A CREATINE UPTAKE ASSAY FOR REPURPOSABLE DRUGS IN SLC6A8-DEFICIENT FIBROBLASTS

AUTHORS

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BACKGROUND

Therapeutic strategies for cerebral creatine transporter deficiency (CTD) include gene therapy, creatine analogues, and small molecules to restore transporter function. We focused on small-molecule approaches aiming at rescue of mutant SLC6A8 transporter protein function.

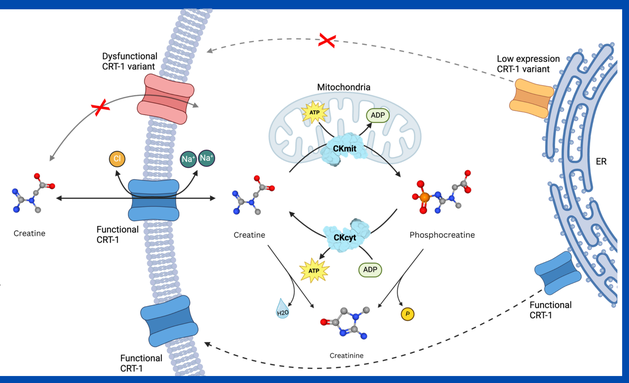
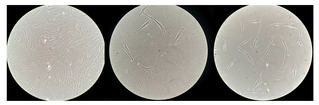


Fig 1. Creatine uptake and metabolism in the cell
Creatine is transported into cells via the Creatine Transporter (CRT-1) and used for ATP homeostasis via Creatine Kinase (CK) enzymes. CRT-1 deficiency can occur when the transporter is not shuttled to the the cell surface (low expression), or when the transporter is dysfunctional at the cell surface.

METHODS

CELL CULTURE

- Human fibroblasts were cultured in 6 well plates (surface area: 9.6 cm² per well) until reaching full confluence.
- Cells were cultured in Minimum Essential Medium (MEM) supplemented with fetal bovine serum (FBS) and antibiotics/antimycotics (penicillin–streptomycin-amphotericin B) to confluence.



PREINCUBATION

- Prior to the experiment, cells were incubated in creatine-free medium (Ham's F-10) for 24 hours without FBS to deplete intracellular creatine levels.
- At the end of the experiment, wells typically yielded approximately 40–60 µg total protein.

DRUG TREATMENT

- Test compounds were dissolved in DMSO or water according to manufacturer's guidelines and added at respective concentrations at 6, 3, or 30 hours prior to the end of the experiment.
- For drugs dissolved in DMSO (Drug 1-5, 7), the highest DMSO concentrations in incubation medium containing 100mM of drug were 0.1%-0.9%
- Drugs dissolved in water: 6, 8
- Drug concentrations:
  - Drugs 1, 3, 4, 7: 10 µM and 100 µM
  - Drug 2 (toxic at 100 µM and 0.9% DMSO): 1 µM and 10 µM
  - Drug 8 (toxic and has inconsistent solubility) at 1, 10 and 100 µM

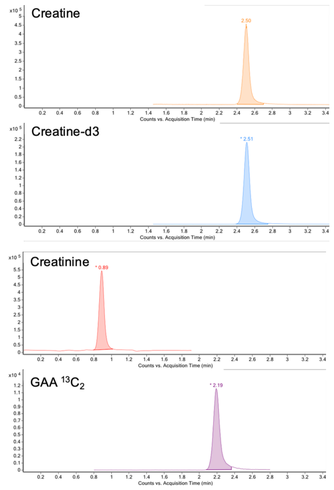
CREATINE UPTAKE ASSAY AND CELL HARVESTING

- Fibroblasts were incubated with D_3 labelled creatine (50 µM) for 6 hours.
- At the end of incubation:

1. Culture medium was removed
2. Cells were washed twice with buffer (DPBS)
3. Cells were trypsinized and collected

MASS SPECTROMETRY (LC-MS)

Cell pellets were extracted with acetonitrile containing a stable isotope-labeled guanidinoacetic acid (GAA) internal standard. Chromatographic separation was achieved via Hydrophilic Interaction Liquid Chromatography (HILIC). Detection and quantification were performed on a triple-quadrupole mass spectrometer operating in positive electrospray ionization mode (ESI+) with targeted Multiple Reaction Monitoring (MRM). Results were normalized for protein content using a BCA protein assay.



SUMMARY & OUTLOOK

Assay Validation: We established a sensitive, robust LC-MS/MS assay for quantifying D3 creatine uptake in cultured human fibroblasts.

Technical Insight: We identified that creatine uptake rates decrease as fibroblast passage numbers increase. Adopting a higher-throughput 24-well format would allow for more concurrent experiments at consistent passage levels.

Experimental Progress: Under current conditions, most compounds did not increase uptake above baseline. Replicate experiments are actively underway to confirm the positive results seen with Drug 4.

Ongoing Experiments: Evaluation of Phenylbutyrate (Drug 8), currently in clinical evalution for SLC6A1 and SLC6A8 deficiency, is in progress.

AIMS

1. Give a detailed description of the experimental protocol used
2. Show results from D3-creatine uptake in a ΔF408 human fibroblast cell line upon exposure to **8 FDA approved drugs** which showed enhanced SLC6A8 activity in experimental HEK293 cells.

