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Ferrocenyl and organic novobiocin derivatives: Synthesis and their *in vitro* biological activity

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ABSTRACT

A focused series of novobiocin derivatives containing a ferrocene unit together with their corresponding organic novobiocin analogues have been synthesized in modest to good yields. These compounds were screened for biological activity against a chloroquine-sensitive strain of Plasmodium falciparum (3D7) and human breast cancer cell line (HCC38). With the exception of compounds **5c** and **5d**, the general trend observed is that incorporation of the ferrocene moiety into novobiocin scaffold resulted in compounds **6a–d**/**6f** showing enhanced activity compared to organic analogues **5a–b** and **5e–f**.

1. Introduction

Despite recent successes and average global decline of malaria infections over the last 15 years, malaria is still a life-threatening disease globally [1,2]. Of the five *Plasmodium* species that are known to infect humans, *Plasmodium falciparum* and *Plasmodium vivax* are attributed to high burden of malaria infections [1,2]. In addition to malaria, cancer is a global problem with an estimated 14 million new cases and 8.2 million deaths reported in 2012 [3]. The major concern is that the use of clinically proven drugs for treatment of both diseases is compromised by associated toxicity and development of multidrug resistant strains [4–6]. Drug resistance is a serious challenge, and it calls for development of new therapeutic agents for clinical management and control of both cancer and malaria.

In the quest for new chemical entities to counter resistance, compounds featuring a coumarin scaffold have attracted immense interest for development of new anticancer and antimalarial agents [7–16]. A typical example of coumarin containing compounds is novobiocin (**1**, Fig. 1), a naturally occurring coumarin antibiotic isolated from *Streptomyces* strains. Novobiocin (**1**) has attracted great attention due to its broad spectrum of biological properties including antimicrobial, anticancer and antimalarial activities [16–18]. It has been found to act by inhibiting pathologically important enzymes such as DNA gyrase [19,20], heat shock protein 90 (Hsp90) and related client proteins [7,21] through the blockade of their ATPase activity.

Considering the relevance of these targets in drug discovery of anti-infective agents and the need for new drug molecules which lack cross-resistance, a significant number of compounds containing structural features of **1** have been extensively investigated and developed as potent agents for bacterial infections [17], cancer [22–24] and malaria [10–12]. Antitumour activity of **1**, albeit poor, was first reported by Neckers and co-workers who demonstrated its potency against the SkBr3 breast cancer cells through the inhibition of Hsp90 [7]. Prior Bia and co-workers had demonstrated that **1** effectively suppressed the growth of *P. falciparum* parasite using chloroquine-resistant FCC_1 strains [18].

Subsequently, Blagg and co-workers explored the preliminary structure-activity relationship (SAR) studies in efforts to identify key structural features of novobiocin type compounds necessary for optimal Hsp90 inhibition and improved antitumour activity [8,9]. Compound KU-174 (**2**), containing a bulkier diaryl appendant in the benzamide side chain (**RHS** = right hand side, Fig. 1), emerged as the most potent compound [8]. Furthermore, the study revealed the presence of a hydrophobic binding pocket in the *C*-terminal domain, which allows the desirable interaction between the benzamide side chain of novobiocin and the *C*-terminus of Hsp90. This binding pocket appeared to be tolerant to larger hydrophobic groups that lead to enhanced affinity and anti-tumour activity [8,9].

The hydrophobic nature and bulkiness of the diaryl moiety in compounds represented by **2** prompted us to explore the overall effects

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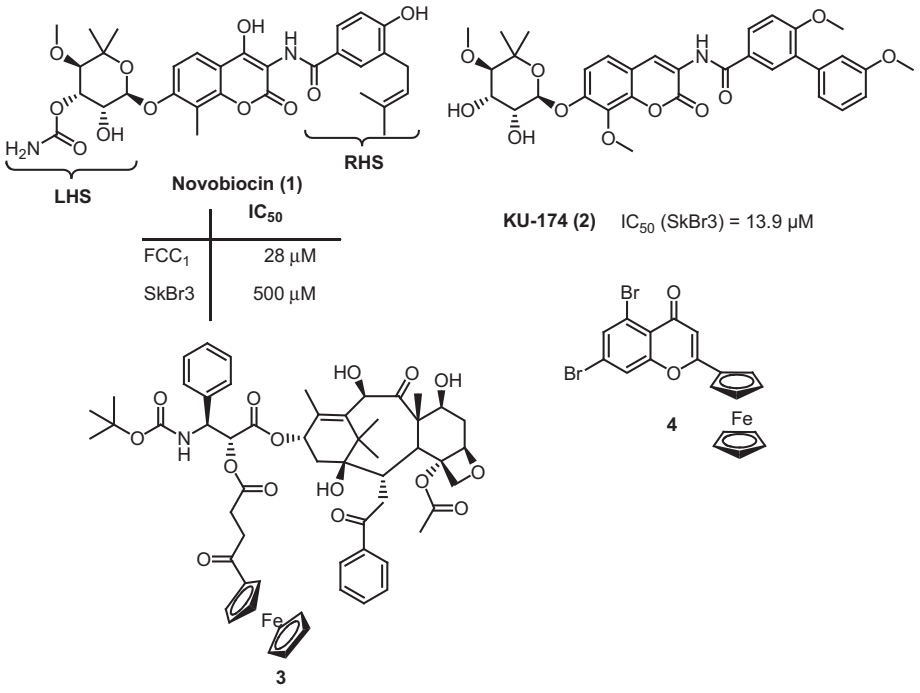
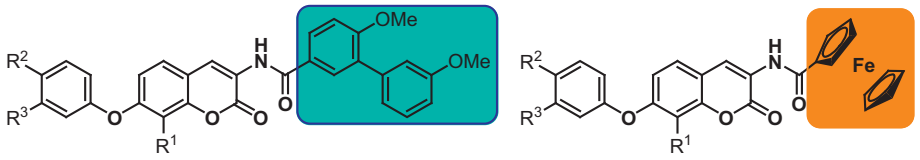


