

Endogenous Endothelial Nitric Oxide Synthase (eNOS) Modulates Oxygen (O₂) Dependent Control of Red Blood Cell (RBC) Energy Metabolism and Antioxidant Status

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Background

- Therapeutic Landscape for Anti-Sickling Drugs**
- Hydroxyurea** induces high O₂ affinity fetal Hb (HbF): inhibits sickling since HbF (α₂γ₂) and HbFS (α₂γ₂β_S) tetramers cannot be incorporated into polymerized HbS fibers.
- Voxelotor** (AKA GBT-440 or Oxbritya) is the first approved aromatic aldehyde antisickling compound that retards HbS polymerization *via* a stable (Schiff base) adduction to Hb that increases Hb O₂ affinity.
 - This approach began with the natural and non-toxic compounds vanillin and **5-HMF**, which did not advance due to pharmacokinetic limitations.
 - Voxelotor clinical efficacy is based on acutely increased Hb levels and reduced hemolysis, that were durable (72wk); though these are surrogate endpoints, aromatic aldehydes may have SCD disease-modifying potential (morbidity and mortality arising from RBC sickling).

5-HMF anti-sickling mechanism

- Deoxy T-state HbS can polymerize: HbO₂ release promotes sickling.
- 5-HMF forms covalent Schiff-base interactions with α-subunit N-terminal αVal1 amines in the Hb α-cleft that stabilize R-state Hb, ↑ Hb O₂ affinity
- 5-HMF also (unlike Voxelotor) inhibits T-state HbS (trans-tetramer) polymerization by weakening intermolecular contacts critical to fiber stability.
- When incubated with SRBCs, 5-HMF inhibits hypoxia-induced sickling and rheological impairment in a dose-dependent manner and limits SRBC dehydration; *in vivo* (Townes SCD mice), 5-HMF protects against hepatic VOC22 and lethal hypoxia.

Barriers to 5-HMF Translation

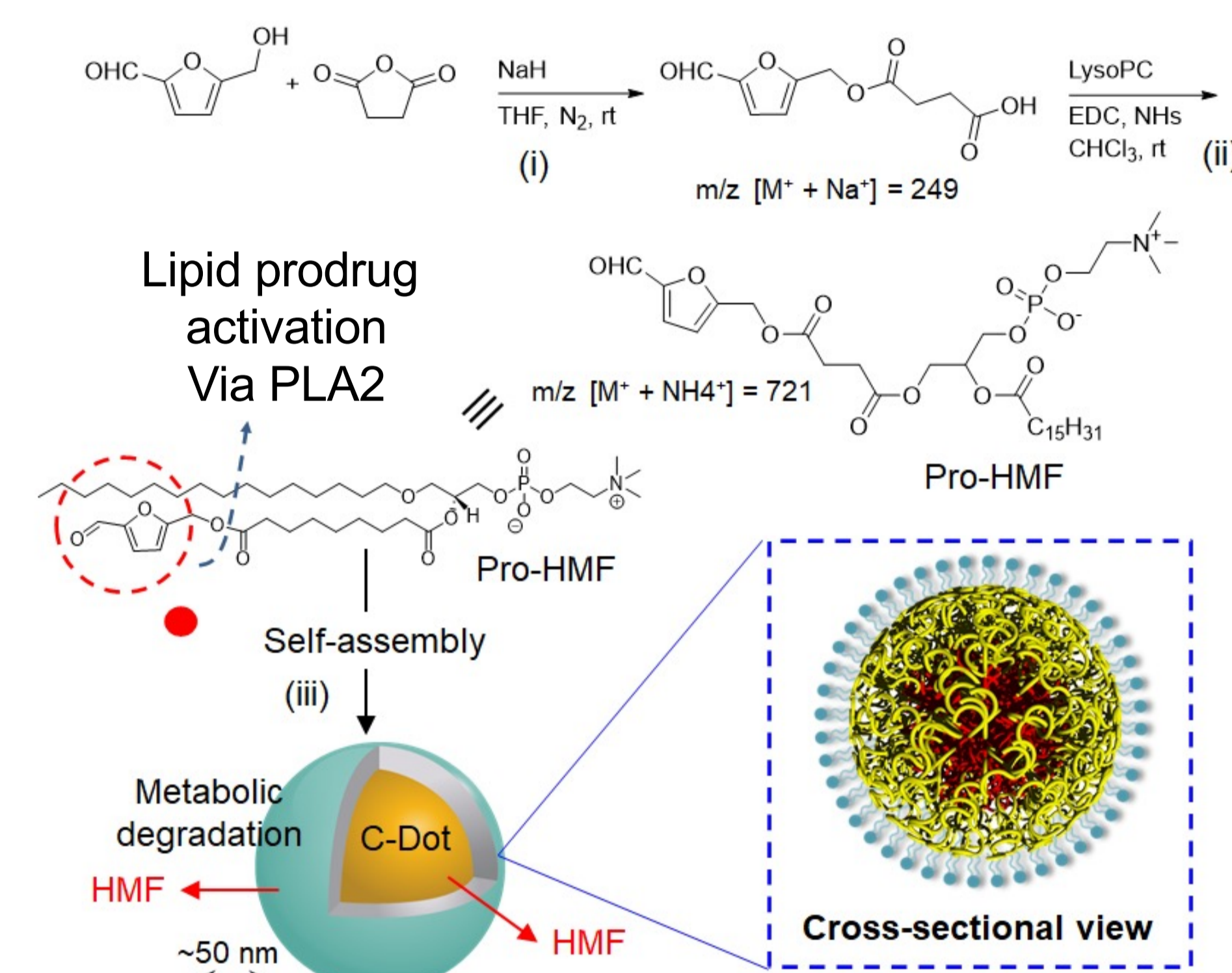
- Despite 5-HMF's potent anti-sickling properties and favorable safety profile (Phase I/II studies (Aes 103, BAX 555): NCT01597401, NCT01871142, NCT01987908), translation has been limited by: (a) high target concentration in blood (Hb, ~ 2.5 mM) and (b) unfavorable pharmacokinetics (PK) (t_{1/2} ~ 1h) arising from metabolism by aldehyde dehydrogenase (ALDH) in the liver (first pass) and (to a lesser extent) in RBCs.

