

In Situ Lipid Interactions of an Anticancer Metal Complex

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ABSTRACT: An integrated multimodal imaging workflow of cryogenic super-resolution fluorescence microscopy and soft X-ray tomography, Orbitrap secondary ion mass spectrometry, and inductively coupled plasma-mass spectrometry has revealed the unexpected targeting of a half-sandwich cyclopentadienyl Rh(III) phenylazopyridine anticancer complex to cellular lipid membranes and lipid droplets. The complex accumulates in plasma membranes with a surprisingly intense switch-on luminescence in living cancer cells, drives remodeling of lipid droplet architecture, and penetrates deeply into lipid-rich tissue environments. DFT modeling shows strong supramolecular interactions between the complex and glycerophosphorylcholine lipids.

Metal-based therapeutic agents, in particular organo-metallic complexes, offer promising opportunities for the discovery of novel drugs.^{1,2} Progress is hampered by the need to identify their molecular targets and modes of action.^{2–4} Many metallodrugs are pro-drugs which undergo ligand exchange and redox reactions in complicated biological media.⁵ Hence mapping the chemical species which are their active pharmacophores is important. Recent advances in multimodal and integrative analytical approaches provide the means to interrogate these species and their interactions with unprecedented spatial, chemical, and mechanistic resolution.^{6,7} These include high resolution luminescence imaging, soft- and hard-X-ray imaging,⁷ secondary ion mass spectrometry,^{8,9} and inductively coupled plasma-mass spectrometry.¹⁰ Important is the emergence of methods to study intact cryo-fixed cells since chemical fixatives and other treatments can affect metal speciation.⁷ The combination of advances in metal speciation together with genomics and proteomics (“metallomics”) offers major advances in systems pharmacology for metallo-drugs.^{10–12}

Here we study a low-spin pseudo-octahedral 4d⁶ Rh(III) complex coordinated to a π -bonded η^5 -cyclopentadienyl, a *N,N*-chelated phenylazopyridine and monodentate chlorido ligands, $[(\eta^5\text{-Cp}^*)\text{Rh}(\text{Me}_2\text{N-phenylazopyridine})\text{Cl}]^+$, **1** (Figure 1a). The complex exhibits micromolar cytotoxic potency across multiple cancer cell lines, with low toxicity toward normal cells, and no cross-resistance with cisplatin.¹³ The azo-bond is locked into the *trans*-E configuration (Figure 1b), unlike the free ligand. The parent phenylazopyridine ligand is a nonemissive azo dye and a safe anti-infective drug (Phenazopyridine).¹⁴

To elucidate the intracellular chemistry of **1** and mode of action, we implemented a multimodal correlative imaging workflow combining super-resolution cryogenic structured illumination microscopy (cryoSIM), soft X-ray tomography (SXT) on vitrified cancer cells, inductively coupled plasma-

mass spectrometry (ICPMS), and Orbitrap secondary ion mass spectrometry (OrbiSIMS) for depth-resolved molecular profiling in tissue,¹⁵ complemented by DFT modeling to characterize supramolecular lipid interactions.

cryoSIM enables visualization of emissive organic dyes and metal complexes in vitrified cells, while SXT allows high-resolution, label-free assessment of organelle morphology. Applying both modalities to the same biological system can afford complementary insights into the localization of drugs and structural outcomes. Human A549 lung adenocarcinoma cells were treated with complex **1**, plunge-frozen, and imaged under cryogenic conditions at beamline B24 (Diamond Light Source) using cryoSIM for correlative analysis. In selected experiments, organelle-specific dyes provided additional subcellular context (Table S1). Surprisingly, complex **1** exhibited intense membrane-associated luminescence (Figure 2c), absent in untreated controls (Figure 2d). Figure 2f shows the dose-dependent intensity of the red fluorescence from the complex localized in the cell membranes.