Fig. 1. Structures of novobiocin (**1**) and KU-174 (**2**), ferrocenyl taxoid (**3**) and flavone (**4**). RHS = right hand side, LHS = left hand side.



	R^1	R^2	R^3
a	H	^{t}Bu	H
b	Me	^{t}Bu	H
c	H	OMe	H
d	Me	OMe	H
e	H	H	OMe
f	Me	H	OMe

Fig. 2. Proposed organic and ferrocenyl derivatives in this study.

upon structural modification of novobiocin derivatives by replacing the diaryl side chain (**RHS**, Fig. 1) with the ferrocene unit to generate new ferrocenyl-based novobiocin derivatives (Fig. 2). Ferrocene is considered as a 'privileged structure' and this has triggered interest in drug discovery and broader field of bioorganometallic chemistry [25,26]. Notable attributes of ferrocene include aromaticity character, favourable redox behaviour, aqueous stability, potential to form reactive oxygen species (ROS), and lack of cytotoxicity effects in human cells [25,26]. The incorporation of inorganic fragments into bioactive organic frameworks remains an appealing strategy to find new compounds for treatment of various ailments [25,26]. A number of important natural compounds with structural modification involving incorporation of the ferrocenyl unit have featured prominently in literature [27]. For example, ferrocenyl taxoid **3** showed promising activity against multidrug resistant colon adenocarcinoma cell lines while ferrocenyl flavone **4** exhibited impressive antivascular activity despite its low cytotoxicity on B16 murine melanoma cells [28,29].

In this study, we limited our attention to a selection of compounds that contain simple substituted aryl moieties in place of the complex noviose sugar moiety (**LHS** = left hand side, Fig. 1). Herein, we endeavoured to identify new novobiocin-like compounds that are effective against both cancer and *P. falciparum* upon the incorporation of an organometallic ferrocene unit. This paper presents the synthesis of ferrocenyl novobiocin derivatives and their *in vitro* biological evaluation as novel anticancer and antiplasmodial agents. Thus far, there have been no literature reports on the antitumour and antiplasmodial effects of ferrocenyl-based novobiocin derivatives.

2. Experimental

2.1. Materials and physical measurements

All chemicals and solvents were purchased from chemical suppliers and were utilized without further purification. The progress of each

reaction was monitored by analytical thin layer chromatography (TLC) using Merck F254 silica gel plates (supported on aluminium sheets) and the plates were visualized under ultraviolet (UV 254 and 366 nm) light or using iodine 'tank'. Compounds were purified by a silica gel column chromatography using Merck Kieselgel 60 Å: 70–230 (0.068–0.2 mm) silica gel mesh. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Biospin 300, 400 or 600 MHz spectrometers, and were referenced internally using residual solvent signals of deuterated DMSO-d_6: 2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C NMR, or deuterated chloroform $CDCl_3$: 7.26 ppm for ^{1}H and 77.2 ppm for ^{13}C NMR at ambient temperature. The chemical shifts were recorded in parts per million (ppm) and the *J*-coupling constants in Herts (Hz). The abbreviations used to describe signal multiplicities are: s = singlet, br s = broad singlet, d = doublet, t = triplet, q = quartet and m = multiplet. High-resolution mass spectra (HRMS) were recorded on a Waters API Q-TOF Ultima spectrometer (Stellenbosch University, Stellenbosch, South Africa). The IR spectra were recorded on PerkinElmer Spectrum 100 FT-IR Spectrometer in the mid-IR range (640–4000 cm^{-1}).

