Identification of a Unique Inhibitor Binding Site on Choline Kinase alpha

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Running title: *Choline kinase – TCD-717 complex structure*

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ABSTRACT

Choline kinase alpha (ChoKα) is an enzyme that is upregulated in many types of cancer and has been shown to be tumorigenic. As such, it makes a promising target for inhibiting tumor growth. Though there have been several inhibitors synthesized for ChoKα, not all of them demonstrate the same efficacy *in vivo*, though the reasons behind this difference in potency are not clear. One particular inhibitor, designated TCD-717, has recently completed phase I clinical trials. Cell culture and *in vitro* studies support the powerful inhibitory effect TCD-717 has on ChoKα, but an examination of the inhibitor's interaction with the ChoKα enzyme has been missing prior to this work. Here we detail the 2.35 Å structure of ChoKα in complex with TCD-717. Examination of this structure in conjunction with kinetic assays reveals that TCD-717 does not bind directly in the choline pocket as do previously characterized ChoKα inhibitors, but rather in a proximal but novel location near the surface of the enzyme. The unique binding site identified for TCD-717 lends insight for the future design of more potent *in vivo* inhibitors for ChoKα.

INTRODUCTION

Human Choline Kinase alpha (ChoKα) is an enzyme responsible for the production of phosphocholine (pCho) in the cell.[1] It catalyzes the gamma-phosphoryl transfer from ATP to choline, yielding pCho and ADP (Fig. 1A), and is the first enzyme in the Kennedy Pathway, which ultimately leads to the production of phosphatidylcholine for membranes. ChoKα is of interest to cancer biologists because it has been found to be upregulated in many cancers, such as breast, lung, colorectal, and prostate,[2] and has been shown to be oncogenic.[3] Further observations that support a link between ChoKα and cancer include: increased intracellular levels of the enzyme are associated with malignant phenotype and poor prognosis[4]; increased ChoKα activity levels correlate with higher tumor grade[5]; and increased availability of pCho in the cell is associated with a more aggressive cancer phenotype.[6] These data evaluated together indicate that ChoKα is a prime target for chemotherapeutic drug design.

We were the first to report the structures of ChoKα1 (hereafter ChoKα; α1 is the full-length 457 residue protein, while α2 is a splice variant missing residues 155-172; the variant studied here is α1) in complex with ADP or pCho. These structural studies revealed a buried choline-binding pocket in the C-terminal domain lined with hydrophobic residues and a more exposed ATP binding site in the N-terminal domain, as well as different enzyme conformations between the pCho and ADP bound enzyme.[7] Recently it was proposed that the ChoKα phosphoryl transfer reaction proceeds via a covalent enzyme intermediate, with Asp306 being the putative catalytic residue,[8] consistent with the fact that its mutation to alanine results in an inactive enzyme.[9] This ping-pong mechanism has likewise been reported for the *Plasmodium falciparum* choline kinase.[10]

TCD-717 is a ChoKα inhibitor that has recently completed phase I clinical trials (https://clinicaltrials.gov/ct2/show/NCT01215864) and is thus of interest to researchers (Fig 1B). Previously, numerous compounds have been examined as ChoKα inhibitors, and for several of

those, crystal structures of the compound bound to ChoKα have been determined (as of January 2018, 14 ChoKα-inhibitor structures were available at RCSB[11-18]). The examined compounds invariably bind at or near the choline-binding site. Additionally, many of the crystalized inhibitors share the characteristic of having a bulky linker between two identical or similar head groups (Fig. 1C), where one of these head groups occupies the choline-binding site. Hemicholium-3 (HC-3, PDB ID: 3G15), a first generation ChoKα inhibitor, exhibits high

Figure 1

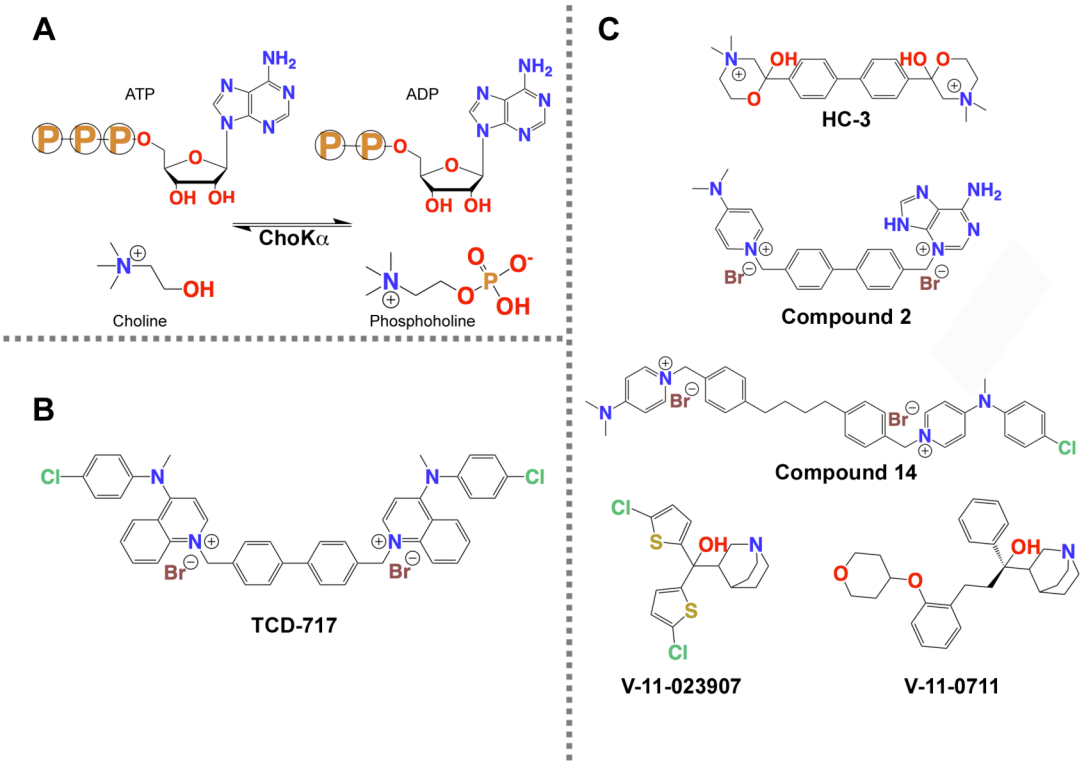


Figure 1. Schematic of ChoKα reaction components and of inhibitors: *A*, Schematic of the reaction catalysed by ChoKα. The gamma-phosphate moiety of ATP is transferred to choline's hydroxyl group to yield ADP and phosphocholine. ChoKα can also use ethanolamine as a substrate. *B*, Structural representation of TCD-717. *C*, Structural representations of the ChoKα inhibitors used for comparisons in this work. All inhibitors have published crystal structures in complex with ChoKα associated with them except V-11-0711, which is derived from V-11-023907.

