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Bisphosphonate-Functionalized Hydroxyapatite Nanoparticles for the Delivery of the Bromodomain Inhibitor JQ1 in the Treatment of Osteosarcoma

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Abstract

Osteosarcoma (OS) is one of the most common neoplasia among children, and its survival statistics have been stagnating since the combinatorial anticancer therapy triad was first introduced. Here, we report on the assessment of the effect of hydroxyapatite (HAp) nanoparticles loaded with medronate, the simplest bisphosphonate, as a bone-targeting agent and JQ1, a small-molecule bromodomain inhibitor, as a chemotherapeutic in different 2D and 3D K7M2 OS in vitro models. Both additives decreased the crystallinity of HAp, but the effect was more intense for medronate because of its higher affinity for HAp. As the result of PO_4^{3-} - NH^+ binding, JQ1 shielded the surface phosphates of HAp and pushed its surface charge to more positive values, exhibiting the opposite effect from calcium-blocking medronate. In contrast to the faster and more exponential release of JQ1 from monetite, its release from HAp nanoparticles followed a zero-order kinetics, but 98% of the payload was released after 48 h. The apoptotic effect of HAp nanoparticles loaded with JQ1, with medronate and with both JQ1 and medronate, was selective in 2D culture: pronounced against the OS cells and nonexistent against the healthy fibroblasts. While OS cell invasion was significantly inhibited by all of the JQ1-containing HAp formulations, that is, with and without medronate, all of the combinations of the targeting compound, medronate, and the chemotherapeutic, JQ1, delivered using HAp, but not HAp alone, inhibited OS cell migration from the tumor spheroids. JQ1 delivered using HAp had an effect on tumor migration, invasion, and apoptosis even at extremely low, subnanomolar concentrations, at which no effect of JQ1 per se was observed, meaning that this form of delivery could help achieve a multifold increase of this drug's efficacy. More than 80% of OS cells internalized JQ1-loaded HAp nanoparticles after 24 h of incubation, suggesting that this augmentation of the activity of JQ1 may be due to the intracellular delivery and sustained release of the drug enabled by HAp. In addition to the reduction of the OS cell viability, the reduction of the migration and invasion radii was observed in

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Notes

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OS tumor spheroids challenged with even JQ1-free medronate-functionalized HAp nanoparticles, demonstrating a definite anticancer activity of medronate alone when combined with HAp. The effect of medronate-functionalized JQ1-loaded HAp nanoparticles was most noticeable against OS cells differentiated into an osteoblastic lineage, in which case they surpassed in effect pure JQ1 and medronate-free compositions. The activity of JQ1 was mediated through increased Ezrin expression and decreased RUNX2 expression and was MYC and FOSL1 independent, but these patterns of gene expression changed in cells challenged with the nanoparticulate form of delivery, having been accompanied by the upregulation of RUNX2 and downregulation of Ezrin in OS cells treated with medronate-functionalized JQ1-loaded HAp nanoparticles.

Graphical Abstract

Keywords

bisphosphonate; calcium phosphate; cancer; JQ1; hydroxyapatite; monetite; nanoparticles; osteosarcoma

1. INTRODUCTION

Osteosarcoma (OS), a malignant tumor of the skeleton, is one of the three most common types of neoplasia among children,¹ with ~50 000 cases reported worldwide for children under 15 years of age.² Although survival of the patients improved drastically following the addition of the combination of systemic chemotherapy and surgical debridement to radiation regimens,³ it has been stagnating ever since, craving for the evolution of more sophisticated treatments. As with osteomyelitis, a problem associated with OS is its localization in regions largely cut off from the vasculature, rendering systemically administered chemotherapeutics chiefly ineffective. Second, OS is pronouncedly metastatic, requiring therapeutic platforms capable of discerning the tumor cells and their micro-populations from the healthy niche in which they thrive. Colloidal formulations of hydroxyapatite (HAp) nanoparticles conjugated with a therapeutic and a targeting agent are designed and developed in this study as a potential basis for one such advanced platform.

With OS being the malignant neoplasm of bone, HAp, the sole inorganic component of all hard tissues in the human body, including bone, presents a natural choice for the drug delivery carrier in the localized treatment of this disease. The approach used in this study stems from the idea that like should cure like and that bone diseases and deformities are best targeted and treated using one or more components of bone itself. Correspondingly, we believe that HAp, the mineral component of hard tissues in the human body, could be an ideal carrier for drug delivery to a variety of, if not all, bone pathologies. Moreover, because of this prime role that HAp plays in the vertebrate world, these times may bring about an

onset in the expansion of scope and imagination with which HAp will be investigated for applications in tissue engineering and drug delivery. Although HAp is a solid compound with obvious deficiencies, for example, negligible tensile strength, low capacity for stable chemical conjugation with active compounds, weak morphological control, and a large propensity for particle aggregation, the fact that it has been selected through the evolution to become the central ingredient of the vertebrate skeleton has appealed to researchers the world over and impelled them to study it for various medical applications other than filling the defected bone. Indeed, over time it has become clear that HAp is a material with an extraordinary array of attractive properties and functions achievable under precise synthesis regimens.⁴ One of the important roles for which HAp nanoparticles have been investigated is that of targeted and sustained deliverers of drugs. As a result, their dominant current biomedical use as the strengthening and osteoconductive component of tissue engineering constructs is expected to be expanded with this entrance into the gene and drug delivery realm.⁵

JQ1, [(*R,S*)-4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-1-thia-5,7,8,9*a*-tetraaza-cyclopenta[*e*]azulen-6-yl]-acetic acid *tert*-butyl ester (Figure 1), is a high-affinity small molecule bromodomain inhibitor discovered in 2010⁶ and intensely researched for its potential clinical applications as a chemotherapeutic for treating cancer.⁷ JQ1 owes its antitumor efficacy to the cocrystal structure with BRD4, a member of the bromodomain and extra-terminal (BET) epigenetic protein family. BRD4 is involved in a number of processes in the mitotic progression cycle, from upregulating the expression of growth-promoting genes to mediating the elongation of RNA during transcription.⁸ Because of the shape complementarity with the acetyl-lysine (Kac) binding pocket of BRD4, JQ1 mimics the structure of Kac inhibitor, blocks BRD4, and prevents its binding to the acetylated histone tails. With the prominent role BRD4 plays in the proliferation of numerous malignant tissues, such an inhibitory activity of JQ1 puts it in the position of a good candidate for an anticancer drug. Because BRD4 assists in anchoring the c-Myc promoter and subsequently signals c-Myc transcription, JQ1 has been also considered as an alternative to scarce direct inhibitors of c-Myc, which suffer from the lack of clear ligand binding sites on this important oncoprotein.⁹ Although JQ1 is a molecule permeable to cells thanks to its comparatively high hydrophobicity, it is also typified by a very short half-life, ~1 h,¹⁰ and its clinical prospect may be tied to the finding of the right conjugates or particulate carriers for it. Here, we report on the investigation of its therapeutic efficacy when delivered to the cells in vitro using HAp nanoparticles. In addition to HAp, the most naturally abundant alkaline calcium phosphate (CaP) phase, we probed the interaction of JQ1 with dicalcium phosphate anhydrous (DCP, also called monetite), a common hydrogenated form of CaP stoichiometry. From the perspective of the targeted delivery, finding the right platform for the delivery of chemotherapeutics is necessary to (a) reduce systemic drug distribution and the associated toxicity, and (b) increase the amount of the drug reaching the target cell or a tissue. HAp nanoparticles are uptaken by the cells with great efficacy, the reason for which they present one of the safest and the most viable alternatives to viral vectors as intracellular carriers of nucleic acids¹¹ and small molecules.¹² In addition to this, HAp nanoparticles per se have exhibited anticancer effects against several cancer cell lines.¹³ Moreover, their inhibitory effect on cell proliferation, caused by decreasing the protein synthesis, not releasing reactive

oxygen species, was reported to be selective, in the 55–90% range for cancer cell lines, for example, MGC803, Os-732, and Bel-7402, and in the 13–30% range for normal cell lines, for example, L-02, MRC-5, and HaCaT.¹⁴ This is yet another reason to explore the synergy between the chemotherapeutic drug and the chemotherapeutic carrier, in this case JQ1 and HAp.

