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European Journal of Pharmacology 435 (2002) 237–244

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Polyglutamation of a novel antifolate, MX-68, is not necessary for its anti-arthritic effect

Kazumi Urakawa*, Masahiko Mihara, Nobuhiro Takagi, Akinori Kawamura, Ken-ichi Akamatsu, Yasuhisa Takeda

Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co., Ltd., 1-135, Komakado, Gotemba, Shizuoka 412-8513, Japan

Received 16 October 2001; received in revised form 19 November 2001; accepted 23 November 2001

Abstract

N-[[4-[(2,4-diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]-carbonyl]-L-homoglutamic acid (MX-68), a derivative of methotrexate, was chemically designed to resist polyglutamation and to have a high affinity for dihydrofolate reductase, in an attempt to reduce the side effects of methotrexate. We confirmed that MX-68 did not undergo polyglutamation and investigated the pharmacological activities of MX-68 compared with methotrexate. (1) In vitro: MX-68 inhibited the activity of dihydrofolate reductase to the same degree as methotrexate-tetraglutamate. MX-68 treatment produced a similar anti-proliferative effect to that of methotrexate. However, the intracellular concentration of MX-68 was much lower than the sum of the levels of methotrexate and its polyglutamate, and the effects of MX-68 disappeared when it was removed from the culture medium. (2) In vivo: Oral administration of MX-68 suppressed the development of collagen-induced arthritis in mice and adjuvant-induced arthritis in rats, in a similar fashion to that of methotrexate. These results indicate that polyglutamation is not essential for the anti-arthritic effect of antifolates. © 2002 Published by Elsevier Science B.V.

Keywords: MX-68; Antifolate; Methotrexate; Polyglutamation; Dihydrofolate reductase; Arthritis model

1. Introduction

The efficacy of methotrexate in refractory rheumatoid arthritis has been demonstrated in many clinical studies (Weinblatt et al., 1985; Williams et al., 1985). Its administration early in the course of the disease is now generally accepted, and it is one of the most commonly used agents for the treatment of rheumatoid arthritis.

The mechanism of action of methotrexate in rheumatoid arthritis is not fully understood. Methotrexate undergoes intracellular polyglutamation by folylpolyglutamate synthetase (Baugh et al., 1973). This enzyme converts methotrexate to polyglutamate forms (methotrexate-polyglutamates) including methotrexate-monoglutamate (methotrexate plus one glutamate), -diglutamate, -triglutamate, and the like, through the repeated addition of a glutamate residue to the glutamate residue of methotrexate. As a result, methotrexate-polyglutamates are kept within cells for longer periods than methotrexate itself (Chabner et al., 1985). In addition, polyglutamation also increases the affinity of methotrexate for its target enzymes such as dihydrofolate reductase (Kumar et al., 1989), thymidylate synthase, and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (Chabner et al., 1985). Nevertheless, it is not known whether polyglutamation essentially participates in the anti-arthritic effect of antifolates.

The intracellular storage of methotrexate as polyglutamate with a consequent decrease in available folates has been postulated to be crucial for the occurrence of adverse events (Ahern et al., 1991; Kamen et al., 1981). To reduce the adverse effects caused by methotrexate-polyglutamates, we synthesized a new antifolate, MX-68 (*N*-[[4-[(2,4-diaminopteridin-6-yl)methyl]-3,4-dihydro-2*H*-1,4-benzothiazin-7-yl]-carbonyl]-L-homoglutamic acid), which was designed to resist polyglutamation by replacing the glutamate residue of methotrexate with a homoglutamate residue. In addition, its aminobenzoic acid moiety was modified to increase its affinity to dihydrofolate reductase (Matsuoka et al., 1997b). In the present study, we confirmed that MX-68 did not undergo polyglutamation and that it potently inhibited dihydrofolate reductase activity. Furthermore, we investigated the in vitro and in vivo properties of MX-68 compared with methotrexate, to consider the role of polyglutamation in the anti-arthritic effect of antifolates, and demonstrated that polyglutamation is not essential for this effect.

* Corresponding author. Tel.: +81-550-87-6735; fax: +81-550-87-5326.
E-mail address: urakawakzm@chugai-pharm.co.jp (K. Urakawa).

0014-2999/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0014-2999(01)01553-9

2. Materials and methods

2.1. Chemicals

MX-68, [9-^{3}H]MX-68, methotrexate-tetraglutamate and Compound 1 were synthesized in the Research Laboratories of Chugai Pharmaceutical (Matsuoka et al., 1997a,b). Methotrexate and [3′, 5′, 7-^{3}H]methotrexate were purchased from Sigma (St. Louis, MO, USA), Amersham (Buckinghamshire, UK), respectively.

