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Pharmacologic basis for the use of dipyridamole to increase the selectivity of intraperitoneally delivered methotrexate

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Summary. Dipyridamole (DP) is an attractive agent with which to increase the selectivity of intraperitoneally delivered methotrexate (MTX). We demonstrated that DP synergistically increased the cytotoxicity of MTX to the human OV 2008 ovarian carcinoma cell line in vitro and that this synergy was highly concentration-dependent. DP did not alter MTX binding in plasma, and vice versa. We found that the two drugs were chemically compatible at concentratioons of <400 μM, which was well above the concentration needed to make continuous i. p. infusion feasible. The ability of OV 2008 cells to accumulate uridine was used as a bioassay for the in vivo activity of DP. When this drug was infused i. p. at 12 mg/m^2 per day, the steady-state peritoneal DP concentrations attained in patients were sufficient for maximal inhibition of uridine uptake, indicating concentrations high enough for synergism with MTX. We found no correlation between total peritoneal protein concentration and either free DP concentration or biologic activity. On the basis of these preclinical and pharmacologic measurements, we conclude that it should be possible to produce selective i. p. biochemical modulation of MTX with DP.

Introduction

Methotrexate (MTX) competitively blocks the enzyme dihydrofolate reductase [27] and depletes intracellular reduced folates that are important in the synthetic pathways of purines and pyrimidines [4]. In addition, MTX may also directly inhibit de novo purine synthesis [1]. Cells can partially protect themselves against MTX toxicity by salvaging nucleoside precursors from the extracellular environment [13] or by increasing the activity of salvage enzymes [25]. Dipyridamole (DP), clinically used as an antiplatelet and vasodilatory agent, interferes with the salvage of nucleosides by blocking their transport across cell membranes [3, 5, 21, 25, 30] and increasing the intracellular amount of MTX polyglutamates [16]. Through these mechanisms, DP can markedly potentiate the cytotoxicity of MTX. This interaction, which is highly concentration-dependent, has been observed in vitro [3, 16, 19, 20, 25]. On the basis of these results, clinical trials testing the concurrent use of MTX and DP have been initiated [11, 12, 24, 26, 28].

Intraperitoneal administration of chemotherapeutic agents can increase drug exposure for tumors confined to the peritoneal cavity [18]. Administration by the i.p. route produces very high peritoneal-to-plasma concentration ratios for many drugs [18]. However, there is a need to develop i.p. chemotherapy regimens with even greater selectivity and efficacy. We explored the possibility of further increasing cytotoxic activity in the peritoneal cavity by coadministration of a modulating agent that interacts synergistically with the cytotoxic compound. The concept is that if both drugs have a high peritoneal-to-plasma concentration ratio and synergy occurs only when the agents are present at high concentrations, the synergistic interaction might be confined to the peritoneal cavity, thus improving the overall selectivity of the program. MTX and DP are attractive agents with which to explore this concept, since we have previously shown that the peritoneal-to-plasma concentration ratios for these two drugs are very high [6, 14].

We have recently reported a phase I trial of the concurrent, continuous i.p. infusion of MTX and DP for 7 days [10]. This report describes additional preclinical studies that support the rationale for the use of this combination for selective i.p. biochemical modulation and pharmacologic evidence that the DP concentrations needed for enhanced selectivity can actually be achieved in patients.

Methods

Chemicals. Pure MTX was obtained as lyophilized powder from the National Cancer Institute. DP (Boehringer Ingelheim Ltd., Ridgefield, Conn) was supplied as a solution of 5 mg/ml water. [3′, 5′, 7-^{3}H]-MTX (20 Ci/mmol) powder and [5,6-^{3}H]-uridine (44 Ci/ml) solution were both obtained from Amersham Corporation (Arlington Heights, Ill). Uridine was obtained as a lyophilized powder from Sigma Chemical Company (St. Louis, Mo).

