User project report

Delivering molecularly targeted radionuclide therapy to alleviate immune suppression in glioblastoma

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Project Title: Delivering molecularly targeted radionuclide therapy to alleviate immune suppression in glioblastoma.

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Background

Patients with glioblastoma (GBM) have a poor prognosis following standard therapy (median survival is 12-15 months), with a 5-year survival rate of only 3-5% [1]. Currently, the standard GBM treatment includes maximal resection (complete resection is achieved extremely rarely due to the diffusely infiltrative nature of these tumours) followed by radiotherapy with concomitant and adjuvant systemic therapies e.g., temozolomide [2]. Despite these aggressive regimens, most patients become refractory to treatment and succumb to disease [3]. Therefore, there is high unmet medical need for new treatment paradigms that lead to more durable remissions.

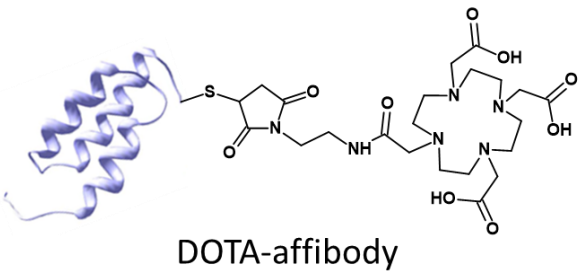
We hypothesise that locoregional administration of molecularly targeted radionuclide therapy with highly cytotoxic radioisotopes (β- or α emitters) associated to EGFR specific vector will selectively induce cell death in EGFR-positive GBM cells, while limiting toxicity in the surrounding normal tissues. Furthermore, we postulate that this therapeutic approach will decrease the immunosuppressive GBM tumour microenvironment (TME) and enhance the sensitivity to checkpoint inhibitors.

To address our hypothesis, we evaluated an EGFR-specific affibody molecule (~7 kDa) radiolabelled with two β- emitters: 177-lutetium (^{177}Lu) and 161-terbium (^{161}Tb) in mice bearing subcutaneous GBM tumours.

Results

Conjugation of DOTA to EGFR affibody molecule ($Z_{EGFR:03115}$)

DOTA has been selected as the suitable chelator for both ^{177}Lu and ^{161}Tb. Before the radiolabelling reaction, the DOTA-$Z_{EGFR:03115}$ conjugate was prepared. The production of the DOTA-$Z_{EGFR:03115}$ conjugate (**Fig. 1**) was performed by attaching a maleimide-functionalised DOTA chelator to the cysteine containing (C-terminus) $Z_{EGFR:03115}$ molecule by slightly modifying a method previously published in the literature [4]. The product was purified by semipreparative RP-HPLC (**Fig. 2**).



DOTA-affibody

Fig. 1. Structure of DOTA-$Z_{EGFR:03115}$ conjugate.

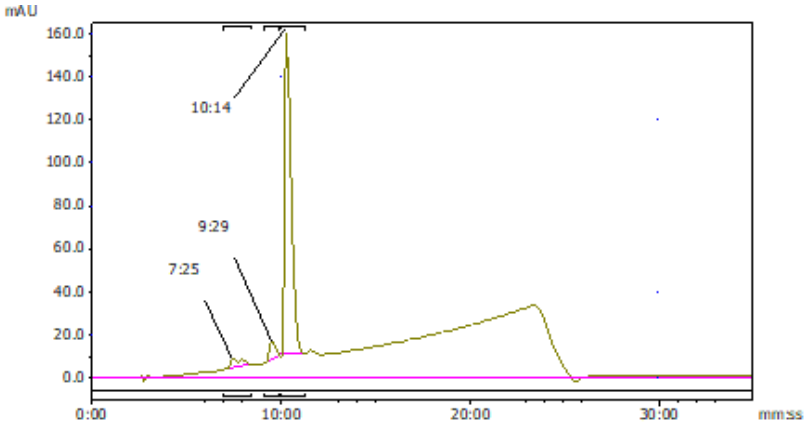


Fig. 2. Representative chromatogram of purified DOTA-$Z_{EGFR:03115}$ registered at 230 nm. Eluents: A= 0.1% TFA in water (v/v); B = 0.1% in acetonitrile (v/v). Column: Zorbax-300SB-C18, 4.6 × 250 mm, 5 µm. Using the same column and elution system, the affibody dimer (control) elutes at 11:58 min:ss and the reduced affibody molecule (monomer) at 10:58 min:ss.

