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Comparative serum albumin interactions and antitumor effects of Au(III) and Ga(III) ions

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ABSTRACT

In the present study, interactions of Au(III) and Ga(III) ions on human serum albumin (HSA) were studied comparatively via spectroscopic and thermal analysis methods: UV–vis absorbance spectroscopy, fluorescence spectroscopy, Fourier transform infrared (FT-IR) spectroscopy and isothermal titration calorimetry (ITC). The potential antitumor effects of these ions were studied on MCF-7 cells via Alamar blue assay. It was found that both Au(III) and Ga(III) ions can interact with HSA, however; Au(III) ions interact with HSA more favorably and with a higher affinity. FT-IR second derivative analysis results demonstrated that, high concentrations of both metal ions led to a considerable decrease in the α -helix content of HSA; while Au(III) led to around 5% of decrease in the α -helix content at 200 μ M, it was around 1% for Ga(III) at the same concentration. Calorimetric analysis gave the binding kinetics of metal–HSA interactions; while the binding affinity (K_a) of Au(III)–HSA binding was around $3.87 \times 10^5 \text{ M}^{-1}$, it was around $9.68 \times 10^3 \text{ M}^{-1}$ for Ga(III)–HSA binding. Spectroscopy studies overall suggest that both metal ions have significant effects on the chemical structure of HSA, including the secondary structure alterations. Antitumor activity studies on MCF7 tumor cell line with both metal ions revealed that, Au(III) ions have a higher antiproliferative activity compared to Ga(III) ions.

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Introduction

Human serum albumin (HSA) is the most abundant and the major ligand binding serum protein, and one of the most important focal point in pharmaceutical industry, since it can bind with various drug molecules and modify their pharmacokinetic properties [1]. The chemical structure of HSA is composed of a single polypeptide chain of 585 amino acids that is divided into three homologous domains (I–III): I (1–195), II (196–383) and III (384–585); and each domain consists of two subdomains (A and B) [2]. Due to its chemical nature, HSA involves in various important physiological and pharmacological processes; therefore, small molecules (of HSA) interactions have been an essential field of research in biochemistry, life sciences, clinical medicine and toxicology for the last decades [2].

Different types of gold and gallium complexes have been synthesized and evaluated in terms of their antitumor potency [3–7].

It was reported that, while gold compounds can affect cancer cell proliferation by inhibiting telomerase and STAT3 [6] or enzyme thioredoxin reductase activity [7]; gallium complexes can exhibit antitumor activity in the presence of phenolate rings [4], amine and pyridine groups by inhibiting the activity of proteasome [8]. Previous studies reported that, *in vitro* interactions of Au(III) complexes with calf thymus DNA are generally weak, different from cisplatin, suggesting these complexes have a weaker binding profile on genomic DNA [9]. However, it is also known that, Au(III) complexes can bind strongly with model proteins, can overcome the cisplatin resistance, and some of the Au(III) compounds have shown high antitumor activity at *in vitro* conditions, indicating different mechanisms take place for Au(III) compounds' antitumor activity compared to cisplatin [9]. Ga(III) has shown antitumor activity against several cancer types and it shows antitumor activity and apoptosis mainly via iron deprivation [10]. On the other hand, it was also reported that, Ga(III) shows low toxicity on many tumor cell types and does not generally induce apoptosis at therapeutic conditions, whereas it shows apoptosis when concentrations of Ga(III) reaches especially high values [10]. Although separate studies of gold and gallium for their antitumor activity are numerous in the literature, studies on comparative antitumor activity of these

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metals are rare, so further studies are needed to discriminate between these two metals for their antiproliferative activity.

In this paper, the interactions of Au(III) and Ga(III) ions with HSA were examined by several spectroscopic and thermal analysis techniques, and differential antitumor activity of these two metal ions on a model tumor cell line were determined. We describe here the effects of Au(III) and Ga(III) bindings on HSA and their antiproliferative activity on tumor cells comparatively, and anticipate that our results will enhance further studies on the antineoplastic properties of Au(III) and Ga(III) ions.

Materials and methods

Materials and sample preparation

All chemical reagents and HSA in lyophilized powder form were purchased from Sigma–Aldrich (USA). Au(III) chloride hydrate, Ga(III) nitrate hydrate and HSA solutions were prepared in a 10 mM Tris (tris(hydroxymethyl)aminomethane)–HCl buffer (pH 7.40) to ensure that the pH of the solutions are stable in all experiments. Powdery HSA stock was stored at +4 °C. Fresh HSA solutions were prepared prior to each experiment.

