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CYTOTOXICITY OF 5-NITRO-2'-DEOXYURIDINE BY *IN VIVO* INHIBITION OF THYMIDYLATE SYNTHETASE

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Thymidylate (dTMP) synthetase (EC 2.1.1.1.45) catalyzes the reductive methylation of 2'-deoxyuridylate (dUMP) to 2'-deoxythymidylate (dTMP) with the concomitant conversion of 5,10-methylenetetrahydrofolic acid (CH_2-H_4folate) to 7,8-dihydrofolic acid. Because this enzyme represents the sole *de novo* pathway for dTMP synthesis, and its activity is required for DNA replication/repair, it is not surprising that it has received much attention as a target for inhibitors with potential chemotherapeutic utility. Recent reviews have been published on the catalytic mechanism (1) and important inhibitors (2) of this enzyme. Indeed, dTMP synthetase is a target for drugs such as 5-fluorouracil (FUra) and 5-fluoro-2'-deoxyuridine (FdUrd) which, after metabolism to the corresponding deoxyribonucleotide, can form a covalent complex with the enzyme with resultant cytotoxicity to proliferating cells. We have recently found that 5-Nitro-2'-deoxyuridylate (NO_2dUMP) is a potent inhibitor of dTMP synthetase which, after formation of a tight reversible complex, forms a covalent bond with the enzyme (3). Indeed, it has been reported that 5-Nitro-2'-deoxyuridine (NO_2dUrd) shows anti-viral activity (4,5) which was attributed to inhibition of dTMP synthetase in infected cells (5). However, to our knowledge the cytotoxicity of this compound has not been investigated. In this paper we report that NO_2dUrd is very cytotoxic to a number of cells grown in culture. Further, the preliminary data provided here strongly suggest that the cytotoxicity is due to *in vivo* inhibition of dTMP synthetase.

Materials and Methods

HTC-4 cells, a subclone of buffalo rat hepatoma tissue culture cells (6), were maintained as suspension cultures in Swims 77 media supplemented with 10% heat treated (56°, 30 min) horse serum. S-49 and S-49/TK$^-$ mouse lymphoma cells and L1210 mouse leukemia cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat treated

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horse serum. The S49/TK$^-$ strain, provided by B. Ullman, was selected for resistance to BrdUrd, and shown to be deficient in thymidine kinase activity by *in vivo* and *in vitro* assays. For growth studies, cells were suspended (6 to 8 x 10^4 cells per ml) in 1 ml medium containing specified supplements or inhibitors and incubated at 37° for 48-72 hr; cell number was determined on a Coulter counter ZB_1 and maximally increased 10- to 20-fold over that innoculated. EC_{50} values refer to the concentration of inhibitor necessary to inhibit cell growth by 50% compared to controls grown under identical conditions except that the inhibitor was omitted; with the aforementioned exceptions, EC_{50} values were determined essentially as described by Geran *et al.* (7). NO_2dUrd, NO_2Urd and NO_2Ura were prepared and purified as described by Huang and Torrence (8).

Results and Discussion

Table 1 shows the EC_{50} values of NO_2dUrd in four cell lines under a variety of conditions. Also provided are EC_{50} values for NO_2Ura, NO_2Urd, FUra and FdUrd. First, it is noted that NO_2dUrd is quite cytotoxic towards S49, L1210 and HTC cells. The EC_{50} values in these lines are 30 to 60-fold greater than for FdUrd, but 6 to 40-fold less than that of FUra. The fact that the S49 strain lacking dThd kinase (S49/TK$^-$) is not affected by NO_2dUrd at concentrations exceeding 100-fold of the EC_{50} of the wild type indicates that phosphorylation to NO_2dUMP by this enzyme is required for the cytotoxic effects of NO_2dUrd. Second, the fact that the cytotoxic effects of NO_2dUrd are completely reversed by 10 μ*M* dThd, only slightly increased by 10 μ*M* dUrd and not affected by 10 μ*M* Urd strongly suggests that dTMP synthetase is the target enzyme inhibited by NO_2dUMP. This is reasonable in view of our recent finding that NO_2dUMP is a potent *in vitro* inhibitor of this enzyme which combines covalently with it in the presence or absence of CH_2-H_4folate (3). It is also of interest to note that both NO_2dUrd and FdUrd are more cytotoxic towards mouse L1210 and S49 cells than towards HTC cells; this is in accord with the observation that HTC cells possess higher levels of dTMP synthetase than do the mouse cell lines tested (9).

Table 1 also provides data on the cytotoxicity of NO_2Urd and NO_2Ura towards these cell lines. As shown, the EC_{50} of NO_2Ura is not reached at concentrations exceeding 0.3 m*M* in L1210 or S49 cells and is 0.25 m*M* in HTC cells. Likewise, NO_2Urd shows little cytotoxicity, having EC_{50} values of *ca.* 0.25 m*M* in all lines tested.

There are certain features of NO_2dUrd which suggest its effect as an anti-tumor agent should be pursued. First, next to FdUrd it is the most potent inhibitor of dTMP synthetase yet reported (2), both *in vitro* and in tissue culture studies. Second, its presumed major catabolic product, NO_2Ura, appears to be relatively non-cytotoxic; this is in striking

Table 1. EC_{50} values (μM) of 5-substituted pyrimidines and corresponding nucleosides [†]

Compound	Cell line			
	S-49	S-49/TK$^-$	L1210	HTC
NO_2dUrd	0.030	>3[‡]	0.033	0.25
NO_2dUrd *plus* 10 μM dThd	>3[‡]	--[§]	>3[‡]	>4[‡]
NO_2dUrd *plus* 10 μM dUrd	0.060	--[§]	0.12	--[§]
NO_2dUrd *plus* 10 μM Urd	0.030	--[§]	--[§]	0.27
NO_2Ura	>300[‡]	--[§]	>300[‡]	250
NO_2Urd	240	--[§]	240	280
FdUrd	0.001	>2	0.0005	0.005
FUra	1.0	--[§]	0.20	1.5

[†]Dose response curves were constructed from a minimum of 12 points in which concentrations of inhibitor spanned 3-log units.

[‡]Highest concentration tested; 50% growth inhibition not observed.

[§]Not tested.

contrast to FdUrd which yields the cytotoxic FUra upon catabolism. Third, unlike FdUMP, potent inhibition of dTMP synthetase by NO_2dUMP does not require the presence of CH_2-H_4folate. This could be of relevance in situations where intracellular folate cofactors are depleted, preventing inhibition of dTMP synthetase by FdUMP (10). Lastly, although not yet unequivocally demonstrated, preliminary evidence suggests that the sole intracellular target of NO_2dUrd is dTMP synthetase. If this proves to be the case, the minimal utility of this compound may be foreseen to be the elucidation of the mechanism of other compounds (*viz.* FUra, FdUrd, methotrexate) which are believed to exert their major cytotoxic effect by direct or indirect inhibition of dTMP synthesis. Should NO_2dUrd exert its cytotoxic effect in animals before it is degraded to the apparently inert NO_2Ura, it could be an effective anti-tumor agent. Such studies are now in progress.

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