Hydrolysis of 3',5'-Dioctanoyl-5-fluoro-2'-deoxyuridine (FdUrd-C8) in Rabbit and Human Plasma and Its Release from Oily Solution

Shoji FUKUSHIMA,* Yoshiki HAYASHI,* Takeo KAWAGUCHI,** Mika KANEKO,* and Masahiro NAKANO*...***

Department of Pharmaceutical Services, Kumamoto University Hospital, *1-1-1 Honjo, Kumamoto, 860, Japan and Faculty of Pharmaceutical Science, Josai University, **1-1 Keyakidai, Sakado, Saitama, 350-02, Japan

(Received March 13, 1989)

Hydrolysis of 3',5'-dioctanoyl-5-fluoro-2'-deoxyuridine (FdUrd-C8), a lipophilic prodrug of 5-fluoro-2'-deoxyuridine (FdUrd), 3'-octanoyl-5-fluoro-2'-deoxyuridine (3'-octanoyl FdUrd) and 5'-octanoyl-5-fluoro-2'-deoxyuridine (5'-octanoyl FdUrd) in the rabbit and human plasma and release of FdUrd-C8 and its hydrolyzed species from Lipiodol Ultra-Fluid (Lipiodol), an oily lymphographic agent, containing FdUrd-C8 were examined. The rates of hydrolysis of FdUrd-C8, 3'-octanoyl FdUrd and 5'-octanoyl FdUrd in the rabbit plasma were fast and almost the same among the three compounds; half lives were 2.4, 3.6 and 3.9 min for 5'-octanoyl FdUrd, FdUrd-C8 and 3'-octanoyl FdUrd, respectively. In the human plasma, however, the rates of hydrolysis were much different among the three compounds and were slower than those in the rabbit plasma; half lives were 11.5, 130 and 1020 min for 5'-octanoyl FdUrd, FdUrd-C8 and 3'-octanoyl FdUrd, respectively. Ratios of the rate constant of 5'-octanoyl FdUrd to that of 3'-octanoyl FdUrd were 1.6 and 85.7 in the rabbit plasma and in the human plasma, respectively. In a release study, although detected species in a release medium were different between the rabbit plasma and the human plasma and the amount of each species reflected the characteristics of esterase in each plasma, the total amounts of compounds released were almost the same both in the rabbit plasma and the human plasma. The existence of bovine serum albumin in a release medium increased the amount of compounds released from a Lipiodol solution to a greater extent than the existence of porcine liver esterase in the release medium. The FdUrd-C8 content in Lipiodol affected the duration of release but not the amount of compounds released.

Keywords — floxuridine, 5-fluoro-2'-deoxyuridine; 3',5'-dioctanoyl-5-fluoro-2'-deoxyuridine; Lipiodol; ester prodrug; esterase; hydrolysis; enzyme specificity; oily solution; sustained release

Introduction

Intra-arterial administration of an oily lymphographic agent, Lipiodol Ultra-Fluid (hereafter abbreviated Lipiodol), containing an anticancer agent, is a new approach to treatment of a hepatic cancer.12 The authors have reported3,4 that Lipiodol containing 3',5'-dioctanoyl-5-fluoro-2'-deoxyuridine (hereafter abbreviated FdUrd-C8), which is a lipophilic prodrug of floxuridine, 5-fluoro-2'-deoxyuridine (hereafter abbreviated FdUrd), exhibited a selective anticancer effect and selective accumulation of FdUrd-C8 and its metabolites in a tumor when injected into the hepatic artery of rabbits bearing VX-2 tumor in the liver; namely, 1) Lipiodol selectively remained at the hepatic cancer area, 2) growth of VX-2 tumor was inhibited for 7 d after injection of Lipiodol containing FdUrd-C8, and 3) elimination of FdUrd-C8 from the tumor was slower than that from nontumorous sites and selectively higher levels of FdUrd-C8 and its metabolites in the tumor were achieved and kept for 72 h after administration. This therapy for the liver cancer, intra-arterial administration of Lipiodol containing FdUrd-C8, has been under clinical study in Japan.

