Nanodelivery Systems Face Challenges and Limitations in Bone Diseases Management

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Bone is a highly dynamic tissue. Hence, diseases affecting bone tissue, such as cancer (primary tumors and metastases), osteoporosis, and genetic conditions present many challenges for traditional therapies, leading to poor therapeutic outcome. Nano-delivery systems are innovative tools used for the treatment of a variety of diseases due to their ability to deliver various therapeutic cargos and specifically target disease sites, including difficult ones like bone. A variety of nano-delivery strategies are recently explored to treat bone-related pathologies, with many resulting in better outcomes than traditional approaches. In this review, the authors cover recent attempts to use nano-delivery systems for the treatment of skeletal-related disorders including primary bone tumors, metastatic bone tumors, and non-cancerous bone disorders. Finally, potential barriers for the translation of such new therapeutics into the clinic and possible methods to overcome these barriers, are discussed.

1. Introduction

Bone diseases encompass a variety of skeletal-related disorders, and can be divided into subclasses including genetic, metabolic, degenerative, traumatic, malignant (metastatic and primary), and myeloma-related disorders. Bone disorders and their potential complications have a major impact on an individual’s life, causing disability, reduced quality of life, and a downward spiral in physical and mental health that for some ultimately results in death. Bone is considered one of the vital organs of the body because the skeleton provides mechanical support for stature and locomotion, protects vital organs, and controls mineral homeostasis. Moreover, recent studies have highlighted the role of bone as an endocrine organ that modulates its own metabolic functions through the production of hormones by bone cells, controlling mineral ion homeostasis (e.g., through Fibroblast growth factor 23 production) and energy balance (e.g., through osteocalcin production). The integrity of the skeleton is maintained by continuous bone remodeling. However, some bone disorders develop from alterations in this physiological process, which can be caused by factors including hormones, age, physical activity, drugs, and comorbidities.

Bone disorders have a high rate of morbidity and mortality, and the majority still lack a clinical solution. Though changes in treatment have improved patients’ survival, the balance between

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Figure 1. Bone remodeling involves the removal of mineralized bone by osteoclast (resorption) followed by the formation of bone matrix by osteoblast (deposition) and subsequent mineralization. The process of bone remodeling maintains bone structure to meet changing mechanical needs and repairs damage in bone matrix. Figure 1 was created using Biorender.com.

side effects and treatment efficacy unfortunately remains a battle. Therefore, there is still a concrete need to develop safer and more effective treatments. Many solutions have been explored, including targeted drug delivery systems, which seem to be a potential winning strategy.\[6\] Nanoparticle (NP)-based approaches combined with the available drugs allow researchers to overcome some of the side effects associated with current therapies.\[6\]

NPs as drug delivery systems have been extensively applied for many different diseases, from tumors to regenerative/inflammatory pathologies.\[7,8\] NPs have proven a favorable platform for specific tissue targeting, since they can develop stable interactions with different ligands, increase drug loading efficiency of both hydrophilic and hydrophobic molecules, and control delivery of micro- and macromolecules. In particular, NPs assure (i) increased drug solubility; (ii) prolonged drug stability as a result of the protective shield that reduces drug metabolism after administration and avoids fast clearance by filtering organs; (iii) enhanced transport ability across cell membranes; (iv) reduction of drug resistance mediated by extrusion pumps; (v) specific delivery of therapeutics to targeted tissue; (vi) controlled release of therapeutic cargo; (vii) reduced systemic adverse effects on healthy tissues or organs; and (viii) multimodal therapies.\[9\]

Due to these properties, nanomedicine has brought forth numerous successful formulations that have reached different stages of clinical trials or approval by the U.S. Food and Drug Administration (FDA), including the recent successful vaccine formulations against SARS-COV-2 by Pfizer and Moderna.\[10\]

In this review, we summarize the progress made in the development of NPs for the treatment of bone disorders, as well as highlight possible strategies to overcome current challenges, as well as potential uses of NPs for malignancies and bone disorders. Finally, we will discuss potential limitations in the translation of such technologies.

1.1. Bone Architecture and Function

Bone, despite its apparent rigid structure, is a remarkably complex and dynamic tissue. Its structure is maintained by the bone remodeling process, in which old or damaged bone is removed by osteoclasts (through bone resorption) and replaced with newly formed bone by osteoblasts (through bone formation) with no change in bone mass or quality (Figure 1).\[11,12\] This plastic homeostasis allows the skeleton to change in size during childhood and provides the bone the unique ability to repair micro- and macro-damages, such as fractures.\[13,14\]

The structure of bone can be divided into the inner trabecular bone and outer cortical bone. Trabecular bone, the "core" of skeletal structure, is very porous and houses the bone marrow, while encompassing only 20% of the total bone mass. Conversely, cortical bone is dense and organized into units called osteons, with lower blood circulation (Figure 2A).\[15\]

Eighty percent of bone mass is composed of a mixture of calcium phosphate salts (hydroxyapatite [HA]) and collagen, making bones a highly mineralized organ.\[16\] This structure makes bones exceptionally resistant to mechanical stress and provides the strength to structurally support the rest of the body (Figure 2B).\[17\]
1.2. Challenges in Bone Treatments

Bones are highly vascularized, but the structure of the vascular network can vary greatly depending on the skeletal site, age, and presence of pathologic conditions affecting the diffusion of the drug from the bloodstream to the target site. Interestingly, the absence of a basal membrane in some bone blood vessels makes them especially permeable to large molecules. This physiological feature can favor NP accumulation within the targeted bone, similar to the enhanced permeability and retention (EPR) effect present in many tumors. On the other hand, the mineralized nature of the bone extracellular matrix poses an exceptional barrier to NP diffusion from blood vessels into the surrounding tissue. In particular, the small gaps between mineralized bone fibers (lamellae) are only a few hundred nanometers large, imposing a threshold (100–300 nm) for the maximum size of NPs targeting the bone. However, even for NPs ranging between 100 and 200 nm or below, the limited space makes tissue penetration challenging.

From a clinical and experimental point of view, differences in bone mineral density, mechanical cues, and biochemical composition between different species (human, dog, sheep, mouse, and rat) need to be considered when choosing an appropriate animal model for bone research. This makes the application of NPs for bone-related diseases far from trivial.

The majority of the NP-based treatment studies reported in the literature are focused on the use of NPs for cancer treatment, and different strategies have been investigated for primary bone tumors and bone metastases.

2. Bone Tumor Diseases

2.1. Bone as the Primary Site of Malignances

Osteosarcoma (OS), chondrosarcoma (CS), and Ewing sarcoma (ES) are the three most common primary bone tumors, accounting for 70% of all cases. They are considered rare tumors and comprise a diverse group of malignant neoplasms. Bone sarcomas are of mesenchymal origin; mesenchymal stem cells (MSCs) can be either the progenitor of tumor cells or stromal cells that participate in tumor development and progression. These tumors can originate in various locations within the bone structure, anywhere from the medullary cavity to the periosteum.

While progress has been made toward improving patient outcomes, the survival rate remains below 30% for patients with metastatic disease. The difficulties in treating bone tumors are...
primarily due to the high biological and anatomical heterogeneity of the cases as well as the complication presented by the high level of metastasis.  

2.1.1. Osteosarcoma

OS is the most common pediatric bone cancer. Approximately 1200 patients are diagnosed with OS annually in the United States, making OS the third most common childhood malignancy, with incidence at a median age of 12 years for girls and 16 years for boys. OS classically develops in the metaphysis of long bones, such as distal femurs and proximal tibiae. This tumor usually arises from malignant primitive MSCs that differentiate into osteoblasts under physiological conditions, but produce a malignant osteoid matrix during progression of malignancies.

OS is known to have a significantly poorer prognosis compared to most other pediatric cancers. The primary reason for treatment failure and recurrence in OS is the high incidence of distant metastatic lesions, which in 80% of cases spread to the lungs.

OS metastasis mainly develops due to low responsiveness to chemotherapy, uncontrolled proliferation of cancer stem cells (CSCs), and drug resistance. Therefore, CSC population represents one of the most important and difficult targets for improvement of the long-term efficacy of chemotherapeutics.

2.1.2. Ewing Sarcoma

ES is the second most common primary bone tumor after OS, representing 10–15% percent of all cases. Like OS, the diagnosis is primarily made among pediatric patients. One of the unique features of this malignancy is that it can originate in either the bone or in soft tissues. The cellular origin of ES remains unclear, and is still a matter of debate. However, recent studies have demonstrated that MSCs are also the most likely cells of origin for this sarcoma.

2.1.3. Chondrosarcoma

CS is the third most common primary tumor of the musculoskeletal system; although improperly classified as a bone
tumor, it can be included in this class because of its similarity to osteosarcomas and proximity to the bone tissue.\[45\] CS is considered a family of different diseases, including primary and secondary CS. It also includes more rare diseases such as dedifferentiated CS, mesenchymal CS, and clear-cell CS.\[46\]

Though all unique, CS diseases share the characteristic of abnormal deposition of cartilage tissue. Due to the increased deposition of extracellular matrix that form a physical barrier against treatments, systemic chemotherapy is generally not considered the most effective therapeutic approach. However, there have been some recent successful attempts at developing NPs for CS treatment, which represent a new frontier, and are the reason we have included it in this review.

2.2. Bone as the Secondary Site of Malignancies

The formation of metastases is a complex, multistep process in which malignant tumor cells spread from their primary tumor lesion to distant organs.\[47\] Tumors most often metastasize to the bone, because its microenvironment helps tumor cells to adhere and proliferate due to the release of survival and growth factors during the process of bone formation and resorption.\[48\]

Most bone metastases (BMs) originate from renal cell carcinoma, prostate cancer, breast cancer, and lung cancer, and their incidence usually depends on the tumor stage at diagnosis.\[49\] Metastatic spread accounts for >90% of all cancer-related deaths in solid malignancies,\[49\] and there has been little improvement in the five-year survival rate over the past decade.\[49\] Therefore, new research needs to be conducted on novel ways to treat BMs.

