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Folate–Cyclodextrin Conjugates as Carriers of the Platinum(IV) Complex LA-12

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Folic acid has emerged as an interesting cell-targeting moiety and a number of drugs have been conjugated to folate. In this context, new conjugates of β-cyclodextrins with folate have been synthesised as drug carriers to improve their selectivity for cells overexpressing the folic acid receptor. In particular, both 3- and 6-functionalised β-cyclodextrins, linked to the α- or γ-carboxylic group of folic acid, have been synthesised and fully characterised. As a proof of concept, the antitumour platinum(IV) complex *cis–trans–cis*-[$PtCl_2(CH_3CO_2)_2$-(adamantylamine)(NH_3)] (LA-12) has been used as a guest drug. The LA-12–cyclodextrin inclusion complexes have been tested on tumour cells. In the presence of cyclodextrin–folate conjugates, LA-12 exhibited IC_{50} values four times smaller than those of LA-12 alone in MDA-MB-231 cells, which overexpress folic acid receptors on their membrane. No improvement of LA-12 cytotoxicity was found in control tumour cells that do not overexpress the folate receptor. Thus, the non-covalent approach, based on inclusion complexes with functionalised cyclodextrins, looks very promising for drug targeting.

Introduction

Cyclodextrins (CyDs) are cyclic oligomers of D(+)-glucopyranosyl units linked by α-1,4 glycosidic bonds. The most common CyDs are α-, β- and γ-CyDs with six, seven or eight glucose units, respectively. The complexing properties of CyDs have been widely investigated and these have found many applications in the pharmaceutical, textile and food industries.[1–3] CyDs can also remove cholesterol from blood, and in 2010 the Food and Drug Administration (FDA) approved the administration of hydroxypropyl β-cyclodextrin in twin girls suffering from a congenital neurodegenerative disease (Niemann–Pick Type C).[4]

The replacement of CyD hydroxyl groups with other functional groups has been shown to improve markedly the field of application of CyDs, and the covalent conjugation of molecules with different properties provides an attractive approach in the building of multifunctional systems with applications in supramolecular, bioinorganic, organic, pharmaceutical and materials chemistry and separation sciences. Thus, a number of CyD conjugates with amines, amino acids, peptides and aromatic systems have been reported over the years.[5] Fascinating compounds, including enzyme mimics, abiotic receptors, fluorescence indicators and molecular actuators have been obtained by shaping the hydrophobic nature and size of the CyD cavity to match the specific requirements of the ad hoc attached molecule. For instance, Sugammadex is a successful CyD derivative designed to selectivity reverse the effect of rocuronium.[6]

CyDs have also been functionalised with folic acid (FA, vitamin B9, (2*S*)-2-[(4-{[(2-amino-4-hydroxypteridin-6-yl)methyl]amino}phenyl)formamido]pentanedioic acid) to give tumour-selective drug carriers or functional food additives.[7–13] Indeed, many studies have identified the isoform α of folate receptor (FR) as an attractive tumour marker, since it is preferentially overexpressed on the surface of cancer cells whereas it is scant in normal tissues.[14] Therefore, FA has emerged as a valuable cell-targeting moiety.[15] FA has two COOH groups on the glutamic residue that can be functionalised through amide bond formation leading to γ- and α-FA derivatives. The γ-carboxylate derivatives are the major products because of steric and electronic effects. It has been demonstrated that the FR recognises folate derivatised at the γ- but not at the α-carboxylate.[16] Therefore, the conjugation with FA is accompanied by a selectivity issue requiring the isolation of the γ-folate derivative.

In early works, FA has been conjugated to randomly methylated β-cyclodextrins (βCyDs), to give per- or mono-functionalised CyDs.[17] Mono-functionalised βCyDs[18] have been used as carriers of doxorubicin modified with an adamantyl residue.

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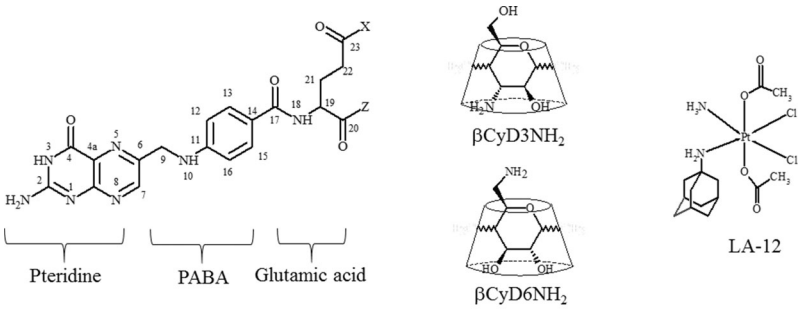
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cplu.201402342. LA-12: *cis–trans–cis*-[$PtCl_2(CH_3CO_2)_2$(adamantylamine)(NH_3)].

Generally, CyD–FA conjugates used as drug carriers were mixtures of different regioisomers.