The parent compound, FdUrd, is expected to be regenerated through two processes; 1) release of FdUrd-C8 from Lipiodol into the body fluid and 2) hydrolysis of FdUrd-C8 to FdUrd by esterase. Since release profiles and rates of hydrolysis are important factors for the anticancer effect of Lipiodol containing FdUrd-C8, factors affecting release profiles of FdUrd-C8 from Lipiodol and rates of hydrolysis of

*** To whom correspondence should be addressed.
FdUrd-C8 and its metabolites (3'-octanoyl FdUrd and 5'-octanoyl FdUrd) in the rabbit and human plasma were examined in this study.

Materials and Methods

Materials — 5-Fluoro-2'-deoxyuridine (FdUrd) was purchased from Heinrich Mack Nachf (West Germany). 3',5'-Diocanoyl-5-fluoro-2'-deoxyuridine (FdUrd-C8) was synthesized according to the method of Nishizawa et al. 3'-Octanoyl-5-fluoro-2'-deoxyuridine (3'-octanoyl FdUrd) was synthesized by acylation of 5'-triethylenepentyl-FdUrd, and subsequent de- triethylene methylation. 5'-Octanoyl-5-fluoro-2'-deoxyuridine (5'-octanoyl FdUrd) was obtained by reacting FdUrd with an equimolar acid anhydride below 0°C and a small amount of 3'-octanoyl-FdUrd was removed by silica gel column chromatography. All the esters were more than 98% pure as observed by high performance liquid chromatography (HPLC). Stock solutions of all esters were prepared in ethanol to give a concentration of 1 mg/ml and stored at 4°C. Lipiodol Ultra-Fluid purchased from Kodama Co., Tokyo, Japan, was a product of Laboratoire Gerbert, Paris, France. Porcine liver esterase (hereafter abbreviated PL-esterase) was purchased from Sigma Chemical Co., U.S.A. The esterase suspension in 3.2 M (NH₄)₂SO₄ solution was diluted with 0.1 M phosphate buffer, pH 7.0, and filtered through a membrane filter (Milliex-HA 0.45 μm, Millipore Co., U.S.A.) and stored at 4°C for no more than 50 h before use. Bovine serum albumin fraction V (hereafter abbreviated BSA) was purchased from Nakarai Chemicals Co., Kyoto. The rabbit plasma was obtained from male New Zealand white rabbits using a heparinized tube and the freshly obtained plasma was adequately mixed and used for experiments. The human plasma was obtained from a healthy volunteer using a heparinized tube and the freshly obtained plasma was used for experiments.

Measurement of Rates of Hydrolysis of FdUrd-C8, 3'-Octanoyl FdUrd and 5'-Octanoyl FdUrd in Rabbit and Human Plasma — Since FdUrd is expected to be regenerated from FdUrd-C8 through two pathways with two steps described by Chart 1, 6) hydrolytic rate constants of three species, FdUrd-C8, 3'-octanoyl FdUrd and 5'-octanoyl FdUrd were examined. A stock solution of FdUrd-C8 or 3'-octanoyl FdUrd or 5'-octanoyl FdUrd in ethanol was introduced into a prewarmed (37°C) plasma (the volume ratio was 1:100 and each volume was adequately set) and the resultant mixture was incubated at 37°C. A sample (200 μl) was withdrawn at appropriate intervals and 1 ml of methanol was immediately added to the sample for precipitation of proteins. The resultant solution was mixed and centrifuged for 10 min at 3000 rpm. Drugs in the supernatant were analyzed with HPLC. HPLC conditions were as follows: column; LiChrosorb RP-18 (Sumitomo Chemical Industry Co., Tokyo), flow rate; 1.0 ml/min, column temperature; 50°C, detector wavelength; 270 nm, mobile phase; a solvent mixture of water containing 0.1% acetic acid and acetonitrile (water-acetonitrile; 98:2, 25:75, 50:50 and 50:50 for FdUrd, FdUrd-C8, 3'-octanoyl FdUrd and 5'-octanoyl FdUrd, respectively). Hydrolytic rate constants were calculated by the linear least squares method.

