

Rapid detection of hypoxia-inducible factor-1-active tumours: pretargeted imaging with a protein degrading in a mechanism similar to hypoxia-inducible factor-1 α

Masashi Ueda · Takashi Kudo · Yuji Kuge · Takahiro Mukai · Shotaro Tanaka · Hiroaki Konishi · Azusa Miyano · Masahiro Ono · Shinae Kizaka-Kondoh · Masahiro Hiraoka · Hideo Saji

Received: 8 December 2009 / Accepted: 29 March 2010
© Springer-Verlag 2010

Abstract

Purpose Hypoxia-inducible factor-1 (HIF-1) plays an important role in malignant tumour progression. For the imaging of HIF-1-active tumours, we previously developed a protein, POS, which is effectively delivered to and selectively stabilized in HIF-1-active cells, and a radioiodinated biotin derivative, (3-¹²³I-iodobenzoyl)norbiotinamide (¹²³I-IBB), which can bind to the streptavidin moiety of POS. In this study, we aimed to investigate the feasibility

M. Ueda
Radioisotopes Research Laboratory, Kyoto University Hospital,
Faculty of Medicine, Kyoto University,
54 Shogoin Kawahara-cho, Sakyo-ku,
Kyoto 606-8507, Japan

M. Ueda · T. Kudo · Y. Kuge · H. Konishi · A. Miyano · M. Ono · H. Saji (✉)
Department of Patho-Functional Bioanalysis,
Graduate School of Pharmaceutical Sciences, Kyoto University,
46-29 Yoshida Shimoadachi-cho, Sakyo-ku,
Kyoto 606-8501, Japan
e-mail: hsaji@pharm.kyoto-u.ac.jp

Y. Kuge
Central Institute of Isotope Science, Hokkaido University,
Kita 15, Nishi 7, Kita-ku,
Sapporo 060-0815, Japan

T. Mukai
Department of Biomolecular Recognition Chemistry,
Graduate School of Pharmaceutical Sciences, Kyushu University,
3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan

S. Tanaka · S. Kizaka-Kondoh · M. Hiraoka
Department of Radiation Oncology and Image-applied Therapy,
Graduate School of Medicine, Kyoto University,
54 Shogoin Kawahara-cho, Sakyo-ku,
Kyoto 606-8507, Japan

of the pretargeting method using POS and ¹²³I-IBB for rapid imaging of HIF-1-active tumours.

Methods Tumour-implanted mice were pretargeted with POS. After 24 h, ¹²⁵I-IBB was administered and subsequently, the biodistribution of radioactivity was investigated at several time points. In vivo planar imaging, comparison between ¹²⁵I-IBB accumulation and HIF-1 transcriptional activity, and autoradiography were performed at 6 h after the administration of ¹²⁵I-IBB. The same sections that were used in autoradiographic analysis were subjected to HIF-1 α immunohistochemistry.

Results ¹²⁵I-IBB accumulation was observed in tumours of mice pretargeted with POS (1.6%ID/g at 6 h). This result is comparable to the data derived from ¹²⁵I-IBB-conjugated POS-treated mice (1.4%ID/g at 24 h). In vivo planar imaging provided clear tumour images. The tumoral accumulation of ¹²⁵I-IBB significantly correlated with HIF-1-dependent luciferase bioluminescence ($R=0.84$, $p<0.01$). The intratumoral distribution of ¹²⁵I-IBB was heterogeneous and was significantly correlated with HIF-1 α -positive regions ($R=0.58$, $p<0.0001$).

Conclusion POS pretargeting with ¹²³I-IBB is a useful technique in the rapid imaging and detection of HIF-1-active regions in tumours.

Keywords Tumour hypoxia · Hypoxia-inducible factor-1 (HIF-1) · Oxygen-dependent degradation (ODD) · Molecular imaging · Pretargeting

Introduction

Insufficient blood supply to a rapidly growing tumour leads to the presence of an oxygen tension below physiological

levels, i.e. hypoxia, in solid tumours [1]. Tumour hypoxia is critically important in tumour physiology and cancer treatment, and it appears to be strongly associated with malignant progression and therapy resistance. The transcription factor, hypoxia-inducible factor-1 (HIF-1) is induced in hypoxic regions. It is a master regulator of the genes that encode for angiogenic and metastatic factors, and plays an important role in tumour progression [2–4]. Thus, noninvasive imaging of HIF-1-active regions in tumours would be useful for characterizing tumours and determining a course of therapy.

HIF-1 is a heterodimer that consists of an oxygen-sensitive alpha subunit (HIF-1 α) and a constitutively expressed beta subunit. In normoxia, two proline residues in the oxygen-dependent degradation (ODD) domain of HIF-1 α are hydroxylated, leading to a proteasomal degradation of HIF-1 α . Under hypoxic conditions, oxygen is the rate-limiting factor for prolyl hydroxylation, resulting in a decreased degradation of HIF-1 α [5, 6]. That is to say, the ODD domain of HIF-1 α is responsible for the regulation of HIF-1 activity. Thus, it is likely that a probe containing the ODD domain and degrading in a manner similar to HIF-1 α could be used to evaluate HIF-1 activity *in vivo*.

We have recently developed proteins in which the protein transduction domain (PTD) is fused to the ODD domain and demonstrated the specificity of these proteins to HIF-1-active cells [7–11]. In a previous study, we also fused the PTD-ODD with a monomeric streptavidin (SAV) to produce a chimeric protein, PTD-ODD-SAV (POS); we also synthesized a radiolabelled biotin derivative, (3-^{123/125}I-iodobenzoyl)norbiotinamide (^{123/125}I-IBB). For nuclear medical imaging, the two were conjugated to give ^{123/125}I-IPOS, the characterization of which has been presented previously [9]. In brief, POS was degraded in an oxygen-dependent manner and a clear tumour image was obtained 24 h after injection of ¹²³I-IPOS. In addition, the tumoral accumulation correlated with HIF-1 activity and the intratumoral distribution of ¹²⁵I-IPOS was heterogeneous and corresponded to hypoxic areas. These findings suggested that ¹²³I-IPOS is a potential probe for the imaging of HIF-1-active tumours. However, due to its large molecular size, ¹²³I-IPOS cleared from the blood slowly; thus, we could not obtain a high tumour-to-normal tissue ratio within a short time after probe injection.

To overcome this problem, we propose a pretargeting method based on the high-affinity interaction between SAV and biotin [12]. This method uses a combination of tumour-seeking molecules and the prompt clearance of low-molecular-weight radiolabelled compounds (effector molecules) that are cleared within minutes from the blood. One of the advantages of the pretargeting method is that it provides a high tumour-to-normal tissue ratio within a short time after injection. In addition, because the effector molecules used in this method are rapidly cleared from

the body, radiation exposure is reduced. Moreover, some recent studies have shown that the tumour uptake of the effector molecules used in the pretargeting method is identical to or even higher than that of directly radiolabelled antibody. The images and therapeutic effects shown in these studies were significantly improved [13–15]. In the present study, we aimed to reveal the effectiveness of the pretargeting method using POS and ¹²³I-IBB in the rapid imaging of HIF-1-active tumour hypoxia.

Materials and methods

Cells and cell culture

FM3A mouse mammary tumour cells were purchased from Health Science Research Resources Bank (Osaka, Japan) and were cultured in 10% fetal bovine serum/RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan). Suit2 human pancreatic tumour cells that express luciferase in response to HIF-1 activity (Suit2/Luc cells) were established by Prof. Kizaka-Kondoh [16]. These cells were authenticated by a multiplex PCR method using short tandem repeat and were maintained in 10% fetal bovine serum/Dulbecco's modified Eagle's medium (Nissui Pharmaceutical). The culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml). Cells were incubated at 37°C in a well-humidified incubator with 5% CO₂ and 95% air.

Preparation of fusion protein

POS was overexpressed in *Escherichia coli* and purified as described in a previous report [9]. Purified POS was then dissolved in Tris-HCl buffer (pH 8.0).

Synthesis

Ammonium ¹²³I-iodide was kindly provided by Nihon Medi-Physics (Hyogo, Japan). Sodium ¹²⁵I-iodide was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All other chemicals used in this study were of reagent grade and are commercially available. ^{123/125}I-IBB and nonradioactive IBB were synthesized as described in a previous report [9, 17]. ¹²³I-IPOS was obtained by incubating ¹²³I-IBB and POS for 1 h, followed by purification on Sephadex G50 columns (GE Healthcare Bioscience, Uppsala, Sweden).

Animal model

Animal studies were conducted in accordance with our institutional guidelines, and the experimental procedures

were approved by the Kyoto University Animal Care Committee. Female C3H/He mice and BALB/c *nu/nu* mice at 5 weeks of age were purchased from Japan SLC (Hamamatsu, Japan). FM3A and Suit2/Luc tumour models were prepared as described in a previous report [9]. The cells were subcutaneously implanted in the right thigh, except in the mice that were to be used for the bioluminescence imaging in which the cells were subcutaneously implanted in both thighs. After tumour implantation, mice were fed an AIN76-A-based, biotin-free diet (Oriental Yeast Company, Tokyo, Japan) in order to prevent dietary biotin from inhibiting the binding of ^{125}I -IBB to POS. The mice were subjected to a tracer study 2 weeks after implantation. The average diameter and average volume of the tumours were 8 mm and 350 mm^3 , respectively.

Biodistribution

^{125}I -IBB (37 kBq) was injected intravenously into FM3A-implanted mice ($n=3-5$), and at 1, 3, 6 and 24 h after injection, the mice were killed. For the pretargeting study, the mice ($n=5$) were injected with 30 μg of POS and 24 h later ^{125}I -IBB was injected intravenously. The mice were killed at the same time points as above. Whole organs were immediately removed and weighed, and their radioactivity was measured with an autowell gamma counter (ARC2000; Aloka, Tokyo, Japan). The results are expressed in terms of the percent injected dose per gram of tissue (%ID/g).

In vivo blocking study

FM3A-implanted mice ($n=5$) were pretargeted with POS (30 μg); 24 h later, ^{125}I -IBB (37 kBq) was injected intravenously, with or without D-biotin (1 nmol) or with unlabelled IBB (500 pmol). Six hours after ^{125}I -IBB administration, the mice were killed. Whole organs were immediately removed and weighed, and their radioactivity was measured with the gamma counter (ARC2000). The results are expressed as %ID/g.

Size-exclusion analysis of radioactive compounds in tumours

FM3A-implanted mice ($n=5$) were pretargeted with POS (30 μg), and 24 h later, ^{125}I -IBB (8.0 MBq) was injected intravenously. The mice were killed 6 h after the injection of ^{125}I -IBB, and the tumours were removed. Extracts were prepared according to a previously reported method [18], with slight modification. In brief, the tumours were homogenized in ice-cold 0.1 M Tris-HCl buffer containing 0.15 M NaCl (pH 6.5), using a Polytron homogenizer (PT10-35, Kinematica, Switzerland). The preparations were then centrifuged at 4°C and $5,000\times g$ for 30 min (Micro Cooling Centrifuge

5922; Kubota, Osaka, Japan). The supernatants were filtered through a 0.46- μm filter (Nacalai Tesque, Kyoto, Japan) and analysed by size-exclusion chromatography using a PD-10 column (GE Healthcare Bioscience).

In vivo imaging

A SPECT-2000H scanner (Hitachi Medical, Tokyo, Japan) equipped with a low-energy, high-resolution, parallel-hole collimator [19, 20] was used. For direct targeting ($n=4$), ^{123}I -IPOS (30 μg , 4.9–7.4 MBq) was injected intravenously into each FM3A-implanted mouse. For the pretargeting method ($n=5$), ^{123}I -IBB (3.1–16 MBq) was injected into a tail vein 24 h after pretargeting with POS (30 μg). Under 2.5% halothane anaesthesia, the mice were placed on a scanner bed in the prone position. Planar images were obtained at 24 h after the injection of ^{123}I -IPOS in mice in the direct-targeted group or at 6 h after the injection of ^{123}I -IBB in mice in the pretargeted group. The image acquisition time was 10 min for both the groups. Regions of interest (ROI) were set on the tumour in the right thigh and the corresponding area in the left thigh.

Comparison between ^{125}I -IBB accumulation and HIF-1 transcriptional activity in the same tumours in mice pretargeted with POS

The Suit2/Luc-implanted mice ($n=5$) were pretargeted with POS (30 μg) and 24 h later, ^{125}I -IBB (37 kBq) was injected intravenously. After 5.5 h, 200 μl of D-luciferin solution (10 mg/ml in PBS; VivoGlo Luciferin, Promega, WI) was injected intraperitoneally. After 20 min, the mice were anaesthetized with 2.5% isoflurane and imaged according to a previously described method [9]. After bioluminescence imaging, the mice were killed and the radioactivity in each tumour was measured.

Autoradiography

Autoradiographic studies were performed in Suit2/Luc-implanted mice ($n=6$). The mice were pretargeted with POS (30 μg) and 24 h later, ^{125}I -IBB (4.1 MBq) was injected intravenously. After another 4 h, pimonidazole (PIMO, 60 mg/kg) was injected intraperitoneally and the mice were killed 2 h later. Autoradiograms were then obtained and analysed according to a previously described method [19–21]. The adjacent section on each autoradiogram was stained with haematoxylin-eosin to identify regions of necrosis.

