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Effect of amide-triazole linkers on the electrochemical and biological properties of ferrocene-carbohydrate conjugates†

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Amide-triazole linker incorporated ferrocene-carbohydrate conjugates were prepared by adopting a regiospecific copper(II)-catalysed 1,3-cycloaddition of ferrocenoyl propargylamide and isopropylidene/acetyl protected carbohydrate azides. Hydrophilic ferrocene glycoside with an amide-triazole linker was synthesised by deacetylation of the hydroxyl groups. All the new compounds were characterised by UV-visible and electrochemical studies and they were found to be stable in organic solvents as well as in the buffer system under physiological conditions (pH = 7.0). The diffusion coefficient (D_f) of the conjugates was also calculated by means of cyclic voltammetric studies. It was observed that while the molecular weight of the compounds had no significant effect on the diffusion coefficient, the hydrophobic/hydrophilic nature of the carbohydrate scaffold displayed varied diffusion coefficient values. Stabilization of the compounds in buffer solution under physiological pH led to almost identical diffusion coefficient values. The compounds derived from xylose and ribose exhibited cytotoxicity on hormone-dependent and hormone-independent breast cancer cell lines, whereas the conjugates derived from glucose and galactose were found to be non-toxic in nature. The compounds did not show any antimicrobial activity against Gram-positive and Gram-negative pathogens.

Introduction

In recent years a plethora of potential biological applications of ferrocene and its derivatives has been reported.[1–8] These include some of the well known ferrocene analogues of conventional drugs for the treatment of breast cancer and malaria, such as ferrocifens, hydroxyferrocifens, ferrocenophanes and ferroquine respectively.[9–14] These examples have provided impetus for the synthesis of ferrocene conjugates for biological applications.[15–17] Facile synthetic procedures using click reaction conditions have made triazoles an attractive functional group for the construction of bioconjugates.[18] Triazoles exhibit several features of biological importance, for instance, non-susceptibility to hydrolytic cleavage or redox modifications, mimicking the hydrogen bond acidity and peptide bond basicity.[19,20] These features have led to the synthesis of several organic and organometallic compounds containing a triazole functionality, a number of them often displaying excellent biological activity.[21–27]

Moreover, several biologically active compounds with triazole as a peptide surrogate have displayed substantial inhibitory activities.[28–34] An amide-triazole linker was identified as one of the components in these compounds responsible for the inhibition properties (Fig. 1). Metzler-Nolte and co-workers

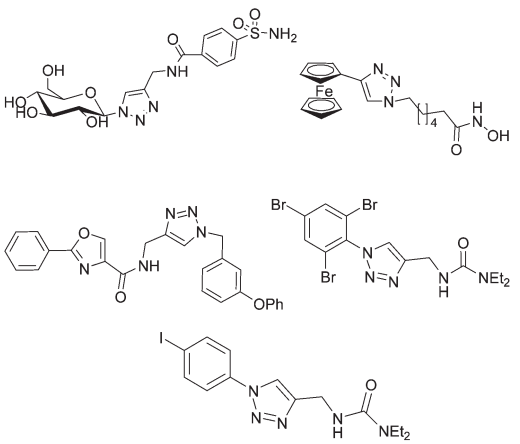

Fig. 1 Some biologically active compounds with amide-triazole linkers.

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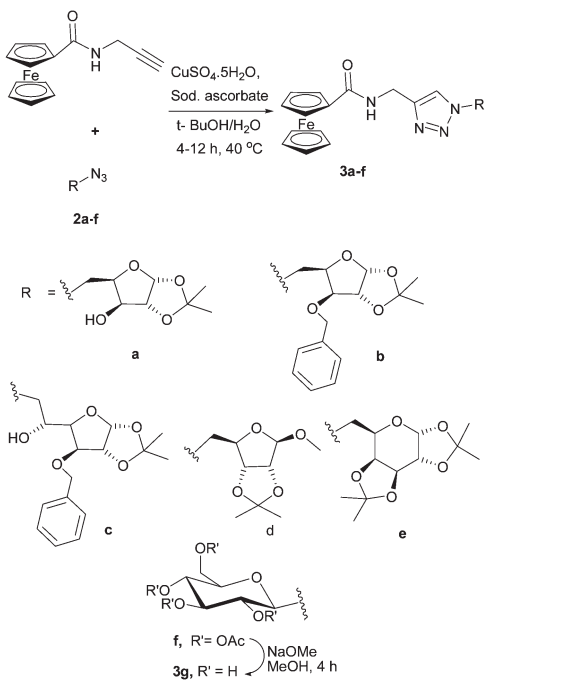
were among the first to report the synthesis of biologically important PNA oligomers and peptides connected through amide-triazole linkers.[35–37] In recent times, click JAHA type ligands consisting of ferrocene conjugates with amide-triazole linkers have been shown to exhibit cytotoxicity towards both hormone-dependent and hormone-independent breast cancer cell lines.[38,39]

Low cost, ready availability, non-toxicity and facile modification into several functional groups of carbohydrate moieties have led to the development of novel synthetic routes for transition metal complexes containing glycoconjugates.[40–44] In addition to these advantages, the hydrophilicity of ferrocene-carbohydrate conjugates can be tuned by protecting and deprotecting the hydroxyl group on the carbohydrate skeleton. Ferrocene-carbohydrate complexes have been at the forefront of scientific research for their use as electrochemical probes[45–48] and biological activity.[49–59] Most of the biologically active ferrocene-carbohydrate conjugates have been prepared from glucose and glucosamine sugars and they have shown significant anti-malarial activities.[54–58]

Our recent study on ferrocene-carbohydrate conjugates connected through triazole and amide linkers has shown reasonable cytotoxicity and antimicrobial activity.[60,61] These results motivated us to study the effect of the amide-triazole linker on the electrochemical and biological properties of such ferrocene conjugates. Herein, we report on the synthesis, characterisation, electrochemical properties of various pentose and hexose sugar ferrocene amide-triazoles **3a–f** (Scheme 1). A detailed electrochemical study has been carried out on all the compounds and the electrochemical diffusion coefficient has been calculated by means of the Randles–Ševčik equation. The anticancer activity of the compounds was determined against A549 (human alveolar adenocarcinoma cells), HeLa (cervical carcinoma cancer cells), MDA-MB-231 (human breast adenocarcinoma cells) and MCF-7 (human breast adenocarcinoma cells) cell lines. The antimicrobial activity of the compounds was screened against both Gram-positive and Gram-negative pathogens.



Scheme 1 Synthesis of ferrocene-carbohydrate amide-triazoles by the click reaction of sugar azide and ferrocenoylpropargylamide.

