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Virtual High-Throughput Screening To Identify Novel Activin Antagonists

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Abstract

Activin belongs to the TGF β superfamily, which is associated with several disease conditions, including cancer-related cachexia, preterm labor with delivery, and osteoporosis. Targeting activin and its related signaling pathways holds promise as a therapeutic approach to these diseases. A small-molecule ligand-binding groove was identified in the interface between the two activin β A subunits and was used for a virtual high-throughput in silico screening of the ZINC database to identify hits. Thirty-nine compounds without significant toxicity were tested in two well-established activin assays: FSH β transcription and HepG2 cell apoptosis. This screening workflow resulted in two lead compounds: NUCC-474 and NUCC-555. These potential activin antagonists were then shown to inhibit activin A-mediated cell proliferation in ex vivo ovary cultures. In vivo testing showed that our most potent compound (NUCC-555) caused a dose-dependent decrease in FSH levels in ovariectomized mice. The Blitz competition binding assay confirmed target binding

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ASSOCIATED CONTENT

Supporting Information

Purity data, solubility data, and toxicity scores of the 39 in silico hits in Supplemental Table 1. Two NUCC-555 analogs toxicity and bioactivity in luciferase assay in Supplemental Table 2. Metabolic stability of NUCC-555 in Supplemental Table 3. Dose-response data of the five functional compounds tested in -338FSH β -Luc L β T2 cells and four functional compounds tested in human liver HepG2 cell line in Supplemental Figure 1. The interaction between activin A and ALK4-ECD-Fc or between activin A/ActRIIA-ECD complex and ALK4-ECD-Fc using the Blitz system in Supplemental Figure 2. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.5b00753.

Author Contributions

J.Z. performed all the biological experiments and data analysis. R.K.M. designed and performed the vHTS. All authors contributed to the experimental design and manuscript writing. All the authors have given approval to the final version of the manuscript.

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of NUCC-555 to the activin A:ActRII that disrupts the activin A:ActRII complex's binding with ALK4-ECD-Fc in a dose-dependent manner. The NUCC-555 also specifically binds to activin A compared with other TGF β superfamily member myostatin (GDF8). These data demonstrate a new in silico-based strategy for identifying small-molecule activin antagonists. Our approach is the first to identify a first-in-class small-molecule antagonist of activin binding to ALK4, which opens a completely new approach to inhibiting the activity of TGF β receptor superfamily members. In addition, the lead compound can serve as a starting point for lead optimization toward the goal of a compound that may be effective in activin-mediated diseases.

INTRODUCTION

Activin belongs to the TGF β superfamily and was first identified as the peptide hormone that stimulates follicle-stimulating hormone (FSH) in the male and female pituitary gland, driving pubertal transition and adult fertility.^{1–4} Activin initiates signal transduction through binding to one of two cell surface type II receptors, RIIA or RIIB. Upon ligand binding, these type II receptors phosphorylate the activin type IB receptor, known as activin-receptor-like kinase 4 (ALK4), the SMADS, which then dissociate from the receptor complex and translocate to the nucleus, where they control cell-specific functions.^{5–8}

In addition to its well-known role in controlling reproductive function, activin is also associated with several disease conditions, including cancer-related cachexia, preterm labor with delivery, and osteoporosis. In late-stage murine cancer models, high circulating activin levels cause apoptosis around the central vein of the liver and the loss of stem cells that line the stomach and intestine, causing the wasting phenotype known as cachexia.^{9–11} In animal models, inhibition of activin using the binding protein follistatin or a soluble RII receptor reverses these adverse effects, even as tumors continue to grow.^{12–16} In humans, increased circulating activin A is observed in cancer patients,^{17–19} and cancer cachexia is associated with an increase in activin A.¹⁹ Activin is also elevated at the end of normal gestation, reaching a peak just prior to, or during labor, in the third trimester. Activin A levels are supraphysiologic in women with idiopathic preterm labor and delivery,^{20,21} and it is predicted that blocking activin A may be a novel approach to prevent preterm labor. Finally, the activin/inhibin/follistatin system has also been shown to regulate bone homeostasis and age-related bone loss.^{22–24} In animal models and a phase I clinical trial, a soluble activin-binding ActRIIA-Fc (either ACE-011 or RAP-011) fusion protein was shown to have an anabolic effect on bone density.^{25,26} Thus, targeting activin may be therapeutic for three

significant human health conditions: cancer-related cachexia, idiopathic preterm labor, and age-related bone loss.

The soluble activin type IIB receptor blocks activin signaling in clinical studies and reverses muscle wasting in cancer cachexia and benefits bone formation;²⁷ however, off-target side effects have limited the clinical potential of activin receptor-based therapeutics to date. Although decoy activin II receptors increase lean body mass and bone mineral density,^{28,29} the bleeding associated with this agent appears to limit its usefulness.³⁰ This lack of translational success is due to lack of selectivity, as the receptor binds to many other TGF β superfamily ligands, including bone morphogenic proteins (BMPs).⁸ Similarly, ALK4 receptor antagonists such as SB-435142 and SB-505124 block activin signaling, but they also interfere with the closely related TGF β superfamily receptors ALK5 and ALK7.^{31,32} Modified activin β A-subunit propeptides bind activin A specifically and achieve more selective blockade of activin signaling; they are still in the experimental stages of development.³³ Two naturally occurring activin antagonists exist, inhibin and follistatin.^{2,34–40} Inhibin has a short half-life,^{41,42} and follistatin is a large macromolecule bionutralizing binding protein not amenable to drug development. Thus, there are no activin antagonists with sufficiently high specificity for clinical use in treating activin-mediated pathologies.

