cPLA2IVA inhibition in basal-like breast cancer: Reduced tumor growth with metabolic, vascular and gene expression changes

Hanna Maja Tunset[1], Eugene Kim[1], Jana Cebulla[1], Muhammad Riyas Vettukattil[1], Astrid Jullumstrø Feuerherm[2], Berit Johansen [2], Tone Frost Bathen[1], and Siver Andreas Moestue[1]

[1]*MR Cancer Group, Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Trondheim, Norway,* [2]*Avexxin AS, Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway*

Target audience: Basic scientists within NMR studies of cancer cell metabolism

Purpose: Basal-like breast cancer (BLBC) is the subtype of breast cancer with poorest prognosis[1]. Currently, no targeted treatment for BLBC is available, and patients are referred to chemotherapy and radiation with variable success. Thus, identifying molecular targets for treating BLBC may be the key to finding effective treatment of this highly aggressive cancer. Interestingly, the gene for cPLA2IVA, *PLA2G4A,* is more highly expressed in BLBC compared to the less aggressive luminal subtype[2,3]. Here, we evaluated the effect of a cPLA2IVA inhibitor on tumor growth, metabolism, vascularization, and gene expression in a xenograft model of BLBC, in order to explicate the role of cPLA2G4A.

Methods: Mice carrying MAS98.12 orthotopic xenografts, representing the BLBC subtype, received the cPLA2IVA inhibitor AVX235[4] (Avexxin AS, Trondheim, Norway) or vehicle (DMSO) by i.p. injection daily for the first week, then every second day. Tumor growth was monitored by caliper measurements. At day 2 (n = 6 for controls or treated with 45 mg/kg AVX235) or 19 (n = 6 for controls or treated with 45 mg/kg AVX235), the mice were sacrificed, and excised tumor tissue was snap frozen in liquid N_2. For High Resolution Magic Angle Spinning Magnetic Resonance Spectroscopy (HR MAS MRS), frozen xenograft tissue (6.4+-3.0 mg) was cut to fit into 30 µl disposable inserts (Bruker BioSpin, Ettlingen, Germany) filled with 3 µl D2O containing 25 mM sodium formate. HR MAS MR spectra were obtained using a Bruker AVANCE DRX-600 spectrometer with a 1H/13C HR MAS probe (Bruker BioSpin). A single-pulse experiment (zgpr; Bruker, spin rate 5 kHz, 5°C, D1 3 s, AQ 3.4s, SW 16 ppm, NS 32) was performed for all samples. Post-processing of spectra included 0.3 Hz exponential line broadening and baseline correction. Data analysis was performed with MATLAB (The Math Works, Natick, MA). HR MAS spectra were mean normalized to minimize differences in the sample weight. Partial least squares discriminant analysis (PLS-DA) was performed (PLS_Toolbox v5.8.3, Eigenvector Research, Manson, WA), and a permutation test was performed (1000 permutations) to evaluate significance[5]. For genome-wide expression analysis, RNA was extracted from tumor tissue using Qiagen RNeasy Mini Kit (Qiagen, Venlo, Netherlands), amplified, reverse transcribed and hybridized on Illumina Human HT-12 v4 Expression BeadChip (Illumina, San Diego, CA). Individual transcripts with a detection p>0.01 for all samples were filtered out. Values were $\log_2$ transformed and quantile normalized, and the significance threshold was set to p<0.05 after Benjamini-Hochberg FDR correction. For µCT, mice (n = 9 for controls, n = 8 for treated with 30 mg/kg AVX235) were sacrificed at day 7 by intracardiac perfusion with Microfil™ (Flow Tech, Inc., Carver, MA), an intravascular CT contrast agent. The tumors were excised and imaged on a Bruker Skyscan 1176 µCT system (9 µm isotropic voxels). Fractional blood volume (FBV), vessel diameter, and distance to nearest vessel (DNV) maps were computed from the images, and two-tailed Mann-Whitney U test (α=0.05) were performed for statistical analysis.

