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RAPID COMMUNICATION

MODULATION OF FLUOROURACIL METABOLISM AND CYTOTOXICITY BY NITROTHIOBENZYLINOSINE

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Fluorouracil (FUra) requires intracellular metabolism to its active nucleotides in order to exert cytotoxicity. FUra can be activated to fluorodeoxyuridine monophosphate (FdUMP), a potent inhibitor of thymidylate synthetase, which can prevent the formation of deoxythymidine monophosphate and subsequently interfere with DNA synthesis [1-3]. In addition, fluorodeoxyuridine triphosphate (FdUTP) can be incorporated into DNA and alter the stability of DNA and the synthesis of DNA replication intermediates [4-6]. FUra can also be metabolized to fluorouridine triphosphate (FUTP) which can then be incorporated into RNA and cause alterations in RNA function [1-3].

We recently showed that the nucleoside transport inhibitor dipyridamole (DP) augmented the toxicity of FUra in a human colon cancer cell line (HCT 116) in both growth inhibition assays and in viability assays measuring colony formation [7]. Inhibition of the salvage of physiologic amounts of thymidine and uridine, however, did not account for the ability of DP to augment FUra cytotoxicity [7]. Because DP can also inhibit the transport of other compounds such as phosphates and sugars [8,9], we wished to test whether FUra toxicity was enhanced by nitrothiobenzylinosine (NBMPR), a highly selective inhibitor of nucleoside transport [8,9]. Our initial studies indicate that NBMPR potentiates FUra cytotoxicity; furthermore, NBMPR produces a selective increase in intracellular FdUMP levels.

The effect of NBMPR on the intracellular accumulation of dThd, Urd and their 5-fluoro-analogs is shown in Fig. 1. NBMPR potently inhibited the uptake of all four nucleosides in a dose-dependent manner.

The effect of NBMPR on FUra toxicity was determined in cell growth experiments measuring growth inhibition following a 72 hr drug exposure. FUra at 5 μM inhibited cell growth to 38% of the control cell number (Fig. 2). NBMPR increased the cytotoxicity of FUra in a dose-dependent manner. For example, 0.05 μM and 2 μM NBMPR given with 5 μM FUra inhibited cell growth to 30% and 19% of the control, respectively, but increasing the concentration of NBMPR from 2 to 5 μM did not result in further augmentation of FUra toxicity (data not shown). Thus, 2 μM NBMPR increased FUra toxicity by two-fold, while NBMPR given without FUra did not inhibit cell growth (Fig. 2). Because thymidine (dThd) in pharmacological concentrations prevented the ability of DP to enhance FUra cytotoxicity [7], the influence of dThd on the cytotoxicity of FUra ± NBMPR was examined. Although NBMPR inhibited the uptake of 25 μM tritiated dThd into HCT 116 cells by 50% compared to control, the actual amount of dThd incorporated into the perchloric acid soluble fraction in the presence of 2 μM NBMPR was substantial (approximately 150 pmoles/10^6 cells, data not shown). As shown in Fig. 2, 25 μM dThd did not reduce the toxicity of FUra, suggesting that thymidylate depletion is not the critical effect in HCT 116 cells. The addition of 25 μM dThd did reverse, however, the augmentation of FUra toxicity produced by NBMPR. The effect of NBMPR

on nucleoside accumulation and modulation of FUra toxicity is very similar to the results obtained with DP and FUra (7), arguing that inhibition of nucleoside transport is indeed the critical effect.

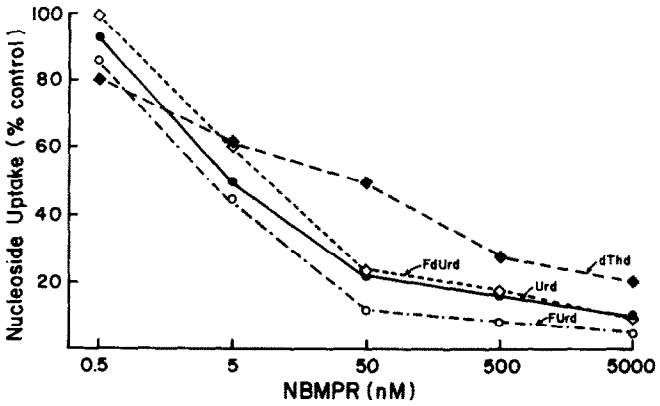

Fig. 1. Effect of NBMPR on nucleoside uptake in HCT 116 cells. Exponentially growing cells were exposed to [^{3}H]nucleoside (1 μM; 1 μCi/nmole) for 1 hr in the presence or absence of NBMPR. The amounts of radiolabeled nucleoside incorporated into the perchloric-acid extractable fraction (in the absence of NBMPR) were: dThd, 86 pmoles/10^6 cells; FdUrd, 452 pmoles/10^6 cells; Urd, 712 pmoles/10^6 cells; and FUrd, 458 pmoles/10^6 cells. The data, presented as percentage of control uptake, are from a single experiment. Similar results were obtained in duplicate experiments. The methodology has been reported previously [7].