Preparation of ^{177}Lu-DOTA-$Z_{EGFR:03115}$

The reaction conditions were tested and confirmed by radiolabelling DOTA-$Z_{EGFR:03115}$ with $^{177}LuCl_3$. The optimised conditions were then be applied to ^{161}Tb. DOTA-$Z_{EGFR:03115}$ was radiolabelled with $^{177}LuCl_3$ (in 0.05M HCl, 70-100 MBq) at pH 5.5 (achieved by the addition of 0.5 M ammonium acetate buffer pH 5.5) to have a theoretical specific activity of 3 MBq/µg. Ethanol (15% v/v) and ascorbic or gentisic acid (ca 11.3 mg/mL) were added as stabilisers to limit the radiolysis of the protein. The mixture was incubated for 15 min at 80°C in a thermoshaker (500 rpm). The selection of the theoretical specific activity, the reaction time and temperature were based on previous work done in our group. The radiometal incorporation was confirmed by ITLC using ITLC-SG strips and citrate pH 5 as mobile phase and by analytical RP-HPLC.
The product was purified either by solid phase extraction (SPE) using an HLB cartridge or by gel filtration using a zeba column (7 kDa MWCO). The radiochemical purity (RCP) was confirmed by ITLC using ITLC-SG strips (to assess the presence of free ^{177}Lu) and by analytical RP-HPLC.
The recovery of the product was approximately 44% and 50-68% from the HLB cartridge and zeba column, respectively. No free ^{177}Lu was detected in the purified product, however few impurities were present, probably from radiolysis, yielding products with RCPs of approximately 92% (**Fig. 3**) and apparent specific activities of 2 MBq/µg.

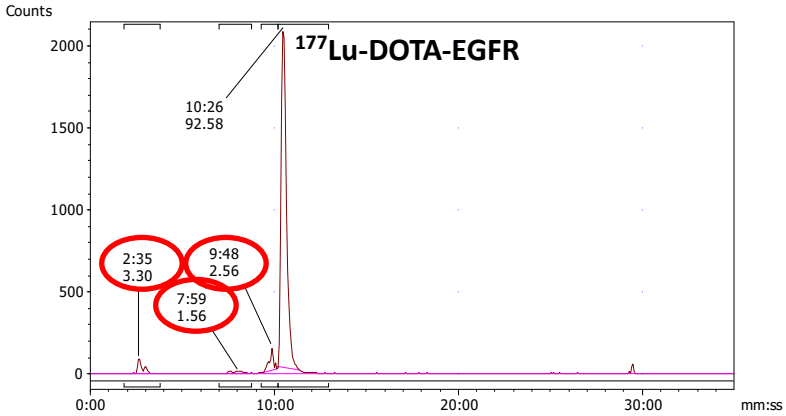


Fig. 3. Representative radiochromatogram of purified ^{177}Lu-DOTA-$Z_{EGFR:03115}$ (Rf = 10:26 min:ss). The radiolysis products are highlighted in red.

Of note: The retention time (Rt) is the top number. The bottom number indicates the ROI%.

After 4 h at 24°C the product, containing gentisic acid (43.5 µg), showed high stability, assessed by RP-HPLC (**Fig. 4**).

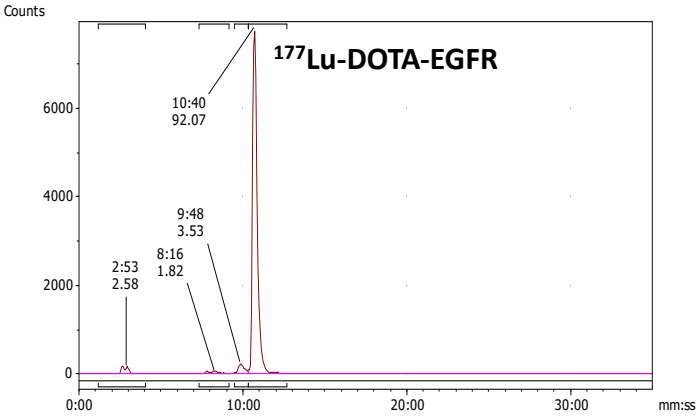


Fig. 4. Representative radiochromatogram of purified ^{177}Lu-DOTA-$Z_{EGFR:03115}$ (Rf = 10:26 min:ss) after 4 h at RT.

