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Simultaneous alteration of de novo and salvage pathway to the deoxynucleoside triphosphate pool by (E)-2'-Deoxy-(fluoromethylene)cytidine (FMdC) and zidovudine (AZT) results in increased radiosensitivity in vitro

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ORIGINAL ARTICLE

Simultaneous alteration of *de novo* and *salvage* pathway to the deoxynucleoside triphosphate pool by (E)-2′-Deoxy-(fluoromethylene)cytidine (FMdC) and zidovudine (AZT) results in increased radiosensitivity *in vitro*

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Abstract

To test whether a thymidine analog zidovudine (=AZT), is able to modify the radiosensitizing effects of (E)-2′-Deoxy-(fluoromethylene)cytidine (FMdC). A human colon cancer cell line Widr was exposed for 48 hours prior to irradiation to FMdC. Zidovudine was added at various concentrations immediately before irradiation. We measured cell survival and the effect of FMdC, AZT and FMdC+AZT on deoxynucleotide triphosphate pool. FMdC results in a significant increase of radiosensitivity. The enhancement ratios (ER=surviving fraction irradiated cells/surviving fraction drug treated and irradiated cells), obtained by FMdC or AZT alone are significantly increased by the combination of both compounds. Adding FMdC to AZT yields enhancement ratios ranging from 1.25 to 2.26. FMdC reduces dATP significantly, with a corresponding increase of TTP, dCTP and dGTP. This increase of TTP, dCTP and dGTP is abolished with the addition of AZT. Adding AZT to FMdC results in a significant increase of the radiosensitizing effect of FMdC. This combination appears to reduce the reactive enhancement of TTP, dCTP and dGTP induced by FMdC while it does not affect the inhibitory effect on dATP.

Several chemotherapeutic compounds have been tested to increase the response of tumor cells to ionizing radiation. Some of these compounds interact at the level of the nucleoside triphosphate (dNTP) pool [1–4]. Perturbations of the dNTP-pool have been shown to result in significant radiation sensitization [1,5–8]. The cellular dNTP-pool depends on *de novo* biosynthesis in which ribonucleotide reductase (RR), dihydrofolate reductase (DHFR) and thymidylate synthetase (TS) are key enzymes, and the *salvage* pathway in which thymidine kinase (TK) plays a major role [9–12]. Hydroxyurea (HU), gemcitabine (dFdC) and more recently (E)-2′-Deoxy-(fluoromethylene) cytidine (FMdC) are known to act as inhibitors of RR. All those compounds have been shown to be able to sensitize tumor cells to ionizing radiation both *in vitro* and *in vivo* [1,8,11–22].

Inhibitors of RR are able to modify the pool of dNTP. The change in the pool, especially the lowering of the dATP, will result in a modification of the activity of the *salvage* pathway especially at the level of TK. One might expect a reactive increase of thymidine incorporation, resulting from an increased activity of thymidine kinase (feedback loop on the salvage pathway) [1,21,23,24]. Hence, we decided to investigate whether, the radiosensitizing effect observed after alteration of the *de novo* pathway by FMdC, could be further modified through manipulation of the salvage pathway, i.e. by presenting an analog of thymidine to TK. The purpose of the salvage pathway is to provide the cell with a low supply of deoxynucleotides (e.g., for DNA repair) during the time interval when the *de novo* synthesis is not or less active. We used the thymidine analog (TA) zidovudine (AZT) as this drug is known to

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interact at the level of TK and is widely used in clinical practice as an inhibitor of reverse transcriptase in the treatment of HIV infection.

The combination of FMdC with a TA, aimed at modifying both the *de novo* pathway and the salvage pathway, has been tested *in vitro* on a human colon cancer cell line (WiDr). We selected a colorectal cancer cell-line because there is usually a high proliferative capacity and hence an increased TK activity, which is known to be a marker of presence of human neoplasia and/or disease progression in many cancer [12,25]. We aimed at defining whether the addition of AZT to FMdC yields increased radiation induced cell death as compared to FMdC alone and whether there is any more change in the NTP and dNTP levels to eventually illustrate our hypothesis.

