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Characterization of 2,3,6,7,10,11-hexahydroxytriphenylene and its effects on cell viability in human cancer cell lines

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Abstract

We synthesized 2,3,6,7,10,11-hexahydroxytriphenylene (HHTP); characterized it by electrochemistry, spectroelectrochemistry and EPR techniques; and evaluated its cytotoxicity to human cancer cell lines. The results revealed that HHTP has accessible higher-oxidation states, especially the tris-semiquinone monoradical. This species is stable and is formed after being stored for months. HHTP exhibited cytotoxic effects on five human cancer cell lines, including glioma and lung cancer cells. The cytotoxic effect was evaluated based on the decrease in cell viability, increases in the percentage of cells with fragmented DNA, and elevated numbers of Annexin V/PI-positive cells after HHTP treatment.

Keywords: semiquinone, hexahydroxytriphenylene, cytotoxicity, cell viability

1. Introduction

The molecule 2,3,6,7,10,11-hexahydroxytriphenylene (HHTP) has a planar and fully conjugated tris-catechol structure. It differs from catechol and ordinary polyphenols because of the fused aromatic rings that stabilize excited species, and in its greater hydrophobicity. Catechol is an important molecule, with many applications and significant toxic effects on living organisms.(Schweigert *et al.* 2001) Catechols are intermediaries in the degradation of aromatic compounds, and although they are omnipresent in nature, they are toxic to some organisms. The mode of action of catechols in cells is not fully understood, but two main properties are involved(Schweigert *et al.* 2001): the capacity to bind to metal ions, forming stable complexes; and the ability to oxidize, forming the semiquinone radical and *o*-benzoquinone. Both properties are responsible for causing irreparable damage to DNA, protein and membranes.(Schweigert *et al.* 2001)

The conjugated electron structure of the planar HHTP also confers interesting electrochemical and spectroscopic properties, which have been little investigated. Ward and co-workers (Barthram *et al.* 1998; Barthram *et al.* 2000; Grange *et al.* 2010) investigated inorganic complexes of HHTP with ruthenium and osmium, and their electrochemical and spectroscopic properties. The trinuclear ruthenium complex is stable in the tris-semiquinone oxidation state, and can exhibit up to six redox processes. A ruthenium-complex film on a conductive glass substrate switched between redox states, and has been proposed as a "smart window".(Grange *et al.* 2010)

Research involving HHTP has increased tremendously, especially in the last two years (Xiang *et al.* 2010; Yuan *et al.* 2012; Guo *et al.* 2013; Adamson *et al.* 2014; Giovanelli *et al.* 2014; Liu *et al.* 2014; Medina *et al.* 2014); most studies have explored the ability of HHTP to form highly ordered architectures. Hmadeh *et al.* (Hmadeh *et al.* 2012) exploited the coordination properties of HHTP to form extended structures with Co(II) and Ni(II), creating porous crystals.

The planar and conjugated structure of triphenylene allows the formation of π - π stacks where molecules assemble as columns, resulting in columnar mesophases. Because of the π - π stacking characteristics, HHTP derivatives have also been intensively investigated as self-assembled thin films, especially in Langmuir-Blodgett films (Karthaus *et al.* 1992; Tsukruk *et al.* 1993; Henderson *et al.* 1997; Vaes *et al.* 1998) and liquid crystal phases (Beattie *et al.* 1992; Manickam *et al.* 2001; Kumar 2004). The columnar stacking properties of triphenylenes were exploited by Ogoshi *et al.* (Ogoshi *et al.* 2009) to generate star-shaped and tadpole-shaped structures, which were used as templates for synthesis of wire-assembled gold nanostructures. Côté *et al.* (Côté *et al.* 2005) exploited the reactivity of the peripheral groups of a triphenylene derivative, reacting HHTP with diboronic acid to obtain covalent organic frameworks (COF).

Kocyigit *et al.* (Kocyigit *et al.* 2010) used HHTP to prepare a Schiff base complexed with metal ions, and suggested that the resulting compounds are highly toxic, but did not mention the possible intrinsic toxicity related to the polyphenol group and polyaromatic nature of HHTP. In the present study, we evaluated the cytotoxicity of free HHTP by determining its effect on cell viability in five human tumor cell lines. Some experiments were compared to its precursor 2,3,6,7,10,11-hexamethoxytriphenylene

(HMTP) to clarify the contribution of the fused aromatic ring in the absence of the phenol group. For better understanding of the chemical and redox properties that could be involved in the cytotoxic effects of HHTP, the results of electrochemistry, UV-vis spectroelectrochemistry, and EPR analyses are shown.

