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Original Articles

Antitumor Activity of 3′,5′-Diesters of 5-Fluoro-2′-deoxyuridine Against Murine Leukemia L1210 Cells

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Summary. *Antitumor activity of several 3′,5′-diesters of 5-fluoro-2′-deoxyuridine (FUdR) against L1210 leukemia cells following intraperitoneal administration was examined. Esters of FUdR with aromatic acid or aliphatic acid of longer chain length were markedly active. Their activities, with respect to ILS_{30}, were as much as 100 times that of unesterified FUdR. 3′,5′-ditoluoyl FUdR also had an improved therapeutic effect: its therapeutic ratio was increased to 8.1, as against 2.0 for FUdR. On the other hand, 3′,5′-diesters of FUdR with aliphatic acid of shorter chain length do not appear to be as active as FUdR. The relationship between the antitumor activity and plasma levels has also been examined. After 3′,5′-diacetyl FUdR, which is one of the drug group showing low cytotoxicity, the plasma concentration rapidly decreased to an unmeasurable level 3 h after dosing. This tendency is similar to that shown in FUdR. On the other hand, with 3′,5′-dipalmitoyl FUdR and 3′,5′-dibenzoyl FUdR, each of which has a marked antitumor effect, plasma concentrations decreased slowly and were maintained for as long as 48 h after dosing. The results show that the cytotoxicity of diesters of FUdR is correlated with the duration of a high plasma level of FUdR.*

Introduction

5-Fluorouracil (FU) is believed to exert its cytotoxic effect through the intracellularly generated metabolite, FUdR monophosphate, which is a powerful inhibitor of the enzyme thymidylate synthetase. This enzyme converts dUMP to dTMP, thereby providing a de novo biosynthetic pathway to a substrate of DNA polymerase [9, 12, 13]. FUdR, through its conversion to the proximate antimetabolite, FdUMP, was markedly effective in inhibiting the proliferation ability of a number of mouse cell lines in vitro [3, 21, 25, 26]. Its activity was up to 100 times higher than that of the mother compound FU on a molar basis. In in vivo studies, however, FUdR has been reported to be less effective than FU in increasing the lifespan of tumor-bearing mice [4, 14, 28]. The reasons for the low chemotherapeutic activity in animals have been reported previously [18]. The cytotoxicity of FUdR depends markedly upon the duration of the exposure of tumor cells to the drugs, and hence was not exerted in mice and humans when the FUdR plasma half-life is very short [6–8, 15, 22]. On contemplating the above property of FUdR, we thought it possible that the cytotoxicity of FUdR would be exerted in vivo in animals as well as in an in vitro system, if plasma concentrations of FUdR could be maintained by giving the depot form of FUdR. In this work, therefore, the antitumor activity of 3′,5′-diester-FUdRs, which are depot forms of FUdR, was examined. This report also confirms the relation between their antitumor activity and the kinetic behavior of the compound after dosing.

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Materials and Methods

Evaluation of Antitumor Activity. Male BDF_1 (C57Bl/6 × DBA/2) mice weighing 19–22 g were purchased from Shizuoka Agricultural Co-operative Association for Laboratory Animals (Hamamatsu, Japan) and a pellet diet (CA-1, CLEA Japan Inc.) and water were offered ad libitum. Each of six animals in each group was inoculated with 10^5 cells of the murine lymphoma L1210 which were maintained in male BDF_1 mice by an intraperitoneal (IP) transplantation every week, and IP injections of the drug were given once daily for 5 days, starting 24 h after transplantation. Thereafter the survival times of the mice were recorded. Antitumor activity of the drugs was evaluated by the increase in lifespan over controls (ILS: T/C,%), ILS_{30} (the dose showing 30% increase in lifespan), maximum ILS, and ILS_{max} (the dose showing maximum ILS) as reported previously [16]. Further, the therapeutic ratio (ILS_{max}/ILS_{30}) was calculated to compare the therapeutic advantage quantitatively.

