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Schedule-dependent synergistic action of tiazofurin and dipyridamole on hepatoma 3924A cells

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Summary. Tiazofurin is an oncolytic nucleoside analog that has shown therapeutic activity in end-stage acute nonlymphocytic leukemia and in chronic granulocytic leukemia in blast crisis. Tiazofurin is anabolized to the active metabolite, TAD, which inhibits IMP dehydrogenase activity, leading to a reduction in guanylate pools and to the cessation of neoplastic cell proliferation. The drug exhibits potent cytostatic and cytotoxic activity against hepatoma 3924A cells in culture. In growth-inhibition and clonogenic assays, the 50% inhibitory concentration of tiazofurin was 3.8 and 4.2 μM, respectively. Dipyridamole, an inhibitor of nucleoside transport, curtails the salvage of nucleosides and bases for nucleotide biosynthesis. Dipyridamole exhibited cytotoxicity against hepatoma 3924A cells, with an LC_{50} of 24 μM and an IC_{50} of 29 μM being recorded. A combination of tiazofurin and dipyridamole provided synergistic cytotoxicity in hepatoma 3924A cells in culture. This synergistic activity was dependent on the order of addition of the drugs. Simultaneous addition of the two drugs produced antagonism, whereas preincubation of cells with tiazofurin or dipyridamole followed by addition of the second drug resulted in synergy. TAD concentrations were significantly higher (129% and 135%) in cells that had been pretreated with tiazofurin or dipyridamole before the addition of the second agent as compared with cells that had been treated simultaneously (113%). These studies indicate the importance of the order of the addition of drugs to obtain a synergistic response in combination chemotherapy and suggest the need for a careful selection of drug modulation in clinical trials of tiazofurin and dipyridamole.

Introduction

Tiazofurin, 2-β-D-ribofuranosylthiazole-4-carboxamide, a C-nucleoside antimetabolite, exhibits potent antitumor activity against murine leukemias and curative activity against Lewis lung carcinoma [14]. The agent is cytotoxic to human lymphoid and lung cancer cells in culture [1, 4]. The mechanism of action of tiazofurin has been shown to be due to its anabolism to an analog of nicotinamide adenine dinucleotide (NAD), TAD, which inhibits IMP dehydrogenase activity, diminishing guanylate pools and resulting in the inhibition of cell growth and proliferation [3, 8, 9, 12]. Tiazofurin displays potent cytotoxicity and antitumor activity against hepatoma 3924A in vitro and in vivo [13]. Recently, tiazofurin has been shown to exhibit therapeutic activity in leukemic patients [15, 16, 19, 20].

The activities of enzymes in the salvage pathways are higher than those of de novo pathways of nucleotide biosynthesis, indicating that a salvage-pathway inhibitor might enhance the cytotoxicity of a drug combination [18]. Dipyridamole, an inhibitor of nucleoside transport, is cytotoxic to hepatoma cells in culture [21].

Synergistic cytotoxicity has been demonstrated for combinations of dipyridamole and acivicin (a glutamine antagonist) in hepatoma cells [21] and in human colon carcinoma cells [6]. Enhancement of the sensitivity of human colon carcinoma cells to growth inhibition by *n*-phosphonacetyl-L-aspartic acid (PALA) has also been reported for dipyridamole [2]. Methotrexate's cytotoxicity toward human colon cancer cells is augmented by dipyridamole [17], as is that of 5-fluorouracil [7]. Recently, potentiation by dipyridamole of Adriamycin's cytotoxicity

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Abbreviations: DP, dipyridamole; HPLC, high-pressure liquid chromatography; IC_{50}, drug concentration inhibiting 50% of cell growth; IMP, inosine monophosphate; LC_{50}, drug concentration inhibiting 50% of cell clonogenicity; PBS, phosphate-buffered saline; TAD, thiazole-4-carboxamide adenine dinucleotide; TR, tiazofurin; TCA, trichloroacetic acid; TOA, tri-*n*-octylamine

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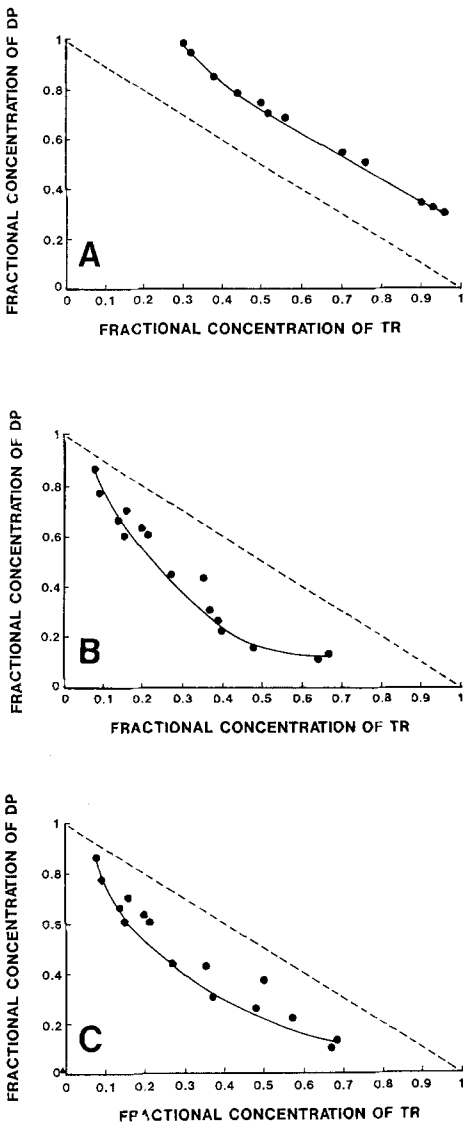

