

Absence of Hypoxanthine:Guanine Phosphoribosyltransferase Activity in Murine Dunn Osteosarcoma¹

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ABSTRACT

The transplantable murine Dunn osteosarcoma has no detectable hypoxanthine:guanine phosphoribosyltransferase (EC 2.4.2.8) activity. This was established from the tumors directly and from tissue culture cell lines derived from the tumor using a variety of assays: *e.g.*, no [³H]hypoxanthine uptake into tumor or tissue culture cells, no conversion of [³H]hypoxanthine to [³H]IMP by cell extracts from tumors or tissue culture cells, no growth of tissue culture cells in hypoxanthine:aminopterin:thymidine medium, and normal growth of these cells in 10 μ M 6-mercaptopurine. Ten human osteosarcomas have been assayed, and two have no apparent hypoxanthine:guanine phosphoribosyltransferase enzyme activity. After high-dose methotrexate treatment *in vivo*, murine tumors could be selectively killed and normal tissues could be spared by using a rescue regimen of hypoxanthine-thymidine-allopurinol.

INTRODUCTION

A major area of controversy surrounding the use of antifolate therapy involves adjuvant treatment of patients with osteosarcoma using high-dose MTX³ followed by LV rescue (6, 16, 19, 20, 22). Not only are the clinical results from this treatment in dispute but, to date, there is no biochemical evidence supporting a selective role for MTX-LV against osteosarcoma cells *versus* normal cells (6). If specific differences could be identified between tumor cells and normal cells, the design of chemotherapy protocols might be rationally modified. MTX inhibits 1-carbon transfers involved in the *de novo* synthesis of both purines and pyrimidines (3, 8). LV provides reduced folates for these same *de novo* pathways but distal to the MTX block of dihydrofolate reductase. The cell can also overcome the effect of MTX on 1-carbon metabolism by the incorporation of pyrimidine and purine molecules into the cell via the salvage pathway enzymes dThd kinase and HPRT (EC 2.4.2.8), respectively. If a tumor cell had altered activity of one or more of these salvage pathways, then rescue from MTX effects with dThd and hypoxanthine rather than with LV might allow MTX to more profoundly affect the tumor. For example, observations of decreased activity of salvage pathway enzymes in L1210 leukemia cells after MTX treatment allowed for more selective rescue from MTX toxicity with a combination of purine and pyrimidine precursors as compared to LV and resulted in enhanced tumor kill (9, 10, 24).

To examine salvage pathways more carefully with respect to osteosarcoma, we have studied the transplanted murine Dunn osteosarcoma because it resembles human osteosarcoma in a number of characteristics, including metastasis to the lungs, surgical control of the transplanted primary being a requirement for cure, and response to adjuvant chemotherapy, including high-dose MTX-LV (11).

MATERIALS AND METHODS

Animals. C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and were fed Charles River Rat, Mouse, and Hamster Formula and water *ad libitum*. Three- to 6-month-old animals were used for these experiments.

Radiolabeled Compounds and Drugs. [¹⁴C]Formate (specific activity, 50 mCi/mmol), [¹⁴C]hypoxanthine (specific activity, 55 mCi/mmol), and [³H]hypoxanthine (specific activity, 4.3 Ci/mmol) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. Sodium phosphoribosyl pyrophosphate, dThd, thymine, adenine, guanine, and hypoxanthine were purchased from Sigma Chemical Co., St. Louis, Mo. MTX and LV were obtained from the National Cancer Institute, Bethesda, Md. HPP and 6-MP were obtained from Burroughs Wellcome and Co., Research Triangle Park, N. C., and Pronase was obtained from Calbiochem-Behring, La Jolla, Calif.

Dunn Osteosarcoma. Animals bearing the transplantable Dunn osteosarcoma were kindly supplied by Dr. Raymond N. Hiramoto, Department of Microbiology, University of Alabama, Birmingham, Ala. The tumor was maintained by serial transplantation in C3H/HeJ mice as described previously (11). Tumor-bearing animals were used 22 to 40 days after *i.m.* injection of 5×10^6 tumor cells.