Reformulated 5-HMF

- We created two complementary 5-HMF prodrugs, integrated as a 'protected' biocompatible composite nanoparticle (5-HMF CompNP) designed to readily fuse with RBCs and be amenable to transdermal delivery (5-HMF grafted phospholipid layered over a sucrose derived graphitic carbon dot core).
- We demonstrated that the prodrugs release 5-HMF through complimentary mechanisms and rates (acutely from the shell by PLA2 and slowly/sustained from the core by enzyme-triggered and peroxide-based degradation).
- Using human SRBCs, we quantified time- and concentration-dependent *in vitro* pharmacodynamic parameters and antisickling potency for these prodrug nano-formulations (alone/combined)

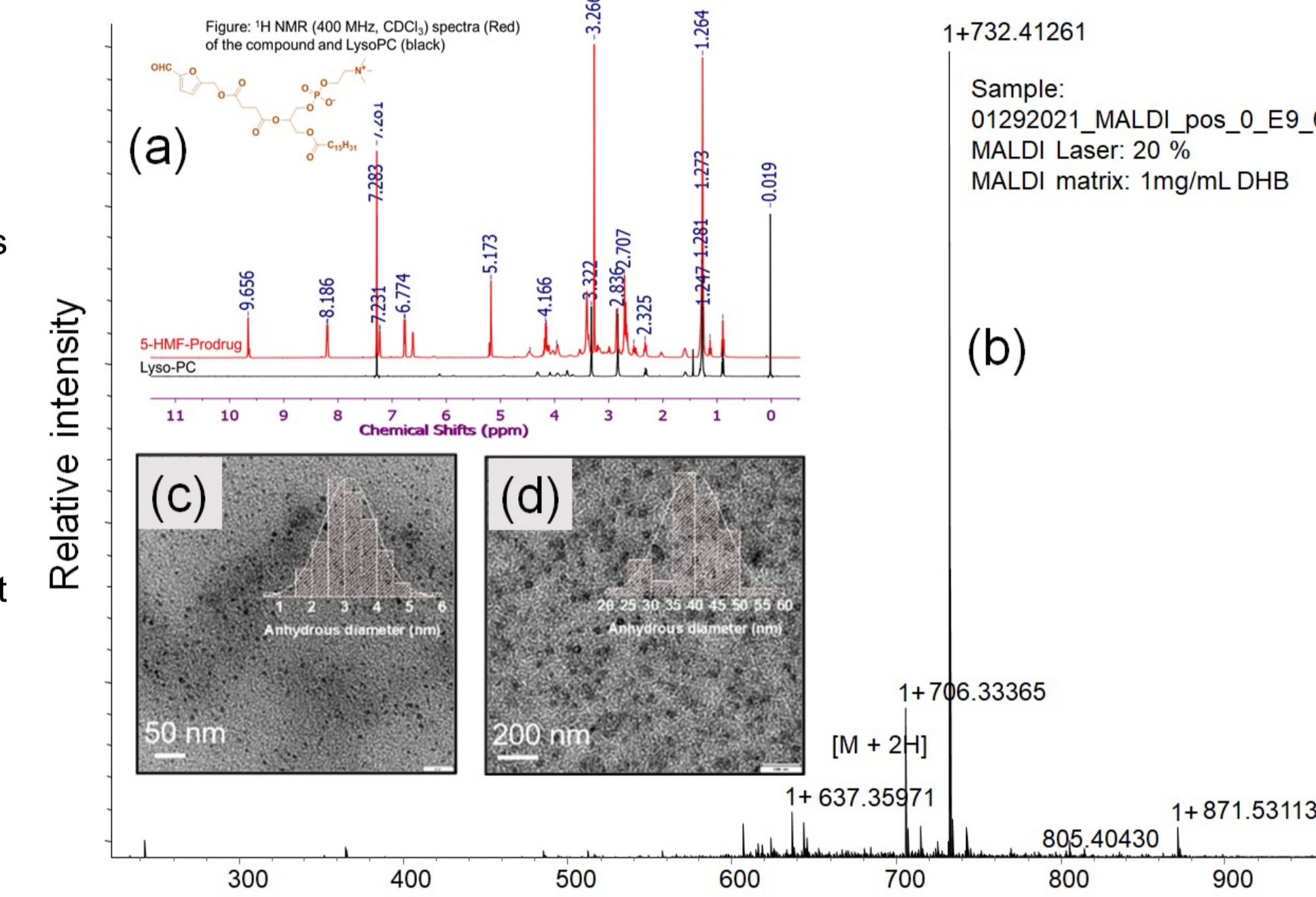
Methods & Results

1 Fabrication & Metabolic Generation of 5-HMF Prodrugs: Carbon Dot (CD) Core & Lipid Prodrug to form a Composite Nanoparticle (CNP)

