Pergamon

0006-2952(95)00228-6

Biochemical Pharmacology, Vol. 50, No. 7, pp. 1095–1098, 1995.

0006-2952/95 $9.50 + 0.00

Short Communication

THE IMP DEHYDROGENASE INHIBITOR MYCOPHENOLIC ACID ANTAGONIZES THE CTP SYNTHETASE INHIBITOR 3-DEAZAURIDINE IN MOLT-3 HUMAN LEUKEMIA CELLS: A CENTRAL ROLE FOR PHOSPHORIBOSYL PYROPHOSPHATE

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(*Received* 20 *September* 1994; *accepted* 17 *May* 1995)

Abstract—Mycophenolic acid, an inhibitor of the enzyme IMP dehydrogenase, antagonizes the CTP synthetase inhibitor 3-deazauridine in its anti-proliferative effects on MOLT-3 human T leukemia cells. No depletion of CTP occurred, and decreased amounts of 3-deazauridine-triphosphate were measured in cells incubated with mycophenolic acid and 3-deazauridine. Most probably, these phenomena are related to the increased amounts of PRPP observed, which can result in an increased pyrimidine biosynthesis *de novo* and, as a consequence, a decreased metabolism of 3-deazauridine via the salvage pathway.

Key words: mycophenolic acid; 3-deazauridine; IMP dehydrogenase; CTP synthetase; phosphorybosyl-pyrophosphate; antagonism

Several studies on IMP IMPDH§ (E.C. 1.1.1.205), the rate-limiting enzyme in the *de novo* biosynthesis of guanine ribonucleotides, as well as studies on CTP synthetase (CTPS; E.C. 6.3.4.2), the rate-limiting enzyme in the biosynthesis of CTP, have shown transformation-associated increased activities of these enzymes in malignant human cells as compared to normal counterpart cells [1–5]. Mycophenolic acid (MPA) and 3-deazauridine (DAU) are potent agents for the inhibition of the activity of IMPDH and CTPS, respectively, leading to growth retardation and cell death and/or differentiation of a variety of cancer cells *in vitro* and *in vivo* [6–11]. It could be hypothesized that simultaneous application of MPA and DAU would give increased effects compared to either drug administered alone. However, we demonstrate herein that after simultaneous application of MPA and DAU on MOLT-3 human leukemic cells, an antagonising effect occurred with respect to the potentials of cell kill and growth arrest.

Materials and Methods

MOLT-3 cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 6% CO_2. All chemicals were obtained from Sigma Chemical Corp. (St Louis, MO, U.S.A.). Stocks of 10 mM MPA or DAU were prepared and stored at −20°C. In the course of this study, two stocks from two different batches of MPA were prepared. The first stock appeared to be approximately 5 times more effective in inhibiting IMPDH in the *in vitro* assay developed by Stet and coworkers [12]. For this reason some experiments were performed with 0.1 μM MPA and others with 0.5 μM MPA, since these concentrations were equally effective in inhibiting IMPDH, and also represent equitoxic doses. Cell numbers were determined using a Coulter counter Z 1000. Cell viability was determined by the trypan blue exclusion method. Intracellular nucleotides were extracted with perchloric acid and analysed by HPLC as described by De Korte *et al.* [13] or according to the method of Plunkett *et al.* [14]. The latter method enabled monitoring of DAU-phosphate compounds. To measure the amounts of DAUTP (detection limit 20 picomoles) reliably, 10 million cells were extracted per sample. Amounts of intracellular PRPP were measured as described by Vogt *et al.* [15]. Amounts of nucleosides in the media were measured as described in ref. [5]. Differences between averaged data were tested with the two-sided Student's *t*-test.

Results

Increasing concentrations of either MPA or DAU resulted in progressive cell kill and a stop to the proliferation of MOLT-3 cells (results not shown). MOLT-3 cells were then treated with a combination of 0.1 μM MPA and 40 μM DAU; in these concentrations either agent killed 20% to 40% of the cells within 72 hours, and the combined agents killed 30% of the cells. During a 72-hour incubation of the MOLT-3 cells within the combined drugs, we observed that the viability and growth of MOLT-3 cells were decreased to a similar degree as cells incubated with MPA alone and to a lesser degree than cells incubated with 40 μM DAU as a single agent (Fig. 1).

The effects of MPA and DAU on ribonucleotide metabolism are depletion of guanine ribonucleotides and cytosine ribonucleotides, respectively. Thus, to investigate the biochemical basis for the antagonising effects of MPA towards DAU on the MOLT-3 cells, we measured the effects of the various drug

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§ Abbreviations: CTPS, cytidine triphosphate synthetase; DAU, 3′-deazauridine; DAUTP, 3′-deazauridine triphosphate; 5FU, 5′-fluorouracil; IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid; MTX, methotrexate; PRPP, phosphoribosyl pyrophosphate.

