Encapsulation of Chemotherapeutic Drug Melphalan in Cucurbit[7]uril: Effects on Its Alkylating Activity, Hydrolysis, and Cytotoxicity

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Supporting Information

ABSTRACT: The formation of inclusion complexes between drugs and macrocycles has proven to be an effective strategy to increase solubilization and stabilization of the drug, while in several cases improving their biological activity. In this context, we explored the formation of an inclusion complex between chemotherapeutic drug Melphalan (Mel) and cucurbit[7]uril (CB[7]), and studied its effect on Mel alkylating activity, hydrolysis, and cytotoxicity. The formation of the inclusion complex (Mel@CB[7]) was proven by absorption and fluorescence spectroscopy, NMR, docking studies, and molecular dynamics simulations. The binding constant for Mel and CB[7] was fairly high at pH 1 \((1.7 \pm 0.7) \times 10^6 \text{ M}^{-1}\) whereas no binding was observed at neutral pH. The Mel@CB[7] complex showed a slightly decreased alkylating activity, whereas the cytotoxicity on the HL-60 cell line was maintained. The formation of the complex did not protect Mel from hydrolysis, and this result is discussed based on the simulated structure for the complex.

1. INTRODUCTION

Melphalan (Mel, Scheme 1) is an antineoplastic drug, which is indicated for the treatment of multiple myeloma and other types of cancer.\(^{1,2}\) Being a drug of the family of nitrogen mustards, its antitumoral effect is related to the alkylation of DNA.\(^{3-5}\) Mel is practically insoluble in water at neutral pH, and it rapidly hydrolyzes in biological media, factors that have an impact on its usability. In this context, a supramolecular approach to improving the overall drug performance, such as the use of cyclodextrins,\(^{6}\) is interesting to explore due to its demonstrated success in pharmaceutical formulations. For example, Evomela is an injectable formulation of Mel that uses a modified \(\beta\)-cyclodextrin (Captisol) to improve its solubility and stability by the formation of an inclusion complex.\(^{5}\)

Cyclodextrins show in general low binding affinities, which is their main downside as a supramolecular solubilizing agent.\(^{6}\) More recently, the family of cucurbit[n]urils macrocycles (CB[n]s, Scheme 1) has emerged as promising candidates for drug delivery applications.\(^{9,10,11}\) Some characteristics that make CB[n]s notable are their low toxicities, solubilizing properties, high thermal stability, high binding affinities, and good solubility in biological fluids.\(^{6,9,10,12-16}\)

Previously, a report by Isaacs and collaborators showed that Mel and other alkylating agents can be efficiently solubilized by acyclic CB[n]s,\(^{17}\) which are very versatile in the binding of several molecules of biomedical relevance. Nevertheless, there is no information regarding how complexation within these macrocycles could affect their stability, alkylating activity, and cytotoxicity. Complexation of drugs by CB[n]s has stimulated much interest over the past decade,\(^{5,10,12,16,19}\) and there are several interesting reports of how complexation affects bioactivity and/or biodistribution.\(^{11,14-16,20}\) Therefore, we were interested in investigating the formation of a supramolecular complex between Mel and CB[n]s and if this process would stabilize it, as it has been shown for several other drugs,\(^{21}\) while maintaining its alkylating activity and cytotoxic effects. For this study, we chose cucurbit[7]uril...
with the encapsulation of the drug. It must be noted that these changes were observed at pH 1 (0.1 M HCl) and no such changes were observed at pH 7 (see Figure S1 in the Supporting Information). These results indicate that the protonation state of Mel is essential for binding. Mel possesses three pKₐ for 2-chloroethylamino, α-carboxylic, and α-amino groups of 1.42, 2.75, and 9.17, respectively. Because binding was observed only at pH 1, the protonation of the 2-chloroethylamino and α-amino groups seems to be essential for a strong binding to the macrocycle. This observation is consistent with previous reports about the gas−dipole interactions between the guest and CB[n] portals, which are lined with carbonyl groups. It is important to note that this interaction with the portals could lead to pKₐ shifts when the drug is encapsulated inside CB[n]s, which was not evaluated in this work. The fact that there is no appreciable binding at pH 7 (zwitterionic species) could be related to a destabilization of the complex because of repulsive interactions with the negative charge density of the carbonyl groups at the portals of CB[7].

