Ca²⁺ Selective Host Rotaxane Is Highly Toxic Against Prostate Cancer Cells

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S Supporting Information

ABSTRACT: New therapies are needed to eradicate androgen resistant, prostate cancer. Prostate cancer usually metastasizes to bone where the concentration of calcium is high, making Ca²⁺ a promising toxin. Ionophores can deliver metal cations into cells, but are currently too toxic for human use. We synthesized a new rotaxane (CEHR2) that contains a benzyl 15-crown-5 ether as a blocking group to efficiently bind Ca²⁺. CEHR2 transfers Ca²⁺ from an aqueous solution into CHCl₃ to greater extent than alkali metal cations and Mg²⁺. It also transfers Ca²⁺ to a greater extent than CEHR1, which is a rotaxane with an 18-crown-6 ether as a blocking group. CEHR2 was more toxic against the prostate cancer cell lines PC-3, 22Rv1, and C4-2 than CEHR1. This project demonstrates that crown ether rotaxanes can be designed to bind a targeted metal cation, and this selective cation association can result in enhanced toxicity.

KEYWORDS: Prostate cancer, rotaxanes, crown ethers, calcium

Prostate cancer is the most often diagnosed cancer in men in North America.¹ Castration-resistant prostate cancer does not respond to androgen deprivation therapy and is difficult to treat. Novel therapies are desperately needed. Ionophores selectively bind metal cations and can be highly toxic, making them attractive for new therapies. The bacterial ionophores salinomycin and monensin are natural antibiotics that deliver selectively K⁺ and Na⁺, respectively, across cellular lipid bilayers. Influx of metal cations can change the Na⁺/K⁺ gradient and intracellular pH, causing cell death. Salinomycin is cytotoxic against several cancer cell lines, including prostate, breast, and colorectal cancer^{2–4} and lymphoma, and has undergone limited clinical trials.^{5–7} Monensin is antiproliferative against colon, lymphoma and myeloma cancer cell lines.^{8–10} Currently, the high toxicities and narrow therapeutic index of bacterial ionophores make them unfit for human usage. As an alternative, synthetic ionophores, such as crown ethers, have been explored as therapeutic agents. Unmodified crown ethers displayed low toxicities in mice when taken orally.¹¹ A hydrophobic crown ether showed antiproliferative activity in the low micromolar range against cervical, breast, colon, pancreatic, and lung carcinoma cells.¹²

To improve the therapeutic index of ionophores, our research group develops highly derivatized ionophores based

on the rotaxane architecture.¹³ [2]Rotaxanes comprise a circular molecule (wheel) threaded over a linearly shaped molecule (axle) with large groups (blocking groups) attached to the axle's ends to keep the wheel threaded. A series of crown ether host rotaxanes (CEHRs), including CEHR1 (Figure 1), were tested as ionophores using phase transfer assays from an aqueous solution into chloroform.¹⁴ A benzyl-18-crown-6 ether was used as a blocking group, which has a known preference for K⁺ ions.^{15,16} CEHR1 not only showed a high percentage of K⁺ transferred (92%), but Mg²⁺ as well (98%). The transfer of Mg²⁺ by B18C6 was not observed. The high transfer efficiency of Mg²⁺ encouraged us to test the cytotoxicity of CEHR1. We found that CEHR1 is highly toxic against ovarian cancer cells (SKOV-3, IC₅₀ = 0.097 ± 0.005 μM in the presence of 10 mM Ca²⁺ and IC₅₀ = 0.33 ± 0.03 μM in the presence of 10 mM Mg²⁺) and that it kills cells through apoptosis.

