Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 25 May 2008, Revised 16 August 2008 Accepted 19 August 2008 Published online 24 September 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/jlcr.1544

# Synthesis of a radioiodinated thymidine phosphorylase inhibitor and its preliminary evaluation as a potential SPECT tracer for angiogenic enzyme expression

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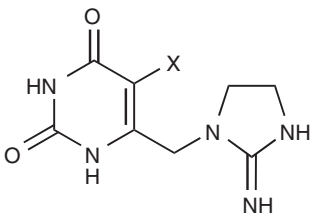
**The expression of thymidine phosphorylase (TP) is strongly associated with angiogenesis in tumors and activation of antitumor agents. We designed a novel 5-$^{125}$I-labeled 6-(2-iminoimidazolidinyl)methyluracil hydrochloride ([$^{125}$I]5I6IMU-HCl) to develop an effective radiotracer for *in vivo* assessment of TP expression in tumors and prognosis of cancer chemotherapy. Radiotracer synthesis was achieved by radioiodination of the precursor, 6-(2-iminoimidazolidinyl)methyluracil at the C-5 position with NCS/radioiodide. After purification by HPLC, [$^{125}$I]5I6IMU-HCl was obtained in high radiochemical yield with satisfactory specific activity. The radiotracer showed high inhibitory potency for the target enzyme and good stability *in vivo*.**

**Keywords:** thymidine phosphorylase (TP); inhibitor; angiogenesis; SPECT; radioiodination

## Introduction

Thymidine phosphorylase (TP) reversibly converts thymidine to thymine and 2-deoxyribose 1-phosphate. It is identical to the angiogenic platelet-derived endothelial cell growth factor.[1,2] High expression of TP is associated with angiogenesis in tumors, malignancy, and overall poor survival.[3] TP is involved in the phospholysis of thymidine nucleoside to cleave the glycosidic bond in the presence of inorganic phosphate. *In vivo*, TP activates fluoropyrimidine-based prodrugs, such as tegafur,[4,5] 5′-deoxy-5-fluorouridine,[6] and capecitabine,[7] to produce 5-fluorouracil. Expression of TP is therefore strongly associated with angiogenesis in tumors and activation of antitumor agents. Noninvasive imaging of TP activity can contribute to the diagnosis of tumor invasiveness, malignancy, and treatment resistance, as well as prognosis of TP-associated cancer chemotherapy.

Various TP inhibitors have been designed and synthesized based on computational estimation of binding modes and energetics of the ligands at the active residues of TP.[8] These include the 5- and 6-substituted uracil analogs (e.g. 6-amino-5-chlorouracil, 6A5CU)[9] and uracils linking to a pyrrolidine group or an imidazolidine group through a methylene carbon at C-6, which best match the active site of TP.[10] Among these inhibitors, 5-X-[6-(2-iminoimidazolidinyl)methyl]uracil (5X6IMU) [X = Cl: (5C6IMU) and X = Br (5B6IMU)] (Figure 1) have the strongest TP inhibitory activity,[10] and therefore we have chosen 5X6IMU as a base and lead compound for a potential tumor imaging tracer. In this study, we have synthesized a radioiodinated 6-[(2-iminoimidazolidinyl)methyl]uracil(5I6IMU), and examined its potential TP inhibitory activity and the biodistribution in mice.




5C6IMU: X= Cl
5B6IMU: X= Br
5I6IMU: X= I
6IMU: X= H

**Figure 1.** Chemical structure of 5-X-[6-(2-iminoimidazolidinyl)methyl]uracil.

## Results and discussion

*N*-halosuccinimides have been successfully utilized for halogenation of pyrimidine nucleosides at C5.[11] An efficient method for

