Synthesis of Novel Targeted Drug Delivery Systems for Cancer Therapy

Antonio Clementi

Technological University Dublin

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Synthesis of Novel Targeted Drug Delivery Systems for Cancer Therapy

Antonio Clementi
Dublin Institute of Technology
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Synthesis of Novel Targeted Drug Delivery

Systems for Cancer Therapy

By

Antonio Clementi

A Thesis Submitted to the Dublin Institute of Technology, for the Degree of Master of Philosophy.

Supervised by Dr. Christine M. O’Connor
Dr. Mary McNamara
Dr. Antonino Mazzaglia
Prof. Maria Chiara Aversa

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May 2008
Abstract

One of the most important goals of pharmaceutical science is localizing the pharmacological activity of the drug at the site of action. Drug delivery systems are molecular tools which without undesired interaction at other sites target a specific drug receptor. In this work a novel folate-cyclodextrin conjugate was synthesised for potential use as a drug delivery system in cancer therapy. Folate was directly covalently bound to an amino derivative of β-Cyclodextrin with a view to decreasing the polydispersity of the carrier system. Ultimately the cyclodextrin cavity will be used to transport drugs to the site of action.

This study also describes the structural characterisation of the novel folate-cyclodextrin conjugate using a range of techniques including electronic, vibrational, NMR, MALDI-MS and ESI-MS spectroscopies, all of which provided evidence that the folate moiety was bound to the cyclodextrin through an amide linkage.

HPLC analysis was employed to study the purity of the conjugate prepared and provided evidence of the formation of α and γ structural isomers. The photostability of the conjugate was assessed using electronic spectroscopy and the formation of particles was detected by light scattering. Preliminary biological evaluation of the tumour targeting device was carried out using HeLa cells and no evidences of cytotoxicity are recorded.
Acknowledgements

Thanks to Dr Christine M. O'Connor for her precious support during this project and for her exceptional human talent. Thanks to Dr Mary McNamara for her guidance and suggesting the most pragmatic way to understand things (just not for science).

Thanks to Dr Antonino Mazzaglia for his help through daily talks. His enthusiasm and life energy have been very important. Prof Maria Chiara Aversa always gave great support during organic synthesis and allowed me to work in the best conditions.

Thanks to all academic staff at the School of Chemical and Pharmaceutical Sciences in DIT, especially to Dr Sarah Rawe, Dr Michael Seery and Dr Claire McDonnell for their contributions to the project. I would also like to remember Dr John Fox, Dr Barry Foley and Dr Declan McCormack. Thanks to the technical staff, Mr Martin Kitson and Ms Annette Callaghan.

I would like to thank Dr Hugh J. Byrne, Ms Louisa Hartnett, and Ms Anne Shanahan of the Focas Institute, DIT.

Thanks to Aversa's team for help during the time spent on organic synthesis: Dr Anna Barattucci and Dr Grazie Cafeo for their contributions to NMR spectra, Prof Placido Giannetto for his suggestions during the lab work and Prof Paola Bonaccorsi. Dr Gianluca Battaglia for his help during the synthetic job.

Thanks to Dr Rosanna Stancanelli, Dr Marta Guardo and Dr Carmela Cannava for HPLC.
Thanks Dr Maria Teresa Sciortino and Dr Maria Giuffré for the biological tests. I like remember Rita Calheiros Cruz (University of Coimbra) for nice talks on biological experiments.

Thanks to Dr Domenico Garozzo’s team (ICTP-CNR Catania) and Dr Alessandro Giuffrida (University of Catania) for mass spectra, Dr Carmelo Corsaro and Dr Spooren Jeroen for high resolution NMR spectra (700 MHz).

Thanks also to Prof Luigi Monsù Scolaro and his staff, especially Dr Giovanna De Luca.

And finally, all graduates and postgraduate members of the MSA lab, Aversa’s team (Lab 49) and Monsù’s team.
Dedication

To my family

to Valeria

and Nanni.
"Science can purify religion from error and superstition. Religion can purify science from idolatry and false absolutes."

Karol Wojtyla
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<table>
<thead>
<tr>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Aminopterine</td>
</tr>
<tr>
<td>br</td>
<td>Broad</td>
</tr>
<tr>
<td>Bn</td>
<td>1,4-Diaminobutane</td>
</tr>
<tr>
<td>CD</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>CDEn</td>
<td>6-Deoxy-6-[1-(2-Amino)Ethylamino]-ß-Cyclodextrin</td>
</tr>
<tr>
<td>CDEn-FA</td>
<td>6-Deoxy-6-[1-(2-Amino)Ethylamino]-ß-Cyclodextrin and folic acid conjugate</td>
</tr>
<tr>
<td>CDTs</td>
<td>6-O-Monotosyl-6-Deoxy-ß-Cyclodextrin</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical Shift</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterium Oxide</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug Delivery System</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolic acid</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>En</td>
<td>1,2-Diaminoethane</td>
</tr>
<tr>
<td>ESI</td>
<td>Electron Spray Ionisation</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FA</td>
<td>Folic Acid</td>
</tr>
<tr>
<td>FR</td>
<td>Folate Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>g</td>
<td>Graft</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic Acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>M</td>
<td>Medium/multiplet</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption/Ionization</td>
</tr>
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<td>MeOH</td>
<td>Methanol</td>
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<td>MP</td>
<td>Melting Point</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MTHF</td>
<td>Methyltetrahydrofolate</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic Therapy</td>
</tr>
<tr>
<td>PTT</td>
<td>Photothermic Therapy</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Retention Factor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>S</td>
<td>Strong/singlet</td>
</tr>
<tr>
<td>T</td>
<td>Triplet</td>
</tr>
<tr>
<td>t&lt;sub&gt;r&lt;/sub&gt;</td>
<td>Retention time</td>
</tr>
<tr>
<td>THF</td>
<td>Tertahydrofolate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Ts</td>
<td>Tosylate</td>
</tr>
<tr>
<td>TTP</td>
<td>Tetraphenylporphyrin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
</tr>
<tr>
<td>VIS</td>
<td>Visible</td>
</tr>
<tr>
<td>W</td>
<td>Weak</td>
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Figure 41. ¹³C-NMR spectrum of 6-o-Monotosyl-6-Deoxy-β-Cyclodextrin (CDTs) in DMSO.

Figure 42. ¹³C-NMR spectrum of 6-Deoxy-6-[1-(2-Amino)-Ethylamino]-β-Cyclodextrin (CDEn) in D₂O.

Figure 43. ¹³C-NMR spectrum of Folate Conjugate (CDEn-FA) in D₂O.

Figure 44. COSY spectrum of Folate Conjugate (CDEn-FA) in D₂O.

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<thead>
<tr>
<th>REAGENT</th>
<th>PURITY</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>extra pure</td>
<td>(Sigma-Aldrich)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>30%</td>
<td>(Sigma-Aldrich)</td>
</tr>
<tr>
<td>Ammonium Hydrogen Carbonate</td>
<td>99%</td>
<td>(Aldrich)</td>
</tr>
<tr>
<td>β-Cyclodextrin</td>
<td>99%</td>
<td>(Xian Hong Chang)</td>
</tr>
<tr>
<td>Chloridric Acid</td>
<td>concentrated</td>
<td>(Fluka)</td>
</tr>
<tr>
<td>Deuterium Oxide</td>
<td>99 Atom D %</td>
<td>(Cambridge Inc.)</td>
</tr>
<tr>
<td>1,2 – Diaminoethane</td>
<td>99%</td>
<td>(Sigma-Aldrich)</td>
</tr>
<tr>
<td>N,N’ – Dicyclohexylcarbodiimide</td>
<td>99%</td>
<td>(Sigma-Aldrich)</td>
</tr>
<tr>
<td>N,N – Dimethylformamide</td>
<td>99.8%</td>
<td>(A.C.S. reagent)</td>
</tr>
<tr>
<td>Dimethylsulphoxide anhydrous</td>
<td>99.9%</td>
<td>(Lab-Scan)</td>
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<tr>
<td>Ethylacetate</td>
<td>98%</td>
<td>(Sigma)</td>
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<tr>
<td>Ethanol</td>
<td>99.9%</td>
<td>(J.T. Baker)</td>
</tr>
<tr>
<td>Folic Acid dihydrate</td>
<td>97%</td>
<td>(Sigma-Aldrich)</td>
</tr>
<tr>
<td>N-Hydroxysuccinimide</td>
<td>98%</td>
<td>(Sigma-Aldrich)</td>
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<td>Methanol</td>
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<td>(Sigma-Aldrich)</td>
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<tr>
<td>Methylsulphoxide-D6</td>
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<td>2-Propanol</td>
<td>Chroma. Grade</td>
<td>(Sigma-Aldrich)</td>
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<tr>
<td>Pyridine</td>
<td>ACS</td>
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</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>98%</td>
<td>(Sigma)</td>
</tr>
<tr>
<td>Sulphuric Acid</td>
<td>ACS</td>
<td>(Sigma)</td>
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</table>
p-Toluenesulphonyl chloride 98% (Aldrich)
Water HPLC 99.9% (Aldrich).
Chapter 1

Introduction
1. 1. Discovery of Cyclodextrins

Cyclodextrins (CDs) are a family of cyclic oligosaccharides obtained from starch by enzymatic degradation. They were discovered in 1891 by Villiers,¹ and isolated in 1903 by Schardinger.² In the 1930s, Freudenberg et al. postulated the cyclic structure of CDs from a crystalline fraction of degraded starch.³, ⁴, ⁵, ⁶ Later, in 1953, these researchers registered the first patent involving CDs which was related to their use in drug formulations.⁷

1. 2. Chemical Structure

Cyclodextrins consist of α(1,4)-linked D(+)glucopyranose units. The most common cyclodextrins are α-, β- and γ-CD having 6, 7 and 8 glucose units respectively, as shown in figure 1. The Greek letter denotes the number of glucose units.¹

![Figure 1. Most common cyclodextrin structures.](image)

The topology can be represented as a toroid with the larger and the smaller openings exposing secondary (OH-2 and OH-3) and primary (OH-6) hydroxyl groups.
respectively. The interior of the toroid is lined by H-3, H-5 and H-6 hydrogens and glycosidic oxygens. The cavity is chiral and is considerably less hydrophilic than an aqueous environment and thus CDs are able to host hydrophobic molecules. The exterior is sufficiently hydrophilic to impart to cyclodextrins water solubility. The toroidal geometry of cyclodextrins is shown in figure 2.\(^{10}\)

\[\text{Primary Hydroxyl Face}\]

\[\text{Secondary Hydroxyl Face}\]

Figure 2. Toroidal geometry of CDs.\(^{10}\)

