# Using Bioorthogonally Catalyzed Lethality Strategy to Generate Mitochondria-Targeting Antitumor Metallodrugs *in vitro* and *in vivo*

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## ABSTRACT

Synthetic lethality was proposed nearly a century ago by geneticists and recently applied to develop precision anticancer therapies. To exploit the synthetic lethality concept in the design of chemical anticancer agents, we developed a bioorthogonally catalyzed lethality (BCL) strategy to generate targeting antitumor metallodrugs both *in vitro* and *in vivo.* Metallodrug **Ru-rhein** was generated from two nontoxic species **Ru-$N_3$** and **rhein-alkyne** *via* exclusive endogenous copper-catalyzed azide alkyne cycloaddition (CuAAC) reaction without the need of an external copper catalyst. The nontoxic species Ru-arene complex **Ru-$N_3$** and **rhein-alkyne** were designed to perform this strategy, and the mitochondrial targeting product **Ru-rhein** was generated in high yield (> 83%) and showed high antitumor efficacy *in vitro*. This BCL strategy achieved a remarkable tumor suppression effect on the tumor-bearing mice models. It is interesting that the combination of metal-arene complexes with rhein *via* CuAAC reaction could transform two nontoxic species into a targeting anticancer metallodrug both *in vitro* and *in vivo*, while the product **Ru-rhein** was nontoxic towards normal cells. This is the first example that exclusive endogenous copper was used to generate metal-based anticancer drugs for cancer treatment. The

anticancer mechanism of **Ru-rhein** was studied and autophagy was induced by increased reactive oxygen species and mitochondrial damage. The generality of this BCL strategy was also studied and it could be extended to other metal complexes such as Os-arene and Ir-arene complexes. Compared with the traditional methods for cancer treatment, this work presented a new approach to generate targeting metallodrugs *in vivo via* the BCL strategy from nontoxic species in the metal-based chemotherapy.


## Keywords

metallodrug; cancer therapy; mitochondrial targeting; bioorthogonally catalyzed lethality; bioorthogonal reaction.


## INTRODUCTION

Synthetic lethality, named by Theodore Dobzhansky in 1946, was an interesting genetic phenomenon originally discovered in *Drosophila melanogaster*: loss of function of either of two gene alone has little effect on *Drosophila* viability, whereas inactivation of both genes simultaneously leads to *Drosophila* death [1]. Synthetic lethality was predicted to be used for the development of anticancer drugs [2-3], and a series of poly ADP-ribose polymerase (PARP) inhibitors such as Olaparib (2014), Rucaparib (2016) and Niraparib (2017), have received FDA approval for use in clinical cancer therapies, verifying that synthetic lethality has provided interesting information for the development and clinical approval of other therapies [4]. The advantage of synthetic lethality-based approach is that it adds a degree of cancer targeting. For example, PARP inhibitors can pose specific cytotoxicity only in cancer cells with a breast cancer (BRCA) gene mutation, no cytotoxicity to the normal cells that do not carry the mutation [5]. However, there are a large number of genetic mutations in cancer cells, it is complicated, time-consuming and expensive to simultaneously screen for two specific genes that can combine and cause lethality.

Inspired by the synthetic lethality feature of *Drosophila*, a bioorthogonally catalyzed lethality (BCL) strategy could be a promising alternative to avoid screening for specific genes and selectively kill cancer cells while minimizing the side effects towards normal cells. Screening two non-toxic chemical entities which only reacted efficiently in cancer cells but not normal cells to produce toxic products would make it easier to achieve the synthetic lethality. Bioorthogonal chemistry has now become

one of the most powerful tools in drug discovery and chemical biology [6-9]. Bioorthogonal chemistry can specifically label and probe a wide variety of biomolecules in living cells and animals [10-13], and used for drug design and synthesis *in situ* [14-17]. It refers to the chemical reactions that can be performed in living cells or tissues without interfering with the biochemical reaction of the organism itself. The copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC) is the most common type of bioorthogonal reactions, most of which use copper as the catalyst [18, 19]. The catalyst Cu species play a key role in the life system and frequently serve as a catalytic cofactor for enzymes that function in antioxidant defense, iron homeostasis, cellular respiration and a variety of biochemical processes [20]. However, the copper concentrations were reported to be significantly elevated in cancerous tissues to normal tissues [21, 22], which rendered the copper ion as a target for cancer treatment [23-24]. Therefore, taking advantage of the higher Cu levels in tumor cells to generate cancer-specific drugs *in situ via* CuAAC will help achieve targeting treatment towards tumor cells and avoid toxicity to normal cells. Up to date, the traditional chemotherapy treatments for cancer include at least two steps: synthesis and purification of certain anticancer drugs from raw materials through a series of organic synthesis and purification, and then injection or orally administration into human body for treatment. It will be a revolutionary breakthrough if the targeting anticancer drugs could be generated precisely in live cancer cells and animals from the nontoxic materials in the tumor microenvironment.

