Supplementary Materials

EC330, a small-molecule compound, is a potential novel inhibitor of leukemia inhibitory factor signaling

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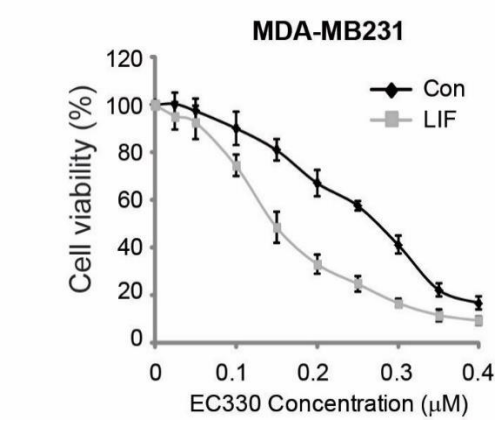
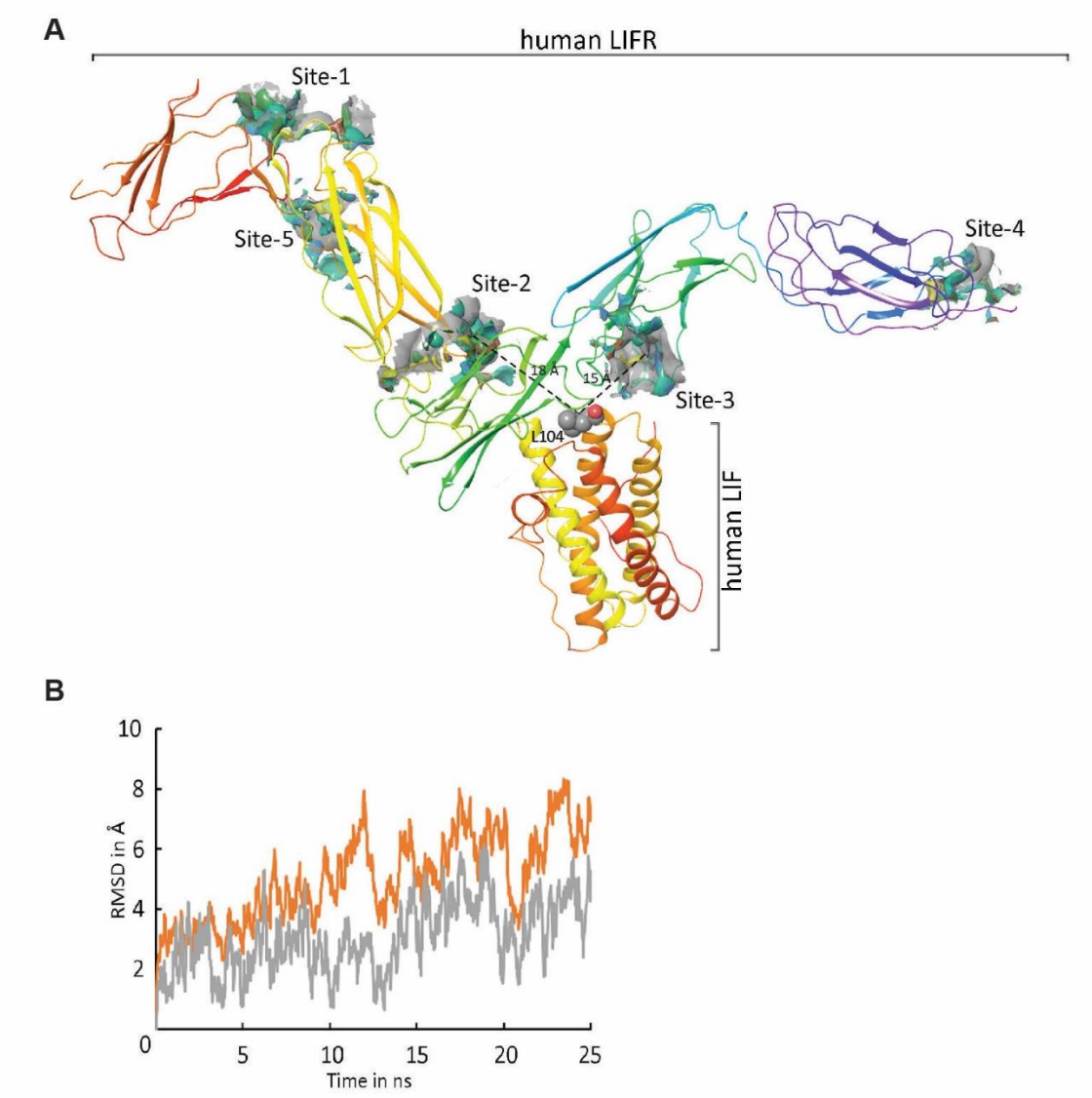
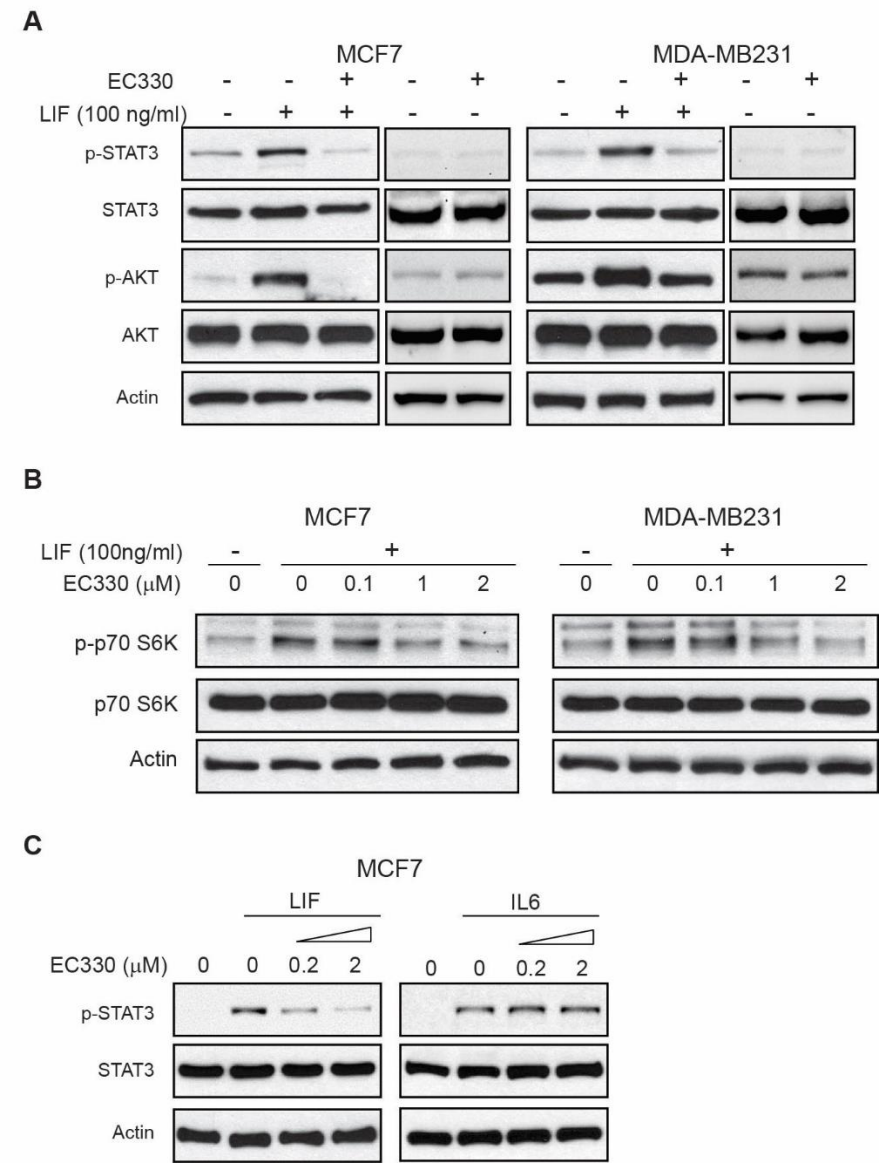
1. Supplementary Figures



