Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 117 (2014) 264–269

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Preliminary investigation of anticancer activity by determining the DNA binding and antioxidant potency of new ferrocene incorporated N,N′,N″-trisubstituted phenylguanidines

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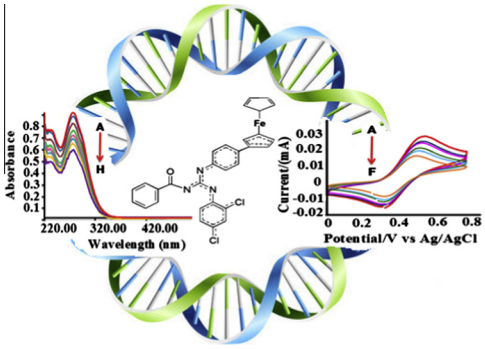
HIGHLIGHTS

- New ferrocenyl phenylguanidines.
- Preliminary investigation of anticancer potency.
- DNA binding studies by UV and CV.
- High binding constant values ($K_b = 10^5$).
- Good free radical scavengers.

GRAPHICAL ABSTRACT

The DNA binding potency of the synthesized guanidines (**1**–**6**) were determined by UV–Vis spectrophotometer and cyclic voltammeter by keeping the concentration of the compounds constant (A) while varying the amount of DNA (B–H). All the compounds showed electrostatic mode of interaction with DNA having impressively high binding constants values (9.9×10^4–1.3×10^5 (M^{-1})). The compounds also showed good antioxidant activities. These two parameters are helpful to design new anticancer drugs.



ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form 30 July 2013
Accepted 2 August 2013
Available online 13 August 2013

Keywords:
Trisubstituted ferrocenyl phenylguanidines
DNA binding
Antioxidant activity

ABSTRACT

Six new bioactive ferrocene based phenylguanidines were successively synthesized and characterized by means of various analytical techniques like elemental analysis, FT-IR, multinuclear (^{1}H and ^{13}C) NMR, UV–Vis spectroscopy and cyclic voltammetry. The interaction of compounds with DNA was investigated by spectroscopic and cyclic voltammetric measurements. The interaction was found to be the electrostatic and the binding constants values were impressively larger. Compounds **f-1**, **f-2**, **f-3** have slight larger binding constant values ranging from 0.8×10^5 to 2.4×10^5 as compared to **g-1**, **g-2** and **g-3** ranging from 7.6×10^4 to 1.1×10^5 which is most probably due to the presence of ferrocene at *para* position where the delocalization of electrons is maximum. Antioxidant activity was determined by UV–Vis spectrophotometer by using DPPH as a free radical. All the compounds exhibit good antioxidant activity and the results so obtained support the structure activity relationship.

Introduction

Guanidines are the organic compounds containing three nitrogens with a central carbon atom and exhibits diverse biological

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http://dx.doi.org/10.1016/j.saa.2013.08.033

applications [1–3]. Nitrogens of the guanidines have the ability to donate electron pair so they can be used as super bases. The guanidines having electron donating groups attached with guanidine moiety have strong basic character due to the more availability of lone pair for donation, so such type of guanidines are less soluble in lipids [4–7]. The introduction of electron withdrawing or conjugated substituents on nitrogen atoms can change the biological properties of the guanidines by making these compound more lipophylic or less basic vice versa. The synthesis of more lipophylic guanidines is a field of interest for so many years because the lipophylic compounds can easily migrate across the cell membrane.

