# Identification of the Mechanism of Action of CYT-0851, An Inhibitor of Monocarboxylate Transporter (MCT) Mediated Lactate Transport

**Abstract # 71**

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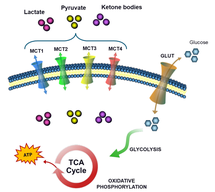
## Background and Rationale

CYT-0851 was originally discovered using a phenotypic screen designed to identify compounds that demonstrated selective cytotoxicity in human lymphoma cell lines, and was rapidly advanced to a Phase 1 clinical trial where responses in solid tumor and lymphoma patients have been observed. To elucidate the mechanism of action (MOA) of CYT-0851, extensive bioinformatic, functional genomic, and molecular characterization was performed. This preclinical work demonstrates that CYT-0851 disrupts lactate transport via inhibition of monocarboxylate transporter (MCT) activity.

**Figure 1: MCTs are Membrane Transporters Fundamental to Cancer Survival**




MCTs are plasma membrane proteins that bi-directionally transport monocarboxylated biomolecules such as lactate, pyruvate, and ketone bodies (**Fig 1**), with MCT1 and MCT4 often upregulated in cancers where they predict poor patient prognosis and increased patient mortality. Metabolic reprogramming is a hallmark of cancer and is characterized by increased dependence on lactate-producing glycolysis to support the metabolic needs of ongoing tumorigenesis. Inhibiting MCT function in glycolytic cancer cells leads to an accumulation of intracellular lactate that impairs glycolysis and inhibits tumor cell growth, thereby making MCTs an attractive target for cancer therapy.

## Results

### CYT-0851 Phenocopies MCT1 Loss in Cell Sensitivity Screen

- A panel of 439 cancer cell lines was treated with a titration of CYT-0851 for 7 days before relative viability assessment.
- CYT-0851 sensitivity for each cell line was tested for its correlation to Cancer Dependency Map gene essentiality scores (**Fig 2**), revealing that CYT-0851 sensitivity phenocopies a dependency on the monocarboxylate transporter MCT1 (*SLC16A1*).
- CYT-0851 sensitivity was compared to gene expression and mutation data across the cell panel, with low expression of the MCT4 monocarboxylate transporter (*SLC16A3*) demonstrating the most significant correlation with sensitivity (**Fig 3**).

**Figure 2: CYT-0851 sensitivity correlation with DepMap essentiality scores across cell panel**

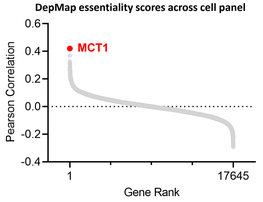



**Figure 3: CYT-0851 sensitivity correlation with RNA expression across cell panel**

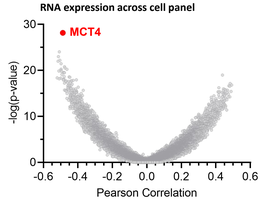
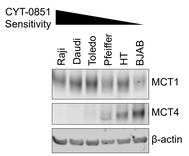
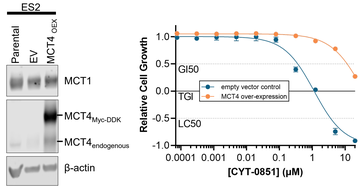



**Figure 4: MCT1 and MCT4 protein expression across a panel of lymphoma cell lines**




- MCT1 and MCT4 protein expression levels were assessed across a cell panel with varying sensitivities to CYT-0851 (**Fig 4**).
- Similar to the RNA-seq analysis above, MCT4 protein expression was inversely correlated with cell line sensitivity to CYT-0851.

**Figure 5: MCT4 over-expression reduces CYT-0851 anti-proliferative effects in ES2 ovarian cancer cell line**







- MCT4 was over-expressed in ES2 cells following lentiviral transduction.
- Cell viability was assessed in empty vector (EV) control and MCT4 over-expressing cells after CYT-0851 treatment and relative cell growth was quantified using the NCI method (**Fig 5**).
- MCT4 over-expression in ES2 cells significantly reduced CYT-0851-mediated anti-proliferative effects.

