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The Nucleotide Prodrug CERC-913 Improves mtDNA Content in Primary Hepatocytes from DGUOK-Deficient Rats

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Abstract:	Loss-of-function mutations in the deoxyguanosine kinase (DGUOK) gene result in a mitochondrial DNA (mtDNA) depletion syndrome. DGUOK plays an important role in converting deoxyribonucleosides to deoxyribonucleoside monophosphates via the salvage pathway for mtDNA synthesis. DGUOK deficiency manifests predominantly in the liver; the most common cause of death is liver failure within the first year of life and no therapeutic options are currently available. In vitro supplementation with deoxyguanosine or deoxyguanosine monophosphate (dGMP) were reported to rescue	

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The Nucleotide Prodrug CERC-913 Improves mtDNA Content in Primary Hepatocytes from DGUOK-Deficient Rats

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Dr. Lawlor is or was recently a member of advisory boards for Audentes Therapeutics, Ichorion Therapeutics, and Solid Biosciences and receives or has recently received research support from these companies. Dr. Lawlor is also a consultant for Audentes Therapeutics, Encoded Therapeutics, AGADA Biosciences, Prothelia, Biomarin,, Kate Therapeutics, Lacerta Therapeutics, Affinia Therapeutics, Modis Therapeutics, Rocket Therapeutics, and Dynacure.

Dr. Thomas is a recent employee of Ichorion Therapeutics and Cerecor.

Mr. Crutcher are recent employees of Ichorion Therapeutics and Cerecor.

Dr. Dimmock was recently a member of the scientific advisory board for Audentes Therapeutics and a consultant for Biomarin.

Dr. Pertusati was a member of scientific advisory board for Ichorion therapeutics.

Mark Vanden Avond has no conflict of interest.

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Vanden Avond*: Data acquisition and interpretation, figure design, and drafting of the manuscript

Meng*: Data acquisition and interpretation, figure design, and drafting of the manuscript

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*Please note that we are requesting co-first authorship for authors vanden Avond and Meng as they both did extensive proportions of the work at all levels.

Abstract

Loss-of-function mutations in the deoxyguanosine kinase (DGUOK) gene result in a mitochondrial DNA (mtDNA) depletion syndrome. DGUOK plays an important role in converting deoxyribonucleosides to deoxyribonucleoside monophosphates via the salvage pathway for mtDNA synthesis. DGUOK deficiency manifests predominantly in the liver; the most common cause of death is liver failure within the first year of life and no therapeutic options are currently available. *In vitro* supplementation with deoxyguanosine or deoxyguanosine monophosphate (dGMP) were reported to rescue mtDNA depletion in DGUOK-deficient, patient-derived fibroblasts and myoblasts. CERC-913, a novel ProTide prodrug of dGMP, was designed to bypass defective DGUOK while improving permeability and stability relative to nucleoside monophosphates. To evaluate CERC-913 for its ability to rescue mtDNA depletion, we developed a primary hepatocyte culture model using liver tissue from DGUOK-deficient rats. DGUOK knockout rat hepatocyte cultures exhibit severely reduced mtDNA copy number (~10%) relative to wild type by qPCR and mtDNA content remains stable for up to eight days in culture. CERC-913 increased mtDNA content in DGUOK-deficient hepatocytes up to 2.4-fold after 4 days of treatment in a dose-dependent fashion, which was significantly more effective than dGMP at similar concentrations. These early results suggest primary hepatocyte culture is a useful model for the study of mtDNA depletion syndromes and that CERC-913 treatment can improve several disease-associated phenotypes in this model.

Synopsis

Treatment of a DGUOK KO rat primary hepatocyte model of mitochondrial DNA (mtDNA) depletion model was with CERC-913 allowed mtDNA recovery exceeding that observed with dGMP treatment.

Introduction

Mitochondrial DNA (mtDNA) depletion syndrome (MDS) is a group of rare autosomal-recessive diseases characterized by reduction of mitochondrial DNA copy number in specific tissues. Genetic causes of MDS are heterogeneous and are principally involved in mtDNA maintenance and replication.

DGUOK mutations account for 14% of MDS cases (Salviati et al 2002), which constitutes the most common genetic abnormality associated with hepatic mitochondrial DNA depletion syndromes (Sezer et al 2015). Patients with DGUOK deficiency typically present with liver dysfunction at birth, with or without neurological impairment, and most die within the first year of life due to liver failure. *DGUOK* is a nuclear gene which encodes mitochondrial deoxyguanosine kinase (DGUOK). DGUOK plays an important role in mtDNA synthesis by phosphorylating purine deoxyribonucleosides to produce deoxyadenosine monophosphate (dAMP) and deoxyguanosine monophosphate (dGMP) (Gower et al 1979).

The pathogenesis of DGUOK deficiency has been studied using patient-derived cell cultures and rodent models, which have provided insights into tissue-specific manifestations of the disease. While mtDNA synthesis can occur during S phase via cytosolic supply of deoxynucleoside triphosphates (dNTPs) irrespective of DGUOK, mutation of DGUOK compromises cell cycle independent mtDNA synthesis (Taanman et al 2003). This has little impact on mtDNA content in tissues undergoing active replication but leads to drastically reduced mtDNA in quiescent tissues, including liver, muscle and brain, since cytosolic dNTP supply is down-regulated. Studies using DGUOK-mutant cell cultures established that treatment of cells with deoxyguanosine and deoxyadenosine, or with dGMP and dAMP, was capable of partially correcting mtDNA content (Taanman et al 2003; Saada 2008; Bulst et al

2009; Bulst et al 2012). While Munro et al (2019) showed a recovery in liver mtDNA content when supplemented with deoxyguanosine + deoxyadenosine, rat (Bennett et al 2016) and mouse models (Zhou et al 2019) of DGUOK deficiency have not studied the impact of dGMP, dAMP, or dGMP + dAMP treatment.

Currently, there are no curative therapies available for DGUOK deficiency (Viscomi and Zeviani 2017). Given that formation of a nucleoside monophosphate intermediate is rate-limiting in nucleic acid synthesis, an emphasis in MDS therapeutic development has been placed on nucleoside monophosphate prodrugs capable of bypassing defective nucleoside kinases. Prodrugs, and ultimately ProTides, are compounds that, when metabolized, are broken down into a pharmacologically active drug, or nucleotide. One such approach employs a phosphoramidate masking group as the foundation of the ProTide nucleotide, providing a functional handle with drug-like properties susceptible to intracellular, enzyme-mediated activation to a nucleoside monophosphate (**Figure 1A**). Preclinical evaluation of ProTide nucleotides is typically limited to *in vitro* models, as high esterase activity in mouse and rat plasma rapidly degrade the ProTide prodrug moiety and precludes the use of *in vivo* rodent models (Rudakova et al 2011; Bahar et al 2012).