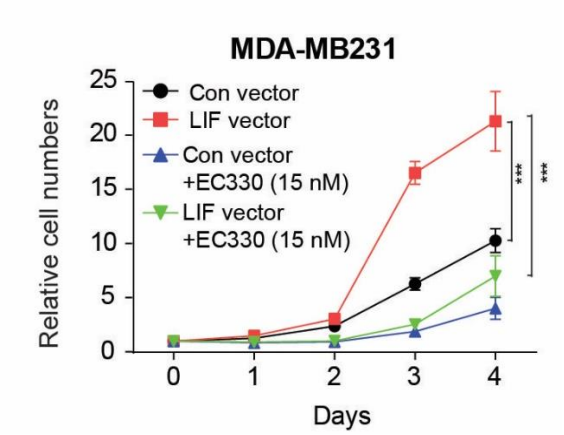
Supplementary Figure S1. Effect of EC330 on cell viability in MDA-MB231 cells with or without ectopic LIF expression. Cell viability was determined by Vi-CELL counter after cells were treated with EC330 at different concentrations for 24 hours. Data are presented as mean ± SD. n=3/group.



Supplementary Figure S2. Potential binding sites on hLIF-R identified through Sitemap and molecular dynamics simulation to determine the binding stability of the EC330 at the site 3 of hLIF-R. **A.** Five prominent binding sites on hLIF-R identified through sitemap program Schrödinger. The distances from L104 to sites 2 and 3 were represented in dashed lines. **B.** The RMSD of LIF-R protein (represented in orange color) and the ligand (represented in grey color).



Supplementary Figure S3. EC330 inhibits the activation of signaling pathways by LIF. **A.** Recombinant human LIF protein (100 ng/ml) increased the levels of p-STAT3 and p-AKT, which was abolished by EC330 (1 μM for 2 hours) in MCF7 and MDA-MB231 cells. **B.** Recombinant human LIF increased the levels of p-p70 S6K in MCF7 (left panels) and MDA-MB 231 (right panels) cells, which was abolished by EC330 (0.1-2 μM for 2 hours). **C.** EC330 inhibited the increase of p-STAT3 induced by LIF but not induced by IL6. MCF7 cells were treated with the recombinant LIF protein (left panels) or IL6 (right panels) together with or without EC330 (0.2-2 μM).



Supplementary Figure S4. EC330 preferentially inhibits the proliferation of MDA-MB231 cells with LIF overexpression. Ectopic LIF expression promoted the proliferation of MDA-MB231 cells, which was largely abolished by EC330 treatment (15 nM).

2. Supplementary materials and methods

2.1 Cell lines and Reagents

Human breast cancer cell lines MCF7 and MDA-MB231 were obtained from American Type Culture C1ollection (ATCC, Manassas, VA). Cell lines with ectopic LIF expression (MDA-MB231-LIF and MCF7-LIF) and control cell lines (MDA-MB231-Con and MCF7-Con) were established in our lab as previously described [1, 2]. All cells utilized were free of mycoplasma contamination confirmed by using a Mycoplasma PCR Detection Kit (Sigma, St. Louis, MO). Short tandem repeat polymorphism analysis (STR) of the cells was used to confirm the identity. Cytokine IL6 was purchased from GenScript. Recombinant human LIF protein was purchased from Millipore.

2.2 Cell viability and proliferation assays

The effect of EC330 on viability of cells with or without ectopic LIF expression was assessed in cells treated with different concentrations of EC330 for 24 hours. Cell viability was determined by the Vi-CELL counter that performs a trypan blue exclusion method. Cell proliferation was determined by counting cell numbers daily for four days using a Vi-Cell counter (Beckman).

