Cancer Chemother Pharmacol (1985) 15: 44–48

Cancer Chemotherapy and Pharmacology

Biochemical mechanisms for the scheduled synergism of (αS, 5S)-2 amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid and 5-fluorouracil in P388 leukemia*

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Summary. A study was made of the in vivo effects of equitoxic doses of AT-125 and 5-FU combination, being administered either simultaneously (% ILS 152) or with a 6-h pretreatment with AT-125 (% ILS 184). To examine the biochemical basis for the scheduled synergism, measurements were made of the concentration of PRPP, the specific activities of CPS II, cytidine, thymidine, uridine, deoxyuridine kinases, and fluorinated nucleotide formation in P388 tumors and the small intestine. Two hours after in vivo simultaneous treatment of mice bearing tumors the concentration of PRPP increased 9- and 6-fold above baseline in the tumor and the small intestine, respectively. In the AT-125 pretreatment arm the concentration of PRPP increased 18- and 7-fold above baseline in the tumor and the small intestine, respectively. CPS II activity was reduced to 28%–18% of control in the tumors in the simultaneous and pretreatment groups, respectively, whereas it remained unchanged in the small intestine. Specific activities of cytidine kinase (5.5 ± 1), thymidine kinase (4.0 ± 1.6), uridine kinase (35.6 ± 6.5), and deoxyuridine kinase (2.4 ± 1.1) nmol/mg protein/h remained unchanged with treatment. In concert with the increased intratumor concentration of PRPP, fluorinated nucleotide formation was proportionally increased in the treatment arms. These results indicate the importance of drug scheduling of the above two agents in treating P388 leukemia.

Introduction

Since the introduction of fluoropyrimidines by Heidelberger in 1957 [11], they have been widely used for the treatment of various solid tumors [10]. Some possible mechanisms of action of these fluoropyrimidines have been explored [1], including RNA- and DNA-directed actions of the 5-FU nucleotides 5-FUTP and 5-FdUMP [7, 17] and cell surface alterations after exposure to 5-FU [15]; however, the exact mode of their antitumor action remains unknown. Recently interest has developed in improving the response of 5-FU by combining it with other chemotherapeutic agents that might alter its metabolism and mechanism of action. On the basis of biochemical considerations, combinations of 5-FU with PALA [16], thymidine [20], methotrexate [6], or folinic acid [8] were evaluated, demonstrating enhanced activity of 5-FU when used in combination with the above agents.

We were interested in the biochemical interaction of 5-FU with AT-125, an inhibitor of selected steps in the pyrimidine and purine de novo biosynthetic system in bacterial and mammalian enzyme systems [13]. Further investigations indicated that AT-125 was an inhibitior of CPS II synthetase and had antitumor activity against L1210 and P388 leukemias in mice and human breast and lung tumor xenografts in athymic mice [19, 9, 12]. It was reported that the rate of decrease of the activities of glutamine-utilizing enzymes following injection of AT-125 was rapid, with a significant decline detected at 10 min. By 2 h, PRPP aminotransferase, CTP synthetase, and CPS II activities had decreased to 2%, 3% and 6%, respectively, of the control values in rat hepatoma. Activities remained at these low levels for 12 h after injection and then slowly returned toward the normal ranges. The inhibition of CPS II by AT-125 diminished the intracellular concentration of pyrimidine nucleotides. As a result of the inhibition of CPS II, the UTP concentration decrease and the incorporation of 5-FUTP into RNA may increase [16]. Furthermore, lowering the concentration of dUMP may potentiate the DNA-directed activity of 5-FU, since dUMP competes with FdUMP for the binding site of thymidylate synthetase [18]. Since AT-125 inhibits CPS II, an *early* step in the de novo pyrimidine biosynthetic pathway, while FdUMP inhibits a *later* step leading to the formation of thymidylate, the combination of the two agents has a biochemical rationale. Furthermore, since the inhibitory action of AT-125 on these enzymes is at its maximum between 2 and 12 h after administration, the scheduling of 5-FU with AT-125 may be important for enhancement of the oncolytic activity in P388 leukemia.