A number of guanidinium compounds have been synthesized which showed strong non-covalent interactions with anionic groups or with DNA through hydrogen bonding and electrostatic interactions due to the involvement of three nitrogens guanidine core thus possesses anticancer activity [8–10]. Scott and his coworkers have studied the biological activities of some variolin and meriolin. Varioline has been found to have modest activities against leukemia cell line. Meriolin (b) displays considerabaly enhanced cyclin-dependent kinase inhibition and cytotoxisity [11]. Some naturally occurring guanidines were screened for their nuclease activity and exhibit cytotoxic properties [12]. Incorporation of ferrocenyl group into organic compounds often enhances the biological action of the resulting products, like ferrocene derivatives of tamoxifen called ferrocifen showed pronounced activity against tamoxifen resistant breast cancer cells [13]. The low cytotoxicity of ferrocene in biological systems, lipophilicity, stability and the π-conjugated system make ferrocene derivatives as decent candidates for investigation of their biological applications [14–17].

Keeping in view, it was thought worthwhile to synthesizing some ferrocene based trisubstituted phenylguanidines having electron withdrawing groups in the form of chlorine, in order to increase the binding potency of the compounds with DNA and make them more free radical scavengers. Before going to expensive cell line study it was decided to preliminary explore their anticancer potency of the compounds by determining the DNA interaction and antioxidant activity, because 'The control over DNA can control different cancerous cell growth' [18] and the free radicals are among the major causes of cancer [19].

Experimental

Materials and apparatus

Ferrocene, 3-nitroaniline, 4-nitroaniline, sodium nitrate, hexadecyltrimetyl ammonium bromide, palladium on charcoal and hydrazine, benzoic acid, thionyl chloride, NH_4SCN, 2,4-dichloroaniline, 3,4-dichloroaniline, 3,5-dichloroaniline and mercury (II) chloride were obtained from Fluka, Switzerland. Analytical grade solvents like diethyl ether, triethylamine, dimethylformamide (DMF), acetone, ethanol and dimethylsulfoxide (DMSO) were purchased from Merck, Germany and used as supplied. The elemental analyses were performed on a LECO-183 CHNS analyzer. The melting temperature was determined on a Bio Cote SMP 10-UK and reported uncorrected. The solid state Fourier transform infrared spectrum was recorded on Bio-Rad Excalibur FT-IR Model FTS 3000 MX. ^1H and ^{13}C NMR spectra were obtained on a Bruker 300 MHz NMR spectrophotometer in $CDCl_3$ using tetra methyl silane as an internal reference. Cyclic voltammetric measurements were performed using Biologsp 300 Potentiostate. Tetrabutylammoniumperchlorate (TBAP) having 99% purity, supplied by Fluka was used as an electrolyte. Measurements were carried out in a single compartment cell with a three electrode configuration, consisting of Ag/AgCl as a reference electrode, a thin platinum wire of thickness 0.5 mm with an exposed end of 10 mm as the counter electrode and a platinum disc as working electrode. UV-Absorption spectra were measured with a UV–Vis spectrometer; Shimadzu 1800. Herring sperm DNA were purchased from Acros Organics UK.

Procedure for the synthesis of compounds (1–6)

The syntheses of the ferrocenyl phenylguanidines were achieved in four steps (Scheme-**1** suppl). In the first step; nitrophenylferrocene (**a** and **b**) were made by the coupling of ferrocene with diazonium salts of nitroaniline using phase transfer catalyst [20]. In the second step; these nitro phenyl ferrocene were reduced into ferrocenylaniline (**c** and **d**) using palladium on charcoal and hydrazine as reducing agent [21]. In the third step three different chloro-substituted thioureas (**e**) were synthesized by the coupling of substituted aniline with thiocynates in acetone [22]. In the fourth step; the benzoylphenylthiourea (**e**) were mixed with the ferrocenyl aniline (**c** and **d**) in dimethyl formamide (DMF) in equimolar ratio with two equivalents of triethylamine (Et_3N). The temperature was maintained below 5 °C using an ice bath and one equivalent of mercuric chloride ($HgCl_2$) was added to the reaction mixture with vigorous stirring. The ice bath was removed after 30 min while the stirring continued overnight. The progress of the reaction was monitored by thin layer chromatography (TLC) till the completion of reaction. Chloroform ($CHCl_3$, 20 ml) was added to the reaction mixture and the suspension was filtered through a sintered glass funnel to remove the mercuric sulfide (HgS) residue. The solvents from filtrate were evaporated under reduced pressure and residue was re-dissolved in dichloromethane (CH_2Cl_2, 20 ml), washed with water (4 × 30 ml) and dried the organic phase over anhydrous magnesium sulfate (MgSO4). The solvent was evaporated and residue was purified by column chromatography to afford ferrocenyl phenylguanidines (**f** and **g**) [23]. Solid and liquid state characterization data is given in Supplemental part.