Cell line and clonogenic assay. The human ovarian carcinoma cell line OV 2008 was used [7]. Cells were maintained in logarithmic growth in RPMI 1640 containing 10% fetal bovine serum and 1% L-glutamine without antibiotics. Cells growing in log phase were harvested with

Abbreviations: MTX, methotrexate; DP, dipyridamole; HPLC, high-performance liquid chromatography

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trypsin, washed with medium, and plated in triplicate onto 60-mm plastic tissue-culture dishes (Corning Glass Works, Corning, NY) at a density of 300 cells/dish in 5 ml culture medium. Varying amounts of MTX and DP were added to the dishes, usually 50 µl 100-fold concentrated stock solution, and the cells were incubated under an atmosphere containing 5% CO_2 for 10 days. Clusters of > 50 cells were counted as one colony; the control dishes generally contained 100 – 150 colonies.

Effect of DP on plasma protein binding of MTX. MTX was added to aliquots of fresh human plasma (from normal volunters) to produce final concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} *M*, with and without 40 µ*M* DP. To each 1-ml sample of MTX-containing plasma, 80 ml tritiated MTX solution (prepared by dissolving 0.09 mmol MTX powder in 1 ml water) was added to obtain 5,000 cpm/sample. The plasma samples were ultrafiltered for 10 min through Centrifree™ micropartition system devices (Amicon Division, W. R. Grace and Co., Danvers, Mass). The ultrafiltrate was added back into the unfiltered plasma, and this cycle was repeated twice. This repetition was necessary to minimize the binding of free MTX to the ultra titration devices. Aliquots of 100 ml ultrafiltered and unfiltered plasma were added to 6 ml 3A70B scintillation fluid (Research Products International Corp., Mount Prospect, Ill) and the radioactivity was measured by scintillation counting over 5 min.

Effect of MTX on plasma protein binding of DP. MTX was added to aliquots of fresh human plasma (from normal volunters) to produce final concentrations of 0, 10, 100, and 500 µ*M*. DP at a final concentration of 20 m*M* was added to all aliquots of plasma. The plasma samples were ultrafiltered for 30 min through the Centrifree™ micropartition devices. DP concentrations were measured by HPLC in both the plasma and the ultrafiltrate [6].

Chemical stability of MTX and DP in combination. The chemical stability of the two drugs mixed together was determined at concentrations of up to 10^{-3} *M*. Immediately after mixing and 24 h later, the mixtures were assessed for the presence of precipitates as well as changes in color and pH. In addition, MTX and DP concentrations in the mixtures were determined by HPLC [23, 29] at 24 h and compared with the corresponding concentrations of the drugs soon after mixing.

Treatment plan and determination of MTX and DP concentrations. The clinical treatment program and drug assays have been reported in detail elsewhere [10]. Briefly, 2.16 mg/m^2 MTX and 12 mg/m^2 DP were given together daily as a continuous i. p. infusion lasting 48 h. The duration of infusion was gradually escalated to 168 h, and when this was found to be well tolerated, the dose of MTX was doubled to 4.32 mg/m^2 per day, with the daily dose of DP kept constant. Peritoneal fluid and blood samples were obtained prior to treatment and every 24 h during the infusion cycle. Total MTX concentrations were determined by an assay measuring the inhibition of dihydrofolate reductase [8]. Total and free DP concentrations were measured by HPLC as previously described [6].

Bioassay of DP. The inhibition of cellular uptake by DP in the patients' peritoneal fluid was measured using a modification of a previously described technique [5]. One million OV 2008 cells were dispersed in 1 ml RPMI 1640 medium in 15-ml centrifuge tubes. After centrifugation, the medium was aspirated and the cell pellet was resuspended in 0.2 ml peritoneal fluid obtained from patients who had attained steady-state drug levels during continuous i. p. infusion. The cells were incubated at 37° C in a 5% CO_2 atmosphere for 10 min, and uridine and tritiated uridine were added to give a final concentration of 10 µ*M* and a specific activity of 10 mCi/ml, respectively. The cell suspension was then reincubated for 30 min and washed three times with 10 ml ice-cold phosphate-buffered saline. We have previously shown that the uptake of uridine was linear over 30 min in OV 2008 cells [5]. The cells were then digested in 1 ml ice-cold 0.1 *N* NaOH for at least 1 h. The radioactivity in a 500-ml aliquot of each sample dissolved in 10 ml Ecoscint (National Diagnostics, Manville, NJ) was quantified by liquid scintillation counting. The control values for uridine uptake were determined froom the ascitic fluid of a non-study patient with carcinoma of unknown primary origin.

