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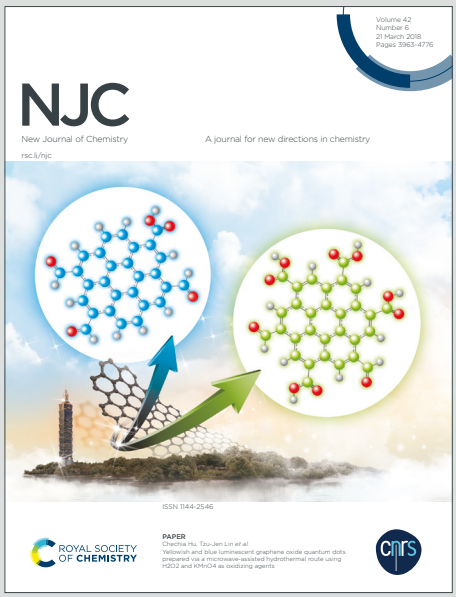

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ARTICLE

Synthesis, Characterization, and Anticancer Activity of Folate γ-Ferrocenyl Conjugates†

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The quest for alternative therapeutic agents with safety and effective profiles is one of the biggest challenges in oncology. The folate-targeted therapy can deliver drugs selectively into malignant cells *via* the Frα receptor, whereas the bioorganometallic chemistry offers potential new drug candidates. Herein, novel folate γ-ferrocene (γ-Fc) conjugates were synthesized through a regiospecific route by reacting amino-terminated glutamate (Glu) γ-Fc residues with pteroyl azide (68-78% yield), and their *in vitro* anticancer activities were evaluated on human cells. The Fc units were attached to protected Glu residues through different linkers using amide coupling and/or "click" reactions. Cyclic voltammetry and UV-vis measurements showed that the triazole group conjugated to the Fc donates electrons to the ferrous center, which facilitates the oxidation of Fc that is responsible for the antiproliferative activity. These results were confirmed by the higher activity of folate γ-triazoleFc **21**, with IC_{50} values at 25.4 μM (± 3.2) on HeLa and 21.7 μM (± 0.3) on MCF-7 cells. This compound was up to 4-fold less toxic on healthy PNT2 cells and Frα-negative PC-3 cancer cells. Blocking experiments with excess of free folic acid inhibited the folates activity suggesting their specific uptake via Frα. Glu residues analogs of folates but lacking the pteroyl moiety were much less toxic on cancer cells than folates. Hence, folates γ-Fc are potential anticancer drug candidates due to their selectivity and enhanced cytotoxicity against Frα-positive malignant cells.

Introduction

Bioorganometallic chemistry has brought new alternatives for medicinal chemistry *via* application of organometallics as therapeutic agents.[1-3] Ferrocene (Fc) derivatives have played a significant role in this area, being applied as antianemic drugs[4] and antiproliferative agents against fungal and bacterial infections,[5-8] malaria,[9] human immunodeficiency virus,[10] and cancer.[7, 11-13] These therapeutical applications are enabled by the exceptional electronic properties of Fc, relatively easy synthesis of derivatives, and high stability in aqueous and aerobic media.[14-17]

Ferrocenyl compounds have shown remarkable *in vitro* and *in vivo* anticancer activities,[18, 19] enabling their use to treat certain types of cancer, including lymphocytic leukemia P-388, breast and colon cancer.[16, 20] Ferrocifen is one of the most relevant Fc-based anticancer drug, in which the β-phenyl ring of tamoxifen – a well-known drug for the treatment of hormone-responsive (ER+) breast cancer – is replaced by a Fc moiety.[21, 22] Ferrocifen has shown excellent results on ER+ and ER- breast cancer, with activity 60-fold higher (IC_{50} = 0.5 μmol against MDA-MB-231 cell line)[22] than tamoxifen (IC_{50} = 29 μmol against MDA-MB-231).[21]

Toxicity of Fc compounds arises from the mild and reversible oxidation of Fc units yielding ferrocenium (Fc^+) cations.[23, 24] This cation generates reactive oxygen species (ROS) in physiological medium, which damage DNA and other essential biomolecules, culminating in cell apoptosis.[23, 24] Potent cytotoxic effects can be observed when Fc is attached to biologically active molecules.[13, 16, 18, 25] Moreover, functional groups adjacent to the Fc unit have a strong influence on the oxidation process by reducing the redox potential (E_p).[16, 26-28] These groups can also interact with biomolecules through secondary interactions to facilitate the charge transfer process and enhance its toxicity.[16, 25] In this context, the triazole scaffold may be crucial in Fc-bioconjugates for anticancer purposes. Triazole group can be used as bioisostere in medicinal chemistry, it is highly stable, and it can interact through hydrogen bonds mimicking amide groups.[29-32] Moreover, 1,4-disubstituted 1,2,3-triazole can be easily obtained using copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) "click" reaction.[32]

Although we had decades of tremendous progress in cancer research, conventional oncolytic agents are commonly associated with significant side effects.[33] In this context, the folate-targeted therapy has induced high expectations to improve the therapeutic index of drugs.[33, 34] This approach relies on the upregulated expression of the folate receptor alpha protein (Frα) in many epithelial tumor cells, including ovary, uterus, endometrium, brain, kidney, lung and breast.[35-37] Consequently, folate ligands can act as molecular Trojan horse to deliver active molecules selectively into tumor cells.[34] In general, folate drug conjugates have shown improved cytotoxic effects and higher drug accumulation in the tumor mass when

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compared to the free drug.[38] Moreover, they usually possess consistent pharmacokinetic behaviors.[37-40]

Folate drug conjugates can be easily prepared by attachment of active molecules to folic acid (FA) via amidation.[41] However, FA has two available carboxyl groups yielding two regioisomers after amidation: folate α-conjugated and folate γ-conjugated.[41] It has been shown that both isomers can associate with Frα at virtually identical levels, but with different *in vivo* pharmacological behaviors.[40, 42-47] In this context, the utilization of regiospecific methods for the synthesis of folate conjugates is crucial to the success of this folate-based therapy.

