Protein Binding and Differential Distribution of Calcitonin in the Sub-compartments of Blood: An in vitro Study

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ABSTRACT
The pharmacodynamics effects of therapeutically administered calcitonin is observed for extended duration much beyond its reported short half-life. The rationale for this pharmacodynamic-pharmacokinetics mismatch of calcitonin is not known. Hence in this study the hypothesis on sub-compartmentalization of calcitonin in blood to explain the extended pharmacodynamics effects of calcitonin was evaluated in vitro using the sheep blood model. A relatively higher proportion of calcitonin concentration was observed in the WBC compartment. The partition coefficient analysis showed levels of calcitonin to be higher on WBC membrane compared to intracellular. Molecular modelling to assess the binding of calcitonin with protein in plasma and WBC membranes indicated physiologically relevant higher affinity with beta-2-microglobulin’s in plasma and glycoproteins (CD44), sushi domains (CD25), fibronectins (CD206) and siglecs (CD22) on WBC membrane. The physiologically relevant higher affinity with several proteins on WBC membrane and plasma can be responsible for extended pharmacodynamics effects observed for therapeutically administered calcitonin.

Key words: Calcitonin, Protein binding, Compartmentalization, Sustained effects, PKPD.

INTRODUCTION
Calcitonin (CT) is an endogenous regulator of serum calcium levels and is primarily produced by the parafollicular C cells in the thyroid gland. CT binds with the calcitonin receptor (CTR; present on osteoclasts) which enhances production of vitamin D and greater retention of calcium to increase the bone density. CT by activating CTR also becomes a potent inhibitor of bone resorption. CT is secreted by the parafollicular C cells in the thyroid gland. CT which consistently seems to extend much beyond its relatively short half-life (40-60 mins). Recent study using nanomaterials and hydrogels have shown controlled release of CT up to 30 days with similarly extended pharmacodynamic effects in experimental model. Several attempts are also being made to enhance the pharmacokinetics of CT to further increase its bioavailability and achieve sustained delivery. The sustained pharmacodynamic effects of CT despite the limitations with its pharmacokinetics calls to question if such attempts to further improve the pharmacokinetics of CT are necessary especially when this can lead to several undesirable pharmacodynamic and adverse effects. Further the variability observed between the pharmacokinetics vs pharmacodynamic effects of CT may parathes to indicate a unique compartmentalization of CT in the blood. Several formulations which carry a risk of increasing the concentration of CT rather than unnecessary efforts to newer CT formulations which carry a risk of increasing the CT concentration. Hence in this study we evaluated the sub-compartmentalization of CT with an in vitro experimental design using pharmacologically relevant CT concentrations.

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MATERIALS AND METHODS
Calcitonin Stock Preparation
Stock solution of salmon Calcitonin (sCT) was prepared by adding 1ml of DMSO (pH3.3) to 1mg sCT (Obtained from Polypeptide Group, CAS-no.: 47931-85-1) and vortexing it for 1 min. A 10µl aliquot of this stock solution was added to 10ml of phosphate buffer saline (PBS) to get a working stock solution of sCT (10 µg/1ml). The working stock solution of sCT was vortexed and store in freezer until use.

Blood sample and fractionation protocol
Intravenous blood samples were collected into heparin coated tubes from three healthy sheep at UCD Research Farm. The blood collection protocol was reviewed and approved by the Institutional Animal Research Ethics Committee (AREC-14-18-Kumar). 10ml of blood sample was transferred to 15 ml falcon tubes and 25µl of working sCT stock solution (calcitonin treated group) or PBS (control group) was added to the blood sample. The blood samples were then incubated at 37°C for one hour following which the blood samples were centrifuged (Rotanta 460/R centrifuge) at 2000xg for 15 min at room temperature (20°C). The plasma, red blood and white blood layers were separated from the centrifuged blood sample into separate tubes. Briefly the plasma was removed first and then the red blood cell (RBC) layer was removed carefully using a transfer pipette leaving about ~0.5 ml of the white blood (buffy coat) layer. Equal volume of Lympoprep™ (Sigma-Aldrich) was added to the remaininguffy coat layer and centrifuged again at 2000xg for 15 min at room temperature (20°C) to isolate the white blood cell (WBC) sample. The isolated RBC component was similarly diluted with equal volume of PBS and centrifuged at 2000xg for 15 min at room temperature (20°C). The supernatant liquid (primary supernatant solution) from centrifugation of WBC or RBC component were also collected into separate tubes and were used for the assay of calcitonin levels using a commercially available ELISA kit (catalogue number S-1155.0001; Peninsula Laboratories).

