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Potentiation of the antitumor activity of 5-trifluoromethyl-2′-deoxyuridine by the use of depot forms of the parent compound

Setsuo Takeda[1], Konstanty Wierzba[1], Jun-ichi Yamashita[1], Hiroshi Matsumoto[1], Hiroyasu Satake[1], Yuji Yamada[1], Norio Unemi[1], Yusuke Wataya[2], and Hikoya Hayatsu[2]

[1] Biological Research Lab., Taiho Pharmaceutical Co., Ltd., 224-2, Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-01, Japan, and
[2] Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

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Summary. 5-Trifluoromethyl-2′-deoxyuridine (CF_3dUrd), an antitumor agent, is known to be short-lived in human plasma. Since its rapid elimination from the bloodstream seems to have descouraged the clinical evaluation of this drug, we explored the potential use of masked derivatives of CF_3dUrd as "depot" forms of the parent compound. First, we observed that the toxicity of CF_3dUrd against HeLA cells in culture was 10^4 times greater for a 24-h treatment as compared with a 1-h treatment at identical concentrations of the drug, which suggests the importance of using a prolonged treatment period. In fact, the divided dosing of CF_3dUrd to L1210-bearing mice was markedly more effective than its single administration. 5′-*O*-Hexanoyl-, N^3-*p*-butylbenzoyl-, 5′-*O*-benzyloxymethyl-, and 3′-*O*-benzyl-CF_3dUrd were found to be effective in maintaining the CF_3dUrd concentration in plasma. The oral doses of these agents required to achieve 50% growth inhibition (ED_{50}) in mice bearing sarcoma 180 tumors were 19, 34, 10, and 13 mg kg^{-1} day^{-1}, respectively, whereas that of CF_3dUrd was 63 mg kg^{-1} day^{-1}. The ED_{50} values for these compounds were inversely correlated with the residence time of CF_3dUrd in plasma. The therapeutic indices of these compounds, calculated as the dose producing a 50% inhibition of body-weight gain (IB_{50}) divided by the ED_{50} value (1.89, 1,21, 1.40, and 2.15, respectively), were significantly higher than that of CF_3dUrd (0.78). Consequently, these depot forms of CF_3dUrd, particularly 3′-*O*-benzyl-CF_3dUrd, are expected to be more useful than the parent compound as antitumor agents.

Introduction

5-Trifluoromethyl-2′-deoxyuridine (CF_3dUrd), originally synthesized by Heidelberger and co-workers [7], has been shown to exert a potent suppressive effect on many transplanted tumors in mice. In particular, its therapeutic index against adenocarcinoma 755 is superior to that of 5-fluoro-2′-deoxyuridine [6]. On the basis of these observations, CF_3dUrd was once used clinically in cancer patients.

It is known that CF_3dUrd is phosphorylated by thymidine kinase to yield 5′-trifluoromethyl-2′-deoxyuridine monophosphate (CF_3dUMP) [2], which is a powerful and irreversible inhibitor of thymidylate synthase [11, 12, 17]. The latter enzyme is essential for DNA synthesis and has been postulated to play a rate-limiting role in this process. CF_3dUMP is consecutively phosphorylated to the di- and triphosphate forms by thymidylate kinase, and CF_3dUrd residues are incorporated into DNA, presumably via the triphosphate [4, 8]. It is believed that the inhibition of thymidylate synthase and the incorporation of CF_3dUrd into DNA play a central role in the antitumor activity of the drug. It is also known that the disappearance of CF_3dUrd from plasma in cancer patients follows first-order kinetics at a half-time of about 18 min [3]. CF_3dUrd has also produced an encouraging number of tumor regressions in patients with advanced breast cancer, but these regressions were difficult to maintain. Therefore, CF_3dUrd has not been evaluated as an anticancer drug [1].

In the present study, we attempted to develop a clinically effective anticancer drug whose structure was based on that of CF_3dUrd but which would be retained for longer periods in body fluids, resulting in the slow release of CF_3dUrd as the active agent.

