SFX-01 targets STAT3 signalling to inhibit stem-like cells in breast cancer patient-derived xenograft tumours

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SFX-01 is a novel therapeutic comprising synthetic sulforaphane (SFN) stabilised within α-cyclodextrin. Breast cancer stem-like cells (CSCs) have been identified in all molecular subtypes and are likely drivers of breast cancer metastasis and treatment resistance. We established previously that CSC activity in ER+ breast cancer represents a source of therapeutic resistance (Simões et al, Cell Reports, 2015).
We investigated SFX-01 effects on breast CSC activity using mammosphere formation efficiency (MFE) and aldehyde dehydrogenase (ALDH) activity using the ALDEFLUOR assay in patient samples and patient-derived xenograft (PDX) tumours. Cells from primary (n=12) and metastatic (n=15) samples were treated with SFX-01 or vehicle control. Using a 2 or 8 week in vivo treatment, early (HBCx34) and metastatic (BB3RC31) ER+ PDX tumours were treated with SFX-01 alone or in combination with tamoxifen or fulvestrant. Tumours were dissociated and MFE and ALDH activity assessed.
SFX-01 in vitro reduced MFE of both primary and metastatic patient samples. TAM and FULV increased MFE and ALDH activity after 2 weeks of treatment in vivo, which was abrogated by combination with SFX-01. TAM+SFX-01 suppressed tumour growth at 8 weeks vs TAM alone in HBCx34 but not BB3RC31. FULV treatment maintained tumour growth suppression at 8 weeks and no additive effect was seen with SFX-01, although MFE and ALDH activity were suppressed. Mechanistically, SFX-01 potently suppressed the increase observed in phospho-STAT3 after anti-estrogen treatments and we are currently investigating STAT3 signalling effectors through RNAseq data analysis.
Our data suggest the potential of SFX-01 for clinically meaningful improvements to endocrine therapy in ER+ breast cancer by reversing CSC mediated resistance.

SFX-01 reduces breast CSC activity in primary (67%, n=8/12) and metastatic (80%, n=12/15) breast cancer patient derived-samples

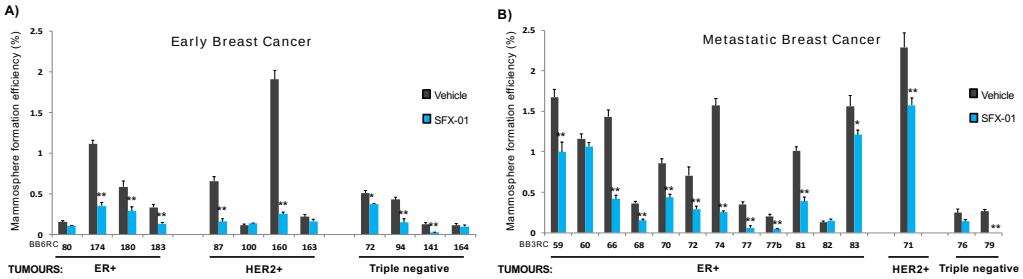

Figure 1: Mammosphere formation efficiency (MFE) of freshly isolated early **(A)** and metastatic (pleural effusions and ascites) **(B)** patient-derived samples cultured in the presence of SFX-01 (5 μM, blue bars) or vehicle control (black bars). MFE data for each individual patient sample is represented. MFE was determined on day 7-9 and calculated by dividing the number of mammospheres formed (≥ 50μm) by the original number of single cells seeded (500 cells/cm2) and is expressed as the mean percentage ± SEM. * p < 0.05; ** p < 0.01

Tamoxifen in combination with SFX-01 reduces breast CSC activity more effectively than tamoxifen alone in ER+ patient samples

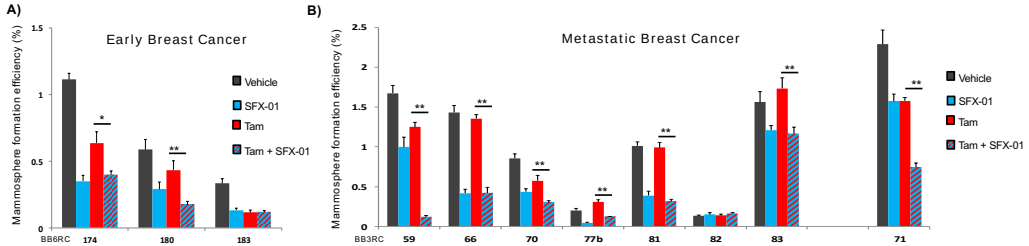

Figure 2: Mammosphere formation efficiency (MFE) of ER+ early **(A)** and metastatic **(B)** patient-derived samples represented in Figure 1. Here, samples were also treated with tamoxifen alone (10^{-6} M, red bars) or in combination with SFX-01 (5 μM, hatched bars). MFE data for each individual patient sample is represented. MFE was determined on day 7-9 and calculated by dividing the number of mammospheres formed (≥ 50μm) by the original number of single cells seeded (500 cells/cm^2) and is expressed as the mean percentage ± SEM. * p < 0.05; ** p < 0.01

SUMMARY

- **SFX-01 targets cancer stem-like cells in early and metastatic patient-derived breast cancer samples**
- **SFX-01 counteracts the effects of anti-estrogens on CSCs in patient-derived breast cancer xenografts**
- **Anti-estrogen activation of STAT3 signalling is inhibited by SFX-01**

SFX-01 prevents tamoxifen enrichment for cells with cancer stem cell properties in patient-derived xenograft tumours

