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Bisphosphonate conjugation for bone specific drug targeting

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ABSTRACT

Bones provide essential functions and are sites of unique biochemistry and specialized cells, but can also be sites of disease. The treatment of bone disorders and neoplasia has presented difficulties in the past, and improved delivery of drugs to bone remains an important goal for achieving effective treatments. Drug targeting strategies have improved drug localization to bone by taking advantage of the high mineral concentration unique to the bone hydroxyapatite matrix, as well as tissue-specific cell types. The bisphosphonate molecule class binds specifically to hydroxyapatite and inhibits osteoclast resorption of bone, providing direct treatment for degenerative bone disorders, and as emerging evidence suggests, cancer. These bone-binding molecules also provide the opportunity to deliver other drugs specifically to bone by bisphosphonate conjugation. Bisphosphonate bone-targeted therapies have been successful in treatment of osteoporosis, primary and metastatic neoplasms of the bone, and other bone disorders, as well as refining bone imaging. In this review, we focus upon the use of bisphosphonate conjugates with antineoplastic agents, and overview bisphosphonate based imaging agents, nanoparticles, and other drugs. We also discuss linker design potential and the current state of bisphosphonate conjugate research progress. Ongoing investigations continue to expand the possibilities for bone-targeted therapeutics and for extending their reach into clinical practice.

1. Introduction

1.1. Bone biology: the ideal bone-targeting drug treats disease and preserves normal bone function

Bones perform many roles in the body such as structure and movement, protection for organs, and mineral storage, and provide the site for blood cell production as bone marrow houses hematopoietic cells generating red blood cells, leukocytes, and platelets. Bones are composed of both organic and inorganic materials, as well as cells that synthesize, remodel, and maintain bone. Bones act as the major storage for calcium and phosphorus in the body through their hydroxyapatite crystal structure, and bone continuously undergoes cycling of deposition and resorption of the osteoid and hydroxyapatite matrix. Minerals are released from bone through resorption by osteoclasts, the cells responsible for breaking down bone matrix, while osteoblasts construct new bone by secreting collagen, chondroitin, and osteocalcin to form osteoid, where hydroxyapatite crystals are deposited to create the hardened bone matrix. Osteocytes, which make up the majority of bone cells, are encased in the bone matrix, sensing pressure to initiate bone resorption by osteoclasts. The balance between bone resorption and formation depends on signals from cytokines, hormones, chemokines, and mechanostimulation, and resorption by osteoclasts is essential for maintenance of normal bone density, trabeculae in spongy bone, and release of minerals. An imbalance in these processes results in pathology of weakened or hyperplastic bone, including common degenerative bone diseases (Florencio-Silva et al., 2015).

Degenerative bone diseases include osteoporosis, which is predicted to affect 61 million men and women by 2020 (Bartl and Gradinger, 2009), as well as Paget's disease and osteogenesis imperfecta. Osteopetrosis is a less common disease of malfunctioning osteoclasts resulting in overly dense but brittle bone. Neoplastic disorders of the bone occur with tumors arising from plasma cells (multiple myeloma), the various bone cell types (*i.e.* osteosarcoma, Ewing's sarcoma, and chondrosarcoma), and also occur as a result of metastasis from tumors originating elsewhere, especially from breast and prostate cancer, the most common origins of metastatic bone neoplasia (De Rosa et al., 2013). The bone microenvironment provides an attractive site for seeding of metastases as it is a rich source of growth factors, and reciprocal interactions between bone and cancer cells result in a so called “vicious cycle of bone metastasis”, during which increased resorption induced by cancer cells further triggers release of growth promoting factors and growth of the tumor (Zheng et al., 2013). Tumors of the bone result in bone lesions, painful osteolysis and risk of pathologic fracture, and

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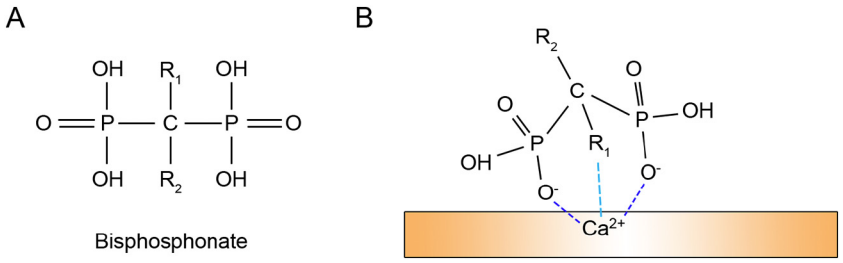


Fig. 1. Bisphosphonate structure and bone binding. A) General bisphosphonate structure consists of a phosphate-carbon-phosphate backbone with variable groups extending from the carbon. B) Bisphosphonates bind bone *via* chelation of calcium ions. Variable binding strengths displayed by bisphosphonates with different side groups suggest further contribution to binding *via* those atoms.

generally have poor prognoses. Along with osteomyelitis, osteoarthritis, and others, these bone-related diseases cause significant morbidity, mortality, and cost to the health care system each year, indicating the need for improved treatment options.

1.2. Drug targeting – bisphosphonate affinity for bone mineral is an ideal foundation for specific targeting

Drug targeting techniques have arisen in the past decades with the goal of increasing drug concentration at the site of injury or disease compared to healthy tissue. Targeting may lower systemic toxicity by allowing reduced dosing and lower off-target drug concentrations. Drug targeting can be passive or specific. Passive targeting can be achieved by extending circulation time, allowing more time for the drug to reach the desired destination. Sites of inflammation and tumors display increased vascular permeability and retention, allowing increased accessibility of molecules in circulation to affected sites (McDonald and Baluk, 2002; Maeda, 2012). In contrast, specific targeting involves determination of a receptor-specific ligand or other property specific to the desired site, allowing concentration or effect only in the desired tissue. As compared to passive targeting, a specific targeting moiety offers greater potential to reduce drug concentration systemically and reduce the total dose required.

Dosing the bone microenvironment is challenging due to poor penetration of most drugs. Attempts to ensure effective concentrations in bone drive high concentrations in other tissues and systemic toxicity. Potential benefits of passive targeting are lost or restricted due to the limited vascular perfusion near the bone surface and thus the unique biochemistry of bone lends itself to specific targeting approaches—high mineral content, receptors and pathways specific to bone cells—have been harnessed for specific targeting with a number of targeting moieties that have been identified (Carbone et al., 2017; Dang et al., 2016). The most studied targeting class of molecule, and focus of this review, is bisphosphonates (BPs). Excepting pathologic microcalcifications in soft tissues, the high concentrations of calcium and phosphorus and their role in the supramolecular structure of bone are unique and create the distinctive target for specific binding of BPs. The exposure of hydroxyapatite during bone remodeling further increases the ability of mineral-binding drugs to localize to sites of high turnover associated with disease. Drugs such as BP take advantage of this characteristic to tightly and specifically bind bone *in vivo*.

1.3. Targeting drug conjugates – defining ideal properties

Targeted drugs bring to mind tyrosine kinase inhibitors uniquely specific to their target's active site or a monoclonal antibody that binds and blocks its unique receptor target; in both cases a single agent and a single target. Targeting drug conjugates offer the potential (and complicating challenges) of bringing together two or more chemical moieties to form one new chemical entity with multiple targets and activities. This review highlights potential drug conjugates that bring two drugs together to drive bone targeting while providing multiple mechanisms of action with the potential for synergistic drug interactions.

An ideal bone-targeting drug conjugate will have the following properties: (i) specific targeting of bone mineral or bone localized cells, (ii) stable to systemic exposure during the time period prior to bone binding, (iii) labile enough to release its drug payloads at times after its bone localization, (iv) the kinetics of drug release drive efficacy with additive or synergistic benefit, (iv) the linker enabling conjugation is nontoxic, (v) efficacy at the bone lesion is achieved with very limited systemic exposure, and (vi) healthy tissue including bone is not adversely affected. The following description of BPs and their properties indicate how well suited they are in the pursuit of an ideal bone-targeting drug.

