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ORIGINAL ARTICLE

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A comparison of the effects of nine folate analogs on early and late murine hematopoietic progenitor cells in vitro

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Abstract *Purpose*: Since the clinical introduction of the antifolates aminopterin (AMT) and methotrexate (MTX) many promising analogs have been developed. A common feature of these compounds is their ability to induce bone marrow suppression. However, few studies have been undertaken on the effect of the folic acid analogs on the cells comprising the hematopoietic system. *Methods*: In this paper we describe the effects of the novel thymidylate synthase (TS) inhibitors raltitrexed (Tomudex, ZD1694), AG337 (nolatrexed, Thymitaq), and the two closely related analogs 5,8-dideazaisofolic acid (IAHQ2a) and 2-desamino-2-methyl 5,8-dideazaisofolic acid (IAHQ2c), the glycinamide-ribonucleosyl (GAR) transformylase inhibitor lometrexol (DDATHF), and the dihydrofolate reductase (DHFR) inhibitors MTX, AMT, trimetrexate (TMTX), and edatrexate (EDX) on purified populations of early and late murine hematopoietic progenitor cells. *Results/Conclusion*: All the antifolates inhibited bone marrow proliferation in suspension cultures and all drugs except DDATHF inhibited colony formation by more mature progenitor cells (CFU-C) in clonogenic assays. The lipophilic agents TMTX and AG337 were most toxic, totally abolishing CFU-C colony formation at high concentrations. When IAHQ2c, raltitrexed, DDATHF, and MTX were investigated further for effects on the immature high proliferative potential colony-forming cells (HPP-CFCs) in semisolid and limiting dilution cultures, none of these agents were found to be toxic to the HPP-CFC, but induced a reversible developmental arrest in the progenitor cell population.

Key words Antifolates · Colony-forming units in culture · High proliferative potential colony-forming cell

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Introduction

The rationale behind the synthesis of novel antifolates has been to develop agents that act more selectively against tumor cells and to overcome the problems associated with drug resistance. These new compounds differ with respect to target enzymes, mode of transport into cells, and/or intracellular metabolism [4].

Antifolates induce perturbations in intracellular folate metabolism, ultimately resulting in inhibition of DNA synthesis [14]. The drugs methotrexate (MTX), aminopterin (AMT), trimetrexate (TMTX), and edatrexate (EDX), all inhibit dihydrofolate reductase (DHFR) [4]. This enzyme is responsible for the conversion of inactive oxidized folates to reduced folates, which are required for synthesis of thymidylate and purines. MTX, AMT, and EDX use the reduced folate carrier (RFC) to enter cells. It has been shown that EDX is a better substrate for the RFC than MTX [28]. These drugs are all polyglutamated intracellularly, the EDX polyglutamates being better DHFR inhibitors than the MTX polyglutamates [13]. TMTX, by contrast, enters cells by passive diffusion and is not polyglutamated [4]. Polyglutamation is considered to be important for the intracellular retention of folates/antifolates. Despite the fact that TMTX is not polyglutamated, it is retained intracellularly for prolonged periods of time. The mechanism behind this retention is unknown.

Another antifolate target is thymidylate synthase (TS), which is responsible for the production of dihydrofolates and the synthesis of thymidylate [13]. The folic acid analogs 5,8-dideazaisofolic acid (IAHQ2a), 2-desamino-2-methyl-5,8-dideazaisofolic acid (IAHQ2c), raltitrexed (Tomudex, ZD1694), and AG337 (nolatrexed, Thymitaq), all inhibit this enzyme [28, 6, 32]. Raltitrexed uses the RFC to enter cells and is polyglutamated [10, 11]. AG337 was synthesized to exhibit a different pharmacological profile than raltitrexed [1] and this drug is lipophilic and lacks the ability to be polyglutamated [32]. In contrast to the lipophilic

TMTX, AG337 rapidly diffuses out of cells if the extracellular concentration decrease. The two closely related analogs IAHQ2a and IAHQ2c were also used in our study. IAHQ2a has a low affinity for the RFC and a slow influx into target cells [27] and very large doses have been required to achieve therapeutic effectiveness in animal models [3, 7, 8, 15, 30]. The 2-desamino-2-methyl analog IAHQ2c was therefore developed, which has a better affinity for the RFC and is more extensively polyglutamated than the parent compound [6, 9].

