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Synthesis, characterization and antitumor activity of novel N-substituted α-amino acids containing ferrocenyl pyrazole-moiety

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ABSTRACT

A series of new *N*-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl] α-amino acids were prepared and characterized by a range of spectroscopic techniques and cyclic voltammetry. The in vitro antitumor activity of all synthesized compounds was investigated against cervix adenocarcinoma HeLa, melanoma Fem-x and myelogenous leukemia K562 cell lines using the MTT method. Tryptophan derivative **1l** exhibited the highest cytotoxic activity in the cell growth inhibition of all three types of cell lines.

1. Introduction

The organometallic ferrocenyl compounds are promising candidates for conjugation with biomolecules due to their stability in aqueous media, the possibility of a large variety of derivatives and favorable electrochemical properties [1]. In the light of these facts, investigation in the field of ferrocene chemistry has seen a drastic increase in attention over the past years, particularly in area peptide mimetic models. Conjugations of ferrocene scaffolds with biomolecules such as DNA, carbohydrates, amino acids and peptides are recognized to provide novel compounds by combining the beneficial properties of each component [2–11]. These bioconjugates of ferrocene with amino acids or peptides are of great interest as structural mimetics of natural peptides for protein folding and construction of highly-ordered assemblies.

Ferrocene has been used as a cytotoxic agent in form of its fericenium salts [12]. Some of its derivatives have exhibited antitumor potential [13–17] with greatly enhanced activity when ferrocene compounds were bound to polymers as prodrugs [18,19]. In an another medicinal application of ferrocene compounds, ferrocifen, a ferrocenyl derivative of hydroxytamoxifen was widely prescribed for the treatment of hormone-dependant breast cancer, as well as ferrociphenol which has been recently reported to show a strong antiproliferative effect [20,21]. Moreover, both compounds were found to possess a specific antiproliferative effect on several malignant melanoma cell lines [22]. The low reduction potential of these compounds is responsible for generating reactive oxygenated species under physiological conditions resulting in anticancer activity [21].

The synthesis and structural characterization of ferrocenyl amino acids and peptide bioconjugates bearing a heterocyclic triazole moiety have been recently reported [23]. Herein, we have focused our attention to synthesize ferrocenyl pyrazole α-amino acids having in mind remarkable pharmacological importance of heterocyclic pyrazole unit as a constitutive part of a number of biologically active compounds. Our intentions were based on the synthetic combination of biomolecules with the known pharmacophores in order to avoid toxic or other unwanted side effects and prepare compounds with favorable properties for pharmacological applications. In continuation with our recent studies on ferrocene derivatives [24,25] we also report in this paper spectral characterization, electrochemical studies and test of their inhibitory effect on tumor growth in order to investigate the structure–reactivity relationship of this new antitumor class of compounds.

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2. Results and discussion

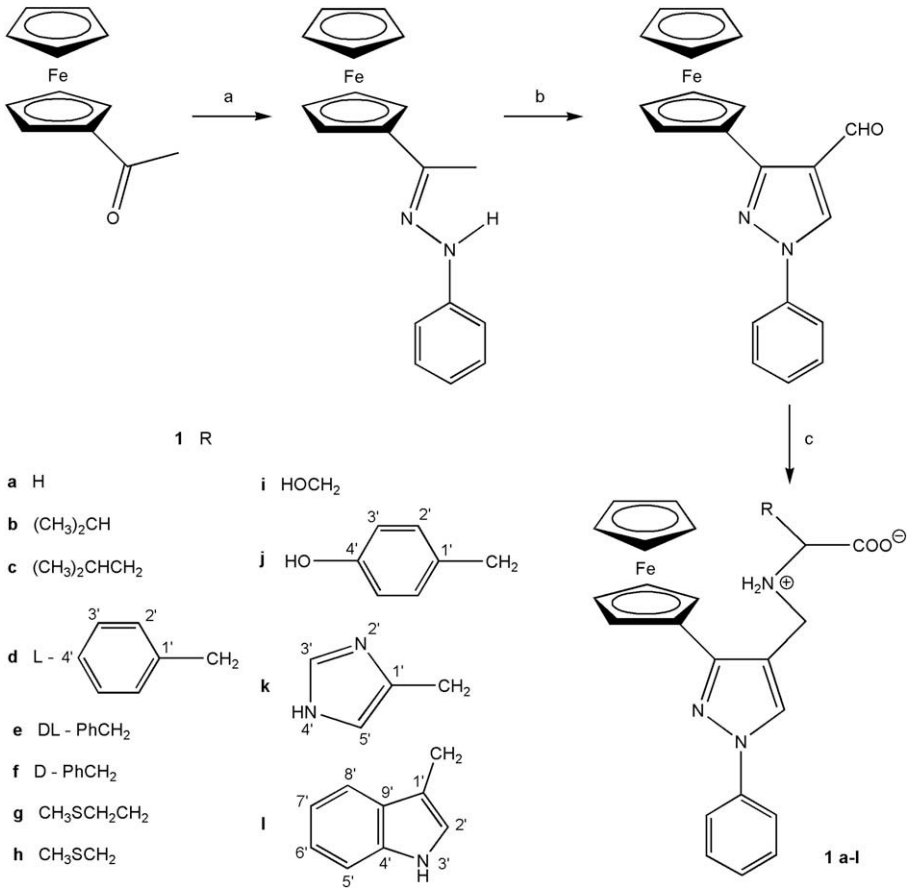
2.1. General synthesis of *N*-[(3-ferrocenyl-1-phenylpyrazol-4-yl)-methyl]α-amino acids

The synthesis of the targeted compounds was performed out according to Scheme 1. Aldehyde precursor 3-ferrocenyl-1-phenylpyrazole-4-carboxaldehyde (FPCA) used for condensation with α-amino acids was prepared from acetylferrocene in a Vilsmeier–Haack formylation by the previously described procedure [28]. In the last step we applied a solventless method [29] combining aldehyde, α-amino acid and NaOH in a porcelain mortar with pestle and aggregating the solid mixture until an orange powder was formed. The condensation was completed by an additional heating to reflux for 2 h in dry methanol. The potential problem with instability of Schiff bases was solved by subsequent reduction with sodium borohydride obtaining desired compounds in satisfactory yields 41–84%. Structures of all new compounds were confirmed by spectral data and elemental analysis.

2.2. IR and NMR spectral studies

The IR spectral region between 2300 and 3600 cm^{-1} is typical for superimposed hydrogen bonded O–H and NH_2^+ stretching bands of multiple fine structure. Weak bands positioned at 2300–2600 cm^{-1} concern to overtones and combinational tones while the group of absorption bands in the region of 2600–2800 cm^{-1} is probably caused by combinational vibrations [30]. The strong bands located between 1612 and 1630 cm^{-1} may be ascribed to largely asymmetric stretchings of the anionic carboxyl group. This band is very characteristic for zwitterionic form of N-substituted α-amino acids since it clearly shows that the carboxyl group is present as the ionized COO^- form. In the IR spectra of all compounds we also find very strong characteristic absorption bands attributed to the pyrazole ring: ν(C=C) and ν(C=N) between 1553 and 1600 cm^{-1} as well as δ(C=C) at around 1505 cm^{-1} [31].