Metallodrugs such as ruthenium-arene (Ru-arene) complexes have aroused considerable interest as the basis for new anticancer agents due to their specificity in cancer cells, high water solubility, clearance properties and low side effects, which represent desirable characteristics for ideal anticancer drugs [25-33]. Results show that the potential target of Ru-arene complexes in the cell might be DNA and their anticancer mechanism are quite different from that of cisplatin [26, 28-31]. Natural product rhein has been used as laxative and stomach drug, exhibited low toxicity towards normal cells [34, 35] and mainly located in mitochondria, which may be helpful to increase the mitochondria accumulation of metal species and ultimately improve the antitumor activity of the metal complex through the mitochondrial targeting if used as ligand. Herein, we described a BCL strategy to generate metallodrugs in cancer cells *in situ via* CuAAC between two non-toxic compounds,

Ru-arene complex **Ru-$N_3$** and mitochondria-targeting **rhein-alkyne** (Figure 1, Scheme S1). The CuAAC reaction mechanism, determination of the BCL product and its yield *in vitro* were studied by ESI-MS and ICP-MS, the mitochondrial-targeting ability, antitumor cytotoxicity, and anticancer mechanism were studied by state-of-the-art chemical, physical techniques and cell biological assays. The generality of the BCL strategy was evaluated by using other metal complexes such as Os-arene and Ir-arene complexes. The application of BCL *in vivo* was also performed in the xenograft A549 tumor-bearing mice model.

## RESULTS AND DISCUSSION

### Design of the BCL Agents

Compound **rhein-alkyne** was synthesized by modifying rhein with mono-propargylamine through the amidation reaction, and **Ru-$N_3$** was obtained by the reaction of dichloro(*p*-cymene) ruthenium dimer with 2,2'-dipyridyl-$N_3$ (Scheme S1). For the BCL strategy, all the experiments such as the mitochondrial-targeting ability, antitumor cytotoxicity, reaction mechanism and determination, anticancer mechanistic studies of BCL product *in vitro* were carried out by incubation the 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$** with a range of cancer cell lines or normal cells, respectively, and the application of BCL *in vivo* was performed in the xenograft A549 tumor-bearing mice model by injection of the 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$**. And all the comparative experiments were repeated by using chemical synthesized product **Ru-rhein** as the same procedures. The comparison reactions between the 1:1 **rhein-alkyne** and **Ru-$N_3$** in the chemical reactor were performed with or without additional Cu catalysts, respectively. Only the reaction with additional Cu catalysts gave the final product **Ru-rhein**, which was purified and characterized by $^1$H NMR, and ESI-MS spectroscopy (Figures S1-S5). Besides, the UV-vis and photoluminescence spectra of **rhein-alkyne** and **Ru-rhein** (10 μM) exhibited that both of them showed good absorption and emission properties ($\lambda_{abs}$=~430 nm, $\lambda_{em}$=~600 nm, Figure S6), which favored monitoring their cell uptake and intracellular distribution by confocal fluorescence imagings. **Ru-rhein** is represented as the BCL product **Ru-rhein** generated *in vitro* or *in vivo*, and the chemical synthesized product is defined as chemical synthesized **Ru-rhein** *infra.*

**Mitochondrial-Targeting of the CuAAC Product**

To verify the possibility of the BCL *via* CuAAC in living cells, the intracellular distribution of **rhein-alkyne** and **Ru-$N_3$** were conducted using confocal microscopy and ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) methods. We have detected the enrichment of **Ru-$N_3$** and **rhein-alkyne** in different organelles after short time of incubation. After incubation of 10 μM **rhein-alkyne** for 4 h or 12 h, the blue fluorescence of **rhein-alkyne** and red fluorescence of Mito-Tracker overlapped well, with the Pearson's correlation coefficient of 0.89 (Figure S7) or 0.90 (Figure 2A). The intracellular amount of Ru was very small after incubation of 10 μM **Ru-$N_3$** for 4 hours, but after 8 or 12 hour-incubation **Ru-$N_3$** could enter cells in large amounts and about 65% or 66 % were enriched in mitochondria (Figure 2B and Figure S8). These suggested that both **rhein-alkyne** and **Ru-$N_3$** were mainly accumulated in the mitochondria of the cells after 8 to 12 h-incubation. Moreover, the cellular location of the chemical synthesized **Ru-rhein** in A549 cells also indicated that the cycloaddition product **Ru-rhein** was enriched in the mitochondria (Figure S9). All these results could serve as a basis for the *in situ* azide-alkyne cycloaddition between **rhein-alkyne** and **Ru-$N_3$**.

**Antitumor Cytotoxicity of BCL Product *in vitro***

We further determined the antitumor cytotoxicities of **rhein-alkyne**, **Ru-$N_3$**, mixture of **rhein-alkyne** and **Ru-$N_3$** (molar ratio of 1:1) towards a range of cancer cell lines or normal LO2 and HLF cells through the MTT assays, with cisplatin as the control group. As shown in Figure 2C, both **rhein-alkyne** and **Ru-$N_3$** exhibited little or no cytotoxicity towards both cancer cells and two normal cell lines, with the $IC_{50}$ values ranging from ~46.2 to over ~100.0 μM. However, the growth of the cancer cells was significantly inhibited and the $IC_{50}$ values was 5.6 μM, as toxic as that of cisplatin (3.3 μM), when 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$** was added to the A549 cell culture (Figure S10). Furthermore, the 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$** also exhibited high cytotoxicity against A2780 and MCF-7 cells, with the $IC_{50}$ values of 10.5 μM and 12.1 μM, respectively. More importantly, the mixture of **rhein-alkyne** and **Ru-$N_3$** exhibited low toxicity against normal LO2 and HLF cells with $IC_{50}$ values of ~53.9 and ~67.3 μM (Figure S11), much lower than that of cisplatin, indicating

high selectivity towards cancer cells. These results suggested that the BCL strategy was realized and the antitumor efficacy was activated when cycloaddition reaction between **rhein-alkyne** and **Ru-$N_3$** occurred in cancer cells *in situ*.

