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Biological evaluation of twenty-eight ferrocenyl tetrasubstituted olefins: Cancer cell growth inhibition, ROS production and hemolytic activity

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Abstract

The antiproliferative effects of twenty-eight tetrasubstituted olefins bearing a ferrocenyl group, including six never-reported compounds, were evaluated against SF-295 (human glioblastoma), HCT-8 (human colon cancer), MDA-MB-435 (human melanoma) and HL-60 (human promyelocytic leukemia) using the MTT test. IC₅₀ values were determined for twenty-three active compounds and of these, ten compounds had IC₅₀ values lower than 2 μM on one or more cell lines. Of all the compounds, only two produced significant amounts of ROS on HL-60 cells, and ROS production and growth inhibition could not be correlated. The ten most antiproliferative compounds were tested for their hemolytic activity on mouse erythrocytes. Five compounds showing high antiproliferative activity and low hemolytic activity were thus identified for further study.

Keywords: Cytotoxicity; Hemolysis; Bioorganometallics; Ferrocene; Ferrocifen; Structure–activity relationship; Cancer cell line

1. Introduction

Ferrocene (Fc) is becoming a popular substituent in medicinal chemistry, and in favorable cases, it can potentiate the biological activity of the parent compound [1], [2]. The recognition that ferrocenyl derivatives of hydroxytamoxifen show strong antiproliferative activity against both estrogen-responsive and estrogen-refractory breast cancer cells [3], [4] has encouraged us to extend this work to a variety of organometallic tetrasubstituted olefins based on the tamoxifen and stilbene skeleton. Structure–activity relationship studies have shown the importance of a ferrocenyl group [5], [6], a phenyl group with protic substitution (OH, NH₂) [7], [8], and a conjugated linker allowing electronic communication between the two for potent antiproliferative effects [9]. In some cases, such as with compounds possessing an acetoxy or amido group [7], [10], preliminary hydrolysis may be necessary to release the active species in cell culture. More recent work shows the interest of the [3]ferrocenophane motif as a new pharmacophore [11], [12], [13].

Most of the literature cited above involves antiproliferative studies on breast and prostate cancer cell lines. In order to expand the scope of this research, we here report the antiproliferative effects of twenty-eight ferrocenyl tetrasubstituted olefins on four tumor types. The antiproliferative effects of all compounds were evaluated by the MTT assay against three cancer cell lines: SF-295 (human glioblastoma), HCT-8 (human colon cancer) and MDA-MB-435 (human melanoma) at a concentration of 25 µg/mL. For the twenty-three compounds showing cell growth inhibition of more than 80%, IC₅₀ values were determined on those three cancer cell lines, along with HL-60 (human promyelocytic leukemia). Ten compounds were identified with IC₅₀ values (after 72 h) below 2 µM for one or more cell lines. For the first time, the hemolytic effects of such ferrocenyl tetrasubstituted olefins were evaluated. Seven compounds displayed measurable hemolytic effects (EC₅₀ below 100 µM), suggesting that their antiproliferative activity could arise from non-specific membrane interactions, which may present challenges in the further development of such molecules. Five compounds, **10**, **11**, **16**, **17** and **28**, exhibited high antiproliferative activity on one or more cell lines and low hemolytic activity, and thus can be considered promising as

anticancer prototypes for further development. ROS production was evaluated on HL-60 cells and does not seem to be linked to the antiproliferative effects.

2. Chemistry

2.1. Compound library

For simplicity, the test compounds can be divided into four series, based on their structure (Chart 1). Series 1 (six compounds) contains molecules where the two phenyl groups lie on opposite carbon atoms of the olefin (vicinal), while Series 2 (seventeen compounds) consist of isomers where the two phenyl groups lie on the same carbon atom (geminal). Series 3 (two compounds) have ferrocenyl group(s) grafted onto a phenyl ring, while the two compounds of Series 4 possess two vicinal ferrocenyl groups directly grafted to the double bond. Substitution on the phenyl rings is varied, involving hydroxyl, methoxy, palmitate, pivalate, $\text{O}(\text{CH}_2)_2\text{Fc}$, NH_2 and/or aminated short carbon chain ($\text{O}(\text{CH}_2)_3\text{N}(\text{CH}_3)_2$) groups. In some cases, the latter organometallic compounds with aminated chains have been isolated as their citrate salts. Finally, we have also included a [3]ferrocenophane derivative with Series 2 geometry and NH_2 substitution.

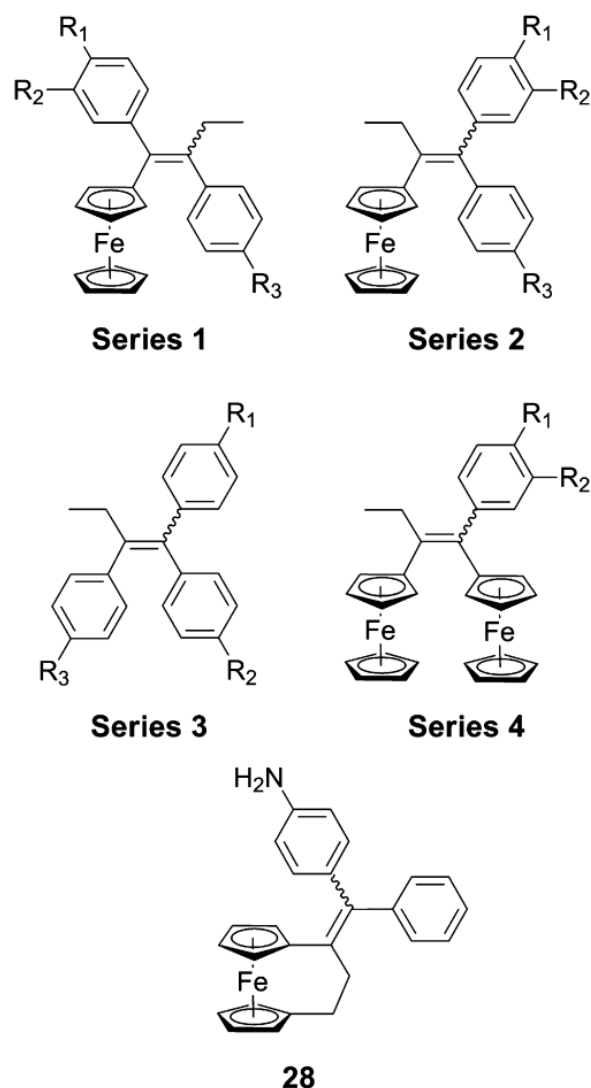
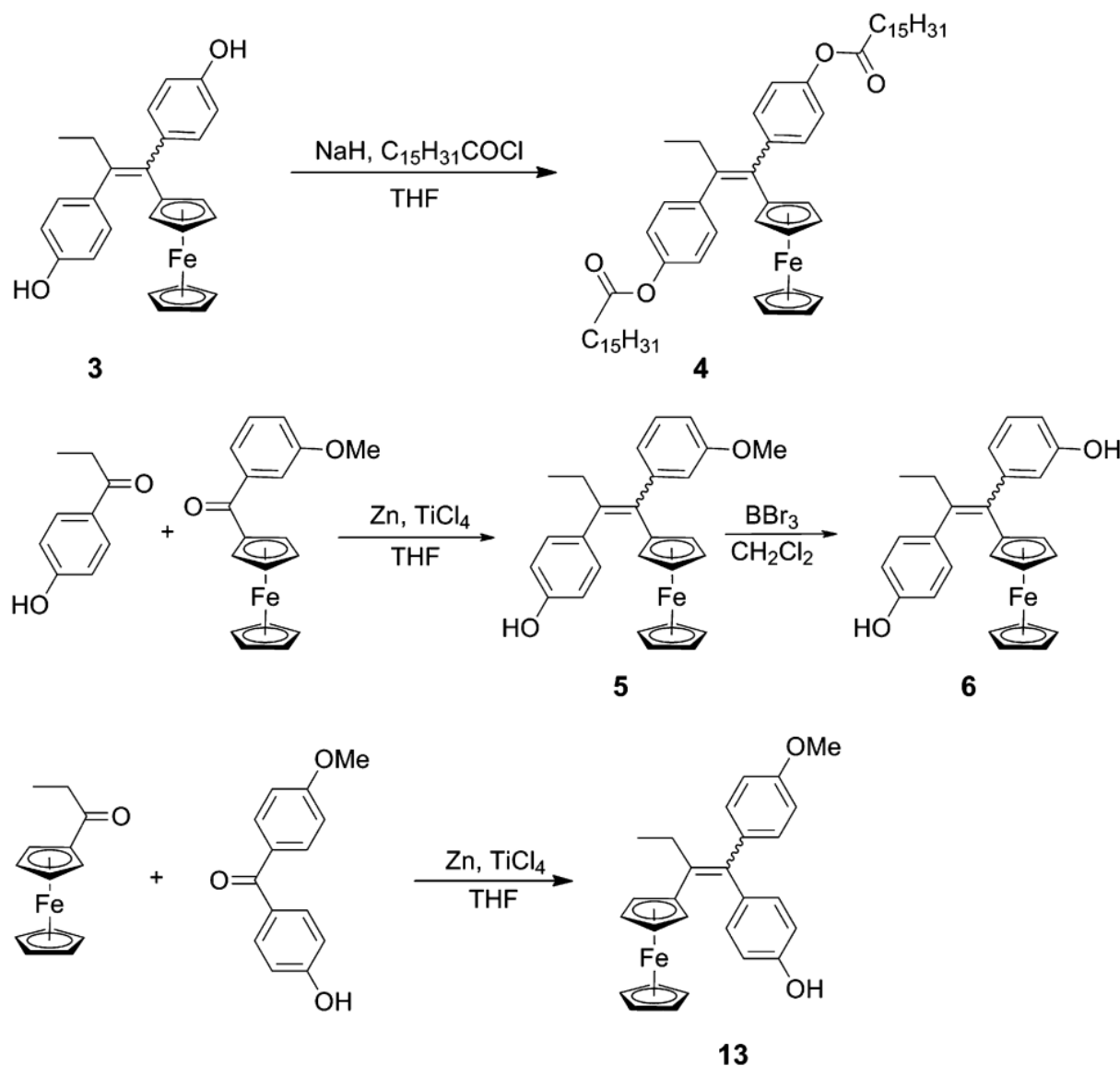


