Original Article
Preclinical evaluation of $^{111}$In-DTPA-INCA-X anti-Ku70/Ku80 monoclonal antibody in prostate cancer

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Abstract: The aim of this investigation was to assess the Ku70/Ku80 complex as a potential target for antibody imaging of prostate cancer. We evaluated the $in$ vivo and $ex$ vivo tumor targeting and biodistribution of the $^{111}$In-labeled human internalizing antibody, INCA-X ($^{111}$In-DTPA-INCA-X antibody), in NMRI-nude mice bearing human PC-3, PC-3M-Lu2 or DU145 xenografts. DTPA-conjugated, non-labeled antibody was pre-administered at different time-points followed by a single intravenous injection of $^{111}$In-DTPA-INCA-X. At 48, 72 and 96 h post-injection, tissues were harvested, and the antibody distribution was determined by measuring radioactivity. Preclinical SPECT/CT imaging of mice with and without the predose was performed at 48 hours post-injection of labeled DTPA-INCA-X. Biodistribution of the labeled antibody showed enriched activity in tumor, spleen and liver. Animals pre-administered with DTPA-INCA-X showed increased tumor uptake and blood content of $^{111}$In-DTPA-INCA-X with reduced splenic and liver uptake. The $in$ vitro and $in$ vivo data presented show that the $^{111}$In-labeled INCA-X antibody is internalized into prostate cancer cells and by pre-administering non-labeled DTPA-INCA-X, we were able to significantly reduce the off target binding and increase the $^{111}$In-DTPA-INCA-X mAb uptake in PC-3, PC-3M-Lu2 and DU145 xenografts. The results are encouraging and identifying the Ku70/Ku80 antigen as a target is worth further investigation for functional imaging of prostate cancer.

Keywords: Ku80/Ku80, INCA-X monoclonal antibody, prostate cancer, tumor targeting, preclinical SPECT/CT

Introduction

Radiolabeled monoclonal antibodies directed to cell-surface antigens specifically expressed by cancer cells have shown a potential for cancer imaging and therapy. Unfortunately, this has not yet been successfully developed and implemented in several malignancies. Prostate cancer is a variable and multifocal disease with a variety of potential tumor-associated imaging biomarkers [1-3]. Yet, the only clinically approved radiolabeled antibody for prostate cancer imaging is ProstaScint®, a murine $^{111}$In-labeled anti-prostate specific membrane antigen (PSMA) antibody that does not bind other prostate-specific antigens like prostatic acid phosphatase (PAP) or prostate-specific antigen (PSA) [4]. A downfall of ProstaScint® is that the antibody binds to the internal domain of PSMA making image interpretation difficult. It is also hindered by high accumulation in necrotic areas and uptake in normal tissues like the gut, liver and kidneys [5]. This illustrates the obvious need for new validated prostate tumor biomarkers to create a basis for $in$ vivo imaging and therapies. Accordingly, there are many antibodies and antibody based conjugates in early stages of clinical development aimed at the selective delivery of therapeutic agents to prostate cancer (http://clinicaltrials.gov NLM Identifiers: NCT00031187, NCT00859781, NCT00-054574, NCT01414283, NCT01414296, NCT-01631552).
The INCA-X antibody used in this study was selected from a large human antibody library [6] and a pool of cancer cell lines using phage display technique and rapid internalization as a selection criteria [7]. INCA-X has been demonstrated to be specific for cell surface exposed epitopes associated with the Ku70/Ku80 complex. The Ku-antigens are part of a protein complex involving at least two proteins, Ku70 (XRCC6) and Ku80 (XRCC5) [8, 9], originally defined as a nuclear auto-antigen [8]. The first described function was its role in DNA double strand break (DSB) repair through non-homologous end-joining (NHEJ) [10, 11]. Interestingly, Ku-deficient mice and men with prostate cancer who undergo castration therapy are hypersensitive to ionizing radiation [12]. This is likely due to decreased levels of the Ku70 protein in prostate cancer cells after castration therapy [13]. In addition to the DNA repair mechanism, the Ku proteins take part in many different processes. Such cellular processes include: V(D)J recombination [14], telomere maintenance [15-17], transcription regulation [18, 19], integrin function [20], a possible receptor for DNA [21], an androgen receptor recycle co-activator [19] and a carrier of proteolytic enzymes [20]. At present, the function and molecular mechanisms behind the Ku complex is not fully understood, though it is known to have pro-survival and pro-invasive roles essential for tumor progression. The Ku70/Ku80 antigen is expressed in the nucleus of all cells. However, several studies have revealed that under certain conditions, and in various tumor cell lines, including: glioma cells, neuroblastoma cells, breast and prostate cancer cell lines, the Ku70/Ku80 antigen relocates to the plasma membrane where it is thought to play a role in invasion, migration and cell adhesion [7, 20, 22-25]. The essential multifunction of the protein complex and its surface expression makes it a suitable target for imaging prostate cancer cells. Moreover, the available surface expression of Ku70/Ku80 complex on tumor cells could be harnessed as a possible marker of patient radiosensitivity after castration therapy and before curative radiotherapy begins. In addition to that, the Ku70/Ku80 tumor-associated antigen could provide a receptor-mediated gateway for the potential to deliver antibody-drug conjugates or radionuclides directly to the prostate cancer cells, potentially reducing the systemic toxicity associated with conventional treatments [7, 26]. INCA-X has been shown to rapidly internalize via endocytosis into a variety of tumor cell lines in vitro and also showed a strong immunotoxic effect on human prostate cancer PC-3 cells (92% inhibition) when conjugated to Saporin [7, 23]. Considering the previous in vitro findings, we went further to investigate the in vivo tumor-targeting potential and whole-body biodistribution of radiolabeled INCA-X in nude mice bearing subcutaneous xenografts of human prostate cancer cell lines. For this investigation we used a preclinical small animal dual modality single-photon emission computer tomography/computer tomography (SPECT/CT) imaging system alongside traditional ex vivo biodistribution studies.

