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Effect of guanosine on antitumor activity of fluorinated pyrimidines against P 388 leukemia

Masaaki Iigo and Akio Hoshi

Pharmacology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji Chuo-ku, Tokyo 104, Japan

Summary. *The antitumor activity of the fluorinated pyrimidines 5-fluorouracil (FUra), 5-fluorouridine (FUrd), and 5-fluoro-2'-deoxyuridine (FdUrd) against P388 leukemia was markedly potentiated by the addition of guanosine (Guo), resulting in therapeutic synergism. Any combination of FUra at 1–20 mg/kg, FUrd at 0.3–1 mg/k, or FdUrd at 1–100 mg/kg with Guo at 100 mg/kg significantly potentiated the activity of FUra, FUrd, or FdUrd, respectively. The potentiation of these fluorinated pyrimidines by guanosine was abolished by the simultaneous administration of cytidine or uridine, but not of thymidine. In particular, cytidine was the strongest inhibitor of antitumor activity of these fluorinated pyrimidines, alone and in combination with guanosine.*

To obtain more effective treatment with the combination of various fluorinated pyrimidines and Guo, the influence of the molar ratios of Guo to the fluorinated pyrimidines on the antitumor activity against P388 leukemia was investigated. The increase in life-span became more pronounced with increasing molar ratios. The optimal molar ratios of Guo/FUra, Guo/FUrd, and Guo/FdUrd were more than 5, 100, and 5, respectively.

Introduction

With a view to improving the therapeutic effect of 5-fluorouracil (FUra), its use in combination with various nucleosides and nucleobases has been examined [6, 7, 13–15, 24, 27]. Some of these increase the activity of FUra, but increase the toxicity to the host simultaneously, so that they do not give a satisfactory improvement in therapeutic effect. Recently, we found that combination of FUra with guanosine (Guo) [10] or guanosine 5'-monophosphate [9, 12] markedly enhanced the antitumor activity of FUra without increasing toxicity to the host in various murine tumor systems, including solid tumors. This potentiation by guanosine may be due to an increase in FU nucleotides caused by both pyrimidine phosphorylase and uridine-cytidine kinase with increased ribose 1-phosphate derived from Guo [5, 11, 17].

Exogenous FUrd and FdUrd can be phosphorylated by uridine-cytidine kinase and thymidine kinase, respectively. On the other hand, FUrd and FdUrd are easily catabolized to FUra by pyrimidine nucleoside phosphorylase [3]. This enzyme activity is higher in tumor tissues than in normal tissues [22]. Therefore, besides FUra, a combination of Guo and FdUrd may also produce a high therapeutic effect. The effect of Guo on the antitumor activity of FUra and its nucleosides was determined.

Offprint requests to: M. Iigo

Materials and methods

Drugs. FUra, FUrd, and FdUrd were kindly supplied by Mitsui Pharmaceuticals, Inc., Tokyo, Japan. Nucleosides were obtained from Sigma Chemical Co. (St Louis, Mo., USA). All compounds were homogenized with 0.5% carboxymethyl cellulose in physiological saline and administered IP.

Animals. Groups of five or six male BDF_1 mice with body weights of 21–23 g (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan) were housed in plastic cages with wood chip bedding and received CA-1 food pellets (CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. All experiments were performed in an animal laboratory with controlled temperature (25° C).

Implantation and treatment of P388 leukemia. A standardized suspension prepared from BDF_1 mice bearing 7-day-old tumors was adjusted to provide 1×10^6 cells/mouse and was injected IP on day 0. Starting 24 h after implantation the mice were given daily treatments IP for 5 consecutive days. The survival times of treated and untreated mice were determined.

Evaluation of antitumor effect. Survival times of treated animals were compared with those of untreated controls in the P388 leukemia system, and the mean, rather than median increase in life-span (ILS) was calculated for statistical analysis. Data were analyzed for significance by means of the two-tailed Student's *t*-test.

