

AWARD NUMBER: W81XWH-18-1-0015

TITLE: Targeted Inhibition of Leukemia Inhibitory Factor (LIF)/LIFR Axis for the Treatment of Triple-Negative Breast Cancer

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REPORT DATE: MAY 2022

TYPE OF REPORT: FINAL Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE MAY 2022		2. REPORT TYPE FINAL		3. DATES COVERED 02/01/2018 - 1/31/2022	
4. TITLE AND SUBTITLE Targeted Inhibition of Leukemia Inhibitory Factor (LIF)/ LIFR Axis for the Treatment of Triple-Negative Breast Cancer				5a. CONTRACT NUMBER W81XWH-18-1-0015	
				5b. GRANT NUMBER BC170312	
				5c. PROGRAM ELEMENT NUMBER	
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				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Texas Health Science Center at San Antonio 7703 Floyd Curl Drive San Antonio, Texas 78229 Evestra, Inc, 6410 Tri-County Parkway, Schertz 78154, TX				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Leukemia inhibitory factor receptor (LIFR) and its ligand LIF play a critical role in cancer progression and therapy resistance. In this DOD funded project, we developed a first-in-class inhibitor of LIFR, EC359, which binds to LIFR and block LIF/LIFR interactions. EC359 treatment exhibited antiproliferative effects, reduced stemness, and promoted apoptosis in triple-negative breast cancer (TNBC) cell lines. Treatment with EC359 attenuated the activation of LIF/LIFR-driven pathways. EC359 also reduced the viability of therapy resistant TNBC models and synergized HDAC inhibitors. EC359 reduced the invasion and decreased metastases of TNBC cells. RNA-seq studies demonstrated oncogenic/survival signaling pathways were attenuated by the EC359 therapy. Importantly, EC359 inhibited the growth of TNBC patient derived explants ex vivo, cell derived xenografts and patient-derived xenografts <i>in vivo</i> . EC359 exhibits distinct pharmacologic advantages, including oral bioavailability, and <i>in vivo</i> stability. Collectively, these data support EC359 as a novel targeted therapeutic that inhibits LIFR oncogenic signaling.					
15. SUBJECT TERMS Breast cancer; TNBC, LIFR, LIF, STAT3, tumor progression					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
Unclassified	Unclassified	Unclassified	Unclassified	59	

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1. INTRODUCTION

Triple negative breast cancer (TNBC) has poor prognosis, lack targeted therapies, contain high percentage of cancer stem cells, and often exhibit therapy resistance. TNBC are more aggressive, and due to lack of targeted therapies, they represent a disproportional share of the breast cancer mortality. TNBC tumors are characterized by high propensity to metastasis, some subtypes of TNBC such as claudin-low are highly enriched with cancer stem cells and frequently exhibit therapy resistance. The propensity of TNBC to metastasize to visceral sites is another major clinical problem and identifying factors that contribute to metastases are needed for advancement of therapies. There is a major need for rationally designed novel therapies that can improve response to TNBC treatment and extend survival.

Leukemia inhibitory factor (LIF) is the most pleiotropic member of the interleukin-6 family of cytokines that signals via the LIF receptor (LIFR). LIF activate multiple signaling pathways including STAT3, MAPK, AKT, and mTOR that are implicated in TNBC progression. Recent evidence implicated tumors exhibit upregulated LIF-LIFR signaling via autocrine and paracrine mechanisms. LIF promotes proliferation and metastasis of breast cancer cells. TNBC cells have higher expression of LIF compared to ER-positive breast cancer cells and overexpression of LIF significantly associated with a poorer relapse free survival in breast cancer patients. LIF signaling play a role in crosstalk between tumor cells and fibroblasts and mediate pro-invasive activation of stromal fibroblasts. LIF-LIFR axis is implicated in the maintenance of stem cells and deregulation of LIF signaling contribute to chemoresistance. Together, these emerging findings strongly suggest that LIF signaling in TNBC may be clinically actionable and that disruption of the LIF signaling has potential to block TNBC progression.

With our expertise in developing novel small molecule inhibitors that disrupt protein-protein interactions and SAR studies using our compound libraries, we have rationally designed and synthesized a first-in class LIF inhibitor compound. The objective of this proposal is to confirm the specificity, test in vivo activity and establish the mechanism, and conduct safety, efficacy, off target screening studies. Our hypothesis is that LIFR signaling play a critical role in TNBC progression and disruption of LIF-LIFR interaction with small molecule inhibitor EC359 will have therapeutic effect.

2. KEYWORDS

Breast cancer; TNBC; LIFR; LIF; STAT3; xenograft; tumor progression;

3. ACCOMPLISHMENTS

Major accomplishments include:

- (1) Identification and characterization of EC359 as a First-in-Class LIFR inhibitor
- (2) Demonstration of the efficacy of EC359 using preclinical xenograft and PDX models of TNBC
- (3) Establishment of the molecular mechanism and signaling studies
- (4) RNA-seq studies and identification of target genes for biomarker analyses
- (5) Establishing the utility of EC359 in treating therapy resistance
- (6) Completion of large-scale synthesis of EC359
- (7) Completion of ADME, PK and preliminary toxicology studies

3-1. What were the major goals of the project?

Aim 1. To establish the efficacy of EC359 using preclinical, syngeneic and PDX models of TNBC.

Aim 2. To establish the molecular mechanism of action of EC359 and to identify potential biomarkers.

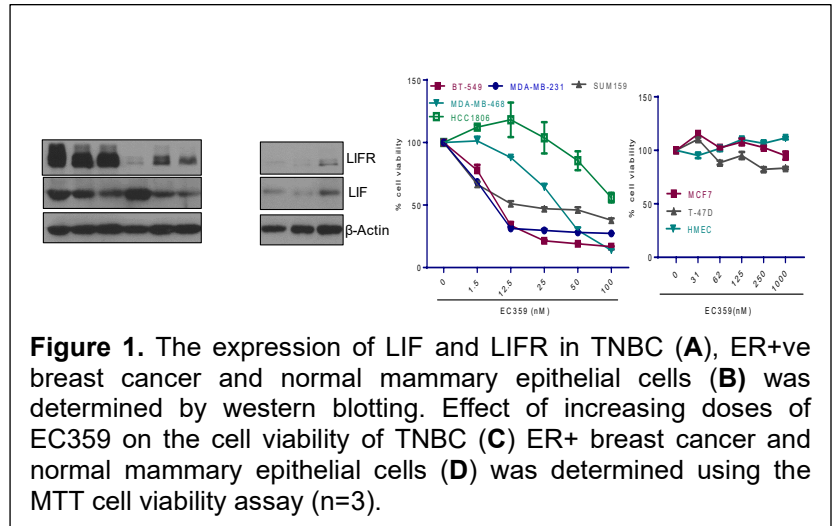
Aim 3. To establish ADME, pharmacokinetics (PK), and toxicology of EC359.

3-2. What was accomplished under these goals?

Major task 1: To establish the efficacy of EC359 using preclinical, and PDX models of TNBC.

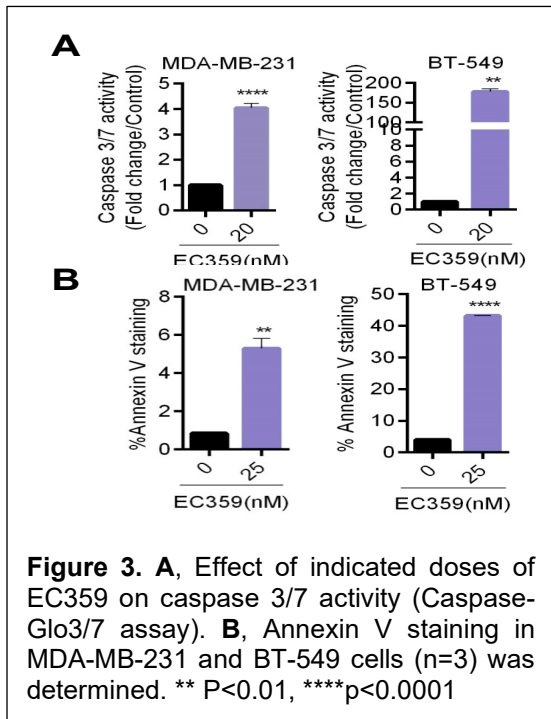
Subtask 1: To characterize the biological activity of EC359 in vitro

EC359 reduced the cell viability of LIF and LIFR expressing cells. We examined the expression of LIF and LIFR in cells that represent various subtypes of TNBC (BT-549, SUM-159, MDA-MB-231, HCC1937, MDA-MB-468, and HCC1806), ER+ve breast cancer (MCF7, and T47D) as well as normal mammary epithelial cells (HMEC). We found that five of the six TNBC cells expressed high levels of LIF and LIFR when compared to ER+ve breast cancer cells and normal cells (Figure 1A,B). Next, we examined the efficacy of EC359 on cell viability of TNBC and ER+ve breast cancer cells. Treatment with EC359 resulted in a significant dose-dependent reduction in the cell viability of TNBC cells (IC₅₀ 10-50 nM) and their inhibition is correlated well with the LIF and LIFR expression levels (Figure 1C). Interestingly ER+ve breast cancer cells which express low levels of LIF and LIFR exhibited low sensitivity to EC359 treatment (IC₅₀ >1000 nM) when compared with TNBC cells (Figure 1D).

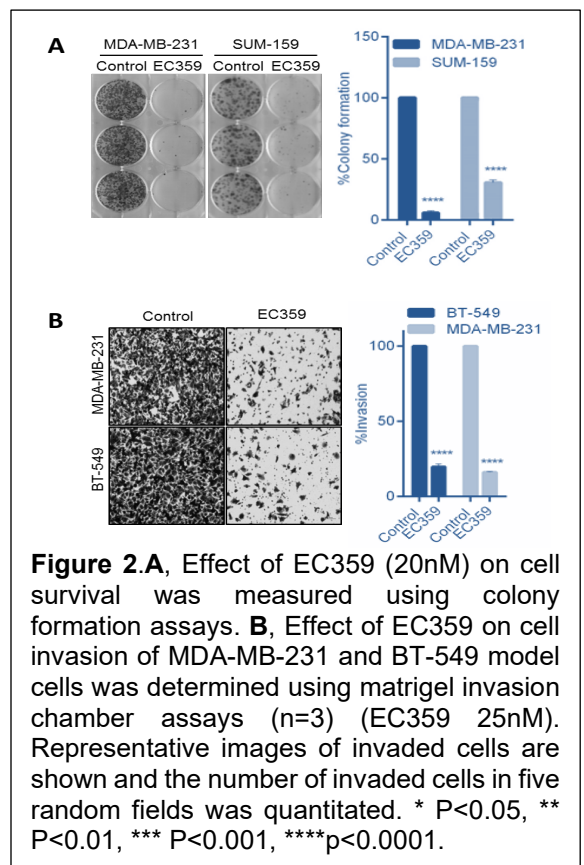


EC359 reduced survival and invasion of TNBC cells.

In clonogenic survival assays, EC359 significantly reduced the colony formation ability of MDA-MB-231 and SUM-159 cells (Figure 2A). Given the important role of the LIF axis in the invasiveness of cancer cells, we examined the effect of EC359 in reducing the invasion of TNBC cells. Matrigel

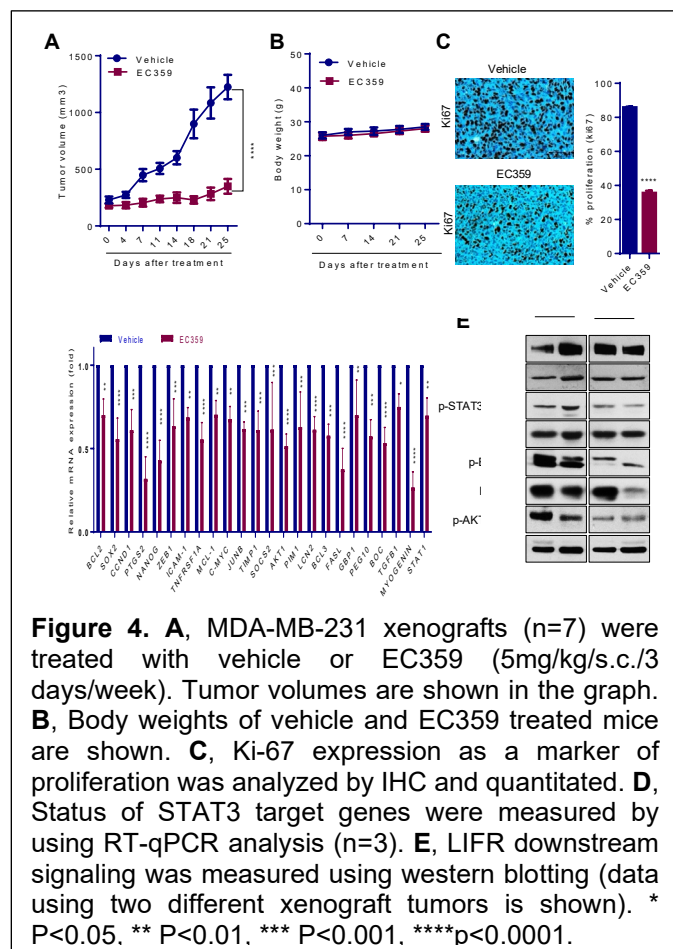


Collectively, these results suggest that EC359 promotes apoptosis of TNBC cells.



EC359 induced apoptosis of TNBC cells: We examined whether EC359 induce apoptosis of TNBC cells using caspase 3/7 activity assay and Annexin V staining assay. EC359 treatment significantly increased caspase 3/7 activity (Figure 3A) and Annexin V positive cells (Figure 3B) in both MDA-MB-231 and BT-549 cells.

Multi-PI contributions: The above work was jointly performed by Dr. Vadlamudi and Dr. Santhamma labs. Dr. Santhamma lab generated needed EC359, and tested its purity and activity. Dr. Vadlamudi lab characterized biological activity using TNBC models.



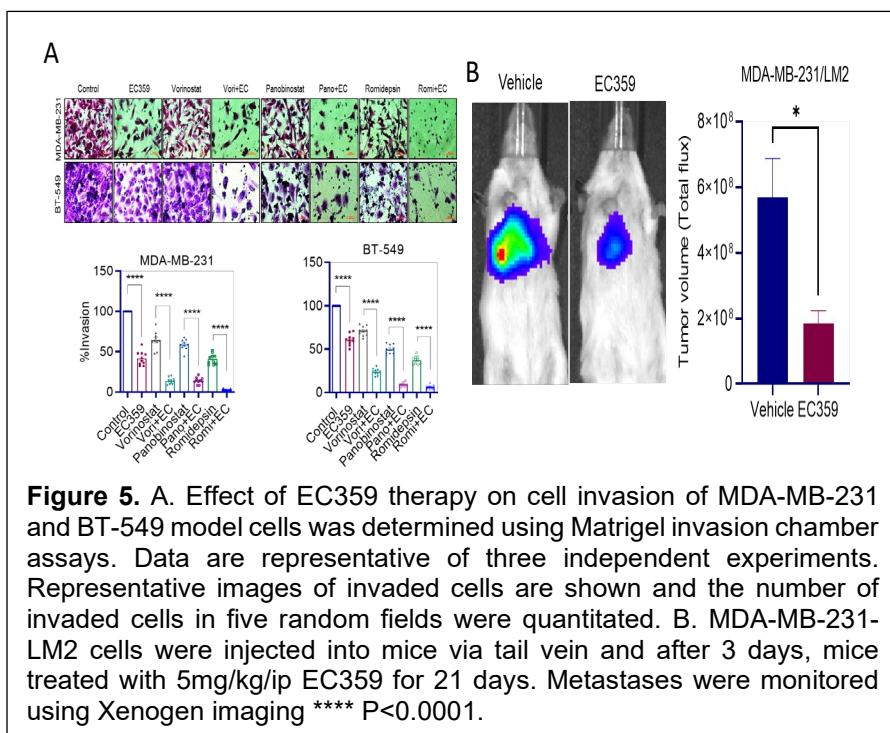
Subtask 2: Test the efficacy of EC359 on blocking TNBC tumorigenesis in vivo

EC359 reduced TNBC xenograft tumor growth in vivo: To test the efficacy of EC359 on *in vivo* tumor progression, we established MDA-MB-231 xenograft tumors in the mammary fat pad of nude mice. Mice were randomized to vehicle (hydroxy methyl cellulose) and EC359 (5 mg/kg/day via subcutaneous injection) 3 days/week. EC359 treatment significantly reduced the tumor progression compared to vehicle (Figure 4A). Mice body weights in the vehicle and EC359 treated groups were similar (Figure 4B) confirming the low toxicity of EC359. Further, EC359 treated tumors exhibited fewer proliferating cells (Ki-67 positive cells) compared to vehicle treated tumors (Figure 4C). Further, RTqPCR analysis confirmed significant decrease in the activation of STAT3 target genes in EC359 treated tumors compared to control (Figure 4D). Western blot analysis confirmed that xenograft tumors express LIFR and LIF (Figure 4E). Further, EC359 treatment substantially reduced the phosphorylation of STAT3 and ERK1/2 pathways in EC359 treated tumors compared to vehicle treated tumors (Figure 4E). Collectively, these results suggest that EC359 has potent anti-tumor activity on TNBC in preclinical models.

Multi-PI contributions: The above work was jointly performed by Dr. Vadlamudi and Dr. Santhamma labs. Dr. Santhamma lab synthesized needed EC359 compound and

optimized process development. Dr. Vadlamudi's lab conducted xenograft studies, tumor measurement and biochemical characterization.

Subtask 3: Effect of EC359 on blocking TNBC tumor progression to metastasis. We examined the potential of EC359 in reducing invasion of TNBC cells. Matrigel invasion assays demonstrated that EC359 was able to reduce the invasion of TNBC models. Further, the combination therapy of EC359+HDACi is more effective than monotherapy in reducing the invasion of MDA-MB-231 and BT-549 cells (Figure 5A). To test the utility of EC359 to block lung metastases in vivo, MDA-MB-23-LM cells were injected in to mice via tail vein and after 3 days, mice were treated with 5mg/kg/ip EC359. Metastases were monitored using Xenogen imaging. Results showed significant reduction in lung metastases upon EC359 treatment (Figure 5B).



Subtask 4: Test the efficacy of EC359 combination therapy in reducing the growth of therapy resistant TNBC. MTT assays demonstrated synergistic activity of EC359 with chemotherapeutic agents, cisplatin, and paclitaxel (Figure 6, top panel). Further, EC359 is also effective in reducing the cell viability of paclitaxel resistant TNBC model cells (Figure 6, bottom panel). Recent studies suggested that HDACi treatment promotes expression and activation of LIFR, which restrains the efficacy of HDACi as TNBC therapeutics and promotes therapy resistance. We examined whether treatment of EC359 enhances the therapeutic efficacy of HDACi on TNBC cells using MTT assays. As shown in Figure 7, addition of EC359 significantly enhanced the efficacy of HDACi in reducing cell viability compared to HDACi as monotherapy. Results showed that the combination index (CI) values were less than 1 in all the three HDACi combinations tested and confirmed that the combination therapy was synergistic (Figure 7A). In clonogenic survival assays, EC359 enhanced HDACi ability to

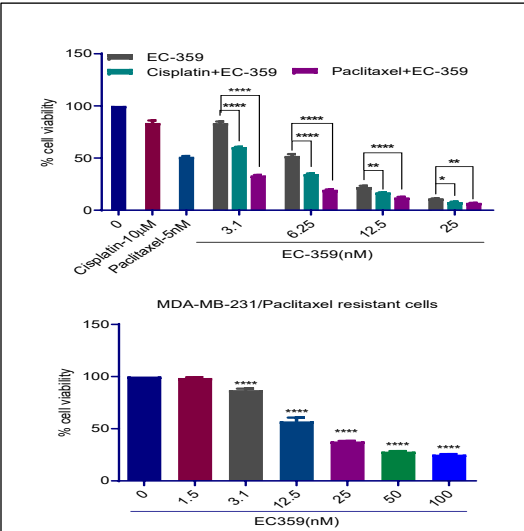


Figure 6: MDA-MB 231 cells or paclitaxel resistant MDA-MB-231 cells were treated with EC359 alone or in combination with cisplatin or paclitaxel and cell viability was measured using MTT assay. **** P<0.0001.

reduce the colony formation of MDA-MB-231 and BT-549 cells (Figure 7B) compared to monotherapy. To test the utility of EC359+HDACi therapy in vivo, we conducted xenograft study

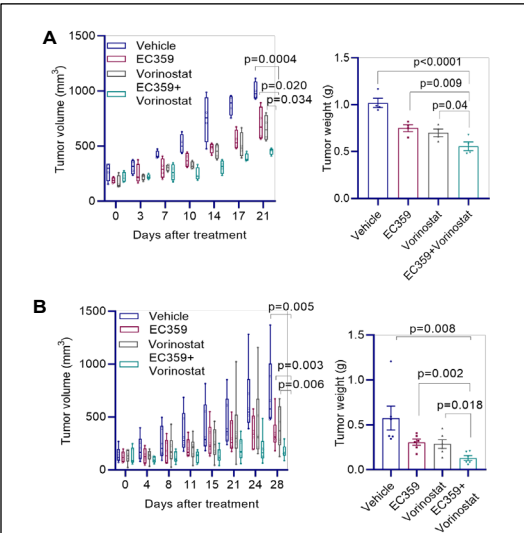


Figure 8: **A**, MDA-MB-231 (n=4) and **B**, MDA-MB-468 (n=6) xenografts in SCID mice were treated with vehicle or EC359 (5 mg/kg/day) or vorinostat (100 mg/kg/day) or in combination. Tumor volume and weight of vehicle and treated tumors were measured.

using two different TNBC cells. MDA-MB-231 and MDA-MB-468 xenografts bearing SCID mice were randomized and treated with vehicle or EC359 (5mg/kg/sc) 3 days/week or HDACi (vorinostat) (100mg/kg/oral) 5 days/week alone or in combination. EC359+HDACi combination treatment resulted in lower tumor volume and smaller tumor weight compared to monotherapy of EC359 or vorinostat (Figure 8A, B). These results suggest that EC359 has potential to reduce therapy resistance by enhancing the efficacy of HDACi to reduce TNBC xenograft tumor growth.

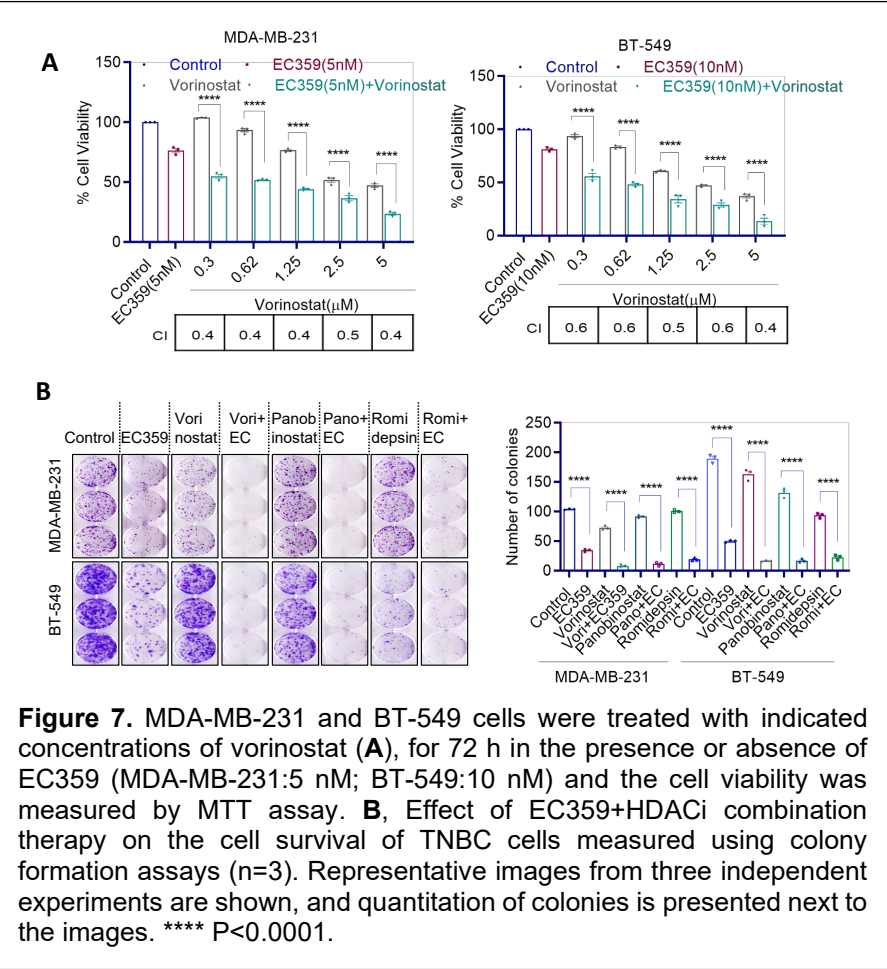
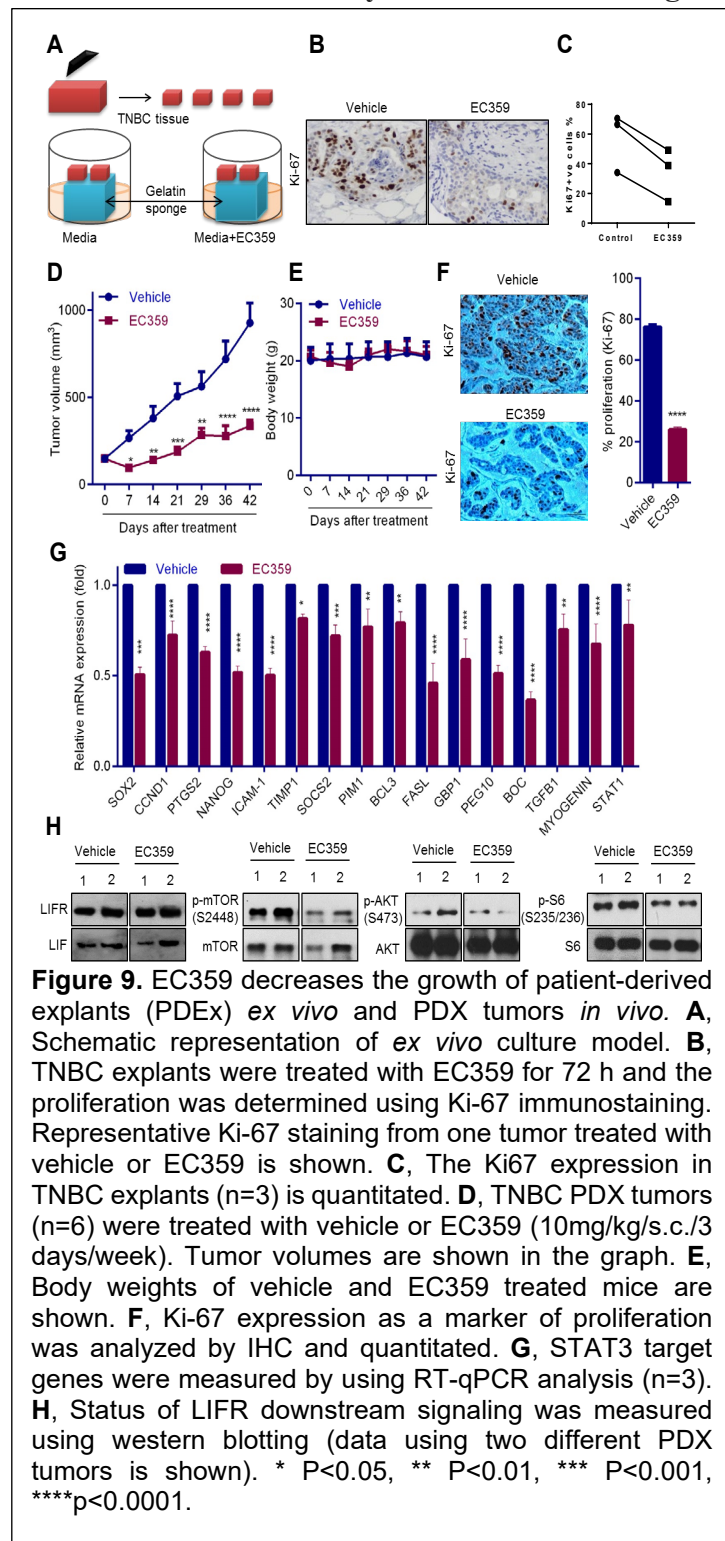


Figure 7. MDA-MB-231 and BT-549 cells were treated with indicated concentrations of vorinostat (**A**), for 72 h in the presence or absence of EC359 (MDA-MB-231:5 nM; BT-549:10 nM) and the cell viability was measured by MTT assay. **B**, Effect of EC359+HDACi combination therapy on the cell survival of TNBC cells measured using colony formation assays (n=3). Representative images from three independent experiments are shown, and quantitation of colonies is presented next to the images. **** P<0.0001.

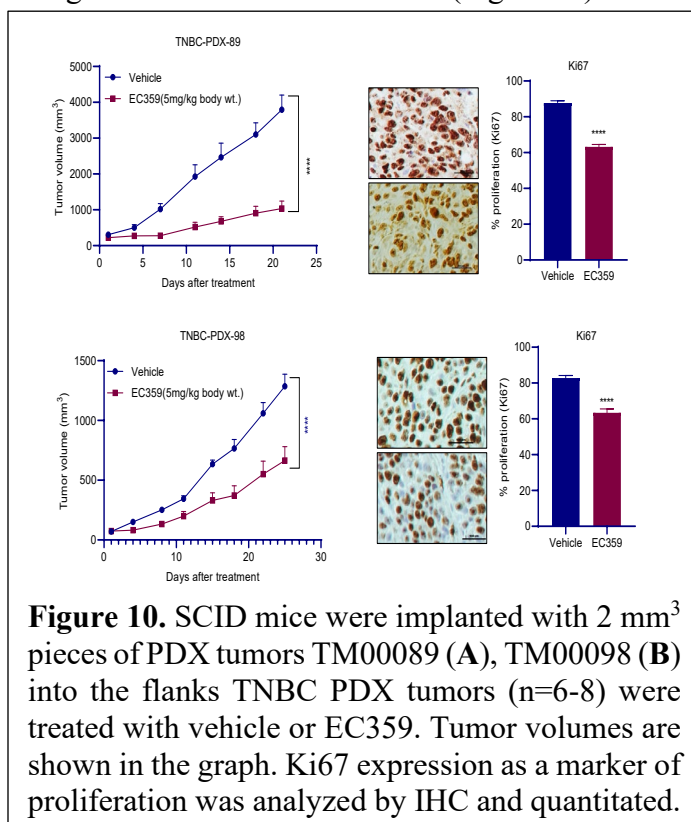
using two different TNBC cells. MDA-MB-231 and MDA-MB-468 xenografts bearing SCID mice were randomized and treated with vehicle or EC359 (5mg/kg/sc) 3 days/week or HDACi (vorinostat) (100mg/kg/oral) 5 days/week alone or in combination. EC359+HDACi combination treatment resulted in lower tumor volume and smaller tumor weight compared to monotherapy of EC359 or vorinostat (Figure 8A, B). These results suggest that EC359 has potential to reduce therapy resistance by enhancing the efficacy of HDACi to reduce TNBC xenograft tumor growth.

Subtask 5: Test the efficacy of EC359 in reducing the growth of TNBC PDX tumors in vivo



showed that EC359 is efficient in reducing the PDX tumor growth compared to vehicle (Figure 10 A,B). Further, EC359 treated tumors showed less proliferation (Ki67 staining) compared to vehicle (Figure 10 A,B). These results suggest that EC359 has therapeutic activity on primary patient derived TNBC explants and PDX tumors.

EC359 has activity against primary patient derived TNBC explants and reduced *in vivo* tumor progression in PDX model. We tested the utility of EC359 using an *ex vivo* culture model of primary breast tumors, which allowed for the evaluation of drugs on human tumors while maintaining their native tissue architecture (Figure 5A). Briefly, surgically extirpated de-identified TNBC tumor tissues were cut into small pieces and placed on gelatin sponge soaked in the culture medium and grown for a short term in the presence of vehicle or EC359 (Figure 5A). Treatment of TNBC explants with EC359 substantially decreased their proliferation (Ki-67 positivity) compared to vehicle treated tumors (Figure 9 B,C). Next, we tested the effect of EC359 on PDX tumor growth *in vivo*. EC359 treatment significantly reduced the tumor progression compared to the vehicle treated control group (Figure 9D) and did not affected body weight (Figure 9E). EC359 treated PDX tumors exhibited fewer proliferating cells compared to vehicle treated tumors (Figure 9F). RT-qPCR analysis confirmed a significant decrease in the activation of STAT3 target genes in EC359 treated mice (Figure 9G). Western blot analysis confirmed that PDX tumors express LIFR and LIF (Figure 9H). Furthermore, EC359 treatment substantially reduced the phosphorylation of mTOR, S6 and AKT in tumors compared to vehicle treated tumors (Figure 9H). We validated these results using two additional PDX models (Figure 10). Results



Major Task 2: To establish the molecular mechanism of action of EC359 and to identify potential biomarkers

Subtask 1: Determine the specificity of EC359 for targeting LIFR

Surface plasmon resonance (SPR) studies confirmed EC359 direct interaction with LIFR. To test whether EC359

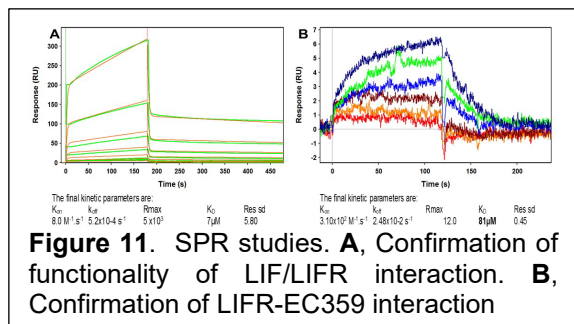


Figure 11. SPR studies. **A**, Confirmation of functionality of LIF/LIFR interaction. **B**, Confirmation of LIFR-EC359 interaction

of studies, results showed EC359 binding to LIFR, but not LIF. Further, EC359 bound to LIFR in a dose dependent manner with K_D 81 μM (Figure 11B). The results confirmed that EC359 is a specific inhibitor of LIF/LIFR complex.

