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Determination of the Rat in Vivo Pharmacokinetic Profile of a Bone-Targeting Dual-Action Pro-Drug for Treatment of Osteoporosis

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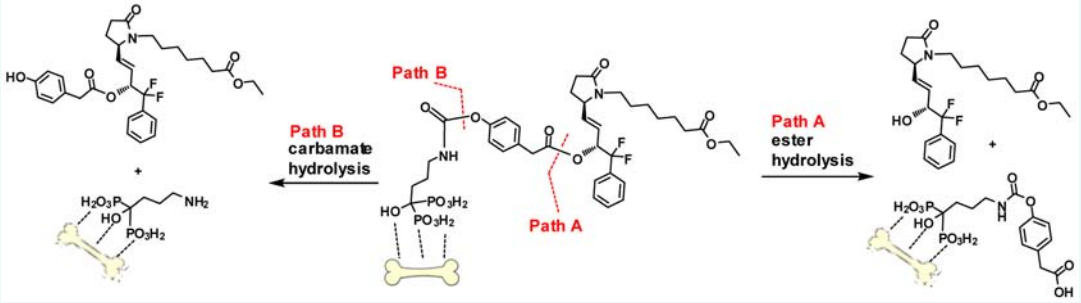
Supporting Information



ABSTRACT: The in vivo hydrolytic pathway of a dual-function bone-targeting EP4 receptor agonist-bisphosphonate pro-drug was deduced from radiolabeling experiments. A ^{14}C labeled pro-drug was used to monitor liberation of the bisphosphonate and results were compared to parallel studies where the EP4 receptor agonist was labeled with ^{3}H. The bone-adsorption of the ^{14}C pro-drug following an IV bolus was about 10% compared to 7.8% for the tritiated pro-drug. The difference in release half-life (5.2 and 19.7 days from ^{3}H and ^{14}C experiments, respectively) indicated that, after binding to bone, the initial hydrolysis occurred at the ester moiety of the linker releasing the EP4 agonist. The conjugate was found to concentrate in more porous, high-surface-area regions of the long bones. Both ^{3}H and ^{14}C experiments indicated a short circulating half-life (1−2 h) in blood.

INTRODUCTION

Osteoporosis is a disease characterized by progressive loss of bone mass and deterioration of bone tissue, leading to bone fragility. Statistically, osteoporosis-related fractures are more common than heart attack, stroke, and breast cancer combined, affecting one-half of women and one-fifth of men in their lifetime.[1]

In a previous report,[2] we described the design, synthesis, and preliminary pharmacokinetic profiles of several conjugate pro-drugs that combined an alendronic acid moiety and a prostaglandin E_2 receptor subtype EP4 agonist moiety. Alendronic acid provided bone-targeting and, putatively, bone resorption inhibition, while local, sustained release of the EP4 receptor agonist was expected to stimulate bone growth.[3−5] A selected conjugate (**C1**, Figure 1), having a bifunctional 4-hydroxyphenylacetic acid linker element where the acid moiety formed an ester with the 15-hydroxy function of the EP4 agonist and the phenol moiety joined to the alendronic acid amino function via a carbamate group, was shown (by monitoring **C1** bearing tritium labeled EP4 agonist moiety) to exhibit a level of bone-targeting and in vivo half-time for release of the EP4 component from the bone-bound conjugate[2] sufficient to warrant further study. Subsequent efficacy studies in an ovariectimized rat model (OVX) of osteoporosis demonstrated that **C1** induced enhanced growth of bone when dosed systemically at 5 or 15 mg/kg once weekly.[6] While the initial design of conjugate **C1** anticipated a more rapid release of the EP4 moiety, we wished to confirm this and to quantify the rates of liberation of the alendronate and EP4 moieties in vivo. Since the EP4-agonist and alendronic acid moieties need to be released in their free forms to exert their biological effects, the relative rate of hydrolysis at the two ends of the linker could affect the final biological effect of this pro-drug. In order to aid in development of **C1** or similar conjugates into a drug to treat and possibly reverse

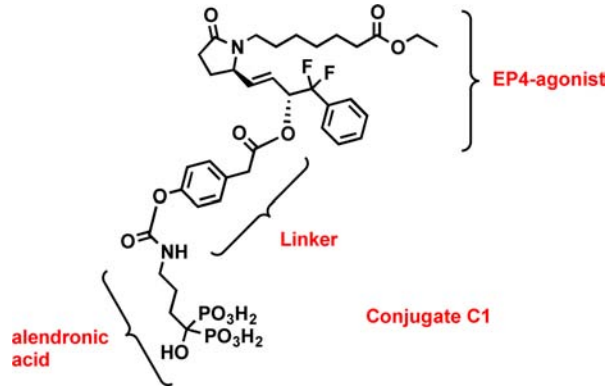


Figure 1. Structure of Conjugate **C1**.

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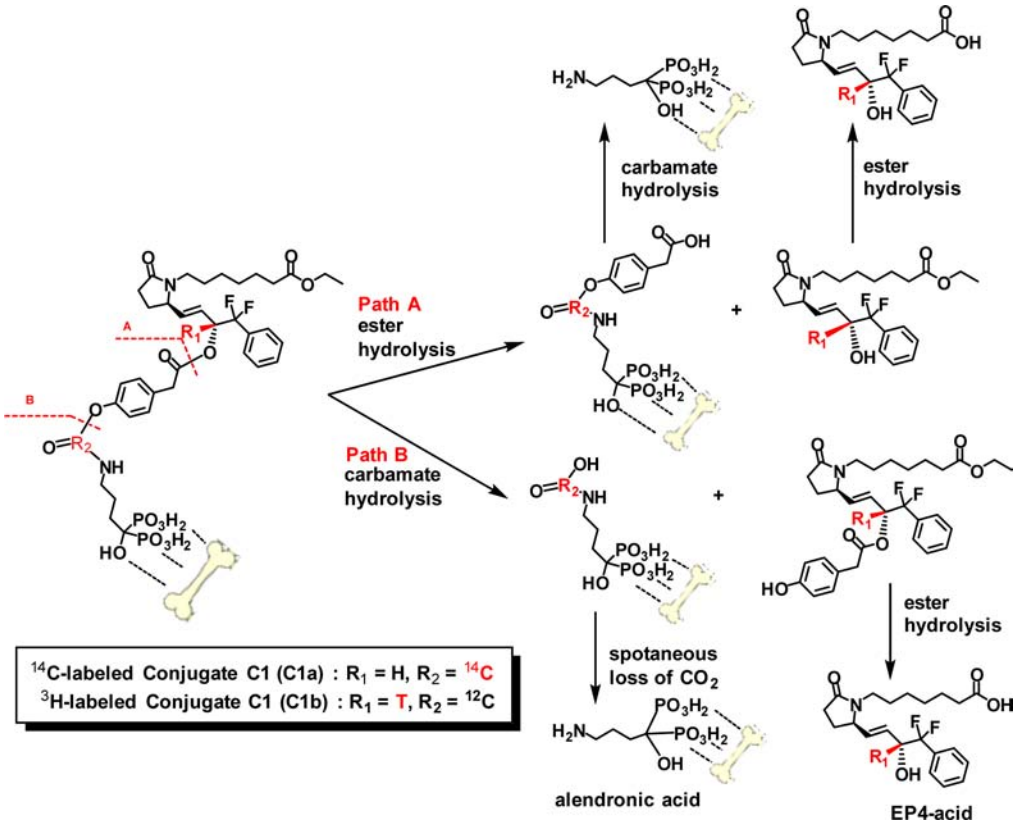

