

N₃-*o*-toluyl-fluorouracil inhibits human hepatocellular carcinoma cell growth via sustained release of 5-FU

Xiaofan Zhang · Julia Li Zhong · Wei Liu ·
Zuhua Gao · Xia Xue · Pan Yue · Limei Wang ·
Cuirong Zhao · Wenfang Xu · Xianjun Qu

Received: 15 June 2009 / Accepted: 2 September 2009 / Published online: 16 September 2009
© The Author(s) 2009. This article is published with open access at Springerlink.com

Abstract

Purpose N₃-*o*-toluyl-fluorouracil (TFU), the prodrug of 5-fluorouracil (5-FU), is the metabolite of N₁-acetyl-N₃-*o*-toluyl-fluorouracil (atofluding). In the present study, we aimed to evaluate the efficacy of TFU on the inhibition of human hepatocellular carcinoma cells via sustained release of 5-FU. The metabolism of TFU underlying the inhibitory effect was also analyzed.

Methods In vitro assays, inhibition of cell growth by TFU was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method. The levels of TFU and 5-FU in the cell culture supernatant fluid were measured by high-performance liquid chromatography (HPLC). In vivo assays, the efficacy of TFU was evaluated in a human hepatocellular carcinoma xenograft mice model after 3 weeks of oral administration. The distributions of TFU and 5-FU in plasma and homogenate tissues including liver, lung and tumor were determined by HPLC.

J. L. Zhong and X. Zhang equally contributed to this work.

X. Zhang · X. Xue · P. Yue · L. Wang · C. Zhao · W. Xu ·
X. Qu (✉)

Department of Pharmacology, School of Pharmaceutical
Sciences, Shandong University, Jinan, China
e-mail: qxj@sdu.edu.cn

W. Liu
Jinan Institute for Drug Control, Jinan, China

Z. Gao
Calgary Laboratory Services, Department of Pathology
and Laboratory Medicine, University of Calgary,
Calgary, AB, Canada

J. L. Zhong
The Base of “111 Project” for Biomechanics and Tissue Repair
Engineering, Bioengineering College, Chongqing University,
Chongqing, China

Results N₃-*o*-toluyl-fluorouracil weakly inhibited the proliferation of SMMC-7721 and PLC/PRF/5 cells in the absence of liver microsomal enzymes. In contrast, the inhibition rates were significantly increased in the presence of these enzymes. HPLC results revealed that TFU was metabolized slowly by liver microsomal enzymes and therefore the concentration of 5-FU was gradually increased with a longer retention time in cell culture supernatant fluid. The efficacy of TFU was confirmed in SMMC-7721 xenografts in Balb/c athymic (nu+/nu+) mice model. TFU treatment induced inhibition of SMMC-7721 growth with few side effects. HPLC results showed that high levels of TFU were still in liver 48 h after the end of oral administration, implying that TFU preferentially accumulated in liver with slow conversion to 5-FU by enzymes. This led to a long-lasting concentration of 5-FU in plasma. Further, a high level of 5-FU was found in tumors with a relatively low level in lungs. These results suggest that the metabolite of TFU was preferentially converted or taken up by tumor cells. The distributions of 5-FU may contribute to its high anti-tumor activity and low adverse reactions in vivo.

Conclusion These results demonstrate that TFU is a promising prodrug of 5-FU for cancer treatment via sustained release of 5-FU in liver.

Keywords N₃-*o*-toluyl-fluorouracil (TFU) ·
5-Fluorouracil (5-FU) · Hepatocellular carcinoma ·
Liver microsomal enzymes · Sustained release ·
Drug distribution

Abbreviations

TFU N₃-*o*-toluyl-fluorouracil
5-FU 5-Fluorouracil

HPLC	High-performance liquid chromatography
NADPH	Nicotinamide adenine dinucleotide phosphate
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
EDTA	Ethylenediamine tetraacetic acid
PBS	Phosphate-buffered saline

Introduction

Since Dushinsky et al. [1] synthesized 5-fluorouracil (5-FU) and discovered its anti-tumor activity in 1957, 5-FU has been widely used in the treatment of various types of cancer including stomach, intestine, liver, etc. [2, 3]. However, its half-life in plasma is very short (15–20 min), thus continuous intravenous administration is needed to maintain therapeutic concentrations [4, 5]. Moreover, the clinical dose of 5-FU is very close to its toxic dosage when given intravenously, resulting in significant toxicity to gastric and intestinal mucosa and bone marrow, making long-lasting treatment difficult [6, 7]. Hence, attempts have been made to develop oral prodrugs with sustained release of 5-FU at equal to or greater than its plasma concentrations that could be obtained by intravenous administration. For instance, S-1 was designed as an oral anti-cancer agent that combines tegafur (FT), 5-chloro-2,4-dihydropyridine (CDHP) and potassium oxonate (Oxo) and that has an increased therapeutic index [2]. N⁴-pentoxycarbonyl-5'-deoxy-5-fluorocytidine (capecitabine) is also an oral fluoropyrimidine precursor that was designed to undergo conversion in liver and tumor to 5-FU. Due to the high activity of thymidine phosphorylase in tumors in converting capecitabine to 5-FU, capecitabine has been shown to have a superior therapeutic index to 5-FU [8].

TFU, the metabolite of N₁-acetyl-N₃-*o*-toluyl-fluorouracil (atofluding), is the prodrug of 5-FU (Fig. 1) [9–11]. Oral administration of TFU significantly delayed the growth of human gastric carcinoma xenografts in Balb/c athymic (nu+/nu+) mice [9, 10]. TFU has been considered to be a candidate fluoropyrimidine derivative that could replace the injectable 5-FU for cancer treatment [11–13]. Our previous studies showed that TFU significantly suppressed the growth of SGC-7901 and MKN-45 cells in vitro in the presence of liver microsomal enzymes but only weak inhibition without it. In the present study, we examined the inhibitory effect of TFU on the proliferation of human hepatocellular carcinoma cells. Further, the metabolism of TFU in addition to the inhibitory action of the drug was also investigated.

