Binding ability properties of β-cyclodextrin dimers linked through their secondary faces towards cancer chemotherapeutics agent methotrexate

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Abstract: The binding abilities properties of two β-cyclodextrin dimers linked through their secondary faces by short, rigid spacer arms towards the cancer chemotherapeutics agent methotrexate have been studied by ITC and NMR (1D and ROESY) experiments. Both dimers are able to bind two molecules of methotrexate with a binding constant between 2.4 and 3.5 times higher than that for native β-cyclodextrin, the dimer having the shortest linker forming the most stable complex

Key words: β-Cyclodextrin dimers – Complexation ability – Methotrexate – Solubility – Calorimetry – NMR – Binding constants.

In general, most drugs need to permeate some biological membranes to have a pharmacological effect, which requires that these molecules must be lipophilic although they also should possess some aqueous solubility [1]. In recent decades, an increasing number of highly lipophilic potential drugs have been developed by high-throughput methods. However, their therapeutic activities are hindered by a poor solubility in water which reduces their bioavailability. In addition, these therapeutic agents can display other limitations for their administration such as irritating effects, low stability towards biological conditions or inadequate organoleptic or physical properties [2]. Cyclodextrins (CDs), a naturally occurring class of cyclic oligosaccharides comprising six (α-CD), seven (β-CD) and eight (γ-CD) D-*gluco*pyranose units linked by α-(1 → 4) bonds, have been extensively investigated as drug complexating agents in order to overcome these faults, especially the lack of solubility [1-6]. These macrocycles possess a relatively rigid thorus-shaped structure defining an inner hydrophobic cavity which allows them to form inclusion complexes in aqueous solution with organic molecules of a lipophilic nature and a suitable size and geometry [7-9]. However, β-CD, the cheapest and most abundant native CD, also shows limited water solubility and it cannot be administered in parenteral formulations due to its nephrotoxicity. Suitable chemical modification has been found to increase the solubility and decrease the toxicity of β-CD [2,4-6], and provides the opportunity of developing site-specific drug delivery systems based on this macrocycle [10].

CD derivatives consisting of two linked CD units have not received much attention as drug complexating agents. These CD dimers usually display improved binding abilities and molecular selectivity as compared with those of native CDs [11,12]. Recently, we have demonstrated the enhanced complexation efficacy towards bile salts of two β-CD dimers linked through their secondary faces with short, rigid spacer arms (*Figure 1*)

[13]. 2–2'CD dimers are less documented than the 6-6' CD dimers and often show higher complexing constants than those bridged by primary faces, due to the fact that guest molecules preferentially penetrate the cavity through the wider secondary rim [13]. Herein, we wish to report their binding abilities towards the cancer chemotherapeutics agent methotrexate (MTX).

MTX is a folic acid analogue of moderate water solubility (12.7 mM at pH 7) [14] which inhibits enzyme dihydrofolate reductase and has a broad spectrum clinical activity against choriocarcinoma, lymphocytic leukemia, breast cancer, osteosarcoma, primary central nervous system lymphoma, and head and neck cancer [15], and can also be used in the treatment of rheumatoid arthritis, psoriasis and inflammatory bowel disease [16].

The inclusion complexations of hosts **1** and **2** with MTX were investigated by Isothermal Titration Calorimetry (ITC) and Nuclear Magnetic Resonance (NMR) techniques. The stoichiometry for the complexation between **1** and **2** and MTX was determined by using the continuous variation method (Job's method) [17] based on the NMR chemical shift displacements of the aromatic hydrogens *a* and *b* of MTX (Figure 1) measured at different host/guest ratios. In both cases, the Job's plots (*Figure 2*) showed a maximum at a molar fraction of 0.67 and symmetrical shapes, indicating that the complexes possessed 1:2 (dimer:MTX) stoichiometry.

ITC provides a direct determination of the enthalpy change of binding (ΔH) and the affinity constant (K), as well as the [drug]/[dimer] ratio when the dimer binding sites are fully saturated (n) which in many cases coincides with the stoichiometry of the complex. We performed ITC experiments in 50 mM sodium phosphate buffer at pH 7.2 and 25 ºC, first, by conventional titration, i.e. the CD derivative placed in the

calorimeter cell and the drug (MTX) in syringe. However, the heat of dilution of MTX (2 mM in syringe) was high and becomes comparable to the heat of interaction. This problem was solved performing the reverse ITC experiments, thus the heats of binding experiment were clearly well differentiated of the dilution heats as can be seen from Figure 3. The stepwise addition of a solution of both CD dimer **1** and **2** and native β-CD to a solution of MTX led to a series of exothermic peaks of decreasing area (*Figure 3*). Calorimetric data can be fitted to the *n* equal and independent binding sites model for a fixed 1:2 (dimer/MTX) stoichiometry, affording the thermodynamic parameters Δ*H* and *K* (*Table I*).