In the ^{1}H NMR spectrum of glycine derivative **1a** two protons of methylene group attached to pyrazole nucleus were present as a singlet at 3.98 ppm. In all other derivatives, as a consequence of the chiral carbon atom of the bonded α-amino acid, these two protons become diastereotopic and give rise to an AB system with a large geminal coupling constant (13.51–14.01 Hz). For the same reason, the presence of chiral carbon center in the α-amino acid generates two chemical nonequivalent diastereotopic sides of the molecule and protons and carbons of the substituted cyclopentadiene ring give more peaks compared to glycine derivative [23]. Even at 500 MHz, not all signals for Cp protons in the ^{1}H NMR spectra are equally well resolved and in the most derivatives (**1c**, **1g**, **1h**, **1j** and **1k**) three complex signals with unresolved couplings and intensities 1:1:2 are observed while phenylalanine and tryptophan derivatives (**1d–f** and **1l**) exhibit four separated signals with 1:1:1:1 intensities. This additional splitting of the ortho and meta ferrocene signals as well as their multiplicities also depend on nature of α-amino acid side chain and cannot be attributed to the chirality of pyrazole-linked α-amino acid alone. The effect of magnetic



Scheme 1. Reagents and conditions: (a) $PhNHNH_2$, EtOH, reflux; (b) DMF, $POCl_3$ (3 equiv.), r.t.; (c) α-amino acid, NaOH, continuous aggregating at rt, then MeOH, reflux, 2 h; $NaBH_4$, 0–5 °C, then rt, 12 h, AcOH.

Table 1
^{1}H NMR chemical shifts of ortho and meta Cp protons.

Compound	*ortho*-H_1	*ortho*-H_2	$\Delta\delta_{ortho}$	*meta*-H_1	*meta*-H_2	$\Delta\delta_{meta}$
1a	4.827	–	4.344	–		
1b	4.910	–	4.321	–		
1c	4.876	4.887	0.011	4.329	–	
1d	4.669	4.707	0.038	4.241	4.263	0.022
1g	4.875	4.885	0.010	4.331	–	
1h	4.849	4.877	0.028	4.334	–	
1i	4.848	–	4.336	–		
1j	4.648	4.701	0.053	4.286	–	
1k	4.731	4.779	0.048	4.295	–	
1l	4.388	4.553	0.165	4.100	4.165	0.065

nonequivalence of both ortho protons of substituted Cp unit is particularly pronounced in compounds containing aromatic or heteroaromatic rings (Table 1). The influence of π-electron system on chemical shift, splitting and multiplicity of ortho and meta Cp protons depends on several factor such as inductive effect, resonance possibility and magnetic anisotropy contributions. Interestingly, there is no appearance of splitting of ortho Cp signals in serine derivative **1i** probably as a consequence of hydrogen bond formation between hydroxyl and carboxylate group causing an alternate conformation with reduced influence of CH_2OH moiety on ferrocene scaffold.

The H atoms of unsubstituted Cp ring are observed as singlets for all compounds. ^{13}C NMR measurements show that the chemical shift of the ortho and meta carbon atoms of substituted and unsubstituted Cp rings exhibit signals which do not vary significantly from one α-amino acid to another. However, five distinct signals in most derivatives indicate magnetically nonequivalent carbon atoms of substituted Cp unit due to presence of chiral center in the α-amino acid moiety. All other signals in ^{1}H and ^{13}C NMR spectra of all compounds are positioned in the expected region of δ-values.

2.3. UV–Vis spectral studies

Electronic spectra of our ferrocene-based compounds were recorded in acetonitrile and methanol in the spectral range 190–1000 nm. Appearance and data recorded in two solvents are, generally, similar. A slight batochromic shift of λ_{max} in MeOH is observed. Due to poor solubility in acetonitrile of most compounds, methanol appeared to be favorable solvent for the spectral recordings.

Since first papers on ferrocene and its spectral characteristics [32,33], numerous other report that ferrocenyl-based compounds absorb (in the absence of a stronger chromofore), generally, as ferrocene alone – in 3–4 main bands. To test the system, especially in view of the observation that the UV–Vis spectra of ferrocene were influenced by the present impurities, external agents etc. [34], we run the spectra of this commercially available compound in both solvents. The bands in AN/MeOH at 199/205 nm, 320/323 nm and 438/441 nm are fairly consistent with the literature data [32,34]. The first band is assigned to $\pi \rightarrow \pi^*$, the other two lie in the region where $d \rightarrow \pi^*$ and d–d interactions are expected [34,35].

Spectra of our compounds (**1a–l**) follow, in general, the pattern of the ferrocene spectrum. The band at ~200 nm is $\pi \rightarrow \pi^*$ ferrocene-based, the second at ~320–360 nm which is about 10 times stronger than for pure ferrocene is MLCT, and the weakest one, which is alike to the corresponding ferrocene band, is probably also d–d origin. But, in these spectra two new close and strong bands appear in the range of ~260–295 nm. Obviously, these bands may belong to other chromofores, such as pyrazole-based moieties. In fact, $\pi \rightarrow \pi^*$ transitions of moderate intensity for pyrazole are reported to be in MeOH at 208 nm [36] and for a number of substituted pyrazole ligands there are even two more transitions, at about 250 and 290 nm [37]. The former high-energy transition in our case might be well overlapped by the stronger ferrocene-based absorption. Besides, the latter two absorptions (~260–295 nm) appear to be the only ones affected by addition of an equivalent amount of NaOH per molecule (**1a–l**) causing deprotonation of the $-NH_2^+-$ group. As a result, the band at ~290 nm is red-shifted for 2–3 nm and increased at the expense of the high-energy one. Thus, it is believed to belong to lower energy $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ pyrazole-moiety transitions which could be affected by the electronic changes in the neighboring atoms.

Finally, it can be concluded that no significant correlation is established between particular spectral data and the structure of **1a–l**. It is not surprising because the changes in substituents exert little effect on absorbing chromofores to which they are not linked by a conjugation system.

2.4. Electrochemistry

Because of low solubility of the investigated compounds in acetonitrile, a common solvent for this type of compounds, our voltammetric experiments were carried out in DMSO and DMF. In the available potential range with 0.1 M TEAP as supporting electrolyte, i.e. −1.9 V to +1.5 V, our compounds display three oxidation processes at potentials >+0.5 V. The first one (~+0.6 V) is well defined oxidation process, the other two at ~+1.2 V and +1.3 V are overlapped multielectron irreversible oxidations followed by chemical reactions of product decomposition. The latter two processes were out of scope of our work.

The main oxidation process (Fig. 1), common for ferrocenyl-based compounds, is one of the most widely studied electrode reactions. It is basically $1 - e^-$ reversible process, with possible kinetic complications depending on the solvent, supporting electrolyte or structural effects of substituents on the ferrocene moiety [38,39]. In this potential range, our compounds, like most others in similar solvents, undergo $1 - e^-$ process accompanied with chemical complications. At Pt electrode, peak separation ΔE_p (i.e. $E_p^a - E_p^c$) varies from 80 mV (20 mV/s) to 140 mV (1 V/s), which is greater than expected for a fully reversible $1 - e^-$ process [40]. Furthermore, the current ratio i_p^a/i_p^c of 1.2–1.4 at low scan rates (<50 mV/s), becoming ~1.1 at higher (⩾0.5 V/s), might be a proof of a slow irreversible chemical reaction coupled to the oxidation process. Leucine (**1c**) and tryptophan (**1l**) derivatives seem to be the most stable in this respect. Namely, as reported for a strong donor system as DMSO, ferrocenyl compounds may be involved in a

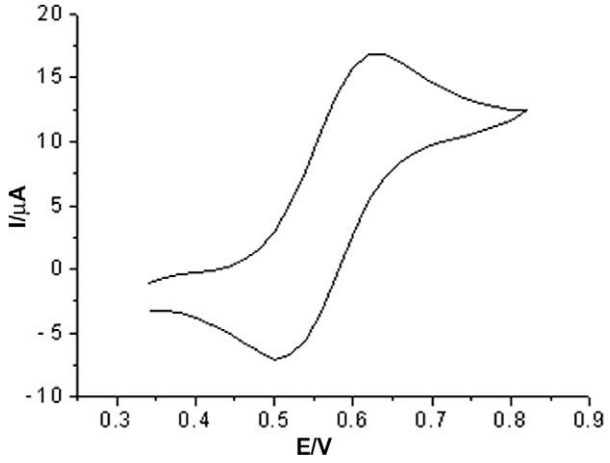


Fig. 1. Cyclic voltammogram for tryptophan derivative (**1l**) in DMSO, 2 mM, Pt, 200 mV/s, air atmosphere.

chemical reaction of a ferricenium cation with solvent molecule to form Fe^{3+} and other decomposition products [39,41]. On the other hand, as reported recently, after this oxidation process carried out in the presence of oxygen a new chemical complication, due to formation of peroxo radical cation is observed, which in turn decomposes rapidly in some polar solvents to form ferrocene in about 50% yield [42].

