The Potential of Cycloaddition Reactions to Generate Cytotoxic Metal Drugs In Vitro

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ABSTRACT

Severe general toxicity issues blight many chemotherapeutics utilised in the treatment of cancers, resulting in the need for more selective drugs able to exert their biological activity at only the required location(s). Toward this aim, we report the development of an organometallic ruthenium compound, functionalised through a η^6-bound arene ligand with a bicyclononyne derivative, able to participate in strain promoted cycloaddition reactions with tetrazines. We show that combination of the ruthenium compound with a di-tetrazine in biological media results in the in situ formation of a dinuclear molecule that is more cytotoxic toward cancer cells than the starting mononuclear ruthenium compound and tetrazine components. Such an approach may be extended to in vivo applications to construct a cytotoxic metallodrug at a tumour site – providing a novel approach toward the turn-on cytotoxicity of metallodrugs in the treatment of cancer.

INTRODUCTION

Many widely used anticancer drugs act through interactions with targets that are not unique to cancer cells. This lack of specificity results in the indiscriminate action of highly cytotoxic chemotherapeutics on cancer cells and healthy cells alike and often results in severe side effects.[1,2] Such lack of specificity is present across virtually every class of chemotherapeutic and has spurred increased efforts towards the development of more selective drugs and protocols for the treatment of cancer. One such route to circumvent general toxicity problems would be the synthesis of a cytotoxic compound at a tumor site/in a cancer cell, which would ensure only cell death at the desired location. Toward this aim, we chose to devise ruthenium-based organometallics from which a more cytotoxic product could be constructed in a physiological environment.

Ruthenium-based anticancer compounds constitute a flourishing area of research with many novel compounds having being developed[3] in attempts to emulate the clinical success of the platinum-based drug cisplatin and subsequent derivatives[4,5] against various cancer types whilst avoiding their associated severe side effects. Ru^{III} prodrugs that are reduced to more active Ru^{II} species in vivo have shown particular promise and two of these, NAMI-A and KP1019, have progressed to clinical trials.[6] Stable Ru^{II} compounds in the form of organometallic species have also proved to be interesting with examples possessing a wide range of activities so far reported.[7,8] The 'piano stool' structure of these organometallics offers considerable scope for structural modification where each ligand may be modulated to allow access to diverse compounds endowed with a wide range of functionality, thus providing an ideal platform for the development of prospective new chemotherapeutics.[7-9] Our research has focused on the [Ru(η^6-arene)Cl_2(PTA)] (RAPTA) family of organometallics which exhibit low cytotoxicity and promising antimetastatic activity in vivo.[10] As part of a recent effort to develop

and study dinuclear RAPTA analogues we investigated the anticancer properties of a small series of arene-linked dinuclear RAPTA analogues (Figure 1).[11] These dinuclear compounds are considerably more cytotoxic toward cancer cells (IC_{50} <15 μM) compared to the negligible cytotoxicity of their mononuclear analogues (IC_{50} >300 μM). Further platinum and ruthenium-based dinuclear metallodrugs (e.g. the platinum compound BBR3610[12] and dinuclear ruthenium organometallics linked via maltol-derive linkers[13]) have also been shown to be significantly more cytotoxic than mononuclear analogues toward tumorigenic cell lines. In light of these observations we postulated whether a dinuclear organometallic Ru^{II} complex could be formed from mononuclear RAPTA species in situ in a cellular environment, thus providing a route via which compounds of low cytotoxicity could be transformed into more cytotoxic species. Such a mechanism could afford a route to tumor selective cytotoxic agents.

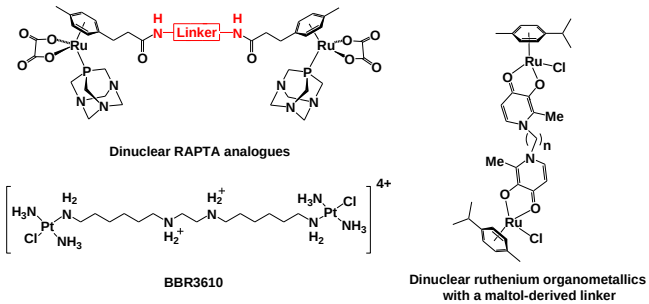


Figure 1. Examples of dinuclear transition metal-based compounds previously investigated for their anticancer properties.

In this context we were interested in the incorporation of bioorthogonal functionality able to participate in strain-promoted cycloaddition reactions into the arene ligand of the RAPTA structure. Strain-promoted cycloaddition reactions have emerged as a powerful tool for the