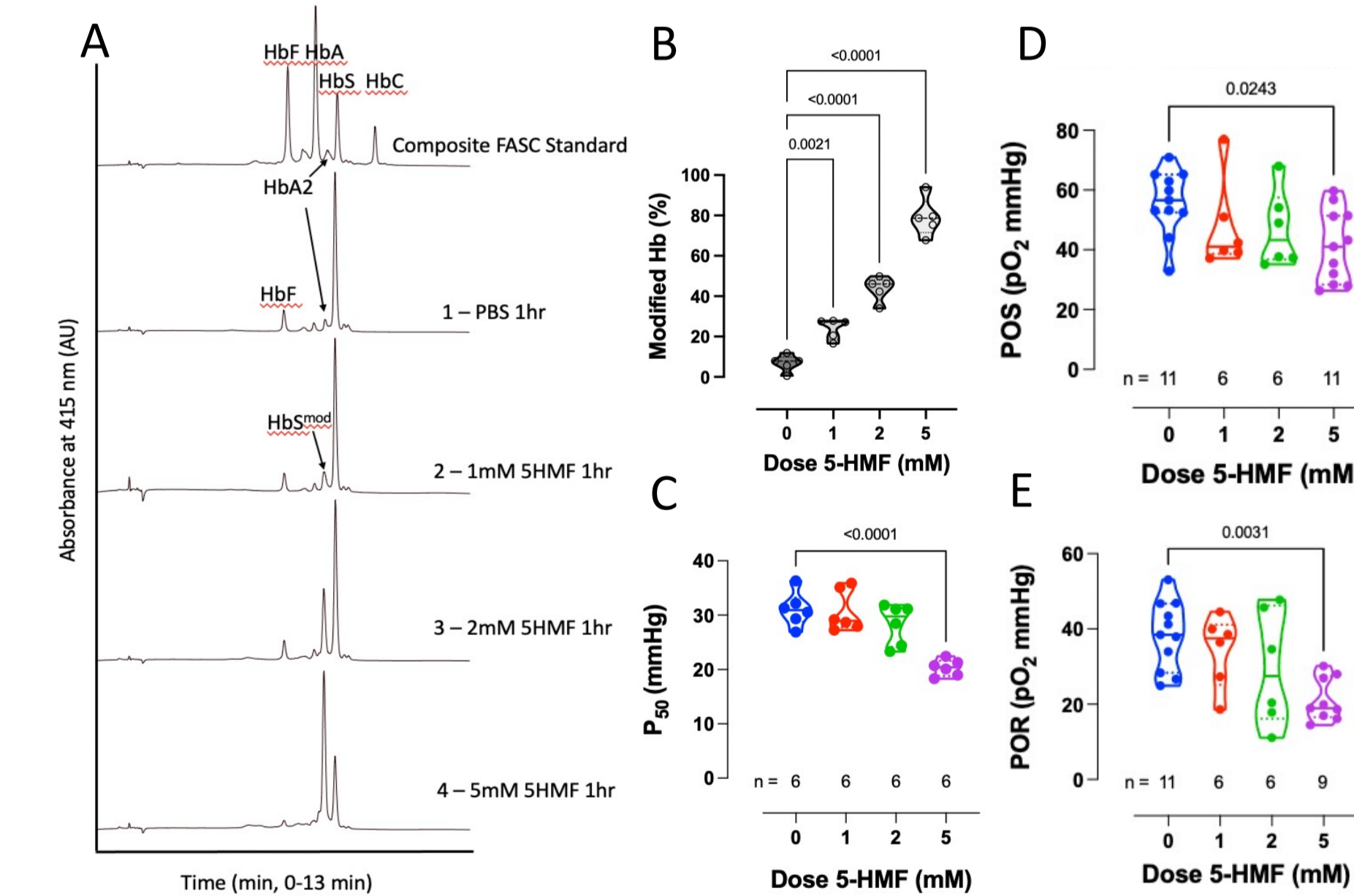


Core-Shell Nanoparticle for Sickled Cells Carrying Anti-Sickling-Prodrug

- (i) 5-HMF/NaH/THF, RT, 12h, 72%
- (ii) lyso-PC/ 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxy-succinimide (NHS), anhydrous chloroform RT, overnight, then
- (iii) L-α-Phosphatidylcholine (5 mM) + 5-HMF lipid (5 mM) at 1:1 molar ratio with carbon dots.