toxicity that prevents its clinical use,[12, 19, 20] but has been used as a scaffolding model for further inhibitor design such as published inhibitors designated compound 2 and compound 14 (PDB IDs: 4BR3 and 4CG8, respectively).[11] Other second-generation inhibitors derived from HC-3, MN58b and TCD-717 (also called RSM-932A), exhibit improved toxicity profiles. MN58b has demonstrated therapeutic efficacy in several cancers such as glioblastoma, colon cancer xenografts, and carcinoma models.[21-23] Whereas MN58b is challenged by toxicity at concentrations close to its therapeutic window, TCD-717 displays a much improved safety profile. Specifically, when TCD-717 was administered to xenograft mice to treat HT29 colon adenocarcinomas, it demonstrated 70% inhibition of tumor growth through the end of treatment.[24] Though there were moderate effects of liver toxicity in daily administrations of drug, a short schedule of weekly i.p. injections showed no associated liver toxicity and was still associated with a 77% reduction in tumor activity.[24] Therefore, while TCD-717 could still have long-term toxicity effects, in the short term it is a very effective anti-tumor agent for a variety of cancers, including not only colon adenocarcinomas but also non-squamous cell lung carcinomas and breast adenocarcinomas.[24] It is also worth noting that there are additional ChoKα inhibitors such as V-11-0711 and V-11-023907.[14] These inhibitors differ from TCD-717 or MN58b because while they still bind in the choline pocket, they are smaller and do not contain symmetrical head groups connected by linkers.

Since TCD-717 has a similar overall structure to many ChoKα co-crystallized inhibitors, in that it contains aromatic ring structures connected by a long linker, it has been assumed that TCD-717 binds in much the same fashion as published inhibitors, i.e. by occupying the length of the choline-binding pocket of ChoKα, as seen with its precursor HC-3. However, whereas kinetic analysis of HC-3 and MN58b revealed that both compounds show inhibition profiles that are not competitive with ATP or Cho, TCD-717 showed uncompetitive behavior.[10] Though no crystal structure has been published for MN58b, comparison of TCD-717 against HC-3

suggested that TCD-717 might bind differently compared to other ChoKα inhibitors; whether this was the case, or what the alternative binding site was, remained unknown. Likewise, there have been multiple suggestions as to why certain inhibitors are more effective than others at arresting cell growth. Evidence suggests that simply preventing pCho production is not sufficient to prevent tumor growth. Alternative mechanisms examine both a scaffolding role of ChoKα or a regulatory role involving Tyr333.[8, 25] The mechanism of how inhibitors might affect either of these roles remains unknown.

Despite the number of inhibitors that have been classified for ChoKα, TCD-717 is the first of these to enter phase I clinical trials and show promise as a cancer treatment. To obtain increased understanding of how ChoKα binds molecules with demonstrated potential therapeutic value, we solved the crystal structure of ChoKα in complex with TCD-717. Comparing this structure to previously published inhibitor-bound ChoKα structures reveals the unexpected result that TCD-717 does not bind directly in the choline pocket as expected, but instead to a unique region outside the catalytic cavity. With the support of kinetic studies, we show that TCD-717 is an effective inhibitor with a unique mode of binding to ChoKα.

MATERIALS AND METHODS

Materials: All chemicals were reagent or molecular biology grade. Platinum *Pfu* polymerase and DNA size markers were from ThermoScientific. dNTPs were from Promega. Restriction endonucleases and Sticky-End Master Mix Ligase were from New England Biolabs. Agarose gel purification, PCR product clean-up, and plasmid mini prep columns were all products of Qiagen. Human liver cDNA library was a product of ResGen (Invitrogen). All oligonucleotides were supplied by IBA GmbH, Göttingen, Germany. Choline and pyruvate kinase were from Sigma. ATP, NADH, phosphoenolpyruvate, and lactate dehydrogenase were purchased from Roche. TCD-717 was obtained from PepTech Corp (Bedford, MA).

Gene Synthesis, Cloning, Protein Expression, and Purification of ChoKα2Δ79 for Kinetic and Structural Analysis: The Δ79N Choline Kinase α1 construct was derived from the previously made GST-tagged Choline Kinase α1 Δ49N vector available in the lab.[7] A DNA amplification primer containing an *Nde*I site was made in the forward (5'-GGA ATT CCA TAT GCC GCC GCA GCC GCC CGC AGA CGA G-3') direction, while T7 Terminator standard primer was used for the reverse direction. These primers were used to amplify ChoKα1 with an N-terminal deletion of 79 residues. The gene was cleaved with *Nde*I and *Bam*HI-HF (New England BioLabs) and subsequently ligated into a modified version of the pET14b expression vector containing an N-terminal His_6 tag followed by SUMO protease site. The resulting DNA construct (verified by Sanger sequencing) was transformed into the Rosetta (DE3)pLysS *E. coli* strain. Cells were grown at 37 ºC in 2xYT medium, which was supplemented with 100 µg/mL Ampicillin (Amp) and 34 µg/mL Chloramphenicol (Chlor). Protein expression was induced with 0.1 – 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) once an OD_{600nm} of 0.6 – 0.7 was reached and cultured overnight at 22 ºC. Cells were harvested by centrifugation, washed with 200 mM KCl, 25 mM Tris pH 7.5, and 10 mM $MgCl_2$, and centrifuged at 5000 rpm for 20 min

before storage at -20^{o} C. After thawing, cells were lysed by sonication in 25 mM Tris pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, 10 mM imidazole pH 7.5, 10% glycerol, 1% Triton X-100, and 1 mM PMSF. Lysed cells were centrifuged at 20,000 rpm for 30 min. Clarified supernatant was loaded onto 5 mL HisTrap HP Ni^{2+} Sepharose column (GE Healthcare), and the column washed with 25 mM Tris pH 7.5, 500 mM NaCl, supplemented with 25 mM and 50 mM imidazole. Protein was eluted in a single fraction in the same buffer supplemented with 500 mM imidazole. The His_6-SUMO tag was cleaved with SUMO protease while dialyzing against 25 mM Tris, pH 7.5, 500 mM NaCl, 10 mM imidazole, and the tag was removed by loading the sample back onto a nickel column. The collected flow-through fraction containing cleaved, purified protein was concentrated to 5 mL and injected onto S-200 gel filtration column (GE Healthcare) equilibrated with 25 mM Tris-HCl pH 7.5, 500 mM NaCl, 3 mM DTT, and 1 mM EDTA. To confirm the purity, collected fractions were analyzed by SDS-PAGE and detected with Coomassie Brilliant Blue staining. All fractions containing purified protein were pooled, concentrated to 36 mg/mL, and stored at -80 ºC.