The potentiality of CyDs as carriers has prompted us to synthesise new CyD derivatives of FA and to separate the different regioisomers. Furthermore, as proof of concept, we tested the ability of CyD–FA conjugates to deliver *cis–trans–cis*-$[PtCl_2(CH_3CO_2)_2(adamantylamine)(NH_3)]$ (LA-12, Figure 1), a complex of Pt^{IV} with 1-adamantylamine (AMA). LA-12 is an antitumour drug[19] that acts as a pro-drug of cisplatin, the latter representing a cornerstone in present-day chemotherapy. Unfortunately, significant side effects, such as nephrotoxicity, neurotoxicity and tumour resistance, have considerably limited the use of cisplatin in cancer therapy. For these reasons, the development of novel platinum-based cancer chemotherapeutics, active even on cisplatin-resistant cancer types, would be highly desirable. One promising new drug is the complex LA-12.[20,21] LA-12 was found to display a high degree of cytotoxicity against both cisplatin-sensitive and cisplatin-resistant ovarian cancer cells. LA-12 reached phase I clinical trials and its oral dose regimens were studied in mouse models and in intrinsically cisplatin-resistant ovarian adenocarcinoma.[22] However, LA-12 is poorly soluble in water (0.03 mg mL^{-1}), which represents a severe limitation for its administration. The inclusion of LA-12 into CyDs, to obtain a pharmaceutical formulation suitable for administration by injection, has been patented.[23] The affinity of the adamantyl group for the CyD cavity is widely recognised.[24] Moreover, an extensive investigation of inclusion complexes between AMA and CyDs has proved that the βCyD cavity is the best suited for fitting AMA (K_a of about 5000 M^{-1}).[25]

Herein, we report the synthesis of both 3- and 6-functionalised βCyDs (βCyD3 and βCyD6) linked to the α- or γ-carboxylic group of the FA to be used as carriers for drugs such as LA-12. In particular, we have synthesised, isolated and characterised the γ and α regioisomers of FA linked to βCyD in position 3 or 6: βCyD3-FAγ (3A-deoxy-3$_A$-(pteroyl-γ-glutamylamine)-2A(*S*),3A(*R*)-βCyD), βCyD3-FAα (3A-deoxy-3A-(pteroyl-α-glutamylamine)-2A(*S*),3A(*R*)-βCyD), βCyD6-FAγ (6A-deoxy-6A-(pteroyl-γ-glutamylamine)-βCyD) and βCyD6-FAα (6A-deoxy-6A-(pteroyl-α-glutamylamine)-βCyD). It is to be stressed that the separation of the two regioisomers of FA is important for the selective interaction with the FR. The 6-derivatives have been reported previously;[18] however, they were used as a mixture of regioisomers.



Compounds	X	Z
βCyD3FAα	βCyD3NH	OH
βCyD3FAγ	OH	βCyD3NH
βCyD6FAα	βCyD6NH	OH
βCyD6FAγ	OH	βCyD6NH

Figure 1. Structures of folate–cyclodextrin conjugates and LA-12.

The secondary side of CyDs has been described as the preferential site for selective binding and catalysis, exhibiting different properties in comparison with the primary side.[26] However, the functionalisation at the secondary side has been less investigated than that at the primary side, in spite of the better synthetic yield of 3-derivatives. The functionalisation at the secondary side through epoxide results in a modification of glucose to altrose.[5] This can be an advantage if the incoming functional side chain has high affinity for the CyD cavity and can be included, as in the case of FA.[27]

Furthermore, we investigated the ability of these compounds to include LA-12 (through different techniques such as NMR and circular dichroism (CD) spectroscopy) and to deliver the drug to tumour cells by determining the antiproliferative activities of LA-12 co-administered with CyD3-FAγ or CyD6-FAγ in FR(+) and FR(−) cell lines.

Results and Discussion

Synthetic aspects

Water-soluble FA–CyD conjugates βCyD6-FAγ, βCyD6-FAα, βCyD3-FAγ and βCyD3-FAα were synthesised through amide condensation reactions starting from 3A-amino-3A-deoxy-2A(*S*),3A(*R*)-βCyD or 6A-amino-6A-deoxy-βCyD and folate *N*-hydroxysuccinimide (FA-NHS). It is known that the activation of FA occurs by 80% to the γ-carboxyl group and by 20% to the α-carboxyl group of the glutamate residue.[11] Therefore, the reaction of amino-βCyD and FA-NHS leads to a mixture of two regioisomers with the folate linked either through its α-carboxyl group or through its γ-carboxyl group. The βCyD–FA regioisomers were isolated through anion-exchange chromatography and fully characterised by ESI-MS, NMR and CD spectroscopy.

NMR spectroscopy

NMR spectra were assigned by 2D COSY, TOCSY, ROESY and HSQC NMR spectroscopy (Figures 1S–8S in the Supporting Information). The NMR spectra were not affected by the βCyD–FA concentration in the analysed sample.

βCyD6-FAs

The ^{1}H NMR spectra of βCyD6-FAs are shown in Figure 2. In the βCyD6-FAα spectrum, the aromatic protons of the *p*-aminobenzoic acid residue (PABA, Figure 1) resonate at $\delta = 6.57$ and 7.57 ppm; H-19 resonates at $\delta = 4.0$ ppm whereas diastereotopic Hs-21 resonate at $\delta = 2.63$ ppm and diastereotopic Hs-22 resonate at $\delta = 1.99$ and 2.16 ppm. The Hs-1 of the βCyD moiety are split into six groups as a consequence of the functionalisation, as observed for other derivatives.[28–30] Also the other protons of the βCyD moiety are spread upon the functionalisation. The diastereotopic 6A protons are shifted upfield (to $\delta = 2.66$ and 3.14 ppm), whereas other 6X protons are shifted upfield or downfield, depending upon the interaction with the aromatic moiety of FA. In particular, two H-6 protons, assigned to the G glucose ring, appear at about 4 ppm. Two H-3 protons (H-3B and H-3X) are shifted upfield at about $\delta = 3.02$ and 2.87 ppm, respectively.

