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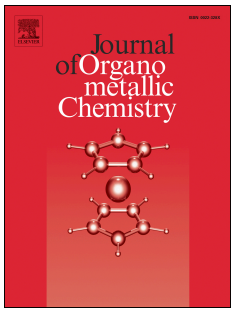

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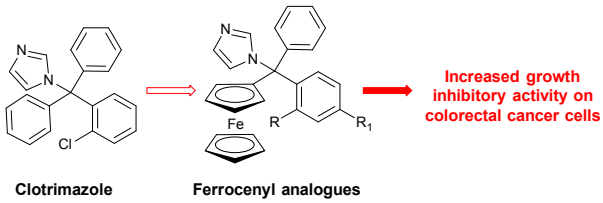
Graphical abstract

The introduction of a ferrocene unit in the clotrimazole scaffold led to increased biological activity compared to the parent drug



Synthesis of the ferrocenyl analogue of clotrimazole drug

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Abstract

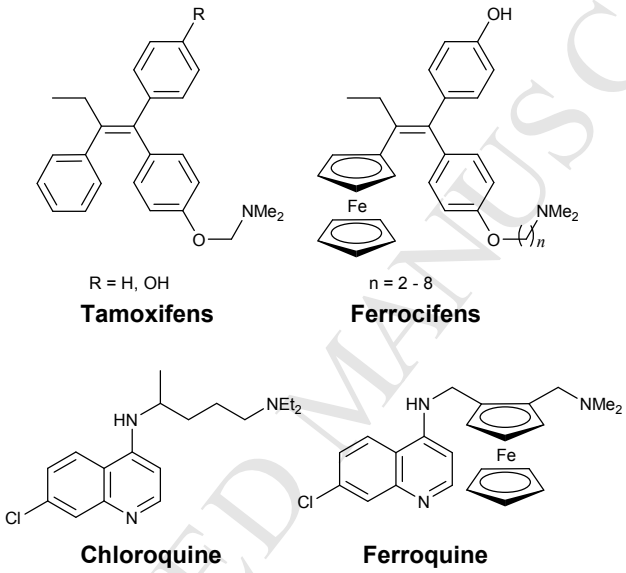
The ferrocenyl analogue of clotrimazole, in which the metallocene fragment replaces one of the phenyl rings in the triphenylmethane system, and a related isomer were prepared through the direct substitution of a methoxygroup in the α-position to ferrocene with imidazole in the key step. The obtained ferrocenyl derivatives were spectroscopically characterized and in a preliminary assay for bioactivity their cell growth inhibitory activity on two different human cancer cell lines was evaluated. In comparison with the parent drug the ferrocene analogues displayed about two-fold increase of cytotoxicity on HT29 colorectal cancer cells, whereas comparable activity was displayed against MCF-7 breast cancer cell line.

1. Introduction

Since the discovery of the anticancer activity of platinum(II) complexes, metal-containing compounds have attracted growing interest as drug candidates due to their peculiar physico-chemical properties and structural diversity.[1] Following the observations that the use of cisplatin and its derivatives in cancer therapy is associated with severe drawbacks,[2] the search for alternative coordination complexes[3] and organometallic compounds[4] with different metals was increasingly stimulated and several highly cytotoxic compounds were disclosed, many of which also showed innovative and metal-specific modes of action.[5] The potential of organometallic compounds for enzyme-inhibitors[6] and non-cancer therapeutics has been also explored,[7] leading to promising results in the field of anti-malarial drugs.[8]

In this context, ferrocene, the prototype of metallocenes, provides an useful platform in bio-organometallic chemistry for the synthesis of a large variety of derivatives with medicinal applications[9] and conjugates with biomolecules[10] due to its stability in aqueous aerobic media, non-toxicity, redox activity and chemical versatility. For its aromatic character and metabolism related to benzene,[11] the ferrocene can be considered a “bio-isosteric” fragment in replacement of a phenyl

ring in known drugs. On the basis of this approach, the Jaouen's group developed a series of ferrocenyl analogues (the "ferrocifen" family) of tamoxifen,[12] a chemotherapeutic agent for patients with hormone-dependent breast cancer, and some of them not only displayed higher antiproliferative effects with respect to the parent drug, but were also active on tumor cell lines lacking the overexpression of estrogen receptor and hence not susceptible to treatment with tamoxifen. A specific mechanism whereby redox activation induces anticancer activity in ferrocifen has been proposed[13] and it seems that the redox properties of iron in the ferrocene moiety also plays a role in ferroquine, an antimalarial drug candidate at clinical stage II derived by the incorporation of the metallocene fragment in the molecule of chloroquine (Scheme 1).[14]



