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CYTOTOXIC MECHANISMS OF 5-FLUOROPYRIMIDINES

RELATIONSHIPS WITH POLY(ADP-RIBOSE) POLYMERASE ACTIVITY, DNA STRAND BREAKAGE AND INCORPORATION INTO NUCLEIC ACIDS

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Abstract—A comparative study of the cytotoxic mechanisms of 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FdUrd) was carried out in Chinese hamster ovary K1 (CHO-K1) cells. The poly(ADP-ribose) polymerase (PADPRP) inhibitor, 3-aminobenzamide (3AB, 3 mM) enhanced the cytotoxicity of FU with a dose enhancement factor at 10% survival of 2. This enhancement was also evident when cells were grown in dThd-free medium, but the IC_{50} for FU was reduced from 50 to 35 μM. In contrast, 3AB did not enhance the cytotoxicity of FdUrd but exerted a small protective effect. The IC_{50} for FdUrd was reduced from 35 to 1.25 μM in dThd-free medium. A 55% reduction in NAD levels was seen within 6 hr of 5.0 μM FdUrd treatment in dThd-free medium, and this reduction persisted over 24 hr. This drop was prevented by co-incubation with 3AB, indicating that PADPRP activation was the cause of the NAD depletion. In contrast, FU treatment had little or no effect on NAD levels. Alkaline elution analysis of cells treated with up to 150 μM FU revealed no DNA strand breaks in mature DNA, but an increase in breaks in nascent DNA. Co-incubation with 3AB had little or no effect on strand break levels. FdUrd (up to 40 μM) produced a dose-dependent increase in both mature and nascent DNA strand breaks. Analysis using a "relative elution" formula demonstrated that 3AB increased the amount of FdUrd-induced strand breaks (at doses ≤5–100 μM) in mature DNA. Whereas FU elution profiles for nascent DNA were biphasic, those for FdUrd were linear. Co-incubation with 3AB increased [^{3}H]FU incorporation into both RNA (by 50%) and DNA (45%). 3AB also enhanced [^{3}H]FdUrd incorporation (by 40%) into RNA but had no effect on incorporation into DNA. These data indicate that in addition to acting as an inhibitor of PADPRP, 3AB exerts other metabolic effects.

The cytotoxic effects of 5-fluorouracil (FU†) have been widely exploited in cancer chemotherapy. FU has a number of metabolic effects. As FUTP it is incorporated into RNA [1, 2], and may inhibit mRNA splicing [e.g. 3, 4]. FdUMP inhibits thymidylate synthase (TS) [5] (which converts dUMP to dTMP) and results in depletion of dTTP pools and increase of dUTP pools. This leads to misincorporation of dUTP and FdUTP into DNA resulting in excision repair [6]. 5-Fluoro-2′-deoxyuridine (FdUrd), which is also used to treat some forms of cancer, is cytotoxic due to the formation of FdUMP [7] and therefore exerts its effects mainly on DNA. However, FdUrd can also be degraded to FU by pyrimidine phosphorylase [8] in which case it can also be incorporated into RNA. It is apparent that the metabolic fates, and presumably the cytotoxic mechanisms, can vary in different cell lines [9–11].

The substituted benzamides have been extensively used as competitive inhibitors of the enzyme poly(ADP-ribose) polymerase (PADPRP, EC 2.4.2.30), a nuclear enzyme which is activated by DNA strand breaks and utilizes NAD as a substrate to modify chromatin proteins by poly(ADP-ribosylation) [12]. 3-Aminobenzamide (3AB) has been shown to enhance the cytotoxicity of a variety of DNA damaging agents, increase the level of DNA strand breaks and increase repair synthesis [e.g. 13–15]. However, recent work has indicated that 3AB has other metabolic effects. For example, 3AB enhanced the cytotoxicity of 6-mercaptopurine and this enhancement correlated with increased phosphoribosylpyrophosphate (PRPP) levels, indicating a synergistic effect of 3AB on inhibition of *de novo* purine synthesis [16].

