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Skeletal accumulation of fluorescently-tagged zoledronate is higher in animals with early stage chronic kidney disease

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MINI-ABSTRACT

This work examines the skeletal accumulation of fluorescently-tagged zoledronate in an animal model of chronic kidney disease. The results show higher accumulation 24-hours post-dose in animals with lower kidney function due to greater amounts of binding at individual surfaces.

ABSTRACT

Purpose/Introduction Chronic kidney disease (CKD) patients suffer from increased rates of skeletal related mortality from changes driven by biochemical abnormalities. Bisphosphonates are commonly used in reducing fracture risk in a variety of diseases yet, their use is not recommended in advanced stages of CKD. This study aimed to characterize the accumulation of a single dose of fluorescently-tagged zoledronate (FAM-ZOL) in the setting of reduced kidney function. **Methods** At 25 weeks of age, FAM-ZOL was administered to normal and CKD rats. 24-hours later, multiple bones were collected and assessed using bulk fluorescence imaging, two-photon imaging, and dynamic histomorphometry. **Results** CKD animals had significantly higher levels of FAM-ZOL accumulation in the proximal tibia, radius and ulna, but not in lumbar vertebral body or mandible, based on multiple measurement modalities. Although the majority of trabecular bone surfaces were covered with FAM-ZOL in both normal and CKD animals, the latter had significantly higher levels of fluorescence per unit bone surface in the proximal tibia. **Conclusions** These results provide a new data regarding how reduced kidney function affects drug accumulation in rat bone.

INTRODUCTION

One in seven individuals within the US is affected by Chronic Kidney Disease (CKD), 60% of these showing measurable reductions in kidney function [1]. CKD-mineral bone disorder (CKD-MBD) is a systemic disorder of altered bone and mineral metabolism that ultimately compromises skeletal integrity and increases fracture risk [2-5]. More striking is that rates of mortality associated with fractures are considerably elevated in CKD individuals compared to other aged-matched patients with fracture [6]. Developing an approach to reduce fracture risk in this population could significantly alter the landscape of health for those with CKD.

Despite a basic understanding of the drivers of bone changes in CKD, treatment for the skeletal manifestations are limited. The most common class of anti-remodeling agent, bisphosphonates, are an effective method to increase bone mass and reduce fracture risk in a multitude of metabolic bone diseases including osteoporosis [7, 8]. The current clinical recommendations by the Kidney Disease Improving Global Outcomes (KDIGO) describe bisphosphonates as an acceptable treatment method in early stage kidney disease (Stage 1-3); but discourage use in CKD patients with biochemical evidence of CKD-MBD such as secondary hyperparathyroidism typically correlated to later stage CKD (Stage 3-5) [9, 10]. The concern is that some of the bisphosphonates (zoledronate and pamidronate) have been shown to cause nephrotoxicity in some instances (e.g., high peak concentrations, rapid infusion rate) [11, 12]. Altered kidney function has also been documented in select animal studies [13, 14]. In addition to documented nephrotoxicity, there is a theoretical concern that the drug will accumulate in the skeleton to levels above those that occur in individuals with normal kidney function [15-18]. This latter concern exists because of the absence of data describing skeletal accumulation of bisphosphonate in the setting of altered kidney function [19]. Therefore, the goal of this study was to utilize an animal model of CKD to test the hypothesis that skeletal accumulation of a fluorescently-labelled bisphosphonate (zoledronate) derivative is altered in the setting of reduced kidney function.

