Electronic Supplementary Material (ESI) for Dalton Transactions.

Supplementary Information

Synthesis and *in vivo* Anticancer Evaluation of Poly(organophosphazene)-based Metallodrug Conjugates

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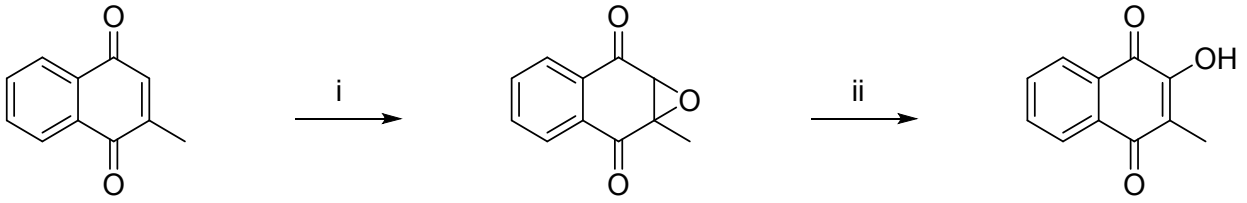
Contents

1 Synthetic Procedures

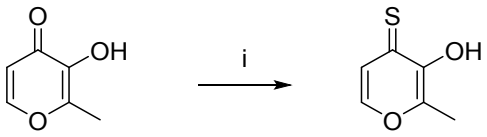
1.1 Synthesis of 2-Hydroxy-3-methyl-1,4-naphthoquinone

The ligand 2-hydroxy-3-methyl-1,4-naphthoquinone was synthesized starting from 2-methyl-1,4-naphthoquinone (menadione) (1.0 g, 5.8 mmol, 1 equiv.), which was dissolved in a 1:4 mixture of water/methanol and cooled to 0 °C. After the addition of NaOH (1.5 mL, 2 M, 3.0 mmol) and H_2O_2 (0.9 mL, 30%, 8 mmol, 1.3 equiv.), the solution was stirred for ten minutes at 0 °C followed by two hours of stirring at room temperature. The mixture was concentrated under reduced pressure and the remaining solution stored at 4 °C to maximize the yield of the intermediate product. The off-white intermediate (long, bright needles) was filtered, washed with water, dried in vacuo, and used without further purification (yield: 81%, 0.89 g). For the subsequent ring-opening reaction 2,3-epoxy-2-methyl-1,4-naphtoquinone (0.7 g, 3.7 mmol, 1 equiv.) and 7 g silica gel were suspended in THF. After the addition of conc. H_2SO_4 (1.4 mL, 96%, 13.7 mmol, 3.7 equiv.), the solvent was removed under reduced pressure (70 °C, 500 mbar) and the residue was extracted with CH_2Cl_2. The organic layer was then washed with a saturated solution of $NaHCO_3$. The combined aqueous layers were acidified using conc. HCl and re-extracted with CH_2Cl_2. The organic solvent was removed under reduced pressure and the yellow, fluffy product was dried in vacuo (yield: .92%, 0.69 g). ^{1}H NMR (500.10 MHz, DMSO-d_6): δ: 1.96 (s, 3H, H_{CH3}), 7.79 (ddd, ^4J(H,H) = 2 Hz, ^{3}J = 8 Hz, ^3J(H,H) = 8 Hz, 1H, H_{ar}), 7.84 (ddd, ^4J(H,H) = 2 Hz, ^3J(H,H) = 8 Hz, ^3J(H,H) = 8 Hz, 1H, H_{ar}), 7.98 (d, ^3J(H,H) = 8 Hz, 1H, H_{ar}), 8.01 (d, ^4J(H,H) = 8 Hz, 1H, H_{ar}), 10.94 ppm (s, 1H, OH).

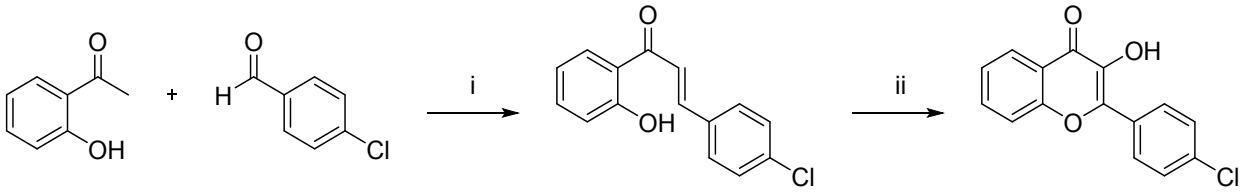


Scheme S1: Synthesis of the naphthoquinone ligand. i) NaOH, H_2O_2, methanol/H_2O 4:1, O °C; ii) H_2SO_4 conc., silica, THF, 500 mbar, 70 °C.

1.2 Synthesis of the Ligands for Complexes 2–4



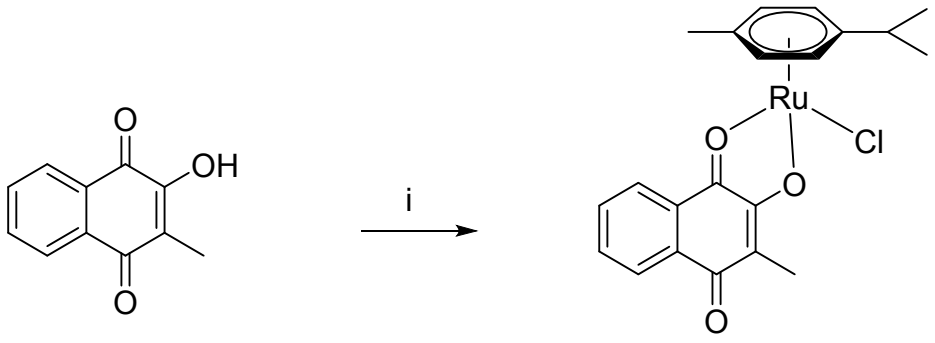
Scheme S2: Commercially available maltol was converted to thiomaltol by reaction with Lawesson's reagent. i) Lawesson's reagent (0.5 equiv.), dry THF, reflux, 4h.



Scheme S3: 2-Hydroxyacetophenone and *p*-chlorobenzaldehyde were reacted to the corresponding hydroxychalcone intermediate in a classic aldol condensation i) NaOH (5 M), CH_3COOH, ethanol. The subsequent ring-closing reaction was performed under Algar-Flynn-Oyamada reaction conditions ii) NaOH (5 M), H_2O_2 (30%), ethanol.

