Ruthenium–arene complexes with NSAIDs: synthesis, characterization and bioactivity†

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Two non-steroidal antiinflammatory drugs indomethacin and mfenamic acid were coordinated to Ru(II)–arenes to afford four new complexes. The cytotoxic activities of the ligands and ruthenium complexes were tested in three human cancer cell lines (K562, A549, MDA-MB-231) and non-tumour human fetal lung fibroblast cells (MRC-5) by MTT assay. Cytotoxicity studies revealed that indomethacin Ru(II)–arene complexes 1 and 3 displayed good cytotoxicity and apparent cytoselective profiles. The IC50 values obtained in leukemia K562 cells were comparable to those of cisplatin (10.3 μM (CDDP), 11.9 μM (1) and 13.2 μM (3)). Flow cytometric analysis of 1 and 3 in triple-negative breast cancer MDA-MB-231 cells revealed an interesting mechanism of action. At IC50 concentrations, 1 and 3 arrested cell cycle progression in S phase and caused rapid accumulation of cells in sub-G1 phase (up to 48%), while Annexin V-FITC/PI staining showed simultaneous occurrence of apoptotic and necrotic cell populations at approximately similar levels of 20%. Measurement of reactive oxygen species (ROS) production by DCFH-DA staining confirmed the potential of 1 and 3 to increase ROS even more than cisplatin. The interaction of the complexes with serum albumins showed their potential ability to bind tightly and reversibly to albumins. The affinity of the complexes to calf-thymus DNA was investigated by UV-vis spectroscopy, viscosity measurements and fluorescence emission spectroscopy for competitive studies of the complexes with ethidium bromide, revealing that their interaction probably occurs via intercalation. Taken together, the results strongly suggest the potential of complexes 1 and 3 to alter cell cycle progression and cause DNA-damage by means of direct DNA-binding or indirectly by ROS production.

Introduction

Rosenberg’s highly significant discovery of cisplatin1 opened the way for the introduction of metal complexes in cancer chemotherapy; since then, scientists have been searching for new potential anticancer compounds. Currently, research in that field is focused on ruthenium(n) complexes.2 Ruthenium complexes have been investigated for use as DNA topoisomerase inhibitors,3 TrxR inhibitors,4 antimicrobial agents,5 molecular probes6 and anticancer agents.7 Also, ruthenium can mimic iron in binding with certain biological molecules, particularly transferrin, and Ru(n) and Ru(m) complexes can display similar ligand exchange kinetics to Pt(n).8 Considering all these characteristics, ruthenium complexes are suitable for medical applications. Three of these, namely KP1339, KP1019 and NAMI, have entered clinical trials, with promising results.2,9,10 A number of ruthenium(n) complexes bearing π-bonded arene ligands have already been developed and have shown promising anticancer activities.11 Because the concentration of oxygen in tumor cells is low, the environment is more reductive than in normal tissue, favouring the active reduced form.8,12

A real revolution in medicine occurred with the discovery of non-steroidal anti-inflammatory drugs (NSAIDs), which have been proven to be biologically active compounds. NSAIDs are one of the most commonly used classes of medication.13 Their main mechanism of action is based on the inhibition of the enzyme cyclooxygenase (COX), more precisely, its two isoforms: COX-1 and COX-2. By this method, they prevent the formation of various prostaglandins responsible for the physiological responses of fever, pain sensation and anti-inflammation.14 Also, NSAIDs have synergistic action on the activity of certain
antitumor drugs and can lead to cell death of a series of cancer cell lines via apoptosis. Metal complexes containing NSAIDs are among the compounds which have received much attention and increasing interest from a medicinal inorganic chemistry viewpoint because they are found in coordination compounds which have active drugs as ligands.

Indomethacin (Hindo, Fig. 1) is a phenylalkanoic acid and is one of the most potent clinically used NSAIDs. It is used to relieve pain, fever and inflammation. Despite the known adverse gastrointestinal side-effects (ulceration and hemorrhage) limiting the doses and use of NSAIDs, Hindo and its Cu(n) complex are widely safely administered in the clinical treatment of acute inflammation and other medical conditions in humans. A series of copper(n) and two tin complexes with indomethacin as a ligand have been reported in the literature.

Mefenamic acid (Hmef, Fig. 1), or N-phenylanthranilic acid, is in clinical use. Mefenamic acid is an effective analgesic and antipyretic agent with relatively mild side-effects, including headaches, diarrhea, vomiting and nervousness. Recently, Cu(n), Co(n), Ni(n), Zn(n), Mn(n) and Sn(n) complexes with mefenamato ligands have been reported.

Recently, Hartinger and Dyson showed in separate studies that oxicam-based NSAIDs, as well as aspirin, coordinate to Ru(n) and Os(n) ions in half-sandwich complexes, providing antitumor-active compounds. Based on these results, the aim of our work presented here was to combine two well-known drugs (NSAIDs) with Ru(n)–arene precursors in order to obtain new half-sandwich Ru(n)–arene complexes with improved cytotoxic activities. This may be a method to alter the purpose of these drugs and to obtain potentially good cytotoxic agents. We synthesized four new complexes and completely characterized them. Furthermore, we investigated their cytotoxic activities by MTT assay. In order to explain the cytotoxicity results, we have analyzed the cell cycle, using flow-cytometric analysis and the annexin V-FITC apoptotic assay, for quantitative analysis of apoptotic and necrotic death of selected cells. We have also measured the production of intracellular reactive oxygen species (ROS) and tested the ability of the compounds to scavenge 1,1-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radicals (•OH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals and to inhibit soybean lipoxigenase (LOX) activity. Additionally, we examined the in vitro affinity of the complexes for bovine serum albumin (BSA) and human serum albumin (HSA) by fluorescence emission spectroscopy as well as the in vitro interaction of the complexes with calf-thymus (CT) DNA by UV-vis spectroscopy, viscosity measurements and fluorescence emission spectroscopy.

Experimental section

Materials and measurements

RuCl₃·3H₂O was purchased from Johnson Matthey (London, UK). Indomethacin and mefenamic acid were purchased from TCI (Tokyo, Japan). Trisodium citrate, NaCl, CT DNA, BSA, HSA, EB, DPPH, ABTS, sodium linoleate, butylated hydroxyltoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), nordihydroguaiaretic acid (NDGA) and caffeine were purchased from Sigma-Aldrich. [Ru(n₆-p-cymene)Cl₂]₂ was prepared according to a published procedure. [Ru(n₆-toluene)Cl₂]₂ was prepared according to a published procedure. Solvents were obtained and used without further purification. Infrared spectra were recorded on a Nicolet 6700 FTIR spectrometer using the ATR technique. NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer. Chemical shifts for ¹H and ¹³C NMR spectra were referenced to residual ¹H and ¹³C present in CDCl₃ or DMSO-d₆. ESI mass spectra of the ruthenium complexes were recorded on a 6210 Time-of-Flight LC-MS instrument (G1969A, Agilent Technologies) in both positive and negative ion modes using CH₃CN/H₂O or CH₃CN as solvents. UV-vis spectra were recorded in solution at concentrations in the range of 10⁻⁵–10⁻³ M on a Hitachi U-2001 dual beam spectrophotometer. Fluorescence spectra were recorded in solution on a Hitachi F-7000 fluorescence spectrophotometer. Viscosity experiments were carried out using an ALPHA L Fungilab rotational viscometer equipped with an 18 mL LCP spindle.

DNA stock solution was prepared by dilution of CT DNA with buffer (containing 150 mM NaCl and 15 mM trisodium citrate at pH 7.0) followed by vigorous stirring at 4 °C for three days; the solutions were maintained at 4 °C for no longer than two weeks. The stock solution of CT DNA gave a ratio of UV absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀) in the range of 1.85 to 1.90, indicating that the DNA was sufficiently free of protein contamination. The concentration of CT DNA was determined by the UV absorbance at 260 nm after 1:20 dilution using c = 6600 M⁻¹ cm⁻¹.