1.4. Bisphosphonates

BPs are a class of molecules consisting of a phosphorus-carbon-phosphorus backbone (Fig. 1). The moieties that branch from the germinal carbon influence the mineral binding and biologic properties of the numerous BPs available. BPs bind bone hydroxyapatite matrix and negatively influence osteoclast activity; they were first identified as potential bone targeting moieties based on their structural similarity to pyrophosphate, a natural regulator of calcium homeostasis. BPs were first described as effectors of calcification and bone resorption in 1968–9 (Russell, 2011) and were shown to prevent osteoporosis in rats only a few years later (Muhlbauer et al., 1971). Although clinical use did not become widespread for many years, BP was first used to prevent hypercalcemia and calcification, and BPs are now extensively used to treat degenerative bone disorders including osteoporosis, Paget's disease, and bone metastases. This class of molecule has been established to bind specifically to hydroxyapatite by coordination between phosphonate groups and the calcium ions of the crystal structure (Fig. 1B). A number of differing BP molecules with varying side chains have been studied and bind hydroxyapatite with differing affinities, suggesting that coordination of other components of the side chains and hydroxyapatite minerals contributes to binding affinities (Cole et al., 2016). While reported binding affinities vary based upon the method of measurement, within a single method comparative affinities between clinically used BP molecules are reported to have 1.17–2.1 fold differences; nitrogen-containing imidazole and primary amino groups, excepting risedronate, providing the highest binding affinity and a corresponding increase in pharmaceutical activity (Cole et al., 2016; van Beek et al., 1998; Lawson et al., 2010). Although higher binding correlates with a stronger clinical effect, BPs with lower binding affinities may provide other advantages in the ability to dissociate and potentially relocate to other bone sites.

BPs inhibit bone resorption by osteoclasts (Carano et al., 1990). The uptake of BP is essentially unique to osteoclasts since the high charge density prevents transit across cell membranes. Osteoclasts ingest BP during bone resorption *via* fluid phase endocytosis along with calcium and other products of bone degradation. BPs then induce apoptosis in osteoclasts; anabolic conversion of non-nitrogen containing BPs (*e.g.* etidronic acid) into ATP analogs disrupt numerous ATP-dependent pathways, and nitrogen containing bisphosphonates (*e.g.* zoledronic acid, ZOL) inhibit farnesyl pyrophosphate synthase (FPPS), which is essential for creation of FPP in the mevalonate pathway (Coxon et al., 2006). This pathway is critical for the prenylation, and thus localization, of small GTPases in the cell, which are necessary for protein trafficking and normal cell function (Coxon et al., 2006). The FPPS

inhibitory mechanism has been confirmed *in vitro* and *in vivo* (Roelofs et al., 2006). In addition to driving apoptosis, BPs have been shown to inhibit the differentiation of osteoclasts (Hughes et al., 1989; Lowik et al., 1988); complimentary activities that substantially reduced bone resorption. Although the mevalonate pathway is necessary for isopentenyl pyrophosphate (IPP) metabolism in all cells, the specific localization to bone and unique uptake by the highly endocytic osteoclasts directs the effects of BPs to these cells. BPs were further demonstrated to have higher binding in areas where active bone resorption is taking place, which exposes more mineral surface of the bone and further concentrates these molecules in osteoclasts (Masarachia et al., 1996). With the exception of the BP conversion to ATP-analogs noted above, clinically used BPs are not metabolized and provide a relatively safe pharmaceutical product with few adverse effects (Russell, 2011; Pazianas and Abrahamsen, 2011). The well-defined BP bone binding, osteoclast uptake and antiresorptive mechanisms and relative safety of BP therapies have stimulated multiple successful therapeutic applications.

1.5. Bisphosphonates in osteoporosis and cancer

BP therapeutics have been successful for treatment of numerous diseases through several usage strategies. They have been used for inhibition of calcification and hypercalcemia, and as inhibitors of bone turnover in osteoporosis, Paget's disease, and malignancy of the bone. BPs effectively treat Paget's disease for extended time periods after few or even one treatment (Vallet and Ralston, 2016). BPs have also been widely exploited for treatment of osteoporosis. The use of BP in osteoporosis has been a fairly recent clinical development, but has become the most commonly prescribed treatment for osteoporosis patients with tens of millions of prescriptions dispensed yearly (Wysowski and Greene, 2013). BPs dramatically increase bone mineral density (BMD) and reduce the risk of hip fracture by about 40%, with similar reductions in vertebral and other non-vertebral fractures. These benefits to osteoporosis patients come without the risks of long term hormone replacement therapy for postmenopausal women (Russell, 2011) and few side effects beyond manageable gastrointestinal distress and extremely rare osteonecrosis of the jaw (Pazianas and Abrahamsen, 2011).

BPs have also been successfully used as adjuvant therapies to treat cancer associated bone disease, reducing pain and skeletal complications. While tumors that have metastasized to bone have a very poor prognosis, treatments to extend survival and quality of life are still highly valued for these patients. Bone is a common site of metastasis, and tumor-induced bone disease is associated with median survival times of less than two years (Coleman, 2006), and breast cancer patients with metastases exhibit bone metastasis in 70% of cases (Kuchuk et al., 2013). BPs were first used in cancer to alleviate bone complications in multiple myeloma patients (Siris et al., 1980), and are now well established drugs to reduce skeletal complications and improve survival in multiple myeloma animal models and human patients (Croucher et al., 2003a; Croucher et al., 2003b; Van Acker et al., 2016). Patients with unresectable benign tumors also experienced improvement in pain as well as improvements in the bone lesion following treatment with ZOL, a BP with a nitrogen-containing side chain (Cornelis et al., 2014).

Besides pain reduction, BP treatment in many metastatic cancers can delay the time to bone metastasis and in some cases reduce tumor growth. Zoledronate has been approved by multiple agencies for the prevention of skeletal related events (SREs) in non-small cell lung cancer. It is prescribed to delay the onset of SREs, reduce the number of SREs, and prevent and palliate pain (Hendriks et al., 2016). BPs prevent metastases to the bone in prostate cancer (Corey et al., 2003; Padalecki et al., 2003), with reduced incidence of skeletal metastasis and increased time to SREs (Saad et al., 2004; Silvestris et al., 2013) and improved disease progression (Russell et al., 2011). In breast cancer patients BPs have been shown to improve bone density associated with age or treatment and the prevention of osteolytic lesions (Van Acker et al., 2016; Neudert et al., 2003), and is considered standard of care. However, results are mixed as other studies show no differences in disease progression with or without BP treatment (Murakami et al., 2014; Wirth et al., 2015; Smith et al., 2014). Effects of BPs on previously established metastases and larger tumors are also less supported than their ability to prevent bone metastasis when treated early (De Rosa et al., 2013; Neudert et al., 2003). However, the reduction of bone turnover *via* BP-induced apoptosis of osteoclasts in many cases reduces the ability of tumors to metastasize to bone.