The activin A crystal structure has been used to identify binding pockets that are predicted to specifically disrupt ligand/ receptor interaction and activin signaling.^{43–47} In this study, we performed an in silico screen based on the activin and follistatin-288 complex crystal structure to identify potential small-molecule activin antagonists. We then tested the antagonistic activity of our initial hit compounds using in vitro, ex vivo, and in vivo assays. Our findings lay the foundation for future work aimed at optimizing these lead compounds as potential small-molecule therapeutics to target cancer-related cachexia and other activin-mediated diseases.

RESULTS

Small-Molecule Binding Site Selection and Protein Preparation for In Silico Studies

We initially used the coordinates of the dimer crystal structure of the activin β A-subunit (2ARV.pdb) at 2.0 Å resolution obtained from the protein crystal structure database (<http://www.rcsb.org/pdb/>) to develop our model for in silico screening. Other crystal structures of the activin A dimer (2 β A-subunits) are available, in complex with either the activin type II receptor or the activin binding protein follistatin.^{44,45} Because the activin β A-subunit structure, particularly the “fingertips”, is altered when the ligand is bound to either the activin receptor or follistatin, we initially chose to use the unbound activin β A-subunit structure (2ARV.pdb) for molecular modeling to screen in silico small-molecule libraries; however, we found that 10 critical residues at the junction of the β A-subunits (residues 47–56) were not resolved in this particular crystal structure. Thus, to consider all the critical residues at the junction, we extracted the activin A dimer structure from various complex crystal structures (activin A in complex with follistatin, 2B0U.pdb; ActRIIB, 1NYS.pdb; and TGF- β 2, 2TGI.pdb) and compared it with the activin β A-subunit structure (2ARV.pdb). The 1NYS.pdb crystal structure having a resolution of 3.05 Å has activin units bound to

ActRIIB. We extracted the activin part and compared it with the unbound activin A structure (2ARV.pdb). The root-mean-square deviation (RMSD) between these two activin structures was found to be 1.7 Å. The 2B0U.pdb crystal structure having a 2.8 Å resolution is a complex structure of activin and follistatin. From this complex, we extracted the activin part and compared it with the unbound β A-subunits structure (2ARV.pdb). We found the RMSD to be 1.2 Å between these two activin structures. Furthermore, we compared the two extracted activin structures from both the complexes (1NYS.pdb and 2B0U.pdb) and found the RMSD between the two structures to be <1.5 Å. The above analysis suggested that the deviation in the activin structure when complexed with other proteins is minimal.

Again, we noticed that many atoms and side chains of the residues of the activin part of 1NYS.pdb have not been resolved, whereas the activin part has been fully resolved in the 2B0U.pdb structure. This observation prompted us to use the fully resolved activin structure extracted from the activin-follistatin complex as our protein target for modeling. Using the site identification (SiteID) module of Tripos software,⁴⁸ we identified a small molecule-binding groove/pocket in the interface of both β -subunits, as shown in Figure 1. This binding site contains critical ALK4 binding residues Met91, Ser60, Ile63, and Met108 identified by mutagenesis studies.⁴⁹ The groove/pocket is shallow with a wide v-shape and also has a hydrophobic core consisting of W, Y, F, and L residues from both β -subunits. As required by the Schrodinger docking tool,⁵⁰ we generated a grid of $12 \times 12 \times 12$ Å, which contained the critical residues Ser60, Ile63, Phe58, and Val62 from one subunit and Trp25, Met91, Met108, and Asn107 from the other, as shown in Figure 1 for further in silico screening.

In Silico Screening Workflow

For our in silico screen, we began with an 18 million compound Zinc database of commercial compounds.⁵¹ Several filters were applied to this library to remove promiscuous, reactive, and other non-druglike molecules to produce a database of 10 million structures (see the Experimental section for details). To this curated library of ~10 million drug-like compounds, the OPLS 2005 force field was set, and the ligand van der Waals radius was scaled to 0.80 Å with partial atomic charges of <0.15 esu. A three-tier docking algorithm⁵⁰ was employed that incorporates virtual high-throughput screening (vHTS), followed by standard precision (SP) and extra precision (XP) docking protocols. The output of this three-tier docking engine was analyzed by considering the interactions of the compounds with the critical residues present at the junction of the β A-subunits as well as the Glide docking scores. We selected 76 compounds having a Glide docking score of <-6.0. To obtain consensus binding poses and to eliminate the noise from the in silico screening experiments as well as to enrich the hit rate with flexible ligand docking tools, we considered an orthogonal docking engine (Surflex) implemented in the Sybyl interface of Tripos.⁴⁸ Glide and Surflex tools have been found to be superior in pose prediction and virtual screening of compound databases.⁵² All 76 structures obtained from Glide docking underwent Surflex (GeomX) docking.⁴⁸ This secondary docking was performed to generate consensus docked poses and scores of the in silico hits but not to assess the superior performance of any of the docking tools. Comparing the binding poses as well as scores obtained from both docking experiments, we found 61 compounds that showed similar binding poses with comparable binding scores. On the basis of commercial availability and

synthetic tractability, we acquired 39 of these compounds from ChemBridge for screening in our in vitro assays (Supporting Information Table 1). The purity and identity of the 39 in silico selected compounds were confirmed by LC/MS (Supporting Information Table 1).

