



(51) International Patent Classification:

A61P 25/28 (2006.01)

C07F 15/00 (2006.01)

A61P 35/00 (2006.01)

(21) International Application Number:

PCT/US2023/078616

(22) International Filing Date:

03 November 2023 (03.11.2023)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/422,506

04 November 2022 (04.11.2022) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH,

TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

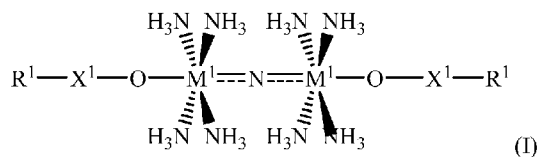
Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

Published:

— with international search report (Art. 21(3))
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(54) Title: PRODRUGS FOR MITOCHONDRIAL CALCIUM UPTAKE INHIBITORS



(57) Abstract: The present technology is directed to a compound of Formula I: or a pharmaceutically acceptable salt and/or solvate thereof, and pharmaceutical compositions or medicaments comprising the compound. The compounds, pharmaceutical compositions, or medicaments are well suited for use as mitochondrial calcium uniporter inhibitors.



PRODRUGS FOR MITOCHONDRIAL CALCIUM UPTAKE INHIBITORS**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application No. 63/422,506, filed on November 4, 2022, the entirety of which is incorporated herein by reference.

U.S. GOVERNMENT RIGHTS

[0002] This invention was made with government support under grant CHE-1750295 awarded by the National Science Foundation. The government has certain rights in the invention.

FIELD

[0003] The present technology generally relates to compounds that inhibit mitochondrial calcium uniporter (MCU).

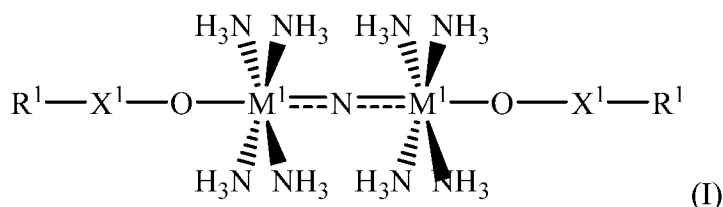
BACKGROUND

[0004] The homeostasis and dynamics of intracellular calcium ions (Ca^{2+}) are involved in a wide range of biological processes.¹⁻³ Among the key organelles responsible for Ca^{2+} regulation are the mitochondria. Mitochondrial Ca^{2+} ($m\text{Ca}^{2+}$) uptake is important for healthy cellular function, but dysregulation of this process can lead to deleterious effects. For example, $m\text{Ca}^{2+}$ overload can trigger cell death^{4,5} and has been implicated in a number of pathological conditions, including neurodegenerative disorders,^{6,7} heart disease,⁸⁻¹⁰ cancer,^{11,12} cystic fibrosis,^{13,14} and ischemic reperfusion injury.¹⁵⁻¹⁷ Ca^{2+} influx into the mitochondria is mediated by the highly selective channel known as the mitochondrial calcium uniporter (MCU).¹⁸⁻²¹ This transmembrane protein resides in the inner mitochondrial membrane (IMM) as a tetrameric assembly comprising of the primary Ca^{2+} -transporting MCU subunit and the regulatory EMRE, MICU1, and MICU2 subunits.²²⁻³¹ Given the role of $m\text{Ca}^{2+}$ overload on various diseases, inhibition of the MCU represents a promising therapeutic strategy.

[0005] There remains a need in the art for effective MCU inhibitors.

SUMMARY

[0006] In an aspect, this disclosure provides a compound according to Formula I



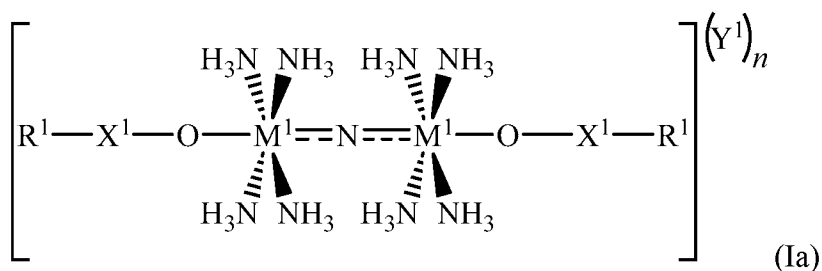
or a pharmaceutically acceptable salt and/or solvate thereof, wherein

M^1 is Ru or Os;

X^1 is —C(O)— or —P(O)(OH)— ; and

R^1 is H, alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl.

In any embodiment herein, the compound of Formula I may be a pharmaceutically acceptable salt according to Formula Ia



or solvate thereof, wherein Y^1 is an anion and n is 1, 2, 3.

[0007] In another aspect, this disclosure is directed pharmaceutical compositions comprising a compound described herein and a pharmaceutically acceptable excipient or pharmaceutically acceptable carrier.

[0008] In another aspect, this disclosure is directed to methods of treating a disease or disorder related to mitochondrial Ca^{2+} overload in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a compound described herein. Diseases or disorders related to mitochondrial Ca^{2+} overload include a neurodegenerative disorder, a cardiovascular disease, a metabolic disease, cancer, a skeletal muscle disease, cystic fibrosis, and ischemic reperfusion injury.

[0009] In yet another aspect, this disclosure is directed to methods of inhibiting mitochondrial calcium uniporter (MCU), comprising administering to a subject an effective amount of a compound described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] **FIG. 1** shows intracellular biochemical events that contribute to ischemia-reperfusion injury (IRI).

[0011] **FIG. 2** demonstrates activation of an exemplary prodrug to the active mitochondrial calcium uniporter (MCU) inhibitor, which blocks mitochondrial calcium uptake.

[0012] **FIG. 3A** is an X-ray crystal structure of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{OPO}_2\text{HCH}_3)_2](\text{CF}_3\text{SO}_3)_3$. **FIG. 3B** is an ^1H NMR spectrum of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{OPO}_2\text{HCH}_3)_2](\text{CF}_3\text{SO}_3)_3$ (500 MHz, DMSO- d_6). **FIG. 3C** is a ^{31}P NMR spectrum of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{OPO}_2\text{HCH}_3)_2](\text{CF}_3\text{SO}_3)_3$ (202 MHz, DMSO- d_6).

[0013] **FIG. 4** is an ^1H NMR spectrum of $[\text{Os}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CCH}_3)_2](\text{CF}_3\text{SO}_3)_3$ (500 MHz, DMSO- d_6).

[0014] **FIG. 5** is an ^1H NMR spectrum of $[\text{Os}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CC}_{10}\text{H}_{16})_2](\text{CF}_3\text{SO}_3)_3$.

[0015] **FIG. 6A** is an X-ray crystal structure of $[(\text{HCO}_2)\text{Ru}(\text{NH}_3)_4(\mu\text{-N})\text{Ru}(\text{NH}_3)_4(\text{O}_2\text{CH})](\text{NO}_3)_3$. **FIG. 6B** is an X-ray crystal structure of $[(\text{CH}_3\text{CH}_2\text{CO}_2)\text{Ru}(\text{NH}_3)_4(\mu\text{-N})\text{Ru}(\text{NH}_3)_4(\text{O}_2\text{CCH}_2\text{CH}_3)](\text{NO}_3)_3$. Outer-sphere ions and solvent molecules are omitted for clarity. Thermal ellipsoids are shown at the 50% probability level. Crystallographic information for both complexes are given in **Table 1**. Interatomic distances and angles are given in **Table 2**.

[0016] **FIGs. 7A-7B** show representative kinetic analysis of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CH})_2](\text{NO}_3)_3$ ("**Compound 1**") at 37 °C via ^1H NMR spectroscopy in MOPS buffered solution with 10% D₂O referenced by dioxane. The proton resonances (**FIG. 7A**) of each species A, B, and L are shown, and the relative concentration of each is plotted over time (**FIG. 7B**) to calculate the rate constants k_1 and k_2 .

[0017] **FIG. 8A** shows the whole cell uptake of Ru265, **Compound 1**, $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CCH}_3)_2](\text{NO}_3)_3$ ("**Compound 2**"), $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CCH}_2\text{CH}_3)_2](\text{NO}_3)_3$ ("**Compound 3**"), and $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{C}(\text{CH}_2)_2\text{CH}_3)_2](\text{OSO}_2\text{CF}_3)_3$ ("**Compound 4**") in HeLa (left) and HEK293T (right) cells after 2h. **FIG. 8B** shows the measurement of cell

uptake in HeLa cells with and without the OCT3 inhibitor decynium-22 after 2h. Data are represented as the mean uptake $\pm$ SD; ****p < 0.005; ns = not significant; n = 3.

[0018] **FIG. 9** shows the dissociation of the axial carboxylate ligands from the ruthenium center, which was monitored at 37 °C via ^1H NM. The dissociation occurs through an $\text{A} \rightleftharpoons \text{B} \rightarrow \text{C}$ process. The relative integrations of each signal were measured for the intact complex (A), mono-substituted complex (B), and the free ligand (L). The relative rate constants were calculated using Equations 1-5 in the examples section below. The rate limiting step was determined to be the first step, with rate constants k_2 being an order of magnitude higher than k_1 . **Compound 1** demonstrated the fastest rate of aquation, which can be attributed to the weaker donor strength of formate compared to the other carboxylate ligands demonstrated by its lower pK_a . In addition, the Ru–O bond length in the crystal structure of **Compound 1** is slightly longer than that of **Compound 3**.

[0019] **FIGs. 10A-10D** show time-dependent mitochondrial Ca^{2+} uptake rates of a) **Compound 1**, b) **Compound 2**, c) **Compound 3**, and d) **Compound 4** at 10 nM in MOPS buffered solution in permeabilized HEK293T cells (1×10^7 cells mL^{-1}). Each time point was prepared from a 400 μM stock of the complexes incubated in 40 mM MOPS buffered solution (pH 7.4) and diluted to 10 nM in $18 \text{ M}\Omega \cdot \text{cm}$ H_2O at the time of the experiment. All uptake rates were normalized to the rate of untreated (control) cells.

[0020] **FIG. 11A** shows mitochondrial Ca^{2+} transients in HeLa cells after treatment with histamine (100 μM) that were pretreated with or without Ru265, **Compound 1**, **Compound 2**, **Compound 3**, or **Compound 4** (50 μM) for 1 h. Data are presented as the mean response $\pm$ SD. **FIG 11B** shows fold increase (F/F_0) of the fluorescence response of each treatment upon addition of histamine (100 μM). Data are presented as the mean response $\pm$ SD.

[0021] **FIG. 12** shows the X-ray crystal structure of Ru265 where the axial chloride ligands are substituted with 1-adamantane carboxylate.

[0022] **FIG. 13** shows time-dependent mitochondrial Ca^{2+} uptake rates of a **Compound 5** at 10 nM in MOPS buffered solution in permeabilized HEK293T cells (1×10^7 cells mL^{-1}). Each time point was prepared from a 400 μM stock of the complexes incubated in 40 mM MOPS buffered solution (pH 7.4) and diluted to 10 nM in $18 \text{ M}\Omega \cdot \text{cm}$ H_2O at the

time of the experiment. All uptake rates were normalized to the rate of untreated (control) cells.

[0023] FIG. 14 shows encapsulation of **Compound 5** into β -cyclodextrin (β -CD).

DETAILED DESCRIPTION

[0024] Before the present disclosure is further described, it is to be understood that the disclosure is not limited to the particular embodiments set forth herein, and it is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Definitions

[0025] The following terms are used throughout as defined below.

[0026] As used herein and in the appended claims, singular articles such as “a” and “an” and “the” and similar referents in the context of describing the elements (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, “such as”) provided herein, is intended merely to better illuminate the embodiments and does not pose a limitation on the scope of the claims unless otherwise stated. No language in the specification should be construed as indicating any non-claimed element as essential.

[0027] As used herein, “about” will be understood by persons of ordinary skill in the art and will vary to some extent depending upon the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art, given the context in which it is used, “about” will mean up to plus or minus 10% of the particular term – for example, “about 10 wt. %” would be understood to mean “9 wt. % to 11 wt. %.” It is to be understood that when “about” precedes a term, the term is to be construed as disclosing

“about” the term as well as the term without modification by “about” – for example, “about 10 wt. %” discloses “9 wt. % to 11 wt. %” as well as disclosing “10 wt. %.”

[0028] The phrase “and/or” as used in the present disclosure will be understood to mean any one of the recited members individually or a combination of any two or more thereof – for example, “A, B, and/or C” would mean “A, B, C, A and B, A and C, B and C, or the combination of A, B, and C.”

[0029] Generally, reference to a certain element such as hydrogen or H is meant to include all isotopes of that element. For example, if an R group is defined to include hydrogen or H, it also includes deuterium and tritium. Compounds comprising radioisotopes such as tritium, C¹⁴, P³² and S³⁵ are thus within the scope of the present technology. Procedures for inserting such labels into the compounds of the present technology will be readily apparent to those skilled in the art based on the disclosure herein.

[0030] In general, “substituted” refers to an organic group as defined below (e.g., an alkyl group) in which one or more bonds to a hydrogen atom contained therein are replaced by a bond to non-hydrogen or non-carbon atoms. Substituted groups also include groups in which one or more bonds to a carbon(s) or hydrogen(s) atom are replaced by one or more bonds, including double or triple bonds, to a heteroatom. Thus, a substituted group is substituted with one or more substituents, unless otherwise specified. In some embodiments, a substituted group is substituted with 1, 2, 3, 4, 5, or 6 substituents. Examples of substituent groups include: halogens (*i.e.*, F, Cl, Br, and I); hydroxyls; alkoxy, alkenoxy, aryloxy, aralkyloxy, heterocyclyl, heterocyclylalkyl, heterocycliloxy, and heterocyclylalkoxy groups; carbonyls (oxo); carboxylates; esters; urethanes; oximes; hydroxylamines; alkoxyamines; aralkoxyamines; thiols; sulfides; sulfoxides; sulfones; sulfonyls; pentafluorosulfanyl (*i.e.*, SF₅), sulfonamides; amines; N-oxides; hydrazines; hydrazides; hydrazones; azides; amides; ureas; amidines; guanidines; enamines; imides; isocyanates; isothiocyanates; cyanates; thiocyanates; imines; nitro groups; and nitriles (*i.e.*, CN).

[0031] Substituted ring groups such as substituted cycloalkyl, aryl, heterocyclyl and heteroaryl groups also include rings and ring systems in which a bond to a hydrogen atom is replaced with a bond to a carbon atom. Therefore, substituted cycloalkyl, aryl, heterocyclyl and heteroaryl groups may also be substituted with substituted or unsubstituted alkyl, alkenyl, and alkynyl groups as defined below.

[0032] Alkyl groups include straight chain and branched chain alkyl groups having from 1 to 12 carbon atoms, and typically from 1 to 10 carbons or, in some embodiments, from 1 to 8, 1 to 6, or 1 to 4 carbon atoms. Alkyl groups may be substituted or unsubstituted. Examples of straight chain alkyl groups include groups such as methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, n-heptyl, and n-octyl groups. Examples of branched alkyl groups include, but are not limited to, isopropyl, iso-butyl, sec-butyl, tert-butyl, neopentyl, isopentyl, and 2,2-dimethylpropyl groups. Representative substituted alkyl groups may be substituted one or more times with substituents such as those listed above, and include without limitation haloalkyl (e.g., trifluoromethyl), hydroxyalkyl, thioalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, alkoxyalkyl, carboxyalkyl, and the like.

[0033] Cycloalkyl groups include mono-, bi- or tricyclic alkyl groups having from 3 to 12 carbon atoms in the ring(s), or, in some embodiments, 3 to 10, 3 to 8, or 3 to 4, 5, or 6 carbon atoms. Cycloalkyl groups may be substituted or unsubstituted. Exemplary monocyclic cycloalkyl groups include, but not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl groups. In some embodiments, the cycloalkyl group has 3 to 8 ring members, whereas in other embodiments the number of ring carbon atoms range from 3 to 5, 3 to 6, or 3 to 7. Bi- and tricyclic ring systems include both bridged cycloalkyl groups and fused rings, such as, but not limited to, bicyclo[2.1.1]hexane, adamantyl, decalynyl, and the like. Substituted cycloalkyl groups may be substituted one or more times with, non-hydrogen and non-carbon groups as defined above. However, substituted cycloalkyl groups also include rings that are substituted with straight or branched chain alkyl groups as defined above. Representative substituted cycloalkyl groups may be mono-substituted or substituted more than once, such as, but not limited to, 2,2-, 2,3-, 2,4-, 2,5- or 2,6-disubstituted cyclohexyl groups, which may be substituted with substituents such as those listed above.

[0034] Cycloalkylalkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to a cycloalkyl group as defined above. Cycloalkylalkyl groups may be substituted or unsubstituted. In some embodiments, cycloalkylalkyl groups have from 4 to 16 carbon atoms, 4 to 12 carbon atoms, and typically 4 to 10 carbon atoms. Substituted cycloalkylalkyl groups may be substituted at the alkyl, the cycloalkyl or both the alkyl and cycloalkyl portions of the group. Representative substituted

cycloalkylalkyl groups may be mono-substituted or substituted more than once, such as, but not limited to, mono-, di- or tri-substituted with substituents such as those listed above.

[0035] Alkenyl groups include straight and branched chain alkyl groups as defined above, except that at least one double bond exists between two carbon atoms. Alkenyl groups may be substituted or unsubstituted. Alkenyl groups have from 2 to 12 carbon atoms, and typically from 2 to 10 carbons or, in some embodiments, from 2 to 8, 2 to 6, or 2 to 4 carbon atoms. In some embodiments, the alkenyl group has one, two, or three carbon-carbon double bonds. Examples include, but are not limited to vinyl, allyl, $-\text{CH}=\text{CH}(\text{CH}_3)$, $-\text{CH}=\text{C}(\text{CH}_3)_2$, $-\text{C}(\text{CH}_3)=\text{CH}_2$, $-\text{C}(\text{CH}_3)=\text{CH}(\text{CH}_3)$, $-\text{C}(\text{CH}_2\text{CH}_3)=\text{CH}_2$, among others. Representative substituted alkenyl groups may be mono-substituted or substituted more than once, such as, but not limited to, mono-, di- or tri-substituted with substituents such as those listed above.

[0036] Cycloalkenyl groups include cycloalkyl groups as defined above, having at least one double bond between two carbon atoms. Cycloalkenyl groups may be substituted or unsubstituted. In some embodiments the cycloalkenyl group may have one, two or three double bonds but does not include aromatic compounds. Cycloalkenyl groups have from 4 to 14 carbon atoms, or, in some embodiments, 5 to 14 carbon atoms, 5 to 10 carbon atoms, or even 5, 6, 7, or 8 carbon atoms. Examples of cycloalkenyl groups include cyclohexenyl, cyclopentenyl, cyclohexadienyl, cyclobutadienyl, and cyclopentadienyl.

[0037] Cycloalkenylalkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of the alkyl group is replaced with a bond to a cycloalkenyl group as defined above. Cycloalkenylalkyl groups may be substituted or unsubstituted. Substituted cycloalkenylalkyl groups may be substituted at the alkyl, the cycloalkenyl or both the alkyl and cycloalkenyl portions of the group. Representative substituted cycloalkenylalkyl groups may be substituted one or more times with substituents such as those listed above.

[0038] Alkynyl groups include straight and branched chain alkyl groups as defined above, except that at least one triple bond exists between two carbon atoms. Alkynyl groups may be substituted or unsubstituted. Alkynyl groups have from 2 to 12 carbon atoms, and typically from 2 to 10 carbons or, in some embodiments, from 2 to 8, 2 to 6, or 2 to 4 carbon atoms. In some embodiments, the alkynyl group has one, two, or three carbon-carbon triple bonds. Examples include, but are not limited to $-\text{C}\equiv\text{CH}$, $-\text{C}\equiv\text{CCH}_3$, $-\text{CH}_2\text{C}\equiv\text{CCH}_3$,

and $\text{-C}\equiv\text{CCH}_2\text{CH}(\text{CH}_2\text{CH}_3)_2$, among others. Representative substituted alkynyl groups may be mono-substituted or substituted more than once, such as, but not limited to, mono-, di- or tri-substituted with substituents such as those listed above.

[0039] Aryl groups are cyclic aromatic hydrocarbons that do not contain heteroatoms. Aryl groups herein include monocyclic, bicyclic and tricyclic ring systems. Aryl groups may be substituted or unsubstituted. Thus, aryl groups include, but are not limited to, phenyl, azulenyl, heptalenyl, biphenyl, fluorenyl, phenanthrenyl, anthracenyl, indenyl, indanyl, pentalenyl, and naphthyl groups. In some embodiments, aryl groups contain 6-14 carbons, and in others from 6 to 12 or even 6-10 carbon atoms in the ring portions of the groups. In some embodiments, the aryl groups are phenyl or naphthyl. The phrase “aryl groups” includes groups containing fused rings, such as fused aromatic-aliphatic ring systems (e.g., indanyl, tetrahydronaphthyl, and the like). Representative substituted aryl groups may be mono-substituted (e.g., tolyl) or substituted more than once. For example, monosubstituted aryl groups include, but are not limited to, 2-, 3-, 4-, 5-, or 6-substituted phenyl or naphthyl groups, which may be substituted with substituents such as those listed above.

[0040] Aralkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to an aryl group as defined above. Aralkyl groups may be substituted or unsubstituted. In some embodiments, aralkyl groups contain 7 to 16 carbon atoms, 7 to 14 carbon atoms, or 7 to 10 carbon atoms. Substituted aralkyl groups may be substituted at the alkyl, the aryl or both the alkyl and aryl portions of the group. Representative aralkyl groups include but are not limited to benzyl and phenethyl groups and fused (cycloalkylaryl)alkyl groups such as 4-indanylethyl. Representative substituted aralkyl groups may be substituted one or more times with substituents such as those listed above.

[0041] Heterocyclyl groups include aromatic (also referred to as heteroaryl) and non-aromatic ring compounds containing 3 or more ring members, of which one or more is a heteroatom such as, but not limited to, N, O, and S. Heterocyclyl groups may be substituted or unsubstituted. In some embodiments, the heterocyclyl group contains 1, 2, 3 or 4 heteroatoms. In some embodiments, heterocyclyl groups include mono-, bi- and tricyclic rings having 3 to 16 ring members, whereas other such groups have 3 to 6, 3 to 10, 3 to 12, or 3 to 14 ring members. Heterocyclyl groups encompass aromatic, partially unsaturated and saturated ring systems, such as, for example, imidazolyl, imidazolinyl and imidazolidinyl

groups. The phrase “heterocyclyl group” includes fused ring species including those comprising fused aromatic and non-aromatic groups, such as, for example, benzotriazolyl, 2,3-dihydrobenzo[1,4]dioxinyl, and benzo[1,3]dioxolyl. The phrase also includes bridged polycyclic ring systems containing a heteroatom such as, but not limited to, quinuclidyl. The phrase includes heterocyclyl groups that have other groups, such as alkyl, oxo or halo groups, bonded to one of the ring members, referred to as “substituted heterocyclyl groups”.

Heterocyclyl groups include, but are not limited to, aziridinyl, azetidiny, pyrrolidinyl, imidazolidinyl, pyrazolidinyl, thiazolidinyl, tetrahydrothiophenyl, tetrahydrofuranly, dioxolyl, furanyl, thiophenyl, pyrrolyl, pyrrolinyl, imidazolyl, imidazoliny, pyrazolyl, pyrazolinyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, thiazolyl, thiazolinyl, isothiazolyl, thiadiazolyl, oxadiazolyl, piperidyl, piperazinyl, morpholinyl, thiomorpholinyl, tetrahydropyranyl, tetrahydrothiopyranyl, oxathiane, dioxyl, dithianyl, pyranly, pyridyl, pyrimidinyl, pyridazinyl, pyrazinyl, triazinyl, dihydropyridyl, dihydrodithiiny, dihydrodithionyl, homopiperazinyl, quinuclidyl, indolyl, indolinyl, isoindolyl, azaindolyl (pyrrolopyridyl), indazolyl, indoliziny, benzotriazolyl, benzimidazolyl, benzofuranly, benzothiophenyl, benzthiazolyl, benzoxadiazolyl, benzoxazinyl, benzodithiiny, benzoxathiiny, benzothiazinyl, benzoxazolyl, benzothiazolyl, benzothiadiazolyl, benzo[1,3]dioxolyl, pyrazolopyridyl, imidazopyridyl (azabenzimidazolyl), triazolopyridyl, isoxazolopyridyl, purinyl, xanthinyl, adeninyl, guaninyl, quinolinyl, isoquinolinyl, quinoliziny, quinoxalinyl, quinazolinyl, cinnolinyl, phthalazinyl, naphthyridinyl, pteridinyl, thianaphthyl, dihydrobenzothiazinyl, dihydrobenzofuranly, dihydroindolyl, dihydrobenzodioxinyl, tetrahydroindolyl, tetrahydroindazolyl, tetrahydrobenzimidazolyl, tetrahydrobenzotriazolyl, tetrahydropyrrolopyridyl, tetrahydropyrazolopyridyl, tetrahydroimidazopyridyl, tetrahydrotriazolopyridyl, and tetrahydroquinolinyl groups. Representative substituted heterocyclyl groups may be mono-substituted or substituted more than once, such as, but not limited to, pyridyl or morpholinyl groups, which are 2-, 3-, 4-, 5-, or 6-substituted, or disubstituted with various substituents such as those listed above.

[0042] Heteroaryl groups are aromatic ring compounds containing 5 or more ring members, of which, one or more is a heteroatom such as, but not limited to, N, O, and S. Heteroaryl groups may be substituted or unsubstituted. Heteroaryl groups include, but are not limited to, groups such as pyrrolyl, pyrazolyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, thiazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, thiophenyl, benzothiophenyl, furanyl, benzofuranly, indolyl, azaindolyl (pyrrolopyridinyl), indazolyl, benzimidazolyl,

imidazopyridinyl (azabenzimidazolyl), pyrazolopyridinyl, triazolopyridinyl, benzotriazolyl, benzoxazolyl, benzothiazolyl, benzothiadiazolyl, imidazopyridinyl, isoxazolopyridinyl, thianaphthyl, purinyl, xanthinyl, adeninyl, guaninyl, quinolinyl, isoquinolinyl, tetrahydroquinolinyl, quinoxalinyl, and quinazolinyl groups. Heteroaryl groups include fused ring compounds in which all rings are aromatic such as indolyl groups and include fused ring compounds in which only one of the rings is aromatic, such as 2,3-dihydro indolyl groups. Representative substituted heteroaryl groups may be substituted one or more times with various substituents such as those listed above.

[0043] Heterocyclalkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to a heterocycl group as defined above. Heterocyclalkyl groups may be substituted or unsubstituted. Substituted heterocyclalkyl groups may be substituted at the alkyl, the heterocycl or both the alkyl and heterocycl portions of the group. Representative heterocycl alkyl groups include, but are not limited to, morpholin-4-yl-ethyl, furan-2-yl-methyl, imidazol-4-yl-methyl, pyridin-3-yl-methyl, tetrahydrofuran-2-yl-ethyl, and indol-2-yl-propyl. Representative substituted heterocyclalkyl groups may be substituted one or more times with substituents such as those listed above.

[0044] Heteroaralkyl groups are alkyl groups as defined above in which a hydrogen or carbon bond of an alkyl group is replaced with a bond to a heteroaryl group as defined above. Heteroaralkyl groups may be substituted or unsubstituted. Substituted heteroaralkyl groups may be substituted at the alkyl, the heteroaryl or both the alkyl and heteroaryl portions of the group. Representative substituted heteroaralkyl groups may be substituted one or more times with substituents such as those listed above.

[0045] Groups described herein having two or more points of attachment (i.e., divalent, trivalent, or polyvalent) within the compound of the present technology are designated by use of the suffix, “ene.” For example, divalent alkyl groups are alkylene groups, divalent aryl groups are arylene groups, divalent heteroaryl groups are divalent heteroarylene groups, and so forth. Substituted groups having a single point of attachment to the compound of the present technology are not referred to using the “ene” designation. Thus, e.g., chloroethyl is not referred to herein as chloroethylene.

[0046] Alkoxy groups are hydroxyl groups (-OH) in which the bond to the hydrogen atom is replaced by a bond to a carbon atom of a substituted or unsubstituted alkyl group as defined above. Alkoxy groups may be substituted or unsubstituted. Examples of linear alkoxy groups include but are not limited to methoxy, ethoxy, propoxy, butoxy, pentoxy, hexoxy, and the like. Examples of branched alkoxy groups include but are not limited to isopropoxy, sec-butoxy, tert-butoxy, isopentoxy, isohexoxy, and the like. Examples of cycloalkoxy groups include but are not limited to cyclopropyloxy, cyclobutyloxy, cyclopentyloxy, cyclohexyloxy, and the like. Representative substituted alkoxy groups may be substituted one or more times with substituents such as those listed above.

[0047] The terms “alkanoyl” and “alkanoyloxy” as used herein can refer, respectively, to -C(O)-alkyl groups and -O-C(O)-alkyl groups, each containing 2–5 carbon atoms. Similarly, “aryloyl” and “aryloyloxy” refer to -C(O)-aryl groups and -O-C(O)-aryl groups.

[0048] The terms “aryloxy” and “arylalkoxy” refer to, respectively, a substituted or unsubstituted aryl group bonded to an oxygen atom and a substituted or unsubstituted aralkyl group bonded to the oxygen atom at the alkyl. Examples include but are not limited to phenoxy, naphthyloxy, and benzyloxy. Representative substituted aryloxy and arylalkoxy groups may be substituted one or more times with substituents such as those listed above.

[0049] The term “carboxylate” as used herein refers to a -COOH group.

