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Crown Ether Host-Rotaxanes as Cytotoxic Agents

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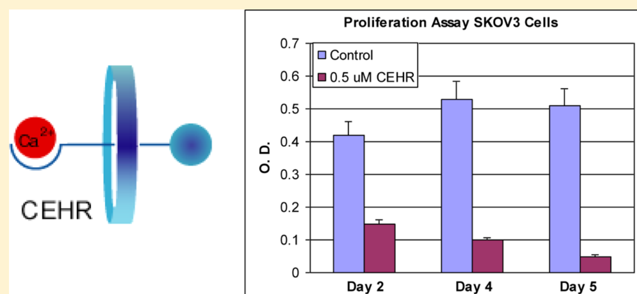
Supporting Information

ABSTRACT: Highly toxic bacterial ionophores are commonly used in veterinary medicine, but their therapeutic index is too narrow for human usage. With the goal of developing ionophores with a broader therapeutic index, we constructed highly derivatized synthetic ionophores. The toxicities of crown ether host-rotaxanes (CEHRs) against the SKOV-3 cell line were measured. The effect of Mg^{2+} or Ca^{2+} on toxicity was explored because changes in the intracellular concentration of these cations can cause cell death through apoptosis. We found that Boc-CEHR is highly toxic and Arg-CEHR is slightly less toxic with IC_{50} values of 0.5 and 6 μM, respectively, in standard growth medium. Increasing the concentration of Ca^{2+} resulted in greater toxicity of the CEHRs, whereas increasing the concentration of Mg^{2+} was less effective on reducing IC_{50}. Cell death occurs mainly through apoptosis. Although preliminary, these results suggest that the CEHRs deliver Ca^{2+} and perhaps Mg^{2+} into cells inducing apoptosis.



KEYWORDS: *apoptosis, calcium, magnesium, rotaxanes, crown ethers*

Bacterial polyether ionophores, such as salinomycin and monensin, are currently used in veterinary medicine as antibacterial and antiparasitic agents and growth promoters. Their mode of action arises from the transport of alkali cations across cellular membranes. The high toxicities and narrow therapeutic index of bacterial ionophores, however, make them unfit for human usage. Synthetic ionophores, such as crown ethers,[1] selectively bind cations based on their size and charge. Early studies of unmodified crown ethers showed that they have low toxicities in mice when taken orally,[2] and hydrophobic crown ethers displayed antiproliferative activity in the low micromolar range against a few cancer cell lines.[3] Promising results from recent investigations of antitumor agents have rekindled the pursuit of ionophores for human therapies. Salinomycin was found to be a 100 times more effective killer of breast cancer stemlike cells than Taxol.[4] Additionally, it induces apoptosis selectively in human cancer cells.[5] Monensin killed tumor cells established from a large range of malignancies[6−9] and prevented the maturation of transforming growth factor β (TGF-β), which is a key event in the pathophysiology of several diseases.[10,11]

Our research group has developed highly derivatized ionophores, based on the rotaxane architecture.[12] These crown ether host-rotaxanes (CEHRs) form complexes with alkali and alkaline cations and transfer them from aqueous solutions into $CHCl_3$. Rotaxanes are a class of compounds highlighting an interlocked wheel and axle with blocking groups on the ends of the axle to keep the wheel threaded (Figure 1).[13]

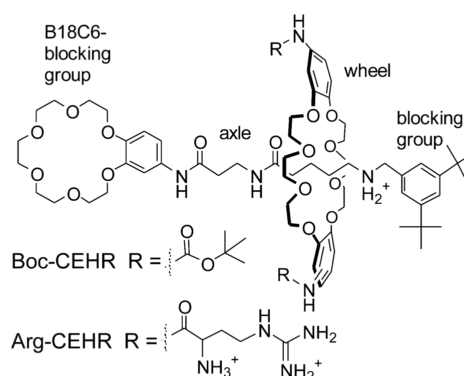


Figure 1. Rotaxanes used in this study and the components of a rotaxane.