Results and discussion

Elemental analysis of the compounds (**1**–**6**) was in good agreement with the calculated values which shows that the compounds are sufficiently pure in bulk. In the FT-IR spectra two peaks, a sharp and a weak, were observed for N—H bonds in the range 3366–3266 cm^{-1}. The C=N stretching frequency in all compounds were observed in the range of 1589–1555 cm^{-1} which is an intermediate between single and double bond, indicating conjugation between all three nitrogen atoms of the guanidine moiety. A sharp C=O stretch was also observed in the range of 1683–1667 $cm^{-1}$and a characteristic peak for Fe—C associated with ferrocene group was observed in the range of 479–468 cm^{-1} for the synthesized compounds.

^1H and ^{13}C NMR spectra of the compounds were recorded relative to TMS as reference standard. All the NMR spectra were taken in $CDCl_3$ at room temperature. In ^1H NMR, the ferrocenyl protons appeared in the range of 4.1–4.8 ppm as three set of signals (singlet, triplet, triplet) with relative integral values of (5:2:2) protons [24]. N—H protons in all compounds appeared as broad singlet in range 10.4–11.6 ppm. All the other aromatic and non-aromatic protons appeared in their specific regions. In ^{13}C NMR spectra, the most down field signals appeared in the range of 176–180 ppm are attributed to the most deshielded carbon of the carbonyl group where as the guanidine carbon (CN_3) appeared relatively up-field in the range of 156–161 ppm. In all the compounds (**1**–**6**), ferrocenyl carbon appeared at 65–84 ppm as four signals with the relative intensities 5:2:2:1 of carbons. All the other aromatic and non-aromatic carbon appeared in their specific regions.

In UV–Vis spectroscopy all the compounds gave four bands, three strong bands in the wavelength range of 200–285 nm while a weak signal in visible region at about 450 nm (Fig. 1). The high absorptivity bands in the UV-region of spectrum can be assigned to the π–π^* transition of aromatic phenyl ring and C=N chromophore. In literature it has been reported that the UV–Vis spectra of ferrocene derivatives give two absorption bands originating from ferrocene moiety. The following three spin-allowed ligand field transitions are expected: $^1A_{1g} \rightarrow a^1E_{1g}$, $^1A_{1g} \rightarrow {}^1E_{2g}$, and $^1A_{1g} \rightarrow b^1E_{1g}$. The first two transitions are unresolved and give rise to the band at about 450 nm and the third transition is responsible for the signal at 325 nm. Both bands are weak owing to the Laporte-forbidden *d–d* character of ligand field transitions [25]. So the weak bands in the test compounds may be assigned to the ferrocene based *d–d* transitions.

The electrochemical properties of the presented compounds were investigated by CV on a glassy carbon electrode in 20% aqueous ethanol, with supporting electrolyte 0.1 M TBAP, in the concentration of 40 μM vs. standard Ag/AgCl, in the cathodic direction from 0.0 V to +0.80 V at the scan rate of 100 mV s^{-1}. Ferrocene moiety is well known (for its derivatives) to undergo easily one-electron oxidation to the ferrocenium ion in a reversible manner. The anodic peaks of compounds appeared in the range of 0.551–0.585 V with corresponding cathodic peaks ranging 0.365–0.399 respectively. For simple ferrocene, oxidation peak was observed at 0.518 V under the same conditions. The result reveals that the electrochemical behavior of the oxidizing moiety of ferrocene can be modulated by changing the electronic properties of the cyclopentadienyl ring. The slight change in the redox behavior of the studied compounds than pure ferrocene is attributed to the electron donating effect of NH in the presence of electronegative chlorine atoms. This group makes the oxidation slightly difficult than ferrocene is evidenced by a positive shift for compounds. In general, the inductive effect works up to three or four bonds, however, the minor change in the position of redox peak due to chloro group suggests that the three nitrogens are in conjugation with each other.

