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TITLE: Exploit Dimethyl Fumarate to Uncover Druggable Vulnerabilities and Prevent Recurrence of ER+ Breast Cancers

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14. ABSTRACT
ZNF217 transcription factor was identified as a target of dimethyl fumarate (DMF) in cell models of aggressive ER+ breast cancer. ZNF217 amplification is associated aggressive tumor features, resistance to endocrine therapy and poor outcome in breast cancer. This evidence indicates that inhibition of ZNF217 will be beneficial, however there are no know direct inhibitors of ZNF217. We show that DMF covalently modifies ZNF217 and inhibits its transcriptional activity. Furthermore, DMF inhibits ZNF217-driven phenotypes such as clonogenic survival and stemness. Cells with higher expression of ZNF217 are more sensitive to ZNF217 inhibition by DMF. Lastly, DMF treatment causes significant tumor regression in a xenograft model. These data suggest that DMF can be the prototype drug upon which to generate improved and more specific inhibitors for ZNF217. Overall, this data could be the basis for a new treatment strategy against aggressive breast cancer, especially to overcome resistance and recurrence.

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Introduction

Our main goal is to identify druggable pathways that govern tamoxifen tolerance, which in turn can be targeted therapeutically to eradicate tamoxifen-tolerant cells and prevent deadly tumor resistance and recurrence from the outset.

While we setup and initiated animal studies to conduct proteomics and RNAseq, we also collected preliminary data from breast cancer cells. Models representing aggressive estrogen receptor (ER)+ breast cancer were employed, such as MCF-7 TamR (tamoxifen resistant), LTED (long-term estrogen deprived, a model of aromatase inhibitor resistance), and MCF-7 Her2 (erbB2/Her2 overexpression model, a model of reduced sensitivity to tamoxifen). These cells models are well established in the field and were obtained from Dr. Rachel Schiff (Baylor).

The transcription factor ZNF217 was identified as a target of dimethyl fumarate (DMF) in these models. ZNF217 amplification is associated with aggressive tumor features, resistance to endocrine therapy and poor outcome in breast cancer. These observations indicate that ZNF217 is a new oncogene in breast cancer and its inhibition will be highly beneficial. However, there are no know direct inhibitors of ZNF217. ZNF217 is a constituent of a core transcriptional complex; it lack a classical active site or druggable domains, therefore it is notoriously difficult to develop inhibitors against such factor. Data included in this report show that DMF covalently modifies ZNF217 and inhibits its transcriptional activity. Furthermore, DMF inhibits ZNF217-driven phenotypes such as clonogenic survival and stemness. Cells with higher expression of ZNF217 are more sensitive to ZNF217 inhibition by DMF. Lastly, DMF treatment causes significant tumor regression in a xenograft model. These data suggest that DMF can be the prototype drug upon which to generate improved and more specific inhibitors for ZNF217. Overall, this data could be the basis for a new treatment strategy against aggressive breast cancer, especially to overcome resistance and recurrence.

Body

Results

The chemical biology approach of iso-TOP-ABPP profiling[1] was conduced in lysates treated with DMF (50μM, 2 hrs) by our collaborator, Dr. Benjamin Cravatt (Scripps). See table listed below for specific DMF targets