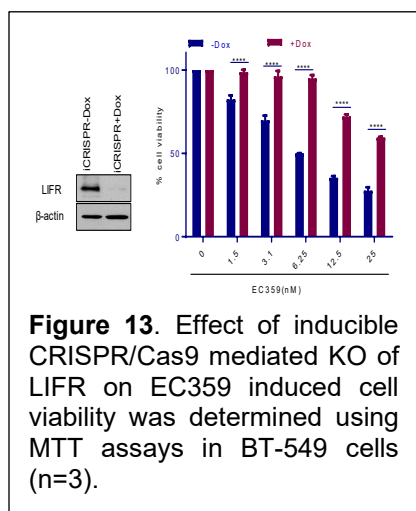


Figure 13. Effect of inducible CRISPR/Cas9 mediated KO of LIFR on EC359 induced cell viability was determined using MTT assays in BT-549 cells (n=3).

Microscale thermophoresis (MST) assays revealed high affinity interaction of EC359 with LIFR. Since SPR studies use immobilized receptor complex, we conducted an orthogonal assay where receptor is not immobilized to verify EC359 binding to the receptor complex. MST is a powerful technique to quantify biomolecule interactions. By combining the precision of fluorescence detection with the variability and sensitivity of thermophoresis, MST provides a flexible, robust and fast way to dissect molecular interactions. MST analysis confirmed direct interaction of EC359 with LIFR with an estimated K_D of 10.2 nM (Figure 12).

CRISPR KO cells confirmed requirement of LIFR for EC359 function. To further confirm the target specificity of EC359, we generated doxycycline inducible LIFR-KO cells using Cas9 stably expressing TNBC cells. Results indicated a reduction of LIFR expression in BT-549 models contributed to the

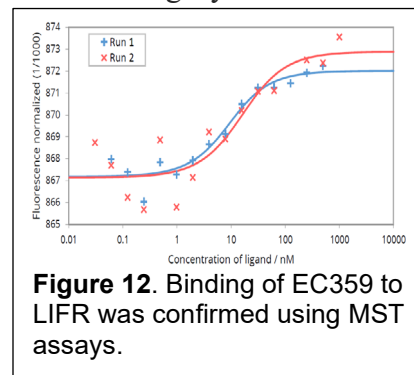


Figure 12. Binding of EC359 to LIFR was confirmed using MST assays.

resistance of the EC359 mediated decrease in cell viability (Figure 13). Collectively, this data suggests that EC359 activity depends on presence of functional LIF/LIFR signaling axis in cells.

Docking studies confirmed EC359 can interact at the LIF-LIFR binding interface.

The site predictions revealed five potential binding sites on the hLIFR in which two sites (2 and 3) are close to the LIF binding interface. The SP docking was performed on all the five sites and docking scores were deduced (Figure 14A). The docking scores towards different binding sites range from -5.8 to -1.6 kcal/mol. It was also observed that EC359 has exhibited more promising scores towards site-3 compared to other sites. The binding poses obtained from the docking were superimposed to the

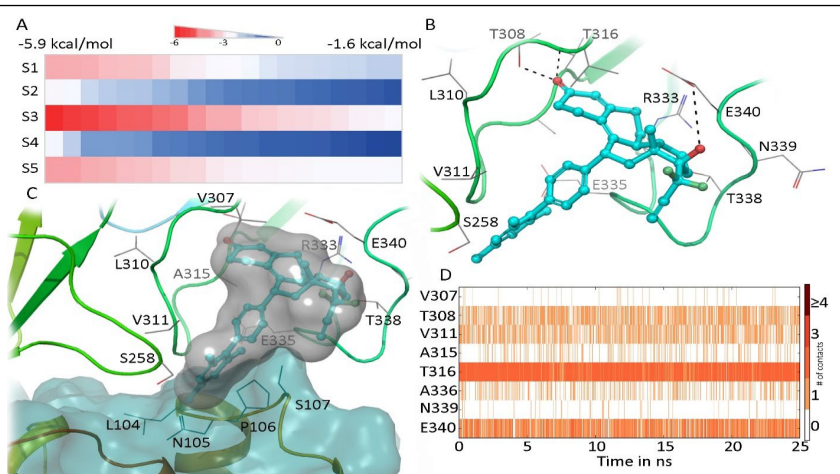
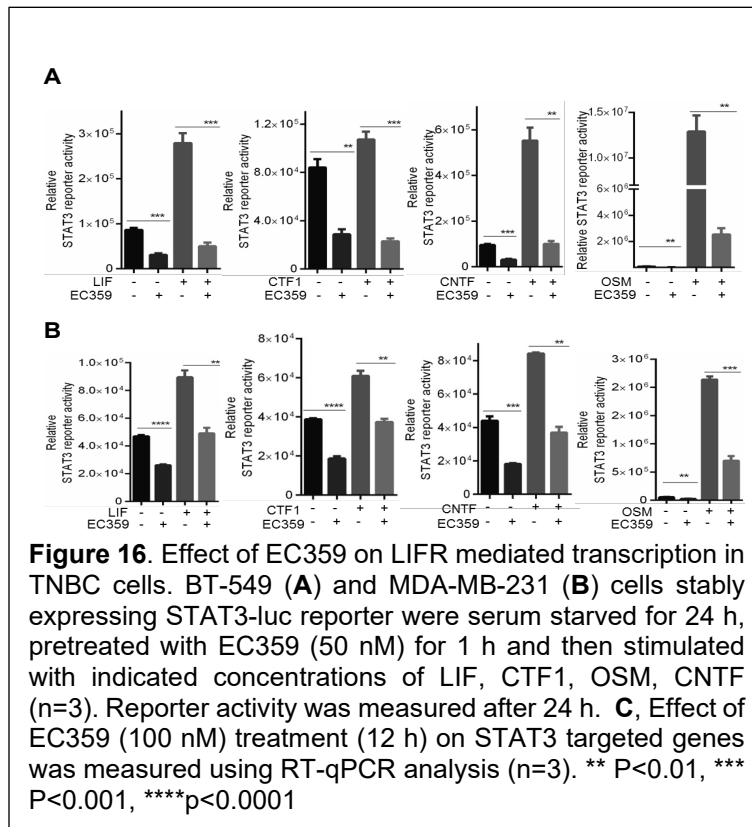
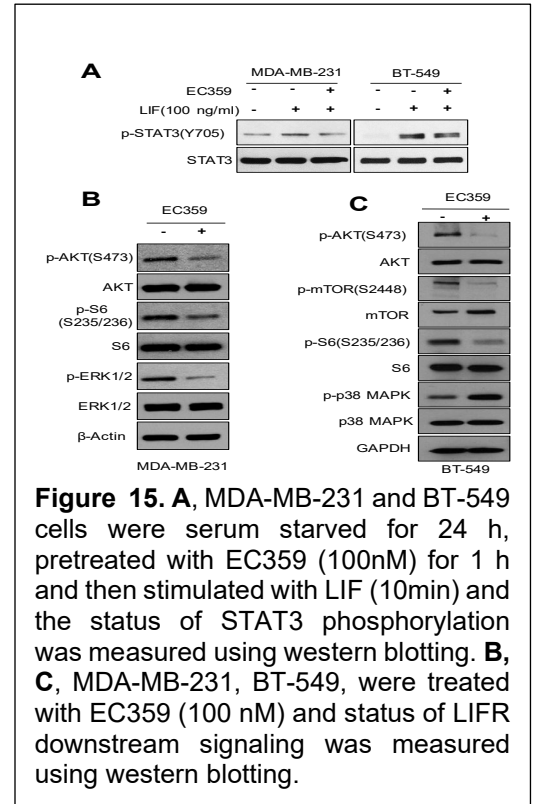


Figure 14. Docking studies. **A**: The docking scores of EC359 at different binding sites. The sites were mentioned as S1 to S5. **B**: Binding of EC359 (represented in blue ball and stick model) with hLIFR (represented in cartoon and line model). The dotted lines representing the hydrogen bonds. **C**: The binding of EC359 in the presence of LIF (represented in blue surface). The binding creates close contacts with residues of LIF. **D**: The protein ligand contacts over a 25 ns of MD.

hLIF-hLIFR complex to see the potential clashes between the ligand and LIF. As expected, the binding poses at site-3 are making steric clashes with residues of LIF. Since the SP docking is a rigid docking method, the ligand induced conformational changes were also studied using IFD by applying flexibility to the surrounding residues. Using standard protocol, the side chains were optimized and 28 poses were generated. The binding energies of all the 28 poses range from -80 kcal/mol to -33 kcal/mol. It was observed that all the generated ligand poses are potentially making steric clashes with hLIF. One of the top scored poses (binding energy = -77 kcal/mol) was critically analyzed for the detailed atomic level of interactions (Figure 14B). In the selected pose, ligand induced conformational changes were observed for the loops close to the LIF binding region. The ligand EC359 was found to sandwich between two loops at the N-terminal of D4 domain by orienting the difluoro-acetylenic group to the bulk solvent. The keto group of the EC359 was found to be involved in two hydrogen bonds with the side chain of T308 and the backbone of T316. Similarly, the hydroxyl group of the ligand was also found to mediate a hydrogen bond with the sidechain of E340. Moreover, van der Waals contacts with the surrounding residues were also found to contribute to the ligand binding. It was observed that EC359 binding to hLIFR would prevent hLIF binding due to steric clashes (Figure 14C). As a final step, the snapshots obtained from the MD simulation were superimposed with the initial pose and RMSD was calculated for protein and ligand separately. The snapshots were analyzed and found that the structural distortions are affected mainly to the loops (connecting separate domains in the LIFR) and the terminal regions. At the same time the ligand is found to remain bound at the binding site even after 25 ns of MD simulation (Figure 14D).

Multi-PI contributions: The above work was jointly performed by Dr. Vadlamudi and Dr. Santhamma labs. Dr. Santhamma lab



completed biophysical studies charactering EC359 interaction with LIFR including SPR and MST studies. Dr. Vadlamudi's lab generated CRISPR KO cells and completed MTT assays.

Subtask 2: Test the effect of effect of EC359 on LIF-LIFR signaling

EC359 reduced LIFR mediated activation of downstream signaling pathways. To further confirm the effect of EC359 on LIF/LIFR downstream signaling pathways, MDA-MB-231 and BT-549 cells were pretreated with vehicle or EC359 and subsequently stimulated with LIF. STAT3 activation was examined using western blotting. EC359 treatment substantially reduced the LIF activation of STAT3 in both BT-549 and MDA-MB-231 cells (Figure 15A). Further, EC359 treatment also substantially decreased the phosphorylation of AKT, mTOR, S6 and ERK1/2 in MDA-MB-231 and BT-549 cells (Figure15 B,C). EC359 treatment also increased the phosphorylation of proapoptotic p38MAPK in BT-549 cells (Figure 15C). These results suggest that EC359 acts as a LIFR inhibitor and attenuates LIFR mediated signaling in TNBC cells.

EC359 inhibited LIFR mediated transcriptional changes. LIF/LIFR activates multiple signaling pathways including JAK/STAT3, MAPK, AKT, and mTOR; all of which are implicated in TNBC progression. To confirm the inhibitory effect of EC359 on LIF/LIFR mediated STAT3 activation, BT-549 cells that stably express STAT3-Luc reporter were pretreated with vehicle or EC359 followed by stimulation with LIF. As expected, LIF treatment significantly increased the STAT3 reporter activity and this activation was inhibited by EC359 treatment (Figure 16A). Since our modeling studies predicted EC359 interaction with the ligand binding interface of LIFR, we examined whether EC359 also blocks signaling by other LIFR ligands such as Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), and Cardiotrophin 1 (CTF1). Results showed that EC359 blocked the OSM, CNTF and CTF1 mediated STAT3 activity in BT-549 cells (Figure 16A). We also confirmed that EC359 has the ability to block LIF, OSM, CNTF and CTF1 mediated STAT3 activation using MDA-MB-231 cells stably expressing STAT3-Luc reporter (Figure 16B).

Multi-PI contributions: The above work was jointly performed by Dr. Vadlamudi and Dr. Santhamma labs. Dr. Santhamma lab synthesized needed EC359. Dr. Vadlamudi’s lab conducted signaling and western studies. Both labs collectively interpreted the data.

Subtask 3: Test the effect of EC359 on TNBC signaling pathways.
Effect of EC359 on TNBC signaling pathways.

To address pathways/genes modulated by EC359 and to determine the mechanisms by which EC359 enhanced the potency of HDAC inhibitors, we performed RNA-seq of BT-549 cells treated with vehicle, EC359, HDACi (Vorinostat) and combination (EC359+HDACi). Comparison of differentially regulated genes (p-adj<0.01, |log2FC|>1) identified 6,341 genes (Figure 5a, b), which were then subdivided into 6 major clusters by unsupervised clustering (Figure 5a). Cluster 1 genes (1,157) were synergistically up-regulated, while Cluster 3 genes (1,501) were synergistically repressed. Cluster 2 genes (661) are up-regulated by HDACi and somewhat suppressed by EC359; while Cluster 4 genes (266) are up-regulated

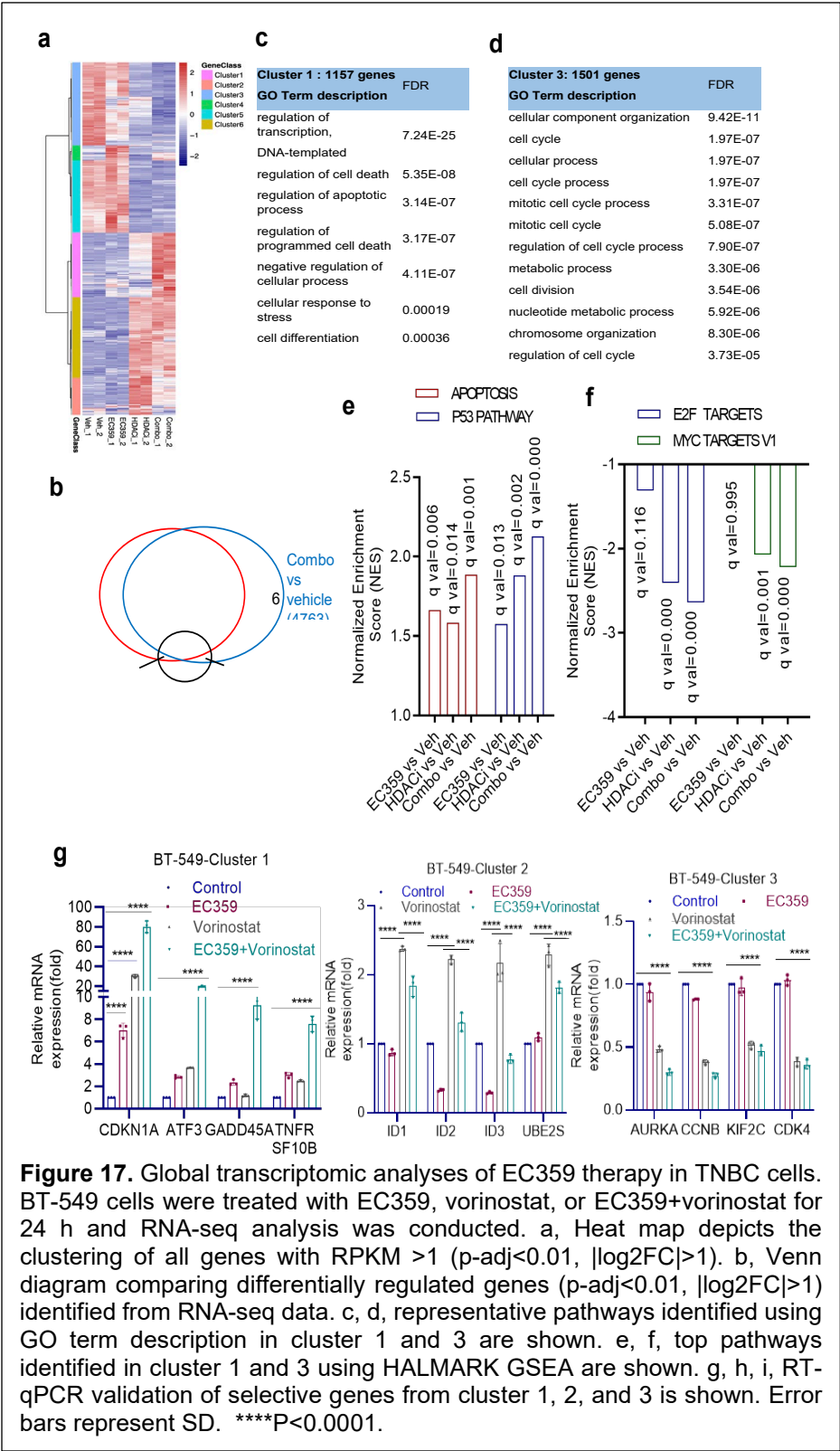


Figure 17. Global transcriptomic analyses of EC359 therapy in TNBC cells. BT-549 cells were treated with EC359, vorinostat, or EC359+vorinostat for 24 h and RNA-seq analysis was conducted. a, Heat map depicts the clustering of all genes with RPKM >1 (p-adj<0.01, |log2FC|>1). b, Venn diagram comparing differentially regulated genes (p-adj<0.01, |log2FC|>1) identified from RNA-seq data. c, d, representative pathways identified using GO term description in cluster 1 and 3 are shown. e, f, top pathways identified in cluster 1 and 3 using HALMARK GSEA are shown. g, h, i, RT-qPCR validation of selective genes from cluster 1, 2, and 3 is shown. Error bars represent SD. ****P<0.0001.

Cluster 1 genes (1,157) were synergistically up-regulated, while Cluster 3 genes (1,501) were synergistically repressed. Cluster 2 genes (661) are up-regulated by HDACi and somewhat suppressed by EC359; while Cluster 4 genes (266) are up-regulated

by EC359 and somewhat suppressed by HDACi. Cluster 6 genes (1,457) are only affected by HDACi and showed little or no effect with EC359 addition; Cluster 5 genes (1,299) are repressed by HDACi and showed no effect with EC359 addition.

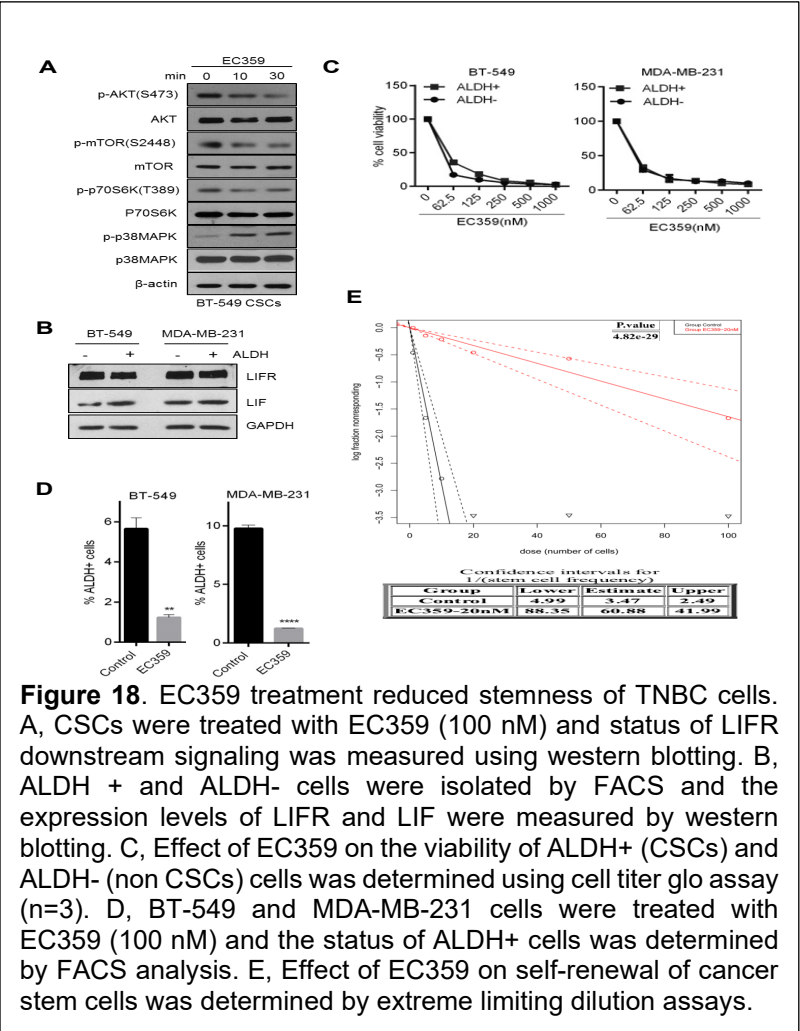
Gene ontology (GO) analyses identified Cluster 1 genes were enriched for signaling pathways associated with the regulation of transcription, cell death, apoptosis, cellular response to stress, and cell differentiation (Figure 17c); Cluster 3 genes were enriched for pathways of cell cycle, mitosis, cell division, and metabolic process (Figure 17d). Accordingly, GSEA results showed enrichment of apoptosis and p53 pathways (Figure 17e), and E2F signaling and Myc pathways (Figure 17f) for Cluster 1 and Cluster 3 genes, respectively.

We then examined the status of STAT3 target genes using custom generated STAT3 induced and repressed gene sets that were defined according to published RNA-seq data obtained from TNBC model cell lines¹⁹. GSEA of these genes revealed that EC359 treatment negatively enriched the STAT3 induced gene set but HDACi treatment showed a trend of positive enrichment of STAT3 induced gene set. Interestingly, when we compared HDACi monotherapy to combination therapy, we observed a negative enrichment of the STAT3 induced gene set suggesting that the addition of EC359 reverted the HDACi mediated upregulation of STAT3 activated genes. On the other hand, both the EC359 and HDACi regulated genes showed a trend for positive enrichment of the STAT3 repressed gene set, however, when compared HDACi monotherapy with combination therapy we observed high positive enrichment of the STAT3 repressed gene set. Altogether, these results suggest the synergistic activity of EC359+HDACi combination therapy in part attributed to the ability of EC359 to repress HDACi mediated aberrant activation of STAT3 target genes and further potentiates the HDACi mediated upregulation of STAT3 repressed genes.

To validate the RNA-seq findings, we conducted RT-qPCR of selective genes in Cluster 1, 2, and 3. As observed in RNA-Seq, expression of genes involved in apoptosis (Cluster 1) were synergistically enhanced by EC359+HDACi therapy compared to EC359 or HDACi treatment alone (Figure 17g). Further, LIF target genes such as ID1, ID2, ID3 (genes identified in Cluster 2) are upregulated by HDACi treatment and are reversed by EC359+HDACi combination therapy (Figure 5h). Similarly, genes involved in cell cycle (Cluster 3) are further downregulated by EC359+HDACi therapy compared to EC359 or HDACi treatment alone (Figure 17i). Altogether, these results suggest that potent activity of EC359+HDACi combination therapy attributed to the ability of EC359 to enhance HDACi mediated apoptotic pathways and inhibit genes involved in cell cycle regulation.

Subtask 4: Test the Effect of EC359 on TNBC Stemness.

EC359 reduced the cell viability and self-renewal of TNBC stem cells. The LIF/LIFR axis plays a vital role in stemness. To test the effect of EC359 on stemness, cancer stem cells (CSCs) were isolated from MDA-MB-231 and BT-549 using ALDH+ flow cytometry sorting. EC359 treatment substantially decreased the phosphorylation of AKT, mTOR, p70S6K, and increased phosphorylation of proapoptotic

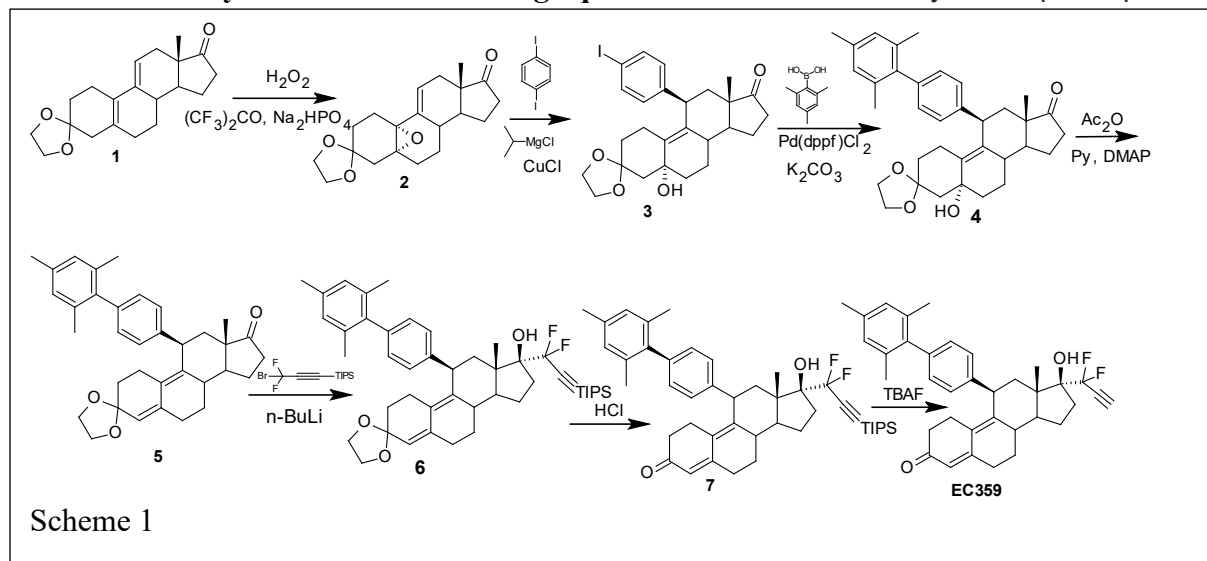


p38MAPK in CSCs (Figure 18A). Western blot analysis showed that ALDH⁺ (CSCs) and ALDH⁻ (differentiated) cells have similar levels of LIFR (Figure 18 B). Further, in cell viability assays, EC359 similarly inhibited both ALDH⁺ and ALDH⁻ cells (Figure 18C). Further, pretreatment of TNBC cells with EC359 significantly reduced the abundance of ALDH⁺ cells (Figure 18D). To further study the effect of EC359 on the self-renewal ability of CSCs, extreme limiting dilution assays (ELDA) were performed. Results showed that EC359 significantly reduced the self renewal of CSCs compared to control (Figure 18E).

Multi-PI contributions: The above work was jointly performed by Dr. Vadlamudi and Dr. Santhamma labs. Dr. Santhamma lab involved on the design of experiment. Dr. Vadlamudi's lab conducted signaling and western studies. Both labs collectively interpreted the data.

Major Task 3: To establish ADME, pharmacokinetics (PK), and toxicology of EC359

Subtask 1: To synthesize EC359 in large quantities. We successfully developed a process for the synthesis of EC-359 so that large quantities can be synthesized efficiently. The medicinal chemistry route for the preparation of EC-359 is shown in Scheme 1.

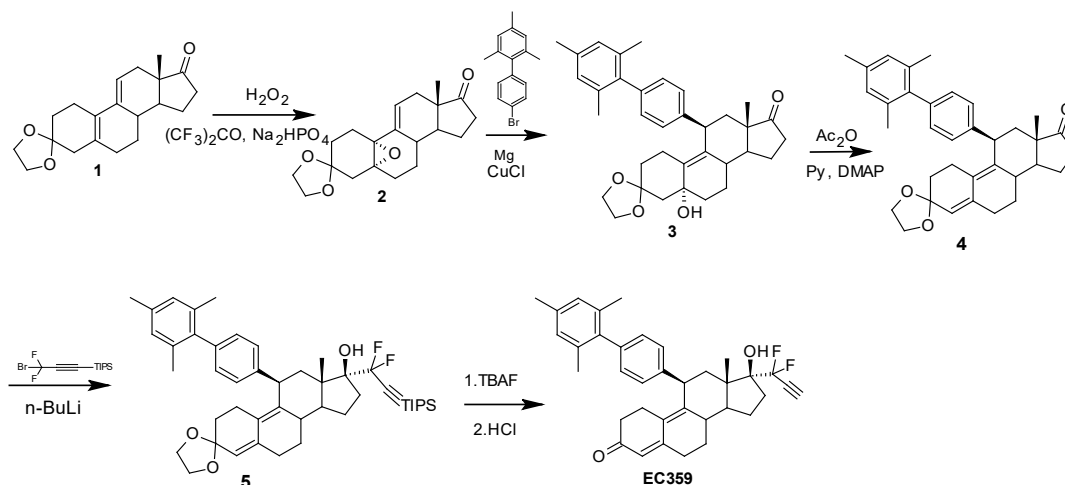


The medicinal chemistry route for the preparation of EC-359 is shown in Scheme 1.

Scheme 1

The medicinal chemistry route consists of 7

steps and involve 5 chromatographic purifications. Moreover, it's lengthy, it involves the use of heavy metal such as palladium in the main synthetic scheme. The need for multiple chromatographies along the synthesis makes it very costly. The overall yield for EC-359 using the medicinal chemistry route was found to be only 28%. To address this, we have designed several synthetic routes and tested for addressing the drawbacks and the route representing the most efficient and viable set of reactions are represented in scheme 2.



All the synthetic steps shown in Scheme 2 has been optimized and it was proved to be the choice of the scheme toward the synthesis of EC359. As an outcome, enough quantities of EC359 with HPLC purity more than 98.5%, required for the preclinical *in vitro* and *in vivo* studies were prepared and made available for the biological studies at Vadlamudi lab. Process development for the large scale synthesis of EC359 is accomplished. Biological activity was tested using various TNBC models in vitro in Vadlamudi lab. The biological activity of EC359 correlated well with the original IC50 values of EC359.

Multi-PI contributions: The above work was performed by Drs. Santhamma lab and Vadlamudi lab. Synthesis was done in Dr. Santhamma lab and the biological activity was tested in Vadlamudi lab.

Subtask2: To establish Absorption, Distribution, Metabolism, Excretion (ADME) of EC359

We have completed ADME studies using CRO and results are summarized in Figure 19. Ames test confirmed that EC359 did not induce an evident (significant) >2 fold increase in the revertant counts at the doses tested (dose related), with the tester strains both with and without metabolic activation according to the evaluation criteria mentioned in OECD guideline no.471. Hence, the compound EC359 is considered non-mutagenic with salmonella typhimurium strains TA98, TA100, TA1535, TA1537 and E.coli combo, both with and without metabolic activation. Among CYP enzymes EC359 inhibits 2D6 hence caution is warranted in concurrent administration that inhibits 2D6 such as Prozac. Metabolic stability and plasma stability are moderate in human with high plasma protein binding (Figure 18). Collectively, the data from these studies indicate EC359 as a druggable candidate for further development after establishing toxicology and safety pharmacology studies.

Multi-PI contributions: The above work was performed by Dr. Santhamma lab and Vadlamudi lab. ADME studies were completed by Dr. Santhamma with the help of CROs. Data analysis and interpretation was done in consultation with Dr. Vadlamudi lab.

Subtask3: Pharmacokinetic (PK) studies of EC359 in vivo.

The goal of these studies are to establish the time needed to obtain therapeutic drug concentration for EC359. We initially optimized the formulation for PK studies. Rats were used to treat orally with a single administration of EC359 formulated in a vehicle containing 58.5% Labrasol ALF, 22.5% Labrafil M, 1944 CS, 9% Capryol 90 and 10 % water q.s. at 0, 2.5, 5,15, 50, 100 and 300 mg/kg sequentially, based on the outcome of previous treatments. We then determined PK. These results showed that EC359 attained good systemic exposure following oral administration. Mean plasma clearance and mean volume of distribution/steady state showed that EC359

demonstrated very lower clearance suggesting 24 h interval may not be suitable for repeated dosing. Plasma half-life was found to be 4.5 h and this could be the reason for slower rate of elimination. Following intravenous bolus, EC359 eliminated as a biexponential decline. The results are summarized in Figure 20 and Table 1.