2.2. Cell lines

IM-9 (B lymphoblast, human) and CCRF-CEM (T lymphoblastic leukemia, human) were purchased from Dainippon Pharmaceutical (Osaka, Japan). Since T lymphocytes are strongly related to the pathogenesis of rheumatoid arthritis (Weyand and Goronzy, 1997), we mainly used CCRF-CEM, which is derived from T lymphocytes. IM-9 was used as a source of human enzyme such as folylpolyglutamate synthetase and dihydrofolate reductase, since a large mass of cells was easily prepared due to the rapid proliferation of these cells. Cells were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 5% (IM-9) or 10% (CCRF-CEM) fetal bovine serum (HyClone Laboratories, Logan, UT, USA), penicillin (100 unit/ml), and streptomycin (0.1 mg/ml) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Estimation of substrate for human folylpolyglutamate synthetase

The crude extract of IM-9 cells was used as a source of human folylpolyglutamate synthetase. Cells were suspended in ice-cold extraction buffer (200 mM Tris–HCl, 10 mM $MgCl_2$, 30 mM KCl, 50 mM 2-mercaptoethanol, pH 8.5) at a concentration of 3×10^7 cells/ml, and lysed by sonication, as described by Jansen et al. (1992). A clear supernatant was obtained after centrifugation at $30{,}000 \times g$ for 30 min and then stored at −80 °C. This fraction was used as human folylpolyglutamate synthetase in the following assay. Protein content was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA, USA).

The activity of the drugs as a substrate for folylpolyglutamate synthetase was assayed according to the method of Moran et al. (1985). The drug (10–100 μM) was incubated with the extract of IM-9 cells (0.42 mg protein) for 2 h at 37 °C in a mixture containing 1 mM L-[3, 4-^{3}H]glutamic acid (NEN, Boston, MA, USA; 148 MBq/mmol), 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 50 mM 2-mercaptoethanol, and 200 mM Tris–HCl (pH 8.5), in a total volume of 0.25 ml. [^{3}H]Folyloligoglutamyl product was isolated by adsorption onto dextran-coated charcoal (Sigma) for 10 min at 0 °C. The charcoal was washed extensively to remove unreacted glutamic acid, and the product was eluted from the charcoal with 60% ethanol containing 4 mM 2-ME and 3 M NH_4OH. After centrifugation at $1800 \times g$ for 10 min, the radioactivity of the supernatant was quantitated using a liquid scintillation counter. The production of polyglutamates is expressed as the amount (nmol) synthesized per 1 h of reaction per 1 mg protein of the IM-9 cell extract.

2.4. Dihydrofolate reductase inhibition study

According to the method of Duch et al. (1982), human dihydrofolate reductase was purified from IM-9 with a methotrexate-agarose column (Sigma), and the inhibitory effects of drugs on dihydrofolate reductase were determined.

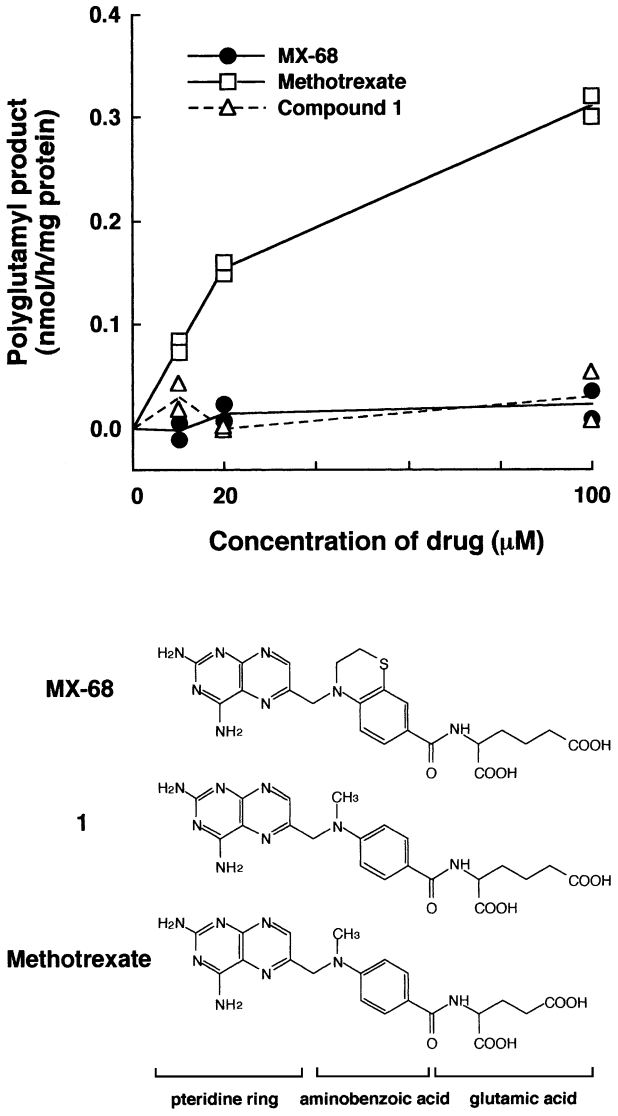

Fig. 1. Substrate activities of MX-68, methotrexate and Compound 1 for human folylpolyglutamate synthetase. Compounds were incubated with the extract of IM-9 cells as a source of human folylpolyglutamate synthetase and with 1 mM [^{3}H]glutamic acid for 2 h. The production of polyglutamates is expressed as the amount (nmol) synthesized per 1 h of reaction per 1 mg protein of the IM-9 cell extract. Each point represents individual data from an experiment performed in duplicate. Chemical structures of MX-68, methotrexate and Compound 1 are shown below the graph.