Chart 1. Compounds studied in this report. $R_1 = \text{H, OMe, OH, OC(O)C}_{15}\text{H}_{31}, \text{OC(O)C(CH}_3)_3, \text{O(CH}_2)_3\text{N(CH}_3)_2$ or $\text{O(CH}_2)_2\text{Fc}$, $R_2 = \text{H, OMe, OH}$, $R_3 = \text{H, OMe, OH, OC(O)C}_{15}\text{H}_{31}, \text{OC(O)C(CH}_3)_3, \text{O(CH}_2)_3\text{N(CH}_3)_2$ or $\text{O(CH}_2)_2\text{Fc}$ (see Table 1, Table 2, Table 3).

2.2. Synthesis

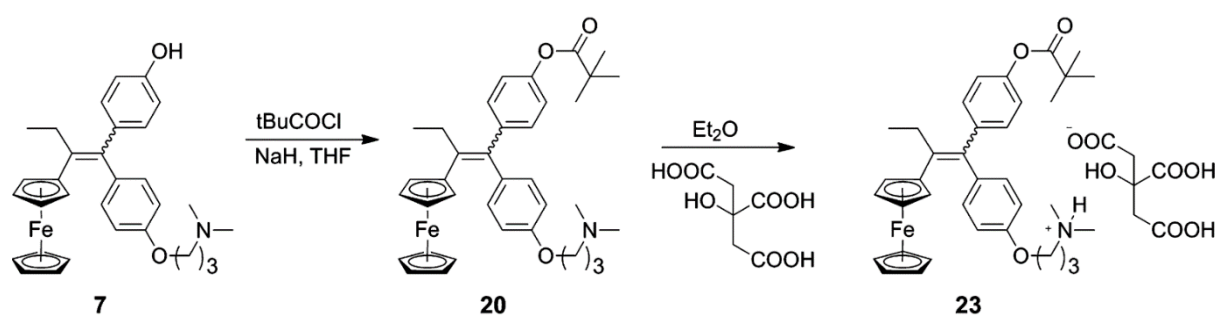
Six new compounds have been synthesized for this study. 1-Ferrocenyl-1,2-bis-(4-palmitoxyphenyl)-but-1-ene (**4**) was obtained in 98% yield by treating 1-ferrocenyl-1,2-bis-(4-hydroxyphenyl)-but-1-ene (**3**), previously synthesized by a McMurry cross-coupling reaction [6], with the long-chain acid chloride and NaH in THF. 1-Ferrocenyl-1-(3-methoxyphenyl)-2-(4-hydroxyphenyl)-but-1-ene (**5**) was synthesized in 52% yield by McMurry cross-coupling between 1-propionyl-4-hydroxybenzene and ferrocenyl-3-methoxyphenyl ketone. The phenol group was deprotected with BBr_3 in CH_2Cl_2 to form 1-ferrocenyl-1-(3-hydroxyphenyl)-2-(4-hydroxyphenyl)-but-1-ene (**6**) in 77% yield. 1-(4-Hydroxyphenyl)-1-(4-methoxyphenyl)-2-ferrocenyl-but-1-ene (**13**) was obtained in 73% yield

via a McMurry cross-coupling reaction between propionyl ferrocenyl ketone and 4-hydroxy-4'-methoxybenzophenone, the latter being formed by treatment of 4,4'-dihydroxybenzophenone with NaH and MeI in DMF (Scheme 1).



Scheme 1. Synthesis of **4**, **5** and **13**; deprotection of **5** to form free phenol **6**.

1-[4-(3-Dimethylaminopropoxy)phenyl]-1-(4-trimethylacetyloxyphenyl)-2-ferrocenyl-but-1-ene (**20**) was synthesized in 76% yield by the treatment of hydroxyferrocifen ($n = 3$) [4] with tBu acid chloride and NaH in THF. 1-[4-(3-Dimethylamoniumpropoxy)phenyl]-1-(4-trimethylacetyloxyphenyl)-2-ferrocenyl-but-1-ene citrate (**23**) was obtained by addition of a solution of citric acid in THF to **20** in a mixture of diethyl ether and THF (Scheme 2).



Scheme 2. Synthesis of compounds **20** and **23**.

3. Results and discussion

3.1. Antiproliferative effects

All the substances herein described were initially evaluated *in vitro* against three cancer cell lines: SF-295 (human glioblastoma), HCT-8 (human colon cancer) and MDA-MB-435 (human melanoma) at a concentration of 25 $\mu\text{g/mL}$ using the MTT assay for mitochondrial activity [14]. Doxorubicin and hydroxytamoxifen were used as positive controls (Fig. 1). With only a few exceptions, all of the compounds provoked total growth inhibition. From this screening, twenty-three compounds were retained for IC_{50} determination on the three cell lines and also on HL-60 human promyelocytic leukemia cells. The five compounds excluded for low activity tended to be those possessing large or stable phenol protecting groups: the dipalmitate **4**, the dimethoxy **12** and the dipivalate **15**. However, it should be noted that the acetoxy derivative **17** showed sub-micromolar IC_{50} values on all cells except MDA-MB-435. This is likely related to the ease of acetyl hydrolysis to release the free phenol in the cell, as previously observed [10]. Also lacking a free phenol, the ferrocene ester **25** was omitted from IC_{50} determination. Contrary to previous studies [15], the *p*-phenol **26** also showed low activity and was not studied further.

For Series 1 (Table 1), although the activities do not vary widely, the di-*p*-phenol **3** was slightly more active than the other compounds, with an average IC_{50} value of 16 μM . This result is consistent with those previously found for a variety of compounds having such geometry on MDA-MB-231 human breast cancer cell lines, where **3** showed a moderate IC_{50} value of 7 μM (after 5 days) [6], [16], similar to that observed for the HL-60 cell line. Compound **6**, also with two phenol groups, likewise demonstrated moderately good activity.

For Series 1 (Table 1) MDA-MB-435, with a mean IC_{50} of 30 μ M, was the most resistant cell line, while the HL-60 leukemia cell was the most sensitive (mean IC_{50} of 10 μ M).

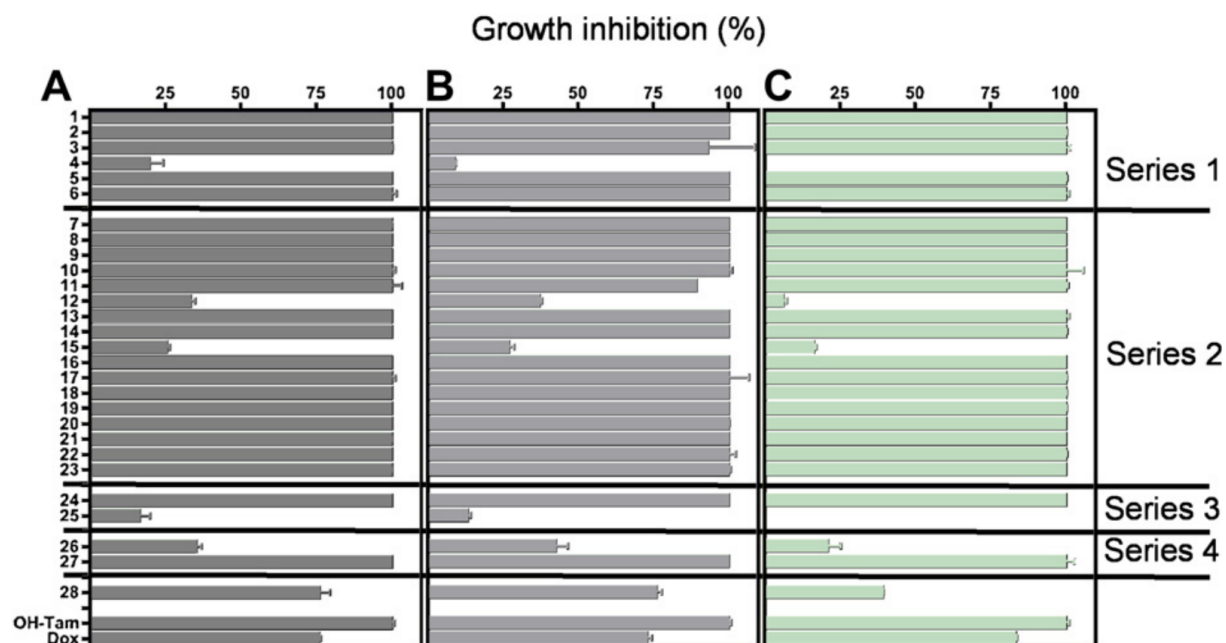
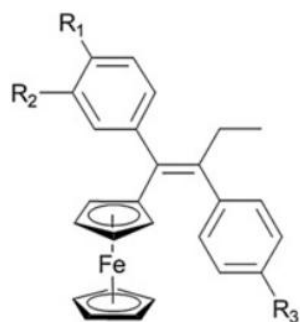


