CNN-Gd\(^{3+}\) Enables Cell Nucleus Molecular Imaging of Prostate Cancer Cells: The Last 600 nm

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ABSTRACT

Molecular imaging is defined as the characterization and measurement of biological processes at the cellular and molecular level. Molecular imaging, therefore, necessitates a sufficient amount of contrast agent within the cell. Consequently, we realized that the intracellular uptake and cell compartment specificity of the commonly used interstitial contrast agent gadolinium (Gd\(^{3+}\)) with a cell-nucleus directed peptide module could be helpful. This modular molecule is characterized by a Gd\(^{3+}\)-complex module that is bound to a transmembrane transport unit (TPU) of human origin and further to a nucleus-directed address module (nuclear localization sequence) resulting in a specific cell nucleus-directed localization. By use of magnetic resonance imaging, Gd\(^{3+}\) could be helpful. This modular molecule is characterized by a Gd\(^{3+}\)-complex module that is bound to a transmembrane transport unit (TPU) of human origin and further to a nucleus-directed address module (nuclear localization sequence) resulting in a specific cell nucleus-directed localization. By use of magnetic resonance imaging, Gd\(^{3+}\) was detected within DU-145 prostate cancer cells after only 10 min. The nuclear localization was confirmed with confocal laser scanning microscopy. The resulting MRI signal enhancement only slightly decreased over the next 48 h compared with an absolute loss of signal enhancement after only 8 h when a random target sequence was used. Therefore, our method seems promising for in vivo application in molecular imaging.

INTRODUCTION

Molecular imaging is defined as the characterization and measurement of biological processes at the cellular and molecular level (1) transmembrane transport of Gd\(^{3+}\) using an amphiphilic transport peptide of human origin (Table 1), which contains a similar peptide sequence to that of the homeodomain of Antennapedia (6). This similar peptide sequence was chosen to minimize the risk of immunizing reactions and is the subject of further in vivo investigations (6). Our modularly constructed CNN-Gd\(^{3+}\)-complex consists of the above described transport peptide which is cleavably covalently linked to the nuclear localization sequence of SV40T-antigen (7) via a disulfide bond. The NLS is in turn linked to the Gd\(^{3+}\)-complex (Fig. 1). The nuclear transport is mediated by soluble cytoplasmic receptors (importins) and uses an active Ran-GDP-system (8). This principle enables the rapid nuclear accumulation (9) of Gd\(^{3+}\)-complex by a CNN\(^{2+}\)-conjugated Gd\(^{3+}\)-complex (CNN-Gd\(^{3+}\)-complex).

MATERIALS AND METHODS

Identification of TPU Structures by Sequence Retrieval System BioComputing

A FASTA search was carried out in the HUSAR Sequence Retrieval System. We searched for peptides of human origin containing sequence homologies to the sequence of the Antennapedia peptide fragment RQIKIW-FQNRRMKWKK. Among several domains, we detected a Smith-Waterman score of: 1B72:A HOMEBOX PROTEIN HOX-B1: 86.667% identity (86.667% unropped) in a 15-amino acid overlap.

![Sequence Alignment](image)

To find the optimal sequence and structural homologues, we selected the amino acid sequence with the above described score of 1B72:A, which was further used as the TPU. In the alignment, identical amino acids were displayed with “.”, and the similarities were displayed with “:”. This sequence was chosen with a view to future in vivo experiments with its promise of a lesser risk of immunizing reactions (6).

Synthesis of the CNN-Gd\(^{3+}\)-Complex and the CNRN-Gd\(^{3+}\)-Complex

To perform solid phase synthesis of peptide modules we used the N-(9-fluorenyl)methoxycarbonyl strategy in a fully automated synthesizer Syro II (MultiSyn Tech, Germany) described by Merriefield (10). The syntheses of TPU transmembrane peptide (Table 1, #3723), the NLS (part of the CNN-Gd\(^{3+}\)-complex) (Table 1, #1552/a), and the random NLS (part of the CNRN-Gd\(^{3+}\)-complex) (Table 1, #1552/b) were performed with an identical procedure.
**Table 1** Biochemical design of the functional modules used in the MRI and CLSM study