We converted them into host-rotaxanes (HRs) by using a synthetic host as one of the blocking groups.[14] Using fluorescein labeling, we showed that HRs deliver Fl-pentapeptides into cells in an energy-independent process since the level of Fl-peptide within the cells was not greatly affected in most cases by lowering the temperature to 4 °C or reducing the level of cellular ATP.[15,16] Recently, Pt-rotaxanes were constructed by attaching Pt to one blocking group.[17] They

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are cytotoxic at the low micromolar level and killed SKOV-3 cells through apoptosis.

In this study, we investigated the toxicities of the CEHRs against SKOV-3 cells with the hypothesis that the CEHRs will increase the intracellular concentration of metal ions, causing cell death. We were especially interested in the toxicities of CEHRs bound to Ca^{2+} because changes in the intracellular levels of Ca^{2+} are linked to apoptosis (programmed cell death).[18−21] Furthermore, the toxicity of monensin depends on the presence of Mg^{2+} or Ca^{2+}[22,23] even though it preferentially binds Na^{+} over alkaline metals[24] (salinomycin prefers K^{+}[25]). Mg^{2+} and Ca^{2+} are nontoxic at their normal concentrations within the body. Ca^{2+} exists at a significantly higher concentration outside cells than within cells. The blood plasma level of Ca^{2+} is around 2 mM, and its intracellular concentration drops to around 0.1 μM. The intracellular and extracellular concentrations of Mg^{2+} are similarly maintained at approximately 1 mM. The percentage of free cytosolic Mg^{2+}, however, is only 6%. A large increase in the intracellular concentration of Mg^{2+} or Ca^{2+} leads to cell swelling and death through necrosis. A moderate increase in the intracellular concentration of Ca^{2+} can lead to apoptosis. The role of Mg^{2+} in apoptosis is less understood than for Ca^{2+}.[26−29] The goal is to develop CEHRs that increase the intracellular concentration of the metal cations to a level that initiates apoptosis but not necrosis.

Because CEHRs bind and transfer Na^{+}, K^{+}, and Mg^{2+} (likely Ca^{2+} as well) into $CHCl_3$, they will likely bind these cations in the growth medium and possibly deliver them into the cells. To determine the toxicity of Mg^{2+} and Ca^{2+} from the rest of metal cations in the solutions, the cells were exposed to increasing concentrations of Mg^{2+} or Ca^{2+}. The toxicities of these solutions with and without a CEHR were compared. A correlation between the concentration of Mg^{2+} or Ca^{2+} and the IC_{50} value for cells exposed to a CEHR would demonstrate that the combination of a CEHR and Mg^{2+} or Ca^{2+} is responsible for cell death. These studies do not provide information on how the combined agents kill cells. It could occur as envisioned via intracellular transport. Alternatively, a CEHR could, for example, make the cells more leaky or effect ion channels.

The cytotoxicities of *t*-butoxycarbonyl (Boc)-CEHR and the newly constructed Arg-CEHR (see the Supporting Information), which was made from Boc-CEHR, were determined. Arginine moieties were attached to the CEHR because the host-rotaxanes contained arginine moieties on the wheel, and they are intracellular transport agents. CEHRs contain several cation-binding sites; however, they only form very weak complexes with metal cations in water. For example, 18-crown-6 ether, which is used as a blocking group, binds Ca^{2+} with $K_{association}$ on the order of 3 M^{-1} in water.[1] The concentration of added $MgCl_2$ and $CaCl_2$ was set as high as possible to promote association without inducing a high level of background (without CEHR) toxicity. The maximum value was set at 40 mM since $CaCl_2$ is moderately toxic at this level (approximately 20% drop in OD). At this concentration, the concentration of a CEHR−metal cation complex will be very low in the aqueous solution. On the other hand, large association constants exist for the CEHR−metal cation complexes in $CHCl_3$.[12] Therefore, association events between a CEHR and a metal cation are more likely to occur closer to the cell surface where the dielectric constant of the medium drops. Standard growth media require Mg^{2+} ($MgSO_4$, 98 mg/L) and Ca^{2+} ($CaCl_2$, 200 mg/L) so all assays solutions contain a baseline level of these alkaline cations. The toxicity of the blocking group (benzyl-18-crown-6 ether) was determined to provide a measure of the importance of the rotaxane architecture for toxicity.