Lometrexol (DDATHF) was the first clinically investigated antifolate which inhibits glycinamide-ribonucleosyl (GAR) transformylase [17], an enzyme involved in the de novo synthesis of purines. The drug binds the RFC but also has high affinity towards the membrane folate receptors (MFR) [2, 12]. DDATHF is efficiently polyglutamated and polyglutamation is essential for expression of GAR transformylase-inhibitory activity.

Hematopoiesis involves the continuous production of large populations of mature blood cells from a small population of hematopoietic stem cells (HSC) present in the bone marrow [16]. The progeny of the HSC are progenitor cells, characterized by increasing lineage-restricted differentiation or commitment. The high proliferative potential colony-forming cell (HPP-CFC) is an immature progenitor cell, which can be detected in the semisolid clonogenic assay. It has multilineage potential and responds to a certain combination of cytokines. The more mature progenitor cells CFU-C (colony-forming units in culture) have a certain degree of lineage commitment and give rise to colonies of granulocytes and macrophages when stimulated with recombinant murine granulocyte-macrophage colony stimulating factor (rmGM-CSF). The early and late bone marrow progenitor cells are central in the short-term and long-term repopulation of the hematopoietic system after injury, and a toxic effect on the progenitor cells often results in significant morbidity or mortality in patients receiving chemotherapy. In the present study we have used purified bone marrow cell populations highly enriched in early (HPP-CFC) and late (CFU-C) bone marrow progenitor cells to delineate the effects of these antifolates on immature cells in the bone marrow.

Materials and methods

Mice/tissue culture reagents

Female Balb/c mice were purchased form Harlan Olac (Oxon, UK). The animals were obtained at 6–8 weeks old and maintained in conventional facilities. IMDM (Iscove's Modified Dulbecco's Medium) was obtained as powdered media from Gibco, Life Technologies (Paisley, Scotland). The medium was supplemented with sodium bicarbonate (3.024 g/l), monothioglycerol (75 μM), and gentamycin (50 μg/ml) prior to use. A single lot of fetal bovine serum (FBS) from Biological Industries (Kibbutz Beth Haernek, Israel) was used throughout the study. MTX, leucovorin (LV), and 5-fluorouracil (5FU) were obtained from Nycomed Pharma (Oslo, Norway). AMT and hypoxanthine/thymidine (HT) were from Sigma Chemicals (St. Louis, Mo., USA), TMTX was a gift from US Bioscience (West Conshohocken, Pa., USA), EDX was obtained from Ciba-Geigy (Basle, Switzerland), DDATHF was from Lilly Research Laboratories (Indianapolis, Ind., USA), raltitrexed (Tomudex, ZD1694) from Zeneca Pharmaceuticals (Macclesfield, UK), and AG337 (nolatrexed, Thymitaq) was a gift from Agouron Pharmaceuticals (La Jolla, Calif., USA). IAHQ2a and IAHQ2c were provided by Prof. John B. Hynes, Medical University of South Carolina, Charleston, S.C., USA.

Growth factors

Recombinant murine granulocyte-macrophage colony stimulating factor (rmGM-CSF) was obtained from Pepro Tech (Rocky Hill, N.J., USA). Recombinant murine interleukin-3 (rmIL-3) was expressed using a baculovirus expression system and purified by anion-exchange, gel filtration, and reversed-phase chromatography. Conditioned medium (CM) from the human bladder carcinoma cell line 5637 (5637 CM) was used as a source of crude stimulating activity and L929 cell conditioned medium (L929 CM) as a source of colony stimulating factor-1 (CSF-1). The growth factors and conditioned media were pretitrated and used at concentrations supporting maximum (plateau) levels of colony formation.

Drug treatment

MTX, AMT, TMTX, EDX, DDATHF, raltitrexed, AG337, IAHQ2a, IAHQ2c, LV, and HT were diluted in phosphate-buffered NaCl (PBS) without Ca^{2+}/Mg^{2+} and added directly to culture dishes in a volume of 10 μl in the CFU-C and HPP-CFC colony assays. In Terasaki culture rescue experiments the cells were seeded in 10 μl medium per well. LV was then added in 10 μl of medium on day 2 of culture. In the XTT proliferation assay performed in 96-well microplates, the drugs were added in 10 μl of medium (final volume 100 μl/well).