[0050] The term “ester” as used herein refers to -COOR⁷⁰ and -C(O)O-G groups. R⁷⁰ is a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl, aralkyl, heterocyclalkyl or heterocycl group as defined herein. G is a carboxylate protecting group. Carboxylate protecting groups are well known to one of ordinary skill in the art. An extensive list of protecting groups for the carboxylate group functionality may be found in *Protective Groups in Organic Synthesis*, Greene, T.W.; Wuts, P. G. M., John Wiley & Sons, New York, NY, (3rd Edition, 1999) which can be added or removed using the procedures set forth therein and which is hereby incorporated by reference in its entirety and for any and all purposes as if fully set forth herein.

[0051] The term “amide” (or “amido”) includes C- and N-amide groups, i.e., -C(O)NR⁷¹R⁷², and -NR⁷¹C(O)R⁷² groups, respectively. R⁷¹ and R⁷² are independently hydrogen, or a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl,

heterocyclalkyl or heterocycl group as defined herein. Amido groups therefore include but are not limited to carbamoyl groups ($-\text{C}(\text{O})\text{NH}_2$) and formamide groups ($-\text{NHC}(\text{O})\text{H}$). In some embodiments, the amide is $-\text{NR}^{71}\text{C}(\text{O})-(\text{C}_{1-5} \text{ alkyl})$ and the group is termed "carbonylamino," and in others the amide is $-\text{NHC}(\text{O})\text{-alkyl}$ and the group is termed "alkanoylamino."

[0052] The term "nitrile" or "cyano" as used herein refers to the $-\text{CN}$ group.

[0053] Urethane groups include N- and O-urethane groups, i.e., $-\text{NR}^{73}\text{C}(\text{O})\text{OR}^{74}$ and $-\text{OC}(\text{O})\text{NR}^{73}\text{R}^{74}$ groups, respectively. R^{73} and R^{74} are independently a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heterocyclalkyl, or heterocycl group as defined herein. R^{73} may also be H.

[0054] The term "amine" (or "amino") as used herein refers to $-\text{NR}^{75}\text{R}^{76}$ groups, wherein R^{75} and R^{76} are independently hydrogen, or a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heterocyclalkyl or heterocycl group as defined herein. In some embodiments, the amine is alkylamino, dialkylamino, arylamino, or alkylaryl amino. In other embodiments, the amine is NH_2 , methylamino, dimethylamino, ethylamino, diethylamino, propylamino, isopropylamino, phenylamino, or benzylamino.

[0055] The term "sulfonamido" includes S- and N-sulfonamide groups, i.e., $-\text{SO}_2\text{NR}^{78}\text{R}^{79}$ and $-\text{NR}^{78}\text{SO}_2\text{R}^{79}$ groups, respectively. R^{78} and R^{79} are independently hydrogen, or a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heterocyclalkyl, or heterocycl group as defined herein. Sulfonamido groups therefore include but are not limited to sulfamoyl groups ($-\text{SO}_2\text{NH}_2$). In some embodiments herein, the sulfonamido is $-\text{NHSO}_2\text{-alkyl}$ and is referred to as the "alkylsulfonylamino" group.

[0056] The term "thiol" refers to $-\text{SH}$ groups, while "sulfides" include $-\text{SR}^{80}$ groups, "sulfoxides" include $-\text{S}(\text{O})\text{R}^{81}$ groups, "sulfones" include $-\text{SO}_2\text{R}^{82}$ groups, and "sulfonyls" include $-\text{SO}_2\text{OR}^{83}$. R^{80} , R^{81} , R^{82} , and R^{83} are each independently a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocycl or heterocyclalkyl group as defined herein. In some embodiments the sulfide is an alkylthio group, $-\text{S-alkyl}$.

[0057] The term “urea” refers to $-\text{NR}^{84}-\text{C}(\text{O})-\text{NR}^{85}\text{R}^{86}$ groups. R^{84} , R^{85} , and R^{86} groups are independently hydrogen, or a substituted or unsubstituted alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, heterocyclyl, or heterocyclalkyl group as defined herein.

[0058] The term “amidine” refers to $-\text{C}(\text{NR}^{87})\text{NR}^{88}\text{R}^{89}$ and $-\text{NR}^{87}\text{C}(\text{NR}^{88})\text{R}^{89}$, wherein R^{87} , R^{88} , and R^{89} are each independently hydrogen, or a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocyclyl or heterocyclalkyl group as defined herein.

[0059] The term “guanidine” refers to $-\text{NR}^{90}\text{C}(\text{NR}^{91})\text{NR}^{92}\text{R}^{93}$, wherein R^{90} , R^{91} , R^{92} and R^{93} are each independently hydrogen, or a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocyclyl or heterocyclalkyl group as defined herein.

[0060] The term “enamine” refers to $-\text{C}(\text{R}^{94})=\text{C}(\text{R}^{95})\text{NR}^{96}\text{R}^{97}$ and $-\text{NR}^{94}\text{C}(\text{R}^{95})=\text{C}(\text{R}^{96})\text{R}^{97}$, wherein R^{94} , R^{95} , R^{96} and R^{97} are each independently hydrogen, a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocyclyl or heterocyclalkyl group as defined herein.

[0061] The term “halogen” or “halo” as used herein refers to bromine, chlorine, fluorine, or iodine. In some embodiments, the halogen is fluorine. In other embodiments, the halogen is chlorine or bromine.

[0062] The term “hydroxyl” as used herein can refer to $-\text{OH}$ or its ionized form, $-\text{O}^-$. A “hydroxyalkyl” group is a hydroxyl-substituted alkyl group, such as $\text{HO}-\text{CH}_2-$.

[0063] The term “imide” refers to $-\text{C}(\text{O})\text{NR}^{98}\text{C}(\text{O})\text{R}^{99}$, wherein R^{98} and R^{99} are each independently hydrogen, or a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocyclyl or heterocyclalkyl group as defined herein.

[0064] The term “imine” refers to $-\text{CR}^{100}(\text{NR}^{101})$ and $-\text{N}(\text{CR}^{100}\text{R}^{101})$ groups, wherein R^{100} and R^{101} are each independently hydrogen or a substituted or unsubstituted alkyl, cycloalkyl, alkenyl, alkynyl, aryl aralkyl, heterocyclyl or heterocyclalkyl group as defined herein, with the proviso that R^{100} and R^{101} are not both simultaneously hydrogen.

[0065] The term “nitro” as used herein refers to an $-\text{NO}_2$ group.

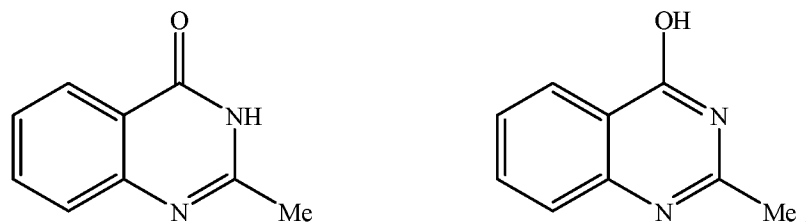
[0066] The term “trifluoromethyl” as used herein refers to $-\text{CF}_3$.

- [0067] The term “trifluoromethoxy” as used herein refers to $-\text{OCF}_3$.
- [0068] The term “azido” refers to $-\text{N}_3$.
- [0069] The term “trialkyl ammonium” refers to a $-\text{N}(\text{alkyl})_3$ group. A trialkylammonium group is positively charged and thus typically has an associated anion, such as halogen anion.
- [0070] The term “isocyano” refers to $-\text{NC}$.
- [0071] The term “isothiocyano” refers to $-\text{NCS}$.
- [0072] The term “pentafluorosulfanyl” refers to $-\text{SF}_5$.
- [0073] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as “up to,” “at least,” “greater than,” “less than,” and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 atoms refers to groups having 1, 2, or 3 atoms. Similarly, a group having 1-5 atoms refers to groups having 1, 2, 3, 4, or 5 atoms, and so forth.
- [0074] As understood by one of ordinary skill in the art, “molecular weight” (also known as “relative molar mass”) is a dimensionless quantity but is converted to molar mass by multiplying by 1 gram/mole or by multiplying by 1 Da – for example, a compound with a weight-average molecular weight of 5,000 has a weight-average molar mass of 5,000 g/mol and a weight-average molar mass of 5,000 Da.
- [0075] As used herein, “complex” and “compound” may be used interchangeably.

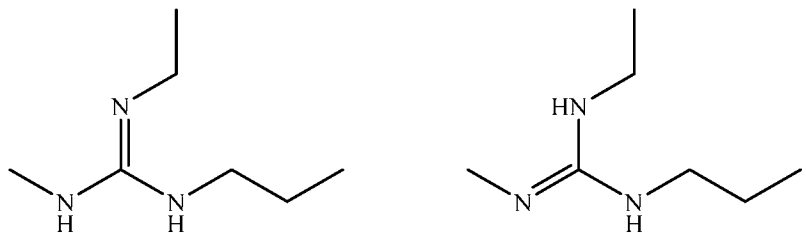
[0076] Pharmaceutically acceptable salts of compounds described herein are within the scope of the present technology and include acid or base addition salts which retain the desired pharmacological activity and is not biologically undesirable (*e.g.*, the salt is not unduly toxic, allergenic, or irritating, and is bioavailable). When the compound of the present technology has a basic group, such as, for example, an amino group, pharmaceutically acceptable salts can be formed with inorganic acids (such as hydrochloric acid, hydroboric acid, nitric acid, sulfuric acid, and phosphoric acid), organic acids (*e.g.*, alginate, formic acid, acetic acid, benzoic acid, gluconic acid, fumaric acid, oxalic acid, tartaric acid, lactic acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid, naphthalene sulfonic acid, and p-toluenesulfonic acid) or acidic amino acids (such as aspartic acid and glutamic acid). When the compound of the present technology has an acidic group, such as for example, a carboxylic acid group, it can form salts with metals, such as alkali and earth alkali metals (*e.g.*, Na⁺, Li⁺, K⁺, Ca²⁺, Mg²⁺, Zn²⁺), ammonia or organic amines (*e.g.*, dicyclohexylamine, trimethylamine, triethylamine, pyridine, picoline, ethanolamine, diethanolamine, triethanolamine) or basic amino acids (*e.g.*, arginine, lysine and ornithine). Such salts can be prepared in situ during isolation and purification of the compounds or by separately reacting the purified compound in its free base or free acid form with a suitable acid or base, respectively, and isolating the salt thus formed.

[0077] Those of skill in the art will appreciate that compounds of the present technology may exhibit the phenomena of tautomerism, conformational isomerism, geometric isomerism, and/or stereoisomerism. As the formula drawings within the specification and claims can represent only one of the possible tautomeric, conformational isomeric, stereochemical or geometric isomeric forms, it should be understood that the present technology encompasses any tautomeric, conformational isomeric, stereochemical and/or geometric isomeric forms of the compounds having one or more of the utilities described herein, as well as mixtures of these various different forms.

[0078] “Tautomers” refers to isomeric forms of a compound that are in equilibrium with each other. The presence and concentrations of the isomeric forms will depend on the environment the compound is found in and may be different depending upon, for example, whether the compound is a solid or is in an organic or aqueous solution. For example, in aqueous solution, quinazolinones may exhibit the following isomeric forms, which are referred to as tautomers of each other:



As another example, guanidines may exhibit the following isomeric forms in protic organic solution, also referred to as tautomers of each other:



Because of the limits of representing compounds by structural formulas, it is to be understood that all chemical formulas of the compounds described herein represent all tautomeric forms of compounds and are within the scope of the present technology.

[0079] Stereoisomers of compounds (also known as optical isomers) include all chiral, diastereomeric, and racemic forms of a structure, unless the specific stereochemistry is expressly indicated. Thus, compounds used in the present technology include enriched or resolved optical isomers at any or all asymmetric atoms as are apparent from the depictions. Both racemic and diastereomeric mixtures, as well as the individual optical isomers can be isolated or synthesized so as to be substantially free of their enantiomeric or diastereomeric partners, and these stereoisomers are all within the scope of the present technology.

[0080] The compounds of the present technology may exist as solvates, especially hydrates. Hydrates may form during manufacture of the compounds or compositions comprising the compounds, or hydrates may form over time due to the hygroscopic nature of the compounds. Compounds of the present technology may exist as organic solvates as well, including DMF, ether, and alcohol solvates among others. The identification and preparation of any particular solvate is within the skill of the ordinary artisan of synthetic organic or medicinal chemistry.

[0081] The terms “patient” or “subject” are used interchangeably to refer to a human or a non-human animal (e.g., a mammal).

[0082] The terms “treat”, “treating”, “treatment” and the like refer to a course of action that eliminates, reduces, suppresses, mitigates, ameliorates, or prevents the worsening of, either temporarily or permanently, a disease, disorder or condition to which the term applies, or at least one of the symptoms associated therewith. Treatment includes alleviation of symptoms, diminishment of extent of disease, inhibiting (e.g., arresting the development or further development of the disease, disorder or condition or clinical symptoms association therewith) an active disease, delaying or slowing of disease progression, improving the quality of life, and/or prolonging survival of a subject as compared to expected survival if not receiving treatment or as compared to a published standard of care therapy for a particular disease.

[0083] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. Also within this disclosure are Arabic numerals referring to referenced citations, the full bibliographic details of which are provided immediately preceding the claims. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the present technology.

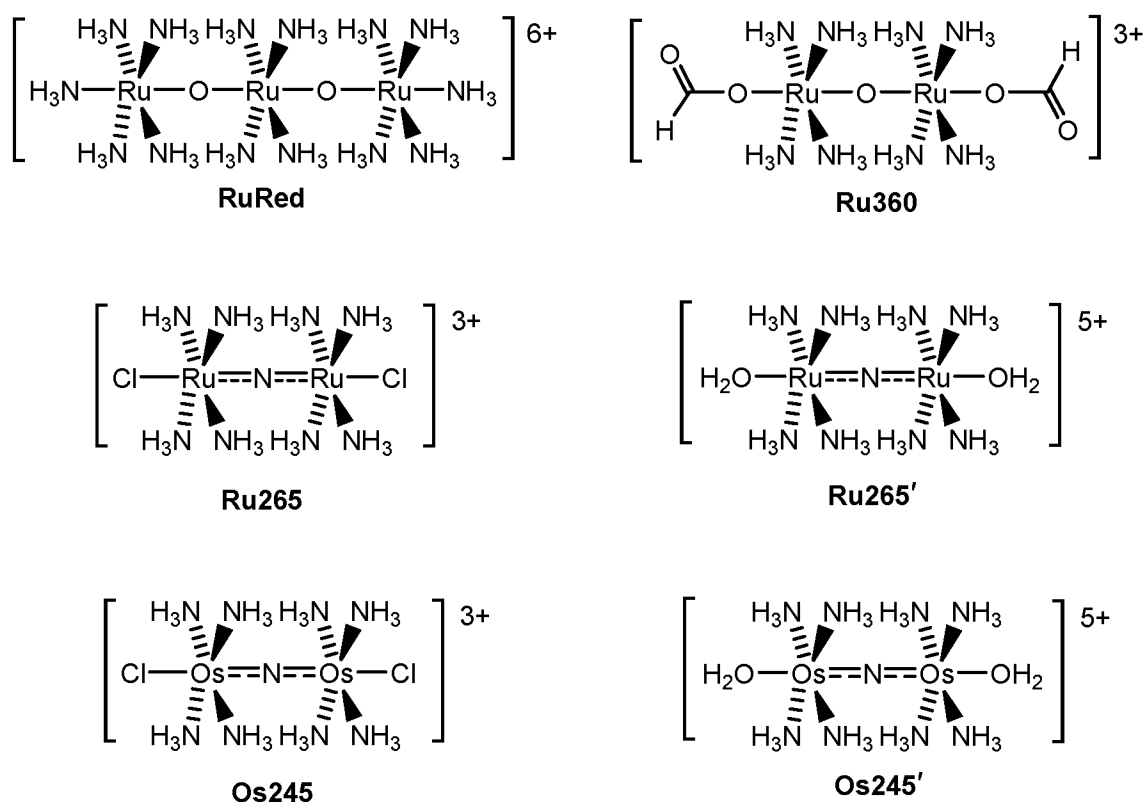
Overview

[0084] To develop new therapeutic agents and tools for studying mCa^{2+} , extensive research efforts have been directed towards the identification of small-molecule MCU inhibitors.³² To date, several organic^{33–39} and inorganic^{40–45} compounds have been discovered to possess MCU-inhibitory activity. Among these compounds, polynuclear Ru complexes are the oldest known MCU inhibitors. The earliest inhibitor of this type is the trinuclear complex ruthenium red (RuRed; **Scheme 1**), which was originally applied as a cytological stain.⁴⁶ Subsequent studies, however, indicated that impurities present within RuRed samples were responsible for the mCa^{2+} uptake inhibitory activity.^{44,47} These efforts ultimately led to the discovery of the dinuclear oxo-bridged complex Ru360 (**Scheme 1**).^{43,44,48} This complex exhibits nM potency as an MCU inhibitor in isolated mitochondria and permeabilized cells. Its use in intact cell systems, however, has yielded inconsistent results – a possible consequence of its poor cell permeability.^{43,45}

[0085] Recently, an analogue of Ru360 called Ru265 was reported (**Scheme 1**).⁴⁰ This compound is structurally similar to Ru360 but contains a bridging nitrido (N^{3-}) ligand

rather than an oxo (O^{2-}) ligand. Notably, this relatively simple modification confers Ru265 with the ability to inhibit mCa^{2+} uptake in intact, non-permeabilized cells, a property that has been leveraged to elicit therapeutic effects in both in vitro and in vivo models of ischemic stroke.⁴⁹ Like Ru360, the axial chloride ligands of Ru265 undergo aquation on the order of minutes under physiological conditions, indicating that the active inhibitor is the diaqua-capped species Ru265' (**Scheme 1**). Accordingly, mutagenesis studies on the DIME region of the MCU, a specific amino acid residue sequence that resides near the pore opening and is highly conserved among different species, as well as molecular docking studies, revealed Ru265' to bind at this site.^{40,41}

Scheme 1. Structures of MCU inhibitors.

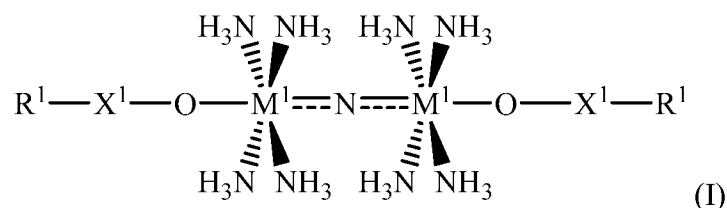


[0086] Given that the axial chloride ligands of Ru265 rapidly dissociate and that the resulting Ru265' is an active MCU inhibitor, modifications to the axial sites of this compound class afford an approach to develop prodrugs that possess improved properties for different biological applications. Specifically, the implementation of less labile axial ligands would yield compounds whose pharmacokinetic, cellular uptake, and targeting properties could be optimized.

Compounds

[0087] The present disclosure relates to compounds that inhibit mitochondrial calcium uniporter (MCU).

[0088] In one aspect, the disclosure is directed to a compound having a structure according to Formula I



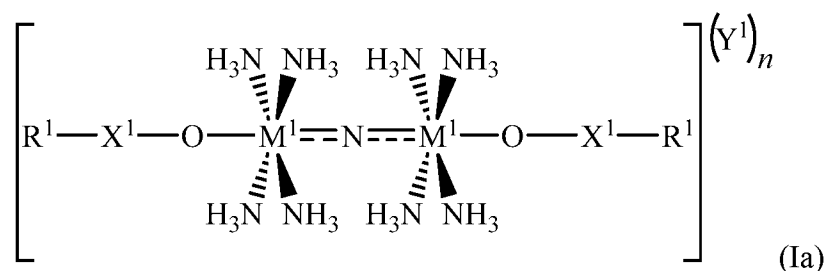
or a pharmaceutically acceptable salt and/or solvate thereof, wherein:

M^1 is Ru or Os;

X^1 is —C(O)— or —P(O)(OH)— ; and

R^1 is H, alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl.

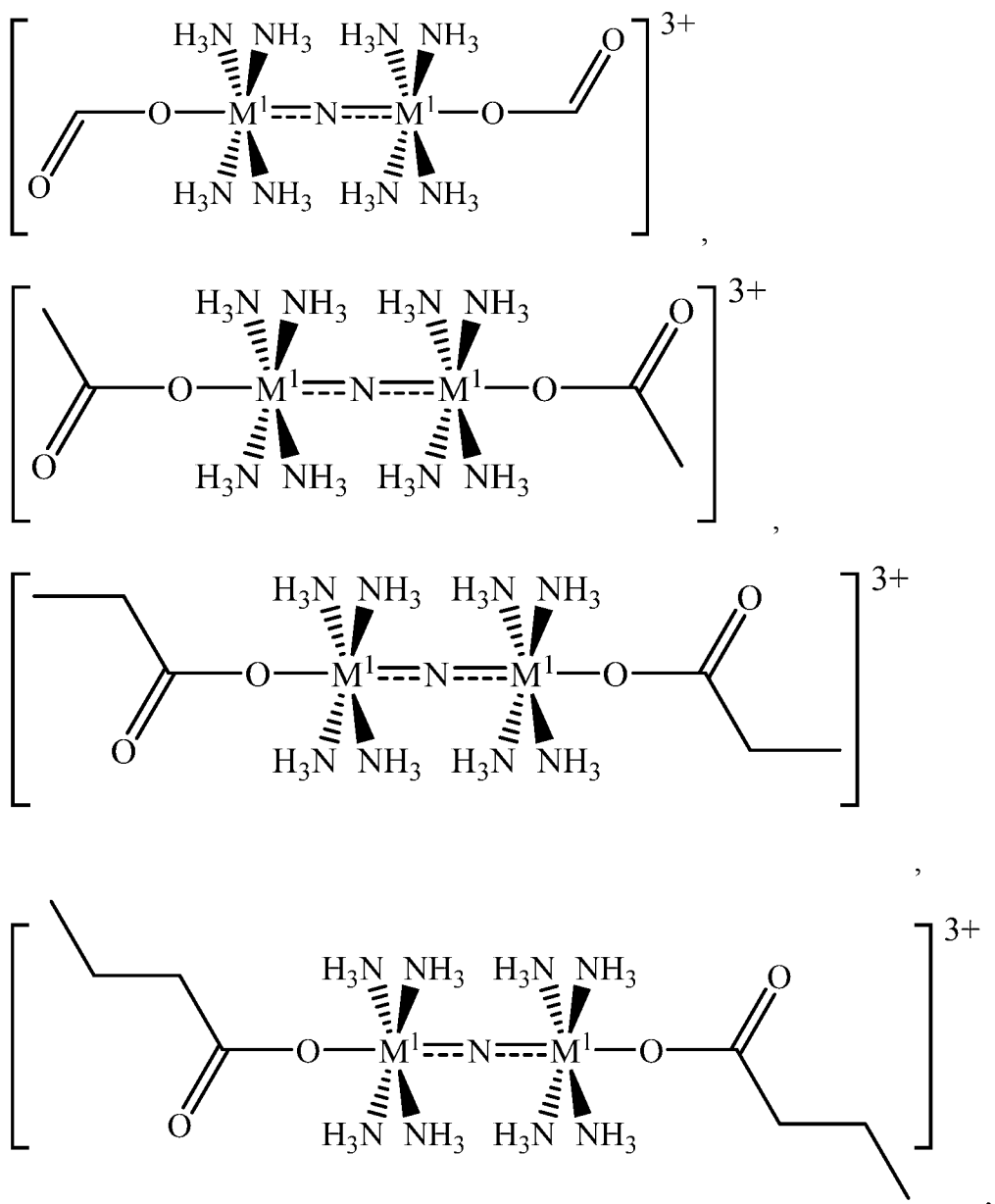
In any embodiment herein, the compound of Formula I may be a pharmaceutically acceptable salt according to Formula Ia

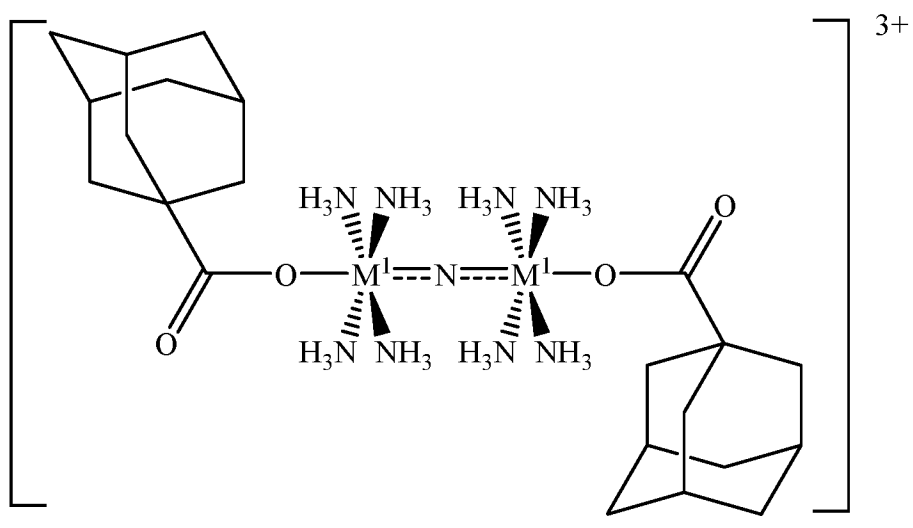
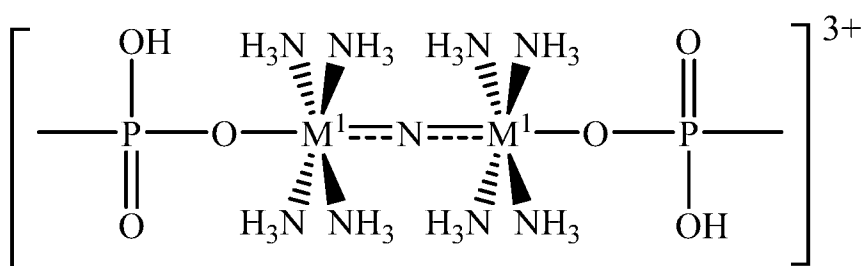
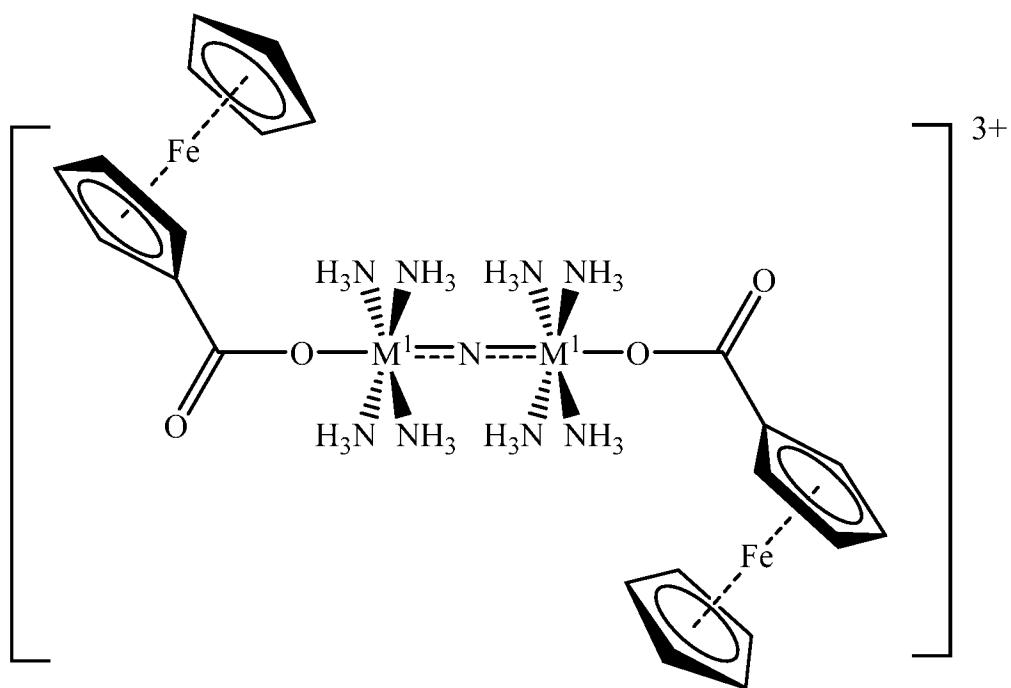


or solvate thereof, wherein Y^1 is an anion and n is 1, 2, or 3. In any embodiment herein, Y^1 may be a halide anion or a triflate anion. In any embodiment herein, Y^1 may be a triflate anion and n is 3.