A. DMF targets identified by iso-TOP-ABPP methodology.

	common DMF targets			new data (in situ 50 uM DMF 2h) DMF sensitive if R>=4						Legacy d
Protein_site	Protein Master_PepExp_pep	SpecCountSum	MaxRatio	TamSens:sol	TamSens:mem	TamResis:sol	TamResis:mem	LTED:sol	LTED:mem	DMF_50
HSPBP1_204	Hsp70-binding protein 1 LLDRDAC(*)DTVR.V	192	20	19.63		20.00		20.00	20.00	20.00
VPS18_806	VPS18 - Vacuolar protein sorting-associated prote	12	6.69	5.58				5.96		13.10
DFFB_194	DFFB - DNA fragmentation factor subunit beta VLC	20	20	12.91				20.00		18.69
CASP2_370	CASP2 - Caspase-2 SDMICGYAC(*)LK.G	20	20	12.91				20.00		8.85
RRM1_492	RRM1 - Ribonucleoside-diphosphate reductase lar	80	20	3.76		20.00		11.03		6.73
ANAPC7_32	ANAPC7 - Anaphase-promoting complex subunit 7	86	20	3.95	2.67	20.00		2.79	3.29	6.34
PGPEP1_14	PGPEP1 - Pyroglutamyl-peptidase 1 YLC(*)DFTYV	42	12.46	6.28		6.21		4.31		7.44
TBCD_850	TBCD - Tubulin-specific chaperone D AGAPDEAV	634	5.1	2.37		2.66		3.84	4.38	5.08
TIGAR_161	TIGAR - Fructose-2,6-bisphosphatase TIGAR EQF	730	20	3.20		3.96		6.12	20.00	7.41
HSF1_153	HSF1 - Heat shock factor protein 1 GKQEC(*)MDS	26								13.26
NRDC_60:62	NRDC - Nardilysin STC(*)SC(*)PDLQPNGQDLGE	10	3.59					3.12		4.08
CPNE3_54	CPNE3 - Copine-3 IKNC(*)LNPQFSKT	180	20	3.87	3.06	20.00	3.51	3.68	5.11	4.32
HDHD3_109	HDHD3 - Haloacid dehalogenase-like hydrolase do	80	4.13	2.24	2.06	2.43	2.84	3.47		11.42
KIAA0391_36	KIAA0391 - Mitochondrial ribonuclease P protein 3	186	20	7.15		13.22	7.37	10.10	20.00	13.55
RIF1_2286	RIF1 - Telomere-associated protein RIF1 ESIPC(*)	12	20					20.00	5.98	4.18
LIG1_895	LIG1 - DNA ligase 1 VREDKQPEQATTSAQVAC(*	38	20	20.00				6.35		11.11
BIRC2_45	BIRC2 Baculoviral IAP repeat-containing protein 2	22	20	19.00				20.00		20.00
CHRAC1_55	CHRAC1 Chromatin accessibility complex protein	572	20	7.69	9.91	5.46	11.27	20.00	20.00	11.72
THNSL1_324	THNSL1 Threonine synthase-like 1	84	20	8.92	20.00			16.42		20.00
AP4E1_1119	AP4E1 AP-4 complex subunit epsilon-1	98	20	4.43		5.18	1.33	7.32	14.48	6.76
CPNE3_54	CPNE3 Copine-3	180	20	3.87	3.06	20.00	3.51	4.80	5.11	3.51
PGLS_32	PGLS 6-phosphogluconolactonase	372	20	3.62		4.33	4.25	6.33	4.91	4.09
THOC1_49	THOC1 THO complex subunit 1	56	20	3.85	2.92		4.10	4.56	11.69	4.15
MAP4_635	MAP4 Microtubule-associated protein 4	86	20	2.49	2.08	2.21	1.99	8.76	2.39	2.52
CRKL_249	CRKL Crk-like protein	284	20	2.98	1.96	2.50	3.04	3.40	3.84	2.74
PGP_297	PGP Phosphoglycolate phosphatase	806	20	2.48	1.39	3.03		4.41		4.24
NUDT8_207	NUDT8 Nucleoside diphosphate-linked moiety X m	152	20	2.60		2.60	2.76	4.21	9.44	-
ROGDI_66	ROGDI Protein rogdi homolog	46	20			20.00		8.99	13.93	-
VDAC3_65	VDAC3 Voltage-dependent anion-selective channe	282	20	20.00	5.93	3.92	4.10	20.00	7.46	4.56
VDAC3_36	VDAC3 Voltage-dependent anion-selective channe	1400	20	11.42	3.44		2.35		3.61	1.85
DFFB_194	DFFB DNA fragmentation factor subunit beta	20	20	12.91				20.00		18.69
DNPEP_327	DNPEP Aspartyl aminopeptidase	168	6.42	2.67	2.67	2.46	2.88	4.52	3.76	2.53
ITPK1_361	ITPK1 Inositol-tetrakisphosphate 1-kinase	70	4.95	2.43		2.38		4.43		-
VDAC3_229	VDAC3 Voltage-dependent anion-selective channe	54	7.96		5.44		2.71		4.19	2.23
SEC63_295	SEC63 Translocation protein SEC63 homolog	124	12.93		4.53		4.00		5.74	17.49
TDRKH_109	TDRKH Tudor and KH domain-containing protein	60	13.46		4.82		5.98		6.86	6.81
UTP6_370	UTP6 U3 small nucleolar RNA-associated protein 6	32	20		4.66		5.41		16.05	3.81
TBL3_728	TBL3 Transducin beta-like protein 3	68	20			20.00	20.00	20.00		-
ZNF217_286	ZNF217 Zinc finger protein 217	158	20	1.09	1.00	1.15	1.45	1.43	20.00	1.23
MKI67_903	MKI67 Antigen KI-67	146	20				17.04	20.00	2.06	1.83
CDKN3_39	CDKN3 Cyclin-dependent kinase inhibitor 3	26	20	20.00		20.00		13.98		-