The Dunn tumor was originally obtained in 1955 from a 21-month-old female C3H mouse, carried in Dr. Lloyd Law's laboratory, which had received an AKR virus inoculate as a newborn. A small primary tumor focus was found in a tail vertebra, and metastatic nodules were present in the lungs and on the surface of the kidneys. Tumor passages were made from the latter source. Dr. Dunn never published a description of this tumor,⁴ although others have done so (25).

Establishing a Dunn Osteosarcoma Cell Line from a Mouse Tumor. A Dunn osteosarcoma was excised from a C3H/HeJ mouse and minced with scissors. The tissue fragments were suspended in cold DME medium and further disrupted with a Potter homogenizer. The cell suspension was frozen at -90° for approximately 1 month. The suspension was thawed, and cells were plated in 60-mm-diameter tissue culture dishes. The cells were grown for 2 weeks in DME medium containing 10% fetal calf serum, 20 mM glutamine, mycostatin (100 units/ml) kanamycin (100 μ g/ml), gentamicin (0.5 mg/ml), penicillin (100 units/ml), and streptomycin (100 μ g/ml). After 2 weeks, no contamination had appeared, and all antibiotics were removed, except for penicillin and streptomycin. Three cell types appeared to grow from the tumor. One type appeared to be a fibroblast-like cell, and a second type appeared to be a flat, round histiocytic-like cell. The third cell type, which comprised approximately 90% of the cells present, was pleomorphic and spindle-shaped, grew in clusters, and piled up. The wild-type Dunn tumor cells

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³ The abbreviations used are: MTX, methotrexate; LV, leucovorin; dThd, thymidine; HPP, allopurinol; HPRT, hypoxanthine:guanine phosphoribosyltransferase; 6-MP, 6-mercaptopurine; DME, Dulbecco's modified Eagle's medium; HAT, hypoxanthine:aminopterin:thymidine.

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⁴ M. K. Deringer, personal communication.

(Dunn-1) were trypsinized and diluted to a concentration of 15 cells/10 ml. They were then plated on a 96-well microtiter plate (Linbro Scientific, Inc., Hamden, Conn.), at 0.2 ml/well. After 2 weeks, colonies of the osteosarcoma-like cells appeared. These colonies were allowed to grow and, when one was reinjected into C3H/HeJ mice, it produced tumors consistent with osteosarcoma on paraffin sections in the same time interval as the original transplanted tumor. This subline (Dunn-1c) was maintained in culture as a clone of the wild-type Dunn cells and was used for these *in vitro* experiments. Subsequently, additional sublines (Dunn 2c to 5c) were also isolated and maintained. All cell cultures were maintained in DME medium with 10% fetal calf serum.

Three other human osteosarcoma cell lines, SaOS (a gift from Dr. Ralph Weichselbaum, Joint Center for Radiation Therapy, Boston, Mass.), and NCI-N377 (a gift from Dr. Adi Gazdar, National Cancer Institute, Bethesda, Md.), were also maintained in DME medium with 10% fetal calf serum.

HPRT Assays. A human RBC hemolysate was used as a control source of HPRT. Whole blood anticoagulated with preservative-free heparin was prepared as described previously (12).

Dunn osteosarcoma lysates were prepared similarly. A piece of tumor was washed with cold DME medium to remove as much blood as possible and then minced with scissors. The tumor fragments were suspended in approximately 2 volumes of cold DME medium and disrupted in a Potter homogenizer. The suspension was filtered through Nitex screen cloth to remove large pieces. Cells were washed 3 times with 0.87% ammonium chloride to lyse RBC. The final pellet was suspended in distilled water, and the suspension was frozen and thawed 4 times, vortexed vigorously, and centrifuged at $20,000 \times g$ for 30 min to remove membranes. The supernatants were assayed for HPRT activity. HPRT activity was similarly determined on cell extracts from wild-type and Dunn-1c cells and from human osteosarcoma cell lines and primary and metastatic human osteosarcomas.

