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New tungstenocenes containing 3-hydroxy-4-pyrone ligands: antiproliferative activity on HT-29 and MCF-7 cell lines and binding to human serum albumin studied by fluorescence spectroscopy and molecular modeling methods

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Abstract

Three new water-soluble tungstenocene derivatives were synthesized and characterized using 3-hydroxy-4-pyrone ligands, which provide aqueous stability to the complexes. The antiproliferative activities of the complexes on HT-29 colon cancer and MCF-7 breast cancer cell lines were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and showed the new tungstenocene derivatives have higher antiproliferative action than tungstenocene dichloride (Cp_2WCl_2 , where Cp is cyclopentadienyl). The binding interactions of the tungstenocenes with human serum albumin (HSA) were investigated using fluorescence spectroscopy and molecular modeling methods. Analysis of the fluorescence quenching spectra indicates that the tungstenocene complexes bind HSA by hydrophobic interactions and hydrogen bonding at fatty acid binding site 6 and drug binding site II. Docking studies provided a description of the hydrophobic interactions and hydrogen bonding by which the tungstenocenes become engaged with HSA. It was determined that the binding affinity of the tungstenocenes for HSA is in the order $\text{Cp}_2\text{WCl}_2 < [\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl} < [\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl} < [\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, consistent with the hydrophobic interactions and the number of hydrogen bonds involved.

Keywords

Tungstenocene dichloride; Anticancer drug; Antitumor agent; MCF-7; HT-29

Introduction

The continuous search for anticancer drugs with high efficacy and low side effects has stimulated the scientific community to explore and apply other “nontraditional” fields in medicinal chemistry. That is the case for organometallic chemistry. The potential application of organometallic chemistry was investigated to target a wide variety of biological targets and this evolved into a new research area, bioorganometallic chemistry [1–3]. Evidence of this is the first report of Köpf and Köpf-Maier [4] on the antitumor activity of titanocene dichloride (Cp_2TiCl_2 , where Cp is cyclopentadienyl).

Metallocene dichlorides were investigated in a series of tumor cell lines and those metallocenes containing titanium, molybdenum, vanadium, and niobium showed significant

antitumor activity on Ehrlich ascites tumor, B16 melanoma, colon 38 carcinoma, and Lewis lung carcinoma cell lines [4–12]. Clinical trials were performed on Cp_2TiCl_2 and showed that it has fewer secondary effects than cisplatin [13–18]. In contrast, tungstenocene dichloride (Cp_2WCl_2) showed only sporadic activity on the above-mentioned cancer cell lines [19]. Consequently, the biological data on Cp_2WCl_2 are very limited and to our knowledge there are no reports on the antitumor activity of tungstenocene derivatives and the corresponding structure–activity relationship.

The bioorganometallic chemistry of molybdenocene dichloride (Cp_2MoCl_2) and its derivatives has been investigated in more detail, and the mechanisms of drug transport to target sites in the cell have been studied [11, 12]. One of the proposed mechanisms of drug transport and drug distribution involves human serum albumin (HSA) [20]. HSA is a protein containing hydrophobic pockets where fatty acids and hydrophobic drugs can be transported from the serum into the cell. Thus, it was hypothesized that HSA transports molybdenocene from the blood to the target site inside the cell [20]. To investigate this hypothesis, our group synthesized water-soluble molybdenocene derivatives containing O, $\dot{\text{O}}$ bidentate chelating ligands in the ancillary positions and their antiproliferative activities and HSA–molybdenocene interactions were investigated [21]. These studies demonstrated that there is no correlation between the binding affinity of molybdenocenes for HSA and cytotoxic activity toward HT-29 and MCF-7 cancer cells [21]. However, this result does not rule out the role of HSA in drug transport because the role of the O, $\dot{\text{O}}$ bidentate ligand also influences the antiproliferative activity and this lack of correlation can be associated with the ancillary ligands.

Our group has also investigated the bioorganometallic chemistry of Cp_2MoCl_2 and water-soluble derivatives containing S,N chelating ligands and compared them with the aforementioned complexes, molybdenocene derivatives containing O, $\dot{\text{O}}$ chelating ligands [22]. We identified that the most active molybdenocene complexes in HT-29 and MCF-7 cancer cell lines are those containing S,N chelating ligands rather than those with O, $\dot{\text{O}}$ chelating ligands [12, 21, 22].

Waern et al. [23] found that molybdenocene derivatives with thiolate ligands are inactive in the V79 Chinese hamster lung cell line because of the inert character of the Mo(IV)-S bonds, producing very robust complexes under physiological conditions. In other studies, Gleeson et al. [24] synthesized a series of benzyl-substituted Cp_2MoCl_2 complexes with methoxy groups at the 4 (4-OMe, **1**), 3,4 [3,4-(OMe)₂, **2**], and 3,4,5 [3,4,5-(OMe)₃, **3**] positions as potential anticancer drugs. The in vitro cytotoxicity studies on human renal cell line Caki-1 at 48 h revealed that **2** and **3** both displayed the same low cytotoxicity IC_{50} values of 290 μM , whereas substantial improvement was observed for molybdenocene **1**, with IC_{50} of 84 μM [24]. Cp_2MoCl_2 has an IC_{50} with regard to the Caki-1 cell line of more than 290 μM .

Surprisingly, the bioorganometallic chemistry of tungstenocene is largely unexplored. To gain insights into the role of the transition metal center in the metallocene and its biological activity as well as the possible mechanisms of transport of these species to the target site, we synthesized several tungstenocene derivatives with O, $\dot{\text{O}}$ bidentate chelating ligands, 3-hydroxy-4-pyrones (kajic acid, maltol, and ethyl maltol), and their antiproliferative activities on HT-29 colon cancer and MCF-7 breast cancer cell lines were investigated.

Maltol is a naturally occurring nontoxic compound and has been studied extensively as an O, $\dot{\text{O}}$ bidentate chelating ligand with many biologically important metals [25–30]. The replacement of the chlorides with 3-hydroxy-4-pyrones (kajic acid, maltol, and ethyl maltol) provides not only aqueous stability and solubility to the complexes but also increase the

hydrophobic character of the corresponding tungstenocene complex. Given the similarity in chemistry between tungsten and molybdenum, we synthesized tungstenocene-(3-hydroxy-4-pyronato) complexes and investigated the HSA–tungstenocene interactions by fluorescence spectroscopy and molecular modeling methods in order to gain insights into the possible mechanism of drug transport to the target sites in the cell. Comparison between molybdenocenes and tungstenocenes can also provide insights into the role of the metal center with regard to the antiproliferative activity of the complexes. We report our results herein.

Results and discussion

Synthesis and characterization

Three water-soluble tungstenocenes were synthesized in aqueous solution according to Scheme 1. The new tungstenocene complexes were synthesized by replacement of the chlorides with O,O' bidentate chelating ligands: maltol, ethyl maltol, and kojic acid. The pH was adjusted to above 5 to ensure ligand deprotonation.