Synthesis of the complexes

Synthesis of K[Ru(n₆-p-cymene)(indo)Cl₂]₁. A solution of Hindo (0.0584 g, 0.163 mmol) in ethanol (5 mL) was neutralized with potassium hydroxide (9.13 mg, 0.163 mmol) and stirred for 1 h at room temperature. After that, a suspension of [Ru(n₆-p-cymene)Cl₂]₂ (0.05 g, 0.08 mmol) in ethanol (5 mL) was added to the ligand solution, and the reaction mixture was precipitated. The precipitate was filtered and dried in vacuo. Yield: 62 mg, 54%. Anal. calc. for C₉₀H₇₂Cl₁₁O₁₄Ru₂K: C, 49.60; H, 4.13; N, 1.99. Found: C, 49.73; H, 4.21; N, 2.09. δ (ppm) H (500 MHz; CDCl₃): 1.31 (CH(C₆H₅)), 19.03 (Z(C₁), Z(C₄)), 28.21 (Z(C₆)), 41.26 (Z(C₇)), 51.24 (Z(C₉)), 58.31 (Z(C₁₀)), 64.82 (Z(C₁₁)), Z(C₁₂)), 66.98 (Z(C₁₃)), 71.91 (Z(C₁₄)), 73.41 (Z(C₁₅)), 73.94 (Z(C₁₆)). δ (ppm) C (125 MHz; DMSO-d₆): 13.49 (Z(C₁₁)), 13.58 (Z(C₁₅)), 13.69 (Z(C₁₆)), 13.76 (Z(C₁₇)), 13.81 (Z(C₁₈)), 13.86 (Z(C₁₉)), 13.91 (Z(C₂₀)), 13.96 (Z(C₂₁)), 13.99 (Z(C₂₂)), 14.01 (Z(C₂₃)), 14.02 (Z(C₂₄)), 14.03 (Z(C₂₅)), 14.04 (Z(C₂₆)), 14.05 (Z(C₂₇)), 14.06 (Z(C₂₈)), 14.07 (Z(C₂₉)), 14.08 (Z(C₃₀)).
129.11 (C5), 131.28 (C15, C17), 134.30 (C13), 135.62 (C14, C18), 139.24 (C8, C16), 156.01 (C2), 168.60 (C10, C12). IR (ATR), \(\nu_{\text{max}}/\text{cm}^{-1}: 2963 (\text{C-H}), 1686 (\text{C-O}), 1453 (\text{C-Car}), 1261 (\text{C-O}), 1226 (\text{C-H}), 841 (\text{C-H}).\) LC-ESI/MS, \(m/z\) (M, %): 592.08 ([M – K – C1 + H]^+), 633.11 ([M – K – Cl + H]^+), 45.

**Synthesis of [NH4][Ru(n=6-ptylene)(mef)Cl2].** A suspension of Hmef (0.04 g, 0.163 mmol) in methanol (5 mL) was added to a suspension of [Ru(n=6-ptylene)Cl2]2 in methanol (5 mL). The reaction mixture was stirred at room temperature for 1 h. The orange suspension was then concentrated (5 mL). The reaction mixture was stirred at room temperature for 2 h. The resulting orange precipitate was filtered and dried in vacuo. Yield: 32.2 mg, 54.12%. Anal. calc. for C22H25Cl2N2O2Ru: C, 49.22; H, 5.45; N, 4.78. Found: C, 49.68; H, 5.13; N, 4.38. 

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**Synthesis of K[Ru(n=6-toluene)(indo)Cl2].** The synthesis of complex 3 is the same as for complex 1 with the use of [Ru(n=6-toluene)Cl2]2 instead of [Ru(n=6-ptylene)Cl2]2. The resulting orange precipitate was filtered and dried in vacuo. Yield: 70 mg, 65%. Anal. calc. for C28H2ClNO2RuKCH3OH: C, 46.90; H, 3.76; N, 2.03. Found: C, 46.60; H, 3.61; N, 2.09. 

**Synthesis of K[Ru(n=6-toluene)(indo)Cl2].** The synthesis of complex 4 is the same as for complex 2 with the use of [Ru(n=6-toluene)Cl2]2 instead of [Ru(n=6-ptylene)Cl2]2. The resulting yellow precipitate was filtered and dried in vacuo. Yield: 32.2 mg, 54.12%. Anal. calc. for C25H32Cl2N2O2Ru2CH3OH: C, 49.22; H, 5.45; N, 4.78. Found: C, 49.68; H, 5.13; N, 4.38. 

**Synthesis of (NH4)[Ru(n=6-toluene)(mef)Cl2].** A suspension of Hmef (0.04 g, 0.163 mmol) in methanol (5 mL) was added to a suspension of [Ru(n=6-toluene)(mef)Cl2]2 in methanol (5 mL). The reaction mixture was stirred at room temperature for 1 h. The orange suspension was then concentrated (5 mL), and solid NH4PF6 (0.03 g) was added. The mixture was stirred at room temperature for 2 h. The resulting orange precipitate was filtered and dried in vacuo. Yield: 51 mg, 55%. Anal. calc. for C25H32Cl2N2O2Ru: C, 53.18; H, 5.67; N, 4.96. Found: C, 53.60; H, 5.69; N, 4.53. 

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wavelength of 570 nm. The IC50 values (concentration of investigated agent that decreases the number of viable cells by 50% in a treated cell population compared to a non-treated control) were estimated from the dose–response curves.

Cell cycle analysis
Flow-cytometric analysis of the cell cycle phase distribution was performed in MDA-MB-231 cells after staining the fixed cells with propidium iodide (PI).47 We examined the effects of complexes 1 and 3, which displayed the most prominent cytotoxicity, in comparison to CDDP as a reference compound.

MDA-MB-231 cells were seeded at a density of 3 × 104 cells per well into 6-well plates (Thermo Scientific Nunc™) in the nutrition medium. After 24 h of growth, the cells were continually exposed to complexes 1 and 3 or CDDP at concentrations corresponding to 0.5 × IC50 and IC50: complex 1 (11 μM and 22 μM), complex 3 (13 μM and 26 μM) and CDDP (8 μM and 16 μM). After 24 h of treatment, cells were collected by trypsinization, washed twice with ice-cold PBS, and fixed for 30 min in 70% ethanol. Fixed cells were washed again with PBS and incubated with RNaseA (1 mg mL⁻¹) for 30 min at 37 °C. Immediately before flow-cytometric analysis, cells were stained with PI at a concentration of 400 μg mL⁻¹. The cell cycle phase distribution was analyzed using a fluorescence activated cell sorting (FACS) Calibur Becton Dickinson flow cytometer and Cell Quest computer software (Becton Dickinson, Heidelberg, Germany).

Annexin V-FITC apoptotic assay
Quantitative analysis of apoptotic and necrotic death of MDA-MB-231 cells induced by complexes 1 and 3 and CDDP as a reference compound were performed using an Annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions (BD Biosciences, Pharmingen San Diego, CA, USA). Briefly, 1 × 10⁶ cells per mL were treated with complexes 1 and 3 and CDDP at concentrations corresponding to 0.5 × IC₅₀ and IC₅₀ for 24 h. After treatment, the cells were collected, washed twice with ice-cold PBS and then resuspended in 200 μL 1× Binding Buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). 100 μL of cell suspension (containing approximately 1 × 10⁶ cells) was transferred to a 5 mL culture tube and mixed with 5 μL of both Annexin V-FITC and PI. The cells were gently vortexed and incubated for 15 min at 25 °C in the dark. After the incubation period, 400 μL of 1× Binding Buffer was added to each sample, which was then analyzed using a FACS Calibur Becton Dickinson flow cytometer and Cell Quest computer software (Becton Dickinson, Heidelberg, Germany).

Measurement of intracellular reactive oxygen species
Generation of reactive oxygen species (ROS) in MDA-MB-231 cells after treatment with complexes 1 and 3 and CDDP was measured using a ROS-sensitive fluorophore, 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich Co.ᵃ⁻ᵇ⁻). Briefly, 3 × 10⁵ MDA-MB-231 cells per well were seeded into 6-well plates (Thermo Scientific Nunc™) in nutrition medium. After 24 h of growth, the cells were treated with complexes 1 and 3 and CDDP at concentrations corresponding to their IC₅₀ values for 4 h. After treatment, the cells were harvested, washed twice with ice-cold PBS, re-suspended in 1 mL of 50 μM DCFH-DA and incubated for 30 min at 37 °C in the dark. After incubation with the dye, the cells were washed with PBS, re-suspended in 300 μL of PBS and immediately analyzed using the FL1 channel of the FACS Calibur Becton Dickinson flow cytometer and using Cell Quest computer software (Becton Dickinson, Heidelberg, Germany). The excitation wavelength used in the measurements was 485 nm, with peak emission measured at 530 nm. Subsequently, the geomean of the DCFH-DA-dependent fluorescence was determined.

