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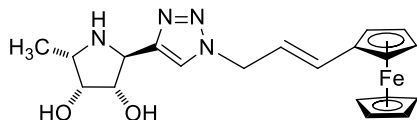
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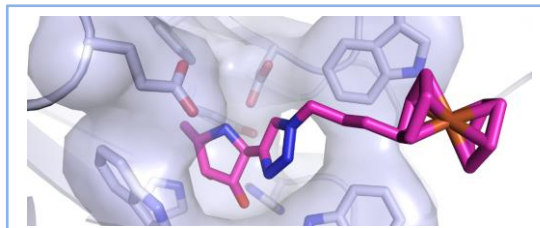
A Second-Generation Ferrocene-Iminosugar Hybrid With Improved Fucosidase Binding Properties

Audrey Hottin^a, Amandine Scandolera^b, Laurent Duca^b, Daniel W. Wright^c, Gideon J. Davies^c and Jean-Bernard Behr



Fucosidase inhibition : $K_i = 0.023 \mu\text{M}$ (bovine kidney)
 $K_i = 0.150 \mu\text{M}$ (*BtFuc2970*)

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A Second-Generation Ferrocene-Iminosugar Hybrid With Improved Fucosidase Binding Properties

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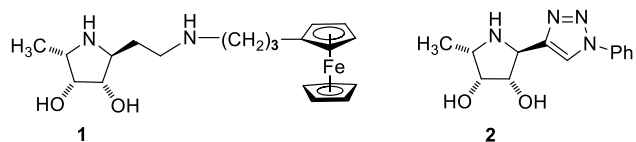
ABSTRACT

The synthesis and the biological evaluation of a new ferrocenyl-iminosugar conjugate designed for fucosidase inhibitory and anticancer activity is described. The compound showed strong affinity for fucosidase from bovine kidney ($K_i=23$ nM) and from *Bacteroides thetaiotaomicron* ($K_i=150$ nM), displaying a 10-fold tighter binding affinity for these enzymes than the previous analogues. The interaction pattern that improves binding has been evaluated through structural analysis of the inhibitor-enzyme complex. The ferrocenyl-iminosugar exhibits significant anticancer activity on MDA-MB-231 and SK-MEL28 cell lines at 100 μ M.

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Glycosylation of proteins is a critical process, which mediates a number of biological events such as enzyme activity, cell-cell or cell-matrix interactions as well as protein targeting.¹ Due to the high diversity of oligosaccharide combinations, this post-translational modification enables very selective biological responses.^{2,3} Glycosylation occurs on diverse proteins displaying various functions (enzymes, cytokines, antibodies) or localization (cell surface, cytoplasm). The biosynthesis of glycoproteins involves specific enzymatic machinery in which glycosyltransferases and glycosidases take a major role in order to add or release sugar units to the growing oligosaccharide chain.⁴ Dysfunction of this process affords proteins with aberrant glycosylation patterns, which contributes to tumor invasion and metastasis through tumour-associated carbohydrate antigens.⁵ Detection of unusual enzymatic activity levels might serve as cancer biomarkers;⁶ for example elevated levels of sialyltransferase are associated with carcinogenesis, proliferation, progression and metastasis, particularly in colorectal carcinomas.⁷ Another enzyme, α -L-fucosidase (AFU), which is responsible for degradation of fucosides and fucose release has been identified as a reliable prognostic biomarker for detecting the development of hepatocellular carcinoma (HCC) in patients with liver cirrhosis.⁸ Indeed, fucose residues participate in many host-cell interactions and, as such, high levels of fucose often correlate with tumor progression as found in: thyroid carcinoma, ovarian carcinoma and colorectal adenocarcinoma. Increased fucosylation of serum glycoproteins has also been observed for patients with breast cancer.⁹ As fucose overexpression occurs in cancer tissues we are exploring the possibility of using fucose mimics, with strong affinity for AFU, as tracers for a selective delivery approach.¹⁰ To this aim, we previously prepared a first series of pyrrolidine-ferrocene conjugates exemplified by **1** (Figure 1), which combined good affinities for AFU thanks to the pyrrolidine scaffold (K_D in the low micromolar range) and revealed anti-proliferative action via the ferrocenyl function.¹¹



