ORIGINAL ARTICLE

Mathematical Modeling of the Effects of CK2.3 on Mineralization in Osteoporotic Bone

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Osteoporosis is an idiopathic disease that affects over 10 million adults in the United States and osteoporotic fractures cost the healthcare system $17 billion each year.1 In million adults in the United States and osteoporotic frac-

ures cost the healthcare system $17 billion each year.1 In osteoporosis, bone mineral density (BMD) and new treatments for this disease are desperately needed. Bone morphogenetic protein 2 (BMP2) is crucial for bone formation. The mimetic peptide CK2.3 acts downstream of BMP2 and increases BMD when injected systemically into the tail vein of mice. However, the most effective dosage needed to induce BMD in humans is unknown. We developed a mathematical model for CK2.3-dependent bone mineralization. We used a physiologically based pharmacokinetic (PBPK) model to derive the CK2.3 concentration needed to increase BMD. Based on our results, the ideal dose of CK2.3 for a healthy individual to achieve the maximum increase of mineralization was about 409 µM injected in 500 µL volume, while dosage for osteoporosis patients was about 990 µM. This model showed that CK2.3 could increase the average area of bone mineralization in patients and in healthy adults.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
- While models for bone turnover and calcium homeostasis exist, no model describes the effect of CK2.3 on osteogenesis. This effect is mediated through the BMP2 signaling pathway.

WHAT QUESTION DID THIS STUDY ADDRESS?
- What is the concentration of CK2.3 needed to increase bone mineral density in osteoporosis patients?

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
- A PBPK model of CK2.3 distribution and binding was developed. In addition, a mathematical model of mineralization allowed the effects of CK2.3 on human bone mineralization to be examined.

HOW MIGHT THIS CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS?
- Modeling both healthy and extremely osteoporotic bone showed that CK2.3 could increase the average area of bone mineralization in both populations. It also estimated doses necessary and therefore aids in the translation of the use of this peptide from the bench to bedside.

Osteoporosis is an idiopathic disease that affects over 10 million adults in the United States and osteoporotic fractures cost the healthcare system $17 billion each year.1 In osteoporosis, the bone mineral density (BMD) is reduced, bone microarchitecture is deteriorated, and the production of proteins and hormones in the body are altered.2 Bone is constantly being remodeled in a dynamic process where osteoblasts are responsible for bone formation and osteoclasts for its resorption.3,4 Bone formation is a multistep process and depends on osteoblasts to deposit osteoid and this osteoid needs to mineralize to develop into new bone tissue. The formation of mineralized nodules in osteogenic cell culture provides a means of assessing mature osteoblast cell function.5 Furthermore, mineralization is one of the major parameters reflecting bone quality and in adult bone mineralization is the major determinant for the rate of bone turnover.6,7 New bone tissue formation can be increased by increasing the number of osteoblasts, increasing osteoblast activity, decreasing the number of osteoclasts, decreasing osteoclast activity, or a combination of any of these factors.

Each mesenchymal progenitor cell (MSC), can differentiate into various cell types (Figure 1a), including osteoblasts, chondrocytes, and adipocytes and is controlled by a variety of growth factors.3,4 Bone morphogenetic protein 2 (BMP2) is one of the major growth factors controlling stem cell fate.8-10 The localization of the BMP receptor type 1a (BMPR1a) on the plasma membrane can drive osteoblast differentiation.11-14 An important mediator of BMP2 signaling is casein kinase II (CK2). CK2 interacts with BMPR1a at the plasma membrane and is released upon BMP2 binding to BMPR1a to further initiating signaling pathways.15,16 CK2.3 a peptide blocking CK2 from interacting with BMPR1a was designed to activate BMP receptors in the absence of BMP2 (Figure 1b). CK2.3 induces osteogenesis and bone formation and increases BMD in vitro and in vivo.15-17 Osteoclasts are formed from hematopoietic stem cells.18 Upon hematopoietic stem cell differentiation into an osteoclast, the main function of the osteoclast is bone resorption. The amount of bone resorption is estimated to be dependent on the ratio of osteoclast to osteoblast concentration in the body.9

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Based on previous research in mice, CK2.3 seems to be a promising option for increasing mineralization and BMD, but its effects on human bone are unknown. Although physiologically based pharmacokinetic (PBPK) models exist on bone remodeling and bone turnover, they focus on calcium homeostasis. BMP2 acts upstream of calcium and regulates multiple other pathways in bone formation and remodeling. Through a PBPK model of CK2.3 distribution and binding to BMPR1a, and a mathematical model of mineralization based on the number of osteoblasts and osteoclasts, the effects of CK2.3 on BMD can be determined. Modeling both healthy and osteoporotic bone will provide further insight into CK2.3’s ability to treat osteoporosis.