The average percentage of cancer cells comprising a malignant tumor ranges from 3% to 15%,¹⁵ highlighting the necessity for finding therapies capable of not only targeting the tumor located within a healthy tissue, but also of distinguishing cancer cells from the healthy ones at the single-cell level. In addition, there is a more pressing medical need to find ways to treat malign metastases than primary tumors,¹⁶ in which case the pervasion of cancer cells across the healthy cell population becomes directly proportional to the difficulty of their eradication. Needless to mention, one of the primary sites of metastases is bone, reiterating the importance of adding the targeting functionality to HAp/drug complexes. Therefore, the drug delivery vehicle composed of HAp nanoparticles as the carrier and JQ1 as the drug was enriched in this study with the medronate ion, the smallest bisphosphonate, as a bone-targeting ligand. Bisphosphonates have been known for their ability to bind to HAp in vivo,¹⁷ the reason for which intravascularly administered medronate conjugated to ^{99m}Tc has been used since the 1970s as the bone-targeting agent in bone scintigraphy.¹⁸ Bound to the bone mineral, bisphosphonates also become uptaken by osteoclasts during the process of bone resorption, an integral part of bone remodeling. This uptake exerts an inhibitory and/or apoptotic effect on their activity,¹⁹ justifying the use of bisphosphonates in the treatment of osteoporosis.²⁰ Conjugated to chemotherapeutics, bisphosphonates have also been used to help the drug target bone cancer,²¹ reducing both the inhibitory IC₅₀ values and the side effects associated with the spread of the drug outside of the target tissue.^{22,23} Previous studies have also shown that specific bisphosphonates, albeit excluding medronate, exhibit natural anticancer properties and can act in synergy with the chemotherapeutic agents.^{24–27} Medronate also shows affinity for the metastatic bone cancer lesions and other areas of abnormal bone development, the reason for which it is being used as a tracer in bone scintigraphy.²⁸ In turn, it also has a high affinity for HAp,²⁹ allowing for a relatively easy functionalization of HAp nanoparticles. The addition of medronate to the synergistic equation involving HAp and JQ1 presents another aspect of this study, whose main goal is to assess the potential of using nanoparticulate HAp, the synthetic version of the bone mineral, as a carrier of a prospective chemotherapeutic in the treatment of OS, a major malignancy of the skeletal system.

2. MATERIALS AND METHODS

2.1. Synthesis of HAp Nanoparticles and Their Loading with Medronate and JQ1

JQ1 (5 mg, 10.9 μ mol) was generously provided by James Bradner (Bradner Laboratory, Harvard Medical School, Boston, MA). It was dissolved in dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Waltham, MA) and divided into 10 mM aliquots, which were frozen at -20°C . When needed for loading, the 25 μL JQ1 aliquots were gently thawed in a 37°C water bath and diluted in ethanol. To synthesize HAp, 400 mL of a 0.06 M aqueous $\text{NH}_4\text{H}_2\text{PO}_4$ (Fisher Scientific) solution containing 25 mL of 28% NH_4OH (Sigma-Aldrich)

was added dropwise to a 400 mL 0.1 M aqueous solution of $\text{Ca}(\text{NO}_3)_2$ (Fisher Scientific), which contained 50 mL of 28% NH_4OH . The beaker was kept heated on a plate at 50 °C and stirred vigorously at 400 rpm. Once the addition of $\text{NH}_4\text{H}_2\text{PO}_4$ had been completed, the suspension was brought to a boil, then immediately removed from the plate and left to air cool at room temperature. Stirring was suspended and the precipitate was left to age along with its supernatant under ambient conditions for 24 h. In case of loading with medronate, this aqueous solution additionally contained 1.7 M methylenediphosphonic acid (Sigma-Aldrich). After 24 h, the precipitate was separated into 50 mL Falcon tubes and centrifuged/washed three times with deionized H_2O (5 min at 5000 rpm), and left to dry in a vacuum oven (Accu Temp-19, Across International) ($p = -20$ mmHg) at 80 °C. A procedure similar to HAp synthesis was used to synthesize JQ1-loaded DCP, but involving (a) 50 mL of 0.25 M $\text{NH}_4\text{H}_2\text{PO}_4$ containing 0.1 mL of concentrated, 28% NH_4OH to make pH 6.8 and (b) 50 mL of 0.33 M CaNO_3 . To load the nanoparticles with JQ1, 1 g of the precipitate was resuspended in 12.5 mL of ethanol containing 10 nM JQ1 using a digital vortex mixer (Fisher Scientific) and let dry in vacuum oven at 80 °C, until the alcoholic solution evaporated. To differ between weakly and stably bound JQ1 loaded via evaporation, lest the encapsulation efficiency (EE) be 100%, JQ1-loaded HAp/DCP was immersed for 1 min in DMSO, and the amount of the drug released to the medium was compared to that initially added. EE was calculated from the following equation, where m_o is the total amount of the drug initially added and m_t is the amount of the drug released to DMSO:

$$\text{EE} = (m_o - m_t) / m_o \times 100 (\%) \quad (1)$$

The drug loading efficiency (LE) was estimated by normalizing the amount of the encapsulated drug (m_d) to the total weight of the carrier (m_c):

$$\text{LE} = m_d / m_c \times 100 (\%) \quad (2)$$

2.2. Physicochemical Characterization

Scanning electron microscopy (SEM) analysis was performed on a JEOL JSM 6320F- FESEM operated in a 1–4 kV voltage range and 8 μA beam current. ImageJ (NIH, Bethesda, MD) was used to derive the average particle size from SEM images. Zeta potential of particles in suspension was measured using a Zetasizer Nano-ZS (Malvern) dynamic light scattering (DLS) device. X-ray diffraction (XRD) was carried out on a Bruker D2 Phaser diffractometer in 10–90° 2θ range, with the step size of 0.002° and 1.5 s of scan time per step. The Scherrer equation applied on the most intense reflections of HAp in the 2θ range used, (211) at 31.86°, was used to estimate the average crystallite size from the diffraction peak half-widths in DIFFRAC.EVA software.

2.3. Cell Culture

K7M2 murine OS cells (ATCC) and mouse primary lung fibroblasts isolated from 9 week old C57B6/J mouse lungs were cultured at 37 °C and 5% CO_2 in MEM- α (Gibco) media supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco) to prevent bacterial and

fungal contamination. All assays were performed on undifferentiated K7M2 cells unless otherwise noted. Osteoblastic differentiation was performed by adding 50 $\mu\text{g}/\text{mL}$ L-ascorbic acid and 10 mM β -glycerophosphate to the cell culture medium.

2.4. Immunofluorescent Staining and Confocal Microscopy

Cells were fixed and stained 48 h after the treatment for nuclei, f-actin, and HAp. Cells were fixed for 5 min in 4% paraformaldehyde (PFA) and washed 3×10 min in PBS, then blocked at room temperature for 1 h in the blocking solution (2% bovine serum albumin, 0.5% Triton-X in PBS), washed 3×10 min with PBS again, and stained with Alexa Fluor 568 phalloidin (1:400), OsteoImage reagent (1:100), and NucBlue Fixed Cell ReadyProbes reagent (Molecular Probes, Life Technologies) for 1 h at room temperature. After the incubation, cells were washed 3×5 min with OsteoImage wash buffer and mounted using Prolong Diamond mounting media (Life Technologies). Images were acquired on a Nikon T1-S/L100 inverted epifluorescent confocal microscope. All of the samples were analyzed in triplicate.

