Comparative analysis of the anti-chikungunya virus activity of novel bryostatin analogs confirms the existence of a PKC-independent mechanism

Running title: CHIKV inhibition by novel bryostatin analogs involves a non-PKC pathway

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Abstract

Previously, we reported that salicylate-based analogs of bryostatin protect cells from chikungunya virus (CHIKV)-induced cell death. Interestingly, 'capping' the hydroxyl group at C26 of a lead bryostatin analog, a position known to be crucial for binding to and modulation of protein kinase C (PKC), did not abrogate the anti-CHIKV activity of the scaffold, putatively indicating the involvement of a pathway independent of PKC. The work detailed in this study demonstrates that salicylate-derived analog **1** and two capped analogs (**2** and **3**) are not merely cytoprotective compounds, but act as selective and specific inhibitors of CHIKV replication. Further, a detailed comparative analysis of the effect of the non-capped versus the two capped analogs revealed that compound **1** acts both at early and late stages in the chikungunya virus replication cycle, while the capped analogs only interfere with a later stage process. Co-dosing with the PKC inhibitors sotrastaurin and Gö6976 counteracts the antiviral activity of compound **1** without affecting that of capped analogs **2** and **3**, providing further evidence that the latter elicit their anti-CHIKV activity independently of PKC. Remarkably, treatment of CHIKV-infected cells with a combination of compound **1** and a capped analog resulted in a pronounced synergistic antiviral effect. Thus, these salicylate-based bryostatin analogs can inhibit CHIKV replication through a novel, yet still elusive, non-PKC dependent pathway.

Key words

Chikungunya virus; Bryostatin; Protein kinase C; Sotrastaurin; Salicylate.

1. Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus (arbovirus) that belongs to the genus *Alphavirus* of the family *Togaviridae* [1]. The symptoms of acute CHIKV infection include fever, arthralgia, and, in many cases, maculopapular rash [2]. In some patients, the acute infection is followed by a chronic stage, characterized by a debilitating polyarthritis, that can last from weeks to years [1]. After many years of sporadic outbreaks following the first reports in 1952, CHIKV recently re-emerged with massive outbreaks in Kenya (2004) and a subsequent rapid spread to most of the Indian Ocean islands and countries of Southeast Asia [3]. In December 2013, the first local transmission of CHIKV in the Americas was reported on the island of St. Martin [4]. Since then, cases have been reported in most of the Caribbean islands, as well as numerous countries in Central and South America resulting in millions of cases, a number which will continue to rise.

Despite several efforts to develop a CHIKV vaccine [5] and to discover selective antiviral compounds [6], vaccines or antiviral drugs are not yet available for prevention or treatment of this infection. Therefore, exploring the possibility of repurposing drugs that are currently in development for the treatment of unrelated conditions as therapy for CHIKV disease provides a potentially expedited path towards therapy.

One such drug, bryostatin 1 (henceforth bryostatin, Fig. 1), is a potent modulator of both conventional (α , β I/ β II, γ) and novel (δ , ϵ , η , θ) PKCs. Bryostatin has progressed into clinical trials as a potential treatment for cancer [7,8], Alzheimer's disease [9] and eradication of HIV reservoirs [10]. In considering novel chemotherapeutic approaches to CHIKV, we recently discovered and reported that bryostatin analogs, incorporating different A- or B-ring functionalities or a salicylate group in place of the AB ring system

[11,12], protect cells from CHIKV-induced cell death with EC₅₀ values in the low μ M range [13,14]. Based on previous reports, in which PKC agonists structurally-distinct from bryostatin demonstrated similar cell protective effects, PKC activation was hypothesized to be involved in the mechanism of action [15,16].

Surprisingly, bryostatin itself had no measureable cell protective activity in the same assay. This was the first indication that the mechanism of action of these analogs might also involve a PKC-independent pathway. To further explore this possibility, analogs were synthesized with a modification (i.e. cap) to the hydroxyl group (OH) at position C26. Such a cap would be expected to greatly reduce or even abolish the ability of the compounds to bind to and thus to modulate PKC activity, as the C26 hydroxyl group of bryostatin-based scaffolds is required for PKC affinity [17]. Interestingly, these capped analogs were still shown to be potent inhibitors of CHIKV-induced cell death, thus providing additional evidence for the existence of a non-PKC-dependent pathway that could be essential for CHIKV replication and that may be druggable [14]. The objective of the present study is to characterize the *in vitro* activity of a lead bryostatin analog (**1**) and two C26-capped analogs (**2** and **3**) (Fig. 1) against CHIKV replication and to further explore whether their anti-CHIKV activity involves a PKC-independent pathway.