Bisphosphonates have also been used to treat primary bone tumors including osteosarcoma (OS). Bisphosphonate use reduces pain and bone lesions in OS patients, increasing quality of life (Hughes, 2009), and although complete clinical studies are few, improvements in disease progression were noted in multiple instances when a BP was used in combination with chemotherapy (Meyers et al., 2011; Conry et al., 2016). In pre-clinical studies, mice with OS given ZOL experienced reduced lung metastases and improved overall survival (Ory et al., 2005; Dass and Choong, 2007) and reduced osteolysis and OS-induced bone formation, but studies have mixed results on reduction of tumor size and metastatic potential (Labrinidis et al., 2009; Ohba et al., 2014a). In a rat OS model, ZOL treatment reduced primary tumor size and bone lysis, and combination with ifosfamide was more effective in preventing tumor recurrence and improving tissue repair (Heymann et al., 2005). Another study by the same group demonstrated that BP treatment resulted in reduced tumor angiogenesis, in addition to the direct induction of apoptosis in tumor cells (Ohba et al., 2014b). Treatment of spontaneous canine OS with pamidronate resulted in pain palliation (Fan et al., 2008) and decreased bone resorption (Fan et al., 2009; Fan et al., 2007), while treatment with ZOL also resulted in improved limb usage and has potential for improving prognosis (Fan et al., 2008; Byrum et al., 2016; Spugnini et al., 2009). In human OS cell lines, MMP-2 and invasion were reduced with alendronate treatment (Cheng et al., 2004). Similar improvements with BP treatment have been observed in other primary bone tumor models including chondrosarcoma, in which osteoclast-mediated bone destruction was reduced with ZOL and tumor size decreased (Otero et al., 2014), while BPs were also cytotoxic to two chondrosarcoma cell lines (Streitbuerger et al., 2011). In Ewing Sarcoma, ZOL reduced cell invasion capability and dissemination of lung metastases, though had no effect on growth of established metastases (Odri et al., 2014), and in a mouse model inhibited primary tumor development in bone, but had no effect on progression (Odri et al., 2010). Bisphosphonates show promising effects on primary bone cancers, but more studies are needed to describe their effects and fully establish treatment benefits.

Bisphosphonates have been demonstrated to have direct anti-cancer properties, and are shown to increase tumor cell death in multiple cancer types *in vitro* and *in vivo* (Sewing et al., 2008; Guenther et al., 2010; Fujita et al., 2012). The mechanism of BP inhibition of cancer is multifold, including induction of apoptosis in tumor cells *in vitro* similar to the mechanism of osteoclast apoptosis, inhibition of HER1/2 receptor signaling, reducing adhesion and invasion of cancer cells (Pickering and Mansi, 2002), inhibition of tumor associated macrophages (Rogers and Holen, 2011), activation of γδ T cells (Coxon et al., 2006; Dieli et al., 2003), and anti-angiogenic properties (Croucher et al., 2003a; Van Acker et al., 2016; Wood et al., 2002; Santini et al., 2007). Despite the tight binding of BPs to bone, success in delivery to extra-skeletal tumor cells has also been demonstrated by encapsulating the drug in liposomes. This was demonstrated in multiple studies with macrophage depletion through liposomal clodronate and nanoparticle delivery of ZOL to extraskeletal tumors, indicating the potential for therapeutic BP use in diverse tumor settings (De Rosa et al., 2013). Additionally, the use of BPs with other chemotherapeutic agents demonstrates a synergistic effect in both bone and soft tissue tumors (Caraglia et al., 2004; Karabulut et al., 2009; Santini et al., 2006). Treatment of mice with a lung cancer model showed paclitaxel and ZOL

displayed a synergistic effect by reducing bone metastasis and prolonging survival (Lu et al., 2008), and Ewing's sarcoma cell lines displayed a synergistic effect when standard chemotherapeutic treatments were combined with BPs (Dos Santos et al., 2014). Prostate cancer cells also displayed a synergistic effect when treated with alendronate and simvastatin, thus inhibiting the mevalonate pathway at two different steps and significantly affecting survival and apoptotic pathways (Rogers et al., 2015). Despite these anti-cancer properties, BP use does not reduce the risk of developing breast cancer in post-menopausal women, suggesting their anti-cancer properties still do not reduce initial development of non-skeletal cancer (Hue et al., 2014). The known clinical applications and anti-neoplastic properties of BP are reviewed in Van Acker et al. (2016), while many clinical trials for the use of BP in cancer patients are still ongoing.

2. Bisphosphonate conjugates

The high bone affinity and specific binding of BPs, as well as the successful clinical use for osteoporosis treatment, has prompted the creation of BP conjugates in order to deliver drugs specifically to bone. We present BP conjugate designs and how these have been applied to cancer, osteomyelitis, osteoporosis, radiation therapies and imaging, in addition to BP-conjugate use in nanoparticles and alternatives to BPs in bone targeting. Among the classes of molecules that have been conjugated for bone delivery are antineoplastic and small molecule drugs, proteins, antibiotics, and imaging agents (Bansal et al., 2004; Gittens et al., 2005a; Herczegh et al., 2002; El-Mabhouh et al., 2006; Reinholz et al., 2010). The variety of conjugated molecules, combined with a variety of possible linkages to the BP molecule, yields a wide array of potential treatments. Conjugation can target the molecule to the bone, reducing systemic exposure and increasing drug half-life and exposure at the site of disease. Conjugates also have the potential for combination therapy synergy, as their BP moiety provides the activities described above in addition to increasing BMD while delivering a second drug to bone. Bisphosphonate conjugates and complexes are not newly realized, as BP complexes with radiolabeled ligands like ^{99m}Tc have been in clinical practice for bone imaging for many years (Subramanian et al., 1975; Bevan et al., 1980; Domstad et al., 1980; Mari et al., 1999; Love et al., 2003) and estrogen conjugates were tested in rats as early as 1996 (Bauss et al., 1996). Despite the decades since conjugate investigation began and demonstrations of bone localization and/or efficacy, most applications still remain at the preclinical stage without advancing to human trials or clinical use. Thus, the promise of this approach can only be realized with its move into drug development and testing in human disease.

2.1. Conjugate design

As seen in Tables 1 and 2, conjugates can have a wide variety of designs that are constrained by three critical elements: (i) the drug payload, (ii) the BP, and (iii) method of conjugation. The choice of the drug payload is defined by the target disease and is typically a compound with proven clinical activity. The chemical properties and potential attachment sites of a drug payload will influence the methods of conjugation possible. The choice of BP moiety will similarly influence the methods of conjugation. In addition, different BPs provide a range of biological activities, bone affinities and anti-resorptive mechanisms of action.

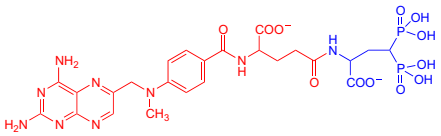
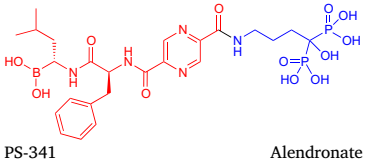
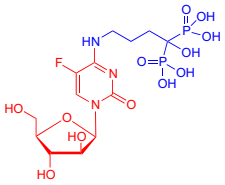
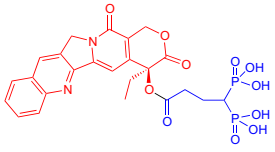
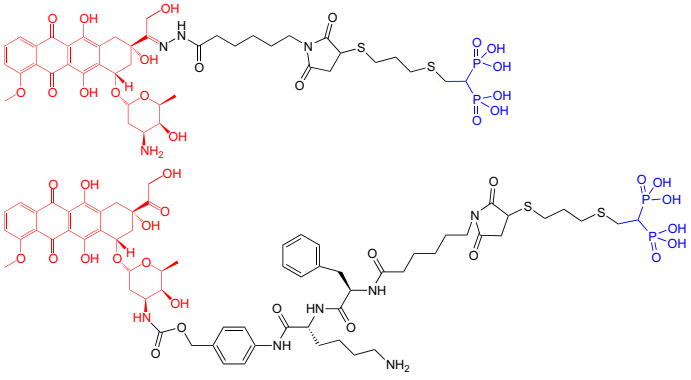
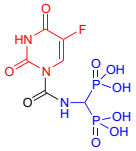
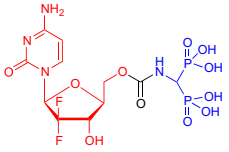
The method for conjugation provides expansion and honing of conjugate function. Drug and BP can be directly conjugated with no linker present (Table 1: 1–5), or small to large linkers may separate the individual drugs (Table 1: 6–11, Table 2). The physical linkage of the BP to its drug counterpart must not interfere with either BP or drug function, and must allow release of moieties in functional forms upon localization. Most investigators have assumed both BP groups should be available for calcium ion chelation, suggesting a side chain R_1 or R_2 (Fig. 1) from the BP carbon backbone is best used for conjugation; however, conjugation to the phosphate has been employed in the only BP conjugate currently being evaluated in the clinic (Table 1: 11). The linker must be stable enough to prevent separation before bone localization, but excessive stability may prevent release of active drug moieties after bone binding. Linkages can be directly from a single BP to a single drug moiety, or can attach a BP to a nanoparticle or polymer structure. The different BP side chains attached to the geminal carbon as well as the phosphates allow diverse linker chemistry.