2.3 Molecular modeling studies

The atomic level interactions of EC330 against human LIF-R (hLIF-R) were deduced by molecular modeling studies. The three-dimensional structure of human LIF (hLIF) –hLIF-R complex was constructed from hLIF - mouse LIF-R (mLIF-R) complex (PDB ID: 2Q7N) by replacing mLIF-R with hLIF-R (PDB ID: 3E0G) [3, 4]. The complex was further energy minimized to avoid residue clashes between the hLIF and hLIF-R. The putative ligand binding sites in hLIF-R were probed

using Sitemap from Schrödinger [5]. The ligand was docked on to the identified binding sites using Glide in SP docking and IFD modes. The best binding pose generated by SP docking was used for the IFD, which was performed by allowing flexibility to the surrounding amino acids (around 6 Å from the center of the ligand). Based on the MM-GBSA score [6] 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 ns.

2.4 Generation of human LIF-LIFR complex

The primary sequences of human (P42702) and mouse (P42703) LIFR share 75 % identity. Structures of hLIF-R (PDB ID 3E0G) and hLIF-mLIF-R (PDB ID 2Q7N) are available in the PDB. The superimposition of hLIF-R and hLIF-mLIF-R was done using SuperPose version 1.0 and the RMSD between the Cα atoms of hLIF-R and mLIF-R is 2.2 Å. After superimposition of hLIF-R to the hLIF-mLIF-R complex, the mLIF-R was removed and hLIF-hLIF-R complex was created. All the glycosylations were also removed from the structures to avoid clashes during the superimposition. The hLIF-LIF-R complex was further energy minimized to avoid the steric clashes between the residues at the interface.

2.5 Energy minimization of hLIF-R

The 'macromodel' module implemented in Schrödinger with a force field OPLS3 was used for the energy minimization of hLIF-LIF-R complex. The default parameters, Polak-Ribier Conjugate Gradient method with maximum iterations of 2500 was used for the energy minimization. Output structure (with an energy -95317 kcal/mol) after the energy minimization was further analyzed for any short contacts especially at the binding interface. It was assumed that the energy minimization

has produced the structural changes that are similar to the structural changes imparted by the protein-protein (ie. hLIF-LIF-R) interactions.

2.6 Protein and ligand preparation

The energy minimized structure of hLIF-R was extracted from the hLIF-LIF-R complex and prepared for molecular docking studies. The 'Protein preparation wizard' and 'Ligprep' modules of Schrödinger was used for the receptor and ligand preparations. During the protein preparation, the bond orders were assigned and disulfide bonds were treated properly. Hydrogen atoms were also added to the polar groups and the structure was optimized and minimized with a RMSD cutoff of 0.30 Å. The ligand EC330 was drawn using 2D sketch option and prepared for the docking studies. During the preparation, multiple states of the ligand were generated at a pH level 7.0±2.0. All of the generated states were used for the docking studies.

2.7 Identification of putative ligand binding sites, molecular docking and MM-GBSA calculations

The putative ligand binding sites on the hLIF-R was identified using Sitemap in Schrödinger. The binding site was defined by placing a 1 Å grid around the entire protein using a site-finding algorithm. The distances between each grid points to the nearby protein atoms were measured and compared to the van der Waals radius of each protein atom. Accordingly, 5 potential sites were identified and shown in **Supplementary Figure 2A**. Around these binding sites, grid was generated with a diameter 20 Å and molecular docking has been carried out. Using SP docking, the binding mode of EC330 towards each binding sites were deduced with default parameters and OPLS3 force field. After molecular docking, the protein ligand complex was introduced to the

hLIF protein and investigated for the possible steric clashes between the EC330 and hLIF. The distance to site-2 (18 Å) and site-3 (15 Å) were measured from a randomly selected residue 'L104' of LIF and found that these sites are close to the LIF binding regions. However, binding mode of EC330 towards any binding sites except site-3 has not made any steric clashes with hLIF. The docking scores of EC330 towards the site-3 ranges from -1.54 to -2.56 kcal/mol. One of the best scored poses was selected for IFD calculations. The protein residues that are 6 Å away from the centroid of the ligand were made flexible during the docking. As a first step of IFD, the Glide docking of the ligand was carried out using a softened potential. By default, 20 poses per ligand was generated. Further side-chain prediction followed by minimization for each protein-ligand complex were performed. A total of 20 poses were generated as a part of IFD calculations and all poses were subjected to MM-GBSA calculations. All of the MM-GBSA calculations were performed using VSGB solvation model with OPLS3 force field.