In this paper, we have investigated in the same cell line (Chinese hamster ovary K1, CHO-K1) the metabolic fates and cytotoxic effects of FU and FdUrd. The role of poly(ADP-ribosylation) reactions in the cytotoxic response has been investigated utilizing 3AB as a PADPRP inhibitor. The results demonstrate fundamental differences in the cytotoxic mechanisms of FU and FdUrd, with respect to their incorporation into nucleic acids and effects on the integrity of DNA. Evidence is presented that FdUrd (but not FU) activates PADPRP, resulting in a rapid depletion of cellular NAD, but inhibition of DNA

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† Abbreviations: FU, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FUrd, 5-fluorouridine; PRPP, phosphoribosylpyrophosphate; PADPRP, poly(ADP-ribose) polymerase; 3AB, 3-aminobenzamide; CHO-K1, Chinese hamster ovary K1 cells; dThd-free, thymidine-free Hams F10 medium; TS, thymidylate synthase.

excision repair mechanisms by 3AB does not enhance the cytotoxicity. Finally, we demonstrate that 3AB, in addition to acting as a PADPRP inhibitor, has another metabolic effect resulting in augmented incorporation of FU and FdUrd into nucleic acids.

MATERIALS AND METHODS

Cell culture. CHO-K1 cells were maintained as a monolayer in Hams F10 medium (supplemented with 10% serum, glutamine and antibiotics) as described previously [17]. Hams F10 contains 3 μM thymidine, an amount which could affect the cytotoxic response of FU and FdUrd, since the drugs exert their toxicity in part by inhibition of TS. Consequently, some experiments were carried out both in normal medium and "thymidine-free" (dThd-free) medium (medium whose only thymidine contribution came from the serum (10% v/v) used. It was observed that the serum used contains ~0.7 μM dThd (personal communication, Mr G. Taylor, this department) and therefore, normal medium contains ~40 times as much dThd as dThd-free medium. dThd-free medium was obtained from Flow Labs (Irvine, U.K.).

Drugs and chemicals. 3AB was obtained from Pfaltz and Bauer (Phase separations, Deeside, U.K.). FU, FdUrd, 5-fluorouridine (FUrd) and other chemicals used in assays (unless otherwise stated) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). FU was dissolved in 0.01 M KOH and FdUrd in water. Both were filter sterilized and stored at −20° in aliquots which were used once and then discarded. 3AB was dissolved in complete medium, filter sterilized and stored at 4° for up to 14 days.

[^{14}C]dThd (51 mCi/mmol) and [^{3}H]dThd (45 Ci/mmol) were obtained from Amersham International (Amersham, U.K.). 5-[6-^{3}H]Fluorouracil (15 Ci/mmol), 5-[6-^{3}H]fluorodeoxyuridine (18 Ci/mmol) and [^{32}P]orthophosphoric acid (carrier free, 9000 Ci/mmol) were obtained from New England Nuclear (Boston MA, U.S.A.).

Drug treatment and clonogenic assays. These were performed as described previously [17] with the concentrations and time intervals as stated in the figure legends.