Table 1
Inhibition of dihydrofolate reductase by MX-68

Compound	IC_{50} (nM)
MX-68	2.05 ± 0.16
Methotrexate	4.47 ± 0.29
Methotrexate-tetraglutamate	2.08 ± 0.28

Inhibitory effects of compounds on human dihydrofolate reductase purified from IM-9 cells were determined. Numbers indicate the IC_{50} values and estimates of the standard error of each value.

In brief, the drug was incubated with dihydrofolate reductase at 32 °C in 100 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 0.2 mM NADPH and 0.07 mM dihydrofolate, in a total volume of 1.0 ml. The change in absorbance was measured at 340 nm. The IC_{50} value was calculated by the four-parameter logistic method using the computer software "GraFit" (Erithacus Software, London, UK). The software provided estimates of the standard error of each value calculated by the matrix inversion method.

2.5. Cell growth inhibition studies

Anti-proliferative effects of drugs were determined either by continuous drug treatment (48 h), or by short-term treatment (18 h) followed by reculture in drug-free medium.

In the first case, CCRF-CEM cells (1×10^4 cells/0.2 ml/well) were cultured with the drug at concentrations of 1–1000 nM in 96-well plates (Falcon, No.3072) for 48 h. [6-^{3}H]Deoxyuridine ([^{3}H]UdR; 37 kBq/well) (Amersham) was added for the last 5 h of culture, and proliferation was assessed by measuring [^{3}H]UdR uptake into the cells.

In the second case, the cells (1×10^6 cells/ml) were exposed to 1000 nM of the drug for 18 h, washed and resuspended in drug-free medium (5×10^5 cells/ml). Subsequent changes in cell number were monitored for a further 48 h.

2.6. Determination of intracellular drug and its polyglutamates

To examine the intracellular contents of drugs and their polyglutamates, CCRF-CEM cells were cultured with radiolabeled drugs.

Cells (1×10^6 cells/ml) were cultured with 1000 nM of [^{3}H]MX-68 or [^{3}H]methotrexate (6.66 GBq/mmol) for 1, 3, 6, or 18 h. Then the cells were washed with phosphate-buffered saline (PBS), and the radioactivity was quantitated as the sum of intracellular drug and metabolite levels, using a liquid scintillation counter.

The intracellular polyglutamation profile of the drug was analyzed according to the method of Krakower and Kamen (1983). CCRF-CEM cells (1×10^6 cells/ml) cultured with 1000 nM of [^{3}H]MX-68 or [^{3}H]methotrexate for 18 h were washed with PBS and suspended in 10 mM Tris–HCl buffer (pH 8.0) containing 5 mM EDTA and 150 mM 2-mercaptoethanol. To extract intracellular drug, the cells were sonicated for 15 min and boiled for 15 min. Cellular debris was removed by centrifugation at 9000 × *g* for 10 min. The drug in the supernatant was analyzed by HPLC on an A-312ODS column (YMC, Kyoto, Japan) with a linear 20–40% acetonitrile gradient in 5 mM PIC-A Reagent (Waters, Milford, MA, USA).

2.7. Animals

Male DBA/1J mice (9 weeks old) and male Lewis rats (6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animals were specific-pathogen free and were kept in cages in a room maintained at 24 ± 2 °C, 55 ± 10% relative humidity. Animals were treated humanely

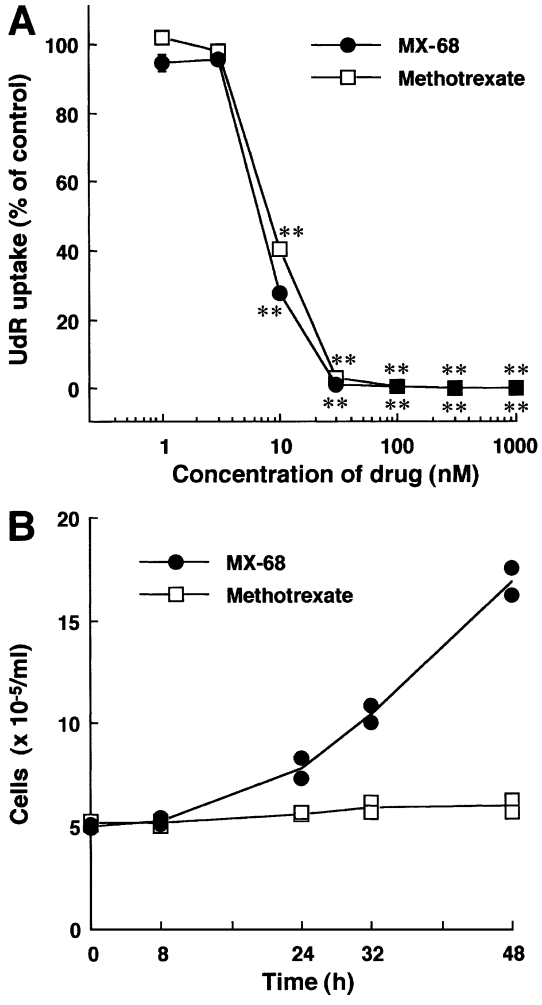

Fig. 2. Inhibitory effects of MX-68 and methotrexate on the growth of CCRF-CEM cells. (A) Continuous drug treatment: cells (1×10^4 cells/0.2 ml/well) were cultured with the drug for 48 h. [^{3}H]UdR was added for the last 5 h of culture. Inhibition is expressed as a percentage of [^{3}H]UdR uptake of control cultures. Each point represents the mean ± S.E. for triplicate cultures. $^{**}P < 0.01$ vs. vehicle control (Dunnett's multiple comparison test). (B) 18-h treatment followed by reculture in drug-free medium: cells (1×10^6 cells/ml) exposed to 1000 nM of the drug for 18 h were washed and resuspended in drug-free medium (5×10^5 cells/ml). Subsequent changes in cell number were monitored for 48 h. Each point represents individual data from an experiment performed in duplicate.