3. Current Treatments for Primary Bone Tumors and Established Bone Metastases

Current treatments for primary bone tumors vary depending on the type and grade of the malignancy.\[40,50,51\] OS and ES tend to be more chemo-sensitive and are primarily treated with neoadjuvant chemotherapy prior to wide surgical resection.\[40\] CS, on the other hand, is generally resistant to both chemotherapy and radiation; thus, the primary treatment is generally wide surgical excision at the time of diagnosis.\[52\] The exception to this is grade 1 CS within the extremities, which is treated with intra-lesion curettage alone due to the low rate of distant metastasis.\[53\] The surgical resection method varies depending on the location and size of the tumor and includes amputation, en bloc excision, and limb salvage techniques.\[54\]

Except for grade 1 CS, surgical resection alone is often not curative due to the development of micro-metastases that can result in tumor recurrence.\[53\] Therefore, surgical resection is often used as a method of palliative treatment to alleviate pain from the tumor mass.\[56\]

Neoadjuvant chemotherapy is used to reduce the tumor mass and number of BMs prior to surgical intervention, while adjuvant chemotherapy is used after surgery to reduce the chance of tumor recurrence.\[57,58\] However, though chemotherapy is one of the first-line treatment options for patients with systemic disease, it is also associated with severe adverse effects including fever, neutropenia, hypersensitivity reactions, and cardiotoxicity due to the non-specific drug biodistribution.\[39\] Moreover, chemotherapy efficacy is also impaired by the bone tissue dense extracellular matrix and by the bone marrow microenvironment which provide a protective niche for cancer cells increasing the development of treatment resistance.\[60\] The most common chemotherapeutics used individually or in combination for primary and metastatic bone tumors are doxorubicin, cisplatin, methotrexate (MTX), cyclophosphamide, and ifosfamide.\[61\] In particular, patients diagnosed with primary and metastatic OS are treated with drug combination therapy (high-dose cycles of doxorubicin, cisplatin, and MTX).\[62–64\] Radiotherapy and radiopharmaceuticals can be used alone or in combination with surgery or chemotherapy. External beam radiotherapy is used to kill cancer cells, reduce bone pain, and decrease fractures.\[65\] The use of radiopharmaceuticals as well as other radiation-enhancing materials in combination with traditional radiotherapy has attracted much attention in the last years thanks to their ability to control the amount of administered radiation, reducing the notorious adverse effects.\[66\] Furthermore, radiopharmaceuticals can be used as theranostic tools to perform both tumor imaging and treatment in a single step.\[66\]

For rare bone malignancies of ES, the most commonly applied therapeutic regimen, known as VDC/IE, is based on a first therapy cycle with vincristine, doxorubicin, and cyclophosphamide, and a second cycle of ifosfamide and etoposide.\[67\] This therapy is normally followed by either radiotherapy or surgery.\[68,69\] Similarly, for the treatment of CS, vincristine, doxorubicin, cyclophosphamide, and etoposide are often used.\[70\] In the case of metastatic CS, vincristine, doxorubicin, and cyclophosphamide are preferred.\[70,71\]

Recently, bisphosphonates (BPs) have been used more frequently in the treatment of bone primary tumors and BMs.\[72,73\] They have been used to increase bone targeting thanks to their ability to enhance binding affinity of calcium ions on HA.\[60\] Three generations of BPs have been approved in the clinic for bone regeneration and bone metastases treatment. Therefore, BPs have been commonly prescribed for prevention and treatment of a variety of bone conditions in which an imbalance between bone formation and bone resorption underlies disease pathology.\[60,71–73\]

Overall, dramatic improvements in treatment regimens have made it possible for most patients with bone tumors in the extremities to avoid full limb amputation, allowing prosthetics or limb salvage surgery use instead to attempt to restore limb function.\[74\]

4. Nanoparticle Treatments for Primary Bone Tumors

4.1. Osteosarcoma

4.1.1. Nanoparticles and Traditional Chemotherapeutics

NPs have been used to improve the efficacy of well-established drugs already used in the treatment of primary bone tumors (Table 1, Figure 4). MTX is a common chemotherapeutic whose mechanism of action involves impairing DNA bases to affect DNA synthesis.\[75\] It has been demonstrated to exert good efficacy against OS in the clinical setting but unfortunately is associated with severe side effects, especially in the pediatric
Table 1. Nanoparticle treatment for primary bone tumors.

| Study | Delivered agent(s) | NP | Drug class | Study design | Results |
|-------|-------------------|----|------------|--------------|---------|
| Osteosarcoma |                     |    |            |              |         |
| Ray et al. [74] | Methotrexate | PLGA coated Mg-Al-layered double hydroxide | Antimetabolite | In vivo (murine model) | • Increased effect of methotrexate with higher accumulation in the tumor.  
• Reduced systemic toxicity compared to free methotrexate |
| Martinez-Carmona et al. [77] | Doxorubicin | Mesoporous silica | Topoisomerase inhibitor | In vitro | • Selective cell toxicity to OS tumor cells compared to healthy pre-osteoblast cells |
| Kallus et al. [80] | Ponatinib, nintedanib, PD173074 | Liposomal formulations | Tyrosine kinase inhibitor | In vitro/in vivo (murine model) | • Anti-tumor effect similar to the free drug on OS cell lines  
• Inhibition of downstream target receptors signaling  
• In vivo tumor growth reduction of 60% compared to free PON |
| Zinger et al. [81] | Ponatinib | Leukosomes | Tyrosine kinase inhibitor | In vitro | • Similar or better cytotoxic effect in comparison with the free Ponatinib  
• Enhanced effect in reducing the phosphorylation of ponatinib molecular targets compared to free ponatinib |
| Wang et al. [89] | Selenite | HA | Cytotoxic agent | In vivo (murine model) | • In vivo, reduced tumor growth of 50%  
• Reduction of lung metastases as well as improving healthy organ functions in vivo compared to empty hydroxyapatite NPs |
| Chen et al. [93] | Salinomycin | Lipid polymer | Diterpen glycoside | In vitro/in vivo (murine model) | • Significantly improved antineoplastic activity toward OS and CSC cells by inhibiting EGFR+ and CD133+ both in 2d and 3d  
• Reduced tumor growth in vivo |
| Gui et al. [95] | All-trans-retinoic acid | Lipid polymer | Retinoid | In vitro/in vivo (murine model) | • Specific cytotoxicity against CD133+ OS cells both in 2d and 3d  
• Suppressed tumor growth in vivo |
| Wang et al. [98] | TRAIL coded plasmids | PAMAM dendrimers | N/A | In vitro/in vivo (murine model) | • Cytotoxicity against human OS cells in vitro  
• Reduced tumor growth in vivo with no toxic effects  
• Achieved immunogenic cell death via thermal ablation resulting in immune antitumor response  
• Extended blood circulation time with preferential accumulation in OS cells  
• Substantially increased apoptosis and autophagy of OS cells both in vitro and in vivo |
| Tuohy et al. [101] | SPION | Cationic liposomes | N/A | In vivo (murine model) | • Substantial reduction in OS cell viability in vitro |
| Yan et al. [103] | All-trans-retinoic acid | Lipid polymer | Retinoid | In vitro/in vivo (murine model) | • MSCs were able to kill OS cells and decrease tumor growth by 68% after two cycles of photoactivation compared to both control groups (PBS and free AlPc54) |
| Ewingsarcoma |                     |    |            |              |         |
| Jordan et al. [112] | SN-38 | Gold | Irotecan metabolite | In vivo (murine model) | • Demonstrates selective toxicity against ES cells expressing EWS-FLI1 mRNA with substantially reduced ES tumor growth in vivo |

(Continued)
Table 1. (Continued).

| Study      | Delivered agent(s) | NP            | Drug class                      | Study design     | Results                                                                 |
|------------|--------------------|---------------|---------------------------------|------------------|-------------------------------------------------------------------------|
| Chondrosarcoma | Trucco et al. [114] | Doxorubicin   | Liposomal formulations          | Topoisomerase inhibitor | Clinical, phase II RCT • The therapy is well-tolerated by patients with a 53% 60-day response rate |

NP = nanoparticles; OS = osteosarcoma; PLGA = poly(lactic-co-glycolic acid); DNA = deoxyribonucleic acid; EGFR = epidermal growth factor receptor; TRAIL = tumor necrosis factor-related apoptosis-inducing ligand; SPION = superparamagnetic magnetite nanoparticles; HA = hydroxyapatite; MSC = mesenchymal stem cells; AMF = alternating magnetic field; Zn–Ph = zinc–phthalocyanine; BSA = bovine serum albumin; PEG = poly(ethylene glycol); PMAN = poly[2-(methylacryloyl)ethyl nicotinate]; TRAIL = tumor necrosis factor-related apoptosis inducing ligand; PAMAM = polyamidoamine; RCT = randomized controlled trial; NR = not recorded

Figure 4. Schematic representation of the different approaches for theranostic nanoparticles delivery to bone primary and metastatic tumors.

population, due to its medullary suppressive activity [76]. To be effective, MTX must be used in very high doses, as well as in combination with other chemotherapeutic drugs [74,76]. To improve MTX pharmacokinetics, Ray et al. [77,78] delivered MTX loaded into PLGA coated Mg-Al-layered double hydroxide NPs in a subcutaneous OS murine model. Compared to an equivalent amount of free MTX, MTX-loaded NPs showed more efficient accumulation within the tumor, significantly increased MTX clearance time (≈fivefold), and longer retention of MTX (fivefold higher half-life), resulting in increased MTX efficacy. In addition, systemic and organ toxicity was reduced compared to free MTX. Finally, these NPs significantly decreased the tumor growth rate, resulting in tumors that were eight times smaller, and prolonging survival time.

Among the different types of NPs, mesoporous silica NPs (MSNs) are a widely used platform for the treatment of several diseases thanks to their tolerability, versatility, easy drug loading, and functionalization [79,80]. In a study by Martinez-Carmona et al. [81] MSNs loaded with doxorubicin were capped with a layer of poly-acrylic acid, allowing for the pH-dependent release of the drug after NP internalization into the endo-lysosomal compartment, avoiding systemic effects. To further increase NP selectivity, concavallin A was used as an active moiety to specifically target tumor cells overexpressing sialic acid on their plasma
membrane. This formulation demonstrated selective cell toxicity to OS tumor cells compared to healthy pre-osteoblast cells in vitro, with an IN50 of 2.5 mg mL\(^{-1}\) of particles for tumor cells compared to 10 mg mL\(^{-1}\) for healthy cells.