DNA binding studies

By UV–Vis spectrophotometer

Stock solutions of the compounds were prepared in 30% aqueous ethanol. DNA stock solution was also prepared. The concentration of DNA was determined spectrophotometrically at 260 nm using molar extinction coefficient $\varepsilon = 6600\ M^{-1}\ cm^{-1}$ [26]. The purity of DNA (free from bound protein) was assessed from the ratio of absorbance at 260 and 280 nm. The A260/A280 ratio was 1.80–1.90, showing that the DNA was sufficiently free from protein. At first the spectra of the compound before the addition of DNA were recorded by taking solvent in the reference cell and solution of the compound in the sample cell. Then the spectra were recorded by adding different concentration of DNA in solutions having constant concentration of the compound. The whole experiment was carried out by keeping the volume and concentration of the compound constant while varying the amount of DNA.

Peaks **1** and **4** were not much affected by the addition of DNA, just a decrease in the absorption take place. Peak **2** showed a slight red shift and **3** exhibited a blue shift. Both the peaks showed pronounced hypochromic effect (Fig. 2) by the incremental addition of DNA. In general a red shift is an indication of intercalation, in which planer groups approaches the nitrogenous bases of DNA and π-stacking takes place between the π electrons of drug and nitrogenous bases of DNA which lead to a decrease in the energy gap between the highest (HUMO) and the lowest (LUMO) molecular orbitals. In present case a very minute bathochromic shift was observed which may be due to partial intercalation or grove binding as classical intercalation lead to a shift of ⩾15 nm.

The hypochromic effect and slight blue shift of peak **3** suggests the electrostatic interaction of its corresponding chromophore with DNA [27]. Thus it was suggested that the compound may interact with DNA by mixed mode of binding (electrostatic and grove binding). In compound **4** the peak **3** also showed a slight red shift so here the grove binding may be dominant over electrostatic interaction (Fig. 3). Further clues about the mode of

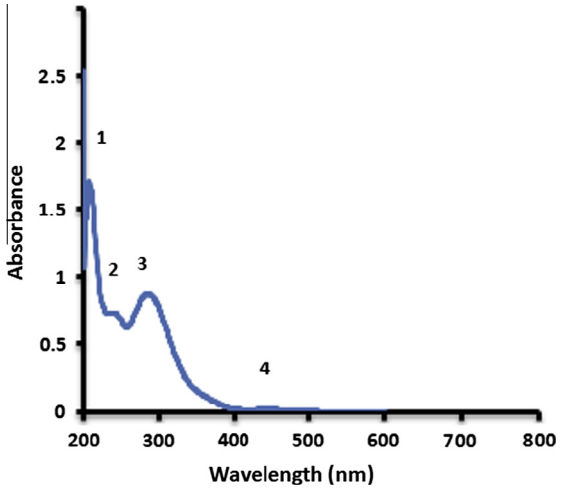


Fig. 1. UV–Vis spectrum of ferroceney phenylguanidine **2**.