XTT proliferation assay

Determination of cellular proliferation was undertaken using the Cell Proliferation Kit II (XTT; Boehringer Mannheim, Mannheim, Germany). The assay is based on the ability of mitochondrial dehydrogenase to metabolize the tetrazolium salt XTT to a formazan dye, whose absorbance can be measured at 450 nm. Cells (1×10^6/ml) were grown in medium supplemented with 10% FBS and rmGM-CSF (20 ng/ml) in 96-well microplates (final volume 100 μl/well). The cells were incubated overnight prior to drug addition (10 μl/well). The cells were subsequently incubated for another 3 days before cell proliferation was assessed.

Colony-forming units in culture assay

Hematopoietic progenitors (CFU-C) were assayed in semisolid agarose cultures. Bone marrow cells were obtained by flushing mouse femurs with IMDM with 10% FBS. 1.5×10^4 fresh or precultured bone marrow cells in 1 ml of IMDM supplemented with 10% FBS, rmGM-CSF (20 ng/ml), and 0.36% agarose (SeaPlaque, Rockland, Me., USA) were plated into 35-mm Petri dishes. After incubation for 7 days at 37 °C in a humidified atmosphere with 5% O_2, 5% CO_2, balance N_2, cultures were scored for colonies (> 50 cells) using a dissecting microscope.

High proliferative potential colony-forming cell assay

HPP-CFCs were assayed in double-layer semisolid agarose cultures in 35-mm Petri dishes. The underlayer contained 1 ml IMDM supplemented with 10% FBS, 7.5% 5637 CM, 15% L929 CM, and 150 ng/ml rmIL-3; 1500 4d5FU Lin^- cells (see below) were added to a 0.5 ml overlayer in IMDM with 10% FBS. The final concentrations of agarose were 0.5% in the underlayer and 0.3% in the

overlayer. After incubation for 14 days at 37 °C in a humidified atmosphere with 5% O_2, 5% CO_2, balance N_2, cultures were scored for colonies (>0.5 mm) using a dissecting microscope. In rescue experiments cultures were incubated for an additional 14 days after addition of the rescue agent.

Enrichment of bone marrow progenitors

5FU (150 mg/kg) was injected into the tail vein of mice. Four days later, the mice were killed and the femoral bone marrow collected. This cell population (4d5FU cells) was then further enriched for immature cells. The bone marrow cells were washed in PBS containing 2% heat-inactivated FBS before incubation at 4 °C for 30 min in a cocktail of antibodies directed against markers on mature cells of the myeloid and lymphoid cell lineages: M1/70.15.11.5.HL (MAC-1 antigen), 53–6.72 (CD8 antigen), GK1.5 (CD4 antigen) and 14.8 (B-cell antigen), each of which were purified from mouse ascitic fluid by protein G chromatography, RB6–8C5 (Gr-1 antigen), which was obtained from Pharmin Gen (San Diego, Calif., USA), and Terr119 (erythrocytes), which was a kind gift from Dr. Tatsuo Kina (Kyoto University, Kyoto, Japan). The cells were then washed three times, and prewashed sheep anti-mouse IgG-coated immunomagnetic beads (Dynal, Oslo, Norway) were added at a cell/bead ratio of 1:2 and incubated at 4 °C for 30 min. Labeled (i.e., Lin^+) cells were removed by a magnetic particle concentrator. A second aliquot of anti-mouse IgG-coated beads was added to the unlabeled cells and the mixture incubated at 4 °C for 30 min. The Lin^+ cells were again removed and the unlabeled cells (4d5FU Lin^- cells) collected.