METHODS

Animals

The Cy/+ Han:SPRD male rat (hereafter referred to as CKD) provides a well-characterized animal model that shows evidence of CKD-MBD by 20-25 weeks of age (~ 50% of normal kidney function and significant elevations in parathyroid hormone) and all clinical CKD-MBD manifestations by 35 weeks of age (~ 10-15% normal kidney function) [20-25]. All procedures conducted in this study were reviewed and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Experimental Design

At 10 weeks of age animals from an in-house breeding colony were identified as normal (NL; n=6) or CKD (n=12) based on blood urea nitrogen (BUN) measures (> 30mg/dL at 10 weeks designated as CKD). Starting at 24-weeks of age animals were fed a casein-based diet (Harlan Teklad TD.04539) which has been shown to produce a more homogeneous development model of CKD by raising phosphorus availability [25]. At 25-weeks of age, normal animals (NL, n=6) and the one half of CKD animals (n=6) were administered a single subcutaneous injection of zoledronic acid conjugated with 5-carboxyfluorescein (FAM-ZOL; 180 µg/kg). This FAM-ZOL compound has previously been administered to rats and shown to allow assessment of bone surface-binding [26]. Unlike other fluorescent bisphosphonates [27], FAM-ZOL at this dose has also been shown to have *in vivo* biological efficacy as determined by measures of BV/TV in an ovariectomy model [26]. An additional group of CKD animals (n=6) was not administered FAM-ZOL and served as a negative control for fluorescence imaging.

All animals were administered calcein via subcutaneous injection (30mg/kg) thirteen and six days before the end of the study, allowing assessment of dynamic bone formation. Calcein blue (Sigma, #M1255), rather than a more traditional calcein green, was chosen to eliminate

spectral overlap with FAM-ZOL [28]. Calcein was mixed to a solution of 0.9% NaCl and 2% NaHCO3 at a concentration of 3%; once mixed the solution was buffered to a pH of 7.4 and then filtered through a 0.2mm sterile filter. Approximately 24 hours post-FAM-ZOL dosing animals were anaesthetized with isoflurane and underwent a brief non-survival surgery within the chest cavity (unrelated to the results presented here) before euthanasia by exsanguination. Long bones were harvested from the right limbs (radius, ulna, tibia), while the right hemi-mandible and the L3 vertebra were also collected. All samples were fixed in NBF (for 24 hours) and then switched to 70% EtOH and stored at room temperature with the exception of L3 which was stored in 70% EtOH the entire time. Bones were stored in amber vials to minimize fluorescence bleaching prior to analysis.

Biochemistries

Blood (~500 µL) was collected from the tail vein prior to euthanasia using capillary tubes. Blood was spun down to collect plasma (about 200 µL) for analysis of BUN (BioAssay systems #DIUR-100), calcium (Pointe Scientific, #C7503-480), and phosphorus (Pointe Scientific, #P7516-500) using colorimetric assays.

Bulk fluorescence imaging

Whole bone fluorescence assessment was conducted on proximal tibia, mandible, radius, ulna, and L3 vertebrae using reflectance epi-fluorescence imaging (IVIS Spectral CT, PerkinElmer). Epi-fluorescence imaging of fluorescently-tagged bisphosphonates has been used previously as an assay of skeletal uptake [26, 29, 30]. The mandible, radius and ulna were all scanned whole. L3 vertebra had the all processes removed leaving just the vertebral body. Proximal tibiae were cut to standard lengths of 10 mm measured from the proximal end of the tibia using a high-speed rotary tool with a diamond-tipped blade (Dremel). Individual scans included a bone from each animal (e.g. ulna from all 18 animals) were run on a single plate to assure uniform scan settings.

Due to the larger size of the mandibles, they were scanned with a (24.4 x 24.4 cm) field of view. All other bone sites were scanned using a 13.8 x 13.8 cm field of view. Due to these differences in field size comparison of mandible data to other sites was not undertaken.

Exposure time per sample plate (comprising of bones from all animals at a given skeletal site) was ~ 1 second using broad emission spectra to enable spectral unmixing through an excitation and emission series combination ranging from 430 – 465 and 500 – 540 respectively. The image series were spectrally unmixed to distinguish the pure FAM-ZOL signal from auto fluorescence and calcein blue signals using standard manufacturer software (Living Image). The imaging system applies two types of corrections for fluorescent imaging: 1) a flat field correction which eliminates pixel-to-pixel sensitivity variations of the detector and distortions in the optical path; and 2) outcome variable is computed as radiant efficiency which eliminates spatial variations in the excitation source across the image by computing the ratio of the emission image to the calibrated excitation image. Calibration of the excitation source is performed daily by the instrument. An average radiant efficiency value ([photons/second]/[μW/cm^2]) was quantified and is reported for each bone.

