***In Vitro* and *In Vivo* Testing of ^{177}Lu -DOTA-Nimotuzumab, a Potential Radioimmunotherapeutical Agent of Cancers**

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Abstract. Nimotuzumab is an anti-cancer agent that belongs to the inhibitor group of anti-epidermal growth factor receptors (*EGFR*). This antibody inhibits the *EGF* protein receptor, which is over-expressed on cancer cells in cancers such as colorectal, brain, pancreas, prostate, *non-small cell lung*, oesophagus, cervical and breast cancer. In order to obtain a higher efficacy agent for therapy of the above-mentioned cancers, nimotuzumab was labeled with beta particle- and gamma-emitter ^{177}Lu . This report discusses the results of our *in vitro* and *in vivo* tests, which included cytotoxicity, clearance, imaging and biodistribution tests. The cytotoxicity test of ^{177}Lu -DOTA-nimotuzumab on A 549 cell lines (lung carcinoma) was found to kill more cancer cells compared to that of unlabeled nimotuzumab. The clearance test of ^{177}Lu -DOTA-nimotuzumab on normal rats showed that the residues of radioactivity were excreted mostly via the urine. The biodistribution and image tests showed that post injection of ^{177}Lu -DOTA-nimotuzumab there was still some radioactivity in a number of organs, such as the kidney, liver and bone. Although the state of radioactivity post injection of ^{177}Lu -DOTA-nimotuzumab in the above-mentioned organs was nearly the same as or slightly lower compared to other radiolabeled monoclonal antibodies, precautions still have to be observed when this product is going to be tested on cancer patients.

Keywords: *Anti EGFR radiolabeled monoclonal antibody; in vitro; in vivo tests; ^{177}Lu -DOTA-nimotuzumab.*

1 Introduction

Nimotuzumab, a humanized monoclonal antibody, is an anti-cancer agent that belongs to the inhibitor group of anti-epidermal growth factor receptors (*EGFR*). This antibody can inhibit epidermal growth factor/human epithelial receptor type-1 (*EGF/HER-1*) receptors that are over-expressed on the surface

of several cancers, such as colorectal, brain, pancreas, prostate, non-small cell lung, oesophagus, cervix and breast cancers [1-3]. In normal conditions, EGF stimulates the normal cells to grow and differentiate. Nimotuzumab inhibits the activation of tyrosine kinase protein and binds to the extracellular domain of the EGFR with an optimal affinity and specificity that in turn will inhibit ligand binding and receptor activation. By blocking this receptor, nimotuzumab prevents the cancer cells from receiving the required signal to grow and differentiate, and hence inhibits metastasis.

Recently, [3] reported that nimotuzumab had been administrated to around 1800 patients worldwide, either in a clinical trial or commercial use, without any major adverse effects such as rash, diarrhoea and conjunctivitis, which were commonly reported for other EGFR-targeting monoclonal antibodies or small molecules [4]. In some European and Asian countries nimotuzumab has been approved for the treatment of several types of cancers such as colorectal, glioma, pancreas, prostate, non-small cell lung, oesophagus, cervix and breast cancers; in America it is yet to be approved.

EGFR has been a key target for the development of many cancer therapeutic agents. It has been reported that these agents, including nimotuzumab, have a higher therapeutic effect when combined with external radiation therapy [3]. Based on this fact, it was considered necessary to develop a targeted radiopharmaceutical that synergises the ability of nimotuzumab to specifically bind to EGFR and halt the proliferation and metastasis of cancer cell with an alpha- or beta-particle emitter that is able to transfer its energy to the surrounding cancer cells, which eventually lyses them. A radioimmunoconjugate, a radionuclide conjugated to the monoclonal antibody that is a targeted radiopharmaceutical, is expected to be more effective compared to the use of nimotuzumab or radiation therapy alone. As a targeted therapy, it is also expected to have fewer adverse effects on surrounding normal tissues or cells.

