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Biochemical and antitumor activity of trimidox, a new inhibitor of ribonucleotide reductase

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Abstract. Trimidox (3,4,5-trihydroxybenzamidoxime), a newly synthesized analog of didox (*N*,3,4-trihydroxybenzamide) reduced the activity of ribonucleotide reductase (EC 1.17.4.1) in extracts of L1210 cells by 50% (50% growth-inhibitory concentration, IC_{50}) at 5 μ*M*, whereas hydroxyurea, the only ribonucleotide reductase inhibitor in clinical use, exhibited an IC_{50} of 500 μ*M*. Ribonucleotide reductase activity was also measured in situ by incubating L1210 cells for 24 h with trimidox at 7.5 μ*M*, a concentration that inhibits cell proliferation by 50% (IC_{50}) or at 100 μ*M* for 2 h; these concentrations resulted in a decrease in enzyme activity to 22% and 50% of the control value, respectively. Trimidox and hydroxyurea were cytotoxic to L1210 cells with IC_{50} values of 7.5 and 50 μ*M*, respectively. Versus ribonucleotide reductase, trimidox and hydroxyurea yielded IC_{50} values of 12 and 87 μ*M*, respectively. A dose-dependent increase in life span was observed in mice bearing intraperitoneally transplanted L1210 tumors. Trimidox treatment (200 mg/kg; q1dx9) significantly increased the life span of mice bearing L1210 leukemia (by 82% in male mice and 112% in female mice). The antitumor activity appeared more pronounced in female mice than in male mice. Viewed in concert, these findings suggest that trimidox is a new and potent inhibitor of ribonucleotide reductase and that it is a promising candidate for the chemotherapy of cancer in humans.

Introduction

Ribonucleotide reductase (EC 1.17.4.1.) is the rate-limiting enzyme of de novo deoxyribonucleoside 5-triphosphate synthesis and as such plays a major role in DNA biosynthesis. The activity of this enzyme is low in resting cells but increases with proliferation and malignant transformation [12, 23, 27]. Of the known inhibitors of ribonucleotide reductase, only hydroxyurea is at present in clinical use [21]; this simple hydroxamate inhibits the growth of various tumor cells in vitro and in vivo and also potentiates the cytotoxic effects of cytarabine [26].

Polyhydroxy-substituted benzohydroxamates have been synthesized as inhibitors of ribonucleotide reductase [13, 14]. One of these compounds, didox (*N*,3,4-trihydroxybenzamide; Fig. 1) has exhibited oncolytic activity against murine leukemias 1210 and P388 as well as against B16 melanoma, Lewis lung carcinoma, Colon 38 carcinoma, CD_8F_1 mammary tumor, and several human tumor xenografts [10]. On the basis of these studies, didox was investigated in phase I and II clinical trials on an acute drug treatment schedule [2, 20, 25]. Although a maximum tolerated dose of 6 g/m^2 was determined in these initial trials, no objective response was observed [2].

Recently, in a search for superior compounds, a polyhydroxybenzoate derivative, trimidox (3,4,5-trihydroxybenzamidoxime HCl; Fig. 1) [15] was synthesized as an analog of didox. In this study, we examined the cytotoxic and biological activities of the new compound trimidox in vitro and in vivo.

Materials and methods

Cytotoxicity studies. Leukemia 1210 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Company, Grand Island, N. Y.) containing 2 m*M* L-glutamine and 1% penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37° C. Logarithmically growing cells (1×10^5 cells/ml) were incubated with various concentrations of trimidox (1–100 μ*M*) for 72 h, and the effect on cell proliferation was determined by counting the cells in a Sysmex microcell counter (Sysmex, Japan).

Assay of ribonucleotide reductase activity. The enzyme activity was measured in extracts of L1210 cells as described elsewhere [18]. This

Abbreviations: Didox, *N*,3,4-trihydroxybenzamide; MTT, {3-[4,5-dimethyl-thiazo-2yl]}-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; trimidox, 3,4,5-trihydroxybenzamidoxime HCl

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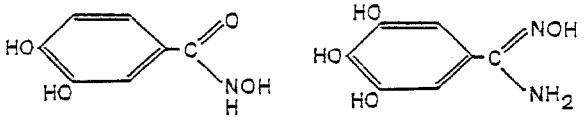

Fig. 1. Structures of didox and trimidox

method utilizes the conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP). Briefly, the assay mixture in 0.34 ml contained 3 μCi [^{3}H]-CDP (specific radioactivity, 14–19 mCi/mmol), 3.3 μmol adenosine triphosphate (ATP), 5.9 μmol $MgCl_2$, 8.8 μmol HEPES-HCl (pH 7.5), trimidox (0–10 μ*M*) or hydroxyurea (0–1 m*M*), 15 μmol dithiothreitol, and enzyme protein (0.4–1.3 mg). The reaction mixture was incubated for 40 min at 30° C and the reaction was terminated by heating at 95° C for 1 min. The products were separated by chromatography on Dowex 50-H$^+$ resin as described elsewhere [18].