Histology

L3, proximal tibia, and tibia diaphyses were infiltrated and embedded in methyl methacrylate as previously described in our laboratory [31]. L3 and proximal tibia were sectioned in the parasagittal plane with a rotary microtome for assessment of trabecular bone; tibia diaphyses were cut in cross-section using a wire saw for assessment of cortical bone. A non-fluorescent mounting medium (Eukitt; Kindler, Freiburg, Germany) was used to cover slip all slides.

Epi-fluorescence microscopy

Sections were imaged on a microscope (Leica DM2700) with an arc lamplight source and quantified using semiautomatic analysis software (Bioquant). Trabecular bone surfaces covered by FAM-ZOL in a region of interest (ROI) of approximately 8 mm^2, located ~0.5 mm distal to the

proximal tibia or cranial L3 growth plates, were measured and normalized to total bone surface. The ROI was drawn to exclude the area directly adjacent to the cortical shell (~0.5mm away from each cortex). Two separate slides were analyzed and the results combined to get percent FAM-ZOL labelled surface value for each animal.

Two-photon microscopy

The fluorescent intensity of FAM-ZOL at individual bone surfaces was assessed by 2-photon microscopy using a Leica TCS SP8 system (Leica Microsystems, Germany) equipped with a mode-locked Ti:Sapphire laser (MaiTai HP DS, Newport Spectra-Physics) tuned to 740nm. Images (12bit) were collected using three NDD detectors (PMT/blue channel- BP 460/50, HyD1/red channel – BP585/40 and HyD2/green channel – BP525/50) and HC PL APO 20x/0.75 IMM CORR CS2 Oil objective lens. Tile images were collected to cover defined ROI sizes of ~ 0.9 x 1.3 mm and ~1.3 x 1.75 mm for vertebrae and proximal tibia, respectively. The ROI locations were chosen in a similar fashion as in the epi-fluorescence measures described above. Images were stitched together using standard software (Fiji) and then the green channel (FAM-ZOL) maximum projection was used to create a composite of the highest fluorescence pixels within the z-plane for each image. The max projection images were analyzed with a customized MATLAB script (**Supplementary Figure 1**). A manually generated mask image defined bone surface and subsequently created an analysis region adjacent to the bone surface to assess pixel intensity. A greyscale value above 1600 (on 12-bit images with a greyscale range of 0 - 4095) was chosen to define FAM-ZOL label. This threshold was calculated by assessing greyscale values for samples absent of FAM-ZOL (CKD without FAM-ZOL) to differentiate bone auto fluorescence from FAM-ZOL. The average greyscale of labeled pixels was calculated as a measure of the concentration of FAM-ZOL within each pixel.

Calcein blue label within the image ROI collected by 2-photon microscopy was analyzed to determine dynamic histomorphometry measures using standard software (Bioquant). Mineral apposition rate (MAR), mineralizing surface (MS/BS,%) calculated by ((0.5*single label) + double

label)/total bone surface), and surface-based bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/year) calculations were made using standard methods [32, 33].

Statistics

All analyses were run using Prism GraphPad software. Data was compared using one-way ANOVA with Tukey post-hoc analysis when appropriate or with t-tests (when two groups were compared). *A priori* α-levels were set at 0.05 to determine statistical significance.

RESULTS

Endpoint serum biochemistries revealed BUN values in both CKD groups were significantly higher compared to NL animals with no difference between the two CKD groups (**Supplementary Table 1**). Neither serum calcium nor serum phosphorus were significantly different among groups (**Supplementary Table 1**).