The overall trend of the βCyD6-FAα spectra suggests the inclusion of the folate moiety into the cavity. Such an inclusion is also supported by the ROESY spectrum (Figure 3S), which shows cross-peak correlations between PABA ring protons and protons H-3 and H-5 inside the βCyD cavity. PABA protons also correlate with the H-3B and H-3X protons that are shifted significantly upfield because of the ring current effect. The overall disposition of the FA is such to bring the PABA ring inside the cavity and the pteryl moiety out of the cavity, next to the upper rim.[27]

In the spectrum of regioisomer βCyD6-FAγ the chemical shifts of the aromatic PABA protons are quite similar to those of the α isomer. In addition, H-9 of FA resonates at $\delta =$ 4.25 ppm, Hs-21 at $\delta = 1.92$ and 2.49 ppm and Hs-22 at $\delta =$ 2.10 and 2.25 ppm. The signals of the CyD protons are spread over a wider range. Overall, the ^{1}H NMR spectra suggest the inclusion of the FA moiety into the CyD cavity. Some H-3 protons are shifted upfield and two H-6 protons are shifted downfield because of the ring current effects. Moreover, H-5A is shifted upfield at $\delta = 3.52$ ppm whereas one H-6A is shifted downfield at $\delta = 4.04$ ppm. Also, the ROESY spectra (Figure 3) suggest the inclusion of the chain into the CyD cavity with correlations between PABA protons and CyD inner protons.

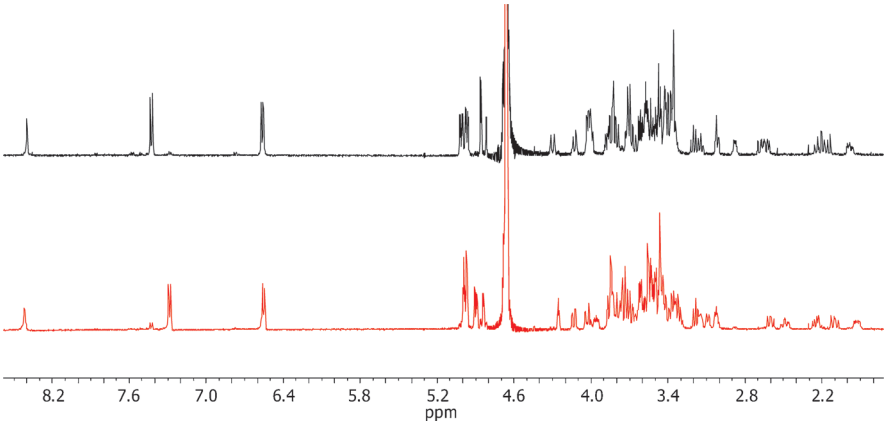


Figure 2. ^{1}H NMR (500 MHz, D_2O) spectra: βCyD6-FAα (black) and βCyD6-FAγ (red).

βCyD3-FAs

Unlike 6-functionalised βCyD6-FA derivatives, the ^{1}H NMR spectra of 3-functionalised βCyD3-FA derivatives (Figure 4) are very different for the α and γ isomers. In the case of βCyD3-FAα, the CyD protons are spread in a way quite similar to those of the βCyD6-FAs thus suggesting the self-inclusion of the side chain. Of the H-1 signals, only the H-1A could be assigned. The function-

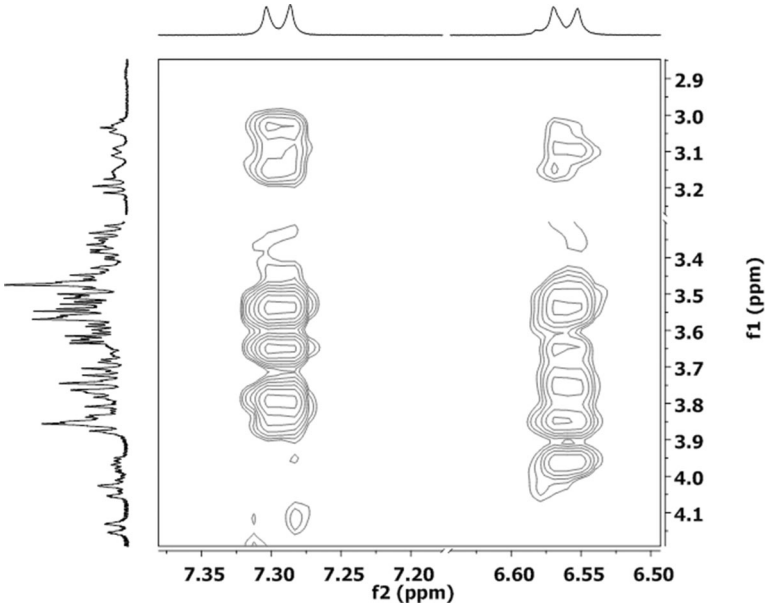


Figure 3. ROESY spectrum (500 MHz) of βCyD6-FAγ in D_2O.

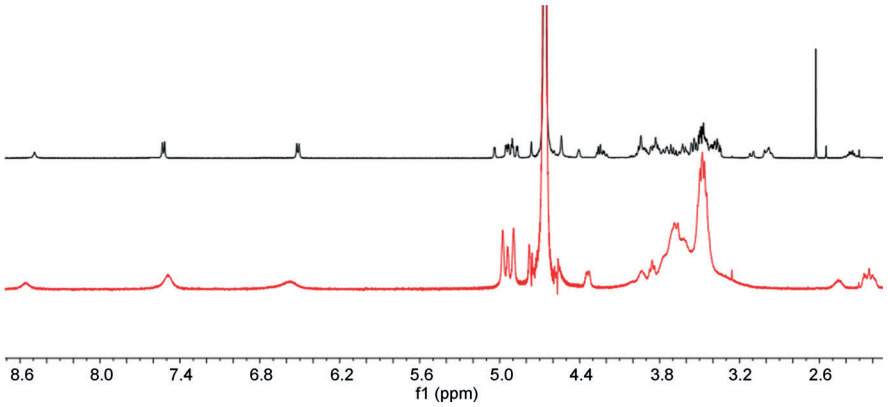


Figure 4. ^{1}H NMR (500 MHz, D_2O) spectra: βCyD3-FAα (black) and βCyD3-FAγ (red).