Linkers between conjugates can include amides, esters, thioesters, or phosphoesters, which are non-specifically cleaved *in vivo*. Proteins conjugated to BP with disulfide linkages early on demonstrated *in vitro* targeting with loss of bone binding upon cleavage by physiological thiols (Zhang et al., 2005). However, the Uludag group showed that disulfide-linked conjugates were not released from a mineral matrix *in vivo* despite cleavage *in vitro,* indicating the importance of confirming linker cleavage in animal models (Wright et al., 2006). Linkers can also have target-specific cleavages, which further refine localization of drug release. These include osteoclast-specific linkages that are sensitive to cathepsin K or MMP, enzymes secreted by osteoclasts during resorption, or acid hydrolysable for release in the resorptive pit formed by osteoclasts (Wang et al., 2005). Hydrolyzable linkers can release a drug from the BP conjugate, though released products must be ensured of full function of both moieties without any toxic byproducts. Conversely, design of a non-hydrolyzable connection of functional moieties can ensure against diffusion of drug from the bone after BP binding. As described above, triphosphate-like nucleoside antimetabolite-BP conjugates were demonstrated to be stable enough in mouse and human serum, ideally allowing the significant part of intact conjugate to bind bone before the chemotherapeutic is released in the bone microenvironment (Ora et al., 2008). Cathepsin-sensitive linkers allow release only upon arriving in an environment with the desired enzyme, as demonstrated *in vitro* with a BP-doxorubicin conjugate with quick release of drug (Hochdorffer et al., 2012). As described in the chemotherapeutics section, agents with cathepsin-sensitive linkers between a BP and chemotherapeutic molecule demonstrate increased efficacy against tumor growth (Miller et al., 2009; Segal et al., 2009), in animal models. The study mentioned above for PGE_2 receptor agonist conjugates used metabolically labile carbamate or 4-hydroxyphenylacetic acid based linkers to achieve their desired drug release time course (Arns et al., 2012), and further study with ^{14}C and ^{3}H allowed dissection of hydrolytic pathways which released drug moieties (Chen et al., 2015). The length of linker may also have effects on drug binding and separation, though this is still unclear as one group reported greater conjugate binding rates upon shorter linkers, others have reported no effect on binding rate (Gittens et al., 2005b; Yewle et al., 2013). Beyond the scope of Table 1 are conjugates with polymers or nanoparticles, greatly increasing the size of the molecules with repeated structure or coated particles. The particle size and other properties of large conglomerates should also be considered, as extravasation may be limited for very large particles (Ishida et al., 1999; Blanco et al., 2015). The possible combinations of BP molecules, drugs, nanoparticles, polymers, and linkers seemingly allow accurate drug delivery systems to diverse targets while preventing systemic toxicity.

2.2. Cancer drug conjugates

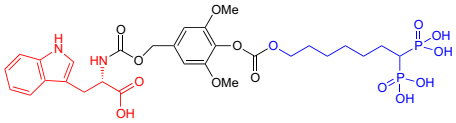
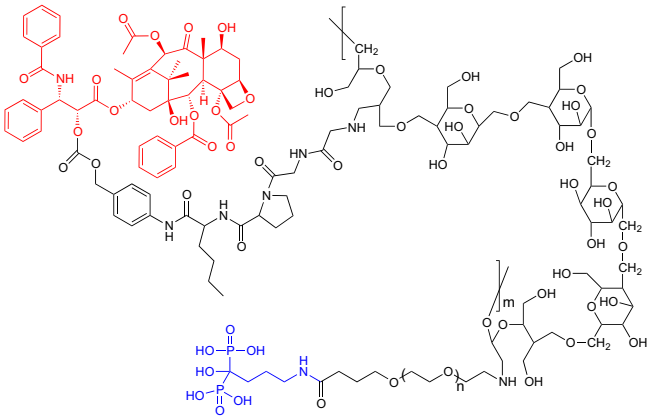
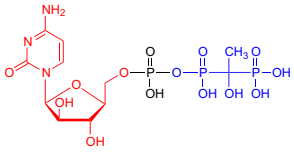
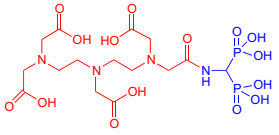
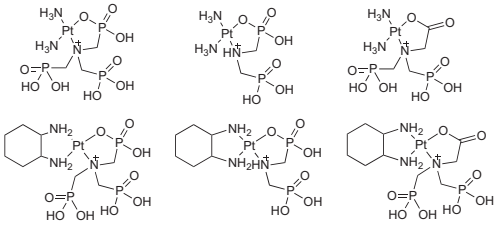
Bone is an attractive target for cancer drug targeting as systemic anti-cancer treatment involves high toxicity and causes widespread adverse side effects, especially for bone neoplasms in which may require higher doses to achieve the necessary concentrations at the site of disease. Bone-targeted cancer chemotherapy has been investigated in multiple studies from *in vitro* studies to animal models. The anti-cancer properties of BPs alone, described above, may offer bifunctional treatment with further antineoplastic drug conjugation, though BP uptake is limited in most cells due to the charged nature of the BP. Many studies

Table 1
Antineoplastic bisphosphonate conjugates.

	Structure	Refs
1	 Methotrexate	(Hosain et al., 1996)
2	 PS-341 Alendronate	(Agyin et al., 2013)
3	 5-FdU Alendronate	(Schott et al., 2012; Weinreich et al., 2012; Schott et al., 2011)
4	 Cisplatin Medronate	(Nakatake et al., 2011)
5	 Camptothecin	(Erez et al., 2008)
6	 Doxorubicin	(Hochdorffer et al., 2012)
7	 5-Fluorouracil Aminomethylene diphosphonate (AMDP)	(El-Mabhouh et al., 2004; El-Mabhouh and Mercer, 2005)
8	 Gemcitabine AMDP	(El-Mabhouh et al., 2006; El-Mabhouh and Mercer, 2008; El-Mabhouh et al., 2011)

(*continued on next page*)

Table 1 (*continued*)

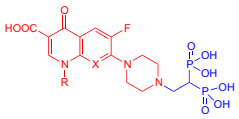
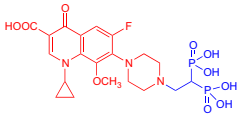
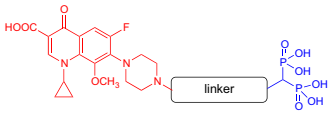
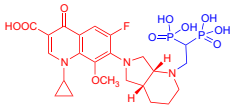
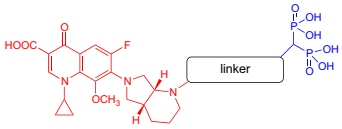
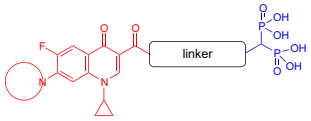
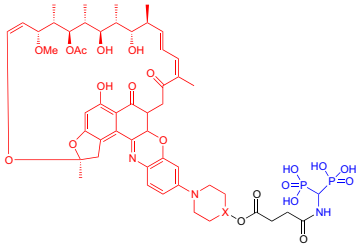
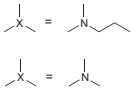
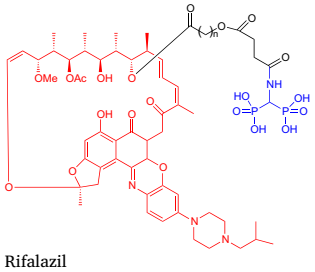
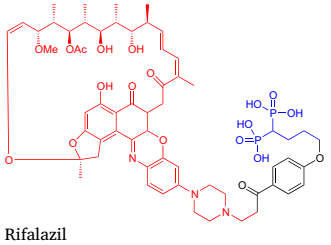
	Structure	Refs
9	 Tryptophan	(Erez et al., 2008)
10	 Paclitaxel Pullulan Alendronate	(Bonzi et al., 2015)
11	 Cytarabine Phosphate Etidronate	(Reinholz et al., 2010; Ora et al., 2008) (NCT02673060)
12	 DTPA AMDP	(El-Mabhouh et al., 2004; El-Mabhouh and Mercer, 2005)
13	 Other types of platinum-containing phosphonates	(Klenner et al., 1990)

Color code: Red – anticancer parent drug; blue – bisphosphonate, generic names provided for clinically used compounds; black - linker.

do not fully explore effects from BPs on their conjugate beyond bone targeting, and many do not explore whether toxicity is reduced by the targeting action. Yet, many studies demonstrate increased efficacy with a BP-antineoplastic conjugate.