Materials and methods

Cell lines and cell culture

The androgen independent human prostate cancer cell lines PC-3 (derived from a human bone metastasis), PC-3M-Lu2 (a metastatic clone of PC-3 cells stably transfected with firefly luciferase gene (luc2)) and DU145 (derived from a human brain metastasis) were chosen for this study since they all express the Ku70 and Ku80 antigens [27]. The PC-3 and DU145 cell lines were purchased from ATCC (Manassas, VA) and cultured as a monolayer in HAM's F12 (PC-3) or RPMI (DU145) medium and supplemented with 10% FBS and 1% penicillin-streptomycin (PEST). PC-3M-Lu2 cells (Caliper, Hopkinton, MA) were cultured as a monolayer in EMEM medium, supplemented with 10% heat inactivated FBS and 1% PEST. All cells were kept at 37°C in an atmosphere of 5% CO₂. Cells were regularly tested for mycoplasma and found free of mycoplasma prior to inoculations.

Prostate tumor model

Male athymic NMRI-nude mice (Taconic Europe, Denmark) aged between six and eight weeks old and with a mean body weight of 37 g (range: 31.5-42 g) were used for this study. The animals were provided autoclaved food and water ad libitum and housed in individually ventilated cages under sterile conditions; maximum of five mice per cage. The tumor model was obtained by subcutaneous injection of PC-3,
PC-3M-Lu2 or DU145 cells into the lower right flank (3-5 × 10^6 cells in 200 µL medium).

Conjugation and ¹¹¹In-labeling

Conjugation with chelator: The INCA-X human monoclonal IgG\sub 1 lambda antibody [6, 28] was supplied by BioInvent (Lund, Sweden). The protein solution (5 mg/mL in PBS) was conjugated with the chelator CHX-A^–DTPA (Macrocyclics, USA) in 0.07 M sodium borate buffer, pH 9.2, using a molar ratio of 3:1 chelator to antibody at 40°C. The reaction was terminated after four hours and CHX-A^–DTPA-INCA-X, from now referred to as DTPA-INCA-X, was separated from free chelate by size-exclusion chromatography on a NAP-5 column (GE Healthcare) equilibrated with 20 mL 0.2 M ammonium acetate buffer, pH 5.5. Conjugated INCA-X was eluted with 1 mL ammonium acetate buffer and aliquoted samples were stored at -20°C.