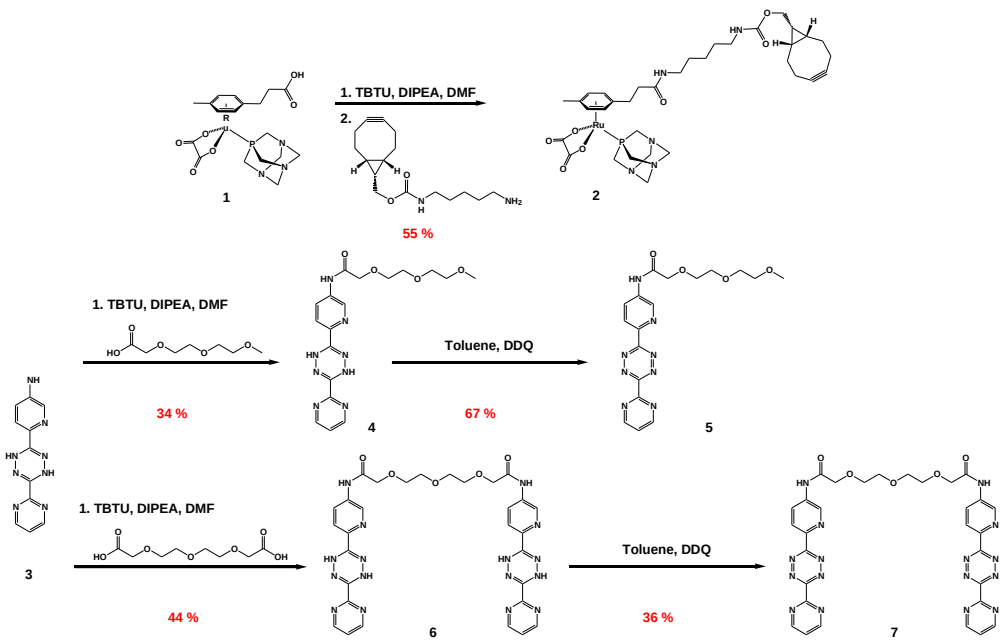
modification and imaging of molecules of interest in a biological environment.[14] The strain-promoted reactions between cyclooctynes and azides,[15] and between trans-cyclooctene[16] or cyclooctynes[17,18] with tetrazines, have been at the forefront of recent advances in this field, resulting in a range of extremely fast bioorthogonal reactions (e.g. a rate constant for reaction between a trans-cyclooctene and a tetrazine of 22000 M^{-1} s^{-1} in CD_3OD at 298 K has been reported[19]). Strain-promoted cycloaddition reactions have been widely employed in cell-based studies to selectively image biomolecules of interest including cell-surface glycans[20] and intracellular and cell surface proteins,[21,22] and in imaging of glycans in live developing zebrafish[23] and tumor imaging in live mice.[24] In addition, strain-promoted cycloaddition reactions with a trans-cyclooctene (TCO) have been employed in the development of a novel doxorubicin (Dox) prodrug strategy based on elimination of Dox from a TCO-Dox conjugate upon reaction with a tetrazine.[25]

We envisaged utilising this chemistry to link two mononuclear, non-toxic organometallic ruthenium compounds to form a cytotoxic dinuclear compound in situ in a cellular environment. Such 'turn-on' cytotoxicity could be utilised in the context of cancer treatment through the spatial localisation of the reactive components either in an extracellular or intracellular environment followed by their reaction to yield the cytotoxic product. Recent reports have demonstrated the cycloaddition of bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN) derivatives with tetrazine derivates with fast reaction kinetics (1245 ± 45 M^{-1} s^{-1}, 55:45 MeOH:H_2O, 298 K).[17] Given the fast reaction kinetics, commercial availability of BCN-derivatives and the synthetic accessibility of tetrazine precursor **3**,[26] we decided to explore whether the combination of a BCN-containing RAPTA derivative with a di-tetrazine linker molecule would yield a more cytotoxic dinuclear ruthenium complex in a cellular environment.

Here, we describe the incorporation of a bicyclo[6.1.0]non-4-yn-9-ylmethanol derivative into the η^6-arene ligand of an organometallic Ru^{II}-arene (RAPTA-type) complex and demonstrate its conjugation to mono- and di-tetrazine derivatives. The BCN-containing RAPTA and the tetrazines conjugate rapidly and in quantitative yield to afford mononuclear and dinuclear ruthenium compounds. Cytotoxicity assays reveal that this conjugation 'activates' the mononuclear RAPTA-BCN derivative to afford a more potent dinuclear compound, providing a novel route toward the in situ 'turn-on' enhancement of cytotoxicity.

RESULTS AND DISCUSSION

BCN-containing RAPTA, **2**, was accessed through the reaction of *N*-(1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl 1,5-diaminopentane (BCN-C_5-NH_2) with the carboxylic acid-containing RAPTA, **1**, using the coupling agent *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) with *N*,*N*-diisopropylethylamine (DIPEA) in DMF (Scheme 1), based on a recently reported route.[11] The ethylene-glycol functionalised tetrazine-precursors **4** and **6** were prepared by reaction of **3** and 2-(2-(2-methoxyethoxy)ethoxy)acetic acid (1:1 ratio) or 3,6,9-trioxaundecanedioic acid (2:1 ratio), respectively, using TBTU and DIPEA in DMF. The resulting tetrazine precursors were then oxidized using DDQ in toluene; chromatographic separation on silica gel yielded the mono-tetrazine and di-tetrazine analogue **5** and **7** respectively.