Measurement of Release Rates — Lipiodol containing FdUrd-C8 (100 μl) was introduced into a prewarmed (37°C) release medium (see below) (10 ml) in a 50 ml volume Ehlenmeyer flask and the mixture was shaken at 37°C. Lipiodol containing FdUrd-C8 sunk because its density was greater than that of the release medium and remained as a single droplet during the experiment. A release medium was a plasma (rabbit or human) or 0.1 M phosphate buffer containing esterase and/or BSA. A sample (200 μl) of the release medium was withdrawn at appropriate intervals and was immediately treated in the same way as in measurement of rates of hydrolysis in a plasma except that the sample was adequately stood in order to hydrolyze all FdUrd-C8 to FdUrd until methanol was added when the release medium contained PL-esterase. The FdUrd-C8 content in Lipiodol was constantly 50 mg/0.1 ml except in the study on the effect of the FdUrd-C8 content in Lipiodol on release profiles.
Chart 1. Hydrolytic Cleavage of FdUrd-C8 to FdUrd
Letters $k_1 - k_4$ represent the pseudo-first-order rate constants for each hydrolysis step.

Results and Discussion

Hydrolysis of FdUrd-C8, 3'-Octanoyl FdUrd and 5'-Octanoyl FdUrd in the Rabbit and Human Plasma

Fig. 1. Hydrolysis of FdUrd-C8, 3'-Octanoyl FdUrd and 5'-Octanoyl FdUrd in the Rabbit Plasma at 37 °C
Averages of three experiments. O, FdUrd-C8; ●, 3'-octanoyl FdUrd; ○, 5'-octanoyl FdUrd.

Fig. 2. Hydrolysis of FdUrd-C8, 3'-Octanoyl FdUrd and 5'-Octanoyl FdUrd in the Human Plasma at 37 °C
Averages of three experiments. O, FdUrd-C8; ●, 3'-octanoyl FdUrd; ○, 5'-octanoyl FdUrd.
TABLE I. Hydrolytic Rate Constants and Half Lives of FdUrd-C8, 3'-Octanoyl FdUrd and 5'-Octanoyl FdUrd in Rabbit and Human Plasma at 37 °C

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rabbit plasma</th>
<th>Human plasma</th>
<th>Site-specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate constant, min⁻¹</td>
<td>Half life, min</td>
<td>Rate constant, min⁻¹</td>
</tr>
<tr>
<td>FdUrd-C8</td>
<td>0.19±0.01</td>
<td>3.6±0.2</td>
<td>0.0052±0.0003</td>
</tr>
<tr>
<td>3'-Octanoyl FdUrd</td>
<td>0.18±0.01</td>
<td>3.9±0.2</td>
<td>0.0007±0.0001</td>
</tr>
<tr>
<td>5'-Octanoyl FdUrd</td>
<td>0.29±0.005</td>
<td>2.4±0.04</td>
<td>0.06±0.0008</td>
</tr>
</tbody>
</table>

n= 3, mean ± SEM.

The time courses of disappearance of FdUrd-C8, 3'-octanoyl FdUrd and 5'-octanoyl FdUrd when they were separately introduced into the rabbit or human plasma are shown in Figs. 1 and 2. They all disappeared following the first order kinetics and the calculated hydrolytic rate constants, k₁ + k₂, k₃, and k₄ (see Chart 1), and corresponding half lives are shown in Table I. The time courses of disappearance of FdUrd-C8 and appearance of hydrolyzed species when FdUrd-C8 was introduced into the rabbit or human plasma are shown in Figs. 3 and 4.