2.2. General synthesis of ferrocene (Fc) containing derivatives 6a–f

An appropriate boc-deprotected 7-aryl-*O*-3-aminocoumarin **9** (1.0 eq) in 30% pyridine/dichloromethane (DCM) solution (2 mL) was allowed to stir at room temperature for 10 min followed by addition of a ferrocene carboxylic acid **11** (2.0 eq) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI·HCl) (3.0 eq). The reaction mixture was allowed to stir at room temperature for 1–3 days. Thereafter, the solvent was removed *in vacuo* to afford the crude product. The crude product was purified by silica gel column chromatography (5% ethyl acetate:hexane) to afford ferrocene containing novobiocin derivatives **6a–f**.

2.2.1. 7-(4-tert-butylphenyl)-O-3-ferrocenylcarboxamidocoumarin (6a)

7-(4-*tert*-Butylphenyl)-*O*-3-aminocoumarin **9a** (0.0800 g, 0.26 mmol), ferrocene carboxylic acid **11** (0.119 g, 0.52 mmol) and EDCI·HCl (0.149 g, 0.78 mmol) afforded **6a** as an orange solid (0.0381 g, 28%). M.p.: 180–182 °C. IR (ν_{max}/cm^{-1}): 3409 (N—H, amide), 1708 (C═O), 1658 (C═O), 1501 (C═C), 1168 (C—O); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.71 (s, 1H, —HC═C—), 8.27 (s, 1H, NH), 7.45 (d, J = 8.6 Hz, 1H, ArH), 7.41 (d, J = 8.9 Hz, 2H, ArH), 7.04–6.95 (m, 3H, ArH), 6.88 (d, J = 2.4 Hz, 1H, ArH), 4.85 (t, J = 1.9 Hz, 2H, FcH), 4.50 (t, J = 1.9 Hz, 2H, FcH), 4.26 (s, 5H, FcH), 1.35 (s, 9H, t-butyl) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 170.0, 159.9, 159.2, 153.0, 151.0, 147.9, 128.8, 127.1 (2C), 123.0, 122.5, 119.8 (2C), 115.5, 114.9, 105.0, 75.2, 71.6 (2C), 70.2 (5C), 68.5 (2C), 34.6, 31.6 (3C) ppm; HRMS (ESI) *m/z* calcd for $C_{30}H_{28}FeNO_4$: 521.1290, found 522.1381 $[M + H]^+$. Anal. calcd for $C_{30}H_{24}FeNO_4{\cdot}H_3CCOCH_3$ C, 68.40; H, 5.74; N, 2.42. Found: C, 68.23; H, 6.03; N, 2.57%.

2.2.2. 7-(4-tert-butylphenyl)-O-8-methyl-3-ferrocenylcarboxamidocoumarin (6b)

7-(4-*tert*-Butylphenyl)-8-methyl-*O*-3-aminocoumarin **9b** (0.0600 g, 0.19 mmol), ferrocene carboxylic acid **11** (0.0856 g, 0.37 mmol) and EDCI·HCl (0.107 g, 0.56 mmol) afforded **6b** as an orange solid (0.0446 g, 45%). M.p.: 188–191 °C. IR (ν_{max}/cm^{-1}): 3389 (N—H), 1712 (C═O), 1698 (C═O), 1506 (C═C), 1170 (C—O); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 8.70 (s, 1H, —HC═C—), 8.32 (br s, 1H, NH), 7.38–7.35 (m, 2H, ArH), 7.28 (d, J = 8.8 Hz, 1H, ArH), 6.92–6.84 (m, 2H, ArH), 6.84 (d, J = 8.6 Hz, 1H, ArH), 4.85 (t, J = 1.9 Hz, 2H, FcH), 4.48 (t, J = 1.9 Hz, 2H, FcH), 4.27 (s, 5H, FcH), 2.42 (s, 3H, CH_3), 1.33 (s, 9H, t-butyl) ppm; ^{13}C NMR (151 MHz, $CDCl_3$) δ_C 170.0, 159.4, 156.7, 154.5, 149.4, 146.7, 126.9 (2C), 125.6, 123.4, 122.5, 118.2 (2C), 117.3, 115.6, 115.6, 75.2, 71.5 (2C), 70.2 (5C), 68.6 (2C), 34.5, 31.6 (3C), 8.8 ppm; HRMS (ESI) *m/z* calcd for $C_{31}H_{25}NO_7$: 535.1446, found 536.1520 $[M + H]^+$. Anal. calcd $C_{31}H_{29}NO_4{\cdot}CH_3OH$ C, 67.73; H, 5.86; N, 2.47. Found: C, 67.54; H, 5.67; N, 2.57%.