In order to get a more precise insight into the extent of each chemical complication, the role of oxygen, solvent and the electrode surface, we performed experiments in DMSO and DMF, at Pt and glassy carbon (GC) electrode, and in the presence of air oxygen and in nitrogen atmosphere. The most pronounced chemical effects were observed in air atmosphere for compounds **1d, 1g, 1i** and **1j**, at low scan rates (Fig. 2). Even in these cases, the newly formed peaks at ~+0.2 V did not exceed 15% of the initial i_p^a. As can be seen from Fig. 2, oxygen affects the voltammogram only slightly. In fact, both chemical complications cannot be observed at scan rates higher than ~500 mV/s. In addition, DMF seems to be less "aggressive solvent", since the newly formed peak current resulting from both kinds of chemical complications did not exceed ~5% of the initial i_p^a at most. In DMF only the formed Fe^{3+} ions can be stabilized into $[FeCl_4]^-$ (reduced at ~+0.1 V) by adding of excess LiCl [41], the peak of this complex reaches about 30% of initial i_p^a.

Other characteristics of this oxidation process, such as current function variations: $i_p^a/v^{1/2}$ and $i_p^a/c \cdot v^{1/2}$ in the applied range of v (20 mV/s–2 V/s) and c (0.5–4.0 mM) are consistent with the electrode process coupled to chemical complications [40]. Finally, the obtained values of $E_{1/2}$ (taken as $(E_p^a + E_p^c)/2$) (reported for Pt in DMSO) varied in a narrow potential range from $E_{1/2} = 0.537$ V (**1d**) to $E_{1/2} = 0.570$ V (**1l**). These potentials could be correlated to electronic transitions at λ_{max} = ~440–450 nm in the UV–Vis spectra of our compounds. In both cases only slight variation of the main process characteristics due to the changes in substituents of ferrocenyl–pyrazolyl-moiety are observed. This is in agreement with a number of reports concerning ferrocenylaminoacids and oligopeptides, where even greater changes than in our compounds did not interfere much with the main process based on ferrocenyl moiety reaction [43–46].

In DMF, the appearance of a new small oxidation peak at potentials ~+0.4 V (close to E_p of unsubstituted ferrocene) at scan rates 100 and 200 mV/s may be a proof that reaction pathway of chemical decomposition leads through ferrocenyl moiety to Fe^{3+} as the final product. Peak potentials and peak current characteristics in DMF are generally similar to those in DMSO, only potentials are about 15 mV more positive. Finally, from a comparison of Pt and GC electrode kinetic data in DMF, a ΔE_p criterion, it appears that the processes at GC are much faster than at Pt (at $v = 500$ mV/s, where the effects of a follow-up chemical reaction were apparently eliminated, we registered ~95 mV at GC vs. ~135 mV at Pt electrode). Besides, Pt surface was more prone to adsorption, therefore, GC electrode appeared to be favorable for these measurements.

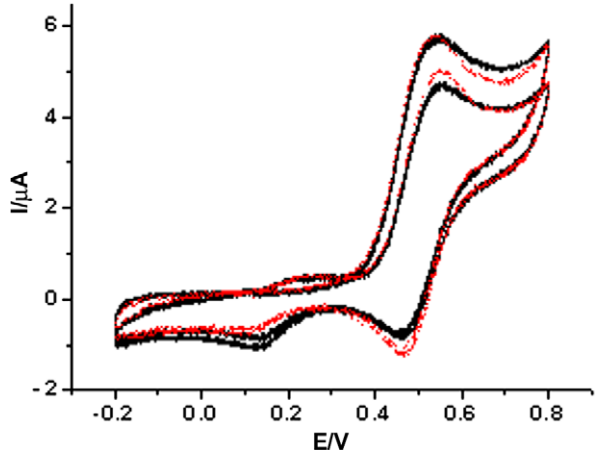


Fig. 2. Cyclic voltammograms for tyrosine derivative (**1j**) in DMSO, 1 mM, GC, 50 mV/s. in nitrogen (- - -) and air (—) atmosphere.

2.5. Biological studies

The in vitro antitumor activities of all synthesized compounds were evaluated by MTT tetrazolium assay dye against cervix adenocarcinoma HeLa, melanoma Fem-x and myelogenous leukemia K562 cell lines. Table 2 represents the results of cytotoxic activity, while Figs. 3 and 4 depict the cytotoxic curves from MTT assay showing the survival of HeLa and K562 cell grown for 72 h in the presence of increasing concentrations of D-phenylalanine (**1f**), methionine (**1g**), histidine (**1k**) and tryptophan (**1l**) derivatives.

Ferrocene alone shows no antitumor activity, but introduction of *N*-phenyl pyrazole unit resulted in a product with powerful cytotoxic properties. A modification of aldehyde precursor (Scheme 1) with different amino acids produced compounds with altered cytotoxic activity compared to the starting material. As shown in Table 2 the malignant cell line K562 was very sensitive while the most active compounds were methionine, histidine and tryptophan derivatives **1g**, **1k** and **1l**, respectively, having a better cytotoxic potential in comparison to the starting precursor 3-ferrocenyl-1-phenylpyrazole-4-carboxaldehyde (FPCA).

It has been previously published that the increase in the lipophilic character on ferrocene results in better cytotoxic activity

Table 2
IC_{50} (μg/ml) for the 72 h of action of investigated compounds and cisplatin on the HeLa, Fem-x and K562 cells determined by MTT test.

Compound	HeLa	FemX	K562
	IC_{50} (μg/ml)		
1a	46.71 ± 5.94	41.42 ± 2.91	41.01 ± 1.56
1b	48.52 ± 1.18	67.04 ± 4.92	61.24 ± 11.37
1c	25.13 ± 7.33	17.36 ± 4.62	11.78 ± 4.63
1d	10.69 ± 5.00	19.14 ± 1.16	7.29 ± 2.86
1e	11.51 ± 2.93	16.82 ± 2.31	6.92 ± 2.04
1f	10.92 ± 2.38	16.67 ± 1.43	7.78 ± 2.84
1g	21.71 ± 2.88	12.66 ± 3.59	6.29 ± 1.15
1h	31.80 ± 4.56	22.16 ± 2.11	11.64 ± 5.92
1i	24.91 ± 1.04	16.37 ± 5.69	12.67 ± 2.07
1j	20.95 ± 2.16	19.88 ± 2.08	8.13 ± 2.65
1k	10.08 ± 1.30	16.76 ± 4.43	6.16 ± 0.43
1l	7.95 ± 1.42	9.78 ± 0.27	6.34 ± 1.24
Ferrocene	>100	>100	>100
FPCA	15.68 ± 0.03	10.11 ± 0.27	7.91 ± 0.43
Cisplatin	2.10 ± 0.20	4.70 ± 0.20	5.90 ± 0.20

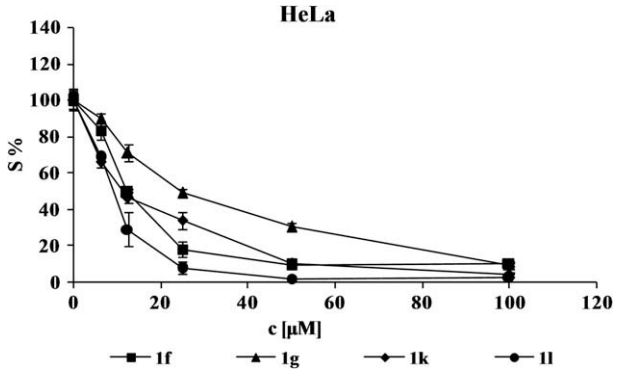


Fig. 3. Representative graphs showing survival of HeLa cell grown for 72 h in the presence of increasing concentrations of **1f**, **1g**, **1k** and **1l**.