Figure 2. Radiolabeled Conjugate **C1** and its possible in vivo hydrolytic pathways.

osteoporosis, we have designed more experiments to study the in vivo pharmacokinetic profile of conjugate **C1**, and we have performed retro-analysis of the previous experiments[2] to elucidate and quantify the hydrolytic pathways that release the two active components from the conjugate.

Due to the abundance of esterases in vivo, and the intrinsic propensity of the ester groups toward hydrolytic cleavage, it would be safe to expect that the EP4-agonist portion of the conjugate would eventually be released in its active (free acid, free hydroxyl) form. However, the same could not be said for the carbamate linkage connecting the alendronic acid portion of the molecule. Phenol carbamates have been used as pro-drugs in the past[7] and are thought to be cleaved by cholinesterases, carboxy-peptidases, and P450 enzymes in the liver, but it was not clear if these activities were expressed in the bone. To discern the hydrolytic pathway, a radiolabeled conjugate (**C1a**) was synthesized with the carbamate-carbonyl labeled with ^{14}C (Figure 2). After the initial binding to bones after IV dosing, the fate of the carbamate linkage could be followed by observing the ^{14}C-label in the isolated bones. If the hydrolysis occurred at the ester linkage (Path A) while the carbamate portion remained unchanged, ^{14}C-radioactivity would be retained in bone. On the other hand, if hydrolysis occurred at the carbamate linkage (Path B), it would give an unstable carbamic acid, which would rapidly and spontaneously lose CO_2, and thus the loss of ^{14}C-radioactivity from the bone. In comparison, radiolabeling of bone with a conjugate ^{3}H-labeled on the prostanoid moiety (**C1b**, Figure 2) would be lost no matter whether the initial hydrolysis occurred via Path A or Path B.[8] Alternatively, loss of radiolabeling could occur by dissociation of the bisphosphonate from bones, but this dissociation is expected to be negligible due to the exceptionally high binding affinity[9] ($K_L = 2.9 \times 10^6$) of bisphosphonates such as alendronic acid to hydroxyapatite, the main mineral component of bones, and the known long half-life (200 days) of alendronate in vivo in the rat.[10] Thus, by comparing the rate of radioactivity loss between ^{3}H- and ^{14}C-labeled conjugates, it would be possible to distinguish between these two hydrolytic pathways and to estimate the relative local bone exposure to the active constituents, EP4 agonist and alendronate, respectively.

RESULTS AND DISCUSSION

Synthesis of ^{14}C-Labeled Conjugate C1 (C1a). The synthesis of ^{14}C-labeled conjugate **C1a** followed a modified procedure from our previous report[2] (Scheme 1). 4-Hydroxyphenylacetic acid (**2**) was converted to its monosilyl protected analogue (**3**) by exhaustive protection followed by selective hydrolysis. Ester coupling with 15-hydroxyl group of the EP4-agonist ethyl ester[11] (**1**) and **3** using dicyclohexylcarbodiimide (DCC) and subsequent silyl deprotection gave the phenol intermediate (**4**). Reaction with [carbonyl ^{14}C]-*p*-nitrophenol chloroformate[12] gave the corresponding *p*-nitrophenol carbonate (**5**), which was coupled with alendronic acid to give the ^{14}C-labeled Conjugate C1 (**C1a**).

Biological Study Methodology. The in vivo experiment with ^{3}H-labeled **C1** (**C1b**, Figure 2) was performed and reported previously.[2] To summarize, 12 female Sprague–Dawley rats were dosed with 10 mg/kg of ^{3}H-labeled Conjugate C1 (**C1b**) in PBS (300–500 μL) via tail vein IV injection. Groups of three rats were euthanized after 6 h, 2 days, 7 days, and 14 days. The tibiae and femora were harvested, cleaned, and dried. The shaft portions were weighed and combusted in a Harvey OX-300 biological oxidizer, and the resulting scintillation mixtures were counted in a Beckman LS

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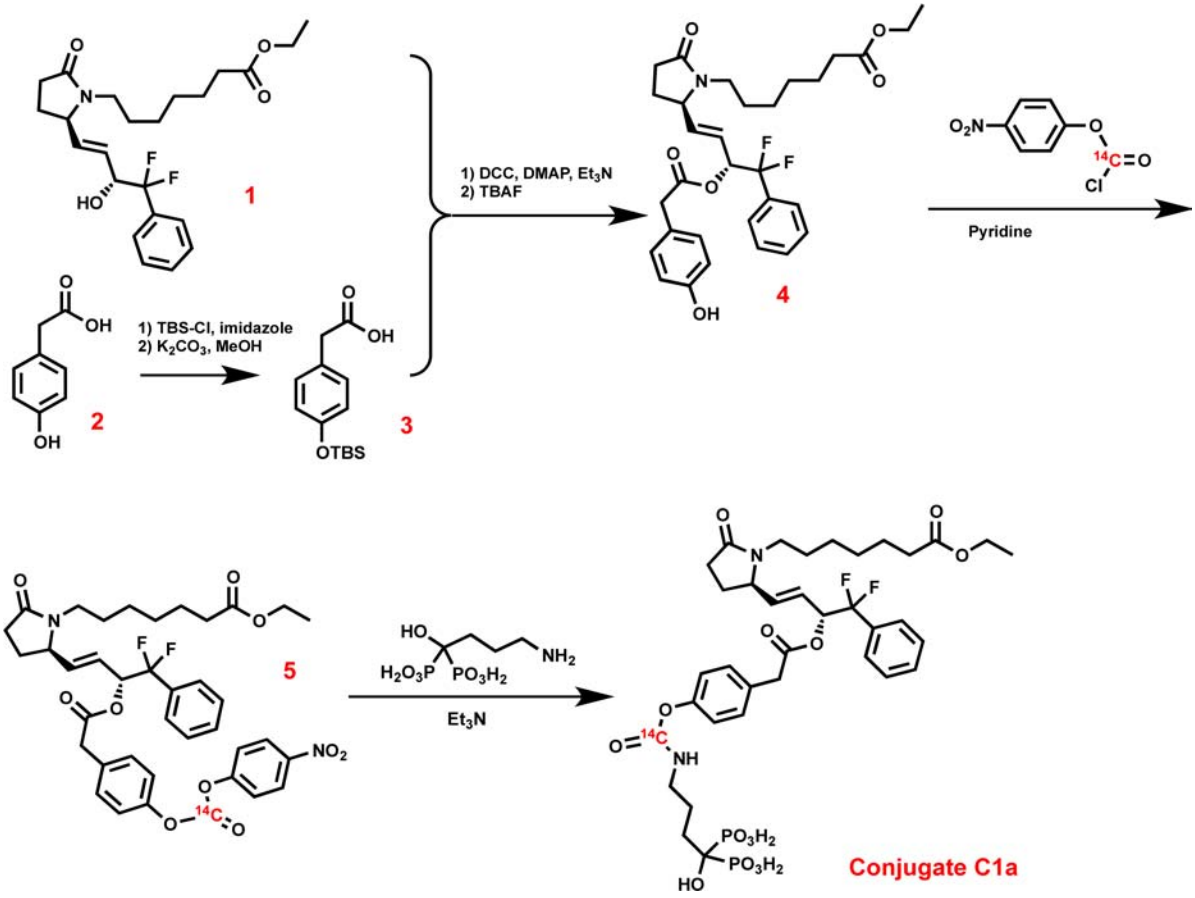
Scheme 1. Synthesis of ^{14}C-Labeled Conjugate C1 (C1a)