The radionuclide that was chosen for radiolabeling nimotuzumab was ^{177}Lu . The choice of this radionuclide was due to its promising physical and chemical properties. Lu-177 emits beta (β) particles with energies of E_{max} 497 keV (78.6%) and 176 keV (12.2%) that are able to penetrate an average of ~1.5 mm depth of tissue, therefore it is suitable for the treatment of small-size cancers. This radionuclide also emits gamma (γ) rays with energies of 113 keV (6.4%) and 208 (11%) keV, which are quite ideal for imaging purposes. Therefore the *in vivo* deposition of such a radioimmunoconjugate can be observed with a γ -camera. The procurement of ^{177}Lu with a relatively high specific radioactivity (~10 Ci/ mg Lu) via nuclear reaction of $^{176}\text{Lu}(n,\gamma)^{177}\text{Lu}$ is relatively easy due to its high cross section of 2100 barn, as reported by Kettering [5].

The aim of this study was to provide the basic data that can be used as a basic consideration for establishing if a clinical trial of ^{177}Lu -DOTA-nimotuzumab in radioimmunotherapy for the treatment of the above-mentioned cancers would be worth pursuing.

2 Materials and Methods

2.1 Materials

All chemicals were used as received without further purification, unless otherwise mentioned in the procedure. Chemicals used in this project included: isotopically enriched $^{176}\text{Lu}_2\text{O}_3$ (60.60%), supplied by Isoflex USA, hydroxysuccinimide-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetraacetic acid (NHS-DOTA), supplied by Macrocyclic, bovine serum albumin (BSA), ethylene diamine tetra acetic acid (EDTA), sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), disodium hydrogen phosphate (Na_2HPO_4), sodium hydroxide and sodium chloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), supplied by Sigma. Hydrochloric acid (HCl) was supplied by Merck. Commercially available Nimotuzumab (TherachimTM) was obtained from Kalbe Farma. Fetal bovine serum (FBS) was supplied by JRS, USA. RPMITM medium was supplied by Gibco. Materials used in this project included EFGR/HER-1 positive cell line (A 549), which was a gift from the Centre for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology (LAPTIAB-BPPT). Normal mice were supplied by the National Veterinary Drug Assay Laboratory. Dialysis cassettes (20 KD MWCO) were obtained from Pierce, and silica gel impregnated glass fibre sheets (ITLC-SG) were purchased from Pall. A protein assay kit was obtained from Bio-Rad. Sephadex-G-25 resin (medium) was purchased from Pharmacia. High-purity water was obtained from Sartorius StedimTM system ($\Omega 18$ Mega Ohm).

The equipment used for analysis included a thin-layer chromatographic scanner (Bio Scan), a plate reader (Bio Tek), a CRC-15R dose calibrator (Capintec), a thermomixer (Eppendorf), and a refrigerated centrifuge (Beckman).

2.2 Methods

Unlike previously reported in Humani, *et al.* [6], where ^{177}Lu -DOTA-nimotuzumab was prepared in a three-step reaction, in this project it was prepared in a two-step reaction (Figure 1). Preparation of ^{177}Lu -DOTA-nimotuzumab with a two-step reaction was carried out by incubating NHS-DOTA and dialysed-nimotuzumab. The purified DOTA-nimotuzumab was then radiolabeled with $^{177}\text{LuCl}_3$ in 0.25 M ammonium acetate pH 5.5.

Figure 1 Reaction scheme of ^{177}Lu -DOTA-nimotuzumab.

2.2.1 Preparation of DOTA-nimotuzumab

The formation of DOTA-nimotuzumab was carried out by adding 3.98 mmol of NHS-DOTA to 0.16 mmol of nimotuzumab in 5 mL of 0.1M phosphate buffer pH 7.5. The mixture was incubated for 24 hours at 4 °C, which was followed by its purification using a dialysis cassette (MMCO 20 KD). After measuring its protein concentration, the DOTA-nimotuzumab was dispensed onto clean vial (5 mg/vial), which was then dried using a freeze dryer. The freeze-dried DOTA-nimotuzumab was then stored at 4-8 °C.

