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Biochemical Characterization and Structure-Based Mutational Analysis Provide Insight into the Binding and Mechanism of Action of Novel Aspartate Aminotransferase Inhibitors

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00914.

Ten supplementary figures that contain additional details about the development and optimization of the GOT1 high-throughput screen, cell-based assays, GOT1 protein production, crystallization, and X-ray structure analysis (PDF)

Notes

The authors declare the following competing financial interest(s): C.A.L. and A.C.K. are inventors on patents pertaining to Kras-regulated metabolic pathways, redox control pathways in pancreatic cancer, and targeting GOT1 as a therapeutic approach. A.C.K. also holds a patent on the autophagic control of iron metabolism and is on the SAB and has ownership interests in Cornerstone Pharmaceuticals and Vescor Therapeutics. L.C.C. owns equity in, receives compensation from, and serves on the Scientific Advisory Boards of Agios Pharmaceuticals and Petra Pharmaceuticals. L.C.C.'s laboratory also receives financial support from Petra Pharmaceuticals. Agios Pharmaceuticals is identifying metabolic pathways of cancer cells and developing drugs to inhibit such enzymes to disrupt tumor cell growth and survival.

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Abstract

Pancreatic cancer cells are characterized by deregulated metabolic programs that facilitate growth and resistance to oxidative stress. Among these programs, pancreatic cancers preferentially utilize a metabolic pathway through the enzyme aspartate aminotransferase 1 [also known as glutamate oxaloacetate transaminase 1 (GOT1)] to support cellular redox homeostasis. As such, small molecule inhibitors that target GOT1 could serve as starting points for the development of new therapies for pancreatic cancer. We ran a high-throughput screen for inhibitors of GOT1 and identified a small molecule, iGOT1-01, with *in vitro* GOT1 inhibitor activity. Application in pancreatic cancer cells revealed metabolic and growth inhibitory activity reflecting a promiscuous inhibitory profile. We then performed an *in silico* docking analysis to study inhibitor—GOT1 interactions with iGOT1-01 analogues that possess improved solubility and potency properties. These results suggested that the GOT1 inhibitor competed for binding to the pyridoxal 5-phosphate (PLP) cofactor site of GOT1. To analyze how the GOT1 inhibitor bound to GOT1, a series of GOT1 mutant enzymes that abolished PLP binding were generated. Application of the mutants in X-ray crystallography and thermal shift assays again suggested but were unable to formally conclude that the GOT1 inhibitor bound to the PLP site. Mutational studies revealed the relationship between PLP binding and the thermal stability of GOT1 while highlighting the essential nature of several residues for GOT1 catalytic activity. Insight into the mode of action of GOT1 inhibitors may provide leads to the development of drugs that target redox balance in pancreatic cancer.

Graphical Abstract

Pancreatic ductal adenocarcinoma (PDA) is projected to become the second most deadly cancer in the United States by 2020, contrasting a relatively low incidence rate.¹ Metastatic PDA is highly resistant to genotoxic chemotherapy, and even the most aggressive regimens provide only a few months of additional survival.² Like most cancers, metabolic processes in PDA cells are altered to facilitate macromolecular biosynthesis and protect against intra- and

extracellular stressors.³ Reactive oxygen species (ROS) are a byproduct of metabolism and represent a notable metabolic stress to PDA cells. Previously, we described a new metabolic pathway in PDA cells that is used to manage ROS through the maintenance of glutathione (GSH) pools.⁴ Inhibition of an essential enzyme in this pathway, cytosolic glutamate oxaloacetate transaminase 1 (GOT1, UniProtKB entry P17174), is profoundly growth inhibitory in PDA cells and tumors but well tolerated by normal pancreatic cells and fibroblasts. GOT1 metabolic pathways have also been shown to play a role in other cancers,^{5–8} and in addition, the concurrent inhibition of the mitochondrial electron transport chain creates a synthetic lethal context for GOT1 inhibition.⁹ Collectively, these results illustrate that small molecule inhibitors of GOT1 would be useful tools for studying and ultimately targeting metabolism in PDA and other cancers.

To date, selective small molecule inhibitors of GOT1 do not exist. Accordingly, we performed a high-throughput small molecule screen using a miniaturized GOT1 protein-based assay. This revealed lead molecules with *in vitro* GOT1 inhibitory activity. Comparison of these inhibitors to genetic GOT1 knockdown in cell-based proliferation assays and metabolomics studies indicated that the lead GOT1 inhibitors exhibit additional activities that precluded a formal assessment of the GOT1 inhibitory activity in cells. *In silico* docking, X-ray crystallography, and thermal shift assays using wild type and mutant enzymes were then employed, which suggested, but were unable to formally conclude, that the GOT1 inhibitors act in a PLP-competitive manner. Collectively, these studies describe a new class of GOT1 inhibitors that may ultimately serve as a lead for the development of drugs that target redox balance in pancreatic cancer.

MATERIALS AND METHODS

Recombinant Protein Expression.

One Shot BL21 (DE3) Star *Escherichia coli* competent cells (Thermo Fisher) were transformed with relevant plasmids and inoculated onto Kanamycin Luria Broth/Agar plates. Two hundred milliliter portions of Terrific Broth starter cultures were used to inoculate 6 × 1.5 L cultures with kanamycin at a concentration of 100 µg/mL. Cultures were grown to an OD₆₀₀ of 0.8 at 37 °C with shaking at 160 rpm and induced with isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. The temperature of the culture was then decreased to 18 °C, and shaking was continued overnight. Cells were harvested the next day by centrifugation using an F9–6×1000 LEX rotor at 6000 rpm for 10 min at 4 °C in a Sorvall Lynx 6000 centrifuge (Thermo Scientific). Bacterial pellets were stored at –80 °C until use.