UV–vis absorbance spectroscopy

HSA solutions were prepared in 10 mM Tris–HCl buffer. Different concentrations (10, 40, 80, 160, 320 μ M) of Au(III) chloride hydrate or Ga(III) nitrate hydrate solutions were mixed with 10 μ M HSA solutions and then incubated for 2 h. After 2 h incubation, UV–vis absorbance spectra of the samples were read by a NanoDrop 2000 Spectrophotometer (Thermo-Scientific, USA). The wavelength range was 220–420 nm.

Fluorescence spectroscopy

HSA solutions were prepared in 10 mM Tris–HCl buffer with a final concentration of 10 μ M. Different concentrations (10, 20, 40, 80, 160, 320, 640, 1280 μ M) of Au(III) chloride hydrate and Ga(III) nitrate hydrate solutions were mixed with HSA solutions and fluorescence readings were taken after 10 min of incubation in an unilluminated room. Excitation wavelength was 290 nm and emission wavelength range was 300–450 nm. Reading was conducted with a SpectraMax M5 Microplate Reader (Molecular Devices, USA) in a quartz cuvette with 1 cm path length.

Isothermal titration calorimetry (ITC)

Calorimetric analysis of metal–HSA bindings was carried out at 25 °C on an iTC₂₀₀ microcalorimeter (Microcal®). The sample cell was filled with 200 μ M of HSA and 4 mM of Au(III) chloride hydrate or Ga(III) nitrate hydrate solutions were injected to the reaction cell. The reaction cell was stirred continuously at 300 rpm to avoid protein foaming. Origin 7.0 software was used for data acquisition and manipulation. ΔG (Gibbs free energy) calculations were made by the following formula [11]:

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad (1)$$

where T is the absolute temperature in Kelvin (298 K) and $R = 8.3151 \text{ J mol}^{-1} \text{ K}^{-1}$.

FT-IR spectroscopy

HSA solutions with a concentration of 250 μ M, and Au(III) chloride hydrate or Ga(III) nitrate hydrate solutions at varying concentrations (100 and 200 μ M at final) were prepared for FT-IR

measurements. HSA and metal solutions were mixed at different ratios and incubated for 2 h. After 2 h incubation, 20 μ L of final mixes were dropped and dried on a 96-well plate at 37 °C for 1 h. After drying, the 96-well plate was utilized in FT-IR transmittance analysis by using Nicolet 6700 FT-Raman Spectrometer (Thermo-Scientific, USA). FT-IR measurements, curve-fitting analysis and basic modifications (e.g., baseline and background corrections) were done by OMNIC™ software. Background corrections for H₂O and CO₂ were carried out for each analysis. Duplicate samples were utilized in each analysis and experiments were repeated for at least two times.

Protein secondary structure analyses were made by using OMNIC™ software. Second derivative analysis of the amide I region (1600–1700 cm^{-1}) was performed to examine how amide I region was affected by exposure to different concentrations of metal solutions. Curve fitting analysis of the amide I region was performed to estimate approximate ratios of the three main subgroups of protein secondary structures with respect to the total protein content, which are adopted at the regions of: 1630 cm^{-1} for β -sheet, 1655 cm^{-1} and 1661 cm^{-1} for α -helix, and 1678 cm^{-1} for β -turns. All tests were done in duplicate.

Cell viability assay

MCF-7 cells (American Type Culture Collection) were cultured in 75 cm^2 culture flasks using Dulbecco's Modified Medium (DMEM; Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco) at 37 °C and 5% CO₂. The viability of them were determined via Alamar blue assay. In 96 well-plate, cells were seeded at the density of 1×10^4 cells per well and cultured at standard conditions prior to metal salt solutions treatment. Varying concentrations of sterilized metal salt solutions (0–1000 μ M) prepared in DMEM were added to the harvested cells, and the cells were then incubated for 24 h at 37 °C and 5% CO₂. Prior to the end of 24 h incubation period (after 21 h incubation under dark conditions), old medium was replaced with a serum free fresh culture medium containing 10% Alamar blue (AbD Serotec, USA). Fluorescence value of each group was measured (Ex = 530 nm, Em = 590 nm) with a M5 Microplate Reader (Molecular Devices, USA). Fluorescence value of each group was divided by the fluorescence value of untreated control and presented as a percentage of the control. Results were analyzed for statistical significance using ANOVA and Tukey tests. Changes were considered significant at $p < 0.05$. Each experiment was performed in quadruplicate ($n = 4$).