2.2.2 Preparation of $^{177}\text{LuCl}_3$

A solution of $^{177}\text{LuCl}_3$ (~ 7.5 Ci/mg Lu) was prepared by irradiating 0.3-0.4 mg of isotopically enriched $^{176}\text{Lu}_2\text{O}_3$ (60.60%) in a multi-purpose GA Siwabessy Reactor for four days. The irradiated target was dissolved in 2 mL of 6 M HCl and 2 mL of H_2O_2 . The mixture was then heated to dryness, and later redissolved with 3 mL of HCl 0.5 M.

2.2.3 Radiolabeling of DOTA-nimotuzumab with ^{177}Lu

The freeze-dried DOTA-nimotuzumab was firstly reconstituted with 1 mL water, which was followed by adding an aliquot of $^{177}\text{LuCl}_3$ (diluted in 0.25 M ammonium acetate, pH 7.5, $\frac{1}{2}$ v/v). The pH of the reaction mixture was then adjusted to 5.5 by addition of either 0.1 M HCl or 0.1 M NaOH. The reaction mixture was incubated at 37 °C for one hour, followed by addition of an excess

amount of 0.05 M EDTA solution (mol EDTA:mol = Lu 50:1). The formation of ¹⁷⁷Lu-DOTA-nimotuzumab was monitored using a TLC system with ITLC-SG as the static phase and saline solution as the mobile phase. The R_fs. for this system were zero for ¹⁷⁷Lu-DOTA-nimotuzumab and one for free ¹⁷⁷Lu (in form of ¹⁷⁷Lu-EDTA).

2.2.4 Purification of DOTA-nimotuzumab

Purification of ¹⁷⁷Lu-DOTA-nimotuzumab was carried out with a Sephadex G-25M column (15x1.2 cm, pre-blocked with 1 mL of 10% BSA, and pre-equilibrated with 0.01 M PBS pH 7.2). An aliquot of raw product of ¹⁷⁷Lu-DOTA-nimotuzumab was loaded onto the Sephadex G-25 column. The column was then eluted with 0.01 M PBS pH 7.2 at ~1 mL/min flow rate. Eluent was retrieved in 0.5 mL fraction and its radioactivity and radiochemical purity were then measured with a dose calibrator and a TLC system respectively. Each fraction associated with ¹⁷⁷Lu-DOTA-nimotuzumab (radiochemical purity > 95%) was pooled, sterilised and then used for further study.

2.2.5 Cell Culture

A549 cell lines (lung carcinoma, positive EFGR/HER-1 cell lines) were cultured in a growth medium that consisted of RPMI with 10% of FBS, and 1% of penicillin-streptomycin in an incubator with an atmosphere of 5% CO₂ at 37 °C. Passage was carried out every two to three days until the numbers of cells met with the cytotoxicity and binding affinity test requirements.

2.2.6 Cytotoxicity Test

A549 cells (~5,000 cells) in RPMI/10 % FBS/1% penicillin-streptomycin (growth medium) were transferred into each well of a sterile 96-Microplate. The plate was then incubated for 24 hours at 37 °C with an atmosphere of 5% CO₂. The ¹⁷⁷Lu-DOTA-nimotuzumab and unlabeled nimotuzumab (50, 100, 200 and 250 µg) in growth medium (triplo) were then added to separate wells on the plate. To all wells was then added a growth medium to give a final volume of 150 µL. The plate was incubated for 24 hours at 37 °C with an atmosphere of 5% CO₂. The growth medium was then removed from each well and the cells were washed with 150 µL PBS. To each well was then added 10 µL of MTT (5 mg/mL) and 90 µL of growth medium, and then the cells were incubated for four hours with an atmosphere of 5% CO₂. Finally, to each well was added 100 µL of SDS 10%, which was followed by incubation in a dark room for 24 hours. The absorbance of each well was then read at 570 nm by using a plate reader (BioTek).