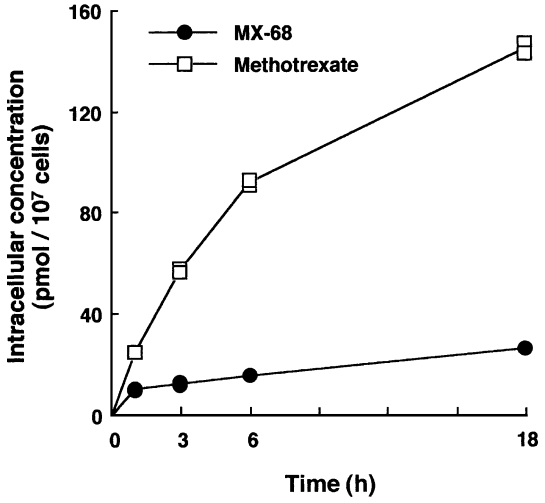

Fig. 3. Drug uptake by CCRF-CEM cells. Cells (1×10^6 cells/ml) were cultured with 1000 nM of [^{3}H]MX-68 or [^{3}H]methotrexate for 1, 3, 6, or 18 h. Then the cells were washed and the radioactivity was quantitated using a liquid scintillation counter. Each point represents individual data from an experiment performed in duplicate.

according to the guideline "General Consideration for Animal Experiments" established by the Experimental Animal Maintenance Committee of Chugai Pharmaceutical.

2.8. Evaluation of inhibitory activity against murine collagen-induced arthritis

Bovine type II collagen (Cosmo Bio, Tokyo, Japan) was dissolved overnight at 4 °C in 0.1 M acetic acid solution to a concentration of 4 mg/ml. Mice were immunized by intradermal injection at the body-tail junction with 0.2 mg of collagen emulsified with an equal volume of complete adjuvant H37 Ra (Difco Laboratories, Detroit, MI, USA). Three weeks later, the mice were boosted with collagen in the same manner. Drugs dissolved in PBS were orally administered three times a week for six weeks from the day of the first immunization with collagen.

The symptoms of arthritis in all four limbs were evaluated with a visual scoring system. Arthritic lesions of limbs were graded on a scale of 0 to 4: 0 (no change); 0.5 (swelling and erythema of one digit); 1.0 (swelling and erythema of two or more digits); 2.0 (mild swelling and erythema of the limb); 3.0 (gross swelling and erythema of the limb); and 4.0 (gross deformity and inability to use the limb). The arthritis score of each animal was the sum of the scores for the four limbs.

2.9. Evaluation of inhibitory activity against rat adjuvant-induced arthritis

Rats were injected at the body-tail junction with 50 μl of 0.7 mg/ml killed *Mycobacterium tuberculosis* H37 Ra (Difco Laboratories) in paraffin liquid. Drugs dissolved in PBS were orally administered five times a week for three weeks from the day of adjuvant injection. The symptoms of arthritis in all four limbs were evaluated with a visual scoring system, as described for the murine collagen-induced arthritis model.

2.10. Statistical analysis

For [^{3}H]UdR uptake data and arthritis scores, the statistical significance of differences was analyzed by Dunnett's test and Steel's test, respectively. Dose dependency was analyzed by Jonckheere's trend test.

3. Results

3.1. Activity as a substrate for folylpolyglutamate synthetase

MX-68 was tested as a substrate for human folylpolyglutamate synthetase, and no polyglutamate was produced