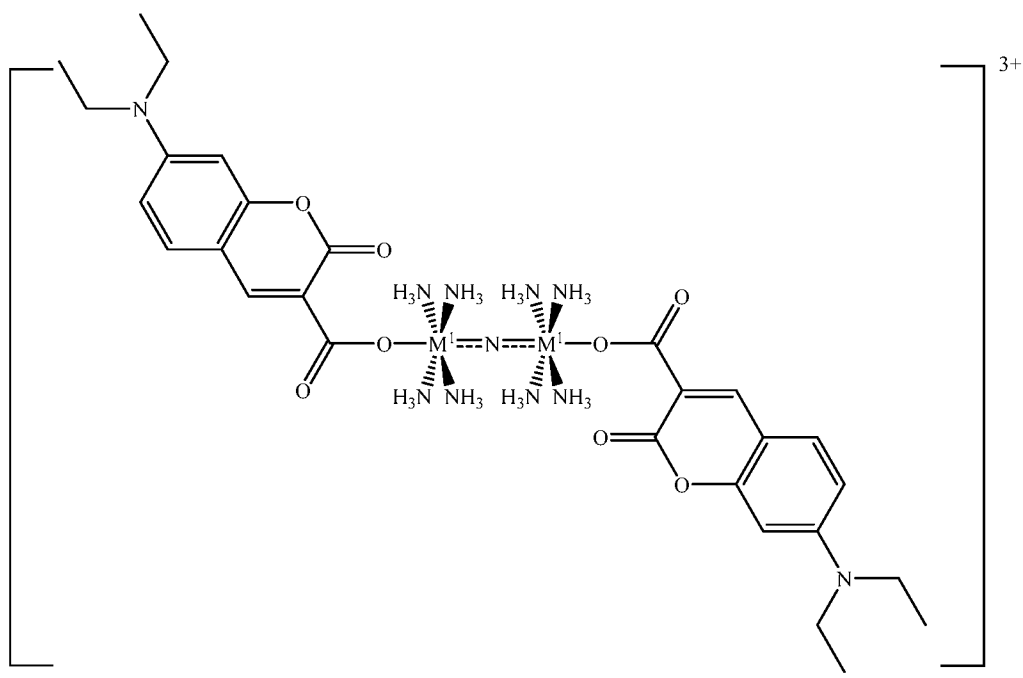
[0089] In one or more embodiments, X^1 is —C(O)— , and R^1 is H or alkyl. In one or more embodiments, X^1 is —P(O)(OH)— and R^1 is H alkyl. In one or more embodiments, R^1 is methyl, ethyl, or propyl. In one or more embodiments, X^1 is —C(O)— and R^1 is cycloalkyl. In one or more embodiments, R^1 is adamantyl.

[0090] In one or more embodiments, the compound is a pharmaceutically acceptable salt or solvate of a pharmaceutically acceptable salt of one of the following:





, or



wherein M^1 is Ru or Os.

[0091] In one or more embodiments, M^1 is Ru. In one or more embodiments, M^1 is Os.

[0092] The compounds described herein are suitable candidates for aquation-activated Ru265 prodrugs. Advantageously, the aquation kinetics of the compounds described herein are substantially slower than those of Ru265, a property that can be leveraged for a time-dependent increase in MCU-inhibitory activity.

Therapeutic Uses

[0093] The present disclosure provides methods for using compounds described herein in the preparation of a medicament for inhibiting MCU. As used herein, the terms “inhibit”, “inhibition” and the like refer to the ability of a compound to decrease the function or activity of a particular target, e.g., MCU. The decrease is preferably at least 50% and may be, for example, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%. The present disclosure also encompasses the use of the compounds described herein in the preparation of a medicament for the treatment of diseases, disorders, and/or conditions that would benefit from inhibition of MCU. As one example, the present disclosure encompasses the use of the compounds described herein in the preparation of a medicament for the treatment of a neurodegenerative disorder, a cardiovascular disease, a

metabolic disease, cancer, a skeletal muscle disease, cystic fibrosis, and/or ischemic reperfusion injury.

[0094] MCU is a transmembrane protein that allows the passage of calcium ions from a cell's cytosol into mitochondria. Its activity is regulated by MICU1 and MICU2, which together with the MCU make up the mitochondrial calcium uniporter complex. The MCU is one of the primary sources of mitochondria uptake of calcium, and flow is dependent on membrane potential of the inner mitochondrial membrane and the concentration of calcium in the cytosol relative to the concentration in the mitochondria. Balancing calcium concentration is necessary to increase the cell's energy supply and regulate cell death. Calcium is balanced through the MCU in conjunction with the sodium-calcium exchanger.

[0095] The dysregulation of mitochondrial calcium (mCa^{2+}) homeostasis has been implicated in several diseases such as, but not limited to, neurodegenerative disorders, cardiovascular diseases, metabolic diseases, cancer, skeletal muscle diseases, cystic fibrosis, and ischemic reperfusion injury. Examples of neurodegenerative disorders include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS) and cerebral ischemic stroke. Examples of cancer include breast cancer, pancreatic cancer, colorectal cancer, liver cancer, melanoma, ovarian cancer, and kidney cancer. A non-limiting example of a metabolic disease includes diabetes.

[0096] Diseases that exhibit a dysregulation of mitochondrial calcium (mCa^{2+}) homeostasis, such as diseases that are related to mitochondrial Ca^{2+} overload, would benefit from treatment using an MCU inhibitor. The compounds described herein act as inhibitors of MCU.

[0097] Accordingly, the compounds described herein may be used in a pharmaceutical composition that includes pharmaceutically acceptable carrier.

[0098] In some embodiments, a pharmaceutical composition for treating a disease or disorder related to mitochondrial Ca^{2+} overload comprises any one of the compounds described herein and a pharmaceutically acceptable excipient.

[0099] The compounds described herein may also be used in methods for treating a disease or disorder related to mitochondrial Ca^{2+} overload in a subject in need thereof. The

methods may include administering to the subject a therapeutically effective amount of a compound described herein.

[0100] In yet another embodiment, the compounds described herein may be used to inhibit mitochondrial calcium uniporter (MCU). The method may include administering to a subject an effective amount of a compound described herein.

Routes of Administration

[0101] In some embodiments, pharmaceutical compositions containing a compound according to this disclosure may be in a form suitable for oral administration. Oral administration may involve swallowing the formulation thereby allowing the compound to be absorbed into the bloodstream in the gastrointestinal tract. Alternatively, oral administration may involve buccal, lingual or sublingual administration, thereby allowing the compound to be absorbed into the blood stream through oral mucosa.

[0102] In another embodiment, the pharmaceutical compositions containing a compound according to this disclosure may be in a form suitable for parenteral administration. Forms of parenteral administration include, but are not limited to, intravenous, intraarterial, intramuscular, intradermal, intraperitoneal, intrathecal, intracisternal, intracerebral, intracerebroventricular, intraventricular, and subcutaneous. Pharmaceutical compositions suitable for parenteral administration may be formulated using suitable aqueous or non-aqueous carriers. Depot injections, which are generally administered subcutaneously or intramuscularly, may also be utilized to release the compounds disclosed herein over a defined period of time.

[0103] Other routes of administration are also contemplated by this disclosure, including, but not limited to, nasal, vaginal, intraocular, rectal, topical (e.g., transdermal), and inhalation.

Pharmaceutical Compositions

[0104] The compounds of the present disclosure may be in the form of compositions suitable for administration to a subject. In general, such compositions are pharmaceutical compositions comprising a compound according to this disclosure or a pharmaceutically acceptable salt thereof and one or more pharmaceutically acceptable excipients. In certain

embodiments, the compound may be present in an effective amount. The pharmaceutical compositions may be used in the methods of the present disclosure; thus, for example, the pharmaceutical compositions comprising a compound according to this disclosure can be administered to a subject in order to practice the therapeutic methods and uses described herein.

[0105] The pharmaceutical compositions of the present disclosure can be formulated to be compatible with the intended method or route of administration. Routes of administration may include those known in the art. Exemplary routes of administration are oral and parenteral. Furthermore, the pharmaceutical compositions may be used in combination with one or more other therapies described herein in order to treat or prevent the diseases, disorders and conditions as contemplated by the present disclosure. In one embodiment, one or more other therapeutic agents contemplated by this disclosure are included in the same pharmaceutical composition that comprises the compound according to this disclosure. In another embodiment, the one or more other therapeutic agents are in a composition that is separate from the pharmaceutical composition comprising the compound according to this disclosure.

[0106] In one aspect, the compounds described herein may be administered orally. Oral administration may be via, for example, capsule or tablets. In making the pharmaceutical compositions that include the compounds of the present disclosure (e.g., a compound of Formula I), or a pharmaceutically acceptable salt thereof, the tablet or capsule includes at least one pharmaceutically acceptable excipient. Non-limiting examples of pharmaceutically acceptable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, polyethylene glycol, cellulose, sterile water, syrup, and methyl cellulose. Additional pharmaceutically acceptable excipients include lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl and propylhydroxy-benzoates.

[0107] In another aspect, the compounds of the present disclosure, or a pharmaceutically acceptable salt thereof, may be administered parenterally, for example by intravenous injection. A pharmaceutical composition appropriate for parenteral administration may be formulated in solution for injection or may be reconstituted for injection in an appropriate system such as a physiological solution. Such solutions may

include sterile water for injection, salts, buffers, and tonicity excipients in amounts appropriate to achieve isotonicity with the appropriate physiology.

[0108] The pharmaceutical compositions described herein may be stored in an appropriate sterile container or containers. In some embodiments, the container is designed to maintain stability for the pharmaceutical composition over a given period of time.

Administering

[0109] In general, the disclosed methods comprise administering a compound described herein, or a composition thereof, in an effective amount to a subject in need thereof. An “effective amount” with reference to a MCU inhibitor of the present disclosure means an amount of the compound that is sufficient to engage the target (e.g., by inhibiting the target) at a level that is indicative of the potency of the compound. For MCU, target engagement can be determined by one or more biochemical or cellular assays resulting in an EC₅₀, ED₅₀, EC₉₀, IC₅₀, or similar value which can be used as one assessment of the potency of the compound. Assays for determining target engagement include, but are not limited to, those described in the Examples. The effective amount may be administered as a single quantity or as multiple, smaller quantities (e.g., as one tablet with “x” amount, as two tablets each with “x/2” amount, etc.).

[0110] In some embodiments, the disclosed methods comprise administering a therapeutically effective amount of a compound described herein to a subject in need thereof. As used herein, the phrase “therapeutically effective amount” with reference to compound disclosed herein means a dose regimen (i.e., amount and interval) of the compound that provides the specific pharmacological effect for which the compound is administered to a subject in need of such treatment. For prophylactic use, a therapeutically effective amount may be effective to eliminate or reduce the risk, lessen the severity, or delay the onset of the disease, including biochemical, histological and/or behavioral signs or symptoms of the disease. For treatment, a therapeutically effective amount may be effective to reduce, ameliorate, or eliminate one or more signs or symptoms associated with a disease, delay disease progression, prolong survival, decrease the dose of other medication(s) required to treat the disease, or a combination thereof. With respect to cancer specifically, a therapeutically effective amount may, for example, result in the killing of cancer cells, reduce cancer cell counts, reduce tumor burden, eliminate tumors or metastasis, or reduce metastatic

spread. A therapeutically effective amount may vary based on, for example, one or more of the following: the age and weight of the subject, the subject's overall health, the stage of the subject's disease, the route of administration, and prior or concomitant treatments.

[0111] Administration may comprise one or more (e.g., one, two, or three or more) dosing cycles.

[0112] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. Also within this disclosure are Arabic numerals referring to referenced citations, the full bibliographic details of which are provided subsequent to the relevant section. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the present technology.

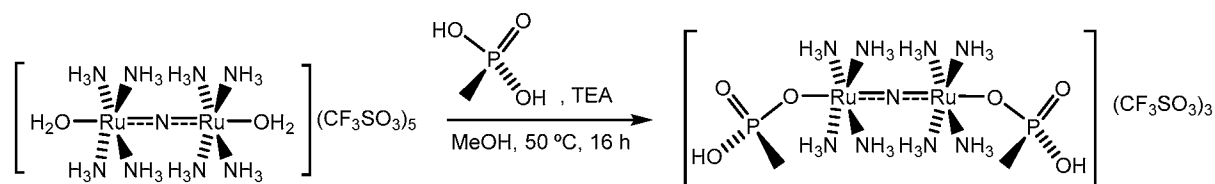
EXAMPLES

[0113] **Example 1: Axial Derivatives of Ru265 as Prodrugs of MCU Inhibition**

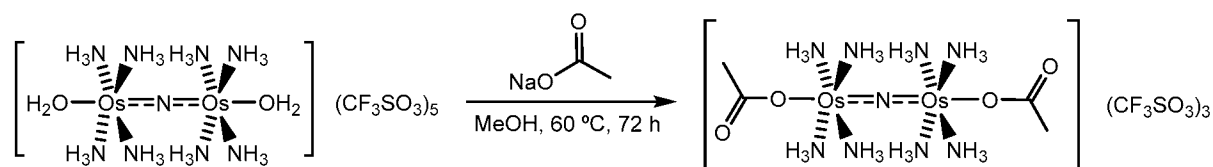
[0114] **Ru265-Phosphonate Complexes: Synthesis and Characterization**

[0115] *Synthesis of $[Ru_2(\mu-N)(NH_3)_8(OPO_2HCH_3)_2](CF_3SO_3)_3$*

[0116] Ru265' (40 mg) was dissolved in 5 mL methanol as a clear, orange solution. Methyl phosphonic acid (36 mg) was dissolved in 10 mL methanol as a clear, colorless solution. From this solution, 2 mL was added to the Ru265' solution as a clear, orange solution. Triethylamine (2 μ L) was added to the solution, and the resulting orange solution was heated for 18 h at 50 °C. The final solution was concentrated to dryness. The resulting yellow solid was washed with 3 $\times$ 3 mL tetrahydrofuran and 3 $\times$ 3 mL diethyl ether. Single, orange crystals of the product were grown by the slow evaporation of methanol over a period of 3 d (**FIG 3A**). Yield: 13 mg. ^1H NMR (500 MHz, DMSO- d_6) δ (ppm) = 3.93 (s, 24H), 1.13 (d, 6H), **FIG. 3B**. ^{31}P (202 MHz, DMSO- d_6) δ (ppm) = 30.18 (q), **FIG. 3C**.

Scheme 2. Synthesis of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{OPO}_2\text{HCH}_3)_2](\text{CF}_3\text{SO}_3)_3$.**[0117] Osmium Derivatives: Synthesis and Characterization****[0118]** *Synthesis of $[\text{Os}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CCH}_3)_2](\text{CF}_3\text{SO}_3)_3$*

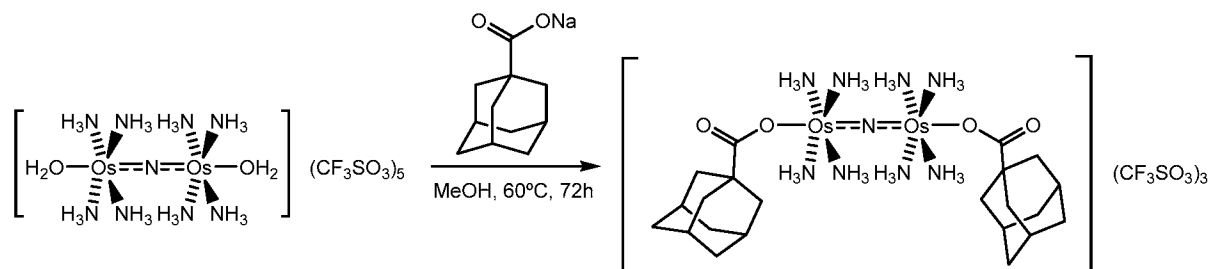
[0119] Os245' (20 mg, 0.015 mmol) was dissolved in 2 mL methanol as a clear, yellow solution. Sodium acetate (5 mg, 0.060 mmol) was dissolved in 3 mL methanol as a clear, colorless solution. Dropwise, the sodium acetate solution was added to the Os245' solution. The resulting clear, yellow solution was stirred at 60 °C for 72 h. The yellow solution was concentrated to dryness and redissolved in 1 mL H_2O . An insoluble, brown precipitate was filtered off, and the resulting yellow filtrate was concentrated to dryness. The resulting yellow powder was collected, washed with 1 mL each of cold water, ethanol, and diethyl ether, and dried in vacuo. Yield: 15.1 mg (0.016 mmol, 89.5%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm) = 4.87 (s, 24H), 1.75 (s, 6H), **FIG. 4**.

[0120] Scheme 3. Synthesis of $[\text{Os}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CCH}_3)_2](\text{CF}_3\text{SO}_3)_3$.**[0121]** *Synthesis of $[\text{Os}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CC}_{10}\text{H}_{16})_2](\text{CF}_3\text{SO}_3)_3$*

[0122] Os245' (20 mg, 0.015 mmol) was dissolved in 2 mL methanol as a clear, yellow solution. Sodium 1-adamantane carboxylate (6 mg, 0.030 mmol) was dissolved in 3 mL methanol as an opaque, white solution. Dropwise, the NaOAd solution was added to the Os245' solution. The clear, yellow solution was warmed to 60 °C and stirred for 72 h. The yellow solution was concentrated to dryness and redissolved in 5 mL THF. The solution was filtered through a 0.2 μm filter. Crystals were formed through the layering of cyclohexane over the THF solution (3:1 cyclohexane:THF) over 3 d. The resulting brownish yellow crystals were collected, washed with 1 mL each of cold water, ethanol, and diethyl ether, and

dried in vacuo for 6 h. Yield: 16.8 mg (0.0126 mmol, 82.6%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ (ppm) = 4.74 (s, 24H), 1.91 (s, 6H), 1.70 (s, 12H), 1.63 (m, 12H), **FIG. 5**.

Scheme 4. Synthesis of $[\text{Os}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CC}_{10}\text{H}_{16})_2](\text{CF}_3\text{SO}_3)_3$.



[0123] Example 2: Carboxylate-Capped Analogues of Ru265 as MCU Inhibitor Prodrugs

[0124] Experimental Section

[0125] Reagents and Materials

[0126] All reagents were obtained commercially and used without further purification. Ru265 was prepared as previously described.⁴⁰ Water ($18\text{ M}\Omega\cdot\text{cm}$) was purified using an ELGA PURELAB flex 2 (High Wycombe, UK).

[0127] Physical Measurements

[0128] 1D-NMR (^1H and $^{13}\text{C}\{^1\text{H}\}$) spectra were acquired at 25°C on a 500 MHz Bruker AV 3HD spectrometer equipped with a broadband Prodigy cryoprobe (Bruker, Billerica, MA). UV-vis spectra were acquired using a Shimadzu UV-1900 spectrophotometer (Shimadzu, Kyoto, Japan) fitted with a temperature-controlled circulating water bath. Elemental analyses (C, H, N) were carried out by Atlantic Microlab Inc. (Norcross, GA). Fluorescence and absorbance of samples in 96-well plates were measured using a BioTek Synergy HT plate reader (Winooski, VT). Graphite furnace atomic absorption spectroscopy (GFAAS) was performed using a PinAAcle 900Z spectrometer (Perkin Elmer, Waltham, MA). Standardized solutions ($0\text{--}200\text{ }\mu\text{g/L}$) of ruthenium were used to generate a calibration curve. The concentrations of all ruthenium stock solutions applied in analytical and biological experiments was verified by GFAAS. Statistical analyses were performed using GraphPad Prism version 9.4.1 by applying a non-paired student's t-test. Curve fitting was also performed using GraphPad Prism version 9.4.1.

[0129] Cell Lines and Culture Conditions

[0130] HeLa and HEK293T cells were obtained from American Type Culture Collection (ATCC, Washington DC) and cultured at 37 °C as adherent monolayers in a humidified atmosphere containing 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, L-glutamine, and 3.7 g/L sodium bicarbonate supplemented with 10% fetal bovine serum (FBS). Cells were tested for mycoplasma contamination bimonthly through the commercial service provided by the College of Veterinary Medicine at Cornell University. All reagents and solutions used in biological studies were sterile filtered through a 0.2 µm filter and maintained under sterile conditions.

[0131] Synthesis of [Ru₂(µ-N)(NH₃)₈(O₂CH)₂](NO₃)₃ (Compound 1)

[0132] Ru265 (100 mg, 0.189 mmol) was dissolved in 15 mL 18 MΩ·cm H₂O. and solid AgNO₃ (160 mg, 0.950 mmol; AESAR) was added to this solution. The reaction mixture was stirred at 50 °C in the dark for 12 h. The resulting cloudy, orange suspension was filtered through celite to remove the precipitated AgCl. The orange filtrate was then treated with solid sodium formate (26 mg, 0.378 mmol; Fluka Chemical Corporation) and stirred at 50 °C for 12 h. After cooling to room temperature, the orange solution was concentrated to dryness under vacuum, leaving an orange solid. This solid was dissolved in 1 mL 10% formic acid in water. Orange crystals of the desired compound were obtained by the vapor diffusion of *p*-dioxane into this solution over the course of 3 d at room temperature. The mother liquor was decanted, and the remaining orange crystals were washed sequentially with 15 mL ethanol, 15 mL methanol, and 15 mL acetone, before drying under vacuum. Yield: 29.7 mg (0.047 mmol, 25%). ¹H NMR (500 MHz, DMSO-d₆) δ (ppm) = 4.01 (s, 24H), 8.12 (s, 2H). ¹³C{¹H} NMR (126 MHz, DMSO-d₆) δ (ppm) = 169.61. IR (ATR) ν (cm⁻¹) = 3295 (s, br), 1606 (m), 1384 (s), 1063 (m), 830 (m). Elemental analysis: calc'd (% for C₂H₂₆N₁₂O₁₃Ru₂·3.5H₂O) C 3.47; H 4.81; N 24.31. Found (%) C 3.74; H 4.72; N 24.11.

[0133] Synthesis of [Ru₂(µ-N)(NH₃)₈(O₂CCH₃)₂](NO₃)₃ (Compound 2)

[0134] The synthesis of **2** was carried out following the procedure described for **Compound 1**, using the same quantities of Ru265 and AgNO₃. In place of sodium formate, sodium acetate (31 mg, 0.378 mmol; Fluka Chemical Corporation) was used, and the crystallization employed 10% acetic acid instead of 10% formic acid. Yield: 26.0 mg (0.040

mmol, 21%). ^1H NMR (500 MHz, DMSO- d_6) δ (ppm) = 3.95 (s, 24H), 1.77 (s, 6H). $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, DMSO- d_6) δ (ppm) = 178.78, 24.98. IR (ATR) ν (cm^{-1}) = 3295 (s, br), 1575 (m), 1384 (s), 1040 (m), 830 (m). Elemental analysis: calc'd (% for $\text{C}_4\text{H}_{30}\text{N}_{12}\text{O}_{13}\text{Ru}_2 \cdot \text{H}_2\text{O}$) C 7.12; H 4.78; N 24.92. Found (%) C 7.09; H 4.65; N 24.71.

[0135] Synthesis of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{CCH}_2\text{CH}_3)_2](\text{NO}_3)_3$ (Compound 3)

[0136] The synthesis of **Compound 3** was carried out following the procedure described for **Compound 1**, using the same quantities of Ru265 and AgNO_3 . In place of sodium formate, sodium propionate (36 mg, 0.378 mmol; AESAR) was used, and the crystallization employed 10% propanoic acid instead of 10% formic acid. Yield: 18.1 mg (0.026 mmol, 14%). ^1H NMR (500 MHz, DMSO- d_6) δ (ppm) = 3.96 (s, 24H), 2.05 (q, 4H), 0.92 (t, 6H). $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, DMSO- d_6) δ (ppm) = 181.74, 30.33, 10.06. IR (ATR) ν (cm^{-1}) = 3295 (s, br), 1565 (m), 1384 (s), 1048 (m), 830 (m). Elemental analysis: calc'd (% for $\text{C}_6\text{H}_{34}\text{N}_{12}\text{O}_{13}\text{Ru}_2 \cdot 1.5\text{H}_2\text{O}$) C 10.13; H 5.24; N 23.62. Found (%) C 10.09; H 4.81; N 23.72.

[0137] Synthesis of $[\text{Ru}_2(\mu\text{-N})(\text{NH}_3)_8(\text{O}_2\text{C}(\text{CH}_2)_2\text{CH}_3)_2](\text{OSO}_2\text{CF}_3)_3$ (Compound 4)

[0138] Ru265 (100 mg, 0.189 mmol) was dissolved in 15 mL 18 M $\Omega \cdot \text{cm}$ H_2O . Solid $\text{AgOSO}_2\text{CF}_3$ (243 mg, 0.950 mmol; Aldrich Chemical Company) was added, and reaction mixture was stirred at reflux for 3 h in the dark. Insoluble AgCl was removed by filtration through celite, and sodium butyrate (41 mg, 0.378 mmol; Aldrich Chemical Company) was added to the orange filtrate, which was then stirred at 50 $^\circ\text{C}$ for 12 h. The orange solution was cooled to room temperature and concentrated to dryness under vacuum to yield an orange solid. This solid was dissolved in 1 mL of methanol and crystallized by allowing diethyl ether to vapor diffuse into this solution at room temperature over the course of 2 d. The resulting orange crystals were washed sequentially with 15 mL acetone and 15 mL diethyl ether, and then dried under vacuum. Yield: 14.7 mg (0.015 mmol, 8%). ^1H NMR (500 MHz, DMSO- d_6) δ (ppm) = 3.92 (s, 24H), 2.01 (t, 4H), 1.45 (q, 4H), 0.83 (t, 6H). $^{13}\text{C}\{^1\text{H}\}$ NMR (126 MHz, DMSO- d_6) δ (ppm) = 163.24, 20.32, 18.67, 13.94. ^{19}F NMR (470 MHz, DMSO- d_6) δ (ppm) = -77.75. IR (ATR) ν (cm^{-1}) = 3295 (m, br), 1603 (m), 1253 (s), 1170 (s), 1033 (m), 830 (w), 632 (m), 516 (w). Elemental analysis: calc'd (% for $\text{C}_{11}\text{H}_{38}\text{N}_9\text{O}_{13}\text{F}_9\text{S}_3\text{Ru}_2 \cdot \text{CH}_3\text{OH}$) C 14.33; H 4.21; N 12.53. Found (%) C 14.09; H 4.28; N 12.30.

[0139] X-Ray Crystallography

[0140] Single crystals of **Compound 3** were obtained via the vapor diffusion of *p*-dioxane into aqueous solutions of the compound in 10% propionic acid at room temperature. **Compound 1** was prepared as triflate salts, and single crystals were obtained via the vapor diffusion of diethyl ether into a methanolic solution of **Compound 1** at room temperature. Low-temperature X-ray diffraction data for **Compound 1** and **Compound 3** were collected on a Rigaku XtaLAB Synergy diffractometer coupled to a Rigaku Hypix detector with either Mo K α radiation ($\lambda = 0.71073$ Å) or Cu K α radiation ($\lambda = 1.54184$ Å), from PhotonJet micro-focus X-ray sources at 100 K. The diffraction images were processed and scaled using the CrysAlisPro software (Rigaku Oxford Diffraction, The Woodlands TX). The structures were solved through intrinsic phasing using SHELXT⁷⁶ and refined against F² on all data by full matrix least squares with SHELXL⁷⁷ following established refinement strategies.⁷⁸ All non-hydrogen atoms were refined anisotropically. All hydrogen atoms bound to carbon were included in the model at geometrically calculated positions and refined using a riding model. Hydrogen atoms bound to oxygen were located in the difference Fourier synthesis and subsequently refined semi-freely with the help of distance restraints. The isotropic displacement parameters of all hydrogen atoms were fixed to 1.2 times the U_{eq} value of the atoms they are linked to (1.5 times for methyl groups). **Compound 1** crystallized in the space group *P*2₁/c; the crystal is twinned and the structure was refined using an HKLF 5 file containing data for two twin domains, with a refined BASF of 0.1824(8). Details of the data quality and a summary of the residual values of the refinements are listed in **Tables 1-3**.

[0141] NMR Kinetics Studies

[0142] Aqueous solutions were prepared to contain 400 μ M complex, 40 mM MOPS buffer (pH 7.4), 10% D₂O for NMR field-locking, and 0.1% *p*-dioxane as an internal reference ($\delta = 3.75$ ppm in D₂O). All data was analyzed as described in the main text using Mestrenova processing software.

[0143] Cytotoxicity Assay

[0144] HeLa and HEK293T cells were seeded in 96-well plates with ~4000 cells/well and incubated overnight. On the following day, the culture media was removed, and cells were treated with media containing varying concentrations of the test complex and incubated for 48 h. The cells were then incubated in DMEM containing 1 mg/mL MTT without FBS for

3 h. Following incubation, the media was removed, and the purple formazan crystals were dissolved in 200 μ L of an 8:1 DMSO/glycine buffer (pH 10) mixture. The absorbance at 570 nm of each well was measured using a BioTek Synergy HT plate reader. The average absorbance of control cells was set to 100% viability, and the average absorbances of treated cells were normalized to the control absorbance. Data were plotted as percent viability versus the log[concentration]. The Hill Equation was applied to the data to determine the half maximal inhibitory concentration (IC_{50}). Data are reported as the average of three independent biological replicates $\pm$ SD.

[0145] Mitochondrial Membrane Potential via JC-1 Assay

[0146] Approximately 1×10^5 HeLa cells were seeded in 35 mm glass-bottomed dishes (MatTek Life Sciences, Ashland, MA) and incubated overnight at 37 °C. On the next day, cells were treated with the desired complex (50 μ M) and incubated for an additional 24 h at 37 °C. The culture media was then removed and replaced with fresh media supplemented with 10 μ M JC-1 dye, followed by incubation in the dark for 30 min at 37 °C. The dye-containing media was removed, and the cells were washed with 2×1 mL phosphate buffered saline (PBS, Corning Life Sciences). The cells were imaged in 1 mL PBS. Control dishes were handled identically to treated dishes. For the positive control dishes, 50 μ M carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) in PBS was used, and the images were collected without the removal of CCCP. The cells were imaged using an EVOS M5000 fluorescence microscope (ThermoFisher, Waltham, MA) with a green fluorescence protein (GFP) filter cube (ex. 457-487/em. 502-538) for the green monomer fluorescence and a Texas red filter cube (ex. 542-582/em. 604-644) for the red J-aggregate fluorescence. The cellular images were analyzed using ImageJ (NIH) and the corrected total cellular fluorescence (CTCF) was calculated using the following formula:

$$CTCF = \text{Integrated density} - (\text{area of cell} \times \text{mean fluorescence of background reading})$$

[0147] For each replicate, the average red/green fluorescence was determined using at least 8 independent cells and was normalized to untreated control cells ($[\text{red/green}]_{\text{control}} = 1$). Data are reported as the average of three independent trials $\pm$ SD.