Fluorescence emission spectra also showed a noticeable decrease in intensity as the concentration of CB[7] in the sample increased (Figure 1, inset), which further supports that the formation of an inclusion complex with CB[7] is taking place.

The binding constant for the Mel@CB[7] complex obtained from the fluorescence titrations was \((K_{11} = \frac{slope}{S_0(1 − slope)}\) (1) (Figure 2). The value for the binding constant with CB[7] is fairly high and falls within the range reported for several benzimidazol-derived drugs. This binding constant \((K_{11})\) can be related to the solubilizing capacity of the macrocycle by a phase-solubility diagram \((\text{drug} \text{ vs macrocycle})\) assuming a 1:1 binding, as depicted by eq 1. \(S_0\) refers to the intrinsic solubility of the drug, whereas the slope is obtained from the linear fit of the data.

The solubility of Mel hydrochloride is reported to be 3.11 mg mL⁻¹, thus, considering the \(K_{11}\) obtained for CB[7] in this work, the simulated slope would be unity. This means that CB[7] is a very good solubilizing agent for Mel and it is close to the slopes reported for acyclic CB[n]s (0.81−1.2). In comparison, \((\text{SBE})_{n}\)−β-CD (Captisol), which possesses a binding constant of 142.7 M⁻¹ with Mel (from phase-solubility diagram), has a simulated slope of 0.6.

The inclusion of Mel inside the cavity of CB[7] is further supported by the ¹H NMR spectra (Figure 3), which show strong downfield shifts for Mel aromatic hydrogens, whereas the signals for the α-carbon hydrogen and the 2-chloroethyl protons are not changed (see Figures S2 and S3 in the Supporting Information for the assignment). Residual peaks from the solvent at around 3.2 ppm prevent the observation of the hydrogens of the methylene group; however, it is clear from the spectra that the aromatic ring is placed inside the cavity, whereas the rest of the molecule sits outside of the macrocycle.

This inclusion mode is consistent with molecular docking studies, which show the preferential inclusion of the aromatic portion of the molecule inside CB[7], with the 2-chloroethylamino group and the α-carbon groups sticking out through the portals (Figure 4). The complex shows favorable binding energy (−5.64 kcal mol⁻¹), which is in line with the high binding constant determined experimentally. It must be emphasized that the value of the binding energy is relative and cannot be correlated directly with the value of the binding constant. However, it is a good indication that the complex is fairly stable. The simulations show that the complex is stabilized by three hydrogen bonds with the carbonyl groups (Figure 4), although hydrophobic interactions and cation−dipole interactions between the protonated amino groups and the portals are certainly contributing to the binding. The docking studies show that the formation of the complex is less favorable at pH 7 than at pH 1 (see Figure S4 in the Supporting Information), but weak interactions in solution cannot be completely ruled out.

Because solvation can largely affect the formation of the complex and its conformation over time, molecular dynamics (MD) simulations were performed for 10 ns to assess the stability of the complex (Figure 5). The results show that Mel remains inside the cavity of CB[7] for the duration of the
simulation and that at least one hydrogen bond is retained throughout the entire time, with sporadic additional hydrogen bonds being formed. It is interesting to note that the conformation of the complex changes very little during the simulation and that the 2-chloroethylamino group is always positioned at the rim of CB[7]. This conformation would maintain the alkylating activity of Mel because this group is responsible for the alkylation of DNA bases.3

Alkylating activity is essential for Mel chemotherapeutic effect. Therefore, we tested if the complexation of Mel by CB[7] altered such property by following the generation of a colored product at 545 nm after reaction with 4-(4-nitrobenzyl)pyridine (NBP), which is based on the alkylation of the pyridine moiety of the reagent giving a chromophore product at basic pH.33 The results in Figure 6 show that there is a slight decrease in the relative alkylating activity of Mel when included inside the cavity of CB[7]; however, this effect is minor. These results agree with the binding mode discussed above from NMR, docking studies, and MD simulations, where the 2-chloroethylamino group is located on the outside of the macrocycle, protruding through one of the portals. Therefore, alkylating activity is roughly maintained.

