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# Protein kinases C as potential host targets for the inhibition of chikungunya virus replication

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ABSTRACT

We have shown previously that prostratin, a non-tumor promoting phorbol ester, inhibits chikungunya virus (CHIKV)-induced cytopathic effects *in vitro*. Prostratin is a potent activator of protein kinases C (PKC), a family of related serine/threonine kinases that regulate many cellular processes such as proliferation and apoptosis. The objective of this study was to explore the mechanism of the anti-CHIKV activity of prostratin. Prostratin reduced the production of infectious virus particles and viral protein accumulation in a dose-dependent manner at a post-entry step during virus replication. The antiviral effect of the compound was cell-dependent, with potent antiviral activity observed in human skin fibroblasts cells, the primary target cells of CHIKV infection. The antiviral activity of prostratin was markedly reduced in the presence of PKC inhibitors, therefore confirming that the antiviral effect results from an activation of PKCs. Together these results showed that PKCs are potential host targets for the inhibition of CHIKV replication.



## 1. Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus that belongs to the genus *Alphavirus* of the family *Togaviridae*. The virus is transmitted by the bite of female mosquitoes of the *Aedes* species (Simon et al., 2011). CHIKV causes chikungunya fever, which is mostly characterized by fever, arthralgia and, sometimes, a maculopapular rash (Singh and Unni, 2011). Although a CHIKV infection is rarely fatal, the disease proceeds in 60% of infected patients into a chronic stage that is characterized by persistent disabling polyarthritis, which can severely incapacitate the patient for weeks up to several years (Simon et al., 2011).

CHIKV was first isolated in 1953 during an epidemic in Tanzania (Lahariya and Pradhan, 2006). In 2004, the global reemergence of CHIKV started in Kenya, after which it spread to several islands in the Indian Ocean (Burt et al., 2012). At the end of 2013, the first local transmission of CHIKV in the Americas has been reported. Since then, most of the Caribbean islands and also many countries in Central and South America have reported many cases of CHIKV infection (Weaver and Forrester, 2015). Therefore, CHIKV is now considered a real health threat to tropical and temperate areas in which *Aedes* mosquitoes thrive.

Despite the widespread and the high morbidity rate of CHIKV infections, there is currently no approved vaccine or antiviral treatment available. Treatment has been limited to analgesics, antipyretics, and anti-inflammatory agents to relieve the symptoms (Lahariya and Pradhan, 2006). A number of classes of compounds that target either a viral or a host factor have been reported to inhibit CHIKV replication *in vitro* (Abdelnabi et al., 2015), but none have progressed towards further development. Therefore, the development of potent antiviral drugs against CHIKV is urgently needed.

Protein kinase C (PKC) is a family of related serine/threonine kinases that regulate many cellular processes such as proliferation, differentiation and apoptosis (Breitkreutz et al., 2007; Corbalán-García and Gómez-Fernández, 2006) through phosphorylation of downstream signaling factors (Breitkreutz et al., 2007; McKernan et al., 2012).

The structure of PKCs consists of a highly conserved catalytic domain at the C-terminus (containing motifs required for ATP/

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substrate-binding and catalysis) and a regulatory domain at the N-terminus (Steinberg, 2008). Most PKCs are activated by diacylglycerol (DAG), which is produced via the hydrolysis of phosphatidylinositol 4,5-bisphosphate by the phospholipase C enzyme. PKC isoforms are classified into three subfamilies based on their regulatory domain structure (Steinberg, 2008). The classical PKCs (α, βI, βII, and γ) are calcium- and DAG-dependent, while the novel PKCs (δ, ε, η, and θ) are only DAG-dependent (Breitkreutz et al., 2007; McKernan et al., 2012; Steinberg, 2008). The third subfamily are the atypical PKCs (ζ and λ/I), which are unresponsive to both calcium and DAG (Breitkreutz et al., 2007; McKernan et al., 2012). Following activation, PKC isoforms undergo translocation from the cytoplasm to the plasma membrane (Steinberg, 2008).

Phorbol-12-myristate 13-acetate (PMA) and prostratin are phorbol esters that were originally isolated from croton oil (*Croton tiglium*) and the tropical plant *Homalanthus nutans*, respectively (Gustafson et al., 1992; Repine et al., 1974). PMA and prostratin are potent activators of PKCs due to their structural similarity to DAG (Breitkreutz et al., 2007; McKernan et al., 2012). Unlike PMA, prostratin has no tumor promoting activity (Szallasi et al., 1992) and is also a known activator of nuclear factor κB (NF-κB) mediated through the activation of IKB kinases. Prostratin was previously reported to inhibit the entry of HIV(Rullas et al., 2004) and to compromise latent HIV viral reservoirs (López-Huertas et al., 2011). Although prostratin was shown to inhibit the replication of CHIKV *in vitro* (Bourjot et al., 2012), the mechanism remains poorly defined.

In this report, we further study the particular characteristics of the antiviral activity of prostratin against CHIKV to shed light on i) the mechanism by which this compound exerts its antiviral activity and ii) the role of PKCs in the cellular antiviral response to CHIKV infection.