Protein Purification.

Bacterial pellets were homogenized by being magnetically stirred at 4 °C in buffer A [20 mM NaH₂PO₄ (pH 7.4) and 0.5 M NaCl], 1:100 protease inhibitor, 1:100 lysozyme, and 1:10000 universal nuclease for 1 h. Cells were lysed by pulsing sonication with a Branson model 250 sonifier at 50% power output in 3 min on/off cycles. The solution was then centrifuged at 16000 rpm for 30 min using an F20–12×50 LEX rotor at 4 °C in a Sorvall Lynx 6000 centrifuge (Thermo Scientific). The supernatant was filtered using a 0.45 µm PES

filter. The clarified lysate was passed over a 5 mL HisTrap HP column (GE Healthcare) using an NGC Quest FPLC system (Bio-Rad) at a flow rate of 1 mL/min with buffer A and eluted using a 20 to 500 mM imidazole gradient over 20 column volumes (CV). The absorbance at 280 nM was monitored, and peak elution fractions were collected and concentrated to 2 mL using an Amicon Ultra-15 centrifugal filter unit with a 10 kDa molecular weight cutoff at 4000 rpm and 4 °C. Material was then injected onto a 16/60 Sephacryl S-200 HR size exclusion column (GE Healthcare) in buffer B [20 mM HEPES (pH 7.5) and 200 mM NaCl]. For wild type GOT1 (WT-GOT1), dimeric material was separated from tetrameric material via SEC, to ensure that proteins were monodisperse. Protein was concentrated to 10–20 mg/mL, aliquoted, and flash-frozen in liquid nitrogen for storage until use.

High-Throughput Screening GOT1/GLOX and Counter Screening GOT1/MDH1 Assays.

GOT1 enzymatic activity was measured utilizing two coupling reactions (Figure 1A,B). For primary screening, GOT1 activity was coupled to aldehyde oxidase 1 (GLOX1) and horseradish peroxidase (HRP). For this reaction, aspartate and α -ketoglutarate (α KG) act as GOT1 substrates and are converted to glutamate and oxaloacetate. The glutamate product serves as a substrate for GLOX1, which is converted to α KG and hydrogen peroxide (H_2O_2). HRP uses H_2O_2 to oxidize nonfluorescent Amplex Red, resulting in the formation of the fluorescent product resorufin that can be readily measured by fluorescence. Using this GLOX1/HRP enzyme-coupled assay, the kinetic parameters and specific activity of recombinant human GOT1 protein were determined and used to optimize a 1536-well plate-based HTS end point assay (Figure S1A–K). Library compounds (15 μ M, DMSO vehicle) at the California Institute for Biomedical Research were screened against GOT1 using nonsaturating (apparent K_M) concentrations of aspartate (4 mM) and α -ketoglutarate (0.5 mM) substrates. Following incubation at 37 °C for 20 min, at which point the rate of reaction was linear under the defined conditions, the fluorescence (excitation at 544 nm, emission at 590 nm) was measured to determine enzyme inhibition activity. The pan-transaminase inhibitor amino-oxyacetate (AOA) was used as a positive control (Z' factor > 0.9) (Figure S1L). The final concentrations of the components of the assay were as follows: 100 mM HEPES buffer, 100 mM KCl, 80 nM GLOX, 0.05 unit/mL HRP, 15 mg/mL Amplex Red, and ~1 nM GOT1 (adjusted from each purification to produce a 20 min linear reaction). GOT1 inhibition activity was confirmed using a lower-throughput secondary assay in which GOT1 activity is coupled to malate dehydrogenase 1 (MDH1). MDH1 catalyzes the conversion of oxaloacetate to malate using NADH (Figure 1B). As a result, enzyme activity can be monitored by the loss of NADH fluorescence (excitation at 350 nm, emission at 460 nm). For this reaction, NADH and MDH1 are added at final concentrations of 250 μ M and 0.3 μ g/mL, respectively.

Cell Lines.

PaTu8902 pancreatic cancer cells were obtained from DSMZ, and DLD1 colon cancer cells were obtained from ATCC. Both lines were maintained in DMEM with 10% fetal bovine serum (FBS). Cell lines were validated by STR profiling and routinely tested for mycoplasma contamination using MycoAlert (Lonza).

PaTu8902 and DLD1 cells were treated overnight with the lentiviral particle-containing 293T supernatant carrying non-targeting (NT) short hairpin (sh)RNAs or those targeting GOT1 (GenBank entry [NM_002079](#)) or GOT2 (GenBank entry [NM_002080](#)). The next day, the medium was changed and 1 $\mu\text{g/mL}$ puromycin was added for 48 h. Stable lines were employed in the colony-forming assays.

Acute Toxicity Assay.

Cells were plated in 96-well format at a density of 6000 cells/well. The next day, cells were treated with iGOT1-01 across a dose range (3.125–200 μM) for 3 h. Drug-containing media were then removed; fresh drug-free media were added, and cell viability was determined via a Cell Titer Glo (Promega) or Alamar Blue (Invitrogen) assay according to the manufacturer's protocol. For Alamar Blue assays, cells were incubated with Alamar Blue-containing media for 2 h at 37 °C after removal of the drug before the fluorescence was read.