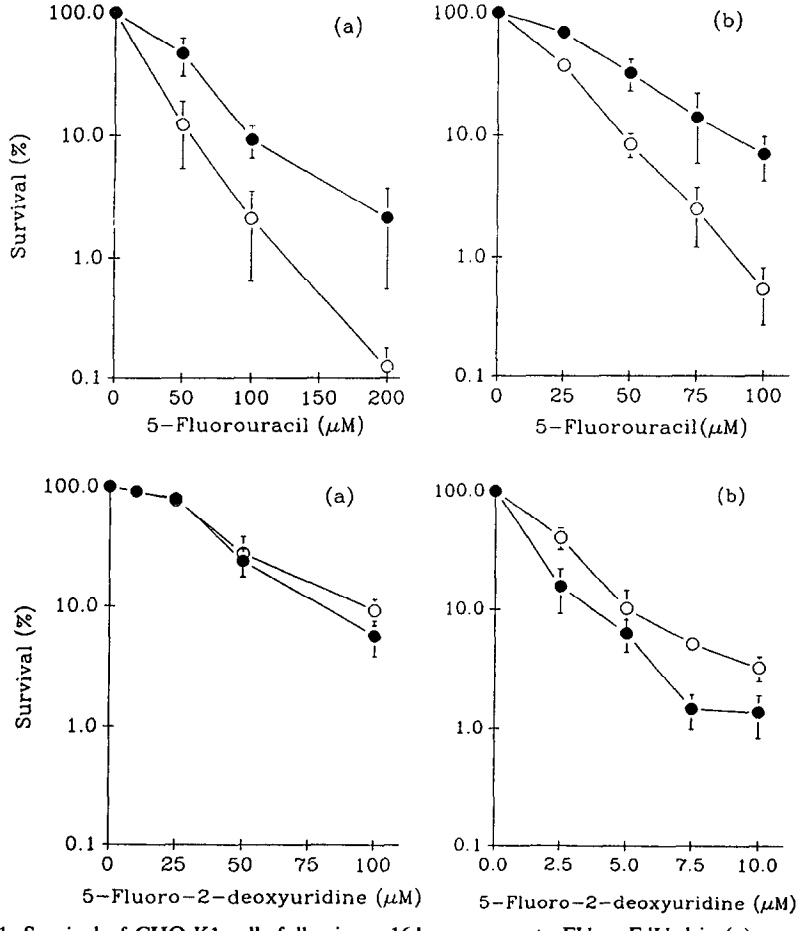

Fig. 1. Survival of CHO-K1 cells following a 16 hr exposure to FU or FdUrd in (a) normal medium and (b) dThd-free medium in the absence (●) or presence (○) of 3 mM 3AB. Results are the means of at least three independent experiments ± SEM.

NAD assays. Cells were seeded at 4×10^5/dish in Thd-free medium and incubated overnight. Drugs were added and at different times thereafter the cells were harvested, NAD extracts prepared and assayed as described previously [18], with the modification that cell extracts were also assayed for protein concentration using Coomassie reagent. Results are the means of three experiments.

Alkaline elution studies. The method used was that of Kohn *et al.* [19], which allows measurement of single strand breaks in mature and nascent DNA. For mature DNA, exponentially growing cells were exposed to [^{14}C]dThd (0.02 μCi/mL) while internal standard cells were exposed to [^{3}H]dThd (0.1 μCi/mL) for 16 hr in dThd-free medium. The radioactive medium was then aspirated and replaced with fresh medium to allow a 6 hr chase period after which drugs were added for a 24 hr exposure (at concentrations stated in the figure legends, except for 3AB, which, when present, was used at a final concentration of 3 mM) to the [^{14}C]dThd-labelled cells. After this period, both drug-treated and internal standard cells were scraped into phosphate-buffered saline (pH 7.2) to form a suspension; internal standard cells were irradiated with 300 cGy X-rays and then both sets of walls were loaded on to filters and lysed prior to elution.

For nascent DNA, exponentially growing cells were exposed to drugs for 24 hr and labelled with similar amounts of [^{14}C]dThd as for mature DNA during the last 4 hr of drug exposure immediately prior to loading onto filters. Similarly, internal standard cells were exposed to [^{3}H]dThd for 4 hr prior to irradiation and were then loaded onto filters.

Incorporation of FU and FdUrd into nucleic acids. Exponentially growing cells in dThd-free medium were exposed simultaneously to [^{3}H]FU (5 μCi/mL) or [^{3}H]FdUrd (4 μCi/mL), and $H_3[^{32}P]O_4$ (10 μCi/mL) for 16 hr before harvesting. Cells were washed in phosphate-buffered saline and treated with a lysis buffer containing 0.01 M Tris (pH 7.4), 0.01 M EDTA, 0.5% sodium dodecyl sulphate and 100 μg/mL proteinase K (BRL, Gaithersburg, MD, U.S.A.). The lysates were digested for 3 hr at 50° and purified by phenol/chloroform extraction [20]. Nucleic acids were precipitated and prepared for analysis by caesium sulphate gradient density centrifugation as described previously [21] to examine incorporation of [^{3}H]FU or [^{3}H]FdUrd, and $H_3[^{32}P]O_4$ into RNA and DNA.