**Investigation on the BCL Product and CuAAC Reaction Mechanism *in vitro***

Based on the high antitumor activity of 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$** in cancer cell lines, we hypothesized a metallodrug with high efficacy was generated in the cancer cells. Several experiments were carried out to determine the BCL product *in vitro.* After the A549 cells were treated with 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$**, **Ru-$N_3$** or **rhein-alkyne** alone, as well as chemical synthesized **Ru-rhein**, respectively, the main ingredient inside the cancer cells were characterized by using the ESI-MS techniques. For comparison, complexes **Ru-$N_3$** or **rhein-alkyne**, and the chemical synthesized **Ru-rhein** were also characterized by ESI-MS techniques in methanol solution, respectively. As shown in Figures S12 and S13, it is interesting to note that after the nontoxic species **Ru-$N_3$** or **rhein-alkyne** enter into the A549 cells, their composition remains unchanged, for the positive-ion MS peak (m/Z) at 322.33 assigned for **rhein-alkyne** or peak at 496.08 for [**Ru-$N_3$** - $PF_6$]$^+$, matching the results well with the samples measured in methanol solutions. Only 1 positive-ion peak (m/Z) at 817.25 was observed for the chemical synthesized **Ru-rhein** in methanol solution (Figure S5), it was assignable to the cycloaddition product [**Ru-rhein** - $PF_6$]$^+$ (817.15). It is remarkable that the same two positive-ion peak (m/Z) at 817.25 and 909.17 were observed for the A549 cells treated with the 1:1 mixture of **rhein-alkyne** and **Ru-$N_3$** (Figure 2A) or with the chemical synthesized **Ru-rhein** (Figure 2B), respectively, and these isotope patterns for peaks at 817.25 were as same as that observed for the chemical synthesized **Ru-rhein** in methanol solution, indicating the formation of cycloaddition product **Ru-rhein** when **rhein-alkyne** and **Ru-$N_3$** encountered each other in A549 cells. The ESI-MS peak at 909.17 might be attributed to the signal of its lysine adduct for [**Ru-rhein** - $PF_6$ - Cl - $H^+$ + lysine]$^+$, which was calculated m/z of 909.34. These results confirmed the successful generation of **Ru-rhein** *in vitro via* alkyne-azide cycloaddition of **rhein-alkyne** and **Ru-$N_3$** without additional Cu catalysts in living cancer cells.

It is worth noting that, without copper catalyst (CuI or $CuSO_4$/NaAsc), the alkyne-azide cycloaddition reaction could not react between **Ru-$N_3$** and **rhein-alkyne** in DMF solution, even under severe conditions of long time and high temperature.

However, such azide-alkyne cycloaddition reaction could react easily without copper catalyst within the intracellular compartment *in situ* and exhibited high reaction yield for **Ru-rhein** in mild conditions, which might be attributed by the higher levels of endogenous copper in tumor cells. It is reported that the content of copper ions in lung cancer patients is 1.3-2.0 times more than that of non-cancerous patients. Scanni [36] found that the copper concentration of normal patients was ~143.03 μg/dL, while the copper levels in cancer patients increased to ~188 ug, about 1.3 times. Diez [37] also pointed out that the copper ion level of Stage III cancer patients was 1.5 times more than that of normal patients, of which concentration was ~150 μg/dL and 100 μg/dL. Thus, we further detected the concentrations of copper species in several cancer and normal cell lines. As shown in Figure S14, the copper concentrations are ~41.3 (A2780), 67.9 (A549), 55.1 (MCF-7), 24.2 (LO2), 21.6 (HLF) ppb/$10^7$ cells respectively, proving that the copper concentrations were indeed higher in cancer cells than that of normal cells. This was consistent with the cytotoxicities of **Ru-rhein**, again verifying our design that the higher levels of copper species could enhance the selectivity against cancer cells and the higher levels of intracellular copper species resulted in higher cytotoxicity of **Ru-rhein** (Table S1). Overall, this suggested that the elevated copper levels in cancer cells might be an important copper source for tumor cells to achieve the BCL in tumor cells.

**Yields of BCL Product Ru-rhein *in vitro***

ICP-MS technique was used for the first time to determine the yields of BCL product **Ru-rhein** *in vitro*. Two concentrations (5 and 10 μM) of **Ru-$N_3$** or 1:1 mixture of **Ru-$N_3$** and **rhein-alkyne** were incubated with A549 cells for 24 h was monitored by ICP-MS techniques, respectively. As shown in Figure 3C, the Ru content in the mitochondria of 5 μM **Ru-$N_3$**-loaded A549 cells was determined to be ~18.7 ng/μg protein, accounted for 62.8% of the total Ru content. When the A549 cells were treated with 5 μM 1:1 mixture of **Ru-$N_3$** and **rhein-alkyne**, the Ru content in the mitochondria was reached up to ~19.3 ng/μg protein, accounted for 77.5% of the total quantity, implied that introduction of rhein into metallodrug could improve the mitochondrial enrichment of Ru species. The yield of **Ru-rhein** *in vitro* was up to ~83.6%, which obtained *via* dividing the total content of **Ru-rhein** in whole A549 cells (24.9 ng) by the total content of **Ru-$N_3$** in whole A549 cells (29.8 ng) ×100%. Similarly, when the A549 cells were incubated with 10 μM **Ru-$N_3$** or 1:1 mixture of

**Ru-$N_3$** and **rhein-alkyne**, respectively, the percentage of Ru in the mitochondria increased from 60.6% to 75.6%, and the yield of **Ru-rhein** *in vitro* was ~88.0%. These results indicated that the existence of rhein could enhance the targeting mitochondrial of Ru species, and higher concentration of 1:1 mixture of **Ru-$N_3$** and **rhein-alkyne** help to generate product in higher yield, both contributed to greatly improve the BCL efficiency.