[0148] Cell Uptake

[0149] HeLa and HEK293T cells were grown to near confluence in 6-well plates. On the day of the experiment, the culture media was removed, and the cells were treated with fresh media containing 0 or 50 μM complex and incubated for 2 h at 37 °C. The culture media was then removed, and the adherent cells were washed with $3 \times 1 \text{ mL}$ of phosphate buffered saline (PBS; Corning Life Sciences), detached with trypsin, and then pelleted by centrifugation ($800 \times g$ for 10 min). The cell pellet was suspended in ice cold lysis buffer (1% w/v 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM EDTA, 50 mM tris(hydroxymethyl)aminomethane (Tris) and 100 mM NaCl; pH = 7.4). The suspension was vortexed for 30 s and incubated on ice for 45 min. The cell lysate was centrifuged to remove precipitated debris and the supernatant was transferred to a clean tube prior to analysis. The Ru content of the lysate was determined using GFAAS and was normalized to the protein content of the sample, which was determined using the bicinchoninic acid (BCA) assay kit following manufacturer instructions (ThermoFisher). Results are reported as the average mass ratio of Ru to protein (pg/ μg) in each sample $\pm$ SD.

[0150] Role of Organic Cation Transporters (OCT) in Cellular Uptake

[0151] Approximately 1×10^5 HeLa cells were seeded in 6-well plates and incubated at 37 °C overnight. On the next day, the culture media was removed, and the cells were treated with fresh media supplemented with the test complex (50 μM) and the OCT3 inhibitor decynium-22 (1 μM). The cells were incubated for 2 h before they were washed, harvested, lysed, and analyzed as described above. Results are reported as the average mass ratio of Ru to protein (pg/ μg) in each sample $\pm$ SD.

[0152] Mitochondrial Ca^{2+} Uptake in Permeabilized HEK293T Cells

[0153] HEK293T cells were grown to near confluency in a 10 cm^2 dish and harvested used 0.05% trypsin + 0.53 mM ethylenediaminetetraacetic acid (EDTA; Corning Life Sciences). The cells were pelleted by centrifugation, suspended in cold PBS supplemented with 5 mM EDTA (pH 7.4), and counted using trypan blue. The cells were pelleted by centrifugation at $800 \times g$ for 5 minutes and resuspended in ice cold KCl solution (125 mM KCl, 20 mM HEPES, 2 mM K_2HPO_4 , 5 mM glutamate, 5 mM malate, 1 mM MgCl_2 , pH 7.2 with KOH) supplemented with 80 μM digitonin and 1 μM thapsigargin. The final solution contained <0.1% DMSO, originating from the digitonin and thapsigargin stocks. The cells were incubated on ice for 15 min and centrifuged at $200 \times g$ for 10 minutes at 4°C. The

pelleted cells were then resuspended in high KCl solution containing 1 μM Calcium Green 5N (ThermoFisher, Waltham, MA) and 2 mM succinate to a final density of 1×10^7 cells mL^{-1} . For each experiment, 100 μL of the cell suspension was placed in each well of a black-walled 96-well plate, treated with the desired concentration of the test complex, and allowed to equilibrate at room temperature for ~ 200 s. The background fluorescence of each well was recorded for 60 s prior to addition of 20 μM CaCl_2 . The change in fluorescence of the dye (ex. 488/em. 528) in response to Ca^{2+} was recorded every 5 s for at least 120 s or until the fluorescence returned to the baseline. The mitochondrial Ca^{2+} uptake rate was calculated as the time constant in the exponential fit of the fluorescence response curve. Control cells that were not treated with compound were handled identically to the treated cells to account for different incubation lengths. The Ca^{2+} uptake rate of treated cells was normalized to that of the control cells (0% inhibition), and each replicate was performed using independently prepared cell suspensions to account for differences in cell count. A bicinchoninic acid (BCA) assay was performed on each cell suspension for every experiment, giving a protein content of ~ 1200 $\mu\text{g mL}^{-1}$ each time. The Hill Equation was used to determine the IC_{50} of MCU inhibition. Data are presented as the average of three independent biological replicates $\pm$ SD.

[0154] Time-Dependent Mitochondrial Ca^{2+} Uptake Experiments

[0155] Aqueous solutions of 400 μM complex were prepared in 40 mM MOPS buffer (pH 7.4; 100-fold excess buffer). The solutions were incubated at 37 $^{\circ}\text{C}$ for 1, 3, and 6 h before being immediately frozen in liquid nitrogen. Each solution was stored at -80 $^{\circ}\text{C}$ until the mitochondrial Ca^{2+} uptake experiments were performed. Each stock solution was then diluted in 18 $\text{M}\Omega\cdot\text{cm}$ H_2O to a final concentration of 10 nM complex in the 96-well plates (200 nM complex before dilution with cell suspension). The mitochondrial Ca^{2+} uptake assay was performed as described above with a concentration of 1×10^7 HEK293T cells mL^{-1} . Data were analyzed as described above and presented as the average mitochondrial Ca^{2+} uptake rate of three independent biological replicates $\pm$ SD.

[0156] Mitochondrial Ca^{2+} Uptake in Intact HeLa Cells using Rhod2AM

[0157] Approximately 5×10^4 HeLa cells were seeded in an 8-well μ -slide (Ibidi USA, Inc., Fitchburg, WI) and incubated overnight at 37 $^{\circ}\text{C}$. The following day, cells were treated with the desired metal complex (50 μM) in DMEM supplemented with 10% FBS for

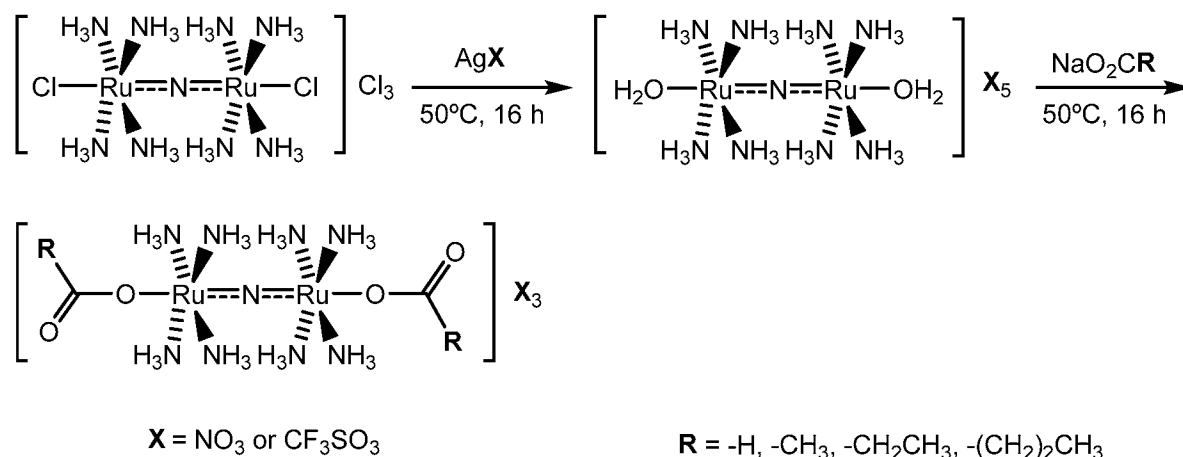
1 h at 37 °C. The culture media was removed, and the cells were washed with 1 × 1 mL PBS before the cells were incubated in extracellular medium (ECM; 135 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂) supplemented with 10 mM glucose, 3.2 mg mL⁻¹ bovine serum albumin (BSA), 0.003% Pluronic F127, and 2 μM Rhod2AM (Molecular Probes) in the dark for 30 min at room temperature. The ECM was then removed, the cells were washed with 1 × 1 mL ECM, and the cells were treated with fresh ECM supplemented with 10 mM glucose and 3.2 mg/mL BSA and incubated for an additional 30 min in the dark at room temperature. The buffer was then removed, and the cells washed with 1 × 1 mL ECM and treated with ECM supplemented with 10 mM glucose and 3.2 mg/mL BSA. The cells were incubated for 15 min at 37 °C before imaging using a Zeiss LSM i710 confocal microscope using a 40× oil objective with an excitation of 561 nm and an emission window of 568-712 nm. After ~ 30 s of baseline recording, histamine (100 μM) was added to the dish and fluorescence images were collected every 3 s to monitor *m*Ca²⁺ uptake. Images were analyzed and quantified using ImageJ and the CTCF was calculated. The average of at least five individual cells were used to determine the average CTCF for each replicate. Results are reported as the average of two independent replicates ± SD.

[0158] Results and Discussion

[0159] Synthesis and Characterization

[0160] To explore the potential of carboxylates as axial ligands for Ru265 prodrugs, the simple smallest carbon chain acids, formate, acetate, propionate, and butyrate, were investigated.

Scheme 5. Synthetic scheme for **Compound 1**, **Compound 2**, **Compound 3**, and **Compound 4**. **Compound 1**, **Compound 2**, and **Compound 3** were synthesized as nitrate salts, while **Compound 4** was synthesized as a triflate salt.



[0161] The general two-step syntheses required to access the formate (**Compound 1**), acetate (**Compound 2**), propionate (**Compound 3**), and butyrate (**Compound 4**) complexes is presented in **Scheme 5**. The reaction was commenced from Ru265 by adding five equiv of either silver nitrate (**Compounds 1–3**) or silver trifluoromethanesulfonate (triflate) (**Compound 4**) at 50 °C for 16 h in water to remove both the inner- and outer-sphere chlorides as insoluble silver chloride. The resulting aqueous solution of Ru265' was then treated with two equiv of the sodium salt of the desired carboxylate, and then allowed to heat at 50 °C for 16 h. The nitrate salts of **Compounds 1–3** were crystallized by the vapor diffusion of p-dioxane into an aqueous solution of the compounds containing 10% of their corresponding carboxylic acid to prevent aquation. For the triflate salt **Compound 4**, vapor diffusion of diethyl ether into a methanolic solution of the crude compound afforded analytically pure material.

[0162] The infrared (IR) spectra present key diagnostic energies. For **Compounds 1–3**, the characteristic energies of the nitrate counter-anion can be observed around 1600 and 1350 cm⁻¹. **Compound 4** shows the characteristic energies of its triflate counter-anion, observed at 1269, 1223, 1145, and 637 cm⁻¹. Each complex demonstrates N–H stretching modes belonging to the primary amine groups near 3400 cm⁻¹. The diagnostic stretching mode of these complexes is that of the asymmetric Ru–N–Ru stretch. For **Compounds 2–4**, this mode is observed at an energy of 1039, 1040, and 1037 cm⁻¹, respectively. **Compound 1** observes this mode at a slightly higher energy of 1064 cm⁻¹. This difference is attributed to the trans-influence of the axial ligands. Given that formate is a much weaker σ-donating ligand (as evidenced by its lower p*K_a*) than the other carboxylates, its weaker Ru–O bond would give rise to a stronger Ru–N bond. The presence of each ligand is confirmed by the C=O stretches, observed as shoulders in all spectra near 1600 cm⁻¹ and 1400 cm⁻¹.

[0163] An analysis of the ^1H nuclear magnetic resonance (NMR) spectra of each of these complexes demonstrate changes in the chemical shifts of the aliphatic region and the equatorial amine ligands. Ru265 has a single chemical shift of 4.15 ppm,⁴⁰ characteristic of its amine ligands. This peak remains in each of **Compounds 1–4**, but the chemical shift moves upfield to chemical shifts near 3.95 ppm. This result is consistent with Ru265', which exhibits an equatorial amine chemical shift at 3.96 ppm. Oxygen-donating ligands, therefore, cause a shift of this peak upfield in the ^1H NMR spectra. Additionally, the chemical shifts of the carboxylate ligands showed a shift upfield when bound to the Ru center of these molecules by about 0.2 ppm. ^{13}C NMR shows the relevant peaks for these carboxylate ligands, and ^{19}F NMR of **Compound 4** is consistent with the presence of triflate counter-anions.

[0164] X-Ray Crystallography

[0165] Via the crystallization methods described above, single crystals of **Compound 3** suitable for X-ray diffraction were obtained. For **Compound 1**, this compound was first converted to the triflate salt and then crystallized from methanol by the vapor diffusion of diethyl ether. The resulting crystal structures are shown in **FIGs. 6A–6B**, crystallographic data collection and refinement data are shown in **Table 1**, and selected interatomic distances and angles are collected in **Tables 1** and **2**, respectively.

Table 1. X-ray crystal data and structure refinement details for **Compound 1** and **Compound 3**.

Compound	1	3
Empirical Formula	$\text{C}_6\text{H}_{29}\text{F}_9\text{N}_9\text{O}_{14}\text{Ru}_2\text{S}_3$	$\text{C}_6\text{H}_{36}\text{N}_{12}\text{O}_{14}\text{Ru}_2$
Formula Weight	920.70	702.57
a (Å)	25.0552(2)	10.41150(10)
b (Å)	10.35730(10)	12.02830(10)
c (Å)	24.9114(3)	20.3198(2)
α (°)	90	90
β (°)	100.8730(10)	104.6280(10)
γ (°)	90	90
V (Å ³)	6348.56(11)	2462.22(4)
Z	8	4
Crystal System	Monoclinic	Monoclinic
Space Group	$P2_1/c$	$P2_1/n$
ρ_{calc} (Mg/m ³)	1.927	1.895
μ (Cu $K\alpha$ /Mo $K\alpha$), mm ⁻¹	10.681	1.310
T (K)	100.0(8)	100.00(10)
2θ range (°)	3.593 – 77.525	2.072 – 26.022
Independent Reflections	27170	4846

R _{int}	0.079	0.0286
Number of Parameters	795	482
Largest diff. peak and hole	2.203/-2.018	1.717/-0.737
GoF	1.041	1.165
R1/wR2 (all data)	0.0896/0.2330	0.0343/0.0866
R1/wR2 (>2 θ)	0.0820/0.2260	0.0333/0.0860

Cu K α λ = 1.54184 Å; Mo K α λ = 0.7073 Å

$R_1 = \Sigma ||F_o| - |F_c|| / \Sigma |F_o|$; $wR2 = \{ \Sigma [w(F_o^2 - F_c^2)^2] / \Sigma (F_o^2)^2 \}^{1/2}$

GoF = $\{ \Sigma [w(F_o^2 - F_c^2)^2] / (n-p) \}^{1/2}$, where n is the number of data and p is the number of refined parameters

Table 2. Selected interatomic distances and angles for **Compound 1** and **Compound 3**.

Selected Interatomic Distances (Å) for Compound 1			Selected Interatomic Distances (Å) for Compound 3		
Ru(1)–O(2)	2.110(6)	Ru(2)–O(3)	2.080(8)	Ru(1)–O(2)	Ru(2)–O(3)
Ru(1)–N(1)	1.756(9)	Ru(2)–N(1)	1.730(9)	Ru(1)–N(1)	Ru(2)–N(1)
Ru(1)–N(2)	2.105(7)	Ru(2)–N(6)	2.117(9)	Ru(1)–N(2)	Ru(2)–N(6)
Ru(1)–N(3)	2.110(7)	Ru(2)–N(7)	2.122(8)	Ru(1)–N(3)	Ru(2)–N(7)
Ru(1)–N(4)	2.107(8)	Ru(2)–N(8)	2.107(8)	Ru(1)–N(4)	Ru(2)–N(8)
Ru(1)–N(5)	2.122(7)	Ru(2)–N(9)	2.117(8)	Ru(1)–N(5)	Ru(2)–N(9)
Selected Bond Angles (°) for Compound 1			Selected Bond Angles (°) for Compound 3		
N(1)–Ru(1)–O(2)	175.9(3)	N(1)–Ru(2)–O(3)	176.0(4)	N(1)–Ru(1)–O(2)	177.49(12)
N(1)–Ru(1)–N(2)	96.1(4)	N(1)–Ru(2)–N(6)	94.6(4)	N(1)–Ru(1)–N(2)	95.28(12)
N(1)–Ru(1)–N(3)	95.0(3)	N(1)–Ru(2)–N(7)	96.0(4)	N(1)–Ru(1)–N(3)	95.96(13)
N(1)–Ru(1)–N(4)	93.4(3)	N(1)–Ru(2)–N(8)	96.0(4)	N(1)–Ru(1)–N(4)	93.08(13)
N(1)–Ru(1)–N(5)	93.8(3)	N(1)–Ru(2)–N(9)	92.3(3)	N(1)–Ru(1)–N(5)	95.09(12)
N(2)–Ru(1)–N(3)	87.3(3)	N(6)–Ru(2)–N(7)	89.7(4)	N(2)–Ru(1)–N(3)	89.92(13)
N(2)–Ru(1)–N(4)	170.4(3)	N(6)–Ru(2)–N(8)	169.5(4)	N(2)–Ru(1)–N(4)	89.95(13)
N(2)–Ru(1)–N(5)	90.0(3)	N(6)–Ru(2)–N(9)	91.4(4)	N(2)–Ru(1)–N(5)	169.61(12)
N(2)–Ru(1)–O(2)	87.2(3)	N(6)–Ru(2)–O(3)	85.9(4)	N(2)–Ru(1)–O(2)	86.50(11)
N(3)–Ru(1)–N(4)	90.7(3)	N(7)–Ru(2)–N(8)	89.8(4)	N(3)–Ru(1)–N(4)	170.94(12)
N(3)–Ru(1)–N(5)	171.0(3)	N(7)–Ru(2)–N(9)	171.6(4)	N(3)–Ru(1)–N(5)	89.51(12)
N(3)–Ru(1)–O(2)	82.8(3)	N(7)–Ru(2)–O(3)	88.0(4)	N(3)–Ru(1)–O(2)	82.25(11)
N(4)–Ru(1)–N(5)	90.6(3)	N(8)–Ru(2)–N(9)	87.6(3)	N(4)–Ru(1)–N(5)	88.99(12)
N(4)–Ru(1)–O(2)	83.2(3)	N(8)–Ru(2)–O(3)	83.5(4)	N(4)–Ru(1)–O(2)	88.70(11)
N(5)–Ru(1)–O(2)	88.5(3)	N(9)–Ru(2)–O(3)	83.7(4)	N(5)–Ru(1)–O(2)	83.15(11)
Ru(1)–N(1)–Ru(2)	176.5(5)			Ru(1)–N(1)–Ru(2)	175.43(19)

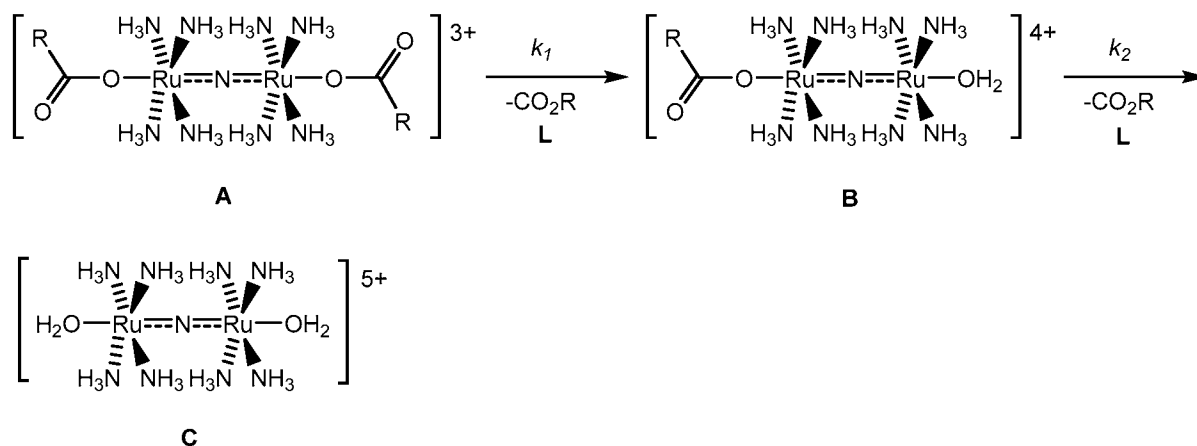
Atoms are labelled as shown in **Figures 6A–6B**. Values in parentheses indicate the standard uncertainty in the last significant figure.

[0166] Both crystal structures reveal the linear Ru(μ -N)Ru core to remain intact with equatorial positions completed by the NH₃ ligands and the axial positions to be occupied by the corresponding carboxylates. The linearity of this motif is reflected by Ru–N–Ru angles are 176.5° and 175.4° for **1** and **3**, respectively. In addition, the Ru–N distances with the bridging nitrido range from 1.745 Å to 1.756 Å in **Compound 1** and **Compound 3**. These values are notably shorter than the Ru–N distances for the equatorial NH₃ ligands, which span 2.089 Å to 2.122 Å. The shorter Ru – N distance with the bridging nitrido is consistent with the multiple bonding character of this interaction. These values are consistent with those of both Ru265 and Ru265', for which the bridging Ru–N distances are 1.742 Å⁴⁰ and 1.739 Å⁶⁵, respectively. The axial Ru–O distances differ between **Compound 1** and **Compound 3**. For **Compound 3**, the bond distances range from 2.076 Å and 2.082 Å, whereas for **Compound 1**, one bond length is in the same range at 2.080 Å and the other is slightly longer at 2.110 Å. Notably, for both complexes these Ru–O distances are slightly shorter than those found in mononuclear Ru-carboxylate compounds, which typically fall within 2.090–2.266 Å.^{66–70} These distances are also slightly longer than the Ru–O distance of the formate ligand in Ru360, reported as 2.033 Å.⁴⁴ In comparing **Compound 1** and **Compound 3**, the longer Ru–O distance in the former may be a consequence of the weaker donor strength of formate compared propionate, a property that is also reflected by the smaller pK_a of formic acid.

[0167] **Kinetics of Dissociation via NMR Spectroscopy**

[0168] With the carboxylate **Compounds 1–4** fully characterized, their aquation kinetics were investigated. As previously noted, the axial chloride ligands of Ru265 are substituted by water within minutes under physiologically relevant conditions.⁶⁵ Thus, the viability of **Compounds 1–4** for prodrugs of Ru265' would require them exhibit substantially slower rates of aquation. To assess these properties, the complexes were incubated in pH 7.4 MOPS-buffered solution at 37 °C and monitored by ¹H NMR spectroscopy to follow the dissociation of the axial ligands over time. All complexes (**A**) underwent a sequential ligand substitution reaction with water to yield Ru265' (**C**) via a detectable monoaquated intermediate (**B**), as shown in **Scheme 6**.

Scheme 6. The aquation pathway of each of the carboxylate–functionalized compounds.



[0169] The dissociation of the axial carboxylate ligands from the ruthenium center was monitored at 37 °C via ^1H NMR through an $\text{A} \rightleftharpoons \text{B} \rightarrow \text{C}$ process. The relative integrations of each signal was measured for the intact complex (A), mono-substituted complex (B), and the free ligand (L), **FIG. 9**. The relative rate constants were calculated using Equations 1-5.⁵ The rate limiting step was determined to be the first step, with rate constants k_2 being an order of magnitude higher than k_1 . **Compound 1** demonstrated the fastest rate of aquation, which can be attributed to the weaker donor strength of formate compared to the other carboxylate ligands demonstrated by its lower $\text{p}K_a$. In addition, the Ru–O bond length in the crystal structure of **Compound 1** is slightly longer than that of **Compound 3**.

[0170] As the aquation reactions progressed, resonances arising from the free ligand (L) and monoaquated species B arose, concomitant with the decay of the intact complex A, as shown in **FIG. 7A** for **Compound 1**. Their relative concentrations were determined by integrating the peaks of their ^1H NMR signals. For **Compound 1**, the relative concentration of Ru265' (C), which is not directly observable by ^1H NMR spectroscopy, was calculated using mass balance (**Equation 4**). For **Compounds 2–4**, the ^1H NMR signals of A and B, overlap, thus requiring analysis using the sum of the concentrations of these species. Rate constants for both the first (k_1) and second (k_2) aquation steps were calculated by fitting the concentration vs time data to the established integrated rate laws for consecutive $\text{A} \rightarrow \text{B} \rightarrow \text{C}$ kinetic process (**Equations 1–5**).⁷¹ A representative data analysis for **Compound 1** are shown in **FIG. 7B**. All tabulated kinetic data are shown in **Table 3**.

$$[\text{A}]_t = [\text{A}]_0 \exp(-k_1 t) \quad (1)$$

$$[\text{B}]_t = [\text{B}]_0 \exp(-k_2 t) + [\text{A}]_0 k_1 (k_2 - k_1)^{-1} \{ \exp(-k_1 t) - \exp(-k_2 t) \} \quad (2)$$

$$[C]_t = [C]_0 + [B]_0 \{1 - \exp(-k_2 t)\} + [A]_0 (1 + \{k_1 \exp(-k_2 t) - k_2 \exp(-k_1 t)\} / (k_2 - k_1)) \quad (3)$$

$$[A]_t + [B]_t + [C]_t = 400 \mu\text{M} \quad (4)$$

$$[L]_t = [B]_t + 2[C]_t \quad (5)$$

Table 3. Aquation rate constants (k_1 and k_2) and half-lives ($t_{1/2}$) of all four derivatives at 37 °C. The pK_a of the conjugate acid of the free ligands are also given.

Complex	$k_1 \times 10^5 \text{ (s}^{-1}\text{)}$	$t_{1/2 \text{ (1)}} \text{ (h)}$	$k_2 \times 10^4 \text{ (s}^{-1}\text{)}$	$t_{1/2 \text{ (2)}} \text{ (h)}$	$pK_a \text{ (HL)}$
1	5.9 ± 0.2	3.3 ± 0.1	3.6 ± 0.3	0.50 ± 0.05	3.75
2	2.0 ± 0.5	9.9 ± 2.4	3.0 ± 1.2	0.7 ± 0.3	4.75
3	2.8 ± 0.1	6.9 ± 0.2	3.3 ± 0.5	0.6 ± 0.1	4.88
4	3.2 ± 0.1	5.9 ± 0.2	2.8 ± 0.2	0.700 ± 0.004	4.82

[0171] For all four complexes, k_1 was measured to be approximately an order of magnitude smaller than k_2 . Thus, aquation of these carboxylate complexes is rate-limited by dissociation of the first ligand. This result is comparable to Ru265, for which aquation of the first chloride ligand is also an order of magnitude smaller than that of the second.⁵⁰ The half-life of carboxylate ligand dissociation among the four complexes is on the order of hours. For comparison, the axial chloride ligands of Ru265 undergo aquation with a half-life of approximately 2 min.⁶⁵ Thus, the prolonged lifetime of the carboxylate-capped compounds portends to their potential use as prodrugs for Ru265'. In comparing the rate constants between **Compounds 1–4**, those of **Compound 1** are several times greater than those of **Compounds 2–4**. The pK_a value of formic acid, the conjugate acid of the carboxylate ligand within **1**, is an order of magnitude smaller than the other alkyl carboxylates employed within **Compounds 2–4**. Thus, the faster aquation rate of **Compound 1** correlates with the poorer basicity and donor strength of formate. Furthermore, as noted above in the discussion of the X-ray crystal structures, the interatomic axial Ru–O distances within **Compound 1** are longer than those found in **Compound 3**. Thus, thermodynamic destabilization of **Compound 1** due to weaker Ru–O interactions appears to give rise to the faster dissociation rates observed within this complex. Collectively, these results demonstrate that axial position inertness of Ru265 analogues can be systematically tuned via the use of ligands of different donor strengths.

[0172] Cytotoxicity and Cellular Uptake

[0173] Key properties of Ru265 that make it valuable as an MCU inhibitor is its limited cytotoxicity and its ability to permeate the cell membrane, a feature that most likely arises from its high redox stability.⁴⁰ To assess the suitability of **Compounds 1–4** in these contexts, their cytotoxic effects and cellular uptake were evaluated in both HeLa and HEK293T cells. The cytotoxicities of these complexes were determined using the colorimetric thiazolyl blue tetrazolium bromide (MTT) assay. With 72 h incubation periods, cell viability of both cell lines remained greater than 90% at concentrations up to 100 μ M. In addition, the JC-1 assay was used to verify that **Compounds 1–4** did not perturb the mitochondrial membrane potential of either cell lines when administered at 50 μ M for 1 h. Having shown that **Compounds 1–4** do not negatively affect cell viability or mitochondrial function, their cellular uptakes were next determined. The four compounds as well as Ru265 were incubated at 50 μ M for 2 h with both HeLa and HEK293T cells, and their intracellular accumulation was quantified by graphite furnace atomic absorption spectroscopy (GFAAS). As shown in **FIG. 8A**, all four complexes and Ru265 are taken up by both cell lines to a similar extent. Thus, the presence of these different carboxylate ligands does not influence cellular uptake. This observation argues against a passive uptake mechanism for which increasing lipophilicity should correlate with enhanced cellular uptake.^{51–53,55} It has been previously reported that Ru265 enters is taken up by cells through the organic cation transporter 3 (OCT3).⁶⁵ This transporter is upregulated in many tissues, including liver, heart, and skeletal muscle,^{72,73} and facilitates the entry of organic cationic species like histamine, choline, dopamine, and norepinephrine.^{74,75} To determine if **Compounds 1–4** may similarly be substrates for the OCT3, their uptake in HeLa cells in the presence of the OCT3-specific inhibitor 1,1'-diethyl-2,2'-cyanine iodide (decynium-22) was determined. As shown in **FIG. 3B**, only the uptake of Ru265 was diminished in the presence of the OCT3 inhibitor. These results suggest that OCT3 only mediates the uptake of Ru265 and not those of the carboxylate-capped analogues.