2.2.7 Imaging Test

The imaging test was carried out by intravenously injecting 200 μCi of ^{177}Lu -DOTA-nimotuzumab (70 μg protein) two rats for each time point. At a determined time posts injection (3, 24, 48, 72 or 96 hours) the rats were anaesthetised and then imaged with a gamma camera.

2.2.8 Biodistribution Test

The biodistribution test was carried out by intravenously injecting 100 μCi (35 μg protein) ^{177}Lu -DOTA-trastuzumab in three normal mice for each time point. At selected time intervals (3, 6, 24, 48, 120, and 144 hrs), three mice were sacrificed. Selected organs were dissected and weighed, and radioactivity measured. The injected dose (% ID) and injected dose per gram (% ID/g) were then calculated from the associated radioactivity of each organ.

2.2.9 Clearance Test

The clearance test was carried out by intravenously injecting 400 μCi of ^{177}Lu -DOTA-nimotuzumab in two rats. Each rat was then kept in a metabolic cage. Urine and faeces of each rat were collected in separate containers. At a determined time period post injection (24, 48, 72, 96, 168, 192, 216, 240 and 264 hrs) urine and faeces were removed onto counting tubes and were counted with a gamma counter.

The animal studies in this project have been approved by the Research Ethical Committee, Faculty of Medicine, Universitas Kristen Maranatha – Immanuel Hospital, under protocol No. 143/ KEP PK UKM – RSI/ VIII/ 2011.

3 Results and Discussions

Unlike the previous report by Humani, *et al.* [6], where ^{177}Lu -DOTA-nimotuzumab was prepared in a three-step reaction, in this project ^{177}Lu -DOTA-nimotuzumab was prepared in a more simple way, in a reaction of only two steps, as shown in Figure 1. First an active ester of NHS-DOTA was conjugated to dialysed nimotuzumab at pH 7.3, which was followed by purification of the DOTA-nimotuzumab product by dialysing in 0.25 M ammonium acetate at pH 7.5. The purified DOTA-nimotuzumab was then kept in liquid or in freeze-dried form, ready to be labeled with ^{177}Lu . The radiolabeling of DOTA-nimotuzumab was carried out by adding $^{177}\text{LuCl}_3$ to 0.25 M ammonium acetate pH 7.5. The pH of the mixture was adjusted to 5.5, followed by heating at 37 $^{\circ}\text{C}$ for one hour. EDTA was then added to the mixture in order to bind free Lu^{3+} . Figure 2 shows the efficiency of the radiolabeling of ^{177}Lu toward-DOTA-nimotuzumab. It can be seen that there was around 14% ^{177}Lu bound to DOTA-nimotuzumab.

Reg	(mm) Start	(mm) Stop	(mm) Centroid	RF	Region Counts	Region CPM	% of Total	% of ROI
Rgn 1	5.4	24.4	14.8	0.074	25249.0	50498.0	14.16	14.49
Rgn 2	77.1	107.3	94.6	0.473	149001.0	298002.0	85.56	85.51
2 Peaks					174250.0	348500.0	97.72	100.00

Figure 2 Radiochromatogram ^{177}Lu -DOTA-nimotuzumab prior purification.

Note: Rf of ^{177}Lu -DOTA-nimotuzumab = 0
Rf of free ^{177}Lu in form of ^{177}Lu -EDTA = 1

Reg	(mm) Start	(mm) Stop	(mm) Centroid	RF	Region Counts	Region CPM	% of Total	% of ROI
Rgn 1	5.4	23.6	14.3	0.071	76405.0	152810.0	98.45	100.00
1 Peaks					76405.0	152810.0	98.45	100.00

Figure 3 Radiochromatogram of purified ^{177}Lu -DOTA-nimotuzumab.