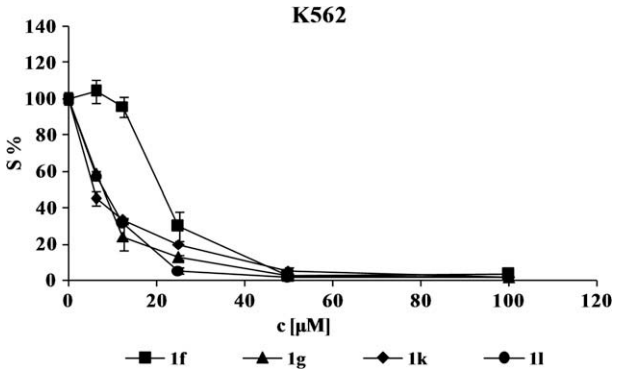


Fig. 4. Representative graphs showing survival of K562 cell grown for 72 h in the presence of increasing concentrations of **1f**, **1g**, **1k** and **1l**.

[47]. In our experiments, opposite to these conclusions, we found that serine and tyrosine derivatives **1i** and **1j** having polar hydroxyl groups proved to be less active than compounds bearing aromatic and especially heteroaromatic rings suggesting that lipophilic character could be diminished in comparison with planar aromatic requirements of amino acid part of molecule. Some interesting findings were reported for a series of *N*-(ferrocenyl) benzoyl dipeptide esters demonstrating the lowering of cytotoxic activity in dependence on increasing of amino acid alkyl chain length [48]. However, all these data come from different cancer cell lines making the difficulty in accordance of their IC_{50} values with our results. The decrease in cytotoxic activity in **1b** and **1h** in comparison with **1c** and **1g**, respectively, could be related to the size and steric effects of the bulky isopropyl group (**1b**) and voluminous sulfur atom (**1h**) closely to aminocarboxylate moiety.

Cytotoxic data for compounds derived from both enantiomers of phenylalanine including the racemic one indicated that there was no significant difference in their activity against all three tested types of cancer cell lines. These results confirmed the important role of the nature of amino acid side chain and steric hindrance for the growth suppressing potential against K562 cell lines. Histidine and tryptophan derivatives **1k** and **1l** appeared to be the most potent in inhibition of all three kinds of cancer cell lines but the activity of **1l** on Fem-x malignant cells was almost identical to FPCA. All other compounds were moderately sensitive towards HeLa and Fem-x cell.

3. Conclusion

Twelve novel *N*-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl] α-amino acids, including both phenylalanine enantiomer derivatives were prepared using standard synthetic protocols. The structures of all compounds were determined by use of spectroscopic, electrochemical and analytical methods. Compounds containing heteroaromatic rings appeared to be the most active against myelogenous leukemia K562 cell lines with a better cytotoxic potential than starting FPCA precursor indicating a pronounced influence of the nature of amino acid moiety and steric effects on cytotoxic activity.

4. Experimental

4.1. Physical measurements

Melting points were determined on a Mel-Temp capillary melting points apparatus, model 1001 and are uncorrected. Optical rotations were measured on a Rudolph Research Analytical automatic polarimeter Autopol IV. Elemental (C, H, N, S) analysis of the samples was carried out by standard micromethods in the Center for Instrumental Analysis, Faculty of Chemistry, Belgrade. Cyclic voltammetric experiments were carried out on an AUTOLAB PST 050 instrument with a Pt-disc and GC-disc (diameter 3 mm) working electrodes, Pt wire counter electrode and a Ag/AgCl reference electrode. All potentials are reported against this electrode. The working electrodes were polished with finest alumina suspension before each set of measurements and when necessary. The measurements were performed in distilled (previously dried on molecular sieves) DMSO and DMF solutions, with 0.1 mol dm^{-3} TEAP as supporting electrolyte. Experiments were carried out either in air or in an inert atmosphere provided by purging nitrogen. Electronic spectra of 1×10^{-4}–1×10^{-3} mol dm^{-3} acetonitrile or methanol solutions of the compounds (**1a**–**l**) were recorded on T 80+ UV/Vis Spectrometer PG Instruments, Ltd., in the spectral range of 190–1000 nm. IR spectra were recorded on a Perkin–Elmer Spectrum One FT-IR spectrometer with a KBr disc. All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer. As a consequence of very poor solubility, all compounds were characterized by NMR in the form of their much better soluble Na-salts by adding finely powdered solid NaOH in NMR tube containing CD_3OD. The full assignments of all reported NMR signals were made by use of 1D and 2D NMR experiments such as HSQC and HMBC heteronuclear correlation techniques.

4.2. General procedure for the preparation of *1a–l*

3-Ferrocenyl-1-phenylpyrazole-4-carboxaldehyde (0.445 g, 1.25 mmol), α-amino acid (1.375 mmol) and NaOH (0.055 g, 1.375 mmol, for **1j** 0.11 g, 2.75 mmol) were milled using a porcelain mortar and pestle to obtain a homogenous orange powder until the release of water was observed. This mixture was transferred into 25 cm^3 of dry methanol and heated to reflux for 2 h. After cooling in an ice bath sodium borohydride (0.057 g, 1.5 mmol) was added in several portions with stirring. The solution was stirred for additional 2 h at room temperature, then diluted with 25 cm^3 of deionized water and left for 12 h, after which time the precipitate had formed by addition of glacial acetic acid. The crude product was collected by filtration, washed with plenty of water and dried over anhydrous $CaCl_2$. The obtained compound was satisfactory pure but very high purity was achieved by its dissolving in methanol solution of 1 equiv, of NaOH followed by precipitation with glacial acetic acid.

4.2.1. *N*-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]glycine monohydrate (*1a*)

Yellow-brown powder; yield: 0.40 g (74%); m.p.: 140–141 °C (Dec.); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 3.34, (s, 2H, CH_2–COO); 3.98, (s, 2H, Pz-CH_2); 4.12, (s, 5H, Fc); 4.34, (t, 2H, *J* = 2.00 Hz, *meta*-Fc,); 4.83, (t, 2H, *J* = 2.00 Hz, *ortho*-Fc); 7.29, (t, 1H, *J* = 7.50 Hz, *p*-phenyl); 7.48, (t, 2H, *J* = 7.50 Hz, *m*-phenyl); 7.75, (d, 2H, *J* = 7.50 Hz, *o*-phenyl); 8.16, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 44.83 (Pz-CH_2); 54.10 (CH_2–COO); 68.58 (2C, *ortho*-Fc); 69.96 (2C, *meta*-Fc); 70.57 (5C, Fc); 79.02 (Fc, ipso); 120.08 (2C, *o*-phenyl); 120.72 (C-4, Pz); 127.40 (*p*-phenyl); 129.23 (C-5, Pz); 130.68 (2C, *m*-phenyl); 141.51 (C-3, Pz); 151.63 (N-subst. phenyl); 179.06 (COO); IR (KBr, cm^{-1}): 3430 ν(O-H); 3084 $\nu(C–H)_{Ar}$; 2926 and 2852 $\nu(C–H)_{Al}$; 2791–2379 $\nu(NH_2^+)$; 1628 $\nu_{as}(COO^-)$; 1599 $\nu(C{=}C)_{Ar}$; 1556 $\nu(C{=}N)_{Ar}$; 1504 $\delta(C{=}C)_{Ar}$; 1464 $\delta(C{=}N)_{Ar}$; 1407 $\nu_s(COO^-)$; 1059 $\delta(C–H)_{ip}$; 757 $\delta(C–H)_{oop}$; 507 and 489 ν_{as}(Cp–Fe––Cp). λ_{max} (MeOH)/nm (log ε/dm^3 mol^{-1} cm^{-1}): 206(4.48), 266(4.18), 286(4.11), ~326sh(4.48), 442(2.69). Anal. Calc. for $C_{22}H_{23}N_3O_3Fe$ (433.28 g/mol): C, 60.99; H, 5.35; N, 9.70. Found: C, 60.78; H, 5.37; N, 9.67%.

