Encapsulation of Mitoxantrone within Cucurbit[8]uril Decreases Toxicity and Enhances Survival in a Mouse Model of Cancer

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S Supporting Information

ABSTRACT: Mitoxantrone was efficiently encapsulated within cucurbit[8]uril in a 2:1 complex where the two mitoxantrone molecules were symmetrically located through both portals of a cucurbit[8]uril cage. The novel complex facilitates increased mitoxantrone uptake in mouse breast cancer cells and decreases the toxicity of the drug in healthy mice. In an orthotopic mouse model of metastatic breast cancer the complex still maintains *in vivo* anticancer activity compared to the free drug, yet provides a statistically significant increase in the survival of these mice compared to conventional mitoxantrone treatment. This new low toxicity formulation offers the possibility to increase mitoxantrone dose and thus maximize efficacy while managing the dose limiting side effects.

KEYWORDS: Cucurbit[8]uril, mitoxantrone, encapsulation, preclinical *in vivo* activity, decreased toxicity, increased survival

Mitoxantrone (see Figure 1) is an anthracenedione derivative that was developed to improve the toxicity

Figure 1. Structure and atom numbering of mitoxantrone.

profile of the anthracycline class of drugs.^{1,2} It is clinically used for the treatment of breast and prostate cancers, lymphomas, and leukemia.^{3–6} Although mitoxantrone is less cardiotoxic than the parent anthracycline drugs, its clinical use is still limited by toxic side-effects, particularly myelosuppression (impaired production of blood cells).^{5,6} Consequently, there

is considerable interest in the development of drug delivery technologies that could reduce the toxicity profile of mitoxantrone. One such technology is encapsulating the drug inside a nontoxic host molecule, for example, macrocyclic cage-like compounds called cucurbit[*n*]uril (Q[*n*]).^{7–9}

Q[*n*] are formed by *n* glycoluril units linked by methylene bridges.^{7–9} These macrocycles have a hydrophobic cavity and hydrophilic portals rimmed by carbonyl groups. Through these portals, a guest molecule can reach the inner hydrophobic cavity. The structural characteristics of Q[*n*] vary, with portal widths from 2.4–11 Å and cavity widths from 4.4–12.6 Å, with an approximate height of 9.1 Å for *n* = 5–10.^{7–9} In this study we report the structure of a mitoxantrone–Q[8] complex in which two molecules of mitoxantrone are symmetrically

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embedded into the Q[8] cavity. Although it has been demonstrated that encapsulation in Q[n] can lower the dose-limiting toxicity of anticancer drugs,^{10–12} and there is one example of an encapsulated drug retaining activity *in vivo*,¹³ evidence that these drugs can enhance survival in an animal model is yet to be reported. The results of this study show that mitoxantrone encapsulation in Q[8] not only rescues mice from acute mitoxantrone-induced toxicity but also provides a statistically significant increase in the survivability of mice bearing a metastatic breast tumor.

Stock solutions of mitoxantrone (Sigma-Aldrich, $\geq 97\%$ purity) were prepared in 99.9% D₂O and then diluted in water, citric acid buffer (pH 2.1), or tris buffer (pH 7.1) to record the NMR spectra. For mitoxantrone, both aliphatic amines will be protonated at pH 7.1, but the anilinic nitrogens will be neutral; however, at pH 2.1, all nitrogens will be protonated. Two pairs of pK_a values have been reported for the four nitrogen groups of mitoxantrone, one centered at 6.0 (anilinium nitrogens) and the other at 8.1 (ammonium nitrogens).¹⁴ Q[7] and Q[8] (synthesized as previously described¹⁵) stock solutions were prepared in the same media and titrations were carried out by adding small aliquots of Q[7] or Q[8] solutions separately into the NMR tube containing mitoxantrone.