Antioxidant activity
The antioxidant activities of complexes 1–4 were evaluated via their ability to scavenge free radicals such as DPPH, hydroxyl and ABTS. Furthermore, their ability to inhibit the activity of soybean lipoygenase was also studied. Each experiment was performed in triplicate, and the standard deviation of absorbance was less than 10% of the mean.

Determination of the reducing activity of the stable radical DPPH.
To an ethanolic solution of DPPH (0.1 mM), an equal volume of an ethanolic solution of the compound was added. The concentration of the solution of the compound was 0.1 mM. Ethanol was also used as a control solution. The absorbance at 517 nm was recorded at room temperature after 20 and 60 min in order to examine the time-dependence of the DPPH radical scavenging activity.⁴⁹⁻ᵃᵇ⁻ The DPPH radical scavenging activity of the compounds was expressed as the percentage reduction of the absorbance value of the initial DPPH solution (RA%). NDGA and BHT were used as reference compounds.

Competition of the tested compounds with DMSO for hydroxyl radicals.
The hydroxyl radicals generated by the Fe³⁺/ascorbic acid system were detected by the determination of formaldehyde produced from the oxidation of DMSO, according to Nash.⁵⁰⁻ The reaction mixture contained EDTA (0.1 mM), Fe³⁺ (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compound (concentration 0.1 mM) and ascorbic acid (10 mM). After 30 min of incubation (37 °C) the reaction was stopped with CCl₄/COOH (17% w/v) and the absorbance at λ = 412 nm was measured. Trolox was used as an appropriate standard. The competition of the compounds with DMSO for ·OH generated by the Fe³⁺/ascorbic acid system, expressed as the percent inhibition of formaldehyde production, was used for evaluation of their hydroxyl radical scavenging activities (·OH%).

Assay of radical cation scavenging activity. ABTS radical cation (ABTS⁺) was produced by reacting an aqueous stock solution (2 mM) of ABTS with 0.17 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS. Although the oxidation of the ABTS commenced immediately, the absorbance became maximal and stable after 6 h. The radical was stable in this form for more than 2 days when stored in the dark.
at room temperature. The ABTS* solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. After addition of 10 μL of diluted compound or standard (0.1 mM) in DMSO, the absorbance reading was taken exactly 1 min after the initial mixing. The radical scavenging activity of the complexes was expressed as the percentage inhibition of the absorbance of the initial ABTS solution (ABTS%). Trolox was used as an appropriate standard.

**Soybean lipooxygenase inhibition study in vitro.** The in vitro soybean lipooxygenase inhibition was evaluated as reported previously. The tested compounds were dissolved in ethanol and were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution (1/9 × 10⁻⁴ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoelic acid at 234 nm was recorded and compared with the appropriate standard inhibitor caffeic acid.

**Albumin binding studies**

The albumin binding studies were performed by tryptophan fluorescence quenching experiments using bovine serum albumin (BSA, 3 μM) or human serum albumin (HSA, 3 μM) in buffer (containing 15 mM trisodium citrate and 150 mM NaCl at pH 7.0). The quenching of the emission intensity of tryptophan residues of BSA at 343 nm or HSA at 351 nm was monitored by using complexes 1–4 (up to the value of r = 0.35). All measurements were performed at room temperature. The obtained data are presented as (η/η₀)₁/₃ versus r, where η is the viscosity of DNA in the presence of the compound and η₀ is the viscosity of DNA alone in buffer solution.

The competitive studies of complexes 1–4 with EB were investigated by fluorescence emission spectroscopy in order to examine if the complexes could displace EB from its CT DNA–EB conjugate. The CT DNA–EB conjugate was prepared by adding 20 μM EB and 26 μM CT DNA in buffer (150 mM NaCl and 15 mM trisodium citrate at pH 7.0). The intercalating effects of the complexes were studied by stepwise addition of a certain amount of a solution of the compound into a solution of the DNA–EB conjugate. The influence of the addition of each compound to the DNA–EB complex solution was obtained by monitoring the changes of the fluorescence emission spectra recorded with an excitation wavelength (λₑₓ) at 540 nm. Complexes 1–4 did not show any fluorescence emission bands at room temperature in solution or in the presence of CT DNA or EB under the same experimental conditions (λₑₓ = 540 nm); therefore, the observed quenching is attributed to the displacement of EB from its EB–DNA conjugate. The Stern–Volmer constants (KₛVm) in M⁻¹ were calculated according to the linear Stern–Volmer equation (eqn (S2), ESI) and the plots of Iₐ/I versus [Q]. The quenching constants (Kᵣ, in M⁻¹ s⁻¹) of the complexes were calculated according to eqn (S3) (ESI) because the fluorescence lifetime of the EB–DNA system is τ₀ = 23 ns.

**Results and discussion**

**Synthesis and characterization of Ru complexes**

Reaction of [Ru(η⁴-arene)Cl₂]₂, namely [Ru(η⁴-p-cymene)Cl₂], or [Ru(η⁴-toluene)Cl₂], with stoichiometric amounts of the corresponding NSAID ligands, indomethacin (Hindo) and mefenamic acid (Hmef), led to the formation of two cymene complexes, [Ru(η⁴-p-cymene)(indol)Cl₂]⁻ and [Ru(η⁴-p-cymene)(mef)Cl₂]⁻, and two toluene complexes, [Ru(η⁴-toluene)(indol)Cl₂]⁻ and [Ru(η⁴-toluene)(mef)Cl₂]⁻, in reasonably good yields. The resulting complexes 1 and 3 precipitated after neutralization with potassium hydroxide, and complexes 2 and 4 precipitated after addition of ammonium hexafluorophosphate to the reaction mixture (Scheme 1). Complexes 1 and 3 showed good solubility in acetonitrile, dimethylsulfoxide, and chloroform and lower solubility in ethanol and methanol. Complexes 2 and 4 showed excellent solubility in all these solvents.

The IR spectra of the free NSAIDs show characteristic absorption bands for the ν(O–H) vibration at about 3500 cm⁻¹. These absorption bands did not appear in the IR spectra of the complexes, indicating ligand coordination via carboxylate anions. Due to this, the coordination absorption bands for carbonyl group stretching at 1650 to 1700 cm⁻¹ in the complexes were also slightly shifted to lower values.
Upon complexation to the metal, the symmetries of \([\text{Ru}(\eta^6-p\text{-cymene})\text{Cl}_2]\) and \([\text{Ru}(\eta^6\text{-toluene})\text{Cl}_2]\) decreased. The cymene and toluene ring protons as well as the protons from the ligand moiety in the complexes exhibited slightly lower chemical shifts in comparison to the starting complexes or the ligands themselves. Signals for protons from the carboxylic group in the \(^1\text{H}\) NMR spectra of all complexes did not appear due to deprotonation and coordination.

The \(^{13}\text{C}\) NMR spectra of the synthesized complexes did not suffer significant changes. Chemical shifts for carbon atoms from the arene moiety were slightly moved to higher values. Values for chemical shifts corresponding to carbon atoms from the carboxylate groups were higher in the complexes compared to the free ligands (Fig. S1–S8, ESI†).

Finally, mass spectra of the complexes were recorded both in positive and negative ion mode. The molecular ion was detected only in the case of complex 3, \(m/z\) values 621.9 in negative mode and 623 in positive mode, while in other cases, only the anionic parts of the complexes with or without Cl ions were detected: \([M - K^+ - 2\text{Cl} + \text{H}]^+ (1, 3)\) and \([M - \text{NH}_4^+ - 2\text{Cl}]^+ (2, 4)\).