Materials and methods

Chemicals and cell cultures

FMdC was kindly provided by Chiron Pharmaceuticals, Inc. (San Francisco, California, USA). Zidovudine (AZT) was obtained from Wellcome Research Laboratories (UK). Cell culture media and supplements were from Gibco BRL (Basel, Switzerland). Fetal calf serum (FCS) was purchased from Fakola AG (Basel, Switzerland).

The cell line WiDr was purchased from American Type Culture Collection (Rockville, Maryland, USA). The cells were maintained in Minimum Essential Medium with 0.85 g/l $NaHCO_3$, supplemented with 10% FCS, 1% non-essential amino-acids (NE-AA), 2 mM L-Glutamine, and 1% penicillin-streptomycin solution. Cells were passaged twice weekly. A test for mycoplasma was routinely performed every 6 months, and found negative for contamination.

Irradiation technique and clonogenic assay

Exponentially growing cells were trypsinized, and seeded in 60×15 mm Falcon Primaria culture flasks with 5 ml medium, allowed to attach and incubated for 24 h before adding the inhibitor of RR. Medium containing the chosen concentration of freshly prepared FMdC was added thereafter and replaced at 24 h. After exposure to this drug, the cells were trypsinized, and resuspended in fresh medium at low density. Cells were plated into 100×20 mm Falcon Primaria culture dishes containing 10 ml medium. After a 3 h incubation in order to obtain cell attachment, the cells were exposed to different concentrations of AZT (25 μM, 50 μM and 100μM) added immediately prior to irradiation.

The cells were irradiated at room temperature with an Oris IBL 137 Cesium source at a dose rate of 80.2 cGy/min. We used a range of single doses from 0 to 8 Gy, using a 2 Gy dose increment. For each radiation dose (0–2–4–6–8 Gy), four dishes were utilized, both for control and drug-exposed cells. The dishes were incubated at 37°C in air and 5% CO_2 for 2 weeks. The cells were fixed in ethanol, stained with crystal violet, and the colonies were manually counted. Colonies of more than 50 cells were considered survivors. All experiments were done in triplicate.

For all the data obtained by clonogenic assays, the surviving fraction of drug treated cells was adjusted for drug toxicity to yield corrected survivals of 100% for unirradiated but drug treated cells. The effect shown is therefore the sensitizing action, after the substraction of the direct cytotoxic effect of each of the drugs.

The impact of the different drugs (FMdC and AZT) and the combination of each on the radiation sensitivity of the WiDr cell line was calculated at different survival levels (2, 20 and 50%).

Analysis of dNTP and NTP pools by gradient elution ion-pair reversed phase high-performance liquid chromatography (HPLC)

Simultaneous quantitation of dNTP and NTP in WiDr cells was performed by gradient elution ion-pair reversed phase HPLC with a modification of a previously described method reported in detail elsewhere [26]. Briefly, exponentially growing WiDr cells were exposed to the drugs in the same experimental conditions as for the clonogenic assays. The cells were trypsinized, washed, centrifuged and resuspended in ice cold ultrapure water (dilution according to cells count) and deproteinized with the same volume trichloroacetic acid (TCA) 6% (final applied concentration = TCA 3%). Acid cell extracts were centrifuged and the resulting supernatants were stored at −80°C prior to analysis. Before the HPLC assay, samples were thawed and aliquots of 100 μl were neutralized with 4.3 μl saturated Na_2CO_3 solution. In the present series of experiments, aliquots of 25 μl were injected onto the HPLC column with satisfactory sensitivity. All experiments were done in triplicate, with the triplication process starting at the cell culture step, to detect variability associated with the culture growth conditions. Results were expressed as the concentration of the four dNTP (expressed in pMole/10^6 cells) and as the absolute levels of the four NTP (as measured by NTP peak areas). The optimization and full validation of the analytical method is described in detail elsewhere [26].

