Supporting Information

Potentiating Bisphosphonate-based Coordination Complexes to Treat Osteolytic Metastases

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1. Experimental

1.1 Materials

Calcium nitrate tetrahydrate [Ca(NO\textsubscript{3})\textsubscript{2}·4H\textsubscript{2}O, 99% pure], calcium chloride hexahydrate [CaCl\textsubscript{2}·6H\textsubscript{2}O, USP grade], zinc nitrate hexahydrate [Zn(NO\textsubscript{3})\textsubscript{2}·6H\textsubscript{2}O, 98% pure], zinc chloride anhydrous [ZnCl\textsubscript{2}, >98% pure], magnesium nitrate hexahydrate [Mg(NO\textsubscript{3})\textsubscript{2}·6H\textsubscript{2}O, 99% pure], etidronic acid 60% aqueous solution (HEDP) and copper sulfate pentahydrate (CuSO\textsubscript{4}·5H\textsubscript{2}O, ACS reagent >98% pure) were purchased from Sigma-Aldrich (St. Louis, MO). Alendronate sodium trihydrate (ALEN, 97% pure) was purchased from TCI America (St. Portland, OR). Alendronate Sodium Tablets-USP (70 mg free equivalent acid) NDC 69097-224-16 and LOT GC70728 supplied from Cipla USA, Inc. (Miami, FL). A stock solution of sodium hydroxide (NaOH, USP grade, 1.5 M) was used for pH adjustments. Distilled water was used as solvent in all syntheses.
Nanopure water from an ARIES Filter Works Gemini High purity water system (18.23 M-Ohm/cm) was used in the preparation of buffers, calibration curves, determination of phase inversion temperature (PIT) and the nanoemulsion synthesis. Sodium chloride (NaCl, ACS reagent >99.0% pure) and sodium phosphate dibasic (Na$_2$HPO$_4$, BioXtra, >99.0% pure) from Sigma-Aldrich (St. Louis, MO), potassium chloride (KCl, 100.1% pure) from J.T. Baker (Phillipsburg, NJ), and potassium phosphate monobasic (KHPO$_4$, HPLC grade, >99.5% pure) from Fluka (Japan), were used as the components to make phosphate buffered saline (PBS) solutions (pH = 7.40). Hydrochloric acid (HCl, 37%) from Sigma-Aldrich (St. Louis, MO) was used to prepare fasted-state simulated gastric fluid (FaSSGF) solutions (pH = 1.60). Polytetrafluoroethylene (PTFE), non-sterile, 0.2 µm, 25 mm syringe filters from Fisher brand, Fisher Scientific (Ireland), were used for filtration during dissolution measurements.

Heptane [CH$_3$(CH$_2$)$_5$CH$_3$, anhydrous 99%] and Brij® L4 [(C$_{20}$H$_{42}$O$_5$)$_n$, average Mn ~362 g/mol] from Sigma-Aldrich (St. Louis, MO), were used to prepare the emulsion for the PIT determination and nanoemulsion synthesis of ALEN-Ca form II.

The cytotoxicity studies were carried out using MDA-MB-231 (ATCC® HTB-26™, Manassas, VA) cell line, fetal bovine serum (FBS, sterile-filtered, suitable for cell culture) from Sigma-Aldrich (St. Louis, MO), Dulbecco’s Modified Eagle’s Medium (DMEM, with 4,500 mg/L glucose and L-glutamine, without sodium bicarbonate, powder, suitable for cell culture) from Sigma-Aldrich (Milwaukee, WI) and penicillin-streptomycin (Pen-Strip, 10,000 units penicillin and 10 mg/mL streptomycin in 0.9% NaCl) from Sigma-Aldrich (St. Louis, MO) were used for cell growth conditions, AlamarBlue® (C$_{12}$H$_7$NO$_4$, suitable for cell culture) from Bio-Rad (Kidlington, Oxford) was used to determine cell proliferation.

1.2 Syntheses

Note: HEDP was added as an auxiliary ligand to decrease the pH below of the pK$_a$’s of the principal ligand in the reactions of ALEN with Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$. These reactions yielded crystalline products with enough single crystal quality for structural elucidation that turn out to be new polymorphs, namely, ALEN-Ca form II, ALEN-Zn form II and ALEN-Mg. When HEDP was not added, the reaction between Ca$^{2+}$ and Zn$^{2+}$ yielded other distinct crystalline products, namely, ALEN-Ca form I and ALEN-Zn form I. For the reaction between ALEN and Mg$^{2+}$ without HEDP, single crystals were not observed.

ALEN-Ca form I. A mixture of ALEN and CaCl$_2·$6H$_2$O with a molar ratio (1:1) was prepared in distilled water at room temperature as follows. The ligand solution was prepared in a 20 mL vial
dissolving 1 mmol (0.2711 g) of solid ALEN in 10 mL of distilled water, and heated at 85.0 °C for 15 min. The metal salt solution was prepared dissolving 1 mmol (0.2191 g) of CaCl₂·6H₂O in 2.5 mL of distilled water. Using a syringe, the metal salt solution was added to the ligand solution and mixed thoroughly. The resulting mixture was then heated at 85°C until crystals appeared (~30 min). After crystals were visually detected, the vial was removed from the heating plate and left undisturbed to allow the crystals to grow. The crystals were then collected by vacuum filtration and air-dried.

**ALEN-Ca form II.** A mixture of ALEN, HEDP and Ca(NO₃)₂·4H₂O with a molar ratio (1:1:1) was prepared in distilled water at room temperature as follows. The ligand solution was prepared in a 20 mL vial dissolving 1 mmol (0.2711 g) of solid ALEN and adding 1 mmol (0.23 mL) of aqueous HEDP in 10 mL of distilled water, and heated at 85°C for 15 min. The metal salt solution was prepared dissolving 1 mmol (0.2362 g) of Ca(NO₃)₂·4H₂O in 2.5 mL of distilled water. Using a syringe, the metal salt solution was added to the ligand solution and mixed thoroughly. The resulting mixture was heated at 85°C until crystals appeared (~1 h). After crystals were visually detected, the vial was removed from the heating plate and left undisturbed to allow the crystals to grow. The crystals were then collected by vacuum filtration and air-dried.