*4.2.2. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-valine monohydrate (**1b**)*

Orange-brown powder; yield: 0.50 g (84%); m.p.: 163 °C (Dec.); $[\alpha]_D^{20} = +17.02$ ($c = 1.116 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 1.03, (d, 3H, $J = 6.50$ Hz, CH_3); 1.05, (d, 3H, $J = 6.50$ Hz, CH_3); 1.93, (octet, 1H, $J = 6.50$ Hz, $(CH_3)_2CH$); 2.97, (d, 1H, $J = 6.50$ Hz, CH–COO); $\delta_A = 4.02$ and $\delta_B = 3.80$, (AB system, 2H, $J_{AB} = 13.51$ Hz, Pz-CH_2); 4.11, (s, 5H, Fc); 4.32, (m, 2H, *meta*-Fc); 4.91, (m, 2H, *ortho*-Fc); 7.29, (t, 1H, $J = 7.50$ Hz, *p*-phenyl); 7.48, (t, 2H, $J = 7.50$ Hz, *m*-phenyl); 7.74, (dd, 2H, $J = 7.50$ and 1.00 Hz, *o*-phenyl); 8.13, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 20.02 (CH_3); 20.60 (CH_3); 33.02 ($(CH_3)_2CH$); 44.32 (Pz-CH_2); 68.79 (*ortho*-Fc); 68.81 (*ortho*-Fc); 69.81 (*meta*-Fc); 69.83 (*meta*-Fc); 70.52 (5C, Fc); 71.87 (CH–COO); 79.19 (Fc, ipso); 120.08 (2C, *o*-phenyl); 121.65 (C-4, Pz); 127.28 (*p*-phenyl); 129.51 (C-5, Pz); 130.65 (2C, *m*-phenyl); 141.57 (C-3, Pz); 152.00 (N-subst. phenyl); 182.41 (COO); IR (KBr, cm^{-1}): 3435 ν(O–H); 3093 ν(C–H)$_{Ar}$; 2965 and 2876 ν(C–H)$_{Al}$; 2642–2386 $\nu(NH_2^+)$; 1630 $\nu_{as}(COO^-)$; 1599 ν(C=C)$_{Ar}$; 1570 ν(C=N)$_{Ar}$; 1505 δ(C=C)$_{Ar}$; 1466 δ(C=N)$_{Ar}$; 1403 $\nu_s(COO^-)$; 1061 δ(C–H)$_{ip}$; 757 δ(C–H)$_{oop}$; 507 and 486 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1}): 204(4.57), 267(4.23), 287(4.15), 325(3.45), 443(2.50). Anal. Calc. for $C_{25}H_{29}N_3O_3Fe$ (475.36 g/mol): C, 63.17; H, 6.15; N, 8.84. Found: C, 63.27; H, 6.13; N, 8.83%.

*4.2.3. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-leucine monohydrate (**1c**)*

Orange-brown powder; yield: 0.48 g (78%); m.p.: 149 °C (Dec.); $[\alpha]_D^{20} = +45.45$ ($c = 1.078 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 0.95, (d, 3H, $J = 6.50$ Hz, CH_3); 1.00, (d, 3H, $J = 6.50$ Hz, CH_3); 1.48, (m, 1H, CH–CH_2–CH); 1.58, (m, 1H, CH–CH_2–CH); 1.87, (nonet, 1H, $J = 6.50$ Hz, $(CH_3)_2CH$); 3.28, (t, 1H, $J = 6.50$ Hz, CH–COO); $\delta_A = 4.04$ and $\delta_B = 3.82$, (AB system, 2H, $J_{AB} = 13.51$ Hz, Pz-CH_2); 4.12, (s, 5H, Fc); 4.33, (m, 2H, *meta*-Fc); 4.88, (m, 1H, *ortho*-Fc); 4.89, (m, 1H, *ortho*-Fc); 7.29, (t, 1H, $J = 7.50$ Hz, *p*-phenyl); 7.48, (dd, 2H, $J = 7.50$ and 1.00 Hz, *m*-phenyl); 7.74, (dd, 2H, $J = 7.50$ and 1.00 Hz, *o*-phenyl); 8.15, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 23.45 (CH_3); 23.57 (CH_3); 26.50 ($(CH_3)_2CH$); 43.81 (Pz-CH_2); 44.92 (CH–CH_2–CH); 64.53 (CH–COO); 68.66 (*ortho*-Fc); 68.77 (*ortho*-Fc); 69.85 (*meta*-Fc); 69.91 (*meta*-Fc); 70.55 (5C, Fc); 79.10 (Fc, ipso); 120.08 (2C, *o*-phenyl); 121.28 (C-4, Pz); 127.32 (*p*-phenyl); 129.40 (C-5, Pz); 130.66 (2C, *m*-phenyl); 141.55 (C-3, Pz); 151.82 (N-subst. phenyl); 183.20 (COO); IR (KBr, cm^{-1}): 3435 ν(O–H); 3084 ν(C–H)$_{Ar}$; 2958 and 2869 ν(C–H)$_{Al}$; 2673–2391 $\nu(NH_2^+)$; 1622 $\nu_{as}(COO^-)$; 1600 ν(C=C)$_{Ar}$; 1555 ν(C=N)$_{Ar}$; 1505 δ(C=C)$_{Ar}$; 1466 δ(C=N)$_{Ar}$; 1409 $\nu_s(COO^-)$; 1059 δ(C–H)$_{ip}$; 755 δ(C–H)$_{oop}$; 507 and 486 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1}): 206(4.52), 267(4.20), 285(4.15), 325(3.45), 443(2.48). Anal. Calc. for $C_{26}H_{31}N_3O_3Fe$ (489.39 g/mol): C, 63.81; H, 6.38; N, 8.59. Found: C, 63.80; H, 6.37; N, 8.59%.