1.3. Physico-Chemical Properties

The physical properties of cyclodextrins are shown in Table 1.1. The increase in diameter from \(\alpha\) to \(\gamma\) reflects the increase in the number of glucopyranose units. It can be seen that the solubility of the cyclodextrins varies in an irregular manner.\(^{11}\) The relatively low solubility of \(\beta\)-CD provided an early reason for its modification. Its annulus is of a size particularly suitable for the inclusion of drug molecules and a more soluble modified form would yield drug complexes with a wide range of applications.
Table 1. Physical Properties of α-CD, β-CD and γ-CD.\textsuperscript{11, 12}

<table>
<thead>
<tr>
<th>Property</th>
<th>α-CD</th>
<th>β-CD</th>
<th>γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of glucopyranose units</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Molecular weight (anhydrous)</td>
<td>972.85</td>
<td>1134.99</td>
<td>1297.14</td>
</tr>
<tr>
<td>Solubility in H\textsubscript{2}O (g per 100 cm\textsuperscript{3})</td>
<td>14.5</td>
<td>1.85</td>
<td>23.2</td>
</tr>
<tr>
<td>Annular diameter measured from C\textsubscript{5} hydrogens, Å</td>
<td>4.7</td>
<td>6.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Partial molar volumes, cm\textsuperscript{3}mol\textsuperscript{-1}</td>
<td>611.4</td>
<td>703.8</td>
<td>801.2</td>
</tr>
<tr>
<td>[α]D at 25°C</td>
<td>+150.5</td>
<td>+162.5</td>
<td>+177.4</td>
</tr>
<tr>
<td>pK\textsubscript{a} at 25°C</td>
<td>12.33</td>
<td>12.20</td>
<td>12.08</td>
</tr>
</tbody>
</table>

1.4. Inclusion Compounds

Due to the structural features of CDs, they are able to form host–guest inclusion complexes with hydrophobic molecules possessing the appropriate size and shape as shown in figure 3. Since the inner surface of the cyclodextrin is more hydrophobic, hydrated cyclodextrins represent a high-energy state that can readily include hydrophobic guest molecules in their cavities. Inclusion compounds can be formed with a wide range of inorganic and organic molecules and have found many industrial applications. One application is in drug delivery whereby using inclusion complexes it is possible to control the release rate of drugs.\textsuperscript{13} CDs have also been used widely as stabilizing and solubilizing systems, enzyme models, catalysts, stationary and mobile phase additives for chiral and isomeric separations,\textsuperscript{14, 15, 16} in environmental processes for water purification, and so on.\textsuperscript{17}
1.5. Functionalised Cyclodextrins

The functionalisation of cyclodextrins allows the molecules to have improved properties and applications as stated above. Cyclodextrins have numerous functional groups and they can undergo a wide variety of chemical reactions.\(^\text{19}\)

The most frequently studied reaction is the electrophilic attack of the hydroxyl groups. The \(\alpha\)-, \(\beta\)-, and \(\gamma\)-CD contain 18, 21 and 24 OHs, respectively. The multiple number of hydroxyl groups enables many possible modifications. There are two primary factors that need to be considered in the modification of the hydroxyl groups of the CDs:

1. the nucleophilicity of the hydroxyl groups;
2. the ability of CDs to form complexes with the reagents used.

The –OH groups in the C6 position are the most basic and often most nucleophilic. The hydroxyl groups in the C2 position are the most acidic, and those at the C3 position are the most inaccessible.\(^\text{20, 21}\)

Figure 4 gives an overview of the methods which can be used for modification of cyclodextrins.\(^\text{22}\)
Chapter 1
Introduction

1·11. normal reactivity, weak base (e.g., pyridine) and a non-complexing electrophile → 6-modified CD

II· reversed reactivity, strong base (deprotonate) and a non-complexing electrophile → 2-modified CD

III· reaction via complex formation use a complexing electrophile → 2-, 3- or 6 modified CD or a mixture of the three

IV· protect 2-position, any base any electrophile → 6-modified CD

V· protect 6-position, weak base any electrophile → 3-modified CD or 2 modified CD

Figure 4. Methods for the selective modification of cyclodextrins in a specific hydroxyl group.22

The term *mono*-substituted derivative usually refers to one substituent per cyclodextrin ring whereas the term *di* or *tri*-substituted CD refers to two or three substituents per glucopyranose unit. CDs with a given substituent present on a particular position of all the glucopyranose units are generally known as per-substituted CDs.

There are two common ways in which the CD hydroxyls groups can be functionalized:

1. monofunctionalization - functionalizing of only one hydroxyl group.

2. per-functionalization – functionalizing of an entire set of hydroxyl groups.

Although *di*- and *tri*-functionalizations exist, they have not been well investigated and are difficult to perform. Monofunctionalization of the CDs have, however, been well studied in the functionalization of these starch derivatives.23, 24

Substitution can be occur by directly through alkylation, acylation and sulfonation. Alternatively, sulfonates, halides and related species may be prepared as
intermediates for the subsequent introduction of other substituents through nucleophilic displacement. Oxidation of the hydroxyl groups of the CDs produces aldehydes and ketones while reactions with acid chlorides. 25

1.6. Monosubstitution of Cyclodextrins

Monofunctionalizations can be achieved by a reaction of the hydroxyl groups with an electrophile. The large number of hydroxyl groups at the three different positions of CDs makes modification at a single desired place. The differences in the chemical properties and reactivity among these sites can be exploited to yield a specific product. 26

Modification of the C6 primary hydroxyl groups on the CD is an easy way to attach a chemical function to the sugar rings of the cyclodextrin. A widely used starting material is mono-6-deoxy-6-tosyl-β-cyclodextrin (CDTs) as depicted in figure 5. A common method for preparation of CDTs involves reaction of β-CD with excess p-toluenesulphonyl chloride, with recrystallisation from water. 27, 28 Due to the importance of these sulphonates acting as intermediates for the further synthesis of other modified CDs, many other publications exist on their preparation. Several nucleophiles can displace the tosyl group on the CD to yield the corresponding modified CD. These include nucleophiles such as iodide, azide, thioacetate, hydroxylamine, aryl or poly-alkylamine. 29, 30, 31, 32

Figure 5. Reaction Scheme for the 6-o-mono-6-deoxy-6-tosyl-β-cyclodextrin synthesis.11
In this work CDTs is used to synthesise an amino CD which is then used to form a conjugate with folic acid as a potential drug delivery system.

Although very few 2-monosubstituted CDs derivatives are known, mono-2-tosyl β-CD has been synthesized using several strategies. m-nitrophenyl tosylate reacts with CD in a DMF/aqueous buffer at pH 10 in a low yield. This reaction proceeds via complex formation to transfer the tosyl group to the 2-position. \(^{33}\)

Monosubstitution at the 3-position is complicated by the fact that the hydroxyl groups at this position are most inaccessible and thus less reactive compared to the highly accessible ones (C-2 and C-6 hydroxyl groups). Most modifications of the C-3 OHs proceeds via the synthesis of manno-mono-2,3-epoxy CD. \(^{34},^{35}\)

1.7. Drug Delivery Systems

One of the most important goals of pharmaceutical science is localizing the pharmacological activity of the drug at the site of action. Drug delivery involves diagnostic and therapeutic application. Drug delivery systems (DDS) are molecular tools which without undesired interactions at other sites target a specific drug receptor. Any aberrant toxicity would be avoided and only the desired therapeutic gain would be produced. \(^{36}\) These molecular systems can also offer controlled drug release. \(^{37}\) Advanced drug delivery systems include a targeting moiety and supplementary active ingredients including the carrier and drug. \(^{38}\) DDS are made from a variety of organic and inorganic compounds such as polymers, lipids (liposomes, nanoemulsions, and solid-lipid nanoparticles), self-assembling amphiphilic molecules, dendrimers and inorganic nanocrystals. \(^{39},^{40},^{41}\) Delivery of the drug–carrier system to the target site
does not however mean that an adequate amount of the free drug will be available at the actual target due to the fact that there are many additional processes that need to take place. These processes include drug release, endocytosis, transport of the drug across the cell membrane, drug-receptor binding, lysosome uptake, nuclear targeting, drug release and drug elimination as shown in Figure 6. 42, 43

![Figure 6](image)

Figure 6. Example of a drug-carrier conjugate or liposome reaching the target cell so the drug reaches the surface of its target cell. 29

1. 8. Cyclodextrins as Drug Carriers

Many drugs form inclusion complexes with cyclodextrins, and the properties of these complexes are usually markedly different from that of the pure drug or pure cyclodextrin. 30 CDs improve the solubilization and stabilization of drugs and they can be potent drug carriers for immediate and delayed delivery. CDs and their
derivatives, especially amphiphilic CDs, also increase drug permeability by direct action on mucosal membranes and enhance drug absorption and/or bioavailability.44 An equilibrium is established between the drug-CD inclusion complex and the free drug which allows delivery of poorly soluble drugs. One of the mechanisms proposed in literature is shown in figure 7 and shows that the drug can be absorbed by the biological membrane directly from the inclusion complex or following dissociation of the complex.45

![Figure 7. Mode of penetration enhancement by CDs in drug delivery to cell membrane.](image)

1.9. Folic Acid as a Targeting Agent

Folic acid (FA) has been developed as a cancer targeting agent and is recognized by tumour cells due to their folate receptors (FR).46 High expression of FRs has been frequently observed in many types of human cancers such as ovarian, endometrial, colorectal, breast, lung, renal cell carcinomas, brain metastases derived from epithelial cancers and neuroendocrine carcinomas. Quantitative studies by Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) confirm elevated
levels of FR in choriocarcinomas, meningiomas, uterine sarcomas, osteosarcomas, non-Hodgkin's lymphomas and premyelocytic leukemias.\textsuperscript{36, 37}

Folic acid is a water soluble vitamin and is a fundamental molecule for basic metabolism. It is needed for the production and maintenance of cells and is required for the synthesis of DNA bases which are in turn needed for DNA replication. \textit{In vivo} folic acid (pteroy-L-glutamic acid, B\textsubscript{12} vitamin) is reduced to 7,8-dihydrofolate (DHF), which is subsequently reduced to 5,6,7,8-tetrahydrofolate (THF) by dihydrofolate reductase. THF is converted to methylene-THF which is then reduced to 5-methyl-5,6,7,8-tetrahydrofolate (5-MTHF).\textsuperscript{38} The structures of some of these metabolites are given in figure 8.\textsuperscript{47} Folate deficiency which is seen as an absence of methylene-THF results in the inhibition of the generation of red blood cells and therefore anemia.

![Figure 8. Overview of folic acid and its metabolic derivatives.\textsuperscript{38}](image)

On the other hand in cancer therapies inhibition of cell growth is favourable. Folate antagonists such as aminopterine (AMP) and methotrexate (MTX) (figure 9) hamper
normal cellular replication and DNA synthesis. It seems that this results came from the inhibition of the enzyme dihydrofolate reductase. Indeed MTX has been in use for more than 50 years to treat many cancers and autoimmune diseases.\textsuperscript{47}

![Figure 9. Competitive antagonists of folic acid where AMP is aminopterine and MTX is methotrexate.](image)

Therefore folate, its metabolites or its antagonists may be suitable agents for anti-cancer therapies. In this work folate conjugated to CD is proposed as an agent to target tumour cells over expressing folate receptors.