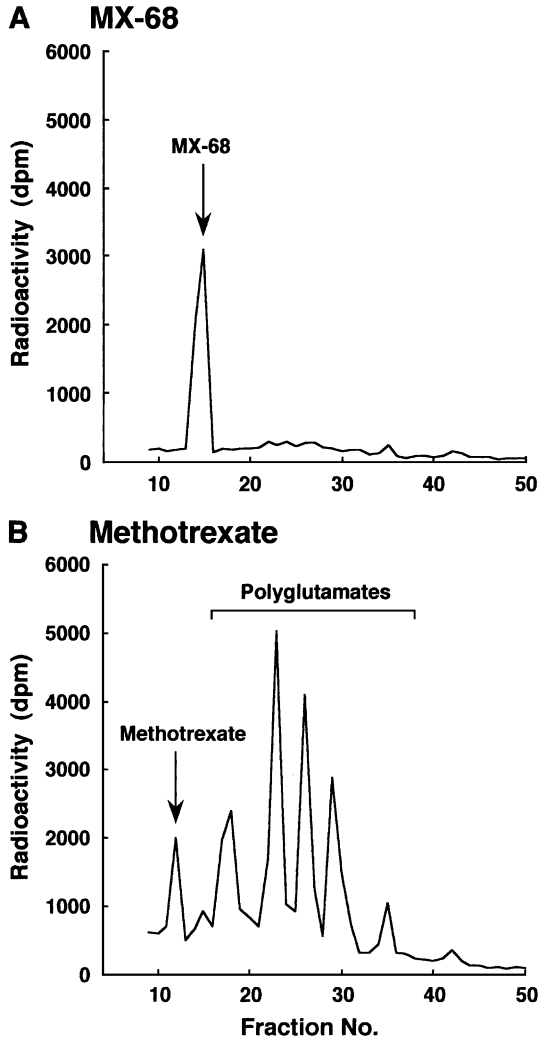

Fig. 4. Analysis of polyglutamate formation in CCRF-CEM cells. Cells were cultured with 1000 nM of [^{3}H]MX-68 (A) or [^{3}H]methotrexate (B) for 18 h, and then the polyglutamation profile of the drug in the extract of the cells was analyzed by HPLC. The experiment was repeated on three occasions, and representative results are shown.

from it. In contrast, methotrexate was utilized as a substrate (Fig. 1). Compound 1, which contained a homoglutamate residue like MX-68 but otherwise resembled methotrexate, was also not a substrate for folylpolyglutamate synthetase (Fig. 1). These results indicate that the homoglutamate residue in MX-68 prevented polyglutamation.

3.2. Inhibition of dihydrofolate reductase

Methotrexate showed an about two-fold stronger inhibition of human dihydrofolate reductase due to polyglutamate formation, with an IC_{50} value of 2.08 nM for methotrexate-tetraglutamate, compared to an IC_{50} value of 4.47 nM for methotrexate. The IC_{50} value for MX-68 was 2.05 nM (Table 1).

3.3. Anti-proliferative effect

Incubation with both MX-68 and methotrexate inhibited the proliferation of CCRF-CEM at concentrations of more than 10 nM, and completely suppressed it at concentrations of more than 100 nM, when cells were continuously exposed to the drug for 48 h (Fig. 2A). Removal of the drug from the culture medium at 18 h demonstrated that the anti-proliferative effect of MX-68 was reversible. In contrast, removal of methotrexate from such cultures did not reverse its inhibitory effect (Fig. 2B).

3.4. Uptake and polyglutamate formation

CCRF-CEM cells were cultured with 1000 nM of [^{3}H]MX-68 or [^{3}H]methotrexate, and the sum of intracellular drug and metabolite levels was determined. The intracellular concentration of MX-68 was much lower than the sum of the levels of methotrexate and its polyglutamate (Fig. 3). After an 18-h culture, the polyglutamation profile of the drug in the extract of the cells was analyzed by HPLC. Although a peak corresponding to MX-68 was detected, no peaks corresponding to polyglutamated MX-68 were detected. In contrast, with methotrexate, there were peaks corresponding to both methotrexate and polyglutamated methotrexate such as methotrexate-monoglutamate, -diglutamate, etc. (Fig. 4).

3.5. Inhibitory activity against murine collagen-induced arthritis

MX-68 and methotrexate were orally administered three times a week for six weeks from the day of the first im-

