Solution, Cell and Animal Studies in Molecular MRI of Estrogen Receptor α

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Purpose: To develop a non-invasive molecular MRI method for detecting, quantifying and investigating the level, activity and degradation of estrogen receptor alpha (ER) in breast cancer cells, tumors and metastases.

Introduction: Estrogens regulate the development and function of the female reproductive organs including the mammary gland through binding to nuclear ER, a transcription factor involved in differentiation and proliferation of cells. Estrogen stimulation of cell division has a key role in the development and progression of a large proportion of breast cancers. Evaluation of the ER level in breast cancer is a well-established tool for assessment of prognosis and for predicting response to endocrine therapy with antiestrogens or aromatase inhibitors. The levels of ER in human breast cancer biopsies span a broad range reaching 1000 fmol/mg cytosol protein. However, the current clinical methods for determining ER are semiquantitative and suffer from technical difficulties and analytical variations which may lead to inconsistent results. Furthermore, these techniques measure the level in a small fraction of the tumor, which may not reflect the actual distribution in the entire tumor. Part of these limitations can be overcome by using a non-invasive molecular imaging technique that will specifically quantify the level and distribution of ER over the whole tumor.

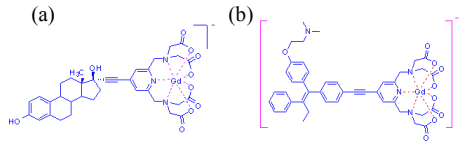

Figure 1. Schematic presentation of EPTA-Gd (a) and TPTA-Gd (b).

Methods: For targeting the ER, two novel contrast agents, composed of a gadolinuim-pyridiniumtetraacetic acid conjugated to 17β-estradiol, EPTA-Gd (1), or to tamoxifen, TPTA-Gd, (Fig.1) were synthesized. The corresponding europium (Eu)-chelates were also synthesized to act as reporters in fluorescence microscopy. Binding affinities to ER were determined by competitive radiometric binding assay. The cellular hormonal-induced activities of the novel contrast agents were tested using the MTT assay. ER and c-myc induced levels were assessed by western blot. **Solution ^{17}O and ^{1}H MRS**: Variable-temperature ^{1}H and ^{17}O transverse relaxation measurements were determined for a solution of PBS buffer and 5% ^{17}O-enriched water in the absence or the presence of EPTA-Gd, at 9.4T Bruker DMX spectrometer. **MRS/MRI of cells and tumors**: T1 and T2 relaxivity measurements were performed on a 4.7T Bruker Biospec spectrometer or a 9.4 T Bruker DMX spectrometer using spin echo with varying TR, and TEs, respectively and both high and standard spatial resolution , as well as MRS. The cell samples: MDA-MB-231 human breast cancer cells engineered to express ER under tetracycline and cultivated on Biosilon beads, were placed in 5mm tubes or in well plates and maintained at 15^{0}C, or continuously perfused at *37 ^{0}C*. In vivo studies were conducted on a Bruker Biospec 9.4T AVANCE II, recording high resolution T2 weighted images and 3D T1 weighted gradient echo images for dynamic studies with the EPTA-Gd injected as a bolus at a dose of 0.03-0.1 mmol/kg. **Fluorescence studies**: Cells on beads were incubated with the novel Eu-contrast agents and scanned by time-resolved fluorescence (TRF) on a wallac VICTOR instrument.

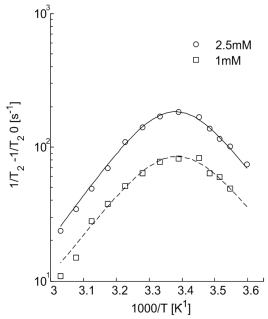

Figure 2. Temperature dependence of the paramagnetic contribution to ^{17}O NMR transverse relaxation rate due to the presence of 1 mM (squares) or 2.5mM (circles) EPTA-Gd measured in PBS buffer at pH 7.0, at 9.4T.

Results:

Biological activity in human breast cancer cells: Both EPTA-Gd and TPTA-Gd showed agonistic activity in a cell system, with pronounced effect of EPTA-Gd and low agonistic effect of TPTA-Gd. EPTA-Gd showed a stimulating growth effect with time- and dose-response in ER+ cell lines, similar to that of E2. TPTA-Gd, although based on the antiestrogen tamoxifen, did not inhibit E2-induced cell growth, and given alone slightly induced cell proliferation. In addition, EPTA-Gd, like E2, induced ER degradation in ER+ cells, indicating binding to ER, and TPTA-Gd had a lower degradation effect on ER level. Western blot analysis demonstrated that both EPTA-Gd and TPTA-Gd stimulate the expression of the proto-oncogene c-myc in ER+ cells with the effect of EPTA-Gd more pronounced.