alisation at position 3 modifies a glucose unit in an altrose residue, thereby increasing the non-equivalence of the sugar units in the NMR spectrum. The $J_{1A,2A}$ value of βCyD3-FAα (about 3 Hz) is smaller than the $J_{1A,2A}$ value (about 7 Hz) of the precursor 3A-amino-3A-deoxy-2A(*S*),3A(*R*)-βCyD.[31] This suggests that the altrose residue, which is in the $^{1}C_4$ conformation in the 3-amino-CyD, has a different conformation ($^{4}C_1$ or $^{O}S_2$) in βCyD3-FAα because of the interaction with the cavity of the side chain, as reported for similar cases.[32]

In addition to the downfield shift of the H-3A proton, as a consequence of the OH substitution by NH, the other two H-3 protons are shifted downfield whereas two H-6 protons are shifted upfield. This trend suggests the inclusion of the aromatic ring, which therefore influences the chemical shifts of CyD inner protons owing to the ring current effect. The ROESY spectra show cross-peak correlations between the PABA aromatic protons and the inner protons of the CyD cavity, thus confirming the self-inclusion of the FA residue. In accord with a self-inclusion mechanism, the spectra do not depend upon the concentration of βCyD3-FAα in the analysed sample.

Unlike the α isomer, the spectra of βCyD3-FAγ show broad signals with less spreading of the signals. Presumptively the moiety is not self-included owing to the glutamate chain and the wide secondary rim of CyD, but some intermolecular interactions may occur leading to colloidal solutions. This is also confirmed by molecular models. Similar colloidal solutions have been reported for related systems.[12]

CD spectroscopy

All compounds were also characterised by CD spectroscopy to further investigate the interaction of FA with the βCyD cavity (Figures 5, 9S and 10S).

βCyD6-FAs

CD spectra of βCyD6-FAs show bands in the absorption region of the folate moiety. The CD spectrum of βCyD6-FAγ (Figure 5) is characterised by an intense positive band at 205 nm, two positive bands at 280 and 310 nm and two small negative bands at 230 and 290 nm. A similar spectrum is obtained for βCyD6-FAα (Figure 9S). The intensities of the CD bands in the CyD-FA conjugates are higher than those in free FA. This behaviour suggests an interaction of the folate moiety with the cavity in accord with the NMR results.[33]

When the competitive guest 1-adamanthanol (ADM) was added to the βCyD6-FA solution, the intensities of the CD bands decreased, which confirms the inclusion of the folate moiety into the cavity. Figure 5 shows the behaviour for βCyD6-FAγ.

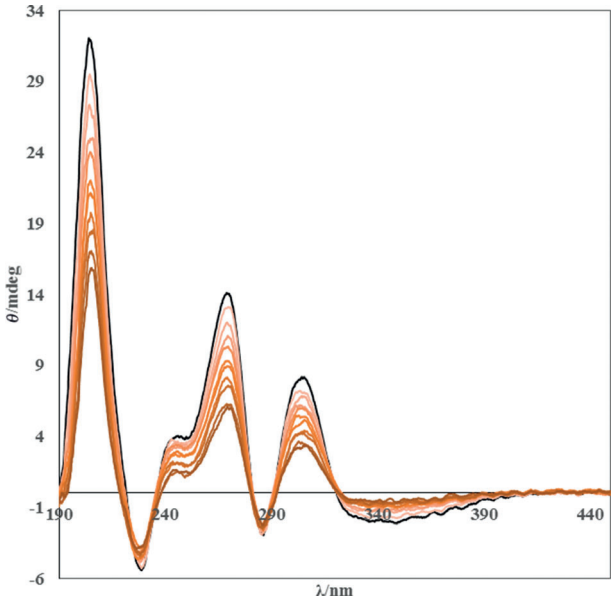


Figure 5. CD spectra of CyD6-FAγ (1×10^{-4} M, black) at increasing concentrations of ADM (red, from 1×10^{-4} to 5×10^{-3} M). Spectra have been corrected for dilution effects.

βCyD3-FAs

βCyD3-FAα has a CD spectrum resembling that of βCyD6-FA (Figure 6). When ADM was added, the CD spectrum of βCyD3-FAα changed significantly and, at high molar ratio (1:10 CyD/ADM), the CD spectrum became similar to that of free FA in keeping with the exclusion of the folate moiety from the cavity.

Unlike βCyD3-FAα, the βCyD3-FAγ regioisomer has a CD spectrum with weak bands and is very similar to that of free FA (Figure 10S): in particular, two small positive bands at 220 and 290 nm. This suggests that the folate moiety does not interact significantly with the cavity or has a different orientation in the cavity. Accordingly, the CD spectrum of βCyD3-FAγ did not change by addition of ADM.

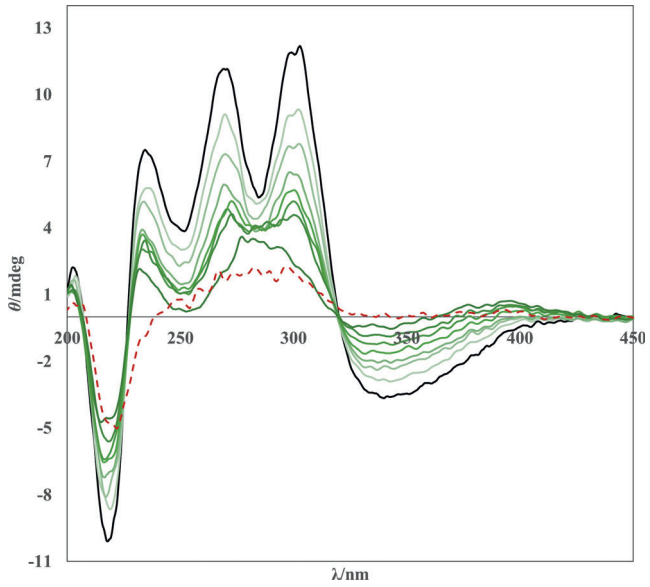


Figure 6. CD spectra of CyD3-FAα (1×10^{-4} M, black) at increasing concentrations of ADM (green, from 1×10^{-4} to 5×10^{-3} M); FA (1×10^{-4} M, red). Spectra have been corrected for dilution effects.