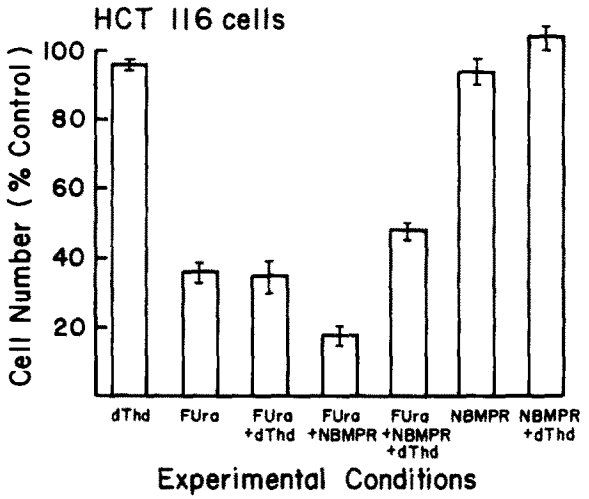

Fig. 2. Modulation of FUra toxicity by NBMPR. Exponentially growing cells were exposed to 5 μM FUra ± 2 μM NBMPR ± 25 μM dThd in medium supplemented with nondialyzed fetal calf serum. After 72 hr of drug exposure, the cells were enumerated with a Coulter counter (see Ref. 8 for methodology). The data are presented as the percentage of control cell number (bars: mean ± S.E., N = 3), with the control cell number 7.2 x 10^5 cells. The data are from a single experiment; similar results were obtained in duplicate experiments.

Because NBMPR could inhibit the transport and, consequently, the efflux of FUrd and FdUrd from HCT 116 cells, the effect of NBMPR on the pattern of FUra metabolism was examined using a modification of Pogolotti's method for complete analysis of FUra metabolites by high performance liquid chromatography using an ion-pairing gradient solvent system

[10]. The distribution of FUra metabolites was measured 4 hr following exposure to FUra ± NBMPR. Free FUra base accounted for over half of the intracellular label. FUrd, FdUrd, FUMP, FdUMP, and FdUTP were not detected. After a 4 hr exposure to 5 μM FUra, FdUMP and FUTP were the major FUra-nucleotides found [Table 1]; the FUTP pool size was over 5-fold larger than the FdUMP pool. The addition of NBMPR produced a 3-fold increase in the FdUMP level from 4.1 to 12.4 pmol/10^6 cells without affecting the FUTP pool size. The ratio of FdUMP:FUTP was increased from 0.18 to 0.65 in the presence of NBMPR. Because dThd was able to reverse the enhancement of FUra toxicity produced by NBMPR, the effect of 25 μM dThd on FUra metabolism in the presence and absence of NBMPR was also studied. dThd reduced the FdUMP over 3-fold to 1.2 pmol/10^6 cells without affecting the amount of FUTP. In addition, dThd not only prevented the FdUMP accumulation in cells exposed to FUra + NBMPR, but reduced the FdUMP levels to below those seen with FUra alone.

Table 1. Distribution of FUra metabolites after 4 hr of drug exposure

Condition	FUra	FdUMP	FUDP	FUTP
5 μM FUra	30.4 ± 8.5*	4.1 ± 0.7	2.9 ± 0.6	23.3 ± 1.9
5 μM FUra + 25 μM dThd	24.7 ± 4.3	1.2 ± 0.6	3.0 ± 1.0	23.7 ± 3.0
5 μM FUra + 2 μM NBMPR	30.5 ± 5.3	12.4 ± 3.2	2.2 ± 0.4	19.3 ± 1.2
5 μM FUra + 2 μM NBMPR + 25 μM dThd	42.5 ± 3.4	1.8 ± 0.2	3.9 ± 1.0	29.3 ± 4.4

*Data are expressed as pmoles/10^6 cells (mean ± S.E., N = 3).