This study was designed to evaluate the utility of a novel ProTide nucleotide of dGMP, CERC-913, in a primary hepatocyte culture model of DGUOK deficiency. DGUOK-deficient hepatocyte cultures display significantly reduced mtDNA content that was restored in a dose-dependent fashion upon treatment with CERC-913. Overall, our work further establishes the usefulness of cell culture systems in understanding the pathogenesis of DGUOK deficiency, while also illustrating the promise of ProTide prodrugs relative to unmodified nucleoside monophosphates as a therapeutic approach to MDS.

Materials and Methods

Preclinical Characterization of CERC-913

A battery of standard preclinical assays was performed to identify the most suitable dGMP ProTide for further study (see Supplementary Materials). A Caco-2 cell line permeability assay was performed to evaluate the likelihood of passive and transporter-mediated diffusion across cellular membranes. Plasma stability assays were performed to determine relative stability of test compounds under physiological conditions. HepG2 (ATCC, HB8065) cells were used to test viability and effects on mitochondrial membrane potential. The Mini-Ames assay and an in vitro micronucleus assay were used to assess the genotoxic potential of CERC-913. Additionally, a pharmacokinetic study in dogs was used to determine plasma exposure and clearance of CERC-913 after oral and IV administration.

Establishment of Primary Hepatocyte Cultures

Primary hepatocytes were isolated from the livers of DGUOK KO and wild type (WT) rats (Bennett et al 2016) of either sex at ages ranging from 4.9 to 12.5 weeks (Shen et al 2012) (See Supplementary Materials). Cells were initially plated using Williams' complete medium, which was changed to HepatoZYME-SFM (Gibco, #1882882) after 1 hour of culture and changed daily. Cell viability was measured using Abcam MTT proliferation kit (Abcam, ab211091).

Efficacy of CERC-913

On the day following cell isolation, CERC-913 (Cerecor) was added to the growth medium of DGUOK KO primary hepatocytes at 50, 100, 200, 400, or 800 μM, with dGMP was used as an agent previously shown to rescue mtDNA depletion *in vitro* (Bulst et al, 2019). mtDNA content was evaluated as previously described (Bennett et al 2016). Subsequent experiments focused

on comparisons of CERC-913 and dGMP at doses of 200, 400, and 800 μM and endpoints

included mtDNA content and western blot. Western blots were performed as previously described (Bennett et al 2016) and using mtCO1 antibody (Abcam, 1D6E1A8, 1:1000) as an indicator of mitochondrial protein expression. Quantification of protein levels normalized to SDHA (Cell Signaling, D6J9M, 1:1000) was performed with Image Lab Software (BIO-RAD).

Statistical Evaluation: Data from all mtDNA experiments was transformed on a logarithmic scale. A ROUT analysis was performed on western blot data to identify potential outliers. The data from mtDNA and western blot experiments was evaluated using a one-way ANOVA with multiple comparisons. A two-way ANOVA analysis was used to evaluate mtDNA timecourse data.

Results

Preclinical characterization of CERC-913

An absorption, distribution, metabolism, and excretion screening campaign of several dGMP ProTides was performed, focusing on the Caco-2 cell line permeability and plasma stability as primary criteria to select a candidate for testing in DGUOK-deficient hepatocytes. The negative charge of nucleoside monophosphates at physiological pH decrease the likelihood of passive diffusion across cell membranes, while plasma phosphatases are capable of rapidly cleaving the phosphate group prior to reaching the desired site of action. The ProTide prodrug approach improves cell permeability and plasma stability by increasing lipophilicity and incorporating a non-hydrolyzable phosphate masking group. CERC-913 was selected as the lead compound based on desirable physicochemical properties, including increased permeability and stability relative to dGMP (**Figure 1B**).

CERC-913 was evaluated for *in vitro* toxicity by assessing cell viability and mitochondrial membrane potential in HepG2 cells. CERC-913 showed no impact on cell viability relative to staurosporine positive control and similarly showed no impact on mitochondrial membrane potential relative to FCCP positive control at the concentrations tested (1 to 3000 uM). Given CERC-913 is a nucleoside-based therapeutic designed as a substrate for DNA synthesis, an evaluation of any potential genotoxic effects was conducted. The results of Ames and *in vitro* micronucleus assays, which tests whether CERC-913 causes DNA mutations, were negative, demonstrating CERC-913 to be non-genotoxic.

Plasma pharmacokinetics (PK) of CERC-913 were assessed in beagle dogs to evaluate the *in vivo* absorption, distribution, metabolism and elimination of CERC-913. A single oral or intravenous dose was given to three animals. The oral half-life ($T_{1/2}$) of CERC-913 in plasma was determined to be roughly 0.7 hours and the oral bioavailability (%F) was 77%. Sofosbuvir (Sovaldi®, Gilead), a revolutionary treatment for Hepatitis C Virus (HCV), was the first approved drug containing a variation of the ProTide phosphoramidate masking group (Sofia et al 2010; Slusarczyk et al 2018) and more recently anticancer ProTides have entered clinical trials (Slusarczyk et al 2014; Blagden et al 2018). CERC-913 contains a ProTide moiety similar to sofosbuvir and other marketed nucleotide prodrugs known to have liver-targeting properties (Sofia et al 2010; Furman et al 2011; Vernachio et al 2011; Murakami et al 2014). When comparing the plasma PK of single 50 mg/kg oral doses of CERC-913 and sofosbuvir in beagle dogs, CERC-913 displayed a slightly improved or comparable exposure profile on all parameters (**Figure 1C**).