To test the CEHR's efficacy against prostate cancer, Ca²⁺ was chosen as the toxin. It is nontoxic at its normal concentrations within the body. Prostate cancer metastasizes to skeletal sites where the Ca²⁺ concentration can be as high as 40 mM.^{17,18} A

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Figure 1. Crown-ether rotaxanes and crown ethers used in this study.

large increase in the intracellular concentration of Ca^{2+} leads to cell swelling and death through necrosis. A moderate increase in the intracellular concentration of Ca^{2+} , however, can lead to apoptosis,^{19,20} as seen with CEHR1.¹⁴ CEHR2 was designed and synthesized (see Supporting Information) to selectively bind and deliver Ca^{2+} by using a benzyl-15-crown-5 ether (B15C5), as one blocking group. 15C5 has a smaller diameter (0.86 to 0.92 Å) than 18C6 (diameter 1.34–1.43 Å),¹⁵ which was used as a blocking group in CEHR1. Crown ethers tend to select cations that more closely match their inner diameters. Thus, B15C5 selectively binds smaller metal cations like Na^+ and Ca^{2+} (atomic radii 0.95 and 0.99 Å, respectively). In this report, we compare the ability of CEHR1 and CEHR2 to bind and transfer metal cations from an aqueous solution into chloroform and measure their cytotoxicity against prostate cancer cell lines PC-3, C4-2, and 22Rv1. We found, as predicted, that CEHR2 significantly transfers Ca^{2+} to a higher percentage than CEHR1 and is more cytotoxic. For each CEHR, the IC_{50} values are very similar for each cell line (around 2 μM for CEHR1 and 0.6 μM for CEHR2), which suggests that the transport mechanism is cell type independent.

Two-phase extraction assays (ions(aq)/ CHCl_3) were performed to obtain transfer efficiencies and a measure of the binding affinities.²¹ The concentrations of the CEHRs, B15C5, and metal cations were set to readily differentiate their transfer efficiencies. Extraction constants (K_e) were determined from the known concentration of the components and the concentration of picrate ion extracted into the CHCl_3 (chl) layer by a host, which is measured using UV/vis absorption spectroscopic analysis (see equations below). The distribution constants (K_d) were taken from the literature.^{22,23} Association constants for the binding of the metal cation by a host in CHCl_3 are the ratio of K_e/K_d . The percentage of metal cation extracted (%T) is equal to the ratio of the concentration of the picrate ion in the chloroform layer versus the total amount of picrate ion measured in the chloroform and aqueous layers. The hosts remained predominantly in the CHCl_3 layer (>93%). Since the concentration of a host was approximately 30-fold

greater than the concentration of a metal cation, the complexes were likely in a 1:1 ratio of host to cation.

$$K_e = [\text{host} \cdot \text{M}^{n+} \cdot n\text{Pic}^-]_{\text{chl}} / [\text{host}]_{\text{chl}} [\text{M}^{n+}]_{\text{aq}} [\text{Pic}^-]_{\text{aq}}^n$$

$$K_d = [\text{M}^{n+} \cdot n\text{Pic}^-]_{\text{chl}} / [\text{M}^{n+}]_{\text{aq}} [\text{Pic}^-]_{\text{aq}}^n$$

$$K_a = K_e / K_d$$

$$\% \text{transfer } (\%T) = [\text{Pic}^-]_{\text{chl}} / ([\text{Pic}^-]_{\text{chl}} + [\text{Pic}^-]_{\text{aq}})$$

The transfer efficiencies (%T) of the hosts interacting with picrate salts are given in Figure 2. Consistent with literature

Figure 2. Percent of picrate ion in CHCl_3 versus the total concentration of picrate ion after extraction from an aqueous phase into CHCl_3 by a host (uncertainty %T $\leq 5\%$). CEHR1 was tested only for the dications (see ref. 13 for the transfer of monocations).

