Published in final edited form as:

Toxicol In Vitro. 2020 June ; 65: 104794. doi:10.1016/j.tiv.2020.104794.

Mitochondrial respiratory chain complex I dysfunction induced by *N*-methyl carbamate *ex vivo* can be alleviated with a cell-permeable succinate prodrug Carbamate toxicity and treatment

Joanna I. Janowska^{a,1,*}, Sarah Piel^{a,1}, Nahima Saliba^a, Claire D. Kim^a, David H. Jang^{a,b}, Michael Karlsson^{a,c,d}, Todd J. Kilbaugh^a, Johannes K. Ehinger^{a,c,e,f}

^aDepartment of Anesthesiology and Critical Care Medicine, Center for Mitochondrial and Epigenomic Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

^bDepartment of Emergency Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

^cLund University, Department of Clinical Sciences Lund, Mitochondrial Medicine, Lund, Skåne 22184, Sweden

^dDepartment of Neurosurgery, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark

^eNeuroVive Pharmaceutical AB, Medicon Village, Lund, Skåne 223 81, Sweden

^fDepartment of Otorhinolaryngology, Head and Neck Surgery, Skåne University Hospital, Lund, Sweden

Abstract

Human exposure to carbamates and organophosphates poses a serious threat to society and current pharmacological treatment is solely targeting the compounds' inhibitory effect on acetylcholinesterase. This toxicological pathway, responsible for acute symptom presentation, can be counteracted with currently available therapies such as atropine and oximes. However, there is still significant long-term morbidity and mortality. We propose mitochondrial dysfunction as an additional cellular mechanism of carbamate toxicity and suggest pharmacological targeting of mitochondria to overcome acute metabolic decompensation. Here, we investigated the effects on mitochondrial respiratory function of *N*-succinimidyl *N*-methylcarbamate (NSNM), a surrogate for carbamate insecticides *ex vivo* in human platelets. Characterization of the mitochondrial toxicity of NSNM in platelets revealed a dose depended decrease in oxygen consumption linked to respiratory chain complex I while the pathway through complex II was unaffected. In intact platelets, an increase in lactate production was seen, due to a compensatory shift towards

*Corresponding author at: The Children's Hospital of Philadelphia, Department of Anesthesiology and Critical Care Medicine, 3401 Civic Center Blvd, Philadelphia, PA 19104, USA. janowskaj@email.chop.edu (J.I. Janowska).

¹Authors contributed equally.

Declaration of Competing Interest

S.P., M.K. and J.K.E. have, or have had, salary from and/or equity interest in NeuroVive Pharmaceutical AB, a company active in the field of mitochondrial medicine. S.P., M.K. and/or J.K.E. have filed patent applications for the use of succinate prodrugs for treatment of lactic acidosis or drug-induced side-effects due to complex I-related impairment of mitochondrial oxidative phosphorylation (WO/2015/155238). S.P. and J.K.E. additionally have filed patent applications for the use of protected carboxylic acid-based metabolites for treatment of mitochondrial disorders (WO/2017/060400, WO/2017/060418, WO/2017/060422).

anaerobic metabolism. Treatment with a cell-permeable succinate prodrug restored the NSNM-induced (100 μ M) decrease in oxygen consumption and normalized lactate production to the level of control. We have demonstrated that carbamate-induced mitochondrial complex I dysfunction can be alleviated with a mitochondrial targeted countermeasure: a cell-permeable prodrug of the mitochondrial complex II substrate succinate.

Keywords

cCarbamates; Cell-permeable succinate; Methyl isocyanate; Mitochondria; NSNM; Respirometry

1. Introduction

Carbamates and organophosphates (OPs) are the most widely used insecticides worldwide, carbamates being the fastest growing (Costa et al., 2008; Transparency Market Research, 2016, 2018). Their primary mechanism of action is acetylcholinesterase (AChE) inhibition resulting in acetylcholine accumulation and overstimulation of the nervous system. The inhibitory effect of OPs and carbamates on AChE is not selective to one species and extends to humans. Around 3 million people are accidentally or intentionally intoxicated with OPs or carbamates, leading to 300,000 deaths annually (Eddleston et al., 2008; Eyer, 2003). Standard treatment in acutely intoxicated patients includes decontamination, supportive care and administration of atropine along with oxime therapy. However, despite timely interventions there is still significant morbidity and mortality. This highlights the critical need to understand the complex pathophysiology and develop alternative/ adjunctive treatments.

Carbamates have been reported to induce mitochondrial dysfunction and oxidative stress in addition to their known cholinergic effects. N-succinimidyl *N*-methylcarbamate (NSNM, a carbamate which shares the unique *N*-methyl carbamate group with other carbamate insecticides including Aldicarb, Carbaryl, Carbofuran, Fig. 1), has been demonstrated to induce mitochondrial-related oxidative stress, inflammation, oxidative DNA damage and apoptosis *in vitro* (Mishra et al., 2008, 2009a; Panwar et al., 2013). Carbamate intoxication *in vivo* results in increased oxidative stress, reduced antioxidant capacity and impaired glucose metabolism. NSNM has also been suggested as an analogue to study the toxicity of methyl isocyanate (MIC) in laboratory settings (Mishra et al., 2008, 2009a, 2009b). MIC, a precursor in carbamate production which can modify biological molecules in a similar fashion to carbamates, has been reported to impair function of mitochondrial complex I (CI) of isolated rat brain and liver mitochondria which could result in decreased ATP production and energetic crisis (Jeevaratnam and Vidya, 1994; Jeevaratnam et al., 1993, 1992). Dysfunctional mitochondria, unable to satisfy bioenergetic demand represents a target for pharmacological development of adjuvant therapies for carbamate and MIC intoxication.

Our group has focused on developing mitochondrial targeted therapeutics for bioenergetic failure, and are investigating promising therapeutics to counteract metabolic crisis occurring in mitochondrial dysfunction (Ehinger et al., 2016; Karlsson et al., 2018; Piel et al., 2018). One therapeutic platform under development includes cell permeable succinate prodrugs for

genetic mitochondrial disorders and drug-induced mitochondrial dysfunction (Ehinger et al., 2016; Piel et al., 2018). Succinate, delivered to the intracellular space, is directly metabolized through complex II (CII) of the respiratory chain and increases ATP production by the mitochondria.