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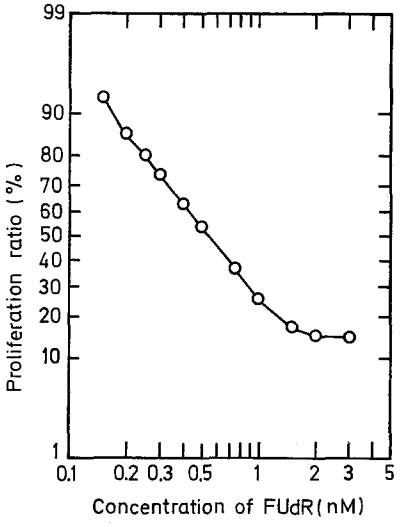


Fig. 1. Dose-response curve of FUdR on the proliferation of murine lymphoma, L5178Y cells

Assay of Plasma Concentration of FUdR. Test compounds were dissolved in physiological saline and were administered to male BDF_1 mice IP. Their doses were 40 μmoles/kg. Blood samples were drawn from the vena cava under ether anesthesia 15, 30, 60 min and 2, 3, 12, 24, and 48 h after dosing. Plasmas were promptly separated and diluted to the desired concentration for assay. Plasma concentrations of FUdR were determined by the bioassay method, because previously reported analytical methods, such as GC/MS or HPLC/MS [6, 22], lacked the sensitivity needed to study the kinetic behavior of FUdR, for which the threshold for antiproliferating effects appears to lie close to or below 0.2 n*M* [18]. L5178Y cells (5×10^4 cells/ml) in exponential growth were incubated at 37° C for 48 h with plasma to be tested for drug concentrations in a CO_2 incubator with a humid atmosphere of 5% CO_2 and 95% air, as described previously [17]. After incubation, the cell proliferation ratio of treated versus control cultures was calculated and the compound plasma concentrations were evaluated as FUdR-equivalent molar concentrations (μ*M*) graphically by the calibration curve, as shown in Fig. 1, proliferation ratios of L5178Y cells treated with FUdR at the graded concentrations being plotted in log-probit table. The sensitivity limit of this method is at least 0.6 μg plasma/ml (2.5 n*M*) as FUdR-equivalent moles; therefore, this method is suitable for studying the kinetic behavior of FUdR.

Results

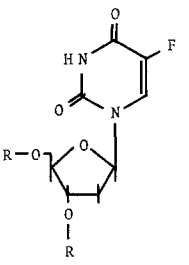
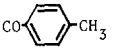
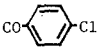
Antitumor Activity of Diesters of FUdR

Chemical structures of the FUdR diesters to be tested are shown in Table 1.

Survival times of mice bearing L1210 cells were increased by IP administration of FUdR, as shown in Fig. 2. An increase in survival time with increasing dose was noted until the dose reached 100 mg · kg^{-1} · day^{-1}, which was the optimal dose in this system; the maximum ILS was 54%. Beyond this level there was a decrease in survival time because of toxicity to the host. The value of ILS_{30}, determined graphically by the dose-response curve, was 50 mg · kg^{-1} · day^{-1} and its therapeutic ratio was 2.0 (Table 2).

Esters of FUdR with aliphatic acid of shorter chain length do not appear to be as active as unesterified FUdR. The ILS_{30} values of 3′,5′-diesters of FUdR with acetyl, propionyl, and butyryl were 100, 140, and 190 mg · kg^{-1} · day^{-1}, respectively. However, the antitumor activity of the ester with

Table 1. Chemical structures of 3′,5′-diester 5-fluoro-2′-deoxyuridines

General structure	Compound no.	−R
	I	-CO-CH_3
	II	-CO-C_2H_5
	III	-CO-C_3H_7
	IV	-CO-C_5H_{11}
	V	-CO-$C_{15}H_{31}$
	VI	-CO- 
	VII	-CO- 
	VIII	-CO- 
	FUdR	-H