METHODS

Experimental methods

Cell culture. C2C12 cells were grown and maintained as previously described.22

Design of peptides. Peptides were designed by our group as previously described.15

Assessing mineralization in vitro. C2C12 cells were grown to 90% confluence in 1.9 cm² 24-well plates. These cells were treated with CK2.3 (0 nM, 50 nM, 100 nM, 250 nM, or 500 nM) in 500 μL DMEM. Our previous data show that 40 nM BMP2 has a similar effect as 100 nM of CK2.3 on mineralization. After 5 days, Von Kossa staining and analysis was performed as described previously.17

Statistical data analysis

For the in vitro assay, single-factor analysis of variance (ANOVA), followed by the Tukey–Kramer post-hoc test was used to analyze all the data. All experiments were repeated three or more times and were normalized to control in each experiment as noted. Chauvenet’s criterion was used to remove the outliers.

Model development

The aim of this study was to develop a mathematical model for bone mineralization based on the concentrations of CK2.3 (Figure 2a) and BMPR1a signaling components (Figure 2b). A PBPK model of the distribution of CK2.3 was used to determine its local dynamic concentration and is used as an input to the bone mineralization model. The
A PBPK model was developed for the CK2.3 dependent activation of BMPR1a. CK2.3 binds to CK2, blocking CK2 from binding to the BMPR1a receptor. If CK2 does not bind to BMPR1a, the receptor can signal MSCs to differentiate into osteoblasts. CK2.3 distribution over time was used to determine the concentration of CK2 that would be available to bind BMPR1a. This was used to produce a value for the signal from the receptors, which was fed back to the mineralization model.

Stages of model development
The PBPK model. Since the primary purpose of CK2.3 is to bind to CK2, only the human organs containing an abundance of CK2 were included in the model. These organs include blood, kidney, liver, heart, and brain. A schematic of the connection between the organs represented in this model is shown in Supplemental Figure 1. Mass balance equations were written around each organ and the binding rate of CK2.3 to CK2 was estimated. The concentration of CK2.3 in the blood was then estimated over time.

Assumptions. Various assumptions were made to develop the equations and simplify the analysis.

- Organs connected with blood flows were modeled as continuous stirred tank compartments connected by pipes with no spatial variation in concentrations.
- CK2 concentration was assumed to have reached a steady state in each organ.
- CK2 is assumed to be only present as a complete protein. The concentration of CK2 in each organ was found using data from the MaxQb database and is displayed in Table 1.

Figure 2 (a) Mineralization areas for various dosages of CK2.3. (a) statistically significant difference from control. (b) statistically significant difference from 50 nM. (c) statistically significant difference from 75 nM. (d) statistically significant difference from 100 nM. (e) statistically significant difference from 250 nM. (f) statistically significant difference from 500 nM. (b) Mineralization areas for various BMPRIa signal quantities.
Table 1 Physiological values of CK2 and BMPR1a in each organ\textsuperscript{23,24}

| Organ of interest | Concentration of CK2 (pM) | Concentration of BMPR1a (pM) |
|------------------|---------------------------|-----------------------------|
| Blood            | 251.1553                  | Blood                       |
| Kidney           | 3.2648*10\textsuperscript{3} | Kidney                      |
| Liver            | 469.3966                  | Liver                       |
| Heart            | 1.9048*10\textsuperscript{3} | Heart                       |
| Brain            | 1.3651*10\textsuperscript{3} | Brain                       |
| Bone marrow      | 1.377*10\textsuperscript{3} | Bone marrow                 |

- The concentrations of the receptor BMPR1a were assumed to be constant, and were derived from the mRNA expressions of the receptor and obtained by the MOPED database and is displayed in Table 1.\textsuperscript{23}
- The binding ratio, k, was assumed to be 1 in every organ. This assumption was made because there are no current data on a partial binding, so it was assumed that if a CK2.3 molecule and a CK2 molecule were both present, they would bind.
- The drug was given intravenously, injected directly into the bloodstream using 500 \textmu L total volume.