In this study, we aimed to further characterize the effect of carbamates on mitochondrial function using NSNM as an analogue, by measuring cellular oxygen consumption and lactate production in human platelets. We further hypothesized that cell permeable succinate prodrugs have potential as a therapeutic countermeasure for carbamate-induced bioenergetic failure.

2. Materials and methods

2.1. Chemicals

The cell-permeable succinate prodrug NV118 was provided by NeuroVive Pharmaceutical AB (Lund, Sweden). All other chemicals were obtained from Sigma-Aldrich (Burlington, MA, USA) and VWR (Bridgeport, NJ, USA).

2.2. Sample acquisition and preparation

The study was carried out in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of the Children's Hospital of Philadelphia (IRB number: 18-015056). After informed consent was acquired, venous blood from healthy volunteers was drawn into K₂EDTA tubes (BD Vacutainer®, NJ, USA) according to clinical standard procedure. Human platelets were isolated as described by Sjovall et al. (2013), with minor modifications. Whole blood was centrifuged (Allegra X-30 Centrifuge, Beckman Coulter, IN, US) at 500g at room temperature (RT) for 15 min and platelet-rich plasma was subsequently collected into 15 ml Falcon tubes. Following a second centrifugation at 3400g at RT for 10 min, the resulting platelet pellet was re-suspended in up to 1 ml of the donor's own platelet-free plasma. Cell counts were performed using an automated hematology analyzer (Medonic M-Series, Clinical Diagnostic Solutions, Inc., Plantation, FL, USA).

2.3. Cellular oxygen consumption

Cellular oxygen consumption was measured using high-resolution respirometry (Oxygraph-2k) with DatLab software version 7 for data recording and analysis (Oroboros Instruments, Innsbruck, Austria). The experiments were performed at 37 °C, with a stirrer speed of 750 rpm and a cell concentration of 200×10^6 platelets per ml in MiRO5 buffer (110 mM sucrose, 0.5 mM EGTA, 3.0 mM MgCl₂, 60 mM K-lactobionate, 10 mM KH₂PO₄, 20 mM taurine, 20 mM HEPES and 1.0 g/L of fatty acid-free bovine serum albumin) as previously described (Sjovall et al., 2013).

Three different respirometry protocols were performed as described below to characterize the effect of NSNM on cellular oxygen consumption of human platelets and assess the ability of the cell-permeable succinate prodrug NV118 to counteract NSNM-induced mitochondrial toxicity. Platelets were used as a source of fresh, viable human mitochondria. The effect of NSNM on mitochondrial-related cellular oxygen consumption was investigated

in intact cells (protocol 1, Fig. 2) and permeabilized cells were used for identification of the site of its toxicity at the mitochondrial respiratory chain (protocol 2, Fig. 3). Lastly, protocol 3 was applied to intact cells to assess the ability of the cell-permeable succinate prodrug NV118 to counteract the NSNM-induced impairment of mitochondrial respiration (Fig. 4).

Protocol 1—After stable routine oxygen consumption (the cellular oxygen consumption with the cells' endogenous substrate supply) was reached, either vehicle (Control, DMSO), or 50 μ M, 100 μ M or 250 μ M of NSNM was added and oxygen consumption was followed for 30 min. Subsequently, the ATP-synthase inhibitor oligomycin (1 μ g/ml) was added to measure LEAK-related oxygen consumption, the oxygen consumption related to the flux of protons through the mitochondrial membrane that is not linked to proton shuttling at the ATP-synthase, as well as cellular oxygen consumption coupled to phosphorylation activity of the ATP-synthase, calculated as the difference in oxygen consumption before and after the addition of oligomycin (Piel et al., 2018). Subsequently, the maximal oxygen consumption capacity of the electron transport system (ETS) uncoupled from phosphorylation pathways was assessed by stepwise titration of the protonophore carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP). Data were corrected for non-mitochondrial cellular oxygen consumption, achieved by addition of the mitochondrial CI inhibitor rotenone (2 μ M), mitochondrial complex III (CIII) inhibitor antimycin A (1 μ g/ml) and mitochondrial complex IV (CIV) inhibitor sodium azide (10 mM).

Protocol 2—After stable routine oxygen consumption was reached, either vehicle (Control, DMSO), 50 μ M, 100 μ M or 250 μ M of NSNM was added and oxygen consumption was followed over 30 min. Most mitochondrial energy substrates do not readily pass the cytoplasmic membrane. Subsequently, to allow for substrate control and detailed characterization of the mitochondrial toxicity induced by NSNM, cells were permeabilized using digitonin. ADP-stimulated maximal oxygen consumption (OXPHOS), LEAK-related oxygen consumption and the maximal oxygen consumption dependent on the ETS and uncoupled from phosphorylation pathways, was measured. OXPHOS and ETS were further assessed for the respective contribution of substrate metabolism through the mitochondrial CI- or CII-pathway, or the combination of both, by sequentially adding mitochondrial complex-specific substrates and inhibitors, as described previously by Sjoval et al. (2013). All cellular oxygen consumption values were corrected for non-mitochondrial cellular oxygen consumption.

Protocol 3—After the effect of vehicle (Control) or NSNM (100 μ M) on routine cellular oxygen consumption was assessed as described in protocol 1, the cell-permeable succinate prodrug NV118 (250 μ M) or vehicle (DMSO) was added. Subsequently, respiration coupled to phosphorylation activity of the ATP-synthase was measured as described for protocol 1. All cellular oxygen consumption values were corrected for non-mitochondrial cellular oxygen consumption (Piel et al., 2018).