### 4.1.2. Nanoparticles and Novel Chemotherapeutics

Tyrosine kinase inhibitors (TKIs) are a relatively new class of drugs able to target specific receptors responsible for cancer progression (Table 1, Figure 4).\[82\] Despite not being used as a current therapeutic option for OS, drugs such as ponatinib (PON), nintedanib, and PD173074 can target signaling pathways involved in OS progression.\[83\] However, these drugs suffer from some critical drawbacks. They are poorly soluble in water, which reduces their maximum dosage and slows their absorption, and can cause severe side effects due to off-target action on filtering organs. In addition, tumor cells can often develop drug resistance mechanisms that either help to expel the drug quickly by overexpressing extrusion pump proteins or upregulate alternative signaling pathways. Kallus et al.\[84\] tried to resolve all these issues by encapsulating these three TKIs into liposomes and polyactic acid NPs. This formulation showed an in vitro anti-tumor effect comparable with the free drug at 72 h and a strong effect downstream of the target pathway. The authors observed decreased toxicity of PON-loaded NPs both at 0.1 and 1 µm compared to the free drug, probably due to slower uptake. When tested in vivo, these NPs showed minimal side effects and, moreover, a 60% greater tumor reduction compared with free PON. The other TKIs have yet to be tested in vivo.

In our recent work, Zinger et al. was able to efficiently encapsulate PON into biomimetic NPs. Like many other drugs with poor water solubility, PON binds to plasmatic proteins in blood circulation when injected intravenously (IV). In this study, the authors exploited the natural ability of bovine serum albumin as natural carrier to bind PON non-covalently, helping to enhance NP loading efficiency (Figure 5).\[85\]

Leukosomes are biomimetic NPs with membrane proteins isolated from leukocytes and integrated on the NP surface.\[86,87\] These leukosome NPs acquire the “self” proprieties of leukocytes (e.g., CD45, CD47), minimizing NPs clearance from the body prior to reaching their intended target. At the same time, adhesion proteins such as integrins and CD11b provide leukosomes with active targeting toward the target proteins expressed by infiltrated blood vessels (Figure 6). These proprieties make these NPs a useful platform for the treatment of heterogeneous pathologies with an inflammatory component, including tumors.\[88–91\] In our work, leukosome PON NPs were ≈140 nm in diameter, had a zeta potential of roughly –2 mV, were suitable for IV administration, had a good polydispersity...
Figure 6. Description of the main components of Leukosomes-PON NPs formulation and their ability in enabling drug loading, long circulation, and tumor targeting capabilities. Figure 6 was created using Biorender.com.

index (0.15), and showed a reliable PON load. In vitro, these NPs were internalized by mouse OS cell lines, and did not show any cytotoxic effect per se, while exerting a similar or better cytotoxic effect in comparison with free PON. Interestingly, PON delivered through leukosomes had an enhanced effect in reducing the phosphorylation of its molecular targets, such as AKT, compared to free PON (80% versus 50%). This suggests that leukosomes can increase PON internalization and retention in OS cells.\[85\]

The use of plant toxic proteins as antitumor agents has been widely reported, since these proteins and several products demonstrated significant role in inhibiting cancer cells alone or in combination with other treatments, compared to chemotherapy. However, their success as therapeutic agents is severely limited by their proteolytic degradation after injection.\[92\] Therefore, the use of polymers and nanoparticles to protect protein therapeutics and direct them against the tumor represent a valid solution. This approach was implemented in a recent work by Yan et al.\[93\] In this study, the authors successfully encapsulated the protein toxin saporin in a dendrimer of poly-boronated polymer that was further coated with poly-(α, β)-DL-aspartic acid, a bone targeting polymer, to enable active targeting against hydroxyapatite in bone (GPSP). These NPs demonstrated improved cytotoxicity compared with free saporin on 143B OS cells and bone metastatic MDA-MB-231 breast cancer cells in vitro. When injected IV in orthotopic OS tumor-bearing mice, the particles efficiently distributed in the bone, with a 50% reduction of their accumulation into the kidneys and almost doubled tumor accumulation compared to non-actively targeted particles. Finally, GPSP efficiently prevented tumor growth, compared to the free drug in both 143B OS model and MDA-MB-231 bone metastatic model. This work highlights how nanomedicine could be applied to re-evaluate many therapeutic agents as new arsenal against OS and other bone tumors.

4.1.3. Nanoparticles and Combined Therapies

Open challenges in tumor therapy include drug resistance, tumor recurrence, and metastasis formation, which significantly contribute to the failure of current cancer treatments. Therefore, the development of effective strategies exploiting different NPs with a variety of molecules (e.g., aptamers, single cargo types, or combinations) offers more chances to better target tumor cells or the CSCs that fuel tumor progression and metastasis formation (Table 1).\[60,94\]

Wang et al.\[95\] tested needle-shaped HA NPs loaded with selenite ions (HANSe), a trace element and cytotoxic agent for cancer cells that induces oxidative stress (ROS).\[96\] HA was utilized to increase selenium sequestration in the bone to favor bone regeneration. HANSe NPs were able to reduce tumor progression when administered intratumorally in an ectopic OS murine model. Overall, this formulation reduced tumor growth 50% by inhibiting the expression of metal matrix proteinase-9 (MMP9) and inducing cell apoptosis, resulting in a reduction of lung metastases as well as improved healthy organ function in vivo compared to empty HA NPs.

Enough evidence suggests that the simultaneous targeting of both CSCs and cancer cells is pivotal in achieving preferable cancer therapeutic efficacy, due to the spontaneous conversion between cancer cells and CSCs. Among CSCs markers, CD133 was initially described as a cell surface marker specific for hematopoietic stem cells and subsequently as a CSC marker across multiple tumor types.\[97\] Together with CD133, the epidermal growth factor receptor (EGFR) pathway plays a critical role in regulating CSCs.\[98\]

Chen et al.\[99\] developed sali-entrapped NPs labeled with CD133 aptamer (Ap-SAL-NPs) that efficiently eliminate CD133+ OS CSCs. Next, the authors formulated dual-targeting NPs by further labeling the Ap-SAL-NPs with EGFR-targeting CL4,
generating NPs able to eliminate both CD133+ and CD133− OS cells. They demonstrated significantly improved cytotoxicity toward OS cells but also improved cytotoxic effect both in 2D (IC₅₀ = 2 μg mL⁻¹ versus 10 μg mL⁻¹ for single targeting) and 3D (fourfold higher) against CSCs thanks to the EGFR and CD133 double targeting. Furthermore, these double targeting NPs were able to substantially reduce tumor growth in an in vivo mouse OS xenograft model, reducing the presence of CD133+ and CD133− cells.

CD133 was similarly exploited as a CSC target by Gui et al.[101] CD133 aptamers were used as an active targeting moiety on lipid polymer hybrid NPs loaded with all-trans-retinoic acid (ATRA, ATRA-PLNP-CD133). ATRA-PLNP-CD133 demonstrated a remarkable specificity for CD133+ OS cells and killing capability, as well as inhibiting their ability to form colonies and spheroids. Moreover, in vivo, the NPs substantially suppressed tumor growth in an ectopic OS murine model.

An advantage offered by NPs is the ability to incorporate molecules, protect them from the environment, and allow cell penetration via endocytosis. This is especially true for nucleic acids; whose high molecular weight and strong negative charge renders them unable to penetrate the cell membrane. Oligonucleotides are also easily degraded by nucleases in the circulation. Among the nucleic acids that selectively trigger cancer cell apoptosis without affecting normal cells, the immune cytokine tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has received significant attention as a cancer therapeutic.[102] However, TRAIL showed low accumulation at target tumor sites, and low efficacy in activating its target pathway.[103] To address this, Wang et al.[104] formulated a polyamidoamine-based dendrimer formulation that was able to electrostatically complex with the TRAIL-coding plasmid. This formulation demonstrated efficient activity against human OS cells in vitro with a twofold higher transfection efficacy and 30% more cytotoxicity than Lipofectamine. Furthermore, these NPs were able to substantially reduce tumor growth (fourfold) in an ectopic murine OS model, with no toxic effects.

Another strategy to enhance NP accumulation at the targeting site that has been studied is superparamagnetic iron oxide NPs (SPIIONS), which are able to concentrate where a magnetic field is applied locally.[105] However, the resulting thermal ablation generated by the magnetic field can also affect healthy cells, and most commercialized formulations have been retracted due to the associated pain and side effects of the treatment.[106] An approach employing magnetic field ablation and SPIIONS was tested to target immune cell death rather than OS cell death.[107] SPIIONS were included on cationic liposomes for local administration in an in vivo ectopic murine OS model. An alternating magnetic field was applied to the tumor to achieve immunogenic cell death via thermal ablation, which enhanced inflammation and subsequently triggered the local immune response. The authors showed a significant reduction of CD206+ monocytes, which are considered tumor-promoting immune cells. Unfortunately, no improvement in overall survival occurred, probably due to the local NP administration, which hampered their diffusion throughout the entire tumor mass. If the author had tried a different route of administration (i.e., IV), they would probably find more improvements in survival rate. Therefore, there is still a need for development of well-tolerated SPIIONS.[108]

However, SPIIONS utility in bone tumors may be limited, because tumor ablation and tissue regeneration must occur simultaneously to favor bone growth.

Similar strategies have been tested combining multifunctional NPs and photodynamic therapy (PDT), a clinically approved cancer therapy,[109] based on a photochemical reaction between a light activatable molecule or photosensitizer, a light source, and molecular oxygen.[110] Many of these PDT-enabling molecules suffer from poor solubility and potential toxicity. For this reason, they are often loaded into NPs to improve their pharmacokinetics and biodistribution. Following PDT, the increase in ROS production rapidly induces tumor cell death but also inflammatory and immune responses.

In a recent study by Yu et al.,[111] the authors developed a drug carrier poly(ethylene glycol)-poly[2-(methylacyryloyl)ethyl nicotinate] (PEG-PMAN) block polymer to efficiently load and deliver zinc phthalocyanine (ZnPc). The resultant NPs loaded with ZnPc (PEG-PMAN/ZnPc, also called PPZ) had extended blood circulation and could preferentially accumulate in OS through the EPR effect. PPZ NPs induced a significant increase of ROS level and toxicity (up to 100-fold) compared to free ZnPc in three different OS cells in vitro. In addition, PDT treatment substantially increased apoptosis and autophagy in a murine orthotopic xenograft OS model, demonstrating that PPZ NPs are a more effective agent for PDT in OS treatment than free ZnPc. Moreover, the authors observed reduced tumor growth and increased accumulation of CD8+ cells within the tumor. However, it is important to note that PDT therapy is not able to penetrate more than few millimeters into a tissue without damaging it. In this successful application, the treatment was administered during surgery, which is invasive and may not be feasible in the clinical scenario.