The HPRT assay used measured the conversion of hypoxanthine to IMP (17). The reaction mixture contained 0.5 μmol of MgCl_2 , 0.1 μmol of sodium phosphoribosyl pyrophosphate, 0.015 μmol of [^{14}C]hypoxanthine, 1- to 25- μl cell extract, and 50 mM Tris-HCl, pH 7.4, in a final volume of 0.1 ml. The mixture was incubated for 20 min at 37° . To stop the reaction, tubes were placed on ice, and 2 μmol of sodium EDTA were added to each tube. The blank reaction contained 0.01 mg of bovine serum albumin, and the positive controls contained 1 to 10 μg of RBC hemolysate.

To determine the amount of [^{14}C]hypoxanthine converted to IMP, 25 μl of each reaction mixture were spotted on DE81 filter paper circles (2 cm in diameter) (Whatman Chemical Separation, Inc., Clifton, N. J.) (4). The filters were allowed to dry completely and then were washed 3 times in 0.001 M ammonium bicarbonate, once in distilled H_2O , and once in methanol. The filters were dried, and radioactivity was determined by scintillation counting.

***In Vitro* Labeling of Dunn-1c Cells and Thin-Layer Chromatographic Analysis.** Dunn-1c cells in log-phase growth were exposed to [^{14}C]formate (25 $\mu\text{Ci/ml}$) and [^3H]hypoxanthine (5 $\mu\text{Ci/ml}$), for 2.5 hr. Nucleic acids from these cells were precipitated with 1.2 N perchloric acid and hydrolyzed to a mixture of bases in a boiling water bath for 1 hr. Samples were neutralized, and precipitated salts were removed by centrifugation. The supernatant was concentrated by lyophilization. Samples were spotted on Eastman 13254 cellulose thin-layer chromatography plates with a fluorescent indicator (2). Authentic standards of adenine, guanine, and thymine were added. The plates were developed in a 70:20:10 (v/v) methanol:HCl:H $_2\text{O}$ system. After drying, plates were cut into 0.5-cm slices, and radioactivity was determined by scintillation counting.

***In Vitro* Cell Growth Studies.** Dunn-1c, SaOS, and NIH osteosarcoma cells were plated on 60-mm plastic dishes in DME containing 10% fetal calf serum. After several hr, attached cells were transferred to either HAT medium or DME containing 10 μM 6-MP. On 3 subsequent days, duplicate plates of each cell type were trypsinized, and cells were counted to monitor growth.

Animal Trial of Treatment with MTX and LV or HAT. C3H/HeJ mice bearing transplanted Dunn osteosarcomas were used 27 days after inoculation, with 5×10^6 tumor cells given i.m. in the left hind leg. Mice were randomly allocated to groups in each experiment. MTX (400 mg/kg) i.p. was followed immediately by either LV (6 mg/kg) or hypoxanthine (50 mg/kg)-dThd (500 mg/kg)-HPP (10 mg/kg) given i.p. Rescue was continued 3 times/day for 8 doses. A second identical treatment was administered beginning after the last rescue dose on Day 3. The maximum tumor diameter was measured with a caliper by both authors. Data were analyzed by a 2-sided Wilcoxon rank-sum test (26).

