Title

Relationship between biodistribution of a novel thymidine phosphorylase (TP) imaging probe and TP expression levels in normal mice

Authors' names

Songji Zhao[1,2], Hua Li[1], Ken-ichi Nishijima[3,4],Yan Zhao[5], Hiromichi Akizawa[6], Yoichi Shimizu[3,4], Kazue Ohkura[7], Nagara Tamaki[5], Yuji Kuge[3,4]

Affiliations and addresses of all authors

[1]Department of Tracer Kinetics & Bioanalysis, Graduate School of Medicine, Hokkaido University, Kita 15 Nishi 7, Kita-ku, Sapporo 060-8638, Japan

[2]Department of Molecular Imaging, Graduate School of Medicine, Hokkaido University, Kita 15 Nishi 7, Kita-ku, Sapporo 060-8638, Japan

[3]Department of Integrated Molecular Imaging, Graduate School of Medicine, Hokkaido University, Kita 15 Nishi 7, Kita-ku, Sapporo 060-8638, Japan

[4]Central Institute of Isotope Science, Hokkaido University, Kita 15 Nishi 7, Kita-ku, Sapporo 060-0815, Japan

[5]Department of Nuclear Medicine, Graduate School of Medicine, Hokkaido University,

Kita 15 Nishi 7, Kita-ku, Sapporo 060-8638, Japan

[6]Laboratory of Physical Chemistry, Showa Pharmaceutical University, 3-3165 Higashi-Tamagawagakuen, Machida, Tokyo 194-8543, Japan

[7]Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido 061-0293, Japan

Address correspondence to

Professor Songji Zhao, MD, PhD

Department of Nuclear Medicine, Tracer Kinetics & Bioanalysis, Graduate Schoool of Medicine, Hokkaido University, Kita 15 Nishi 7, Kita-ku, Sapporo 060-8638, Japan

E-mail: zsi@med.hokudai.ac.jp, Telephone: 81-11-706-5085, Fax: 81-11-706-7155

Type of article

Original Article

Abstract

Objective Thymidine phosphorylase (TP) is a key enzyme in the pyrimidine nucleoside salvage pathway and its expression is upregulated in a wide variety of solid tumors. In mice, we previously observed high and specific accumulation levels of our TP imaging probe, radioiodinated 5-iodo-6-[(2-iminoimidazolidinyl)methyl]uracil (IIMU) not only in high-TP-expressing tumors, but also in the liver and small intestine. To clarify the reason for the high accumulation levels of radioiodinated IIMU in the liver and small intestine, we investigated the expression levels of TP in mice in comparison with the biodistribution of radioiodinated IIMU (^{123}I-IIMU).

Methods BALB/cCrSlc mice were injected with ^{123}I-IIMU, and the radioactivity levels [%ID/g (normalized to a mouse of 25 g body weight)] in the tissues of interest were determined 0.5, 1, 3 and 24 h after the injection (n=5, each time point). To determine the expression levels of TP, BALB/cCrSlc and ddy mice (n=3/each strain) were euthanized, and the heart, liver, lung, spleen, kidney, stomach, small intestine, large intestine and brain were collected. The mRNA and protein expression levels of TP in these organs were examined by quantitative reverse transcription-polymerase chain reaction and western blot analyses, respectively.

Results In BALB/cCrSlc mice administered ^{123}I-IIMU, markedly high radioactivity levels were observed in the liver [1.568±0.237 (%ID/g)] and small intestine [0.506±0.082 (%ID/g)], whereas those in the other tissues were fairly low [< 0.010±0.003 (%ID/g)] 30 minutes after the injection. The highest expression levels of TP mRNA were also observed in the liver and small intestine among the tissues tested. Immunoblotting showed intense immunoreactive bands of the TP protein for the liver and small intestine, whereas no notable bands were detected for other tissues. Similar expression profiles of TP mRNA and protein were observed in ddy mice.

Conclusion We confirmed TP expression in various tissues of mice at the mRNA and protein levels: high TP expression levels were observed in the liver and small intestine. These high TP expression levels are consistent with the high accumulation levels of ^{123}I-IIMU in these tissues. Our results may provide important information about the physiological accumulation of ^{123}I-IIMU, which may be useful for the clinical diagnostic imaging of TP.

