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6-[(2-Iminopyrrolidinyl)methyl]-5-[^{125}I]iodouracil as a potential thymidine phosphorylase-targeted radiopharmaceutical: synthesis and preliminary biological evaluation

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Thymidine phosphorylase (TP) is expressed at higher levels in many types of malignant tumors than in adjacent nonneoplastic tissues. The aim of this study was to develop a radiolabeled TP inhibitor, 6-[(2-iminopyrrolidinyl)methyl]-5-[^{125}I]iodouracil ([^{125}I]1) as a TP-targeted radiopharmaceutical. No-carrier-added [^{125}I]1 was synthesized by halogen exchange of the corresponding bromide (2). After purification by reverse-phase HPLC, [^{125}I]1 showed a radiochemical purity of over 97%. When administered to normal mice, [^{125}I]1 showed a rapid clearance from the blood and a low accumulation in the thyroid and stomach, indicating good *in vivo* stability against deiodination. By coinjection of unlabeled 1, the uptakes in the TP-expressing normal tissues, small intestine and liver were significantly reduced, suggesting TP-specific modes of accumulation of [^{125}I]1. These findings suggest that [^{125}I]1 possesses the required properties for *in vivo* imaging of TP activity.

Keywords: thymidine phosphorylase; tumor; radiolabeled inhibitor

Introduction

Thymidine phosphorylase (TP), which is identical to platelet-derived endothelial cell growth factor, catalyzes the reversible phosphorolysis of thymidine into thymine and 2-deoxy-D-ribose-1-phosphate.[1] Many types of malignant tumors express higher levels of TP than adjacent nonneoplastic tissues, possibly promoting angiogenesis, tumor growth, invasion and metastasis.[2–6] Indeed, enhanced expression of TP is associated with a poor prognosis in cancer patients.[7–9] This enzyme is also involved in the catabolism of anti-cancer agents. TP is thought to catalyze not only the inactivation of cytotoxic pyrimidine analogs such as trifluorothymidine but also the metabolic activation of 5-fluorouracil and its prodrugs.[10,11] Therefore, *in vivo* imaging of TP activity would be helpful in estimating the tumor characteristics and in determining treatment strategies.

As a tracer of TP activity, an ^{18}F-labeled TP substrate, 5′-deoxy-5′-[^{18}F] fluorothymidine ([^{18}F]DFT), was synthesized recently and evaluated *in vitro*.[12] Although [^{18}F]DFT was taken up by tumor cells, the uptake was not correlated with TP activity. Furthermore, the accumulated radioactivity effluxed completely from the tumor cells by 1 h. These phenomena could be due to the diverse cellular fates of [^{18}F]DFT including the generation of the readily diffusible metabolites. These observations suggest that it may be difficult to image TP activity by approaches using radiolabeled TP substrate analogs.

Several TP inhibitors have been developed as therapeutic agents. Among them, 5-halogenated 6-[1-(2-iminopyrrolidinyl)-methyl]uracils (Cl: TPI, I: **1**, Br: **2**; Figure 1) have advantages over previously described inhibitors such as 6-amino-5-chlorouracil.[13] These have a 1000-fold higher inhibitory activity than 6-amino-5-chlorouracil and do not inhibit other enzymes involved in pyrimidine nucleoside metabolism. Experiments using recombinant enzymes showed that TPI and **2** are tightly and irreversibly bound to the active site of TP as mimics of the substrate transition state.[14–16] Furthermore, the potency of these inhibitors was demonstrated *in vivo*. In nude mice bearing TP-expressing tumors, administration of TPI caused a significant reduction in tumor growth rate and suppressed metastasis to the liver or lung.[17–19] TAS-102, which combines trifluorothymidine with TPI at a 1:0.5 molar ratio, is currently undergoing clinical trials as a new anti-tumor drug preparation.[20,21] From these observations, TP inhibitors could be considered as candidate compounds for *in vivo* imaging of TP activity. In this

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study, a radiolabeled TP inhibitor, 6-[(2-iminopyrrolidinyl)-methyl]-5-[^{125}I]iodouracil ([^{125}I]**1**; Figure 1), was synthesized, and the distribution properties of [^{125}I]**1** in normal mice were evaluated.