The new water-soluble tungstenocene complexes were characterized by spectroscopic methods, NMR, IR and UV–vis spectroscopy, and by cyclic voltammetry. The ^1H NMR spectra of the three compounds prove the presence of two equivalent η^5 -Cp rings and the coordinated O,O' chelating ligand. The equivalency of the Cp rings suggests that the O,O' bidentate chelating ligand occupies the ancillary positions, residing in the plane bisecting the Cp–W–Cp angle. The IR spectra of the complexes showed a C–H stretching band from Cp at approximately $3,100\text{ cm}^{-1}$. The characteristic C=O bands from the free maltol, ethyl maltol, and kojic acid ligands appear at $1,652$ – $1,662\text{ cm}^{-1}$, but in the new complexes (3-hydroxy-4-pyronato derivatives) they appear at lower wavenumbers ($1,605$ – $1,600\text{ cm}^{-1}$) owing to the coordination of carbonyl oxygen to W(IV).

The new tungstenocene species containing maltolato, ethyl maltolato and kojato ligands are stable in aqueous solution and buffer solution at pH 7.4 for extended periods (more than 48 h) without notable decomposition as monitored by ^1H NMR spectroscopy, whereas Cp_2WCl_2 has very limited solubility in water and dissolution is achieved by sonication, which is accompanied by chloride hydrolysis and decomposition in a few hours (less than 3 h), as explained later.

In D_2O at pH ~ 3.4 , Cp_2WCl_2 requires sonication for 1 h to induce partial dissolution. The ^1H NMR spectrum shows two singlets at 5.97 ppm (major species) and 5.77 ppm, with less than 1 % decomposition. In pure D_2O but at pH 7.4, Cp_2WCl_2 shows signals for the free cyclopentadiene as well as three singlets at 6.24, 5.98, and 5.87 ppm (major species), which may be attributed to Cp signals of tungstenocene species, but the species were not characterized further owing to their low stability. In 25 mM tris(hydroxymethyl)aminomethane- d_1 (Tris- d_7)/10 mM NaCl buffer solution at pH 7.4, Cp_2WCl_2 shows three singlets at 6.24, 5.98, and 5.64 ppm (major species). We believe the species at 5.64 ppm is a “ Cp_2W –Tris” species, analogous to the species formed by Cp_2MoCl_2 in Tris buffer [31]. In addition, signals corresponding to free cyclopentadiene are observed and indicate decomposition of the complex.

Conversely, in 25 mM Tris- d_1 /10 mM NaCl buffer solution at physiological pH, the new tungstenocene-(3-hydroxy-4-pyronato) species were stable and did not show any significant change that could be attributed to replacement of the O,O' bidentate chelating ligand by chloride or Tris buffer in 48 h. In D_2O /10 mM NaCl solution at pH < 4 , none of the new tungstenocenes undergo O,O' bidentate ligand replacement by the chlorides.

In the UV–vis spectra, in buffer solution, the new complexes exhibit absorption bands at 210–215 nm, which are attributed to π – π^* transitions from Cp rings. The d – d transition in Cp_2WCl_2 at 340 nm is redshifted in the new species to 420 nm for $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$. The free ligands, maltol, ethyl maltol, and kojic acid, exhibit two absorption bands around 220 and 270 nm, which are attributed to n – π^* and π – π^* intraligand transitions (Table 1S, Figs. S1, S2). These bands are redshifted in $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, supporting formation of the complex.

Electrochemical characterization

The redox behavior of the tungstenocene complexes was investigated by cyclic voltammetry. In 100 mM KCl, Cp_2WCl_2 showed irreversible redox behavior with an oxidation potential at 185 mV (Fig. 1). On the other hand, irreversible redox behaviors and higher oxidation potentials were observed for $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$: 569, 575, and 581 mV, respectively. Thus, the latter organometallic species are stabler toward oxidation in an aqueous environment than Cp_2WCl_2 . The redox behaviors of the new species can be attributed to the 3-hydroxy-4-pyronato ligands, which afford more aqueous stability to $\text{Cp}_2\text{W(IV)}$ than does chloride. Secondary chemical reactions and/or the formation of stable metal–oxo species may explain the irreversible redox behaviors of the new species [12, 21].

The electrochemical behaviors of the new complexes were also investigated in an aqueous environment but under physiological conditions, 100 mM Tris buffer solution at pH 7.4 (Figs. 2, S3). The new complexes showed oxidation peaks at potentials higher than the potential of the oxidation peak of Cp_2WCl_2 , also suggesting higher stabilities in aqueous solution under physiological conditions. The voltammograms exhibit two oxidation peaks. The first peak, at more negative potential, is attributed to the formation of W(V) or stable metal–oxo species and the second peak, at more positive potential, is attributed to the irreversible oxidation of 3-hydroxy-4-pyronato ligands. This assignment is based on the following arguments. The voltammograms of the complexes in KCl (Fig. 1) showed the formation of W(V) species assigned to potentials analogous to those observed for the first peak in 100 mM Tris buffer. The free ligands, in 100 mM Tris buffer solution, showed irreversible oxidation peaks (see the electronic supplementary material) that appear at potentials similar to the potentials of the second peak in Fig. 2. Cp_2WCl_2 showed a small broad peak at 635 mV, which most likely belongs to a “ $\text{Cp}_2\text{W-Tris}$ ” species, as observed for Cp_2MoCl_2 in Tris buffer [31].

In acetonitrile (100 mM Bu_4NPF_6), Cp_2WCl_2 shows a reversible oxidation peak ($E_{\text{pa}} = 0.47$ V; Fig. 3), which involves one-electron transfer attributed to $\text{W}^{\text{V/IV}}$ species, and a second, small reversible wave at 1.0 V, most likely corresponding to the solvated species $([\text{Cp W}^{\text{IV}}(\text{NCMe})\text{Cl}]^+ / [\text{Cp}_2\text{W}^{\text{V}}(\text{NCMe})\text{Cl}]^{2+})$ as previously reported [32]. $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ showed two oxidation peaks. The first one corresponds to irreversible oxidation of W^{IV} to W^{V} in the range 0.67–0.73 V. Bulk electrolysis of previously reported tungstenocene complexes has shown that a one-electron oxidation ($\text{W}^{\text{IV/V}}$) process (1 faraday/mol) occurs in this range, 0.2–0.7 V [32]. The second one, the reversible redox couple in the range 0.89–0.92 V, is attributed to the 3-hydroxy-4-pyronato ligands. Although this second redox couple could be attributed to some kind of solvated species $([\text{Cp W}^{\text{IV}}(\text{NCMe})\text{L}]^+)$, where L is 3-hydroxy-4-pyronato, the fact that $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ are stable in acetonitrile without any change in composition or ligand replacement suggests that the 3-hydroxy-4-pyronato ligands may be responsible for this second redox couple.