Keywords: thymidine phosphorylase expression, 5-iodo-6-[(2-iminoimidazolidinyl)methyl]uracil (IIMU), liver, small intestine, normal mouse tissues

Introduction

Thymidine phosphorylase (TP), also known as the platelet-derived endothelial cell growth factor (PD-ECGF), is a key enzyme in the pyrimidine nucleoside salvage pathway that catalyzes the reversible phosphorylation of thymidine to thymine and 2-deoxy-D-ribose, and its expression is upregulated in a wide variety of solid tumors [1]. Several experimental data indicate that the enzymatic activity of TP is indispensable for its angiogenic effect [2,3]. High TP expression levels are associated with an increased microvessel density in various human tumors [4,5]. It is also reported that TP expression correlates well with tumor malignancy, infiltration, and metastasis and overall poor survival [6,7]. Moreover, TP is essential for the bioactivation of 5-fluorouracil and its prodrugs, including doxifluridine and capecitabine [8,9]. Previous studies using the transfectants of TP cDNA and immunohistochemistry have indicated that a high expression level of TP is significantly associated with the response to the treatment with 5-fluorouracil or its prodrugs [8,9]. Accordingly, in vivo imaging of TP activity will contribute not only to the diagnosis of tumor angiogenesis and invasiveness but also to the prognosis and clinical evaluation of cancer chemotherapy using fluoropyrimidine-based anticancer drugs.

To develop a radioprobe for in vivo TP imaging, a radiolabeled uracil-based TP inhibitor, 5-iodo-6-[(2-iminoimidazolidinyl)methyl]uracil (IIMU), was designed and

synthesized by our group [10]. In our previous studies, IIMU showed a high TP inhibitory potency and ^{125}I-labeled IIMU (^{125}I-IIMU) exhibited rapid blood clearance and urinary excretion properties in tumor-bearing mice [11]. We demonstrated specific accumulation of radioiodinated IIMU at high levels in a high TP-expressing tumor (A431, a human epidermoid carcinoma) in in vitro and in vivo experiments. These findings indicate that the accumulation of radioiodinated IIMU in tumor cells is TP-specific and directly corresponds to TP expression levels [11,12]. On the other hand, in tumor-bearing mice (BALB/c athymic nude mice) we found high accumulation levels of radioiodinated IIMU in the liver and small intestine, although those in other normal tissues were very low [11]. In this study, to clarify the reason for the high accumulation levels of radioiodinated IIMU in the liver and small intestine, we investigated the expression levels of TP in various tissues of mice in comparison with the biodistribution of radioiodinated IIMU (^{123}I-IIMU).

Materials and Methods

Animals

The entire experimental protocol was approved by the Laboratory Animal Care and Use Committee of Hokkaido University (approval number 13-0057) and performed in accordance with the Guidelines for Animal Experiments at the Graduate School of Medicine, Hokkaido University. Eight-week-old healthy male BALB/cCrSlc and ddy mice (supplied by Japan SLC, Inc., Hamamatsu, Japan) were used in the following experiments after a one-week acclimatization period. The institutional laboratory housing provided a 12-hour light/dark cycle and met all the criteria of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Biodistribution of ^{123}I-IIMU

^{123}I-IIMU was synthesized by a method previously reported [10]. The radiochemical purity of ^{123}I-IIMU was more than 99%. BALB/cCrSlc mice were injected with a saline solution of ^{123}I-IIMU (2.5 MBq/0.1ml/mouse). These mice were sacrificed 0.5, 1, 3 and 24 h after the injection (n=5, each time point), and the tissues of interest, such as the muscle, heart, liver, lung, spleen, kidney, stomach*, small intestine*,

large intestine* (* including contents) and brain, and blood were collected. Tissues and blood samples were weighed, and their radioactivity was measured using a gamma counter (1480 WIZARD 3"; Wallac Co., Ltd.). Radioactivity in the tissues was expressed as the percentage of injected dose per gram of tissue (%ID/g), which was normalized to a mouse of 25 g body weight.

Expression levels of TP

BALB/cCrSlc (n=3) and ddy (n=3) mice were euthanized and perfused transcardially with cold PBS. The heart, liver, lung, spleen, kidney, stomach, small intestine, large intestine and brain were collected and frozen in liquid nitrogen. The expression levels of TP mRNA and protein in these tissues were examined by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses, respectively.