Proliferation Assay.

PaTu8902 and DLD1 cells were plated in 96-well white-walled plates in growth medium at a density of 500 cells/well. The following day, vehicle (DMSO or PBS), AOA (in PBS), or iGOT1-01 (in DMSO) was added. Cells were grown for 5–8 days and assayed by the Cell Titer Glo (Promega) assay according to the manufacturer's instructions. Cell viability is presented relative to vehicle-treated wells.

Colony-Forming Assay.

Colony-forming assays (CFAs) were performed as described previously.⁴ PaTu8902 and DLD1 cells with stable doxycycline-inducible (iDox) shRNAs targeting GOT1, GOT2, or NT were plated into six-well plates at a density of 500 cells/well in triplicate. Dox-containing (1 $\mu\text{g/mL}$) medium was added 24 h after cell seeding, and media (with or without Dox) were replaced with fresh media every 48 h until the end point. At the end point (8 days for PaTu8902, 10 days for DLD1), cells were fixed with pure MeOH and stained with crystal violet, and colonies were imaged and quantified. GOT1 and GOT2 knockdowns were validated by Western blot analysis.

Western Blot Analysis.

Stable iDox-shNT, -shGOT1, and -shGOT2 cells were cultured with or without Dox medium, as described for CFAs. On the day that cells were stained in CFA assays, protein lysates were collected with RIPA buffer (Sigma) containing protease inhibitor cocktail (Sigma/Roche) and phosphatase inhibitor (Sigma). Protein samples (10–40 μg) were resolved on NuPAGE Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked in Tris-buffered saline (Bio-Rad) containing 5% nonfat dry milk (Bio-Rad) and 0.1% Tween 20 (Sigma) (TBS-T buffer) and incubated with the primary antibody overnight at 4 °C. The membranes were then washed with TBS-T buffer followed by exposure to the appropriate HRP-conjugated secondary antibody for 1 h and visualized on film (GeneMate) using enhanced chemiluminescence (ECL) detection (Amersham). The following antibodies were used: anti-aspartate aminotransferase (anti-GOT1) at a 1:1000 dilution (Abcam, ab17193), anti-GOT2 at a

1:1000 dilution (Abcam, ab171739), and loading control vinculin at a 1:10000 dilution (Cell Signaling). The anti-rabbit IgG, HRP-linked secondary antibody (Cell Signaling) was used at a 1:10000 dilution and hybridized for 1 h at room temperature.

Metabolomics Analysis.

Isotope tracing metabolomic analysis was performed as described previously.¹⁰ Briefly, PaTu8902 cells were grown as detailed above and then transferred into glutamine-free (with 10 mM glucose) DMEM containing 10% dialyzed FBS and supplemented with 2 mM [U-¹³C]glutamine overnight. Polar metabolites were extracted with 2 mL of a MeOH/H₂O mixture (4:1) for 30 min on dry ice, scraped, transferred to 2 mL tubes, and centrifuged (30 min at 21000g), and the supernatants were dried under vacuum. Samples were resuspended in 15 μ L of high-performance liquid chromatography grade water. Five micro-liters of each sample was injected and analyzed using a 5500 QTRAP triple-quadrupole mass spectrometer (AB/Sciex) coupled to a Prominence UFLC system (Shimadzu).

GOT1/GLOX Assay for Specific Activity.

GOT1 or mutant GOT1 protein (300 ng) was preincubated at 37 °C in a buffer containing 200 mM HEPES (pH 7.4) and 200 mM KCl. A separate reaction mix containing 4 mM aspartate, 0.5 mM α KG, 80 nM GLOX, 0.5 unit of HRP (Sigma-Aldrich), and 1.285 μ g of Amplex Red Reagent (Invitrogen) was also prepared and prewarmed at 37 °C. WT-GOT1 or mutants were then added to the reaction mix to bring the final volume to 100 μ L. The production of resorufin was measured kinetically using a Tecan I-control Infinite 200 Pro plate (excitation at 544 nm, emission at 590 nm) for 15 min with 15 s interval reads. To relate H₂O₂ to relative fluorescence units (RFU), an H₂O₂ dilution series was generated from 16.6 pg to 8.5 ng in 200 mM HEPES (pH 7.4) and 200 mM KCl. The dilution series was added to the Amplex Red reaction mix supplemented with 4 mM aspartate and 0.5 mM α KG, dispensed into a 96-well plate, and incubated in the dark for 5 min at 37 °C, at which point a single end point read was taken. Interpolation using the H₂O₂ standard curve was used to determine velocities (micromolar per minute per milligram) for WT-GOT1 or mutant GOT1 enzymes.

In Silico Modeling.

Docking was performed using the WT-GOT1 crystal structure [Protein Data Bank (PDB) entry 3II0]. Model coordinates were prepared for docking using the protein preparation wizard within Maestro 11 by adding hydrogens and generating protonation states using EPIK at pH 7.0 $\pm$ 2.0.^{11–13} Hydrogen bond assignments and torsion angles were optimized using PROPKA, and a restrained minimization was performed using the OPLS3 force field.^{14–17} Ligands were prepared with LIGPREP from SMILES using an OPLS3 force field modified using EPIK.^{11–13,15–18} Molecules were desalted and featured all tautomers. A glide grid of 8000 $\text{\AA}^3$ was centered on residues previously identified as being near the PLP binding site, including F19, E142, and D223 as identified in the GOT1 crystal structure. Glide docking was performed using the SP protocol.¹⁹ Ligand poses were evaluated on the basis of Emodel and Glide scores.