Scheme 1. Ferrocenyl analogues of active drugs

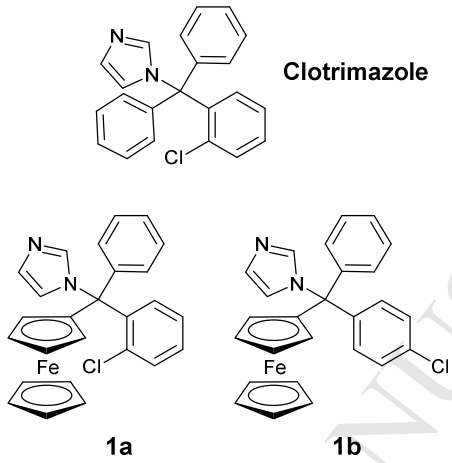
Other established drugs, as the antiandrogen nilutamide,[15] anticancer paclitaxel,[16] antimalarial artemisinin,[17] have been modified by using ferrocene as a substituent in their core structure, and the biologically activity is usually retained or enhanced.

Clotrimazole is a broad-spectrum antimycotic drug containing an imidazole ring linked to a triphenylmethyl group, a structural motif that has recently considered as pharmacophore in the design of novel antimalarial agents.[18] Clotrimazole also displays antiproliferative effects on different cancer cells, related with its activity as potent inhibitor of glycolysis.[19] Coordination compounds of clotrimazole with different metals[20] and ruthenium(II) organometallic derivatives[21] are reported, many of them with increased biological activity with respect to the original drug.

Although ferrocenylphenylimidazole[22] and some ferrocenyl(alkyl)azoles have been prepared and evaluated for their antitumor activity,[23] ferrocenyl derivatives of clotrimazole have not previously considered.

Here we describe the synthesis of two ferrocenyl analogues of clotrimazole, **1a** and **1b** (Scheme 2), in which the metallocene fragment replaces one of the phenyl rings in the triphenylmethane system, and the evaluation of their cell growth inhibitory effect on two different human cancer cell lines.

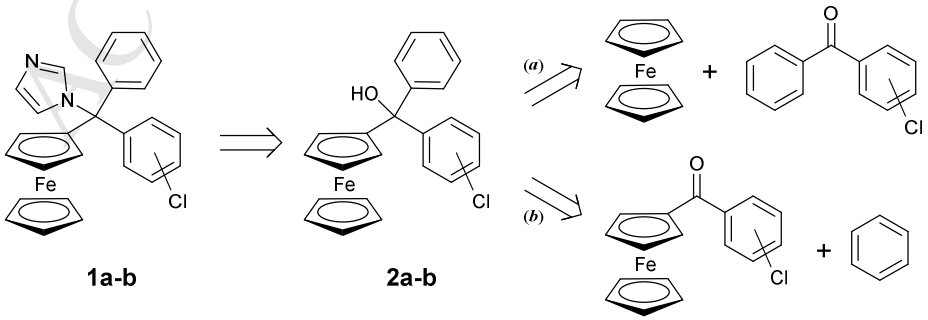
Although the inspiring molecule of clotrimazole contains an atom of chlorine in position 2, the isomeric compound **1b** was also prepared in order to detect possible effects on the biological activity related with the position of the chlorine.