Immunohistochemistry

The slides used in the autoradiographic study were subjected to dual fluorescent immunostaining for HIF-1 α

and PIMO. The sections were fixed in 2% paraformaldehyde and ice-cold methanol, blocked with Protein Block Serum Free (Dako, Glostrup, Denmark), and treated with anti-human/mouse HIF-1 α polyclonal antibody (R&D Systems, Minneapolis, MN) as a primary antibody. The specific signals were detected using an Alexa Fluor 568-conjugated F(ab')₂ fragment of goat anti-rabbit antibody (Invitrogen, San Diego, CA). Thereafter, after washing with PBS, FITC-conjugated mouse IgG₁ monoclonal antibody (Chemicon, Temecula, CA) was treated according to the manufacturer's protocol for PIMO staining. The sections were then dried and coverslipped using an antifade reagent (ProLong Gold; Invitrogen). To evaluate the specificity of the HIF-1 α signal, negative control rabbit immunoglobulin (Dako) was incubated with the adjacent sections, instead of the primary antibody. To evaluate the specificity of the PIMO signal, tumour sections derived from PIMO-untreated mice were stained following the same protocol. Fluorescent microscopic images were obtained by BIOREVO BZ-9000 (Keyence, Osaka, Japan).

Image analysis

The autoradiograms were quantified using MultiGauge software (ver. 3.0, Fuji Photo Film, Tokyo, Japan) and the immunohistochemical images were analysed using BZ analyser software (Keyence). ROIs were set in identical positions in both images on the basis of the x- and y-position of each ROI displayed in both software programs. The necrotic regions were excluded from the analysis. We set 10–14 ROIs (area 1 mm²) on the autoradiogram of each tumour and then transferred them to the corresponding immunohistochemical image. The radioactivity in each ROI was expressed in terms of percent injected dose. The expression densities of HIF-1 α and PIMO were determined in terms of the percentage of the positively stained region.

Statistical analyses

The two groups were compared using the Mann-Whitney *U* test, and correlation coefficients were assessed using the Spearman rank analysis. A chi-squared test was used to compare the correlation coefficients between ¹²⁵I-IBB accumulation and HIF-1 α expression and PIMO adduct formation. Values of *p* < 0.05 were considered statistically significant.

Results

Radiosynthesis of ^{123/125}I-IBB and ¹²³I-IPOS

¹²³I-IBB and ¹²⁵I-IBB were obtained in the absence of a carrier and with radiochemical yields of 29% and 65% and radiochemical purities of greater than 95% and greater than 94%, respectively. ¹²³I-IPOS was obtained with a radiochemical yield of 90% and a radiochemical purity of greater than 99%.

Biodistribution

An examination of the biodistribution of ¹²⁵I-IBB alone revealed that the tumour-to-blood ratio was less than 1 at all time points. This indicated that ¹²⁵I-IBB did not accumulate in the tumours. The radioactivity in the stomach was low indicating the resistance of ¹²⁵I-IBB to deiodination in vivo (Table 1). In contrast, in the pretargeted group, the tumour-to-blood ratio was greater than 1 as early as 1 h after the injection of ¹²⁵I-IBB and the tumour-to-blood ratio increased in a time-dependent manner (Table 2). The blood clearance in both groups was comparable. In the pretargeted group, tumour accumulation of ¹²⁵I-IBB 6 h after injection was 1.58 \pm 0.42%ID/g, which was more than 30-fold higher than for ¹²⁵I-IBB alone (0.05 \pm 0.01%ID/g). The radioactivity in the normal tissues decreased more rapidly than in the

Table 1 Biodistribution of ¹²⁵I-IBB in FM3A-implanted mice

Organ	Time after injection (h)			
	1	3	6	24
Blood	1.12 \pm 0.43	0.33 \pm 0.01	0.15 \pm 0.00	0.03 \pm 0.01
Liver	6.36 \pm 1.70	1.21 \pm 0.04	0.20 \pm 0.04	0.02 \pm 0.01
Heart	1.27 \pm 0.31	0.26 \pm 0.01	0.07 \pm 0.04	0.02 \pm 0.02
Lung	1.14 \pm 0.22	0.30 \pm 0.04	0.10 \pm 0.03	0.02 \pm 0.01
Kidney	5.54 \pm 0.74	1.06 \pm 0.23	0.35 \pm 0.18	0.07 \pm 0.02
Stomach	0.62 \pm 0.13	0.35 \pm 0.23	0.33 \pm 0.24	0.12 \pm 0.10
Intestine	32.61 \pm 8.24	24.73 \pm 12.12	10.69 \pm 4.93	0.95 \pm 0.64
Tumour	0.53 \pm 0.14	0.13 \pm 0.04	0.05 \pm 0.01	0.01 \pm 0.01
Muscle	0.84 \pm 0.18	0.15 \pm 0.05	0.03 \pm 0.02	0.01 \pm 0.01
Tumour/blood ratio	0.49 \pm 0.14	0.39 \pm 0.09	0.36 \pm 0.09	0.57 \pm 0.57

Organ uptake values are means \pm SD (*n*=3–5) in units of percent injected dose per gram of tissue, except for the stomach (percent injected dose) and tumour/blood ratio

Table 2 Biodistribution of ¹²⁵I-IBB in FM3A-implanted mice pretargeted with POS

Organ	Time after injection (h)			
	1	3	6	24
Blood	1.28±0.22	0.58±0.05	0.37±0.05	0.05±0.01
Liver	10.16±0.98	5.56±0.74	4.07±0.38	0.57±0.15
Heart	1.77±0.11	0.90±0.12	0.59±0.08	0.06±0.02
Lung	1.98±0.24	0.95±0.13	0.67±0.07	0.08±0.01
Kidney	25.09±1.42	11.58±2.46	5.78±1.02	0.30±0.04
Stomach	0.63±0.15	0.56±0.17	0.34±0.20	0.08±0.05
Intestine	22.45±3.40	20.11±7.22	8.66±6.13	0.76±0.42
Tumour	2.73±0.67	2.03±0.16	1.58±0.42	0.59±0.23
Muscle	1.04±0.16	0.47±0.13	0.30±0.11	0.04±0.02
Tumour/blood ratio	2.15±0.39	3.51±0.26	4.23±0.81	13.13±4.58

Organ uptake values are means ±SD (n=5) in units of percent injected dose per gram of tissue, except for the stomach (percent injected dose) and tumour/blood ratio

tumour. The radioactivity in the tumour at 24 h after injection was the second highest among the organs examined and both the tumour-to-blood and tumour-to-muscle ratios further increased (Table 2).

Tumour uptake of ¹²⁵I-IBB was significantly reduced to 46% following treatment with nonradioactive IBB (Table 3). Concomitant treatment with D-biotin also resulted in a 37% decrease in tumour uptake of ¹²⁵I-IBB (data not shown). ¹²⁵I-IBB accumulation in other tissues, except the blood, decreased following treatment with nonradioactive IBB.

Size-exclusion analysis of radioactive compounds in tumours

The recovery of radioactivity from tumour homogenates was 95.7±7.2%, and that from the PD-10 columns was 85.5±6.9%. A major proportion of the radioactivity was eluted in a macromolecular fraction (78.7±6.7%).

Table 3 Blocking study of the biodistribution of ¹²⁵I-IBB in FM3A-implanted mice pretargeted with POS

Organ	Vehicle	IBB treatment
Blood	0.36±0.18	0.48±0.10
Liver	5.26±0.61	2.58±0.69*
Heart	0.76±0.09	0.38±0.08
Lung	1.09±0.25	0.48±0.12
Kidney	12.15±5.03	2.44±0.41**
Stomach	0.82±0.48	0.25±0.04
Intestine	9.04±3.77	5.06±2.30
Tumour	1.95±0.63	0.90±0.18*
Muscle	0.51±0.06	0.16±0.04***
Tumour/blood ratio	5.49±1.12	1.93±0.38****

Organ uptake values are means±SD (n=5) in units of percent injected dose per gram of tissue, except for the stomach (percent injected dose) and tumour/blood ratio

*p<0.005, **p<0.001, ***p<0.0005, ****p<0.0001 vs. vehicle group

In vivo imaging

Figure 1a shows the accumulation of ¹²³I-IBB in the POS-pretargeted tumour. The tumour was clearly visualized 6 h after ¹²³I-IBB injection, and the calculated tumour-to-muscle ratio was 4.6±1.4. The image was comparable to that obtained 24 h after ¹²³I-IPOS injection, using the direct-targeting method (Fig. 1b; tumour-to-muscle ratio 6.9±1.6). Compared to the direct targeting method, with the pretargeting method, radioactivity decreased in the liver, but increased in the intestine. These findings were consistent with the biodistribution study data.

¹²⁵I-IBB accumulation vs. HIF-1 transcriptional activity in tumours in mice pretargeted with POS

Figure 2 shows a positive correlation between HIF-1-induced luciferase bioluminescence and ¹²⁵I-IBB accumulation 6 h after injection in tumours in mice pretargeted with POS (R=0.84, p<0.01).

Regional distribution of ¹²⁵I-IBB in the POS-pretargeted tumour

Dual fluorescent immunohistochemistry revealed the presence of HIF-1α- and PIMO-positive hypoxic areas in the tumour (Fig. 3a, b). The sections derived from PIMO-untreated mice were not stained with anti-PIMO antibody, and the HIF-1α signal was not detected in the negative control antibody-treated sections (data not shown). The autoradiogram showed that the distribution of ¹²⁵I-IBB in the tumour was heterogeneous (Fig. 3c) and, for the most part, corresponded to the HIF-1α- and PIMO-positive hypoxic areas. However, there were also a few regions where the signal for HIF-1α or PIMO was positive, but where ¹²⁵I-IBB had not accumulated (Fig. 3d, e). High-magnification merged imaging of HIF-1α and PIMO immunostaining revealed that HIF-1α-positive regions and

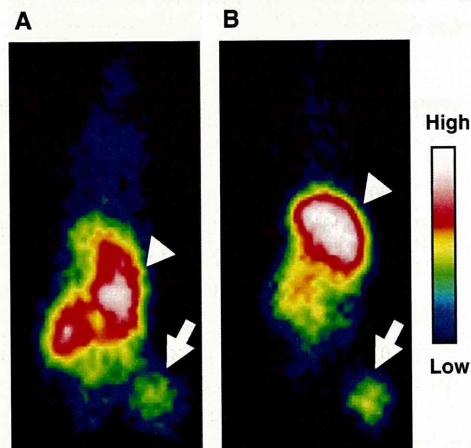


Fig. 1 Typical planar images of FM3A-implanted mice acquired in the pretargeting method (**a**) and the direct-targeting method (**b**). Images were acquired 6 h after injection of ^{125}I -IBB in the pretargeting method and 24 h after injection of ^{123}I -IPOS in the direct targeting method. Tumours are clearly visualized (arrows) in a similar fashion in both images (arrowheads **a** intestine, **b** liver)

PIMO-positive regions were not always identical; some regions were positive for both, while other regions did not match (Fig. 3f). Therefore, we compared correlations between ^{125}I -IBB accumulation and the expression of HIF-1 α and PIMO. As shown in Fig. 4, significant positive correlations were found between the regional accumulation of ^{125}I -IBB in POS-pretargeted tumour sections and the expression of both hypoxic markers (HIF-1 α $R=0.58$, $p<0.0001$; PIMO $R=0.34$, $p<0.005$). The correlation coefficient between ^{125}I -IBB and HIF-1 α was greater than that with PIMO, although the difference was not statistically significant ($\chi^2=3.13$, $p=0.077$).

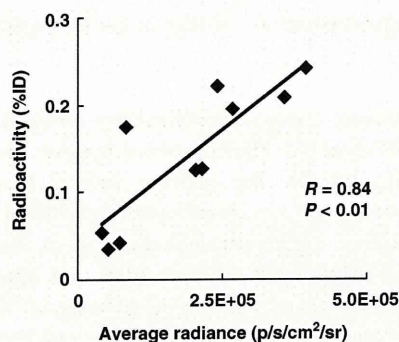


Fig. 2 Correlation between ^{125}I -IBB accumulation and HIF-1 transcriptional activity in the same tumour in mice pretargeted with POS. Mice carrying tumours with the HIF-1-dependent luciferase reporter gene in both thighs were used. The ordinate represents accumulated radioactivity (%ID), and the abscissa represents HIF-1-dependent luciferase bioluminescence. The correlation coefficient (R) was 0.84, indicating a highly significant correlation ($p<0.01$)

Discussion

In the study reported here we showed rapid tumour imaging following pretargeting using POS and $^{123/125}\text{I}$ -IBB. Using the pretargeting method, the tumoral accumulation (1.6% ID/g) and the tumour-to-blood ratio (4.2) 6 h after injection were found to be comparable to the data obtained 24 h after ^{125}I -IPOS injection (1.4%ID/g, 5.1) [9]. The scintigraphic images of mice targeted with ^{123}I -IPOS were very similar to those of prior studies, in which high signal levels were detected in the tumour, although the highest activity was seen in the liver even 24 h after injection [9]. In contrast, the images of the pretargeted mice clearly showed that ^{123}I -IBB accumulated in the tumour more rapidly and cleared much more promptly from the body (with the exception of the intestine) than ^{123}I -IPOS. These results indicate that the pretargeting method allows a shortening of the waiting time to one-fourth that required for direct targeting to obtain an adequate image. Clinical application of the pretargeting method in the future will contribute to a reduction in radiation exposure of the patient.

Recently it has been suggested that HIF-1 activates a number of genes, such as VEGF, erythropoietin, matrix metalloproteinase and glucose transporter, and that the expression level of HIF-1 correlates with a poor prognosis in many tumours [22–26]. Furthermore, the activation of such genes has an important role with respect to aggressive cancer phenotypes. Therefore, visualization and tumour therapy targeting for HIF-1 activation may be an important supplement to imaging of hypoxia per se when characterizing tumours. Some hypoxia imaging probes, including ^{18}F -fluoromisonidazole (^{18}F -FMISO), 1- α -D-(5-deoxy-5- ^{18}F -fluoroarabinofuranosyl)-2-nitroimidazole (^{18}F -FAZA) and ^{64}Cu -diacetyl-bis(N^4 -methylthiosemicarbazone) (^{64}Cu -ATSM), have been developed [27]. They detect physically low oxygen pressure (<10 mmHg) and are useful in predicting the efficacy of radiotherapy [28]. However, the mechanisms underlying hypoxic accumulation of such probes are not dependent on HIF-1 expression [29]. Previously reported HIF-1-activity imaging systems required exogenous reporter gene transcription, which presents difficulties in practice [30, 31]. In contrast, our approach could assess HIF-1-activity directly, via a two-step targeting of the probes.