1.3 Synthesis of Complex 1 Chlorido[2-methyl-3-(oxo-κO)-1,4-naphthoquinonato-κO1](η6-p-cymene)ruthenium(II)

Complex **1** was synthesized according to the general procedure using 2-hydroxy-3-methyl-1,4-naphthoquinone (302 mg, 1.61 mmol, 1 equiv.) which was deprotonated with NaOMe (95 mg, 1.76 mmol, 1.1 equiv.) and reacted with bis[dichlorido(η^6-*p*-cymene)Ru(II)] (439 mg, 0.72 mmol, 0.45 equiv.). After precipitation from dichloromethane/*n*-hexane the product was isolated as dark blue crystals (Yield: 69%, 450 mg). ^{1}H NMR (500.10 MHz, $CDCl_3$, 25 °C) δ = 1.38 (dd, ^4J(H,H) = 7 Hz, ^3J(H,H) = 13 Hz, 6H, $H_{CH3,Cym}$), 2.15 (s, 3H, H_{CH3}), 2.44 (s, 3H, $H_{CH3,Cym}$), 2.73–2.93 (m, 1H, $H_{CH,Cym}$) 5.95 (d, ^3J(H,H) = 6 Hz, 1H, $H_{ar,Cym}$), 6.24 (d, ^3J(H,H) = 6 Hz, 1H, $H_{ar,Cym}$), 7.55 (ddd, ^4J(H,H) = 2 Hz, ^3J(H,H) = 8 Hz, ^3J(H,H) = 8 Hz, 1H, H_{ar}), 7.73 (ddd, ^4J(H,H) = 2 Hz, ^3J(H,H) = 8 Hz, ^3J(H,H) = 8 Hz, 1H, H_{ar}), 8.00–8.05 ppm (m, 2H, H_{ar}); ^{13}C NMR (125.75 MHz, $CDCl_3$, 25 °C) *δ* = 9.0 (CH_3), 19.22 ($CH_{3,Cym}$), 22.9 & 23.0 ($2CH_{3,Cym}$), 32.6 (CH_{Cym}), 69.3 & 70.3 ($CH_{ar,Cym}$), 72.7 & 73.7 ($CH_{ar,Cym}$), 88.2 ($C_{ar,Cym}$), 91.3 ($C_{ar,Cym}$), 124.2 (C3), 126.8 (C5), 128.0 (C8), 131.6 (C7), 133.0 (C4a), 136.4 (C6), 170.2 (C2), 183.5 (C4), 195.5 ppm (C1). elemental analysis calcd. (%) for $C_{21}H_{21}ClO_3Ru$: C 55.08, H 4.62, O 10.48; found: C 54.73, H 4.59, O 10.84.



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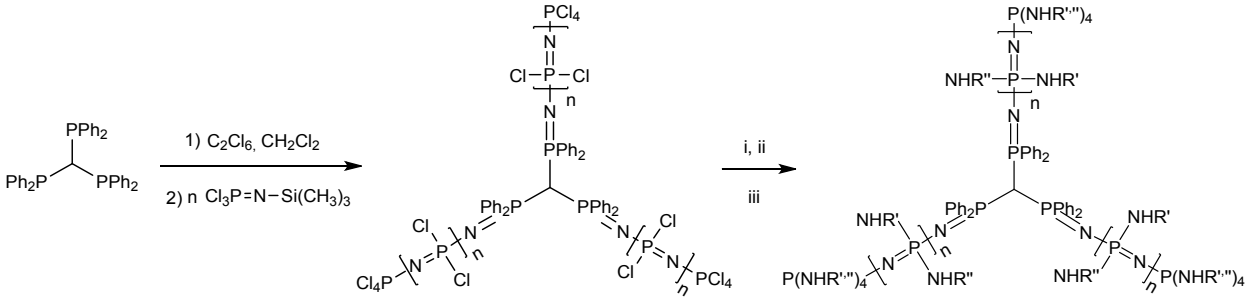
Scheme S4: Synthesis of complex **1**. i) NaOMe, Methanol, [dichlorido(η^6-*p*-cymene)Ru(II)]$_2$

1.4 Synthesis of the Poly(organo)phosphazene

The star branched poly(dichlorophosphazene) $[NPCl_2]_n$ was synthesized according the literature procedures.[1] Briefly: in a glove box at room temperature 1,1,1-tris(diphenylphosphino)methane (35 mg, 0.06 mmol) was chlorinated with C_2Cl_6 (48.1 mg, 0.20 mmol, 3.3 equiv.) in anhydrous CH_2Cl_2 overnight, yielding three ionic $[RPh_2PCl]^+$ species with Cl^- as counter ions. The monomer $Cl_3PNSi(CH_3)_3$[2] (2.07 g, 9.23 mmol, for n_{arm}=50) was dissolved in anhydrous CH_2Cl_2 (2 mL), the phosphine solution was then added and the mixture was stirred for 24 h.

Tertbutyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (1.83 g, 7,39 mmol) was dissolved in THF and Et_3N (0.75 g, 1.1 mL, 1 equiv) was added. The poly(dichlorophosphazene) solution was slowly added to the THF solution and the mixture was stirred for 24 h. An excess amount of Jeffamine M-1000 (15.70 g, 15.70 mmol) was dissolved in THF and Et_3N (1.70 g, 2.2 mL, 1 equiv), added to the solution containing the partially substituted polyphosphazene, and stirred for 24 h. After filtration and removal of the solvent at reduced pressure, the polymer was purified by dialysis (12 kDa cutoff) in H_2O for 24 h, followed by dialysis in ethanol for 72 h. Removal of the solvent at reduced pressure gave a waxy solid. Yield: 6.71 g, 50%. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.12 (br, 11.16H), 1.42 (s, 9H), 3.37(s, 7.4H), 3.63 (m, 167.7H) ppm; {1H}^{31}P NMR (121 MHz, $CDCl_3$, δ): 1.08 ppm. $M_{n,theo}$ = 2.16 × 10^5 g mol^{-1}, SEC (linear polystyrene (PS) standards): M_n = 1.84 × 10^4 g mol^{-1}, M_w/M_n = 1.60, DLS (H_2O): d_h = 10.3 nm.



Scheme S5. i) tertbutyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (R'), Et_3N, THF, 24 h; ii) Jeffamine M-1000 (R''), Et_3N, THF, 24 h; iii) CH_2Cl_2/TFA (2:1), 1h; iv) Et_3N, methanol abs., complexes **1–4**, 24 h. Ratio R':R''= 29:71.