Cytotoxicity studies

The antiproliferative activity of the tungstenocene complexes was evaluated in the HT-29 colon cancer cell line and the hormone-dependent MCF-7 breast cancer cell line at 72 h of exposure using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [33, 34]. Cp_2WCl_2 has been basically neglected as a potential anticancer drug owing to low response and sporadic activity on Ehrlich ascites tumor, B16 melanoma, colon 38 carcinoma, and Lewis lung carcinoma cell lines [19]. Thus, the role of the O, $\dot{\text{O}}$ bidentate chelating ligands in terms of solubility, stability, hydrophobicity, and antiproliferative activity was investigated in order to elicit structure–activity relationships. Additionally, the antiproliferative activities of the corresponding molybdenum isostructural complexes have been reported, and consequently comparison between tungsten and molybdenum complexes can afford insights into the role of the metal center with regard to the biological activity [21]. Table 1 summarizes the antiproliferative data, including FeCp_2BF_4 as a reference compound [35, 36]. The cytotoxic activity of FeCp_2BF_4 has been studied thoroughly [35].

In the HT-29 cell line it was observed that the new watersoluble tungstenocene complexes are at least one order of magnitude more cytotoxic than Cp_2WCl_2 . Nevertheless, their cytotoxic activities remain low and do not exceed that of FeCp_2BF_4 . Yet, it is clear that the 3-hydroxy-4-pyronato ligands imparted more cytotoxic activity than chloride. Not surprisingly, these data correlate with the aqueous stability and redox behaviors of the complexes. $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ are stable in aqueous solution for an extended time, whereas Cp_2WCl_2 is barely soluble in water and decomposes in a few hours. Additionally, $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ have higher oxidation potentials in buffer solution than Cp_2WCl_2 , suggesting these species are more robust in a physiological environment.

In the MCF-7 cell line, the cytotoxicity data are also somewhat correlated to the stability of the complexes and again the IC_{50} values are not better than for FeCp_2BF_4 . Dose–response curves are depicted in Fig. 4. $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ is one of the least active species in this cell line but still is more cytotoxic than Cp_2WCl_2 . However, the enhancement in terms of antiproliferative activity attributed to the 3-hydroxy-4-pyronato ligands is small. In general terms, replacement of chloride by the O, $\dot{\text{O}}$ bidentate ligands in tungstenocene does not improve significantly the cytotoxic activity for the MCF-7 cell line, whereas for the HT-29 cell line, the enhancement is slightly more significant. But these species do not reach the expected IC_{50} values, in the micromolar range, to be useful as chemotherapeutic drugs.

Our research group has reported the antiproliferative activity of Cp_2MoCl_2 , $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$, and $\text{Cp}_2\text{Mo}(\text{malonate})$ complexes on HT-29 colon cancer and MCF-7 breast cancer cell lines [21]. The IC_{50} values for Cp_2MoCl_2 , $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$, and $\text{Cp}_2\text{Mo}(\text{malonate})$ in the HT-29 colon cancer cell line are 2.6 (± 0.3), 1.31 (± 0.02), and 1.9 (± 0.3) mM, respectively, whereas for the MCF-7 breast cancer cell line none of the complexes exhibited antiproliferative effects. Instead, they showed proliferative and estrogenic effects [21]. Comparing Mo(IV) species with W(IV) species in the HT-29 cell line it is clear that Cp_2MoCl_2 is more cytotoxic than Cp_2WCl_2 . This can be explained in terms of the solubility and aqueous stability. At physiological pH, $\text{Cp}_2\text{MoCl}_2(\text{aq})$ ($\text{Cp}_2\text{Mo}(\text{H}_2\text{O})(\text{OH})^+ / [\text{Cp}_2\text{Mo}(\mu\text{-OH})_2\text{MoCp}_2]^{2+}$) is stable, whereas $\text{Cp}_2\text{WCl}_2(\text{aq})$ decomposes in 3 h or less.

In contrast to Cp_2MoCl_2 and Cp_2WCl_2 , $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ are stable at pH 7.4. In terms of the stability of the complexes to decomposition (Cp protonolysis, O, $\dot{\text{O}}$ ligand replacement), both molybdenocenes and tungstenocenes are robust at physiological pH. The oxidation potentials for Mo(IV) species are higher than those for W(IV) species, indicating that

Cp_2MoCl_2 and $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$ are stabler to oxidation than their tungstenocene counterparts [21]. However, $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ (also applies for $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$) has anti-proliferative activity on the MCF-7 cell line, whereas $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$ has proliferative activity. In this scenario the role of the metal center becomes important. We could envisage or speculate that W(IV) is more redox active under physiological conditions than Mo(IV), most likely inducing a more cytotoxic effect in the cell than Mo(IV). But other factors must be considered. The MCF-7 cell line is an estrogen-receptor-positive cell line. MCF-7 cells process estrogen in the form of estradiol, via estrogen receptor in the cell cytoplasm, using estradiol as a natural ligand to estrogen receptor to activate cell transcription. Thus, estrogen is a natural ligand to estrogen receptor to promote proliferation of estrogen-receptor-positive cells such as MCF-7 cells. The proliferative activities of the molybdenocenes have been attributed to their estrogen-like effects on hormone-dependent cancer cell lines with possible coordination interactions with the cysteine residues of the estrogen receptor [21, 37–39]. This estrogenic effect is not observed for the tungstenocene analogs. For comparison, we have synthesized $\text{Cp}_2\text{W}(\text{malonate})$, but this compound has limited stability, and an analytically pure compound to afford satisfactory elemental analysis has not been obtained. Nonetheless, the $\text{Cp}_2\text{W}(\text{malonate})$ IC_{50} values for the HT-29 and MCF-7 cell lines are 0.79 and 0.41 mM, respectively, suggesting higher cytotoxic activity than for the molybdenum counterpart, which is proliferative. However, this tungstenocene species has not been isolated in an analytically pure form and a reliable comparison cannot be made.

HSA–tungstenocene interactions

Fluorescence quenching spectral analysis—HSA is a single polypeptide with 585 amino acids with three homologous α -helical domains (I, II, and III), and each domain is subdivided into two subdomains (A and B) [40–43]. There are two principal drug binding sites located in subdomains IIA and IIIA, sites I and II (Fig. 5). With regard to protein fluorescence, only three amino acids have fluorophores: tryptophan, tyrosine, and phenylalanine. However, the intrinsic fluorescence of HSA is mainly attributed to the single tryptophan (Trp214) which is located in subdomain IIA, where one of the drug binding sites resides, in particular for metal complexes [40–43].

The intensity of the fluorescence of a substance can be affected or influenced by many factors, such as molecular rearrangements, collisions, complex formation, and energy transfer between two or more species, leading to what is called a fluorescence quenching spectrum. Thus, to study the HSA–metal binding interactions, the fluorescence quenching spectra were obtained at three different temperatures with an excitation wavelength of 280 nm and the emission recorded from 300 to 400 nm.

