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Discovery and optimization of aspartate aminotransferase 1 inhibitors to target redox balance in pancreatic ductal adenocarcinoma

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy that is extremely refractory to the therapeutic approaches that have been evaluated to date. Recently, it has been demonstrated that PDAC tumors are dependent upon a metabolic pathway involving aspartate aminotransferase 1, also known as glutamate-oxaloacetate transaminase 1 (GOT1), for the maintenance of redox homeostasis and sustained proliferation. As such, small molecule inhibitors targeting this metabolic pathway may provide a novel therapeutic approach for the treatment of this devastating disease. To this end, from a high throughput screen of ~800,000 molecules, 4-(1*H*-indol-4-yl)-*N*-phenylpiperazine-1-carboxamide was identified as an inhibitor of GOT1. Mouse pharmacokinetic studies revealed that potency, rather than inherent metabolic instability, would limit immediate cell- and rodent xenograft-based experiments aimed at validating this potential cancer metabolism-related target. Medicinal chemistry-based optimization resulted in the identification of multiple derivatives with >10-fold improvements in potency, as well as the identification of a tryptamine-based series of GOT1 inhibitors.

Deregulation of metabolism is a hallmark feature of cancer.[1–5] The rewiring of metabolic programs in cancer cells can serve a range of functions. For example, cancer cells alter metabolism in order to facilitate bioenergetics and biosynthesis, to manage cellular stressors and challenges to redox balance and/or to regulate gene expression.[5] The role of metabolic deregulation in cancer significantly depends on context. Tissue of origin, tissue architecture, degree of oxygenation, as well as other factors influence the metabolic programs of a particular cancer. Thus, small molecule pharmacological probes that target defined components of metabolic pathways/processes have the potential to address specific cancer metabolism-related hypotheses that are challenging to query using genetic methods. Further, such probe molecules may serve as starting points for drug discovery programs aimed at targeting cancer metabolism, as part of drug combination therapeutic approaches for the treatment of refractory tumor types.[6–8] As cancer metabolism targets are frequently found to be overexpressed in tumor cells, which therefore results in the need for higher levels of target engagement in tumor versus non-diseased cell types, a challenge to this approach is the identification of cancer metabolism targets that selectively sensitize cancer cells without impacting non-diseased cell types.

PDAC is a lethal cancer type that is projected to become the second leading cause of cancer-related deaths in the United States by 2020 and, at present, has a 5-year survival rate of ~6%.[9,10] Recently, several PDAC cell lines were demonstrated to be reliant on a KRAS-regulated non-canonical glutamine (Gln) metabolism pathway that enables proliferation and tumor growth.[11] Specifically, KRAS-dependent down-regulation of glutamate dehydrogenase (GLUD1) and upregulation of GOT1 in PDAC results in Gln-derived aspartate being converted to oxaloacetate (OAA) by GOT1 in the cytoplasm, which is subsequently converted to pyruvate through a series of reactions that ultimately lead to the generation of NADPH, which is used to maintain redox balance.[11] GOT1 knockdown in PDAC

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was demonstrated to stunt growth *in vitro* and *in vivo*, whereas GOT1 knockdown in normal cells had no effect on proliferation.[11] As such, GOT1 inhibitors may provide a much-needed therapeutic approach for selectively targeting PDAC.

A biochemical assay-based screen of ~800,000 compounds at the California Institute for Biomedical Research resulted in the identification 4-(1*H*-indol-4-yl)-*N*-phenylpiperazine-1-carboxamide (**1a**) as an inhibitor of GOT1 enzyme activity (Fig. 1).[12]

The synthesis of the urea-based compounds described below was readily accomplished by addition of aryl- or alkyl-isocyanates to 1-(aryl)piperazines or tryptamine in the presence of triethylamine (Schemes 1 and 2). The structural integrity of the identified screening hit was corroborated by re-synthesis of **1a**, which was demonstrated to possess a confirmed IC_{50} of 85 μM in an MDH coupled GOT1 enzymatic assay.[13]