Since nucleoside transport inhibitors can block the influx and efflux of nucleosides [8,9], inhibition of FdUrd efflux might be a potential mechanism by which NBMPR produced an increase in FdUMP levels. In order to test this possibility, HCT 116 cells were prelabeled with [6-^{3}H]FdUrd to permit incorporation of [6-^{3}H]FdUMP intracellularly. The [6-^{3}H]FdUrd was then removed, and fresh medium containing either no drugs, NBMPR, dThd, or NBMPR plus dThd was then added. The intracellular retention of FdUMP and the appearance of FdUrd in the medium 1 hr later are shown in Fig. 3. Under control conditions, over 70% of the initial FdUMP was lost by 60 min. NBMPR maintained the FdUMP at 60% of the initial pool size. 25 μM dThd, in contrast, accelerated the loss of FdUMP from the cell, with less than 5% of the FdUMP pool retained after 60 min whether or not NBMPR was present. In parallel, NBMPR delayed the appearance of FdUrd in the medium. In the presence of 25 μM dThd, however, a larger amount of FdUrd was detected extracellularly compared to control.

We have shown that the nucleoside transport inhibitor NBMPR augments the cytotoxicity of FUra and produces a selective 3-fold increase in intracellular FdUMP pools. The mechanism by which NBMPR increases the levels of FdUMP appears to be blockade of the efflux of FdUrd. Both the enhancement of FUra cytotoxicity and the selective buildup of FdUMP produced by NBMPR were reversed by 25 μM dThd. 25 μM dThd increased the loss of FdUMP from cells and the appearance of FdUrd in the medium even in the presence of NBMPR. Therefore, competition of dThd with FdUrd for dThd kinase is a likely explanation for the diminished FdUMP levels. Because dThd did not protect cells from the toxicity exerted by FUra alone, these findings suggest that NBMPR is altering the mechanism by which FUra causes cytotoxicity. Nucleoside transport inhibitors may provide a novel means of enhancing the cytotoxicity of FUra through increased FdUMP accumulation.

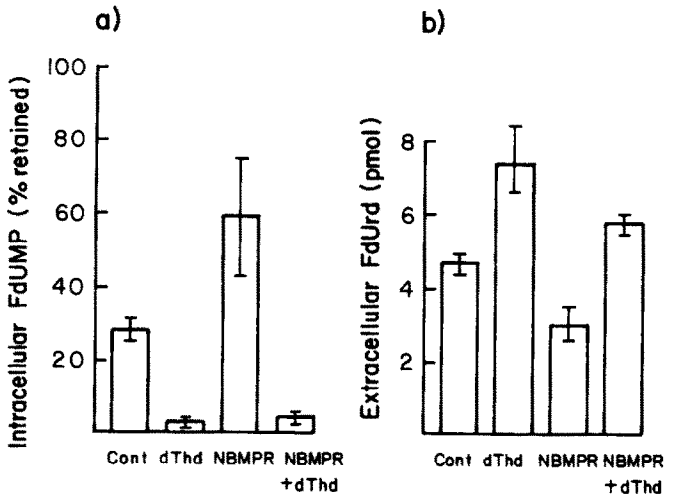

Fig. 3. The effect of NBMPR on the retention of intracellular FdUMP in HCT 116 cells. Exponentially growing cells were prelabeled with 8 μCi [6-^{3}H]FdUrd (20 Ci/mmol) for 4 hr. The medium was then aspirated and the cells were washed 3 times with phosphate buffered saline to remove the extracellular label. Fresh medium containing either no drugs (control), 2 μM NBMPR, 25 μM dThd, or 2 μM NBMPR plus 25 μM dThd was then added. The cells and the medium were extracted with PCA immediately and 60 min thereafter. The neutralized acid-soluble fractions were lyophilized and reconstituted in distilled water. The intracellular constituents were analyzed by HPLC to determine the FdUMP levels using the methodology referred to previously [10]. The extracellular constituents were analyzed by HPLC using a reverse phase column with water as the solvent to determine the amount of FdUrd appearing in the medium. At the zero time point, the FdUMP pool was 7pmoles/10^6 cells, and <0.5 pmoles FdUrd were present in the medium. Fig. 3a represents the intracellular FdUMP pool 60 min following removal of the label under the various conditions as a percentage of the FdUMP pool measured at the zero time point (mean + range, N=2). Fig. 3b represents the amount of FdUrd present in the medium 60 min following removal of the extracellular label under the various conditions. The data are presented as the pmoles of FdUrd per 10^6 cells per dish (mean + range, N=2).

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