2.5. Flow Cytometry

In addition to fluorescent cell staining, nanoparticle uptake was analyzed using flow cytometry (Becton Dickinson FACSVerse). K7M2 OS cells were grown to confluency in aforementioned growth conditions in 24-well plates before 5 mg/mL of HAp or JQ1-loaded HAp nanoparticles was added to them. After 24 h incubation at 37 °C, the cells were rinsed with PBS and trypsinized using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA). The trypsinized cells were fixed in 4% PFA for 10 min, then centrifuged at 2000 rpm for 5 min and washed once with PBS. HAp nanoparticles were then stained with OsteoImage agent for 30 min and then washed with PBS. Non GFP/FITC expressing cells were gated using no particle treated control cells, while GFP positive gate was determined using a line of constitutively active GFP-expressing fibroblasts created earlier by transfecting human lung fibroblasts with a monomeric eGFP/N1 plasmid and selecting GFP positive cells using 0.3 $\mu\text{g}/\text{mL}$ G418, also called gentamicin. Constitutively GFP expressing fibroblasts were trypsinized using 0.25% trypsin-EDTA, but were not fixed with PFA because PFA would quench the GFP signal. For this reason, they were used as positive controls in a live form. All of the samples were analyzed in duplicate.

2.6. MTT Viability Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was prepared according to the manufacturer's instructions (Vybrant MTT Cell Proliferation Assay Kit V-13154). Cells were plated as described in section 2.3, cultured until confluency, and challenged with either 5 mg/mL of HAp nanoparticles or 1 μM JQ1 (unless otherwise noted) and incubated at 37°C with 5% CO₂. The assay was performed after 24, 48, and 72 h according to the manufacturer's instructions, and absorbance was measured at 540 nm using a microplate reader (FLUOstar Omega, BMG LABTECH).

2.7. Tumor Spheroid Migration and Invasion Assays

The hanging drop method was used to form tumor spheroids for migration assays. In total, 2×10^3 K7M2 cells in 20 μL of the culture medium were pipetted in one drop onto the lid of a 100 cm dish. Forty drops were plated onto one lid, and the dish itself was filled with 10 mL of sterile PBS. Once all of the drops were plated, the lid was inverted back onto the dish and spheroids were allowed to grow in the humidified chamber for 4 days at 37 °C with 5% CO_2 . After 4 days, the spheroids were harvested from the dish lids and used in the migration assay. Tumor spheroids for the invasion assay were formed in 96-well low-adhesion plates (Corning). Two $\times 10^4$ cells were seeded into 96-well plates and incubated at 37 °C with 5% CO_2 for 4 days. After 4 days, the spheroids were removed individually from 96-well plates for use. For the migration assays, wells in a 24-well plate were coated with 0.1% gelatin for 30 min at 37 °C. Wells were then washed with PBS three times. A single spheroid was placed into the gelatin coated well with 1 mL of the culture medium. Five mg/mL of HAp nanoparticles or 1 μM of JQ1 was then added to the wells and incubated at 37 °C with 5% CO_2 . After 4 days, the migrating distance of the leading edge of the cells migrating out of the spheroid was measured using ImageJ (NIH, Bethesda, MD). For the invasion assays, a single spheroid was imbedded into 100 μL of Matrigel (Corning) in a 48-well plate. The Matrigel was allowed to polymerize at 37 °C for 30 min. After 30 min, the complete culture media were added to wells and incubated at 37 °C with 5% CO_2 for 4 days. The area of the invading cells into the extracellular matrix (ECM) and the area of the tumor spheroid after 4 days of treatment were then measured using ImageJ.

3. RESULTS AND DISCUSSION

3.1. Physicochemical Properties of HAp Nanoparticles Loaded with Medronate (BP) and/or JQ1

The sizes and morphologies of HAp nanoparticles synthesized alone and in the presence of different additives are displayed in Figure 2a and b–d, respectively. Interestingly, individual additives, JQ1 and medronate (BP), affected HAp particle size and shape, respectively (Figure 2b,c), whereas their combined addition affected neither the particle size nor shape (Figure 2d). Thus, JQ1 increased the particle size of HAp from 18.4 nm on average to 30.5 nm, retaining the round, but sharply edged shape of the particles (Figure 2c). In contrast, the addition of BP elongated HAp particles, but had no effect on the average particle size (20 nm) (Figure 2b). Previous studies have shown that the elongation of HAp particles is facilitated at low Ca/P molar ratios.³⁰ Even at identical supersaturations, solutions with lower $[\text{Ca}]/[\text{H}_x\text{PO}_4^{3-x-}]$ yield faster step migration kinetics during crystal growth,³¹ implying that the natural tendency of hexagonal crystalline symmetries toward particle elongation becomes augmented at low Ca/P molar ratios. By binding to Ca^{2+} ions on the particle surface (Figure 3a) and disabling them from acting as binding sites for the growth units from the solution, BPs effectively decrease the Ca/P ratio and promote particle elongation, presumably along the most favorable, *c* axis of the hexagonal crystal lattice of HAp. Additionally, most prismatic, (*hk*0) faces display an excess of Ca^{2+} ions, as opposed to the basal, (001) plane that displays an excess of OH^- and PO_4^{3-} groups.³² By binding exclusively to Ca^{2+} ions, the surface concentration of BP molecules is expected to be greater on (*hk*0) faces, blocking their growth and promoting the elongation of the particles in the

(001) crystallographic direction. JQ1, in contrast, is expected to bind to HAp through electrostatic attraction between the three potentially protonated nitrogen atoms comprising the tetraazacyclopenta[e]azulene ring and the triply charged PO_4^{3-} ions of HAp, exerting an opposite effect on the Ca/P surface molar ratio (Figure 3b). It is expected that the three sp^2 hybridized nitrogen atoms of the azulene ring would be the most probable charge carriers in the molecule. Correspondingly, Figure 1b shows that the highest electrostatic potential regions in the molecular structure of JQ1 exist around these three nitrogen atoms, in addition to the alkoxy region of the *tert*-butyl ester terminus. The compound is incapable of undergoing carbocation at the 4-chlorophenyl ring because chlorine is covalently bound and its dissociation would be entailed by rapid hydrogenation. Also, hydrolysis of the *tert*-butyl ester, forming negatively charged carboxylate group free to bind to Ca^{2+} ions of HAp, would require more reactive solvents, while the single nitrogen with a lone pair involved in the π system is less alkaline than its three sp^2 counterparts and thus less prone to protonation, given that the latter would have a more disruptive effect on the aromaticity of the ring. Although theoretical, density functional studies delineated hydrogen-bonded interactions, involving the OH^- group of HAp, as dominant in binding ibuprofen to HAp,³³ they ignored the diffusivity and the intense exchange of this group across the solid/solution interface. Despite the electrostatic interaction with HAp, the main effect of the physisorption of JQ1 is exhibited through increasing the hydrophobicity of the surface and thus affecting the growth habit of individual particles. All of the particles were in the nanosized range (<100 nm, Figure 2f), including those of DCP, the additional CaP phase prepared for comparison purposes and loaded with JQ1 (Figure 2e,f).

Zeta potential curves as the function of pH display the characteristic trend of decrease in value and a shift toward negative values as pH is increased (Figure 4). This is explained by the increase in the concentration of negatively charged OH^- ions and the corresponding decrease in the concentration of free protons in the double charge layer as pH is increased. JQ1 binds with its protonated azulene ring to the surface phosphates of HAp, shielding them and thus effectively pushing the surface charge to more positive values. Correspondingly, the zeta potential of HAp/JQ1 is more positive and/or less negative at any given pH than that of pure HAp (Figure 4). In contrast, bisphosphonates are expected to bind to Ca^{2+} ions on the surface of HAp. As a result, the point of zero charge (PZC) of medronate-loaded HAp (HAp/BP) is pushed to a significantly lower value of 2.3 as compared to that of 3.9 for HAp and 5.3 for HAp/JQ1 (Table 1).