1. 10. Folic Acid Conjugates

Folic acid receptors can be direct targets, involving antagonist drugs, or indirect targeting tools for delivery involving competitive drugs. This explains the several strategies used for folate conjugation. The conjugation of folic acid to therapeutic agents including alkylating agents, taxols, platinum compounds, and fluorouracil has been investigated.\textsuperscript{40, 41, 48}

Folic acid has also been conjugated to polyethyleneglycol (PEG) to successfully deliver a wide variety of compounds including chemotherapeutic agents, oligonucleotides, photosensitizers, polymers and dendrimers.\textsuperscript{42, 49, 50, 51, 52, 53}

A β-Cyclodextrin-polyethyleneglycol-folic acid conjugate (CD-PEG-FA) has been produced from the reaction between CD-PEG-NH\textsubscript{2} and FA. CD-PEG-NH\textsubscript{2} was
obtained through the intermediate CDTs and CD-PEG. The conjugate with folic acid was proposed as an active tumour targeting molecule. Another conjugate folate-PEG-folate-graft-polyethyleneimine (FPF-g-PEI) has also been reported and is described in the literature as a potential gene carrier. Recently synthetic procedures for the production of nanoparticles of FA conjugates have been reported.

I. 11. Aims and Objectives of This Work

The aim of this work is the development of new vehicles as drug delivery systems for cancer therapy applications. The synthetic strategy followed is based on the production of a new cyclodextrin-folate conjugate (CDEn-FA) which involves reaction between an ethylenediamine modified CD and folic acid. Similar molecules have been reported by Caliceti at al. using a polymer spacer (PEG) between the β-cyclodextrin and the folate. However in the work presented here FA will be directly bound to CDEn in an attempt to eliminate the polydispersity observed in CDs modified with macromolecular species such as PEG. In this way we will design a molecular system with a controlled number of binding sites (i.e. targeting moiety, CD cavity, metal coordination sites) and with properties of recognition towards receptor proteins. CDEn-FA can then be exploited for the intracellular delivery of organic and inorganic drugs or for the delivery of photosensitizers or metal nanoparticles for use in Photodynamic Therapy (PDT) of tumours.

The objectives of this work are thus to:

• Synthesise CDEn-FA.
• Characterise all products and reagents using a range of techniques including electronic, vibrational, NMR, MALDI-MS and ESI-MS spectroscopies.

• Investigate the photostability of CDEn-FA.

• Evaluate the biological properties of CDEn-FA.
Chapter 2

Synthesis and Characterisation of Cyclodextrin Derivatives
Chapter 2

Synthesis and Characterisation of Cyclodextrin Derivatives

2.1. Introduction

As mentioned in Chapter 1 the syntheses of CD derivatives carried out in this work are based on well known reactions of β-CD. 59, 60

6-O-(p-Tosyl)-β-Cyclodextrin (CDTs) is a common and extensively investigated precursor of many cyclodextrin derivatives made from the reaction between p-Tosyl Chloride and β-CD in aqueous NaOH. 11, 61, 62, 63, 60 The reaction is carried out at 4°C with stirring overnight with a preliminary filtration step to remove the excess of unreacted chemicals. 64 Reverse phase chromatography can be used to purify the material. 65

6-(2-Aminoethyl)amino-6-deoxy-β-Cyclodextrin (CDEn) can be obtained from the CDTs on reaction with an excess of ethylenediamine. 66, 67, 68, 69, 70, 71, 72, 73 After reflux at 75°C for 4 hours, the cooled product can be precipitated from acetone and recrystallised from a water-methanol mixture. 74

There are no reports in the literature of methods for the preparation of a derivative with folate directly connected to CD. Caliceti et al. reported a method for the preparation of a FA-PEG-CD product. The procedure involved the synthesis of CD-PEG-NH₂ by reaction of monotosyl-β-cyclodextrin with excess of 700 Da diamino-PEG. The second step involved the synthesis of CD-PEG-FA by reaction of CD-PEG-NH₂ with folic acid. However the polyethyleneglycol linkage gave polydispersity to the material. 54 A similar synthetic route was presented in the preparation of the folate-poly(ethylene glycol)-poly(L-lysine) conjugate (FOL-PEG-PLL). It is a derivative for gene delivery applications, and its preparation involved a peptide bond between the carboxyl function of the folic acid and the terminal amine group. 50
Chapter 2 Synthesis and Characterisation of Cyclodextrin Derivatives

The most important technique used to investigate CDs and their derivatives is Nuclear Magnetic Resonance (NMR) spectroscopy. Fluorescence, UV-vis spectroscopy, calorimetry, etc. can play a role in measuring complexation energetics with CDs, but usually provide only very indirect and qualitative information about inclusion modes and geometries. NMR spectroscopy has become the most important method for structural elucidation of CD, for controlling the intricate synthetic modifications of the CDs by modern preparative methods. 9

The NMR experiment exploits the magnetic properties of nuclei to provide information on molecular structure. Only atoms with spin nuclear quantum numbers different than zero can show magnetic properties. Common magnetic nuclei are $^1$H, $^{13}$C, $^{15}$N, $^{19}$F, $^{29}$Si and $^{31}$P. When a strong magnetic field of strength $B_0$ is turned on along a direction designated as the $z$ axis, the energies of the nuclei are affected so that there is a group of nuclei aligned in the $+z$ ($B_0$) direction and another group aligned in the opposite direction $-z$ ie the energy levels split. The difference in energy of this representation is $\Delta E = \gamma \hbar B_0 / 2\pi$, where $\gamma$ is the gyromagnetic ratio and $\hbar$ is Planck’s constant. Therefore the frequency $\nu = (\gamma / 2\pi) B_0$. 75 The signal of resonance for each atom depends on the local environment. So, for example, a hydrogen of the methyl group will give a different response than a hydrogen of the hydroxyl group from the same molecule. The NMR scale is set up by reference to the tetramethylsilane (TMS) peak at zero Hz. The chemical shift $\delta$ (ppm) is the common unit used in NMR spectroscopy and is the difference between the frequency of the reference line divided by the reference frequency: $\delta = 10^6 \cdot (\nu-\nu_{\text{ref}})/\nu_{\text{ref}}$. 76

Light can be described in terms of photons or as an electromagnetic wave. The Beer-Lambert law is used to describe the absorption of the light such that $A =$
εel, where A is the absorbance, c is the concentration of sample in units of molarity, l is the pathlength in centimeters and ε is the molar extinction coefficient (mol\(^{-1}\) dm\(^3\) cm\(^{-1}\)). On the absorption of light there is a change in the system from the ground state to the excited state. This transition depends on the wavelength of light used and so electronic transitions occur in the UV-VIS range, whereas vibrational transitions occur in the infrared range.

The infrared region (IR or MIR) extends from 4000 cm\(^{-1}\) (2.5 μm) to 400 cm\(^{-1}\) (25 μm). IR spectroscopy is a rapid, sensitive, easy to handle technique providing many different sampling methods for gases, liquids and solids and it is possible to obtain both qualitative and quantitative information. The standard format of an IR spectrum is transmittance [%T] versus wavenumber [cm\(^{-1}\)], where transmittance is the ratio of the intensity of transmitted light to the intensity of the incident light. With organic compounds, characteristic vibrations of the various functional groups occur usually between 4000 and 1500 cm\(^{-1}\).

Electronic transitions occur in the UV-VIS range and so adsorption of radiation in this range excites an electron from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). The visible region corresponds to 400-800 nm whereas the ultraviolet region is beyond the visible in the 200-400 nm range. The spectra are often described as bands rather than peaks and the wavelength at an absorption maximum is referred to as the \(\lambda_{\text{max}}\) of the band. From this information it is possible to estimate the molar extinction coefficient of a material according to the Beer-Lambert law. In this work electronic spectroscopy is used to study the stability of folic acid and its derivative.
Mass spectral analyses involve the formation of gaseous ions from an analyte and subsequent measurement of the mass-to-charge ratio (m/z) of these ions. The mass spectrometer separates the ions generated upon ionization according to their mass-to-charge ratio (or a related property) to give a graph of ion abundance versus m/z. When Electrospray Ionization Mass Spectroscopy (ESI-MS) is used, a strong electric field is applied to the capillary carrying the analyte solution and the spray is produced at atmospheric pressure. Spraying under these conditions produces highly charged droplets whose charge is determined by the polarity of the field applied to the capillary. Desolvation of the droplets is aided by a counter-current flow of warm nitrogen gas.

Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight Mass Spectrometry (MALDI-TOF MS) is a technique in which a co-precipitate of an UV-light absorbing matrix and a molecule is irradiated by a nanosecond laser pulse. Most of the laser energy is absorbed by the matrix, which prevents unwanted fragmentation of the molecule. The ionized molecules are accelerated in an electric field and enter the flight tube. During the flight in this tube, different molecules are separated according to their mass-to-charge ratio and reach the detector at different times. In this way each molecule yields a distinct signal. The matrix consists of crystalline materials such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix) and 2,5-dihydroxybenzoic acid (DHB). A solution of one of these is prepared often in a mixture of water and an organic solvent such as acetonitrile (ACN) or ethanol. Trifluoroacetic acid (TFA) may also be added.
The method is used for detection and characterization of molecules with molecular masses between 400 and 350,000 Da. It is a very sensitive method, which allows the detection of low quantities of sample with an accuracy of 0.1 - 0.01 %. ESI is best suited for analysis of monodispersed molecules because of complications arising from the formation of multiply charged ions. Alternatively, MALDI-TOF MS is ideally suited for characterizing polydispersed oligomers.

2.2. Instrumentation

All reactions were monitored by thin layer chromatography (TLC) carried out on precoated silica-gel 60F 254 plates (E. Merck, ref. # 5554). UV detection was used employing a CN-6T Vilber Lourmat UV lamp at 365 nm (6 W) and 254 nm (6 W).

Melting points were measured using the Stuart melting point SM P3 apparatus.