$K_i = 0.29 \mu\text{M}$ (fucosidase)
 (77% inhibition of MDA-MB-231
 cell growth at $50 \mu\text{M}$)

$K_i = 4.8 \text{ nM}$ (fucosidase)

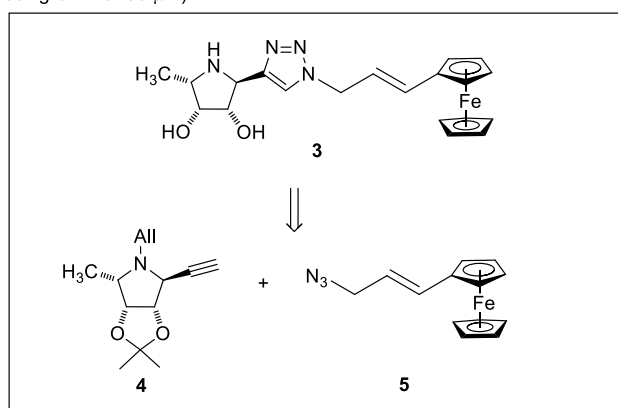
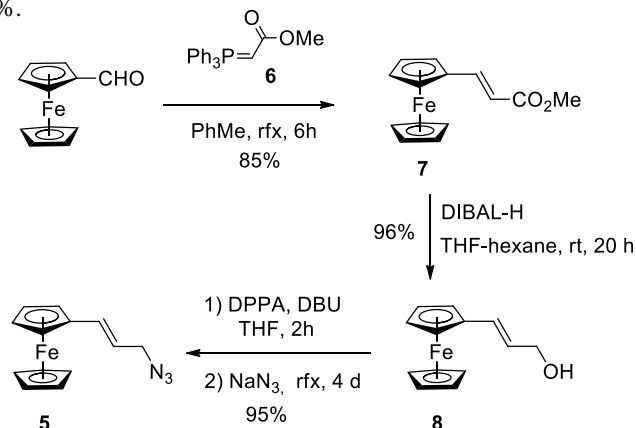


Figure 1.

In mammals, it is believed that the damaging effect of iron is related to RedOx reactions, producing reactive Oxygen Species (ROS), which in turn, oxidize biomolecules such as lipids, proteins or DNA.¹² Thus, it is reasonable to assume that Fc or its derivatives may act as low molecular weight iron carriers and their transport in close proximity to tumour, via the fucosidase-

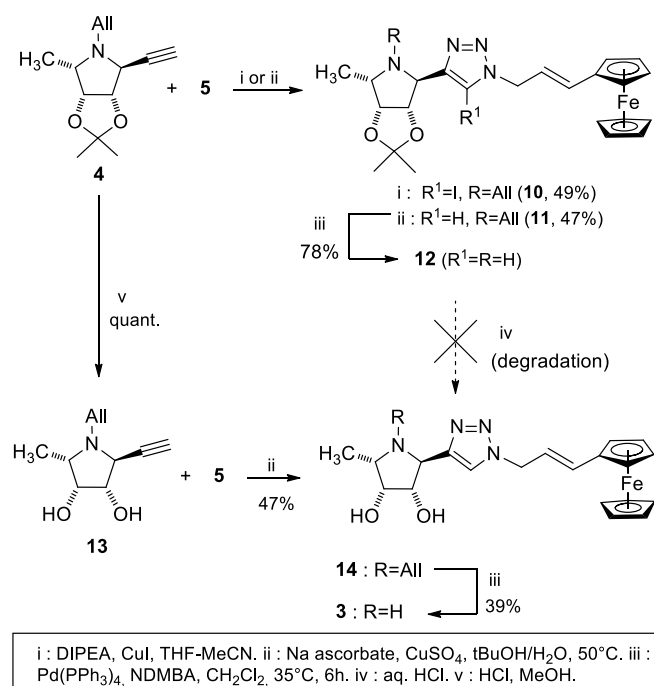
targeting pyrrolidine could localize their deleterious effect. The success of such a strategy relies on the strong affinity of the 5-membered dihydroxypyrrolidine for fucosidase. To improve these binding potencies, the insertion of a hydrophobic substituent at position C-2 of the pyrrolidine moiety was recently attempted with success.¹³⁻¹⁵ A triazole linker between the pyrrolidine and the hydrophobic aglycon permitted to optimize interactions, affording dissociation constants in the nanomolar range (Fig 1, compound **2**). With this in mind, we designed the new pyrrolidine-ferrocene conjugate **3** which features a triazole linker between the pyrrolidine and the ferrocene motifs. In addition, a conjugated double bond was inserted in the structure, in the aim of increasing the hydrophobic character of the linker, but also to modulate the redox properties of the conjugated Fc pharmacophore and possibly improve the production of ROS.