Figure 5a demonstrates that medronate exerts an effect on the formation of HAp by decreasing its crystallinity. Thus, HAp/BP exhibits a completely amorphous XRD pattern, as opposed to HAp, which exhibits a polycrystalline one. That this effect is typical for bisphosphonates in general was verified by substituting medronate with alendronate and observing the same effect, albeit lessened in intensity. Even at a 4 times higher concentration, alendronate does not diminish the crystallinity of HAp as much as medronate (Figure 5a,b). HAp precipitated with 7 mM alendronate does not become completely amorphized (Figure 5b), as is the case with HAp precipitated with 1.7 mM medronate (Figure 5a). It is usually assumed that the bisphosphonates containing a nitrogenated side chain, for example, alendronate, bind to HAp stronger than medronate as the simplest, side-chain-free bisphosphonate;³⁴ however, it is possible that their polar nature is attracted to the

polar aqueous medium too, thus weakening the interaction with the growing crystal surface and having less of an amorphizing effect on it. It is also often being said that bisphosphonates do not interfere with the mineralization process on the atomic scale, but have an inhibitory^{35–38} and apoptotic³⁹ effect on the osteoclasts instead, alongside restraining the proliferation of osteocytes.^{40,41} These data oppose this view by demonstrating a drastic effect that bisphosphonates have on the crystallization process. By binding strongly to the surface of the growing crystals, they may block their growth, be it aggregational or diffusional, and contribute to the overall reduction of crystallinity. The amorphization effect due to the addition of alendronate up to 28 mM was present but to a drastically lower extent in a previous HAp/BP coprecipitation study, the reason being the 10× higher concentrations of ionic precursors used in it.⁴² By using lower Ca^{2+} and PO_4^{3-} concentrations in this study, the rate of the crystal growth is lowered, increasing the intensity of the effect BP exerts on it. JQ1, in contrast, does not decrease the crystallinity of HAp or DCP precipitated in its presence (Figure 5c) anywhere as noticeably as BP does, the reason being its comparatively hydrophobic nature and low affinity for HAp, as demonstrated by their minimal binding in water. Therefore, the average crystallite size of HAp drops from 34.4 to 21.4 nm when coprecipitated with JQ1, whereas it adopts a completely amorphous structure when coprecipitated with BP.

The release of JQ1 from HAp particles is not sustained, given that 98% of the drug payload was released in the first 48 h (Figure 6). This is a consequence of the pronounced hydrophobicity of the drug. The only hydrophilic region, capable of stably binding to CaP, is the methyl side chain forming after the dissociation of the chloride ion. In contrast to bisphosphonates, whose central portions of the molecule engage in double chelation with Ca^{2+} ions on the surface of HAp, such bonds are not formable between solid HAp and JQ1. As the result, the adsorption of JQ1 onto HAp is weak, leading to the relatively fast release of the drug into the liquid medium. Still, the burst release of the drug was not prominent and the release rate followed a linear function in the first 48 h (Figure 6). In an attempt to extend the release, HAp as the hydroxylated form of CaP forming under neutral and alkaline conditions was substituted with DCP, a hydrogenated form of CaP forming under acidic conditions. The rationale for this comparison was that the precipitation at alkaline pH would yield a higher surface ratio between Ca^{2+} and PO_4^{3-} ions than the precipitation at acidic pH, partly because of the higher Ca/P molar ratio of HAp than that of DCP (1.67 vs 1) and partly because the dominance of free OH^- groups over free protons in alkaline conditions would favor Ca^{2+} on the particle/solution interface, whereas the dominance of free protons over free OH^- groups in acidic conditions would favor PO_4^{3-} as the terminal groups. It is for this reason that biological molecules, almost always negatively charged, bind well to HAp. With JQ1 presumably interacting with HAp through its protonated azulene ring, it was expected that it should bind better to PO_4^{3-} than to Ca^{2+} and that DCP would provide for a better physisorption substrate than HAp. Additionally, the probability for neutralization of the charge carried by $-\text{NH}^+$ groups on the azulene ring is lower at the low pH conditions under which DCP is synthesized. Concordantly, it was previously shown that higher Ca/P ratios in HAp directly coincide with a greater affinity for platinum complexes with bisphosphonates,⁴³ the reason being a more effective binding of phosphonate groups to Ca^{2+} than to PO_4^{3-} .

However, as seen from Figure 6, the release of JQ1 was even faster from DCP than from HAp, counteracting the hypothesis that surface group termination control could be used to modify the intensity of the desorption of the drug. Concordantly, the efficiencies of both loading and “encapsulation” of JQ1 onto HAp were by an order of magnitude higher than those onto DCP (Table 2). Indirectly, this proves the structural and compositional volatility of the surface of aqueously dispersed CaP particles. Specifically, CaPs are typified by the heavily hydrated and diffusive atomic layers at the surface, which allow it to rearrange itself in response to the pH, the ionic composition of the medium, and other physicochemical parameters of the solution at the particle interface in a process that plays an essential role in bone remodeling.⁴⁴ The surface turnover of more acidic DCP would thus be higher in the alkaline release solution (pH 7.4) than that of HAp, explaining the faster release of the drug. The latter rate, however, became significantly reduced when JQ1 was coprecipitated with the solid, despite the lower EE under such conditions. The release from DCP under such conditions becomes more sustained (Figure 6), presumably because of the greater degree of surface entrapment of the drug or its incorporation inside the hydrated layers of DCP structure. Under such conditions, the lower Ca/P ratio of DCP may promote stronger binding and delayed desorption of JQ1 as compared to HAp. Evaporation from alcoholic solutions still resulted in larger loading efficiencies and was used to load JQ1 onto HAp for all of the biological tests elaborated next.

3.2. Biological Properties of HAp Nanoparticles Loaded with Medronate (BP) and/or JQ1

3.2.1. Cancer Cell versus Primary Cell Viability—As seen from Figures 7 and 8, the apoptotic effect of drug-loaded HAp nanoparticles is selective, pronounced against the OS cells and nonexistent against the healthy fibroblasts. K7M2 OS cells demonstrate a decreased viability when treated with JQ1 or HAp/BP on all 3 days of the treatment, and with HAp/JQ1 or HAp/BP/JQ1 after the longest, 72 h treatment (Figure 7a). Considering this, HAp nanoparticles in combination with medronate (BP) have an anticancer activity equal to that of the pure anticancer drug, JQ1. A comparison between the two shows no statistically significant difference on days 1 and 3, while the reduction in the OS cell viability is significantly larger ($p < 0.05$) for HAp/BP than for JQ1 on day 2 of the treatment. This indicates that, in total, a combination of the carrier and the targeting ligand in this case has a greater cytotoxic efficacy than the therapeutic drug alone. On the third and final day of the treatment, the reduction of the cell viability was significant in all cell populations except those treated with pure, unloaded HAp. Interestingly, HAp particles loaded with both JQ1 and BP were not as effective as those containing only BP and in the same range as for those containing only JQ1, demonstrating that the synergy between the drug and the targeting ligand can also be detrimental for the therapeutic efficacy of both. These findings are in agreement with the previous reports of the ability of bisphosphonates to induce cancer cell apoptosis and inhibit the cell invasion.⁴⁵ The effect was particularly intense for zoledronate,^{46,47} the bisphosphonate with the strongest bone-binding properties,⁴⁸ as well as other bisphosphonates that contain nitrogenated side chains. Viability tests run against K7M2 OS cells also evidenced the ability of HAp to increase the cytotoxicity of JQ1, given that HAp/JQ1 delivered more than 3 orders of magnitude lower concentration of JQ1 (0.624 nM) than that administered as a part of the JQ1-only treatment (5 μ M) and yet produced a comparable effect on the viability at the final, 72 h time point (Figure 7a). Administered at

the same concentration as that delivered using HAp, JQ1 had no effect on the OS cells (Figure 7b).

On the other hand, the apoptotic effect against the regular primary fibroblasts was present, albeit to a very minor extent ($p = 0.043$) and only in cells treated with pure JQ1 after 48 h of the particle treatment (Figure 8). Neither of the HAp particles loaded with BP and JQ1 exhibited the apoptotic effect on primary cells, indicating their selective apoptotic activity against cancer cells. Pure, unloaded HAp nanoparticles had neither an effect on the cancer cells nor on the healthy ones in terms of their viability.