Martella et al.[112] proposed multi-modal keratin NPs functionalized with the photosensitizer Chlorin-e6 (Ce6) and loaded with the chemotherapeutic drug Paclitaxel (PTX), known as PTX-Ce6@Ker NPs. This combination can kill OS cells with PTX and eradicate chemo-resistant cells with photodynamic therapy. The internalization of PTX-Ce6@Ker NPs into different human OS cell lines (MG63, SaOS-2, and U-2 OS) was confirmed by an altered cell morphology and changes in beta-tubulin organization due to PTX. In this paper, the authors also demonstrated the efficacy of this combined treatment in both 2D and 3D in vitro models. The PTX cytostatic effect and the ROS oxidative damage upon light irradiation resulted in a massive cell death in all the human OS cell lines tested. This combined approach that differs from other targeted therapies is a promising new strategy that could improve treatment efficacy and prognosis, in particular in relapsed OS patients.

Using a multi-modal application, Lenna et al.[113] exploited the intrinsic properties of MSCs to migrate and infiltrate into the tumor stroma. The study tested the efficacy of the photoactivation of MSCs loaded with poly-methyl methacrylate core-shell fluorescent NPs (FNPs) functionalized with the photosensitizer tetra-sulfonated aluminum phthalocyanine (AlPcS4) in vitro and in vivo. In vitro, MSCs were able to internalize AlPcS4@FNPs without loss or alteration of motility or viability, and after photoactivation to the MSCs, induced high levels of OS cell death in both 2D and 3D co-cultures. A substantial decrease in both MSC and OS cell viability was observed at all co-culture ratios used.
(MSC:OS 1:1, 1:3, or 1:7). The authors also investigated the in vivo effect of photo-stimulation treatment on intratumorally injected AlPcS4@FNPVs loaded in MSCs in an ectopic OS mouse model. They showed that MSCs were able to kill OS cells and decrease tumor growth by 68% after two cycles of photoactivation, compared to both control groups (PBS and free AlPcS4). Moreover, NP internalization into MSCs not only improved the local NP accumulation at the target site, but also reduced the side effects when compared to AlPcS4@FNP alone. These findings suggested that MSCs may be an effective trojan horse for targeted NP delivery, alone or in combination with other OS treatment modalities.\cite{115,114}

Encapsulation of multiple chemotherapeutics with different molecular targets into NPs can allow for a synergistic effect, resulting in decreased tumor growth and reduced side effects with lower dosage requirements.\cite{115} Based on this approach, Prasad et al. used HA methacrylate NPs with a ceramic core and dextran shell for combinatorial delivery of MTX and DOX against human osteosarcoma OMG-63 cells.\cite{116} This formulation showed sequential extended release of MTX followed by DOX in vitro for 10 days, in both acidic and alkaline buffers. These dual drug-loaded NPs showed a synergistic cytotoxic effect in vitro, as well as higher induction of OS cells apoptosis compared to the free drug. However, no direct comparison was made with the free drug, limiting interpretation of the result. Overall, these NPs provide a potential bone void filling material, as well as a platform for sequential delivery of DOX and MTX for the treatment of bone cancer.

4.2. Rare Bone Malignancies

The majority of the studies focused their attention on NPs for OS therapy, since it is the most common primary bone tumor. However, some studies attempted the use of NPs on other bone malignancies, such as ES and CS (Table 1, Figure 4).

4.2.1. Ewing’s Sarcoma

Gold NPs (AuNPs) have been used in a wide variety of drug delivery applications thanks to their chemical stability, low toxicity, and multiple therapeutic as well as diagnostic applications.\cite{117} A recent work by Jordan et al.\cite{118} demonstrated the potential of AuNPs for the delivery of active irinotecan metabolite SN-38 for ES. The authors conjugated SN-38 with the oligonucleotide complementary to EWS-FLI1 mRNA, an mRNA that is specifically expressed by ES. When the AuNPs are internalized, the EWS-FLI1 mRNA attaches to its complementary oligonucleotide conjugated to SN-38, resulting in drug-oligo dissociation from NPs into the cells’ cytosol. This innovative mRNA-targeting strategy provides a new, epigenetically based level of specific drug delivery. This system demonstrated selective toxicity in ES cells expressing the target EWS-FLI1 mRNA, and substantially reduced ES tumor growth in an ectopic murine model in vivo.

4.2.2. Chondrosarcoma

Despite CS being considered a rare cancer, many clinical trials are investigating combinatorial treatments in order to identify promising novel treatments (e.g., belinostat and SGI-110; guadecitabine; pazopanib; AG-120; Regorafenib; sirolimus and cyclophosphamide; nivolumab and nab-rapamycin).\cite{119} One Phase I clinical trial has tested the efficacy of NPs loaded with doxorubicin and temsirolimus against CS. Temsirolimus inhibits the mechanistic target of rapamycin (mTOR) kinase receptor, which is involved in cell metabolism and growth. This receptor and its downstream signaling pathway is often overexpressed in tumors, making it an interesting potential target for novel antitumor strategies.\cite{120} In the Phase I trial, 15 patients with relapsed/refractory sarcoma were evaluated.\cite{121} Among the 9 patients treated, 3 had CS. The response rate, defined as stable or improved disease for 60 days, was 33%. Patients had a reduction of mTOR signaling and a reduction in expression of aldehyde dehydrogenase, a marker of CSCs and therefore of tumor progression, in post-treatment biopsies. The drug was well tolerated, no dose-limiting toxicities were observed, and the maximum tolerated dose was not reached, thus a Phase II trial has been designed and enrollment has started. The results of this early-stage trial are encouraging and will hopefully lead to a novel NP-based treatment against CS, and spark further interest in developing new NPs for rare and often overlooked cancers with similar poor outcomes, with great benefit for the patients.

5. Nanoparticle Treatments for Bone Metastases

The 5-year survival rate of patients with BM is still low (20%) with no effective treatment available.\cite{122} Targeting of the metastatic niche is difficult due to the absence of the vascularization and desmoplasia found in primary solid tumors.\cite{123} This environment hinders the use of passive targeting by EPR in the design and application of NPs. Moreover, the heterogeneous origin of BMs results in cells with different molecular profiles, making it difficult to find universal markers.\cite{123,124} Consequently, different NPs must be used to target each subtype of BMs, making treatment more challenging. Thus, new strategies are needed to improve drug delivery and targeting to BMs through NPs (Figure 4, Table 2).

5.1. Nanoparticles and Chemotherapeutic Drugs

Despite all the challenges mentioned above, some strategies have been explored for the delivery of chemotherapeutics/molecules loaded into NPs for BM therapy, representing a promising and exciting frontier of research (Table 2).\cite{60}

Adjei et al.\cite{125} utilized PLGA-NPs that also targeted the bone marrow in order to inhibit BM formation and increase the delivery and efficacy of paclitaxel for BM treatment. Some studies have suggested that NPs with a neutral surface charge have prolonged circulation time, reduced opsonization, less toxicity, and a reduced tendency to aggregate in a biological environment compared with cationic NPs.\cite{127} Using a PC-3M-luc cell-induced osteolytic intraosseous model of prostate cancer (PCa), the authors demonstrated the ability of neutrally charged NPs to accumulate both in the primary tumor within the bone marrow and in the metastasized tibia, whereas anionic or cationic NPs did not accumulate. The authors showed that a single IV administration
of paclitaxel-loaded NPs reduced both BM progression and bone loss compared to the free drug without causing acute toxicity in mice. The prevention of bone loss is important because bone loss is often the mechanism by which prostate BMs can invade the extracellular matrix (ECM), causing additional metastases and secondary tumor growth. However, further studies looking at optimal doses and regimens of drug-loaded NPs for BMs are needed. Hence, this work highlights an effective strategy for bone marrow targeting that could have significantly broader therapeutic implications in treating BMs.\(^\text{125}\)

In an attempt to find new strategies for the treatment of metastatic PCa, Gaur et al.\(^\text{126}\) developed chitosan NPs that deliver miR-34a, which is involved in PCa progression and metastasis. Overexpression of miRNA-34a induces a non-canonical form of autophagy, as well as apoptosis, resulting in inhibition of cell proliferation and promotion of cell death. Thus, the authors showed the same in PCa cell lines with metastatic potential overexpressing miRNA-34a. In an in vivo xenograft model of PCa BMs, intra-femoral administration of these NPs demonstrated inhibition of BM growth compared to a subcutaneous model, implicating the anti-tumorigenic effects on both tumor cells and bone microenvironment while preserving bone integrity. Therefore, the induced expression of miR-34a in PCa cells resulted in an “anti-proliferative” state. Together, these results provide a