Results

Effect of various nucleosides on antitumor activity of FUra, FUrd, and FdUrd

P388 leukemia was treated by consecutive daily IP injection for 5 days of FUra (3 mg/kg), FUrd (0.3 mg/kg), or FdUrd (30 mg/kg), singly or in combination with various nucleosides (100 mg/kg).

The combination of FUra plus Guo, inosine, adenosine, deoxyguanosine, thymidine, and xanthosine significantly enhanced the antitumor activity over that of FUra alone, but

Table 1. Effect of pyrimidine and purine nucleosides on antitumor activity of 5-fluorouracil against P388 leukemia

Treatment	Mean ± SD	ILS (%)	P^a
FUra	14.5 ± 0.8	45	
FUra + cytidine	10.5 ± 0.5	5	< 0.001
FUra + deoxycytidine	14.5 ± 0.5	45	NS[b]
FUra + uridine	13.7 ± 2.1	37	NS
FUra + deoxyuridine	14.8 ± 0.4	48	NS
FUra + thymidine	16.8 ± 0.8	68	< 0.001
FUra + adenosine	17.8 ± 2.3	78	< 0.01
FUra + deoxyadenosine	15.3 ± 0.8	53	NS
FUra + guanosine (Guo)	20.2 ± 2.6	102	< 0.001
FUra + deoxyguanosine	17.0 ± 0.6	70	< 0.001
FUra + inosine	18.8 ± 0.8	88	< 0.001
FUra + xanthosine	16.0 ± 0.9	60	< 0.05

Drugs were given IP on 5 consecutive days to groups of six BDF_1 mice. Doses of FUra and various nucleosides were 3 and 100 mg/kg, respectively. Mean survival time of untreated control mice was 10.0 ± 0.0 days

[a] Statistics were evaluated by the two-tailed Student's *t*-test by comparison with FUra alone

[b] NS, not significant

Table 2. Effect of pyrimidine and purine nucleosides on antitumor activity of 5-fluorouridine against P388 leukemia

Treatment	Mean ± SD	ILS (%)	P^a
FUrd	14.2 ± 0.4	42	
FUrd + cytidine	9.7 ± 0.8	− 3	< 0.001
FUrd + deoxycytidine	14.8 ± 1.6	48	NS[b]
FUrd + uridine	11.2 ± 1.0	12	< 0.001
FUrd + deoxyuridine	14.0 ± 2.0	40	NS
FUrd + thymidine	16.0 ± 1.2	60	< 0.01
FUrd + adenosine	14.7 ± 1.2	47	NS
FUrd + deoxyadenosine	13.5 ± 1.2	35	NS
FUrd + guanosine (Guo)	17.3 ± 1.5	73	< 0.001
FUrd + deoxyguanosine	14.8 ± 0.4	48	NS
FUrd + inosine	15.8 ± 1.2	58	< 0.01
FUrd + xanthosine	14.2 ± 0.8	42	NS

Drugs were given IP on 5 consecutive days to groups of six BDF_1 mice. Doses of FUrd and various nucleosides were 0.3 and 100 mg/kg, respectively. Mean survival time of untreated control mice was 10.0 ± 0.6 days

[a] Statistics were evaluated by the two-tailed Student's *t*-test by comparison with FUrd alone

[b] NS, not significant

the antitumor activity of FUra was decreased significantly by cytidine (Table 1).

In the case of FUrd, Guo, thymidine, and inosine potentiated the antitumor activity significantly compared with that of FUrd alone. Conversely, the combination with cytidine or uridine decreased the activity of FUrd significantly (Table 2).

On the other hand, the antitumor activity of FdUrd was markedly enhanced by Guo and inosine, but it was decreased significantly by cytidine, uridine, deoxyuridine, and thymidine (Table 3).

Thus, the antitumor activity of these three fluorinated pyrimidines was markedly potentiated by guanosine, potentiation by inosine being the next most marked. In contrast, antagonism of the activity of each of the fluorinated pyrimidines was seen when cytidine was administered simultaneously.