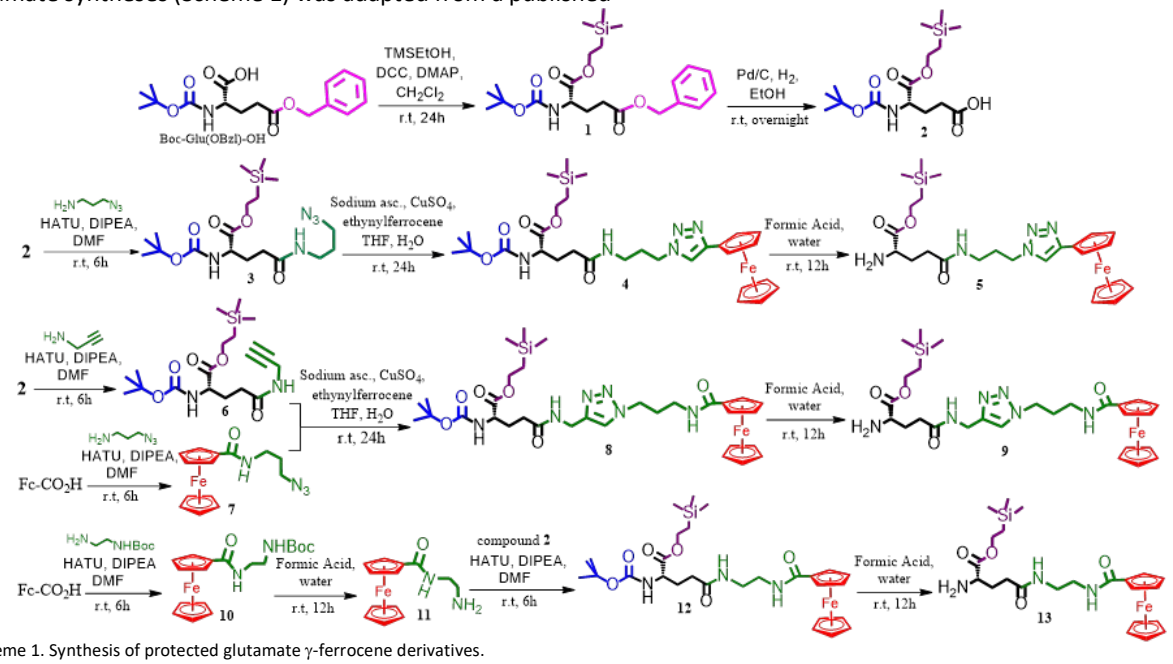
Herein, the synthesis, characterization and anticancer activity of folates γ-Fc are reported for the first time. The Fc units were connected to protected glutamate (Glu) residues *via* different linkers using amide coupling and "click" reactions. Folates were synthesized *via* a regioselective route by reacting protected Glu γ-Fc residues with pteroyl azide (Pte-N_3). Three analogs were synthesized varying the functional group close to the Fc: (i) one triazole group conjugated to the Fc, (ii) one triazole group distal and one amide group conjugated to the Fc, and (iii) only amide groups conjugated and distal to the Fc. The effect of the triazole group was rationalized by comparing the electronic properties of each compound with their anticancer activity on human cells. The cancer-targeting ability and enhanced cytotoxic effect of folates γ-Fc were investigated by comparing their activity in healthy and cancerous cells with Glu γ-Fc residues lacking the Pte moiety.

Results and discussion

Synthesis of folate γ-Fc and Glu γ-Fc derivatives

Regioselective synthesis of folate γ-Fc conjugates was performed by reacting a pteroyl intermediate with protected glutamate residues containing a Fc unit at the γ position. The glutamate syntheses (Scheme 1) was adapted from a published methodology,[48] which starts with the commercial Glu derivative L-Glutamic acid, N-[(1,1-dimethylethoxy)carbonyl]-, 5-(phenylmethyl) ester (Boc-Glu(OBzl)-OH). In the first step, the α-carboxylic acid group of Boc-Glu(OBzl)-OH was protected with 2-(trimethylsilyl)ethanol (TMSEtOH) *via* Steglich esterification. Compound **1** was obtained in 90% yield, after purification with column chromatography on silica gel. Formation of compound **1** was supported by the new signals in the ^{1}H NMR spectrum assigned to the silyl ester unit at 4.21 (*m*, COC*H*$_2$, 2H), 1.00 (*m*, C*H*$_2$Si(CH$_3$)$_3$, 2H) and 0.04 ppm (*s*, Si(C*H*$_3$)$_3$, 9H) (SI, Figure S1). The γ-benzyl ester of compound **1** was selectively removed *via* Pd/C-catalyzed hydrogenation reaction yielding compound **2** in 83% yield, after purification with column chromatography. Full deprotection was supported by ^{1}H NMR spectrum due to the absence of the peaks at 7.34 and 5.11 ppm assigned to the aromatic (Ar-*H*, 5H) and methylene protons (OCOC*H*$_2$, 2H) of the benzyl ester protecting group (SI, Figure S4). Compound **2** has one protected amine group with *tert*-butyloxycarbonyl (Boc) group, one protected α-carboxyl with a silyl ester, and one free γ-carboxylic acid that enables regiospecific γ-conjugation of linkers containing the Fc unit.

To obtain the Glu-Fc derivative bearing one triazole group conjugated to the Fc unit, compound **2** was functionalized with 3-azido-1-propanamine through HATU-mediated amide coupling. Compound **3** was obtained in 71% yield after purification with column chromatography on silica gel. Compound **3** was identified by ^{1}H NMR (SI, Figure S7) through the presence of the multiplet peak at 3.40-3.33 ppm assigned to the methylene groups adjacent to the azide (N$_3$C*H*$_2$, 2H), and the signal of the new amide group (HNC*H*$_2$, 2H). Subsequent CuAAC "click" reaction between compound **3** and ethynylferrocene, using sodium ascorbate and copper sulfate, afforded compound **4** in 84% yield. Compound **4** was purified by flash chromatography on silica gel, and its formation was supported by ^{1}H NMR spectrum (SI, Figure S10) through the



Scheme 1. Synthesis of protected glutamate γ-ferrocene derivatives.