### Table 2. Nanoparticles treatment for metastatic bone tumors.

| Study          | Metastatic cancer investigated | Delivered agent | NP               | Drug class                  | Study design               | Results                                                                 |
|----------------|-------------------------------|----------------|------------------|----------------------------|----------------------------|-------------------------------------------------------------------------|
| Adjei et al.\(^\text{119}\) | Prostate cancer              | Paclitaxel      | PLGA             | Antimicrotubule agent      | In vivo (murine model)     | • Increased tumor targeting  
• Reduced bone metastasis progression  
• Prevention of bone loss without causing acute toxicity in mice compared to the rapid-release of Cremophor EL (PTX-CrEL) free drug  
• Inhibition of metastatic tumor growth while preserving bone integrity  
• Increased doxorubicin cytotoxicity in vitro  
• Maintenance of bone structure integrity while reducing tumor size sixfold in vivo  
• Reduced doxorubicin cardiotoxicity  
• Increased tumor targeting specificity  
• Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Gaur et al.\(^\text{121}\)       | Prostate cancer              | miRNA-34a       | Chitosan         | Tumor suppressor            | In vivo (murine model)     | • Inhibition of metastatic tumor growth while preserving bone integrity  
• Increased doxorubicin cytotoxicity in vitro  
• Maintenance of bone structure integrity while reducing tumor size sixfold in vivo  
• Reduced doxorubicin cardiotoxicity  
• Increased tumor targeting specificity  
• Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Ye et al.\(^\text{122}\)         | Lung cancer                  | Doxorubicin     | DOX-hyd-PEG-ALN  | Topoisomerase inhibitor     | In vitro/in vivo (murine model) | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Ross et al.\(^\text{124}\)       | Breast cancer                | Docetaxel       | Docetaxel micelles targeting integrin αvβ3 | Antimicrotubule agent | In vivo (murine model)     | • Increased doxorubicin cytotoxicity in vitro  
• Maintenance of bone structure integrity while reducing tumor size sixfold in vivo  
• Reduced doxorubicin cardiotoxicity  
• Increased tumor targeting specificity  
• Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Jie et al.\(^\text{127}\)        | Breast cancer                | N/A             | Photothermal Nax WO3 | N/A            | In vitro/in vivo (murine model) | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Li et al.\(^\text{118}\)         | Breast cancer                | IR780           | PLGA             | N/A                        | In vitro/in vivo (murine model) | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Jiang et al.\(^\text{129}\)      | Breast cancer                | ICG, zolendronate | ICG/Fe3O4/PLGA-ZOL | Bisphosphonate               | In vivo (murine model)     | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Yang et al.\(^\text{137}\)       | Breast cancer                | PTT             | Arginine         | N/A                        | In vitro/in vivo (murine model) | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Wang et al.\(^\text{138}\)       | Breast cancer                | SN-38 PTT Alendronate Fe | PDA             | Topoisomerase I             | In vitro/in vivo (murine model) | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Wang et al.\(^\text{139}\)       | Breast cancer                | PTT/CQ          | PEG- alendronate-functionalized and chloroquine (CQ)-loaded PDA | Antimalarials | In vitro/in vivo (murine model) | • Reduced tumor burden (twofold) and bone degradation compared with free drug in vivo  
• NIR irradiation-dependent cytotoxicity in vitro  
• Inhibition of tumor growth without systemic side effects in vivo  
• Irradiation-dependent cytotoxicity in vitro  
• Decreased tumor growth in vivo  
• Higher accumulation in the tibia  
• Inhibition of tumor growth and bone resorption  
• Inhibition of tumor growth  
• Efficient accumulation in the bones  
• Tumor size and weight reduction |
| Kotagiri et al.\(^\text{141}\)   | Breast cancer                | Titanocene      | PDT              | N/A                        | In vitro/in vivo (murine model) | • Efficient accumulation in the bones  
• Tumor size and weight reduction |

NP = nanoparticles; PLGA = poly(lactic-co-glycolic acid); DOX-hyd-PEG-ALN = doxorubicin-poly (ethylene glycol)-alendronate; NaxWO3 = oxygen vacancy-rich tungsten bronze NPs; ICG/Fe3O4/PLGA-ZOL = superparamagnetic iron oxide and indocyanine green - entrapped poly-lactide-co-glycolide modified by zolendronate; NR = not recorded; N/A = not applicable
new understanding of the biological effects of miR-34a as a treatment for metastatic prostate cancer. This highlights the clinical potential of this miRNA delivery platform for BM treatment, but future work moving toward clinical studies should systematically study dosage and treatment frequency, potentially with patient stratification based on disease stages, in order to obtain optimal therapeutic outcomes especially in the long term.

Another strategy is to consider features of the BM microenvironment to improve NP affinity for bone; for example, BPs are a popular choice as active-targeting agents. Wei-Liang Ye et al. synthesized doxorubicin-loaded multifunctional NPs combining pH-sensitive polymers with BPs. These NPs were able to reduce the IC$_{50}$ of doxorubicin by 50% on an A549 human lung cancer cell line compared to the free drug, 48 h after treatment. In addition, the authors showed that IV administration of NPs in A549-bearing nude mice not only increased drug accumulation in BMs but also resulted in a sixfold reduction of BM volume. Moreover, the bone mineral density was similar to healthy bone, and the cardiac toxicity of doxorubicin was significantly reduced.

Current evidence suggests that the use of BP-targeted chemotherapy has increased treatment efficacy compared to standard chemotherapy. To address BM targeting limitations, Ross et al. directly targeted integrin $\alpha v \beta 3$ in breast cancer BMs. Using a docetaxel-loaded NPs targeting integrin $\alpha v \beta 3$ (AV3-MPs), they observed a reduction of BMs, less bone destruction, and less side effects compared to the same doses of free docetaxel. Furthermore, mice treated with $\alpha v \beta 3$-MP-docetaxel exhibited a significant decrease (twofold) in BM growth compared to free drug. Overall, the authors propose a new approach to enhance delivery of chemotherapeutics to $\alpha v \beta 3$-expressing cancer cells as a bone-specific therapeutic strategy.

5.2. Nanoparticles and Combined Therapies

Combining NP systems with photo-thermal therapy (PTT) for multifunctional treatment has also been tested in BMs (Table 2). PTT refers to the activation of photosensitizing agents by near-infrared laser irradiation to generate heat for thermal ablation of cancer cells. So far, PTT and combinational therapies including PTT have been investigated in animal models with lung, bone, or lymph node metastasis of many types of cancer, and showed promising therapeutic efficiency. NPs carrying PTT enablers increase accumulation at the tumor site, reducing side effects on healthy cells. Due to their high therapeutic efficacy, ability to penetrate deeply into the tissue, and low invasiveness compared to surgical treatment, PTT has been successfully applied in the clinic as therapy for neck and head tumors. Most of these current photothermal nanotherapeutics are constructed with inorganic nanomaterials made of noble metals, carbon nanotubes, or graphene, which significantly hampers their clinical applications due to their potential long-term toxicity. To overcome these limitations, PTN requires more sophisticated design and engineering with precise targeting, powerful imaging, and synergistic effects to improve the therapeutic outcome of patients with cancer metastasis.

Using this approach, Jie et al. developed novel oxygen vacancy-rich tungsten bronze NPs (NaxWO3). These NPs did not show toxicity toward human embryonic kidney 293T cells, but they observed significant dose-dependent cytotoxicity against breast cancer 4T1 cells, showing excellent PTT conversion ability and photothermal stability in vitro. Moreover, strong tumor ablation was observed in vivo, and complete tumor inhibition was achieved without recurrence for 2 weeks in an ectopic 4T1 tumor mouse model. Finally, intratibial breast cancer BMs were inhibited, resulting in decreased bone destruction and tumor volume. Mechanistically, the authors demonstrated that NaxWO3+PTT treatment increased apoptotic gene expression and downregulated osteoclastic gene expression, leading to decreased osteoclast differentiation. In addition, after NaxWO3+PTT administration, recurrence was not observed after 2 weeks following percutaneous 4T1 tumor removal, confirming the desirable PTT effects of novel NaxWO3 NPs against cancer cells.

Moreover, photothermally triggered IR780 combined with PLGA NPs (IR780@PLGA NPs) has been used to treat breast cancer BMs. IR780@PLGA NPs combined with PTT achieved exceptional cytotoxicity and inhibition of BM growth both in vitro and in vivo. The authors speculated that the effects of the IR780@PLGA NPs were mainly due to intra-tumor injection resulting in an increased accumulation in the tumor. Moreover, intra-tumor injection avoided biodegradation and off-target distribution. Compared to therapies such as surgery and chemotherapy, IR780@PLGA NPs combined with PTT showed promising therapeutic effects while inhibiting bone damage with low cytotoxicity.

In a similar work, Jiang et al. recently fabricated NPs based on superparamagnetic iron oxide ($\text{Fe}_3\text{O}_4$) and indocyanine green (ICG)-entrapped PLGA modified with zoledrionate (ICG/Fe3O4@PLGA-ZOL) for PTT of breast cancer tibial BMs. ICG is an FDA-approved water-soluble tricarbocyanine dye that exhibits absorption and emission maxima in the near-infrared region. ICG and Fe$_3$O$_4$ can convert this light into heat, while NPs with Fe$_3$O$_4$ and zoledrionate can target a specific site in the bone under an external magnetic field. By using both methods simultaneously, higher accumulation in the tibia and greater antitumor activity of these NPs was achieved in vivo. Furthermore, the NP accumulation in the medullary cavity of the tibia allows the treatment of deep BM lesions. ICG/Fe3O4@PLGA-ZOL NPs were able to inhibit tumor cell growth and bone resorption by the dual actions of bone targeting and PTT, suggesting a great potential as cancer theranostics in the near future.

Another interesting material used in combination with PTT treatment is polydopamine (PDA). This is a synthetic bio-compatible analog of melanin, which can be used to form either multifunctional core–shell nanostructure in NPs formulations or used as a tunable coating. Its versatility was shown by Yang et al. where they focused on the optimization of arginine-doped PDA NPs (SMNP) toward improved properties and functions especially enhancing the photothermal efficiency. The authors reported that even a small percentage (0.21%) of arginine in the PDA NPs (SMNP-1) was able to increase their NIR absorbance and PTT efficiency by $\approx60\%$, demonstrating high cell death in vitro and the ability to destroy the tumor tissue structure in vivo in breast cancer bone metastases models compared with conventional PDA-based synthetic melanin agents.
Among the works that employed PDA, a recent study by Wang et al.\textsuperscript{138} focused on the use of PDA NPs functionalized with alendronate, as bone active targeting agent, loaded with the antitumor irinotecan active metabolite SN-38 (PDA-ALN/SN38). These NPs were able to target hydroxyapatite in vitro and efficiently heated up upon NIR irradiation. Furthermore, PDA-ALN/SN38 demonstrated to be more effective in killing MDA-MB-231 breast cancer cells and PC-9 human lung adenocarcinoma cells compared to SN-38 treatment and PTT individually. Their efficacy was demonstrated also in vivo using orthotopic bone metastatic murine models, in which NIR irradiated PDA-ALN/SN38 showed a tenfold and fivefold tumor reduction than free SN-38 alone or empty PDA-ALN NPs irradiated, respectively. Moreover, when these NPs were combined with iron as MRI contrast agent (PDA/Fe-ALN), they demonstrated remarkable accumulation in osteolytic lesions in an orthotopic bone metastasis murine model. Overall, this formulation enhanced the magnetic resonance contrast in bone tumors and efficiently suppressed bone tumor growth as well as tumor-induced osteolysis.

In another study, the authors showed how PTT associated to autophagy inhibition could be a strategy to suppress bone tumors and metastasis formation.\textsuperscript{139} Using polyethylene glycol-conjugated alendronate-functionalized and chloroquine (CQ)-loaded PDA NPs (PPA/CQ), they assessed how these NPs efficiently accumulate into the bone tissues, especially into the osteolytic lesions around tumors. CQ was able to inhibit both autophagy in MDA-MB-231 breast cancer cells and differentiation and activation of osteoclasts reducing tumor-associated bone resorption with high efficiency. The data revealed that PPA/CQ mediated PTT reduced cancer cell viability with 20% higher efficiency than PPA- and PDA-mediated ones. Thus, it efficiently inhibited tumor growth of 50% compared to PPA/CQ alone and osteolysis in an in vivo model of breast cancer, enhancing the PTT ability of killing cancer cells.