NMR spectra were recorded with an AM 300 Brucker spectrometer and a Varian Mercury 300 Spectrometer. \(^1\)H NMR spectra were recorded at 300 MHz and \(^13\)C NMR spectra at 75.5 MHz. A Bruker Avance spectrometer was used for high resolution spectra, operating at the 700 MHz \(^1\)H resonance frequency and 175 MHz \(^13\)C resonance frequency. Samples were dissolved in D\(_2\)O or DMSO. The \(^1\)H NMR reference peaks were the water peak, which gave a signal at 4.76 ppm, and the DMSO peak at 2.50 ppm. The \(^13\)C NMR reference was 1,4-Dioxane as an internal standard, which gave a signal at 67.19 ppm. Solutions were typically from 1 to 12 mmol dm\(^{-3}\). All spectra were recorded at room temperature.
Infrared spectra were recorded with the Perkin-Elmer Spectrum BX FT-IR spectrometer. The Spectrum BX can operate in ratio, single-beam or interferogram modes. The optical system gives data collection over a total range of 7800 to 100 cm$^{-1}$, with a maximum resolution of 1 cm$^{-1}$. Several mid infrared detectors are available. DTGS (Deuterated Triglycine Sulphate) or LiTaO$_3$ (lithium tantalite) detectors are standard and there is also the option of using MCT (Mercury Cadmium Telluride) or PAS (photoacoustic) detectors. The electronics system is based on the Motorola 68340 Integrated Processor. The instrument is connected to a PC and controlled using the Spectrum. Spectra were recorded of solid samples as KBr discs or Nujol mulls over the range of 4000-400 cm$^{-1}$ with background correction.

Electronic spectra were measured using a Hewlett Packard 8453 UV-VIS spectrophotometer. The HP 8453 spectrophotometer is a single beam, microprocessor-controlled UV-Visible spectrophotometer with collimating optics. The detector is a diode-array (1024 photodiodes), with a spectral range from 190 to 1100 nm, a resolution of 2 nm and a slit width of 1 nm. The light source for the UV region is a deuterium lamp with a shine-through aperture. The lamp emits over the 190 nm to approximately 800 nm wavelength range. The light source for the visible and short wave near-infrared (SWNIR) wavelength range is a low-noise tungsten lamp. This lamp emits light over the 370 nm to 1100 nm wavelength range. There is a temperature control unit also available with peltier technology. The instrument is connected to a PC with HP UV-Visible software. Quartz cells of 1 cm and 4 mm were employed for the measurements. All spectra were recorded at room temperature with solutions of $10^{-4}$ mol dm$^{-3}$. 
Electrospray ionisation mass spectrometry (ESI-MS) was used for the characterisation and purity determination of the samples. The instrument used was a Thermo LXQ linear trap, with potential full scan at nozzle of 4.5 kV, flow 5 μL/min and sheath gas nitrogen at 20 a.u. (arbitrary unit). The system can be used to determine molecular masses in the range 50 - 100000 Da, with an accuracy of 20 ppm. The samples were introduced into the mass spectrometer in solution and ionised at atmospheric pressure. Solutions of 10^4 mol dm^-3 were used.

MALDI-TOF analyses were performed on a Perseptive (Framingham, MA) Voyager STR instrument equipped with delayed extraction technology. Ions were formed by a pulsed UV laser beam (nitrogen laser, 337 nm) and accelerated through 24 kV. Samples were diluted in CHCl₃ and mixed 1:1 v/v with the matrix solution obtained by dissolving 2,5-dihydroxybenzoic acid (DHB) in CH₃OH/0.1% trifluoroacetic acid/CH₃CN (1:1:1 by volume) at a concentration of 30 mg/mL. Exactly 1 μL of this mixture was deposited onto a stainless steel 100 sample MALDI plate and allowed to dry at room temperature before running the spectra in the positive polarity.

2.3. Synthesis

2.3.1. Synthesis of 6-o-Monotosyl-6-Deoxy-β-Cyclodextrin (CDTs)

β-cyclodextrin (10.01 g, 7.61 mmol) was dissolved in NaOH (100 cm³, 0.4 M) at 0°- 4°C to which was added p-toluenesulphonyl chloride (3.00 g, 15.79 mmol). The mixture was vigorously stirred for 4 - 5 hours at constant temperature. The mixture was filtered to remove the excess p-toluenesulphonyl chloride and the pH was reduced to 6.5 by addition of 1M HCl. A precipitate formed in the solution
which was then left at 4°C for 24 hours. The product was recovered by filtration, washed with acetone and recrystallised from water. The material was dried under vacuum at 60°C for at least 4 hours.\textsuperscript{11, 61, 62, 63, 60, 64}

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\textbf{\(\beta\)-CD}}; \node (b) at (2,0) {\textbf{TsCl}}; \node (c) at (4,0) {\textbf{CDTs}}; \node (d) at (2,0) {+}; \node (e) at (2,0) {0 - 4°C \hspace{1cm} 0.4 M NaOH \hspace{1cm} + \hspace{1cm} HCl}; \end{tikzpicture}
\end{center}

\textbf{Figure 10.} Synthesis of 6-o-Monotosyl-6-Deoxy-\(\beta\)-cyclodextrin (CDTs).

\textbf{Yield:} 1.86 g, 18\% (literature value 17\%).\textsuperscript{64}

\textbf{m.p.:} 179°C with slow decomposition (literature value 179°C with slow decomp.).\textsuperscript{64}

\textbf{TLC:} \(R_f\) CDTs = 0.7, solvent system 7:5:5:4 ethylacetate:ethanol:water:ammonium sulphate; detection by dipping in 5\% sulphuric acid/ethanol and heating. Analysis of the product showed no parent CD present at an \(R_f\) of 0.1-0.5.

\(R_f\) CDTs = 0.49, solvent system 7:5:5:1 ethylacetate:ethanol:water:ammonia 30\%; detection by dipping in 5\% sulphuric acid/ethanol and heating. Analysis of the product showed no parent CD present at an \(R_f\) of 0.38.

\textbf{\(^1\)H-NMR (DMSO) \(\delta\) (ppm):} 7.74 (d), 7.42 (d), 5.77 (br), 5.71 (br), 4.83 (m.), 4.49 (br), 3.63 (br), 3.56 (br), 3.49 (br), 3.45 (br), 3.30 (br), 2.42 (s).

\textbf{\(^{13}\)C NMR (DMSO) \(\delta\) (ppm):} 144.86, 132.68, 129.93, 127.62, 101.96, 81.52, 73.09, 72.75, 72.45, 68.02, 60.04, 21.27.

\textbf{FT-IR (Nujol) \(\nu\) (cm\(^{-1}\)):} 2943 (s), 2902 (s), 2848 (s), 2727 (w), 2360 (w), 1653 (m), 1463 (s), 1377 (s), 1158 (m), 1028 (m), 837 (w), 721 (w), 668 (m), 579 (w), 442 (s).

Detailed spectra are reported in the Appendix Section.
2.3.2. Synthesis of 6-Deoxy-6-[1-(2-Amino)-Ethylamino]-β-Cyclodextrin (CDEn)

CDTs (9.64 g, 6.73 mmol) was dissolved in 1,2-diaminoethane (50 cm³, 0.75 mol) and refluxed with vigorous stirring under nitrogen for 24 hours at 70°C. The mixture was then concentrated under vacuum and gave a light yellow viscous oil. The oil was dissolved in a minimum volume of water-methanol 3:1 (100 cm³). The solution was added to acetone at -15°C obtained by mixing with liquid nitrogen. A white precipitate formed. The product was recovered by filtration and purified over a porous ceramic plate overnight. It was recrystallised from water and dried at 60°C for 4 hours. 66, 67, 68, 69, 70, 71, 72, 73, 74

![Reaction Scheme]

Figure 11. Preparation of 6-Deoxy-6-[1-(2-Amino)-Ethylamino]-β-Cyclodextrin (CDEn).

Yield: 6.25 g, 68% (literature value 65%).

m.p.: 230°C with slow decomposition (value is not present in literature).

TLC: R₅ CDEn = 0.65; solvent system 5:3:4:1 methanol : chloroform : water : ammonia; detection by dipping in 5% sulphuric acid/ethanol and heating. Analysis of the product showed no parent CDTs present at an R₅ of 0.92 or En at an R₅ of 0.53.
R<sub>f</sub> CD<sub>E</sub>n = 0.19 solvent system 7:7:5:4 ethyl acetate : 2-propanol : concentrated ammonium hydroxide : water; detection by dipping in 5% sulphuric acid/ethanol and heating.

$^1$H NMR (D<sub>2</sub>O) δ (ppm): 4.93 (br), 3.76 (br), 3.71 (br), 3.50 (br), 3.47 (br), 3.41 (br), 2.65 (br), 2.54 (br), 2.06 (br).

$^{13}$C NMR (D<sub>2</sub>O) δ (ppm): 105.10, 104.08, 86.62, 84.40, 75.59, 74.55, 74.27, 73.85, 68.34, 62.76, 52.30, 48.02, 42.03.

FTIR (Nujol) ν (cm<sup>-1</sup>): 3735 (w), 2918 (s), 2852 (s), 2360 (w), 1653 (m), 1559 (w), 1456 (s), 1377 (s), 1156 (m), 1032 (m), 501 (w).

UV-VIS, λ<sub>max</sub> (nm): 210.

ESI Mass Spectra: m/z = 1177.6 (M+H)<sup>+</sup>.

Detailed spectra are reported in the Appendix Section.

2.3.3. Synthesis of CD<sub>E</sub>n-FA Conjugate

CD<sub>E</sub>n (0.2000 g, 0.15 mmol) was dissolved in a minimum volume of pyridine (15 cm<sup>3</sup>). Folic acid (0.0330 g, 0.07 mmol) and N-hydroxysuccinimide (0.0350 g, 0.31 mmol) were dissolved in DMSO (10 cm<sup>3</sup>) and added to the CD<sub>E</sub>n solution. Dicyclohexylcarbodiimide (0.0630 g, 0.31 mmol) was added. The solution was stirred in the dark overnight under nitrogen at 20°C. The solution was then warmed to 45°C for two hours and was added to acetone (50 cm<sup>3</sup>) at -20°C. The precipitate which formed was left to stand overnight. About 0.1 g of crude product (maximum yield 0.24 g) was recovered by filtration and washed with acetone, acetonitrile and diethylether.
Figure 12. Preparation of CDEn-FA conjugate.

Purification of the material was carried out by column chromatography (15 x 150 mm) to remove any unreacted starting materials and undesired products using CM Sephadex C25 40-125μ (Sigma) with gradient elution by 0-0.2M NH₄HCO₃ (total volume 300 cm³). Over 80 fractions of 1.5 cm³ were collected and followed by TLC. The fractions with Rf values for CDEn and folic acid were discarded. All other fractions were combined and a solid material recovered by rotary evaporation. This material was further purified by column chromatography (20 x 300 mm) on Sephadex G-25 20-80μ to remove high molecular weight by-products. The eluent was deionized water (150 cm³) and fractions of 0.5 cm³ were collected. A material was recovered for further investigation from fractions with an Rf value as given below.

**Yield:** 0.0070 g, 3%.

**m.p.:** 200°C (slow decomposition).

**TLC:** Rf CDEn-FA = 0.53, solvent system 5:1:3:2 propanol : ethyl acetate : water : ammonia; detection by dipping in 5% sulphuric acid/ethanol and heating and UV lamp.
Rf(CDEn-FA) = 0.58 solvent system 5:3:1 propanol : water : ammonia; detection by dipping in 5% sulphuric acid/ethanol and heating and UV lamp.