Table I. Effect of FMdC (30 nM), AZT (range 25–100 μM) and the combination on the plating efficiency of WiDr cells *in vitro*. Data are given for as the mean value plus or minus standard error for 100 cells plated. All experiments have been done in triplicate.

| Control | FMdC 30 nM | AZT | | | FMdC+AZT 25 μM | FMdC+AZT 50 μM | FMdC+AZT 100 μM |
		25 μM	50 μM	100 μM			
102+1.5	87.2+11.1	100.2+1.2	96.2+3.0	80.5+5.8	84.9+5.5	73.5+11.2	43.2+10.9

Statistical analysis

Data are presented as the mean + the standard error of three independent experiments. Surviving fractions were compared using a two-sided paired t-test. The difference was considered significant if a 0.05 p-value was reached. Dose response curves (from 0 to 8Gy), were fitted using a second degree polynomial regression analysis, yielding a linear quadratic equation. The curve fitting was obtained using Statview 5.0 software on a MacIntosh G3 computer. From this linear quadratic equation we calculated the ER values at 50%, 20% and 2% survival levels.

Results

Effect of FMdC and AZT on clonogenicity

At a concentration of 30 nM there was no major impact of FMdC on the plating efficiency (PE) of WiDr cells as compared to untreated controls. The data with AZT alone or combined to FMdC are tabulated in Table I. At higher concentrations of AZT (50 and 100 μM) there is a significant drop in clonogenicity induced by the combination as compared to each drug alone.

Effect on the radiation dose response curve

As illustrated in Figure 1, the use of FMdC alone and AZT alone reduction of the shoulder of the dose response indicating a radiation sensitizing effect. The combination of AZT and FMdC, however, yields a significant increase of the radiation sensitivity of the WiDr cell line as compared to each drug alone combined to irradiation.

The calculated enhancement ratios (ER) at different survival levels obtained from the linear quadratic fitting of the curves are shown in Figure 2. At the low concentration of FMdC (30 nM) the ER values for survival levels ranging from 2 to 50% are ranging from 1.18 to 1.28. AZT alone yields radiosensitization especially at higher doses of AZT (50 and 100 μM). The largest effect, based on the ER value, is obtained at clinical relevant radiation doses, i.e. in the initial part of the radiation dose response curve (see progression of the calculated ER values with increasing survival level). When FMdC is combined

with AZT, the ER values are significantly increased compared to each drug alone.

Effect of AZT and FMdC on dNTP pool measured by HPLC

In order to obtain a clear cut and reproducible effect of FMdC on the pools it was decided – based on our previous published results – to use 50 nM and 100 nM instead of 30 nM which was the concentration used in the clonogenic assays.

All the data are grouped under Figure 3. The HPLC analysis confirms our previous data: we observe a highly significant reduction of the dATP level with a corresponding increase of the dCTP and TTP levels. As far as the NTP levels are concerned, we reiterate the previous results i.e. a global increase in the NTP levels which may in part be explained by cell cycle redistribution [1].
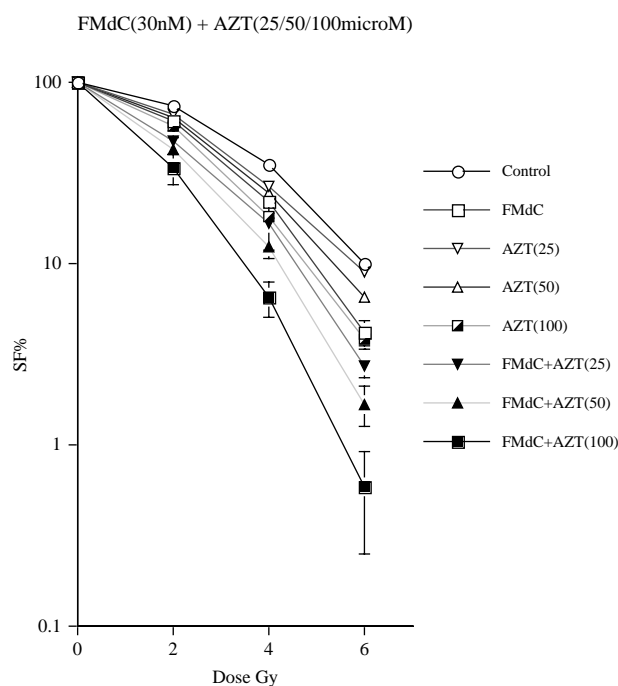