SKOV-3 cells were exposed to low micromolar concentrations of CEHRs, and their proliferation was monitored over 5 days (details are given in the Supporting Information). MTS assays were performed to determine cell viability at days 2, 4, and 5 (Figure 2). The MTS assay is a colorimetric assay that measures cell viability through the enzymatic reduction of a tetrazolium dye. Changing the concentration of $MgCl_2$ and $CaCl_2$, without the presence of a CEHR, altered the proliferation rate of the cells, especially for higher concentrations of $CaCl_2$. When compared to cells grown in standard growth media, cells exposed to higher concentrations of $MgCl_2$ showed a similar or a greater cell population (+20 to −10% change in OD), whereas cells exposed to higher concentrations

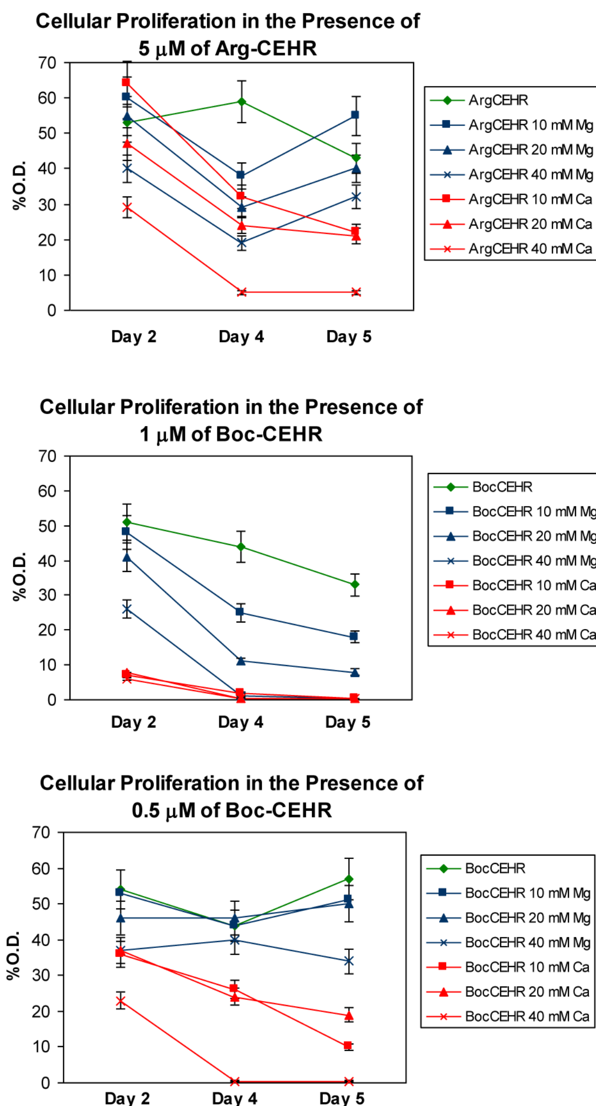

Figure 2. MTS assays were performed to provide a measure of cellular viability of SKOV-3 cells exposed to the CEHRs. The percent optical densities were derived by calculating the ratio of optical densities of solutions containing a CEHR and salts to the same solutions without the CEHR and multiplying by 100%. Results represent the average of four separate experiments.