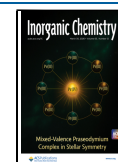
Strikingly, even at a high concentration of 300 μM (10 $\times$ IC₅₀, 1 h treatment), **1** did not induce detectable structural damage, and treated cells retained morphology comparable to that of untreated controls (X-ray mosaics in Figure S1; cryoSXT in Movies S1 and S2; compare cryoSXT Movie S1 for control cells, with Movie S2 for treated cells). Consistent behavior was observed across independent experiments at IC₅₀ concentration (30 μM), confirming a reproducible and selective interaction of **1** with lipid-rich membrane domains.

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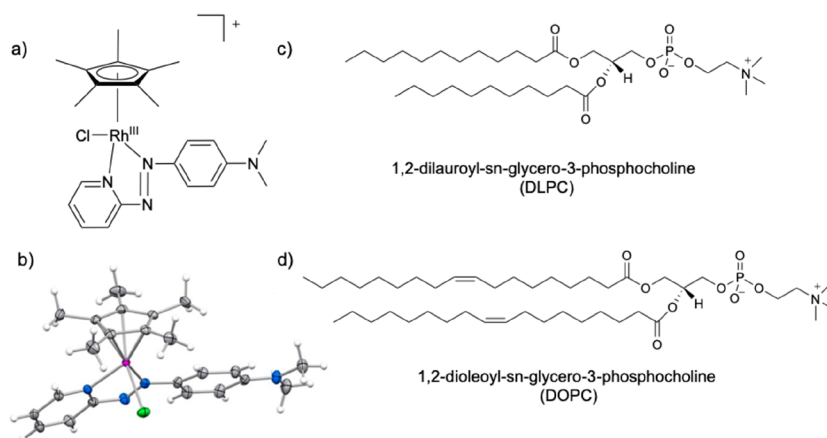


Figure 1. Complex 1 and phospholipids. (a) $[\eta^5\text{-Cp}^*\text{Rh}(\text{4-Me}_2\text{N-phenylazpy})\text{Cl}]^+$; (b) X-ray crystal structure of $1\cdot\text{PF}_6$, thermal ellipsoids drawn at 50% probability, counteranion omitted, showing the *trans* (E) N=N azo bond configuration. Rh, purple; N, blue; Cl, green. Phospholipids (c) DLPC and (d) DOPC.

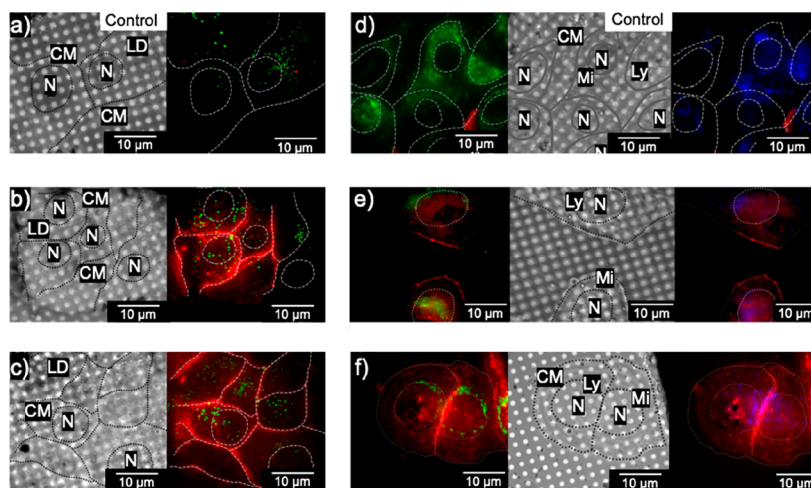


Figure 2. Correlated bright-field (left) and cryoSIM fluorescence (right) images of two representative areas of cryo-fixed human A549 lung cancer cells on Quantifoil carbon-gold grids. (a, d) Untreated (control) cells or (b, c) cells treated with $10 \times \text{IC}_{50}$ 1 (300 μM , 1 h, 310 K in DMEM), or (e, f) $1 \times \text{IC}_{50}$ 1 (30 μM , 1 h, 310 K in DMEM). Both treatment conditions show cell membrane localization. Stains: LipidSpot ($\lambda_{\text{ex/em}} = 427/585$ nm) (a, b, c), green emission; MitoTracker ($\lambda_{\text{ex/em}} = 490/516$ nm) (d, e, f), green emission; LysoTracker ($\lambda_{\text{ex/em}} = 373/422$ nm) (d, e, f), blue emission. Rh complex treatment ($\lambda_{\text{ex/em}} = 561/605$ nm), red emission. Lipid droplets, LD; nucleus, N; cell membrane, CM; lysosomes, Ly.