**Mechanistic Studies of the Cell Death by BCL Product Ru-rhein *in vitro***

Several cell biological assays were used to investigate the mechanism of the cell death induced by **Ru-rhein** *in vitro*. Since the **Ru-rhein** mainly localized in mitochondria, we supposed that the cell death might be related to mitochondrial death pathways. Mitochondria is a major source and may act as a point of integration for death signals originating from both the extrinsic and intrinsic pathways [38], such as an elevation of reactive oxygen species (ROS) levels, variations in mitochondrial membrane potential and the generation of autophagy. The impact of **Ru-rhein** on intracellular ROS level, mitochondrial membrane potential and autophagy-related protein expression was investigated. When the A549 cells were treated with **Ru-rhein**, the levels of intracellular ROS increased and the mitochondrial membrane potential was disturbed severely with a concentration-dependent manner (Figures S15-S17). The excess ROS caused obvious oxidative stress and mitochondria dysfunction in A549 cells exposed to **Ru-rhein**, we explored whether the cytotoxic effect of **Ru-rhein** was related to the induction of autophagy.

We further examined the autophagy by using confocal laser scanning microscopy, transmission electron microscope (TEM) and western blot analysis. Monodansylcadaverine (MDC) staining is a specific assay for autolysosomes, which is dependent on a second ubiquitin-like protein-binding system (located on the membrane of the autophagy vesicle, which can be specifically bound to it) [39]. As shown in Figure 4A, positively dot-like structures were observed both in the peripheral region of the nucleus and selectively aggregated in the autophagy vesicles when A549 cells were treated with **Ru-rhein** for 12 h, while no blue fluorescence was observed in the cytoplasm in the control group without **Ru-rhein** incubation, indicating that **Ru-rhein** induced the formation of autophagosomes. **Ru-rhein**-induced autophagy was also monitored by TEM (Figure 4B). Numerous double-membraned cytosolic autophagic vacuoles in A549 cells were observed after

treatment with **Ru-rhein** for 24 h, while such autophagic vacuoles were not found in the control group without drug treatment. These results verified that significant autophagy occurred in A549 cells after treatment of **Ru-rhein**.

Moreover, microtubule-associated protein light chain 3 (LC3), as a mammalian homolog of yeast Atg8, has been used as a specific marker to detect autophagy, occurrence of autophagy will render LC3 conjugate to phosphatidylethanolamine and target to autophagic membranes [40]. Western blotting experiments were then performed using an anti-LC3 antibody to verify the expression of related autophagy proteins for A549 cells after treated with **Ru-rhein**. After treatment with cisplatin, **rhein-alkyne** and **Ru-$N_3$** for 24 h, the expression of LC3-II in A549 cells was almost negligible compared to that in the positive control HCQ (Figure 4C). However, an obvious increase in LC3-II protein expression was observed when A549 cells were treated with **Ru-rhein**, and the ratio of LC3-I to LC3-II expression was 1.3 times higher than that in the control group without drug treatment (Figure 4D), proving the induction of autophagy by **Ru-rhein**, which was consistent with the results of confocal imagings of MDC and TEM images. Taken together, these evidences indicated that **Ru-rhein** mainly caused autophagic cell death of A549 cells through alterations in oxidative stress and mitochondria membrane potential. Similarly, the main mechanism of chemical synthesized **Ru-rhein** to cause cell death was also autophagy induced by increased reactive oxygen species and mitochondrial damage (Figure S18). These results also indicated that the BCL strategy enabled the formation of mitochondria-targeted antitumor metallodrug **Ru-rhein** *in vitro*. In addition, we also investigated the effects of **Ru-rhein** treatment on cell cycle and apoptosis of A549 cells, which showed **Ru-rhein** did not cause cell death by blocking the cell cycle and only caused a small amount increased population of early apoptotic cells (Figures S19 and 20).

**Application of BCL *in vivo***

Since BCL was achieved successfully in cancer cells *in vitro*, we further investigated whether the BCL could be triggered by endogenous Cu in A549 tumor-bearing mice models. The mice were divided randomly into 4 groups (6 mice/group) and intratumorally injected with **Ru-rhein** (1:1 mixture of **Ru-$N_3$** and **rhein-alkyne**), **rhein-alkyne**, **Ru-$N_3$** and saline once every three days for 18 days. As shown in Figures 5A and 5C, the growth of the tumors was significantly inhibited after the mice

were treated with **Ru-rhein**, while the mice treated with **rhein-alkyne**, **Ru-$N_3$** or saline showed rapid growth of the tumors. These results suggested that **Ru-rhein** displayed high anticancer efficacy and achieved BCL *in vivo*, which was consistent with the *in vitro* results. Besides, the body weight of the mice treated with different samples showed no obvious change during the cancer treatment (Figure 5B), indicating that **Ru-rhein** displayed little toxicity to the mice.

The haematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining assays were further performed to evaluate the efficacy of different treatments (Figure 5D). The images of H&E-stained tumor tissue exhibited distinct necrosis of tumor cells treated by **Ru-rhein**, and the images obtained from the TUNEL staining also showed the highest level of cell death in the tumor tissue of the mice treated with **Ru-rhein**. While the images of H&E-stained tumor tissue and the TUNEL staining after treated with the control groups (**rhein-alkyne**, **Ru-$N_3$** and saline) could not exhibit obvious tumor necrosis. Besides, the chemical synthesized **Ru-rhein** could also induce high level of cell death in the tumor tissue and significantly inhibit the growth of the tumor *in vivo* (Figure S21). Taken together, these results suggested that **Ru-rhein** exhibited satisfactory therapeutic effects *in vivo*, proving the BCL strategy can also be applied in living mice.