B.

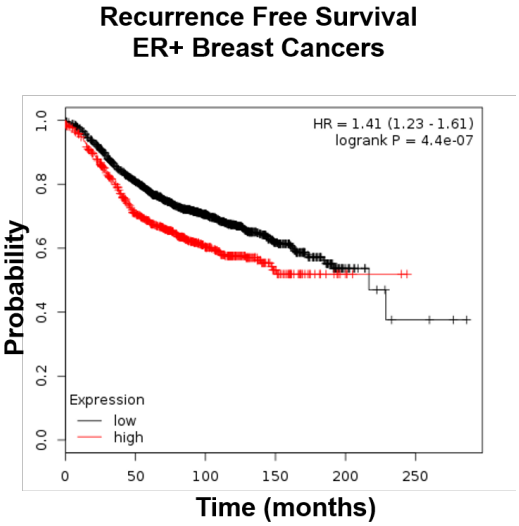

Figure 1. (A) The list of DMF targets identified in breast cancer cells. Kaplan-Meier analysis to identify the role of ZNF217 in recurrence free survival (RFS) of ER+ breast cancers. Higher expression of ZNF217 (red line) leads to reduced RSF (data generated with KMplotter).

identified in breast cancer cells. An R-value >4, indicates a DMF-specific ligandable hotspot in the proteome (Fig. 1A). Upon investigating these targets for relevance to breast cancer, we decided to focus on ZNF217. ZNF217 is a constituent of a core transcriptional complex that includes CoREST, histone deacetylase 1/2, lysine demethylase 1, and the C-terminal binding protein 1/2[2]. ZNF217 amplification is associated with aggressive tumor features, resistance to endocrine therapy, and poor outcome in breast cancer[2-5]. More recently, Weaver and colleagues reported that stiff stroma increases breast cancer risk by inducing the oncogene ZNF217[6]. This evidence indicates that ZNF217 inhibition can be of therapeutic value against aggressive tumors, but can also be used for chemoprevention.

Kaplan-Meier survival analysis supports the existing literature and indicates that high ZNF217 levels are associated with shorter recurrence free survival (Fig. 1B).

Breast cancer cell lines were examined for ZNF217 expression levels. Both MCF-7 Her2 cells and LTED cells display increased levels of ZNF217 compared to control cells (Fig. 2). Based on this data, ZNF217-related phenotypic changes were carried out using the MCF-7 Her2/MCF-7 Neo model, with additional validations in the LTED/MCF-7 model.

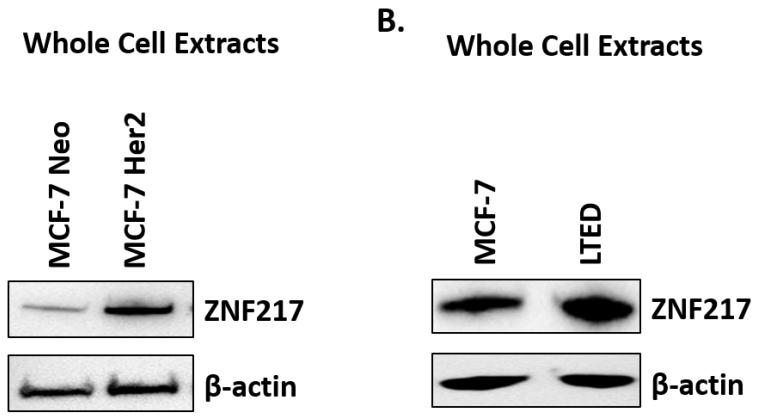

Figure 2. ZNF217 expression levels by immunoblotting in (A) MCF-7 Neo controls and MCF-7 Her2 overexpressing cells, and (B) in parental MCF-7 and LTED cells.