Figures 6 and S5 present the fluorescence quenching spectra of HSA upon addition of Cp_2WCl_2 and its derivatives. Upon analysis of these figures, it is evident that the quenching of the HSA fluorescence intensity is dependent on the concentration of tungstenocene, but the tungstenocene complexes can be separated according to their quenching behaviors. Cp_2WCl_2 causes moderate quenching of the HSA fluorescence intensity without shifting the position of the maximum emission wavelength of 336 nm, whereas $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ cause strong quenching of the HSA intrinsic fluorescence intensity along with a redshift of the maximum emission wavelength. The strong quenching and redshift suggest that the microenvironment around Trp214 is changing, and this is indicative of HSA–tungstenocene binding interactions. Upon examination of the solid-state structure of HSA, it can be observed that Trp214 is located in subdomain IIA but near the interface between subdomains IIA and IIIA [40–43]. Thus, Trp214 was used as a sensor to monitor local perturbations and conformational changes

within the protein. Particularly important, the complexes that contain 3-hydroxy-4-pyronato ligands are more hydrophobic and showed stronger hydrophobic interactions with HSA than did Cp_2WCl_2 as discussed in “Molecular modeling–docking studies.”

To elicit the mechanism responsible for the quenching of the HSA intrinsic fluorescence intensity, the Stern–Volmer equation (Eq. 1) was used to analyze the data: [44–50].

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q], \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively, k_q is the biomolecular quenching constant, τ_0 is the fluorescence lifetime in the absence of the quencher (for a biopolymer it is on the order of 10^{-8} s^{-1}), $[Q]$ is the concentration of the quencher, and K_{SV} is the Stern–Volmer quenching constant.

There are two types of quenching: dynamic and static. Dynamic quenching depends on the diffusion of a quencher toward a fluorophore; therefore, they are in contact only during the lifetime of the excited state. Static quenching is related to the formation of the complex and binding interactions between the fluorophore and the quencher [44–50]. One way to determine the quenching mechanism is by examining the behavior of the fluorophore in the presence of the quencher at different temperatures. Therefore, the fluorescence quenching spectra of HSA were obtained at three different temperatures, and were analyzed using the Stern–Volmer equation (Eq. 1). A linear Stern–Volmer plot is indicative of a single quenching mechanism, either static or dynamic [50]. In fact, our Stern–Volmer plots for the HSA–tungstenocene interactions were linear (Fig. S6).

Dynamic quenching depends upon diffusion; hence, the quenching constants increase when the temperature is increased. On the other hand, static quenching is characterized by a decrease in the quenching constants when the temperature increases, owing to the stability of HSA–tungstenocene complexes decreasing. Also, a static quenching is observed when the value of k_q is larger than the limiting diffusion collision quenching constant, K_D , of a biomolecule with various quenchers ($2.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) [42–50]. Table 2 presents the Stern–Volmer quenching constants for the HSA–tungstenocene interactions obtained using the fluorescence quenching spectra at three different temperatures.

Table 2 shows that all the k_q values are larger than the diffusion collision quenching constant, K_D , of a biomolecule ($2.0 \times 10^{10} \text{ L/mol s}$). This indicates that these are static quenchings and indicates there is HSA–tungstenocene complex formation. Additionally, the k_q and K_{SV} values of the tungstenocene-3-hydroxy-4-pyronato derivatives are one order of magnitude larger than for Cp_2WCl_2 . But, as mentioned previously, K_{SV} and k_q are a function of temperature. In this regard, with exception of $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, as a general trend, the increase in temperature increases the K_{SV} and k_q values, suggesting a dynamic quenching. $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ showed the opposite behavior. These conflicting results prompted us to further use the fluorescence quenching spectra and the K_{SV} values to calculate the corresponding thermodynamic parameters, as described below.

The change in stability of the HSA–tungstenocene interaction (complex) as a function of temperature is clearly reflected in the K_{SV} values and it is a function of the intermolecular forces holding the complex together. To pinpoint the type of interactions in which HSA–tungstenocene is engaged, using the van't Hoff equation, $\ln K = -\Delta H^0/RT + \Delta S^0/R$, we determined the thermodynamic parameters for these interactions (Table 3) and created van't Hoff plots (Fig. S7).

Upon examination of Table 3, it is evident that all tungstenocene complexes demonstrated spontaneity in the process (negative ΔG^0) and the HSA-W(IV) interactions of the new tungstenocene complexes are stabler than those for Cp_2WCl_2 . Cp_2WCl_2 , $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ have positive values of both ΔH^0 and ΔS^0 . $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ has negative ΔH^0 and positive ΔS^0 . Ross and Subramanian [51] have conceptualized a model for ligand binding to proteins based on thermodynamic parameters and intermolecular forces contributing to the stability. Their model proposes the following. $\Delta H^0 > 0$ and $\Delta S^0 > 0$ implies hydrophobic interactions; $\Delta H^0 < 0$ and $\Delta S^0 < 0$ suggests van der Waals and hydrogen bonding; $\Delta H^0 \sim 0$ (slightly positive or negative) and $\Delta S^0 > 0$ implies electrostatic and ionic interactions. Therefore, the complexes are mainly engaged in hydrophobic interactions but, the small ΔH^0 values also imply electrostatic/ionic interactions. In particular, for $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, the negative ΔH^0 suggests it becomes engaged in electrostatic/ionic interactions and hydrogen bonds to a greater extent than Cp_2WCl_2 , $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, as discussed for the docking studies described in “Molecular modeling–docking studies.” Further-more, on the basis of the ΔG^0 values, HSA-metal interactions are stabler for tungstenocene containing the 3-hydroxy-4-pyronato ligands than for Cp_2WCl_2 . This is most likely a result of hydrophobic interactions caused by the 3-hydroxy-4-pyronato ligands.

The aforementioned behaviors are also reflected in the quenching spectra, where the new tungstenocene derivatives caused a redshift of the maximum emission wavelength. In addition, the ΔS^0 values for $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ are greater than that for Cp_2WCl_2 . This is additional evidence regarding the hydrophobic interactions of the 3-hydroxy-4-pyronato ligands in the binding pocket. The ΔS^0 values suggest higher stability as a result of hydrophobic interactions of the complexes with the surrounding amino acids, pushing water molecules out of the hydrophobic pocket and as a result the water molecules are arranged more randomly. To this end, the thermodynamic results correlate well with the hydrophobic character imparted by the 3-hydroxy-4-pyrone groups on tungstenocene complexes which promotes hydrophobic interactions within the protein hydrophobic binding sites. In fact, binding interactions between maltol and HSA have been investigated by fluorescence spectroscopy [52]. It was found that maltol becomes engaged in hydrophobic interactions in subdomain IIA, drug binding site I, in a hydrophobic pocket near Trp214 [52].

Molecular modeling–docking studies—HSA has several binding sites for endogenous compounds such as fatty acids and exogenous substances such as drugs and metal ions. As mentioned previously, there are two primary drug binding sites in HSA, site I and site II in subdomains IIA and IIIA, respectively (Fig. 5) [40–43, 51]. In addition to these drug binding sites, there are seven common binding sites that are occupied by medium-chain and long-chain fatty acids [53]. There are five additional binding sites on HSA for medium-chain fatty acids that may be occupied only in the presence of a high concentration of fatty acids [53]. To gain insight and understanding into the binding interactions between tungstenocenes and HSA as a transport protein, and at which binding site the metallocene is located, molecular modeling methods were employed.