Experimental section

General information

Optical rotations were determined on a JASCO J-120 polarimeter. ^{1}H spectra were recorded for $CDCl_3$ and DMSO solutions on 300 MHz (Avance 300) and 500 MHz (Innova 500) instruments. Chemical shifts for protons were reported using tetramethylsilane (TMS) as the internal reference. ^{13}C NMR spectra were recorded at 75.5 MHz on an Avance 300 instrument and the carbon shifts are referenced to the ^{13}C signal of $CDCl_3$ at 77.0 ppm. Coupling constants (*J*) were expressed in Hz. Infrared spectra were recorded on a Thermo Nicolet Nexus 670 spectrometer using KBr discs. Melting points were determined on a Toshniwal melting point apparatus and are uncorrected. Ferrocenoyl propargylamide was prepared according to literature procedures.[62] Water soluble amide-triazole **3g** was prepared by the deacetylation of **3f** according to the literature procedure. The UV-visible spectra were recorded on a Varian Cary 500 spectrophotometer over the range 200–550 nm using 1 cm path length cuvettes. Cyclic voltammetry (CV) was performed with a conventional three-electrode configuration consisting of glassy carbon as a working electrode, platinum as an auxiliary electrode and an Ag/AgCl reference electrode. The cyclic voltammograms were recorded in anhydrous methanol in the presence of 0.1 M tetrabutylammonium perchlorate (TBAP) as the supporting electrolyte with a scan rate of 0.1 V s^{-1}, using a CHI620 model electrochemical analyzer at room temperature.

General procedure for the preparation of ferrocene-carbohydrate amide-triazoles

Ferrocenoyl propargyl amide (0.20 g, 1.39 mmol) and azido sugars (**3a–f**) (1.39 mmol) were suspended in a 1 : 1 mixture of water and *tert*-butyl alcohol (8 mL). To this solution, sodium ascorbate (0.05 g, 0.2 mmol) and $CuSO_4{\cdot}5H_2O$ (0.02 g, 0.09 mmol) were added. The heterogeneous mixture was stirred at 40 °C, until the disappearance of the starting materials. The reaction mixture was quenched with saturated NH_4Cl solution and extracted with CH_2Cl_2 (2 × 5 mL). The combined organic layers were dried over Na_2SO_4 and the crude mass, obtained by the removal of the solvent under vacuum, was purified by column chromatography on alumina.

5-Deoxy-1,2-*O*-isopropylidene-5-(4-methyl amido ferrocenyl-1*H*-1,2,3-triazole-1-yl)-α-D-xylofuranose (3a). Treatment of 5-azido-5-deoxy-1,2-*O*-isopropylidene-α-D-xylofuranose (0.3 g, 1.39 mmol) (**2a**) and ferrocenoyl propargylamide (0.2 g,

1.39 mmol) resulted in **3a** as an orange solid. Yield: 0.41 g (69%); mp: 70 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −13.14; IR (KBr, ν_{max}/cm^{-1}) = 3434, 3107, 2982, 1656, 1069; ^1H NMR (300 MHz, $CDCl_3$, Me_4Si): δ (ppm) = 1.30 (s, 3H, CH_3 of CMe_2), 1.40 (s, 3H, CH_3 of CMe_2), 4.13 (s, 5H, C_5H_5), 4.19–4.23 (m, 1H, H-5), 4.34 (t, 2H, J = 1.70 Hz, C_5H_4), 4.46–4.50 (m, 1H, H-5), 4.57–4.60 (m, 4H, CH_2NH, H-3 and H-4), 4.71–4.80 (m, 3H, C_5H_4, H-2), 5.30 (s, 1H, OH), 5.99 (d, 1H, J = 3.21 Hz, H-1), 6.93 (t, 1H, J = 5.09 Hz, NH), 7.85 (s, 1H, triazole); ^{13}C (75.5 MHz, $CDCl_3$): δ (ppm) = 26.14, 26.77, 34.56, 48.67, 68.15, 68.24, 69.73, 70.69, 74.35, 75.06, 79.19, 85.3, 105.1, 111.93, 124.02 (C_5 triazole), 144.86 (C_4 triazole), 171.09 ppm; ESI-MS (in CH_3OH): m/z = 483 $[M + H]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 264 (5244), 307 (1196), 442 (220) nm; Anal. calc. for $C_{22}H_{26}FeN_4O_5$ (482.1): C 54.79, H 5.43, N 11.62; found: C 54.66, H 5.29, N 11.47.

3-*O*-Benzyl-5-deoxy-1,2-*O*-isopropylidene-5-(4-methyl amido ferrocenyl-1*H*-1,2,3-triazol-1-yl)-α-D-xylofuranose (3b). Treatment of 5-azido-3-*O*-benzyl-5-deoxy-1,2-*O*-isopropylidene-α-D-xylofuranose (**2b**) (0.42 g, 1.39 mmol) and ferrocenoyl propargylamide (0.2 g, 1.39 mmol) resulted in **3b** as an orange solid. Yield: 0.56 g (79%); mp: 65 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −20.37; IR (KBr, ν_{max}/cm^{-1}) = 3417, 3090, 2984, 2931, 1730, 1641, 1530,1076; ^1H NMR (300 MHz, $CDCl_3$, Me_4Si): δ (ppm) = 1.30 (s, 3H, CH_3 of CMe_2), 1.40 (s, 3H, CH_3 of CMe_2), 3.97 (d, 1H, J = 3.02 Hz, H-2), 4.14 (s, 5H, C_5H_5), 4.33 (t, 2H, J = 2.26 and 1.62 Hz, C_5H_4), 4.51–4.54 (m, 3H, CH_2NH and H-5), 4.57–4.62 (m, 3H, C_5H_4 and H-5), 4.65–4.7 (m, 4H, H-3, H-4 and CH_2Ar), 5.90 (d, 1H, J = 3.02 Hz, H-1), 6.43 (t, 1H, J = 6.04 and 5.28 Hz, NH), 7.32–7.41 (m, 5H, Ar), 7.61 (s, 1H, triazole) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 26.10, 26.65, 34.82, 49.17, 66.05, 68.19, 69.62, 70.39, 71.85, 78.69, 81.40, 81.85, 105.12, 111.98, 123.39, 127.89, 128.25, 128.61, 136.71 (C_5 triazole), 144.85 (C_4 triazole), 170.39 ppm; ESI-MS (in CH_3OH): m/z = 573 $[M + H]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 262 (5610), 305 (960), 447 (220) nm; Anal. calc. for $C_{29}H_{32}FeN_4O_5$ (572.17): C 60.85, H 5.63, N 9.79; found: C 60.51, H 5.84, N 9.67.