Materials and methods

Chemicals

N₃-*o*-toluyl-fluorouracil was synthesized by acylation of 5-FU with 2-methylbenzoyl chloride in pyridine at room temperature as described previously [11]. TFU was dissolved in dimethylsulfoxide (DMSO) for the in vitro assay and in 5% amyllum for in vivo study.

Preparation of liver microsomal enzymes

Liver microsomal enzyme fraction was prepared from the livers of male Sprague-Dawley rats which were intraperitoneally injected with phenobarbital [14]. The post-mitochondrial fraction (S-9) was obtained from the liver homogenate by centrifugation and then mixed with 0.1 M phosphate buffer with 125 μM NADPH (nicotinamide adenine dinucleotide phosphate). Two microliters of the mixture was applied to each well of 96-well plates when cells were incubated in the presence of liver microsomal enzymes [9, 10].

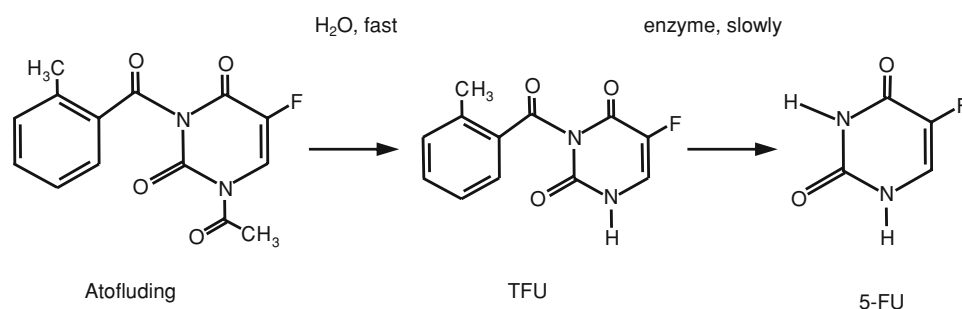
Cell lines and cell culture

The human hepatocellular carcinoma cell lines SMMC-7721 and PLC/PRF/5 were purchased from Shanghai Cell Bank, the Institute of Cell Biology, China Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, and 10 mM HEPES buffer at 37°C in a humid atmosphere (5% CO₂–95% air) and were harvested by brief incubation in 0.02% (w/v) EDTA in PBS (ICN, Aurora, OH, USA).

Inhibition of cell proliferation in vitro

Hepatocellular carcinoma cells (2.5 × 10⁴ per well) seeded in 96-well plates were exposed to different concentrations of TFU (5–200 μg ml⁻¹) in the absence or presence of liver microsomal enzymes for the indicated time. 5-FU (20 μg ml⁻¹; Institute for Drug Control of China, Beijing) was used as the positive control. To analyze the metabolism of TFU in vitro, the cell culture supernatant fluid was collected from each well at the indicated time and the concentrations of TFU and its metabolite 5-FU were analyzed by high-performance liquid chromatography (HPLC) as described below. The wells were then washed with PBS and cell viability was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay as described elsewhere [15]. Triplicate experiments with triplicate samples were performed.

Fig. 1 Scheme for the metabolism of atofluding to 5-FU via TFU



Extraction of TFU and 5-FU from supernatant fluid of cell culture

One hundred microlitre of NaH_2PO_4 (0.5 M) and 2.5 ml acetic ether were added to 500 μl of supernatant fluid. The mixture was vortexed for 3 min, then centrifuged (3,000 rpm, 5 min). Two milliliters was transferred to a clean tube thereafter and evaporated under nitrogen at 50°C . The resulting residue was reconstituted with 200 μl of mobile phase (acetonitrile–water, 50:50, v/v) for HPLC measurement [11, 16].

HPLC conditions

The contents of TFU and 5-FU in cell culture supernatant fluid, plasma and tissue homogenates were measured at 258 nm by HPLC using an SPD-10Avp Shimadzu pump and an LC-10Avp Shimadzu UV–vis detector. Samples were chromatographed on a 4.6×250 mm reverse phase stainless steel column packed with 5 μm particles (Venusil XBP C-18, Agela, China) eluted with a mobile phase consisting of 50:50 (v/v) mixture of acetonitrile and water at a flow rate of 1 ml min^{-1} [11, 16]. Under these conditions, the method of HPLC was established (Fig. 2). The linearity of the method was evaluated by analyzing nine calibration standards in triplicate over the nominal concentration range of $0.05\text{--}300 \mu\text{g ml}^{-1}$ for TFU and 5-FU. The correlation coefficients obtained using $1/x^2$ weighted linear regressions were better than 0.9977 for TFU and 0.9985 for 5-FU (Fig. 2).

Inhibition of tumor growth in vivo

The in vivo efficacy of TFU and distribution of drugs were evaluated in SMMC-7721 xenografts mice model [9, 10]. Balb/c athymic (nu+/nu+) female mice, 4–6 weeks of age, were purchased from Animal Center of China Academy of Medical Sciences (Beijing, China). The animals were housed under pathogen-free conditions. The research protocol was in accordance with the institutional guidelines of Animal Care and Use Committee at Shandong University. SMMC-7721 cells (1×10^7) were suspended in 100 μl of

Matrigel (Collaborative Biomedical, Bedford, USA) and were injected subcutaneously into the right anterior flank of mice. After 7 days, when the tumor volume reached approximately $0.1\text{--}0.2 \text{ cm}^3$, then 0, 25, 50, 100 mg kg^{-1} of TFU in 0.5 ml of 5% amylum were orally administered. 5-FU (25 mg kg^{-1}) was injected via the tail vein as a positive control [3]. Drugs were administered 6 days per week for three consecutive weeks. Mice were killed 48 h after the last dose. Tumor, liver and lung were harvested and blood samples were collected from the postorbital venous plexuse for drug analysis as described below. The rate of tumor growth inhibition was defined as a ratio to the control (without TFU) tumor weight.