2. Materials and methods

All the reagents and solvents were of analytical grade. Anhydrous FeCl_3 was obtained as described in the literature (Manickam *et al.* 2001). 1,2-Dimethoxybenzene (Veratrol) and BBr_3 were purchased from Aldrich and used as received. The solvents were purchased from commercial sources (Sigma-Aldrich and Vetec) and used as received.

2.1 Synthesis

Precursor 2,3,6,7,10,11-hexamethoxytriphenylene (HMTP): In a 500-mL round-bottom flask, 231 mmol (37.5 g) anhydrous FeCl_3 was dissolved in 188 mL dichloromethane and 6 mL trifluoroacetic acid. Under stirring, 90 mmol (12.5 g) veratrol (1,2-dimethoxybenzene) dissolved in 63 mL dichloromethane was slowly added dropwise. The mixture was stirred for 24 h. The resulting deep-blue solid was separated by filtration, washed with dichloromethane, and dried in a desiccator under vacuum over calcium chloride. The isolated solid was suspended in 65 mL methanol and stirred for 10 min while gas emerged and the color of the solid changed to green. The solid was filtered and washed with methanol and water. Then, the solid was dissolved in 65 mL dichloromethane, and 2.5 g

silica was added to the mixture, which was heated and refluxed for 1 h. The suspension was filtered and dried in a desiccator over calcium chloride, and kept in the dark for 4 days. After that, the HMTP was extracted in a soxhlet with 125 mL dichloromethane for 24 h. The dichloromethane solution was rotoevaporated to dryness, and the solid was suspended in 20 mL methanol and kept in a freezer (-16 °C) for 4 days. Finally, the crystalline solid was filtered and dried in a desiccator over calcium chloride. This procedure furnished 751 mg of the product (6.1%). ¹H-NMR: (200 MHz, 30 °C, CDCl₃, TMS) δ (ppm): 4.11 (18 H) and 7.70 (6H). IR ν_{max} (cm⁻¹): 2985, 2935, 2825, 1620, 1519, 1463, 1417, 1263, 1207, 1157, 1047, 833, 777, 624, 538.

2,3,6,7,10,11-hexahydroxytriphenylene (HHTP): 1.47 mmol (600 mg) of 2,3,6,7,10,11-hexamethoxytriphenylene was suspended in 30 mL dichloromethane maintained at -80 °C under continuous stirring. Then, 0.01 mol (2.57 g) of BBr₃ dissolved in 24 mL anhydrous dichloromethane was slowly added dropwise. This mixture was stirred overnight while the temperature naturally rose to room temperature. After that, 10 mL water was added to the mixture and the dichloromethane was removed by rotoevaporation. The solid formed in this process was filtered and recrystallized in water. This procedure furnished 225 mg of the product (47%). ¹H-NMR: (200 MHz, 30 °C, DMSO-d₆, TMS) δ (ppm): 7.63 (0.99 H) and 9.35 (1.00 H). IR ν_{max} (cm⁻¹): 3300, 1616, 1541, 1508, 1456, 1242, 1180, 854 and 578. MS m/z: 323.18 (M⁺ [C₁₈H₁₁O₆]⁺); 646.98 (M₂⁺ [C₃₆H₂₃O₁₂]⁺); 970.73 (M₃⁺ [C₅₄H₃₅O₁₈]⁺). CHN: C₁₈H₁₂O₆.H₂O experimental (calculated): %C: 62.74 (63.16), %H: 4.03 (4.12), and %N: 0.55 (0.0).