In the rabbit plasma (Fig. 1), rates of hydrolysis of three compounds were fast and were almost the same among three compounds; half lives were 2.4, 3.6 and 3.9 min for 5'-octanoyl FdUrd, FdUrd-C8 and 3'-octanoyl FdUrd, respectively. The ratio of k₄ to k₃ (k₄/k₃) was 1.6 and site-specificity of esterase in the rabbit plasma on 5'site and 3'site of monoesters of FdUrd was small.

![Fig. 3. Time Course of Disappearance of FdUrd-C8 and Appearance of Its Hydrolyzed Species in the Rabbit Plasma at 37 °C](image)

Averages of three experiments. ○, FdUrd-C8; ▽, FdUrd; 3'-octanoyl FdUrd and 5'-octanoyl FdUrd were not detected.

![Fig. 4. Time Course of Disappearance of FdUrd-C8 and Appearance of Its Hydrolyzed Species in the Human Plasma at 37 °C](image)

Averages of three experiments. ○, FdUrd-C8; ●, 3'-octanoyl FdUrd; ▽, FdUrd; 5'-octanoyl FdUrd.
In the human plasma (Fig. 2), however, rates of hydrolysis of three compounds were slower than those in the rabbit plasma and were much different among three compounds; half lives were 11.5, 130 and 1020 min for 5'-octanoyl FdUrd, FdUrd-C8 and 3'-octanoyl FdUrd, respectively. The ratio of $k_4$ to $k_5$ ($k_4/k_5$) was 85.7 and site-specificity of esterase in the human plasma on 5'site and 3'site of monoesters of FdUrd were larger than that of esterase in the rabbit plasma.

These results reflected on the species appeared when FdUrd-C8 was introduced into the rabbit and human plasma. In the rabbit plasma (Fig. 3), neither 3'-octanoyl FdUrd nor 5'-octanoyl FdUrd were detected and only FdUrd was detected. The rates of hydrolysis of 5'-octanoyl FdUrd and 3'-octanoyl FdUrd were so fast that they were rapidly hydrolyzed to FdUrd following their production from FdUrd-C8 in the rabbit plasma. In the human plasma (Fig. 4), however, 5'-octanoyl FdUrd was detected at a low concentration and was not detected at 4 h but 3'-octanoyl FdUrd and FdUrd were detected at much higher concentrations than 5'-octanoyl FdUrd. Although 5'-octanoyl FdUrd was rapidly hydrolyzed to FdUrd following its production from FdUrd-C8 because the rate of hydrolysis was fast (half life of 11.5 min), 3'-octanoyl FdUrd was accumulated following its production from FdUrd-C8 since its rate of hydrolysis was slow (half life of 1020 min). Assuming that all of 5'-octanoyl FdUrd produced from FdUrd-C8 was hydrolyzed to FdUrd in 4 h and that FdUrd produced from 3'-octanoyl FdUrd was ignored when FdUrd-C8 was introduced into the human plasma, then the ratio of 3'-octanoyl FdUrd to FdUrd would approximate $k_2/k_3$, $k_2/k_1$ was about 3.6.

Release Study

It may be considered that FdUrd-C8 dissolved within Lipiodol cannot be hydrolyzed by esterases in the aqueous medium and that FdUrd-C8 released from Lipiodol into a release medium can be hydrolyzed by esterases in the release medium. Therefore 3'-octanoyl FdUrd, 5'-octanoyl FdUrd and FdUrd appeared in the release medium in the release study may be considered to be hydrolyzed products of FdUrd-C8 released from Lipiodol.

However, no species could be detected when FdUrd-C8 in Lipiodol was equilibrated in a release medium of 0.1 M phosphate buffer (data are not shown) probably because the solubility of FdUrd-C8 was very small in the buffer. If so, some solubilizers are necessary for release of FdUrd-C8 from Lipiodol.

