Crystal Structure of the Human Fatty Acid Synthase Enoyl-Acyl Carrier Protein-Reductase Domain Complexed with Triclosan Reveals Allosteric Protein-Protein Interface Inhibition*

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Background: Enoyl reductase (hER) is a catalytic domain in cancer-associated human fatty acid synthase (FAS) and is inhibited by triclosan.

Results: Triclosan binds at the protein-protein interface (PPI), causing distant active site changes.

Conclusion: Triclosan is an allosteric PPI inhibitor.

Significance: Triclosan can be used to develop PPI FAS inhibitors for anti-cancer therapy.

Human fatty acid synthase (FAS) is a large, multidomain protein that synthesizes long chain fatty acids. Because these fatty acids are primarily provided by diet, FAS is normally expressed at low levels; however, it is highly up-regulated in many cancers. Human enoyl-acyl carrier protein-reductase (hER) is one of the FAS catalytic domains, and its inhibition by drugs like triclosan (TCL) can increase cytotoxicity and decrease drug resistance in cancer cells. We have determined the structure of hER in the presence and absence of TCL. TCL was not bound in the active site, as predicted, but rather at the protein-protein interface (PPI). TCL binding induces a dimer orientation change that causes downstream structural rearrangement in critical active site residues. Kinetics studies indicate that TCL is capable of inhibiting the isolated hER domain with an IC50 of ~55 μM. Given the hER-TCL structure and the inhibition observed in the hER domain, it seems likely that TCL is observed in the physiologically relevant binding site and that it acts as an allosteric PPI inhibitor. TCL may be a viable scaffold for the development of anti-cancer PPI FAS inhibitors.

Type I fatty acid synthase (FAS)2 is a lipogenic enzyme that synthesizes the long chain saturated fatty acids used to make membranes, lipid signaling molecules, and membrane protein anchors (1). FAS is one large polypeptide containing seven catalytic domains including the β-ketoacyl synthase (KS), malonyl acetyl transferase, dehydratase (DH), enoyl-acyl carrier protein-reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and thioesterase domains (1). Functional FAS is a homodimer with the KS, DH, and ER domains forming the interface (2). Crystal structures of several human FAS catalytic domains have been solved previously including the KS-malonyl acetyl transferase didomain (3) and the thioesterase domain (4–6). However, an x-ray crystal structure of the human ER domain (hER) is currently unavailable.

Human FAS is associated with a variety of diseases and adverse health conditions including obesity, diabetes (7), hepatic steatosis (8), inflammation, and cancer (9). Its relationship with cancer pathogenesis has attracted the most attention (9, 10). In humans, diet is the primary source of fatty acids, and FAS is expressed at very low levels. However, this enzyme is dramatically up-regulated in many cancers including breast, prostate, colon, bladder, pancreatic, and lung. Tumors become dependent on FAS activity to maintain growth rates despite high circulating fatty acid levels (11).

FAS was initially identified as an antigenic marker of poor prognosis and has been associated with recurrence and decreased survival in thirteen different cancers. It was later classified as a metabolic oncogene (reviewed in Ref. 11). Overexpression of FAS in benign cell lines increased both anchorage-dependent and independent growth, as well as survival, although it was insufficient to induce tumorigenic transformation alone (12, 13). FAS overexpression has also been linked to drug resistance (14). FAS inhibition has been shown to delay cancer progression in prostate and lung cancers, to sensitize previously resistant cells to chemotherapy, and to enhance cytotoxicity in breast and ovarian cancers (15–22). Several studies have demonstrated that FAS inhibition induces selective apoptosis in breast and prostate cancer cells with little effect on healthy cells, both in vitro and in vivo (18, 23–25). There are currently no FDA-approved anti-cancer FAS inhibitors on the market.
Human Enoyl Reductase Domain Complexed with Triclosan

Several FAS inhibitors targeted to various domains have been developed with mixed results (reviewed in Refs. 10 and 11). Drugs that target the KS domain such as cerulenin and C75 are prone to cross-reactivity with carnitine palmitoyltransferase-1, resulting in significant weight loss. Green tea polyphenols target the KR domain, but the high IC_{50} and poor bioavailability make translating it to human patients infeasible. The irreversible thioesterase-targeting drug orlistat suffers from poor solubility and low oral bioavailability. The antibacterial agent triclosan, which targets bacterial ER proteins, has also been shown to inhibit FAS via the ER domain.