Table 3. Effect of pyrimidine and purine nucleosides on antitumor activity of 5-fluoro-2′-deoxyuridine against P388 leukemia

Treatment	Mean ± SD	ILS (%)	P^a
FdUrd	19.0 ± 0.6	68	
FdUrd + cytidine	14.5 ± 1.4	28	< 0.001
FdUrd + deoxycytidine	18.2 ± 0.4	61	NS[b]
FdUrd + uridine	16.0 ± 0.6	42	< 0.001
FdUrd + deoxyuridine	17.2 ± 0.4	52	< 0.001
FdUrd + thymidine	17.8 ± 1.0	58	< 0.05
FdUrd + adenosine	18.2 ± 0.4	61	NS
FdUrd + deoxyadenosine	18.5 ± 0.5	64	NS
FdUrd + guanosine (Guo)	23.5 ± 1.5	108	< 0.001
FdUrd + deoxyguanosine	19.5 ± 0.8	73	NS
FdUrd + inosine	21.0 ± 0.9	86	< 0.001
FdUrd + xanthosine	20.2 ± 1.5	78	NS

Drugs were given IP on 5 consecutive days to groups of six BDF_1 mice. Doses of FdUrd and various nucleosides were 30 and 100 mg/kg, respectively. The mean survival time of untreated control mice was 11.3 ± 0.8 days

[a] Statistics were evaluated by the two-tailed Student's *t*-test by comparison with FdUrd alone

[b] NS, not significant

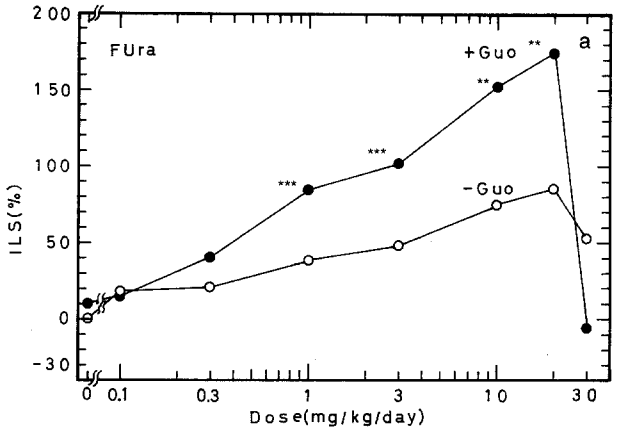
Effect of guanosine on the antitumor activity of FUra, FUrd, and FdUrd

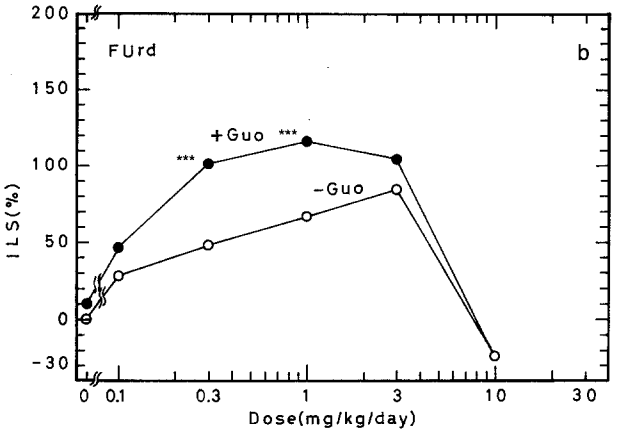
As shown in Fig. 1, survival time of animals was increased with increasing doses of FUra, FUrd, and FdUrd up to doses of 20, 3, and 100 mg/kg, respectively. Therapeutic effects in combination therapy with Guo (100 mg/kg) and FUra at 3−20 mg/kg, FUrd at 0.3−1 mg/kg, or FdUrd at 100 mg/kg were higher than the maximum effect of FUra, FUrd, or FdUrd monotherapy, respectively, and resulted in therapeutic synergism (these combinations yielded maximum effects greater than were achievable with monotherapy). The shape of the survival curve of mice treated with FUrd was different from that for the other two compounds; in this case the optimal dose was decreased.