Table 1. IC_{50} Values for the Rotaxanes against SKOV3 Cells[a] after 4 Days of Exposure

	added $MgCl_2$ (mM)				added $CaCl_2$ (mM)		
rotaxane[b]	0	10	20	40	10	20	40
Arg-CEHR IC_{50} (μM)	5.0 ± 0.2	2.8 ± 0.7	3.3 ± 0.5	2.6 ± 0.5	4.6 ± 0.6	4.5 ± 0.5	1.6 ± 0.1
Boc-CEHR IC_{50} (μM)	0.42 ± 0.03	0.33 ± 0.03	0.14 ± 0.04	0.10 ± 0.03	0.097 ± 0.005	0.08 ± 0.01	0.050 ± 0.007

[a]Cells were grown in standard growth media. [b][Arg-CEHR] = 5 μM, and [Boc-CEHR] = 1 μM.

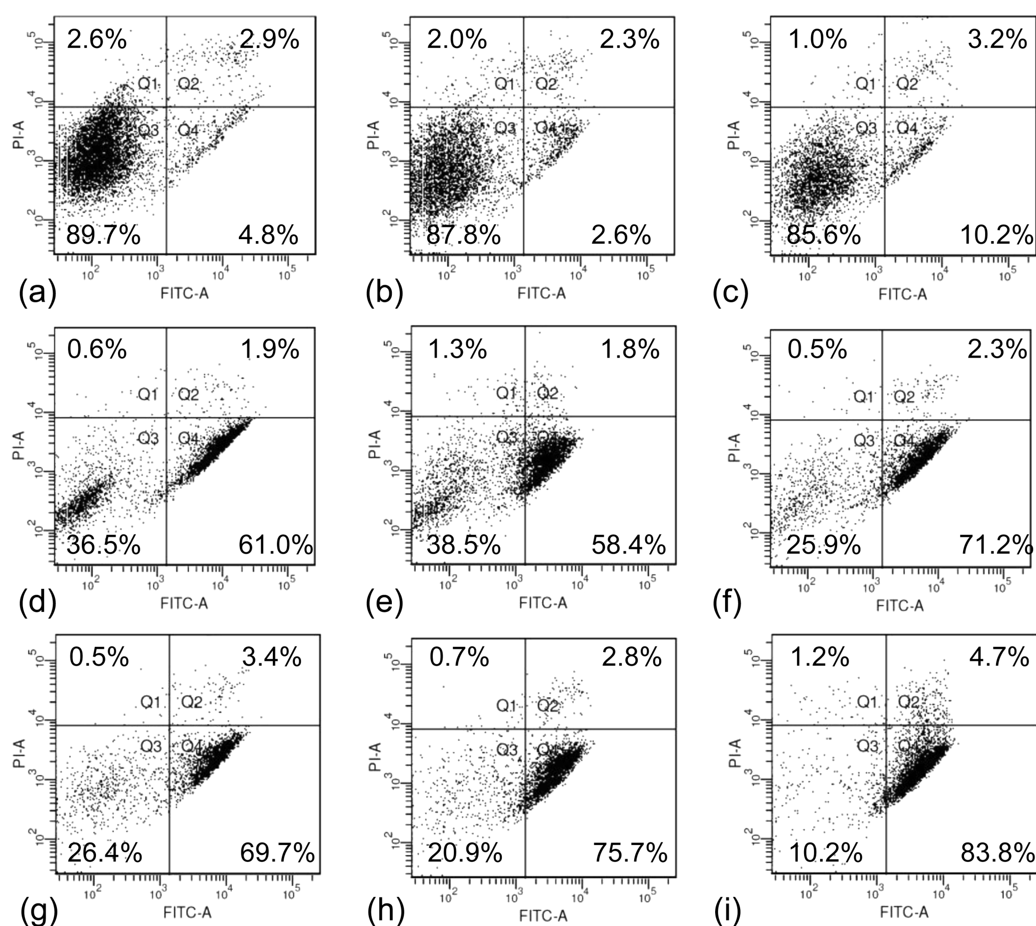

Figure 3. Percent change in the number of induced apoptotic SKOV-3 cells caused by a 4 day exposure to (a) 0.2% DMSO, (b) added $MgCl_2$ (20 mM), (c) added $CaCl_2$ (10 mM), (d) Arg-CEHR (5 μM), (e) Arg-CEHR (5 μM) and added $MgCl_2$ (20 mM), (f) Arg-CEHR (5 μM) and added $CaCl_2$ (10 mM), (g) Boc-CEHR (1 μM), (h) Boc-CEHR (1 μM) and added $MgCl_2$ (20 mM), and (i) Boc-CEHR (1 μM) and added $CaCl_2$ (10 mM). Cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) and then analyzed with dual flow cytometric analysis. Necrotic cells are in the upper left quadrant (Q1), late apoptotic/necrotic cells are in the upper right quadrant (Q2), living cell populations are clustered in the lower left quadrant (Q3), and cells in early apoptosis are in the lower right quadrant (Q4).

of $CaCl_2$ showed a reduction in cell population (−10 to −20% change in OD). Therefore, toxicities were determined by comparing the number of cells still adhered after exposure to solutions that contained a CEHR and an added salt to solutions that contained the same concentration of added salt, giving percent optical density values (% OD).

Arg-CEHR is toxic in the standard growth medium at the 5 μM level, giving a % OD of approximately 50%, which was maintained over the 5 days of monitoring. Its concentration was not increased since we wanted to observe the effect of added alkaline salt. Increasing the concentration of $MgCl_2$ lowered the % OD of the cells. There is a consistent drop in % OD by day 4, but the cell population begins to recover by day 5. The addition of $CaCl_2$ resulted in a more toxic solution (except for 10 mM $CaCl_2$ day 2) than with the addition of $MgCl_2$. Furthermore, the recovery in the cell population was not observed by day 5. The lowest % OD value for Arg-CEHR was observed for solutions that contained highest concentration of $CaCl_2$ at 40 mM.