3-*O*-Benzyl-6-deoxy-1,2-*O*-isopropylidene-6-(4-methyl amido ferrocenyl-1*H*-1,2,3-triazol-1-yl)-α-D-glucofuranose (3c). Treatment of 3-*O*-benzyl-6-azido-6-deoxy-1,2-*O*-isopropylidene-α-D-glucofuranose (**2c**) (0.33 g, 1.39 mmol) and ferrocenoyl propargylamide (0.2 g, 1.39 mmol) resulted in **3c** as an orange solid. Yield: 0.42 g (77%); mp: 65 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −17.86; IR (KBr, ν_{max}/cm^{-1}) = 3384, 3090, 2984, 2931, 1743, 1635, 1531, 1076, 1024; ^1H NMR (300 MHz, $CDCl_3$, Me_4Si): δ (ppm) = 1.31 (s, 3H, CH_3 of CMe_2), 1.43 (s, 3H, CH_3 of CMe_2), 3.25 (d, 1H, J = 5.28 Hz, OH), 3.87–3.91 (m, 1H, H-5), 4.13 (s, 5H, C_5H_5), 4.15 (d = 2H, J = 5.28 Hz, CH_2Ar), 4.33 (t, 3H, J = 2.26 Hz, C_5H_4), 4.40–4.55 (m, 2H, CH_2NH), 4.58–4.65 (m, 3H, C_5H_4, H-4), 4.65–4.67 (m, 2H, H-3 and H-2) 4.71–4.74 (m, 2H, H-6), 5.93 (d, 1H, J = 3.02 Hz, H-1), 6.36 (t, 1H, J = 6.04 Hz, NH) 7.28–7.46 (m, 5H, Ar), 7.69 (s, 1H, triazole); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 26.25, 26.77, 34.79, 50.14, 53.84, 67.67, 68.27, 69.67, 70.51, 72.16, 80.23, 81.05, 81.54, 82.15, 105.19, 111.98, 123.92, 127.80, 128.29, 128.63. 137.08 (C_5 triazole), 144.61 (C_4 triazole), 170.43; ESI-MS (in CH_3OH): m/z = 603 $[M + H]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 264 (4016), 306 (938), 442 (162) nm; Anal. calc. for $C_{30}H_{34}FeN_4O_6$ (602.18): C 59.81, H 5.69, N 9.30, Found: C 59.09, H 5.47, N 8.91.

Methyl-5-deoxy-2,3-*O*-isopropylidene-5-(4-methyl amido ferrocenyl-1*H*-1,2,3-triazol-1-yl)-β-D-ribofuranoside (3d). Treatment of 5-azido-5-deoxy-1-*O*-methyl-2,3-*O*-isopropylidene-β-D-ribofuranoside (**2d**) (0.23 g, 1.39 mmol) and ferrocenoyl propargylamide (0.2 g, 1.39 mmol) resulted in **3d** as an orange solid. Yield: 0.49 g (80%); mp: 180–185 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −12.06; IR (KBr, ν_{max}/cm^{-1}) = 3399, 3135, 2989, 2945, 1635, 1519, 1106; ^1H NMR (300 MHz, $CDCl_3$, Me_4Si): δ (ppm) = 1.28 (s, 3H, CH_3 of CMe_2), 1.44 (s, 3H, CH_3 of CMe_2), 3.39 (s, 3H, OCH_3), 4. 13 (s, 5H, C_5H_5), 4.34 (t, 2H, J = 2.26 and 1.51 Hz, C_5H_4), 4.41 (m, 2H, CH_2NH), 4.52–4.57 (m, 2H, H-5), 4.62–4.67 (m, 4H, C_5H_4, H-3 and H-4), 4.75 (d, 1H, J = 5.28 Hz, H-2), 5.0 (s, 1H, H-1), 6.39 (t, 1H, J = 5.28 and 3.77 Hz, NH), 7.71 (s, 1H, triazole); ^{13}C NMR (75.5 MHz, $CDCl_3$ + DMSO): δ (ppm) = 24.92, 26.35, 34.88, 53.16, 55.60, 68.13, 69.68, 70.51, 75.51, 81.74, 84.95, 85.18, 110.01, 112.92, 122.77 (C_5 triazole), 145.18 (C_4 triazole), 170.49; ESI-MS (in CH_3OH): m/z = 497 $[M + H]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 264 (4038), 308 (908), 449 (134) nm; Anal. calc. for $C_{23}H_{28}FeN_4O_5$ (496.14): C 55.66, H 5.69, N 11.29; found: C 55.26, H 5.14, N 10.93.

6-Deoxy-1,2 : 3,4-di-*O*-isopropylidene-6-(4-methyl amido ferrocenyl-1*H*-1,2,3-triazol-1-yl)-α-D-galactopyranose (3e). Treatment of 6-azido-6-deoxy-1,2 : 3,4-*O*-diisopropylidene-α-D-galactopyranose (**2e**) (0.28 g, 1.39 mmol) and ferrocenoyl propargylamide (0.2 g, 1.39 mmol) resulted in **3e** as an orange solid. Yield: 0.38 g (76%); mp: 140 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −31.7; IR (KBr, ν_{max}/cm^{-1}) = 3109, 2984, 2921, 1641, 1379, 1219, 1080, 1007, 814; ^1H NMR (500 MHz, $CDCl_3$, Me_4Si): δ (ppm) = 1.27 (s, 3H, CH_3 of CMe_2), 1.34 (s, 3H, CH_3 of CMe_2), 1.39 (s, 3H, CH_3 of CMe_2), 1.48 (s, 6H, CH_3 of CMe_2), 4.14 (s, 5H, C_5H_5), 4.16–4.20 (m, 2H, CH_2NH), 4.30–4.32 (m, 3H, C_5H_4 and H-6), 4.45 (dd, 1H, J = 5.9 and 7.9 Hz, H-6), 4.57 (d, 1H, J = 3.99 Hz, H-5), 4.60–4.64 (m, 3H, H-4, H-3 and H-2), 4.66–4.68 (t, 2H, J = 2.0 Hz, C_5H_4), 5.48 (d, 1H, J = 4.9 Hz, H-1), 6.43 (m, 1H, NH), 7.75 (s, 1H, triazole); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 24.43, 24.82, 25.93, 34.94, 50.42, 67.04, 68.04, 68.17, 69.67, 70.23, 70.43, 70.73, 71.05, 96.19, 108.97, 109.88, 123.59 (C_5 triazole), 156.25 (C_4 triazole), 170.32; ESI-MS (in CH_3OH): m/z = 553 $[M + H]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 262 (4500), 307 (966), 447 (162) nm; Anal. calc. for $C_{26}H_{32}FeN_4O_6$ (552.16): C 56.53, H 5.84, N 10.14; found: C 56.91, H 5.42, N 9.98.