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presence of the peaks between 4.93-4.00 ppm assigned to the Fc aromatic protons, as well as the singlet peak at 7.50 ppm characteristic of 1 4-disubstituted 1,2,3-triazole proton. The Boc group of compound **4** was removed with formic acid to afford the amide-terminated compound **5** in quantitative yield, after washing with diethyl ether. Full deprotection was supported by ^{1}H NMR spectrum (SI, Figure S14) due to the absence of the Boc methyl protons signal at 1.45. The formation of compound **5** was further confirmed by mass spectrometry by its molecular ion peak $[M+H]^+$ at 540.22 m/z (calculated for $C_{25}H_{37}FeN_5O_3Si$: 540.21 m/z) (SI, Figure S17).

The Glu-Fc derivative containing one conjugated amide group to the Fc unit and one distal triazole group was obtained using a similar approach. Compound **2** was reacted with propargylamine via HATU-mediated amidation to afford compound **6** in 96% yield, after purification with column chromatography. Compound **6** was identified by ^{1}H NMR (SI, Figure S18) through the presence of the peaks at 6.38, 5.05, and 2.23 ppm, which are assigned to the newly formed amide group (CON*H*), methylene group adjacent to the amide (CONHC*H*$_2$) and terminal alkyne proton, respectively. The Fc motif was prepared by coupling ferrocenecarboxylic acid (Fc-CO_2H) with 3-azido-1-propanamine using HATU as the coupling agent. Compound **7** was obtained in 88% yield, after purification by flash chromatography on silica gel. Compound **7** was identified by ^{1}H NMR (SI, Figure S21) through the presence of the multiplet peaks at 3.49-3.40 ppm assigned to the methylene groups adjacent to the azide (N_3CH_2) and newly formed amide group (HNC*H*$_2$). Compound **6** and **7** were combined using CuAAC "click" reaction to afford compound **8** in 83% yield, after purification by flash chromatography on silica gel. The formation of compound **8** was supported by ^{1}H NMR spectrum (SI, Figure S24) through the presence of the peaks assigned to the Fc aromatic protons and the singlet peak at 7.66 ppm assigned to the triazole proton. The Boc group of compound **8** was selectively removed after treatment with formic acid to afford compound **9** in quantitative yield. Formation of compound **9** was supported by ^{1}H NMR spectrum (SI, Figure S28) through the absence of the signal at 1.37 ppm assigned to the methyl protons of the Boc group. Formation of compound **9** was further confirmed by mass spectrometry by its molecular ion peak $[M+H]^+$ at 597.22 m/z (calcd for $C_{27}H_{40}FeN_6O_4Si$: 597.23 m/z) (SI, Figure S31).

In the third Glu-Fc derivative (**13**) both units were attached via amide bonds using an ethylenediamine linker. For this, Fc-CO_2H reacted with N-Boc-ethylenediamine via HATU-mediated coupling yielding compound **10** in 84% yield. Formation of compound **10** was evidenced by the ^{1}H NMR spectrum (SI, Figure S32) through the presence of the peaks between 4.79-4.02 ppm assigned to the Fc protons, as well as the signals at 3.42-3.24 and 1.42 ppm assigned to the methylene (C*H*$_2$NH) and methyl protons of the Boc group, respectively. The Boc group of compound **10** was removed after treatment with formic acid to afford compound **11** in quantitative yield after washing with diethyl ether. Full deprotection was supported by ^{1}H NMR spectrum (SI, Figure S35) through the absence of the Boc group signal at 1.42 ppm. Subsequent condensation between compound **2** and compound **11** afforded compound **12** in 88% yield, after purification with column chromatography on silica gel. Formation of compound **12** was supported by ^{1}H NMR spectrum (SI, Figure S38) through the presence of signals from both units (Fc and Glu), as well as the peak at 7.06 ppm assigned to the newly formed amide group. The Boc group of compound **12** was removed with formic acid yielding compound **13** in quantitative yield. Full deprotection was proven by ^{1}H NMR spectrum (SI, Figure S42) by the absence of the signal at 1.42 ppm assigned to the Boc group. Mass spectrometry confirmed the formation of compound **13** by its molecular ion peak $[M+H]^+$ at 502.18 m/z (calcd for $C_{23}H_{35}FeN_3O_4Si$: 502.18 m/z) (SI, Figure S45).

Pteroyl azide compound (Pte-N_3) **14** was prepared (Scheme 2) following a methodology published by Jin Luo *et al.*[49] Briefly, folic acid (FA) was reacted with excess of trifluoroacetic anhydride yielding N2,10-bis(trifluoroacetyl)pyrofolic acid **15**. This compound is a mixture of diacylated anhydride and diacylated carboxylic acid. Subsequent addition of water to compound **14** afforded N10-(trifluoroacetyl)pyrofolic acid **16** in quantitative yield from FA. N10-(trifluoroacetyl)pyrofolic acid was reacted with hydrazine to eliminate the Glu moiety yielding pteroyl hydrazide **17** in 98% yield. Pteroyl hydrazine **17** was transformed into Pte-N_3 **14** via nitroso transfer reaction using *tert*-butyl nitrite and potassium thiocyanate, followed by diazotization under acidic conditions. Pte-N_3 **14** was obtained in 68% yield after purification by precipitation in 2-propanol. Pte-N_3 **14** can react with nucleophiles (e.g., the previous mentioned amine-terminated Glu γ-Fc compounds) due to the presence of the acyl azide group.