Of note: The stability of the purified product will be evaluated in PBS and mouse serum in upcoming studies.

Preparation of ^{161}Tb-DOTA-$Z_{EGFR:03115}$

DOTA- $Z_{EGFR:03115}$ was radiolabelled with $^{161}TbCl_3$ (in 0.05M HCl, 100-160 MBq) at pH 4.5 (achieved by the addition of 0.5 M ammonium acetate buffer pH 4.5) to have a theoretical specific activity of 3 MBq/µg. Ethanol (15% v/v) and gentisic acid (23 mg/mL) were added as stabilisers to limit the radiolysis of the protein. The mixture was incubated for 15 min 75°C in a thermoshaker (500 rpm). The radiometal incorporation was confirmed by ITLC using ITLC-SG strips and 50 mM EDTA pH 5 as mobile phase and by analytical RP-HPLC. No free ^{161}Tb was detected by ITLC in the product, however few impurities were present in the HPLC trace, probably from radiolysis, yielding products with RCPs of approximately 92% (**Fig. 5**) and apparent specific activities of 3 MBq/µg. The lower reaction temperature, compared to ^{177}Lu, allowed a complete incorporation of the radiometal with the production of less degradation products. Therefore, no further purification of the product was required.

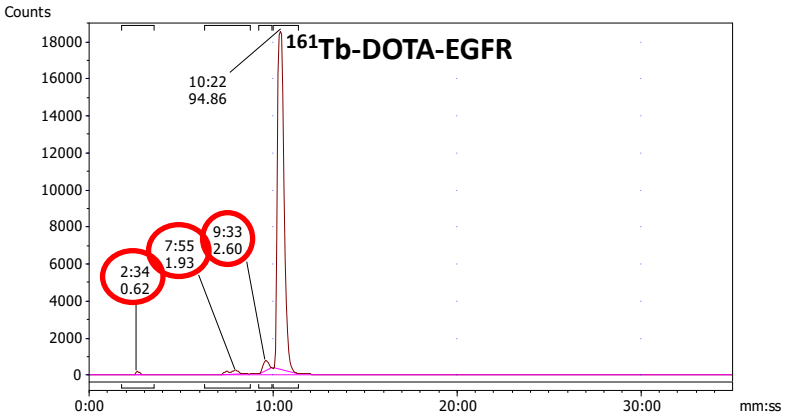


Fig. 5. Representative radiochromatogram of purified ^{161}Tb-DOTA-$Z_{EGFR:03115}$ (Rf = 10:22 min:ss). The radiolysis products are highlighted in red.

^{161}Tb-DOTA-Z$_{EGFR:03115}$ stability

Initially, the benefits of the addition of a stabiliser to the purified were tested. A comparison between a solution of ^{161}Tb-DOTA-Z$_{EGFR:03115}$ with and without sodium ascorbate (10 mg) was performed (**Fig. 6**).