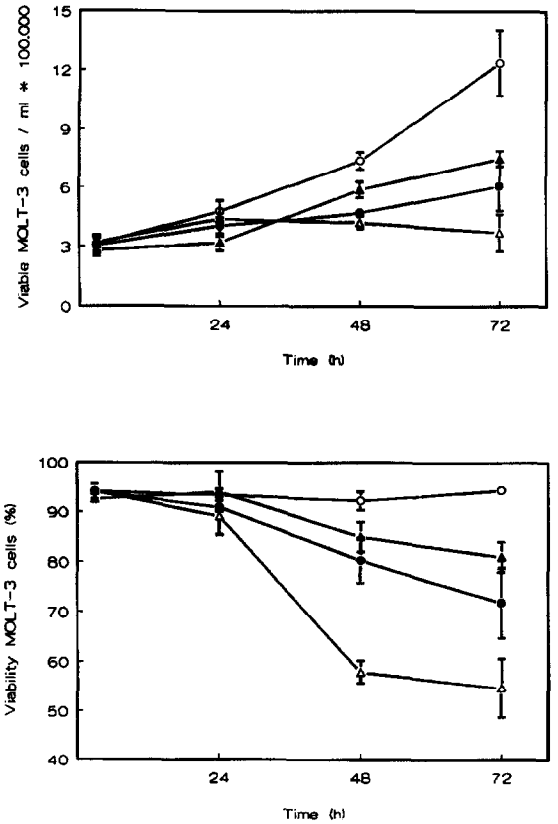

Fig. 1. Growth and viability of MOLT-3 cells treated with MPA and/or DAU. Growth (upper part) and viability (lower part) of MOLT-3 human T-leukemia cells during treatment with 0.1 μM MPA (solid triangles) or 0.1 μM MPA and 40 μM DAU (solid circles) or 40 μM DAU (open triangles) compared with control cells (no drugs added; open circles). Mean of three independent experiments ±2 times SD.

combinations on the intracellular ribonucleotide pools. Treatment of MOLT-3 cells with MPA gave a decrease in the intracellular guanine ribonucleotide pools, independent of the presence of DAU. Treatment with DAU gave a small decrease in the amount of UTP and a severe depletion of CTP (Fig. 2). Although definitive proof is still forthcoming, the decrease in UTP after incubation with DAU may be explained by a decrease in the conversion of uridine into UMP through the competition of DAU towards uridine at the levels of transport and phosphorylation by uridine kinase. As is further shown in Fig. 2, the ratio of UTP over CTP is considerably increased. CTP synthetase is rate-limiting in CTP synthesis, and maintains the ratio between UTP and CTP pools [16]. Therefore, it is possible to conclude from the increased UTP/CTP ratio with the decreased CTP pools in MOLT-3 cells incubated with DAU alone that inhibition of CTP synthetase activity had occurred. Thus, the effects of either MPA or DAU in MOLT-3 cells were similar to those described for other cell types [6–11]. Because GTP acts as a cofactor for CTP synthetase [17–19], an additional depletion of CTP in cells incubated with a combination of MPA and DAU might have been expected when compared to cells incubated with DAU alone. However, in MOLT-3 cells incubated with the combination of 0.1 μM MPA and 40 μM DAU, no significant differences from untreated MOLT-3 cells were observed either in the amounts of UTP or CTP or in the UTP over CTP ratio (Fig. 2). This observation indicated that enzyme CTP synthetase was not substantially inhibited in cells treated with MPA and DAU. Therefore, to further investigate the mechanism underlying the absence of CTP synthetase inhibition, we incubated cells with 0.5 μM MPA and 40 μM DAU or 40 μM DAU alone and measured the amounts of DAUTP formed. We observed a decreased biosynthesis of DAUTP in MOLT-3 cells treated with MPA and DAU compared to cells treated with DAU alone (Fig. 3). Thus, cells exposed to MPA and DAU simultaneously apparently are unable to synthesize the amounts of DAUTP required to deplete CTP pools. As is explained in the Discussion section below, we hypothesized that this phenomenon may have resulted from the complementary stimulation of the pyrimidine *de novo* biosynthesis pathway after accumulation of PRPP. To test this hypothesis, we measured the amounts of PRPP in MOLT-3 cells 24 hours after incubation with 0.5 μM MPA or 0.5 μM MPA and 40 μM DAU, and compared them with control cells. Figure 4 shows that in cells treated with MPA or the combination of MPA and DAU, accumulation of PRPP to significant supranormal levels ($p < 0.01$), indeed occurred.