Fig. 1. Single dose antiproliferative activity of the ferrocenyl tetrasubstituted olefins evaluated against SF-295 (glioblastoma, A), HCT-8 (colon cancer, B) and MDA-MB-435 (melanoma, C) cell lines (at 25 μ g/mL) using the MTT assay after 72 h incubation. Data presented as a percentage of growth inhibition relative to untreated cells. Doxorubicin (Dox, 5 μ g/mL) and hydroxytamoxifen (OH-Tam, 25 μ g/mL) were used as positive controls.

Series 2 is not only the largest series, but also contains some of the most active compounds of those tested (Table 2). We have previously observed that a geminal arrangement of the phenyl groups yields superior activity compared to their vicinal isomers on the MDA-MB-231 hormone-independent cell line [6], [16]. The cell selectivity profile for Series 2 is similar to that observed for Series 1, with HL-60 the most sensitive, (average IC_{50} of 2 μ M) and MDA-MB-435 as by far the most resistant cell line, (average IC_{50} of 14.2 μ M). Remarkably however, MDA-MB-435 is sensitive to the compounds possessing aminated side chains, which all demonstrate low micromolar activity. Originally thought to be a metastatic breast cancer cell line, recent gene studies suggest that this is a malignant melanoma line related to M14 [17]. The activity of the aminated compounds is unexpected, as MDA-MB-435, regardless of its histological provenance, is considered an estrogen receptor-negative cell line, while the activity of molecules (such as tamoxifen) possessing such side chains is attributed to estrogen–receptor interactions.

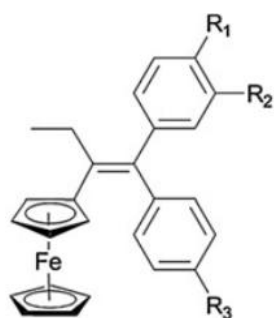
Table 1. Antiproliferative effects for Series 1.



Series 1

	R1	R2	R3	IC ₅₀ (CI _{95%}) of compounds after 72 h, in μM			
				SF-295	HCT-8	MDA-MB-435	HL-60
1	MeO	H	OH	23 \pm 3	19 \pm 3	32 \pm 9	11 \pm 3
2	OH	H	MeO	20 \pm 1	15 \pm 4	26 \pm 4	8 \pm 3
3	OH	H	OH	17 \pm 3	11 \pm 3	27 \pm 3	7 \pm 1
4	OC(O)	H	OC(O)	nd	nd	nd	nd
	C ₁₅ H ₃₁		C ₁₅ H ₃₁				
5	H	MeO	OH	20 \pm 2	16 \pm 3	28 \pm 2	13 \pm 2
6	H	OH	OH	15 \pm 1	8 \pm 3	36 \pm 5	11 \pm 3

nd - not determined. Data correspond to IC₅₀ values and 95% confidence interval obtained from non-linear regression.

Table 2. Antiproliferative effects for Series 2.**Series 2**

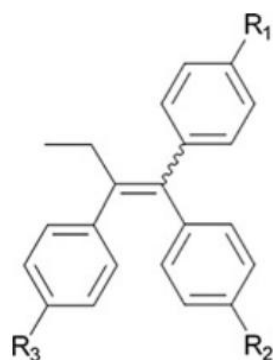
	R1	R2	R3	IC ₅₀ (CI _{95%}) of compounds after 72 h, in μM			
				SF-295	HCT-8	MDA-MB-435	HL-60
7	OH	H	O(CH ₂) ₃ NMe ₂	2.9 ± 0.9	1.4 ± 0.2	5.3 ± 0.7	1.9 ± 3
8	H	OH	H	4.0 ± 0.6	3.1 ± 0.5	12 ± 1	2.0 ± 0.2
9	H	OH	OH	13 ± 22	2 ± 1	32 ± 2	1.15 ± 0.07
10	NH ₂	H	H	2.6 ± 0.7	3 ± 1	19 ± 6	1.6 ± 0.2
11	H	H	H	1.0 ± 0.2	0.9 ± 0.1	16 ± 4	1.04 ± 0.04
12	MeO	H	MeO	nd	nd	nd	nd
13	MeO	H	OH	7 ± 1	6.6 ± 0.9	18 ± 2	2.2 ± 0.1
14	OC(O)CMe ₃	H	OH	9 ± 2	6 ± 4	16 ± 4	4 ± 2
15	OC(O)CMe ₃	H	OC(O)CMe ₃	nd	nd	nd	nd
16	OC(O)CH ₃	H	OH	1 ± 2	1 ± 2	29 ± 4	0.94 ± 0.04
17	OC(O)CH ₃	H	OC(O)CH ₃	1 ± 2	2 ± 1	27 ± 4	0.90 ± 0.04
18	H	H	OH	2.9 ± 0.5	3.7 ± 0.7	23 ± 5	2.5 ± 0.3
19	O(CH ₂) ₃ NMe ₂	H	O(CH ₂) ₃ NMe ₂	2.3 ± 0.3	1.3 ± 0.7	1.2 ± 0.1	1.0 ± 0.2
20	OC(O)CMe ₃	H	O(CH ₂) ₃ NMe ₂	4 ± 1	2.8 ± 0.5	6.3 ± 0.9	4 ± 3
21	O(CH ₂) ₃ NMe ₂	H	Amino citrate	1.9 ± 0.2	0.88 ± 0.04	0.83 ± 0.08	0.52 ± 0.07
22	OH	H	Amino citrate	2.1 ± 0.4	0.82 ± 0.06	3.0 ± 0.2	0.66 ± 0.06
23	OC(O)CH ₃	H	Amino citrate	7 ± 2	4.4 ± 0.8	7.1 ± 0.7	5 ± 4

Indeed, those compounds possessing aminated side chains, and especially two such chains, tend to be the most active overall. For example, **19** and **21** (where **21** is the citrate salt of **19**) are notable in having an average IC₅₀ value over all cell lines below 2 μM . Compound **22**, the citrate salt of hydroxyferrocifen ($n = 3$) is also quite active. These three molecules have previously been evaluated for their antibacterial and antifungal properties and **19**, in

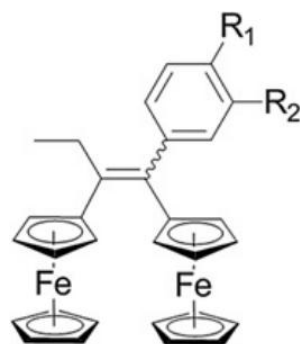
particular, shows excellent inhibitory properties against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (<12.5 µg/mL) [18]. Compound **19** also shows good activity against MDA-MB-231 cells, with IC₅₀ = 0.45 µM [19]. The activity of many Series 2 molecules, particularly phenols, has previously been attributed to oxidative metabolism to quinone methide species. Lacking free phenols, compound **19** and **21** would not be expected to form such electrophilic species in the cell. Instead, the mechanism of such compounds is thought to be related to metal coordination, possibly that of Zn²⁺ [19].

Comparing the antiproliferative effects of **7–9**, it seems that the aromatic substitution pattern does not play an important role, contrasting with previous results found on MDA-MB-231 cells [15] (*vide infra*). Concerning protection of the hydroxyl groups, the presence of the less bulky substituent (acetyl, in compounds **16** and **17**), in comparison to pivaloyl (in **14** and **15**) leads to an increase in biological activity.

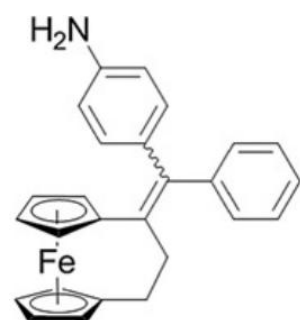
In the other series, only two compounds were evaluated for their IC₅₀ values. The ferrocene ether **24** inhibited cell growth with an average IC₅₀ of 10 µM, being most active against the HCT-8 cell line (Table 3). The diferrocene **27**, with an *m*-phenol, inhibited cell growth with an average IC₅₀ of 15 µM, being most active against HL-60 cell line. This contrasts with the previous observation that the *para*-substituted derivative is slightly more active on MDA-MB-231 human breast cancer cells, (IC₅₀ = 2.8 µM) after 5 days, than the *meta* compound, (3.5 µM) [15]; here the trend is reversed. We also evaluated an example of a recently discovered series of molecules possessing a [3]ferrocenophane group [11]. Compound **28**, the cyclic derivative of **10**, showed potent activity on SF-295, HCT-8 and HL-60 cells, but very low activity on the melanoma cells. The strong antiproliferative effects on the first three cell lines is consistent with previous results on the MDA-MB-231 and PC-3 cell lines, where IC₅₀ values in the nanomolar region were obtained [11].

