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Trimidox, an inhibitor of ribonucleotide reductase, synergistically enhances the inhibition of colony formation by Ara-C in HL-60 human promyelocytic leukemia cells

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Abstract

Ribonucleotide reductase is the rate-limiting enzyme for the *de novo* synthesis of deoxynucleoside triphosphates and therefore represents a good target for cancer chemotherapy. Trimidox (3,4,5-trihydroxybenzamidoxime) was identified as a potent inhibitor of this enzyme and was shown to significantly decrease deoxycytidine triphosphate (dCTP) pools in HL-60 leukemia cells. We now investigated the ability of trimidox to increase the antitumor effect of 1-β-D-arabinofuranosyl cytosine (Ara-C). Ara-C is phosphorylated by deoxycytidine kinase, which is subject to negative allosteric regulation by dCTP. Therefore, a decrease of dCTP may cause increased Ara-C phosphorylation and enhanced incorporation of Ara-C into DNA. Ara-C incorporation indeed increased 1.51- and 1.89-fold after preincubation with 75 and 100 μM trimidox, respectively. This was due to the significantly increased 1-β-D-arabinofuranosyl cytosine triphosphate pools (1.9- and 2.5-fold) after preincubation with trimidox. We also investigated the effects of a combination of trimidox and Ara-C on the colony formation of HL-60 cells. A synergistic potentiation of the effect of Ara-C could be observed, when trimidox was added. Trimidox, which decreases intracellular deoxynucleoside triphosphate concentrations thus leading to apoptosis, enhanced the induction of apoptosis caused by Ara-C. We conclude, that trimidox is capable of synergistically enhancing the effects of Ara-C and therefore this drug combination might be further tested in animals.

Keywords: Ara-C; Ribonucleotide reductase; HL-60; Combination chemotherapy

1. Introduction

Ribonucleotide reductase (RR; EC 1.17.4.1) is the rate-limiting enzyme for *de novo* formation of deoxyribonucleotides and plays an important role in DNA synthesis. The enzyme activity was shown to be significantly increased in tumor cells and was linked with malignant transformation and proliferation [1–3]. The enzyme was, therefore, considered to be a good target for cancer chemotherapy [4,5]. A number of compounds inhibiting this enzyme were synthesized. Inhibitors of RR, such as hydroxyurea, gemcitabine, fludarabine, and chlorodeoxyadenosine are now used to treat various malignancies [6–9].

A novel group of inhibitors of RR are polyhydroxy-substituted benzoic acid derivatives [10–13]. Among these compounds, trimidox is a very effective and promising enzyme inhibitor and has demonstrated excellent anticancer activity in animal tumor models [14,15]. Trimidox was also shown to enhance the *in vivo* effect of adriamycin [14]. It depleted deoxynucleoside triphosphate (dNTP) pools and inhibited the growth of various tumor cell lines

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Abbreviations: dNTP, deoxynucleoside triphosphate; dCTP, deoxycytidine triphosphate; Ara-C, 1-β-D-arabinofuranosyl cytosine; Ara-CTP, 1-β-D-arabinofuranosyl cytosine triphosphate.

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more effectively than hydroxyurea [15,16]. Incubation of HL-60 cells with 50 μM trimidox for 24 hr caused a decrease of dCTP to 39% of control values [16].

Trimidox, such as other inhibitors of RR, might be also used in the treatment of leukemia patients. Because Ara-C is widely used in the treatment of acute myeloid leukemia (AML) as well as chronic myeloid leukemia patients, we now investigated the combination effects of trimidox and Ara-C in the human promyelocytic leukemia HL-60 cell line. Ara-C, when phosphorylated to its triphosphated form, competes with dCTP for incorporation into newly synthesized DNA. In addition, we showed previously, that treatment of HL-60 cells with trimidox significantly decreases intracellular dCTP pools [16]. Ara-C has to be phosphorylated to 1-β-D-arabinofuranosyl cytosine triphosphate (Ara-CTP) in order to be active. Ara-CTP is then incorporated into DNA and causes DNA fragmentation, strand breaks and apoptosis of tumor cells. The enzyme deoxycytidine kinase is responsible for this phosphorylation step of Ara-C. Deoxycytidine kinase activity is feed back inhibited by dCTP; therefore, we postulate that addition of trimidox to Ara-C treatment may result in increased Ara-CTP concentrations and Ara-C incorporation into DNA, leading to enhanced antitumor effects.