- This mixture was vortexed, left at RT for 30 m, then probe sonicated (10m: amp 1, on 2 sec and off 1 sec.), purified by dialysis 10KDa MWCO for 2-3 days; then lyophilized and redispersed in 3 mL of 1X PBS.



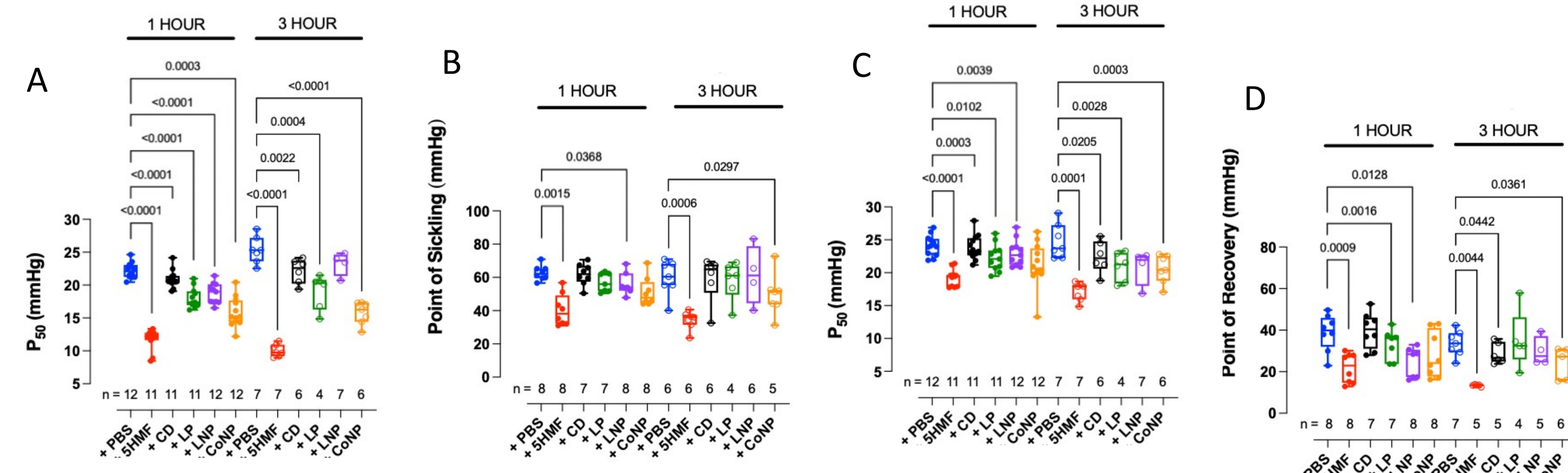
2 5-HMF Pharmacokinetics (PK) & Pharmacodynamics (PD)