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Abbreviation: AT-125, (αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; 5-FU, 5-fluorouracil; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; PRPP, phosphoribosyl pyrophosphate; 5-FUMP, 5-fluorouridine monophosphate; 5-FUDP, 5-fluorouridine diphosphate; 5-FUTP, 5-fluorouridine triphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate: ATP, adenosine triphosphate; CPS II, carbamylphosphate synthetase II; PCA perchloric acid

* Presented in part at the Seventy-fourth Annual Meeting of the American Association for Cancer Research, San Diego, California, May 1983

Materials and methods

Chemicals AT-125 and 5-FU were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. PRPP, ATP, UMP, UDP, UTP, thymidine, cytidine, uridine, and deoxyuridine were purchased from the Sigma Chemical Co. (St. Louis, Mo). 5-Fluoro [2-^{14}C]uracil (9.1 mCi/mmol) was obtained from SRI International, Stanford, Calif. Sodium [^{14}C]bicarbonate (57.5 mCi/mmol) was purchased from New England Nuclear (Boston, Mass). 5-FU was dissolved in 0.9% saline immediately before use, and AT-125 was also dissolved in 0.9% saline; the pH was adjusted to 7.0–7.2.

Antitumor activity studies. P388 leukemia was obtained from American Type Culture Collection, Rockville, Md 20852. P388 leukemia cells (1×10^5) were injected IP to BDF_1 mice, and 24 h later chemotherapeutic agents were administered SC the doses being the same as the toxicological does. Mice treated with saline were used as control. Percent increase in life span (% ILS) of the mice treated with drugs was used as an index for antitumor activity.

P388 leukemic cells were carried in vivo and for experimental purposes tumor-bearing animals were sacrificed by cervical dislocation. Then the ascitic fluid was removed and the cells counted with a Coulter counter. The viability of the leukemic cells was tested with the Trypan blue exclusion test.

Tumor supernatant preparation for enzyme assays. Leukemic mice were sacrificed by cervical dislocation; tumor cells were aspirated and homogenized for 10 s with a polytron homogenizer in 1 vol. of a buffer consisting of 30% dimethylsulfoxide, 5% glycerin, 0.1 *M* Tris-HCL (pH 8,4) 0.1 *M* KCl, 0.2 m*M* EDTA, and 0.2 m*M* dithiothreitol. After centrifugation for 3 min at 12000 xg the supernatants were immediately used for assay. All dispensations were made at 4° C.

Biochemical parameters

Nucleoside kinases. Measurements of nucleoside kinase activities were carried out by incubation at 37° C for 15 min in a reaction mixture consisting of 5 µl ^{14}C-labeled precursor (cytidine 485 mCi/mmol, thymidine 54 mCi/mmol, uridine 52.4 mCi/mmol, deoxyuridine 58.4 mCi/mmol, 0.25 mCi), 5 µl 0.01 *M* ATP, $MgCl_2$ in 0.05 *M* Tris (pH 8.4) or buffer alone and 5 µl tumor supernatant. The reaction was terminated by adding 20 µl 2 *N* HCl followed by centrifugation at 12000 *g* for 3 min. Samples (5 µl) were subjected to ascending chromatography on Whatman No. 3M paper for 18 h using isopropyl alcohol: EDTA (saturated solution): toluene: 14MNH_4OH (320:44:40:4, v/v) as solvent for the measurement of pyrimidine nucleoside kinase. Chromatographic extraction and measurement of radioactivity were carried out as previously reported [14]. Enzyme activity was expressed as nanomoles of product formed per hour per milligram of protein.

PRPP assay. The method of Tax and Veerkamp [21] was modified as reported earlier [4]. Mice bearing P388 leukemia received 'simultaneous' or 'scheduled' treatment with AT-125 and 5-FU. Two hours after drug administration the animals were anesthetized with ether and the tumor cells were quickly aspirated from the peritoneum, centrifuged at 600 *g* for 15 min to pellet the cells, and homogenized in 5% PCA (tumor: PCA, 1:3 v/v). To each 1 ml homogenate, 50 µl 1 *M* potassium phosphate (pH 8.5) and 70 µl 40% KOH were added to bring the pH to 7–7.2. The neutralized homogenates were spun at 12000 *g* for 10 s in an Eppendorff microcentrifuge and immediately frozen in dry ice until use.