Compound	Assays	Result
EC359	Mutagenicity testing- S. typhimurium TA98, TA100, TA1535 and E.coli WP2 uvrA + E. coli WP2[pKM101] strains	No mutagenicity
EC359	Cardiotoxicity assessment (hERG): EC359 against hERG membrane using a fluorescence polarization assay	No liability
EC359	CYP inhibition: In vitro assessment of Cytochrome P450 Inhibition potential for EC359 using human liver microsomes (1A2, 2C9, 2C19, 2D6, 3A4)	2D6 inhibition
EC359	Hepatocyte stability: In vitro evaluation of EC359 compound for metabolic stability using cryopreserved human, mouse, rat and dog hepatocytes	Human & Mouse-moderate Rat & Dog - low
EC359	Microsomal stability: In vitro evaluation of EC359 compound for metabolic stability using cryopreserved human, mouse, rat and dog liver microsomes	Human & Mouse-moderate Rat & Dog- low
EC359	Plasma protein binding (%)	Human- 99.98; Mouse- 99.63; Rat- 99.89; Dog- 99.83
EC359	Plasma stability (% remaining at 60 min)	Human-113.92; Mouse- 104.91; Rat- 108.64; Dog- 105.61
EC359	Solubility: pION, kinetic, thermodynamic	Low <10 µg/mL
EC359	Metabolite identification	Major metabolic pathway- Phase I metabolism; No glucuronide metabolites as evidenced by low CL in UDPGA
EC359	Caco2 -permeability & efflux transporter substrate activity	Low permeable; No efflux transporter substrate activity
EC359	Off-target binding study (CERE screen)	GR and hERG were identified as off targets, however, IC 50 of binding with these receptors - up to 10uM and 30uM no GR and hERG binding respectively
EC359	LIF and LIFR binding - Thermophoresis method	Kd LIFR- 10.2nM; Kd LIF- No binding up to 5micromM

Figure 19. ADME studies of EC359

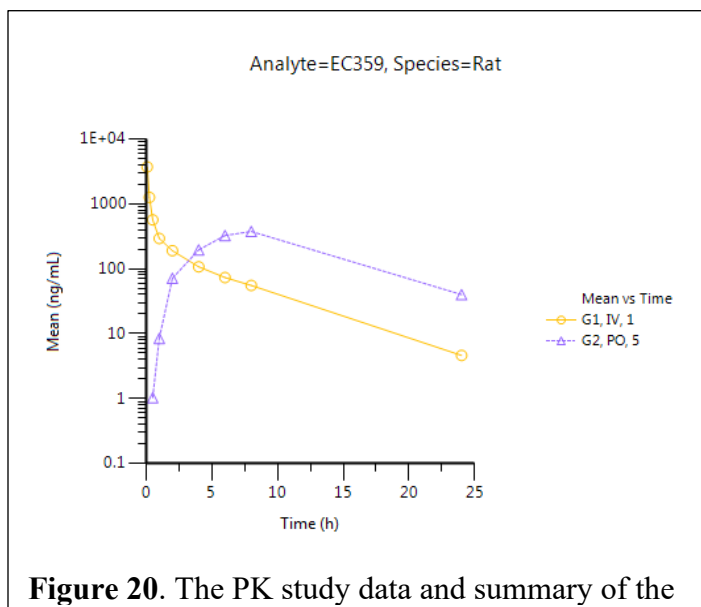


Figure 20. The PK study data and summary of the

Table 1. PK data for EC359.

C₀: Back extrapolated concentration at time zero; NR: Not reported due to improper elimination phase; @: Median

Treat	Group (Dose)	T _{max} @ (h)	C ₀ /C _{max} (ng/mL)	AUC _{last} (ng.h/mL)	AUC _{inf} (ng.h/mL)	Cl (mL/min/kg)	V _{ss} (L/kg)	V _d (L/kg)	T _{1/2} (h)	%F	MRT (h)
EC 359	G1_IV (1 mg/kg)	NA	6469.44±2531.87	2364.85±204.03	2395.0±210.85	7.0 ± 0.58	1.47 ± 0.17	2.71 ± 0.16	4.49 ± 0.28	-	3.17 ± 0.27
	G2-PO (5 mg/kg)	8	383.62 ± 220.66	3657.84 ± 1536.39	NR					31	10.51 ± 1.75

Subtask4: MTD and Toxicology Studies for EC359

We have successfully synthesized the gram scale of EC359 in-house and used for MTD/DRF studies and GLP-tox study requirements in rodent and non-rodent species. We have completed single Maximum Tolerable Dose (MTD) Study Followed by Repeated Dose (7 Days) Range Finding Study of EC359 in Sprague Dawley Rats. Routine cage-side observations were made for all animals once a day for general signs of toxicity (considering the peak period of anticipated effects after dosing) and twice a day to check morbidity and mortality. The rats were exposed to the test item at 0, 2.5, 5, 15, 50, 100 and 300 mg/kg. The animals were weighed on the day of dosing and 48 h after dosing respectively. Blood was collected for clinical pathology investigations at pre-dose and on day 3 after each dose during the single dose phase of the study.

Clinical Signs: All animals treated at all dose levels survived the treatment and observation period. Animals treated at 0, 2.5 and 5 mg/kg, with a single oral dose of the test item were normal and were free from all visible clinical signs. Animals treated @ 15 mg/kg were dull following 1 h post treatment and persisted for around 2 hours. Dullness was observed in animals treated @ 50 and 100 mg/kg starting 1 h post treatment and continued till the end of the day. However, all animals were normal from post-treatment day 1 onwards. Animals treated @ 300 mg/kg were weak with abnormal gait and were lying prostrate starting 1 hour post-dose until 72 h post dose however, with little signs of recovery.

Body Weight (up to 300 mg/kg). Normal body weight gain was observed following treatment at around 48 h in animals treated up to 0, 2.5, 5, 15 and 50 mg/kg as compared to respective pre-exposure weights. There was no body weight gain in animals treated @ 100 mg/kg. Animals treated @ 300 mg/kg revealed a marginal decrease (< 10%) in body weight following 48 h post dose.

Clinical Pathology (up to 300 mg/kg): No visible changes in the tested hematological and clinical chemistry parameters were observed in the samples collected after 72 hours as compared to pre-dose and control in animals treated at 0, 2.5, 5, 15, 50, 100 and 300 mg/kg.

Gross Pathology (up to 50 mg/kg): No abnormalities were detected during the gross pathological examination of animals treated at 0, 2.5, 5, 15 and 50 mg/kg. The 100mg dose the animals were dull post-dose for 24 hours. The 300mg/kg dosed animals were dull for 4days to recover.

Conclusion: Single oral dosing of EC359 produced toxicity including test article-related clinical signs at 100 and 300 mg/kg/dose, however, clinical signs being severe at 300 mg/kg. The MTD for this phase of the study was 100 mg/kg. Oral administration of EC359 for 7 consecutive days produced excessive toxicity along with significant decreases in body weight and feed consumption at 100 mg/kg resulting in early termination of the rats on Day 6. At 30 mg/kg, dullness and piloerection were observed. The dose of 10 mg/kg did not reveal any abnormalities attributable to treatment. The liver, kidneys, adrenals, thymus, spleen, stomach and bone marrow were identified as potential target organs. Based on findings, the MTD of EC359 following repeated oral administration for 7 consecutive days was 30 mg/kg in SD rats under the tested conditions.

3-3. What opportunities for training and professional development has the project provided?

During the entire duration, this project provided training opportunities for 10 students/fellows (two graduate students, two post-doctoral fellows, two undergraduate students and four high school students). All the students/fellows were trained in conducting breast cancer research using BCa model cells, preclinical models, and interpreting the data. In addition, post-doctoral fellow was given opportunity to train graduate students; this provided an opportunity to sharpen their mentoring skills. All students were participated in weekly project meetings and presented their research once in a month as oral presentation. In addition, students/fellows were provided several professional development opportunities including participation in journal clubs, attendance of grand rounds, and attendance of ethics seminar series. Further, post doc and graduate students attended AACR and SABCS annual meeting to present the research findings.

3-4. How were the results disseminated to communities of interest?

We published this study results as three peer reviewed manuscripts (1) Molecular Cancer therapeutics and (2) Communications biology (3) review article in Genes and Diseases. These manuscripts were attached at the end of the report.

4. IMPACT

4-1. What was the impact on the development of the principal discipline(s) of the project?

Our data demonstrated that EC359 is a highly potent LIFR inhibitor and demonstrated the specificity of EC359 for inhibition of LIF–LIFR interaction, confirmed the mechanism of action and on target specificity. EC359 also possesses favorable pharmacologic features. Our PDX tumor study demonstrated utility of EC359 in treating human TNBC tumors. Since EC359 is a small, stable molecule, it is amenable for translation to clinical trials.

4-2. What was the impact on other disciplines?

LIF and LIFR are over-expressed in multiple solid tumors. Considering the wide deregulation of LIF/LIFR axis in multiple tumors, a small molecule LIFR inhibitor EC359 may have utility in treating other solid tumors including ovarian cancer, endometrial cancer, colon cancer, and pancreatic cancer all which exhibit deregulated LIF/LIFR signaling.

4-3. What was the impact on technology transfer?

Nothing to Report.

4-4. What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS

5-1. Changes in approach and reasons for change.

Nothing to Report.

5-2. Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

5-3. Changes that had a significant impact on expenditures

Nothing to Report.

5-4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report.

6. PRODUCTS

6-1. Publications, conference papers, and presentations

1. Viswanadhapalli S, et al. Development of a first-in-class small molecule inhibitor (EC359) targeting oncogenic LIF/LIFR signaling for the treatment of triple negative breast cancer. Proceedings of San Antonio Breast Cancer Symposium. Poster Session 2: Tumor cell and molecular biology: Novel/Emerging Therapeutic Targets; Date/Time: Thursday, December 6, 2018 - 7:00 am. Poster #P2-06-02
2. Viswanadhapalli S, et al. Therapeutic utility of EC359 for targeting oncogenic LIFR signaling in triple negative breast cancer 2019. *Proceedings of American Association for Cancer Research*.
3. Viswanadhapalli S, Luo Y, Sareddy GR, et al. EC359: A First-in-Class Small-Molecule Inhibitor for Targeting Oncogenic LIFR Signaling in Triple-Negative Breast Cancer. *Mol Cancer Ther*. 2019;18(8):1341–1354. doi:10.1158/1535-7163.MCT-18-1258
4. Viswanadhapalli S, et al. Development of a first-in-class small molecule inhibitor (EC359) targeting oncogenic LIF/LIFR signaling for the treatment of triple negative breast cancer. Proceedings of San Antonio Breast Cancer Symposium. Poster Session 2: Tumor cell and molecular biology: Novel/Emerging Therapeutic Targets; Date/Time: Thursday, December 6, 2018 - 7:00 am. Poster #P2-06-02.
5. Viswanadhapalli S, et al. Novel combination therapy for treating TNBC using LIFR and HDAC Inhibitors. Proceedings: AACR Annual Meeting 2020; April 27-28, 2020 and June 22-24, 2020; Philadelphia, PA. DOI: 10.1158/1538-7445.AM2020-562 Published August 2020.
6. Viswanadhapalli S, Dileep KV, Zhang KYJ, Nair HB, Ratna K. Vadlamudi, Targeting LIF/LIFR signaling in cancer, *Genes & Diseases*, 2021, ISSN 2352-3042, <https://doi.org/10.1016/j.gendis.2021.04.003>. (<https://www.sciencedirect.com/science/article/pii/S235230422100057X>)
7. Li M, Viswanadhapalli S, Santhamma B, Pratap UP, Luo Y, Liu J, Altwegg KA, Tang W, Liu Z, Li X, Ebrahimi B, Yan H, Zou Y, Konda S, Sareddy GR, Xu Z, Chen Y, Rao MK, Brenner AJ, Kaklamani VG, Tekmal RR, Ahmed G, Raj GV, Nickisch KJ, Nair HB, Vadlamudi RK. LIFR inhibition enhances the therapeutic efficacy of HDAC inhibitors in triple negative breast cancer. *Commun Biol*. 2021 Oct 29;4(1):1235. doi: 10.1038/s42003-021-02741-7. PMID: 34716410; PMCID: PMC8556368.

6-2. Website(s) or other Internet site(s)
Nothing to Report.

6-3. Technologies or techniques
Nothing to Report.

6-4. Inventions, patent applications, and/or licenses

Nothing to Report.

6-5. Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7-1. What individuals have worked on the project?

Name	Project Role	Effort (mo.)	Site	Contribution to Project	Other Funding Support and Changes in the Reporting Period
Dr. Bindu Santhamma	Initiating PI	3.0	Evestra	Chemical synthesis, process development	1R44CA235991-01 (5.0 months)
Migdalís Cintron-Miguel	Co-Investigator	1.85	Evestra	Analytical method development, analytical measurements	None to report (no longer working at Evestra)
Gulzar Ahmed	Co-Investigator	0.3	Evestra	Chemical synthesis, conjugation chemistry	1R44CA235991-01 (4.0 months)
Rebecca Huff	Administrative assistant	0.48	Evestra	Budgetary support	None to report (no longer working at Evestra)
Angel Alcala	Administrative assistant	0.6	Evestra	Logistical support	1R44CA235991-01 (2.75 months)
Ratna Vadlamudi	Partner PI	1.8	UTHSCSA	Experiment design, project supervision, data analysis, Partner PI coordination, data presentation	R01 CA223828 (0.90) DOD-BCRP:W81XWH-18-1-0021 (0.6 mo) R01 CA239227-01(0.56 mo)
Tekmal Rajeshwar Rao	Co-investigator	0.96	UTHSCSA	Experiment design and execution, data analysis and presentation	R01 CA223828 (0.48) Pardee foundation (0.36 mo)
Suryavathi Viswanadhapalli	Post-doctoral fellow	4.8	UTHSCSA	Experiment design and execution, data analysis and presentation	DOD BCRP: W81XWH-18-1-0021(1.2mo); Pardee foundation (5.6 mo)
Uday Pratap	Post-doctoral fellow	4.0	UTHSCSA	Experiment execution, data analysis and presentation	None to report
Behnam Ebrahimi	Pre-doctoral fellow	3.0	UTHSCSA	Experiment execution, data analysis and presentation	None to report

7-2. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

7-3. What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

8-1. COLLABORATIVE AWARDS

This Progress Report is jointly authored by **Dr. Bindu Santhamma (Initiating PI)** and **Dr. Ratna Vadlamudi (Partner PI)** and provides details for the work performed in their respective laboratories. An identical copy of this Report will be submitted by Dr. Bindu Santhamma and Dr. Vadlamudi independently as their respective Progress Report.

8-2. Quad Chart

Not applicable.

9. APPENDICES

Reprints of four Abstracts

Reprint of manuscript 1

Reprint of manuscript 2

Reprint of manuscript 3

1) [P2-06-02] Development of a first-in-class small molecule inhibitor (EC359) targeting oncogenic LIF/LIFR signaling for the treatment of triple negative breast cancer

Authors: Viswanadhapalli S, Luo Y, Sareddy GR, Santhamma B, Zhou M, Li M, Pratap UP, Altwegg KA, Li X, Srinivasan U, Ma S, Chang A, Riveros AC, Zhang KY, Dileep KV, Pan X, Murali R, Bajda M, Raj G, Brenner A, Manthathi V, Rao M, Tekmal RR, Nair HB, Nickisch KJ, Vadlamudi RK

Room: Hall 1

Date/Time: Thursday, December 6, 2018 - 7:00 am

Session Info: Poster Session 2: Tumor cell and molecular biology: Novel/Emerging Therapeutic Targets (7:00 AM-9:00 AM)

Development of a first-in-class small molecule inhibitor (EC359) targeting oncogenic LIF/LIFR signaling for the treatment of triple negative breast cancer

Viswanadhapalli S, Luo Y, Sareddy GR, Santhamma B, Zhou M, Li M, Pratap UP, Altwegg KA, Li X, Srinivasan U, Ma S, Chang A, Riveros AC, Zhang KY, Dileep KV, Pan X, Murali R, Bajda M, Raj G, Brenner A, Manthathi V, Rao M, Tekmal RR, Nair HB, Nickisch KJ, Vadlamudi RK UT Health and Mays Cancer Center, San Antonio; Evestra, Inc., San Antonio; Instituto de Química, Ciudad de, Mexico; RIKEN Center for Biosystems Dynamics Research, Yokohama, Japan; Cidars-Sinai Medical Center, Los Angeles; Jagiellonian University, Cracow, Poland; UT Southwestern, Dallas

Background: Leukemia inhibitory factor (LIF) and its receptor LIFR are over-expressed in multiple solid tumors and play a key role in tumor growth, progression, and resistance to standard anti-cancer treatments. Triple-negative breast cancer (TNBC) lacks targeted therapies and represents a disproportional share of breast cancer (BCa) mortality. TNBC exhibits autocrine stimulation of the LIF/LIFR axis and overexpression of LIF is associated with poorer relapse-free survival in BCa patients. LIF signaling also promotes maintenance of stem cells. Therefore, targeting the LIF/LIFR axis may have therapeutic utility in TNBC.

Methods: We rationally designed a small organic molecule (EC359) that emulates the LIF/LIFR binding site and functions as a LIFR inhibitor from a library of compounds. *In silico* docking studies were used to identify the putative interaction of the EC359 and LIF/LIFR complex. Direct binding of EC359 to LIFR was confirmed using surface plasmon resonance (SPR) and microscale thermophoresis technique (MST) assays. *In vitro* activity was tested using Cell-Titer Glo, MTT, invasion, and apoptosis assays. Mechanistic studies were conducted using Western blot, reporter gene assays, and RNA-seq analysis. Xenograft, patient-derived xenograft (PDX), and patient-derived explant (PDEX) models were used for preclinical evaluation and toxicity.

Results: Molecular docking studies showed that EC359 interacts at the LIF/LIFR binding interface. SPR and MST studies confirmed direct interaction of EC359 to LIFR. EC359 reduced the growth of TNBC cells with high potency (IC₅₀ 50-100nM) and promoted apoptosis. Further, EC359 treatment reduced invasion and stemness of TNBC cells. EC359 activity is dependent on the expression levels of LIFR and showed little or no activity on TNBC cells that have low levels of LIFR or ER+ve BCa cells. Further, EC359 significantly reduced the viability of cisplatin and taxane-resistant TNBC cells and enhanced the efficacy of HDAC inhibitors. Mechanistic and biochemical studies showed that EC359 interacts with LIFR and effectively blocking LIF/LIFR interactions. EC359 also blocked LIFR interactions with other LIFR ligands such as oncostatin M, ciliary neurotrophic factor, and cardiotrophin-1. EC359 treatment attenuated the activation of LIF/LIFR driven pathways including STAT3, mTOR, AKT, and MAPK. RNA-seq analysis identified regulation of apoptosis as one of the important pathway modulated by EC359. In TNBC xenograft and PDX assays, EC359 significantly reduced tumor progression. Further, using human primary BCa PDEX cultures, we demonstrated that EC359 has the potential to substantially reduce the proliferation of human BCa. Pharmacologically, EC359 exhibited high oral bioavailability and long half-life with a wide therapeutic window.

Conclusions: EC359 is a novel targeted therapeutic agent that inhibits LIF/LIFR oncogenic signaling in TNBC via a unique mechanism of action. EC359 has the distinct pharmacologic advantages of oral bioavailability, *in vivo* stability, and is associated with minimal systemic side effects. (DOD BCRP grant #BC170312) **Session:** Poster Session 2: Tumor cell and molecular biology: Novel/Emerging Therapeutic Targets (7:00 AM-9:00 AM **Date/Time:** Thursday, December 6, 2018 - 7:00am


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Activity: Abstract Submission

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Therapeutic utility of EC359 for targeting oncogenic LIFR signaling in triple negative breast cancer
Short Title:

LIFR therapy for breast cancer

Author Block: Suryavathi Viswanadhapalli¹, Mengxing Li¹, Yiliang Luo¹, Gangadhara R Sareddy¹, Bindu Santhamma², Mei Zhou³, Shihong Ma⁴, Rajni Sonavane⁴, Uday P. Pratap¹, Kristin A. Altwegg¹, Annabel Chang⁴, Alejandra Chávez-Riveros⁵, Kalarickal V. Dileep⁶, Kam Y. Zhang⁶, Marek Bajda⁷, Ganesh V. Raj⁴, Andrew Brenner¹, Vijaya Manthathi², Manjeet Rao⁸, Rajeshwar R. Tekmal¹, Hareesh B. Nair², Klaus J. Nickisch², Ratna K. Vadlamudi¹.
¹UT Health Science Ctr. at San Antonio, San Antonio, TX; ²Evestra, Inc, San Antonio, TX; ³Second Xiangya Hospital, San Antonio, TX; ⁴UT Southwestern, Dallas, TX; ⁵Instituto de Química, Mexico; ⁶Center for Biosystems Dynamics Research, RIKEN, Yokohama, Japan; ⁷Jagiellonian University Medical College, Cracow, Poland; ⁸Greehey Children's Cancer Research Institute, San Antonio, TX

Abstract:

Background: Leukemia inhibitory factor receptor (LIFR) and its ligand LIF play a major critical role in cancer progression, metastasis, stem cell maintenance, and therapy resistance. Recent studies in breast cancer have shown that feedback activation of LIFR limits response to histone deacetylase (HDAC) inhibitors and induce resistance. We rationally designed a small molecule (EC359) that emulates the LIF-LIFR binding site and functions as a LIFR inhibitor from a library of compounds. Here, we tested the utility of EC359 as a monotherapy and to effectively block LIF-LIFR interactions in overcoming resistance to HDAC inhibitors.

Methods: We have used multiple triple negative breast cancer (TNBC) models that represent all six types of TNBC. *In vitro* activity was tested using Cell-Titer Glo, MTT, invasion, and apoptosis assays. Mechanistic studies were conducted using western blot, reporter gene assays, and RNA-seq analysis. Xenograft, patient-derived xenograft (PDX), and patient-derived explant (PDX) models were used for preclinical evaluation and toxicity.

Results: EC359 treatment exhibited anti-proliferative effects, reduced invasiveness and stemness, and promoted apoptosis in all six TNBC cell lines. The activity of EC359 is dependent on LIF and LIFR expression and CRISPR mediated knockdown of LIFR significantly abolished EC359 activity. Treatment with EC359 attenuated the activation of LIF-LIFR driven pathways including STAT3, mTOR, and AKT. EC359 significantly reduced tumor progression in TNBC xenografts, PDX models and reduced proliferation in patient derived primary TNBC explants. In MTT based cell viability assays, addition of EC359 enhanced efficacy of SAHA compared to monotherapy of SAHA. In clonogenic survival assays, EC359 significantly enhanced ability of SAHA to reduce the colony formation compared to monotherapy. Mechanistic studies using three different TNBC models using western blot analysis and reporter gene assays confirmed activation of LIFR signaling pathway upon SAHA treatment and its blockage by EC359. Treatment of TNBC PDX explants with EC359 enhanced ability of SAHA to substantially decrease the proliferation (Ki-67 positivity) compared to monotherapy treated tumors.

Conclusions: Collectively, these data support EC359 as a novel targeted therapeutic that inhibits LIFR oncogenic signaling as a monotherapy or in combination with HDAC inhibitors.

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Author Disclosure Information:

S. Viswanadhapalli: None. **M. Li:** None. **Y. Luo:** None. **G. Sareddy:** None. **B. Santhamma:** ; Evestra, Inc. **M. Zhou:** None. **S. Ma:** None. **R. Sonavane:** None. **U.P. Pratap:** None. **K.A. Altwegg:** None. **A. Chang:** None. **A. Chávez-Riveros:** None. **K.V. Dileep:** None. **K.Y. Zhang:** None. **M. Bajda:** None. **G.V. Raj:** None. **A. Brenner:** None. **V. Manthathi:** ; Evestra, Inc. **M. Rao:** None. **R.R. Tekmal:** None. **H.B. Nair:** ; Evestra, Inc. **K.J. Nickisch:** ; Evestra, Inc. **R.K. Vadlamudi:** None.

Sponsor (Complete):

Category and Subclass (Complete): ET06-03 HDAC and methyltransferase inhibitors

Research Type (Complete): Translational research

Organ Site/Structures (Complete):

*Primary Organ Site: Breast cancer

*Choose Chemical Structure Disclosure Option:

YES, and I WILL DISCLOSE. Compounds with defined structures were used, and I WILL DISCLOSE them in my presentation.

*Please explain reason for not disclosing (maximum 250 characters with spaces): : NA

*Reference or patent application number : NA

Keywords/Indexing (Complete): Breast cancer ; Histone deacetylase inhibitor ; Small molecule inhibitor ; Signal transducers and activators of transcription (STAT)

Financial Support for Attendance (Complete):

Submission Fee (Complete): Your credit card order has been processed on Wednesday 14 November 2018 at 11:16 PM.

Status: Complete

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P3-11-08. Targeting LIFR enhances the activity of HDAC inhibitors for the treatment of triple negative breast cancer
 (<https://www.sabcs.org/2019-SABCS>)

December 12, 2019, 5:00 PM - 7:00 PM □ Hall 1

Authors

Suryavathi Viswanadhapalli , Mengxing Li , Bindu Santhamma , Uday P Pratap , Yiliao Luo , Junhao Liu , Kristin A Altwegg , Xiaonan Li , Hui Yan , Zhenming Xu , Andrew Brenner , Gangadhara R Sareddy , Rajeshwar R Tekmal , Hareesh B Nair , Klaus J Nickisch and Ratna K Vadlamudi . UT Health San Antonio, San Antonio, TX; Evestra Inc., San Antonio, TX

Abstract

Background: Triple-negative breast cancer (TNBC) is a heterogeneous disease. TNBC lacks targeted therapies and represents a disproportional share of the breast cancer (BC) mortality rate. Histone deacetylase inhibitors (HDACIs) are emerging as promising multifunctional agents in TNBC to elicit cytotoxic actions. Recent studies have shown that cancer cells elucidate feedback activation of leukemia inhibitory factor receptor (LIFR) which in turn curtails response to HDACIs. We developed a first-in-class inhibitor of LIFR, EC359 that directly interacts with LIFR and effectively blocks LIFR downstream signaling. Here, we examined whether the novel LIFR inhibitor, EC359, has the ability to counteract negative effects of LIFR signaling to enhance HDACIs therapeutic efficacy in the treatment of TNBC.

Methods: We tested multiple HDACIs currently in clinical trials including vorinostat, panobinostat, romidepsin, and givinostat using multiple TNBC models. The effect of combination therapy of HDACIs and EC359 on TNBC cell viability and invasion was examined using MTT assays and matrigel invasion assays respectively. The efficacy of combination therapy on cell survival and apoptosis was determined using clonogenic assays and Caspase 3/7 assays, respectively. Mechanistic studies were performed using Western blotting, qRT-PCR, and reporter gene assays. The efficacy of combination therapy *in vivo* was examined using Xenograft, patient-derived xenograft (PDX), and patient-derived explant (PDEX) models.

Results: We demonstrated that the treatment of TNBC models with HDACIs increased the expression of LIFR. Immunohistochemistry analyses of breast tumors using tissue microarrays revealed significant expression of LIFR in TNBC samples. Knockdown of LIFR or treatment with a small molecule inhibitor of LIFR (EC359) significantly enhanced the efficacy of HDACIs in reducing cell viability, colony formation ability, and invasiveness as well as promoted apoptosis compared to monotherapy of HDACIs or EC359 in TNBC cell lines. Mechanistic studies, reporter gene assays and biochemical studies using multiple TNBC models exhibited activation of the LIFR signaling pathway upon HDACIs treatment but was attenuated by EC359+HDACI combination therapy. Treatment of human breast tumors utilizing PDEX assays showed that EC359 enhanced the ability of HDACIs to decrease the proliferation (Ki-67 positivity) compared to monotherapy. Furthermore, using TNBC xenografts and PDX models, we demonstrated that EC359 treatment enhanced the ability of HDACIs to reduce *in vivo* tumor growth compared to monotherapy.

Conclusions: Our results suggest that the combination therapy of HDACIs and EC359 provides greater therapeutic efficacy than monotherapy. In addition, treatment with EC359 can overcome the feedback activation of LIFR currently observed in the treatment of TNBC with HDACIs.

Abstract 562: Novel combination therapy for treating TNBC using LIFR and HDAC Inhibitors

Suryavathi Viswanadhapalli, Mengxing Li, Bindu Santhamma, Uday P. Pratap, Yiliao Luo, Junhao Liu, Kristin A. Altwegg, Xiaonan Li, Ahmed Gulzar, Hui Yan, Zhenming Xu, Andrew Brenner, Gangadhara R. Sareddy, Manjeet K. Rao, Rajeshwar R. Tekmal, Hareesh B. Nair, Klaus J. Nickisch and Ratna K. Vadlamudi

DOI: 10.1158/1538-7445.AM2020-562 Published August 2020

Proceedings: AACR Annual Meeting 2020; April 27-28, 2020 and June 22-24, 2020; Philadelphia, PA

Abstract

Background: Triple-negative breast cancer (TNBC) lacks targeted therapies and represents a disproportional share of the breast cancer (BC) mortality rate. TNBC exhibits autocrine stimulation of the LIF/LIFR axis and overexpression of LIF is associated with poorer relapse-free survival in BC patients. Histone deacetylase inhibitors (HDACIs) are emerging as promising multifunctional agents in TNBC to elicit cytotoxic actions. Recent studies have shown that cancer cells elicit feedback activation of leukemia inhibitory factor receptor (LIFR) which in turn curtails response to HDACIs. We developed a first-in-class inhibitor of LIFR, EC359 that directly interacts with LIFR and effectively blocks LIFR downstream signaling. The objective of this study is to examine the therapeutic efficacy of combination therapy using preclinical and patient-derived xenograft (PDX) models.

Methods: We tested utility of combination therapy using multiple HDACIs that are currently in clinical trials along with EC359. The effect of combination therapy was evaluated using MTT, invasion, colony formation, and Caspase3/7 assays. Mechanistic studies were performed using Western blotting, qRT-PCR, and STAT3 reporter assays. The efficacy of combination therapy *in vivo* was examined using xenograft, PDX, and patient-derived explant (PDEx) models.

Results: Immunohistochemical analyses of breast tumors using tissue microarrays revealed significant expression of LIFR in TNBC tissues. Treatment of TNBC model cells with four different HDACIs increased the expression of LIFR. LIFR inhibitor EC359 at nM concentration is additive to HDACIs in reducing cell viability. Knockdown of LIFR or treatment with EC359 significantly enhanced the efficacy of HDACIs in reducing the cell viability, colony formation ability, and invasiveness as well as promoted apoptosis compared to monotherapy in TNBC model cells. On the contrary, treatment with STAT3 inhibitor requires μ M concentrations to reduce the cell viability of TNBC cells and is not additive to HDACIs. Mechanistic studies utilizing STAT3 reporter gene assays and biochemical studies using multiple TNBC model cells exhibited activation of the LIFR signaling pathway upon HDACIs treatment but was attenuated by EC359 therapy. Treatment of human TNBC utilizing PDEx assays showed that EC359 enhanced the ability of HDACIs to decrease proliferation (Ki-67 positivity) compared to monotherapy. Using TNBC xenografts and PDX models, we demonstrated that EC359 treatment enhanced the ability of HDACIs to reduce *in vivo* tumor growth compared to monotherapy.

Conclusions: Our results suggest that the combination therapy of HDACIs and EC359 provides therapeutic utility in overcoming the limitation of feedback activation of LIFR observed in the treatment of HDACIs in treating TNBC. Supported by DOD BCRP grant W81XWH-18-1-0016 (R.K. Vadlamudi; K.J. Nickisch)

Citation Format: Suryavathi Viswanadhapalli, Mengxing Li, Bindu Santhamma, Uday P. Pratap, Yiliao Luo, Junhao Liu, Kristin A. Altwegg, Xiaonan Li, Ahmed Gulzar, Hui Yan, Zhenming Xu, Andrew Brenner, Gangadhara R. Sareddy, Manjeet K. Rao, Rajeshwar R. Tekmal, Hareesh B. Nair, Klaus J. Nickisch, Ratna K. Vadlamudi. Novel combination therapy for treating TNBC using LIFR and HDAC Inhibitors [abstract]. In: Proceedings of the Annual Meeting of the American Association for Cancer Research 2020; 2020 Apr 27-28 and Jun 22-24. Philadelphia (PA): AACR; Cancer Res 2020;80(16 Suppl):Abstract nr 562.

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EC359: A First-in-Class Small-Molecule Inhibitor for Targeting Oncogenic LIFR Signaling in Triple-Negative Breast Cancer



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Abstract

Leukemia inhibitory factor receptor (LIFR) and its ligand LIF play a critical role in cancer progression, metastasis, stem cell maintenance, and therapy resistance. Here, we describe a rationally designed first-in-class inhibitor of LIFR, EC359, which directly interacts with LIFR to effectively block LIF/LIFR interactions. EC359 treatment exhibits antiproliferative effects, reduces invasiveness and stemness, and promotes apoptosis in triple-negative breast cancer (TNBC) cell lines. The activity of EC359 is dependent on LIF and LIFR expression, and treatment with EC359 attenuated the activation of LIF/LIFR-driven pathways, including STAT3, mTOR, and AKT.

Concomitantly, EC359 was also effective in blocking signaling by other LIFR ligands (CTF1, CNTF, and OSM) that interact at LIF/LIFR interface. EC359 significantly reduced tumor progression in TNBC xenografts and patient-derived xenografts (PDX), and reduced proliferation in patient-derived primary TNBC explants. EC359 exhibits distinct pharmacologic advantages, including oral bioavailability, and *in vivo* stability. Collectively, these data support EC359 as a novel targeted therapeutic that inhibits LIFR oncogenic signaling.

See related commentary by Shi et al., p. 1337

Introduction

Leukemia inhibitory factor (LIF) is the most pleiotropic member of the IL6 family of cytokines (1). LIF signaling is mediated via

the LIF receptor (LIFR) complex, which is comprised of LIFR and glycoprotein 130 (gp130; ref. 2). The LIFR does not have intrinsic tyrosine kinase activity. Both LIFR and gp130 constitutively associate with the JAK–Tyk family of cytoplasmic tyrosine kinases. Consequently, LIF binding to the LIFR complex activates multiple signaling pathways, including JAK/STAT, MAPK, AKT, and mTOR (2–4). LIF and LIFR are widely expressed in many solid tumors (1, 5–7) and their overexpression is often associated with poor patient prognosis (8, 9). In addition, high circulating LIF levels correlate with tumor recurrence (10).

The LIF/LIFR axis acts on multiple aspects of cancer biology to promote tumor growth, metastasis, and therapy resistance (11). LIF is a key regulator of cancer stem cells (CSC; ref. 11), plays a role in stem cell maintenance (12, 13), regulates self-renewal and pluripotency (12), and is associated with chemoresistance (10, 14). LIF functions as a growth factor to promote growth and invasion (15). Recent evidence indicates upregulated LIF–JAK–STAT3 signaling via autocrine and paracrine mechanisms in tumors (10, 16, 17). However, lack of any small-molecule inhibitors (SMI) that block LIF/LIFR signaling represents a major knowledge gap and critical barrier for advancement of LIF/LIFR-targeted cancer therapy.