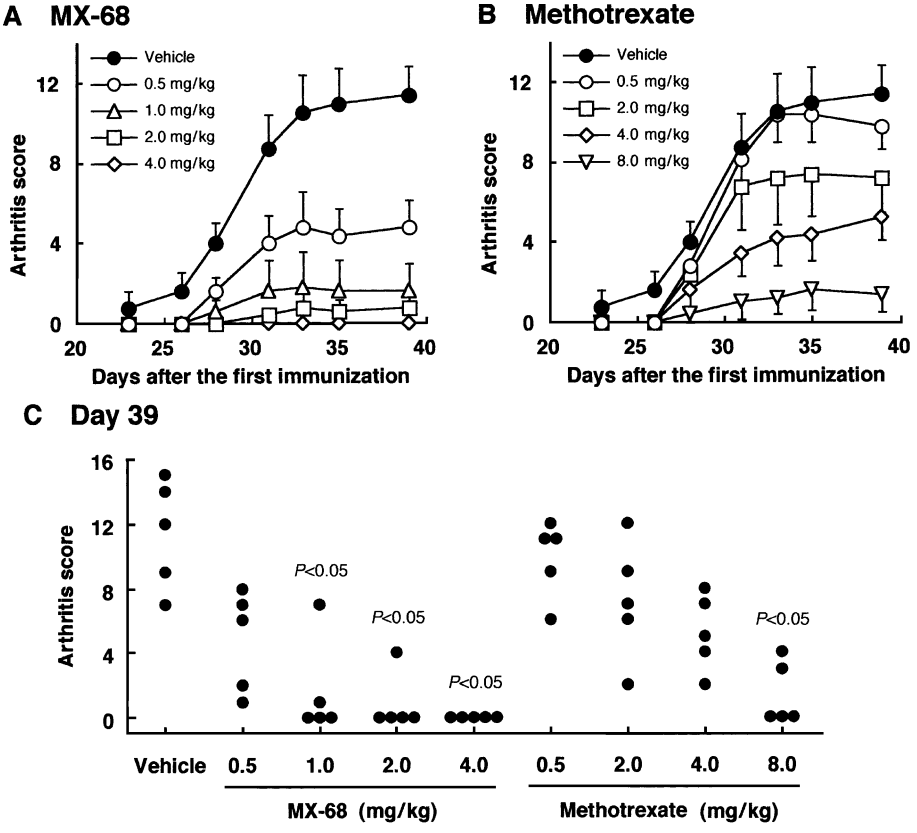

Fig. 5. Suppressive effects of MX-68 and methotrexate on collagen-induced arthritis in mice. MX-68 and methotrexate were orally administered three times a week for six weeks from the day of the first immunization with collagen. (A, B) Time course of arthritis development in MX-68- and methotrexate-treated mice: each point represents the mean ± S.E. (error bar) arthritis scores (five mice per group). (C) Arthritis score on Day 39: each point represents the score of individual mice. The significance of the difference between the scores of the control and treatment groups on the day when the score of control group reached a maximum (Day 39) was analyzed by Steel's test.

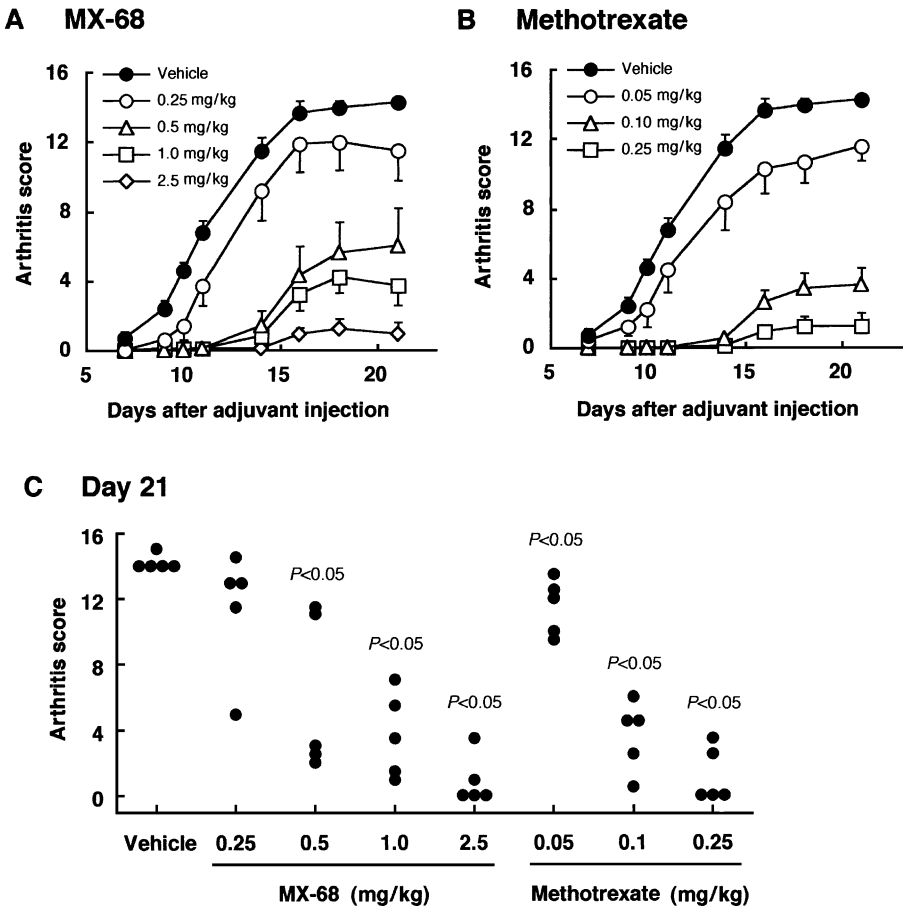

Fig. 6. Suppressive effects of MX-68 and methotrexate on adjuvant-induced arthritis in rats. MX-68 and methotrexate were orally administered five times a week for three weeks from the day of adjuvant injection. (A, B) Time course of arthritis development in MX-68- and methotrexate-treated rats: each point represents the mean ± S.E. (error bar) arthritis scores (five rats per group). (C) Arthritis score on Day 21: each point represents the score of individual rats. The significance of the difference between the scores of the control and treatment groups on the day when the score of the control group reached a maximum (Day 21) was analyzed by Steel's test.

munization with collagen (Day 0). The arthritis score of the vehicle-treated group increased gradually and reached a maximum on Day 39. The efficacy of the drug was statistically evaluated using the arthritis score for each group on Day 39. MX-68 significantly suppressed the development of arthritis in a dose-dependent manner, causing complete suppression at 4.0 mg/kg (Fig. 5).

3.6. Inhibitory activity against rat adjuvant-induced arthritis

MX-68 and methotrexate were orally administered five times a week for three weeks from the day of adjuvant injection (Day 0). The arthritis score of the vehicle-treated group increased gradually and reached a maximum on Day 21. The efficacy of the drug was statistically evaluated using the arthritis score for each group on Day 21. MX-68 significantly suppressed the development of adjuvant-induced arthritis in a dose-dependent manner (Fig. 6).