In the present study, we therefore, elucidated the combination effects of trimidox and Ara-C in HL-60 human promyelocytic leukemia cells. We examined the inhibitory effects on tumor cell colony growth of this combination. As trimidox was shown to induce apoptosis in HL-60 cells, we also investigated the induction of apoptosis by the combination of trimidox and Ara-C [17]. In addition Ara-CTP formation and Ara-C incorporation into DNA after treatment of HL-60 cells with both compounds was determined to elucidate the metabolic mechanisms, which are involved in this drug combination.

2. Materials and methods

2.1. Chemicals and supplies

Trimidox was synthesized as described [12] and was a gift of Dr. Bart van't Riet of Molecules for Health. All other chemicals and reagents were commercially available and of highest purity.

2.2. Cell culture

The human promyelocytic leukemia HL-60 cell line was purchased from ATCC (American Type Culture Collection). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (GIBCO, Grand Island Biological Co.), and with 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2. Cell counts were determined using the microcellcounter CC-108 (Sysmex). Cells growing in logarithmic phase of growth were used for all the studies described below.

2.3. Clonogenic assay

Cells (3×10^3 per plate) in the logarithmic phase of growth were plated in 1 mL RPMI medium containing 0.33% agar type VII (Sigma) and 15% fetal calf serum. Cultures were exposed to 0.5 and 0.75 μM of trimidox and to 0.5, 0.75, 1, and 3 nM Ara-C for 7 days at 37° in a humidified atmosphere containing 5% CO_2. Drugs were applied as single drugs and in combination. Colonies (>50 cells) were counted using an inverted microscope at 30× magnification. All experiments were repeated three times.

2.4. Hoechst dye 33258 and propidium iodide double staining

Cells were incubated with 10 and 15 μM trimidox and with 5 and 10 nM Ara-C alone or in combination for 8 hr. Hoechst 33258 (HO, Sigma) and propidium iodide (PI, Sigma) were added directly to the cells to final concentrations of 5 and 2 μg/mL, respectively. After 1 hr incubation at 37°, the cells were examined by fluorescence microscopy (Zeiss Axiovert 35) with Dapi filters. Cells were photographed on Kodak Ektachrome P1600 film and differentiated into three groups: viable, apoptotic (cells exhibiting early signs of apoptosis were counted as apoptotic), and necrotic cells [18].

2.5. Ara-C incorporation into DNA

HL-60 cells were incubated with 10, 25, 50, 75, and 100 μM trimidox for 24 hr. After resuspending in fresh medium, cells were incubated with 0.1 μM ^{3}H radiolabelled (specific activity 32 Ci/mmol; Amersham Pharmacia Biotech) Ara-C for 2 hr. Aliquots of 1×10^7 cells were centrifuged at 200 *g* for 5 min, washed with PBS, centrifuged, and resuspended in DNA lysis buffer (EDTA 0.5 M, Tris 1 M, Sarcosine 5%, pH 8.0). Then 20 U RNAse was added and incubated at 37° for 1 hr. Finally 150 μg proteinase K was added and incubated at 37° overnight. In 1×10^7 cells, 219 ± 83 pmol Ara-C were incorporated.

Then DNA was isolated according to standard procedures [19] and the radioactivity was measured using a Packard betacounter. Counts were corrected according to the total DNA concentrations in the respective sample.

2.6. Ara-CTP determination

HL-60 cells were incubated with 10, 25, 50, 75, and 100 μM trimidox for 24 hr. After resuspending in fresh medium, the cells were incubated with 10 μM Ara-C for another 4 hr. Then Ara-CTP concentrations were determined by HPLC as earlier described [20]. Untreated control cells formed 25.6 ± 0.5 μM Ara-CTP per 10^7 cells.

2.7. Statistical calculations

The calculations of dose–response curves and determinations of synergism or antagonism were performed using the ''Calcusyn'' software designed by Chou and Talalay (Biosoft, Ferguson) [21]. Significant differences were calculated by *t*-test using the Prism 3.0 software (GraphPad software Inc.).

3. Results

3.1. Clonogenic assay

The combination effects of Ara-C and trimidox were tested employing a clonogenic assay. HL-60 cells were seeded in the presence of the drugs in semisolid agar and the colony formation of HL-60 cells was ascertained. The number of colonies (more than 50 cells pro colony) formed in the presence and absence of the drugs after 7 days were counted. Results are shown in Fig. 1. The combinations of 0.5 and 0.75 μM trimidox with 0.5–3 nM Ara-C yielded synergistic effects. These relatively low IC_{50} values were found due to the long incubation period of 7 days. It was necessary to observe the colony growth for such a relatively long period due to the growth time of the control colonies. The combination indices according to the equation of Chou and Talalay were <1 for all combinations tested. These results indicate significant synergistic inhibition of colony formation by the combination of both drugs (data not shown).