Scheme 2. Ferrocenyl analogues of clotrimazole

2. Results and discussion

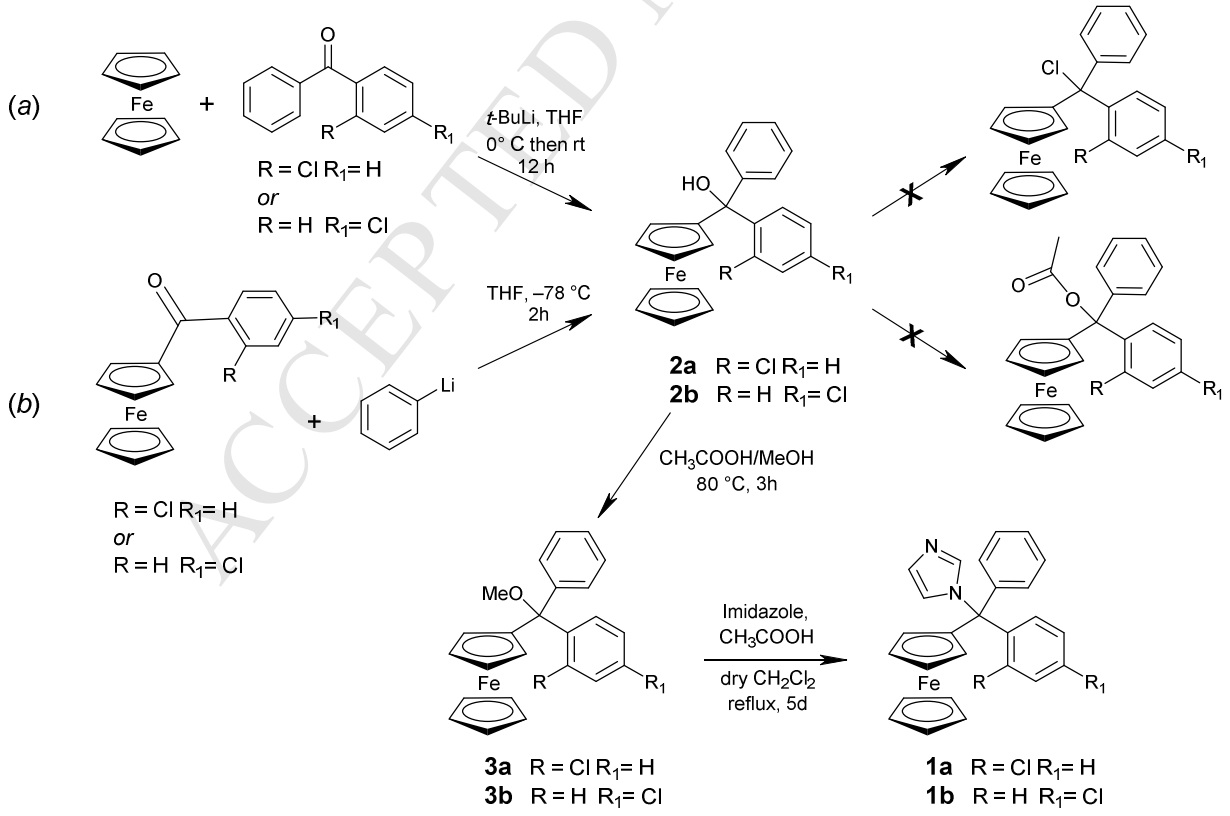
In the synthesis of clotrimazole and related derivatives the introduction of the heteroaromatic ring on the quaternary carbon center is usually accomplished by reaction of a suitable triphenylchloride with imidazole in the presence of a base and, according to this route, the ferrocenyl carbinols **2a-b** were then identified as key intermediate in the synthesis of the target compounds **1a-b**. Among the different pathways deduced from retrosynthetic analysis (Scheme 3), we firstly resorted into (***a***) as a more convenient way to the tertiary alcohols **2a-b** for the commercial availability of the starting benzophenones.



Scheme 3. Retrosynthetic analysis for ferrocenyl analogues of clotrimazole

Metalation of ferrocene with *tert*-BuLi in THF followed by reaction with 2-chloro- or 4-chlorobenzophenone for 12 h gave the expected alcohols **2a-b** in moderate yield (55-60%), that did not increase changing the reaction solvent or the reagent stoichiometry. In spite of the additional step required for the preparation of the starting ferrocenylketones (by Friedel-Crafts acylation of ferrocene[24]), the alternative reaction of 2- or 4-chlorobenzoylferrocene with a phenyl anion source (route (*b*) in Scheme 1) was also evaluated. The use of phenylmagnesium bromide was not effective, but the reaction in the presence of phenyllithium proceeded smoothly resulting in good yield (72-78%) of target alcohols (Scheme 4).

Ferrocenyl alcohols **2a-b** were characterized by their spectroscopic properties and in the ^{1}H-NMR spectra the metallocenic moiety was easily identified from the usual pattern for monosubstituted ferrocenes, with a singlet accounting for five protons on the unsubstituted cyclopentadienyl ring and distinct narrow multiplets for the protons on the other ring, in the typical region between 4.0 and 4.5 ppm. In $CDCl_3$ the resonances of phenyl protons appeared as complex multiplets for **2a** while only two singlets were observed for **2b**, indicating a magnetic near equivalence for both the substituted and unsubstituted rings in the diarylmethane moiety of the molecule. However, when the spectrum of **2b** was registered in $(CD_3)_2SO$ the expected pattern for a *para*-disubstituted benzene was observed, partially overlapped to the resonances of the other aromatic protons.



Scheme 4. Synthesis of the ferrocenyl analogues of clotrimazole

The conversion of alcohols **2a-b** into the ferrocenyl analogues of clotrimazole **1a-b** was attempted by treatment with $SOCl_2$ followed by *in situ* reaction with imidazole according a procedure reported for triphenylmethane derivatives,[18] but extensive degradation of the starting alcohols was observed.

Alternative ferrocene substrates for direct reaction with imidazole were then considered since it is well known that α-ferrocenylalkylderivatives with suitable leaving groups easily undergo the displacement of such groups by a broad variety of nucleophiles with retention of configuration and in mild conditions. This peculiar reactivity, originally demonstrated for α-ferrocenylalkyl trimethylammonium salts and α-ferrocenylacetates,[25] has been also reported for α-ferrocenyl alcohols with phosphines[26] and some *C*- and *N*-nucleophiles.[27]

The reaction of **2a** or **2b** with imidazole in water at 100 °C[27b] or in the presence of acid catalysis[26,27a] did not gave any product and the use of carbonyldiimidazole as alternative source of the nitrogen ring[22] was also unsuccessful.