Triclosan (TCL) and its role in cancer is currently a contentious topic (reviewed in Ref. 26). It is a common antibacterial agent in personal care products, and studies have revealed that it can be detected in urine, semen, and breast milk (27–33), although there is no evidence of accumulation in animal models or humans (27). A number of studies implicate it in estrogenic activity, although there is no evidence of accumulation in animal models or humans (27). A number of studies implicate it in estrogenic activity, although there is no evidence of accumulation in animal models or humans (27).

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Here we present, for the first time, the x-ray structure of the homodimeric hER domain, alone and in complex with TCL. The structure is highly similar to the ER domain in the full-length porcine FAS (pFAS) crystal structure (42) performed previously; however, no experimental structural data regarding TCL binding to hER are currently available.

For the apo hER crystals, the protein samples were thawed on ice, spiked with potassium NADP^{+} in Tris, pH 8, and TCL in ethanol to final concentrations of 5 and 2 mM, respectively, and allowed to incubate overnight at 4 °C. The protein was concentrated to 5 mg/ml as determined by UV using an 280-nm extinction coefficient of 1.3 (mg/ml)^{-1} cm^{-1}. It was then frozen at −80 °C to preserve activity until the kinetics assays were performed. hER used for crystallization was concentrated to 5 mg/ml and treated with 25 μl of 1 M iodoacetic acid (IAA), 1 M NaOH per mg of protein. The reaction was incubated at room temperature in the dark for 30 min. The protein was buffer exchanged into Q buffer (20 mM Tris, 500 mM NaCl, 10% (w/v) glycerol, pH 8) and concentrated. The concentrated protein was loaded onto a Superdex 75 16/60 column (GE Healthcare) equilibrated in size exclusion buffer. The hER-containing fractions were pooled.

The hER used for kinetics was buffer exchanged into storage buffer (20 mM Tris, 20 mM NaCl, 2 mM TCEP, 1 mM EDTA, 5% (w/v) glycerol, pH 8) and concentrated to −5 mg/ml as determined by UV using an 280-nm extinction coefficient of 1.3 (mg/ml)^{-1} cm^{-1}. It was then frozen at −80 °C to preserve activity until the kinetics assays were performed. hER used for crystallization was concentrated to 5 mg/ml and treated with 25 μl of 1 M iodoacetic acid (IAA), 1 M NaOH per mg of protein. The reaction was incubated at room temperature in the dark for 30 min. The protein was buffer exchanged into Q buffer (25 mM Tris, 1 mM NaCl, 2 mM EDTA, 10% (w/v) glycerol, pH 8.2) and loaded onto a 5-ml HiTrap Q column (GE Healthcare) equilibrated in Q buffer. The column was washed with 3 column volumes of Q buffer, followed by a gradient of Q buffer with 500 mM NaCl. The fractions containing IAA-treated hER (IAA-hER) were pooled, and buffer was exchanged into storage buffer, aliquoted, and frozen at −80 °C until the crystallization trials were performed.

hER Crystallization, Data Collection, and Structure Solution—IAA-hER was used for all crystallization experiments because unmodified hER did not crystallize. IAA-hER was crystallized in several conditions and space groups. No significant differences were observed between the different space groups; therefore, the highest resolution data sets were selected for discussion (described below). Two structures, a TCL-free hER (apo hER) and a TCL-bound hER (hER-TCL) were determined. TCL was manufactured by Calbiochem (Merck Millipore). The initial crystallization hits for both structures were obtained using the MCGS screen (Microlytic, Burlington, MA).

For the apo hER crystals, the protein samples were thawed on ice, spiked with potassium NADP^{+} in Tris, pH 8, and TCL in ethanol to final concentrations of 5 and 2 mM, respectively, and allowed to incubate overnight at 4 °C. The protein was concen-
trated to 6.5 mg/ml as determined by a Bio-Rad protein assay calibrated with IAA-treated hER. Crystals were obtained using hanging drop vapor diffusion (43). Drops were composed of 2 μl of hER in NADP+ and TCL and 2 μl of precipitant solution (100 mM BisTris, pH 6.5, 100 mM MgCl2, 19% (w/v) PEG 3350) suspended over 500 μl of precipitant solution. Crystals were cryoprotected by soaking for 30 min in 100 mM BisTris, pH 6.5, 100 mM MgCl2, 25% (w/v) PEG 3350, 20% glycerol. Data were collected on Beamline 5.0.1 at the Advanced Light Source through the collaborative crystallography program. A total of 360 usable images were collected at a 0.9774 Å wavelength and a detector distance of 230 mm.