Radiolabeling of DTPA-INCA-X

DTPA-INCA-X in ammonium acetate buffer (50 µL, ~1 mg/mL), pH 5.5, was mixed with a predetermined amount of ¹¹¹InCl\sub 3 (~50 MBq, Mallindkrodt, The Netherlands). After incubation at room temperature for one hour, the labeling was terminated and equilibrated with PBS. Labeling efficiency was monitored with ITLC strips (Biodex, USA) and eluted with 0.2 M citric acid. In this system, the radiolabeled conjugate remains at the origin line, while free indium migrates with the front of the solvent. The radioactivity distribution was determined with a PhosphorImager system (Perkin Elmer, Welle- sley, MA, USA) using the Optiquant as quantification software (Perkin Elmer).

The labeling yield of ¹¹¹In-DTPA-INCA-X was greater than 95% as determined by thin layer chromatography and required no further purification.

Flow cytometry

The antibody binding, Ku70 and Ku80 cell surface expression were analyzed by flow cytometry (FACS). PC-3 cells were incubated for one hour with individual antibodies at a concentration of 10 µg/mL in PBS containing 0.5% w/v bovine serum albumin. Primary antibodies were INCA-X (human IgG\sub 1 lambda), anti-Ku70 (mouse IgG, clone N3H10, Abcam, Cambridge, UK), and anti-Ku80 (mouse IgG, clone 111, Abcam). Bound antibody was detected with anti-human Ig APC or anti mouse Ig APC (Jackson Immuno Research, USA). Dead cells were excluded by SYTOX Green Nucleic Acid Stain (Molecular Probes). All incubations were performed on ice. Cells were analyzed with a FACSCalibur (BD Bioscience, California, USA).

Binding specificity of ¹¹¹In-DTPA-INCA-X

PC-3 cells were cultivated in a Petri dish with a diameter of 3.5 cm to a cell density of 2-5 × 10^5 cells per dish. Labeled conjugates (4 µg/dish) were added to two groups of Petri dishes containing PC-3 cells. A 100-fold excess of non-radiolabeled (cold) INCA-X was pre-administered to one group of dishes 30 minutes before the labeled conjugate was added. The cells were incubated for two hours at 37°C and incubation media was collected. The cells were washed three times with cold serum-free medium and treated with 0.5 mL complete medium was added to every dish and the cells were re-suspended. The cell suspension was collected for radioactivity measurements. Cell-associated radioactivity (C) was measured with an automated gamma counter (1480 Wizard OY; Wallac) in parallel with 1 mL corresponding incubation medium (M). The percentage (%) of added radioactivity bound to cells was calculated as = C × 100%/(C+M).

Internalization and retention of ¹¹¹In-DTPA-INCA-X

The internalization rate of the labeled conjugate was determined on PC-3 cells. Shortly, PC-3 cells were seeded in 6 well plates at 5 × 10^5 cells/well one day prior to the experiment. The cells were incubated for three hours at 4°C on ice with 4 µg/dish of ¹¹¹In-labeled INCA-X. Thereafter, the incubation medium was discarded and the cells were washed three times using ice-cold serum-free medium. After the addition of 1 mL medium, the cells were further incubated at 37°C. At designated time points, up to three days, one group of three dishes was analyzed for cell-associated radioactivity. Medium was collected, cells were washed three times with ice-cold serum-free medium (these two steps were omitted for the time-point 0 hour) and treated with 0.5 mL of 4 M urea solu-
tion containing 0.2 M glycine buffer, pH 2.5, for five minutes on ice. The acid fraction was collected and the cells were washed with an additional 0.5 mL acid solution. The radioactivity in the acid wash fraction was considered membrane-bound radioactivity. After the addition of 0.5 mL of 1 M NaOH, the cells were incubated at 37°C for 30 minutes and the basic solution was collected. The cell dishes were washed with an additional 0.5 mL of basic solution. The radioactivity in the alkaline fractions was considered internalized radioactivity. The radioactivity content of the samples was measured as mentioned above.

