Tumor Targeted Delivery of Glutamine Antagonist: Use of CES1$^{-/-}$ Mice

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ABSTRACT

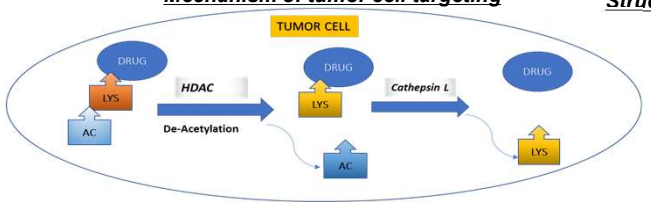
- 6-diazo-5-oxo-L-norleucine (DON), a potent glutamine antagonist, broadly blocks glutamine utilizing reactions critical for the synthesis of nucleic acids, amino acids, proteins and the generation of alpha-ketoglutarate essential for energy metabolism.[1]
- DON has shown robust efficacy in multiple preclinical cancer models and exploratory clinical studies. Although promising, development of DON was halted due to its dose-limiting gastrointestinal (GI)-toxicities, as the GI system is highly dependent on glutamine utilization.
- Given DON's promising efficacy, we developed novel tumor cell-targeted glutamine antagonists intended to circulate intact in plasma and be preferentially biotransformed to DON in tumor cells.
- Using a well-defined screening paradigm, we discovered compound **6**, (isopropyl 2-(6-acetamido-2-(adamantane-1-carboxamido) hexanamido)-6-diazo-5-oxohexanoate), that showed stability in plasma, liver and intestinal homogenates, yet was readily cleaved to DON in P493B human tumor cells. When directly compared to DON, compound **6**, exhibited 55-fold enhanced P493B cell-to-plasma ratio. In a time-dependent study, compound **6** showed sustained DON delivery to P493B cells while maintaining minimal release in human plasma. Moreover, in a cell proliferation assay, compound 6 showed dose-dependent inhibition of P493B cell growth.
- Using plasma from CES1-/- mice, wild-type mice and human, we confirmed that compound **6** exhibited similar stability in CES1-/- mice and human plasma but not in wild-type mice plasma. We then performed pharmacokinetic evaluation in C57BL/6 CES1-/- mice bearing flank murine EL4 tumors.
- Following subcutaneous dosing (1 mg/kg DON equivalent), compound **6** exhibited excellent pharmacokinetics with a ~5-fold higher DON tumor exposures (AUC = 5.1 nmol*h/g) versus plasma (1.1 nmol*h/mL) and a 11-fold higher tumor exposures versus GI-tissues (toxicity site; AUC = 0.45 nmol*h/mL).
- These studies describe discovery of a tumor targeted glutamine antagonist. In addition, we introduced a murine model, that recapitulates human metabolism and can be broadly utilized in prodrug development. Future studies will investigate the dose dependent efficacy and safety of compound 6 in tumor bearing C57BL/6 CES1-/- mice.

METHODS

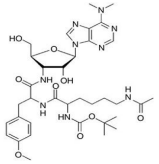
- **Chemistry**: A series of DON prodrugs with ε-acetylated lysine on DON's amine were synthesized for selective activation by tumor-enriched proteases such as histone deacetylases (HDACs) and cathepsins (e.g. Cathepsin B and L) based on a recently reported strategy for prodrug of puromycin.[2]
- **Tissue stability**: In vitro plasma and tissue homogenate (liver and GI tissue) stability assays were performed as previously reported.[3] Briefly, prodrugs were spiked in plasma or swine tissue homogenates and percent remaining at 1 h was determined via LC-MS/MS.
- **Tumor cell/plasma partitioning**: Human cancer cells (e.g. DU4475, H69, P4P3B) were incubated with test compounds (assay concentration: 20 µM) in 1 mL human plasma for 1 h. Cells were pelleted, and both plasma and cells were analyzed for DON release by LC-MS/MS, as we have described previously.[4]
- **Cell viability:** Assay was performed using CellTiter 96® AQueous One Solution Cell Proliferation reagents (Promega, USA). Briefly, P493B lymphoma cells were plated in 96 well plates at a density of 20000 cells/well. Cells were allowed to proliferate for 72 h in the presence of test compounds. Thereafter, 20 µL of CellTiter 96™ AQueous (Promega #3580) was added per well and incubated for 2 h. Absorbance was measured at 490 nm.
- **Pharmacokinetic study**: CES1$^{-/-}$ mice were injected with EL4 mouse lymphoma cells via subcutaneous (SC) injection (1×10^6 cells in 0.2 mL of phosphate-buffered saline) on the flank of each mouse. When tumors grew to a mean volume of around 400 mm^3, **6** was administered subcutaneously to mice (n=3 mice per time-point, 2 males and 1 female) at a dose of 3.2 mg/kg (1 mg/kg DON equivalent dose). Plasma, tumor and jejunum tissues were collected for quantification of both DON and intact prodrug. Bioanalysis of the samples was performed using LC-MS/MS.