**Note:** Employing Ca(NO₃)₂ for the synthesis of ALEN-Ca form II promotes the formation of higher quality single crystals, compared to when CaCl₂ is employed. Both metal salts can be employed for either synthesis, ALEN-Ca forms I or II under their respective synthetic conditions. The only difference is the morphology and quality of the resulting crystals when employing Ca(NO₃)₂ for ALEN-Ca form II.

**ALEN-Zn form I.** A mixture of ALEN and ZnCl₂ with a molar ratio (1:1) was prepared in distilled water at room temperature as follows. The ligand solution was prepared in a 20 mL vial dissolving 1 mmol (0.2711 g) of solid ALEN in 10 mL of distilled water, and heated at 85°C for 30 min. The metal salt solution was prepared dissolving 1 mmol (0.1363 g) of ZnCl₂ in 2.5 mL of distilled water. Using a syringe, the metal salt solution was added to the ligand solution and mixed thoroughly. The resulting mixture was heated at 85°C until crystals appeared (~1 h). After crystals were visually detected, the vial was removed from the heating plate and left undisturbed to allow the crystals to grow. The crystals were then collected by vacuum filtration and air-dried.
ALEN-Zn form II. A mixture of ALEN, HEDP and ZnCl₂ with a molar ratio (1:1:1) was prepared in distilled water at room temperature as follows. The ligand solution was prepared in a 20 mL vial dissolving 1 mmol (0.2711 g) of solid ALEN and adding 1 mmol (0.23 mL) of aqueous HEDP in 10 mL of distilled water, and heated at 85°C for 15 min. The metal salt solution was prepared dissolving 1 mmol (0.1363 g) of ZnCl₂ in 2.5 mL of distilled water. Using a syringe, the metal salt solution was added to the ligand solution and mixed thoroughly. The resulting mixture was heated at 85°C until crystals appeared (20 min.). After crystals were visually detected, the vial was removed from the heating plate and left undisturbed to allow the crystals to grow. The crystals were then collected by vacuum filtration and air-dried.

ALEN-Mg. A mixture of ALEN, HEDP and Mg(NO₃)₂·6H₂O with a molar ratio (1:1:1) was prepared in distilled water at room temperature as follows. The ligand solution was prepared in a 20 mL vial dissolving 1 mmol (0.2711 g) of solid ALEN and adding 1 mmol (0.23 mL) of aqueous HEDP in 10 mL of distilled water, and heated at 85°C for 15 min. The metal salt solution was prepared dissolving 1 mmol (0.2564 g) of Mg(NO₃)₂·6H₂O in 2.5 mL of distilled water. Using a syringe, the metal salt solution was added to the ligand solution and mixed thoroughly. The resulting mixture was heated at 85°C until crystals appeared (~ 1 h). After crystals were visually detected, the vial was removed from the heating plate and left undisturbed to allow the crystals to grow. The crystals were then collected by vacuum filtration and air-dried.

1.3 Polarized Optical Microscopy

Optical micrographs were recorded with a Nikon Eclipse Microscope LV100NPOL, equipped with a Nikon DS-Fi2 camera and NIS Elements BR software version 4.30.01.

1.4 Dissolution Profiles

Dissolution profile in PBS

Stock Solution: Standard stock solution of ALEN was prepared by dissolving 100 mg of the drug in a 100 mL volumetric flask with PBS. More dilute solutions were obtained by appropriate dilution from this stock solution (see Calibration Curve section).
**Calibration Curve:** Accurately measured aliquots of the ALEN stock solution were transferred into a series of 25 mL volumetric flasks to achieve a concentration range between 0.05-0.7 mg/mL. Each solution was completed to the 25 mL mark with PBS.

**Generation of ALEN-Cu complex:** To generate the ALEN-Cu complex, 4 mL of the diluted ALEN solutions were transferred into a series of 25 mL volumetric flasks to achieve an ALEN-Cu concentration range between 0.008-0.11 mg/mL. To each flask, 20 mL of 2.5 mM CuSO₄ solution was added, homogenized and completed to volume with nanopure water. The absorbance of the ALEN-Cu complex was measured at 231 nm against a reagent blank prepared by the addition of 4 mL PBS buffer and 20 mL of the CuSO₄ solution in a 25 mL volumetric flask and completed to volume with nanopure water.

**Dissolution Profile:** Dissolution profiles were recorded for ALEN, Alendronate Sodium Tablets-USP (generic form of Fosamax®), ALEN-Ca form I, ALEN-Ca form II, ALEN-Zn form I, ALEN-Zn form II, and ALEN-Mg. Dissolution tests were performed in 100 mL of PBS buffer (pH = 7.40), controlling temperature at 37°C and stirring at 150 rpm. For the ALEN-based BPCCs, ALEN and tablets, 70 mg of the solid were ground using a mortar and pestle. The powder was added to the PBS solution at the beginning of the dissolution under stirring. Samples of 1.6 mL were collected after 0, 5, 10, 15, 20 and 25 s to record the dissolution for the deglutition profile. For the complete dissolution profile, aliquots of the exact volume were collected from 1-6 min, in one-minute intervals. After the six-minute period, aliquots for 10, 15 and 20 min time points were collected. After collection, the aliquots were filtrated using a PTFE filter. The filtered solutions were placed in 10 mL volumetric flasks. To produce the ALEN-Cu complex, 7.4 mL of the 2.5 mM CuSO₄ solution was added and completed to volume with nanopure water. The absorbance of the formed ALEN-Cu complex was measured at 231 nm against a reagent blank. The reagent blank was prepared by adding 1.6 mL PBS buffer, with 7.4 mL of the 2.5 mM CuSO₄ solution and completed to volume with nanopure water in a 10 mL volumetric flask. Absorbance measurements were performed on an Agilent Technologies Cary Series UV-Vis Spectrophotometer, Cary 100 UV-Vis model; using the UV Cary Scan software version v.20.0.470. All measurements were performed with a 400-200 nm scan.