Whole-body in vivo imaging studies

SPECT imaging was carried out on a preclinical dual-modality SPECT/CT system (Nano SPECT/CT plus, Bioscan Inc., Washington, DC) equipped with four gamma cameras and multi pinhole apertures (NSP-106 mouse collimator) with each pinhole measuring 1 mm in diameter. Two NMRI-nude mice bearing PC-3M-Lu2 subcutaneous tumors were designated as either predose group 1 or non-predose group. Five NMRI-nude mice bearing DU145 subcutaneous tumors were designated as: predose group 1, predose group 2, predose group 3, or the non-predose group. Under isofluorane anesthesia, PC-3M-Lu2 and DU145 tumor-bearing mice in predose group 1 were pre-administered intravenously approximately 330 µg of DTPA-conjugated, non-labeled INCA-X 24 hours before the second tail vein injection of $^{111}$In-DTPA-INCA-X (10-15 MBq [270-405 µCi], 20 µg INCA-X). DU145 tumor-bearing mice in group 2 and group 3 were pre-administered non-labeled DTPA-INCA-X (330 µg) at 48 hours before (group 2) or immediately before (group 3) the second tail vein injection of $^{111}$In-DTPA-INCA-X (same amount as group 1). The non-predose group was given a single tail vein injection of $^{111}$In-DTPA-INCA-X (10-15 MBq [270-405 µCi], 20 µg INCA-X). All mice imaged via preclinical SPECT/CT were sacrificed by isofluorane overdose 48 hours post-injection. The PC-3 tumor-bearing mice were sacrificed at 48 hours (including all predosed mice), 72 hours and 96 hours post-injection. Selected organs, blood and tumor were collected and placed in pre-weighed plastic vials. The labeled antibody distribution was determined by measuring radioactivity using an automated gamma counter along with known standards. The tissue and tumor uptake was decay-corrected and calculated as the mean percentage of injected activity per gram of tissue (%IA/g), tumor-to-organ (T/O) and organ-to-blood (O/Bl) ratios and as the standard deviation (SD) of the mean values.

Ex vivo biodistribution studies

Biodistribution studies were performed with and without a pre-administered dose of cold DTPA-conjugated antibody in male NMRI-nude mice bearing human DU145 ($n = 7$) or PC-3 ($n = 15$) subcutaneous xenografts. The mice were divided into groups of three to four mice per group and the studies began when the tumors reached a volume larger than 50 mm$^3$. Under isofluorane anesthesia, mice grouped to receive a pre-administered dose of cold antibody were given an intravenous injection of DTPA-INCA-X (1.1.6 mg) 24 hours prior to the tail vein injection of $^{111}$In-DTPA-INCA-X (0.3-0.4 MBq [8-11 µCi], 15 µg of INCA-X). The other group of mice received a single tail vein injection of $^{111}$In-DTPA-INCA-X (0.3-0.4 MBq [8-11 µCi], 15 µg of INCA-X). The predosed and non-predosed DU145 tumor-bearing mice were sacrificed at 48 hours post-injection. The PC-3 tumor-bearing mice were sacrificed at 48 hours (including all predosed mice), 72 hours and 96 hours post-injection. Selected organs, blood and tumor were collected and placed in pre-weighed plastic vials. The labeled antibody distribution was determined by measuring radioactivity using an automated gamma counter along with known standards. The tissue and tumor uptake was decay-corrected and calculated as the mean percentage of injected activity per gram of tissue (%IA/g), tumor-to-organ (T/O) and organ-to-blood (O/Bl) ratios and as the standard deviation (SD) of the mean values.

Ethic statement

All animal studies were approved and performed in strict accordance with the guidelines set by the Malmö-Lund Ethical Committee, Lund University for the use and care of laboratory animals (Permit Number: M40-11). All efforts were made to strictly minimize animal suffering.

Results

$^{111}$In-DTPA-INCA-X anti-Ku70/Ku80 McAb in prostate cancer

INCA-X has been shown to bind to and internalize into PC-3 human prostate cancer cells in

$^{111}$In-DTPA-INCA-X anti-Ku70/Ku80 McAb in prostate cancer
In-DTPA-INCA-X anti-Ku70/Ku80 McAb in prostate cancer

We confirmed the binding results by FACS, illustrating INCA-X binding to the surface of PC-3 cells (Figure 1A). The anti-Ku70 binding on the surface of PC-3 cells (Figure 1C) was weak compared to that of the anti-Ku80 binding (Figure 1B) when assayed via FACS.