2.4. Lactate production

First, 2×10^8 platelets were incubated in 1.0 ml of PBS containing glucose (10 mM) in the Roto-Therm Mini Plus (Benchmark Scientific, Edison, NJ, USA) at 37 °C and lactate

concentration in the medium was measured every 2 h for a total of 6 h using the Lactate Plus Lactate Meter (Nova Biomedical, MA, USA). After the first measurement ($t = 0$ h), either vehicle (Control, DMSO) or NSNM (100 μM) were added and after 2 h of exposure treatment with the cell-permeable succinate prodrug NV118 (21.5 μM) or vehicle (DMSO) was initiated and repeated every 30 min. The lactate production slope ($\mu\text{mol lactate} \times 2 \times 10^8 \text{ platelets}^{-1} \times \text{h}^{-1}$) was calculated by linear regression from onset of treatment with NV118 (2 h) until the end of the assay (6 h) as previously described (Ehinger et al., 2016). The mechanism of action of the cell-permeable succinate prodrug, viability of the cell preparation and preserved glycolytic activity in the presence of the drug was confirmed by comparison of two additional controls: NSNM-intoxicated platelets which were co-incubated with antimycin A, with and without NV118.

2.5. Data analysis

Each experiment of this study was performed with a group size of 5 replicates per group ($n = 5-8$) and data are expressed as mean $\pm$ SD. Statistical analyses were performed using Graph Pad PRISM software (GraphPad software version 7, CA, USA). For analysis of statistical difference one-way ANOVA was performed. Dunnet's *post hoc* test was applied for evaluation of the effect of increasing doses of NSNM on cellular oxygen consumption (Figs. 2–3) and Tukey's multiple comparison test was performed for evaluation of the treatment effect of the cell-permeable succinate prodrug (Figs. 3–4). A p -value of 0.05 was accepted to indicate statistical difference. No blinding or randomization was performed.

3. Results

3.1. NSNM dose-dependently inhibits mitochondrial respiration in intact human platelets

NSNM induced dose-dependent inhibition of cellular respiration in intact human platelets (Fig. 2). Compared to control, 30 min exposure of platelets to 50 μM , 100 μM and 250 μM NSNM caused 11%, 31% and 56% decrease in routine oxygen consumption respectively (Fig. 2A). The coupled oxygen consumption, calculated as a difference between oxygen consumption before and after oligomycin addition, presented a similar pattern of inhibition (50 μM : 12%, 100 μM : 26%, 250 μM : 47%, Fig. 2B). The lowest among the tested doses of NSNM that significantly reduced both routine and coupled oxygen consumption was 100 μM ($p < .01$). LEAK oxygen consumption, measured after addition of ATPase inhibitor oligomycin, was decreased in NSNM-treated cells but significantly different from control only at 250 μM ($p < .05$, data not shown). Moreover, the maximal FCCP stimulated oxygen consumption was significantly decreased compared to control with all doses of NSNM (50 μM : 20%, 100 μM : 40%, 250 μM : 63%) (Fig. 2C). In summary, NSNM is dose-dependently inhibiting integrated mitochondrial respiration in intact human platelets.

3.2. NSNM reduces complex I-linked respiration in permeabilized human platelets

To better characterize the nature of the mitochondrial inhibition induced by NSNM, control and cells intoxicated with increasing doses of NSNM were permeabilized with digitonin and additions of saturating concentrations of mitochondrial complex-specific substrates and inhibitors were made sequentially as described above. Maximal, ADP-stimulated cellular oxygen consumption in the presence of substrates that are metabolized through the

mitochondrial CI-pathway (CI, CIII and CIV; OXPHOS_{CI-linked}) was significantly decreased with increasing doses of NSNM compared to control (50 μ M: 33% ($p < .05$), 100 μ M: 36% ($p < .05$) and 250 μ M: 55% ($p < .001$), Fig. 3A). Maximal inducible oxygen consumption not coupled to ADP phosphorylation (hence only reflecting the capacity of the electron transport chain; ETS) in the presence of the CI inhibitor rotenone and succinate, a substrate that is metabolized through the mitochondrial CII-pathway (CII, CIII and CIV; ETS_{CII-linked}), did not change (Fig. 3D). Only the highest dose of NSNM investigated in this study caused significantly lower oxygen consumption in both maximal, ADP-stimulated cellular oxygen consumption and maximal non coupled ETS in the presence of substrates that are metabolized both through mitochondrial CI and II (OXPHOS_{CI+II-linked}, ETS_{CI+II}) (Fig. 3B–C). LEAK-related oxygen consumption, the oxygen consumption related to the flux of protons through the inner mitochondrial membrane that is not linked to proton shuttling of the ATP-synthase, was unchanged (data not shown). In summary, presence of NSNM is inhibiting the pathway CI-CIII-CIV, while leaving the pathway CII-CIII-CIV unaffected.

3.3. The cell-permeable succinate prodrug NV118 attenuates toxic effects of NSNM on mitochondrial metabolism

The lowest inhibitory dose of NSNM on routine and coupled cellular oxygen consumption of intact cells (100 μ M) was selected for further characterization of NSNM induced mitochondrial toxicity and evaluation of the cell-permeable succinate prodrug intervention strategy. Intact human platelets exposed to 100 μ M NSNM and treated with the cell-permeable succinate prodrug NV118 (250 μ M) achieved a significant increase in routine oxygen consumption exceeding control levels (Fig. 4A). The increase in routine oxygen consumption was coupled to phosphorylation activity by the ATP-synthase and reached $7.19 \text{ pmol O}_2 \times \text{s}^{-1} \times 10^8 \text{ platelets}^{-1}$ in controls, $5.18 \text{ pmol O}_2 \times \text{s}^{-1} \times 10^8 \text{ platelets}^{-1}$ in non-treated NSNM intoxicated cells and $8.17 \text{ pmol O}_2 \times \text{s}^{-1} \times 10^8 \text{ platelets}^{-1}$ in NV118 treated NSNM intoxicated cells (Fig. 4B).