The conversion of **2a** or **2b** into the corresponding acetates was then tried, without success, in standard conditions using acetic anhydride in combination with different bases (pyridine, Et_3N, Et_3N/DMAP) and reactions of esterification in the presence of Lewis acids,[28] with microwave irradiation or in solvent-free conditions[29] were also carried out, but the formation of the expected products was not detected in any case.

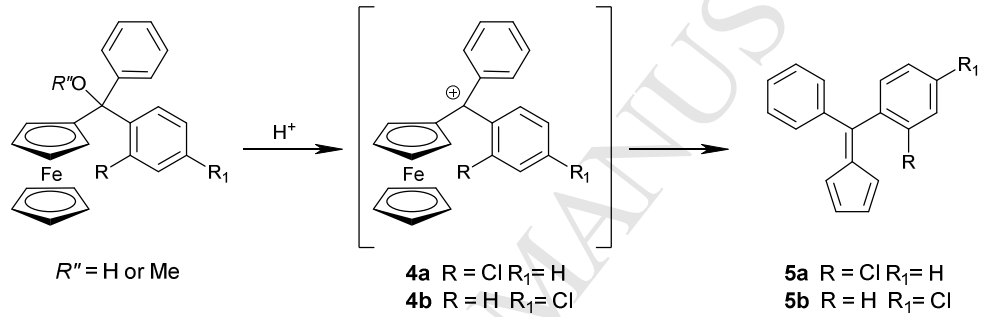
These discouraging results, otherwise in agreement with the reported unreactivity of sterically hindered tertiary ferrocenylalcohols,[27a,30] prompted us to look for different derivatives and, pleasantly, we were able to obtain the methyl ethers **3a** and **3b** in nearly quantitative yield by simple treatment of **2a** or **2b** with a mixture of acetic acid and methanol.

In spite of the exchange reaction of ferrocenyl α-methoxy substituent by nucleophiles is limited, at the best of our knowledge, to just two examples[31,32] on ferrocenes bearing the ether functionality on a secondary carbon, we succeeded into the synthesis of the target compounds **1a** and **1b** by treatment of ethers **3a** and **3b** with imidazole under acid catalysis. Although in acidic medium the protonation of the *N*-basic center of imidazole involves the use of large excess of this reagent, the acid catalysis was found strictly necessary. Attempts to decrease the reaction time using high-boiling solvents led to degradation of the substrate. Alcohols **2a** or **2b** in about 18-20 % yield were detected in the reaction mixture unless dry CH_2Cl_2 is used, so confirming the reported sensitivity of α-methoxyferrocenes to hydrolysis, further increased under acid catalysis.[32]

The described synthesis of the ferrocenyl analogues of clotrimazole, though moderately efficient in the last step (44% yield), provides an additional example in the short list of nucleophilic substitutions on fully α-substituted ferrocenes. The presence of the imidazole ring in the target compounds **1a** and **1b** was associated to three additional resonances in the aromatic region of the

^{1}H-NMR spectrum and to a sensible upfield shift for the ^{13}C-NMR signal of the quaternary carbon with respect to the oxygenated parent compounds. In the ESI-MS spectra of **1a** and **1b** the base peak derived from the preferential loss of the imidazole fragment from the molecule and molecular ion was hardly detected (<1%). Acquisition of MS spectra in SIM mode allowed us to better detect the molecular ion, so confirming the identity of the products.

A careful inspection of the reaction mixture of **3a** or **3b** with imidazole revealed the presence of side products identified as previously unreported diphenylfulvene derivatives **5a** or **5b**, resulting from Nesmeyanov fragmentation[33] of ferrocenyl carbenium ions **4a**-**b**, whose formation is favoured by the acidic medium (Scheme 5). The identification of **5a** an **5b** was mainly based on their ^{1}H-NMR spectra that displayed characteristic resonances with a complex *AA'XX'* coupling pattern for protons in the cyclopentadiene ring.[34]



Scheme 5. Fulvenes from ferrocenyl derivatives

Ferrocenyl analogues of clotrimazole **1a** and **1b** were preliminarly assayed for their cell growth inhibition activity on two different human cancer cell lines in comparison with clotrimazole. The cell models were chosen on the basis of the reported antiproliferative effects of clotrimazole[19] on breast cancer and colon cancer cell lines. As shown in Table 1 clotrimazole was more active in breast cancer cell MCF-7 than in colon cancer cell line HT29, although its activity (GI_{50} 21.44 μM) was lower than a classical anticancer chemotherapeutic drug, such as 5-fluorouracil (5-FU, GI_{50} 2.98 μM).

The growth inhibitory effect of clotrimazole on MCF-7 is in agreement with reported data[19a] and the present result on HT29 is comparable with that observed in murine colon adenocarcinoma cells.[19b]

Compounds **1a** and **1b** were active on MCF-7 cell line with a potency similar to that of the parent drug. Conversely, GI_{50} values more than halved compared to clotrimazole (Table 1) were determined for ferrocenyl derivatives **1a** and **1b** on HT29 cancer cell line and no significant difference related with the isomeric position of the chlorine atom in the molecules was observed.

