

Synthesis of Novel Heterocyclic Ferrocenyl Chalcones and Their Biological Evaluation

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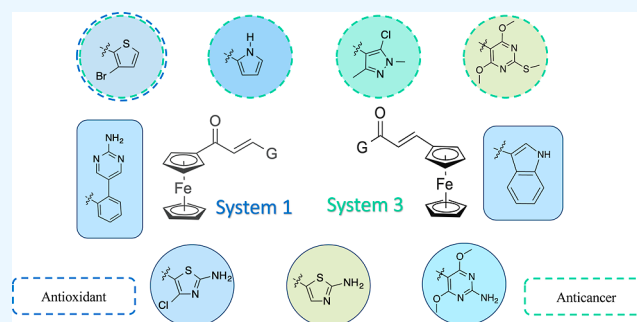


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ABSTRACT: Breast cancer is currently the most commonly diagnosed cancer, with 287,850 new cases estimated for 2022 as reported by the American Cancer Society. Therefore, finding an effective treatment for this disease is imperative. Chalcones are α,β -unsaturated systems found in nature. These compounds have shown a wide array of biological activities, making them popular synthetic targets. Chalcones consist of two aromatic substituents connected by an enone bridge; this arrangement allows for a large number of derivatives. Given the biological relevance of these compounds, novel ferrocene-heterocycle-containing chalcones were synthesized and characterized based on a hybrid drug design approach. These heterocycles included thiophene, pyrimidine, thiazolyl, and indole groups. Fourteen novel heterocyclic ferrocenyl chalcones were synthesized and characterized. Herein, we also report their cytotoxicity against triple-negative breast cancer cell lines MDA-MB-231 and 4T1 and the noncancer lung cell line MRC-5. System 3 ferrocenyl chalcones displayed superior anticancer properties compared to their system 1 analogues. System 3 chalcones bearing five-membered heterocyclic substituents (thiophene, pyrazole, pyrrole, and pyrimidine) were the most active toward the MDA-MB-231 cancer cell line with IC_{50} values from 6.59 to 12.51 μM . Cytotoxicity of the evaluated compounds in the 4T1 cell line exhibited IC_{50} values from 13.23 to 213.7 μM . System 3 pyrazole chalcone had consistent toxicity toward both cell lines ($IC_{50} \sim 13 \mu\text{M}$) as well as promising selectivity relative to the noncancer MRC-5 control. Antioxidant activity was also evaluated, where, contrary to anticancer capabilities, system 1 ferrocenyl chalcones were superior to their system 3 analogues. Antioxidant activity comparable to that of ascorbic acid was observed for thiophene-bearing ferrocenyl chalcone with $EC_{50} = 31 \mu\text{M}$.



INTRODUCTION

Breast cancer is currently the most commonly diagnosed cancer, with 287,850 new cases estimated for 2022, as reported by the American Cancer Society.¹ Among the types of breast cancer, triple-negative breast cancer (TNBC) is one of the most aggressive. Because TNBC does not overexpress the estrogen receptor, progesterone receptor, or HER2, it is significantly harder to diagnose and treat. Around 15% (~28,000 cases) of all breast cancer cases are of the triple-negative subtype, making it crucial to find effective treatment for this disease.

Chalcones are conjugated systems, in which two aromatic rings are linked by a α,β -unsaturated ketone (Figure 1). They can be found naturally or can be synthetically obtained and have a large number of known biological activities, such as antitumor activity against human breast cancer, inhibitory activity against certain enzymes, and antioxidant, anti-inflammatory, hypoglycemic, antihepatotoxic, antimicrobial, and antimalarial activities.^{2–10} Chalcones have been used as templates and precursors for many compounds, by adding

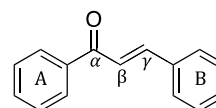


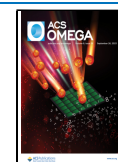
Figure 1. Chalcone scaffold with two aromatic rings (A,B) bridged by α,β -unsaturated ketone.

functional groups, changing the stereochemistry, or by modifications of the substituents. The organometallic ferrocene moiety has been introduced into the chalcone framework as either ring A or B. These ferrocenyl chalcones are divided into two groups: system 1 and system 3. Ferrocene as ring A at the 1 position corresponds to system 1, while

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ferrocene as ring B at the 3 position corresponds to system 3 (Figure 2). The ferrocene scaffold is widely applied in

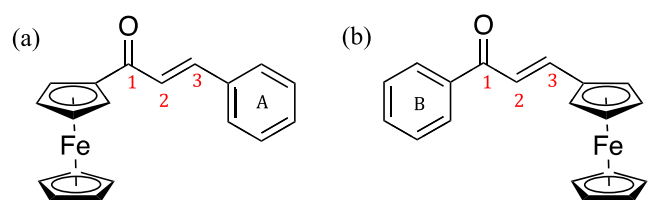


Figure 2. (a) System 1 ferrocenyl chalcones and (b) system 3 ferrocenyl chalcones.

biological systems and is used in the design of potential drugs given its favorable chemical properties.^{11,12} The most popular example of a ferrocene-bearing potential drug is tamoxifen's analogue, ferrocifen. Tamoxifen is a drug currently used to treat breast cancer, and by replacing one phenyl group with ferrocene, its biological activity improved significantly.¹³ This potential new drug is currently in clinical trials for the treatment of various cancers.¹⁴ Ferrocifen and its analogues have been extensively studied for their ferrocene-conferred properties.^{15–17} Among them is its capacity to be oxidized to ferrocenium (Fe^{3+}), for example, by hydrogen peroxide, which would produce a hydroxyl radical in the Fenton reaction. This hydroxyl radical has potential biological applications.^{18,19} Moreover, ferrocenium can be reduced back to ferrocene by NADH or other reducing enzymes.²⁰ This mechanism would mostly affect cancer cells due to the overproduction of hydrogen peroxide, which is not common in normal healthy cells.²¹ The oxidized and reduced species can follow different mechanisms of action, which makes them ideal scaffolds to treat aggressive diseases such as TNBC, among other multidrug-resistant illnesses.

Ferrocene is one of the aromatic substituents, and the second aromatic substituent also plays an important role in the biological and chemical properties of the resulting ferrocenyl chalcone. It has been reported that substitution with heterocycles generally provides pharmacological and pharmacokinetic benefits as drug candidates for further development.²² Heterocyclic substitution may cause changes in the degree of ionization of compounds in physiological pH, resulting in variations of basicity and lipophilicity.²³ Biological activities and pharmaceutical uses attributed to heterocycles as part of active molecules include antitubercular, analgesic, antipyretic, anti-inflammatory, antiplatelet, anti-HIV, antagonist, CNS depressant, antifungal, antibacterial, antioxidant, and anticancer activities.^{24–28} Amid the many prominent anticancer scaffolds that have replaced one or both aromatic rings in organic chalcones include diazepines, indoles, triazoles, thiazoles, thiophenes, and imidazolones, among many others.^{29–32} These compounds exhibited IC_{50} values ranging from 0.16 to 11 μM toward breast, lung, colon, cervix, leukemia, and cancer cell lines.^{31,33–35} Including ferrocene in

the chalcone moiety has been explored toward the search for novel anticancer agents. Among those studied are furan, pyrazole, and piperazine-bearing ferrocene–chalcone hybrids with IC_{50} values as low as 1 μM toward MDA-MB-231.^{36–40} This study aims to further broaden the library of ferrocene–chalcone hybrids by synthesizing 15 novel compounds bearing azoles, pyrimidines, pyrrole, and indole moieties. Compounds were obtained through a Claisen–Schmidt condensation reaction (Scheme 1) and characterized via ^1H NMR, ^{13}C NMR, IR, and UV–vis spectroscopy. Compounds' cytotoxicity toward the TNBC cell lines MDA-MB-231 and 4T1 were studied to establish the structural–activity relationship (SAR) between systems and the corresponding heterocycles. Selectivity of the evaluated compounds was assessed in the noncancer cell line MRC-5.