Probably the different chain length in the α and γ isomers determines a different disposition of the FA moiety in the case of βCyD3 derivatives. In the α isomer the chain is longer and the moiety could interact intramolecularly with the cavity. In the γ isomer intermolecular interactions might be preferred, as also suggested by the NMR data.

Interaction with LA-12

The ability of βCyD–FA conjugates to form inclusion complexes with LA-12 was investigated. LA-12 is a promising new antitumour platinum(IV) complex containing AMA. Moreover, being a platinum(IV) complex it is rather inert and does not undergo ligand substitution in the presence of FA conjugates as confirmed by NMR spectra.

The adamantane residue present in LA-12 is known to have high affinity for the βCyD cavity and a number of drugs have been functionalised with adamantane to form inclusion complexes with CyDs.[34] A well-known case is that of doxorubicin, although in this case functionalisation has led to reduced pharmacological activity.[18] The inclusion complexes of LA-12 with CyD–FA conjugates were investigated by NMR spectroscopy through addition of a concentrated dimethyl sulfoxide (DMSO) solution of LA-12 to an aqueous solution of CyD–FAs. The stability of the inclusion complexes was measured as a function of time. The ROESY spectrum (Figure 7) showed that the signals of the adamantane residue at 1.90 and 1.60 ppm have cross-peak correlations with the signals in the range 3.90–3.40 ppm at which CyD protons H-3 and H-5, residing inside the βCyD cavity, resonate. Therefore, the NMR data support the formation of an inclusion complex between LA-12 and the CyD cavity with displacement of the self-included folate moiety.

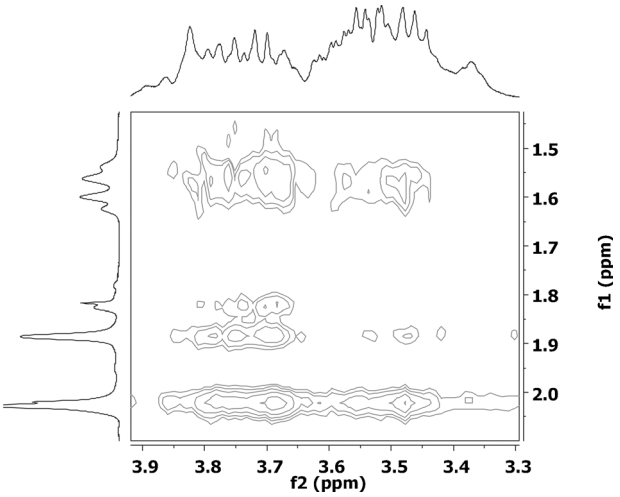


Figure 7. ROESY spectrum (500 MHz) of βCyD6-FAγ with LA-12 in D_2O.

Antiproliferative activity

To verify if the functionalised βCyDs can favour the cellular uptake of LA-12, we investigated the antiproliferative activity of LA-12 in the presence of βCyD–FA. Cell proliferation assays were performed on cancer cell lines differing in the degree of FR expression.[14,35,36] MCF-7 cells were used as negative control. βCyDs at 50 μM were not cytotoxic.

The most remarkable results were obtained with MDA-MB-231 cells, for which the IC_{50} values (Table 1) of LA-12 co-administered with βCyD6-FAγ (1:5) were notably smaller than those obtained with LA-12 alone or LA-12 co-administered with unfunctionalised βCyD (1:5). As expected, in the control cell line (MCF-7) no variation in cytotoxicity was observed upon co-administration of LA-12 with βCyD or βCyD–FA. The effect of co-administration with βCyD–FAs was also observed in the case of A2780 cells for which the degree of FR overexpression is lower than that of MDA-MB-231.[37] T47-D cells seemed to be more sensitive to the presence of CyD–FAs than to CyD but with a lower activity than that of LA-12.

Table 1. Summary of IC_{50} values [μM] of CyD–FAs on human tumour cell lines.

Cyclodextrin conjugates[a]	A2780	MDA-MB-231	T47-D	MCF-7
LA-12	0.59 ± 0.15	2.40 ± 0.45	1.61 ± 0.36	4.29 ± 0.86
βCyD3-FAγ–LA-12 (1:1)	0.51 ± 0.13	3.02 ± 0.36	1.61 ± 0.41	3.88 ± 0.81
βCyD3-FAγ–LA-12 (5:1)	0.34 ± 0.18	0.70 ± 0.12[b]	1.48 ± 0.11	4.07 ± 0.71
βCyD6-FAγ–LA-12 (1:1)	0.80 ± 0.22	2.39 ± 0.45	1.99 ± 0.62	4.17 ± 0.62
βCyD6-FAγ–LA-12 (5:1)	0.29 ± 0.10[b]	0.84 ± 0.28[d]	2.45 ± 0.30	5.59 ± 0.49
βCyD–LA-12 (1:1)	0.46 ± 0.21	2.20 ± 0.45	3.25 ± 0.23	5.40 ± 0.36
βCyD–LA-12 (5:1)	0.58 ± 0.16	2.79 ± 0.20	3.76 ± 0.75	5.52 ± 0.57

The values are the mean ± standard deviation [μM] of 4–7 data points. [a] The ratio CyD/LA-12 is given in parentheses. [b] CyD3-FA–LA-12 (5:1) versus LA-12, $p = 0.005$. [c] CyD6-FAγ–LA-12 (5:1) versus LA-12, $p = 0.010$. [d] CyD6-FAγ–LA-12 (5:1) versus LA-12, $p < 0.001$.