Peritoneal protein concentrations. Peritoneal protein concentrations were determined using the method of Bradford [2]. The determinations were done on the pretreatment ascites (if present) or on an aspirate obtained immediately after the start of instillation of the chemotherapeutic drugs (if no ascites was present) and from peritoneal fluid aspirates obtained during the infusion process. The standard curves were derived from aliquots of bovine serum albumin.

Results

Synergistic cytotoxicity of MTX and DP for human ovarian carcinoma cell line OV 2008

Using a clonogenic assay with the OV 2008 cell line, no cytotoxicity was produced by MTX alone, even at concentrations of up to 100 µ*M*. This was the expected result, as fetal calf serum has been shown to contain hypoxanthine and thymidine at concentrations sufficient to prevent, in part, MTX-induced starvation for deoxyribonucleotides [13, 22]. DP alone produced no cytotoxicity at concentrations up to 20 m*M*.

Figure 1 shows that 0.02 µ*M* MTX alone produced no toxicity and that in the presence of this concentration of MTX, DP at concentrations of up to 4 µ*M* also produced no cell kill. However, as the concentration of MTX was increased from 0.02 to 0.07 µ*M*, the dose-response curve for DP became progressively steeper. In the presence of 0.03 µ*M* MTX, a DP concentration of 4 µ*M* was required to produce a 1-log cell kill; in the presence of 0.04 µ*M* MTX, < 0.5 µ*M* DP was required, and in the presence of 0.07 µ*M* MTX, < 0.1 µ*M* DP was required to reduce cell survival to < 10%. This result indicates a very potent synergistic interaction between MTX and DP and demonstrates that it is very highly dependent on the concentration of both agents. Repetition of the experiment presented in Fig. 1 demonstrated variation in the individual IC_{50} for each curve, but the extent of the synergistic interaction and the high degree of concentration de-

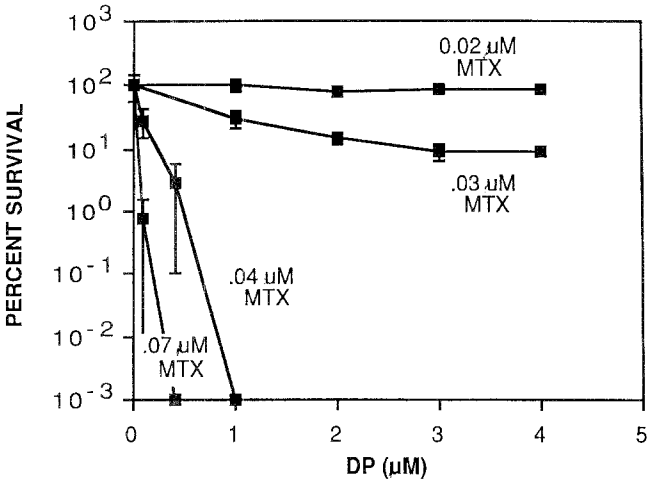

Fig. 1. Dose-response curves for the killing of clonogenic OV 2008 cells by DP in the presence of increasing concentrations of MTX. Each data point represents the mean value determined from three plates; vertical bars represent SD

pendence was repeatedly observed in each of three such experiments.

If the potentiation of MTX was due primarily to DP-induced inhibition of nucleoside membrane transport, one might expect that once all transport sites were saturated by DP [9], a further increase in DP concentration would not additionally augment the MTX effect. When DP concentrations of >4 μ*M* were tested, there was in fact no further potentiation of MTX-induced cytotoxicity at MTX concentrations ranging from 0.01 to 0.025 μ*M*.

Effect of DP on protein binding of MTX

The ability of DP to affect the plasma protein binding of tritiated MTX was tested over the MTX concentration range of 0.01 – 100 μ*M*. The protein binding of MTX at these concentrations in the absence of DP ranged from 53% to 61%, and in the presence of 10 μ*M* DP it ranged from 54% to 60%. At an MTX concentration of 1 μ*M*, 54% ± 2% ($n = 5$) was free, non-protein-bound drug in the absence of DP; 54% ± 4% ($n = 2$) was free MTX in the presence of 16 μ*M* DP; and 56% ± 7% (n = 3) was free drug in the presence of 40 μ*M* DP.