The ¹H NMR resonances from the aromatic and aliphatic protons of mitoxantrone were assigned using standard one- and two-dimensional experiments. The encapsulation of drugs into Q[n] can be monitored by NMR spectroscopy. The mode of encapsulation can be understood by observing the chemical shift changes of the guest resonances upon addition of Q[n].^{16,17} It has been established that resonances from guest protons located inside the Q[n] cavity shift upfield. The protons that are positioned near the center of the host Q[n] cavity are expected to exhibit the largest upfield shifts. In comparison, small downfield shifts are observed for resonances from guest protons located close to but outside of the Q[n] portal.^{16,17} The resonances from the mitoxantrone aromatic protons shifted downfield (0.02 ppm for the H2,3 protons and 0.11 ppm for the H6,7 protons) upon Q[7] addition to the mitoxantrone solution (see SI Figure S1), indicating that the aromatic ring system was not encapsulated. Consistent with this conclusion were the observed upfield shifts from the aliphatic Hc (0.15 ppm) and Hd (0.24 ppm) resonances upon Q[7] addition, suggesting that only the aliphatic side-chains can be encapsulated in Q[7].

Solutions of approximately 4:1, 2:1, and 1:1 ratios of mitoxantrone to Q[8] were obtained by adding small aliquots of Q[8] into the solution of mitoxantrone in water, citric acid buffer (pH 2.1), or tris buffer (pH 7.1). In all cases a significant upfield shift (1.18 ppm) was observed for the H2,3 resonance and a downfield shift (0.17 ppm) was noted for the H6,7 resonance of mitoxantrone (see Figure 2). This indicates the H2,3 protons are located deep within the Q[8] cavity, but with the H6,7 protons positioned outside of the Q[8] portal. At a ratio $R = 4$ (mitoxantrone to Q[8]), two sets of resonances were observed, one due to the free mitoxantrone and the other to the Q[8]-bound mitoxantrone. This indicates mitoxantrone encapsulation in Q[8] is in slow exchange (on the NMR time scale). The resonances from the Q[8] protons shifted upfield (0.10 to 0.19 ppm) upon encapsulation of mitoxantrone. The chemical shift changes for the resonances from both the free drug and Q[8]-bound drug are given in SI Table 1. The dominant 2:1 binding mode (mitoxantrone/Q[8]) was confirmed at $R = 1$ by the observation of approximately

Figure 2. ¹H NMR spectrum of free mitoxantrone (A) and with added Q[8] at ratios R (mitoxantrone to Q[8]) = 4.0 (B), 2.0 (C), and 1.0 (D), in pH 2.1 citric acid buffer in D₂O. Resonances from free and bound mitoxantrone and Q[8] are indicated.

equally intense resonances from protons from both the free Q[8] and mitoxantrone-bound Q[8]. Analysis of the quenching of the mitoxantrone fluorescence as a function of added Q[8] gave a mitoxantrone–Q[8] binding constant of $5.9 \times 10^{10} \text{ M}^{-2}$, and confirmed the 2:1 binding stoichiometry (see SI Figures S2 and S3). The 2:1 binding stoichiometry was also confirmed using a Job's plot (SI Figure S4). As identical (within experimental error) changes in chemical shift were observed for the mitoxantrone H6,7 and H2,3 protons upon encapsulation in Q[8] in either citric acid or tris buffer, it is concluded that all nitrogens are protonated at pH 7.

Molecular models were constructed using the HyperChem program¹⁸ to understand the binding mode of mitoxantrone in Q[8]. The molecular model shown in Figure 3 is consistent

Figure 3. Molecular model of two mitoxantrones symmetrically encapsulated through both portals of Q[8].

with the changes in chemical shifts observed in the NMR spectra upon addition of Q[8]. The observation of only one resonance for each of the Q[8] protons at $R = 2$ indicates that the two mitoxantrone molecules are symmetrically encapsulated with respect to Q[8]. The H2,3 protons of mitoxantrone are located deep in the cavity, while the H6,7 protons are located outside of the Q[8] portal, and the carbonyl rimmed portals of the Q[8] would be stabilized by hydrogen bonding and electrostatic attractions with the positively charged drug. The mitoxantrone–Q[8] inclusion complex is also stabilized by the π – π stacking interactions between the two encapsulated drugs.