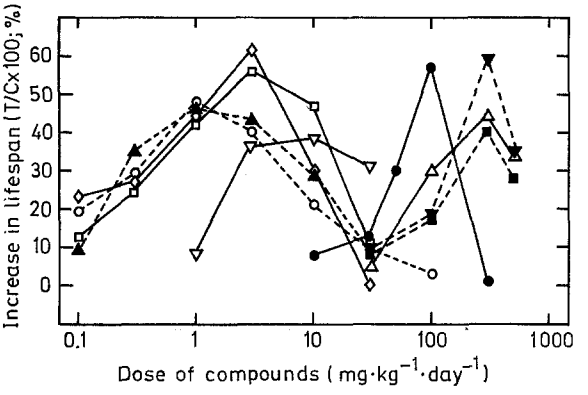


Fig. 2. Dose-response curves of diesters of 5-fluoro-2′-deoxyuridine against murine L1210 leukemia cells. Starting 24 h after implantation with L1210 cells, the mice received the compounds IP, as shown by symbols indicated below, once daily for 5 days. Control mice were treated with saline. Thereafter, the survival times of the mice were recorded, and the increase in lifespan (ILS) was calculated on the basis of the mean survival time of treated versus the control animals. (——●——), FUdR; (——△——), diacetyl-FUdR; (--▼--), dipropionyl-FUdR; (--■--), dibutyryl-FUdR; (——▽——), dihexanoyl-FUdR; (——□——), dipalmitoyl-FUdR; (--○--), dibenzoyl-FUdR; (——◊——), ditoluoyl-FUdR; (--▲--), dichlorobenzoyl-FUdR

Table 2. Antitumor activity of 3′,5′-diester-5-fluoro-2′-deoxyuridines and 5-fluoro-2′-deoxyuridine against L1210 lymphoma cells

Compound		ILS_{30}	ILS_{max}	Max. ILS (%)	Therapeutic ratio
		($mg \cdot kg^{-1} \cdot day^{-1}$)			
FUdR		50	100	54	2.0
I	(-diacetyl)	100	300	44	3.0
II	(-dipropionyl)	140	300	59	2.1
III	(-dibutyryl)	190	300	40	1.6
IV	(-dihexanoyl)	2.3	10	38	4.3
V	(-dipalmitoyl)	0.45	3.0	56	6.7
VI	(-dibenzoyl)	0.30	1.0	47	3.3
VII	(-ditoluoyl)	0.37	3.0	60	8.1
VIII	(-dichlorobenzoyl)	0.25	1.0	45	4.0

aliphatic acid of intermediate chain length (hexanoyl) is approximately 20 times higher than that of FUdR. The most active agent in the aliphatic ester series was the longer-chain acid ester (palmitoyl). Its ILS_{30} value was 0.45 $mg \cdot kg^{-1} \cdot day^{-1}$, which was less than one-hundredth that of FUdR. On the other hand, the optimal dose was greater, relative to FUdR, than that giving an ILS_{30}, and therefore the therapeutic ratio was increased to 6.7 from 2.0.

Esters of FUdR derived from other than aliphatic acids, aroyl esters, were also markedly active against L1210 cells. The ILS_{30} values of 3′,5′-diesters of FUdR with benzoyl, toluoyl and chlorobenzoyl were 0.30, 0.37, and 0.25 $mg \cdot kg^{-1} \cdot day^{-1}$, respectively. Especially, 3′,5′-ditoluoyl FUdR had an advantage in the therapeutic ratio over others, due to low toxicity, and the value was 8.1, which was the best among them. Antitumor activity of diesters of FUdR against adenocarcinoma-755 tumors has been reported by Nishizawa et al. [25]. From their data recorded with the range of 10–40 $mg \cdot kg^{-1} \cdot day^{-1}$, they could not distinguish the advantage that could be found at the lower doses, such as 0.3 or 1.0 $mg \cdot kg^{-1} \cdot day^{-1}$.

Plasma Levels of Compounds

Applying the bioassay technique used with L5178Y cells, which are highly sensitive to FUdR, we performed the measurement of drug concentration in plasma (Fig. 3). At 15 min after administration of FUdR plasma concentration of active metabolites derived from it was 12 μ*M* as FUdR-equivalent molars, but it rapidly declined monoexponentially, with an observed half-life of 5 min. This tendency was similar to that of earlier published findings on the time course of FU in plasma [7, 22]. Following 3′,5′-diacetyl FUdR, which has a low antitumor activity the time course of the plasma concentration is close to that of FUdR; a very rapid decrease of plasma concentration is observed within the first 3 h.