Equations. A basic mass balance differential equation was used for each organ. Each equation has three terms describing a different cause for changing the concentration of the mimetic peptide. The equations are structured as shown in Eq. 1:

\[
\text{Time derivative of concentration} = \text{Flow in} - \text{Flow out} - \text{amount bound to CK (1)}
\]

The flow in and flow out terms are different for the blood than they are for the other organs. For the blood, these terms depend on the summation of the blood flow in and out of the other organs. Eq. 2 represents the flow in – flow out for the blood, while Eq. 3 represents the flow in – flow out for the other organs.

\[
\begin{align*}
\text{(Flow in – Flow out)}_{\text{blood}} &= \sum Q_c - \left( \sum Q \right)_{\text{blood}} \quad (2) \\
\text{(Flow in – Flow out)}_{i} &= Q_c - \left( c_{\text{blood}} - c_i \right) \quad (3)
\end{align*}
\]

where \(Q\) is molar flow rate, \(c\) is concentration of CK2.3, and \(i\) represents the organ compartment that is being described. Since the basic equation for each organ compartment, excepting blood, is the same, \(i\) can stand for any of these compartments.

The remaining term for each equation represents the amount of CK2.3 that binds to CK2. CK2.3 binding to CK2 correlates to depletion of the CK2.3 molecules. Each organ has a different amount of CK2, so CK2.3 degrades at a different rate depending on its location. As an initial estimate, the rate of binding is assumed to be \(k = 1\), but can later be changed to fit experimental data. Thus, the complete equations are shown in Eqs. 4 and 5:

\[
\frac{dc}{dt_{\text{blood}}} = \left( \sum Q_c - \left( \sum Q \right)_{\text{blood}} - k \ast \left( P_{\text{blood}} \ast c_{\text{blood}} \right) / V_{\text{blood}} \right) \quad (4)
\]

\[
\frac{dc}{dt_i} = (Q_c - c_i) - k \ast P_i \ast c_i / V_i \quad (5)
\]

where \(P\) is concentration of CK2 in the blood and \(V\) is volume.

Model for bone turnover

A mathematical model was developed to represent bone turnover in humans. The amount of mineralization within a bone was dependent on the number of osteoblasts and the number of osteoclasts. The number of osteoblasts was dependent on the BMPR1a signal and the number of osteoclasts was dependent on the number of osteoblasts.\textsuperscript{3} The BMPR1a signal was dependent on the concentration of CK2.3 in the blood as well as the concentration of BMPR1a and CK2 in the bone. A MatLab (MathWorks, Natick, MA) code was written in order to incorporate the values from the PBPK model and obtain ideal doses and dose ranges. Experimental data were collected by using C2C12 cells to relate CK2.3 dosage and bone mineralization (Figure 2a). C2C12 cells are frequently used as a model for BMP2-dependent mineralization and the results obtained seemed to correlate with the results obtained in MSCs.\textsuperscript{15,16,21,22,26} Moreover, C2C12 cells express BMPR1a at high levels, while other BMP receptors are only expressed at low levels. C2C12 cells also respond with upregulation of BMPR1a to stimulation with BMP2, suggesting BMPR1a is the receptor crucial for BMP2 stimulation.\textsuperscript{27} The data were used to determine the relationship between mineralization and the signal produced by BMPR1a receptors.

Stages of model development.

1. Develop an equation for the BMPR1a signal released in the C2C12 cells as a function of CK 2.3 present in the blood.
2. Develop simple model for change in area of bone mineralization.
3. From the signal released, determine formation of pre- and active osteoblasts.
4. Determine the number of osteoclasts from number of osteoblasts.
5. Predict depletion constant as a function of mineralization constant.
6. Model mineralization area produced by active osteoblasts.
7. Predict mineralization rate of osteoblasts.
8. Develop model for bone cycle in healthy and osteoporotic human bone.

Assumptions. Various assumptions were made to characterize the equations and simplify the analysis.

- The doses used for the model were obtained by multiplying the doses used in mouse research by a scalar of 1.936*10\textsuperscript{7}, derived from a mass fraction of mouse and human weights, in order to obtain the human equivalent.\textsuperscript{28}
- Studies have shown that only the BMPR1a located on the cell surface outside of caveolae and in clathrin-coated pits (CCPs) are affected by CK2.3. 30% of BMPR1a receptors are located on the...
surface of the cell, while around 15% are located in CCPs. Therefore, 30–45% of receptors are susceptible to the effects of CK2.3. Thus, 40% of the BMPR1a receptors in each organ of our model were available for binding and signaling.

- The MEK/ERK pathway is not involved in adipogenesis for CK2.3. Therefore, it was determined that the entire signal from this pathway goes towards osteogenesis.
- 100% of the signal was assumed to go to the formation of preosteoblasts in a 1:1 ratio, such that each unit of signal results in the formation of one preosteoblast.
- 50% of the signal from the BMPR1a receptors that are unavailable to be bound by CK2 goes toward osteogenesis. This number was derived from Moseychuk et al. CK2 interacts on BMPR1a at two sites and can induce osteogenesis as well as adipogenesis and depends on the site it is released from.
- 95% preosteoblast become active osteoblast.
- Based on the experimental and previous data, a cubic correlation between CK2.3 concentration and CK2.3 signal should exist. Extremely osteoporotic bone: 5:1. Healthy bone: 5:4. Working with healthy bone, it was assumed that as the signal increased, the proportion of osteoclasts to osteoblasts decreased because the signal is promoting osteoblast production. To ensure that the equation displayed the correct ratio, the denominator of the first term must equal 1 at steady state. Therefore, a value equal to 1 subtracted from 0.30, or 0.70 must be added to the signal.