A recent immunohistochemical study has demonstrated the lack of complete colocalization between HIF-1 α and PIMO. Sobhanifar et al. have reported that HIF-1 is expressed at higher levels of oxygen than those that allow PIMO metabolism and binding [32]. Thus, we compared the correlation between ^{125}I -IBB accumulation and HIF-1 α - and PIMO-positive regions. As shown in Fig. 4, the correlation coefficient between ^{125}I -IBB and HIF-1 α was greater than that between ^{125}I -IBB and PIMO, although the

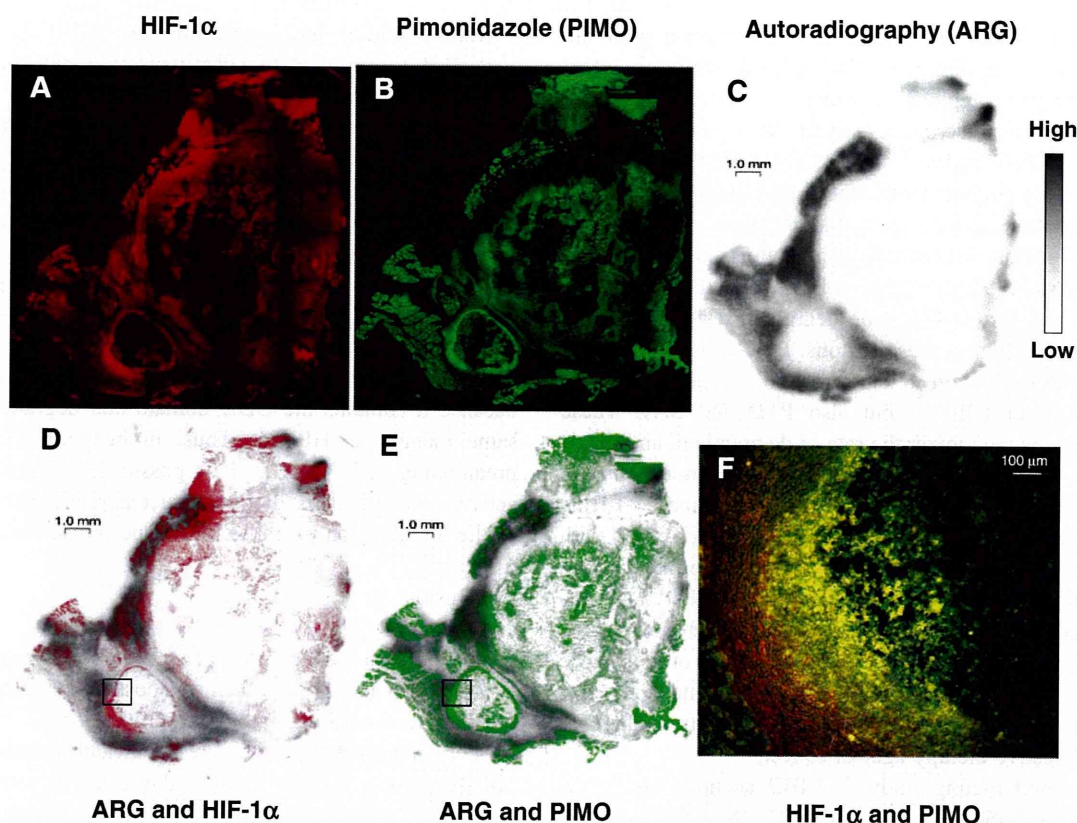


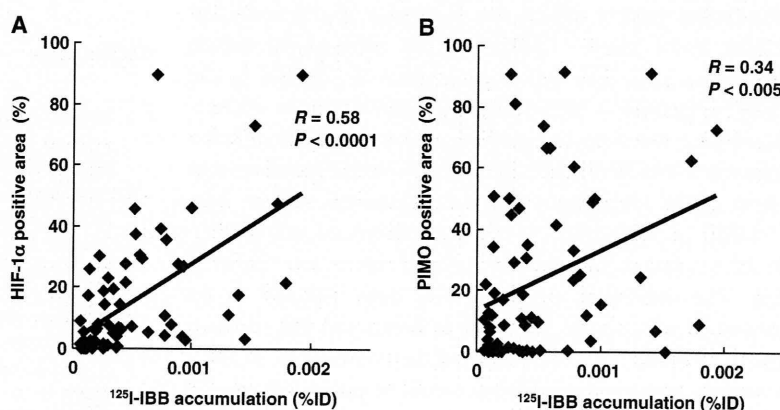
Fig. 3 Comparison between the intratumoral distribution of ¹²⁵I-IBB and the HIF-1α- and PIMO-positive hypoxic regions in POS-pretargeted tumours. **a–c** Typical images of HIF-1α immunostaining (**a**), PIMO immunostaining (**b**) and from autoradiography (**c**) in the same section. **d, e** Autoradiography image merged with HIF-1α

immunostaining (**d**) and PIMO immunostaining (**e**) images. **f** High-magnification merged image of HIF-1α immunostaining and PIMO immunostaining (from insets in **d** and **e**). Some regions are both positive (*yellow*) and others do not match (*red and green*)

difference between the two correlation coefficients was not statistically significant. Therefore, the regions showing ¹²⁵I-IBB accumulation potentially corresponded to the HIF-1α-positive regions and to the PIMO-positive regions but to a lesser degree. However, in the present study, the HIF-1α-positive region was mainly detected both in the PIMO-

positive regions and in the surrounding regions (Fig. 3f). This result is consistent with those of a previous study, which showed that carbonic anhydrase 9, a HIF-1-regulated protein, generally colocalizes with PIMO though displaying greater extension in the direction of perfused areas [33]. Due to the overlap, the region showing ¹²⁵I-IBB accumu-

Fig. 4 Correlation between areas showing ¹²⁵I-IBB accumulation and HIF-1α- and PIMO-positive areas in POS-pretargeted tumours. A total of six sections (one section per tumour) were analysed. Quantitative analysis of autoradiograms provided data on ¹²⁵I-IBB accumulation. ¹²⁵I-IBB accumulation was positively correlated with HIF-1α and PIMO expression (HIF-1α $R=0.58$, $p<0.0001$; PIMO $R=0.34$, $p<0.005$)



lation also showed a weak positive correlation with the PIMO-positive regions. On the other hand, there were a few HIF-1 α -negative regions in which ^{125}I -IBB accumulated (Fig. 3d). This discrepancy could be explained by the delivery of POS and/or ^{125}I -IBB. Since both probes were delivered by blood flow, they potentially tended to accumulate in regions rich in blood vessels, i.e. HIF-1 α -negative regions. Because the degradation rate of POS was relatively slow [9], an excess of POS delivered to HIF-1 α -negative regions could not be degraded, resulting in ^{125}I -IBB accumulation in these regions. POS contains not only the essential domain related to the oxygen-dependent degradation of HIF-1 α , but also PTD and SAV. These modifications may lower the rate of degradation, and this is a drawback of POS. However, it should be noted that by using the pretargeting method with POS and ^{123}I -IBB, radioactivity accumulation was significantly correlated with the HIF-1 α -positive region (Fig. 4a). Furthermore, accumulation of ^{123}I -IBB was correlated with HIF-1 transcriptional activity in the tumours in mice pretargeted with POS (Fig. 2). Thus, our approach would detect not only hypoxic regions, but also HIF-1-active regions, and thus this approach could prove important to the qualitative diagnosis of and effective therapy against cancer.

In the pretargeting study, ^{125}I -IBB accumulated in the tumours of POS-pretargeted mice. The accumulation was inhibited by nonradioactive IBB and D-biotin. Moreover, most of the radioactivity in the tumour existed as macromolecules. Taken together, the radioactivity in the tumour was caused by the binding of ^{125}I -IBB to the SAV moiety of POS. Thus, ^{125}I -IBB has the ability to bind to SAV, not only in vitro but also in vivo. It has been reported that the expression of HIF-1 is not ubiquitous, but heterogeneous, and is small in tumours [34]. Accumulation of the probe in tumours harbouring HIF-1-dependent reporter genes has been reported to be approximately 1%ID/g [34–36], which is comparable to the results of the present study.

In the biodistribution study of ^{125}I -IBB alone, accumulation of radioactivity was highest in the intestine. This tissue distribution pattern reflects the behaviour of radioiodinated biotin itself, since ^{125}I -IBB is not cleaved by serum biotinidase [17]. The high accumulation of ^{125}I -IBB in the intestine resulted in high background activity in the abdominal region; therefore, the imaging of abdominal HIF-1-active tumours would be difficult. In this case, biotin derivatives that show faster clearance from the abdominal region than ^{125}I -IBB are required. However, the background radioactivity in the thoracic region and cervicofacial region was generally low. The expression of HIF-1 has been reported to be associated with a poor prognosis in breast and lung cancers and in head and neck squamous cell carcinomas [26, 37, 38]; therefore, our imaging approach would be particularly useful for detecting HIF-1 activity in these tumours.

Recent studies have revealed that HIF-1 levels are controlled not only at the post-transcriptional level (that is, the oxygen-dependent regulation of the HIF-1 α) but also at the transcriptional and translational levels [39, 40]. In a region where the transcription and translation of HIF-1 α increases, an excess amount of HIF-1 α is likely to saturate the ubiquitin/proteasome degradation system and result in the upregulation of HIF-1, even under mildly hypoxic conditions. This may be one reason why the threshold of oxygen pressure for HIF-1 stabilization varies and depends upon the character of the organs or tumours. In such regions, the POS degradation rate would also decrease, because it contains the ODD domain and degrades in the same manner as HIF-1 α . Thus, in imaging with POS-pretargeting and ^{123}I -IBB, it is possible to depict HIF-1-active regions that result from either hypoxic stabilization or the upregulation of transcription and translation.

Conclusion

Using the pretargeting method, clear tumour images were obtained in a shorter time than was possible with the direct-labelling method. Intratumoral accumulations of ^{125}I -IBB in the POS-pretargeted tumours were significantly correlated with HIF-1 α -positive regions. These findings demonstrate that the pretargeting method with POS and ^{123}I -IBB is effective for the rapid imaging of HIF-1-active tumour hypoxia.

Acknowledgments The authors would like to thank Nihon Medi-Physics for providing ammonium ^{123}I -iodide. This work was supported in part by “R&D of Molecular Imaging Equipment for Malignant Tumor Therapy Support” of the New Energy and Industrial Technology Development Organization (NEDO), Japan, a Health Labour Sciences Research Grant for Research on Advanced Medical Technology from the Ministry of Health, Labour and Welfare of Japan, and a Grant-in-Aid for Exploratory Research (17659010) and a Grant-in-Aid for Young Scientists (B) (21791187) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflicts of interest None.

References

1. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* 1989;49:6449–65.
2. Semenza GL. Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochem Pharmacol* 2000;59:47–53.
3. Wenger RH. Cellular adaptation to hypoxia: O₂-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O₂-regulated gene expression. *FASEB J* 2002;16:1151–62.
4. Ballinger JR. Imaging hypoxia in tumors. *Semin Nucl Med* 2001;31:321–9.
5. Kizaka-Kondoh S, Inoue M, Harada H, Hiraoka M. Tumor hypoxia: a target for selective cancer therapy. *Cancer Sci* 2003;94:1021–8.

6. Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O₂ tension. *Am J Physiol* 1996;271:C1172–80.
7. Harada H, Kizaka-Kondoh S, Li G, Itasaka S, Shibuya K, Inoue M, et al. Significance of HIF-1-active cells in angiogenesis and radioresistance. *Oncogene* 2007;26:7508–16.
8. Harada H, Kizaka-Kondoh S, Hiraoka M. Optical imaging of tumor hypoxia and evaluation of efficacy of a hypoxia-targeting drug in living animals. *Mol Imaging* 2005;4:182–93.
9. Kudo T, Ueda M, Kuge Y, Mukai T, Tanaka S, Masutani M, et al. Imaging of HIF-1-active tumor hypoxia using a protein effectively delivered to and specifically stabilized in HIF-1-active tumor cells. *J Nucl Med* 2009;50:942–9.
10. Kizaka-Kondoh S, Konse-Nagasawa H. Significance of nitroimidazole compounds and hypoxia-inducible factor-1 for imaging tumor hypoxia. *Cancer Sci* 2009;100:1366–73.
11. Kizaka-Kondoh S, Tanaka S, Harada H, Hiraoka M. The HIF-1-active microenvironment: an environmental target for cancer therapy. *Adv Drug Deliv Rev* 2009;61:623–32.
12. Green NM. Avidin. 3. The nature of the biotin-binding site. *Biochem J* 1963;89:599–609.
13. Goldenberg DM, Rossi EA, Sharkey RM, McBride WJ, Chang CH. Multifunctional antibodies by the Dock-and-Lock method for improved cancer imaging and therapy by pretargeting. *J Nucl Med* 2008;49:158–63.
14. Sharkey RM, Cardillo TM, Rossi EA, Chang CH, Karacay H, McBride WJ, et al. Signal amplification in molecular imaging by pre-targeting a multivalent, bispecific antibody. *Nat Med* 2005;11:1250–5.
15. Sharkey RM, Karacay H, Litwin S, Rossi EA, McBride WJ, Chang CH, et al. Improved therapeutic results by pretargeted radioimmunotherapy of non-Hodgkin's lymphoma with a new recombinant, trivalent, anti-CD20, bispecific antibody. *Cancer Res* 2008;68:5282–90.
16. Kizaka-Kondoh S, Itasaka S, Zeng L, Tanaka S, Zhao T, Takahashi Y, et al. Selective killing of hypoxia-inducible factor-1-active cells improves survival in a mouse model of invasive and metastatic pancreatic cancer. *Clin Cancer Res* 2009;15:3433–41.
17. Foulon CF, Alston KL, Zalutsky MR. Synthesis and preliminary biological evaluation of (3-iodobenzoyl)norbiotinamide and ((5-iodo-3-pyridinyl)carbonyl)norbiotinamide: two radiolabeled biotin conjugates with improved stability. *Bioconjug Chem* 1997;8:179–86.
18. Motta-Hennessy C, Sharkey RM, Goldenberg DM. Metabolism of indium-111-labeled murine monoclonal antibody in tumor and normal tissue of the athymic mouse. *J Nucl Med* 1990;31:1510–9.
19. Ishino S, Kuge Y, Takai N, Tamaki N, Strauss HW, Blankenberg FG, et al. ^{99m}Tc-Annexin A5 for noninvasive characterization of atherosclerotic lesions: imaging and histological studies in myocardial infarction-prone Watanabe heritable hyperlipidemic rabbits. *Eur J Nucl Med Mol Imaging* 2007;34:889–99.
20. Ishino S, Mukai T, Kuge Y, Kume N, Ogawa M, Takai N, et al. Targeting of lectinlike oxidized low-density lipoprotein receptor 1 (LOX-1) with ^{99m}Tc-labeled anti-LOX-1 antibody: potential agent for imaging of vulnerable plaque. *J Nucl Med* 2008;49:1677–85.
21. Ueda M, Iida Y, Tominaga A, Yoneyama T, Ogawa M, Magata Y, et al. Nicotinic acetylcholine receptors expressed in the ventral posterolateral thalamic nucleus play an important role in anti-allodynic effects. *Br J Pharmacol* 2010;159:1201–10.
22. Harada H, Xie X, Itasaka S, Zeng L, Zhu Y, Morinibu A, et al. Diameter of tumor blood vessels is a good parameter to estimate HIF-1-active regions in solid tumors. *Biochem Biophys Res Commun* 2008;373:533–8.
23. Marignol L, Coffey M, Lawler M, Hollywood D. Hypoxia in prostate cancer: a powerful shield against tumour destruction? *Cancer Treat Rev* 2008;34:313–27.
24. Ke HL, Wei YC, Yang SF, Li CC, Wu DC, Huang CH, et al. Overexpression of hypoxia-inducible factor-1alpha predicts an unfavorable outcome in urothelial carcinoma of the upper urinary tract. *Int J Urol* 2008;15:200–5.
25. Miyake K, Yoshizumi T, Imura S, Sugimoto K, Batmunkh E, Kanemura H, et al. Expression of hypoxia-inducible factor-1alpha, histone deacetylase 1, and metastasis-associated protein 1 in pancreatic carcinoma: correlation with poor prognosis with possible regulation. *Pancreas* 2008;36:e1–9.
26. Trastour C, Benizri E, Ettore F, Ramaoli A, Chamorey E, Pouyssegur J, et al. HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. *Int J Cancer* 2007;120:1451–8.
27. Mees G, Dierckx R, Vangestel C, Van de Wiele C. Molecular imaging of hypoxia with radiolabelled agents. *Eur J Nucl Med Mol Imaging* 2009;36:1674–86.
28. Dunphy MP, Lewis JS. Radiopharmaceuticals in preclinical and clinical development for monitoring of therapy with PET. *J Nucl Med* 2009;50 Suppl 1:106S–21S.
29. Krohn KA, Link JM, Mason RP. Molecular imaging of hypoxia. *J Nucl Med* 2008;49 Suppl 2:129S–48S.
30. He F, Deng X, Wen B, Liu Y, Sun X, Xing L, et al. Noninvasive molecular imaging of hypoxia in human xenografts: comparing hypoxia-induced gene expression with endogenous and exogenous hypoxia markers. *Cancer Res* 2008;68:8597–606.
31. Yeom CJ, Chung JK, Kang JH, Jeon YH, Kim KI, Jin YN, et al. Visualization of hypoxia-inducible factor-1 transcriptional activation in C6 glioma using luciferase and sodium iodide symporter genes. *J Nucl Med* 2008;49:1489–97.
32. Sobhanifar S, Aquino-Parsons C, Stanbridge EJ, Olive P. Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. *Cancer Res* 2005;65:7259–66.
33. Li XF, Carlin S, Urano M, Russell J, Ling CC, O'Donoghue JA. Visualization of hypoxia in microscopic tumors by immunofluorescent microscopy. *Cancer Res* 2007;67:7646–53.
34. Serganova I, Doubrovin M, Vider J, Ponomarev V, Soghomonyan S, Beresten T, et al. Molecular imaging of temporal dynamics and spatial heterogeneity of hypoxia-inducible factor-1 signal transduction activity in tumors in living mice. *Cancer Res* 2004;64:6101–8.
35. Hsieh CH, Kuo JW, Lee YJ, Chang CW, Gelovani JG, Liu RS. Construction of mutant TKGFP for real-time imaging of temporal dynamics of HIF-1 signal transduction activity mediated by hypoxia and reoxygenation in tumors in living mice. *J Nucl Med* 2009;50:2049–57.
36. Wen B, Burgman P, Zanzonico P, O'Donoghue J, Cai S, Finn R, et al. A preclinical model for noninvasive imaging of hypoxia-induced gene expression; comparison with an exogenous marker of tumor hypoxia. *Eur J Nucl Med Mol Imaging* 2004;31:1530–8.
37. Ioannou M, Papamichali R, Kouvaras E, Mylonis I, Vageli D, Kerenidou T, et al. Hypoxia inducible factor-1 alpha and vascular endothelial growth factor in biopsies of small cell lung carcinoma. *Lung* 2009;187:321–9.
38. van den Broek GB, Wildeman M, Rasch CR, Armstrong N, Schuurung E, Begg AC, et al. Molecular markers predict outcome in squamous cell carcinoma of the head and neck after concomitant cisplatin-based chemoradiation. *Int J Cancer* 2009;124:2643–50.
39. North S, Moenner M, Bikfalvi A. Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. *Cancer Lett* 2005;218:1–14.
40. Skinner HD, Zheng JZ, Fang J, Agani F, Jiang BH. Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1alpha, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling. *J Biol Chem* 2004;279:45643–51.

RESEARCH PAPER

Nicotinic acetylcholine receptors expressed in the ventral posterolateral thalamic nucleus play an important role in anti-allodynic effects

M Ueda^{1,2}, Y Iida³, A Tominaga², T Yoneyama², M Ogawa⁴, Y Magata⁴, H Nishimura⁵, Y Kuge^{2*} and H Saji²

¹Radioisotopes Research Laboratory, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan,

²Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan,

³Bioimaging Information Analysis, Graduate School of Medicine, Gunma University, Maebashi, Japan, ⁴Laboratory of Genome-Bio Photonics, Photon Medical Research Center, Hamamatsu University School of Medicine, Hamamatsu, Japan, and

⁵Radioisotope Laboratory, Kyoto Prefectural University of Medicine, Kyoto, Japan

Background and purpose: Much interest is currently being focused on the anti-nociceptive effects mediated by nicotinic acetylcholine (nACh) receptors, including their location and mechanism of action. The purpose of this study was to elucidate these issues using 5-iodo-3-(2(S)-azetidylmethoxy)pyridine (5IA), a nACh receptor agonist, and [¹²⁵I]5IA.

Experimental approach: We partially ligated the sciatic nerve of Sprague-Dawley rat to induce neuropathic pain [Seltzer's partial sciatic nerve ligation (PSL) model]. We then examined the changes in nACh receptor density in the CNS using [¹²⁵I]5IA autoradiography and the involvement of nACh receptors in anti-nociceptive effects in the region where changes occurred.

Key results: Autoradiographic studies showed that the accumulation of [¹²⁵I]5IA and the number of nACh receptors in the thalamus of PSL rats were increased about twofold compared with those in the sham-operated rats. No change was observed in other brain regions. Rats injected in the ventral posterolateral thalamic nucleus (VPL) with 5IA demonstrated a significant and dose-dependent anti-allodynic effect and this effect was completely antagonized by mecamylamine, injected with 5IA, into the VPL. The blockade of nACh receptors in the VPL by mecamylamine decreased by 70% the anti-allodynic effect of 5IA, given i.c.v. Moreover, mecamylamine given intra-VPL by itself, induced significant hyperalgesia.

Conclusions and implications: Our findings suggest that the nACh receptors expressed in the VPL play an important role in the anti-allodynic effects produced by exogenous and endogenous agonists.

British Journal of Pharmacology (2010) **159**, 1201–1210; doi:10.1111/j.1476-5381.2009.00613.x; published online 5 February 2010

Keywords: nicotinic acetylcholine receptor; thalamus; ventral posterolateral thalamic nucleus; 5IA; anti-allodynic effect; neuropathic pain

Abbreviations: 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; MPE, maximal possible effect; nACh, nicotinic acetylcholine; NRM, nucleus raphe magnus; PPTg, pedunculopontine tegmental nucleus; VPL, ventral posterolateral thalamic nucleus

Introduction

Numerous studies have shown that nicotinic acetylcholine (nACh) receptor agonists possess analgesic effects (Decker

et al., 2004; Jain, 2004). Because these effects are not antagonized by opioid receptor antagonists and arise from the activation of nACh receptors (Bannon *et al.*, 1998a; nomenclature follows Alexander *et al.*, 2008), much interest has been focused on nACh receptors as a novel target of anti-nociceptive drugs that do not involve the opioid system. Although morphine is a very well known and effective analgesic, its usefulness for patients with neuropathic pain is limited (Bannon *et al.*, 1998c; Sindrup and Jensen, 1999). In contrast, nACh receptor agonists are able to ameliorate neuropathic pain by affecting systems other than the opioids (Bannon *et al.*, 1998b,c; Decker *et al.*, 1998). Therefore,

Correspondence: Hideo Saji, Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan. E-mail: hsaji@pharm.kyoto-u.ac.jp

*Present address: Central Institute of Isotope Science, Hokkaido University, Sapporo 060-0815, Japan.

Received 24 April 2009; revised 22 July 2009; accepted 18 October 2009

identification of the sites of action of nACh receptor agonists and the elucidation of their anti-allodynic mechanisms are desirable.

One of the most studied nACh receptor-acting analgesics is ABT-594. This compound is a selective and potent agonist with high affinity for $\alpha 4\beta 2$ -nACh receptors (predominant in the brain), low affinity for $\alpha 7$ -nACh receptors and no affinity for $\alpha 1\beta 1\gamma\delta$ -nACh receptors (Donnelly-Roberts *et al.*, 1998; Holladay *et al.*, 1998). By virtue of this selectivity, ABT-594 has analgesic efficacy with an improved therapeutic window compared with non-selective agonists such as epibatidine (Bannon *et al.*, 1998b).

Recently, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine (5-iodo-A-85380, 5IA), an analogue of ABT-594, was synthesized (Saji *et al.*, 2002). It is also an $\alpha 4\beta 2$ -nACh receptor-specific agonist (Ueda *et al.*, 2008) with a relatively good safety profile (Vaupel *et al.*, 2003; Ueda *et al.*, 2004). In addition, radiolabelled 5IA, [$^{123/125}$ I]5IA, was also developed (Saji *et al.*, 2002) and was reported to be a promising ligand for imaging nACh receptors in rodents and in humans (Saji *et al.*, 2002; Mamede *et al.*, 2004; Mamede *et al.*, 2007; Oishi *et al.*, 2007; Brasic *et al.*, 2009). Non-radioactive 5IA and [$^{123/125}$ I]5IA can be considered the same compound with regard to their biodistribution or metabolism. Therefore, the pharmacokinetics, receptor occupancy and binding potential (an index of nACh receptor density) of 5IA are able to be measured readily using [$^{123/125}$ I]5IA. Studies using 5IA have the advantage that pharmacodynamic effects can be compared directly with the pharmacokinetic profile of the compound.

Previous studies have demonstrated the up-regulation of spinal muscarinic acetylcholine receptors (Chen and Pan, 2003) and thalamic cannabinoid CB₁ receptors (Siegling *et al.*, 2001) in neuropathic pain models. It has been reported that the up-regulation of muscarinic and CB₁ receptors contributes to the increased analgesic efficacy of each agonist. As nACh receptor agonists are also effective against neuropathic pain, up-regulation of nACh receptors may also occur in such models. We hypothesized that brain regions where changes in the density or function of nACh receptors occurred would play an important role in anti-nociceptive effects, provided that such changes were found under neuropathic conditions. In the present study, we used a partial sciatic nerve ligation (PSL) model of neuropathic pain (Seltzer *et al.*, 1990) and examined the changes in nACh receptors in the PSL rats using [125 I]5IA. Furthermore, based on the result, we investigated the involvement of the nACh receptors expressed in the region where changes occurred as a result of the model of neuropathic pain.

Methods

Animals

Animal care and experimental procedures were conducted in accordance with our institutional guidelines, and the experimental procedures were approved by the Kyoto University Animal Care Committee.

Male Sprague-Dawley rats weighing 200–250 g were purchased from Japan SLC Co., Ltd. (Hamamatsu, Japan). The

rats were kept at a constant ambient temperature under a 12 h light/dark cycle with free access to food and water.

