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Biochemical Pharmacology, Vol. 40, No. 8, pp. 1925–1928, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Characterization of repair inhibition by methotrexate of ethylmethanesulfonate- and ultraviolet irradiation-induced DNA damage in Chinese hamster cells*

(*Received* 29 *November* 1989; *accepted* 11 *May* 1990)

As an inhibitor of dihydrofolate reductase (DHFR†), thymidylate synthase (TS), and aminoimadazolecarboxamide-phosphoribosyl transformylase (AICAR), methotrexate (MTX) interferes with the synthesis of adenylate, guanylate, and thymidylate [1]. Through depletion of nucleotide pools, MTX causes inhibition of semiconservative DNA synthesis. Recently, we demonstrated that treatment of cells with MTX also leads to inhibition of DNA synthesis required for the repair of ethylmethanesulfonate (EMS)- or UV-induced DNA damage [2]. MTX was compared to a drug combination known to block the DNA synthesis step of excision repair, hydroxyurea + Ara-C (H/A). Although MTX was found to inhibit the repair of both types of damage, its efficacy was equivalent to H/A after EMS-but not UV-induced damage. These data suggested that MTX was more efficacious at inhibiting the short patch mode of repair. The ability of MTX to affect repair was prevented by coadministration of hypoxanthine (HX) and thymidine (TdR), indicating that MTX-induced nucleotide depletion was responsible for repair inhibition.

To further understand the mechanism by which MTX inhibits these forms of DNA repair, we have studied the dependency of EMS repair inhibition on both pretreatment time and concentration of MTX. The basis for the selectivity with which MTX inhibited EMS repair more effectively than UV repair has also been examined. We found that MTX in combination with Ara-C was as effective as H/A in causing inhibition of UV repair.

* From a dissertation presented by A.H.B. to the Department of Pharmacology, the George Washington University Graduate School of Arts and Sciences, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Abbreviations: DHFR, dihydrofolate reductase; AICAR, aminoimidazolecarboxamide-phosphoribosyl transformylase; MTX, methotrexate; SSB, single strand breaks; HU, hydroxyurea; Ara-C, cytosine arabinofuranoside; H/A, HU + Ara-C; EMS, ethylmethanesulfonate; UV, ultraviolet irradiation; CHO, Chinese hamster ovary; REB, rad equivalent breaks; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TS, thymidylate synthase; HX, hypoxanthine; and TdR, thymidine.

Methods

Tissue culture reagents were obtained from Gibco (Grand Island, NY). Tissue culture flasks were obtained from Corning (Corning, NY) and tissue culture dishes from Costar (Cambridge, MA). Unless otherwise indicated, all other reagents were obtained from Sigma (St. Louis, MO).

Cell culture and drug exposure conditions. The AA8 CHO cell line used in this work was grown in monolayer in DMEM supplemented with dialyzed calf serum (5%, v/v), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°. In all experiments, cells were allowed to recover for at least 8 hr after plating before drug treatment was commenced.

For EMS repair experiments, 600,000 cells were plated into 25 cm^2 flasks, 8 hr later MTX was added, and the cells were incubated for an additional 16 hr. Exposure to EMS was carried out by first transferring 5 of the 10 mL of medium present in each treatment flask to sterile tubes; the transferred medium, which were kept at 37°, was used to refeed the cells after treatment with EMS. EMS was then added to the cells to achieve a final concentration of 1.8 mg/mL. One hour later, the drug-containing medium was replaced with the medium removed earlier. For UV repair experiments, 600,000 cells were plated into 10-cm dishes. Eight hours later, MTX was added to the cells. Sixteen hours later (immediately prior to irradiation), the medium was transferred to sterile tubes and was used to refeed the cells immediately after exposure to 10 J/m^2 of 254 nm UV light delivered from a germicidal lamp. For both UV and EMS repair experiments, repair was terminated by replacing the medium with ice-cold PBS and keeping the flasks or dishes on ice until harvesting the cells for alkaline elution analysis.