The main problem that Mel has as a drug is its instability in aqueous media due to rapid hydrolysis at neutral pH.24,34,35 Evomela is reported to be stable for 1 h after reconstitution at room temperature.8,36 To assess if complexation within CB[7] protected the drug from hydrolysis, we performed a series of experiments where Mel was incubated at physiological temperature, and subsequently hydrolysis products were quantified by high-performance liquid chromatography (HPLC) based on previous reports from the literature.24,35,37 In the case of the CB[7] complex, before the analysis, Mel was released from CB[7] using adamantylamine (ADA) as a competitor due to its high binding constant (1.2 × 10^{10} M^{-1}).38 Release from the macrocycle is necessary for quantification because the extinction coefficient of the complex is lower than that for free Mel as shown in Figure 1. In these experiments, it is also important to consider that Mel will hydrolyze somewhat during sample preparation and during the HPLC run. Therefore, control experiments were performed for

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**Figure 3.** 1H NMR spectra of Mel, CB[7], and Mel@CB[7] complex (1 equiv of CB[7]) in DCl/D2O (1:20).

**Figure 4.** Simulated structure for the Mel@CB[7] complex from docking studies. Color code for Mel atoms: C, cyan; O, red; N, blue; and H, gray. The structure of Mel corresponds to the fully protonated form at pH 1. Hydrogen bonds are indicated in the figure.
nonincubated samples and the small amounts of hydrolyzed products detected were subtracted from the incubated samples (see Figures S5 and S6 in the Supporting Information). Loss of the chlorine atoms leads to their replacement by hydroxyl groups. Therefore, there are two main hydrolysis products, the monohydroxy (MOH) and the dihydroxy (DOH) derivatives of Mel, though other products have been identified by mass spectrometry (MS). The chromatogram in Figure 7 corresponds to a representative experiment, which shows that Mel incubation produces a single hydrolysis product with a retention time of 3.6 min. This product is the same for Mel or the Mel@CB[7] complex and was attributed to the MOH derivative based on mass spectral analysis (see Figures S7 and S8 in the Supporting Information). Note that the DOH derivative can be detected by MS but at a relatively low abundance, indicating that it is a minor product. Comparisons of the integrated areas of the chromatogram peaks for Mel and MOH yielded a hydrolysis ratio of 15.7 ± 2.5% for Mel and 11.8 ± 2.7% for Mel@CB[7]. These two values are the same within error, indicating that CB[7] complexation does not protect Mel from hydrolysis. It is noteworthy that Mel hydrolysis is strongly pH-dependent and higher rates of hydrolysis are observed at neutral or basic pH. This behavior is consistent with the proposed mechanism of hydrolysis, involving a nucleophilic attack of the unprotonated amino group toward one of the chlorine-bearing carbon atoms. The results obtained herein are in line with previous discussions about the binding mode within CB[7]; thus, the exposure of the 2-chloroethylamino group to the solvent does not change its reactivity toward hydrolysis. This is different than previously reported slowing of the rate of hydrolysis by Captisol because in that case the 2-chloroethylamino group is embedded within the hydrophobic cavity of the macrocycle, and this is clearly a limitation for the CB[7] complex. Although hydrolysis is not prevented, alkylating activity was almost unmodified, which is still a good antecedent for its therapeutic action.
Finally, the cytotoxicity of Mel and Mel@CB[7] complex was assessed in human leukemia cell line (HL-60) as a model for its therapeutic action. The cytotoxicity assays shown in Figure 8 revealed that there is no significant difference between the efficacies of Mel and its CB[7] complex in inducing cancer cell death. Samples in the presence of only CB[7] showed no cytotoxicity, as reported for several cell lines.\textsuperscript{15,18} It is important to emphasize that even when the alkylating activity was slightly decreased and its hydrolysis was not prevented, the Mel@CB[7] complex performs as well as the drug by itself, but CB[7] encapsulation offers enhanced solubility. One can speculate that because the binding of Mel to CB[7] was observed only at acidic pH and not at pH 7, encapsulation could help improve drug delivery for an oral formulation of Mel, as the drug would be released after passing through the stomach.

