Cancer Chemother Pharmacol (2002) 50: 111–116
DOI 10.1007/s00280-002-0478-7

ORIGINAL ARTICLE

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Osteosarcoma cells, resistant to methotrexate due to nucleoside and nucleobase salvage, are sensitive to nucleoside analogs

Received: 11 January 2002 / Accepted: 2 May 2002 / Published online: 14 June 2002

Abstract *Purpose:* To test a novel strategy for overcoming intrinsic resistance to methotrexate (MTX) in osteosarcoma (OS) due to nucleoside and nucleobase salvage (NS). *Methods:* Four OS cell lines, found to be highly resistant to MTX, were tested to determine the dominant mechanism of resistance. Sensitivity to MTX was tested in the presence of dialyzed serum or the transport inhibitor dipyridamole (DP) to confirm the contribution of NS to MTX resistance. We then investigated whether increased NS activity could be exploited using cytotoxic nucleoside analogs. *Results:* Like other cell types, OS cells are capable of circumventing inhibition of de novo nucleotide synthesis by relying on NS. MTX, at concentrations as high as 1 m*M* did not inhibit cell growth in culture medium supplemented with undialyzed serum. In contrast, when NS was inhibited by DP or in medium depleted of nucleosides and nucleobases, sensitivity to MTX was seen at nanomolar concentrations. In medium with dialyzed serum, thymidine and hypoxanthine provided dose-dependent protection from MTX toxicity at concentrations similar to those seen in human plasma. No evidence of other significant mechanisms of resistance were found. All four cell lines were sensitive to 3-day exposures to cytarabine (IC_{50} 0.22 to 2.88 μ*M*) and vidarabine (IC_{50} 0.09 to 0.95 μ*M*). *Conclusions:* Salvage of de novo nucleotide synthesis inhibition by extracellular thymidine and hypoxanthine, at physiologically relevant concentrations, contributes to resistance to MTX in OS. However, this same process may impart a collateral sensitivity to nucleoside analogs. These findings support clinical trials for patients with OS using nucleoside analogs, either alone or in combination.

Keywords Methotrexate · Osteosarcoma · Drug resistance · Ara-C · Ara-A

The project described was supported in part by the Fazzi Fellowship Fund. B.A.K. is an American Cancer Society Professor.

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Introduction

Osteosarcoma (OS) is a rare bone tumor predominantly affecting children and young adults. As many as two-thirds of patients with OS can be cured with a combination of surgery and intensive multiagent chemotherapy, provided no clinically detectable metastases were present at the time of initial diagnosis [28]. For those patients who do have detectable metastases, as well as those who relapse or progress during or after treatment, the prognosis is dramatically inferior [29]. Innovative therapeutic approaches are urgently needed for these patients.

Methotrexate (MTX) plays a prominent role in many therapeutic regimens used to treat patients with OS. As a classical antifolate, MTX inhibits cell growth by interfering with folate-dependent enzymatic reactions necessary for de novo thymidine and purine synthesis. Decreased transport of MTX across the cell membrane through the reduced folate carrier or increased expression of the target enzyme dihydrofolate reductase are thought to account for much of intrinsic and acquired clinical resistance to MTX [11]. However, the redundancy of cellular pathways for the synthesis of DNA precursors provides another natural mechanism of resistance to pharmacologic inhibition of de novo nucleotide synthesis. A wide variety of neoplastic cell lines have been shown to be capable of circumventing the toxicity of MTX by salvaging preformed nucleosides and nucleobases [2, 12, 13, 15, 31, 38, 41, 44] which are

abundantly available in the normal extracellular milieu, and may be even more prevalent in the local tumor microenvironment [17].

Dipyridamole (DP), an inhibitor of facilitated nucleoside transport [32], is synergistic with antifolates in vitro [1, 3, 5, 10, 17, 21, 32, 35, 41, 44]. However, clinical trials of DP in combination with MTX have not shown overwhelming success [43, 46], most likely due to a high degree of protein binding, which severely limits the concentration of free DP [24]. Rather than inhibiting nucleobase salvage (NS) to increase MTX cytotoxicity, we sought to test whether increased NS could be pharmacologically exploited using commonly used antineoplastics. Because some nucleoside analogs share uptake mechanisms with natural compounds [9, 36, 37, 45], we tested their ability to inhibit OS cell growth.

We report here that four OS cell lines, highly resistant to MTX, were sensitive to two cytotoxic nucleoside analogs in vitro at concentrations that can be achieved in vivo. These findings suggest a clinically useful strategy for patients with relapsed or refractory OS.