CPS II assay. CPS II activity was determined according to a previously described method [5] by measuring the incorporation of $NaH^{14}CO_3$ into *N*-[^{14}C]carbamyl-*L*-aspartate. To 1500-µl Eppendorff reaction vessels were added 10 µl 50 m*M* HEPES buffer, pH 7.2, containing 62.5 m*M* ATP, 75 m*M* $MgCl_2$, 37.5 m*M* *L*-aspartic acid, 8.2 m*M* *L*-glutamine, and 12.5% glycerin; 5 µl either HEPES buffer or 0.05 *M* 5-chloro-4-oxo-*L*-norvaline; and 10 µl tumor supernatant. After addition of 5 µl $NaH^{14}CO_3$ (1 mCi/ml) to the underside of the lids, vessels were centrifuged at 12000 *g* for 1 s to mix reactants and incubated at 37° C for 15 min. The reaction was terminated by heating at 95° for 2 min, after which 50 µl 2 *N* HCl was added to dissipate the unreacted [^{14}C]bicarbonate. Endogenous ATCase quantitatively converted the newly formed [^{14}C]carbamyl phosphate to *N*-[^{14}C]carbamyl-*L*-aspartate. The lids were cut off, and the vessels were submerged in vials containing 15 ml Aquasol and counted at 85% efficiency in a scintillation spectrometer. CPS II activity was taken as the difference in radioactivity obtained in reaction vessels lacking and containing the *L*-glutamine antagonist *L*-CONV(5,chloro-4-oxo-*L*-norvaline; 2-amino-5-chlorolevulinic acid). Confirmation that the differential radioactivity was solely *N*-[^{14}C]carbamyl-*L*-aspartate was accomplished by chromatography on a column of high-resolution anion-exchange resin. After overnight dialysis, the dialysed fluid was centrifuged for 3 min at 12000 *g* and immediately used for assays. All dispensations were made at 4° C.

Incorporation of [2-^{14}C]5-FU into nucleotides. Mice with P388 leukemia were divided into two groups of six. One group received AT-125 (12 mg/kg) and 5-FU (28.4 mg/kg) SC simultaneously, while the other group received AT-125 followed 6 h later by 5-FU. Cold 5-FU was spiked with 10 µCi [2-^{14}C]5-FU (SA 9.1 mCi/mmol) for 5-FU treatment of the tumor-bearing animal. Two hours after 5-FU administration mice were anesthetized with ether, and tumors were quickly removed, pelleted, weighed, and homogenized in 5% PCA (tumor: PCA, 1:3 v/v). PCA homogenates of tumor were centrifuged at 12000 *g* for 1 min in an Eppendorff centrifuge, after which the supernatant fluid was neutralized with 4 *N* KOH, kept on ice for 20 min, and centrifuged at 10000 *g* at 4° C to remove $KClO_4$. Neutralized extracts were stored at −80° C until use.

Measurement of nucleotides concentration. UMP, UDP, and UTP were separated by high-performance liquid chromatograph using a Model 6000 A solvent delivery system, and a Model U6K universal injector (Water Associates, Milford, Mass). Peak areas, retention time, and concentrations based on standards were calculated with a Model 720 System Controller and a Model 730 data module (Water Associates); a Partisil-10 SAX column (0.45 × 25 cm)

Table 1. Antitumor activity studies with AT-125 and 5-FU in P388 leukemia mice

Treatment* Group	Survival days (Mean ± SD	% Increase in life span
Saline control	8.2 ± 0.63	100
AT-125 and 5-FU simultaneously	12.5 ± 1.58	152
AT-125, 6 h later 5-FU	15.1 ± 1.8	184

* A minimum number of 6 mice were used in each group. 1×10^5 cells were injected into each mouse and drugs (5-FU [28.4 mg/kg] and AT-125 [12 mg/kg]) were administered 24 h after tumor injection into mice in the schedules which are outlined above

(Whatman Inc., Clifton, NJ) was used. Separation was accomplished by elution for 10 min at 1 ml/min with 0.005 *M* KH_2PO_4, pH 2.8, after which the flow rate was increased to 2 ml/min and a linear gradient of 0.005 *M* KH_2PO_4 (pH 2.8) to 0.5 *M* KH_2PO_4 (pH 4.8) was maintained over 20 min. Absorbance was monitored at 254 nm, and the fractions of eluate were collected every 30 s. UMP, UDP, and UTP were co-eluted with [^{14}C]5-FUMP, [^{14}C]5-FUDP, and [^{14}]5-FUTP, respectively.