Sample extraction

The samples of liver, lung and tumor were weighed and homogenized with 0.9% NaCl solution (1:3, w/w) in an ice bath. Blood samples were centrifuged for 15 min (4,000 rpm) in cold (4°C) and then the plasma was obtained. Two hundred microliters of NaH_2PO_4 (0.5 M) and 2.5 ml of acetic ether were added to 500 μl of the homogenate solution (plasma sample, 200 μl). The mixture was vortexed for 3 min, then centrifuged (3,000 rpm, 5 min) [16, 17]. TFU and 5-FU extractions and HPLC measurement were carried out as described above.

Statistical analysis

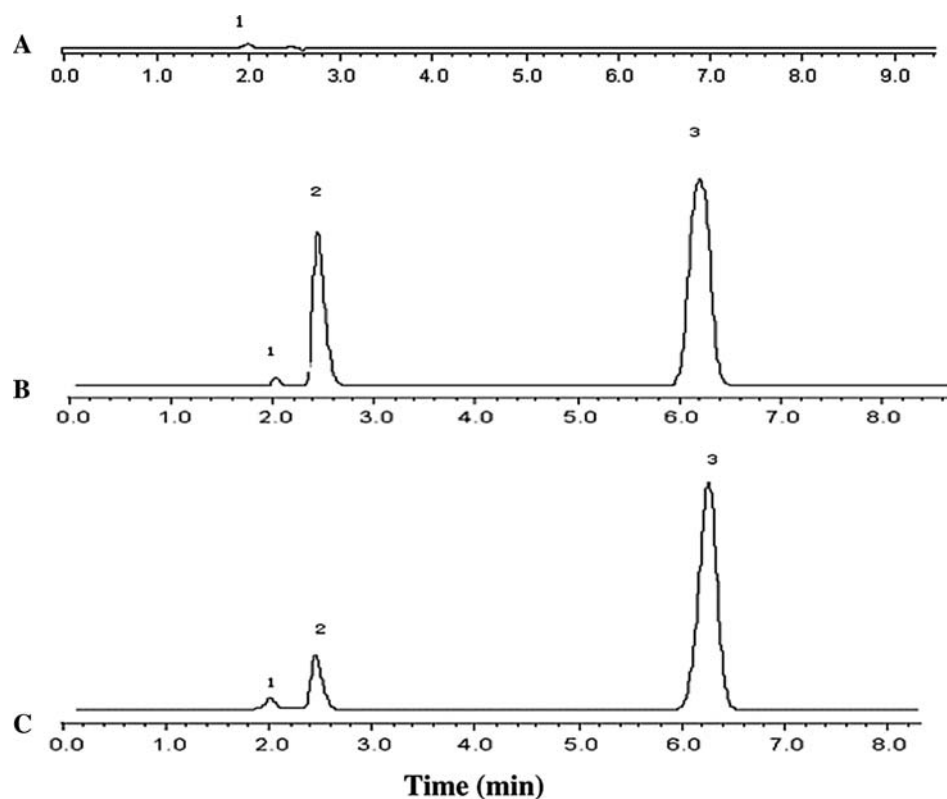
Data were described as the mean \pm SD, and analyzed by Student's two-tailed *t* test. The limit of statistical significance was $P < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS Inc., Chicago, IL).

Results

Anti-proliferative effects of TFU in vitro

Human hepatocellular carcinoma SMMC-7721 cells were treated with TFU for up to 120 h and then subjected to the MTT assay. As shown in Fig. 3 (top left) in the absence of

Fig. 2 Examples of chromatograms for TFU and 5-FU from control cell culture medium (a), reference standard (b) and medium from cell culture with liver microsomal enzymes (c). After incubation with various concentrations of TFU, the medium from cell culture was extracted and the drug content was determined by HPLC on SPD-10Avp Shimadzu pump and an LC-10Avp Shimadzu UV-vis detector as described in “Materials and methods”. The linearity of the method was evaluated by analyzing nine calibration standards in triplicate over the nominal concentration range of 0.05–300 $\mu\text{g ml}^{-1}$ for TFU and 5-FU. The correlation coefficients obtained using $1/x^2$ weighted linear regressions were better than 0.9977 for TFU and 0.9985 for 5-FU. 1 DMSO, 2 5-FU, 3 TFU



liver microsomal enzymes, incubation with TFU (5–200 $\mu\text{g ml}^{-1}$) did not significantly inhibit the growth of SMMC-7721 cells, but significance was seen with higher concentrations and longer incubation times (100–200 $\mu\text{g ml}^{-1}$, 96–120 h, the inhibition rates range from 16.5 to 20.4%, $P < 0.05$ vs. relevant control).

The inhibitory effect of TFU on the proliferation of SMMC-7721 cells was then examined in the presence of liver microsomal enzymes. The inhibition rates were greatly increased at all concentrations (5–200 $\mu\text{g ml}^{-1}$) and incubation times (24–120 h), being 52–79% in SMMC-7721 cells (Fig. 3, top right).

Similar proliferation profiles were observed for PLC/PRF/5 cells exposed to TFU in the absence and presence of liver microsomal enzymes (Fig. 3, bottom).

TFU sustained release of 5-FU in vitro

To analyze the metabolism of TFU mediated by liver microsomal enzymes, the levels of TFU and its metabolite 5-FU in the medium from SMMC-7721 cell culture were examined by HPLC. As shown in Fig. 4 (top left), TFU was maintained at relative stable levels in all concentrations and incubation times in the absence of liver microsomal enzymes. However, a reduced level of TFU was measured at doses of 100 and 200 $\mu\text{g ml}^{-1}$ at longer incubation time (72–120 h, $P < 0.05$ vs. 0 h control),

probably catalyzed by the cytosolic enzymes in cancer cells [18]. Therefore, the much lower levels of its metabolite 5-FU were detected in cell culture medium (Fig. 4, top right).