RESULTS AND DISCUSSION

Synthesis. System 1 ferrocenyl chalcones 1–8 (Table 1) were prepared from acetyl ferrocene and the corresponding heterocyclic aldehyde. These compounds were synthesized via the basic Claisen–Schmidt aldol condensation reaction (Table 2). All reactions were carried out under solvent-free conditions first, looking toward a greener approach. If these conditions were not successful, a solvent was then used. Compounds 1–3 bearing bromothiophene, pyrrole, and pyrazole heterocycles were obtained under solvent-free conditions, while 4–8 were obtained using a solvent either under reflux or at room temperature. Moderate to good yields were obtained for most compounds, with the lowest yield being compounds 4 and 7. For thiazolyl-bearing chalcone 4, the stability of the heterocycle played an important role in the reaction conditions. The amino-substituted thiazolyl group is easily oxidized given the nature of its heteroatoms. Because the amino group has a deactivating effect on the aldehyde toward a nucleophilic attack, stronger reaction conditions were needed; however, this resulted in decomposition of both the starting material and the product, producing lower yields. In addition, the resulting chalcone slowly decomposed, as it is not thermally stable and is photosensitive. On the other hand, for indole-bearing chalcone 7, similar obstacles were encountered as the corresponding aldehyde was moisture-sensitive and decomposed before reacting. Compound 7 was obtained from the protected aldehyde acetyl-indole carboxaldehyde, which resulted in a mixture of products and lower yields. As for compounds 5–6, as illustrated in Scheme 2, pyrimidine aldehydes with *o*-Cl substituents were used as a starting material. Varying bases and solvents resulted in no product formation. In search of a stronger base, *t*-BuOK/BuOH was used with no success. Nevertheless, when using *t*-BuOK in MeOH as the solvent, a product was obtained and properly characterized. It was observed that the methoxide species produced an in situ nucleophilic substitution on the *o*-Cl substituents, resulting in *o*-methoxylated ferrocenyl chalcones. In the case of compound 5, the use of a strong base caused the decomposition of the

Scheme 1. General Scheme of Claisen–Schmidt Aldol Condensation Reaction for System 1 and System 3 Heterocyclic Ferrocenyl Chalcones

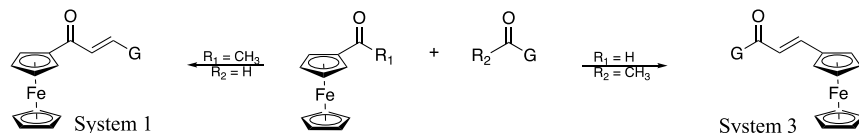


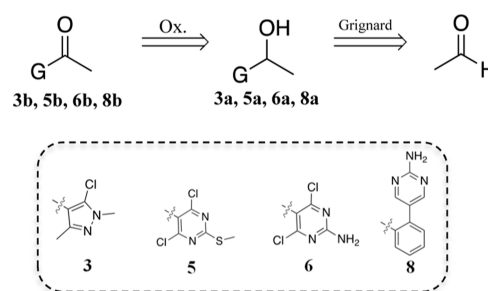
Table 1. System 1 and System 3 Contain Ferrocenyl Chalcones Bearing Heterocyclic Substituents

	System 1	System 3
	1	1'
	2	2'
	3	3'
	4	4'
	5	5'
	6	6'
	7	7'
	8	8'

corresponding aldehyde, resulting in trace yields and secondary products. The starting material for compound 6 resisted strong basic conditions, yielding the 1,5 *o*-methoxylated product.

It is noteworthy to mention that *o*-Cl-substituted chalcone formation could be detected by TLC, but it decomposed immediately, hinting that *o*-OCH₃ substituents have a stabilizing effect on the resulting product.

System 3 ferrocenyl chalcones 1'–7' (Table 1) were obtained similarly from ferrocene carboxaldehyde and corresponding heterocyclic acetophenone with overall similar

Scheme 2. Retrosynthetic Analysis for Non-commercially Available Acetophenones

yields. However, following TLC analysis, it became apparent that the reactions proceeded in higher yields, but purification of the final product was challenging and resulted in yields lower than anticipated. In terms of reactivity, this was to be expected given the electronic nature of the heterocyclic aldehydes used for system 1, which are less reactive than the ketones used for system 3.

Although for system 3 chalcones, the reactivity was manageable, for compounds 3', 5', 6', and 8', the starting material was not commercially available or economically feasible. The acetophenones were synthesized from aldehydes used for system 1 ferrocenyl chalcones. Thus, the aldehydes were reacted with methyl Grignard and subsequently oxidized to obtain the target ketones (Scheme 2). Alcohols 3a and 8a were obtained by reacting aldehydes with MeMgBr in THF at –10 °C (Table 3). At this same temperature, the methyl

Table 3. Synthetic Conditions of Grignard Reactions for the Corresponding Heterocyclic Alcohols

entry	Grignard	solvent	temp (°C)	time (h)	yield (%)
3a	MeMgBr	THF	–10	4.5	93
5a	MeMgBr	DCM	–78	4.5	75
6a	MeMgBr	THF	–78	4.5	81
8a	MeMgBr	THF	–10	4.5	65

Grignard reagent attacked the *o*-Cl-substituted carbon in pyrimidines 5 and 6, yielding multiple side-products including one and/or both ortho positions methylated. The corresponding *o*-Cl-substituted alcohols 5a and 6a were obtained at –78 °C, a temperature at which nucleophilic substitution by the Grignard reagent was avoided completely.

As for the oxidation of these alcohols, the optimized conditions are reported in Table 4. Non-amino-substituted 3b and 5b were obtained using the mild oxidizing agent pyridinium chlorochromate (PCC) and purified by column chromatography. However, for amino-containing 6 and 8,

Table 2. Synthetic Conditions for System 1 Ferrocenyl Chalcones (1–8) and System 3 Ferrocenyl Chalcones (1'–8')

entry	base	solvent	temp	time	yield (%)	entry	base	solvent	temp	time	yield (%)
1	NaOH		rt	15 min	75	1'	KOH aq	EtOH	rt	1 h	52
2	KOH		70 °C	15 min	48	2'	NaOH		70 °C	15 min	60
3	NaOH		70 °C	15 min	55	3'	NaOH aq	EtOH	Rt	3 h	34
4	KOH aq	EtOH	reflux	4 h	13	4'	NaOH	MeOH	reflux	4 h	40
5						5'	KOH	MeOH	rt	4 h	17
6	<i>t</i> -BuOK	MeOH	reflux	24 h	26	6'					
7	KOH	THF	reflux	24 h	15	7'	NaOH		70 °C	15 min	38
8	NaOH aq	EtOH	rt	4 h	57	8'					

Table 4. Synthetic Conditions of Oxidation Reactions for the Corresponding Heterocyclic Ketones

entry	oxidizing agent	solvent	temp	time (h)	yield (%)
3b	PCC	DCM	−10 °C-rt	6	92
5b	PCC	DCM	−10 °C-rt	6	48
6b	H ₂ Cr ₂ O ₇	Et ₂ O	−10 °C-rt	16	72
8b	H ₂ Cr ₂ O ₇	Et ₂ O	−10 °C-rt	16	

purification via column chromatography was not effective. To assess this synthetic obstacle, the crude oil from the preceding Grignard reaction was oxidized using chromic acid. This stronger oxidizing agent was utilized to oxidize the remaining aldehyde to carboxylic acid and the corresponding alcohol to ketone. Purification of **6b** and **8b** was achieved by extraction, and they were readily used for the synthesis of the corresponding system 3 ferrocenyl chalcones **6'** and **8'**. For compounds **5'** and **6'**, ketones **5b** and **6b** were reacted with ferrocene carboxaldehyde using methanol (MeOH) as the solvent, similarly to systems 1, 5 and 6, to obtain analogous *o*-methoxylated products. However, 1,5 *o*-methoxylated **6'** was not obtained. Instead, only one ortho position was methoxylated. This comes as a result of the weaker KOH base being used in comparison to *t*-BuOK used for system 1. In this case, using a stronger base resulted in the decomposition of ketone **6B**; hence, the reaction did not proceed. For system 3, product **8'** was likely achieved as observed by TLC; nevertheless, the traces of this product as well as the starting material decomposed almost immediately.