$^1$H NMR (D$_2$O) $\delta$ (ppm): 8.65 (s), 7.70 (d), 6.67 (d), 4.97 (br), 4.54 (m), 3.94 (s), 3.77 (br), 3.70 (br), 3.60 (br), 3.53 (br), 3.45 (br), 2.65 (br), 2.23 (br), 2.08 (br), 1.87 (br).

$^{13}$C NMR (D$_2$O) $\delta$ (ppm): 181.46, 178.07, 167.94, 163.04, 150.92, 146.96, 144.98, 129.13, 115.26, 112.70, 101.98, 83.33, 81.00, 75.87, 73.31, 71.79, 68.30, 60.02, 55.71, 49.19, 45.81, 35.67, 33.80, 28.11.

FTIR (Nujol) $\nu$ (cm$^{-1}$): 3851 (w), 3735 (w), 3687 (w), 3583 (m), 2920 (s), 2853 (s), 2726 (m), 2360 (w), 1652 (m), 1462 (s), 1377 (s), 1155 (m), 1031 (m), 721 (s), 470 (s).

UV-VIS, $\lambda_{max}$ (nm): 194, 281, 354.

ESI Mass spectra: m/z = 1600.7.

MALDI $z/m$: 1602.6 (M+2H$^+$)$^+.$

Detailed spectra are reported in the Appendix Section.

2.4. Discussion

In this section NMR shifts are assigned following the numbering system reported in Figure 13 and Figure 14.
Figure 13. Numbering of CD derivative.

Figure 14. Numbering of substituents on the CD derivatives: a) CDTs; b) CDEn; c) CDEnFA.
A data summary of $^1$H-NMR measurements is reported on the following Table 2 and an overview of the $^{13}$C-NMR analysis is reported on Table 3 and 4.
### Table 2. $^1$H NMR spectroscopic data for β-CD and all CD Derivatives.

| Sample     | $H_1$($p$) | $H_2$ | $H_3$ | $H_4$ | $H_5$ | $H_6$ | $OH_2$ | $OH_3$ | $OH_6$ | $H_7$ | $H_8$ | $H_9$ | $H_{10}$ | $H_{11}$ | $H_{12}$ | $H_{13}$ | $H_{14}$ | $H_{15}$ | $H_{16}$ | $H_{19}$ | $H_{20}$ | $H_{22}$ | $H_{23}$ | $H_{25}$ | $H_{27}$ | $H_{28}$ |
|------------|------------|-------|-------|-------|-------|-------|--------|--------|--------|-------|-------|-------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| βCD        | 4.82       | 3.28  | 3.57  | 3.31  | 3.53  | 3.62  | 5.73   | 5.68   | 4.46   |       |       |       |         |         |         |         |         |         |         |         |         |         |         |         |
| CDTs       | 4.83       | 3.30  | 3.56  | 3.45  | 3.49  | 3.63  | 5.77   | 5.71   | 4.49   | 7.74  | 7.42  | 2.42  |         |         |         |         |         |         |         |         |         |         |         |
| CDEn       | 4.93       | 3.47  | 3.71  | 3.41  | 3.50  | 3.76  | 2.06   | 2.65   | 2.54   |       |       |       |         |         |         |         |         |         |         |         |         |         |         |         |         |
| CDEn-FA    | 4.97       | 3.53  | 3.70  | 3.45  | 3.60  | 3.77  | 2.08   | 2.65   | 2.23   | 1.87  | 3.94  | 7.70  | 6.67   | 4.54   | 8.65   |         |         |         |         |         |         |         |         |         |         |

* Samples were dissolved in DMSO-d$_6$ solvent. Others sample were dissolved in D$_2$O solvent.
The $^1$H-NMR spectrum obtained for β-CD in DMSO in this study showed the anomeric hydrogen H1(β) as a doublet at 4.82 ppm, with signals for the H2, H3, H4, H5 and H6 protons at 3.28, 3.61, 3.35, 3.55 and 3.62 ppm respectively. The results obtained for β-CD show agreement with the pattern of values published by Schneider et al., who reported H1(β), H2, H3, H4, H5 and H6 at 4.82, 3.29, 3.64, 3.34, 3.59 and 3.64 ppm respectively. The same author reports chemical shift values for OH groups 5.52 (OH2), 5.48 (OH3) and 4.26 (OH6) ppm. Again there is good agreement with the results found in this study for OH2, OH3 and OH6 at 5.73, 5.68 and 4.46 ppm respectively. Protons involved in hydrogen bonding are much more deshielded than free protons and therefore appear in different regions of the $^1$H-NMR spectrum.

The resonances of OH2 and OH3 were found to appear lowest field due to the formation of intramolecular bonds (Figure 15).

The $^1$H-NMR spectrum of CDTs showed in the aromatic region the characteristic pattern of a tosyl system (two sets of doublets at 7.74 ppm and 7.42 ppm). Both doublet groups integrated for 2 protons which is equivalent to the total...
number of protons (four) available in the aromatic ring of the tosyl group. The same values are reported in the literature. A singlet found at 2.42 ppm was ascribed to the three methyl protons of the tosyl group. The 6-hydroxyl substitution was also confirmed by the decrease in the integration of the peaks at 4.49-4.52 ppm from 7.74 to 7.42. These results also compare favourably with the literature. The $^1$H-NMR spectrum of CDEn does not show any tosyl group signals which suggests successful amination of CDTs through nucleophilic substitution by the diaminooethane group. The anomeric proton has a chemical shift of 4.93 ppm and signals at 2.65 and 2.54 ppm show the presence of methylene hydrogens (H8, H9) from the diaminooethane group. The signal for H1 has been reported at 4.90 or 5.10 ppm and the signal for H8 has been reported in the range 2.70-2.90 ppm. The results found in this work show good agreement with the literature.

The conjugate CDEn-FA gives a spectrum as shown in Figure 16.
There are no reports in the literature of the synthesis of CDEn-FA and therefore there are no spectroscopic data available. Bonechi et al.\textsuperscript{86} published a paper on an NMR investigation of folic acid and reported chemical shift values for the aromatic protons H25, H19, H20 at 8.75, 7.75 and 6.74 ppm respectively. \textsuperscript{87, 88} The \textsuperscript{1}H-NMR spectrum of CDEn-FA reported here does show signals in the region 8.7-6.5 ppm which can be assigned to these aromatic protons. However there are three signals for each aromatic proton. This suggested that the folic acid moiety is present in three different configurations and that FA is probably bonded to CDEn through both carboxylic functions generating γ and α conjugates and there may also be some impurity of free FA.
This is further supported by looking at the signal for the anomeric hydrogen H1 at -5 ppm which integrates for 14 protons and suggests the presence of two CD molecules. Integration of the peaks in the region 4 – 3 ppm assigned to the protons H5, H3, H4, H2 and H6 of CD also suggest two CD units.

Further evidence for the presence of α and γ conjugates was found using ROESY spectroscopy and Figure 17 shows the spectra obtained. It was possible to assign respectively H19α, H19γ and H20α, H20γ by correlation of the signals belonging to the same carboxylic acid groups of both conjugates. It was also possible to show that the phenyl group of the folate moiety can interact with the CD cavity by correlation of the peaks assigned to H19 and H20 of FA (6.59 and 7.51 ppm respectively) and the peaks assigned to the hydrogens of the CD cavity. However deep inclusion of the FA group within the cavity can be excluded since no cross-peak between the proton of pterine ring (H25 at 8.65 ppm) and the CD cavity was detected. It may be possible that formation of a supramolecular adduct between CDEn-FA and unbound FA (free) has formed.
Figure 17. ROESY spectra of CDEn-FA in D₂O.
Table 3. $^{13}$C NMR spectroscopic data for β-CD and all CD derivatives (C1-C6).

<table>
<thead>
<tr>
<th>Sample</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C1'</th>
<th>C2'</th>
<th>C3'</th>
<th>C4'</th>
<th>C5'</th>
<th>C6'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD*</td>
<td>102.20</td>
<td>72.64</td>
<td>72.02</td>
<td>81.70</td>
<td>73.23</td>
<td>60.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDTs*</td>
<td>101.96</td>
<td>72.78</td>
<td>72.45</td>
<td>81.52</td>
<td>73.09</td>
<td>60.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68.02</td>
</tr>
<tr>
<td>CDEn</td>
<td>105.10</td>
<td>74.55</td>
<td>73.85</td>
<td>84.40</td>
<td>75.59</td>
<td>62.76</td>
<td>104.08</td>
<td>74.27</td>
<td>86.62</td>
<td>68.34</td>
<td>48.02</td>
<td></td>
</tr>
<tr>
<td>CDEn-FA</td>
<td>101.98</td>
<td>73.31</td>
<td>71.79</td>
<td>81.00</td>
<td>75.87</td>
<td>60.02</td>
<td>101.98</td>
<td></td>
<td>83.33</td>
<td>68.30</td>
<td>45.81</td>
<td></td>
</tr>
</tbody>
</table>

* Samples were dissolved in DMSO-d$_6$ solvent. Others sample were dissolved in D$_2$O solvent.
Table 4. $^{13}$C NMR spectroscopic data for β-CD and all CD derivatives (C7-C29).

| Sample   | C7    | C8    | C9    | C10   | C11   | C12   | C13   | C14   | C15   | C16   | C17   | C18   | C19   | C20   | C21   | C22   | C23   | C24   | C25   | C26   | C27   | C28   | C29   |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| CDTs†    | 132.68| 127.62| 129.93| 144.86| 21.27 |
| CDEn     | 52.30 | 42.03 |
| CDEn-FA  | 49.19 | 35.67 | 178.07| 33.81 | 28.11 | 55.71 | 181.46| 163.04| 115.26| 129.13| 112.70| 150.92| 45.81 | 146.96| 144.98| 167.94|       |       |       |       |       |

† Samples were dissolved in DMSO-d$_6$ solvent. Others sample were dissolved in D$_2$O solvent.
The spectrum of β-cyclodextrin shows a signal for the highly deshielded anomeric C1 at 102.20 ppm and a signal for the highly shielded C6 atom at 60.36 ppm. Signals for the atoms C2 and C3 as well as C5 are located in the region 72 ppm to 73 ppm. The signal assigned to C4 is clearly separated from other signals and is found at 81.70 ppm. Schneider et al.\textsuperscript{9} reported the chemical shifts of C1 and C6 at 102.58 ppm and 61.17 ppm respectively which agrees reasonably well with results reported here. For the atoms C2, C3, C4 and C5, Schneider et al.\textsuperscript{9} recorded values of 72.67, 73.89, 81.94, 72.89 ppm respectively. From this study chemical shifts at 72.75, 72.45, 81.52 and 73.09, can be similarly assigned.