findings, B15C5 preferred Na^+ to the other cations and only transferred a small percentage of Ca^{2+} or Mg^{2+} , which were tested at a lower concentration, vide infra. Incorporating the B15C5 moiety into a rotaxane dramatically increased the amount of metal cations transferred. The percent transfer of monocations was increased 4-fold to 8-fold. A similar selectivity was observed for CEHR2 ($\text{Na}^+ > \text{K}^+ > \text{Cs}^+ > \text{Li}^+$) as found with B15C5, which suggests that the cations likely bind to its B15C5 blocking group. To more easily differentiate the ability of CEHR2 and CEHR1 to transfer Ca^{2+} and Mg^{2+} , the concentrations of the CEHRs and cations were lowered to a 1 mM concentration of a CEHR and 30 μM of picrate salts. CEHR2 transferred approximately 1.5 times more Mg^{2+} ($67 \pm 1\%$) and 2.5 times more Ca^{2+} ($99 \pm 5\%$) when compared to CEHR1 (42% and 38%, respectively). Association constants for the complexes in CHCl_3 were calculated. The alkaline dications were held more tightly (for CEHR2, $\text{Log}K_a = 10.5$ and 7.9 for Ca^{2+} and Mg^{2+} , respectively) than the alkali metal cations ($\text{Log}K_a$ s from 2.7 to 3.6).

The efficient transfer of Ca^{2+} and the strong CEHR– Ca^{2+} complex bodes well for high cytotoxicity. The picrate counterion, however, enhances the transfer process by promoting complexation in the aqueous solution. In the cellular proliferation assays, picrate salts were not used. Thus, the concentration of a CEHR–metal cation complex will be very low in the aqueous solution. However, the tight complexes that occur in CHCl_3 are driven mainly by favorable interactions between the oxygen atoms of a crown ether and the metal cation. 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 requires Mg^{2+} (MgSO_4 , 98 mg/L) and Ca^{2+}

(CaCl₂, 200 mg/L) so all assays solutions contain a baseline level of these alkaline cations. To promote Ca²⁺ transfer, we added CaCl₂ to a concentration of 10 mM, which was not noticeably toxic without the presence of a CEHR. The toxicities of the blocking groups (B18C6 and B15C5) were determined to provide a measure of the importance of the rotaxane architecture for toxicity.

The cytotoxicities of CEHR1, CEHR2, B18C6, and B15C5 against the castration resistant, prostate cancer lines PC-3, 22Rv1, and C4-2 were determined. The cells were seeded in microtiter wells and exposed to low micromolar concentrations of CEHRs (10 μ M to 0.1 μ M) and high micromolar concentrations of B16C8 and B15C5 (100 μ M to 12 μ M). For CEHR1 and CEHR2, five samples were run for each concentration. The B15C5 and B18C6 assays were run in triplicates. Cellular proliferation was monitored over 5 days (details are given in [Supporting Information](#)). MTS assays were performed to determine cell viability at days 2 and 5 ([Figure 3](#)).

Figure 3. MTS assays were performed to provide a measure of cellular viability of PC-3, C4-2, and 22Rv1 cells exposed to the B15C5 and B18C6 (100 μ M) and the CEHRs (2 μ M). The percentage of live cells was derived by calculating the ratio of optical densities of solutions containing a CEHR or a crown ether to the same solutions without a host and multiplying by 100%. Error bars represent the 95% confidence limits.

For the control experiments, cells were exposed to 10 mM CaCl₂ and 0.2% DMSO, which is the highest concentration added to an assay solution from a host's stock solution. The percentage of live cells was calculated from the ratio of the average absorbance of an assay solution to background solution multiplied by 100%.