Additionally, lactate production, a hallmark of anaerobic metabolism occurring in mitochondrial dysfunction or oxygen shortage was measured in intact human platelets exposed to NSNM. NSNM (100 μ M) increased lactate production in human platelets significantly by $0.12 \text{ } \mu\text{mol lactate} \times 2 \times 10^8 \text{ platelets}^{-1} \times \text{h}^{-1}$ compared to controls ($p < .001$) (Fig. 5). Treatment of NSNM intoxicated cells with the cell-permeable succinate prodrug NV118, started at 2 h of exposure and was repeated every 30 min, reduced NSNM-induced lactate production significantly (NSNM: $0.25 \text{ } \mu\text{mol lactate} \times 2 \times 10^8 \text{ platelets}^{-1} \times \text{h}^{-1}$; NSNM + NV118: $0.18 \text{ } \mu\text{mol lactate} \times 2 \times 10^8 \text{ platelets}^{-1} \times \text{h}^{-1}$, $p < .05$) (Fig. 5). The mechanism of action of the cell-permeable succinate prodrug, the viability of the cell preparation and preservation of glycolytic activity in the presence of the drug was confirmed by comparison of lactate production of cells exposed to NSNM and co-incubated with the mitochondrial respiratory chain complex III inhibitor antimycin A, with or without NV118. Lactate production was similar between both groups (data not shown), demonstrating that a downstream block of electron transport abolishes the NV118 effect, and that glycolysis again can be upregulated as a response. In summary, the cell-permeable succinate prodrug

NV118 attenuates the NSNM-induced toxic effects on mitochondrial respiration and cellular lactate production.

4. Discussion

In the current study we have demonstrated direct mitochondrial toxicity by NSNM, a carbamate analogue previously also reported as a substitute of MIC for *in vitro* studies. Intact human platelets from healthy volunteers were exposed to NSNM, and exhibited decreased cellular oxygen consumption coupled to phosphorylation by the ATP-synthase in a dose-dependent fashion. Further in-depth analysis revealed inhibition of solely the CI-linked cellular oxygen consumption, while CII and all downstream complexes were spared. Additionally, impaired mitochondrial function was confirmed by increased platelet lactate production upon NSNM exposure indicating a shift from aerobic to anaerobic metabolism. Treatment of NSNM-intoxicated platelets with the cell-permeable succinate prodrug NV118 bypassed CI inhibition and restored cellular oxygen consumption coupled to phosphorylation by the ATP-synthase and significantly attenuated the NSNM-induced increase in lactate production (Fig. 6).

Carbamate insecticides (Fig. 1) are readily available and widely used which makes exposure to these agents common. Their acute severe toxicity is largely related to inhibition of cholinesterase enzymes resulting in cholinergic symptoms with seizures and respiratory failure leading to death. Current treatment is based on the use of anticholinergic drugs (atropine), oxime therapy and supportive care. However, it has been proposed that both acute and chronic exposure to carbamates may cause long-term neuropathy, depression, increase risk of cancer, altered immune response and endocrine disorders unrelated to acetylcholinesterase inhibition, possibly due to mitochondrial dysfunction and oxidative stress (Akbar et al., 2012; Beard et al., 2013; Branch and Jacqz, 1986; Dickoff et al., 1987; Gummin et al., 2018; Indira et al., 2013; Lee et al., 2007; Schulte-Oehlmann et al., 2011; Smalley, 1970; Umehara et al., 1991; Yang et al., 2000; Zheng et al., 2001). Biochemical afflictions induced by carbamates, such as altered energy metabolism, excessive oxygen and nitrogen free radical production, depletion of intracellular antioxidant levels, mitochondrial respiratory chain dysfunction and DNA damage have been reported in several species including humans (Barlow et al., 2005; Gera et al., 2011; Gupta and Goad, 2000; Rai and Sharma, 2007; Zeljezic et al., 2008). Neurological deficits observed in carbofuran-treated rats were directly correlated to impaired mitochondrial function leading to oxidative stress along with antioxidants depletion in the brain (Kamboj et al., 2006, 2008). To our knowledge, this is the first time a direct inhibitory effect of the carbamate NSNM on CI- but not CII-linked oxygen phosphorylation pathways has been demonstrated. The advantage of the methods used in this study, is that effects on mitochondrial function is tested on actively respiring human mitochondria within their cellular context, in contrast to previous studies on carbamate toxicity where isolated mitochondria were used, or enzymatic activities of respiratory complexes was measured (Akbar et al., 2012; Kamboj et al., 2008; Moreno et al., 2007). The results of this study support that counteracting mitochondrial dysfunction with mitochondrial targeted therapies may present a novel adjunctive treatment strategy.

Lactate accumulation is a hallmark of upregulated glycolysis occurring in cells with impaired oxidative phosphorylation as a result of mitochondrial dysfunction or shortage of oxygen. Thus, lactate build-up found during intoxication of platelets with NSNM indicates a shift towards glycolysis and confirmed mitochondrial dysfunction (Fig. 6 A). The combined approach of assessing lactate production and mitochondrial respiration with high-resolution respirometry is a physiologically relevant method to study mitochondrial mechanistic toxicity and to evaluate and screen mitochondrial targeted therapies.

The observed mitochondrial toxicity phenotype was subsequently treated with a first-of-a-kind mitochondrial-targeted drug: cell-permeable succinate, NV118. Succinate, a substrate which supports CII-linked respiration while bypassing impaired CI, is delivered to the cytoplasm using a prodrug strategy. We showed that this effectively restores impaired mitochondrial respiration coupled to ATP production secondary to NSNM intoxication and attenuates increased lactate generation (Fig. 6B). Mitochondrial targeted therapies for drug- and chemical poisoning have gained increasing attention in recent years (Du et al., 2019; Lee and Boelsterli, 2014; Lee et al., 2015; Saito et al., 2010). Others have previously demonstrated that succinate improves energy metabolism and prevents cell-death *in vitro* and *in vivo* in other conditions such as traumatic brain injury, sepsis, mitochondrial disease and drug-induced mitochondrial intoxication (Ehinger et al., 2016; Giorgi-Coll et al., 2017; Hinke et al., 2007; Jalloh et al., 2017; Malaisse et al., 1997; Nowak et al., 2008; Piel et al., 2018; Protti et al., 2007). Increased cell-permeability and potentially organ delivery with the prodrug strategy makes this compound class an attractive adjuvant therapeutic option for chemical threats (Ehinger et al., 2016; Piel et al., 2019).