Cell Lysis Protocol
500µl of cell sample (RBC or WBC) was added to 1.5ml Eppendorf’s tube and centrifuged at 500xg for 5 min (Any supernatant was carefully transferred to the respective primary supernatant solution). 100µl of the cell pellet was resuspend in 300µl of Celllytic™ MT reagent (Sigma-Aldrich) and incubated on a shaker for 15 min at 37°C. Following this the sample was resuspended in 300µl of PBS and was used for quantification of calcitonin levels using the ELISA kit.

Quantification of calcitonin levels
Calcitonin was quantified using a commercially available Competitive ELISA kit. (catalogue number S-1155.0001; Peninsula Laboratories). All samples were stored at -80°C until used for analysis. Briefly, the reagents and samples were prepared as per the manufacturer’s instructions to establish the standard curve (Figure 1) and quantification of calcitonin levels.17 The levels of calcitonin in the samples following the cell lysis protocol described above was considered as the total intracellular calcitonin concentration in RBC or WBC. While the levels of calcitonin in the supernatant prior to cell lysis step was considered as the calcitonin concentration bound to cell surface (RBC-S or WBC-S). While the levels of calcitonin in the plasma was considered as total calcitonin (bound and unbound) concentration in the plasma. The calcitonin concentration is expressed as pg/ml and the calcitonin distribution is expressed in percent, with the total sum of calcitonin concentration in all five compartments [plasma, RBC, WBC, RBC-surface (RBC-S) and WBC-surface (WBC-S)] considered as 100 percent.

Partition coefficient of calcitonin in sub compartments of the blood
The partition coefficient of calcitonin was calculated by taking ratio of the calcitonin concentration in the respective compartments as reported previously.14

Protein structure and molecular docking analysis:
The protein data bank (PDB; https://www.rcsb.org/) was searched for major reported 3D structures of human plasma proteins and proteins on WBC membrane. 37 different plasma proteins and 30 different proteins on WBC membrane were identified and used for the analysis of molecular interactions (number of hydrogen bonds) with human-calcitonin (hCT) or salmon-calcitonin (sCT) using the Chimera software as reported before for other protein-protein interactions.18-22 The molecular interactions of sCT and hCT with the human calcitonin receptor ectodomain (PDB ID: 5II0) was considered as reference to quantify relative affinity of rest of the interactions. The reported EC50 values of sCT and hCT against human calcitonin receptor for cAMP production were used as reference value to generate the simulated dose response curves as reported previously.21-23

Statistical analysis
The data are presented as Mean ± SD of three independent experiments in each group and each experiment was conducted in triplicates. The data was analysed by two-way ANOVA using GraphPad Prism software (version 8).

RESULTS
In this study we evaluated the following five sub-compartments of the blood i.e., 1) plasma, 2) RBC, 3) WBC, 4) RBC-Surface (RBC-S) and 5) WBC-Surface (WBC-S). Differential distribution of calcitonin within these sub-compartments was observed (Figure 1). In the control group, majority (~ 82%) of calcitonin was located on WBC surface (WBC-S), with the rest of the calcitonin equally distributed between the plasma and samples were prepared as per the manufacturer’s instructions to establish the standard curve (Figure 1) and quantification of calcitonin levels.17 The levels of calcitonin in the samples following the cell lysis protocol described above was considered as the total intracellular calcitonin concentration in RBC or WBC. While the levels of calcitonin in the supernatant prior to cell lysis step was considered as the calcitonin concentration bound to cell surface (RBC-S or WBC-S). While the levels of calcitonin in the plasma was considered as total calcitonin (bound and unbound) concentration in the plasma. The calcitonin concentration is expressed as pg/ml and the calcitonin distribution is expressed in percent, with the total sum of calcitonin concentration in all five compartments [plasma, RBC, WBC, RBC-surface (RBC-S) and WBC-surface (WBC-S)] considered as 100 percent.

