Antagonism of SF-1 as a potential targeted therapy for malignant Leydig cell tumors

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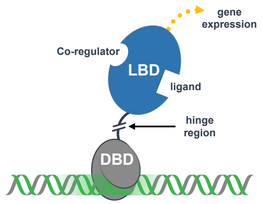
Introduction 1

- Leydig cell tumors (LCT) belong to the family of sex cord stromal tumors (SCST), a collection of tumors formed in the supporting tissue within the ovaries or testes. In adult patients about 10% of LCT are malignant and metastasis is common. Non-resectable metastatic disease is poorly responsive to radiation and chemotherapy[1].
- The orphan nuclear receptor SF-1 (NR5A1) is a specific expression marker of LCT and SCST. SF-1 is necessary for development of fetal Leydig cells and regulates adult Leydig progenitor cell formation and/or survival[2].
- Significant data support a role for SF-1 in adrenocortical cancer (ACC). To address the need for a targeted therapy in ACC, Orphagen has identified potent small molecule antagonists to SF-1[3].
- Using R2C, a rat Leydig tumor cell line, we demonstrate that small molecule antagonists of SF-1 inhibit LCT proliferation *in vitro* and *in vivo*.
- SF-1 antagonist, OR-449, is in IND-enabling studies for a Phase I trial start at the end of 2022 and provides a novel targeted therapy for the treatment of ACC, LCT and other SCST.

Figure 1: NR control of gene expression



LBD: ligand-binding domain;
DBD: DNA-binding domain

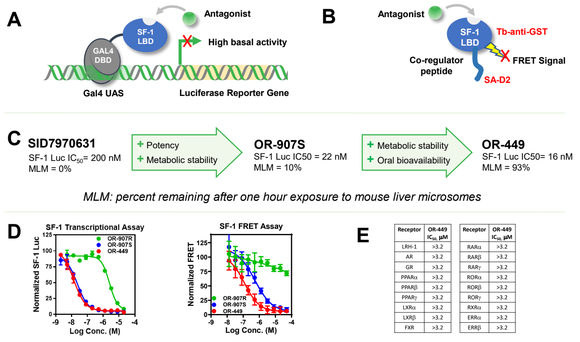
Figure 2: SF-1 is a specific marker for LCT and SCST

Tumor type	SF-1 staining	ref
Leydig cell tumors	positive	4
testicular germ cell tumors	negative	5
ovarian Sertoli cell tumor	Positive	6
granulosa cell tumor	Positive	7

OR-449: A Specific Antagonist of SF-1 2

- Assay methodology: Transcription from SF-1 ligand-binding domain (**Figure 3A**) and coactivator recruitment assay based on FRET signal between terbium-anti-GST-labeled SF-1 LBD and streptavidin (SA)-D2-labeled co-regulator peptide (**Figure 3B**).
- Discovery of OR-449: Initial hits were identified by HTS[8]. Medicinal chemistry at Orphagen identified OR-907S, a potent antagonist with limited metabolic stability, and subsequently OR-449 (**Figure 3C, 3D**).
- Ligand pharmacology: OR-907R (SF-1 Luc IC_{50} =2.5 μM) and OR-907S are enantiomers. Relative potencies are comparable in transcription and FRET assays (**Figure 3D**).
- OR-449 is inactive at a panel of nuclear receptor transcriptional assays (**Figure 3E**).

Figure 3: OR-449 Assay Methodology, Discovery, and Pharmacology

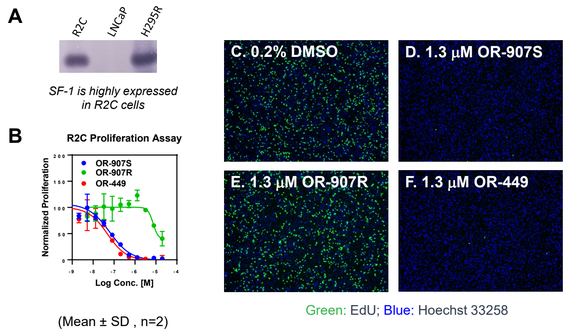


Receptor	OR-449 IC_{50} μM	Receptor	OR-449 IC_{50} μM
LRH-1	>3.2	RARα	>3.2
AR	>3.2	RARβ	>3.2
GR	>3.2	RARγ	>3.2
PPARα	>3.2	RORα	>3.2
PPARβ	>3.2	RORβ	>3.2
PPARγ	>3.2	RORγ	>3.2
LXRα	>3.2	RXRα	>3.2
LXRβ	>3.2	ERRα	>3.2
FXR	>3.2	ERRβ	>3.2