2. Materials and methods

2.1. Cells and viruses

African green monkey kidney cells, Vero A cells (ATCC CCL-81) and Buffalo green monkey kidney cells, BGM cells (ECACC 90092601) 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). The virus propagation and antiviral assays were performed in the same medium except that it was supplemented with 2% FBS instead of 10%. All cell cultures were maintained at 37°C in an atmosphere of 5% CO₂ 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). Sindbis virus (SINV, strain HRsp, GenBank J02363.1) and the Semliki Forest virus (SFV, Vietnam strain, GenBank EU350586.1) belong to the collection of viruses at the Rega Institute of Medical Research, Belgium. The virus stocks were prepared in Vero A cells and stored at -80°C.

2.2. Compounds

Bryostatin analogs (1-3) were synthesized as described before [11,12,14]. T-705 was obtained from Boc Sciences (New York, USA). Go 6976 was purchased from Abcam Biochemicals (Cambridge, UK) and sotrastaurin from Axon Medchem BV (The Netherlands). 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. Arbidol was a kind gift by Prof. S. Polyak (University of Washington, Seattle, USA) and was dissolved as a 10 mg/ml solution in 100% ethanol after which it was 10-fold diluted with sterile water before use.

2.3. Cytopathic effect (CPE) reduction assay

Vero A cells were seeded at a density of 2.5×10^4 in 96-well tissue culture plates (BD Falcon) and were allowed to adhere overnight. The next day, dilution series of the compounds was prepared in the medium starting with a concentration of 50 μ M, after which the cultures were infected with CHIKV-899, SINV or SFV. 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 toxicity of the compounds was determined by treating uninfected cells with the same serial dilutions of the compound used in the antiviral assay. After five days of incubation, the 50% cytotoxic/cytostatic concentration (CC_{50}), which is the concentration of compound that is required to reduce cell viability by 50%, was determined using the MTS/PMS method.

2.4. Quantitative reverse transcription PCR (qRT-PCR)

Extracellular viral RNA was isolated from 150 μ l supernatant using the NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany), while the intracellular viral RNA was isolated using the Cells-to-cDNA™ lysis buffer (Life Technologies, Bleiswijk, The Netherlands). The sequences of primers and probe used in qRT-PCR were described before [18]: forward primer 5'-CCGACTCAACCATCCTGGAT-3', reverse primer 5'-GGCAGACGCAGTGGTACTTCCT-3', probe 5'-FAM-TCCGACATCATCCTCCTTGCTGGC-TAMRA. The one-step, quantitative RT-PCR was performed in a total volume of 25 μ l, containing 13.94 μ l H₂O, 6.25 μ l master mix (Eurogentec, Belgium), 0.375 μ l of forward

primer, 0.375 µl of reverse primer (final concentration of each primer 150 nM), 1 µl of probe (final concentration 400 nM), 0.0625 µl reverse transcriptase (Eurogentec, Belgium) and 3 µl RNA sample. The reaction was quantified using the Applied Biosystems 7500 Fast Real-Time PCR System (Branchburg, NJ, USA) using the following conditions: 30 min at 48°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For quantification, standard curves were generated each run using 10-fold dilutions of a CHIKV standard cDNA.

2.5. Virus yield assay

Vero A cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well. Next day, cells were treated with a serial dilution of the selected compound and then infected with CHIKV-899 (MOI 0.001). After 2h of infection, the cells were washed to remove non-adsorbed virus, treated again with the same serial dilutions of compounds and incubated for 48h. At the end of the incubation period, supernatant was collected and virus RNA was quantified by real-time qRT-PCR (**section 2.4.**), while the amount of infectious progeny virus was determined by titration assay (CCID₅₀/ml) as described before [18].

2.6. Delay of treatment assay

Vero A cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/well and incubated overnight. Two hours prior to CHIKV-899 infection, the selected compound (15 µM of analog 1, 20 µM of analog 2, 20 µM of analog 3, 300 µM of T-705 or 75 µM of arbidol) was added at the condition -2 hours. Subsequently, at time point 0, the medium of all wells was removed and the cells were infected with virus with MOI of 1 for 1 hour at 37°C. The compounds were added at the previously mentioned concentrations at 0, 2, 4 and 6 hours after infection. Following 24h of incubation, cells

were lysed the intracellular RNA was quantified by qRT-PCR as described before (section 2.4.).