Our synthetic strategy toward Fc-pyrrolidine **3** exploited the Copper(I)-catalyzed Azide-Alkyne Cycloaddition (CuAAC) of ethynylpyrrolidine **4** with Ferrocenylpropeneazide **5** as the key step (Figure 1).¹⁶ Ethynylpyrrolidine **4** was synthesized starting from D-ribose, by following a 7-step procedure as described in the literature.¹⁵ The preparation of Ferrocenylpropeneazide **5** started with a standard Wittig reaction between commercial Ferrocenecarboxaldehyde and the stabilized ylide **6** to afford exclusively the expected *trans*-alkene **7** in 85% yield (Scheme 1).¹⁷ Insertion of the azide function in methyl 3-ferrocenylpropenoate **7** was envisioned via the corresponding alcohol **8**. Surprisingly, the selective reduction of the ester function in **7** proved tricky. No reaction occurred when an ethereal solution of diisobutylaluminium hydride (DIBAL-H) or LiAlH_4 was added to a solution of **7** at room temperature. When heating at 50°C , only the isobutyl ester of 3-ferrocenylpropenoate was isolated with DIBAL-H (no reaction with LiAlH_4), the latter being formed by an unexpected transfer of an isobutyl group from the isobutyl-aluminium reagent to the methyl ester **7**.¹⁸ Changing to a solution of DIBAL-H in hexane proved beneficial. Whereas no reaction occurred when DIBAL-H/hexane was added to a stirred solution of **7** at room temperature in a standard manner, a clean conversion was observed by changing the experimental protocol. The allylic alcohol **8** was obtained in high yield (96%) when a solution of Fc-ester in THF was added, in reverse order, to a stirred solution of DIBAL-H/hexane, at room temperature. Finally, targeted Fc-azide **5** was obtained by direct conversion of alcohol **8** to the azide with diphenylphosphorazidate and DBU in the presence of sodium azide according to the procedure described by Thompson *et al.*¹⁹ The whole sequence afforded pure ferrocenylpropeneazide **5** in three steps from commercial Fc-CHO with an overall yield of 77%.



Scheme 1. Synthesis of ferrocenyl-azide **5**.

The CuAAC reaction of Fc-azide **5** with ethynylpyrrolidine **4** was first performed in a nonaqueous medium (THF/MeCN) with copper(I) iodide (2 equivalents) and a large excess of diisopropylethylamine (DIPEA). Instead of the expected product **11**, the iodo-triazole **10** was isolated in 49% yield. Compound **10** could result from the Huisgen cycloaddition between Fc-azide **5** and an iodo-acetylide intermediate formed *in situ* by reaction of terminal alkyne **4** with copper(I) iodide.^{20,21} To circumvent this difficulty we turned to the standard CuAAC procedure reported by Sharpless, which uses copper(II) salts (CuSO₄) in the presence of sodium ascorbate as the reducing agent.²² When the reaction was held in water/*tert*-butanol for 48h at 50°C, the desired triazole **11** was isolated in 47% yield after purification by chromatography over silica gel. After *N*-allyl deprotection with Pd(PPh₃)₄ in the presence of *N,N*-dimethylbarbituric acid (78% yield), we struggled with the final hydrolysis of the isopropylidene group. Under the standard conditions also used for other ferrocene derivatives (1M aqueous hydrochloric acid)^{10,11} we observed the degradation of the starting material, with no formation of the expected product **3**. The reaction medium became dark blue during the reaction, revealing the possible formation of ferrocenium species. The same observation was made in a control reaction in which ferrocenylalcohol **8** was stirred in the presence of acid, supporting the instability of the unsaturated Fc moiety in the presence of HCl. A variety of other methods were tested for the deprotection of the acetonide (with FeCl₃, BCl₃ or Er(OTf)₃ as the reagents), but all proved unsuccessful. To tackle this issue, hydrolysis of the isopropylidene was performed upstream the synthetic route, directly on substrate **4** to yield dihydroxypyrrrolidine **13** in quantitative yield. Reaction of alkyne **13** with azide **5** under the conditions stated above (CuSO₄, sodium ascorbate), afforded *N*-allyl triazole **14**. Final deprotection of **14** with Pd(PPh₃)₄ and *N,N*-dimethylbarbituric acid afforded pure Fc-imosugar **3** after purification by silica gel chromatography.