mtDNA depletion in DGUOK KO primary hepatocytes

The impact of primary hepatocyte culture on mtDNA depletion was assessed to ensure that DGUOK-derived primary hepatocytes displayed a useful degree of mtDNA depletion. Primary hepatocyte cell cultures were isolated from livers of DGUOK KO and wild type (WT) rats (Bennett et al 2016) of either sex as previously described (Shen et al 2012). The mtDNA content from WT and DGUOK KO primary hepatocyte cultures was assessed daily to determine whether mtDNA content was variable over time and to identify useful timepoints for treatment studies. MtDNA content was assessed by RTqPCR (described in supplemental methods) comparing mitochondrial specific primers relative to nuclear primers. As cell yields and viability were variable between isolations from different animals (n = 3 animals per genotype in this experiment), mtDNA copies were compared to age- and timepoint-matched cultures from WT rats. Over the 8-day period of culture following primary hepatocyte isolation, DGUOK KO primary hepatocyte cultures showed a consistent proportion of mtDNA depletion ranging from 7.1-10.6% of WT values during this period ($p<0.0001$, **Figure 2A**), similar to the 90% reduction in mtDNA that was reported in liver tissue from this DGUOK KO rat colony (Bennett et al 2016). WT cultures showed stable mtDNA copies for the first 4 days in culture, with a decrease in mtDNA copies after day 4. This was accompanied by a subjective decrease in cellularity in these WT cultures (**Figure 2B**) that suggests that the mtDNA decrease was due to cell loss. Pilot cell cultures followed past 8 days showed poor viability, establishing that later timepoints with this culture system were impractical for our purposes. Subsequent experiments were performed with key timepoints including 4 and 8 days in culture to account for changes in cell viability while also allowing the maximum duration of treatment to observe therapeutic effects.

Establishment of CERC-913 effective dose in primary hepatocyte cultures

Based on the demonstration of stable mtDNA depletion over 8 days in culture, dose escalation experiments were performed with a focus on mtDNA recovery after 4 days in culture. A dose

escalation strategy incorporating 0 μM, 50 μM, 100 μM, 200 μM, 400 μM, and 800 μM CERC-913 per well was used with media containing CERC-913 changed daily starting at day 1 following isolation. When compared to the relative mtDNA copy number DGUOK KO hepatocytes receiving no CERC-913 (355.6 mtDNA copies), 50 μM CERC-913 did not significantly increase mtDNA copy number (668.3 mtDNA copies, $p = 0.13$), while 100-800 μM CERC-913 significantly increased relative mtDNA copy number levels (**Figure 3A**). This increase was dose-dependent with an increase at 100 μM (772.4 mtDNA copies, $p < 0.05$) and an increase which plateaued at 200, 400, and 800 μM (1042, 1033, and 1048 mtDNA copies, respectively, $p < 0.01$). Based on these findings, additional studies were performed focusing on doses of 0 μM, 200 μM, 400 μM, and 800 μM CERC-913.

Assessment of CERC-913 efficacy in comparison to dGMP

A focused experiment was performed to establish whether CERC-913 provided greater mtDNA recovery than dGMP, previously dGMP + dAMP had been shown to improve mtDNA content in DGUOK-deficient cells (Taanman et al 2003; Saada 2008; Bulst et al 2009; Bulst et al 2012). In our hands, dGMP + dAMP did not significantly improve mtDNA recovery (1.4 fold increase) beyond what was observed using dGMP alone (1.7 fold increase; **Supplementary Figure 1**) (Camara et al., 2012. Equivalent doses (0 μM, 200 μM, 400 μM, and 800 μM) were tested for both CERC-913 and dGMP and mtDNA content was assessed after 4 and 8 days in culture (**Figure 3B and 3C**). In comparison to the mtDNA copy numbers observed in KO control hepatocytes (413.6 and 335.1 mtDNA copies for four and eight days, respectively), dGMP did not significantly increase relative mtDNA copy numbers at any dose at 4 days (200, 400, 800 μM; 398.5, 443.4, and 586.1 mtDNA copies, respectively) and only significantly increased relative mtDNA copy numbers at the 800 μM dose at eight days (200, 400, 800 μM; 424.4,

511.3, and 663.7 mtDNA copies, respectively, $p<0.05$). CERC-913 significantly increased relative mtDNA copy numbers at 200, 400 and 800 μM at 4 days (1018.7, 927.9, and 975.5 mtDNA copies, respectively, $p<0.05$) and 8 days (1109.2, 1202.0, and 1294.8 mtDNA copies, respectively, $p<0.0001$) when compared to KO control hepatocytes after 4 and 8 days in culture. For all treatment conditions and timepoints, the relative mtDNA copy numbers remained significantly different in comparison to untreated wild type (WT) hepatocytes at 4 and 8 days (4181.7 and 2215.7 mtDNA copies, respectively, $p<0.0001$). When comparing mtDNA content after treatment between CERC-913 and dGMP conditions, CERC-913 treated cells showed significantly higher mtDNA content at 200 μM at 4 and 8 days in comparison to dGMP treated cells and a similar difference was observed at 8 days when comparing the 400 μM dose of CERC-913 versus dGMP ($p<0.05$). There was no significant difference in mtDNA content when comparing CERC-913 and dGMP treated cells at 800 μM doses at either timepoint.

Assessment of additional efficacy endpoints with CERC-913 treatment

The impact of CERC-913 treatment on protein expression and cell viability was assessed to determine whether additional efficacy endpoints beyond mtDNA content are improved over 8 days of treatment in this cell culture system. Cell viability at 4 and 8 days in culture was assessed using an MTT assay in cultures treated with 0, 200, 400 and 800 μM of CERC-913. As noted in our earlier experiments, cell viability was more consistent after 4 days in culture, with nonsignificant trends toward fewer cells in all conditions at 8 days. Treatment with CERC-913 did not significantly affect cell viability at any dose (**Figure 4A**). With respect to protein expression, western blots were used to assess the expression of subunit 1 of cytochrome c oxidase, the mitochondrial electron complex IV (mtCO1), using protein isolates from six different treatment experiments. Expression levels of key proteins were highly variable between different

primary cell culture isolations (each derived from different animals), suggesting that a demonstration of statistically significant improvements in protein expression using this culture system will likely be challenging. While the level of mtCO1 protein was consistently decreased in untreated DGUOK cells in comparison to WT (**Figure 4B,** $p<0.01$), extensive variation in the overall amount of protein expression in WT and DGUOK KO cells complicated the analysis of treatment-related increases in mtCO1. This variation between isolations was also observed in proteins commonly used as loading controls. For instance, the WT culture from our second isolation in this experiment showed very high mtCO1 protein levels but essentially undetectable GAPDH and SDHA protein levels.