Docking studies were performed for tungstenocenes and HSA using the program SYBYL 8.1 [54]. Table 4 summarizes the hydrogen bonds and van der Waals/hydrophobic and hydrogen bonding interactions. The interactions listed are those within a distance of 4 Å or less. The docking studies were performed using automatic flexible docking algorithm with default settings and using Proto-Mol-based methods [54, 55]. The stablest HSA–tungstenocene interaction was chosen by selecting the structure with the highest total score, which represents the highest affinity in terms of $-\log K_d$ values (see Table 5) [55]. The structures with the highest scores are those with the highest affinity. Table 5 presents the –

$\log K_d$ values of the two stablest docking structures. In the docking studies, the fatty acids were removed from the protein; therefore, the possibility of ligand exchange between the fatty acid and the complex was excluded.

The docking studies performed for HSA and tungstenocenes revealed that between the stablest docking structures, only $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ occupies drug binding site II, located in subdomain IIIA, whereas none of the remaining tungstenocene complexes occupy drug binding site I or drug binding site II. Instead, they occupy one of the seven common binding sites for fatty acids, site 6 in subdomain IIA (Fig. 5). Site 6, where Cp_2WCl_2 , $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ are docked, is more hydrophobic (it has a higher content of hydrophobic amino acids) whereas in drug binding site II, the complex is well surrounded by a combination of hydrophobic and hydrophilic residues. In this regard, the Cp rings are surrounded principally by hydrophobic amino acids and the kojato is surrounded by hydrophilic ones. Site 6 is located at the interface between subdomains IIB and IIA (Fig. 7). This docking study also showed that $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ has higher affinity for HSA than do the other complexes: $\text{Cp}_2\text{WCl}_2 < [\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl} < [\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl} < [\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$. This trend can be explained in terms of noncovalent binding interactions, as discussed below.

In site 6, Cp_2WCl_2 and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ are not engaged in hydrogen bonding, whereas $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ is engaged in hydrogen bonding between Lys212 and the ketonic oxygen of the maltolato ligand (Figs. 7, S8). This ketonic oxygen is also involved in O,Ö chelate bonding to $\text{Cp}_2\text{W}(\text{IV})$. This hydrogen bonding apparently forces $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ to be in a different orientation inside the binding pocket (about 45°) with respect to Cp_2WCl_2 and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ (Fig. 7). It is not clear why $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ does not become engaged in hydrogen bonding, but the steric hindrance of the ethyl substituent on the pyrone may explain this behavior. The Cp rings in Cp_2WCl_2 and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ are surrounded by the same set of amino acids, mostly hydrophobic (Fig. 7). The ethyl maltolato ligand is located at the entrance of the pocket, pointing away from the cavity where it can interact with the solvent.

In contrast to Cp_2WCl_2 , $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ occupies drug binding site II in subdomain IIIA. The Cp rings have van der Waals interactions mainly with Leu407, Leu430, Leu 453, Phe403, Tyr411, Val433, Gly434, Cys392, and Cys438. Residues Leu387, Leu453, Ile388, Pro384, Val344, and Ala449 are surrounding the six-membered ring of kojate. Particularly interesting, $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ forms two hydrogen bonds. One hydrogen bond involves the carbonyl oxygen of Met446 and the hydrogen of the hydroxyl group of kojate and the second involves the N–H of Glu450 and the oxygen of the hydroxyl group. The additional hydroxyl group on the pyrone ring for the kojato ligand versus the ethyl maltolato and maltolato ligands, suggests it needs a more polar environment with a combination of hydrophilic and hydrophobic residues, which is not available in site 6 but is readily available in drug binding site II [53]. Figure 8S presents a detailed representation of the amino acids involved in the binding cavity.

In the second stablest docking structures, Cp_2WCl_2 , $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ remained in the same binding site (site 6), except that the orientation of the complex differs. In marked contrast, $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ occupies a different binding site (site 7) in subdomain IIA. Even in this site, the complex becomes engaged in three hydrogen bonds. One involves Lys199 and the alkoxy oxygen of the kojato ligand, whereas the hydroxyl group of the kojato ligand is engaged in two hydrogen bonds with His242. These involve the N–H group of the imidazole ring and the oxygen of the carbonyl group of His242. This alkoxy oxygen is also engaged in O,Ö chelate bonding to $\text{Cp}_2\text{W}(\text{IV})$. Even

though this structure is less stable than the one in drug binding site II, the number of hydrogen bonds suggests that it is a stable docking structure and it cannot be ruled out.

There are two reports concerning to the binding interactions of functionalized titanocenes and HSA [38, 56]. Molecular modeling methods were used to study the docking of Titanocene Y (bis[(*p*-methoxybenzyl)cyclopentadienyl]titanium(IV) dichloride) into a free fatty acid cavity (palmitate was removed, creating a free site for binding). Docking studies showed that Titanocene Y fits well into this cavity without any steric hindrance created by the organometallic group (Cp_2TiCl_2). A hydrogen bond was identified between one of the oxygens of the *p*-methoxybenzyl group and Ser285 as well as polar contacts and van der Waals interactions with Ala256, Phe68, Leu20, Ala149, Pro150, and Arg255. These residues belong to subdomains IA, IB, and IIA, and Titanocene Y resides in site 7 [38]. However, owing to the hydrophobic nature of the complex, the residues involved in this docking are different from those of $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, which are more hydrophilic.

In other docking studies, a series of functionalized titanocenes were investigated by molecular modeling methods using two algorithms: docking at predetermined drug binding sites I and II and an automatic docking algorithm defined by the program [56]. This study demonstrated that functionalized titanocenes can bind in predetermined drug binding sites I and II and that these dockings are stabilized by a series of hydrogen bonds and van der Waals interactions. In contrast, using the automatic docking algorithm, the study showed that the titanocenes bind at two different sites not previously identified as drug binding sites I and II. Instead, they become engaged in binding interactions with residues from both binding site I and binding II. The site occupied by the titanocenes when this algorithm is used does not belong to any of the seven fatty acid binding sites.

Materials and methods

Materials

Cp_2WCl_2 (97 %) and the ligands, malonic acid disodium monohydrate salt (98 %; $\text{C}_3\text{H}_2\text{Na}_2\text{O}_4 \cdot \text{H}_2\text{O}$), 3-hydroxy-2-methyl-4-pyrone (99 %; maltol, $\text{C}_6\text{H}_6\text{O}_3$), kojic acid ($\text{C}_6\text{H}_6\text{O}_4$), and 2-ethyl-3-hydroxy-4*H*-pyran-4-one (99 %; ethyl maltol, $\text{C}_7\text{H}_8\text{O}_3$), were obtained from Sigma-Aldrich. Water was previously deionized, and degassed with nitrogen. The pH was adjusted with 1.0 M KOH. The tungstenocene derivatives were purified by column chromatography, using lipophilic Sephadex (20–100 lm) as the stationary phase and methanol as the mobile phase; both were purchased from Sigma-Aldrich.

The IR spectra were recorded with a PARAGON 1000 PC FT-IR spectrometer (PerkinElmer) with samples as compressed KBr disks. Data collection was performed using the software program Spectrum RX (version 3.02). The NMR spectra were obtained with a 500 MHz Bruker spectrometer. Elemental analyses were performed by Atlantic Microlab. UV–vis spectra were recorded with a JASCO V-560 double-beam spectrophotometer at room temperature. Solutions of Cp_2WCl_2 , tungstenocene derivatives, and ligands at 5.0×10^{-4} M were prepared in a buffer solution of 0.1 M Tris–HCl/10 mM NaCl at pH 7.4.