Boc-CEHR is significantly more toxic than Arg-CEHR against SKOV-3 cells. Only a 1 μM solution of Boc-CEHR was required to give a % OD value of 50% after 2 days of exposure. Increasing the concentration of $MgCl_2$ in the presence of Boc-CEHR correlates with an increase in the toxicity of the solutions. Unlike with Arg-CEHR, solutions with Boc-CEHR and added $MgCl_2$ were toxic throughout the 5 days of exposure. Increasing the concentration of $CaCl_2$ greatly increased the toxicity of Boc-CEHR to a point where the % OD was essentially zero. Therefore, the concentration of Boc-CEHR was lowered to 0.5 μM, and the assay was repeated. At this lower concentration, the addition of $MgCl_2$ did not significantly increase the toxicity of Boc-CEHR, except at its

highest concentration of 40 mM by day 5. Adding 10 or 20 mM $CaCl_2$ increased the toxicity of Boc-CEHR, and this toxicity increased linearly over 5 days. In the presence of 40 mM $CaCl_2$, Boc-CEHR was highly toxic at 0.5 μM. In the control study, benzyl-18-crown-6 ether is not toxic up to 40 μM in normal growth media. This shows the importance of the rotaxane architecture for the observed toxicities of the CEHRs.

To obtain a more accurate measure on the effect of alkaline cation concentration on the toxicities of the CEHRs, IC_{50} values were calculated for adhered SKOV-3 cells exposed to Arg-CEHR (5 μM) or Boc-CEHR (1 μM) for 4 days (Table 1). Raising the concentration of $MgCl_2$ increased the toxicity of Arg-CEHR; however, the toxicity levels out between 10 and 40 mM of added $MgCl_2$. With the addition a $CaCl_2$, a significant drop in toxicity was only observed at the highest concentration of $CaCl_2$ added. The IC_{50} values for Boc-CEHR were lowered consistently with an increase in the concentration of $MgCl_2$ or $CaCl_2$.

