Poster 1232

# PKC Agonist Exposure Sufficient to Activate T Cells *In Vivo* also Causes Coagulopathy


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## Background

- Activation of latent HIV reservoir is part of a strategy for HIV cure as it should enable the elimination of infected cells by immune-mediated clearance mechanisms and facilitate long-term remission or cure.[1]
- Protein kinase C (PKC) agonists, such as Prostratin and Bryostatin, are highly effective at activating latent HIV.[2-3]
- DAG lactones are a class of small molecules that activate PKCs.[4]
- Effective use of PKC agonists is limited by their severe toxicity, with a mechanism not clearly elucidated.[5-6]

## Objectives

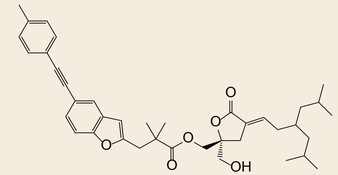
- To elucidate the primary mechanism of toxicity of the novel small molecule PKC agonist, C-232A.
- To develop a predictive *in vitro* screening platform to inform on potential toxicity of future PKC agonists.

## Methods

- PKC translocation by small molecule agonists was assessed by fluorescent microscopy of GFP-labeled PKC in A549 cells and by immunostaining endogenous PKC in Jurkat cells.
- *Ex vivo* activation of HIV transcription by qPCR and CD69 expression was assessed by FACS in CD4+ T cells treated with PKC agonists from ART-suppressed HIV-infected donors.
- Dose escalation PK/PD studies were conducted in rats and rhesus macaques by IV infusion of C-232A.
  - Systemic exposure was determined by measuring C-232A plasma levels with an LC-MS/MS method.
  - Activation markers and cytokines were measured by flow cytometry and multiplex immunoassay.
  - Investigational toxicology endpoints were assessed including hematology, coagulation and anatomic pathology.
- Flow cytometry and light transmission aggregometry were utilized to assess *in vitro* platelet activation and function.

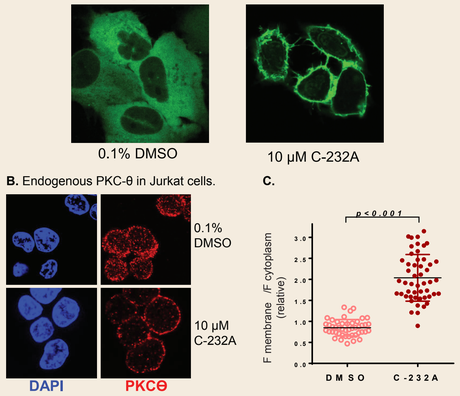
## Results

### Figure 1. Structure of the novel small molecule PKC agonist, C-232A.



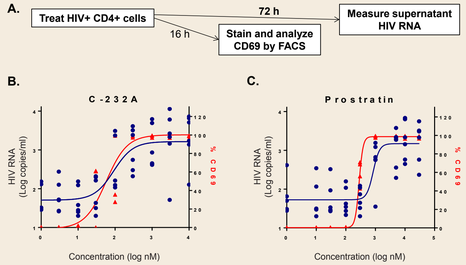
## Results (Cont'd)

### Figure 2. C-232A induces PKC-θ translocation.



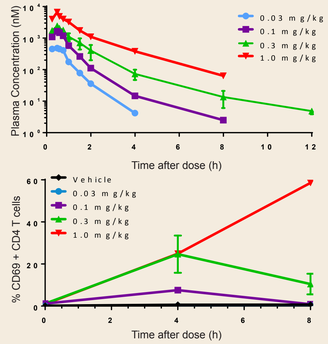

A. Confocal microscopy images of A549 cells expressing GFP-labeled PKC-θ after treatment with vehicle or C-232A.
B. Confocal microscopy images of immunostained endogenous PKC-θ in Jurkat cells after treatment with vehicle or C-232A.
C. Quantification of endogenous PKC-θ relative fluorescence in Jurkat cellular membrane over cytoplasm.

### Figure 3. Prostratin and C-232A induce HIV transcription and CD69 activation in HIV-infected donor T cells *ex vivo*.




*A. Ex vivo* assay workflow for donor T cells treated with PKC agonists.
B. Dose-dependent C-232A induced HIV transcription and CD69 activation.
C. Dose-dependent Prostratin induced HIV transcription and CD69 activation.

### Figure 4. Dose escalation PK/PD study of C-232A by IV infusion in rhesus macaques results in both T cell activation and severe toxicity.




| Dose (mg/kg) | $AUC_{last}$ (nM•h) | $C_{max}$ (nM) |
|---|---|---|
| 0.03 | 489 | 481 |
| 0.1 | 1521 | 1650 |
| 0.3 | 3033 ± 709 | 2447 ± 268 |
| 1.0 | 8315 | 6700 |

C.

| Dose (mg/kg) | Clinical Signs |
|---|---|
| 1.0 | **6.5 hours postdose:** animal appeared pale and hypoactive with discolored skin and swelling around the eyes<br>**8 hours postdose:** animal humanely sacrificed per veterinarian request |

A. C-232A plasma level versus time curves and corresponding pharmacokinetic parameters.
B. C-232A induced CD69 expression on T-cells over time postdose.
C. Clinical signs observed in animal administered a dose level of 1 mg/kg C-232A.

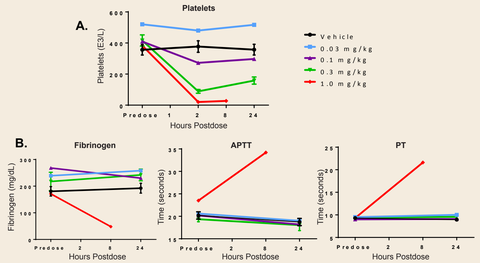
Similar PK and clinical signs were observed in PK/PD studies of C-232A in rats (data not presented).