6500 scintillation counter. Blood samples were collected for the 6 h rats at 0.5, 1, 2, and 5 h. For each blood sample, plasma was separated by centrifugation, and proteins were removed by acetonitrile (ACN) precipitation. The supernatant was added to a scintillation cocktail and counted to obtain the circulating drug level.

In subsequent ^{14}C experiments, the same methodology was used except a lower dose of **C1b** (5 mg/kg) was used and the experiment was extended to 28 days.[13] The experiments were arranged in a staggered fashion (Figure 3). Groups of three rats

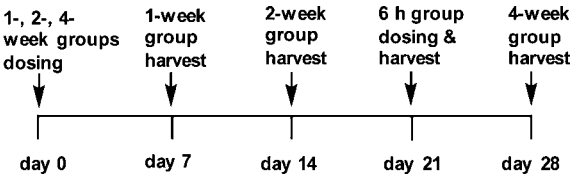


Figure 3. Experiment schedule for dosing ^{14}C-labeled conjugate **C1a**.

were euthanized after 6 h, 7 days, 14 days, and 28 days; the femora were harvested, cleaned, dried, and weighed. In this case the bone shaft and large joint portions were analyzed separately in a Harvey OX-300 biological oxidizer, and the resulting scintillation mixtures were counted in a Beckman LS 6500 scintillation counter. The instrument was set to automatically correct for counting efficiency, quenching, and chemiluminescence, and the results are presented in DPM (disintegrations per minute) (Table 1). Serial blood samples were collected for the 6 h rats at 0.5, 1, 2, 4, and 6 h (Table 3). The whole blood was analyzed directly in the biological oxidizer and the resulting scintillation mixture was counted to obtain the circulating drug-equivalent levels.

The counting data from the bones were normalized with the bone sample weight and the total radioactivity dosed (DPM/[mg of sample weight]/μCi dosed). When both femur shafts were analyzed, the results were averaged within each animal. To get an estimate on the total bone uptake, it was assumed that 8% of the total body weight was bones, and that the compound distributed evenly among the bones. Although these assumptions are overly simplistic, they should be reasonable approximations when doing relative comparisons. The results from three animals at each time point were analyzed and plotted with *GraphPad Prism* v 5.0 (Graphpad Software Inc., La Jolla, CA, USA). To determine if the ^{3}H and ^{14}C data were statistically different, the data from matching time points were subjected to Student *t*-tests,[14] and statistically different results ($P < 0.05\%$) were marked with an asterisk. The initial bone-bound drug concentrations (Y_0) were given by *GraphPad Prism* using *one phase decay* algorithm directly.

However, the calculation of half-life values was not as straightforward. *GraphPad Prism* used the following model in its calculation:

$$Y = (Y_0 - \text{Plateau})^{(-K*X)} + \text{Plateau}$$

Due to the limited span of both the ^{3}H and ^{14}C experiments, there were not enough data points toward the tail-end of the experiments to show the slow decrease in bone-bound drug concentration. The program thus assumed that there was a

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Table 1. Pharmacokinetic Results after Dosing ^{3}H- and ^{14}C-Labeled Conjugate C1

	^{3}H experiments (with **C1b**)				^{14}C experiments (with **C1a**)			
rat	time	animal weight (g)	femora shaft average DPM/mg/μCi dosed	fraction of initial dose in bones[a] (%)	time	animal weight (g)	femora shaft average DPM/mg/μCi dosed	fraction of initial dose in bones[a] (%)
1	6 h	218	9.57	7.59	6 h	305	8.24	9.14
2		218	8.16	6.46		310	10.09	11.38
3		244	8.69	7.71		294	9.15	9.78
4	2 days	270	6.72	6.60	7 days	259	6.56	6.18
5		255	6.74	6.25		285	6.81	7.05
6		262	6.88	6.56		295	6.16	6.61
7	7 days	257	2.86	2.68	14 days	289	5.03	5.29
8		250	3.13	2.84		279	3.49	3.54
9		270	2.99	2.93		271	5.81	5.72
10	14 days	330	0.80	0.96	28 days	317	2.71	3.13
11		308	1.68	1.88		N.R.[b]	3.58	4.14
12		304	2.10	2.33		319	3.27	3.78
	Y_0 = 7.8 ± 0.4% $t_{1/2}$ = 5.2 days				Y_0 = 10.3 ± 0.5% $t_{1/2}$ = 19.7 days			

[a]Extrapolated from the DPM/mg/μCi value, assuming total bone weight was 8% of the body weight, and that distribution of radioactivity were homogeneous throughout the bones. [b]Body weight was mistakenly not recorded, using the average body weight of rats 10 and 12 in calculation.