Table 3. Antiproliferative effects for Series 3, 4 and compound **28**.**Series 3**

	R1	R2	R3	IC ₅₀ (CI _{95%}) of compounds after 72 h, in μM			
				SF-295	HCT-8	MDA-MB-435	HL-60
24	OH	-O(CH ₂) ₂ Fc	OH	11 \pm 1	6 \pm 3	13 \pm 17	9 \pm 1
25	-O(CH ₂) ₂ Fc	-O(CH ₂) ₂ Fc	H	nd	nd	nd	nd

**Series 4**

26	OH	H	-	nd	nd	nd	nd
27	H	OH	-	13 \pm 1	11 \pm 3	27 \pm 3	8.4 \pm 4



28	NH ₂	H	-	1.0 \pm 0.8	0.5 \pm 0.3	>61.68	<0.12
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nd – not determined. Data correspond to IC₅₀ values and 95% confidence interval obtained from non-linear regression.

In total, ten compounds were identified with IC₅₀ values lower than 2 μM on one or more cell lines: **7, 9, 10, 11, 16, 17, 19, 21, 22** (Series 2) and **28** (ferrocenophane). In order to compare

the relative merit of each compound, the mean IC_{50} value over all twenty-eight compounds was calculated for each cell line. The deviations from the mean values are shown in Fig. 2. It is clear from the graph that Series 1 and Series 4 compounds have a lower than average activity on all cell lines. In Series 2, six compounds, **7**, **8**, **11**, **19**, **21** and **22**, showed better than average activity for all cell lines, while **10**, **13**, **16**, **17** and **18** showed better than average activity on all cell lines except for melanoma. Indeed the MDA-MB-435 cell line showed the widest range of responses to the test compounds. Compound **28**, with a ferrocenophane moiety, showed the best activity against SF-295, HCT-8 and HL-60 of all the molecules, while **21** (diamine salt) showed the best activity against MDA-MB-435.

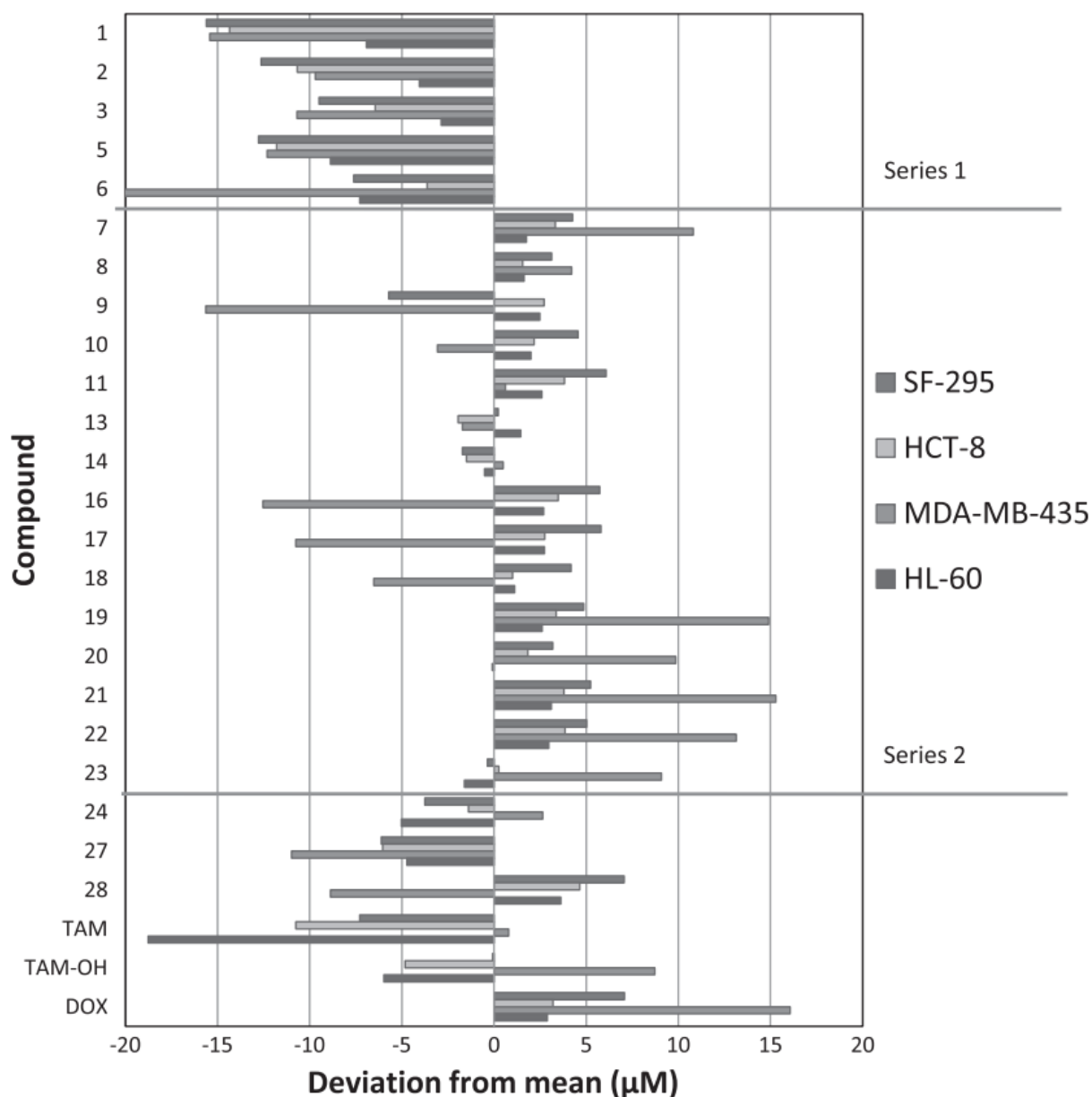


Fig. 2. Deviation from mean IC₅₀ for all compounds. The zero value represents the average IC₅₀ value for the entire set of compounds. Positive values for the bars indicate higher activity than average; negative values for the bars indicate lower activity than average.

3.2. ROS production

The molecules were further tested for their ability to induce ROS generation in HL-60 cells. Cells were incubated for 1 h at 5 μ M concentration of each compound. Surprisingly, only **8** and **13** led to significant ROS generation compared to the negative control (set to 100%), and this ROS generation was substantially lower than the pro-oxidant activity of β -lapachone (Fig. 3). Under these conditions, ROS production does not correlate with antiproliferative effects, suggesting a mechanism of action independent of oxidative stress on the HL-60 cells. There have been very few studies on ROS production for the ferrocifen-type molecules. One study showed ROS production after 10 min by the ferrocifen **7** in MCF-7 and MDA-MB-231 cells [20], while no ROS production was observed in melanoma cells [21].