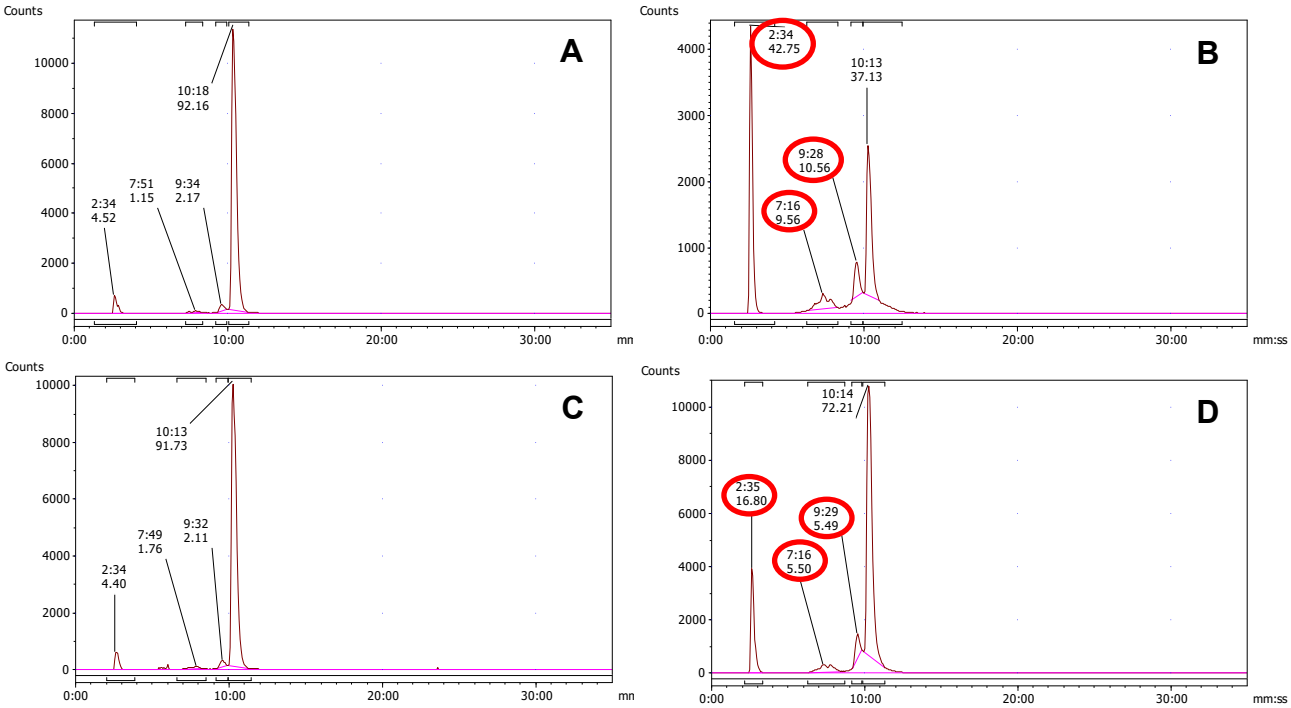


Fig. 6. Representative radiochromatograms of freshly prepared ^{161}Tb-DOTA-Z$_{EGFR:03115}$ (A and C) and after 3-4 days at room temperature with (D) or without (B) ascorbate.

In the presence of a stabiliser, the product retains a RCP of more than 72% compared to 37% when no radioprotectant was added after days from the preparation. The main radiolysis product is highly hydrophilic and elutes with the solvent front (2:35 min:ss) and was not unchelated ^{161}Tb since no free ^{161}Tb was detected by ITLC analysis (**Fig. 7**).

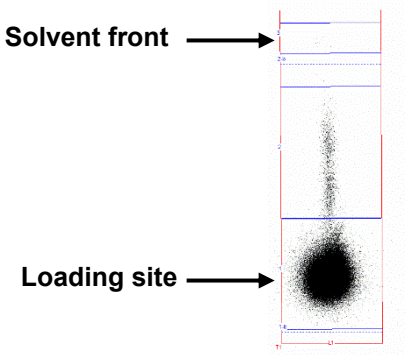


Fig. 7. Representative ITLC analysis of 4 days old ^{161}Tb-DOTA-Z$_{EGFR:03115}$ with ascorbate on an ITLC-SG strip and using 50 mM ETDA pH 5 as mobile phase. ^{161}Tb-DOTA-Z$_{EGFR:03115}$ stays at the loading site (Rf =0) while uncoordinated ^{161}Tb runs with the solvent front (Rf =1). Radiolysis products have Rf=0.3-0.5.

To try clarifying the nature of the degradation products, the peaks were collected by HPLC and analysed by ITLC (**Fig. 8**).

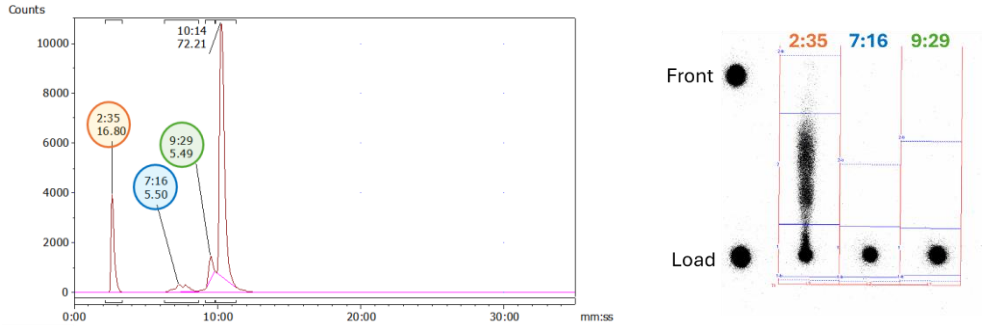


Fig. 8. Chromatogram showing the three separate peaks collected (left) and then analysed by ITLC (right).