Total RNA was extracted using an RNA isolation kit (RNAqueous®-4PCR, Applied Biosystems) and protocols specified by the manufacturer. Total RNA was reversely transcribed using a cDNA reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Quantitative real-time PCR was performed to measure TP and GAPDH mRNA expression levels using the 7500

Real-Time PCR System (Applied Biosystems) with TaqMan Universal Master Mix II (Applied Biosystems) and primer-probe sets of TaqMan Gene Expression Assays (TP, Mm00460357_ml; GAPDH, Hs99999905_ml; Applied Biosystems). Four twofold dilutions of heart cDNA samples were used to determine the relative quantities of TP and GAPDH mRNA in each tissue sample, and the ratio of the mRNA equivalents of TP and GAPDH was then calculated as the level of TP gene expression after normalization with that of the heart. Triplicate samples were used for quantitative real-time PCR analysis for each tissue.

In western blot analysis, protein extracts were separated on 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. After blocking with TBST buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5 ml/L Tween 20) plus 5% skim milk, the membranes were incubated with a rabbit polyclonal anti-mouse TP primary antibody or a rabbit monoclonal anti-human GAPDH primary antibody (Cell Signaling Technology Inc., Beverly, MA). The polyclonal antibody to TP was raised in rabbits against three different peptides unique to mouse TP (Japan Bio Services): C-EPRQLPELIRLKRD (residues 18-31), C-WPKAWHQQLVDKHST (residues 90-104) and CSGSPTQRRQLLPH (residues 347-360) of TP protein sequence (471 amino acid

residues, National Center for Biotechnology Information database). After repeated washings, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare UK Ltd., Amersham, UK). Immunoreative bands were detected using freshly prepared ECL plus™ western blotting detection reagents (GE Healthcare) and the luminescent image analyzer, LAS-4000 mini (Fujifilm, Tokyo, Japan). GAPDH was used as the protein loading control for immunoblotting.

Results

Biodistribution of ^{123}I-IIMU

Table 1 and Figure 1A show the distribution of ^{123}I-IIMU in BALB/cCrSlc mice. Thirty minutes after the ^{123}I-IIMU injection, markedly high radioactivity levels were observed in the liver [1.568 ± 0.237 (%ID/g)] and small intestine [0.506 ± 0.082 (%ID/g)], and these high levels remained until 3 h after the injection. The radioactivity level was low but significant in the large intestine [0.057 ± 0.016 (%ID/g)] and increased over time. The radioactivity levels in the blood, plasma, muscle, heart, lung, spleen and brain were fairly low [< 0.010 ± 0.003 (%ID/g)].

Expression levels of TP mRNA and protein

The mRNA and protein expression levels of TP in BALB/cCrSlc and ddy mice are shown in Figs. 1B and C, and Fig. 2. In the BALB/cCrSlc mice (Fig. 1B), TP mRNA was found to be highly expressed in the liver and small intestine. The ratios of the TP mRNA expression levels in the liver and small intestine to that in the heart were 2.6 ± 0.7 and 2.5 ± 0.9, respectively. The TP mRNA expression levels were moderately high in the kidney, lung, brain and large intestine and relatively low in the spleen and stomach (Fig. 1B). Immunoblotting showed intense immunoreactive bands of the TP protein in the liver and small intestine of BALB/cCrSlc mice (Fig. 1C). No significant bands were visible in the lanes for the heart, lung, spleen, kidney, stomach, large intestine and brain samples (Fig. 1C).

Similar expression profiles with high TP mRNA and protein expression levels in the liver and small intestine were observed in ddy mice (Fig. 2).

Discussion

In this study, markedly high radioactivity levels were observed in the liver and small intestine, and these high levels remained until 3 h after the ^{123}I-IIMU injection (Table 1 and Fig. 1A). High expression levels of TP mRNA and protein were also

observed in the liver and small intestine (Figs. 1B and C, and Fig. 2). Thus, the distribution of ^{123}I-IIMU is consistent with the expression levels of TP in the liver and small intestine.