Microwell clonogenic assay/statistics

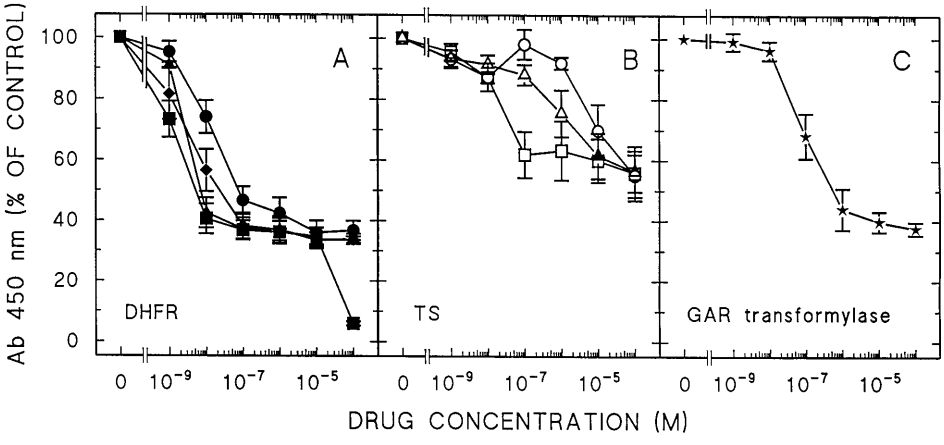
In the microwell clonogenic assay, five 4d5FU Lin^- cells (corresponding to 0.3 progenitor cells/well) were seeded per well in Terasaki plates in 10 µl IMDM medium supplemented with 10% FBS and 5637 CM, L929 CM, and rmIL-3 ± antifolates. On day 2 of culture PBS, LV (final concentration = 1 m*M*) or HT (final concentration of hypoxanthine = 100 µ*M* and thymidine = 16 µ*M*) were added to the cultures, giving a final volume of 20 µl. Colonies were counted after 14 days of incubation. During counting we discriminated between large colonies completely covering the wells (HPP-CFC derived colonies) and small colonies (non-confluent wells), probably deriving from more mature progenitor cells. When appropriate, statistical significance between treatment groups was assessed by one-way analysis of variance (ANOVA) and a multiple comparison procedure based on the Dunnett's test.

Results

The effect of the different antifolates on the proliferation of unfractionated murine bone marrow cells was measured by the XTT proliferation assay. The TS inhibitors IAHQ2a, IAHQ2c, and raltitrexed were the least potent inhibitors of proliferation, inducing significant inhibition at 10 µ*M* (30%), 1 µ*M* (25%), and 100 n*M* (38%), respectively (Fig. 1B). The TS inhibitors did not induce more than 45% inhibition at the highest drug concentration (100 µ*M*). The GAR transformylase inhibitor DDATHF induced significant inhibition at 100 n*M* (32%), and 62% at the highest drug concentration (Fig. 1C). The most potent inhibitors were MTX, AMT, TMTX, and EDX, which all bind DHFR (Fig. 1A). Significant inhibition of proliferation was observed at 10 n*M* of MTX and AMT, inducing 26% and 58% inhibition, respectively, and at 1 n*M* EDX and TMTX, inducing 19% and 27% inhibition, respectively. TMTX induced 94% inhibition at 100 µ*M*, while the other agents did not induce more than 66% inhibition at this high drug concentration (Fig. 1A).

The effect of the antifolates on the clonogenicity of murine CFU-C was assayed in semisolid agarose cultures (Fig. 2). Bone marrow cells (1.5×10^4/culture) were incubated in rmGM-CSF-supplemented cultures in the presence or absence of antifolates. In this assay the GAR transformylase inhibitor DDATHF did not induce significant inhibition of colony formation (Fig. 2C). The TS inhibitors IAHQ2a (10 µ*M*), IAHQ2c (1 µ*M*), AG337 (1 µ*M*), and raltitrexed (100 n*M*) induced 30%, 39%, 67%, and 74% inhibition, respectively (Fig. 2B). AG337 totally abolished colony formation at

Fig. 1 Effect of different antifolates on proliferation of murine bone marrow cells measured in the XTT proliferation assay. Bone marrow cells (1×10^6/ml) were incubated for 3 days in 100-µl liquid cultures containing rmGM-CSF (20 ng/ml) and (**A**) the dihydrofolate reductase inhibitors methotrexate (*MTX*; ●), aminopterin (*AMT*; ▲), edatrexate (*EDX*; ◆), or trimetrexate (*TMTX*; ■), (**B**) the thymidylate synthase inhibitors IAHQ2a (○), IAHQ2c (△), or raltitrexed (□), or (**C**) the glycinamide-ribonucleosyl transformylase inhibitor lometrexol (DDATHF) (*). The cells were preincubated overnight prior to the addition of each of the drugs. After 3 days of incubation cellular proliferation was determined by the XTT proliferation kit. Each data point represents the mean ± SE of three separate experiments. Some of the SE bars are hidden by the symbols. *SE* standard error of the mean