Figure 1. Dose reponse curve of WiDr cell line irradiated *in vitro*; upper curves illustrating the effect of AZT alone; lower curves FMdC (30 nM/48 h) alone versus the combination with the various concentrations of AZT. The data are plotted with corresponding standard error (often contained within the size of the symbols used).
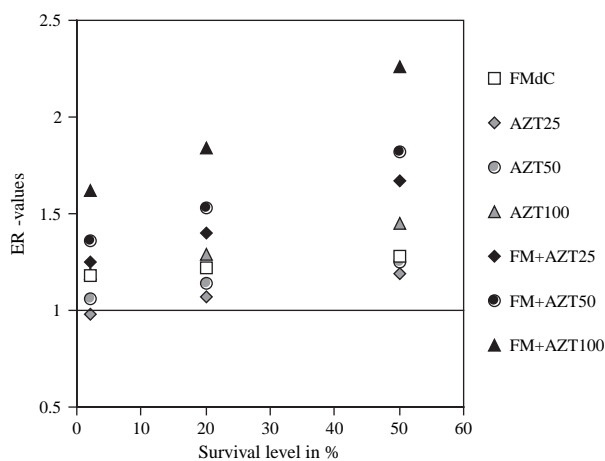
Figure 2. Effect of FMdC, AZT or the combination on the ER-values, calculated from the linear quadratic equation, obtained at survival levels of 2%, 20% and 50%.

AZT at the lowest concentration (25 μM) does not influence the dNTP levels compared to untreated controls but at this low concentration, the combination with 50 nM FMdC yields a significant drop of dCTP and TTP levels compared to FMdC treated cells (data not shown). The combination of higher doses of AZT with 50 nM FMdC results in a significant decrease of dCTP and TTP as compared to FMdC alone (Figure 3). In fact the reactive increase of dCTP and TTP by FMdC is abbrogated if FMdC is used in presence of AZT. The changes in dGTP are less clear cut. On the other hand, AZT does not seem to affect the clear decrease of dATP induced by FMdC, but the levels of dATP are close to the lowest detectable levels.

In summary, the combination of FMdC, known to interact at the level of RR resulting in especially a dATP drop and rise in TTP and dCTP, to AZT results in a significant lowering of the levels of these latter dNTP while for dGTP the effect is less consistent.

Discussion

Radiation sensitivity of cell lines depends among other factors on the pool of dNTP [1,6–8,27]. This pool is regulated through two different pathways, the *de novo* biosynthesis and the pyrimidine *salvage* pathway. In the first pathway Ribonucleotide Reductase (RR), Dihydrofolate Reductase (DHFR), and Thymidylate Synthetase (TS) are key enzymes. The enzyme RR is, however, the rate-limiting step in the *de novo* pathway, whereas for the *salvage* pathway thymidine kinase (TK) is the key enzyme [12]. Thymidine kinase is subject to feedback regulation by dNTP, i.e. a reduction of dNTP results in an increased activity of the *salvage* pathway (positive feedback loop).

One of the first compounds active at the level of RR to be used in a clinical setting has been Hydroxyurea (HU). More recently, drugs such as gemcitabine (2′,2′-difluoro-2′-deoxycytidine =dFdC) and (E)-2′-Deoxy-(fluoromethylene) cytidine (FMdC) have been developed as new inhibitors of RR [28,29]. These compounds are able to lower the dNTP-pool. Gemcitabine (dFdC) is under active investigation as a radiosensitizer in pancreatic cancer [7,8]. On the other hand, FMdC has been shown to be on a variety of cell lines such as colon (WiDr), cervix (C4-1, C-33-A, SiHa, Hela), and breast (MCF-7) cancer cell lines [1,17,30–33]. In some of those published data, radiosensitization has been highlighted [1,5,18–20]. The present experiments aimed at demonstrating that radiosensitization observed at very low levels of FMdC (30 nM) can substantially be modified by interacting at the level of the salvage pathway.