2.2.3. 7-(4-methoxyphenyoxy)-O-3-ferrocenylcarboxamidocoumarin (6c)

7-(4-Methoxyphenyl)-*O*-3-aminocoumarin **9c** (0.0950 g, 0.34 mmol), ferrocene carboxylic acid **11** (0.154 g, 0.67 mmol) and EDCI·HCl (0.192 g, 1.0 mmol) afforded **6c** as a dark red solid (0.0867 g, 52%). M.p.: 114–115 °C. IR (ν_{max}/cm^{-1}): 3394 (N—H), 3054 (C—H), 2928 (C—H), 1690 (C═O), 1667 (C═O), 1502 (C═C), 1168 (C—O); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 8.70 (s, 1H, —HC═C—), 8.26 (s, 1H, NH), 7.43 (d, J = 8.6 Hz, 1H, ArH), 7.06–7.01 (m, 2H, ArH), 6.96–6.90 (m, 3H, ArH), 6.83 (d, J = 2.4 Hz, 1H, ArH), 4.82 (t, J = 2.0 Hz, 2H, FcH), 4.47 (t, J = 1.9 Hz, 2H, FcH), 4.26 (s, 5H, FcH), 3.83 (s, 3H, OCH_3) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 170.0, 160.5, 159.2, 156.9, 151.0, 148.6, 128.8, 123.0, 122.4, 121.8 (2C), 115.2 (2C), 114.9, 114.6, 104.4, 77.2, 75.1, 71.6 (2C), 70.2 (5C), 68.5 (2C), 55.8 ppm; HRMS (ESI) *m/z* calcd for $C_{27}H_{22}FeNO_5$: 495.0769, found 496.0851 $[M + H]^+$. Anal. calcd $C_{27}H_{21}FeNO_5{\cdot}CH_3OH$: C, 63.77; H, 4.78; N, 2.66. Found: C, 63.99; H, 4.90; N, 2.64%.

2.2.4. 7-(4-methoxyphenyoxy)-O-8-methyl-3-ferrocenylcarboxamidocoumarin (6d)

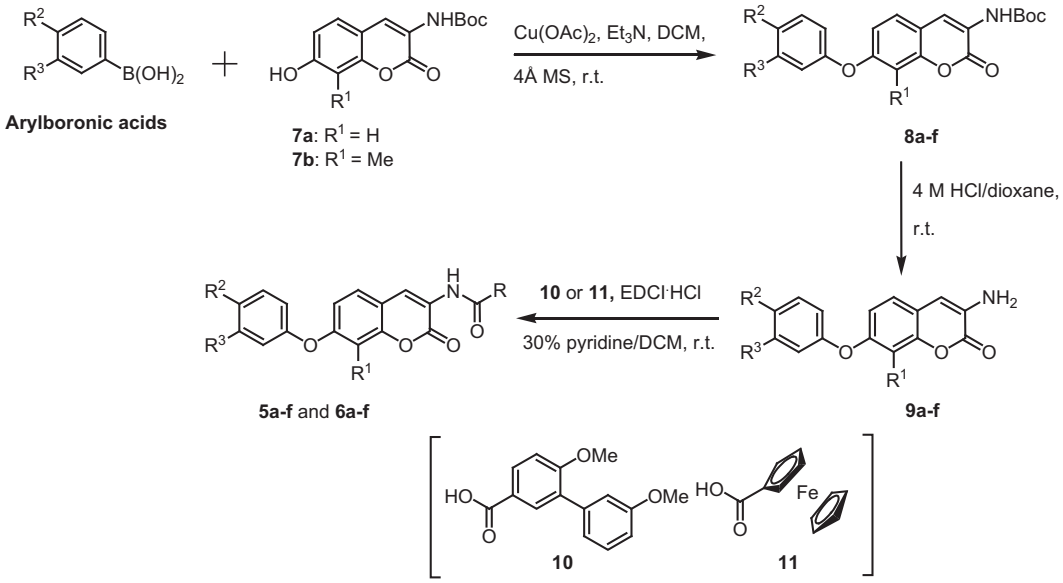
7-(4-Methoxyphenyl)-*O*-8-methyl-3-aminocoumarin **9d** (0.0750 g, 0.25 mmol), ferrocene carboxylic acid **11** (0.116 g, 0.50 mmol) and EDCI·HCl (0.145 g, 0.76 mmol) afforded **6d** as an orange solid (0.0245 g, 19%). M.p.: 167–169 °C. IR (ν_{max}/cm^{-1}): 3407 (N—H), 1704 (C═O), 1667 (C═O), 1499 (C═C), 1202 (C—O); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 8.70 (s, 1H, NH), 8.33 (br s, 1H, —HC═C—), 7.28 (d, J = 3.3 Hz, 1H, ArH), 6.99–6.95 (m, 2H, ArH), 6.94–6.89 (m, 2H, ArH), 6.75 (d, J = 8.6 Hz, 1H, ArH), 4.86 (t, J = 1.9 Hz, 2H, FcH), 4.51–4.48 (m, 2H, FcH), 4.29 (s, 5H, FcH), 3.83 (s, 3H, OCH_3), 2.45 (s, 3H, CH_3) ppm; ^{13}C NMR (151 MHz, $CDCl_3$) δ_C 170.0, 159.5, 157.6, 156.2, 150.0, 149.3, 125.5, 123.4, 122.3, 120.5 (2C), 116.4, 115.1 (3C), 114.3, 75.2, 71.5 (2C), 70.2 (5C), 68.5 (2C), 55.8, 8.7 ppm; HRMS (ESI) *m/z* calcd for $C_{28}H_{24}FeNO_5$: 509.0926, found 510.1004 $[M + H]^+$. Anal. calcd $C_{28}H_{23}FeNO_5{\cdot}CH_3OH$: C, 64.34; H, 5.03; N, 2.59. Found: C, 64.77; H, 4.59; N, 2.66%.