Materials and methods

Reagents. The synthesis of four CF_3dUrd derivatives has been described elsewhere [18–20]. [6-^{3}H]-Fluorodeoxyuridine monophosphate ([6-^{3}H]-FdUMP) was enzymatically synthesized from [6-^{3}H]-5-fluororacil ([6-^{3}H]-FUra; New England Nuclear, Boston) using a homogenate-supernatant of mouse sarcoma 180 (S180) tumor that contained deoxy-

Abbreviations: CF_3dUrd, 5-trifluoromethyl-2′-deoxyuridine; CF_3dUMP, 5′-trifluoromethyl-2′-deoxyuridine-5′-monophospate; S180, sarcoma 180; L1210, L1210 leukemia; k_{el}, elimination rate constant; $T^1/_2$, half-life time; AUC, area under the curve; ILS, increase in life span; TS, thymidylade synthase; FdUMP, 5-fluoro-2′-deoxyuridine-5′monophosphate; FUra, 5-fluorouracil

Offprint requests to: S. Takeda

uridine phosphorylase and thymidine kinase. [6-^{3}H]-FdUMP was separated by thin-layer chromatography on PEI-cellulose and had a radioactivity of 12.9 Ci/mmol. All other chemicals were commerical products.

Evaluation of cytotoxicity. HeLa and S180 cells were grown as monolayers in 6-cm petri dishes containing Eagle's minimum essential medium supplemented with 10% calf serum in a CO_2 incubator maintained at 37° C for 7 days. Cells in the exponential growth phase were seeded in the culture medium to a density of 1×10^4 cells/4 ml per plate on day 0. On day 1, the cells were treated at 37° C with various concentrations of the compounds. After treatment, cells were centrifuged at 900 *g* for 10 min, resuspended in fresh culture medium, and then cultured. Cell counts were carried out on day 7.

Evaluation of antitumor activity. S180 and L1210 cells were used as experimental tumor models. S180 (5×10^6 cells) was implanted s.c. into 5-week-old male ICR mice (Japan Clea Inc., Tokyo, Japan), and L1210 (1×10^5 cells) was implanted i.p. into 7-week-old male CDF_1 mice (Shizuoka Laboratory Animal Agricultural Cooperative, Shizuoka, Japan). Groups consisting of seven mice each were used for the individual dose levels tested. CF_3dUrd was dissolved in physiological saline for i.p. injection. The depot compounds were suspended in 0.5% carboxymethyl cellulose solutions containing 0.1% Tween-80 and were given p.o. at a volume of 0.1 ml/10 g body weight. Drugs were given once a day for 7 consecutive days (days 1–7). The percentage of inhibition of the growth of S180 tumors was calculated by comparing the average tumor weight determined on day 10 in treated mice with that measured in animals that had received vehicle only (controls). The percentage of increase in life span (ILS) of animals bearing L1210 leukemia was calculated from the mean duration of survival in the treated group as compared with that in the control group, in which leukemic mice were treated with vehicle alone.

Therapeutic evaluation. The therapeutic index (IB_{50}/ED_{50}), which represents the relationship between the antitumor effect and the toxicity of a drug, was calculated for each compound as the ratio of the dose required to achieve 50% growth inhibition in the S180 test (ED_{50}) to the dose producing a 50% inhibition of body-weight increase in the host mice (IB_{50}). The IB_{50} values were determined from the average body-weight gain recorded for the treated mice during the test period (days 0–10) with that noted for the control animals.

Assay of the plasma concentration of CF_3dUrd released from its derivatives. Test compounds were given orally at a dose of 50 mg/kg to male ICR mice. Groups of three mice each were evaluated at the desired time points. The animals had been deprived of food for 18 h prior to drug administration. Blood samples were collected from mice under ether anesthesia at 0.5, 1, 2, 4, 8, and 12 h after drug administration and were centrifuged to separate the plasma. CF_3dUrd in the plasma was identified and measured by high-performance liquid chromatography (LC-3A liquid chromatograph, Shimazu, equipped with a UV-detector operating at 254 nm). Samples of 100 µl plasma were deproteinized by the addition of 100 µl methanol-concentrated HCl (120 : 1, v/v) followed by centrifugation, and 10 µl samples of the supernatants were then injected onto a µ-Bondapak C_{18} column (30 cm × 4.0 mm, Waters Associates). The mobile phase consisted of 10 mM KH_2PO_4 and a linear gradient of methanol (from 0 to 80% rate of increase, 4%/min). The flow rate was 1.5 ml/min. Under these conditions, CF_3dUrd was detectable as a discrete peak (retention time, 10.9 min) that was easily distinguishable from those corresponding to the masked derivatives and other substances in the plasma.

