Identification and Mechanistic Characterization of a Peptide Inhibitor of Glycogen Synthase Kinase (GSK3 β) Derived from the Disrupted in Schizophrenia 1 (DISC1) Protein

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ABSTRACT

Glycogen Synthase Kinase 3-beta (GSK3 β) is a critical regulator of several cellular pathways involved in neuroplasticity and is a potential target for neurotherapeutic development in the treatment of neuropsychiatric and neurodegenerative diseases. The majority of efforts to develop inhibitors of GSK3 β have been focused on developing small molecule inhibitors that compete with ATP through direct interaction with the ATP binding site. This strategy has presented selectivity challenges due to the evolutionary conservation of this domain within the kinome. The Disrupted in Schizophrenia (DISC1) protein, has previously been shown to bind and inhibit GSK3 β activity. Here, we report the characterization of a 44-mer peptide derived from human DISC1 (hDISCtide) that is sufficient to both bind and inhibit GSK3 β in a non-competitive mode that is distinct from classical ATP competitive inhibitors. Based on multiple independent biochemical and biophysical assays, we propose that hDISCtide interacts at two distinct regions of GSK3 β : an inhibitory region that partially overlaps with the binding site of FRATide, a well-known GSK3 β binding peptide, and a specific binding region that is unique to hDISCtide. Taken together, our findings present a novel avenue for developing a peptide-based selective inhibitor of GSK3 β .

KEYWORDS

Glycogen Synthase Kinase 3 β (GSK3 β), Disrupted in Schizophrenia 1 (DISC1), Enzyme Kinetics, FRATide, Axin

INTRODUCTION

Approximately 16% of the world's population suffers from neuropsychiatric disorders every year¹. While current treatments are efficacious, a significant proportion of patients either suffer from adverse side-effects or show decreased responsiveness to current treatments. Most major classes of neuropsychiatric drugs share similar mechanisms of action, which limits the therapeutic options for patients who do not respond well to the existing pharmaceuticals. In order to address this need, identifying novel molecular targets is a priority. One likely target is the multifunctional constitutively active Ser/Thr enzyme, glycogen synthase kinase 3-beta (GSK3 β), which participates in the regulation of multiple cellular pathways including those responsible for cell proliferation, cell architecture, and neurodevelopment³. As a result of this diversity, a dysregulation of GSK3 β has been implicated in various conditions, including mood disorders, Alzheimer's disease, diabetes, and cancer⁴. More than 40 GSK3 β substrates have been confirmed to date, and over 500 other potential substrates await validation^{5,6}. Clinical translation of GSK3 β inhibitory molecules for neuropsychiatric diseases face three major challenges that include poor selectivity among closely related kinases and other CNS targets, blood-brain barrier (BBB) penetrability, and more significantly, chronic toxicity⁷. Although tremendous advances have been made in the development of highly selective GSK3 β inhibitors^{7,8}, there still exists an opportunity to address novel mechanisms of inhibition that may overcome certain limitations, such as high cellular ATP levels and selectivity across the kinase.

As a basally active enzyme involved in a plethora of signaling pathways, GSK3 β activity has multi-layered regulatory mechanisms that include post-translational modification, substrate priming, and protein complex formation. Regulation of the Wnt/ β -catenin signaling pathway relies on partitioning GSK3 β into distinct complexes. At rest, a subset of GSK3 β is localized within a multi-protein unit called the destruction complex where the scaffold protein, Axin, binds to both GSK3 β and its substrate β -catenin. GSK3 β activity is enhanced ~24,000 fold towards Axin-bound β -catenin compared to free β -catenin⁹. GSK3 β acts to hyper-

phosphorylate β -catenin which is unstable and is then targeted for degradation. In the presence of an external Wnt signal, Axin and GSK3 β are sequestered into another protein complex called the signalosome where GSK3 β activity is inhibited by the phosphorylated C-terminal tail of the Wnt co-receptor LRP5/6 occupying its substrate-binding site¹⁰. As a result, more stable β -catenin accumulates in the cytoplasm and translocates to activate Wnt-responsive genes in the nucleus. Both overabundant and insufficient activity of the Wnt/ β -catenin pathway is linked to abnormal neurodevelopmental processes that contribute to the development of several psychiatric diseases¹¹.

The psychiatric disease risk factor, Disrupted in Schizophrenia 1 (DISC1), is a scaffold-like protein that binds and inhibits GSK3 β 's function in the Wnt/ β -catenin pathway to modulate neuroprogenitor proliferation in the mouse model¹². Moreover, DISC1's inhibition of GSK3 β seems to be specific to the Wnt/ β -catenin pathway. In the same study, manipulating DISC1 expression had no effect on the inhibitory phosphorylation of Ser9 on GSK3 β , which occurs in other signaling pathways involving GSK3 β ¹². Additionally, not all GSK3 β substrates are affected by the changes of DISC1 expression¹². Therefore, DISC1-mediated inhibition of GSK3 β offers an innovative approach in designing novel GSK3 β inhibitors that are pathway and substrate selective. In the present study, we investigated the inhibition mechanism of a 44-residue peptide from human DISC1 (hDISCtide) that is sufficient to both interact with and inhibit GSK3 β 's kinase activity. Our biochemical and biophysical data provide basis to an inhibition model whereby hDISCtide possesses two distinct regions that interact with GSK3 β , one that acts as an anchor, and the other one which is necessary for inhibition.

RESULTS AND DISCUSSION

Previously in an *in vitro* assay, a 44-residue region of the mouse DISC1 protein was shown to inhibit and bind to human GSK3 β ¹². Here, we confirmed that the equivalent human sequence (hDISCtide), which is 70% identical, also inhibits human GSK3 β in an *in vitro* kinase assay (Figure 1A). Using a phospho-primed glycogen synthase peptide (GSP2) as the substrate, hDISCtide inhibited recombinant GSK3 β with an IC₅₀ of 95 nM (Figure 1B). We also confirmed that hDISCtide could physically interact with GSK3 β as assessed through a surface plasmon resonance (SPR) assay. In order to ensure a stable baseline, we generated recombinant N- and C-terminal double His-tagged GSK3 β (His-GSK3 β -His). Using this SPR assay, hDISCtide exhibited very slow binding kinetics to GSK3 β with minimal dissociation after 10 minutes (Figure 1C). The surface of the SPR chip was regenerated after each injection so that fresh protein could be captured for each injection of hDISCtide. The sensorgrams at various hDISCtide concentrations were fitted to the OneToOne model (Equation 1, $\chi^2 = 0.88$), with a k_a of 495 (± 31.4) M⁻¹s⁻¹ and k_d of 2.49x10⁻⁴ ($\pm 8.91 \times 10^{-6}$) s⁻¹. Due to the slow binding kinetics, the binding affinity cannot be estimated from the binding equilibrium dissociation constant ($K_D = k_d/k_a$). Additionally, we were unable to obtain saturation binding at a practical concentration range to measure affinity by steady-state analysis. To cross reference the K_D value of hDISCtide with GSK3 β we developed a microscale thermophoresis (MST) assay, which detects binding based on changes in molecular motions along a temperature gradient. Thus, MST differs from SPR in that binding is detected from free molecules in solution, whereas SPR requires one molecule to be immobilized. Using this MST assay the K_D of hDISCtide to GSK3 β was measured to be 5.7 μ M (Figure 1D).