The percentages of live cells for cells exposed to 2 μ M CEHRs and 100 μ M crown ethers are shown in [Figure 3](#). B18C6 is not toxic up to a 100 μ M concentration (>95% of live cells). Similarly, B15C5 is not toxic after 2 days of exposure; however, it appears to be slightly toxic after 5 days (80% to 85% of live cells). This finding is consistent with the smaller crown ether preferentially delivering the toxin Ca²⁺. The CEHRs are highly toxic as compared to the crown ethers. By day two, CEHR2 was significantly more toxic than CEHR1. At day 5, however, while CEHR2 maintained its toxicity, CEHR1 demonstrated a higher percentage of cell death. To obtain a more accurate measure of the toxicities of the CEHRs, IC₅₀ values were calculated for adhered PC-3, 22Rv1, and C4-2 cells exposed to the reagents for 2 and 5 days ([Table 1](#)). After 2 days, CEHR2 was three to five times more toxic than CEHR1. This gap narrows to two to three times more toxic for CEHR2 than CEHR1 at day 5. CEHR1's IC₅₀ values drop over time, whereas CEHR2 remains more constant. There appears to be a slight recovery of cell growth for C4-2 cells in the presence of CEHR2. The IC₅₀ values are independent of cell type. For example, at day 2, the average IC₅₀ value for CEHR1 was 2.5 $\pm$ 0.3 μ M and for CEHR2 was 0.7 $\pm$ 0.1 μ M. This finding suggests that the CEHRs act as transporters of Ca²⁺ or other cations across the cellular membrane.

Fluo-8am was used to obtain relative levels of free intracellular Ca²⁺. It is a cell-permeable fluorescent probe that hydrolyzes within cells and binds free Ca²⁺, producing a unique fluorescent signal ([Supporting Information](#)). The concentration of the CEHRs for these assays had to be set at values whereby the amount of Ca²⁺ within cells could be raised above control levels without causing significant cell death. We found that setting the concentrations of CEHR1 and CEHR2 at 4 and 1 μ M, respectively, and approximately twice their IC₅₀ values, resulted in a reproducible, enhanced level of intracellular Ca²⁺ without extensive cell death within 4 h. The measured fluorescence intensities were divided by the number of viable cells and averaged ([Figure 4](#)). We also tested whether this assay was sensitive to the concentration of extracellular Ca²⁺. CaCl₂ concentration was kept low (0.5 to 2 mM) to minimize cell death.

At the concentration of CEHRs approximately twice their IC₅₀ values, cells in the presence of CEHR1 contained a similar to slightly more amounts of intracellular Ca²⁺ than in the presence of CEHR2. The levels of intracellular Ca²⁺ for cells without a CEHR were significantly lower. To determine whether changing the concentration of extracellular Ca²⁺ from 0.5 to 2 mM altered the level of intracellular Ca²⁺, the fluorescence per cells (FPC) values for each CEHR and control

Table 1. IC₅₀ Values for the CEHRs against Prostate Cancer Cells^a

rotaxane	day 2			day 5		
	PC-3	22Rv1	C4-2	PC-3	22Rv1	C4-2
CEHR1 IC ₅₀ (μ M)	2.2 $\pm$ 0.2	2.7 $\pm$ 0.4	2.71 $\pm$ 0.09	1.6 $\pm$ 0.3	1.5 $\pm$ 0.2	2.20 $\pm$ 0.04
CEHR2 IC ₅₀ (μ M)	0.77 $\pm$ 0.09	0.78 $\pm$ 0.08	0.56 $\pm$ 0.06	0.42 $\pm$ 0.06	0.57 $\pm$ 0.09	0.97 $\pm$ 0.03

^aCells were grown in standard growth media with enhanced CaCl₂ (10 mM).

Figure 4. Intracellular levels of Ca²⁺ were measured using Fluo-8am as the fluorescent probe. Fluorescence intensities were obtained by dividing the measured fluorescence by the number of cells. Error bars were obtained by averaging five FPC values per condition.