Table I – Apparent thermodynamic parameters for the binding of MTX to dimers **1** and **2** and β-CD obtained by ITC in 50 mM sodium phosphate buffer at pH 7.2 and 25 ºC.

CD derivative	$K \times 10^{-3}$ (M^{-1})	$-\Delta H$ (kJ mol^{-1})	$T\Delta S^0$ (kJ mol^{-1})
β-CD	0.54 ± 0.03[a]	27.2 ± 0.4	-10.45 ± 0.6
1	1.9 ± 0.2	24.5 ± 0.6	-5.8 ± 0.5
2	1.3 ± 0.3	24.3 ± 0.8	-7.1 ± 1.3

[a] Reported *K* value 380 M^{-1} (Ref. 18)

The binding constants for the complexation between dimers **1** and **2** and MTX are 3.5 and 2.4 times, respectively, higher than that for the native β-CD. The thermodynamic profile for both CD dimers is very similar. The inclusion compensation is in both cases enthalpy driven showing a similar favorable Δ*H* term of approximately 24 kJ mol^{-1} which is partially compensated by a moderate decrease in entropy (-5.8 and -7.1 kJ $mol^{-1}$for **1** and **2**, respectively). The binding of MTX to the native β-CD is also driven by favorable enthalpic gain ($-\Delta H$ = 27.2 kJ mol^{-1}), accompanied by a entropic loss ($T\Delta S°$ = -10.4 kJ mol^{-1}) higher than for the CD dimers **1** and **2**. These results indicate

that the stronger binding of MTX by the linked 2-2'-CD dimers with respect to the native β-CD is thermodynamically accomplished by a decrease of about 50% of the favorable enthalpic gain (-Δ*H*) *per* binding cavity and a stronger reduction of about 60-70% of the unfavorable entropic loss *per* CD unit (TΔ*S*°).

Table II – Selected chemical shift for MTX (δ, ppm) and chemical shift differences (Δδ, ppm) for MTX in the presence of CD dimer **1** and **2**

	δ	δ (Δδ)	
Hydrogen	MTX	**1** + MTX	**2**+ MTX
a	8.58	8.21 (-0.37)	8.19 (-0.39)
e	7.62	7.62 (0.00)	7.62 (0.00)
d	6.79	6.61 (-0.18)	6.62 (-0.17)
b	4.80	4.86 (+0.06)	4.84 (+0.04)
c	3.12	3.28 (+0.16)	3.25 (+0.13)
			7.36 (-0.04)[a]

[a] Chemical shift for aromatic proton for **2** (δ 7.40 ppm in the absence of CD dimer)

In order to obtain a deeper insight on the supramolecular structure of the complexes, 1D and 2D NMR experiments were carried out in D_2O. When ^1H NMR spectra of MTX both in the absence and the presence of the dimers were compared, large shift changes were observed for some protons of the drug (*Table II*, *Figure 1*). Thus, upon interaction with dimers **1** and **2** we observed a shielding effect on the aromatic protons *a* (-0.37 and -0.39 ppm, respectively) and *d* (-0.18 and -0.17 ppm, respectively). By contrast, the methyl group *c* shifted downfield (+0.16 and +0.13, respectively). Much smaller shifts were detected for protons *b* (+0.06 and +0.04 ppm, respectively) and *h* (-0.05 and -0.09 ppm, respectively), while negligible or no shifts were noticed for hydrogen atoms *e*, *f* and *g*. It is interesting to highlight the different behaviour shown by the two aromatic protons of the aminobenzoic moiety (atoms *d* and *e*).