2.2. Apparatus and measurements

UV-vis spectra were obtained from dimethylformamide (DMF) solutions placed in quartz cuvettes, using a Shimadzu UV 2450 spectrometer in the transmission mode in the range of 190 to 1100 nm. The Bruker Vertex 70 spectrophotometer was used to acquire FTIR spectra in the range of 4000 to 400 cm^{-1} . Spectra of the compounds embedded in KBr pellets were acquired in the transmission mode. The electrochemical experiments were performed on an Ivium Compactstat potentiostat/galvanostat in a three-electrode cell; the Ag/Ag^+ (0.01 mol L^{-1} in CH_3CN) reference electrode was used for experiments conducted with 0.1 mol L^{-1} TBAClO_4 electrolyte solution in DMF. Squarewave voltammetry experiments were conducted with the following parameters: potential step = 0.5 mV, amplitude = 40 mV, and frequency 40 or 80 Hz. UV-vis spectroelectrochemistry experiments were conducted using the Ivium Compactstat potentiostat/galvanostat coupled with the Agilent 8453 spectrophotometer, and an ALS SEC-C Thin Layer Quartz Glass Spectroelectrochemical cell kit with a Pt gauze working electrode. EPR spectra were obtained from solid samples in a Bruker E500 Spectrometer in the x-band region (9.5 GHz) at room temperature and at 77 K. For EPR experiments, HHTP was analyzed in three forms: freshly prepared, aged for more than 8 months, and an oxidized sample. The oxidized sample of HHTP was generated by the reaction of HHTP with a stoichiometric amount (1:1) of H_2O_2 (30% solution). After stirring for 5 min, the solid was filtered and dried under vacuum. The Thermo Fisher Scientific Inc. LTQ XL Linear Ion Trap Mass Spectrometer was used to record the ESI mass spectra of the compounds at a concentration of 10^{-2} mg mL^{-1} in dichloromethane. The Bruker DPX 200 spectrometer operating at 4.7 Tesla was used to acquire the ^1H -NMR (200.13 MHz) spectra of the compounds in CDCl_3 and $\text{DMSO}-d_6$; the signals were assigned to the TMS reference at 303 K.

2.3 Cell culture conditions

We used freshly prepared HHTP samples in all biological-activity experiments. The different tumor cell lines were kindly provided by Prof. Mari C. Sogayar of the Chemistry Institute, University of São Paulo, Brazil. These cells were cultured in DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco) and 50 $\mu\text{g mL}^{-1}$ gentamicin (Sigma-Aldrich). The cells were maintained at 37 °C in a 5% CO₂ atmosphere. The HHTP or HMTP compounds were dissolved in DMSO, and cisplatin (cisDDP) was dissolved in 0.9% NaCl solution. For cell treatments, all compounds were further diluted in DMEM medium at 5.0, 10 and 25 $\mu\text{mol L}^{-1}$. Cultures were incubated for 48 h. Control conditions consisted of cells maintained in culture medium plus each vehicle (equivalent to 10 $\mu\text{mol L}^{-1}$ treatment).

2.4 Cell viability analysis

Cell viability was evaluated through the crystal violet staining assay, as described by Kueng *et al.* (Kueng *et al.* 1989). Briefly, cells were seeded in 96-well culture plates and incubated for adhesion during 24 h. Then, the normal medium was replaced by medium containing the above-mentioned concentrations of HHTP, HMTP or cisplatin, and incubated for 48 h. After treatment, the medium was removed, and the cells were washed with phosphate saline buffer (PBS) solution and fixed with 100% methanol during 10 min. The cells were immersed in a solution of 0.2% crystal violet in 2% ethanol for 3 min, and then washed with PBS until the excess crystal violet was cleared. Then, an elution step was performed with 0.05 mol L^{-1} sodium citrate diluted in 50% ethanol, during 10 min. The absorbance was determined in a microplate reader (Infinite 200, Tecan Group) at 540 nm. The results were expressed as a percentage of viable cells compared to the control (100%).

2.5 Determination of hypodiploid DNA content

DNA fragmentation was evaluated through the SubG1 analysis using propidium iodide, as described by Riccardi and Nicoletti (Riccardi and Nicoletti 2006). Human glioma cells (4×10^4 cells/plate) were incubated in 12-well plates (24 h, 37 °C). The cells were then treated with $10 \mu\text{mol L}^{-1}$ of HHTP or HMTP for 48 h. After centrifugation, the pellet was washed twice with PBSA and incubated with a cold staining solution of $50 \mu\text{g mL}^{-1}$ propidium iodide, 0.1% Triton X-100, 0.1% sodium citrate, and 0.2 mg mL^{-1} RNase A (30 min, in the dark). The samples were run in a FACSCalibur System, and the DNA histograms obtained were analyzed to measure the proportion of sub-G1 hypodiploid cells, using WinMDI 2.9 software (developed by Joseph Trotter).