**Generality of the BCL Strategy**

To probe the general applicability of the BCL, Os-arene and Ir-arene complexes were used to form BCL products *via* azide-alkyne cycloaddition between two nontoxic species of **rhein-alkyne** and **M-$N_3$** (M=Os, Ir) in cancer cells (Scheme S1). And the chemical synthesized complexes were characterized by $^1$H NMR, and ESI-MS spectroscopy (Figures S22-S27). The antiproliferative activities of the 1:1 mixture of **rhein-alkyne** and **M-$N_3$** (M=Os, Ir) against several cell lines were determined (Table S2), both the **rhein-alkyne** and **M-$N_3$** (M=Os, Ir) complexes exhibited poor or no cytotoxicity, while the cycloaddition products **M-rhein** (M=Os, Ir) displayed high cytotoxicities with $IC_{50}$ values decreasing to 13.3 μM. This not only confirmed the successful cycloaddition reaction between **rhein-alkyne** and **M-$N_3$** (M=Os, Ir) species in living cells, but also showed the enhanced cytotoxicities towards cancer cells. These results indicated the potential generality of the BCL strategy, and this

strategy might be extended to a wider range for developing metallodrugs for cancer chemotherapy.

## CONCLUSIONS

Inspired by the synthetic lethality, we selected two non-toxic components which could generate efficient anticancer species only in cancer cells *in situ* without external catalyst, such strategy is named as the bioorthogonally catalytic lethality strategy (BCL). Catalytic products **M-rhein** (M = Ru, Os or Ir) were generated *in vitro* by adopting exclusive endogenous copper-catalyzed azide alkyne cycloaddition (CuAAC) reaction between **M-$N_3$** and mitochondria-targeting **rhein-alkyne** without an additional copper catalyst. This BCL strategy generated targeting metallodrugs with high antitumor efficacy both *in vitro* and *in vivo* and could be extended to a wider range for metallodrugs cancer therapy. Moreover, this BCL strategy could be realized the remarkable tumor suppression effect on the tumor-bearing mice models. The combination of metal-arene complexes with rhein *via* CuAAC reaction could transform two nontoxic species into targeting high anticancer metallodrugs **M-rhein**, which are nontoxic to normal cells. The autophagy was the main cause for the cancer cell death and this was consistent with the fact that **Ru-rhein** is a mitochondria-targeting antitumor metallodrug. The generality of this strategy was also studied by replacing of the metal species by nontoxic osmium or iridium precursors. This strategy not only avoided complicated pre-synthesis of the metallodrugs but also was expected to overcome the decomposition and inactivation of pre-synthesized cycloaddition product *in vitro* and *in vivo*. Generation of metallodrugs *in vivo* by the BCL strategy might be a new approach compared with the traditional treatment pattern of anticancer metallodrugs, might provide a new pattern and platform for precise, targeted and efficient anticancer chemotherapy, especially for metal-based drugs.

## SUPPLEMENTARY DATA

Supplementary data are available at NSR online.


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## AUTHOR CONTRIBUTIONS

J. Z., H.-K.L. and Z.-J.G. proposed and supervised the project. X.-X.L., C.-G.Q., J. Z., H.-K.L. and Z.-J.G. conceived and designed the experiments. X.-X.L., Q.T. and M.-D.L. carried out the synthesis and most of the structural characterizations. X.-X.L., C.-G.Q., Q.T., Y.-X.D., M.-D.L. and J.-W.D. carried out biological experiments, including the cell and mice experiments. X.-X.L., C.-G.Q., Z.S., Y.Q., J. Z., H.-K.L. and Z.-J.G. co-wrote the manuscript. All authors discussed the results and participated in analyzing the experimental results.

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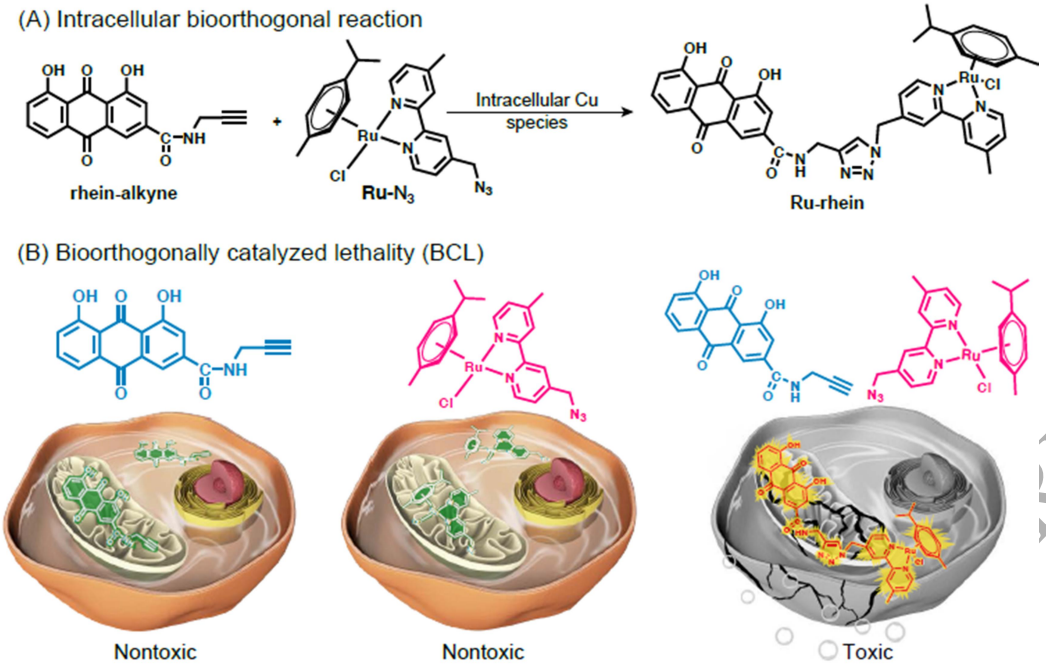
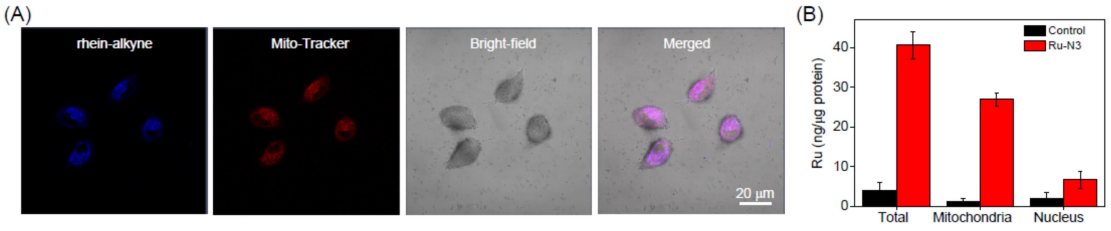