RESULTS

Clonogenic survival assays

Figure 1 shows the clonogenic survival of CHO-K1 cells following a 16 hr exposure to FU in the presence and absence of 3 mM 3AB: (a) when cells were grown in normal medium and (b) in dThd-free medium. 3AB potentiated the cytotoxicity of FU with a dose enhancement factor at 10% survival of 2-fold, and this effect was not altered when the assay was carried out in dThd-free medium. Whereas the IC_{50} (the concentration of drug required to decrease survival by 50%) for FU alone was 50 μM in normal medium, it was reduced to 35 μM in dThd-free medium.

Figure 1 also shows survival following a 16 hr exposure to FdUrd in the presence and absence of 3AB in normal (c) and dThd-free medium (d), respectively. 3AB did not potentiate the cytotoxicity of FdUrd but had some protective effect. Note that the IC_{50} was reduced from 35 to 1.25 μM when dThd-free medium was used.

NAD assays

A rapid drop in cellular NAD levels within 2 hr (attributable to PADPRP activation) is known to occur after certain types of DNA damage [e.g. 13]. The effect of FdUrd treatment on cellular NAD levels, when cells were grown in dThd-free medium, is shown in Table 1. There was a significant depletion in cellular NAD content following 6 and 24 hr exposure to FdUrd, and this was abolished by co-incubation with 3AB. For example, 2.5 μM FdUrd (which reduced survival to 20% in dThd-free medium) had caused a 23% decrease by 6 hr and had reached 53% by 24 hr. In contrast, FU treatment had little or no effect on NAD levels (results not shown).

Table 1. Effect of FdUrd on cellular NAD levels

Time (hr)	Treatment*	NAD (pmol/mg protein)
6	Control	2698 ± 137
6	2.5 μM FdUrd	2082 ± 93
6	5.0 μM FdUrd	1230 ± 63
6	5.0 μM FdUrd + 3AB	2262 ± 185
6	3AB	2797 ± 339
24	Control	2894 ± 93
24	2.5 μM FdUrd	1406 ± 143
24	5.0 μM FdUrd	1216 ± 230
24	5.0 μM FdUrd + 3AB	2445 ± 187
24	3AB	4283 ± 202

Cells were treated with FdUrd in the presence or absence of 3AB for the times indicated, and then harvested and prepared for NAD and protein assays.
* When included, 3AB was used at a final concentration of 3 mM.
Values are means ± SEM.