SF-1 Antagonists Inhibit R2C Cell Proliferation *In Vitro* 3

- SF-1 expression in a rat Leydig tumor cell was determined by immunoblotting (**Figure 4A**) using the N1665 anti-SF-1 antibody. H295R, an ACC cell line, and LNCaP cells are positive and negative controls, respectively.
- Dose response inhibition of EdU incorporation in R2C cells by SF-1 antagonists. 6-FAM azide labelled EdU+ cells were quantified by a fluorescent plate reader. Average fluorescence from DMSO (100%) or 10 μM cycloheximide (0%) treated wells are used to normalize EdU+ (**Figure 4B**). OR-449 and OR-907S are equally active, as in Figure 3D.
- R2C cells were cultured with either DMSO, OR-907S, OR-907R or OR-449 (**Figure 4C-F**) for 3 d. EdU+ cells were labeled with 6-FAM azide (green) on a Hoechst 33258 (blue) background.

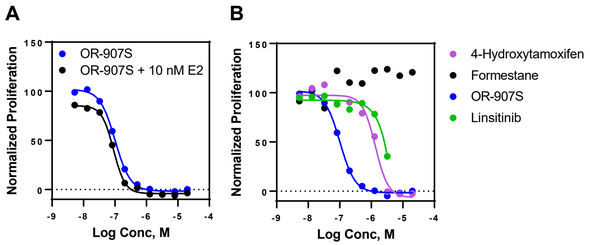
Figure 4: R2C Proliferation *In Vitro*



Novel Mechanism of SF-1 Antagonists 4

- It was suggested that LCT survival is mediated by SF-1 as a part of insulin-like growth factor 1 (IGF-1) signaling transduction pathway, which resulted in the regulation of aromatase expression and estrogen-dependent proliferation[9].
- Potency and inhibition efficacy of SF-1 antagonists are not shifted by the treatment of 17β-estradiol (E2), suggesting the inhibitory effect of SF-1 antagonists is independent of hormone regulated cellular response (**Figure 5A**).
- Formestane, an aromatase inhibitor, linsitinib, an IGFIR/IR inhibitor, or 4-hydroxytamoxifen, an estrogen receptor inhibitor, do not inhibit R2C proliferation potently, suggesting a novel inhibitory mechanism of SF-1 antagonists to LCTs (**Figure 5B**).

Figure 5: R2C Proliferation Shows a Unique SF-1 Dependency

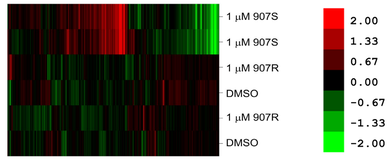


R2C cells were incubated with test compounds, with or without 10 nM 17β-estradiol (E2) for two days. EdU was added the last 16 h to label S-phase cells.

Regulation of Gene Expression by SF-1 Antagonist *In Vitro* 5

- R2C cells were cultured with 0.1% DMSO, 1 μM OR-907S, or 1 μM OR-907R for 3 days (N=2/group). Gene expression was determined by RNA sequencing.
- Heatmap analysis (**Figure 6A**) showed that gene expression patterns for DMSO and 1 μM OR-907R (inactive enantiomer) are similar and differ significantly from OR-907S (active enantiomer). RPKM data from 15,349 detectable rat genes were used in analysis by hierarchical clustering. RPKM values for each gene were log-2-transformed.
- KEGG pathway analysis (**Figure 6B**) demonstrates that compared to OR-907R, OR-907S treatment affects multiple cancer-related pathways (p<0.001).

