

# PreS2-TML peptide or guanidinium modified Gd-DOTA exhibits efficient cellular uptake

Markus Wolf<sup>a\*</sup>, Ulrike Bauder-Wüst<sup>a</sup> and Rüdiger Pipkorn<sup>b</sup>

<sup>a</sup> Department of Radiopharmaceutical Chemistry, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

<sup>b</sup> Peptide Synthesis Facility, German Cancer Research Center (DKFZ), TP3 Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany.

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**Abstract.** The majority of magnetic resonance contrast agents are restricted to the extracellular domains. For the development of novel, intracellular magnetic resonance contrast agents, we have designed Gd-DOTA derivatives comprising PreS2-TML peptide or ethylguanidinium as carrier moiety. Initial in vitro cell uptake studies with Jurkat cells revealed efficient contrast agent uptake for imaging purposes, in the range of 0.04 fmol/cell (PreS2-TML peptide) to 0.2 fmol/cell (ethylguanidinium) following 2 h incubations at 100  $\mu$ M.

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To enhance magnetic resonance imaging (MRI) contrast between different tissues or between specific tissue compartments, a variety of intra- or extravascular paramagnetic contrast agents are available, e.g., the gadolinium(III) chelation complex [Gd(DTPA)(H<sub>2</sub>O)]<sup>2-</sup> (commercial name: Magnevist®; generic name: gadopentetate dimeglumine; DTPA = diethylenetriamine-N,N,N',N'',N'''-pentaacetic acid) or [Gd(DO3A-butrol)(H<sub>2</sub>O)] (Gadovist® or gadobutrol; DO3A-butrol = 1,4,7-tris(carboxymethyl)-10-(1,2,4-trihydroxy-but-3-yl)-1,4,7,10-tetraazacyclododecane).<sup>1</sup> A principal barrier to the development of MRI contrast agents for investigating biological questions is the delivery of agents across cellular membranes. The majority of MRI contrast agents are restricted to the extracellular domains, and there are few examples of membrane permeable MR contrast agents reported in the literature.<sup>2-7</sup> Recently, we have reported about polyamine-based MRI contrast agents with facilitated intracellular tumor uptake.<sup>8</sup> To achieve a detectable contrast enhancement via T1-weighted MRI, on the order of 10<sup>7</sup> - 10<sup>8</sup> Gd(III) complexes (0.017 - 0.17 fmol) per cell need to be internalized.

Our present research is focussing on the development of novel intracellular MRI contrast agents.

It has been reported that an amphipatic alpha-helix between the amino acids 41 and 52 of the PreS2-domain of hepatitis-B virus surface antigens mediates cell permeability and per se can act as a shuttle for peptides and functional proteins.<sup>9</sup> Membrane translocation occurs in an energy-independent manner.<sup>9</sup> Nucleic acids

with guanidinium modification exhibit efficient cellular uptake.<sup>10</sup>

We propose that conjugation of the PreS2-TML peptide or a guanidinium group to Gd-DOTA mediates efficient uptake into mammalian cells. The chemical structure of the conjugates is outlined in figure 1.

The DOTA-ethylguanidinium conjugate was obtained by condensation of DOTA-tris(tert. butylester) and *N,N*'-Bis-Boc-protected ethylguanidin in the presence of the coupling reagent reagent HATU (O-(7-Azabenzotriazol-1-yl)-*N,N,N,N'*-tetramethyluronium hexafluorophosphate). The protecting groups were cleaved with trifluoroacetic acid (TFA)/H<sub>2</sub>O/triisopropylsilane (95:2.5:2.5). Reversed-phase HPLC purification of the crude product yielded the pure conjugate.

DOTA-PreS2-TML was synthesized automatically on Rink amide resin, according to the Merrifield strategy. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids were coupled in a stepwise manner to the corresponding resin.<sup>11</sup> 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in *N*-methylpyrrolidone (NMP) was used as coupling agent. Fmoc groups were removed using 20% piperidine/NMP. The compounds were cleaved from the resin with trifluoroacetic acid (TFA)/H<sub>2</sub>O/triisopropylsilane (95:2.5:2.5). Reversed-phase HPLC purification of the crude products yielded the pure ligand which was characterized by analytical

reversed-phase HPLC and ion-spray mass spectrometry (see table 1) to reveal identity and purity.