^{1}H and ^{17}O NMR solution studies: *T1 and T2 relaxivities* in physiological solution at 9.4T and 15 °C were: EPTA-Gd: r1=6.8 and r2=16.7(mM^{-1}s^{-1}) ; TPTA-Gd: r1=4.7and r2=50 (mM^{-1}s^{-1}). Temperature dependent studies of the paramagnetic contribution of EPTA-Gd to the water ^{17}O NMR transverse relaxation rate (R_{2p}) (Figure 2), as well as to the ^{1}H relaxation rates in these solutions, yielded kinetic parameters and mechanism of the water exchange and relaxation enhancement in this ligand. The temperature dependent curve is characterized by a fast-exchange region where R_{2p} decreases with temperature, and by a slow exchange region, where R_{2p} increases with temperature. The data was analyzed by a non-linear least-square fitting to equations based on scalar relaxation mechanism (2) with several adjusted parameters. The water exchange rate was $^{17O}k_{ex}^{298}$= (1.1±0.3)x10^7 s^{-1}, with an enthalpy of activation of ΔH= 61.4±7.7 kJ mol^{-1} indicating a relatively fast water exchange rate. The mechanism suggested proton exchange via hydrogen bonding rather than a whole water molecule exchange.

***Fluorescence studies*:** TRF measurements of EPTA-Eu in the same cell system have revealed specific binding of EPTA-Eu to ER+ cells as compared to ER- cells, with binding affinity in the micromolar range and Kd of 0.57±0.07 uM.

^{1}H MRS/MRI studies in human breast cancer cells: T_1 measurements in the presence and absence of EPTA-Gd (n=9) or TPTA-Gd (n=5) and after washout indicated higher enhancement and significant differences in T1, between treated ER+ cells and the control ER+ cells as compared to the treated ER- cells and the ER- control cells, specifically after removal of the free and nonbound contrast agent from the cell system by washing with standard medium (Fig.3). T2 measurements also indicated significant differences after washout of the contrast agent in comparison to control cells. Concentration dependent MRS experiments performed on perfused cells confirmed the MRI results indicating significantly higher relaxivity in ER+ cells due to the binding to ER and consequently marked decrease in the rotational correlation time of the paramagnetic ligand.

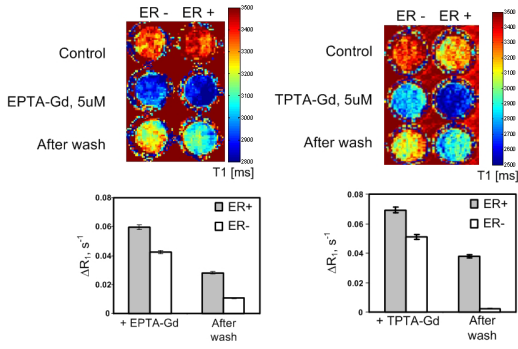

Figure 3. T1 maps of ER+ and ER- MDA-MB-231 cells cultivated on Biosilon beads using standard medium, in the presence of 5 uM EPTA-Gd or TPTA-Gd, and after washout.

In vivo MRI studies of ER+ MDA-MB-231 tumors implanted in mice: EPTA-Gd was found to be non toxic, with a half-life clearance time of ~0.5h after bolus injection of 0.1 mmol/kg EPTA-Gd to the tail vein. Experiments on tumor bearing mice have indicated higher retention time in peripheral region of the tumor, as compared to the muscle suggesting binding of the ligand to ER in this region.

Conclusion: We have characterized by various methods the solution chemistry of two novel ER-targeted Gd(III)-based contrast agents, as well as their biological activity and MRI parameters in cells and tumors . These agents demonstrated selective binding with high affinity to ER and significant enhancement of the water T1 and T2 relaxivity in ER+ as compared to ER- systems, and served to identify ER localization *in vivo*.

References 1. Gunanathan C et al., Bioconjug Chem. 2007 Sep-Oct;18(5):1361-5, **2.** Caravan P et al., Inorg. Chem. 2007 46 (16):6632-39.

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