For the hER-TCL, the initial protein samples were prepared as described above with 5 mM potassium NADP+, and 2 mM TCL. The first round of crystals were grown via hanging drop vapor diffusion using the proportions described above with a precipitant solution of 3.2 M NaCl, 100 mM imidazole, pH 8. The entire drop was transferred into 50 μl of precipitant solution and crushed using a seed bead (Hampton Research, Aliso Viejo, CA). The next round of crystals were grown in the same manner using 2 μl of 6 mg/ml hER in 5 mM NADP+, 2 mM TCL, 1.5 μl of precipitant (2.5 M NaCl, 100 mM imidazole, pH 8), and 0.5 μl of a 106 seed crystal dilution over 500 μl precipitant. These crystals were again crushed with a seed bead. The final crystals were grown using 2 μl of 5 mg/ml hER in 20 mM NADP+, 2 mM TCL, 1.5 μl of precipitant (2.625 M NaCl, 100 mM imidazole, pH 7.5), and 0.5 μl of a 106 seed crystal dilution over 500 μl of precipitant. The crystals were cryoprotected by soaking 20 min in 2.75 M NaCl, 100 mM imidazole, 20 mM potassium NADP+, 24.5% (w/v) sucrose, pH 7.5. Data from two crystals were collected in-house at 1.5418 Å using a Rigaku FR-E+ SuperBright microfocus rotating anode generator with Varimax HF optics and an R-AXIS IV++ area detector (Rigaku, The Woodlands, TX). For each crystal, 360 images were collected with a detector distance of either 140 or 115 mm.

All data were processed using Mosflm (44), followed by symmetry identification in Pointless and scaling/merging in Aimless (45). The data statistics are listed in Table 1. Because of the limitations of the hER-TCL space group, a complete data set could not be obtained with a single crystal. Therefore, the data from two separate crystals were merged for downstream analysis. The initial molecular replacement was performed in Phaser (46) implemented within Phenix (47) using the pFAS ER domain monomer, chain A, and residues 1529–1867 (Protein Data Bank code 2vz8) (42). Human and porcine ER domains have an identical number of residues. Sigma weighted mFo − DFo and 2mFo − DFo. Fourier maps were generated from the molecular replacement model, and the structures were rebuilt manually in COOT (48). Feature-enhanced maps (49) implemented in Phenix were also used to improve the signal and remove bias. The model was then subjected to rigid body refinement and iterative cycles of coordinate, atomic displacement parameter, and occupancy refinement in phenix.refine (47), as well as ligand placement and manual model refinement in COOT. Translation, liberation, and screw rotation groups were selected using the TLSMD server (50), and translation, liberation, and screw rotation refinement was applied in the final refinement cycle. Subsequent structures used this rebuilt hER model for molecular replacement followed by the same refinement protocol. The final refinement statistics are listed in Table 1. Domain movement analysis was performed in DynDom (51). The porcine ER to hER-TCL and apo hER to hER-TCL comparisons were performed using default values; however, to account for subtler movements, the apo hER to porcine ER comparison used a window length of 11 and a ratio of 0.9. The PISA server (52) was used to calculate interfaces and ligand binding energies.

**hER Kinetics**—The hER kinetics assay was adapted from previously published protocols (53). Briefly, each reaction contained 150 μg of unmodified hER, 280 μM sodium NADPH, 100 mM Bicine, 100 μM EDTA, 1 mM DTT, pH 7.5. The total reaction volume was 150 μl. The hER was incubated with varying concentrations of TCL (0–100 μM) or an equivalent volume of 100% ethanol for 10 min prior to the reaction. The reaction was initiated with the addition of 600 μM crotonyl-CoA, and the reaction progress was recorded as the loss of UV absorbance at 340 nm over 300 s using a Shimadzu UV-2501PC spectrophotometer (Shimadzu Scientific, Columbia, MD) with a 1-cm path length. The data were analyzed using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA). The data presented are the means ± S.D. of triplicate measurements. The percentages of standard error of all the data were less than 10%.