Discussion

In MOLT-3 cells incubated with MPA and DAU, the inhibition of CTP synthetase was decreased compared to cells incubated with DAU alone (Fig. 2). DAU must be converted into DAUTP before it can inhibit CTP synthetase [9, 11]. The initial steps in this process are uptake by facilitated transport, followed by conversion into DAUMP through the action of uridine kinase (E.C. 2.7.1.48) [21]. Uridine and cytidine are substrates competing with DAU for both transport [20] and phosphorylation by uridine kinase [21, 22]. Therefore, the presence of different concentrations of these nucleosides in the media could provide an explanation for the decreased biosynthesis of DAUTP from DAU in MOLT-3 cells after incubation with MPA and DAU compared to incubation with DAU alone. However, the concentrations of uridine (0.5 μM) and cytidine (<0.1 μM) in the media were similar in all experiments.

MPA does not have to be metabolised for the inhibition of IMPDH to occur and reaches its site of action through diffusion [23, 24]. A possible explanation for the effects observed with the combination of MPA and DAU may be a decreased consumption of PRPP by the purine *de novo* pathway. Increased PRPP pools stimulate the *de novo* pyrimidine synthesis pathway [25]. Thus, after inhibition of the purine *de novo* pathway, the increased PRPP pools stimulated the pyrimidine synthesis *de novo* pathway, resulting in increased amounts of *de novo* synthesized uracil nucleotides. This stimulation of pyrimidine *de novo* biosynthesis after inhibition of the *de novo* purine biosynthesis pathway has been studied previously, and is called "complementary stimulation" [26–28]. In the context of this study, "complementary antagonism" seems to be a more appropriate term. Uridine kinase is subject to feedback inhibition by CTP and UTP [22]. As the activity of uridine kinase was decreased by feedback inhibition due to the increase in biosynthesis of pyrimidine ribonucleotides *de novo*, the amounts of DAU metabolised into DAUMP through the action of uridine kinase were decreased, finally resulting in a decreased synthesis of DAUTP. A difference in the amounts of DAUTP synthesized in MOLT-3 cells incubated with either DAU or MPA and DAU becomes manifest only after two hours (Fig. 3). Effective accumulation of PRPP in cells of the closely related cell line MOLT-f4 likewise did not occur prior to several hours after the application of MPA [29]. This observation supports our hypothesis regarding the mechanism underlying the antagonising effect of MPA towards DAU in MOLT-3 cells.

Because the antagonistic activity of MPA towards the toxic activity of DAU seems to depend on an inability to form DAUTP, a sequential application of DAU followed by incubation with MPA might optimize the efficacy of the drug combination. As the accumulation of PRPP appears in fact crucial to the phenomenon of antagonism between MPA and DAU observed in this study, this might have further important implications. Drugs used to treat a variety of malignancies in humans (e.g., mercaptopurine, 6-thioguanine, and fluoro-uracil) must first be converted into ribosyl-phosphate compounds in order to be effective. Because these conversions require PRPP, the rate