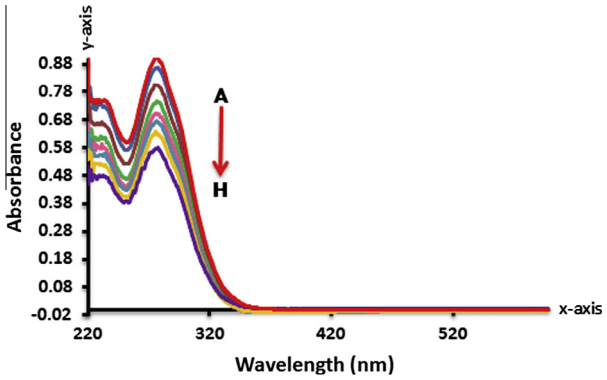


Fig. 2. UV–Vis spectrum of compound **1** in the absence (A) and presence of (B 30; C 40; D 50; E 60; F 70; G 80; H 90 μM) of DNA.

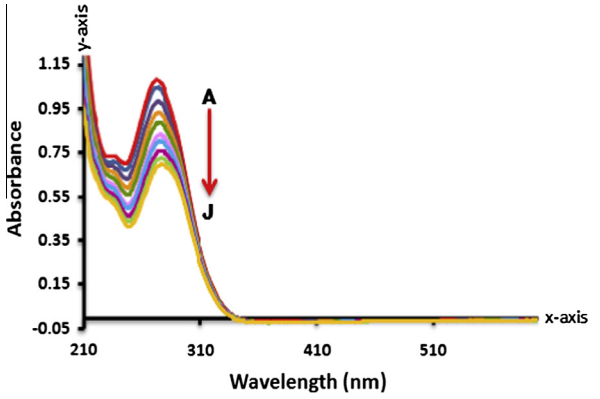


Fig. 3. UV–Vis spectrum of compound **6** in the absence (A) and presence of (B 30; C 40; D 50; E 60; F 70; G 80; H 90; I 100; J 110 μM) of DNA.

Table 1
Binding constant values for ferrocenyl guanidines (**1**–**6**), simple phenylguanidines and ferrocenyl anilines.

Compounds	Binding constant (M^{-1}) (UV)	Binding constant (M^{-1}) (CV)
f-1	1.22×10^5	1.31×10^5
f-2	1.6×10^5	2.4×10^5
f-3	0.8×10^5	1.1×10^5
g-1	9.9×10^4	1.1×10^5
g-2	0.1×10^5	0.84×10^5
g-3	9.9×10^4	7.61×10^4
N,N′-diphenyl-N″-benzoylguanidine (A)	1.6×10^3	5.6×10^3
N-phenyl-N′-(2,4-dichlorophenyl)-N″-benzoylguanidine (B)	7.8×10^3	1.4×10^4
4-Ferrocenyl aniline [24]	2.02×10^3	3.85×10^3
3-Ferrocenyl aniline [30]	9.3×10^3	–

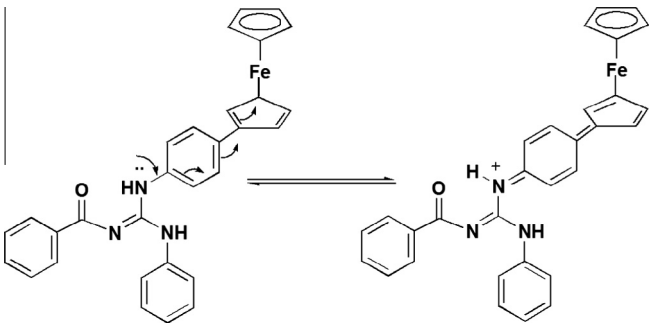
interaction can be obtained from the binding const values and also from the cyclic voltammetric measurements.

Based upon the decrease in absorbance at the λ_{max}, the values of DNA binding constant for these compounds have been calculated according to the following host guest equation I [27].

$$\frac{A_o}{A - A_o} = \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} + \frac{\varepsilon_G}{\varepsilon_{H-G} - \varepsilon_G} \frac{1}{k[DNA]} \quad (1)$$

where A_o and A is the absorbance of the free compound and compound–DNA adduct respectively and, ε_G and $\varepsilon_{H\text{-}G}$ is the molar extinction coefficient of the free compound and compound–DNA adduct respectively. The slope to intercept ratio of the plot $A_o/(A - A_o)$ versus 1/[DNA] yields the binding constants of these compounds and are reported in Table 1.