Among the different subtypes of breast cancer, 60%–70% are estrogen receptor (ER) positive (ER⁺ breast cancer), and 15%–24% are triple-negative breast cancer (TNBC; ref. 18). TNBC is more aggressive, and due to the lack of targeted therapies, represents a disproportional share of the breast cancer mortality (19, 20). TNBC exhibit high propensity for metastasis, with some subtypes such as claudin-low that are highly enriched for

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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Mol Cancer Ther 2019;18:1341–54

doi: 10.1158/1535-7163.MCT-18-1258

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CSCs, and frequently exhibits therapy resistance (19, 20). In breast cancer cells, LIF/LIFR signaling activates multiple signaling pathways including STAT3, AKT, and mTOR pathways and contributes to activation of mTOR downstream targets such as p70S6K and 4EBP1 (4). LIF/LIFR signaling promotes tumor progression of both ER⁺ breast cancer and TNBC cells (21–23). In addition, LIF mRNA levels were elevated in invasive breast carcinomas compared with the normal breast tissues (24). Overexpression of LIF is significantly associated with a poorer relapse-free survival in patients with breast cancer (4).

In this study, we report the development of a novel LIFR inhibitor EC359 that selectively binds LIFR and blocks binding of ligands attenuating LIFR oncogenic signaling. Using molecular modeling, *in vitro*, and *in vivo* assays, we demonstrated that EC359 interacts with LIFR and inhibits cell viability of TNBC cells that express both LIF and LIFR. In addition, EC359 reduced the invasion and stemness of TNBC cells, and promoted apoptosis. In xenograft and patient-derived xenograft (PDX) assays, EC359 significantly reduced the tumor progression. This study represents the first report detailing the development of a first-in-class inhibitor of LIF/LIFR.

Materials and Methods

Cell lines and reagents

Human breast cancer cells MCF7, T47D, MDA-MB-231, BT-549, SUM-159, HCC1937, MDA-MB-468, HCC1806, and normal mammary epithelial cells (HMEC) were obtained from ATCC, were maintained as per ATCC guidelines, and used from early passages (<10 passages after thawing). All model cells utilized were free of *Mycoplasma* contamination and were confirmed by using the Mycoplasma PCR Detection Kit purchased from Sigma. Short tandem repeat polymorphism analysis of the cells was used to confirm the identity at University of Texas Health San Antonio (UTHSA) core facilities. CSCs isolated from TNBC cells were maintained in MammoCult medium along with the supplements according to the manufacturer's instructions (StemCell Technologies). The GAPDH, p-ERK1/2, ERK1/2, p-p70S6K, p70S6K, p-S6, S6, p-Akt(S473), Akt, p-p38 MAPK, p38 MAPK, p-mTOR(S2448), mTOR, p-STAT3(Y705), and STAT3 antibodies were purchased from Cell Signaling Technology. LIF and LIFR antibodies were purchased from Santa Cruz Biotechnology. β -Actin and all secondary antibodies were purchased from Sigma. ALDEFLUOR assay kit was obtained from StemCell Technologies. The Ki-67 antibody was purchased from Abcam. LIFR Knockout (KO) model cells were generated using Genescript CRISPR gRNA Constructs (Genescript-s64729-LIFR CRISPR guide RNA 1; Genescript-s64731-LIFR CRISPR guide RNA 2) and transfecting them into Cas9 stably expressing BT-549 cells followed by puromycin selection. EC359 and EC330 were synthesized using the detailed synthetic protocol described in the patent WO 2016/154203 A1 (Evestra Inc.). Characterization of EC330 and EC359 produced was described in the Supplementary Methods.

Western blotting and biotin pull-down assays

For Western blotting, cells were lysed in RIPA buffer (Thermo Fisher Scientific) containing protease and phosphatase inhibitors. Total cellular lysates were mixed with 4 $\times$ SDS sample buffer and run on SDS-PAGE, and transferred onto nitrocellulose membranes and blots were developed using antibodies and the ECL Kit (Thermo Fisher Scientific). Avidin-biotin pull down was

performed as described previously (25). Briefly, BT-549 total cellular lysates and purified LIFR was incubated with Biotin-control or Biotin-EC359 overnight and incubated with NanoLink Streptavidin Magnetic Beads (Solulink) for 1 hour at room temperature. The binding of EC359 to LIFR was confirmed by Western blotting. Intensity of signaling bands in Western blots were quantitated using ImageJ program (NIH, Bethesda, MD).

Cell invasion assays

The effect of EC359 on cell invasion of TNBC cells was determined by using the Corning BioCoat Growth Factor Reduced Matrigel Invasion Chamber assay. MDA-MB-231 and BT-549 cells were treated with vehicle or EC359 (25 nmol/L) for 22 hours and invaded cells in all the treatment conditions were determined according to the manufacturer's protocols.

Extreme limiting dilution assays

CSCs from MDA-MB-231 and BT-549 cells were sorted using established stem cell marker ALDH using the ALDEFLUOR kit and flow cytometry. CSCs were cultured in MammoCult medium with the supplements as per manufacturer's instructions. The effect of EC359 on self-renewal of CSCs was determined by ELDA. Briefly, CSCs were seeded in decreasing numbers (100, 50, 20, 10, 5, and 1 cells/well) in 96 well ultra-low attachment plates and treated with vehicle or EC359. After 10 days, the number of wells containing spheres per each plating density was recorded and stem cell frequency between control and treatment groups was calculated using ELDA analysis software (<http://bioinf.wehi.edu.au/software/elda/>).

Cell viability, clonogenic, and apoptosis assays

The effect of EC359 on cell viability of TNBC cells was assessed by using MTT assay as described previously (25). TNBC cells were seeded in 96-well plates (1×10^3 cells/well) and after overnight incubation cells were treated with varying concentrations of EC359 for 5 days. To test the effect of EC359 on the viability of CSCs and non CSCs, CellTiter-Glo assays were performed (Promega). Briefly, cells were seeded in 96-well, flat, clear-bottom, opaque-wall microplates and treated with vehicle or EC359 for 3 days. The total ATP content as an estimate of total number of viable cells was measured on an automatic Fluoroskan Luminometer. For clonogenic survival assays, cells were seeded in triplicates in 6-well plates (500 cells/well), after overnight incubation cells were treated with vehicle or EC359 for 5 days and after 2 weeks, colonies that contain ≥ 50 cells were counted and used in the analysis. The effect of EC359 on apoptosis was measured by Annexin V/PI staining and Caspase-3/7 activity assay as described previously (25, 26). Briefly, MDA-MB-231 and BT-549 cells were seeded in 96-well plates (2×10^3 /well), after overnight incubation cells were treated with vehicle or EC359 (20 nmol/L) for 72 hours. After treatment, equal amount of caspase-3/7 substrate containing solution was added to the media, and luciferase activity was measured using luminometer according to the manufacturer's protocol (Promega).

qRT-PCR

Reverse transcription (RT) reactions were performed by using SuperScript III First Strand kit (Invitrogen), according to the manufacturer's protocol. Real-time PCR was done using Sybr-Green on an Illumina Real-Time PCR system. Primer sequences were included in the Supplementary Table S1.

Surface plasmon resonance studies

Binding profiles of EC359 to LIF/LIFR were evaluated using surface plasmon resonance (SPR). Recombinant human LIF was purchased from R&D Systems (catalog no. 7734-LF-500) and human LIFR-Fc was purchased from Speed Biosystems (catalog no. YCP1132). Sensor chips were purchased from ForteBio (www.fortebio.com). Detailed SPR protocol was provided in the Supplementary Methods.

Microscale thermophoresis assays

A serial dilution of the ligand (EC359) was prepared in a way to match the final buffer conditions in the reaction mix (10 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005 % Tween-20, 10% DMSO). The highest concentration of ligand was 2.00 μ mol/L and the lowest 61.0 pmol/L. Five microliters of each dilution step was mixed with 5 μ L of the fluorescent molecule. The final reaction mixture, which was loaded in capillaries, contained a respective amount of ligand (max. conc: 1.00 μ mol/L; min. conc: 30.5 pmol/L) and constant 5 nmol/L fluorescent molecule (protein target LIFR-labeled fluorescent dye- NHS chemistry). Thermophoretic movement of fluorescently labeled protein with EC359 was performed using on a Monolith NT.115 Pico at 25°C, with 7% LED power and 60% Laser power (Nanotemper Technologies).

Molecular modeling studies

The atomic level interactions of EC359 against human LIFR (hLIFR) were studied by molecular modeling. The existing structural information of LIFR was utilized for the studies. The partial structure of human LIFR (hLIFR; domains D1–D5; PDB ID: 3E0G) and the structure of human LIF (hLIF) in complex with the partial murine LIFR (mLIFR; domains D1–D5) have been reported in the Protein Data Bank (PDB ID: 2Q7N; refs. 27, 28). As a preliminary step, the sequence and structural similarities of both of these LIFRs were deduced. Furthermore, the three-dimensional structure of hLIF–hLIFR complex was constructed from hLIF–mLIFR by replacing mLIFR. The complex was energy minimized to avoid the residue clashes between the hLIFR and hLIF. From the minimized complex, the hLIFR was again separated and prepared for the docking studies. Because there was no information available on the ligand-binding sites, the whole receptor was probed using Sitemap (Schrödinger) to detect possible binding sites (29). Two steps of molecular docking were performed such as standard precision (SP) and induced fit (IFD) on the identified binding sites. The purpose of SP docking was to detect the binding strength and orientations of ligand at respective binding sites. On the basis of the docking scores, the sites were ranked. Later, an appropriate ligand pose was selected and flexible docking (IFD) was performed by allowing flexibility to the surrounding amino acids (around 6 Å from the center of the ligand). On the basis of the MM-GBSA (30) score and visual inspection an appropriate pose was selected and subjected to molecular dynamics simulation (MDS) to estimate the residence time of the ligand over a period of 25 nanoseconds. The detailed description of methods used in the study was included in the Supplementary Methods and Supplementary Figs. S2 and S3.

Reporter gene assays

For STAT3-luc assays, MDA-MB-231 and BT-549 cells were stably transduced with STAT3-firefly luciferase reporter lentivirus purchased from Cellomic Technology. STAT3-luc reporter-

expressing cells were serum starved for 24 hours, pretreated with EC359 for 1 hour, and then stimulated with LIF or other indicated ligands for 24 hours. Cells were lysed in passive lysis buffer, and the luciferase activity was measured by using the dual-luciferase reporter assay system (Promega) using luminometer.

In vivo xenograft studies

All animal experiments were performed after obtaining University of Texas Health San Antonio (UTHSA) Institutional Animal Care and Use Committee (IACUC) approval, and all the methods were carried out in accordance with IACUC guidelines. MDA-MB-231 cells (2×10^6) were mixed with equal volume of growth factor-reduced Matrigel and implanted in the mammary fat pads of 8-week-old female athymic nude mice as described previously (31). After tumor establishment, and achievement of measurable size, mice were randomized into control and treatment groups ($n = 8$ tumors per group). Control group received vehicle (hydroxymethylcellulose) and the treatment group received EC359 (5 mg/kg/day) 3 days per week subcutaneously. All mice were monitored daily for adverse toxic effects. Tumor growth was measured with a caliper at 3–4 day intervals, and volume was calculated using a modified ellipsoidal formula: tumor volume = $1/2(L \times W^2)$, where L is the longitudinal diameter and W is the transverse diameter. At the end of the experiment, mice were euthanized, and tumors were excised, and processed for histologic and biochemical studies.

Patient-derived xenograft model

The TNBC tissue was obtained from a deidentified surgical specimen (F0) just after surgery from a patient with invasive ductal carcinoma (pT3 pN2a pM) via UTHSA PDX Core. The tumor tissues were divided into three parts; the first part was snap frozen and stored in liquid nitrogen, the second part was fixed in 10% buffered formalin and processed for histologic characterization, and the third part was placed in ice-cold PBS, cut into small pieces (3–5 mm³), and engrafted into mammary fat pad of NCI SCID/NCr mice. PDX tumor was confirmed negative for ER, PR, HER2 by the Pathology core. Tumors from early passages were dissected into small pieces and implanted into the flanks of SCID mice. The mice were then randomized when they reached tumor volume of approximately 150 mm³ into control or treatment groups ($n = 6$ tumors per group). The control group received vehicle (hydroxymethylcellulose) and the treatment group received EC359 (10 mg/kg/day) 3 days per week subcutaneously. At the end of the treatment, tumors were excised and processed for histologic studies, protein, and RNA analysis.

Patient-derived explant studies

TNBC tissues were collected from discarded surgical samples from UT Southwestern Medical Center (UTSW, Dallas, TX) patients for research purposes after obtaining the written informed consent and in accordance with institutional review board-approved protocol (STU-032011–187). All the studies were conducted in accordance with the Declaration of Helsinki. Tissues were processed and excised into small pieces and cultured on gelatin sponges for 24 hours in medium containing 10% FBS as described previously (25). Tissues were treated with vehicle or EC359 in culture medium for 72 hours and fixed in 10% buffered formalin at 4°C overnight and subsequently processed into

paraffin blocks. Sections were then processed for IHC analysis for Ki-67.

Immunohistochemistry (IHC)

IHC analysis was performed as described previously (25). Briefly, sections were blocked with normal goat serum (Vector Laboratories) followed by incubation overnight with Ki-67 (1:100) primary antibody and subsequent secondary antibody incubation for 30 minutes at room temperature. Immunoreactivity was visualized by using the DAB substrate and counterstained with hematoxylin (Vector Laboratories). Percent of Ki-67-positive proliferating cells was calculated in five randomly selected microscopic fields.

Pharmacokinetic studies and bioavailability studies

A pharmacokinetic study of EC359 was conducted in both mice and rats following intravenous and oral administration of the compound (GVK Bio). Intravenous formulation (5 mg/kg) was prepared as described: A required volume 0.1 mL of DMSO stock (20 mg/mL) was taken in an Eppendorf tube then 0.100 mL of DMSO was added and vortexed, then sonicated, followed by addition of 1.800 mL 10% Solutol in PBS, vortexed, and probe sonicated approximately 1–2 minutes to make a final solution of 1 mg/mL concentration. $t_{1/2}$, $AUC_{0-1\text{st hr}}$, $AUC_{0-1\text{st hr}}$, AUC_{extra} , CL , V_d , $MRT_{0-1\text{st hr}}$ and RSQ were measured using LC/MS-MS. For oral dosing (10 mg/kg) volume, 0.200 mL of DMSO stock (20 mg/mL) was taken in an Eppendorf tube then 1.800 mL 10% Solutol in PBS was added, vortexed, and probe sonicated approximately 1–2 minutes to make a solution of 2 mg/mL concentration. C_{max} , T_{max} , $AUC_{0-1\text{st hr}}$, $AUC_{0-1\text{st hr}}$, AUC_{extra} , $F\%$, $MRT_{0-1\text{st hr}}$ and RSQ were measured. GR antagonism assays were performed using SelectScreen Biochemical Nuclear Receptor Profiling Service (Invitrogen).

Statistical analysis

All the statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Software). A Student t test was used to assess statistical differences between control and EC359-treated groups. All the data represented in bar graphs are shown as mean $\pm$ SE. A P less than 0.05 was considered significant.

Data and materials availability

All data supporting the conclusions are included in the paper and/or in the Supplementary Materials.

Results

Optimization and generation of lead LIFR inhibitor EC359

We initially synthesized several compounds to target LIFR signaling using rationalized design based on crystal structure of LIF/LIFR. Within this series of compounds, one compound (EC330) showed higher potency (Fig. 1A, left). In cell viability assays (CellTiter Glo luminescent assay) using cancer cells, EC330 inhibited growth at approximately $IC_{50} \sim 50$ nmol/L (Fig. 1B). The reported X-ray crystallographic studies of LIF suggested a four α -helix bundle topology with a compact core predominantly composed of hydrophobic residues contributed by the four α -helices (32). Initial structure-activity relationship studies in our laboratory have shown the following structural features are necessary for the LIF inhibitory action: (i) difluoro-acetylenic function at the 17-alpha position and (ii) 4'-substitution at the

11-phenyl ring. Because the EC330 has a steroidal backbone, we investigated the binding of EC330 to steroid receptors such as glucocorticoid receptor (GR). EC330 showed some antagonism to GR (79.8 nmol/L), which may elicit unwanted toxicity. Therefore, we pursued medicinal chemistry modifications, which retained its potency on LIFR, while reduced steroidal-receptor interactions. Additional SPR studies and subsequent synthetic efforts resulted in the development of EC359 (Fig. 1A, right). To examine whether optimization of EC359 retained its activity on par with the initial lead compound EC330, we conducted several studies. Receptor-binding studies revealed EC359 has more desirable characteristics than EC330 including lack of affinity to GR (Fig. 1C). In cell viability assays, EC359 showed significant inhibitory activity on par with EC330 in BT-549 model cells (Fig. 1B).

SPR studies confirmed EC359 direct interaction with LIFR

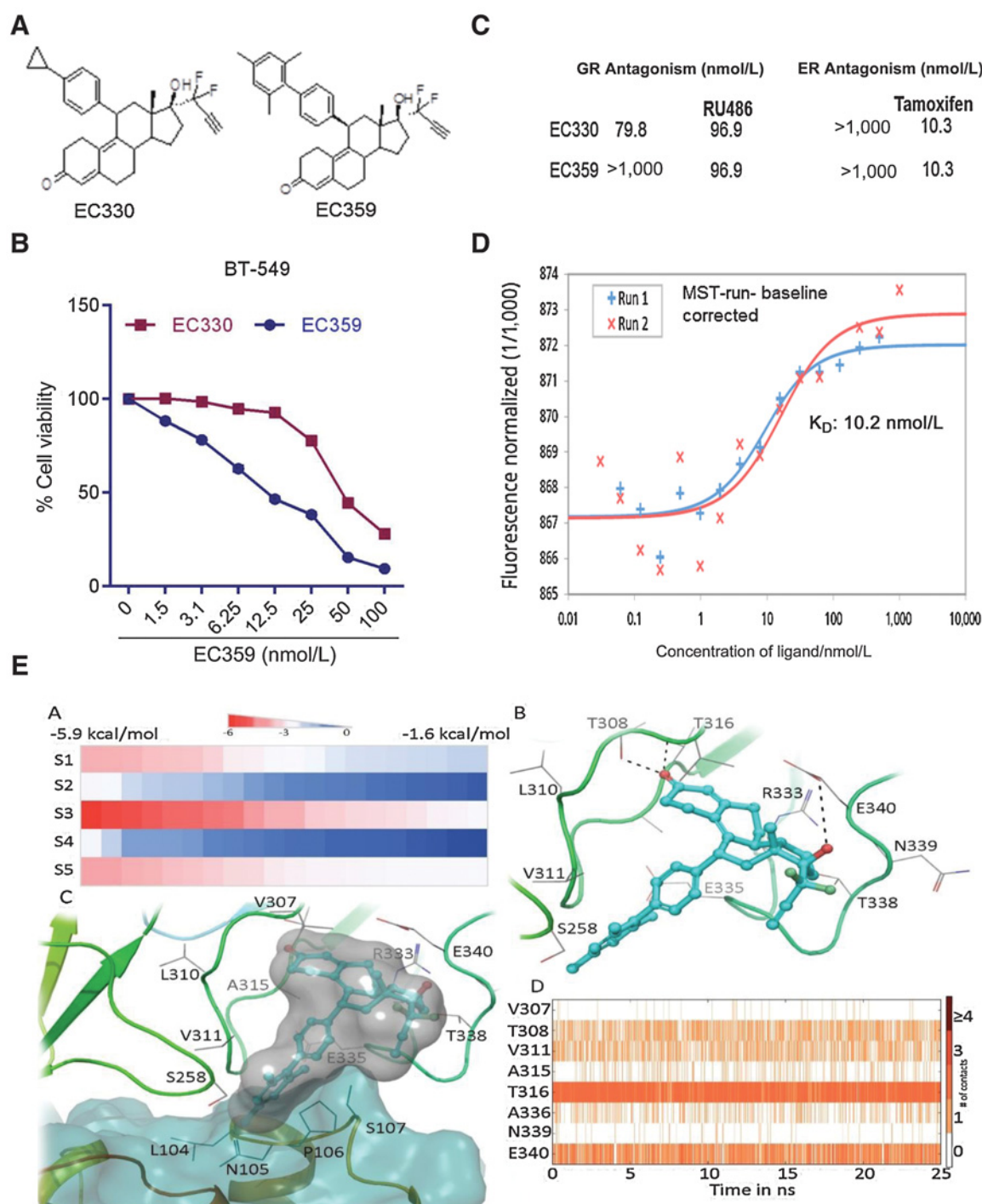
To test whether EC359 directly bind to LIFR complex, binding profiles of EC359 to LIF/LIFR were evaluated using SPR. Two sets of studies were performed: (i) to verify the integrity of recombinant proteins, the interaction between LIFR and LIF was studied; (ii) small-molecule binding to LIF/LIFR by either immobilizing LIFR or LIF onto a sensor chip was tested. Results from the first set of studies confirmed the integrity of recombinant LIF and LIFR; LIF bound to immobilized LIFR-Fc with a binding constant of 7 $\mu\text{mol/L}$ (Supplementary Fig. S1A). In the second set of studies, results showed EC359 binding to LIFR, but not LIF. Furthermore, EC359 bound to LIFR in a dose-dependent manner with $K_d = 81$ $\mu\text{mol/L}$ (Supplementary Fig. S1B). The results confirmed that EC359 is a specific inhibitor of LIF/LIFR complex.

MST assays revealed high-affinity interaction of EC359 with LIFR

Ligand binding to the immobilized receptor in SPR technique will be insensitive to ligand-induced structural changes, and thus the measured affinity by SPR may obscure true (*in vivo* equivalent) affinity of the drug. Hence, we conducted an orthogonal assay, namely MST, where the receptor is not immobilized to verify EC359 binding to the receptor complex. MST is a powerful technique to quantify biomolecular interactions. By combining the precision of fluorescence detection with the variability and sensitivity of thermophoresis, MST provides a flexible, robust, and fast way to dissect molecular interactions (33, 34). MST analysis confirmed direct interaction of EC359 with LIFR with an estimated K_d of 10.2 nmol/L (Fig. 1D). To further demonstrate that EC359 directly interacts with LIFR, we generated biotinylated EC359. Biotin addition did not affect EC359 biological activity (Supplementary Fig. S1C). Using biotin-EC359, we examined whether it interacts with LIFR. Purified LIFR protein or BT-549 cellular lysate was incubated with biotin-EC359 and its ability to interact with LIFR was determined using avidin pull-down assay followed by Western blotting. Results elucidated that EC359 interacts with LIFR (Supplementary Fig. S1D and S1E).

Docking studies suggested EC359 can interact at the LIF-LIFR binding interface

The site predictions revealed five potential binding sites on the hLIFR (Supplementary Fig. S4) in which two sites (2 and 3) are close to the LIF-binding interface (Supplementary Fig. S4). The SP docking was performed on all the five sites and docking scores were deduced (Fig. 1EA). The docking scores toward different

**Figure 1.**

Characterization of EC359. **A**, Schematic representation of the structure of EC330 and EC359. **B**, Dose-response curve of EC330 and EC359 determined using MTT assay on BT-549 cells ($n = 3$). **C**, Ability of EC359 to interact with other steroid receptors was analyzed by *in vitro* binding assays. **D**, Binding of EC359 to LIFR was confirmed using MST assays as described in Materials and Methods. **EA**, The docking scores of EC359 at different binding sites. The sites were mentioned as S1 to S5. **EB**, Binding of EC359 (represented in blue ball and stick model) with hLIFR (represented in cartoon and line model). The dotted lines represent the hydrogen bonds. **EC**, The binding of EC359 in the presence of LIF (represented in blue surface). The binding creates close contacts with residues of LIF. **ED**, The protein ligand contacts over 25 nanoseconds of MD.

binding sites range from -5.8 to -1.6 kcal/mol. It was also observed that EC359 has exhibited more promising scores toward site-3 compared with other sites. The binding poses obtained from the docking were superimposed to the hLIF-hLIFR complex to see the potential clashes between the ligand and LIF. As expected, the binding poses at site-3 are making steric clashes with residues of LIF (Supplementary Fig. S5). Because the SP docking is a rigid docking method, the ligand-induced conformational changes were also studied using IFD by applying flexibility to the surrounding residues. Using standard protocol, the side chains were optimized and 28 poses were generated. The binding energies of all the 28 poses range from -80 kcal/mol to -33 kcal/mol (Supplementary Fig. S6). It was observed that all the generated ligand poses are potentially making steric clashes with hLIF. One of the top scored poses (binding energy = -77 kcal/mol) was critically analyzed for the detailed atomic level interactions (Fig. 1EB). In the selected pose, ligand-induced conformational changes were observed for the loops close to the LIF-binding region (Supplementary Fig. S7). The ligand EC359 was found to sandwich between two loops at the N-terminal of D4 domain by orienting the difluoro-acetylenic group to the bulk solvent. The keto group of the EC359 was found to be involved in two hydrogen bonds with the side chain of T308 and the backbone of T316. Similarly, the hydroxyl group of the ligand was also found to mediate a hydrogen bond with the sidechain of E340. Moreover, van der Waals contacts with the surrounding residues were also found to contribute to the ligand binding. It was observed that EC359 binding to hLIFR would prevent hLIF binding due to steric clashes (Fig. 1EC). As a final step, the snapshots obtained from the MD simulation were superimposed with the initial pose and RMSD was calculated for protein and ligand separately. The snapshots were analyzed and found that the structural distortions are affected mainly to the loops (connecting separate domains in the LIFR) and the terminal regions. At the same time the ligand is found to remain bound at the binding site even after 25 nanoseconds of MD simulation. The protein ligand contacts over 25 nanoseconds of MD simulation are shown in (Fig. 1ED; Supplementary Fig. S8).

EC359 has favorable pharmacologic features

We then conducted pharmacokinetic analysis of EC359 using various established tests (Supplementary Fig. S9). Results from intravenous dosing studies using 5 mg/kg in rats indicated a mean C_0 of 74669.11 ng/mL, $t_{1/2}$ (hours) of 3.86 hours, AUC_{0-last} (ng-hour/mL) of 15,544.36, and AUC_{0-inf} (ng-hour/mL) of 15,573.91. Results from oral dosing studies using 10 mg/kg in rats indicated a mean C_{max} (ng/mL) of 919.50, T_{max} (h) of 2.67, AUC_{0-last} (ng-hour/mL) of 3,792.26, and AUC_{0-inf} (ng-hour/mL) of 3,876.82. Ames test confirmed that EC359 did not induce an evident (significant) >2-fold increase in the revertant counts at the doses tested (dose related), in the tester strains both with and without metabolic activation according to the evaluation criteria mentioned in OECD guideline no.471. Hence, the compound EC359 is considered nonmutagenic with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and *E.coli* combo, both with and without metabolic activation. In hERG cardiotoxicity screening, up to 30 μ M/L concentration of EC359 did not show 50% inhibition, hence no liability. Among CYP enzymes, EC359 inhibits 2D6, therefore, caution is warranted in concurrent administration of drugs that inhibits 2D6 such as Prozac. Metabolic stability and plasma stability are moderate in human with

high plasma protein binding. Good bioavailability (pharmacokinetics) was observed in both intraperitoneal and oral dosing (Supplementary Fig. S9). Collectively, the data from these studies indicate EC359 has specific on-target activity (pharmacodynamics) and suggests EC359 as a druggable candidate for further development.

EC359 reduced the cell viability of LIF- and LIFR-expressing cells

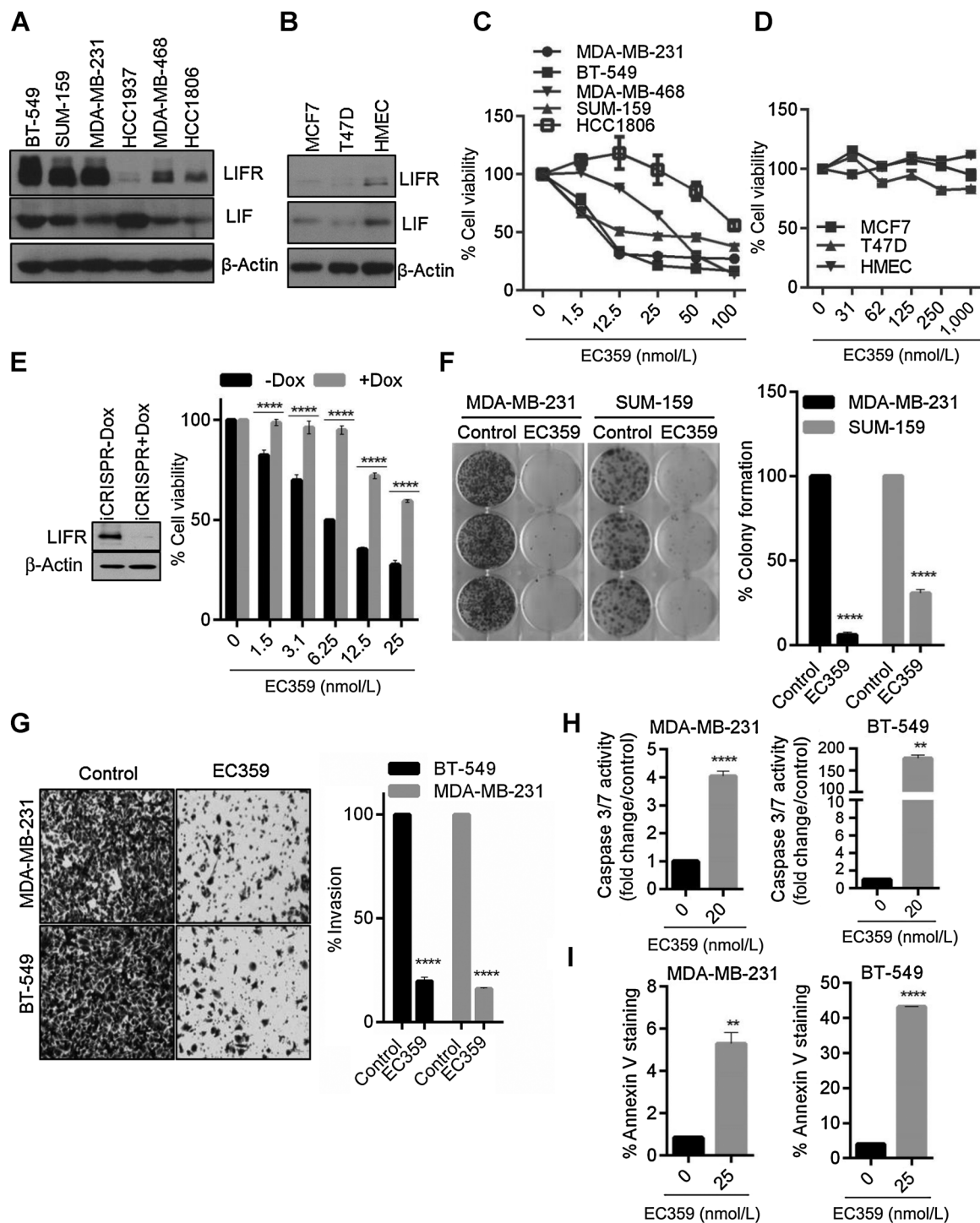
We first examined the expression of LIF and LIFR in cells that represent various subtypes of TNBC (BT-549, SUM-159, MDA-MB-231, HCC1937, MDA-MB-468, and HCC1806), ER⁺ breast cancer (MCF7 and T47D) as well as normal mammary epithelial cells (HMEC). We found that five of the six TNBC cells expressed high levels of LIF and LIFR when compared with ER⁺ breast cancer cells and normal cells (Fig. 2A and B). Next, we examined the efficacy of EC359 on cell viability of TNBC and ER⁺ breast cancer cells. Treatment with EC359 resulted in a significant dose-dependent reduction in the cell viability of TNBC cells (IC_{50} = 10–50 nmol/L) and their inhibition is well correlated with LIF and LIFR expression levels (Fig. 2C). Interestingly ER⁺ breast cancer cells that express low levels of LIF and LIFR exhibited low sensitivity to EC359 treatment (IC_{50} > 1,000 nmol/L) when compared with TNBC cells (Fig. 2D). To further confirm the target specificity of EC359, we generated doxycycline-inducible LIFR-KO cells using Cas9 stably expressing TNBC cells. Results indicated a reduction of LIFR expression in BT-549 models contributed to the resistance of the EC359-mediated decrease in cell viability (Fig. 2E). Collectively, these data suggest that EC359 activity depends on presence of functional LIF/LIFR signaling axis in cells.