Results and discussion

Chemistry

The synthesis of **2** has been performed by alkylation of 2-iminopyrrolidine hydrochloride with **4** in the presence of NaOEt in DMF.[22,23] In the synthesis of TPI, the use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)/MeOH in place of NaOEt/DMF led to the improvement of chemical yields.[23] As shown in Scheme 1, 2-iminopyrrolidine hydrochloride was alkylated with **4** in the presence of DBU in MeOH to give the radiolabeling precursor **2** (25%). Alkylation of 2-iminopyrrolidine hydrochloride with **3** in the presence of DBU in MeOH resulted in the desired compound **1** in 47% yield.

[^{125}I]**1** was synthesized by halogen exchange of the corresponding bromide **2** (Scheme 2). The reaction was carried out at 145°C. In reverse-phase HPLC analyses, the required [^{125}I]**1** and precursor bromide **2** showed well-separated peaks as shown in Figure 2. Purification by HPLC with concomitant UV and radioactivity detection confirmed the identity of [^{125}I]**1** by comparison with its corresponding unlabeled analog **1**. [^{125}I]**1** was obtained with a radiochemical yield varying from 8 to 17% and was not optimized. Radiochemical purity exceeded 97% with a specific activity above 5.0 GBq/μmol, based on the limits of detection of the HPLC UV detector.

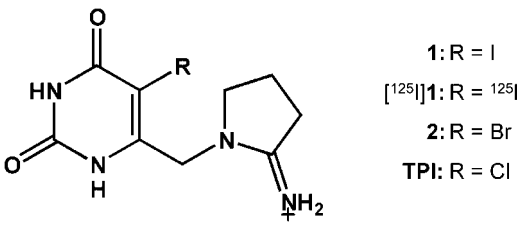


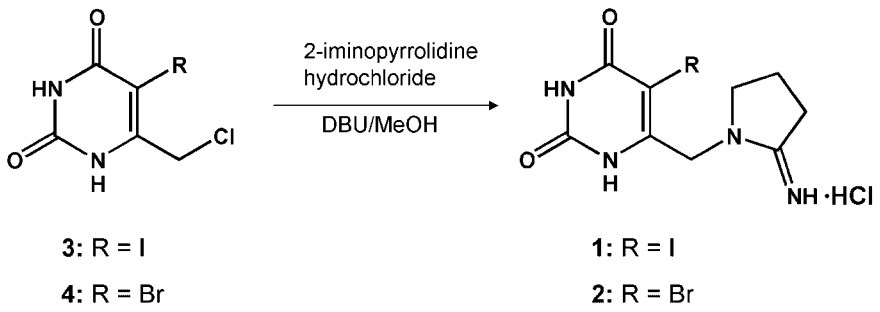
Figure 1. Chemical structures of TP inhibitor and its radioiodinated analog.

In vitro stability

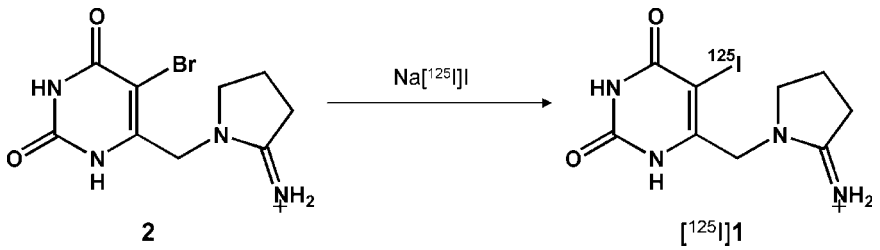
Table 1 shows the *in vitro* stability of [^{125}I]**1** in buffered solutions at different pH. When incubated in phosphate-buffered saline (pH 7.4), the radiochemical purity of [^{125}I]**1** gradually decreased to about 60% within 24 h. On the other hand, under mildly acidic conditions (pH 4.8, acetate-buffered saline) there was no measurable decomposition of [^{125}I]**1** over a 24-h incubation period. Therefore, acetate-buffered saline (pH 4.8) was used as an injection medium for the *in vivo* studies.

Tissue distribution in mice

To evaluate the radioactivity pharmacokinetics, [^{125}I]**1** was administered to normal mice (Table 2). [^{125}I]**1** showed a rapid clearance from the blood and a marked accumulation in the small intestine, kidney and liver. At 24-h postinjection, 80 and 10% of the injected radioactivity were recovered in the urine and feces, respectively. The radioactivity levels in the stomach and thyroid were low, indicating the good *in vivo* stability against deiodination. It is reported that TP is highly expressed in the small intestine and liver in mice.[24–26] Thus, the accumulation of [^{125}I]**1** in these organs should be mediated by TP binding. In fact, coinjection of unlabeled **1** (3 mg/kg) significantly reduced the accumulation in the small intestine by over one-tenth and in the liver by over one-twentieth (Figure 3). On the other hand, the level of [^{125}I]**1** in the kidney, which should participate in the excretion of [^{125}I]**1**, was not altered by coinjection of **1**, suggesting a nonspecific accumulation. This is consistent with the reported findings that TP activity in the kidney was low.[24,26] More recently, a radioiodinated TP inhibitor, 6-[(2-iminoimidazolidinyl)methyl]-5-[^{125}I]iodouracil ([^{125}I]5I6IMU),



Scheme 1.