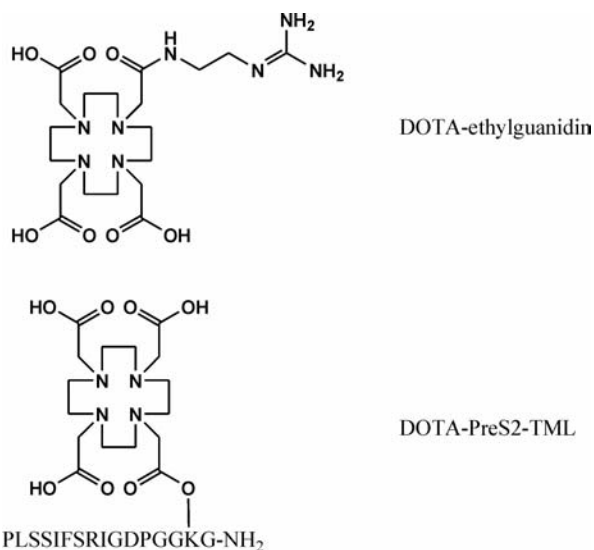


Figure 1: Chemical structure of the ligands.

To a solution of the free ligand in water (16–32 mM) was added gadolinium(III)acetate (1.3 equiv.). The reaction mixture was heated to 80°C and stirred for 3 hr. The reaction mixture was then cooled to ambient temperature, and the pH was adjusted to 11 with aqueous ammonium hydroxide. The mixture was filtered through a 0.2 µm syringe filter, and freeze dried to yield a white solid.

Compds	Chemical formula/ amino acid sequence	[M+H] <sup>+</sup>
DOTA-ethylguanidin	C <sub>19</sub> H <sub>36</sub> N <sub>8</sub> O <sub>7</sub>	489.01
Gd-complex of DOTA- ethylguanidin	C <sub>19</sub> H <sub>33</sub> N <sub>8</sub> O <sub>7</sub> Gd	644.4
DOTA-PreS2-TML	PLSSIFSRIGDPG GK(DOTA)G-NH <sub>2</sub>	1973.52
Gd-complex of DOTA-PreS2-TML	C <sub>86</sub> H <sub>138</sub> N <sub>25</sub> O <sub>28</sub> Gd	2127.01

Table 1. Chemical formulas and mass spectrometry data .

Jurkat cells were obtained from the German Cancer Research Center (DKFZ, Heidelberg) tumor bank and grown at 37 °C as stock cultures in RPMI 1640 supplemented with 10% fetal calf serum and 1% glutamine as standard medium under a 5% CO<sub>2</sub> atmosphere (all components: Pan Biotech GmbH, Aidenbach, Germany). For contrast agent uptake studies 10<sup>7</sup> cells were incubated for 2 h with medium containing 0, 10, 25, 50 or 100 µM of the selected gadolinium complex. Afterwards the medium was removed, the cells were washed twice, resuspended in culture medium and counted. The gadolinium content of the cells was determined by ICP-MS. Samples

containing 3 × 10<sup>6</sup> harvested cells were digested with 50% concentrated HNO<sub>3</sub> (Superselect; Merck, Darmstadt, Germany) under microwave heating with a Mars 5 apparatus (CEM GmbH, Kamp-Lintfort, Germany). To each sample 100 µL of an aqueous solution of rhodium chloride (1 µg/mL) were added so that Rh-103 could be used as an internal quantification standard. Gd-160 measurements were performed with the ICP-MS instrument mentioned above. The gadolinium concentrations were determined using standard curves created prior to cell analyses.

The uptake of the complexes **Gd-1** and **Gd-2** after 2-h incubation was concentration dependent (Figure 1) and reached values in the range 0.04 - 0.02 fmol/cell for the 100 µM incubation.

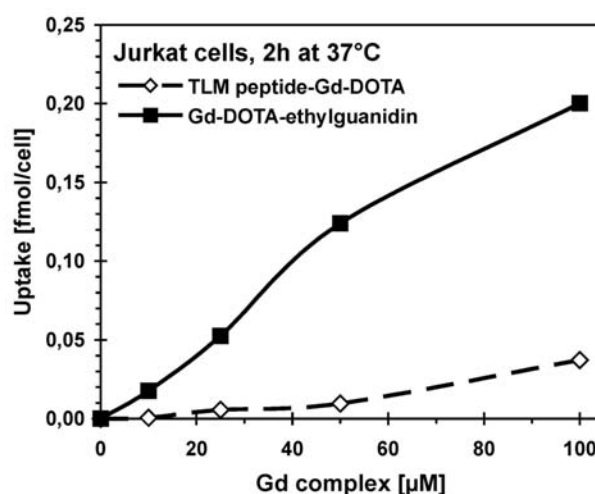


Figure 2: Intracellular uptake of the gadolinium complexes into Jurkat cells following 2 h incubations at 37°C.

The data presented here support the further evaluation of these complexes as contrast agents in magnetic resonance imaging.

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