Inhibition of CI is one of the most commonly reported toxic effect of chemicals and drugs on mitochondrial function (Degli Esposti, 1998; Wallace and Starkov, 2000). These include other commonly used pesticides, such as paraquat, glyphosate and chlorpyrifos (Bus and Gibson, 1984; Lee et al., 2012; Mesnage et al., 2015), industrial agents, such as MIC (Jeevaratnam and Vidya, 1994; Jeevaratnam et al., 1993; Jeevaratnam et al., 1992), but also pharmacological agents, such as the antidiabetic drug metformin, the nucleotide reverse transcriptase inhibitor efavirenz and the cancer treatment cisplatin (Brunmair et al., 2004; Lee and Boelsterli, 2014; Piel et al., 2015; Vuda and Kamath, 2016). As such, cell-permeable succinate prodrugs present a potential pharmacological treatment strategy for chemicals and drugs with a similar reported mitochondrial CI-related toxic profile as NSNM. Improving the function of a common toxicological target, the mitochondria, with cell-permeable succinate prodrugs, rather than targeting a single chemical agent or drug hence could be widely applicable as a treatment against chemical and drug intoxication, and may serve as adjuvants to other antidotes and countermeasures.

The study has certain limitations. Even though human platelets are an excellent source of viable human mitochondria and have been shown to mirror mitochondrial function from other tissues (Tyrrell et al., 2016, 2017), there are still important differences in the metabolic profile between platelets and organs with high bioenergetic demand, such as the nervous system, the major target organ for carbamate and OP toxicity. However, we believe that this work complements *in vitro* and *in vivo* studies performed in rodents, as there are relevant differences in metabolism between species. NV118 is a prodrug of succinate optimized for

in vitro use with the main application to deliver succinate to intact cells in respiration (oxygen consumption) protocols, and the compounds' relative instability has limited the available experimental approaches to short-term experiments. Further, upon intracellular prodrug hydrolysis, formaldehyde is released which, at high concentration, has an inhibitory effect on glycolysis (Garcia-Sancho, 1985; Tiffert et al., 1984). For *in vitro* experiments dependent on glycolysis and lactate production as an output measure NV118 should be carefully titrated to improve mitochondrial respiration without inhibiting glycolysis. The doses of NV118 used in the present study effectively attenuated lactate production without apparent inhibitory effects on glycolysis, as evidenced by restored lactate production following downstream inhibition of the respiratory chain, at CIII, using antimycin A. To further develop this pharmaceutical concept, more inert byproducts are preferable, and for *in vivo* testing, more stable compounds are required. Such prodrugs of succinate are under development (Piel et al., 2019). Other potential effects of succinate administration, such as oxidative stress or modification of oxygen sensing pathways (succinate accumulation may stabilize HIF-1 α) (Selak et al., 2005), also need to be addressed.

We conclude that the carbamate NSNM exerts mitochondrial inhibition through CI, and that this can be attenuated by cell-permeable prodrugs of succinate. This is a new mechanism in carbamate toxicity, and we have demonstrated that it can be successfully targeted using cell-permeable prodrugs of succinate.

Acknowledgments

The authors would like to thank Dr. Fred Henretig for his immense knowledge and support; Martha Sisko for her dedication to our program; and the many volunteers from the pediatric intensive care unit, especially the nurses for their willingness to volunteer and the excellence they bring to their jobs on a daily basis. Finally, we would like to thank Drs. Jett and Spriggs for their encouragement and guidance and the many members of the CounterAct Program who work tirelessly for all our sake.

Funding information

This work is supported by the CounterAct Program, National Institute of Neurologic Disorders and Stroke [Kilbaugh; R21 NS103826].

Abbreviations

AChE	acetylcholinesterase
CI	complex
CII	complex II
CIII	complex III
CIV	complex IV
CV	complex V/ATP-synthase
ETS	electron transport system
FCCP	protonophore carbonyl cyanide p-(trifluoromethoxy) phenylhydrazine

MIC	methyl isocyanate
NAD⁺/NADH	nicotinamide adenine dinucleotide
NSNM	N-succinimidyl <i>N</i> -methylcarbamate
OPs	organophosphates
OXPHOS	oxidative phosphorylation
Q	quinone

References

- Akbar SM, Sharma HC, Jayalakshmi SK, Sreeramulu K, 2012 Methylparathion and carbofuran-induced mitochondrial dysfunction and oxidative stress in *Helicoverpa armigera* (Noctuidae: Lepidoptera). *Pestic. Biochem. Physiol* 103, 31–37.
- Barlow BK, Lee DW, Cory-Slechta DA, Opanashuk LA, 2005 Modulation of antioxidant defense systems by the environmental pesticide maneb in dopaminergic cells. *Neurotoxicology* 26, 63–75. [PubMed: 15527874]
- Beard JD, Hoppin JA, Richards M, Alavanja MC, Blair A, Sandler DP, Kamel F, 2013 Pesticide exposure and self-reported incident depression among wives in the agricultural health study. *Environ. Res* 126, 31–42. [PubMed: 23916637]
- Branch RA, Jacqz E, 1986 Subacute neurotoxicity following long-term exposure to carbaryl. *Am. J. Med* 80, 741–745. [PubMed: 3083676]
- Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhausl W, Fornsinn C, 2004 Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? *Diabetes* 53, 1052–1059. [PubMed: 15047621]
- Bus JS, Gibson JE, 1984 Paraquat: model for oxidant-initiated toxicity. *Environ. Health Perspect* 55, 37–46. [PubMed: 6329674]
- Costa LG, Giordano G, Guizzetti M, Vitalone A, 2008 Neurotoxicity of pesticides: a brief review. *Front. Biosci* 13, 1240–1249. [PubMed: 17981626]
- Degli Esposti M, 1998 Inhibitors of NADH-ubiquinone reductase: an overview. *Biochim. Biophys. Acta (BBA)* 1364, 222–235. [PubMed: 9593904]
- Dickoff DJ, Gerber O, Turovsky Z, 1987 Delayed neurotoxicity after ingestion of carbamate pesticide. *Neurology* 37, 1229–1231. [PubMed: 3037439]
- Du K, Ramachandran A, Weemhoff JL, Woolbright BL, Jaeschke AH, Chao X, Ding WX, Jaeschke H, 2019 Mito-tempo protects against acute liver injury but induces limited secondary apoptosis during the late phase of acetaminophen hepatotoxicity. *Arch. Toxicol* 93, 163–178. [PubMed: 30324313]
- Eddleston M, Buckley NA, Eyer P, Dawson AH, 2008 Management of acute organophosphorus pesticide poisoning. *Lancet* 371, 597–607. [PubMed: 17706760]
- Ehinger JK, Piel S, Ford R, Karlsson M, Sjoval F, Frostner EA, Morota S, Taylor RW, Turnbull DM, Cornell C, Moss SJ, Metzsch C, Hansson MJ, Fliri H, Elmer E, 2016 Cell-permeable succinate prodrugs bypass mitochondrial complex I deficiency. *Nat. Commun* 7, 12317. [PubMed: 27502960]
- Eyer P, 2003 The role of oximes in the management of organophosphorus pesticide poisoning. *Toxicol. Rev* 22, 165–190. [PubMed: 15181665]
- Garcia-Sancho J, 1985 Pyruvate prevents the ATP depletion caused by formaldehyde or calcium-chelator esters in the human red cell. *Biochim. Biophys. Acta* 813, 148–150. [PubMed: 3970916]
- Gera N, Kiran R, Mahmood A, 2011 Carbofuran administration induces genotoxic effects in epithelial cells across crypt-villus axis in rat intestine. *Pestic. Biochem. Physiol* 100, 280–283.