Figure 6: OR-907R, OR-907S and DMSO R2C Gene Expression



B

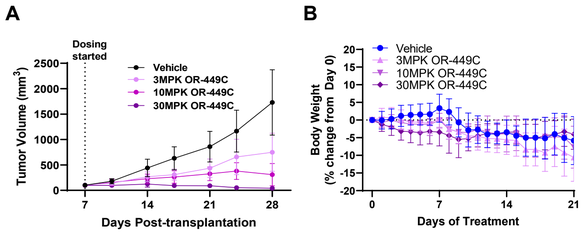
Pathway Name	Gene number O/C	rawP	adjP
Drug metabolism - cytochrome P450	18/30	1.58e-13	2.21e-11
Metabolism of xenobiotics by cytochrome P450	15/26	3.90e-11	2.73e-09
Glutathione metabolism	15/39	5.05e-08	2.36e-06
Cell cycle	20/97	1.81e-07	1.86e-05
Oocyte meiosis	16/79	3.94e-06	0.0002
Progesterone-mediated oocyte maturation	13/61	1.78e-05	0.0006
ECM-receptor interaction	10/35	0.0002	0.0056
Steroid hormone biosynthesis	7/18	0.0002	0.0056
p53 signaling pathway	9/41	0.0003	0.0077

Genes are selected based on the Tukey P-Value (< 0.05) and Fold Change (> 2 up / down); C: the number of reference genes in the category; O: the number of genes regulated by OR-907S in the category; rawP: p-value from hypergeometric test; adjP: p-value adjusted by the multiple test adjustment.

OR-449 Inhibits R2C Tumor Growth *In Vivo* 6

- R2C cells ($5x10^5$) were implanted subcutaneously in the right flank of male Nu/Nu immunocompromised mice. Mice were randomly assigned to groups of 12 bearing similarly sized tumors. The mean tumor volume at the start of dosing (7 days post implantation) was $100mm^3$.
- Mice were dosed with OR-449 (3, 10, or 30 mg/kg) or vehicle by daily oral gavage for 21 days.
- OR-449 (30 mg/kg) completely suppressed tumor growth compared to vehicle-treated mice (**Figure 7A**).
- OR-449 was well-tolerated as demonstrated by similar body weight changes compared to vehicle mice (**Figure 7B**).

Figure 7: *In Vivo* R2C Tumor Growth and Body Weight



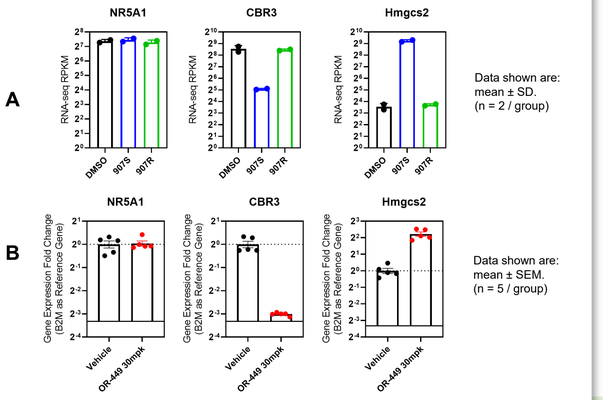
Tumor volume was calculated as: (length x width^2) x 0.5. Mean tumor volume at the start of dosing (7 days post implantation) was $100mm^3$. Data shown are mean ± SD. (n=10-12/group)

Regulation of Gene Expression by OR-449 *In Vivo* 7

R2C tumor-bearing mice were randomly assigned to groups of 5 bearing similarly sized tumors. The mean tumor volume at the start of dosing (14 days post implantation) was ~250mm^3. Mice were dosed with OR-449 (30 mg/kg, PO) or vehicle for 3 days. Tumor mRNA was isolated on Day 4.

Gene regulation by OR-449 in R2C tumors *in vivo* (**Figure 8B**) follows a similar pattern as gene regulation by OR-907S treatment in R2C cells *in vitro* (**Figure 8A**), supporting the hypothesis that OR-449 is regulating tumor gene expression through SF-1.

Figure 8: Gene Regulation by OR-449 *In Vivo* Is Consistent with OR-907S *In Vitro*



Conclusions 8

- OR-449, a specific antagonist of SF-1, inhibits proliferation of R2C tumor cells in culture and completely blocks R2C tumor growth as a xenograft
- OR-907S responsive genes identified in R2C cell culture track with OR-449 responsive genes in R2C tumors, consistent with SF-1 acting as the drug target for OR-449-mediated control of growth.
- Medical therapy for metastatic LCT is limited. These results highlight SF-1 antagonism as a novel targeted therapeutic approach with potential utility in the treatment of ACC, LCT and other SCST.
- OR-449 is currently in IND-enabling studies in order to enter the clinic by the end of 2022.

References & Acknowledgements 9

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