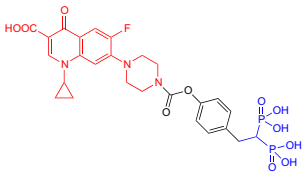
A number of *in vitro* studies show increased efficacy when conjugating a traditional cancer chemotherapeutic with a BP. However, many also lack convincing data for conjugate function. An early study showed that BP and methotrexate linked together *via* a peptide bond successfully localized to bone (Table 1: 1) (Hosain et al., 1996; Sturtz et al., 1992). Later, another BP-methotrexate conjugate was shown to induce apoptosis in OS cells *in vitro* but at a rate similar to the standard methotrexate OS treatment (Yang et al., 2014a). The Roy group demonstrated that BP conjugated proteasome inhibitors (Table 1: 2) exhibit strong toxicity on multiple myeloma cell lines (Agyin et al., 2013). Neubauer's group reported that a 5F-deoxyuridine- alendronate conjugate (Table 1: 3) showed increased hydroxyapatite binding and cytotoxicity to cancer cells, but did not demonstrate separation of the two drug moieties (Schott et al., 2012). The same conjugate was tested in a gastric adenocarcinoma cell line and showed slightly higher efficacy against cancer than non-malignant cells, but less sensitivity than to separate drug components, again indicating lack of release of individual components from the conjugate (Weinreich et al., 2012). Another *in vitro* study created a paclitaxel, alendronate, and pullulan conjugate with a cathepsin-K sensitive linker (Table 1: 10) which assembled into a colloidal sphere and showed higher antiproliferative activity with the BP than without in metastatic breast cancer and OS cells (Bonzi et al., 2015). The Suemune group created and tested *in vitro* a dialkylbisphosphonate platinum complex (Table 1: 4) with the idea to bring the antitumor effects of platinum to bone metastases (Nakatake et al., 2011). A doxorubicin-BP conjugate with cathepsin-B or acid-sensitive linkers (Table 1: 6) showed stability in plasma with quick release of drug, but only one of the compounds showed a higher mouse MTD in mice than doxorubicin alone and efficacy has not been investigated (Hochdorffer et al., 2012).

Table 2
Antimicrobial bisphosphonate conjugates.

	Structure		References
1	 Norfloxacin: X = C, R = C_2H_5 Enoxacin: X = N, R = C_2H_5 Ciprofloxacin: X = C, R = cyclopropyl		(Herczegh et al., 2002) (Houghton et al., 2008)
2	 Gatifloxacin		(Houghton et al., 2008)
3	 Gatifloxacin		
4	 Moxifloxacin		
5	 Moxifloxacin		
6	 Fluoroquinolone esters		(Tanaka et al., 2008)
7	 Benzoxazinorifamycines	 AMDP	(Reddy et al., 2008)
8	 Rifalazil	AMDP	
9	 Rifalazil		

(*continued on next page*)

Table 2 (*continued*)

	Structure	References
10	 Ciprofloxacin	(Sedghizadeh et al., 2017)

Color code: Red – antimicrobial parent drug; blue – bisphosphonate; black - linker.

The relevance of cell-based studies is an important question, and the authors accept the data referenced above but want to suggest caution in its interpretation. Fig. 1 illustrates the high charge to mass ratio of BPs that prevent their uptake into cells and accounts for osteoclast specific uptake driven by the bone resorption process unique to these cells when growing on bone mineral. Cell-based studies permit high concentrations and/or long term exposure to BPs that may enable pinocytosis mediated levels of effective BP concentrations, but one would not expect *in vivo* exposures at these concentrations or time durations to be possible. Moreover, with BP conjugates the kinetics of release of the drug payload into cell culture media must be known to understand the results of cell based experiments. One should expect the hydrolysis of drug conjugates into cell culture media to be driven by different enzymes or chemical conditions than those experienced *in vivo*. Thus, while cell culture can provide some knowledge about the conjugate with a specific cell type, the desired properties of a bone targeted drug conjugate must be tested *in vivo*.

Many studies went beyond cell culture to pre-clinical animal models to ensure bone localization and antitumor efficacy. Mercer's group found a 5-fluorouracil BP conjugate (Table 1: 7, 12) showed accumulation in bone with rapid clearance of unbound conjugate (El-Mabhouh et al., 2004), and labeling this 5-FU or diethylenetriaminepentaacetic acid (DTPA) BP conjugate with ^{188}Re showed bone accumulation for potential combination therapy (El-Mabhouh and Mercer, 2005). The same group created a gemcitabine/BP conjugate (Table 1: 8) labeled with ^{99m}Tc or ^{188}Re, which was demonstrated to bind bone *in vitro* and localize to bone *in vivo*. The amide link allowed potential cleavage to release local concentrations of the drug in attempt to reduce the toxicity of gemcitabine and ^{188}Re when administered systemically (El-Mabhouh et al., 2006; El-Mabhouh and Mercer, 2008). This gemcitabine-BP conjugate reduced the size and number of bone metastases in a mouse metastatic breast cancer model (El-Mabhouh et al., 2011). Shabat's group created a esterolytic BP-camptothecin conjugate (Table 1: 5) which bound hydroxyapatite and hydrolyzed under physiological conditions to release the free drug (Erez et al., 2008). Another structural design of the conjugates, where phosphate group of BP was used to link to nucleoside-5′-monophosphate, thus providing an analog of nucleoside triphosphate capable of releasing both components at the bone, had been reported (Reinholz et al., 2010). The etidronate-cytarabine compound MBC-11 (Table 1: 11) increased BMD and reduced incidence of bone metastases in mouse breast cancer and multiple myeloma models (Reinholz et al., 2010) significantly outperforming the control groups treated with free cytarabine, free etidronate or the combination of free cytarabine and etidronate. These types of conjugates were demonstrated to hydrolyze in mouse and human serum with a half-life in a scale of hours, suggesting time for bone binding with a release of the antineoplastic drug in the bone microenvironment (Ora et al., 2008). MBC-11 is one of the few examples of a BP conjugate that has advanced to oncology clinical trials (NCT02673060). The Phase I study for patients with cancer-induced bone disease treated with MBC-11 is completed with maximum tolerated dose and indications of efficacy reported (Zinnen et al., 2017).