Results and discussion

UV–vis absorbance spectra

Absorption peaks of HSA were observed at 280 nm (Fig. 1). While presence of Au(III) at increasing concentrations causes changes in the actual spectrum of HSA with an increase in the total intensity of absorbance at 280 nm, Ga(III) did not exhibit a considerable difference on the HSA spectra in general. This result may indicate stronger binding characteristics and higher binding affinity for Au(III), since Au(III) has a higher impact on the spectra of HSA, probably by significantly altering the actual geometry of this protein. While the actual spectrum of HSA is highly altered at increasing concentrations of Au(III), the total intensity of absorption spectra increased gradually with the increase in Au(III) content. Such an increase in the absorption spectra may indicate the numbers of aromatic acid residues of HSA which are extended into aqueous environment are increased gradually [12]. This outcome may occur due to the exposure of a large hydrophobic pocket (i.e., IIA subdomain of HSA), which contains a tryptophan (Trp-214), to

Fig. 1. UV-vis absorbance spectra of (A) Au(III)-HSA and (B) Ga(III)-HSA interactions for varying metal ion concentrations (0, 10, 40, 80, 160, 320 μM), within 260–300 nm.

Fig. 2. Fluorescence spectra of (A) Au(III)-HSA and (B) Ga(III)-HSA interactions for varying metal ion concentrations (0, 10, 40, 80, 160, 320, 640, 1280 μM), within 300–450 nm.

the surrounding aqueous environment [12,13]. Therefore, UV-vis absorbance spectra revealed that the chemical structure of HSA seems to be more altered by the addition of Au(III), rather than Ga(III).

Fluorescence emission spectra

Fluorescence emission peaks of HSA were observed at 345 nm (Fig. 2). The main three fluorophores of HSA protein are known as tryptophan, tyrosine and phenylalanine [14]. The fluorescence of HSA is mostly triggered by the presence of tryptophan, because phenylalanine has a very low quantum yield and the fluorescence that comes from tyrosine is drastically quenched by the presence of an amino group, a carboxyl group or a tryptophan near to the tyrosine [15]. As shown in Fig. 2, the presence of both metal salts caused a quenching in the fluorescence spectrum of HSA along with a spectral shift toward shorter wavelengths. The blue-shift in some of the emission spectra can be attributed to the electron density shift from the benzene ring to the pyrrole ring upon excitation [16], and it suggests metal ions bind to serum protein through electrostatic interactions [17]. Finally, Au(III) is inferred to be better than Ga(III) in quenching the fluorescence, which may also suggest a stronger interaction existing between Au(III) and HSA.

Thermal analysis

ITC final thermograms revealed that, both Au(III)-HSA and Ga(III)-HSA reactions are exothermic, and Au(III)-HSA interactions are much more favorable than Ga(III)-HSA interactions in room temperature (Fig. 3, Table 1). K_a values revealed that, Au(III)-HSA binding has a much higher affinity than Ga(III)-HSA binding,

and this finding is in agreement with the UV-vis absorbance spectroscopy and fluorescence spectroscopy results. If we compare ΔH and ΔS values of these samples, Au(III)-HSA binding is enthalpically more favorable but entropically less favorable than Ga(III)-HSA binding. Both reactions were conducted under constant pressure and temperature conditions. ΔG values of Au(III)-HSA and Ga(III)-HSA bindings were negative, hence these reactions are exergonic and are likely to occur spontaneously through forward direction at ambient conditions. Although the homogeneity of both reactions evades the accurate prediction of reaction spontaneities, Au(III)-HSA binding seems to have a higher spontaneity over Ga(III)-HSA binding [18]. Furthermore, since ΔH , ΔS and ΔG values were all negative in both reactions, the reactions are enthalpy driven and it reveals that non-covalent interactions (e.g., electrostatic, van der Waals) are dominant between Au(III)-HSA and Ga(III)-HSA bindings [19].

In brief, Au(III)-HSA and Ga(III)-HSA bindings are expected to be spontaneous at ambient conditions, Au(III)-HSA binding is much more favorable and much stronger than Ga(III)-HSA binding, and our UV-vis spectroscopy and fluorescence spectroscopy results are consistent with the thermal analysis results.

FT-IR spectra

FT-IR spectroscopy was performed on HSA samples to determine protein secondary structure alterations due to Au(III) or Ga(III) binding with HSA. Curve fitting analysis was performed for amide I region to show how protein secondary structures were altered at high concentrations of Au(III) and Ga(III) ions. Four defined regions to represent three main groups of protein secondary structures (α -helix, β -sheets and β -turns) were chosen for curve fitting analysis

Table 1

Thermodynamic parameters obtained by ITC analysis for the binding of HSA with Au(III) or Ga(III) ions.