Establishing a pattern between the synthetic conditions for the preparation of system 1 and system 3 analogues, beyond what was aforementioned, was not possible given that the physical and chemical properties of each heterocycle are very different even between systems. The optimized conditions reported in Table 2 take into consideration the highest yield of the pure product obtained. Some of the attempted reaction conditions appeared to have higher crude yields but were arduous to purify. Several subsequent purifications yielded the pure product in lower yields.

Ferrocenyl chalcones were confirmed via ¹H NMR and ¹³C NMR where characteristic signals were assigned. In ¹H NMR, ferrocene signals appear between 4 and 5 ppm, alkene signals appear around 6–8 ppm, and heteroaryl signals appear at 6–9 ppm. Coupling constants (*J*) were determined and lay between 14 and 16 Hz, indicative of a *trans* conformation. As for ¹³C NMR, ferrocene signals are found in the 70–80 ppm range, while alkene and aromatic signals were observed anywhere between 100 and 175 ppm. Carbonyl signals varied between 180 and 195 ppm. IR spectra showed α,β -unsaturated system signals with carbonyls appearing around 1650 cm^{−1} and C=C alkene signals appearing around 1550 cm^{−1}. UV–vis absorption spectra of chalcones in DMSO at 50 μ M show two absorption peaks, at λ 300–365 nm with signals corresponding to the ferrocene moiety and at λ 400–500 nm with signals corresponding to the α,β -unsaturated system.

BIOASSAYS

The antioxidant activities of all synthesized ferrocenyl chalcones were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The half-maximal effective concentration (EC₅₀) of the compounds at micromolar concentrations (μ M) is reported in Table 5. A general trend demonstrated superior antioxidant activity for system 1

Table 5. EC₅₀ Values after 15 min of Incubation of Ferrocene–Chalcone Compounds 1–8 and 1'–8' with 50 μ M Solution of DPPH Free Radical

entry	EC ₅₀ (μ M)	entry	EC ₅₀ (μ M)
1	31 ± 1	1'	>300
2	>121 ^a	2'	>300
3	119 ^a	3'	>300
4	86 ^a	4'	>300
5	^b	5'	>300
6	>300	6'	^b
7	>300	7'	>300
8	65 ± 1	8'	^b
l-ascorbic acid	34 ± 1	paclitaxel	>300

^aResult obtained by interpolation in the equation for the best fit of the linear regression line instead of the dose–response curve analysis at GraphPad Prism6. ^bAssay was not performed for this compound. L-Ascorbic acid was used as control to validate the protocol.

ferrocenyl chalcones. System 3 derivatives did not show any antioxidant activity <300 μ M. Compounds 1, 2, 3, 4, and 8 displayed EC₅₀ < 300 μ M. Only bromo thiophene–ferrocenyl chalcone 1 had an effective concentration comparable to that of ascorbic acid with an EC₅₀ value of 31 ± 1. A noteworthy observation from this study is that most compounds that showed antioxidant capabilities (1–4) are five-membered heterocycles (thiophene, pyrrole, pyrazole, and thiazole, respectively). The most potent antioxidant agent was sulfur heterocycle thiophene. Sulfur's remarkable antioxidant potential comes as a result of its ability to expand its octet. Sulfur has the ability to accept and stabilize free radicals, making it an ideal heteroatom to include when searching for strong antioxidant agents. In fact, including sulfur in molecular structures has been used as a technique to improve the antioxidant activity of certain aromatic compounds.⁴¹ Moreover, primary amines are mostly beneficial to the antioxidant activity. Aromatic amines have the ability to donate protons that react with free radicals, which endows them with antioxidant properties.^{42,43} Antioxidants whose radical scavenging activity abilities come from hydrogen donation are considered to be primary antioxidants. On the other hand, those that quench radical species by virtue of their electronic properties are considered secondary antioxidants.⁴⁴ The superior antioxidant potential of system 1 derivatives comes as a result of system 1 ferrocenyl chalcones being more resistant to oxidation than system 3 chalcones, as reported by Wu et al. Higher polarization of the carbonyl bond is observed when adjacent to ferrocene; furthermore, the electron-withdrawing effect of the carbonyl makes the iron 2+ center more electron deficient and less likely to oxidize. On the other hand, system 3 analogues tend to be prone to oxidation.⁴⁵

Cytotoxicity of compounds was evaluated in the triple-negative MDA-MB-231 breast cancer cell line. Cytotoxicity was determined after 72 h of incubation at various concentrations (Figure S54). Prescreening of the compounds was performed at 0.1, 1, 10, and 100 μ M concentrations. The data suggest that four system 1 chalcones (S54a), three five-membered heterocycles (1–3), and one polycyclic (8) heterocycle showed 100% inhibition at 100 μ M concentration. Nevertheless, at ≤10 μ M, no system 1 ferrocenyl chalcone had a significant cytotoxic effect.

Prescreening of system 3 ferrocenyl chalcones as anticancer agents toward the MDA-MB-231 cell line (S54b) showed that

all tested compounds except for indole ferrocenyl chalcone **7'** had 100% cell growth inhibition at 100 μM concentrations. However, those compounds that showed some cytotoxicity at 10 μM concentration were selected for the determination of the half-maximal inhibitory concentration (IC_{50}) (Figure 3).

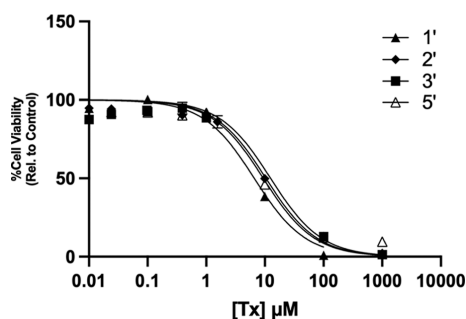


Figure 3. Cell viability percentage relative to the control MDA-MB-231 cancer cells treated with system 3 ferrocenyl chalcones at various concentrations.

Selected heterocyclic ferrocenyl chalcones were mostly those bearing five-membered heterocycles (thiophene, pyrrole, and pyrazole) **1'**–**3'**. Moreover, six-membered methylthio pyrimidine **5'** was also selected.