To investigate the in vitro targeting capabilities of radiolabeled INCA-X, blocking assays were performed on the PC-3 prostate cancer cell line. From these tests, we show that the binding of $^{111}$In-DTPA-INCA-X to PC-3 cells was specific since the uptake of $^{111}$In-DTPA-INCA-X was significantly blocked by the excess of cold INCA-X ($P<0.0001$) (Figure 2A). The cellular processing of $^{111}$In-DTPA-INCA-X is illustrated in Figure 2B. Table 1. Biodistribution (%IA/g) of $^{111}$In-DTPA-INCA-X in nude mice bearing PC-3 tumors

<table>
<thead>
<tr>
<th></th>
<th>$^{111}$In-DTPA-INCA-X</th>
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<tbody>
<tr>
<td></td>
<td>48 h (predose, n = 3)</td>
</tr>
<tr>
<td>Tumor</td>
<td>13.09 ± 1.04</td>
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<tr>
<td>Blood</td>
<td>21.38 ± 1.33</td>
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<tr>
<td>Spleen</td>
<td>12.06 ± 1.55</td>
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<tr>
<td>Lungs</td>
<td>12.98 ± 5.86</td>
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<tr>
<td>Kidneys</td>
<td>10.39 ± 0.65</td>
</tr>
<tr>
<td>Liver</td>
<td>8.42 ± 0.29</td>
</tr>
<tr>
<td>Muscle</td>
<td>3.26 ± 1.25</td>
</tr>
</tbody>
</table>

Figure 1. Flow cytometry of PC-3 cells. (A) INCA-X. (B) Anti-Ku80 and (C) anti-Ku70. Dotted lines represent non-binding isotype control antibody and solid lines represent the primary antibody.

Figure 2. In vivo binding specificity and internalization of $^{111}$In-DTPA-INCA-X on PC-3 cells. (A) Blocking assay showing the binding specificity of $^{111}$In-DTPA-INCA-X to PC-3 cells. (B) Cellular retention and internalization of $^{111}$In-DTPA-INCA-X on PC-3 cells. Error bars represent mean ± standard error. ****: $P<0.0001$.
111In-DTPA-INCA-X anti-Ku70/Ku80 McAb in prostate cancer

Figure 2B. The internalization rate of 111In-DTPA-INCA-X in PC-3 cells increased over time and had internalized by approximately 40% by 72 hours after interrupted incubation. The retention of the conjugate displayed the same pattern and seemed to continue to increase after 72 hours when the assay was stopped.

Biodistribution and preclinical SPECT/CT imaging of 111In-DTPA-INCA-X in PC-3 and PC-3M-Lu2 tumor-bearing mice

The 111In-DTPA-INCA-X antibody biodistribution in the major organs, blood and tumors of mice bearing subcutaneous PC-3 tumors was ascertained from the %IA/g values (Table 1 and Figure 3A). The biodistribution in non-predosed mice revealed the majority of activity in the spleen (consistent with SPECT images of PC-3M-Lu2 xenografted mice; Figure 4), followed by the liver, tumor and kidneys. Low to moderate uptake in other organs was also noted. Antibody localization to the tumor nearly doubled from approximately 8 %IA/g (SD 3) at 48 hours to 15 %IA/g (SD 9) at 96 hours post-injection of 111In-DTPA-INCA-X (Table 1). The mean uptake values presented in Table 1 also show localization to the spleen, which increased over time from the first measurement of 101 %IA/g (SD 18) at 48 hours to 174 %IA/g (SD 23) at 96 hours post-injection. There was a marked decrease of activity from all other tissues and blood with time, showing the antibody is not retained in non-specific organs (Table 1). Notably, the organ-to-blood ratios in Table 2 increased over time in the major organs and tumor, indicating there was an active uptake. But, as illustrated in Table 3, the tumor-to-organ ratios also increased over time in all organs, except in the spleen, thus confirming tumor specificity. Despite the high uptake in the spleen of non-predosed mice, 111In-DTPA-
INCA-X accumulated well in tumors, with a tumor-to-blood ratio around 30 by 96 hours post-injection (Table 3).

Interestingly, mice pre-administered with cold DTPA-INCA-X displayed significantly higher accumulation of activity in the tumors (P = 0.03) increasing from 8 %IA/g (SD 3) to 13 %IA/g (SD 1) (Table 1 and Figure 5A), and significantly reduced activity in the spleen (P = 0.002) from 101 %IA/g (SD 18) to 12 %IA/g (SD 2) (Table 1 and Figure 5B) at 48 hours post-injection of $^{111}$In-DTPA-INCA-X. The tumor-to-organ uptake ratios of mice pre-administered cold DTPA-INCA-X were improved in comparison with the mice that only received $^{111}$In-DTPA-INCA-X (Table 3), yet the blood concentration increased (Table 1 and Figure 3B). The SPECT/CT images of mice with and without a predose of DTPA-INCA-X (Figure 4) effectively mirror the ex vivo biodistribution results (Table 1). The image of the non-predosed mouse shown in Figure 4 reveals the majority of activity is in the spleen (white arrow) with fairly homogenous, low activity in the major blood rich organs and slightly higher uptake in the red bone marrow (red arrows). Conversely, the SPECT/CT images also show that by pre-administering a dose of cold DTPA-INCA-X 24 hours prior to the labeled antibody, we could significantly boost the accumulation of $^{111}$In-DTPA-INCA-X in the tumors (tumor circled in yellow).