Further modifications to BP-chemotherapeutic conjugates were designed in pre-clinical studies to add additional functions and increased refinement to drug delivery and efficacy. Polymeric systems can improve drug solubility and allow multiple drug and targeting agents conjugated to a single polymer. N-(2-hydroxypropyl)-methacrylamide (HPMA), a water-soluble and non-immunogenic polymer used as a carrier for low molecular weight drugs can be conjugated to BP with strong binding to bone *in vivo* (Pan et al., 2008). With the idea that an HPMA polymer allows accumulation in tumor sites due to increased extravasation, an HPMA-paclitaxel and alendronate conjugate was created with a cathepsin B-sensitive linker, a protease produced by proliferating endothelial and prostate cancer cells. This conjugate inhibited migration in cells (Miller et al., 2009) and reduced intratibial tumor growth beyond what was seen in untargeted moieties, increased apoptosis in cancer cells, reduced vascularization of the tumor, and exhibited no toxicity compared to significant toxic effects with free drug (Miller et al., 2011). With a different polymer strategy, an alendronate-paclitaxel conjugate was formed with self-assembling PEG micelles also with improved safety and efficacy over free paclitaxel (Miller et al., 2013). An HPMA, TNP-470, and alendronate conjugate with cathepsin K-sensitive linkers, an enzyme employed by osteoclasts, also showed a large reduction in OS tumor growth over that of separate drugs, as well as dramatically reduced side effects associated with systemic anti-angiogenic agent TNP-470 treatment (Segal et al., 2009; Segal et al., 2011). In a non-bone targeting antineoplastic application, a conjugate of alendronate and glucomannan was used for specific uptake by and elimination of highly endocytic tumor-associated macrophages, while alendronate alone did not show this effect (Zhan et al., 2014). Additional cancer drugs have been encased in nanoparticles with BP targeting moieties, and are discussed below. Overall, many applications of BP conjugates with antineoplastic agents have been demonstrated in cells and animal cancer models showing successful bone targeting and increased efficacy over free drugs.

2.3. Osteomyelitis

Osteomyelitis is an infection of the bone that requires long term and at times invasive treatments which are not always successful in eliminating infection, resulting in a chronic or recurring disease. Inflammation and necrosis may compromise bone vasculature and further reduce delivery of untargeted drugs. Targeted antibiotics have the potential to substantially improve treatments for this disease. The first reported (Herczegh et al., 2002; Houghton et al., 2008; Tanaka et al., 2008) (Table 2:1) bisphosphonate conjugated fluoroquinolones efficiently bound bone, but were predictably unable to release the fluoroquinolone moiety. Further conjugate development produced prodrug type compounds (Table 2: 3,5,6) which displayed efficient bone binding, release of active drug, and were successful in preventing osteomyelitis where free fluoroquinolone was not (Herczegh et al., 2002; Houghton et al., 2008; Tanaka et al., 2008). These *in vivo* studies into osteomyelitis were not extensive and mainly focused on preventing establishment of infection, requiring further investigation into

conjugate treatment of established infections. Reported BP-fluoroquinolone antimicrobial activity is complex and varies with specific pathogen, length and nature of a linker/spacer between two moieties, bone affinity, linker stability and release kinetics, suggesting that conjugates stable in circulation and able to release the antibiotic at the bone surface have more chances to become clinically useful drugs. Recent study by the Ebetino group demonstrated that a single high dose of a BP-ciprofloxacin conjugate (Table 2: 10) showed a 99% reduction in bacterial load in an animal osteomyelitis model (Sedghizadeh et al., 2017). Another antibiotic class, benzoxazinorifamycins (Table 2: 7–9), were also conjugated to bind bone and demonstrated to be releasable, resulting in presence over a longer time than free drug, and were effective before and after establishment of infection (Reddy et al., 2008). However, to our knowledge no anti-infective BP conjugates have undergone clinical studies.

2.4. Osteoporosis and other targets

Other treatment applications have also explored the use of BP conjugates. Although degenerative bone disease is standardly treated with BPs, many have explored conjugating BPs to further inhibit bone resorption in pre-clinical studies. Conjugated molecules include those involved in the bone turnover balance, but which may have other effects when treated systemically. One example is a BP conjugate with osteoprotegerin (OPG), a RANKL inhibitor preventing formation of osteoclasts, which accumulated in bone with twice the concentration of non-targeted OPG, and to an even greater extent in an osteoarthritis rat model with active bone remodeling (Doschak et al., 2009). Another important regulator of bone turnover is estrogen, but systemic treatment may increase risk of breast or uterine cancers. Estradiol-BP conjugates were also explored with one compound showing successful estrogenic activity in the bones and not uterus, while others showed no effect (Bauss et al., 1996; Morioka et al., 2010). Calcitonin, which acts through calcitonin receptors on osteoclasts to reduce resorption, is also taken up by other cells and has a short biological half-life resulting in little effect from systemic administration. Calcitonin retained activity and bound hydroxyapatite when conjugated (Bhandari et al., 2010), and the conjugate showed superior ability to maintain bone volume and density in an ovariectomized rat model compared to free calcitonin (Bhandari et al., 2012). A PEGylated calcitonin-BP conjugate further improves stability in circulation and bone targeting (Yang et al., 2014b). Another regulatory molecule that lacks efficacy when administered systemically is parathyroid hormone (PTH). A PTH peptide was conjugated to a BP molecule at its N-terminus and was shown to maintain its activity in cells (Yewle et al., 2013). To stimulate regeneration of bone after osteoporotic losses, a BP was conjugated with prostaglandin E_2 for its anabolic effects on bone. This conjugate was designed with differing linkers, allowing selection of a desired release of free PGE_2, and showed increased rates of bone growth over free PGE_2 (Gil et al., 1999). Later, a BP conjugate with a prostaglandin E_2 EP4 receptor subtype agonist was developed for its benefits of increased stability compared to PGE_2 and without the systemic side effects of the agonist alone (Arns et al., 2012). The agonist-BP conjugate reversed osteopenia in an ovariectomized rat model (Liu et al., 2015). The Sawyer group found a Src kinase inhibitor with anti-resorptive activity, and adding a targeting moiety showed *in vivo* protection against hypercalcemia (Violette et al., 2001; Shakespeare et al., 2003).

More applications for BP conjugates include anti-inflammatory delivery, slow release gels, and dental treatment. Synthesis of BP conjugates to corticosteroids has been explored, allowing the potential for applying BP to inflammatory bone diseases such as osteoarthritis (Page et al., 2001). An anti-inflammatory drug diclofenac was conjugated with BP and demonstrated greater bone accumulation and release at a lower effective dose and without gastrointestinal side effects expected for an anti-inflammatory (Hirabayashi et al., 2002; Hirabayashi et al., 2001). Beyond these few studies, there is a surprising lack of investigation into anti-inflammatory conjugate drug delivery for osteoarthritis, for which current treatments often result in undesirable systemic effects. Bisphosphonate use has also been studied in the application of oral bone growth, and some conjugates have been studied for their effects on tooth enamel. The Wang group found that an aldendronate-beta cyclodextrin conjugate bound hydroxyapatite in tooth enamel (Liu et al., 2007), and simvastatin acid complexed with alendronate beta-cyclodextrin conjugate was effective in preserving bone long term (Lee et al., 2011; Price et al., 2013). A poly(amido amine)-alendronate conjugate also promotes HA mineralization while binding strongly to tooth enamel (Wu et al., 2013). Another application for a BP conjugate is slow release hydrogels. Linking a BP with a polymer allows incorporation into a hydrogel for slow release applications, which is composed of ECM component hyaluronan allowing natural degradation. An unconjugated small molecule drug alone diffuses too quickly from the gel, while conjugates have more desirable release kinetics (Varghese et al., 2009). These many applications give a glimpse into the possibilities for diverse applications of BP conjugates and drug-targeting.