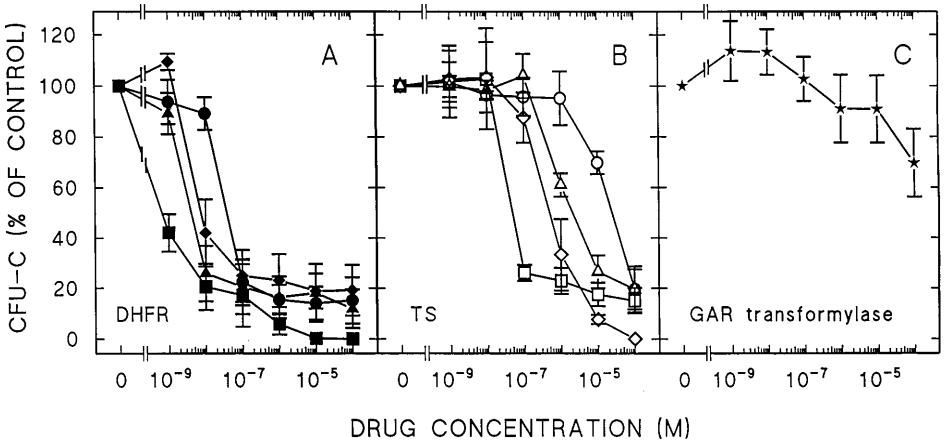


Fig. 2 Effect of different antifolates on the clonogenicity of murine colony-forming units in culture. Bone marrow cells (1.5×10^4) were plated in 35-mm Petri dishes in 1-ml semisolid agarose cultures containing rmGM-CSF and different concentrations of each of the drugs: **A** the dihydrofolate reductase inhibitors methotrexate (*MTX*; ●), aminopterin (*AMT*; ▲), edatrexate (*EDX*; ◆), or trimetrexate (*TMTX*; ■), **B** the thymidylate synthase inhibitors IAHQ2a (○), IAHQ2c (△), raltitrexed (□), or AG337 (◇), **C** the glycinamide-ribonucleosyl transformylase inhibitor lometrexol (DDATHF) (*). Colonies were scored after 7 days incubation. Each data point represents the mean ± SE of three separate experiments. Some of the SE bars are hidden by the symbols. *SE* standard error of the mean

100 μ*M*, while the other TS inhibitors did not induce more than 85% inhibition. Again the DHFR inhibitors were most potent, significant inhibition being observed with 100 n*M* MTX, 10 n*M* EDX and AMT, and 1 n*M* TMTX inducing 78%, 75%, 80%, and 83% inhibition, respectively (Fig. 2A). TMTX totally abolished colony formation at the highest drug concentration, while the other DHFR inhibitors did not induce more than 88% inhibition.

The effects of MTX, IAHQ2c, raltitrexed, and DDATHF on immature progenitor cells were investigated in the HPP-CFC colony assay using 4d5FU Lin$^-$ cells (1500/culture). The effect on colony formation with 1 μ*M* and 100 μ*M* of each of the antifolates was only evident as reduced size of formed colonies (Fig. 3). The colonies in the DDATHF-treated cultures were slightly larger than with the other drugs. When LV or HT was added to the antifolate-treated cultures at day 2, the colonies subsequently formed were of the same size as in control cultures (Fig. 4).

The direct effects of MTX, IAHQ2c, raltitrexed, and DDATHF on HPP-CFC were investigated in microwell clonogenic cultures. Limiting dilution experiments were first performed to quantify the cloning efficiency of the 4d5FU and 4d5FU Lin$^-$ cells. Clonogenic cells stimulated by 5637 CM, L929 CM, and rmIL-3 were detected in a ratio of about 1.5% and 7% for the two cell populations, respectively. To assure that the assay was indeed clonogenic, we seeded five 4d5FU Lin$^-$ cells/well (corresponding to 0.3 clonogenic cells/well). Colonies were counted after 14 days of incubation. When counting colonies, we discriminated between large colonies derived from HPP-CFC (confluent wells) and small colonies (non-confluent wells, >10 cells). In control cultures almost all colonies were of the HPP-CFC type (Table 1).

At a concentration of 1 μ*M*, all the drugs induced a switch towards formation of only small colonies. The addition of LV or HT at day 2 of culture again resulted in an increase in the number of large colonies and a decrease in the number of small colonies in the antifolate-treated cultures when compared with unrescued cultures.