2 Characterization

2.1 NMR Measurements

NMR spectra of the monomer, poly(dichlorophosphazene) and substituted polymer(organo)phosphazene were measured on a Bruker 300 MHz spectrometer. The signal of $CDCl_3$ was used as internal reference for the ^1H NMR measurements. The $\{^1H\}^{31}P$ NMR measurements were carried out at 121 MHz and 85% phosphoric acid was used as external standard. NMR spectra of the final compounds and organometallic precursors were recorded on a Bruker FT-NMR Avance III™ 500 MHz instrument at 500.10 MHZ for ^1H NMR and 202.44 MHz for $\{^1H\}^{31}P$ NMR measurements.

2.1.1 ^1H NMR Spectrum of Complex 1

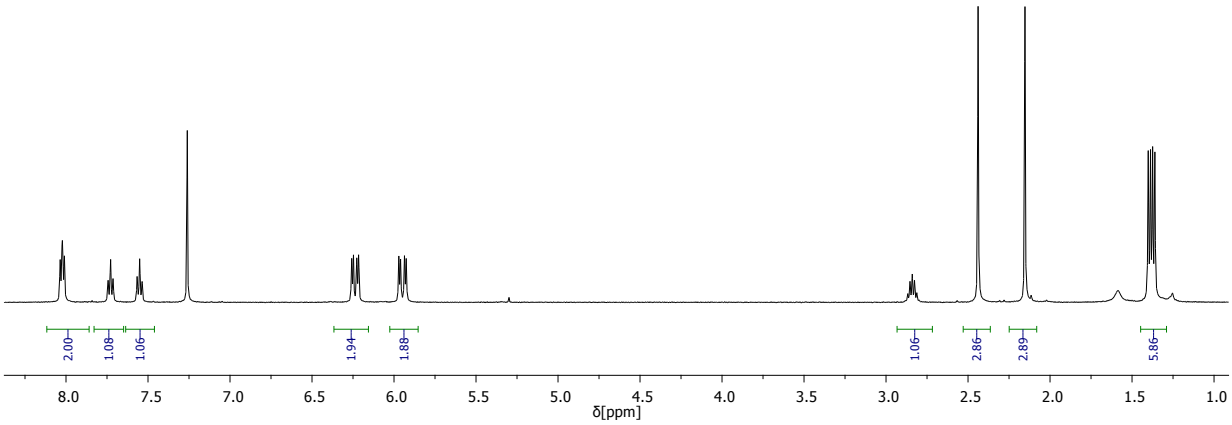


Figure S1: ^1H NMR spectrum of complex **1** recorded in $CDCl_3$.

2.1.2 ^{13}C NMR Spectrum of Complex 1

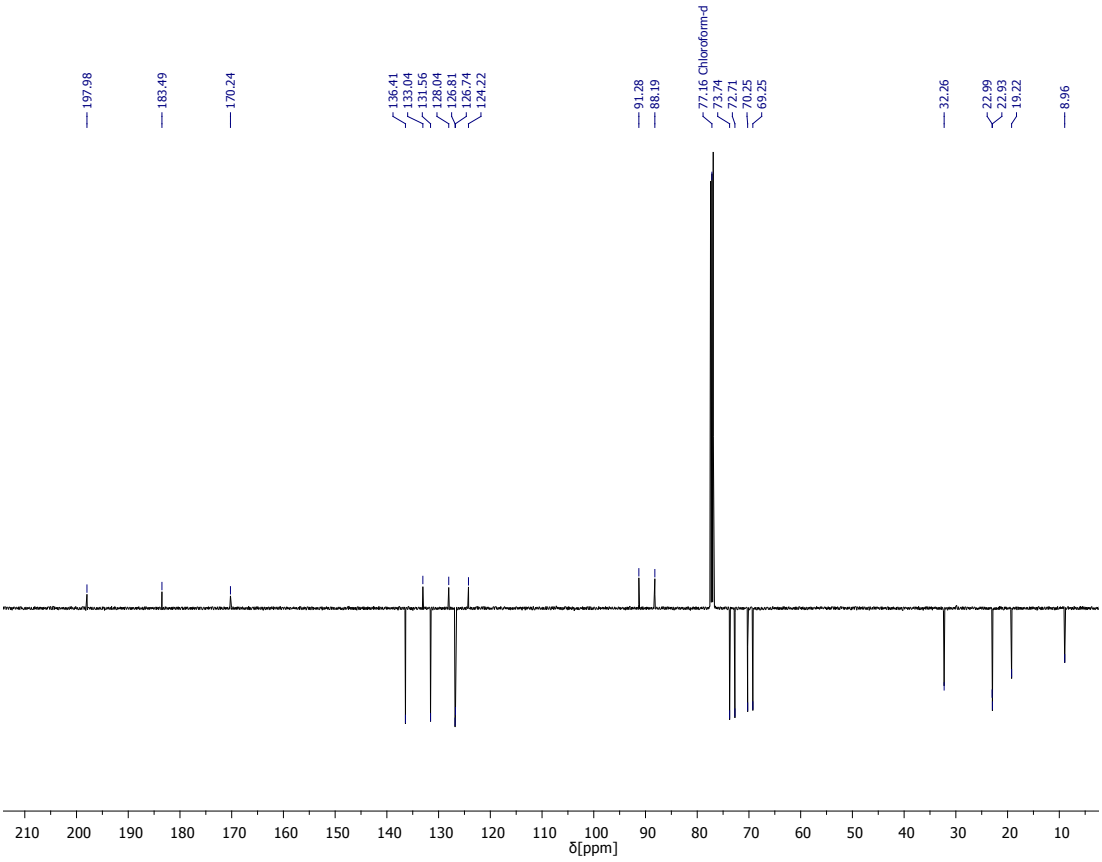


Figure S2: ^{31}C NMR spectrum of complex **1** recorded in $CDCl_3$.