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<tr>
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<td>Transport-peptide</td>
<td>TPU (human) $\text{H}_2\text{N-TQVKWFQNRMKQKK-(Cys-CO-NH}_2\text{)-(SH)-CONH}_2^{**}$</td>
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<tr>
<td>#1552a</td>
<td>Random</td>
<td>NLS-[SV40-antigen] $\text{H}_2\text{N-PKKKRKV-(Cys-CO-NH}_2\text{)-(SH)-CONH}_2^{**}$</td>
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<tr>
<td>#1552b</td>
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<td>NLS (CNN)-conjugated-Gd$^{3+}$-complex</td>
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**MRI**

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<td>#1552b</td>
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<td>#1552 af</td>
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**CLSM**

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<td>#1552 bf</td>
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**Localization of the NLS-Gd$^{3+}$-Complex by CLSM.** To perform fluorescence microscopic studies, DU-145 cells and lymphocytes ($5 \times 10^5$) were incubated for 24 h in Quadruprim plus (Heraeus, Germany) containing sterile glass coverslips. After two wash cycles with MEM, the cells were incubated with $\text{ALEXA-CNN}^{\text{FITC}}$-Gd$^{3+}$-complex (Table 1, #1552/af) and $\text{ALEXA-CNN}^{\text{FITC}}$-Gd$^{3+}$-complex (Table 1, #1552/bf). To increase the contrast of optical sections, 12–20 single exposures were averaged. Parameters of the image acquisition were adapted to show signal intensities in accordance with the visual microscopic image.

**Measurement of Nuclear Gd$^{3+}$ Concentration Using ICP-MS**

The nuclear uptake of CNN-Gd$^{3+}$ was examined by a mass spectrometry method. Gd$^{3+}$ concentration measurements were carried out by a high resolution element mass spectrometer (Finnigan MAT ELEMENT2, Bremen, Germany) with ICP (ICP-MS) at a resolution ($\Delta m \times m^{-1}$) of 4000. The instrument was equipped with a self-aspirating 100 $\mu$l min$^{-1}$ PFA-Nebulizer and spray chamber, standard injector, and torch. Instrument and operation parameters were as follows: plasma power, 1100 W; cool gas flow, 15.5 liters $\times$ min$^{-1}$; auxiliary gas flow, 1 liter $\times$ min$^{-1}$; sample gas flow, 1 liter $\times$ min$^{-1}$; mass window, 850%; search window, 800%; integration win-
Molecular Modeling

Because no experimental data for these modules were available, spatial models were generated based on homologous data. The objective of the following spatial model is a representation of the relative magnitudes of the component units and an approximation of their respective structures for the purposes of visualization and is, as such, not exactly representative of the molecular structure. The spatial model of the bioconjugate was formed by manual connection of the molecular modules (TPU, NLS, and DPTH). The FASTA search option of the PDB (11) was used to identify sequences that show high similarity with the TPU (KMTTRQTFWHR!H!KHC) and the NLS (PKKRRKVV). In the case of TPU, the crystal structure of the site-specific recombinase, XerD (PDB entry, 1A0P; 217–231: QMTTRQTFWHR!KHYA) was taken as a template for which an 85% identity in a 13-amino acid overlap was shown. For NLS, a part of the crystal structure of the tissue transglutaminase (PDB entry, 1KV3: 598–605: PKQKRKLV) was taken, which in turn showed a 71% identity in a 7-amino acid overlap. Although the sequences are too short to provide highly reliable spatial structures, this approach seems to be justified to generate models for the purpose of visualization. The biopolymer option of the INSIGHTII module was applied to mutate the required amino acids. A minimization using the AMBER-Force field was followed by a short molecular dynamics simulation in aqueous solution to relax the constructed model. The Gd$^{3+}$ complex was taken from the Cambridge Structural Database (entry, heqba; Ref. 12). The aforementioned molecular modules were connected using INSIGHTII software (Accelrys, San Diego, CA). INSIGHTII was also used to produce the graphical representations of the bioconjugates.

MRI

DU-145 Cell Uptake of the CNN-Gd$^{3+}$-Complex and CNRN-Gd$^{3+}$-Complex Compared With That of Magnesvit. DU-145 cells were harvested and divided into tubes (Falcon; Becton Dickinson; number of cells, $4 \times 10^6$...
cells/tube). The CNN-Gd\(^{3+}\)-complex (Table 1, #1552/a), the CNRN-Gd\(^{3+}\)-complex (Table 1, #1552/b), and the Magnevist were each dissolved in MEM in a concentration of 0.5 mM and were then incubated for 10, 20, and 30 min up to 3 h. After centrifugation of the tubes (800 rpm \(\times\) 10 min), the incubation medium (supernatant) was removed, and the cells (pellet) were washed twice with MEM without conjugates to remove all unbound Gd\(^{3+}\)-DTPA (Magnevist), CNN-Gd\(^{3+}\)-complex (Table 1, #1552/a), and CNRN-Gd\(^{3+}\)-complex (Table 1, #1552/b).