Materials and methods

Materials

Dialyzed and undialyzed fetal calf serum (FCS) and RPMI 1640 culture medium without folic acid were purchased from Gibco-Invitrogen Corporation (Carlsbad, Calif.). Hanks' buffered saline solution, trypsin-EDTA, sodium pyruvate, glutamine, MEM non-essential amino acids, MTX, doxorubicin, cytarabine (cytosine arabinoside, Ara-C), and vidarabine (adenine arabinoside, Ara-A) were all obtained from Sigma Chemical Co. (St. Louis, Mo.). [3′,5′,7-^{3}H(N)]MTX and 5-methyl-[3′,5′,7,9-^{3}H]tetrahydrofolic acid were obtained from Moravek Biochemicals (Brea, Calif.).

Cell cultures

The four human OS cell lines studied, Saos-2, MG-63, U-2 OS, and G-292 are commercially available. They were maintained in sterile plastic tissue culture flasks at 37°C in an atmosphere containing 5% CO_2 in growth medium (folic acid-free RPMI 1640, with penicillin and streptomycin, 3.5 g/l glucose, minimal essential amino acids, 2 m*M* glutamine, 1 m*M* sodium pyruvate) supplemented with undialyzed FCS and 50 n*M* *d,l*-5-methyltetrahydrofolate (5MeTHF) as the only folate source. This concentration of folate is similar to the physiologic human plasma folate concentration (i.e. about 25 n*M* *l*-5MeTHF) and is well below the 2 μ*M* concentration of folic acid in standard tissue culture media. This growth medium with 15% undialyzed FCS contains approximately 0.6 μ*M* thymidine and 3.3 μ*M* hypoxanthine, similar to the concentrations seen in normal human plasma [15, 16]. The medium was changed and cells were split as needed for experiments and to maintain healthy cellular growth.

Cytotoxicity

Cells were plated in 96-well tissue culture plates, at a concentration of 10^4 to 4×10^4 cells/ml in 100 μl growth medium supplemented with 15% FCS (either dialyzed or undialyzed, as indicated in the Results), and 50 n*M* 5MeTHF. All experimental conditions included three to eight replicates per experiment and were repeated on multiple days. After 24 h incubation at 37°C in an atmosphere containing 5% CO_2, various drugs or combinations thereof were added to each well, as indicated in the Results. In some experiments the medium containing drug was removed after 24 h and replaced with fresh drug-free medium. The cells were incubated for an additional 3 to 5 days, until the cells in control wells reached confluence. Over the course of each assay, the growth of cells in culture medium with dialyzed FCS was similar to the growth of cells in medium with undialyzed FCS on the same plates (data not shown). Viable cells were then quantitated with a colorimetric assay that uses their ability to convert the tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega, Madison Wis.) to a water-soluble formazan product that can be detected spectrophotometrically in a microplate reader. Light absorbance was found to be linearly proportional to cell count across the range of cell numbers in our experiments. For each condition, cell counts are expressed as percentages of the mean absorbance in control wells (i.e. those not exposed to drug). The drug concentrations capable of 50% growth inhibition relative to control cells (IC_{50}) were determined using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, Calif.). The data were fitted to a sigmoidal dose-response curve, with a variable slope, with the formula $Y = \text{bottom} + (\text{top}–\text{bottom})/(1 + 10\hat{}((\log IC_{50}–X)*\text{HillSlope}))$, where Y is the cell count as a percentage of the mean cell counts in control wells, Bottom and Top are the lowest and highest cell counts, and X is the logarithm of drug concentration.

Accumulation and metabolism of MTX and 5MeTHF

Intracellular MTX accumulation by the four OS cell lines was measured after a 24-h exposure to [^{3}H]-MTX at a final extracellular concentration of 1 μ*M*, as previously described [6]. Accumulation of labeled 5MeTHF was determined in separate experiments at an extracellular concentration of 50 n*M* [6]. Intracellular MTX and 5MeTHF polyglutamates were then separated by HPLC on a reverse-phase C_{18} column coupled to a Flo-Beta in-line scintillation counter and a UV spectrophotometer, as previously detailed [20]. Authentic MTX and folylpolyglutamates were used as internal standards.

Cellular content of dihydrofolate reductase (DHFR)

Total intracellular MTX binding capacity was analyzed by measuring [^{3}H]MTX binding in whole cell extract, using techniques previously described [19]. Because DHFR is the only intracellular protein with significant affinity for non-polyglutamylated MTX in this assay, and each DHFR molecule can bind one molecule of MTX, this method allows calculation of picomoles DHFR per 10^6 cells [19].