Results

Antitumor acitivity parameters

The effects of AT-125 and 5-FU in combination administered either simultaneously or with AT-125 6 h before 5-FU to mice carrying P388 leukemia are shown in Table 1. The scheduled method of administration of the drugs generated a significantly improved % ILS (184) compared with treatment with both drugs simultaneously.

Biochemical parameters

Levels of PRPP, CPS II, cytidine kinase, thymidine kinase, uridine kinase, and deoxyuridine kinase were determined in tumor and small intestine of mice with P388 leukemia (Table 2). Tumor-bearing animals were divided into three groups of ten: saline alone; AT-125 and 5FU administered simultanously; and AT-125 given 6 h before 5-FU.

Tumor PRPP concentrations increased 9-fold (AT-125 and 5-FU administered simultaneously) or 18-fold (AT-125 administered prior to 5-FU) above the saline-treated baseline. In comparison, a normal tissue represented by small intestinal mucosa in this study demonstrated an increase in PRPP levels, which did not differ significantly according to the schedule of administration of AT-125 and 5-FU, there being a 6-fold vs a 7-fold increase in PRPP concentration with simultaneous vs scheduled administration, respectively.

Following treatment with either scheduled or simultaneous therapy with AT-125 and 5-FU, the CPS II activity in the tumor was inhibited to 80% of baseline, whereas the small intestinal mucosa was not inhibited with either treatment method.

Following drug treatment the specific activities of cytidine kinase (5.5 ± 1), thymidine kinase (4.0 ± 1.6), uridine kinase (35.6 ± 6.5), and deoxyuridine kinase 2.4 ± 1.1) of the tumors did not demonstrate any substantial inhibition compared with the baseline values.

The incorporation of [2-^{14}C]5-FU into various species of [2-^{14}C]fluororibonucleotides in the tumors were examined. The percent ratios of labeled fluororibonucleotide to the total fluororibonucleotide following the two treatment schedules are shown in Table 3. There was an approximate two-fold increase in the percent ratios of the mono-, di- and triphosphate fluororibonucleotide in the sequential arm of the study versus the simultaneous treatment.

Discussion

Combination chemotherapy with 5-FU has been used widely [2, 3]. Many such combinations are empirically designed. The purpose of this study is to examine the combination of the two selected agents based on the known biochemical mode of action. Moreover, it is our intention to demonstrate that the appropriate scheduling of such agents would synergize an antitumor activity of the agents.

Table 2. Biochemical studies with 5-FU and AT-125 in mice bearing P388 leukemia[a]

Biochemical parameters	Saline		AT-125 and 5-FU simultaneously		AT-125, 6 h later 5-FU	
	Tumor	Small intestine	Tumor	Small intestine	Tumor	Small intestine
PRPP (μM)	5 ± 2.4	2.1 ± 1.2	49.3 ± 6.9	12.6 ± 3.7	92.4 ± 10.5	15.9 ± 2.5
CPS II (nmol/mg protein/h)	574 ± 200	112 ± 113	166 ± 22	124 ± 15	109 ± 45	94 ± 17
Cytidine kinase (nmol/mg protein/h)	5.5 ± 1.1		5.2 ± 0.9		5.7 ± 2.0	
Thymidine kinase (nmol/mg protein/h)	4.0 ± 1.6		1.8 ± 0.3		3.9 ± 1.4	
Uridine kinase (nmol/mg protein/h)	35.6 ± 6.5		29.2 ± 7.5		29.2 ± 14.8	
Deoxyuridine kinase (nmol/mg protein/h)	2.4 ± 1.1		0.9 ± 0.2		1.7 ± 0.6	