2.2.5. 7-(3-methoxyphenyoxy)-O-3-ferrocenylcarboxamidocoumarin (6e)

7-(3-Methoxyphenyl)-*O*-3-aminocoumarin **9e** (0.0780 g, 0.28 mmol), ferrocene carboxylic acid **11** (0.127 g, 0.55 mmol) and EDCI·HCl (0.158 g, 1.0 mmol) afforded **6e** as a dark red solid (0.056 g, 41%). M.p.: 180–183 °C. (IR ν_{max}/cm^{-1}): 3400 (N—H), 1693 (C═O), 1668 (C═O), 1513 (C═C), 1158 (C—O); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 8.71 (s, 1H, NH), 8.28 (s, 1H, —HC═C—), 7.46 (d, J = 8.6 Hz, 1H, ArH), 7.29 (t, J = 8.2 Hz, 1H, ArH), 6.98 (dd, J = 8.5 and 2.3 Hz, 1H, ArH), 6.93 (d, J = 2.2 Hz, 1H, ArH), 6.75 (dd, J = 8.3 and 2.2 Hz, 1H, ArH), 6.66 (dd, J = 8.0 and 1.8 Hz, 1H, ArH), 6.63 (t, J = 2.3 Hz, 1H, ArH), 4.84 (t, J = 1.8 Hz, 2H, FcH), 4.49 (t, J = 1.8 Hz, 2H, FcH), 4.26 (s, 5H, FcH), 3.80 (s, 3H, OCH_3) ppm; ^{13}C NMR (151 MHz, $CDCl_3$) δ_C 170.1, 161.2, 159.2, 159.1, 156.7, 150.9, 130.6, 128.9, 122.9, 122.7, 115.8, 115.2, 112.1, 110.4, 106.1, 105.6, 75.0, 71.6 (2C), 70.2 (5C), 68.5 (2C), 55.6 ppm; HRMS (ESI) *m/z* calcd for $C_{27}H_{22}FeNO_5$: 495.0769, Found 496.0835 $[M + H]^+$. Anal. calcd $C_{27}H_{21}FeNO_5$: C, 65.47; H, 4.27; N, 2.83. Found: C, 65.88; H, 4.50; N, 2.70%.

2.2.6. 7-(3-methoxyphenyoxy)-O-8-methyl-3-ferrocenylcarboxamidocoumarin (6f)

7-(3-Methoxyphenyl)-*O*-8-methyl-3-aminocoumarin **9f** (0.080 g, 0.27 mmol), ferrocene carboxylic acid **11** (0.124 g, 0.54 mmol) and EDCI·HCl (0.155 g, 0.81 mmol) afforded **6f** as an orange solid (0.0615 g, 45%). M.p.: 162–164 °C. IR (ν_{max}/cm^{-1}): 3396 (N—H), 1716(C═O), 1663 (C═O), 1510 (C═C), 1170 (C—O); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 8.71 (s, 1H, NH), 8.32 (s, 1H, —HC═C—), 7.31 (d, J = 8.5 Hz, 1H, ArH), 7.23 (t, J = 8.7 Hz, 1H, ArH), 6.89 (d, J = 8.5 Hz, 1H, ArH), 6.67 (dd, J = 8.2 and 2.5 Hz, 1H, ArH), 6.54–6.51 (m, 2H, ArH), 4.85 (t, J = 1.9 Hz, 2H, FcH), 4.48 (t, J = 1.9 Hz, 2H, FcH), 4.27 (s, 5H, FcH), 3.79 (s, 3H, OCH_3), 2.39 (s, 3H, CH_3) ppm; ^{13}C NMR (151 MHz, $CDCl_3$) δ_C 170.1, 161.2, 159.3, 158.3, 155.9, 149.3, 130.5, 125.7, 123.2, 122.7, 117.8, 116.4, 116.0,



Scheme 1. Synthesis of target organic **5a–f** and ferrocenyl **6a–f** derivatives.

