MR Imaging of U87ΔEGFR Human Glioma Tumor Xenografts Using Targeted Signal-Amplifying Enzymatic System

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Introduction: A truncated and constitutively active form of the EGF receptor variant III (EGFRvIII) is a major determinant of tumor growth and poor prognosis in glioblastoma multiforme (GBM) [1]. To test a system for targeted imaging of EGFRvIII we investigated the retention of peroxidase-generated products of a paramagnetic molecular substrate di(tyramido)-DTPA(Gd) (diTyr-DTPA(Gd), Fig. 1) in U87ΔEGFR human glioma xenografts. F(ab')$_2$ fragments of humanized anti-EGFRvIII monoclonal antibody (mAb) EMD72000 were conjugated to deglycosylated horseradish peroxidase (HRP) and glucose oxidase (GOX) and used as a self-complementing enzymatic signal amplification system [2] for EGFRvIII targeted imaging. We anticipated that receptor expression sites will exhibit a prolonged MR signal enhancement due to the formation of polymerized products of diTyr-DTPA(Gd) oxidation by mAb conjugates [2].

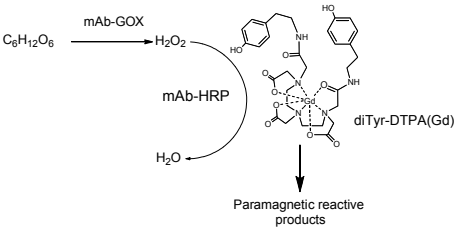

Fig. 1 – Peroxidase substrate diTyr-DTPA(Gd) reaction with the mAb-conjugated enzyme pair (GOX/HRP).

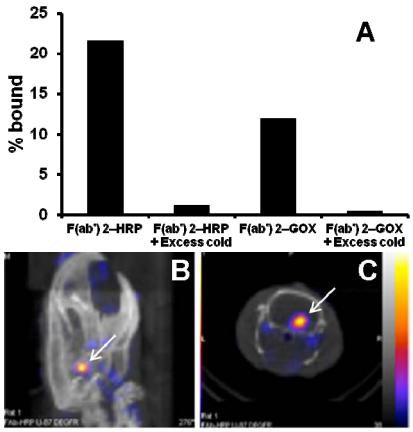

Fig. 2 – Specificity of ^{99m}Tc-labeled conjugates in A) U87ΔEGFR cell assay; SPECT/CT images of rat brain in the B) sagittal and C) axial orientations showing accumulation of ^{99m}Tc-labeled antibody conjugates in U87ΔEGFR tumors. Arrows indicate position of glioma.

Methods: The paramagnetic substrate diTyr-DTPA(Gd) was synthesized as described in [3]; F(ab')$_2$ fragments of mAb were linked to deglycosylated and hydroxylamine-capped HRP or GOX via bisaromatic hydrazone bonds and purified by size-exclusion HPLC. The purified conjugates were characterized in human glioma U87ΔEGFR cell culture and optimal ratios of HRP and GOX conjugates were determined to provide the maximum signal with low cytotoxicity. F(ab')$_2$-GOX and F(ab')$_2$-HRP were also modified with NHS-MAG$_3$, for radiolabeling with ^{99m}Tc to study the binding and uptake of the conjugates *in vitro* and *in vivo*. For *in vivo* studies, $5{\cdot}10^4$ U87ΔEGFR cells were stereotaxically implanted in the brains of RNU rats. T1-weighted (T1-WT) spin-echo MRI at 3T was performed with the following parameters: TR/TE=700ms/8.2ms, FOV=2.56cm×2.56cm, matrix = 256×256, NEX=4. Ten days after tumor implantation, each animal was imaged on two occasions under isoflurane anesthesia. 1) Day 1 – a pre-contrast image was acquired followed by IV injection of 0.1 mmol/kg diTyr-DTPA(Gd). T1-WT images were then acquired over a 1.5-h period. 2) Day 2 – anti-EGFRvIII conjugates (100 µg mAb/animal) were injected IV. Four hours later, a pre-contrast image was acquired followed by IV injection of 0.1 mmol/kg diTyr-DTPA(Gd). T1-WT images were acquired over a 2-h period. Animals were sacrificed and frozen brain sections were stained for peroxidase activity and EGFR expression.

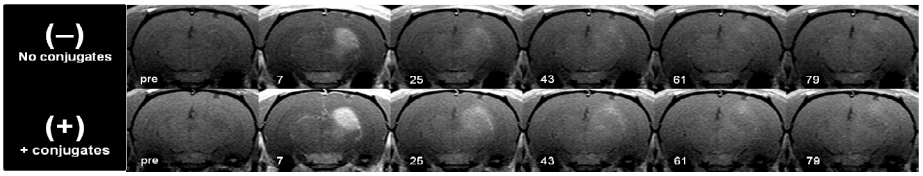

Fig. 3 – U87ΔEGFR cell-surface binding and internalization of the conjugate mixture at the optimized HRP/GOX ratio.