Kinetic Assay of Choline Kinase alpha: Choline kinase activity was assayed spectrophotometrically using a modified pyruvate kinase/lactate dehydrogenase-coupled system. The reaction buffer contained 100 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.25 mM NADH, four units of pyruvate kinase, and seven units of lactate dehydrogenase; final reaction mixture also contained purified enzyme and substrates to a total volume of 1 ml. For determination of steady-state kinetic parameters in the presence of inhibitors, the concentration of ATP and choline were kept constant at 500 μM. 100 nM ChoKα1 was incubated with TCD-717 at various concentrations prior to beginning the assay. It was found that without pre-incubation, the measured inhibition was less robust and inconsistent across multiple measurements, especially for the choline kinase rate (Fig. S1B). The reaction

performed at 37 °C was initiated by the addition of choline. ADP formation was followed spectrophotometrically using a Varian Cary 50 spectrophotometer by measuring the decrease of NADH at 340 nm. An IC_{50} curve was determined by analyzing the data in GraphPad Prism 6.0.

Crystallization of Choline Kinase alpha in complex with TCD-717: All crystals were grown at 20 ºC using sitting drop vapor diffusion method. Crystals of the apo form of ChoKα1-Δ79 were grown in 0.1 M MES pH 6.5 and 20% PEG 3350 for TCD-717 soaking. Drops of 2 µl protein at 10 mg/ml were set at a 1:1 ratio with reservoir solution. Crystals took 2-3 days to appear as large flat sheets. 72-48 hours prior to data collection, TCD-717 was soaked by mixing a 50 mM solution of 100% DMSO-resuspended compound 1:4 with reservoir buffer. A volume of 0.5 µl of this solution was added directly to the drop containing the crystal. Within 2 hrs of adding the pale yellow TCD-717 solution, crystal color had visibly darkened compared to buffer surrounding it. Prior to data collection, the inhibitor-soaked crystals of ChoKα1-Δ79 were cryoprotected in mother liquor containing 30% ethylene glycol.

Data Collection and Structure Determination of ChoKΔ79 bound to TCD-717: Diffraction data for ChoKα1-Δ79 with TCD-717 was obtained at the Life Sciences Collaborative Access Team ID beamlines 21-ID- G at the Advanced Photon Source, Argonne National Laboratory (wavelength, 0.979 Å; temperature, 100 K) (refer to Table 1 for data collection and refinement statistics**)**. Data processing was executed using XDS.[26] The inhibitor-bound structure of ChoKα1-Δ79 was solved using MOLREP[27] molecular replacement software using the highest-resolution available structure of ChoKα1 [4CG8] as a model. Further refinement of the model was done using REFMAC5 software.[28] All figures of structures were generated using MacPyMOL (PyMOL™ Molecular Graphics System, version1.7.2.3; Schrodinger, LLC).

RESULTS

TCD-717 is a sub-micromolar inhibitor of ChoKα in vitro.

It has been noted that ChoKα has a relatively high ATPase activity of 4.5 sec^{-1}, along with a higher choline kinase activity of 71 sec^{-1}.[14] For the 79 residues N-terminally truncated ChoKα enzyme used for this study, at saturating substrate concentrations (2 mM Choline/ATP) we observed an even higher ATPase rate (26.5 ± 0.9 sec^{-1}), and a choline kinase rate of 91.1 ± 3.1 sec^{-1} after correction for the ATPase rate. TCD-717 completely inhibits the ATPase rate at high concentrations (Fig. 2A), suggesting that either the binding of the compound competes with