## Results, continued

### MCT4 Loss Sensitizes Cell Lines to CYT-0851 in CRISPR Screen

**Figure 6: CRISPR screen to identify CYT-0851 sensitizers**

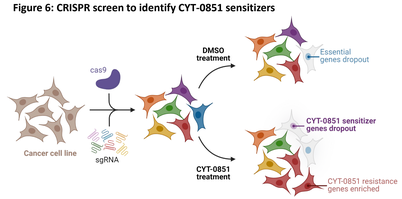



**Table 1: Cell lines used in CRISPR screen**

| Cell Line | Indication | CYT-0851 $IC_{50}$ (μM) |
|---|---|---|
| **Pfeiffer** | Lymphoma | 0.52 |
| **T47D** | Breast Cancer | 5.86 |
| **SU8686** | Pancreatic Cancer | >20 |

**Figure 7: MCT1/4 protein expression in CRISPR screen cell lines**

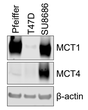



**Figure 8: CRISPR screen hits**

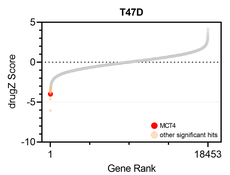
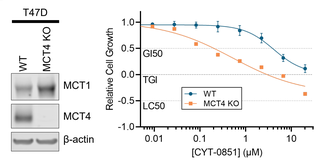






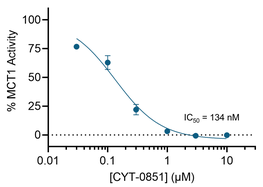
**Figure 9: CRISPR screen hit validation in T47D cell line**




- A whole exome CRISPR dropout screen was performed to identify genes that sensitize cell lines to CYT-0851. Cell lines were treated with DMSO or CYT-0851 before gRNA barcode sequencing (**Fig 6**).
- Three cell lines with varying sensitivities to CYT-0851 were utilized in the CRISPR screen (**Table 1, Fig 7**).
- Screen hits (FDR <0.2) were identified using the drugZ algorithm (**Fig 8**).
- MCT4 was the most significant hit consistent between the SU8686 and T47D cell lines, sensitizing both lines to CYT-0851 treatment.
- MCT4 knockout in T47D cells enhanced CYT-0851-mediated cell growth inhibition, validating the CRISPR screen hit (**Fig 9**).

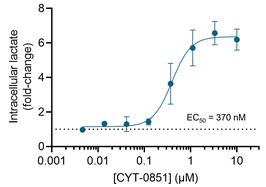
### CYT-0851 Inhibits MCT Transporter Activity

**Figure 10: CYT-0851 inhibits MCT1 substrate import**




- Canine MDCKII cells expressing human MCT1 were treated with a titration of CYT-0851, and import of the MCT1 substrate, 2-TPGA was measured by LC-MS (**Fig 10**).
- CYT-0851 inhibits 2-TPGA import with an $IC_{50}$ = 134 nM.

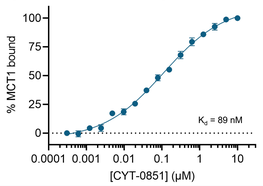
**Figure 11: CYT-0851 induces lactate accumulation**




- Daudi human lymphoma cells were treated with a titration of CYT-0851 for 4 h, then intracellular lactate was measured via LactateGlo (**Fig 11**).
- CYT-0851 induces lactate accumulation with $EC_{50}$=370 nM.

### CYT-0851 Directly Binds Human MCT1

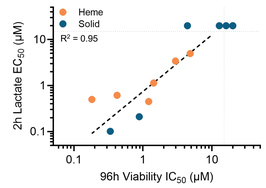
**Figure 12: CYT-0851 direct binding to MCT1 measured by MST**




- Microscale thermophoresis (MST) monitors the solution phase change in protein fluorescence and mobility in response to a temperature gradient created with an infrared laser.
- The method is compatible with detergent solubilized proteins and enables measurement of direct ligand binding.
- Purified hexahistidine-tagged MCT1/BSG complex was mixed with a hexahistidine interacting fluorescent dye and MST was monitored as a function of compound concentration.
- CYT-0851 binds to MCT1/basigin with $K_d$ = 89 nM.