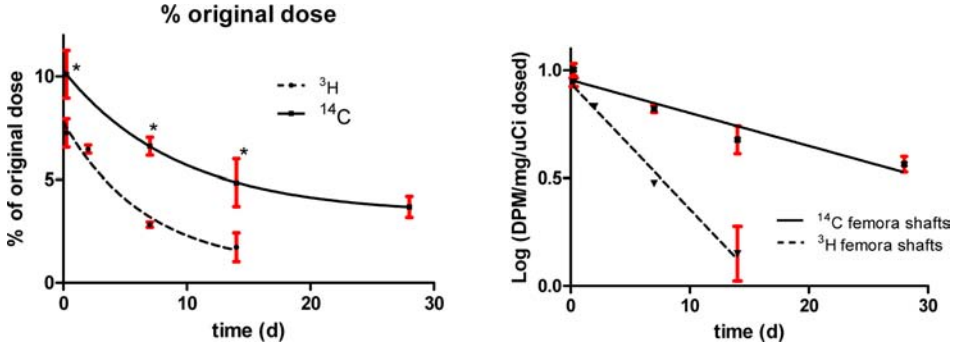

Figure 4. Comparison of remaining bone-bound radioactivity.

"plateau" at approximately the value of the last reading, and focused the calculation on the early half of the data set. To correct for this bias, one can manually set the "plateau" value to zero in the "constraint" tab. An alternative was to do a semilog transformation on the *Y*-axis to make the graph linear, and then do a linear fit. The half-life can be calculated from the slope of the graph. Both these methods gave similar results.[15]

In our original report[2] the results from the two sets of tibiae and femora of each rat were averaged to calculate the [DPM/mg bone sample] value, and then multiplied by 8% of the respective body weight to get the total bone-bound radioactivity. The results from three rats were again averaged before the plotting and calculations. In the retro-analyses, the femora data were separated from the tibiae data so that the spatial distribution could be evaluated, and only the femora data were used when comparing with the ^{14}C-experiment data.

Pharmacokinetic Profile of Conjugate C1. The results from ^{14}C and retro-analyzed ^{3}H experiment results from femora shafts are summarized in Table 1 and Figure 4. Although a Student *t*-test indicated there was a statistically significant difference in the initial bone uptake after 6 h (7.8 ± 0.4% for **C1b** versus 10.3 ± 0.5% for **C1a**), the difference was small and could be attributed to other factors, such as the differences in experimental animals and sample analyses.[16] It is equally possible that a small amount of ester hydrolysis occurred (pathway A), with partial loss of the ^{3}H label, prior to binding to bone. Irrespectively, a more pronounced difference was observed for the release half time of the conjugate **C1a** versus **C1b**. The $t_{1/2}$ value calculated from the percentage radioactivity remaining was 5.2 days from ^{3}H experiment, and 19.7 days from the ^{14}C experiment.

It is clear that the ^{14}C-label was eliminated at a much slower rate than the ^{3}H-label, suggesting that the majority of initial hydrolysis occurs at the ester linkage (Path A in Figure 2). Thus, the EP4-portion of the conjugate was released first (with concomitant loss of ^{3}H-labeling), while the bone-bound alendronic acid portion remained connected with the 4-hydroxyphenylacetic acid linker via the carbamate group bearing the ^{14}C-label. Subsequently, the carbamate is hydrolyzed causing the slower loss of ^{14}C-activity from bones.

To evaluate the chemical stability of **C1**, a solution of unlabeled conjugate **C1** in pH 7.4 PBS buffer was incubated at 37 °C, and analysis showed ~3%/day hydrolysis ($t_{1/2}$ ~22 days in a one-phase decay model). Notably, the initial hydrolysis observed was at the *carbamate linkage* and not at the linking ester bond judged by LC-MS analyses and by comparison with authentic samples. Thus, in vivo, one can postulate that, after binding to bone, significant enzymatic hydrolysis takes place first at the ester group of the 4-hydroxyphenylacetic acid linker, while cleavage of the carbamate group follows and may mostly be hydrolyzed uncatalyzed by its intrinsic reactivity with water at physiological pH.

Spatial Distribution of the Conjugate. Significant variations of radioactivity were observed between different bones (tibiae vs femora) and different sections of bones (shafts

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Table 2. Spatial Distribution of Radioactivity

	^{3}H experiments (with **C1b**)				^{14}C experiments (with **C1a**)			
rat	time	tibiae shaft DPM/mg/ μCi dosed	femora shaft DPM/mg/ μCi dosed	ratio[a]	time	femora shaft DPM/mg/ μCi dosed	femora joint DPM/mg/ μCi dosed	ratio (large joint/shaft)
1	6 h	5.89	9.57	1.6	6 h	8.24	20.32	2.5
2		5.99	8.16	1.4		10.09	24.37	2.4
3		5.05	8.69	1.7		9.15	24.61	2.7
4	2 days	4.43	6.72	1.5	7 days	6.56	17.02	2.6
5		4.32	6.74	1.6		6.81	17.55	2.6
6		1.64	6.88	4.2		6.16	15.32	2.5
7	7 days	1.77	2.86	1.6	14 days	5.03	10.96	2.2
8		1.50	3.13	2.1		3.49	9.70	2.8
9		1.72	2.99	1.7		5.81	11.45	2.0
10	14 days	1.21	0.80	0.7	28 days	2.71	7.14	2.6
11		0.68	1.68	2.5		3.58	7.35	2.1
12		1.19	2.10	1.8		3.27	8.28	2.5
			average	**1.9**			**average**	**2.4**

[a]Ratio of [DPM/mg bone/μCi dosed] between femora and tibiae.

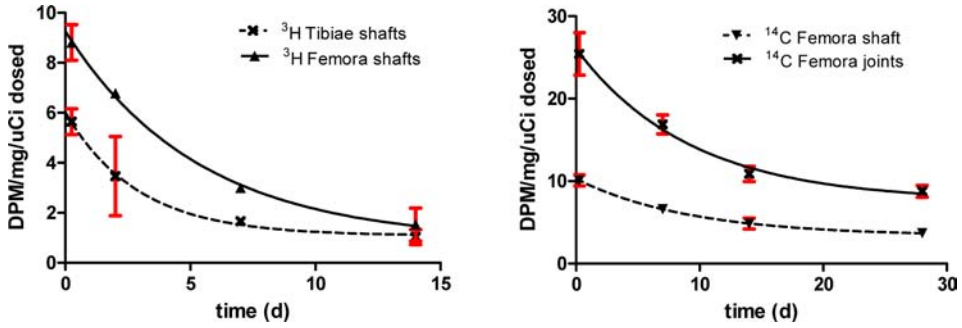

Figure 5. Spatial distribution and release of radioactivity from bones after dosing of conjugates **C1b** (left panel) and **C1a** (right panel).

vs large joints in femora). The results were summarized in Table 2 and Figure 5.