As stated above, the use of radiotherapy against primary bone tumors and bone metastases is one of the staples for the treatment of these neoplasms.\textsuperscript{140} However, radiopharmaceuticals per se are often unable to accumulate into the tumor lesions, causing toxic effects in the other tissues (especially kidney). Thus, to increase the targeting specificity, radiopharmaceuticals have been conjugated with antibodies or other active ligands. Despite the advantages of targeted radiopharmaceuticals, the use of NPs to encapsulate them together with other therapeutic agents offers the possibility of novel combined treatments as well as unexpected radiopharmaceutical-drug synergy. An example is represented by the study of Kotagiri et al.\textsuperscript{141} In this work, the hydrophobic antitumor drug Titanocene (TC)\textsuperscript{142} was loaded in albumin NPs (TC-HSA) as a targeting strategy against bone metastatic breast cancer murine model. After the administration of TC-HAS, mice were injected with the FDA-approved radiopharmaceutical fluorodeoxyglucose (FDG).\textsuperscript{143} FDG works both as a radiopharmaceutical and as a photo-activator, which enables TC photodynamic cytotoxicity. The authors demonstrated the ability of TC-HSA and FDG to accumulate in mice lower limbs bones, where the metastases tended to localize. Furthermore, this strategy was able to reduce almost 70% of bone metastases, leaving only well-defined metastatic foci. This treatment could help to reduce the metastatic burden and make bone metastases operable and less painful. This study is the first example of how employing NPs to improve radiotherapy goes beyond the simple tumor targeting that can be addressed by simpler radiopharmaceutical–ligand conjugate, and instead uncovers new synergies between radiotherapy and other treatments, ultimately leading to innovative therapies.

### 6. Other Bone Disorders

There is a plethora of bone diseases that can occur at or before birth, such as genetic abnormalities and developmental defects. There are also many diseases that will affect the skeleton later in life, such as osteogenesis imperfecta, osteoporosis, Paget’s disease, and osteopetrosis. Due to the low prevalence of these diseases in the population (<1%), and the heterogeneity in their etiology, they still lack effective treatments (Table 3).\textsuperscript{144–152}

Recently, NPs have been among the new strategies evaluated as possible therapies, but their application is limited due to the complexity and low population prevalence of these diseases. Current studies focus more on the unique nanostructures or development mechanisms of the bone pathology that could be potential therapeutic targets, rather than attempting to use NPs to specifically target each disorder (Table 4).\textsuperscript{151}

#### 6.1. Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a genetic disorder that causes a decreased amount of type I collagen.\textsuperscript{154,155} Normally, type I collagen is an important component of bone, skin, tendons, dentin, and sclera. This protein is composed of a triple helix structure: one alpha-1 chain (coded by COL1A1) and one alpha-2 chain (coded by COL1A2).\textsuperscript{156–158} The large majority of genetic mutations occur in the COL1A1 or COL1A2 genes.\textsuperscript{156,159,160} The decreased amount of collagen can result from either decreased secretion or the production of abnormal collagen subunits, and can cause an insufficient production of osteoid, leading to abnormal bone remodeling. The disease affects 1/20 000 individuals at birth; mutations are either inherited by autosomal dominance or occur via a de novo mutation. No resolute cures are currently available for OI.\textsuperscript{161–165}

Clinically, patients present with bone fragility, frequent bone fractures, ligamentous laxity, short stature, scoliosis, codfish vertebrae, basilar invagination, oclecran apophyseal avulsion fractures, coxa vara, and congenital anterolateral radial head dislocations. Non-orthopedic signs and symptoms include blue sclera, dysmorphic and triangular shaped facies, hearing loss, brownish opalescent teeth, hypermetabolism, thin skin, mitral valve prolapse, and aortic regurgitation.\textsuperscript{154,155,159,166–168}

#### 6.2. Osteopetrosis

Osteopetrosis is an extremely rare metabolic disease in the bone that results in defective osteoclast resorption of immature bone.\textsuperscript{169} As a result of reduced bone resorption, dense bone and obliterated medullary canals form, resulting in an increased risk of fracture.\textsuperscript{170} Patients with this disorder are also more prone to cranial nerve palsies due to the overgrowth of skull forams, osteomyelitis due to the lack of marrow vascularization and
Table 3. Table depicts the imbalance that occurs in each of the bone disorders.

| Pathophysiology          | Bone resorption | Bone formation | Serum Ca²⁺ | Serum PO₄³⁻ | ALP |
|--------------------------|-----------------|----------------|------------|-------------|-----|
| Osteogenesis imperfecta  | Abnormal        | Abnormal       | N          | N           | N/↑ |
| Osteopetrosis            | ↓↓              | ↑              | N          | N           | N   |
| Paget’s Disease          | ↑↑              | ↑↑             | N/↑        | N/↑         | ↑↑  |
| Osteoporosis             | ↑↑              | ↑              | N          | N/↑         | N   |

↑ = increased, ↓ = decreased, N = normal

Table 4. Nanoparticles treatment for bone disorders.

| Study                  | Clinical applications | Delivered agent | NP | Drug class | Study design | Results                                                                 |
|------------------------|-----------------------|-----------------|----|------------|--------------|-------------------------------------------------------------------------|
| Wu et al. [208]        | Osteogenesis imperfecta, osteoporosis | Copper          | MSN | N/A        | In vitro     | • Significant promotion of osteogenic differentiation of hBMSCs and upregulated bone-related gene expression |
| Kim et al. [210]       | Osteogenesis imperfecta, osteoporosis | SiRNA           | MSN | N/A        | In vitro     | • Mesoporous bioactive NP can effectively deliver SiRNA to inhibit osteoclast activity |
| Curtin et al. [212]    | Osteogenesis imperfecta, osteoporosis | BMP-2           | nHA | N/A        | In vitro     | • Enhanced osteogenesis was observed following nHA-BMP2 transfection both in 2D and 3D |
| Song et al. [213]      | Osteoporosis, osteoporosis | miRNA           | Vascular endothelial cell exosomes | N/A | In vitro/in vivo (Murine model) | • Inhibited osteoclast induction in vitro • Inhibited osteopetrosis development in vivo |
| Conner et al. [214]    | Paget’s Disease, osteogenesis imperfecta, osteoporosis | Alendronate, pamidronate | Gold | Bisophosphonate | In vitro | • Concentration-dependent effect on osteoclastic and osteoblastic viability with the delivery of bisphosphonate functionalized gold NP |
| Hu et al. [216]        | Osteoporosis          | B-estradiol      | MSN | Estrogen derivative | In vitro | • Efficiently enhances osteoconductive activity in an in vitro model |
| Cao et al. [217]       | Osteoporosis          | BMP-2           | chitosane | N/A | In vivo (rabbit model) | • Induce angiogenesis and bone formation in a critically sized bone defect in an in vivo model |
| Ignjatovic et al. [218] | Osteoporosis         | Cobalt          | HA  | N/A        | In vitro/in vivo (Rat model) | • Decreased viability of osteoblastic cells • Demonstrated regeneration of mandibular osteoporotic bones in an in vivo model |
| Alghamdi et al. [220]  | Osteoporosis          | Bisphosphonates | Calcium phosphate | Bisphosphonate | In vivo (Rat model) | • Simultaneous targeting of bone formation and bone resorption to synergistically improve bone-implant integration in an osteoporotic rat model |
| Weitzmann et al. [221] | Osteoporosis          | Polyethylene glycol | MSN | N/A        | In vitro/in vivo (Murine model) | • Effectively promoted osteoblast activity while inhibiting osteoclast activity both in vivo and in vitro |

NP = nanoparticle; MSN = mesoporous silica nanoparticles; BMP-2 = bone morphogenic protein-2; nHA = nano-hydroxyapatite; hBMSC = human bone marrow stromal cells; NR = not recorded; N/A = not applicable

impaired white blood cell function, coxa vara, and carpal tunnel syndrome. [171–173]

The pathophysiology of the disease varies. In the autosomal recessive form, the proton pump or chloride channel is dysfunctional, resulting in pancytopenia, hepatosplenomegaly, and infection. However, this form is fatal at an early age without a bone marrow transplant. The intermediate autosomal recessive form results in carbonic anhydrase II or chloride channel dysfunction. [171–174] Finally, the benign, but autosomal dominant form of the disease is due to chloride channel dysfunction. [171,175]

6.3. Paget’s Disease

Paget’s disease results in abnormal bone remodeling due to an imbalance between osteoclasts and osteoblasts, and thus causes excessive bone resorption and abnormal bone formation. [176] No definite cause has yet been elucidated, but it is believed to be due to a viral infection, most likely paramyxovirus or respiratory syncytial virus. [177,178] Histologically, numerous large osteoclasts with multiple nuclei are observed, with virus like inclusion bodies within the osteoclasts. [179–182]
Epidemiologically, Paget’s disease affects 1–2% of the population, peaking in the 5th decade of life, and is most common in Caucasians. The most commonly affected sites include the femur, pelvis, tibia, skull, and spine. Orthopedic symptoms include bone pain, long bone bowing, fractures, large joint osteoarthritis, and secondary sarcomas. Other symptoms include high output heart failure. Among the patients that are diagnosed with Paget’s disease, less than 1% will go on to develop Paget’s sarcoma.

### 6.4. Osteoporosis

Osteoporosis is an age-related disease that results in an overall decrease in bone mass. This condition is secondary to an uncoupling of osteoclast and osteoblast activity that leads to the disruption of the bone microarchitecture. As a result, poorly treated osteoporosis leads to high rates of fragility fractures, which most commonly involve the distal radius, axial spine, hip, and pelvis.

Currently, 10 million Americans and 200 million people worldwide are estimated to have osteoporosis. Within the United States, 1.5 million fragility fractures occur each year. There is a four-to-one female predominance in the incidence of osteoporosis. Osteoporosis is most commonly diagnosed through dual energy X-ray absorptiometry (DEXA) scan, which evaluates bone mineral density (BMD) in the lumbar spine and the hip. A T score less than −2.5, which is BMD greater than 2.5 standard deviations below the peak BMD of an average 25-year-old individual, is considered diagnostic for osteoporosis.