Surgical operation

The PSL neuropathic pain model was established according to a previously published method (Seltzer *et al.*, 1990). Under sodium pentobarbital (50 mg·kg⁻¹, i.p.) anesthesia, the right sciatic nerve was exposed just distal to the branch leading to the posterior biceps femoris/semiotendinosus muscles. A 7–0 silk suture was inserted into the nerve and tightly ligated so that the dorsal 1/3–1/2 of the nerve was trapped in the ligature. In the sham-operated rats, the sciatic nerve was exposed, but was left intact.

After recovery from the surgery, the rats were implanted with stainless steel guide cannulae (o.d. 0.7 mm, i.d. 0.38 mm) under sodium pentobarbital (50 mg·kg⁻¹, i.p.) anesthesia for i.c.v. or intra-ventral posterolateral thalamic nucleus (VPL) administration. The rats were placed into a stereotaxic apparatus (SR-5, Narishige Co., Ltd., Tokyo, Japan) and unilaterally implanted with a guide cannula above the lateral ventricle (0.8 mm posterior and 1.5 mm lateral to bregma, 2.0 mm below the outer surface of the skull) for i.c.v. administration or above the left VPL, which was contralateral to the nerve ligation (2.4 mm posterior and 3.3 mm lateral to bregma, 2.0 mm below the outer surface of the skull) for intra-VPL administration. The stereotaxic coordinates were determined following an atlas (Paxinos and Watson, 2005). The guide cannulae were held firmly in place using dental acrylic cement. After surgery, the rats were individually returned to their cages and left to recover for 5 days or more until the experiments.

von Frey filament test

Just before and 2 weeks after the PSL, tactile sensitivity was measured by the up-down method using calibrated von Frey filaments ranging from 0.07 to 26 g (North Coast Medical, Morgan Hill, CA, USA), in a previously described method with slight modifications (Marcil *et al.*, 2006). Briefly, for testing, the rats were individually placed on an elevated wire mesh floor. After a habituation period of 15–30 min, the tactile stimulus was applied to the middle plantar area of the each paw by placing the von Frey filament perpendicular to the surface of the paw. The filament was held in this position with enough force to cause slight bending. Each trial involved 10 applications of filaments every 1–2 s. The threshold was determined as the filament of the lowest stiffness at which the rat responded (quick paw flick) in one or more of the trial. The rats that showed a lower threshold postoperatively than preoperatively in both paws were considered as demonstrating allodynia and were used in the following studies.

Effect of 5IA and/or mecamlamine on tactile allodynia

For i.c.v. administration, an injection cannula (o.d. 0.35 mm, i.d. 0.18 mm) was inserted 5.0 mm below the surface of the skull along the guide cannula. Then, various concentrations of 5IA (1–10 nmol in 5 μ L) or vehicle were infused at 5 μ L per rat with a constant rate of 10 μ L·min⁻¹ using a microsyringe

pump (EP-60; Eicom Corporation, Kyoto, Japan). The cannula was retained in place for an additional 1 min to prevent backflow of the drugs. When required, mecamlamine (5 mg·kg⁻¹) was injected subcutaneously 30 min before i.c.v. administration of 5IA.

For intra-VPL administration, an injection cannula was inserted 6.2 mm below the surface of the skull. Subsequently, vehicle, 5IA (1–50 nmol in 0.5 µL), mecamlamine (1–10 nmol in 0.5 µL) or a mixed solution of 5IA and mecamlamine (10 nmol each in 0.5 µL) was infused at 0.5 µL per rat with a constant rate of 0.4 µL·min⁻¹. The cannula was retained in place for an additional 1 min.

For double injections, the cannula for intra-VPL administration was inserted intra-VPL, and mecamlamine (10 nmol in 0.5 µL) or vehicle was infused in the same way as described above. Five minutes later, the cannula for i.c.v. administration was inserted, and 5IA (10 nmol in 5 µL) or vehicle was infused as described above. Both cannulae were retained in place for an additional 1 min.

Just before and 15, 30, 60, 90 and 120 min after administration, tactile allodynia was evaluated using the von Frey filament test. The results were expressed as a percentage of the maximal possible effect (% MPE) according to the following formula:

$$\%MPE = \frac{\text{post-drug threshold} - \text{pre-drug threshold}}{\text{cut-off (26 g)} - \text{pre-drug threshold}} \times 100$$

Inclined plane test

The inclined plane test was carried out using a sliding apparatus (Medical Agent Co., Ltd., Kyoto, Japan), as described previously (Okada *et al.*, 2002). This procedure was used to evaluate the effects of anti-allodynic drugs on motor function (Fukui *et al.*, 2001; Okada *et al.*, 2002; Yasuda *et al.*, 2005). Intraperitoneal administration of baclofen, which causes muscle relaxation, significantly reduced the slope angle at which rats were no longer able to maintain their position and slid down the table, thus indicating the appropriateness of this procedure (Fukui *et al.*, 2001). Each rat was placed on a stainless steel plate inclined at 30°, and the angle of the plate was increased at a rate of 2°·s⁻¹. The maximum angle of the plane at which the rat maintained its body position without sliding down was determined.

The rats were habituated to the procedure three times per day. After 2 days of habituation, the test was performed three times, and the mean of the last two values was taken as a control. Soon after measuring the control value, drugs or vehicle were administered via each route, as described above, and the maximum angle before sliding down was determined at 15, 30, 60, 90 and 120 min after administration. Drugs were used at the maximum concentration for each administration route. The data are represented as a percentage of the control value.

Ex vivo autoradiography

Ex vivo autoradiography was performed using a previously described method (Kanegawa *et al.*, 2006) with slight modifications. Three groups of rats, a sham-operated group, a 2 week

and a 1 month post-PSL group, were used in this experiment. Each group consisted of four to seven animals.

Each rat was injected with 2 MBq of [¹²⁵I]5IA via its tail vein and killed 60 min after radioligand injection. Their brains were quickly removed, frozen in hexane (–80°C) and cut into 20 µm-thick coronal sections with a cryomicrotome (CM3000, Leica, Germany). The sections were exposed to imaging plates (BAS-UR, Fuji Photo Film Co., Ltd., Tokyo, Japan) with ¹²⁵I autoradiographic microscale standards (Amersham Biosciences, Buckinghamshire, UK). After 25 h exposure, the [¹²⁵I]5IA autoradiograms were obtained using a bioimaging analyzer (BAS 3000, Fuji Photo Film Co., Ltd., Tokyo, Japan), and quantitative densitometric analyses were performed with dedicated software (Image Gauge ver. 3.1, Fuji Photo Film Co., Ltd., Tokyo, Japan). Regions of interest were drawn over eight brain regions bilaterally [frontal cortex, striatum, hippocampus, thalamus, pedunculopontine tegmental nucleus (PPTg), nucleus raphe magnus (NRM), locus coeruleus and cerebellum]. Data were represented as the mean of the ligated and contralateral side.

In vitro autoradiography

Three groups of rats were used as described for the *ex vivo* autoradiography. Each group consisted of five to seven animals. The rats were killed and their brains were removed and frozen immediately. Each frozen brain was cut into 20 µm-thick coronal sections, thaw-mounted onto gelatin-coated glass slides and kept frozen at –80°C until use.

A method for the autoradiographic determination of receptor density was previously published (Tanaka *et al.*, 1993; Suzdak *et al.*, 1994). A binding assay was conducted using a modification of a previously described method (Mukhin *et al.*, 2000). The assay buffer used was 50 mM Tris-HCl (pH 7.0) containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂ and 1 mM MgCl₂. The sections were incubated with 23.75–380 pM [¹²⁵I]5IA (specific activity: 220 Ci·mmol⁻¹) in the same buffer for 1 h at 25°C, then rinsed twice in ice-cold buffer for 5 min each time, and once in distilled water for 1 min, and dried under a cold air stream. Non-specific binding was determined in the presence of 300 µM (–)-nicotine. The sections were exposed to the imaging plates with ¹²⁵I standards for 20 h. The autoradiograms were obtained and the quantitative analyses were performed in a similar way to that described above. Values for the maximum density of binding sites (B_{max}) were gained from saturation binding isotherms (one-site binding) of specific binding by means of nonlinear curve fitting (Prism 5.00, GraphPad Software, San Diego, CA, USA).

Statistical analyses

After the behavioural experiments, the rats were killed, and their brains were removed and frozen immediately. Coronal sections (20 µm) including the VPL were prepared and thaw-mounted onto gelatin-coated glass slides and subjected to Nissl staining. The placements of the tips of the injection cannulae were confirmed by using the atlas (Paxinos and Watson, 2005). Only data from rats with the injection cannulae correctly placed in the VPL were used for the statistical analyses.

The analyses of the data from the von Frey filament test and the inclined plane test were performed using two-way analysis of variance (ANOVA) with repeated measures. If there was a significant difference, a *post hoc* one-way ANOVA followed by a Tukey-Kramer multiple comparison test was performed using each treatment combination as an independent group. Analyses of the data from the autoradiographic studies were performed using a one-way ANOVA followed by a Tukey-Kramer multiple comparison test. Differences were considered significant when $P < 0.05$.

Materials

5IA and [¹²⁵I]5IA were synthesized according to a previously published method (Saji *et al.*, 2002). Mecamylamine hydrochloride, a nACh receptor antagonist, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The sodium pentobarbital for injection was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). All drugs were administered in physiological saline solution. All other chemicals used were of reagent grade.

Results

Anti-allodynic effect of i.c.v. administered 5IA

In the present study, the rats presented a bilateral tactile allodynia following the PSL. The paw withdrawal thresholds decreased from 13.4 ± 0.9 g to 3.1 ± 0.3 g and from 13.4 ± 1.0 g to 4.2 ± 0.4 g in the ligated paws and the contralateral paws respectively. First, we evaluated an anti-allodynic effect of i.c.v. administered 5IA, as there has been no previous report regarding whether 5IA is able to palliate neuropathic pain. Figure 1A shows the anti-allodynic effect observed in the ligated paws. Two-way ANOVA demonstrated significant main effects of treatment ($F_{3,90} = 5.56$, $P = 0.007$) and time ($F_{4,90} =$

7.51 , $P < 0.001$) and a significant interaction between treatment and time ($F_{12,90} = 2.85$, $P = 0.003$). Injection of 5IA (10 nmol) increased % MPE values significantly at 15 min after administration (Vehicle: -3.4 ± 2 , 10 nmol: 38 ± 6). Although the effect peaked at 15 min after administration and decreased gradually, significant increases in % MPE values were also observed in the 3 nmol- and 10 nmol-treated groups 30 min after administration. Similar results were also observed in the contralateral paws (data not shown).

Next, we examined an effect of mecamylamine, a blood-brain barrier permeable nACh receptor antagonist, on the anti-allodynic effect of 5IA. Significant main effects of treatment ($F_{3,95} = 6.75$, $P = 0.003$) and time ($F_{4,95} = 5.61$, $P < 0.001$) and a significant interaction between treatment and time ($F_{12,95} = 7.44$, $P < 0.001$) were found. Mecamylamine ($5 \text{ mg}\cdot\text{kg}^{-1}$, s.c.) blocked the effect of 5IA completely and the % MPE values were reduced to levels obtained after treatment with vehicle (Figure 1B).

Changes in the accumulation of [¹²⁵I]5IA in vivo

We compared [¹²⁵I]5IA accumulation between the PSL model rats and the sham-operated rats using an *ex vivo* autoradiographic method. Representative autoradiograms are shown in Figure 2A. The thalamic signals of the PSL groups were stronger than those of the sham-operated group, while the cortical and hippocampal signals were similar among the three groups. There was no difference between the ligated and contralateral sides of the PSL model rats. A significant increase (170%) in the accumulation of [¹²⁵I]5IA was observed only in the thalamus in both PSL groups and no detectable change was seen in other regions (Figure 2B).

Measurement of the density of nACh receptors in vitro

We performed an autoradiographic saturation assay using [¹²⁵I]5IA as a radioligand. The saturation binding curves are

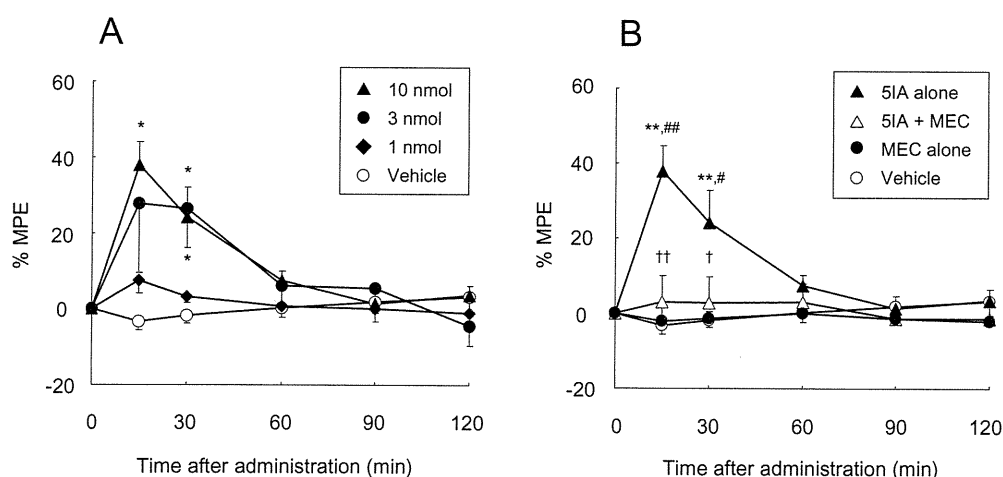


Figure 1 (A) Effect of i.c.v. administered 5IA on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect (% MPE). Each point represents the mean \pm SEM of the ligated paws of five to six animals per group. * $P < 0.05$ versus vehicle at the same time point. (B) Effect of mecamylamine (MEC, $5 \text{ mg}\cdot\text{kg}^{-1}$, s.c., 30 min prior) on the anti-allodynic effect induced by 5IA (i.c.v.). Data are presented as a percentage of the maximum possible effect. Each point represents the mean \pm SEM of the ligated paws of five to six animals per group. ** $P < 0.01$ versus vehicle, # $P < 0.05$, ## $P < 0.01$ versus MEC alone, and † $P < 0.05$, †† $P < 0.01$ versus 5IA alone at the same time point. 5IA, 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine; MPE, maximal possible effect.