Assay of excision repair by alkaline elution. The time-course of excision repair was monitored by using alkaline elution to measure SSB at various time points following the induction of DNA damage. This technique to follow repair is based on the fact that the excision of damaged regions creates single-strand breaks (SSB) in the DNA, whereas the filling of gaps and their ligation to the parent DNA serves to remove the breaks. In the presence of inhibitors of DNA repair synthesis, such as H/A, a large accumulation of SSB results due to the build up of excision gaps along the DNA [3–5]. The alkaline elution procedure used in

this work has been described [2] and is essentially that as detailed by Kohn *et al.* [5].

Results and Discussion

Coadministration of HX and TdR was shown previously to prevent completely repair inhibition by MTX after EMS treatment, demonstrating the role of nucleotide depletion in this response [2]. To determine whether inhibition of EMS repair by MTX could be attributed primarily to purine or thymidine nucleotide depletion, the relative contributions of HX and TdR to attenuate inhibition of EMS repair were determined. As shown in Fig. 1, TdR was more efficacious than HX in reversing repair inhibition in MTX-treated cells. This observation suggests that, relative to the effects on dATP and dGTP pools, depletion of dTTP is highly associated with inhibition of EMS repair.

The dependency of repair inhibition on MTX concentration and pretreatment time was also examined. A 16-hr pretreatment with 10 μM MTX has been shown previously to inhibit EMS repair [2]. To determine the importance of these two variables, inhibition of EMS repair was measured using: (a) shorter pretreatment times with 10 μM MTX, and (b) the 16-hr pretreatment time at progressively lower MTX concentrations. As shown in Figs. 2 and 3, inhibition of EMS repair by MTX was sensitive to changes in both parameters. Figure 2 shows that at doses above 0.5 μM MTX, inhibition of repair became significant. This finding suggests that at lower doses of MTX, residual biosynthesis of dNTPs may have taken place, allowing repair to proceed. We previously determined [2] that the ability of MTX and EMS to act synergistically to kill cells was also dependent on MTX concentration. Therefore, the efficacy of MTX to inhibit EMS repair appears to correlate with the ability of the agents to synergistically kill cells.

The effect of shorter pretreatment times is shown in Fig. 3. Pretreatment times of 1 and 2 hr were insufficient to inhibit repair synthesis, whereas longer pretreatment times were found to be more effective. This dependency of repair

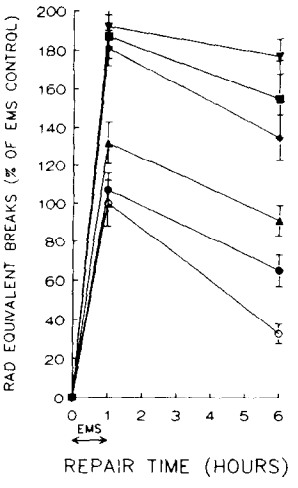

Fig. 2. Dependence of inhibition of EMS repair on MTX concentration. Cells were pretreated for 16 hr with 0, 0.05, 0.1, 0.5, 1, or 10 μM MTX. They were then exposed to EMS, and SSB were measured at various times as described in Methods. Results were normalized with respect to the number of REB measured at the 1-hr time point of the EMS alone treatment group, which was assigned a value of 100%. The actual REB measured for the control group was 371 ± 44. MTX concentrations: (○) 0 μM (EMS only); (●) 0.05 μM; (▲) 0.1 μM; (◆) 0.5 μM; (▼) 1 μM; and (■) 10 μM. Each point is the mean (±SE) of two to three observations from three independent experiments.

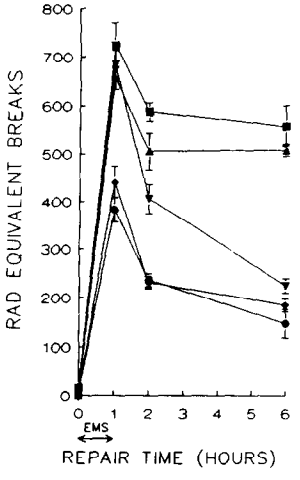

Fig. 1. Ability of HX and/or TdR to prevent inhibition of repair by MTX. Cells were pretreated for 16 hr with 10 μM MTX with or without 100 μM HX and/or 10 μM TdR. Cells were then exposed to EMS, and SSB were measured at various times as described in Methods. Key: (●), control (EMS only); (■) MTX; (▼) MTX + TdR; (▲) MTX + HX; and (◆) MTX + HX + TdR. Each point is the mean (±SE) of two observations from two independent experiments.