### RESULTS AND DISCUSSION

**Overall Structure**—The hER domain construct contained residues 1529–1867, which are divided into two subdomains (Fig. 1, A and B). The first is the NADPH binding domain con-
sisting of residues 1651–1794. The second is a noncontiguous substrate binding domain that includes residues 1530–1650 and 1795–1858. The overall fold of the hER domain is very similar to the equivalent domain in the full-length pFAS structure (42). The NADPH binding domain adopts a traditional Rossmann fold with a five-strand parallel $\beta$-sheet ($\beta8–\beta12$) sandwiched between several helices. Helix $\alpha6$ bridges into the substrate binding domain. The substrate binding domain fold is similar to oxidoreductases with a twisted seven-strand $\beta$-sheet wrapping around $\alpha6$ and flanked by several helices and an additional two-strand sheet. The structure exists as a homodimer in solution (Fig. 1C). Although there is some variation in the dimer orientations of our structures (discussed in detail below), the interface occurs along the outer edge of the $\beta12$ strands of both monomers, extending the Rossmann fold sheet, with the individual parallel sheets oriented anti-parallel to one another. In this study, we determined the crystal structures of the TCL-free hER (apo hER) and hER complexed with the inhibitor TCL (hER-TCL). All the crystals were crystallized with IAA-hER. There are six Cys residues in the hER construct; however, most of them showed no density consistent with carboxymethylation, and none were located near the NADPH binding site or at the dimer interface. The crystals were also co-crystallized in the presence of NADP$^+$; however, no difference density for NADP$^+$ was apparent in any of the structures.

**Apo Structure**—Apo hER was solved in the P1 space group to 1.7 Å resolution with two molecules in the unit cell (Fig. 1C). A number of regions could not be resolved from the density and were excluded (chain A residues 1529, 1550–1557, 1725–1726, 1747–1750, 1771–1782, and 1862–1867 and chain B residues 1530, 1550–1559, 1771–1781, 1801–1804, and 1866–1867). No significant differences were observed between the monomers in the unit cell (C$_{RMSD}$ 0.407 Å). The dimer interface area could not be accurately calculated because of the unmodeled regions. This structure was also solved in the P21 and P21212 space groups at lower resolutions. No significant difference in the monomeric structure, dimer orientation, or unmodelable loop regions were observed (data not shown). It should also be noted that all these data were co-crystallized in the presence of TCL; however, TCL was never observed in the structure.

**TCL Co-crystal Structure**—The hER-TCL complex was solved in P1 to 1.84 Å resolution with four molecules in the unit cell. The molecules formed a dimer of dimers with chains A and B forming one biological homodimer and chains C and D forming the other. Like the apo structure, there were several regions that lacked density and were excluded (chain A residues 1529, 1550–1551, 1725–1726, 1747–1750, 1771–1782, and 1862–1867; chain B residues 1530, 1550–1559, 1771–1781, 1801–1804, and 1866–1867; chain C residues 1529, 1550–1557, 1771–1781, 1801–1804, and 1866–1867; chain D residues 1529, 1550–1557, 1724–1726, 1772–1779, 1846–1847, and 1862–1867). A carboxymethylated cysteine was modeled into chain C at residue 1548 at the cusp of a disordered region. This is the only evidence of carboxymethylation seen in any of the structures, which is unsurprising given the disorder in this region. An imidazole molecule, a critical component of this crystallization condition,
was found buried in the cleft between the two subdomains of each monomer in the ASU.

Little difference was seen between the individual monomers, with the hER-TCL monomers superposed individually onto the apo hER monomers, minimal difference was seen. The Cα RMSDs ranged between 0.73 and 0.97 Å (Fig. 1B). The significant difference between the apo hER and hER-TCL structures is the shift in the dimer orientation (Fig. 1C). There is an 11.8° rotational shift and a −0.9 Å translation along the rotational axis. It should be noted that another hER-TCL structure at much lower resolution was solved from a completely different crystallization condition (data not shown). Despite polar opposite crystallization conditions, this structure crystallized in the same space group with nearly identical unit cell dimensions. No significant difference in the monomer structure or dimer orientation was observed. Given this result and the fact that the apo hER dimer is unchanged in the other space groups, it seems likely that the change in dimer orientation can be attributed to TCL binding. Additionally, because all the apo structures were co-crystallized in the presence of TCL and showed no density for the molecule, the observed TCL is probably not the result of simple adventitious binding.