2,3,4,6-Tetra-*O*-acetyl-6-(4-methyl amido ferrocenyl-1*H*-1,2,3-triazol-1-yl)-β-D-glucopyranoside (3f). Treatment of 1-deoxy-azido-2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (**2f**) (0.28 g, 1.39 mmol) and ferrocenoyl propargylamide (0.2 g, 1.39 mmol) resulted in **3f** as an orange solid. Yield: 0.38 g (76%); mp: 95 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −36.4; IR (KBr, ν_{max}/cm^{-1}) = 3109, 2984, 2921, 1641, 1379, 1219, 1080, 1007, 814; ^1H NMR (300 MHz, $CDCl_3$, Me_4Si): δ (ppm) = 1.86 (s, 3H, OCH_3), 2.03 (s, 6H, OCH_3), 2.06 (s, 3H, OCH_3), 3.97–4.01 (m, 1H, H-5), 4.11 (s, 5H, C_5H_5), 4.24–4.30 (m, 1H, H-6), 4.35

(d, 2H, J = 1.77 Hz, C_5H_4), 4.53–4.60 (m, 1H, H-6), 4.67–4.74 (m, 4H, C_5H_4 and CH_2NH), 5.23 (t, 1H, H-4), 5.39–5.46 (m, 2H, H-2 and H-3), 5.86 (d, 1H, J = 8.49 Hz, H-1), 6.49 (t, 1H, J = 5.09 and 5.28 Hz, N*H*), 7.90 (s, 1H, triazole); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 20.12, 20.44, 20.58, 34.72, 61.46, 67.58, 68.01, 68.23, 69.61, 70.32, 70.50, 72.59, 74.97, 75.29, 85.66, 121.15 (C_5 triazole), 145.75 (C_4 triazole), 168.68, 169.20, 169.88, 170.43; ESI-MS (in CH_3OH): m/z = 641 $[M + H]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 225 (5100), 307 (1168), 447 (220) nm; Anal. calc. for $C_{28}H_{32}FeN_4O_{10}$ (640.14): C 52.51, H 5.04, N 8.75. Found: C 52.19, H 5.30, N 8.87.

6-(4-Methyl amido ferrocenyl-1*H*-1,2,3-triazol-1-yl)-β-D-glucopyranoside (3g). The acetylated 1,2,3-triazole **3f** was dissolved in dry MeOH (10 mL) and the solution was made alkaline with a freshly prepared solution of NaOMe in methanol (1 M, 1 mL). The resulting solution was stirred at room temperature until the formation of the product, as indicated by TLC (4 h). After completion of the reaction, the solvent was removed under vacuum and the crude product was purified by column chromatography using a methanol–ethyl acetate mixture. The pure compound was dissolved in water and lyophilised. Yield: 0.632 g (76%); mp: 95 °C; $[\alpha]_D^{34}$ (c 0.2, CH_3OH) = −5.7; IR (KBr, ν_{max}/cm^{-1}) = 3423, 2925, 1633, 1381, 1290, 1099, 1047, 814; ^{1}H NMR (300 MHz, DMSO, Me_4Si) δ (ppm) = 1.25 (bs, 4H, *OH*), 3.46–3.66 (m, 4H, H-6, H-5, H-4), 3.76–3.86 (m, 2H, H-3,H-2), 4.15 (s, 5H, C_5H_5), 4.35 (t, 2H, J = 1.7 Hz, C_5H_4), 4.55 (d, 2H, J = 1.7 Hz CH_2NH), 4.82 (t, 2H, J = 1.7 Hz, C_5H_4), 5.5 (d, 1H, J = 9.1 Hz, H-1), 7.91 (s, 1H, triazole), 8.19 (m, 1H, N*H*); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) = 27.76, 28.47, 29.21, 37.56, 66.85, 67.37, 68.57, 68.71, 69.72, 77.42, 127.65, 130.30 (C_5 triazole), 131.23 (C_4 triazole), 166.44; ESI-MS (in CH_3OH): m/z = 495 $[M + Na]^+$; UV-Vis in CH_3OH: λ_{max} [ε(dm^3 mol^{-1} cm^{-1})] = 264 (2760), 307 (644), 444 (132) nm; Anal. calc. for $C_{20}H_{24}FeN_4O_6$ (472.10): C 50.36, H 5.12, N 11.86. Found: C 49.98, H 5.31, N 11.15.

Biological studies

***In vitro* cytotoxicity testing.** The cytotoxicity of the test compounds was assessed according to literature procedures.[63] Cell lines used for testing *in vitro* cytotoxicity included HeLa derived from human cervical cancer cells (ATCC No. CCL-2), A549 derived from human alveolar adenocarcinoma epithelial cells (ATCC No. CCL-185), MDA-MB-231 derived from human breast adenocarcinoma cells (ATCC No. HTB-26) and MCF7 derived from human breast adenocarcinoma cells (ATCC No HTB-22) were obtained from the American Type Culture Collection, Manassas, VA, USA. All the tumour cell lines were maintained in a modified DMEM medium supplemented with 10% fetal bovine serum, along with 1% non-essential amino acids without L-glutamine, 0.2% sodium hydrogen carbonate, 1% sodium pyruvate and 1% antibiotic mixture (10 000 units penicillin and 10 mg streptomycin per mL). The cells were washed and resuspended in the above medium and 100 μL of this suspension was seeded in 96 well-bottom plates. The cells were maintained at 37 °C in a humidified 5% CO_2 incubator (Model 2406 Shellab CO_2 incubator, Sheldon, Cornelius, OR). After incubation for 24 h the cells were treated for 2 days with test compounds at concentrations ranging from 0.1–100 μm in DMSO (1% final concentration) and were assayed at the end of the second day. Each assay was performed with two internal controls: (1) an IC_0 with cells only, (2) an IC_{100} with media only. After incubation for 24 h, the cells were subjected to the MTT colorimetric assay (5 mg mL^{-1}). The effect of the different test compounds on the viability of the tumour cell lines was measured at 540 nm on a multimode reader (Infinite® M200, Tecan, Switzerland). The IC_{50} values (50% inhibitory concentration) were calculated from the plotted absorbance data for the dose–response curves. The assay was performed using doxorubicin and cisplatin as standards and 1% DMSO as a vehicle control. To avoid DMSO toxicity, the values obtained for the DMSO control were subtracted from those of the test compounds. IC_{50} values (in μM) are expressed as the average of two independent experiments.