To determine the sequence specificity of the inhibition and binding to GSK3 β , we next examined a peptide composed of the same amino acids as hDISCtide but synthesized in a scrambled order. This peptide did not show any efficacy as an inhibitor of GSK3 β (data not shown). Subsequently, several mutagenesis analyses were performed to evaluate the impact of different amino acid substitutions on GSK3 β inhibition. A single-site mutation of A220P completely abolished hDISCtide inhibition, whereas

S216P reduced inhibition potency (Figure 1E, G). A peptide with a double mutation of F210A and F212A was unable to inhibit GSK3 β (Figure 1E, G).

To determine whether hDISCtide acts as a general kinase inhibitor, its effect on a cyclin dependent kinase 5 (CDK5), a close homolog of GSK3 β , was examined. CDK5 shows 24.5% identity and 38.1% similarity in amino acids compared to GSK3 β . Most reported GSK3 β inhibitors that are not selective also affect CDK5 activity¹³. To assess how selective hDISCtide is to GSK3 β , we used an ADP-Glo assay to test the inhibition potency of hDISCtide against CDK5 in a reaction containing ATP (at approximate K_m values) and the CDK5 substrate peptide (PKTPKKAKKL) derived from histone H1. For comparison, we included an assay containing the small molecule non-selective inhibitor of GSK3 β , roscovitine. hDISCtide did not show any inhibition against CDK5, whereas, roscovitine showed potent inhibition (Figure 1F). Thus, hDISCtide appears to possess selectivity against GSK3 β , and therefore the inhibition mechanism of hDISCtide is likely distinct from that of a general kinase inhibitor.

Simple inhibition modality can be described based on the affinity of the inhibitor to different enzyme states along the reaction coordinate of the enzyme. The inhibitor can bind exclusively to the free enzyme (E) (competitive inhibition), exclusively to the enzyme-substrate complex (ES) (uncompetitive inhibition), or to both species (noncompetitive inhibition). In noncompetitive inhibition, the degree to which the inhibitor changes the binding affinity of the substrate to the enzyme is described by the constant α . When α is significantly larger than 1, the inhibitor completely excludes the binding of the substrate. When $\alpha=1$, the inhibitor has the same affinity as the free enzyme and ES complex. When $\alpha>1$, the inhibitor binds with a higher affinity to the free enzyme. When $\alpha<1$, the inhibitor binds with a higher affinity to the ES complex¹⁴.

To investigate the inhibition modality of hDISCtide against GSK3 β , we evaluated how different concentrations of hDISCtide influence the V_{max} and K_m of the two substrates (ATP and GSP2) used in our kinase assays. To determine if hDISCtide can compete for ATP binding, GSK3 β enzymatic reactions were carried out with varying hDISCtide and ATP concentrations in the absence of GSP2 using a time-resolved

fluorescence energy transfer (TR-FRET) kinase assay (Supplementary Figure 1A). Similarly, to determine if hDISCtide can compete directly with the substrate peptide GSP2, GSK3 β enzymatic reactions were carried out with varying hDISCtide and GSP2 concentrations while the ATP concentration was fixed at the approximate K_m value of 15 μ M using the ADP-Glo assay (Supplementary Figure 1B). Both datasets were fitted to the Michaelis-Menten equation, followed by an estimation of the K_m and V_{max} for the respective substrate at each hDISCtide concentration. As a control, we also performed a similar kinetic analysis with CHIR-99021, a well-established, ATP competitive, small molecule inhibitor of GSK3 β , using the TR-FRET assay with varying hDISCtide and ATP concentration in the absence of GSP2 (Supplementary Figure 1C). First, pure competitive inhibitors should have no effect on V_{max} ¹⁴, as expected and observed for CHIR-99021 (Figure 2C-i). Since $^{ATP}V_{max}$ and $^{GSP2}V_{max}$ both decreased curvilinearly with increasing hDISCtide concentration, we can conclude that hDISCtide does not directly compete with ATP or GSP2 substrate (Figure 2A-i, 2B-i)¹⁴. Second, uncompetitive inhibitors should have no effect on the ratio V_{max}/K_m ¹⁴. Since this ratio decreases curvilinearly for the three datasets, we can rule out uncompetitive inhibition (Figure 2A-iii, 2B-iii, 2C-iii)¹⁴. Finally, the α constant was deduced from the K_m replots. $^{ATP}K_m$ increased curvilinearly with increasing hDISCtide concentration, indicating non-competitive inhibition with $\alpha > 1$ ¹⁴ (Figure 2A-ii). In comparison, the linearity of the $^{ATP}K_m$ replot for CHIR-99021 is a signature trend for a pure ATP competitive inhibitor (Figure 2C-ii). Conversely, $^{GSP2}K_m$ decreased curvilinearly with increasing hDISCtide concentration, indicating non-competitive inhibition with $\alpha < 1$ ¹⁴ (Figure 2B-ii). In summary, hDISCtide is a non-competitive inhibitor relative to both ATP and GSP2. Based on the α constants, hDISCtide has a higher affinity for free GSK3 β compared to ATP-bound GSK3 β . In contrast, hDISCtide has a higher affinity for the GSP2-bound GSK3 β compared to an enzyme where the substrate peptide binding pocket is unoccupied.

Since hDISCtide is a non-competitive inhibitor relative to both ATP and the substrate peptide GSP2, the binding site of hDISCtide likely involves regions outside of the ATP and substrate binding pockets. The C-terminal domain of GSK3 β contains a protein binding site where two well-characterized

peptides (AxinGID and FRATide) bind^{9, 15}. Both of these peptides are derived from scaffold proteins that localize subsets of cellular GSK3 β to different compartments of a cell. The crystal structures of GSK3 β /AxinGID and GSK3 β /FRATide show that the peptides partially share the same binding site at the C-terminal domain of GSK3 β (Figure 5A-B, highlighted in blue)^{9, 15}. AxinGID binds GSK3 β as a single helix (Figure 5A), whereas FRATide binds GSK3 β as two α -helices bent at a 90 degree angle (Figure 5B)^{9, 15}. The plasticity of the peptide-binding loop can accommodate the two unique conformations of AxinGID and FRATide. To test the hypothesis that hDISCtide shares binding site with AxinGID or FRATide at a similar location on GSK3 β , we sought to compare their binding kinetic parameters with that of hDISCtide measured by SPR. First, we confirmed the binding of either AxinGID or FRATide to GSK3 β using a Biolayer Interferometry (BLI) assay, which follows similar biophysical assay principles compared to SPR. BLI yielded a K_D of 3.6 μ M and 63.6 μ M for FRATide and AxinGID respectively (Figure 3A, Supplementary Figure 2A). The low affinity of AxinGID was likely due to the inclusion of only residues that were observed to be ordered in the crystal structure⁹. Removing the flexible flanking residues appear to affect its affinity.