### MTT assay

The cytotoxic activities of the novel ruthenium(II) complexes 1–4, as well as the free NSAID ligands, indomethacin and mefenamic acid, in comparison to cisplatin (CDDP) were investigated by MTT assay. The study was performed in three human cancer cell lines (K562, A549, MDA-MB-231) and in one human non-tumor cell line (MRC-5). The results obtained after 72 h of continuous drug action are presented as IC\(_{50}\) values (\(\mu\text{M}\)) (Table 1) provided from cell survival diagrams (Fig. 2). Complexes 1–4 exhibited generally higher cytotoxic activity compared to free NSAIDs, Hindo and Hmef, although the IC\(_{50}\) values of complexes varied in the micromolar range from 11.9 to 275.7 \(\mu\text{M}\) depending on the cell line. Complexes 1 and 3, carrying indomethacin ligands, showed the highest cytotoxic potential. The human myelogenous leukemia K562 cell line proved to be the most sensitive to the actions of 1 and 3 (IC\(_{50}\) values: 11.9 \(\mu\text{M}\) and 13.2 \(\mu\text{M}\), respectively). These values were comparable to cisplatin (IC\(_{50}\) = 10.3 \(\mu\text{M}\)), and were significantly lower than the IC\(_{50}\) values obtained for complexes 2 (IC\(_{50}\) = 96.4 \(\mu\text{M}\)), and 4 (IC\(_{50}\) = 133 \(\mu\text{M}\)). Interestingly, MDA-MB-231 cells exhibited much greater sensitivity toward 1 and 3 than CDDP-resistant lung carcinoma A549 cells and non-tumor MRC-5 cells. The results for MRC-5 indicated that this cell line was at least 4 times less sensitive to 1–4 than to CDDP. The NSAIDs exhibited poor activity, up to 300 \(\mu\text{M}\), while Hmef could be considered inactive in MRC-5. A structure–activity comparison revealed that

### Table 1. IC\(_{50}\) values (\(\mu\text{M}\)) obtained after 72 h of continuous drug action

<table>
<thead>
<tr>
<th>Compound</th>
<th>K562 (\mu\text{M})</th>
<th>A549 (\mu\text{M})</th>
<th>MDA-MB-231 (\mu\text{M})</th>
<th>MRC-5 (\mu\text{M})</th>
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<tbody>
<tr>
<td>1</td>
<td>11.9 ± 4.4</td>
<td>45.5 ± 2.7</td>
<td>22 ± 3.6</td>
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<td>96.4 ± 2.2</td>
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<td>142.4 ± 9.3</td>
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<td>Hindo</td>
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<tr>
<td>Hmef</td>
<td>143.9 ± 4.1</td>
<td>217.3 ± 46.7</td>
<td>237.9 ± 18.8</td>
<td>&gt;300</td>
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<tr>
<td>CDDP</td>
<td>10.3 ± 1.2</td>
<td>13.6 ± 1.8</td>
<td>15.9 ± 2.1</td>
<td>9.3 ± 0.9</td>
</tr>
</tbody>
</table>

\(\alpha\) IC\(_{50}\) values (\(\mu\text{M}\)) are presented as the mean ± SEM of three independent experiments. >300 denotes that IC\(_{50}\) was not obtained in the range of concentrations tested up to 300 \(\mu\text{M}\).
the NSAID Hindo enhanced the cytotoxicity of the resulting ruthenium(II)–arene complexes 1 and 3 despite having poor activity itself. On the other hand, \( \pi \)-bonded arene ligands such as \( p \)-cymene (in complexes 1 and 2) or toluene (in complexes 3 and 4) appeared to have no significant impact on the \textit{in vitro} cytotoxic potential of the complexes. Triple-negative breast cancer cells such as MDA-MB-231 are characterized by high metastatic potential and lack of specific molecular targets for effective therapy; thus, the results of the present study indicated that ruthenium-based “combi-molecules” may represent a promising alternative for treatment of this type of cancer (Table S1, ESI†).

**Cell cycle analysis**

The capability of complexes 1, 3 and CDDP to induce cell cycle alterations was examined by flow cytometry after 24 h of continual treatment and staining with PI. Triple-negative breast carcinoma MDA-MB-231 cells were chosen to further investigate the mechanism of action of complexes 1 and 3. The results are presented in Fig. 3 as diagrams of cell distribution over the cell cycle phases. At low \( 0.5 \times \text{IC}_{50} \) concentrations, both 1 and 3 affected cell cycle progression in a manner different from that of cisplatin, causing slight accumulation of cells in G2-M phase and a decrease in G1 phase. Cell cycle changes progressed at higher concentrations of the tested complexes 1 and 3 (\( \text{IC}_{50} \)) and were characterized by rapid accumulation of cells in sub-G1, up to 48.5% (1) and 47.09% (3) compared to the control (0.03%). There was a decrease in the cell population in the G2-M and G1 phases and arrest in the S phase of the cell cycle, up to 18.66% (1) and 20.95% (3) versus the control (14.24%). Obviously, cells that could not progress through S phase following action of 1 and 3 (at \( \text{IC}_{50} \)) entered cell death and accumulated in sub-G1 phase. Formation of the Sub-G1 or hypodiploid peak is considered to be a hallmark of fragmented DNA.56,57 Further UV-vis spectroscopic studies also
demonstrated the potential of complexes 1 and 3 to bind to DNA (Table 4) and support these findings. The potential of different metal ions conjugated to NSAIDs to damage DNA and alter the cell cycle was previously demonstrated. Recent investigation by Kasparkova and colleagues of new Pt(n) conjugates carrying the NSAID diclofenac demonstrated their potential to act as a so-called “combi-molecule” which combines the DNA-binding properties of the metal center and antitumor properties of NSAID ligands for cytotoxic activity. The results of the present study strongly suggested the ability of complexes 1 and 3 to cause damage to DNA and arrest DNA replication, either through direct DNA-binding or by an indirect mechanism.

**Annexin V-FITC apoptotic assay**

Because MDA-MB-231 cells exhibited particular sensitivity to the cytotoxic action of complexes 1 and 3 (IC\textsubscript{50} value), showing an intense Sub-G1 peak, we further investigated whether the apoptotic or necrotic changes underlie the mechanism of cell death. The potential of the investigated compounds or CDDP to induce apoptosis/necrosis in MDA-MB-231 cells was assessed by flow cytometry following Annexin-V-FITC/PI dual staining after 24 h of continual drug treatment. The results (Fig. 4A) show the percentage of early apoptotic cells, labeled FITC(+)/PI(−), late apoptotic cells, FITC(+)/PI(+), and necrotic cells, FITC(−)/PI(+).

The obtained experimental data indicated that both complexes 1 and 3 as well as CDDP initiated apoptosis in a concentration-dependent manner. At 0.5 × IC\textsubscript{50} concentrations, 1 and 3 caused 7.96% and 6.73% of FITC(+)/PI(−) staining, respectively. These values were comparable to those of cisplatin, where 6.75% of cells were apoptotic, versus 4.41% in the control. As the dose of the compounds increased, the cells shifted from the healthy state, FITC(−)/PI(−), to either early apoptotic, FITC(+)/PI(−), or necrotic, FITC(+)/PI(+). Particularly significant dose-dependent effects on the cell redistribution between quadrants were observed after treatment with complexes 1 and 3. At IC\textsubscript{50} concentrations of 1 and 3, percentages of early apoptotic cells increased up to 20%, which is 3 times more than for the cells treated with CDDP. Simultaneously, FITC(+)/PI(+) staining, characteristic for necrosis, increased up to 20%. Results of the Annexin-V-FITC/PI apoptosis assay are in accordance with the cell cycle study, which confirmed that under the same treatment conditions (IC\textsubscript{50}, 24 h), more than 40% of cells underwent DNA-fragmentation and accumulated in Sub-G1 phase as either apoptotic or necrotic cells.