Discussion

DGUOK deficiency is an MDS resulting in devastating morbidity, primarily manifesting in the liver, along with death for which there is no currently available therapeutic option. Prior work has supported the notion of bypassing DGUOK by supplementing cells with nucleoside monophosphates, but delivery and uptake of nucleoside monophosphates to tissues *in vivo* in rodent models is limited. This study focused on the design of a liver-specific model of DGUOK deficiency to evaluate the efficacy of a novel ProTide prodrug of dGMP. CERC-913 was designed to resist dephosphorylation and passively diffuse across cell membranes to a greater extent than dGMP. To circumvent the poor rodent plasma stability of ProTides (McGuigan et al 2010; Vernachio et al 2011; Bahar et al 2012; Siegel et al 2017), a primary hepatocyte cell culture model was developed using liver tissue from DGUOK KO rats (Bennett et al 2016). The model was evaluated for its ability to recapitulate mtDNA depletion phenotype observed in DGUOK-deficient patients and assess the efficacy of CERC-913 relative to dGMP.

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4 DGUOK deficiency has been studied *in vitro* using patient-derived fibroblast and myoblast
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6 cultures, and *in vivo* using transgenic modeling in mice and rats. Cellular models have been
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8 essential in establishing how *DGUOK* mutations result in mtDNA depletion. Studies using
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10 DGUOK-mutant cell cultures have also established that treatment of cells with deoxyguanosine
11
12 and deoxyadenosine, or with dGMP and dAMP, is capable of partially correcting mtDNA content
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14 (Taanman et al 2003; Saada 2008; Bulst et al 2009; Bulst et al 2012). A recent study using
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16 DGUOK-mutant, iPSC-derived hepatocytes found that mitochondrial respiratory phenotypes
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18 were improved by treatment with nicotinamide adenine dinucleotide (NAD) (Jing et al 2018).
19
20 With respect to *in vivo* models, our group has previously reported a *DGUOK* KO rat model with
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22 a mild phenotype despite an approximately 90% reduction in mtDNA copy number in liver
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24 tissues (Bennett et al 2016), that has since been used to evaluate nicotinamide riboside as a
25
26 means of increasing NAD+ levels, which significantly improved the function of several
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28 mitochondrial electron transport chain (ETC) complexes I, III, IV, and V (Jing et al 2018). The
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30 use of this rat model in interventional studies, however, is complicated by the almost complete
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32 lack of behavioral or pathological abnormalities in the animals. Additionally, a mouse model of
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34 DGUOK deficiency has recently been described that develops a more severe disease
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36 phenotype, including weight loss, lipofuscin accumulation in the liver, and an approximately 99%
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38 reduction in mtDNA in the liver, with smaller relative decreases in other organs (Zhou et al
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40 2019). Overall, each of these model systems has reinforced the impact of DGUOK on mtDNA
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42 content while highlighting the difficulty of recapitulating the severe clinical phenotypes observed
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44 in humans at equivalent levels of mtDNA deficiency. The DGUOK KO rat model provided liver
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46 tissue for primary hepatocyte culture in this study, allowing an assessment of mtDNA depletion,
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48 cell viability and protein expression.
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58 Previous studies of bypass therapy in DGUOK deficiency demonstrate that nucleoside
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60 supplementation can be an effective pharmacological approach to DGUOK deficiency *in vitro*
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(Buchaklian et al 2012; Camara et al 2014). However, the cell types employed in prior studies may not provide an ideal context for evaluating kinase bypass, since active nuclear DNA replication during S phase could enable importation of cytosolic nucleoside metabolites for mtDNA synthesis and compensate for mutant DGUOK. Furthermore, improved intracellular delivery of dGMP is required to make kinase bypass more practical. Nucleoside monophosphates, such as dGMP, are negatively charged, making them highly polar and incapable of passively diffusing across cellular membranes. Plasma phosphatases rapidly degrade nucleoside monophosphates prior to reaching the desired site of action. ProTide nucleotides, including CERC-913, display enhanced stability in human plasma and improved membrane permeability relative to nucleoside monophosphates. Such properties allow ProTides to cross cellular membranes intact, after which a carefully orchestrated intracellular activation process reveals the active monophosphate metabolite (Furman et al 2011; Vernachio et al 2011; Serpi et al 2013; Murakami et al 2014; Maize et al 2017).

In this study, we compared CERC-913 to dGMP with respect to their ability to improve mtDNA content in primary DGUOK KO hepatocyte cultures. CERC-913 performed significantly better than equivalent doses of dGMP, which had much less of an impact on mtDNA content in hepatocytes than previously observed in patient-derived fibroblasts. The hepatocyte model provides additional insight into the context-dependency of mtDNA depletion. The ability to import cytosolic nucleosides into mitochondria requires active DNA replication during S-phase and can compensate for insufficient de novo mtDNA synthesis. Our model further demonstrates that quiescent cell-types, unable to enter S-phase, are particularly vulnerable to mutations in mitochondrial nucleoside kinases, as evidenced by the severe (>90%) mtDNA depletion in DGUOK KO hepatocytes relative to WT hepatocytes. We also attempted to establish additional efficacy endpoints related to functional recovery of DGUOK KO hepatocytes, including cell viability and expression of mtCO1. Unfortunately, variation between replicates (including WT

control samples) limited the usefulness of these studies, and so no clear-cut therapeutic effects could be demonstrated using these assays. This variation was likely in part due to the limitations in experimental time-course due to short-term hepatocyte viability in cell culture.

The ProTide nucleotide exemplifies a validated approach to kinase bypass that has enabled the development and approval of multiple drug candidates. A single, daily 400 mg oral dose of the ProTide sofosbuvir establishes liver concentrations of roughly ~100 µM in HCV patients at steady-state (Babusis et al 2018). In our model, CERC-913 induced significant rescue of mtDNA depletion at effective concentrations as low as 100 µM and PK experiments in dog demonstrated an improved plasma exposure profile relative to sofosbuvir. The data generated in our hepatocyte model help toward understanding a therapeutic threshold to further develop pharmacokinetic-pharmacodynamic (PKPD) relationships for translation to DGUOK-deficient patients.

In addition to severe liver dysfunction, DGUOK-deficient patients can also experience serious and life-threatening neurological symptoms that often result in early mortality, even following successful liver transplantation. Another critical feature of developing a substrate replacement therapy for DGUOK deficiency is determining the ability of dGMP ProTides to reach the CNS and provide the opportunity to rescue or impede neurological deterioration. Further nonclinical evaluation of CERC-913 as a therapy for DGUOK deficiency, including its ability to cross the blood-brain barrier in appreciable amounts, is currently underway.