Antimicrobial activity

The antimicrobial activity of the compounds was determined by a modified microtiter broth dilution method[64] against different pathogenic reference strains, including Gram-positive bacteria (*Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96, *S. aureus* MLS16 MTCC 2940, *Micrococcus luteus* MTCC 2470), Gram-negative bacteria (*Klebsiella planticola* MTCC 530, *Escherichia coli* MTCC 739 and *Pseudomonas aeruginosa* MTCC 2452) and *Candida albicans* MTCC3018, procured from the Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. Sterile blank discs (6.0 mm dia., HiMedia Laboratories Pvt. Ltd., Mumbai, India) were impregnated with the test compounds at a dose of 1.4–300 μg mL^{-1} and allowed to dry at room temperature in a laminar air flow chamber. The sterile discs containing different test compounds were placed individually on the surface of the medium in Petri plates, containing Müller-Hinton agar seeded with 0.1 mL previously prepared microbial suspensions individually containing 1.5 × 10^8 cfu mL^{-1} (equal to 0.5 McFarland). Standard antibiotic solutions of neomycin (for bacterial strains), fluconazole (for *Candida albicans*) and the well containing methanol (vehicle control) served as positive and negative controls, respectively. The plates were incubated for 24 h at 37 °C and the minimum inhibitory concentration (MIC) values were determined as the lowest concentration of the test compound, which resulted in a significant decrease in the inoculum viability (>95%) at which no coloration was observed. All experiments were carried out in duplicate and the mean values were considered.

Results and discussion

The ferrocene-carbohydrate amide-triazoles were prepared by the click reaction between ferrocenoyl propargylamide (**1**) and various sugar azides (**2a–f**) in the presence of sodium ascorbate and copper(II) sulfate in a 1 : 1 mixture of ^{t}BuOH and water at 40 °C for 4 h (Scheme 1). The reaction of per-*O*-acetyl

glucoside azide (**2f**) with ferrocenoyl propargylamide was slow compared to the terminal carbohydrate azides (**2a–e**). The water soluble amide-triazole (**3g**) was prepared by the deacetylation of compound **3f** using sodium methoxide in methanol.[45,46]

All the compounds were characterised by ^{1}H and ^{13}C NMR spectroscopy. The protons of the unsubstituted cyclopentadiene ring appeared as a singlet at 4.1 ppm, while those for the substituted Cp ring appeared as a triplet/multiplet around 4.3 ppm and 4.6 ppm in the ^{1}H NMR spectra. Except for the compound **3d**, the anomeric proton of the sugar moieties was observed as a doublet around 5.7–5.9 ppm. In the case of compound **3d**, a singlet was observed for the anomeric carbon at 5.6 ppm. The characteristic peaks corresponding to the amide proton and the triazole protons were observed at around 6.4 ppm and 7.9 ppm, respectively. For the compound **3e**, the amide and the triazole protons were observed at 6.9 and 8.1 ppm, respectively.

In the ^{13}C NMR spectra, the *gem*-dimethyl groups appeared as singlets around 24 ppm and 26 ppm. The amide carbonyl carbon appeared at 170 ppm and the triazole carbons were observed in the regions 120 ppm and 140 ppm.

Except for **3g**, all the compounds were characterised by the [M + H] peak in the mass spectra. For the compound **3g**, the [M + Na] peak was observed in the mass spectra. For all the compounds, the characteristic IR bands for –NH and –CO stretching were observed around 3385–3417 cm^{-1} and 1635 cm^{-1} respectively.

The UV-visible spectra of ferrocene conjugates were recorded in methanol over a wavelength range 250–550 nm (Fig. 2). All the compounds exhibited two absorption maxima around 260 nm and 310 nm corresponding to the $Fe(a_{1g}) \rightarrow Cp(e_{1g})$ charge transfer due to the amide-triazole functionality.[60,61] In addition to these two absorption maxima, a characteristic broad band was observed around 450 nm due to the symmetry forbidden $Fe(a_{1g}) \rightarrow Fe(e_{1g})$ transitions.

The stability of the compounds in aqueous media was determined by recording the UV-visible spectra in aqueous DMSO and DMSO-buffer solutions under physiological conditions (pH 6.0–7.4). No significant change in the intensity of the spectral band in the region 250 and 335 nm indicates the stability of the compounds in aqueous solutions beyond 8 h.

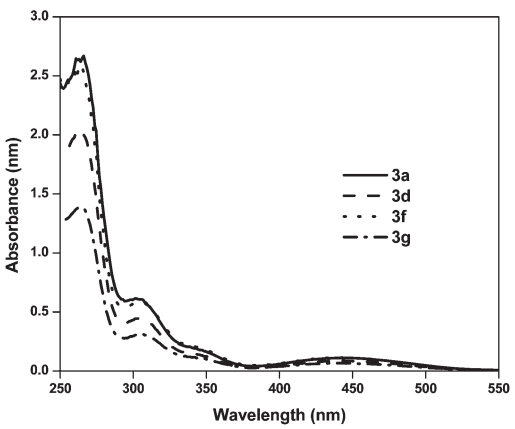


Fig. 2 UV-Vis spectrum of ferrocene conjugates **3a**, **3d**, **3f** and **3g** in 0.5 mM methanol solution.

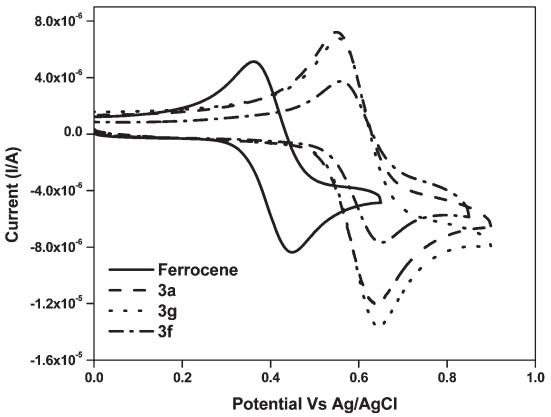


Fig. 3 Cyclic voltammograms of ferrocene conjugates **3a**, **3f** and **3g** in 1.0 mM methanol solution at 25 °C.