Our in vivo and ex vivo tests confirmed that by pre-administering non-labeled, DTPA-conjugated INCA-X before intravenous injection of the labeled antibody, we were able to significantly reduce the off-target binding of the labeled antibody and increase the tumor targeting capability of $^{111}$In-DTPA-INCA-X to PC-3 and PC-3M-Lu2 human xenografts in nude mice.

**Biodistribution and preclinical SPECT/CT imaging of $^{111}$In-DTPA-INCA-X in DU145 tumor-bearing mice**

The biodistribution results of $^{111}$In-DTPA-INCA-X, as determined from %IA/g in various organs

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**Table 2. Organ-to-blood ratios in nude mice bearing PC-3 tumors**

<table>
<thead>
<tr>
<th></th>
<th>48 h (predose, n = 3)</th>
<th>48 h (n = 4)</th>
<th>72 h (n = 4)</th>
<th>96 h (n = 4)</th>
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<tbody>
<tr>
<td>Tumor</td>
<td>0.61</td>
<td>1.61</td>
<td>6.07</td>
<td>30.22</td>
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<tr>
<td>Spleen</td>
<td>0.56</td>
<td>21.67</td>
<td>64.82</td>
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<tr>
<td>Lungs</td>
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<td>1.89</td>
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<td>Kidneys</td>
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<tr>
<td>Liver</td>
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<td>3.94</td>
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<tr>
<td>Prostate</td>
<td>0.61</td>
<td>1.78</td>
<td>2.73</td>
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<tr>
<td>Muscle</td>
<td>0.15</td>
<td>0.16</td>
<td>0.27</td>
<td>1.35</td>
</tr>
<tr>
<td>Brain</td>
<td>0.03</td>
<td>0.35</td>
<td>0.74</td>
<td>1.22</td>
</tr>
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</table>

**Table 3. Tumor-to-organ ratios in nude mice bearing PC-3 tumors**

<table>
<thead>
<tr>
<th></th>
<th>48 h (predose, n = 3)</th>
<th>48 h (n = 4)</th>
<th>72 h (n = 4)</th>
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<td>Spleen</td>
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<tr>
<td>Lungs</td>
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<td>0.96</td>
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<td>Kidneys</td>
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<td>Liver</td>
<td>1.55</td>
<td>0.41</td>
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<td>Prostate</td>
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<td>Muscle</td>
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<tr>
<td>Brain</td>
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<td>8.15</td>
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<td>Blood</td>
<td>0.61</td>
<td>1.61</td>
<td>6.07</td>
<td>30.22</td>
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**Figure 4.** SPECT/CT images of nude mice bearing PC-3M-Lu2 xenografts. Posterior projections of mice injected with (A) $^{111}$In-DTPA-INCA-X (10-15 MBq (270-405 µCi), 20 µg INCA-X) and (B) pre-administered intravenously cold DTPA-INCA-X (approx. 330 µg) followed one day later by $^{111}$In-DTPA-INCA-X (same amount as in (A)). The mice were imaged 48 hours post-injection of $^{111}$In-DTPA-INCA-X. Tumors are circled in yellow, white arrow points to spleen and red arrows indicate activity in red bone marrow.
and tumor of nude mice bearing DU145 tumors (Figure 3C), show strong similarities to the results of nude mice bearing PC-3 tumors (Figure 3B). Similar to our findings in non-predosed mice bearing PC-3 tumors, the majority of activity in mice bearing human DU145 xenografts was also concentrated to the spleen at 48 hours post-injection of $^{111}$In-DTPA-INCA-X (16 %IA/g (SD 6)). Low to marginal uptake values were noted in the kidney (4 %IA/g (SD 1)), liver (4 %IA/g (SD 1)), lungs (3.47 %IA/g (SD 0.41)) and blood pool (1.87 %IA/g (SD 0.44)) (Figure 3C). Moreover, by intravenously pre-administering cold DTPA-INCA-X 24 hours prior to $^{111}$In-DTPA-INCA-X, we were able to block the off target uptake and 'redirect' the antibody to the tumors (Figures 3C and 5).