Folate γ-Fc conjugates **18**, **19** and **20** were obtained after coupling Pte-N_3 **14** with the respective amine-terminated Glu γ-Fc residues (**5**, **9** and **13**), using 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD) as base (Scheme 2). All three folates γ-Fc were obtained in reasonable reaction yields (60% for folate **18**, 56% for **19** and 58% for **20**) after purification by column chromatography on silica gel with a mixture of dichloromethane, methanol and triethylamine as eluent (solvent mixture gradient from 99:0:1 to 79:20:1). Formation of folates was confirmed by ^{1}H NMR spectroscopy (SI, Figures S46, S51 and S55) through the presence of the signals from both

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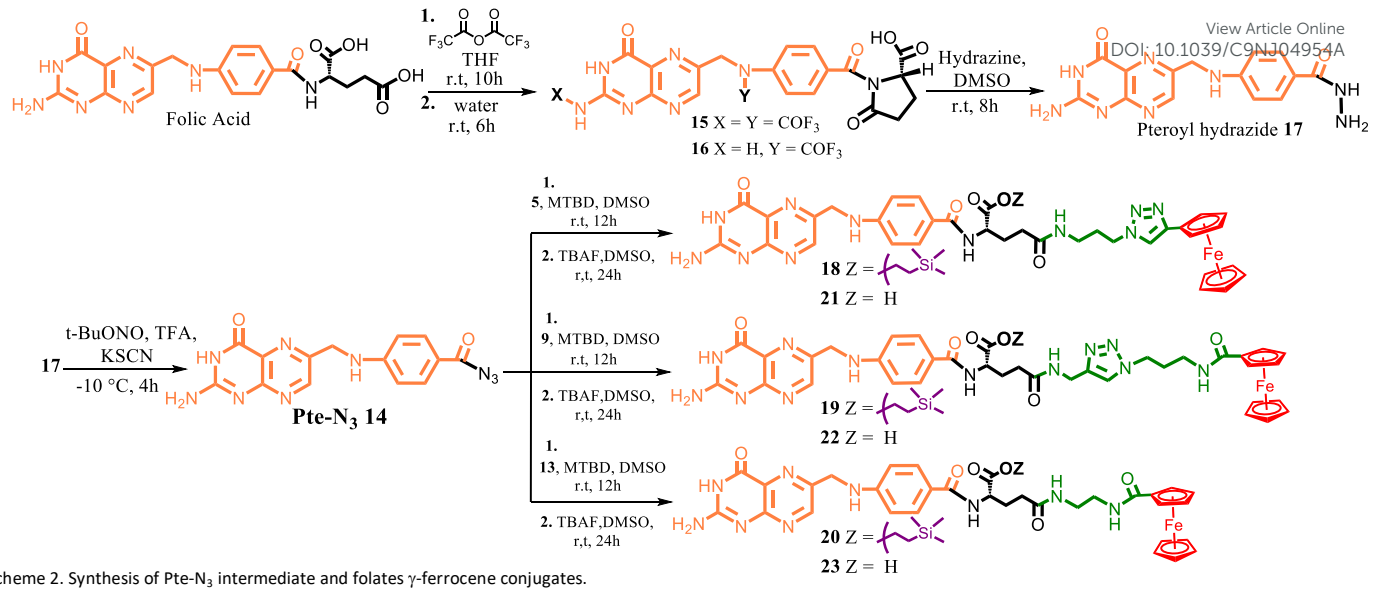

Scheme 2. Synthesis of Pte-N_3 intermediate and folates γ-ferrocene conjugates.

pteroyl and Glu γ-Fc moieties. Folates have characteristic signals around 8.65, 7.66 and 6.64 ppm, which are assigned to the C(7)-*H* of the pterin ring, and two aromatic protons Ar-*H*, respectively. Fc presents its signals around 4.68-4.02 ppm. The Glu moiety presents its signals near 4.35-4.32ppm (HNC*H*), and between 2.23-1.92 ppm assigned to the methylene groups.

Subsequent deprotection of the α-silyl ester of folates **18**, **19**, and **20** using tetrabutylammonium fluoride (TBAF) (solution 1.0 M in THF) afforded the final folates **21**, **22** and **23** (Scheme 2). Utilization of dried TBAF was crucial for these reactions. Otherwise, degradation of the Fc moiety was observed probably due to the formation of hydrofluoric acid. Folate γ-Fc **21** has one triazole group conjugated to the Fc unit, and it was obtained in 68% yield. Folate γ-Fc **22** has one conjugated amide group to the Fc unit and one distal triazole group (72% yield). Folate γ-Fc **23** has two amide groups distal and conjugated to the Fc unit (78% yield). All folates were purified by precipitation in diethyl ether:acetone (80:20), followed by washing with cold HCl solution pH 2 (3×10 mL), cold water (3×10 mL) and cold acetonitrile (3×10 mL). Full deprotection was supported by ^{1}H NMR spectrum (Figure 1) through the absence of the signals assigned to the silyl ester unit near 4.36 (COCH_2), 0.95-0.93 ($CH_2Si(CH_3)_3$) and 0.00 ppm ($Si(CH_3)_3$), as well as the presence of the signal around 11.5 ppm assigned to the α-carboxylic acid proton (see the expanded spectra in the SI, Figure S60, S65 and S70). Formation of the folate γ-Fc conjugates was also confirmed by high-resolution ESI-QTOF mass spectrometry. Folate γ-Fc **21** presented its molecular ion peak $[M+H]^+$ at 734.2252 m/z (calcd for $C_{34}H_{35}FeN_{11}O_5$: 734.2245 m/z) (SI, Figure S63), folate γ-Fc **22** $[M+H]^+$ at 791.2253 m/z (calcd for $C_{36}H_{38}FeN_{12}O_6$: 791.2259 m/z) (SI, Figure S68), and folate γ-Fc **23** $[M]^-$ at 694.1859 m/z (calcd for $C_{32}H_{33}FeN_9O_6$: 694.1830 m/z) (SI, Figure S73).