110.4, 109.1, 104.5, 75.1, 71.6 (2C), 70.2 (5C), 70.2 (2C), 68.6, 55.5, 8.8 ppm; HRMS (ESI) *m/z* calcd for $C_{28}H_{24}FeNO_5$: 509.0926, found 510.1004 $[M + H]^+$. Anal. calcd $C_{28}H_{23}FeNO_5{\cdot}CH_3OH$ C, 64.34; H, 5.03; N, 2.59. Found: C, 64.46; H, 4.61; N, 2.66%.

2.3. Growth inhibition assays

2.3.1. HCC38 breast cancer cell inhibition assay

The toxicity of novobiocin and novobiocin analogues was assessed in the human breast cancer HCC38 cell line (ATCC CRL-2314) using a WST-1 cell proliferation kit (Roche) carried out according to manufacturer's instructions. All compounds were dissolved to make a final concentration of 100 mM in DMSO. Cells of equal density (4×10^4 cells/mL at 50 μL/well) were seeded in a 96 well plate and treated the following day with a range of compound concentrations (0, 0.32, 1.6, 8, 40, 200, 1000 μM). After 72 h, media containing compounds were removed from the wells and 5 μL of a 5 mg/mL WST-1 reagent in 100 μL of medium was added to each of the wells and incubated for 4 h prior to reading absorbance at 450 nm. The cell viability was determined by cleavage of the tetrazolium salt, WST-1, to formazan by metabolically active cells to produce a colored product. This viability assay was carried out in triplicate and the dose response and half-maximal inhibitory concentrations (IC_{50}) determined by non-linear regression using GraphPad Prism 4.

2.4. In vitro antiplasmodial assay

The 3D7 strain of *Plasmodium falciparum* was routinely cultured in medium consisting of RPMI1640 containing 25 mM Hepes (Lonza), supplemented with 0.5% (w/v) Albumax II (Thermo Fisher Scientific), 22 mM glucose, 0.65 mM hypoxanthine, 0.05 mg/mL gentamicin and 2–4% (v/v) human erythrocytes. Cultures were maintained at 37 °C under an atmosphere of 5% CO_2, 5% O_2, 90% N_2. To assess antiplasmodial activity, three-fold serial dilutions of test compounds in culture medium were added to parasite cultures (adjusted to 2% parasitaemia, 1% haematocrit) in 96-well plates and incubated for 48 h. Duplicate wells per compound concentration were used. Parasite lactate dehydrogenase (PLDH) enzyme activity in the individual wells was determined by removing 20 μL of the parasite cultures and mixing it with 125 μL colorimetric substrate solution containing 0.18 M L-lactic acid, 0.13 mM acetylpyridine adenine dinucleotide, 0.39 mM nitrotetrazolium blue chloride, 0.048 mM phenazineethosulphate and 0.16% (v/v) Triton X-100 in 44 mM Tris buffer (pH 9). Color development was monitored by measuring absorbance at 620 nm in a Spectramax M3 plate reader (Molecular Devices). Absorbance values were converted to % parasite viability relative to untreated control cultures and plotted against log[compound] to derive IC_{50} values by non-linear regression, using GraphPad Prism (v. 5.02) software.

2.5. In vitro cytotoxicity assay

HeLa cells (Cellonex) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Lonza) supplemented with 10% fetal calf serum and antibiotics (penicillin/streptomycin/amphotericin B) at 37 °C in a 5% CO_2 incubator. Cells were plated in 96-well plates at a cell density of 2×10^4 cells/well and grown overnight. Serial dilutions of test compounds were incubated with the cells for an additional 24 h, and cell viability in the wells assessed by adding 20 μL resazurin toxicology reagent (Sigma-Aldrich) for an additional 2–4 h. Fluorescence readings (excitation 560 nm, emission 590 nm) obtained for the individual wells were converted to % cell viability relative the average readings obtained from untreated control wells. Plots of % cell viability *vs.* log [compound] were used to determine IC_{50} values by non-linear regression using GraphPad Prism (v. 5.02).