Scheme 2.

was synthesized.[27] The distribution patterns of [^{125}I]5I6IMU in normal mice were similar to those of [^{125}I]**1**, but at present the effect of [^{125}I]5I6IMU cannot be elucidated because the specific accumulation of [^{125}I]5I6IMU was not determined.

Experimental

General

Chemical reagents and solvents were of commercial quality and were used without further purification unless otherwise noted. All melting points (m.p.) were determined on a Yanaco m.p. apparatus and are uncorrected. ^{1}H-NMR spectra were obtained on a Varian Unity 400 (400 MHz), and the chemical shifts are reported in parts per million downfield from tetramethylsilane. Infrared (IR) spectra were recorded with a Shimadzu FTIR-8400 spectrometer. Mass spectra were obtained with a JEOL JMS DX-610 (FAB MS) or an Applied Biosystems Mariner System 5299 spectrometer (ESI MS). The progress of the reaction was monitored by TLC on Silica gel 60F 254 plates (Merck).

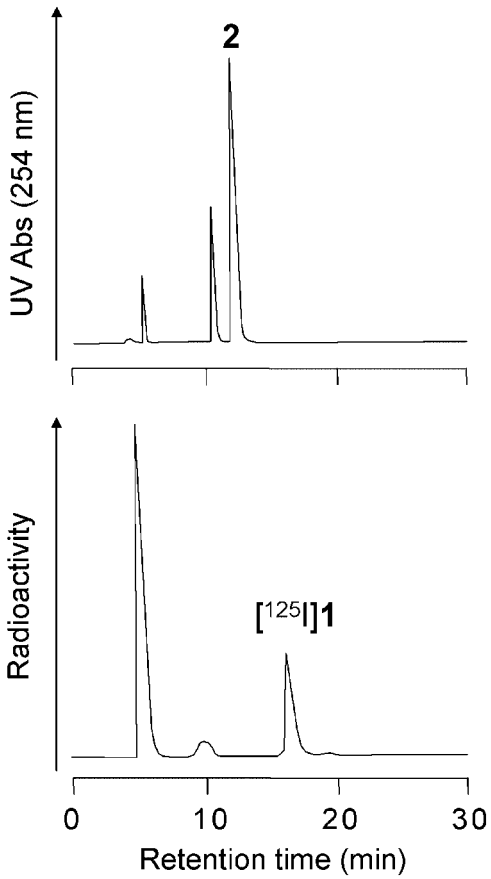


Figure 2. Typical HPLC profiles of the radiolabeling reaction mixture. The analysis was performed with a Cosmosil 5C$_{18}$-PAQ column (4.6 × 250 mm) eluted with 0.1% aqueous TFA at a flow rate of 0.8 mL/min. Under these conditions, the retention times were 17–18 min for **1** and 12–13 min for **2**.

Chemistry

*6-[(2-Iminopyrrolidinyl)methyl]-5-iodouracil hydrochloride (**1**)*

2-Iminopyrrolidine hydrochloride was synthesized from 2-pyrrolidinone as described previously.[28] 6-Chloromethyl-5-iodouracil (**3**) was prepared by halogenation of 6-chloromethyluracil with *N*-iodosuccinimide in DMF.[22,29] To a solution of **3** (143 mg, 0.50 mmol) and DBU (152 mg, 1.0 mol) in 1.0 mL of MeOH was added 2-iminopyrrolidine hydrochloride (72.5 mg, 0.60 mmol) and heated under reflux for 1 h. After cooling in an ice bath, the precipitate was collected by filtration and dissolved in 3 M aqueous HCl (1.0 mL) at 90°C. Insoluble material was filtered out, and the filtrate was added EtOH (10 mL) and allowed to stand at room temperature, and the precipitate was collected by filtration to give **1** (78.4 mg, 47%) as a white solid (m.p. > 250°C). ^{1}H-NMR (400 MHz, DMSO) δ ppm: 2.06 (2 H, q, J = 7.5 Hz), 2.86 (2 H, t, J = 7.7 Hz), 3.58 (2 H, t, J = 7.2 Hz), 4.58 (2 H, s), 9.17 (1 H, s), 9.61 (1 H, s), 11.34 (1 H, s), 11.55 (1 H, s). IR (KBr) cm^{-1}: 2970, 1670, 1655, 1420. ESI(+)-MS (*m/z*) calcd. for $C_9H_{11}IN_4O_2$ 335.0000[M+H]$^+$, found 334.9970[M+H]$^+$. Anal. calcd for $C_9H_{12}ClIN_4O_2$ C: 29.17, H: 3.26, N: 15.12, found C: 29.18, H: 3.41, N: 14.95.