Electrochemistry

The electrochemical characterisation of the compounds was performed in methanol by means of cyclic voltammetry (CV) in 1.0 mM methanol solution using a three electrode cell consisting of a glassy carbon working electrode, platinum wire auxiliary electrode and Ag/AgCl reference electrode with a scan rate of 0.1 V s^{-1}. The cyclic voltammograms of the compounds are shown in Fig. 3. All the ferrocene-carbohydrate conjugates exhibited one electron reversible oxidation wave with respect to the Ag/AgCl electrode in the presence of 0.1 M tetrabutyl ammonium perchlorate (TBAP) as the supporting electrolyte. The compounds exhibited peak separation potentials ranging from 89–97 mV and current ratios equal to unity (Table 1).

The cyclic voltammetry experiments were also conducted in DMSO and DMSO buffer solutions (phosphate buffer pH = 7.0). It was observed that in DMSO solution, the compounds exhibited a quasi-reversible wave (Fig. 4) with an oxidation potential ranging from 623–626 mV. Except for **3g**, all the compounds exhibited an additional reversible wave around 110–280 mV, resulting from the expulsion of the Cp ring due to the attack of the nucleophile at the Fe(III) centre.[65] In the case of **3g**, only the quasi-reversible wave for the ferrocenium ion was observed, the absence of the reversible wave may be due to the stabilization of the molecule by the free hydroxyl groups of the compound.[66,67]

On addition of 0.1 M phosphate buffer solution to a 1.0 mM DMSO solution (1 : 1) of the compounds, a reversible oxidation wave corresponding to the ferrocenium ion was observed with $E_{1/2}$ values ranging from 496–561 mV (Fig. 5). It was also observed that, in the buffer solution, the compounds were unaffected by the nucleophilic solvent molecules as indicated by the absence of an extra oxidation peak. The peak

Table 1 Electrochemical data of compounds **3a–f**

Compound	E_{pa}(mV)	E_{pc}(mV)	ΔE_p(mV)	$\Delta E_{1/2}$(mV)	i_a/i_c
In methanol					
Ferrocene	477	406	71	441	0.99
3a	638	549	89	594	1.07
3b	645	551	94	598	1.05
3c	644	550	94	597	1.05
3d	644	552	92	598	1.06
3e	647	550	97	599	1.07
3f	644	555	89	600	1.07
3g	651	559	92	605	1.02
In DMSO + buffer					
3a	555	461	92	509	1.06
3b	556	466	90	510	1.01
3c	556	464	92	510	1.02
3d	540	452	88	496	1.05
3e	556	464	92	510	1.06
3f	556	473	83	514	1.01
3g	611	512	99	561	1.01

$\Delta E_P = (E_{pa} - E_{pc})$ and $\Delta E_{1/2} = 0.5(E_{pa} + E_{pc})$, where E_{pa} and E_{pc} are the anodic and cathodic peak potentials, respectively. The electrode system consists of a glassy carbon working electrode, platinum auxiliary electrode and Ag/AgCl reference electrode. Scan rate: 100 mV s^{-1}.

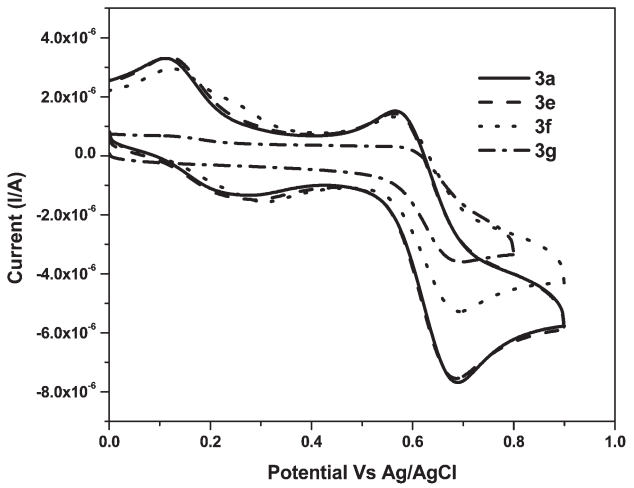


Fig. 4 Cyclic voltammograms of ferrocene conjugates **3a**, **3e**, **3f** and **3g** in 1.0 mM DMSO solution at 25 °C.

separation values were obtained in the range 88–99 mV and the current ratios approached unity. The stabilization of the conjugates in buffer solutions leads to a slight decrease in the electrode potential compared to the DMSO solvent.[66,67] In the case of compound **3g**, a slight variation in the $E_{1/2}$ value was observed due to the interaction of solvent molecules with the free hydroxyl groups of the compound.

Determination of diffusion coefficients

In general, it is observed that the diffusion coefficient decreases with an increase in the molecular weight and size of the compound.[68] However, a similar trend was not observed in the case of carbohydrate conjugates due to their different

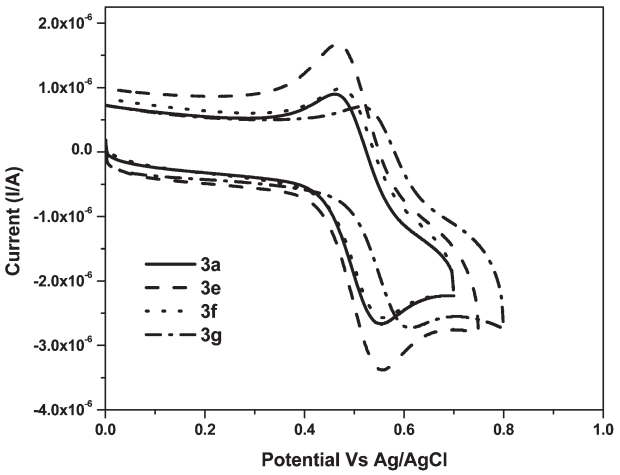


Fig. 5 Cyclic voltammograms of ferrocene conjugates **3a**, **3e**, **3f** and **3g** in 1.0 mM DMSO solution containing 0.1 M phosphate buffer (pH = 7.4) at 25 °C.

geometries and interaction of these molecules with the solvent system.[46] It has been mentioned by Vargas-Berenguel and co-workers that the diffusion coefficients of the carbohydrate derived ferrocene conjugates were independent of the molecular weight of the compound.[46] The objective of the present study was to observe the trend of the diffusion coefficient of ferrocene conjugates containing various sugars linked through amide-triazole linkers. Another point of interest originates from the lack of substantial literature evidence on the study of the diffusion coefficient of ferrocene-carbohydrate conjugates with different sugar moieties in polar and buffer solutions.