2.5. Radiation therapy and imaging

Additional widely studied BP targeting applications are radiation therapy (RT) and clinical imaging. Radiation therapy using BP targeting has been effectively used to treat pain associated with bone metastasis in many cancer patients. Phosphonate-based radiopharmaceuticals with an α, β, or γ emitting radionuclide quickly accumulate in bone and deliver RT locally, providing pain palliation (Lange et al., 2016). Samarium-153-ethylene diamine tetramethylene phosphonate has been shown to effectively reduce pain associated with bone metastases (Hirabayashi and Fujisaki, 2003). ^{188}Re is also an attractive radionuclide for therapy as it behaves similarly to ^{99m}Tc (discussed below), yet exhibits toxicity when administered systemically, suggesting a targeted therapy would be beneficial. This was addressed by the Blower group with a $^{188}Re(CO)_3$-dipicolylamine-alendronate conjugate, which accumulated in areas of high bone metabolism (Torres Martin de Rosales et al., 2010). As mentioned previously, a ^{188}Re-labeled gemcitabine/BP conjugate combines strategies to both bind bone, deliver a chemotherapeutic, and deliver RT (El-Mabhouh and Mercer, 2008). To further increase binding of a BP-radiolabel conjugate to bone, the Rosch group added an albumin binding agent to increase circulation time and reduce rapid loss of the agent after treatment, with application for both radiation therapy and imaging (Meckel et al., 2016). Many other radionuclides have been complexed with BPs and have undergone preclinical studies for targeted radiation therapy of bone neoplasms (Cole et al., 2016).

Besides treatment with RT, the use of bone targeting for medical imaging also has been successfully established for clinical use. High contrast bone imaging is important for the identification of bone metastases and diagnosis of metabolic and other bone disorders. ^{99m}Tc-linked BPs are actively used in bone scintigraphy to image areas of high bone turnover. The ability of BPs to be linked to a gamma-emitting technetium isotope and their affinity for sites of high bone turnover with quick elimination from soft tissue has supported their use in imaging, and radiopharmaceuticals coupled to BPs are widely used in bone scans to identify and evaluate bone issues (Verbeke et al., 2002). Clinically available conjugates include ^{99m}Tc hydroxyethylidene disphosphonate, ^{99m}Tc methylene disphosphonate, and ^{99m}Tc hydroxymethylene disphosphonate (Cole et al., 2016; Wang et al., 2005), and ^{99m}Tc -(bis)alendronate-DTPA conjugates are still in development (El-Mabhouh et al., 2004; Chadha et al., 2013). Bisphosphonate conjugates with a DOTA core were also designed for imaging and allow easy radiolabeling by complexation of a metal isotope (Vitha et al., 2008), and other chelation agents are being investigated as well (Cole et al., 2016). The Hermann group developed a BP-^{68}Ga imaging system for PET (Holub et al., 2015), indicating that more imaging systems are still

in progress and can further advance current techniques.

Fluorescent conjugates for research applications and for greater resolution than scintigraphy have also been created. Fluorescent imaging allows a longer signal life than scintigraphy but is limited by depth of signal in tissue in humans, suggesting the desired application should determine the technique (Zaheer et al., 2001; Kashemirov et al., 2008; Sun et al., 2016). A pamidronate-pullulan conjugate with attached fluorescent or MR imaging moieties was demonstrated to be successful for binding hydroxyapatite and accumulating in regenerating bone tissue (Liu et al., 2012). MRI and PET imaging could also benefit from improved bone imaging, and conjugation to a chelator such as DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), BPAMD (4-([bis-(phosphonomethyl))carbamoyl]methyl)-7,10-bis(carboxy methyl)-1,4,7,10-tetraazacyclododec-1-yl)acetic acid), or NOTA (1,4,7-triazacyclononane-1,4,7-trisacetic acid) is also required for these imaging techniques (Cole et al., 2016). For X-ray based imaging, gold nanoparticles were coated with alendronate, increasing contrast for the imaging of damaged bone or microcalcifications associated with breast cancer (Ross and Roeder, 2011). Conjugation strategies allow the development of targeted imaging agents for the imaging technique of choice. Many current BP conjugate imaging techniques using radionuclides and others are reviewed in Cole et al. (2016).

2.6. Nanoparticles

Another exploitation of BP targeting is their use in nanoparticles, as nanoparticles with attached BP targeting moieties can act as drug delivery systems to bone. Nanoparticles can carry a large amount of drug, protect the drug from proteasomal or enzymatic degradation, extend the time of circulation while the BP targets the particle to bone, and prevent de-functionalization of the drug through conjugation techniques. New technologies and polymers have significantly enhanced the possibilities for nanoparticle encased drugs and delivery systems. Coating or encapsulating drugs allows virtually any drug class to be bone targeted *via* BP without the possibility of deactivating the delivered drug through a chemical conjugation process. However, one concern with large molecule conjugates is the ability to extravasate, which is size limited but required to reach hydroxyapatite surface for binding and is not fully addressed in many studies. More advanced and comprehensive studies are required with nanoparticles, which go beyond proof of bone binding to fully study efficacy. A number of materials can be used to enclose a drug of interest, and combinations of materials can further refine delivery. HPMA polymer as described above, is highly biocompatible and is used with small molecule drugs to enhance retention time. Poly(D,L-lactic-*co*-glycolic acid) (PLGA) has been approved by the FDA for drug delivery systems due to its biocompatibility, biodegradability, and its low toxicity (Carbone et al., 2017; Dang et al., 2016). Polyethylene glycol (PEG) shielding of a drug molecule allows "stealth", or evasion of detection by the immune system for longer circulation and less macrophage uptake. Particles then coated with a BP have seen successful bone targeting. PLGA nanoparticle-BP conjugates were found to be biocompatible (Cenni et al., 2008), and PEG could be coupled to two agents at opposite ends of its polymer chains, such as alendronate for targeting and paclitaxel for treatment, while localizing to the bone (Clementi et al., 2011).

Many BP nanoparticle conjugates were found to efficiently target to bone and improve drug efficacy in pre-clinical studies. PLGA nanoparticles containing docetaxel and coated with ZOL were demonstrated to target to bone (Ramanlal Chaudhari et al., 2012), and PLGA nanoparticles conjugated with alendronate and PEG showed increasing absorption with increased alendronate concentration (Choi and Kim, 2007). A PLGA-alendronate conjugate in which doxorubicin was encapsulated conferred a higher reduction in bone metastases than free drug (Pignatello et al., 2012; Salerno et al., 2010). Similarly, blending of PLGA, PEG, and alendronate into nanoparticles resulted in long circulation and high targeting capacity. These nanoparticles loaded with bortezomib (a highly toxic proteasome inhibitor used for multiple myeloma treatment) resulted in enhanced survival and decreased tumor burden, although this was only compared to particles without drug encapsulated (Swami et al., 2014). The Hammond group encapsulated doxorubicin in liposomes which were then coated with a "layer-by-layer" method to create a nanoparticle with an aqueous polyelectrolyte, poly(acrylic acid), side-chain functionalized with alendronate subsequently electrostatically-assembled in a nanoparticle coating. These nanoparticles were effectively taken up by OS cells and effective in xenograft mouse models (Morton et al., 2014). Hydroxyapatite-based biodegradable mPEG-PLGA nanoparticles of risedronate were found to have increased bioavailability and significant effects on bone turnover indicators in an osteoporotic model system (Rawat et al., 2016). In another technique, the Wang group created a polyrotaxane using click chemistry, allowing varying numbers of BP molecules and addition of imaging or additional therapeutic agents (Hein et al., 2010).

Instead of polymers, liposomes and micelles can also be bone-targeted with BP. Liposomes containing doxorubicin displaying BP head groups bind to hydroxyapatite and were more toxic to OS cells *in vitro* than drug-containing liposomes without BP targeting or drug alone (Anada et al., 2009; Wu and Wan, 2012). The Uludag group demonstrated liposomes decorated with BP have hydroxyapatite affinity, and these thiolBP-functionalized liposomes were retained to a greater degree in mineral-containing scaffolds in an animal implant model (Wang et al., 2012). Nonetheless, cell-based studies have the same limitations discussed above for cancer drug conjugates. However, a doxorubicin-loaded alendronate-targeting micelle showed decreased toxicity and delay in tumor growth in a mouse model (Ye et al., 2015). Combining the benefits of encased drug delivery with BP targeting may further enhance the capabilities of drug delivery to bone.