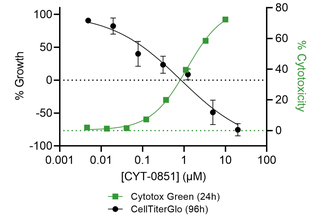
### CYT-0851 Cytotoxicity Correlates with Lactate Accumulation

**Figure 13: CYT-0851-induced lactate accumulation correlates with cell growth inhibition**




- A panel of cell lines were treated with a titration of CYT-0851, and intracellular lactate accumulation was assessed after 2 hours of treatment and cell viability after 96 hours.
- CYT-0851-mediated lactate accumulation $EC_{50}$ significantly correlated with cell viability $IC_{50}$ (**Fig 13**).
- Lactate accumulation assays were also performed in isogenic paired cell lines described above (**Fig 5, Fig 9**), and CYT-0851-induced lactate accumulation was observed only in cell lines exhibiting growth inhibition (data not shown).

**Figure 14: CYT-0851-induced anti-proliferative effects are cytotoxic**




- The ES2 ovarian cancer cell line was treated with a titration of CYT-0851, and cytotoxicity was assessed after 24 hours of treatment with Cytotox Green and cell viability after 96 hours with CellTiterGlo (**Fig 14**).
- CYT-0851 induced a clear cytotoxic response as measured in both assays.

### CYT-0851 MCT Selectivity Profile Versus Other MCT Inhibitors

**Figure 15: Development of a human MCT-overexpressing in vitro system to assess MCT inhibitor selectivity**

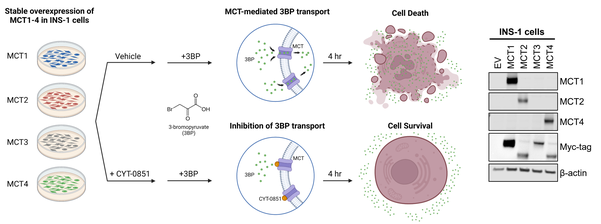



**Figure 16: CYT-0851-mediated inhibition of 3BP import in MCT-overexpressing INS-1 cell lines**

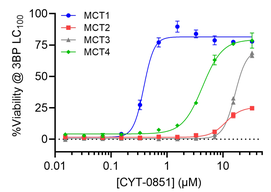



**Table 2: Summary of MCT inhibitor cellular potency and selectivity against MCT1-4**

| Inhibitor | Reported Selectivity | MCT1 | MCT2 | MCT3 | MCT4 |
|---|---|---|---|---|---|
| CYT-0851 | | +++ | - | + | ++ |
| AZD3965 | MCT1/2 | ++++ | ++ | - | - |
| BAY-8002 | MCT1/2 | ++ | ++ | - | - |
| MCT4i | MCT4 | - | - | ++++ | ++++ |

- Human MCT1-4 were stably expressed in INS-1 rat pancreatic islet β-cells, which are deficient in endogenous MCT expression. 3-Bromopyruvate (3BP), a highly reactive and cytotoxic monocarboxylate, can enter the INS-1 cells via the overexpressed MCT isoform, resulting in rapid cell death. However, prevention of 3BP transport into the cell by an MCT inhibitor can prevent 3BP-mediated cell death (**Fig 15**).
- INS-1 cells over-expressing an MCT paralog were treated with a titration of 3BP to determine the lowest concentration that results in 100% cell killing ($LC_{100}$). The INS-1 cells were then pre-treated with a titration of CYT-0851 before exposure to the $LC_{100}$ concentration of 3BP specific for that MCT-over-expressing cell line, and rescue of cell viability was measured (**Fig 16**).
- Paralog selectivity was determined for known MCT inhibitors, with relative potency against MCT1-4 shown (**Table 2**).

### CYT-0851 Induces Metabolic Switch

**Figure 17: Measurement of CYT-0851 induced metabolic changes**

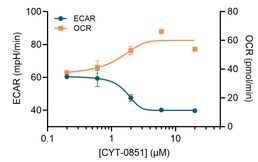
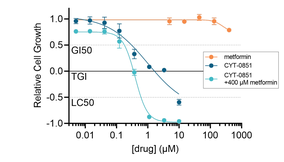



**Figure 18: The cytotoxic effect of CYT-0851 is enhanced when used in combination with metformin**




- Utilization of glycolysis and oxidative phosphorylation (OXPHOS) were measured by the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), respectively, in Raji lymphoma cells via the Seahorse XF Analyzer.
- CYT-0851 treatment results in a dose-dependent decrease in ECAR and a concomitant increase in OCR (**Fig 17**), suggesting an increased cellular dependence on and utilization of OXPHOS.
- Raji cells were treated with a titration of CYT-0851, metformin, or the combination of both drugs, cell viability was measured, and data plotted using the NCI method (**Fig 18**). Metformin at concentrations known to inhibit OXPHOS had little effect on cell viability as a single agent, but when combined with CYT-0851 profoundly enhanced cytotoxicity.
- Similar results were observed with other inhibitors of oxidative phosphorylation, such as IACS-010759.