3.2.2. K7M2 Osteosarcoma Single Cell and f-Actin Morphology—Addition of HAp nanoparticles, with or without JQ1 and/or BP, decreased the elongation of the OS cells (Figure 9), suggesting their transition toward a more osteoblastic phenotype, as in agreement with our previous observation that this effect is inducible with HAp nanoparticles in MC3T3 cells.^{49,50} It is conceivable that a therapeutic benefit of HAp against OS may be tied to its ability to drive the OS cells toward an osteoblastic, more quiescent phenotype as compared to its fibroblastic precursor. Cultured osteoblasts do not divide, as opposed to their fibroblastic precursors, which may benefit the treatment in vivo. In general, pluripotency presents one of the greatest hindrances to successful cancer therapies, and cancer stem cells, involved in remission and drug resistance, present one example.⁵¹ The ability of the carrier to drive the cells toward a less pluripotent and more differentiated phenotype can be a positive contribution to the therapeutic effect of the carried drug.

3.2.3. Inhibition of Migration and Invasion of K7M2 Osteosarcoma Tumor Spheroids—Migration and invasion are crucial events in the pathology of metastatic cancers. While these two events are not identical, both processes involve many of the same molecular mechanisms. However, the ability of a cell type to migrate does not reflect its ability to invade the surrounding tissues, and compounds that affect one process may not affect the other one to an equal extent. Therefore, the effects that JQ1 alone and JQ1 delivered with the use of HAp nanoparticles have on the OS cell migration and invasion were studied in parallel in an in vitro 3D tumor spheroid model. In general, this 3D model mimics the in vivo behavior of a solid OS tumor and predicts the response of cancer cells to the treatment better than does the traditional, 2D culture. Assays assessing the distance of the migration of cells exiting the tumor showed that HAp/BP/JQ1 and HAp/JQ1 nanoparticles reduced the average radius of the migration of cells away from the center of the tumor spheroid equally. HAp/BP nanoparticles reduced the distance of the cell migration more than HAp/BP/JQ1 and HAp/JQ1 nanoparticles did, and the treatment with JQ1 only was the most effective (Figure 10, Table 3). The treatment with JQ1 was significantly more effective than the treatments with HAp/BP/JQ1 and HAp/JQ1, but it was insignificantly more effective as compared to the treatment with HAp/BP ($p = 0.0813$), conforming to the comparatively high anticancer activity of HAp/BP seen in the 2D assay (Figure 7). Still, no treatment could completely halt the cell migration from the tumor mass.

In contrast to the migration assay, the invasion assay is based on observing the difference between the invasion of the tumor spheroid through regular Matrigel and through Matrigel seeded with the nanoparticles. Figure 11 demonstrates the effects different nanoparticles had

on the invasion of K7M2 cells through 3D Matrigel. Results show that HAp/BP and HAp alone did not significantly reduce the area of cellular invasion as compared to the untreated control, while HAp/JQ1 and HAp/BP/JQ1 reduced it to a similar degree. This indirectly eliminates the possibility of competition between the two surface sorbates, JQ1 and BP, for the ionic species (Ca^{2+} ---BP and PO_4^{3-} ---JQ1) on the HAp nanoparticle surface. Both the cell migration and the invasion radii for spheroids treated with pure HAp were insignificantly ($p > 0.05$) different as compared to the negative control group and lower for HAp particles containing any combination of the two therapeutic payloads, BP, JQ1, and BP/JQ1. Still, JQ1 treatment was the most effective in preventing the invasion of OS cells into the ECM (Figure 11, Table 3). Again, however, no treatment could completely suppress the invasive behavior of OS cells from the tumor mass. While the JQ1-loaded HAp nanoparticles could to some degree reduce both the ability of OS cells to migrate and their ability to invade the ECM, none of the particles reduced the area of the tumor spheroid itself. Interestingly, while the difference between JQ1-loaded HAp particles and JQ1 only in terms of reducing the migration was only 50–100 μm , that is, $\sim 50\%$ versus 70% , respectively, the ability of JQ1 alone to reduce the invasion was far greater than its ability to reduce the migration when compared to drug-loaded HAp nanoparticles (Figure 11, Table 3). Additionally, only JQ1-treated spheroids showed a reduction in the area of the tumor itself (Figure 11). This observation agrees with a previous study demonstrating that JQ1 dramatically shrank OS tumors *in vivo*.⁵² Our work, however, shows that, despite the ability of JQ1 to reduce the tumor volume, it cannot completely prevent either the migration or the invasion of OS cells from the tumor mass into the surrounding ECM, thereby increasing the likelihood that, despite the reduction of the tumor size, the reoccurrence of OS is likely at a distant site due to the continual migratory/invasive behavior of the remaining OS cells.

While the drug-loaded HAp nanoparticles were seemingly less effective than the JQ1 only treatment, the dosages of the two treatment types were not completely comparable. A 5 mg/mL dose of JQ1-loaded HAp particles released a total of 0.624 nM of JQ1 over 2 days, whereas cells and spheroids in JQ1 only treatments were treated with 1 μM total JQ1. As seen from Figure 7b, the viability of OS cells was unaffected when treated with 0.624 nM JQ1, which was expected given that this concentration is by 2 orders of magnitude lower than the 50–100 nM IC_{50} range reported for the BRD4 inhibition by JQ1.⁵³ However, when treated with the same concentration of JQ1 delivered using HAp particles, the cytotoxic effect was obvious, suggesting the positive effects of using nanoparticulate HAp as a carrier. Despite more than 3 orders of magnitude difference between the amount of JQ1 released from HAp particles and the amount of JQ1 used in drug-only treatments, JQ1 coupled to HAp nanoparticles was relatively effective in reducing the OS cell migration, less effective at halting the invasion, and ineffective in reducing the tumor volume. One possibility as to why JQ1 is effective at such minimal concentrations when attached to HAp nanoparticles is that the release of JQ1 from HAp takes place over 2 days (Figure 6). Given that the half-life of JQ1 is ~ 1 h, its effectiveness is short-term; however, when loaded onto HAp nanoparticles, its effect is prolonged beyond that of the JQ1 treatment alone even at the minimal concentration released. Another possibility is the uptake of HAp particles by the OS cells. HAp nanoparticles by themselves are easily uptaken by OS cells and elicit no intrinsic toxicity (Figure 12). Some portion of HAp/JQ1 particles localized perinuclearly

(Figure 12d) after being trafficked by the vesicular compartments to this region of the cell where most endosomal escape events take place.⁵⁴ Quantification of HAp nanoparticle uptake, with and without the drug load, using fluorescence cell sorting showed that the majority of K7M2 OS cells, specifically 88.6% for HAp and 82.6% for HAp/JQ1, uptake detectable levels of particles after 24 h of coincubation (Figure 13). These percentages were comparable to the percentage of transfected, constitutively eGFP-expressing K7M2 cells detected at the child, P3 gate, while the slightly lower uptake efficiency of JQ1-loaded HAp may be merely an artifact caused by the competition of the drug and the dye for adsorption onto the HAp particle surface. The uptake of the drug-loaded nanoparticles would ensure that the OS cells are dosed with the drug, which otherwise, due to its hydrophobicity, might segregate in aqueous media before being internalized by the cells. In this scenario, JQ1 takes advantage of the high uptake rate of HAp nanoparticles as well as of their ability, as effective gene delivery carriers,^{4,5} to neutralize the endosomal proton pumps and protect the drug from premature degradation en route to the nuclear region that is the site of action of BRD4 and other members of the BET protein family targeted by JQ1. The uptake pathway and the mechanism by which HAp nanoparticles deliver JQ1 are thus expected to bear resemblance to those by which they deliver the exogenous genetic material to the nucleus during transfection events.