The ITLC analysis showed that by staying at the loading site, like ^{161}Tb-DOTA-Z$_{EGFR:03115}$, the products eluting at 7:16 and 9:29 min:ss are of protein nature and, being radioactive, contain the chelator. Showing no activity eluting with the mobile phase front, the peak eluting at 2:35 min:ss is confirmed not to be free ^{161}Tb. Additionally, this early peak is a mixture of at least 3 small and hydrophilic products (probably of peptide nature) and, since they are radioactive, all contain the chelator.

Stability studies

Based on the short biological half-life of affibody molecules *in vivo* (i.e. <1 h), the stability of ^{161}Tb-DOTA-Z$_{EGFR:03115}$ was tested up to 24 h and not longer.

Stability in PBS

The purified product, containing ascorbate (10 mg), was incubated in PBS pH 7.4 at 24°C. The samples (n=3) were analysed by ITLC (to assess the degree of demetallation) and HPLC (to confirm the RCP) after 1h and 24 h. ^{161}TbCl$_3$ was used as control. No free ^{161}Tb was detected at any time point (**Table 1**). Analysed by HPLC, the samples contained ^{161}Tb-DOTA-Z$_{EGFR:03115}$, of decreasing purity over time (Table 1), and the previously observed degradation products (**Fig. 8**) which amounts increased over time.

Table 1.

Time [h]	RCP ^{161}Tb-DOTA-Z$_{EGFR:03115}$ [%]	Free ^{161}Tb [%]
0	92	0
1	91.70±0.06	0
24	84.30±2.47	0

Stability in mouse serum

Purified ^{161}Tb-DOTA-Z$_{EGFR:03115}$, containing ascorbate (10 mg), was incubated in mouse serum at 37°C. The samples (n=3) were analysed by ITLC (to assess the degree of demetallation) and HPLC (to confirm the RCP) after 24 h. ^{161}TbCl$_3$ was used as control. No free ^{161}Tb was detected by ITLC analysis.

Before the HPLC analysis, the mouse serum samples (n=3) were processed by adding ethanol followed by DMF to precipitate the serum proteins. After 24 h, 69.12% of the activity was associated with serum proteins (**Table 2**). The supernatants were analysed by HPLC after filtration and showed a RCP of the product of 55.47±3.6% (**Fig. 9**).

Table 2.

	$^{161}TbCl_3$ [% of initial activity]	**^{161}Tb-DOTA-$Z_{EGFR:03115}$ [% of initial activity]**
Pellet from EtOH	41.89	55.53±2.69
Pellet from DMF	53.63	13.59±1.33
Supernatant	4.48	30.88±1.4

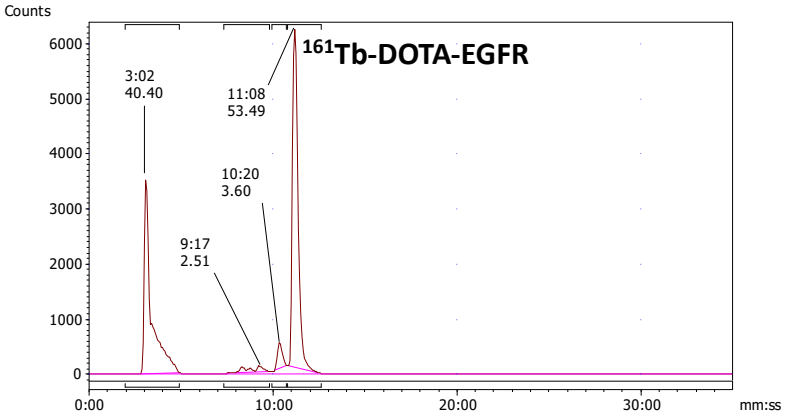


Fig. 9. Mouse serum stability of ^{161}Tb-DOTA-$Z_{EGFR:03115}$ (24 h): representative radiochromatogram of the supernatant after the precipitation of the serum proteins.

Of note: Alternative stabilisers to ethanol and gentisic acid will be investigated in the future aiming to preserve a higher degree of product integrity.

References

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