The concentrations of TFU and 5-FU were then examined in the presence of liver microsomal enzymes. The levels of TFU were gradually decreased (Fig. 4, bottom left) and its metabolite 5-FU was increased slowly and maintained the high levels for up to 120 h (bottom right). These results implied that TFU was metabolized slowly and the liver microsomal enzymes appeared to be important for conversion of TFU to 5-FU.

Similar profiles of metabolism of TFU were observed in PLC/PRF/5 cells exposed to TFU. Figure 5 shows the concentrations of TFU and its metabolite 5-FU determined by HPLC in cell culture medium from this cell line. In the absence of liver microsomal enzymes, TFU was maintained at relative stable level (Fig. 5, top left) and the low concentration of its metabolite 5-FU (top right) was found in the cell culture medium. In contrast, the levels of TFU were decreased gradually (Fig. 5, bottom left) and the concentrations of its metabolite 5-FU were increased significantly in the presence of liver microsomal enzymes (bottom right).

We also examined the concentrations of 5-FU, the positive control in this experiment, in the medium from SMMC-7721 culture after single treatment. The level of

Fig. 3 Growth inhibition of SMMC-7721 cells (*top*) and PLC/PRF/5 (*bottom*) induced by TFU in the absence (*left*) or presence (*right*) of liver microsomal enzymes in vitro. Cells were treated with various concentrations of TFU for up to 120 h. Viable cells were evaluated by MTT assay and denoted as a percentage of untreated control at the concurrent time point. The bars indicate mean \pm SD ($n = 3$)

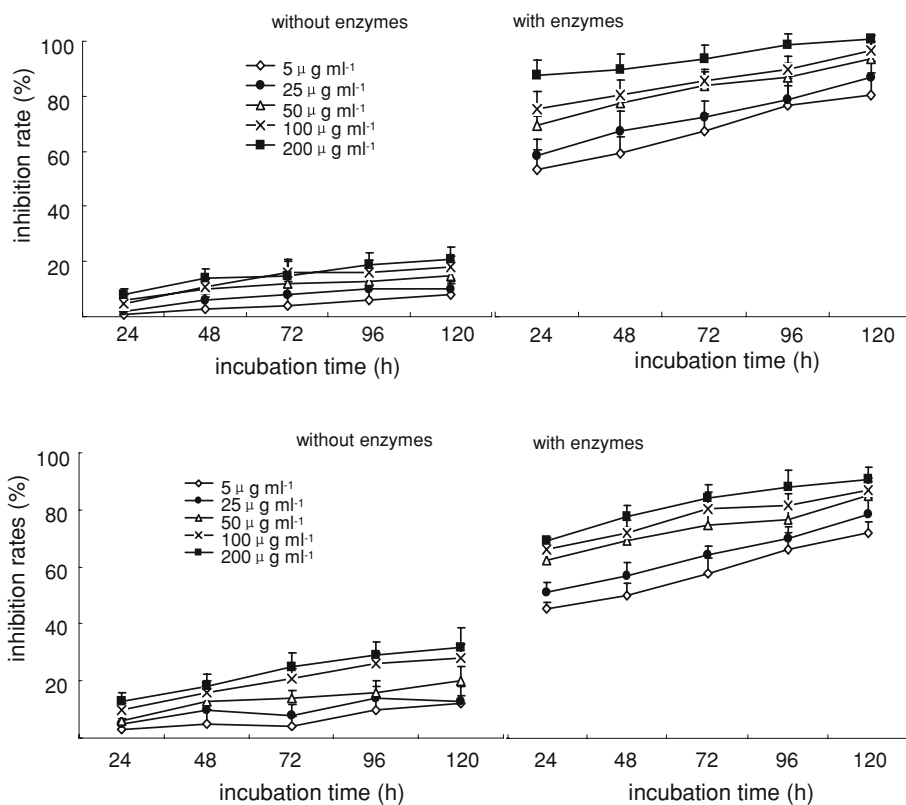
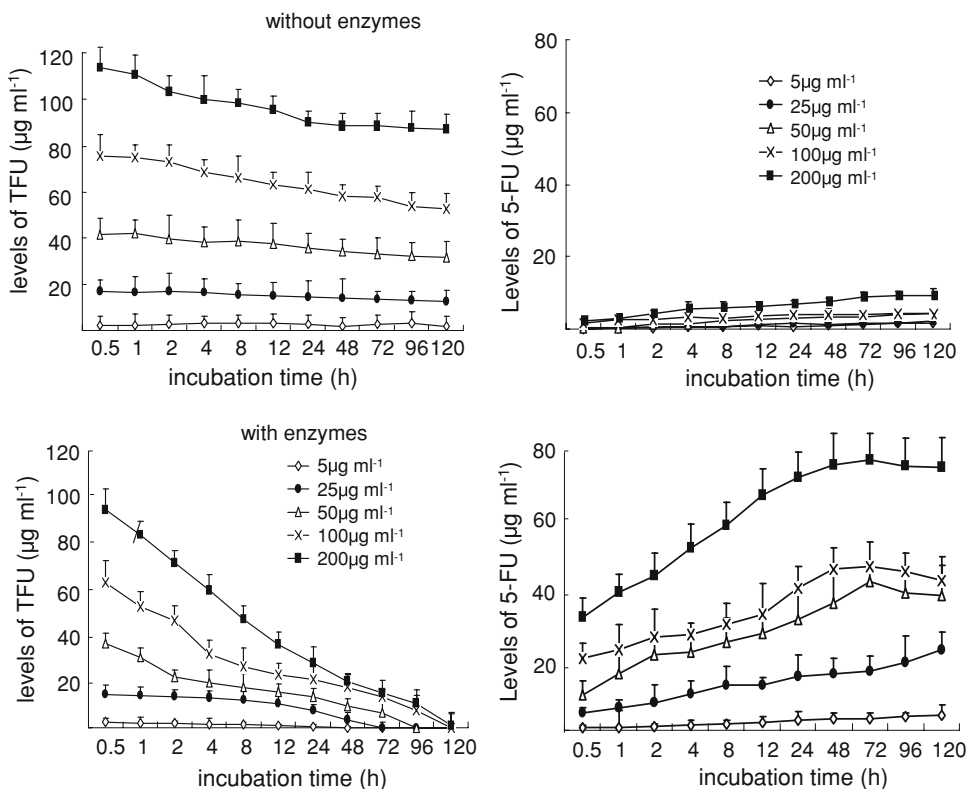