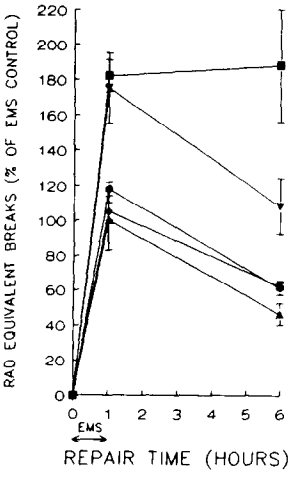

Fig. 3. Dependence of inhibition of EMS repair on pretreatment time with MTX. Cells were pretreated for 0, 1, 2, 9, or 16 hr with 10 μM MTX. They were then exposed to EMS, and SSB were measured at various times as described in Methods. Results were normalized with respect to the number of REB measured at the 1-hr time point of the EMS alone treatment group, which was assigned a value of 100%. The actual REB measured for the control group was 452 ± 79. MTX pretreatment times: (▲) 0 hr (EMS only); (●) 1 hr; (◆) 2 hr; (▼) 9 hr; and (■) 16 hr. Each point is the mean (±SE) of two to three observations from four independent experiments.

on MTX pretreatment time could be interpreted in several ways. One possibility is that the depletion of nucleotides to levels that result in repair inhibition is not a rapid event. Another interpretation is that longer incubation times allow for a greater portion of the cell population to enter S-phase, the stage during which dNTP depletion is expected to be greatest. During S-phase, TS is most active, which leads to tetrahydrofolate depletion and cessation of nucleotide synthesis [1]. In addition, the demands imposed by DNA replication imply that dNTP utilization is also greatest during S-phase. Thus, with longer incubation times, more cells enter S-phase, causing nucleotide levels to drop to the point at which DNA repair synthesis ceases. This would lead to the dependency of repair inhibition on MTX pretreatment time which was observed.

As discussed earlier, relative to H/A, MTX was found to be less effective at inhibiting UV repair than EMS repair. To investigate this observation further, MTX was compared with HU (which also causes nucleotide depletion) to determine whether UV repair in these cells showed a general insensitivity to nucleotide depletion, or if it was insensitive to the dNTP perturbations induced specifically by MTX. In addition, the possibility that MTX in combination with Ara-C may lead to enhanced inhibition of UV repair was also investigated. The results are shown in Fig. 4. When used alone, HU resulted in greater inhibition of repair than MTX, but much less than that produced by HU + Ara-C. In combination with Ara-C, both HU and MTX caused equivalent inhibition of UV repair. The observation that maximal inhibition could only be achieved in the presence of Ara-C supports the notion that UV repair could not be fully blocked by nucleotide depletion alone. The ability of HU and Ara-C in combination to have enhanced capability to inhibit repair is due to the depression of dCTP pools by HU. The lowering of dCTP pools by HU enhances the incorporation of Ara-CTP into DNA [6]. The observation that MTX and Ara-C in combination was as effective as HU and Ara-C suggests that MTX causes a reduction in dCTP pools large enough to increase the utilization of Ara-C. Cadman and Eiferman [7] found in L1210 cells that MTX given prior to Ara-C leads to increased Ara-CTP formation, incorporation into DNA, and synergistic cytotoxicity. Their findings are consistent with the data presented here, which demonstrate that MTX had a dramatic impact on the ability of Ara-C to inhibit UV repair over the entire 6-hr repair period. The ability of MTX and Ara-C to cause inhibition of UV repair could have important implications concerning the possible use of MTX to increase the effectiveness of clinically relevant DNA damaging compounds, because the repair of the lesions introduced by many of these agents is handled through UV-like (long patch) repair mechanisms. Evidence for this comes from the observation that XP and other UV repair deficient cells are hypersensitive to a number of clinically active DNA damaging agents, including cisplatin, nitrogen mustard, busulfan, and mitomycin C [8–10]. Therefore, the results shown in Fig. 4 would suggest that MTX/Ara-C combinations would be a potentially useful avenue by which tumor cell kill could be maximized.