Evaluation of **1a** in preliminary ADME PK studies indicated that the parent compound of this series is stable in mouse plasma, with >95% remaining following 4 h of incubation. In addition, **1a** was found to have reasonable bioavailability and exposure properties in the mouse following oral administration of a 20 mg/kg dose ($t_{1/2}$ = 0.7 h, C_{max} = 4133 ng/mL, $AUC_{(0-24\ hr)}$ = 11,734 h * ng/mL).[14] Based on these findings, it was rationalized that potency, rather than inherent exposure or metabolic stability properties, would limit sufficient target engagement by **1a** to enable its use as an *in vivo* probe for the evaluation of GOT1 as a potential PDAC drug target. This limitation was the primary focus of an initial medicinal chemistry effort aimed at optimizing potency and establishing a structure-activity relationship for **1a**.

Initial attempts to modify the structure of **1a** were focused on replacement of the indole ring, which could represent a liability as a result of potential oxidization by cytochrome P450 enzymes to oxindole and hydroxyindole metabolites.[15] Representative derivatives that consist of simple substitution on the indole ring to replacement with alternative aryl, heteroaryl or alkyl groups are shown in Table 1. A complete list of derivatives that were synthesized and tested are shown in Supplementary Table 1. The observed GOT1 inhibitory activity of these analogs, as determined using the MDH coupled GOT1 enzymatic assay, indicate that replacement of indole by phenyl, heteroaryl or simple alkyl group substituents ablates activity (Table 1). These findings, combined with the lack of observed activity for compounds **1j** and **1k**, indicate that the nature and relative geometry of the hydrogen bond donor of the indole ring system is essential for GOT1 inhibition activity.

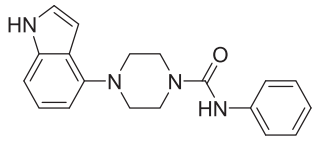
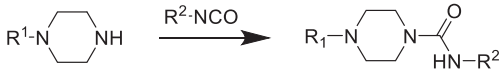
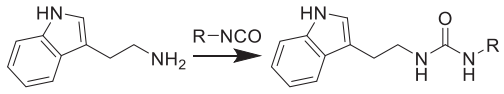


Fig. 1. HTS GOT1 inhibitor hit **1a**.

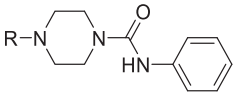


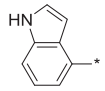
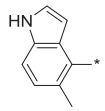
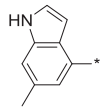
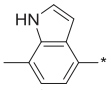
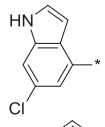
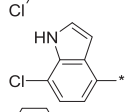
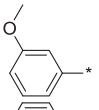
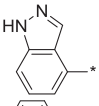
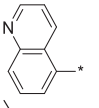
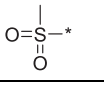
Scheme 1. Synthesis of **1a**, **1g–1i**, **1l–1w**, **2a–2j**, **2l**, **2n–2p**, **2t–2as**, **2aw**, **2ba**, **4c** and **4f**. Reagents and conditions: (i) 1.1 eq. TEA, DCM, R.T., 15 m. 20–80%.



Scheme 2. Synthesis of **3a–j**. Regents and conditions: (i) 1.1 eq. TEA, DCM, R.T., 30 min, 10–50%.

Table 1
GOT1 inhibition activity of compounds **1a–n**.

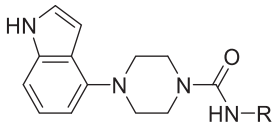


Compound	R	IC_{50}[a] (μM)
1a		85
1b		>100
1c		>100
1d		41
1e		46
1f		>100
1g		>100
1h		>100
1i		>100
1j		>100
1k		>100
1l		>100
1m		>100
1n		>100

[a] Data are reported as mean of n = 3 determinations.