To confirm the iso-TOP-ABPP data that DMF engages ZNF217 in breast cancer cells, an additional chemical biology approach was utilized. A DMF-probe was designed to replicate the biological activity of DMF[7]. It contains an alkyne functional group, which can be used as a bio-orthogonal tag for downstream applications, as we have previously reported[7]. A schematic representation of the experimental approach is shown in Fig. 3A. In MCF-7 Her2 cells, treatment of cell lysate with DMF-probe, generates a covalent adduct that can be precipitated and identified by immunoblotting (Fig. 3B). Importantly, pretreatment with DMF, inhibits interaction with the DMF-probe, indicating that both DMF and the probe modify the same site on the ZNF217 protein (Fig. 3B, last lane).

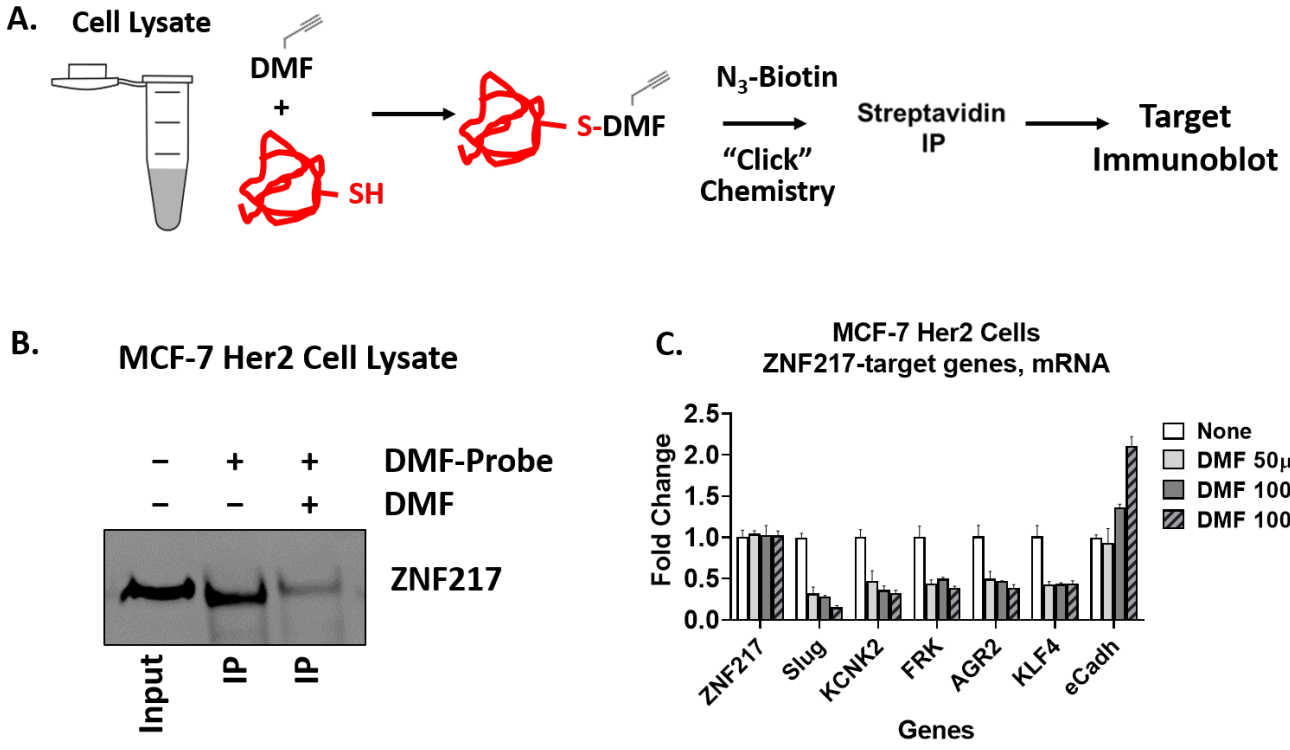

Figure 3. (A) Scheme for target identification using the DMF-probe in cell extracts. Cell lysates were incubated with the DMF-probe, cross-linked by click chemistry to an azido-biotin reporter tag, allowing IP using streptavidin beads to capture biotinylated protein. (B) ZNF217 protein is identified from reactivity of the DMF-probe. DMF was preincubated for 30min (last lane), followed by DMF-probe for another 30min. (C) ZNF217 target genes are inhibited or derepressed (eCadh) by DMF in MCF-7 Her2 cells. mRNA is quantified by RT-QPCR.

Given that ZNF217 is a transcription factor, we examined a number of genes regulated by ZNF217 as reported by Krig et al[8]. While there is no effect on ZNF217 itself, there is a significant reduction from DMF treatment in Slug, KCNK2, FRK, AGR2 and KLF4 gene expressions (Fig. 3C). ZNF217 was shown to repress eCadherin[9], and consistent with this report, we find that DMF inhibition of ZNF217 leads to eCadherin derepression (Fig. 3C).