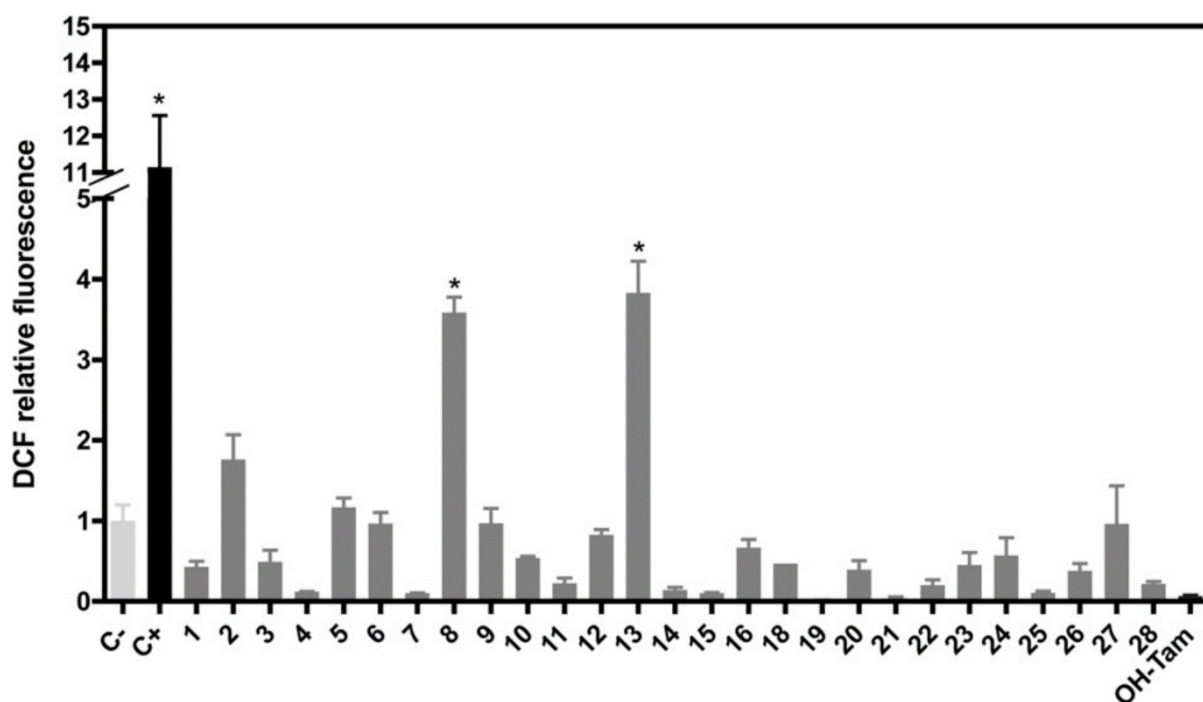
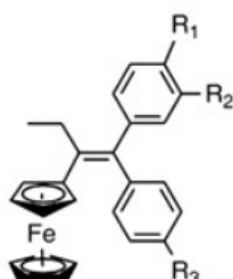
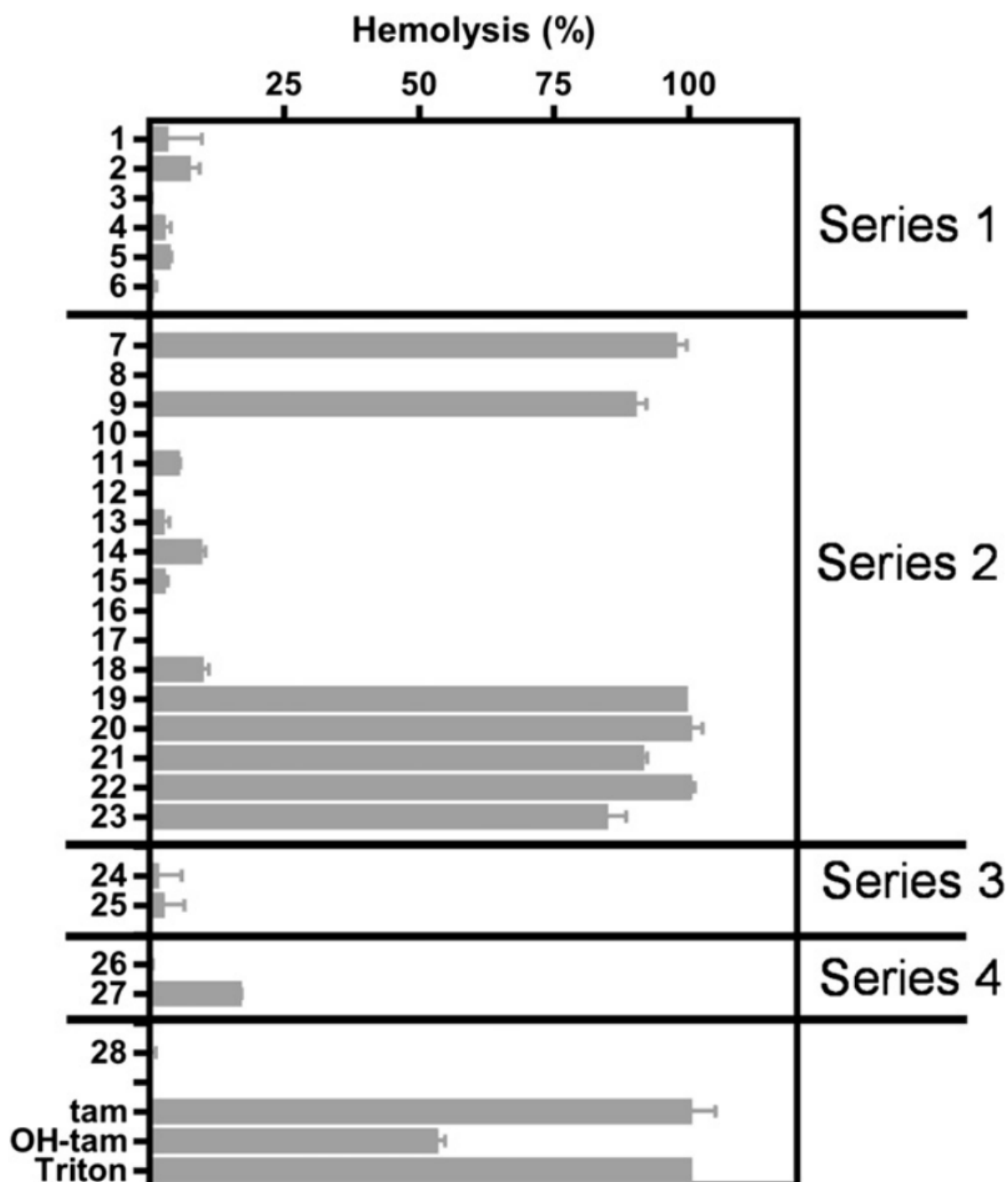


Fig. 3. Reactive oxygen species (ROS) level detected by 2',7'-dichlorodihydrofluorescein diacetate (H₂-DCF-DA) flow cytometry in HL-60 cells after treatment with the ferrocenyl tetrasubstituted olefins during 1 h β -lapachone (5 μ M) was used as a positive control. Cells were stained with H₂-DCF-DA, and five thousand events were analyzed. Data correspond to mean \pm standard error of the mean from two independent experiments performed in triplicate. * p < 0.05, ANOVA followed dunnett test.

3.3. Hemolytic activity

Tamoxifen and its derivatives strongly partition into biomembranes, leading to changes in the framework of the erythrocyte membrane and its cytoskeleton, which may result in lysis of the red blood cells [22], [23]. The affinity for membranes is highly influenced by even small modifications in the chemical structure; for example, hydroxytamoxifen was shown to be three times less hemolytic than tamoxifen [23]. Indeed, tamoxifen has been associated with hemolytic anemia in patients taking the drug [24], [25], [26]. Although the biochemical mechanism involved in tamoxifen-induced hemolysis has not been fully elucidated, metabolism to hydroxytamoxifen seems to moderate this effect.

Hemolytic properties have never been evaluated for the ferrocenyl derivatives of tamoxifen and stilbene. Thus, in a second set of experiments, all the compounds and relevant standards (triton, tamoxifen and hydroxytamoxifen) were evaluated for hemolytic activity in order to verify whether the observed antiproliferative effects could be related to unspecific membrane damage (Fig. 4). Along with tamoxifen ($EC_{50} = 8.64 \mu\text{M}$), seven compounds, all belonging to Series 2, present lytic properties on mouse erythrocytes [**7** ($EC_{50} = 17.74 \mu\text{M}$), **9** ($70.06 \mu\text{M}$), **19** ($26.33 \mu\text{M}$), **20** ($6.77 \mu\text{M}$), **21** ($25.90 \mu\text{M}$), **22** ($5.45 \mu\text{M}$) and **23** ($4.69 \mu\text{M}$)]. The hemolytic activity of the compounds: **23** > **22** > **20** > tamoxifen > **7** > **21** > **19** > **9**, seems to be associated with the presence of aminated side chains, either *N,N*-dimethylpropylamine and/or a quaternary ammonium salt, both with surfactant characteristics. On the other hand, hydroxytamoxifen, the active metabolite of tamoxifen, which is responsible for the antiestrogenic effects in the breast, was not found to be hemolytic ($EC_{50} = 213.7 \mu\text{M}$).



7: $R_1 = \text{OH}$, $R_2 = \text{H}$, $R_3 = \text{O}(\text{CH}_2)_3\text{NMe}_2$

9: $R_1 = \text{H}$, $R_2 = R_3 = \text{OH}$

19: $R_1 = R_3 = \text{O}(\text{CH}_2)_3\text{NMe}_2$, $R_2 = \text{H}$

20: $\text{OC}(\text{O})\text{CMe}_3$, $R_2 = \text{H}$, $R_3 = \text{O}(\text{CH}_2)_3\text{NMe}_2$

21: $R_1 = \text{O}(\text{CH}_2)_3\text{NMe}_2$, $R_2 = \text{H}$, $R_3 = \text{O}(\text{CH}_2)_3\text{NHMe}_2$ citrate

22: $R_1 = \text{OH}$, $R_2 = \text{H}$, $R_3 = \text{O}(\text{CH}_2)_3\text{NHMe}_2$ citrate

23: $R_1 = \text{OC}(\text{O})\text{CMe}_3$, $R_2 = \text{H}$, $R_3 = \text{O}(\text{CH}_2)_3\text{NHMe}_2$ citrate

Fig. 4. Single dose hemolysis of the ferrocenyl tetrasubstituted olefins on mouse erythrocytes 2% at 250 $\mu\text{g}/\text{mL}$, after 1 h incubation. Data presented as a percentage of hemolysis relative to positive control (100%). Triton X-100 (1%), tamoxifen (tam, 250 $\mu\text{g}/\text{mL}$) and hydroxytamoxifen (OH-tam, 250 $\mu\text{g}/\text{mL}$) were used as positive controls.