Scheme 2. Synthesis of hybrid molecule **3**.

We determined the affinity of ferrocenyl-pyrrolidine **3** for fucosidase from bovine kidney and *Bacteroides thetaiotaomicron* (*BtFuc2970* α -L-fucosidase, a surrogate for human GH29

fucosidases with sequence identities of 27% and 28% towards FUCA1 and FUCA2, respectively). Pleasingly, compound **3** showed strong affinity for fucosidases, with $K_i = 23$ nM for the protein from bovine kidney and $K_i = 150$ nM towards *BtFuc2970*. Ferrocenyl-iminocyclitol **3** binds to bovine kidney α -L-fucosidase much more tightly than the reported homologue **1** ($K_d = 0.29$ μ M).¹¹ The introduction of the triazole linker in place of the linear aminopentyl chain improved the stability of the fucosidase-pyrrolidine complex by more than one order of magnitude.

In order to elucidate the structural factors that determine the potency of inhibition of fucosidase, we determined the X-ray crystal structure of *BtFuc2970* liganded with compound **3** (Figure 2, PDB code 5HDR). As expected, the binding mode of **3** in the enzymatic active site is through an *E*₃ conformation, as observed previously for other *fuco*-configured pyrrolidine inhibitors.^{11,15,23} Curiously, **3** appears to be a tighter binder of mammalian α -L-fucosidases than *BtFuc2970* (see kinetic data above), while in contrast the affinity of **1** for both bovine kidney α -L-fucosidase and *BtFuc2970* are comparable (0.29 μ M vs. 0.46 μ M, respectively).¹¹ The cause of this discrepancy is uncertain and may arise due to solvent effects, or perhaps more likely due to specific differences in the 3-dimensional structures of the enzymes. It is well documented that primary sequence differences exist between bacterial and mammalian α -L-fucosidases, resulting likely in differences in loop architecture near the GH29 family enzymatic active site.^{24,25} Interactions made between *fuco*-configured inhibitors and these loop regions of GH29 enzymes may underlie the observed discrepancy in K_i values. This highlights the pressing need for 3 dimensional structures of mammalian α -L-fucosidases in order to determine the effect these regions may have on inhibitor potency.

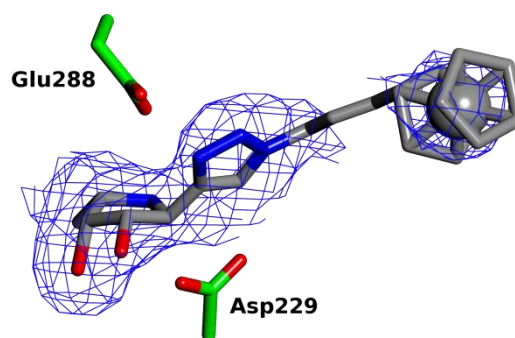


Figure 2. Compound **3** lying in the active site of *BtFuc2970*; the map shown is a likelihood-weighted $F_o - F_c$ map calculated before the incorporation of phases from compound **3** in refinement, contoured at 2σ . The catalytic nucleophile Asp²²⁹ and acid/base Glu²⁸⁸ residues are annotated. The absence of strong density for the "linker" region reflects greater mobility, as observed previously for other fucosidase ligand complexes. The figure was drawn using CCP4MG²⁶.