The results of the 3D anticancer tests carried out on K7M2 spheroids and those of the 2D test carried out on the same cells in the plated form differed, mainly in terms of the more intense activity of JQ1-loaded HAp nanoparticles observed in the 3D test as opposed to the 2D one. This observation illustrates the fact that the efficacy of action of therapeutic agents in general greatly depends on the context in which their interaction with the target cells is being initiated. This adds to the previously observed apoptotic, antiproliferative, and anti-invasive effects of bisphosphonates⁵⁵ and JQ1⁵⁶ as cell-dependent. On the other hand, the reduction of the cell migration and invasion radii was observed in both HAp/BP/JQ1 and HAp/BP treated groups, reconfirming the finite anticancer activity of HAp combined with medronate observed in the above-mentioned testing on plated K7M2 cells (Figures 7 and 8). BPs, specifically zoledronate, were observed to inhibit the invasion of different tumor cell lines by preventing the translocation of Ras homologue family member A (RHOA) from the cytoplasm to the cell membrane, thus disorganizing the actin cytoskeleton, reducing the number of stress fibers as well as the cell motility.⁵⁷ However, this can explain the hindered migration, but not invasiveness of K7M2 cells subjected to HAp/BP treatment. A more probable scenario, thus, involves the dominant inhibition of MMPs 2 and 9 activity, previously pinpointed as the reason for hindered migration of zoledronate-treated Ewing's sarcoma cells through a 3D Matrigel membrane *in vitro*.⁵⁸ It is possible that this lack of inhibition of invasion by HAp/BP treatment is one reason that zoledronate failed to improve the therapeutic outcomes when combined with chemotherapy in recent phase III clinical trials, despite the preclinical data that suggested otherwise.⁵⁹

3.2.4. Gene Expression in K7M2 Osteosarcoma Cells Challenged with

Nanoparticles—Figure 14 displays the results of gene expression analysis in K7M2 OS cells treated with different HAp nanoparticles or JQ1 alone. JQ1 was originally identified as an inhibitor of c-MYC;⁶⁰ however, subsequent work has shown that inhibition of Myc is not

necessarily the main mediator of JQ1 activity. Recent work has shown that JQ1 can suppress other gene targets, such as FOSL1 and RUNX2 in OS cells.^{61,62} Despite the results demonstrating that in other tumor models JQ1 activity is c-Myc dependent, our results show that, similar to previous work on JQ1 and osteosarcoma, the activity of JQ1 in OS is MYC independent. Figure 14a shows that rather than suppressing the MYC expression, JQ1 and HAp/BP slightly increased the expression of MYC in K7M2 cells. This increase of MYC expression has been noted in previous studies of JQ1 treatment of OS cells.⁶³ Here, the expression of MYC in cells treated with HAp/JQ1 nano-particles was similar to the control. Only in cells treated with HAp/BP/JQ1 did the MYC expression significantly decrease by 24 h. If JQ1 activity in most OSs is MYC independent, then it has been postulated that the activity of JQ1 could be mediated through the suppression of RUNX2 and FOSL1. Interestingly, our results demonstrate that for all of the JQ1-loaded HAp compositions and for JQ1 alone, there was no significant difference in FOSL1 expression between the treated cells and the control, although HAp only treated particles showed an increase in FOSL1 expression (Figure 14b).

JQ1 was previously observed to downregulate the expression of osteogenic markers, including RUNX2, and hinder the osteogenic differentiation.⁶⁴ That this effect could be reversed through the codelivery with HAp/BP was insinuated by a study in which osteogenic differentiation was promoted by the latter particles.⁶⁵ Our data conform to these findings; JQ1 only treatment reduced the expression of RUNX2 in OS cells (Figure 14c), as in agreement with previous results and expected given the fact that RUNX2 is a direct target of BRD4 inhibition by JQ1.⁶⁶ In contrast, there was a significant increase in the expression of RUNX2 in OS cells treated with for HAp/JQ1, HAp/BP, and HAp/BP/JQ1, an effect that may be due to the ability of HAp to induce osteogenic differentiation, although HAp itself did not evoke the same effect (Figure 14c). Ezrin is a member of the ERM (ezrin, radixin, moesin) protein family and functions as a linker between f-actin and cell membrane proteins.⁶⁷ Upregulated Ezrin expression has been associated with enhanced metastatic progression and observed in both murine (e.g., K7M2) and human OS cell lines.^{68,69} Ezrin expression in OS cells treated with HAp/JQ1 particles was unaffected as compared to the control (Figure 14d). Cells treated with HAp/BP, however, displayed a significant increase in Ezrin expression, but JQ1 only treatment induced a much greater Ezrin expression than any of the drug-loaded nano-particle treatments. HAp/BP/JQ1 treatment even caused a significant reduction of Ezrin expression as compared to the control (Figure 14d). While an increase in Ezrin expression would suggest a more aggressive phenotype, it is possible that this gene upregulation can also contribute to BETi activity. Obviously, in the case of JQ1, despite the increased expression of Ezrin, JQ1-treated OS cells exhibit the largest decrease in cell migration (Figure 10) and are the least invasive too (Figure 11), suggesting that too much Ezrin, past a certain threshold, can be detrimental despite the previous studies showing that metastatic cells depend on overexpressed Ezrin.⁷⁰ It is also possible that JQ1 alone as compared to JQ1 bound to HAp in combination with medronate affect gene expression of OS cells differently, with JQ1 affecting OS cell viability through overexpression of Ezrin, while HAp/BP/JQ1 works by inhibiting Ezrin expression. This antagonism may be linked to the different effects the active, phosphorylated Ezrin and the inactive, dephosphorylated Ezrin exert on cells.⁷¹ We also examined the total amount of f-actin in OS cells treated with

JQ1 and with different drug-loaded nanoparticles because Ezrin is a direct physical link between f-actin and cell membrane proteins. Figure 14e shows that the amount of actin present in OS cells matched the expression pattern seen in the quantification of Ezrin expression; however, the isotropic organization of f-actin microfibers measured using FibrilTool was unaffected regardless of the increase or decrease in the amount of actin in the cell (data not shown).

3.2.5. Nanoparticle Treatment Effect on Differentiated K7M2 Osteosarcoma Cells

—Although MTT results showed that JQ1 at 1 μ M was more effective than any of the drug-loaded nanoparticles in reducing the viability of K7M2 OS cells, these findings should be taken with reservation, one reason being that, unlike OS tumors in vivo, the OS cells in vitro do not retain all of the characteristics of the original tumor. For example, OS tumors are characterized by the production of osteoid (immature bone) by the malignant cells, and this characteristic is not present in the K7M2 cell line, which is often described as fibroblastic rather than osteoblastic. This phenotypic inclination was morphologically evidenced in Figure 9. Therefore, to assess the efficacy of the different drug-loaded HAp nanoparticles against OS cells differentiated toward an osteoblastic phenotype, we differentiated K7M2 cells for 7 days to induce the osteogenic marker expression. This differentiation was confirmed by the higher expression of the osteogenic transcription factor, RUNX2, in the differentiated population (Figure 15a). Comparison of MTT assays run on undifferentiated and differentiated K7M2 cells shows that, while JQ1 alone was greatly effective against the undifferentiated cells (Figure 7), only HAp/BP/JQ1 had a significant effect on cell viability in the differentiated population (Figure 15b). K7M2 cells differentiated toward an osteoblastic phenotype engage in the increased production of mineral nodules, which bone-mineral-targeting BP molecules on the HAp/BP/JQ1 nanoparticle surface are expected to have an affinity for. Such an affinity may promote the greater uptake of HAp/BP/JQ1 nanoparticles and augment the apoptotic effect caused by JQ1. The transition of K7M2 to an osteoblastic phenotype is, in turn, accompanied by the suppression of its lytic antagonist, which is paralleled by the increased expression of osterix, a transcription factor deficient in undifferentiated cells and required for osteoblast differentiation and bone formation.⁷² Although this phenotypic change can be expected to lower the particle uptake propensity, the totality of its pharmacodynamic effects is difficult to envisage.

4. SUMMARY

In this study, we functionalized HAp nanoparticles with medronate (BP) as a bone-targeting moiety and JQ1, a small-molecule bromodomain inhibitor, as an anticancer chemotherapeutic and tested them in different 2D and 3D OS in vitro models. As compared to the traditional treatment, consisting of systemic chemotherapy, radiation, and surgical excision, JQ1 delivery using HAp nanoparticles functionalized with a targeting agent presents a novel therapeutic approach, not researched before. In 2D culture assays, JQ1-loaded HAp nanoparticles exhibited a promising selectivity, having been more toxic to OS cells than to primary fibroblasts. If the bone cells surrounding the tumor were relatively immune to the therapy effects as compared to the OS cells, this would provide for a crude form of targeting.