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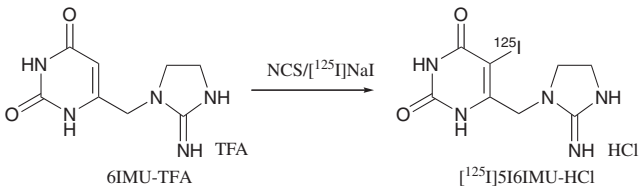
direct electrophilic radioiodination of arenes has been developed using *N*-chlorosuccinimide (NCS)/radioiodide.[12] We conducted 5-iodination of a uracil derivative by NIS or the NCS/radioiodide method. The precursor, 6-(2-iminoimidazolidinyl)-methyluracil(6IMU), was given as a HBr salt (6IMU-HBr) by the reaction of the corresponding diamine and BrCN.[13] After conversion of 6IMU-HBr into the TFA salt (6IMU-TFA), the TFA salt was treated with NIS in $CH_3CN$-$H_2O$ at 50°C for 1 h to give 5I6IMU-TFA, which was readily converted to the HCl salt (5I6IMU-HCl) through an HPLC column using a solvent system of 0.01 M HCl–MeOH (95:5) in 93% total yield. Radioiodination of 6IMU-TFA was accomplished by an electrophilic substitution with NCS/[$^{125}$I]NaI under the same conditions, and the desired product, [$^{125}$I]5I6IMU-HCl, was effectively isolated through reverse-phase HPLC (Scheme 1, Figure 2).

*N*-radioiodosuccinimide (N*IS) may be a source of electrophilic iodide, because N*IS can be generated *in situ* by reacting radioiodide with NCS. Total radiochemical yield after purification




**Scheme 1.** Radiosynthesis of [$^{125}$I]5I6IMU-HCl.

was 83%, and the specific activity was 52.9 GBq/μmol. Chemical and radiochemical purity of the product was >99%. HPLC analysis of radiolabeled 5I6IMU stored in ethanolic solution at 4°C revealed excellent radiochemical stability over 12 days, indicating that refrigerated ready-to-inject solutions of 5I6IMU-HCl can be stored for <12 days within <1.5% change or decomposition. The present method provides sufficient amounts of radiolabeled product for *in vitro* as well as *in vivo* evaluations.

The TP inhibitory effect of 5I6IMU-HCl was assessed by the TP-catalyzed conversion of thymidine into thymine according to the previous procedure.[13] The standard TP inhibitor 6-amino-5-chlorouracil (6A5CU) and highly effective inhibitor 5-bromo-6-[(2-iminoimidazolidinyl)methyl]uracil hydrobromide (5B6IMU-HBr) were also examined as the reference compounds. The $IC_{50}$ values estimated for 5I6IMU-HCl, 5B6IMU-HBr, and 6A5CU were $4.3 \times 10^{-3}$ μM, $4.2 \times 10^{-3}$ μM, and 1.80 μM, respectively. Thus, the inhibitory potency of 5I6IMU-HCl was nearly identical to that of 5B6IMU-HBr, the most efficient inhibitor. These results showed that the substitution of bromide with iodide at the 5-position of the uracil ring did not affect the TP inhibitory potency. A guanidine group on 6-methylene bridged uracil ring may enhance inhibitory potency by tight binding into the active site of TP.

Biodistribution of [$^{125}$I]5I6IMU-HCl in normal mice is shown in Table 1. [$^{125}$I]5I6IMU-HCl showed fast blood clearance. Accumulation in the liver was observed. This can be interpreted as indicative of the physiological TP expression in this organ.[14] Radioactivity in stomach and thyroid were low, indicating good *in vivo* stability of the compound. [$^{125}$I]5I6IMU-HCl also showed rapid excretion. Urinary excretion until each sampling time was as follows: 29.6±7.51 (3 h), 40.0±4.01 (8 h), and

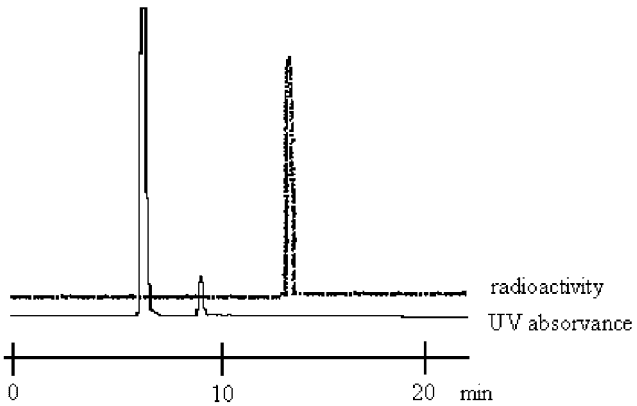



**Figure 2.** Chromatogram from the HPLC separation of [$^{125}$I]5I6IIMU-HCl. Upper trace shows radioactivity response; [$^{125}$I]5I6IMU-HCl retention time 13.7 min. Lower trace shows absorbance at 254 nm response; 6IMU-HCl retention time 6.6 min and 5CI6IMU-HCl retention time 9.2 min.