*5-Bromo-6-[(2-iminopyrrolidinyl)methyl]uracil hydrochloride (**2**)*

5-Bromo-6-(chloromethyl)uracil (**4**) was prepared in a previously described manner.[22,29] To a solution of **4** (1.01 g, 4.22 mmol) and DBU (1.61 g, 10.6 mol) in 10 mL of MeOH was added 2-iminopyrrolidine hydrochloride (498 mg, 4.12 mmol) and heated under reflux for 2.2 h. After cooling in an ice bath, the precipitate was collected by filtration and dissolved in 3 M aqueous HCl (10 mL) at 90°C. An insoluble material was filtered out and the filtrate was added with EtOH (60 mL) and allowed to stand at room temperature, and the precipitate was collected by filtration to give **2** (337 mg, 25%) as a white solid (m.p. > 250°C). ^{1}H-NMR (400 MHz, DMSO) δ ppm: 2.05 (2 H, q, J = 7.7 Hz), 2.87 (2 H, t, J = 7.7 Hz), 3.59 (2 H, t, J = 7.1 Hz), 4.65 (2 H, s), 9.32 (1 H, s), 9.70 (1 H, s), 11.44 (1 H, s), 11.67 (1 H, s). IR (KBr) cm^{-1}: 3030, 2970, 1699, 1659, 1423. ESI(+)-MS (*m/z*) calcd. for $C_9H_{11}N_4O_2Br$ 287.0138[M+H]$^+$, found 287.0114[M+H]$^+$. Anal. Calcd. for $C_9H_{12}BrClN_4O_2$ C: 33.41, H: 3.74, N: 17.32, found C: 33.64, H: 3.83, N: 17.31.

*6-[(2-Iminopyrrolidinyl)methyl]-5-[^{125}I]iodouracil ([^{125}I]**1**)*

To a solution of **2** (50 μg) in 10 μL of 0.1% aqueous HCl was added carrier-free Na[^{125}I]I (1.1 MBq, 629 GBq/mg in 0.1 M NaOH; American Radiolabeled Chemicals Inc.) and concentrated

Table 1. *In vitro* stability of [^{125}I]**1** in phosphate-buffered saline (pH 7.4) or acetate-buffered saline (pH 4.8) at 37°C

Incubation media	Radiochemical purity (%)		
	6 h	12 h	24 h
Phosphate-buffered saline (pH 7.4)	93.4 ± 2.6	79.2 ± 1.2	58.2 ± 5.3
Acetate-buffered saline (pH 4.8)	99.9 ± 0.0	97.4 ± 1.0	94.8 ± 1.6

Each value represents the mean ± s.d. of three samples.

Table 2. Biodistribution of radioactivity after intravenous injection of [^{125}I]**1** into mice

Tissue	Uptake (%ID/g)				
	30 min	1 h	3 h	6 h	24 h
Blood	0.6 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Spleen	0.8 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.3	0.3 ± 0.1
Pancreas	0.8 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.0
Stomach	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.4	0.9 ± 0.1	0.1 ± 0.1
Small intestine	5.1 ± 1.0	2.7 ± 0.3	2.0 ± 1.4	0.5 ± 0.3	0.0 ± 0.0
Colon	1.2 ± 0.1	0.9 ± 0.0	0.8 ± 0.4	0.6 ± 0.4	0.1 ± 0.0
Kidney	3.2 ± 1.8	2.3 ± 1.1	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Liver	23.6 ± 4.5	14.1 ± 3.0	5.4 ± 5.2	1.2 ± 0.5	0.0 ± 0.0
Heart	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
Lung	0.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Muscle	0.6 ± 0.1	0.8 ± 0.7	0.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
Thyroid[a]	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	0.7 ± 0.1	0.6 ± 0.3
Urine[a]					80.0 ± 15.5
Feces[a]					9.7 ± 7.2

Each value represents the mean ± s.d. of three to five animals.
[a]Expressed as % injected dose.