According to the literature, the membrane-interacting properties may explain some side effects observed during tamoxifen therapy such as hemolytic anemia. However these properties may also be related to anticancer effects, since lysis was not restricted to erythrocytes, being also observed in cancer cell lines [27], [28]. Indeed, the cell membrane is recognized as a target for the cytotoxicity of tamoxifen and derivatives [22], [23], [29]. Present data reinforce the hypothesis that membrane-disrupting properties contribute to anticancer effects for some of these compounds, nonetheless, the precise antiproliferative mechanisms are likely more complex and involve different targets, being also highly dependent on the structural requirements.

Table 4 summarizes the toxicity index, defined here as the ratio between EC_{50} (hemolytic activity) and IC_{50} (cytotoxicity) for the ten compounds identified with an IC_{50} value lower than 2 μM on one or more cell lines. Larger values indicate better specific toxicity and point to the most suitable compounds for future development. On average, compound **11**, 2-ferrocenyl-1,1-diphenyl-but-1-ene, showed an interesting combination of growth inhibition and low hemolytic activity. Compound **28** showed the best combination of high antiproliferative activity and low hemolytic activity; in fact, the hemolytic activity was so low (no activity at the upper concentration of 250 $\mu\text{g}/\text{mL}$), that only the lower limits of the ratios could be calculated. Compounds **10** and **17**, both possessing a *p*-OH group, and compound **16**, with two protected phenol groups, also show promising activity with low hemolytic activity.

Table 4. Selectivity index relating growth inhibition and hemolytic effects.

Compounds	Cell lines (Ratio EC ₅₀ /IC ₅₀ mM)			
	SF-295	HCT-8	MDA-MB-435	HL-60
7	0.06	0.13	0.03	0.09
9	0.05	0.36	0.02	0.61
10	2.44	2.50	0.31	3.85
11	6.25	7.69	0.41	6.25
16	4.00	4.55	0.19	5.88
17	3.85	2.56	0.18	5.56
19	0.12	0.20	0.22	0.26
21	0.14	0.29	0.31	0.50
22	0.03	0.07	0.02	0.08
28^a	>6.25	>14.29	>0.10	>50.00

^a Only lower values are given, because no hemolytic effects were observed at the highest concentration of 250 µg/mL.

4. Conclusions

We have here presented the antiproliferative effects on human cancer cell lines, the hemolytic effects on mouse erythrocytes and the capacity of ROS production on HL-60 cells of twenty-eight ferrocenyl derivatives based on the tamoxifen or stilbene skeleton. By expanding the panel of cell lines, we discovered that the MDA-MB-435 melanoma cell line is highly resistant to the tested ferrocene compounds, except where aminated side chains are present. The antiproliferative effects of these compounds, however, may be related to membrane disruption, as these compounds display hemolytic activity at low micromolar concentrations. ROS generation was displayed by only two inactive compounds, and thus does not seem to be important for the antiproliferative effects of the ferrocenyl compounds.

This work also allows us to take a second look at compounds that were previously abandoned due to less than ideal activity in previous cell culture assays. One of the most interesting compounds appears to be the simple ferrocenyl diphenyl **11**. Unable to form a quinone methide without preliminary hydroxylation, this compound exhibited only moderate activity on MDA-MB-231 breast cancer cells, where the formation of electrophilic metabolites appears important. However, in the assays reported here, this compound demonstrated sub-

micromolar activity for all cell lines, except melanoma. Furthermore, the lack of a phenol group may be advantageous for *in vivo* activity. For example, one of the difficulties in obtaining benefit from natural polyphenols found in the diet is their metabolism to more polar compounds and fast elimination from the body [30]. The non-hydroxylated **11** would be thus expected to possess a longer *in vivo* lifetime and greater bioavailability than phenol analogs; this should also be the case for **16**, where the phenol groups are protected. These lipophilic compounds are also particularly suitable for lipophilic nano-emulsion formulation. Furthermore, hydroxylation of **11** or deprotection of **16** in the liver may further enhance their efficacy, analogous to the case of tamoxifen/hydroxytamoxifen. These attractive features, coupled with the low hemolytic activity recognized here for the first time, suggests that **11** is a good candidate for formulation and *in vivo* examination, which is currently being pursued.

5. Experimental

5.1. Chemistry

All reactions were performed under argon using standard Schlenk techniques. Anhydrous THF was obtained by distillation from sodium/benzophenone. Thin layer chromatography was performed on silica gel 60 GF254. The preparative HPLC separations were performed on a Shimadzu apparatus with a Nucleodur C18 column (length of 25 cm, diameter of 3.2 cm, and particle size of 10 μM) using acetonitrile as an eluent. The analytical HPLC controls were performed on a Shimadzu apparatus with a Nucleodur C18 column (length of 15 cm, diameter of 0.45 cm, and particle size of 5 μM) using acetonitrile as an eluent. Infrared spectra were obtained on an IRFT BOMEM Michelson-100 spectrometer or a JASCO FT/IR-4100 as a KBr plate. ^1H and ^{13}C NMR spectra were recorded on a 300 MHz Bruker spectrometer. Mass spectrometry was performed with a Nermag R 10-10C spectrometer. Elemental analyses were performed by the microanalysis service of CNRS at Gif sur Yvette. HRMS measurements were performed with an LTQ-Orbitrap XL of Thermo Fischer used in positive mode with an electrospray source. The synthesis of compounds **1** [16], **2** [16], **3** [6], **7** [4], **8** [15], **9** [15], **10** [31], **11** [15], **12** [3], **14** [18], **15** [18], **16** [11], **17** [10], **18** [4], **19** [18], **21** [18], **22** [18], **24** [32], **25** [32], **26** [15], **27** [15], and **28** [11] have been previously reported in the literature.

5.1.1. 1-Ferrocenyl-1,2-bis-(4-palmitoxyphenyl)-but-1-ene (**4**)

A suspension of 60% sodium hydride in oil (0.5 g, 12.5 mmol) was added in portions to a stirred solution of diphenol **3** (2.12 g, 5 mmol) in dry THF (30 mL). The stirring was

maintained for 10 min and palmitoyl chloride was slowly added (3.024 g, 11 mmol). The reaction mixture was stirred for 3 h and then ethanol (5 mL) was added. After 1 h, the mixture was poured into water, dichloromethane was added, and the mixture was decanted. The aqueous phase was extracted with dichloromethane, the combination of organic layers was dried under magnesium sulfate, concentrated under reduced pressure, and chromatographed with dichloromethane as the eluent. The yellow solid was recrystallized from ether to furnish **4** in a 98% yield. ^1H NMR (300 MHz, CDCl_3): δ 0.69 (t, $J = 7.4$ Hz, 3H, CH_3), 0.81 (t, $J = 6.7$ Hz, 6H, CH_3), 1.09–1.60 (m, 48H, CH_2), 1.65–1.79 (m, 4H, CH_2), 1.99 (q, $J = 7.4$ Hz, 2H, CH_2), 2.36–2.45 and 2.48–2.58 (m, 4H, CH_2), 3.24 and 4.11 (t, $J = 1.9$ Hz, 2H, C_5H_4), 3.87 and 4.15 (t, $J = 1.9$ Hz, 2H, C_5H_4), 3.91 and 3.98 (s, 5H, Cp), 6.73 and 7.08 (d, $J = 8.7$ Hz, 2H, C_6H_4), 6.76 and 7.10 (d, $J = 8.7$ Hz, 2H, C_6H_4), 6.86 and 7.15 (d, $J = 8.7$ Hz, 2H, C_6H_4), 6.92 and 7.26 (d, $J = 8.7$ Hz, 2H, C_6H_4). ^{13}C NMR (75 MHz, CDCl_3): δ 12.7 (CH_3), 14.1 (2CH_3), 22.7 (2CH_2), 25.0 (2CH_2), 29.1 (2CH_2), 29.3 (2CH_2), 29.4 (2CH_2), 29.5 (2CH_2), 29.7 ($6 \times 2 \text{CH}_2$), 30.6 (CH_2), 31.9 (2CH_2), 34.5 (2CH_2), 68.2 ($2\text{CH C}_5\text{H}_4$), 69.1 (5CH Cp), 69.3 ($2\text{CH C}_5\text{H}_4$), 85.7 (C_{ipso}), 121.0 ($2\text{CH C}_6\text{H}_4$), 121.5 ($2\text{CH C}_6\text{H}_4$), 129.9 ($2\text{CH C}_6\text{H}_4$), 130.4 ($2\text{CH C}_6\text{H}_4$), 134.4 (C), 139.4 (C), 139.6 (C), 140.6 (C), 149.4 (C), 149.5 (C), 172.2 (CO), 172.3 (CO). IR (KBr, $\nu \text{ cm}^{-1}$): 3097, 2919, 2850 (CH_2 , CH_3), 1759 (CO). MS (CI, NH_3) m/z : 901 [$\text{M} + \text{H}$] $^+$, 918 [$\text{M} + \text{NH}_4$] $^+$. Anal. Calcd for $\text{C}_{58}\text{H}_{84}\text{FeO}_4$: C, 77.30; H, 9.39. Found: C, 77.03; H, 9.27.