In our previous studies of tumor-bearing mice, we found high accumulation levels of radioiodinated IIMU in the liver and small intestine, and the radiotracer accumulation was markedly reduced in blocking experiments [11,13]. These results suggested that the high accumulation levels in the liver and small intestine are also attributable to the binding of radioiodinated IIMU to physiologically expressed TP. However, the TP expression levels in these tissues were not elucidated and it was not clear whether the high accumulation levels of radioiodinated IIMU in these tissues depend on the TP expression levels. Therefore, we performed the present study to clarify the mechanism of radioiodinated IIMU accumulation in the liver and small intestine. On the other hand, the in vivo kinetics of radiopharmaceuticals generally depend on the protein level. In the present study, we clarified the relationship between the accumulation levels of radioiodinated IIMU and TP expression at protein levels in normal tissues of mice. To further confirm the results, we simultaneously investigated the mRNA expression levels of TP in various tissues of normal mice. Consequently, we observed high expression levels of TP mRNA and protein in the liver and small

intestine of our mice that were consistent with the high accumulation levels of radioiodinated IIMU in these tissues. In our previous study of normal ddy mice, we also found high accumulation levels of radioiodinated IIMU in the liver and small intestine [10], which was consistent with findings in normal BALB/cCrSlc mice in the present study. Together with the results of our previous blocking experiments [11, 13], the high accumulation levels in the liver and small intestine can be attributable mainly to the binding of radioiodinated IIMU to physiologically expressed TP. However, to clarify the accumulation levels of radioiodinated IIMU in relation to TP expression, further experimental studies, including blocking studies with nonradioiodinated IIMU in normal mice are necessary. The accumulation levels of radioiodinated IIMU in the liver of BALB/cCrSlc mice was considerably higher than that those of ddy mice [10] 24 h after the injection. A possible explanation for the discrepancy may be the difference in the rearing environment. BALB/cCrSlc mice were reared in conventional cages, whereas ddy mice were individually reared in metabolic cages. The differences in the rearing environment of mice might cause the different accumulation levels of radioiodinated IIMU in the liver (24 h postinjection), although the exact reason for the discrepancy remains unclear. On the other hand, the gradually increased levels of ^{123}I-IIMU in the large intestine may indicate that ^{123}I-IIMU was partially excreted via

the hepatobiliary system. However, regarding the intestine, to clarify the accumulation levels of radioiodinated IIMU in relation to TP expression, further experimental studies of the accumulation levels of radioiodinated IIMU in the intestine without contents are strongly required.

As for the expression or activity of TP in normal tissues including the liver and small intestine, Haraguchi et al. previously reported the tissue distribution of TP activity and indicated that those in the liver and small intestine were higher than those in other tissues of C57BL/Swiss mice [14]. Using rabbits, Friedkin and Roberts also found that the TP activities in the liver and small intestine tissues were higher than those in other tissues [15]. Most recently, the presence and amount of TP in healthy human tissues were assessed by western blotting and ELISA by Boschetti et al. [16]. They observed high TP expression levels in the liver and an intermediate TP expression level in the intestinal mucosa. Our observations are consistent with these previous findings. Our present study is the first to show the distributions of TP expression at the mRNA and protein levels in normal mice, although there are some reports of TP activity in normal mice and rabbit tissues [14,15].

In conclusion, the present study confirmed the TP expression in various tissues of mice at the mRNA and protein levels; high TP expression levels were observed in the

liver and small intestine, which are consistent with the high accumulation levels of radioiodinated IIMU in these tissues. Our results may provide important information about the physiological accumulation of ^{123}I-IIMU, which may be useful for the clinical diagnostic imaging of TP.

References

1. Bronckaers A, Gago F, Balzarini J, Liekens S. The dual role of thymidine phosphorylase in cancer development and chemotherapy. Med Res Rev. 2009;29(6):903–953.
2. Moghaddam A, Zhang HT, Fan TP, Hu DE, VeC Lees, Turley H, et al. Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc Natl Acad Sci USA 1995; 92(4):998–1002.
3. Miyadera K, Sumizawa T, Haraguchi M, Yoshida H, Konstanty W, Yamada Y, et al. Role of thymidine phosphorylase activity in the angiogenic effect of platelet derived endothelial cell growth factor/thymidine phosphorylase. Cancer Res. 1995; 55(8):1687–1690.
4. Harino Y, Imura S, Kanemura H, Morine Y, Fujii M, Ikegami T, et al. Role of tumor angiogenesis in gallbladder carcinoma: with special reference to thymidine phosphorylase. Int J Clin Oncol. 2008; 13(5):452–457.
5. Han HS, Hwang TS. Angiogenesis in gastric cancer: importance of the thymidine phosphorylase expression of cancer cells as an angiogenic factor. Oncol Rep. 2007; 17(1):61–65.
6. Takayama T, Mugiya S, Sugiyama T, Aoki T, Furuse H, Liu H, et al. High levels of

thymidine phosphorylase as an independent prognostic factor in renal cell carcinoma. Jpn J Clin Oncol. 2006; 36(9):564–569.