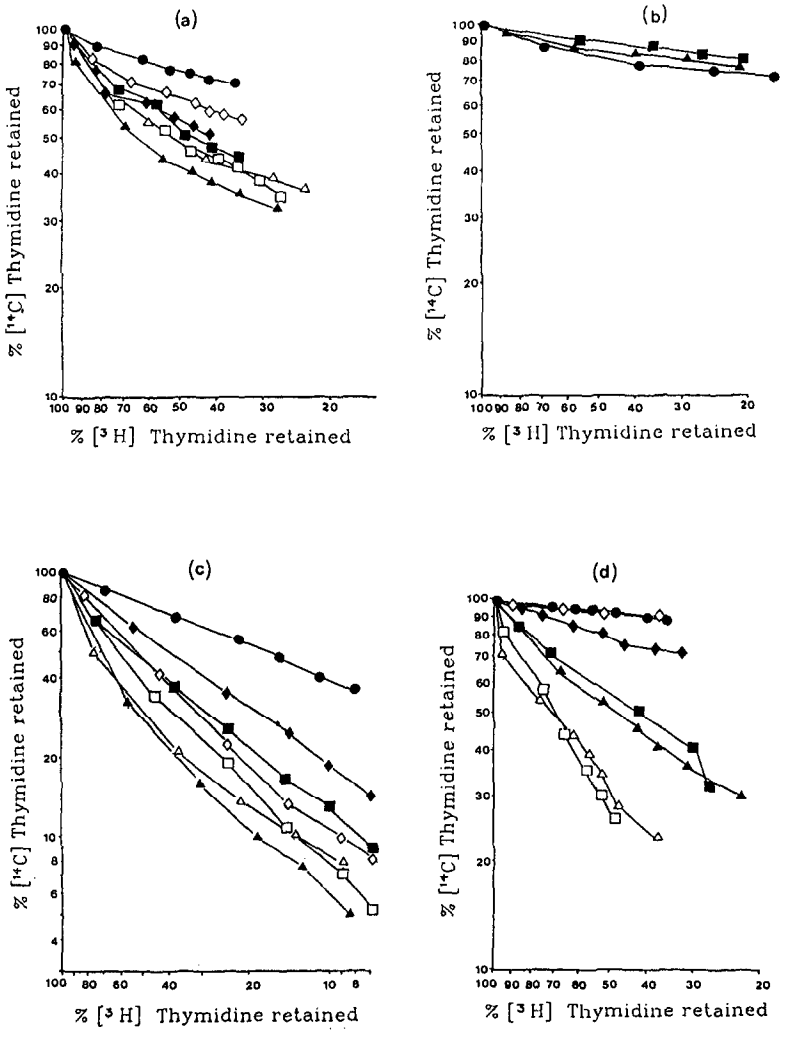

Fig. 2. Alkaline elution profiles for FU-treated cells. (a) Profile for nascent DNA following a 24 hr exposure of cells to FU including labelling during the last 4 hr of drug exposure. (●) Control (untreated); (◆) 50 μM; (■) 100 μM; (▲) 150 μM. (Profile for 3AB alone was identical to that for the untreated control.) (◇) 3AB + 50 μM; (□) 3AB + 100 μM; (△) 3AB + 150 μM. (b) Profile for mature DNA after labelling for 16 hr followed by a 6 hr chase period and then a 24 hr exposure to FU. (●) Control; (■) 5 μM; (▲) 100 μM. Profiles for FdUrd-treated cells (timing of labelling and drug treatments were identical to those for FU-treated cells). (c) Profile for nascent DNA; (●) control (untreated); (◆) 3 μM; (■) 10 μM; (▲) 40 μM; (◇) 3AB + 3μM; (□) 3AB + 10 μM; (△) 3AB + 40 μM. (d) Profile for mature DNA (●) control; (◆) 1 μM; (■) 10 μM; (▲) 20 μM; (◇) 3AB + 1 μM; (□) 3AB + 10 μM; (△) 3AB + 20 μM. Results shown are from one experiment.

Alkaline elution studies

Figure 2a shows that FU treatment (0–150 μM) produced a dose-dependent increase in the number of single strand breaks (which includes alkali-labile lesions) in nascent DNA. Co-incubation with 3AB caused little or no effect on the amount of DNA strand breaks produced over the same dose range. When mature DNA was analysed (Fig. 2b) the profiles showed that doses of up to 100 μM FU (which reduced survival to <10%) had no effect on the rate of elution of mature DNA compared to untreated cells.

FdUrd produced a dose-dependent increase in strand breaks in both nascent and mature DNA (Fig. 2c and d). This increase was apparent up to doses of 40 μM, after which increasing the concentration of FdUrd did not further increase the DNA damage. Co-incubation with 3AB increased the amount of FdUrd-induced strand breaks in mature DNA only at the higher doses of FdUrd tested. This increase in strand breaks in the presence of 3AB was also seen when nascent DNA was studied, but the effect was not as marked. To analyse in more detail the effect of 3AB on the number of FdUrd-induced strand breaks in nascent and mature DNA, a "relative elution" formula was used [21]. Figure 3a