Fig. 4 – T1-WT sequential rat brain images of U87ΔEGFR xenografts after the injection of diTyr-GdDTPA; (–) temporal washout of diTyr-GdDTPA with no conjugate pre-injection; (+) washout of diTyr-GdDTPA following IV injection with anti-EGFRvIIIconjugates in the same animal matched slices (time in minutes).

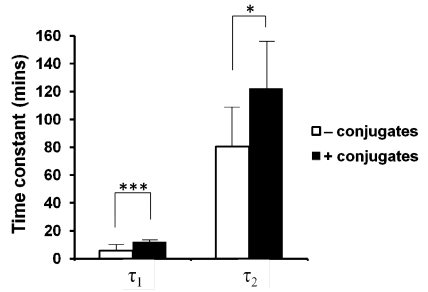

Fig. 5 – DTCs for diTyr-DTPA(Gd) in the absence (n = 4) and presence (n = 3) of conjugates in U87ΔEGFR human glioma xenografts. Both τ_1 and τ_2 showed significant conjugate-dependent differences (*p=0.01, ***p=0.0001).

Results and Discussion: Both ^{99m}Tc-labeled antibody conjugates showed specific binding to U87ΔEGFR cells *in vitro* (Fig. 2A) and *in vivo* (Fig. 2BC). The binding was inhibited by the excess of cold mAb conjugates (Fig. 2A). Cell-binding and internalization studies showed that 85% of total cell-bound conjugates were retained on the surface at 4°C, whereas ~80% conjugates were internalized at 37°C (Fig. 3). T1-WT images showed significantly higher initial enhancement of the tumor pre-injected with conjugates (Fig. 4, (+) 7 min) than that observed in the same animal without pre-injection of conjugates (Fig. 4 (–) 7 min). The retention period of contrast agent was much longer on Day 2 in rats pre-injected with conjugates (Fig. 4 (+)) over the same time period compared to Day 1. The washout of the contrast agent was best modeled using a biexponential decay equation: $P(t) = s_1{\cdot}exp(-t/\tau_1) + s_2{\cdot}exp(-t/\tau_2)$. Bioelimination of diTyr-DTPA(Gd) was quantified by fitting the temporal signal-intensity decay for each day in the absence and presence of anti-EGFRvIII conjugates. In the current study, a short (τ_1) and long (τ_2) decay time constant (DTC) was observed as compared to Gli36ΔEGFR-bearing rats reported by us previously [4]. Gli36ΔEGFR displayed a monoexponential MR signal decay on Day 1 and a biexponential decay on Day 2 whereas U87ΔEGFR tumors showed biexponential decay on both days. Both the DTCs (τ_1 and τ_2) were significantly longer on Day 2 compared to Day 1 (Fig. 5) corresponding to the presence of paramagnetic products due to the conjugate co-localization at the EGFRvIII target sites. The DTCs (τ_1 and τ_2) on Day 2 for both Gli36ΔEGFR and U87ΔEGFR animals were not significantly different (Table 1) indicating a similarity of conjugate accumulation in both tumor models which resulted in a similar bimodal washout of the contrast agent.

	τ_1 (min)	τ_2 (min)
U87Δ (Day 1)	6±3	81±28
U87Δ (Day 2)	12±0.4	122±35
Gli36Δ (Day 2)	17±9	127±44

Table 1 – Comparison of decay time constants between Gli36ΔEGFR [4] and U87ΔEGFR human gliomas.

Conclusion: Administration of EGFRvIII-targeted mAb conjugates resulted in specific binding to U87ΔEGFR cells of which at least ~20% remained on the surface enabling the reaction with the contrast agent. Following conjugate administration *in vivo* (Day 2), the retention of contrast agent was attributed to the longer DTCs compared to that of Day 1. These longer DTCs are consistent with enzyme-mediated retention of the paramagnetic products in the EGFRvIII-overexpressing cells in the tumor.

References: [1] Hu, J *et al*. (2011) PNAS **108**:15984-15989; [2] Bogdanov, A., *et al.* (2007). Bioconjug Chem **18**: 1123-1130; [3] Querol, M., *et al.* (2007). Chembiochem **8**: 1637-1641; [4] Shazeeb, M.S., *et al.* (2011). Cancer Res **71:** 2230-2239.