**Modeling the signal.** From the concentration of CK2.3 in the blood, the BMPR1a signal that was released was calculated. For this calculation it was assumed that if no CK2 was present in the bone, 40% of the BMPR1a would release a signal. Eq. 6 represents the amount of CK2.3 signal released in the MSCs.

\[
\text{CK2.3 Signal} = 0.4 \times R_{\text{bone}} - (P_{\text{bone}} - C_{\text{blood}})
\]  

where R is BMPR1a concentration, P is CK2 concentration in the bone, and c is CK2.3 concentration in the blood. This equation represents the signal produced from the addition of CK2.3. Furthermore, the total osteoblast differentiation signal (S) is calculated utilizing the aforementioned CK2.3 signal and the steady state signal, as estimated from Moseychuk et al. This is shown in Eq. 7.

**Total Osteoblast Differentiation Signal** \(S = \text{CK2.3 Signal} + 0.5 \times (0.6 + R_{\text{bone}})\)  

To understand and determine the equations of mineralization, it is essential to understand osteoblast differentiation under normal conditions. This was achieved by solving for the total osteoblast differentiation signal in Eq. 7 when CK2.3 Signal is equal to zero (since CK2.3 is not naturally found in the body). This produced a value of 0.30 pM. The equation for the area of mineralization was determined in Eq. 9:

\[
A_M = S_{\text{OB}} \times r_M
\]  

\[
S_{\text{OB}} = \text{Signal Forming Active Osteoblasts (pM)}
\]

\[
r_M = \text{Mineralization Constant (pixels}^2 \text{pM}^{-1})
\]

100% of the signal was assumed to go to the formation of preosteoblasts in a 1:1 ratio and 95% of preosteoblasts then become active osteoblasts. Based on this literature and previously stated assumptions, the equation for active osteoblasts is shown below in Eq. 10:

\[
AOB = S \times PPO \times POA = 0.95 \times S + r_M
\]  

\[
S = \text{Signal from BMPR1a Receptors Activated by CK2.3 (pM)}
\]

\[
PPO = \text{Proportion of Signal Becoming Pre-Osteoblasts = 1}
\]

\[
POA = \text{Proportion of Pre-Osteoblasts Becoming Active Osteoblasts} = 0.95
\]

From Eq. 8, the following equation for the area of depletion was determined and is shown below in Eq. 11:

\[
A_D = S_{\text{DC}} \times r_D
\]  

\[
S_{\text{DC}} = \text{Signal Forming Active Osteoclasts}
\]

\[
r_D = \text{Depletion Constant (pixels}^2 \text{pM}^{-1})
\]

The number of active osteoclasts in healthy bone when the bone cycle is at steady state is 1.25 times the number of osteoblasts, for a ratio of 5:4. In severely osteoporotic bone this ratio becomes 5:1. Working with healthy bone, it was assumed that as the signal increased, the proportion of osteoclasts to osteoblasts decreased because the signal is promoting osteoblast production. To ensure that the equation displayed the correct ratio, the denominator of the first term must equal 1 at steady state. Therefore, a value equal to 1 subtracted from 0.30, or 0.70 must be added to the signal.

\[
AOC = \frac{1.25}{S + 0.7023} \times AOB = \frac{1.25}{S + 0.7023} \times 0.95 \times S
\]

Eq. 13 represents the area of depletion. This was derived from Eqs. 11 and 12:

\[
A_D = \left(\frac{1.25}{S + 0.7023} \times 0.95 \times S\right) \times r_D
\]

By substituting Eqs. 11 and 13 into Eq. 8, the overall equation became Eq. 14:

\[
\Delta A = 0.95 \times S \times r_M - \left(\frac{1.25}{S + 0.7023} \times 0.95 \times S\right) \times r_D
\]  

At steady state, the total area change is assumed to be zero and the signal produced is 0.30 pM. Solving for
These simulations allowed for examination of the effects of CK2.3 on mineralization. The files for both models can be found attached as Supplementary Data.