In terms of activity toward MDA-MB-2 (Table 6), $1' > 5' > 2' > 3'$ with IC_{50} values of 6.59, 9.30, 10.51, and 12.51 μM ,

Table 6. IC_{50} Values for System 3 Ferrocenyl Chalcones **1'**–**3'** and **5'**

entry	IC_{50} (μM)	
	MDA-MB-231	4T1
1'	6.59 \pm 1	32.90 \pm 1
2'	10.41 \pm 1	76.51 \pm 1
3'	12.51 \pm 2	13.23 \pm 1
5'	9.30 \pm 1	213.7 \pm 2

respectively. Again, the most potent derivative was **1'** S-heterocycle bromo thiophene–ferrocenyl chalcone. Additionally, good activity was also observed for N-heterocycles. It is a noteworthy observation that heterocycles bearing primary amines did not exhibit cytotoxicity. These data suggest that substitution with primary amines was detrimental for activity toward this cancer cell line. Compounds **1'**–**3'** and **5'** were further evaluated in 4T1 cancer cell lines (Figure 4). In human cell line MDA-MB-231, IC_{50} values were $< 20 \mu\text{M}$. However,

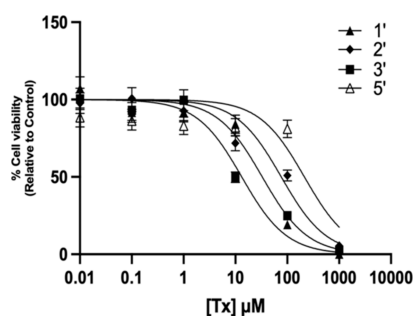


Figure 4. Cell viability percentage relative to the control of 4T1 cancer cells treated with system 3 ferrocenyl chalcones at various concentrations.

in the 4T1 mouse cell line, $\text{IC}_{50} < 100 \mu\text{M}$ with the exception of **5'** (Table 6). Only compound **3'** was consistent in toxicity on both cell lines with $\text{IC}_{50} < 15 \mu\text{M}$. For **1'**–**2'** and **5'**, the toxicity varied between cell lines, suggesting that the mechanism of action of these compounds varies according to the physiology, genotype, and phenotype of the cell and would need to be further studied.

The most promising compounds against MDA-MB-231 cells were advanced to further cell viability screenings against additional cell lines. The compounds were tested against the lung noncancer cell line MRC-5 to establish cancer cell selectivity (Figure 5). Compounds **1'**, **2'**, and **3'** exhibit a

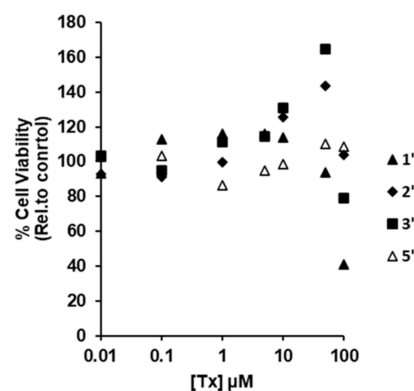


Figure 5. Cell viability percentage relative to control of the treatment with the heterocyclic ferrocenyl chalcones **1'**, **2'**, **3'**, and **5'** tested at concentrations 100, 50, 10, 5, 1, 0.1, and 0.01 μM against the noncancer lung cell line MRC-5.

similar trend of inducing cell proliferation in the midrange of concentrations tested. While compound **2'** returns to $\sim 100\%$ cell viability at 100 μM , compound **3'** induces $\sim 20\%$ viability inhibition, whereas compound **1'** induces nearly 60% inhibition. The cell viability data for compound **1'** could not be fit to determine an IC_{50} value due to the proliferative and antiproliferative behavior displayed throughout the concentration range, but it can be estimated to be between 50 and 100 μM . Compound **5'** does not induce any significant proliferation or inhibition throughout the concentration range examined. Based on its relatively potent activity against MDA-MB-231 and 4T1 cells and minor antiproliferative behavior against MRC-5 cells, compound **3'** displays promising cancer cell selectivity.

Rationalizing on the SAR of the evaluated ferrocenyl chalcone derivatives in the MDA-MB-231 cancer cell line, the anticancer activity of system 3 derivatives suggests an oxidative mechanism of action. As ferrocene is oxidized, it can participate in Fenton reactions, which has been proposed as one of the main mechanisms of action of ferrocenyl derivatives.⁴⁶ Supporting this theory, a correlation between system 1 antioxidant activity and system 3 cytotoxicity in the MDA-MB-231 cell line can be observed for five-membered heterocycles **1'**–**3'**. Nevertheless, deviation of the trend is observed when the heterocycle is substituted with primary amines. As previously established, aromatic amines are considered primary antioxidants and will have radical scavenging properties regardless of the system. Only system 1 ferrocenyl chalcone derivatives showed antioxidant activity as secondary antioxidants. This further supports an oxidative mechanism toward MDA-MB-231 cells. If the activity of

system 3 derivatives comes from the ability to participate in oxidative Fenton reactions, the substitution with primary antioxidants, such as amines, would counteract the formation of reactive oxygen species and render the compound inactive. In the case of the evaluated compounds in the 4T1 cell line, additional studies must be conducted in order to gain mechanistic insight.

CONCLUSIONS

Thirteen novel ferrocenyl chalcones bearing heterocyclic substituents thiophene, pyrrole, pyrazole, and pyrimidine were synthesized. Both system 1 and system 3 chalcones were synthesized and characterized. Their antioxidant capacity was evaluated and compared among system 1 and system 3 ferrocenyl chalcones. It was found that most system 1 ferrocenyl chalcones were moderate antioxidants. Compound 1, bromo thiophene-substituted ferrocenyl chalcone was the best antioxidant agent with an EC_{50} value of 31 μM , comparable to that of ascorbic acid. Because free radicals have been linked to cancer, heart disease, and degenerative conditions, potent antioxidants are pertinent to the search for the treatment of these diseases.⁴⁷ Contrarily, system 3 ferrocenyl chalcones demonstrated superior biological activity toward the TNBC cell line to its system 1 analogue. Among the studied heterocycles showing promising anticancer activity are thiophene, pyrrole, pyrazole, and pyrimidine system 3 ferrocenyl chalcones. System 3 thiophene-bearing ferrocenyl chalcone was the most active compound in the MDA-MB-231 cell line with an IC_{50} value of 6.58 μM , while in the 4T1 cell line, pyrazole 3' was the most active with an IC_{50} value of 13.23 μM . System 3 pyrazole ferrocenyl chalcone showed cytotoxicity toward both evaluated TNBC cell lines as well as favorable cell selectivity, representing a promising anticancer agent structure. Additional studies must be conducted in various breast cancer cell lines in order to further explore the substituents' effect on the cytotoxicity toward the search for superior anticancer agents. However, the SAR in this cancer cell line elucidated that in addition to S-heterocycles, all nonamino-substituted N-heterocycles showed good anticancer activity.

EXPERIMENTAL SECTION

Instrumentation. Proton (^1H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 500 MHz NMR spectrometer (Billerica, Massachusetts, United States) using deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$) as the solvent. Chemical shifts were recorded in units of parts per million (ppm, δ) downfield from the peak of the internal standard (TMS). ^1H NMR data are reported as follows: chemical shift (ppm), multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m)], and integration. ^{13}C NMR data are reported by chemical shift (ppm). Ultraviolet–visible (UV–vis) spectra were obtained using a Cary 300 UV–vis spectrophotometer (Agilent Technologies, Santa Clara, California, United States). All UV–vis spectra for the samples were collected at 50 μM in DMSO. Mass spectra were obtained using an AB SCIEX 4800 MALDI-TOF/TOF mass spectrometer in positive ion reflector mode (Framingham, Massachusetts, United States). The matrix for all samples examined by MALDI-TOF MS was composed of 5 mg/mL α -cyano-4-hydroxycinnamic acid and was prepared in a solvent composed of acetonitrile and water in a 1:1 (v/v) ratio with

trifluoroacetic acid at 0.1% (v/v). 1 μL of the matrix was placed on the MALDI wells and allowed to crystallize. Then, 1 μL of sample solutions (10 μM in 200 proof ethanol) was placed on top of the crystallized matrix and allowed to crystallize. The mass range was set from 200 to 1000 m/z . MS Excel was used to tabulate the data, and IsoPro 3.1 was used to obtain the theoretical spectra of the species identified. The theoretical and experimental intensities were normalized to a maximum signal of 100. Note that for all ferrocene species, two ion forms were detected: the Fe(III) form (instrumentally formed) and the Fe(II) form + H⁺ ion adduct. Multiwell plate absorbance was measured in a Tecan Infinite M200 PRO plate reader. Cells were grown in a Revco Elite III RCO5000T-5-ABC incubator. Cell counting was performed by the trypan blue method (1:5) with a hemocytometer. Culture viability monitoring was performed by using a Nikon Eclipse TS-100 microscope.