Fig. 1 A–C. Cytotoxicity of the combination of TR and DP toward hepatoma 3924A cells in culture. Hepatoma 3924A cells growing in the logarithmic phase were incubated for 48 h with TR (0.25–10 μM) and/or DP (2.5–10 μM) or with saline and cell growth was determined in triplicate assays by counting the cells in a Coulter counter as detailed in Materials and methods. **A** Simultaneous addition of the two agents. **B** Preincubation of cells with TR for 3 h before the addition of DP. **C** Preincubation of cells with DP for 3 h prior to the addition of TR

toward HeLa cells in vitro and sarcoma 180 cells in vivo has been reported [11].

The present study was aimed at elucidating the cytotoxic effect of combinations of dipyridamole and tiazofurin on hepatoma 3924A cells in culture. Unlike acivicin, which inhibits the de novo synthesis of both purine and pyrimidine nucleotides, tiazofurin selectively blocks the de novo synthesis of guanylates including guanosine triphosphate (GTP) and deoxyguanosine triphosphate (dGTP). 5-Fluorouracil and PALA preferentially reduce the de novo synthesis of pyrimidine nucleotides. Given that tiazofurin is now undergoing clinical trials and that our prior studies in tiazofurin-resistant hepatoma 3924A cells have demonstrated enhanced salvage capacity of guanylates [10], we hypothesized that the combination of a salvage-pathway inhibitor (dipyridamole) and tiazofurin should reduce the chances for the development of resistant cell populations. We also examined the influence of the order of addition of the drugs on their resultant cytotoxicity toward hepatoma 3924A cells in culture.

Table 1. Cytotoxicity of TR and DP toward hepatoma 3924A cells in culture

Compounds	Growth-inhibition assay IC_{50} (μM)	Clonogenic assay LC_{50} (μM)
TR	3.8	4.2
DP	29.0	24.0

Materials and methods

Materials

Tiazofurin (TR) was kindly provided by Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). Dipyridamole (DP) was supplied by Sigma Chemical Company (St. Louis, Mo.). [5-^{3}H]-TR (specific activity, 1.0 Ci/mmol) was procured from Research Triangle Institute (Research Triangle Park, N. C.). Materials for the preparation of cell-culture medium were obtained from Grand Island Biological Company (Grand Island, N. Y.). Partisil 10-SAX columns and HPLC apparatus consisting of a system controller, a data module, model 510 pumps, and a model 440 absorbance detector were obtained from Waters Associates (Milford, Mass.).

Methods

Cytotoxicity studies. Hepatoma 3924A cells were grown in McCoy's 5A medium supplemented with 10% fetal calf serum in an atmosphere containing 95% air and 5% CO_2 as described elsewhere [21]. For examination of the effect of drugs on cell growth, cells growing in the logarithmic phase (1×10^5 cells/ml; 5 ml/flask) were continuously exposed to various concentrations of drugs (TR and/or DP) or saline for 48 h and the numbers of cells were then determined in a Coulter counter [21]. Under the conditions of culture, hepatoma 3924A cells exhibited an average doubling time of 14 h. The effect of TR on hepatoma cell survival was examined as detailed elsewhere [21]. Briefly, 500 cells were seeded in flasks along with various concentrations of TR and/or DP or saline for 7 days; the colonies were stained with crystal violet, and the surviving fraction of treated cells was calculated as a percentage of the surviving colonies formed by untreated cells.

For evaluation of the effectiveness of the combinations, a dose-response curve was obtained for each drug and four or five concentrations were selected for combinations. The fractional inhibitory concentration of each drug was calculated by dividing the concentration of the inhibitor present in the combination by the amount of inhibitor that would be required to exert the same degree of inhibition by itself, and the results were then plotted [5]. The effects of the two compounds were additive when the points fell on a straight line, connecting unity on the ordinate with unity on the abscissa. Deviations to the left of this theoretical line indicated synergism; deviations to the right represented interference or antagonism between the drugs. By drawing intersecting straight lines through the experimental points, one arrives at a point where the combined fractional inhibitory concentrations reach a minimum, which represents the point of maximal effectiveness [5].