Release of FdUrd-C8 and Its Hydrolyzed Compounds from Lipiodol Containing FdUrd-C8 in the Rabbit and Human Plasma

Release profiles of FdUrd-C8 and its hydrolyzed species from Lipiodol containing FdUrd-C8 in the rabbit and human plasma are shown in Figs. 5 and 6, and the total amounts (summation of four species on a molar basis) of released compounds are shown in Fig. 7.

Detected species in each plasma reflected the characteristics of esterase observed in the hydrolysis study. In the rabbit plasma (Fig. 5), since rates of hydrolysis were rapid and site-specificity was not large, FdUrd-C8 released from Lipiodol was hydrolyzed rapidly to FdUrd resulting in high concentrations of FdUrd and low concentration of FdUrd-C8, 3'-octanoyl FdUrd and 5'-octanoyl FdUrd. In the human plasma (Fig. 6), however, since the rate of hydrolysis of FdUrd-C8 at 5'site was about 4 times the rate of hydrolysis of FdUrd-C8 at 3'site, FdUrd-C8 was hydrolyzed more slowly than in the rabbit plasma.

Fig. 5. Release Profiles of FdUrd-C8 and Its Hydrolyzed Species from Lipiodol Containing FdUrd-C8 into the Rabbit Plasma at 37°C

Averages of three experiments. ○, FdUrd-C8; □, 3'-octanoyl FdUrd; ●, 5'-octanoyl FdUrd; ▽, FdUrd.
higher than that at 3' site and 3'-octanoyl FdUrd was very slowly hydrolyzed to FdUrd and 5'-octanoyl FdUrd was rapidly hydrolyzed to FdUrd, the main detectable species was 3'-octanoyl FdUrd. FdUrd was detected at a lower concentration than 3'-octanoyl FdUrd and concentrations of 5'-octanoyl FdUrd were much lower.

In spite of the difference between the rabbit and human plasma in their detectable species, the total amounts of released compounds were almost the same (Fig. 7). This indicates that release rates of FdUrd-C8 from Lipiodol were almost the same in both the rabbit and human plasma.

As to the factors affecting release of FdUrd-C8 from Lipiodol, two factors, rate of hydrolysis of FdUrd-C8 to FdUrd which is easily soluble in water and solubilization of FdUrd-C8 itself with some solubilizers in a plasma (i.e. albumin), should be considered. The effect of these two factors was examined.

**Effect of Esterase Activity and/or BSA on Release of FdUrd-C8 from Lipiodol**

PL-esterase almost completely hydrolyzed FdUrd-C8 to FdUrd within 5 min in 0.1 M phosphate buffer containing 5 U/ml PL-esterase (data are not shown) and this activity was much higher than those in the rabbit and human plasma.

Figure 8 shows the effect of PL-esterase concentration in 0.1 M phosphate buffer on release profiles of FdUrd-C8 from Lipiodol. The total amounts of compounds released were increased as the activity of esterase in the release medium was increased and the release profile exhibited zero order kinetics. However, the total amounts of compounds released in these media were much lower than those in the rabbit and human plasma.

**Fig. 8.** Release Profiles of FdUrd-C8 from Lipiodol into the Phosphate Buffer Containing PL-esterase

Averages of three experiments. Content of PL-esterase: ○, 5 U/ml; ●, 10 U/ml; ⊘, 20 U/ml.
Hydrolysis and Release of FdUrd-C8

Fig. 9. Effect of BSA on Release Profiles of FdUrd-C8 from Lipiodol

Averages of three experiments. Release medium: ○, phosphate buffer containing PL-esterase (5 U/ml) and BSA (5%); ●, phosphate buffer containing BSA (5%); □, phosphate buffer containing PL-esterase (5 U/ml).

plasma in spite of the activity of esterase in the buffer containing PL-esterase being much higher than those in the rabbit and human plasma.