FIGURES

Figure 1: Properties of CERC-913. (A) ProTide prodrug activation pathway for CERC-913 to yield dGMP. (B) Physiochemical properties of CERC-913 in comparison to dGMP. [a]A-B/B-A with efflux inhibitor, [b]recovery <2%, [c]% remaining @ 2 hours in rat, dog and human, ND = Not Detectable at any time point later than 5 minutes post-incubation. (C) PK parameters of CERC-913 in dogs after a single 50 mg/kg oral dose. CERC-913 compares favorably to sofosbuvir, the first-ever approved ProTide nucleotide as an oral therapy with liver-targeting properties. [d]First dose, [e]Steady-state, [f]Estimated (no IV dosing arm included).

Figure 2: Primary cell culture of WT and DGUOK KO hepatocytes. (A) mtDNA content in WT vs. DGUOK KO primary hepatocytes over 8 days in culture. Statistical analysis was performed by running an analysis of variance on Log10 transformed relative mtDNA copy number values. Data are represented as mean ± SEM (B) Relative cellularity of WT and DGUOK KO primary hepatocytes after 4 and 8 days in culture. ****p<0.0001.

Figure 3: Effects of CERC-913 treatment on mtDNA content in primary hepatocyte cultures. (A) Impact of escalating CERC-913 dose on mtDNA content in DGUOK KO hepatocyte cultures after 4 days in culture. (B) Comparison of treatment-related mtDNA increases between CERC-913 and dGMP after 4 days in culture. (C) Comparison of treatment-related mtDNA increases between CERC-913 and dGMP after 8 days in culture. Data are stated as relative mtDNA copy number, but statistical analysis was performed by analysis of variance (ANOVA) on Log10 transformed mtDNA relative copy number values. Data are represented as mean ± SEM. Symbols identify statistical significance with different comparisons: *Compared to WT control, #Compared to KO control, †Compared to CERC 200, and ‡Compared to CERC 400. The number of symbols denotes level of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

Figure 4: Effects of CERC-913 treatment on other cellular endpoints in primary hepatocyte cultures. (A) Impact of effective CERC-913 doses on cell viability in DGUOK KO hepatocyte cultures. (B) Impact of effective CERC-913 doses on mitochondrial protein expression in DGUOK KO hepatocyte cultures isolated in 6 different sets of animals, with cells collected at 4 and 8 days in culture. mtCO1 is used as an example of a mtDNA-encoded protein that is likely to be affected by differences in mtDNA copy number. GAPDH and SDHA are nuclear-encoded proteins meant to serve as loading controls. Note that the WT cell culture from isolation 2 shows highly abnormal protein expression patterns with respect to all of these proteins at 4 and 8 days. The values from this specimen are not included in the graphs or statistical analysis shown due to these aberrations, and so the "WT Control" condition in the graph represents n=5 whereas all other graphed conditions represent n=6 isolations. Data are represented as mean $\pm$ SEM. $^{**}p<0.01$, $^{***}p<0.001$.

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Figure 1

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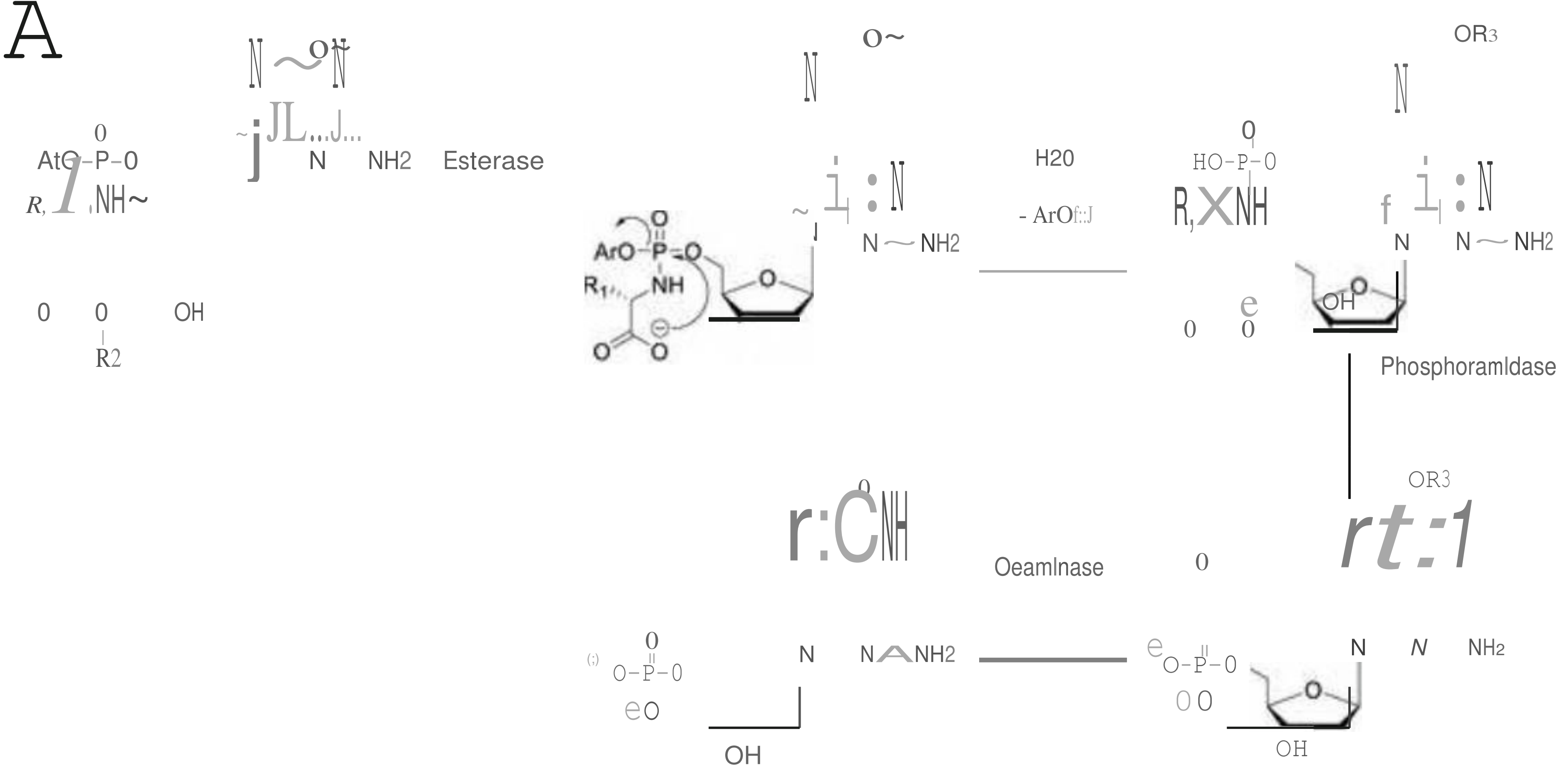