2.7. Bisphosphonate alternatives

While not the focus of this review, it is of value to mention the limitations of BPs have prompted investigation into other bone targeting moieties. Although adverse effects are rare, long term inhibition of osteoclasts with BP use can lead to osteonecrosis of the jaw, nephrotoxicity, hypocalcemia, and ocular dysfunction (Prommer, 2009). Bisphosphonates also have low oral bioavailability, with less than 1% absorbed from oral dosing, and cause gastrointestinal irritation in some patients. Low adherence to oral drug regimens is also a problem with many patients (Russell, 2011), but association of the molecule to bone is rapid and dissociation is low and can take years (Cremers and Papapoulos, 2011). However, long-term effects may not be desirable in some cases, as long-term use can cause hardening of the bone leading to brittleness and osteonecrosis of the jaw. Studies show that non-osteoclast bone cells do not take up BP bound to matrix (Schindeler and Little, 2005) and non-osteoclast cells experience no detectable protein prenylation effects under conditions which strongly inhibit the same pathway in osteoclasts (Coxon et al., 2006), suggesting other cells do not take up BP as osteoclasts are able, reducing off-target effects of the drug. The generally low toxicity, well-established mechanism, and easy handling of BP create a facile drug development pathway that has not encouraged development of other targeting systems, many of which require high production cost. Yet, a few other bone-targeting strategies have been developed and have undergone preliminary studies, and a few examples such as denosumab (Tsourdi et al., 2011) have clinical successes. Monoclonal antibodies (Ebb et al., 2012; Branstetter et al., 2012), small molecule inhibitors (Coleman et al., 2004; Gooding and Edwards, 2016), RNAi (Wang and Grainger, 2012; Soutschek et al., 2004), tetracycline derivatives (Wang et al., 2005; Cai et al., 2012; Skinner and Nalbandian, 1975), acidic peptides (Tormo et al., 2013; Wang et al., 2014; Kasugai et al., 2000), and other biological and synthetic bone-binding molecules (Jahnke et al., 2015; Yoshida et al., 2002; Low and Kopecek, 2012) have been demonstrated to successfully bind and target therapeutics to bone. No other molecule class has been

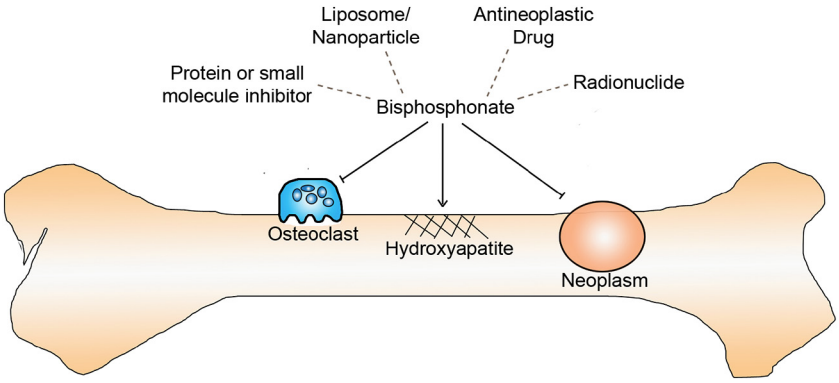


Fig. 2. Schematic of bone-targeting bisphosphonate conjugates and effects of bisphosphonates. Bisphosphonates alone inhibit osteoclasts, bind hydroxyapatite crystal structure, and negatively affect neoplasms. The addition of a conjugated drug may increase the efficacy of these functions or add further modes of action. This representation does not encompass all functions of bisphosphonates or conjugated moieties.

demonstrated to have all of the properties that BPs bring to bone drug-targeting: high bone affinity, anti-neoplastic effects, inhibition of bone resorption, pharmacokinetic stability, and accessible chemistry for application of conjugates (Fig. 2).

3. Future prospects

The criteria for ideal bone-targeted therapies presented in section 1.3, and the compounds reviewed suggest both a promising direction and a lack of critical information. Articles often report the synthesis of a conjugates and demonstrate hydroxyapatite affinity without additional *in vitro* work. Those with *in vitro* data often lack *in vivo* proof-of-concept. Those that report efficacy have not followed up with pharmacokinetic or toxicology studies. As a result, we have many promising bone-targeted approaches with little understanding of how they effect *in vivo* bone affinity, drug release kinetics, possible synergies of BP-drug payload interactions, mechanisms of action, tissue distribution, impacts on maximum-tolerated-dose, toxicity and the critical parameters needed to optimize a therapeutic for human use. Such information would be a great benefit to the rational design of future conjugates.

Three bisphosphonate-based conjugates have been in human clinical trials. The last BP conjugate approved for clinical use in imaging was ^{99}Tc-MDP (Cole et al., 2016), which was patented in China in 1995 (Chinese Patent No. ZL94113006.1). Osteodex is a poly-bisphosphonate containing dextran, alendronate and guanidine with preclinical efficacy reported (Meurling et al., 2009; Holmberg et al., 2010; Daubine et al., 2011) and completion of a phase I trial in metastatic castration resistant prostate cancer—NCT01595087—with recruitment of a phase IIb study underway and interim data indicating stabilization of bone turnover markers. MBC-11 (compound 11 in Table 1) reported reduction of cancer induced bone lesions in a number of patients at the conclusion of a phase I study in cancer induced bone disease (Zinnen et al., 2017). These examples have brought the approach into the clinic, and if successful will be the first therapeutic BP-conjugates approved for human use. We anticipate more compounds in future clinical testing; and hope that future pre-clinical and clinical research includes endpoints that inform us on the missing elements outlined above and approach the ideal bone-targeted therapies.

The development of polymers, nanoparticles, and associated conjugates is still ongoing and material science has the potential to identify new molecules that may target bone. The combination of conjugate and other treatment strategies allows for a huge variety of applications as well as the ability to increase targeting and efficacy with multifaceted conjugate designs. Numerous studies for the delivery of systemically toxic drugs to bone *via* targeted nanoparticles have arisen and point to the many possibilities for improvement of current drugs and development of new systems. Combining BPs with conventional therapies for increased targeting and cytotoxicity in the case of cancer cells has the potential to provide synergistic effects for improving many treatments. Bone neoplasms, osteomyelitis and osteoarthritis could all benefit greatly with improvements to current treatments. Treatment of osteomyelitis with targeted antibiotics may greatly reduce the morbidity associated with the time scale of the infection and long term treatment requirements. Osteoarthritis could benefit from research in bone targeting anti-inflammatory agents. Degenerative, neoplastic, and additional bone disorders all stand to gain significant progress in treatment with future advances in bone-targeted therapeutics and BP conjugates.

4. Conclusions

Bone targeted therapeutics have the potential to significantly improve treatments for bone associated diseases and neoplasms. The high mineral content of the bone hydroxyapatite matrix and tissue-specific cells provide a highly specific environment for multiple drug-targeting strategies. Substantial progress has been made with the use of BPs to directly treat degenerative bone disorders as well as emerging evidence for improvement in neoplastic bone disease. Bisphosphonates are now being additionally investigated as targeting moieties for the directing of conjugated drugs or nanoparticles to the bone microenvironment. Multiple drug classes and nanoparticle encapsulations have demonstrated success in specific delivery of drug to bone and improved efficacy of treatment. Hydrolysable and target-specific linkers between BP and the conjugated particle allow release of drug upon bone binding. Numerous drugs, nanoparticles, and conjugation techniques have shown significant promise in drug targeting and efficient treatment of bone maladies. The elements of ideal bone-targeting therapies are known, with continuing BP conjugate studies and trials further advances will be made in the treatment of bone disorders.

Conflict of interest statement

Kristen B. Farrell is an employee of MBC Pharma Inc.

Alexander Karpeisky and Shawn Zinnen are officers and part owners of MBC Pharma Inc. and founders of Osteros Biomedica.

Douglas H. Thamm has nothing to declare.

Transparency document

The Transparency document associated with this article can be found, in online version.

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