Results and discussion: Tumor size was significantly reduced in AVX235 treated mice from day 5 and throughout the treatment period (Fig. 1A), with a mean growth inhibition of 65 % at day 19. For metabolic analysis, PLS-DA based on HR MAS MRS spectra was performed. At day 2, the model showed clear discrimination (specificity = 83 %, sensitivity 83%, p=0.004 by permutation test) between treated and control groups (Fig.1B). This was mainly attributed to higher phosphocholine (PCho) and lower glycerophosphocholine (GPC) levels in treated samples. PCho is precursor of the cPLA2IVA substrate phosphatidylcholine (PtdCho). cPLA2IVA converts PtdCho to lysoPtdCho, which is further metabolized to GPC. Treatment induced GPC decrease is indicative of a better prognosis[6] and is in agreement with a lowering of cPLA2IVA activity. The products of cPLA2IVA enzymatic reaction, mainly arachidonic acid and lysophospholipids such as lysoPtdCho, are both involved in tumorigenic and angiogenic signaling[7]. Further, the production of the tumorigenic and angiogenic lysophosphatidic acid is dependent on cPLA2IVA generated lysoPCho/other lysophospholipids or direct conversion from phosphatidic acid. We speculate that cPLA2IVA inhibition leads to reduced levels of these angiogenic metabolites. However, the sensitivity of HR MAS MRS does not allow for detection of these metabolites. Thus, we examined effects of cPLA2IVA inhibition on vascular features by using µCT at day 7. The resulting images show marked alteration in tumor vasculature (Fig. 1C, 1D). Treated tumors were less vascularized (FBV: control=6.0±1.6%, treated=4.1±1.5%, p=0.03), had fewer large vessels (mean vessel diameter: control=106.0±10.8 µm, treated=93.2±6.4 µm, p=0.03), and contained larger avascular regions (DNV: control=52.9±15.7 µm, treated=87.1±16.0 µm, p=0.002). It is likely that this results in unfavorable, hypoxic conditions. After 19 days, the metabolic distinction between groups was lost, which may be caused by compensatory mechanisms in the complex choline metabolic pathway. Interestingly, the timing of gene expression changes did not parallel metabolic findings. In the gene expression analysis, only one gene (*ZBF36*, coding for a post-translational degradation factor), was differentially expressed at day 2. However, after 19 days, the expression of 17 genes was significantly altered, possibly indicating that the vascular changes influence gene expression. 14 of 17 genes were up-regulated compared to controls, and these are mostly associated with translation or transcription. Also, *FAM162,* involved in regulation of apoptosis and *CREBZF,* a positive regulator of the tumor suppressor p53, were up-regulated in the treated group. The three down-regulated genes *YWHAE, LMNA, a*nd *PMVK* are linked with signal transduction, nuclear membrane stability, and cholesterol synthesis, respectively. Taken together, the data seem to imply that the cells have a general increase in protein production at the later time point, and a possible shift of program towards cell death. We hypothesize that cPLA2IVA inhibition first has direct metabolic effects in the tissue, followed of alterations of the vasculature, which may induce the late changes in gene expression.

Conclusion: Inhibition of cPLA2IVA in a human-derived BLBC model significantly inhibits growth and favorably alters the metabolism, vascular features, and gene expression of tumors. Thus, cPLA2IVA inhibition may represent a novel strategy for targeted treatment.

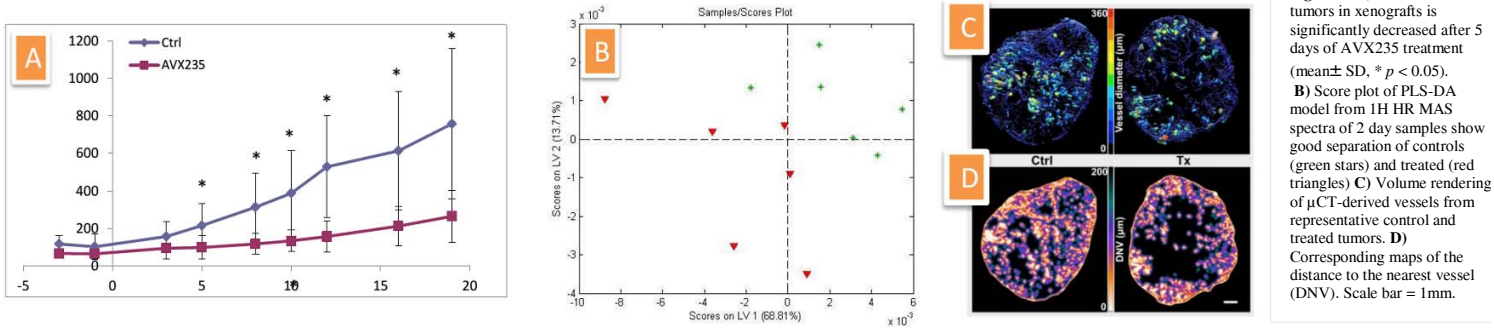

Figure 1. A) Growth of BLCB(tumors in xenografts is significantly decreased after 5 days of AVX235 treatment (mean± SD, * p < 0.05). **B)** Score plot of PLS-DA model from 1H HR MAS spectra of 2 day samples show good separation of controls (green stars) and treated (red triangles) **C)** Volume rendering of µCT-derived vessels from representative control and treated tumors. **D)** Corresponding maps of the distance to the nearest vessel (DNV). Scale bar = 1mm.

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