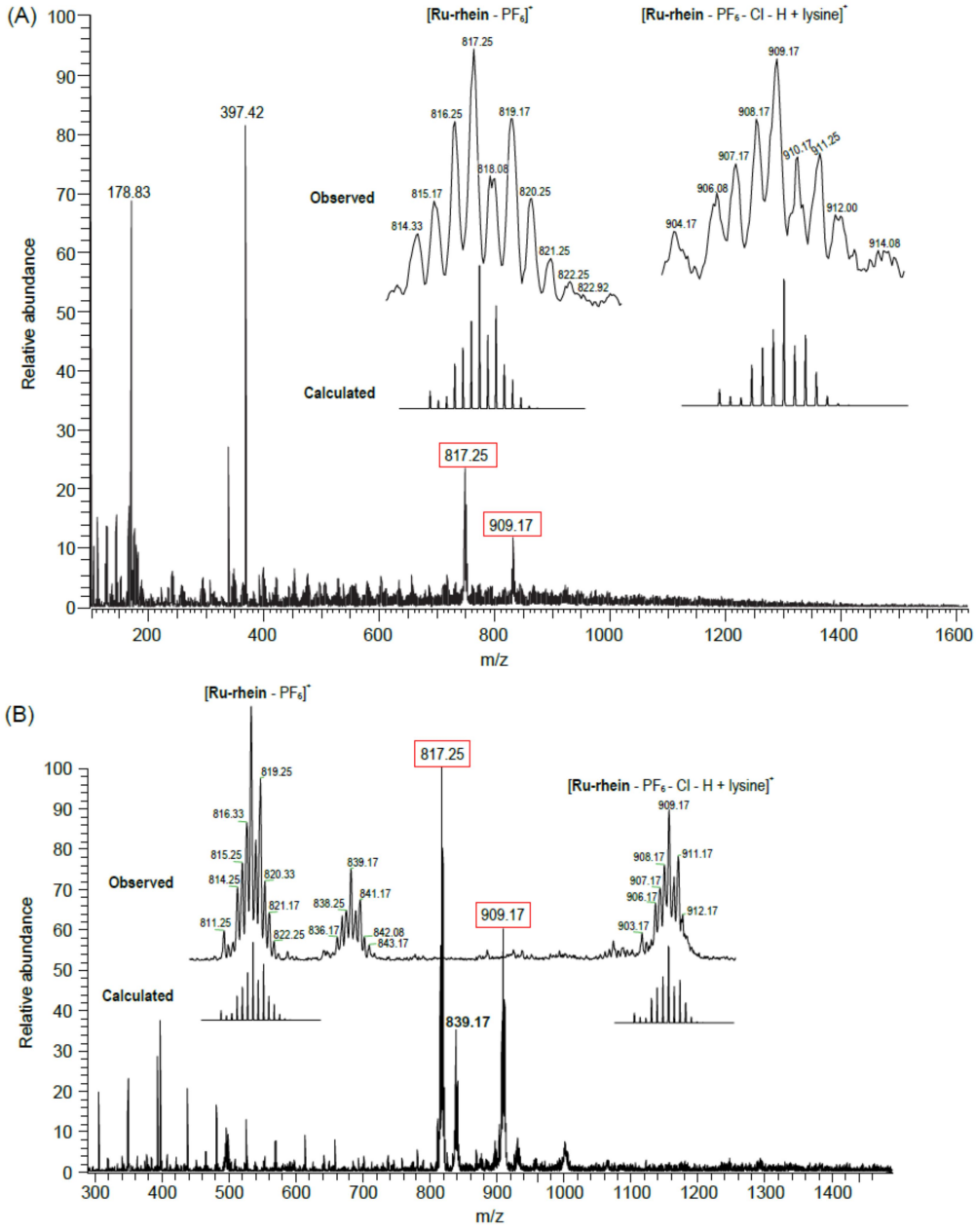

**Figure 1**. (A) By utilizing the high efficiency and selectivity of bioorthogonal reaction, the natural product derivative **rhein-alkyne** and ruthenium precursor **Ru-$N_3$** were designed to generate the anticancer metallodrug **Ru-rhein** *in situ* with copper species as the catalyst. Besides, the higher copper levels in tumor cells might help realize the bioorthogonal reaction and kill the tumor cells selectively without the need of an external copper catalyst; (B) Bioorthogonally catalyzed lethality (BCL) between **rhein-alkyne** and **Ru-$N_3$** in tumor cells. Both **rhein-alkyne** and **Ru-$N_3$** exhibited poor or no cytotoxic activities, once they encounter inside the cancer cells, the *in-situ* cycloaddition product **Ru-rhein** *via* bioorthogonal reaction exhibited high cytotoxicity against cancer cells, realizing the BCL.