2.7. Entry assay using CHIKV pseudoparticles (CHIKVpp)

CHIKV pseudoparticles (CHIKVpp), which are lentiviral vesicles with CHIKV envelope glycoproteins expressed on the surface, were prepared as previously reported [19]. BGM cells were seeded in white 96-well tissue culture plates (ViewPlate-96, PerkinElmer, Belgium) at a density of 2.5×10^4 cells/well in assay medium and left to adhere overnight. The next day, serial dilutions of the compounds were added to the cells followed by infection with the appropriate dilution of CHIKVpp. Arbidol was used as a positive control entry inhibitor. On day three post-infection, cells were lysed and the firefly luciferase activity in the cell lysate was detected using the Luciferase Assay System kit as described by the manufacturer (Promega, The Netherlands).

2.8. Reversal of anti-CHIKV activity by PKC inhibitors

Vero A cells were seeded in 96-well tissue culture plates at a density of 2.5×10^4 cells/well and were allowed to adhere overnight. The following day, cells were treated with serial dilutions of the selected PKC inhibitor (sotrastaurin or Go 6976) starting at 16 μ M. Immediately after, 12 μ M of analog 1, 2 or 3 was added to cells followed by infection with CHIKV-899 (final MOI 0.001). T-705 (60 μ M) was included in this assay as a negative control. The effect of the PKC inhibitors on the antiviral activity of the tested bryostatin analogs was assessed by the MTS/PMS method.

2.9. Matrix combination study

Combination study was performed by treating Vero A cells with a matrix of 2-fold dilution series of analog 1 and either analog 2 or 3 after which the cells were infected with CHIKV-899 at MOI of 0.001. After 5 days of incubation, cell viability was quantified

180 by the MTS/PMS method. Data were then analyzed at the 95% confidence level with the
181 MacSynergy II template based on the method of Prichard and Shipman [20,21].

182 **2.10. PKC-binding assays**

183 Affinity of analogs 1, 2 and 3 for selected human PKC isoforms (obtained from Life
184 Technologies) was determined via heterogeneous competitive binding assay with [3H]-
185 PDBu as described before [22].

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3. Results

3.1. Bryostatins analogs are selective inhibitors of CHIKV replication

First, to determine whether the selected analogs are selective inhibitors of CHIKV replication or more broadly exert antiviral activity against alphaviruses, their effect on virus-induced cytopathic effect (CPE) in Vero A cells by two other representative alphaviruses, Sindbis (SINV) and Semliki forest virus (SFV), was assessed using the MTS/PMS method. Moderate to no activity was observed against SINV and SFV whereas their potent anti-CHIKV activity was confirmed in parallel (Table 1, Fig. 2A). Compounds **2** and **3** were shown, using the MTS/PMS method and microscopic examination, to exhibit minimal adverse effects on cell viability at the highest tested concentration (50 μ M) (Table 1, Fig. 2B). Although the highest tested concentration of analog **1** (50 μ M) did not result in cytotoxicity using the MTS/PMS method, cells treated with that concentration showed some morphological changes.

To further corroborate the selective anti-CHIKV activity of compounds **1**, **2**, and **3**, their dose-response effect was determined in a virus yield assay [18] using both quantitative RT-PCR and end-point titration for infectious virus content. Significant reduction of both viral RNA replication as well as the production of infectious virus progeny was observed with increasing concentrations of the three compounds (Fig. 2C and D).

3.2. Bryostatin analogs differently affect the CHIKV replication cycle

To determine whether the compounds affect either an early or late stage (or both) of the CHIKV replication cycle, a delay-of-treatment assay was performed. Compound **1** and the capped analogs maintained their antiviral activity against CHIKV when added to the cells up to 6 and 4 hours post-infection, respectively. This indicates that these compounds inhibit one or more late stages of virus replication (Fig. 3A). Furthermore, compound **1** and the capped analogs were evaluated for their potential inhibitory

activity in a CHIKV pseudoparticle (CHIKVpp) entry assay in buffalo green monkey (BGM) cells. In parallel, arbidol, a known early-stage inhibitor of CHIKV infection, was used as a reference compound. Interestingly, compound **1** clearly inhibited viral entry ($EC_{50} = 4.3 \pm 0.5 \mu M$, Fig. 3B), whereas the capped analogs did not elicit any effect in this assay (even at the highest non-toxic concentrations tested). This implies that the mechanism of action of compound **1** involves multiple stages of the virus replication cycle, and/or that it acts by means of more than one mechanism of action, one of which may be specific for the capped analogs.