Figure 2

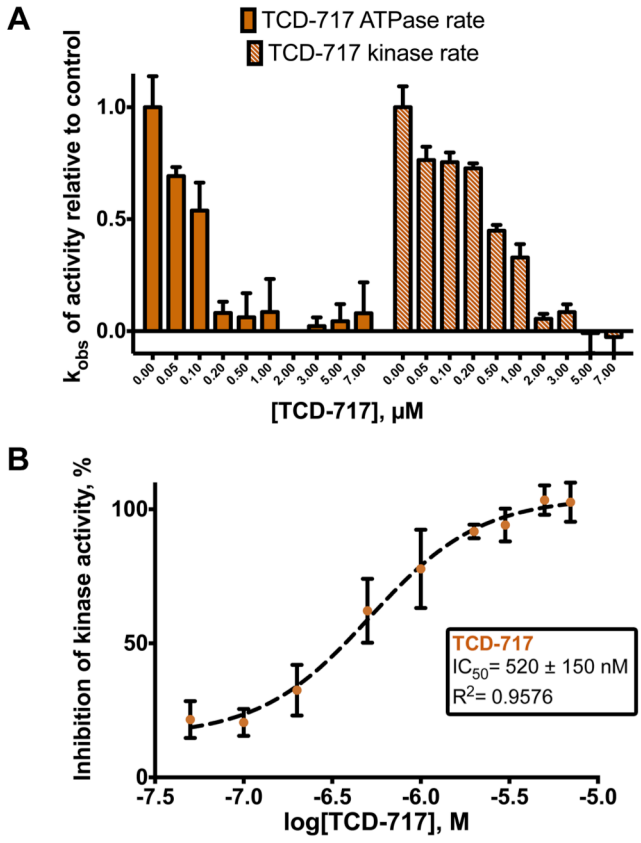


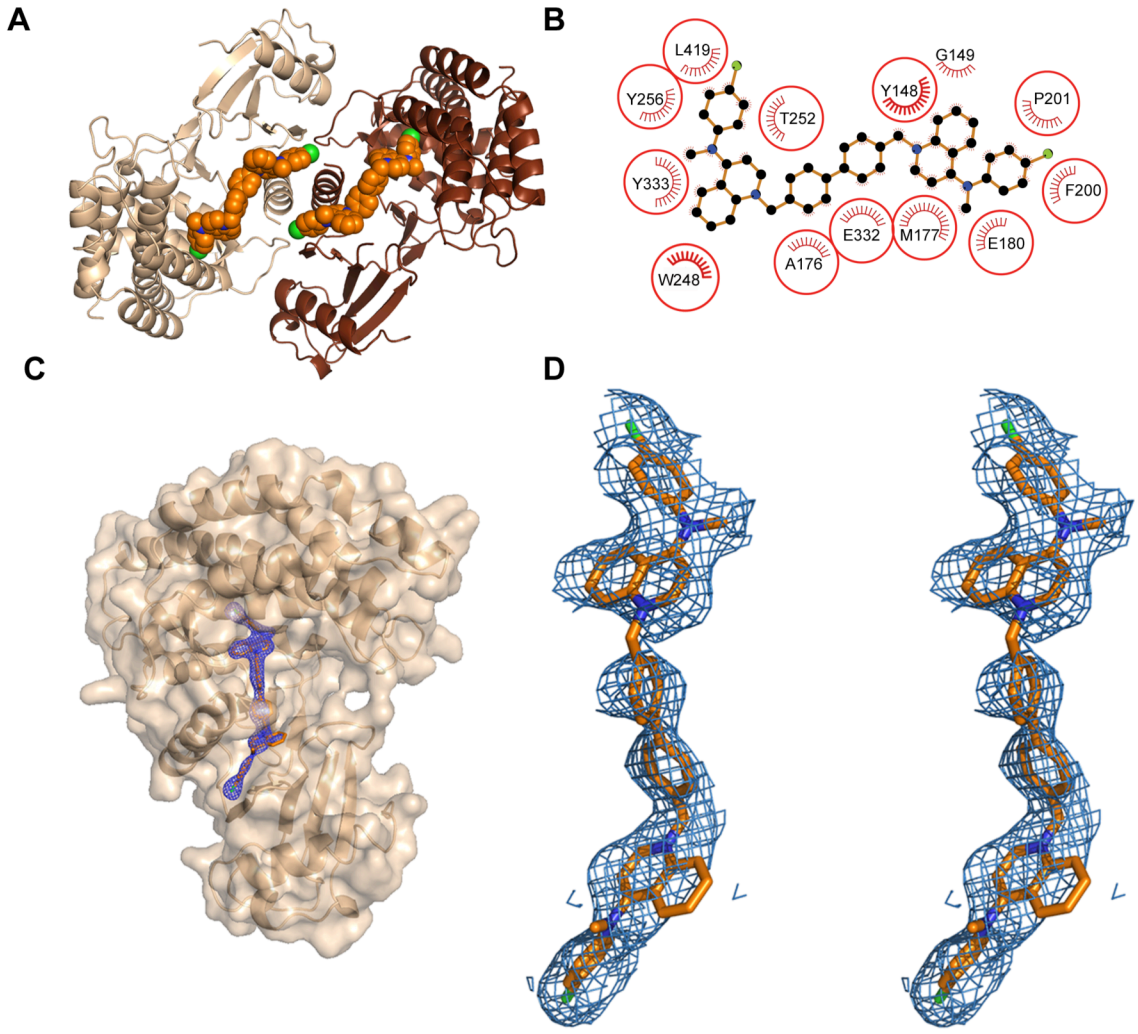
Figure 2. Kinetic parameters of TCD-717 and effect on ChoKα. *A*, Effect of TCD-717 on the ATPase rate and choline kinase rate of the ChoKα enzyme. TCD-717 ultimately ablates both activities of the enzyme at high concentrations of inhibitor *C,* Dose-response and calculated IC_{50} value for TCD-717 in the presence of 1 nM ChoKα enzyme.

ATP, or that binding of the compound stabilizes an enzyme conformation that does not accommodate ATP binding; we will present data that suggests the latter scenario is more likely. TCD-717 also fully inhibits the choline kinase rate, giving an IC_{50} value of approximately 500 nanomolar, (Fig. 2B). TCD-717 displayed low levels of solubility in aqueous buffers, and hence all kinetic experiments were conducted in the presence of 0.02% DMSO (higher DMSO concentrations affected the ATPase rate of the enzyme and did not resolve solubility issues, see Fig. S1A). High concentrations of inhibitor led to precipitation of the compound, making it difficult to assess accurate k_{on}/k_{off} rates by SPR or K_d using NMR or MicroScale Thermophoresis. Also due to these difficulties with solubility, as well as the need to pre-incubate TCD-717 prior to executing the kinetic experiments, it was difficult to obtain a reliable model to describe the mechanism of inhibition for the compound. Further studies would be necessary to accurately explain the inhibitory mechanism of TCD-717.

Visualizing TCD-717 binding to ChoKα: To obtain the structure of ChoKα in complex with TCD-717 we soaked apo crystals with the compound, collected a diffraction data set to 2.35 Å resolution, and refined the model to R_{work} = 20.7% / R_{free}=26.7% (see Table 1 for data collection and refinement statistics). The physiological oligomeric state of ChoKα is a dimer, and in our crystals a dimer builds the asymmetric unit. We observed TCD-717 bound to each protomer (Fig. 3A). Occupancy of the molecule was found to be 0.9 by Phenix Refine, consistent with dual conformations for the side chain of Trp248 (more about this below). Analysis of the enzyme-inhibitor interactions reveals that TCD-717 exclusively uses hydrophobic interactions to drive its inhibition with the enzyme (Fig. 3B). Most notably, Tyr148, Ala176, Met177, Glu180, Phe200, Pro201, Trp248, Thr252, Tyr256, Glu332, Tyr333, Phe341, and Leu419 serve to orient and hold TCD-717 in place. Instead of binding in the buried choline-binding pocket, unambiguous electron density for TCD-717 was observed towards the surface of the enzyme

(Fig. 3C). This electron density was evident from the unbiased map obtained from the data (Fig.

Figure 3



3D). Each ChoKα protomer is comprised of a distinct N-terminal and a C-terminal domain; the symmetric TCD-717 molecule spans the two domains.

Inspection of the published structures of several ChoKα inhibitors (Fig. 1B) shows that these molecules contain a tertiary amine group designed to mimic the similar structure present in the substrate molecule choline. Therefore, it is not surprising that the structures of ChoKα in complex with these molecules show that the tertiary amine group of the inhibitors is positioned