(C)

| Cell line | $IC_{50}$ values (μM) for 48 h | | | |
|---|---|---|---|---|
| | rhein-alkyne | Ru-$N_3$ | Ru-rhein | Cisplatin |
| A549 | 51.2 ± 0.6 | >100 | 5.6 ± 1.6 | 3.3 ± 1.5 |
| A2780 | 46.2 ± 1.4 | >100 | 10.5 ± 0.7 | 6.0 ± 1.2 |
| MCF-7 | 60.7 ± 2.1 | >100 | 12.1 ± 0.9 | 5.5 ± 0.6 |
| HLF | 55.2 ± 1.3 | >100 | 67.3 ± 1.4 | 12.7 ± 0.6 |

**Figure 2**. The successful cycloaddition reaction between **rhein-alkyne** and **Ru-$N_3$** in A549 cells. (A) Confocal microscopy images of A549 cells incubated with **rhein-alkyne** (10 μM) for 12 h, then co-localized with Mito-Tracker Deep Red. The Pearson correlation coefficients were 0.90, respectively. $\lambda_{ex}$=405 nm, $\lambda_{em}$=520-700 nm for **rhein-alkyne**; $\lambda_{ex}$=543 nm, $\lambda_{em}$= 580-700 nm for Mito-Tracker. Scale bar: 20 μm; (B) Cellular uptake of Ru in A549 cells with 10 μM **Ru-$N_3$** determined by ICP-MS (ng/μg protein), verifying the colocalization of **rhein-alkyne** and **Ru-$N_3$** in the mitochondria; (C) The $IC_{50}$ (μM) values for **rhein-alkyne**, **Ru-$N_3$**, **Ru-rhein**, and cisplatin against cancer and normal (HLF, LO2) cell lines for 48 h, indicating that **Ru-rhein** exhibited enhanced cytotoxicity towards cancer cells and low toxicity to normal cells, confirmed the successful *in situ* BCL in cancer cells.




(C)

| | | Mitochondria | Nucleus | Cytosol | Total | Mito/Total (%) | Yield (%)[a] |
|---|---|---|---|---|---|---|---|
| 5 μM | **Ru-$N_3$** | 18.7 ± 1.6 | 4.4 ± 1.4 | 6.8 ± 1.9 | 29.8 | 62.8 | 83.6 |
| | **Ru-rhein** | 19.3 ± 2.7 | 2.1 ± 1.0 | 3.5 ± 1.2 | 24.9 | 77.5 | |
| 10 μM | **Ru-$N_3$** | 29.4 ± 2.1 | 5.7 ± 0.6 | 13.4 ± 1.4 | 48.5 | 60.6 | 88.0 |
| | **Ru-rhein** | 32.3 ± 1.7 | 4.9 ± 0.9 | 5.5 ± 2.3 | 42.7 | 75.6 | |

[a]Yield = Total (**Ru-rhein**) / Total (**Ru-$N_3$**) × 100%

**Figure 3.** ESI-MS results of A549 cells after treated with 10 μM **Ru-rhein** without Cu(I) catalysts (A) and 10 μM chemical synthesized **Ru-rhein** (B) for 12 h. The m/Z

peak 817.25 is assigned as [**Ru-rhein** - $PF_6$]$^+$ (calculated 817.29). The m/Z peak 909.17 is assigned as [**Ru-rhein** - Cl **-** $PF_6$ - H + lysine]$^+$ (calculated 909.34). The similar m/Z peaks at 817.25 of (A) and (B) indicated the successful *in situ* CuAAC between **Ru-$N_3$** and **rhein-alkyne** in A549 cells without additional Cu(I) catalysts; (C) Cellular uptake of Ru in A549 cells with 5 and 10 μM **Ru-$N_3$** or **Ru-rhein** incubation determined by ICP-MS (ng/μg protein).