Cell Viability Assay. MDA-MB-231. We evaluated the therapeutic effect that our synthesized compounds had on TNBC cells with the CellTiter 96 Nonradiative Assay (Promega) as per the manufacturer's instructions. Briefly, MDA-MB-231 (TNBC cell line) cells were seeded on 96-well plates (4000 cells/well) and maintained with Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), under standard cell conditions (37 $^\circ\text{C}$, 5% CO_2 , humid atmosphere) for 24 h. Next, cells were treated with DMSO or each compound, with concentrations ranging from 100 μM to 10 nM. Cells were incubated with treatments for 72 h under standard cell conditions. After treatment, 15 μL of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) dye (0.5 mg/mL) was added to each well (with dead cells, living cells, and 100 μL of treatments with media), and the plates were incubated for 3 h. The reaction was stopped with the Kit's stop solution buffer, and the plate was incubated for another hour. The cell viability of TNBC cells upon the treatment of each compound was analyzed after reading absorbances at 570 nm and calculating the % of living cells (relative to 100 nM of DMSO-treated cells).

4T1. We evaluated the therapeutic effect that our synthesized compounds had on TNBC cells (4T1) with the CellTiter 96 Nonradioactive Assay (Promega) as per the manufacturer's instructions. Briefly, 4T1 (TNBC cell line) cells were seeded on 96-well plates (4000 cells/well) and maintained with Gibco's RPMI medium supplemented with 10% FBS (Invitrogen), under standard cell conditions (37 $^\circ\text{C}$, 5% CO_2 , humid atmosphere) for 24 h. Next, cells were treated with DMSO or each compound, with concentrations ranging from 100 μM to 10 nM. Cells were incubated with treatments for 72 h under standard cell conditions. After treatment, 15 μL of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) dye (0.5 mg/mL) was added to each well (with dead cells, living cells, and 100 μL of treatments with media), and the plates were incubated for 3 h. The reaction was stopped with Kit's stop solution buffer, and the plate was incubated for another hour. The cell viability of TNBC cells upon the treatment of each compound was analyzed after reading absorbances at 570 nm and calculating the % of living cells (relative to 100 nM DMSO-treated cells).

MRC-5. A thawed stock of MRC-5 cells was washed with 1 \times PBS and resuspended in phenol red DMEM (supplemented with 10% FBS and 1% penicillin–streptomycin) and then seeded in a 100 \times 20 mm (complete, O.D. \times H) Petri dish and

grown in a 5% (v/v) CO₂ humidified atmosphere at 37 °C. At least three passages were performed to ensure the integrity of the cells. After this point, cells were collected, thoroughly washed with 1× PBS, and then resuspended in phenol red-free DMEM (supplemented with 10% FBS, 1% penicillin–streptomycin, and 2.4 mM L-glutamine) at 2.0 × 10⁵ cells/mL concentration. A volume of 50 μL of cells was seeded into 96-well plates. Cells were incubated for 24 h. Stock solutions of heterocyclic ferrocenyl chalcones were prepared fresh in DMSO at concentrations of 50,000, 5000, 2500, 500, 250, 50, 5, and 0.5 μM. The MRC-5 cells were treated with 48 μL of media and 2 μL of the compound stock solutions to obtain final concentrations of 100, 50, 10, 5, 1, 0.1, and 0.01 μM in a final percentage of 2% (v/v) DMSO. The cells are tolerant toward this percentage of DMSO. Attempts were made to work with compounds at concentrations above 100 μM, but they resulted in significant precipitation within the cell wells interfering with the assay. Control wells in the plates consisted of one lane of cells treated with media alone including the 2% (v/v) DMSO as the measure of 100% viable cell growth in the media. Another control lane consisted of no cells with media including the 2% (v/v) DMSO as a measure of the background. A third control lane consisted of no cells in which the highest concentration of each compound was added. Due to the solution color of these compounds overlapping with the solution color of the formazin product, this lane was used to correct for the compound absorbance at the different dose concentrations. Cells were incubated for 68 h. A 25 μL MTT solution (5 mg/mL in 1× PBS, pH 7.4) was added. After 4 h, 50 μL of 4% (w/v) SDS (prepared in 0.1 M Tris buffer at pH 11) was added to solubilize the purple formazan product crystals and left overnight in the incubator. The plates were read at the wavelength of 570 nm (absorbance of the formazan product at pH > 10.0) and 800 nm (as a background correction) using a Tecan plate reader. The cytotoxic/antiproliferative behavior of the compounds was evaluated in MRC-5 cells. The absorbance of all wells was compared to the absorbance of the untreated cells with the MTT set as the 100% viable cell standard and the absorbance of the no-cell control set as the 0% viable cell standard. Nonlinear regression in Origin 8.5 was utilized to fit the growth curves using the pharmacology dose–response equation to determine the IC₅₀ and the corresponding standard deviation. All samples were tested six times with at least two biological replicates.

Antioxidant Assay. The radical scavenging assay was carried out in triplicate with the following protocol, which is an adaptation from the protocol reported by Sharma and co-workers, which was previously used at our research laboratory.⁴⁸ Ferrocenyl chalcones were dissolved in 99.9% methanol and serially diluted in the same solvent to concentrations from 300 to 0.1 mM. 50 μL of each sample dilution was transferred to a flat-bottom clear 96-well plate, and 50 μL of freshly prepared DPPH (100 mM) solution in methanol was added. Immediately after DPPH solution addition, the plate was inserted in the Infinite M200 PRO (Tecan) plate reader and the absorbance at 517 nm was determined after 15 min of incubation. In the same plate, three positive control wells (containing 50 mM DPPH solution without test compound solution) and three negative control wells (containing only methanol) were tested for comparison. Because our ferrocenyl chalcone presented absorbance at 517 nm, 50 mL of each sample dilution was transferred to the plate also in triplicate, but instead of DPPH solution, 50 mL of

methanol was added to eliminate the interference associated with this absorbance. Ascorbic acid, which is a commonly known antioxidant, was used as the standard for the assay. The absorbance of the positive control wells was taken as 100%, and the results are expressed as a percentage of this control. Dose–response curves were generated by using GraphPad Prism (v 6.0) biostatistics software to determine the EC₅₀. The EC₅₀ was defined as the effective concentration of the tested compound to decrease to 50% of the free DPPH radical concentration after 15 min of incubation.

Synthetic Methods. *Synthesis of (1) 3-(3-Bromothiophen-2-yl)-1-ferrocenylprop-2-en-1-one.* Acetylferrocene (0.25 mmol), 3-bromothiophene-2-carboxaldehyde (0.30 mmol), and crushed NaOH (0.50 mmol) were manually stirred. This solvent-free reaction mixture was manually stirred in a 50 mL round-bottomed flask for a period of 15 min at room temperature. The reaction was monitored by thin-layer chromatography (TLC) using 8:2 hexane/ethyl acetate as the mobile phase. The solid was filtered by suction using cold water and hexane. The solid was then recrystallized from ethanol and water. ¹H NMR (in ppm, CDCl₃, 500 MHz) 4.25 (s, 5H), 4.62 (s, 2H), 4.91 (s, 2H), 7.00 (d, 1H), 7.09 (d, 1H), 7.37 (d, 1H), 7.95 (d, 1H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 69.9, 70.2, 73.0, 80.6, 116.5, 124.0, 127.2, 131.4, 132.0, 135.4, 192.4. MALDI-TOF (positive): *m/z* 399.88, {[C₁₇H₁₃BrFe(III)OS]}⁺. UV–vis (DMSO): ε_{332 nm} = 14,840 M⁻¹ cm⁻¹; ε_{419 nm} = 3950 M⁻¹ cm⁻¹.