Protein Crystallization.

Novel crystallization conditions were obtained for WT-GOT1 at 16 mg/mL by sparse matrix screening using a 1:1 ratio of protein to mother liquor via sitting drop vapor diffusion (Figure S2). An initial crystallization hit of 25% (w/v) polyethylene glycol 3350 and 0.1 M sodium acetate trihydrate (pH 4.5) was identified after 48 h at 21 °C. The hit was further optimized by additive screening. Final crystallization conditions included 5% Cymal.²⁰ Cryoprotected crystals were soaked in mother liquor supplemented with 20% glycerol and flash-frozen in liquid nitrogen.

T110A (30 mg/mL) mutant crystals were grown using the same method as described above (Figure S2). Crystals were nucleated in a mother liquor of 25% (w/v) polyethylene glycol 3350 and 0.1 M HEPES (pH 7.5) after 48 h at 21 °C. Cryoprotected crystals were soaked in mother liquor supplemented with 20% ethylene glycol and flash-frozen in liquid nitrogen.

T110A/S256A (38 mg/mL) mutant crystals were nucleated from a solution containing 20% (w/v) polyethylene glycol monomethyl ether 2000, 0.1 M Tris (pH 8.5), and 0.2 M trimethylamine N-oxide dehydrate after 48 h at 21 °C (Figure S2). To increase size, crystals were microseeded using the dilution method.²¹ Cryo-protected crystals were soaked in mother liquor supplemented with 20% glycerol and flashfrozen in liquid nitrogen.

Several attempts were made to obtain an iGOT1-074-8- bound structure using co-crystallization and soaking. Crystals were soaked with iGOT1-074-8 over a concentration range of 0.15–0.3 mM for 5 h to 7 days and then analyzed for diffraction. Ultimately, we were not able to successfully generate inhibitor-bound crystals for any of the GOT1 proteins.

Structure Determination and Analysis.

Diffraction data were collected at the APS (Argonne National Laboratory, Lemont, IL) using LS-CAT 21. Multiple data sets were collected using a MAR300 and Dectris Eiger 9M detectors at a wavelength of 1.00 Å and 100 K. Diffraction data were indexed, processed, and scaled using the HKL2000 software suite.^{22,23} Structures were determined by molecular replacement using MOLREP with WT-GOT1 (PDB entry 3II0) as the search model.^{24,25} The models were built and refined using REFMAC5 followed by placement of water molecules using COOT.^{23,26,27} T110A-GOT1 diffraction data showed twinning, and the structure was determined using detwinning methods and restrained refinement within the CCP4 suite.²³ In the T110A/S256A mutant structure, no density was observed for iGOT1-074-8. The final model statistics are listed in Table 1.

Thermal Shift Assays.

WT-GOT1 and its derived mutants bat 1 mg/mL were mixed in a 1000:1 ratio with Sypro Orange dye (Sigma-Aldrich) and plated. Samples were processed using a Bio-Rad CFX C100 Touch quantitative polymerase chain reaction and run using the FRET assay settings with a heating ramp of 0.3 °C/s cycling from 4 to 100 °C. Analysis was performed using the Bio-Rad CFX Manager Software (version 3.1, Bio-Rad).

RESULTS

Identification of Novel GOT1 Inhibitors.

A library of 800000 small molecules was screened in single-point format at 15 μM (Figure S3) in the GOT1/GLOX/HRP assay (Figure 1A).²⁸ Setting a threshold Z score of -3 , corresponding to at least 50% GOT1 inhibition, gave a 0.1% hit rate and resulted in 850 hits. These putative hits were first confirmed in triplicate and then counter-screened against GLOX/HRP. Compounds with inhibitory activities in the GLOX/HRP assay similar to those in the GOT1/GLOX/HRP assay were assumed to be false positives that inhibited the coupling enzymes. From these collective efforts, we identified the hit compound iGOT1-01 (Figure 1C and Figure S3), which had an IC_{50} of 11.3 μM in the GOT1/GLOX/HRP assay (Figure 1D). This was ~ 3 -fold more potent than its activity in the GLOX/HRP assay [31.5 μM (Figure S4A)].

GOT1 and GLOX can both utilize glutamate, which was assumed to account for the activity of iGOT1-01 in the GLOX/HRP assay. To examine the GOT1 inhibitory activity of iGOT1-01 in the absence of GLOX, we developed an additional GOT1 assay that employs malate dehydrogenase 1 (MDH1) as the coupling enzyme (Figure 1B). In this assay, the oxaloacetate product from the GOT1 reaction is the substrate for MDH1, which oxidizes NADH to NAD^+ . The loss of NADH fluorescence is used as a readout of GOT1 activity. In the GOT1/MDH1 assay, iGOT1-01 had an IC_{50} of 84.6 μM (Figure S4B). No inhibitory activity was observed for iGOT1-01 against MDH1 alone at 100 μM (Figure S4C).

We then used mass spectrometry-based metabolomics approaches to monitor changes in cellular metabolism associated with the GOT1 inhibitory activity of iGOT1-01. To this end, we first determined the maximal concentration of the inhibitor that cells would tolerate over a 3 h period. The 3 h time point represents the length of time required to turn over the intracellular pool of the GOT1 substrate aspartate, whose accumulation we previously illustrated to provide a robust readout for GOT1 inhibition.⁴ Application of iGOT1-01 to a pancreatic cancer cell line (PaTu8902) and a colon cancer cell line (DLD1) at the largest soluble dose (200 μM) for 3 h revealed little to no toxicity using two readouts of cell viability (Figure 2A,B).