While no major ultrastructural alterations were detectable during the first hour of exposure, the emergence of punctate red luminescence indicated localized accumulation of the complex, suggesting the initiation of early molecular events that may precede downstream cytotoxic responses occurring on longer time scales.

The downstream metabolic consequences were investigated by quantifying lipid droplet (LD) morphology. LDs play crucial roles in cell proliferation.¹⁶ They contain mostly triacyl glycerols, and also cholesteryl esters with embedded surface proteins including lipid droplet metabolism enzymes. Designs for luminescent metal complexes as stains for LDs have been reported, e.g., in refs 17 and 18.

Lipid droplet-rich fractions were isolated from A549 cells treated with complex 1, and Rh content was determined by ICPMS (details in Section S2.15, Figure S2). Accumulation of Rh was ca. 3-fold higher at $5 \times \text{IC}_{50}$ compared to $1 \times \text{IC}_{50}$ (Figure S2). LipidSpot staining, widefield fluorescence imaging and FIJI analysis revealed significant enlargement of LDs in Rh(III)-treated cells relative to controls, with increases in LD diameter of ca. 3.7-fold and volume of ca. 48-fold, but a ca. 4-

fold decrease in LD number for cells treated with 1 (Figure 3d,e, SI Section S2.15). LDs can grow by acquiring lipids from the ER, or by fusing with other lipid droplets dependent on cell death-inducing DNA fragmentation factor-like effector (CIDE) proteins.¹⁶ The marked increase in droplet diameters and volumes induced by complex 1 are consistent with fusion or coalescence of smaller droplets into fewer, enlarged lipid stores. Notably the Rh species in the LDs did not give rise to detectable luminescence.

Remarkably, for a cytotoxic anticancer compound, cryogenic soft X-ray studies on vitrified A549 cells treated with complex 1 ($10 \times \text{IC}_{50}$, 300 μM) revealed that their ultrastructure is almost indistinguishable from controls. Tomogram reconstructions clearly resolved the nucleus, mitochondria, lipid droplets, endosomes, and endoplasmic reticulum, with little evidence of rupture or organelle-loss. Across multiple preparations, 1-treated cells consistently preserved intact architecture suitable for semiquantitative analysis. The luminescence observed for complex 1 in cells was unexpected. Luminescent half-sandwich Rh(III) complexes are rare.¹⁸ Typically they lack emission due to thermally accessible metal-centered (MC) states.¹⁹ TD-