7. Chen LC, Hsueh C, Tsang NM, Liang Y, Chang KP, Hao SP, et al. Heterogeneous ribonucleoprotein k and thymidine phosphorylase are independent prognostic and therapeutic markers for nasopharyngeal carcinoma. Clin Cancer Res. 2008; 14(12):3807–3813.
8. Schwartz EL, Baptiste N, Wadler S, Makower D. Thymidine phosphorylase mediates the sensitivity of human colon carcinoma cells to 5-fluorouracil. J Biol Chem. 1995; 270(32):19073–19077.
9. Tokunaga Y, Hosogi H, Hoppou T, Nakagami M, Tokuka A, Ohsumi K. Prognostic value of thymidine phosphorylase/platelet-derived endothelial cell growth factor in advanced colorectal cancer after surgery: evaluation with a new monoclonal antibody. Surgery. 2002;131(5):541–547.
10. Takahashi M, Seki K, Nishijima K, Zhao S, Kuge Y, Tamaki N, et al. Synthesis of a radioiodinated thymidine phosphorylase inhibitor and its preliminary evaluation as a potential SPECT tracer for angiogenic enzyme expression. J Label Compd Radiopharm. 2008; 51(11):384–387.
11. Akizawa H, Zhao S, Takahashi M, Nishijima K, Kuge Y, Tamaki N, et al. In vitro

and in vivo evaluations of a radioiodinated thymidine phosphorylase inhibitor as a tumor diagnostic agent for angiogenic enzyme imaging. Nucl Med Biol. 2010; 37(4):427–432.

12. Li H, Zhao S, Jin Y, Nishijima K, Akizawa H, Ohkura K, et al. Radiolabeled uracil derivative as a novel SPECT probe for thymidine phosphorylase: suppressed accumulation into tumor cells by target gene knockdown. Nucl Med Commun. 2011;32(12):1211–1215.
13. Nishijima K, Zhao S, Zhao Y, Feng F, Shimizu Y, Abo N, et al. Preparation and evaluation of [^{123}I]IIMU for SPECT imaging of thymidine phosphorylase expression in tumors. J Label Compd Radiopharm. 2013;56:S349.
14. Haraguchi M, Tsujimoto H, Fukushima M, Higuchi I, Kuribayashi H, Utsumi H, et al. Targeted deletion of both thymidine phosphorylase and uridine phosphorylase and consequent disorders in mice. Mol Cell Biol. 2002;22(14):5212–5221.
15. Friedkin M, Roberts D. The enzymatic synthesis of nucleosides. I. Thymidine phosphorylase in mammalian tissue. J Biol Chem. 1954;207(1):245–256.
16. Boschetti E, D'Alessandro R, Bianco F, Carelli V, Cenacchi G, Pinna AD, et al. Liver as a source for thymidine phosphorylase replacement in mitochondrial neurogastrointestinal encephalomyopathy. PLoS One. 2014;9(5):e96692.

Figure legends

Figure 1. Biodistribution of ^{123}I-IIMU and expression levels of TP mRNA and protein in BALB/cCrSlc mice.

A) Biodistribution of ^{123}I-IIMU, %ID/g; %ID/g (normalized to a mouse of 25 g body weight), B) expression levels of TP mRNA, and C) western blot analysis of TP protein with GAPDH as loading control. Data are expressed as mean ± SD.

Figure 2. Expression levels of TP mRNA and protein in ddy mice.

A) Expression of TP mRNA and B) western blot analysis of TP protein with GAPDH as loading control. Data are expressed as mean ± SD.

Table 1. Biodistribution of ^{123}I-IIMU in BALB/cCrSlc mice

Tissue	^{123}I-IIMU uptake level [%ID/g (normalized to a mouse of 25 g body weight)]			
	0.5 h n=5	1 h n=5	3 h n=5	24 h n=5
Blood	0.010 ± 0.003	0.003 ± 0.000	0.001 ± 0.000	0.002 ± 0.000
Plasma	0.008 ± 0.003	0.003 ± 0.000	0.001 ± 0.000	0.002 ± 0.000
Muscle	0.005 ± 0.001	0.002 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Heart	0.003 ± 0.001	0.001 ± 0.000	0.000 ± 0.000	0.001 ± 0.000
Liver	1.568 ± 0.237	1.476 ± 0.072	1.525 ± 0.173	0.969 ± 0.094
Lung	0.012 ± 0.004	0.004 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Spleen	0.007 ± 0.003	0.002 ± 0.000	0.001 ± 0.000	0.001 ± 0.000
Kidney	0.026 ± 0.012	0.008 ± 0.007	0.002 ± 0.000	0.003 ± 0.000
Stomach	0.018 ± 0.010	0.018 ± 0.009	0.011 ± 0.002	0.019 ± 0.003
Small intestine	0.506 ± 0.082	0.538 ± 0.070	0.465 ± 0.037	0.347 ± 0.028
Large intestine	0.057 ± 0.016	0.058 ± 0.009	0.064 ± 0.006	0.100 ± 0.012
Brain	0.001 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