Studies on the metabolism of TR. For examination of the metabolism of TR, hepatoma cells growing in the exponential phase (6×10^6 cells/flask)

Table 2. Effect of TR and DP on the colony-forming ability of hepatoma 3924A cells in culture

Concentration (μM)		Survival of cells (%)			
TR	DP	TR alone (A)	DP alone (B)	Combination	
				Predicted (A × B/100)	Observed
2.5	2.5	84	104	87	65
2.5	5.0	84	103	87	64
2.5	10.0	84	94	79	68
5.0	2.5	25	104	26	11*
5.0	5.0	25	103	26	10*
5.0	10.0	25	94	24	6*
7.5	2.5	8	104	8	2*
7.5	5.0	8	103	8	2*
7.5	10.0	8	94	8	1*

[a] Hepatoma cells in culture were preincubated with TR for 6 h and then incubated with DP for 7 days in an atmosphere containing 95% air and 5% CO_2 as detailed in Materials and methods

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

were incubated with 10 μM [5-^{3}H]-TR (specific activity, 9.6 mCi/mmol) and/or DP at 37° C in an atmosphere containing 95% air and 5% CO_2 for 6 h. Cells were immediately centrifuged, washed once with cold PBS, extracted with 10% TCA, and immediately neutralized with TOA in freon as described elsewhere [10]. An aliquot of the neutralized extract was analyzed on a Partisil 10-SAX column using an ammonium phosphate buffer system as previously described [8, 10]. Under the conditions of the assay, TAD eluted at 19 min.

Results and discussion

The cytotoxicity of TR and DP toward hepatoma 3924A cells in culture was evaluated by growth-inhibition and clonogenic assays as shown in Table 1. The results suggest that the cytostatic concentrations of the two drugs were similar to the cytotoxic concentrations. However, the cytotoxicity of TR was 6- to 7-fold that of DP.

The effect of the combination of TR and DP on cell growth is plotted in Fig. 1. The fractional inhibitory concentrations of DP and TR were determined according to the method of Elion et al. [5]. Simultaneous addition of the two drugs (Fig. 1 A) produced an antagonistic effect. When TR was added first, followed 3 h later by DP, a synergistic effect was observed (Fig. 1 B) at optimal TR and DP concentrations of 2.5 and 10 μM, respectively. When DP was added first and TR was added 3 h later, synergistic cytotoxicity was again noted at optimal TR and DP concentrations of 2.5 and 10 μM, respectively (Fig. 1 C).

For further examination of the effect of pretreatment time, hepatoma 3924A cells were incubated with tiazofurin for periods of up to 12 h, after which dipyridamole was added and the effect on cell growth was examined. The results indicate that incubation of cells with TR for periods of 3–9 h prior to the addition of DP provided synergy (data not shown). The effect of TR and DP on the colony-forming ability of hepatoma 3924A cells is shown in Table 2. A synergistic response was obtained when cells were pretreated with TR prior to the addition of DP.

For elucidation of the reasons for the synergistic activity of TR and DP, the concentration of TAD, the active metabolite of TR, was quantitated in cells that had been exposed to the drug or drugs (Table 3). The results indicated a significant increase in the concentration of TAD when hepatoma 3924A cells were preincubated with either TR or DP as compared with the simultaneous addition of both compounds. The uptake of TR was not significantly affected by the order of addition of the two drugs (data not given).

Table 3. Influence of DP on the metabolism of TR to TAD in hepatoma 3924A cells in culture[a]

Order of addition of drugs	TAD concentration (pmol/g cells)	TR alone (%)
TR alone	150±3	100
Simultaneous addition of TR and DP	169±6	113
Preincubation with DP followed 3 h later by exposure to TR	203±6	135*
Preincubation with TR followed 3 h later by exposure to DP	193±3	129*

[a] Hepatoma 3924A cells growing in the exponential phase were incubated in quadriplicate with radiolabeled TR (10 μM) or DP (10 μM) for 6 h in an atmosphere containing 95% air and 5% CO_2. Cells were harvested and processed as detailed in Materials and methods

* Significantly different from the results obtained using TR either alone or simultaneously with DP ($P < 0.05$)

Protection of hepatoma 3924A cells from TR's cytotoxicity by guanosine indicates that the inhibition of de novo purine nucleotide biosynthesis by TAD might be overcome by salvaging guanosine [13]. It was hypothesized that as DP is a salvage-transport inhibitor [21], it should prevent salvage nucleotide biosynthesis and thereby provide synergistic activity. We also examined the influence of the order of addition of TR and DP on the resultant cytotoxicity. The results suggest that the order of addition is important for the cooperative action of the two compounds. The results further indicate that inhibition of the salvage pathway by DP may require some time and, thus, the simul-

taneous addition of the two agents would not provide optimal conditions for synergistic cytotoxicity.

Since TR has demonstrated significant activity in the treatment of leukemia [15, 20], it is important that effective combinations of TR be elucidated to enhance its therapeutic values and to prevent the emergence of resistant neoplastic cells. There is potential to reopen TR trials in solid tumors, depending on their capacity to form high levels of TAD.

The novel aspects of this study include the following: (1) synergistic activity was demonstrated for TR and DP in a solid tumor line, hepatoma 3924A, and (2) this synergistic activity was dependent on the order of addition of the two agents.

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