Synthesis of (2) 1-Ferrocenyl-3-(1H-pyrrol-2-yl)prop-2-en-1-one. For the synthesis of product 2, the reaction was carried out under solvent-free conditions at 70 °C. Acetylferrocene (0.25 mmol), 1H-pyrrole-2-carbaldehyde (0.25 mmol), and NaOH (0.50 mmol) were manually stirred. The reaction was monitored by TLC using 8:2 hexane/ethyl acetate as the mobile phase. The crude product was then extracted by using ethyl acetate and water. For purification purposes, the crude product was passed through a silica gel column with 8:2 hexane/ethyl acetate. ¹H NMR (δ in ppm, CDCl₃, 500 MHz) 4.20 (s, 5H), 4.55 (s, 2H), 4.89 (s, 2H), 6.34 (q, 1H), 6.72 (d, 1H), 6.98 (d, 1H), 7.68 (d, 1H), 8.73 (s, 1H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 69.7, 70.2, 72.3, 81.0, 111.5, 114.2, 117.1, 122.3, 129.5, 130.8, 192.3. MALDI-TOF (positive): *m/z* 304.94, {[C₁₇H₁₅Fe(III)NO]}⁺. UV–vis (DMSO): ε_{371 nm} = 17,750 M⁻¹ cm⁻¹; ε_{490 nm} = 1420 M⁻¹ cm⁻¹.

Synthesis of (3) 3-(3-Chloro-2,5-dimethyl-3H-2λ⁴-pyrazol-4-yl)-1-ferrocenylprop-2-en-1-one. For the synthesis of product 3, the reaction was carried out under solvent-free conditions at 70 °C. Acetylferrocene (0.25 mmol), 3-chloro-2,5-dimethyl-3H-2λ⁴-pyrazol-4-carbaldehyde (0.25 mmol), and NaOH (0.25 mmol) were manually stirred. The reaction was monitored by TLC using 8:2 hexane/ethyl acetate as the mobile phase. Afterward, the violet solid resulting from the reaction was vacuum-filtered and rinsed with cold water and hexane. The crude product was purified via column chromatography using 8:2 hexane/ethyl acetate. ¹H NMR (δ in ppm, CDCl₃, 500 MHz): 2.42 (s, 3H), 3.82 (s, 3H), 4.20 (s, 5H), 4.56 (s, 2H), 4.86 (s, 2H), 7.06 (d, 1H), 7.61 (d, 2H). ¹³C NMR (δ in ppm, CDCl₃, 125 MHz): 14.0, 36.2, 69.7, 70.1, 72.6, 80.9, 112.7, 121.4, 128.5, 129.6, 148.6, 193.0. MALDI-TOF (positive): *m/z* 367.98, {[C₁₈H₁₇ClFe(III)N₂O]}⁺. UV–vis (DMSO): ε_{315 nm} = 14,410 M⁻¹ cm⁻¹; ε_{386 nm} = 4320 M⁻¹ cm⁻¹; ε_{490 nm} = 1270 M⁻¹ cm⁻¹.

Synthesis of (4) (E)-3-(2-Aminothiazol-5-yl)-1-ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware

assembled with a magnetic bar and condensation column and sealed with a septum. For the synthesis of product **4**, 0.30 mmol of acetylferrocene and 0.25 mmol of 2-amino-5-formylthiazole were dissolved in 1 mL of EtOH. Aqueous solution of KOH was added dropwise (1 mL). This reaction was stirred vigorously under reflux for 4 h. The reaction was stopped with cold water and extracted using ethyl acetate. The crude product was purified by silica column chromatography in 6:4 hexane/ethyl acetate. ^1H NMR (δ in ppm, CDCl_3 , 500 MHz): 4.17 (s, 5H), 4.55 (s, 2H), 4.84 (s, 2H), 6.46 (d, 1H), 7.34 (s, 1H), 7.76 (d, 1H), 9.72 (s, 2H). ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz): 69.7, 70.1, 72.6, 80.5, 120.8, 126.0, 131.5, 144.9, 181.0, 192.6. MALDI-TOF (positive): m/z 337.93, $\{[\text{C}_{16}\text{H}_{14}\text{Fe(III)N}_2\text{OS}]\}^+$. UV-vis (DMSO): $\epsilon_{325\text{ nm}} = 25,700\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{392\text{ nm}} = 9360\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (6) (E)-3-(2-Amino-4,6-dimethoxypyrimidin-5-yl)-1-ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware assembled with a magnetic bar and condensation column and sealed with a septum. Then, 0.30 mmol of acetylferrocene and 0.25 mmol of 2-amino-4,6-dichloropyrimidine-5-carbaldehyde with 1 mmol of *t*-butoxide were dissolved in 1 mL of methanol and heated to reflux. This reaction was stirred vigorously for 24 h. The reaction was stopped with cold water, and the crude product was filtered. The crude product was purified through column chromatography using 7:3 hexane: ethyl acetate. ^1H NMR (δ in ppm, CDCl_3 , 500 MHz): 4.01 (s, 6H), 4.19 (s, 5H), 4.51 (s, 2H), 4.87 (s, 2H), 5.03 (s, 2H), 7.28 (d, 1H), 7.95 (d, 1H). ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz): 54.3, 69.8, 70.2, 72.2, 81.8, 92.8, 122.4, 130.4, 161.1, 170.3, 194.3. MALDI-TOF (positive): m/z 393.01, $\{[\text{C}_{19}\text{H}_{19}\text{Fe(III)N}_3\text{O}_3]\}^+$. UV-vis (DMSO): $\epsilon_{365\text{ nm}} = 27,720\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (7) 1-H-3-Indole-1-ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware assembled with a magnetic bar and condensation column and sealed with a septum. Under an anhydrous N_2 atmosphere, 0.30 mmol of acetylferrocene and 0.25 mmol of *N*-acetylindole-3-carboxaldehyde with 1 mmol of potassium hydroxide were weighed and dissolved in 1 mL of THF and heated under reflux. This reaction mixture was stirred vigorously for 24 h. The reaction was stopped with cold water, and the crude product was filtered. The crude product was purified through column chromatography using 8:2 hexane: ethyl acetate followed by recrystallization in ethanol: water mixture. ^1H NMR (δ in ppm, CDCl_3 , 500 MHz): 4.20 (s, 5H), 4.60 (s, 2H), 4.99 (s, 2H), 7.22 (d, 1H), 7.33 (m, 2H), 7.47 (d, 1H), 7.61 (s, 1H), 8.08 (m, 2H), 8.64 (s, 1H). ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz): 69.7, 69.9, 72.4, 81.2, 112.1, 114.3, 119.2, 120.6, 121.6, 123.4, 125.6, 129.9, 134.9, 137.3, 193.7. MALDI-TOF (positive): m/z 355.01, $\{[\text{C}_{21}\text{H}_{17}\text{Fe(III)NO}]\}^+$. UV-vis (DMSO): $\epsilon_{290\text{ nm}} = 11,790\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{375\text{ nm}} = 6550\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (8) 4-(2-(2-Aminopyrimidin-5-yl)phenyl) 1-Ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware assembled with a magnetic bar. Then, 0.30 mmol of acetylferrocene and 0.25 of 2-(2-aminopyrimidin-5-yl)-benzaldehyde were added and dissolved in 1 mL of EtOH. An aqueous solution of KOH was added dropwise (1 mL). This reaction mixture was stirred vigorously at room temperature for 4 h. The reaction was stopped with cold water, and the crude product was filtered. The crude product was purified by recrystallization from an acetone and water mixture. ^1H NMR (δ in ppm, CDCl_3 , 500 MHz): 4.21 (s, 5H),