In the initial ^{3}H experiment the shaft portion of both tibiae and femora were analyzed, and the femora shafts were found to have about twice the radioactivity of the tibiae shafts per unit mass. In the ^{14}C experiments the shafts and large joints portion of the femora were analyzed, and the unit-mass radioactivity of large joints was 2−3-fold higher than that of the shafts. The difference in morphology was likely responsible. The large joints are more porous and contain trabecular bone, with much more surface area, more capillaries, and more blood flow than in bone shafts. Similar differences exist between tibiae and femora shafts. Since the alendronic acid portion of conjugate **C1** was expected to bind to the exposed bone surface, it was understandable that more of it would bind to the large joints than to the shafts, and more to femora than to tibiae.

Circulating Drug Equivalents in Blood. Circulating drug levels were measured and are expressed as percentages of the 0.5 h level so that the ^{3}H and ^{14}C results can be compared (Table 3 and Figure 6). Although there are visual differences in the rate of elimination, the *P* value was only 0.06, which rendered the two rates statistically indistinguishable. The quality of fit in the semilog plot was also low, especially for the 0.5 h data points. To account for the apparently higher terminal blood concentration in ^{3}H experiments, we postulate that there was probably some hydrolysis of the conjugates while circulating in the blood, especially when the conjugate passes through the liver, prior to binding to bone. The liberated EP4 moiety (which would bear the ^{3}H labeling) has a moderate

Table 3. Circulating Drug in Blood

time (h)	rat	^{3}H experiment (normalized[a])	^{14}C experiment (normalized[a])
0.5	1	100	100
	2	100	100
	3	100	100
1	1	62.2	43.4
	2	50.8	43.3
	3	61.4	40.8
2	1	32.8	15.2
	2	21.6	19.5
	3	34.1	14.1
4	1	-	8.1
	2	-	10.8
	3	-	8.8
5	1	26.1	-
	2	25.1	-
	3	27.8	-
6	1	-	6.2
	2	-	11.1
	3	-	5.9

[a]Data are presented as fractions of the 0.5 h levels.

cLogP[17] (cLogP = 3.75 as the ethyl ester, 2.48 as the fully hydrolyzed acid). It could be expected to circulate in the bloodstream with a respectable half-life. On the other hand, the alendronic acid portion (which would bear the ^{14}C labeling) has very low cLogP (−2.23 with the linker portion attached as carbamate, −4.1 as the carbamic acid) and would be expected

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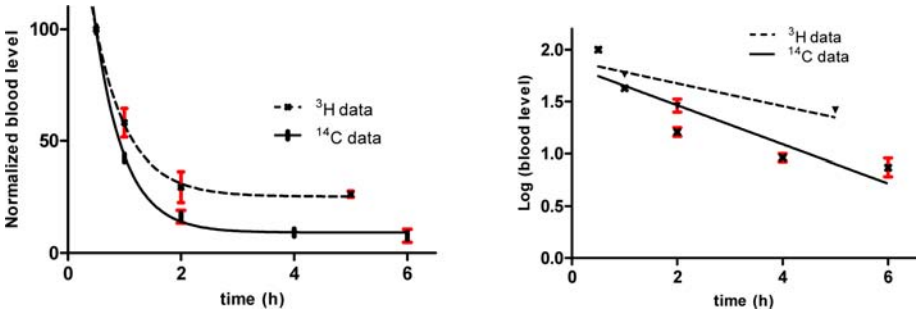

Figure 6. Circulating drug level in blood (same data set in linear plot and semilog plot).

to be rapidly excreted into the bile and/or bladder. Due to the possible complications, it was not appropriate to fit the data into a simple one-phase decay model, or to estimate the half-life from the model. Nonetheless we can conclude qualitatively that the conjugate was eliminated from the circulating blood relatively quickly, with 1−2 h nominal half-life.

CONCLUSIONS

Double labeling of the unique dual pro-drug conjugate **C1** with tritium in the EP4 agonist moiety and ^{14}C in the linker attaching the alendronic acid moiety has allowed us to evaluate the systemic stability and elimination profile of the conjugate, to determine the amount of bone uptake and integrity of the bone-bound conjugate, and to quantify the differential release of the active components, the bone growth stimulating EP4 agonist and the bone resorption inhibitor alendronic acid. As expected, the conjugate was found to be stable enough in rat plasma to allow significant bone uptake in its intact form (overall 7.8% of the original ^{3}H dose and 10.3% of the ^{14}C dose). The difference was not large but could reflect some partial hydrolysis in the blood before **C1** reached the bone. The degree of uptake into bones correlates with the bone surface area, with most being found in the trabecular bone. The conjugate that was not taken up in bone was rapidly cleared from the systemic circulation largely via the liver, again as anticipated. Once bound to bone, the conjugate **C1** liberated the EP4 agonist moiety, as evidenced by loss of tritium from the bones, with a half time for release of 5.2 days, compatible with once per week dosing, which has shown efficacy in rat models of osteoporosis.[4] Given the relative 7.8% uptake of tritium representing the EP4 agonist component and the half time for release of 5.2 days, one can calculate that ca. 45 nM (or 18 μg/kg) of the agonist would be released, on average, per day after a dose of 5 mg/kg **C1**. Similarly, based on 10.2% uptake and 19.2 day half time for release one can calculate that ca. 15 nM (or 3.8 μg/kg) of free alendronate would be released (attached to bone), on average, per day after a dose of 5 mg/kg **C1**. This is somewhat more than the exposure that can be calculated for the clinical dose of alendronate (10 mg/day) given its very low oral bioavailability of 0.7% and systemic bone uptake of about 50% in humans[18] that would equate to about 0.7 μg/kg/day taken up in bone.

Thus, **C1** targets bone and releases the active components in a balanced manner and at a rate that should allow simultaneous anabolic stimulation of osteoblast mediated bone growth and inhibition of osteoclast mediated bone resorption. The data from this pharmacokinetic study will be useful to inform the interpretation of the results from longer-term efficacy studies carried out with **C1** in the rat OVX model that are now underway.

