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5-FLUORO-2-PYRIMIDINONE, A LIVER ALDEHYDE OXIDASE-ACTIVATED PRODRUG OF 5-FLUOROURACIL

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Abstract—5-Fluorouracil (5-FU) is an effective antitumor agent used in treating various cancers. Because of its metabolism by intestinal and other cells, 5-FU has an inconsistent bioavailability that limits its oral use. 5-Fluoro-2-pyrimidinone (5-FP), a 5-FU prodrug, was synthesized and found to be converted to 5-FU by aldehyde oxidase, an enzyme present in high concentrations in the livers of mice and humans but not in the gastrointestinal tract. Using BDF1 mice, the pharmacokinetics of 5-FP were studied and compared with those of 5-FU. The bioavailability of 5-FP given orally was 100% at a dosage of 25 mg/kg and 78% at a dosage of 50 mg/kg. The half-lives of both doses of 5-FP were at least 2-fold longer than the half-lives of the same doses of 5-FU, and the clearance rates of 5-FP were 3-fold slower. 5-FP was converted rapidly to 5-FU *in vivo*. The resulting 5-FU was measured at a steady-state level of 40–70 μM in plasma, at a dosage of 25 mg/kg, that was sustained for at least 4 hr. Also, when given orally, 5-FP was shown to have potent activity against Colon 38 tumor cells and P388 leukemia cells in mice. The therapeutic index of 5-FP was similar to that of 5-FU in these mouse tumor models. The potential clinical use of 5-FP as a prodrug of 5-FU should be considered.

Key words: aldehyde oxidase; antitumor; fluoropyrimidinone; fluorouracil; prodrug

Hepatic metastasis is still one of the major causes of morbidity and mortality in patients with colorectal carcinoma [1, 2]. 5-FU‡ is the drug most widely employed to treat colorectal cancer metastasized to the liver, is administered usually by systemic intravenous therapy, and is regarded generally as the most active agent against metastatic colorectal carcinoma as well as against other types of cancers [3]. The principle action of 5-FU is generally accepted as being inhibition of thymidylate synthase [4], and its incorporation into RNA and DNA [5–7]. Once FdUMP is phosphorylated to FdUTP and incorporated into DNA, its removal by uracil DNA glycohydrolase can cause DNA breaks [7]. 5-FU has a short plasma half-life, high total body clearance, and a high hepatic extraction ratio [8, 9]; however, the variable bioavailability among patients has limited its oral usage [10]. The major cause of differences in the bioavailability of 5-FU among individuals could be due to the metabolism of 5-FU in the gastrointestinal (GI) tract. We have shown previously [11, 12] that hepatic aldehyde oxidase possesses a novel activity that will oxidize 5-substituted-2-pyrimidinone deoxyriboses to their 5-substituted-deoxyuridine counterparts. 5-FP was shown by others to be converted to 5-FU by liver aldehyde oxidase [13, 14]. In our previous study [11], aldehyde oxidase activity was not detected in gastrointestinal tissue. This suggests the potential of 5-FP to serve as an oral prodrug of 5-FU, and thus, we hypothesize, that 5-FP may provide a more consistent bioavailability of 5-FU. Significantly, the potential use of 5-FP as a prodrug of 5-FU was not discussed by others reporting the aldehyde oxidase conversion of 5-FP to 5-FU [13, 14]. The present study describes the synthesis of 5-FP, the distribution of aldehyde oxidase in mouse tissue and the kinetics of the oxidation of 5-FP to 5-FU, the pharmacokinetics of 5-FP in mice, and the toxicity and antitumor activity of 5-FP in mice.

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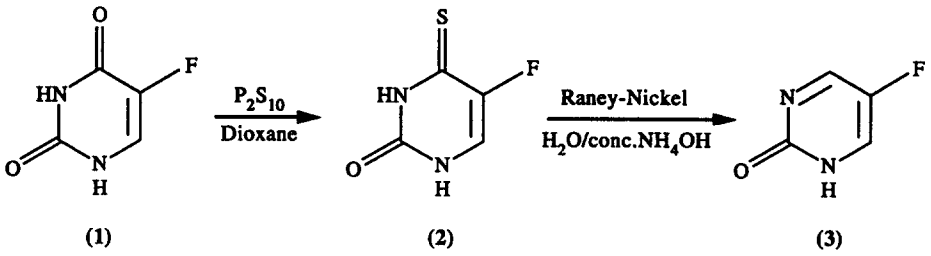
‡ Abbreviations: 5-FU, 5-fluorouracil; 5-FP, 5-fluoro-2-pyrimidinone; IPdR, 5-iodo-2-pyrimidinone-2′-deoxyribose; IUdR, 5-iodo-deoxyuridine; EPdR, 5-ethynyl-2-pyrimidinone deoxyribose; and TCA, trichloroacetic acid.