RATIONALE

Mechanism of tumor cell targeting



Structure of Boc-Lys(Ac)-Puro



RESULTS

Chemistry

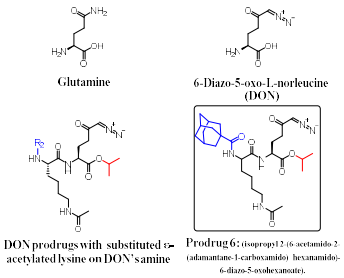


Figure 1: *Chemical structures of glutamine analogs and prodrugs*

In Vitro Metabolic Stability and Tumor-cell Partitioning of 6

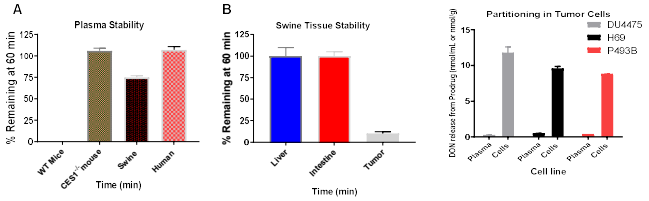


Figure 2: *In vitro metabolic stability studies in (A) plasma from wild-type (WT) mice, CES1$^{-/-}$ mice, swine and human and (B) swine tissues including liver (metabolic site) gut (toxic site) and P493B tumor cells (target site)*

Figure 3: *Human tumor cell-to-plasma partitioning of **6** in three different tumor cell lines*

In Vitro Bioactivation Mechanism, Stability with hRecombinant Enzymes and Tumor Cell Viability of 6

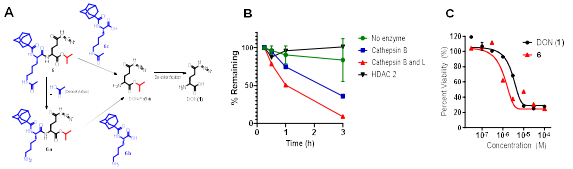
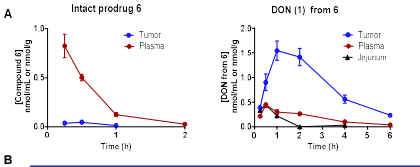


Figure 4: ***(A)****Metabolite identification and biochemical activation pathway in Tumor Cells. (B) Bioactivation of 6 in presence of human recombinant enzymes (C) Cell viability of P493B cells using DON or Prodrug **6***

In Vivo Pharmacokinetics of 6 in CES1$^{-/-}$ Mice



B

6			
Matrix	C_{max} (nmol/mL or nmol/g)	T_{max} (h)	AUC_{0-t} (nmol.h/mL or nmol.h/g)
Plasma	0.82±0.12	0.25	0.40
Tumor	0.05 ± 0.01	0.50	0.03
Jejunum	BLQ	BLQ	BLQ
DON (1) from 6			
Matrix	C_{max} (nmol/mL or nmol/g)	T_{max} (h)	AUC_{0-t} (nmol.h/mL or nmol.h/g)
Plasma	0.44±0.04	0.50	1.06
Tumor	1.55 ± 0.19	1.00	5.07
Jejunum	0.45 ± 0.18	0.50	0.45

Figure 5: *(A) Pharmacokinetic profile of prodrug and DON release from prodrug in EL4 tumor-bearing CES1$^{-/-}$ mice (B) Pharmacokinetic parameters.*

CONCLUSIONS

- *Successfully synthesized ε-acetylated lysine prodrug on DON's amine of which prodrug **6** was the lead*
- ***6**, showed stability in plasma, liver and intestinal homogenates, but was readily cleaved to DON in multiple tumor cells providing a 40- 55-fold enhanced tumor cell-to-plasma ratio*
- *The mechanism of **6** bioactivation was shown to involve both cathepsin B and L.*
- *Using CES1$^{-/-}$ mice that recapitulated human metabolism, we showed **6** preferentially bioactivated in tumor affording 5- and 11- fold higher tumor exposures versus plasma and intestinal tissues, respectively*

ACKNOWLEDGEMENT:

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REFERENCES

1. Structural basis for the active site inhibition mechanism of human kidney-type glutaminase (KGA), Scientific reports. **2014**, 4: 3827.
2. Synthesis and Preclinical Evaluation of a Highly Improved Anticancer Prodrug Activated by Histone Deacetylases and Cathepsin L. *Theranostics* **2016**, 6: 808-816.
3. N-substituted prodrugs of mebendazole provide improved aqueous solubility and oral bioavailability in mice and dogs. *Journal of medicinal chemistry* **2018,** 61: 3918-3929.
4. Tumor-targeted delivery of 6-Diazo-5-oxo-L-norleucine (DON) using substituted acetylated lysine prodrugs. *Journal of medicinal chemistry* (Accepted).