*4.2.4. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-phenylalanine monohydrate (**1d**)*

Yellow-orange powder; yield: 0.50 g (76%); m.p.: 156–157 °C (Dec.); $[\alpha]_D^{20} = +13.99$ ($c = 1.072 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 2.82, (dd, 1H, $J = 8.50$ and 13.50 Hz, Ph-CH_2); 3.12, (dd, 1H, $J = 5.50$ and 13.50 Hz, Ph-CH_2); 3.47, (dd, 1H, $J = 5.50$ and 8.50 Hz, CH); $\delta_A = 3.96$ and $\delta_B = 3.77$, (AB system, 2H, $J_{AB} = 14.01$ Hz, Pz-CH_2); 4.05, (s, 5H, Fc); 4.24, (m, 1H, *meta*-Fc); 4.26, (m, 1H, *meta*-Fc); 4.67, (m, 1H, *ortho*-Fc); 4.71, (m, 1H, *ortho*-Fc); 7.19, (t, 1H, $J = 7.50$ Hz, 1H at 4′); 7.27, (m, 3H, 2H at 3′ and *p*-phenyl); 7.34, (dd, 2H, $J = 7.50$ and 1.00 Hz, 2H at 2′); 7.46, (t, 2H, $J = 7.50$ Hz, *m*-phenyl); 7.63, (dd, 2H, $J = 7.50$ and 1.00 Hz, *o*-phenyl); 7.82, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 41.71 (Ph-CH_2); 43.92 (Pz-CH_2); 67.31 (CH–COO); 68.59 (*ortho*-Fc); 68.62 (*ortho*-Fc); 69.80 (*meta*-Fc); 69.85 (*meta*-Fc); 70.50 (5C, Fc); 79.01 (Fc, ipso); 120.09 (2C, *o*-phenyl); 121.34 (C-4, Pz); 127.29 (*p*-phenyl); 127.40 (4′); 129.19 (C-5, Pz); 129.47 (2C, 3′); 130.59 (2C, *m*-phenyl); 130.71 (2C, 2′); 141.08 (1′); 141.45 (C-3, Pz); 151.76 (N-subst. phenyl); 181.92 (COO); IR (KBr, cm^{-1}): 3447 ν(O–H); 3085 ν(C–H)$_{Ar}$; 2926 and 2854 ν(C–H)$_{Al}$; 2731–2389 $\nu(NH_2^+)$; 1621 $\nu_{as}(COO^-)$; 1600 ν(C=C)$_{Ar}$; 1568 ν(C=N)$_{Ar}$; 1504 δ(C=C)$_{Ar}$; 1456 δ(C=N)$_{Ar}$; 1410 $\nu_s(COO^-)$; 1060 δ(C–H)$_{ip}$; 755 δ(C–H)$_{oop}$; 507 and 486 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1}): 206(4.52), 267(4.11), 287(3.94), ~326(3.40), 445(2.44). Anal. Calc. for $C_{29}H_{29}N_3O_3Fe$ (523.40 g/mol): C, 66.55; H, 5.58; N, 8.03. Found: C, 66.43; H, 5.60; N, 8.05%.

*4.2.5. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]DL-phenylalanine monohydrate (**1e**)*

Yield: 0.49 g (75%).

*4.2.6. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]D-phenylalanine monohydrate (**1f**)*

Yield: 0.49 g (75%); $[\alpha]_D^{20} = -14.13$ ($c = 1.054 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v).

*4.2.7. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-methionine monohydrate (**1g**)*

Orange-brown powder; yield: 0.49 g (77%); m.p.: 155–156 °C (Dec.); $[\alpha]_D^{20} = +41.21$ ($c = 1.092 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 1.92, (m, 1H, CH–CH_2); 2.00, (m, 1H, CH–CH_2); 2.10, (s, 3H, CH_3–S); 2.67, (t, 2H, $J = 7.50$ Hz, CH_2–S); 3.34, (t, 1H, $J = 6.50$ Hz, CH–CH_2); $\delta_A = 4.04$ and $\delta_B = 3.84$, (AB system, 2H, $J_{AB} = 13.51$ Hz, Pz-CH_2); 4.12, (s, 5H, Fc); 4.33, (t, 2H, $J = 1.00$ Hz, *meta*-Fc); 4.87, (t, 1H, $J = 1.00$ Hz, *ortho*-Fc); 4.88, (t, 1H, $J = 1.00$ Hz, *ortho*-Fc); 7.29, (t, 1H, $J = 7.50$ Hz, *p*-phenyl); 7.48, (t, 2H, $J = 7.50$ Hz, *m*-phenyl); 7.74, (d, 2H, $J = 7.50$ Hz, *o*-phenyl); 8.16, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 15.44 (CH_3–S); 32.15 (S–CH_2); 35.03 (CH–CH_2); 43.93 (Pz-CH_2); 64.79 (CH–COO); 68.70 (*ortho*-Fc); 68.75 (*ortho*-Fc); 69.87 (*meta*-Fc); 69.90 (*meta*-Fc); 70.54 (5C, Fc); 79.12 (Fc, ipso); 120.09 (2C, *o*-phenyl); 121.25 (C-4, Pz); 127.32 (*p*-phenyl); 129.44 (C-5, Pz); 130.65 (2C, *m*-phenyl); 141.55 (C-3, Pz); 151.83 (N-subst. phenyl); 182.18 (COO); IR (KBr, cm^{-1}): 3436 ν(O–H); 3082 ν(C–H)$_{Ar}$; 2918 and 2853 ν(C–H)$_{Al}$; 2658–2406 $\nu(NH_2^+)$; 1619 $\nu_{as}(COO^-)$; 1600 ν(C=C)$_{Ar}$; 1558 ν(C=N)$_{Ar}$; 1505 δ(C=C)$_{Ar}$; 1464 δ(C=N)$_{Ar}$; 1409 $\nu_s(COO^-)$; 1059 δ(C–H)$_{ip}$; 756 δ(C–H)$_{oop}$; 507 and 486 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon$/dm^3 mol^{-1} cm^{-1}): 207(4.00), 217(4.04), 267(4.03), 288(4.00), 327(3.76), 444(2.53). Anal. Calc. for $C_{25}H_{29}N_3O_3SFe$ (507.43 g/mol): C, 59.17; H, 5.76; N, 8.28; S, 6.32. Found: C, 59.25; H, 5.77; N, 8.30; S, 6.32%.

*4.2.8. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]S-methyl-L-cysteine monohydrate (**1h**)*

Orange powder; yield: 0.46 g (75%); m.p.: 156–157 °C (Dec.); $[\alpha]_D^{20} = +0.98$ ($c = 1.023 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 2.12, (s, 3H, CH_3–S); 2.79, (dd, 1H, $J = 13.50$ and 7.50 Hz, S–CH_2); 2.94, (dd, 1H, $J = 13.50$ and 5.00 Hz, S–CH_2); 3.41, (dd, 1H, $J = 7.50$ and 5.00 Hz, CH); $\delta_A = 4.09$ and $\delta_B = 3.91$, (AB system, 2H, $J_{AB} = 14.01$ Hz, Pz-CH_2); 4.12, (s, 5H, Fc); 4.33, (t, 2H, $J = 1.50$ Hz, *meta*-Fc); 4.85, (m, 1H, *ortho*-Fc); 4.88, (m, 1H, *ortho*-Fc); 7.29, (td, 1H, $J = 7.50$ and 1.00 Hz, *p*-phenyl); 7.48, (dd, 2H, $J = 7.50$ Hz, *m*-phenyl); 7.74, (dd, 2H, $J = 7.50$ and 1.00 Hz, *o*-phenyl); 8.21, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 16.10 (CH_3–S); 39.55 (S–CH_2); 43.76 (Pz-CH_2); 64.05 (CH–COO); 68.65 (*ortho*-Fc); 68.74 (*ortho*-Fc); 69.89

(*meta*-Fc); 69.94 (*meta*-Fc); 70.57 (5C, Fc); 79.07 (Fc, ipso); 120.05 (2C, *o*-phenyl) 121.08 (C-4, Pz); 127.34 (*p*-phenyl); 129.46 (C-5, Pz); 130.67 (2C, *m*-phenyl); 141.54 (C-3, Pz); 151.81 (N-subst. phenyl); 180.59 (COO); IR (KBr, cm^{-1}): 3436 ν(O–H); 3092 ν(C–H)$_{Ar}$; 2921 and 2853 ν(C–H)$_{Al}$; 2746–2385 $\nu(NH_2^+)$; 1628 $\nu_{as}(COO^-)$; 1599 ν(C=C)$_{Ar}$; 1557 ν(C=N)$_{Ar}$; 1505 δ(C=C)$_{Ar}$; 1465 δ(C=N)$_{Ar}$; 1411 $\nu_s(COO^-)$; 1057 δ(C–H)$_{ip}$; 756 δ(C–H)$_{oop}$; 507 and 486 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon/dm^3\,mol^{-1}\,cm^{-1}$): 204(4.57), 267(4.20), ~287(4.11), ~327sh(3.43), 444(2.50). Anal. Calc. for $C_{24}H_{27}N_3O_3SFe$ (493.40 g/mol): C, 58.42; H, 5.52; N, 8.52; S, 6.50. Found: C, 58.35; H, 5.50; N, 8.52; S, 6.49%.