Hence, the diffusion coefficient (D_f) was calculated, based on the reversible redox behaviour, in methanol and in buffer solutions, according to literature procedures.[68] In order to calculate the diffusion coefficient, the corresponding anodic peak currents (i_a) were obtained by recording the cyclic voltammograms at various scan rates ranging from 20–100 mV (see ESI†). The straight line obtained (Fig. 6) due to the diffusion-controlled electrochemical process shows that the current is directly proportional to the scanning rate $v^{1/2}$. In this case, the diffusion coefficients (D_f) of **3a–g** were obtained from the Randles–Ševčik relationship:[68]

$$i_a = 0.4463 \times n^{3/2} F^{3/2} A\ (RT)^{-1/2} D_f^{1/2} C v^{1/2}$$

At 25 °C, the RT value becomes 2480 J mol^{-1}, and by using the Faraday constant of F = 96 500 C mol^{-1}, the above equation can be written as

$$i_a = 2.69 \times 10^5 \times A D_f^{1/2} C v^{1/2}$$

where i_a is the anodic peak current, A is the area of the electrode (m^2), C is the concentration of the electrochemically active species (mol m^{-3}), n is the number of electrons (for ferrocene, n = 1), v expresses the sweep rate (V s^{-1}), and the diffusion coefficient, D_f (m^2 s^{-1}) of **3a–g** thus calculated.

It was observed from the calculations that the compounds display variable diffusion coefficients with an increase in the

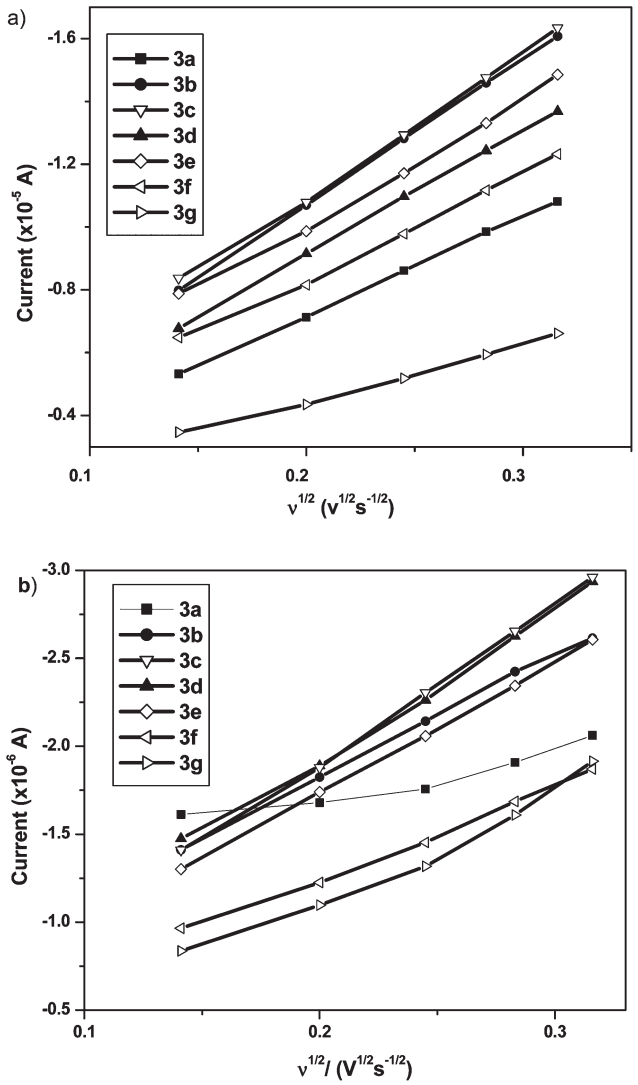


Fig. 6 Plots of anodic peak current of ferrocene-carbohydrate conjugates in (a) methanol and (b) DMSO-buffer solutions.

molecular weight of the compound (Table 2). In methanol, the compounds with free hydroxyl groups (**3a** and **3g**) exhibited low diffusion coefficients compared to the other compounds and the D_f value was further decreased as the number of free hydroxyl groups increased, which is evident from the low D_f value of compound **3g**. The compounds containing an *O*-benzyl group (**3b** and **3c**), showed significantly high D_f values. Compounds **3d**, **3e** and **3f** with completely protected sugar skeletons (other than benzyl groups) showed D_f values in the range 4.0–5.8 × 10^{-10} m^2 s^{-1}. The compound with an acetyl protected sugar (**3f**) exhibited a high D_f value compared to the compound with the free hydroxyl group. The lower D_f value of **3g** may be due to stabilization by the non-covalent interactions such as hydrogen-bonding with the solvent molecules. From this, we can conclude that the electrochemical diffusion of the ferrocene-carbohydrate conjugates depends on the nature of the protecting groups as well as the structure of the carbohydrate moiety.

In DMSO-buffer solution, under physiological pH, the diffusion coefficient of the compound was found to be almost ten times lower than in the methanol. For all the compounds, the D_f value was found to be around 0.1–0.18 × 10^{-10} m^2 s^{-1}, which indicated a slow diffusion rate due to the stabilization of the compounds in the buffer system. In conclusion, it was observed that in the buffer solution, the geometry, the spatial arrangement and the type of carbohydrate scaffold had no appreciable effect on the diffusion coefficient of the amide-triazole linked ferrocene-carbohydrate conjugates.

Biological evaluation

***In vitro* cytotoxicity (MTT assay).** The cytotoxicity of the ferrocene-carbohydrate amide-triazoles **3a–f** was assessed on the basis of the measurement of the *in vitro* growth in 96 well plates by cell-mediated reduction of tetrazolium salt to form water insoluble formazan crystals according to the literature procedures.[63] These compounds were tested for cytotoxicity against the different test cell lines up to a concentration of 100 μM. Ferrocene was also tested on these cell lines for comparison purposes. The concentration of the compounds at which 50% of the cell growth was inhibited (IC_{50}) was calculated and shown in Table 3.

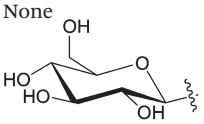
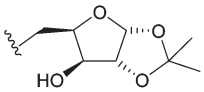
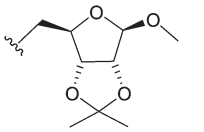
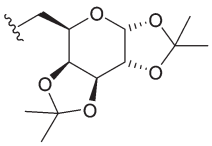
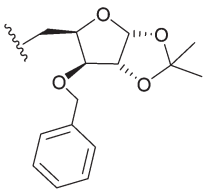
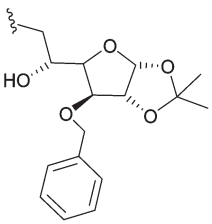
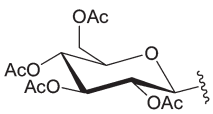
From the literature, it is known that the compounds with amide-triazole linkers exhibited cytotoxicity towards hormone-dependent and hormone-independent breast cancer cell lines.[39] However, it was observed that in the case of ferrocene-carbohydrate amide-triazoles, except for compounds **3b** and **3d**, all the compounds were non-toxic towards the tested cell lines. It was interesting to note that although ferrocene exhibited cytotoxicity towards the cell lines, the ferrocene conjugates containing amide-triazoles derived from galactose (**3e**) and glucose (**3c**, **3f** and **3g**) did not show any cytotoxicity on the cell lines.