Results

OS cells are highly resistant to MTX in complete medium

Growth of the four OS cell lines was not inhibited by continuous exposure to MTX at concentrations as high as 3 m*M* when the cells were grown in culture medium supplemented with 15% undialyzed FCS. As a positive control, a 24-h exposure to doxorubicin was inhibitory (Fig. 1) in this same culture medium.

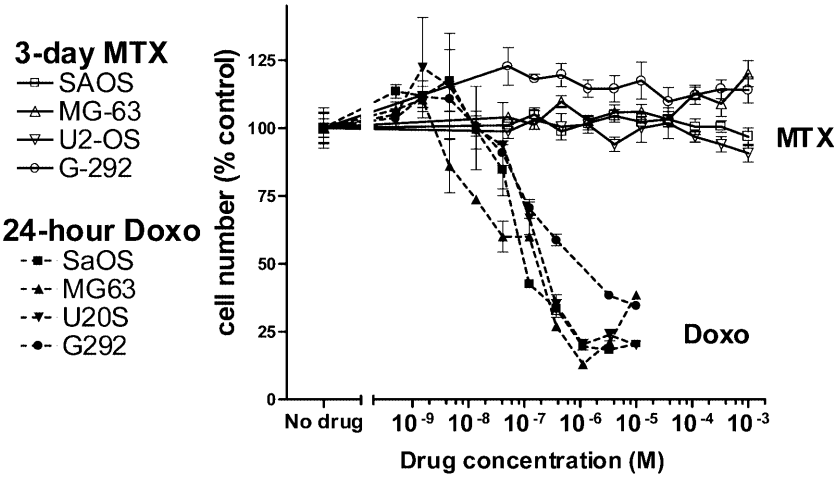

Fig. 1. Sensitivity of OS cells to MTX and doxorubicin. Dose-response curves for four OS cell lines exposed to varying concentrations of MTX for 3 days (*solid lines*) or doxorubicin for 24 h (*dashed lines*) in culture medium supplemented with undialyzed serum. Cell numbers are expressed as percentages of the number of cells in control wells not exposed to MTX or doxorubicin. Each point represents the mean of at least six replicates; error bars indicate the standard error of the mean. For all cell lines, cell growth was not inhibited by continuous exposure to 1 m*M* MTX but was inhibited by a 24-h exposure to 1 μ*M* doxorubicin

NS is confirmed to contribute to MTX resistance in these OS cells

We found no evidence of impaired MTX transport or increased DHFR expression to explain this high degree of resistance (Table 1). DHFR content in the four OS cell lines was not significantly greater than that reported [19] in 20 clinical OS samples (0 to 120 pmol/g wet weight, or approximately 0 to 0.120 pmol/10^6 cells). Moreover, intracellular MTX after a 24-h exposure to only 1 μ*M* drug was in molar excess in relation to the DHFR content by a ratio ranging from 59:1 to 219:1.

Decreased metabolism of MTX to polyglutamates, known to contribute to MTX resistance in other human sarcoma cell lines [23], was observed in these OS cells. However, the absolute amount of MTX-polyglutamates remained in excess of DHFR content. In addition, a parallel decrease was noted in metabolism of 5MeTHF to polyglutamates (Table 1), potentially negating any contribution to MTX resistance by reducing intracellular concentrations of the protective folylpolyglutamates.

All four cell lines were sensitive to MTX, with IC_{50} values in the low nanomolar range, when NS was inhibited by the addition of 1 μ*M* DP (Table 2). DP alone did not inhibit cell growth, and did not affect the IC_{50} of 3-day exposure to doxorubicin (Table 3). Similar growth inhibition by MTX was seen when the cells were grown in culture medium supplemented with dialyzed, rather than undialyzed FCS (Table 2).

Neither thymidine nor hypoxanthine alone, each at a concentration of 10 μ*M*, was sufficient to prevent the toxicity of MTX in culture medium with dialyzed FCS (Fig. 2). However, in the presence of 10 μ*M* hypoxanthine, a concentration-dependent protective effect of thymidine was seen (Fig. 2A). The concentration of thymidine necessary to restore growth to 50% of that seen in control cells not exposed to MTX was between 0.03 and 0.26 μ*M* for the four cell lines. Hypoxanthine provided a similar dose-dependent protective effect, when cells were grown in the presence of excess thymidine (Fig. 2B).