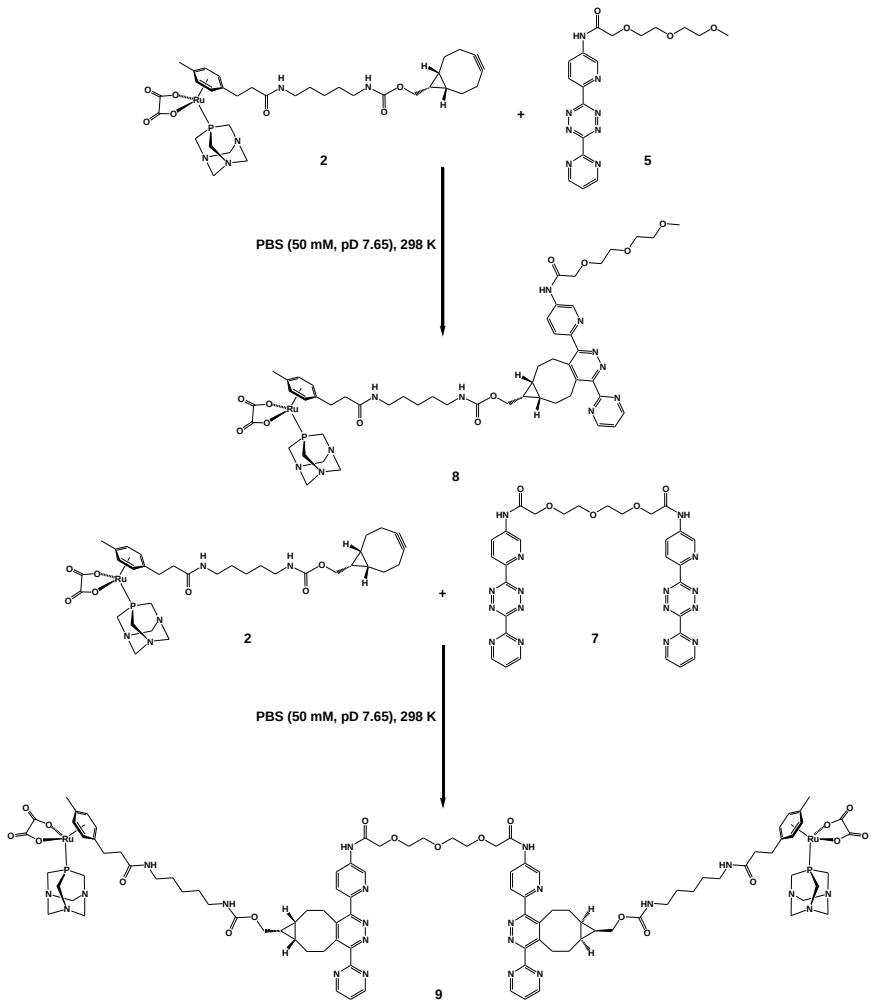
Scheme 1. Synthesis of the BCN-containing RAPTA derivative **2**, and the mono- and di-tetrazines **5** and **7**.

All compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, high-resolution mass spectrometry and HPLC/LC-MS.

Tetrazines possess a range of stabilities in biological media, with the general trend being the more electron-rich the tetrazines the greater the stability, but the lower the reactivity toward cycloaddition reactions.[27] We examined the stability of tetrazines **5** and **7** in both phosphate-buffered saline (PBS, 50 mM) and in RPMI cell culture media containing 10 % fetal bovine serum (FBS) at 37°C by monitoring the change in absorbance at 515 nm for **5** and 525 nm for **7**. For **5** in PBS, the decrease in absorbance at 515 nm fit well to a single exponential decay function revealing a half-life of 82 min (Figure S16). ^{1}H NMR spectra of **5** in PBS revealed the complete loss of the original tetrazine resonances over this time with concomitant colour change

of the solution from red to yellow (Figure S17). Ditetrazine **7** is considerably more stable in PBS with a more gradual linear loss of absorbance at 525 nm (Figure S18) and the half life for this compound was found to be significantly longer (>23 h). Analysis of **5** in RPMI with 10% FBS by UV-Vis was more complex (Figure S19); following an initial induction period a rapid loss in absorbance at 515 nm is observed, reaching a minimum value after approximately 50 min. This decay in absorbance did not fit well to various decay functions – indicative of multiple processes which presumably include decomposition of the tetrazine and reaction with components on the RPMI/FBS solution. Analysis of **7** in RPMI + 10% FBS was complicated by the slow formation of a precipitate. The reactivity of the tetrazines reported here follows observations of high reactivity of tetrazines with similar core structures in PBS and FBS solutions.[27] However, given that the reactions between **2** and **5** or **7** are extremely fast compared to tetrazine decomposition in PBS or RPMI media cytotoxicity studies were undertaken.

The reaction of the BCN-containing RAPTA derivative, **2**, with the mono- and di-tetrazines **5** and **7** was investigated in aqueous solution (Scheme. 2).



Scheme 2. The cycloaddition reaction between complex **2** and tetrazines **5** and **7**.

^{1}H NMR spectroscopy was used to probe the reaction between tetrazines **5** and **7** and the BCN-RAPTA adduct, **2**, in PBS (50 mM in D_2O, pD 7.65, 298 K). On addition of **2** (0.2 ml, 12 mM) to a solution of **5** (0.2 ml, 12 mM) evolution of gas was immediately observed, indicating the release of N_2. ^{1}H NMR of the solution before and after (2 min) addition of **2** showed complete transformation of the tetrazine resonances to product peaks consistent with the sole formation of the desired cycloaddition product **8** (Figure 2).