[0174] **Mitochondrial Ca^{2+} Uptake Inhibition in Permeabilized Cells**

[0175] Given the favorable cellular uptake and lack of cytotoxicity of **Compounds 1–4**, their MCU-inhibitory properties in digitonin-permeabilized HEK293T cells was investigated following established protocols. In the presence 1 μ M of either Ru265 or **Compounds 1–4**, complete abrogation of $m\text{Ca}^{2+}$ uptake was observed in these permeabilized cells. The administered concentrations for each compound were varied to obtain dose

response curves for MCU inhibition from which 50% inhibitory concentration (IC₅₀) values were determined. All four complexes exhibit nanomolar potency, but are approximately two-fold less active than Ru265 (**Table 4**).

Table 4. IC₅₀ values of MCU-mediated *m*Ca²⁺ uptake inhibition for Ru265 and derivatives in permeabilized HEK293T cells (1 × 10⁷ cells/mL).

Complex	IC ₅₀ (nM)
Ru265 ^[a]	8.6 ± 2.2
1	14.7 ± 2.9
2	18.8 ± 1.2
3	19.1 ± 1.7
4	18.7 ± 0.1

^[a] Reference 30

[0176] Kinetics of MCU Inhibition

[0177] Although **Compounds 1–4** are slightly less potent than Ru265, their ability to aquate within the hour timescale to yield Ru265' prompted us to investigate their time-dependent MCU-inhibitory properties. Before assessing MCU inhibition in permeabilized HEK293T cells, each complex was incubated in pH 7.4 MOPS buffer solution for 1, 3, or 6 h at 37 °C. After these incubation periods, the aged solutions were diluted to yield a 10 nM concentration of the compounds, and their *m*Ca²⁺ uptake inhibitory properties were determined. The transient curves of these experiments, showing the fluorescence response of the Calcium Green 5N sensor, demonstrate that *m*Ca²⁺ uptake is increasingly impaired for complexes that were preincubated for a longer time period. The normalized *m*Ca²⁺ uptake rates as a function of buffer preincubation time periods are shown in **FIGs. 10A-10B**. These data reveal all four carboxylate complexes to attain the same MCU-inhibitory activity as Ru265 after an incubation period of 6 h. These results suggest that axial ligand modification of Ru265 provides an effective means of generating aquation-activated prodrugs for this compound.

[0178] Intact Cell Mitochondrial Ca²⁺ Uptake Inhibition

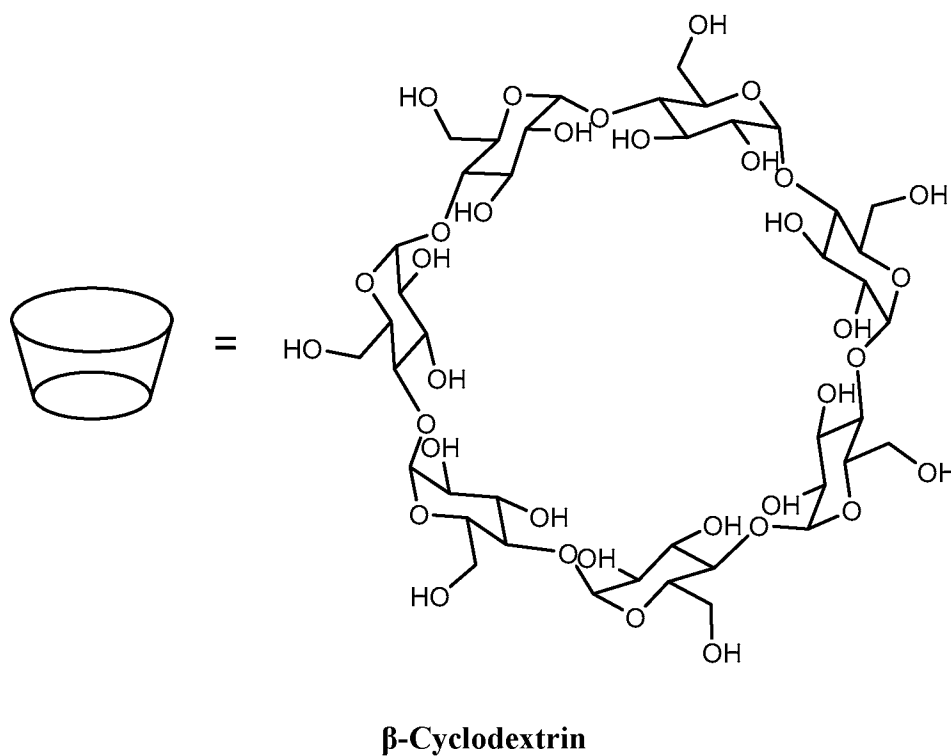
[0179] Having demonstrated that **Compounds 1–4** are active MCU inhibitors in permeabilized cells, their ability to operate in intact cell systems like Ru265 were investigated.⁴⁰ The mitochondria-localizing dye Rhod-2-AM was incubated with HeLa cells that were treated with each complex for 1 h at 37 °C. Histamine was added to the cells to stimulate mitochondrial Ca²⁺ uptake, and the fluorescence response was measured and

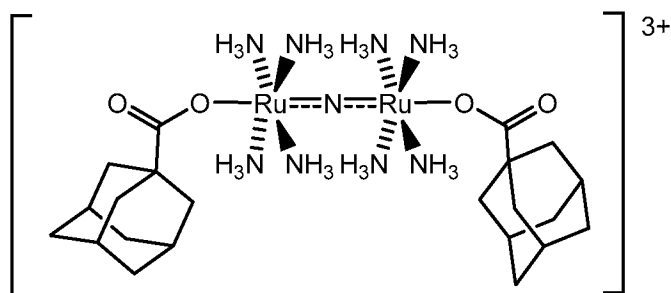
compared to nontreated cells. As was expected, Ru265 and the derivatives discussed in this work showed a decreased fluorescence response in comparison to untreated cells, as shown in **FIGs. 11A-11B**. These results demonstrate that these compounds maintain their potent inhibitory activity in intact cell systems.

[0180] Example 3: Ru265 with Adamantane for Supramolecular Chemistry with Cyclodextrin

[0181] A new derivative of Ru265 was synthesized by the axial substitution of the chloride ligand for 1-adamantane carboxylate (**Compound 5**). The adamantane carboxylate groups are known to complex into cyclodextrin molecules, leveraging the possibility for an MCU inhibitor participating in supramolecular drug delivery. The complex was purified by the diffusion of hexane into a concentrated solution of crude material in THF and isolated as single, orange crystals (**FIG. 12**).

Scheme 7. Structure of β -cyclodextrin and Compound 5



**Compound 5****[0182] Mitochondrial Calcium Uptake Studies**

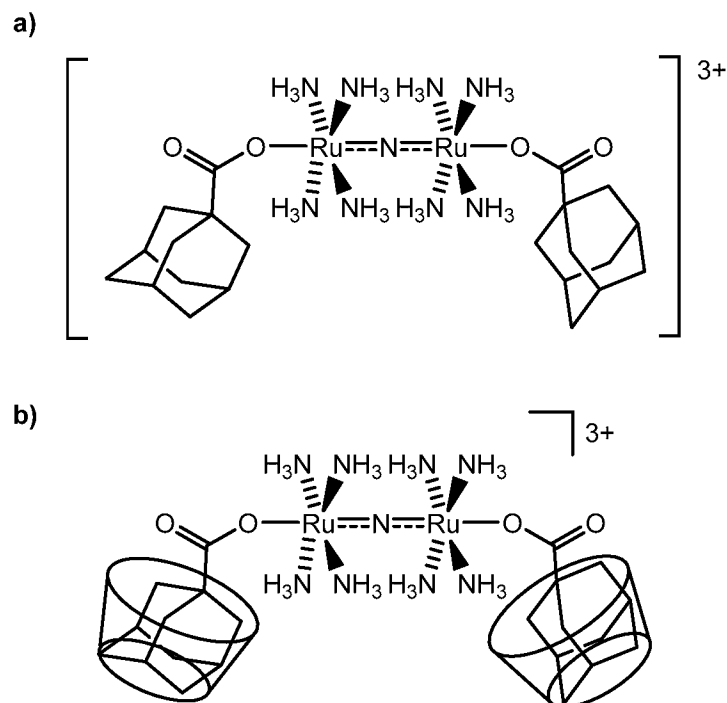
[0183] The MCU-inhibitory properties of this complex were examined in permeabilized HEK293T cells as previously described. The adamantane ligand decreased the potency of the complex (**Table 5, FIG. 13**). Seemingly, the substitution of the axial ligand of Ru265 decreases the potency. In addition, this compound exhibited prodrug capabilities at 25 nM over 6 h incubation at 37 °C.

Table 5. IC₅₀ values of MCU-mediated *m*Ca²⁺ uptake inhibition for Ru265 and **Compound 5** in permeabilized HEK293T cells (1 × 10⁷ cells/mL).

Complex	IC ₅₀ (nM) – HEK293T (10 ⁷ cells mL ⁻¹)
Ru265	8.6 ± 2.2
Compound 5	50.7 ± 0.6

[0184] Encapsulation into β-CD

[0185] The encapsulation of **Compound 5** was examined by NMR spectroscopy. First, two eq. of β-CD were added to 1 eq. **Compound 5** and stirred in D₂O for 1 h. Spectra were then taken, and downshift adamantane resonances were observed, suggesting encapsulation. Then, the same solutions were subjected to Diffusion-Ordered NMR spectroscopy (DOSY, **FIG. 14**). Each solution displayed a different diffusion coefficient, further suggesting that the adamantane ligands have been encapsulated into β-CD.

Scheme 8. Encapsulation of **Compound 5** into β -CD.

[0186] This work presents compounds that demonstrate a prodrug approach to MCU inhibition through simple ligand dissociation of the inhibitor Ru265. In addition, an adamantane-derivative of Ru265 has shown an ability to encapsulate into the supramolecular complex β -CD. These compounds and their properties can be leveraged as potential therapeutics to fight ischemic reperfusion injury and related diseases. Both the prodrug approach and supramolecular drug delivery have the potential to offer significant improvements to the efficacy of Ru265 as an MCU inhibitor.

[0187] While certain embodiments have been illustrated and described, a person with ordinary skill in the art, after reading the foregoing specification, can effect changes, substitutions of equivalents and other types of alterations to the compounds of the present technology or salts, pharmaceutical compositions, derivatives, prodrugs, metabolites, tautomers or racemic mixtures thereof as set forth herein. Each aspect and embodiment described above can also have included or incorporated therewith such variations or aspects as disclosed in regard to any or all of the other aspects and embodiments.

[0188] The present technology is also not to be limited in terms of the particular aspects described herein, which are intended as single illustrations of individual aspects of the present technology. Many modifications and variations of this present technology can be

made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods within the scope of the present technology, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing descriptions. Such modifications and variations are intended to fall within the scope of the appended claims. It is to be understood that this present technology is not limited to particular methods, reagents, compounds, compositions, labeled compounds or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting. Thus, it is intended that the specification be considered as exemplary only with the breadth, scope and spirit of the present technology indicated only by the appended claims, definitions therein and any equivalents thereof.

[0189] The embodiments, illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms “comprising,” “including,” “containing,” etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the claimed technology. Additionally, the phrase “consisting essentially of” will be understood to include those elements specifically recited and those additional elements that do not materially affect the basic and novel characteristics of the claimed technology. The phrase “consisting of” excludes any element not specified.

[0190] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

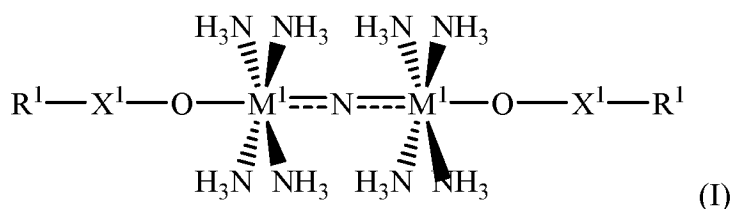
[0191] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed

range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, *etc.* As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, *etc.* As will also be understood by one skilled in the art all language such as “up to,” “at least,” “greater than,” “less than,” and the like, include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member.

[0192] All publications, patent applications, issued patents, and other documents (for example, journals, articles and/or textbooks) referred to in this specification are herein incorporated by reference as if each individual publication, patent application, issued patent, or other document was specifically and individually indicated to be incorporated by reference in its entirety. Definitions that are contained in text incorporated by reference are excluded to the extent that they contradict definitions in this disclosure.

[0193] The present technology may include, but is not limited to, the features and combinations of features recited in the following lettered paragraphs, it being understood that the following paragraphs should not be interpreted as limiting the scope of the claims as appended hereto or mandating that all such features must necessarily be included in such claims:

A. A compound of Formula I



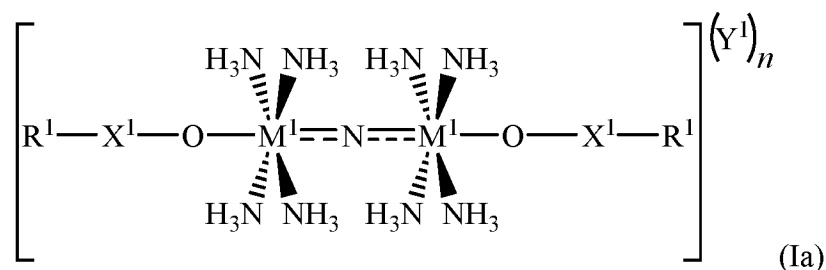
or a pharmaceutically acceptable salt and/or solvate thereof, wherein

M^1 is Ru or Os;

X^1 is —C(O)— or —P(O)(OH)— ; and

R^1 is H, alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl;

optionally where the compound of Formula I is according to Formula Ia

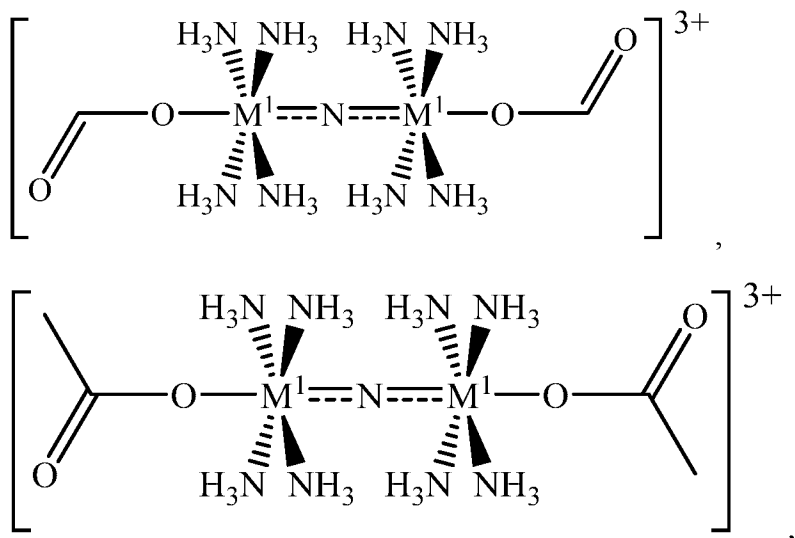


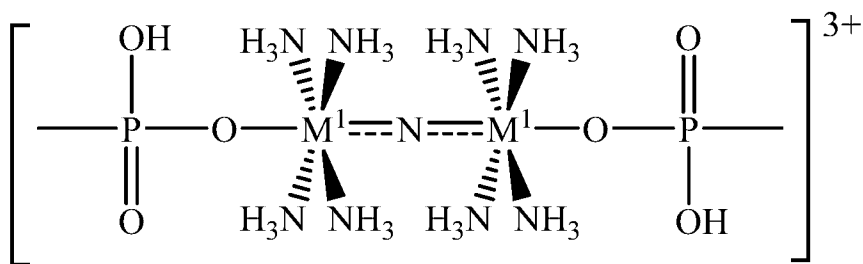
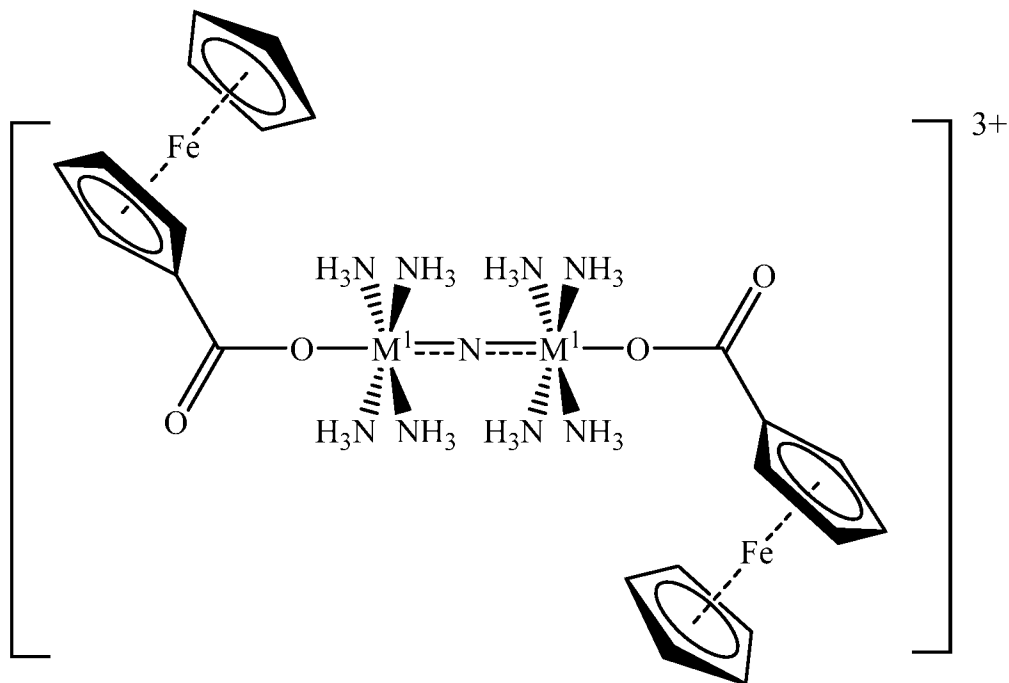
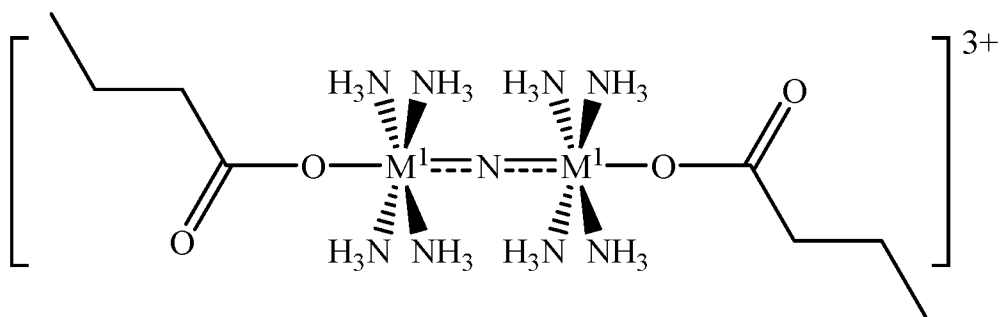
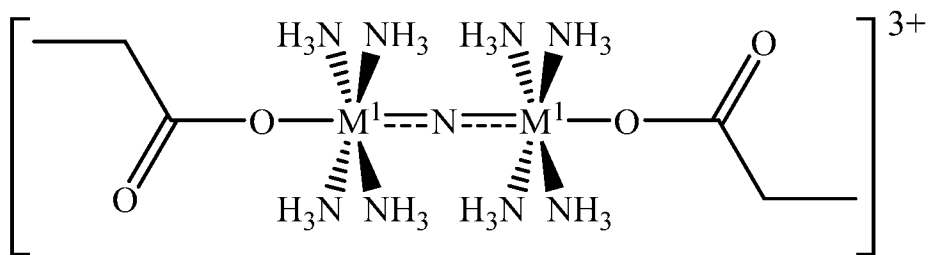
or solvate thereof, wherein

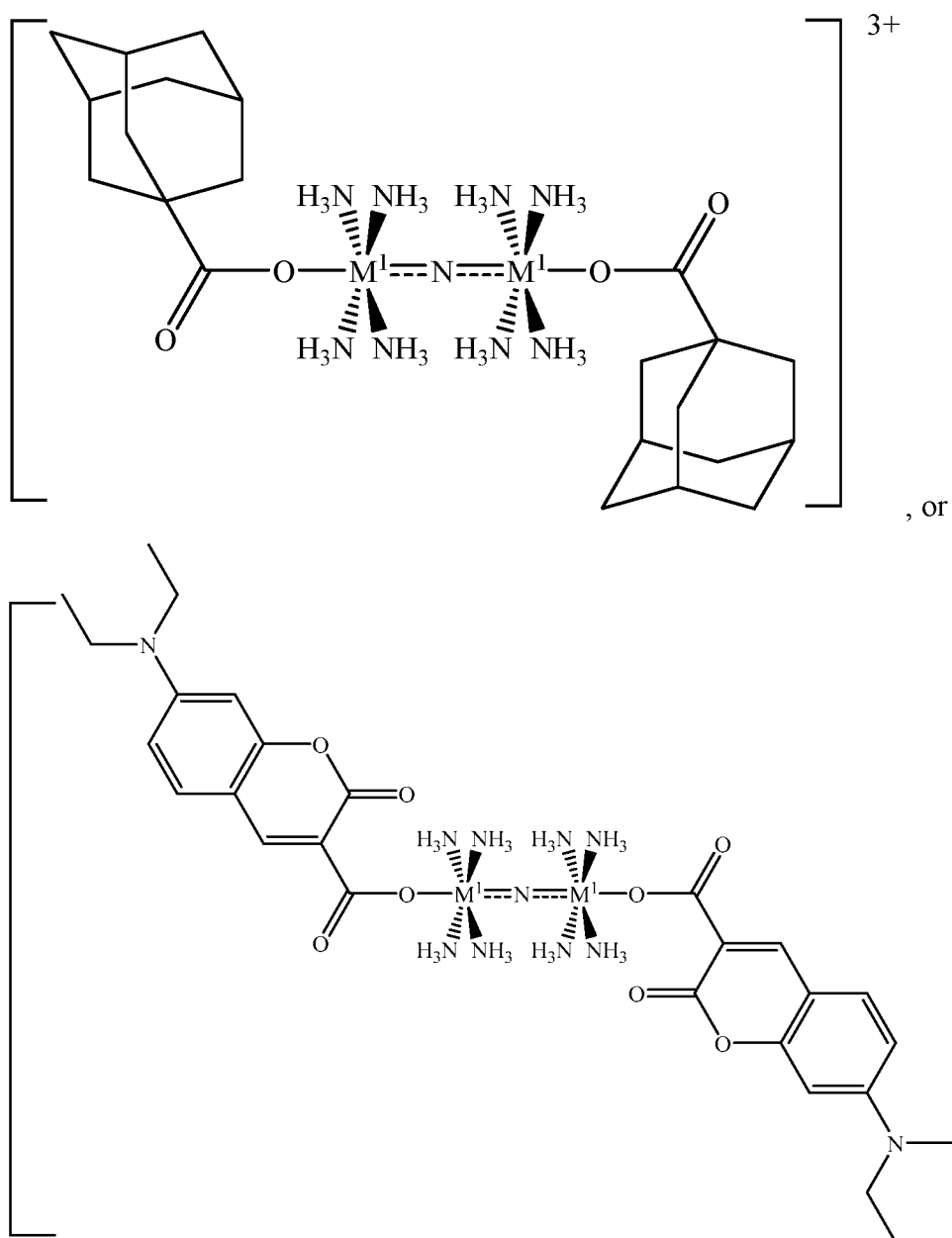
Y^1 is an anion; and

n is 1, 2, 3.

- B. The compound of Paragraph A, wherein X^1 is $-\text{C}(\text{O})-$, and R^1 is H or alkyl.
- C. The compound of Paragraph A, wherein X^1 is $-\text{P}(\text{O})(\text{OH})-$ and R^1 is H alkyl.
- D. The compound of any one of Paragraphs A-C, wherein R^1 is methyl, ethyl, or propyl.
- E. The compound of Paragraph A, wherein X^1 is $-\text{C}(\text{O})-$ and R^1 is cycloalkyl.
- F. The compound of Paragraph E, wherein R^1 is adamantyl.
- G. The compound of Paragraph A, wherein the compound is a pharmaceutically acceptable salt or solvate of a pharmaceutically acceptable salt of one of the following:







H. The compound of any one of Paragraphs A-G, wherein M^1 is Ru.

I. The compound of any one of Paragraphs A-G, wherein M^1 is Os.

J. A pharmaceutical composition comprising

a compound of any one of Paragraphs A-I; and
a pharmaceutically acceptable carrier.

K. A pharmaceutical composition comprising

an effective amount of a compound of any one of Paragraphs A-I for treating a
disease or disorder related to mitochondrial Ca^{2+} overload; and

a pharmaceutically acceptable excipient.

- L. The pharmaceutical composition of Paragraph K, wherein the disease related to mitochondrial Ca^{2+} overload is selected from a neurodegenerative disorder, a cardiovascular disease, a metabolic disease, cancer, a skeletal muscle disease, cystic fibrosis, and ischemic reperfusion injury.
- M. The pharmaceutical composition of Paragraph L, wherein the neurodegenerative disorder is selected from the group consisting of Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS) and cerebral ischemic stroke.
- N. The pharmaceutical composition of Paragraph L, wherein the cancer is selected from the group consisting of breast cancer, pancreatic cancer, colorectal cancer, liver cancer, melanoma, ovarian cancer, and kidney cancer.
- O. A method of treating a disease or disorder related to mitochondrial Ca^{2+} overload in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the compound of any one of Paragraphs A-I.
- P. The method of Paragraph O, wherein the disease related to mitochondrial Ca^{2+} overload is selected from a neurodegenerative disorder, a cardiovascular disease, a metabolic disease, cancer, a skeletal muscle disease, cystic fibrosis, and ischemic reperfusion injury.
- Q. The method of Paragraph P, wherein the neurodegenerative disorder is selected from the group consisting of Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS) and cerebral ischemic stroke.
- R. The method of Paragraph P, wherein the cancer is selected from the group consisting of breast cancer, pancreatic cancer, colorectal cancer, liver cancer, melanoma, ovarian cancer, and kidney cancer.
- S. The method of any one of Paragraphs O-R, wherein the subject is a human.

T. A method of inhibiting mitochondrial calcium uniporter (MCU), the method comprising administering to a subject an effective amount of the compound of any one of Paragraphs A-I for inhibiting MCU.

[0194] Other embodiments are set forth in the following claims, along with the full scope of equivalents to which such claims are entitled.