EC359 reduced survival and invasion and induced apoptosis of TNBC cells

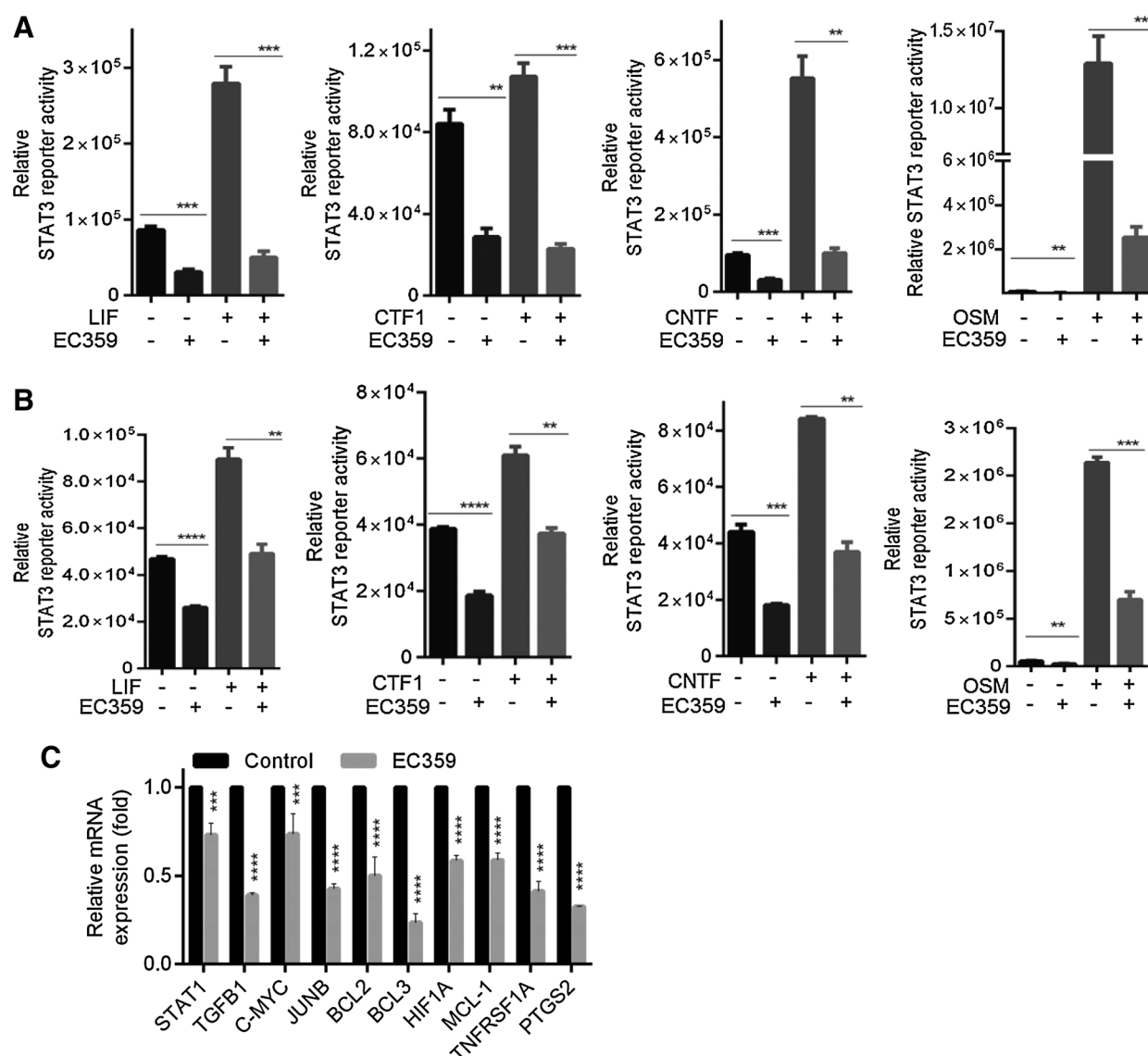
We next examined the efficacy of the EC359 on the survival of TNBC cells. In clonogenic survival assays, EC359 significantly reduced the colony-forming ability of MDA-MB-231 and SUM-159 cells (Fig. 2F). Given the important role of the LIF axis in the invasiveness of cancer cells, we examined the effect of EC359 in reducing the invasion of TNBC cells. Matrigel invasion assays demonstrated that EC359 significantly reduced the invasion potential of MDA-MB-231 and BT-549 cells (Fig. 2G). Furthermore, we examined whether EC359 induced apoptosis in TNBC cells using caspase 3/7 activity assay and Annexin V staining assay. EC359 treatment significantly increased caspase-3/7 activity (Fig. 2H) and Annexin V-positive cells (Fig. 2I) in both MDA-MB-231 and BT-549 cells. Collectively, these results suggest that EC359 exhibits significant inhibitory activity on invasion and promotes apoptosis of TNBC cells.

EC359 inhibited LIFR-mediated transcriptional changes

LIF/LIFR activates multiple signaling pathways including JAK/STAT3, MAPK, AKT, and mTOR; all of which are implicated in TNBC progression. To confirm the inhibitory effect of EC359 on LIF/LIFR-mediated STAT3 activation, BT-549 cells that stably express STAT3-Luc reporter were pretreated with vehicle or EC359 followed by stimulation with LIF. As expected, LIF treatment significantly increased the STAT3 reporter activity and this activation was inhibited by EC359 treatment (Fig. 3A). Because our modeling studies predicted EC359 interaction with the ligand-binding interface of LIFR, we examined whether EC359 also blocks signaling by other LIFR ligands such as Oncostatin M

**Figure 2.**

EC359 decreases cell viability, colony formation, and invasion, and promotes apoptosis of TNBC cells that express LIF and LIFR. The expression of LIF and LIFR in TNBC (A), ER⁺ breast cancer and normal mammary epithelial cells (B) was determined by Western blotting. Effect of increasing doses of EC359 on the cell viability of TNBC (C), ER⁺ breast cancer and normal mammary epithelial cells (D) was determined using the MTT cell viability assay ($n = 3$). E, Effect of inducible CRISPR/Cas9-mediated KO of LIFR on EC359-induced cell viability was determined using MTT assays in BT-549 cells ($n = 3$). F, Effect of EC359 (20 nmol/L) on cell survival was measured using colony formation assays. G, Effect of EC359 (25 nmol/L) on cell invasion of MDA-MB-231 and BT-549 model cells was determined using Matrigel invasion chamber assays ($n = 3$). Representative images of invaded cells are shown, and the number of invaded cells in five random fields was quantitated. Effect of indicated doses of EC359 on caspase-3/7 activity (Caspase-Glo3/7 assay; H) and Annexin V staining in MDA-MB-231 and BT-549 cells ($n = 3$) was determined (I). **, $P < 0.01$; ****, $P < 0.0001$.

**Figure 3.**

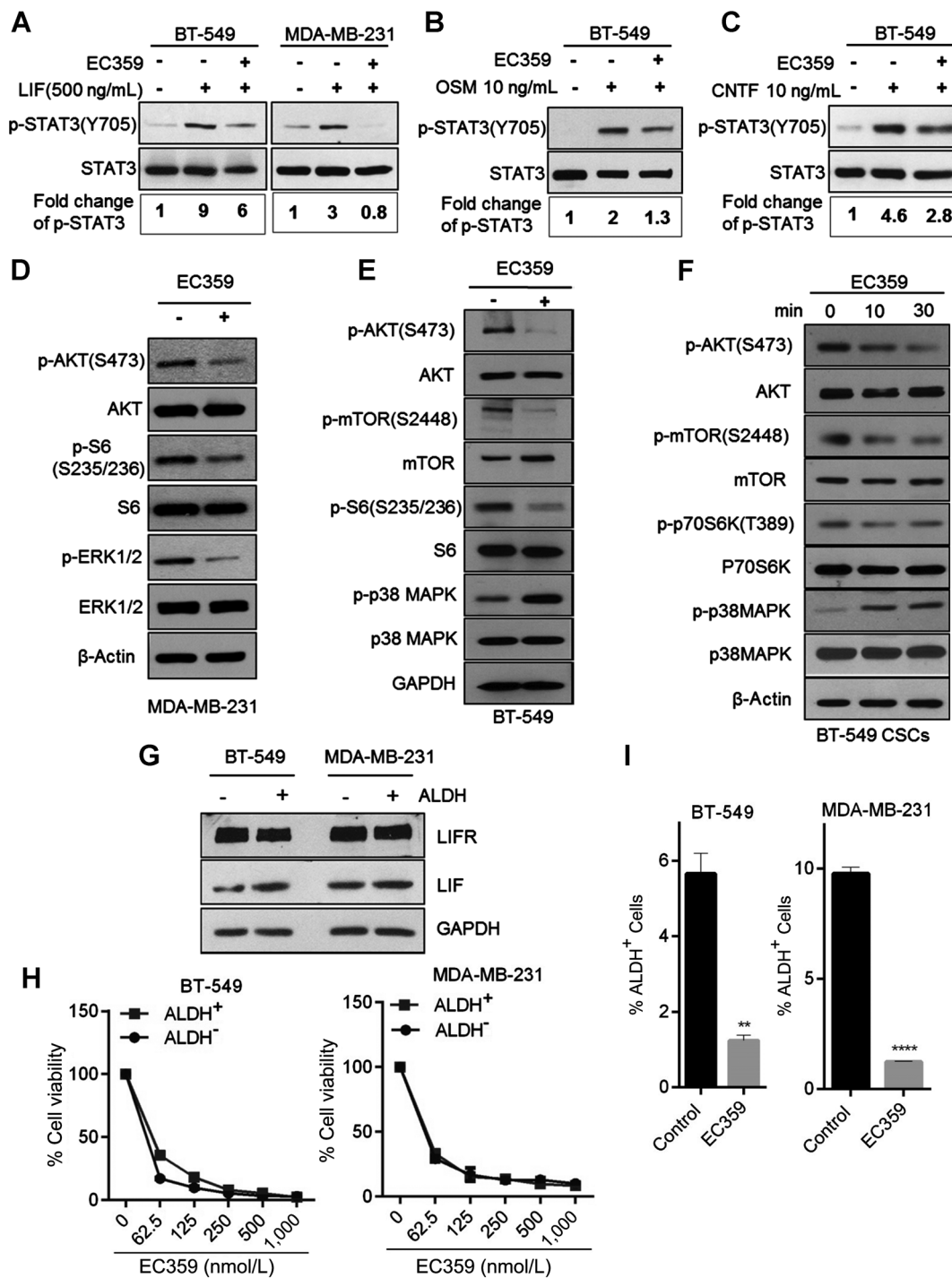
Effect of EC359 on LIFR-mediated transcription in TNBC cells. BT-549 (A) and MDA-MB-231 (B) cells stably expressing STAT3-luc reporter were serum starved for 24 hours, pretreated with EC359 (50 nmol/L) for 1 hour, and then stimulated with LIF, CTF1, OSM, and CNTF ($n = 3$). Reporter activity was measured after 24 hours. C, Effect of EC359 (100 nmol/L) treatment (12 hours) on STAT3-targeted genes in BT549 cells was measured using qRT-PCR analysis ($n = 3$). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

(OSM), Ciliary Neurotrophic Factor (CNTF), and Cardiotrophin 1 (CTF1). Results showed that EC359 blocked the OSM, CNTF, and CTF1-mediated STAT3 activity in BT-549 cells (Fig. 3A). We also confirmed that EC359 has the ability to block LIF-, OSM-, CNTF-, and CTF1-mediated STAT3 activation using MDA-MB-231 cells stably expressing STAT3-Luc reporter (Fig. 3B). In qRT-PCR assays using BT-549 cells, EC359 treatment significantly reduced the expression of several known STAT3 target genes (Fig. 3C).

EC359 reduced LIFR-mediated activation of downstream signaling pathways

To further confirm the effect of EC359 on LIF/LIFR downstream signaling pathways, MDA-MB-231 and BT-549 cells were pre-

treated with vehicle or EC359 and subsequently stimulated with LIF. STAT3 activation was examined using Western blotting. EC359 treatment substantially reduced the LIF activation of STAT3 in both BT-549 and MDA-MB-231 cells (Fig. 4A). EC359 also reduced the STAT3 activation by OSM and CNTF (Fig. 4B and C). In addition, EC359 treatment substantially decreased the phosphorylation of AKT, mTOR, S6, and ERK1/2 in MDA-MB-231 and BT-549 cells (Fig. 4D and E). EC359 treatment also increased the phosphorylation of proapoptotic p38MAPK in BT-549 cells (Fig. 4E). We confirmed whether alteration in downstream signaling seen upon EC359 treatment such as STAT3 occurs in the cell line that has a doxycycline-inducible deletion of the LIFR. Results showed that KO of LIFR significantly reduced the STAT3 activation (Supplementary Fig. S10A). Furthermore,

**Figure 4.**

EC359 inhibits LIFR downstream signaling and reduces stemness of TNBC cells. **A**, MDA-MB-231 and BT-549 cells were serum starved for 24 hours, pretreated with EC359 (100 nmol/L) for 1 hour, and then stimulated with LIF (10 minutes), and the status of STAT3 phosphorylation was measured using Western blotting. STAT3 phosphorylation was quantitated using ImageJ program, normalized to total STAT3, and shown as fold induction over control cells. BT-549 cells were serum starved for 24 hours, pretreated with EC359 (100 nmol/L) for 1 hour, and then stimulated with OSM (10 ng; **B**) and CNTF (10 ng; **C**) for 10 minutes, and the status of STAT3 phosphorylation was measured using Western blotting. **D–F**, MDA-MB-231, BT-549, and CSCs were treated with EC359 (100 nmol/L), and status of LIFR downstream signaling was measured using Western blotting. **G**, ALDH⁺ and ALDH⁻ cells were isolated by FACS, and the expression levels of LIFR and LIF were measured by Western blotting. **H**, Effect of EC359 on the viability of ALDH⁺ (CSCs) and ALDH⁻ (non-CSCs) cells was determined using CellTiter Glo assay ($n = 3$). **I**, BT-549 and MDA-MB-231 cells were treated with EC359 (100 nmol/L), and the status of ALDH⁺ cells was determined by FACS analysis. **, $P < 0.01$; ****, $P < 0.0001$.

stimulation of LIFR KO cells with LIF did not activate STAT3 in this model. However, EC359 is able to block LIF-mediated STAT3 activation in LIFR-expressing control cells. These results confirm that the downstream effects seen in EC359 are due to its effects on LIFR and that STAT3 is a downstream effector of LIFR in TNBC cells (Supplementary Fig. S10A). These results suggest that EC359 acts as a LIFR inhibitor and attenuates LIF and other LIFR ligand-mediated signaling in TNBC cells.

EC359 reduced the cell viability and self-renewal of TNBC stem cells

The LIF/LIFR axis plays a vital role in stemness (6, 12). To test the effect of EC359 on stemness, CSCs were isolated from MDA-MB-231 and BT-549 using ALDH⁺ flow cytometry sorting. EC359 treatment substantially decreased the phosphorylation of AKT, mTOR, p70S6K, and increased phosphorylation of proapoptotic p38MAPK in CSCs (Fig. 4F). Western blot analysis showed that ALDH⁺ (CSCs) and ALDH⁻ (non-CSCs) cells have similar levels of LIFR (Fig. 4G). Furthermore, in cell viability assays, EC359 similarly inhibited both ALDH⁺ and ALDH⁻ cells (Fig. 4H). To further study the effect of EC359 on the self-renewal ability of CSCs, extreme limiting dilution assays (ELDA) were performed. Results showed that EC359 significantly reduced the self-renewal of CSCs compared with control (Supplementary Fig. S10B). Furthermore, pretreatment of TNBC cells with EC359 significantly reduced the abundance of ALDH⁺ cells (Fig. 4I).

EC359 reduced TNBC xenograft tumor growth *in vivo*

To test the efficacy of EC359 on *in vivo* tumor progression, we established MDA-MB-231 xenograft tumors in the mammary fat pad of nude mice. Mice were randomized to vehicle (hydroxymethylcellulose) and EC359 (5 mg/kg/day via subcutaneous injection) 3 days/week. EC359 treatment significantly reduced the tumor progression compared with vehicle (Fig. 5A). The body weights of mice in the vehicle and EC359-treated groups remained unchanged (Fig. 5B) confirming the low toxicity of EC359. Moreover, EC359-treated tumors exhibited fewer proliferating cells (Ki-67-positive cells) compared with vehicle-treated tumors (Fig. 5C). In addition, qRT-PCR analysis confirmed significant decrease in the activation of STAT3 target genes in EC359-treated tumors compared with vehicle (Fig. 5D). Western blot analysis confirmed that xenograft tumors express LIFR and LIF (Fig. 5E). Furthermore, EC359 treatment substantially reduced the phosphorylation of STAT3, ERK1/2, and AKT in tumors compared with vehicle-treated tumors (Fig. 5E). Collectively, these results suggest that EC359 has potent antitumor activity on TNBC in preclinical models.

EC359 has activity against primary patient-derived TNBC explants and reduced *in vivo* tumor progression in PDX model

We tested the utility of EC359 using an *ex vivo* culture model of primary breast tumors, which allowed for the evaluation of drugs on human tumors while maintaining their native tissue architecture (Fig. 6A). Briefly, surgically extirpated deidentified TNBC tissues were cut into small pieces and placed on gelatin sponge soaked in the culture medium and grown for a short term in the presence of vehicle or EC359 (Fig. 6A). Treatment of TNBC explants with EC359 substantially decreased their proliferation (Ki-67 positivity) compared with vehicle-treated tumors (Fig. 6B and C). Next, we tested the effect of EC359 on PDX tumor growth *in vivo*. EC359 treatment significantly reduced the tumor progres-

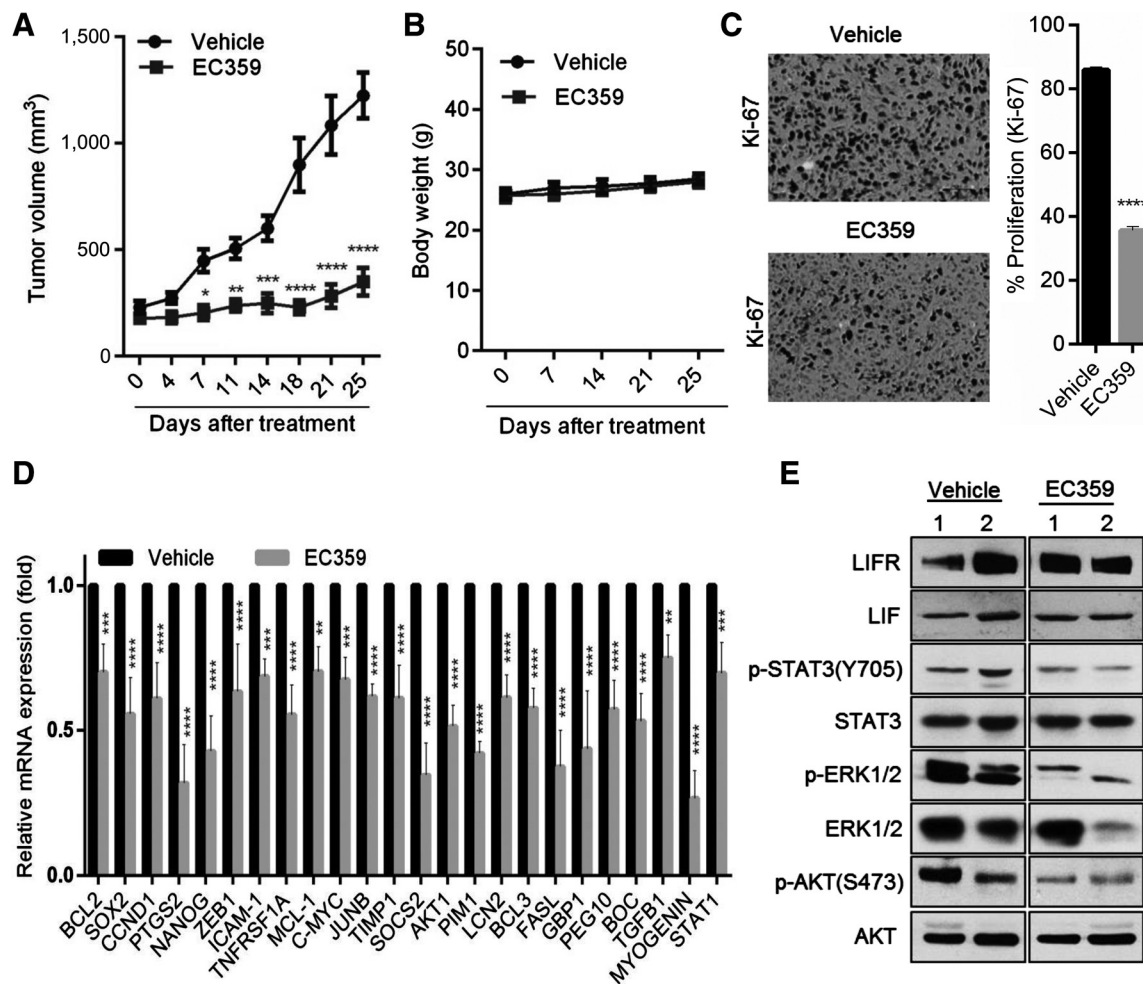
sion compared with the vehicle-treated control group (Fig. 6D) and did not affect the body weight (Fig. 6E). EC359-treated PDX tumors exhibited fewer proliferating cells compared with vehicle-treated tumors (Fig. 6F). qRT-PCR analysis confirmed a significant decrease in the activation of STAT3 target genes in EC359-treated mice (Fig. 6G). Western blot analysis confirmed that PDX tumors express LIFR and LIF (Fig. 6H). Furthermore, EC359 treatment substantially reduced the phosphorylation of mTOR, S6, and AKT in tumors compared with vehicle-treated tumors (Fig. 6H). These results suggest that EC359 has therapeutic activity in primary patient-derived TNBC explants and PDX tumors.

Discussion

LIF is the most pleiotropic member of the IL6 family of cytokines (4) that signals via the LIFR (5). Recent evidence suggested tumors exhibit upregulated LIF/LIFR signaling via autocrine and paracrine mechanisms (1, 5–7). However, lack of specific inhibitors targeting the LIF/LIFR axis represents a critical barrier in the field. In this study, we rationally designed a small organic molecule, EC359 that emulates the LIF/LIFR-binding site and functions as a first-in-class LIFR inhibitor from a library of compounds. Using multiple TNBC cells, we demonstrated that EC359 decreases cell viability, invasion, and promotes apoptosis. Mechanistic studies using Western blot, reporter gene assays, and qRT-PCR confirmed significant reduction of activation of LIF/LIFR-mediated pathways. Utilizing PDX, and patient-derived explant (PDEX) models, we demonstrated the *in vivo* efficacy of EC359.

The molecular modeling and SPR suggests the putative binding site is at the interface of LIF and LIFR. EC359 may display longer resident time (i.e., slower k_{off}) in the LIF/LIFR complex as suggested by the molecular model. Reasonable (about 30 minutes) residence time ($1/k_{off}$) suggests it may display biological activity *in vitro* and *in vivo*. However, potency of EC359 may depend on the concentration of LIF. During the SPR assay, we noted that EC359 was incompletely dissolved in the running buffer. Thus, due to poor solubility of the inhibitor in running buffer (inhibitor precipitates at concentrations 25–50 μ mol/L in 5% DMSO) derived kinetic constants must be considered approximate; spiking at 2 μ mol/L (green color) suggest that the compound may be aggregated; microaggregation affects not only the transport property of the ligands, but also the off-rate. Thus, the weaker binding affinity derived from SPR studies is likely due to poor solubility of the compound, which may be attributed as limitation of SPR technique. Nonetheless, results from SPR show that EC359 is specific to LIFR.

Recently, the MST technique has been widely used for characterizing protein–ligand interactions. MST offers a unique advantage over conventional isothermal titration calorimetry (ITC); unlike SPR, in MST, the target is not immobilized, and ligand-binding is independent of size or physical properties of ligands. MST analysis indicated higher binding affinity (K_d) between LIF and LIFR (1.36 nmol/L) than LIFR and EC359 (10.2 nmol/L). Also, the longer residence time/slower k_{off} in SPR demonstrates this pertinent biological effect. These values were consistent with high nanomolar potency of EC359 *in vitro* and *in vivo*. The discrepancy in the binding affinity measured between SPR and MST assays may be due to either difference in steady-state binding (MST) versus kinetic binding (SPR), or drug-induced structural changes upon binding; structural changes at the binding site, or

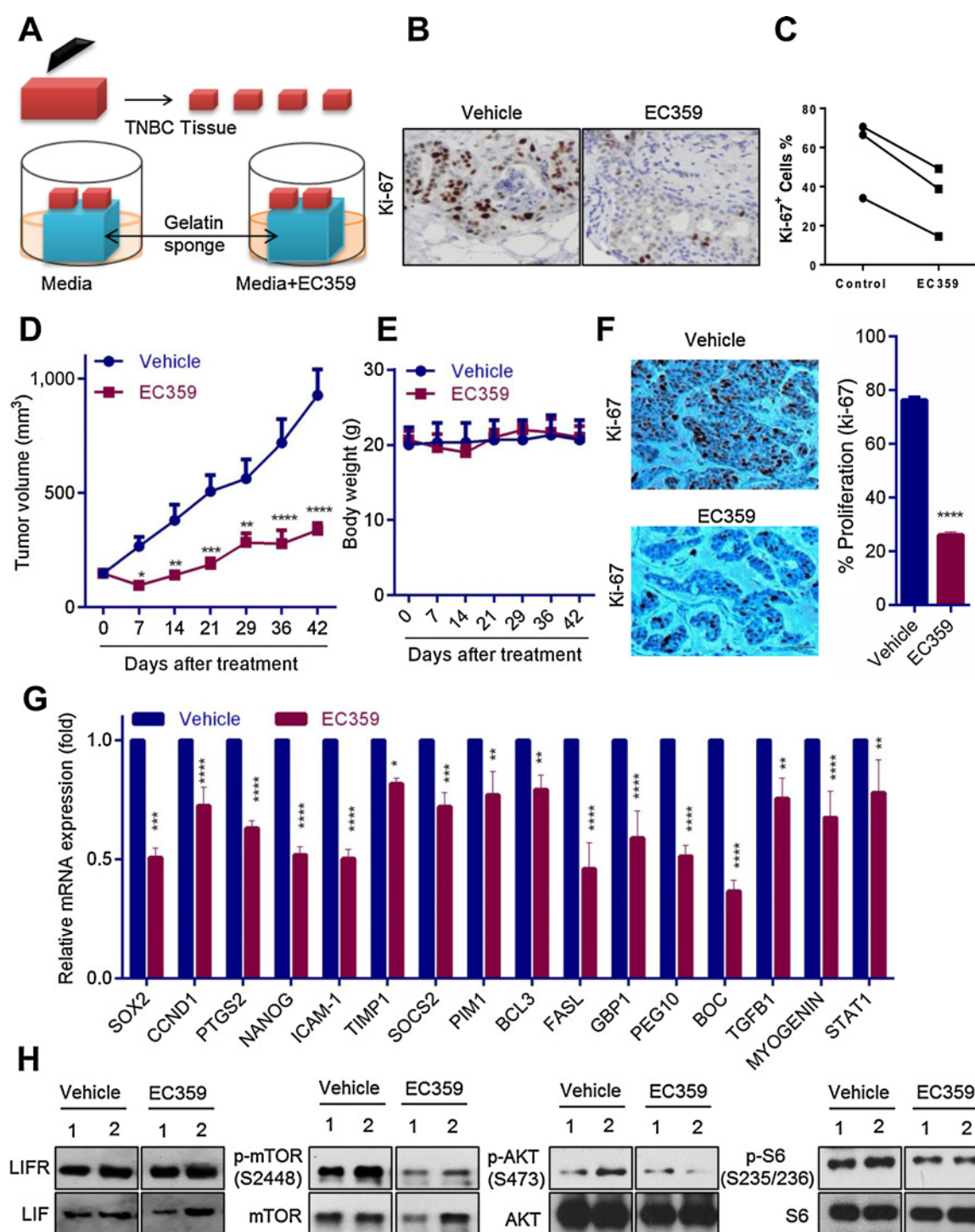
**Figure 5.**

EC359 inhibits the growth of TNBC xenograft tumors. **A**, MDA-MB-231 xenografts ($n = 8$) were treated with vehicle or EC359 (5mg/kg/s.c./3 days/week). Tumor volumes are shown. **B**, Body weights of vehicle and EC359-treated mice are shown. **C**, Ki-67 expression as a marker of proliferation was analyzed by IHC and quantitated. **D**, Status of STAT3 target genes was measured using qRT-PCR analysis ($n = 3$). **E**, LIFR downstream signaling was measured using Western blotting (data using two different xenograft tumors are shown). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

both. Despite the differences in SPR and MST techniques, our results show that EC359 directly binds and disrupts the LIFR signaling complex.

The LIF/LIFR axis exhibits differential effects, which depended on the cell type, including stimulating or inhibiting cell proliferation, differentiation, and survival (4, 11, 24). LIFR is also reported to function as a metastasis suppressor through the Hippo-YAP pathway (35) and confer a dormancy phenotype in breast cancer cells disseminating to bone (36). It should be noted that presence of LIF is important for LIFR activation as we have determined in our SPR analysis. Hence studies using low LIF-expressing cell lines such as MCF-7 or T47D may not have an overly active LIF/LIFR signaling. However, LIFR signaling is complex as multiple ligands activate LIFR including LIF, CNTF, OSM, and CTF1. Despite the ability of LIF to activate JAK1/STAT3, PI3K/AKT, and MAPK pathways in these cell lines, differences in signaling outcome may, in part, arise from differential levels of activation of these three pathways, multiple ligands to LIFR, and differences in tumor microenvironment (TME; refs. 1, 37).

Earlier studies revealed that LIF, CTF1, and OSM share an overlapping binding site located in the Ig-like domain of LIFR and different behaviors of LIF, CTF1, and OSM can be related to the different affinity of their site for LIFR (38). Our modeling studies predicted that EC359 will interact at the LIF-LIFR binding interface and block interaction of LIF to LIFR. In agreement with published studies, our reporter assays and Western blot analyses showed that EC359 has the ability to block the signaling mediated by other cytokines (CTF1, CNTF, and OSM) that interact LIFR at LIF/LIFR interface. Blockage of LIFR by EC359 can leverage additional benefit of interfering the LIFR-JAK-STAT pathway by all known four LIFR ligands. We speculate that the unique ability of EC359 to bind the common ligand-binding site blocks multiple ligands' interactions with LIFR offers an advantage over other biologics or small molecules that can only target either of these ligands alone. This may also account the apparent differences in the activity seen by EC359 in TNBC and ER⁺ breast cancer as TNBC expresses higher levels LIFR ligands compared with ER⁺

**Figure 6.**

EC359 decreases the growth of primary patient-derived TNBC explants *ex vivo* and PDX tumors *in vivo*. **A**, Schematic representation of *ex vivo* culture model. **B**, TNBC explants were treated with EC359 for 72 hours, and the proliferation was determined using Ki-67 immunostaining. Representative Ki-67 staining from one tumor treated with vehicle or EC359 is shown. **C**, The Ki67 expression in TNBC explants ($n = 3$) is quantitated. **D**, TNBC PDX tumors ($n = 6$) were treated with vehicle or EC359 (10 mg/kg/s.c./3 days/week). Tumor volumes are shown. **E**, Body weights of vehicle and EC359-treated mice are shown. **F**, Ki-67 expression as a marker of proliferation was analyzed by IHC and quantitated. **G**, STAT3 target genes were measured by using qRT-PCR analysis ($n = 3$). **H**, Status of LIFR downstream signaling was measured using Western blotting (data using two different PDX tumors are shown). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

breast cancer. In SPR studies, we found that the presence of ligand LIF further enhanced LIFR interaction with EC359 compared with LIFR alone. Because the ER⁺ breast cancer cells lack or possess low levels of LIF and LIFR, the increased fold difference in activity (sensitivity) of EC359 toward TNBC cells may reflect presence of increased ligand/receptor levels in TNBC. Furthermore, EC359 is unable to block OSM, CTF1, and CNTF, interactions with their natural receptors (OSMR/gp130, LST/gp130, CNTFR/gp130 respectively); therefore, EC359 is less likely to affect the physiologic signaling of CTF1, CNTF, and OSM. As a consequence, the issue of toxicity is less likely to occur. Accordingly, in xenograft studies, we did not observe toxicity over the course of EC359 treatment. However, future studies using formal toxicity protocols are needed to address the toxicity concerns and is beyond the current scope of this work.

Breast cancer cells often exhibit autocrine stimulation of LIF–LIFR axis. Some subtypes of TNBC such as claudin-low are highly enriched for CSC markers (39, 40). The LIF promoter is hypermethylated in normal breast epithelial cells, but extensively demethylated during breast cancer progression (5). TNBC cells have higher expression of LIF and LIFR compared with ER⁺ breast cancer cells and overexpression of LIF is significantly associated with a poorer relapse-free survival in patients with breast cancer (4). Together, these emerging findings strongly suggest that LIF signaling in TNBC may be clinically actionable and that disruption of the LIF signaling cascade has potential to block progression of subtypes of TNBC that exhibit a LIF/LIFR autocrine loop.

LIF activates multiple signaling pathways via LIFR including STAT3, MAPK, AKT, and mTOR (3, 4)—all are implicated in cancer progression. Tumors exhibit upregulated LIF–JAK–STAT3 signaling via autocrine and paracrine mechanisms (1, 5–7). LIF signaling also plays a role in crosstalk between tumor cells and fibroblasts, and mediates the proinvasive activation of stromal fibroblasts (9). LIF/LIFR signaling is implicated in modulation of multiple immune cell types present in tumor microenvironment (TME) including T-eff, T-reg, macrophages (41), and myeloid cells, which results in immune suppression (42). In our studies using TNBC model cells, we found that EC359 substantially reduced the activation of STAT3, MAPK, AKT, and mTOR; and significantly delayed tumor progression *in vivo*. However, our mechanistic studies are limited to EC359 effects on epithelial cells; future studies are needed to clearly examine the effect of EC359 on TME.

LIF and LIFR are overexpressed in multiple solid tumors (5, 7, 43). While LIF can act on a wide range of cell types, LIF knockout mice have revealed that many of these actions are not apparent during ordinary development (1), indicating a potential therapeutic window for LIF/LIFR axis inhibitors in addition to less toxicity in normal adult tissues. Considering the importance of the LIF/LIFR axis in cancer, humanized anti LIF antibody (MSC-1) that blocks LIF signaling is being tested in a phase I clinical trial mode to determine its safety and tolerability (ClinicalTrials.gov, NCT03490669). Given the wide deregulation of the LIF/LIFR axis in multiple tumors, the small-molecule LIFR inhibitor EC359 may have utility in treating other solid tumors including glioblastoma, ovarian cancer, colon cancer, and pancreatic cancer all of which exhibit dysregulated LIF/LIFR signaling. Our studies only examined

the utility of EC359 using TNBC models. Future studies are needed to further evaluate the effects of EC359 in other cancer models and to examine any potential beneficial effects of EC359 on TME.