The SPECT/CT images of DU145 tumor-bearing mice were consistent with the ex vivo biodistribution data in regards to predose group 1 versus non-predosed animals. Namely, the majority of activity was concentrated to the spleen of non-predosed animals (Figure 6A, white arrow) 48 hours post-injection of the labeled antibody. The image of predose group 1 animal, which was pre-administered cold DTPA-INCA-X 24 hours prior to administering $^{111}$In-DTPA-INCA-X, showed increased localization to the tumor (Figure 6C, circled in yellow); however, there was background activity from circulating antibody in highly vascularized organs and blood. For that reason, we conducted an additional study to determine whether administering the predose of cold antibody at different time-points would affect the outcome. Therefore, mice bearing DU145 xenografts were pre-administered cold DTPA-INCA-X 48 hours (predose group 2; Figure 6D) or directly before (predose group 3; Figure 6B) the radiolabeled antibody was intravenously administered. The animals were imaged with the SPECT/CT camera 48 hours after $^{111}$In-DTPA-INCA-X administration. The images with the highest tumor uptake were collected from mice pre-administered DTPA-INCA-X 48 hours prior to the labeled INCA-X (predose group 2; Figure 6D). Mice predosed directly before $^{111}$In-DTPA-INCA-X was administered (predose group 3; Figure 6B), had less circulating activity than observed in predose group 1 animals (Figure 6C), but still more than observed in the predose group 2 animals (Figure 6D). Yet, regardless of when the predose was administered, the spleen of all animals pre-administered cold DTPA-INCA-X was effectively blocked and there was an obvious increased accumulation of $^{111}$In-DTPA-INCA-X in all of the tumors (Figures 5 and 6, tumors circled in yellow).

Discussion

INCA-X is a human antibody originally selected from a large naïve antibody library, using selective tumor up regulated antigens and internalization as selection and screening criteria. In this paper we confirmed INCA-X binding to the surface of viable PC-3 cells (Figure 1). The pres-
ence of the proteins was documented through specific anti-peptide antibodies, anti-Ku70 (amino acids 506-541) and anti-Ku80 (amino acids 610-705). The Ku70 and Ku80 proteins are known to take part in a plethora of essential functions in the eukaryotic cell, with their chaperon function in the Ku-dependent NHEJ repair pathway of DSB as the most documented feature [19, 29]. One hallmark of tumors is genomic instability often manifested as DSB. DNA double strand breaks are induced by several different endogenous and exogenous factors and thus mammalian cells have developed a number of repair systems to meet the challenge of DNA damage, of which the Ku70/Ku80 associated NHEJ pathway is very important. Ku70/Ku80 proteins are generally associated with the nucleus, yet are frequently found in cytoplasm and on the cell surface. In non-malignant cells, Ku70 is also reported to have a function at the cell surface and acts as a receptor for *Rickettsia conori* uptake into non-phagocytic mammalian cells [30, 31] or as a receptor for DNA [13].