- Giorgi-Coll S, Amaral AI, Hutchinson PJA, Kotter MR, Carpenter KLH, 2017 Succinate supplementation improves metabolic performance of mixed glial cell cultures with mitochondrial dysfunction. *Sci. Rep* 7, 1003. [PubMed: 28432362]
- Gummin DD, Mowry JB, Spyker DA, Brooks DE, Osterthaler KM, Banner W, 2018 2017 annual report of the American association of poison control centers' National Poison Data System (NPDS): 35th annual report. *Clin. Toxicol. (Phila)* 56, 1213–1415. [PubMed: 30576252]
- Gupta RC, Goad JT, 2000 Role of high-energy phosphates and their metabolites in protection of carbofuran-induced biochemical changes in diaphragm muscle by memantine. *Arch. Toxicol.* 74, 13–20. [PubMed: 10817662]
- Hinke SA, Martens GA, Cai Y, Finsi J, Heimberg H, Pipeleers D, Van de Castele M, 2007 Methyl succinate antagonises biguanide-induced AMPK-activation and death of pancreatic beta-cells through restoration of mitochondrial electron transfer. *Br. J. Pharmacol* 150, 1031–1043. [PubMed: 17339833]
- Indira M, Andrews MA, Rakesh TP, 2013 Incidence, predictors, and outcome of intermediate syndrome in cholinergic insecticide poisoning: a prospective observational cohort study. *Clin. Toxicol. (Phila)* 51, 838–845. [PubMed: 24047461]
- Jalloh I, Helmy A, Howe DJ, Shannon RJ, Grice P, Mason A, Gallagher CN, Stovell MG, van der Heide S, Murphy MP, Pickard JD, Menon DK, Carpenter TA, Hutchinson PJ, Carpenter KL, 2017 Focally perfused succinate potentiates brain metabolism in head injury patients. *J. Cereb. Blood Flow Metab* 37, 2626–2638. [PubMed: 27798266]
- Jeevaratnam K, Vidya S, 1994 In vitro and in vivo effects of methyl isocyanate on rat brain mitochondrial respiration. *Arch. Environ. Contam. Toxicol* 27, 272–275. [PubMed: 8060173]
- Jeevaratnam K, Vidya S, Vaidyanathan CS, 1992 In vitro and in vivo effect of methyl isocyanate on rat liver mitochondrial respiration. *Toxicol. Appl. Pharmacol* 117, 172–179. [PubMed: 1471148]
- Jeevaratnam K, Vidya S, Vaidyanathan CS, 1993 Role of lipid peroxidation in impairment of mitochondrial function at complex I by methyl isocyanate treatment of rats in vivo. *Biochem. Mol. Biol. Int.*
- Kamboj A, Kiran R, Sandhir R, 2006 Carbofuran-induced neurochemical and neurobehavioral alterations in rats: attenuation by N-acetylcysteine. *Exp. Brain Res* 170, 567–575. [PubMed: 16307259]
- Kamboj SS, Kumar V, Kamboj A, Sandhir R, 2008 Mitochondrial oxidative stress and dysfunction in rat brain induced by carbofuran exposure. *Cell. Mol. Neurobiol* 28, 961–969. [PubMed: 18340526]
- Karlsson M, Pukenas B, Chawla S, Ehinger JK, Plyler R, Stelow M, Gabello M, Hugerth M, Elmer E, Hansson MJ, Margulies S, Kilbaugh T, 2018 Neuroprotective effects of cyclosporine in a porcine pre-clinical trial of focal traumatic brain injury. *J. Neurotrauma.*
- Lee KK, Boelsterli UA, 2014 Bypassing the compromised mitochondrial electron transport with methylene blue alleviates efavirenz/isoniazid-induced oxidant stress and mitochondria-mediated cell death in mouse hepatocytes. *Redox Biol.* 2, 599–609. [PubMed: 25460728]
- Lee WJ, Sandler DP, Blair A, Samanic C, Cross AJ, Alavanja MC, 2007 Pesticide use and colorectal cancer risk in the agricultural health study. *Int. J. Cancer* 121, 339–346. [PubMed: 17390374]
- Lee JE, Park JH, Shin IC, Koh HC, 2012 Reactive oxygen species regulated mitochondria-mediated apoptosis in PC12 cells exposed to chlorpyrifos. *Toxicol. Appl. Pharmacol* 263, 148–162. [PubMed: 22714038]
- Lee KK, Imaizumi N, Chamberland SR, Alder NN, Boelsterli UA, 2015 Targeting mitochondria with methylene blue protects mice against acetaminophen-induced liver injury. *Hepatology* 61, 326–336. [PubMed: 25142022]
- Malaisse WJ, Nadi AB, Ladriere L, Zhang TM, 1997 Protective effects of succinic acid dimethyl ester infusion in experimental endotoxemia. *Nutrition* 13, 330–341. [PubMed: 9178284]
- Mesnage R, Arno M, Costanzo M, Malatesta M, Seralini GE, Antoniou MN, 2015 Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose roundup exposure. *Environ. Health* 14, 70. [PubMed: 26302742]