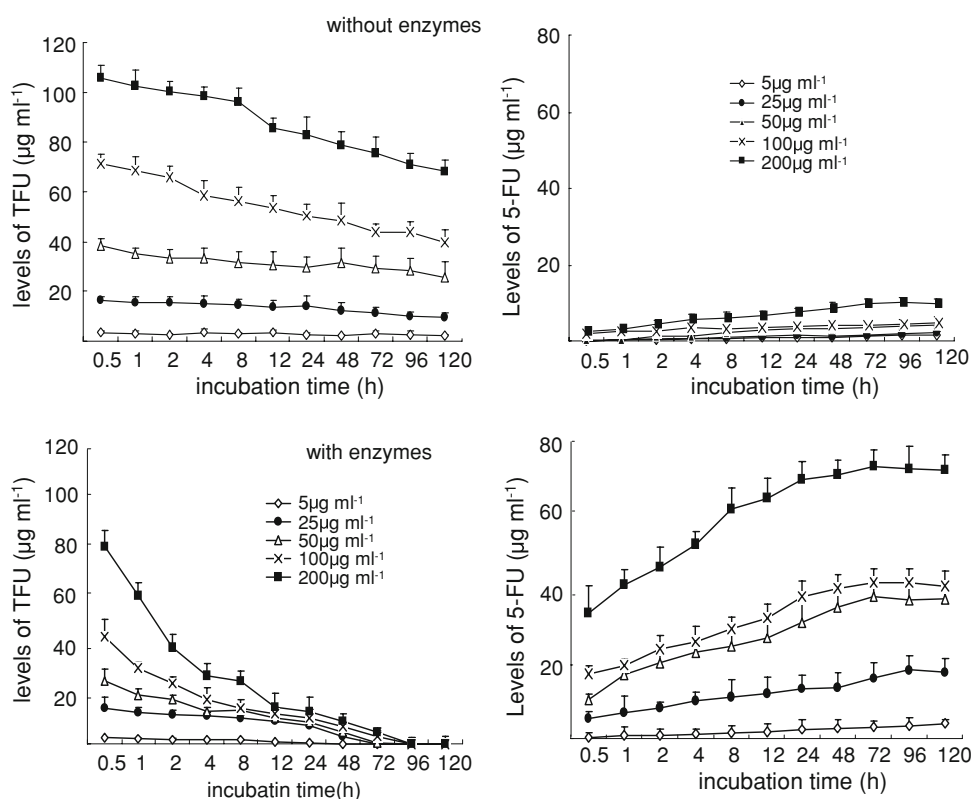
Fig. 4 Levels of TFU and 5-FU in the medium from SMMC-7721 cell culture in the absence (*top*) or presence (*bottom*) of liver microsomal enzymes in vitro. SMMC-7721 cells were exposed to various concentrations of TFU for up to 120 h. Levels of TFU (*left*) and its metabolite 5-FU (*right*) in cell culture medium at individual time points were analyzed by HPLC as described in Fig. 2. The bars indicate mean \pm SD ($n = 3$)



5-FU was decreased rapidly within 120 min incubation, indicating that 5-FU was degraded by the cytosolic enzymes in cancer cells [18]. The degradation of 5-FU was

more pronounced in the presence of liver microsomal enzymes than in the absence of enzymes (Fig. 6). Similar result was found with PLC/PRF/5 cells (data not shown).

Fig. 5 Levels of TFU and 5-FU in the medium from PLC/PRF/5 cell culture in the absence (*top*) or presence (*bottom*) of liver microsomal enzymes *in vitro*. PLC/PRF/5 cells were exposed to various concentrations of TFU for up to 120 h. Levels of TFU (*left*) and its metabolite 5-FU (*right*) in the cell culture medium at individual time points were analyzed by HPLC as described in Fig. 2. The bars indicate mean \pm SD ($n = 3$)



Efficacy of TFU administration in mice

We evaluated the efficacy of TFU in hepatocellular carcinoma xenografts in Balb/c athymic (nu+/nu+) mice. As shown in Table 1, the growth of SMMC-7721 tumor was significantly delayed after 3 weeks of oral administration. At doses of 25, 50 and 100 mg kg^{-1} of TFU, the inhibition rates were 38.9, 51.9 and 60.8%. Suppression of SMMC-7721 tumor growth was dose dependent (25 mg kg^{-1} , $P < 0.05$ vs. untreated group; 50 and 100 mg kg^{-1} , $P < 0.01$ vs. untreated group). TFU treatment was generally well tolerated by mice with less than 20% reduction in body weight (50 and 100 mg kg^{-1} , $P > 0.05$ vs. untreated group). In contrast, a significant body weight loss was observed (25.9%, $P < 0.01$ vs. untreated group) and three mice died during the continuous administration of 5-FU via the tail vein (Table 1).