Figure 1: Distribution (percent) and concentration (pg/ml) of calcitonin in sub-compartments [plasma, RBC, WBC, RBC-Surface (RBC-S) and WBC-Surface (WBC-S)] of blood. The standard curve used for the estimation of calcitonin concentration is shown with the equation used inset in the graph. For the estimation of calcitonin distribution, the total concentration of calcitonin in all the five compartments was considered as hundred percent. The data are presented as Mean ± SD of three independent experiments in each group and each experiment was conducted in triplicates. The data between control and calcitonin treated group are statistically (p<0.05) significant.
and RBC compartments (Figure 1). Following exposure of whole blood to therapeutically achieved concentration (2.5 ng/ml) of calcitonin, its distribution within the blood compartments was significantly altered (Figure 1). Following external addition of calcitonin, an equal distribution (~ 30% each) of calcitonin in plasma, WBC and WBC-surface was observed with only less than 8% of calcitonin associated with RBC. The differences in concentration of calcitonin (pg/ml) in the sub-compartments (plasma, RBC, WBC, RBC-S and WBC-S) between the control (4.6-6.6; 4.6-7.6; 0.7-3; 2.2-3.3 and 46.1-99.6 respectively) and calcitonin treated (566.7-581.7; 7.3-11.7; 703.3-930.2; 138.4-262.9; 736.2-976.2 respectively) group were statistically (p<0.05) significant (Figure 1). The total concentration of calcitonin in whole blood and plasma of the control group was 88±27 pg/ml and 6±1 pg/ml respectively. While in the calcitonin treated group the concentration of calcitonin in whole blood and plasma was 2400±73 pg/ml and 575±8 pg/ml respectively.

The partition coefficient between the different sub-compartments of the blood was assessed (Figure 2). The partition coefficient between the RBC-Plasma, WBC-Plasma, RBC-WBC-membrane(rm), WBC-WBC-membrane(wm) differed significantly (p<0.05) between the control and calcitonin treated group (Figure 2). As the absolute concentration of calcitonin in the RBC compartment was very small, the partition coefficient difference in this compartment is of negligible clinical interest. However the variability in the partition coefficient of the WBC compartment indicated significant association of calcitonin with the WBC membrane. This was further evident from despite similar concentration (~ 40%) of calcitonin both within WBC and on WBC membrane, a higher partition coefficient favouring calcitonin accumulation on the WBC membrane was observed in the calcitonin treated group. Among all the partition coefficients, the K_{wm} was most significantly influenced under higher calcitonin concentration, with the dynamics changing from intracellular accumulation of calcitonin at lower concentration (control group) to a marginal preference of cell surface association at higher concentration (calcitonin treated group).

Calcitonin is reported to show reasonable plasma protein binding (~ 40%). Hence to address the interaction of calcitonin with plasma protein or proteins on WBC-membrane, molecular docking analysis was performed to study the potential interaction of both salmon and human calcitonin with 37 major plasma proteins and 30 major proteins on WBC membrane (Figure 3A). Molecular docking analysis showed four-fold higher affinity of salmon calcitonin against human calcitonin receptor ectodomain compared to the human calcitonin (Figure 3B, C, Table 1). hCT was observed to have higher affinity with the following proteins in plasma (3MRK (Alpha-Fetoprotein), 1DUZ (Beta-2-microglobulin)) and on WBC membrane [5VKJ (CD22), 6YIO (CD25)]. While the sCT showed significant affinity with the following proteins in plasma (1I4F (Beta-2-microglobulin), 1DUZ (Beta-2-microglobulin)) and on WBC membrane [4PZ3 (CD44), 5XTS (CD206)] (Figure 3D, Table 1). Both hCT and sCT were observed to significantly bind with several plasma as well as WBC membrane proteins with variable affinities (Figure 3A). However sCT was observed to have superior affinity to WBC membrane proteins, while hCT in contrast showed superior affinity to plasma proteins (Figure 3A).