Since FRATide showed higher affinity binding to GSK3 β in the BLI assay, we sought next to perform a comparative analysis of the binding kinetics of FRATide and hDISCtide to GSK3 β using the SPR-based assay. In contrast to the slow binding kinetics observed for hDISCtide, FRATide bound GSK3 β with a fast on- and off-rate. Unlike hDISCtide, which never reached saturation in our experimental setting, the binding sites for FRATide were saturated within two minutes of injection (Figure 3B). We were able to measure the K_D by steady-state and kinetic analysis (Figure 3B). The affinity of FRATide to GSK3 β was determined to be 1.9 μ M by steady-state analysis (Figure 3C). The kinetic data did not fit the standard OneToOne model, but instead fitted best to a OneToOne TwoState model (Equation 2). The OneToOne TwoState model describes the presence of an initial weaker interaction event, followed by a subsequent change to a stronger interaction. The initial weaker interaction event could be contributed by GSK3 β binding to only one of the two helices of FRATide, followed by the stronger interaction formed with the

additional contacts from the second FRATide helix. Nevertheless, the affinity obtained from this kinetic model was 3.2 μM , which is comparable to that obtained from steady-state analysis (1.9 μM).

Taking advantage of the substantial difference in GSK3 β binding kinetics between hDISCtide and FRATide that could be measured with the SPR assay, we next asked whether FRATide can compete with hDISCtide for binding at a similar site. Since hDISCtide binding kinetics were slow, we first incubated GSK3 β with 4 μM of hDISCtide for 20 mins in solution before capturing the GSK3 β -hDISCtide complex on the sensor chip. After immobilization, subsequent injection of hDISCtide (4 μM) showed no detectable binding, confirming that the binding sites for hDISCtide on GSK3 β had been saturated (Figure 3D – red). In contrast, FRATide was still able to interact with the GSK3 β -hDISCtide complex, and presented similar binding kinetics (Figure 3D – blue) when compared to FRATide interacting with GSK3 β alone (Figure 3B). This observation indicates that FRATide can still access its binding site on hDISCtide-bound GSK3 β , suggesting that the FRATide and hDISCtide binding sites do not overlap. To further test the notion that FRATide and hDISCtide bind at distinct sites on GSK3 β , we investigated how FRATide influences hDISCtide inhibition of GSK3 β . Since FRATide binding to GSK3 β blocks its activity toward non-phospho-primed substrates (e.g. Axin), but does not affect GSK3 β 's activity toward phospho-primed substrates like that of glycogen synthase¹⁶, we reasoned that FRATide should not affect the inhibition potency of hDISCtide in our GSK3 β activity assay using a glycogen synthase-derived peptide substrate. We first confirmed that FRATide on its own does not inhibit GSK3 β (Supplementary Figure 2B). However, contrary to our expectation, the inhibition of GSK3 β by hDISCtide was abolished in the presence of FRATide (Figure 3E). To explore this effect more, a double titration experiment was performed. In the presence of 0.5 μM FRATide, the IC_{50} of hDISCtide increased ~3-fold. At concentrations of 2.5 μM and above, FRATide completely abolished the inhibition of hDISCtide even at 100 μM (Figure 3F). Thus, these data show that the binding of FRATide blocks the ability of hDISCtide to form an inhibitory interaction with GSK3 β .

The contradictory interpretation from the SPR and ADP-Glo assays might be explained by considering the size of the peptides. Given that the size of hDISCtide (44 a.a.) is twice as long as that of FRATide (25 a.a.), the protein-protein-interface (PPI) of GSK3 β /hDISCtide could be larger than that of GSK3 β /FRATide particularly if hDISCtide binds in an extended conformation. FRATide may disrupt the region of the PPI of GSK3 β /hDISCtide that is essential for its inhibitory function while other regions of hDISCtide remain bound to GSK3 β . In this proposed model, there are two distinct functional regions of hDISCtide: one that acts as an anchor and forms specific interactions with GSK3 β and one that is required for its inhibitory function. Our previously published studies are consistent with this model. When overlapping 15-mer peptide segments spanning the mouse DISCtide region were tested for GSK3 β binding, only residues from 211 to 225 showed GSK3 β binding by SPR; however, these truncated peptides did not inhibit GSK3 β kinase activity¹². In our hands, we also observed that the equivalent human 15-mer, hDISCtide(209-225), only bound, but did not inhibit human GSK3 β (data not shown). This indicates that the regions flanking the central 15-mer are essential for GSK3 β inhibition. To identify which end of the central 15-mer was responsible for hDISCtide inhibitory activity, several truncated hDISCtide peptides were evaluated using the ADP-Glo assay. From the N-terminus, 13 residues were dispensable to the inhibition potency, whereas trimming the C-terminus by just four residues greatly reduced the inhibition potency (Figure 4A-C). Based on this data, we propose that hDISCtide(209-225) comprises the anchor that interacts with a unique site on GSK3 β (Figure 5C –“GB”), whereas hDISCtide(226-236) is the region (Figure 5C – “GI”) responsible for GSK3 β kinase inhibition. The presence of FRATide interferes with the interactions between hDISCtide-GI and GSK3 β (Figure 5C).

The crystal structure of GSK3 β trapped in a catalytically competent conformation (PDB:4NU1) shows that three regions are important for catalysis: 1) the glycine loop (cyan), which changes conformation upon substrate peptide binding; 2) the C-loop (yellow), which provides key specificity to substrate peptides and engages in conformational change upon substrate peptide binding; 3) the activation loop (red), which forms the substrate peptide binding surface opposite to the C-loop. (Figure 5A)¹⁰. Since our kinetic data

show that hDISCtide inhibition is non-competitive with ATP or the substrate peptide, hDISCtide could inhibit GSK3 β by preventing the above three regions of GSK3 β from forming a catalytic competent conformation (Figure 5C-i). hDISCtide inhibition can only occur if hDISCtide-GI can interact with its binding site on GSK3 β (Figure 5C-i orange oval). The method by which FRATide overcomes hDISCtide's inhibition of GSK3 β could occur in one of two ways: 1) FRATide could physically block the hDISCtide inhibitory binding site (Figure 5C-ii); or 2) FRATide binding could induce a conformational change in the hDISCtide-GI binding site (Figure 5C-iii). The crystal structure of GSK3 β /FRATide shows that FRATide binds at the C-terminal domain of GSK3 β clamped by a helix (pepHelix: 262-273) and a loop (pepLoop: 285-299) (Figure 5B, segment coloured in blue). These regions can potentially interact directly or indirectly with hDISCtide-GI while hDISCtide-GB interacts with regions outside of this FRATide binding site.