Distributions of 5-FU and TFU in mice

The distributions of TFU and its metabolite 5-FU in plasma, liver, lung and tumor were measured by HPLC. As expected, high levels of TFU were detected in liver, while TFU was not detected in plasma, lung and tumor, indicating that the drug was preferentially accumulated and metabolized in liver (Table 2). We analyzed the concentration of 5-FU in the same samples. The plasma 5-FU

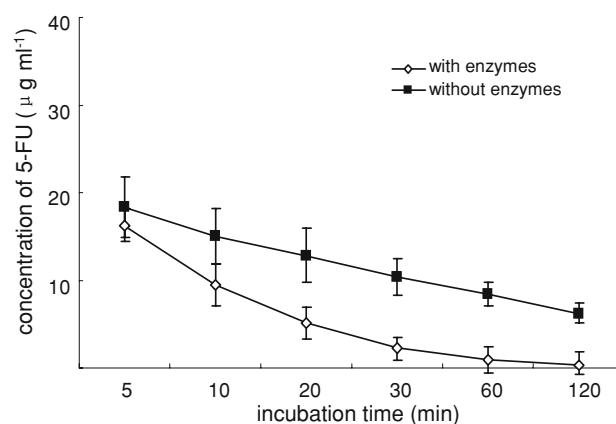


Fig. 6 Levels of 5-FU in culture medium from SMMC-7721 culture after a single exposure. SMMC-7721 cells were exposed to 5-FU (20 $\mu\text{g ml}^{-1}$) in the absence or presence of liver microsomal enzymes for the indicated time. 5-FU was measured by HPLC as described in Fig. 2. The bars indicate mean \pm SD ($n = 3$)

remained relatively at stable levels (13.52–15.82 ng ml^{-1}) although the drug administration ended 48 h before. 5-FU content was high in liver, suggesting that 5-FU accumulated after TFU degradation. Importantly, higher content of 5-FU was found in SMMC-7721 xenografts and relatively lower levels were measured in lungs (Table 2), indicating that the tumor was the main site of action of 5-FU. In contrast, 5-FU was not detected in plasma, liver, lung or

Table 1 The inhibitory effects of TFU on SMMC-7721 xenograft in Balb/c athymic (nu+/nu+) mice

Dosage (mg kg ⁻¹)	Mice survived (<i>n</i>)	Body weight ^a (g; mean ± SD)	Tumor weight ^b (g; mean ± SD)	Tumor growth inhibition (%)
TFU				
0	10	22.32 ± 2.33	3.37 ± 0.54	–
25	10	22.81 ± 1.84	2.05 ± 0.69*	38.9
50	10	20.74 ± 1.63	1.62 ± 0.61**	51.9
100	10	20.13 ± 2.12	1.32 ± 0.62**	60.8
5-FU				
20	7	16.51 ± 2.23	1.53 ± 1.03**	54.5

Established tumors (0.1–0.2 cm³) were treated orally with TFU 7 days post-SMMC-7721 implant as described in “Materials and methods”

* $P < 0.05$, ** $P < 0.01$ versus untreated group

^a Body weight was measured after finishing drug administration

^b Tumor measurements were made after mice killed

Table 2 The distribution of 5-FU and TFU in different tissues and SMMC-7721 xenografts in Balb/c athymic (nu+/nu+) mice ($n = 10$)

Dosage (mg kg ⁻¹)	Content of TFU (μg g ⁻¹)				Content of 5-FU (μg g ⁻¹)			
	Plasma (ng ml ⁻¹)	Lung	Liver	Tumor	Plasma (ng ml ⁻¹)	Lung	Liver	Tumor
TFU								
0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
25	Nd	Nd	10.21 ± 1.38	Nd	13.52 ± 0.36	42.08 ± 5.17	168.24 ± 20.11	154.89 ± 19.28
50	Nd	Nd	36.54 ± 3.54	Nd	14.87 ± 0.53	53.12 ± 6.33	214.93 ± 27.59	197.81 ± 15.36
100	Nd	Nd	59.63 ± 4.87	Nd	15.82 ± 0.43	68.34 ± 9.35	280.30 ± 37.21	264.78 ± 20.73
5-FU								
25	–	–	–	–	Nd	Nd	Nd	Nd

TFU was orally given for three consecutive weeks. The measurement of TFU and 5-FU was performed 48 h after the last dose. 5-FU was injected via tail vein as a positive control

Nd concentration of drug was not detectable

tumor in animals at this time point (48 h after the last dose of 5-FU) when 5-FU was intravenously given (Table 2).

Discussion

N₃-*o*-toluyl-fluorouracil is the metabolite of N₁-acetyl-N₃-*o*-toluyl-fluorouracil (atofluding) [19]. Previously, atofluding had been proven effective against many types of tumors including intestinal, gastric and esophageal carcinoma in phase III clinical trials [19]. However, its development was discontinued due to the instability of the preparation [9, 10]. Further studies indicated that the acetyl group is prone hydrolysis into TFU rapidly by fluorine at the C₅ position (Fig. 1), impairing quality control for the preparation. In contrast, TFU is stable and therefore a significant level of TFU was detected in serum rather than atofluding itself in clinical trials [19]. Our previous studies also showed that its metabolite 5-FU was detected at 50 h after single oral atofluding [9, 10, 20]. Thereafter, we presumed

that TFU would be the prodrug of 5-FU. Direct application of TFU may be superior to atofluding.