Note: Rf of ^{177}Lu -DOTA-nimotuzumab = 0
Rf of free ^{177}Lu in form of ^{177}Lu -EDTA = 1

In order to obtain a radiopharmaceutical that has radiochemical purity $> 95\%$ (the minimum radiochemical purity for a good radiopharmaceutical) [7] the ^{177}Lu -DOTA-nimotuzumab was then purified by a size exclusion column (Sephadex G25 M). Figure 3 shows the radiochromatogram of the purified ^{177}Lu -DOTA-nimotuzumab. It can be seen that the radiochemical purity of the ^{177}Lu -DOTA-Nimotuzumab increased to 98.5% after purification; this product would be used for further testing.

A cytotoxicity test was carried out in order to determine the ability of ^{177}Lu -DOTA-nimotuzumab to kill cancer cells that expressed EFGR/HER-1 such as A-549 cell lines (positive EFGR/HER-1 lung carcinoma).

Figure 4 shows the absorbance of A-549 cells that were treated with either unlabeled nimotuzumab or ^{177}Lu -DOTA-nimotuzumab. It can be seen that the A-549 cells that were treated with ^{177}Lu -DOTA-nimotuzumab gave a lower absorbance at any given concentration compared to the absorbance of the A-549 cells lines that were treated with unlabeled nimotuzumab. The more significant one was at 250 ppm of ^{177}Lu -DOTA-nimotuzumab, where the absorbance of A-549 cells treated with ^{177}Lu -DOTA-nimotuzumab was significantly lower compared to the ones treated with unlabeled nimotuzumab. Absorbance is proportional to the concentration of the formed formazan, which in turn is proportional with the number of living cells [8]. Hence, it can be suggested that ^{177}Lu -DOTA-nimotuzumab is more effective in killing cancer cells compared to its counterpart of unlabeled nimotuzumab.

Figure 4 Absorbance of A-549 cells treated with unlabeled nimotuzumab and ^{177}Lu -DOTA-nimotuzumab.

A clearance test was aimed at finding out the tendency of how fast or how slow and in which way (urine or faeces) the radioactivity of the radiopharmaceutical was excreted post injection. This test was preformed by intravenously injecting a certain amount of ¹⁷⁷Lu-DOTA-nimotuzumab in two rats, which were then kept in metabolic cages. After certain time periods, urine and faeces were collected and then counted with a gamma counter. The percentage of radioactivity that was excreted either through urine or faeces was calculated by dividing the counts at the measured time with standard count multiplied by 100. The percentage of radioactivity excreted from urine and faeces up to 264 hrs post injection of ¹⁷⁷Lu-DOTA-nimotuzumab can be seen in Table 1.

Table 1 The percentage of radioactivity excreted from urine and faeces up to 240 hrs post injection of ¹⁷⁷Lu-DOTA-nimotuzumab in normal rats.

No.	Time(hours)	% Radioactivity	
		Urine	Faeces
1	0 – 24	1.43	0.56
2	24 – 48	3.09	2.58
3	48 – 72	3.31	0.68
4	72 – 96	2.86	0.68
5	96 – 168	9.18	0.68
6	168 – 192	2.26	0.42
7	192 – 216	2.71	0.53
8	216 – 240	2.86	0.55
9	240 – 264	2.20	0.36
Subtotal		29.94	6.89

From Table 1 it can be seen that clearance of radioactivity post injection of ¹⁷⁷Lu-DOTA-nimotuzumab occurred mostly via the urine. At any measured time the excretion of radioactivity via the urine varied between 2 and 9%. On the other hand, excretion of radioactivity via the faeces was mostly < 1% at any measured time. The total radioactivity excreted via urine and faeces 264 hrs post injection of ¹⁷⁷Lu-DOTA-trastuzumab was 29.94 and 6.89% respectively. The tendency of excretion of radioactivity via the urine was probably due to the negative charge of the injected radioimmunoconjugate.