There are at least three good rationales for modulating TK in our experimental setting. First, ionizing radiation is known to induce TK transcription and enzymatic activity in human cells [34]. Second, it has been shown that cellular radioresistance, at least in a rat glioma cell line, is related to the expression of the thymidine kinase gene [35]. Third, in the case of inhibition of one of the key enzymes in the *de novo* pathway, there will be an increase of TK activity-as a result of the lowering of one of the dNTP-and hence an increased capacity of phosphorylation of thymidine analogs such as AZT, resulting in incorporation and hence potentially radiosensitization [14,36,37]. The utmost importance of TK in radiation response makes it an attractive target to interact with either by reduction of TK expression by targeted mutagenesis and antisense strategies, or direct modulation of TK itself. We decided to investigate this latter strategy. We did already demonstrate the proof of principle in an earlier paper using the combined effect of FMdC and iodo-deoxy-uridine, but decided to use a compound more readily available for clinical use [23].

From the research in AIDS, it is currently known that the phosphorylation and hence anti-HIV activity of AZT, can be substantially increased by modulation of *de novo* pyrimidine biosynthetic pathway by methotrexate and 5-fluoro-2′-deoxyuridine, especially at the level of DHFR and TS, respectively [12,21,36–38]. Based on this mechanism, some investigators are combining AZT, 5-fluorouracil and leucovorin in the treatment of metastatic colorectal cancer [12,39–41].