Acknowledgments

This study was supported (in part) by the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program, Ministry of Education, Culture, Sports, Science and Technology, Japan. We also thank Nihon Medi-Physics Co., Ltd., for providing ^{123}I-NaI.

Competing interests We declare no other potential conflict of interest relevant to this article.

Authors' contributions

SZ and HL contributed to the study concept and design; carried out all aspects of the in vitro and in vivo studies; performed data collection, statistical analysis, data interpretation and literature research; and drafted the manuscript. KN contributed to the synthesis and provision of ^{123}I-IIMU. HA, YS, KO and NT participated in discussion and data interpretation, and provided some important advice. YK contributed to the study concept and design, confirmed all study results, performed statistical analysis and interpretation of data, and critically revised the manuscript. All the authors read and approved the final manuscript.

Figure 1

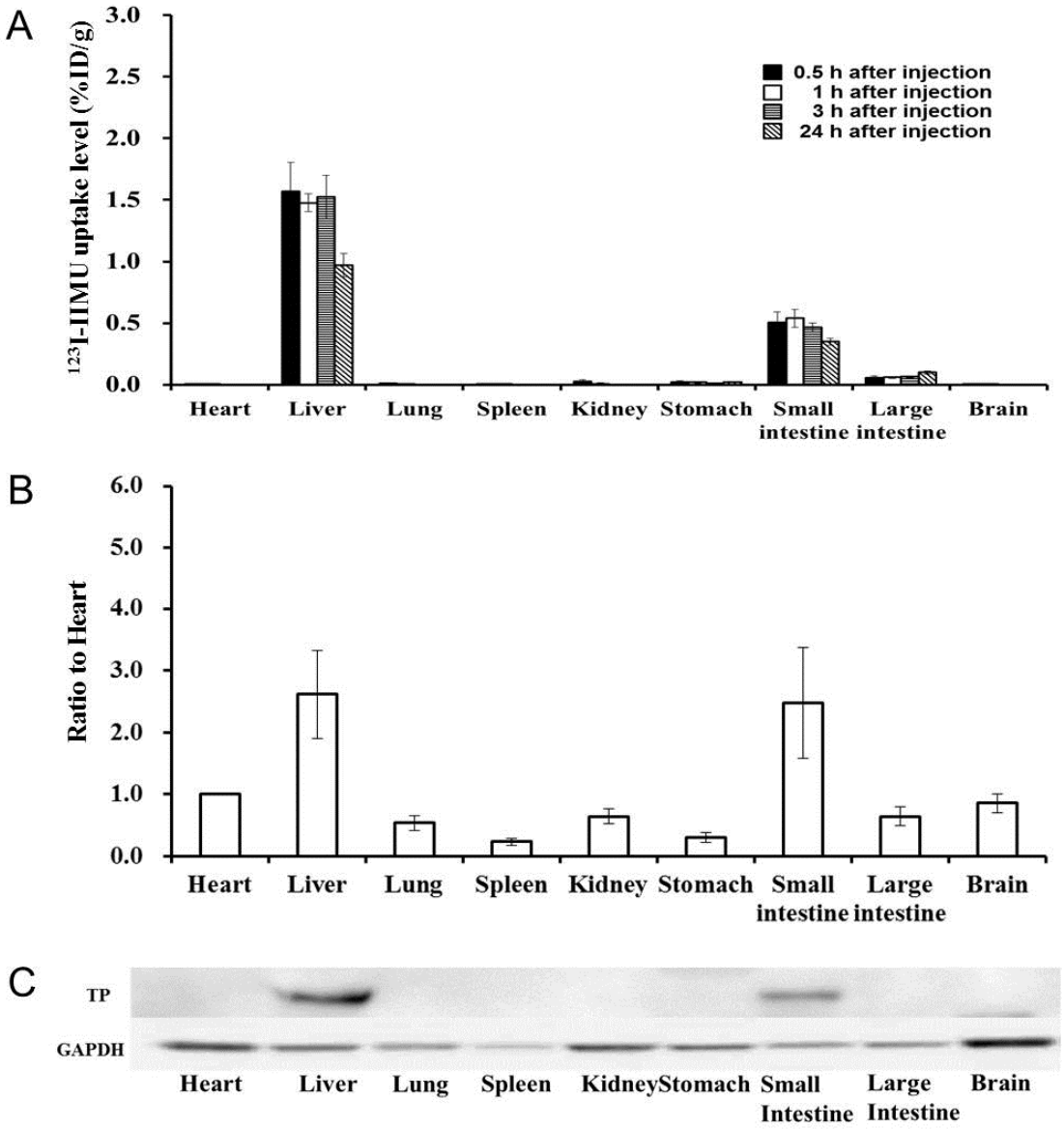
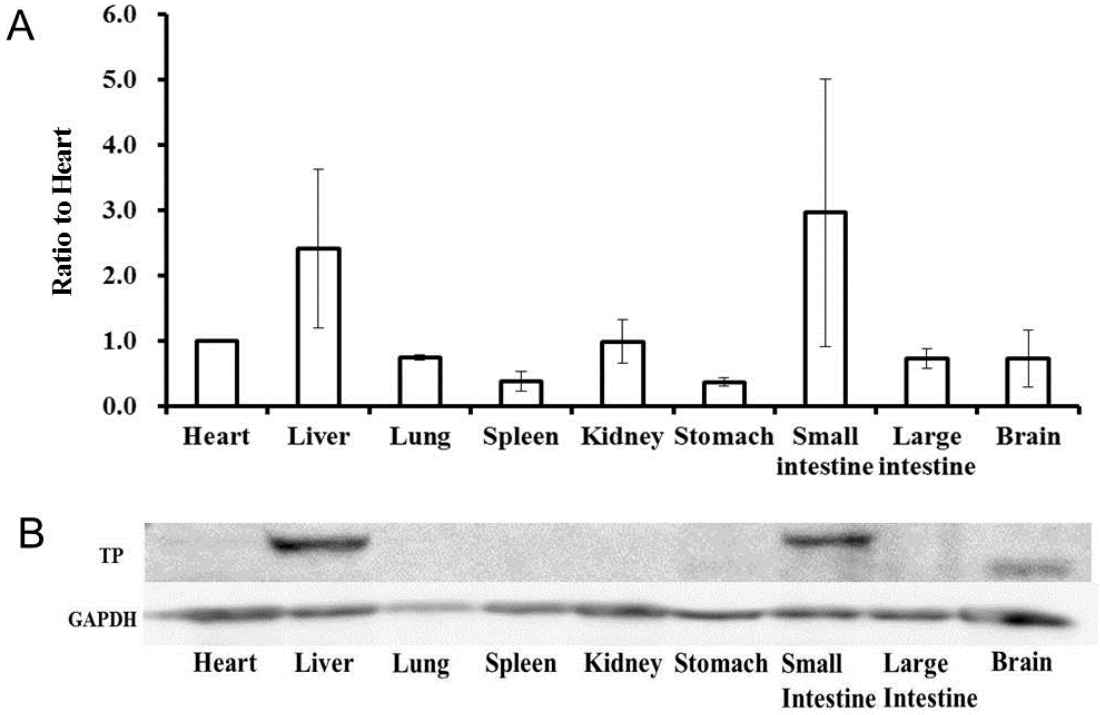

A
^{123}I-IIMU uptake level (%ID/g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5 h after injection
1 h after injection
3 h after injection
24 h after injection
Heart
Liver
Lung
Spleen
Kidney
Stomach
Small intestine
Large intestine
Brain
B
Ratio to Heart
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Heart
Liver
Lung
Spleen
Kidney
Stomach
Small intestine
Large intestine
Brain
C
TP
GAPDH
Heart
Liver
Lung
Spleen
Kidney
Stomach
Small Intestine
Large Intestine
Brain

Figure 2


A
Ratio to Heart
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Heart
Liver
Lung
Spleen
Kidney
Stomach
Small intestine
Large intestine
Brain
B
TP
GAPDH
Heart
Liver
Lung
Spleen
Kidney
Stomach
Small Intestine
Large Intestine
Brain