Cells actively utilizing NS pathways are collaterally sensitive to nucleoside analogs

Ara-C and Ara-A, in 3-day continuous exposure, were each active against all four cell lines (Table 3). This

Table 1. MTX and MeTHF uptake and metabolism and DHFR content. Total intracellular [^{3}H]MTX content was determined by scintillation counting after a 24-h exposure to 1 μ*M* [^{3}H]MTX. 5Me-[^{3}H]THF content was similarly determined after exposure to 50 n*M* 5Me-[^{3}H]THF. MTX and 5MeTHF polyglutamates (MTX-PG and MeTHF-PG, respectively) were separated by HPLC, and the fraction with a polyglutamate chain length of two or more is shown in the Table. DHFR was measured as total intracellular MTX binding capacity in whole cell extracts

Cell line	[^{3}H]MTX uptake (pmol/10^6 cells)	MTX-PG (%)	[^{3}H]5MeTHF uptake (pmol/10^6 cells)	MeTHF-PG (%)	DHFR content (pmol/10^6 cells)
Saos-2	3.33 ± 0.07	68	4.44 ± 0.83	39	0.058
MG-63	6.46 ± 0.11	49	7.36 ± 2.63	36	0.110
U-2 OS	6.59 ± 0.84	26	4.28 ± 0.58	47	0.127
G-292	10.7 ± 3.46	26	5.63 ± 1.00	49	0.047

Table 2. Resistance of OS cells to MTX depends on NS. Concentrations of MTX required to inhibit 50% of control cell growth in 3-day continuous exposure in the presence of undialyzed or dialyzed serum, and 0 or 1 μ*M* DP as indicated. In standard culture medium, all four cell lines were highly resistant to MTX. Sensitivity was only seen in culture medium depleted by dialysis of nucleosides and nucleobases, or in the presence of the transport inhibitor DP. Values are IC_{50} values in nanomoles/liter with 95% confidence intervals in parentheses

Cell line	DP 0		DP 1 μ*M*
	Undialyzed serum	Dialyzed serum	Undialyzed serum
Saos-2	$>10^6$	2.24 (1.3–3.9)	7.5 (5.1–11.0)
MG-63	$>10^6$	3.80 (2.6–5.5)	9.8 (4.8–19.8)
U-2 OS	$>10^6$	2.95 (1.9–4.6)	25.7 (6.3–105)
G-292	$>10^6$	5.25 (2.7–10.0)	35.5 (11.6–109)

Table 3. Sensitivity of OS cells to doxorubicin, Ara-C and Ara-A. Concentrations required to inhibit 50% of control cell growth in 3-day continuous exposure in the presence or absence of 1 μ*M* DP, as indicated. Under conditions where the cells demonstrated resistance to MTX, all four lines were sensitive to doxorubicin, Ara-C and Ara-A. DP had no effect on doxorubicin sensitivity, but protected the cells against Ara-A and Ara-C. Values are IC_{50} values in micromoles/liter with 95% confidence intervals in parentheses (*n.d.* not done)

Cell line	Doxorubicin		Ara-C		Ara-A	
	DP 0	DP 1 μ*M*	DP 0	DP 1 μ*M*	DP 0	DP 1 μ*M*
Saos-2	0.13 (0.09–0.21)	0.14 (0.08–0.25)	0.22 (0.17–0.28)	0.68 (0.60–0.76)	0.09 (0.07–0.11)	1.7 (1.04–2.78)
MG-63	0.07 (0.04–0.13)	0.07 (0.07–0.15)	0.27 (0.21–0.34)	>100	0.20 (0.17–0.25)	9.12 (4.39–19.0)
U-2 OS	0.24 (0.11–0.53)	0.18 (0.17–0.36)	1.12 (0.72–1.76)	n.d.	0.57 (0.45–0.72)	>30
G-292	0.72 (0.47–1.12)	0.76 (0.39–1.33)	2.88 (2.31–3.60)	n.d.	0.95	>30