Discussion

The cytotoxic effects of MTX on human and murine bone marrow cells have been studied in in vitro cultures

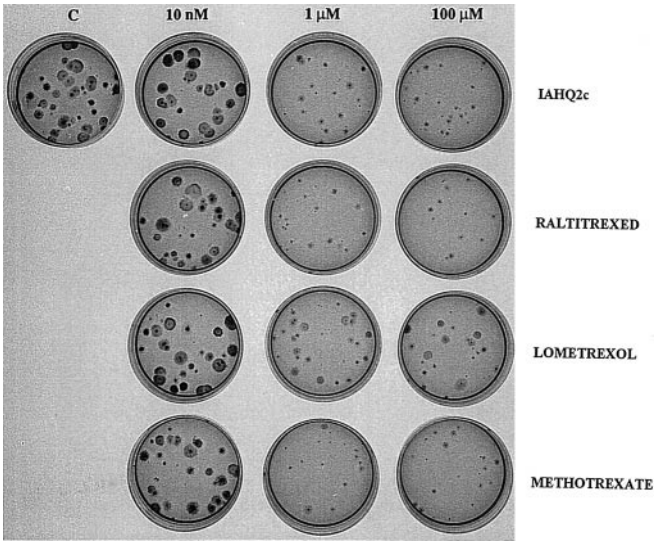


Fig. 3 The effect of methotrexate (*MTX*), IAHQ2c, raltitrexed, and lometrexol on murine high proliferative colony-forming cells. 1.5×10^3 4d5FU Lin$^-$ cells were incubated in 35-mm Petri dishes in the presence of PBS (C = control) or 10 n*M*, 1 μ*M*, or 100 μ*M* MTX, IAHQ2c, raltitrexed or lometrexol (DDATHF) in 1.5-ml double-layered semisolid agarose cultures containing 5637 CM, L929 CM, and rmIL-3. After 14 days of incubation the colonies were stained overnight with *p*-iodonitrotetrazolium violet

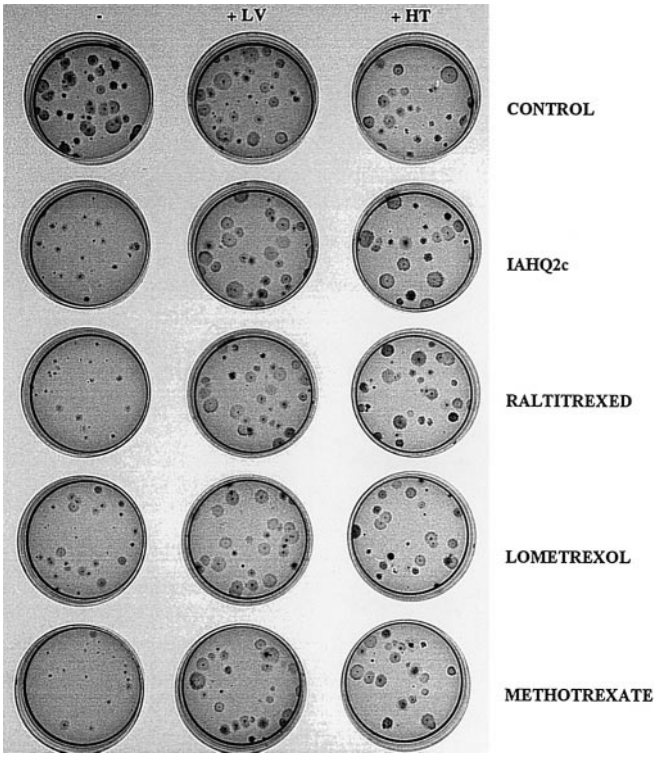


Fig. 4 The effect of delayed leucovorn (*LV*) or hypoxanthine plus thymidine (*HT*) rescue on HPP-CFC colony formation in methotrexate- (*MTX*), IAHQ2c-, raltitrexed-, and lometrexol (DDATHF)-treated cultures. 1.5×10^3 4d5FU Lin$^-$ cells were incubated in 35-mm Petri dishes in the presence of PBS (C = control) or 1 μ*M* MTX, IAHQ2c, raltitrexed or lometrexol, in 1.5-ml double-layered semisolid agarose cultures containing 5637 CM, L929 CM, and rmIL-3. On day 2 of incubation cultures received PBS (C), LV (final concentration 1 m*M*) or HT (final concentration of hypoxanthine 100 μ*M* and thymidine 16 μ*M*) in a volume of 100 μl. After an additional 14 days of incubation the colonies were stained overnight with *p*-iodonitrotetrazolium violet

of granulocyte precursors [19, 20, 21, 31]. These experiments, performed in the 1970s and the 1980s, were limited by the poorly developed in vitro culture techniques available at the time. Since then, there have been major advances in the purification and characterization of hematopoietic growth factors, and an improvement of in vivo and in vitro techniques for growing both early and late hematopoietic precursors. In this study we have made use of these advances to obtain more information about the action of the novel antifolates on different bone marrow cell populations.