In the hER-TCL structure, a single TCL molecule is located between the respective dimers, away from the active site. TCL is bound against the dimer’s extended Rossmann fold sheet (Fig. 1C). Because the region around the TCL binding site was most ordered in the AB dimer, it is the basis of all subsequent observations and discussions of hER-TCL. TCL engages solely in van der Waals interactions with hydrophobic side chain residues from both chain A (Leu1753, Leu1780, Ile1784, and Phe1791) and B (Leu1753, Leu1780, and Phe1791) (Fig. 2, A and B).

The hER-TCL binding mode is significantly different from bacterial ER-TCL binding, exemplified by the prototypical E. coli ER, EnVM, in an EnVM-triclosan-NAD⁺ complex (Protein Data Bank code 1qsg) (54). EnVM and hER are not overly similar, with 13.3% sequence identity. Secondary structural superposition yields a Cα RMSD of 2.9 Å across 105 residues, primarily in the NAD(P)H binding domain. When looking at the EnVM-TCL binding site, the A ring of TCL is stacked against the NAD⁺ nicotinamide ring (Fig. 2, C and D). In addition to several hydrophobic interactions, the oxygens of TCL also hydrogen bond with both the protein and the NAD⁺ ribose sugar, a feature not seen in the hER-TCL complex. Additionally, there is a significant difference in the TCL torsion angles between human and bacterial ER-bound TCL (Fig. 2, B and D). Idealized TCL Φ1 (C4-C5-O7-C8) and Φ2 (C5-O7-C8-C9) torsions are 93.1° and −173.7°, respectively. Human ER-bound TCL had an average Φ1 of 49.6 ± 1.6° and a Φ2 of −131.0 ± 2.3° obtained from the two dimers. The EnVM-TCL complex had an average Φ1 of −95.5 ± 4.4° and an average Φ2 of 175 ± 4.5°, respectively, obtained from the eight monomers in the asymmetric unit. These contortions highlight the differences seen between the two TCL binding modes.

Comparison of hER to Full-length pFAS — The x-ray structure of pFAS was solved previously in the presence (holo) and absence (apo) of NADP⁺ (Protein Data Bank codes 2vz9 at 3.3 Å and 2vz8 at 3.22 Å resolution, respectively) (42). When comparing the sequences (residues 1529–1867), the porcine ER domain (pER) is 77% identical and 84% similar to hER. It was for this reason that pER was used as our molecular replacement model. Comparison of holo pER chain A with apo hER chain A produced a Ca RMSD of 1.3 Å across 290 residues. Comparison with hER-TCL chain A showed a Ca RMSD of 1.6 Å (284 residues) (Fig. 3A). However, when aligning the nucleotide binding domain (residues 1651–1794) alone, the RMSD of pER to apo hER and hER-TCL is 0.664 and 0.798 Å, respectively. The difference can primarily be attributed to an intradomain shift in the exterior helices and two-strand sheet of the substrate binding domain, with pER forming a more compact structure (Fig. 3A). When this compact structure is put in the context of the full-length FAS, the more compact arrangement of pER can easily be attributed to the presence of the DH and KR domains, which abut the shifted regions (Fig. 3B). The structural effects of the adjacent catalytic domains in the full-length pFAS structure are also apparent in the unmodelable hER regions. Several of these disordered regions are ordered in porcine FAS because they are adjacent to the other catalytic domains. The most notable are residues 1550–1560 that lie next to the KR domain and residues 1723–1726 and 1771–1779 that are close to the DH domain dimer (Fig. 3B). Although these domains are nearby, they have relatively small direct interfaces of 408 and 158 Å² for the KR and DH domains, respectively. The space between pER and the adjacent domains ranges from ~5 to 13 Å and probably contains a large number of water-mediated interactions that are not observed in the relatively low resolution structure.