In pancreatic cancer cells grown in culture, our previous results using nonradioactive carbon 13 (^{13}C)-labeled glutamine metabolomic profiling illustrated that GOT1 substrate aspartate is predominantly derived from glutamine.⁴ In addition, we demonstrated that shRNA-mediated GOT1 knockdown led to the accumulation of aspartate and predominantly the $\text{M} + 4$ isotopologue of aspartate. Thus, to analyze the GOT1 inhibitory activity of iGOT1-01 in cells, we used [^{13}C]glutamine tracing metabolomics and followed glutamine-derived aspartate, specifically, and the glutamine-derived metabolome, generally, in PaTu8902 cells across range of iGOT1-01 concentrations (Figure 2C–E). Nonsupervised hierarchical clustering of the [^{13}C]glutamine tracing metabolomics data revealed widespread changes to the glutamine-derived metabolome at 200 μM iGOT1-01 (Figure 2C). This was not observed for smaller doses, which all cluster with the vehicle-treated cells. Glutamine-derived metabolites from cells treated with the small molecule pan-transaminase control AOA

clustered independently of those from iGOT1-01-treated cells with one important exception, aspartate isotopologues.

In pancreatic cancer cells, inhibition of GOT1 preferentially leads to accumulation of the M + 4 aspartate isotopologue from [¹³C]glutamine. In contrast, inhibition of GOT2 (the mitochondrial GOT isoform) leads to a drop in the level of M + 4 aspartate, as aspartate is the product of the GOT2-mediated reaction. Because GOT2 is upstream of GOT1 in pancreatic cancer glutamine metabolism, dual GOT1/GOT2 inhibition is reflected by a GOT2 inhibition signature.⁴ This is evident using the pan-transaminase inhibitor and positive control AOA (Figure 2E and Figure S5A). Indeed, we observed that iGOT1-01 treatment of pancreatic cancer cells led to a drop in the level of all aspartate isotopologues, in a manner similar to that seen with AOA (Figure 2E). Thus, these data suggest iGOT1-01 also has GOT2 inhibitory activity in cells. Because GOT2 inhibition precludes the assessment of GOT1 inhibition, we were unable to determine the degree of GOT1 inhibitory activity in cells using this assay. Next, we tested the growth inhibitory potential of iGOT1-01 in PaTu8902 and DLD1 cells (Figure 2F) and compared it to that of AOA (Figure 2G). DLD1 cells were more sensitive to both inhibitors. Previously, we demonstrated the growth of pancreatic cancer cells was repressed following shRNA-mediated inhibition of GOT1 or GOT2.⁴ In contrast, here we found that the growth of DLD1 cells was not impacted by GOT1 or GOT2 knockdown (Figure 2H–K and Figure S5B–G). Given that iGOT1-01 behaved more like AOA in terms of its impact on the metabolome (Figure 2C) and cellular selectivity (Figure 2F,G), these data suggest that dual inhibition of GOT1 and GOT2 in cells exhibits a profile different from that of single-isoform inhibition. Alternatively, because at large doses growth of both cell lines was fully inhibited, pleiotropic inhibitory activity against multiple transaminases or PLP-dependent enzymes may account for these results.

***In Silico* Analysis.**

Given the high concentration of iGOT1-01 required in cells and the observed incongruence with the isoform-selective shRNA experiments, we set forth to analyze how our lead scaffold was interacting with GOT1. Such insight could be used to identify regions on the molecule that could be optimized for isoform selectivity by medicinal chemistry-based approaches. For the *in silico* analysis, we opted to use iGOT1-790-5, which was determined to be a modestly more potent iGOT1-01 analogue (Figure S6A,B).

On the basis of our *in silico* studies, the top pose (−9.6 kcal/ mol) suggested that iGOT1-790-5 bound competitively within the pyridoxal 5′-phosphate (PLP) site (Figure 3). As a baseline comparator, PLP was docked with the top pose yielding a score of −8.1 kcal/ mol, suggesting that the predicted pose of iGOT1-790-5 was significant. Several interactions were predicted between GOT1 and iGOT1-790-5, including hydrogen bonding among Tyr226, Trp141, Asp223, Asn195, and Gly39, three of which were also reported to bind PLP.^{29,30} In this structure, PLP displayed polar contacts with Trp141, Asp223, Tyr226, Gly109, Thr110, Ser256, Phe257, and Lys259.

Kinetic Analysis.

To eliminate the competition between iGOT1-790-5 and endogenous PLP, a series of mutants were designed (T110A, S256A, and T110A/S256A) to ablate PLP binding and open the active site for iGOT1-790-5. Residues were chosen that had side-chain polar contacts with the PLP phosphate and did not appear to play a role in the docked pose of iGOT1-790-5. Additionally, we postulated that reducing the overall polar surface area of the GOT1 active site may be beneficial for the binding of iGOT1-790-5 because of its hydrophobicity. It is noteworthy that we did not select to mutate the active site lysine residue (Lys259), which covalently binds and holds PLP and would formally preclude PLP binding, based on the notion that Lys259 is much closer to interactions that may be essential to iGOT1-790-5 binding.