solution independent of cell type were averaged for each concentration of Ca²⁺ (e.g., for CEHR₂ in 0.5 mM CaCl₂, $FPC_{0.5M} = FPC_{PC-3, 0.5M} + FPC_{22Rv1, 0.5M} + FPC_{C4-2, 0.5M}/3$). $FPC_{0.5M}$, FPC_{1M} , and FPC_{2M} per CEHR and control were similar and without an apparent trend (For CEHR₂, $FPC_{0.5M, 1M, 2M Ca} = 100 \pm 20$; CEHR₁ $FPC_{0.5M, 1M, 2M Ca} = 80 \pm 11$; and control $FPC_{0.5M, 1M, 2M Ca} = 50 \pm 8$). Thus, the FPC values for 0.5, 1, and 2 M CaCl₂ solutions were combined for each CEHR (and control) and cell line to determine whether the intracellular level of Ca²⁺ depended upon cell line. The level of Ca²⁺ within PC-3 cells ($FPC_{CEHR2} = 119 \pm 13$ and $FPC_{CEHR1} = 89 \pm 11$) was greater than for 22Rv1 ($FPC_{CEHR2} = 94 \pm 20$; $FPC_{CEHR1} = 80 \pm 5$) and C4-2 ($FPC_{CEHR2} = 85 \pm 9$; $FPC_{CEHR1} = 72 \pm 11$) cells. The control PC-3 cells, however, also contained a greater FPC value ($FPC_{control} = 58 \pm 8$) than for 22Rv1 ($FPC_{control} = 45 \pm 3$) and C4-2 ($FPC_{control} = 46 \pm 8$). Therefore, PC-3 cells could have been more susceptible to extracellular levels of Ca²⁺ in this assay. Whether or not the fluorescence intensity was dependent on cell line, the results of

the assays clearly show that the CEHRs increased the level of intracellular Ca²⁺. Furthermore, the independence of the extracellular Ca²⁺ concentration on FPC indicates that the CEHRs do not operate through reducing membrane integrity. If the CEHRs were making holes in the outer cellular membrane, the intracellular level of Ca²⁺ at 2 M should be significantly greater than at 0.5 M.

CEHR1 was found to be more cytotoxic against ovarian cancer cells than prostate cancer cells. For example, its IC₅₀ value for SKOV-3 was 0.1 μ M,¹⁴ whereas for the investigated prostate cancer cells, it was on average 2.5 μ M. If CEHR1 is truly a transporter and not reliant on a cellular mechanism, the IC₅₀ values should be more similar. Using Ca²⁺ as the toxin could be the differentiating factor. Prostate cancer involves the deregulation of Ca²⁺ homeostasis and the overexpression of the Ca²⁺-sensing receptor.²⁴ Furthermore, prostate cancer cells tend to metastasize to bone. For example, 74% metastatic, castration resistant, prostate cancers have bone metastasis.²⁵ However, the incidence of bone metastasis in ovarian cancer is rare, occurring around 2% of the cases.²⁶ Our observations are consistent with these findings. Low concentration of delivered Ca²⁺ by CEHR1 kills ovarian cancerous cells, but may promote the growth of prostate cancer. We did not observe, however, an enhanced growth of the prostate cancer cells with the lowest levels of a CEHR. The maximum cell growth was around 75% to 90% of the background level for samples containing the lowest concentrations of CEHR's at day 2 and day 5 (Supporting Information). Although the low level delivery of Ca²⁺ did not noticeably promote the growth of the prostate cancer cells, this possibility should be considered when nutrients are converted into drugs.

Ionophores hold promise as new drugs to overcome the resistances observed with traditional drugs. Natural ionophores are currently too toxic to be used in humans. They will likely need to be modified in terms of their physical properties and the cations they carry to be safe and effective. We have shown that a rotaxane decorated with a crown ether blocking group can be designed to selectively bind and transport Ca²⁺ from aqueous solutions into chloroform. More importantly, this preference for Ca²⁺ appears to manifest itself in enhanced toxicities against androgen resistant, prostate cancer cells. CEHRs raise the level of intracellular Ca²⁺, which is consistent with Ca²⁺ acting as the toxin. Although CEHR2 shows a preference for Ca²⁺, we will attempt to improve its selectivity for prostate cancer cells by targeting the PSMA marker.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.6b00347.