**Measurement of intracellular reactive oxygen species**

To investigate whether induced cytotoxic and apoptotic effects of synthesized ruthenium(n)-arene complexes 1 and 3 are related to ROS production, we measured the intracellular ROS levels in MDA-MB-231 cells after 4 h of treatment with the tested complexes 1 and 3 or CDDP at concentrations corresponding to their IC\textsubscript{50} values. As illustrated in Fig. 5A, the ROS level did not change significantly after 4 h exposure to CDDP. However, after 4 h of treatment with complexes 1 and 3, slight changes in the shapes of the fluorescent signal curves can be noted. Fluorescence of DCFH-DA in the treated cells is red-shifted toward a higher intensity compared to the control cells (Fig. 5B). This shift implies modest production of intracellular reactive oxygen species for 3.9% of cells treated with 1 and 5.42% of cells treated with 3; these values are higher than those of the control cells (2%) and cisplatin (1.88%) (Fig. 5C). However, a study of the scavenging abilities of the tested complexes for DPPH, superoxide radical and hydroxyl radical showed that complex 1 exhibited the highest ROS scavenging properties. Certainly, additional studies are needed in order to precisely address the features of the tested complexes that dominate the mechanisms of their cytotoxic action. The noticeable changes in ROS levels under treatment at an early time point (4 h) suggest that these complexes exhibit a strong, \[ \text{IC}_{50} \text{ concentrations, 1 and 3 caused 7.96\% and 6.73\% of FITC(+)/PI(−) staining, respectively. These values were comparable to those of cisplatin, where 6.75\% of cells were apoptotic, versus 4.41\% in the control. As the dose of the compounds increased, the cells shifted from the healthy state, FITC(−)/PI(−), to either early apoptotic, FITC(+)/PI(−), or necrotic, FITC(+)/PI(+). Particularly significant dose-dependent effects on the cell redistribution between quadrants were observed after treatment with complexes 1 and 3. At IC}_{50} \text{ concentrations of 1 and 3, percentages of early apoptotic cells increased up to 20\%, which is 3 times more than for the cells treated with CDDP. Simultaneously, FITC(+)/PI(+) staining, characteristic for necrosis, increased up to 20\%. Results of the Annexin-V-FITC/PI apoptosis assay are in accordance with the cell cycle study, which confirmed that under the same treatment conditions (IC}_{50}, 24 h), more than 40\% of cells underwent DNA-fragmentation and accumulated in Sub-G1 phase as either apoptotic or necrotic cells.} \]

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Fig. 4  (A) Bar graph of apoptosis and necrosis, quantified by FACS, after Annexin V-FITC and PI labelling. (B) Dot plot diagrams following 24 h treatment of MDA-MB-231 cells with complexes 1 and 3 or CDDP at concentrations corresponding to 0.5 × IC_{50} and IC_{50}. Representative dot plots of three independent experiments are given, presenting intact cells in the lower-left quadrant, FITC(-)/PI(-); early apoptotic cells in the lower-right quadrant, FITC(+)/PI(-); late apoptotic or necrotic cells in the upper-right quadrant, FITC(+)/PI(+); and necrotic cells in the upper-left quadrant, FITC(-)/PI(+).
Fig. 5 Effects of complexes 1, 3 and CDDP on intracellular ROS levels. MDA-MB-231 cells were treated for 4 h with the tested compounds at concentrations corresponding to their IC_{50} values and subjected to flow cytometry-based oxidative stress analysis for measurement of the ROS levels. Intracellular oxidative stress is indicated on the horizontal axis and corresponds to the fluorescence intensity of DCFH–DA. The vertical axis shows the cell numbers. (A) Representative histograms of one of three independent experiments, presenting the percentage of increase of ROS accumulation in treated cells relative to control cells; (B) enhancement of intracellular ROS accumulation, observed via the shift of the signal curve obtained for the treated cells (presented as a line) to the right compared with that of the control cells (presented as a filled curve); (C) bar graph presenting the mean fluorescence intensities of DCFH–DA of three independent experiments.
multiple-layer cytotoxic effect, possibly triggered by ROS release and afterwards by induction of cell death. Previous literature data on the \textit{in vitro} antitumor effects of NSAID agents, such as acetylsalicylic acid (aspirin), reported their potential to synergistically enhance the cytotoxicity of cisplatin or other chemotherapeutic anticancer agents by different mechanisms, including cell cycle alteration or induction of apoptosis through oxidative stress and mitochondrial dysfunction.\textsuperscript{59,61–63} The results of the present study signify that NSAIDs conjugated with ruthenium–arenes represent a promising class of agents with unique \textit{in vitro} anti-tumor features resulting from the combined properties of both ruthenium–arenes and NSAIDs.

**Antioxidant activities of the complexes**

Compounds that can scavenge free radicals or inhibit their production have potential applications in the treatment of inflammation\textsuperscript{49a,b} because free radicals are species involved in the inflammatory process. Within this context, the \textit{in vitro} antioxidant activities of complexes 1–4, \textit{i.e.} the \textit{in vitro} scavenging activities of complexes 1–4 towards DPPH, ABTS and hydroxyl radicals, and their ability to inhibit the activity of soybean lipoxygenase have been studied and have been compared to the well-known antioxidant agents NDGA, BHT, trolox and caffeic acid, which were used as standard reference compounds (Table 2).

The ability of complexes 1–4 to scavenge DPPH radicals (which is usually closely related to antiaging, anticancer and anti-inflammatory activity)\textsuperscript{49a,b} was time-independent because no significant differences were observed after treatment for 20 min and 60 min. On average, complexes 1–4 were better DPPH-scavengers than the corresponding free NSAIDs Hindo and Hmef but less active than the reference compounds BHT and NDGA. Complexes 1 and 3, bearing indomethacin ligands, were more potent than their analogues 2 and 4, respectively. Complex 1 was found to be the best DPPH-scavenger among the present complexes.

The scavenging of hydroxyl radicals (**OH) usually indicates that the compounds may offer relief from the presence of reactive oxygen species.\textsuperscript{49a,b} The scavenging ability of the complexes towards hydroxyl radicals is significantly high; all complexes 1–4 were more active than the corresponding free NSAIDs, Hindo and Hmef, and more active than the reference compound trolox. Complexes 1 and 2, which contain the arene p-cymene, are the most active hydroxyl-scavengers among the present compounds.

The scavenging of cationic ABTS radicals (ABTS\textsuperscript{**}) is often used as a marker of total antioxidant activity.\textsuperscript{49b} Regarding the ABTS scavenging ability of complexes 1–4, the complexes are more active than the corresponding free NSAIDs, Hindo and Hmef, but less active than the reference compound trolox. Complexes 1 and 3 bearing indomethacin as a ligand are better ABTS-scavengers than their menenamato analogues 2 and 4, respectively. Complex 1 is the most active ABTS-scavenger among complexes 1–4.

Compounds that can inhibit the activity of LOX may be considered as potential antioxidants or free radical scavengers because lipoxygenation is a procedure which usually occurs via a carbon-centered radical.\textsuperscript{64} In comparison with the reference compound caffeic acid, complexes 1–4 are very potent LOX inhibitors, with the indomethacin complexes 1 and 3 being the most active compounds.

In general, complexes 1–4 are better radical scavengers than the corresponding free NSAIDs Hindo and Hmef, suggesting that their coordination to Ru(n) results in enhanced scavenging activity. In comparison with reported metal–NSAID analogues,\textsuperscript{17,22,29–33,65–69} the present complexes 1–4 are significantly active DPPH-, ABTS- and hydroxyl-scavengers and LOX-inhibitors. Although the number of the present compounds is rather low to clarify the structural factors that lead to enhanced antioxidant activity, we suggest that the complexes bearing indomethacin and/or p-cymene are the most potent compounds; as a result, complex 1 was found to be the most active compound. Furthermore, the complexes are more active scavengers of hydroxyl and ABTS radicals than of DPPH radicals; this scavenging selectivity for hydroxyl and ABTS radicals has been previously reported in the literature,\textsuperscript{70–72} especially for metal–NSAID complexes.\textsuperscript{17,22,29–33,65–69}

**Interaction with biomolecules**

**Interaction of the complexes with albumins.** The most important role of the serum albumins (SAs) is the transporta-
tion of ions and drugs towards their biological targets, \textit{i.e.} cells and tissues.\textsuperscript{50} Within this context, the study of the interaction of biologically potent compounds (such as the reported complexes 1–4) with SAs can be considered as an approach for the exploration of potential biological activity and applications. As a result of this interaction, the biological properties of