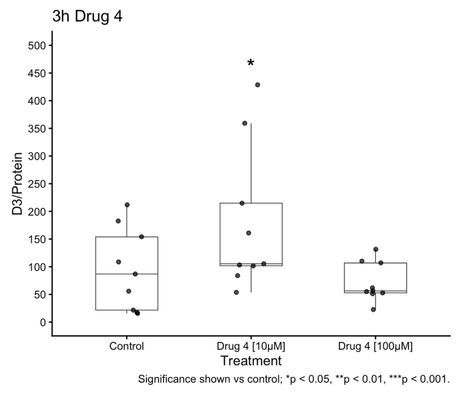
PREVIOUS WORK

1. Using an AI-based screening tool, we identified 7 repurposable drug candidates that enhanced SLC6A8 activity in ΔF408-transfected HEK 293 cells, measured by single-cell patch-clamp analysis (reported previously). One additional drug candidate, Drug 8, enhanced SLC6A8 activity in other SLC6A8 variants.
2. Developed a sensitive LC–MS/MS assay to quantify creatine uptake in human control and SLC6A8-deficient fibroblasts, including ΔF408. We assessed control fibroblasts and found a Km of 10-15 micromolar and a saturation of uptake at 50 - 100 micromolar of D3-creatine during a 6-hour incubation time. We used these parameters for testing drug response in a ΔF408 human fibroblast cell line (purchased from Coriell Biobank GM 27874).

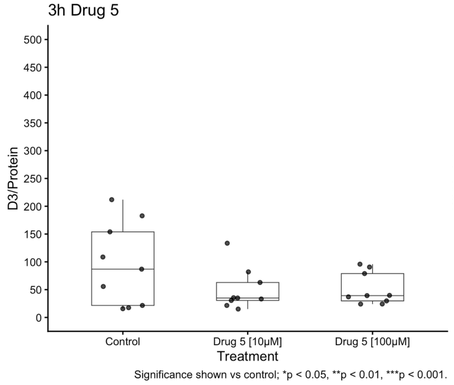
RESULTS

Drug	*Concentration (µM) n= # experiments*			*Exposure time (hours)*				*Comment*
	1 µM	*10 µM*	*100 µM*	*3 h*	*6 h*	*30 h*	*78 h*	
1	–	*n = 11*	*n = 11*	*n = 3*	*n = 6*	*n = 3*	*ip*	*No significant treatment effect*
2	*n = 11*	*n = 11*	–	*n = 3*	*n = 6*	*n = 3*	*ip*	*No significant treatment effect*
3	–	*n = 11*	*n = 11*	*n = 3*	*n = 6*	*n = 3*	*ip*	*Decrease at 100 µM, 3 h*
4	–	*n = 7*	*n = 7*	*n = 3*	*n = 4*	–	*ip*	*Increase at 10 µM, 3 h; Repeat experiments in progress*
5	–	*n = 10*	*n = 10*	*n = 3*	*n = 5*	*n = 2*	*ip*	*Decrease at 10 and 100 µM, 30 h*
6	–	*n = 10*	*n = 10*	*n = 3*	*n = 5*	*n = 2*	*ip*	*Decrease at 10 and 100 µM, 30 h*
7	–	*n = 5*	*n = 5*	–	*n = 3*	*n = 2*	*ip*	*Inconsistent solubility and toxicity; Experiments on hold*
8	*ip*	*ip*	*ip*	*ip*	*ip*	*ip*	*ip*	*In Progress*

ip = In Progress



Significance shown vs control; *p < 0.05, **p < 0.01, ***p < 0.001.



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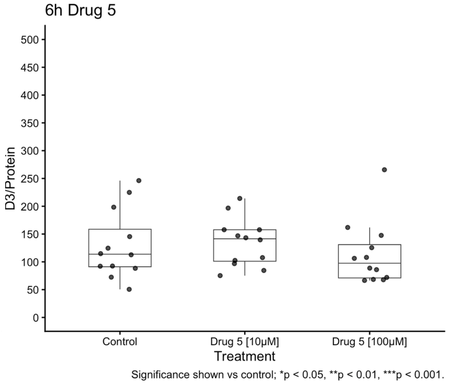

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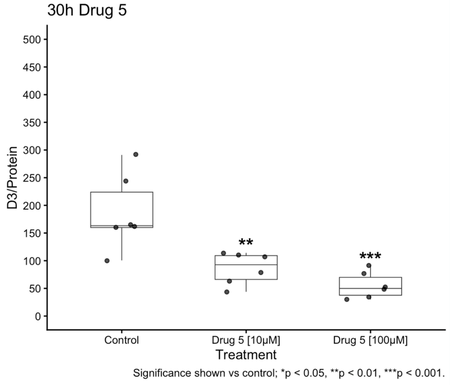

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DISCUSSION

Statistical Analysis: 25 data sets across 7 drugs were analyzed via linear regression using RStudio.
Drug 4 (Early Response): Showed a small, statistically significant increase in D_3-Creatine uptake at 3 hours with 10 µM, but not at 100 µM. Replicate experiments are needed to confirm this concentration-specific effect.
Drug 5 (Delayed Decrease): showed no significant effect at 3 hours or 6 hours, but significantly reduced D_3-Creatine uptake after 30 hours at both 10 µM and 100 µM.

Trends in Other Drugs:
Drugs 3 and 7: Showed significant *reduction* in uptake at early time points (3 hours and 6 hours respectively).
Drugs 6 and 7: Produced significant *reductions* in D_3-Creatine levels by the 30 hour mark.

Non-Responders:
Drugs 1 and 2: Showed no significant effect across any tested timepoints or concentrations.

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