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Chemotherapeutic bone-targeted bisphosphonate prodrugs with hydrolytic mode of activation

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Abstract—Osseous tissues are considered to be limited as therapeutic target sites due to their biological properties. We have designed and synthesized two kinds of hydrolytically activated chemotherapeutic prodrugs containing bisphosphonate, a bone-targeting moiety. The first can be conjugated to drug molecules with an available hydroxy group; the drug is attached to the bisphosphonate component through an ester-labile linkage. The second is for use with drug molecules with amine functional group. In this case, a self-immolative linker is used to attach the drug to the bisphosphonate component through a carbonate-labile linkage. The concept was demonstrated using the drugs camptothecin, which has a hydroxy functional group, and tryptophan, which is a model molecule for a drug with amine functionality. Both prodrugs showed significant binding capability to hydroxyapatite, the major component of bone, and were hydrolytically activated under physiological conditions.

Chemotherapeutic prodrugs with capability to target certain tissues or cell types may enhance the potency or eliminate the side effects of drugs.[1–4] Although many prodrug approaches have been demonstrated, osseous tissues are considered to be limited as target sites due to their biological properties. In contrast to other tissues, the blood flow rate in bones is very low, because they mainly consist of an inorganic compound $Ca_{10}(PO_4)_6(OH)_2$, termed hydroxyapatite (HAP). Recently a promising drug delivery system with a bisphosphonate (BP) moiety for targeting osseous tissues was proposed.[5] Bisphosphonates have high affinity for HAP and calcified tissues are the main targets for accumulation after administration of bisphosphonates into the body.[6,7] Several examples of anticancer drugs linked to a bisphosphonate moiety have been reported in the scientific literature.[8] Most of them failed to show any improved anticancer effect, however, when tested in vivo in mice.[8,9] In these compounds, the chemotherapeutic drug was linked to the bisphosphonate through a stable chemical linkage that did not allow release of the active drug. Interestingly, when anti-inflammatory drugs were conjugated to a bisphosphonate moiety through an ester linkage, promising results were observed in animal-model experiments.[10–12] Therefore, we assume that a bisphosphonate moiety conjugated to a chemotherapeutic agent through a hydrolyzable linker will target the drug conjugate to the osseous tissue, where the active parent drug will be slowly released through the hydrolysis of the linkage. Here we report the design, synthesis, and in vitro evaluation of two new prodrugs, based on known chemotherapeutic drugs, attached to bisphosphonate moiety through a hydrolyzable linkage. The linkage was spontaneously hydrolyzed at a physiological pH (half-life time of hours to days) and active drug was released. Both prodrugs showed significant binding capability to hydroxyapatite, the major component of bone.

In order to demonstrate our concept, we chose to conjugate a bisphosphonate moiety to the known chemotherapeutic drug camptothecin.[13] In clinical trials, camptothecin was not effective due to its extremely low aqueous solubility and severe toxic side effects.[14] Several new chemical derivatives of camptothecin that overcome some of the disadvantages of the parent compound are now being evaluated in clinical trials.[2] Camptothecin is an ideal candidate for conjugation with bisphosphonate as this moiety will impart both water solubility and target the compound to bone.

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Camptothecin can be esterified with bisphosphonate-butyric acid to form an esterolytic-activated prodrug, as illustrated in Figure 1. The prodrug is termed Camdronate; we chose the suffix 'dronate' since most bisphosphonate-based drugs follow this nomenclature.[5]

Camdronate was synthesized as illustrated in Figure 2. Bisphosphonate carboxylic acid **1** was prepared as previously described.[11] Chlorination of **1** with oxalyl chloride generated acylchloride-tetraethyl-bisphosphonate **2**. Then camptothecin was acetylated with acylchloride **2** to give ester **3**, which was deprotected with trimethylsilylbromide to afford Camdronate. The phosphonic acid moiety was transformed into its water-soluble sodium salt by the addition of an appropriate amount of sodium hydroxide (until the pH of the solution reached 9).

Next, we evaluated the hydrolytic stability of the ester linkage in Camdronate under physiological conditions. Camdronate was incubated in RPMI cell-medium (pH 7.4) at 37 °C and the release of free camptothecin was monitored by RP-HPLC. Camdronate was gradually hydrolyzed to release free camptothecin with a $t_{1/2}$ of 40 h (Fig. 3).

Camdronate was then evaluated by cell-growth inhibition assay using a cancerous acute lymphoblastic leukemia (ALL) Jurkat cell line (Fig. 4). Camdronate was 10-fold less active (IC_{50} 2.1×10^{-8}) than free camptothecin (IC_{50} 1.4×10^{-9}). This was expected since the masking of the 20-hydroxy group of camptothecin by an ester generates a prodrug with reduced anticancer activity. The prodrug can be recovered upon the hydrolysis of the ester linkage. No cytotoxicity was detected for the bisphosphonate-butyric acid moiety under the assay conditions.

Since Camdronate is designed for bone targeting, we sought for a simple model system to simulate bone tissue. Hydroxyapatite (HAP) is a commercially available calcium mineral. A suspension of HAP in aqueous media was previously used as a bone mod-

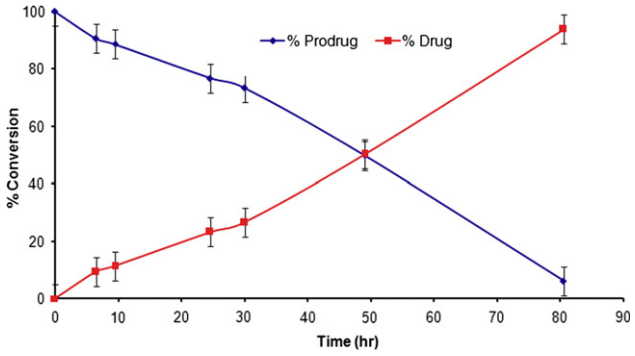

Figure 3. Hydrolysis of Camdronate (blue) to release camptothecin (red) in RPMI cell-medium, 37 °C.

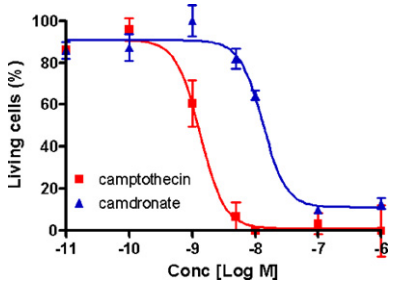

Figure 4. Growth inhibition activity of Camdronate (blue) and camptothecin (red) on Jurkat cells after a 96-h incubation over a range of concentrations of drug/prodrug.

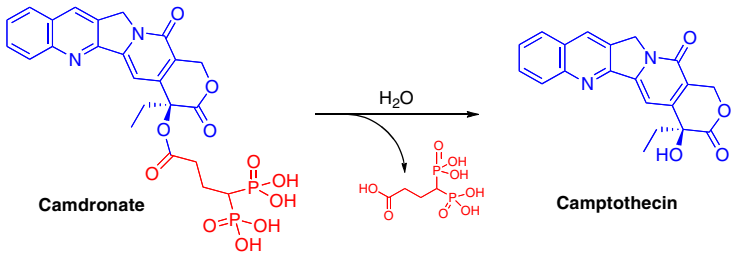

Figure 1. Hydrolysis of Camdronate under physiological conditions releases free, active camptothecin.

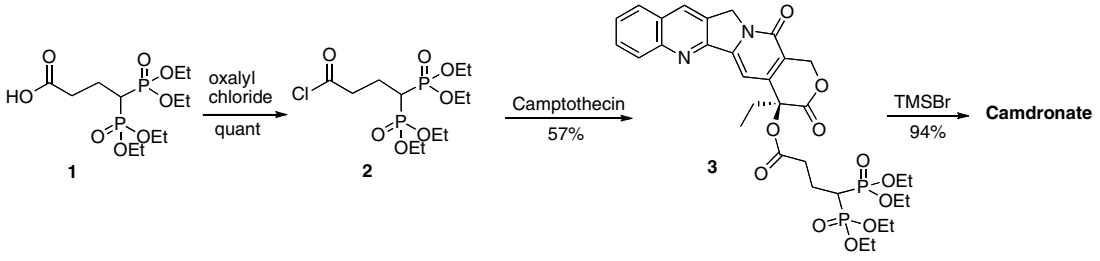

Figure 2. Chemical synthesis of Camdronate.

el.[16] An aqueous solution of Camdronate was vigorously stirred with HAP for 1 h to allow binding of the bisphosphonate moiety with the calcium mineral. The mixture was filtered and the precipitate was washed with water to remove any unbound Camdronate. The precipitate (HAP and any bound Camdronate) was then incubated in phosphate buffer saline, pH 7.4 (PBS), at 37 °C and the hydrolytic release of camptothecin to the solution was monitored by RP-HPLC. The camptothecin was slowly released through hydrolysis over 300 h (Fig. 5, blue plot). In order to ensure that the unconjugated camptothecin molecule had no affinity for HAP, a control experiment was performed in which the HAP suspension was mixed with camptothecin and then filtered and washed. No camptothecin was detected in the HAP precipitate from this experiment (green). In contrast, when the Camdronate/HAP ratio was doubled, the total amount of free camptothecin released was correspondingly increased (red).