In order to investigate the targeting ability and enhanced cytotoxic effect of the folates γ-Fc, the protecting groups of compounds **4**, **8**, and **12** were removed (Scheme 3). The resultant compounds **24**, **25** and **26** have analogs structures of folates γ-Fc but lacking the Pte moiety. L-Glutamate and L-

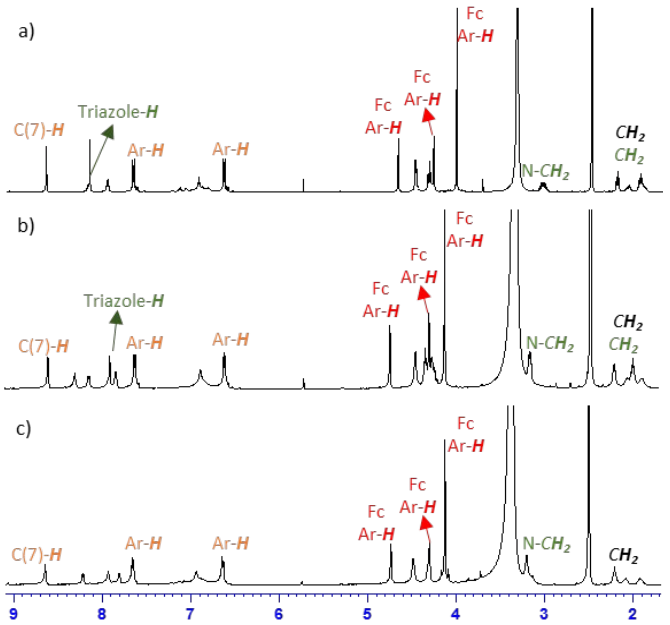

Figure 1. ^{1}H NMR spectra of a) folate γ-Fc **21** (500 MHz, DMSO-D6), b) folate γ-Fc **22** (400 MHz, DMSO-D6), and c) folate γ-Fc **23** (500 MHz, DMSO-D6).

glutamine are essential bioactive compounds for the proper functioning of both healthy and cancerous cells.[50] Hence, their derivatives may have antagonistic effects on cancers, being used as antineoplastic agents or targeting groups.[50] Some of these compounds have great anticancer properties, and they have already been employed in clinical trials, such as azaserine, acivicin and thalidomide.[51] Azaserine and acivicin are inhibitors of key enzymes in the glutamine metabolism, but notorious side effects limited their use.[50]

Unprotected Glu γ-Fc compounds were obtained in two sequential deprotection reactions (Scheme 3): (i) silyl ester removal with TBAF, and (ii) Boc deprotection with formic acid in water. Glu γ-Fc compounds were obtained in good yields, being 84% for Glu γ-Fc **24**, 93% for Glu γ-Fc **25**, and 80% for Glu γ-Fc **26**. Full deprotections were supported by ^{1}H NMR spectra (SI,

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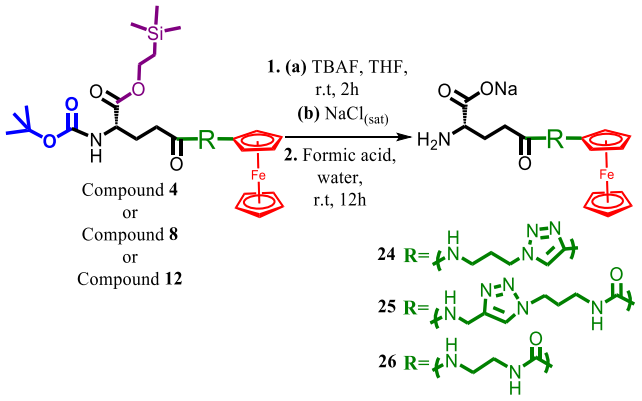

Scheme 3. Synthesis of Glu γ-Fc compounds

Figures S75, S79, and S83) due to absence of the peaks of the Boc and silyl ester. Formation of Glu γ-Fc **24**, **25**, and **26** were confirmed by ^{13}C NMR and mass spectrometry. All compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, two-dimensional [^{1}H-^{13}C]-HSQC NMR, and mass spectrometry. The spectra and assignments are detailed in the SI.

Electrochemical Characterization

Since the redox behavior of ferrocenyl compounds is responsible for their biological activity, cyclic voltammetry (CV) was employed to study the charge transfer process of such compounds. Cyclic voltammograms of folates γ-Fc (Figure 2a) and Glu γ-Fc (Figure 2b) revealed reversible Fc/Fc^{+} redox waves near the expected potential of Fc derivatives (Table 1). As expected, compounds with triazole group conjugated to the Fc (Folate γ-Fc **21** and Glu γ-Fc **24**) exhibited lower E_p (274 and 251 mV, respectively) than the amide-Fc derivatives (**22**, **23**, **25**, and **26**).[29, 30, 52] These results confirm that the triazole group adjacent to Fc donates electronic density to the redox center, facilitating its oxidation. Moreover, the chemically reversible (I_{pc}/I_{pa}=1 and E_{pc}-E_{pa} ≤ 60 mV) nature of the redox waves of all compounds indicates their high stability in physiological medium.[25]

UV-vis characterization

Glu γ-Fc derivatives exhibited two absorptions bands in the UV-vis spectra around 265 and 400-450 nm (Figure 3a), which are characteristic of Fc compounds.[53-55] These bands are assigned to the Fe(a_{1g})-Cp(e_{2g}) charge transfer (or π-π* transitions) and symmetry forbidden Fe(a_{1g})-Fe(e_{1g}) transitions (or *d-d* transitions), respectively.[53-55] The absorption intensities around 400-450 nm were weak due to being symmetry forbidden transitions.[54, 55] The wavelength of maximum absorbance (λ_{max}) from *d-d* transitions of compound **24** was observed at higher wavelength (442 nm) when compared to compounds **25** and **26** (around 410 nm) (Table 1). Again, these results suggest that the triazole group conjugated to the Fc unit is able to donate electrons to the Fc complex, inducing a bathochromic shift in the *d-d* absorption of Fc. These observations are in line with those from CV in which lower E_p values were observed for compounds **21** and **24**.

Table 1. Electrochemical and UV-vis data.

Compound	E_{pc} (mV)	E_{pa} (mV)	E_P (mV)	E_{pc}-E_{pa} (mV)	λ_{max} (nm)
folate γ-Fc **21**	296	252	274	44	[a]
folate γ-Fc **22**	434	386	410	48	[a]
folate γ-Fc **23**	420	381	401	39	[a]
Glu γ-Fc **24**	279	222	251	57	442
Glu γ-Fc **25**	421	368	394	53	411
Glu γ-Fc **26**	410	368	389	42	410

[a] *d-d* absorption of Fc is overlapped with π-π* of folates.