We next focused on evaluation of the unsubstituted phenyl amide region of **1a** which, as a result of the potential for release of aniline containing metabolites, represents a genotoxicity liability. Analogs containing mono- or di-substitution of the phenyl group were synthesized (Scheme 1) and evaluated using the MDH-coupled GOT1 enzymatic assay (Table 2 and Supplementary Table 2). In addition, analogs in which phenyl is replaced by alkyl, aryl, or heteroaryl substituents were similarly prepared and evaluated. In general, introduction of electron-withdrawing groups on the phenyl ring resulted in improved observed potency against GOT1, as exemplified by the relative activities of **2b** as compared to **2c**. Encouragingly, as demonstrated by the observed activities of **2c**, **2d**, and **2g**, ~10-fold enhancement in potency was achieved

Table 2
GOT1 inhibition activity of compounds **2a–t**.



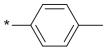
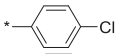
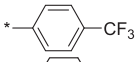
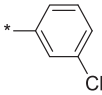
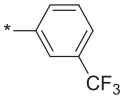
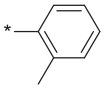
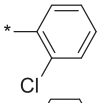
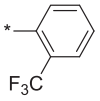
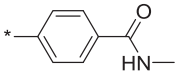
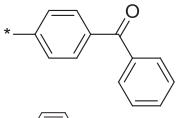
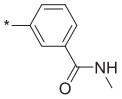
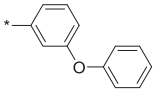
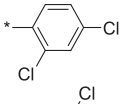
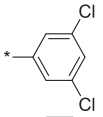
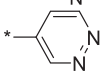
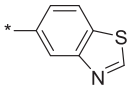
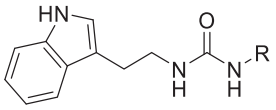
Compound	R	IC_{50}[a] (μM)
2a		>100
2b		>100
2c		8.2
2d		16
2e		>100
2f		52
2g		14
2h		>100
2i		>100
2j		>100
2 k		>100
2l		43
2m		>100
2n		39
2o		56
2p		22
2q		>100
2r		>100
2s		>100

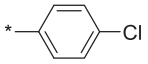
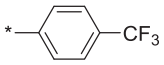
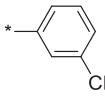
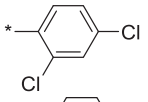
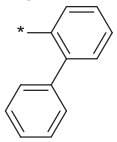
Table 2 (*continued*)

Compound	R	IC_{50}[a] (μM)
2t		76

[a] Data are reported as mean of n = 3 determinations.

Table 3
GOT1 inhibition activity of compounds **3a–f**.



Compound	R	IC_{50}[a] (μM)
3a		>100
3b		36
3c		39
3d		62
3e		32
3f		34

[a] Data are reported as mean of n = 3 determinations.

by introducing an electron withdrawing substituent at either the *meta*- or *para*-position of the *N*-phenyl group. Indeed, **2c** and **2d** represent molecules that are currently being evaluated in cell-based metabolomics, cell-based selective toxicity and protein co-crystallization experiments. As illustrated by the observed potencies of **2o** and **2p**, bi-substitution failed to significantly improve activity over the corresponding mono-substituted derivative. *ortho*-Substitutions were not found to be tolerated, demonstrated by **2h**, **2i** and **2j**. Further, as demonstrated by the observed activities of **2l** and **2n**, the wide variety of substituents tolerated at the distal position of this series suggests that the binding pocket may accommodate additional functionality at this position, which may afford additional beneficial binding modes and improved affinity.