3.2. Induction of apoptosis by Ara-C and trimidox

The induction of apoptosis was determined by Hoechst–propidium iodide double staining as described in the methods section. Results shown in Fig. 2 are percentages of apoptotic cells after 8 hr of drug exposure. All cells exhibiting even early signs of apoptosis were counted as apoptotic. The percentage of cells in apoptosis was significantly ($P < 0.05$) higher than the predicted values for additive induction of apoptosis. We have previously extensively studied the induction of apoptosis by trimidox in HL-60 cells [7]. Only one relatively low drug concentration of trimidox (15 μM) was selected on purpose in order to show the mechanism of additive induction of apoptosis by trimidox in combination with Ara-C (10 nM).

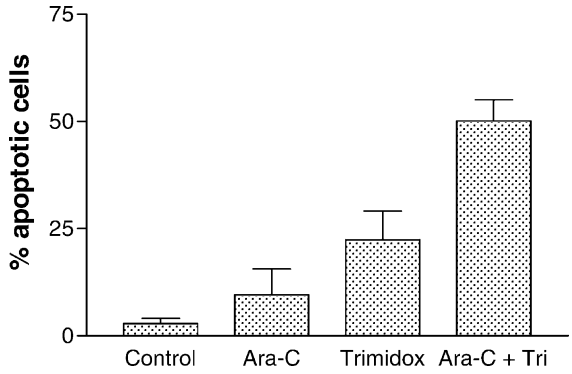

Fig. 2. Induction of apoptosis by Ara-C and trimidox in HL-60 cells. Cells were incubated with 10 nM Ara-C and 15 μM trimidox for 8 hr. Then, apoptotic cells were determined by fluorescence microscopy after Hoechst–propidium iodide staining. Data are means ± SEM of three determinations.

3.3. Ara-C incorporation into DNA

We measured the incorporation of radiolabelled Ara-C into the DNA of HL-60 cells. As shown in Fig. 3, preincubation of cells with 75 and 100 μM trimidox for only 24 hr significantly increased the incorporation of Ara-C into DNA. Preincubation with 75 μM trimidox caused a 1.5-fold ($P < 0.05$) and incubation with 100 μM trimidox caused a nearly 2-fold increase (189% of control, $P < 0.05$) of the Ara-C activity incorporated into DNA, when compared with control cells (Ara-C treatment alone).

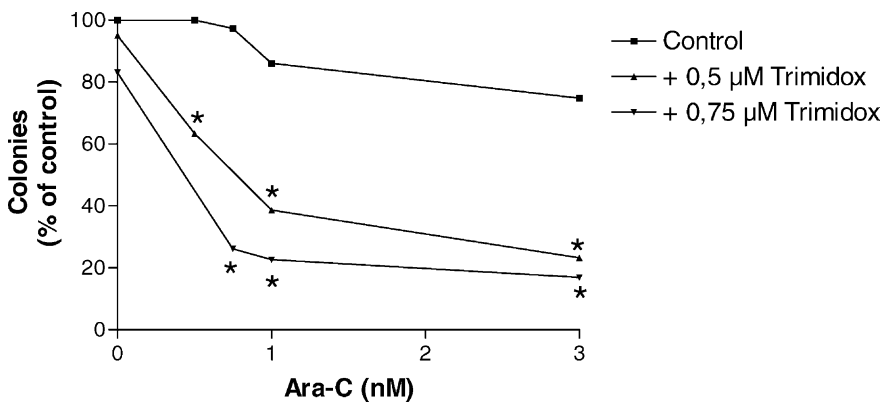

Fig. 1. Effect of trimidox and Ara-C on the colony formation of HL-60 cells. Cells were incubated for 7 days, then the number of the colonies was counted. Data are means of three determinations. SDs were within 5%. Synergistic combination effects according to Chou and Talalay (combination index <1) are marked with an asterisk (*).

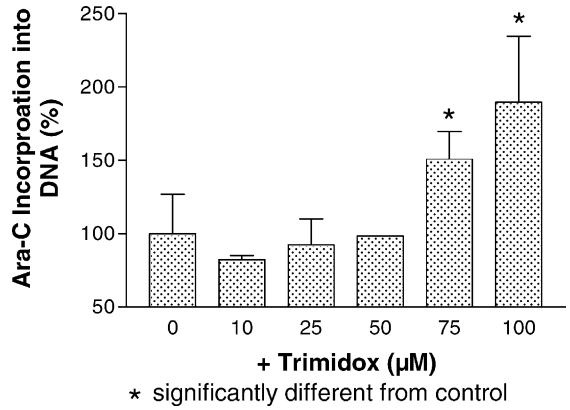

Fig. 3. Ara-C incorporation into DNA with and without trimidox preincubation. Cells were preincubated with various concentrations of trimidox for 24 hr, then trimidox was washed out and cells were incubated with 0.1 μM Ara-C for 2 hr. Data are means ± SEM of three determinations.