MRI used a 1.5-T whole body Siemens Magnetom Vision Plus with a standard circular polarized head coil. The test tubes were firmly positioned parallel to each other totally submerged in a water bath. The imaging protocol consisted of an axial T1-weighted spin echo sequence (TR, 600 ms; TE, 15 ms; scan time, 45 s). The field of view was 200 mm \(\times\) 200 mm, using a 256 \(\times\) 256 imaging matrix and two acquisitions. Slice thickness was 2 mm, resulting in a pixel size of 0.79 \(\times\) 0.78 mm. T1 and T2 relaxation times within the pellets of the three tubes (CNN-Gd\(^{3+}\), CNRN-Gd\(^{3+}\), and Magnevist) were measured to evaluate the intracellular relaxivity of the respective contrast agents (\(R = 1/\ T1\)). The T1 relaxation time was measured by an inversion recovery sequence (TR, 5000 ms; TE, 76 ms; TI, 25–4000 ms; 17 different TI values; scan time, 15 \(\times\) 25 s; field of view, 160 mm \(\times\) 160 mm; matrix, 132 \(\times\) 256; slice thickness, 7 mm; pixel size, 1.21 \(\times\) 0.63 mm). T2 relaxation time was measured by a multi echo sequence (TR, 5000 ms; 16 TE values; 30–245 ms; field of view, 300 mm \(\times\) 300 mm; matrix, 256 \(\times\) 256; slice thickness, 5 mm; pixel size, 0.98 \(\times\) 0.98 mm; scan time, 21 min 21 s). Signal intensity measurements were obtained from DU-145 carcinoma cells and background. A tube with DU-145 cells, incubated in MEM without contrast agent, was used as a control. In this way, the DU-145 cells were tested for uptake of the Gd\(^{3+}\)-complex-transporter when bound to either a NLS sequence (#1552/a) or a random sequence (#1552/b). As a control, the same procedure was performed in nontumor cells (lymphocytes). Because of a signal intensity maximum in prostate cancer cells and lymphocytes after 3 h incubation, efflux measurements were begun after this time period. For this, both cell types were washed with conjugate-free MEM to remove all Gd\(^{3+}\)-complexes. This procedure was repeated hourly until no signal increase compared with the control tube (DU-145 prostate cancer cells or lymphocytes in MEM without contrast agent) could be detected in T1-weighted sequences.

All experimental sequences were performed three times.

RESULTS AND DISCUSSION

Our predominant aim was to deliver the Gd\(^{3+}\)-complex into the cell nucleus. This had been achieved previously using the plasma membrane translocation peptide, HIV-1 tat (2). This viral protein possesses
nuclear import characteristics (13). We were also able to confirm the good uptake characteristics of the HIV-1 tat peptide. However, the HIV-1 tat peptide possesses not only a transactivating effect on the long terminal repeat promoter but also can induce apoptosis in hippocampal neurons (5, 14, 15). As a consequence of these transactivating effects of HIV-1 tat peptide, we chose a different method and examined human TPUs, which show a comparable transport efficiency (Table 1, #1552).

In parallel, we used molecular modeling to obtain the most appropriate spatial visualization of the conjugate (Fig. 1). Although it is clear that the presented spatial structures of the bioconjugate are one approximate configuration of the many which flexible molecules such as peptides may exhibit, they represent to some extent a realistic spatial model; an all-atom model of the complete molecules is presented, the shapes of the component modules are as have been reported for homologous structures, the relative sizes of the modules are correct, and the physicochemical characteristics of the surface are represented.