Collectively, this work provides new insights on how GSK3 β is regulated through its association with DISC1. Further investigation is required to understand the inhibition mechanism of DISC1 against GSK3 β within a relevant cell type in the context of the full-length protein. DISC1 regions outside of the 44 residue DISCtide could interact with other proteins and influence which subcellular GSK3 β pool that can be inhibited by DISC1. For example, GSK3 β could exist in the destruction complex in the absence of Wnt signal, the signalosome in the presence of Wnt signal, or remain free in the cytoplasm. Addressing which pool of GSK3 β DISC1 could interact and inhibit will shed light on the temporal involvement of DISC1 in the regulation of Wnt signaling – does DISC1 also modulate the basal level of GSK3 β activity or only during Wnt activation? Moreover, it would be important to consider other DISC1 interactors that have been found to regulate Wnt-GSK3 β / β -catenin signaling and modulate neural progenitor proliferation during early embryonic cortical development, such as Dixdc1¹⁷. Dixdc1 binds at the C-terminus (596-852) of DISC1 and has a calpain homology domain that interacts with actin. It would be interesting to determine if Dixdc1 influences DISC1's inhibition of GSK3 β that occurs at the N-terminus. Some short DISC1 isoforms that contain the GSK3 β inhibitory region, but lack the Dixdc1 binding region, have been reported to be upregulated in the hippocampus of schizophrenic patients¹⁸. If Dixdc1 affects DISC1's inhibition of

GSK3 β , different ratios of long and short DISC1 isoform will influence the overall Wnt/ β -catenin signaling and the pathophysiology of schizophrenia.

As the minimal GSK3 β inhibitory region of DISC1, hDISCtide has therapeutic potential for rescuing the loss of GSK3 β inhibitory function upon loss of DISC1. Moreover, since the binding site of hDISCtide partially overlaps with that of AxinGID and FRATide, hDISCtide can potentially disrupt GSK3 β association with their respective scaffold proteins, Axin and FRAT. AxinGID is derived from Axin that is a key component in the Wnt/ β -catenin signaling pathway. This work suggests that hDISCtide can potentially normalize Wnt signaling by inhibiting Axin-dependent GSK3 β phosphorylation of β -catenin. FRATide is a fragment from FRAT, which was initially thought to segregate GSK3 β binding away from Axin when the Wnt/ β -catenin pathway is activated¹⁶. However, FRAT was subsequently found to be non-essential for the Wnt/ β -catenin pathway in higher organisms¹⁹. Instead, the main function of FRAT appears to be a shuttle that carries nuclear GSK3 β to the cytoplasm when the PI3K/AKT/mTORC1 signaling pathway is activated^{20, 21}. By changing the subcellular localization, FRAT can control GSK3 β activity towards cytoplasmic or nuclear substrates in response to PI3K/AKT/mTORC1 signalling. More recently, high expression of FRAT has been reported to confer rapamycin resistance in several cancer cell lines by reducing the population of nuclear GSK3 β that catalyzes inhibitory phosphorylation of nuclear proteins that repress cell growth²¹. Therefore, it is tempting to argue that hDISCtide could also normalize the PI3K/AKT/mTORC1 signaling pathway by preventing FRAT-dependent shuttling of GSK3 β out of the nucleus and promotes GSK3 β phosphorylation of its nuclear substrates.

GSK3 β has attracted substantial pharmaceutical investments due to its implication in multiple diseases including type 2 diabetes, Alzheimer's disease (AD), bipolar disorder, and cancer. A recent paper outlining AstraZeneca's 20+ years of experience in developing small molecules that target GSK3 β in hope to treat AD showed that work on their best clinical candidates were terminated due to toxicity²². Our work presents a novel therapeutic strategy in designing peptide-based inhibitors of GSK3 β . Our data indicates that hDISCtide is a non-competitive inhibitor to both ATP and the substrate peptide GSP2. In particular,

inhibitors that compete with the substrate peptide binding site offer new guidance for drug design that are more specific for GSK3 β ²³. However, peptide-based CNS therapeutics face their own challenges since they have short half-lives and a low penetrability of the BBB. Intensive research is currently underway to develop new strategies that will allow therapeutic peptides to be specifically transported to brain tissues. An example of this is peptide modification (N-terminal acylation, glycosylation, halogenation) used to increase BBB perviousness and peptide delivery through the use of nanoparticles which have shown recent success^{24, 25}. Future structural and cell-based studies will be necessary to evaluate the efficacy and further develop hDISCtide as a therapeutic for CNS disorders.

METHODS

Peptides

All peptides used in this study were synthesized by NeoBioLab or Genscript. FRATide: DPHRLQLQLVLSGNLIKEAVRRLHS (GenScript); AxinGID: VEPQKFAEELIHRLEAVQ (Genscript); hDISCtides sequences are as listed in Figures 1A, G, 4C.

Small Molecule Kinase Inhibitors

CHIR-99021 was purchased from Stemgent (04-0004-10). Roscovitine was purchased from Calbiochem (186692-46-6).

GSK3 β Cloning, Expression, and Purification

Four different sources of GSK3 β was used in this study: 1) GST-GSK3 β purchased from BP-Biosciences (BPS Biosciences Cat 40007); 2) GSK3 β made in house for the competition experiment between hDISCtide and FRATide by ADP-Glo assays; 3) His-GSK3 β -His made in house for the binding assays using SPR; 4) 6X-HisGSK3 β purchased from Signalchem (G09-10H-05) for BLI and MST experiments. *Cloning:* The codon optimized human GSK3 β gene was synthesized and subcloned into the pST44 backbone containing an N-terminal histidine tag (pST44-HisNGSK3 β)²⁶. An additional histidine tag was inserted at the C-terminus of the reading frame (pST44-HisNGSK3 β His).