The influence of molar ratio on the therapeutic activity of the combination of the fluorinated pyrimidines and Guo is shown in Fig. 2. Doses of FUra, FUrd, and FdUrd were optimal in combination treatment and at 20, 1, and 100 mg/kg, respectively. The ILS increased with increasing molar ratios. Guo/FUra and Guo/FdUrd molar ratios of more than 5 achieved the best therapeutic results. On the other hand, the Guo/FUrd molar ratio did not affect the antitumor activity until it reached 30, and a Guo/FUrd molar ratio of 100 was necessary for the potentiation.

Reversal studies

FUra at 3 mg/kg gave a 45% ILS and FUra at 3 mg/kg plus Guo at 100 mg/kg gave a 102% ILS ($P < 0.001$). However, the combined effect of FUra plus Guo was reversed by cytidine and uridine, but not by thymidine (Fig. 3. I). Cytidine at 100 mg/kg almost completely abolished the antitumor effect of





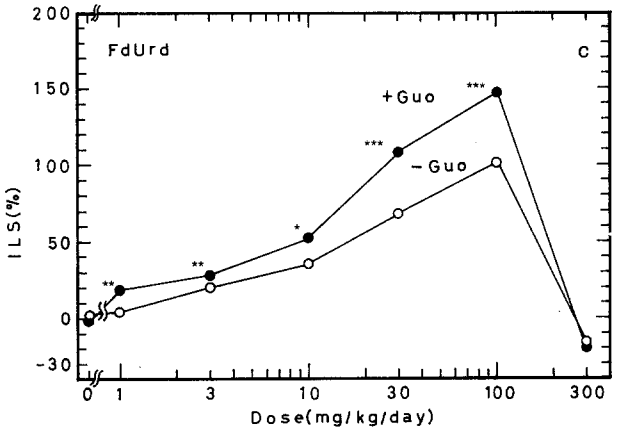


Fig. 1a–c. Effect of guanosine (100 mg/kg) on the antitumor activity of FUra (**a**), FUrd (**b**), and FdUrd (**c**). Drugs were given IP on 5 consecutive days to groups of five BDF_1 mice. * Comparison with fluorinated pyrimidine monotherapy, $P < 0.05$; ** comparison with fluorinated pyrimidine monotherapy, $P < 0.01$; *** comparison with fluorinated pyrimidine monotherapy, $P < 0.001$

FUra, but in the case of uridine the antitumor effect of FUra was maintained.

FUrd at 0.3 mg/kg gave a 42% ILS and the antitumor activity of FUrd was also potentiated by Guo (73% ILS, $P < 0.01$). This potentiation by Guo was reversed by any of the nucleosides except xanthosine and deoxyguanosine, but in

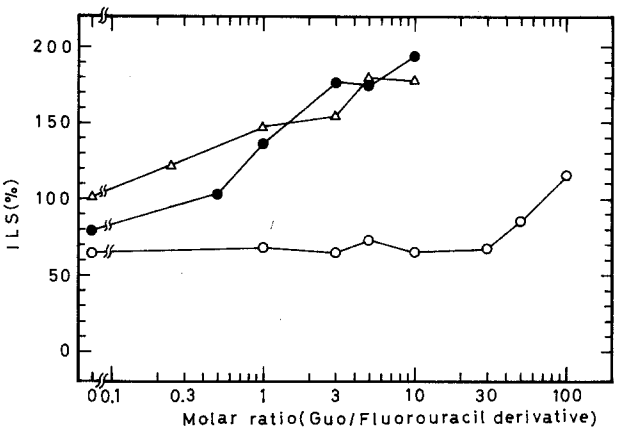


Fig. 2. Antitumor activity of fluorinated pyrimidines in combination with guanosine at various molar ratios. Doses of FUra (●), FUrd (○), and FdUrd (△) were 20, 1, and 100 mg/kg, respectively. Drugs were given IP for 5 consecutive days to groups of six BDF_1 mice

most cases the antitumor activity of FUrd was preserved. Cytidine and uridine completely abolished the antitumor effect of FUrd (Fig. 3. II).

