EGF receptor expression imaging using antibody-conjugated enzymes and a paramagnetic substrate.

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INTRODUCTION. Recently, several classes of anticancer agents specifically inhibiting EGFR-mediated signal transduction were clinically tested in an attempt to decrease invasive tumor progression while inducing cell apoptosis in advanced pancreatic, colorectal, non-small cell lung and head and neck cancers. Several clinical trials use anti-EGFR regimens alone or in combination with other therapies [e.g., monoclonal antibodies (cetuximab, panitumumab, matuzumab) or receptor tyrosine kinase inhibitors (erlotinib and gefitinib)]. We previously described several paramagnetic chelates that undergo rapid oxidation and oligomerization in the presence of peroxidase and myeloperoxidase [1,2]; (MRamp effect). The current research extended these studies into EGF-receptor imaging *in vivo* using the therapeutic anti-EGFR antibody (matuzumab, αEGFR)-targeted enzyme delivery. By using these targeted enzymes in a combination with the bifunctional paramagnetic electron donor compound (i.e. carrying two reactive residues of 5-hydroxytryptamide per one chelate), we tested the specificity of antibody conjugate delivery and the sensitivity to EGFR expression *in vivo*.

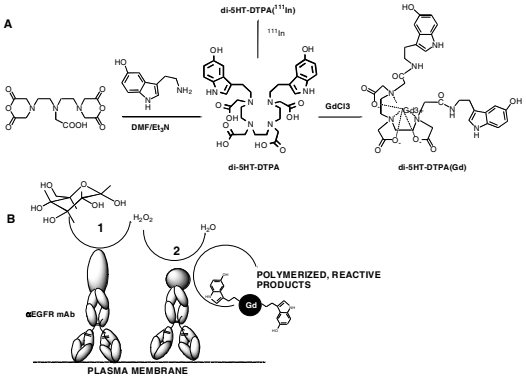

Fig. 1. **A** - Synthesis of di-5HT-DTPA and metal chelates. **B**- HRP/GO targeted coupled catalysis in the presence of glucose. Di-5HT-DTPA(Gd) is used as a reducer of oxidized HRP.

METHODS. Peroxidase substrate di-5HT-DTPAGd was synthesized as in Fig. 1A [3], chelated with Gd and purified by C18-HPLC. Enzymes were covalently conjugated to αEGFR mAb using bisaromatic hydrazone bond method (SANH/SFB (Pierce) and purified on a Superdex 200 FPLC column (GE); Fig 1B. Conjugates were tested using ELISA in A431 and SW620 (negative control) cell culture using ABTS as HRP substrate. Radioimmunoassays were performed by incubating cells in the presence of various conjugate concentrations and di-5HT-DTPA[^{111}In]. Enzymatically coupled HRP/GO-mediated relaxivity changes in the presence of glucose were measured at 0.47T using 0.1-2.5 mM di-5HT-DTPAGd (Table 1). Mice (*nu/nu*) were implanted with $2 \cdot 10^6$ A431 or SW620 cells s.c. in the femoral muscle area. Biodistributions were performed in groups of n=3. Animals were imaged under 1.5% isofluorane anesthesia at two weeks after the implantation. MR images were acquired using a Bruker Biospin 2.0T/45 cm imaging spectrometer equipped with ±20G/cm self-shielded gradients. A baseline set of T1-weighted, spin-echo (SE) MRIs was obtained prior to the injection of the antibody conjugates. TR/TE = 400/8.0 ms, FOV = 3 cm x 3 cm, matrix = 256x128, NEX = 8; 12 images acquired consecutively over a 3-h period after the injection of 0.2 mmol/kg di-5HT-DTPA(Gd) via a tail vein catheter. The animals were imaged again at 18h after antibody-conjugate administration using the same dose of the contrast agent. IHC of frozen sections was performed using αEGFR and anti-CD31 mAbs labeled with digoxigenin followed by anti-digoxigenin-AP conjugates.

Table 1. Properties of 5HT-DTPAGd as a MRamp substrate in the presence of the enzymes covalently linked to αEGFR (0.5 μg mAb and 5mM glucose in PBS, pH 7.2.)