Protein Overexpression and Purification: All constructs were transformed in BL21(DE3)star. Seed cultures were inoculated in 1-2 L TB media. The cultures were induced with 0.15 mM IPTG at OD=0.1-0.2 at 16-17 °C for 35-40 hours. Cells were resuspended in lysis buffer (50 mM Hepes pH 7.2, 50 mM NaCl, 5% Glycerol, 1 mM PMSF) and lysed by cell disruption (Constant systems LTD 0.75KW, two passes with 25K Psi) at 4 °C. The lysate was centrifuged at 40,000 x g for 40-60 mins. The supernatant was diluted 1:10 in dilution buffer (50 mM Hepes pH 7.2, 5% glycerol, 50 mM NaCl) and loaded on an SP-Sepharose-FF column equilibrated with Buffer A (50 mM Hepes pH 7.2) and washed for 20-40 column volume. Bound proteins were eluted in a linear gradient with 100% Buffer B (Buffer A + 1 M NaCl). Fractions containing

GSK3 β were pooled and loaded onto a NiNTA column equilibrated with Buffer C (50 mM Tris pH 7.5, 300 mM NaCl, 12.5 mM imidazole) and washed for 10-20 column volumes. His-GSK3 β was eluted in a 100% step gradient with Buffer D (Buffer C + 250 mM imidazole). Protein was pooled and cleaved with TEV at 1:10 and dialyzed in 50 mM Hepes pH 7.2, 500 mM NaCl, 5 mM beta-mercaptoethanol overnight. For some batches, cleaved GSK3 β was already pure enough. Otherwise, an additional size-exclusion chromatography was performed with buffer (50 mM Hepes pH 7.2, 500 mM NaCl, 2 mM MgCl₂, 1 mM TCEP). Pure GSK3 β was flash-frozen in liquid nitrogen and stored at -80 °C. For SPR studies, His-GSK3 β -His protein was stored without TEV cleavage.

Surface Plasmon Resonance (SPR)

SPR studies were conducted using the Reichert 2-channel system (Model: 2SPR). The Nickel Nitrilotriacetic Acid (Ni-NTA) Sensor Chip (Reichert CA# 13206065) was first prepared by flowing a NiSO₄ solution overtop to capture the Ni⁺ ions which primed it for protein capture. SPR assay was conducted in running buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.01% Tween 20, 1 mM TCEP). Between 500-2000 RU of His-GSK3 β -His were captured onto the Ni-coated surface. Once the baseline was stable (<5 RU/min), analytes were injected at 25 μ l/min for 2 mins association time and 2 mins dissociation time. The NiNTA chip surface was regenerated with the stripping buffer (20 mM NaPhos pH 8.5, 300 mM NaCl, 50 mM EDTA) or the regeneration buffer (50 mM Hepes pH 7.2, 300 mM NaCl, 250 mM imidazole) and 50 mM NaOH solution. Each regeneration solution was injected at 100 μ l/min for 2 mins. Each chip could be regenerated for up to ~10 times before the capture level decreased significantly. Results were analyzed and fitted using TraceDrawer 1.6.1 (Ridgeview Instruments AB). For datasets that required regeneration after every injection, local fitting of Bmax for each curve was performed to account for differences in capture level.

ADP-Glo Kinase Assay

Reagents for the ADP-Glo assay were purchased from Promega (Cat V9102). For GSK3 β kinase assay, reactions were performed with 9 μ M GSK3 β substrate peptide (YRRAAVPPSPSLSRHSSPHQ S(PO₃H₂)EDEEE), 15 μ M ATP, 10-25 nM GSK3 β in reaction buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 0.01% Brij-35, 3 mM DTT). The protein, substrate, and GSK3 β ligands were preincubated for 15 minutes before the addition of ATP. Reactions were allowed to proceed for 30 minutes. ADP production was monitored by luminescence as described in the Promega manual.

For CDK5 kinase assay, the reaction contains 10 nM CDK5-p25 (a gift from Kosik lab), 40 μ M ATP, 20 μ M cdk5 substrate peptide (PKTPKKAKKL, Anaspec #60026-1). The reaction was allowed to proceed for 25 minutes. The luminescence due to kinase activity was quantified using a luminescence plate reader as per the manufacturer's recommendations (Envision).

Time-Resolved Fluorescence Energy Transfer (TR-FRET) Assay

Reagents for TR-FRET assays were purchased from Perkin Elmer. Reactions were performed with 50 nM ULight-Glycogen Synthase (Ser641/pSer657) peptide (Perkin Elmer #TRF0131-M), 15 μ M ATP, 1 nM GSK3 β in the same reaction buffer as above. The protein, substrate, and GSK3 β ligands were preincubated for 15 minutes before the addition of ATP. Reactions were allowed to proceed for 25 minutes. Reaction termination and detection were performed using 1X LANCE detection buffer with 20 mM EDTA and 2 nM LANCE Ultra Europium anti-phospho Glycogen Synthase (Ser641) (Perkin Elmer #TRF0220-M).

Microscale Thermophoresis (MST) and Bioilayer Interferometry (BLI) Assays

The MST assays were performed as described previously²⁷. Similarly, the BLI assays were conducted as described²⁸, with the use of biotinylated 6X-His-GSK3 β as the recombinant protein.

Data Analysis

All data were analyzed using GraphPad Prism 8.0.1 (GraphPad Software Inc.).