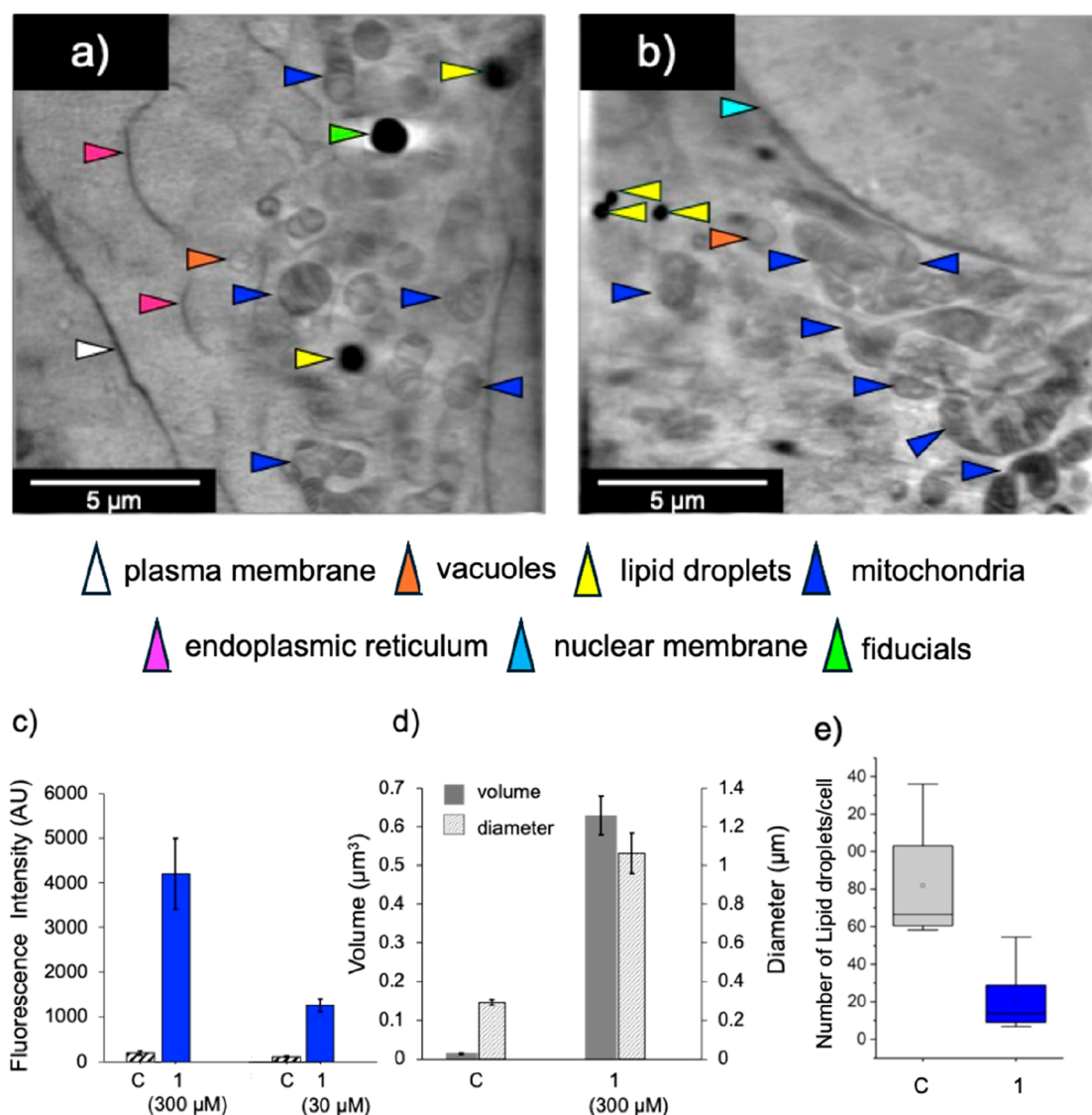


Figure 3. Effect of complex **1** on organelles in lung cancer cells. Soft X-ray tomograms of vitrified cells: (a) control cells and (b) cells after treatment with **1** ($10 \times \text{IC}_{50}$, 300 μM , 1 h), showing intact nuclei, mitochondria, lipid droplets, and ER, with the overall ultrastructure closely resembling that of untreated controls. (c) Quantification of red fluorescence in images from treated ($n = 10$ cells) and untreated ($n = 59$ cells) cells, across multiple independent grids. (d) Quantification of LD volume and diameter of treated and untreated cells. (e) Number of LDs after treatment with 300 μM **1**. Cells were stained with LipidSpot. Lipid droplets were segmented from LipidSpot-stained images and quantified for number, diameter, and volume with consistent thresholding across conditions, significant ca. 3.7-fold increase in mean diameter relative to controls. 3D cryoSIM reconstructions showed a ca. 48-fold increase in average droplet volume.

DFT calculations on **1** (gas phase) showed that the S_1 state is primarily $\pi \rightarrow \pi^*$ on the Me_2N -azpy unit with $\leq 30\%$ MLCT character (Figure 4a,b). Simulated emission at ~ 650 nm matches the experimental maximum, supporting azopyridine as the emissive center (Figures S3–S6). While parent phenylazopyridine is nonfluorescent,²⁰ substituents such as NMe_2 in **1** can influence electron donation to the $\text{N}=\text{N}$ bond. The stereochemistry of the $\text{N}=\text{N}$ bond can have a major effect on its chemical and optical properties.²¹ For the free ligands, photoisomerization from the ground-state *trans* to excited state *cis* $\text{N}=\text{N}$ configuration plays a role in the emission. The CNNC dihedral angle modulates the S_0 – S_1 gap, impacting emission. Free ligands undergo *trans*–*cis* photoisomerization, but in complex **1**, the azo bond is locked in the *trans* (E) configuration, which stabilizes the emission behavior.