**Dissolution profile in FaSSGF**
**Stock Solution**: Standard stock solution of ALEN was prepared by dissolving 100 mg of the drug in a 100 mL volumetric flask with FaSSGF. More dilute solutions were obtained by appropriate dilution from this stock solution (see Calibration Curve section).

**Calibration Curve**: Accurately measured aliquots of the ALEN stock solution were transferred into a series of 25 mL volumetric flasks to achieve a concentration range between 0.05-0.7 mg/mL. Each solution was completed to the 25 mL mark with FaSSGF.

**Generation of ALEN-Cu Complex**: To generate the ALEN-Cu complex, 3 mL of the diluted ALEN solutions were transferred into a series of 25-mL volumetric flasks to achieve an ALEN-Cu concentration range between 0.006-0.084 mg/mL. To each flask, 15 mL of 2.5 mM CuSO₄ solution and 5 mL of PBS was added, homogenized and completed to volume with nanopure water. The absorbance of the formed ALEN-Cu complex was measured at 225 nm against a reagent blank prepared by the addition of 3 mL FaSSGF, 5 mL of PBS buffer and 15 mL of the CuSO₄ solution in a 25 mL volumetric flask and completed to volume with nanopure water.

**Dissolution Profile**: Dissolution profiles were recorded for ALEN, Alendronate Sodium Tablets-USP (generic form of Fosamax®), ALEN-Ca form I, ALEN-Ca form II, ALEN-Zn form I, ALEN-Zn form II, and ALEN-Mg. Dissolution tests were performed in 100 mL of FaSSGF (pH = 1.60), controlling temperature at 37°C and stirring at 150 rpm. For the ALEN-based BPCCs, ALEN and tablets, 70 mg of the solid were grinded using a mortar and pestle. The powder was added to the FaSSGF solution at the beginning of the dissolution under stirring. Aliquots of 1.2 mL were collected after 0, 5, 10, 15, 20 and 25 s to record the early-state dissolution profile. For the complete dissolution profile, aliquots of the exact volume were collected from 1-6 min, in one-minute intervals. After the six-minute period, aliquots for 10, 15 and 20 min time points were collected. After collection, the aliquots were filtrated using a PTFE filter. The filtered aliquots were placed in 10 mL volumetric flasks. To produce the ALEN-Cu complex, 2 mL of PBS and 6 mL of the 2.5 mM CuSO₄ solution was added and completed to volume with nanopure water. The absorbance of the formed ALEN-Cu complex was measured at 225 nm against a reagent blank. The reagent blank was prepared adding 1.2 mL FaSSGF solution, with 2 mL PBS buffer and 6 mL of the 2.5 mM CuSO₄ solution and completed to volume with nanopure water in a 10-mL volumetric flask. Absorbance measurements were performed on an Agilent Technologies Cary Series UV-Vis Spectrophotometer, Cary 100 UV-Vis model; using the UV
Cary Scan software version v.20.0.470. All measurements were performed with a 400-200 nm scan.

1.5 Phase inversion temperature (PIT) Determination

A solution of ALEN with a concentration of 10 mg/mL was prepared with nanopure water. In a 20 mL vial, 11 mL of the ALEN solution was added with 3 mL of heptane and 0.9 mL of Brij® L4. The mixture was homogenized with an IKA T10 Basic Ultra Turrax (IKA Works Inc., Wilmington, NC), for 30 sec at a speed of “4” (14,450 rpm equivalent). The experimental set up was assembled using a jacketed beaker with a 20.3 cm (8”) stainless steel RTD temperature probe (VWR®, VWR International). The conductivity of the content was measured with a Fisherbrand Accumet BasicAB30 conductivity meter (Fisher Scientific UK, Loughborough, UK). The bath temperature was controlled with a Julabo F32-ME Refrigerated/Heating Circulator (JULABO GmbH, Seelbach, Germany). Both the vial and the bath contained stir bars stirring at 300 rpm using a VWR® Professional Hot Plate Stirrer (97042-714, VWR®, VWR International). The internal temperature of the emulsion was allowed to reach 2.0°C in the bath before starting the measurements. A temperature profile started at 2°C and end at 62°C at a heating rate of 1°C/min. The conductivity of the mixture was recorded in 1-degree intervals.

1.6 Nanoemulsion Synthesis of nano-Ca@ALEN form II

Temperature Profile

The nanoemulsion synthesis was conducted in a Crystalline (Technobis Crystallization Systems, Alkmaar, Netherlands). Two reactors from the Crystalline were used to complete the synthesis. The temperature profile for the first reactor consisted on setting the initial temperature of the emulsion in the reaction vial at 7°C for 30 min with a 1,250 rpm stirring. Tuning with nanopure water was performed before placing the reaction vial in the reactor. For the second reactor, temperature was set at 45°C before tuning with nanopure water. Temperature was held for 30 min under stirring at 1,250 rpm before heating at 85°C. Addition of the metal salt solution to the reaction vial was performed after reaching 85°C (see Synthesis section). Temperature was held for 30 min allowing the reaction to occur.