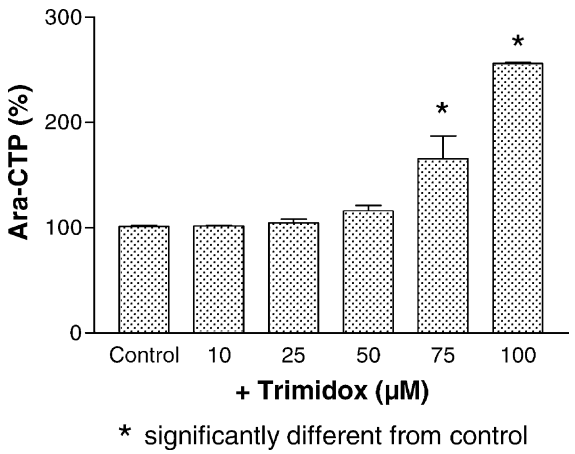

Fig. 4. Ara-CTP formation with and without trimidox preincubation. Cells were preincubated with various concentrations of trimidox for 24 hr, then trimidox was washed out and cells were incubated with 10 μM Ara-C for 4 hr. Data are means ± SEM of three determinations.

3.4. Ara-CTP concentrations after trimidox exposure

HL-60 cells were preincubated with 10–100 μM trimidox for 24 hr, then 10 μM Ara-C was added for 4 hr. After preparation of cell extracts according to the method described in the methods section, Ara-CTP concentrations were determined by HPLC. As shown in Fig. 4, preincubation of the cells with 75 and 100 μM trimidox caused a significant ($P < 0.05$) increase of intracellular Ara-CTP pools. Pool sizes increased to 167 and 256% of control values after incubation with 75 or 100 μM trimidox, respectively.

4. Discussion

Plagemann *et al.* [22] were the first to show that the metabolism of Ara-C can be enhanced by hydroxyurea in hepatoma cells. Ara-C is mainly used in the treatment of AML, but it was also shown that a combination of low dose Ara-C with interferon enhances the treatment outcome in patients suffering from chronic myeloid leukemia [23,24].

Extensive studies of Gandhi and coworkers suggested to modulate the Ara-C metabolism by coadministration with fludarabine, which is also an inhibitor of RR [25–27]. They could confirm their *in vitro* findings in patients suffering from AML and Gandhi's FLAG (fludarabine, Ara-C, G-CSF) protocol is now widely used for the treatment of myeloid leukemia patients [28–30].

It was also shown by Nandy *et al.* [31] that inhibition of RR by isoindole derivatives is capable of causing drug synergy with Ara-C in Ara-C sensitive as well as resistant cells and induces apoptosis in leukemia cells. Other authors have previously described that inhibition of *de novo* dNTP synthesis can enhance Ara-C sensitivity and even reverse Ara-C resistance [32,33]. As treatment failure is mainly caused by the development of resistance, successful modulation of Ara-C resistance can be crucial for the survival of many patients.

These results and the promising effects of *de novo* inhibition of dNTPs by inhibition of RR and salvage inhibition by Ara-C in leukemia cells, prompted us to investigate the combination effects of trimidox, a new inhibitor of RR with Ara-C. In addition it was shown earlier by Howell *et al.* [34], that didox, a dihydroxy-substituted benzohydroxamic acid derivative, which is chemically related to trimidox and also inhibits RR activity, can increase the incorporation of Ara-C into the DNA of HL-60 cells. A more than 2-fold increase of Ara-C incorporation into DNA could be observed after pretreatment of HL-60 cells with 100 μM didox. As didox also depletes intracellular dNTP concentrations significantly, these effects might be due to the increased Ara-CTP formation and subsequently to the increased Ara-C incorporation [34]. These results are similar to the effects seen in this study with the more promising compound trimidox. We now demonstrate that trimidox synergistically enhances the metabolism and the colony growth inhibitory effects of Ara-C in leukemia cells. After preincubation with trimidox, we could show that the main Ara-C metabolite Ara-CTP, as well as the incorporation of Ara-C into DNA was significantly increased. As both compounds induce apoptosis, we also investigated the induction of programmed cell death after treatment with trimidox and Ara-C. Increased apoptotic effects could be observed. Therefore, further *in vivo* studies can be recommended in order to test whether the described drug combination might become an additional treatment option for leukemia patients.

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