**DISCUSSION**

Compartmentalization within the blood may be beneficial for less stable drugs, as protein binding in the sub-compartments will facilitate sustained pharmacodynamic effects of the drug.24,25 We report here such a behaviour for calcitonin, which in this study was observed to show selective affinity to the WBC sub-compartments both in the control as well as calcitonin treated groups. To the best of our knowledge such higher sub-compartmentalization of calcitonin in WBC is not previously reported. The specific sub-compartmentalization of calcitonin in WBC and plasma may explain the extended duration of pharmacodynamic effects of therapeutically administered calcitonin despite its short half-life. The observations from this study has several implications to both development of novel calcitonin formulations and as well as clinical use of currently available calcitonin formulations.5,9,11,12

Blood consists of cellular (RBC, WBC and platelets) and fluid (Plasma) compartments and drugs have equal opportunity to be uniformly diffused into all the sub-compartments.26 However selective molecular

![Figure 2: Partition coefficient (K) of calcitonin between various sub-compartments [blood (b), plasma (p), RBC (r), WBC (w), RBC Surface (rm) and WBC Surface (wm)]. The data are presented as Mean ± SD of three independent experiments in each group and each experiment was conducted in triplicates. The data between control and calcitonin treated group are statistically (p<0.05) significant for all categories except K_{bop}.](image)

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<thead>
<tr>
<th>PDB ID</th>
<th>Human calcitonin (nM)</th>
<th>Salmon calcitonin (nM)</th>
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<tr>
<td>*Receptor (5II0)</td>
<td>7.2±1.3</td>
<td>1.4±1.2</td>
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<td>2.31±0.42</td>
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<td>1.46±0.42</td>
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<td>5VKJ</td>
<td>2.66±0.48</td>
<td>1710.8±488.8</td>
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* human calcitonin receptor ectodomain (PDB ID: 5II0)
interactions of the drug will preferentially facilitate presence of the drug in specific sub-compartments. The molecular interactions could be with specific proteins, other biomolecules, selective transporters or channels on cell membrane or in plasma. In this study we observed that calcitonin was predominantly (~82%) present in the WBC surface in the control group. The calcitonin concentration on WBC surface was ~15 times higher than that observed in the plasma in the control group. This observation is of interest in interpreting the literature on calcitonin pharmacokinetics as most studies in humans have reported a calcitonin concentration of 5-15 pg/ml in plasma or serum, which based on observation from this study may represent <8% of total calcitonin concentration in the whole blood. Hence the reported values of calcitonin concentration in plasma or serum in our opinion should be readdressed considering the potential of calcitonin to sub-compartmentalization in WBC.

The plasma concentration of calcitonin (6±1 pg/ml) observed in the control group in this study was far lower than that reported in sheep (165.13±12.53 pg/ml) by other studies. However these previous studies have used less sensitive/specific calcitonin assays. Also it is not known if breed specific differences can contribute to the variance observed in calcitonin concentrations. Another likely explanation for observing lower concentration of calcitonin in this study could be poor sensitivity of our assay against sheep specific calcitonin in contrast to its higher sensitivity against salmon or human calcitonin. Hence the absolute values of calcitonin levels reported by this study in the control group should be interpreted with caution. However this potential sensitivity issue should not affect our interpretations of relative distribution of calcitonin between the various sub-compartments.

Hence the absolute values of calcitonin levels reported by this study for observing lower concentration of calcitonin in this study could not be explained. The plasma concentration of calcitonin (6±1 pg/ml) observed in the control group in this study was far lower than that reported in sheep (165.13±12.53 pg/ml) by other studies. However these previous studies have used less sensitive/specific calcitonin assays. Also it is not known if breed specific differences can contribute to the variance observed in calcitonin concentrations. Another likely explanation for observing lower concentration of calcitonin in this study could be poor sensitivity of our assay against sheep specific calcitonin in contrast to its higher sensitivity against salmon or human calcitonin. Hence the absolute values of calcitonin levels reported by this study in the control group should be interpreted with caution. However this potential sensitivity issue should not affect our interpretations of relative distribution of calcitonin between the various sub-compartments.