Nanoemulsion Synthesis of nano-Ca@ALEN form II.
The pre-homogenized emulsions prepared for the PIT determination were used to perform the nano-Ca@ALEN form II nanoemulsion synthesis. The emulsion was homogenized before being transferred to the reaction vial. From this mixture, 2.5 mL were transferred to a Crystalline reaction vial with a stir bar, sealed with a reflux cap. The vial was placed in the first reactor at 7°C for 30 min under 1,250 rpm continuous stirring. The vial was transferred to the second reactor at 45°C, where the emulsion was stirred for 30 min at 1,250 rpm before heating to 85°C. The metal salt solution was prepared dissolving 4 mmol (0.9448 g) of Ca(NO₃)₂·4H₂O in 10 mL of nanopure water. A volume of 2.5 mL of the metal salt solution was added to the reaction vial via syringe after reaching 85°C. The solution was allowed to continue stirring at 1,250 rpm for 30 min at 85°C. Once the reaction completed, the reaction vial was left undisturbed for 1 h before analyzing the supernatant from the water phase.

1.7 Dynamic Light Scattering (DLS) Measurements

Aliquots of the supernatant from the water phase presumed to contain nano-Ca@ALEN form II nanoparticles were analyzed in a Malvern Panalytical Zetasizer NanoZS (Spectris PLC, Surrey, England) equipped with a He-Ne orange laser (633 nm, max 4 mW). Data was analyzed with Malvern software, version 7.12. The Zetasizer software automatically optimizes the built-in attenuator distance and the number of runs per measurement. The amount of run time was held constant at 10 sec., each measurement was performed in triplicate. The reaction vial from the nanoemulsion synthesis was undisturbed for 1 h prior to analysis. Aliquots of 50 µL of the supernatant from the water phase were transfer in disposable polystyrol/polystyrene cuvettes (REF: 67.754, 10 x 10 x 45 mm, Sarsted, Germany) in a 1:20 dilution ratio with nanopure water. The prepared samples remained undisturbed near the Zetasizer for 30 min prior to the measurements. Size measurements were performed after 2 min of sample equilibration inside the instrument at room temperature (25°C). The refractive index used for the sample was 1.334, which correspond to ALEN in water. This value was determined by measuring an aliquot of ALEN stock solution with a Mettler Toledo Refracto 30GS (Mettler Toledo, Columbus, OH).

1.8 Cytotoxicity Assay
Cell Culture Methods. MDA-MB-231 cell lines were incubated at 37.0°C with 5.0 % CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 5.0 % fetal bovine serum (FBS) and 1.0 % penicillin-streptomycin (Pen-Strep). Cells were passaged once a week at 80.0 % confluence and media was exchanged twice per week.

Seeding. At an 80% confluency, cells were planted at a density of 2.5×10⁵ cells/mL in a 96 well plate and incubated at 37.0 °C with 5.0 % CO₂ for 24 h. Each treatment was performed in triplicate with ALEN or ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN (nanocrystals) at various concentrations. Three well plates were prepared for each time point (24, 48, and 72 h) per treatments with ALEN and ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN (nanocrystals).

Treatments. Cells were treated with ALEN or ALEN-Ca form II (bulk), using serial dilutions between 145.0-0.00 µM, and incubated for 24, 48 and 72 h (37.0°C, 5.0 % CO₂). Also, cells were treated with nano-Ca@ALEN form II (nanocrystals), using 2.0 mL of nano-Ca@ALEN form II (nanocrystals) and incubated for 24, 48 and 72 h (37.0°C, 5.0 % CO₂). For the control groups (0.00 µM) only media (DMEM, 1.0 % Pen-Strep) was used. All experiments were performed in triplicate.

AlamarBlue® Assay. After each time point, cells were treated with 100.0 µL of AlamarBlue® (10% in DMEN, 1.0 % Pen-Strep), and incubated for 4 h (37.0°C, 5.0 % CO₂). The fluorescence was measured at 560.0 nm of excitation and lambda max 590.0 nm of emission. The dose response curves (% cell survival vs. concentration) for ALEN and ALEN-Ca form II (bulk) were employed to calculate the half maximal effective concentration (IC₅₀) values. The percentage of live cells was obtained for each experiment considering the viability of the control group (100.0 %) compared to the cells treated with ALEN and ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN (nanocrystals) solutions.

2. Raman Vibrational Spectroscopy

Raman spectra were recorded at room temperature in a Thermo Scientific DXR Raman microscope, equipped with a 532 nm laser, 400 lines/nm grating, and 50 µm slit. The spectra
were collected over the range of 3,400 to 100 cm\(^{-1}\) by averaging 32 scans with 5 sec exposures in the OMNIC for Dispersive Raman software version 9.2.0. Figures S2.1-S2.5 depict overlay of the experimental Raman spectra of the ALEN-based BPCCs and ALEN.

**Figure S2.1.** Raman spectra overlay of “as received” ALEN (bottom, blue), and the synthetized ALEN-Ca form I BPCC (top, red).

**Figure S2.2.** Raman spectra overlay of “as received” ALEN (bottom, blue), and the synthetized ALEN-Ca form II BPCC (top, red).
**Figure S2.3.** Raman spectra overlay of “as received” ALEN (bottom, blue), and the synthetized ALEN-Zn form I BPCC (top, red).

**Figure S2.4.** Raman spectra overlay of “as received” ALEN (bottom, blue), and the synthetized ALEN-Zn form II BPCC (top, red).
Figure S2.5. Raman spectra overlay of “as received” ALEN (bottom, blue), and the synthetized ALEN-Mg BPCC (top, red)

3. Micro-powder X-ray Diffraction (PXRD)

Powder diffractograms were collected for all polycrystalline samples using a Rigaku XtaLAB SuperNova single micro-focus Cu-Kα radiation (λ = 1.5417 Å) source equipped with a HyPix3000 X-ray detector in transmission mode operating at 50 kV and 1 mA. Powder samples were mounted in MiTeGen micro loops. Powder diffractograms were collected at 100 K over an angular 2θ range between 6 – 60° with a step of 0.01° using the Gandalfi move experiment for powders. Data was analyzed within the CrystAlisPRO software (v1.171.39.45c). Figures S3.1-S3.5 depict overlay of experimental powder pattern of the ALEN-based BPCCs and ALEN.
**Figure S3.1.** Powder X-ray diffractogram overlay of “as received” ALEN (bottom, blue) and synthetized ALEN-Ca form I BPCC (top, red).
Figure S3.2. Powder X-ray diffractogram overlay of “as received” ALEN (bottom, blue) and synthetized ALEN-Ca form II BPCC (top, red).