To evaluate if the piperazine linkage to the indole moiety is essential for activity, a series of alternative linkage strategies, including tryptamine derivatives, were designed, synthesized (Scheme 2) and tested using the MDH coupled GOT1 enzymatic assay (Table 3, Supplementary Tables 3 and 4). While the unsubstituted phenyl derivative **3a** showed no significant activity, inclusion of electron-withdrawing groups on phenyl in the context of the tryptamine core, again improved activity and resulted in the identification of active analogs within this context. Interestingly, in contrast to the relative observed activities of **2c** and **2o**, the

observed equipotent activities of **3b** and **3e** demonstrates that *ortho*-substitution is tolerable within the tryptamine series. Indeed, even phenyl substitution at the *ortho* position (**3f**) was well tolerated, consistent with the existence of an expansive, hydrophobic binding pocket. Overall, the observed activities within the tryptamine series strongly suggests that the linkage between the indole and phenyl moieties is not constrained and that other linking chemistries may further enhance GOT1 inhibitory and physicochemical properties.

In summary, we have developed two novel series of phenylurea-based compounds that inhibit GOT1 enzymatic activity with potencies in the low micromolar IC_{50} range. Future directions will include further exploration of the piperazine series at the *meta* or *para* positions of the phenyl moiety, in order to explore the binding pocket around this region in more detail, which may improve observed potencies. In addition, optimization of the linkage between the indole and phenyl moieties will be prioritized, given the structural differences between tryptamine and indole-4-piperazine and the observed relative activities. Finally, effort will be required to improve the overall metabolic stability of these compound series, in order to mitigate potential C_{max}-driven toxicities that might be predicted for a metabolic target. Overall, these compounds represent first-in-class non-covalent inhibitors of GOT1 and potent derivatives may serve as leads for the development of potential therapeutics for the treatment of PDAC.

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A. Supplementary data

Supplementary data (synthetic methodology, compound characterization and additional data tables) associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.061.

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12. *GLOX-coupled GOT1 assay:* GOT1 enzymatic activity was measured utilizing two coupling reactions: GOT1/GLOX and GOT1/MDH1. In the first method, GOT1 reaction was coupled to GLOX and HRP. In this reaction, aspartate and alpha-ketoglutarate are provided as substrates to GOT1 at K_M concentrations (4 mM and 0.5 mM, respectively), which are then converted to glutamate and oxaloacetate. The glutamate serves as a substrate for GLOX which is converted to alpha-ketoglutarate and H_2O_2. In the presence of HRP, H_2O_2 oxidizes non-fluorescent Amplex red producing Resorfin which is readily measured by fluorescence (530 nm Ex/550 nm Em). The final concentration of the components of the assay are as follows: HEPES buffer 100 mM, KCL 100 mM, GLOX 80 nM, HRP 0.05 U/mL, Amplex Red (15 μg/mL), GOT1 (~1 nM, adjusted from each purification to produce 20 min linear reaction). The reaction components are mixed except for GOT1, which is prepared in a separate buffer; all buffers are kept on ice prior to dispensing. For measuring the reaction in the HTS format, first the compounds are transferred to microplates (384 or 1536) then the two components of the assay are added. The plates are incubated for 20 min, which is during the linear part of the reaction at 37 °C prior to measurement of fluorescence.
13. *MDH1-coupled GOT1 assay:* In the second method, the GOT1 reaction is coupled to MDH1. MDH1 catalyzes the conversion of oxaloacetate to malate by using NADH as a source of proton. Therefore, for monitoring the enzyme activity, loss of NADH fluorescence (340 nm Ex/445 nm Em) is measured. For the GOT1/MDH1 coupling, NADH and MDH1 are added at final concentrations of 250 μM and 0.3 μg/mL, respectively. The other details are same as described for GOT1/GLOX assay.
14. *Pharmacokinetic studies:* Female CD1 mice (9 weeks old, Charles River) were dosed by oral gavage at a dose of 20 mg/kg (5 mL/kg). At 0.5-, 1-, 4-, 8- and 24-h time points (n = 3 for each time point) 250 μL of whole blood was collected via retro-orbital sinus into a heparinized capillary tube, centrifuged at 12,000 rpm for 3 min and the resulting plasma was stored frozen at −80 °C prior to analysis. Mice were deprived of food overnight and had food returned after the 4-h bleed time point. Drug levels in plasma were determined using an AB Sciex API 3000 LC/MS/MS system, based on observed analyte area.
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