EXPERIMENTAL PROCEDURES

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance II 600 MHz spectrometer using a TCI cryoprobe, an Avance III 500 MHz spectrometer using a TXI inverse probe, or an Avance III 400 MHz spectrometer using a BBOF + ATM probe. NMR data processing was performed with *MestRecNova* software (MestreLab Research, v 6.0.4−5850). The spectra were referenced to the corresponding solvent signals.[19] LC-MS were recorded with an ESI ion source on an Agilent 6200 Time-of-Flight spectrometer coupled with Agilent 1200 series front-end. Analytical thin-layer chromatography (TLC) was performed on aluminum plates precoated with silica gel 60F-254 as the adsorbent (EMD). The developed plates were air-dried, exposed to UV light and/or dipped in $KMnO_4$ solution[20] and heated. Flash chromatography was performed on a BioTage Isolera instrument using HP-silica cartridges from BioTage or SiliCycle Inc. Derivatized silica was obtained from SiliCycle Inc. Tetrahydrofuran (THF) was distilled from Na and benzophenone under nitrogen. Dichloromethane (DCM) was distilled from CaH_2 under nitrogen. Pyridine, triethylamine (TEA), and diisopropylethylamine (DIPEA) were distilled from CaH_2 under nitrogen. Other reagents and solvents were obtained from commercial vendors and used as received.

Intermediates **4**, **5**, and conjugate **C1** have been synthesized previously using alternative routes and conditions.[2] The NMR spectra of **4**, **5**, and **C1** were compared with those from the original route and were found to be substantially identical.

2-(4-*tert*-Butyldimethylsiloxyphenyl)acetic acid (3). 2-(4-Hydroxyphenyl)acetic acid (**2**, 3.06 g, 20.1 mmol) was dissolved in anhydrous THF (40 mL) in a 100 mL round-bottom flask. Imidazole (3.43 g, 50.4 mmol, 2.5 equiv) was added and the mixture was stirred until all solids dissolved. The solution was cooled to 0 °C (ice/water bath) and TBDMS-Cl (6.36 g, 42.2 mmol) in THF (10 mL) was added dropwise. The cold bath was removed and the mixture was stirred at room temperature overnight (∼16 h). The mixture was diluted with hexanes to ∼100 mL and filtered through a Celite pad (3 mm bed on a 40 mL sintered glass funnel). The filter cake was washed with methyl *tert*-butyl ether (MTBE, 30 mL), and the filtrate was concentrated to dryness. The residue was purified by flash chromatography (50 g HP-Silica cartridge from BioTage, 2% to 10% EtOAc/hexanes linear gradient) to give the **bis-TBDMS-3**, 6.74 g (colorless oil, yield 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, J = 8.6 Hz, 2H), 6.78 (d, J = 8.5 Hz, 2H), 3.53 (s, 2H), 0.97 (d, J = 3.0 Hz, 9H), 0.84 (d, J = 3.1

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Hz, 9H), 0.22 (s, 6H), 0.17 (s, 6H) ppm. HRMS: calculated for $C_{20}H_{36}O_3Si_2$, 380.2203; observed m/z (ESI^+) 381.2276 ([M + H]$^+$), 778.4735 ([2M+NH_4]$^+$).

The above material was added to a suspension of K_2CO_3 (4.91 g, 35.5 mmol) in THF (20 mL) and MeOH (15 mL). The mixture was stirred at room temperature for 1.5 h, at which time TLC indicated completed reaction (mini-workup with EtOAc/1 M HCl before TLC; developing solution 1:1 hexanes/MTBE). The mixture was filtered through a pad of Celite (3 mm bed on a 60 mL sintered glass funnel) and rinsed down with 1:1 MTBE/MeOH (40 mL). The filtrate was concentrated, and the residue was partitioned between water (60 mL, adjusted to pH 2−3 with 1 M HCl) and MTBE (40 mL × 3). The combined organic was washed once with brine (20 mL, acidified with 1 mL 1 M HCl) and concentrated. The residue was purified by flash chromatography (10% to 100% MTBE/hexanes gradient on a 100 g BioTage HP-Silica cartridge) to give **3** as colorless thick oil, 4.40 g (yield 94%; solidified upon standing at room temperature). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13 (d, J = 8.5 Hz, 2H), 6.79 (d, J = 8.5 Hz, 2H), 3.57 (s, 2H), 0.98 (s, 9H), 0.19 (s, 6H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$) δ 178.05, 155.09, 130.49, 126.04, 120.30, 40.38, 25.81, 18.33, −4.28 ppm. HRMS: calculated for $C_{14}H_{22}O_3Si$, 266.1338; observed m/z (ESI^-) 265.1227 ([M-H]$^-$), 569.2065 ([2M+K−H]$^-$).

Ethyl 7-((*R*)-2-(4,4-Difluoro-3-(2-(4-hydroxyphenyl)-acetoxy)-4-phenylbut-1-en-1-yl)-5-oxopyrrolidin-1-yl)-heptanoate (4). Alcohol **1** (432 mg, 1.02 mmol), acid **3** (430 mg, 1.61 mmol), DMAP (a few crystals, ∼2 mg, ∼2 mol %), and pyridine (190 μL, 2.4 mmol) were dissolved in dry DCM (5 mL). DCC (324 mg, 1.57 mmol) was added to the above solution as solid. TLC after 10 min indicated completed conversion (developing solvent 1:1 EtOAc/hexane). The reaction mixture was filtered, and the filter cake was washed with MTBE (5 mL). The filtrate was concentrated to dryness and the residue was dissolved in MTBE (20 mL) and washed with [1:1 0.5 M citric acid/water] (20 mL). The aqueous was extracted once more with MTBE (10 mL) and the combined organic was concentrated to dryness. The residue was purified by flash chromatography (50 g BioTage HP-Silica cartridge, 25% to 100% EtOAc/hexanes gradient) to give **(TBDMS)-ester 4** as viscous oil, 650 mg (yield 95%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48−7.41 (m, 1H), 7.41−7.33 (m, 4H), 7.00 (d, J = 8.4 Hz, 2H), 6.75 (d, J = 8.5 Hz, 2H), 5.75−5.65 (m, 1H), 5.61 (dd, J = 15.4, 6.7 Hz, 1H), 5.50 (dd, J = 15.4, 8.4 Hz, 1H), 4.11 (q, J = 7.1 Hz, 2H), 3.99 (td, J = 8.0, 5.3 Hz, 1H), 3.65 (dd, J = 11.0, 8.8 Hz, 1H), 3.52 (d, J = 2.8 Hz, 2H), 3.47−3.38 (m, 1H), 2.61 (ddd, J = 13.6, 8.6, 5.2 Hz, 1H), 2.36−2.30 (m, 2H), 2.27 (t, J = 7.6 Hz, 2H), 2.14 (dt, J = 16.1, 7.8 Hz, 1H), 1.94 (d, J = 9.9 Hz, 1H), 1.69 (d, J = 13.4 Hz, 1H), 1.60 (dt, J = 15.5, 7.6 Hz, 4H), 1.48−1.15 (m, 10H), 1.10 (t, J = 12.1 Hz, 1H), 0.98 (s, 9H), 0.19 (s, 6H). HRMS: calc'd for $C_{37}H_{51}F_2NO_6Si$, 671.3454; observed m/z (ESI^+) 672.3488 ([M + H]$^+$).