B

Physicochemical Property	CERC-913	dGMP
Caco-2 P.., (x 10-er	0.812.1	0.3/0.Sb
Plasma Stability (r/d/h)c	0/ 95/ 99	NO

C

PK Parameter	CERC·913d	Sofosbuvir•
Cmu (JJg/mL)	7.3	6.2
T, (h)	1.3	0.5
AUCft..... (µg"hlml)	12.5	6.9
T,t2 (h)	0.7	0.6
Bioavail:ability (%F)	77%	-10%1

Figure 2

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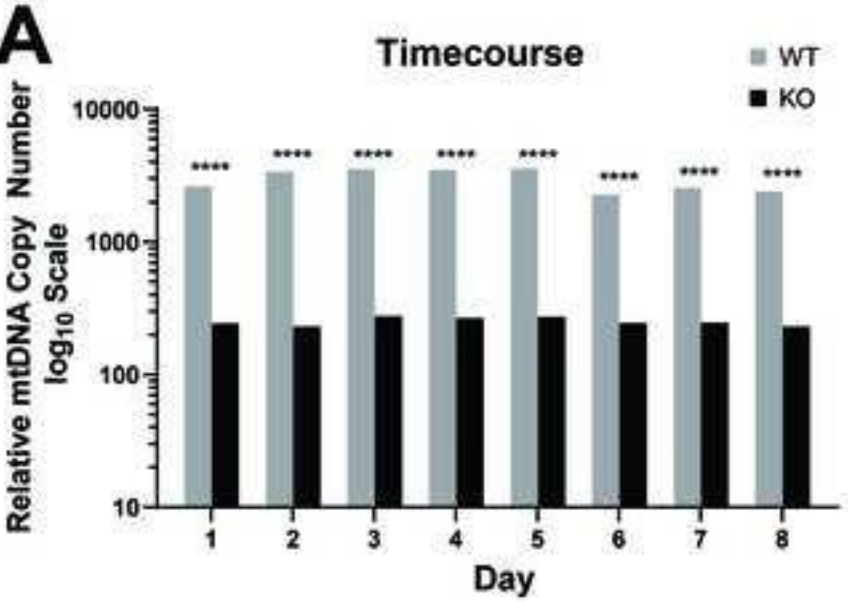