3.3. The anti-CHIKV activity of the capped analogs is PKC-independent

A fixed concentration ($12 \mu M$) of compound **1** or the capped analog **2** or **3** were combined with different concentrations of either the pan-PKC inhibitor sotrastaurin (Fig. 4A) or the conventional-selective PKC inhibitor Go 6976 (Fig. 4B). The antiviral activity of these combinations was quantified in a CPE-reduction assay. The antiviral activity of compound **1** was inhibited in a dose-dependent manner by both PKC inhibitors, whereby the effect of the pan-PKC inhibitor was more pronounced (Fig. 4A). In contrast, the activity of compounds **2** and **3** was not markedly affected by the presence of either of the PKC inhibitors (Fig. 4).

Furthermore, the PKC binding affinity of compounds **1**, **2**, and **3** was quantified using a heterogeneous competitive binding assay. The binding affinity of compound **1** proved to be the greatest for the novel PKC isoform δ , followed by the novel isoforms ϵ and θ , and the conventional isoform βI (Table 2). For compounds **2** and **3**, the affinity for both the conventional and novel isoforms of PKC was greatly reduced, as the K_i values were 10^2 to 10^3 fold higher than for analog **1**. This further differentiates the capped analogs from prototype compound **1** (Table 2).

3.4. Combination of analog 1 with a capped analog showed a synergistic antiviral effect

A matrix combination study was performed according to the method of Prichard and Shipman [20] in which multiple concentrations of compound **1** were combined with multiple concentrations of either **2** or **3** and the antiviral effect of these different combinations was quantified by MTS/PMS method. Synergy plots were then generated at the 95% confidence level with the MacSynergy II template [21]. Surprisingly, both combination studies (**1** with **2** and **1** with **3**) resulted in a robust synergistic antiviral effect (Fig. 5).

4. Discussion

Protein kinase C (PKC) is a family of related serine/threonine kinases that regulate many cellular processes such as proliferation, differentiation and apoptosis [23,24] through phosphorylation of downstream signaling factors [23,25]. Most PKCs are activated by diacylglycerol (DAG), which is produced via the hydrolysis of phosphatidylinositol 4,5-bisphosphate by the phospholipase C enzyme.

Bryostatin-1 is a potent activator of PKCs due to its structural similarity to DAG [25].

Bryostatin-1 has been reported as a promising compound to eradicate latent HIV viral reservoirs [10,26]. The anti-HIV activity of bryostatin-1 was shown to be dependent on

both PKC α and PKC δ [10]. We previously reported that novel salicylate-based bryostatin

analogs protect cells from chikungunya virus (CHIKV)-induced cell death. Interestingly,

capping the hydroxyl group at position C26, a crucial position for binding to PKC, did not

reduce the anti-CHIKV activity of the scaffold. This gave an initial indication of the

involvement of a PKC-independent pathway in the anti-CHIKV activity of these

compounds. This is confirmed in the present study by detailed comparative analysis of

the anti-CHIKV activity of the non-capped analog (**1**) and the capped analogs (**2** and **3**).

Compound **1** was selected as it was the most active of the salicylate-based analogs in the

inhibition of CHIKV-induced cell death. Because the uncapped parent precursor of **2** and

3 was cytotoxic and exhibited minimal selectivity against CHIKV, it was not studied

further. The differently capped analogs **2** and **3** provided comparable cell protective

effects competitive with the best leads to date, suggesting that variation in the ester

versus ether capping groups which did significantly diminish PKC affinity did not affect

cell protective activity. As expected from studies on the importance of C26 for PKC

binding, both capped analogs exhibit significantly reduced PKC-affinity. That these very

270 different capping groups gave similar cell protective effects indicates that variations in
271 the capping group is tolerated for protective activity but not for PKC binding.

272 We first demonstrated that these compounds selectively inhibit CHIKV with no or little
273 antiviral activity against two other related alphaviruses. In addition, the compounds
274 reduced virus yield in CHIKV-infected cells in a dose-response manner, indicating that
275 these compounds are not merely cytoprotective, but has a selective inhibitory effect on
276 CHIKV replication. Delay of treatment and CHIKVpp entry assays showed that
277 compound **1** inhibit CHIKV replication both at early and late stages, while the capped
278 analogs only interfere with a later stage. This provided a first evidence that the non-
279 capped analog may have an additional or different mechanism of action.