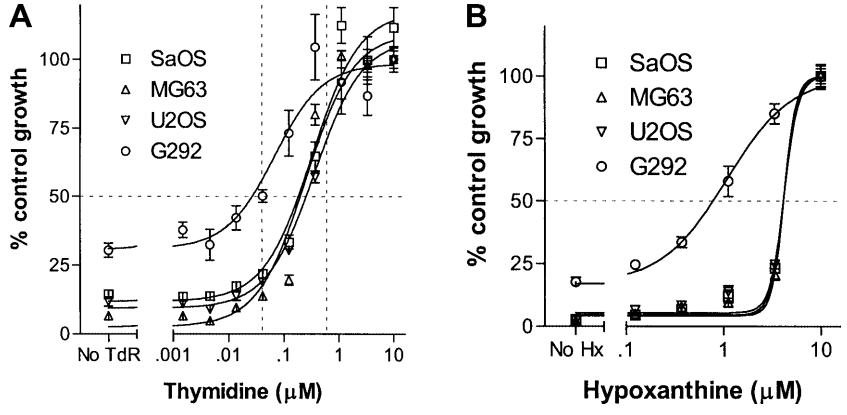

Fig. 2A, B. Dose-response curves for the protection of OS cells against the toxicity of 10 μ*M* MTX by thymidine (**A**) and hypoxanthine (**B**). Cells were incubated in medium supplemented with dialyzed serum and excess hypoxanthine (10 μ*M*) when testing thymidine rescue (**A**), or excess thymidine (10 μ*M*) when testing hypoxanthine rescue (**B**). MTX was added at a final concentration of 10 μ*M*. Cell numbers are expressed as percentages of the number of cells in control wells, i.e. those wells not exposed to MTX. Each point represents the mean of at least three replicates; error bars indicate the standard error of the mean. Dotted vertical lines in **A** indicate the published range of normal serum thymidine [15, 16]

toxicity was not affected by growth in culture medium with undialyzed FCS, conditions under which MTX had no inhibitory effect on cell growth. The addition of DP protected the four cell lines against the toxic effects of Ara-A and Ara-C, significantly increasing the IC_{50} in the cell lines tested (Table 3).

Discussion

The potential for cells to circumvent MTX cytotoxicity through salvage of thymidine and hypoxanthine from the extracellular fluid has been demonstrated in a variety of normal [15, 16, 17, 35] and neoplastic cell types in vitro [2, 12, 13, 15, 31, 38, 41, 44]. We confirmed the contribution of NS to MTX resistance in OS cells, showing MTX sensitivity only in dialyzed serum or when the cells were exposed to a pharmacologic inhibitor of nucleoside transport. The striking finding that the steep portions of the dose-response curves for protection against MTX toxicity by thymidine and hypoxanthine overlap with the published normal ranges of serum thymidine (0.04 to 0.6 μ*M*) and hypoxanthine (0.2 to 2.6 μ*M*) [15, 16] provides further evidence that this

process could contribute to clinical MTX resistance in vivo.

The strategy of inhibiting NS with DP to overcome MTX resistance due to this mechanism has been investigated in both preclinical models [32] and clinical trials [14, 43, 46]. However, increased hematologic toxicity was observed in some of these trials, probably due to the dependence of hematopoietic stem cells on NS, rather than de novo synthesis [34]. Moreover, the avid binding of DP to the plasma protein alpha$_1$-acid glycoprotein [24] limits available free drug concentrations and clinical efficacy. Newer analogues of DP are being developed that are more potent inhibitors of thymidine uptake and have less affinity for alpha$_1$-acid glycoprotein [7].

We now show data supporting the use of a novel strategy targeting cells resistant to MTX by exploiting, rather than inhibiting, NS. The four OS cell lines we examined were sensitive to the purine analog Ara-A and the pyrimidine analog Ara-C. Sensitivity was seen under conditions where MTX was ineffective even at millimolar concentrations. Although the drug concentrations necessary to inhibit OS growth were higher than those necessary to inhibit some acute myeloid leukemia (AML) cell lines (see, for example, references 9 and 22), the inhibitory concentration for OS cells was less than the 5 μM steady-state plasma concentration that can be safely achieved in humans receiving ara-C as a continuous infusion [4].

The combination of Ara-C with fludarabine (a fluorinated analog of Ara-A) has been used for patients with hematologic malignancies with acceptable toxicity [8, 18, 26, 30, 40, 42]. To our knowledge, this regimen has never been given to patients with OS or other solid tumors. In addition, the cytidine analog gemcitabine has shown activity in soft tissue sarcomas [25, 33, 39] and may prove useful for patients with OS. Merimsky et al., for example, describe six patients with OS who failed therapy including high-dose MTX [27]. Four of these derived clinical benefit from gemcitabine, although there were no clinical responses.

The data reported here support further clinical trials to determine whether patients with refractory or relapsed OS will benefit from treatment including nucleoside analogs.

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