[a] Cells bearing P388 leukemia were administered saline or 5-FU (28.4 mg/kg) and AT-125 (12 mg/kg) simultaneously or AT-125 (12 mg/kg) and 6 h apart 5-FU (28.4 mg/kg), the animals were killed and tumors were removed and processed separately for each biochemical parameter as described in *Materials and methods* and assayed. Small intestine was removed, cleaned with 0.9% ice-cold saline, incized with a sharp blade. The intestinal mucosa was carefully teased from the muscular portion of the bowel and processed as described above

Table 3. Effect of scheduling of the combination chemotherapy on nucleotides

	Incorporation of [2-^{14}C]5-FU into nucleotides[a]	
	AT-125 and 5-FU simultaneously (nmol/10^6 cells)	AT-125, 6 h later 5-FU (nmol/10^6 cells)
$\frac{^{14}\text{C-5FUMP}}{\text{5FUMP+UMP}} \times 100$	9.5 ±1.8	16.6±3.2
$\frac{^{14}\text{C-5FUDP}}{\text{5-FUDP+UDP}} \times 100$	3.26±0.9	6.3±0.86
$\frac{^{14}\text{C-5FUTP}}{\text{5-FUTP+UTP}} \times 100$	1.53±0.37	4.8±0.64

[a] Minimum of six mice were used for each group. Mice with P388 leukemia were divided into two groups and one group was treated with AT-125 (12 mg/kg) and 5-FU (28.4 mg/kg) IP, simultaneously, and the other group received AT-125 followed 6 h later by 5-FU spiked with 10 μCi [2-^{14}C]5-FU. Two hours after 5-FU administration mice were anesthetized with ether, and tumors were quickly removed are homogenized in 5% PCA. PCA homogenates of tumor were centrifuged at 12000 *g* for 1 min and neutralized with 4 *N* KOH, kept on ice for 20 min, and centrifuged at 10000 *g* at 4°C to remove $KClO_4$. Neutralized extracts were used for the measurement of nucleotides

Thus, we examined the combination of 5-FU and AT-125 in tumor-bearing animals.

We selected in vivo doses of 5-FU and AT-125 which, when these were administered as single agents, produced minimal toxicity. The toxicity was increased marginally when 5-FU and AT-125 were given concurrently, but increased substantially when AT-125 was administered 6 h prior to 5-FU at the given equimolar doses. Similarly, the antitumor activity of the combination consisting of AT-125 administered 6 h before 5-FU was superior to that of the two drugs given together (% ILS = 184 vs 162).

To elucidate the mechanism of the observed synergism an examination of CPS II and selected pyrimidine salvage enzymes were made. Intratumor CPS II activity was profoundly and equally inhibited with either arm of treatment, to 20% of baseline values, but small intestinal mucosa did not show any CPS II inhibition following drug treatment. With the inhibition of CPS II, tumoral phosphoribosyl pyrophosphate increased following treatment with the combination of AT-125 and 5-FU, but the tumoral PRPP pool was more marked with the scheduled than with the simultaneous treatment. Such an enhancement of PRPP pool sizes cannot be explained solely on the basis of CPS II inhibition in the tumor. Additionally, following treatment, small intestinal mucosa also demonstrated an increase in PRPP concentrations, which did not correlate with the observed CPS II activity in that organ site.

Finally, we examined the incorporation of [2^{14}C]5-FU in the tumor following treatment with 5-FU and AT-125 simultaneously and when AT-125 was administered 6 h before 5-FU. It was of interest to observe that the percent ratio of ^{14}C-5-fluororibonucleotide to the total unlabeled 5-fluororibonucleotide and respective ribonucleotide in the mono-, di-, and triphosphate species was approximately two-fold higher in the sequential than in the simultaneous chemotherapy arm.

Thus, we have demonstrated in this paper that the combination of 5-FU and AT-125 is biologically synergistic in enhancing the antitumor activity in vivo, and moreover, that with appropriate scheduling of the agents it is possible to maximize the antitumor effect of the agents. The synergism is in keeping with the increased tumoral PRPP pool size and correlates with the enhanced incorporation of ^{14}C-5-FU into ^{14}C-fluoronucleotides.

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Received October 9, 1984/Accepted December 11, 1984