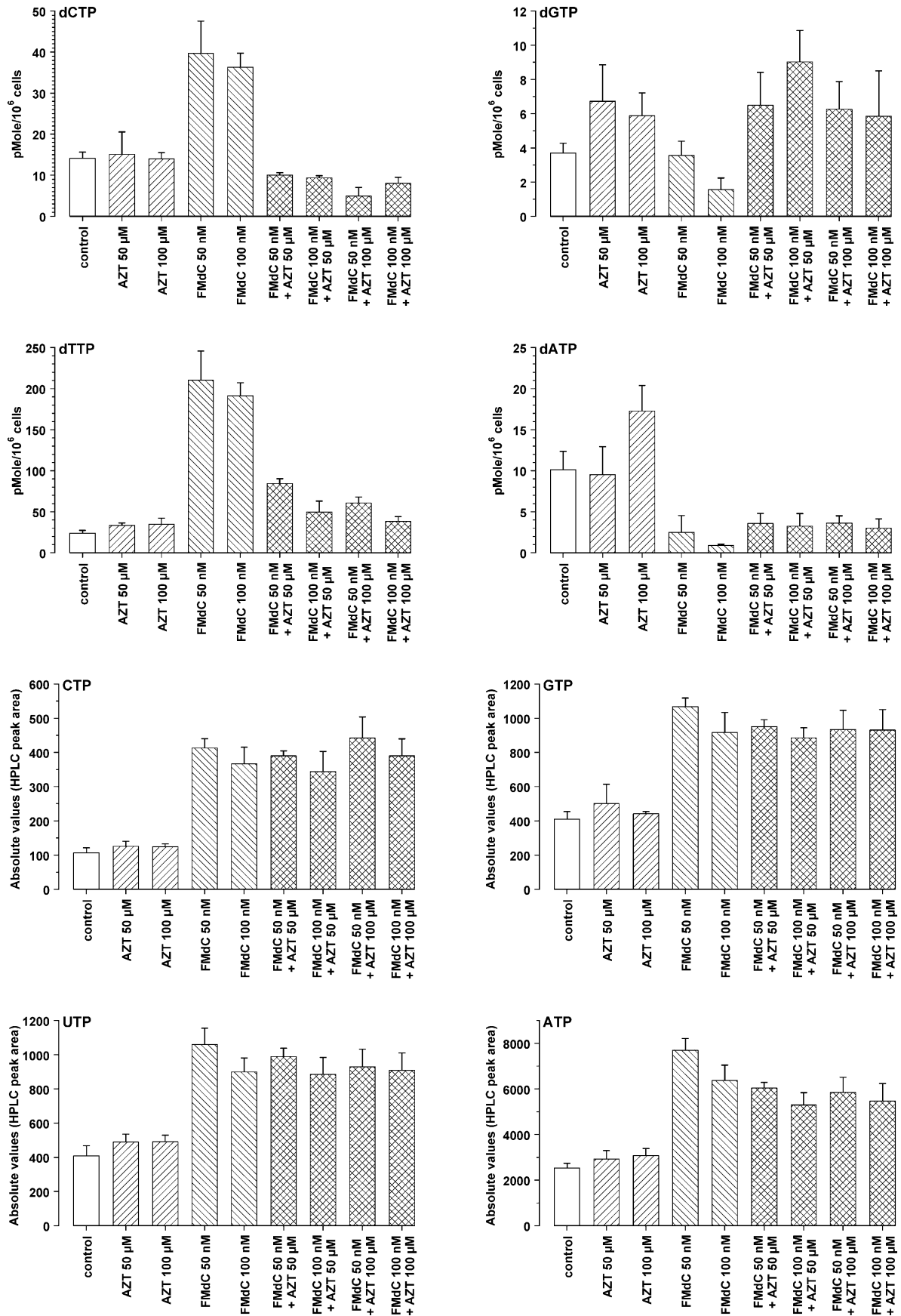Kuo et al. highlighted the importance of simultaneous modulation of the *de novo* and salvage path-
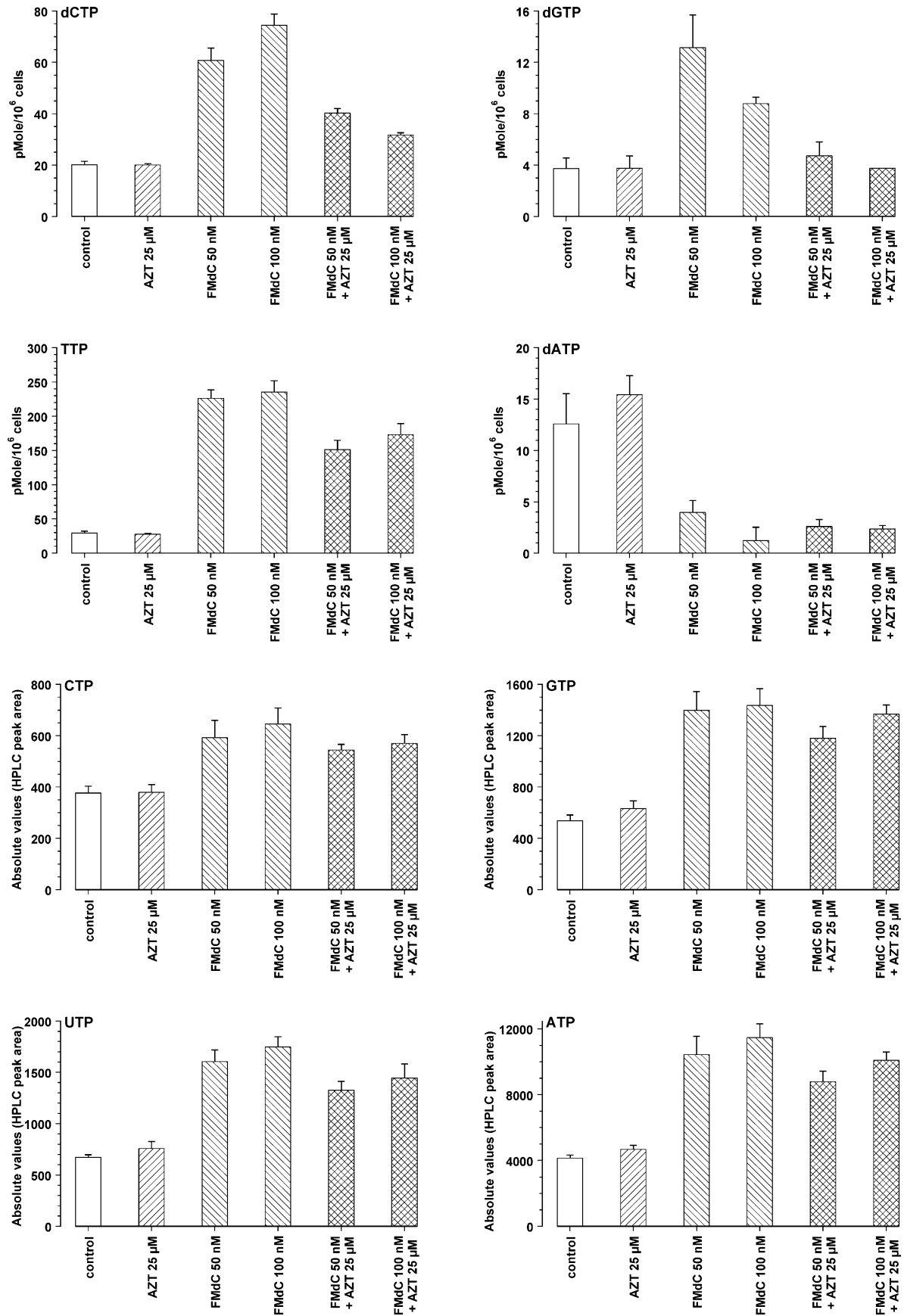
Figure 3 *(Continued)*