Figure S3.3. Powder X-ray diffractogram overlay of “as received” ALEN (bottom, blue) and synthetized ALEN-Ca form I BPCC (top, red).
**Figure S3.4.** Powder X-ray diffractogram overlay of “as received” ALEN (bottom, blue) and synthetized ALEN-Zn form II BPCC (top, red).

**Figure S3.5.** Powder X-ray diffractogram overlay of “as received” ALEN (bottom, blue) and synthetized ALEN-Mg BPCC (top, red).
4. Single Crystal X-ray Diffraction (SCXRD)

The crystalline products of the hydrothermal reactions were observed under the microscope using polarized light to assess their crystal quality. Suitable single crystals were mounted in MiTeGen micro loops for structure elucidation. Structural elucidation was performed in either of two instruments. Crystal structure for ALEN-Zn form I was collected in a Bruker AXS SMART APEX-II single crystal diffractometer equipped with a Monocap collimator and APEX-II CCD detector with a Mo-Kα (λ = 0.71073 Å) radiation source operating at 50 kV and 40 mA. The data collection was carried out at 100 K using an Oxford Cryosystems Cryostream 700 cooler. The following crystal structures, ALEN-Ca forms I and II, ALEN-Zn form II and ALEN-Mg were collected with a Rigaku XtaLAB SuperNova single micro-focus Cu-Kα radiation (λ = 1.5417 Å) source equipped with a HyPix3000 X-ray detector in transmission mode operating at 50 kV and 1 mA within the CrystAllisPRO software (v1.171.39.45c). The data collection was carried out at 100 K using an Oxford Cryosystems Cryostream 800 cooler. All crystal structures were solved by direct methods. The refinement was performed using full-matrix least squares on F² within the Olex2 (v1.2) software. All non-hydrogen atoms were anisotropically refined. Figures S4.1-S4.10 display the molecular structure, asymmetric unit and ball stick representation for the refined structures. Figures S4.11-S4.15 depict overlay of the simulated and experimental powder patterns for all structures solved. Figures S4.16-S4.18 show distinct overlays of the simulated powder diffraction pattern overlay for other previously known structures of ALEN coordination complexes in comparison to ones solved within this work.¹⁻⁸ Simulated PXRDs were extracted from the crystallographic information files (CIF files) obtained from the Cambridge Structural Database or obtained within this work.
**Figure S4.1.** Molecular structure of (a) the asymmetric unit, and (b) crystalline packing of ALEN-Ca form I BPCC along c-axis.

**Figure S4.2.** Ball-stick representation (atoms labeled) showing the connectivity between Ca atom and ALEN to form the ALEN-Ca form I BPCC.
**Figure S4.3.** Molecular structure of (a) the asymmetric unit, and (b) crystalline packing of ALEN-Ca form II BPCC along c-axis.

**Figure S4.4.** Ball-stick representation (atoms labeled) showing the connectivity between Ca atom and ALEN to form the ALEN-Ca form II BPCC.
**ALEN-Zn form I**

(a) Molecular structure of (a) the asymmetric unit, and (b) crystalline packing of ALEN-Zn form I BPCC along $a$-axis.

**Figure S4.5.**

(b) Ball-stick representation (atoms labeled) showing the connectivity between Zn atom and ALEN to form the ALEN-Zn form I BPCC.

**Figure S4.6.**
**Figures S4.7.** Molecular structure of (a) the asymmetric unit, and (b) crystalline packing of ALEN-Zn form II BPCC along $a$-axis.

**Figures S4.8.** Ball-stick representation (atoms labeled) showing the connectivity between Zn atom and ALEN to form the ALEN-Zn form II BPCC.
Figure S4.9. Molecular structure of (a) the asymmetric unit, and (b) crystalline packing of ALEN-Mg BPCC along \( \alpha \)-axis.

Figure S4.10. Ball-stick representation (atoms labeled) showing the connectivity between Mg atom and ALEN to form the ALEN-Mg BPCC.
Figure S4.11. Simulated (bottom, blue) and experimental (top, red) powder pattern overlay of ALEN-Ca form I BPCC.

Figure S4.12. Simulated (bottom, blue) and experimental (top, red) powder pattern overlay of ALEN-Ca form II BPCC.
Figure S4.13. Simulated (bottom, blue) and experimental (top, red) powder pattern overlay of ALEN-Zn form I BPCC.

Figure S4.14. Simulated (bottom, blue) and experimental (top, red) powder pattern overlay of ALEN-Zn form II BPCC.
**Figure S4.15.** Simulated (bottom, blue) and experimental (top, purple) powder pattern overlay of ALEN-Mg BPCC.
Figure S4.16. Simulated powder diffraction pattern overlay of synthesized BPCCs (from bottom to top); ALEN-Ca forms I and II (blue and orange), ALEN-Ca forms I and II (light grey and yellow), ALEN-Mg (light blue), contrasted with the reported structures of ALEN coordination complexes ALEN-Cr-Mo (light green, QOBKUP), ALEN-Li (navy blue, EJEZUP), ALEN-Zn (mahogany, ICUVAE), ALEN-Ni (dark grey, ICUTUW), ALEN-Co (brown, ICUTOQ), ALEN-Mn (dark turquoise, ICUVEI), and ALEN-Cd (dark green, ICUVIM). Synthesized BPCCs are not isostructural to any of these previously reported structures.
Figure S4.17. Simulated powder diffraction pattern overlay of synthesized BPCCs containing calcium (Ca$^{2+}$) (from bottom to top); ALEN-Ca forms I and II (blue and orange, respectively), contrasted with other reported structures of ALEN coordination complexes containing the same metal. Reported ALEN coordination complex EMIDIN$^4$ (purple) is isomorphic to ALEN-Ca form II, while the reported complex LILQII$^5$ (red) is different to any other BPCC.
Figure S4.18. Simulated powder diffraction pattern overlay of isostructural synthesized BPCCs (from bottom to top); ALEN-Zn form II and ALEN-Mg (yellow and light blue), contrasted with other isostructural reported structures of ALEN coordination complexes ALEN-Co (light purple, ICUVOS)\textsuperscript{3}, ALEN-Cu (aqua, FAZVOT)\textsuperscript{6}, and ALEN-Ni (fuchsia, ACOZUP)\textsuperscript{7}. 
Figure S4.19. Simulated powder diffraction pattern overlay of synthesized BPCC (from bottom to top); ALEN-Zn form I (light grey), contrasted with other isostructural reported structure of ALEN coordination complex ALEN-Mn (olive green, GIKPEW)\(^8\).
5. Thermogravimetric Analysis (TGA)