2.1.3 ^{1}H NMR Spectrum of the Boc-protected Polymer

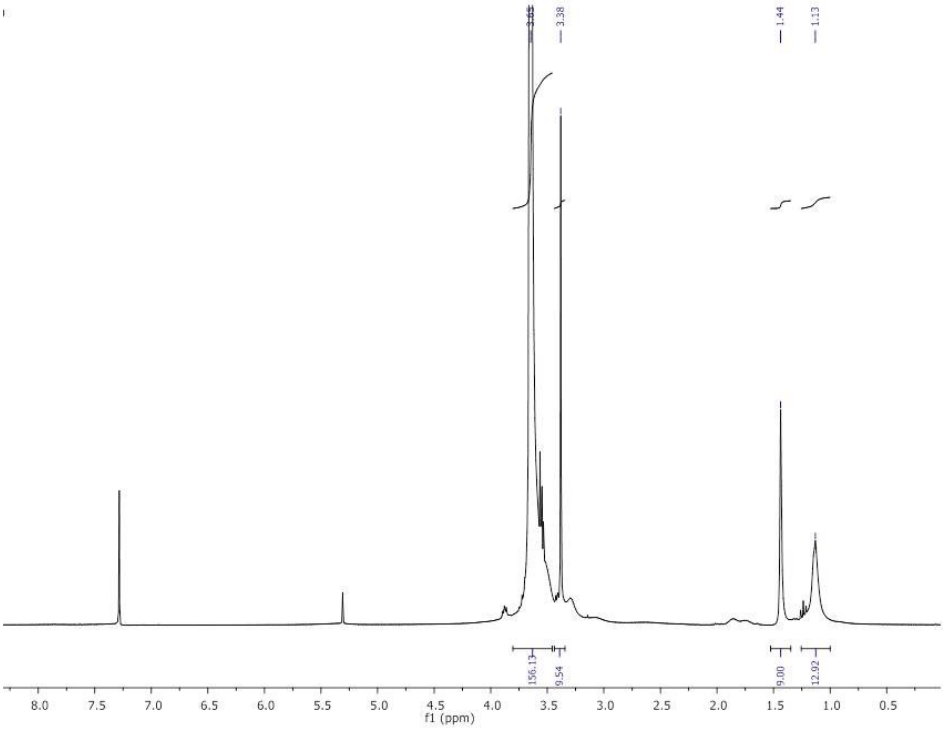
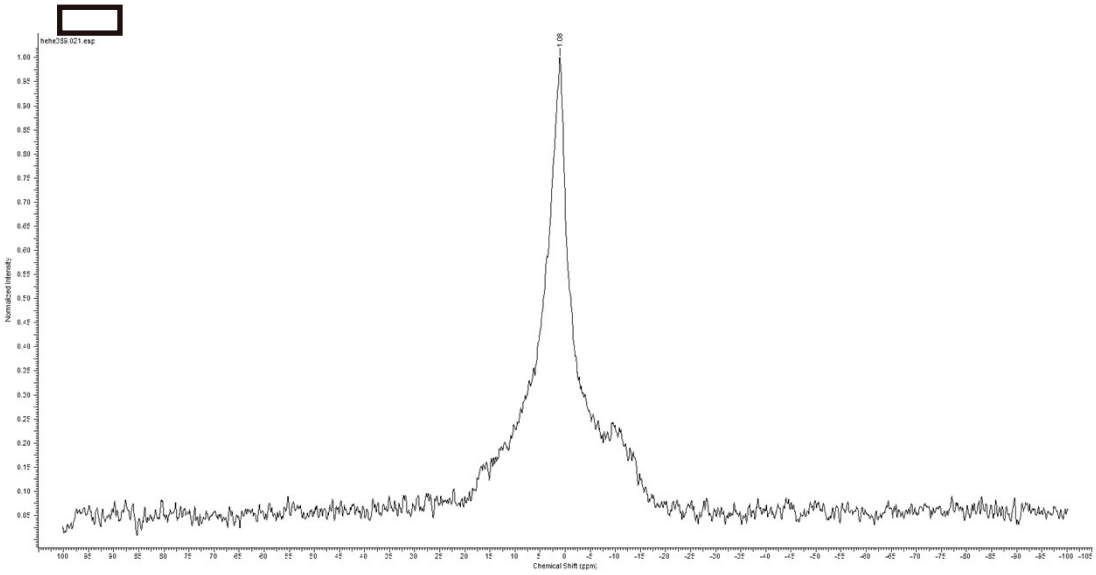


Figure S3: ^{1}H NMR spectrum of the BOC-protected polymer recorded in $CDCl_3$.



2.1.4 {^{1}H}^{31}P NMR Spectrum of the Boc-protected Polymer

Figure S4: {^{1}H}^{31}P NMR spectrum of the BOC-protected polymer recorded in $CDCl_3$.

2.1.5 {^{1}H}^{31}P NMR Spectrum of Conjugate 2a

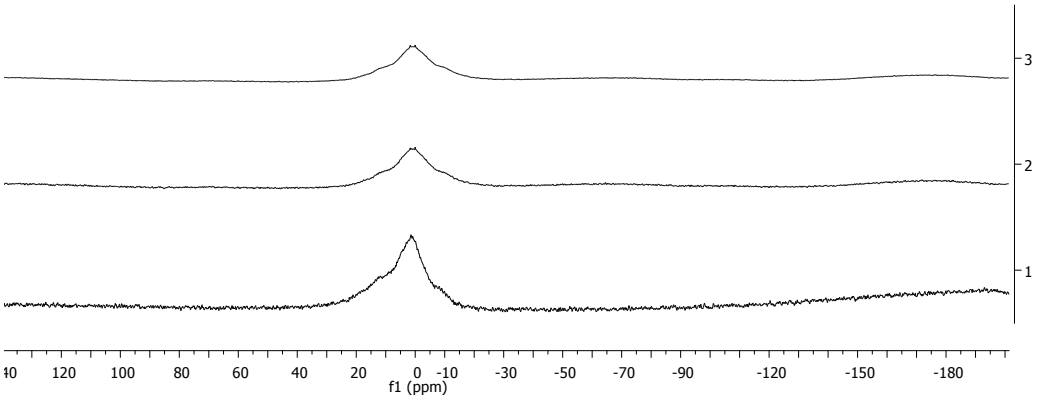


Figure S5: {^{1}H}^{31}P NMR Spectra of conjugate **2a** in aqueous solution; samples drawn and measured after one day (1), one week (2), and 4 weeks (3).

2.2 UV-Vis Investigations

A stock solution of complex **1** in DMSO (2 mM) was prepared and diluted with ammonium acetate buffer (50 mM, pH 7.0) or citric acid buffer (50 mM, pH 6.0, 5.0, 4.0, and 3.0) to a final concentration of 20 µM in 1% DMSO/buffer. An aqueous solution of conjugate **1a** (2 mM ruthenium) was diluted with ammonium acetate buffer or citric acid buffer (all 50 mM) to obtain samples of pH 7.0, 6.0, 5.0, 4.0 and 3.0. pH values were measured with an EcoScan pH 6 pH-meter equipped with a Eutech Instruments Ag/AgCl pH electrode calibrated with Alfa Aesar Specpure standard buffer solutions at pH 4.00, 7.00, and 10.00. UV−Vis spectra were recorded in 1 h intervals for 24 h at 25 °C on a PerkinElmerLambda 35 UV−Vis spectrophotometer.