4. Discussion

In the present study, we showed that MX-68 did not undergo polyglutamation, potently inhibited dihydrofolate reductase activity and cell proliferation, and suppressed the development of collagen-induced arthritis in mice and adjuvant-induced arthritis in rats, in a similar manner to methotrexate. These results indicate that non-polyglutamatable antifolate can also express its efficacy in vivo, and that polyglutamation is not essential for the anti-arthritic effect of antifolates.

MX-68 was not utilized as a substrate by human folylpolyglutamate synthetase in the enzyme assay. This was supported by the fact that polyglutamated MX-68 was not detected in the extract of CCRF-CEM cultured with MX-68. These results indicate that the molecular design of MX-68 was correct, where the glutamate residue of methotrexate was substituted with a homoglutamate residue, based on the report that folylpolyglutamate synthetase catalyzed the formation of polyglutamates at the γ position of the glutamate

residue of methotrexate (Galivan, 1980). Furthermore, this is also supported by the result that no polyglutamate was produced from Compound 1, which was different from methotrexate only in the glutamate residue.

In order to enhance the pharmacological efficacy of MX-68, the compound was designed to have increased affinity for dihydrofolate reductase through modification of the aminobenzoic acid moiety, based on Oefner's binding model between methotrexate and dihydrofolate reductase (Oefner et al., 1988). We speculated that filling a hydrophobic open space between the dihydrofolate reductase and the aminobenzoic acid moiety of methotrexate with a fixed hydrophobic substituent would cause tighter and energetically more favorable binding to dihydrofolate reductase (Matsuoka et al., 1997b). In fact, the inhibitory activity of MX-68 on human dihydrofolate reductase was about twice as potent as that of methotrexate, and comparable to that of methotrexate-tetraglutamate. Probably as a result of this activity, MX-68 treatment exerted a similar anti-proliferative efficacy to that of methotrexate treatment in vitro (Fig. 2A). Not only measurement of [^{3}H]UdR uptake but also MTT assay demonstrated the effects of MX-68 (unpublished data). Moreover, we have confirmed that the inhibitory effects of MX-68 on the proliferation of peripheral blood mononuclear cells from healthy volunteers, human endothelial cells from the umbilical vein, and synovial fibroblastic cells from patients with rheumatoid arthritis were almost identical to those of methotrexate (Mihara et al., 1996).

Several investigators have suggested that the inhibition of dihydrofolate reductase might not be essential for the efficacy of methotrexate in rheumatoid arthritis, and that the inhibition of AICAR transformylase is important (Cronstein et al., 1993; Baggott et al., 1993). It was reported that the inhibitory activity of methotrexate-tetraglutamate on human AICAR transformylase was 2500-fold more potent than that of methotrexate (Chabner et al., 1985); however, MX-68 had the same weak potency to inhibit human AICAR transformylase as methotrexate (manuscript in preparation). Therefore, the fact that MX-68 markedly suppressed the development of arthritis in animal models strongly suggests that the inhibitory activity on dihydrofolate reductase is predominant for the anti-arthritic effects of antifolates.

In the present study, methotrexate suppressed the development of arthritis in rats at a much lower dose than it did in mice, although MX-68 showed anti-arthritic effects in rats at a similar dose (per body weight) as in mice. One reason may be that methotrexate is a better substrate for rat folylpolyglutamate synthetase than for the mouse equivalent (Moran et al., 1985). These results suggest that polyglutamation intensifies the effect of methotrexate. Having undergone polyglutamation, a large quantity of methotrexate was stored in cells (Fig. 3) and sustained its effects even after its disappearance from the extracellular space (Fig. 2B). These are probably the causes of the potent in vivo efficacy of methotrexate.

However, the intracellular accumulation of polyglutamates is postulated to cause the adverse effects of methotrexate (Ahern et al., 1991; Kamen et al., 1981). Long-term methotrexate therapy is associated with some serious adverse effects such as hepatic dysfunction and bone marrow suppression, and so toxicity rather than lack of efficacy appears to be the major factor limiting its clinical use (Alarcon et al., 1989). Although we could not compare the toxicity of the two drugs, because the drugs did not appear to be toxic during the experimental period, MX-68 is expected to have fewer adverse effects related to polyglutamation. Moreover, since MX-68 rapidly leaves cells, the discontinuation of administration is expected to result in the drug washing out of the body without delay when an adverse effect occurs. Therefore, MX-68 might be safer than methotrexate and can be administered daily to patients with rheumatoid arthritis, if necessary.

There are some patients who tolerate methotrexate, and several mechanisms of resistance have been considered. Impairment of methotrexate-polyglutamate formation is one of them (Koizumi and Allegra, 1992), and MX-68 does not need the process of polyglutamation for its anti-arthritic effect. Therefore, MX-68 may be efficacious in a subgroup of methotrexate-resistant patients.

In summary, MX-68 is a unique antifolate that does not form polyglutamates. The efficacy of MX-68 proves that polyglutamation is not essential for the anti-arthritic effect of antifolates. Moreover, MX-68 is expected to be a superior anti-rheumatic drug with less toxicity.

References

Ahern, M.J., Kevat, S., Hill, W., Hayball, P.J., Harley, H., Hall, P., 1991. Hepatic methotrexate content and progression of hepatic fibrosis: preliminary findings. Ann. Rheum. Dis. 50, 477–480.