The structural modification with ferrocene resulted in the enhancement of cytotoxic activity of the organometallic analogues in comparison with the parent drug in HT29 cells. This effect might be

related with the redox properties of iron in the ferrocene moiety[12,14] and its ability to generate cytotoxic reactive oxygen species (ROS) in proper cellular conditions.

Indeed, it has been suggested that cancer cells are more vulnerable to treatment with agents that generate ROS due to altered metabolism and mitochondrial functions.[35] Colon cancer cell lines accumulate high level of redox cycling metals and show high level of endogenous oxidative stress.[36] Further studies are necessary to evaluate the role of oxidative stress in induction of cytotoxicity by ferrocene derivatives.

Table 1. Growth inhibition assay on different cancer cell lines[a]

Compound	GI_{50} (μM)[b,c]	
	HT29 (HTB-38)	***MCF-7***(HTB-22)
Clotrimazole	64.19	21.44
1a	27.51	23.84
1b	28.13	20.44
5-FU[d]	5.71	2.98

[a]Growth inhibition was measured with colorimetric MTT assay (see experimental). [b]Values are reported as GI_{50}, the concentration of the compound required to cause 50% inhibition of cell growth. [c]Concentration-response curves are reported in supplemental material.[d]5-Fluorouracil.

3. Conclusion

Two ferrocenyl analogues of clotrimazole, in which the metallocene unit replaces one of the phenyl ring in the triarylmethane system, were prepared and their growth inhibitory activity assayed on two different human cancer cell lines MCF-7 and HT29. In spite of some chemical inertness of the intermediate tertiary ferrocenylalcohol, the corresponding ferrocenyl methyl ether was identified as a suitable substrate for the direct nucleophilic substitution with imidazole. In comparison with the parent drug the ferrocene derivatives displayed about two-fold increase of cytotoxicity on HT29 colorectal cancer cells, whereas comparable activity was displayed against MCF-7 breast cancer cell line and further studies are in progress to elucidate the molecular basis of the observed selectivity.

4. Experimental

4.1. General

^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance™ 400 spectrometer at 400.13 and 100.62 MHz, respectively. Chemical shifts (δ) are given as ppm relative to the residual solvent peak and coupling constants (*J*) are in Hz. In the NMR assignment Cp and Cp' refers to substituted

and unsubstituted cyclopentadienyl ring, respectively. Ferrocene, 2-chlorobenzophenone and 4-chlorobenzophenone were purchased from Aldrich. Column chromatography was performed on silica gel 60 (Merck, 40-63 μm) using the specified eluents. High resolution mass spectra (HR-MS) were obtained on a Thermofisher Orbitrap QExactive instrument with ESI ionization mode, using 3.2V cone voltage and 300 °C source temperature. Melting point are uncorrected. Curve-fitting and GI_{50} analysis was performed with the GraphPad Prism software (San Diego, California, USA).

4.2. Friedel-Craft acylation of ferrocene

To a suspension of $AlCl_3$ (413 mg, 3.1 mmol) and ferrocene (500 mg, 2.7 mmol) in CH_2Cl_2 (10 mL) at 0 °C, 2-chlorobenzoylchloride (353 μL, 2.8 mmol) or 4-chlorobenzoylchloride (350 μL, 2.7 mmol) in CH_2Cl_2 (3 mL) was added dropwise within 20 min. The mixture was warmed to room temperature and stirred overnight. After quenching at 0 °C by dropwise addition of ice-cold water (5 mL, **Caution**: gas evolution), the reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with satd. $NaHCO_3$ (2 × 50 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4 and taken to dryness to give a residue which was purified by column chromatography on silica gel (*n*-hexane:AcOEt 90:10 v/v). *2-Chlorobenzoylferrocene*: (715 mg, 2.2 mmol, 82% yield),red crystals (from *n*-hexane), mp 111-112 °C, ^{1}H-NMR ($CDCl_3$): δ 4.28 (s, 5H, Cp'H), 4.60 (t, *J* = 2.0, 2H, CpH), 4.75 (t, *J* = 2.0, 2H, CpH), 7.35-7.40 (m, 2H, ArH), 7.46 (dd, *J* = 8.0 and 1.2, 1H, ArH), 7.51 (dd, *J* = 7.4 and 1.6, 1H, ArH),; ^{13}C-NMR ($CDCl_3$): δ 70.13 (Cp'H), 71.10 (CpH), 72.89 (CpH), 78.54 (Cp), 126.20 (ArH), 128.64 (ArH), 130.28 (ArH), 130.70 (ArH), 131.05 (Ar), 139.51 (Ar), 198.66 (CO). HRMS calcd for $C_{17}H_{14}ClFeO$ $[M+H]^+$ 325.00826, found 325.00791.
4-Chlorobenzoylferrocene: (702 mg, 2.2 mmol, 80% yield), red crystals (from *n*-hexane), mp 119-120 °C, ^{1}H-NMR ($CDCl_3$): δ 4.21 (s, 5H, Cp'H), 4.62 (t, *J* = 1.6, 2H, CpH), 4.89 (t, *J* = 1.6, 2H, CpH), 7.46 (d, *J* = 8.4, 2H, ArH), 7.87 (d, *J* = 8.4, 2H, ArH); ^{13}C-NMR ($CDCl_3$): δ 70.34 (Cp'H), 71.51 (CpH), 72.80 (CpH), 78.42 (Cp), 128.59 (ArH), 129.58 (ArH), 137.81 (Ar), 138.11 (Ar), 197.81 (CO). HRMS calcd for $C_{17}H_{14}ClFeO$ $[M+H]^+$ 325.00826, found 325.00848.