The amide-triazole **3a**, derived from xylofuranose, did not show cytotoxicity on any cell lines, whereas compound **3b**, derived from the benzylated xylofuranose, exhibited cytotoxicity on the hormone-independent breast cancer cell line, MDA-MB-231, with an IC_{50} value of 2.82 μM, (so far this happens to be the best IC_{50} value reported for ferrocene-carbohydrate conjugates). In addition to this, the compound also exhibited cytotoxicity on the hormone-dependent breast cancer cell lines like MCF-7 and A549 with IC_{50} values of 11.31 μM and 7.01 μM, respectively.

Ribofuranoside amide-triazole (**3d**) was selectively active on the breast cancer cell lines. This compound exhibited cytotoxicity on the hormone-dependent breast cancer cell line, MCF-7, with an IC_{50} value of 3.18 μM. The compound exhibited very good cytotoxicity on the hormone-independent breast cancer cell line, MBA-MD-231, with an IC_{50} value of 7.31 μM. Moreover, it is interesting to note that, irrespective of the linker used, the ferrocene-carbohydrate conjugates containing the ribofuranoside moiety displayed almost similar IC_{50} values for the MBA-MD-231 cell lines (Table 4).

It was found that among all the sugar derivatives screened for the present study, the amide-triazole linked

Table 2 Influence of the molecular weight of compounds on the diffusion coefficient (D_f) in methanol and DMSO-buffer solutions

Compound	Molecular weight (mg mmol^{-1})	Carbohydrate scaffold	D_f in methanol ($\times 10^{-10}$ m^2 s^{-1})	D_f in DMSO-buffer ($\times 10^{-10}$ m^2 s^{-1})
Ferrocene	186	None	164	—[a]
3g	472		1.0	0.10
3a	482		3.0	0.18
3d	496		4.9	0.11
3e	552		5.8	0.18
3b	572		6.8	0.22
3c	602		7.0	0.23
3f	640		4.0	0.09

[a] Not determined.

Table 3 Cytotoxicity of ferrocene amide-triazoles

	IC_{50} (μM)[b]			
Compound[a]	A549	HeLa	MDA-MB-231	MCF-7
Ferrocene	21.3 (±0.13)	28.3 (±0.09)	35.2 (±0.15)	54.8 (±0.05)
3b	7.01 (±0.11)	>100	2.82 (±0.14)	11.31 (±0.03)
3d	>100	>100	7.31 (±0.13)	3.18 (±0.10)
Doxorubicin[c]	1.21 (±0.10)	0.45 (±0.07)	0.50 (±0.11)	1.05 (±0.04)
Cisplatin[c]	0.11 (±0.07)	0.20 (±0.13)	0.21 (±0.05)	0.41 (±0.10)

[a] For compounds **3a**, **3c**, **3e–g**, IC_{50} values of >100 μM was observed. [b] Values are the mean of two independent experiments, and the standard deviation values are given in brackets. [c] Standard.

ferrocene-carbohydrate conjugates with a hydroxyl protected furanose ring structure displayed significant cytotoxicity.

Table 4 Effect of linker on IC_{50} values against MDA-MB-231 in the case of ribofuranoside containing ferrocene conjugates

Linker	IC_{50} (μM) for MDA-MB-231
Amide[a]	7.29
Triazole[b]	7.35
Amide-triazole	7.31

[a] Ref. 61. [b] Ref. 60.

Antimicrobial activity

It is well known that various microbial strains often develop resistance to antibiotics.[69,70] A metal-specific mode of action of some metallocene drugs has been envisaged to counter such microbial resistance.[16] Edwards and co-workers have synthesised and studied the antimicrobial activity of ferrocenyl

penicillin and ferrocenyl cephalosporin derivatives.[71–74] In recent times, the group of Metzler-Nolte have reported the antimicrobial activity of metallocene and half-metallocene derivatives of the conventional drug platensimycin.[75–78] In addition, certain metallocene-peptide conjugates have also exhibited antimicrobial activity.[78–81] These findings have motivated us to determine the antimicrobial activity of the ferrocene-carbohydrate conjugates. During the course of our study, we observed that some of the ferrocene-carbohydrate conjugates displayed antimicrobial properties.[60,61] In the continuation of the study, we decided to extend our investigation towards finding the effect of the amide-triazole linker attached to the ferrocene conjugates on antimicrobial activities.

It was observed that the standard utilized in the present study, neomycin, exhibited an MIC value of 18.7 μg mL^{-1} against all the test bacterial strains and flucanozole showed an MIC value of 18.7 μg mL^{-1} on *Candida albicans*. However, none of the test compounds (**3a–g**) exhibited any inhibition towards both Gram-positive and Gram-negative bacteria as well as *Candida albicans* even at a concentration of 300 μg mL^{-1}. It can be concluded that this set of compounds is not suitable as antimicrobial agents.

Conclusions

In conclusion, herein we described the synthesis and complete electrochemical characterisation of ferrocene-carbohydrate conjugates connected through amide-triazole linkers. The compounds were found to be stable in aqueous and buffer systems under physiological conditions. The electrochemical studies indicated that the amide-triazole linkers were more electron withdrawing compared to simple amide and triazole functionalities. The electrochemical calculations indicated that in the methanol solvent, the diffusion coefficient of the compounds depends on the protecting groups and the geometry of the carbohydrate. On the other hand, in buffer solution under physiological pH, the diffusion coefficient was independent of the geometry and hydrodynamic radius of the compounds. Among seven ferrocene-carbohydrate conjugates under study, only two compounds exhibited cytotoxicity. Interestingly, the water soluble compound **3g** did not exhibit cytotoxicity. Particularly, compound **3b** and compound **3d** show promising cytotoxicity against hormone-independent (IC_{50} of 2.82 μM) and hormone-dependent cancer cells (IC_{50} of 3.18 μM), respectively, which were found to be one of the best IC_{50} values compared to those reported for other known ferrocene-carbohydrate conjugates. These results indicated the importance of sugar scaffolds in designing ferrocene-carbohydrate conjugates. Further experiments to develop new ferrocene-carbohydrate conjugates with different linkers and their biological studies are underway.

Acknowledgements

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