The mouse breast cancer cell line 4T1.2 was employed to characterize the effects of mitoxantrone and mitoxantrone–

Q[8] on cancer cells. Cells were seeded at 8000 cells/well in 4-well chambered cover glasses (ThermoScientific NuncGermany) and allowed to attach overnight under incubation at 37 °C in 5% CO₂. Live imaging was then performed on a Zeiss LSM 780 laser scanning confocal microscope with a 20× objective (Plan-Apocromat, 0.8 numerical aperture) and scanning speed of 7 (scan time of 3.87 s). Drug was added and wells were imaged simultaneously over a 4 h period. Cells were imaged in wide-field at a depth focus of 1.72 μm, and excitation/detection parameters were 568 nm/568–712 nm. Zen 2.3 Blue edition imaging software (Carl Zeiss, Germany) was used for image analysis. The comparative cell uptake of mitoxantrone and mitoxantrone–Q[8] is shown in Figure 4

Figure 4. Top panel: Cellular accumulation and distribution of mitoxantrone and mitoxantrone–Q[8] after 4 h treatment. 4T1.2 cells were exposed to 5 μM of each treatment (with respect to mitoxantrone; Mito–Q[8] also contained 2.5 μM Q[8]) and analyzed by confocal microscopy. Scale bar, 50 μm. Bottom panel: The fluorescence intensity/cell was analyzed at various time points for each treatment during live cell imaging. The fluorescence intensity was quantitated at three separate slide positions for each treatment. Error bars are SEM.

where the distribution of both drug formulations appears similar at the 4 h time point, primarily indicative of extra-nuclear localization. Previous investigations have revealed that mitoxantrone distributes preferentially to lysosomes of cells,¹⁹ and the use of a lysotracker stain to label lysosomes reveals that mitoxantrone–Q[8] also preferentially distributes to this organellar location (SI Figure S5).

Significantly, the confocal microscopy results suggest the mitoxantrone–Q[8] complex is more readily taken up by the cells than free mitoxantrone. As it is possible that the mitoxantrone fluorescence increases upon encapsulation in Q[8], the effect of Q[8] encapsulation was examined at both pH 2.1 and 7.1. In both cases the fluorescence was partially quenched, by approximately 25% at pH 2.1 and 55% at pH 7.1 (SI Figure S2). Consequently, it is concluded that either the mitoxantrone–Q[8] complex more readily accumulates in the cancer cells or that the Q[8] facilitates the uptake of

mitoxantrone without being directly taken up itself. The cellular uptake of the mitoxantrone–Q[8] complex is consistent with the results of a very recent study where a fluorescent dye–Q[7] covalent conjugate was shown to be taken up by live HT22 cells, whereas the free dye was not cell permeable.²⁰ Furthermore, an enhancement of *in vitro* and *in vivo* uptake of coumarin-6 in the presence of Q[7] has also been reported.²¹

Since the fluorescence intensity of uptake indicates higher cellular accumulation of the mitoxantrone–Q[8] complex compared to free mitoxantrone, a MTT growth inhibition assay was employed to monitor the biological outcome of drug treatment (SI Figure S6). The growth inhibitory effects of anthracenediones are closely linked to nuclear accumulation of the drug and subsequent topoisomerase-II mediated DNA damage.²² The similar potency observed for both mitoxantrone and mitoxantrone–Q[8] implies that both treatments ultimately result in a similar degree of topoisomerase-mediated DNA damage.

Regardless of observations with respect to isolated cells, the most rigorous preclinical tests of drug anticancer effects require animal models. The 4T1.2 cell line is an aggressive orthotopic breast cancer model that spontaneously metastasizes to lung and bone, recapitulating the human disease.²³ In order to prepare for testing of mitoxantrone formulations in this model, nontumor bearing female Balb/c mice (9 weeks old) were treated with drugs at 2 mg/kg (with respect to the mitoxantrone component; Q[8] contributed an extra 3 mg/kg where mitoxantrone–Q[8] treatment was employed) over a two week period and body weight was monitored daily, see Figure 5.

Figure 5. Body weight change in response to drug treatment. Groups of four mice were subjected to the indicated treatment twice weekly for 2 weeks (drug injection days; 0, 3, 7, 10). All treatments were administered intravenously by tail vein injection. The vehicle consisted of 0.9% saline, and drug treatments were 2 mg/kg with respect to mitoxantrone and 3 mg/kg with respect to the Q[8] component. Data represents the mean change in body weight from baseline. Error bars are SEM.