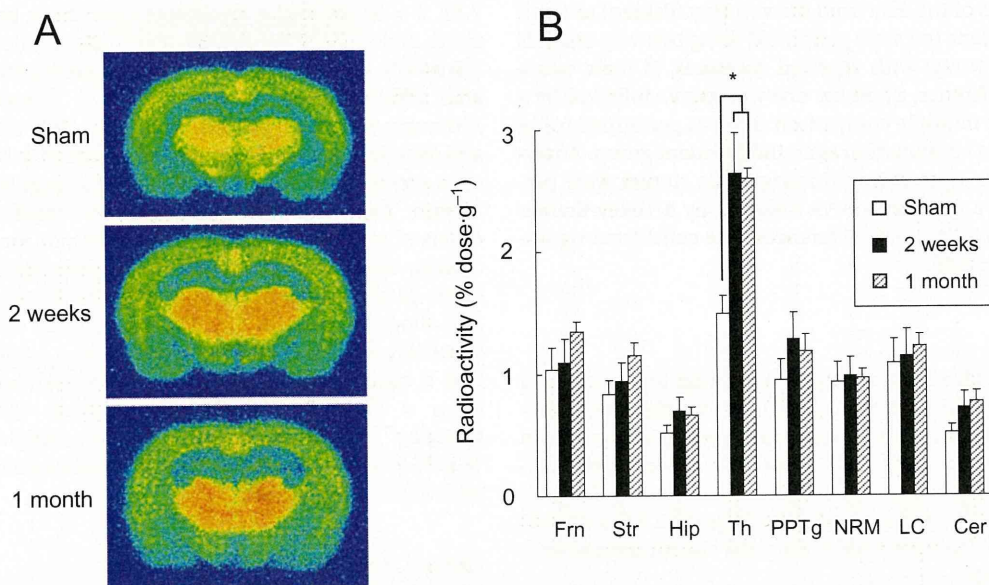


Figure 2 (A) Representative autoradiograms of brain regions from sham-operated and PSL rats given [¹²⁵I]5IA, i.v. (B) Changes in the regional accumulation of [¹²⁵I]5IA in Seltzer's PSL model of neuropathic pain. A significant increase was observed only in the thalamus, and no detectable change occurred in other regions. Each column represents the mean \pm SEM of four to seven animals per group. * $P < 0.05$ versus sham. PSL, partial sciatic nerve ligation; 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; Frn, frontal cortex; Str, striatum; Hip, hippocampus; Th, thalamus; PPTg, pedunculopontine tegmental nucleus; NRM, nucleus raphe magnus; LC, locus coeruleus; Cer, cerebellum.

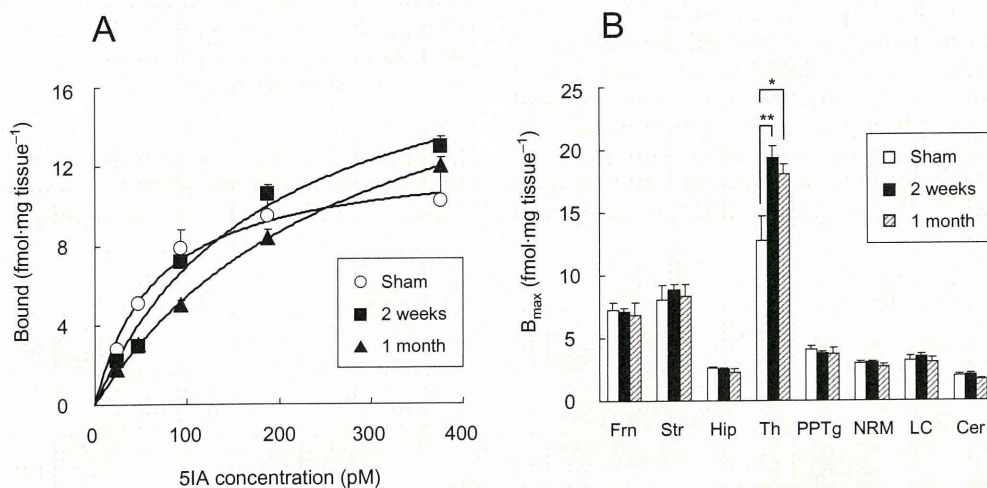


Figure 3 (A) Saturation curves of [¹²⁵I]5IA binding to sham-operated and PSL rat thalamus determined *ex vivo*. Each point represents the mean \pm SEM of five to seven animals per group. The B_{max} values estimated by nonlinear regression analysis of these mean data were 12.5, 19.9 and 21.2 fmol/mg protein in sham-operated, 2 week post-PSL, and 1 month post-PSL group respectively. (B) Changes in nACh receptor density in brain regions of rats with Seltzer's PSL model of neuropathic pain. A significant increase was observed only in the thalamus, and no detectable change occurred in other regions. Each column represents the mean \pm SEM of five to seven animals per group. * $P < 0.05$, ** $P < 0.01$ versus sham. PSL, partial sciatic nerve ligation; 5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; Frn, frontal cortex; Str, striatum; Hip, hippocampus; Th, thalamus; PPTg, pedunculopontine tegmental nucleus; NRM, nucleus raphe magnus; LC, locus coeruleus; Cer, cerebellum.

presented in Figure 3A. As with the *ex vivo* autoradiography, both PSL groups showed a significant increase in B_{max} value that occurred in the thalamus only (Figure 3B). The percentage increase in B_{max} values in the thalamus (150%) was similar to those observed in the *ex vivo* autoradiographic findings.

Anti-allodynic effect of 5IA given intra-VPL

Based on previous reports (Kupers and Gybels, 1993; Derbyshire *et al.*, 1997; Gybels, 2001), we predicted that the VPL was involved in the expression of anti-nociceptive effects and investigated the association between nACh receptors expressed in the VPL and the anti-allodynic effect of 5IA.

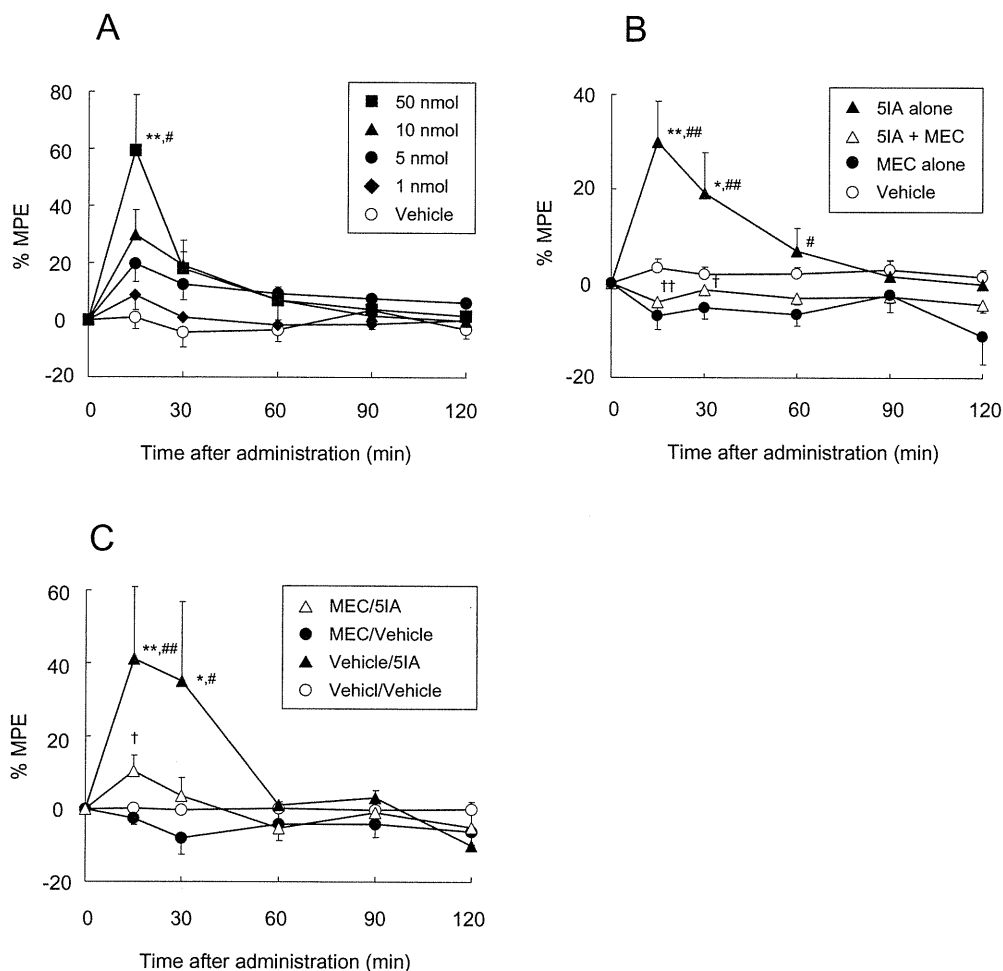


Figure 4 (A) Effect of 5IA injected intra-VPL on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect (% MPE). Each point represents the mean \pm SEM of the ligated paws of five to six animals per group. * $P < 0.05$, ** $P < 0.01$ versus vehicle, and # $P < 0.05$ versus 1 nmol of 5IA at the same time point. (B) Effect of mecamlamine (MEC, 10 nmol in 0.5 μ L) administered concurrently with 5IA in an intra-VPL manner on the anti-allodynic effect produced by 5IA (10 nmol in 0.5 μ L, intra-VPL). Data are presented as a percentage of the maximum possible effect. Each point represents the mean \pm SEM of the ligated paws of five to six animals per group. * $P < 0.05$, ** $P < 0.01$ versus vehicle, # $P < 0.05$, ## $P < 0.01$ versus MEC alone, and † $P < 0.05$, †† $P < 0.01$ versus 5IA alone at the same time point. (C) Effect of mecamlamine (MEC, 10 nmol in 0.5 μ L, intra-VPL, 5 min prior) on the anti-allodynic effect produced by 5IA (10 nmol in 5 μ L, i.c.v.). Data are presented as a percentage of the maximum possible effect. Each point represents the mean \pm SEM of the ligated paws of four to seven animals per group. * $P < 0.05$, ** $P < 0.01$ versus vehicle/vehicle, # $P < 0.05$, ## $P < 0.01$ versus MEC/vehicle, and † $P < 0.05$ versus vehicle/5IA at the same time point. 5IA, 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine; MPE, maximal possible effect; VPL, ventral posterolateral thalamic nucleus.

Figure 4A shows the anti-allodynic effects that occurred in the ligated paws of rats that received 5IA intra-VPL. Two-way ANOVA demonstrated significant main effects of treatment ($F_{4,115} = 3.97$, $P = 0.014$) and time ($F_{4,115} = 13.6$, $P < 0.001$) and a significant interaction between treatment and time ($F_{16,115} = 3.11$, $P < 0.001$). There was a significant difference between the 50 nmol-treated group and the vehicle- or 1 nmol-treated groups. Although one-way ANOVA revealed a significant difference between the data at 30 min after administration ($P = 0.028$), the *post hoc* Tukey test showed no significant difference.

Subsequently, we injected a mixed solution of 5IA and mecamlamine (10 nmol each) intra-VPL and performed the von Frey filament test. Two-way ANOVA demonstrated signifi-

cant main effects of treatment ($F_{3,85} = 11.34$, $P < 0.001$) and time ($F_{4,85} = 5.22$, $P = 0.001$) and a significant interaction between treatment and time ($F_{12,85} = 4.05$, $P < 0.001$). Mecamlamine antagonized the 5IA-induced anti-allodynic effect completely, and furthermore, the % MPE values of mecamlamine-treated rats tended to decrease compared with those of the vehicle-treated rats, although the difference was not significant (Figure 4B).