5.1.2. 1-Ferrocenyl-1-(3-methoxyphenyl)-2-(4-hydroxyphenyl)-but-1-ene (**5**)

Titanium chloride (12.43 g, 7.2 mL, 65.5 mmol) was added dropwise to a suspension of zinc powder (8 g, 122.4 mmol) in dry THF (300 mL) at 10 °C. The mixture was heated at reflux for 2 h. A second solution was prepared by dissolving *m*-methoxybenzoylferrocene [16], (6.4 g, 20 mmol) and 4'-hydroxypropiophenone (3.0 g, 20 mmol) in dry THF. This latter solution was added dropwise to the first solution and then the reflux was continued for 2 h. After cooling to room temperature, the mixture was stirred with water and dichloromethane. The mixture was acidified with diluted hydrochloric acid until dark color disappeared and was decanted. The aqueous layer was extracted with dichloromethane and the combination of organic layers was dried on magnesium sulfate. After concentration under reduced pressure, the crude product was chromatographed on silica gel column with dichloromethane as the eluent. The residue was recrystallized from an ether/petroleum ether solution to furnish **5** as an orange solid (4.55 g, 52% yield). ^1H NMR (300 MHz, CDCl_3): δ 0.70 and 0.95 (t, $J = 7.4$ Hz, 3H, CH_3), 1.98 and 2.80 (q, $J = 7.4$ Hz, 2H, CH_2), 3.29 (t, $J = 1.8$ Hz, 2H, C_5H_4), 3.60 and 3.80 (s, 3H, OCH_3), 3.87 (t, $J = 1.8$ Hz, 2H, C_5H_4), 3.93 and 3.97 (s, 5H, Cp), 5.33 (s, 1H,

OH), 6.87–6.96 (m, 5H, H_{arom}), 7.11 (d, $J = 8.5$ Hz, 2H, C₆H₄), 7.35 (t, $J = 7.4$ Hz, 1H, H_{arom}). ¹³C NMR (75 MHz, CDCl₃): δ 12.8 (CH₃), 30.8 (CH₂), 55.3 (OCH₃), 68.1 (2CH C₅H₄), 69.2 (5CH Cp), 69.3 (2CH C₅H₄), 86.1 (C_{ipso}), 111.9 (CH_{arom}), 115.3 (CH_{arom}), 115.4 (2 CH_{arom}), 122.2 (CH_{arom}), 128.9 (CH_{arom}), 130.3 (2 CH_{arom}), 134.6 (C), 135.6 (C), 139.4 (C), 143.8 (C), 154.4 (C), 159.3 (C). IR (KBr, ν cm⁻¹): 3428 (OH), 3093, 2964, 2928, 2866 (CH₂, CH₃). MS (EI, 70 eV) m/z : 438 [M]⁺, 409 [M-Et]⁺, 373 [M-Cp]⁺, 121 [FeCp]⁺. Anal. Calcd for C₂₇H₂₆FeO₂: C, 73.98; H, 5.97. Found: C, 74.03; H, 5.99.

5.1.3. 1-Ferrocenyl-1-(3-hydroxyphenyl)-2-(4-hydroxyphenyl)-but-1-ene (**6**)

Compound **5** (3.96 g, 9.03 mmol), was dissolved in dry dichloromethane (60 mL) at 0 °C and boron tribromide (6.79 g, 2.56 mL, 27.1 mmol) was added. The mixture was stirred overnight at room temperature. The solution was slowly poured into a mixture of water and ice and extracted with ethyl acetate. The combined organic layers were washed with water, dried over magnesium sulfate and concentrated under reduced pressure. The mixture was chromatographed on a silica gel column with dichloromethane as the eluent and the residue was recrystallized from chloroform to furnish **6** as an orange solid (2.93 g, 77% yield). ¹H NMR (300 MHz, acetone-d₆): δ 0.64 and 0.86 (t, $J = 7.4$ Hz, 3H, CH₃), 1.95 (q, $J = 7.4$ Hz, 2H, CH₂), 3.22 and 4.04 (t, $J = 1.9$ Hz, 2H, C₅H₄), 3.80 and 4.08 (t, $J = 1.9$ Hz, 2H, C₅H₄), 3.88 and 3.91 (s, 5H, Cp), 6.36–6.49 (m, 8H, H_{arom} of minor isomer), 6.65–6.79 (m, 3H, C₆H₄ with OH in meta of major isomer), 6.82 (d, $J = 8.7$ Hz, 2H, C₆H₄ with OH in para of major isomer), 6.95 (d, $J = 8.7$ Hz, 2H, C₆H₄ with OH in para of major isomer), 7.16 (t, $J = 7.7$ Hz, 1H, C₆H₄ with OH in meta of major isomer), 7.88 and 8.24 (s, 1H, OH), 7.90 and 8.26 (s, 1H, OH). ¹³C NMR (75 MHz, acetone-d₆): δ 13.1 (CH₃), 31.5 (CH₂), 68.5 (2CH C₅H₄), 69.8 (5CH Cp), 70.0 (2CH C₅H₄), 87.0 (C_{ipso}), 114.2 (CH_{arom}), 116.2 (2CH C₆H₄), 117.5 (CH_{arom}), 121.7 (CH_{arom}), 129.8 (CH_{arom}), 131.0 (2CH C₆H₄), 135.0 (C), 135.6 (C), 140.0 (C), 144.9 (C), 157.2 (C), 158.1 (C). IR (KBr, ν cm⁻¹): 3433 (OH), 2958, 2927, 2861 (CH₂, CH₃). MS (EI, 70 eV) m/z : 424 [M]⁺, 395 [M-Et]⁺, 359 [M-Cp]⁺, 121 [CpFe]⁺. HRMS (FAB, C₂₆H₂₄FeO₂: [M]⁺) calcd: 424.1126, found: 424.1107. Anal. calcd for C₂₆H₂₄FeO₂: C, 73.59; H, 5.7. Found: C, 73.41; H, 5.61.

5.1.4. 1-(4-Hydroxyphenyl)-1-(4-methoxyphenyl)-2-ferrocenyl-but-1-ene (**13**)

Titanium chloride (6.04 g, 3.5 mL, 31.8 mmol) was added dropwise to a suspension of zinc powder (3.9 g, 59.7 mmol) in dry THF (150 mL) at 10 °C. The mixture was heated at reflux for 2 h. A second solution was prepared by dissolving propionyl ferrocene [33] (2.42 g, 10 mmol) and 4-hydroxy-4'-methoxybenzophenone [34] (2.28 g, 10 mmol) in dry THF. This latter solution was added dropwise to the first solution and the reflux was continued for 2 h.

After cooling to r.t., the mixture was stirred with water and dichloromethane. The mixture was acidified with diluted hydrochloric acid until the dark color disappeared and was decanted. The aqueous layer was extracted with dichloromethane and the combination of organic layers was dried on magnesium sulfate. After concentration under reduced pressure, the crude product was chromatographed on silica gel column with dichloromethane as the eluent and recrystallized from an ether/pentane solution to furnish 3.19 g (yield: 73%) of **13** in an undetermined 3/2 ratio of *Z* and *E* isomers. ¹H NMR (300 MHz, CDCl₃): δ 0.94 (t, *J* = 7.4 Hz, 3H, CH₃), 2.47 (q, *J* = 7.4 Hz, 2H, CH₂), 3.70 and 3.74 (s, 3H, OCH₃), 3.87 (s, 2H, C₅H₄), 4.00 (s, 2H, C₅H₄), 4.05 (s, 5H, Cp), 4.53 and 4.59 (s, 1H, OH), 6.61 and 6.68 (d, *J* = 8.5 Hz, 2H, C₆H₄), 6.71 and 6.79 (d, *J* = 8.5 Hz, 2H, C₆H₄), 6.85 and 6.89 (d, *J* = 8.5 Hz, 2H, C₆H₄), 7.03 and 6.99 (d, *J* = 8.5 Hz, 2H, C₆H₄). ¹³C NMR (75 MHz, CDCl₃): δ 15.4 (CH₃), 27.9 (CH₂), 55.1 (OMe), 68.0 (2CH C₅H₄), 69.1 (5CH Cp), 69.3 (2CH C₅H₄), 87.1 (C_{ipso}), 113.5 and 113.6 (2CH C₆H₄), 115.0 and 115.1 (2CH C₆H₄), 130.4 and 130.6 (2CH C₆H₄), 131.0 and 131.1 (2CH C₆H₄), 136.7 (C), 137.1 (C), 137.2 and 137.7 (C), 137.5 (C), 153.8 (C), 157.9 (C). IR (KBr, ν cm⁻¹): 3418 (OH), 2933, 2964 (CH₂, CH₃). MS (EI, 70 eV) *m/z*: 438 [M]⁺, 409 [M-Et]⁺, 373 [M-Cp]⁺, 121 [CpFe]⁺. HRMS (EI, 70 eV, C₂₇H₂₆FeO₂: [M]⁺) calcd: 438.1283, found: 438.1287. Anal. calcd for C₂₇H₂₆FeO₂: C, 73.98; H, 5.97. Found: C, 73.83; H, 6.05.