Pharmacokinetic evaluation of CF_3dUrd plasma concentrations following the administration of its derivatives. CF_3dUrd plasma concentrations were examined for kinetic analysis. The elimination-rate constants (K_{el}) and corresponding half-lives ($t_{1/2}$) were calculated from the terminal elimination phase using least-squares regression. The area under the CF_3dUrd plasma concentration-time curve (AUC) was calculated according to the trapezoidal rule using all the experimental data points. The final AUC values were calculated for $t = 0$ to infinity, including the residual AUC that was estimated from the last experimentally determined concentration divided by a corresponding elimination-rate constant [13]. Based on the elimination-rate constant, the time required to obtain CF_3dUrd concentrations corresponding to the IC_{90} values was calculated.

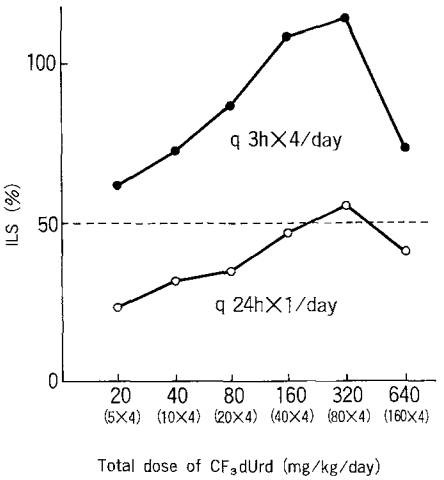

Fig. 1. Influence of the treatment schedule on the antitumor activity of CF_3dUrd against L1210 leukemia. The animals were given equivalent daily doses of CF_3dUrd either as a single daily injection (○——○) or as four divided doses injected at 3-h intervals (●——●)

Thymidylate synthase assay. The thymidylate synthase (TS) assay was done according to the method of Spears et al. [15, 16], in which the binding of FdUMP to TS protein is measured. The tissues were minced with scissors, homogenized, and sonicated at 4° C in 3 vol. of 200 mM TRIS-HCl buffer (pH 7.4, containing 20 mM 2-mercaptoethanol, 15 mM cytidylate, and 100 mM NaF), and cytosols were prepared by centrifugation at 105,000 *g* for 60 min. Then, 8 pmol [6-^{3}H]-FdUMP (50 µl in 50 mM potassium phosphate buffer, pH 7.4) was added to 50 µl cytosol plus 25 µl of a cofactor solution consisting of 50 mM potassium phosphate buffer (pH 7.4) supplemented with 20 mM 2-mercaptoethanol, 100 mM NaF, 15 mM cytidylate, 2% bovine serum albumin, 2 mM terahydrofolic acid, 16 mM sodium ascorbate, and 9 mM formaldehyde. The tubes were incubated in triplicate at 30° C for 20 min. Cold 1.0 *N* perchloric acid was added at 4° C, and the preparation was centrifuged at 3,000 rpm for 15 min to remove unbound labeled FdUMP. Each precipitate was washed three times with 0.5 *N* perchloric acid. The final precipitates were dissolved in 0.5 ml formic acid and ACS-II (Amersham, Japan), and the radioactivity was measured. The TS level was expressed in picomoles of FdUMP bound per gram of tissue.

Results

Treatment schedule and activity of CF_3dUrd

The antitumor activity of CF_3dUrd given on different treatment schedules was measured. The in vitro experiments using HeLa cells in culture showed that the growth rate of cells that had been treated with CF_3dUrd was impaired. The IC_{50} values were found to be 8.5×10^{-5} M (25.2µg/ml) for the 1-h exposure and 5.1×10^{-9} M (0.0015 µg/ml) for the 24-h continuous exposure. Thus, a comparison of the IC_{50} values reveals that the growth-inhibitory effect of CF_3dUrd was 4 orders of magnitude greater for the continuous 24-h exposure as compared with the acute 1-h exposure. It therefore appears that sustained treatment is far more effective than acute treatment in suppressing cell growth.