$\text{Cp}_2\text{W}(\text{malonate})$ was dissolved in a mixture of water/methanol (1:1) containing 0.2 % formic acid prior to mass-spectral analysis. A Bruker Daltonics Esquire 6000 instrument was used to record the mass-spectral data. Electrospray positive ion mode was used as the ionization mode during the mass spectrometry experiment. The experimental intensities were reported relative to the intensity of the parent peak in the mass spectrum.

The HT-29 colon cancer cell line and the MCF-7 breast adenocarcinoma cell line were purchased from American Type Culture Collection (ATCC). HT-29 and MCF-7 cells were

maintained at 310 K and 95 % air/5 % CO₂ in their respective media. The growth medium for HT-29 cells was McCoy's 5A complete medium supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) antibiotic/antimycotic. The growth medium for MCF-7 cells was Eagle's minimum essential medium supplemented with 10 % (v/v) fetal bovine serum, 1 % (v/v) antibiotic/antimycotic, nonessential amino acids, and 0.01 mg/mL bovine insulin. MTT and Triton X-100 used for the cytotoxic assay were obtained from Sigma. All MTT manipulations were performed in a dark room.

Synthesis and characterization of tungstenocene derivatives

Cp₂WCl₂ (150 mg, 0.390 mmol) was placed in a threeneck round-bottom flask of 100-mL volume with 25 mL of deionized water and the solution was degassed. The solution was heated until the complex dissolved completely forming a violet solution. Then, 0.390 mmol of the corresponding ligand (maltol, ethyl maltol, kojic acid, and malonate) was added and the pH was adjusted with 0.1 M KOH. For ethyl maltol and maltol, the pH was adjusted to 5.5 and 6.0, respectively, whereas for kojic acid and malonate the pH was adjusted to 7.6 and 9.0, respectively. The reaction mixture was stirred overnight at room temperature under a nitrogen atmosphere and an amber solution was formed with a minor amount of black precipitate, and the final pH decreased by around 3 pH units in each case. The liquid phase was decanted, the solvent was removed under a vacuum, and the resulting solid was purified by column chromatography, using lipophilic Sephadex as the solid phase and methanol as the solvent. To achieve acceptable elemental analysis, the samples were dried under a vacuum for 3 h at 303 K. Higher temperatures led to decomposition of the complexes. Cp₂W(malonate) is highly hygroscopic and decomposes in the presence of oxygen. Satisfactory elemental analysis of Cp₂W(malonate) was not obtained owing to decomposition. The mass spectrum was recorded instead.

[Cp₂W(maltolato)]Cl

¹H NMR [500 MHz, dimethyl-*d*₆ sulfoxide (DMSO-*d*₆)] δ (ppm): 8.36 [d, 1H, H(5)], 7.05 [d, 1H, H(6)], 5.91 (s, 10H, Cp), 2.36 (s, 3H, CH₃)

¹³C NMR [125 MHz, DMSO-*d*₆, 2,2-dimethylsila-pentane-5-sulfonic acid (DSS)] δ (ppm): 187.44 [C(4)], 161.92 [C(6)], 157.91 [C(2)], 157.12 [C(3)], 110.90 [C(5)], 98.56 (Cp), 15.05 (CH₃).

IR (KBr, cm⁻¹): 3,086 (C–H, Cp), 3,044 (C–H), 1,605, 1,548 (C=O), 1,478, 1,430 (C=C), 1,270, 1,208 (C–O), 925 (C–H, Cp), 846 (C–H), 564 (W–O).

Anal. Calc. for C₁₆H₁₅WO₃Cl: C, 40.49; H, 3.19. Found: C, 40.07; H, 3.10.

[Cp₂W(ethyl maltolato)]Cl

¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 8.39 [d, 1H, H(5)], 7.06 [d, 1H, H(6)], 5.91 (s, 10H, Cp), 2.71 (q, 2H, CH₂), 1.14 (t, 3H, CH₃).

¹³C NMR (125 MHz, DMSO-*d*₆, DSS) δ (ppm): 187.74 [C(4)], 161.87 [C(6)], 161.30 [C(2)], 157.20 [C(3)], 110.89 [C(5)], 98.55 (Cp), 21.95 (CH₂), 11.33 (CH₃).

IR (KBr, cm⁻¹): 3,046 (C–H, Cp), 2,974 (C–H), 1,602, 1,550 (C=O), 1,470, 1,426 (C=C), 1,256, 1,190 (C–O), 1,000 (C–H, Cp), 836 (C–H), 558 (W–O).

Anal. Calc. for: C₁₇H₁₇WO₃Cl: C, 41.79; H, 3.51. Found: C, 41.43; H, 3.45.

[Cp₂W(kojato)]Cl

[Cp₂W(kojato)]Cl ¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 8.36 [s, 1H, H(5)], 6.99 [s, 1H, H(2)], 5.92 (s, 10H, Cp), 5.63 (s, 1H, OH), 4.50 (s, 2H, CH₂).

¹³C NMR (125 MHz, DMSO-*d*₆, DSS) δ (ppm): 190.74 [C(4)], 172.43 [C(6)], 164.09 [C(3)], 144.65 [C(2)], 107.27 [C(5)], 98.55 (Cp), 59.73 (CH₂).

IR (KBr, cm⁻¹): 3,216 (OH), 3,105 (C–H, Cp), 3,065 (C–H), 1,600, 1,550 (C=O), 1,472, 1,430 (C=C, Cp), 1,274, 1,188 (C–O), 925 (C–H, Cp), 849 (C–H), 583 (W–O).

Anal. Calc. for: C₁₆H₁₅WO₄Cl: C, 39.17; H, 3.08. Found: C, 39.01; H, 2.97.

Cp₂W(malonate)

¹H NMR (500 MHz, DMSO-*d*₆) δ (ppm): 5.86 (s, 10H, Cp), 3.08 (s, 2H).

IR (KBr, cm⁻¹): 3,104 (C–H, Cp), 1,644 (C=O), 1,430 (C=C, Cp), 1,386 (C–O), 970 (C–H, Cp), 844 (C–H), 564 (W–O).

Electrospray ionization mass spectrometry (positive mode), *m/z* (relative intensity): Cp₂W(malonate)⁺ 415 (79.1), 416 (55.1), 417 (100), 419 (83.1).

Stability studies by NMR spectroscopy

Solutions (1 × 10⁻³ M) of Cp₂WCl₂, [Cp₂W(maltolato)]Cl, [Cp₂W(ethyl maltolato)]Cl, and [Cp₂W(kojato)]Cl in D₂O at pH 7.4 and 25 mM Tris-*d*₁₁/10 mM NaCl buffer solution at pH 7.4 were used for ¹H NMR experiments. Likewise, 1 × 10⁻³ M solutions of Cp₂W(maltolato)]Cl, [Cp₂W(ethyl maltolato)]Cl, and [Cp₂W(kojato)]Cl in D₂O/10 mM NaCl pH < 4 were used for the NMR experiments. Further, 0.01 M NaOD was used to adjust the pH. For Cp₂WCl₂ in D₂O at pH ~ 3.4, the exact concentration was not determined accurately owing to the low solubility of the tungstenocene in an aqueous environment.