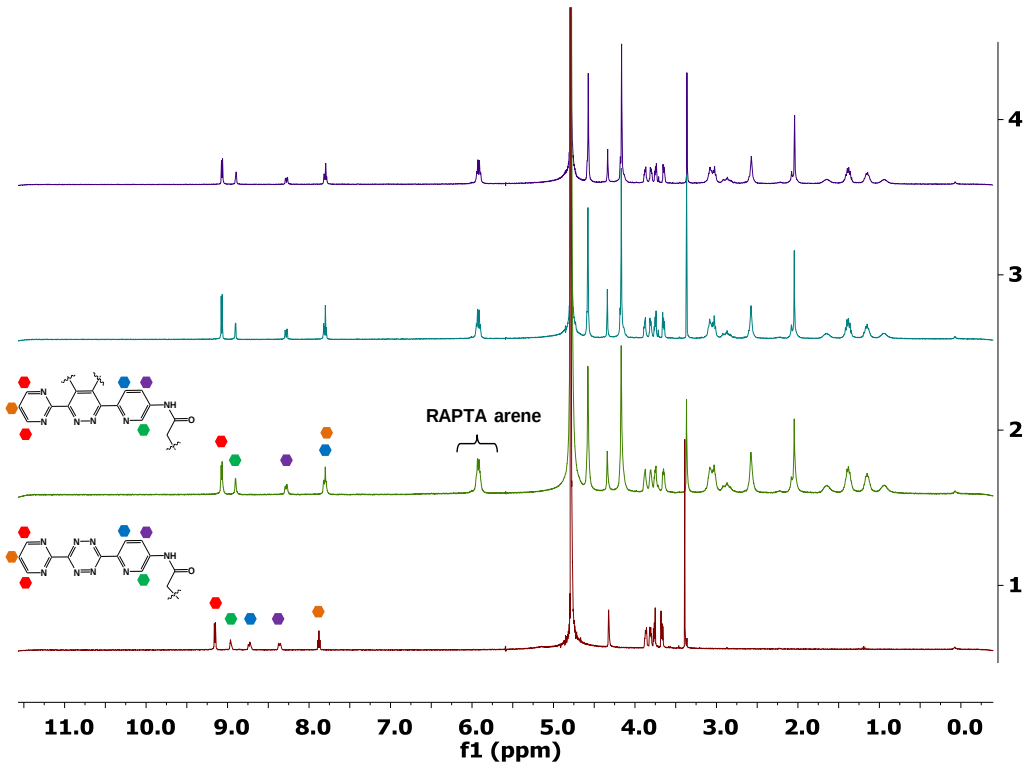


Figure 2. ^{1}H NMR spectra (400 MHz) of the reaction between **2** (12 mM) and **5** (12 mM) in PBS (50 mM, D_2O, pD 7.65, 298 K). 1) **5** alone. 2) **5** following the addition of **2** (1 eq) (2 min). 3) after 20 min. 4) after 105 min. Aromatic resonances of tetrazine **5** and the cycloddition product **8** are labelled.

Reaction of **2** with the di-tetrazine, **7**, under the same conditions, also led to the desired dinuclear cycloaddition product **9** in quantitative yield (Figure S13). In addition, the reaction between **2** and **7** was also performed at a concentration of 20 μM in H_2O (unbuffered, 310 K, 12 h). Analysis of the reaction products by ESI-MS revealed the complete conversion of the starting components to **9**, highlighting the utility of the cycloaddition reaction at very low concentrations. The reaction between **2** and tetrazines **5** and **7** was too fast to monitor by UV-Vis spectroscopy, consistent with the high rate constants for the reaction between related

tetrazines and BCN-derivatives previously reported (1245 M^{-1} s^{-1} in 55:45 MeOH:H_2O, 298 K)[17] and the expectation that these rate constants are enhanced in purely aqueous conditions.

To assess the potential of the cycloaddition reactions with tetrazines to modify the structure (and biological activity) of **2** in cellular media the cytotoxicities of **2**, **5** and **7** were evaluated in the A2780 cell line, as were the cycloaddition products **8** and **9** preformed through incubation of **2** and **5** or **7** in H_2O or by in situ formation of the dinuclear species in the cell culture (Table 1). The cytotoxicities of tetrazines **5** and **7** were negligible with both possessing an IC_{50} value of >400 μM. The BCN-RAPTA complex **2** is more cytotoxic with an IC_{50} value of 12 μM. The IC_{50} values of the preformed cycloaddition compounds **8** and **9** are different to that of **2**. The dinuclear compound **9** is, as anticipated, more cytotoxic than the mononuclear complex, with a 2.4 fold decrease in IC_{50} value (with respect to Ru concentration) whilst the preformed mononuclear compound **8** is less cytotoxic. The dinuclear cycloaddition product, **9**, formed in situ has a similar IC_{50} to that when preformed, 2.5 and 2.6 μM respectively. In contrast, the mononuclear complex **8** formed in situ is more cytotoxic than the preformed mononuclear compound with an IC_{50} value of 5.7 μM compared to the IC_{50} value of >20 μM for the preformed adduct. The increased cytotoxicity of **8** formed in situ potentially indicates that the individual components **2** and **5** may be separately internalised, transported then combine in an intracellular location different to the intracellular destinations of preformed **8**, leading to different biological activity.