MATERIALS AND METHODS

Chemicals. 5-FP was synthesized originally by Helgeland and Laland in 1964 [15]. We synthesized [2-^{14}C]-5-FP from [2-^{14}C]-5-FU [compound 1; 2.1 mCi in 18 mL of a 1:1 dilution of H_2O and ethanol (v/v); Moravek Biochemicals Inc., Brea, CA]. The solution was transferred to a 25-mL round-bottom flask, evaporated to dryness at room temperature, and then co-evaporated with dry dioxane (3 × 1 mL) under reduced pressure. To the residue, 13.8 mg (0.11 mmol) of non-radioactive 5-FU, 34 mg phosphorous pentasulfide (P_2S_{10}, 0.08 mmol) [16] and 4 mL dioxane were added. The solution was heated to reflux with stirring for 3 hr, and then was filtered while hot; the resulting solid was washed three times with 1 mL dioxane. The filtrate and washings were combined and evaporated under reduced pressure to yield crude [2-^{14}C]-5-fluoro-4-thiouracil (compound 2), which was used for the next reaction without further purification.



(1) (2) (3)

Scheme 1.

A solution of compound 2 in 8 mL of H_2O and concentrated ammonium hydroxide (5:4, v/v) was heated to reflux wth stirring [17]. Raney-Nickel® (20 mg) was then added to the mixture [18], and after 3 hr the reaction was essentially complete. The hot solution was filtered and washed several times with hot water. The washings were combined and evaporated to dryness *in vacuo*. The residue was passed through a short silica gel (1.2 g) column and eluted with a chloroform:ethyl acetate:ethanol mixture (CH_2Cl_2:AcOEt:EtOH, 2:2:1, by vol.). The fractions containing the target compound were combined and evaporated under reduced pressure to obtain the crude [2-^{14}C]-5-FP (compound 3).

The progress of the reactions described above was monitored by thin-layer chromatography (EM precoated silica gel sheets containing a fluorescent indicator) using unlabeled samples previously synthesized in our laboratory as references. The respective R_f values in CH_2Cl_2:EtOAc:EtOH (4:4:1, by vol.) are as follows: compound 1: R_f 0.67; compound 2: R_f 0.77; compound 3: R_f 0.38. Compound 3 was further purified by HPLC using a Partisil SAX-10 column with water as the mobile phase before animal studies were initiated. The purified 5-FP had a UV absorbance maximum at 316 nm.

IPdR and its analogs were synthesized by Dr. T. J. Bardos and coworkers at the State University of New York, Buffalo, NY [19].

Animals. Female BDF1 mice (weighing 18–20 g) were purchased from Charles River Laboratories (Portage, MI) and were used when they were 8–10-weeks-old for the toxicity studies, for the maintenance of transplanted tumors, and for pharmacokinetics studies. All animals were cared for according to N.I.H. guidelines.

Evaluation of toxicity of 5-FU and 5-FP in BDF1 mice. To study the toxicity of 5-FU and 5-FP, female BDF1 mice were divided into several groups of five mice each. Drugs, at indicated doses, were dissolved in sterile saline and given orally every day for the first 5 days. Then, the mice were observed for a period of 40 days. The toxicities were determined based on the number of survivors, and the 50% lethal dose (LD_{50}) was calculated.

In vivo *evaluation against P388-R mouse leukemia cells and Colon 38 cells in BDF1 mice.* P388-R mouse leukemia cells (10^6 cells per mouse) that were resistant to Adriamycin [20] were injected i.p. into BDF1 mice. Drug was administered orally to the mice on days 1–5, and control mice were fed sterile saline.