The above material (650 mg, 0.97 mmol) was dissolved in THF (5 mL) and tetrabutylammonium fluoride (TBAF, 1 M in THF, 1.1 mL, 1.1 mmol) was added and the mixture was stirred at room temperature for 5 min. The reaction mixture was partitioned between water (20 mL) and MTBE (20 mL), and the aqueous was extracted once more with MTBE (10 mL). The combined organic was concentrated to dryness, and the residue was purified by flash chromatography (25g BioTage HP-Silica cartridge, 30% to 100% EtOAc/Hexanes gradient) to give a brown oil, which slowly solidified upon standing at room temperature to give phenol **4**, 504 mg (yield 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53−7.40 (m, 5H), 7.06 (d, J = 8.5 Hz, 2H), 6.78 (d, J = 8.5 Hz, 2H), 6.70 (s, 1H), 5.76−5.65 (m, 1H), 5.58 (dd, J = 15.5, 5.1 Hz, 1H), 4.92 (ddd, J = 15.4, 8.9, 1.1 Hz, 1H), 4.15 (q, J = 7.1 Hz, 2H), 3.93 (td, J = 8.4, 4.0 Hz, 1H), 3.53 (d, J = 1.2 Hz, 2H), 3.50−3.37 (m, 1H), 2.41 (ddd, J = 13.6, 8.3, 5.1 Hz, 1H), 2.35−2.24 (m, 4H), 2.18−2.08 (m, 1H), 1.67−1.55 (m, 2H), 1.54−1.43 (m, 1H), 1.42−1.14 (m, 10H). HRMS: calc'd for $C_{31}H_{37}F_2NO_6$, 557.2589; observed m/z (ESI^-) 556.2579 ([M-H]$^-$), 1113.5195 ([2M-H]$^-$).

Ethyl 7-((*R*)-2-(4,4-Difluoro-3-(2-(4-(((4-nitrophenoxy)-carbonyl-14C)oxy)phenyl)acetoxy)-4-phenylbut-1-en-1-yl)-5-oxopyrrolidin-1-yl)heptanoate (5). Phenol **4** (127 mg, 228 μmol), pyridine (50 μL, 627 μmol, 2.75 equiv), and a few crystals of DMAP (∼1 mg, 8 μmol, 0.04 equiv) were dissolved in dry THF (1.5 mL) and cooled to 0 °C (ice−water bath). 4-Nitrophenyl chloroformate (70 mg, 347 μmol, 1.53 equiv) was dissolved in dry THF (0.3 mL), and added to the reaction mixture slowly. The reaction was allowed to proceed at room temperature overnight, and then quenched with 2 mL of 0.5 M citric acid. The layers were separated, and the aqueous was extracted with MTBE (2 mL). The combined organic was concentrated by rotary evaporation, and the residue was purified by flash chromatography (4 g SiliCycle HP-Silica cartridge, 0% to 5% MeOH in DCM as the eluent) to give PNP-carbonate **5** as a thick oil (158.7 mg, yield 96%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.36−8.28 (m, 2H), 7.54−7.47 (m, 2H), 7.46−7.41 (m, 1H), 7.41−7.32 (m, 4H), 7.24−7.19 (m, 4H), 5.75−5.67 (m, 1H), 5.63 (dd, J = 15.4, 6.8 Hz, 1H), 5.48 (dd, J = 15.4, 8.5 Hz, 1H), 4.11 (q, J = 7.1, 2H), 4.05−3.94 (m, 1H), 3.63 (d, J = 1.9 Hz, 2H), 3.43 (ddd, J = 13.8, 8.6, 7.3 Hz, 1H), 2.58 (ddd, J = 13.7, 8.6, 5.2 Hz, 1H), 2.39−2.21 (m, 4H), 2.20−2.08 (m, 1H), 2.04 (s, 2H), 1.67−1.52 (m, 1H), 1.45−1.14 (m, 9H). HRMS: calc'd for $C_{38}H_{40}F_2N_2O_{10}$, 722.2651; observed m/z (ESI^+) 723.2720 ([M + H]$^+$).

The same reaction was conducted by ViTrax Inc. as a customer synthesis using ^{14}C-labeled 4-nitrophenyl-chloroformate to provide ^{14}C-labeled carbonate **5** (40 mg, 56 μmol) in 74% yield; specific activity 17.8 mCi/mmol. LRMS (provided by ViTrax): calc'd for $C_{37}{}^{14}CH_{40}F_2N_2O_{10}$: 724.27; observed m/z (ESI^+) 725.06 ([M + H]$^+$).

Conjugate C1. A stock solution of alendronic acid triethylamine salt was prepared by mixing alendronic acid (0.5 g, 2 mmol), water (3 mL), and triethylamine (560 μL, 4 mmol). The mixture was stirred and sonicated until homogeneous. The solution was adjusted to pH ∼8.4 with a pH meter, and diluted with 5 mL DMF to give a final concentration of about 0.25 M. 180 μL of this solution (45 μmol) was placed in a 2 mL HPLC vial, ^{14}C-carbonate **5** (0.1 mg/μL in DMF, 75 μL, 10 μmol, specific activity 17.8 mCi/mmol, 178 μCi) was added and the solution was stirred at room temperature for 1.5 h. The reaction mixture was diluted with 0.1% formic acid (FA) in water (600 μL; final pH ∼ 3−4) and loaded onto a weak anion exchange column (WAX column, 150 mg diethylamino-derivatized silica packed in a 1 mL SPE cartridge, activated by passing 0.1 M HCl/MeOH, 4 column bed volumes (CV); then 0.1% FA in water, 4 CV × 2). It was sequentially eluted with 0.1% FA (1 mL × 2), 50% MeOH/0.1% FA (1 mL × 2), MeOH (1 mL), and MeOH/0.1 M HCl (1 mL × 2). An aliquot of each fraction was analyzed by scintillation counting, and the fractions containing most of the radioactivity were pooled and diluted with 3× water (∼12 mL

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total). The mixture was load in two portions to a 1000 mg C18 SPE (HoneyWell; activated with MeOH, 3 CV; water, 6 CV). The SPE was eluted with water (5 mL × 2), 20% MeOH (5 mL), 50% MeOH/[40 μL 1 M NaOAc buffer, pH 7] (5 mL), and MeOH (5 mL × 2). The fractions were monitored by counting radioactivity in the aliquots, and the fractions with most of the radioactivity were pooled and concentrated to dryness under a stream of nitrogen to give conjugate **C1** as a thin film of white solid, 88 μCi (radioactive yield ~50%).