A more sophisticated form of targeting was attempted to be achieved with the use of BP as a surface ligand. Surprisingly, it was found out that BP itself, when delivered using HAp, selectively attacked and obliterated cancer cells while having no negative effect on the viability of healthy fibroblasts. Simultaneously, all of the combinations of the targeting compound, BP, and the chemotherapeutic compound, JQ1, delivered using HAp, but not HAp alone, inhibited OS cell migration from the tumor spheroids. Reduction of cell migration observed in HAp/BP/JQ1-treated OS spheroids as well the sole ability of HAp/BP/JQ1 to reduce the viability of differentiated OS cells further confirmed the utility of BP as a component of the drug/carrier couple, even though, as we see, the roles of the targeting agent, the therapeutic, and the carrier may get mixed up and overlapped due to their synergistic entwinement. The positive effects of this synergy are evidenced by the consistently favorable effect achieved by HAp/BP/JQ1: not only did it reduce OS cell migration and viability of differentiated OS cells more than any other HAp composition, but it also selectively necrotized OS cells, just like HAp/JQ1 and HAp/BP did. Clinical trials assessing the effect of bisphosphonates in combination with chemotherapy and surgery in the treatment of OS have had unsatisfactory outcomes,⁵⁹ indicating that their use in a targeting role, codelivered with a chemotherapeutic drug, may provide for a more prospective option. OS cell invasion was significantly inhibited by all of the JQ1-containing formulations, that is, HAp/JQ1 and HAp/BP/JQ1, including pure JQ1. Because of the more than 3 orders of magnitude lower concentration of JQ1 in JQ1-loaded HAp nanoparticles, with or without medronate, they were not as effective as 1 μ M JQ1 in both 2D and 3D assays; at the same time, however, there was an increase in the effect of JQ1 delivered with the use HAp nanoparticles as compared to JQ1 delivered alone and in the same concentration. The fact that such extremely low concentrations of JQ1 had an effect on tumor migration, invasion, and apoptosis means that loading the drug onto HAp could help achieve a multifold increase of its efficacy.

There are more advantages to the delivery of JQ1 using appropriate carriers as compared to the delivery of JQ1 alone. JQ1 has a very short half-life in vivo, ~1 h, so its benefits are limited as a clinical treatment. However, loading JQ1 onto HAp nanoparticles can extend its biodistribution by delaying its release and assuring that it is not metabolized prematurely. Also, HAp is uptaken by cells easily, without toxic aftereffects. The flow cytometry analysis showed that the majority of OS cells internalize HAp and JQ1-loaded HAp nanoparticles, suggesting that the observed augmentation of the activity of JQ1 when delivered using HAp may be due to the facilitated uptake and sustained release of the drug. Dosing of the cells through the drug-loaded particle uptake protects the drug from premature release and hydrophobic aggregation, meaning that less drug can be used to achieve the same pharmacodynamic effect. Also, for OS cells that have been differentiated toward an osteoblastic phenotype, HAp/BP/JQ1 was the only effective treatment, which could be due to multiple effects, including the possibility that the mineral nodules produced by osteoblastic cells are targeted by BPs, thereby increasing the particle uptake despite the fact that K7M2 osteoblasts are relatively quiescent cells as compared to their more lytic precursors. It is possible that for the mixed tumor types that OS are composed of, the osteoblastic, bony part of the tumor may not be particularly affected by JQ1 and that the combination of HAp, BP, and drug will prove itself more potent.

The comparatively low loading efficiency of hydrophobic JQ1 onto ionic HAp has been a definite downside of the therapy conceived hereby, and being able to increase this efficiency would certainly increase the therapeutic profile of the particles. One way of increasing the therapeutic effectiveness of HAp would be to provide a more stable complex with JQ1. This is challenging given the hydroxylated crystal structure of HAp and the heavily hydrated and diffusive Stern layer that intrinsic hydroxylation causes in combination with triply charged phosphates and chaotropic calcium ions; this facilitates bone remodeling in vivo, but makes stable covalent conjugations with organics virtually impossible. One possible solution would be to explore combinations of HAp with additional phases at the aqueous interface. Cholesterol molecules, which undergo epitaxy with HAp,⁷³ or their vesicular formulations enveloping HAp nanoparticles, for example, phospholipids, may provide an anchoring region for hydrophobic JQ1 to bind more stably to. The combination of ultrafine HAp nanoparticles and mono- or bilayer amphiphiles as Pickering emulsions presents yet another largely unexplored theme, and both of these will be the focus of our future studies on this topic. Regardless of our success in these endeavors, the story of HAp in drug delivery to OS is far from over. This train is too late to be stopped now.

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Figure 1. 3D structural models of JQ1 molecule employing CPK coloring scheme and drawn using Jmol, showing the molecular surface of the compound (a) and its electrostatic potential contours (b).

Figure 2. SEM images of pure HAp particles (a), HAp particles loaded with medronate (HAp/BP) (b), HAp particles loaded with JQ1 (HAp/JQ1) (c), HAp particles loaded with both medronate and JQ1 (HAp/JQ1/BP) (d), and DCP particles loaded with JQ1 (DCP/JQ1) (e), along with the average particle size for each of the particle types (f). Error bars represent standard deviation.

Figure 3. Interaction of medronate with Ca^{2+} (a) and JQ1 with PO_4^{3-} (b) ions on the particle surface of HAp.

Figure 4. Zeta potential versus pH curves for pure HAp particles and HAp particles loaded with medronate (HAp/BP) and JQ1 (HAp/JQ1). Error bars representing standard deviation are invisible with the naked eye.

Figure 5. XRD patterns demonstrating the effects of medronate (a), alendronate (b), and JQ1 (c) on the crystal structure of HAp (a–c) and DCP (c). HAp and DCP diffraction peaks are indexed with “ Δ ” and “ $\circ$ ”, respectively.

Figure 6. JQ1 release curves from HAp and DCP nanoparticles loaded with JQ1 via evaporation (solid line) and coprecipitation (dashed line). Error bars represent standard deviation ($n = 3$).

Figure 7.

Mitochondrial dehydrogenase activity indicative of K7M2 mouse OS cell viability following the 24, 48, and 72 h treatments with pure JQ1 (1 μ M), pure HAp nanoparticles, HAp nanoparticles loaded with medronate (HAp/BP), HAp nanoparticles loaded with JQ1 (HAp/JQ1), and HAp nanoparticles loaded with both medronate and JQ1 (HAp/BP/JQ1). The negative control, that is, cells subjected to no particle treatment, are marked with C-, while the positive control, containing no cells, only the culture medium, is marked with C+. (b) Mitochondrial dehydrogenase activity indicative of K7M2 mouse OS cell viability following the 24, 48, and 72 h treatments with pure JQ1 at the concentration equivalent to that delivered using HAp/JQ1 and HAp/JQ1/BP nanoparticles (0.624 nM). No effect on OS cell viability was observed when they were treated with 0.624 nM JQ1 (b), but when treated with the same concentration of JQ1 delivered using HAp particles, the effect was obvious (a). Bars and error bars represent averages and standard deviations, respectively. Data points statistically significantly lower ($p < 0.05$) as compared to the negative control (C-) are marked with an asterisk.

Figure 8.

Mitochondrial dehydrogenase activity indicative of primary mouse fibroblast cell viability following the 24, 48, and 72 h treatments with pure JQ1, pure HAp nanoparticles, HAp nanoparticles loaded with medronate (HAp/BP), HAp nanoparticles loaded with JQ1 (HAp/JQ1), and HAp nanoparticles loaded with both medronate and JQ1 (HAp/BP/JQ1). The negative control, that is, cells subjected to no particle treatment, are marked with C-. Bars and error bars represent averages and standard deviations, respectively. Data points statistically significantly lower ($p < 0.05$) as compared to the negative control (C-) are marked with an asterisk.