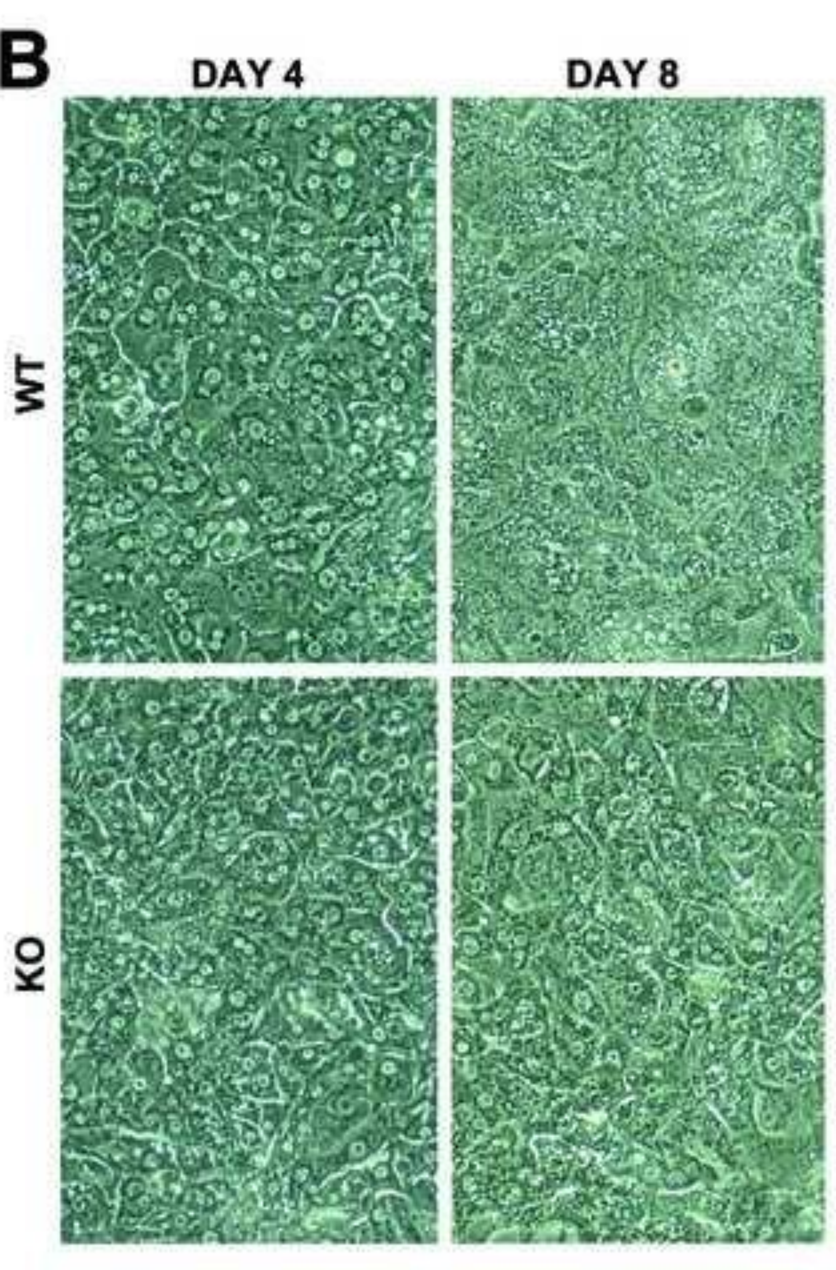

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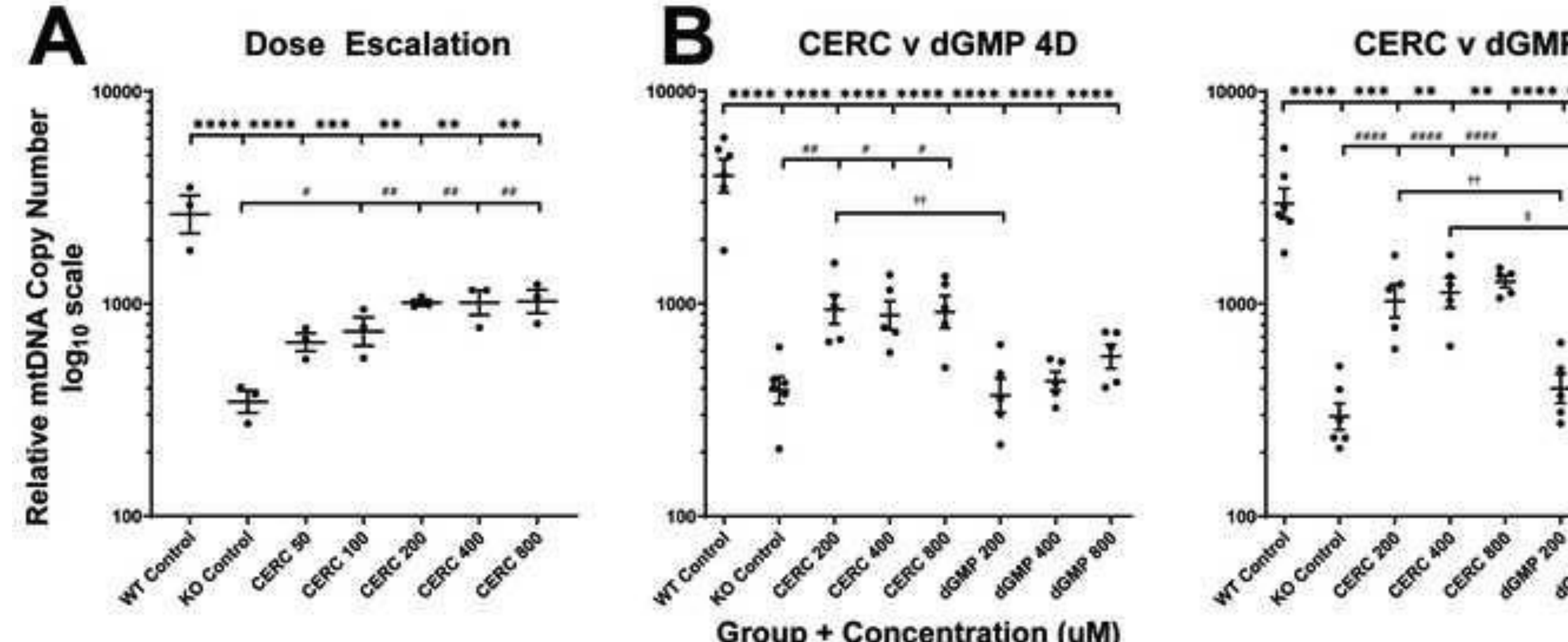

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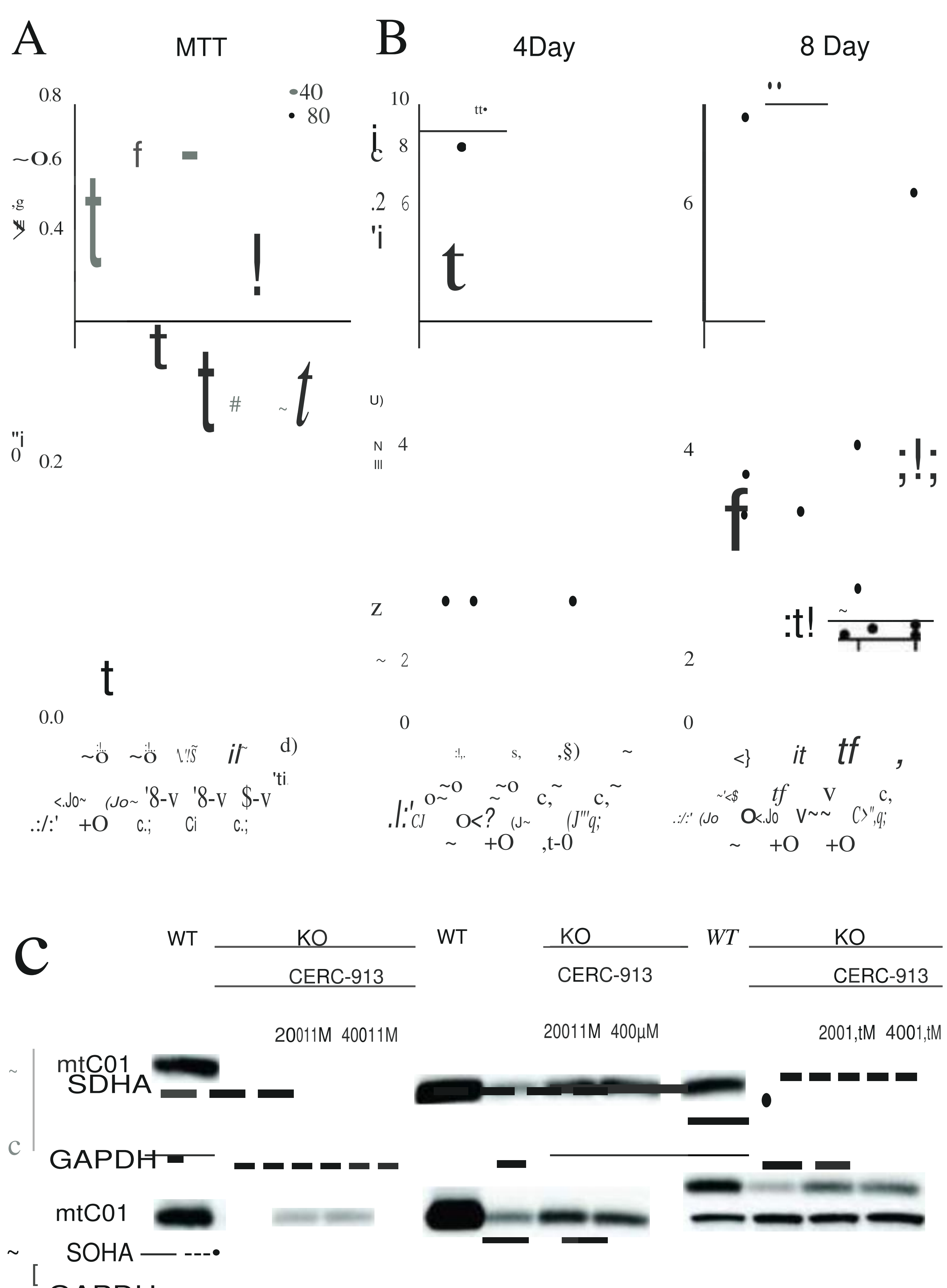