Cytotoxicity assay

Biological activity was determined using the MTT assay originally described by Mossman [32] but using 10 % Triton in 2-propanol as a solvent for the MTT formazan crystals [33]. HT-29 and MCF7 cells were maintained at 310 K and 95 % air/5 % CO₂ in McCoy's 5A (ATCC) complete medium, which had been supplemented with 10 % (v/v) fetal bovine serum (ATCC) and 1 % (v/v) antibiotic/antimycotic (Sigma). Asynchronously growing cells (HT-29 or MCF-7) were seeded at 10,000 cells per well in 96-well plates containing 100 μ L of complete growth medium, and allowed to recover overnight. The solutions of the complexes were prepared first by dissolving the corresponding tungstenocene in the medium. Then, 25 μ L of the medium per well was added to the first two columns (controls) and 25 μ L of solutions of the complexes per well, dissolved in the medium, was added to rest of the plate. Each column had a different concentration of the complex, starting from 0.01 to 0.00014 M, and then it was incubated by 72 h. After incubation, 50 μ L per well of a solution of MTT (2.4 mg/mL) dissolved previously in the medium was added and the mixture was incubated for 2 h to produce insoluble purple formazan dye inside the mitochondria from living cells. When the incubation was completed, the cell medium was removed and the cells were rinsed with cold phosphate buffer solution. Then, 180 μ L per well of Triton solution (10 %) in 2-propanol was added and the mixture was incubated until the purple formazan crystals dissolved. The absorbance of the resulting purple solutions was measured at 490 nm, with background subtraction at 630 nm, in an ELx808 microplate reader (BioTek instruments). The IC₅₀ values of the complexes were calculated by fitting

the data to a sigmoidal dose–response plot (variable slope) using the software program Prism 3.0. The IC_{50} values were the average of four independent experiments. The standard deviation (S_x) was calculated as follows: $S_x = AS_y/0.434$, where A is the IC_{50} value and S_y is the error in the y-axis (viability) provided by Prism 3.0.

Electrochemistry

Electrochemical characterization of Cp_2WCl_2 , tungstenocene derivatives, and ligands was conducted using an Epsilon voltammetric analyzer with a BASi C3 cell stand, using the software program Epsilon EC-2000XP. The samples were dissolved in acetonitrile with 0.1 M Bu_4NPF_6 as the supporting electrolyte at a concentration of 0.001 M for the complex and ligands or in aqueous solution using 0.1 M Tris–HCl/10 mM NaCl pH 7.4 or 0.1 M KCl as the supporting electrolytes at a concentration of 5.0×10^{-4} M for the complex and ligands. Glassy carbon (BAS model MF-2012) was used as the working electrode, Ag/AgCl in 3.0 M NaCl was used as the reference electrode, and a platinum wire was used as the auxiliary electrode. The working electrode was polished with 0.05- μ m alumina slurry for 1–2 min, and then rinsed with double-distilled and deionized water. This cleaning process was done before each cyclic voltammetry experiment. The sweep was performed from 1,500 to –0.1 mV for complexes dissolved in acetonitrile and from 1,000 to 0 mV for complexes dissolved in buffer solution with a scan rate of 100 mV/s and sensitivity of 100 μ A.

Protein binding studies

HSA solution (1 mL, 2.7×10^{-6} M) was titrated with 14- μ L aliquots of solutions of the complex (6.0×10^{-4} M) to reach a molar ratio of metal to protein of 1:3. The fluorescence quenching spectra were obtained at three different temperatures with an excitation wavelength of 280 nm and 300–400 nm for emission using a Shimadzu RF-5301 PC spectrofluorophotometer equipped with a temperature controller. The fluorescence spectra were corrected for dilution. We determined that the dilution factor was small and did not change the fluorescence spectra significantly. However, the quenching spectra were not corrected for the inner-filter effect, neither for excitation nor for emission, since the maximum absorbances of the tungstenocene species in such dilute solution (approximately 2.68×10^{-6} M) are between 0.001 and 0.01. In these dilute conditions, using the formula for correction of the inner-filter effect [$F_{corr} = F_{obs} \text{ antilog}(A_{exc} + A_{em})/2$] has neither a significant effect nor is needed, according to the standard quenching analysis procedure [49].

Molecular modeling–docking studies

The geometries of the tungstenocene derivatives were optimized using density function theory with the B3LYP hybrid functional and the 6-31G** basis set using Gaussian 03 [57]. These structures were subsequently used for the docking studies with HSA. The crystal structure of the HSA complex with decanoic acid (Protein Data Bank code 1E7E) was used for the docking studies. The complexed decanoic acid was removed from the coordinate file as was the water molecule, and the protein structure was minimized with the Powell method with the Kollman all-atom force field and the AMBER charges. Finally, the protein structure was prepared for the docking runs using the Biopolymer Structure Preparation Tool with default settings as implemented in SYBYL 8.1 [54]. The docking mode was defined with Surflex-Dock [55], which employs an automatic flexible docking algorithm, with default settings and using ProtoMol, a threshold of 0.15, and a bloat of 5. This comprises an empirical scoring function and a search engine to dock ligands into the protein binding sites using a ProtoMol-based method [55].

Conclusions

Three water-soluble tungstenocene complexes were synthesized, structurally characterized by spectroscopic methods, and their cytotoxicity properties were investigated. First, the 3-hydroxy-4-pyronato ligands provide higher stability to tungstenocene in an aqueous environment at physiological pH and in buffer conditions. The higher stability is evidenced by the higher oxidation potentials of the tungstenocene complexes over Cp_2WCl_2 .

The cytotoxicity study allowed us to investigate the role of the ligand and metal center in their antiproliferative action. In both cell lines, the 3-hydroxy-4-pyronato ligands enhance the cytotoxic activity of the resulting tungstenocene complexes, but the effect is less pronounced in the MCF-7 cell line. In comparison with the molybdenocene analogs, the tungstenocene complexes are one order of magnitude more cytotoxic in the HT-29 cell line than Cp_2MoCl_2 and $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$. None of the new complexes exceed the antiproliferative activity of FeCp_2BF_4 , and their IC_{50} values are not in the micromolar range to be useful as anticancer drugs. More intriguing are the MCF-7 results. In this cell line, the tungstenocenes have antiproliferative activities, whereas the molybdenocene counterparts have proliferative and most likely estrogenic properties. It is clear from these data that tungstenocenes do not have estrogenic effects on the MCF-7 breast cancer cell line.