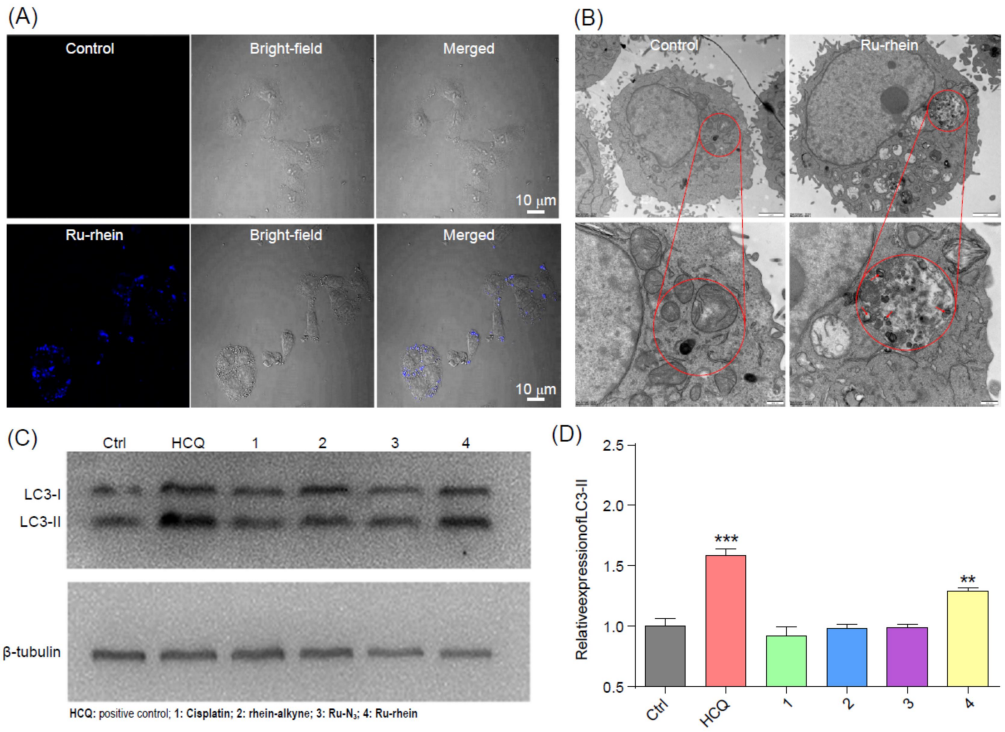



**Figure 4.** The cell death mechanism after treatment with **Ru-rhein**. (A) A549 cells were stained with 5 μM monodansylcadaverine (MDC) for 20 min after treatment with **Ru-rhein** (5 μM) separately for 24 h and then analyzed by fluorescence microscopy. The increased fluorescence indicated the **Ru-rhein**-induced formation of autophagosomes. $\lambda_{ex}$=405 nm, $\lambda_{em}$=430-600 nm for MDC. Scale bar: 10 μm. (B) Transmission electron microscopy imagings showed numerous autophagic vacuoles (red arrows) in A549 cells after treated with 5 μM **Ru-rhein** for 24 h. (C, D) The autophagy-related protein expression of LC3 induced by 5 μM cisplatin (**1**), **rhein-alkyne** (**2**), **Ru-$N_3$** (**3**) and **Ru-rhein** (**4**) respectively in A549 cells for 24 h, observed by western blot analysis. HCQ treatment as the positive control induced autophagy. The data represent the mean±SEM of three different experiments. **$p$<0.01, ***$p$<0.001. These results indicated that **Ru-rhein** mainly caused autophagic cell death of A549 cells.

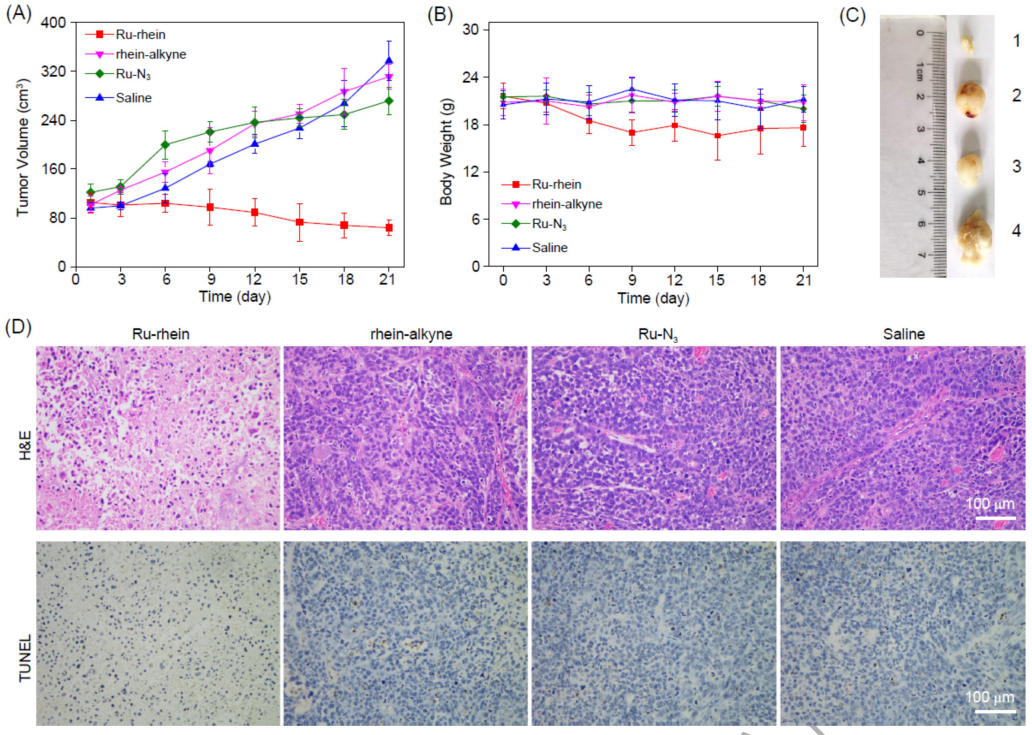



**Figure 5.** The successful BCL *in vivo*. Nude mice bearing A549 tumors were intratumorally injected with various samples at a dosage of 8.0 mg/kg body weight once every three days. The samples included **Ru-rhein**, **rhein-alkyne**, **Ru-$N_3$** and saline. (A) Changes in tumor volume during 18 days of the indicated treatments. Data are the mean ± SD (n = 6). (B) Changes in mice weight during 18 days of the indicated treatments. Data are the mean ± SD (n=6). (C) Photographs of tumors of each group obtained on the 18$^{th}$ day of treatment. 1: **Ru-rhein**; 2: **rhein-alkyne**; 3: **Ru-$N_3$**; 4: Saline. (D) Detection of cell death in the tumor tissues after treatment with H&E and TUNEL staining assay after treatment with different samples. Scale bars are 100 μm.