<table>
<thead>
<tr>
<th>Compound</th>
<th>RA%, 20 min</th>
<th>RA%, 60 min</th>
<th>**OH%</th>
<th>ABTS%</th>
<th>LOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34.23 ± 0.35</td>
<td>33.26 ± 0.66</td>
<td>96.89 ± 0.33</td>
<td>88.11 ± 0.92</td>
<td>29.76 ± 0.12</td>
</tr>
<tr>
<td>2</td>
<td>13.67 ± 0.35</td>
<td>15.16 ± 0.42</td>
<td>96.58 ± 0.11</td>
<td>78.21 ± 0.27</td>
<td>34.56 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>26.58 ± 0.41</td>
<td>25.42 ± 0.17</td>
<td>93.41 ± 0.16</td>
<td>82.54 ± 0.23</td>
<td>27.34 ± 0.55</td>
</tr>
<tr>
<td>4</td>
<td>8.82 ± 0.47</td>
<td>9.24 ± 0.48</td>
<td>93.56 ± 0.38</td>
<td>72.61 ± 0.86</td>
<td>40.54 ± 0.73</td>
</tr>
<tr>
<td>Hindo</td>
<td>20.32 ± 0.73</td>
<td>24.65 ± 0.47</td>
<td>91.34 ± 0.74</td>
<td>79.64 ± 0.30</td>
<td>34.46 ± 0.59</td>
</tr>
<tr>
<td>Hmef</td>
<td>5.72 ± 0.08</td>
<td>11.74 ± 0.20</td>
<td>92.31 ± 0.44</td>
<td>66.32 ± 0.38</td>
<td>48.52 ± 0.88</td>
</tr>
<tr>
<td>NDGA</td>
<td>81.02 ± 0.18</td>
<td>82.60 ± 0.17</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>BHT</td>
<td>31.30 ± 0.30</td>
<td>60.00 ± 0.38</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
<tr>
<td>Trolox</td>
<td>Not tested</td>
<td>Not tested</td>
<td>82.80 ± 0.13</td>
<td>91.8 ± 0.17</td>
<td>Not tested</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Not tested</td>
<td>Not tested</td>
<td>Not tested</td>
<td>600 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>
the compounds may alter when bound to albumins, or novel alternative pathways or mechanisms of activity and transportation may be revealed.\textsuperscript{73} Therefore, the interaction of complexes 1–4 with HSA and its homologue BSA were studied by fluorescence emission spectroscopy. The solutions of HSA and BSA exhibit intense fluorescence emission bands at $\lambda_{em,max} = 352$ nm and $343$ nm, respectively, with an excitation wavelength at $295$ nm; this band is due to the presence of tryptophans, \textit{i.e.} the tryptophan at position 214 in HSA and the tryptophans at positions 134 and 212 in BSA.\textsuperscript{50}

The quenching in the fluorescence emission spectra of the SAs induced by the presence of the complexes was low for the complexes in the case of HSA (the quenching of the initial HSA fluorescence emission ($\Delta I/I_0$) reached $\sim 49.3\%$ in the presence of complex 3, Fig. 6A) and was much more pronounced for BSA (the quenching of the initial BSA fluorescence emission ($\Delta I/I_0$) reached $74.8\%$ in the presence of complex 2, Fig. 6B). This quenching of the SA fluorescence emission band may be ascribed to possible changes in the tryptophan environment of SA and, subsequently, changes in the secondary structure of albumin arising from the binding of each complex to SA.\textsuperscript{53a}

The quenching constants ($k_q$) of the interactions of the complexes with the albumins were calculated (Table 3) from the corresponding Stern–Volmer plots (Fig. S9 and S10, ES1$^\dagger$) and Stern–Volmer quenching equation (eqn (S2) and (S3), ES1$^\dagger$), where the fluorescence lifetime of tryptophan in SA was taken as $\tau_0 = 10^{-8}$ s;\textsuperscript{52} the quenching constants are significantly higher than the value of $10^{10}$ M$^{-1}$ s$^{-1}$, suggesting that the interaction of the complexes with the albumins takes place \textit{via} a static quenching mechanism,\textsuperscript{52} which indicates the formation of a new conjugate between each complex and the albumin. The $k_q$ constants of complexes 1–4 may show significant SA-quenching ability for the complexes; this effect was more intense in the case of BSA, with complex 1 showing the highest $k_q$ for HSA and complex 2 for BSA. The derived $k_q$ constants for complexes 1–4 are within the range found for a series of metal-complexes bearing NSAIDs as ligands.\textsuperscript{17,22,29–33,65–69}

The SA-binding constants ($K$) of the complexes have been determined (Table 3) from the corresponding Scatchard plots (Fig. S11 and S12, ES1$^\dagger$) and the Scatchard equation (eqn (S4), ES1$^\dagger$). The $K$ constants of complexes 1–4 are all relatively high, similar to their respective free NSAIDs, and are in the range of the values calculated for metal–NSAID complexes.\textsuperscript{17,22,29–33,65–69}

Among the present complexes, complexes 4 and 1 bear the highest $K$ constants for HSA and BSA, respectively. Considering the structural factors present in complexes 1–4, it seems that complexes 1 and 2 bearing $\eta^6$-cymene ligands show higher binding activity for BSA than their analogues 3 and 4 bearing $\eta^6$-toluene ligands. In the case of HSA, we cannot confidently suggest a structural factor that leads to higher binding affinity.

The SA-binding constants of complexes 1–4 lie in the range of $4.24 \times 10^8$ to $4.49 \times 10^8$ M$^{-1}$ (Table 3) and are high enough to reveal the potential binding of the complexes to SAs in order to enable transfer towards potential biotargets. Additionally, the $K$ constants are significantly lower than the value of $10^{15}$ M$^{-1}$, \textit{i.e.} the binding constant of avidin with diverse compounds,\textsuperscript{74} which is the strongest known non-covalent reversible interaction,\textsuperscript{53c} this comparison may indicate reversible binding of the complexes to the SAs and may reveal the potential for release upon arrival at the desired target.

**Interaction of the complexes with CT DNA.** Covalent binding or noncovalent interactions are the most common interactions between metal complexes and double-stranded DNA. Covalent binding takes place when nitrogen atoms of DNA-bases displace one or more labile ligands of the complex, while the noncovalent interactions are: (i) intercalation, \textit{i.e.} $\pi \rightarrow \pi$ stacking interactions between the complex and DNA nucleobases, (ii) electrostatic interactions, namely Coulomb forces developed between the complexes and the phosphate groups of DNA, and (iii) groove-binding, attributed to van der Waals or hydrogen-bonding or hydrophobic bonding interactions along the grooves of the DNA helix.\textsuperscript{75a} The potential anticancer and/or anti-inflammatory activities of NSAIDs and their complexes are often related to their DNA-binding behavior.\textsuperscript{17,75b} Within this context,
bases between the aromatic chromophores of the complexes and DNA-complexes percentage of hypochromism observed for the mefenamato UV-vis spectroscopic titration studies. Of course, the significant (Table 4). Safe conclusions cannot be merely derived from viscosity measurements were performed.

Table 3 The albumin quenching and binding constants for complexes 1–4

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{(HSA)}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{(HSA)}$ (M$^{-1}$)</th>
<th>$k_{(BSA)}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{(BSA)}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hindo$^{22}$</td>
<td>7.80(±0.60) × 10$^{12}$</td>
<td>2.22(±0.19) × 10$^{4}$</td>
<td>7.68(±0.28) × 10$^{12}$</td>
<td>8.95(±0.40) × 10$^{5}$</td>
</tr>
<tr>
<td>Hme$^{10}$</td>
<td>7.13(±0.34) × 10$^{12}$</td>
<td>1.32(±0.15) × 10$^{4}$</td>
<td>2.78(±0.20) × 10$^{12}$</td>
<td>1.35(±0.22) × 10$^{5}$</td>
</tr>
<tr>
<td>1</td>
<td>6.10(±0.29) × 10$^{12}$</td>
<td>2.15(±0.08) × 10$^{4}$</td>
<td>1.25(±0.05) × 10$^{12}$</td>
<td>4.49(±0.30) × 10$^{5}$</td>
</tr>
<tr>
<td>2</td>
<td>5.46(±0.19) × 10$^{12}$</td>
<td>9.79(±0.34) × 10$^{4}$</td>
<td>1.70(±0.08) × 10$^{12}$</td>
<td>3.63(±0.15) × 10$^{5}$</td>
</tr>
<tr>
<td>3</td>
<td>2.04(±0.13) × 10$^{12}$</td>
<td>9.44(±0.40) × 10$^{4}$</td>
<td>4.85(±0.11) × 10$^{12}$</td>
<td>5.30(±0.18) × 10$^{4}$</td>
</tr>
<tr>
<td>4</td>
<td>4.32(±0.29) × 10$^{12}$</td>
<td>4.24(±0.12) × 10$^{4}$</td>
<td>1.30(±0.04) × 10$^{12}$</td>
<td>2.63(±0.10) × 10$^{5}$</td>
</tr>
</tbody>
</table>

the DNA-binding affinities of complexes 1–4 were studied in vitro by UV-vis spectroscopy, viscosity measurements and their ability to displace the typical DNA-intercalator EB.