**Table 1.** Biodistribution of [$^{125}$I]5I6IMU-HCl in normal mice (%dose/g tissue)

| Organ | 0.5 h | 1 h | 3 h | 24 h |
|---|---|---|---|---|
| Blood | 0.13±0.04 | 0.06±0.01 | 0.03±0.01 | 0.02±0.01 |
| Plasma | 0.12±0.02 | 0.05±0.02 | 0.04±0.01 | 0.02±0.01 |
| Muscle | 0.08±0.02 | 0.03±0.01 | 0.01±0.00 | 0.00±0.00 |
| Stomach | 0.29±0.06 | 0.15±0.04 | 0.23±0.08 | 0.48±0.27 |
| Heart | 0.07±0.03 | 0.02±0.00 | 0.02±0.01 | 0.01±0.00 |
| Spleen | 0.11±0.03 | 0.04±0.01 | 0.03±0.01 | 0.02±0.01 |
| Liver | 33.78±3.83 | 31.41±4.14 | 27.33±7.31 | 5.00±1.77 |
| Kidneys | 0.68±0.40 | 0.52±0.48 | 0.25±0.13 | 0.05±0.02 |
| Lung | 0.17±0.04 | 0.06±0.01 | 0.04±0.01 | 0.01±0.01 |
| Small intestine | 7.11±0.85 | 6.25±0.63 | 6.41±1.15 | 4.97±0.53 |
| Large intestine | 1.06±0.29 | 0.75±0.13 | 1.16±0.22 | 1.70±0.27 |
| Brain | 0.01±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| Fat | 0.10±0.03 | 0.02±0.01 | 0.02±0.01 | 0.02±0.01 |
| Bladder | <0.01 | <0.01 | <0.01 | 0.00±0.00 |
| Thyroid | 0.01±0.00 | 0.01±0.00 | 0.04±0.01 | 0.31±0.12 |

Each value represents the percentage injected dose/gram tissue except in the thyroid and bladder (% injected dose/organ) and is the mean±S.D. for five mice.

57.9 ± 4.74 (24 h) % injected dose. A small amount of injected dose (8.10 ± 4.16%) was excreted in the feces until 24 h post-injection.

These results suggest that $^{125}$I-labeled 5I6IMU-HCl may serve as a novel SPECT tracer for the evaluation of angiogenic enzyme activity in tumors.

## Experimental

### Materials and methods

*Materials*

Chemicals and solvents were purchased as follows: 6-chloromethyluracil from Tokyo Kasei Kogyo (Tokyo, Japan); TP from *Escherichia coli* (*E. coli*) from Sigma; [$^{125}$I]NaI (642.8 GBq/mg) from Perkin Elmer Life and Analytical Sciences (Boston, MA).

The proton nuclear magnetic resonance ($^1$H NMR) spectra were recorded on a JEOL ECP-400 spectrometer (JEOL, Tokyo, Japan). Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard. Mass spectra were recorded on a JMS-110 (JEOL).

*Syntheses*

*Synthesis of 6IMU-TFA*: To a solution of 395 mg (3.73 mmol) of cyanogen bromide in 2.7 mL water was added 341 mg (1.85 mmol) of 6-[(2-aminoethyl)amino]methyluracil, and this was stirred for 1 h at 50°C. The precipitate was collected by filtration and washed with water and MeOH to give 535 mg of 6IMU as a crude HBr salt quantitatively. The product was converted to 6IMU-TFA and purified simultaneously through a reverse-phase HPLC column using a solvent system consisting of 0.1% TFA/MeOH = 95:5. (250 × 20.0 mm i.d. Inertsil ODS-3 (GL Sciences), flow rate, 3.0 mL/min). mp 213–214°C. $^1$H NMR ($CD_3OD$) δ: 3.59–3.78 (4H, m), 4.30 (2H, s), 5.51 (1H, s). FAB-MS *m/z*: 210 $(M+H)^+$. Anal. Calcd for $C_{10}H_{12}N_5O_4F_3$: C, 37.16; H, 3.74; N, 21.67. Found: C, 37.24; H, 3.78; N, 21.45.