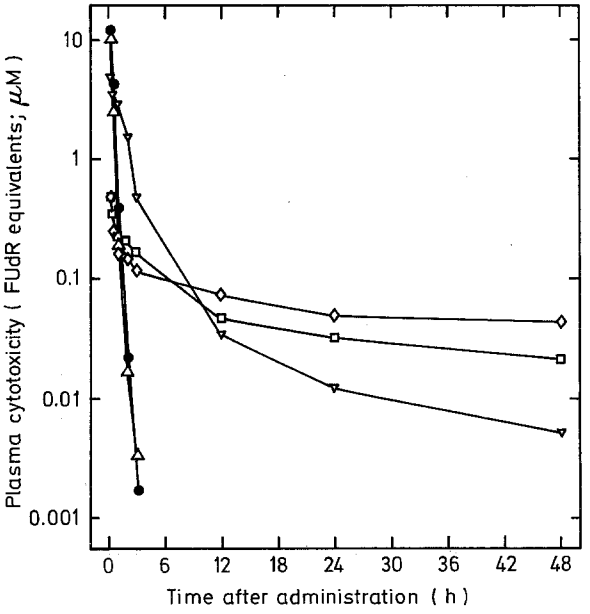


Fig. 3. Plasma concentrations of active metabolites after administrations of diesters of FUdR. Each compounds was administered intraperitoneally at 40 μmol/kg. At the indicated time after administration, plasma concentrations were determined by a bioassay method. Each values being is an average from five mice and is expressed as FUdR-equivalent molar concentration (μ*M*) (——●——), FUdR; (——△——), diacetyl-FUdR; (——▽——), dihexanoyl-FUdR; (——□——), dipalmitoyl-FUdR; (——◇——), ditoluoyl-FUdR

Thereafter plasma concentrations fall to unmeasurable levels (less than 2.5 n*M*). On the other hand, following, in 3′,5′-dipalmitoyl FUdR and 3′,5′-dibenzoyl FUdR, plasma concentrations at 15 min were lower than those of 3′,5′-diacetyl FUdR or FUdR, but they decreased slowly and were still retained the levels of 0.02 and 0.04 μ*M*, respectively, for as long as

48 h after a single dose. After 3′,5′-dibutyryl FUdR, its retention in the plasma is between that after 3′,5′-diacetyl FUdR and that after 3′,5′-dipalmitoyl FUdR. That is, the plasma concentration 48 h after dosing remained at the level of 0.05 μ*M*, which was enough to give 99% inhibition of proliferation of L5178Y cells cultured in medium containing FUdR for 24 h [18]. The above results confirm that the maintenance of a high level of FUdR over a considerable period produces an enhanced antitumor effect.

Discussion

In in vitro studies, FUdR is more active than FU against several tumor lines and is a better precursor of 5-fluoro-2′-deoxyuridine 5′-monophosphate, an active form of the drug. In mice or in human subjects, however, FUdR is not much more effective than FU, partly because of the rapid degradation of FUdR to FU by pyrimidine nucleoside phosphorylases [2, 23]. Chemical modifications of FUdR designed to render the drug resistant to phosphorolytic cleavage have not been successful [2, 19], nor have attempts to inhibit nucleoside phosphorylase activity with other compounds [1, 20, 24], indicating that other factors than the rapid degradation of FUdR to FU are involved. Recently, we demonstrated that the cytotoxicity of FUdR depended markedly upon the exposure time [18]. This high time dependency may contribute in part to the low chemotherapeutic effect in mice or humans, when plasma concentrations of the drug declined rapidly to noneffective levels within a few hours after dosing [7, 12]. These findings, therefore, indicate that the cytotoxicity of FUdR might be increased if a high level of the drugs in plasma were maintained by administration of a depot form of FUdR or by continuous infusion of FUdR itself. There are many reports of clinical studies of FUdR given by continuous intraarterial administration [10, 11].

However, Sullivan and Miller have claimed a marked increase in the toxicity of FUdR when it is given as a long infusion [29, 30], and similar claims have been made by Burrows et al. [5]. In the present study, we have succeeded in enhancing the activity of FUdR by introduction of ester on C-3′ and -5′ of its sugar moiety; in particular, esters of FUdR with aliphatic acid of longer chain length or with aromatic acid were markedly active against L1210 cells. It is also noteworthy that these compounds are effective over a relatively broad dose range, indicating a good therapeutic index. These esters were also observed to act as FUdR precursors for slow release of higher and more persistent levels of FUdR than are obtained with direct administration of FUdR. The rate of FUdR release from the esters might be controlled by modifying the ester groupings. Although the mechanisms of action of these compounds remain unknown, they are able to suppress in vivo tumor growth, presumably by being slowly anabolized in vivo to an active form and inhibiting thymidylate synthetase as FUdR does. If further investigations extend the search for new FUdR derivatives, the present data will be used to guide the design of more potent and less toxic antitumor agents.

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