Effect of MTX on protein binding of DP

The ability of MTX to affect the plasma protein binding of DP was tested over the MTX concentration range of 0 – 500 μ*M* in the presence of 20 m*M* DP. The free, non-protein-bound fraction of total DP stayed constant, from 93% to 97%, over the MTX concentration range.

Chemical stability of MTX and DP in combination

No problems related to chemical stability were reported when MTX and DP were given i. v. in a clinical trial [28]. However, continuous i. p. instillation requires the co-mixing of both agents at much higher concentrations in the drug reservoir. Therefore, we tested stability under the conditions anticipated in the reservoir. When the drugs were mixed in 0.9% NaCl at room temperature at concentrations of 500 μ*M* MTX and 500 m*M* DP, a precipitate formed within 24 h. No precipitate was observed at drug concentrations of $\leqslant 400$ μ*M* each. HPLC quantitation revealed no loss of either drug over an 8-day period in mixtures containing 400 μ*M* MTX and 400 μ*M* DP. The pH of a solution of both drugs at 10^{-3} μ*M* was 4.5 soon after mixing, and it remained the same at 24 h. Thus, with the exception of precipitation at high concentrations, there was no evidence that the two drugs were reacting chemically with each other.

Bioassay of DP concentrations attained in vivo

Based on the preclinical information presented above, we conducted a phase I/pharmacokinetic study of constant i. p. infusion of MTX and DP for periods of up to 7 days to assess the feasibility of using DP to produce selective i. p. modulation of MTX activity. The clinical details of this trial and the MTX and DP concentrations attained in the peritoneal cavity have been reported elsewhere [10]. The mean MTX concentration in the peritoneal cavity was 7.80 ± 3.63 μ*M* at the highest infusion rate tolerated (4.32 mg/m^2 per day), and the mean total DP concentration was 30.4 ± 9.7 μ*M* (at an infusion rate of 12 mg/m^2 per day).

To determine whether the in vivo biologic activity of the DP concentrations attained was sufficient to produce synergy with MTX, we measured the ability of peritoneal fluid samples obtained at steady state to inhibit the uptake of [^{3}H]-uridine into OV 2008 cells in vitro. Inhibition of uridine uptake by patient samples was compared with that produced by an ascitic fluid to which graded amounts of DP were added, which was used as a standard for all patient samples. Figure 2 shows this standard curve for the inhibition of [^{3}H]-uridine uptake into OV 2008 cells. The uridine uptake decreased as the DP concentration was increased to 30 μ*M*, after which it plateaued at about 10% of control valaues. MTX at 22.2 μ*M* did not affect uridine uptake or further affect the latter when it was co-incubated with 50 μ*M* DP.

Uridine uptake was reduced to a mean of 16% ± 11% of control values in peritoneal fluid samples obtained from eight separate courses of treatment. Thus, the mean biologically active DP concentration in the peritoneal cavity was sufficiently high for maximal inhibition of the uptake of a representative nucleoside, an effect associated with marked enhancement of MTX cytotoxicity (Fig. 1). Concurrent measurements were made of free DP concentration by HPLC and DP activity by bioassay on peritoneal fluid samples from 11 separate courses of therapy obtained at a time when steady state had been reached. Uridine uptake tended to decrease with increasing free DP concentrations in the peritoneal cavity, but there was no statistically significant linear correlation between these parameters.

Peritoneal protein concentrations

The peritoneal protein concentrations measured during ten courses of chemotherapy infusion were quite variable both within and between patients (Fig. 3). In five patients a small amount of peritoneal fluid was obtainable prior to drug instillation. The mean pretreatment peritoneal protein concentration was 17.9 ± 16.1 mg/ml. During the course of the i. p. infusion, the protein concentrations ranged from a low of 0.03 mg/ml to a high of 52 mg/ml. The percentage of the total peritoneal DP that was protein-