4.58 (s, 2H), 4.86 (s, 2H), 5.25 (s, 2H), 7.03 (d, 1H), 7.32 (d, 1H), 7.46 (m, 2H), 7.76 (d, 1H), 7.78 (d, 1H), 8.32 (s, 2H). ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz): 69.7, 70.1, 72.8, 80.5, 124.0, 125.5, 127.5, 128.3, 130.0, 130.5, 134.0, 136.6, 138.7, 158.4, 162.2, 192.5. MALDI-TOF (positive): m/z 409.02, $\{[\text{C}_{23}\text{H}_{19}\text{Fe(III)N}_3\text{O}]\}^+$. UV-vis (DMSO): $\epsilon_{300\text{ nm}} = 21,710\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{340\text{ nm}} = 11,410\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{401\text{ nm}} = 6270\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (1') 1-(3-Bromothiophen-2-yl)-3-ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware assembled with a magnetic bar. Then, 0.25 mmol of ferrocenecarboxaldehyde and 0.25 of 2-acetyl-3-bromothiophene were added and dissolved in 1 mL of EtOH. An aqueous solution of KOH 2 M was added dropwise (1 mL). This reaction was stirred vigorously at room temperature for 1 h. The reaction was stopped with cold water, and the crude product was filtered. The product was purified by silica column chromatography in 9:1 hexane/ethyl acetate. ^1H NMR (in ppm, CDCl_3 , 500 MHz) 4.23 (s, 5H), 4.60 (s, 2H), 4.89 (s, 2H), 6.97 (d, 1H), 7.09 (d, 1H), 7.01 (d, 1H), 7.35 (d, 1H), 7.93 (d, 1H); ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz) 69.8, 70.1, 72.9, 80.5, 116.3, 123.9, 127.0, 131.2, 131.9, 135.2, 192.2. MALDI-TOF (positive): m/z 399.86, $\{[\text{C}_{17}\text{H}_{13}\text{BrFe(III)-OS}]\}^+$. UV-vis (DMSO): $\epsilon_{335\text{ nm}} = 13,790\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{507\text{ nm}} = 9770\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (2') 3-Ferrocenyl-1-(1H-pyrrol-2-yl)prop-2-en-1-one. For the synthesis of product **2'**, the reaction was carried out under solvent-free conditions at 70 °C. Ferrocenecarboxaldehyde (0.25 mmol), 2-acetylpyrrole (0.25 mmol), and NaOH (0.5 mmol) were added to a round-bottom flask and manually agitated for 15 min. The reaction was stopped with cold water, and the crude product was filtered. The product was purified by silica column chromatography in 8:2 hexane/ethyl acetate. ^1H NMR (δ in ppm, CDCl_3 , 500 MHz) 4.20 (s, 5H), 4.55 (s, 2H), 4.88 (s, 2H), 6.34 (d, 1H), 6.71 (m, 1H), 6.72 (d, 1H), 6.98 (s, 1H), 7.68 (d, 1H), 8.72 (s, 1H); ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz) 69.7, 70.2, 72.6, 81.0, 111.5, 114.2, 117.1, 122.3, 129.5, 130.8, 192.9. MALDI-TOF (positive): m/z 304.95, $\{[\text{C}_{17}\text{H}_{15}\text{Fe(III)NO}]\}^+$. UV-vis (DMSO): $\epsilon_{337\text{ nm}} = 18,440\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{487\text{ nm}} = 2700\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (3') 1-(3-Chloro-2,5-dimethyl-3H-2 λ^4 -pyrazol-4-yl)-3-ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware assembled with a magnetic bar. Then, 0.25 mmol of ferrocenecarboxaldehyde and 0.25 mmol of 1-(5-chloro-1,3-dimethyl-1H-pyrazol-4-yl)ethan-1-one were combined and dissolved in 1 mL of EtOH. An aqueous solution of 2 M NaOH 2 M was added dropwise (1 mL). This reaction mixture was stirred vigorously at room temperature for 4 h. The reaction was stopped with cold water, and the crude product was filtered. The product was purified by silica column chromatography in 9:1 hexane/ethyl acetate. ^1H NMR (δ in ppm, CDCl_3 , 500 MHz) 2.42 (s, 3H), 3.82 (s, 3H), 4.20 (s, 5H), 4.56 (s, 2H), 4.86 (s, 2H), 7.06 (d, 1H), 7.01 (d, 2H). ^{13}C NMR (δ in ppm, CDCl_3 , 125 MHz): 14.0, 36.2, 69.7, 70.1, 72.6, 80.87, 112.7, 121.4, 128.5, 129.6, 148.6, 193.0. MALDI-TOF (positive): m/z 367.98, $\{[\text{C}_{18}\text{H}_{17}\text{ClFe(III)N}_2\text{O}]\}^+$. UV-vis (DMSO): $\epsilon_{318\text{ nm}} = 15,410\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{474\text{ nm}} = 4529\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (4') (E)-1-(2-Aminothiazol-5-yl)-3-ferrocenylprop-2-en-1-one. This reaction was set up on dried glassware assembled with a magnetic bar. Then, 0.25 mmol of ferrocenecarboxaldehyde and 0.25 mmol of 1-(2-amino-1,3-

thiazol-5-yl)ethan-1-one were added and dissolved in 1 mL of MeOH. An aqueous solution of 2 M NaOH 2 M was added dropwise (1 mL). This reaction mixture was stirred vigorously under reflux for 4 h. The reaction was stopped with cold water, and the crude product was extracted using EtOAc/H₂O. The product was recrystallized from acetone and water. ¹H NMR (δ in ppm, CDCl₃, 500 MHz): 4.20 (s, 5H), 4.49 (s, 2H), 4.75 (s, 2H), 7.19 (d, 1H), 7.32 (s, 2H), 7.56 (s, 1H), 8.05 (d, 2H). ¹³C NMR (δ in ppm, CDCl₃, 125 MHz): 68.9, 69.4, 70.8, 79.6, 118.9, 131.1, 142.3, 147.0, 174.3, 179.6. MALDI-TOF (positive): *m/z* 337.95, {[C₁₆H₁₄Fe(III)N₂OS]}⁺. UV-vis (DMSO): $\epsilon_{365\text{ nm}} = 16,210\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{493\text{ nm}} = 2850\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (5') *(E)-1-(4,6-Dimethoxy-2-(methylthio)pyrimidin-5-yl)-3-ferrocenylprop-2-en-1-one*. This reaction was set up on dried glassware assembled with a magnetic bar and condensation column and sealed with a septum. Then, 0.30 mmol of ferrocenecarboxaldehyde and 0.25 of 1-(4,6-dichloro-2-(methylthio)pyrimidin-5-yl)ethan-1-one with 1 mmol of NaOH were dissolved in 1 mL of MeOH. This reaction was stirred vigorously for 4 h at room temperature. The reaction was stopped with cold water, and the crude product was filtered. The crude product was purified through silica column chromatography using 7:3 hexane: ethyl acetate. ¹H NMR (δ in ppm, CDCl₃, 500 MHz): 2.60 (s, 3H), 3.99 (s, 6H), 4.16 (s, 5H), 4.47 (s, 2H), 4.51 (s, 2H), 6.53 (d, 1H), 7.28 (d, 1H). ¹³C NMR (δ in ppm, CDCl₃, 125 MHz): 14.1, 54.5, 69.1, 69.9, 71.6, 78.4, 100.5, 125.4, 147.7, 167.2, 171.5, 190.5. MALDI-TOF (positive): *m/z* 424.00, {[C₂₀H₂₀Fe(III)-N₂O₃S]}⁺. UV-vis (DMSO): $\epsilon_{317\text{ nm}} = 16,610\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{435\text{ nm}} = 7050\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (7') *1-H-1-Indole-3-ferrocenylprop-2-en-1-one*. For the synthesis of product 7', the reaction was carried out under solvent-free conditions at 70 °C. Ferrocenecarboxaldehyde (0.25 mmol), 3-acetylindole (0.25 mmol), and NaOH (1.0 mmol) were added to a round-bottom flask and manually agitated for 15 min. The reaction was stopped with cold water, and the crude product was filtered. The product was purified by silica column chromatography in 8:2 hexane/ethyl acetate. ¹H NMR (δ in ppm, DMSO, 500 MHz) 4.19 (s, 5H), 4.49 (s, 2H), 4.81 (s, 2H), 7.21 (m, 2H), 7.32 (d, 1H), 7.5 (m, 2H), 8.33 (d, 1H), 8.57 (s, 1H), 12.01 (s, 1H); ¹³C NMR (δ in ppm, DMSO, 125 MHz) 69.2, 69.8, 70.9, 80.4, 112.6, 118.0, 122.1, 122.2, 122.3, 123.4, 126.4, 134.4, 137.3, 141.0, 183.8. MALDI-TOF (positive): *m/z* 355.01, {[C₂₁H₁₇Fe(III)NO]}⁺. UV-vis (DMSO): $\epsilon_{337\text{ nm}} = 57,140\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{476\text{ nm}} = 6010\text{ M}^{-1}\text{ cm}^{-1}$.