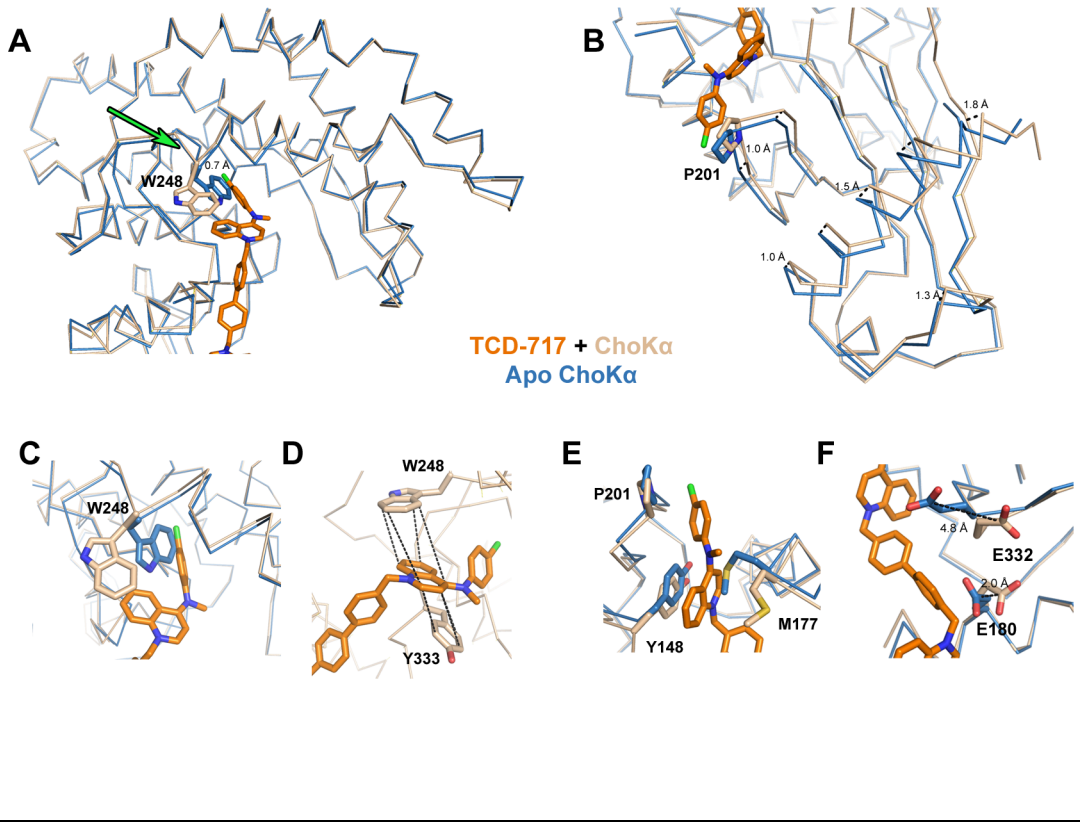
in the active site close to where the tertiary amine of choline is observed. Surprisingly, this is not the case of TCD-717, despite also possessing this tertiary amine moiety.

To confirm the unexpected binding mode of TCD-717, we collected three additional diffraction data sets of apo ChoKα soaked with the inhibitor. While these data sets were of slightly lower resolution (but all better than 2.5 Å), all presented unambiguous electron density for TCD-717 at the same binding site discussed above. We also tested crystals generated by co-crystallizing ChoKα and TCD-717 (at 2 mM), but these had no inhibitor bound.

Conformational changes in ChoKα are required for TCD-717 binding*:* Overlay of ChoKα−TCD-717 and apo ChoKα (PDB ID: 2I7Q) reveals that the overall structure of the enzyme is little changed with an r.m.s.d. of 0.41 Å over 291 common residues. However, closer inspection reveals both gross secondary structure movements across domains as well as significant side chain adjustments are undergone in order to accommodate the inhibitor.

In the C-terminal domain (Arg212-Val457), where deeper binding of TCD-717 takes place, most striking is the rotation of the Trp248 side chain, which allows one of the TCD-717 chlorophenyl moieties to occupy its former position as seen in the apo structure. This flip is further evidence for the incomplete occupancy of TCD-717, as electron density for the side chain can be seen in both orientations in the structure. In addition to the side chain rotation of Trp248, its Cα-atom is pushed outwards by 0.7 Å (Fig. 4A, green arrow). This is a notable change as Trp248 is part of α-helix 4, and binding of TCD-717 requires the distortion and displacement of nearby residues in the helix. Likewise, most of the N-terminal domain (Asp80-Ser211) is shifted by 1.0-1.8 Å due to TCD-717's stacking effect on Pro201 (Fig. 4B). Specifically, there is movement of the N-terminal α-helix by ~1.4 Å and a shift of the β-sheet formed by residues Leu197-Leu241 by ~1.2 Å.

Figure 4



A closer examination of the newly positioned Trp248 side chain shows a ~100° rotation compared to the apo structure (Fig. 4C). This shift, coupled with a ~35° rotation of Tyr333 (a residue with a putative regulatory role[9]), acts to sandwich the TCD-717 quinolone ring (Fig. 4D). The opposite side of the molecule interacts with the N-terminal domain. Here a strand-loop-strand motif is pushed outwards, with the loop Pro201 changing its Cα-atom position by 1.0 Å, thereby allowing sufficient space for the chlorophenyl group to stack above it. In addition, Tyr148 and Met177 change their side chain position, and, in the case of Tyr148, also its Cα-atom position by 1.0 Å, to allow for stacking the second quinolone moiety of TCD-717 (Fig. 4E). The biphenyl linker moiety of the compound makes few interactions with the enzyme, acting mostly to position the head groups for interactions with the N- and C-terminal domains, but does disrupt the side chains of both Glu180 and Glu332, displacing them by 1.8 Å and 5.3 Å,

respectively (Fig. 4F). Since Glu332 has been implicated in Mg^{2+} co-ordination and therefore ATP binding, disruption of this interaction could contribute to the inhibited enzyme's reduced ATPase rate and thus overall inhibition of the enzyme.[12] In sum, several significant main chain and side chain conformational changes are required to allow for the binding of TCD-717 to ChoKα.