Alarcon, G.S., Tracy, I.C., Blackburn Jr., W.D., 1989. Methotrexate in rheumatoid arthritis. Toxic effects as the major factor in limiting long-term treatment. Arthritis Rheum. 32, 671–676.

Baggott, J.E., Morgan, S.L., Ha, T.-S., Alarcon, G.S., Koopman, W.J., Krumdieck, C.L., 1993. Antifolates in rheumatoid arthritis: a hypothetical mechanism of action. Clin. Exp. Rheumatol. 11 (Suppl. 8), S101–S105.

Baugh, C.M., Krumdieck, C.L., Nair, M.G., 1973. Polygammaglutamyl metabolites of methotrexate. Biochem. Biophys. Res. Commun. 52, 27–34.

Chabner, B.A., Allegra, C.J., Curt, G.A., Clendeninn, N.J., Baram, J., Koizumi, S., Drake, J.C., Jolivet, J., 1985. Polyglutamation of methotrexate. Is methotrexate a prodrug? J. Clin. Invest. 76, 907–912.

Cronstein, B.N., Naime, D., Ostad, E., 1993. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. J. Clin. Invest. 92, 2675–2682.

Duch, D.S., Edelstein, M.P., Bowers, S.W., Nichol, C.A., 1982. Biochemical and chemotherapeutic studies on 2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-d]pyrimidine (BW 301U), a novel lipid-soluble inhibitor of dihydrofolate reductase. Cancer Res. 42, 3987–3994.

Galivan, J., 1980. Evidence for the cytotoxic activity of polyglutamate derivatives of methotrexate. Mol. Pharmacol. 17, 105–110.

Jansen, G., Schornagel, J.H., Kathmann, I., Westerhof, G.R., Hordijk, G.J., Van der Laan, B.F.A.M., 1992. Measurement of folylpolyglutamate synthetase activity in head and neck squamous carcinoma cell lines and clinical samples using a new rapid separation procedure. Oncol. Res. 4, 299–305.

Kamen, B.A., Nylen, P.A., Camitta, B.M., Bertino, J.R., 1981. Methotrexate accumulation and folate depletion in cells as a possible mechanism of chronic toxicity to the drug. Br. J. Haematol. 49, 355–360.

Koizumi, S., Allegra, C.J., 1992. Enzyme studies of methotrexate-resistant human leukemic cell (K562) subclones. Leuk. Res. 16, 565–569.

Krakower, G.R., Kamen, B.A., 1983. In situ methotrexate polyglutamate formation in rat tissues. J. Pharmacol. Exp. Ther. 227, 633–638.

Kumar, P., Kisliuk, R.L., Gaumont, Y., Freisheim, J.H., Nair, M.G., 1989. Inhibition of human dihydrofolate reductase by antifolyl polyglutamates. Biochem. Pharmacol. 38, 541–543.

Matsuoka, H., Kuroki, T., Yano, K., Takeda, Y., 1997a. Synthesis of tritiated *N*-[4-(2,4-diaminopteridine-6-methyl)-3,4-dihydro-2*H*-1,4-benzothiazine-7-carbonyl]-L-homoglutamic acid. J. Labelled Compd. Radiopharm. 39, 363–368.

Matsuoka, H., Ohi, N., Mihara, M., Suzuki, H., Miyamoto, K., Maruyama, N., Tsuji, K., Kato, N., Akimoto, T., Takeda, Y., Yano, K., Kuroki, T., 1997b. Antirheumatic agents: novel methotrexate derivatives bearing a benzoxazine or benzothiazine moiety. J. Med. Chem. 40, 105–111.

Mihara, M., Urakawa, K., Takagi, N., Moriya, Y., Takeda, Y., 1996. In vitro and in vivo biological activities of a novel nonpolyglutamable antifolate, MX-68. Immunopharmacology 35, 41–46.

Moran, R.G., Colman, P.D., Rosowsky, A., Forsch, R.A., Chan, K.K., 1985. Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. Mol. Pharmacol. 27, 156–166.

Oefner, C., D'arcy, A., Winkler, F.K., 1988. Crystal structure of human dihydrofolate reductase complexed with folate. Eur. J. Biochem. 174, 377–385.

Weinblatt, M.E., Coblyn, J.S., Fox, D.A., Fraser, P.A., Holdsworth, D.E., Glass, D.N., Trentham, D.E., 1985. Efficacy of low-dose methotrexate in rheumatoid arthritis. N. Engl. J. Med. 312, 818–822.

Weyand, C.M., Goronzy, J.J., 1997. The molecular basis of rheumatoid arthritis. J. Mol. Med. 75, 772–785.

Williams, H.J., Willkens, R.F., Samuelson Jr., C.O., Alarcon, G.S., Guttadauria, M., Yarboro, C., Polisson, R.P., Weiner, S.R., Luggen, M.E., Billingsley, L.M., Dahl, S.L., Egger, M.J., Reading, J.C., Ward, J.R., 1985. Comparison of low-dose oral pulse methotrexate and placebo in the treatment of rheumatoid arthritis. A controlled clinical trial. Arthritis Rheum. 28, 721–730.