Table 1. IC_{50} values of Ru^{II} compounds **2**, **8** and **9** and tetrazines **5** and **7** in the human ovarian carcinoma (A2780) cell line after 72 h (MTT assay).

Compound	IC_{50} (complex concentration, μM) A2780 cell line
2	12 ± 1
5	> 400
7	> 400
8 (preformed)	> 20
9 (preformed)	2.5 ± 0.4
8 (formed in situ)	5.7 ± 1
9 (formed in situ)	2.6 ± 0.2

CONCLUSIONS

In conclusion, in this proof-of-principle study we have shown that through the development of a RAPTA compound containing a bicyclononyne derivative appended to the arene ligand, and its strain-promoted cycloaddition reaction with a di-tetrazine in biological media, a more cytotoxic dinuclear compound can be accessed. Moreover, the higher cytotoxicity of the large mononuclear complex **8** formed in situ, relative to the preformed sample, potentially indicates that the cycloaddition reactions may proceed inside the cell leading to the modified cytotoxicity of the complex. For in vivo applications it may be envisaged that chemotherapy with such cycloaddition-based systems may proceed via the sequential treatment with the ruthenium complex, e.g. **2**, then once accumulation had reached sufficient levels the cycloaddition partner, e.g. **5** or **7**, would then be administered to initiate a cytotoxic response. Much work is needed to

optimize such a study, including the development of a less cytotoxic ruthenium component in place of **2**. However, our work demonstrates that the combination of functionalised tetrazines and RAPTA derivatives containing functionality able to participate in strain-promoted cycloaddition reactions holds significant potential in the development of organometallic systems with turn-on cytotoxicity. This approach could be extended to many other classes of drug and drug-like compounds.

EXPERIMENTAL SECTION

Materials: All commercially purchased materials were used as received. *N*-(1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl 1,5-diaminopentane (BCN-C_5-NH_2) was purchased from SynAffix (Oss, Netherlands). Dichloromethane, diethyl ether and toluene were dried and degassed prior to use using a PureSolv solvent purification system (Innovative Technology INC). *N*'*N*-dimethylformamide (99.8%, Extra Dry, Acroseal®) and acetone (99.8%, Extra Dry, Acroseal®) were obtained from Acros Organics and methanol (anhydrous, 99.8%) was purchased from Sigma-Aldrich. H_2O was obtained from a Milli-Q Integral 5 purification system. Thin-layer chromatography was carried out on silica plates (Merck 5554), visualised under UV irradiation (254 nm), with iodine staining or using potassium permanganate dip. Where required, compounds were purified using either manual chromatography using silica gel (SiliCycle R12030B) or a Varian 971-FP flash chromatography system using pre-packaged silica gel columns (Luknova). **Instrumentation and methods:** ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker Avance II 400 spectrometer (^{1}H at 400 MHz, ^{13}C at 101 MHz and ^{31}P at 162 MHz). Spectra are referenced internally to residual solvent peaks (D_2O: ^{1}H δ 4.79, ^{13}C δ unreferenced; DMSO-d_6: ^{1}H δ 2.50, ^{13}C δ 39.52; $CDCl_3$: ^{1}H δ 7.26, ^{13}C δ 77.16); ^{31}P NMR spectra are reported relative to an 85% H_3PO_4 external reference. Electrospray-ionisation mass

spectra (ESI-MS) were acquired on a Q-Tof Ultima mass spectrometer (Waters) operated in the positive ionization mode and fitted with a standard Z-spray ion source equipped with the Lock-SprayTM interface. The samples were diluted in $CH_3CN/H_2O/HCOOH$ (50:49.9:0.1, ~10^{-5} M) and 5 µl was introduced into the mass spectrometer by infusion at a flow rate of 20 µl/min with a solution of $CH_3CN/H_2O/HCOOH$ (50:49.9:0.1). Experimental parameters were set as follows: capillary voltage: 3.5 kV, sample cone: 35 V, source temperature: 80°C, desolvation temperature: 200°C, acquisition window: *m/z* 300-1500 in 1 s. External calibration was carried out with a solution of phosphoric acid at 0.01% introduced through an orthogonal ESI probe. Data from the Lock-Spray were used to calculate a correction factor for the mass scale and provide accurate mass information of the analyte. Data were processed using the MassLynx 4.1 software. LC-MS data was recorded on an AutoPrep system (Waters) equipped with: 2525 binary gradient module, column/fluidics organizer, sample manager 2767, PDA detector and ZQ4000 ESI-MS. Electrospray, positive ionization mode was employed with a cone voltage of 25 volts, a source temperature of 100 °C and a mass range of *m/z* 200-1500 in 2 sec. HPLC analysis was performed on an Agilent 1260 Infinity HPLC equipped with DAD detector; an Agilent Hi-Plex H, 300*7.7 column was utilised. UV-Vis absorbance spectra were recorded on a JASCO V-550 UV/VIS spectrophotometer (using Spectra Manager software (version 1.53.01)) and also on a SpectroMax M5e multi-mode microplate reader (using SoftMax Pro software (version 6.2.2)). Melting points were measured using a Stuart Scientific SMP3 apparatus and are uncorrected. A SpectroMax M5e multi-mode microplate reader was used to record the absorbance of solutions contained in 96-well plates (using SoftMax Pro software (version 6.2.2)).