Synthetic protocols for the materials, plots used to calculate IC₅₀ values, cellular assay protocols, and a representative figure showing cells exposed to Fluo-8am (PDF)

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Notes

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REFERENCES

- (1) Hotte, S. J.; Saad, F. Current management of castrate-resistant prostate cancer. *Curr. Oncol.* **2010**, *17*, S72–S79.
- (2) Kim, K. Y.; Yu, S. N.; Lee, S. Y.; Chun, S. S.; Choi, Y. L.; Park, Y. M.; Song, C. S.; Chatterjee, B.; Ahn, S. C. Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization. *Biochem. Biophys. Res. Commun.* **2011**, *413*, 80–86.
- (3) Oak, P. S.; Kopp, F.; Thakur, C.; Ellwart, J. W.; Rapp, U. R.; Ullrich, A.; Wagner, E.; Knyazev, P.; Roidl, A. Combinatorial treatment of mammospheres with trastuzumab and salinomycin efficiently targets HER2-positive cancer cells and cancer stem cells. *Int. J. Cancer* **2012**, *131*, 2808–2819.
- (4) Wang, Y. Effects of salinomycin on cancer stem cell in human lung adenocarcinoma A549 cells. *Med. Chem.* **2011**, *7*, 106–111.
- (5) Naujokat, C.; Steinhart, R. Salinomycin as a drug for targeting human cancer stem cells. *J. Biomed. Biotechnol.* **2012**, *2012*, 9506–9558.
- (6) Kopp, F.; Hermawan, A.; Oak, P. S.; Herrmann, A.; Wagner, E.; Roidl, A. Salinomycin treatment reduces metastatic tumor burden by hampering cancer cell migration. *Mol. Cancer* **2014**, *13*, 16–21.
- (7) Scherzad, A.; Hackenberg, S.; Schramm, C.; Froelich, K.; Ginzkey, C.; Hagen, R.; Kleinsasser, N. Geno- and cytotoxicity of salinomycin in human nasal mucosa and peripheral blood lymphocytes. *Toxicol. In Vitro* **2015**, *29*, 813–818.
- (8) Park, W. H.; Kim, E. S.; Jung, C. W.; Kim, B. K.; Lee, Y. Y. Monensin-mediated growth inhibition of SNU-C1 colon cancer cells via cell cycle arrest and apoptosis. *Int. J. Oncol.* **2003**, *22*, 377–382.
- (9) Park, W. H.; Seol, J. G.; Kim, E. S.; Kang, W. K.; Im, Y. H.; Jung, C. W.; Kim, B. K.; Lee, Y. Y. Monensin-mediated growth inhibition in human lymphoma cells through cell cycle arrest and apoptosis. *Br. J. Haematol.* **2002**, *119*, 400–407.
- (10) Park, W. H.; Kim, E. S.; Kim, B. K.; Lee, Y. Y. Monensin-mediated growth inhibition in NCI-H929 myeloma cells via cell cycle arrest and apoptosis. *Int. J. Oncol.* **2003**, *23*, 197–204.
- (11) Hendrixson, R.; Mack, M.; Palmer, R.; Ottolenghi, A.; Ghirardelli, R. Oral Toxicity of Cyclic Polyethers-12-Crown-4, 15-Crown-5, and 18-Crown-6 in Mice. *Toxicol. Appl. Pharmacol.* **1978**, *44*, 263–268.
- (12) Marjanovic, M.; Kralj, M.; Supek, F.; Frkanec, L.; Piantanida, I.; Smuc, T.; Tusek-Bozic, L. Antitumor potential of crown ethers: Structure-activity relationships, cell cycle disturbances, and cell death studies of a series of ionophores. *J. Med. Chem.* **2007**, *50*, 1007–1018.
- (13) Wang, X.; Zhu, J.; Smithrud, D. B. Synthesis and Investigation of Host-[2]Rotaxanes That Bind Metal Cations. *J. Org. Chem.* **2010**, *75*, 3358–3370.