Figure 3 (*Continued*)

Figure 3. dNTP and NTP levels obtained from HPLC analysis of WiDr cells exposed to FMdC (50–100 nM) and AZT at a concentration of 25 (Figure 3A) and 50–100 μM (Figure 3B). These concentrations were selected based on previous data aimed at optimizing the interpretation of the HPLC technique [1,23,26]. FMdC induces an accumulation of ATP as a result of a cell cycle redistribution effect [1,23]. AZT alone does not change the ATP pool. The major drop in dATP after addition of FMdC is not modified by the addition of AZT. The increase in TTP induced by FMdC is significantly reduced by the addition of AZT. AZT alone has no effect as compared to controls. The increase of dGTP pool and dCTP pool with FMdC is reduced by the addition of AZT. AZT alone has no effect.

way. They demonstrated the effect of interaction of hydroxyurea (HU) and iododeoxyuridine (IdUrd) on the radiation sensitivity of the 647V cell line [42]. HU is an inhibitor of the M2 subunit of RR. This drug lowers dATP, or at higher concentrations, acts as a more general inhibitor of deoxynucleotide biosynthesis. Inhibitors of RR are known to be able to modify the radiation response both *in vitro* and *in vivo* [1,5,8,13,18–20,22,43–45].The addition of IdUrd to HU results in an increased radiosensitization because the thymidine salvage pathway is stimulated resulting in an increased incorporation of IdUrd [42]. This thymidine analogue, incorporated in the dNTP pool after phosphorylation by TK, has been shown to sensitize tumor cells to ionizing radiation both *in vitro* and *in vivo* [3,4,15,42,46–49]. The radiosensitizing capacity depends on the incorporation into DNA [7]; this incorporation may be augmented by decreasing competing TTP pools through feedback inhibition of RR [6]. Therefore, exploiting the inhibition of RR with a positive feedback regulation on TK through a reduced dATP may result in a net increase of incorporation of AZT, and hence an increased susceptibility of DNA to single-strand breaks from radiation-induced free radicals. The combination of FMdC and AZT applied simultaneously yielded in our hands a significant increase of the radiation sensitivity of WiDr cells as compared to either compound separately. We tentatively explain the observation by speculating that incorporation of phophorylated forms of AZT in DNA may result in chain termination and DNA synthesis inhibition.

We have already published the potential of AZT alone in specific experimental conditions to protect cells against ionizing irradiation to a small degree (ER about 1.1) [50]. However, as shown in the present experiments, the combination of an inhibitor of RR such as FMdC used at concentrations not altering the PE, to a thymidine analogue such as AZT, provide a powerful pharmacological tool to obtain at least *in vitro* a significant increase of the sensitivity of a WiDr cell line to ionizing radiation. Moreover, these levels of ER's are obtained at a clinically relevant radiation dose level of 2 Gy, and at concentrations of drugs which are clinically achievable in humans.

In conclusion, these experiments provide supplementary arguments in favor of the importance of purine and pyrimidine de novo and salvage pathways in the radiation response. As human tumor cells have markedly elevated enzyme activity in these pathways, there might be a differential effect with this biomodulation between tumor and normal cells. The colorectal model, as chosen in the present experiments, is an ideal model because of the well known difference in TK and RR activity as compared to normal cells [11,12]. However, it remains to be investigated whether this difference in enzymatic activity between normal and tumor cells can serve as a basis for a therapeutic differential effect exploitable in a clinical setting. Nevertheless, based on the present *in vitro* data, the combination of FMdC and AZT deserves *in vivo* experiments.

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