A preliminary screen of the effects of the different antifolates on bone marrow cell proliferation was undertaken using the XTT proliferation assay, which measures the ability of mitochondrial dehydrogenase to metabolize the tetrazolium salt XTT to a formazan dye (orange color). All antifolates significantly inhibited bone marrow cell proliferation in these suspension cultures (Fig. 1). The proliferation assay, however, does not discriminate between effects on mature or immature cells. The direct effect of the antifolates on progenitor cells in the bone marrow was therefore evaluated in semisolid clonogenic assays.

We have found that EDX is a more potent inhibitor of CFU-C colony formation than MTX (Fig. 2A). In accordance with these findings, EDX has been shown to have characteristics that favor transport into both tumor cells and normal cells when compared with MTX [5, 28]. The intracellular accumulation of EDX in normal epithelial cells has also been shown to be greater than that of MTX [5]. These properties of EDX might contribute to the higher inhibitory potency of this drug towards bone marrow progenitors. The toxicity profile of EDX in phase I and II trials is very similar to that observed with MTX, one of the most frequent toxicities being myelosuppression. In a phase II study, which compared EDX with MTX in patients with head and neck cancer, no definitive clinical advantage over MTX was found.

In our study, the lipophilic agent TMTX was the most potent inhibitor of CFU-C colony formation and differed from the other DHFR inhibitors in totally abolishing CFU-C colony formation at high concentrations (Fig. 2A). The newly synthesized potent TS inhibitor AG337 is also lipophilic, enters cells readily without the RFC, and drug potency is not dependent on polyglutamation [29]. AG337 mimicked TMTX, but differed from the other non-lipophilic TS inhibitors by inducing total inhibition of CFU-C colony formation at high concentrations (Fig. 2B).

Of the TS inhibitors, raltitrexed was the most potent inhibitor of CFU-C and induced the same degree of

Table 1 Effect of methotrexate (*MTX*), IAHQ2c, raltitrexed, and lometrexol on formation of high proliferative potential colony-forming cell (*HPP-CFC*) colonies in microwell suspension cultures of 4d5FU Lin$^-$cells. 4d5FU Lin$^-$ cells (5 cells/culture) were incubated with 1 μ*M* MTX, IAHQ2c, raltitrexed, or lometrexol (DDATHF) in Terasaki cultures stimulated with 5637 CM, L929 CM, and rmIL-3. On day 2 of culture PBS (control), leucovorin (*LV*; final concentration 1 m*M*) or hypoxanthine plus thymidine (*HT*; final concentration of hypoxanthine 100 μ*M* and thymidine 16 μ*M*) were added to the cultures in a volume of 10 μl, giving a final volume of 20 μl. After an additional 14 days of incubation a total of 180 (no rescue) or 120 (LV or HT rescue) wells were scored for large colonies (HPP-CFC; confluent wells) and small colonies (non-confluent wells, > 10 cells) in each treatment group

	No rescue		+LV		+HT	
Treatment	Small	Large	Small	Large	Small	Large
Control	2.2	17.2	8.3	15.0	4.2	14.2
IAHQ2c	15.6	0.0	7.5	10.0	2.5	10.0
Raltitrexed	7.8	0.0	7.5	11.7	2.5	10.0
Lometrexol	15.6	0.0	7.5	13.3	1.7	15.8
Methotrexate	13.6	0.0	7.5	11.7	5.8	10.0

inhibition as MTX (Fig. 2A,B). Raltitrexed is rapidly transported into cells by the reduced folate carrier and extensively polyglutamated [28]. Experiments in cell cultures have shown that 95% of this drug is present intracellularly in the polyglutamated form within 4 h of raltitrexed exposure, resulting in potent and prolonged inhibition [11]. This agent is currently in phase III testing in combination with other drugs [28]. Raltitrexed is now a registered drug for colorectal cancer in many countries, and it has been shown to produce similar antitumor response rates with lower toxicity than conventional 5FU/LV chemotherapy [28].