TFU conversion and sustained release of 5-FU are very important for its activity evaluation. Many derivatives of fluoropyrimidine are metabolized primarily in the liver by microsomal enzymes and then release their metabolite 5-FU into circulation [21, 22]. For instance, the bio-activation of capecitabine to 5-FU is considered to take place in three steps by different enzymes in the liver including carboxylesterase, cytidine deaminase and thymidine phosphorylase [23]. The pharmacokinetic studies in patients showed that the highest plasma level of 5-FU was detected 1 h after administration and maintained an active level for 1.5 h [23]. In the current study, we mimicked the catalytic procedure of TFU in vitro and found that TFU maintained high concentrations in the absence of liver microsomal enzymes. However, a slight reduction in TFU was observed with long incubation times. The cytosolic enzymes in tumors may also be involved in metabolism of the fluoropyrimidine derivatives [8, 18, 23]. Therefore, we presumed

that TFU was also degraded by the cytosolic enzymes in medium of cell culture in the absence of liver microsomal enzymes. In the presence of liver microsomal enzymes, the concentrations of TFU were gradually reduced and consequently the levels of 5-FU were increased, indicating that the sustained release of 5-FU was mainly metabolized by liver microsomal enzymes. The conversion of TFU and sustained release of 5-FU were confirmed by the measurement of plasma drug concentrations in mice bearing SMMC-7721 tumors. The concentration of 5-FU was detected in plasma 48 h after finishing oral administration, indicating that the long-lasting plasma 5-FU was released from TFU. The maintenance of the long-lasting plasma 5-FU is probably the main reason for its high anti-tumor activity *in vivo* [9].

The distribution and accumulation of 5-FU in tumors are also very important for the evaluation of anti-tumor activity of TFU. In this study, we examined the levels of drugs in plasma, liver, lung and tumor. High levels of TFU and its metabolite 5-FU were detected in liver, indicating that TFU preferentially accumulated in liver and was slowly metabolized to 5-FU [9, 10]. Therefore, prolonged levels of 5-FU were detected in plasma. Many studies have shown that prolonged continuous steady-state concentrations of 5-FU are superior to intermittent bolus injections in patients receiving adjuvant therapy [9]. The administration of intermittent bolus injections caused many serious toxicities [9]. Therefore, various prodrugs of 5-FU which continuously release 5-FU have been developed including S-1, capecitabine and TFU [2, 9, 23]. Importantly, 5-FU levels appear to be high in tumors and relatively low in lungs, indicating that the metabolite of TFU is preferentially converted or taken up by tumor cells. Tumor was the main site of action of 5-FU. These distributions of the metabolite 5-FU may contribute to the high therapeutic index of TFU *in vivo*.

The bio-activation of TFU to 5-FU has remained unknown. Because liver microsomal enzymes contain various forms of reductase and oxygenase [21, 22], the conversion of TFU may associate with the hydrolytic activity in liver microsomal enzymes [9]. Liver microsomal enzymes are the NADPH-dependent reductase [24]. The activity of reductase is initiated by donating electrons from NADPH consumption [14, 25]. By the bio-transformation pathways in liver, 5-FU is slowly released into blood and then preferentially distributed in tumor. Due to the high activity of the many 5-FU-associated enzymes in tumor tissues, *i.e.*, dihydropyrimidine dehydrogenase, thymidylate synthase, orotate phosphoribosyl transferase, and thymidine phosphorylase, 5-FU is preferentially accumulated and converted to its active form in tumor cells [2, 8, 9]. In addition, some of the cytosolic enzymes including cytidine deaminase have been found in high levels in

tumors [23, 26]. These enzymes are responsible for the intracellular activation of the fluoropyrimidine from the intermediate metabolite to the active form 5-FU [23, 26, 27]. We presume that these cytosolic enzymes are also involved in converting the intermediate metabolite of TFU to 5-FU in tumors. A high level of 5-FU was found in tumors. The bio-transformation pathways of TFU to 5-FU and the distributions of the intermediate metabolites need to be further investigated.

In conclusion, treatment of TFU in the presence of liver microsomal enzymes and oral administration of TFU in mice induced inhibitory effects on proliferation of hepatocellular carcinoma cells and tumors. Measurement of drug concentrations revealed that the inhibitory effect of TFU was associated with sustained release of 5-FU from TFU degraded by liver microsomal enzymes. The results presented here provide strong support for the effective development of TFU as a promising fluoropyrimidine derivative for cancer treatments.

Acknowledgments This study was supported by National Natural Science Foundation of China (30672485) and Chongqing Municipal Natural Science Foundation of China to Dr. JL Zhong.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. Dushinsky R, Plevin E, Heidelberger C (1957) The synthesis of 5-fluoropyrimidines. *J Am Chem Soc* 79:4559–4560
2. Shirasaka T (2009) Development history and concept of an oral anticancer agent S-1 (TS-1): its clinical usefulness and future vistas. *Jpn J Clin Oncol* 39:2–15
3. Yuan F, Qin X, Zhou D, Xiang QY, Wang MT, Zhang ZR, Huang Y (2008) *In vitro* cytotoxicity, *in vivo* biodistribution and anti-tumor activity of HPMA copolymer-5-fluorouracil conjugates. *Eur J Pharm Biopharm* 70:770–776
4. Felici A, Loos WJ, Verweij J, Cirillo I, de Bruijn P, Nooter K, Mathijssen RH, de Jonge MJ (2006) A pharmacokinetic interaction study of docetaxel and cisplatin plus or minus 5-fluorouracil in the treatment of patients with recurrent or metastatic solid tumors. *Cancer Chemother Pharmacol* 58:673–680
5. El-Khoueiry AB, Lenz HJ (2006) Should continuous infusion 5-fluorouracil become the standard of care in the USA as it is in Europe? *Cancer Invest* 24:50–55
6. Martinez J, Martin C, Chacon M, Korbenfeld E, Bella S, Senna S, Richardet E, Coppola F, Bas C, Hidalgo J, Escobar E, Reale M, Smilovich AM, Wasserman E (2006) Irinotecan, oxaliplatin plus bolus 5-fluorouracil and low dose folinic acid every 2 weeks: a feasibility study in metastatic colorectal cancer patients. *Am J Clin Oncol* 29:45–51
7. Arai W, Hosoya Y, Haruta H, Kurashina K, Saito S, Hirashima Y, Yokoyama T, Zuiki T, Sakuma K, Hyodo M, Yasuda Y, Nagai H, Shirasaka T (2008) Comparison of alternate-day versus consecutive-day treatment with S-1: assessment of tumor growth