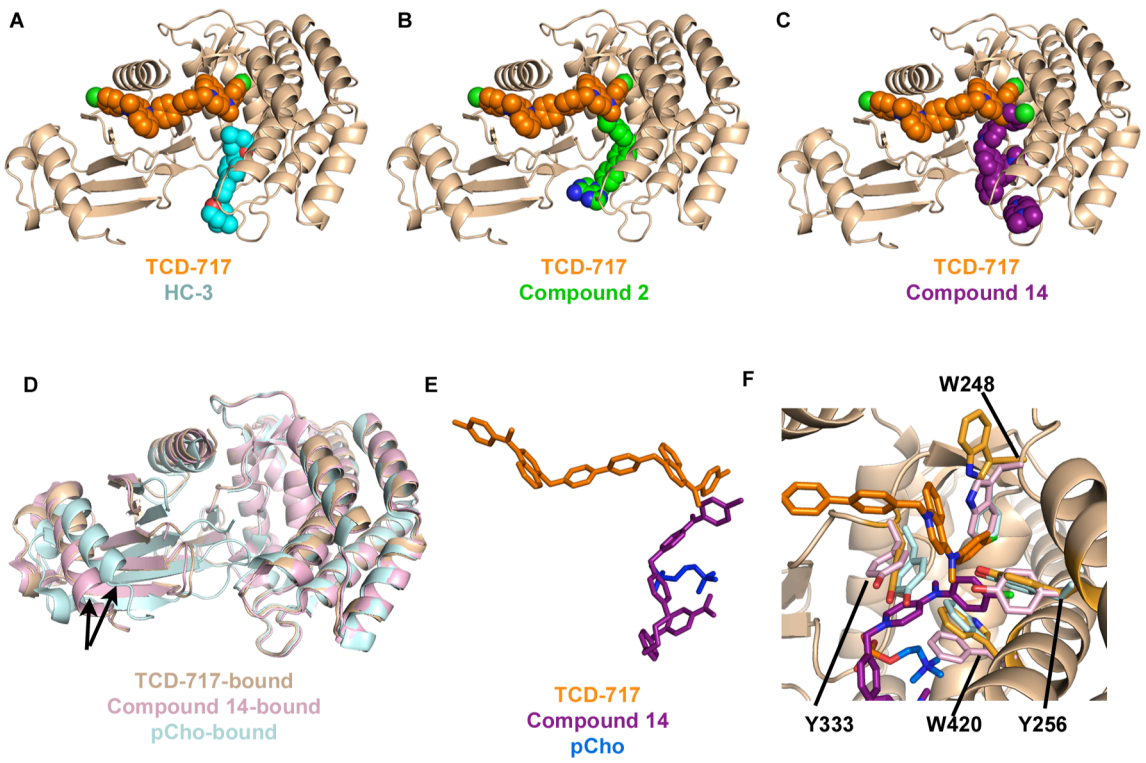
These observations may rationalize our finding that TCD-717 is a slow-acting inhibitor. Adding 1 μM of TCD-717 directly to the kinetic reaction buffer containing 1 nM ChoKα shows limited inhibition of the choline kinase rate, though the ATPase rate is reduced (Fig. S1 B & C). On the other hand, pre-incubating 100 μM TCD-717 with 100 nM of the enzyme for less than 1 hour and then doing a 100-fold dilution into the reaction buffer so that the final TCD-717 concentration is again 1 μM and ChoKα is 1 nM shows marked inhibition. This suggests a slow k_{on} rate, which is consistent with the requirement for multiple conformational changes in both lobes of ChoKα in order to accommodate the inhibitor.

TCD-717 binds at a different site than all previously reported ChoKα inhibitors: Figure 1 shows the chemical structure of the substrates of the choline kinase reaction and of several inhibitors of ChoKα. A common motif of the inhibitors is a protonated tertiary amine group that would mimic that present in choline. Indeed, the distance of the tertiary nitrogen atom of choline (as present in the pCho structure, PDB ID: 2CKQ) to the tertiary nitrogen atom present in the inhibitors HC-3 (PDB ID: 3G15), compound 2 (4BR3), compound 14 (4CG8), V-11-023907 (4DA5) is a relatively short 1.5, 1.5, 3.2, 1.5 and Å, respectively.[11, 12, 14] Hence, for these compounds, it seems that the protonated tertiary amine group determines the positioning of the molecules such that this group occupies essentially the same binding site. In contrast, the analogous distance for TCD-717 is 8.7 Å, exemplifying not only the unique binding mode of

TCD-717 relative to all previously examined ChoKα inhibitors, but also suggesting a different mode of inhibition.

HC-3 and compound 2 share the same bi-phenyl linker group as TCD-717, though HC-3 is a symmetrical molecule with oxazonium rings on either side whilst compound 2 contains a group that mimics the adenine moiety of ATP and another with a tertiary amine on a pyridinium

Figure 5



ring (Fig. 1C). These both bind the length of the choline-binding pocket whilst TCD-717 binds in a site remote from these (Fig. 5 A&B).

Compound 14 does not have a bi-phenyl linker, and like compound 2 has non-identical head groups; one of which is the pyridinium ring seen on compound 2 and one which contains a chlorophenyl group attached to a similar pyridinium ring (Fig. 1C). Though a chlorophenyl group is likewise present in TCD-717, the two molecules bind in different locations, with a distance of 7.3 Å between the chlorine atoms present in the two molecules (Fig. 5C). Because they share

the same head group in the general region of choline binding, we further compared the modes of binding of TCD-717 vs. compound 14.

The overlay of the compound 14-bound structure and our TCD-717-bound structure has an r.m.s.d of 0.39 Å across 308 common residues, indicating that the overall architecture adopted by ChoKα is not dramatically different, regardless of the nature of the inhibitor. However, overlay of the phosphocholine-bound structure (PDB ID: 2CKQ) with either the compound-14 or TCD-717 bound structures give r.m.s.d.s of 0.70 Å over 251 residues and 0.87 Å over 272 residues, respectively. The discrepancy for the TCD-717-bound structure is due in large part to the contraction of the two lobes due to TCD-717 binding, as previously discussed. The N-terminal lobe of ChoKα is not as expanded as in the pCho-bound structure (Fig. 5D, black arrows). When considering the overlay of TCD-717/compound 14/pCho in the C-terminal lobe aligned structures, TCD-717 does not superimpose on pCho's location in the enzyme, while compound 14 does (Fig. 5E). Therefore, while TCD-717 is adjacent to the choline-binding pocket, we did not interpret it as actually binding in the site. In contrast, compound 14 can be said to occupy the same site.

Supporting this interpretation is an examination of the aromatic residues in proximity to the choline-binding site. Notably, Trp248 does not adopt an alternative conformation to allow compound 14's chlorophenyl ring to fit, although it does so for TCD-717's (Fig. 5F). Likewise, Tyr256 and Trp420 rotate to stack compound 14, but remain in the same positions for the TCD-717 and pCho-bound structures. Finally, Tyr333 shows some degree of transposition between all three overlayed structures, although its side chain only shows marked rotation for the TCD-717 bound structure. Therefore we have concluded that while TCD-717 may disrupt the ability of choline to bind, it does not directly out-compete the substrate for space, unlike previously published compounds.

Discussion

Here we demonstrate that the potent inhibitor TCD-717 binds to ChoKα in a different manner than many of the other ChoKα inhibitors for which a structure has been reported. Whereas canonical ChoKα inhibitors bind deep inside the choline pocket, TCD-717 wraps around the active site, forming a connection between the N- and C-terminal domains of the enzyme and potentially restricting the conformational dynamics of the enzyme. HC-3 is the parent molecule for most ChoKα inhibitors, including TCD-717,[12, 24] and thus it was assumed TCD-717 binds ChoKα similarly to its sister derivatives; however, we have here demonstrated that this is not the case. It is interesting that due to both its potency and low toxicity, it was TCD-717 that was chosen among HC-3 derivatives as the inhibitor that would proceed to clinical trials. This was likely due to many factors besides the potency of enzymatic inhibition, including cell permeability, arrest of cell proliferation, *in vivo* bioavailability, target specificity and binding affinity, among others.