ZNF217 covalent modification by DMF is predicted to occur on a cysteine residue. Iso-TOP-ABPP data indicated that cysteine 286 is the site of modification. Interestingly, although ZNF217 contains eight zinc fingers, cysteine 286 is not part of any of them. This led us to hypothesize that DMF inhibits ZNF217 activity, not by modifying one of the zinc fingers, thereby preventing binding to DNA, but rather by disrupting a critical interaction of the transcriptional core that ZNF217 belongs to. The experiments to address this hypothesis are ongoing.

ZNF217 was reported to sustains proliferation, resistance to cell death, cancer stem cells, epithelial to mesenchymal transition (EMT), invasion, and metastasis[2-5]. We examined a number of these phenotypes in MCF-7 Her2 cells that express elevated levels of ZNF217 compared to MCF-7 Neo control cells. We find that DMF reduced cell viability in a dose dependent manner, but there was no difference between the two cell lines

(Fig. 4D). However, on mammosphere growth and clonogenic assay (Fig. 4B-C), there is a significant difference in sensitivity to DMF between the two cell lines. MCF-7 Her2 cells with elevated ZNF217 are more sensitive to DMF alone at equivalent doses compared to the MCF-7 Neo cells (Fig. 4A and 4B). This suggests that higher ZNF217 levels renders MCF-7 Her2 cells more susceptible to DMF, especially on phenotypes driven by ZNF217, such stemness and clonogenic survival. ER+ tumors with elevated Her2 often develop tamoxifen resistance. As expected MCF-7 Her2 cells are less responsive to tamoxifen shown in Fig. 4C (second set of bars). This also indicates that the more aggressive MCF-7 Her2 cell line has a larger tamoxifen-tolerant population, which we established is a precursor to therapy resistance and tumor recurrence[10]. DMF specifically targets these cells and sensitizes them to 4-hydroxytamoxifen (4OHT) in a dose dependent manner (Fig. 4C). We are corroborating these findings in a second model using the LTED cells (data not shown).