Interactions of **1** with the model membrane phospholipids dioleoylphosphatidylcholine (DOPC, Figures 4c, and S7;

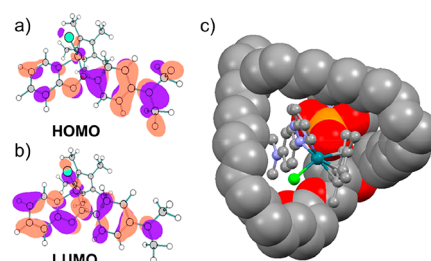


Figure 4. Electronic states of complex **1** and lipid binding. (a) HOMO and (b) LUMO from TD-DFT calculations ($\omega\text{B97X-D3}$ functional). (c) DFT model of supramolecular adduct with glycerophosphorylcholine (spacefill) wrapped around **1** (ball and stick). Rh, turquoise.

solvent growth: Movies S3 and S4) and dilauroylphosphatidylcholine (DLPC, Figure S8) were explored by DFT modeling. For DLPC, in a hydrophilic model (Figure S8a), the lipid quaternary ammonium substituent interacts with the chlorido ligand of **1** ($N-Cl = 4.96 \text{ \AA}$), and phosphate oxygens contact C3/C4 pyridine protons (2.17 and $2.53 \text{ \AA}$). Together with lipid chain interactions, this results in a formation energy of 50 kJ/mol . In contrast, in the hydrophobic model (Figure S8b), the lipid chains wrap around Cp^* and azopyridine rings, giving a more favorable formation energy of 70 kJ/mol . The DOPC supramolecular complex was even more stable (119 kJ/mol), with lipid chains nearly in contact around the Rh complex ($C15-C17 = 4.3 \text{ \AA}$) and a phosphate group near the Me_2N -phenyl unit ($C4-PO = 3.8 \text{ \AA}$; $PO-N = 3.73 \text{ \AA}$, Figure S7).

We investigated the penetration of complex **1** into veal brain homogenate (VBH) as a lipid-rich surrogate that mirrors the structural complexity and fluidity of native tissue, using OrbiSIMS. This combines the depth spatial precision of SIMS ($<10 \text{ nm}$) with the ultrahigh mass resolution and accuracy of Orbitrap detection ($R = 240,000$), an even more powerful combination than time-of-flight (ToF)-SIMS.²² This can provide unambiguous identification of intact metal complexes, their fragments, and endogenous biomolecules.²³

A solution of **1** deposited onto veal brain homogenate on a silicon wafer was dried, and analyzed using a 20 keV Ar^+ cluster ion beam optimized to minimize surface damage while preserving molecular ion signal.²⁴ Depth-profiling revealed persistent and colocalized signals for both $[Rh]^+$ and intact **1** across the full tissue homogenate thickness, sectioned with $10 \text{ }\mu\text{m}$ thickness (Figures 5, S9, and S10, Table S2). Additional