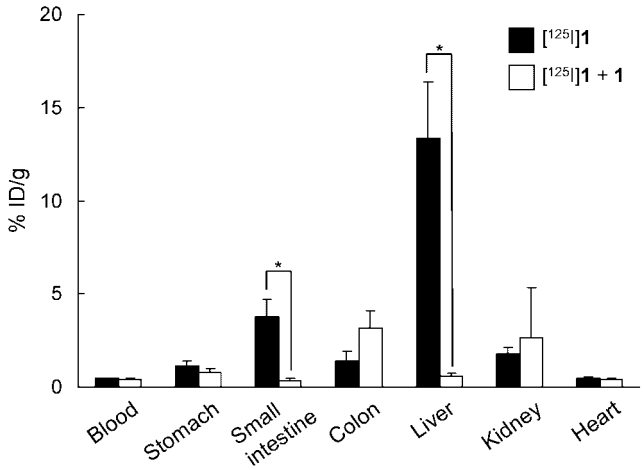


Figure 3. Effects of coinjection of the cold compound **1** on the tissue distribution of radioactivity at 1-h postinjection of [^{125}I]**1** into mice. Each value represents the mean ± s.d. of four to five animals. Significant differences from control (without cold compound) were identified with the unpaired *t*-test (asterisk, $P < 0.001$).

in vacuo. The residue was heated at 145°C for 25 min. After cooling to room temperature, [^{125}I]**1** was purified by HPLC performed with a Cosmosil 5C_{18}-PAQ column (4.6 × 250 mm) eluted with 0.1% aqueous TFA at a flow rate of 0.8 mL/min. The radiochemical purity and specific activity were determined by HPLC using the same conditions.

In vitro stability

[^{125}I]**1** was dissolved with 20 mM phosphate-buffered saline (pH 7.4) or 20 mM acetate-buffered saline (pH 4.8), and the solutions were incubated at 37°C. After 6, 12 and 24 h of incubation, samples were drawn and the radiochemical purity was determined by HPLC.

Tissue distribution in mice

Animal experiments were conducted in accordance with our institutional guidelines and were approved by the Animal Care and Use Committee, Kyushu University. One hundred microliters of [^{125}I]**1** solution (7 kBq) in 20 mM acetate-buffered saline (pH 4.8) was injected intravenously into 7-week-old male ddY mice (26–36 g). The mice were sacrificed by exsanguination under ether anesthesia at 30 min and 1-, 3-, 6- and 24-h postinjection ($n = 3$–5). Blood was collected by heart puncture and the tissues were harvested. The radioactivity in the samples was counted with an auto-gamma-counter (ARC-605II, Aloka) and the tissues were weighed. The tissue uptake of the radioactivity was expressed as a percentage of injected dose per gram of tissue (%ID/g). In the other group of mice, a blocking experiment to determine the TP-specific uptake of the radiotracer was carried out. [^{125}I]**1** (7 kBq) was coinjected with unlabeled **1** (3 mg/kg). One hour later the mice were sacrificed ($n = 4$–5) and the tissue uptake of the radioactivity was expressed as %ID/g.

Statistical analysis

Statistical analysis was performed by applying the unpaired *t*-test. $P < 0.05$ was considered to be statistically significant.

Conclusion

For *in vivo* imaging of TP activity, we developed a radioiodinated TP inhibitor, 6-[(2-iminopyrrolidinyl)methyl]-5-[^{125}I]iodouracil ([^{125}I]**1**). As a radiolabeling precursor, 5-bromo-6-[(2-iminopyrrolidinyl)methyl]uracil (**2**) was prepared by alkylation of 2-iminopyrrolidine hydrochloride with 5-bromo-6-(chloromethyl)uracil (**4**) in the presence of DBU in MeOH. Radiochemical synthesis of [^{125}I]**1**, at high radiochemical purity and at high specific activity, was accomplished by halogen exchange of compound **2**, followed by purification by reverse-phase HPLC. When administered to normal mice, [^{125}I]**1** showed a significant uptake in TP-rich regions (small intestine and liver), which was blocked by coinjection of cold compound **1**. Very low stomach and thyroid uptakes indicated that [^{125}I]**1** was not deiodinated *in vivo*. These results suggest that the newly synthesized [^{125}I]**1** could become a potential agent for *in vivo* imaging of TP activity

in tumors. The results using TP-expressing tumor cells will be reported in the future.

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