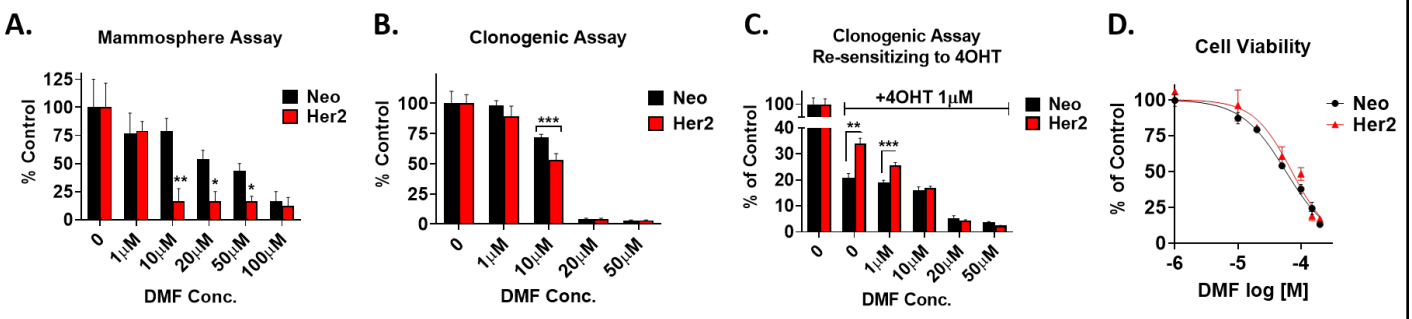

Figure 4. (A) Mammosphere assay in MCF-7 Neo (black) and Her2 cells (red). Cells are seeded in suspension on low attachment plates in de-differentiating conditions. Methyl cellulose is added to prevent cell aggregation. DMF is added at the indicated doses. After 7 days of treatment, cells that grew to establish mammospheres >50μm in diameter are counted. (B-C) Clonogenic assay is conducted in MCF-7 Neo (black) and Her2 cells (red). Cells are seeded at low density, and treated twice weekly with (B) DMF alone or (C) DMF + 4OHT (1μM). After 2 weeks, colonies are fixed and stained in crystal violet. (D) Cell viability is measured MCF-7 Neo (black) and Her2 cells (red) after 3 days of treatment with DMF. Viable cells are fixed and stained in crystal violet. After solubilizing, the absorbance is measured at 562nm and data is normalized to % of control. Data plotted with Graphpad. *p< 0.05, ** p<0.01, *** p<0.001.

Given the positive outcome of the *in vitro* studies, we evaluated DMF's anti-tumor activity in a xenograft model using the aggressive MCF-7 Her2 cells. DMF treatment significantly attenuated tumor growth (Fig. 5A). At the end of the study, tumors were excised and ZNF217 target genes were quantified by RT-QPCR. A similar pattern of regulation by DMF observed *in vitro* (Fig. 3C) is also present in tumors (Fig. 5B). Overall, our data indicate that DMF treatment induces tumor regression *in vivo*, at least in part, due to inhibition of ZNF217.

Additional studies are ongoing to fully understand the biological consequence of ZNF217 modification and inhibition by DMF, especially with regard to endocrine therapy resistance and recurrence. In conclusion, DMF inhibition of ZNF217 ameliorates a number of aggressive phenotypes and induces tumor regression. DMF can be the starting point for medicinal chemistry efforts to generate more specific inhibitors for ZNF217 and address an unmet need in the field.