Compound Screening Using Cell-Based Assays

The molecules were first screened on the basis of their solubility in the L β T2 cell culture medium. Of the initial 39 molecules, 30 were soluble up to 200 μ M and were subsequently tested for biological function in cell-based assays. Soluble compounds were assayed for toxicity using an L β T2 cell viability assay. The L β T2 cell line, a line derived from a mouse pituitary gonadotrope tumor⁵³ was chosen for toxicity tests because activin A regulates the cell cycle in most cell types with the exception of the gonadotrope cell. The L β T2 cell line was also used to assess the biological function of our molecules; this is based on the major biological function of activin, which is to stimulate the production of the hormone FSH.^{53–57} Of the 30 soluble compounds, 11 were found to reduce cell viability <20% at 100 μ M and were thus considered to have low toxicity (Supporting Information Table 1). To test biological function, L β T2 cells were stably transfected with the –338 promoter region of the FSH β gene conjugated to a luciferase reporter (–338FSH β -Luc), allowing us to monitor FSH production by measuring the luciferase amount, as previously described.^{54–58} The –338FSH β -luc L β T2 stable cell line was used for initial functional screening of the 11 low-toxicity compounds. Follistatin-288 is a natural antagonist of activin A ($IC_{50} = 0.15 \pm 0.03$ nM) and was used as the positive control in the L β T2 luciferase assay.⁴⁰ There were five compounds that showed dose-dependent antagonistic activity against activin A-stimulated FSH β promoter activation in –338FSH β -luc L β T2 cells (Table 1, Supporting Information Figure 1A).

Activin A also induces HepG2 cell apoptosis,⁵⁹ which is one of the phenotypes of cancer cachexia in the mouse model.⁶⁰ To further evaluate our activin antagonists, we measured the ability of the compounds to reverse activin-mediated apoptosis in HepG2 human liver cells. The HepG2 cell apoptosis induced by activin A can be reversed by Fst288, which served as a positive control for this assay.¹⁵ The IC_{50} of Fst288 in the HepG2 functional assay was 0.76 ± 0.53 nM. We tested the five compounds that showed activity in our L β T2 luciferase assay and found that four compounds reversed the effect of activin A on HepG2 cells in a dose-dependent manner (Table 2, Supporting Information Figure 1B). In particular, NUCC-474 and NUCC-555 showed greater antagonistic activity than other compounds in both assays and were further assessed as lead compounds (Tables 1 and 2). Since the action of activin differs between the two cell types, namely, stimulation of hormone gene expression in the absence of cell cycle regulation and stimulation of apoptosis (exit from cell cycle), these assays provide powerful screening tools toward the identification of lead compounds that regulate activin.

Inhibition of Ex Vivo Ovarian Cell Proliferation

To continue our characterization of the two lead compounds, we chose to test their efficacy in an activin A-regulated ovarian follicle granulosa cell assay. In this cell type, activin stimulates cell proliferation,⁶¹ which can be blocked by follistatin and inhibin A.^{15,36} Ovaries from 4-day-old CD1 mice were cultured with activin A alone or together with

follistatin, inhibin, NUCC-555, or NUCC-474 for 4 days. On the final day of culture, the ovaries were treated with BrdU for 24 h to assess proliferation. The BrdU signal was increased by ~60% in activin A-treated ovaries, reflecting the expected mitogenic effect of activin A.^{62,63} Ovaries that were treated with either NUCC-555 or NUCC-474 showed less activin A-stimulated proliferation (Figure 2). In this organ culture assay, 200 μ M of NUCC-555 completely inhibited exogenous activin A-mediated ovarian cell proliferation, similar to that seen with Fst288 (4 nM) and inhibin A (8 nM), and NUCC-474 at 200 μ M only partially inhibited exogenous activin A-mediated ovarian cell proliferation.

In Vivo Functional Antagonism of Activin-Stimulated FSH in Ovariectomized Mice

Ovariectomized mice have high serum FSH levels caused by the loss of negative feedback to the pituitary from ovarian inhibin, allowing activin A in the pituitary to constitutively stimulate FSH production.^{35,64} We used 12-week-old ovariectomized mice for in vivo functional testing of our lead activin antagonist NUCC-555. Animals received subcutaneous injections of 15, 30, or 60 mg/kg of NUCC-555 every 12 h for a total of 4 doses, or Fst288 (200 μ g/kg) every 12 h for a total of 3 doses. These dosing regimens were based on our and others' experience.⁶⁵ The histopathology of the kidney and liver tissue was unchanged in NUCC-555-treated animals, as observed with H&E staining (Figure 3A). The mice also displayed normal behavior after NUCC-555 injection. FSH levels in the solvent control, NUCC-555, and Fst288-treated animals were measured by ELISA, with levels in the control animals set to 100%. In Fst288-treated animals, the FSH level decreased almost 60% (Figure 3B). Treatment of mice with 30 or 60 mg/kg NUCC-555 resulted in a 70% decrease in FSH level, similar to the effect seen with Fst288 treatment (Figure 3B). NUCC-555 at the 15 mg/kg dose had no significant effect on FSH levels compared with control-treated mice (Figure 3B).

Inhibition of NUCC-555 to Activin A Binding with Its Receptors

As a TGF β superfamily member, activin initiates signal transduction through binding to one of two cell surface type II receptors, RIIA or RIIB. Upon ligand binding, this ligand receptor complex can further recruit activin type IB receptor, also known as activin-receptor-like kinase 4 (ALK4), which phosphorylates the intracellular signaling proteins SMAD.^{8,66,67} We developed an assay using the Blitz binding system equipped with Dip and Read⁶⁸ to measure the interaction between the ActA/ActRIIA-ECD complex and ALK4-ECD-Fc. The interaction between the activin A and ALK4-Fc with or without ActRIIA-ECD (negative and positive controls, respectively) was measured (Supporting Information Figure 2). Assay details are described in the Materials and Animals section. No interaction between activin and ALK4-Fc was measured (as expected),⁶⁹ but there was strong interaction when binding was measured in the presence of the ActRIIA-ECD (Supporting Information Figure 2), which matched previous reports.^{67,69} NUCC-555 is a nearly complete antagonist to complex binding with ALK4-ECD-Fc at 25 μ M (complex concentration of 1.6 μ M) (Figure 4A). Furthermore, NUCC-555 inhibited this interaction in a dose-dependent manner (Figure 4A). To know whether NUCC-555 could interfere with activin binding to ActRIIA, the Blitz binding system was used to measure the interaction between activin A and ActRIIA-ECD-Fc with or without NUCC-555 presence. The NUCC-555 could weakly interfere with activin A binding with ActRIIA-ECD-Fc at 25 μ M (Figure 4B).

Selectivity Testing for NUCC 555

Like activin, myostatin (GDF8) is a TGF β superfamily member that can signal via ALK4, and its actions can also be blocked by follistatin-288.⁷⁰ To determine the binding affinity of NUCC-555 with activin A or myostatin, we performed the Blitz binding assay with both proteins to see whether NUCC-555 blocks the myostatin/ ActRIIA-ECD complex binding with ALK4-ECD-Fc. NUCC-555 completely blocked activin A/ActRIIA complex binding with ALK4 (Figure 5A) at 25 μ M, but showed no blocking activity to the myostatin/ ActRIIA complex binding with ALK4 at the same dose (Figure 5B). The lack of antagonism for NUCC-555 to myostatin was also confirmed by the functional selectivity of NUCC-555 for activin in the L β T2 assay.³³ In this assay, the IC₅₀ of NUCC-555 for activin A was 5.37 $\pm$ 4.01 μ M, which is almost 10-fold lower than the measured IC₅₀ of NUCC-555 for myostatin (47.92 $\pm$ 34.02 μ M) (Figure 5C).

To further confirm these data, we examined the ligand-binding pocket at the interface of two myostatin subunits. The shape and size of the pockets are very similar in these two molecules, but there are subtle residue differences present in each of the binding pockets (Figure 6). NUCC-555 interacts with Trp25, Trp28, Phe55, Tyr93, Lys103, and Asn107 in the activin A binding pocket, showing three potential hydrogen bonds with Lys103 and Asn107 and a π - π interaction with Trp35 (Figure 6A). In comparison, the docking of NUCC-555 in the myostatin binding pocket showed hydrophobic interactions with Pro56 and Ile98, and one potential hydrogen bond with Trp31 was observed (Figure 6B).

DISCUSSION

In this study, we used our earlier crystal structure of activin A⁴⁵ to identify a previously unappreciated binding pocket at the interface of both β A-subunit monomers that could provide a binding site for activin-selective ligands. This activin ligand binding pocket has high affinity for the follistatin N-terminal region (ND)⁴⁵ and activin type I receptor (ALK4),⁴⁹ emphasizing its role in mediating activin signaling and providing support for this pocket as a drug target.

We used a three-tier in silico screening strategy to identify candidate small-molecule antagonists of activin A targeting this unique binding pocket. Our panel of in vitro toxicity, functional screening methods, and biophysical binding assays provided a robust and efficient way to conduct initial screening of potential activin antagonists informed by in silico candidates. Using L β T2 cells (parent line and with a stably integrated FSH β promoter-luciferase gene complex)⁵⁴ and an apoptosis assay using the HepG2 human liver cell line,⁵⁹ two compounds were shown to be soluble, have low toxicity, and have low micromolar IC₅₀ activity (designated NUCC-474 and NUCC-555). Further testing of the two lead antagonists in an activin-dependent cell proliferation assay using ex vivo ovarian explants led to the selection of NUCC-555 as the molecule for in vivo assessment in an ovariectomized mouse model to evaluate FSH response.^{35,64} Importantly, NUCC-555 blocked FSH secretion in this model in a dose-dependent manner, confirming the validity of the in vitro assays in a well-established in vivo model of activin function. We further confirmed that NUCC-555 binds the activin complex using biophysical technique Blitz label-free assay. Compound NUCC-555 blocked activin/ ActRIIA complex binding with ALK4 in the Blitz label-free

binding assay, further supporting our view that the functional effects measured in our cellular assays are caused by NUCC-555 preventing the activin/ActRIIA complex from binding ALK4. To confirm NUCC-555 as a robust hit series and not a singleton, we purchased 14 commercially available close analogs. We found that two compounds showed low toxicity and a bioactivity similar to NUCC-555 in the -338 FSH β luc L β T2 cell based assay (Supporting Information Table 2).

Because the activin pathway is known to drive fertility/ infertility as well as cancer-related cachexia and other diseases, strategies that block activin signaling have been developed. Soluble ActRIIA-Fc or ActRIIB-Fc blocks activin signaling and reverses cancer cachexia-associated wasting and increases bone formation in mice;^{16,71} however, because of the broad binding affinity of ActRIIB to many TGF β ligands, such as GDF8 (myostatin), GDF11, activin A, activin B, activin AB, Nodal, and BMP7 (also called OP-1),⁶⁶ the soluble ActRIIA-Fc or ActRIIB-Fc fusion protein would be expected to produce undesirable side effects, such as bleeding,³⁰ that would limit its clinical acceptance. No adverse effects were identified in the mice treated with NUCC-555 in our efficacy studies (Figure 3A). To further verify the selectivity of NUCC-555 to activin A, we chose to examine myostatin (GDF8), one of the TGF β superfamily members that shares the same signaling pathway as activin A and the same binding protein, follistatin.⁷⁰ NUCC-555 strongly inhibited activin/ActRIIA complex binding to ALK4 at 25 μ M, whereas there was no inhibition of myostatin/ ActRIIA complex binding to ALK4 at the same drug concentration in the Blitz binding system equipped with the “dip and read” probe sensor. In addition, our 338FSH β -luc L β T2 in vitro cell-based luciferase assay also confirmed that NUCC-555 had a 10-times greater antagonistic function for activin when compared with myostatin.

To further confirm these data, we explored the ligand binding pocket at the interface of two myostatin subunits and showed that there are subtle residue differences present in each of the binding pockets that provide the specificity of NUCC-555 for activin. Thus, NUCC-555 not only strongly antagonizes function against activin A but also has selectivity for binding to activin A over other TGF β superfamily members, such as myostatin. This provides an important area for future exploration in drug discovery efforts to minimize off-target effects that could lead to toxicity in vivo.

To our knowledge, our approach is the first to identify a first-in-class small molecule antagonist of activin binding to ALK4, which opens a completely new approach to inhibiting the activity of TGF β receptor superfamily members. To date, inhibition of these pathways has been achieved by targeting the kinase activities of ALK4, -5, and -7 using ATP-competitive kinase inhibitors such as SB-435142 and SB-505124. In addition to their inherent poor selectivity for specific TGF β receptor superfamily members, ATP competitive kinase inhibitors such as SB-435142 and SB-505124 also exhibit off-target class effects that depend on the kinase inhibition profile of each inhibitor that can lead to dose-limiting toxicities.^{72,73} Our approach of targeting the ligand-receptor interface interferes with the system at the point of cascade initiation and is therefore expected to lead to a broader therapeutic window for the novel class of small molecules described herein.

CONCLUSION

Overall, in this study, we have identified a selective small molecule antagonist of activin action through a streamlined platform linking in silico analysis with robust biological validation, including in vivo efficacy. Small-molecule antagonists have several advantages over broadly acting antagonists such as soluble ActRII-Fc that are currently in various stages of clinical trials. First, a highly selective activin antagonist will have fewer off-target effects that would be expected with our compound compared to soluble ActRIIA-Fc or ActRIIB-Fc, which will block a variety of ligands that utilize a relatively few number of receptor targets. Second, small molecules typically provide a rapidly tunable core structure that ultimately enables molecular optimization necessary for future therapeutic success (e.g., pharmacodynamics and pharmacokinetic profiles, ADMET). Third, we anticipate that our in silico approach can be applied to other members of the TGF β superfamily to create new classes of highly specific small molecule antagonists that can be used to modulate TGF β signaling in health and disease. These data provide important proof-of-concept to our in silico screening strategy, from initial identification to verification of relevant in vivo efficacy. This initial integrated approach also lays a strong foundation for further lead optimization through medicinal chemistry guided by iterative bioassays with the goal of developing new therapeutics for cancer-related cachexia or other diseases related to high activin serum levels.

EXPERIMENTAL METHODS

Materials and Animals

Purified activin A, inhibin A, follistatin-288 (Fst288), and ActRIIA- ECD were produced by our lab as previously described.^{45,74} Myostatin (GDF8), ALK4-ECD-Fc, and ActRIIA-ECD-Fc were purchased from R&D (Minneapolis, MN). All in silico screening compounds were purchased from Chembridge (San Diego, CA). All 39 compounds were tested for purity and identity by LC/MS prior to testing. All compounds displayed the $M + 1$ ion in ESI⁺ consistent with the expected structure. Thirty-five of the compounds had purity >95% by LC, and data for all 39 are given in Supporting Information Table 1. CD1 mice and ovariectomized mice were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures involving mice were approved by the Northwestern University Animal Care and Use Committee. Mice were housed and bred in a barrier facility within Northwestern University's Center of Comparative Medicine (Chicago, IL, USA) and were provided with food and water ad libitum. Temperature, humidity, and photoperiod (14L/10D) were kept constant.

In Silico Filtering of the Small Molecule Database for Ligand Preparation

The ZINC database,⁵¹ which contains approximately 18 million commercially available compounds, was used for virtual high-throughput screening. All compounds in the ZINC library were subjected to a panel of PAINS substructures filters with Smiles ARbitrary Target Specifications (SMARTS) strings^{75–77} to eliminate promiscuous and non-drug-like molecules that interfere with functionality, including reactive functional groups, lipophilic chains of seven or more carbon atoms, crown ethers, disulfides, excessive acidic groups ($n >$

3), thiols, epoxides, aziridines, hydrazones, thioureas, thiocyanates, benzylic quaternary nitrogens, thioesters cyanamide, β -lactones, di- and triphosphates, phosphines, phosphonic acids, sulfonic acids, sulfonyl halides, boronic acids, and more than two nitro groups. Filtering generated a list of ~10 million commercially available compounds for further screening. Before screening this 10 million compound data set with our earlier defined small molecule ligand binding pocket, it was subjected to the LigPrep module of Schrödinger⁵⁰ in OPLS2005 force field at pH 7.0 ± 1 retaining the specific chirality. A low-energetic 3D structure for each molecule was generated in this ligand preparation panel.

Protein Preparation for Docking

The Glide docking engine implemented in the Schrödinger software⁵⁰ suite was utilized. The activin A structure was subjected to Prime validation to correct for irrelevant side chains, missing atoms, and undesired orientations of Asn, Gln, or His residues and to replace the “b-values” with optimized potential for liquid simulations (OPLS) charges. Next, the protein preparation (Prot-Prep) module was used to prepare and refine the structure to generate the “receptor”, the portion of the activin A dimer to be used for small-molecule docking. The three-tier docking engine of the Schrödinger software program is built upon a grid-based algorithm that requires grid generation in the active site of the target protein. A $12 \times 12 \times 12$ Å grid was generated considering the critical residues Ser60, Ile63, Phe58, and Val62 from the wrist region of one β A subunit and Trp25, Met91, Met108, and Asn107 from the finger region of the other subunit (Figure 1). The depth of the ligand binding pocket was found to be ~10 Å with a volume of 1100 Å³. The computed average length and volume of a set of 1800 known drug molecules available in the drug database using the QikProp module of Schrödinger software was found to be within this limit.⁵⁰ The binding pocket is shallow with a wide v-shape and a hydrophobic core consisting of Trp, Tyr, Phe, and Leu residues from both β A-subunits. For the Surflex docking,⁴⁸ the protein preparation tool was used as implemented in the Sybyl interface. The hydrogens were added in the hydrogen bonding orientations, Gasteiger charges were assigned, and irrelevant torsions were eliminated. Using the residues identified through the SiteID module,⁴⁸ a residue-based protomol was generated for Surflex docking.

Solubility Testing

Candidate compounds were dissolved in DMSO to a final concentration of 50 mM. The compounds were diluted in cell growth media (DMEM-F12 with 5% FBS, 1% penicillin/streptomycin [P/S], and 4.5 g/mL D-glucose; Invitrogen, Grand Island, NY) to a final concentration of 200 μ M for 24 h. Precipitate formation was confirmed visually under an upright light microscope.

Toxicity Assay

The L β T2 immortalized pituitary gonadotrope cell line was used to test the toxicity of candidate compounds; the gonadotrope is the only known cell type in which activin does not regulate the cell cycle as it does in liver cells (apoptotic)^{11,59,78} and granulosa cells (mitogenic).^{62,63,79,80} Cells were plated at 5×10^4 cells per well in 96-well plates, cultured for 24 h in cell growth media (DMEM-F12 with 5% FBS, 1% P/S, and 4.5 g/mL D-glucose), and then treated with candidate compounds at 10 μ M or 100 μ M in cell growth media for 24

h. After 24 h, 20 μ L of MTT reagent (Promega, Madison, WI) was added to each well and incubated for another 2–3 h to assess cell viability. Plates were read at 490 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT).

Luciferase Assay

Although the cell cycle is not regulated by activin in gonadotrope cells, it does stimulate the production of the hormone FSH. L β T2 cells were stably transfected with the –338 promoter region of the FSH β gene conjugated to a luciferase reporter (–338FSH β -Luc), as previously described.^{54,56,57,81–84} Activin stimulates FSH promoter-driven luciferase activity in –338FSH β -Luc-transfected L β T2 cells, which we used as a readout in the initial functional screening of the candidate compounds. The well-known activin antagonist follistatin^{40,85} was used as a control. –338FSH β -Luc L β T2 cells were plated at 5×10^4 cells/well in 96-well plates for 24 h, then treated with activin A (0.2 nM) alone plus the same amount of DMSO as the solvent control, or with 0.4–100 μ M of each compound dissolved in DMSO, or 0.05–4 nM of Fst288 for 6 h at 37 °C in serum-free, phenol red-free DMEM-F12 supplemented with 1% P/S. Following treatment, cells were lysed, and the luciferase activity was measured using a luciferase assay kit (Promega, WI) and a Synergy HT Multi-Mode Microplate Reader (BioTek, VT). The assay conditions were standardized for activin and its antagonists.⁵⁴

Liver Apoptosis Assay

The human hepatoma-derived cell line HepG2 was used as a secondary screen of liver apoptosis.⁵⁹ HepG2 cells were maintained in DMEM with 10% FBS, 1% P/S, pyruvate, and glutamine-Max (Invitrogen, NY). The cells were plated at 5×10^4 cells/well in 96-well plates and treated with 1 nM activin A alone plus the same amount of DMSO as the solvent control, or with 1.6–200 μ M of each compound dissolved in DMSO, or 0.5–8 nM of Fst288 for 96 h at 37 °C in DMEM supplemented with 0.2% FBS, 1% P/S, pyruvate, and glutamine-Max.⁵⁹ On the last day of treatment, 20 μ L of MTT reagent was added to each well, and the cells were incubated for another 0.5–1 h to assess cell viability as described in the toxicity assay.

Ovary Ex Vivo Culture and BrdU Assay

Ovaries dissected from 4-day-old CD1 mice were cultured on 0.4- μ m membrane inserts (EMD Millipore Co, Billerica, MA) in DMEM/F12 with 0.03% BSA⁸⁶ and treated with 1 nM activin A alone with the same amount of DMSO as the solvent control, 200 μ M of the test compound, 4 nM Fst288, or 8 nM inhibin A as positive controls for 4 days. At the end of treatment, cultured ovaries were treated with 10 μ M BrdU for another 24 h. The ovaries were harvested and stored in –80 °C for dot blot analysis using a modified version of a previously published protocol,⁸⁷ as follows. Genomic DNA was isolated from thawed tissues following the Qiagen total DNA isolation protocol (Valencia, VA). Genomic DNA was diluted to 10 ng/ μ L, and 150 ng was denatured by incubation in a 96 °C water bath for 5 min, followed by rapid cooling in an ice bath. The single-stranded DNA was dot-blotted onto a nitrocellulose membrane and fixed by ultraviolet cross-linking in a Stratilinker (Stratagene, La Jolla, CA). The membrane was incubated with mouse anti-BrdU monoclonal antibody (1:2000 dilution; Sigma, St. Louis, MO) overnight at 4 °C, followed by incubation

with HRP-conjugated antimouse IgG antibody (Invitrogen, NY) for 1 h at room temperature. BrdU-labeled DNA was detected by ECL (GE HealthCare Life Sciences, Pittsburgh, PA) and exposure to X-ray film (Kodak, Rochester, NY) for various lengths of time, and then quantified using densitometric analysis with ImageJ Imaging Software (version 1.40g).