## 2. Materials and methods

### 2.1. Cells, viruses and compounds

Buffalo green monkey kidney (BGM, ECACC 90092601) cells and African green monkey kidney cells (Vero cells, ATCC CCL-81) were maintained in minimal essential medium (MEM Rega-3, Gibco, Belgium) supplemented with 10% fetal bovine serum (FBS, Gibco, Belgium), 1% L-glutamine (Gibco, Belgium) and 1% sodium bicarbonate (Gibco, Belgium). Human embryonic lung fibroblasts (HEL, ATCC CCL-75) were generously provided by Professor G. Andrei (Rega Institute for Medical Research, Leuven, Belgium) and were maintained in MEM (Gibco, Belgium) supplemented with 10% FBS (Gibco, Belgium), 1% HEPES (Gibco, Belgium), 1% non-essential amino acids (NEAA, Gibco, Belgium), 1% streptomycin/penicillin and 1% sodium pyruvate (Gibco, Belgium). The virus propagation and antiviral assays were performed in the same medium except that it was supplemented with 2% FBS instead of 10%. Human skin fibroblast cells (ATTC CRL-2522) were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, GE Healthcare HyClone, Singapore), supplemented with 10% FBS (GE Healthcare HyClone, Singapore) and 1% penicillin/streptomycin (Gibco, Singapore). All cell cultures were maintained at 37 °C in an atmosphere of 5% $CO_2$ and 95%–99% relative humidity.

CHIKV Indian Ocean strain 899 (CHIKV-899; GenBank FJ959103.1) is a lab-adapted strain that was a kind gift of Professor C. Drosten (University of Bonn, Germany). The CHIKV-899 stock was prepared in Vero A cells and stored at −80 °C. Full-length CHIKV and O'nyong'nyong virus (ONNV) infectious clones were produced using similar standard molecular biology techniques as previously described (Tsetsarkin et al., 2006). CHIKV isolates from Singapore (SGP011), Reunion Island (LR2006 OPY1) and the Caribbean (CNR20235), and ONNV were tagged with Gaussia luciferase (Gluc) from marine copepod *Gaussia princeps* at a site in between the non-structural and structural genes. Viral stocks of infectious clones were produced in Vero E6 cells (ATCC CRL-1586), titrated by plaque assays and stored at −80 °C.

Prostratin was purchased from LC Laboratories (USA), Go 6976 and BAPTA-AM from Abcam Biochemicals, rottlerin from Enzo Life Sciences BVBA (Belgium), sotrastaurin from Axon Medchem BV (The Netherlands), Ro-32-0432 and bisindolylmaleimide II from Santa Cruz Biotechnology. All compounds were dissolved in analytical grade DMSO to yield 10 mg/ml stocks. The compounds were protected from light and were stored at −20 °C until used.

### 2.2. Determination of $CCID_{50}$ per ml

The infectivity of a particular cell line for CHIKV was quantified by means of end-point titration. Briefly, the selected cell types were seeded in 96-well microtiter plates at a density of $2.5 \times 10^4$ cells/well (except for HEL cells, a density of $8 \times 10^3$ cells/well was used) and were allowed to adhere overnight. The next day, 6 parallel 10-fold serial dilutions of the virus were prepared. After 5 days of incubation, the cells were examined microscopically for virus-induced cytopathic effects (CPE). A well was scored positive if any traces of virus-induced CPE were observed compared to the uninfected controls. For assays performed in CRL-2522 cells, the $CCID_{50}$ assay was done by first seeding $3 \times 10^4$ Vero E6 cells per well in 96-well microtiter plates overnight. Cells were then treated with serial dilutions of the supernatant collected from infected CRL-2522 cells prepared in serum-free DMEM for 1.5 h, before being refreshed with complete medium. After 4 days of incubation at 37 °C, cells were fixed with 10% formalin (Sigma-Aldrich) and stained with crystal violet. The $CCID_{50}$/ml was calculated using the method of Reed and Muench (1938) and is defined as the virus dose that would infect 50% of the cell cultures.

### 2.3. Cytopathic effect (CPE) reduction assay

Cells were seeded at a density of $2.5 \times 10^4$ for BGM and Vero cells, and $8 \times 10^3$ cells/well for HEL cells in 96-well tissue culture plates (BD Falcon) and were allowed to adhere overnight. The next day, dilution series of the compound was prepared in the medium, after which the cultures were immediately infected with CHIKV-899 at the desired multiplicity of infection (MOI: 0.01, 0.005, 0.001, 0.0005). On day 5 post-infection, the antiviral effect was quantified using the MTS/PMS method as described by the manufacturer (Promega, The Netherlands). The cells were checked by microscope for minor signs of virus-induced cytopathic effects or compound-induced adverse effects on cell and monolayer morphology. The 50% effective concentration ($EC_{50}$), which is defined as the concentration of compound that is required to inhibit virus-induced cell death by 50%, was determined using logarithmic interpolation. In parallel, the 50% cytotoxic/cytostatic concentration ($CC_{50}$), which is the concentration of compound that is required to reduce cell viability by 50%, was determined in non-infected cells using the MTS/PMS method to measure the cell viability.

### 2.4. Antiviral effect of prostratin against selected alphaviruses in human fibroblasts

A cell viability assay was first performed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Singapore) as previously described (Ching et al., 2015). Briefly, semi-white 96-well microtiter plates were seeded with CRL-2522 at a density of $5 \times 10^3$ cells per well. Next day, cells were treated with a serial dilution of prostratin starting with concentration of 100 μM in