The spectrum of CDTs shows a signal for the C1 atom at 101.96 ppm. Chemical shifts for C6 and C6' can be observed at 60.04 and 68.02 ppm respectively. For the atoms C2, C3, C4 and C5, literature\textsuperscript{9} data are 71.1, 73.1, 82.4 and 74.1 ppm respectively. For the atoms C2 and C3, Petter et al.\textsuperscript{64} reported signals in the range from 73.3 to 71.4 ppm. In this study signals for the atoms C2, C3, C4 and C5 are 74.55, 73.85, 84.40 and 75.59 ppm respectively are observed and they fit the literature data trend. Signals for the aromatic carbon atoms of the tosyl group can be seen in the range 127 to 145 ppm. The peak for the methyl substituent C11 is at 21.24 ppm. Schneider et al.\textsuperscript{9} found peaks at 101.08 ppm assigned to C1, 81.00 ppm assigned to C4, 72.30 ppm assigned to C5 and 68.60 ppm assigned to C6' all of which are in agreement with the experimental data recorded here.

The spectrum of CDEn shows chemical shifts values for the C1 atom at 105.10 and at 104.08 ppm for the C1' atom. The resonance of the substituted carbon atom (C6') which appears at 68.02 ppm in the spectrum of CDTs was greatly shifted to 48.02 ppm. The C6' carbon atom bonded to the tosyl group in CDTs is much more
deshielded than when this atom is bonded to the amine group in the CDEn. This fact constituted evidence that the successful replacement of the tosyl group by the amine group occurred. The carbon atoms C2, C3, C4 and C5 gave in the spectrum of CDEn signals in similar position to those observed in the spectrum of CDTs. Additional signals were detected at 52.30 ppm and 42.03 ppm, which were assigned to C8 and C9 of the diaminooethane group respectively. These results are in good agreement with those reported by Potter et al.\textsuperscript{89} and Fernández et al.\textsuperscript{85}.

The $^{13}$C-NMR spectrum of CDEn-FA has not been reported previously and assignments are based on data reported for folic acid.\textsuperscript{86} It is evident that there is a change of chemical shift for the C1 atom from 105 ppm in CDEn to 102 ppm in CDEn-FA. For the atoms C2, C3, C4, C5 and C6 the observed values are similar to those of CDEn.

Assignment of peaks in the region 120 – 180 ppm was not possible due to weak signals and therefore a poor signal to noise ratio. Peaks at 49.19 and 35.67 ppm can be assigned to C8 and C9 respectively. The decrease in frequency can be explained in terms of a new charge distribution caused by formation of a peptide bond. The CH$_2$- groups C12, C13 and C23 are detected at 33.80, 28.11 and 45.81 ppm respectively. Chemical shifts are detected at 30.4, 26.1 and 45.9 ppm respectively. The aromatic carbons C18, C19, C20 and C21 gave signals at 115.26, 129.13, 112.70 and 150.92 can be assigned to atoms of folic acid. For folic acid, Bonechi et al.\textsuperscript{86} reported signals at 121.3, 129.0, 111.2 and 150.8 ppm, which are in good agreement with results obtained for CDEn-FA. Atoms of the petirinic group C25, C26, C27 and C28 gave signals at 146.96, 144.98, 150.92 and 167.94 ppm respectively. Folic acid\textsuperscript{86} gives chemical shift values for these atoms at 148.6, 153.8, 156.2 and 161.3 ppm.
Table 5 shows the IR frequencies obtained for β-CD and all derivatives with assignments. 

<table>
<thead>
<tr>
<th>β-CD</th>
<th>CDTs</th>
<th>CDEn</th>
<th>CDEn-FA</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(cm⁻¹)</td>
<td>(cm⁻¹)</td>
<td>(cm⁻¹)</td>
<td>(cm⁻¹)</td>
<td></td>
</tr>
<tr>
<td>2968</td>
<td>2943</td>
<td>2918</td>
<td>2920</td>
<td>-CH₂- asymmetric stretch</td>
</tr>
<tr>
<td>2895</td>
<td>2902</td>
<td></td>
<td></td>
<td>“” “”</td>
</tr>
<tr>
<td>2727</td>
<td>2727</td>
<td>2726</td>
<td></td>
<td>-OH stretch</td>
</tr>
<tr>
<td>2847</td>
<td>2848</td>
<td>2852</td>
<td>2853</td>
<td>-CH₂- symmetric stretch</td>
</tr>
<tr>
<td>1653</td>
<td>1653</td>
<td>1653</td>
<td>1653</td>
<td>OH- bending</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1559</td>
<td>N-H bending vibrations</td>
</tr>
<tr>
<td>1463</td>
<td>1463</td>
<td>1456</td>
<td>1462</td>
<td>-CH₂- scissor mode</td>
</tr>
<tr>
<td>1377</td>
<td>1377</td>
<td>1377</td>
<td>1377</td>
<td>-CH₂- symmetric bend</td>
</tr>
<tr>
<td>1156</td>
<td>1158</td>
<td>1156</td>
<td>1155</td>
<td>Secondary and tertiary alcohol modes</td>
</tr>
<tr>
<td>1028</td>
<td>1028</td>
<td>1032</td>
<td>1031</td>
<td>C-O stretching (cyclic alcohols)</td>
</tr>
<tr>
<td>837</td>
<td></td>
<td></td>
<td></td>
<td>Para-Disubstituted Benzene mode</td>
</tr>
<tr>
<td>721</td>
<td>721</td>
<td>721</td>
<td></td>
<td>Rocking</td>
</tr>
</tbody>
</table>

The spectrum of CDTs shows a peak at 837 cm⁻¹ which can be assigned to vibrations of the paradisubstituted benzene ring of the tosyl group.

The spectrum of CDEn shows a signal at 1559 cm⁻¹ assigned to the N-H bending vibration.
These results suggest successful synthesis of CDTs and CDEn. The spectrum of CDEn-FA does not show peaks assigned to previous intermediates.

Electronic spectroscopy was used for spectroscopic comparison of CDEn and CDEn-FA. The spectrum of CDEn shows $\lambda_{\text{max}}$ at 210 nm. The spectrum of the conjugate shows new bands at 194, 281 and 354 nm. Off et al.\textsuperscript{91} reported absorption peaks for the folic acid at 280 and 350 nm which agrees reasonably well with the bands recorded here and suggests the presence of a folic acid moiety in the conjugate.

The mass spectrum (ESI-MS) of CDEn gave confirmation of the molecular weight from the peak $[M+H]^+$ at 1177.6 m/z. The spectrum shows an additional base peak at 1219.6 m/z $[M+H]^+$ possibly caused by the presence of a diamine derivative such as CDEn$_2$.

MALDI and ESI-MS spectra of the conjugate CDEn-FA are given in appendix G. These spectra confirm the formation of the conjugated product with signals assigned to CDEnFA$+H^+$ and CDEnFA$+2H^+$ observed at m/z 1600.7 and 1602.6 respectively.
Chapter 3

Photostability Studies
3.1. Photostability of Folic Acid

Previous studies on folic acid have shown that on heating for 10 hours at pH 4-12 the stability decreased with decreasing pH. Photodegradation of folic acid (FA) by ultraviolet radiation is a well-documented photochemical reaction. FA is a photosensitive compound that undergoes photolysis by UV. The photodegradation of FA is divided into three phases leading to the formation of specific photoproducts such as p-aminobenzoyl-L-glutamic acid, 6-formylpterin and pterin-6-carboxylic acid. Off et al. reported that when FA is exposed to UV radiation, its absorption spectrum changes as shown in Figure 18. As can be seen there is a decrease in the intensity of the peak at 280 nm and an increase in intensity of the peak at 350 nm together with a shift in wavelength.

![Absorption spectra of 10^-3 M folic acid after 0, 20, 40 and 60 min of UVA exposure at pH 7.](image)

Figure 18. Absorption spectra of 10^-3 M folic acid after 0, 20, 40 and 60 min of UVA exposure at pH 7.

Folic acid is a polyprotic system with three major equilibrium constants (pK_{a1}=2.38; pK_{a2}=3.38; pK_{a3}=4.83) with a dissociation sequence NH\(^+\) (C28\(^-\)), \(\alpha\)-COOH (C15) and \(\gamma\)-COOH (C11), respectively. The spectrum of a folate solution changes
significantly with irradiation time in the presence of $O_2$ at different pH values. In acid media spectra are almost indistinguishable while in alkaline media spectra are very different. When the irradiated solutions of folate (pH 10 ÷ 11) are stored in the dark, further changes can also be observed. 95

3.2. Chromatographic and Spectroscopic Study

The CDEn-FA conjugate was evaluated by High-performance liquid chromatography (or high pressure liquid chromatography) (HPLC) and UV-VIS analyses to gain an understanding of the stability and the purity of the product.

HPLC is a column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. 96 The mixture to be separated is transferred to a column with a solvent or a solvent mixture (eluent/mobile phase). The column is a tube, in most cases of stainless steel, filled with the stationary phase. The separation occurs on the column. Under optimal conditions the components to be separated pass through the stationary phase at different rates and leave the column after different times. The components (the solutes) are registered by a detector. This information is passed on to the data evaluation unit and the output is a chromatogram. The number of peaks is equal to the number of separated components in the sample, and the area is proportional to the amount. HPLC equipment consists at least of an eluent delivery system (pump), an injector, a column, a detector and a data evaluation system. 97
3.3. Experimental

A Shimadzu HPLC system was used equipped with an SCL-10A VP controller system, a Shimadzu LC-10 AD VP solvent delivery module, a SPD-M10A VP UV/Vis photodiode array detector (PDA) and controlled by EZSTART v7.2 SP1 Chromatography Software. A Rheodyne 8125 injector with a 20 µL loop was used. The stationary phase was a Supelco column RP C-18 (4.6 x 25 mm) with a mobile phase of phosphate buffer solution (PBS) (10 mM, pH 2.27): acetonitrile (ACN) 8:92 v/v. Measurements were carried out with a flow rate 0.5 mL/min at room temperature (25°C). HPLC analysis was carried out repetitively in order to optimise the conditions, until chromatographic reproducibility was detected.
As previously mentioned the detection system was a PDA unit (Figure 20) which is equipped with a deuterium (D₂) lamp and a tungsten halogen (W) lamp. The light from the two lamps is mixed by a half mirror, reflected onto a concave mirror, and introduced into a flow cell. A shutter or a filter before the cell is automatically inserted into the light path at each measurement of the dark signal (dark current), and at the automatic wavelength calibration and automatic wavelength check. The light which has passed through the flow cell is gathered on a slit by another mirror and introduced to the spectrophotometer. The light resolved into its spectral compositions by a holographic grating in the spectrophotometer forms a spectral image on a photodiode array. 512 photodiode elements are lined up on the surface of the photodiode array. One element is assigned every 1.2 nm of the spectra in the range of 190 to 800 nm.