There was a statistically significant overall difference in bulk tissue radiant efficiency (fluorescence intensity) between CKD and NL groups for radius, ulna, and proximal tibia (**Table 1, Figure 1**) while there was no significant difference in the L3 or mandible (**Table 1, Figure 1**). At all three skeletal sites having significant differences, CKD animals had higher radiant efficiency compared to NL.

Surface-based quantification using epi-fluorescence microscopy, revealed NL animals had approximately 85 - 95% of trabecular surfaces covered with FAM-ZOL in proximal tibia and vertebra, respectively (**Figure 2**). There was no significant difference among groups for trabecular surface covered by FAM-ZOL in the vertebra (**Figure 2**). CKD animals also had significantly higher surface coverage on the periosteal surface of the tibial diaphysis (**Table 2**).

Two-photon max projection mosaic scans were used to determine FAM-ZOL fluorescence intensity. Proximal tibia trabecular bone FAM-ZOL label intensity was significantly higher in CKD

animals compared with NL (p=0.043) (**Figure 3).** A similar trend was noted in the vertebra although differences did not reach statistical significance.

Dynamic histomorphometric measures in proximal tibia showed no significant difference between groups for MAR, MS/BS, or BFR (**Table 3**).

DISCUSSION

This study was designed to characterize how alterations in kidney function affect the distribution and accumulation of a single dose of fluorescently-tagged zoledronate (FAM-ZOL). Our results document that animals with CKD (having about 50% of normal kidney function) accumulate significantly higher amounts of FAM-ZOL in the proximal tibia, radius, and ulna, but not L3 or the mandible, as assessed 24 hours after a single dose. In both normal and CKD animals, the majority of trabecular bone surfaces were covered with FAM-ZOL. The higher accumulation in CKD animals was driven by greater amounts of FAM-ZOL bound at a given surface, rather than having more surfaces bound with compound relative to normal.

In animals with normal kidney function, bisphosphonate bind with high affinity to hydroxyapatite. Most binding occurs within the first 6 hours [34]. Any drug not bound in the skeleton is excreted, unmetabolized by the kidney [2]. Based on this knowledge, we chose to examine the skeletal accumulation of FAM-ZOL 24-hours post dose. Our normal animals showed between 80 and 100% of trabecular surfaces in the tibia and vertebra were labelled with FAM-ZOL. This data is in contrast to some of the earliest work examining alendronate localization which showed that only a fraction of bone surfaces were labelled – with particular attention drawn to resorption surfaces [35]. This latter study is prominently cited in the literature to support the idea that bisphosphonate binding is preferential to active surfaces [36-38]. Recent work, although not quantifying amount of surface label, suggest robust surface labeling by fluorescent-risedronate consistent more in line with the current work [39, 40]. There are a number of potential reasons for these discrepancies across studies. The difference in type of bisphosphonate (alendronate versus

zoledronate) and method of assessment could both explain differences from our current work, although another possible explanation is the age of the animals (earlier work used eight-day old animals while the current work used skeletally mature animals). Our results from cortical bone were less clear, with periosteal surfaces showing 50-90% coverage and endocortical surfaces showing 20-40%.

Given the majority of trabecular surfaces were covered with FAM-ZOL in the normal animals, we hypothesized that higher bulk levels of accumulation in animals with reduced kidney function were due to greater accumulation at given sites. Indeed, using 2-photon microscopy we measured average fluorescence on surfaces within a defined region of interest in the proximal tibia trabecular bone. This assay revealed significantly greater average fluorescence, suggestive of a greater amount of FAM-ZOL on the surface, in animals with CKD (Figure 3). Previous work with fluorescent-tagged risedronate and alendronate have analyzed accumulation depth [39, 40], where the compound penetrates beyond the exposed surface into the matrix via canaliculi forming a band of variable thickness at surfaces. Similar depth measures were not conducted in this work. Our results show that FAM-ZOL accumulation on a given surface, rather than accumulation over a greater length of surface, is the main difference in trabecular bone between normal and CKD conditions.