ABBREVIATIONS

ADP-GLO – Adenosine Di Phosphate Glow

ADP – Adenosine Di Phosphate

BBB – Blood Brain Barrier

CNS – Central Nervous System

DISC1 – Disrupted in Schizophrenia 1

FRAT – Frequently Rearranged in Advanced T Cell Lymphoma

GS - Glycogen Synthase

GSK3 β – Glycogen Synthase Kinase 3 Beta

GST – Glutathione S-transferase

NiNTA – Nickel Nitrilotriacetic Acid

SP-FF – SP Sepharose Fast Flow

SP-HP – SP Sepharose High Performance

SPR – Surface Plasmon Resonance

TEV – Tobacco Etch Virus

Wnt - Wingless/Integrated

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REFERENCES

1. Kessler, R. C.; Chiu, W. T.; Demler, O.; Merikangas, K. R.; Walters, E. E., Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry* **2005**, *62* (6), 617-27.
2. Ritchie, H.; Roser, M., Mental Health. In *Our World in Data*, 2019.
3. Patel, P.; Woodgett, J. R., Glycogen Synthase Kinase 3: A Kinase for All Pathways? *Curr Top Dev Biol* **2017**, *123*, 277-302.
4. Beurel, E.; Grieco, S. F.; Jope, R. S., Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* **2015**, *148*, 114-31.
5. Cormier, K. W.; Woodgett, J. R., Recent advances in understanding the cellular roles of GSK-3. *Fl1000Research* **2017**, *6*.
6. Sutherland, C., What Are the bona fide GSK3 Substrates? *Int J Alzheimers Dis* **2011**, *2011*, 505607.
7. Liang, S. H.; Chen, J. M.; Normandin, M. D.; Chang, J. S.; Chang, G. C.; Taylor, C. K.; Trapa, P.; Plummer, M. S.; Para, K. S.; Conn, E. L.; Lopresti-Morrow, L.; Lanyon, L. F.; Cook, J. M.; Richter, K. E.; Nolan, C. E.; Schachter, J. B.; Janat, F.; Che, Y.; Shanmugasundaram, V.; Lefker, B. A.; Enerson, B. E.; Livni, E.; Wang, L.; Guehl, N. J.; Patnaik, D.; Wagner, F. F.; Perlis, R.; Holson, E. B.; Haggarty, S. J.; El Fakhri, G.; Kurumbail, R. G.; Vasdev, N., Discovery of a Highly Selective Glycogen Synthase Kinase-3 Inhibitor (PF-04802367) That Modulates Tau Phosphorylation in the Brain: Translation for PET Neuroimaging. *Angew Chem Int Ed Engl* **2016**, *55* (33), 9601-5.
8. Wagner, F. F.; Bishop, J. A.; Gale, J. P.; Shi, X.; Walk, M.; Ketterman, J.; Patnaik, D.; Barker, D.; Walpita, D.; Campbell, A. J.; Nguyen, S.; Lewis, M.; Ross, L.; Weiwer, M.;

- An, W. F.; Germain, A. R.; Nag, P. P.; Metkar, S.; Kaya, T.; Dandapani, S.; Olson, D. E.; Barbe, A. L.; Lazzaro, F.; Sacher, J. R.; Cheah, J. H.; Fei, D.; Perez, J.; Munoz, B.; Palmer, M.; Stegmaier, K.; Schreiber, S. L.; Scolnick, E.; Zhang, Y. L.; Haggarty, S. J.; Holson, E. B.; Pan, J. Q., Inhibitors of Glycogen Synthase Kinase 3 with Exquisite Kinome-Wide Selectivity and Their Functional Effects. *ACS Chem Biol* **2016**, *11* (7), 1952-63.
9. Dajani, R.; Fraser, E.; Roe, S. M.; Yeo, M.; Good, V. M.; Thompson, V.; Dale, T. C.; Pearl, L. H., Structural basis for recruitment of glycogen synthase kinase 3 β to the axin-APC scaffold complex. *EMBO J* **2003**, *22* (3), 494-501.
 10. Stamos, J. L.; Chu, M. L.; Enos, M. D.; Shah, N.; Weis, W. I., Structural basis of GSK-3 inhibition by N-terminal phosphorylation and by the Wnt receptor LRP6. *eLife* **2014**, *3*, e01998.
 11. Bem, J.; Brozko, N.; Chakraborty, C.; Lipiec, M. A.; Kozinski, K.; Nagalski, A.; Szewczyk, L. M.; Wisniewska, M. B., Wnt/ β -catenin signaling in brain development and mental disorders: keeping TCF7L2 in mind. *FEBS Lett* **2019**.
 12. Mao, Y.; Ge, X.; Frank, C. L.; Madison, J. M.; Koehler, A. N.; Doud, M. K.; Tassa, C.; Berry, E. M.; Soda, T.; Singh, K. K.; Biechele, T.; Petryshen, T. L.; Moon, R. T.; Haggarty, S. J.; Tsai, L. H., Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3 β / β -catenin signaling. *Cell* **2009**, *136* (6), 1017-31.
 13. Bhat, R. V.; Budd Haeberlein, S. L.; Avila, J., Glycogen synthase kinase 3: a drug target for CNS therapies. *J Neurochem* **2004**, *89* (6), 1313-7.
 14. Copeland, R. A., Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists. *Methods of biochemical analysis* **2005**, *46*, 1-265.

15. Bax, B.; Carter, P. S.; Lewis, C.; Guy, A. R.; Bridges, A.; Tanner, R.; Pettman, G.; Mannix, C.; Culbert, A. A.; Brown, M. J.; Smith, D. G.; Reith, A. D., The structure of phosphorylated GSK-3 β complexed with a peptide, FRATtide, that inhibits beta-catenin phosphorylation. *Structure* **2001**, *9* (12), 1143-52.
16. Thomas, G. M.; Frame, S.; Goedert, M.; Nathke, I.; Polakis, P.; Cohen, P., A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of axin and beta-catenin. *FEBS Lett* **1999**, *458* (2), 247-51.
17. Singh, K. K.; Ge, X.; Mao, Y.; Drane, L.; Meletis, K.; Samuels, B. A.; Tsai, L. H., Dixdc1 is a critical regulator of DISC1 and embryonic cortical development. *Neuron* **2010**, *67* (1), 33-48.
18. Nakata, K.; Lipska, B. K.; Hyde, T. M.; Ye, T.; Newburn, E. N.; Morita, Y.; Vakkalanka, R.; Barenboim, M.; Sei, Y.; Weinberger, D. R.; Kleinman, J. E., DISC1 splice variants are upregulated in schizophrenia and associated with risk polymorphisms. *Proc Natl Acad Sci U S A* **2009**, *106* (37), 15873-8.
19. van Amerongen, R.; Nawijn, M.; Franca-Koh, J.; Zevenhoven, J.; van der Gulden, H.; Jonkers, J.; Berns, A., Frat is dispensable for canonical Wnt signaling in mammals. *Genes Dev* **2005**, *19* (4), 425-30.
20. Bechard, M.; Trost, R.; Singh, A. M.; Dalton, S., Frat is a phosphatidylinositol 3-kinase/Akt-regulated determinant of glycogen synthase kinase 3 β subcellular localization in pluripotent cells. *Mol Cell Biol* **2012**, *32* (2), 288-96.
21. He, L.; Fei, D. L.; Nagiec, M. J.; Mutvei, A. P.; Lamprakis, A.; Kim, B. Y.; Blenis, J., Regulation of GSK3 cellular location by FRAT modulates mTORC1-dependent cell growth and sensitivity to rapamycin. *Proc Natl Acad Sci U S A* **2019**, *116* (39), 19523-19529.