The aim of this study was to make a first evaluation of the Ku70/Ku80 in vivo availability, up regulation and essential function as a target candidate for antibody imaging. Using $^{111}$In labeled, DTPA-conjugated INCA-X antibody, we confirmed the binding and internalization of the antibody on PC-3 cells (Figure 2), previously reported with $^{125}$I labeled antibody on PL45 pancreatic cell line [7]. When injected into animals with xenografts of human prostate cancer cell lines known to express the target antigen in vivo, a significant tumor uptake could be seen with SPECT/CT imaging only after pre-administering non-labeled antibody (Figures 4 and 6B-D). This finding differed from our in vitro
blocking tests on the PC-3 cell line where the 
$^{111}$In-DTPA-INCA-X uptake on PC-3 cells was sig-
nificantly blocked with a predose of cold INCA-X
(Figure 2A). Specific accumulation of $^{111}$In-DTPA-INCA-X in the human prostate cancer-based
 xenografts was observed after the pre-adminis-
tration of cold DTPA-INCA-X, whereas uptake in
other organs was negligible and eliminated with
time, except for the spleen. In animals pre-
administered DTPA-INCA-X, there was a shift of
activity from the spleen to the blood pool and
tumor (Figure 3B and 3C), indicating a block of
a dominating off target effect. The shift of activity
indicates that $^{111}$In-DTPA-INCA-X antibody is
capable of targeting human prostate cancer-
based xenografts in mice that first receive a
predose of cold antibody. Some antigen in the
spleen appears to be rapidly targeted but also
easily blocked with a suitable pre-administered
dose of DTPA-conjugated, non-labeled anti-
body. The nature of this binding is preliminary
interpreted as an off target binding (cross reac-
tivity) rather than an indication of the expres-
sion of available Ku70/Ku80 antigen on murine
cells. Using immune precipitation with INCA-X
attached to magnetic beads via Protein G and
extracts of 1% NP 40 solubilized tissue, INCA-X
could be shown to readily precipitate Ku70/
Ku80 complex from all human cell lines tested
[7] but not from any mouse tumor cell line or
from solubilized mouse spleen. These results
indicate that the antibody does not bind the
mouse Ku70/Ku80 complex and thus the local-
ization seen with the labeled DTPA-INCA-X is
the result of a low avidity binding to another
epitope.

The biodistribution data of mice bearing PC-3
xenografts (Table 1) showed the uptake of
labeled antibody in the spleen and tumor were
both increased with time post-injection of
$^{111}$In-DTPA-INCA-X and reached a maximum
after 96 hours when the study was ended. The
differences in the biodistribution and SPECT
images between the more aggressive PC-3/
PC-3M-Lu2 tumor-bearing mice and the DU145
tumor-bearing mice, with regards to the signifi-
cant level of activity redirected to the tumors
and the clarity of predose group 1 SPECT imag-
es, could be due to differences in the expres-
sion of the Ku70/Ku80 antigen on the surface
of the cells. The Ku70/Ku80 expression on
both cell lines is thought to be similar [27], yet
it is feasible that the more aggressive cells
have a higher cell surface expression than that
on the less aggressive cell lines.

Various biomarkers have been mentioned as
imaging and therapeutic candidates. PSMA is a
clinically valid tumor biomarker for prostate
cancer; however, its true impact in the clinical
setting has not been made apparent. ProstaScint® is currently the only clinically
approved radiolabeled antibody for imaging
prostate cancer, yet one of the downfalls of
ProstaScint® is that it targets the internal
domain of PSMA making image interpretation
complex and thus results in a high number of
false positives and false negatives [32, 33].
There is now more focus on targeting the external
domain of PSMA, which shows promise
[34]. Obviously there is room for new approach-
es, and at least four antibody-drug conjugates
targeting prostate cancer are in the early stage
of clinical development: anti-PSMA from
Progenics Pharmaceuticals, Inc. (http://clinical-
trials.gov NLM identifier: NCT01414283), anti-
secondary lymphoid tissue chemokine, SLC-
44A2, (ASG-5ME) and anti-prostate stem cell
antigen monoclonal antibody, AGS-1C4D4,
from Astellas Pharma (http://clinicaltrials.gov
NLM identifiers: NCT01228760 and NCT00-
519233, respectively) and anti-TROP2 (IMMU-
44A2, (ASG-5ME) and anti-prostate stem cell
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}
In-DTPA-INCA-X anti-Ku70/Ku80 McAb in prostate cancer

ings, we believe that the INCA-X antibody defines a target worth further evaluation. As this was the first study assessing the INCA-X antibody in a living system, the difference between uptake of labeled antibody with and without a pre-administered dose of DTPA-INCA-X in human prostate cancer cell lines and in nude mice bearing human prostate cancer-based xenografts needs to be further investigated. Nevertheless, the results are encouraging to go further and try to improve the tumor uptake by testing newly generated antibodies directed to the Ku70/Ku80 antigen.

Conclusion

The $^{111}$In-DTPA-INCA-X antibody can target and internalize into subcutaneous xenografts of prostate cancer cell lines in mice pre-administered non-labeled, DTPA-conjugated INCA-X antibody, thus identifying the Ku70/Ku80 antigens as targets worth further investigation.

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In-DTPA-INCA-X anti-Ku70/Ku80 McAb in prostate cancer


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