Thermographs were recorded in a TGA Q500 (TA Instruments Inc.). Thermographs of ALEN and the ALEN-based BPCCs were collected using a temperature range of 30 – 700°C at 5°C/min under a N₂ gas purge (60 mL/min). In all cases, ~10 mg of powder sample was thermally treated. Data was analyzed with TA Universal Analysis software version 4.3A. Figures S5.1-5.5 depict overlay of thermographs for the ALEN-based BPCCs and ALEN. For the thermal decomposition of the “as received” ALEN, a low temperature (100 °C) weight loss (14.67 %) was observed, which corresponds to the dehydration of the ligand (trihydrate). Subsequently, at higher temperature (225-400 °C) a weight loss of 27.33 % was observed, which corresponds to the degradation of ALEN.

![Thermogravimetric Analysis Graph](image)

**Figure S5.1.** TGA analysis of ALEN-Ca form I BPCC shows a low temperature (140-190°C) weight lost (5.29 %), which was attributed to the decomposition of ALEN, subsequently at higher temperature (290-700°C) a weight loss of 17.77 % occurred, which was attributed to the degradation of calcium.
**Figure S5.2.** TGA analysis of ALEN-Ca form II BPCC shows a low temperature (260-300°C) weight lost (12.14 %), which was attributed to the decomposition of ALEN, subsequently at higher temperature (300-700°C) weight loss of 26.39 % occurred, which was attributed to the degradation of calcium.

**Figure S5.3.** For the ALEN-Zn form I BPCC, a low temperature (200-250°C) weight lost (5.09 %) was observed, which is attributed to the decomposition of ALEN. Subsequently, at higher temperature (300-700°C) the degradation of zinc occurs as accounted by a weight loss of 16.34 %.
Figure S5.4. For the ALEN-Zn form II BPCC, a low temperature (150-300°C) weight lost (12.30 %) was observed, which is attributed to the decomposition of ALEN. Subsequently, at higher temperature (300-700°C) the degradation of zinc occurs as accounted by a weight loss of 25.56 %.

Figure S5.5. TGA analysis of ALEN-Mg BPCC shows a low temperature (260-300°C) weight lost (12.51 %), which was attributed to the decomposition of ALEN. Subsequently at higher temperature (360-700°C) a weight lost (23.48 %) occurred, which was attributed to the degradation of magnesium.

Micrographs and X-ray microanalysis (SEM-EDS) were recorded with a JEOL JSM-6480LV scanning electron microscope with an Evenhart Thomley secondary electron imagining (SEI) detector and an energy dispersive X-ray analysis (EDAX) Genesis 2000 detector. Images were taken with an acceleration voltage of 20 kV, an electron beam of 11 mm width, with a spot size value of 36, SEI signal and high vacuum mode. Figures S6.1.1-S6.1.5 depict the energy dispersive spectra for the synthesized ALEN-based BPCCs. Figures S6.2.1-S6.2.5 represent scanning electron micrographs of the ALEN-based BPCCs showing clusters and single crystals.

6.1. Energy Dispersive Spectra

ALEN-Ca form I

Figure S6.1.1. Energy dispersive spectra of ALEN-Ca form I BPCC displaying the presence of atoms (carbon, nitrogen, oxygen and phosphorous) present in ALEN and the metal (calcium).
ALEN-Ca form II

Figure S6.1.2. Energy dispersive spectra of ALEN-Ca form II BPCC displaying the presence of atoms (carbon, nitrogen, oxygen and phosphorous) present in the ligand and the metal (calcium).

ALEN-Zn form I

Figure S6.1.3. Energy dispersive spectra of ALEN-Zn form I BPCC displaying the presence of atoms (carbon, nitrogen, oxygen and phosphorous) present in the ligand and the metal (zinc).
**ALEN-Zn form II**

Figure S6.1.4. Energy dispersive spectra of ALEN-Zn form II BPCC displaying the presence of atoms (carbon, nitrogen, oxygen and phosphorous) present in the ligand and the metal (zinc).

**ALEN-Mg**

Figure S6.1.5. Energy dispersive spectra of ALEN-Mg BPCC displaying the presence of atoms (carbon, nitrogen, oxygen and phosphorous) present in the ligand and the metal (magnesium).
6.2. Scanning Electron Micrographs

**ALEN-Ca form I**

Figure S6.2.1. Scanning electron micrographs of (a) ALEN-Ca form I BPCC single crystal at x100 magnification, (b) crystals of ALEN-Ca form I BPCC at x100 magnification, and (c) a single crystal of ALEN-Ca form I BPCC at x130 magnification.