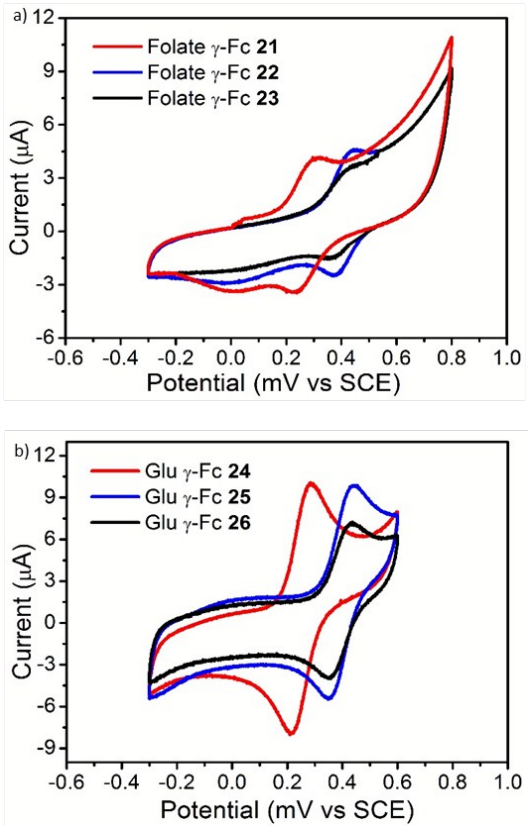

Figure 2. Cyclic voltammetry of (a) folates γ-Fc and (b) Glu γ-Fc in PBS (0.1 M, pH 7.4).

Folates γ-Fc showed two absorptions at 365 and 280 nm (Figure 3b), assigned to π-π* transition localized on the pterin ring and π-π* transition of the pterin ring, respectively.[56, 57] The absorption from *d-d* transitions of Fc between 400-450 nm were not observed because these low intensities bands are overlapped with the broad absorption of the folate moiety around 365 nm.

Anticancer activity

Frα has been seen as a promising biomarker to deliver drugs selectively into cancer cells with notorious avidity (K_D = 1-10 nM).[40] Folate-drug conjugates are internalized into malignant cells via the natural and nondestructive endosomal route, without loss of binding affinity.[37, 40] Although Frα is also present in healthy cells, its location is restricted to the apical surface of the polarized epithelial cells, and the receptor is not exposed to molecules in the bloodstream.[40, 58, 59] On the other hand, Frα

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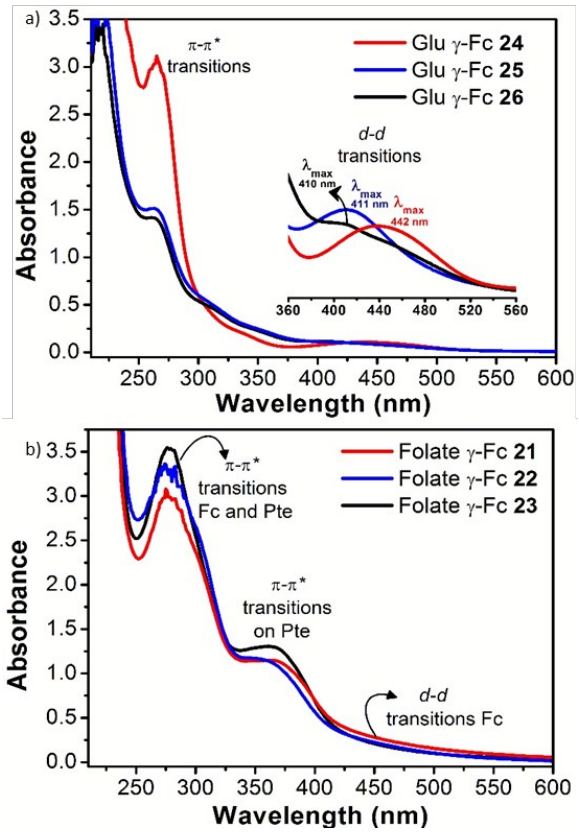

Figure 3. UV-vis spectra of a) Glu γ-Fc and b) folates γ-Fc in PBS (0.1M, pH 7.4).

can be found at very high levels in many epithelial tumors, with expression up to 100 times greater.[60-64]

In vitro cytotoxicity of folates γ-Fc and Glu γ-Fc was investigated using cultured monolayers of human healthy or cancerous cell lines: PNT2 (normal prostate epithelial cells), HeLa (cervical cancer), MCF7 (breast adenocarcinoma), and PC-3 (prostate adenocarcinoma). PNT2 and PC-3 are Frα-negative, whereas HeLa and MCF7 are Frα-positive. Cell viability was determined by colorimetric assay using the Cell Counting Kit-8 (CCK-8), after incubation of the cells for 48 h with appropriate concentrations of the ferrocenyl compounds.

Cell viability was plotted against the compounds concentration (Figure 4), and the corresponding IC_{50} values are given in table 2. All folates exhibited substantial toxicity against cancer cells. In general, folate γ-Fc **21** showed higher activity on cancer cells than the other folates. The IC_{50} values for compound **21** were 21.7 μM (± 0.3) on MCF-7 and 25.4 μM (± 3.2) on HeLa cells. These values were close to the IC_{50} of cisplatin (positive control, Table 2), suggesting the ability of folate **21** to be applied as an anticancer drug.

To verify the targeting ability of folates γ-Fc, their *in vitro* cytotoxicity was assessed on normal PNT2 cells and Frα-negative PC-3 cells. Folates **21** and **22** were less active on such cells. Folate γ-Fc **21** was up to 4-fold less toxic than in MCF-7 cells, with IC_{50} at 87.2 μM (± 4.0) on PNT2 cells and 62.0 mM (± 4.9) on PC-3 (Table 2). Folate γ-Fc **23** was more toxic against PC-3 cells rather than the Frα-positive HeLa and MCF7 cells, suggesting a low affinity of this compound towards Frα.