In summary, our data demonstrated that EC359 is a highly potent and specific LIFR inhibitor. EC359 blocked LIF/LIFR physical and functional interaction, signaling, and reduced cell viability of LIF/LIFR-expressing TNBC cells both *in vitro* and *in vivo*. EC359 represents a exciting new mechanism to modulate LIF/LIFR oncogenic functions. Because EC359 is a small, stable molecule, it is amenable for translation to clinical trials for patients with TNBC as either monotherapy or in combination with current standard of care.

Disclosure of Potential Conflicts of Interest

G.V. Raj is a founder at EtrixRx and GaudiumRx, reports receiving a commercial research grant from Bayer, has received speakers' bureau honoraria from Bayer, Janssen, Astellas, and Pfizer, is a consultant/advisory board member for Bayer and Janssen. K.J. Nickisch has ownership interest (including stock, patents, etc.) from Evestra. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

We thank Jessica Perry (Ob/Gyn UT Health San Antonio) for proofreading of the manuscript. MST studies were performed by 2bind GmbH, Germany. K.V. Dileep thanks Japan Society for the Promotion of Science for a postdoctoral fellowship. This study was supported by the DOD BCRP grant W81XWH-18-1-0016 (to R.K. Vadlamudi and K.J. Nickisch), DOD BCRP grant W81XWH-16-0294 (to R.R. Tekmal), NCI Cancer Center Support Grant P30CA054174-17, Max and Minnie Tomerlin Voelcker Fund (to G.R. Sareddy), and NIH grant 1R01CA179120-01 (to R.K. Vadlamudi and M. Rao).

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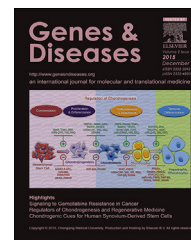
Received November 6, 2018; revised March 12, 2019; accepted May 16, 2019; published first May 29, 2019.

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REVIEW ARTICLE

Targeting LIF/LIFR signaling in cancer

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Received 7 January 2021; received in revised form 5 April 2021; accepted 9 April 2021

KEYWORDS

LIF;
 LIFR;
 LIFR inhibitor;
 STAT3;
 Targeted therapy

Abstract Leukemia inhibitory factor (LIF), and its receptor (LIFR), are commonly over-expressed in many solid cancers and recent studies have implicated LIF/LIFR axis as a promising clinical target for cancer therapy. LIF/LIFR activate oncogenic signaling pathways including JAK/STAT3 as immediate effectors and MAPK, AKT, mTOR further downstream. LIF/LIFR signaling plays a key role in tumor growth, progression, metastasis, stemness and therapy resistance. Many solid cancers show overexpression of LIF and autocrine stimulation of the LIF/LIFR axis; these are associated with a poorer relapse-free survival. LIF/LIFR signaling also plays a role in modulating multiple immune cell types present in tumor micro environment (TME). Recently, two targeted agents that target LIF (humanized anti-LIF antibody, MSC-1) and LIFR inhibitor (EC359) were under development. Both agents showed effectiveness in preclinical models and clinical trials using MSC-1 antibody are in progress. This article reviews the significance of LIF/LIFR pathways and inhibitors that disrupt this process for the treatment of cancer.

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Abbreviations: leukemia inhibitory factor, (LIF); LIF receptor, (LIFR); programmed death-ligand 1, (PD-L1); cancer stem cells, (CSCs); tumor micro environment, (TME); oncostatin M, (OSM); ciliary neurotrophic factor, (CNTF); cardiotrophin 1, (CTF1); humanized Anti-LIF antibody, (MSC-1); breast cancer, (BCa); endometrial cancer, (ECa); ovarian cancer, (OCA); prostate cancer, (PCa); colorectal cancer, (CRC); pancreatic ductal adenocarcinoma, (PDAC); AKT, protein kinase B; HER2, human epidermal growth factor receptor 2; JAK, Janus kinase; mitogen activated protein kinase, (MAPK); mammalian target of rapamycin, (mTOR); signal transducer and activator of transcription 3, (STAT3); triple negative breast cancer, (TNBC).

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Peer review under responsibility of Chongqing Medical University.

<https://doi.org/10.1016/j.gendis.2021.04.003>

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Please cite this article as: S. Viswanadhapalli, K.V. Dileep, K.Y.J. Zhang et al., Targeting LIF/LIFR signaling in cancer, Genes & Diseases, <https://doi.org/10.1016/j.gendis.2021.04.003>

Introduction

Leukemia inhibitory factor (LIF) is the most pleiotropic member of the interleukin-6 family of cytokines.¹ LIF mediates its signaling using membrane receptor complex that is comprised of LIFR and glycoprotein 130 (gp130).² LIF first binds to LIFR which leads to the formation of high affinity functional complex with gp130. The LIFR does not have an intrinsic tyrosine kinase activity. However, LIFR and gp130 constitutively associate with JAK-Tyk family of cytoplasmic tyrosine kinases, which facilitates downstream signaling.³ Tumors exhibit upregulated LIF/LIFR-JAK-STAT3 signaling via autocrine and paracrine mechanisms.^{4–7} LIF promotes activation of mTORC1/p70S6K signaling pathway enhancing tumor growth and inhibit DNA damage responses.⁸ LIF/LIFR signaling promotes tumorigenesis and metastasis of breast cancer by enhancing AKT-mTOR signaling.⁹ LIFR regulates tumor angiogenesis through ERK/IL-8 pathway.¹⁰ LIF/LIFR signaling negatively regulates tumor-suppressor p53 through STAT3/ID1/MDM2 axis.¹¹ LIFR signaling is complex as multiple ligands such as Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), and Cardiotrophin 1 (CTF1) also have the potential to interact with LIFR and activate its downstream signaling.⁷ Earlier studies revealed that LIF, CTF1, and OSM share an overlapping binding site located in the Ig-like domain of LIFR and different behaviors of LIF, CTF1, and OSM can be related to the different affinity of their site for LIFR.¹² In this review, we summarize the emerging significance and functions of LIF/LIFR signaling in cancer. We will also discuss recent therapeutic strategies that are being developed in targeting LIF/LIFR axis in cancer.

Significance of LIF/LIFR signaling in cancer

LIF/LIFR axis is implicated in tumor growth and progression by acting on multiple aspects of cancer biology.¹³ LIF functions as a growth factor in pancreas carcinoma cells⁴ and high LIF expression in pancreatic ductal adenocarcinoma (PDAC) was associated with shorter overall survival.¹⁴ Aberrant production of LIF in the pancreas is restricted to pathological conditions and circulating LIF correlates well with tumor response to therapy.¹⁵ LIFR expression identifies highly malignant melanocytic lesions at an early stage; and LIFR is associated with unfavorable prognosis in melanoma.¹⁶ LIFR also serves as a novel prognostic biomarker for gallbladder cancer.¹⁷

Histone methyltransferase KMT2D sustains prostate carcinogenesis and metastasis by epigenetically activating LIFR.¹⁸ The LIF promoter is hypermethylated in normal breast epithelial cells, but extensively demethylated during Breast Cancer (BCa) progression.⁵ LIF promotes proliferation and metastasis of BCa cells, and overexpression of LIF is commonly associated with poorer relapse free survival in BCa patients.⁹ Triple Negative Breast Cancer (TNBC) cells exhibit a higher expression of LIF and LIFR compared to that of ER + BCa cells and the overexpression of LIF is significantly associated with a poorer relapse free survival in BCa patients.⁹ BCa progression increases the expression of OSM, LIF, OSMR, LIFR and gp130. The secretion of OSM and LIF by both epithelial and stromal cells may further contribute to BCa progression by autocrine and paracrine manner.²⁰

LIFR expression is upregulated in colorectal cancer (CRC) and represents a potential therapeutic target.¹⁰ Overexpression of LIF is associated with poor prognosis in CRC.¹¹ LIF is also frequently overexpressed in osteosarcoma, and promotes the growth and invasion by activating STAT3 pathway.²¹ PI3K subunit p110 α controls the expression of LIFR α via c-Myc and miR-125b axis to promote Medulloblastoma cell proliferation.¹⁹ LIF/LIFR/STAT axis maintain programmed death-ligand 1 (PD-L1) protein stability and promote metastasis-associated gene expression and may serve as a prognostic biomarker and potential therapeutic target for prostate cancer.²²

Furthermore, LIF/LIFR axis exhibits differential effects in various cell types including stimulating or inhibiting cell proliferation, differentiation and survival depending on the cell type.^{9,13,23} LIF promotes gastric cancer cell proliferation, migration, and invasion via the LIFR-Hippo-YAP pathway and inhibited apoptosis. LIF inhibits the activity of the Hippo pathway by reducing phosphorylation of YAP, increased YAP nuclear translocation, and increased cell proliferation.²⁴ LIFR is also reported to function as a metastasis suppressor through the Hippo-YAP pathway²⁵ and confer a dormancy phenotype in BCa cells disseminating to bone.²⁶ Despite the ability of LIF to activate JAK1/STAT3, PI3K/AKT and MAPK pathways in these cells, differences in signaling outcome may in part arise from differential levels of activation of these three pathways, multiple ligands to LIFR and differences in tumor micro environment (TME).^{1,27} Collectively, these findings implicate that LIF binding to LIFR complex activates multiple signaling events and in many solid cancers LIF/LIFR signaling promote cancer progression (Fig. 1).

LIF/LIFR signaling in tumor microenvironment

LIF/LIFR signaling plays a role in modulating multiple immune cell types present in tumor micro environment (TME) including T-eff, T-reg, macrophages,²⁸ and myeloid cells which leads to immune suppression.²⁹ LIF regulates CXCL9 in tumor-associated macrophages and prevents CD8⁺ T cell tumor-infiltration impairing anti-PD1 therapy. The combination of LIF neutralizing antibodies with the inhibition of the PD1 immune checkpoint promotes tumor regression, immunological memory, and an increase in overall survival.³⁰ LncAMPc upregulates LIF/LIFR expression to stimulate the Jak1-STAT3 pathway to maintain PD-L1 protein stability and immunosuppression.²²

Ovarian Cancer (OCa) cells secrete LIF and are implicated in the development of immune deficiency in the peritoneal cavity of OCa patients.^{28,31} Obesity signals (such as estrogen, leptin) promote endometrial cancer (ECa)³² and function as potent inducers of LIF.^{33,34} LIF is an established E2-responsive gene in the uterus.³⁵ LIF signaling promotes crosstalk between tumor cells and fibroblasts, and mediate pro-invasive activation of stromal fibroblasts.³⁶ Obese people have unusually high levels of leptin; and leptin increases p-STAT3, LIF, LIFR, levels in cultured human endometrial cells.³⁴

Tumors upregulate LIF/LIFR signaling via autocrine and paracrine mechanisms.^{4–6} LIF functions both as paracrine or autocrine growth factor for breast, kidney and prostate

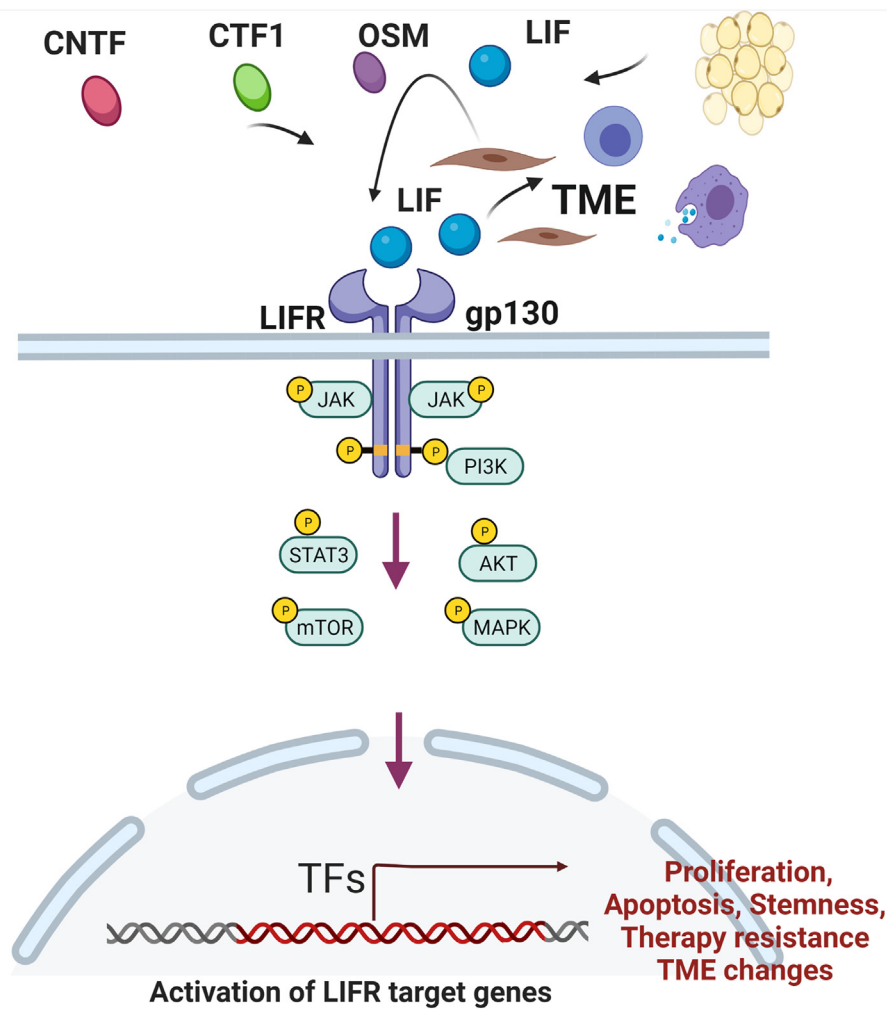


Figure 1 LIF/LIFR oncogenic signaling network. LIF/LIFR signaling promotes tumor progression by regulating multiple signaling aspects including activation of STAT3 signaling, mTOR, AKT, MAPK. LIF/LIFR axis promotes cell survival, enhances stemness and inhibits apoptosis. Further LIF/LIFR axis regulates multiple aspects of tumor microenvironment including macrophage signaling. Further, autocrine or paracrine loops of LIF/LIFR signaling also drives cancer progression.

cancers.¹³ TGF-beta induces the self-renewal capacity of glioma stem cells through the SMAD-dependent induction of LIF.³⁷ Hypoxia induces LIF expression in human cancer cells.³⁸ LIF signaling plays a role in crosstalk between tumor cells and fibroblasts, and mediate pro-invasive activation of stromal fibroblasts.³⁶ LIF is produced by OCa associated mesenchymal stem cells, induces tumor cell JAK-STAT signaling via LIFR, increases the percentage of cancer stemlike cells, and promotes tumor growth.³⁹ Autocrine loop involving LIF, LIF Receptor, and STAT4 drives sustained fibroblast production of inflammatory mediators.⁴⁰

Role of LIF/LIFR axis in stemness and therapy resistance

The evidence for the role of LIF/LIFR axis in its contribution to therapy resistance is well supported by literature. Several studies demonstrated that cancer stem cells (CSCs) are highly tumorigenic, spared by chemotherapy, sustain tumor growth, and are implicated in tumor recurrence.^{41–44} LIF/

LIFR axis is a key regulator of CSCs¹³; further, LIF-STAT3 axis is implicated in stem cell self-renewal and pluripotency⁴⁵ and in the regulation of the transcription factors SOX2 and NANOG that play a critical role in stemness.⁴⁶ Recent studies showed that the switch from migration to proliferation was regulated by BM-MSC-secreted LIF/LIFR/p-ERK/pS727-STAT3 signaling to promote early disseminated cancer cells mesenchymal–epithelial transition and premetastatic niche formation.⁴⁷ TGFβ is shown to promote BCa stem cell self-renewal through an Interleukin-like EMT Inducer (ILEI)/LIFR signaling axis.⁴⁸ Glioma-initiating cells (GICs) are responsible for the initiation/recurrence of gliomas and TGF-beta/LIF signaling induces the self-renewal capacity and prevents the differentiation of GICs.³⁷

High circulating LIF levels correlate well with tumor recurrence and contribute to chemoresistance.^{6,49} Overexpression of LIF is an important mechanism for the attenuation of p53, which promotes chemoresistance in CRCs.¹¹ LIF is a commonly upregulated gene in carboplatin- and paclitaxel-resistant cells and its expression correlates with poor outcome in ECa patients.⁵⁰ Additionally,

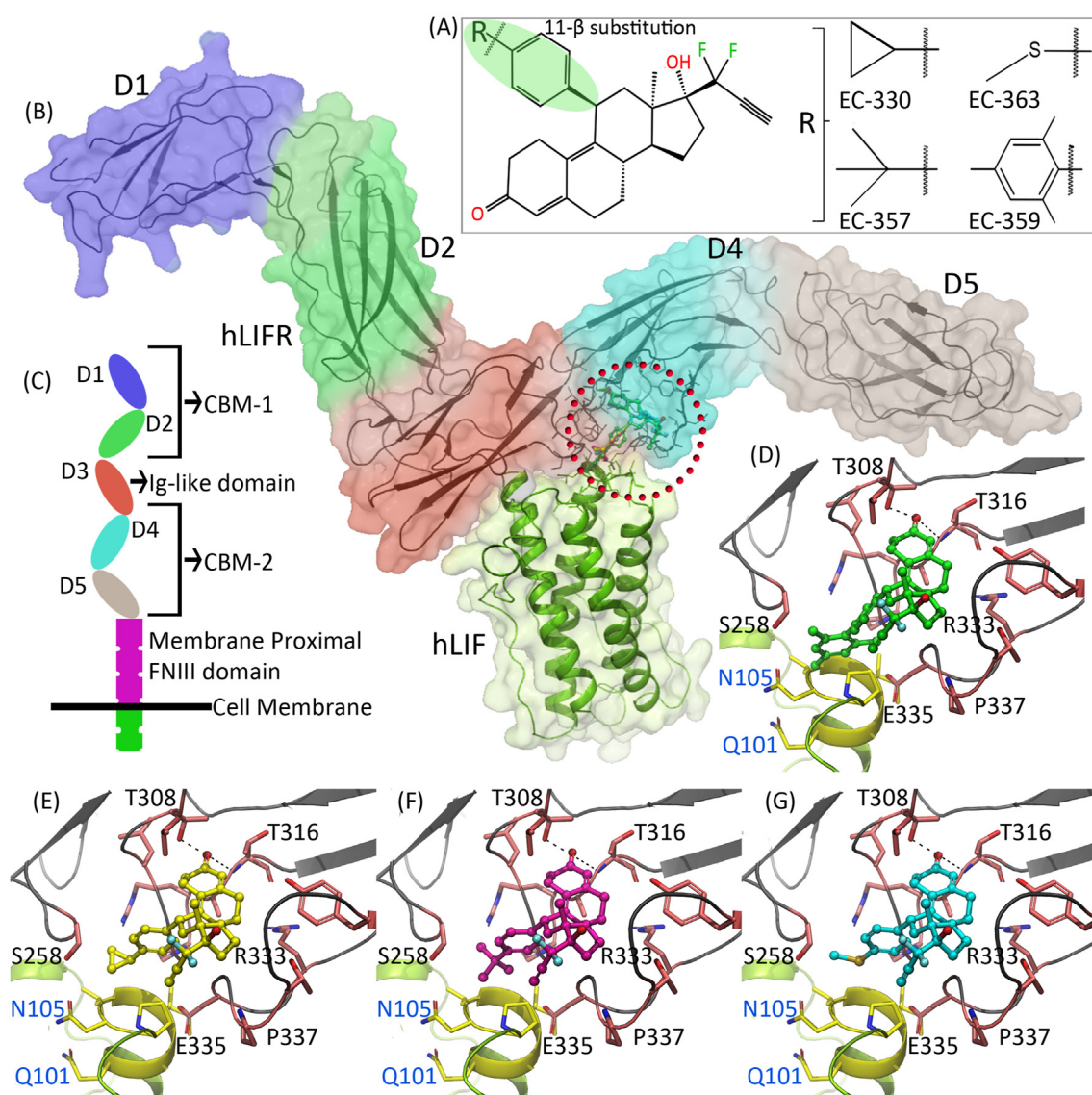


Figure 2 Binding mode of LIFR inhibitors. (A) Structures of the compounds that preferentially block the interactions of LIF with LIFR. The 11- β position is shown in green color. (B) Three dimensional structure of hLIF–LIFR complex. Domains in hLIFR are displayed in blue (domain D1), green (domain D2), red (domain D3), cyan (domain D4) and gray (domain D5) color surfaces and as a black ribbon. The hLIF is displayed in green ribbon and white surface. The red circle in the hLIFR denotes potential binding pocket. (C) Domain representation of LIFR receptor illustrated in cartoon. (D–G) Binding modes of EC359 (represented as green ball and stick), EC330 (yellow), EC357 (violet), and EC363 (cyan). hLIFR is shown in black ribbon and pink stick, hLIF is shown in yellow ribbon and sticks.

LIF/LIFR signaling contributes to drug-resistant OCa.⁵¹ Autocrine and paracrine LIF signaling is shown to promote chemoresistance in cholangiocarcinoma by up-regulating Mcl-1 via a novel STAT3-PI3K/AKT-dependent pathway.⁶ Androgen deprivation therapy (ADT) upregulates EGFR–LIFR signaling that activates succinate-CoA ligase GDP-forming beta subunit (SUCLG2), which subsequently stimulates the metabolic changes associated with neuroendocrine differentiation and aggressive prostate cancer phenotype.⁵²

Elevated circulating LIF is a marker of poor response to neo-adjuvant treatment in oesophageal adenocarcinoma (OAC) and targeting LIF signaling enhances neoadjuvant treatment responses.⁵³ LIFR mediated activation of STAT3

confers resistance to T-DM1 in HER2-positive breast cancer.⁵⁴ LIF promotes nasopharyngeal carcinoma (NPC) tumorigenesis and serum LIF levels predict local recurrence and radiosensitivity.⁸ Collectively, the ability of LIF/LIFR axis to promote therapy resistance can be attributed to its propensity to enhance stemness, promoting survival and reduce apoptosis.

Structural features of LIFR for blocking ligand signaling

To understand the structural features of LIFR, we conducted homology modeling of the human LIFR (hLIFR)

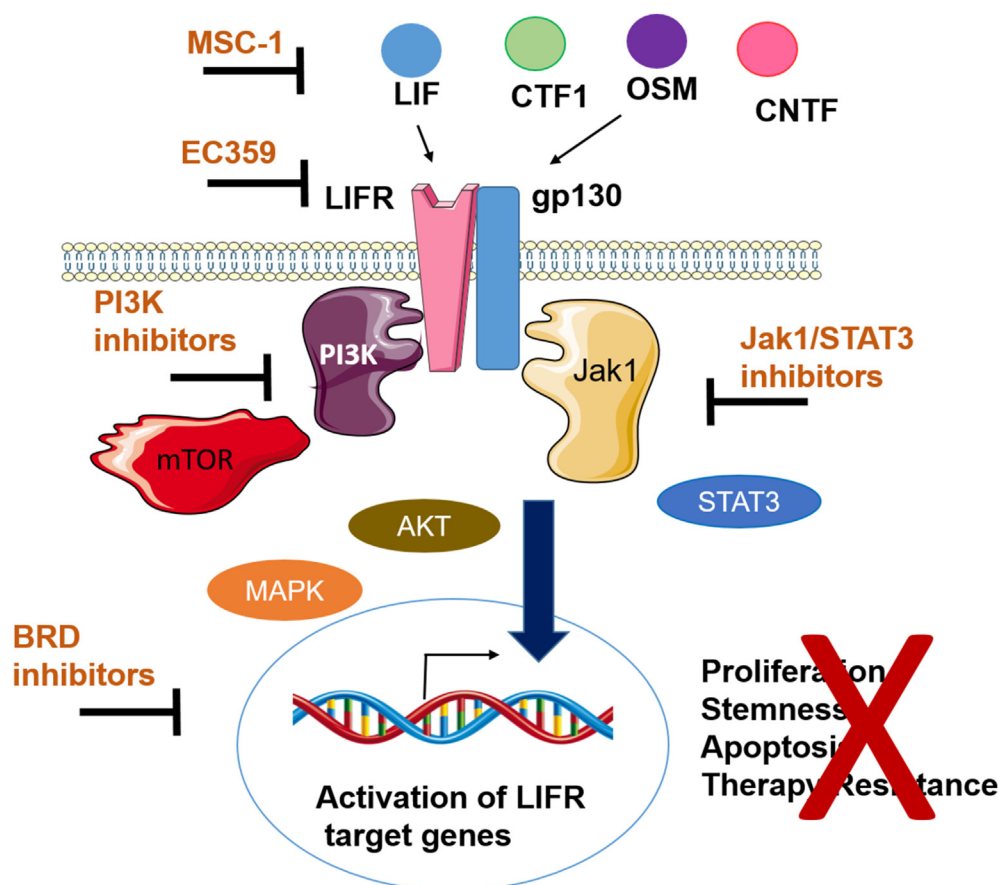


Figure 3 Schematic depicting the drugs that inhibit LIF/LIFR downstream signaling. Anti LIF antibody MSC-1 or LIFR inhibitor EC359 can be used to directly interfere LIF/LIFR signaling. Targeting LIF/LIFR signaling using inhibitors of LIFR activated pathways including Jak1/STAT3 inhibitors, PI3K inhibitors or BRD inhibitors may also be useful to interfere LIF/LIFR signaling.

based on human LIF–mouse LIFR complex (hLIF–mLIFR). This model suggested the presence of two cytokine-binding modules (CBM-1 and 2). The CBM-1 is constituted by domains D1 and D2, whereas the CBM-2 is constituted by domains D4 and D5. The D1 and D2 domains are connected by a short linker. The two CBMs are separated by an Ig-like domain, D3 is packed across the end of the D2 domain through loops originating from the end of D2 domain. D3 domain is connected to the D4 domain through loops. Similarly, D4 domain is also connected to D5 domain through loops. Although three-membrane proximal fibronectin type-III (FNIII) domains are present in the LIFR, they were not resolved in previously reported crystal structures (PDB IDs: 2q7n, 3e0g and 1pvh). Due to the high sequence identity between the mLIFR and hLIFR, it was no surprise that our modeled hLIFR structure also adopted the extended zigzag conformation seen in mLIFR (Fig. 2B and C). A saddle-shaped protein–protein interacting (PPI) interface is constituted by a four-helix bundle from LIF, and one sheet (D3) and loops (D4) from LIFR. Potential binding pockets are found on both the LIF and LIFR in the vicinity of the PPI interface (Fig. 2B, red circle). We surmise that small molecules that could interact with these binding pockets may interfere with LIF–LIFR interactions. Our previous molecular docking studies suggested that ligands such as EC359, EC330, EC357 and EC363 are able to

bind to the hLIFR at the PPI interface and block interactions with LIF (Fig. 2D–G).^{55,56} *In silico* binding energy calculations ranked the affinity of these compounds towards hLIFR in the order of EC359 > EC330 > EC357 > EC363. Similarly, our previous structure activity relationship studies suggested that the 11- β substitution in the ligands is the determining factor for the blockade of LIF–LIFR interactions. Ligands with a bulkier substitution displayed more steric clashes with LIF and exhibited higher inhibiting properties compared to those with a smaller substitution. Collectively, these modeling studies helped to design and develop potent small molecule inhibitors for targeting LIFR.

Therapeutic strategies targeting LIF/LIFR

Analyses of LIF KO mice have revealed that many of the LIF actions are not apparent during normal development,¹ indicating a potential therapeutic window for LIF/LIFR axis inhibitors in addition to less toxicity in normal adult tissues. A recent study demonstrated that LIF is a key paracrine factor from stromal cells acting on cancer cells; and LIF blockade or genetic LIFR deletion slows tumor progression, and augments the efficacy of chemotherapy to prolong survival of PDAC.¹⁵ It is suggested that LIF serves as

a target to limit neural remodeling in pancreatic cancer, which contributes to poorer quality of life with increased metastatic progression and LIF-blocking antibody reduced intratumoral nerve density.⁵⁷ Another study also confirmed that blockade of LIF by neutralizing antibodies represents an attractive approach to improving therapeutic outcome in KRAS driven pancreatic cancer.⁵⁸

Concomitantly, antibodies to LIF significantly suppressed growth of breast, kidney and prostate cancer cells suggesting that LIF acts as either a paracrine or an autocrine growth factor for these solid cancers.¹³ Considering the importance of the LIF/LIFR pathway, Northern Biologics/Celgene recently developed a humanized Anti-LIF antibody (MSC-1) that blocks LIF signaling, and its utility is being tested in a phase I clinical trial to determine its safety and tolerability (ClinicalTrials.gov, NCT03490669). A recent study examined the antitumor effect of immunization against LIF and LIFR. This study found that immune-mediated blockage of LIF and LIFR delayed/prevented breast tumor growth and immunotherapy of LIFR was a more efficient approach in the inhibition of tumor growth compared to immunotherapy of LIF.⁵⁹

Recently, several small molecules that preferentially target LIF/LIFR axis were identified including EC330, EC357, and EC363 (US patent 10,053,485) (Fig. 2A). Of these molecules, EC330 efficiently inhibited LIF/LIFR mediated proliferation and tumor growth and molecular docking studies suggested EC330 target LIFR.⁵⁵ Further optimization of EC330 to emulate the LIF binding site in LIFR and to improve PK properties resulted in the development of a first-in-class LIFR inhibitor (EC359) (Fig. 2A).⁵⁶ The specificity of EC359 to target LIFR and its activity was rigorously tested using biophysical assays (SPR, MST, modeling, biotin pull down), and CRISPR KO cells. EC359 also has the ability to block signaling mediated by other cytokines (CTF1, CNTF, and OSM) that interact LIFR at LIF/LIFR interface.⁵⁶ We speculate that the unique ability of EC359 to bind to common ligand binding site which blocks multiple ligands interaction with LIFR, offers an advantage over biologics or small molecules that can only target either of these ligands alone (Fig. 3). Modeling studies predicted that EC359 will interact at the LIF–LIFR binding interface and block the interaction of LIF to LIFR (Fig. 2). EC359 showed effectivity in pre-clinical xenograft and PDX models and has substantiated PK properties.⁵⁶ Utilizing pancreatic cell line-derived organoids, EC359 is also shown to be effective in targeting oncogenic LIF/LIFR signaling in pancreatic tumor stroma.⁶⁰ Since EC359 is a small, stable molecule; it is amenable for translation in cancer patients as monotherapy, or in combination with current standard of care.⁵⁶

HDAC inhibition upregulates LIFR expression, activates JAK1-STAT3 signaling and JAK1 or BRD4 inhibition sensitizes BCa to HDAC inhibitors, implicating combination inhibition of HDAC with JAK1 or BRD4 as potential therapies for BCa (Fig. 3).⁶¹ Recent study showed that LIF functions in parallel with IL-6 to promote OCa growth and that therapeutic targeting of a single cytokine axis may be less effective.³⁹ Therefore, combination therapy of LIF/LIFR inhibitors along with blockage of JAK/STAT pathway may be beneficial (Fig. 3).

Conclusion and future perspectives

In closing, LIF/LIFR signaling is implicated in the cancer progression and deregulation of LIF/LIFR axis occurs in many solid cancers. Preclinical studies using *in vitro* and *in vivo* models confirmed oncogenic functions of LIF/LIFR in tumor progression, stemness, therapy resistance, and alterations in tumor microenvironment. Humanized Anti-LIF antibody (MSC-1) therapeutics for targeting LIF are in Phase I clinical trials to test the efficacy of LIF antibody as a therapeutic in cancer (Fig. 3). However, LIFR can be activated by multiple ligands in addition to LIF, activating LIFR downstream pathways. Because cancer cells express multiple ligands that activate LIFR, it may be prudent to develop inhibitors that target common binding pocket for all the ligands in LIFR. EC359 is a rationally designed organic molecule that can emulate the LIF/LIFR binding site which functions as a first-in class LIFR inhibitor blocking its interactions with multiple ligands (Fig. 3). In preclinical studies EC359 showed effectivity; however, future studies are needed to better the understanding of LIF/LIFR signaling mechanisms in solid cancer cells using global approaches, validating efficacy of LIF/LIFR axis inhibitors in multiple solid tumor models and establishing LIF/LIFR signaling effects in modulating tumor micro environment.

Authors contribution

S.V; D.K.V; K.Y.J.Z; H.B.N; R.K.V: Conceptualization, Writing – Reviewing and Editing. All authors approved the final manuscript.

Funding

This work was supported by the DOD BCRP grant W81XWH-18-1-0016 (R.K.V); W81XWH-18-1-0015 (H.B.N); NCI R44CA235991 (H.B.N); NCI Cancer Center Support Grant P30CA054174-17; Elsa U. Pardee foundation grant 166675-44096 (S.V), NIH grant 1R01CA179120-01 (R.K.V).

Conflict of Interests

H.B.N is an employee of Evestra Inc. All other authors declare no conflicts of interest.

Acknowledgements

We thank Jessica Perry for proof reading the manuscript.

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LIFR inhibition enhances the therapeutic efficacy of HDAC inhibitors in triple negative breast cancer

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Histone deacetylase inhibitors (HDACi) are identified as novel therapeutic agents, however, recent clinical studies suggested that they are marginally effective in treating triple negative breast cancer (TNBC). Here, we show that first-in-class Leukemia Inhibitory Factor Receptor (LIFR α) inhibitor EC359 could enhance the therapeutic efficacy of HDACi against TNBC. We observed that both targeted knockdown of LIFR with CRISPR or treatment with EC359 enhanced the potency of four different HDACi in reducing cell viability, cell survival, and enhanced apoptosis compared to monotherapy in TNBC cells. RNA-seq studies demonstrated oncogenic/survival signaling pathways activated by HDACi were attenuated by the EC359 + HDACi therapy. Importantly, combination therapy potently inhibited the growth of TNBC patient derived explants, cell derived xenografts and patient-derived xenografts in vivo. Collectively, our results suggest that targeted inhibition of LIFR can enhance the therapeutic efficacy of HDACi in TNBC.