RESULTS

Studies with Dunn Osteosarcoma Cells *In Vitro*. In order to more easily examine purine-biosynthetic pathways, a Dunn osteosarcoma cell line and subclones were established from a C3H/HeJ mouse bearing a transplanted tumor. *De novo* and salvage purine precursor synthesis was first studied by examining incorporation of tracer amounts of [^{14}C]formate and [^3H]hypoxanthine, respectively, into the Dunn-1c cell line by thin-layer chromatographic analysis. [^{14}C]Formate was incorporated into guanine, adenine, and thymine in Dunn-1c, as determined from radiochromatograms of thin-layer chromatography analyses of cellular nucleic acids. In contrast to the [^{14}C]formate incorporation, there was no [^3H]hypoxanthine incorporation into the Dunn-1c cells (Chart 1). Direct assays for the presence of HPRT activity were then made in extracts of freshly dispersed Dunn tumor cells as well as from extracts of tissue culture cells of the original tumor (Dunn-1) and the clone (Dunn-1c) (Table 1). There was no evidence of HPRT activity in the Dunn cell lines, nor was HPRT activity present in extracts of transplantable Dunn tumors (Table 1). Control HPRT assays (RBC) were linear with respect

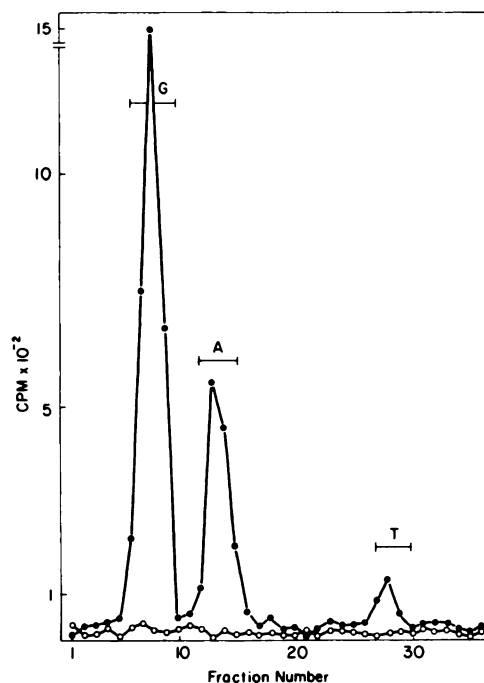


Chart 1. Thin-layer chromatographic analysis of *in vitro* labeled Dunn-1c cells. [^{14}C]Formate (25 $\mu\text{Ci/ml}$) and [^3H]hypoxanthine (5 $\mu\text{Ci/ml}$) were added to log-phase cultures for 2.5 hr. The amount of acid-precipitable [^{14}C]formate incorporated into 1×10^7 cells was 358,000 cpm. Approximately 6,000 cpm were applied to this radiochromatogram, and recovery was greater than 90%. Cell suspensions were prepared for thin-layer chromatographic analysis as in "Materials and Methods." The R_i is shown of authentic standards, with guanine (G), adenine (A), and thymine (T) added to the preparation. ●, controls; ○, drug treatments.

to time of incubation and protein concentration. Dunn tumor extracts assayed at a 10-fold higher hypoxanthine concentration still showed no evidence of HPRT activity. To ascertain whether there was an inhibitor of HPRT activity in these cells, extracts of a transplanted Dunn tumor were added to RBC lysates, and activity was measured. These Dunn extracts had no effect on RBC HPRT activity. To control for the possible degradation of [³H]IMP to [³H]inosine, which would not be retained on the DE81 filters, HPRT assays were also performed at pH 10 after pre-heating cell lysates at 60° for 10 min to minimize nucleotidase activity (17). There was no change in HPRT activity under these conditions.

Three human osteosarcoma cell lines, NIH, NCI-N377, and SaOS, all express HPRT activity (Table 1) and incorporate [³H]hypoxanthine into nucleic acids. Ten human osteosarcomas were also assayed for HPRT activity (Table 1). Two specimens (Specimens 2 and 6) had undetectable HPRT activity.