Figure 9. Morphologies of single K7M2 OS cells (blue, nucleus; red, f-actin) in untreated, control population and in populations challenged with different nanoparticle treatments: pure JQ1, pure HAp nanoparticles, HAp nanoparticles loaded with medronate (HAp/BP), HAp nanoparticles loaded with JQ1 (HAp/JQ1), and HAp nanoparticles loaded with both medronate and JQ1 (HAp/BP/JQ1).

Figure 10.

Inhibition of cell migration from the OS tumor spheroids by HAp nanoparticles alone (HAp), HAp nanoparticles loaded with JQ1 (HAp/JQ1), with JQ1 and medronate (HAp/BP/JQ1), and with medronate only (HAp/BP). All drug-loaded HAp nanoparticles inhibited cell migration from the tumor mass equally as compared to the untreated control and HAp only, but were not as effective as JQ1 alone in inhibiting migration. (A) Untreated control; (B) HAp only; (C) HAp/JQ1; (D) JQ1 only; (E) HAp/BP; and (F) HAp/BP/JQ1. (G) Cells were treated with 5 mg/mL of particles or 1 μ M of JQ1. Average distance of the longest leading edge of cell migration from center of the tumor mass, $n = 6$. Distance of the migration was measured using ImageJ. Scale bar 100 μ m. All experiments were repeated at least twice. Error bar shows standard deviation. Data points significantly different in value as compared to the untreated control (no particle, $p < 0.05$) are topped with an asterisk.

Figure 11.

Inhibition of the OS cell invasion of the ECM by HAp nanoparticles alone (HAp), HAp nanoparticles loaded with JQ1 (HAp/JQ1), with JQ1 and medronate (HAp/BP/JQ1), and with medronate only (HAp/BP). Only HAp/JQ1 and HAp/BP/JQ1 could slightly inhibit the invasion of the ECM by the OS cells as compared to the untreated control. HAp/BP did not inhibit the cell invasion significantly more than the HAp only. JQ1 most significantly inhibited the invasion of ECM by the OS cells. (A) Untreated control; (B) HAp only; (C) HAp/BP; (D) JQ1; (E) HAp/JQ1; and (F) HAp/BP/JQ1. (G) Average of the area of invading OS cells into the ECM. (H) Area of the main tumor mass after the treatment. Cells were treated with 5 mg/mL of particles or 1 μ M of JQ1. Area covered by the OS cells away from the tumor mass was measured using ImageJ. Area of the tumor mass was also measured using ImageJ. Scale bar 100 μ m. All experiments were repeated at least three times. Error bars show standard deviations. Data points significantly different in value as compared to the untreated control (no particles, $p < 0.05$) are topped with an asterisk.

Figure 12.

Uptake of the bare and functionalized HAp nanoparticles by the OS cells. Both types of nanoparticles are easily uptaken by the OS cells, provoking no toxicity. (A) No particle control; (B) HAp only; (C) HAp/BP; (D) HAp/JQ1; (E) HAp/BP/JQ1; and (F) JQ1 only. The f-actin microfilaments (phalloidin) are stained in red, cell nuclei (DAPI) in blue, and HAp nanoparticles in green. Arrow in (D) denotes perinuclear localization of HAp/JQ1 particles.

Figure 13.

Flow cytometry analysis of HAp and HAp/JQ1 nanoparticle uptake. Panels a, c, e, and g represent all, parent scattering events (P1) for the negative control (a), for the transfected, constitutively eGFP expressing K7M2 cells as the positive control (c), for K7M2 cells incubated with fluorescent green HAp nanoparticles for 24 h prior to the measurement (e), and for K7M2 cells incubated with fluorescent green HAp/JQ1 nanoparticles for 24 h prior to the measurement (g). Panels b, d, f, and h represent scattering events detected at the child, P3 gate for the negative control (b), for the transfected, constitutively eGFP expressing K7M2 cells as the positive control (d), for K7M2 cells incubated with fluorescent green HAp nanoparticles for 24 h prior to the measurement (f), and for K7M2 cells incubated with

fluorescent green HAp/JQ1 nanoparticles for 24 h prior to the measurement (h). The parent (P1) and the child (P3) gates were set using regular, nonfluorescing K7M2 cells and transfected, constitutively eGFP expressing K7M2 cells, respectively. The percentage of events detected at both the parental, P1, and the child, P3, gate is indicative of the percentage of cells internalizing detectable levels of fluorescing HAp and HAp/JQ1 nanoparticles (i). Bars in (i) represent averages ($n = 2$), while error bars represent standard deviation.

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Figure 14.

Effect of JQ1 and different HAp nanoparticle formulations on gene expression in K7M2 OS cells. Cells were treated with 5 mg/mL of nanoparticles or 1 μ M of JQ1 for 24 h, and transcript levels were quantitated by qPCR. (A) Expression of c-MYC was reduced only in K7M2 cells treated with HAp/BP/JQ1 nanoparticles. (B) Expression of *FOSL1* was not significantly decreased by any of the treatments. (C) JQ1 reduced the expression of RUNX2, while HAp/JQ1, HAp/BP/JQ1, and HAp/BP significantly increased the expression of RUNX2. (D) JQ1 only and HAp/BP increased the expression of Ezrin, while HAp/BP/JQ1 decreased it and HAp/JQ1 had no effect on it. (E) Quantitation of the total f-actin of OS cells treated with different HAp nanoparticle formulations and JQ1 alone. Average fluorescence intensity of f-actin was measured using ImageJ. All experiments were done in triplicate. Data points significantly higher in value as compared to the untreated control (no particle, p

< 0.05) are topped with “^”. Data points significantly lower in value as compared to the untreated control (no particle, $p < 0.05$) are topped with “v”.

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Figure 15.

(A) Increased expression of the osteogenic transcription factor, Runx2, in K7M2 OS cells after 7 days of differentiation. (B) MTT assay of K7M2 OS cells differentiated toward an osteoblastic phenotype. K7M2 cells were differentiated for 7 days toward an osteoblastic phenotype, then treated with different nanoparticle formulations or JQ1 only for 24 h. The MTT assay run after 24 h shows that for differentiated cells, only HAp/BP/JQ1 had any significant effect on the OS cell viability. All experiments were done in triplicate. Error bars represent standard deviation. Cell viabilities significantly lower in value as compared to the untreated control (no particle in (B), $p < 0.05$) are topped with an asterisk.

Table 1

PZC Values for Pure HAp, HAp Loaded with Medronate (HAp/JQ1), and HAp Loaded with JQ1 (HAp/JQ1)

sample	point of zero charge
HAp/BP	2.3
HAp	3.9
HAp/JQ1	5.3

Table 2Loading and Encapsulation Efficiencies for JQ1 on Different Calcium Phosphate Carriers (HAp, DCP)^a

calcium phosphate	loading efficiency	encapsulation efficiency
HAp	$57 \times 10^{-4}\%$	25.1%
DCP	$5 \times 10^{-4}\%$	8.4%

^aNote: Encapsulation efficiency is a standard term and does not account for the fact that loading on HAp and DCP happens strictly via physisorption, not encapsulation or entrapment.

Table 3

Average Distance of Cell Migration and Average Area of Cell Invasion into the ECM by OS Cells from Tumor Spheroid Mass

treatment	migration distance $\pm$ SD (μm)	invasion area $\pm$ SD (mm^2)
no particles	559.07 $\pm$ 50.66	1.538 $\pm$ 0.339
HAp	563.00 $\pm$ 131.82	1.351 $\pm$ 0.318
HAp/BP	345.46 $\pm$ 55.89	1.240 $\pm$ 0.171
JQ1	292.17 $\pm$ 37.56	0.212 $\pm$ 0.048
HAp/JQ1	403.70 $\pm$ 78.39	1.040 $\pm$ 0.137
HAp/BP/JQ1	397.27 $\pm$ 118.20	0.925 $\pm$ 0.237