Competitive blocking experiments were carried out using 1 mM of FA in the growth medium. In this condition, the activities of folates **21** and **22** against HeLa and MCF7 cells were much lower (Table 2). However, the IC_{50} values on PNT2 and PC-3 cells did not quantitatively change. These findings support the ability of these compounds to bind selectively to Frα, with a low level of unspecific uptake via the other folate protein carriers, such as the reduced folate carrier (RFC). RCF is found in almost all cells, but it may have low affinity for oxidized folates (K_m around 150-200 μM).[36, 65] Therefore, folates γ-Fc **21** and **22** can reach and damage cancer cells without harming healthy cells.

The Glu γ-Fc residues showed similar behavior of folates, being Glu γ-Fc **24** the most active against cancer cells. Except on PC-3 cells where Glu γ-Fc **26** showed the lower IC_{50} (Table 2). However, the IC_{50} values for Glu γ-Fc residues were much lower than the folates (Table 2). In this context, Lingling Shan *et al.* described the synthesis of a bifunctional folate-paclitaxel (PTX) prodrug conjugate and evaluated its anticancer properties. PTX is a widely used oncolytic agent, but it is highly lipophilic with poor pharmacokinetics. Attachment of the folate ligand to PTX enhanced the tumor accumulation and efficacy. Folate-PTX was five times more effective on MDA-MB-231 cells (human breast adenocarcinoma) than the free drug. Moreover, the conjugate was 2-fold less toxic on 293T normal cells (derivative of human embryonic kidney), although it is still highly toxic on healthy cells (IC_{50} = 4.4 nM).[66] These observations are in line with the results presented here, in which the folate targeting group enhanced the antiproliferative effect of the ferrocenyl moieties.

Interestingly, compound Glu γ-Fc **26** showed a high selectivity index against prostate cancer cells presenting a IC_{50} value of 31.6 μM against PC-3 that is about 12 times higher than the IC_{50} value on PNT2 (371.8 μM). This finding suggests that this compound might be a good candidate for selective prostate cancer therapy and will be subject of further investigation.

As predicted by CV and UV-vis measurements, compounds bearing one triazole group conjugated to the Fc are more toxic. These compounds exhibited lower E_p's (Figure 2, Table 1) and bathochromic shifts in the UV-vis absorption above 400 nm (Figure 3, Table 1). These data support the electron donating effect of the conjugated triazole group on the Fc unit. Hence, these compounds are readily oxidized, increasing the ROS levels, and inducing cell death.

All these findings revealed the promising effects of donor groups to the Fc on the anticancer activity. Although folate **21** showed great potential to be applied as an anticancer drug, it has to be further studied to evaluate its pharmacokinetic behavior. Overall, the combination of folate therapy with bioorganometallic chemistry appears to be a relevant approach to design new drugs with safety and effective profiles.

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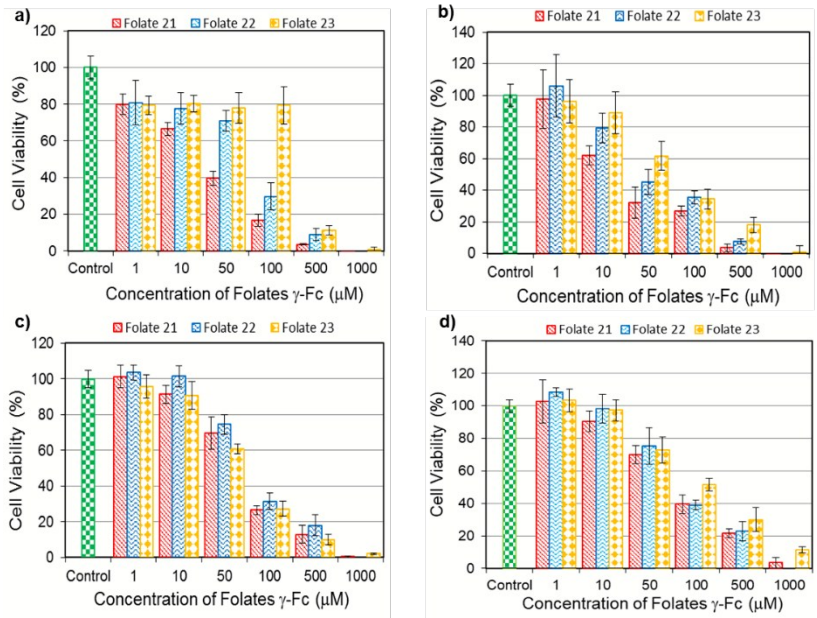

Figure 4. Cell viability of folates on (a) HeLa cells, (b) MCF7 cells, (c) PC-3 cells, and (b) PNT2 cells.

Table 2. IC_{50} of ferrocenyl compounds

Cell line	Cisplatin	Folate γ-Fc **21**	Folate γ-Fc **22**	Folate γ-Fc **23**	Folate γ-Fc **21** + 1mM FA	Folate γ-Fc **22** + 1mM FA	Folate γ-Fc **23** + 1mM FA	Glu γ-Fc **24**	Glu γ-Fc **25**	Glu γ-Fc **26**
HeLa	17.3 μM (± 3.7)	25.4 μM (± 3.2)	56.2 μM (± 6.7)	170.8 μM (± 1.60)	60.2 μM (± 5.2)	76.2 μM (± 8.2)	235.0 μM (± 18.4)	144.1 μM (± 11.0)	169.6 μM (± 6.4)	982.3 μM (± 5.3)
MCF7	11.1 μM (± 0.5)	21.7 μM (± 0.3)	54.2 μM (± 16.4)	80.6 μM (± 12.2)	102.3 μM (± 11.0)	95.5 μM (± 0.4)	170.1 μM (± 5.6)	84.3 μM (± 1.7)	167.7 μM (± 12.0)	313.2 μM (± 18.0)
PC-3	6.9 μM (± 0.15)	62.0 μM (± 4.9)	66.8 μM (± 6.8)	54.3 μM (± 5.1)	72.5 μM (± 5.0)	72.6 μM (± 8.2)	53.6 μM (± 2.3)	64.9 μM (± 9.3)	461.5 μM (± 24.2)	31.8 μM (± 2.7)
PNT2	18.8 μM (± 3.9)	87.2 μM (± 4.0)	101.3 μM (± 15.0)	138.9 μM (± 12.8)	80.0 μM (± 6.3)	93.8 μM (± 10.2)	143.7 μM (± 13.4)	324.0 μM (± 35.5)	358.4 μM (± 30.4)	371.8 μM (± 16.0)

The IC_{50} values are the mean of two experiments done in triplicate.