Next, we generated and analyzed the activity of the mutant GOT1 structures, relative to that of WT-GOT1. Our results showed that WT-GOT1 was active, albeit with a specific activity ($27.1 \mu\text{mol min}^{-1} \text{mg}^{-1}$) that was lower than the literature value ($70.83 \mu\text{mol min}^{-1} \text{mg}^{-1}$).³¹ The relative activities of GOT1 mutants, compared to that of the wild type enzyme, were determined by comparison of the specific activity at the apparent K_M of each substrate. Each single mutant appeared to retain partial activity. The T110A mutant trended toward being less active than the S256A mutant; however, the difference between the two was not significant (Figure 4). We interpreted this to mean that each single mutant retained some degree of binding to PLP, although the affinity for PLP was decreased substantially. Furthermore, introduction of the double mutant T110A/S256A completely ablated enzyme activity and allowed us to assume there was no PLP bound in the active site (Figure 4 and Figure S6C,D).

X-ray Structure Analysis.

We performed X-ray protein co-crystallographic studies using wild type and mutant variants of GOT1 and a GOT1 inhibitor further optimized for solubility and into which bromine was substituted to facilitate phase determination [iGOT1-074-8 (Figure S6E)]. Initial efforts with the wild type protein failed to yield a co-crystal structure with electron density corresponding to bound iGOT1-074-8. On the basis of relative catalytic activities, we predicted that the T110A/S256A double mutant would not possess bound endogenous PLP and that this would facilitate the observation of iGOT1-074-8 bound within the GOT1 PLP binding site. Efforts to co-crystallize either the T110A or S256A single mutant with iGOT1-074-8 resulted in structures that contained electron density within the PLP binding site corresponding to bound PLP but not the iGOT1-074-8 ligand. Consistent with the observed ablation of catalytic activity displayed by the T110A/S256A double mutant (Figure 4), efforts to co-crystallize this form of the protein resulted in a structure that lacked electron density within the PLP binding site that would correspond to either a bound molecule of PLP or the iGOT1-074-8 ligand. Presumably, the transient and/or low-affinity nature of the GOT1—iGOT1-074-8 interaction precluded observation of the bound state of this interaction. Crystal structure statistics are listed in Table 1.

The WT-GOT1 structure is a homodimer, each of which contains PLP. An overlay of the monomers reveals a high degree of similarity with a RMSD of $0.14 \text{ \AA}$ (Figure S7A).

Similarly, WT and T110A mutant structures share a high degree of structural similarity [RMSD of 0.50 Å (Figure S7B)]. In contrast to that of WT-GOT1, the structure of the T110A mutant has six monomers in the asymmetric unit with partial density for the PLP cofactor in the active site (Figure S7C). Overall, each chain and their PLP cofactors are highly similar [RMSD of 0.15–0.23 Å (Figure S7D)]. In the double mutant structure, there is only one monomer per asymmetric unit, also with a high degree of structural similarity to WT-GOT1, yet without bound PLP [RMSD of 0.35 Å (Figure 5A and Figure S8)].

Overall, the structural results support the enzymatic activity data (Figures 4 and 5 and Figures S7 and S8). We observed lower activity for the T110A mutant protein than for the WT protein, which correlated with a decrease in the electron density for PLP in the T110A mutant when compared to WT (Figure 5B,C). In the double mutant structure, there was no density for PLP, confirming the abolishment of enzymatic activity (Figure 5D).

Thermal Shift Assay and Compound Titration.

Thermal shift assays (TSA) were utilized to biophysically characterize recombinant human WT-GOT1 and its mutants. The assay functions by protein denaturation over a temperature gradient, where during protein unfolding, exposed hydrophobic regions bind a dye and fluoresce due to solvent relaxation effects. The melting profile of WT-GOT1 displayed multiple peaks ($dRFU/dt$ vs temperature), with major peaks at 50, 60, and 70 °C, indicative of multiple species in solution or loosely associated domains (Figure 6A). Size exclusion chromatography (SEC) provided supporting evidence for both a dimeric species and a tetrameric species in solution for the WT protein (Figure S9A). Dimeric and tetrameric species were separated and again subjected to protein melting, both yielding identical profiles, indicating an active equilibrium (data not shown).

In contrast to the WT protein, the T110A and S256A mutants were observed by SEC to be exclusively dimeric in solution (Figure S9A). Via comparison of the TSA results of GOT1 variants (in the absence of iGOT1-074-8), both T110A and S256A GOT1 mutants displayed a minor melting peak near 40 °C, likely corresponding to apoprotein, free of PLP (Figure 6A). The major peak for both single mutants melted at a much higher temperature, near 70 °C. This species likely corresponds to PLP-bound GOT1. Further supporting this hypothesis, the melting profile for the T110A/S256A mutant displayed a single uniform melting peak at 41.5 °C (Figure 6A). A titration of T110A/S256A with PLP at high concentrations shifted the melting profile from 40 to 70 °C, supporting the assignment of these peaks as the PLP-unbound and -bound forms (Figure 6B).

WT-GOT1 and its mutants were also assessed in the presence of iGOT1-074-8. For WT-GOT1, the addition of iGOT1-074-8 stabilized the protein, shifting the thermal profile toward a single peak at 72 °C (Figure 6C). Importantly, this result supported the conclusion that iGOT1-074-8 directly binds GOT1. For both single mutants, increasing concentrations of iGOT1-074-8 increase the peak intensity for the unbound state at 40 °C, supporting the hypothesis that iGOT1-074-8 acts through displacement of PLP (Figure 6D,E). However, it did not appear that iGOT1-074-8 fully displaced PLP in this experiment, as the bound form is still observed. Addition of iGOT1-074-8 to the double mutant appeared as a small shoulder peak at 47 °C (Figure 6F), supporting the notion that iGOT1-074-8 binds the

enzyme directly and does not have the requirement for a transitional GOT1—PLP complex. Overall, the thermal analysis supports the binding of iGOT1-074-8 to GOT1 through the displacement of PLP; however, this interaction is likely transient or weak as we did not observe full conversion of the target to an unbound PLP state (Figure 6 and Figure S9B–F).