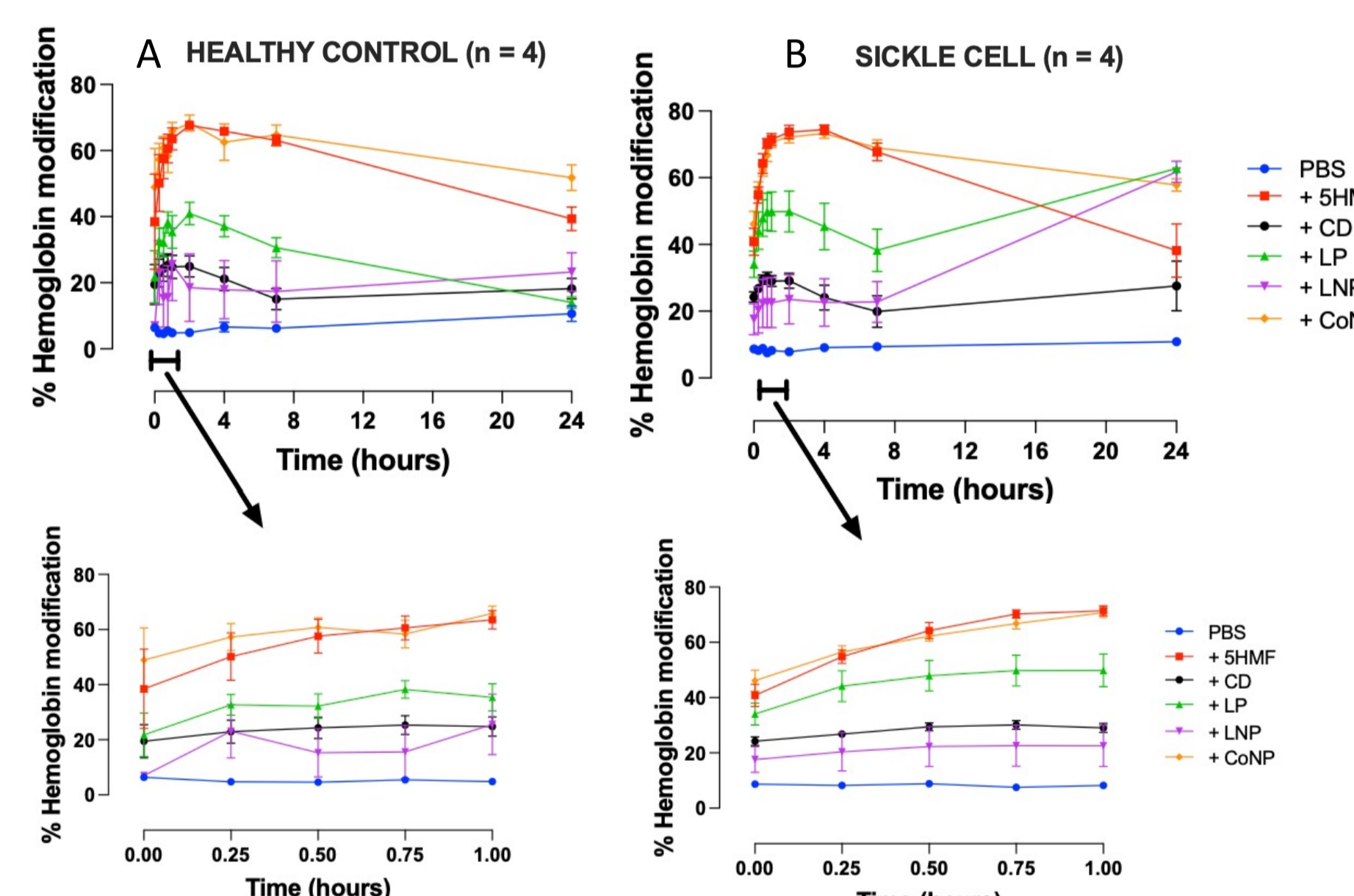
- Human SCD RBCs were incubated with 5-HMF or 5-HMF prodrug formulations (as above, n=6);
- after either 1 or 3h, we performed either: oxygen dissociation curves (ODC) or laser ektactometry to quantify either p50 or POS and POR (both, as above).
- Consistent with PK data, the 5-HMF CoNP prodrug formulation exhibited the greatest efficacy with regard to p50 during (A) O₂ unloading (simulating systemic circulatory transit), and correspondingly leading to (B) a significantly lower pO₂ for hypoxia-induced point of sickling (POS) (e.g., improved hypoxia tolerance).
- Similarly, the CoNP exhibited the greatest efficacy with regard to p50 during (C) O₂ loading (simulating pulmonary circulatory transit), and correspondingly leading to (D) a significantly lower pO₂ for reoxygenation induced point of recovery (POR) (e.g., HbS de-polymerization appears to initiate at lower pO₂).