RESULTS

A dose–response curve for CK2.3 was performed in C2C12 cells (Figure 2a). For this, C2C12 cells were stimulated with CK2.3 and osteoblast activity was measured using the Von Kossa assay. C2C12 cells are frequently used to study BMP2 signaling and osteoblast differentiation and activity. C2C12 cells are a cell line that can be used to mimic BMP2-dependent mesenchymal progenitor cell differentiation.8,15,22,30–32 Interestingly, higher doses of the peptide decrease mineralization. This effect may be due to peptide toxicity. Alternatively, higher concentrations of the peptide may direct the C2C12 cells into a different lineage. Next, a model for mineralization and PBPK model for CK2 signaling was developed (Figures 2b, 3). Using this model for the input of the PBPK model, we found the effective dose range for CK2.3 by running the PBPK model with CK2.3 injection doses ranged between 0 and 2 mM in a 500 μl total injection volume. Running the PBPK model with multiple CK2.3 doses within that range further narrowed the effective range down. From these simulations the ideal CK2.3 dose for a human was then estimated (Figure 4). Figure 4a shows the simulation between 200 and 1,000 μM CK2.3 in a 500 μl total injection volume. As can be seen from the data, the ideal dose of CK2.3 was estimated to be 409 μM. Moreover, the simulation showed that without an injection of CK2.3, healthy bone has an average area of mineralization of 0 pixels². This represents the rate of mineralization, being equal to the rate of resorption, resulting in no net change in bone volume. Similarly, the model was run for osteoporotic bone using the signal output from CK2.3 doses ranging from 0 to 16 mM. Figure 4b shows the simulation from 600–1,600 μM in a 500 μl injection volume in osteoporotic bone. According to the model, without injecting CK2.3, severely osteoporotic bone has an average area of mineralization of −820 pixels². According to our model, injecting CK2.3 into osteoporotic bone can decrease the area of degradation significantly, thereby slowing the negative effects of degradation. For increasing doses of CK2.3, the signal levels increase to about 0.31 pM before leveling off. However, unlike the BMPR1a signal levels, the area of mineralization actually begins to decrease at high levels of CK2.3 instead of leveling off, possibly indicating cytotoxicity of the drug. The mineralization at 600 μM was used as an estimate for the initial value because the model does not accurately predict the response at smaller doses. In this model, no dose of CK2.3 was able to raise mineralization above 0 pixels². However, a dose of 990 μM was able to increase the mineralization to −437 pixels². The mimetic peptide CK2.3 has been shown to increase osteoblast formation in vitro and in vivo15–17 and has potential to treat or prevent osteoporosis in humans; however, the ideal dose of
CK2.3 that could be useful was unknown. Using the PBPK model, the effect of CK2.3 levels off at C24 M in a healthy individual. At this dose, it takes the CK2.3 ~25 min to be completely distributed throughout the body and block CK2 from binding to BMPR1a (Figure 5). Depending on the dose, this signal could be augmented for as many as 500 min before CK2.3 drops below the level necessary to produce additional signal. Blood has the highest concentration of CK2, while the liver has the smallest. As a result, CK2.3 is able to block all of the CK2 in the liver longer than it is able to block the CK2 in the blood. The blood, kidney, liver, heart, and brain are the only organs that have a significant concentration of CK2; they are, however, not the only organs with BMPR1a receptors. The other BMPR1a receptors would not be affected by CK2.3, since there is no CK2 to prevent these BMPR1a receptors from signaling. The output signal from the PBPK can thus be thought of as the additional signal released as a result of adding CK2.3.

**DISCUSSION**

According to the model, injections of CK2.3 can increase mineralization in extremely osteoporotic bone nearly to levels of normal healthy bone. Patients who have osteoporosis could use CK2.3 doses around 990 μM to increase mineralization levels to nearly that of a healthy bone. While this dose may seem high when compared to those in the previous literature, it should be noted that previous research was conducted on cell cultures and mice, so the scale increase in the model is accompanied by a similar increase in dose. It should also be noted that our model is based on a single bolus injection of CK2.3. Other methods of dosage, such as continuous dosing through an i.v. line or even multiple injections spaced over a few hours, may improve the mineralization above the predicted values.

Both the PBPK and the mathematical bone model were developed with several assumptions. As a result, the model only examined the effects of CK2.3 on mineralization in healthy and extremely osteoporotic bone. In the future, our model could be extended and merged with other models that focus on calcium homeostasis such as the model by Peterson and Riggs or Komarova et al.

Our model reflects the effects of CK2.3 and not the actual bone formation process. In addition, our model is not valid for small doses of CK2.3. In spite of the limitations of the model, the model can be useful in making initial dosage decisions.
estimates in humans to increase bone mineralization with CK2.3 peptide.

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