In the in vivo experiment, L1210-bearing mice were treated with CF_3dUrd and the prolongation of their survival was measured. Figure 1 shows the survival of the

Table 1. Therapeutic evaluation of CF_3dUrd derivatives

Compound and its molecular weight	R_1	R_2	R_3	ED_{50} (mg/kg/day)	IB_{50} (mg/kg/day)	Therapeutic index (IB_{50}/ED_{50})
CF_3dUrd (M. W. 296.2)	-H	-H	-H	63	49	0.78
5'-*O*-Hexanoyl-CF_3dUrd (M. W. 394.3)	$-C-(CH_2)_4CH_3$ (O)	-H	-H	19	36	1.89
N^3-*p*-Butylbenzoyl-CF_3dUrd (M. W. 456.4)	-H	-H	$-C-C_4H_9$ (O)	34	41	1.21
5'-*O*-Benzyloxymethyl-CF_3dUrd (M. W. 416.3)	$-CH_2OCH_2$	-H	-H	10	14	1.40
3'-*O*-Benzyl-CF_3dUrd (M. W. 386.3)	-H	$-CH_2$	-H	13	28	2.15

The mean weight±standard deviation in tumors of the control group was 2.37±0.57g.

animals either after i.p. administration of CF_3dUrd once a day for 7 days or after the injection of an equivalent total daily dose at four 3-h intervals for 7 days. The ILS at the maximum tolerated dose of 320 mg kg^{-1} day^{-1} was 123% for the animals treated by divided dosing and 57% for those given the collective dose. Clearly, the divided dosing resulted in a significantly longer survival of the mice than did the collective dosing.

Therapeutic evaluation of CF_3dUrd derivatives

The chemical structures of four CF_3dUrd derivatives and their therapeutic evaluation in animals bearing S180 solid tumors are shown in Table 1. These four compounds were the most effective among the 5'-*O*-acyl [18], N^3-acyl [18], *O*-alkoxyalkyl [19], and *O*-alkyl [20] derivatives of CF_3dUrd we have thus for prepared. The antitumor activity (ED_{50}) of these compounds were 2–6 times higher than that of CF_3dUrd. Their toxicity (IB_{50}) was somewhat higher than that of CF_3dUrd. Overall, the therapeutic indices (IB_{50}/ED_{50}) of these derivatives, especially 3'-*O*-benzyl-CF_3dUrd, were significantly greater than that of CF_3dUrd.

Effect of CF_3dUrd on HeLa and S180 cell growth in vitro

The cytotoxicity of CF_3dUrd was determined according to the ability of the drug to prevent the growth of experimental cells as compared with untreated controls. By addition of the drug at varying concentrations to the culture dishes, the IC_{90} value of CF_3dUrd (the concentration required to inhibit cell proliferation by 90% during the 7-day incubation period) was determined. The IC_{90} value found for CF_3dUrd against HeLa cells was 0.07 μg/ml. Almost the same value (0.06 μg/ml) was found for the drug against S180 cells in culture.

Plasma levels of CF_3dUrd after oral administration of CF_3dUrd derivatives

The plasma samples collected after oral administration of the CF_3dUrd derivatives were analyzed by high-performance liquid chromatography. All four derivatives gave rise to the generation of CF_3dUrd in plasma. The plasma concentration of CF_3dUrd was measured as a function of time following a single p.o. dose of CF_3dUrd and its derivatives at a fixed dose of 50 mg/kg (Fig. 2).

The peak plasma concentration of CF_3dUrd (8.15 μg/ml) was reached at 30 min (the first datum point in the curve), and the concentration rapidly decreased within the first 4 h. Thereafter, the plasma concentration fell to unmeasurable levels (less than 0.02 μg/ml). In contrast, although the plasma CF_3dUrd concentration achieved following dosing with 5'-*O*-benzyloxymethyl-CF_3dUrd was lower at 30 min than that obtained after CF_3dUrd administration, the subsequent decrease was much slower, resulting in levels higher than the IC_{90} value as late as at 12 h. The other three derivatives also showed prolonged maintenance of plasma levels of CF_3dUrd. On the basis of the time course shown in Fig. 2, kinetic parameters for the change in plasma

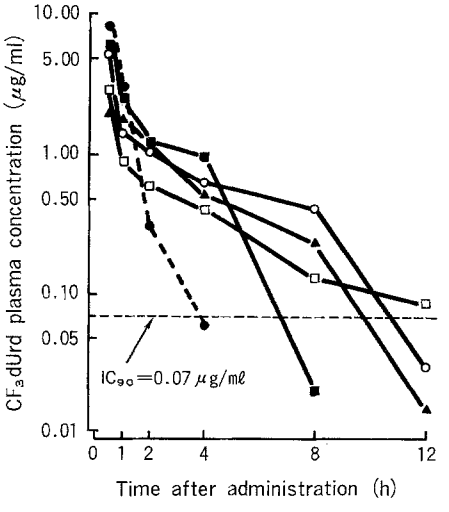

Fig. 2. Time course of CF_3dUrd plasma concentrations following p.o. administration of 50 mg/kg CF_3dUrd (●----●) 5'-*O*-hexanoyl-CF_3dUrd (○——○) N^3-*p*-butylbenzoyl-CF_3dUrd (■——■), 5'-*O*-benzyloxymethyl-CF_3dUrd (□——□), and 3'-*O*-benzyl-CF_3dUrd (▲——▲) to normal ICR/JCL mice

Table 2. Kinetic parameters for the change in plasma CF_3dUrd concentration following single oral doses of 50 mg/kg CF_3dUrd and its depot forms

Compound	Paramters				
	K_{el} (h^{-1})	$t_{1/2}$ (h)	$t_{0.07}$ (h)	AUC ($\mu g\ ml^{-1}\ h$)	Maximal concentration observed ($\mu g/ml$)
CF_3dUrd	1.389	0.50	3.65	6.96	8.15
5′-*O*-Hexanoyl-CF_3dUrd	0.336	2.06	10.82	8.96	5.32
N^3-*p*-Butylbenzoyl-CF_3dUrd	0.699	0.99	6.60	9.65	6.03
5′-*O*-Benzyloxymethyl-CF_3dUrd	0.208	3.33	12.05	5.33	2.93
3′-*O*-Benzyl-CF_3dUrd	0.301	2.30	11.50	6.95	2.06

K_{el}, Overall apparent elimination-rate constant; $t_{1/2}$, biological half-life; $t_{0.07}$, time during which the concentration of CF_3dUrd stays above 0.07 $\mu g/ml$; AUC, total area under the plasma-concentration curve

CF_3dUrd levels were estimated (Table 2). The overall rate of CF_3dUrd elimination from the plasma (K_{el}) correlated excellently with the ED_{50} value for the compounds (Fig. 3). On the other hand, neither the AUC values nor the observed peak concentrations showed any significant correlation with the anticancer activity as expressed as the ED_{50} value.

Thus, the overall elimination rate of CF_3dUrd seems to be a main determinant of the antitumor activity exhibited by these reagents. As shown in Fig. 3, the ED_{50} values for the CF_3dUrd derivatives correlated inversely with the time required to maintain an effective plasma concentration of CF_3dUrd.

Effect of a derivative on TS activity in tumor tissues

As a measure of the efficacy of depot drug, the TS activity in the tumor tissues was determined following the administration of 3′-*O*-benzyl-CF_3dUrd, the most useful CF_3dUrd derivatives tested in our experiment, to S180-bearing mice. As shown in Table 3, mice given 3′-*O*-benzyl-CF_3dUrd orally at 20 mg/kg showed greatly reduced TS levels for 8 h after dosing. Even at 24 h, the level remained low.

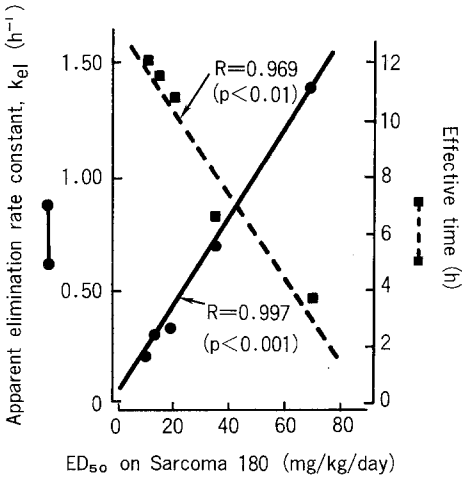

Fig. 3. Correlation between the antitumor effect (ED_{50}), the overall apparent elimination-rate constant (K_{el}), and the time (h) during which the CF_3dUrd concentration remained above 0.07 $\mu g/ml$ ($t_{0.07}$) following p.o. administration of CF_3dUrd and its four derivatives

