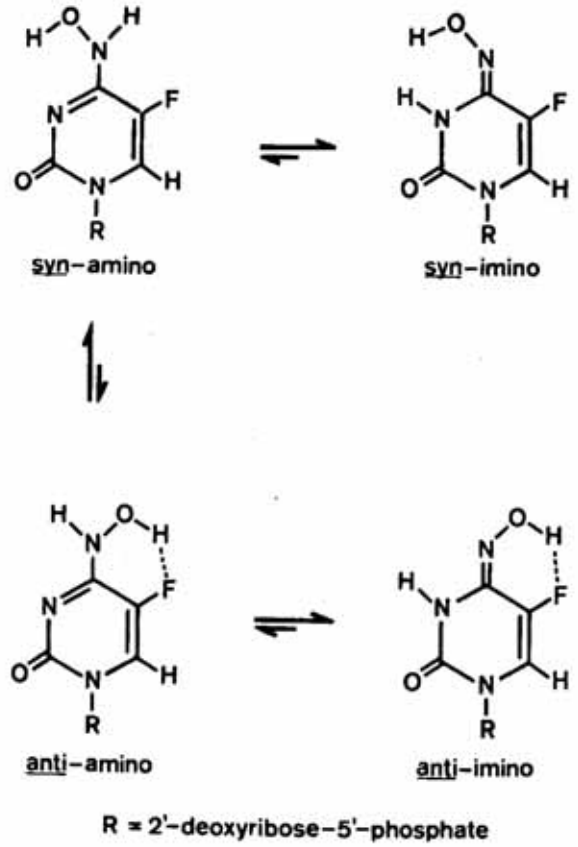

Fig. 4. Amino-imino and syn-anti equilibria for N^4-OH-FdCMP and hypothetical stabilization of the syn rotamers by intramolecular hydrogen bonding [15].

It should be noted that a crucial role has been ascribed to dUMP pyrimidine $C_{(4)}$=O and a non-dissociated N(3)-H groups in the specificity of enzyme binding, *via* an active centre asparagine residue and an ordered water molecule, of the pyrimidine moiety [20, 21]. This phenomenon, resulting in the discrimination by thymidylate synthase active centre between dUMP and dCMP, with the above mentioned asparagine residue proposed to stabilize, by hydrogen bonding, the partial negative charge developed on O^4 of covalently bound dUMP [22], has been suggested to be mechanism-based [20]. Since strong mechanism-based inactivation by FdUMP or 4-thio-FdUMP of thymidylate synthase has been shown to depend on a non-dissociated N(3)-H group [23], the active centre asparagine appears to be involved in the interaction, as proposed by Fig. 5. The same is probably true for N^4-OH-FdCMP and N^4-OH-dCMP, as (i) the most stable appear to be their imino forms [18, 19 and references therein], with the non-dissociated N(3)-H and $C_{(4)}$=N-OH [imitating the $C_{(4)}$=O] groups (Fig. 4), and (ii) comparison of thymidylate synthase inactivation by N^4-OH-dCMP and dCMP showed a lack of activity of the latter [15], indicating again strong demand for the structure involving N(3)-H and $C_{(4)}$=N- groups.

In view of the foregoing, the mechanism of apparent sensitivity of thymidylate synthase inactivation by 4-substituted FdUMP analogues to variations of nonconservative amino-acid residue(s) in the enzyme active center is probably related to the mechanism of pyri-

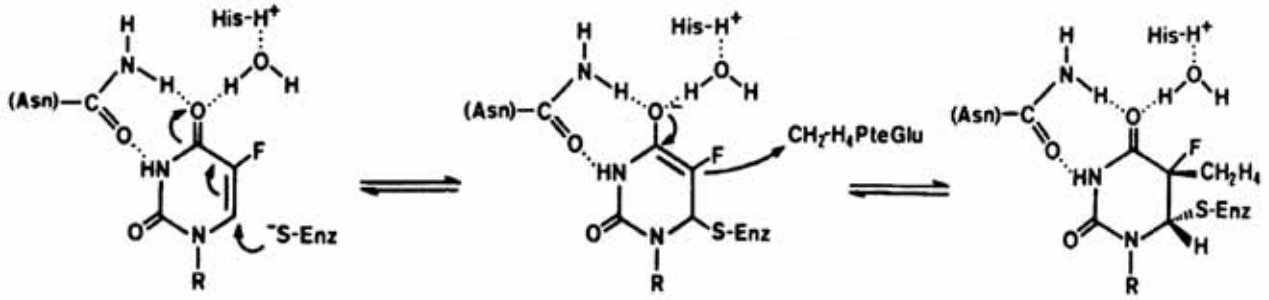

Fig. 5. Possible involvement of the active centre asparagine in thymidylate synthase inactivation by FdUMP (R = 2'-deoxyribose-5'-phosphate).

midine recognition and the crucial role of the $C_{(4)}$=O group in the latter. An additional support for this interpretation comes from the finding that specificity of 2-thio-FdUMP for inactivation of thymidylate synthases 1 - 5 (for enzyme sources see Fig. 3, legend) paralleled that of FdUMP [16, 24]. The exact nature of this phenomenon, potentially exploitable in chemotherapy, remains to be elucidated.

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