Figure 9 shows the effect of BSA on release profiles. The total amounts of compounds released were highly increased when the release medium contained BSA whether PL-esterase was contained or not. Figure 10 shows the detectable species when the release medium was 0.1 M phosphate buffer containing only BSA. FdUrd-C8 was detected at the highest concentration and other species were detected at a lower concentration. This indicates that FdUrd-C8 was solubilized by BSA and BSA also had esterase activity.

From these results (Figs. 5—10), it is suggested that release of FdUrd-C8 from Lipiodol depends on its solubility in the release medium affected by serum albumin or other factors and the appearance rate of hydrolyzed species depends on the esterase activity in the release medium.

Fig. 10. Release Profiles of FdUrd-C8 and Its Hydrolyzed Species from Lipiodol into the Phosphate Buffer Containing BSA (5%)

Averages of three experiments. ○, FdUrd-C8; ●, 3'-octanoyl FdUrd; □, 5'-octanoyl FdUrd; ▽, FdUrd.

Fig. 11. Effect of FdUrd-C8 Content in Lipiodol on Release Profiles of FdUrd-C8 from Lipiodol into the Phosphate Buffer Containing PL-Esterase

FdUrd-C8 content in Lipiodol: ○, 50 mg/0.1 ml; ●, 25 mg/0.1 ml; □, 12.5 mg/0.1 ml.

Fig. 12. Effect of FdUrd-C8 Content in Lipiodol on Release Profiles of FdUrd-C8 from Lipiodol into the Human Plasma

FdUrd-C8 content in Lipiodol: ○, 50 mg/0.1 ml; ●, 25 mg/0.1 ml.
Effect of FdUrd-C8 Content in Lipiodol on Release Profiles

Since it has been reported that the anticancer effect of Lipiodol containing FdUrd-C8 was dependent on the FdUrd-C8 content in Lipiodol, its effect on the release profiles was examined in the PL-esterase solution and in the human plasma (Figs. 11 and 12).

The total amounts of compounds released were almost equal among three contents both in the PL-esterase solution and in the human plasma and the release profile nearly followed zero order kinetics, although the quantity was much different between the two release media. These results suggested that duration of release of FdUrd-C8 from Lipiodol increased as the FdUrd-C8 content in Lipiodol was increased and that the degree of duration might be related to the content dependent anticancer effect of FdUrd-C8 in Lipiodol.

General Discussion

Lipiodol was located in the blood vessels, an extra-cellular space and an intra-cellular space of the liver tumor after administration into the hepatic artery. In the present study, we examined release of FdUrd-C8 from Lipiodol and hydrolysis of FdUrd-C8 in the rabbit and human plasma in order to comprehend the fate of FdUrd-C8 in Lipiodol which was located in the blood vessel and in an extra-cellular space. From the study, it was found that release of FdUrd-C8 from Lipiodol is sustained and that duration of release is prolonged as the FdUrd-C8 content in Lipiodol is increased. It was also found that the amount of FdUrd-C8 released from Lipiodol is the same both in rabbits and humans, but the amounts of hydrolyzed species from FdUrd-C8 were different between the rabbit plasma and human plasma depending on the characteristics of the esterase.

Permeability of these species; FdUrd-C8, 3'-octanoyl FdUrd, 5'-octanoyl FdUrd and FdUrd through a cancer cell membrane is important for anticancer effects of FdUrd-C8 in Lipiodol. Although high extraction of FdUrd by the liver after administration of a FdUrd solution into the hepatic artery has been reported, permeability or extraction of FdUrd-C8, 3'-octanoyl FdUrd and 5'-octanoyl FdUrd has not been examined and must be examined in the future.

On the other hand, the fate of FdUrd-C8 in Lipiodol which is located in an intra-cellular space is not clear; whether FdUrd-C8 exists in Lipiodol or not and how the characteristics of esterase in a cancer cell is. These are the problems to be examined in the future.

References