In MRI, an increased intracellular signal intensity in DU-145 cells could be detected after only 10 min incubation with our nuclear Gd$^{3+}$-delivery system CNN-Gd$^{3+}$-complex (Fig. 4; Table 1, #1552/a; whole body 1.5 T Siemens Magnetom, standard circular polarized head coil). Because of the inability of MRI to recognize different cellular compartments, CLSM was further used to confirm the nuclear localization of the Gd$^{3+}$-complex. Because of the higher sensitivity of CLSM compared with MRI, lower concentrations of the CNN-FITC-Gd$^{3+}$-complex (Table 1, #1552/af) could be used in CLSM (CLSM, 100 pM; MRI, 0.5 mM). In CLSM, a nuclear fluorescence signal was detected that would suggest that the CNN-Gd$^{3+}$-complex accumulated mainly at this site (Table 1, #1552/a). If, by way of comparison, Magnevist alone was used as a contrast agent in MRI, there was no signal change above that of the DU-145 cells that had been incubated solely in MEM. The measured relaxivity ($R$) within the DU-145 cell pellets changed by more than a factor of 5 after incubation with our Gd$^{3+}$-complex transporter ($R = 0.00354$) as compared with that after incubation solely with Magnevist ($R = 0.00069$). The mass spectrometrically measured higher concentration of Gd$^{3+}$ within the nucleus compared with that in the cytoplasm (factorial difference, 40,000) could be explained by the interaction with Ran-GDP and importins. To show the nuclear spec-

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**Fig. 4.** Upper part, graph of MR signal intensity versus time after incubation with the Gd$^{3+}$-[DTPH]$_4$-HN-K-K-NLS-C/S-C-TPU (CNN-Gd$^{3+}$; red dashed circles) and the Gd$^{3+}$-[DTPH]$_4$-HN-K-K-Random-C/S/C-TPU (CNNR-Gd$^{3+}$; blue dashed squares) for DU-145 human prostate cancer cells. Data represent three independent experiments. Lower part, axial T1-weighted MR images of the cell pellets consisting of $40 \times 10^6$ cells. MEM was used as cell culture medium.
ificity of the CNN-Gd\textsuperscript{3+}-complex, a CNRN-Gd\textsuperscript{3+}-complex (random NLS; Table 1, #1552/b) was used resulting in a slightly higher MRI signal enhancement after 3 h compared with that after using the specific NLS.

After the signal intensity had reached its maximum after 3 h, only a slight decrease was then observed over the next 45 h when the CNN-Gd\textsuperscript{3+}-complex was used (Fig. 4). A possible explanation could be the lack of efflux of the CNN-Gd\textsuperscript{3+}-complex out of the nucleus (Table 1, #1552/a). In contrast, the CNRN-Gd\textsuperscript{3+}-complex (Table 1, #1552/b) could not enter the nuclear space and remained in the cytoplasm (Fig. 5b). The random sequence did not represent a suitable substrate for karyophilic proteins (importins). Therefore, efflux was possible, and a complete reduction of signal enhancement could be observed after only 8 h (Fig. 4). In CLSM, dual staining of both the ALEXA-CN\textsubscript{R}N-FITC-Gd\textsuperscript{3+}-complex and ALEXA-CN\textsubscript{R}N-FITC-K-Random-C-S\textsubscript{S}-C-TPU\textsubscript{ALEX}A conjugate consisting of the human TPU and a Gd\textsuperscript{3+}-[DTPH]\textsubscript{4}-HN-K\textsubscript{F\textsubscript{ITC}}-K-NLS-C-S\textsubscript{S}-C-TPU\textsubscript{ALEX}A conjugate consisting of the human transmembrane carrier TPU covalently attached to a FITC-labeled peptide random sequence was added to the culture medium 30 min before CLSM analysis of living DU-145 cells at a concentration of 100 pM. Fluorescence signals were detected exclusively within the cytoplasm, whereas the nuclei remained unstained.

Fig. 5. a, CLSM optical section of living DU-145 prostate cancer cells incubated for 30 min with the modular transport peptide Gd\textsuperscript{3+}-[DTPH]\textsubscript{4}-HN-K\textsubscript{F\textsubscript{ITC}}-K-NLS-C-S\textsubscript{S}-C-TPU\textsubscript{ALEX}A conjugate consisting of the human TPU and a Gd\textsuperscript{3+}-[DTPH]\textsubscript{4}-HN-K\textsubscript{F\textsubscript{ITC}}-K-Random-C-S\textsubscript{S}-C-TPU\textsubscript{ALEX}A conjugate consisting of the human transmembrane carrier TPU covalently attached to a FITC-labeled peptide random sequence was added to the culture medium 30 min before CLSM analysis of living DU-145 cells at a concentration of 100 pM. The green fluorescence signal reveals a distinct nuclear localization of the peptide. b, a Gd\textsuperscript{3+}-[DTPH]\textsubscript{4}-HN-K\textsubscript{F\textsubscript{ITC}}-K-Random-C-S\textsubscript{S}-C-TPU\textsubscript{ALEX}A conjugate consisting of the human transmembrane carrier TPU covalently attached to a FITC-labeled peptide random sequence was added to the culture medium 30 min before CLSM analysis of living DU-145 cells at a concentration of 100 pM. Fluorescence signals were detected exclusively within the cytoplasm, whereas the nuclei remained unstained.