For elucidating the changes in ribonucleotide reductase activity in situ, exponentially growing L1210 cells (5×10^5 cells/ml; 10 ml) were incubated with 7.5 μ*M* trimidox or PBS for 24 h at 37° C in a humidified atmosphere containing 5% CO_2. In separate experiments, L1210 cells were also incubated with PBS or trimidox (100 μ*M*) for 2 h at 37° C. The cells were then pulsed with [U-^{14}C]-cytidine (1.25 μCi, 2.8 μ*M*) for 30 min at 37° C. Cells were collected by centrifugation at 400 *g* for 5 min, washed twice with PBS, and processed to extract total genomic DNA, and the radioactivity was determined [4, 22].

Chemosensitivity assay. L1210 cells (2×10^5 cells/ml) were incubated in 96-well microtiter plates with various drug concentrations (1–100 μ*M*) for 48 h in RPMI 1640 medium. The reduction of the tetrazolium compound MTT was analyzed with an assay kit (Promega). The absorbance was recorded using a Beckman Biomek 1000 Work Station (Beckman Instruments Co., St. Louis, Mo.) [16].

Antitumor activity in vivo. L1210 cells (1×10^5/mouse) were transplanted intraperitoneally into groups of six $B_6D_2F_1$ male or female mice weighing between 17 and 20 g. Animals were housed in cages, and food and water were provided ad libitum. At 24 h after tumor implantation, mice were treated intraperitoneally once a day for 9 days with sterile saline or trimidox (25–200 mg/kg). Body weights of mice were recorded thrice weekly.

Results

The growth-inhibitory effect of hydroxyurea, didox, and trimidox on proliferating L1210 cells in culture is presented in Table 1. After a 72-h exposure of cells, hydroxyurea, didox, and trimidox exhibited 50% growth-inhibitory (IC_{50}) values of 50, 13, and 7.5 μ*M*, respectively. The cytotoxic effects of hydroxyurea, didox, and trimidox as determined by the tetrazolium-based cytotoxicity assay showed an IC_{50} value of 87 μ*M* for hydroxyurea, whereas didox and trimidox exhibited IC_{50} values of 41 and 12 μ*M*, respectively, showing that in molar terms, trimidox is the most potent cytotoxic agent among the three compounds tested (Table 1).

Inhibition of ribonucleotide reductase activity

L1210 cell extracts were incubated with various concentrations of trimidox and assayed for the enzyme activity. The results show that trimidox inhibited the activity of ribonucleotide reductase with an IC_{50} value of 5 μ*M*; however, under the same experimental conditions, hydroxyurea had an IC_{50} value of 500 μ*M*. Earlier studies have demonstrated that incorporation of radiolabeled cytidine into DNA correlates well with the activity of ribonucleotide reductase [24]. Thus, as a measure of in situ ribonucleotide reductase activity, when L1210 cells were incubated with 7.5 μ*M* trimidox (IC_{50}) for 24 h and then pulsed for 30 min with [^{14}C]-cytidine, 78% inhibition of cytidine incorporation into DNA was observed. When cells were incubated for 2 h at 37° C with 100 μ*M* trimidox and then pulsed with [^{14}C]-cytidine, cytidine incorporation into DNA was inhibited by 50% (Fig. 2).

Table 1. Cytotoxicity of ribonucleotide reductase inhibitors to L1210 cells in culture

	IC_{50} (μ*M*)[a]	
Agent	Growth inhibition	MTT assay
Hydroxyurea	50	87
Didox	13	41
Trimidox	7.5	12

L1210 cells (1×10^5 cells/ml) were incubated in 96-well microplates with various drug concentrations for 72 h. The reduction of the tetrazolium compound MTT was analyzed as described in Materials and methods

[a] Data represent mean values for 3 assays ($P < 0.05$)

Table 2. Antitumor activity of trimidox in L1210 leukemia-bearing mice[a]

	% Increase in life span	
Dose (mg/kg)	Male mice	Female mice
25	21*	36*
50	37*	38*
100	37*	52*
200	82*	112*

* Significantly different from the control value ($P < 0.05$)

[a] Groups of 6 mice each were treated with the indicated dose for a period of 9 days. Data represent mean values for 2 experiments

Antitumor activity of trimidox

Groups of male and female $B_6D_2F_1$ mice were intraperitoneally implanted with murine leukemia 1210 cells and then treated with saline or trimidox daily for 9 days. The mean survival time of mice injected with saline was 8 days; this was significantly increased in a dose-dependent fashion in both male and female mice by treatment with trimidox (Table 2). The results suggest that the antitumor activity of trimidox may be more pronounced in female mice, which showed an increase in life span of 112% at a dose of 200 mg/kg, whereas at the same dose, male mice exhibited an 82% increase in survival time. However, hydroxyurea given on a similar schedule at a dose of 2000 mg/kg produced an increase in life span of only 62% [11]. These studies in concert, suggest that trimidox is a more potent inhibitor of ribonucleotide reductase that exerts powerful antitumor activity in comparison with hydroxyurea.