Involvement of nACh receptors in the VPL in the anti-allodynic effect induced by i.c.v. 5IA administration

To explore to what extent nACh receptors in the VPL were involved in the anti-allodynic effects arising from central

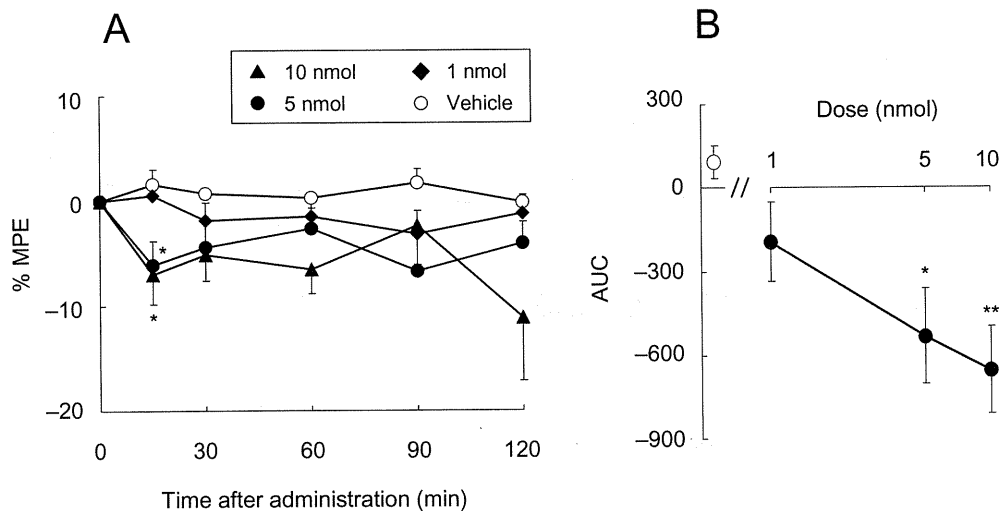


Figure 5 (A) Effect of mecamlamine injected intra-VPL on neuropathic tactile allodynia. Data are presented as a percentage of the maximum possible effect (% MPE). Each point represents the mean \pm SEM of the ligated paws of five to six animals per group. * $P < 0.05$ versus vehicle at the same time point. (B) Dose-response curve of mecamlamine-induced hyperalgesia. The open symbol represents the AUC of the vehicle-treated group, and the closed symbols represent that of each concentration of the mecamlamine-treated group. * $P < 0.05$, ** $P < 0.01$ versus vehicle. AUC, area under the curves; MPE, maximal possible effect; VPL, ventral posterolateral thalamic nucleus.

nACh receptor activation, we evaluated the effect of intra-VPL administered mecamlamine on the anti-allodynic effects produced by i.c.v. administered 5IA. The results from the ligated paws are illustrated in Figure 4C. Two-way ANOVA demonstrated significant main effects of treatment ($F_{3,105} = 7.91$, $P = 0.001$) and time ($F_{4,105} = 5.65$, $P < 0.001$) and a significant interaction between treatment and time ($F_{12,105} = 2.49$, $P = 0.008$). The rats administered vehicle intra-VPL followed by 10 nmol of 5IA i.c.v. (Vehicle/5IA group) demonstrated significantly elevated % MPE values at 15 and 30 min after administration. This result was consistent with the anti-allodynic effect produced by i.c.v. administered 5IA (Figure 1). Pretreatment with intra-VPL mecamlamine (10 nmol) significantly inhibited the effect of i.c.v. administered 5IA (10 nmol). The % MPE values for rats treated with mecamlamine alone (MEC/Vehicle group) also tended to decrease compared with those of the vehicle treated rats, although the difference was not significant.

Hyperalgesic effect of intra-VPL administered mecamlamine

To examine the effects of mecamlamine on tactile allodynia in more detail, we injected multiple doses of mecamlamine intra-VPL and performed the von Frey filament test. Data from the ligated paws are shown in Figure 5A. There was a significant main effect of treatment ($F_{3,95} = 6.77$, $P = 0.003$) but no effect of time nor interaction between time and treatment. The *post hoc* Tukey test demonstrated a significant difference between vehicle-treated group and 5 nmol- or 10 nmol-treated groups. The calculated area under the curves of each group clearly demonstrated the dose-dependent hyperalgesic effects of mecamlamine (Figure 5B).

Effects of 5IA and mecamlamine on motor function

We conducted an inclined plane test to evaluate the effects of 5IA and mecamlamine on general motor functions. The

Table 1 Effects of 5IA on motor functions

Drug	Time after administration				
	15 min	30 min	60 min	90 min	120 min
i.c.v.					
Vehicle	98 \pm 1	102 \pm 4	98 \pm 2	92 \pm 3	94 \pm 3
5IA	93 \pm 2	97 \pm 3	88 \pm 4	96 \pm 3	93 \pm 1
Intra-VPL					
Vehicle	99 \pm 3	99 \pm 2	97 \pm 4	100 \pm 2	97 \pm 3
5IA	100 \pm 4	97 \pm 2	99 \pm 2	97 \pm 1	102 \pm 2

Data are expressed as a percentage of the control level ('angle of sliding'), which was determined before drug administration in each animal, and represent the mean \pm SEM of five to six animals per group. No significant difference was observed between the two groups (two-way ANOVA with repeated measures).

5IA, 5-iodo-3-(2(S)-azetidylmethoxy)pyridine; VPL, ventral posterolateral thalamic nucleus.

maximum angle that the rats were able to endure before slipping down the plane was not significantly different between the vehicle and drug-treated groups at any time point (Table 1, data not shown for mecamlamine).

Discussion and Conclusions

In the present study, we examined the changes in central nACh receptor density in a rat model of neuropathic pain and the involvement of nACh receptors in anti-nociceptive effects in the region where changes occurred. First, we evaluated the anti-allodynic effect of 5IA after i.c.v. administration and found that the anti-allodynic effect was dose-dependent. Because the effect was completely antagonized by mecamlamine, the anti-allodynic effect of 5IA must occur via nACh receptors. No significant difference was observed between the

vehicle and 5IA treated groups in the inclined plane test suggesting that 5IA exhibited anti-allodynic effects without affecting motor functions.

We found that bilateral up-regulation of thalamic nACh receptors occurred in the PSL model of neuropathic pain. This is the first report of up-regulation of thalamic nACh receptors under chronic painful conditions. Because the up-regulation of both muscarinic and cannabinoid CB₁ receptors have been reported to contribute to the increased analgesic efficacy of each agonist (Siegling *et al.*, 2001; Chen and Pan, 2003), up-regulated nACh receptors may also contribute to the potentiation of anti-allodynic effects produced by nACh receptor agonists and attenuate neuropathic pain. Consistent with Seltzer's report (Seltzer *et al.*, 1990), the unilateral PSL caused bilateral tactile allodynia in the present study; and thus, the up-regulation must occur bilaterally. In the present study, we performed an autoradiographic saturation assay to determine the regional B_{max} of nACh receptors. As no report concerning autoradiographic saturation assays using [¹²⁵I]5IA has been published, it is important to validate the method used. Doura *et al.* (2008) performed quantitative autoradiography using [¹²⁵I]5IA, but, in their study, they incubated brain slices with only a single concentration of [¹²⁵I]5IA and did not perform a saturation assay. Thus, a direct comparison between our present data and theirs is not possible. Nevertheless, the ratios of the thalamus to the striatum or the cortex of our present data were consistent in the data reported by Doura *et al.* (2008), suggesting that our method was valid.

A previous PET study showed that the increase in cerebral blood flow in the VPL positively correlated with pain intensity, suggesting involvement of the VPL in pain transmission (Derbyshire *et al.*, 1997). Furthermore, electrical stimulation of the VPL has produced pain alleviation in both rat models and patients with neuropathic pain (Kupers and Gybels, 1993; Gybels, 2001). These findings suggest that the VPL is involved in the expression of anti-nociceptive effects. Indeed, we demonstrated that 5IA administered locally into the VPL significantly and dose-dependently reversed tactile allodynia. This effect was antagonized by coadministered mecamlamine. As no significant difference was observed between the vehicle and 5IA treated groups in the results of an inclined plane test, the changes in % MPE values observed in the 5IA treated groups were considered to reflect the analgesic effect, not motor dysfunction. These findings suggest that the nACh receptors expressed in the VPL were involved in the anti-allodynic effect that occurred after nACh receptor agonist administration. This was consistent with the finding that blockade of nACh receptors in the VPL caused a decrease in the anti-allodynic effect of i.c.v.-administered 5IA. Moreover, the intra-VPL injection of mecamlamine alone induced significant decreases in % MPE values. Mecamlamine is not an inverse agonist, but a non-competitive antagonist (Jensen *et al.*, 2005). Thus, during neuropathic pain, an intrinsic anti-allodynic mechanism by which ACh activates nACh receptors expressed in the VPL may be present and antagonism of these receptors by mecamlamine causes the hyperalgesic effect. That is to say, the nACh receptors expressed in the VPL may participate in anti-allodynic effects produced not only by exogenous but also endogenous agonists.

The present findings do not negate the involvement of nACh receptors in anti-allodynic effects outside of the VPL. Previous pharmacological studies reported that the central sites involved in nACh receptor-mediated antinociception were the NRM and the PPTg. The antinociception produced by nicotinic stimulation of the PPTg or the NRM depended upon muscarinic cholinergic, 5-hydroxytryptaminergic and adrenergic systems at the level of the lumbar spinal cord (Iwamoto and Marion, 1993). Curzon *et al.* (1998) showed that microinjection of nACh receptor agonists (epibatidine and A-85380) into the NRM produced antinociception against heat stimuli, and these effects were prevented by coadministration of mecamlamine into the NRM. Indeed, we demonstrated that VPL blockade by mecamlamine before i.c.v. administration of 5IA decreased the anti-allodynic effect by up to approximately 70%, not 100%. Thus, the remainder of the anti-allodynic effect is possibly caused by the binding of 5IA to the NRM and/or the PPTg.

Recently, Mogg *et al.* (2004) have reported that 5IA can functionally activate the $\alpha 4\beta 2$ -nACh and $\alpha 6\beta 2$ -nACh receptors. However, Perry *et al.* have reported that the nACh receptors expressed in the thalamus are mainly of the $\alpha 4\beta 2$ sub-type (Perry *et al.*, 2002) and that the $\alpha 6$ subunit is present in less than 4% of the thalamic nACh receptors (Perry *et al.*, 2007). Therefore, the anti-allodynic effect we reported was probably mediated via the $\alpha 4\beta 2$ -nACh receptors, at least when 5IA was administered into the VPL. Whereas many results have shown that $\alpha 4$ subunit-containing nACh receptors play an important role in nACh receptor-mediated antinociception (Bitner *et al.*, 1998; Marubio *et al.*, 1999; Bitner *et al.*, 2000), the contribution of the $\alpha 6\beta 2$ -nACh receptors to the anti-nociceptive effect is unknown. As A-186253 has been developed as a specific antagonist of the $\alpha 4\beta 2$ -nACh receptors (Itier *et al.*, 2004), it may be possible to investigate $\alpha 6\beta 2$ -nACh receptor-mediated antinociception using both 5IA and A-186253.

In summary, we have demonstrated for the first time that up-regulation of thalamic nACh receptors occurs in a model of chronic pain. Moreover, we found that intra-VPL administration of 5IA attenuated tactile allodynia, dose-dependently. This effect was completely antagonized by co-administered mecamlamine. The blockade of nACh receptors in the VPL by mecamlamine caused a decrease in the anti-allodynic effect of i.c.v.-administered 5IA. These findings indicate that the nACh receptors expressed in the VPL are a potential site of the anti-nociceptive action produced by 5IA. Furthermore, mecamlamine, given intra-VPL, induced a hyperalgesic effect. This effect is likely to be responsible for the mecamlamine antagonism of the intrinsic anti-allodynic mechanism induced by endogenous acetylcholine. These findings suggest that the nACh receptors expressed in the VPL play an important role in the anti-allodynic effects produced by exogenous and endogenous agonists.

Acknowledgements

We thank Dr Takayuki Nakagawa (Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University) for his technical support and invaluable discussion regarding the behavioural tests. This work was

supported in part by a Grant-in-Aid for Scientific Research (A) (19209041) and a Grant-in-Aid for Young Scientists (B) (19790869) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Smoking Research Foundation.

Conflicts of interest

The authors state that they have no conflicts of interest.