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Breast cancer (BC) is the most common cancer in women with an estimated 281,550 new cases and about 43,600 women are expected to die in 2021 from BC in the U.S. (American Cancer Society, Cancer Facts & Figures 2021). Among the different subtypes of BC, 15–24% are triple-negative breast cancer (TNBC)¹. TNBC is more aggressive, and due to limited targeted therapies, represents a disproportional share of the BC mortality^{2,3}. There is a critical need for rationally designed therapeutics that can improve response to TNBC treatment.

Epigenetic changes are implicated in the progression of many cancers including TNBC^{4–6}. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) determine the acetylation status of histones. HATs and HDACs affect gene expression; and inhibitors of HDACs (HDACi) cause growth arrest, differentiation, and/or apoptosis of many cancers⁷. The FDA has approved histone deacetylase inhibitors (HDACi), a class of small-molecular therapeutics as anticancer agents for various cancers including T-cell lymphoma and multiple myeloma. HDACi, such as vorinostat, romidepsin, and panobinostat are being tested for treating TNBC in clinical trials. Although HDACi monotherapy showed good promise in treating hematological malignancies, they are marginally effective in treating solid tumors such as TNBC^{5,8}.

Leukemia inhibitory factor receptor (LIFR, also referred as LIFR α , LIF Receptor Subunit Alpha, CD118)⁹ and its ligand LIF¹⁰, are widely expressed in many solid tumors including BC¹¹, and their overexpression is often associated with poor prognosis for patients. Recent studies suggested LIF–LIFR axis as a promising therapeutic target for cancer therapy^{12,13}. LIF binding to LIFR complex comprised of LIFR and glycoprotein 130 (gp130)⁹, triggers LIFR signaling to activate multiple signaling pathways including JAK, STAT, MAPK, AKT, and mTOR^{9,11,14}, all of which are implicated in TNBC progression. Recent studies have elucidated that HDACi treatment promotes expression and activation of LIFR, which restrains the utility of HDACi for BC treatment¹⁵. Targeting LIFR may serve as a unique approach to enhance the efficacy of HDACi for treatment of TNBC. We have rationally designed, synthesized and selected a lead small organic molecule, EC359 that can emulate the LIF–LIFR binding site and, therefore, function as a LIFR inhibitor¹⁶. We hypothesized that EC359 will have utility in enhancing the efficacy of HDACi by inhibiting LIFR oncogenic signaling.

Here, we examined the therapeutic utility of EC359 in improving the efficacy of HDACi in TNBC models. Our results showed that multiple HDACi that are currently in clinical trials induce expression of LIFR which compromise the efficacy of HDACi treatment. Utilizing LIFR knockout cell lines, we provided genetic evidence on the essential role of LIFR in HDACi-mediated aberrant activation of oncogenic/survival signaling pathways. Using *in vitro* and *in vivo* models, we demonstrated the synergistic effect of EC359 + HDACi combination therapy. Mechanistically, EC359 inhibited HDACi induced LIFR oncogenic signaling pathways.

Results

HDACi treatment induces LIFR expression. A recent study using whole-genome microarray discovered that HDACi (vorinostat) treatment induces expression of LIFR which reduces the efficacy of HDACi therapy¹⁵. We tested the generality of this finding by treating TNBC cells with four different HDACi (vorinostat, panobinostat, romidepsin, and givinostat) which are currently in clinical trials. Western blotting results using five different TNBC cells confirmed that treatment with all four HDACi indeed induced expression of LIFR (Fig. 1a and Supplementary Data Fig. 1a). RT-qPCR assays confirmed upregulation of LIFR mRNA expression (Fig. 1b). Further, HDACi

induced LIFR expression is functional as measured by its downstream activation of STAT3 phosphorylation (Fig. 1a and Supplementary Fig. 1a) and STAT3-Luc reporter activity (Fig. 1c). We next determined the expression of LIFR ligands in TNBC cells. Western blot analysis confirmed expression of LIF in TNBC model cells (Fig. 1d). Further, RT-qPCR assays showed that TNBC cells also express other LIFR ligands, specifically OSM and CNTF (Supplementary Fig. 1b). In addition, gp130 which functions as a co-receptor of LIFR is also expressed in TNBC cells (Supplementary Fig. 1b) and HDACi treatment did not alter its expression (Supplementary Fig. 1c). Collectively, these findings confirmed that HDACi aberrantly activates the expression of LIFR and abundant expression of LIFR ligands in TNBC cells may contribute to autocrine signaling via LIFR.

HDACi-mediated STAT3 activation requires the presence of LIFR. To confirm the direct role of LIFR in HDACi-mediated activation of STAT3, we knocked out (KO) the expression of LIFR using the CRISPR/Cas9 system and then treated cells with HDACi. LIFR-KO substantially reduced the induction of STAT3 phosphorylation mediated by HDACi (Fig. 1d). In MTT cell viability assays, LIFR-KO increased the potency of HDACi in reducing cell viability (Fig. 1e). Further, in colony formation assays, LIFR-KO enhanced the ability of HDACi to reduce the cell survival of TNBC cells (Fig. 1f). Collectively, these results suggest that HDACi-mediated STAT3 activation requires the presence of LIFR.

LIFR inhibitor EC359 synergizes with HDACi to decrease TNBC cell viability. We examined whether treatment with EC359 enhances the therapeutic potency of HDACi on TNBC cells using MTT cell viability assays. As shown in Fig. 2a and Supplementary Fig. 2a, b, addition of EC359 enhanced the potency of HDACi in reducing cell viability compared to HDACi as monotherapy. Results showed that the combination index (CI) values were <1 in all the three HDACi combinations tested and confirmed that the combination therapy was synergistic. We also compared the utility of STAT3 inhibitor (NSC 74859) in combination with HDACi. Results indicated that STAT3 inhibitor mediated reduction in cell viability of TNBC cells requires μ M concentrations and is not synergistic to HDACi (Supplementary Fig. 3a, b). On the contrary, EC359 at nM concentrations is synergistic to HDACi in reducing cell viability of TNBC cells.

EC359 enhances potency of HDACi in biological assays *in vitro*. We then evaluated the utility of EC359 + HDACi therapy using several biological assays. In clonogenic survival assays, EC359 enhanced HDACi ability to reduce the colony formation of MDA-MB-231 and BT-549 cells compared to monotherapy (Fig. 2b). Matrigel invasion assays demonstrated that combination therapy of EC359 + HDACi is more effective in reducing the invasion of MDA-MB-231 and BT-549 cells compared to monotherapy (Fig. 2c, d). We next examined the utility of EC359 + HDACi combination therapy in enhancing apoptosis using both Annexin V-PI and Caspase 3/7 assays. Results showed that EC359 increased HDACi ability to promote apoptosis in both assays (Fig. 2e). LIF-STAT3 axis is implicated in stem cell self-renewal and pluripotency¹⁷. Further, LIF signaling is implicated in the regulation of the transcription factors SOX2 and NANOG which play a critical role in stemness¹⁸. Analysis of TCGA databases revealed that high expression of LIFR and LIF correlates with the expression of mammary stemness gene set (Supplementary Fig. 4a). Treatment of CSCs with EC359 enhanced the ability of HDACi in reducing the cancer stem cells (CSCs) viability, and mammosphere formation (Supplementary

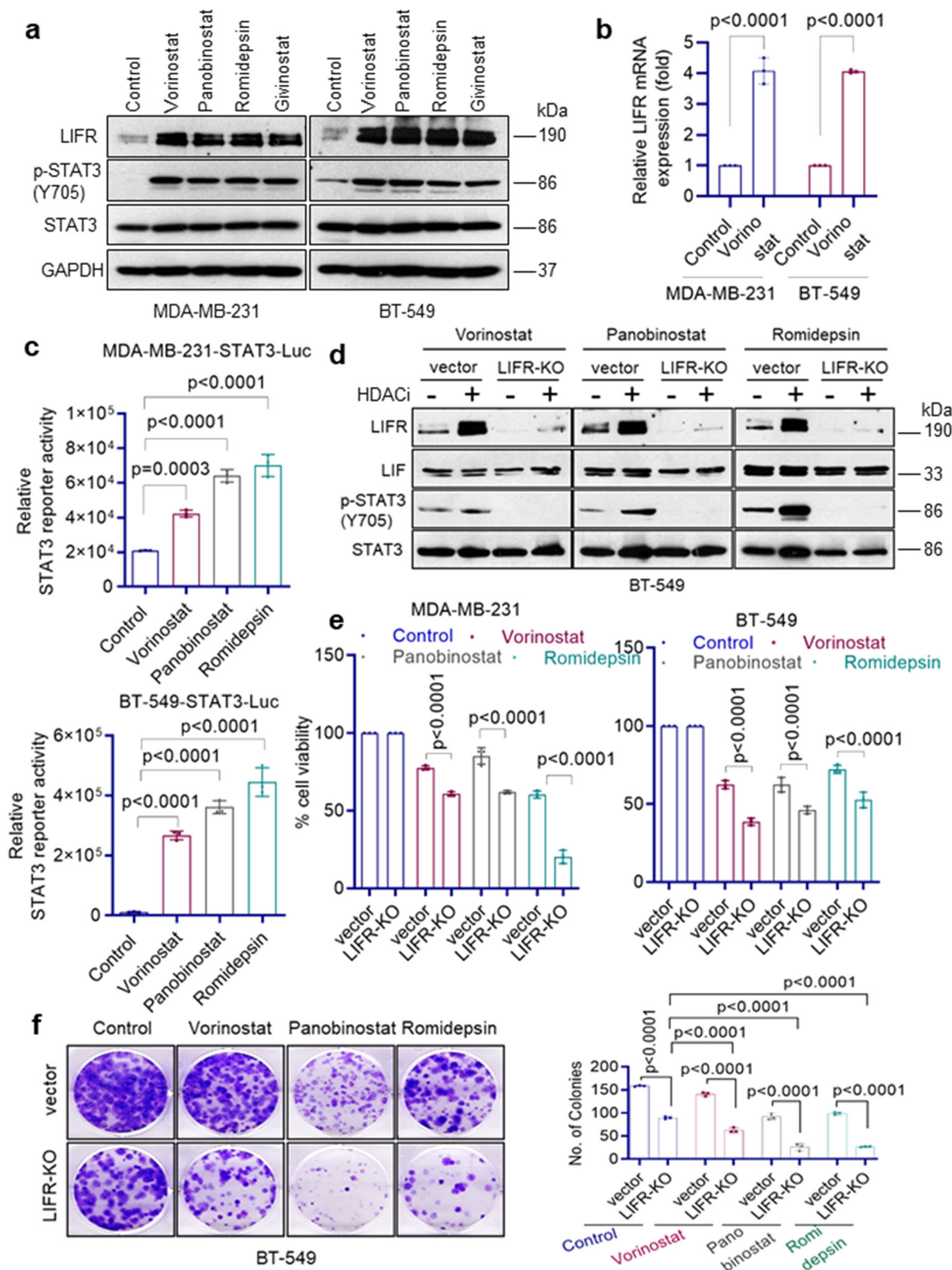


Fig. 4b-d). These results suggest that EC359 has potential to enhance the ability of HDACi to reduce cell survival, invasion, stemness, and enhance apoptosis of TNBC cells.

LIFR expression is upregulated in TNBC tissues. Recent studies showed that LIFR expression is upregulated during tumor

progression of many cancers and contributes to therapy resistance^{12,13}. Here, we examined whether LIFR expression is upregulated in BC using tissue microarrays that consist of various subtypes of BC and benign breast tissues. LIFR expression is higher in TNBC compared to benign, ER + and HER2 + BC (Fig. 3a). These results suggest that upregulation of LIFR expression occurs in TNBC.

Fig. 1 HDACi treatment-induced STAT3 activation via LIFR expression. **a** TNBC model cells (MDA-MB-231 and BT-549) were treated with indicated HDACi (vorinostat: 10 μ M; panobinostat: 1 μ M; romidepsin: 1 μ M; givinostat: 1 μ M) for 24 h and expression of LIFR, p-STAT3(Y705), and STAT3 were determined using Western blotting. **b** MDA-MB-231 and BT-549 cells were treated with vorinostat (10 μ M) for 10 h and levels of LIFR were measured by RT-qPCR. RT-qPCR data were normalized to GAPDH and data are representative of three independent experiments ($n = 3$). **c** MDA-MB-231 and BT-549 cells stably expressing STAT3-luc reporter were treated with indicated HDACi and reporter activity was measured after 24 h. Data are representative of three independent experiments ($n = 3$). **d** BT-549 vec or BT-549 LIFR-KO cells were treated with indicated HDACi (vorinostat: 10 μ M; panobinostat: 1 μ M, romidepsin: 1 μ M) for 10 h and induction of LIFR, LIF, and p-STAT3(Y705) was measured by Western blotting. The effect of LIFR-KO on the activity of HDACi was determined using MTT cell viability assay (**e**) and clonogenic survival assay (**f**). **e, f** Data are representative of three independent experiments ($n = 3$). Error bars represent SD. In **b, e**, and **f**, p -values were calculated using two-way ANOVA. In **c**, p -values were calculated using one-way ANOVA.

EC359 enhances HDACi efficacy in xenograft assays. To test the utility of EC359 + HDACi therapy in vivo, we conducted xenograft study using two different TNBC cells. MDA-MB-231 and MDA-MB-468 xenografts bearing SCID mice were randomized and treated with vehicle or EC359 (5 mg/kg/ip) 3 days/week or HDACi (vorinostat) (100 mg/kg/oral) 5 days/week alone or in combination. EC359 + HDACi combination treatment resulted in lower tumor volume and smaller tumor weight compared to monotherapy of EC359 or vorinostat (Fig. 3b, c). Mice treated with EC359 + HDACi combination exhibited no overt signs of toxicity. IHC analyses revealed that MDA-MB-468 xenografts treated with EC359 + HDACi combination showed decreased proliferation as measured by Ki67 compared to vehicle and monotherapy (Fig. 3d). IHC analyses also revealed that MDA-MB-468 xenografts treated with HDACi have increased STAT3 phosphorylation and treatment with EC359 + HDACi combination attenuated STAT3 phosphorylation (Supplementary Fig. 5). These results suggest that EC359 has potential to enhance the efficacy of HDACi to reduce TNBC xenograft tumor growth.

EC359 blocks HDACi-mediated LIFR downstream signaling pathways. We next conducted mechanistic studies using four different TNBC models and three HDACi including vorinostat, panobinostat, and romidepsin. Western blot analysis demonstrated activation of LIFR signaling pathway upon HDACi treatment as evidenced by increased expression of LIFR and activation of its downstream signaling pathways including STAT3 (Fig. 4a, b), AKT, and mTOR (Fig. 4b). Interestingly, pretreatment of EC359 resulted in blockage of LIFR activated downstream signaling in all three HDACi treatments (Fig. 4a, b). Importantly, in STAT3 reporter assays, as expected HDACi treatment stimulated the STAT3-Luc reporter, but pretreatment with EC359 abrogated HDACi induced activation of STAT3 (Fig. 4c, d). Collectively, these findings suggest that HDACi treatment induces expression of LIFR, which in turn contributes to the upregulation of LIFR downstream signaling, however, EC359 is efficacious in reducing HDACi-mediated aberrant LIFR signaling.

EC359 reduce HDACi-mediated activation of growth-promoting genes and enhance expression of pro-apoptotic genes. To determine the mechanisms by which EC359 enhanced the potency of HDAC inhibitors, we performed RNA-seq of BT-549 cells treated with vehicle, EC359, HDACi (Vorinostat), and combination (EC359 + HDACi). Comparison of differentially regulated genes (p -adj < 0.01, $|\log_2FC| > 1$) identified 6341 genes (Fig. 5a, b), which were then subdivided into 6 major clusters by unsupervised clustering (Fig. 5a, Supplementary Fig. 6a). Cluster 1 genes (1157) were synergistically upregulated, while Cluster 3 genes (1501) were synergistically repressed (Supplementary Fig. 6a). Cluster 2 genes (661) are upregulated by HDACi and somewhat suppressed by EC359; while Cluster 4 genes (266) are upregulated by EC359 and somewhat suppressed by HDACi. Cluster 6 genes (1457) are only affected by HDACi and showed

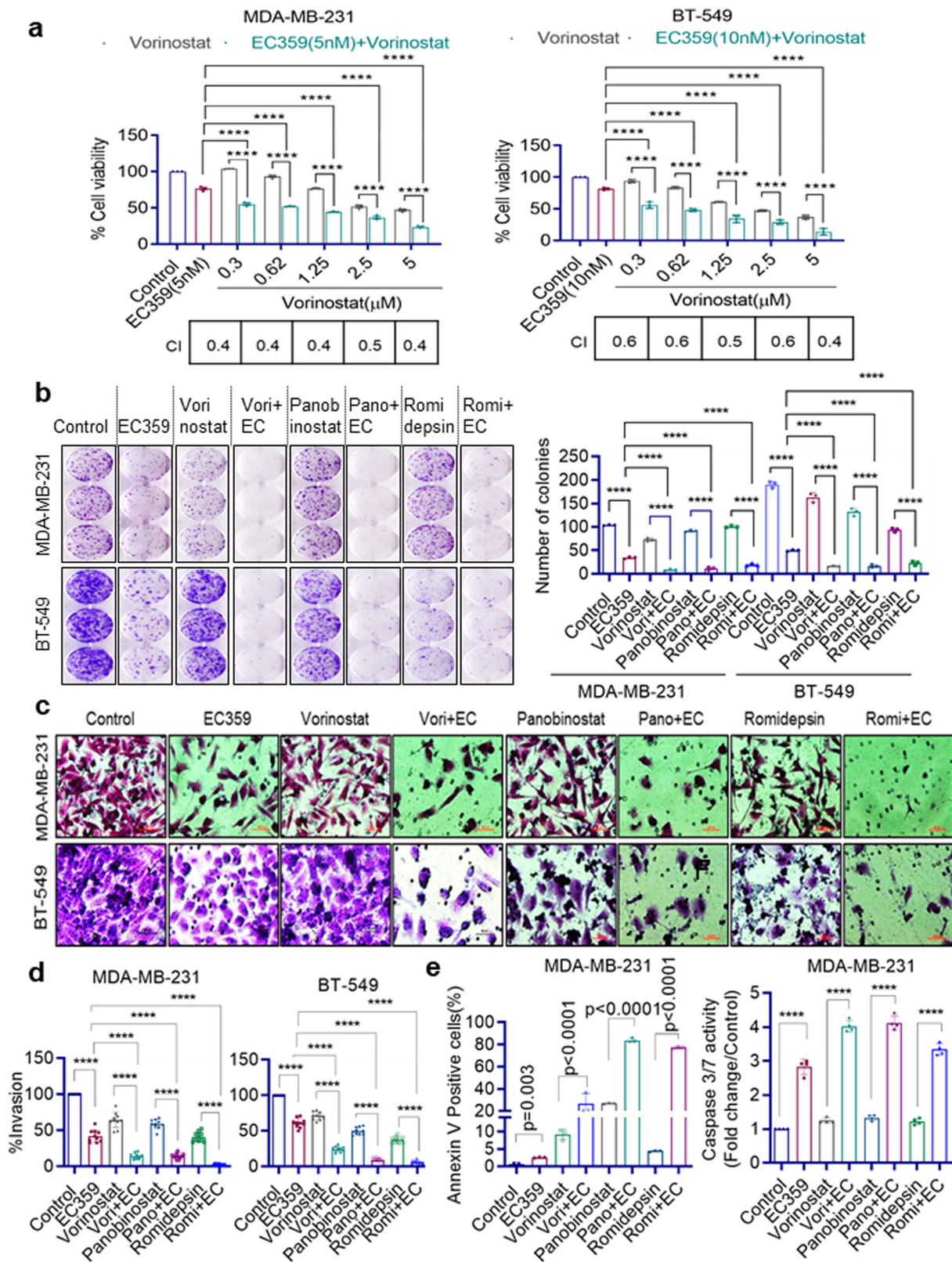
little or no effect with EC359 addition; Cluster 5 genes (1299) are repressed by HDACi and showed no effect with EC359 addition (Supplementary Fig. 6a).

Gene ontology (GO) analyses identified Cluster 1 genes were enriched for signaling pathways associated with the regulation of transcription, cell death, apoptosis, cellular response to stress, and cell differentiation (Fig. 5c); Cluster 3 genes were enriched for pathways of cell cycle, mitosis, cell division, and metabolic process (Fig. 5d). Accordingly, GSEA results showed enrichment of apoptosis and p53 pathways (Fig. 5e), and E2F signaling and Myc pathways (Fig. 5f) for Cluster 1 and Cluster 3 genes, respectively.

We then examined the status of STAT3 target genes using custom-generated STAT3 induced and repressed gene sets that were defined according to published RNA-seq data obtained from TNBC model cell lines¹⁹. GSEA of these genes revealed that EC359 treatment negatively enriched the STAT3 induced gene set but HDACi treatment showed a trend of positive enrichment of STAT3 induced gene set (Supplementary Fig. 5b). Interestingly, when we compared HDACi monotherapy to combination therapy, we observed a negative enrichment of the STAT3 induced gene set suggesting that the addition of EC359 reverted the HDACi-mediated upregulation of STAT3 activated genes (Supplementary Fig. 5b). On the other hand, both the EC359 and HDACi regulated genes showed a trend for positive enrichment of the STAT3 repressed gene set, however, when compared HDACi monotherapy with combination therapy we observed high positive enrichment of the STAT3 repressed gene set (Supplementary Fig. 5c). Altogether, these results suggest the synergistic activity of EC359 + HDACi combination therapy in part attributed to the ability of EC359 to repress HDACi-mediated aberrant activation of STAT3 target genes and further potentiates the HDACi-mediated upregulation of STAT3 repressed genes.

To validate the RNA-seq findings, we conducted RT-qPCR of selective genes in Cluster 1, 2, and 3. As observed in RNA-Seq, expression of genes involved in apoptosis (Cluster 1) was synergistically enhanced by EC359 + HDACi therapy compared to EC359 or HDACi treatment alone (Fig. 5g). Further, LIF target genes such as ID1, ID2, ID3²⁰ (genes identified in Cluster 2) are upregulated by HDACi treatment and are reversed by EC359 + HDACi combination therapy (Fig. 5h). Similarly, genes involved in cell cycle (Cluster 3) are further downregulated by EC359 + HDACi therapy compared to EC359 or HDACi treatment alone (Fig. 5i). Altogether, these results suggest that potent activity of EC359 + HDACi combination therapy attributed to the ability of EC359 to enhance HDACi-mediated apoptotic pathways and inhibit genes involved in cell cycle regulation.

EC359 enhances efficacy of HDACi therapy in PDX derived explant assays. We used ex vivo culture models of PDX tumors (PDX-derived explants, PDEs) to evaluate the efficacy of EC359 + HDACi treatment. PDEs maintain the native tissue architecture and better recapitulate the heterogeneity of TNBC in



a laboratory setting. We utilized three distinct TNBC PDX tumors and three HDACi (vorinostat, panobinostat, romidepsin) in this assay (Fig. 6a–c). The efficacy of EC359 + HDACi treatment on the proliferation was measured using Ki67 staining. Combination treatment of TNBC PDX tissues revealed that

EC359 enhanced ability of all three HDACi in reducing the proliferation (Ki67 positivity) compared to monotherapy treated tumors (Fig. 6b, c). These results suggest that combination of EC359 + HDACi is more effective in reducing proliferation of TNBC PDEs compared to monotherapy.

Fig. 2 EC359 synergistically enhanced HDACi ability to reduce cell viability, colony formation, invasion, and to induce apoptosis of TNBC cells. MDA-MB-231 and BT-549 cells were treated with indicated concentrations of vorinostat (**a**), for 72 h in the presence or absence of EC359 (MDA-MB-231: 5 nM; BT-549: 10 nM) and the cell viability was measured by MTT assay ($n = 3$). Combination Index (CI) values with respect to different concentrations were shown in the bottom of each graph. **b** Effect of EC359 + HDACi combination therapy on the cell survival of TNBC cells was measured using colony formation assays ($n = 3$). Representative images from three independent experiments are shown on the left panel, and quantitation of colonies is presented on the right panel. **c** Effect of EC359 + HDACi combination therapy on cell invasion of MDA-MB-231 and BT-549 cells was determined using matrigel invasion chamber assays. Data are representative of three independent experiments ($n = 3$). Representative images of invaded cells are shown and the number of invaded cells were quantitated (**d**). **e** Effect of EC359 + HDACi therapy on apoptosis was determined using Annexin V-PI staining and Caspase 3/7 activity (Caspase-Glo 3/7[®] assay) in MDA-MB-231 cells. Data are representative of three independent experiments ($n = 3$). Error bars represent SD. The combination index (CI) of EC359 + HDACi therapy was determined using Chou-Talalay method. p -values were calculated using two-way ANOVA. **** $p < 0.0001$.

EC359 enhances efficacy of HDACi therapy in PDX models.

We next evaluated *in vivo* efficacy of EC359 and HDACi combination using two TNBC-PDX models. Results showed that EC359 and HDACi (vorinostat) combination therapy is more efficient in reducing the PDX tumor growth compared to EC359 or HDACi monotherapy (Fig. 7a, b). Western blot analysis of tumor lysates showed that PDX mice treated with HDACi have increased expression of LIFR. Interestingly, EC359 and HDACi combination therapy substantially reduced the HDACi-mediated increase in LIFR expression (Fig. 7c). Further, EC359 + HDACi treated tumors showed less proliferation (Ki67 staining) compared to EC359 or HDACi monotherapy (Fig. 7d, e). Collectively, these results suggest that EC359 + HDACi combination therapy is highly effective in reducing the progression of TNBC PDX tumors *in vivo*.

Discussion

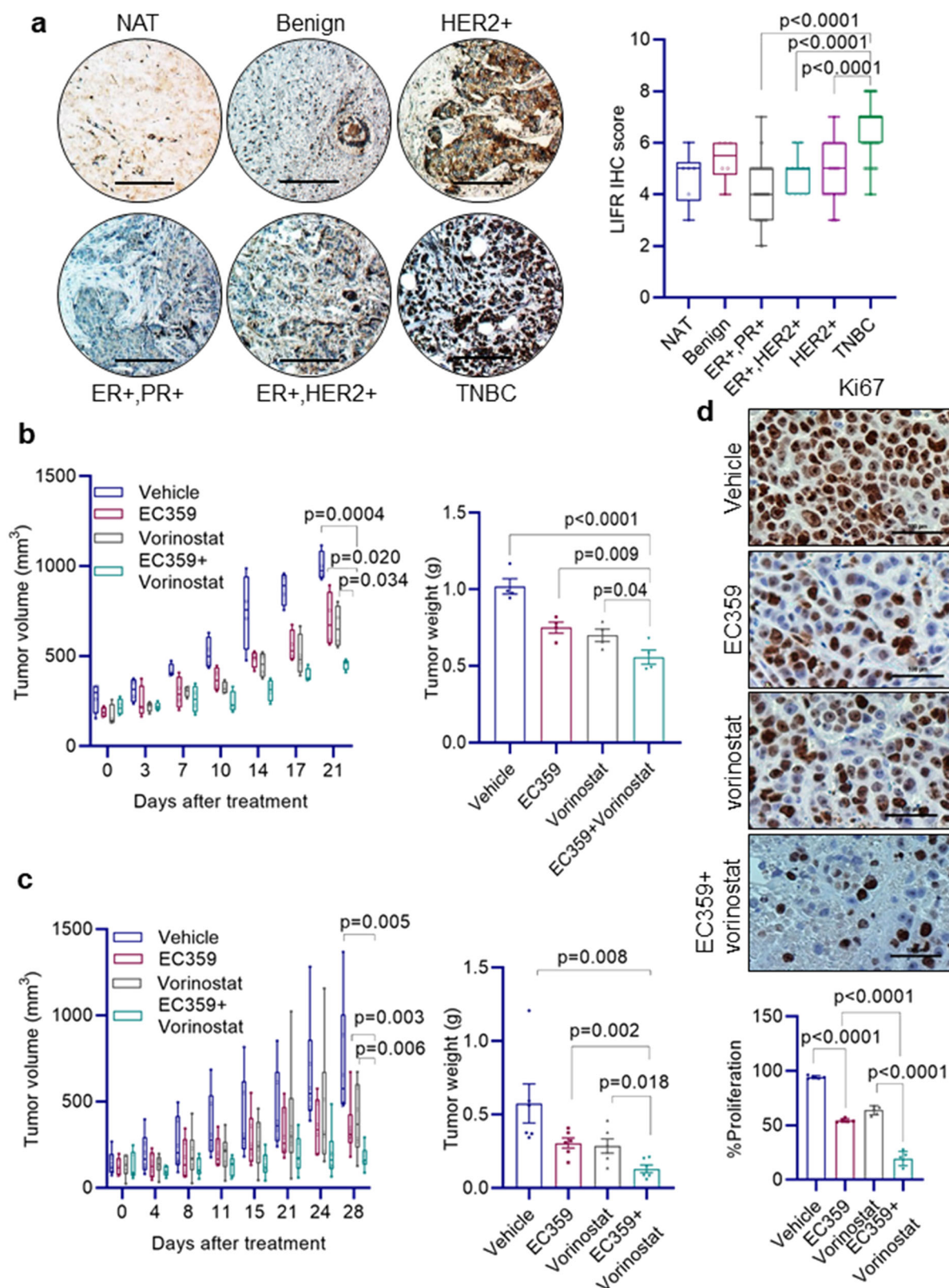
Epigenetic changes are implicated in the progression of many cancers including TNBC. The FDA has approved HDACi as a class of anticancer agents, for treatment of various cancers. While clinical trials with HDACi in TNBC are ongoing, recent studies suggest that HDACi could have limited efficacy in TNBC due to aberrant activation of LIFR signaling. Here, we tested the hypothesis that a first-in-class LIFR inhibitor, EC359, will have utility in enhancing the potency of HDACi by reducing LIFR oncogenic signaling. The utility of the combination therapy of EC359 with HDACi was tested using multiple TNBC cell lines, cell line-derived xenografts, patient-derived xenograft, and PDX-derived explants. Our findings indicate that EC359 was synergistic with HDACi in multiple TNBC models. Mechanistic studies using multiple TNBC models, CRISPR KO, and RNA-seq, models established the importance of blocking LIFR signaling to enhance the utility of HDACi for treating TNBC.

HDACi have shown promise in treating hematological malignancies. Several HDACi targeting class I, II, and IV HDACs are currently under development for use as anticancer agents⁸. However, their efficacy in treating solid cancers such as TNBC, as monotherapy is limited^{5,8}. Preclinical trials using HDACi such as panobinostat, vorinostat, and entinostat have shown that these epigenetic agents exert an anti-proliferative effect on TNBC cells^{4,5}. Further, studies showed that HDACi therapeutic outcomes against TNBC improved when they are used in combination with existing chemotherapies, kinase inhibitors, and autophagy inhibitors⁵. Vorinostat is a pan-HDACi that induces apoptosis in several types of hematological and solid tumor cells^{5,21}. A recent study showed that HDACi promote activation of LIFR that restrains the efficacy of HDACi in BC¹⁵ and concurrent inhibition of BRD4 or JAK sensitizes TNBC to HDACi¹⁵. Our study further extended these observations using four distinct HDACi (vorinostat, romidepsin, givinostat, and panobinostat) and provide evidence that HDACi treatment aberrantly activates LIFR signaling. Our earlier studies confirmed the specificity of

EC359 using MST, SPR, and LIFR KO TNBC models¹⁶. Further, EC359 has limited activity in ER⁺ BC cells, however, it showed potent activity in TNBC models suggesting EC359 effects may be subtype-specific and are independent of steroid receptors such as ER and PR. In this study, we provide evidence that HDACi-mediated activation of STAT3 was abolished in LIFR KO cells and that blocking LIFR signaling using a first-in-class LIFR inhibitor, EC359, enhances HDACi therapy.

The LIFR does not have any intrinsic tyrosine kinase activity, however, LIFR constitutively associates with the JAK-Tyk family of cytoplasmic tyrosine kinases and LIF binding to LIFR complex activates the JAK/STAT pathway⁹. Furthermore, the LIF/LIFR axis can activate multiple signaling pathways including STAT3, MAPK, AKT, and mTOR^{11,14} all of which are implicated in TNBC progression. Recent studies suggest the critical role of LIF/LIFR signaling in TNBC progression. TNBC exhibits autocrine stimulation of the LIF-LIFR axis, and overexpression of LIF is associated with poorer relapse-free survival in BC patients¹¹. Our results showed that TNBC cells express LIFR co-receptor gp130 and multiple LIFR ligands, thus has the potential to create autocrine/paracrine signaling loops. Accordingly, TNBC exhibited high endogenous STAT3 phosphorylation compared to ER⁺ BC¹⁶ and EC359 treatment reduced STAT3 phosphorylation in TNBC cells. LIF and LIFR expression occur more prominently in TNBC compared to ER⁺ BC¹⁶. Our results using breast tumor tissues are also in agreement with published studies that TNBC tumors have higher level of LIFR. Further, our mechanistic studies demonstrated that EC359 was effective in reducing the aberrant LIFR signaling induced by HDACi treatment in TNBC cells.