Imaging and biodistribution tests were set up in order to measure the uptake of ¹⁷⁷Lu-DOTA-nimotuzumab in the targeted organ (cancer cells) and non-target organs. In these experiments the uptake of ¹⁷⁷Lu-DOTA-nimotuzumab was only determined in non-target organs. An imaging test was carried out by injecting 100 µCi of ¹⁷⁷Lu-DOTA-nimotuzumab in 2 rats/group. At designated times (3, 24, 48, 72 and 96 hrs) post injection the rats were anaesthetised and then imaged with a γ-camera. The images are shown in Figures 5, 6, 7, 8, and 9.

Figure 5 Image of a rat 3 hours post injection of ^{177}Lu -DOTA-nimotuzumab.

Figure 6 Image of a rat 24 hours post injection of ^{177}Lu -DOTA-nimotuzumab.

Figure 7 Image of a rat 48 hours post injection of ^{177}Lu -DOTA-nimotuzumab.

Figure 8 Image of a rat 72 hours post injection of ^{177}Lu -DOTA-nimotuzumab.

Figure 9 Image of a rat 96 hours post injection of ^{177}Lu -DOTA-nimotuzumab.

It can be seen from the above images that some radioactivity still remained in which seemed to be a hepatic area 96 hrs post injection of ^{177}Lu -DOTA-nimotuzumab. As the gamma camera images cannot give quantitative data, a biodistribution test was then performed on the mice. The biodistribution results in normal mice up to 144 hrs post injection of ^{177}Lu -DOTA-nimotuzumab are shown in Figure 10.

Figure 10 Biodistribution of ^{177}Lu -DOTA-nimotuzumab in normal mice.

It can be seen in Figure 10 that there were still some radioactive residues in organs such as liver (~6%), kidney (~5%) and bone (~3%) 144 hrs post injection of ^{177}Lu -DOTA-nimotuzumab. These results are nearly the same as those of MOv18 monoclonal antibody, which was radiolabeled with ^{177}Lu using benzyl-DOTA (Bz-DOTA) as bifunctional chelating agent, as reported by Zacchettia [9]. The radioactive residues were reported to be around 5, 3 and 7% in liver, kidney and bone of nu/nu tumour-bearing A431MK mice, respectively, and around 5, 2, and 4% in liver, kidney and bone nu/nu A43FR tumour-bearing mice, respectively 144 hrs post injection of ^{177}Lu -Bz-DOTA- MOv18 [9,10]. However, the radioactive residue in the mice livers 144 hours post injection of ^{177}Lu -DOTA-nimotuzumab was found to be slightly lower (~6%) compared to that of the radioactive residue of ^{177}Lu -p-SCN-Bz-DOTA-cetuximab in nu/nu tumour-bearing A431 mice (~8%), as reported by Lars R. Perk [10].

The traces of radioactivity in the liver of the normal mice up to 144 hours post injection of ^{177}Lu -DOTA-nimotuzumab indicate that some radioimmunoconjugate was metabolised or catabolised in this organ. This is not an unusual phenomenon, since the liver is known as an organ where high molecular weight molecules such as monoclonal antibody (150-180 K Dalton), metabolised or catabolised. However, a good radioimmunoconjugate is expected not to retain a high radioactive residue in non-target organs such as liver, lung and other sensitive organs, so that these organs are not exposed to unnecessary radiation. Therefore, if this radioimmunoconjugate is going to be tested on cancer patients, precautions have to be observed.

4 Conclusions

In vitro and *in vivo* testing of ^{177}Lu -DOTA-nimotuzumab with a radiochemical purity of > 95% has been performed. The cytotoxicity test on A-549 cells (lung carcinoma) showed that ^{177}Lu -DOTA-nimotuzumab kills more cancer cells compared to unradiolabeled nimotuzumab. The clearance test of ^{177}Lu -DOTA-nimotuzumab on normal mice showed that the radioactive residue was excreted mostly via the urine. The image and biodistribution tests showed that there were still some radioactive residues in a number of organs.

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