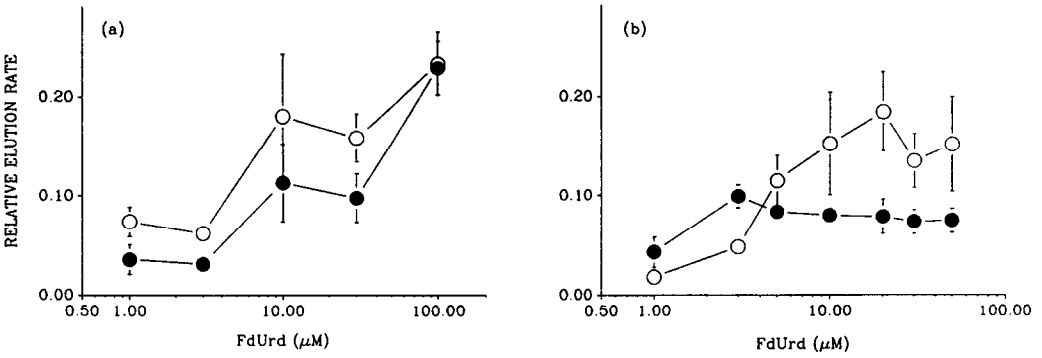

Fig. 3. Relative elution rates for FdUrd-treated cells. These were calculated from mean retention values (from at least three experiments ± SEM). The formula used for relative elution is log (retention FdUrd ± 3AB treated sample)—log (retention of control sample). A retention value of 0.8 [^{3}H]dThd was used (similar results were obtained using a retention value of 0.5; however, this would have excluded one of the graphs used because of variability of elution rates between experiments). Although the values yielded are −ve, the relative elution values are shown as +ve for simplicity of presentation. (●) FdUrd alone; (○) FdUrd + 3AB (a) in nascent and (b) in mature DNA.

shows that 3AB treatment produced a small but significant increase in the amount of FdUrd-induced strand breaks in nascent DNA over the dose range 1–50 μM. Figure 3b demonstrates that in mature DNA, 3AB increased the amount of breaks, but only at doses ≥5 μM. The results for the ±3AB relative elution values in Fig. 3 were verified as significantly different by the Student's paired *t*-test ($P \leq 0.024$ at 3, 10 and 30 μM FdUrd for Fig. 3A; $P = 0.067$, 0.028 and 0.080, respectively, at 10, 30 and 50 μM FdUrd for Fig. 3B). When the elution profiles obtained for nascent DNA from FU- and FdUrd-treated cells were compared, a consistent difference in the shape of the profiles was observed. Whereas FU-treated cells gave distinctly biphasic profiles (e.g. Fig. 2a) indicative of the selective accumulation of small DNA fragments (as was also observed by Grem *et al.* [22]), FdUrd-treated cells produced more linear profiles (e.g. Fig. 2c), demonstrating a more heterogeneous distribution of size classes of DNA.

Incorporation of [^{3}H]*FU and* [^{3}H]*FdUrd into nucleic acids*

Since it was possible that 3AB could modulate the cytotoxicity of FU and FdUrd by altering their metabolic fates, their incorporation into nucleic acids was measured. Cells were simultaneously labelled with [^{32}P]orthophosphoric acid together with either [^{3}H]FU or [^{3}H]FdUrd, and the results expressed as ^{3}H/^{32}P ratios. Co-incubation with ^{32}P allows for experimental differences in nucleic acid synthesis during labelling to be accounted for. It was established that 3AB *per se* had no effect on ^{32}P incorporation into trichloroacetic acid-precipitable counts (results not shown). 3AB significantly increased the incorporation of [^{3}H]FU into DNA and RNA as compared to controls (Fig. 4). For RNA, 3AB increased the ratio by about 50% and for DNA by about 45%. Analysis of the ratios indicates that about seven times as much FU is incorporated into RNA as compared to DNA. 3AB also increased the incorporation of [^{3}H]FdUrd into RNA (Fig. 4) by about 40% but had little or no effect on the incorporation into DNA. In contrast to FU, only 1.3 times as much FdUrd is incorporated into RNA as compared to DNA.