**ALEN-Ca form II**

Figure S6.2.2. Scanning electron micrographs of (a), ALEN-Ca form II crystal clusters at x350 magnification, (b) ALEN-Ca form II BPCC single crystals and clusters at x650 magnification, and (c) a single crystal of ALEN-Ca form II BPCC at x2,000 magnification.
**ALEN-Zn form I**

Figure S6.2.3. Scanning electron micrographs of (a) ALEN-Zn form I BPCC clusters at x170 magnification, (b) crystals and clusters of ALEN-Zn form I BPCC at x190 magnification, and (c) a single crystal of ALEN-Zn form I BPCC at x400 magnification.

**ALEN-Zn form II**

Figure S6.2.4. Scanning electron micrographs of (a) ALEN-Zn form II BPCC clusters and single crystals at x130 magnification, (b) single crystals of ALEN-Zn form II BPCC at x250 magnification, and (c) single crystals of ALEN-Zn form II BPCC at x550 magnification.
**Figure S6.2.5.** Scanning electron micrographs of (a) ALEN-Mg clusters and single crystals at x130 magnification, (b) single crystal view of ALEN-Mg clusters at x850 magnification, and (c) an isolated single crystal of ALEN-Mg at x1,000 magnification.
7. Dissolution Profiles for ALEN-based BPCCs

Figures S7.1-S7.4 depict the absorption spectra with the respective calibration curve for the ALEN-Cu complex quantification in FaSSGF and PBS. Figures S7.5-S7.8 represent the deglutition and complete dissolution profiles in FaSGGF and PBS for ALEN, the ALEN Sodium Tablets-USP (generic form of Fosamax®), and the ALEN-based BPCCs.

Calibration Curves

![Absorbance spectra of ALEN-Cu complex](image)

**Figure S7.1.** Absorbance spectra of ALEN-Cu complex presenting a $\lambda_{\text{max}}$ at 231 nm in PBS in the concentration range (0.008-0.11 mg/mL) employed to construct a calibration curve.
Figure S7.2. Calibration curve of ALEN-Cu complex for ALEN quantification for ALEN-based BPCCs in PBS.

Figure S7.3. Absorbance spectra of ALEN-Cu complex presenting a $\lambda_{\text{max}}$ at 225 nm in FaSSGF in the concentration range (0.006-0.084 mg/mL) to construct a calibration curve.
Figure S7.4. Calibration curve of ALEN-Cu complex for ALEN quantification for ALEN-based BPCCs in FaSSGF.

Dissolution profiles

Figure S7.5. Early stage dissolution profile (deglutition) in PBS for ALEN reagent (blue), Alendronate Sodium tablets (red), ALEN-Ca form I (cyan), ALEN-Ca form II (green), ALEN-Zn form I (orange), ALEN-Zn form II (purple) and ALEN-Mg (navy blue) as quantified by the ALEN-Cu complexation method.
For the simulated drug release in physiological pH conditions, dissolution testing was conducted in PBS (pH = 7.40). After comparing the complete dissolution profile in this media for each compound analyzed (Figure S7.6), results demonstrate that the ALEN Sodium Tablets-USP (generic form of Fosamax®) have a higher dissolution rate, reaching the maximum release of the ALEN content (80%) in 25 s. Dissolution for ALEN as a reagent resulted in a slower release but reached the similar amount of dissolved drug as the tablets (~80 %) in 10 min. The ALEN-based BPCCs showed a lower solubility, dissolution rate and equilibrium solubility compared to the tablets and the reagent in this media. The coordination complexes reached a maximum release after 6-10 min and kept stable through 20 min. ALEN-Mg showed a higher dissolution rate between the coordination complexes, releasing a maximum of 63% of the ALEN. The crystal phases containing calcium, presented moderate dissolution rates and release of the drug, showing a 52% and 48% of ALEN dissolved for ALEN-Ca forms I and II, respectively. Coordination complexes with zinc, revealed lower dissolution rates and drug release, which correspond to a percent of ALEN dissolved of 43% and 35% for ALEN-Zn forms I and II, respectively.

Figure S7.6. Complete dissolution profile in PBS for ALEN reagent (blue), Alendronate Sodium tablets (red), ALEN-Ca form I (cyan), ALEN-Ca form II (green), ALEN-Zn form I (orange), ALEN-Zn form II (purple) and ALEN-Mg (navy blue) as quantified by the ALEN-Cu complexation method.
Figure S7.7. Early stage dissolution profile in FaSSGF for ALEN reagent (blue), Alendronate Sodium tablets (red), ALEN-Ca form I (cyan), ALEN-Ca form II (green), ALEN-Zn form I (orange), ALEN-Zn form II (purple) and ALEN-Mg (navy blue) as quantified by the ALEN-Cu complexation method.

To verify the stability of the coordination complexes at its maximum release, a complete dissolution profile in FaSSGF (pH = 1.60) was performed. This dissolution profile (Figure S7.8) showed that ALEN reaches the maximum release of the drug (100%) faster (10 s.) compared to the other compounds. The ALEN Sodium Tablets-USP (generic form of Fosamax®) achieved a 100% release in 25 s. As observed in these results, ALEN is released from the tablets in the stomach leading to poor absorption in the intestines (up to 10%). All ALEN-based BPCCs presented lower dissolution and equilibrium solubility compared to ALEN and the commercial tablets. In the case of the coordination complexes with calcium coordinates, a higher solubility and dissolution rate was observed among other ALEN-based BPCCs. The maximum release of the ALEN content from their structure was 100% for each after 15 min. ALEN-Mg presented a moderate dissolution rate and solubility in the media, reaching an average of 45% as its maximum release of ALEN after 10 min. The moderate to lower dissolution rates correspond to the coordination complexes containing zinc, which reached a maximum release of 66% and 35% for ALEN-Zn forms I and II, respectively.
Figure S7.8. Complete dissolution profile in FaSSGF for ALEN reagent (blue), Alendronate Sodium tablets (red), ALEN-Ca form I (cyan), ALEN-Ca form II (green), ALEN-Zn form I (orange), ALEN-Zn form II (purple) and ALEN-Mg (navy blue) as quantified by the ALEN-Cu complexation method.
8. Phase Inversion Temperature (PIT) Determination

Figure S8.1 depicts the PIT determination curve for an aqueous ALEN solution in heptane and Brij L4®.