2.6 Apoptosis assay

Apoptosis was determined in human glioma cells using the Annexin V-FITC and PI staining. Cells were seeded onto 12-well plates and treated with HHTP ($10 \mu\text{M}$) or equal volume of DMSO (vehicle-control group) for 48 h. Both floating and adherent cells were collected. After centrifugation, the pellet was resuspended in $100 \mu\text{L}$ of binding buffer, and stained with $5 \mu\text{L}$ of Annexin V-FITC and $5 \mu\text{L}$ of PI for 15 min at 25°C in the dark. Samples were diluted in $400 \mu\text{L}$ of binding buffer and analyzed by flow cytometry. For each measurement, at least 10,000 cells were acquired using an Accuri C5[®] cytometer (BD).

3 Results and discussion

The ^1H -NMR spectra of the precursor HMTP and the HHTP were clean, exhibiting two signals corresponding to the aryllic and methylic/hydroxylic hydrogens of the products (Figs. SI01, SI02). The HHTP spectra also showed a water-hydrogen signal that, in addition to the FTIR spectrum and CHN result, suggest the presence of water in the sample. The ESI-MS spectrum (Fig. SI03) showed a more intense peak at (m/z) 323, which corresponds to the deprotonated molecular ion with the formula $[\text{C}_{18}\text{H}_{11}\text{O}_6]^-$, in addition to peaks at 647 and 971. Isolation of the peak at 647 and 20 V cone voltage generated the peak at $m/z = 323$ (Fig. SI04). The peaks at 647 and 971 were attributed to the presence of dimers and trimers, respectively, of HHTP, revealing the tendency of HHTP to aggregate in the gaseous phase due to π - π stacking.

Since catechols have the ability to oxidize, forming the semiquinone radical and *o*-benzoquinone, which cause irreparable damage and lead to cytotoxic effects, we characterized HHTP by electrochemistry, spectroelectrochemistry, and EPR to elucidate its redox properties. The cyclic voltammetry (CV) of HHTP in DMF (Fig. 1A), starting in the positive potential direction, showed three very intense anodic peaks over 0.1 V (0.6 vs SHE); a cathodic peak at -0.8 V, characteristic of an irreversibly adsorbed species; and other low-intensity current peaks. The squarewave voltammetry (SWV) showed the same intense current peaks (Fig. 1B). The -0.8 V cathodic peak was three times more intense than each of the anodic peaks over 0.1 V, indicating that the reduction related to the three-step oxidation occurred in a single step in an adsorbed species. The low-intensity current peaks are probably associated with species generated in chemically-electrochemically coupled reactions in the presence of water in the electrolyte solution. The cathodic peak behavior of an adsorbed species is very similar to the description by Barthram *et al.* (Barthram *et al.*

1998). Therefore, the three oxidation processes were attributed to HHTP processes: 1. $\text{CatCatCat} \rightarrow \text{CatCatSq}$; 2. $\text{CatCatSq} \rightarrow \text{CatSqSq}$; and 3. $\text{CatSqSq} \rightarrow \text{SqSqSq}$; (where Cat. = catechol and Sq = semiquinone forms of three residues present in HHTP). In the reduction direction, we postulated the single 3-electron process, $\text{SqSqSq} \rightarrow \text{CatCatCat}$.

Although in the CV experiment we could distinguish three oxidation steps, using UV-vis spectroelectrochemistry we observed a single process, which decreased the π - π^* bands below 300 nm and formed a broad band in the 400 - 500 nm region (Fig. 1C). Because these three oxidation processes are irreversible, chemically-electrochemically coupled reactions occur at the time scale of the measurement. In the experiments, we applied a fixed potential of 1.0 V and the spectra were recorded in sequence, with 1-s intervals. The result indicated that only a single equilibrium occurred, revealed by the isosbestic point at 339 nm (Fig. 1C). We assume that the process observed in the spectroelectrochemical experiment is $\text{CatCatCat} \rightarrow \text{SqSqSq}$. According to Grange *et al.* (Grange *et al.* 2010) the tris-semiquinone SqSqSq form of HHTP is a monoradical. Considering this, the broad band observed in the 400 - 500 nm region is associated with the radical transition.