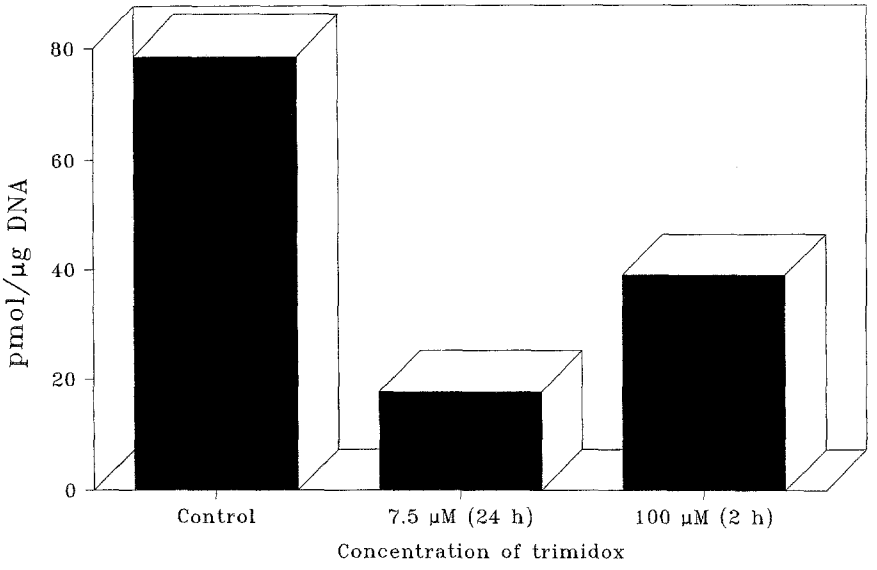

Fig. 2. Inhibition of ribonucleotide reductase in situ by trimidox. L1210 cells were incubated at 37° C with trimidox (7.5 µ*M* for 24 h or 100 µ*M* for 2 h) and then pulsed with [^{14}C]-cytidine for 30 min. Cytidine incorporation into DNA was determined as described in Materials and methods. Data represent mean values for 3 determinations

Discussion

Ribonucleotide reductase consists of two protein subunits, the nonheme iron subunit and the effector-binding subunit [9]; these can be inhibited independently. Drug combinations simultaneously targeting both ribonucleotide reductase subunits exhibit synergistic cytotoxicity [7, 8]. As no cross-resistance has been observed between agents targeting either subunit [3, 6], this enzyme is a sensitive target for cancer therapy [5]. Inhibitors of ribonucleotide reductase, such as hydroxyurea, combined with cytarabine provide synergistic cytotoxicity by inhibiting de novo pyrimidine nucleotide synthesis [19]. Pretreatment of cytarabine-resistant cells with hydroxyurea renders the cells once again sensitive to cytarabine [1]. However, thus far, only hydroxyurea has been used in the clinical setting, although it is a weak inhibitor of ribonucleotide reductase. It has some activity in leukemia, but its lack of chemical stability and rapid plasma clearance constitute major disadvantages [17]. Didox has been shown to be a strong inhibitor of ribonucleotide reductase and tumor growth in murine tumor models [10]. In spite of its impressive preclinical activity, this compound has been found to have a relatively short half-life in phase I/II clinical trials [2, 10], suggesting the necessity of its frequent administration to obtain effective antitumor activity. In early studies with didox, significant gastrointestinal (nausea, vomiting, and diarrhea) and hepatic toxicities were observed at the lower doses; therefore, didox was given as a 36-h infusion in an attempt to induce more prolonged inhibition of ribonucleotide reductase [2]. The lack of a clinical response to didox in the limited number of patients with refractory tumors might relate to the resistant nature of the tumors or to the inadequate plasma concentrations of didox reached in that study [2]. The relative lack of toxicity noted in the phase II trials of didox in patients with advanced breast cancer suggests that a higher dose may be required.

Trimidox was synthesized as part of a program initiated to obtain superior inhibitors of ribonucleotide reductase [11]. The present studies demonstrate that this compound exhibits potent inhibitory activity against ribonucleotide reductase both in vitro and in situ. We also compared its potency with that of hydroxyurea and didox. Among these three agents, trimidox was the most potent inhibitor of ribonucleotide reductase activity. In addition, trimidox significantly increased (by a factor of 2) the survival period of mice bearing L1210 tumors. Dose-dependent antitumor activity was noted for trimidox in mice implanted with L1210 cells. The antitumor activity appeared to be more pronounced in female mice than in male mice, and future studies should elucidate the reasons for this observation.

Novel aspects of this study include the elucidation for the first time of the biochemical, cytotoxic, and antitumor activity of a new and effective ribonucleotide reductase inhibitor, trimidox. This investigation should be helpful in the design of new inhibitors of ribonucleotide reductase.

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