

¹¹¹In-labeled Nimotuzumab Modified with Nuclear Localization Sequences (NLS) for Targeting Herceptin® Resistant Breast Cancer Cells



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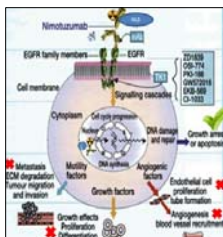
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Abstract

Objective: Nimotuzumab (Nmb) is a monoclonal antibody selectively targeting cancer cells overexpressing epidermal growth factor receptor (EGFR). Abnormal up-regulation of EGFR is found to be present in breast cancer (BC) cells which are resistant to trastuzumab (Herceptin®) and is associated with poor prognosis. Currently, there are few agents that target EGFR on BC cells with acquired resistance to trastuzumab. Thus, our objective is to modify an already established anti-EGFR antibody (Nmb) with an Auger emitting radioisotope Indium-111 to target cells that are either intrinsically resistant or acquire resistance to Herceptin. **Methods:** Nmb was derivatized with sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) for reaction with NLS-peptides and labeled with Indium-111 using benzyl-diethylenetriaminepentaacetic acid (bn-DTPA). The immunoreactivity of ¹¹¹In-NLS-Nmb was determined by its ability to displace the binding of Nmb in MDA-MB-468 human breast cancer cells. Cellular uptake and nuclear localization were evaluated in MDA-MB-468 and MCF-7 cells, which express high and very low levels of EGFR, respectively, by cell fractionation. Finally, the cytotoxicity of ¹¹¹In-Nmb and ¹¹¹In-NLS-Nmb was studied by clonogenic assays. **Results:** The characterization of ¹¹¹In-NLS-Nmb by instant thin layer chromatography (ITLC) and SDS-PAGE showed there were 2.09±2.21 bn-DTPA and 8.5±0.20 NLS per antibody. Furthermore, the radiochemical purity and specific activity was found to be 99.23±0.12% and 350±9.50 MBq/mg respectively. The dissociation constant for binding of ¹¹¹In-NLS-Nmb to MDA-MB-468 cells was 9.8±0.86 nM. In the case of ¹¹¹In-NLS-Nmb 31.2±2.59% of the radioactivity associated with MDA-MB-468 cells was found in the nucleus compared to only 16.0±0.63% for ¹¹¹In-Nmb. For comparison, ¹¹¹In-EGF had only 2.4±0.11% internalized in the nucleus. Moreover, the internalization was significantly lower in MCF-7 cells which express low levels of EGFR similar to that on normal epithelial tissues. The clonogenic survival of MDA-MB-468 cells exposed for 3 days to 20nM of Nmb, NLS-Nmb, ¹¹¹In-Nmb and ¹¹¹In-NLS-Nmb was 60±7.04%, 61±6.5%, 21±5.3%, and 2.8±0.16% respectively. The CS of MCF-7 cells exposed to ¹¹¹In-NLS-Nmb (40 nM) was 82.6±6.4% vs. 80.0±1.6% for ¹¹¹In-Nmb (p>0.05) and 95±19.4% for Nmb (p>0.05). Preservation of selectivity for abnormal EGFR overexpression of the Nmb conjugate supports further development of this potent conjugate despite ubiquitous EGFR expression in normal tissues.

Background

In the past our laboratory has developed a novel radiotherapeutic agent, ¹¹¹In-labeled human EGF (¹¹¹In-hEGF) which exploits the overexpression of EGFR found in 30-50% of cases of ER-negative, hormone resistant and poor prognosis breast cancers (BC)^(1,2). Although ¹¹¹In-hEGF exhibited potent cytotoxic effects towards EGFR-overexpressing MDA-MB-468 cells alone or in combination with other drugs such as gefitinib, it had a very short half-life (24-36 mins) in mice and was also eliminated very rapidly from the blood after intravenous injection in BC patients. This rapid elimination resulted in low tumour uptake. As an alternative, in this study we now examine the EGFR-dependent cytotoxicity of the humanized IgG, mAb Nmb (h-R3) (YM-BioSciences) labeled with ¹¹¹In which is expected to have a longer half-life and greater tumour uptake. Nmb binds to the extracellular domain of EGFR and blocks ligand activation, resulting in inhibition of EGFR activation and cancer cell proliferation as well as production of VEGF. Severe side-effects are rarely observed with Nmb, in contrast to the currently approved high-affinity anti-EGFR antibodies, cetuximab (Erbix[®]) and panitumumab (Vectibix[®]). The severe side-effects with the other antibodies are predominantly caused by binding to EGFR in normal tissues. Nmb's improved safety profile is a result of selectively targeting cancer cells overexpressing EGFR due to intermediate affinity. The selective targeting of cancer cells makes Nmb the preferred anti-EGFR antibody for conjugation.



Proposed model of action of ¹¹¹In-NLS-Nmb

Objectives

Hypothesis

¹¹¹In-NLS-Nmb (EGFR mAb) will be able to deliver lethal, DNA damaging doses of radiation specifically to the nucleus of trastuzumab resistant and EGFR-overexpressing BC cells while minimizing toxicity towards normal tissues.