Annexin V assays were performed to determine the cause of cell death (Figure 3). Annexin V is a phospholipid binding protein that when linked to a fluorophore is used to detect early stage apoptotic cells, which involves the loss of plasma membrane. The loss of membrane, however, also occurs with necrotic processes. Thus, propidium iodide, which stains dead cells, is used in conjunction with Annexin V. Viable cells are not stained with Annexin V or PI, early apoptotic cells are stained by only Annexin V, and cells in late apoptosis or dead cells are stained by both agents.

Adhered cells were exposed to Arg-CEHR (5 μM) or Boc-CEHR (1 μM) for 2 or 4 days. To set thresholds, a control population of SKOV-3 cells was exposed to a small amount of DMSO (0.1%). This was the highest percentage of DMSO in the assay solutions, which occurred in the assay containing Arg-CEHR at 5 μM. Each population of cells was harvested. The relative fluorescence intensity of these cells was quantified by multicolor flow cytometry (BD FacsAria). The threshold for background fluorescence for Annexin V and PI was set at a position that included 5% of cells incubated with 0.1% DMSO alone and exposed to both Annexin V and PI (Figure 3A). Debris was eliminated from analysis by a preset gate, which was set by analyzing untreated and unstained cells. These thresholds were then applied to each cell population.

We initially chose to investigate the cell death mechanism of each rotaxane (Arg-CEHR, 5 μM, and Boc-CEHR, 1 μM) at day 2 (Supporting Information) and day 4 (Figure 3) with $MgCl_2$ and $CaCl_2$ at 20 mM. Exposing cells to 20 mM $CaCl_2$, however, showed significant cell death by day 4 (21% Annexin V positive and 4% Annexin V/PI positive). Therefore, the concentration of added $CaCl_2$ was lowered to 10 mM. For all of the conditions investigated, only a very small percentage of cells were stained with PI only (0.5−2.2%) except for cells exposed to Boc-CEHR and 10 mM $CaCl_2$ at day 2 (4.1%). Similarly, only a low percentage of cells were positive to both PI and Annexin V (1.8−7.3%). Most cells when exposed to Arg-CEHR or Boc-CEHR were either unaffected by the agents or harvested in the early stages of apoptosis. By day 2, 25−50% of the cells were in early stage apoptosis, and by day 4, this fraction increased to around 75%. Boc-CEHR outperformed Arg-CEHR, and it is slightly more toxic in the presence of added $MgCl_2$ or $CaCl_2$. Thus, the number of dead and dying cells roughly matches the results obtained through the MTS assay.

Herein, we demonstrate that CEHRs are cytotoxic toward SKOV-3 ovarian cancer cells in the low micromolar range within 2 days of exposure. The percentage of dead cells was either maintained or increased throughout a 5 day period. The toxicities of the CEHRs increased consistently with an increase in the concentration of $CaCl_2$. With the addition of $MgCl_2$, Boc-CEHR demonstrated enhanced toxicities, whereas Arg-CEHR produced inconsistent results. The proliferation assay showed that solutions of Arg-CEHR with added $MgCl_2$ are initially more toxic than standard growth medium, but the cell population recovered by day 5. Thus, the enhanced toxicity of Arg-CEHR caused by the addition of $MgCl_2$ is more sensitive to the exposure time than for solutions containing Arg-CEHR and $CaCl_2$. The majority of cells died through an apoptotic mechanism. The percentage of cells in early state apoptosis increased from day 2 (25−50%) to day 4 (58−84%), which is consistent with the results obtained in the MTS assays. The percentage of necrotic cells or cells undergoing late stage of apoptosis stayed at 7% or less. These results show that the CEHRs operate as expected: they enhance the intracellular concentration of Mg^{2+} and Ca^{2+} to induce apoptosis without necrosis. For drug development, the CEHRs will be built to bind Ca^{2+} more tightly at physiological concentration of 2 mM (not up to 40 mM as used in this study). The CEHRs will also be further derivatized with a targeting agent to selectively guide them to diseased cells.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic protocols for the materials, plots used to calculate IC_{50} values, and apoptosis results for a 2 day exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CEHR, crown ether host-rotaxane; Boc, *t*-butoxycarbonyl

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