The binding constant values calculated for the ferrocenyl phenylguanidines were compared with the guanidines themselves having no ferrocene which were found to be lower than the presented compounds. Hence, the presence of ferrocene is concluded to enhance the DNA binding activity of guanidines. This may be due to the fact that in the presence of ferrocene the delocalization of lone pair of nitrogen extended to cyclopentadienyl ring of ferrocene and the nitrogen become more polarized, stable and favorable for electrostatically bind with negatively charged DNA (Scheme 2). The binding constants results showed that the compounds having ferrocene at *para*-position have slight larger binding ability as compared to the *meta*-ferrocenyl phenylguanidines. This may be due to more delocalization of electron when the cyclopentadienyl ring of ferrocene is at *para* position. The binding constant values were also compared with the ferrocenyl anilines (**c** and **d**). High binding constant values revealed that the guanidine core having three nitrogens is more likely to bind electrostatically or through hydrogen bonding with DNA as compared to ferrocenyl anilines having a single NH [28].



Scheme 2. Increase in conjugation due to the presence of ferrocene at *para* position.

By cyclic voltammetry

Stock solutions of the compounds of known concentrations were prepared in 30% aqueous dimethyl sulfoxide (DMSO). The voltammogram of the compounds solution was recorded after flushing out oxygen by purging with argon gas for 10 min just prior to each experiment. The procedure was then repeated for the systems with a constant concentration of the tested compound and varying concentration of DNA [29]. The working electrode was cleaned after every electrochemical assay.

The binding potency of the compounds with DNA was also checked on cyclic voltammeter to find further clues about binding mode. By the addition of DNA, the anodic peak is shifted in the negative going direction and I_{pa} is also dropped by 20%. The substantial diminution in peak current is attributed to the formation of the slowly diffusing compound–DNA supramolecular complex due to which concentration of the free compound, mainly responsible for the transfer of current is lowered. The mode of compound–DNA interaction can be judged from the variation in peak potential (Figs. 4 and 5).

In general, the positive shift called anodic shift in formal potential is caused by the intercalation of the drug into the double helical structure of DNA, while negative shift is observed for the electrostatic interaction of the cationic drug DNA backbone [29]. The obvious negative peak potential shift in the CV behavior of the compounds by the addition of DNA is attributed to the electrostatic interaction between the compound and the DNA. On the similar lines, the negative peak potential shift called cathodic shift in the CV behavior of all studied guanidines is related to the electrostatic interaction of positively charged ferrocenium state with the negatively charged DNA.

Iron of ferrocene is easier to oxidize in the presence of DNA because its oxidized form is more strongly bound to DNA than its reduced form (neutral form). This can also be explained by equation II [29].

$$E_b^0 - E_f^0 = 0.059 \log \left(\frac{K_{red}}{K_{oxi}} \right) \quad (2)$$

where E_0^f and E_0^b are the formal potentials of the ferrocenyl phenylguanidines couple in the free and bound forms respectively. For a shift of −17 mV caused by the addition of 40 M DNA into 3 mM compound **1**, a ratio of K_{red}/K_{oxi} was calculated as 0.37, which indicates the stronger interaction of the oxidized form of the compound with DNA than the reduced form.