22. Bhat, R. V.; Andersson, U.; Andersson, S.; Knerr, L.; Bauer, U.; Sundgren-Andersson, A. K., The Conundrum of GSK3 Inhibitors: Is it the Dawn of a New Beginning? *J Alzheimers Dis* **2018**, *64* (s1), S547-S554.
23. Eldar-Finkelman, H.; Licht-Murava, A.; Pietrokovski, S.; Eisenstein, M., Substrate competitive GSK-3 inhibitors - strategy and implications. *Biochim Biophys Acta* **2010**, *1804* (3), 598-603.
24. Salameh, T. S.; Banks, W. A., Delivery of therapeutic peptides and proteins to the CNS. *Adv Pharmacol* **2014**, *71*, 277-99.
25. Morimoto, B. H., Therapeutic peptides for CNS indications: Progress and challenges. *Bioorg Med Chem* **2018**, *26* (10), 2859-2862.
26. Tan, S.; Kern, R. C.; Selleck, W., The pST44 polycistronic expression system for producing protein complexes in Escherichia coli. *Protein Expr Purif* **2005**, *40* (2), 385-95.
27. Bernard-Gauthier, V.; Mossine, A. V.; Knight, A.; Patnaik, D.; Zhao, W. N.; Cheng, C.; Krishnan, H. S.; Xuan, L. L.; Chindavong, P. S.; Reis, S. A.; Chen, J. M.; Shao, X.; Stauff, J.; Arteaga, J.; Sherman, P.; Salem, N.; Bonsall, D.; Amaral, B.; Varlow, C.; Wells, L.; Martarello, L.; Patel, S.; Liang, S. H.; Kurumbail, R. G.; Haggarty, S. J.; Scott, P. J. H.; Vasdev, N., Structural Basis for Achieving GSK-3beta Inhibition with High Potency, Selectivity, and Brain Exposure for Positron Emission Tomography Imaging and Drug Discovery. *J Med Chem* **2019**, *62* (21), 9600-9617.
28. Patnaik, D.; Pao, P.-C.; Zhao, W.-N.; Silva, M. C.; Hylton, N. K.; Chindavong, P. S.; Pan, L.; Tsai, L.-H.; Haggarty, S. J., Exifone is a Potent HDAC1 Activator with Neuroprotective Activity in Human Neuronal Models of Neurodegeneration. *bioRxiv* **2020**, 2020.03.02.973636.

29. Soares, D. C.; Carlyle, C. C.; Bradshaw, N. J.; Porteous, D. J., DISC1: Structure, Function, and Therapeutic Potential for Major Mental Illness. *ACS Chem. Neurosci.* **2011**, 2 (11), 609-632.
30. Sanchez-Pulido, L.; Ponting, C. P., Structure and evolutionary history of DISC1. *Hum Mol Genet* **2011**.
31. Singh, K. K.; De Rienzo, G.; Drane, L.; Mao, Y.; Flood, Z.; Madison, J.; Ferreira, M.; Bergen, S.; King, C.; Sklar, P.; Sive, H.; Tsai, L. H., Common DISC1 polymorphisms disrupt Wnt/GSK3beta signaling and brain development. *Neuron* **2011**, 72 (4), 545-58.

FIGURE LEGENDS

FIGURE 1. A) Schematics of the full-length DISC1 isoform. hDISCtide (residues 193-236) from the full-length protein is the most potent GSK3 β inhibitory region of DISC1. The N-terminal region of DISC1 is predicted to be disordered, whereas the C-terminal domains contain several regions with high helical content that can self-associate or interact with other proteins²⁹. Two stretches of the sequence are similar to the UVR-like domain, which is a conserved protein-protein interaction domain folded into an anti-parallel first identified in the bacterial endonuclease³⁰. No other sequence similarity was noted in other regions of the protein²⁹. A83V, R264Q, L607F, and S704C are disease-associated variants of DISC1. A83V, R264Q, and L607F have been shown to impact Wnt/GSK3 β signaling while S704C does not have any effect³¹. **B) hDISCtide inhibition of GSK3 β by ADP-Glo assay.** hDISCtide inhibits GSK3 β with an IC₅₀ of 95 nM. The reaction contains 10 nM GSK3 β , 15 μ M ATP, and 9 μ M of the substrate peptide GSP2. Reaction was allowed to proceed for 20 min. The data were fitted with the equation for sigmoidal dose-response (variable slope) to estimate the IC₅₀ ($R^2=0.9411$). **C) hDISCtide binds GSK3 β with slow kinetics.** ~500-2000 RU of double His-tagged GSK3 β was captured on a Ni-NTA chip. The concentration of hDISCtide injected was ranged from 1.79-28.6 μ M. The surface was regenerated after every injection as little dissociation of the peptide was observed. The data were fitted to a OneToOne model with $k_a=495 \text{ M}^{-1}\text{s}^{-1}$ and $k_d=2.49 \times 10^{-4} \text{ s}^{-1}$ ($\chi^2=0.88$). **D) Binding affinity of hDISCtide to GSK3 β measured by microscale thermophoresis assay.** Fraction of GSK3 β bound with hDISCtide. The data were fitted with the equation for sigmoidal dose-response (variable slope) to estimate the K_D of 5.7 μ M ($R^2=0.9720$). **E) hDISCtide inhibition is sequence specific.** Percent GSK3 β inhibition by 10 μ M of hDISCtide and its mutants. Single site mutation of S216P reduces the inhibition potency while A220P shows no inhibition. The double mutant F210A_F212A completely abolished hDISCtide inhibition. **F) hDISCtide does not inhibit CDK5, a structurally similar kinase to GSK3 β .** Roscovitine, a small molecule general kinase inhibitor, is the positive control of the CDK5 assay. Percent CDK5 inhibition by 11 μ M of hDISCtide and roscovitine. **G)**

Sequences of hDISCtide for Figure E. Top sequence is the wild-type sequence. Highlighted in red are the mutated residues.

FIGURE 2 Replots of the Michaelis-Menten kinetic parameters show that hDISCtide is a non-competitive inhibitor of GSK3 β with respect to ATP and GSP2. **A)** To determine if hDISCtide can compete directly with ATP, GSK3 β enzymatic reactions were carried out in the absence of GSP2. $^{ATP}V_{max}$ and $^{ATP}K_m$ were obtained from data fitted to the Michaelis-Menten equation (Supplementary Figure 1A). **i)** Replots of $^{ATP}V_{max}$ as a function of increasing hDISCtide concentration. $^{ATP}V_{max}$ decreases curvilinearly with increasing hDISCtide concentration, indicating an absence of competition¹⁴. **ii)** Replots of $^{ATP}K_m$ as a function of increasing hDISCtide concentration. $^{ATP}K_m$ appears to increase curvilinearly with increasing hDISCtide concentration, leading us to conclude that the mechanism of inhibition is non-competitive with $\alpha > 1$ ¹⁴. **iii)** Replots of $^{ATP}V_{max}/^{ATP}K_m$ showing the ratio decreases curvilinearly with increasing hDISCtide concentration, thus ruling out un-competitive inhibition¹⁴. **B)** To determine if hDISCtide can compete directly with the substrate peptide GSP2, GSK3 β enzymatic reactions were carried out with a fixed concentration of ATP and varying concentrations of hDISCtide and GSP2. $^{GSP2}V_{max}$ and $^{GSP2}K_m$ were obtained from data fitted to the Michaelis-Menten equation (Supplementary Figure 1B). **i)** Replots of $^{GSP2}V_{max}$ as a function of increasing hDISCtide concentration. $^{GSP2}V_{max}$ decreases curvilinearly with increasing hDISCtide concentration, indicating an absence of competition¹⁴. **ii)** Replots of $^{GSP2}K_m$ as a function of increasing hDISCtide concentration. $^{GSP2}K_m$ appears to decrease curvilinearly with increasing hDISCtide concentration, leading us to conclude that the mechanism of inhibition is non-competitive with $\alpha < 1$ ¹⁴. **iii)** Replots of $^{GSP2}V_{max}/^{GSP2}K_m$ showing the ratio decreases curvilinearly with increasing hDISCtide concentration, thus ruling out un-competitive inhibition¹⁴. **C)** As a comparison, similar mechanistic analyses were performed with the well-established ATP competitive small molecule inhibitor of GSK3 β , CHIR-99021. GSK3 β enzymatic reactions were carried out in the absence of GSP2. $^{ATP}V_{max}$ and $^{ATP}K_m$ were obtained from data fitted to the Michaelis-Menten equation (Supplementary Figure 1C). **i)** Replots of