On the other hand, the antitumor activity of FdUrd plus Guo was reversed by cytidine, uridine, and adenosine. Of these, cytidine was the strongest inhibitor of the antitumor activity of FdUrd (Fig. 3III).

Thus, none of the pyrimidine and purine nucleosides enhanced the antitumor activity more than the combination of fluorinated pyrimidines and Guo, and cytidine and uridine completely reversed the potentiation. The pattern of reversal of potentiation by Guo with nucleosides in the FUrd experiment was different from that in the FUra and FdUrd experiments.

Discussion

FUra and FdUrd have been used extensively in the adjuvant chemotherapy of various human tumors. To achieve potentiation of the therapeutic effect, combinations of normal metabolites with FUra have been examined [6, 7, 13–15, 24, 27]. Many of these combinations increase not only the antitumor activity but also toxicity to the host at the same time. Only a combination of FUra with guanosine potentiates the antitumor activity without increasing toxicity to the host in the L1210 leukemia system, and the combination shows a higher therapeutic ratio than FUra monotherapy [10, 11]. This combination was also active against P388 leukemia. Moreover, FdUrd and FUrd are markedly potentiated by guanosine, as shown in this study. Potentiation by guanosine is generally observed with fluoropyrimidine derivatives (FUra, FUrd, and FdUrd). Similarly, antagonism of potentiation by guanosine of the effects of these three fluorinated pyrimidines was observed when cytidine was administered simultaneously. Though the site of action in vitro differs among these three compounds [16, 29], the patterns of inhibition and reversal with nucleosides are similar in vivo. Metabolic changes of the compounds in the body are thought to be important for an understanding of antitumor activity in vivo.

Several mechanisms of action have been proposed for the fluorinated pyrimidines FUra, FUrd, and FdUrd. FUra and FdUrd are converted to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which binds irreversibly to thymidylate