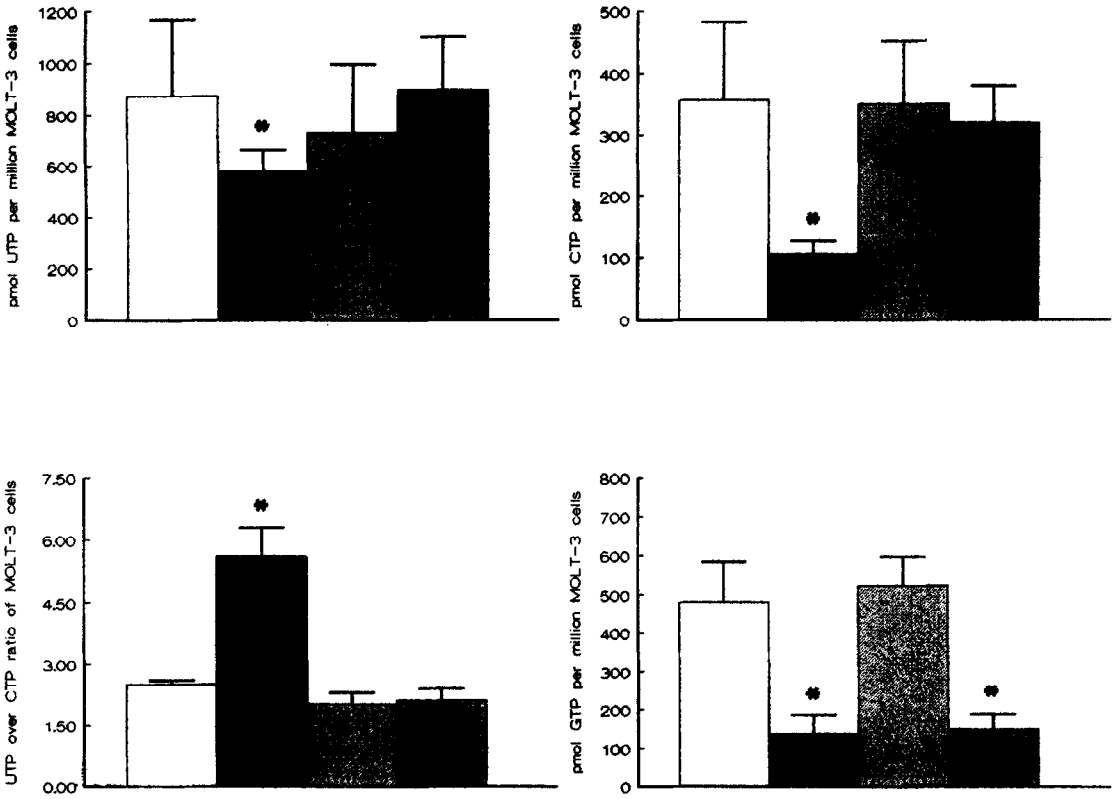

Fig. 2. The effects of MPA and DAU on GTP, UTP, and CTP pools. Amounts of intracellular GTP, UTP, and CTP measured by HPLC and the ratio of UTP over CTP in MOLT-3 cells, 24 hours after the onset of the experiments. The bars indicate the mean ±2 times *SD* of three independent experiments. From left to right in each panel: MOLT-3 cells untreated, 40 μM DAU, treated with a combination of 0.1 μM MPA and 40 μM DAU, and treated with 0.1 μM MPA, respectively. Note the difference in the *Y* axes of either panel. * = significant difference ($p < 0.01$).

at which they take place might be increased with a time-scheduled application of inhibitors of *de novo* ribonucleotide biosynthesis pathways. In such a way "complementary stimulation" of the formation of the active forms of the drugs might be accomplished. One example of the possible application of such a strategy is the scheduled administration of methotrexate (inhibition of the purine *de novo* pathways with increases of PRPP) followed by 5-fluorouracil (5FU) (enhanced conversion of 5FU with PRPP to the active form), which is of greater efficacy than simultaneous administration of MTX and 5FU or administration of either drug as a single agent [30]. Studies on the benefits of scheduling inhibitors of purine and pyrimidine ribonucleotide biosynthesis are now in progress in our laboratories.

Acknowledgement—The authors express their gratitude for the generous financial support provided by the Foundation for Paediatric Cancer Research, grant 89-01.

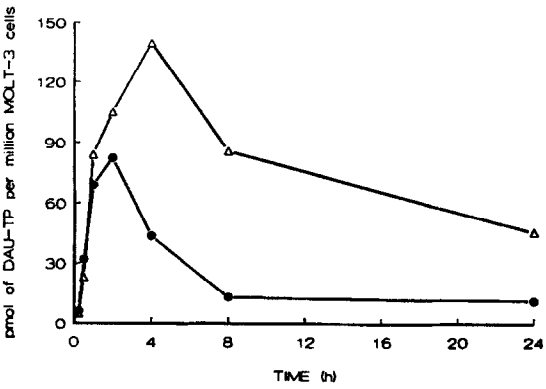

Fig. 3. The synthesis of DAUTP. Amounts of intracellular DAU-TP synthesized by MOLT-3 cells treated with 40 μM DAU (open triangles) or MOLT-3 cells treated with a combination of 0.5 μM MPA and 40 μM DAU (solid circles). Single representative experiment. Amounts of intracellular DAUTP were determined by HPLC according to the method described by Plunkett *et al.* [13].

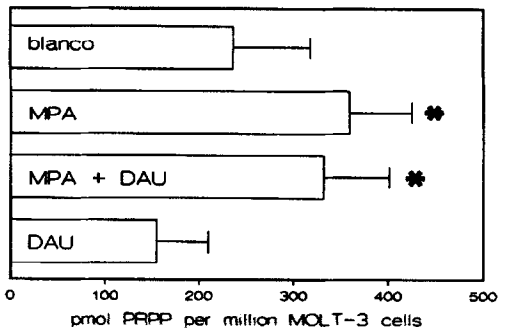

Fig. 4. The sizes of PRPP pools. Intracellular PRPP pools in MOLT-3 cells, 24 hours after the start of the experiments. The bars represent the average values of four independent experiments ±2 times *SD*. PRPP was measured as described in ref. [14]. * = significant increase ($P < 0.01$).

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