Chemotherapeutic drugs with an available amine functional group, such as doxorubicin or melphalan, generally form hydrolytically stable derivatives upon masking of their reactive amines through an amide or a carbamate linkage. Such a doxorubicin-based prodrug did not show any therapeutic effect when tested against human tumor xenografts.[9] In order to extend our concept to additional drugs with an amine functional group, we sought to link the bisphosphonate through a hydrolyzable bond to a short self-immolative linker for subsequent attachment to the amine group of the drug (Fig. 6).

As model molecule for a drug with an available amine functional group, we chose the amino acid (L)-tryptophan. The chemical structure of a tryptophan prodrug (Trypdronate) is shown in Figure 7. The bisphosphonate

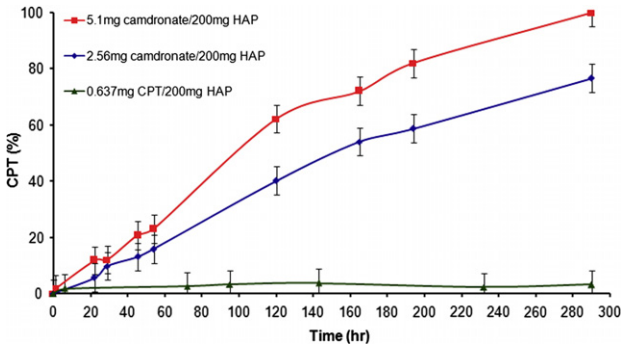

Figure 5. Release of camptothecin from HAP-Camdronate under physiological conditions.

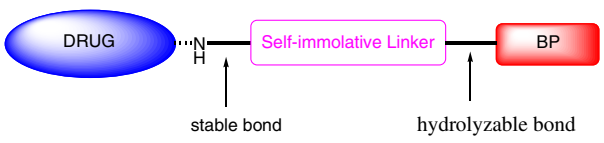

Figure 6. Schematic structure of a bisphosphonate prodrug of a drug with an amine functional group.

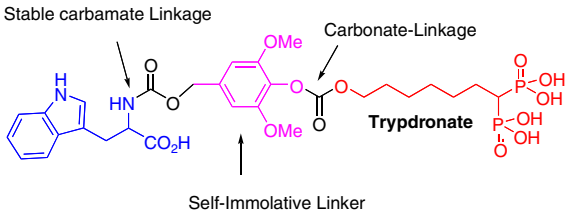

Figure 7. Chemical structure of Trypdronate.

moiety was linked through a labile carbonate bond to the self-immolative moiety, 4-hydroxy-3,5-dimethoxybenzyl-alcohol, which was further attached through a stable carbamate linkage to the amine group of tryptophan.

The bisphosphonate moiety is designed to target the drug conjugate to the osseous tissue, where the active parent drug will be released through the hydrolysis of the carbonate linkage and a spontaneous 1,6-elimination followed by decarboxylation. A detailed proposed release mechanism is presented in Figure 8.

Trypdronate was synthesized as shown in Figure 9. Tetrabenzyl-bisphosphonate **4** (prepared as published before[11]) was reacted with protected bromohexanol to generate compound **5**, which was deprotected to yield alcohol **6**. Carbonate **9** was obtained by selective silyl-protection of **7** to afford phenol **8**, which was acylated with 4-nitrophenyl chloroformate. Next, alcohol **6** was coupled with carbonate **9** to form compound **10**, which was deprotected in the presence of amberlyst-15 to give alcohol **11**. The latter was acylated with 4-nitrophenyl chloroformate to generate carbonate **12**, which was coupled with tryptophan to afford compound **13**. Catalytic hydrogenation of **13** removed the benzyl protection groups of the bisphosphonate to afford Trypdronate.

The stability of Trypdronate was then evaluated under physiological conditions. It was incubated in PBS (pH 7.4) at 37 °C and the release of tryptophan was monitored by RP-HPLC. Trypdronate was slowly hydrolyzed over seven days to release free tryptophan with a $t_{1/2}$ of 90 h (Fig. 10a). Next, we evaluated the binding ability of Trypdronate in our bone model. An aqueous solution of Trypdronate was vigorously stirred with HAP for 1 h to allow binding of the bisphosphonate moiety with the calcium mineral. The mixture was filtered and the precipitate was washed with water to remove any unbound Trypdronate. The precipitate was then incubated in PBS (pH 7.4) at 37 °C and the hydrolytic release of tryptophan to the solution was monitored by RP-HPLC. The Trypdronate was slowly hydrolyzed over 170 h (Fig. 10b, blue plot). Free tryptophan has no affinity for HAP as shown by a control experiment in which the HAP suspension was mixed with tryptophan and then filtered and washed. No release of tryptophan was observed from the HAP precipitate in the control experiment (Fig. 10b, green). As observed with the Camdronate, when the Trypdronate/HAP ratio was

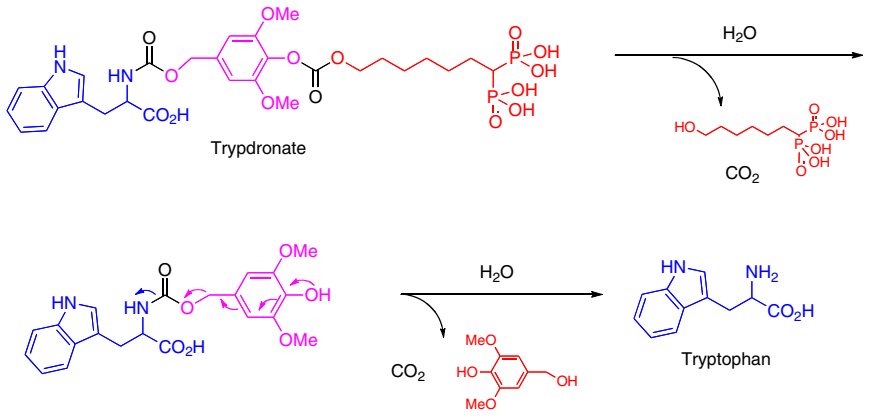

Figure 8. Proposed mechanism of release of tryptophan via hydrolysis of the carbonate linkage of Trypdronate and spontaneous 1,6-elimination.

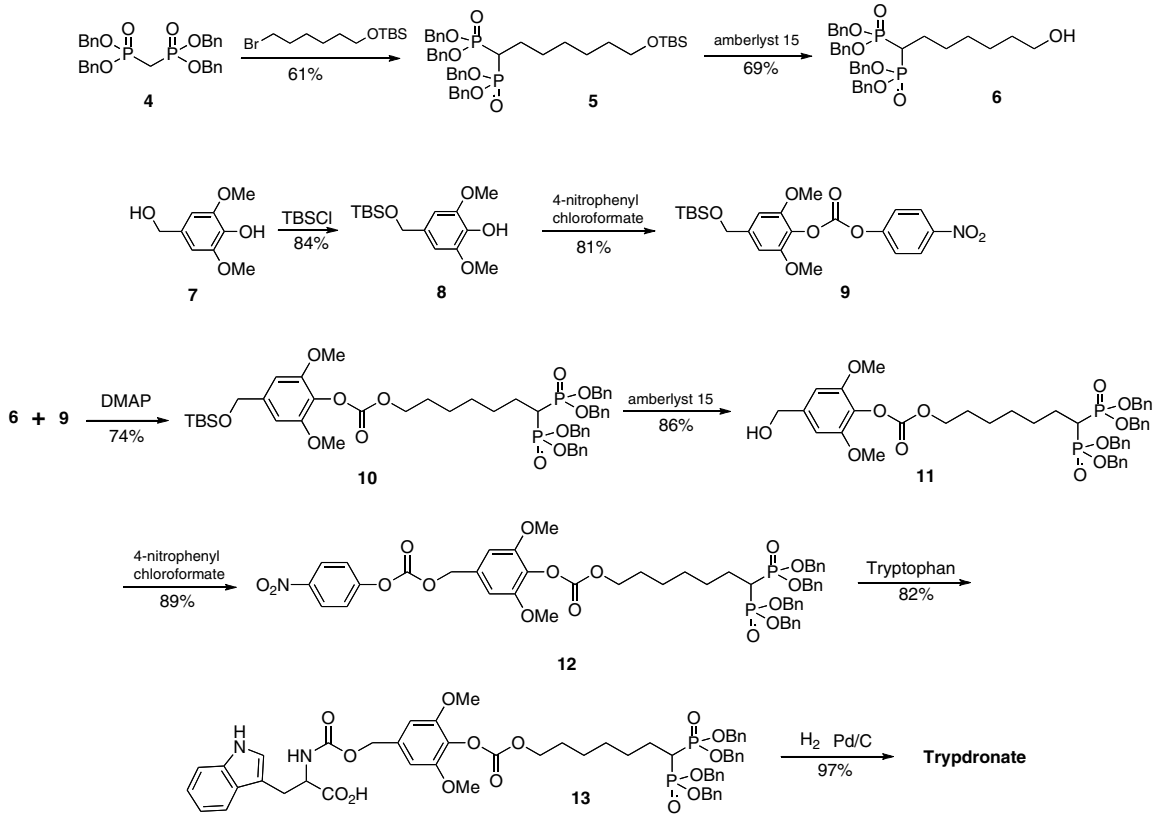

Figure 9. Chemical synthesis of Trypdronate.

doubled, the total amount of free tryptophan released was correspondingly increased (red).