Thermodynamic parameters	Au(III)	Ga(III)
K_a (binding affinity) (M^{-1})	$3.87 \times 10^5 \pm 4.9 \times 10^4$	$9.68 \times 10^3 \pm 5.4 \times 10^2$
ΔH (enthalpy change) (cal/mol)	$-1.911 \times 10^4 \pm 255$	$-1.061 \times 10^4 \pm 231$
ΔS (entropy change) (cal/mol $^\circ$)	-38.5	-17.3
ΔG (Gibbs free energy change) (kJ/mol)	-31.88	-22.74

Fig. 3. ITC final thermograms of (A) Au(III)–HSA and (B) Ga(III)–HSA bindings.

at amide I region, and the percentage differences of these groups with respect to different concentrations of metal ions were determined. Fig. 4 shows a representative spectrum of the amide I region following the application of curve fitting analysis. As demonstrated in Table 2, α -helix content of HSA decreased for both metal ions in a concentration dependent manner (from 55% to 50% for Au(III), from 55% to 54% for Ga(III)), and the decrease in Au(III) samples is higher than the decrease in Ga(III) samples. When comparing the percentile changes for the groups of protein secondary structures at increasing concentrations of metals, while both β -sheets and β -turns contents increased in Au(III) samples, only β -turns content increased gradually for Ga(III) samples.

In conclusion, it was observed that both metal ions bind with HSA and alters its secondary structure at high concentrations, but Au(III)–HSA binding has much more influence on the protein's chemical structure than Ga(III)–HSA binding.

Antitumor activities of Au(III) and Ga(III) ions on MCF-7 cell line

The potent antitumor activity of Au(III) and Ga(III) ions were tested against human breast cancer (MCF-7) cells *in vitro* by Alamar

Fig. 4. Representative spectrum of the amide I region as a result of curve fitting analysis, showing the distribution of protein secondary structures that are analyzed in this study.

Table 2

Curve fitting analysis results of 250 μ M HSA incubated with Au(III) or Ga(III) ions at different concentrations. The wavenumber values at each row represent where the maximal peak values for the defined group occur. The percentage values at each row represent the means of area under curve values for the defined group with respect to the total surface area at amide I region. The area-percentage values are the averages of duplicate samples (means $\pm$ SEM).

	β -Sheets	α -Helix	β -Turns
HSA only	1595 cm^{-1} 24.98% $\pm$ 0.73	1633–1657 cm^{-1} 54.96% $\pm$ 0.9	1682 cm^{-1} 20.05% $\pm$ 1.63
100 μ M Au(III)	1599 cm^{-1} 27.61% $\pm$ 0.62	1635–1659 cm^{-1} 50.57% $\pm$ 0.47	1685 cm^{-1} 21.81% $\pm$ 0.15
200 μ M Au(III)	1598 cm^{-1} 27.22% $\pm$ 0.58	1633–1658 cm^{-1} 49.62% $\pm$ 3.53	1683 cm^{-1} 23.15% $\pm$ 0.58
100 μ M Ga(III)	1601 cm^{-1} 24.51% $\pm$ 0.19	1634–1658 cm^{-1} 54.19% $\pm$ 1.58	1684 cm^{-1} 21.29% $\pm$ 0.05
200 μ M Ga(III)	1601 cm^{-1} 24.67% $\pm$ 0.31	1635–1658 cm^{-1} 53.81% $\pm$ 0.44	1683 cm^{-1} 21.47% $\pm$ 0.3

Fig. 5. *In vitro* cytotoxicity results of MCF-7 cells after incubating with different concentrations of Au(III) and Ga(III) ions. The cells were incubated for 24 h.

blue assay. Cells were exposed to different concentrations of these ions for 24 h. As shown in Fig. 5, cytotoxicity profiles of Au(III) and Ga(III) ions are very different. Although Au(III) has shown antitumor activity starting with concentrations of 250 µM in a concentration dependent manner, none of the tested concentrations of Ga(III) exhibited significant antiproliferative activity against MCF-7 cells.

Conclusions

In the present study, *in vitro* characterization of Au(III)–HSA and Ga(III)–HSA interactions were made by several spectroscopic and thermal analysis methods, and cell viability assay was performed to determine differential antitumor activity of these two ions on a model tumor cell line. In brief, our findings suggest that Au(III) has a better HSA binding profile and it has a better antiproliferative activity compared to Ga(III). This report highlights the importance of *in vitro* characterizations of Au(III)–HSA and Ga(III)–HSA bindings, elucidates the HSA binding properties of Au(III) and Ga(III) ions, and discriminates the antitumor activity differences of these two ions on a model tumor cell line. For Au(III)–HSA and Ga(III)–HSA interactions *in vivo* conditions, further studies should be done to determine the true potential of Au(III)–HSA and Ga(III)–HSA complexes for antineoplastic purposes.

Conflict of interest statement

The authors declare no conflict of interest.

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