References

- Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* 153 (Suppl. 2): S1–S209.
- Bannon AW, Decker MW, Curzon P, Buckley MJ, Kim DJ, Radek RJ *et al.* (1998a). ABT-594 [(R)-5-(2-azetidylmethoxy)-2-chloropyridine]: a novel, orally effective antinociceptive agent acting via neuronal nicotinic acetylcholine receptors: II. In vivo characterization. *J Pharmacol Exp Ther* 285: 787–794.
- Bannon AW, Decker MW, Holladay MW, Curzon P, Donnelly-Roberts D, Puttfarcken PS *et al.* (1998b). Broad-spectrum, non-opioid analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors. *Science* 279: 77–81.
- Bannon AW, Decker MW, Kim DJ, Campbell JE, Arneric SP (1998c). ABT-594, a novel cholinergic channel modulator, is efficacious in nerve ligation and diabetic neuropathy models of neuropathic pain. *Brain Res* 801: 158–163.
- Bitner RS, Nikkel AL, Curzon P, Arneric SP, Bannon AW, Decker MW (1998). Role of the nucleus raphe magnus in antinociception produced by ABT-594: immediate early gene responses possibly linked to neuronal nicotinic acetylcholine receptors on serotonergic neurons. *J Neurosci* 18: 5426–5432.
- Bitner RS, Nikkel AL, Curzon P, Donnelly-Roberts DL, Puttfarcken PS, Namovic M *et al.* (2000). Reduced nicotinic receptor-mediated antinociception following in vivo antisense knock-down in rat. *Brain Res* 871: 66–74.
- Brasic JR, Zhou Y, Musachio JL, Hilton J, Fan H, Crabb A *et al.* (2009). Single photon emission computed tomography experience with (S)-5-[(123)I]iodo-3-(2-azetidylmethoxy)pyridine in the living human brain of smokers and nonsmokers. *Synapse* 63: 339–358.
- Chen SR, Pan HL (2003). Up-regulation of spinal muscarinic receptors and increased antinociceptive effect of intrathecal muscarine in diabetic rats. *J Pharmacol Exp Ther* 307: 676–681.
- Curzon P, Nikkel AL, Bannon AW, Arneric SP, Decker MW (1998). Differences between the antinociceptive effects of the cholinergic channel activators A-85380 and (+/-)-epibatidine in rats. *J Pharmacol Exp Ther* 287: 847–853.
- Decker MW, Curzon P, Holladay MW, Nikkel AL, Bitner RS, Bannon AW *et al.* (1998). The role of neuronal nicotinic acetylcholine receptors in antinociception: effects of ABT-594. *J Physiol Paris* 92: 221–224.
- Decker MW, Rueter LE, Bitner RS (2004). Nicotinic acetylcholine receptor agonists: a potential new class of analgesics. *Curr Top Med Chem* 4: 369–384.
- Derbyshire SW, Jones AK, Gyulai F, Clark S, Townsend D, Firestone LL (1997). Pain processing during three levels of noxious stimulation produces differential patterns of central activity. *Pain* 73: 431–445.
- Donnelly-Roberts DL, Puttfarcken PS, Kuntzweiler TA, Briggs CA, Anderson DJ, Campbell JE *et al.* (1998). ABT-594 [(R)-5-(2-azetidylmethoxy)-2-chloropyridine]: a novel, orally effective analgesic acting via neuronal nicotinic acetylcholine receptors: I. In vitro characterization. *J Pharmacol Exp Ther* 285: 777–786.
- Doura MB, Gold AB, Keller AB, Perry DC (2008). Adult and periadolescent rats differ in expression of nicotinic cholinergic receptor subtypes and in the response of these subtypes to chronic nicotine exposure. *Brain Res* 1215: 40–52.
- Fukui M, Nakagawa T, Minami M, Satoh M (2001). Antinociceptive effects of intracerebroventricularly administered P2 purinoceptor agonists in the rat. *Eur J Pharmacol* 419: 25–31.
- Gybels J (2001). Thalamic stimulation in neuropathic pain: 27 years later. *Acta Neurol Belg* 101: 65–71.
- Holladay MW, Wasicak JT, Lin NH, He Y, Ryther KB, Bannon AW *et al.* (1998). Identification and initial structure-activity relationships of (R)-5-(2-azetidylmethoxy)-2-chloropyridine (ABT-594), a potent, orally active, non-opiate analgesic agent acting via neuronal nicotinic acetylcholine receptors. *J Med Chem* 41: 407–412.
- Itier V, Schonbachler R, Tribollet E, Honer M, Prinz K, Marguerat A *et al.* (2004). A-186253, a specific antagonist of the alpha 4 beta 2 nAChRs: its properties and potential to study brain nicotinic acetylcholine receptors. *Neuropharmacology* 47: 538–557.
- Iwamoto ET, Marion L (1993). Adrenergic, serotonergic and cholinergic components of nicotinic antinociception in rats. *J Pharmacol Exp Ther* 265: 777–789.
- Jain KK (2004). Modulators of nicotinic acetylcholine receptors as analgesics. *Curr Opin Investig Drugs* 5: 76–81.
- Jensen AA, Frolund B, Liljefors T, Krosgaard-Larsen P (2005). Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. *J Med Chem* 48: 4705–4745.
- Kanegawa N, Kiyono Y, Kimura H, Sugita T, Kajiyama S, Kawashima H *et al.* (2006). Synthesis and evaluation of radioiodinated (S,S)-2-(alpha-(2-iodophenoxy)benzyl)morpholine for imaging brain norepinephrine transporter. *Eur J Nucl Med Mol Imaging* 33: 639–647.
- Kupers RC, Gybels JM (1993). Electrical stimulation of the ventroposterolateral thalamic nucleus (VPL) reduces mechanical allodynia in a rat model of neuropathic pain. *Neurosci Lett* 150: 95–98.
- Mamede M, Ishizu K, Ueda M, Mukai T, Iida Y, Fukuyama H *et al.* (2004). Quantification of human nicotinic acetylcholine receptors with ¹²³I-SIA SPECT. *J Nucl Med* 45: 1458–1470.
- Mamede M, Ishizu K, Ueda M, Mukai T, Iida Y, Kawashima H *et al.* (2007). Temporal change in human nicotinic acetylcholine receptor after smoking cessation: SIA SPECT study. *J Nucl Med* 48: 1829–1835.
- Marcil J, Walczak JS, Guindon J, Ngoc AH, Lu S, Beaulieu P (2006). Antinociceptive effects of tetrodotoxin (TTX) in rodents. *Br J Anaesth* 96: 761–768.
- Marubio LM, del Mar Arroyo-Jimenez M, Cordero-Erausquin M, Lena C, Le Novere N, de Kerchove d'Exaerde A *et al.* (1999). Reduced antinociception in mice lacking neuronal nicotinic receptor subunits. *Nature* 398: 805–810.
- Mogg AJ, Jones FA, Pullar IA, Sharples CG, Wonnacott S (2004). Functional responses and subunit composition of presynaptic nicotinic receptor subtypes explored using the novel agonist 5-iodo-A-85380. *Neuropharmacology* 47: 848–859.
- Mukhin AG, Gundisch D, Horti AG, Koren AO, Tamagnan G, Kimes AS *et al.* (2000). 5-Iodo-A-85380, an alpha4beta2 subtype-selective ligand for nicotinic acetylcholine receptors. *Mol Pharmacol* 57: 642–669.
- Oishi N, Hashikawa K, Yoshida H, Ishizu K, Ueda M, Kawashima H *et al.* (2007). Quantification of nicotinic acetylcholine receptors in Parkinson's disease with (123)I-SIA SPECT. *J Neurol Sci* 256: 52–60.
- Okada M, Nakagawa T, Minami M, Satoh M (2002). Analgesic effects of intrathecal administration of P2Y nucleotide receptor agonists UTP and UDP in normal and neuropathic pain model rats. *J Pharmacol Exp Ther* 303: 66–73.
- Paxinos G, Watson C (2005). *The Rat Brain in Stereotaxic Coordinates*, 5th edn. Elsevier Academic Press: San Diego, CA.
- Perry DC, Xiao Y, Nguyen HN, Musachio JL, Davila-Garcia MI, Keller KJ (2002). Measuring nicotinic receptors with characteristics of alpha4beta2, alpha3beta2 and alpha3beta4 subtypes in rat tissues by autoradiography. *J Neurochem* 82: 468–481.

- Perry DC, Mao D, Gold AB, McIntosh JM, Pezzullo JC, Kellar KJ (2007). Chronic nicotine differentially regulates alpha6- and beta3-containing nicotinic cholinergic receptors in rat brain. *J Pharmacol Exp Ther* 322: 306–315.
- Saji H, Ogawa M, Ueda M, Iida Y, Magata Y, Tominaga A *et al.* (2002). Evaluation of radioiodinated 5-iodo-3-(2S)-azetidinylmethoxy pyridine as a ligand for SPECT investigations of brain nicotinic acetylcholine receptors. *Ann Nucl Med* 16: 189–200.
- Seltzer Z, Dubner R, Shir Y (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain* 43: 205–218.
- Siegling A, Hofmann HA, Denzer D, Mauler F, De Vry J (2001). Cannabinoid CB(1) receptor upregulation in a rat model of chronic neuropathic pain. *Eur J Pharmacol* 415: R5–R7.
- Sindrup SH, Jensen TS (1999). Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. *Pain* 83: 389–400.
- Suzdak PD, Foged C, Andersen KE (1994). Quantitative autoradiographic characterization of the binding of [³H]tiagabine (NNC 05-328) to the GABA uptake carrier. *Brain Res* 647: 231–241.
- Tanaka K, Fukuuchi Y, Gomi S, Takashima S, Mihara B, Shirai T *et al.* (1993). Reduction in second-messenger ligand binding sites after brain ischemia – autoradiographic Bmax and Kd determinations using digital image analysis. *Brain Res Bull* 32: 49–56.
- Ueda M, Iida Y, Kitamura Y, Kawashima H, Ogawa M, Magata Y *et al.* (2008). 5-Iodo-A-85380, a specific ligand for alpha4beta2 nicotinic acetylcholine receptors, prevents glutamate neurotoxicity in rat cortical cultured neurons. *Brain Res* 1199: 46–52.
- Ueda M, Iida Y, Mukai T, Mamede M, Ishizu K, Ogawa M *et al.* (2004). 5-[¹²⁵I]Iodo-A-85380: assessment of pharmacological safety, radiation dosimetry and SPECT imaging of brain nicotinic receptors in healthy human subjects. *Ann Nucl Med* 18: 337–344.
- Vaupel DB, Tella SR, Huso DL, Mukhin AG, Baum I, Wagner VO *et al.* (2003). Pharmacology, toxicology, and radiation dosimetry evaluation of [I-123]5-I-a-85380, a radioligand for in vivo imaging of cerebral neuronal nicotinic acetylcholine receptors in humans. *Drug Dev Res* 58: 149–168.
- Yasuda T, Miki S, Yoshinaga N, Senba E (2005). Effects of amitriptyline and gabapentin on bilateral hyperalgesia observed in an animal model of unilateral axotomy. *Pain* 115: 161–170.

Imaging of HIF-1-Active Tumor Hypoxia Using a Protein Effectively Delivered to and Specifically Stabilized in HIF-1-Active Tumor Cells

Takashi Kudo¹, Masashi Ueda^{1,2}, Yuji Kuge¹, Takahiro Mukai³, Shotaro Tanaka⁴, Maki Masutani¹, Yasushi Kiyono^{2,5}, Shinae Kizaka-Kondoh⁴, Masahiro Hiraoka⁴, and Hideo Saji¹

¹Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan;

²Radioisotopes Research Laboratory, Faculty of Medicine, Kyoto University Hospital, Kyoto University, Kyoto, Japan; ³Department of Biomolecular Recognition Chemistry, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan;

⁴Department of Radiation Oncology and Image-Applied Therapy, Kyoto University Graduate School of Medicine, Kyoto University, Kyoto, Japan; and ⁵Biomedical Imaging Research Center, University of Fukui, Fukui, Japan

Hypoxia-inducible factor-1 (HIF-1) plays an important role in malignant tumor progression and in the development of resistance to radiotherapy. We designed a novel fusion protein (PTD-ODD-SAV [POS]) consisting of a protein transduction domain (PTD), streptavidin (SAV), and a portion of the oxygen-dependent degradation domain (ODD) of HIF-1 α that confers the same oxygen-dependent regulation as HIF-1 α on POS. (3-¹²³I-iodobenzoyl)norbiotinamide (¹²³/¹²⁵I-IBB) was conjugated to the SAV moiety of POS to synthesize ¹²³/¹²⁵I-IBB-labeled POS (¹²³/¹²⁵I-IPOS). The purpose of this study was to evaluate the feasibility of ¹²³I-IPOS as an imaging probe for HIF-1-active tumor hypoxia. **Methods:** After a 24-h incubation of ¹²⁵I-IPOS with various tumor cell lines under either normoxic (20% O₂) or hypoxic (0.1% O₂) conditions, the intracellular radioactivity was investigated. Then, the biodistribution of ¹²³/¹²⁵I-IPOS was examined with tumor-implanted mice, and an in vivo imaging study was performed. The tumoral accumulation of ¹²⁵I-IPOS was compared with HIF-1 activity using the mice carrying tumors with the HIF-1-dependent luciferase reporter gene. Furthermore, the intratumoral localization of ¹²⁵I-IPOS was examined by the autoradiographic study, and then the same slide was subjected to immunostaining for pimonidazole, which is the hypoxic marker. **Results:** The ratios of radioactivity in hypoxic cells to that in normoxic cells were more than 2. These results indicate incorporation of ¹²⁵I-IPOS into these cells and degradation of ¹²⁵I-IPOS by normoxic tumor cells. In the biodistribution study, ¹²⁵I-IPOS accumulated in the tumor (1.4 \pm 0.3 percentage injected dose per gram) 24 h after administration. At that time, ¹²⁵I-IPOS showed high tumor-to-blood and tumor-to-muscle ratios (5.1 \pm 0.3 and 14.0 \pm 3.9, respectively). The tumors were clearly visualized by in vivo imaging 24 h after ¹²³I-IPOS injection (tumor-to-muscle ratio was 9.6). The tumoral accumulation of ¹²⁵I-IPOS correlated with HIF-1 activity ($R = 0.71$, $P < 0.05$),

and its intratumoral distribution coincided with the hypoxic regions. **Conclusion:** ¹²³I-IPOS is a potential probe for the imaging of HIF-1 activity in tumors. Given the role of HIF-1 in tumor biology, its detection may be considered an indicator of aggressive cancer phenotypes.

Key Words: molecular imaging; oncology; radiopharmaceuticals; hypoxia-inducible factor-1 (HIF-1); tumor hypoxia; oxygen-dependent degradation (ODD); protein transduction domain (PTD)

J Nucl Med 2009; 50:942–949

DOI: 10.2967/jnumed.108.061119

In solid tumors, hypoxia, or an oxygen tension below physiologic levels, develops as abnormal proliferation outstrips the blood supply (1). This hypoxic region is involved in tumor malignancy and proliferation and results in the development of resistance to radiotherapy (2–4). Hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates the cellular response to hypoxia, induces several genes that mediate these changes in tumor behavior (5,6). Indeed, HIF-1-active or hypoxic cells have recently been shown to play crucial roles in angiogenesis and radioresistance (7). Finally, the expression level of HIF-1 correlates with a poor prognosis in many tumors (8,9). Thus, imaging HIF-1-active regions in a tumor has the potential to provide, in a noninvasive manner, insight into those aspects of tumor biology most relevant to treatment design.

HIF-1 is a heterodimer that consists of the oxygen-sensitive α -subunit (HIF-1 α) and the constitutively expressed β -subunit (HIF-1 β). Under normoxic conditions, HIF-1 α is hydroxylated by prolyl hydroxylases on the proline residues in the oxygen-dependent degradation domain (ODD). The hydroxylated proline residues accelerate ubiquitination of ODD

Received Dec. 14, 2008; revision accepted Feb. 16, 2009.

For correspondence or reprints contact: Hideo Saji, Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimo-Adachi-cho, Sakyo-ku, Kyoto 606-8501, Japan.

E-mail: hsaji@pharm.kyoto-u.ac.jp

COPYRIGHT © 2009 by the Society of Nuclear Medicine, Inc.