UV-vis spectroscopy is used as a preliminary method to examine the DNA-binding mode and to calculate its strength. Any changes observed in the DNA-band or the intraligand transition bands of the complexes may reveal the existence of interactions and their possible modes. The UV-vis spectra of a CT DNA solution in the presence of complex 4 at increasing $r$ values are shown representatively in Fig. 7A. The band at $\lambda_{\text{max}} = 258$ nm exhibits a slight hypochromism accompanied by a red-shift up to 260 nm, suggesting the existence of interactions between CT DNA and the complex leading to the formation of a new complex-DNA conjugate$^{76}$ and resulting in stabilization of the CT DNA double-helix.$^{77}$ The behaviour of CT DNA in the presence of the other complexes is quite similar.

In the UV-vis spectra of complex 1 (Fig. 7B), the intraligand band observed at 321 nm exhibited in the presence of CT DNA showed slight hypochromism of up to 7%. Similar changes were observed in the UV-vis spectra of complex 3 in the presence of DNA (Table 4). In the UV-vis spectra of complex 4 (Fig. 7C), upon addition of CT DNA, the intraligand band located at 282 nm (I) presented a significant hyperchromism up to 20% followed by a significant red-shift of 15 nm; upon addition of CT DNA, the second band located at 349 nm (II) presented an intense hypochromism up to 50% followed by elimination. Similar spectroscopic features were observed in the UV-vis spectra of complex 2 in the presence of DNA (Table 4). Safe conclusions cannot be merely derived from UV-vis spectroscopic titration studies. Of course, the significant percentage of hypochromism observed for the mefenamato complexes 2 and 4 can be attributed to $\pi \rightarrow \pi$ stacking interactions between the aromatic chromophores of the complexes and DNA-bases$^{53,54,55}$ and may be consistent with an intercalative binding mode, leading to stabilization of the DNA helix.$^{77}$ However, in order to clarify the DNA-binding modes of complexes 1–4, DNA-viscosity measurements were performed.

The DNA-binding constants ($K_b$) of complexes 1–4 (Table 4) were calculated using the Wolfe–Shimura equation$^{55a}$ (eqn (S5), ESI†) and the corresponding plots of [DNA]/[$c_A - c_i$] versus [DNA] (Fig. S13, ESI†). The $K_b$ constants of complexes 1–4 (Table 4) are relatively high and suggest strong binding of the complexes to CT DNA. Considering the structural factors present in complexes 1–4, it seems that the complexes bearing $\eta^5$-p-cymene ligands (1 and 2) show higher DNA-affinity than their analogues bearing $\eta^5$-toluene ligands (3 and 4); also, the complexes bearing mefenamato ligands (2 and 4) are better...
Table 4 UV–vis spectral features of the interaction of complexes 1–4 with CT DNA. UV–band (λ in nm) (percentage of the observed hyper-/hypo-chromism (ΔA/Δλ, %), blue-/red-shift of the λmax (Δλ, nm)) and DNA-binging constants (Kb)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Band (ΔA/Δλ, a Δλb)</th>
<th>Kb (M−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hindo22</td>
<td>314 (sh) (−10, 0)</td>
<td>3.37(±0.23) × 105</td>
</tr>
<tr>
<td>Hmef28</td>
<td>324 (−10, 0)</td>
<td>1.05(±0.02) × 105</td>
</tr>
<tr>
<td>1</td>
<td>321 (−7, 0)</td>
<td>2.85(±0.17) × 105</td>
</tr>
<tr>
<td>2</td>
<td>296 (+40, +12); 350 (−50, εlm)</td>
<td>3.69(±0.40) × 105</td>
</tr>
<tr>
<td>3</td>
<td>321 (−3, 0)</td>
<td>3.24(±0.22) × 104</td>
</tr>
<tr>
<td>4</td>
<td>282 (+20, +15); 349 (−50, εlm)b,c</td>
<td>3.15(±0.12) × 105</td>
</tr>
</tbody>
</table>

a “+” denotes hyperchromism, “−” denotes hypochromism, b “+” denotes red-shift, “−” denotes blue-shift. c “εlm” = eliminated.

DNA-binders than those bearing indomethacin ligands (1 and 3), with complex 2 having the highest Kb constant (3.69(±0.40) × 105 M−1) among the complexes. The Kb constants of complexes 1–4 are within the range found for other metal–NSAID complexes.17,22,29–33,65–69 Further, the Kb constants are similar to or higher than that of the classical intercalator EB (1.23 × 105 M−1) as calculated by Dimitrakopoulou et al.78

The interaction of complexes 1–4 with CT DNA was also monitored via DNA-viscosity measurements in the presence of increasing amounts of the complexes. As is known, the relative DNA-viscosity (ηr/η0) is sensitive to relative DNA-length changes (Lr/L0) occurring in the presence of a DNA-binder because they are correlated by the equation Lr/L0 = (ηr/η0)1/3.79 In the case of intercalation, the DNA-viscosity will increase, while in the case of non-classical intercalation, i.e. groove-binding or electrostatic interaction, the DNA-viscosity may decrease slightly or remain unchanged.

The changes in the viscosity of a CT DNA solution (0.1 mM) were studied in the presence of increasing amounts of complexes 1–4 (up to the value of r = 0.35). For all complexes, the DNA-viscosity showed a considerable increase upon their addition (Fig. 8). This increase may be attributed to intercalation of the complexes between DNA-bases because in the case of intercalation, the separation distance of the DNA bases will increase in order to accommodate the intercalating compounds and, subsequently, the DNA-viscosity will increase.79

The existing conclusion of intercalation may clarify and enhance the preliminary conclusions derived from the UV–vis spectroscopic studies.

EB is a typical DNA-intercalator; its intercalation between DNA-bases via its planar phenanthridine ring results in the formation of the EB–DNA conjugate, which exhibits an intense fluorescence emission band at 592 nm with λex = 540 nm. The displacement of EB from the EB–DNA conjugate induced by a compound that can intercalate DNA may indirectly verify the intercalating ability of the compound. In such a case, quenching of the EB–DNA fluorescence emission band will appear upon addition of the DNA-intercalating compound.51,55a

The EB–DNA conjugate was completely formed after pretreatment of EB ([EB] = 20 μM) and DNA ([DNA] = 26 μM) for 1 h in buffer solution. The fluorescence emission spectra of the EB–DNA conjugate were recorded for increasing amounts of the

Fig. 8 Relative viscosity (ηr/η0)1/3 of CT DNA (0.1 mM) in buffer solution (150 mM NaCl and 15 mM trisodium citrate at pH 7.0) in the presence of increasing amounts of complexes 1–4 (r = [complex]/[DNA]).

Fig. 9 (A) Fluorescence emission spectra (λex = 540 nm) for EB–DNA ([EB] = 20 μM, [DNA] = 26 μM) in buffer solution in the absence and presence of increasing amounts of complex 2 (up to the value of r = 0.35). The arrow shows the changes of intensity upon increasing amounts of 2.