LIF/LIFR axis is implicated in tumor growth by modulating several pathways implicated in tumor progression²² including maintenance of stem cells^{17,18}, development of chemoresistance^{23,24} and invasion²⁵. Tumors upregulate LIF/LIFR signaling via autocrine and paracrine mechanisms^{24,26,27}. Several recent studies demonstrated that LIF blockade slow tumor progression, augment the efficacy of chemotherapy¹², and improves therapeutic outcome¹³. Another study using a soluble version of the LIFR as a ligand trap demonstrated that blocking of LIF signaling slows tumor progression in a mouse model of pancreatic cancer²⁸. Since TNBC cells express multiple LIFR ligands (LIF, OSM, and CNTF) and co-receptor gp130, aberrant induction of LIFR by HDACi has the potential to contribute to LIFR autocrine signaling loop. LIFR is also reported to function as a metastasis suppressor through the Hippo-YAP pathway²⁹ and confer a dormancy phenotype in breast cancer cells disseminating to bone³⁰. LIFR signaling is complex as multiple ligands activate LIFR including LIF, CNTF, OSM, and CTF1, and LIF/LIFR axis promote TNBC progression¹⁶. The differences in signaling outcome in different studies may in part arise from differential levels of activation of these pathways, multiple ligands to LIFR, and differences in tumor micro environment (TME)^{31,32}. Recently published studies indeed support oncogenic



role of LIF/LIFR signaling in cancer progression^{16,33,34}. Our results from RNA-seq studies also suggest that the beneficial effect of the EC359 + HDACi involves regulation of multiple genes that involved in several pathways including apoptosis, metabolism, and cell cycle.

Recent studies suggested LIF/LIFR axis as a promising clinical target for cancer therapy^{12,13}. Considering the importance of LIF/

LIFR pathway, Northern Biologics Company recently developed a humanized Anti-LIF antibody (MSC-1) that blocks LIF signaling, and its utility is being tested in phase I clinical trial mode to determine its safety and tolerability (ClinicalTrials.gov, NCT03490669). However, lack of small molecule inhibitors that block LIF/LIFR signaling represents a major knowledge gap. Further developing a small molecule inhibitor targeting LIF/LIFR

Fig. 3 LIFR is highly expressed in TNBC and EC359 + HDACi combination therapy reduced TNBC xenograft tumor growth. **a** Breast tissue microarray consisting of normal adjacent tissue (NAT, $n = 6$), benign ($n = 6$), ER⁺PR⁺ ($n = 50$), ER + HER2+ ($n = 11$), HER2+ ($n = 30$), and TNBC ($n = 61$) samples were subjected to immunohistochemical staining using LIFR antibody and the intensity and positivity of LIFR staining was quantitated. Scale bar represents 100 μ m. **b** MDA-MB-231 ($n = 4$) and **c** MDA-MB-468 ($n = 6$) xenografts in SCID mice were treated with vehicle or EC359 (5 mg/kg/day) or vorinostat (100 mg/kg/day) or in combination. Tumor volume and weight of vehicle and treated tumors were measured. **d** the proliferation of MDA-MB-468 xenograft tumors was determined using Ki67 immunostaining. Representative Ki67 staining from each treatment condition is shown in the upper panel and quantification plot is shown in the lower panel. In **a**, p -value was calculated using one-way ANOVA. Error bars represent SD. In **b**, **c**, and **d**, p -values were calculated using t test and two-way ANOVA.

is very cost effective. Recently, we developed a first-in-class inhibitor of LIFR, EC359. Our results using multiple *in vitro*, *ex vivo*, and *in vivo* TNBC models and PDX models demonstrated the utility of EC359 in enhancing the efficacy of HDACi for treating TNBC.

TNBC are very heterogeneous tumors and often exhibits autocrine stimulation of LIFR axis by alternating the expression of ligands. Endoplasmic reticulum stress and hypoxia are few of the hallmarks of TNBC³⁵. Hypoxia induces LIF expression in human cancer cells³⁶. LIF promotes proliferation and metastasis of BC cells, and overexpression of LIF is commonly associated with poorer relapse-free survival in BC patients¹¹. Further, increased expression of alternative LIFR ligands such as OSM, CNTF, and CTF1 was reported in TNBC. EC359 was shown to inhibit the LIFR activation by all LIFR ligands including LIF, OSM, CNTF, and CTF1¹⁶. Since LIFR activates multiple downstream signaling pathways including STAT3, AKT, and mTOR, blockage of STAT3 alone may not enhance HDACi efficacy. Similarly, use of BRD4 inhibitors alone may not increase the efficacy of HDACi as TNBC utilize multiple ways to activate LIF-LIFR signaling. In support of this, our results suggest that EC359 is highly effective in enhancing therapeutic utility of HDACi for treating TNBC.

In summation, our results using multiple TNBC models suggest that aberrant activation of LIFR occurred upon HDACi treatment and further demonstrated the utility of LIFR inhibitor EC359 in enhancing HDACi therapy. Since HDACi and LIFR inhibitors are currently in clinical development, the findings from this study suggest that a combination of the two agents may provide additional clinical benefit in the treatment of TNBC.

Methods

Cell culture and reagents. Human TNBC cells (MDA-MB-231, BT-549, MDA-MB-468, HCC1806, and HCC70) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as per ATCC guidelines. All the model cells utilized were free of mycoplasma contamination. STR DNA profiling was used to confirm cell identity. Antibodies for GAPDH, p-S6, S6, p-Akt (S473), Akt, p-mTOR (S2448), mTOR, p-STAT3 (Y705), and STAT3 were purchased from Cell Signaling Technology (Danvers, MA). LIF and LIFR (LIFR α , CD118) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). β -actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). The Ki67 antibody was purchased from Abcam (Cambridge, MA). LIFR knockout (KO) model cells and synthesis of EC359 were done using protocol in our earlier publication¹⁶. Vorinostat, panobinostat, romidepsin, and givinostat were purchased from MedChemExpress (Monmouth Junction, NJ).

Cell viability, colony formation, invasion, and apoptosis assays. The effects of vehicle (DMSO), EC359, and HDACi alone or in combination on cell viability were measured using the MTT cell viability assay¹⁶. HDACi and EC359 concentrations used in the assays were based on our earlier studies¹⁶, dose-response curves using TNBC models, and published studies¹⁵. The combination index (CI) of EC359 + HDACi therapy was determined using Chou-Talalay method³⁷. Apoptosis was measured using Annexin V-PI staining (BioLegend, San Diego, CA) and Caspase-Glo[®] 3/7 assays (Promega, Madison, WI) according to manufacturer's protocol. For colony formation assays, TNBC model cells (500 cells/well) were seeded in triplicate in 6-well plates, treated with indicated drugs for 5 days, and allowed to grow for 14 days. The cells were fixed in ice-cold methanol and stained with 0.5% crystal violet solution. Colonies that contain ≥ 50 cells were counted and used in the analysis. The effect of combination therapy on cell invasion was studied using the Corning[®] BioCoat[™] Growth Factor Reduced Matrigel[®] Invasion Chamber assay

(Corning, Corning, NY). MDA-MB-231 and BT-549 cells were treated with vehicle or EC359 or HDACi or combination for 22 h and invaded cells were determined and quantitated using the manufacturer's protocol.

Western blotting and RT-qPCR. Whole-cell lysates were prepared using RIPA buffer as previously described¹⁶. Total proteins were mixed with SDS sample buffer and subjected to SDS-PAGE. All primary antibodies for western blotting were done using 1:1000 dilution. Secondary antibodies for western blotting were diluted 1:1000 for anti-mouse antibodies and 1:2000 for anti-rabbit antibodies. Blots were developed using the ECL kit (Thermo Fisher Scientific, Waltham, MA). RT-qPCR was performed using SuperScript III First Strand kit (Invitrogen, Carlsbad, CA) and by using SYBR Green on an Illumina Real-Time PCR system. Primer sequences of the genes used were included in Supplementary Table 1.

Luciferase reporter assays. Generation of MDA-MB-231 and BT-549 cells stably expressing a STAT3-luciferase reporter was earlier described¹⁶. For reporter assays, cells were serum starved for 24 h, treated with EC359 or HDACi or combination therapy for 24 h, and reporter activity was measured. Cells were lysed in passive lysis buffer, and the reporter activity was measured using the dual-luciferase reporter assay system (Promega, Madison, WI).

CSCs cell viability and mammosphere formation assays. Cancer stem cells (CSCs) from BT-549 cells were sorted using the ALDEFLUOR kit (STEMCELL Technologies, Cambridge, MA) and flow cytometry. CSCs were cultured in MammoCult medium as per manufacturer's instructions. The effect of EC359 + HDACi combination therapy on the viability of CSCs was measured using Cell Titer-Glo assays (Promega, Madison, WI, USA). For CSC mammosphere assays, single-cell suspensions of CSCs were seeded in 24-well ultra-low attachment plates (100 cells/well) in triplicate and treated with vehicle or EC359 or vorinostat or EC359 + vorinostat for 7 days, and the newly formed spheres were counted.

RNA-seq and differential expression analysis. The effect of EC359 + HDACi combination therapy on global transcriptome was determined by RNA-sequencing using established protocol³⁸. Total RNA from BT-549 cells treated with vehicle, EC359, vorinostat, and EC359 + vorinostat was prepared using RNeasy mini kit (Qiagen, Valencia, CA). Illumina TruSeq RNA Sample preparation and sequencing were performed at Greehey Children's Cancer Research Institute Genome Sequencing Facility (UTHSA) using standard illumina HiSeq 3000 sequencing protocol. The raw reads were aligned to the reference human genome (UCSC hg19) with TopHat2³⁹. Genes were annotated (using NCBI RefSeq) and quantified by HTSeq⁴⁰, and DESeq⁴¹ was used to identify differentially expressed genes and genes with fold change >1 and multiple-test adjusted p value <0.01 were used for interpreting the biological pathways. Gene Set Enrichment Analysis was performed using stand-alone distribution (<http://www.gsea-msigdb.org/gsea/index.jsp>)⁴². STAT3 induced gene set and a STAT3 repressed gene set were custom-generated using published TNBC model RNA-seq data¹⁹. For GSEA, due to the size limitation of gene set (<500), we choose the top 500 STAT3 induced genes and 341 STAT3 repressed genes ($p < 0.01$, $\log_2FC < -0.5$). These 2 gene sets are close to our RNA-seq data (both cell lines are TNBC mesenchymal cells). RNA-seq data have been deposited in the GEO database under a GEO accession number GSE163249.

Animal studies. All animal experiments were performed using UTHSA IACUC approved protocol. For xenograft tumor assays, 2×10^6 MDA-MB-231 ($n = 4$) or MDA-MB-468 ($n = 6$) cells were mixed with an equal volume of matrigel and implanted in the mammary fat pads of 6-week-old female athymic nude mice¹⁶. Once tumors reached measurable size, mice were divided into control and treatment groups. Mice bearing TNBC PDX tumors were purchased from Jackson laboratory (TM00089; TM00096; TM00098) and TNBC PDX line UTPDX0001 establishment was earlier described¹⁶. When tumors reached ~ 750 mm³ they were dissected into 2 mm³ pieces and implanted into the flanks of 6-week-old female SCID mice. When the tumor volume reached ~ 150 mm³, mice were randomized for treatment. The control group received vehicle and the treatment groups received EC359, or vorinostat or EC359 + vorinostat. EC359 and vorinostat doses were chosen based on earlier published studies^{15,16}. The mice were monitored daily for adverse toxic effects and tumor volume was measured every 3–4 days using

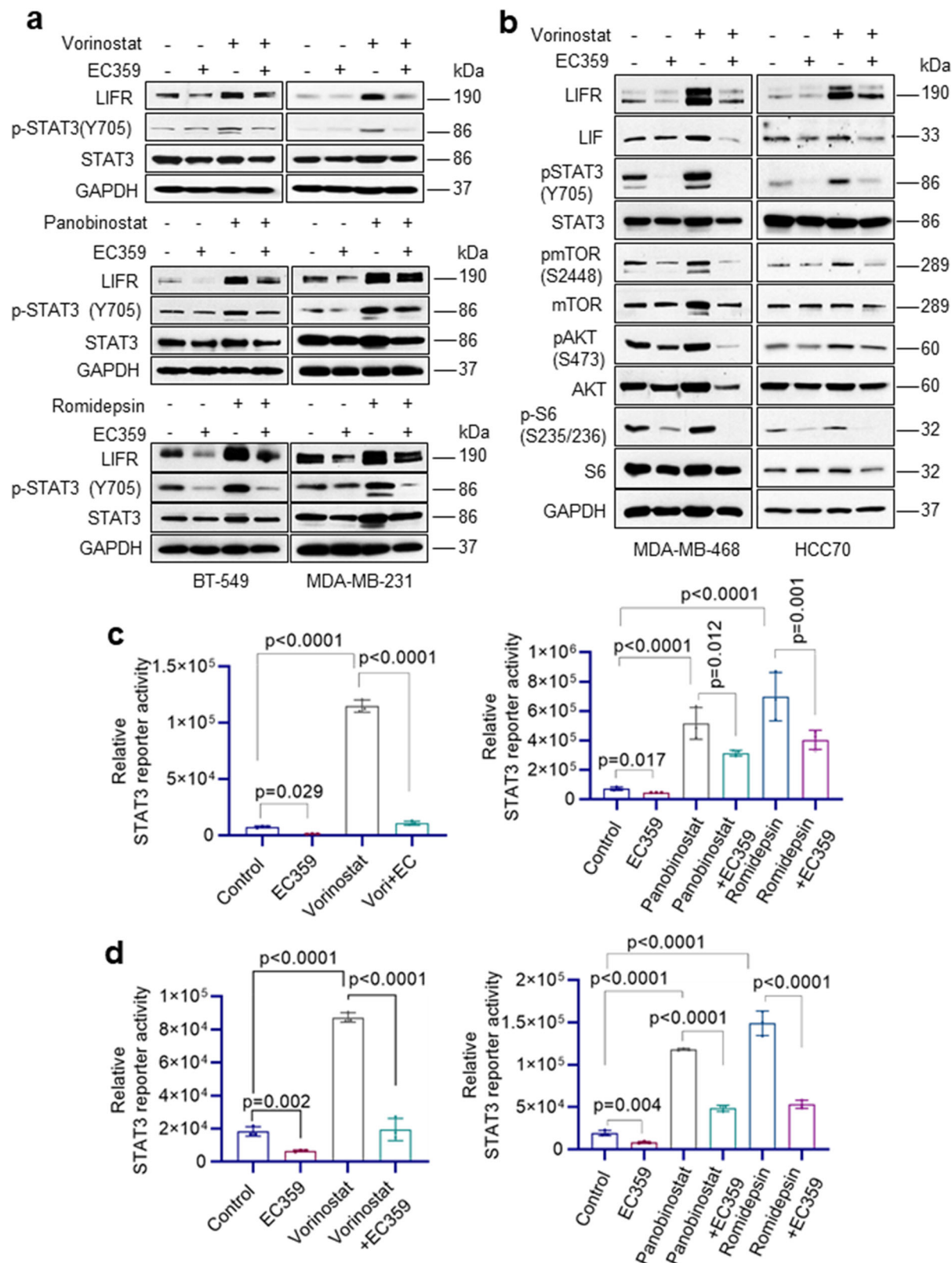


Fig. 4 EC359 inhibits HDACi induced LIFR signaling in TNBC cells. a BT-549 and MDA-MB-231 cells were treated with EC359 (100 nM) or HDACi (vorinostat (10 μ M) or panobinostat (500 nM) or romidepsin (100 nM)) alone or in combination for 10 h. Effect of EC359 on HDACi induced LIFR downstream signaling was measured using western blotting. **b** MDA-MB-468 and HCC70 cells were treated with EC359 (100 nM) or vorinostat (10 μ M) alone or in combination. Effect of EC359 on vorinostat-induced LIFR downstream signaling was measured using western blotting. Effect of EC359 on HDACi induced STAT3-Luc activity in BT-549 (**c**) and MDA-MB-231 (**d**) cells was determined using reporter assays. Data are representative of three independent experiments ($n = 3$). Error bars represent SD. In **c**, **d**, p -values were calculated using two-way ANOVA.

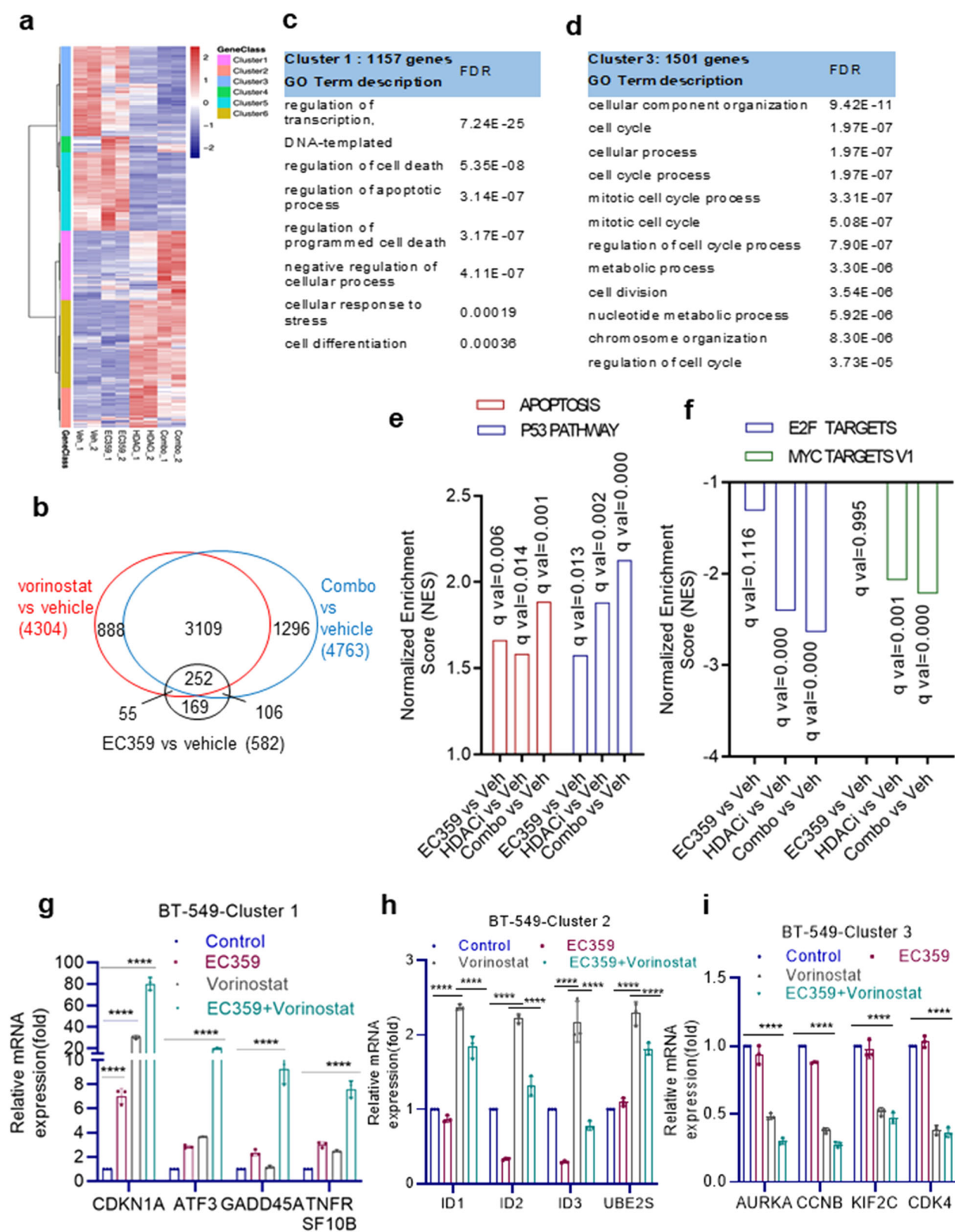


Fig. 5 Global transcriptomic analyses of EC359 + HDACi combination therapy in TNBC cells. BT-549 cells were treated with EC359, vorinostat, or EC359 + vorinostat for 24 h, and RNA-seq analysis was conducted. **a** Heat map depicts the clustering of all genes with RPKM > 1 ($p\text{-adj} < 0.01$, $|\log_2FC| > 1$). **b** Venn diagram comparing differentially regulated genes ($p\text{-adj} < 0.01$, $|\log_2FC| > 1$) identified from RNA-seq data. **c, d** representative pathways identified using GO term description in Cluster 1 and 3 are shown. **e, f** Top pathways identified in Cluster 1 and 3 using HALMARK GSEA are shown. **g–i** RT-qPCR validation of selective genes from Cluster 1, 2, and 3 is shown. Error bars represent SD. **** $P < 0.0001$.

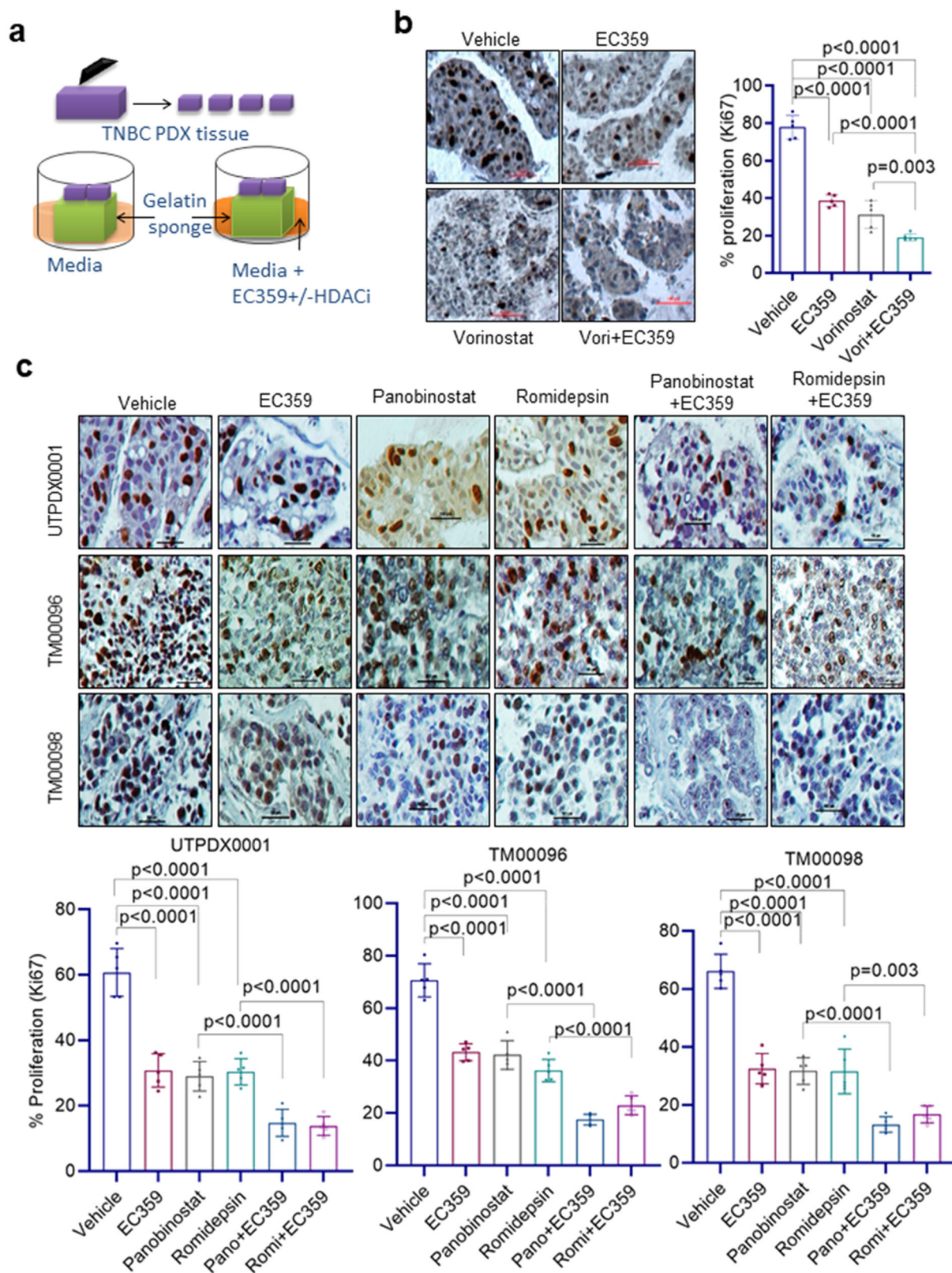


Fig. 6 EC359 enhance the ability of HDACi to decrease the proliferation of patient-derived xenograft explants (PDE) ex vivo. **a** Schematic representation of ex vivo culture model. **b** TNBC PDX explants (UTPDX0001) were treated with EC359 (500 nM), vorinostat (10 μ M), or combination for 72 h and the proliferation was determined using Ki67 immunostaining. Data are representative of three independent experiments ($n = 3$). **c** TNBC explants from three different PDX tumors (UTPDX0001, TM00096; TM00098) were treated with EC359 (500 nM), panobinostat (100 nM), romidepsin (50 nM) or combination for 72 h and the proliferation was determined using Ki67 immunostaining. Representative Ki67 staining from each treatment condition is shown. Data are representative of three independent experiments ($n = 3$). The number of Ki67-positive cells from five different images were counted and plotted as histogram. Error bars represent SD. In **b**, and **c**, p -values were calculated using one-way ANOVA.

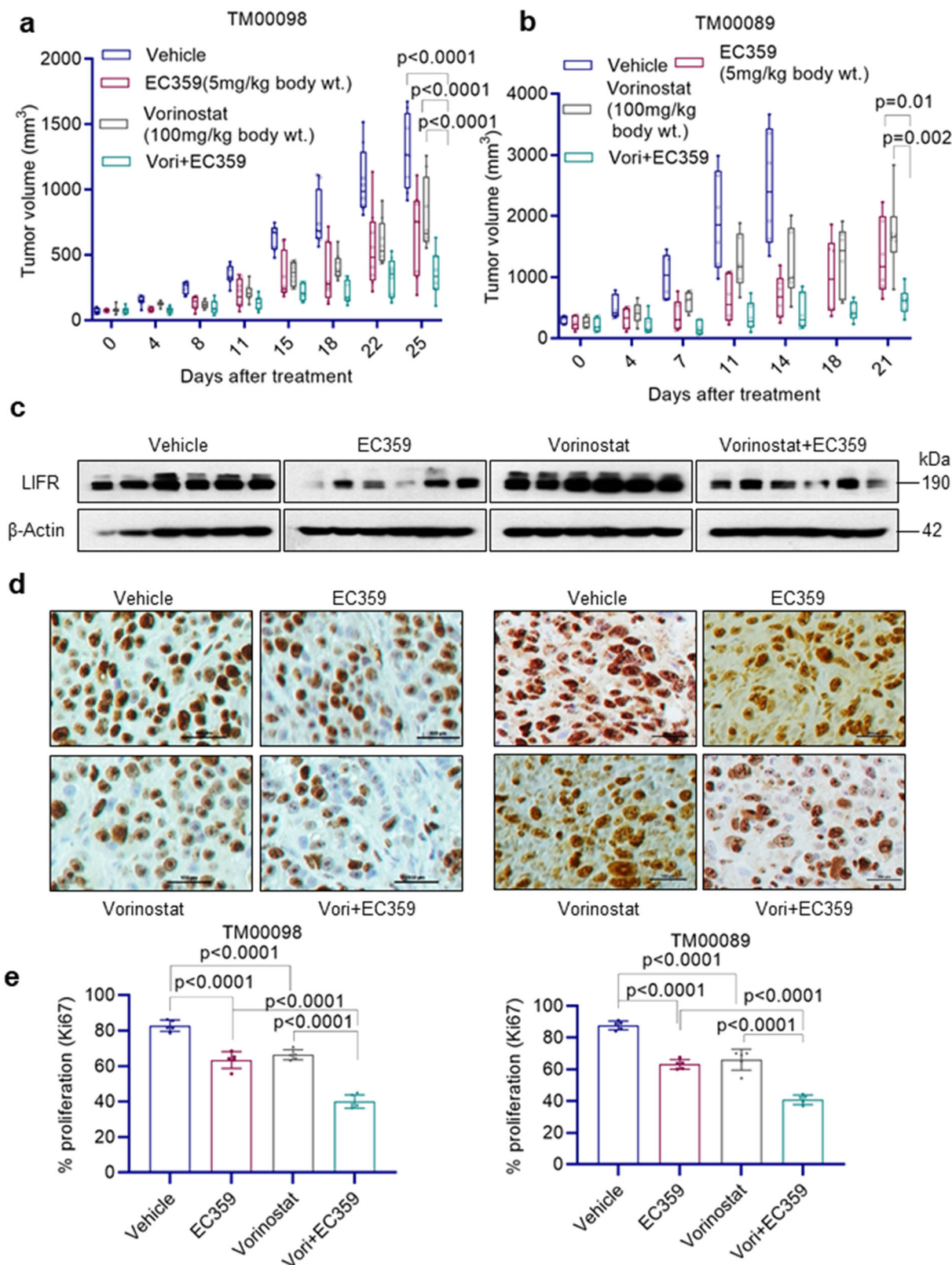


Fig. 7 EC359 enhanced HDACi ability to reduce tumor growth in PDX models of TNBC. **a, b** Female SCID mice were implanted with 2 mm³ pieces of PDX tumors TM00098 (**a**) TM00089 (**b**) into the flanks and randomized for treatment when the tumor volume reached ~150 mm³. TNBC PDX tumors ($n = 6-8$) were treated with vehicle or EC359 or vorinostat or combination. Tumor volumes are shown in the graph. Control mice in panel **b** were euthanized on day 14 because of higher tumor volume. **c** Total protein lysates from vehicle and treated TM00089 PDX tumors were analyzed for levels of LIFR using western blotting. **d, e** Ki67 expression as a marker of proliferation was analyzed by IHC and quantitated. Error bars represent SD. In **a**, p -values were calculated using two-way ANOVA. In **b**, p -values were calculated using multiple unpaired t test. In **e**, p -values were calculated using one-way ANOVA.

calipers. At the end of each experiment, the mice were euthanized, and the tumors were removed, weighed, and processed for histological studies and protein analysis. IHC analysis was performed using published protocol¹⁶. Primary antibodies were incubated overnight with Ki67 (1:100) or Phospho STAT3 (Y705) antibody (1:50) and subsequent secondary antibody incubation for 30 min at room temperature. Percentage of Ki67-positive proliferating cells was calculated in five randomly selected microscopic fields.

PDX-derived explant (PDE) studies. Excised PDX tumor samples (UTPDX0001; TM00096; TM00098) were processed, and cultured ex vivo as previously described^{43,44}. Briefly, PDX tumor samples were dissected into 2 mm³, and then incubated on gelatin sponges for 24 h in culture medium containing 10% FBS, followed by treatment with either vehicle or HDACi or EC359 or in combination for 72 h. Treated tumor tissues were then fixed in 10% formalin at 4 °C overnight and then processed into paraffin-embedded blocks.

Tissue microarray (TMA) and immunohistochemistry analysis. BC tissue microarrays (TMA cat# BR1503g and BR487c) were purchased from US Biomax, Inc. (Rockville, MD). TMAs were probed using LIFR antibody. IHC analysis was conducted⁴⁵ and immunoreactivity was visualized using DAB substrate and counterstained with hematoxylin (Vector Laboratories Inc., Burlingame, CA). Tissue arrays were scored using Allred Scoring system⁴⁶. Briefly, the LIFR staining intensity was scored on a scale between zero and three and the proportion of positive stained cells was rated as one between 0 and 1%, two between 1 and 10%, three between 10 and 33%, four between 33 and 66%, and five between 66 and 100%. Control rabbit IgG staining was used as a negative control. The sections were scored by two independent evaluators blinded to the patient's clinical status.

Statistics and reproducibility. Statistical differences between groups were analyzed with either a *t*-test or ANOVA as appropriate using GraphPad Prism 9 software. A value of *p* < 0.05 was considered as statistically significant. The combination index (CI) was calculated using the Chou-Talalay method³⁷. For animal studies, sample size of tumors/treatment was derived using effect information from previous studies and calculations were based on a model of unpaired data power = 0.8; *p* < 0.05. All in vitro assays were performed in biological replicates in technical triplicate.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Source data for the graphs and charts are provided in Supplementary Data 1. The RNA-seq data have been deposited in the GEO database (accession number GSE163249). Unedited western blot images are available in the Supplementary Information (Supplementary Figs. 7–17).

Received: 19 February 2021; Accepted: 1 October 2021;

Published online: 29 October 2021

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Acknowledgements

We thank Perry Jessica (Ob/Gyn UT Health San Antonio) for proofreading the manuscript. This study was supported by the DOD BCRP grant W81XWH-18-1-0016 (R.K.V.; K.J.N.); and DOD BCRP grant W81XWH-18-1-0021(Z.X.; R.K.V.); NCI Cancer Center Support Grant P30CA054174-17; NIH CTSA UL1TR002645; Voelcker fund young investigator award (G.R.S.) CPRIT Pre-doctoral Fellowship (K.A.A.), UT Foundation/ Shelby Tengg Foundation award 166120/44090 (S.V.), Elsa U. Pardee Foundation grant 166675-44096 (S.V.), NIH grant 1R01CA179120-01 (R.K.V.; M.R.), VA Grant I01BX004545 (R.K.V) and CPRIT RP160732 (Y.C.). GCCRI Genome Sequencing Facility is supported by NIH Shared Instrument grant 1S10OD021805-01.

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Competing interests

The authors declare the following competing interests: Authors including Bindu Santhamma, Swapna Konda, Gulzar Ahmed, Hareesh B. Nair, and Klaus J. Nickisch are current employees of Evestra Inc. All other remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s42003-021-02741-7>.

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Peer review information *Communications Biology* thanks Komaraiah Palle and the other, anonymous, reviewers for their contribution to the peer review of this work. Primary Handling Editor: Eve Rogers. Peer reviewer reports are available.

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