![Figure 20. Schematic diagram of a Photodiode Array Detector.](image)

The UV-VIS analysis was carried out on a Hewlett Packard 8453 UV-VIS spectrophotometer. The details of the UV-VIS system have been previously been
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described in Chapter 2. CDEn-FA was studied to evaluate its stability after irradiation by a UV/VIS light source at physiological pH. Samples of folic acid (500 μM in PBS 10 mM, pH=7.00) and the conjugate (160 μM in Millipore purified water) were irradiated with a halogen lamp (15 W) at room temperature for 5 hours with stirring. The irradiating beam was filtered through a 1 cm glass cell filled with water to remove the IR and UV component. During the investigation UV-VIS spectra were recorded to monitor any change of electronic spectra due to irradiation.

3.4. Results and Discussion on HPLC.

A HPLC study of folic acid as reference was not carried out since the elution conditions which must be used for successful resolution of the CDEn-FA conjugate cannot be applied to solutions of folic acid. In the literature, many researchers studied folic acid by ion-pairing HPLC in very polar solvent systems. Folates have also been investigated by HPLC using a prevalent ratio of organic solvent systems or a gradient elution method at different pH values. The results obtained in this study for CDEn-FA are shown in Figure 21.
The chromatogram shows two main peaks at 2.78 (peak 1) and 3.22 (peak 2) minutes and a minor peak at 3.80 (peak 3) minutes. Two overlapping peaks were detected at 4.38 (peak 4) and 4.96 (peak 5) minutes. These results are summarised in Table 6 below.

Table 6. HPLC results for CDEn-FA at two different flow rates with an injection volume of 10 µL and detection at 285 nm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flow [mL/min]</th>
<th>Peak 1 tR [min]</th>
<th>Peak 2 tR [min]</th>
<th>Peak 3 tR [min]</th>
<th>Peak 4 tR [min]</th>
<th>Peak 5 tR [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDEn-FA</td>
<td>0.700</td>
<td>2.74</td>
<td>3.21</td>
<td>3.81</td>
<td>4.40</td>
<td>5.00</td>
</tr>
<tr>
<td>CDEn-FA</td>
<td>0.300</td>
<td>4.40</td>
<td>5.00</td>
<td>5.52</td>
<td>5.87</td>
<td>6.83</td>
</tr>
</tbody>
</table>

The HPLC study of CDEn-FA gave satisfactory reproducibility in terms of retention time. The first peak was well resolved with a tR at 2.7 minutes. A second well resolved peak was located at 3.2 minutes. Peak number 3 is very low in terms of intensity (tR = 3.8 minutes). Peak number 4 (tR = 4.3 minutes) is easily detected and there is also a broad peak (number 5) with tR = 5.0 minutes. However the presence of
five peaks in the chromatogram suggests that the material is impure or it may have degraded.

In an attempt to identify each component obtained on separation of CDEn UV-Vis spectra were obtained of each product exiting the HPLC column and results are given in Figure 22 to Figure 26. Spectra were also obtained of FA, CDEn and CDEn-FA and are shown in Figure 27 below and results are summarised in Table 7.

Figure 22. UV-VIS spectrum of first compound from HPLC showing maximum absorbance at 275 nm.
Figure 23. UV-VIS spectrum of second compound from HPLC with maximum absorbance at 198 nm, 285 nm and 365 nm.

Figure 24. UV-VIS spectrum of third compound from HPLC with maximum absorbance at 197 nm, 285 nm and 365 nm.
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Figure 25. UV-VIS spectrum of fourth compound from HPLC with maximum absorbance at 275 nm and 365 nm.

Figure 26. UV-VIS spectrum of fifth compound from HPLC with maximum absorbance at 198 nm, 285 nm and 365 nm.
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Figure 27. UV-VIS spectra of Folic Acid, CDEn and CDEn-FA in PBS solution.

The electronic spectrum of CDEn-FA shows bands centered at 262 and 365 nm respectively and it should be very similar to the spectrum of FA because the CDEn can not shows a UV-Vis spectra for the absence of chromophor groups. The bands can be assigned to the electronic transitions in the pteridine moiety and the phenyl ring respectively. 114

Table 7. Peak assignment of the CDEn-Fa chromatogram.

<table>
<thead>
<tr>
<th>Peak</th>
<th>CDEn</th>
<th>FA</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
<th>Peak 5</th>
</tr>
</thead>
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<td>365</td>
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52
From the results obtained it is very difficult to reach any conclusive assignments. Both CDEn and FA absorb in the 200 to 220 nm region and therefore it is not possible to determine which peak in the chromatogram corresponds to CDEn or FA or the conjugate CDEn-FA. Also, in this region could be happen some solvent absorption effects like peak 1 and 4, where there are off scale absorption phenomena. However peak 5 can be tentatively assigned to the conjugate. The spectrum shows clearly absorption features of both CDEn and FA. In particular the absorption at 198 nm consistent with the presence of FA should be noted. Of all five spectra recorded only those associated with peaks 3 and 5 in the chromatogram show this absorption but the intensity is too weak for the spectrum of peak 3.

Peak 1 in the chromatogram can possibly be assigned to some metabolite of FA since there is no absorption in the region above 300 nm which would be expected for FA itself. This peak can also not be assigned to CDEn since the cyclodextrin derivative does not absorb in this region.

It is difficult to determine the origin of peaks 2, 3 and 4 in the chromatogram since all three show absorption features of both FA and CDEn. Further analysis is required eg via LC-MS experiments to determine the structure of the compounds giving rise to these peaks in the chromatogram.

3.5. Results and Discussion on Photodegradation Test.

Photodegradation tests were carried out on samples by recording UV-VIS spectra every hour. Figure 28 and Figure 29 show the spectra obtained over the UV-VIS range of 200 – 700 nm.
Figure 28. UV-Vis spectra Photostability of FA (160 μM in Phosphate Buffer Solution, 10 mM, pH= 7.40) over a 5 hour period. The arrows show isosbestic point.
In this study the electronic spectrum of FA shows significant changes after exposure to a visible light source over a 5 hour period. There are increases in intensity of the band centred at 281 nm. There is also an increase in intensity together with a slight decrease in wavelength of the band centered at 352 nm. This may seem at first glance to contradict the work of Off et al. who reported a decrease in the intensity of the peak at 280 nm. However these workers exposed FA to UV radiation and in our work no UV component was used.

The spectrum of CDEn-FA does not show significant changes, in terms of absorbance and wavelength shifts, after irradiation experiments using the visible light source. As shown in Figure 29, the band at 265 nm undergoes only a very small increase in intensity after 1 h of irradiation. No change was registered for the band...
centered at 360 nm. These results suggest that FA is photodegradable. However, stability has been very much improved in the conjugate material CDEn-FA which may be important if the material is to be used as a drug delivery agent.
Chapter 4

Biological Evaluation
4.1. Introduction

The biological study was required in order to evaluate the performance of the new folate conjugate "in vitro". The design of this conjugate is based on a potential application in drug delivery for cancer pathology and therefore the biocompatibility becomes a predominant factor.

Many researchers reported studies on cytotoxicity and uptake of drug delivery systems based on folate receptors. To understand the cytotoxic effect of the CDEn-FA product in a human cancer cell line HeLa cells were used. HeLa cells are an immortal cell line derived from uterine cervical cancer cells and are used in medical research. These cells are models of a human cancer cell with high transfection capacity and have a reliable and constant over-expression of the folate receptor. They contain a membrane folate-binding protein (FBP) which is involved in the uptake of free folate. HeLa cells have also been used to study inorganic nanostructured-folates.

Cytology evaluation is the analysis of cells under a microscope. Cellular changes are used for the diagnosis of disease, to study the cellular anatomy, function and chemistry. Trypan blue is a standard assay used in the measurement of the viability of cells. It is a reliable and fast test. Trypan blue, a diazo dye shown in Figure 30, is a vital stain used to selectively colour dead tissues or cells blue. Living cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell Trypan blue is not absorbed. However, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope.
Figure 30. Trypan blue molecule.\textsuperscript{125}

HOEST 33342 is useful for monitoring cell death by apoptosis.\textsuperscript{126} Apoptosis and necrosis are two major processes by which cells die. Apoptosis is an active, genetically regulated disassembly of the cell from within. Disassembly creates changes in the phospholipid content of the cytoplasmic membrane outer layer. Necrosis normally results from a severe cellular disruption.\textsuperscript{127}

4.2. Experimental

HeLa cells were obtained from the American-Type Culture (ATC) collection and propagated at 1:6 ratio using Dulbecco’s modification of Eagle’s minimal essential medium supplemented with 10% new born calf serum, 100 μ/mL of penicillin and 100 μg/mL of streptomycin, at 37° C, in 5% CO\textsubscript{2} atmosphere. The cells were incubated for 24 hours.

The percentage of cell death was evaluated by microscopy in a Burker chamber, using the trypan blue exclusion standard assay. In a 24-well plate the cell solution was transferred with a density of 1.5 x 10\textsuperscript{5} cells/well.

The procedure used for was as follows:

1. 0.5 ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of 1 x 10\textsuperscript{5} to 2 x 10\textsuperscript{5} cells per ml) were placed in a screw cap test tube.
2. 0.1 ml of 0.4% Trypan Blue Stain was added and mixed thoroughly.

3. The solution was allowed to stand for 5 min at 30°C.

4. The hemocytometer was filled for cell counting.

5. The cells were studied under a microscope, to observe if the non-viable cells were stained and the viable cells excluded the stain. HeLa cells were treated for 3 hours with CDEn-F A (10^{-4} M) and FA (10^{-4} M in ratios of 2:10 and 3:10 and afterwards they were collected and layered on poly lysinated slides. After 10 min, the cells were stained with HOECHST 33342 fluorescent DNA-binding dye at 50 mM/mL (Sigma) and analyzed (objectives 40x) by using a rhodamine filter (red emission) and a DAPI filter (blue emission) on a Biomed fluorescence microscope (Leitz, Wetzlar, Germany).

4.3. Results and Discussion

The following visuals (Figure 31 to Figure 34) were obtained from the microscopy analysis of CDEnFA and FA as control at different ratios using Rhodamine filter (A), DAPI filter (B) and a combination of images is shown in C.
Chapter 4

Biological Evaluation

Figure 31. HeLa cells treated with CDEn-FA 2:10 in according to the procedure decrypted.

Figure 32. HeLa cells treated with CDEn-FA 3:10 in according to the procedure decrypted.

Figure 33. HeLa cells treated with FA 2:10 (control) in according to the procedure decrypted.

Figure 34. HeLa cells treated with FA 3:10 (control) in according to the procedure decrypted.
During preliminary biological testing, HeLa cells were not affected when they were treated with CDEn-FA. The images produced when using the Rhodamine filter (A) for CDEn-FA and FA at all ratios studied showed a diffuse background confirming that both compounds were not located specifically in a cellular region. This was also supported by combining the images (C) and no red spots were detected. The conjugate seams have a better performance on the folate receptor recognition than free folic acid.