Synthesis.

Complex **1**[11] and compound **3**[26] were synthesized according to literature procedures.

Complex 2: *N*,*N*-diisopropylethylamine (DIPEA) (31 µl, 0.18 mmol) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) (29 mg, 0.09 mmol) were added to a suspension of **1** (46 mg, 0.09 mmol) in DMF (1 ml) then the mixture was briefly sonicated then stirred for 5 min. The yellow suspension was then added to a solution of *N*-(1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyloxycarbonyl 1,5-diaminopentane (BCN-C_5-NH_2) (25 mg, 0.09 mmol) in DMF (1 ml) and the yellow suspension was stirred to form a yellow solution within 20 min. After 1 h the solution was filtered through cotton wool and the filtrate was diluted with acetone (15 ml) to yield a precipitate. The suspension was cooled to 4°C and the filtrate isolated by centrifugation then concentrated under reduced pressure to a volume of 1 ml. Diethyl ether (15 ml) was added to the filtrate to yield a solid (40 mg) that was collected by centrifugation. The filtrate was left at 4°C for 16 h to yield further solid that was also collected (18 mg). The combined solids were dissolved in MeOH (2.5 ml), filtered then diethyl ether was added until precipitation began. The suspension was left at 4°C for 16 h to yield further solid that was collected by centrifugation then dried under reduced pressure to yield the desired product as a yellow powder (38 mg, 0.049 mmol, 55 %). Compound **2** is water soluble (>25 mM), was stable over 4 months stored under N_2 at -18°C and is stable in aqueous solution for several days (>72 h); ^{1}H NMR (400 MHz, D_2O) δ 5.96 (m, 4H, Ar), 4.59 (s, 6H, PTA), 4.18 (s, 8H, PTA and -CH_2-O-), 3.08-3.13 (m, 4H, 2 x –C*H*$_2$NHCO-), 2.61 (s, 4H, -C*H*$_2$-C*H*$_2$-CO-), 2.20-2.33 (m, 6H), 2.08 (s, 3H, -CH_3), 1.43-1.61 (m, 7H), 1.20 (m, 2H), 0.97 (m, 2H); ^{31}P{^{1}H} NMR (D_2O, 162 MHz): δ = -33.4; ^{13}C NMR (101 MHz, D_2O) δ 173.6, 166.0, 158.7, 100.2, 98.7, 96.4, 88.5 (d, 2C, J = 3.0 Hz, Ar), 87.7 (d, 2C, J = 3.0 Hz Ar), 70.7 (d, 3C, J = 6.0 Hz, PTA), 63.5, 48.6 (d, 3C, J = 15.0 Hz), 40.3, 39.2, 35.7, 28.7, 28.3, 27.9, 23.3, 20.8, 19.8, 17.3; HRMS (ES^+) *m*/*z* found

772.2440 $[M + H]^+$ $C_{34}H_{49}N_5O_7PRu$ requires 772.2423. LCMS (Vydac C18 5 mm (218TP54), 4.6x150 mm (Waters) column): t_R = 16.2 min.

Compound 4: TBTU (274 mg, 0.853 mmol) and DIPEA (247 µl, 1.418 mmol) was added to a solution of 2-(2-(2-methoxyethoxy)ethoxy)acetic acid (152 mg, 0.853 mmol) in DMF (1 ml) under an atmosphere of N_2 and stirred for 20 min. The solution was then added to a suspension of **3** (179 mg, 0.704 mmol) in DMF (3 ml) then stirred. After 18 h a further TBTU (374 mg, 1.16 mmol) and 2-(2-(2-methoxyethoxy)ethoxy)acetic acid (152 mg, 0.853 mmol) was added followed by reduction of the solvent volume to 1.5 ml under vacuum and stirring for a further 24 h. The reaction mixture was dried under reduced pressure, diluted by the addition of DCM (50 ml), extracted with H_2O (2 x 20 ml) followed by drying of the organic layer with Na_2SO_4. The organic layer was concentrated to 5 ml then directly loaded onto a silica gel column (25 g) and the crude product was isolated (DCM and 2% MeOH). The residue was then further purified via column chromatography on silica gel (80 g) (DCM (100%) to DCM and MeOH (2%)). The eluted product fractions were collected and subject to a further column chromatography on silica gel (80 g) (DCM (100%) to DCM and MeOH (2%)) to yield the product as an orange-red solid (100 mg, 0.241 mmol, 34 %); R_F (silica, DCM-MeOH, 90:10) : 0.37; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.14 (s, 1H, amide N*H*), 8.82 (d, *J* = 5.0 Hz, 2H), 8.69 (s br, 2H), 8.33 – 8.29 (m, 2H), 8.01 (d, *J* = 8.5 Hz, 1H), 7.36 (t, *J* = 5.0 Hz, 1H), 4.13 (s, 2H, -O-C*H*$_2$-CONH-), 3.85 – 3.54 (m, 8H, 2 x –O-C*H*$_2$C*H*$_2$-O-), 3.36 (s, 3H, C*H*$_3$), 1.88 (m, 1H); ^{13}C{^{1}H} NMR ($CDCl_3$, 101 MHz): δ = 169.3, 157.5, 156.4, 146.1, 145.7, 142.5, 140.0, 135.9, 127.6, 121.8, 121.6, 71.8, 71.5, 71.0 , 70.4, 70.2, 59.1; HRMS (ES^+) *m*/*z* found 437.1674 $[M + Na]^+$ $C_{18}H_{22}N_8O_4Na$ requires 437.1662, m.p. 105 - 107 °C.