The freshly prepared HHTP exhibited no signal in the EPR spectrum. The HHTP treated with 1 equivalent stoichiometric amount of hydrogen peroxide led to an EPR active species, which exhibited a signal with $g = 2.00402$, which at room temperature had a peak-to-peak width = 0.56 mT (Fig. SI05), characteristic of an organic free radical. With the hydrogen peroxide added in a 1:1 ratio, HHTP would lead to the CatCatSq form, but considering the spectroelectrochemical result and the oxidation processes occurring at closely similar potential values, we assume that the SqSqSq monoradical was also detected

in the EPR experiment, contributing to the signal observed. The formation of a radical is not exclusive to the catechol residue of HHTP. The methoxylated HMTP also exhibited an EPR signal (Fig. SI06) with $g = 2.00426$ and peak-to-peak width = 0.57 mT at room temperature after treatment with hydrogen peroxide. This shows that the aromatic fused ring stabilizes the oxidized form as a radical and contributes to HHTP reactivity. A very similar EPR signal was obtained by aging the HHTP for months, as dried samples in vials. The aged HHTP also showed a very intense and narrower signal with $g = 2.00234$ and a peak-to-peak width = 0.4 mT (Fig. 1D), which was attributed to the SqSqSq monoradical form of HHTP. As the HHTP is easily converted to the semiquinone form and a radical is highly reactive with biomolecules, the cytotoxicity of HHTP based on its effects on cell viability in human cancer cell lines was investigated.

In order to assess the effects of HHTP on cell viability, we evaluated the effects of HHTP treatment on five human cell lines, including glioma and lung-cancer cells. The cells were exposed to different concentrations of HHTP ($5 - 25 \mu\text{mol L}^{-1}$), and then the cellular viability was determined by crystal violet staining. After treatment for 48 h, a significant decrease in the number of viable cells was observed in all tumor cell lines, compared to the controls (Fig. 2A), reaching more than 35% reduction in A549 and U87MG cells, and around 20-27% reduction in A172, T98G and NCI-H23 cells with the lowest concentration of HHTP ($5 \mu\text{mol L}^{-1}$). The effects of HHTP at the highest concentration evaluated ($25 \mu\text{mol L}^{-1}$) followed the same profile, reaching more than 58% reduction in A549 and U87MG cells, and around 36-47% reduction in A172, T98G and NCI-H23 cells. Similar results were obtained with a manual cell-counting method (Fig. SI06).

All tumor cell lines used in this study grow as adherent cultures, and their usual morphology is polygonal, slightly elongated and sometimes with cytoplasmic filaments. Figure 2B shows the morphological effects of HHTP on U87MG cells; these effects were similar for all tumor cells analyzed. Morphological changes were clearly observed after 48 h of HHTP treatment, since DMSO alone has no effect (Fig. 2B, panel A). The number of cells was clearly reduced and some changes in the cell volume were observed, with cells appearing more flattened (Fig. 2B, panel C and Fig. 2B, panel D). These data were consistent with the results for cell viability.

The cellular cytotoxicity of HHTP was also compared to a standard chemotherapy drug, cisplatin. Cisplatin (cisDDP) is widely used in the treatment of a variety of human malignancies, and has been tested in combination with temozolomide in patients with recurrent glioblastoma.(Brandes *et al.* 2004; Silvani *et al.* 2004) Also, some studies have shown that the use of cisplatin can benefit patients with temozolomide-refractory high-grade malignant glioma.(Zustovich *et al.* 2009) The human glioma cell lines (U87MG, T98G and A172) were treated separately with HHTP and cisplatin at concentrations ranging from 5 to 25 $\mu\text{mol L}^{-1}$ for 48 h. As shown in Figure 3, the in vitro cytotoxicity of HHTP was very similar to that observed for cisplatin at all drug concentrations evaluated.

In addition, we evaluated the proportion of sub-G1 hypodiploid cells, using a flow cytometry analysis. This method can detect cells that have lost some of their DNA in the late stage of apoptosis, following endonuclease activity. Using the nucleic-acid stain propidium iodide (PI), the number of hypodiploid cells undergoing this process can be counted in the sub-G1 region of the PI histogram. The cells were treated with 10 $\mu\text{mol L}^{-1}$ of HHTP for 48 h. As shown in Figure 4, the proportion of sub-G1 hypodiploid cells after

HHTP treatment increased, from 2.6% (control conditions) to approximately 10.8%, in both tumor cell lines.