When using the DAPI filter (B) for CDEn-FA and FA at all ratios studied no damage to the nucleic cell or no change in the morphology was observed. These results suggest a very low cytotoxicity for CDEn-FA. This encourages further experimentation on cell systems to develop CDEn-FA as a drug delivery system.
Chapter 5

Conclusion and Future Work
5.1. Final Overview of Results

A new material CDEn-FA was synthesized and characterized using NMR, IR and Mass Spectroscopy.

From the results obtained it is clear that two structural isomers (α- and γ-) of CDEn-FA were synthesized. Another adduct between conjugate and free FA was detected and was not removed after the purification process.

HPLC studies gave further support to the spectroscopy results and also suggest the presence of CDEn-FA and a further adduct.

Exposure of the new material to visible radiation showed that it is substantially more photostable than free FA at physiological pH values.

A preliminary biological evaluation of the cyclodextrin-folate conjugate was carried out to understand its effect on HeLa cell lines. No evidence of toxicity are observed.

At this stage there are no evidence that the material can cross the cell wall.

The photostability tests and the biological evaluation results encourage further studies on this material for applications in drug delivery systems for cancer therapy or diagnosis.

5.2. Future Work

The first goal of any future work is the separation of the two isomeric forms of CDEn-FA possibly by a preparative HPLC method. In this way trace products may be removed and the α and γ isomers of CDEn-FA separated. Investigation of the material by NMR diffusion and Light Scattering experiments will give further information on its aggregation properties by competitive complexation with...
adamantanol. Further biological tests and drug solubility properties will also be considered in future investigations.

Protonation and metal complexation equilibria can be investigated by Capillary Electrophoresis and potentiometric titration studies. The coordination properties of the conjugate towards different transition metal ions, such as ruthenium and platinum will be studied. Folic acid has been shown to form a coordination complex with nickel, the structure of which is shown below.

(Figure 35. Detail of the folic acid aromatic unit coordination to Ni²⁺)

In this work it was shown that CDEn-FA could not cross the cell membrane cell. It was localized at cell wall. Therefore there is a need to study different functionalised CDs with more affinity for biological membranes which may help delivery into the cytoplasm region. Amphiphilic molecules incorporating other CD derivatives and porphyrin systems will be studied with a view to developing a system which can successfully delivery drugs into the cells. Such versatility of the CDEn-FA can then be exploited for intracellular delivery of photosensitisers in photodynamic
therapy of tumours, organic and inorganic drugs used in conventional anticancer therapy, metal nanoparticles.
Figure 36. $^1$H-NMR spectrum of β-Cyclodextrin in DMSO.
Figure 37. $^1$H-NMR spectrum of 6-o-Monotosyl-6-Deoxy-β-cyclodextrin (CDTs) in DMSO.
Figure 38. $^1$H-NMR spectrum of 6-Deoxy-6-[1-(2-Amino)Ethylamino]-β-Cyclodextrin (CDEn) in D$_2$O.
Figure 38. 1H-NMR spectrum of Folate Conjugate (CDEn-FA) in D$_2$O.
B. $^{13}$C-NMR spectra

Figure 40. $^{13}$C-NMR spectrum of β-Cyclodextrin in DMSO.
Figure 41. $^{13}$C-NMR spectrum of 6-o-Monotosyl-6-Deoxy-β-Cyclodextrin (CDTs) in DMSO.
Figure 42. $^{13}$C-NMR spectrum of 6-Deoxy-6-[1-(2-Amino)-Ethylamino]-β-Cyclodextrin (CDEn) in D$_2$O.
Figure 4.3. $^1$H-NMR spectrum of Polysialate Conjugate (CDHn-FA) in D$_2$O.
C. COSY spectra

Figure 44. COSY spectrum of Folate Conjugate (CDEn-FA) in D₂O.
D. ROESY spectra

Figure 45. ROESY spectrum of Folate Conjugate (CDEn-FA) in D$_2$O.
E. TOCSY spectra

Figure 46. TOCSY spectrum of Folate Conjugate (CDEn-FA) in D₂O.
Figure 47. IR Spectrum of β-Cyclodextrin in nujol.
Figure 48. IR Spectrum of 6-o-Monotosyl-6-Deoxy-β-Cyclodextrin (CDTs) in nujol.
Figure 49. IR Spectrum of 6-Deoxy-6-[(2-Amino)Ethylamino]-β-Cyclodextrin (CDEn) in nujol.
Figure 51. IR Spectrum of Folic Acid (FA) in nujol.
Figure 52. ESI Mass Spectrum of 6-Deoxy-6-[(2-Amino)-ethylamino]-β-Cycloextrin (CDEn).
Figure S4. MALDI Mass Spectrum of Folate Conjugate (CDEn-FA).
Targeted Drug Delivery Systems for Cancer Therapy

A. Clementi, G. O'Connor, M. McNamara, F. A. Mazzaglia, M. C. Aversa, A. Gielniak

ABSTRACT

The use of cyclophanes (CD) in drug delivery has advanced in recent years and this may be attributed to their biocompatibility and well-established synthesis. Chemical modification of CDs has shown to admit the physicochemical properties and the host capacity for a variety of drugs. CD has been widely used in the early stages of pharmaceutical applications because of its ready availability and its many uses suitability for a wide range of drugs. Chemical modification of CD has proven to enhance specific interactions, microenvironmental stability and reduced toxicity in previous studies. Finally, developments are over-represented in several human cancers including breast, renal and cervical cancer. This property has been utilized to develop tumor selective anti-neoplastic drugs. Finalite has been used to chemotherapeutic drugs and serve tumor cells with huge appetite for drugs, finally micelles pull the drug to the tumour. However, the direct conjugation of finalite to the bioactive drug can lead to the loss of targeting or alter the function of the conjugate. Finalite-cyclophane bisconjugates have been prepared with polyethylene glycol (PEG) linkers, however, the conjugates partially prevent drug degradation. The study describes the synthesis and characterization (UV, FT-IR, NMR, MALDI-TOF and ESI-MS) of a novel finalite-cyclophane bisconjugate (COE FA). As mentioned previously it was found that direct conjugation of finalite to the bioactive drug is lost of targeting or alteration of the function of the conjugate and most of the conjugates to date cannot be further modified to improve targeting or anti-tumour activity. Preliminary biological evaluation of the tumour targeting device will be discussed.

INTRODUCTION

Cyclophanes easily form inclusion compounds with a wide range of organic and inorganic molecules. One application is in drug delivery by the formation of inclusion complexes e.g. in combination with different drugs. It is possible to control the release rate of drugs. Targeted drug delivery systems are molecular tools which without unintended interaction at other sites target a specific drug receptor. Any adverse toxicity would be avoided and only the desired therapeutic gain would be produced. Finalite and FA (FA) has been chosen as a cancer targeting agent and it is recognised by the tumour cells by finalite receptors (FR). Conjugates of finalite are extremely potent selective agents that target tumour cells expressing the high affinity finalite receptor. The aim of this work is to develop a new nanovehicle as a drug delivery systems for cancer pathology applications.

SYNTHETIC STRATEGY

Figure 1. Formation of inclusion CD-complex with a guest molecule

Figure 2. Structure of Finalite Acid Mechanism of targeting in cancer (FA)

Figure 3. Schematic representation of the synthetic route to the formation of COE FA

SYNTHETIC STRATEGY

The monochemistry analysis does not show cyclophaneity of the COE FA material.

Figure 4. Fluorescence microscopy analysis (merging images from Minimum and DNA filter) of Hela cells. The cells were treated with COE FA at 2 μM, 10 μM, 100 μM, FA acid (as control) at 310°C were stained with HOCHEST 33342 dye in phosphate buffer solution (10 mM, pH 7.4).

Figure 5. Absorption spectra of the CD (free, COE FA complex, COE FA complex and COE FA complex with finalite)

Figure 6. UV-Vis absorption spectra of COE FA and COE FA with finalite

Figure 7. NH overtone (N-H/3300 cm−1)

Figure 8. NH overtone (N-H/3300 cm−1)

Figure 9. NH overtone (N-H/3300 cm−1)

DISCUSSION OF RESULTS

H-NMR was assigned by COSY (H-NMR). The H-NMR shows three groups of signals (three for each aromatic proton) which are representative of the finalite and portion in three different configurations as shown in Figure 5. By ROESY (H-NMR) it was possible to assess the geometry of the finalite molecules which can interact with the cavity of the CD. ESI-MS confirms the formation of the COE FA conjugate as shown in Figure 1. UV-Vis absorption analysis of COE FA and product COE FA show different absorption spectra as shown in Figure 6. The HPLC-FLD has measured the stability and purity of the product. The material presents traces of COE FA and FA not reacted. Preparative HPLC experiments are in progress to optimize the purification. During preliminary biological testing, two cells were not affected when they were treated with COE FA. These initial biological evaluations indicate for further implementation in cell systems to develop a drug targeting vehicle.

CONCLUSION

In summary, COE FA was synthetized with an attempt to eliminate the cyclophaneity of the modified CD. This fact is vital in the design and characterization (thermodynamic properties, photophysical, etc. ) of new multifunctional host-guest systems having different sites of complication. By designing a molecular system with a controlled number of binding sites (i.e. targeting moiety, CD cavity, metal coordination environment) it will be possible to modulate the properties of recognition towards receptor proteins. Such versatility of the COE FA can be exploited in the macromolecular delivery of photosensitizers (Photodynamic Therapy of Tumours, PDT), organic and inorganic drugs (conventional anticancer therapy, metal nanoparticles (Photothermal Therapy of Tumours, PTT))

AKNOWLEDGEMENTS


Dr Rosario Biancari for HPLC, Pharmaco-chemical Dept. University of Messina, Italy

Dr Natale Timona Scionti for the biological tests. Dept of Microbiological Genetics and Molecular Sciences, University of Messina, Italy

Dr Camillo Cienfante and Dr Giuseppe Gennaro for high resolution NMR spectra (100 MHz). Dept of Physicals, University of Messina

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[4] UV-Vis absorption analysis of COE FA and product COE FA show different absorption spectra as shown in Figure 6. The HPLC-FLD has measured the stability and purity of the product. The material presents traces of COE FA and FA not reacted. Preparative HPLC experiments are in progress to optimize the purification.

[5] During preliminary biological testing, two cells were not affected when they were treated with COE FA. These initial biological evaluations indicate for further implementation in cell systems to develop a drug targeting vehicle.

[6] In summary, COE FA was synthetized with an attempt to eliminate the cyclophaneity of the modified CD. This fact is vital in the design and characterization (thermodynamic properties, photophysical, etc. ) of new multifunctional host-guest systems having different sites of complication. By designing a molecular system with a controlled number of binding sites (i.e. targeting moiety, CD cavity, metal coordination environment) it will be possible to modulate the properties of recognition towards receptor proteins. Such versatility of the COE FA can be exploited in the macromolecular delivery of photosensitizers (Photodynamic Therapy of Tumours, PDT), organic and inorganic drugs (conventional anticancer therapy, metal nanoparticles (Photothermal Therapy of Tumours, PTT))

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