REFERENCES

- (1) Pozzan, T.; Rizzuto, R.; Volpe, P.; Meldolesi, J. Molecular and Cellular Physiology of Intracellular Calcium Stores. *Physiol. Rev.* **1994**, *74* (3), 595–636.
- (2) Berridge, M. J.; Bootman, M. D.; Roderick, H. L. Calcium Signalling: Dynamics, Homeostasis and Remodelling. *Nat. Rev. Mol. Cell Biol.* **2003**, *4* (7), 517–529.
- (3) Clapham, D. E. Calcium Signaling. *Cell* **2007**, *131* (6), 1047–1058.
- (4) Carafoli, E.; Brini, M. *Calcium Signalling and Disease: Molecular Pathology of Calcium*; 2007; Vol. 45.
- (5) Orrenius, S.; Gogvadze, V.; Zhivotovsky, B. Calcium and Mitochondria in the Regulation of Cell Death. *Biochem. Biophys. Res. Commun.* **2015**, *460* (1), 72–81.
- (6) Celsi, F.; Pizzo, P.; Brini, M.; Leo, S.; Fotino, C.; Pinton, P.; Rizzuto, R. Mitochondria, Calcium and Cell Death: A Deadly Triad in Neurodegeneration. *Biochim. Biophys. Acta - Bioenerg.* **2009**, *1787* (5), 335–344.
- (7) Abeti, R.; Abramov, A. Y. Mitochondrial Ca²⁺ in Neurodegenerative Disorders. *Pharmacol. Res.* **2015**, *99*, 377–381.
- (8) Gustafsson, Å. B.; Gottlieb, R. A. Heart Mitochondria: Gates of Life and Death. *Cardiovasc. Res.* **2008**, *77* (2), 334–343.
- (9) Santulli, G.; Xie, W.; Reiken, S. R.; Marks, A. R. Mitochondrial Calcium Overload Is a Key Determinant in Heart Failure. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (36), 11389–11394.
- (10) Zhou, B.; Tian, R. Mitochondrial Dysfunction in Pathophysiology of Heart Failure. *J. Clin. Invest.* **2018**, *128* (9), 3716–3726.
- (11) Vultur, A.; Gibhardt, C. S.; Stanis, H.; Bogeski, I. The Role of the Mitochondrial Calcium Uniporter (MCU) Complex in Cancer. *Pflugers Arch. Eur. J. Physiol.* **2018**, *470* (8), 1149–1163.
- (12) Delierneux, C.; Kouba, S.; Shanmughapriya, S.; Potier-Cartreau, M.; Trebak, M.; Hempel, N. Mitochondrial Calcium Regulation of Redox Signaling in Cancer. *Cells*

- 2020, 9 (2), 432.
- (13) Rimessi, A.; Pozzato, C.; Carparelli, L.; Rossi, A.; Ranucci, S.; de Fino, I.; Cigana, C.; Talarico, A.; Wieckowski, M. R.; Ribeiro, C. M. P.; Trapella, C.; Rossi, G.; Cabrini, G.; Bragonzi, A.; Pinton, P. Pharmacological Modulation of Mitochondrial Calcium Uniporter Controls Lung Inflammation in Cystic Fibrosis. *Sci. Adv.* **2020**, 6 (19).
 - (14) Rimessi, A.; Vitto, V. A. M.; Patergnani, S.; Pinton, P. Update on Calcium Signaling in Cystic Fibrosis Lung Disease. *Front. Pharmacol.* **2021**, 12 (3), 1–12.
 - (15) Halestrap, A. P. Calcium, Mitochondria and Reperfusion Injury: A Pore Way to Die. *Biochem. Soc. Trans.* **2006**, 34 (2), 232–237.
 - (16) Shintani-Ishida, K.; Inui, M.; Yoshida, K. Ischemia-Reperfusion Induces Myocardial Infarction through Mitochondrial Ca²⁺ Overload. *J. Mol. Cell. Cardiol.* **2012**, 53 (2), 233–239.
 - (17) Sanderson, T. H.; Reynolds, C. A.; Kumar, R.; Przyklenk, K.; Huttemann, M. Molecular Mechanisms of Ischemia-Reperfusion Injury in Brain: Pivotal Role of the Mitochondrial Membrane Potential in Reactive Oxygen Species Generation. *Mol. Neurobiol.* **2013**, 47 (1), 9–23.
 - (18) Kirichok, Y.; Krapivinsky, G.; Clapham, D. E. The Mitochondrial Calcium Uniporter Is a Highly Selective Ion Channel. *Nature* **2004**, 427 (6972), 360–364.
 - (19) Kamer, K. J.; Mootha, V. K. The Molecular Era of the Mitochondrial Calcium Uniporter. *Nat. Rev. Mol. Cell Biol.* **2015**, 16 (9), 545–553.
 - (20) Rizzuto, R.; De Stefani, D.; Raffaello, A.; Mammucari, C. Mitochondria as Sensors and Regulators of Calcium Signalling. *Nat. Rev. Mol. Cell Biol.* **2012**, 13 (9), 566–578.
 - (21) Nemani, N.; Shanmughapriya, S.; Madesh, M. Molecular Regulation of MCU: Implications in Physiology and Disease. *Cell Calcium* **2018**, 74 (May), 86–93.
 - (22) De Stefani, D.; Raffaello, A.; Teardo, E.; Szabó, I.; Rizzuto, R. A Forty-Kilodalton Protein of the Inner Membrane Is the Mitochondrial Calcium Uniporter. *Nature* **2011**, 476 (7360), 336–340.
 - (23) Oxenoid, K.; Dong, Y.; Cao, C.; Cui, T.; Sancak, Y.; Markhard, A. L.; Grabarek, Z.; Kong, L.; Liu, Z.; Ouyang, B.; Cong, Y.; Mootha, V. K.; Chou, J. J. Architecture of the Mitochondrial Calcium Uniporter. *Nature* **2016**, 533 (7602), 269–273.
 - (24) Fan, C.; Fan, M.; Orlando, B. J.; Fastman, N. M.; Zhang, J.; Xu, Y.; Chambers, M. G.; Xu, X.; Perry, K.; Liao, M.; Feng, L. X-Ray and Cryo-EM Structures of the Mitochondrial Calcium Uniporter. *Nature* **2018**, 559 (7715), 575–579.
 - (25) Baradaran, R.; Wang, C.; Siliciano, A. F.; Long, S. B. Cryo-EM Structures of Fungal

- and Metazoan Mitochondrial Calcium Uniporters. *Nature* **2018**, 559 (7715), 580–584.
- (26) Nguyen, N. X.; Armache, J. P.; Lee, C.; Yang, Y.; Zeng, W.; Mootha, V. K.; Cheng, Y.; Bai, X. chen; Jiang, Y. Cryo-EM Structure of a Fungal Mitochondrial Calcium Uniporter. *Nature* **2018**, 559 (7715), 570–574.
- (27) Fan, M.; Zhang, J.; Tsai, C. W.; Orlando, B. J.; Rodriguez, M.; Xu, Y.; Liao, M.; Tsai, M. F.; Feng, L. Structure and Mechanism of the Mitochondrial Ca^{2+} Uniporter Holocomplex. *Nature* **2020**, 582 (7810), 129–133.
- (28) Wang, Y.; Nguyen, N. X.; She, J.; Zeng, W.; Yang, Y.; Bai, X. chen; Jiang, Y. Structural Mechanism of EMRE-Dependent Gating of the Human Mitochondrial Calcium Uniporter. *Cell* **2019**, 177 (5), 1252-1261.e13.
- (29) Vais, H.; Payne, R.; Paudel, U.; Li, C.; Foskett, J. K. Coupled Transmembrane Mechanisms Control MCU-Mediated Mitochondrial Ca^{2+} Uptake. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, 117 (35), 21731–21739.
- (30) Wang, C.; Baradaran, R.; Long, S. B. Structure and Reconstitution of an MCU–EMRE Mitochondrial Ca^{2+} Uniporter Complex. *J. Mol. Biol.* **2020**, 432 (20), 5632–5648.
- (31) Zhuo, W.; Zhou, H.; Guo, R.; Yi, J.; Zhang, L.; Yu, L.; Sui, Y.; Zeng, W.; Wang, P.; Yang, M. Structure of Intact Human MCU Supercomplex with the Auxiliary MICU Subunits. *Protein Cell* **2021**, 12 (3), 220–229.
- (32) Woods, J. J.; Wilson, J. J. Inhibitors of the Mitochondrial Calcium Uniporter for the Treatment of Disease. *Curr. Opin. Chem. Biol.* **2020**, 55, 9–18.
- (33) Santo-Domingo, J.; Vay, L.; Hernández-SanMiguel, E.; Lobatón, C. D.; Moreno, A.; Montero, M.; Alvarez, J. The Plasma Membrane $\text{Na}^+/\text{Ca}^{2+}$ Exchange Inhibitor KB-R7943 Is Also a Potent Inhibitor of the Mitochondrial Ca^{2+} Uniporter. *Br. J. Pharmacol.* **2007**, 151 (5), 647–654.
- (34) Thu, V. T.; Kim, H. K.; Long, L. T.; Lee, S. R.; Hanh, T. M.; Ko, T. H.; Heo, H. J.; Kim, N.; Kim, S. H.; Ko, K. S.; Rhee, B. D.; Han, J. NecroX-5 Prevents Hypoxia/Reoxygenation Injury by Inhibiting the Mitochondrial Calcium Uniporter. *Cardiovasc. Res.* **2012**, 94 (2), 342–350.
- (35) Schwartz, J.; Holmuamedov, E.; Zhang, X.; Lovelace, G. L.; Smith, C. D.; Lemasters, J. J. Minocycline and Doxycycline, but Not Other Tetracycline-Derived Compounds, Protect Liver Cells from Chemical Hypoxia and Ischemia/Reperfusion Injury by Inhibition of the Mitochondrial Calcium Uniporter. *Toxicol. Appl. Pharmacol.* **2013**, 273 (1), 172–179.
- (36) Arduino, D. M.; Wettmarshausen, J.; Vais, H.; Navas-Navarro, P.; Cheng, Y.;

- Leimpek, A.; Ma, Z.; Delrio-Lorenzo, A.; Giordano, A.; Garcia-Perez, C.; Médard, G.; Kuster, B.; García-Sancho, J.; Mokranjac, D.; Foskett, J. K.; Alonso, M. T.; Perocchi, F. Systematic Identification of MCU Modulators by Orthogonal Interspecies Chemical Screening. *Mol. Cell* **2017**, *67* (4), 711–723.
- (37) Kon, N.; Murakoshi, M.; Isobe, A.; Kagechika, K.; Miyoshi, N.; Nagayama, T. DS16570511 Is a Small-Molecule Inhibitor of the Mitochondrial Calcium Uniporter. *Cell Death Discov.* **2017**, *3* (1), 1–7.
- (38) Di Marco, G.; Vallese, F.; Jourde, B.; Bergsdorf, C.; Sturlese, M.; De Mario, A.; Techer-Etienne, V.; Haasen, D.; Oberhauser, B.; Schleege, S.; Minetti, G.; Moro, S.; Rizzuto, R.; De Stefani, D.; Fornaro, M.; Mammucari, C. A High-Throughput Screening Identifies MICU1 Targeting Compounds. *Cell Rep.* **2020**, *30* (7), 2321–2331.
- (39) De Mario, A.; Tosatto, A.; Hill, J. M.; Kriston-Vizi, J.; Ketteler, R.; Vecellio Reane, D.; Cortopassi, G.; Szabadkai, G.; Rizzuto, R.; Mammucari, C. Identification and Functional Validation of FDA-Approved Positive and Negative Modulators of the Mitochondrial Calcium Uniporter. *Cell Rep.* **2021**, *35* (12), 109275.
- (40) Woods, J. J.; Nemani, N.; Shanmughapriya, S.; Kumar, A.; Zhang, M.; R. Nathan, S.; Thomas, M.; Carvalho, E.; Ramachandran, K.; Srikantan, S.; B. Stathopoulos, P.; J. Wilson, J.; Madesh, M. A Selective and Cell-Permeable Mitochondrial Calcium Uniporter (MCU) Inhibitor Preserves Mitochondrial Bioenergetics after Hypoxia/Reoxygenation Injury. *ACS Cent. Sci.* **2019**, *5* (1), 153–166.
- (41) Woods, J. J.; Rodriguez, M. X.; Tsai, C. W.; Tsai, M. F.; Wilson, J. J. Cobalt Amine Complexes and Ru265 Interact with the DIME Region of the Mitochondrial Calcium Uniporter. *Chem. Commun.* **2021**, *57* (50), 6161–6164.
- (42) Unitt, J. F.; Boden, K. L.; Wallace, A. V.; Ingall, A. H.; Coombs, M. E.; Ince, F. Novel Cobalt Complex Inhibitors of Mitochondrial Calcium Uptake. *Bioorganic Med. Chem.* **1999**, *7* (9), 1891–1896.
- (43) Matlib, M. A.; Zhou, Z.; Knight, S.; Ahmed, S.; Choi, K. M.; Krause-Bauer, J.; Phillips, R.; Altschuld, R.; Katsube, Y.; Sperelakis, N.; Bers, D. M. Oxygen-Bridged Dinuclear Ruthenium Amine Complex Specifically Inhibits Ca²⁺ Uptake into Mitochondria in Vitro and in Situ in Single Cardiac Myocytes. *J. Biol. Chem.* **1998**, *273* (17), 10223–10231.
- (44) Emerson, J.; Clarke, M. J.; Ying, W. L.; Sanadi, D. R. The Component of “Ruthenium Red” Responsible for Inhibition of Mitochondrial Calcium Ion Transport. *Spectra*,

- Electrochemistry, and Aquation Kinetics. Crystal Structure of μ -O-
[(HCO₂)(NH₃)₄Ru]₂Cl₃. *J. Am. Chem. Soc.* **1993**, *115* (25), 11799–11805.
- (45) Nathan, S. R.; Pino, N. W.; Arduino, D. M.; Perocchi, F.; MacMillan, S. N.; Wilson, J. J. Synthetic Methods for the Preparation of a Functional Analogue of Ru360, a Potent Inhibitor of Mitochondrial Calcium Uptake. *Inorg. Chem.* **2017**, *56* (6), 3123–3126.
- (46) Moore, C. L. Specific Inhibition of Mitochondrial Ca⁺⁺ Transport by Ruthenium Red. *Biochem. Biophys. Res. Commun.* **1971**, *42* (2), 298–305.
- (47) Broekemeier, K. M.; Krebsbach, R. J.; Pfeiffer, D. R. Inhibition of the Mitochondrial Ca²⁺ Uniporter by Pure and Impure Ruthenium Red. *Mol. Cell. Biochem.* **1994**, *139* (1), 33–40.
- (48) Ying, W. L.; Emerson, J.; Clarke, M. J.; Sanadi, D. R. Inhibition of Mitochondrial Calcium Ion Transport by an Oxo-Bridged Dinuclear Ruthenium Ammine Complex. *Biochemistry* **1991**, *30* (20), 4949–4952.
- (49) Novorolsky, R. J.; Nichols, M.; Kim, J. S.; Pavlov, E. V.; J Woods, J.; Wilson, J. J.; Robertson, G. S. The Cell-Permeable Mitochondrial Calcium Uniporter Inhibitor Ru265 Preserves Cortical Neuron Respiration after Lethal Oxygen Glucose Deprivation and Reduces Hypoxic/Ischemic Brain Injury. *J. Cereb. Blood Flow Metab.* **2020**, *40* (6), 1172–1181.
- (50) Woods, J. J.; Spivey, J. A.; Wilson, J. J. A [¹H, ¹⁵N] Heteronuclear Single Quantum Coherence NMR Study of the Solution Reactivity of the Ruthenium-Based Mitochondrial Calcium Uniporter Inhibitor Ru265. *Eur. J. Inorg. Chem.* **2022**, *2022* (6), 1–7.
- (51) Testa, B.; Crivori, P.; Reist, M.; Carrupt, P. A. The Influence of Lipophilicity on the Pharmacokinetic Behavior of Drugs: Concepts and Examples. *Perspect. Drug Discov. Des.* **2000**, *19*, 179–211.
- (52) Leeson, P. D.; Davis, A. M. Time-Related Differences in the Physical Property Profiles of Oral Drugs. *J. Med. Chem.* **2004**, *47* (25), 6338–6348.
- (53) Leeson, P. D.; Springthorpe, B. The Influence of Drug-like Concepts on Decision-Making in Medicinal Chemistry. *Nat. Rev. Drug Discov.* **2007**, *6* (11), 881–890.
- (54) Hughes, J. D.; Blagg, J.; Price, D. A.; Bailey, S.; DeCrescenzo, G. A.; Devraj, R. V.; Ellsworth, E.; Fobian, Y. M.; Gibbs, M. E.; Gilles, R. W.; Greene, N.; Huang, E.; Krieger-Burke, T.; Loesel, J.; Wager, T.; Whiteley, L.; Zhang, Y. Physiochemical Drug Properties Associated with in Vivo Toxicological Outcomes. *Bioorganic Med. Chem. Lett.* **2008**, *18* (17), 4872–4875.

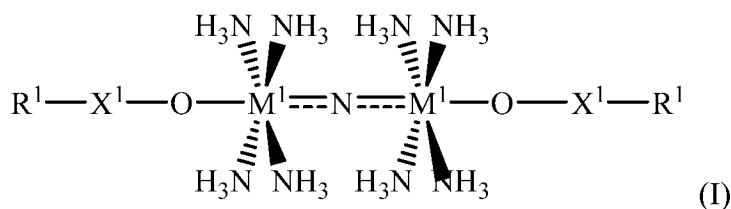
- (55) Waring, M. J. Defining Optimum Lipophilicity and Molecular Weight Ranges for Drug Candidates-Molecular Weight Dependent Lower Log D Limits Based on Permeability. *Bioorganic Med. Chem. Lett.* **2009**, *19* (10), 2844–2851.
- (56) Gleeson, M. P.; Hersey, A.; Montanari, D.; Overington, J. Probing the Links between in Vitro Potency, ADMET and Physicochemical Parameters. *Nat. Rev. Drug Discov.* **2011**, *10* (3), 197–208.
- (57) Liu, X.; Testa, B.; Fahr, A. Lipophilicity and Its Relationship with Passive Drug Permeation. *Pharm. Res.* **2011**, *28* (5), 962–977.
- (58) Johnson, T. W.; Gallego, R. A.; Edwards, M. P. Lipophilic Efficiency as an Important Metric in Drug Design. *J. Med. Chem.* **2018**, *61* (15), 6401–6420.
- (59) Johnstone, T. C.; Lippard, S. J. The Effect of Ligand Lipophilicity on the Nanoparticle Encapsulation of Pt(IV) Prodrugs. *Inorg. Chem.* **2013**, *52* (17), 9915–9920.
- (60) Zheng, Y. R.; Suntharalingam, K.; Johnstone, T. C.; Yoo, H.; Lin, W.; Brooks, J. G.; Lippard, S. J. Pt(IV) Prodrugs Designed to Bind Non-Covalently to Human Serum Albumin for Drug Delivery. *J. Am. Chem. Soc.* **2014**, *136* (24), 8790–8798.
- (61) Awuah, S. G.; Zheng, Y. R.; Bruno, P. M.; Hemann, M. T.; Lippard, S. J. A Pt(IV) Pro-Drug Preferentially Targets Indoleamine-2,3-Dioxygenase, Providing Enhanced Ovarian Cancer Immuno-Chemotherapy. *J. Am. Chem. Soc.* **2015**, *137* (47), 14854–14857.
- (62) Abu Ammar, A.; Raveendran, R.; Gibson, D.; Nassar, T.; Benita, S. A Lipophilic Pt(IV) Oxaliplatin Derivative Enhances Antitumor Activity. *J. Med. Chem.* **2016**, *59* (19), 9035–9046.
- (63) Hallett, A. J.; Placet, E.; Prieux, R.; McCafferty, D.; Platts, J. A.; Lloyd, D.; Isaacs, M.; Hayes, A. J.; Coles, S. J.; Pitak, M. B.; Marchant, S.; Marriott, S. N.; Allemann, R. K.; Dervisi, A.; Fallis, I. A. Exploring the Cellular Uptake and Localisation of Phosphorescent Rhenium: Fac -Tricarbonyl Metallosurfactants as a Function of Lipophilicity. *Dalt. Trans.* **2018**, *47* (40), 14241–14253.
- (64) Konkankit, C. C.; Vaughn, B. A.; Huang, Z.; Boros, E.; Wilson, J. J. Systematically Altering the Lipophilicity of Rhenium(i) Tricarbonyl Anticancer Agents to Tune the Rate at Which They Induce Cell Death. *Dalt. Trans.* **2020**, *49* (45), 16062–16066.
- (65) Woods, J. J.; Lovett, J.; Lai, B.; Harris, H. H.; Wilson, J. J. Redox Stability Controls the Cellular Uptake and Activity of Ruthenium-Based Inhibitors of the Mitochondrial Calcium Uniporter (MCU). *Angew. Chemie - Int. Ed.* **2020**, *59* (16), 6482–6491.
- (66) Matteoli, U.; Menchi, G.; Bianchi, M.; Piacenti, F.; Ianelli, S.; Nardelli, M. Structure

- and Catalytic Activity of Phosphine-Substituted Ruthenium Carbonyl Carboxylates. *J. Organomet. Chem.* **1995**, 498 (2), 177–186.
- (67) Malik, K. Z.; Robinson, S. D.; Steed, J. W. Ruthenium Carboxylate Complexes $[\text{Ru}(\text{O}_2\text{CR})_2(\text{H}_2\text{O})(\text{Me}_2\text{SO})_3]$ ($\text{R} = \text{Me}, \text{CF}_3$)-Synthesis and X-Ray Crystal Structures. *Polyhedron* **2000**, 19 (13), 1589–1592.
- (68) Hiatt, N. P.; Lynam, J. M.; Welby, C. E.; Whitwood, A. C. Ruthenium Carboxylate Complexes as Easily Prepared and Efficient Catalysts for the Synthesis of β -Oxopropyl Esters. *J. Organomet. Chem.* **2011**, 696 (1), 378–387.
- (69) Carmona, D.; Viguri, F.; Pilar Lamata, M.; Ferrer, J.; Bardají, E.; Lahoz, F. J.; García-Orduña, P.; Oro, L. A. Ruthenium Amino Carboxylate Complexes as Asymmetric Hydrogen Transfer Catalysts. *Dalt. Trans.* **2012**, 41 (34), 10298–10308.
- (70) Jeschke, J.; Gäbler, C.; Korb, M.; Rüffer, T.; Lang, H. Ruthenium Carboxylate Complexes as Efficient Catalysts for the Addition of Carboxylic Acids to Propargylic Alcohols. *Eur. J. Inorg. Chem.* **2015**, 2015 (18), 2939–2947.
- (71) Gellene, G. I. Application of Kinetic Approximations to the $\text{A} \rightleftharpoons \text{B} \rightarrow \text{C}$ Reaction System. *J. Chem. Educ.* **1995**, 72 (3), 196–199.
- (72) Verhaagh, S.; Schweifer, N.; Barlow, D. P.; Zwart, R. Cloning of the Mouse and Human Solute Carrier 22a3 (Slc22a3/SLC22A3) Identifies a Conserved Cluster of Three Organic Cation Transporters on Mouse Chromosome 17 and Human 6q26-Q27. *Genomics* **1999**, 55 (2), 209–218.
- (73) Gründemann, D.; Schechinger, B.; Rappold, G. A.; Schömig, E. Molecular Identification of the Corticosterone-Sensitive Extraneuronal Catecholamine Transporter. *Nat. Neurosci.* **1998**, 1 (5), 349–351.
- (74) Koepsell, H.; Lips, K.; Volk, C. Polyspecific Organic Cation Transporters: Structure, Function, Physiological Roles, and Biopharmaceutical Implications. *Pharm. Res.* **2007**, 24 (7), 1227–1251.
- (75) Ogasawara, M.; Yamauchi, K.; Satoh, Y. I.; Yamaji, R.; Inui, K.; Jonker, J. W.; Schinkel, A. H.; Maeyama, K. Recent Advances in Molecular Pharmacology of the Histamine Systems: Organic Cation Transporters as a Histamine Transporter and Histamine Metabolism. *J. Pharmacol. Sci.* **2006**, 101 (1), 24–30.
- (76) Sheldrick, G. M. Crystal Structure Refinement with SHELXL. *Acta Crystallogr. Sect. C Struct. Chem.* **2015**, 71, 3–8.
- (77) Sheldrick, G. M. A Short History of SHELX. *Acta Crystallogr. Sect. A Found. Crystallogr.* **2008**, 64, 112–122.

- (78) Müller, P. Practical Suggestions for Better Crystal Structures. *Crystallogr. Rev.* **2009**, *15*, 57–83.

WHAT IS CLAIMED IS:

1. A compound of Formula I



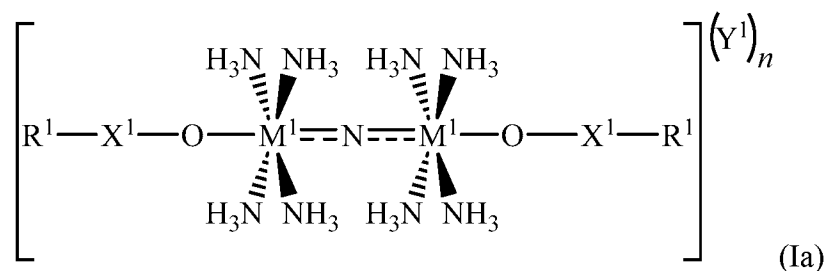
or a pharmaceutically acceptable salt and/or solvate thereof, wherein

M^1 is Ru or Os;

X^1 is ---C(O)--- or ---P(O)(OH)--- ; and

R^1 is H, alkyl, cycloalkyl, heterocyclyl, aryl, or heteroaryl;

optionally wherein the compound of Formula I is a pharmaceutically acceptable salt according to Formula Ia

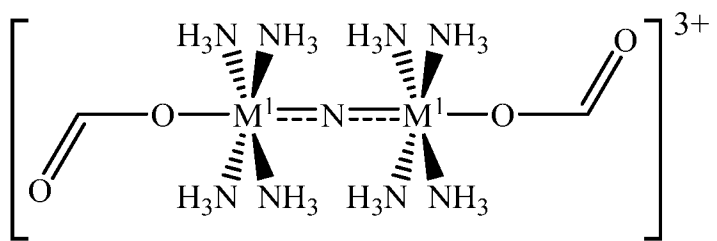


or solvate thereof, wherein

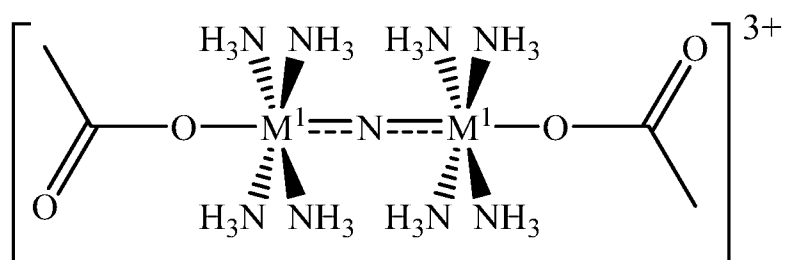
Y^1 is an anion; and

n is 1, 2, 3. .

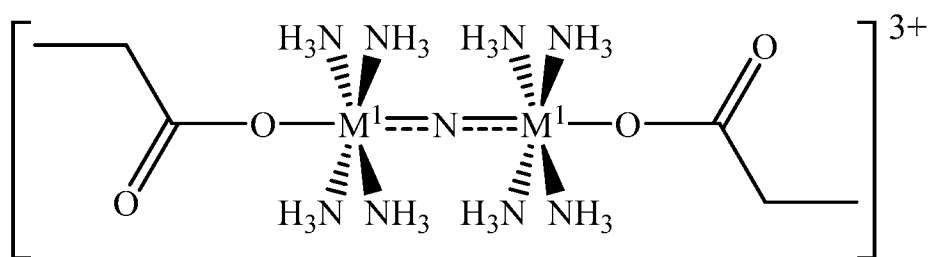
2. The compound of claim 1, wherein X^1 is ---C(O)--- , and R^1 is H or alkyl.
3. The compound of claim 1, wherein X^1 is ---P(O)(OH)--- and R^1 is H alkyl.
4. The compound of claim 1, wherein R^1 is methyl, ethyl, or propyl.
5. The compound of claim 1, wherein X^1 is ---C(O)--- and R^1 is cycloalkyl.
6. The compound of claim 5, wherein R^1 is adamantyl.
7. The compound of claim 1, wherein the compound is a pharmaceutically acceptable salt or solvate of a pharmaceutically acceptable salt of one of the following:



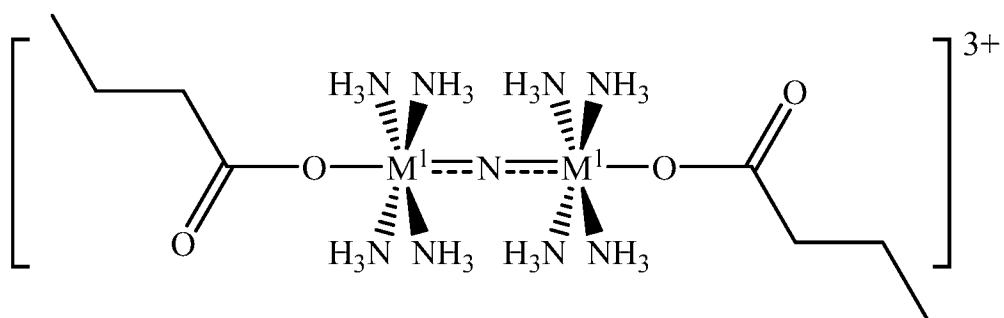
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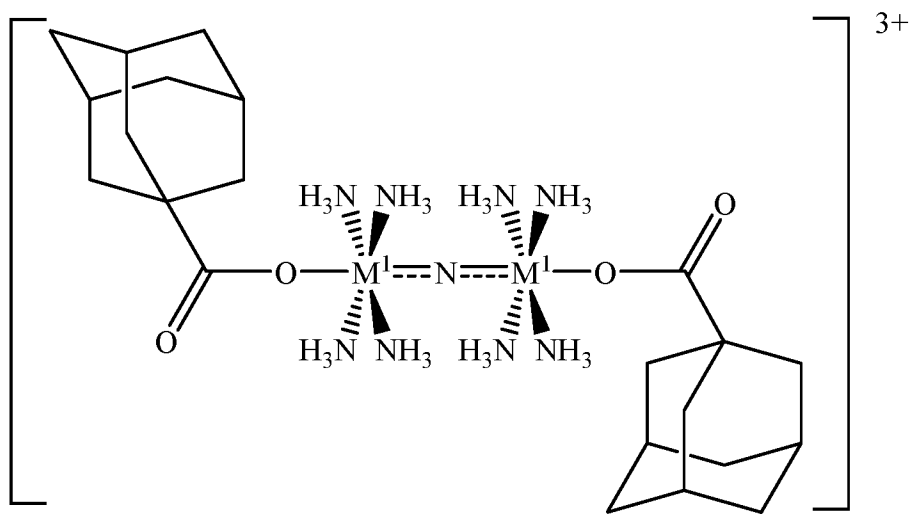
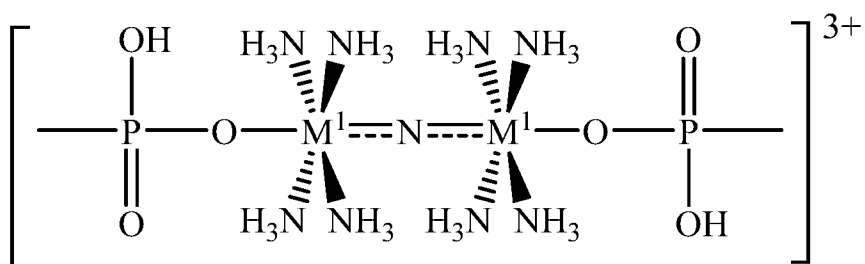
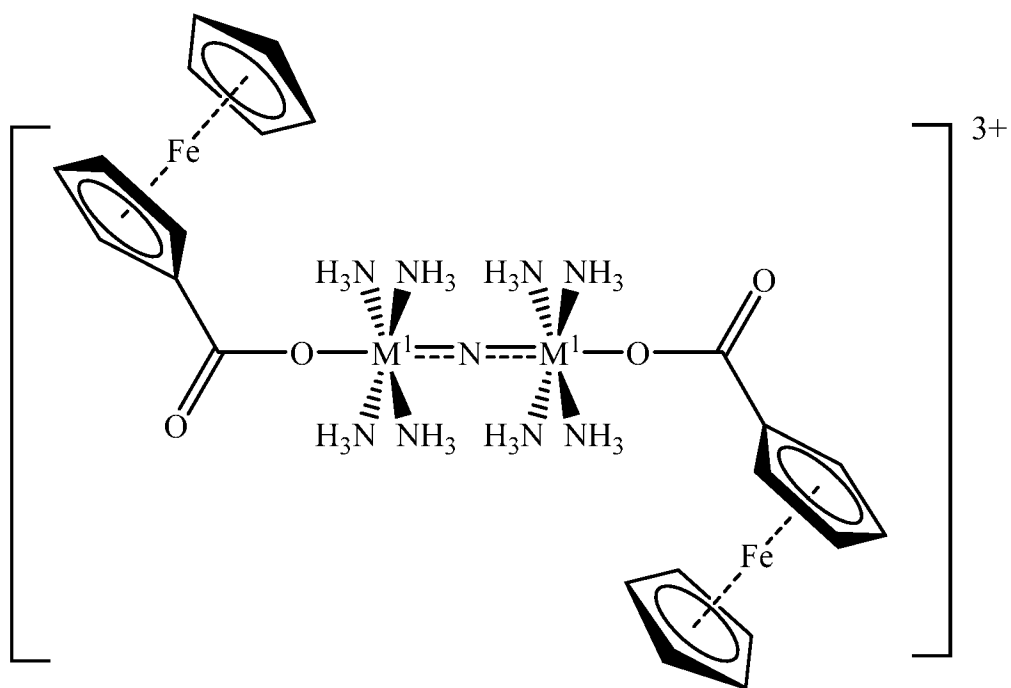
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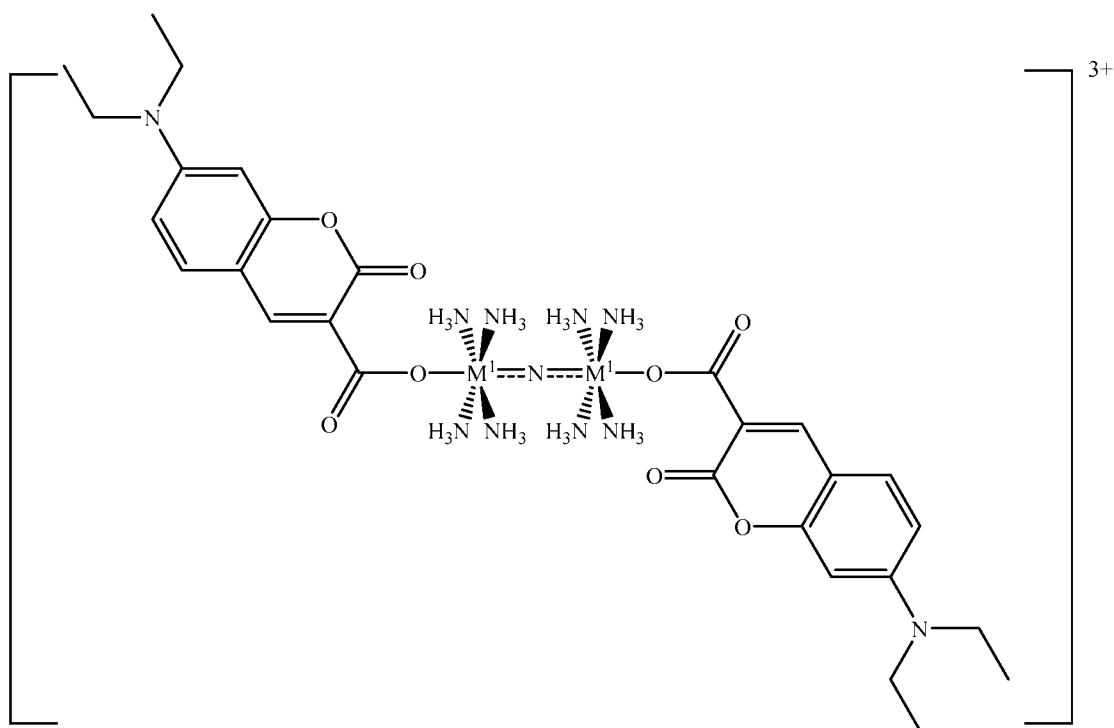
,