Based upon the decayin peak current of all the studied compounds by the addition of different concentrations of DNA (30–60-μM, B–E) the binding constant values were calculated according to the following equation.

$$\frac{1}{[DNA]} = \frac{K(1 - A)}{1 - (I/I_0)} \quad (3)$$

where K is binding constant, I and I_0 are the peak currents with and without DNA and A is proportionality constant. The plot of 1/[DNA] versus $1/(1 - I/I_0)$ yielded binding constant values (Table 1). For the determination of binding site size the following equation was used.

$$\frac{C_b}{C_f} = K \left\{ \frac{\text{free base pairs}}{s} \right\} \quad (4)$$

where s is the binding site size in terms of base pairs. Measuring the concentration of DNA in terms of base pairs, the concentration of the base pairs can be expressed as.

$$\frac{C_b}{C_f} = K \left\{ \frac{DNA}{2s} \right\} \quad (5)$$

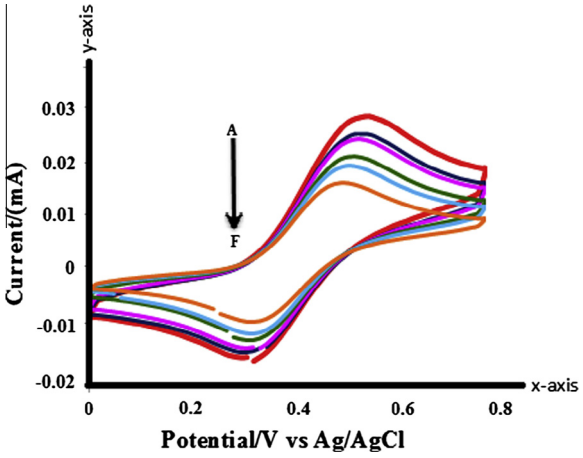


Fig. 4. Voltammogram of 3 mM compound **1** in the absence (**A**) and presence of (**B** 20; C 40; D 60; E 80 μM) of DNA.

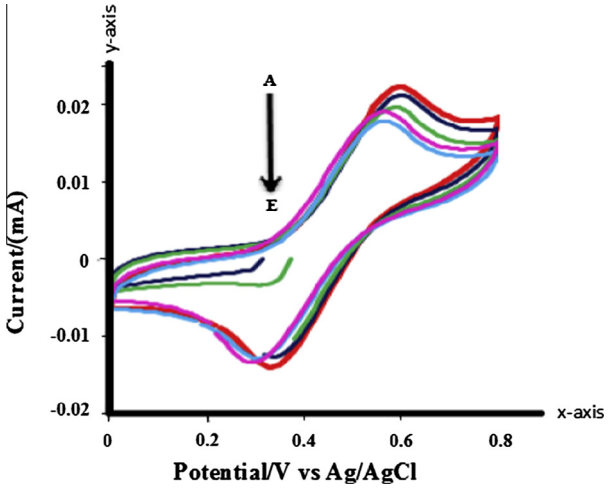


Fig. 5. Voltammogram of 3 mM compound **3** in the absence (A) and presence of (B 20; C 40; D 60; E 80; F 100 μM) of DNA.

C_f and C_b denote the concentration of the free and DNA-bound species respectively. The C_b/C_f ratio was determined by the equation given below.

$$\frac{C_b}{C_f} = \frac{I_0 - I}{I} \quad (6)$$

where I and I_0 represent the peak currents of the drug in the presence and absence of DNA. Putting the value of K as calculated according to the equation III, the binding site size of 0.88 bp was obtained from the plot of C_b/C_f versus [DNA]. The small value of ***s*** indicates a dominant electrostatic interaction of ferrocenyl phenyl guanidine **1** with DNA.

Antioxidant activity

Free radical scavenging activity (antioxidant activity) of the ferrocenyl phenylguanidines (**1**–**6**) was performed by using 167 μM concentrations of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in ethanol [30–32]. The absorbance of DPPH without any additions was stable over 30 min. Ethanol was purified and dried before use according to the standard protocol. Test samples of 3.125, 6.25, 12.5, 25, 50, 100 μg/ml concentrations of the compounds with