Colon 38 cells were transplanted s.c. into female BDF1 mice [21]. After 2 weeks, mice with 0.2 to 1 cm^3 tumors were selected for drug studies. The mice were then given 5-FP and 5-FU p.o. daily for 5 days as described above. Tumor dimensions were measured according to the methods previously described [21]. The tumor weights were monitored every 1–2 days for 2 weeks in the mice treated with or without drug.

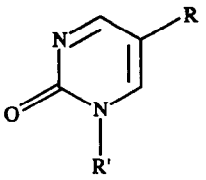
Tissue preparation. Mouse tissues were homogenized with an electric homogenizer as previously described [11]. The homogenate was centrifuged at 10,000 *g* for 10 min, filtered through cheesecloth, then dialyzed, and stored at −80° before use.

Assay conditions for the conversion of 5-FP to 5-FU. The mixture for the assay of the conversion of 5-FP to 5-FU contained 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 180 μM IPdR or 5-FP and approximately 0.01 mg of total protein from the 10,000 *g* supernatant of tissue homogenate in a final volume of 500 μL and incubated for 10 min at 37°, unless specified otherwise. Proteins were removed by precipitation with 15% (final concentration) TCA followed by low speed centrifugation. TCA was neutralized using a chlorotrifluoroethane (freon): trioctylamine (55:45) extraction mixture [21]. Samples were analyzed on an RP-18 HPLC column (Alltech, Deerfield, IL) using an acetonitrile/ammonium acetate buffer, as previously described [11].

Pharmacokinetic study of 5-FU and 5-FP. [^{14}C]-5-FU or [^{14}C]-5-FP was administered to female BDF1 mice either i.v. through the tail vein or p.o. Mice were randomized into two groups (2–3 animals per time point; each animal was bled only twice): one group received 25 mg/kg and the other group received 50 mg/kg of either 5-FU or 5-FP. Both drugs were dissolved in sterile saline, and the animals received 0.2 mL drug each. Blood was collected from the retro-orbital sinus using a heparinized capillary tube at 2, 5, 15, and 30 min and 1, 2, 4, and 24 hr after drug injection.

The blood collected was centrifuged immediately, and the plasma was separated and frozen at −20°. The frozen plasma was pooled due to the small

Table 1. Kinetic constants for the conversion of 5-FP to 5-FU and their analogs by rat liver aldehyde oxidase



R	R'	Substrate abbreviation	K_m* (μM)	V_{max}* (nmol/mg/min)	Product abbreviation
F	H	5-FP	220 ± 40	8 ± 3	5-FU
I	H	5-IP†	100 ± 30	18 ± 3‡	5-IU†
I	dR†	IPdR	150 ± 20	11 ± 2‡	IUdR
C≡C	dR	EPdR	77 ± 9	20 ± 4‡	EUdR

* Values are means ± SEM of at least three experiments.
† dR = deoxyribose; 5-IP = 5-iodopyrimidinone; and 5-IU = 5-iodouracil.
‡ Data were taken from our previous publication [11].

volumes of plasma obtained and then extracted using TCA followed by freon : trioctylamine extraction as described above. The 5-FU and 5-FP in the extracted samples were separated by HPLC, using a Partisil-10 SAX column (25 cm × 4.6 mm; Whatman International Ltd., Maidstone, U.K.) with 10 mM potassium phosphate buffer (pH 6.8) as the mobile phase. Fractions were collected in vials, and radioactivity was counted after addition of scintillation fluid (Ecoscint, National Diagnostics, Atlanta, GA).

Pharmacokinetic analysis. Plasma concentration versus time curves were fitted to a non-compartmental model of drug distribution. Pharmacokinetic modeling and parameter estimation were performed using the nonlinear regression program PCNONLIN (Statistical Consultants, Lexington, KY). Areas under the curve (AUC), volumes of distribution at the steady state, total body clearance rates, and mean residence times were calculated using standard pharmacokinetic equations [22].

RESULTS

Activity of aldehyde oxidase in different tissues. Our previous publication [11] indicated that aldehyde oxidase activity, which converts IPdR to IUdR, was localized mainly in the liver in rats and humans. Since our current studies were performed mainly in mice, we analyzed mouse tissue homogenates for aldehyde oxidase activity and found enzyme activities in liver homogenates similar to those in rats and humans: 8–12, 5–15, and 2–7 nmol/mg protein/min for mice, rats and humans, respectively. Enzyme activity was minimal in mouse intestine, brain and bone marrow (data not shown).