Lead Compound Functional Specificity Testing

Myostatin (GDF8) and activin A can signal via the same receptors, ActRII and ALK4.^{33,70} Although only activin A stimulates FSH promoter activity in gonadotrope cells in vivo, myostatin can also stimulate the FSH promoter in the L β T2 cell line in vitro.³³ The luciferase assay described above was therefore used to test the specificity of response to the lead compounds. -338FSH β -Luc L β T2 cells were plated at 5×10^4 cells/well in 96-well plates. The cells were treated with 0.2 nM activin A, 0.2 nM myostatin alone plus the same amount of DMSO as solvent control, or together with test compounds at 0.8–100 μ M for 6 h as described above. Following treatment, the cells were lysed, and the luciferase activity was measured using the Promega kit described above.

Animal Treatment and Serum Collection

Ovariectomized, 12-week-old CD1 mice were purchased from Jackson Laboratories. The mice were injected subcutaneously with 200 μ g/kg Fst288,⁸⁵ various doses of compound NUCC-555 (15, 30, or 60 mg/kg), or vehicle solution (TBS or DMSO) twice daily for 1.5 days (Fst288, 3 doses total) or 2 days (NUCC-555, 4 doses total). After injection, mice were sacrificed for serum and tissue collection. The serum was stored at -20 °C until analysis.

Histology

Fresh kidney and liver tissue collected from ovariectomized mice that received four doses of either the vehicle solution or 60 mg/kg NUCC-555 were fixed with Modified Davidson's fixative (Electron Microscopy Science Inc., Hatfield, PA) for 5 days at 4 °C and then processed and embedded in paraffin. H&E staining was performed by the Histology Core (Northwestern University P50 Ovarian Histology Core) using standard methods.

FSH ELISA

FSH was measured in serum samples from ovariectomized mice treated with vehicle, Fst288, or NUCC-555 using an FSH ELISA kit purchased from CUBIO (Wuhan, China), following the manufacturer's protocol. The sensitivity of the assay is 0.35mIU/mL. Plates were read using a Synergy HT Multi-Mode Microplate Reader (BioTek, VT) at 450 nm.

Competition Binding Assay

The Blitz binding system is housed in the Keck Biophysics Facility (Northwestern University) and is equipped with a Dip and Read Protein A biosensor⁶⁸ (ForteBio, CA, USA). Blitz binding assays were performed to measure binding between the activin A/ActRIIA-ECD complex with ALK4-ECD-Fc, the myostatin/ActRIIA-ECD complex with ALK4-ECD-Fc, or activin A with ActRIIA-ECD-Fc. The activin A and ActRIIA were premixed at a 1:2 molar ratio to a final concentration of 1.6 μ M in PBS buffer. The myostatin and ActRIIA mixture was prepared the same as the activin A and ActRIIA

mixture. The ALK4-Fc in 50 ng/mL concentration was immobilized by the protein A biosensor for 120 s. The protein A biosensor immobilized by Alk4-Fc was dipped into the activin A/ ActRIIA complex mixture solution either with the solvent control or with different doses (0.4, 1.6, 6.25, or 25 μ M) NUCC-555 for 120 s association, and 120 s dissociation in PBS buffer. The real-time wavelength shift was recorded by ForteBio software. The competition binding assay between the myostatin/ActRIIA complex and ALK4-ECD-Fc or between the activin A and ActRIIA-Fc was performed using the same methods. A single dose of NUCC-555 25 μ M was used for the competition assay.

Statistical Analyses

Values are reported as the means $\pm$ SD. IC₅₀ values were determined on the basis of the sigmoidal dose-response (variable slope) curve using Prism software (Version 6.0, GraphPad Software, San Diego, CA). Dot blot densitometry quantification comparisons and FSH level comparisons were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test. A *p* value <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

ActRII	activin type II receptor
ALK4	activin-like kinase 4, activin type IB receptor
TGFβ	transforming growth factor β
BMP	bone morphogenic protein
GDF	growth and differentiation factor
FSH	follicle-stimulating hormone
Fst	follicle-stimulating hormone
ELISA	enzyme-linked immunosorbent assay
ECD	extra cellular domain

RMSD	root-mean-square deviation
vHTS	virtual high-throughput screening
SP	standard precision
XP	extra precision

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Figure 1.

Docking site in the activin A dimer used for in silico screening of small-molecule antagonists. The junction between the two βA subunits of activin A, which can interact with follistatin 288 N-terminal domain (ND), forms a pocket that also interacts with activin type I receptor (ALK4). This pocket was used in in silico screening of small molecule antagonists of activin A.

Figure 2.