DISCUSSION

Although exogenous dThd slightly decreased the cytotoxicity of FU and FU + 3AB, it did not alter the 2-fold enhancement by 3AB. In contrast, the IC_{50} for FdUrd was reduced from 35 μM in normal medium to 1.25 μM in dThd-free, indicating that TS inhibition is its main cytotoxic mechanism. In this case, 3AB did not enhance FdUrd toxicity but slightly reduced it. These data suggest that the mechanism by which 3AB potentiates FU toxicity in CHO-K1 cells is one to which inhibition of TS makes little or no contribution.

Our data is consistent with that of Roobol *et al.* [9], who demonstrated that exogenous dThd protects L1210 cells from FdUrd-induced cytotoxicity as the salvage pathway comes into operation to relieve TS inhibition, but has no effect on FU cytotoxicity. In a cell line whose FU cytotoxicity is wholly RNA directed, dThd can actually increase incorporation of FU into RNA and enhance cytotoxicity. Spiegelman *et al.* [23] showed that dThd protects FU from catabolism and thus augments its ribonucleotide incorporation; however, this was clearly not the case in our cell line.

The rapid NAD drop (within 6 hr) induced by FdUrd treatment is reminiscent of that seen with other DNA damaging agents, e.g. dimethyl sulphate [13]. In agreement with our results, Yingnian *et al.* [24] demonstrated that by 24 hr 10 μM FdUrd had caused NAD depletion in human FL amniotic cells which was prevented by 3AB. Table 1 shows that 3AB completely prevented the FdUrd-induced NAD

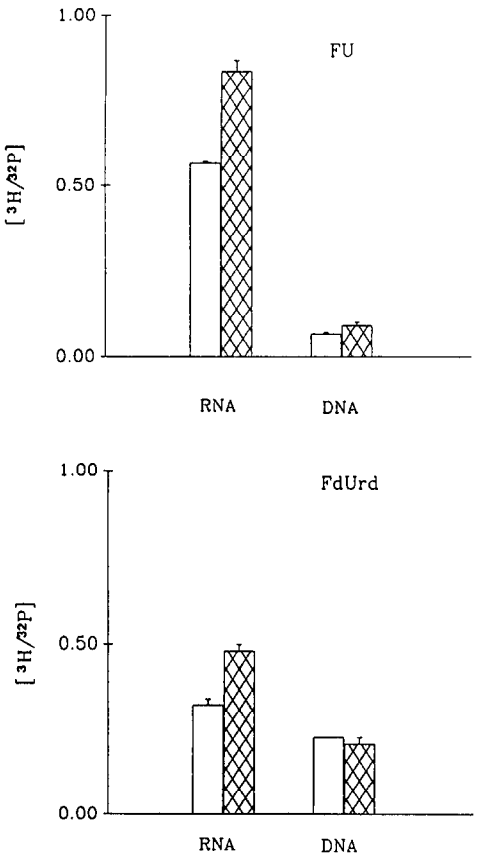

Fig. 4. Incorporation of FU and FdUrd into RNA and DNA. Cells were treated with [^{3}H]FU and ^{32}P, or [^{3}H]-FdUrd and ^{32}P, in the absence or presence of 3AB (3 mM) for 16 hr to follow incorporation of radiolabel into RNA and DNA. Results were averaged from three independent experiments, each performed in triplicate. The results of paired *t*-tests (comparing the incorporation of ^{3}H in the presence and absence of 3AB) for both DNA and RNA were significant at the 95% confidence level. Representative histograms show the [^{3}H]FU/^{32}P and [^{3}H]FdUrd/^{32}P ratios (±SEM) for DNA and RNA (open bars show the ratios for cells treated with FU or FdUrd alone; cross-hatched bars show the ratios for the cells co-incubated with 3AB).

depletion indicating that FdUrd treatment causes the type of DNA damage that activates PADPRP.