The conversion of 5-FP to 5-FU by aldehyde oxidase in mouse liver homogenate was explored, and the results are shown in Table 1. Both the K_m and relative V_{max} values of 5-FP as the substrate for aldehyde oxidase were determined. These values were compared with those measured in a previous study [11] using 5-IP, EPdR and IPdR as substrates. Methyl or ethyl groups at the 5-position were not substrates of aldehyde oxidase (data not shown).

Pharmacokinetics of 5-FP and 5-FU in mice. HPLC analysis of the plasma of mice treated with 25 mg/kg of 5-FP (Fig. 1) demonstrates that conversion of 5-FP to 5-FU had largely taken place by 1 hr. These figures were derived by trapezoidal analysis of the peaks from the elution profiles of the [^{14}C]-labeled drug obtained from the HPLC. This is a representative experiment that was repeated once with overall identical results. However, it was impossible to combine the two experiments because of slight variations in actual sampling times.

Table 2 summarizes the pharmacokinetics of 5-FP and 5-FU administration to BDF1 mice from the experiment shown in Fig. 1. The values shown for 5-FP are calculated for the amount of 5-FP in the plasma of mice. The values for 5-FU derived from 5-FP are not included in the table. The clearance rates of 5-FU were three times higher and the half-lives at least 2-fold shorter than those of 5-FP. The bioavailability of 5-FP was 100% at a dose of 25 mg/kg and 78% at 50 mg/kg. The bioavailability of 5-FU was 89% at 25 mg/kg. The volumes of distribution were slightly higher for 5-FU than for 5-FP at both doses.

Toxicity of 5-FU and 5-FP in BDF1 mice. The toxicities of 5-FU and 5-FP, given orally to BDF1 mice, are shown in Table 3. The drugs were administered daily for 5 days and followed for 40 days. 5-FP was at least 2-fold less toxic than 5-FU based on the LD_{50} determinations. Body weights were identical among all groups, with no decreases over time seen in drug-treated animals (data not shown).

Effects of 5-FU and 5-FP on mice bearing leukemia cells. The effects of 5-FU and 5-FP on survival time of mice inoculated with 10^6 mouse P388-R leukemia cells [22] were evaluated. Mice were inoculated

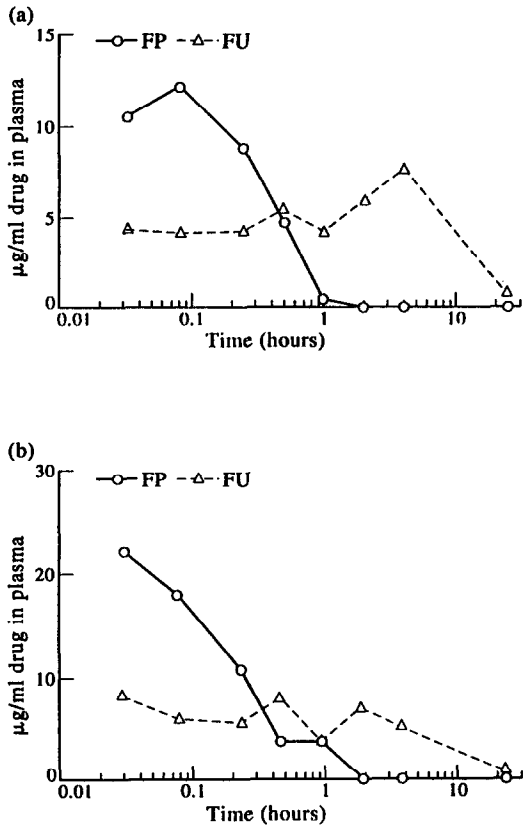


Fig. 1. Conversion of 5-FP to 5-FU in mice administered 5-FP. Mice were given 25 mg/kg [^{14}C]-5-FP either p.o. (A) or i.v. (B). The values were derived from HPLC elution profiles of mouse plasma samples.