The molar ratio between CyD and LA-12 plays an important role. Indeed, when CyD–FAs and LA-12 were administered in a 1:1 ratio, the cytotoxicity of LA-12 did not change. This result is in accord with a higher concentration of host molecule promoting the formation of the inclusion complex with beneficial effect on the antiproliferative activity.

These data confirm the ability of the folate derivatives of CyDs to act as targeted drug carriers when the drug can form inclusion complexes with CyDs.

Conclusion

The synthesis of water-soluble folic acid (FA)-conjugated β-cyclodextrins (βCyDs) as new targeting drug systems has been reported. In particular, βCyD was modified at the primary or secondary side with FA. The different regioisomers (FA binding in the α or γ position of the glutamic chain) were successfully isolated and characterised.

These derivatives are able to include the promising anticancer agent LA-12 and improve its solubility in water. Furthermore, LA-12, when co-administered with βCyD3-FAγ or βCyD6-FAγ, showed IC_{50} values significantly lower than that of LA-12 alone or LA-12 co-administered with βCyD in FR(+) cancer cells.

To the best of our knowledge this is the first report of pure regioisomers of βCyD–FA conjugates used as drug carriers. Furthermore this is the first time that FA was conjugated to βCyDs at the secondary side (βCyD3-FA derivatives). The functionalisation with FA in the γ position encumbers the self-inclusion of the FA side chain and makes the cavity fully available for the inclusion of the guest. In addition, the 3-derivatives can be obtained in higher yields than the 6-derivatives. The results suggest that the new FA derivatives are very promising as targeted carriers for antitumour drugs in FR(+) cells.

Experimental Section

Materials

All chemicals obtained from commercial sources were used without further purification: β-cyclodextrin (βCyD, Fluka), folic acid (FA, Sigma–Aldrich) and anhydrous *N*,*N*-dimethylformamide (DMF) (Aldrich). 6A-Amino-6A-deoxy-βCyD and 3A-amino-3A-deoxy-2A(*S*),3A(*R*)-βCyD were synthesised from the corresponding tosylates as previously described.[28,38]

Folate *N*-hydroxysuccinimide (FA-NHS) was synthesised according to the method of Lee and Low.[39] Folic acid (500 mg, 1.13 mmol) was dissolved in DMSO (10 mL) in the presence of triethylamine (TEA, 20 μL) and reacted with *N*-hydroxysuccinimide (NHS, 30 mg, 1.13 mmol) and dicyclohexylcarbodiimide (233 mg, 1.13 mmol) for 12 h at room temperature in the dark. The reaction mixture was filtered to remove the precipitated side product dicyclohexylurea, and the filtrate treated with diethyl ether to allow precipitation of FA-NHS. The yellow-orange precipitate was collected by filtration of the mother liquor and stored at −20 °C.

LA-12 was synthesised as reported elsewhere.[19]

DEAE and CM Sephadex 25 (Sigma–Aldrich) were used for column chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates (60-F254, Merck, Darmstadt, Germany). Products were detected on TLC by UV spectroscopy or by an anisaldehyde test.

Stock solutions of LA-12 were prepared in DMSO. Inclusion complexes of LA-12 and CyDs were prepared by mixing the stock solution of LA-12 with aqueous solutions of CyDs.

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 25 °C with a Varian UNITY PLUS-500 spectrometer at 499.9 and 125.7 MHz, respectively. The NMR spectra were obtained by using standard pulse programs from the Varian library. The 2D experiments (COSY, TOCSY, HSQC and ROESY) were acquired using 1 K data points, 256 increments and a relaxation delay of 1.2 s. The spectra were referred to the solvent signal. For clarity, the sugar units in CyD derivatives are labelled A–G counter-clockwise starting from the modified ring (denoted as A) and viewing from the primary hydroxyl groups.

Circular dichroism spectroscopy

CD measurements were performed on a JASCO J-1500 spectropolarimeter. The spectra represent the average of 10 scans and were recorded at 25 °C on freshly prepared aqueous solutions. FA solutions were prepared in water at pH 8.0.

Mass spectrometry

ESI-MS measurements were performed by using a Finnigan LCQ DECA XP PLUS ion-trap spectrometer operating in the negative-ion mode and equipped with an orthogonal ESI source (Thermo Electron Corporation, USA). Xcalibur software was used for the elaboration of mass spectra.

Cell culture

Ovarian carcinoma A2780 and ductal breast epithelial carcinoma T47-D cells were grown as monolayers in RPMI medium (Euroclone, Pero, Italy) supplemented with 10% foetal bovine serum (FBS, Euroclone). The medium of T47-D was supplemented with insulin (5 U mL^{-1}). Breast carcinoma MDA-MB-231 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Euroclone) supplemented with 10% FBS and 1% penicillin–streptomycin (Euroclone).

Determination of antiproliferative activity by the MTT assay

Cell lines were plated (180 μL of a suspension of appropriate concentration) into flat-bottomed 96-well microtitre plates. After 6–8 h of incubation, cells were treated with the selected complexes (five solutions (20 μL) in a dilution ratio of 1:5 (starting from 10 μM concentration) and processed by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described elsewhere.[40] The stock solutions of LA-12 were freshly prepared in DMSO (1%). The final concentration of DMSO in the cell culture medium did not exceed 0.2%. CyDs and their FA derivatives were used starting from 50 μM concentration. LA-12 and CyDs were administered together.