The influence of drug dose on its pharmacokinetic parameters is widely reported in the literature. Hence the observation of significant changes to the sub-compartmentalization of calcitonin at a higher but therapeutically relevant concentration although previously unreported is not surprising. Even at the therapeutically relevant concentration of calcitonin the major sub-compartments of relevance were plasma and WBC, as the concentration of calcitonin in the RBC compartment was very low to be pharmacodynamically significant. Although this study didn't analyse the calcitonin levels in the platelet compartment, but accounting the total calcitonin added with levels of calcitonin in different sub-compartments evaluated in, our opinion the platelet compartment is an insignificant entity for influencing pharmacodynamic effects of calcitonin. Between the plasma and WBC, this study observed a marginally higher presence of calcitonin in the WBC compartment at therapeutic concentration. This preferential presence of calcitonin in the WBC compartment was observed on WBC surface rather than intracellular, as observed from the partition coefficient parameters. The partition coefficient between WBC-Plasma (Kw/w) as well as WBC-WBC-surface(Kw/w) were higher than one, which validate our interpretation on preferential presence of calcitonin on WBC membranes.

Calcitonin is reported to be marginally (~40%) bound to plasma proteins. Hence in this study we presumed that calcitonin may similarly bind to proteins on WBC membrane as well. Most literature attribute plasma protein binding of drug to albumin due its abundance in the plasma, despite the possibility of many other plasma proteins also binding with calcitonin. While several studies have reported drugs binding to proteins on RBC membrane the literature on drugs bound to WBC membrane is relatively scant. In contrast to the general assumption of higher binding of calcitonin to albumin in plasma, we observed that both hCT and sCT had weaker affinity to albumin compared to other proteins in plasma and WBC membrane. In the plasma both hCT and sCT were observed to have superior affinity to beta-2-microglobulin's, although the physiological significance of this is unknown. The binding of calcitonin to proteins on WBC membrane is not previously reported. On the WBC membrane hCT and sCT were observed to have relatively higher affinity towards glycoproteins (CD44), sushii domains (CD25), fibronectins (CD206) and siglec (CD22). The binding of calcitonin with several proteins in plasma and WBC membrane at physiologically relevant affinities suggest that these interactions can favour sustained release of calcitonin to achieve its extended pharmacodynamics effects much beyond its currently known short half-life. Future studies to take advantage of these various calcitonin-protein interactions will have therapeutic merits and should be investigated.

In summary, this study reports a novel observation on sub-compartmentalization of calcitonin on WBC membranes at therapeutically relevant concentrations, which can contribute to sustained availability of calcitonin for its pharmacological effects. This study also reports several novel calcitonin-protein interactions in plasma and WBC-membrane, which merits further studies to enhance therapeutic efficacy of calcitonin.

ACKNOWLEDGEMENT

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Figure 3: Molecular interaction of human (hCT) and salmon (sCT) calcitonin with human calcitonin receptor, various plasma and WBC membrane proteins. A) affinity of hCT and sCT with human calcitonin receptor (highlighted in light purple), various plasma (highlighted in orange) and WBC (highlighted in light green) membrane proteins. Scale: dark green represented strong affinity, sky blue represented medium affinity, red represented weak affinity. B) Molecular docking image of hCT and sCT with human calcitonin receptor ectodomain (PDB ID: 5II0). Yellow lines represent hydrogen bonds between calcitonin and its receptor. C) Simulated dose response curve of hCT and sCT based on its efficacy to produce cAMP levels following activation of calcitonin receptor. The data are presented as Mean ± SD of three simulation performed at ± 1, 2 and 3 sigma deviations. D) Representative images showing molecular interaction of hCT and sCT with selected plasma (3MRK, 1DUZ, 1IFF) and WBC (5VKJ, 6YIO, 4PZ3, 5XTS) membrane proteins. Yellow lines represent hydrogen bonds between calcitonin and its target protein.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES