Targeting therapeutics to bone by conjugation with bisphosphonates

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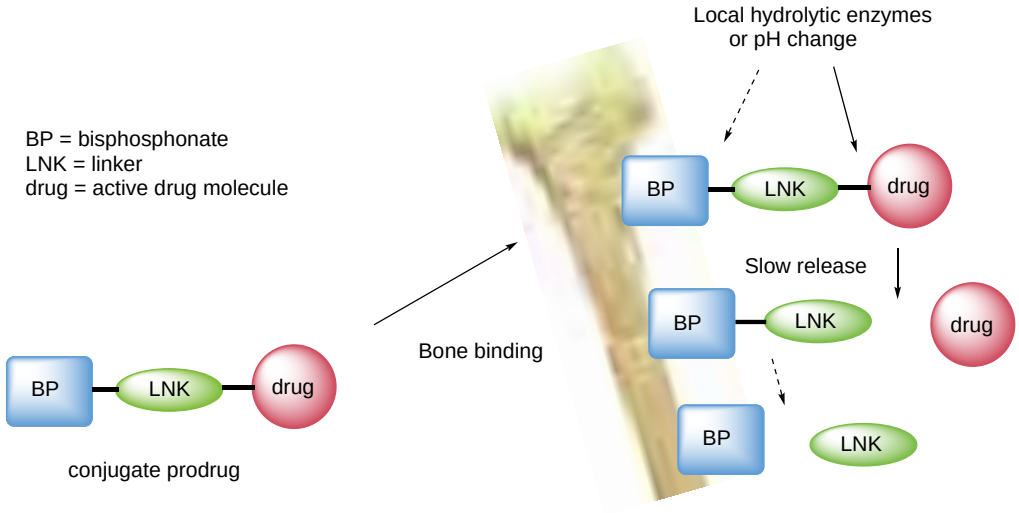
Graphical Abstract: Targeting therapeutics to bone by conjugation with bisphosphonates



Abstract:

Bisphosphonates target and bind avidly to the mineral (hydroxyapatite) found in bone. This targeting ability has been exploited to design and prepare bisphosphonate conjugate prodrugs to deliver a wide variety of drug molecules selectively to bones. It is important that conjugates be stable in the blood stream and that conjugate that is not taken up by bone is eliminated rapidly. The prodrugs should release active drug at a rate appropriate so as to provide efficacy. Radiolabelling is the best method to quantify and evaluate pharmacokinetics, tissue distribution, bone uptake and release of the active drug(s). Recent reports have described bisphosphonate conjugates derived from the antiresorptive drug, alendronic acid and anabolic prostanoid drugs that effectively deliver prostaglandins and prostaglandin EP4 receptor agonists to bone and show enhanced anabolic efficacy and tolerability compared to the drugs alone. These conjugate drugs can be dosed infrequently (weekly or bimonthly) whereas the free drugs must be dosed daily.

Introduction:

Treating diseases of the bone can be difficult due to relatively low vascularization and the physical barriers of penetrating the tissue. High doses of systemic drugs may be necessary to provide efficacy in bone and in the case of drugs with important systemic effects or side effects these may limit or exclude their use in treating conditions of the bones. Many researchers in the past have attempted to identify chemical structural elements that may preferentially bind to the hydroxyapatite mineral in bone as a way to target drugs to bone [1, 2]. Some polyhydroxy containing molecules such as tetracyclines [3] or poly-acidic peptides [4, 5], polymers [6, 7], hydroxylated heterocycles [8] and monophosphonates [9] have been found to bind to apatite and have been studied but binding to hydroxyapatite may in fact impair or block biological activity of such compounds. One structure type that has found considerable success as a bone targeting moiety is the class of drugs known as bisphosphonates. Bisphosphonates and hydroxy-bisphosphonates represent a non-hydrolyzable analog of pyrophosphate and bind avidly to hydroxyapatite. Once complexed to hydroxyapatite the bisphosphonates are essentially irreversibly bound and can only be liberated in the course of bone turnover by osteoclasts, cells that dissolve the mineral in bone with hydrochloric acid as part of the bone resorption process [10]. Aminoalkyl 1-hydroxy-1,1-bisphosphonates such as alendronic acid (Fosamax^{TM}) are effective antiresorptive drugs and have been in wide use for decades [11]. Bisphosphonates have been used as a vehicle to selectively deliver proteins [12], liposomes [13], nanoparticles [14] and small molecules to bone by virtue of their bone binding properties and can serve as a point of attachment for a wide variety of drugs and active molecules

including proteasome inhibitors [15], anticancer drugs [16, 17], analgesics [18] and antibiotics [19–21].

Bisphosphonates such as alendronate are very polar molecules and are poorly absorbed following oral dosing and are rapidly eliminated from the body if they do not bind first to bone [22–24]. Radiolabelling studies have shown that 40 - 50% of alendronate is taken up by bones after systemic dosing [23]. Conjugates with bisphosphonates are expected to also not be well absorbed and are usually studied after IV dosing.

An ideal drug-bisphosphonate prodrug for bone-targeted delivery would have the following properties:

1) Be tethered by a linker element that is stable in the blood stream such that within a short time (< 1 day) the molecule will either bind to bone intact or be largely eliminated intact from the body,
2) Be inactive in so far as the activity of the drug to be delivered is concerned,
3) Once bound to bones, release the active drug in a slow and sustained manner. An ideal half time for release of about 4-7 days would support infrequent dosing of once a week or twice a month,
4) Be sufficiently potent so that the overall dose of conjugate (and body load of bisphosphonate in bones) would be relatively low and within the scale of doses of bisphosphonates that have been shown to be safe in the past,
5) The drug to be delivered is on its own poorly distributed to bone, or if dosed as a free drug in the systemic circulation, exhibits unacceptable side effects that limit its use.

To quantitatively assess stability and integrity of a drug-bisphosphonate conjugate prodrug it is preferable to radiolabel the drug (and also, ideally, the bisphosphonate or linker component). The radiolabel(s) can then be used to quantitatively assess tissue distribution, bone uptake and elimination after dosing, and to demonstrate release of the bone-bound drug to determine the half-time for drug release. While many researchers have designed and synthesized bisphosphonate-drug conjugates and some have evaluated their effects in vivo (*vide supra*), very few have evaluated biodistribution and elimination of the prodrug in vivo or quantified bone exposure or drug release in the bones.

One class of drugs that have been successfully targeted to bones both via bisphosphonates (and also via conjugation to polyanionic polymers [6]) are the bone anabolic prostaglandins related to prostaglandin E_2 (PGE_2). PGE_2 was shown to be anabolic for bone in animals [25, 26] and in humans [27]. However, PGE_2 interacts with 4 distinct receptors in the body and exhibits many other physiological effects including muscle contraction and relaxation, hypotension and various gastro-intestinal effects that render its use for bone impractical [28].

Bone targeting prodrugs of prostaglandins E_2 and E_1.

Some reports have described targeting PGE_1 to bone using an fluorescene isothiocyanate(FITC)-labeled hydroxymethylpolyacrylamide copolymer(P)–Asp8 conjugate (P-Asp8-FITC) [6] where the PGE_1 was coupled as an ester to the polymer and the fluorescein was used to gain a measure of bone attachment. Polyaspartate is known to bind to bone mineral due to its polycarboxylate structure. The polypeptide was a potential substrate for cathepsin K (the major protease secreted by bone resorbing osteoclast cells) and cleavage of the polymer could

facilitate liberation of PGE_1 especially in areas of high bone turnover. Those authors found that a single injection of P-Asp8-FITC-PGE_1 in rats resulted in enhanced bone formation (relative to vehicle or P-Asp8-FITC) measured 4 weeks later but absolute amounts of polymer that reached the bone and of PGE_1 that was delivered were not defined [6].

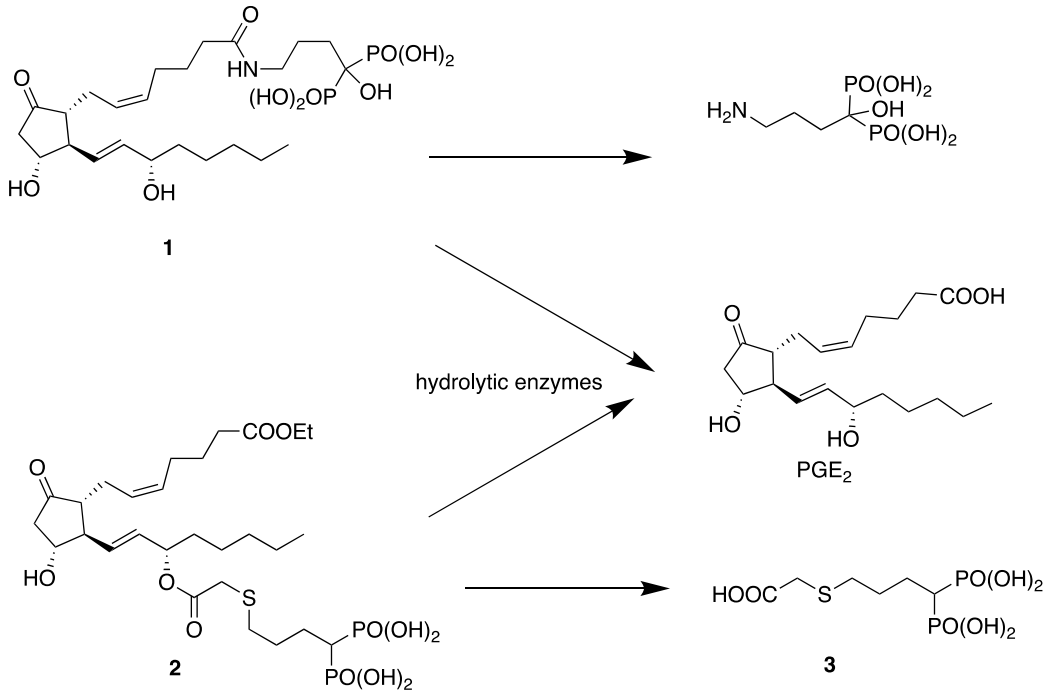


Figure 1: Bone targeting bisphosphonate conjugates derived from PGE_2 [29]

In another study, several bone-targeting prodrugs of PGE_2 were prepared with the intent of devising a dual action prodrug that would release both PGE_2 as an anabolic agent and an antiresorptive bisphosphonate [29] (Figure 1). One conjugate (**1**) was prepared where alendronic acid was coupled to PGE_2 through an amide bond and this conjugate was radiolabelled with ^{3}H on PGE_2 and ^{14}C on alendronate to allow tracking in vivo. **1** was dosed to rats and found to be well taken up by bones (about 12 - 15% of administered dose), but tracking of the radiolabels over 4 weeks showed that the tritium (representing PGE_2) was not

released and both labels remained unchanged presumably due to the stability of the amide bond in vivo. In the same study a second conjugate (**2**) was prepared where PGE_2 was conjugated with a bisphosphonate via an ester bond at the 15-hydroxy group on PGE_2. Compound **2** was found to be quite stable in blood and a radiolabelled version (synthesized from tritiated PGE_2) was shown to be taken up into bones (about 3 - 3.5 % of administered dose) and then to release the ^3H label with a half-time of about 7 days. Whereas PGE_2 was dosed intraperintonealy at 6 mg/kg (its maximum tolerated dose), the conjugate **2** could be dosed intravenously up to 100 mg/kg without overt side effects. Dosing **2** intravenously at 10 and 100 mg/kg once weekly for 28 days resulted in a dramatic anabolic effect and was shown to be more effective as a bone growth stimulant than PGE_2 dosed daily at its maximum tolerated dose. A mixture of PGE_2 and **3** (5 mg/kg each) was dosed once weekly to show the benefit of conjugation and showed no significant anabolic or antiresorptive effects at those doses. Unfortunately, while prodrug **2** was a potent and well tolerated anabolic agent it did not demonstrate antiresorptive activity and was difficult to prepare on larger scale due to the sensitivity of PGE_2 (a tendency for β–elimination of the 9-hydroxyl group under acidic or basic conditions and facile isomerization of the 5,6 double bond).

Subsequent to these studies, Machwate et al. [30] demonstrated that PGE_2 exerted its anabolic effect in bone through agonism at the EP4 receptor subtype and a number of very potent and highly selective EP4 receptor agonists were subsequently identified [28]. One of these compounds (**4**) (Figure 2) was much more stable that PGE_2 both chemically and metabolically, was found to be orally absorbed [31], and was tested in rats for bone effects. Compound **4** dosed orally at 0.5 mg/kg for 28 days demonstrated anabolic effects similar to PGE_2 dosed at 3

mg/kg (R. Young and G. Rodan, unpublished results). Unfortunately, EP4 selective agonists were found to retain some of the systemic and GI side effects of PGE_2 and thus far have not been developed further for treatment of osteoporosis.

Bone targeting bisphosphonate conjugates delivering EP4 receptor agonists. The option of preparing a bone-targeting bisphosphonate conjugate as a prodrug to deliver an EP4 agonist to bones was revisited in a recent series of studies. In particular, the enhanced stability of the selective agonists **4** and its analog **5** [32] allowed synthesis of prodrug conjugates designed to liberate both the EP4 agonist and an active

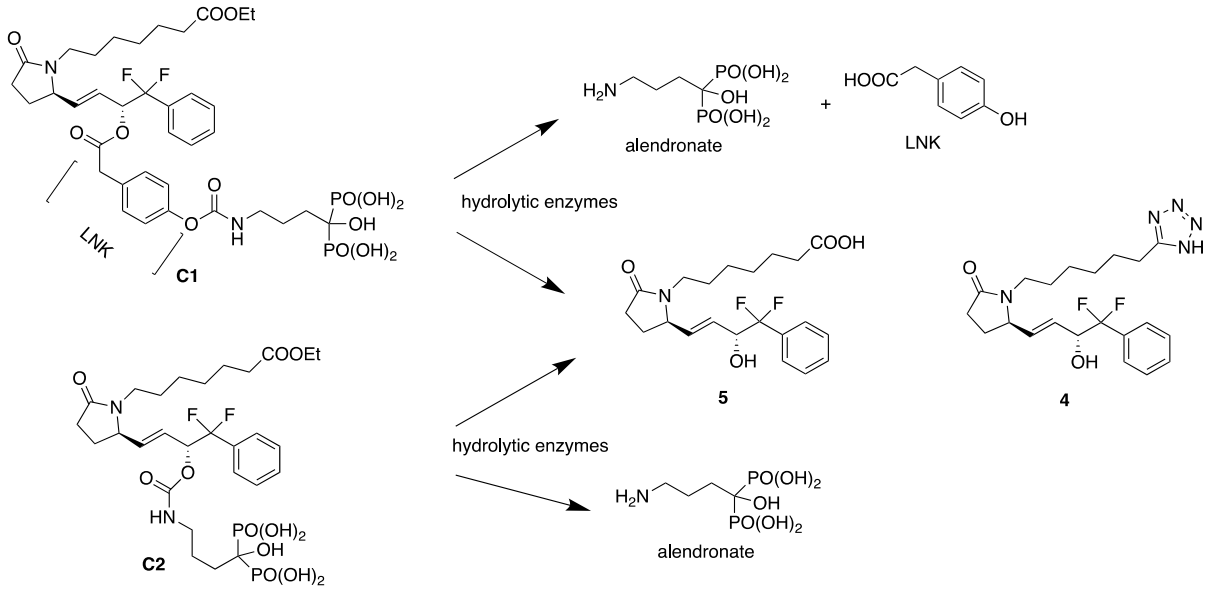


Figure 2: Bone targeting bisphosphonate conjugate prodrugs based on selective EP4 agonist **5**.

bisphosphonate, alendronate. Two conjugates were reported [33] (Figure 2). One conjugate (**C1**) employed a bifunctional linker group (LNK), 4-hydroxyphenylacetic acid, where the carboxyl function of LNK was esterified to the 15-hydroxyl group of **5** and the phenolic group on

LNK was attached to alendronic acid via a carbamate group. A second conjugate (**C2**) was prepared where **5** (as ethyl ester) and alendronate were directly coupled via a carbamate functionality. It was anticipated that the carbamate functions could be hydrolyzed in vivo based on the known in vivo conversion of carbamate prodrugs such as bambuterol [34]. In the case of **C1,** ester hydrolysis (as demonstrated for PGE2 conjugate **2**) would liberate **5** while carbamate hydrolysis would liberate alendronate. In the case of **C2,** hydrolysis of the carbamate would liberate both parts simultaneously (Figure 2). Synthesis of conjugate **C1** was straightforward aided by the stability of **5**. The pharmacokinetics and biodistribution of **C1** and **C2** in rats was also reported [35]. **C1** was prepared in tritium labeled (on the EP4 agonist moiety) and in dual (^{3}H/^{14}C) labelled forms with tritium label in the EP4 agonist (**5**) part of the molecule and ^{14}C incorporated into the carbamate function. It was expected that the rate of hydrolysis of the ester bond joining **5** to the linker could be considerably faster than hydrolysis of the carbamate moieties in **C1** or **C2**. Radiolabelled **C2** was prepared from tritium-labelled **5** (as ethyl ester) to provide a compound that was tracked in mono-labelled form in vivo. Thus, it was possible to demonstrate good stability of **C1** and **C2** in blood plasma (only 5-10% hydrolysis after 24 hours incubation) and to show that the prodrugs bound rapidly and irreversibly to bone powder after a few minutes contact. Labelled **C1** and **C2** were both dosed intravenously to rats and the biodistribution and elimination of the labels were tracked in feces, bones and blood. Uptake of intact into bones **C2** was quite good (9.4% of administered dose) whereas uptake of **C1** was somewhat less (5.9%). For both **C1** analysis of bones after 6 hours showed both ^{3}H and ^{14}C labels in a ratio similar to that in the dosed drugs indicating the conjugates had survived intact before binding. The radioactivity corresponding to the portion of both **C1** and **C2** that did not

bind to bones was rapidly cleared from the systemic circulation with an elimination half-time of < 0.5 h and in each case, elimination was presumably via the liver (most of the label was found in feces). For **C1**, the $^{3}H/^{14}C$ ratios found in blood and bones was similar to that originally dosed. In the case of the experiment with **C2** [33], the tritium label in bone diminished slowly with an estimated half-time of 28 days. This was quite slow but indicated that the prodrug might be amenable to a once a month dosing regimen. In the case of **C1,** the tritium label was reduced in the bones at a much faster rate than the ^{14}C found in the linker [35]. The half-times for loss of label were 5 days and 22 days respectively. It is interesting to note that the loss of label from the bones for both drugs appeared to be biphasic and more rapid during the first two weeks and then slowing considerably. This may possibly be due to new bone overgrowing the bound prodrug and eventually isolating the bound conjugate from access to hydrolytic enzymes.

In a recent study, another EP4 agonist-alendronate prodrug conjugate (**6**) was reported [36] where the two drugs were linked via a peptide linker (Figure 3). The peptide linker was based on Cbz-Leu-Phe-(7-amino-4-methylcoumarin), a known dipeptide substrate of cathepsin K, the proteolytic enzyme secreted by bone-resorbing osteoclasts. The carboxybenzyloxycarbonyl (Cbz) N-capping group was substituted with an acetic acid group on the Cbz and was attached to **6** via an ester bond as was done in **C1**. The peptide was designed such that cathepsin K would cleave the C-terminal amide to liberate a 4-aminobenzyloxycarbamyl alendronate moiety which would then self-immolate to liberate active alendronate (Figure 3). This novel conjugate was synthesized in dual radiolabelled form with tritium on the EP4 agonist and ^{14}C in the carbamyl group attached to alendronate. In this manner one could monitor both hydrolytic events by loss

of label in the bones after binding. Unfortunately, the conjugate was found to be only moderately stable in rat plasma with 44% liberation of tritium (i.e. **5**) in 4 hrs.

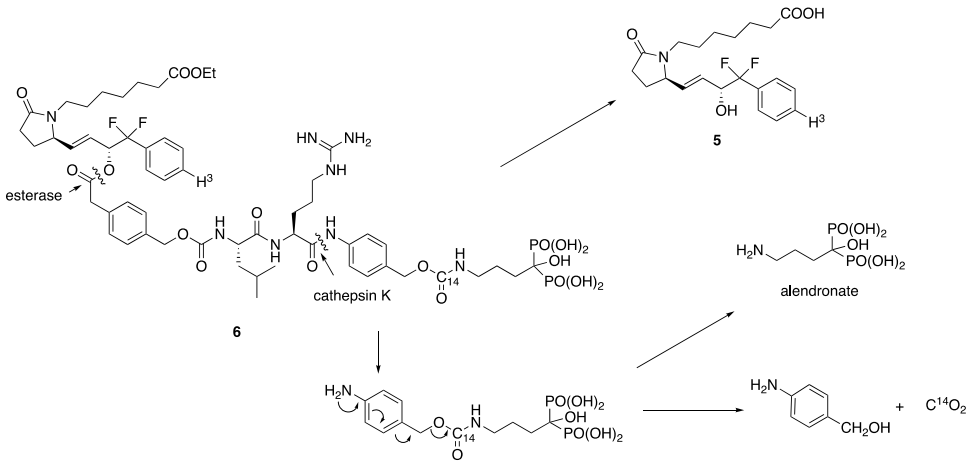


Figure 3: Bone targeting prodrug conjugate liberating alendronate by action of cathepsin K [36]

and 53 % over 24 hrs. Nonetheless the dual labelled conjugate was dosed to rats and notably, the ratio of labels found in the blood and liver were essentially the same as dosed, suggesting the conjugate was largely intact until eliminated. In contrast, the ratio of labels in the bones after 6 h indicated about 50% of **5** was lost prior to binding of the conjugate to bone and while 5% of the ^{14}C label was found in bones 6 h post dose, only about 2.5% of the ^{3}H label was found. The decay (release) of labels was monitored and the loss of ^{3}H proceeded with a 4.4 day T½ (much as was seen with **C1**). However, the release of the ^{14}C label was also quite fast (T½ 5 days) indicating that the proteolytic cleavage of the peptide was quite efficient. While, in the end, the conjugate was not deemed suitable for a follow up efficacy trial, due to the significant loss of the EP4 agonist before bone binding, it did demonstrate the feasibility of using cathepsin K as an enzymatic trigger to release a bone-bound drug from a conjugate bound to bone. This could lead others to use the technique in the future.

In vivo Anabolic Efficacy studies with EP4 agonist-bisphosphonate conjugate prodrugs

The stability and pharmacokinetic results obtained with conjugates **C1** and **C2** indicated that they were viable candidates for follow up efficacy studies in the ovariectomized rat model of osteoporosis. In an initial curative 6-week study [37] female rats were ovariectimized (had their ovaries surgically removed) and were kept 7 weeks post operation to allow for osteopenia to be established. Six groups of 6-8 rats were tested including one group that had undergone a sham operation (sham group) and an ovariectomized (OVX) group that were dosed IV with vehicle (vehicle control). In other groups, **C1** was dosed at 5 mg/kg (low dose) and 25 mg/kg (high dose) once weekly. However, in the high dose group the first two doses at 25 mg/kg was not well tolerated (local irritation and hind limb swelling) and this dose was subsequently reduced to 15 mg/kg biweekly until the end of the study. Another group was dosed iv, once weekly, with a mixture of 2.5 mg/kg of **5** (as ethyl ester, hereafter referred to as EP4a) and 2.5 mg/kg the linker unit bound to alendronic acid (LNK-ALN) to test the effect of conjugation. The sixth group was dosed subcutaneously and daily with 4 mg/kg PGE_2 as a positive control. At 12 days and 2 days before termination, the rats were injected with Calcein green to fluorescently mark the bones to assess rates of growth.

Figure 4: **C1** effects in bone in rat ovariectomized (OVX) rat assay: Representative images from microCT from the proximal tibial metaphysis. From left to right: sham rat, OVX rat and OVX rat treated with **C1** conjugated drug.

The results showed curative anabolic effects (Figure 4). Weekly administration of **C1** conjugate dose-dependently increased bone volume in trabecular bone, and partially or completely reversed OVX-induced bone loss in the tibial metaphysis and in the lumbar vertebrae and improved vertebral mechanical strength. The conjugate also dose-dependently stimulated endocortical woven bone formation and intracortical remodeling in cortical bone. The low dose treatment increased the mechanical strength while the high dose also increased strength but compromised bone material properties. Conjugation between the EP4a and ALN-LK components was crucial to the drug's anabolic efficacy. The low dose gave results comparable to that of the PGE_2 positive control despite the fact that the maximum dose of EP4a was only 10% of the PGE_2 dose on a weekly basis. This study with **C1** conjugate represented the first time that a combined therapy using an anabolic agent and the anti-resorptive compound ALN has shown significant anabolic effects which reversed established osteopenia.

In a second and longer (12 week) curative OVX rat study [38] **C1** was dosed iv at 5 mg/kg once weekly (C1 high) and twice monthly (C1 low) and **C2** was dosed at 15 mg/kg twice monthly (C2 high) and once monthly (C2 low). These groups were compared to OVX rats dosed with a mixture of alendronate and **5** ethyl ester (EP4a) (0.75 mg/kg each dosed every two weeks, a dose calculated to mimic the degree of exposure of rats to **5** and ALN in the **C2** high dose experiment). There were also with sham (operated but non-OVX) and vehicle OVX rat controls.

In this study results from the **C1** weekly dose recapitulated the results obtained in the first study but the twice-monthly dose group showed no significant effects on cortical and trabecular bone. The **C2** gave minimal effects at either dose as did the unconjugated mixture. It thus appeared that the amount of **5** and ALN released from **C2** was too small to exert a measurable effect and that the **C1** dose of 5 mg/kg was optimal. Because **5** was released at the same time as the alendronate, it also appears that they functionally antagonized each other and therefore showed no significant effects, either anabolic or antiresorptive, on bone.

Conclusions: The studies discussed in this review have shown that a bisphosphonate can provide a viable vector for targeting drugs to bones and that it is very important to characterize the stability of such conjugates in the blood stream and to also to monitor the amount of conjugate that reaches and binds to the bone as well as the rate at which the prodrug releases its components. The percentage of dose that binds to bones can vary quite considerably (as seen from 2.5 to about 10%) and generally is less than observed for alendronate itself. Also, these conjugates tend to be rapidly eliminated in feces whereas alendronate is largely eliminated in the urine. These differences may correlate with polarity and possibly with plasma protein binding as the conjugates are generally much more lipophilic than the bisphosphonate alone. In the best conditions such as was found with **C1**, the bone targeted prodrug approach can deliver drugs to bone and provide sustained local release, efficacy and avoid unwanted systemic side effects. [38].

Future prospects: The next step for the use of bisphosphonates to target therapeutics to bone is to advance drugs such as **C1** into human clinical trials to definitively provide human proof of

principle and to demonstrate efficacy and tolerability in treating human diseases of the bone. A logical indication for **C1** would be osteoporosis but the hurdles to bring a new drug for osteoporosis to the market are immense. Very large and long duration clinical trials are needed to support developing a new osteoporosis drug with such a high level of expense and risk that only the largest pharmaceutical companies can contemplate such a project. An alternative and exciting indication may be advancing **C1** for treatment for the osteogenesis imperfecta (OI), otherwise known as brittle bone disease, an orphan congenital disease which afflicts thousands of children and where treatment options are few. Alendronate is used as an antiresorptive to treat OI in children [39] and several bone anabolic biologic drugs such as teriparatide [40] and the sclerostin antibody BPS804 [41] have shown promise in clinical trials to build density bone in adult OI patients. A targeted combination drug such as **C1** may have a useful role to play in treatment of OI.

As linker technologies improve, bisphosphonates will be used to target other drugs to bone including anticancer drugs and antibiotics. The key to success will be to build prodrugs that are very stable in the bloodstream, are well taken up by bone and any drug not taken up should be rapidly eliminated intact in an inactive form. The bone-bound prodrug will then give sustained release of active agent and should thus achieve at least a ten-fold dose (exposure) advantage compared to dosing free drug alone. We are now developing new, very stable linker technologies that spontaneously (but slowly) release a wide variety of drug molecules and do not require enzymatic cleavage. These linkers will be “tunable” to provide a wide range of half-ties of release and further revolutionize the field.

Highlights:

- PGE_2 receptor EP4 agonists are anabolic in bones but retain some side effects
- EP4 agonists conjugated with bisphosphonates target bones selectively
- EP4 agonist-alendronate conjugates are effective bone anabolics when dosed weekly

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