**Figure S8.1.** Nanoemulsion PIT determination of aqueous ALEN solution, showing the phase inversion occurs at approximately ~ 17°C (dashed line). Phase inversion starts at 9°C and ends at 24°C as depicted by the light orange region.
9. DLS Measurements

Tables S9.1-S9.3 summarize the DLS parameters and values after analyzing three independent PIT-nanoemulsion synthesis yielding nano-Ca@ALEN form II nanoparticles. Figures S9.1-S9.3 depict the DLS spectra showing the particle size distribution of the synthesized nano ALEN-based BPCC from the three synthesis attempts.

Table S9.1. Dynamic light scattering parameters and values after analyzing the PIT-nanoemulsion synthesis of nano-Ca@ALEN form II.

<table>
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<tr>
<th>Run</th>
<th>Size (d.nm)</th>
<th>% Intensity</th>
<th>St Dev (d.nm)</th>
<th>PDI</th>
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Figure S9.1. DLS spectra showing particle size distribution (d. nm) for each run (red, blue and green) and an average of 383.9 d. nm (purple) of nano-Ca@ALEN form II.
Table S9.2. Dynamic light scattering parameters and values after analyzing the PIT-nanoemulsion synthesis of nano-Ca@ALEN form II.

<table>
<thead>
<tr>
<th>Run</th>
<th>Size (d.nm)</th>
<th>% Intensity</th>
<th>St Dev (d.nm)</th>
<th>PDI</th>
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Figure S9.2. DLS spectra showing particle size distribution (d. nm) for each run (red, blue and green) and an average of 495.3 d. nm (purple) of nano-Ca@ALEN form II.
Table S9.3. Dynamic light scattering parameters and values after analyzing the PIT-nanoemulsion synthesis of nano-Ca@ALEN form II.

<table>
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<th>Run</th>
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<th>% Intensity</th>
<th>St Dev (d.nm)</th>
<th>PDI</th>
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Figure S9.3. DLS spectra showing particle size distribution (d. nm) for each run (red, blue and green) and an average of 482.2 d. nm (purple) of nano-Ca@ALEN form II.
10. Polarized Optical Microscopy/Powder X-ray Diffraction (nano-Ca@ALEN)

Agglomerated nanocrystals of nano-Ca@ALEN from II were mounted in 10 µm MiTeGen micro loop. Optical micrographs were recorded with a Nikon Eclipse Microscope LV100NPOL, equipped with a Nikon DS-Fi2 camera and NIS Elements BR software version 4.30.01. Powder X-ray diffraction analysis parameters were maintained the same as in Section 3. Figure S.10.1 shows the representative sample mounted in the micro loop. Figure S.10.2 depicts the PXRD overlay of ALEN, ALEN-Ca form II bulk crystals and nano-Ca@ALEN nanocrystals.

![Figure 10.1](image)

**Figure 10.1.** Polarized optical micrographs of nano-Ca@ALEN form II agglomerated nanocrystals mounted in a 10 µm MiTeGen micro loop, observed at (a) 10x magnification and (b) 20x magnification.

![Figure 10.2](image)

**Figure 10.2.** Powder X-ray diffractogram overlay of “as received” ALEN (black), ALEN-Ca form II bulk crystals (red), and nano-Ca@ALEN form II nanocrystals (light blue).
11. Cytotoxicity Assay and IC\textsubscript{50} Determination

Treatments were performed at 24, 48, and 72 h for ALEN, ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN form II (nanocrystals). Cells were incubated in a Thermo Scientific Forma Steri-Cycle i160 CO\textsubscript{2} Incubator with Stainless Steel Chamber. Cell viability were assayed with the AlamarBlue\textsuperscript{®} method and based on difference in fluorescence between treated and non-treated cultures. The fluorescence was measured in an Infinite M200 PRO Tecan Microplate Reader (\lambda\textsubscript{exc} = 570 nm, \lambda\textsubscript{em} = 590 nm) at room temperature. The dose response curves (% cell survival vs. concentration) for ALEN, ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN (nanocrystals) were employed to calculate the half-maximal inhibitory concentration (IC\textsubscript{50}) and the percentage of relative cell viability (%RCV) values for the treatments using GraphPad Prism 8. Table S11.1 and S11.2 summarize the values obtained for cell live percentage and IC\textsubscript{50} determinations, and relative cell viability (%) of the cell line treated respectively. Figures S11.1 and S11.2 depict the IC\textsubscript{50} curves and relative cell viability (%) graphs respectively.

Table S11.1. Minimum percentage of cell live and IC\textsubscript{50} values after 24, 48 and 72 h of treatment with ALEN, ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN form II (nanocrystals) using the MDA-MB-231 cell line.

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<td>72 h</td>
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<td>ALEN-Ca form II (bulk crystals)</td>
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<td>72 h</td>
<td>15.50</td>
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</table>
**Figure S11.1.** IC$_{50}$ curves for ALEN (top) and ALEN-Ca form II (bulk crystals, bottom) using MDA-MB-231 cell line at (a) and (d) 24 h, (b) and (e) 48 h, (c) and (f) 72 h, respectively.

**Table S11.2.** Relative cell viability (%) of MDA-MB-231 cell line treated with ALEN, ALEN-Ca form II (bulk crystals) and nano-Ca@ALEN form II (nanocrystals) at 7.5 µM, 15 µM, and 30 µM for 24, 48 and 72 h of treatment.

<table>
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<th>Time Points</th>
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<th>15 µM</th>
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<td>100 ± 7</td>
<td>104 ± 7</td>
<td>44 ± 3</td>
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12. References