The self-immolative linker used in the Trypdronate design is based on 4-hydroxy-3,5-dimethoxybenzyl-alcohol (**7**). This molecule is linked with the bis-phosphonate component (the nature of the binding site or its constant affinity is not known[18]) through a carbonate linkage that hydrolyzed under physiological conditions with a $t_{1/2}$ of 90 h. The hydrolysis rate of the carbonate bond can be controlled through the nature of the substituent on the aromatic ring of the self-immolative linker **7**. Substituents of different electron-donating/withdrawing capabilities will decrease or increase the hydrolysis rate of the Trypdronate prodrug. We recently demonstrated similar substituent effects on the disassembly rate of a self-immolative molecular dendritic system.[15] Tryptophan was used as model for a drug with an available amine functional group, since it is a simple molecule that was applied before for such a purpose.[17] Chemotherapeutic drugs like doxorubicin would require modification in the synthesis of their drug-bisphosphonate conjugate, since the synthetic pathway presented in Figure 9 is not entirely compatible with their structure (for example, the removal of the benzyl protecting groups from **12** to yield **13** under catalytic hydrogenation conditions will reduce the doxorubicin

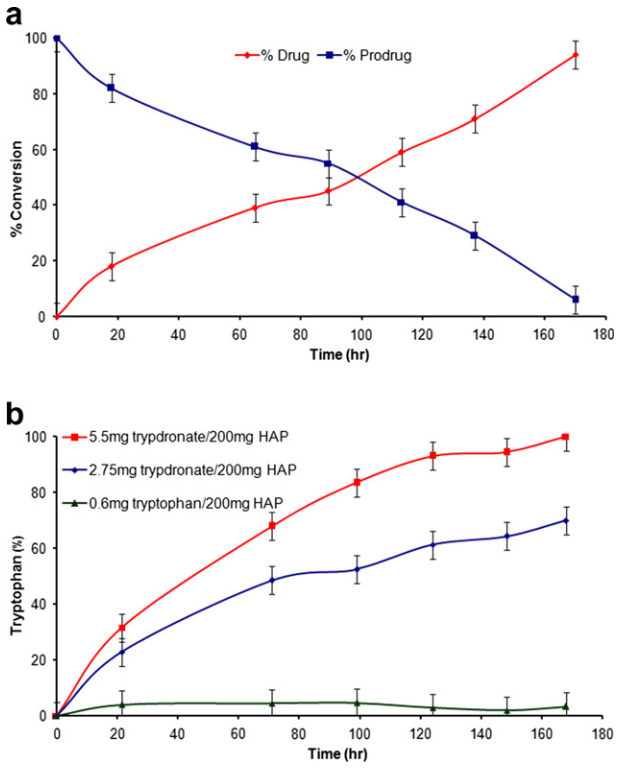

Figure 10. (a) Hydrolysis of Trypdronate (blue) to release tryptophan (red) in PBS (pH 7.4) at 37 °C. (b) Release of tryptophan from HAP/Trypdronate under physiological conditions (PBS, pH 7.4, 37 °C). The maximum loading of Trypdronate per 100 mg HAP was calculated to be 0.625 mg.

molecule, too). However, the hydrolytic release mechanism of the active drug from the bisphosphonate conjugate is likely to be identical.

In conclusion, we have demonstrated two options for construction of hydrolytically activated chemotherapeutic prodrugs containing a bisphosphonate bone-targeting moiety. The first option is applicable for drug molecules with an available hydroxy group. The drug is attached to the bisphosphonate component through an ester-labile linkage. The second option is suitable for drug molecules with available amine functional group. In this case, a self-immolative linker is used to join the drug and the bisphosphonate component through a carbonate-labile linkage. The concept was demonstrated using camptothecin and tryptophan. Both prodrugs bound to HAP, a model for bone, and were hydrolytically activated under physiological conditions. In the Jurkat cell line, the prodrug Camdronate was 10-fold less toxic than free camptothecin. We are currently developing a suitable animal model for in vivo evaluation of Camdronate.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.029.

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