- Mishra PK, Panwar H, Bhargava A, Gorantla VR, Jain SK, Banerjee S, Maudar KK, 2008 Isocyanates induces DNA damage, apoptosis, oxidative stress, and inflammation in cultured human lymphocytes. *J. Biochem. Mol. Toxicol* 22, 429–440. [PubMed: 19111005]
- Mishra PK, Raghuram GV, Panwar H, Jain D, Pandey H, Maudar KK, 2009 Mitochondrial oxidative stress elicits chromosomal instability after exposure to isocyanates in human kidney epithelial cells. *Free Radic. Res* 43, 718–728. [PubMed: 19513903]
- Mishra PK, Samarth RM, Pathak N, Jain SK, Banerjee S, Maudar KK, 2009 Bhopal gas tragedy: review of clinical and experimental findings after 25 years. *Int. J. Occup. Med. Environ. Health* 22, 193–202. [PubMed: 19819837]
- Moreno AJ, Serafim TL, Oliveira PJ, Madeira VM, 2007 Inhibition of mitochondrial bioenergetics by carbaryl is only evident for higher concentrations — relevance for carbaryl toxicity mechanisms. *Chemosphere* 66, 404–411. [PubMed: 16860847]
- Nowak G, Clifton GL, Bakajsova D, 2008 Succinate ameliorates energy deficits and prevents dysfunction of complex I in injured renal proximal tubular cells. *J. Pharmacol. Exp. Ther* 324, 1155–1162. [PubMed: 18055880]
- Panwar H, Jain D, Khan S, Pathak N, Raghuram GV, Bhargava A, Banerjee S, Mishra PK, 2013 Imbalance of mitochondrial-nuclear cross talk in isocyanate mediated pulmonary endothelial cell dysfunction. *Redox Biol.* 1, 163–171. [PubMed: 24024149]
- Piel S, Ehinger JK, Elmer E, Hansson MJ, 2015 Metformin induces lactate production in peripheral blood mononuclear cells and platelets through specific mitochondrial complex I inhibition. *Acta Physiol (Oxford)* 213, 171–180.
- Piel S, Ehinger JK, Chamkha I, Frostner EA, Sjoval F, Elmer E, Hansson MJ, 2018 Bioenergetic bypass using cell-permeable succinate, but not methylene blue, attenuates metformin-induced lactate production. *Intensiv. Care Med. Exp* 6, 22.
- Piel S, Janowska J, Ehinger JK, Karlsson M, Kilbaugh TJ, 2019 Mitochondrial targeted biofuels as countermeasures against chemical threats, A44. *Crit. Care (A1677–A1677)*.
- Protti A, Carre J, Frost MT, Taylor V, Stidwill R, Rudiger A, Singer M, 2007 Succinate recovers mitochondrial oxygen consumption in septic rat skeletal muscle. *Crit. Care Med* 35, 2150–2155. [PubMed: 17855829]
- Rai DK, Sharma B, 2007 Carbofuran-induced oxidative stress in mammalian brain. *Mol. Biotechnol* 37, 66–71. [PubMed: 17914167]
- Saito C, Zwingmann C, Jaeschke H, 2010 Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine. *Hepatology* 51, 246–254. [PubMed: 19821517]
- Schulte-Oehlmann U, Oehlmann J, Keil F, 2011 Before the curtain falls: endocrine-active pesticides—a German contamination legacy. *Rev. Environ. Contam. Toxicol* 213, 137–159. [PubMed: 21541850]
- Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E, 2005 Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* 7, 77–85. [PubMed: 15652751]
- Sjoval F, Ehinger JK, Marelsson SE, Morota S, Frostner EA, Uchino H, Lundgren J, Arnbjornsson E, Hansson MJ, Fellman V, Elmer E, 2013 Mitochondrial respiration in human viable platelets—methodology and influence of gender, age and storage. *Mitochondrion* 13, 7–14. [PubMed: 23164798]
- Smalley HE, 1970 Diagnosis and treatment of carbaryl poisoning in swine. *J. Am. Vet. Med. Assoc* 156, 339–344. [PubMed: 5461037]
- Tiffert T, Garcia-Sancho J, Lew VL, 1984 Irreversible ATP depletion caused by low concentrations of formaldehyde and of calcium-chelator esters in intact human red cells. *Biochim. Biophys. Acta Biomembr* 773, 143–156.
- Transparency Market Research, 2016 Insecticides Market to Reach US\$3.90 billion by 2023 - Global Industry Analysis, Size, Share, Growth, Trends and Forecast 2015–2023, 3 11, 2016 ed.
- Transparency Market Research, 2018 Global Insecticides Market: Advent of Novel Formulations and Innovative Strategies by Players to Accentuate Growth, 03 1, 2018 ed.

- Tyrrell DJ, Bharadwaj MS, Jorgensen MJ, Register TC, Molina AJA, 2016 Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: implications for a minimally invasive biomarker of mitochondrial health. *Redox Biol.* 10, 65–77. [PubMed: 27693859]
- Tyrrell DJ, Bharadwaj MS, Jorgensen MJ, Register TC, Shively C, Andrews RN, Neth B, Dirk Keene C, Mintz A, Craft S, Molina AJA, 2017 Blood-based bioenergetic profiling reflects differences in brain bioenergetics and metabolism. *Oxidative Med. Cell. Longev* 2017, 7317251.
- Umehara F, Izumo S, Arimura K, Osame M, 1991 Polyneuropathy induced by m-tolyl methyl carbamate intoxication. *J. Neurol* 238, 47–48. [PubMed: 2030373]
- Vuda M, Kamath A, 2016 Drug induced mitochondrial dysfunction: mechanisms and adverse clinical consequences. *Mitochondrion* 31, 63–74. [PubMed: 27771494]
- Wallace KB, Starkov AA, 2000 Mitochondrial targets of drug toxicity. *Annu. Rev. Pharmacol. Toxicol* 40, 353–388. [PubMed: 10836141]
- Yang PY, Tsao TC, Lin JL, Lyu RK, Chiang PC, 2000 Carbofuran-induced delayed neuropathy. *J. Toxicol. Clin. Toxicol* 38, 43–46. [PubMed: 10696923]
- Zeljezic D, Vrdoljak AL, Kopjar N, Radic B, Milkovic Kraus S, 2008 Cholinesterase-inhibiting and genotoxic effects of acute carbofuran intoxication in man: a case report. *Basic Clin. Pharmacol. Toxicol* 103, 329–335. [PubMed: 18699799]
- Zheng T, Zahm SH, Cantor KP, Weisenburger DD, Zhang Y, Blair A, 2001 Agricultural exposure to carbamate pesticides and risk of non-Hodgkin lymphoma. *J. Occup. Environ. Med* 43, 641–649. [PubMed: 11464396]

Fig. 1. Chemical structures of *N*-methyl carbamates. Chemical structures and IUPAC designations of the carbamates NSNM (N-succinimidyl *N*-methylcarbamate), Aldicarb (2-methyl-2-(methylthio) propionaldehyde O-(methylcarbamoyl) oxime), Carbaryl (1-naphthyl *N*-methylcarbamate), and Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate) are depicted, common N-carbamate group is highlighted in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Effect of N-succinimidyl *N*-methylcarbamate (NSNM) on cellular oxygen consumption of intact human platelets with endogenous substrates. (A) Routine cellular oxygen consumption, (B) cellular oxygen consumption coupled to phosphorylation pathways, here referred to as coupled oxygen consumption, and (C) cellular maximal electron transport system (ETS) capacity were measured in intact human platelets exposed to increasing doses of NSNM or its vehicle (Control) for 30 min. NSNM dose-dependently inhibited integrated mitochondrial routine, coupled and maximal ETS-related oxygen consumption in intact

human platelets. $n = 5-6$. Data are expressed as mean $\pm$ SD. One-way ANOVA with Dunnet's *post hoc* test was used for analysis of differences to Control with * = $p < .05$, ** = $p < .01$ and *** = $p < .001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Characterization of the mitochondrial toxic effect of N-succinimidyl *N*-methylcarbamate (NSNM) on cellular oxygen consumption in permeabilized human platelets. (A) Cellular complex I-linked and (B) complex I + II-linked, maximal ADP-stimulated oxygen consumption as well as (C) complex I + II-linked and (D) complex II-linked maximal electron transport capacity (ETS) was evaluated in permeabilized human platelets following exposure to increasing doses of NSNM or its vehicle (Control) for 30 min. In the presence of NSNM the integrated function of mitochondrial complexes I-III-IV pathways was reduced while mitochondrial complexes II-III-IV pathways were unaffected $n = 5-8$. Data are expressed as mean $\pm$ SD. One-way ANOVA with Dunnet's *post hoc* test was used for analysis of differences to Control with * = $p < .05$, ** = $p < .01$ and *** = $p < .001$.

Fig. 4.

Effect of the cell-permeable succinate prodrug NV118 on routine and coupled cellular oxygen consumption of intact human platelets intoxicated with N-succinimidyl *N*-methylcarbamate (NSNM). Intact human platelets were exposed to NSNM (100 μM) or its vehicle (Control) for 30 min. Subsequently, one out of two groups receiving NSNM was treated with the cell-permeable succinate prodrug NV118 (250 μM) and (A) routine cellular oxygen consumption and (B) coupled cellular oxygen consumption was evaluated in controls, untreated NSNM-intoxicated cells and NSNM-intoxicated cells treated with

NV118. Coupled oxygen consumption is defined as the cellular oxygen consumption linked to phosphorylation by the ATP synthase. Treatment with the cell-permeable succinate prodrug following exposure to NSNM improved the impaired cellular routine and coupled oxygen consumption. $n = 5$. Data are expressed as mean $\pm$ SD. One-way ANOVA with Tukey's post hoc test was performed for analysis of differences between each group with ** = $p < .01$.

Fig. 5.

Effect of the cell-permeable succinate prodrug NV118 on lactate production of intact human platelets intoxicated with N-succinimidyl *N*-methylcarbamate (NSNM). 2×10^8 platelets were incubated in PBS containing glucose (10 mM) at 37 °C and lactate concentration in the medium was measured every 2 h for a total of 6 h using the Lactate Plus Lactate Meter (Nova Biomedical, MA, USA). Either vehicle (Control, white circle) or NSNM (dark blue circle, 100 μM) were added after the first measurement ($t = 0$ h). After 2 h of exposure treatment with the cell-permeable succinate prodrug (light blue circle, 21.5 μM) was initiated in one out of two NSNM groups and was repeated every 30 min. Data are expressed as lactate (μmol) produced per cell number per hour and was calculated from onset of treatment with NV118 (2 h) until the end of the assay (6 h). Co-treatment with the cell-permeable succinate prodrug attenuated NSNM-induced increased lactate production. $n = 5$. All data are expressed as mean $\pm$ SD. One-way ANOVA with Tukey's *post hoc* test was used for analysis of differences between each group. * = $p < .05$. *** = $p < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Schematic illustration of the metabolic rescue with the cell-permeable succinate prodrug NV118 in human platelets exposed to N-succinimidyl N-methylcarbamate. (A) N-succinimidyl N-methylcarbamate (NSNM) induces inhibition of complex I (CI). As a result, ATP production by complex V (CV/ATP-synthase) is decreased causing increased glycolysis to compensate for the reduced mitochondrial ATP production. Pyruvate metabolism is shifted towards lactate generation to meet the increased NAD⁺ demand, which is accompanied by intracellular and extracellular acidification. (B) Cell-permeable succinate prodrugs, such as NV118, can pass the cell membrane independent of active transporters. Through intracellular metabolism succinate is released and metabolized by complex II (CII). Oxidation of succinate at CII restores downstream electron flow, oxygen consumption and proton translocation across the inner mitochondrial membrane, which is linked to ATP generation. CIII, complex III. CIV, complex IV. NAD⁺/NADH, nicotinamide adenine dinucleotide. Q, Q-junction.