IC_{50} values were calculated on the basis of the analysis of single concentration–response curves. The Cricket Graph III software was used to analyse the concentration–response curves.

Statistical analysis

The Mann–Whitney test for non-parametric data was used for statistical analysis. The number of data points for each experimental group ranged from 4 to 7.

Synthesis of 3A-deoxy-3A-(pteroyl-glutamylamine)-2A(*S*),3A(*R*)-βCyD (βCyD3-FA)

FA-NHS (237 mg, 0.4 mmol) and 3A-amine-3A-deoxy-2A(*S*),3A(*R*)-βCyD (50 mg, 44.6 μmol) were dissolved in dry DMF (10 mL), TEA (10 μL) was added and the reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated to dryness in vacuo. The product was suspended in Milli-Q water (3.0 mL) and purified by anion-exchange liquid chromatography (DEAE Sephadex A-25, HCO_3^- form) using a linear gradient of NH_4HCO_3 (0–0.3 M). Two products, corresponding to α and γ regioisomers of the conjugate CyD3-FA, were obtained.

βCyD3-FAγ: Yield: 20%. ^{1}H NMR (500 MHz, D_2O): $\delta = 8.67$ (s, 1H; H-7 of FA), 7.50 (m, 2H; H-13 and H-15 of FA), 6.56 (m, 2H; H-12 and H-16 of FA), 5.04–4.74 (m, 7H; Hs-1 of CyD), 4.56 (s, 2H; H-9 of FA), 4.58 (m, 1H; H-3 of CyD), 4.34 (m, 1H; H-19 of FA), 3.98–3.10 (m, 41H; Hs-6, Hs-5, Hs-4, Hs-3, Hs-2 of CyD), 2.42 (m, 1H; H-22 of FA), 2.22 ppm (m, 3H; Hs-21 and H-22′ of FA); CD (H_2O), λ ($\Delta\varepsilon$): 220 (1.1), 290 nm (1.2); ESI-MS: *m/z*: 777.2 $[M-2H]^{2-}$, 1555.5 $[M-H]^-$; TLC: 0.68 (PrOH/AcOEt/H_2O/NH_3 4:2:4:1).

βCyD3-FAα: Yield: 45%. ^{1}H NMR (500 MHz, D_2O): $\delta = 8.48$ (s, 1H; H-7 of FA); 7.52 (d, $J = 8.4$ Hz, 2H; H-13 and H-15 of FA); 6.52 (d, $J = 8.2$ Hz, 2H; H-12 and H-16 of FA); 5.04 (d, $J = 2.8$ Hz, 1H; H-1 of CyD); 4.95 (d, $J = 2.9$ Hz, 1H; H-1 of CyD); 4.93 (d, $J = 3.4$ Hz, 1H; H-1 of CyD); 4.91 (m, 2H; H-1 of CyD); 4.87 (d, $J = 3.3$ Hz, 1H; H-1 of CyD); 4.76 (bs, 1H; H-1A of CyD), 4.54 (m, 2H; H-9 of FA); 4.41 (bs, 1H; H-3A of CyD), 4.33–4.13 (m, 2H; H-3G, H-3X of CyD and H-19 of FA); 4.06–3.30 (m, 36H; H-6, H-5A, H-5, H-4, H-3, H-2 of CyD); 3.11 (d, $J = 11.1$ Hz, 1H; H-6Y of CyD); 3.04–2.90 (m, 2H; H-5A and H-6_aY of CyD), 2.49 (m, 2H; Hs-22 of FA); 2.25 ppm (m, 2H; Hs-21 of FA); CD (H_2O), λ ($\Delta\varepsilon$): 220 (−2.9), 235 (2.3), 270 (3.3), 300 nm (3.5); ESI-MS: *m/z*: 777.4 $[M-2H]^{2-}$, 1555.4 $[M-H]^-$; TLC: 0.68 (PrOH/AcOEt/H_2O/NH_3 4:2:4:1).

Synthesis of 6A-deoxy-6A-(pteroyl-γ-glutamylamine)-βCyD (βCyD6-FAγ) and 6A-deoxy-6A-(peteroyl-α-glutamylamine)-βCyD (βCyD6-FAα)

FA-NHS (220 mg, 0.3 mmol) and 6A-amino-6A-deoxy-βCyD (110 mg, 97 μmol) were dissolved in dry DMF (4 mL), TEA (20 μL) was added and the reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated to dryness in vacuo and the solid residue was dissolved in Milli-Q water (3 mL) and purified by ion-exchange liquid chromatography (DEAE Sephadex, HCO_3^- form) using a linear gradient of NH_4HCO_3 from 0 to 0.3 M. Two products, corresponding to the α and γ regioisomers of the conjugate βCyD6-FA, were obtained.