3. CONCLUSIONS
Mel was effectively encapsulated inside CB[7], which was demonstrated by changes in the absorption and fluorescence spectra, NMR, docking studies, and MD simulations. The binding mode corresponded to the inclusion of the aromatic ring inside the cavity, whereas the \( \alpha \)-amino, \( \alpha \)-carboxylic, and 2-chloroethylamino groups protruded through the portals. Stabilization of the complex was due to a combination of hydrogen bonding, hydrophobic interactions, and cation–dipole interactions. The protonation state of Mel was fundamental for the binding, being observed experimentally only for the fully protonated form at pH 1. It must be emphasized that Mel hydrochloride is viable for an injectable formulation (Alkeran). Encapsulation of Mel inside CB[7] could hold promise for oral intake, where the complex might be stabilized. The formation of the Mel@CB[7] inclusion complex showed a slight decrease for the alkylating activity, but the cytotoxicity was not affected, as shown for the HL-60 cell line. On the other hand, hydrolysis was not prevented as shown for the encapsulation of Mel in the \( \beta \)-cyclodextrin derivative (Evomela), and this is proposed to be due to the binding mode within the macrocycle. In CB[7], the aromatic ring is inside the cavity of the macrocycle with the 2-chloroethylamino group placed outside of the cavity, whereas for the \( \beta \)-cyclodextrin derivative, this group remains inside the cavity, slowing down hydrolysis.

4. EXPERIMENTAL SECTION

4.1. Chemicals. Melphanal (Mel), cucurbit[7]uril (CB[7]), adamantylamine (ADA), and bis(cyclopentadienyl) cobalt(III) hexafluorophosphate (Cob\(^+\)) were obtained from Sigma and used without further purification. Hydrochloric acid (37%), formic acid (100%), acetic acid (100%), sodium acetate, and sodium phosphate salts were obtained from Merck. 4-(4-Nitrobenzyl)pyridine (NBP) was obtained from Chem-Impex International. Ultrapure water from a Milli-Q water purification system was used to prepare all of the solutions (resistivity of 18.2 M\( \Omega \) cm).

4.2. Sample Preparation. Stock solutions of Mel (1 mg mL\(^{-1}\)) were prepared by dissolving the drug in ethanol/HCl solution (99:1). Diluted samples were prepared in 0.1 M HCl (pH = 1) or 10 mM phosphate buffer, pH 7 (pH meter Hanna HI2221). Final concentrations were determined by their UV–vis absorption spectra using a molar extinction coefficient of (4.9 ± 0.2) \( \times \) 10\(^5\) M\(^{-1}\) cm\(^{-1}\) at 260 nm in 0.1 M HCl, which was determined in this work.

Stock solutions of CB[7] were prepared in water (\( \approx \)1 mM) and titrated against a known concentration of Cob\(^+\) by UV–vis spectroscopy according to the method reported in the literature.\textsuperscript{40} ADA stock solutions (10 mM) were prepared in water.

4.3. Absorption and Fluorescence Measurements. The association of Mel (16 \( \mu \)M) to CB[7] (0–50 \( \mu \)M) was measured by absorption and fluorescence spectroscopy. Adsorption was measured on a HP8453 spectrophotometer using 1 cm pathlength cuvettes. Fluorescence emission spectra were obtained by exciting the samples at 260 nm (5 nm bandwidth) using a LS55 PerkinElmer fluorimeter. The temperature was kept at 25 \( ^\circ \)C using a waterbath. Binding isotherms built from the fluorescence data were adjusted using numerical analysis as reported previously.\textsuperscript{41,42}

4.4. NMR Measurements. Mel (2.5 mg) was dissolved in 500 \( \mu \)L of DCl/D\(_2\)O (1:20) with the aid of sonication in the absence or presence of 1 equiv of CB[7]. The NMR spectra were obtained using a Bruker Avance III HD instrument working at 400 MHz.