AG337 and IAHQ2c were more potent CFU-C inhibitors than IAHQ2a (Fig. 2B). IAHQ2c is the 2-desamino-2-methyl analog of IAHQ2a, and has been found to be a more potent inhibitor of cultured cells in spite of being a weaker inhibitor of TS than IAHQ2a [7]. The reason for this is probably that IAHQ2c is an excellent substrate for folylpolyglutamate synthetase, and has better affinity for the reduced folate transporter than IAHQ2a.

The TS and DHFR inhibitors were more inhibitory in the CFU-C assay than in the proliferation assay (Fig. 1A,B; Fig. 2A,B). A possible explanation for this may be the different incubation times used, 3 days incubation in the proliferation assay and 7 days in the CFU-C assay. It is also difficult to compare these two sets of experiments, since the XTT proliferation assay measures the activity of a mitochondrial enzyme, whilst the CFU-C assay directly measures progenitor cell proliferation.

Another issue that is important to address is the problem of thymidine/purine salvage in the different assay systems. Dying cells may release these salvage components and the undialyzed serum used in the media may be a source of thymidine/purines. In the proliferation assay dying cells could contribute to salvage due to the relatively high cell concentration used (1×10^6 cells/ml). In the CFU-C and HPP-CFC clonogenic assay, salvage would probably be less prominent because of the lower cell concentrations (1.5×10^4 cells/ml and 1×10^3 cells/ml, respectively) and because the cells are immobilized in a semisolid matrix. The problem with salvage from dying cells is probably neglectable in the microwell clonogenic cultures, where only 5 cells/well/20 μl were seeded (250 cells/ml). We would like to emphasize that the observed biological effects of the antifolates on proliferation and differentiation of bone marrow progenitor cells in the experimental systems used are a result of the balance between antifolates and folates/salvage components in the cultures, and not a property which can be associated with a specific antifolate concentration.

The only GAR transformylase inhibitor included in these studies, DDATHF, differs from the other antifolates in that it inhibits purine synthesis. In contrast to the TS and DHFR inhibitors, DDATHF did not induce significant inhibition of CFU-C colony formation (Fig. 2C) but rather a significant inhibition of proliferation of bone marrow cells (Fig. 1C). The lack of inhibitory effect on the CFU-C progenitors is difficult to explain. We could speculate that a longer incubation time is needed for DDATHF to exert its action in the CFU-C assay. This phenomenon deserves further investigation. In contrast to these findings, DDATHF has, in some phase I studies, shown severe toxicity against the bone marrow [18, 22, 23]. This was surprising, since much higher doses had been used in early animal studies, but there were indications that the effect of DDATHF in animal models was strongly influenced by LV and folic acid [24, 25]. Other more recent phase I studies indicate that the toxicity seems to depend on the dose and treatment regimen, with repeated dosing of the drug inducing cumulative toxicity [28]. Protocols involving concomitant administration of folic acid or the use of LV as an antidote have shown favorable effects.

When a selection of the antifolates (MTX, IAHQ2c, raltitrexed, and DDATHF) were investigated for effects on the immature progenitors HPP-CFC in the semisolid clonogenic assay, they all induced a reduction in colony size and not a reduction in colony numbers even at very high drug concentrations (1 μ*M* and 100 μ*M*; Fig. 3). A reduction in colony numbers would imply that the agents were directly toxic to the immature HPP-CFC. On the other hand, a reduction in colony size could be due to a suppressive effect of the drugs towards the HPP-CFC themselves or later progenitors developing from the HPP-CFCs. The finding that 2 days delayed LV or HT administration again resulted in growth of large colonies indeed implied that the drugs did not induce death of the HPP-CFC, but rather a reversible cytostatic effect on these immature progenitors and/or later progenitor cells. The same reversible reduction in colony size was seen when looking at growth of single HPP-CFC in the microwell clonogenic assay (Table 1). An earlier study also showed that low and intermediate concentrations (10 n*M* and 1 μ*M*) of the lipophilic TMTX induced the same reversible action on the HPP-CFC and/or later progenitors but that high concentrations (100 μ*M*) of this agent totally abolished HPP-CFC colony formation [28].

In conclusion the antifolates MTX, IAHQ2c, raltitrexed, and DDATHF share the surprising property of inducing a completely reversible cytostatic effect on the HPP-CFC themselves, and/or progenitors deriving from these immature cells, as shown in the HPP-CFC clonogenic assay with delayed LV or HT rescue. However, at high concentrations the lipophilic antifolates TMTX and AG337 are unique in displaying irreversible cytotoxicity towards the CFU-C progenitors by totally abolishing colony formation in the CFU-C assay.

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