The biological consequences of this apparent lack of HPRT activity in Dunn tumor cells were tested in the following drug experiments. 6-MP, a drug which must be activated by HPRT in order to be cytotoxic, was added to Dunn-1c cells, and their growth was compared to that of untreated cells (Chart 2A). The addition of 10 μM 6-MP had no effect on the growth of Dunn-1c tumor cells. The growth of the human osteosarcoma cell line SaOS, which contains HPRT activity (Table 1), was inhibited more than 90% by the same concentration of 6-MP. Dunn-1c cells were then exposed to HAT medium. HAT medium has no cytotoxic effect on cells with intact salvage pathways, since the inhibition of *de novo* 1-carbon transfers by aminopterin is overcome by the incorporation of dThd and hypoxanthine via dThd kinase and HPRT, respectively. If one or the other salvage enzyme is missing, however, then cells will not proliferate or will die in HAT medium. Greater than 90% of the Dunn-1c cells were killed after a 3-day exposure to HAT medium (Chart 2B). If adenine (5 × 10⁻⁵ M) is added to the HAT medium, Dunn-1c cell growth is restored to normal. HAT medium had no effect on the growth of 3 other osteosarcoma cell lines, all of which had detectable HPRT activity (Table 1).

Animal Treatment Trial. Since the Dunn tumor cells lack

Table 1
HPRT activity in osteosarcoma

Source of tissue or cells	HPRT activity (nmol/min/mg of protein)
Normal human RBC	0.85
Mouse Dunn osteosarcoma	
Transplanted tumors (n = 3)	
Dunn-1	<0.01
Dunn-1c	<0.01
Human osteosarcoma cell lines	
NIH	0.66
NCI-N377	0.40
SaOS	0.17
Primary tumors	
1	0.12
2	<0.01
3	0.10
4	0.05
5	0.08
6	<0.01
7	0.01
8	0.02
9	0.01
10	0.10

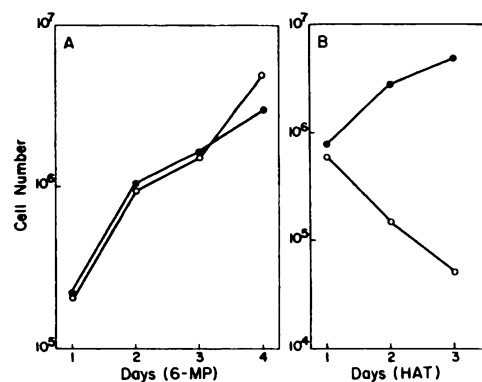


Chart 2. Log-phase cultures of Dunn-1c were grown on 60-mm plastic dishes in DME medium containing 10% fetal calf serum. In A, 10 μM 6-MP were added to one set of cultures, and growth rate was compared to that of untreated cells over the next 4 days. In B, HAT medium was added to one set of cultures, and growth rate was compared to that of untreated cells over the next 3 days. The cell count at each point in both A and B is the average of duplicate dishes. ●, controls; ○, drug treatments.

HPRT activity, they should also be susceptible to killing *in vivo* by a HAT-like combination of drugs. In clinical practice, MTX almost always replaces aminopterin. We therefore undertook a therapeutic trial in an attempt to exploit this purine salvage pathway deficiency by selectively killing cells that lack HPRT activity. C3H/HeJ mice were used 27 days after i.m. injection of 5 × 10⁶ Dunn tumor cells into the left hind leg. Both treatment groups received MTX (400 mg/kg) i.p. followed immediately by i.p. rescue with either LV (6 mg/kg) or hypoxanthine (50 mg/kg)-dThd (500 mg/kg)-HPP (10 mg/kg) (Table 2). On Day 3, after 8 doses of either LV or hypoxanthine-dThd-HPP, a second identical course of treatment was given to each group, respectively. In 2 separate experiments, animals rescued with hypoxanthine-dThd-HPP had a significant decrease in tumor size as compared to animals rescued with LV (Table 2).