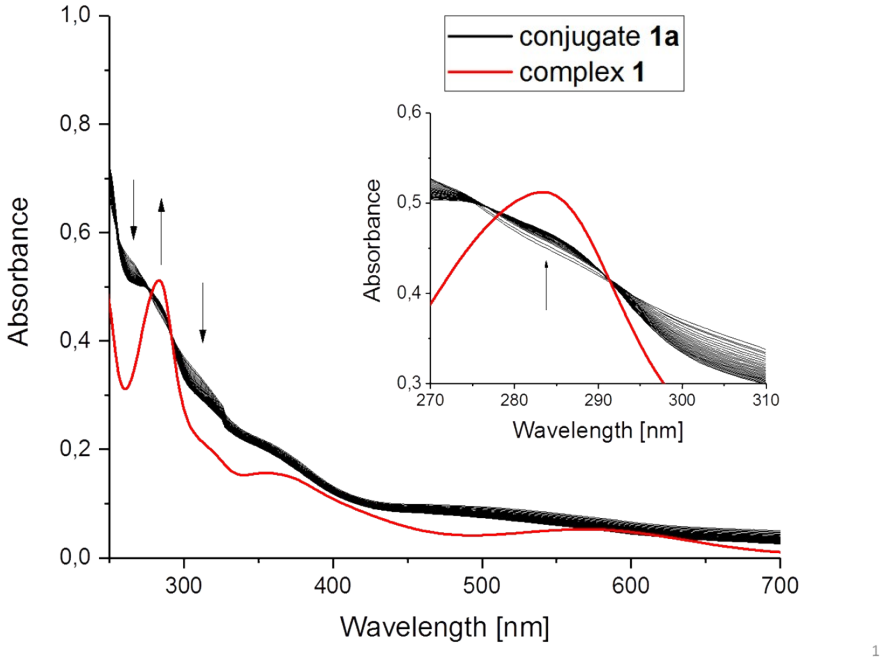


Figure S6: UV-Vis spectra of **1** and **1a** incubated at 37 °C for 24 h at pH 3.

2.3 DLS

The zeta potential measurements and the dynamic light scattering (DLS) measurements were carried out with a Malvern ZetaSizer Nano-ZS analyzer (Malvern Instruments, UK) in a disposable capillary cell and polystyrene cuvette, respectively, at 25 °C. To yield a 1 mg mL^{-1} concentration, the samples were dissolved in deionized H_2O and filtered through a 0.2 mm nylon filter. For the DLS, the 4 mW HeNe Laser was set at λ = 633 nm with the detector angle at 173° for backscattering measurements, and the volume particle size distribution was used to determine the hydrodynamic diameter.

2.4 ICP-MS Measurements

Table S1: ICP-MS parameters

ICP-MS Agilent 7500ce	
RF power (W)	1560
Cone material	Nickel
Carrier gas ($L \cdot min^{-1}$)	0.92–0.97
Make up gas ($L \cdot min^{-1}$)	0.22–0.27
Plasma gas ($L \cdot min^{-1}$)	15
Monitored isotopes	^{101}Ru, ^{102}Ru, ^{103}Rh, ^{115}In, ^{185}Re
Dwell time (s)	0.3
Number of replicates	10

The instrument was tuned on a daily basis to achieve maximum sensitivity.

Sample preparation

After the indicated drug treatment, mice were anesthetized and blood as well as urine was collected by heart and bladder punctuation, respectively. Serum was separated from the cellular blood compartment (CBC) by centrifugation at 3000 rpm for 10 min for two times and stored at -20 °C. In addition, samples of tissues (kidney, liver, lung, tumor) were collected and also stored at -20 °C for quantitative metal determination. Digestion of tissue (approx. 20 mg or 50 µL gravimetrically) was performed in nitric acid (2 mL, 20%, 4.6 M) using a microwave system (Discover SP-D, CEM Microwave Technology, Germany). The following microwave parameters were used: 200 °C, 5 min ramp time, 6 min hold time and 300 W maximal power. Digested samples were diluted with Milli-Q water resulting in nitric acid concentrations <4% and ruthenium/rhodium concentrations <15 $\mu g \cdot kg^{-1}$.

2.5 SEC-ICP-MS Analysis

Conjugate **2a** was diluted with a 10 mM ammonium acetate buffered solution to give a final ruthenium concentration of 150 µM for UV-Vis detection and 0.5 µM for ICP-MS detection. The samples were subjected to SEC-UV-Vis spectroscopy and SEC-ICP-MS, respectively. A size exclusion column (10 mm × 300 mm, 13 µm) (Superdex^{TM} 200, 10/300 GL, GE Healthcare) with an approximate bed volume of 24 mL and a linear separation range of 10–600 kDa was used. Analysis was carried out on an Agilent 1260 Infinity System (Agilent, Waldbronn, Germany) controlled by the Agilent OpenLAB CDS ChemStation Edition Rev. C.01.06[61] software. The injection volume in both experimental set-ups was 50 µL and elution was performed in isocratic mode with an aqueous solution of ammonium acetate (10 mmol·L^{-1}, pH 7.4) as mobile phase at a flow rate of 0.3 mL·min^{-1}. The column was calibrated with bovine serum albumin (66 kDa) and uracil (112.09 g·mol^{-1}) using UV–Vis detection at 240 nm. The column was coupled via a PEEK tubing directly to a MicroMist nebulizer of the ICP-MS. The ruthenium trace was recorded using the Agilent MassHunter Chromatography software package (Workstation Software, Version B.01.01, Build 123.11, Patch 4, 2012). The instrumental parameters of the ICP-MS are given in **Table S1**, in **Section 2.4**.

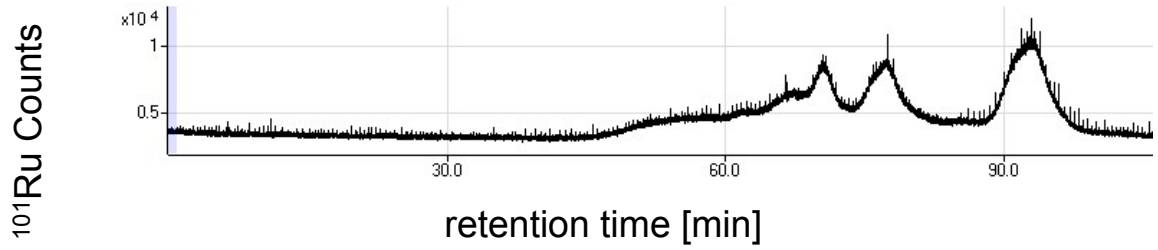


Figure S7: Degradation of conjugate **2a** due to acid-containing mobile phase (H_2O, 0.1 vol% formic acid) evaluated with SEC-ICP-MS analysis (^{101}Ru), no buffer used. The chromatogram was recorded directly without prior incubation. Different peaks might correspond to released drug, complex **2** still attached to linker sidechains or smaller fractions of the original conjugate.