Experimental

Information about the chemicals, experimental and characterization techniques for all compounds are detailed in the SI. All compounds were characterized by mass spectrometry, ^{1}H, ^{13}C, and 2-D NMR spectroscopy. Supplementary cytotoxicity data are also available in the SI.

Electrochemical measurements

CV's were carried out in a VersaSTAT 3 potentiostat (Princeton Applied Research) with three electrodes: glassy carbon working electrode, Pt auxiliary electrode, and a reference saturated calomel electrode (SCE). Phosphate buffer (PBS) (0.1 M, pH 7.4) was used as supporting electrolyte. Voltammograms were collected at a scan rate of 100 $mV.s^{-1}$, using a concentration of the test compounds at 10 mM. The Ep's were calculated by the mean of E_{pc} (cathodic potential) and E_{pa} (anodic potential).

Cell culture

Human cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For investigation of the folates cytotoxicity, cells were grown for 5 days in folate-free RPMI 1640 medium to induce the expression of Frα. The folate-free medium contains heat-inactivated FBS as the only source of folates around 3 nM, a

value at the low end of the physiological concentration in human serum.[45, 67] The cells were kept in 75 cm^2 flasks, at 37 °C in a humidified incubator with 5% CO_2. Culture medium was changed every 48 h. Cells were passaged using 0.25% trypsin-0.02% EDTA solution with 80-90% of confluence.

In vitro cytotoxicity testing (CCK-8) calorimetric assay

Cells were seeded into 96-well plates with 200 μL of culture medium in a final density of 8×10^3 cells/well. After incubation for 24 hours, solutions of the test compounds (in culture medium) at concentrations ranging from 1 to 1000 μM were added. Aqueous stock solutions of the folates were prepared by adding equimolar amounts of sodium hydroxide yielding the water-soluble carboxylate salts. After 48 hours, the growth medium was removed and the cells were washed with PBS (0.1 M, pH 7.4) (2 × 100 μL). Cells were incubated for additional 4 h after addition of 100 μL of fresh culture medium and 10 μL of Cell Counting Kit-8 (CCK-8). The absorbance of each well was recorded in a microplate reader model FlashScan 530 Analitic Jena at 450 nm. The relative cell viability was expressed as a percentage relative to the control cells. The assay was performed using cisplatin as positive control. IC_{50} vales were determined using the GraphPad Prism software. For folates γ-Fc, competitive blocking experiments were performed with addition of FA 1 mM into the culture medium. All experiments were repeated minimum twice to assure reproducibility.

Conclusions

Three folates γ-Fc were synthesized for the first time and their anticancer activity was assessed against a panel of four human cell lines. Folates were obtained via a regiospecific route in good reaction yields (68-78%). Compounds were designed to have one triazole or amide group conjugated to the Fc unit. CV and UV-vis measurements showed that the triazole group conjugated to the Fc donates electrons to the iron center, which facilitates the oxidation of Fc that is responsible for the antiproliferative activity. In fact, folate γ-triazoleFc **21** was the most active compound with IC_{50} values of 25.4 μM against HeLa and 21.7 μM against on MCF-7 cells. This compound was up to 4-fold less toxic on healthy PNT2 cells and Frα-negative PC-3 cancer cells. Glu residues analogs of folates but lacking the Pte moiety were much less toxic on cancer cells than folates. Hence, folate γ-Fc **21** is a potential new anticancer drug candidate due to its specificity and enhanced cytotoxicity against Frα-positive malignant cells. However, this compound has to be further studied to evaluate its pharmacokinetic behavior. Overall, the combination of folate therapy with Fc compounds appears to be a relevant approach to design new drugs with enhanced therapeutic index.

Conflicts of interest

There are no conflicts to declare.

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Novel folate γ-ferrocene conjugates were synthesized through a regiospecific route and showed selectivity and enhanced cytotoxicity against Frα-positive malignant cells.

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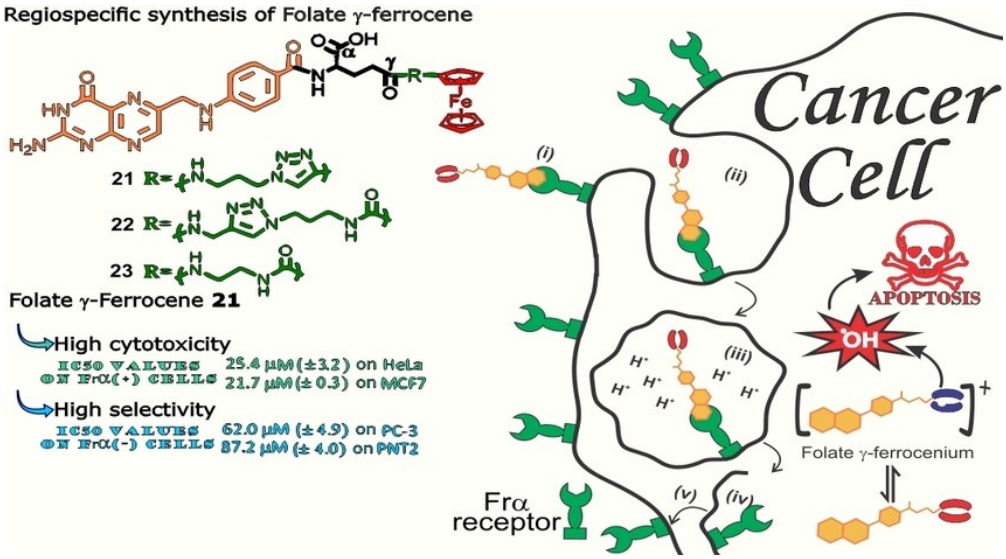

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