3. Results and discussion

3.1. Chemistry

As illustrated in Scheme 1, the synthesis of the targeted novobiocin analogues commenced with phenolic coumarin derivatives **7a–b**, which were accessible through the methods which have previously been reported in the literature [30,31]. Chan-Lam coupling of **7a–b** with commercially available aryl boronic acids afforded the boc-protected 7-aryl-*O*-3-acetamidocoumarins **8a–f** as white solids in 11–45% yields [32]. Deprotection of **8a–f** under acidic conditions affected the free amines **9a–f**, which were subsequently coupled *via* modified standard amidation conditions with diaryl carboxylic acid **10** or ferrocene carboxylic acid **11** to produce the final novobiocin derivatives **5a–f** and **6a–f** in the yields ranging between 13 and 71%, respectively [9]. Compounds **5a–f** (Supplementary information) have previously been synthesized and tested against SkBr3 and MCF-7 breast cancer cell lines by Kusuma et al. [33] Herein, these compounds were included to serve as references in biological assays. In all cases, the structural identity of

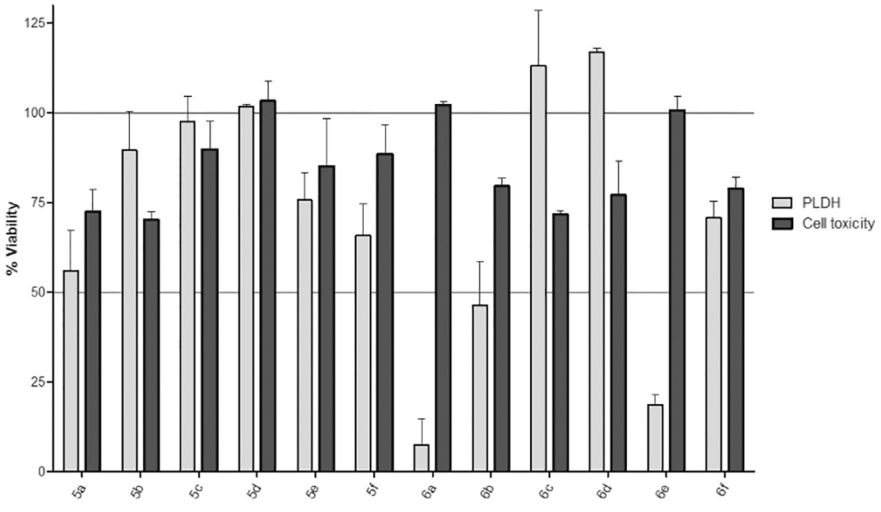


Fig. 3. *In vitro* antiplasmodial and cell toxicity (HeLa cell viability) data for compounds **5a–f** and **6a–f**.

each compound was confirmed by ^{1}H and ^{13}C NMR spectroscopy including mass spectrometry. The signals corresponding to the coumarin nucleus coupled with aryl ethers were consistent with those reported in previous studies (Supplementary information) [33]. ^{1}H NMR spectra of the ferrocenyl novobiocins **6a–f** revealed the characteristic proton signals due to the ferrocenyl rings appearing as two triplets and one singlet between δ 4.26 and 4.87 ppm. In ^{13}C NMR spectra, the carbon atom signals of the Cp rings appeared in the range δ 68.5–75.2 ppm.

3.2. *In vitro* biological evaluation

3.2.1. *Plasmodium falciparum parasite inhibition and cytotoxicity assays*

The *in vitro* screening of the prepared compounds began with cytotoxicity (HeLa cells) and malaria parasite lactate dehydrogenase (PLDH) assays, which were conducted at the initial concentration of 20 μM solutions of each compound in duplicates, and chloroquine (CQ) was included as a control drug. Using the PLDH assay, the antiplasmodial activity of the compounds was realized against the chloroquine-sensitive *P. falciparum* parasite strain, 3D7. The results have been summarized and displayed in Table S1 (Supporting information) and Fig. 3. In general, the majority of compounds showed weak inhibition or no significant effects at 20 μM. The initial screening data revealed only two ferrocenyl based compounds, **6a** and **6e**, which showed sufficient growth inhibition of the *P. falciparum* parasite below 20% parasite viability.

The initial screening data (Table S1) led to determining of the IC_{50} values of compounds **6a** and **6e**, and this was conducted by plotting a curve of percentage parasite cell viability against the logarithm of the compound concentration (Fig. 4). However, both compounds **6a** and **6e** appeared moderately active against the *P. falciparum* parasite strain 3D7 with IC_{50} values above 9 μM. All compounds displayed minimal growth inhibition of HeLa cells at 20 μM with > 70% of cell viability was often observed. Compounds **6a** and **6e** displayed no cytotoxicity at maximum tested concentration (200 μM). Furthermore, the cell viability data suggested that inhibition growth of *P. falciparum* was independent of general toxic effect (HeLa cell viability was > 70%).