It is well known that one of the earlier events of apoptosis includes the loss of membrane phospholipid asymmetry, with translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the cell surface. It has been established that PS externalization is regulated by activation of scramblases coupled with inactivation of flippases, through caspase-dependent apoptotic pathways (Balasubramanian *et al.* 2007; Leventis and Grinstein 2010; Suzuki *et al.* 2013; Segawa *et al.* 2014). The presence of PS on the plasma membrane promotes the phagocytic recognition, engulfment and destruction of apoptotic cells (de Cathelineau and Henson 2003; Segawa and Nagata 2015). Therefore, we performed double-staining assays in glioma cell lines using Annexin-V (which has a high affinity for PS) and PI (which stains only membrane-damaged cells). Our results showed that treatment with HHTP significantly reduced the percentage of viable cells (around 20%) and increased the percentage of Annexin V/PI-positive cells compared with control cells (1,2% versus 9,0%), in both tumor cell lines (Fig. 5A and Fig. 5B). These results suggest that HHTP induces cell death by apoptotic mechanisms.

In an additional experiment, the cytotoxic differences between HHTP and its precursor HMTP were compared. The reduction of cytotoxicity of the non-phenol HMTP in comparison to HHTP was evident, since glioma cells treated with 5 $\mu\text{mol L}^{-1}$ HMTP for 48 h showed no significant difference in cell viability compared to the control (Fig. SI08a). However, viability was affected at higher concentrations of HMTP, reaching 35% reduction at the highest concentration of HMTP (25 $\mu\text{mol L}^{-1}$) (Fig. SI08a). Also, the proportion of sub-G1 hypodiploid cells after HMTP treatment was slightly increased (around 3.7%), only at the highest concentration of HMTP used (Fig. SI08b).

Our results revealed that HHTP has a similar effect to other polyphenolic compounds, such as curcumin, apigenin, genistein, (–)epigallocatechin and (–)epigallocatechin-3-gallate, which can induce cell death by apoptosis in glioma and lung-tumor cells and can increase the anti-neoplastic activity of chemotherapeutic drugs. (Zanotto-Filho *et al.* 2015; Das *et al.* 2010; De Oliveira *et al.* 2010; Lu *et al.* 2011) Cell surface exposure of phosphatidylserine is a feature of cells dying through the process of apoptosis, so it is interesting to analyze the time taken for the observed phosphatidylserine translocation induced by HHTP in comparison to other polyphenolic compounds. Here, we showed that HHTP induces increase in the percentage of Annexin V-positive cells (proportion of approximately 9%) in both glioma cells (U87MG and T98G) after 48h of treatment. Zanotto and co-workers showed no significant differences in the Annexin V-positive cell population in U251MG glioma cells after treatment with curcumin for the same period of time (48h), however treatment with curcumin for 120h promotes an increase (approximately 12 %) of Annexin V-stained cells. (Zanotto-Filho *et al.* 2015) In another study, El Bachá and co-workers observed the treatment of glioblastoma GL-15 cells with catechol (1,2 dihydroxybenzene) for 48h leads to increase in the percentage of cells labeled with annexin in around 20 % (De Oliveira *et al.* 2010), but at catechol concentration 60 times higher than we used here for HHTP compound (600 μ M versus 10 μ M). In addition, we observed a relationship between cytotoxic activity and the HHTP properties, especially with respect to the ability of the polyphenol and conjugated aromatic rings to stabilize the oxidized radical form, demonstrating a potential adjuvant use of HHTP for chemotherapy.

4 Conclusions

HHTP exhibited mainly the catechol redox properties, with accessible higher oxidation states, but improved by the presence of fused aromatic rings. In solution, the free HHTP reached the tris-semiquinone monoradical state, as demonstrated by UV-vis spectroelectrochemistry. The chemically oxidized or aged samples of HHTP exhibited a typical radical EPR signal. The radical form of HHTP is probably responsible for its high cytotoxicity. Different tumor cell lines, including glioma and lung cells, lost their viability after treatment with HHTP, showing increases in the percentages of hypodiploid and Annexin V/PI-positive cells. The results revealed that free HHTP has an intrinsic cytotoxic effect on human tumor cell lines, and although the detailed mechanisms underlying the cytotoxic effects of HHTP on tumor cells warrant further research, the present results suggest that HHTP may induce cell death, probably by apoptotic pathways.