4.3. Synthesis of 1-[(2-chlorophenyl)]-1-phenyl-1-hydroxymethylferrocene, 2a

Procedure A: Ferrocene (500 mg, 2.68 mmol) was dissolved in anhydrous THF (10 mL) under argon atmosphere and 1.7 M pentane solution of *t*-BuLi (1.6 ml, 2.68 mmol) was slowly added at 0°C under stirring. After 1 h 2-chlorobenzophenone (550 mg, 2.54 mmol) was added and the reaction mixture left to stand overnight at room temperature under stirring. The reaction was then quenched with H_2O and extracted with AcOEt (10 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4 and taken to dryness under vacuum. The residue was purified by column chromatography on silica gel (*n*-hexane-AcOEt 97:3 v/v) to give **2a** (565 mg, 1.40 mmol, 55% yield) as a yellow solid, mp = 129-130 °C; ^{1}H-NMR ($CDCl_3$): δ 3.78 (bs, 1H, CpH), 4.00 (s, 1H, -

OH), 4.19 (s, 5H, Cp'H), 4.26 (s, 1H, CpH), 4.33 (s, 2H, CpH), 7.16-7.20 (m, 3H, ArH), 7.27-7.38 (m, 3H, ArH); ^{13}C-NMR ($CDCl_3$): δ 67.78 (CpH), 68.05 (CpH), 68.64 (CpH), 68.90 (Cp'H), 70.30 (CpH), 78.54 (C-q), 98.12 (Cp), 125.96 (ArH), 126.86 (ArH), 127.02 (ArH), 127.27 (ArH), 128.82 (ArH), 130.36 (ArH), 131.28 (ArH), 133.29 (Ar), 144.28 (Ar), 145.06 (Ar). HRMS calcd for $C_{23}H_{19}ClFeO$ 402.04738, found 402.04758.

Procedure B: To a solution of 2-chlorobenzoylferrocene (200 mg, 0.62 mmol) in 10 mL of anhydrous THF, PhLi (350 µl of 1.8M solution in Et_2O, 0.63 mmol) was added at –78 °C and the mixture was allowed to stir for 2 h at this temperature. The reaction was quenched with H_2O and extracted with AcOEt (6 mL × 3). The organic layers were pooled, washed with brine and dried over Na_2SO_4. Evaporation of the solvent gave a residue that was purified by column chromatography on silica gel (*n*-hexane-AcOEt 97:3 v/v) to give pure **2a** (180 mg, 0.45 mmol, 72% yield).

4.4. Synthesis of 1-[(4-chlorophenyl)]-1-phenyl-1-hydroxy-methylferrocene, 2b

Reaction of ferrocene (500 mg, 2.68 mmol) and 4-chlorobenzophenone (550 mg, 2.54 mmol) according procedure A described above gave title compound **2b** as a yellow solid (543 mg, 1.35 mmol, 53%yield), mp = 115-116 °C ; ^{1}H-NMR (d_6-DMSO): δ 3.99 (s, 1H, CpH), 4.01 (s, 5H, Cp'H), 4.05 (s, 1H, CpH), 4.21 (s, 2H, CpH), 5.89 (s, 1H, -OH), 7.19-7.28 (m, 7H, ArH), 7.33 (d, *J* = 8.4, 2H, ArH); ^{13}C-NMR (d_6-DMSO): δ 67.96 (CpH), 67.99 (CpH), 69.00 (Cp'H), 77.03 (C-q), 97.63 (Cp), 127.03 (ArH), 127.46 (ArH), 127.56 (ArH), 129.38 (ArH), 131.50 (Ar), 147.84 (Ar), 148.52 (Ar). HRMS calcd for $C_{23}H_{19}ClFeO$ 402.04738, found 402.04746.