### 7. Current Treatments for Other Bone Disorders

Current standard of care for OI includes fracture prevention and treatment. Fracture prevention involves early decreasing deformities and reduce the risk of fractures. Among the fracture prevention treatments, BPs are often given to reduce fracture rate and pain. The BPs act by inhibiting osteoclasts, resulting in increased cortical diameter and cancellous bone volume. This approach is also applied for Paget’s disease and osteoporosis as first-line therapy, in order to prevent future fragility and fracture by decreasing osteoclastic bone resorption. Despite their therapeutic benefits, BPs are systemically administered, increasing the time of exposure, which can cause side effects such as osteonecrosis of the jaw and atypical fractures.

Other strategies for OI therapy rely on growth hormones and bone marrow transplantation, and have been used with limited success. Moreover, surgical intervention with fixation is applied only for OI-related fracture treatment. Bone marrow transplant, high dose 1,25-dihydroxy vitamin D and/or interferon gamma-1B is the therapy most commonly used for osteopetrosis treatment, while fractures often result in casting, or surgical treatment.

A supportive care approach based on anti-inflammatory treatments can alternatively be used for Paget’s disease, either alone or combined with BPs. Calcitonin is the second line of treatment, causing osteoclasts to shrink in size and decreasing their bone resorption activity. Surgical treatment is necessary for patients with degenerative joint diseases or long bones with bowing and/or impending pathological fractures. However, treatment is not curative, and ultimately aims to solely decrease the morbidity associated with the disease.

### 8. Nanoparticle Treatments for Other Bone Disorders

#### 8.1. Osteogenesis Imperfecta

Due to the limited treatment options, research is being conducted to improve the imbalance between bone resorption and bone formation (Table 4). One way to modify this imbalance is to reduce the activity of osteoclasts. There are three main ways to target osteoclasts: through their recruitment to the site, their activity, and the time that they remain viable. Thus, gold NPs (AuNPs) has been shown to efficiently target and inhibit osteoclast formation and improve localized delivery of BPs to osteoclasts. In particular, it has been demonstrated that AuNPs have improved the delivery of alendronate, one of the currently used BPs, but other BPs have yet to be investigated. In addition, because osteoblasts also play a role in the imbalance of bone remodeling, the utility of targeting osteoblasts with AuNPs should be further investigated.

Though not specifically investigated for osteogenesis imperfecta treatment, the activity of osteoclasts has been shown to be reduced using mesoporous silica NPs (MSNs). This was achieved by incorporating calcium ions into MSNs such as mesoporous bioactive glass nanospheres. Glass NPs have been proven to promote bone formation due to the release of calcium ions that support osteoblast proliferation and differentiation.

In addition, the MSNs were able to reduce osteoclast activity by delivering siRNAs designed to downregulate genes involved in osteoclastogenesis (i.e., c-fos, c-fos, cathepsin-K, tartrate-resistant acid phosphatase, and nuclear factor of activated T cells cytoplasmic 1). siRNAs can also be used to decrease the expression of nuclear factor kappa B (NF-kB), which is the osteoclast activation mediated receptor, ultimately inhibiting the activity of osteoclasts. However, these NPs have limited utility for genetic diseases, as they are only able to release siRNA for 3 days, reducing the possibility for clinical application.

HA-NPs have also been investigated in bone disorders due to their similar structure to the inorganic composition of bone minerals. Curtin et al. used HA-NPs to deliver bone morphogenetic protein 2 (BMP-2) to induce stem-cell mediated bone formation. The resulting nHA-BMP2NPs were uniquely suited for applications in bone tissue engineering, being bioactive and osteoconductive at the same time. Using an in vitro model, the authors first evaluated the transfection ability of these NPs compared to a commercially available kit (e.g., Lipofectamine 2000, a calcium phosphate [CaP] transfection kit). Interestingly, transfection using the HA-NPs was also more efficient in human MSCs compared to rat MSCs. Enhanced osteogenesis was observed following HA-BMP2 transfection (plasmid- DNA encoding BMP2) in both 2D and 3D cultures when using low levels of pBMP2, demonstrating their intrinsic capacity for promoting bone formation. When nHA-BMP2 particles were combined with the additional osteogenesis-promoting and osteoconductive nature of
collagen-nHA scaffolds, the inherent bone forming capacity was considerably enhanced.\cite{210,211} However, additional studies need to be performed in vivo to determine if sustained target delivery can be achieved.

The major limitations of RNAi molecule delivery strategies are based on siRNA instability, enzymatic degradation, and immune recognition; these can be overcome by NPs, which also improve transportation efficiency across the cell membrane compared to other carriers. An example of this approach is the implementation of CRISPR-Cas9 technology with an NP delivery system. Non-viral vector-based therapeutic genome editing with CRISPR/Cas9 in vivo has started a new era in the application of molecular therapy for the treatment of many genetic diseases. Despite the exciting potential of this strategy, some studies reported unwanted off-target effects. Therefore, CRISPR-Cas9 still requires further basic research and optimization before being applied to the clinic, and we believe that emerging delivery strategies will solve the limitations of delivery efficiency, biosafety, and scale-up production in the near future.\cite{232–234}

8.2. Osteopetrosis

Thus far, studies evaluating the use of NPs to treat osteopetrosis are sparse (Table 4). Studies have focused on the role of bone-secreted exosomes in maintaining bone homeostasis. Exosomes are a type of extracellular vesicle containing various cargos; in effect, they are natural NPs. Song et al. developed vascular endothelial cell exosomes that were able to inhibit osteopetrosis. Moreover, when delivering miRNA (miR-155) using the vascular endothelial cell exosomes, osteoclast induction was suppressed in vitro. In vivo, this approach inhibited osteopetrosis development in a murine model in which mice underwent ovariectomy to cause hormone-dependent osteopetrosis: specifically, exosomes were able to increase, two to threefold, the bone density, bone surface, and number of trabeculae in treated mice compared with untreated mice, as well as decreasing the number of TRAP positive cells (which are a marker of osteoblasts) by \(\approx50\%\).\cite{235} By targeting pathways that are important in disease development and progression, it is possible to treat or halt disease progression.

8.3. Paget’s Disease

Current studies on the use of NPs to treat Paget’s disease are summarized in Table 4. This disease is rare in humans, and it is difficult to identify an animal model that appropriately recapitulates the disease. Due to these limitations, the use of NPs to treat Paget’s disease has yet to be investigated. However, there are a few promising preliminary experimental findings that are worth discussing.

Conner et al. examined the effect of AuNPs functionalized with BPs on osteoclasts. AuNPs were functionalized with alendronate and pamidronate, and when murine pre-osteoclasts and osteoblasts were treated with the NPs, no changes in the viability of the osteoclasts were observed in vitro. This suggests that the imbalance between osteoclasts and osteoblasts may be able to be altered through the use of BP-functionalized AuNPs.\cite{236}

Similarly, Pascaud et al. used BPs as an antiresorptive drug. The authors studied the application of tiludronate, which was adsorbed on nanocrystalline HA. This study identified the mechanism of BPs adsorption on nanocrystalline HA with different surface composition, as well as how BPs adsorption on the nanocrystalline HA affects phosphate ion release. They were able to identify that phosphate ion were released by dissolution of nanocrystals, the maturation of nanocrystalline HA, and the release of surface phosphate groups. Moreover, the adsorption was strongly influenced by the nature and composition of the nanocrystals, suggesting that certain nanomaterials would be superior over others in the treatment of Paget’s disease.\cite{237}

8.4. Osteoporosis

Due to limitations related to the current treatment, extensive research is being conducted for safe alternative treatment of osteoporosis with NPs (Table 4). One method consists of using nanomaterials for effective therapeutic delivery. A study by Hu et al. demonstrated that use of \(\beta\)-estradiol-loaded MSN NPs is an efficient method for enhancing the osteoconductive potential of this nanomaterial in an in vitro model. Using a layer-by-layer assembly technique, they found that MSNs can effectively serve as a long term nano-reservoir type drug-delivery system that efficiently regulates bone homeostasis.\cite{238}

Using NPs for the regulation of bone remodeling is another area of research in the treatment of osteoporosis. A study by Cao et al. demonstrated, using an in vivo rabbit model, that BMP-2-loaded chitosan NPs placed in critically sized bone defects can completely bridge the defects in as little as 2 weeks. They found that these NPs can efficiently induce angiogenesis and bone formation.\cite{239} Although this study did not directly investigate an osteoporosis model, recent trends in osteoporotic treatment target bone formation as an effective alternative to the traditional approach of preventing bone resorption.\cite{240} Thus, utilizing BMP-2 loaded NPs to promote bone formation is an effective, novel approach for the direct treatment of osteoporosis.

Ignjatovic et al. introduced cobalt into the crystal lattice of HA to confer it superparamagnetic properties.\cite{241} The authors showed in vitro that HA-NPs were biocompatible with and not toxic to epithelial Caco-2 cells, whereas they substantially decreased viability and deformed the cytoskeleton and cell morphology of MC3T3-E1 osteoblastic cells in direct proportion with the content of cobalt ions incorporated. In vivo, this method of substitution led to an increase in osteogenesis and replacement of osteoporotic bone in a rat model. The most promising results were observed when HA-NPs with the largest content of cobalt ions (12 wt%) were implanted in the bone defect, resulting in increased angiogenesis, vascularization, migration, and proliferation of bone-forming cells as well as increased alkaline phosphatase activity and bone density.

A similar technique was exploited by Alghamdi et al. utilizing titanium implants coated with BP-loaded calcium phosphate NPs (nCaP), which successfully promoted new bone formation while also decreasing osteoclast activity in an in vivo rat osteoporotic model.\cite{242} Overall, this strategy demonstrated that the simultaneous targeting of bone formation (by nCaP) and bone resorption (by BP) using nCaP/BP surface coatings synergistically improved bone-implant integration, especially in osteoporotic conditions.
A study by Weitzmann et al. demonstrated that bioactive silica NPs with an electron-dense cobalt ferrite magnetic core decorated with polyethylene glycol (NP1-MNP_PEG) had the ability to promote osteoblast activity while inhibiting osteoclast activity in vitro and in vivo. The authors investigated the capacity of these NPs to reverse bone loss in aged mice, as a model of human senile osteoporosis. They observed that NP1-MNP_PEG NPs increased bone mineral density, bone volume, and biochemical markers of bone formation in comparison with osteocalcin vehicle as negative control, without damaging the organs. Thus, this method may be an effective novel method for counteracting bone loss. These findings show promising data to guide future research into incorporating NPs for the prevention and treatment of osteoporosis.

9. Challenges in Nanotechnology

Remarkable success has been achieved in the nanotechnology field, especially in the development of NPs as drug delivery systems. These formulations have revolutionized the therapeutic field in the past few decades, and several NP formulations have been approved for clinical trials or achieved FDA approval. However, many challenges to introducing NPs into clinical applications still lie ahead (Figure 7).

9.1. Safety

One challenge to NP use is safety, highlighting the need for better tools and methods to assess NP toxicity and carcinogenicity. NP toxicity is highly dose- and exposure-dependent, and therefore they are generally used at concentrations far below those which are considered harmful. However, the long-term effect of NP bioaccumulation inside the body needs to be further investigated, especially in the healthy host. In addition, despite the existence of numerous NP-like products already on the market, there are still some scientific/methodological gaps in the knowledge of potential health and safety hazards associated with some of the materials used. Currently, there are no international standards for NP-specific risk assessments, including data requirements and testing strategies. This may be due to assessments being laborious and costly. While manufacturers are committed to evaluating the safety of NP products and eager to implement the necessary measures to ensure safety, due to
the lack of regulation, each manufacturer utilizes different tools and assessments related to safety. Therefore, stricter precautionary procedures and guidelines are needed for clinical NP applications.[244–249]

9.2. Commercialization

Another key obstacle for NP manufacturing is commercialization, which is the reason why few formulations are in pre-clinical studies. Despite what has been learned so far about NP features, their complexity in composition and structure compared to free small molecule drugs make the scale-up and reproducibility challenging. Most approved formulations are still based on a bottom-up approach, where different components are assembled under specific conditions, triggering their self-assembly and final formulation. Unfortunately, this approach can be difficult to scale up due to the many variables that need to be finely controlled and tuned.[244–249] The shared goal of chemistry, manufacturing, and controls (CMC) and good manufacturing practices (GMPs) is to ensure that a product consistently meets a predetermined standard of quality. Therefore, a well-planned, carefully designed manufacturing practice and batch-to-batch reproducibility are necessary requirements for the transition from the preclinical to clinical application. In particular, the scale-up of more complex NPs creates additional challenges, and existing unit operations or development of novel manufacturing processes need to be revised.[244–248,250]

9.3. Storage

The complexity of NP formulations also poses substantial concerns for their stability under storage, a factor that is often overlooked in the preclinical research setting. For long-term storage, freezing and lyophilization are the most commonly used methods for a wide range of NPs. Unfortunately, crystallization and vacuum dehydration can damage macromolecules, decreasing NP stability unless appropriate cryoprotectants are used. Since the physical stability of these nanomaterials, including those for mRNA delivery, is not well known, storage remains a critical step to allow commercialization. This problem has recently been faced for the Pfizer COVID-19 vaccine, which required cold temperature storage for its stability. Therefore, it is urgently necessary to study and understand the various conditions for long-term storage, such as temperature and physical states (aqueous, freezing, or lyophilized), in order to provide a basis for future clinical applications.[244–249,251]

9.4. FDA Approval

Another challenging issue is the regulatory approval of NPs by the FDA due to the absence of specific guidelines for NP production. The FDA believes that evaluations of safety, effectiveness, public health impact, or regulatory status for NPs should consider any unique properties and behaviors that the nanotechnology application may impart. Some NPs have received FDA approval, and are in early stages of development or in clinical trials, but most of them are based on conventional drugs that are already FDA approved and are simple formulations (e.g., albumin-bound rapamycin NPs, paclitaxel albumin NPs (Abraxane), nab-paclitaxel/rituximab-coated NPs, remdesivir (GS-5734) and neurosivir (NA-831) NPs.[7,10,252] However, current research is ongoing into the formulation of new and more complex NP platforms for future therapeutic options. Therefore, case-by-case investigations are required to harness the tremendous potential of these nanotherapeutics. A comprehensive set of guidelines for regulatory approval is urgently needed to expedite the evaluation and approval of nanotherapeutic treatments.[244–249,251]

9.5. In Vitro and in Vivo Models

Currently, most studies still rely exclusively on the use of in vitro and in vivo models. Though in vivo models are well-established, widely used, and provide information on biodistribution targeting as well as systemic and organ toxicity of NPs, they are not able to fully reproduce all bone disease features. Most in vivo studies rely on subcutaneous tumor models that are simple and reproducible but not able to replicate the tumor microenvironment. Considering the high impact of metastases on cancer mortality, models that reproduce human metastasis are also essential for the understanding of NPs penetration and targeting. To overcome the discrepancy between preclinical studies and clinical trials, animal models that more closely mimic the heterogeneity and anatomical histology of human tumors have been developed, such as genetically engineered mouse models, cell line-derived xenografts, and patient-derived xenografts.[254,255] These models represent the most powerful tool to study tumor heterogeneity and cancer-associated mechanisms and satisfy the requirements of an effective preclinical tool. They have been used for targeted drug treatment studies, drug screening, and research into resistance mechanisms, confirming their accurate predictive value, fidelity, and stability. Though the application of these mouse models in tumor research is associated with a lot of advantages, they also face challenges. They rely on immunodeficient mice and are technically time-consuming and costly.[254,255]

To achieve more translational results, the development and biological evaluation of NPs should be performed in a multidisciplinary framework that combines formulation, scale up potential, stability under storage, and use of disease models that closely resemble the pathological conditions present in the clinical setting. In this context, several innovative in vitro models have been successfully employed.[156] These models are designed to include the 3D, dynamic, and stromal components normally present in the tumor. Among these 3D models, cellular spheroids have been used extensively. Spheroids help to recapitulate the structure of solid tumors, creating a hypoxic, often necrotic center and a more vital external layer of cells. Spheroids can be cultured in suspension, but in several cases, spheroids have been integrated within an ECM-mimicking hydrogel. This ECM is composed either from synthetic polymers (e.g., Poly–caprolactone, alginates), natural polymers (collagens and proprietary formulations such as Matrigel), or a combination of both. The use of these structured models allows tumor cells to interact with ECM components. Moreover, researchers are then able to tune the porosity and stiffness of the gel to mimic a more aggressive tumor.[257,258]
Another layer of complexity is provided by utilizing co-cultures of two or more cell types, which allows the reconstruction of the stromal niche often found in bone tumors in an in vitro setting. This niche includes endothelial cells, osteoclasts, and osteoblasts. Different cell types found within the tumor can influence tumor growth and respond to therapy, making it important to explore not only each cell type, but also how the cell types influence each other. Finally, the use of dynamic culture conditions through plate stirrers or microfluidic chips exposes the cells to physiological shear stresses, forcing them to adapt and migrate in a similar way to what occurs clinically.\[18\]

Recently, efforts have been focused on the development of biomimetic “organ/tumor-on-a-chip” models. These models replicate the tumor structure, microenvironment, and biology, and therefore have the potential to address many important biological questions. Although still in early stages, these systems have already offered additional insights into NP cell binding, accumulation, and diffusion.\[259–261\] Several technical challenges (e.g., co-culture systems; representation of normal metabolism, and physiology) still need to be addressed for on-chip platforms before they can be widely adopted and translate to the clinical phase. This would not only make the data more meaningful, but also increase the chances of formulations reaching clinical testing and the clinic.

**10. Conclusions and Future Perspectives**

In this review, we discussed the different NPs commonly used for bone-related diseases. Despite all the progress that has been made and the exciting advances that have occurred in the development of various multifunctional drug delivery platforms, these NPs are not ready for the clinic. Most are still at the cellular and animal experimental stage, and will face a long transitional period before clinical application.

Also, several obstacles impede the study of bone diseases, presenting real challenges to overcome. These include tissue that is hard to manipulate, rare diseases, difficulty obtaining tumor tissue fragments from human patients (e.g., for BMs), and the limited number of animal models that effectively resemble the corresponding human bone disease. Even though recent NPs delivery systems have shown promising results in in vitro or ex vivo studies, it is fundamental to evaluate the platforms in appropriate and more realistic bone tumor murine models that can closely mimic the human tumors, offering better insight into NP efficacy.

From a therapeutic perspective, the biggest challenge to meet in NPs development remains the ability to maximize drug accumulation at the target site and to minimize the dosage and systemic adverse effects.

From a fabrication standpoint, NP formulations tend to include the presence of materials similar to native bone structure (such as HA or its analogs). This approach is inherently biomimetic and ensures NP affinity for the target tissue and efficient absorption.

Active targeting strategies have been investigated to exploit more specific target ligands; for example, using BPs thanks to their ability to be integrated within the bone mineralized matrix. Despite the enhanced accumulation at the target sites, there are still key factors such as target tissue accessibility, disease-dependent anatomical and physiological barriers, and formulation stability which are limiting the application of these nanoformulations. In addition, it is imperative to understand the optimal targeting ligand density on the surface of each formulation. Through extensive experimentation and by taking a disease-driven approach for the NP-based platforms development, it will be possible to build comprehensive preclinical data sets that best predict efficacy for patient sub-groups and support the translation to the clinic.

Evidently, not all drug delivery strategies are equally promising. For these reasons, we believe that in the near future, combined therapies can provide a synergistic therapeutic effect, prolonged therapeutic window, reduced drug resistance, limited side effects, and systemic exposure to healthy tissues. Bone-targeted NPs could be used in combination with immunotherapy to specifically deliver checkpoint inhibitors, antibodies, and immune-modulating agents (antigens/adjuvants), increasing their therapeutic effect. Considering the indispensable role that immunotherapy plays in cancer treatment, its combination with other therapeutic options such as PTT or chemotherapy could increase the final outcomes. Moreover, advances in technology have driven innovation and progress in radiation oncology. Thus, radiotherapy in combination with an NP-based imaging agent administration could not only improve the treatment efficacy as well as the toxicity reduction, but also could better monitor radiotherapy treatment response in real time and further improve personalized treatment.

In conclusion, we expect that nanotechnology will play more important roles in the future treatment of bone tumors and disorders. We anticipate that better optimized nanocarriers will be developed after overcoming some of the above-mentioned challenges.

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**Conflict of interest**

The authors declare no conflict of interest.
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