NAD depletion in cells treated with FdUrd may contribute to its cytotoxic mechanism. Cohen and Barankiewicz [25] showed that a rapid depletion of NAD (caused by *N*-methyl-*N'*-nitro-nitroso-guanidine treatment) led to a sharp drop in ATP levels, which was non-recoverable. Since 3AB actually reduced FdUrd-induced cytotoxicity, it is probable that the NAD drop contributes to the cytotoxic mechanisms of FdUrd, and by extrapolation of other chemotherapeutic agents which are TS inhibitors.

Ingraham *et al.* [6] have shown that FdUTP and dUTP can be excised effectively from DNA. The specific TS inhibitor, CB3717, produces a dose-dependent increase in DNA strand breaks in mature DNA [26], and this was attributed to inhibition of the repair of spontaneous DNA damage. This would arise because of the dUTP/dTTP pool imbalance leading to reiterative misincorporation of dUTP into repair patches. A similar mechanism can be attributed to FdUrd, with the addition that FdUTP misincorporation into repair patches will also occur. The absence of such FU-induced strand breaks in mature DNA at doses of FU which are highly cytotoxic (e.g. 100 μM reduced survival to <10%) indicates that the metabolic perturbations caused by FU are not sufficient to produce this type of DNA damage. This data is in concurrence with that of Yin and Rustum [11] who found that in HCT-8 cells whose growth was inhibited by >99% by FU treatment, there was negligible damage to mature DNA. In contrast, 1 μM FdUrd (which only reduced survival to ~60%) produced measurable damage in mature DNA.

The observation that there is a marked increase in FdUrd-induced strand break levels, particularly in mature DNA, in the presence of 3AB implicates poly(ADP-ribosylation) reactions in the repair mechanisms of this damage, consistent with the NAD depletion observed. However, this is not correlated with enhancement of FdUrd cytotoxicity by 3AB. These results suggest that single strand DNA breaks are not in fact the lethal lesion, and that toxicity is mediated by other pathways (e.g. unbalanced growth). This contrasts with the consistent correlation between increased DNA strand breaks levels and enhanced cytotoxicity observed with other DNA damaging agents used in conjunction with 3AB [e.g. 13, 27].

The misincorporation of dUTP and FdUTP at the replication fork [28] would lead to post-replication excision repair and the consequent appearance of nascent DNA strand breaks and retardation of chain elongation. The data presented here demonstrate nascent DNA damage with both FU and FdUrd as has been observed in FU- and CB3717-treated cells [22, 26, 29]. Additionally, they highlight differences in the distribution of damage induced in nascent DNA by FU and FdUrd, as shown by the marked changes in the shapes of the elution profiles. These data are the first direct comparison of FU and FdUrd in a single cell line, where differences in the cytotoxic mechanisms have been confirmed by "thymidine rescue" experiments.

The increased incorporation of [^{3}H]FU into DNA and RNA by co-incubation with 3AB indicates that 3AB augments the anabolism of the base analogue, and this presumably is the mechanism whereby 3AB potentiates FU cytotoxicity. The increased incorporation of FdUrd into RNA must result only from the conversion of FdUrd to FU in the cell, mediated by pyrimidine phosphorylase. These data, together with the observation that 3AB did not affect the cytotoxicity of FUrd (unpublished results) also pinpoint, by logical deduction, the metabolic effect of 3AB that results in increased incorporation of FU into nucleic acids. Thus, 3AB modulates either the activity of phosphoribosyl transferase (which catalyses the conversion of FU to FUMP in the presence of PRPP), or the activity of nucleoside phosphorylase (which converts FU to FUrd). An

effect of 3AB on phosphoribosyl transferase activity possibly due to, for example, an antifolate effect resulting in increased PRPP levels would be consistent with our previous observations [16], showing a synergistic effect of 6-mercaptopurine and 3AB on PRPP levels.

The pleiotropic metabolic fates and effects of FU and FdUrd lead to diverse cytotoxic effects which are either DNA- or RNA-directed. We have demonstrated here that poly(ADP-ribosylation) reactions participate in the DNA damage response and may contribute to the cytotoxic mechanisms. The role of PADPRP-induced NAD depletion in the cytotoxic mechanisms of TS inhibitors merits further investigation.

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