Objectives

1. Development and characterization of ¹¹¹In-NLS-Nmb
2. Analyze receptor binding properties by using direct radioligand binding assays
3. Cell internalization and nuclear localization studies
4. Clonogenic assays to evaluate ¹¹¹In-NLS-Nmb effects

Breast Cancer Cell Lines

1. MDA-MB-468: High EGFR expression: Insensitive to trastuzumab
2. MDA-MB-231:
3. TrR1: Intermediate EGFR expression: Low sensitivity to trastuzumab
4. TrR2: } Low EGFR expression: Insensitive to trastuzumab
5. MCF-7:

EGFR expression (receptors/cell): High (0.5-1.0 x 10⁶), Intermediate (0.2-0.7 x 10⁶) and Low (<0.2 x 10⁶)

Results

Construction and Characterization of ¹¹¹In-NLS-Nmb

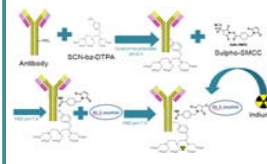


Fig.1. Diagram showing construction of ¹¹¹In-NLS-Nmb

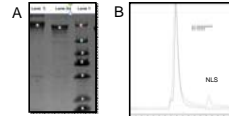


Fig.2. (A) SDS PAGE shows the conjugation of Nmb with NLS (8.5±0.20NLS per antibody) indicated by the band shift. Lane 2a shows the un conjugated antibody and lane 3 shows the NLS-Nmb (B) HPLC chromatogram of Nmb (Black) and ¹¹¹In-NLS-Nmb (Green). This indicates that conjugate is pure and no aggregates were formed during conjugation.

Radioligand Binding Assay

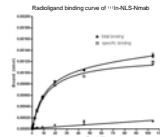


Fig.3. Characterization of the binding of ¹¹¹In-NLS-Nmb to EGFR on MDA-MB-468 cells by radioligand saturation binding assays. The dissociation constant for binding of ¹¹¹In-NLS-Nmb to MDA-MB-468 cells was 9.8±0.862 nM and Bmax was 0.00121nmol.

Internalization Assay

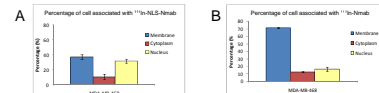


Fig.4. Internalization assay with respect to the radioactivity associated to the cells. (A) Internalization of ¹¹¹In-NLS-Nmb by MDA-MB-468 BC cells. (B) Internalization of ¹¹¹In-Nmb by MDA-MB-468 cells.

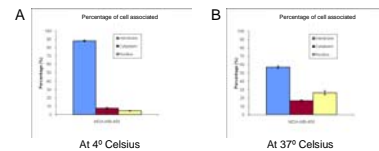


Fig.5. Internalization of ¹¹¹In-NLS-Nmb at 4°C and 37°C in MDA-MB-468 cells with respect to the radioactivity associated with the cells (A) shows internalization at 4°C and (B) shows internalization at 37°C.

Internalization Assay

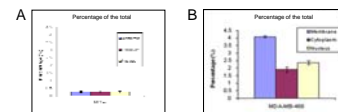


Fig.6. Internalization of ¹¹¹In-NLS-Nmb in (A) MCF-7 BC cell line and (B) MDA-MB-468 with respect to the total amount of radioactivity added to incubation media.

Clonogenic Assay

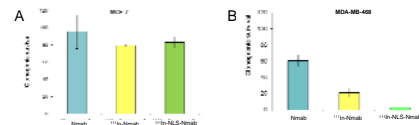
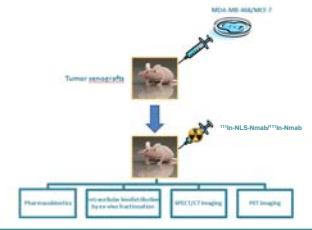


Fig. 7. Clonogenic survival of cells treated for 3 days with Nmb or ¹¹¹In-Nmb or ¹¹¹In-NLS-Nmb (A) MCF-7 cells (B) MDA-MB-468 cells.

Future Studies

1. *In vitro* studies
 1. Do further clonogenics on Herceptin resistant cell lines such as MDA-MB-231, TrR1, TrR2
 2. Gamma H2AX assay to assess DNA damage
 3. Confocal microscopy studies to visualize nuclear importation
2. *In vivo* studies with ¹¹¹In-NLS-Nmb in Breast Cancer Xenografts
 1. Biodistribution studies in athymic mice to evaluate tumour and normal tissue localization
 2. PET imaging studies to measure tumor response



Conclusions

- ❖ The binding of ¹¹¹In-NLS-Nmb to EGFR on MDA-MB-468 cells was conserved and the NLS promoted higher nuclear uptake compared to ¹¹¹In-Nmb without NLS.
- ❖ NLS-peptides routed ¹¹¹In-Nmb into the nucleus of EGFR-positive human BC cells, rendering the radiopharmaceutical lethal through the emission of nanometer range Auger electrons.
- ❖ The greater cytotoxic potency of ¹¹¹In-NLS-Nmb compared to Nmb *in vitro* and its ability to discriminate between cells with overexpressed EGFR (e.g. MDA-MB-468) compared to normal levels of EGFR (e.g. MCF-7) suggests that it could be an effective targeted radiotherapeutic agent for EGFR-positive and trastuzumab-resistant BC in humans.

References and Acknowledgements

1. Harris AL, Nicholson S, Sainsbury R, Wright C, Farnon J. Epidermal growth factor receptor and other oncogenes as prognostic markers. *J Natl Cancer Inst Monogr.* 1992(11):181-7.
2. Sainsbury JRC, Farnon JR, Needham GK. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet.* 1987;1(8547):1398-402.
3. Spicer J. Technology evaluation: Nimotuzumab, the center of molecular immunology/YM BioSciences/Oncoscience. *Curr Opin Mol Ther.* 2005;7(2):182-91.
4. Snyder LC, Astsaturov I, Weiner LM. Overview of monoclonal antibodies and small molecules targeting the epidermal growth factor receptor pathway in colorectal cancer. *Clin Colorectal Cancer.* 2005;5(SUPPL. 2)
5. Allan DGP. Nimotuzumab: Evidence of clinical benefit without rash. *Oncologist.* 2005;10(9):760-1.



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