The pER dimer has a Ca RMSD of 2.3 Å (547 residues) with apo hER and 3.3 Å (526 residues) with hER-TCL chains A and B.
When the intradomain shift between pER and apo hER is calculated using DynDom, a 12.1° rotation and a 0.5 Å translation are observed. A 23.6° rotation is observed between the pER dimer and hER-TCL dimer. This number is the product of the intradomain shift between hER and pER, unrelated to TCL binding, is noted with a red oval. B, the intradomain shift shown in the context of full-length pFAS. The pFAS DH domain is colored gray, and the KR domain is colored maroon. C, superposition of the TCL-binding and NADPH-binding regions from apo hER, TCL-bound hER, and pER (residues 1760–1800). The proteins are colored as described for A, with different shades indicating different chains of the respective homodimers. The relevant side chains, TCL, and NADPH are shown as sticks. The TCL and NADPH are colored as follows: carbon, yellow; oxygen, red; nitrogen, blue; phosphorus, orange; and chlorine, green. The dotted lines indicate disordered regions. D, the TCL-binding and NADPH-binding regions from apo hER, TCL-bound hER, and pER in the context of pFAS. The DH domain is colored gray. The figures were generated in PyMOL.

When focusing on the NADPH binding site from pER and the TCL-binding site in hER-TCL, a number of observations can be made (Fig. 3C). Residues Lys1771 and Asp1797 are candidate catalytic residues because of their strict conservation in all type I FAS sequences and their proximity to NADPH for hydride transfer (42). TCL binding appears to reorient the strands leading to these residues, causing downstream shifts in the loops and helices harboring these critical residues. Notably, there is a shift in Asp1797. In apo hER, Asp1797 extends into the empty NADPH binding site, and the residue shifts ~3 Å upon TCL binding. When comparing pER to hER-TCL, the Asp1797 side chain moves a more conservative 1 Å further from the NADP. In addition, when comparing pER and hER-TCL, the loop containing Lys1771 and Phe1772 shifts 3.8 Å. Phe1772 orients toward the hydrophobic residues of the chain B α12 helix. The Lys1771 side chain reorients into the solvent channel and is now too disordered to model. Neither of these residues was observed in apo hER structure, because of disorder. Residues 1774–1779 of pER form a short coil/helix, whereas in apo hER and hER-TCL, this region is disordered. In the hER structures, this area lies along a solvent channel. In pER, this area is flanked by the DH domain (Fig. 3D). Even in the presence of disorder, visible conformational changes upon TCL binding are apparent. Taken overall, it is plausible that TCL binding could reorient the NADPH binding site into a catalytically unfavorable conformation, leading to inhibition of ER activity.

**hER Kinetics**—Kinetics were performed on unmodified hER to assess its activity and inhibition by TCL. The published specific activity of the ER partial reaction in full-length human FAS purified from HepG2 cells is 89.3 nmol NADPH min⁻¹ mg⁻¹ (55). The specific activity of hER at the reaction conditions used was 1.86 ± 0.08 nmol NADPH min⁻¹ mg⁻¹. Given the differing molecular weights and that higher concentrations of both protein and crotonyl CoA were used in our assay, this represents a greater than 48-fold reduction in activity. This observation is not entirely surprising. The x-ray structures indicate that the critical NADPH binding site is dynamic in the absence of the adjacent catalytic domains. Additionally, in the context of full-length pFAS, the fatty acid substrate is held and oriented in the active site by the ACP domain. With the presence of this coordinating protein, there would have been minimal evolutionary pressure to develop a high affinity substrate binding site. The isolated domain in solution may not have sufficient affinity to bind the crotonyl-CoA substrate without a large excess present. This supposition is supported by the inability to reach maximal velocity at the concentrations tested (up to 1.2 mM crotonyl CoA), making accurate calculation of \( K_{m} \) impossible.

IAA-hER, which was used for crystallization, showed no change in overall activity compared with unmodified ER; however, the modification did preserve the activity for a longer period when stored for several days at 4 °C. The cysteine carboxymethylation seems to improve the long term stability of hER, which may be why the IAA-hER crystallized, whereas the unmodified hER did not.

Even with reduced activity, TCL inhibition could be observed. Following a 10-min preincubation of hER with NADPH and TCL, the TCL IC_{50} was determined to be 54.7 ± 1.4 μM. This is relatively consistent with previous studies that determined the human FAS ER partial reaction IC_{50} of TCL to be ~90 μM using SKBr-3 breast cancer cell lysates (37). In the same study, TCL displayed time-dependent inhibition exhibiting a pseudo-first order rate constant of 4.7 M⁻¹ s⁻¹ (37). Unfortunately, because of the reduced activity, an accurate rate constant could not be calculated for hER.