4.5. Structure Optimization and Molecular Docking. Mel in different protonation states and CB[7] were constructed using Gaussian 03\textsuperscript{41} and optimized using the B3LYP method and 6-31G** base set.\textsuperscript{4} The partial charges of the compounds were corrected using ESP methodology. Topology and parameters for all structures were obtained using the SwissParam server.\textsuperscript{42}

Molecular dockings of Mel inside CB[7] were carried out using AutoDock 4.0 suite software.\textsuperscript{43} The grid maps were calculated using the autogrid4 subprogram and were located in the center of CB[7]. The volumes for the grid maps were 70 \( \times \) 70 \( \times \) 70 points with a grid-point spacing of 0.375 \( \AA \). The autotors option was used to define the rotating bonds in the ligand. The following parameters were employed in the Lamarckian genetic algorithm dockings: initial population of 1500 random individuals with a population size of 150 individuals; 2.5 \( \times \) 10\(^6\) energy evaluations, a maximum number of 27 000 generations, a mutation rate of 0.02, and a cross-over rate of 0.80. The docked complexes were built picking the lowest docked-energy binding positions with a relatively high number of conformations.

4.6. Molecular Dynamics Simulations. Mel@CB[7] complexes in different protonation states were solvated by a
and incubated for 24 h. After the treatment, cell viability was measured on an Hitachi Elite LaChrom HPLC system using an isocratic mobile phase of acetonitrile and 0.1% formic acid in water (32:68), RP-18 endcapped column (5 μm, 250 x 4 mm², Merck), 1 mL min⁻¹ flow, and 260 nm for the detection wavelength (L-2455 diode array detector). Control experiments with nonincubated samples were performed to take into account the hydrolysis of Mel during the analysis (preparation and HPLC column run), and the small amounts of hydrolyzed products detected were subtracted from the incubated samples.

4.8. Hydrolysis. The measurement of the hydrolysis degree of Mel was adapted from previously reported methods. Mel (100 μM) in 0.1 M HCl was incubated at 37 °C for 3 h in the absence or presence of 1 equiv of CB[7]⁴. After incubation, ADA (200 μM) was added to the samples containing CB[7] to release Mel from the macrocycle. Hydrolysis products were measured on an Hitachi Elite LaChrom HPLC system using an isocratic mobile phase of acetonitrile and 0.1% formic acid in water (32:68), RP-18 endcapped column (5 μm, 250 x 4 mm², Merck), 1 mL min⁻¹ flow, and 260 nm for the detection wavelength (L-2455 diode array detector). Control experiments with nonincubated samples were performed to take into account the hydrolysis of Mel during the analysis (preparation and HPLC column run), and the small amounts of hydrolyzed products detected were subtracted from the incubated samples.

4.9. Cytotoxicity Assay. HL-60 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were seeded in 96-well plates at a density of 3 x 10⁴ cells/well. Mel or Mel@CB[7] were added at a final concentration of 200 μM and incubated for 24 h. After the treatment, cell viability was determined by the MTT assay (10% v/v of 5 mg mL⁻¹ MTT solution was added to each well and incubated for 2 h). Then, the formazan crystals formed by the reaction between metabolically active cells and MTT were dissolved by adding a solution of 10% sodium dodecyl sulfate in 0.01 M HCl into each well. The plate was left overnight in an incubator to finally read its absorbance at 570 nm using a Biotek Synergy HT microplate reader.

**ACKNOWLEDGMENTS**

We wish to thank CONICYT for the financial support through their FONDECYT research program (grant no. 1160443). Also, we would like to thank FONDECYT grant no. 1161375 that supported the computational calculations. J.R.-D. thanks CONICYT-CHIA/Doctorado Nacional/2015-21150894. We thank Prof. Verónica Arancibia for her kind access to their HPLC instrumentation. We also thank Pablo Barrias and CONICYT FONDEQUIP/UHPLC MS/MS EQM 120065 for mass spectrometry analysis.

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**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01335.

Absorption spectra, NMR spectra, docking studies at pH 7, HPLC chromatograms, and mass spectra (PDF)

**ASSOCIATED CONTENT**

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