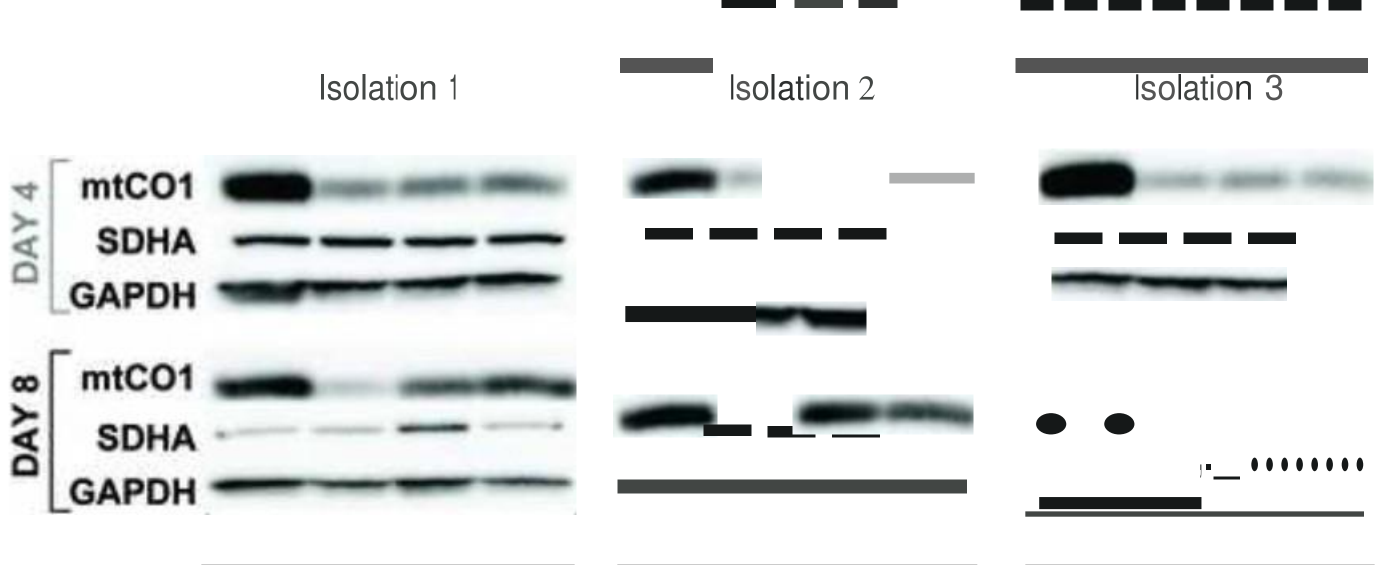

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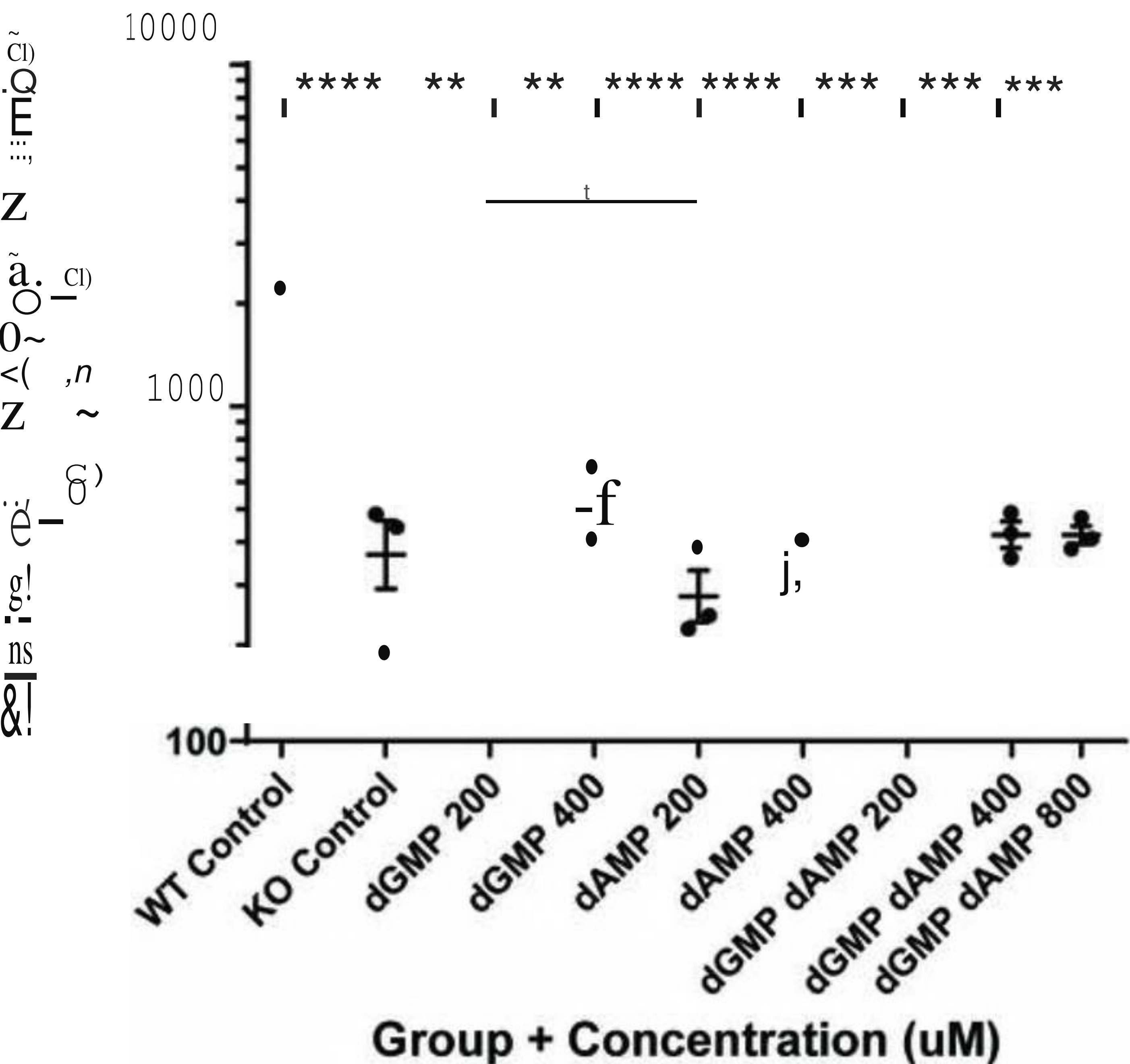

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