To understand what makes TCD-717 effective *in vivo*, it is interesting to consider some of the proposed mechanisms *for in* vivo efficacy of ChoKα inhibitors beyond simply the depletion of pCho. As previously mentioned, Tyr333 undergoes a slight rotation upon TCD-717 binding. This residue has been proposed to have a regulatory effect for downstream proliferation effects. TCD-717 binding may affect these other signaling pathways and thus tumor progression by prohibiting phosphorylation of this residue.[9]

Several residues adopt disfavored rotamer positions in order to accommodate TCD-717. Though Tyr148 and Trp248 have poor electron density, there is still clear evidence they occupy the space as modeled. Furthermore, electron density for Tyr256 clearly shows an alternative rotamer conformation. Notably, there is no electron density for residues 150-177. This loop is unresolved in all published structures[29] and corresponds to the alternative splicing that gives rise to the ChoKα2 isoform. As our structures were solved and kinetics performed using the

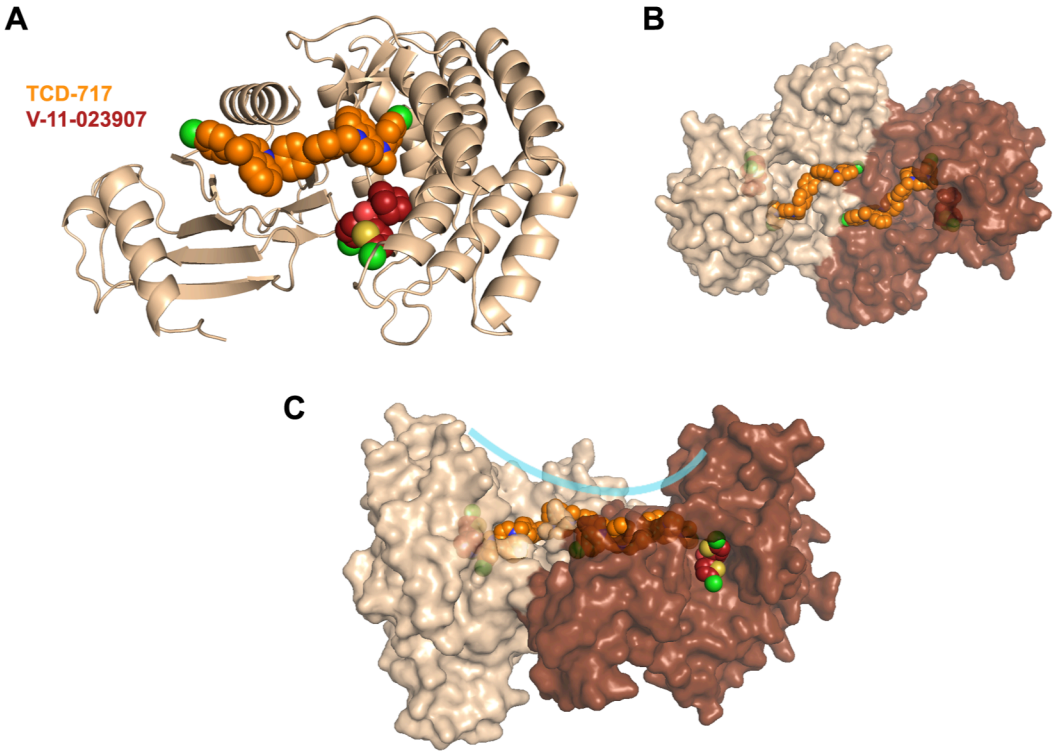
ChoKα1 isoform, it is possible this loop is at least partially responsible for slowing the on/off rate of the inhibitor. DIMPLOT analysis of the TCD-717 bound structure versus the apo form (PDB ID: 3G15) shows no differences in the dimer interface (data not shown).

However, another possibility emerges when considering a ChoKα inhibitor developed by Vertex Pharmaceuticals called V-11-0711. This inhibitor is similar in structure to V-11-023907 (Fig. 1B) for which a crystal structure in complex with ChoKα is available (PDB ID: 4DA5). V-11-0711 has been reported to have an IC_{50} of 20 nM,[8] which is 30-fold lower compared to the value we determined for TCD-717. Yet, whereas V-11-0711 – as expected by inhibiting the choline kinase reaction – reduced the amounts of pCho in HeLa cells, the effect on cells was confined to a reversible growth arrest.[8] In contrast, these same researchers report that knockdown of ChoKα by siRNA resulted in significant apoptotic cell death. In contrast, the less potent ChoKα inhibitor TCD-717 has been reported to have good *in vivo* efficacy in regards to apoptosis.[24] This invites the question as to why the higher-affinity ChoKα inhibitor V-11-0711 is less effective *in vivo* compared to TCD-717. Since the pCho levels are reduced in cells exposed to V-11-0711, product bioavailability cannot be the main reason.

In considering this clinically relevant question, it is important to realize that a non-enzymatic role in promoting cancer cell proliferation has been attributed to ChoKα by several groups.[8, 9, 30] If simply inhibiting the choline kinase reaction is not the main mechanism of cancer cell inhibition, it is possible that the molecule operates by perturbing another ChoKα function, potentially one that involves protein-protein interactions. When using crystal structures to consider the different binding modes of the *in vivo* effective TCD-717 to the parent molecule V-11-023907 of *in vivo* ineffective V-11-0711 (Fig. 6A), a striking difference becomes apparent. Whereas V-11-023907 binds deep within the enzyme and does not protrude from its surface (Fig. 6B), TCD-717 binds along a cleft on the surface, thereby changing its surface properties at that face of the enzyme (Fig. 6C). Notably, both compounds induce similar enzyme

conformations, having an r.m.s.d. of 0.42 Å over 297 common residues. In other words, though binding of TCD-717 or V-11-023907 (and thus presumably V-11-0711 binding) results in the same enzyme conformation, they present very different surfaces in the cell. We postulate that a

Figure 6



main factor behind the efficacy to TCD-717 comes from its unique binding mode at the surface, which acts to disturb a yet-to-be-identified interactor of ChoKα. Likewise, the potency of TCD-717 may be a combination of both its surface-affecting properties and its interaction with Tyr333, which was linked to cSrc-phosphorylation and downstream cancer progression.[9]

In conclusion, here we show that TCD-717, a ChoKα inhibitor currently in clinical trials, binds at a unique surface site on the enzyme and acts to connect the N- and C-terminal domains. In order to accommodate TCD-717, the enzyme must undergo a slight expansion and several side chains must change their conformation, which may explain the slow binding of the compound to ChoKα. In contrast, all previously structurally analyzed inhibitors of ChoKα were

seen to bind along the choline pocket. The protrusion of TCD-717 from the enzyme, in contrast to the buried nature of smaller inhibitors such as V-11-0711, may act to perturb a non-enzymatic role of ChoKα that requires a protein partner.