,



, or



8. The compound of any one of claims 1-7, wherein M¹ is Ru.
9. The compound of any one of claims 1-7, wherein M¹ is Os.
10. A pharmaceutical composition comprising
 - a compound of any one of claims 1-7; and
 - a pharmaceutically acceptable carrier.
11. A pharmaceutical composition comprising
 - an effective amount of a compound of any one of claims 1-7 for treating a disease or disorder related to mitochondrial Ca²⁺ overload; and
 - a pharmaceutically acceptable excipient.
12. The pharmaceutical composition of claim 11, wherein the disease related to mitochondrial Ca²⁺ overload is selected from a neurodegenerative disorder, a cardiovascular disease, a metabolic disease, cancer, a skeletal muscle disease, cystic fibrosis, and ischemic reperfusion injury.
13. The pharmaceutical composition of claim 12, wherein the neurodegenerative disorder is selected from the group consisting of Alzheimer's disease (AD), Parkinson's disease

(PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS) and cerebral ischemic stroke.

14. The pharmaceutical composition of claim 12, wherein the cancer is selected from the group consisting of breast cancer, pancreatic cancer, colorectal cancer, liver cancer, melanoma, ovarian cancer, and kidney cancer.
15. A method of treating a disease or disorder related to mitochondrial Ca^{2+} overload in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the compound of any one of claims 1-7.
16. The method of claim 15, wherein the disease related to mitochondrial Ca^{2+} overload is selected from a neurodegenerative disorder, a cardiovascular disease, a metabolic disease, cancer, a skeletal muscle disease, cystic fibrosis, and ischemic reperfusion injury.
17. The method of claim 15, wherein the neurodegenerative disorder is selected from the group consisting of Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS) and cerebral ischemic stroke.
18. The method of claim 15, wherein the cancer is selected from the group consisting of breast cancer, pancreatic cancer, colorectal cancer, liver cancer, melanoma, ovarian cancer, and kidney cancer.
19. The method of any one of claims 15-18, wherein the subject is a human.
20. A method of inhibiting mitochondrial calcium uniporter (MCU), the method comprising administering to a subject an effective amount of the compound of any one of claims 1-7 for inhibiting MCU.