Table 3. Toxicity of 5-FU and 5-FP given orally to BDF1 mice

Compound	Dose (mg/kg)	Death/ Total	LD_{50} (mg/kg) daily
5-FU	50	1/5	
	75	3/5	70
	100	4/5	
5-FP	100	0/5	
	150	1/5	180
	200	5/5	

Five animals were used in each group.

Table 4. Effects of 5-FU and 5-FP on survival time of mice bearing leukemia P388-R cells*

Group	Dose† (mg/kg)	Survival (days) ± SD	ILS‡ (%)
Control		12.8 ± 1.6	
5-FU	25	13.4 ± 1.1	5
	50	16.0 ± 1.0	25
5-FP	50	12.6 ± 0.9	0
	100	16.6 ± 0.9	30

Five animals were used in each group. Values are averages of two experiments.

* P388-R cells (10^6), which are resistant to Adriamycin, were inoculated into each mouse i.p.

† Tumor injection was given on day 0, and drug treatments were given daily on days 1–5.

‡ Increase in life span compared with mice not treated with drug.

with tumor cells and then treated daily with 5-FP or 5-FU for 5 days. Two weeks after tumor inoculation, the survival times of the mice were compared (Table 4). Mice treated with 5-FP exhibited survival times similar to those treated with 5-FU, although twice as much 5-FP was required to achieve this effect.

Oral treatment of mouse Colon 38 tumors with 5-FU and 5-FP. BDF1 female mice bearing s.c.

Table 2. Pharmacokinetic behavior of 5-FP and 5-FU administered orally to BDF1 mice

	5-FP Administration		5-FU Administration	
	25 mg/kg	50 mg/kg	25 mg/kg	50 mg/kg
AUC (μg·mL/hr)	7.4 ± 0.7	25.9 ± 2.8	2.5 ± 0.3	5.7 ± 1.1
$T_{1/2}$ (hr)	0.2 ± 0.03	0.4 ± 0.05	0.1 ± 0.02	0.10 ± 0.03
CL_T (mL/hr)	67.9 ± 6.5	48.6 ± 4.2	198.3 ± 27.2	176.3 ± 35.8
AUMC (μg·mL/hr)	2.2 ± 0.5	13.5 ± 3.2	0.3 ± 0.1	0.8 ± 0.4
MRT (hr)	0.3 ± 0.04	0.5 ± 0.07	0.1 ± 0.03	0.1 ± 0.04
V_{SS} (L/kg)	1.0 ± 0.06	1.0 ± 0.05	1.4 ± 0.1	1.2 ± 0.2
Bioavailability	100%	78%	89%	—

These values (means ± SEM) were based on 3–6 mice per group. Abbreviations:
AUMC: area under the first moment vs concentration curve;
AUC: area under the concentration vs time curve;
CL_T: clearance rate;
MRT: mean residence time (AUMC/AUC = MRT);
$T_{1/2}$: half-life; and
V_{SS}: calculated volume of distribution at steady state.

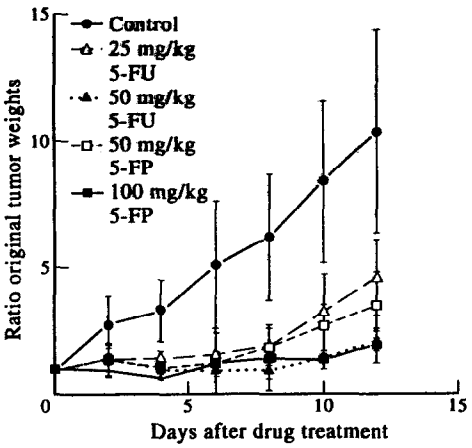


Fig. 2. Treatment of mouse carcinoma (Colon 38) with 5-FU and 5-FP. BDF1 female mice bearing s.c. transplants of Colon 38 cell tumors were selected and treated, as described in Materials and Methods. Tumor sizes were monitored and converted to tumor weights on days 1, 3, 5, 7, and 12, and the ratios of starting weights were plotted against the day after the drug treatment. The starting weights of tumors (average weights: 0.3 g) were assigned the value of 1. Since several tumors often formed at the site of injection, the weights of all tumors were combined. Five mice were included in each group. Error bars represent ± SEM.