4.5. Synthesis of 1-[(2-chlorophenyl)]-1-phenyl-1-methoxy-methylferrocene, 3a

A sample of **2a** (200 mg, 0.50 mmol) was dissolved in 20 mL of MeOH/AcOH 8:1 v/v mixture and heated at 80°C until complete conversion of the substrate was obtained (2-3 h). The reaction mixture was taken to dryness to give **3a** as a yellow solid (184 mg 0.44 mmol, 88% yield) used without further purification. Mp = 121-122 °C; ^{1}H-NMR ($CDCl_3$): δ 3.38 (s, 3H, -OMe), 3.76 (bs, 1H, CpH), 4.16 (s, 5H, Cp'H), 4.17-4.20 (m, 2H, CpH), 4.24-4.26 (m, 1H, CpH), 7.22-7.24 (m, 2H, ArH), 7.26-7.34 (m, 6H, ArH), 7.58 (m, 1H, ArH); ^{13}C-NMR ($CDCl_3$): δ 52.68 (-OMe), 66.97 (CpH), 67.98 (CpH), 69.12 (CpH), 69.23 (Cp'H), 71.05 (CpH), 84.18 (C-q), 94.87 (Cp), 125.66 (ArH), 126.75 (ArH), 127.07 (ArH), 127.81 (ArH), 128.89 (ArH), 131.65 (ArH), 132.21 (ArH), 134.42 (Ar), 141.43 (Ar), 144.33 (Ar). HRMS calcd for $C_{24}H_{21}ClFeO$ 416.06303, found 416.06280.

4.6. Synthesis of 1-[(4-chlorophenyl)]-1-phenyl-1-methoxy-methylferrocene, 3b

Treatment of **2b** with MeOH/AcOH mixture as described above gave title compound **3b** in 90% as a yellow solid, mp = 111-112 °C; ^{1}H-NMR ($CDCl_3$): δ 3.21 (s, 3H, -OMe), 3.91 (bs, 2H, CpH), 4.08 (s, 5H, Cp'H), 4.21 (bs, 2H, CpH), 7.26-7.41 (m, 9H, ArH); ^{13}C-NMR ($CDCl_3$): δ 52.19 (-

OMe), 67.69 (CpH), 67.83 (CpH), 69.23 (Cp'H), 69.53 (CpH), 69.64 (CpH), 83.75 (C-q), 93.38 (Cp), 127.19 (ArH), 127.43 (ArH), 128.60 (ArH), 130.00 (ArH), 132.78 (Ar), 143.79 (Ar), 144.39 (Ar). HRMS calcd for $C_{24}H_{21}ClFeO$ 416.06303, found 416.06083.

4.7. Synthesis of 1-[(2-chlorophenyl) -1-phenyl-1-ferrocenylmethyl]-1*H*-imidazole, 1a

To a solution of **3a** (180 mg, 0.43 mmol) in anhydrous CH_2Cl_2 (20 mL) imidazole (290 mg, 4.26 mmol) and glacial acetic acid (400 µl) were added and the mixture was stirred under reflux for 5 days. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2 (5 mL × 3). Collected organic layers were dried on Na_2SO_4, filtered off and concentrated in a rotary evaporator to give a residue that was purified by column chromatography on silica gel. Elution of the column with *n*-hexane gave *6-phenyl-6'-(2-chlorophenyl)fulvene*, **5a** (16 mg, 0.06 mmol, 14% yield) as a pale orange oil, ^{1}H-NMR ($CDCl_3$): δ 5.95 (bdt, 1H), 6.47 (bdt, 1H), 6.52 (bdt, 1H), 6.63 (bdt, 1H), 7.33-7.40 (m, 9H).

Further elution of the column with *n*-hexane-AcOEt 60:40 v/v afforded pure **1a** (85 mg, 0.19 mmol, 44% yield) as yellow solid, mp = 139-140°C; ^{1}H-NMR ($CDCl_3$): δ 3.87 (bs, 1H, CpH), 4.04 (s, 6H, Cp'H and CpH), 4.25 (bs, 1H, CpH), 4.35 (bs, 1H, CpH), 6.44 (dd, *J* = 1.2 and 8.0, 1H, ArH), 7.13 (m, 2H, ArH and Im-H), 7.23 (bs, 1H, Im-H), 7.26 (dd, *J* = 1.2 and 8.0, 1H, ArH), 7.31 (dd, *J* = 1.6 and 8.4, 1H, ArH), 7.40-7.42 (m, 3H, ArH), 7.49-7.51 (m, 2H, ArH), 7.89 (s, 1H, Im-H); ^{13}C-NMR ($CDCl_3$): δ 67.22 (CpH), 68.91 (CpH), 69.74 (Cp'H), 70.14 (CpH), 71.28 (C-q), 72.64 (CpH), 95.25 (Cp), 121.38 (Im-H), 126.72 (ArH), 127.27 (Im-H), 127.83 (ArH), 128.30 (ArH), 128.79 (Ar-H), 129.59 (ArH), 130.47 (ArH), 131.83 (Ar-H), 134.96 (Ar), 139.10 (Im-H), 140.18 (Ar), 142.24 (Ar). HRMS calcd for $C_{23}H_{18}ClFe$ $[M\text{-}Im]^+$ (100% *relative intensity*) 385.04464, found 385.03906; calcd for $C_{26}H_{21}ClFeN_2$ $[M]^+$ (SIM mode) 452.07426, found 452.07370.