DISCUSSION

The murine Dunn osteosarcoma spontaneously maintains a HPRT- phenotype. When Dunn tumors are grown *in vitro*, either as the original transplanted wild-type tumor or after selection of cloned cell lines, these cells fail to incorporate [³H]hypoxanthine, are not growth inhibited by exposure to 6-MP, will not grow in HAT medium, and have no detectable HPRT activity. Karyotypic analysis of tumor cells by Q-banding shows no evidence of a gross X-chromosome deletion.⁵ We are not aware of other spontaneous HPRT-deficient tumor models, although 6-thioguanine-resistant T-lymphocytes, presumed to arise *in vivo*, have recently been identified in human peripheral blood (1). It has been suggested that one determinant of resistance in blast cells from patients with acute lymphocytic leukemia (who are treated with drugs including 6-MP) is reversion to a HPRT- phenotype (21). The recent isolation of HPRT complementary DNA and genomic clones (5, 18) should facilitate a molecular analysis of these Dunn tumor cells. In fact, preliminary analysis of Dunn-1c cells indicates that the HPRT gene is present and that revertants to a HPRT+ phenotype can be isolated in HAT medium.⁶ In addition, other Dunn sublines (Dunn 2c to 5c) have similar phenotypes.⁶

Our animal treatment results show that, after MTX treatment, rescue with hypoxanthine-dThd-HPP rather than with LV of the mouse bearing an HPRT-deficient tumor produces a selective

⁵ H. T. Abelson and R. Tantravahi, unpublished results.

⁶ H. T. Abelson and C. Gorka, unpublished results.

Table 2

Treatment of C3H/HeJ mice bearing Dunn osteosarcoma

Values are the average tumor diameters in each group of mice. Estimated error in measurement was $\pm 4\%$.

Treatment	Maximum tumor diameter (cm)			<i>p</i>	% of change in tumor diameter from Day 1 to Day 7
	Before first treatment; Day 1	Before second treatment; Day 3	1 wk after first treatment; Day 7		
Experiment 1					
MTX-LV (4) ^a	2.2	2.4	2.3	0.04	+4
MTX-hypoxanthine-dThd-HPP (4)	2.3	2.3	1.5		
Experiment 2					
None (3)	2.5	2.4	2.9	1.0	+16
MTX-LV (8)	2.5	2.6 (7) ^b	2.7		
MTX-hypoxanthine-dThd-HPP (8)	2.3	2.5 (7) ^c	1.8	0.0006	+8
					-22

^a Numbers in parentheses, number of mice in each group.^b One mouse died; tumor diameter, 2.8 cm (excluded).^c One mouse died; tumor diameter, 1.7 cm (excluded).

antitumor effect by essentially duplicating HAT medium in the animal. Since the C3H/HeJ mouse bone marrow is not deficient in HPRT activity, the preformed pyrimidine, dThd, and purine hypoxanthine can be incorporated via the salvage pathways dThd kinase and HPRT, respectively. HPP is required in C3H/HeJ mice to inhibit liver xanthine oxidase so that hypoxanthine remains available for incorporation.⁶ The addition of 5×10^{-5} M adenine to HAT medium restores normal growth to Dunn-1c cells, indicating the presence of adenine phosphoribosyltransferase activity in Dunn-1c cells. In vivo, however, purine salvage via adenine phosphoribosyltransferase is not effective, since plasma adenine levels are low and the selective antitumor effect of hypoxanthine-dThd-HPP rescue is not overcome.

These results might help to clarify some of the present confusion that exists with respect to the role of high-dose MTX-LV therapy for human osteosarcoma. Our results suggest that some human osteosarcomas, like the Dunn osteosarcoma, may lack HPRT activity. If there is a subset of patients with osteosarcoma whose tumors have no detectable HPRT activity, then effective treatment in these patients with MTX-LV may be related to lack of purine salvage in the tumor via HPRT. It should be possible, therefore, to enhance selective tumor cytotoxicity in these patients by concurrent administration of MTX and dThd (7, 13–15, 23), analogous to what we have demonstrated for the Dunn osteosarcoma. Concurrent infusion of MTX with dThd alone has been shown to improve antitumor activity in L1210 leukemia (23). Assay of osteosarcoma at the time of initial surgery could identify those patients whose tumors exhibit this specific biochemical difference from normal cells and for whom a rational chemotherapeutic approach can be undertaken.

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