4.2.9. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-serine monohydrate (**1i**)

Yellow powder; yield: 0.40 g (69%); m.p.: 167 °C (Dec.); $[\alpha]_D^{20} = -6.18$ ($c = 1.133 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 3.35, (dd, 1H, J = 6.00 and 4.50 Hz, C*H*–COO); 3.76, (dd, 1H, J = 10.75 and 6.00 Hz, CH–C*H*$_2$); 3.85, (dd, 1H, J = 10.75 and 4.50 Hz, CH–C*H*$_2$); δ_A = 4.09 and δ_B = 3.92, (AB system, 2H, J_{AB} = 14.01 Hz, Pz-C*H*$_2$); 4.12, (s, 5H, Fc); 4.34, (m, 2H, *meta*-Fc); 4.85, (m, 2H, *ortho*-Fc); 7.29, (t, 1H, J = 7.50 Hz, *p*-phenyl); 7.48, (t, 2H, J = 7.50 Hz, *m*-phenyl); 7.75, (d, 2H, J = 7.50 Hz, *o*-phenyl); 8.20, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 43.92 (Pz-*C*H$_2$); 65.12 (CH$_2$–OH); 66.71 (*C*H–COO); 68.57 (*ortho*-Fc); 68.64 (*ortho*-Fc); 69.92 (*meta*-Fc); 69.94 (*meta*-Fc); 70.57 (5C, Fc); 79.08 (Fc, ipso); 120.07 (2C, *o*-phenyl) 121.19 (C-4, Pz); 127.34 (*p*-phenyl); 129.38 (C-5, Pz); 130.67 (2C, *m*-phenyl); 141.56 (C-3, Pz); 151.69 (N-subst. phenyl); 180.07 (COO); IR (KBr, cm^{-1}): 3431 ν(O–H); 3073 ν(C–H)$_{Ar}$; 2961 and 2852 ν(C–H)$_{Al}$; 2703–2462 $\nu(NH_2^+)$; 1628 $\nu_{as}(COO^-)$; 1598 ν(C=C)$_{Ar}$; 1554 ν(C=N)$_{Ar}$; 1504 δ(C=C)$_{Ar}$; 1463 δ(C=N)$_{Ar}$; 1401 $\nu_s(COO^-)$; 1057 δ(C–H)$_{ip}$; 758 δ(C–H)$_{oop}$; 508 and 487 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon/dm^3\,mol^{-1}\,cm^{-1}$): 198(4.08), 267(3.60), 286(3.34), 325(2.90), 446(1.96). Anal. Calc. for $C_{23}H_{25}N_3O_4Fe$ (463.31 g/mol): C, 59.63; H, 5.44; N, 9.07. Found: C, 59.87; H, 5.42; N, 9.10%.

4.2.10. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-tyrosine monohydrate (**1j**)

Orange-brown powder; yield: 0.56 g (83%); m.p.: 168 °C (Dec.); $[\alpha]_D^{20} = +7.97$ ($c = 0.502 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 2.66, (dd, 1H, J = 13.50 and 9.00 Hz, CH–C*H*$_2$); 3.04, (dd, 1H, J = 13.50 and 4.50 Hz, CH–C*H*$_2$); 3.46, (dd, 1H, J = 9.00 and 4.50 Hz, C*H*–COO); δ_A = 3.97 and δ_B = 3.80, (AB system, 2H, J_{AB} = 14.01 Hz, Pz-C*H*$_2$); 4.07, (s, 5H, Fc); 4.29, (m, 2H, *meta*-Fc); 4.65, (m, 1H, *ortho*-Fc); 4.70, (m, 1H, *ortho*-Fc); 6.63, (d, 2H, J = 8.00 Hz, 2H at 2′); 7.03, (d, 2H, J = 8.00 Hz, 2H at 3′); 7.27, (t, 1H, J = 7.50 Hz, *p*-phenyl); 7.49, (t, 2H, J = 7.50 Hz, *m*-phenyl); 7.67, (dd, 2H, J = 7.50 and 1.00 Hz, *o*-phenyl); 7.85, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 40.81 (Ph-*C*H$_2$); 43.89 (Pz-*C*H$_2$); 67.41 (*C*H–COO); 68.56 (2C, *ortho*-Fc); 69.84 (*meta*-Fc); 69.94 (*meta*-Fc); 70.51 (5C, Fc); 78.96 (Fc, ipso); 119.59 (2C, 3′); 120.01 (2C, *o*-phenyl); 121.27 (C-4, Pz); 125.92 (1′); 127.21 (*p*-phenyl); 129.01 (C-5, Pz); 130.67 (2C, *m*-phenyl); 131.14 (2C, 2′); 141.42 (C-3, Pz); 151.67 (N-subst. phenyl); 165.58 (4′); 182.25 (COO); IR (KBr, cm^{-1}): 3430 ν(O–H); 3092 ν(C–H)$_{Ar}$; 2923 and 2853 ν(C–H)$_{Al}$; 2691–2369 $\nu(NH_2^+)$; 1612 $\nu_{as}(COO^-)$; 1599 ν(C=C)$_{Ar}$; 1563 ν(C=N)$_{Ar}$; 1516 and 1505 δ(C=C)$_{Ar}$; 1464 δ(C=N)$_{Ar}$; 1409 $\nu_s(COO^-)$; 1057 δ(C–H)$_{ip}$; 755 δ(C–H)$_{oop}$; 507 and 486 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon/dm^3\,mol^{-1}\,cm^{-1}$): 201(4.80), 224sh(4.46), 268(4.32), 284(4.28), 3.26sh(3.54), 445(2.58). Anal. Calc. for $C_{29}H_{29}N_3O_4Fe$ (539.40 g/mol): C, 64.57; H, 5.42; N, 7.79. Found: C, 64.73; H, 5.40; N, 7.82%.

4.2.11. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-histidine acetate trihydrate (**1k**)