The fluorescence quenching spectra of HSA with the tungstenocenes showed that the interaction is static, and on the basis of the thermodynamic parameters, the species become engaged mainly in hydrophobic and electrostatic/hydrogen bond interactions. To explain the thermodynamic parameters of the HSA–tungstenocene interactions, docking studies were performed. These studies revealed that the complexes occupy site 6 and drug binding site II. The most hydrophilic complex, $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, docks on site II, which is more polar and hydrophilic than site 6 [53]. Thus, $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ behaves differently from the other tungstenocenes and becomes engaged in two hydrogen bonds in drug binding site II. The negative ΔH° value for $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ further supports the presence of hydrogen bonds in site II. Sites 6 and II are located in subdomains IIA and IIIA, respectively, and the tryptophan is located in subdomain IIA but at the interface between both subdomains. Thus, the changes in tryptophan fluorescence correspond to changes in the protein conformation as a result of drug binding.

Finally, the affinity of the tungstenocenes for HSA can be explained in terms of the number of hydrogen bonds holding the complex together. The highest affinity is exhibited by $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$, where two hydrogen bonds (in addition to hydrophobic interactions caused by the pyrone ring) were detected, followed by $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ (with one hydrogen bond plus hydrophobic interactions), $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ (hydrophobic interaction caused by the pyrone ring), and Cp_2WCl_2 (lack of pyrone ring).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Cyclic voltammograms of Cp_2WCl_2 (1), $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ (2), $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ (3), and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ (4) in 0.1 M KCl (Cp is cyclopentadienyl). The scan rate was 100 mV/s and the complex concentration was 5.0×10^{-4} M. The reference electrode was Ag/AgCl in 3.0 M NaCl

Fig. 2. Cyclic voltammograms of Cp_2WCl_2 (1), $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ (2), $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ (3), and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ (4) in 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.4). The reference electrode was Ag/AgCl in 3.0 M NaCl and the scan rate was 100 mV/s

Fig. 3. Cyclic voltammograms of Cp_2WCl_2 (1), $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$ (2), $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ (3), and $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ (4) in acetonitrile (0.1 M Bu_4NPF_6). The reference electrode was Ag/AgCl in 3.0 M NaCl and the scan rate was 100 mV/s

Fig. 4.
Dose-response curves of Cp_2WCl_2 and its derivatives for the MCF-7 breast cancer cell line at 72 h of exposure

Fig. 5. Cartoon diagram of the crystal structure of human serum albumin (HSA). Site 6 is occupied by Cp_2WCl_2 , $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$, and $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ and drug binding site II is occupied by $[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$

Fig. 6. Fluorescence quenching spectra of HSA with different complexes: **a** Cp_2WCl_2 and **b** $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$

Fig. 7. Docking representations of the amino acids in the HSA binding site for Cp₂WCl₂, [Cp₂W(maltolato)]Cl, [Cp₂W(ethyl maltolato)]Cl, and [Cp₂W(kojato)]Cl

Scheme 1.
Synthesis of the water-soluble tungstenocenes

Table 1

Cytotoxic activity of tungstenocene dichloride and tungstenocene derivatives against HT-29 colon cancer and MCF-7 breast cancer cell lines at 72 h of drug exposure

Complex	IC ₅₀ mM (±SD)	
	HT-29	MCF-7
Cp ₂ WCl ₂	10 (1)	0.63 (0.01)
[Cp ₂ W(maltolato)]Cl	0.54 (0.02)	0.33 (0.01)
[Cp ₂ W(ethyl maltolato)]Cl	0.35 (0.02)	0.19 (0.04)
[Cp ₂ W(kojato)]Cl	0.20 (0.01)	0.59 (0.03)
FeCp ₂ BF ₄	0.180 (0.010)	0.150 (0.004)

Each value is an average of six measurements on four independent experiments

Cp cyclopentadienyl, *SD* standard deviation

Table 2

Stern-Volmer quenching constants for the tungstenocene–human serum albumin (HSA) system at different temperatures

Complex	<i>T</i> (K)	<i>K</i> _{SV} (L/mol)	<i>k</i> _q (L/mol s)
Cp ₂ WCl ₂	288	4,044.4	4.04 × 10 ¹¹
	310	4,772.0	4.77 × 10 ¹¹
	317	5,104.0	5.10 × 10 ¹¹
[Cp ₂ W(ethyl maltolato)]Cl	288	14,238.0	1.42 × 10 ¹²
	310	16,392.0	1.64 × 10 ¹²
	317	17,546.0	1.75 × 10 ¹²
[Cp ₂ W(kojato)]Cl	303	15,738.0	1.57 × 10 ¹²
	310	15,043.0	1.50 × 10 ¹²
	317	14,147.0	1.41 × 10 ¹²
[Cp ₂ W(maltolato)]Cl	288	11,945.0	1.19 × 10 ¹²
	310	15,076.0	1.51 × 10 ¹²
	317	16,578.0	1.66 × 10 ¹²

Table 3

Thermodynamic parameters for tungstenocene–HSA interactions

Complex	<i>T</i> (K)	ΔH^0 (kJ/mol)	ΔS^0 (J/mol K)	ΔG^0 (kJ/mol)
Cp ₂ WCl ₂	288	5.96	89.71	−19.87
	310			−21.85
	317			−22.48
[Cp ₂ W(ethyl maltolato)]Cl	288	5.28	97.83	−22.88
	310			−25.03
	317			−25.71
[Cp ₂ W(kojato)]Cl	303	−6.07	60.33	−24.35
	310			−24.77
	317			−25.20
[Cp ₂ W(maltolato)]Cl	288	8.39	107.17	−22.48
	310			−24.83
	317			−25.58

Table 4

Van der Waals and polar contacts between tungstenocenes and HSA at distance of 4 Å or less

Cp ₂ WCl ₂	[Cp ₂ W(maltolato)]Cl	[Cp ₂ W(ethyl maltolato)]Cl	[Cp ₂ W(kojato)]Cl
Arg209	Arg209	Arg209	Ser342
Lys212	Lys212 (one hydrogen bond)	Lys212	Val344
Val235	Val235	Val235	Pro384
Ala213	Ala213	Ala213	Leu387
Val216	Val216	Val216	Ile388
Ser232		Ser232	Asn391
Val231			Cys392
Asp324	Asp324	Asp324	Phe403
			Leu407
Val325	Val325	Val325	Arg410
			Tyr411
Leu327	Leu327		Leu430
Gly328	Gly328	Gly328	Val433
Leu331	Leu331		Gly434
Phe228	Phe228	Phe228	Cys438
			Met446 (hydrogen bond O(Met)-H(kojato))
			Ala449
			Glu450 (hydrogen bond N-H(Glu)-O(kojato))
			Leu453
			Arg485
			Leu453

Table 5

Affinity constants of tungstenocenes for the two stablest structures inside HSA

Cp_2WCl_2	$[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$	$[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$	$[\text{Cp}_2\text{W}(\text{kojato})]\text{Cl}$
$-\log K_d = 3.94$ (stablest)	$-\log K_d = 6.19$ (stablest)	$-\log K_d = 5.01$ (stablest)	$-\log K_d = 7.35$ (stablest)
$-\log K_d = 3.40$	$-\log K_d = 4.47$	$-\log K_d = 4.69$	$-\log K_d = 6.96$

 K_d is the dissociation constant