Discussion

The present results clearly show that the cell-killing effect of CF_3dUrd strongly depends on the duration of the drug's action rather than on the short-term achievement of a high drug level (Fig. 1). An optimal clinical effect may therefore be expected following the continuous administration of a low concentration of CF_3dUrd to the tumor-bearing patient. In fact, during an early clinical trial, Ansfield and Ramirez [1] observed that CF_3dUrd was more effective when given in a series of intermittent injections.

This finding has led us to examine derivatives of CF_3dUrd bearing masking groups. Such derivatives may slowly release CF_3dUrd, the active principle, at a rate sufficient for the maintenance of its effective concentration in the body fluids over an extended period. Also, we desired to create an effective drug against solid tumors. Therefore, we used the S180 solid-tumor system for drug screening. The four CF_3dUrd derivatives that were used in the present study were found to be the most promising agents among the many CF_3dUrd derivatives we have thus for tested [18–20]. These four reagents showed antitumor properties more efficient than those of the parent drug CF_3dUrd (Table 1). Furthermore, they demonstrated a characteristic CF_3dUrd-releasing ability, exactly as we had

Table 3. Inhibition of TS in tumors following the oral administration of 3′-*O*-benzyl-CF_3dUrd to sarcoma 180-bearing mice

Time after drug administration (h)	TS activity as expressed by the FdUMP-binding activity (pmol/g tumor)	Inhibition of TS (%)
1	6.22±0.80*	86.9
2	5.96±1.05*	87.5
4	6.86±1.09*	85.6
8	12.09±2.61*	74.6
24	18.79±2.14*	60.5
Controls	47.60±5.98	0

Male ICR/JCL mice (6 weeks old) received 20 mg/kg 3′-*O*-benzyl-CF_3dUrd p.o. on day 10 following the s.c. transplantation of 5×10^6 sarcoma 180 cells on day 0. At various intervals after drug administration, 5 mice per group were killed and the tumors were rapidly excised, frozen, and stored at –60° C; 5 untreated mice served as controls. The TS activity in tumor tissues was measured by the [6-^{3}H]-FdUMP-TS binding assay (data represent mean values ± SD)

* $P < 0.001$

anticipated (Table 2, Fig. 2). In our judgement, therefore, these compounds are potentially more useful as agents against solid tumors than is the parent drug CF_3dUrd.

The K_i value for CF_3dUMP in the inhibition of TS has been reported to be 3.8×10^{-8} M (0.011 μg/ml) [12]. Therefore, the plasma concentrations of CF_3dUrd released from the tested derivatives, which lay above the 10^{-6} M level during the effective period, could have been sufficient to inhibit TS activity in the tumor cells. In fact, we observed that 3′-*O*-benzyl-CF_3dUrd inhibited TS activity in the tumor tissue by 75%–90% for 8 h after the oral administration of its ED_{50} dose to S180 tumor-bearing mice. The levels of thymidine kinase, the anabolic enzyme responsible for the transformation of CF_3dUrd into CF_3dUMP, and TS have been were reported to be higher in tumor tissues than in normal tissues of either rodents [9, 14] or humans [5, 10]. Therefore, a slow release of CF_3dUrd from these CF_3dUrd compounds would result in a selective suppression of tumor-cell growth, whereas the toxicity toward normal cells should be minimal due to the low level of these target enzymes in the latter.

The slow release and the lack of a sudden rise in the maximal plasma concentration of the active compound was associated with low toxicity as expressed by the corresponding therapeutic indices. Elucidation of the mechanism and kinetics of their biotransformation to CF_3dUrd and further studies on their antitumor activity against a wider range of tumor models will enable a thorough evaluation and, eventually, a clinical trial of these compounds. In addition, the present study provides a biochemical screening method for the search for more effective antitumor agents among CF_3dUrd derivatives.

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