**TCL Inhibition Mode**—TCL inhibition of FAS was initially believed to act as a traditional active site inhibitor, similar to bacterial ER (38). However, in our structure, the hER TCL binding site is located away from the active site at the protein-pro-
tein interface, indicating an allosteric mode of inhibition. On a basic kinetics level, there are significant differences seen between human and bacterial ERs. The hER domain and the FAS ER partial reaction had TCL IC_{50} values in the 55–90 μM range. TCL binding in bacterial enzymes is extremely tight, with K_{i} values in the low micromolar to picomolar range (reviewed in Ref. 56). The supposition that hER uses an allosteric mechanism is also supported by the estimated binding energies calculated in PISA. The estimated binding energy of TCL in bacterial EnvM is approximately −15 kcal/mol. Binding energy calculations of TCL docked into an hER homology model in a "bacterial" manner generate a theoretical binding energy of −5.73 kcal/mol (38). The TCL binding energy in our allosteric site is estimated to be approximately −12 kcal/mol, indicating that the allosteric binding site might be more energetically favorable.

The allosteric model is also consistent with TCL inhibition time dependence (37). The drug would need time to insert itself into the dimer interface for its full inhibition effects to be seen. When observing the TCL binding site in the context of full-length FAS, there is some solvent accessibility from the bulk solvent and between the adjacent domains that would allow the inhibitor to insert itself in between the monomers with minimal structural rearrangement. It should be noted that TCL shows slow onset inhibition in bacterial *Francisella tularensis* FabI ER; however, this observation is associated with a loop closure around the inhibitor and produces tight binding (0.44 nM K_{i}) (57). In contrast, hER has no equivalent structural change and a comparatively low binding affinity.

Given the location of TCL in the dimer interface and the significant impact on the dimer orientation, it seems likely that TCL is acting as a PPI inhibitor. PPI inhibitors are a growing class of small molecule inhibitors that act through modulating the interface of protein complexes. They tend to be more hydrophobic, with more aromatic rings, fewer rotatable bonds, and fewer hydrogen bondable groups (58), similar to TCL. The most efficient PPI inhibitors tend to be larger molecules. PPI inhibitors with lower molecular weights like TCL usually have K_{d} values in the micromolar range (58). PPI inhibitors do not necessarily induce large conformational changes but rather act through local rearrangements (59). Comparisons with pER indicate that TCL binding could plausibly reorient the NADPH binding site into a catalytically unfavorable conformation, leading to inhibition of the ER activity.

It should be reiterated that despite significant effort to crystallize the protein with NADP^{+}, no density was seen in the structures. If the TCL is capable of binding in the active site in a bacterial fashion, the NADP^{+} would need to be present for this to occur. Therefore, the active site binding model could theoretically be plausibly. However, the kinetic data and the PPI inhibitor-like characteristics of the TCL binding site support the hypothesis of an allosteric inhibition mechanism. Additionally, the observed TCL binding site is energetically favorable over the calculated active site binding model. Therefore, it seems likely that the crystal structure binding site is the physiologically relevant one.

**Conclusions**—Studies have repeatedly demonstrated that TCL inhibits human FAS (36–41); however, no determination regarding the binding mode has ever been performed. In this study, we present for the first time the x-ray crystal structure of the human FAS ER domain, both alone and in the presence of its inhibitor TCL. TCL inhibition of FAS was initially believed to act as a traditional active site inhibitor, similar to its mode of inhibition in bacterial ER. However, our structural and kinetic data support an allosteric PPI inhibition model. TCL induces dimer orientation changes that result in downstream reorientation of catalytic residues in the NADPH binding site. If this is the actual mode of inhibition, then TCL may be a viable scaffold to design larger molecule that might have more inhibitory potential. The ER dimer interface is 1359 Å^{2} in pFAS. This opens up a lot of potential interaction area to exploit for future inhibitor design. The chemical structure could potentially be adjusted to remove the controversial xenoestrogenic properties and improve its safety *in vivo*. Several TCL derivatives have already been developed to combat TCL-resistant bacteria (57, 60–62), presenting a pool of potential candidate compounds to develop structure-activity relationship profiles. Understanding and exploiting the TCL inhibition mechanism is the first step toward developing a novel, viable anti-cancer FAS inhibitor.

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