4.8. Synthesis of 1-[(4-chlorophenyl)-1-phenyl-1-ferrocenylmethyl]-1*H*-imidazole, 1b

A solution of **3b** (200 mg, 0.48 mmol) in anhydrous CH_2Cl_2 (20 mL) was reacted with imidazole (325 mg, 4.78 mmol) and acetic acid (480 µl) for 5 days and the reaction mixture worked up as above to give a residue that was purified by column chromatography on silica gel. Elution of the column with *n*-hexane gave *6-phenyl-6'-(4-chlorophenyl)fulvene*, **5b** (20 mg, 0.08 mmol, 16% yield) as a pale orange oil, ^{1}H-NMR ($CDCl_3$): δ 6.25-6.28 (m, 2H), 6.60-6.62 (m, 2H), 7.25-7.31 (m, 4H), 7.35-7.40 (m, 5H).

Further elution of the column with *n*-hexane-AcOEt 60:40 v/v afforded pure **1b** (95 mg, 0.21 mmol, 44% yield) as a yellow solid, mp = 135-136°C; ^{1}H-NMR ($CDCl_3$): δ 3.96 (bs, 1H, CpH), 3.98 (bs, 1H, CpH), 4.00 (s, 5H, Cp'H), 4.32 (bs, 2H, CpH), 7.00-7.03 (m, 3H, 2 × ArH and 1 × Im-H), 7.09 (dd, *J* = 2.0 and 8.0, 2H, ArH), 7.11 (s, 1H, Im-H), 7.30 (d, *J* = 8.4, 2H, ArH), 7.33-7.35 (m, 3H, ArH), 7.59 (s, 1H, Im-H); ^{13}C-NMR ($CDCl_3$): δ 68.51 (CpH), 68.68 (CpH), 69.62 (Cp'H), 70.53

(CpH), 70.72 (C-q), 70.77 (CpH), 94.19 (Cp), 121.14 (Im-H), 127.86 (ArH), 127.96 (ArH), 127.98 (ArH), 128.34 (ArH), 128.48 (ArH), 129.95 (ArH), 134.08 (Ar), 138.77 (Im-H), 142.62 (Ar), 143.42 (Ar). HRMS calcd for $C_{23}H_{18}ClFe$ $[M\text{-}Im]^+$ (100% *relative intensity*) 385.04464, found 385.03898; calcd for $C_{26}H_{21}ClFeN_2$ $[M]^+$ (SIM mode) 452.07426, found 452.07371.

4.9. Human Cell Cultures

Human colorectal adenocarcinoma cell line HT29 (ATCC number: HTB-38), and human mammary adenocarcinoma MCF-7 (ATCC number: HTB22) were cultured as previously reported.[37]

4.10. Cell growth inhibition assay

Stock solutions of compounds **1a**, **1b** and 5-fluorouracil (5-FU, positive control) were prepared in 9:1 dimethylsulfoxide (DMSO)/ Dulbecco Modified Eagle Medium (DMEM). Cancer cell lines (2.5-3.0 x 10^3 cells/0.33cm^2) were plated in 96 well plates "Nunclon TM Microwell TM" (Nunc), and incubated at 37 °C in the culture medium (DMEM + 10% fetal bovine serum). After 24 h, 10 µl of a solution of **1a**, **1b** or 5-FU at the suitable concentration were added to each culture well containing 90 µl of culture medium, so that the final concentration of DMSO was 1%. Concentrations of in the range 0.01 - 100µM (0.01, 0.1, 1.0, 10 and 100 µM) were tested.

Cells treated with 1% of DMSO were used as negative control. Microplates were incubated at 37 °C in humidified atmosphere of 5% CO_2, 95% air for 3 days, and cytotoxicity was measured with colorimetric assay based on the use of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).[38] Optical densities were read on a multiwell scanning spectrophotometer (PlateReader AF2200, Eppendorf, Milan, Italy), using a wavelength of 570 nm. Each value was the average of 6-8 wells. The GI_{50} value was calculated according to NCI[39] thus, GI_{50} is the concentration of test compound when 100 x $(T - T_0)/(C - T_0) = 50$ (where T is the optical density of the test well after a 72 h period of exposure to test compound, T_0 is the optical density at time zero, C is the DMSO control optical density). The cytotoxicity effect was calculated according to NCI when the optical density of treated cells was lower than the T_0 value using the following formula: 100 x $(T\text{-}T_0) / T_0$.

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- The ferrocenyl analogue of clotrimazole and an isomeric compound were synthesized
- The ferrocenyl derivatives were spectroscopically characterized
- The key synthetic step involves nucleophilic substitution of a tertiary alcohol
- Compounds were tested on two cancer cell lines for antiproliferative activity