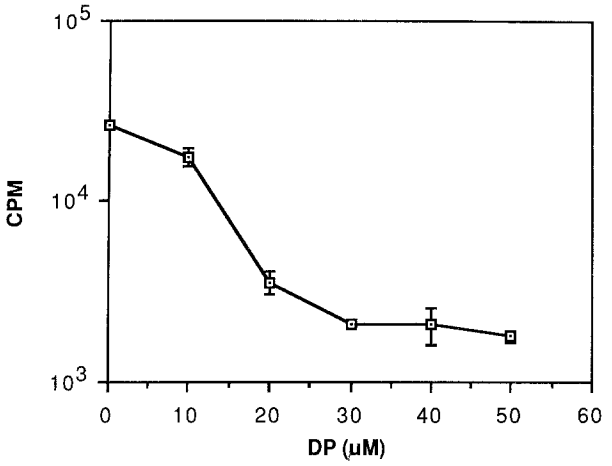

Fig. 2. Inhibition of [^{3}H]-uridine uptake (expressed as cpm) by OV 2008 cells according to increasing concentrations of DP. Each point represents the mean of three samples; error bars represent SD

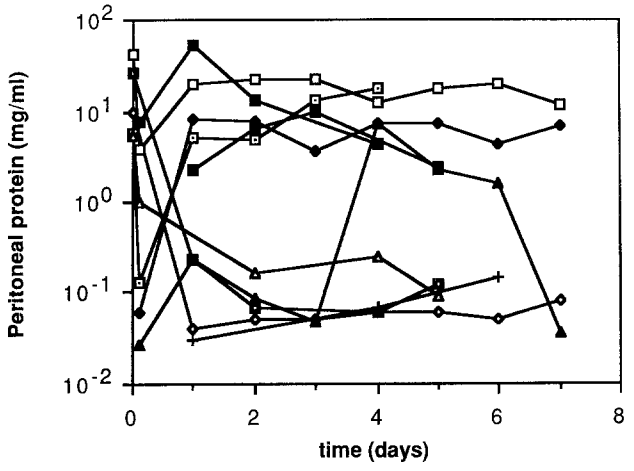

Fig. 3. Peritoneal protein concentrations during continuous i.p. infusion of MTX and DP are plotted for ten courses. Each curve represents the protein profile from one course of therapy

bound tended to increase with peritoneal protein concentration, but there was no statistically significant linear correlation between these parameters. The free DP peritoneal clearance tended to increase with increasing peritoneal protein concentration, whereas the total DP peritoneal clearance stayed constant with increasing peritoneal protein concentration; however, there was no significant linear correlation between either total or free peritoneal DP clearance and peritoneal protein concentration.

Discussion

We found that DP markedly potentiated the cytotoxicity of MTX to the OV 2008 cell line in vitro and that this interaction was very dependent on the concentration of each agent. Although in vivo the concentrations of MTX and DP required for synergy and the extent of the latter may be substantially different, the fact that our previous studies had demonstrated the feasibility of maintaining very high local concentrations of both MTX and DP in the peritoneal activity relative to the plasma [6, 10, 14] made these two agents attractive as candidates to test the concept of selective i.p. biochemical modulation.

The ability of DP to enhance the toxicity of MTX is thought to be primarily due to blockade of nucleoside and nucleobase salvage [25], although inhibition of MTX efflux may also play some role [3]. The efficacy of DP as modulator of MTX toxicity is in part a function of the thymidine and hypoxanthine concentrations available extracellularly. In the OV 2008 clonogenic assay, small amounts of thymidine and hypoxanthine are contributed by the fetal calf serum (T. K. C. Chan, unpublished data). Likewise, sufficient amounts of thymidine are present in human plasma to offset, in part, the toxicity of MTX [13, 15; Chan TKC, unpublished data]. Thus, although the in vivo situation is undoubtedly somewhat different than the conditions under which synergy was demonstrated in vitro, there is reason to anticipate that DP can modulate MTX toxicity in vivo. In fact, several recent clinical trials [11, 12, 24, 26, 28] have demonstrated that DP can increase the toxicity of MTX when both are given systemically.