Compound 5: **4** (84 mg, 0.203 mmol) was suspended in anhydrous toluene (10 ml) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (92 mg, 0.405 mmol) was added. The suspension was stirred for 1 h at 120°C followed by evaporation of the solvent under reduced pressure. The desired product was isolated by column chromatography on silica gel (80 g) (DCM (100 %) to DCM and MeOH (98:2) over 7 minutes then to DCM and MeOH (94:6) at 40 minutes) as a pink powder (56 mg, 0.136 mmol, 67 %). **5** is soluble in H_2O at a concentration of >24 mM; R_F (silica, DCM-MeOH, 90:10) : 0.5; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 9.37 (s, 1H, amide N*H*), 9.14 (d, *J* = 5.0 Hz, 2H, Ar), 9.01 (d, *J* = 2.5 Hz, 1H, Ar), 8.80 (d, *J* = 8.5 Hz, 1H, Ar), 8.66 (dd, *J* = 8.5, 2.5 Hz, 1H, Ar), 7.59 (t, *J* = 5.0 Hz, 1H, Ar), 4.20 (s, 2H, -O-C*H*$_2$-CONH-), 3.86 – 3.58 (m, 8H, 2 x –O-C*H*$_2$C*H*$_2$-O-), 3.37 (s, 3H, CH_3); ^{13}C{^{1}H} NMR ($CDCl_3$, 101 MHz): δ = 169.8, 163.5 x 2, 159.7, 158.6, 150.4, 144.8, 142.6, 137.6, 127.4, 125.8, 122.6, 71.8, 71.6, 70.8, 70.5, 70.1; HRMS (ES$^+$) *m*/*z* found 435.1516 $[M + Na]^+$ $C_{18}H_{20}N_8O_4Na$ requires 435.1505, m.p. 146 - 149 °C ; HPLC (Agilent Hi-Plex H, 300*7.7 column): t_R = 8.2 min.

Compound 6: 3,6,9-trioxaundecanedioic acid (186 mg, 0.837 mmol) was added to a solution of DIPEA (0.56 ml, 3.22 mmol) in DMF (1 ml) and stirred at room temperature for 5 min followed by the addition of TBTU (646 mg, 2.01 mmol). The solution was stirred for 30 min followed by the addition of **3** (426 mg, 1.68 mmol); the suspension was stirred for 19 h then dried under reduced pressure. The resulting solid was purified by column chromatography on silica gel (DCM to 12% MeOH) to yield the desired product as a sticky orange solid (258 mg, 0.372 mmol, 44 %); R_F (silica, DCM-MeOH, 93 :7) : 0.2; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (m, 6H, Ar and amide NH), 8.59-8.64 (m, 4H, Ar and NH), 8.32 (s, 2H, NH), 8.25 (dd, 2H, *J* = 8.5, 2.5 Hz, Ar), 7.98 (d, 2H, *J* = 8.5 Hz, Ar), 7.36 (t, 2H, *J* = 5.0 Hz, Ar), 4.16 (s, 4H, -O-C*H*$_2$-CONH-), 3.82 (s, 8H, -O-C*H*$_2$-C*H*$_2$-O-); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.5, 157.5, 156.4, 145.9, 145.7,

142.9, 139.6, 135.5, 127.6, 122.0, 121.6, 71.1 70.7, 70.5; HRMS (ES^+) *m*/*z* found 695.2676 $[M + H]^+$ $C_{30}H_{31}N_{16}O_5$ requires 695.2664.