DISCUSSION

Pancreatic cancer cells depend on the GOT1 pathway for survival and growth, and the targeting of this pathway may provide new, selective drugs. We ran an HTS campaign for GOT1 inhibitors and identified iGOT1-01 as a micromolar inhibitor of GOT1 (Figure 1C and Figure S3). Glutamine carbon tracing experiments using mass spectrometry-based metabolomics indicated that iGOT1-01 inhibited GOT2 (Figure 2), which precluded an analysis of the GOT1 inhibitory activity because GOT2 is upstream of GOT1 in glutamine metabolism.⁴ Pancreatic cancer cells are also dependent on GOT2 activity, and a dual GOT^{1/2} inhibitor may have additional utility. Indeed, GOT1 and GOT2 share complete conservation of the active site moieties that interact with PLP (Figure S10) and potentially the inhibitors described herein. In addition, dependence on GOT activity is a feature of several types of cancer, and inhibition of mitochondrial metabolism sensitizes cancer cells to GOT inhibition.^{5–9} Nevertheless, the GOT2 inhibitory activity and perhaps additional transaminase or PLP-dependent enzyme off-target activities of iGOT1-01 indicate that a more selective inhibitor will be necessary to test the activity of GOT1 inhibition in preclinical pancreatic cancer models. To this end, the iGOT1-01 drug scaffold may provide a useful starting point.²⁸

In GOT1, multiple hydrogen bond networks (direct and extended) are coupled to the phosphate pyridinyl nitrogen and 3-hydroxy of PLP, inducing its electrophilicity. Residues Thr110 and Ser256 face the phosphate group. Lys259 faces the PLP aldehyde to form a Schiff base making an internal aldimine. Residues Tyr226 and Asp195 make hydrogen bonds to the PLP hydroxyl group. Asp223 makes a hydrogen bond to the pyridinyl nitrogen of the PLP cofactor. Altogether, these interactions result in the activation and stabilization of PLP (Figure 5B). As previously described, an extended hydrogen bond network involving residues Asp222, His143, and His189 and water molecules affects the protonation state of the PLP pyridine nitrogen and its activation.^{29,30} Herein, we demonstrate PLP binding can be disrupted by disturbing the hydrogen bond network within the phosphate group, either partially or entirely, resulting in reduced or ablated GOT1 enzymatic activity.

It was interesting to note that single mutation of either Thr110A or Ser256A resulted in an altered oligomeric state of the protein, eliminating the tetrameric species compared to the WT protein. While these residues reside near the dimer interface, they do not directly disrupt dimerization. Comparing the structure of T110A or T110A/S256A to that of the WT does reveal a shift in the C-terminal helix (371–411) that sits at the dimer interface of tetrameric WT-GOT1 (Figure S8B).

The mutational studies have provided evidence for the displacement of PLP by iGOT1-074-8 (Figure 6). Addition of iGOT1-074-8 to either the S256A or T110A mutant results in a melting profile that mimics the PLP-free state for the T110A/S256A mutant.

Docking results support the finding that iGOT1-074-8 operates through direct competition for the active site. These results are tempered by an inability to generate a compound-bound crystal structure in any of these trials. Future studies will be required to enhance compound affinity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

GOT1	glutamate oxaloacetate transaminase 1
PDA	pancreatic ductal adenocarcinoma
PLP	pyridoxal 5-phosphate
ROS	reactive oxygen species
GSH	glutathione
ITPG	isopropyl β -D-1-thiogalactopyranoside
AOA	amino-oxyacetate
SEC	size exclusion chromatography
HTS	high-throughput screening
GLOX	glutamate oxidase
HRP	horseradish peroxidase
MDH1	malate dehydrogenase 1
FBS	fetal bovine serum
CFA	colony-forming assay
ECL	enhanced chemiluminescence

RMSD

root-mean-square deviation

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Figure 1. High-throughput screening strategy and structure of the GOT1 inhibitor. (A) GLOX/HRP-coupled GOT1 assay. (B) Orthogonal MDH1-coupled GOT1 assay used to confirm GOT1 inhibition activity. (C) Chemical structure of GOT1 inhibitor hit iGOT1-01. (D) Concentration-dependent inhibition of GOT1 activity by iGOT1-01, as determined using the GLOX/HRP-coupled assay ($IC_{50} \sim 11.3 \mu M$).