In conclusion, this work characterized inhibition of EMS repair by MTX with respect to MTX concentration and exposure time. The data demonstrated that relatively high doses of MTX are required to achieve maximal inhibition of repair. In addition, the dependency on MTX pretreatment time indicates that cell-cycle perturbations induced by prolonged MTX exposure may be important to produce this response. Finally, we report that MTX in combination with Ara-C caused inhibition of UV repair as efficaciously as H/A.

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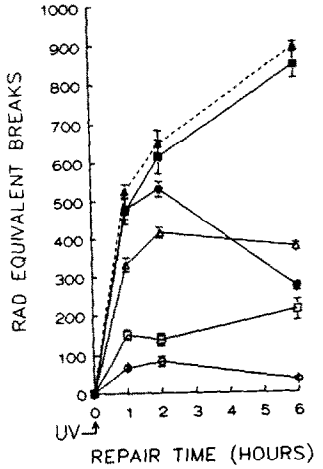

Fig. 4. Inhibition of UV repair by MTX and HU alone and in combination with Ara-C. Cells were pretreated with: (●) 50 μM Ara-C, 1 hr; (□) 10 μM MTX, 16 hr; (■) 10 μM MTX, 16 hr, with 50 μM Ara-C present during the final hour; (△) 2 mM HU for 1 hr; (▲) 2 mM HU + 50 μM Ara-C for 1 hr; or (○) untreated. The cells were then exposed to UV, and SSB were measured at various times as described in Methods. Each point is the mean (±SE) of two observations from four independent experiments.

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Biochemical Pharmacology, Vol. 40, No. 8, pp. 1928–1931, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Stimulation of inositol phosphate production in clonal HSDM1C1 cells by endothelins and sarafotoxin

(*Received* 30 *November* 1989; *accepted* 5 *June* 1990)

Endothelin (ET*), a polypeptide of 21 amino acid residues, has been isolated recently from porcine vascular endothelial cells and has been shown to be identical in structure to human endothelin [1, 2]. Human and porcine ETs have been denoted as ET_1 [1, 2]. Rat ET (ET_3), on the other hand, has a slightly different amino acid sequence to ET_1 but still has a high degree of homology with the latter peptide [1, 2]. Subsequently ETs have been shown to be some of the most potent vasoconstrictor substances known to date and are capable of eliciting powerful responses *in vivo* and *in vitro* in a variety of animal/tissue preparations [1–5]. Although Yanagisawa *et al.* [1] originally proposed that perhaps ET_1 produced its biological actions by facilitating the entry of extracellular calcium into the cell by interacting with Ca^{2+} channels, it has been shown that ET_1 does not bind directly to voltage-sensitive Ca^{2+} channels, N-type Ca^{2+}-channels, or to sodium channels [5–7]. On the contrary, preliminary evidence, using cultured vascular smooth muscle cells [8–10], has indicated that ET_1 receptors may be coupled to the phosphoinositide (PI) turnover signalling mechanism resulting in mobilization of intracellular and/or extracellular Ca^{2+} [9, 10].

Sarafotoxin S6b(SRFT) is also a 21 amino acid-containing peptide which has been purified from snake venom [11, 12]. SRFT has been shown to have a high degree of homology with ET_1 and, like the latter, is a potent vasoconstrictor [11, 12]. SRFT receptors in the rat brain and heart appear to mediate PI turnover [11, 12], and ET_1 and SRFT appear to have similar properties in terms of receptor binding activity and stimulation of PI hydrolysis in these tissues [13].