- inhibition and toxicity reduction in gastric cancer cell lines in vitro and in vivo. *Int J Clin Oncol* 13:515–520
8. Guichard SM, Mayer I, Jodrell DI (2005) Simultaneous determination of capecitabine and its metabolites by HPLC and mass spectrometry for preclinical and clinical studies. *J Chromatogr B Anal Technol Biomed Life Sci* 826:232–237
 9. Liu J, Li X, Cheng YN, Cui SX, Chen MH, Xu WF, Tian ZG, Makuuchi M, Tang W, Qu XJ (2007) Inhibition of human gastric carcinoma cell growth by treatment of N_3 -*o*-toluyl-fluorouracil as a precursor of 5-fluorouracil. *Eur J Pharmacol* 574:1–7
 10. Liu J, Xu WF, Cui SX, Zhou Y, Yuan YX, Chen MH, Wang RH, Gai RY, Makuuchi M, Tang W, Qu XJ (2006) The inhibition of human gastric carcinoma cell growth by the atofluding derivative N_3 -*o*-toluyl-fluorouracil. *World J Gastroenterol* 12:6766–6770
 11. Sun W, Zhang N, Li A, Zou W, Xu W (2008) Preparation and evaluation of N_3 -*o*-toluyl-fluorouracil-loaded liposomes. *Int J Pharm* 353:243–250
 12. Zou W, Sun W, Zhang N, Xu W (2008) Enhanced oral bioavailability and absorption mechanism study of N_3 -*o*-toluyl-fluorouracil-loaded liposomes. *J Biomed Nanotechnol* 4:1–9
 13. Sun W, Zou W, Huang G, Li A, Zhang N (2008) Pharmacokinetics and targeting property of TFu-loaded liposomes with different sizes after intravenous and oral administration. *J Drug Target* 16:357–365
 14. Das M, Rastogi S, Khanna SK (2004) Mechanism to study 1:1 stoichiometry of NADPH and alkoxyphenoxazones metabolism spectrophotometrically in subcellular biological preparations. *Biochim Biophys Acta* 1675:1–11
 15. Chen MH, Cui SX, Cheng YN, Sun LR, Li QB, Xu WF, Ward SG, Tang W, Qu XJ (2008) Galloyl cyclic-imide derivative CH1104I inhibits tumor invasion through suppressing matrix metalloproteinase activity. *Anticancer Drugs* 19:957–965
 16. Licea-Perez H, Wang S, Bowen C (2009) Development of a sensitive and selective LC–MS/MS method for the determination of α -fluoro- β -alanine, 5-fluorouracil and capecitabine in human plasma. *J Chromatogr B Anal Technol Biomed Life Sci* 877:1040–1046
 17. Musende AG, Eberding A, Wood C, Adomat H, Fazli L, Hurtado-Coll A, Jia W, Bally MB, Guns ET (2009) Pre-clinical evaluation of Rh2 in PC-3 human xenograft model for prostate cancer in vivo: formulation, pharmacokinetics, biodistribution and efficacy. *Cancer Chemother Pharmacol* 64:1085–1095
 18. Ozawa S, Hamada M, Murayama N, Nakajima Y, Kaniwa N, Matsumoto Y, Fukuoka M, Sawada J, Ohno Y (2002) Cytosolic and microsomal activation of doxifluridine and tegafur to produce 5-fluorouracil in human liver. *Cancer Chemother Pharmacol* 50:454–458
 19. Li Q, Feng FY, Han J, Sui GJ, Zhu YG, Zhang Y, Zhang ZH, Li L, Wang PH, Zhou MZ, Zhang YC (2002) Phase III clinical study of a new anticancer drug atofluding. *Ai Zheng* 21:1350–1353
 20. Xu W, Zhang Z, Castaner J (2001) Atoflluding. *Drugs Future* 26:935–938
 21. Tabata T, Katoh M, Tokudome S, Nakajima M, Yokoi T (2004) Identification of the cytosolic carboxylesterase catalyzing the 5'-deoxy-5-fluorocytidine formation from capecitabine in human liver. *Drug Metab Dispos* 32:1103–1110
 22. Peet CF, Enos T, Nave R, Zech K, Hall M (2005) Identification of enzymes involved in phase I metabolism of ciclesonide by human liver microsomes. *Eur J Drug Metab Pharmacokinet* 30:275–286
 23. Vainchtein LD, Rosing H, Schellens JH, Beijnen JH (2009) A new, validated HPLC–MS/MS method for the simultaneous determination of the anti-cancer agent capecitabine and its metabolites: 5'-deoxy-5-fluorocytidine, 5'-deoxy-5-fluorouridine, 5-fluorouracil and 5-fluorodihydrouracil, in human plasma. *Bio-med Chromatogr*. doi:10.1002/bmc.1302
 24. Teichert J, Baumann F, Chao Q, Franklin C, Bailey B, Hennig L, Caca K, Schoppmeyer K, Patzak U, Preiss R (2007) Characterization of two phase I metabolites of bendamustine in human liver microsomes and in cancer patients treated with bendamustine hydrochloride. *Cancer Chemother Pharmacol* 59:759–770
 25. Rastogi S, Das M, Khanna SK (2002) A novel approach to study the activity and stoichiometry simultaneously for microsomal pentoxyresorufin-*O*-dealkylase reaction. *FEBS Lett* 512:121–124
 26. Ribelles N, López-Siles J, Sánchez A, González E, Sánchez MJ, Carabantes F, Sánchez-Rovira P, Márquez A, Dueñas R, Sevilla I, Alba E (2008) A carboxylesterase 2 gene polymorphism as predictor of capecitabine on response and time to progression. *Curr Drug Metab* 9:336–343
 27. Toi M, Bando H, Horiguchi S, Takada M, Kataoka A, Ueno T, Saji S, Muta M, Funata N, Ohno S (2004) Modulation of thymidine phosphorylase by neoadjuvant chemotherapy in primary breast cancer. *Br J Cancer* 90:2338–2343