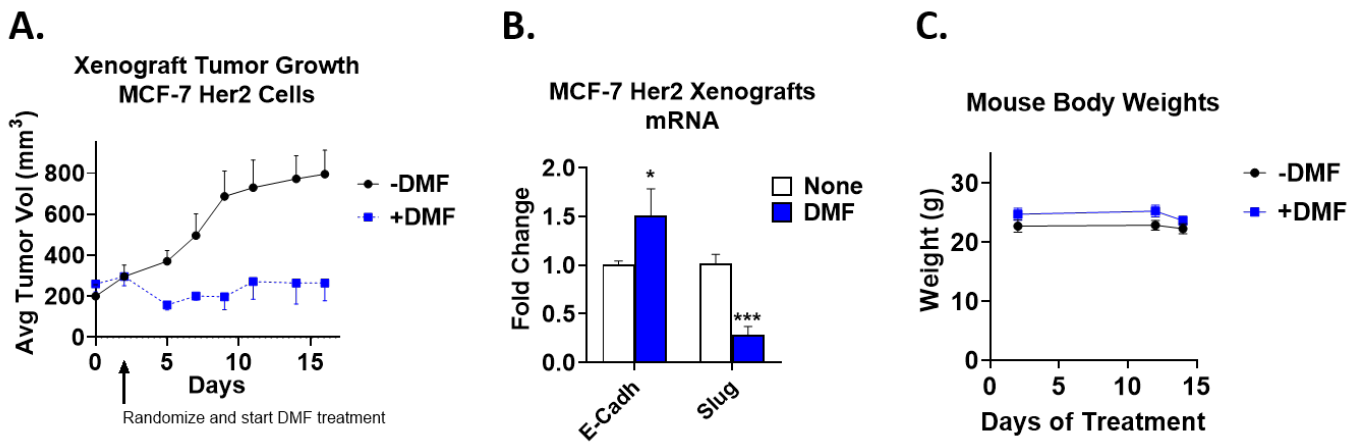

Figure 5. (A) DMF inhibits tumor growth. MCF-7 Her2 cells were injected into the mammary fat pads of female athymic nude mice. Once tumors initiated, mice were randomized to receive DMF (30mg/kg daily, 5 days per week) or vehicle control. Tumor volume was calculated as (length x width^2) x π/2. (B) Tumors at the end of the study in (A) were excised and RNA was isolated. Gene expression is quantified by RT-QPCR. (C) Mouse weights during the course of the study are shown.

Key Research Accomplishments

- Initiated and completed the first round of *in vivo* studies. Tumor samples are sent to collaborator for proteomic analysis. Next, we are preparing tumor samples for RNA sequencing.
- Identified an important target of DMF, ZNF217, which is relevant to aggressive ER+ breast cancer disease, including endocrine resistance and recurrence.
- ZNF217 inhibition by DMF ameliorates a number of aggressive phenotypes *in vitro*.
- ZNF217 inhibition by DMF induces tumor regression *in vivo*.
- Working towards completing a manuscript based on the data presented above.

Reportable Outcomes

1. Progress in identifying a druggable target of DMF relevant to endocrine therapy resistance and recurrence.
2. Ongoing chemical proteomics analysis followed by RNA sequencing will unveil new druggable targets and pathways that we will develop as presented above.

Conclusion

Our progress is on track and follows the approved SOW.

References

1. Roberts, A.M., Ward, C.C. & Nomura, D.K. Activity-based protein profiling for mapping and pharmacologically interrogating proteome-wide ligandable hotspots. *Current opinion in biotechnology* **43**, 25-33 (2017).
2. Lee, D.F., Walsh, M.J. & Aguilo, F. ZNF217/ZFP217 Meets Chromatin and RNA. *Trends Biochem Sci* **41**, 986-988 (2016).
3. Vendrell, J.A.*, et al.* ZNF217 is a marker of poor prognosis in breast cancer that drives epithelial-mesenchymal transition and invasion. *Cancer Res* **72**, 3593-3606 (2012).
4. Cohen, P.A., Loudig, O., Liu, C., Albanese, J. & Fineberg, S. The ZNF217 Biomarker Predicts Low- and High-Risk Oncotype DX((R)) Recurrence Score in ER-Positive Invasive Breast Cancers. *Front Pharmacol* **10**, 524 (2019).
5. Littlepage, L.E.*, et al.* The transcription factor ZNF217 is a prognostic biomarker and therapeutic target during breast cancer progression. *Cancer Discov* **2**, 638-651 (2012).
6. Northey, J.J.*, et al.* Stiff stroma increases breast cancer risk by inducing the oncogene ZNF217. *J Clin Invest* **130**, 5721-5737 (2020).
7. Kastrati, I.*, et al.* Dimethyl Fumarate Inhibits the Nuclear Factor kappaB Pathway in Breast Cancer Cells by Covalent Modification of p65 Protein. *The Journal of biological chemistry* **291**, 3639-3647 (2016).
8. Krig, S.R.*, et al.* Identification of genes directly regulated by the oncogene ZNF217 using chromatin immunoprecipitation (ChIP)-chip assays. *The Journal of biological chemistry* **282**, 9703-9712 (2007).
9. Huang, H.N.*, et al.* Chromosome 20q13.2 ZNF217 locus amplification correlates with decreased E-cadherin expression in ovarian clear cell carcinoma with PI3K-Akt pathway alterations. *Hum Pathol* **45**, 2318-2325 (2014).
10. Kastrati, I.*, et al.* The NF-kappaB Pathway Promotes Tamoxifen Tolerance and Disease Recurrence in Estrogen Receptor-Positive Breast Cancers. *Mol Cancer Res* **18**, 1018-1027 (2020).

Appendices

None