4 5-HMF Prodrugs & CNP PD

As for our PK data, effect upon p50 and POS/POR progress from 1h to 3h. NB: No prodrug/NP formulation resulted in hemolysis > 1-2%.



3 5-HMF Prodrugs & CNP PK



- RBCs from (A) controls, or (B) SCD patients were incubated with: PBS, 5-HMF, carbon dots (CD), 5-HMF lipid prodrug (LP), 5-HMF lipid prodrug nanoparticles (LNP), or 5-HMF composite nanoparticles (CoNP), all equimolar for 5-HMF (5mM).
- Sequentially (at 0.25, 0.5, 0.75, 1, 2, 4, 6 or 24h) % 5-HMF adducted Hb was determined sequentially (as above).
- Relative efficacy was (high to low): free CoNP, 5-HMF, LP, CD, LNP. N.B. Effect for LP, LNP & CoNP is sustained beyond that of free 5-HMF; effect for LP, LNP & CD is progressive beyond 24h.

- We reformulated **5-HMF** to address translation limitations arising from unfavorable pharmacokinetics.
- We created two complementary **5-HMF prodrugs** that are integrated as a 'protected' biocompatible composite nanoparticle (**5-HMF CompNP**) designed to readily fuse with RBCs and be amenable to transdermal delivery (5-HMF grafted phospholipid layered over a sucrose derived graphitic carbon dot core).
- We demonstrated that the **prodrugs release 5-HMF through complimentary mechanisms and rates** (acutely from the shell by PLA2 and slowly/sustained from the core by enzyme-triggered and peroxide-based degradation).
- Using human SRBCs, we **quantified time- and concentration-dependent in vitro pharmacodynamic parameters and antisickling potency** for these prodrug nano-formulations (alone/combined)

Next Steps

- Define 5-HMF CompNP pharmacokinetics (PK) and pharmacodynamics (PD) in vivo in Townes SCD mice**
 - 5HMF-adducted Hb via HPLC
 - O₂ equilibration curves (Hemeox)
 - inhibition of sickling (LORRCA Oxygenscan (point of sickling and recovery))
 - RBC morphometry after ex vivo exposure to 4% O₂
- Determine benefit conferred by 5-HMF CompNP in vivo in Townes SCD mice during hypoxic stress**
 - Vulnerability to acute lethal hypoxia (FiO₂ 5%)
 - Time to death
 - Organ injury during sublethal hypoxia (FiO₂ 8%)

Assay List
Complete Blood Count, % reticulocytes, plasma free Hb, d-dimer, blood smear (Wright's stain)
Complete Metabolic Profile (renal panel, liver panel, LDH, lactate, pH, blood gases)
Cytokine profile (IL-1β, IL-6, TNF-α, MCP1)
Scored Histopathology: brain, heart, lung, liver, kidney (H&E, TUNEL, pimidazole)

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