transplants of Colon tumor 38 cells were treated orally with either 5-FP or 5-FU. Mice showed similar reductions in tumor size whether treated with 5-FP or 5-FU (Fig. 2). When given a dose of either 50 or 100 mg/kg, the latter dose correlated with the greatest reduction in tumor size for 5-FP or 5-FU treatments. At 50 mg/kg, the tumor sizes regressed through day 7, until reaching a slightly larger size than the original by day 16. The untreated mice had tumor sizes 10 times larger than the original tumor sizes by day 12. At no time did tumor weights exceed 3.6 g. 5-FU and 5-FP were administered to the mice in equitoxic doses (Table 3). Figure 2 shows one representative experiment, which was repeated with similar results. Due to variations in initial tumor sizes and mouse ages, the experiments could not be totalled together.

DISCUSSION

Our studies showed that 5-FP could be converted to 5-FU *in vivo* in the liver, which is rich in aldehyde oxidase [11, 12]. We found that the enzymatic conversion of 5-FP to 5-FU was absent in intestines and bone marrow homogenates and supported the idea that 5-FP was converted to 5-FU in liver before being transported to other target sites. Our pharmacokinetic results indicated that 5-FP was converted to 5-FU in mice, with higher 5-FP plasma concentrations at the early time points, again supporting the idea that 5-FP could be converted to 5-FU *in vivo* (Fig. 1). 5-FU, derived from 5-FP, was present in plasma in higher amounts than 5-FP after 1 hr and was present in significant amounts even after 24 hr, when 5-FP was given orally or intravenously. This extended presence of 5-FU was not seen in mice treated with 5-FU. In view of the difference in substrate behavior of aldehyde oxidase in different species [13, 14], studies in human subjects *in vivo* will be necessary to confirm whether the superior pharmacokinetics of 5-FP over 5-FU seen in mice is relevant in humans.

Several enzymes related to the metabolism and catabolism of 5-FU, such as orotate phosphoribosyltransferase, pyrimidine nucleoside phosphorylases and dihydrouracil dehydrogenase, were reported to be located in the gastrointestinal tract and appear to be light-cycle dependent [23]. Therefore, the individual variability and circadian rhythms of these intestinal enzymes may play a significant role in modulating the plasma levels of 5-FU given orally. However, 5-FP before being converted to 5-FU was not metabolized by these enzymes (unpublished results). The only enzyme that utilizes 5-FP as a substrate, aldehyde oxidase, was found not to be light-cycle dependent (data not shown) and is present predominantly in the liver. The absence of the metabolism of 5-FP in intestinal cells was substantiated using the colonic cell line, Caco-2 [24]. No metabolism of 5-FP could be detected, whereas 5-FU was readily metabolized in Caco-2 monolayer cultures [25, 26]. Therefore, the variable bioavailability among individuals given oral doses of 5-FU may not be seen in oral delivery of 5-FP. Also, because of the lack of metabolism seen with Caco-2 colonic cells, lower GI toxicity would be expected with oral use of 5-FP than is seen currently in patients treated with 5-FU. It is not uncommon for the bioavailability of 5-FU to approach 100% in mice, even though it is lower in humans, so our results are not unexpected.

We found that 5-FP was as effective as 5-FU against both P388-R mouse leukemia cell transplants (Table 4) and against Colon 38 mouse tumors (Fig. 2). The toxicity of 5-FP after a 5-day administration was at least 2-fold lower than that of 5-FU (Table 3). The relative potency of the antitumor activity of 5-FP was approximately 2-fold less than that of 5-FU; however, when taking the toxicity into account, the therapeutic index of 5-FP was similar to that of 5-FU.

Based on our results presented in this paper, 5-FP holds promise of being a future drug that may substitute for 5-FU treatment of various cancers. Additionally, oral administration of 5-FP may also be as effective as 5-FU in the treatment of cancer. Since the prodrug activation site is localized mainly in the liver, the use of 5-FP in the treatment of liver-associated cancers should be explored. Additionally, the oral use of 5-FP may decrease some of the systemic toxicity seen with i.v. 5-FU treatment and would eliminate the need for expensive hospitalization for the i.v. therapy currently used. Detailed studies of the pharmacokinetics and pharmacodynamics of 5-FP are under investigation.

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