This shift changes pattern roughly matches that described for the interaction of MTX with the native β-CD [18], and has been used to propose the inclusion of the pteridine residue, the methyl group and partially the aminobenzoic moiety in the cavity of the macrocycles [18,19]. We carried out 2D ROESY NMR experiments to study the structure of the formed complexes. This technique provides a snapshot of the protons that are close in the space. In detail, the pteridine aromatic hydrogen *a*, which gives the largest shift change of the drug, does not show any cross peak with the dimers, suggesting that residue is not included in the cavity, but perhaps located within the shielding cone of the MTX benzene ring or the π-system of the linker. By contrast, the methyl group *c* (but not the methylene *b*) and both the aromatic protons *d* and *e* (the latter showing no shift change upon the complexation) are in spatial proximity to hydrogen H3, located within the β-CD cavity. Atoms *d* and *e* also interact with the macrocycle protons H2, which are localised on the cavity secondary, wider opening rim. Thus, the 2D ROESY NMR data suggest that the binding of MTX with dimers **1** and **2** may not follow the commonly proposed inclusion structure for the binding of this drug to native β-CD. Considering all the NMR results together, an interaction where the aminobenzoic moiety shallowly caps the macrocyclic secondary rim might be feasible. At the same time, the upfield changes induced in the NMR chemical shifts observed for the pteridine aromatic hydrogen suggest that additional interactions between that moiety and some other parts of the complex (whether the MTX benzene or the linker moieties of the dimer) should also be present. Such binding mode is different from that described for native β-CD [18, 19] and is in agreement with a less penetration of the guest in the cavity as suggested by a less favorable enthalpic gain of the complexation.

In summary, the two β-CD dimers presented in this study have demonstrated to be good hosts for the cancer chemotherapeutics agent MTX. According to ITC, both dimers form a complex with two molecules of MTX simultaneously with binding constants which are between 2.4 and 3.5 times higher than that for native β-CD, the dimer having the shortest linker forming the most stable complex. NMR (1D and ROESY) experiments suggest that MTX could adopt U shape conformation partly inserting the aminobenzoic ring but remaining capping the macrocyclic secondary rim of the β-CD unit.

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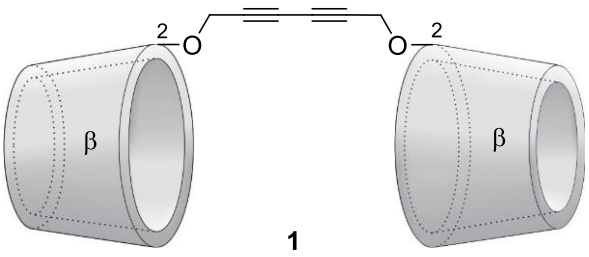
FIGURES



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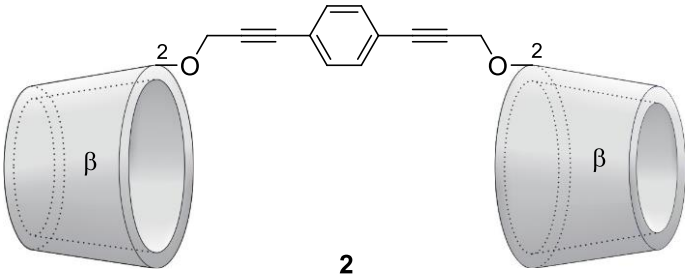

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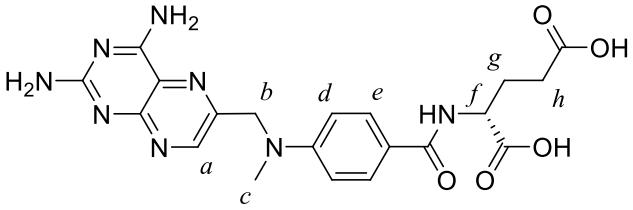


Methotrexate (MTX)