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Figure captions

Fig. 1. (A) Cyclic voltammogram of HHTP at 20 mV s^{-1} . (B) Squarewave voltammogram in -1.5 to 1.0 V direction and reverse. (C) UV-vis spectroelectrochemical evolution of HHTP solution while a 1.0 V potential was applied. (D) EPR spectrum of aged HHTP sample at 77 K . Inset: Structure of the tris-semiquinone form of HHTP. * corresponds to the internal standard chromium-doped magnesium oxide signal.

Fig. 2. Effects of HHTP on cell viability and morphology. (A) Viability of the tumor cell lines after HHTP treatment. Values represent the mean $\pm$ SD of the percentage of viable cells compared to the control (data represent three independent experiments, each in triplicate). Significant differences were found in the percentage of viable cells at all HHTP concentrations tested (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (B) Morphological assessment of U87MG cells after treatment with HHTP for 48 h . Optical microscopy analysis ($\times 230$). (Panel A) Control - DMSO vehicle; (Panel B) HHTP = $5 \text{ } \mu\text{mol L}^{-1}$; (Panel C) HHTP = $10 \text{ } \mu\text{mol L}^{-1}$; (Panel D) HHTP = $25 \text{ } \mu\text{mol L}^{-1}$.

Fig. 3. Effects of HHTP and cisplatin on cell viability. Viability of the glioma cell lines after HHTP treatment in comparison to cisplatin treatment. HHTP and cisplatin were used at different concentrations (5 to $25 \text{ } \mu\text{mol L}^{-1}$) for 48 h . (A) U87MG cells; (B) T98G cells; (C) A172 cells. Values represent the mean $\pm$ SD of the percentage of viable cells compared

to the controls (data represent three independent experiments, each in triplicate, *** = $p < 0.001$).

Fig. 4. Effects of HHTP on DNA fragmentation. Fragmented DNA content of glioma cells treated with HHTP ($10 \mu\text{mol L}^{-1}$ for 48 h). Data represent three independent experiments, each in triplicate (*** = $p < 0.001$).

Fig. 5. Apoptosis experiment using Annexin-V/PI double-staining method by flow cytometer. Human glioma cells were treated with HHTP ($10 \mu\text{mol L}^{-1}$) or DMSO (vehicle-control group) for 48 h. After that, cells were processed in Ca^{2+} rich buffer with FITC conjugated annexin-V and propidium iodide, and samples were analyzed by flow cytometer. (A-D) Representative scatter plots of PI (y-axis) vs Annexin V (x-axis) in HHTP-treated glioma cells. Viable cells are shown in the lower left quadrant, Annexin V-positive cells are shown in the lower right quadrant, Annexin V/PI-positive cells are shown in the upper right quadrant, and PI-positive cells are shown in the upper left quadrant. (A) Control U87MG cells, (B) U87MG treated with HHTP (C) Control T98G cells (D) T98G treated with HHTP (E) Quantification of apoptotic U87MG cells. (F) Quantification of apoptotic T98G cells; Data represent three independent experiments, each in triplicate (** = $p < 0.01$; *** = $p < 0.001$).

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135x124mm (300 x 300 DPI)

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122x31mm (300 x 300 DPI)

Fig. 4. Effects of HHTP on DNA Fragmentation. Fragmented DNA content of glioma cells treated with HHTP (10 $\mu\text{mol L}^{-1}$ for 48 h). The data represent three independent experiments, each in triplicate (***) = $p < 0.001$).

121x70mm (300 x 300 DPI)

Fig. 5. Apoptosis experiment using Annexin-V/PI double-staining method by flow cytometer. Human glioma cells were treated with HHTP ($10 \mu\text{mol L}^{-1}$) or DMSO (vehicle-control group) for 48 h. After that, cells were processed in Ca^{2+} rich buffer with FITC conjugated annexin-V and propidium iodide, and samples were analyzed by flow cytometer. (A-D) Representative scatter plots of PI (y-axis) vs Annexin V (x-axis) in HHTP-treated glioma cells. Viable cells are shown in the lower left quadrant, Annexin V-positive cells are shown in the lower right quadrant, Annexin V/PI-positive cells are shown in the upper right quadrant, and PI-positive cells are shown in the upper left quadrant. (A) Control U87MG cells, (B) U87MG treated with HHTP (C) Control T98G cells (D) T98G treated with HHTP (E) Quantification of apoptotic U87MG cells. (F) Quantification of apoptotic T98G cells; Data represent three independent experiments, each in triplicate (** = $p < 0.01$; *** = $p < 0.001$).
270x149mm (300 x 300 DPI)