Orange-brown powder; yield: 0.31 g (41%); m.p.: 178 °C (Dec.); $[\alpha]_D^{20} = +4.50$ ($c = 1.111 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 1.90, (s, 3H, C*H*$_3$, acetate); 2.86, (dd, 1H, J = 14.50 and 8.00 Hz, CH–C*H*$_2$); 3.09, (dd, 1H, J = 14.50 and 5.50 Hz, CH–C*H*$_2$); 3.46, (dd, 1H, J = 8.00 and 5.50 Hz, C*H*–COO); δ_A = 4.01 and δ_B = 3.82, (AB system, 2H, J_{AB} = 13.51 Hz, Pz-C*H*$_2$); 4.07, (s, 5H, Fc); 4.29, (m, 2H, *meta*-Fc); 4.73, (m, 1H, *ortho*-Fc); 4.78, (m, 1H, *ortho*-Fc); 6.89, (s, 1H at 5′); 7.28, (t, 1H, J = 7.50 Hz, *p*-phenyl); 7.47, (t, 2H, J = 7.50 Hz, *m*-phenyl); 7.53, (d, 1H, J = 2.00 Hz, 1H at 3′); 7.70, (dd, 2H, J = 7.50 and 1.00 Hz, *o*-phenyl); 8.02, (s, 1H, Pz); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 24.40 (CH$_3$, acetate); 31.96 (CH–*C*H$_2$); 43.84 (Pz-*C*H$_2$); 65.63 (*C*H–COO); 68.61 (2C, *ortho*-Fc); 69.87 (2C, *meta*-Fc); 70.53 (5C, Fc); 79.04 (Fc, ipso); 120.03 (2C, *o*-phenyl) 121.25 (5′ and C-4, Pz, overlapped); 127.30 (*p*-phenyl); 129.34 (C-5, Pz); 130.64 (2C, *m*-phenyl); 134.33 (1′); 136.00 (3′); 141.50 (C-3, Pz); 151.76 (N-subst. phenyl); 180.62 (COO, acetate); 181.67 (COO); IR (KBr, cm^{-1}): 3415 ν(O–H); 3144 ν(N–H)$_{imidazole}$; 3087 ν(C–H)$_{Ar}$; 3009 ν(C–H)$_{imidazole}$; 2924 and 2853 ν(C–H)$_{Al}$; 2791–2465 $\nu(NH_2^+)$; 1701 $\nu(COO^-)_{acetate}$; 1631 $\nu_{as}(COO^-)$; 1599 ν(C=C)$_{Ar}$; 1556 ν(C=N)$_{Ar}$; 1505 δ(C=C)$_{Ar}$; 1463 δ(C=N)$_{Ar}$; 1410 $\nu_s(COO^-)$; $^{1053\ \delta(C-H)}$$_{ip}$; 757 δ(C–H)$_{oop}$; 502 and 485 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon/dm^3\,mol^{-1}\,cm^{-1}$): 208(4.59), 267(4.23), 286(4.23), ~328sh(4.48), 444(2.57). Anal. Calc. for $C_{28}H_{35}N_5O_7Fe$ (609.45 g/mol): C, 55.18; H, 5.79; N, 11.49. Found: C, 55.16; H, 5.78; N, 11.53%.

4.2.12. N-[(3-ferrocenyl-1-phenylpyrazol-4-yl)methyl]L-tryptophan dihydrate (**1l**)

Orange-brown powder; yield: 0.58 g (80%); m.p.: 148–149 °C (Dec.); $[\alpha]_D^{20} = -46.00$ ($c = 1.000 \times 10^{-3}$ g/cm^3, MeOH/CH_2Cl_2, 1/1 v/v); ^{1}H NMR (500 MHz, CD_3OD, Na salt): 2.97, (dd, 1H, J = 14.50 and 9.50 Hz, CH–C*H*$_2$); 3.37, (dd, 1H, J = 14.50 and 4.00 Hz, CH–C*H*$_2$); 3.68, (dd, 1H, J = 9.50 and 4.00 Hz, C*H*–COO); δ_A = 3.93 and δ_B = 3.72, (AB system, 2H, J_{AB} = 14.01 Hz, Pz-C*H*$_2$); 3.97, (s, 5H, Fc); 4.10, (m, 1H, *meta*-Fc); 4.16, (m, 1H, *meta*-Fc); 4.39, (m, 1H, *ortho*-Fc); 4.55, (m, 1H, *ortho*-Fc); 7.02, (t, 1H, J = 7.50 Hz, 1H at 7′); 7.12, (t, 1H, J = 7.50 Hz, 1H at 6′); 7.17, (s, 1H at 2′); 7.25, (t, 1H, J = 7.50 Hz, *p*-phenyl); 7.38, (d, 1H, J = 8.00 Hz, 1H at 8′); 7.42, (t, 2H, J = 7.50 Hz, *m*-phenyl); 7.46, (d, 2H, J = 7.50 Hz, *o*-phenyl); 7.66, (s, 1H, Pz); 7.81, (d, 1H, J = 8.00 Hz, 1H at 5′); ^{13}C NMR (125 MHz, CD_3OD, Na salt): 31.44 (CH–*C*H$_2$); 43.87 (Pz-*C*H$_2$); 65.71 (*C*H–COO); 68.31 (*ortho*-Fc); 68.49 (*ortho*-Fc); 69.70 (*meta*-Fc); 69.84 (*meta*-Fc); 70.44 (5C, Fc); 78.84 (Fc, ipso); 112.60 (8′); 113.23 (1′); 119.93 (7′); 119.97 (2C, *o*-phenyl); 120.04 (5′); 121.10 (C-4, Pz); 122.57 (6′); 124.79 (2′); 127.20 (*p*-phenyl); 128.94 (C-5, Pz); 129.09 (9′); 130.59 (2C, *m*-phenyl); 138.54 (4′); 141.36 (C-3, Pz); 151.68 (N-subst. phenyl); 182.40 (COO); IR (KBr, cm^{-1}): 3420 ν(O–H); 3371 ν(N–H)$_{indole}$; 3081 ν(C–H)$_{Ar}$; 2925 and 2853 ν(C–H)$_{Al}$; 2747–2383 $\nu(NH_2^+)$; 1621 $\nu_{as}(COO^-)$; 1599 ν(C=C)$_{Ar}$; 1570 ν(C=N)$_{Ar}$; 1504 δ(C=C)$_{Ar}$; 1459 δ(C=N)$_{Ar}$; 1411 $\nu_s(COO^-)$; 1060 δ(C–H)$_{ip}$; 749 δ(C–H)$_{oop}$; 507 and 492 ν_{as}(Cp–Fe–Cp). λ_{max} (MeOH)/nm ($\log \varepsilon/dm^3\,mol^{-1}\,cm^{-1}$): 209(4.63), 217(4.62), 270(4.25), 288(4.20), ~327sh(3.43), 444(2.50). Anal. Calc. for $C_{31}H_{32}N_4O_4Fe$ (580.46 g/mol): C, 64.15; H, 5.58; N, 9.67. Found: C, 64.16; H, 5.58; N, 9.67%.

4.3. In vitro studies

4.3.1. Drugs and solutions

The MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved (5 mg/ml) in phosphate buffer saline pH 7.2 and filtered (0.22 μm) before use. The RPMI 1640 cell culture medium, fetal bovine serum (*FBS*), and MTT, were purchased from Sigma Chemical Company, USA.

4.3.2. Cell lines

Cervix adenocarcinoma HeLa and melanoma Fem-x cell lines were maintained in monolayer culture, and myelogenous leukemia K562 cells in suspension culture, in nutrient medium RPMI 1640, with 10% (inactivated at 56 °C) FBS, 3 mM of L-glutamine, and antibiotics.

4.3.3. Treatment of cell lines

Stock solutions (10 mM) of compounds were made in dimethylsulfoxide (DMSO), and were dissolved in corresponding medium to the required working concentrations. Target cells HeLa, (2000 cells per well), Fem-x (2000 cells per well), or K562 cells (3000 cells per well) were seeded into wells of a 96-well flat-bottomed microtitre plate. Twenty hours later, after the cell adherence, 50 μl of the investigated compounds was added to cells in final concentrations (6.25, 12.5, 25, 50, and 100 μM), except in the control wells, where only nutrient medium was added to the cells. Exceptionally compounds were applied to the suspension of leukemia K562 cells 2 hours after the cell seeding. The intensity of agents action on cancer cell survival was determined 72 h later by MTT test [26], modified by Ohno and Abe [27]. Briefly, 20 μl of MTT (5 mg/ml) dye was added to each well. After incubation for further 4 h, 100 μl of 10% SDS were added to extract the insoluble product formazan, resulting from conversion of the MTT dye by viable cells. The number of viable cells in each well is proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 570 nm. To achieve cell survival (%), absorbance at 570 nm of a sample with cells grown in the presence of various concentrations of agent was divided with absorbance of control sample (the absorbance of cells grown only in nutrient medium), having subtracted from absorbance of a corresponding sample with target cells the absorbance of the blank. IC50 is used as the measure of the toxic agents action and is determined from the graph $S(\%) = f(c)$, as the concentration of the agent which induces decrease in cell survival to 50%.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2009.08.013.

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