3.2.2. *Anti-proliferative activity*

The same compounds were also subjected to biological assay evaluation against the human triple negative breast cancer cell line (HCC38) with Paclitaxel utilized as a positive control and novobiocin **1** as a reference compound (Table 1). At first glance, the general pattern that emerged from screening assay results is that ferrocene containing derivatives **6a–f**, with exception of **5c** and **5d**, showed higher potency compared to their non-ferrocenyl derivatives **5a–f** (Table 1). For example, compound **6a** showed ~10-fold increased activity compared to its non-ferrocenyl counterpart, **5a**, and parental novobiocin (**1**). Similarly, **6b** had higher activity than **5b** with ~26-fold improvement. Although it showed reduced activity compared to its non-ferrocenyl counterpart, the activity of compound **6c** was significantly superior to that of the parental novobiocin (**1**) with ~10–15 times increased potency. The lack of antitumour activity observed for most of the non-ferrocenyl derivatives against the HCC38 breast cancer cell line in our case appeared to be far more consistent with the findings by Kusuma et al. [33] and co-workers who also demonstrated poor anti-proliferative effects for these compounds against SkBr3 and MCF7 breast cell lines. Despite being active against HCC38, both **5c** and **5d** were inactive against both SkBR3 and MCF7 breast cancer lines [33].

The poor activity of compound **6e**, which appeared to be inconsistent with the general trend observed for other compounds from this series, amongst other reasons, could be attributed to poor solubility in aqueous medium. Since we limited our study to specific novobiocin-like compounds containing ferrocene unit, a realistic structure-activity relationship (SAR) could not be clearly defined. Nevertheless, the presence of the methyl substituent at position 8 of the coumarin framework in the non-ferrocenyl analogues resulted in reduced activity. On the contrary, in ferrocenyl analogues (**6b**, **6d** and **6f**) the methyl substituent in the same position led to enhanced activity. Within the ferrocene-series, ferrocene congeners devoid of methyl substituent at position 8 showed less potency against the HCC38 cell line. Previous studies [8] have shown that upon methylation of position 8 the anti-proliferative effect is enhanced, suggesting that 8-methyl is important for activity and in most cases compounds showed ~10-fold enhanced potency compared to hydrogen containing analogues [8].

4. Conclusions

In summary, the results presented by this work highlight that the incorporation of ferrocenyl moiety on the benzamide side of novobiocin resulted in compounds with enhanced biological activity. Ferrocenyl analogues showed superior potency than their non-ferrocenyl counterparts against human breast cancer cells and *P. falciparum* strain. Additionally, the ferrocenyl derivatives did not manifest any significant cytotoxic effects against HeLa cells when assayed under similar conditions as the *P. falciparum* cultures, suggesting that these compounds may display a degree of selectivity for parasitic cells over human cells.

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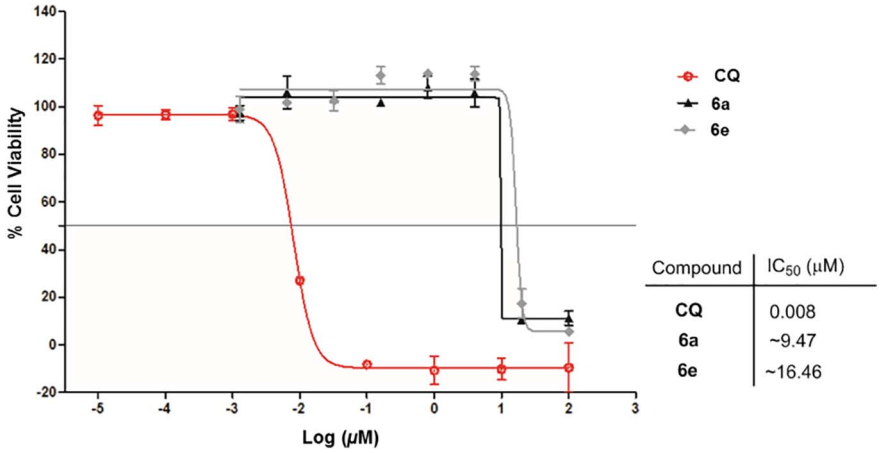

Fig. 4. Percentage viability of the *P. falciparum* parasite at different concentrations and related IC_{50} values of **6a** and **6e**. IC_{50} value represents concentration of the compound effecting 50% inhibition of parasitic cell growth.

Table 1
In vitro anticancer activity of compounds **5a–f** and ferrocenyl derivatives **6a–f**[a].

Compound	IC_{50} (μM) HCC38	Compound	IC_{50} (μM) HCC38
Paclitaxel	0.029		
1	192.0		
5a	197.4	**6a**	20.7
5b	223.1	**6b**	8.73
5c	0.36	**6c**	13.2
5d	2.12	**6d**	1.06
5e	–[b]	**6e**	261.5
5f	> 1000	**6f**	8.01

[a] Activity of compounds is reported as IC_{50} values representing concentrations of the compounds that effected 50% inhibition of cellular growth. Data are expressed as average μM concentration resulting from at least two independent experiments.
[b] The IC_{50} value(s) could not be determined.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2017.04.014.

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