βCyD6-FAγ: Yield: 40%. ^{1}H NMR (500 MHz, D_2O): $\delta = 8.42$ (s, 1H; H-7 of FA), 7.29 (d, $J = 8.4$ Hz, 2H; H-13 and H-15 of FA), 6.55 (d, $J = 8.3$ Hz, 2H; H-12 and H-16 of FA), 4.99 (m, 2H; H-1 of CyD), 4.97 (m, 2H; H-1A and H-1 of CyD), 4.91 (d, 1H; H-1 of CyD), 4.89 (d, $J = 3.6$ Hz, 1H; H-1 of CyD), 4.84 (d, $J = 3.1$ Hz, 1H; H-1 of CyD), 4.69 (m, 2H; H-9 of FA), 4.26 (t, $J = 3.2$ Hz, 1H; H-19 of FA), 4.13 (d, $J = 11.2$ Hz, 1H; H-6X of CyD), 4.04 (d, $J = 14.4$ Hz, 1H; H-6_b A of CyD), 3.96 (m, 1H; H-3Y of CyD), 3.89–3.28 (m, 34H; H-6, H-5A, H-5, H-4, H-3, H-2 of CyD), 3.20 (t, $J = 9.2$ Hz, 1H; H-4A of CyD), 3.14 (m, 1H; H-3G of CyD), 3.09 (d, $J = 9.8$ Hz, 1H; H-3G of CyD), 3.03 (m, 2H; H-3X of CyD), 2.60 (dd, $J = 14.4$, 10.9 Hz, 1H; H-6_a A of CyD), 2.49 (m, 1H; H-21b of FA), 2.25 (dd, $J = 16.2$, 7.4 Hz, 1H; H-22b of FA), 2.10 (dd, $J = 16.2$, 12.5 Hz, 1H; H-22a of FA), 1.93 ppm (dd, $J = 13.6$, 7.4 Hz, 1H; H-21a of FA); ^{13}C NMR (125 MHz, D_2O): $\delta = 147.40$ (C-7 of FA), 128.3 (C-13 and C-15 of FA), 113.7 (C-12 and C-16), 104–101 (C-1 of CyD), 87.0 (C-4A), 82.9–81.2 (Cs-4), 80.8–76.2 (C-5, C-3), 63.6–60.7 (Cs-6), 62.2 (C-6X of CyD), 58.6 (C-19 of FA), 48.9 (C-9 of FA), 41.8 (C-6A), 73.6 (C-3Y of CyD), 72.3 (C-3G of CyD), 73.4 (C-3X of CyD), 32.5 (C-22 of FA), 28.7 ppm (C-21 of FA); CD (H_2O), λ ($\Delta\varepsilon$): 204 (38.1), 230 (−2.3), 250 (−3.1), 275 (9.2), 306 nm (4.4); ESI-MS: *m/z*: 777.2 $[M-2H]^{2-}$, 1555.2 $[M-H]^-$; TLC: 0.56 (PrOH/AcOEt/H_2O/NH_3 4:2:4:1).

βCyD6-FAα: Yield: 12%. ^{1}H NMR (500 MHz, D_2O): $\delta = 8.40$ (s, 1H; H-7 of FA), 7.43 (d, $J = 8.7$ Hz, 2H; H-13 and H-15 of FA), 6.57 (d, $J = 8.6$ Hz, 2H; H-12 and H-16 of FA), 5.02 (d, $J = 3.6$ Hz, 1H; H-1 of CyD), 5.00 (d, $J = 3.6$ Hz, 1H; H-1G of CyD), 4.98 (d, $J = 3.8$ Hz, 1H; H-1 of CyD), 4.96 (d, $J = 3.0$ Hz, 1H; H-1 of CyD), 4.86 (d, $J = 3.5$ Hz, 2H; H-1A and H-1B of CyD), 4.82 (m, 1H; H-1X of CyD), 4.69 (m, 2H; H-9 of FA), 4.30 (d, $J = 14.8$ Hz, 1H; H-5A of CyD), 4.13 (d, $J = 10.9$ Hz, 1H; H-6_bG of CyD), 4.02 (m, 2H; H-6_aG of CyD and H-19 of FA), 3.99–3.30 (m, 34H; H-6, H-5, H-4, H-3, H-2 of CyD), 3.20 (m, 1H; H-4A of CyD), 3.15 (m, 1H; H-6_bA of CyD), 3.02 (m, 1H; H-3B of CyD), 2.88 (m, 1H; H-3X of CyD), 2.71–2.66 (m, 1H; H-6A of CyD), 2.61 (m,1H; H-22b of FA), 2.23 (m, 1H; H-22 of FA) 2.16 (m, 1H; H-21_b of FA), 1.98 ppm (m, 1H; H-21 of FA); ^{13}C NMR (125 MHz, D_2O): $\delta = 145.91$ (C-7 of FA), 134.3 (C-13 and C-15 of FA), 112.2 (C-12 and C-16), 104–101 (C-1 of CyD), 82.9–81.2 (Cs-4), 80.8–76.2 (C-5, C-3, C-2), 68.5 (C-2A), 63.6–60.7 (Cs-6), 58.9 (C-19 of FA), 47.5 (C-9 of FA), 35.0 (C-22 of FA), 27.5 ppm (C-21 of FA); CD (H_2O), λ ($\Delta\varepsilon$): 205 (23.1), 225 (−4.4), 275 (3.1), 305 nm (2.5); ESI-MS: *m/z*: 777.3 $[M-2H]^{2-}$, 1555.5 $[M-H]^-$; TLC: 0.50 (PrOH/AcOEt/H_2O/NH_3 4:2:4:1).

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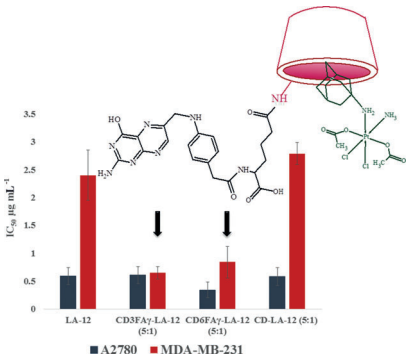
Drugs included: The antitumor drug *cis–trans–cis*-[$PtCl_2(CH_3CO_2)_2$(adamantylamine)(NH_3)] (LA-12) co-administered with 3- or 6-functionalised β-cyclodextrin conjugated with γ-folate (βCyD3-FAγ or βCyD6-FAγ) as an inclusion complex showed IC_{50} values significantly lower than that of LA-12 alone or LA-12 co-administered with βCyD in folate receptor-overexpressing cancer cells (see figure).



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Folate–Cyclodextrin Conjugates as Carriers of the Platinum(IV) Complex LA-12