5.1.5. 1-[4-(3-Dimethylaminopropoxy)phenyl]-1-(4-trimethylacetyloxyphenyl)-2-ferrocenylbut-1-ene (20)

In a Schlenk flask, phenol **7** [4] (2.36 g, 4.64 mmol) was dissolved into anhydrous THF and sodium hydride (0.222 g, 5.6 mmol, 60% suspension in oil) was added. After stirring 10 min, trimethylacetyl chloride (1.12 g, 1.14 mL, 9.3 mmol) was added and the reaction mixture was stirred for 3 h. 5 mL of ethanol was added and the stirring was continued for 1 h in order to destroy the excess of trimethylacetyl chloride. The mixture was poured into water, extracted twice with dichloromethane and concentrated under reduced pressure. Then the residue was chromatographed on a silica gel column with a solution of 10% triethylamine in chloroform to yield **20** in 76% yield as a dark orange oil containing a 50/50 mixture of *Z* and *E* isomers. ¹H NMR (300 MHz, CDCl₃): δ 0.94 (t, *J* = 7.3 Hz, 3H, CH₃), 1.26 and 1.28 (s, 9H, ^tBu), 1.82–1.98 (m, 2H, CH₂), 2.21 and 2.23 (s, 6H, NMe₂), 2.37–2.58 (m, 4H, CH₂N + CH₂), 3.83 (s, 2H, C₅H₄), 3.89 and 3.93 (t, *J* = 6.5 Hz, 2H, CH₂O), 3.97–4.07 (m, 7H, C₅H₄+Cp), 6.66 and 6.77 (d, *J* = 8.7 Hz, 2H, C₆H₄), 6.81 and 6.94 (d, *J* = 8.7 Hz, 2H, C₆H₄), 6.87 and 6.96 (d, *J* = 8.7 Hz, 2H, C₆H₄), 7.02 and 7.12 (d, *J* = 8.7 Hz, 2H, C₆H₄). ¹³C NMR (75 MHz, CDCl₃): δ 15.4 and 15.5 (CH₃), 27.1 (3CH₃ ^tBu), 27.3 (CH₂), 27.9 and 28.1 (CH₂), 39.1 (C ^tBu), 45.2

(2CH₃ NMe₂), 56.4 (CH₂N), 66.0 (CH₂O), 68.1 and 68.2 (2CH C₅H₄), 69.2 (5CH Cp), 69.3 (2CH C₅H₄), 86.8 (C_{ipso}), 114.1 and 114.2 (2CH C₆H₄), 121.0 and 121.2 (2CH C₆H₄), 130.2 and 130.5 (2CH C₆H₄), 130.9 and 131.0 (2CH C₆H₄), 136.7 (2C), 136.9 and 137.6 (C), 142.1 and 142.3 (C), 149.2 and 149.3 (C), 157.4 (C). IR (KBr, ν cm⁻¹): 3093, 3035, 2966, 2870, 2816, 2765 (CH₃, CH₂), 1751 (CO). MS (CI, NH₃) m/z : 594 [M + H]⁺.

5.1.6. 1-[4-(3-Dimethylamoniumpropoxy)phenyl]-1-(4-trimethylacetyloxyphenyl)-2-ferrocenyl-but-1-ene citrate (**23**)

Compound **20** (0.593 g, 1 mmol) was dissolved into 10 mL of THF and 40 mL of diethyl ether was added. A solution of citric acid (0.21 g, 1 mmol), in THF (5 mL) was added dropwise into the first solution. A yellow precipitate was immediately formed. After stirring for 20 min, the solution was stripped off by syringe and the yellow solid was washed with 10 mL of diethyl ether and dried under vacuum to furnish **23** in 96% yield. ¹H NMR (300 MHz, methanol-d₄): δ 0.91 and 0.93 (t, J = 6.0 Hz, 3H, CH₃), 1.23 and 1.26 (s, 9H, ^tBu), 2.17 (s broad, 2H, CH₂), 2.45–2.58 (m, 2H, CH₂), 2.62 (d, J = 15.4 Hz, 2H, CH₂), 2.73 (d, J = 15.4 Hz, 2H, CH₂), 2.82 (s broad, 2H, CH₂N), 3.20 (s broad, 6H, NMe₂H⁺), 3.79 (s broad, 2H, C₅H₄), 3.88–4.06 (m, 9H, C₅H₄ + Cp + CH₂O), 6.54–7.23 (m, 8H, C₆H₄). ¹³C NMR (75 MHz, methanol-d₄): δ 15.8 (CH₃), 25.8 (CH₂), 27.5 (3 CH₃ ^tBu), 29.0 and 29.1 (CH₂), 39.4 (C ^tBu), 40.1 (CH₂), 43.8 (NMe₂H⁺), 44.8 (CH₂N), 66.1 (CH₂O), 69.1 and 69.3 (2CH, C₅H₄), 70.1 and 70.4 (2CH, C₅H₄), 70.2 (5CH, Cp), 74.4 (C citrate), 87.9 (C_{ipso}), 115.1 and 115.3 (2CH C₆H₄), 115.3 and 115.5 (2CH C₆H₄), 131.5 and 131.7 (2CH C₆H₄), 132.0 and 132.2 (2CH C₆H₄), 137.3 (C), 137.9 (C), 139.3 (C), 139.6 (C), 143.6 (C), 143.9 (C), 174.9 (2 CO), 178.9 and 178.8 (CO), 179.4 (CO). IR (KBr, ν cm⁻¹): 3421 (OH), 3082, 3039, 2970, 2873 (CH₃, CH₂), 1736 (CO). Anal. calcd for C₄₂H₅₁FeNO₁₀·H₂O: C, 62.76; H, 6.64; N, 1.74. Found: C, 62.42; H, 6.62; N, 1.64.

5.2. Biology

5.2.1. Cell culture

Antiproliferative activity of all compounds was evaluated for four human tumor cell lines obtained from the National Cancer Institute (Bethesda, MD, USA): HL-60 (promyelocytic leukemia), MDA-MB-435 (melanoma), SF-295 (CNS glioblastoma) and HCT-8 (colon carcinoma). Cells were grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, 100 μ g/mL streptomycin and 100 U/mL penicillin at 37 °C in a 5% CO₂ atmosphere.

5.2.2. Sample preparation

Each compound was dissolved in DMSO to obtain a concentration of 5 mg/mL. For the experiments, the vehicle concentration, DMSO, was kept at a maximum of 0.1%.

5.2.3. MTT test

The growth inhibition activity of all compounds was tested against four tumor cell lines using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Aldrich Co., St. Louis, MO/USA) reduction assay [14]. For all experiments, cells were plated in 96-well plates in the following concentrations (cells/mL): SF-295: 0.1×10^6 ; HCT-8: 0.7×10^5 ; MDA-MB-435: 0.1×10^6 ; HL-60: 0.3×10^6 . The substances previously dissolved in DMSO were serially diluted in RPMI to obtain final concentrations ranging from 0.048 to 25 $\mu\text{g/mL}$ and incubated during 72 h. Control groups received the same amount of DMSO. After 69 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL). Three hours later, the MTT formazan product was dissolved in 150 μL of DMSO, and absorbance was measured at 595 nm (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, CA, USA). Doxorubicin (0.01–5 $\mu\text{g/mL}$) was used as positive control.

5.2.4. Measurement of the generation of reactive oxygen species

Intracellular reactive oxygen species (ROS) accumulation was monitored using 2',7'-dichlorodihydrofluorescein diacetate, which is converted to highly fluorescent dichlorofluorescein in the presence of intracellular ROS [35]. After 1 h of incubation with 5 μM of each compound, cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{-DCF-DA}$) (20 μM) and incubated at 37 °C for 30 min in the dark. β -Lapachone (5 μM) was used as positive control for ROS generation. Cells were then harvested, washed, resuspended in PBS and analyzed immediately by flow cytometry at excitation and emission wavelengths of 490 and 530 nm, respectively [36]. ROS generation was presented as DCF relative fluorescence.

5.2.5. Hemolysis

The methodology of Costa-Lotufo et al. (2002) [37] was used, allowing us to evaluate the potential of the test substance in causing injuries to the plasma membrane of the cell, either in the formation of pores or the total rupture. Blood was collected from the orbital plexus of Swiss mice (*Mus musculus*), being diluted in 30 volumes of NaCl 0.85% containing CaCl_2 10

mM. The erythrocytes had been washed 2 times by centrifugation (1500 rpm/3 min) and finally suspended at 2% in NaCl 0.85% containing CaCl₂ 10 mM. The assays had been carried through in 96 well-plates. The substances previously dissolved in DMSO were serially diluted in NaCl 0.85% containing CaCl₂ 10 mM to obtain final concentrations of 1–250 µg/mL and incubated during 1 h under constant agitation at room temperature (26 ± 2 °C). Triton X-100 (1%) was used as positive control. The samples were, then, centrifuged (5000 rpm/3 min) and the released hemoglobin was measured colorimetrically at 540 nm.

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