Lead compounds inhibit activin-stimulated granulosa cell proliferation in ex vivo ovary culture. Ovaries from 4-day-old CD1 mice were cultured and treated with activin A alone or with the known activin antagonists inhibin and follistatin or with the lead candidate small molecule antagonists NUCC-555 and NUCC-474. BrdU was added on the last day of ovary culture. DNA dot blots were carried out, and the density of each dot was calculated by ImageJ. Data are reported from three independent experiments. Asterisks represent statistically significant differences between groups ($p < 0.05$). A oneway ANOVA followed by Tukey's multiple comparisons test was used for the statistical analysis.

Figure 3. NUC-555 treatment of ovariectomized mice. (A) Kidney and liver morphology of ovariectomized mice following injection of NUCC-555. H&E staining of the liver and kidney from ovariectomized mice after treatment with either vehicle or 60 mg/kg NUCC-555 administered twice daily for 2 days, for a total of 4 doses. (B) NUCC-555 antagonizes activin-stimulated FSH in ovariectomized mice. Twelve-week-old ovariectomized mice were injected subcutaneously with vehicle, Fst288, or NUCC-555 at the indicated doses twice daily for 1.5 or 2 days. Mice received a total of 3 doses of Fst288

and 4 doses of NUCC-555. The serum FSH level in each treatment group was measured by FSH ELISA. The average FSH level in vehicle-treated mice was set at 100%. A total of 5–7 mice were used for each treatment group, and 10 mice were used for the control group. Letters correspond to statistically significant differences between groups. Using one-way ANOVA followed by Tukey's multiple comparisons test, $p < 0.05$ was considered statistically significant.

Figure 4.

NUCC-555 interferes with activin A binding to its receptors. (A) NUCC-555 interferes with the activin A/ActRIIA-ECD complex binding to ALK4-ECD-Fc. The Blitz system equipped with dip and read protein A biosensor was used to monitor the binding between the activin A/ActRIIA-ECD complex with Alk4-ECD-Fc with or without NUCC-555 present. The dose ranges (0.4, 1.6, 6.25, and 25 μ M) of NUCC-555 were chosen on the basis of the IC_{50} in prior bioassays. The complex of activin A/ActRII-ECD was mixed as a 1:2 ratio to a final concentration of 1.6 μ M. (B) NUCC-555 interferes with activin A binding to ActRIIA-ECD-

Fc. The Blitz system equipped with a dip and read protein A biosensor was also used to monitor the binding between activin A and ActRIIA-ECD-Fc with or without NUCC-555 present. A single dose of NUCC-555 (25 μ M) was chosen for the competition binding. The final activin A concentration was 1.6 μ M.

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Figure 5.

NUCC-555 selectivity was tested between activin A and myostatin. (A) Control NUCC-555 antagonism of activin/ActRIIA-ECD binding to ALK4-ECD-FC. A single dose 25 μ M of NUCC-555 was chosen for the competition binding using the Blitz system as described previously (activin A/ActRIIA-ECD complex and ALK4-ECD-Fc with or without NUCC-555 present). (B) NUCC-555 does not block myostatin/ActRIIA-ECD binding with ALK4-ECD-Fc. The same method was also used to monitor the binding between the myostatin/ActRIIA-ECD complex and ALK4-ECD-Fc with or without NUCC-555 present.

A single dose, 25 μM , of NUCC-555 was chosen for the competition binding. (C) NUCC-555 inhibits activin A NOT myostatin signaling in the -338FSH β -Luc L β T2 cell line. The mouse pituitary stable cell line, -338FSH β -Luc L β T2 cells, was treated with activin A (0.2 nM) or myostatin (0.2 nM) alone or with an equal amount of DMSO as a solvent control, or with different doses of NUCC-555 from 0.4 to 100 nM for 6 h. Triplicate for each treatment and three individual experiments were performed. The IC_{50} of the NUCC-555 antagonism function for activin A was $5.37 \pm 4.01 \mu\text{M}$, and the IC_{50} of the NUCC-555 antagonism function for myostatin was $47.92 \pm 34.02 \mu\text{M}$. IC_{50} values were determined on the basis of the sigmoidal dose-response (variable slope) curve using Prism software.

Figure 6.

Comparison of the docking pockets of activin A and myostatin for the NUCC-555 binding. (A) The docked pose of NUCC-555 in activin A dimer (2B0U.pdb). (B) The docked pose of NUCC-555 in myostatin dimer (3HH2.pdb). Magenta dotted bonds represent potential hydrogen bonds.

Table 1Primary Function Tests of Low Toxicity Compounds in the –338FSH β -Luc L β T2 Cell Assay

Compound ID	Structure	Luc assay IC ₅₀
Fst 288	N/A	0.15 $\pm$ 0.03 nM
NUCC-474		4.42 $\pm$ 2.05 μ M
NUCC-475		23.64 $\pm$ 13.12 μ M
NUCC-478		>100 μ M
NUCC-479		4.18 $\pm$ 1.67 μ M
NUCC-527		>100 μ M
NUCC-530		4.25 $\pm$ 2.46 μ M
NUCC-553		>100 μ M
NUCC-555		3.67 $\pm$ 1.80 μ M
NUCC-558		>100 μ M
NUCC-559		>100 μ M

Compound ID	Structure	Luc assay IC ₅₀
NUCC-560		>100 μ M

Table 2

Secondary Functional Tests of Lead Compounds in the Human Liver HepG2 Cell Line Assay

Compound ID	Structure	Apoptosis Assay IC ₅₀
Fst 288	N/A	0.76 ± 0.53 nM
NUCC-474		23.2 ± 8.49 μM
NUCC-475		>200 μM
NUCC-479		47.3 ± 13.3 μM
NUCC-530		43.9 ± 12.6 μM
NUCC-555		12.1 ± 5.96 μM