2.8 Molecular dynamics simulation

The binding stability of the EC330 at the site-3 was investigated using molecular dynamics (MD) simulations. The best binding pose (hLIF-R-EC330 complex) selected from the IFD studies was prepared by soaking into an orthorhombic box (volume 1156533 $Å^3$) consisting 34898 TIP3P water molecules and 3 Na+ ions. The force field used, total time for the simulations were OPLS2005 and 25 ns respectively. Intermediate structures (snapshots) saved at each 25 ps were superimposed to the original structure (reference structure) and deduced the RMSD of ligand and protein with respect to the reference frame. The RMSD of ligands and proteins were deduced and finally plotted against time. The average RMSD for protein was found to be 5.01 ± 1.4 Å at the same time the average RMSD found for the ligand is 3.0 ± 1.2 Å (**Supplementary Figure 2B**).

Further investigations on the snapshots revealed that the large RMSD of the protein is due to the deviations in the loops that connect different domains (D1-D5). Even though the ligand exhibited a RMSD close to 3.0 Å, the hydrogen bond observed in the molecular docking was found to be stable throughout the simulations.

2.9 Western blot and Avidin-biotin pull-down assays

The cell extracts were prepared and western blot assays were performed as previously described [7]. The following antibodies were used: anti-p-STAT3 (Cell Signaling Technology), anti-STAT3 (Santa Cruz), anti-p-AKT (Cell Signaling Technology), anti-AKT (Santa Cruz), anti-p-p70S6 kinase (Cell Signaling Technology), anti-p70S6K (Cell Signaling Technology), anti-LIF (R&D), anti-LIF-R (Santa Cruz) and anti β-actin (Sigma) antibodies. Avidin-biotin pull-down assays were performed as described previously [8]. In brief, cellular lysates of MCF7-LIF cells were incubated with Avidin-Biotin-EC330 or Control-Avidin beads for 2 hours followed with pull down using Avidin beads (NanoLink Streptavidin Magnetic Beads; Solulink). The interaction of LIFR with Avidin-Biotin-EC330 was analyzed by Western blot assays.

2.10 Migration Assays

The trans-well system (24 well, 8μM pore size, BD Biosciences) was used to measure cell migration ability as previously described [7, 9]. The cells diluted by medium without FBS were seeded on the upper chambers. The lower chambers were filled with medium containing 10% FBS. Cells on the lower chamber surface were stained at 24 hours after seeding and were counted by image J software.

2.11 *In Vivo* xenograft tumorigenesis assays

All animal experiments were performed with the approval of IACUC of Rutgers State University of New Jersey. Mice were housed according to national and institutional guidelines for animal care. For subcutaneous (*s.c.*) xenograft tumor assays, cancer cells were injected (*s.c.*) to 6-week-old BALB/c nude mice to form xenograft tumors. When tumors reached the size of ~30 mm^3, mice were treated with EC330 (1mg/kg) or PBS (vehicle) *via* intraperitoneal (*i.p.*) injection 5 times/week. n≥6/group. All mice were monitored daily for adverse toxic effects. The tumor was measured with a caliper every 3 days, and the tumor volume was calculated as following: 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 experiments, mice were euthanized and tumors were excised, weighed and processed for histological studies.

2.12 Statistical analysis

Statistical differences between groups were analyzed with either Student's *t*-test or Two-way ANOVA as appropriate using GraphPad Prism 6 software (GraphPad Software, SanDiego, CA). All data are presented as mean ± SD. A value of $p< 0.05$ was considered to be statistically significant.

3. Supplementary references

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