Compound 7: **6** (259 mg, 0.373 mmol) and DDQ (347 mg, 1.53 mmol) were suspended in toluene (18 ml) and the mixture heated at reflux for 3 h. The red suspension was dried under reduced pressure and the desired product isolated by column chromatography on silica gel (100 % DCM to 20 % MeOH) as a dark red powder (94 mg, 0.136 mmol, 36 %). **7** is soluble in H_2O at a concentration of >1 mM; R_F (silica, DCM-MeOH, 85 :15) : 0.45; ^1H NMR (400 MHz, DMSO-d_6) δ 10.35 (s, 2H, amide NH), 9.17 (d, 4H, *J* = 5.0 Hz, Ar), 9.12 (d, 2H, *J* = 2.5 Hz, Ar), 8.62 (d, 2H, *J* = 8.5 Hz, Ar), 8.47 (dd, 2H, *J* = 8.5, 2.5 Hz, Ar), 7.82 (t, 2H, *J* = 5.0 Hz, Ar), 4.24 (s, 4H, -O-C*H*$_2$-CONH-), 3.75 (d, 8H, -O-C*H*$_2$-C*H*$_2$-O-); ^{13}C NMR (101 MHz, DMSO-*d*6) δ 169.6, 162.7, 162.6, 159.0, 158.4, 144.0, 141.9, 137.8, 126.8, 125.1, 122.9, 70.4, 70.2, 69.7; HRMS (ES^+) *m*/*z* found 691.2349 $[M + H]^+$ $C_{30}H_{27}N_{16}O_5$ requires 691.2350; m.p. 187 °C; HPLC (Agilent Hi-Plex H, 300*7.7 column): t_R = 10.1 min.

General procedure for the non-isolated preparation of 8/9: For NMR studies **8** was prepared through the incubation of **2** (12 mM) and **5** (1 eq.) in PBS (50 mM, D_2O, pD 7.65, 298 K). **9** was also prepared through the incubation of **5** (7.2 mM) and **7** (0.5 eq.) in PBS (50 mM, D_2O, pD 7.65, 298 K). For cell studies stock solutions of **8** and **9** were prepared through the co-incubation of **2** (200 μM) and **5** (1 eq.) or **7** (0.5 eq.) in H_2O at 310 K for 12 h then analysed with ESI-MS. These solutions were used without further isolation/purification; HRMS (ES^+) of **8** *m*/*z* found 589.6945 $[M + H + Na]^{2+}$ $C_{52}H_{69}N_{11}O_{11}PRuNa$ requires 589.6923; HRMS (ES^+) of **9** *m*/*z* found 726.5654 $[M + 3H]^{3+}$ $C_{98}H_{125}N_{22}O_{19}P_2Ru_2$ requires 726.5679.

Cell culture and inhibition of cell growth: The A2780 cell line was obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). RPMI-1640 media and

penicillin streptomycin solution were obtained from Life Technologies, fetal bovine serum (FBS) was obtained from Sigma. Cells were cultured in RPMI-1640 with GlutaMAX media containing 10% FBS and penicillin at 37°C and 5% CO_2. Cytotoxicity was determined using the MTT assay.[28] Cells were seeded in flat-bottomed 96-well plates by the addition of cells as a suspension in media containing 10% FBS (100 μl per well, approximately 4300 cells) and pre-incubated for 24 h. Preformed stock solutions of **8** and **9** were prepared as described previously, all other stock solutions were prepared in H_2O immediately prior to use. Stock solutions were then diluted by the addition to the culture medium then sequentially diluted to yield compound solutions of the required concentrations. Aliquots (100 μl) of these stock solutions were added to plate wells to yield final compound concentrations in the range 0 μM to 100 μM/400 μM. The 96-well plates were then incubated for a further 72 h followed by the addition of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution (20 μl, 5 mg/mL in H_2O) to each well and the plates incubated for a further 2 h. The culture medium was then aspirated and the formazan precipitate produced by mitochondrial dehydrogenases of living cells was dissolved by the addition of DMSO (100 μl) to each well. The absorbance of the resultant solutions at 590 nm, which is directly proportional to the number of surviving cells, was recorded using a microplate reader. The percentage of surviving cells was determined by measurement of the absorbance of wells corresponding to untreated control cells. The reported IC_{50} values are based on the mean values from three independent experiments; each concentration level per experiment was evaluated in triplicate, and those values are reported in Table 1.

ASSOCIATED CONTENT

Supporting Information. LC-MS and HPLC chromatograms for all compounds, NMR spectra for all compounds, supplementary mass spectra and UV-Vis stability data for tetrazines **5** and **7** is provided as supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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

Notes

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ABBREVIATIONS

BCN, bicyclo[6.1.0]non-4-yn-9-ylmethanol; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; Dox, doxorubicin; FBS, fetal bovine serum; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate buffered saline; RAPTA, [Ru(η^6-arene)Cl_2(PTA)];

RPMI, Roswell Park Memorial Institute medium; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TCO, trans-cyclooctene;

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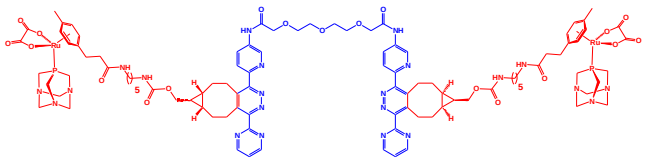
SYNOPSIS

The strain-promoted cycloaddition between a Ru^{II} organometallic compound, functionalised at the arene ligand with a bicyclononyne derivative, and a di-tetrazine, yields the formation of a dinuclear organometallic compound in biological media. The dinuclear compound exhibits increased cytotoxicity against tumorigenic cells relative to its constituent components, yielding a novel route toward their cytotoxic activation in cellulo.



Increased cytotoxicity