Figure 1

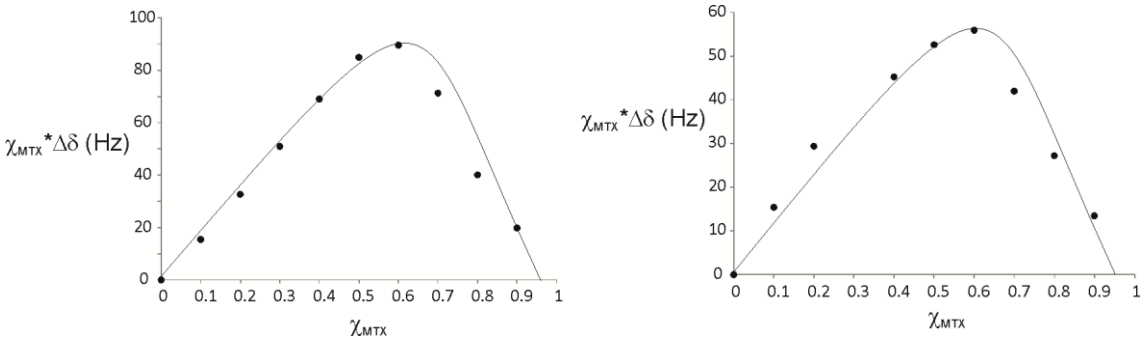


Figure 2

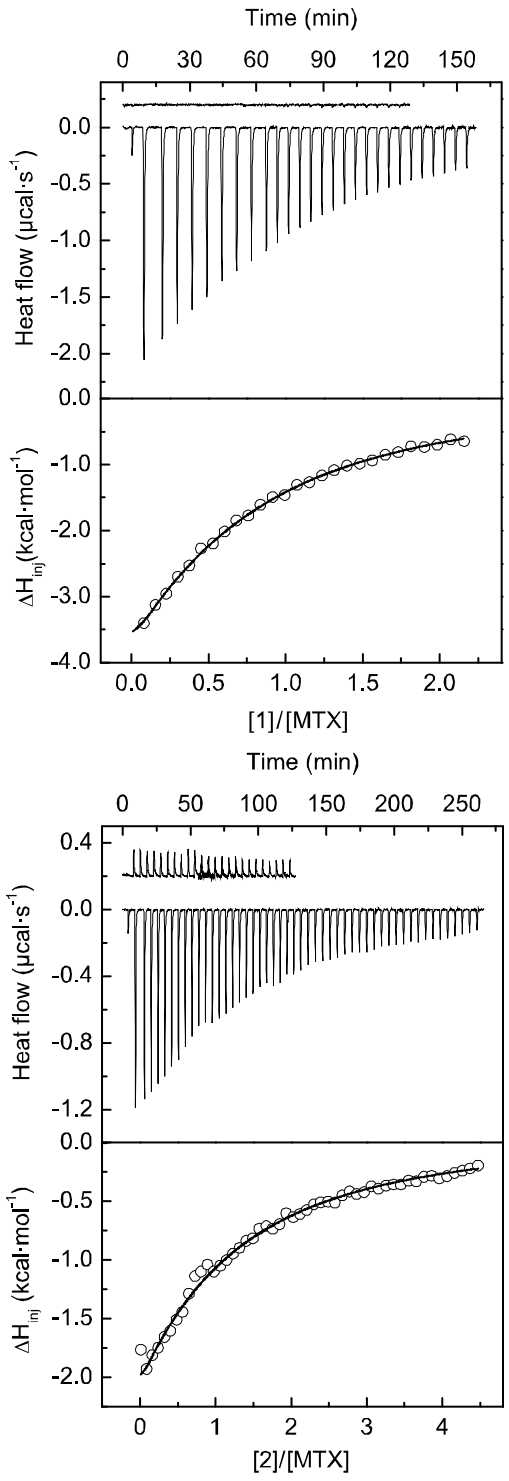
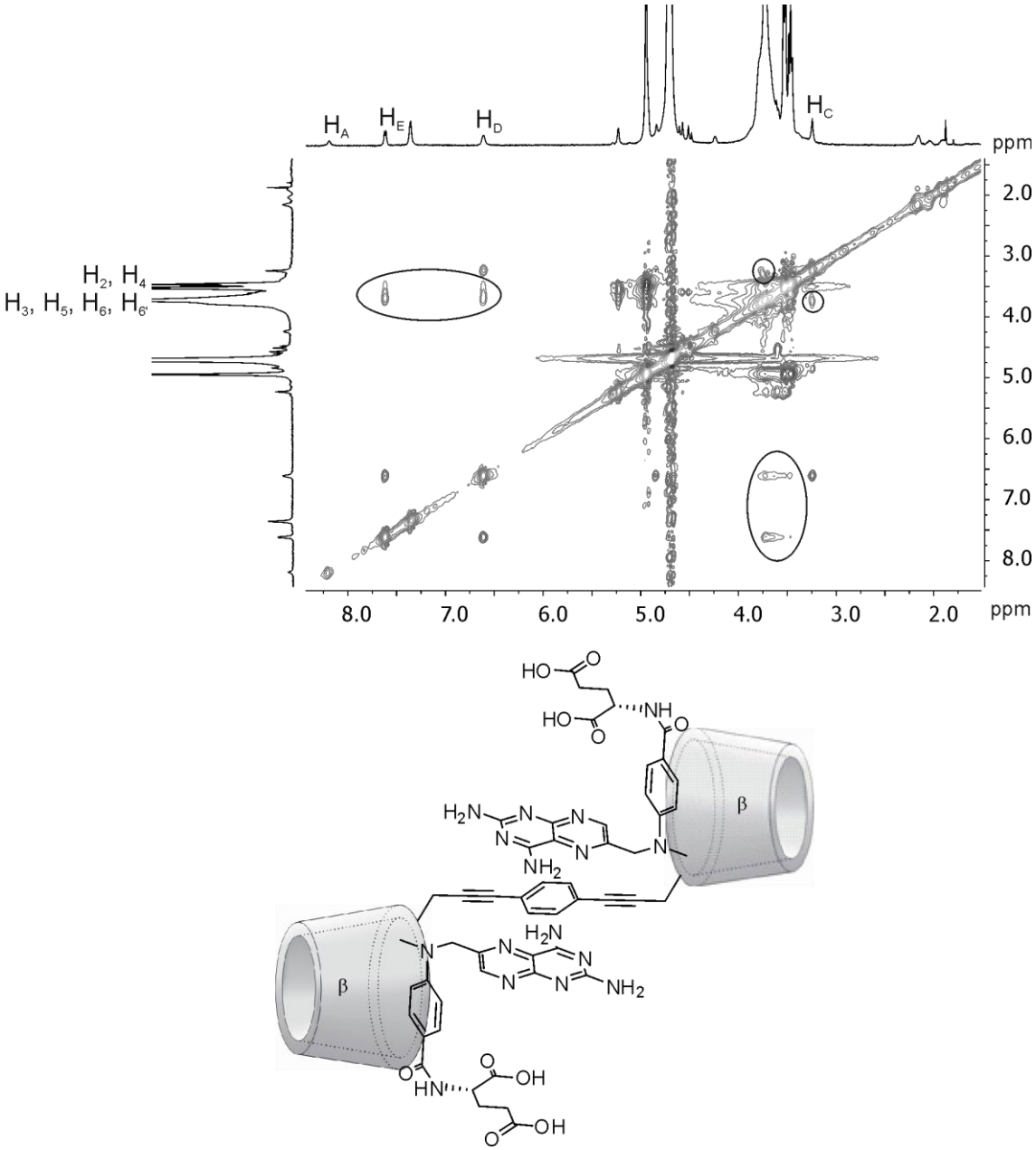