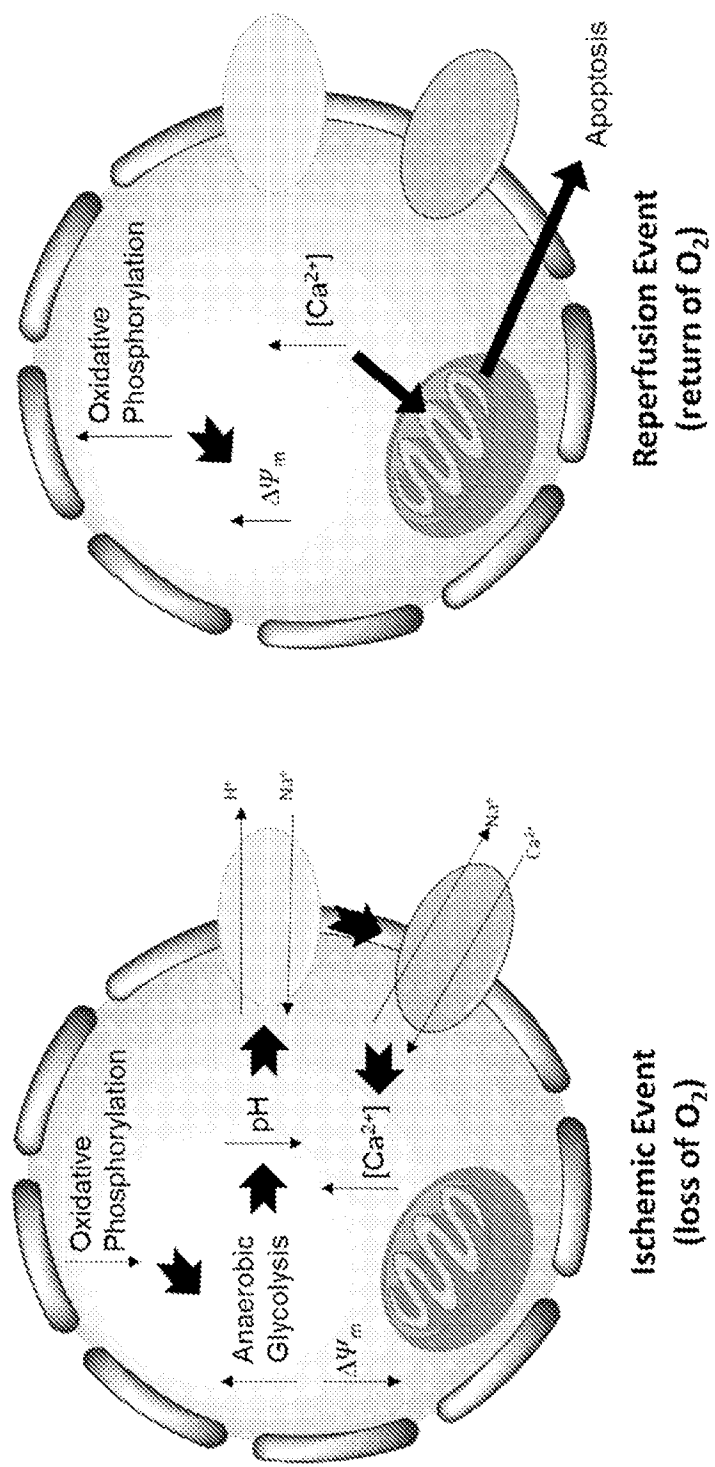


FIG. 1

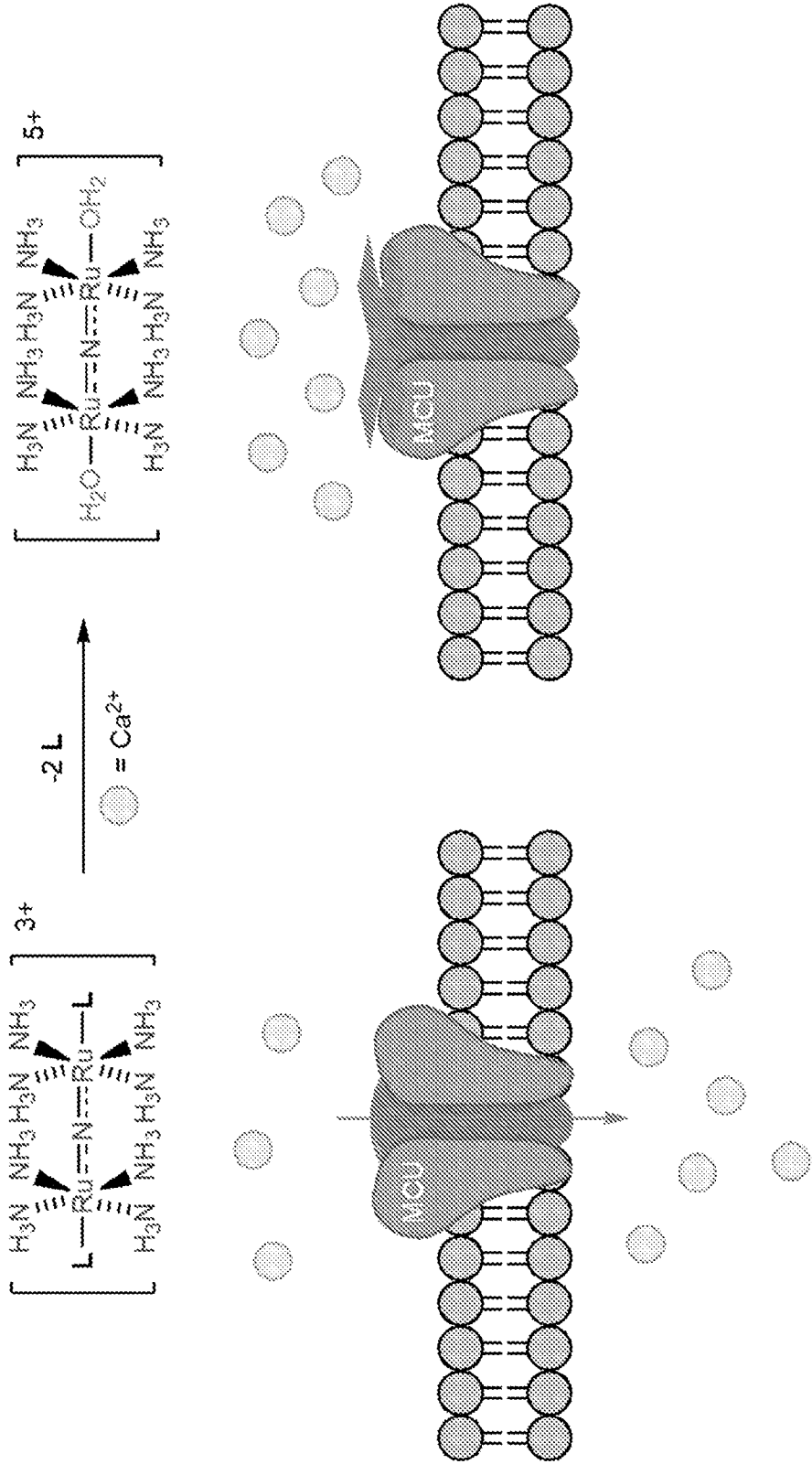


FIG. 2

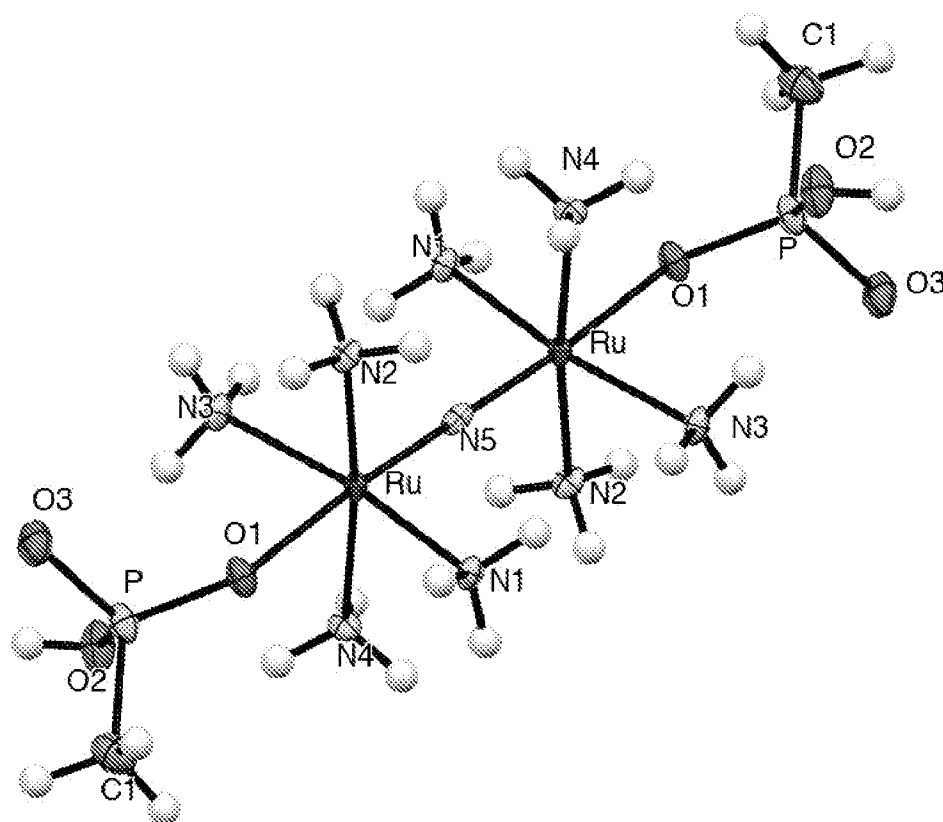


FIG. 3A

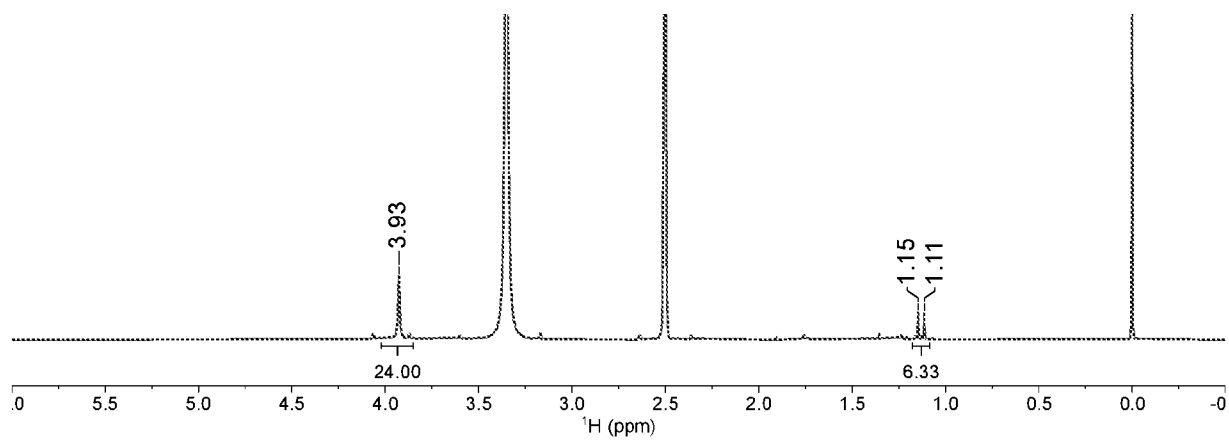


FIG. 3B

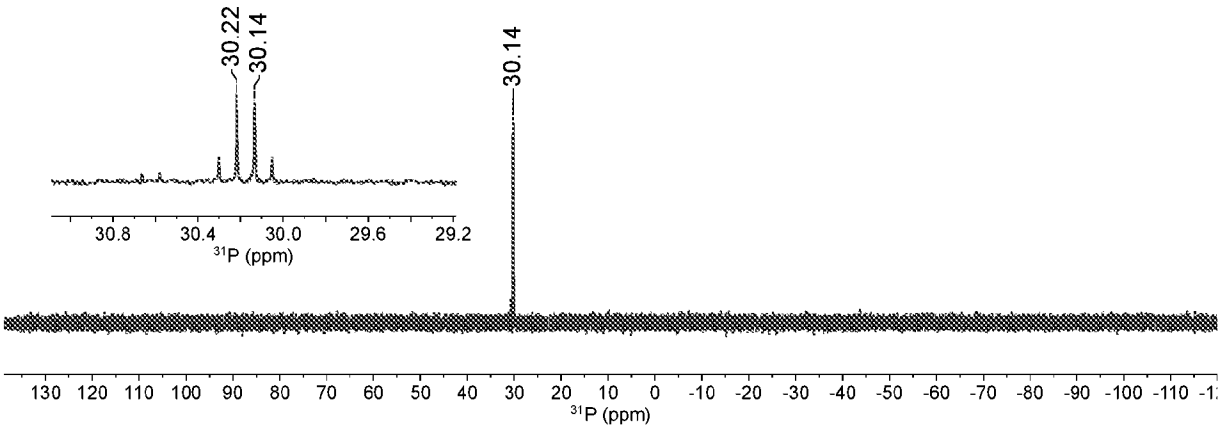


FIG. 3C

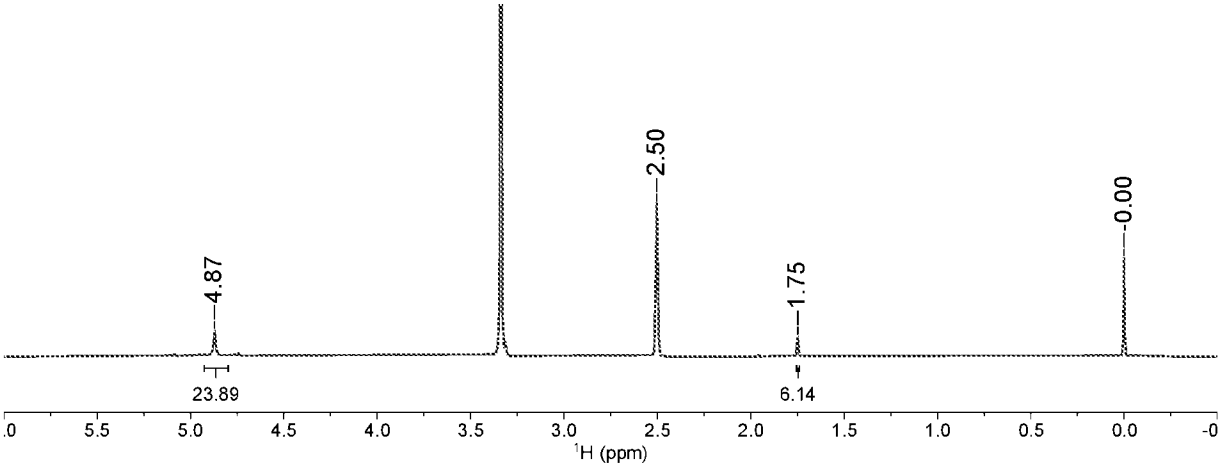


FIG. 4

5/15

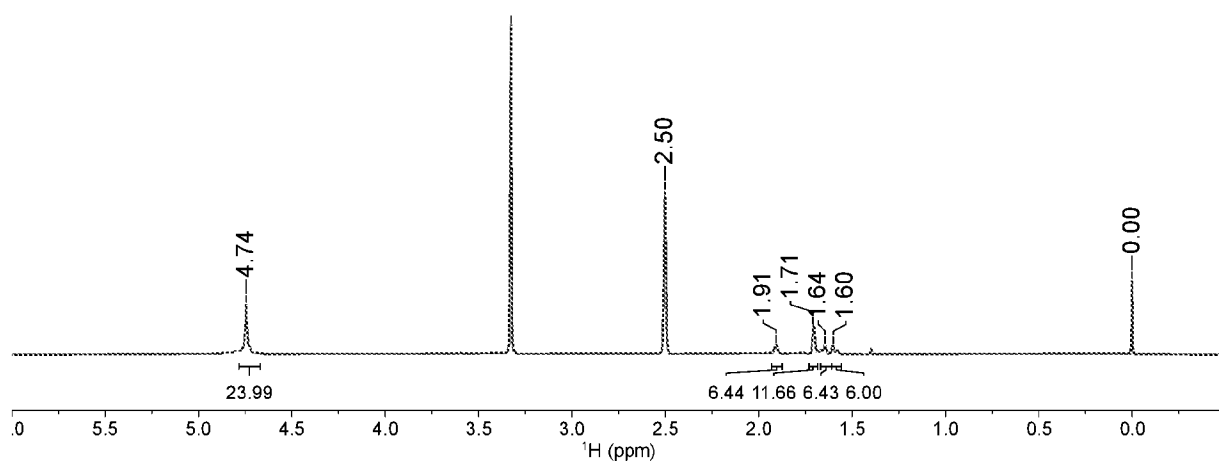


FIG. 5

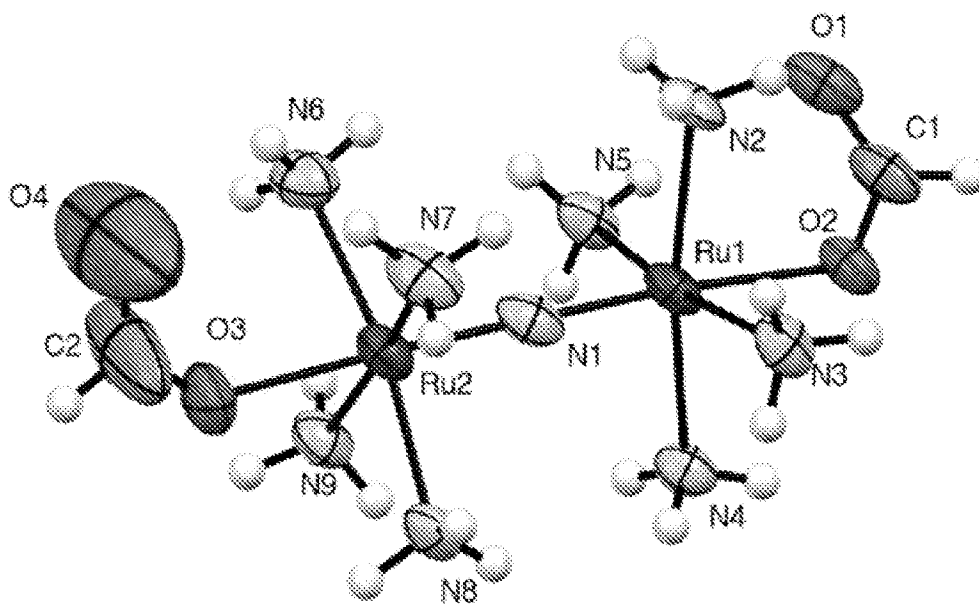


FIG. 6A

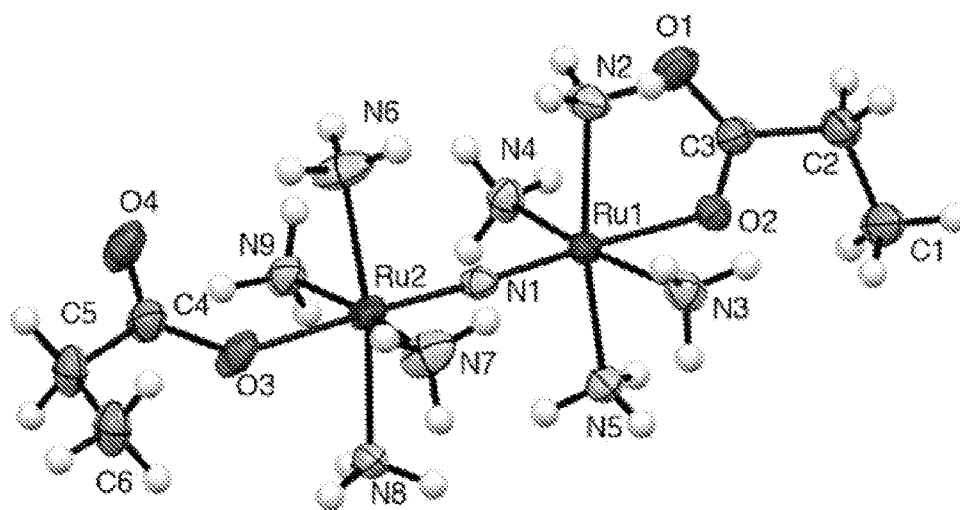


FIG. 6B

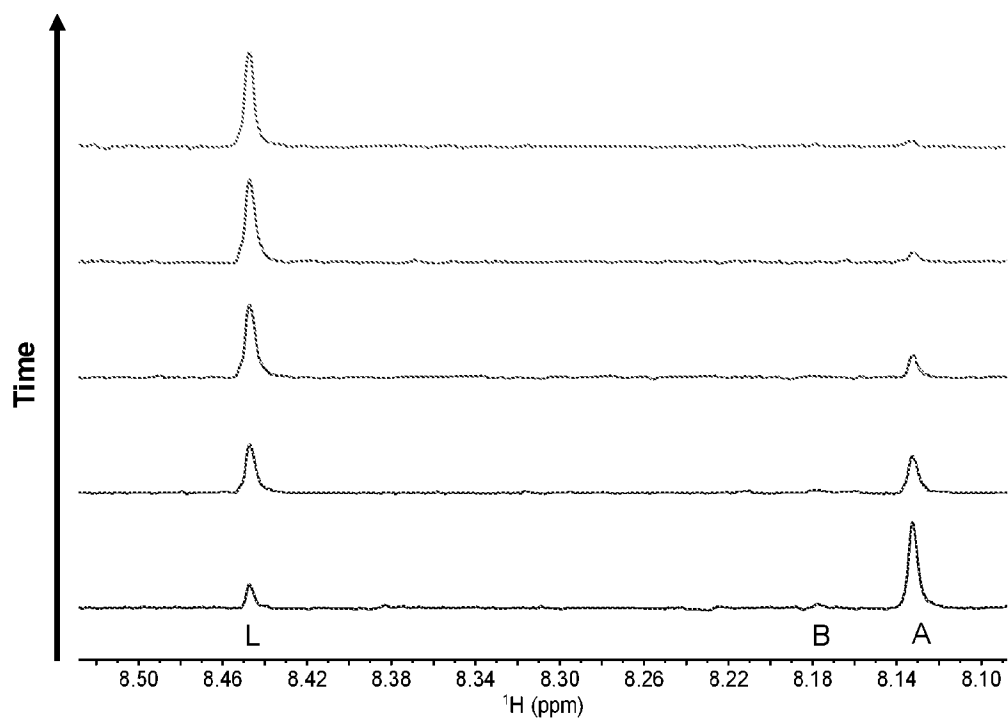


FIG. 7A

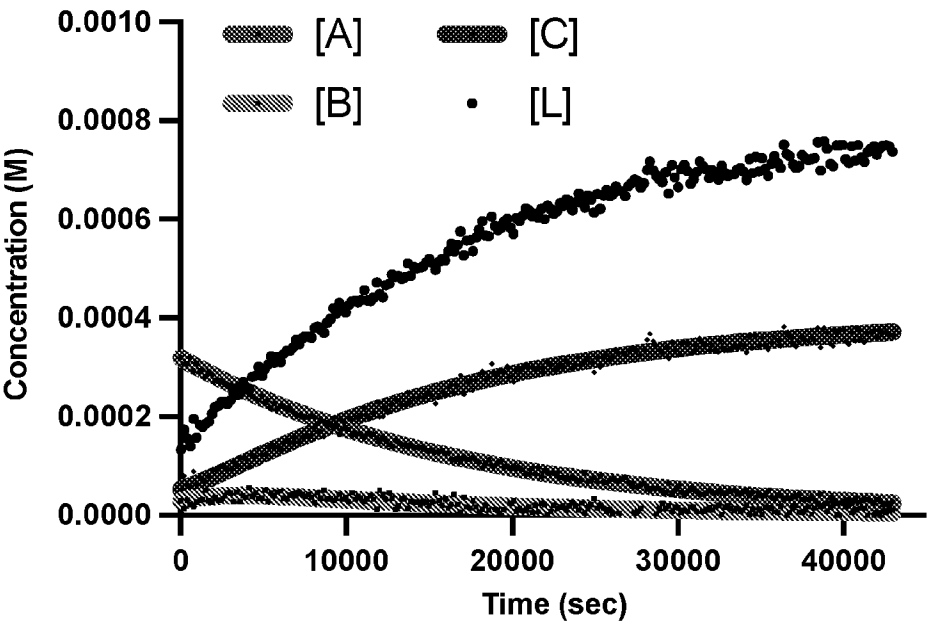


FIG. 7B

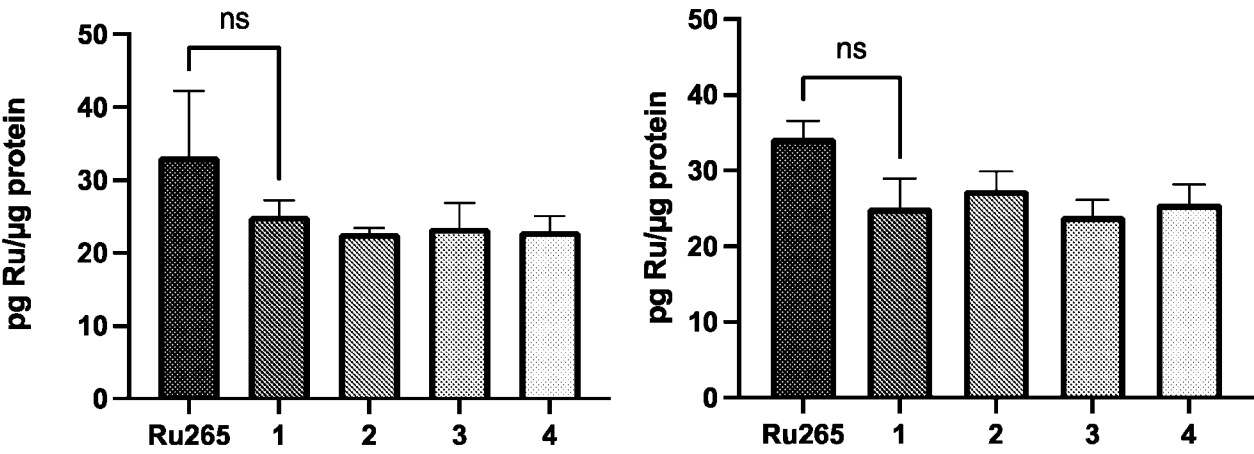


FIG. 8A

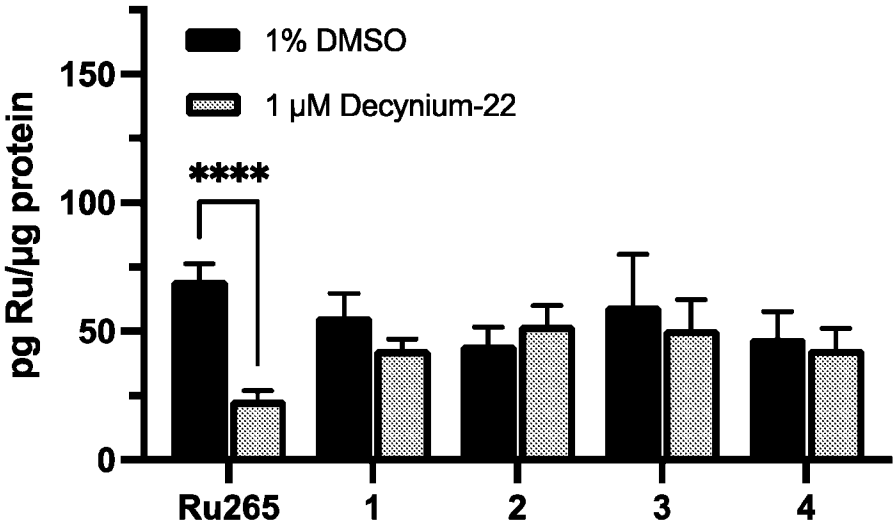


FIG. 8B

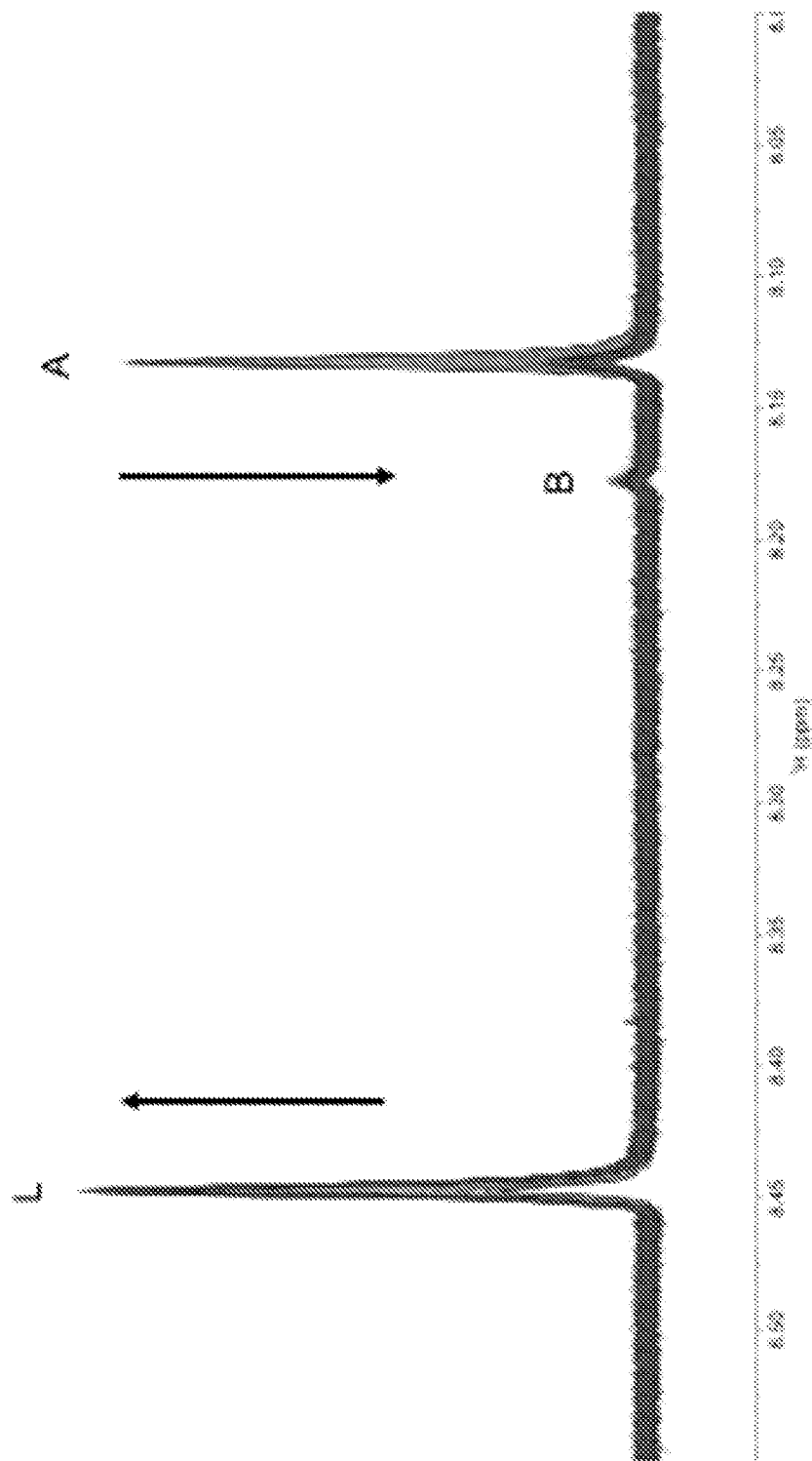


FIG. 9

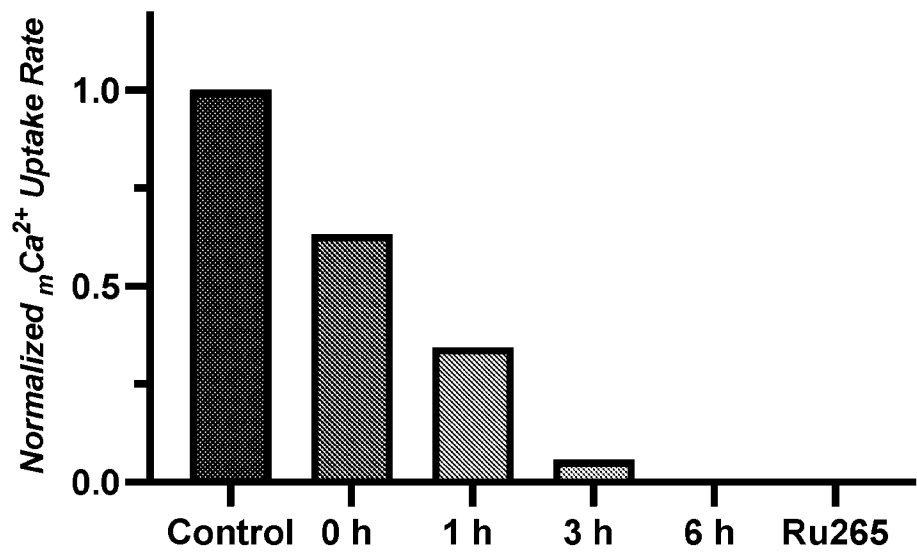


FIG. 10A

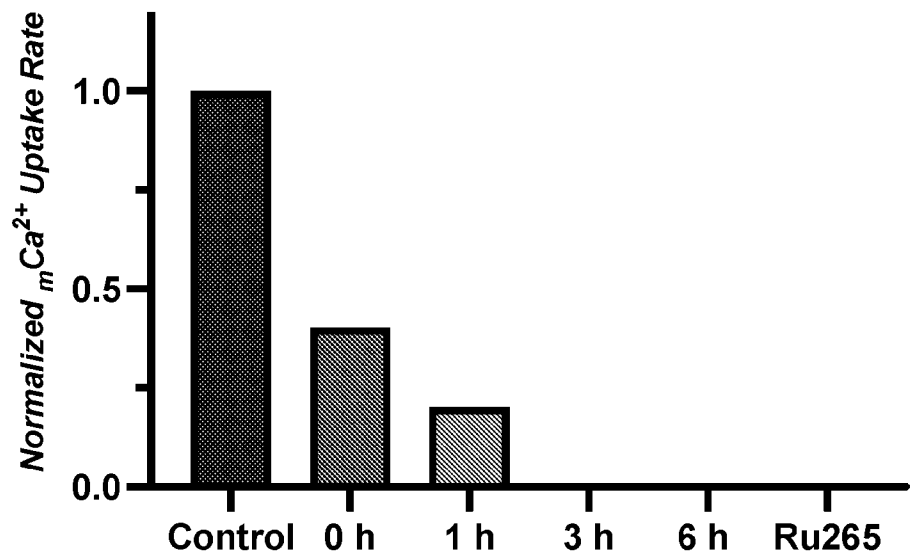


FIG. 10B

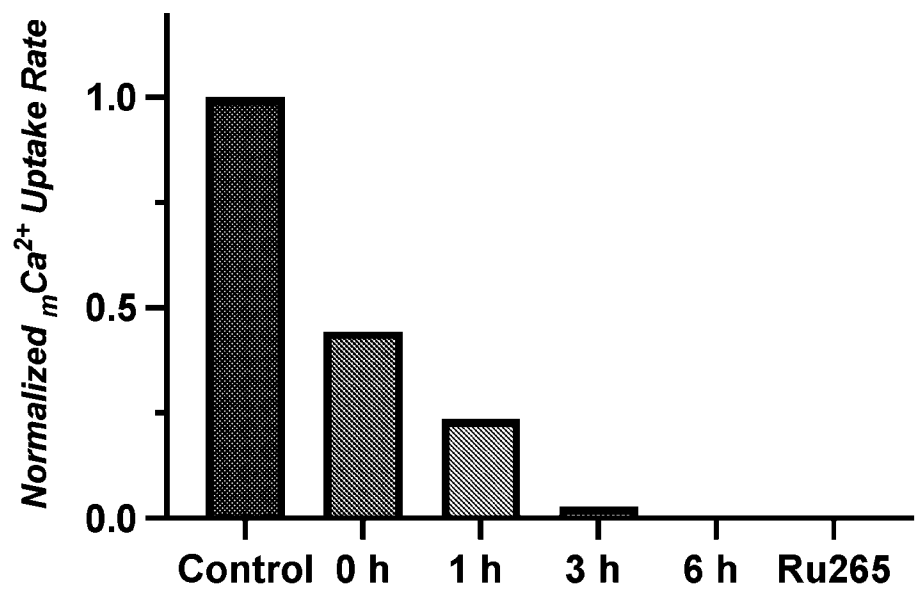


FIG. 10C

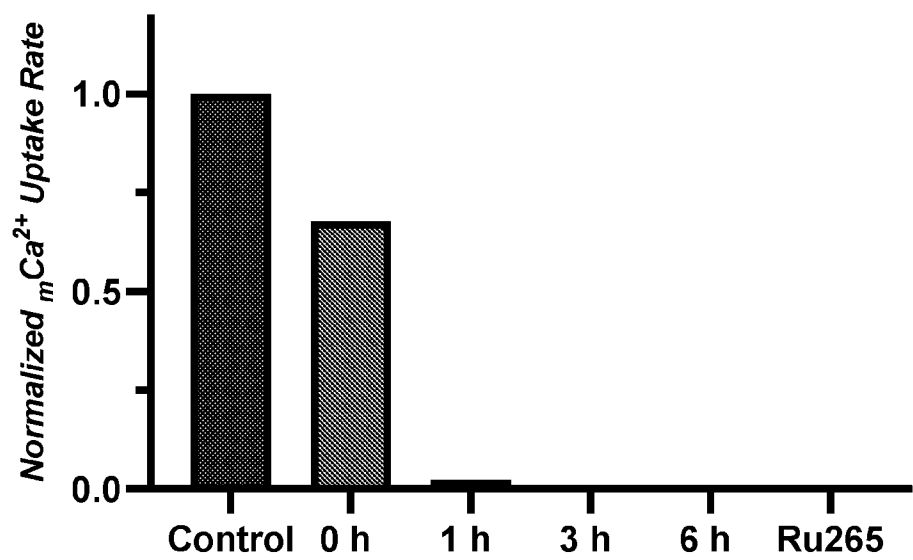


FIG. 10D

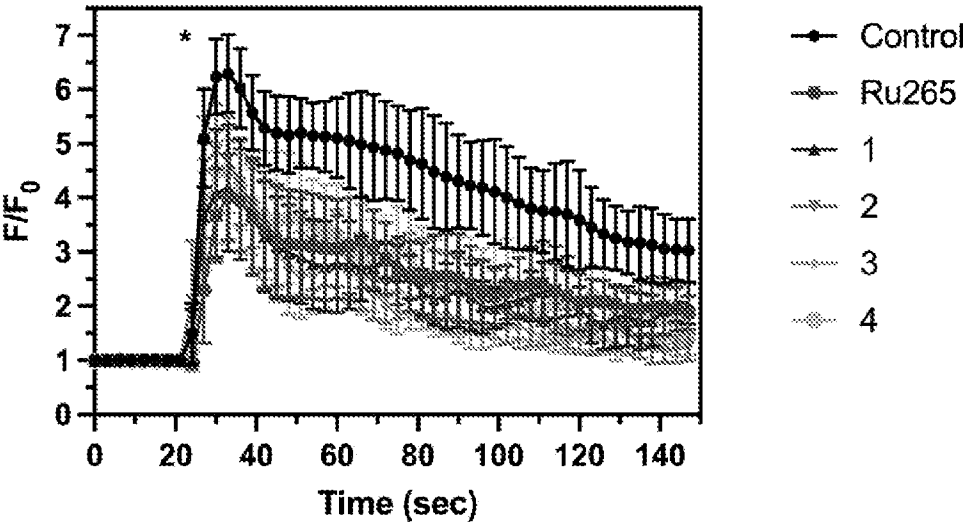


FIG. 11A

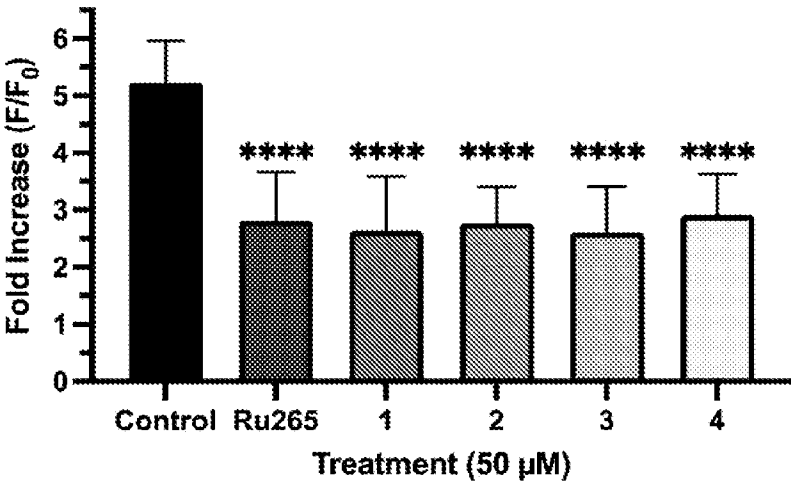


FIG. 11B

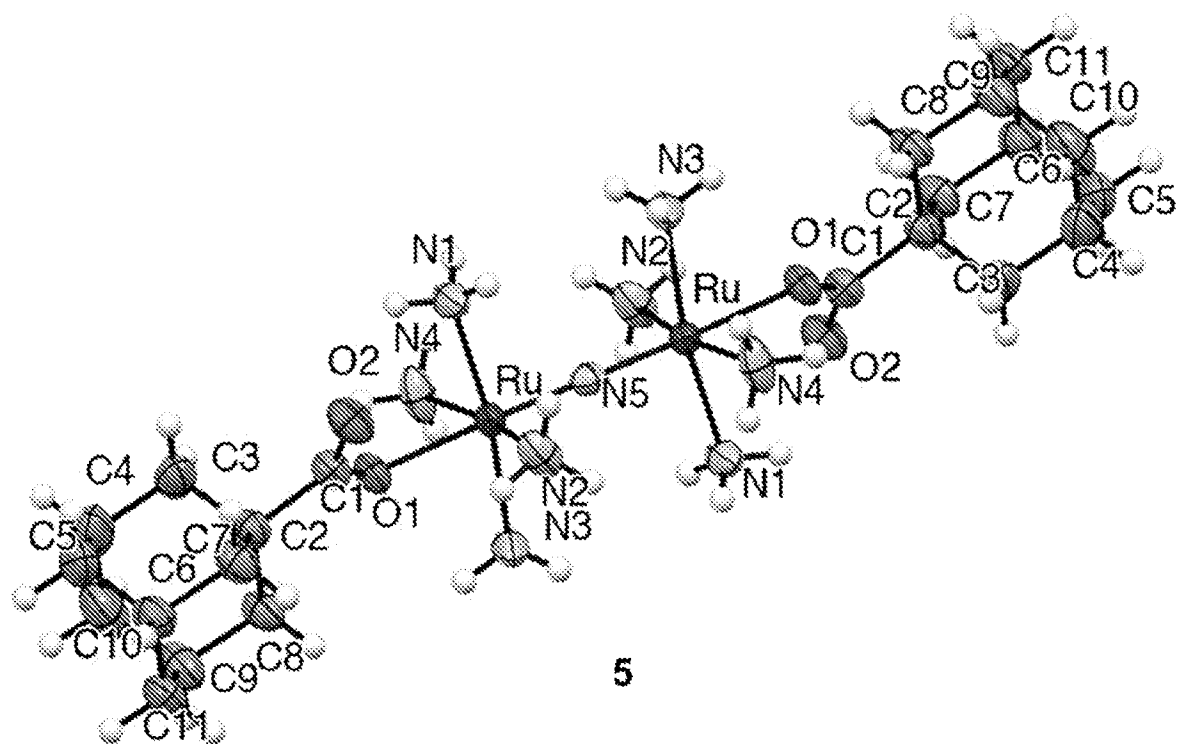


FIG. 12

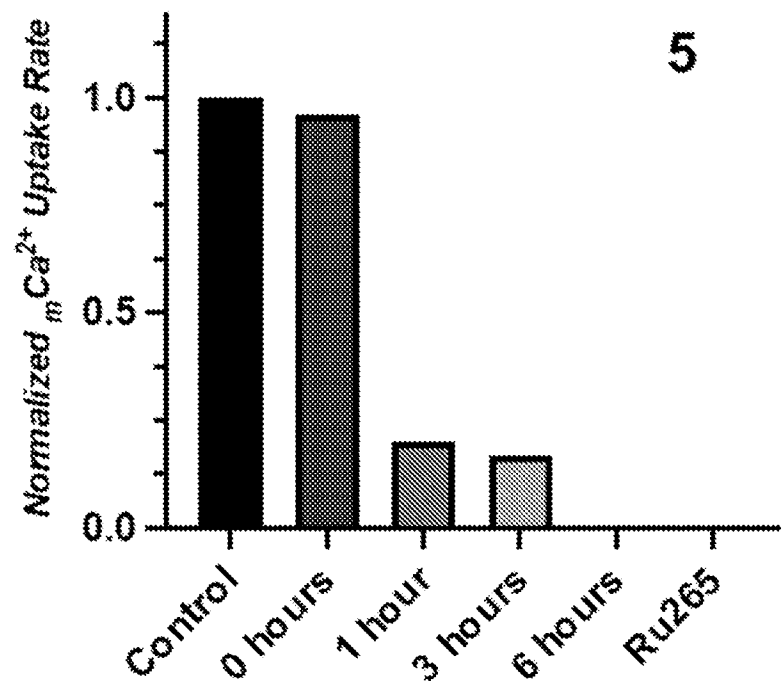


FIG. 13

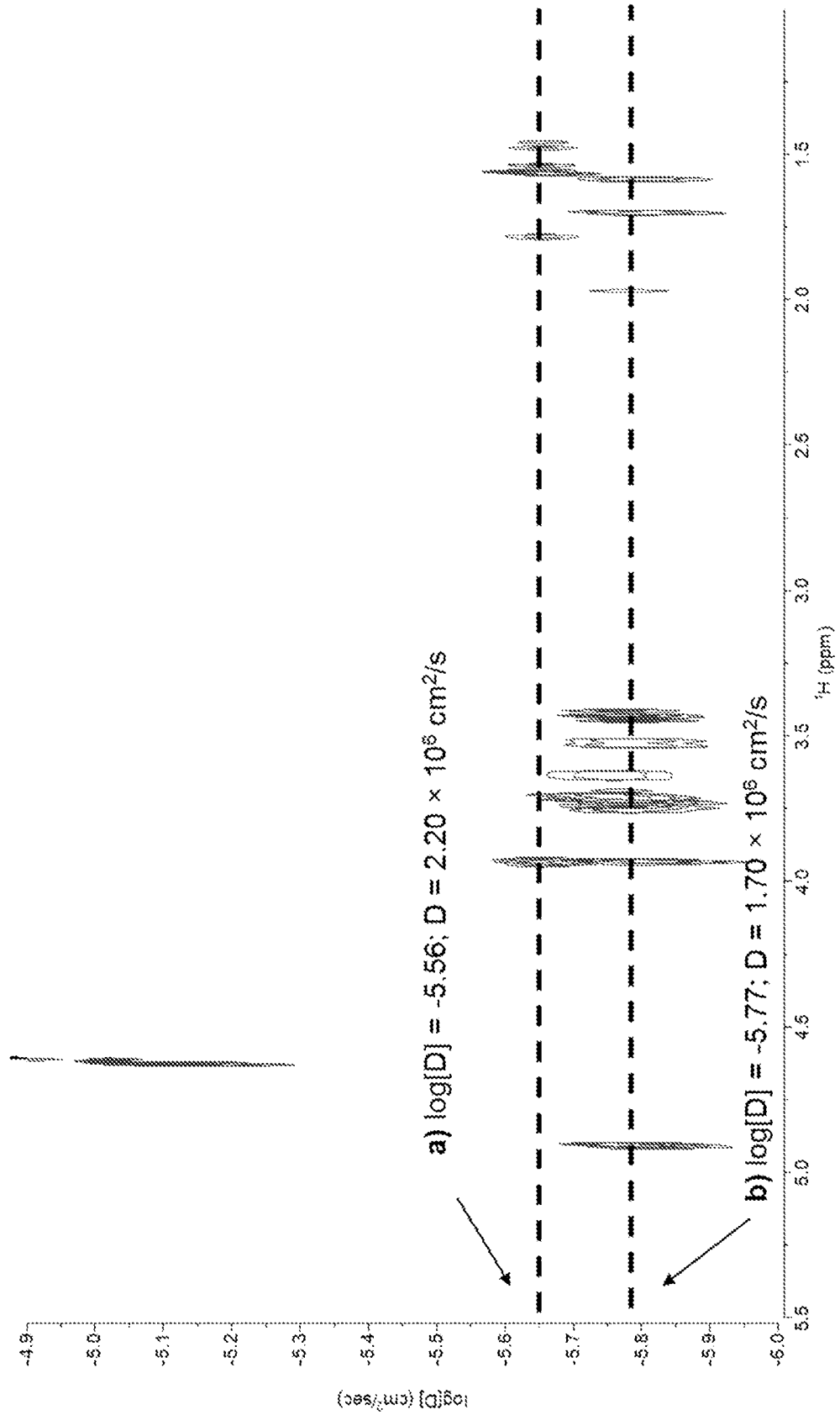


FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2023/078616

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A61P 25/28** (2023.01); **A61P 35/00** (2023.01); **C07F 15/00** (2023.01)CPC: **C07F 15/0053**; **C07F 15/0026**; **A61P 35/00**; **A61P 25/28**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2019/0247427 A1 (CORNELL UNIVERSITY et al.) 15 August 2019 (15.08.2019) entire document	1, 2, 8, 10-18, 20
A	CN 113029918 A (ZHEJIANG HOSPITAL) 25 June 2021 (25.06.2021) see machine translation	1, 2, 8, 10-18, 20
A	ZAZUETA et al., Inhibitory Properties of Ruthenium Amine Complexes on Mitochondrial Calcium Uptake, Journal of Bioenergetics and Biomembranes, Vol. 31, No. 6, 1999 [retrieved on 25 January 2024]. Retrieved from the Internet: <URL: https://link.springer.com/article/10.1023/A:1005464927366 >. Pgs. 551-557	1, 2, 8, 10-18, 20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

25 January 2024 (25.01.2024)

Date of mailing of the international search report

15 March 2024 (15.03.2024)

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. **571-273-8300**

Authorized officer

MATOS
TAINA

Telephone No. **571-272-4300**

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: **19**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-18 and 20 are drawn to compounds of Formula I or a pharmaceutically acceptable salt and/or solvate thereof; pharmaceutical compositions thereof, methods of treating thereof, and methods of inhibiting thereof.

The first invention of Group I+ is restricted to a compound of shown Formula I or a pharmaceutically acceptable salt and/or solvate thereof, wherein M1 is Ru; X1 is -C(O)-; and R1 is H; pharmaceutical compositions thereof; methods of treating thereof; and methods of inhibiting thereof. The first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. Specifically, the first named invention was selected based on the first listed element for each of the variables presented in the claims (M1, X1, R1 – claim 1). It is believed that claims 1, 2, 8, 10-18 and 20 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the above embodiment.

Applicant is invited to elect additional formula(e) for each additional compound to be searched in a specific combination by paying an additional fee for each set of election. Each additional elected formula(e) requires the selection of a single definition for each compound variable. An exemplary election would be a compound of shown Formula I or a pharmaceutically acceptable salt and/or solvate thereof, wherein M1 is Os; X1 is -C(O)-; and R1 is H; pharmaceutical compositions thereof; methods of treating thereof; and methods of inhibiting thereof. Additional formula(e) will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the “+” group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulae do not share a significant structural element requiring the selection of alternatives for the compound variables M1, X1, and R1 and accordingly these groups lack unity a priori.

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

Additionally, even if Groups I+ were considered to share the technical features of a compound of the core structure of Formula I or a pharmaceutically acceptable salt and/or solvate thereof; a pharmaceutical composition thereof; a method of treating a disease or disorder related to mitochondrial Ca²⁺ overload in a subject in need thereof; and a method of inhibiting mitochondrial calcium uniporter (MCU), these shared technical features do not represent a contribution over the prior art as disclosed by US 2019/0247427 A1 to Cornell University et al.

US 2019/0247427 A1 to Cornell University et al. teach a compound of the core structure of Formula I or a pharmaceutically acceptable salt and/or solvate thereof (Fig. 1B, compound C-2, see shown structure); a pharmaceutical composition thereof (Para. [0036], pharmaceutical compositions that contain any of the above described ruthenium complexes dispersed in a pharmaceutically acceptable carrier); a method of treating a disease or disorder related to mitochondrial Ca²⁺ overload in a subject in need thereof (Para. [0041], method for treating or preventing a disease or condition that operates by calcium transport through the mitochondrial calcium uniporter (MCU); Para. [0010], protect cardiomyocytes from the mCa²⁺ overload); and a method of inhibiting mitochondrial calcium uniporter (MCU) (Para. [0041], method for treating or preventing a disease or condition that operates by calcium transport through the mitochondrial calcium uniporter (MCU) ... inhibits the MCU).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1, 2, 8, 10-18, 20**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.