The reaction was repeated in 100−200 μL scales (with respect to DMF stock solution of **5**) to convert all ^{14}C-carbonate **5** to conjugate **C1**. The pooled product was dissolved in water and purified again with a C18 SPE (sequential elution with water, then 50% MeOH/water, then 100% MeOH). Three of the most radioactive fractions were combined and concentrated to dryness to give conjugate **C1** as white solid, 26.3 mg (yield 54%), radioactivity 360 μCi (radioactive yield 36%), specific activity 16.7 mCi/mmol. Purity 99.1% (HPLC-UV).

NMR and HRMS characterization were done on non-radioactive material synthesized by the same methods: ^{1}H NMR (400 MHz, D_2O) δ 7.57−7.31 (m, 5H), 7.16 (d, J = 8.4 Hz, 2H), 6.99 (d, J = 8.4 Hz, 2H), 5.84−5.57 (m, 2H), 5.03 (dd, J = 14.2, 9.0 Hz, 1H), 4.17−3.94 (m, 3H), 3.73 (d, J = 15.0 Hz, 1H), 3.62 (d, J = 14.9 Hz, 1H), 3.15 (t, J = 6.6 Hz, 2H), 3.12−3.02 (m, 1H), 2.64−2.43 (m, 1H), 2.26 (q, J = 7.5 Hz, 4H), 2.16−1.99 (m, 1H), 1.99−1.64 (m, 4H), 1.59−0.92 (m, 12H). HRMS: calc'd for $C_{36}H_{48}F_2N_2O_{14}P_2$, 832.2549; observed m/z 833.2650 (ESI^+, $[M + H]^+$), 831.2495 (ESI^-, $[M\text{-}H]^-$).

Biology. All animal experiments were carried out in compliance with animal care guidelines and policies of the Canadian Council on Animal Care and under protocols approved by the Simon Fraser University Animal Care Committee.

Female Sprague−Dawley rats (Charles River) were about 13 weeks old when received. They were acclimatized to the facility and operators, and were randomly divided to four groups of three. The dosing solutions were prepared by mixing labeled and unlabeled Conjugate **C1** in PBS (phosphate-buffered saline, pH 7.2). In ^{3}H-labeled experiments, the final dosing level was 10 mg/kg, and 14.7 μCi/rat (specific activity about 5.2 mCi/mmol). The injection volume was 1 mL/kg body weight (ca. 200−300 μL). In ^{14}C-labeled experiments, the dosing was done at 5 mg/kg, and 5 μCi/rat for 7, 14, and 28 day experiments (specific activity about 3.6 mCi/mmol); and 5 mg/kg and 1 μCi/rat (specific activity 0.67 mCi/mmol) for the 6 h experiments. The dosing was done with a fixed volume of 500 μL. The bolus injection and blood sampling were done via the tail vein.

Measurement of Bone-Bound Radioactivity. At specific time points, three rats were euthanized by CO_2-suffocation, and long bones were harvested. Soft tissues from the bones were removed with a scalpel, and the bones were placed in a vacuum desiccator over Drierite for 1 day. The bones were then cut into sections of shafts and joints, and further dried until constant weight (Δ < 1 mg over a 2 h period).

Sections of the bones (200 ± 50 mg) were combusted in a Harvey OX-300 biological oxidizer using the 4 min program, and the collected scintillation mixture was counted on a Beckman Coulter LS-6500 scintillation counter. The counting time was 1 min.

Analysis of Blood Samples. Blood samples (0.5 mL per time point per rat) were taken from the 6 h group of rats at 0.5, 1, 2, 5 h (for ^{3}H-experiments), or 0.5, 1, 2, 4, 6 h (for ^{14}C-experiments), and placed in heparinized microcentrifuge tubes.

Blood samples from the ^{3}H experiments (200 μL) were centrifuged at ~5000 × g until plasma had separated. 100 μL of the plasma was diluted with acetonitrile (ACN, 100 μL) and centrifuged again. 50 μL of the supernatant was added to 15 mL of scintillation mixture (Amersham Biosciences, cat. no. NBCS104) and counted on a Beckman Coulter LS-6500 scintillation counter. The counting time was 1 min.

Blood samples from the ^{14}C experiments (0.5 mL) were frozen in liquid nitrogen and the solid was divided into two portions of roughly equal sizes. Each portion was placed on a piece of quantitative filter paper (~30 mg) and placed in the porcelain ladle. Samples were allowed to warm up to room temperature before being analyzed in a Harvey OX-300 biological oxidizer using the 4 min program. The collected scintillation mixture was counted on a Beckman Coulter LS-6500 scintillation counter. The counting time was 1 min. The readings from the two halves of the sample were corrected for the blank, and summed to give the original radioactivity.

■ ASSOCIATED CONTENT

Supporting Information

NMR and HRMS spectra for key intermediates and C1 conjugate. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00160.

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Author Contributions

G.C. conducted the ^{14}C-labeled experiments and the retro-analysis of the ^{3}H results. S.A. conducted the original ^{3}H-labeled experiments. R.Y. provided guidance in both experiments.

Notes

The authors declare no competing financial interest.

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(8) Although Figure 2 depicts the hydrolysis of the ethyl ester as a second step in both pathways A and B (for simplicity), the experiments in this study would offer no evidence for this sequence of events. However, earlier studies showed that the EP4 agonist ethyl ester (**1**) is hydrolyzed rapidly in rat plasma (within 30 min)[2] and is likely that the ethyl ester hydrolysis would occur prior to conjugate cleavage in either case.
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(13) In the prior ^{3}H-experiment, release of the EP4 agonist moiety showed $t_{1/2}$ of 5.2 days.[2] We anticipated a longer half-time for cleavage of the carbamate moiety in the subsequent ^{14}C experiment and therefore the 2-day time point was replaced with a 28-day time point to have a more even spacing and better capture the pharmacokinetic profile. The 5 mg/kg dose was used for the ^{13}C experiment to better reflect the effective therapeutic dose[6] and this small difference would not be expected to have a significant impact on bone uptake.
(14) http://www.physics.csbsju.edu/stats/t-test_bulk_form.html.
(15) The forced ″plateau = 0″ method gave $t_{1/2}$ of 4.8 and 24.0 days for ^{3}H and ^{14}C, respectively. The semilog plot method gave $t_{1/2}$ of 5.2 and 19.7 days, respectively.
(16) The original report (ref 2) gave 6 h bone uptake as 5.9% instead of 7.8%. This was calculated from the average of tibiae and femora data. The current retro-analysis only used the femora data.
(17) Calculated with *ChemBioDraw Ultra* (Perkin Elmer) v 13.0.2.3021.
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