Next, the anti-proliferative effect of **3** was analyzed using the hormone-independent breast cancer cell line MDA-MB-231 and the human melanoma cell line SK-MEL28. Ferrocenyl-azide **5**, lacking the pyrrolidine carrier, as well as first-generation ferrocenyl-pyrrolidine **1**, which had previously been assayed towards MDA-MB-231 cell lines,^{10,11} were also tested here as controls. The results reported on Figure 3 clearly demonstrate that the tested ferrocenyl compounds **1**, **3** and **5** exhibit anti-proliferative effect on both breast cancer and melanoma cell lines. Compound **1** displayed a clear dose-response behavior, especially on MDA-MB-231 cells, and showed a significant

effect even at 12.5 μM concentration of drug. In the presence 100 μM of **1** MDA-MB-231 and SK-MEL28 cell viability decreased by 80%. The triazole-substituted analogue **3** proved less active, with only 20% cell death at 100 μM on both cell lines. However, with SK-MEL28, compound **3** also showed a dose-response effect. Interestingly, **3** exhibited the same profile as ferrocenyl-azide **5**, which reflects the exclusive role of the ferrocenyl structure in the anti-proliferative activity.

These results show that the conjugation of the ferrocene pharmacophore to the pyrrolidine carrier is not detrimental to its antiproliferative action. Moreover, the introduction of a triazole linker in the structure of hybrid pyrrolidine-ferrocene **3** increases cell-viability, when compared to amino-tethered **1**. However, it is important to note that *in vitro* tests do not precisely reflect *in vivo* performance. Thus, despite its moderate biological effect when compared to compound **1**, the increased affinity of compound **3** for fucosidase could be a strong advantage for targeting cancer tissues in a complex biological environment.

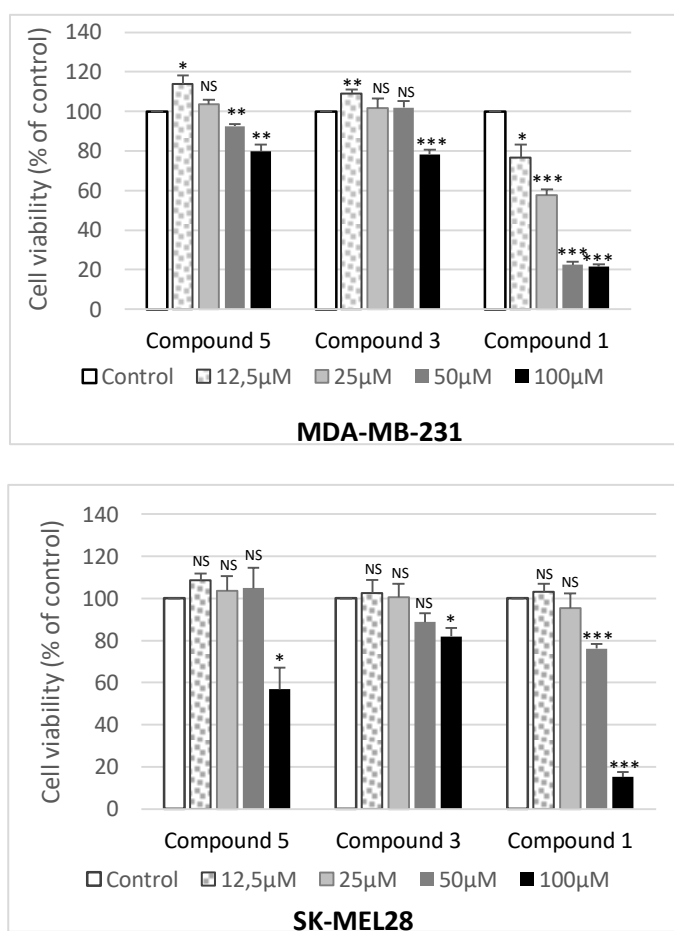


Figure 3. Evaluation of cell viability following 24h treatment with compounds **1,3,5** using human MDA-231 breast cancer and SK-MEL28 melanoma cells lines. Cells were treated for 24h with or without compounds then cell viability was evaluated using MTT assay.

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Supplementary Material

General experimental procedures for the synthesis of all new compounds, procedures for crystal structure determination and for biological assays.