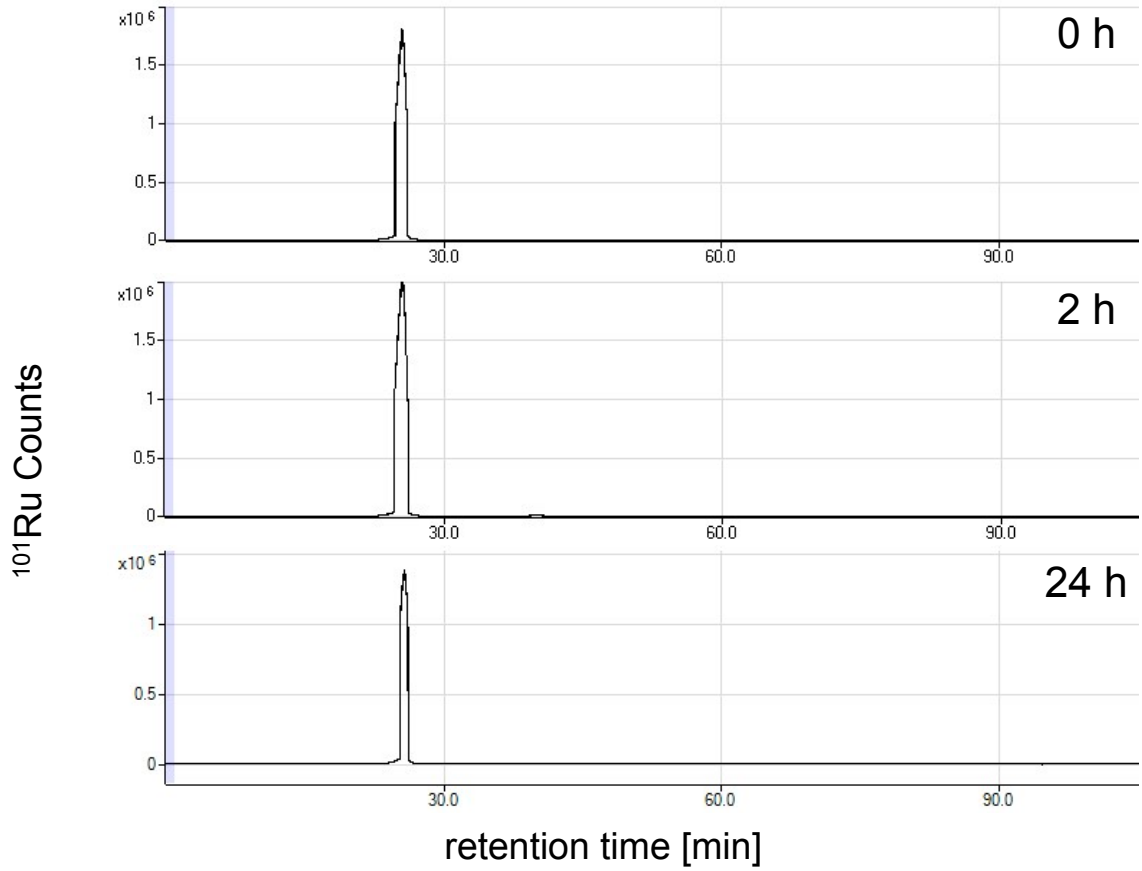


Figure S8: SEC-ICP-MS analysis of conjugate **2a**, co-incubated at 37 °C with a 10-fold excess of BSA. Sample measured after 0, 2, and 24 h of incubation (0.5 μM ruthenium, 10 mM ammonium acetate buffer). No transfer of active ruthenium-containing moiety to HSA was observed (no ruthenium traces were detected at retention times different from the signal corresponding to conjugate **2a** at 28 min even after 24 h).

3 Biological Investigations

3.1 Applied Dosages of Conjugates 1a–3a

Table S2: Overview of dosages used for animal experiments

Dosages	**Conjugate 1a**	**Conjugate 2a**	**Conjugate 3a**
Total amount	100 mg·kg^{-1}	100 mg·kg^{-1}	100 mg·kg^{-1}
Complex load	5.4 mg compound **1**·kg^{-1}	30 mg compound **2**·kg^{-1}	13 mg compound **3**·kg^{-1}
Metal load	1.2 mg Ru·kg^{-1}	7.5 mg Ru·kg^{-1}	3.2 mg Rh·kg^{-1}
Total amount	50 mg·kg^{-1}	50 mg·kg^{-1}	50 mg·kg^{-1}
Complex load	2.7 mg compound **1**·kg^{-1}	15 mg compound **2**·kg^{-1}	6.5 mg compound **3**·kg^{-1}
Metal load	0.6 mg Ru·kg^{-1}	3.8 mg Ru/kg	1.6 mg Rh·kg^{-1}
Total amount	-	25 mg·kg^{-1}	-
Complex load		7.7 mg compound **2**·kg^{-1}	
Metal load		1.9 mg Ru·kg^{-1}	

3.2 Organ Distribution – Metal levels

Table S3: Ruthenium und rhodium levels in [ng·mg^{-1}], data collected via ICP-MS measurements after microwave-assisted digestion of blood and tissue samples (measurement errors are within <5% RSD).

	Ruthenium levels [ng·mg^{-1}]				**Rhodium levels [ng·mg^{-1}]**	
Conjugate	**1a**		**2a**		**3a**	
Metal dose	1.2 mg·kg^{-1}	1.9 mg·kg^{-1}	3.75 mg·kg^{-1}	7.5 mg·kg^{-1}	1.6 mg·kg^{-1}	3.25 mg·kg^{-1}
Cellular blood compartment	0.70	0.19	2.05	3.97	0.09	0.34
	0.35	0.49	1.67	4.60	0.14	0.28
Kidney	4.82	2.46	4.53	9.13	3.29	4.44
	4.64	2.42	4.93	9.05	3.11	7.97
Liver	2.58	20.11	23.48	55.35	7.36	11.67
	2.74	15.02	28.67	55.70	6.53	14.30
Lung	0.35	1.27	1.87	4.74	0.57	1.46
	0.34	0.79	2.20	4.68	0.74	1.71
Serum	1.12	1.41	2.46	5.95	0.32	0.59
	1.09	1.87	4.02	8.15	0.28	0.76
Urine	0.18	0.31	0.54	-	0.54	0.26
	0.68	0.55	-	-	0.27	1.04