(B) Plot of the relative fluorescence intensity of EB–DNA (%I/I0) at λem = 592 nm vs. r = [complex]/[DNA] in buffer solution (150 mM NaCl and 15 mM trisodium citrate at pH 7.0) in the presence of complexes 1–4 (quenching up to 18.5% of the initial EB–DNA fluorescence for 1, 11.3% for 2, 27.5% for 3 and 18.5% for 4).
Table 5 Percentage of EB–DNA fluorescence quenching ($\Delta$I/I$_0$, %), Stern–Volmer constants ($K_{SV}$) and quenching constants of EB–DNA fluorescence ($k_q$) for complexes 1–4

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Delta$I/I$_0$ (%)</th>
<th>$K_{SV}$ (M$^{-1}$)</th>
<th>$k_q$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIndo$^{22}$</td>
<td>87.5</td>
<td>$3.62(±0.09) \times 10^7$</td>
<td>$1.57(±0.04) \times 10^{12}$</td>
</tr>
<tr>
<td>HMeF$^{10}$</td>
<td>80.0</td>
<td>$1.38(±0.06) \times 10^7$</td>
<td>$6.87(±0.26) \times 10^{12}$</td>
</tr>
<tr>
<td>1</td>
<td>81.5</td>
<td>$8.96(±0.30) \times 10^4$</td>
<td>$3.90(±0.13) \times 10^{12}$</td>
</tr>
<tr>
<td>2</td>
<td>88.7</td>
<td>$7.71(±0.26) \times 10^4$</td>
<td>$3.35(±0.12) \times 10^{12}$</td>
</tr>
<tr>
<td>3</td>
<td>72.5</td>
<td>$3.75(±0.13) \times 10^4$</td>
<td>$1.63(±0.06) \times 10^{12}$</td>
</tr>
<tr>
<td>4</td>
<td>81.5</td>
<td>$6.67(±0.23) \times 10^4$</td>
<td>$2.90(±0.10) \times 10^{12}$</td>
</tr>
</tbody>
</table>

Compounds (representatively shown for complex 2 in Fig. 9A up to $r = 0.35$). The complexes under study did not show any appreciable fluorescence emission bands at room temperature in solution or in the presence of EB or CT DNA under the same experimental conditions, i.e. with $\lambda_{ex} = 540$ nm. Therefore, the observed quenching upon addition of complexes 1–4 is significant (up to 88.7% of the initial EB–DNA fluorescence emission intensity, Table 5 and Fig. 9B) and can be attributed to the displacement of EB from the EB–DNA conjugate and its replacement by the complexes; thus, binding of the complexes at the DNA-intercalation sites may be indirectly concluded.

The quenching of the EB–DNA fluorescence is in good agreement with the linear Stern–Volmer equation (eqn (S2), ESI$^{1}$), as shown in the corresponding Stern–Volmer plots ($R \sim 0.99$, Fig. S14, ESI$^{1}$). The $K_{SV}$ constants of the complexes (Table 5) are in the range reported for other metal–NSAID complexes, with complex 1 having the highest $K_{SV}$ constant among the complexes. The quenching constants of the compounds ($k_q$) in regard to their competition with EB were calculated according to eqn (S3) (ESI$^{1}$), where the fluorescence lifetime of the EB–DNA system has the value $\tau_0 = 23$ ns.$^{55b}$

The derived $k_q$ constants (Table 5) are significantly higher than $10^{10}$ M$^{-1}$ s$^{-1}$, suggesting that the quenching of the EB–DNA fluorescence by the complexes takes place via a static mechanism which leads to the formation of a new conjugate, obviously between DNA and each complex.$^{51}$

Conclusions

The paper presented here showed that two chosen NSAIDs, HIndo and HMeF, in the reaction with Ru(II)–arene complexes were bound monodentately via a carboxylate group. This was confirmed by NMR and IR spectroscopy results as well as by mass spectrometry. Cytotoxicity studies revealed that the NSAIDs ligand HIndo, despite having poor activity itself, when coordinated to ruthenium(II)–arenes such as 1 and 3, enhanced the cytotoxicity of the resulting complexes; meanwhile, the π-bonded arenes $p$-cymene (1) and toluene (2) did not seem to impact the in vitro cytotoxic potential of the complexes. A study of the mechanisms of action in CDDP resistant breast carcinoma MDA-MB-231 cells, using flow cytometry, revealed that complexes 1 and 3 arrested the cell cycle in S phase and caused rapid DNA-fragmentation and accumulation in sub-G1 phase. Even more than 40% of cells underwent either apoptotic or necrotic cell death after 24 h action of 1 and 3 at IC$_{50}$. HIndo certainly provided features that improved the cytotoxic activity of the resulting complexes 1 and 3. Intercalation may be the most likely mode of interaction with DNA, as revealed by in vitro DNA-viscosity experiments with CT-DNA and EB-displacement experiments. The indomethacin-ruthenium complexes are better “DNA-binders” than the corresponding free indomethacin ligands. Additionally, fragmentation of DNA may be partially caused by indirect pathways, such as ROS production. Measurements of intracellular ROS production in MDA-MB-231 cells after 4 h of treatment with 1 and 3 at IC$_{50}$ showed increased ROS levels, suggesting that the complexes exhibit multiple-layer cytotoxic effects that are also triggered by ROS release. The biological actions of most NSAIDs, including HIndo, customarily involve selective inhibition of cyclooxygenases (COX-1 and COX-2).$^{80,81}$ However, literature data provide evidence that the pharmacokinetic properties of NSAIDs may be also related to their ability to interact with membrane phospholipids and/or to disrupt membrane permeability, suggesting that effects at the cell membrane level may be an additional mechanism of action and toxicity of ruthenium-indomethacin complexes 1 and 3.$^{82}$

In the present study, complexes 1–4 were also tested in regard to their ability to scavenge DPPH, ABTS and hydroxyl radicals, to inhibit soybean lipoxigenase activity and to bind to serum albumins. The results showed that, in general, the complexes are more active radical scavengers and LOX inhibitors than their corresponding free NSAIDs. The interaction of the complexes with albumins showed their potential ability to bind tightly and reversibly to albumins, enabling them to be transferred to and released at their potential biological targets.

In the expanding field of rational drug discovery, ruthenium complexes represent a promising class of small molecules that can be optimized to more specifically target tumour cells. The results of the present study of ruthenium(II)–arene complexes conjugated to indomethacin contribute to the development of so-called “combi-molecules” that may exhibit stronger or more specific antitumor effects than their single complexes or ligands alone. The present results may be considered to be promising for further biological studies and potential applications.

Abbreviations

- K562: Human myelogenous leukemia cells
- A549: Human lung adenocarcinoma cells
- MDA-MB-231: Human breast adenocarcinoma cells
- MRC-5: Non-tumor human lung fibroblast cells
- MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
- RPMI 1640: Roswell Park Memorial Institute nutrient medium (1640)
- CDDP: cis-Diaminedichloroplatinum(II), cisplatin
- DCFH-DA: 2’,7’-Dichlorodihydrofluorescein diacetate
- DNA: Deoxyribonucleic acid
ROS Reactive oxygen species
PK1019 Indazolium-trans-tetrachlorobis-(1H-indazole)ruthenate(ni)
PK1339 Sodium-trans-tetrachlorobis-(1H-indazole)ruthenate(ni)
NAMI Sodium-transimidazoliumdimethylsulfoxidetetrachlororuthenate(ni)
TrxR Thioredoxin
NSAID Non-steroidal anti-inflammatory drug
COX Enzyme cyclooxygenase
Hmef Mefenamic acid
Hindo Indomethacin
ABTS+ 1,1-Diphenyl-picrylhydrazyl
Hmef Mefenamic acid
DPPH 1,1-Diphenyl-2-picrylhydrazyl
Hmef Mefenamic acid
ABTS*+ 2,2‘-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radicals
LOX Soybean lipoxygenase
HSA Bovine serum albumin
CT DNA Calf-thymus deoxyribonucleic acid
UV-vis spectroscopy Ultraviolet-visible spectroscopy
EDTA Ethylenediaminetetraacetic acid
BHT Butylated hydroxytoluene
NDGA 4,4‘-N,N-diacetate-(p-cymene)
HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
DMSO Dimethyl sulfoxide
FCS Fetal calf serum
PI Propidium iodide
RNaseA Ribonuclease A
EDTA Ethylenediaminetetraacetic acid
SA Serum albumin

Conflicts of interest
The authors declare no conflict of interest.

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