The development of TCD-717 as a second-generation inhibitor of ChoKα derived from HC-3 demonstrates the unpredictable nature of drug development. Here we show that TCD-717 binds on a unique surface compared to its parent molecule, uncovering a novel mode of interaction despite the fact that its starting scaffold was well characterized and known to interact differently. TCD-717's potential role as a disruptor of the putative scaffolding role of ChoKα likewise demonstrates that simple inhibition of enzymatic reactions is not the only target to consider when developing new inhibitors for enzymes that also possess protein-protein interaction functions. As inhibition of ChoKα has the potential to treat a wide variety of cancers, examining drugs that selectively target this protein is crucial to better understanding of its role in disease progression, as both a mediator of protein binding functions and as an enzyme.

Supporting Information: Figure illustrating controls and experimental read-out for the kinetic experiments described in the text (Figure S1).

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: SK prepared the Chokα clone, expressed and purified the enzyme, and executed the kinetic and crystallographic studies. EJD provided the TCD-717. AL supervised SK, participated in x-ray data collection, processing and refinement, and in the structural analysis. SK and AL wrote the paper with input from EJD.

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Table 1: Data collection and refinement statistics	
Structure	ChoKα +TCD-717
PDB ID	5W6O
Data Collection	
Space Group	$P2_12_12_1$
Cell dimensions	
a, b, c (Å)	55.95 122.35 131.40
α, β, γ (°)	90 90 90
Resolution (Å)	30.0 - 2.34 (2.48 - 2.34)[a]
R_{merge} (%)	9.6 (90.7)
I/σI	13.35 (1.80)
$CC_{1/2}$	99.8 (55.5)
Completeness (%)	94.0% (91.4%)
Reflections	166,378
Unique Reflections	36,492
Refinement	
Resolution (Å)	2.35
R_w/R_f (%)	20.7/26.7
No. Atoms	
Protein	5,941
Inhibitor	104
Water	173
Buffer Components	143
R.M.S.D	
Bond Length (Å)	0.012
Bond Angles (°)	1.504
Ramachandran Plot (%)	
Most Favored*	96.15
Additionally Allowed	3.85
Generously Allowed	0
Disallowed	0

[a] Highest resolution shell in parenthesis

Figure Legends:

Figure 3. TCD-717 interaction with ChoKα is driven by hydrophobic interactions across the N- and C- terminal domains. *A,* Dimer of ChoKα in complex with TCD-717. The inhibitor lies near the dimer interface (tan/brown). *B,* Ligplot diagram shows key interactions responsible for TCD-717 binding. It is chiefly hydrophobic interactions that orient TCD-717 in place. *C,* Refined electron density contoured to 1σ within 1.6 Å of TCD-717 in monomer B of ChoKα. TCD-717 is clearly present in the monomer, and spans both the C- (top) and N- (lower) terminal lobes of the enzyme. *D*, A stereo view of the unbiased F_o-F_c electron density of TCD-717, contoured to 1σ within 1.8 Å of TCD-717.

Figure 4. Binding of TCD-717 induces conformational changes compared to the unbound ChoKα structure. *A,* Overlays of the apo (slate, PDB ID: 2I7Q) and TCD-717-bound (tan) ChoKα structures show gross distortions in the overall structure. Here the C-terminal lobe is affected by the binding of TCD-717. Most notably, the side chain of Trp248, indicated by the green arrow, moves to accommodate the inhibitor, thus shifting its α-carbon by 1.0 Å and distorting the α-helix that begins with Trp248. *B,* The N-terminal lobe is affected by the binding of TCD-717. Pro201 stacks with the chlorophenyl ring opposite that of Trp248, shifting a loop and thus the β-sheets it orients by about 1.0 Å. This effect radiates out to the N-terminal α-helix, which undergoes a shift of as much as 1.7 Å. Most of the N-terminal lobe (approx. residues Asp80-Ser211) is shifted by at least 1.0 Å compared to the apo structure. R.M.S.D. for the overlayed structures is 0.42 Å across 289 residues. *C,* Closer examination of Trp248 reveals a ~135^o shift in the side chain along with a 1.0 Å of the backbone. *D,* Hydrophobic stacking drives

the binding of TCD-717. The shift of Trp248, along with Tyr333, helps orient TCD-717 in the C-terminal binding groove. *E,* The N-terminal lobe also contains residues that undergo side-chain orientation shifts, most notably Met177, which serves to stack the opposite face of TCD-717 along with Tyr148 and previously discussed Pro201. *F,* Glu180 and Glu332 undergo dramatic conformational shifts in their side chains of 2.0 and 4.8 A, respectively, to allow the biphenyl linker to fit.

Figure 5. TCD-717 binds at a unique site compared to previously published inhibitors. The overlays of TCD-717 (orange) with previously published inhibitors *A,* HC-3 (cyan) or *B,* Compound 2 (green). Each of these inhibitors binds in the choline active site, clearly distant from TCD-717's binding site. *C,* The overlay of TCD-717 (orange) and published compound 14 (purple) also reveals a different binding mode. Though compound 14 shares the chlorophenyl group present on TCD-717, it does not bind across both the N- and C- terminal lobes. *D,* An overlay of the B monomer from the TCD-717-bound structure (wheat), compound 14-bound structure (pink), and pCho-bound (light blue) structure shows the dramatic shift in the N-terminal lobe for the uninhibited, pCho-bound enzyme. *E*, Examination of only the inhibitors or substrate show that while compound 14 occupies the same space as pCho, TCD-717 does not superimpose. *F*, A close-up of the chlorophenyl head group from the overlay of TCD-717 (orange), compound 14 (purple), and pCho (blue) show that the inhibitors do not disrupt the choline binding pocket in the same manner. Though Trp248 must shift to accommodate TCD-717 (residue side chains in bright orange), it is in the same position as the pCho-bound structure in the case of compound 14 (residue side chains in pink). However, Tyr256 and Trp420 are shifted differently in the compound 14-bound structure but not the TCD-717-bound structure. Finally, Tyr333 shows some translocation in the case of compound 14 but rotation of the side chain hydroxyphenyl group in the case of TCD-717.

Figure 6. TCD-717 binding changes the surface presented by ChoKα, unlike the smaller inhibitor V-11-023907. *A,* V-11-023907 (red) binds deep in the choline-binding pocket of ChoKα. *B,* Overlays of both inhibitors nestled in surface view show clearly that V-11-023907 is not accessible to solvent and binds in a different location compared to TCD-717. *C,* A 90° rotation of the ChoKα dimer shows that while V-11-023907 is buried deep within the protein, TCD-717 is surface-exposed and thus has the potential to disrupt protein-protein binding mediated by a putative ChoKα scaffold, indicated here by a cyan line.

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