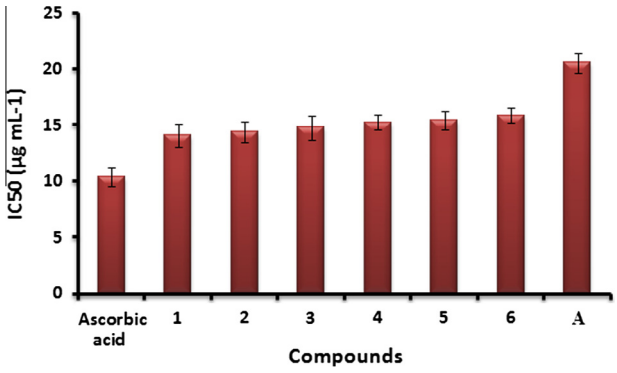
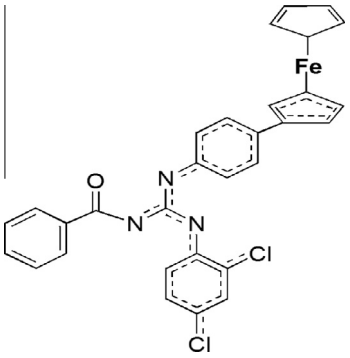


Fig. 6. *In vitro* Antioxidant assay chart of synthesized ferrocenyl phenylguanidines (**1**–**6**) with a comparison to ascorbic acid (standard) and N,N′-diphenyl-N″-benzoylguanidine (**A**).



Scheme 3. Extended resonance due to the presence of ferrocene and electronegative chlorine atoms.

constant DPPH concentration were prepared in ethanol and buffered at pH 6. At first the spectra of the DPPH solution before the addition of compound was recorded by taking solvent in the reference cell and solution of the analyte in the sample cell. Then the spectra were recorded for different concentration of compounds in solutions having constant concentration of the DPPH. The whole experiment was carried out by keeping the volume and concentration of the DPPH constant while varying the amount of compounds concentration. The procedure was repeated by using the ascorbic acid as standard.

Antioxidants had the ability to trap the free radicals. Highly reactive free radicals and oxygen species are being found in biological systems from different sources. These free radicals are responsible for the oxidation of nucleic acids, proteins, lipids or DNA and thus antioxidant inhibits the oxidative mechanisms leading to degenerative diseases. The synthesized ferrocenyl phenylguanidines have been analyzed by the DPPH assay. Antioxidants react with DPPH and can produce 1,1-diphenyl-2-picrylhydrazine. Due to odd electron, DPPH gives a strong absorption band at 517 nm. In the presence of a free radical tested samples and this assay is useful as a primary screening system. The activity of tested compound (**1**–**6**) is reported here as IC_{50} values (Fig. 6). Guanidines are the compound having two NH protons, and having the ability to scavenge the free radical by losing the protons. After trapping the free radical, the compound itself becomes a free radical, which may be stabilized by the extended resonance due to the presence of ferrocene and chloro substituents on phenyl ring or by the formation of a dimer of guanidines. Results revealed that all the compounds have slight high activity as compared to plenylguanidines without ferrocene. This may be due to resonance

stabilization of the resulting free radical on the cyclopentadienyl ring of ferrocene or on the phenyl ring having chlorine atoms (Scheme 3), so the structure activity relation is quite justified that electron withdrawing groups and extended conjugation makes the guanidines resonatically more stable and polarized to electrostatically bind with the DNA.

Conclusion

The work presented in the present paper describes the synthesis of the six new ferrocene incorporated phenylguanidines and their structural characterization by means of different analytical techniques. High binding constant values and good antioxidant activity reveal that the incorporation of ferrocene and electronegative chlorine atoms can enhance the biological activities of guanidines by making the compounds stable and more lipophylic. High binding constant values and good antioxidant activities are those parameters which are required for the design of new anticancer drugs.

Acknowledgements

Rukhsana Gul is grateful to the Department of Chemistry, Quaid-i-Azam University Islamabad and Higher Education Commission (HEC) of Pakistan for their support.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2013.08.033.

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