Bisphosphonates are highly effective at reducing fracture risk [7, 8]. Dosing instructions for zoledronic acid recommend reduced dosing in patients with creatinine clearance <60 ml/min, and ‘use not recommended’ in patients with creatinine clearance <30 ml/min. This is based on the idea that transient high circulating levels of drug have been linked to nephrotoxicity. Yet there also exists a theoretical concern that reduced drug clearance, due to reduced kidney function, could lead to higher accumulation in the skeleton beyond what would occur in someone with normal renal function. The focus of the current study is on the latter. Our data suggest similar bone surface coverage, but more accumulation at a given skeletal site in the setting of moderate kidney impairment. Although these data provide some insight into the kinetics of skeletal

accumulation in animals with compromised kidney function, there is no direct in vivo evidence that this higher accumulation has a physiological consequence. *In vitro* studies illustrate the potential for negative effects of bisphosphonate accumulation, with low concentrations of numerous bisphosphonates having beneficial effects on osteocyte viability while higher concentrations can induce cell death [41, 42]. There exists no evidence that similar accumulation-based effects exist for remodeling suppression. Future studies that not only quantify levels of accumulation but also effects on physiological outcomes (remodeling and/or bone mechanical properties) will be essential to paint a more complete picture.

CKD can manifest with wide range of bone turnover rates [4, 43] and whether high or low remodeling rates exist can affect various properties of the bone. The Cy/+ used in the current work has been shown to be a high-remodeling rate model [23], driven by the hyperparathyroidism [44] that begins to manifests at least by 20 weeks of age and progressively worsens [24, 25]. The current work, for the first time assessed bone remodeling rates in this model at 25 weeks of age, finding non-significant differences between CKD and normal animals (**Table 3**). The most likely reason for this finding is not that the effect is not manifesting, but rather that the sample size (which was powered for looking at FAM-ZOL) was insufficient for dynamic histomorphometry (increasing sample size to n=12/group would raise the power from its current 40% to 73%). Future work to determine if higher rates of remodeling (such as occur in advanced disease in this and other models) affect binding are warranted.

The execution of our study had some limitations. First, the analysis focused on an analog of only one bisphosphonate, zoledronate. With the longest half-life and known stable binding affinity to bone [45, 46], zoledronate seemed to be a logical one to test for distribution and accumulation studies. Nevertheless, each bisphosphate possesses different characteristics and varying properties meaning that the results found here cannot be generalize to other bisphosphonates. Subcutaneous (SC) administration of drug was used in the current study while intravenous (IV) administration is used in patients. Based on a small pilot study, we found SC and

IV dosing results in comparable levels of FAM-ZOL in normal animals **(Supplementary Figure 2)**. It is also important to note that the addition of a bulky fluorescent tag on zoledronate has the potential to affect its binding characteristics and localization and thus it is not possible to definitively know that it behaves similar to the native compound. Additionally, this study focuses on the distribution and accumulation of FAM-ZOL at a single time point, 24 hours post dose. Therefore, the results should not be extrapolated to shorter or longer time frames.

In conclusion we have shown that the binding at 24 hours of fluorescently-tagged zoledronate differ in the context of reduced kidney function. In both normal and CKD animals the fluorescently-tagged zoledronate covered the majority of bone surfaces, yet in the setting of early stage CKD there is greater accumulation at a given surface in some, but not all bones.

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Figure Legends

Figure 1: Fold difference in bulk fluorescence (average radiant efficiency) of CKD animals relative to normal animals across multiple bones assessed. * $p < 0.05$ versus normal animals based on t-test analysis of absolute values.

Figure 2. Surface based FAM-ZOL coverage of trabecular bone in the proximal tibia (A) and vertebra (B) from epi-fluorescence microscopy analysis. Graph data is presented as the mean and standard deviation along with individual data points. * $p < 0.05$.

Figure 3. Two-photon assessment of FAM-ZOL intensity at trabecular bone surfaces of the proximal tibia (A) and vertebra (B). Average pixel intensity of CKD animals was significantly higher than normal (NL) for the proximal tibia but not the vertebra. Graph data is presented as the mean and standard deviation of greyscale values (12-bit images with a greyscale range of 0 - 4095) along with individual data points. (C) shows representative proximal tibia images for NL and CKD. The left panel displays the original FAM-ZOL images captured on the two-photon microscope and then the right panel displayed the thresholded FAM-ZOL signal that was quantified. * $p < 0.05$.

Supplementary Figure 1. Analysis of two photon images. A) Raw image of green channel detector. B) MATLAB Input image with bone area as white and black for bone marrow. C) Analysis region with non-analyzed regions shown in grayscale. D) Pixels above FAM-ZOL threshold.

Supplementary Figure 2. Bulk fluorescence assessment of whole radius and tibia from normal animals administered FAM-ZOL by either intravenous (IV) or subcutaneous (SC) injection (n=4 per dose route). There was no significant difference in the mean group values between the two routes for either the radius or tibia sites ($p > 0.60$).

TABLE 1. Bulk fluorescence total radiant efficiency (fluorescence intensity, [photons/s]/[μW/cm^2]**)**

	NL – FAM-ZOL	**CKD – FAM-ZOL**	**CKD as a % of NL**	**T-test**
Proximal tibia	$7.23 \times 10^9 \pm 2.31 \times 10^9$	$10.94 \times 10^9 \pm 1.73 \times 10^9$	+ 51%	0.0102
Radius	$9.13 \times 10^9 \pm 5.32 \times 10^9$	$21.13 \times 10^9 \pm 6.67 \times 10^9$	+ 131%	0.0057
Ulna	$18.6 \times 10^9 \pm 7.95 \times 10^9$	$38.0 \times 10^9 \pm 13.4 \times 10^9$	+ 104%	0.0125
L3	$3.04 \times 10^9 \pm 1.46 \times 10^9$	$3.85 \times 10^9 \pm 1.74 \times 10^9$	-	0.4071
Mandible	$23.7 \times 10^9 \pm 29.1 \times 10^9$	$64.9 \times 10^9 \pm 45.2 \times 10^9$	-	0.0889

All data presented as mean and standard deviation. μW, micro watts.

TABLE 2. Tibia cortical bone FAM-ZOL surface label

	NL – FAM-ZOL	CKD – FAM-ZOL	T-test
Tibial periosteal surface FAM-ZOL coverage, %	55.39 ± 8.60	88.22 ± 11.76	0.0003
Tibial endocortical surface FAM-ZOL coverage, %	17.19 ± 12.45	38.93 ± 20.49	0.0505

All data presented as mean and standard deviation.

TABLE 3. Dynamic histomorphometry of proximal tibia trabecular bone

	NL – FAM-ZOL	CKD – FAM-ZOL	T-test
Mineral apposition rate, μm/day	1.55 ± 0.40	1.86 ± 0.19	0.133
Mineralizing surface/bone surface, %	18.83 ± 8.65	26.52 ± 4.09	0.077
Bone formation rate, $\mu m^3/\mu m^2$/year	113 ± 82	178 ± 33	0.099

All data presented as mean and standard deviation.

SUPPLEMENTAL TABLE 1. Serum biochemistries

	NL – FAM-ZOL	CKD	CKD – FAM-ZOL	ANOVA
BUN (mg/dL)	21.96 ± 2.61	41.01 ± 4.44 *	39.61 ± 4.38 *	< 0.001
Calcium (mg/dL)	7.77 ± 1.22	7.43 ± 1.22	8.44 ± 1.12	0.3493
Phosphorus (mg/dL)	5.87 ± 0.62	6.18 ± 0.64	6.19 ± 1.03	0.7300

All data presented as mean and standard deviation. * $p < 0.05$ versus NL-FAM-ZOL.

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