*Synthesis of 5I6IMU-HCl*: To a solution of 30.2 mg (0.09 mmol) of 6IMU-TFA in 1.5 mL aqueous acetonitrile ($CH_3CN/H_2O$ = 2:1) was added 25.3 mg (0.11 mmol) of NIS, and this was stirred for 2 h at room temperature. After removal of the solvent, the residue was subjected on a reverse-phase HPLC column (250 × 20.0 mm i.d. Inertsil ODS-3 (GL Sciences)) using a mobile phase consisting of 0.01 M HCl/MeOH = 95:5 at 3.0 mL/min. The desired product, 5I6IMU as an HCl salt, was obtained in 93% yield (32.2 mg, 0.08 mmol). mp 185°C (dec). $^1$H-NMR ($CD_3OD$) δ: 3.67 (4H, s), 4.49 (2H, s). FAB-MS *m/z*: 336 $(M+H)^+$. Anal. Calcd for $C_8H_{11}N_5O_2ClI + 1/3H_2O$: C, 25.45; H, 3.11; N, 18.55; Cl, 9.39; I, 33.61. Found: C, 25.37; H, 2.92; N, 18.20; Cl, 9.39; I, 34.06.

*Radiolabeling*

Dry acetone (50 μL) containing NCS (15 μg) was added to sodium [$^{125}$I]NaI (18.5 MBq) in a reaction vial, and the mixture was allowed to stand for 10 min at room temperature. Acetone was then removed completely under a stream of $N_2$. A solution of 6IMU-TFA (100 μg) in 40 μL of aqueous acetonitrile ($CH_3CN/H_2O$ = 2:1) was added to the residue, and the capped vial was heated for 1 h at 50°C. After removal of the solvent, the crude product was converted to radiolabeled 5I6IMU-HCl and purified simultaneously by reversed-phase HPLC using a solvent system containing hydrochloric acid. Preparative HPLC was performed on a 250 × 10.0 mm i.d. Phenomenex Luna C18 column eluting with a mobile phase consisting of 0.01 M HCl/MeOH = 95:5 at a flow rate of 2.0 mL/min. The radioactive fraction corresponding in retention time to authentic 5I6IMU-HCl (13.7 min) was effectively separated from the HCl salt of the starting material (6IMU) and by-product, 5-chlorinated uracil derivative 5C6IMU-HCl. The purified product [$^{125}$I]5I6IMU-HCl (15.4 MBq) was dried under vacuum and dissolved in sterile saline.

The radiochemical purity of the labeled compound was determined by analytical HPLC, which was performed on a 250 × 4.6 mm i.d. Inertsil ODS-3 column (GL Sciences) eluted with 0.01 M HCl/MeOH = 95:5 at 0.7 mL/min. For calculations of specific activity, standard solutions of unlabeled compounds were used to compare the area under UV absorption.

*TP inhibitory potency*

The phosphorolysis of thymidine by *E. coli* TP was measured by HPLC analysis. The reaction mixture consisted of a total volume of 950 μL containing 10 mM Tris-HCl buffer (pH 7.3) (850 μL), 0.1 M potassium phosphate (pH 7.1) (100 μL), TP (0.11 U), and test compound (final concentrations: $10^{-4}$–$10^{-10}$ mol/L). After 2 min of incubation at 37°C, the mixture was added 50 μL of 0.1 M thymidine and incubated at 37°C for 5 min, then quenched with 0.5 M NaOH solution (300 μL). The thymidine was separated from thymine and quantified by HPLC analysis.

*Biodistribution studies*

All procedures involving animals were performed in accordance with the Institutional Guidelines of Hokkaido University and the current laws in Japan. Biodistribution studies were performed by intravenous administration of [$^{125}$I]5I6IMU-HCl (radioactive dose 37 kBq/saline 0.1 mL/mouse) to 9-week-old female ddY mice (28.6–34.9 g). At 0.5, 1, 3, and 24 h after the administration, mice were sacrificed ($n$ = 5 for each time point). Urine and feces were collected from the mice assigned to the 24 h group. Samples of blood and the organs of interest were excised and weighed, and radioactivity was measured with an auto well gamma counter.

## Conclusion

This work represents successful synthesis of a TP inhibitor-based radiotracer with great potential for noninvasive tumor imaging. Further studies to establish the usefulness of 5I6IMU as a TP-targeting SPECT tracer for assessment of tumor invasiveness are currently in progress.

## Acknowledgement

This work was supported in part by Grants-in-Aid for Scientific Research and High Technology Research Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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