### Biomarker & Combination Hypotheses

**Figure 19: Additional synthetic lethal hits generated from SU8686 CYT-0851 sensitivity CRISPR screen**

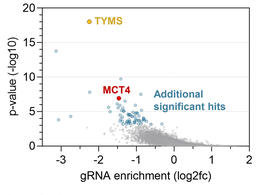



**Figure 20: CYT-0851 synergizes with 5-fluorouracil in Panc0403 pancreatic cancer cell model**

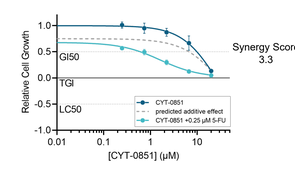



**Figure 21: Gene sets enriched from hits in SU8686 screen**

| |
|---|
| **GO Terms** *(# of hits that overlap)* |
| *Mitochondrion (39)* |
| *Mitochondrial Respiratory Chain (12)* |
| ***Reactome Terms*** |
| *Respiratory Electron Transport (11)* |
| *TCA Cycle & Electron Transport (12)* |
| ***Human Phenotype Ontology*** |
| *Acid/Base Homeostasis (19)* |
| *Serum Lactate (13)* |

- In addition to MCT4, several other significant synthetic lethal hits were identified in the CYT-0851 sensitivity CRISPR screens; the *TYMS* gene was one of the top hits (**Fig 19**).
- Thus, the combination of 5-fluorouracil (5-FU) with CYT-0851 was tested in vitro, resulting in synergistic cell growth inhibition in a subset of cell lines (**Fig 20**). Synergy score calculated using Lehar, et al. method (Nat Biotech 2009*)*.
- Gene set enrichment analysis of the CRISPR screen hits identified metabolism and metabolic pathways **(Fig 21),** suggesting genetic alterations or pharmacological inhibition of these pathways could represent viable biomarker or combination strategies.
- In addition to this work, metabolomic and transcriptomic assays were performed in cell lines treated with CYT-0851, with analyses ongoing. These data may provide additional hypotheses for CYT-0851 patient selection biomarkers.

## Conclusions

- CYT-0851 was discovered in a phenotypic screen. Through bioinformatic and functional genomic analyses, and molecular characterization it was determined to be an inhibitor of monocarboxylate transporter (MCT) activity.
- Inhibiting MCT function in glycolytic cancer cells leads to an accumulation of intracellular lactate that impairs glycolysis and inhibits tumor cell growth, thereby making MCTs an attractive target for cancer therapy.
- CYT-0851 inhibits the import of MCT ligands into cells, and induces dose-dependent intracellular lactate accumulation that correlates with cytotoxicity.
- CYT-0851 directly binds to MCT1 with a $K_d$ of 89 nM and exhibits potent inhibition of MCT1 relative to other MCT paralogs. However, in contrast to other known MCT inhibitors, CYT-0851 demonstrates the ability to inhibit both MCT1 and MCT4. Determination of direct binding to other MCT paralogs is ongoing.
- MCT4 expression inversely correlates with lactate accumulation and the extent of CYT-0851 cytotoxicity in cells.
- CYT-0851 reduces glycolytic activity, possibly inducing a dependence on alternate pathways, such as oxidative phosphorylation, for energy production. Consistent with this, combined treatment of CYT-0851 with OXPHOS inhibitors results in a significant enhancement of cytotoxicity compared to single agent treatment.
- CYT-0851 is currently being evaluated in a Phase 2 study as a monotherapy for the treatment of heme and solid tumors, and in combination with anti-metabolite drugs in a Phase 1b study (NCT03997968).
- MCT1 and MCT4 expression levels, factors that commit cancers to glycolysis, and other mitochondrial and metabolic genes are being evaluated as potential patient selection biomarkers.

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*Authors contributed equally to this work