$1/^{ATP}V_{app}$ as a function of increasing hDISCtide concentration. Varying hDISCtide concentration has no effect on $1/^{ATP}V_{app}$, indicating pure competitive inhibition¹⁴. **ii)** Replots of $^{ATP}K_m$ as a function of increasing CHIR-99021 concentration. $^{ATP}K_m$ increases linearly with increasing CHIR-99021 concentration, a signature trend for ATP competitive inhibitors. **iii)** Replots of $^{ATP}V_{max}/^{ATP}K_m$ showing the ratio decreases curvilinearly with increasing hDISCtide concentration, thus ruling out un-competitive inhibition¹⁴.

FIGURE 3 FRATide and hDISCtide partially shares binding site. A) FRATide binds GSK3 β with a K_D of 3.8 μ M measured by the BLI assay. The equilibrium dissociation constant (K_D) was estimated by steady-state analysis using the response signal at equilibrium for each FRATide concentration (Supplementary Figure 3). The data were fitted globally with the 1:1 binding model with the equation $Response=(R_{max}*Conc.)/K_D + Conc)$, from which the K_D is estimated at the concentration where 50% of R_{max} is reached. **B-C)** Real-time binding analysis of FRATide to GSK3 β by SPR. FRATide binding to GSK3 β has faster kinetics compared to hDISCtide. ~500-2000 RU of double his-tagged GSK3 β was captured on a NiNTA chip. The concentrations of FRATide injected were 0.2, 0.35, 0.7, 1.5, 4, 8 μ M. The data was fitted to a OneToOne TwoState model with $k_{a1}=9940\text{ M}^{-1}\text{s}^{-1}$, $k_{d1}=6.50\times10^{-2}\text{ s}^{-1}$, $k_{a2}=1.62\times10^{-2}\text{ M}^{-1}\text{s}^{-1}$, $k_{d2}=8.09\times10^{-3}\text{ s}^{-1}$ ($\chi^2=0.44$), $K_D=3.2\text{ }\mu\text{M}$. The K_D from equilibrium analysis is 1.9 μ M. **D) Real-time analysis of the co-binding of hDISCtide and FRATide to GSK3 β by SPR.** 3 $\mu\text{g/ml}$ (~64 nM) double his-tagged GSK3 β was incubated with 4 μM of hDISCtide before capturing (~553 RU). Injecting 4 μM of hDISCtide did not generate any response (red). Subsequent injection of 4 μM FRATide gave a response with comparable kinetics as that observed on a surface captured with unbound GSK3 β in B (blue). **E-F) FRATide abolishes hDISCtide inhibitory activity against GSK3 β .** E) ADP-Glo luminescent assay to compare GSK3 β activity in the presence of FRATide and/or hDISCtide. 25 μM of FRATide and/or hDISCtide was added to each condition containing 25 nM of GSK3 β , 9 μM GSP2, 100 μM ATP in reaction buffer. F) Double titration curves of hDISCtide and FRATide. FRATide at a concentration of 2.5 μM and

higher eliminated inhibition potency of hDISCtide against GSK3 β . At 0.5 μ M concentration, FRATide increased the IC₅₀ of hDISCtide ~3 fold.

FIGURE 4. Inhibitory action of hDISCtide truncations on GSK3 β activity. Different truncations of hDISCtide were tested for their ability to inhibit GSK3 β using the ADP-Glo assay. The N-terminal 13 residues were dispensible to hDISCtide inhibition potency whereas trimming the C-terminus by 4 residues were detrimental to the inhibitory action of hDISCtide. **A)** Dose-response inhibition of hDISCtide and its truncated constructs against GSK3 β . **B)** Percent inhibition of GSK3 β by the different hDISCtide truncation constructs. **C)** Sequences of the hDISCtide constructs for panels 5A and 5B.

FIGURE 5. Crystal structures of GSK3 β in different conformations. **A)** Catalytically competent conformation of GSK3 β . GSK3 β bound to the scaffold peptide Axin (magenta). The catalytic conformation of the enzyme consists of the glycine-rich loop (cyan), the C-loop (yellow), and the activation loop (red). The scaffold binding regions consist of a helix and a loop (blue). The substrate peptide binding pocket is bound by the pseudo-substrate peptide formed by the N-terminus (residues 6-10) with a phosphorylated Ser9. The pseudo substrate peptide, ADP, and the transition analog AIF3 are shown in stick model (PDB:4NU1). **B)** GSK3 β bound to the scaffold peptide FRATide (magenta) (PDB:1GNG). **C)** Models of hDISCtide inhibition mechanism. GSK3 β activity depends on the three regions forming a catalytic conformation (Figure 6A), represented by the orange rectangle. hDISCtide could inhibit activity by preventing the formation of a catalytic competent GSK3 β , as represented by an orange oval. hDISCtide has two binding regions: hDISCtide-GB and hDISCtide-GI. hDISCtide-GI binds to the orange oval to block GSK3 β from forming catalytic competent conformation (i.e. orange rectangle). FRATide only affects the binding of hDISCtide-GI but not hDISCtide-GB. It can either block hDISCtide-GI from interacting to its binding site or changes the conformation of the GI binding site such that hDISCtide-GI can no longer bind.

Figure 1

A Variable hDISCtide and ATP, no GSP2

B Variable hDISCtide and GSP2, constant ATP

C Variable CHIR-99021 and ATP, no GSP2

Figure 2

Figure 3

Figure 4

Figure 5