- (14) Smithrud, D. B.; Wang, X.; Tarapore, P.; Ho, S. Crown Ether Host-Rotaxanes as Cytotoxic Agents. *ACS Med. Chem. Lett.* **2013**, *4*, 27–31.
- (15) Izatt, R. M.; Pawlak, K.; Bradshaw, J. S.; Bruening, R. L. Thermodynamic and Kinetic Data for Macrocyclic Interaction with Cations and Anions. *Chem. Rev.* **1991**, *91*, 1721–1785.
- (16) Izatt, R. M.; Bradshaw, J. S.; Nielsen, S. A.; Lamb, J. D.; Christensen, J. J. Thermodynamic and Kinetic Data for Cation-Macrocyclic Interaction. *Chem. Rev.* **1985**, *85*, 271–339.
- (17) Berger, C. E.; Rathod, H.; Gillespie, J. I.; Horrocks, B. R.; Datta, H. K. Scanning electrochemical microscopy at the surface of bone-resorbing osteoclasts: evidence for steady-state disposal and intracellular functional compartmentalization of calcium. *J. Bone Miner. Res.* **2001**, *16*, 2092–2102.
- (18) Silver, I. A.; Murrills, R. J.; Etherington, D. J. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp. Cell Res.* **1988**, *175*, 266–276.
- (19) Moffitt, K. L.; Martin, S. L.; Walker, B. From sentencing to execution - the processes of apoptosis. *J. Pharm. Pharmacol.* **2010**, *62*, 547–562.
- (20) Duke, R.; Witter, R.; Nash, P.; Young, J.; Ojcius, D. Cytolysis Mediated by Ionophores and Pore-Forming Agents - Role of Intracellular Calcium in Apoptosis. *FASEB J.* **1994**, *8*, 237–246.
- (21) Helgeson, R. C.; Weisman, G. R.; Toner, J. L.; Tarnowski, T. L.; Chao, Y.; Mayer, J. M.; Cram, D. J. Host-Guest Complexation 0.18. Effects on Cation Binding of Convergent Ligand Sites Appended to Macrocyclic Polyethers. *J. Am. Chem. Soc.* **1979**, *101*, 4928–4941.
- (22) Koenig, K. E.; Lein, G. M.; Stuckler, P.; Kaneda, T.; Cram, D. J. Host-Guest Complexation 0.16. Synthesis and Cation Binding Characteristics of Macrocyclic Polyethers Containing Convergent Methoxyaryl Groups. *J. Am. Chem. Soc.* **1979**, *101*, 3553–3566.
- (23) BatinicHaberle, I.; Spasojevic, I.; Crumbliss, A. L. Second-sphere coordination of ferrioxamine B and association of deferriferrioxamine B, $\text{CH}_3(\text{CH}_2)_4\text{NH}_3^+$, NH_4^+ , K^+ , and Mg^{2+} with synthetic crown ethers and the natural ionophores valinomycin and nonactin in chloroform. *Inorg. Chem.* **1996**, *35*, 2352–2359.
- (24) Vaz, C. V.; Rodrigues, D. B.; Socorro, S.; Maia, C. J. Effect of extracellular calcium on regucalcin expression and cell viability in neoplastic and non-neoplastic human prostate cells. *Biochim. Biophys. Acta, Mol. Cell Res.* **2015**, *1853*, 2621–2628.
- (25) Koo, K. C.; Park, S. U.; Kim, K. H.; Rha, K. H.; Hong, S. J.; Yang, S. C.; Chung, B. H. Prognostic Impacts of Metastatic Site and Pain on Progression to Castrate Resistance and Mortality in Patients with Metastatic Prostate Cancer. *Yonsei Med. J.* **2015**, *56*, 1206–1212.
- (26) Sehouli, J.; Olschewski, J.; Schotters, V.; Fotopoulou, C.; Pietzner, K. Prognostic role of early versus late onset of bone metastasis in patients with carcinoma of the ovary, peritoneum and fallopian tube. *Ann. Oncol.* **2013**, *12*, 3024–3028.