Time (min)
0 30 60 90 120 150
Heat flow (µcal·s-1)
0.0 -0.5 -1.0 -1.5 -2.0
0.0 -1.0 -2.0 -3.0 -4.0
ΔHinj(kcal·mol-1)
0.0 0.5 1.0 1.5 2.0
[1]/[MTX]
Time (min)
0 50 100 150 200 250
Heat flow (µcal·s-1)
0.4 0.0 -0.4 -0.8 -1.2
0.0 -0.5 -1.0 -1.5 -2.0
ΔHinj (kcal·mol-1)
0 1 2 3 4
[2]/[MTX]

Figure 3


H_A
H_E
H_D
H_C
ppm
H_2, H_4
H_3, H_5, H_6, H_6'
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7.0
6.0
5.0
4.0
3.0
2.0
HO
O
NH
H_2N
N
NH_2
β
OH
HN

Figure 4

FIGURE CAPTIONS

Figure 1 – β-CD dimers and methotrexate.

Figure 2 –Graphical plots of $\Delta\delta^*\chi_{MTX}$ obtained from the observed chemical shift displacement of H_a of MTX induced by the increase of the concentration of dimer **1** (left) and dimer **2** (right) versus χ_{MTX}, where χ_{MTX} is the molar fraction of MTX.

Figure 3 – Calorimetric titrations of 200 μM MTX with 10 μL injections of 2.1 mM **1** (top) and 2.0 mM **2** (bottom) in 50 mM sodium phosphate buffer at pH 7.2 and 25 ºC. The top panel shows the raw calorimetric data, denoting the amount of generated heat (negative exothermic peaks) following each injection of the dimer. The upper panel corresponds to the dimer dilution experiment. The area under each peak represents the amount of heat released upon binding of MTX to the dimers. Note that, as the titration progresses, the area under the peaks gradually becomes smaller due to an increased complexation of the drug by the hosts. This area was integrated and plotted against the molar ratio dimer:MTX. The smooth solid lines represent the best fit of the experimental data to the model of *two* equal and independent binding sites for the dimer.

Figure 4 –Top: 2D-ROESY spectrum (500 MHz, D_2O, 25 ºC, 200 ms of mixing time) for equimolar mixtures (2 mM) of dimer **2** and MTX. Cross peaks involving protons c, d and e of MTX are highlighted in circle. Bottom: Proposal structure of the complex.

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