3.3 Anticancer Activity in vivo

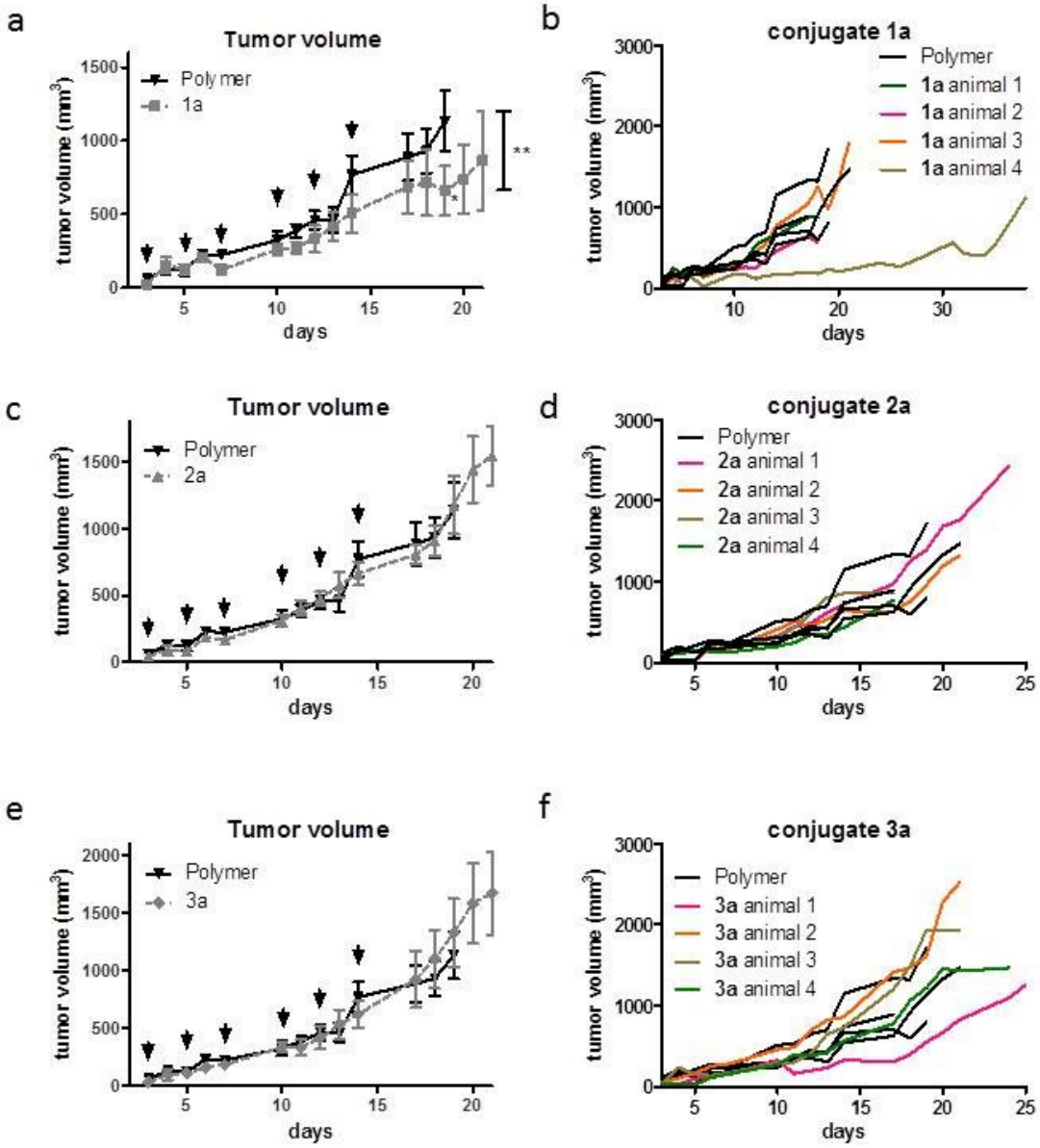


Figure S9: Anticancer activity of **1a–3a**. Murine CT-26 cells ($5x10^5$) were injected subcutaneously into the right flank of female Balb/c mice (n=4 per group). Animals were treated intravenously with **1a**, **2a** and **3a**. The applied doses were 50 $mg \cdot kg^{-1}$ for **1a** and **3**a, and 25 $mg \cdot kg^{-1}$ for 2a on day 3, 5, 7, 10, 12, and 14. Tumor volume is depicted as mean ±SD for treatment with unloaded polymer or with **1a** (a), **2a** (c) and **3a** (e). Additionally, tumor growth curves of single CT-26-bearing animals under treatment with unloaded polymer or **1a** (b), **2a** (d) and **3a** (f) are shown. **p<0.01 statistically different from unloaded polymer by two-way ANOVA (day 3-19). * p<0.05 statistically different from unloaded polymer (at day 19) by Bonferroni post test