However, to date the physiological actions of ET_1 and SRFT, such as tissue contractions [1–5], *in vivo* hemodynamic and vascular effects [5, 11, 12], and the biochemical effects of these peptides, such as PI turnover and Ca^{2+} mobilization [11, 12], have been conducted on isolated organs, tissue slices, cells in primary culture (after multiple passages in some cases) or using *in vivo* preparations. In the present study we have identified functional receptors for ET_1, ET_3 and SRFT in an immortal cell line, mouse fibrosarcoma cells (HSDM1C1), using PI turnover as an index of receptor activation. This is an important finding since clonal cell lines have several advantages over primary cultures of tissues and, therefore, HSDM1C1 cells may prove useful in further regulatory studies on the ET_1/SRFT receptor-coupled PI turnover system.

Materials and Methods

Murine fibrosarcoma cells (from the American Type Culture Collection) were cultured in Ham's F10 medium supplemented with 15% donor horse serum and 2.5% fetal calf serum in 150 cm^2 Costar sterile culture flasks at 37° in a humidified atmosphere of 10% CO_2 and 90% air. The medium was changed every 3 days, and the cells were passaged by trypsinization every 5 days and subcultured at a density of 0.2–0.3 × 10^6/well in 24-well plates. The cell inositol phospholipids were labeled at 37° with 0.45 to 0.8 μCi/mL of *myo*-[^{3}H]inositol (American Radiochemical Co.; 15 Ci/mmol) for 2 days.

For the phosphoinositide turnover experiments, the culture medium was aspirated, cells were washed gently with 2 mL of oxygenated Krebs-bicarbonate buffer (pH 7.4), and the cells were challenged with 0.25 to 0.5 mL of the drug solution made in Krebs buffer containing 10 mM LiCl. The cells were incubated for 30 min at 37° with the test compounds or buffer alone, and the assays were terminated by the addition of 1 mL of ice-cold chloroform/methanol (1 : 2, v/v). For antagonism studies, the putative antagonists were added to the cells 30 min prior to addition of agonists. After 5 min, the cell lysates were transferred to Biovials, and 0.3 mL of chloroform and 0.3 mL of water were added to the lysates. The contents of the vials were shaken for 5 sec and then left to stand at 23° for 1 hr in order to separate the aqueous and organic phases. After this time, 0.8 mL of the upper aqueous phase was transferred to Econo-columns containing 1 mL of AG-1-X8 anion exchange resin (200–400 mesh in formate form; Bio-Rad). The free *myo*-[^{3}H]inositol was eluted from the columns with 8–10 mL of unlabeled *myo*-inositol (20 mM) and discarded. Total [^{3}H]inositol phosphates retained on the resin were then eluted off using 4 mL of 1 M ammonium formate and 0.1 formic acid and counted and quantified as previously described [14–16]. The concentration–response curves were analyzed using an iterative curve-fitting computer program [17] and the concentrations of the agonists required to produce 50% of the maximal response (EC_{50} values) obtained.

ET_1, ET_3, SRFT, bradykinin (BK), BK-antagonist and other peptides were purchased from Peninsula Laboratories and *cis*-dioxolane, histamine, glutamate, γ-aminobutyric acid (GABA), serotonin, norepinephrine, U69593 and 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) were purchased from Research Biochemicals Inc. and the Sigma Co.

Results and Discussion

Initial exploratory studies using a fixed concentration of test compounds (10 μM) showed that while ET_1, ET_3 and SRFT inducdd a 4.5- to 5.6-fold stimulation of PI turnover in HSDM1C1 cells (Fig. 1), several other neuroactive agents were inactive in this system (Table 1). The only other substances shown to be active in this system were BK and the muscarinic agonist, *cis*-dioxolane (CD) (Table 1). Detailed concentration–response studies revealed that ET_1, ET_3, SRFT, BK and CD possessed agonist activity, with

* Abbreviations: ET, endothelin; BK, bradykinin; PI, phosphoinositide; IP, inositol phosphate; and SRFT, sarafotoxin S6b.