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the ALEXA 546 Fluor-labeled TPU (Table 1, #1552/bf) could not enter the cell nucleus and remained outside within the cytoplasm, resulting in a mixed fluorescence signal (ALEXA 546 Fluor, red; FITC, green; Fig. 5b).

In contrast, the Gd\textsuperscript{3+}-[DTPH]\textsubscript{4}-HN-K\textsubscript{FITC}-K-NLS-C-S\(\equiv\)S-C-TPU\textsubscript{ALEXA} (Table 1, #1552/af) was proven to be located within the nucleus (Fig. 5a). Some cells in Fig. 5a can be seen not to have taken gadolinium up into the nucleus, which could be explained as follows. The transport of CNN-Gd\textsuperscript{3+} into the nucleus is an active Ran-GDP-dependent process and will not take place in cells functionally damaged during preparation but still apparently morphologically intact. However, the transport from the extracellular space into the cytoplasm is a passive process and would continue to take place even in functionally damaged cells. Additionally, some asynchronicity between cells with respect to the rates of nuclear uptake can be assumed. A slightly mixed fluorescence signal was also detected in the cytoplasm, whereas additionally a distinct sole green fluorescence signal (FITC) was observed within the nucleus (Fig. 5a). The cytoplasmic cleavage of the disulfide bond between the two modules ALEXA 546 Fluor-tagged TPU and the FITC-tagged NLS-Gd\textsuperscript{3+}-complex is followed by the effective nuclear import of the NLS-Gd\textsuperscript{3+}-complex\textsubscript{FITC} (Table 1, #1552/af; Fig. 5a). No evidence of cytotoxicity was observed after incubation with CNN- or CNRN-Gd\textsuperscript{3+}-complexes for 72 h.

Our results suggest a promising specific and noninvasive method for the visualization of the cell nucleus in MRI. The basic Gd\textsuperscript{3+}-complex (Magnevist) cannot enter the cell in concentrations sufficient for an MRI signal enhancement. However, after using high Gd\textsuperscript{3+}-complex concentrations without a specific delivery system in combination with a long incubation period (100 h), intracellular Gd\textsuperscript{3+}-complex was measured in amounts sufficient for Gd\textsuperscript{3+}-NCT (16). These time and dose requirements seem barely conceivable with respect to future NCT in humans. However, the use of Gd\textsuperscript{3+} in NCT would be advantageous because of the large neutron capture cross-section of \textsuperscript{157}Gd\textsuperscript{3+}, which leads to irreparable DNA damage after neutron irradiation (17). Therefore, the CNN-Gd\textsuperscript{3+}-complex would make a suitable target for neutrons in NCT to the greatest effect when situated in the cell nucleus. Additionally, the potential to trace the CNN-Gd\textsuperscript{3+}-complex directly by MRI would be useful for evaluating possible effects of Gd\textsuperscript{3+}-NCT.

It is also conceivable that the CNN-Gd\textsuperscript{3+}-complex could simultaneously take on a diagnostic as well as a therapeutic role. To perform a highly efficient chemos- and radiotherapy, it is indispensable to know the DNA repair enzyme activity in tumors (18). Determining such enzyme activity levels in MRI before further treatment was commenced would be helpful in the decision-making process.

A contrast agent called EgadMe (19) was applied by microinjection into the cell nucleus to measure galactosidase activity. Water access to the first coordination sphere of Gd\textsuperscript{3+} was blocked with a substrate that could be removed by enzymatic cleavage. After cleavage, Gd\textsuperscript{3+} can interact directly with water protons to increase the MR signal. Galactopyranose was used as a blocking group, which in turn enabled the measurement of the activity of galactosidase. A similar method could potentially be used to visualize alkyltransferase, decisive for the outcome of chemotherapy (19), in the nucleus by first preventing water access to the first coordination sphere of a Gd\textsuperscript{3+}-complex with a suitable substrate, which when enzymatically cleaved would lead to water access and a resulting increase in signal intensity in MRI.

In conclusion, the CNN-Gd\textsuperscript{3+} complex is an example of a nonviral and microinjection-free method for delivery of Gd\textsuperscript{3+} into the cell nucleus.

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