### Table 1. C-232A induces selected cytokine and chemokines in rhesus after IV administration.

| Cytokine (pg/mL) | Cytokine levels in plasma at 4 hr post dose | | | | |
|---|---|---|---|---|---|
| | Vehicle | 0.03 mg/kg | 0.1 mg/kg | 0.3 mg/kg | 1 mg/kg |
| IL-6 | 87 | 43 | 126 | 76 | 3872 |
| IL-8 | 216 | 275 | 2531 | 375 | 6153 |
| IL-1β | 43 | 45 | 70 | 59 | 343 |
| IL-1RA | 117 | 169 | 775 | 2265 | 4471 |
| MIP-1α | 228 | 208 | 160 | 187 | 1111 |
| MIP-1β | 183 | 131 | 428 | 707 | 3913 |
| I-TAC | 112 | 88 | 427 | 365 | 5602 |
| MIG | 89 | 55 | 64 | 55 | 658 |
| MCP-1 | 756 | 1900 | 3250 | 6117 | 13620 |
| VEGF-A | 160 | 107 | 161 | 81 | 330 |
| IL-18 | 165 | 114 | 193 | 145 | 351 |
| IFN-γ | 54 | 43 | 75 | 53 | 200 |
| IFN-α | 166 | 90 | 143 | 112 | 187 |
| TNF-α | 48 | 56 | 39 | 19 | 28 |
| IL-10 | 88 | 42 | 80 | 54 | 82 |

**Table 1.** Multiplex immunoassays were performed to analyze cytokine and chemokine levels in plasma samples collected from plasma of both vehicle control and C-232A groups 4 h after IV administration. IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17A, and IL-23 were measured but not induced by C-232A.

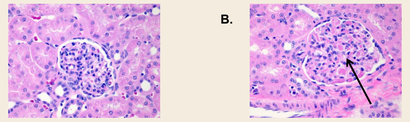
### Figure 5. Abnormal hematology and coagulation parameters in rhesus macaques administered C-232A indicate platelet activation, aggregation and excessive clotting.




A. C-232A administration causes rapid dose-dependent decreases in circulating platelet levels.
B. Administration of 1 mg/kg C-232A by IV infusion causes an abnormal coagulation panel, with decreased circulating clotting factors (fibrinogen) and increased clotting times (activated partial thromboplastin time [APTT] and prothrombin time [PT]).

Similar hematology and coagulation findings were observed in PK/PD studies of C-232A in rats (data not presented).

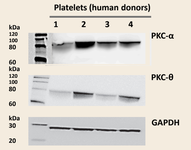
### Figure 6. IV infusion of C-232A at 1 mg/kg manifests in hemorrhage and thrombi across multiple organs in the rhesus macaque, a hallmark of disseminated intravascular coagulation (DIC).




A. Representative image of a normal glomerulus from an H&E stained kidney section of the monkey administered 1 mg/kg C-232A.
B. Representative image of multiple glomerular thrombi from an H&E stained kidney section of the monkey administered 1 mg/kg C-232A.

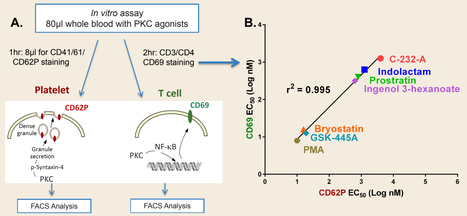
Histopathology analysis in the rhesus macaque did not reveal characteristics of cytokine release syndrome, such as immune cell infiltration or edema.
Similar histopathology findings were observed in PK/PD studies of C-232A in rats (data not presented).

### Figure 7. Certain PKC isoforms are expressed in platelets.



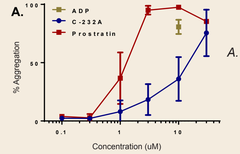

A. Western blot analysis for PKC-α, PKC-θ and GAPDH expression in platelets isolated from four healthy human donors.

### Figure 8. An *in vitro* platform in whole blood predicts C-232A and Prostratin platelet toxicity relative to pharmacodynamic potency.




A. Workflow for measuring platelet (CD62P) and T cell (CD69) activation in whole blood treated with PKC agonists.
B. Several classes of PKC agonists induce CD69 on T cells and CD62P on platelets with similar potency.

### Figure 9. C-232A and Prostratin induce platelet aggregation.




*A. In vitro* platelet aggregation caused by Prostratin, C-232A and an assay positive control (adenosine diphosphate [ADP]).

## Conclusions

- The primary mechanism of C-232A toxicity in rhesus macaques, as well as rats (*data not shown*), is mediated by platelet activation, aggregation and coagulopathy. The *in vitro* data presented here, as well as published *in vivo* data[6], suggests a similar toxic mechanism of action for Prostratin.
- Platelet activation is a critical safety liability associated with PKC agonists and should be carefully monitored in any preclinical or clinical studies. The *in vitro* screening tools presented here should inform on the platelet activation potential of PKC agonists prior to *in vivo* studies.

## References


**1.** Sengupta S, et al. *Immunity* 2018. 48(5): 872-895. **2.** Jiang G, et al. *AIDS Res Hum Retroviruses* 2015. 31(1): 4-12. **3.** Kulkosky J, et al. *Blood* 2001. 98: 3006-3015. **4.** Hamer D, et al. *J Virology* 2003. 77(19): 10227-10236. **5.** Gutierrez C, et al. *AIDS* 2016. 30(9): 1385-92. **6.** Brown S, et al. *United States Patent Application Publication* 2011. US 2011/0224297 A1.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