Figure 2.

iGOT1-01 activity in cell-based assays. (A and B) Impact of iGOT1-01 on cell viability using two independent readouts in pancreatic cancer cells (PaTu8902 in black) or colon cancer cells (DLD1 in red) after 3 h. Relative cell viability as measured by (A) Cell Titer Glo and (B) Alamar Blue. (C) Heat map of altered metabolites ($P < 0.1$) as measured by mass spectrometry-based metabolomics for $[^{13}\text{C}]$ glutamine tracing in PaTu8902 cells after treatment with the compound for 3 h. Data are arranged using two-dimensional unsupervised hierarchical clustering. Red represents metabolites that are more abundant, and green those that are less abundant. The levels of aspartate and glutathione metabolite are commonly decreased in iGOT1-01 (200 μ M) and positive control AOA-treated cells, as highlighted in the metabolite cluster in the red box. (D) Volcano plot of all of the metabolomics data for 200 μ M iGOT1-01-treated PaTu8902 cells relative to the vehicle. Data for Asp isotopologues are colored red. (E) iGOT1-01 exhibits a dose-dependent decrease in the level of aspartate isotopologues from ^{13}C -labeled glutamine that is comparable to that seen upon treatment with AOA (positive control). *** $P < 0.001$. Dose—response curves for (F) iGOT1-01 and (G) AOA in pancreatic cancer cells (PaTu8902 in black) and colon cancer cells (DLD1 in red). (H–K) GOT1 or GOT2 knockdown using two independent doxycycline (Dox)-inducible (iDox) shRNAs inhibits colony formation in the PaTu8902 pancreatic cancer cells (black) but not in the DLD1 colon cancer cells (red). WT, wild type; NT, nontargeting shRNA. *** $P < 0.001$.

Figure 3.

In silico prediction of iGOT1-790-5 bound to WT-GOT1. iGOT1-790-5 (purple, shown as sticks) is predicted to bind within the PLP site (gray, shown as sticks). The presented pose showed the highest score (-9.6 kcal/mol). Thr110 and Ser256 interact with PLP but are not predicted to interact with iGOT1-790-5.

Figure 4.

WT-GOT1 and mutant GOT1 enzymatic activity. Bar graph comparing specific activities of WT vs mutant GOT1 samples based on the coupled Amplex Red assay. The GOT1 double mutant clearly shows no activity ($0.02 \mu\text{mol min}^{-1} \text{mg}^{-1}$ vs background detected at $0.04 \mu\text{mol min}^{-1} \text{mg}^{-1}$).

Figure 5.

WT-GOT1 and mutant GOT1 crystal structure overlay data. (A) Overlay of WT (gray), T110A (salmon), and T110A/S256A (cyan) GOT1 monomer crystal structures. (B) The $2F_o - F_c$ electron density map (gray) contoured at 1σ around PLP in the WT-GOT1 structure. Residues around the PLP site are shown as sticks. The hydrogen bond distance is shown between Ser256 and PLP (2.8 Å). (C) The $2F_o - F_c$ electron density map (gray) contoured at 1σ around PLP in the T110A mutant structure, refined with 70% occupancy for PLP in the active site. Partial density is present for PLP compared to the WT structure. Residues around the PLP site are shown as sticks. The hydrogen bond distance is shown between Ser256 and PLP (2.6 Å). (D) No density was observed for the PLP cofactor in the T110A/S256A mutant structure. Residues around the PLP site are shown as sticks. Water molecules (red pluses) were observed in the PLP site.

Figure 6.

Melting curves for WT-GOT1 and its mutants in the presence of iGOT1-074-8. (A) WT-GOT1 and mutant protein melting profiles. (B) Melting profile for the T110A/S256A GOT1 double mutant across a PLP titration. Melting profiles for (C) WT, (D) T110A, (E) S256A, and (F) T110A/S256A GOT1 proteins across a range of iGOT1-074-8 concentrations.

Table 1.**Data Collection and Refinement Statistics**

	WT	T110A	S256A/T110A
Diffraction Statistics			
wavelength (Å)	0.9787	0.9787	0.9787
space group	P212121	C2	P6522
<i>a</i> , <i>b</i> , <i>c</i> (Å)	91.03, 96.86, 97.87	257.70, 148.61, 83.10	84.08, 84.08, 210.12
<i>α</i> , <i>β</i> , <i>γ</i> (deg)	90, 90, 90	90, 90, 90	90, 90, 120
resolution (Å)	50–2.10 (2.15–2.10) ^a	50–3.00 (3.08–3.00) ^a	50–1.7 (1.74–1.70) ^a
no. of molecules in the asymmetric unit	2	6	1
total no. of reflections	48527	59429	46568
no. of unique reflections	3512	4281	3272
completeness (%)	99.92 (99.44) ^a	99.77 (97.32) ^a	99.76 (97.32) ^a
<i>I</i> /σ(<i>I</i>)	30.01 (4.52) ^a	9.42 (1.71) ^a	47.62 (5.01) ^a
<i>R</i> _{merge} ^b	0.07 (0.32) ^a	0.09 (0.61) ^a	0.08 (0.51) ^a
Refinement Statistics			
<i>R</i> _{work} / <i>R</i> _{free} (%) ^c	20.93/26.93	18.86/21.21	17.90/22.87
no. of protein Atoms	6,430	18,406	3,653
no. of waters	102	10	237
no. of PLPs	2	6	-
no. of PEGs	2	3	2
no. of PO ₄ ³⁻	-	-	1
RMSD for bond lengths (Å ²)	0.013	0.016	0.022
RMSD for bond angles (deg)	1.57	1.93	2.16
PDB entries	6DND	6DNA	6DNB

^aValues in parentheses correspond to those for the highest-resolution shell.

^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$, where *I* is the integrated intensity of given reflection.

^c $R_{\text{work}} = \sum_{hkl} \|F_{\text{obs}} - F_{\text{calc}}\| / \sum_{hkl} |F_{\text{obs}}|$. Five percent of the reflections were excluded from the *R*_{free} calculation.