Synthesis of (3a) *1-(5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)ethan-1-ol*. This reaction was set up on dried glassware assembled with a magnetic bar under a N₂ atmosphere. 2.00 mmol of 3-chloro-2,5-dimethyl-3H-2 λ^4 -pyrazol-4-carbaldehyde was dissolved in 3 mL of anhydrous THF. 2 mL of MeMgBr 3 M reagent was added dropwise, and the mixture was stirred for 4 h at -10 °C. The reaction was stopped with cold water and neutralized with a saturated solution of ammonium chloride. The product was extracted using EtOAc/H₂O and was used for the next step without further purification. ¹H NMR (δ in ppm, CDCl₃, 500 MHz) 1.41 (d, 3H), 2.17 (s, 3H), 3.52 (s, 1H), 3.61 (s, 3H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 13.1, 22.7, 35.5, 61.9, 118.7, 124.9, 146.1.

Synthesis of (5a) *1-(4,6-Dichloro-2-(methylthio)pyrimidin-5-yl)ethan-1-ol*. This reaction was set up on dried glassware assembled with a magnetic bar under a N₂

atmosphere. 2.00 mmol of 4,6-dichloro-2-methylsulfanylpyrimidine-5-carbaldehyde was dissolved in 3 mL of anhydrous THF. 2 mL of MeMgBr 3 M reagent was added dropwise, and the mixture was stirred for 4 h at -10 °C. The reaction was stopped with cold water and neutralized with a saturated solution of ammonium chloride. The product was extracted using EtOAc/H₂O and purified by silica column chromatography in 9:1 hexane/ethyl acetate. ¹H NMR (δ in ppm, CDCl₃, 500 MHz) 1.54 (d, 3H), 2.50 (s, 3H), 3.44 (s, 1H), 5.35 (q, 1H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 14.3, 20.7, 65.9, 127.5, 160.0, 171.3.

Synthesis of (6a) *1-(2-Amino-4,6-dichloropyrimidin-5-yl)ethan-1-ol*. This reaction was set up on dried glassware assembled with a magnetic bar under a N₂ atmosphere. 2.00 mmol of 2-amino-4,6-dichloropyrimidine-5-carbaldehyde was dissolved in 3 mL of anhydrous THF. 3 mL of MeMgBr 3 M reagent was added portionwise, and the mixture was stirred for 4 h at -78 °C. The reaction was stopped with cold water and neutralized with a saturated solution of ammonium chloride. The product was extracted using EtOAc/H₂O and purified by silica column chromatography in 8:2 hexane/ethyl acetate. ¹H NMR (δ in ppm, DMSO, 500 MHz) 1.41 (d, 3H), 2.17 (s, 3H), 3.52 (s, 1H), 3.61 (s, 3H); ¹³C NMR (δ in ppm, DMSO, 125 MHz) 21.3, 64.5, 120.7, 160.6, 161.0.

Synthesis of (8a) *1-(2-(2-Aminopyrimidin-5-yl)phenyl)ethan-1-ol*. This reaction was set up on dried glassware assembled with a magnetic bar under a N₂ atmosphere. 2.00 mmol of 2-(2-aminopyrimidin-5-yl)benzaldehyde was dissolved in 3 mL of anhydrous THF. 3 mL of MeMgBr 3 M reagent was added portionwise, and the mixture was stirred for 4 h at -78 °C. The reaction was stopped with cold water and neutralized with a saturated solution of ammonium chloride. The product was extracted using EtOAc/H₂O and was used for the next step without further purification. ¹H NMR (δ in ppm, CDCl₃, 500 MHz): 1.25 (d, 3H), 4.71 (o, 1H), 5.10 (d, 1H), 6.72 (s, 2H), 7.14 (dd, 1H), 7.28 (td, 1H), 7.38 (td, 1H), 7.61 (dd, 1H), 8.21 (s, 2H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 25.7, 65.0, 123.1, 126.5, 127.3, 128.3, 130.2, 134.2, 145.4, 157.9, 163.0.

Synthesis of (3b) *1-(5-Chloro-1,3-dimethyl-1H-pyrazol-4-yl)ethan-1-one*. The crude product of 1-(5-chloro-1,3-dimethyl-1H-pyrazol-4-yl)ethan-1-ol was dissolved in dry DCM, and 5 mmol of PCC was added at -10 °C. The reaction mixture was stirred for 6 h under a N₂ atmosphere and allowed to reach room temperature. The reaction mixture was filtered through Celite and extracted using EtOAc/H₂O and purified by silica column chromatography in 8:2 hexane/ethyl acetate. ¹H NMR (δ in ppm, CDCl₃, 500 MHz) 2.43 (s, 3H), 2.50 (s, 3H), 3.81 (s, 3H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 15.3, 30.1, 36.4, 117.2, 130.3, 151.1, 191.9.

Synthesis of (5b) *1-(4,6-Dichloro-2-(methylthio)pyrimidin-5-yl)ethan-1-one*. 1.5 mmol portion of 1-(4,6-dichloro-2-(methylthio)pyrimidin-5-yl)ethan-1-ol was dissolved in dry DCM and 5 mmol of PCC was added at -10 °C. The reaction mixture was stirred for 6 h under a N₂ atmosphere and allowed to reach room temperature. The reaction mixture was filtered through Celite and extracted using EtOAc/H₂O and purified by silica column chromatography in 8:2 hexane/ethyl acetate. ¹H NMR (δ in ppm, CDCl₃, 500 MHz) 2.58 (s, 3H), 2.60 (s, 3H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 14.9, 31.4, 128.0, 156.9, 174.4, 196.6.

Synthesis of (6b) *1-(2-Amino-4,6-dichloropyrimidin-5-yl)ethan-1-one*. The crude product of 1-(2-amino-4,6-

dichloropyrimidin-5-yl)ethan-1-ol was dissolved in 5 mL of Et₂O, and 3 mL of 1.25 M H₂Cr₂O₇ was added dropwise at –10 °C. The reaction mixture was stirred for 16 h and allowed to reach room temperature. The reaction was neutralized with NaOH 2 M and extracted using Et₂O/H₂O. The crude product was used for the next step. ¹H NMR (δ in ppm, CDCl₃, 500 MHz) 2.59 (s, 3H), 5.63 (s, 2H); ¹³C NMR (δ in ppm, CDCl₃, 125 MHz) 31.5, 123.0, 157.9, 161.0, 197.0.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.3c01830>.

¹H NMR, ¹³C NMR, IR, and UV–vis spectra for **1**, **2**, **3**, **4**, **6**, **7**, **8**, **1'**, **2'**, **3'**, **4'**, **5'**, and **7'**. ¹H NMR and ¹³C NMR spectra for **3a**, **5a**, **6a**, **8a**, **3b**, **5b**, and **6b** (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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