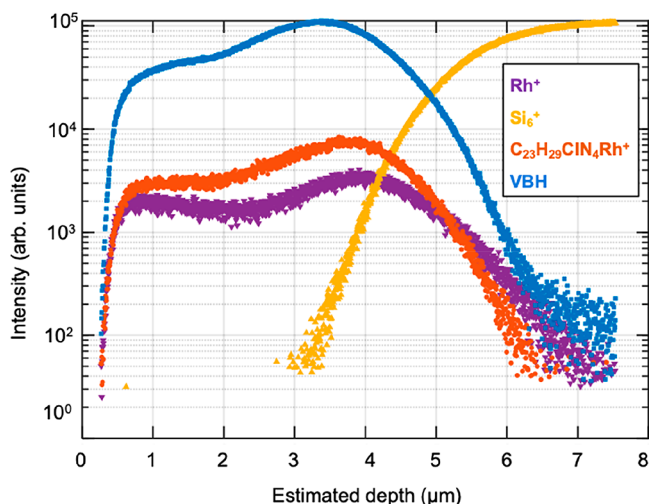


Figure 5. Penetration of complex **1** into a model tissue (VBH). OrbiSIMS positive-ion depth profiles obtained using a $20 \text{ keV Ar}_{2200}^+$ ion beam. Complex **1**, orange; $[Rh]^+$, purple; $[Si_6]^+$, yellow; and [VBH], blue, where [VBH] is the sum of ion fragments characteristic of choline headgroups of phosphorylcholine lipids: $[C_5H_{15}PNO_4]^+$, $[C_5H_{14}NO]^+$, and $[C_5H_{12}N]^+$.

signals for choline-containing lipids (VBH, $[C_5H_{14}NO]^+$, m/z 104.1070) (Table S3) and the underlying silicon substrate ($[Si_6]^+$) served as depth benchmarks. Sputter-yield volumes were calibrated using an organic standard (Irganox).²⁵

Ion dose–response analysis showed that the intensity of these ions changes as the sample is increasingly sputtered. Following an initial transient region attributed to stabilization

of the surface charge, fragment Rh^+ and **1** ions were detected at a high-intensity throughout the homogenate identified by the choline headgroup ions. At the interface with the silicon wafer their intensities fall sharply with a concomitant rise in Si_6^+ . The similar ion intensity depth profiles for **1** and the VBH marker ions indicate that **1** is uniformly distributed throughout the depth of the homogenate tissue. Importantly, intact **1** was consistently detected across the homogenate depth, indicating that the complex remains chemically stable and structurally intact under these biologically relevant, lipid-rich conditions.

This work has revealed the multitargeting of a chelated half-sandwich Rh(III) complex to lipid membranes and lipid droplets in human lung cancer cells, and provided insights into its strong molecular lipid interactions. Lipids represent underexplored targets for metallo-anticancer drugs. We envisage that machine learning incorporating the type of data reported here will eventually pave the way for the application of artificial intelligence to aid metallodrug design as it has done for targeted organic drugs.²⁶ However, the complexity of both the thermodynamics and kinetics of metallodrug activation and speciation are formidable concepts for current AI technology.

■ ASSOCIATED CONTENT

Data Availability Statement

All data underlying this study are openly available for download, free of charge, as noted in the SI for cryoSXT and SXT in Section S2.10 and for OrbiSIMS in Section S2.13.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.inorgchem.6c00104>.

Materials, experimental procedures, instruments and methods, fluorescence data, OrbiSIMS data, X-ray mosaic images, Rh content, DFT calculations and models, and TD-DFT absorption spectra (PDF)
 DLPC_hydrophilic_interaction.pdb: DFT model of hydrophilic interaction of DLPC and **1** (PDB)
 DLPC_hydrophobic_interaction.pdb: DFT model of hydrophobic interaction of DLPC and **1** (PDB)
 DOPC_hydrophobic_interaction.pdb: DFT model of DOPC wrapped around **1** (PDB)
 Movie S1: cryoSXT of control cells (AVI)
 Movie S2: cryoSXT of treated cells (AVI)
 Movie S3: DFT of solvent growth for **1** without lipid (MP4)
 Movie S4: DFT of solvent growth for **1** with lipid (MP4)

Accession Codes

Deposition Number 2454589 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via the joint Cambridge Crystallographic Data Centre (CCDC) and Fachinformationszentrum Karlsruhe Access Structures service.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

cryoSXT, cryogenic soft X-ray tomography; cryoSIM, super-resolution fluorescence structured illumination microscopy (at cryogenic temperatures); DFT, density functional theory; DOPC, dioleoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; ESI-MS, electrospray ionization mass spectrometry; ICPMS, inductively coupled plasma mass spectrometry; OrbiSIMS, secondary ion mass spectrometry with Orbitrap detection; TD-DFT, time-dependent density functional theory; ToF-SIMS, time-of-flight secondary ion mass spectrometry

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