Reactants (solutions in PBS)	R_1/[Gd] (0.47T, 40°) [mM· s] $^{-1}$	R_1 increase	Kinetic constants, [4] (k1+k-1)/s^{-1}
Di-5HT-DTPA-Gd	4.35	-	NA
Di- 5HT-DTPA-Gd/ αEGFR-HRP	4.6	1.07	<0.00002
Di-5HT-DTPA-Gd(PBS) / αEGFR-HRP/ αEGFR-GO	9.7	2.20	0.0075±0.0020

RESULTS AND DISCUSSION. Covalent conjugates of αEGFR mAb, HRP and GO had an average mass of 410 kD. In the presence of di-5HT-DTPA(Gd) and glucose the conjugates showed rapid conversion of the paramagnetic substrate into the products with a 2.2-times increase of the average R1/Gd values (Table 1). Cell culture experiments showed that conjugates binding to EGFR+ A431 cells was highly specific, saturable and that the binding of reaction products with the cells (40 pmol converted ^{111}In-labeled substrate/mg cell protein) was occurring only if both conjugates (HRP and GO) were bound to the cell-surface receptors. Biodistribution in tumor-bearing animals showed that 25-times more αEGFR mAb antibody (4.2±0.3% injected dose) was bound to A431 tumors compared to a control SW620 tumor (0.16%). For antibody conjugates the ratio of tumor-accumulated doses was 12-fold (A431/SW620). MRI experiments were conducted such that each animal served as its own control. Animals were initially injected with the substrate alone and the temporal dynamics of MR signal elimination from the tumor showed that almost no T1-contrast remained after 24h (Fig.2A). When the same animals were administered the antibodies and then re-injected with di-5HT-DTPA(Gd), the temporal dynamics of T1-signal enhancement and retention in the A431 tumor margin (Fig 2 B, arrow) was significantly increased – at 2.5 hours and at later time points (up to 24 h after the injection) – as compared to animals receiving substrate alone. Immunohistochemical corroboration revealed that the spatial distribution of EGFR overexpression closely resembled the pattern of T1-signal enhancement in the tumors (arrows). EGFR-positive tumor margin was positive for markers of angiogenesis and glucose oxidase (the evidence of conjugate delivery). No enhancement of the tumor margin was observed in control SW620 tumors which do not overexpress EGFR. These experiments suggest that receptor targeting using paramagnetic "MR stains" allow investigation of receptor-specific imaging signatures using MRI. We anticipate that the use of therapeutic antibodies in imaging could help identify patient candidates that could benefit from anti-EGFR therapies as well as serve as a tool for treatment monitoring.

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Funded by NIH RO1 EB000858. EMD72000 was generously provided by Merck KaGA (Darmstadt, Germany).

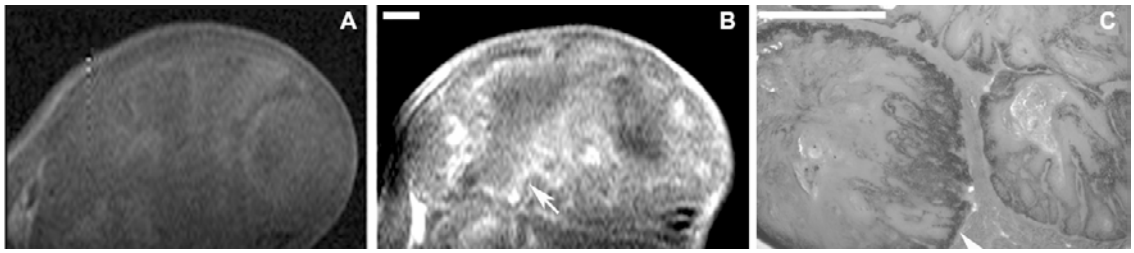

Fig. 2 **A**- T1w SE image of A431 xenograft obtained after the injection of: A- 0.2 mmol/kg di-5-HT-DTPA(Gd) alone ; **B**- after the sequential injection of 20 μg of αEGFR mAb conjugates followed by di-5-HT-DTPA(Gd). **C**- anti-EGFR staining of tumor section. Arrows point to tumor margin that shows EGFR overexpression (bright signal in B, IHC staining in C). Bars=1 mm.