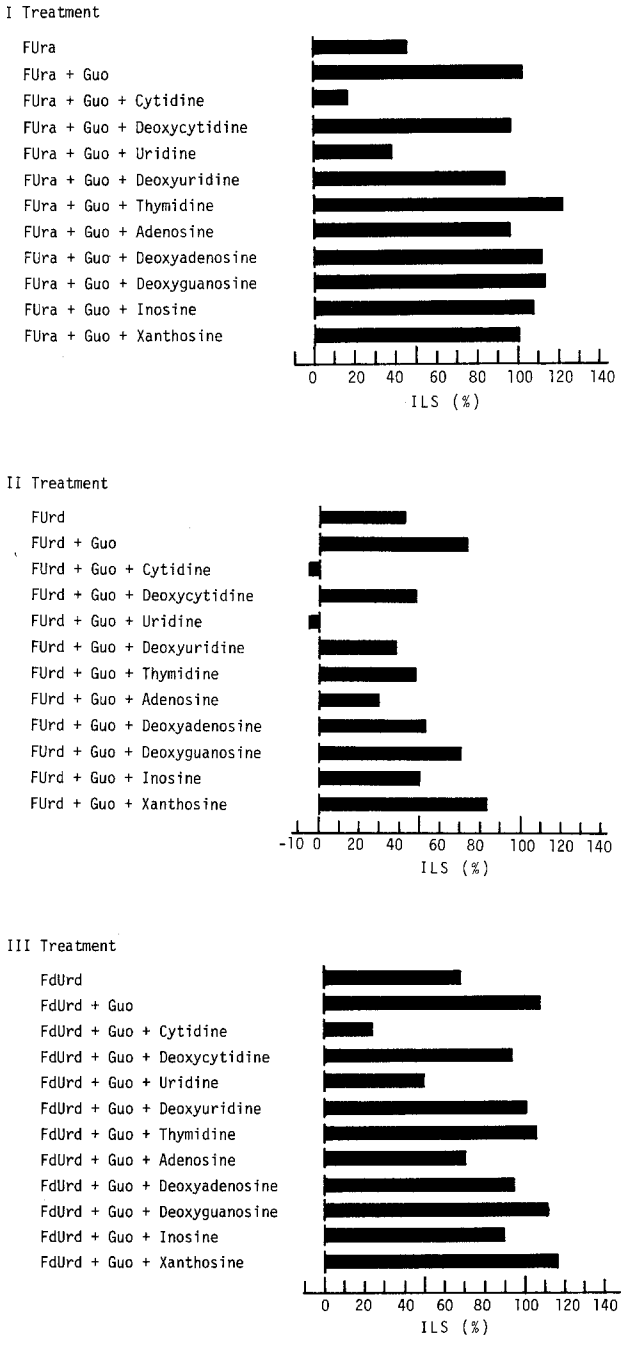


Fig. 3. Effect of pyrimidine and purine nucleosides on antitumor activity of various fluorinated pyrimidines plus guanosine. Doses of FUra *(I)*, FUrd *(II)*, FdUrd *(III)*, and various nucleosides were 3, 0.3, 30, and 100 mg/kg. Drugs were given IP for 5 consecutive days to groups of six BDF_1 mice

synthetase and inhibits DNA synthesis [1, 8, 19, 25, 26, 28]. The fluorinated pyrimidines are also incorporated into RNA and disrupt processing [2, 18, 20, 21, 23]. There is a highly significant relationship between incorporation of FUra into RNA and loss of clonogenic survival [20]. This relationship is maintained even in the presence of excess thymidine, which bypasses the effect of FdUMP on DNA synthesis. These findings suggest that the formation of FUra-RNA is the major mechanism of cytotoxic action in some cell lines. Furthermore, in this study, the potentiation by guanosine was found to be maintained even in the presence of excess thymidine, and this result also supports the concept that RNA, rather than the DNA-directed action of FUra, is the most important determinant of its activity.

Guanosine increases ribose 1-phosphate pools, which results in FUra phosphorylation to 5-fluorouridine 5′-monophosphate (FUMP), thereby increasing total intracellular FUra accumulation as nucleotides and incorporation into RNA [5]. In fact, guanosine stimulates the incorporation of FUra into RNA, but not in the presence of cytidine in sarcoma 180 cells [11]. The reduction of FUra phosphorylation in cytidine-treated cells presumably results from increased inhibiton of uridine-cytidine kinase caused by increased CTP pools, but the exact effect of cytidine on FUra metabolism at the molecular level is not clear as yet.

A large proportion of FdUrd is rapidly degraded to FUra by pyrimidine nucleoside phosphorylase [3] and incorporated into RNA [19]. Therefore, the mechanism of potentiation of the antitumor activity of FdUrd by Guo in vivo may be similar to that of FUra.

FUrd was also potentiated by guanosine, but the mechanism of its potentiation may be different from that of FUra and FdUrd, because ribose 1-phosphate is unnecessary for FUrd. To achieve significant potentiation ($P < 0.05$) of the effect of FUrd (1 mg/kg) a Guo/FUrd molar ratio of more than 50 (Guo = 54 mg/kg) was required, while the molar ratios needed for potentiation of the effects of FUra (20 mg/kg) and FdUrd (100 mg/kg) were more than 1.0 (Guo = 43 mg/kg) and 0.25 (Guo = 29 mg/kg), respectively. To potentiate the antitumor activity of FUrd, guanosine was required in a higher molar ratio than for FUra and FdUrd, but the amounts of guanosine administered per body weight were similar for all three compounds. The excess guanosine may produce a decrease in pyrimidine pools [4], inhibition of 5′-'cap' formation in small nuclear RNA species [30], and an increase in the drug transport into tumor cells, besides an increased ribose 1-phosphate level.

At any rate, guanosine enhanced the in vivo antitumor activity of the three fluorinatied pyrimidines without increasing its toxicity to the host.

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