Weight loss provides a reliable indicator of drug-mediated toxicity. Mitoxantrone treatment resulted in gradual weight decline over the study period reaching a maximum of ~15% by 2 weeks. In contrast the group of mice treated with mitoxantrone–Q[8] maintained their body weight throughout the treatment period, indicative of protection from drug-mediated toxicity. In order to test whether cucurbituril complexes are able to biodistribute to tumor tissue and exert a therapeutic effect, tumor-bearing mice were treated with the two different drug formulations. All animal experiments were performed in compliance with relevant laws and institutional guidelines and were approved by the La Trobe University

Animal Ethics Committee. Mice were injected in the fourth mammary fat pad with 1×10^5 4T1.2 cells, and once tumors became palpable on day 8, drug treatment was initiated. Drug treatments were given twice weekly for 2 weeks and tumors measured with digital calipers. Animals were monitored and culled when they reached an ethical end point of weight loss >20% baseline, primary tumor >1500 mm³, or signs of metastatic distress. Body weight loss followed the trend shown for the treatments in Figure 5 (data not shown) indicating that the toxicity benefits of mitoxantrone–Q[8] are also applicable to tumor-bearing mice. Treatment with both mitoxantrone and the mitoxantrone–Q[8] complex induced a partial tumor growth inhibition that was not significantly different between the two treatment groups (Figure 6).

Figure 6. Change in primary mouse tumor volume in response to drug treatment. The vehicle consisted of 0.9% saline, and drug treatments were 2 mg/kg with respect to mitoxantrone and 3 mg/kg with respect to the Q[8] component. Treatments consisted of saline vehicle ($n = 10$), mitoxantrone ($n = 11$) and mitoxantrone–Q[8] ($n = 10$) given twice weekly for 2 weeks as indicated by orange arrows. In the mitoxantrone group, two mice were culled on day 19, and one mouse was culled on day 21 due to toxicity issues. *One way ANOVA p value < 0.05 for mitoxantrone and mitoxantrone–Q[8] groups compared to vehicle. Error bars are SEM.

To determine how drug treatment translates to the overall survivability of mice, animals were monitored up to 33 days post tumor inoculation. In this time period the 4T1.2 primary tumor undergoes metastasis primarily to lung and bone. Strikingly, the mice treated with the mitoxantrone–Q[8] complex all survived until day 26, by which point the untreated and mitoxantrone-treated groups had survival rates of 30% and 60%, respectively (Figure 7). This represents a statistically

Figure 7. Kaplan–Meier survival of tumor-bearing mice in response to drug treatment. Drug treatments were 2 mg/kg with respect to mitoxantrone and 3 mg/kg with respect to Q[8]. Mice were culled when they reached an ethical end point of weight loss >20% baseline, primary tumor >1500 mm³, or signs of metastatic distress. Log-rank (Mantel–Cox) Test; vehicle vs mitoxantrone–Q[8], ** p value 0.0033; vehicle vs mitoxantrone ns, mito vs mitoxantrone–Q[8], * p value 0.0316.

significant survival benefit for mice treated with mitoxantrone–Q[8] compared to either of the other two treatments. This survival benefit is most likely due to the lower toxicity induced by mitoxantrone–Q[8] in comparison to mitoxantrone, while maintaining efficacy against the primary tumor. In this model it is not possible to determine whether mitoxantrone–Q[8] has increased efficacy with respect to metastatic lesions. However, this can be assessed in follow-up experiments where primary tumors can be surgically excised and drug treatment then initiated.

The results of this study demonstrate that, although the aromatic ring system of mitoxantrone does not bind in the cavity of Q[7], it is readily encapsulated in a 2:1 symmetric binding mode in Q[8]. A major goal of cancer therapy is to improve the long-term survival of cancer patients while maintaining patient quality of life. This analysis of mitoxantrone–Q[8] indicates that the drug complex has the potential to meet these requirements more effectively than mitoxantrone. Using this new low toxicity formulation, it may now be possible to increase the dose of mitoxantrone to maximize efficacy while managing the dose limiting effects. Furthermore, and surprisingly, Q[8] encapsulation increased the rate and level of cellular uptake of mitoxantrone. This may further increase the potential of cucurbit[n]uril as drug delivery vehicle.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.7b00090.

Experimental details, table of ¹H NMR chemical shifts of mitoxantrone and mitoxantrone bound to Q[8], and Figures S1–S6 (PDF)

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Notes

The authors declare no competing financial interest.

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