In the presence of a fixed concentration of MTX, the DP dose-response curve reached a plateau at concentrations above approximately 4 μ*M*. This can be explained by the fact that nucleosides can enter cells by at least two mechanisms (reviewed in [9]). A substantial fraction of the uptake is due to saturable facilated diffusion that is inhibited by DP, and the remainder takes place by DP-independent mechanisms such as simple diffusion. Thus, any further elevation in DP concentration above the level at which facilated diffusion of nucleosides is totally blocked would not be expected to further increase MTX cytotoxicity. This maximal inhibition of cellular nucleoside uptake was apparent in our bioassay (Fig. 2), where uridine uptake reached a plateau at about 10% of control values at high peritoneal DP concentrations. The DP concentration required to augment MTX toxicity in vitro was lower than that required to inhibit uridine uptake; however, this is attributable to differences in protein binding of DP in tissue-culture medium vs human plasma and to the fact that the K_i for DP is known to vary somewhat for different nucleosides.

Based on the concentration-dependent synergy between MTX and DP and our demonstration that MTX and DP were chemically stable when mixed together at 400 μ*M* each, we proceeded to test the concept of selective i.p. biochemical modulation of MTX by DP in patients, using a solution containing up to 20 μ*M* MTX and 50 μ*M* DP (i.e., 4.32 mg/m^2 MTX and 12 mg/m^2 DP dissolved in 1 l fluid) [10]. When MTX was given at 4.32 mg/m^2 per day and DP, at 12 mg/m^2 per day by continuous i.p. infusion, the mean steady-state peritoneal concentration of MTX was 7.80 μ*M* and that for DP was 30.4 μ*M*. The mean plasma concentratioons were 0.109 and 0.57 μ*M* for MTX and DP, respectively.

Based on the dose-response curves derived from our clonogenic assays (Fig. 1), the concentrations of the drugs in the peritoneal cavity were much higher than those necessary to achieve significant enhancement of cytotoxicity. This was borne out by both the bioassay and clinical results. Uridine uptake into OV 2008 cells was reduced to a mean of 16%±11% of control values when the cells were exposed to peritoneal fluid obtained from

patients during the chemotherapy infusion cycle. Thus, the bioassay corroborated the measurement of peritoneal concentrations of DP and demonstrated that the concentration of biologically active DP in the peritoneal cavity was sufficient to produce biochemical modulation of MTX. This program produced 3 minor responses in 13 patients evaluable for response [10], an encouraging response rate, since most of these patients were heavily pretreated and had drug-resistant tumors. The biological activity of most drugs depends on the non-protein-bound, free fraction of total drug that is present in the vicinity of the recipient cell. We found that in the peritoneal cavity an average of 39% of the DP occurred as free drug, whereas in the plasma $<1\%$ was free DP [10]. Thus, by virtue of the difference in protein binding, the peritoneal/plasma concentration ratio for biological active DP was $>2{,}300$, whereas the ratio for total drug was only 53. We found that DP did not influence the plasma protein binding of MTX over a wide MTX concentration range and at DP concentrations of up to 40 μM in vitro. Similarly, we found that MTX did not affect the plasma protein binding of DP over a wide MTX concentration range and at a DP concentration of 20 mM. Thus the displacement of MTX or DP from protein-binding sites would not seem to account for the synergy between these two agents in vitro and would not be expected to contribute to differential exposure for the peritoneal cavity and plasma when the drugs are given by the i. p. route.

Because of the importance of DP protein binding, we sought to determine whether protein concentrations were changing in the peritoneal cavity during the course of infusion and whether the percentage of free DP or the DP clearance could be related to the total peritoneal protein concentration. We found that peritoneal protein concentrations were highly variable within and between patients, both initially and during the course of the i.p. infusion. In our patients, 2 l protein-free 0.9% saline was initially given i.p. as rapidly as possible; thereafter, 1 l saline per day was given i.p. by constant infusion for the duration of the treatment cycle. The protein levels at the end of the loading dose tended to be lower than those measured prior to the institution of chemotherapy. This was expected, since the fluid diluted the protein in the peritoneal cavity. During the continuous infusion, protein levels did not vary in any consistent manner. In addition, we found no significant correlation between protein concentration and either the fraction of DP that was free or the peritoneal clearance of DP calculated from the quotient of the infusion rate and the steady-state DP concentration, although such a correlation was expected. In the plasma, DP is largely bound to alpha-1 acid glycoprotein [17]; a significant correlation might have been observed if the level of this specific protein had been measured in the peritoneal cavity.

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