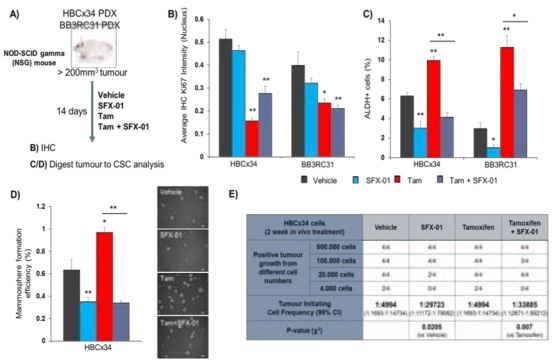

Figure 3: **A)** BB3RC31 and HBCx34 patient derived xenografts (PDXs) treated *in vivo* for 14 days with SFX-01 (300mg/kg/day, oral gavage) in the presence or absence of tamoxifen (10mg/kg/day, oral gavage). HBCx34 model was kindly provided by Dr Elisabetta Marangoni (Institute Curie, Paris). **B)** Quantification of Ki67 expression determined by immunohistochemistry showing that tamoxifen but not SFX-01 significantly decreases proliferation marker Ki67. **C)** Percentage of ALDH-positive cells was determined with ALDEFLUOR assay. ALDH-positive cells were discriminated from ALDH-negative cells using the ALDH inhibitor, DEAB. Mouse cells were excluded from the FACS analysis with anti-mouse MHC Class I (H-2Kd) antibody. **D)** Mammosphere formation efficiency was determined on day 7-9 and calculated by dividing the number of mammospheres formed (≥ 50μm) by the original number of single cells seeded (500 cells/cm^2) and is expressed as the mean percentage of mammosphere formation. Representative micrographs are shown (scale bar 50 μm). **E)** Secondary transplantation of 500K, 100K, 20K and 4K cells after in vivo treatments. Experiment was carried out in NSG mice with 90-day slow-release estrogen pellets. Tumor growth (>75 mm3) was assessed at day 90 and is represented as mice positive for growth/mice tested for each cell number tested. ELDA of tumor-initiating cell frequency is shown. Data are represented as mean ± SEM. * p < 0.05; ** p < 0.01

SFX-01 inhibits tumour growth compared to tamoxifen alone and prevents formation of micrometastasis in the lungs

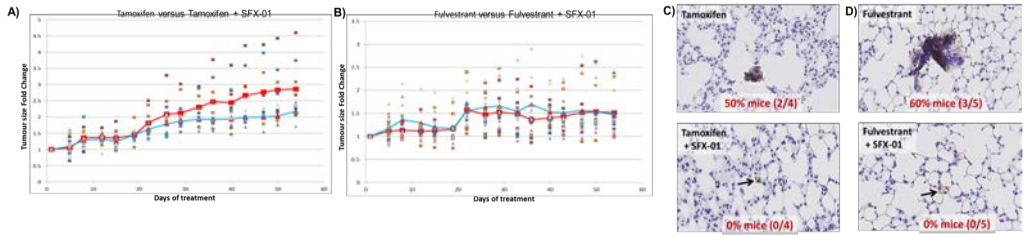

Figure 4: **A/B)** Tumour growth of HBCx34 PDX tumours treated *in vivo* for 56 days with tamoxifen (10mg/kg/day, oral gavage) or fulvestrant (200 mg/kg/week, subcutaneous injection) in the presence or absence of SFX-01 (300mg/kg/day, oral gavage). Individual tumours (n=10) treated with tamoxifen (**A**) or fulvestrant (**B**) are represented by squares and tumours treated in combination with SFX-01 are represented by triangles. Red line shows average tumour growth for tamoxifen (**A**) or fulvestrant (**B**) and blue line shows average tumour growth for the combination treatment with SFX-01. **C/D)** Mice lungs were stained with anti-human mitochondrial antibody and micrometastases with at least 10 cells were counted. Percentage of mice bearing micrometastases for each treatment group is shown.

SFX-01 targets STAT3 signalling, which is activated by anti-estrogen therapy

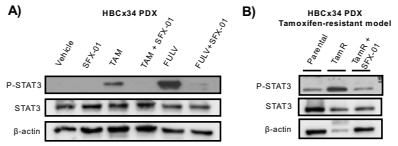

Figure 5: **A)** phospho-STAT3 and total STAT3 protein expression levels determined by Western Blot in HBCx34 PDX treated *in vivo* for 56 days with SFX-01 in the presence or absence of tamoxifen or fulvestrant. β-actin was used as a reference for the loading control. **B)** Protein expression levels in tamoxifen-resistant (TAMR) HBCx34 PDX treated in vivo for 56 days with SFX-01.

STAT3-related genes are up-regulated in anti-estrogen resistant ALDH+ cells and are associated with worse outcomes for ER+ breast cancer patients

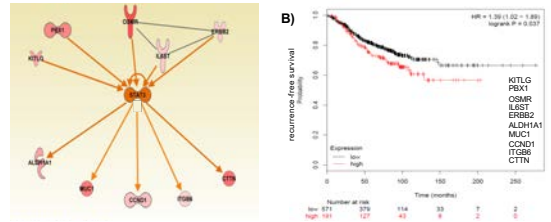

Figure 6: **A)** Ingenuity Pathway Analysis (IPA) of STAT3-related genes differentially expressed in ALDH+ cells of metastatic patient derived cells treated with anti-estrogen treatments predict activation of STAT3 signalling pathway. **B)** Kaplan-Meier analysis demonstrates that elevated expression of the STAT3 10-gene signature is significantly associated with shortened recurrence-free survival in ER+ breast cancer patients. The gene expression data is from published microarray datasets available in KMplotter (www.kmplot.com).

CONCLUSION

SFX-01 may be of potential benefit in combination with anti-estrogens to overcome endocrine resistance of ER+ tumours