3.4 Morphological Changes of Organs and Tissues

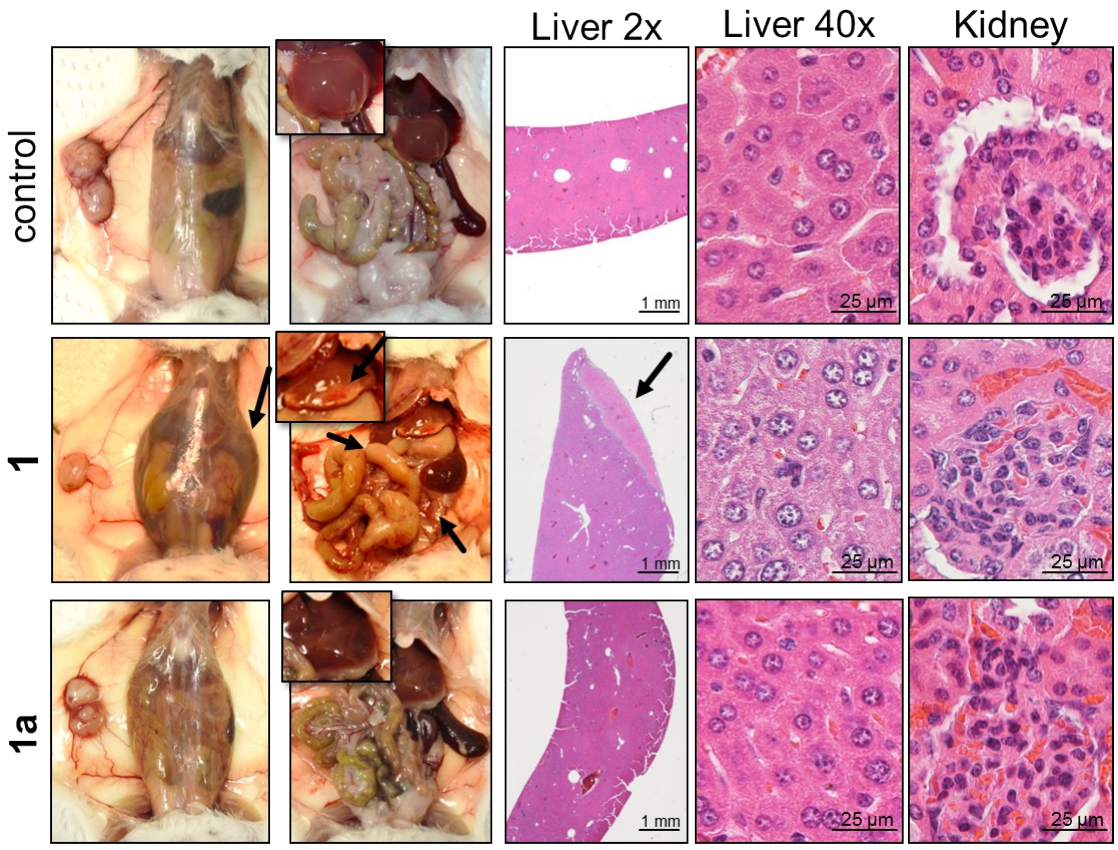


Figure S10: Representative pictures from CT-26 tumor-bearing animals treated with the indicated substances. Animals were treated with **1** (30 mg·kg^{-1} i.p.) and **1a** (50 mg·kg^{-1} i.v.) and sacrificed on day 17 and 24, respectively. Local adverse effects of treatment with **1** are indicated with arrows in pictures taken during dissection (ascites, inflated intestine, lesions on liver, growth of additional tissue) and photographs of hematoxylin/eosin-stained, paraffin-embedded liver and kidney tissue (arrow). In contrast, no macroscopic or microscopic tissue damage was found after treatment with **1a**.

3.5 Histological Evaluation of Tissue Samples

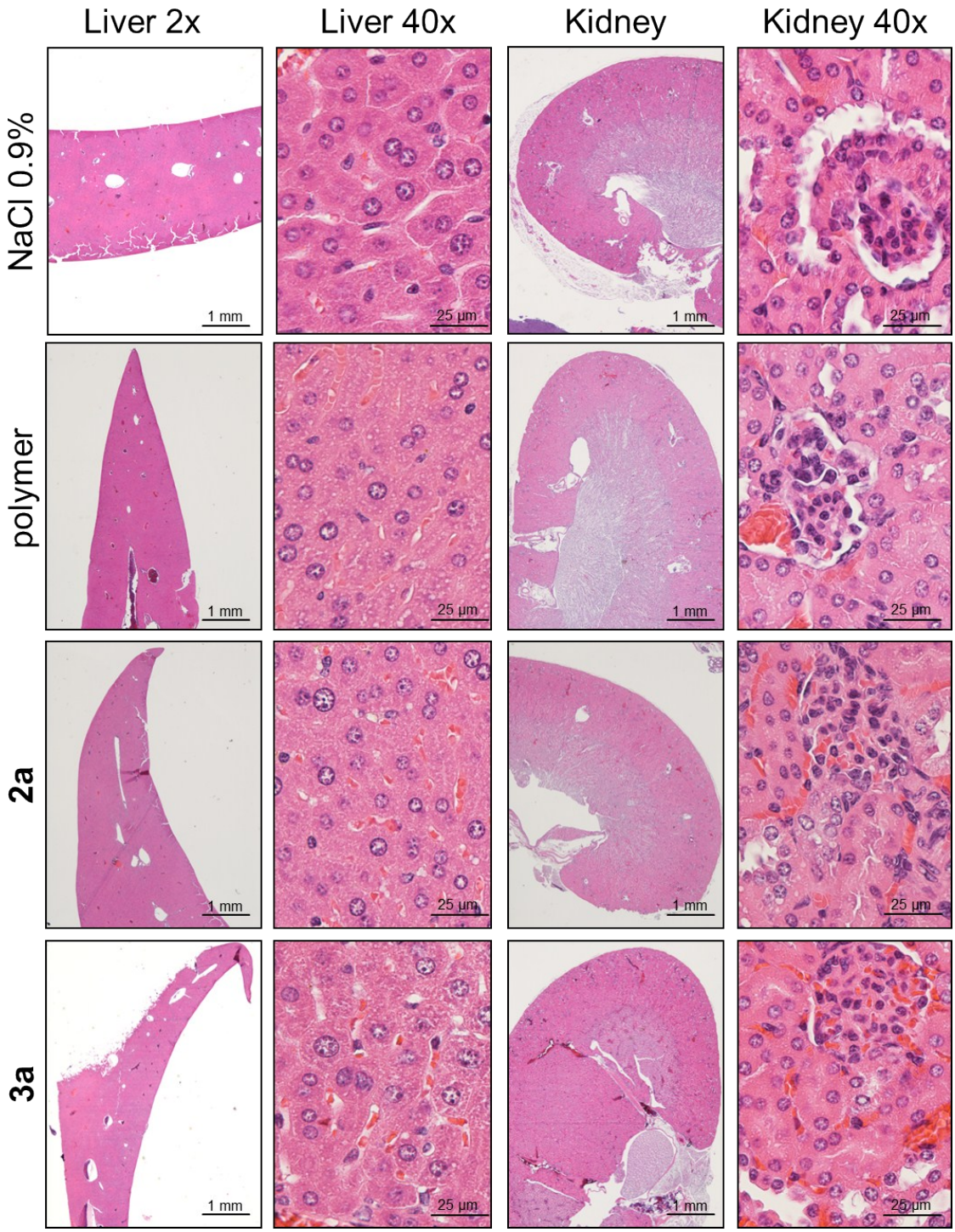


Figure S11: Histological evaluation of liver and kidney tissue after drug treatment. Animals were treated intravenously with 0.9% NaCl, unconjugated polymer, 25 mg·kg^{-1} and 50 mg·kg^{-1} of **2a** and **3a**, respectively, on day 3, 5, 7, 10, 12, and 14. Animals were sacrificed on day 24, 19, 17 and 25 and organs were formalin-fixed, paraffin-embedded and stained with hematoxylin/eosin.

4 References

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[2] B. Wang, E. Rivard, I. Manners, *Inorg. Chem.* **2002**, *41*, 1690–1691.