280 To further explore the involvement of PKC activation in the anti-CHIKV effect of these
281 compounds, a combination study was performed with PKC inhibitors. The activity of
282 compound **1** is counteracted by addition of either a pan or selective classical PKC
283 inhibitor while that of the capped analogs is not. This implicates, at least partially, PKC
284 activation in the mechanism of action of analog **1**, whereas the anti-CHIKV activity of the
285 capped analogs appears to be independent of PKC. This finding also matches with the
286 fact that the capped analogs showed markedly reduced affinity to conventional and
287 novel PKC isoforms in a heterogeneous competitive binding assay.

288 To elucidate whether the capped and non-capped analogs have different mechanisms of
289 action, a matrix combination study was performed according to the method of Prichard
290 and Shipman [20]. Remarkably, the combination of compound **1** with either capped
291 analogs resulted in a pronounced synergistic antiviral effect. This provides strong
292 evidence that compound **1** and the capped analogs **2** and **3** have different but inter-
293 related mechanisms of action.

294 Collectively, salicylate-based bryostatin analogs have been developed that inhibit CHIKV
295 replication through a novel, yet still elusive, non-PKC dependent pathway. Currently, it
296 remains unclear whether compound 1 shares the same elusive mechanism of action as
297 compounds 2 and 3 in addition to modulation of PKC, or whether the capped and non-
298 capped scaffolds target unique but intertwined mechanisms of action. To this end,
299 further studies are ongoing to unravel the precise molecular mechanism by which the
300 capped bryostatin analogs inhibit CHIKV replication. Unveiling the identity of that
301 molecular target may reveal a promising new druggable pathway in the quest for
302 inhibitors of CHIKV replication.

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Figures

Fig. 1. Bryostatin 1, salicylate-derived analog 1, and C26-capped analogs 2 and 3.

Fig. 2. Bryostatin analogs (1, 2, and 3) are specific and selective inhibitors of CHIKV replication. **A)** Dose-response effect of analogs 1, 2 and 3 on CHIKV-induced cell death in Vero A cells as quantified by the MTS/PMS method. **B)** Dose-response effect of analogs 1, 2, and 3 on cell viability of Vero A cells as quantified by MTS/PMS method. **C)** Dose-response effect of analogs 1, 2 and 3 on the release of CHIKV particles in the supernatant by treated, infected Vero A cells as determined by qRT-PCR (RNA). **D)** Dose-response effect of analogs 1, 2 and 3 on the release of CHIKV particles in the supernatant by treated, infected Vero A cells as determined by titration for infectious progeny virus particles (TCID₅₀/ml). Data presented are mean values $\pm$ SD of at least three independent experiments.

Fig. 3. Bryostatin analogs (1, 2, and 3) differently affect the CHIKV replication cycle. **A)** Delay-of-treatment study was done by adding 15 μ M of analog 1, 20 μ M of analog 2 or 20 μ M of analog 3 to Vero A cells prior to (-2h), at the time of (0h), or after infection (2, 4, and 6 h) with CHIKV strain 899 (MOI=1). After 24h of infection, the intracellular viral RNA was quantified by qRT-PCR. Arbidol (75 μ M) and T-705 (300 μ M) were included as reference compounds for early and late stage inhibition, respectively. **B)** Evaluation of the dose-response effect of analogs 1, 2 and 3 on the entry of CHIKV pseudoparticles (CHIKVpp) in BGM cells. On day 3 post-infection, the firefly luciferase signal in the cellular lysate was quantified. Arbidol was included as early-stage reference compound. Data are mean values $\pm$ SD of at least three independent experiments.

Fig. 4. Dose-response effect of PKC inhibitors on the antiviral activity of compound 1 and the capped analogs. The dose-response effect of two PKC inhibitors **A)**

sotrastaurin (pan-PKC inhibitor) and **B**) Gö6976 (conventional-selective PKC inhibitor) was assessed in the presence of a fixed concentration of either compound **1** (12 μ M), **2** (12 μ M), or **3** (12 μ M) which, as such, results in 100% inhibition of virus-induced cell death. Subsequently, the cells were immediately infected with CHIKV (MOI 0.001). T-705 (60 μ M) was included as a reference compound. On day 5 after infection, a time at which untreated, infected cells show signs of massive cytopathic effects, cell viability was quantified using the MTS/PMS method. Data presented are mean values $\pm$ SD of at least three independent experiments (** $P < 0.01$, *** $P < 0.001$, unpaired T-test).

Fig. 5. Combination study of compound 1 with either capped analog 2 or 3. A combination matrix of 2-fold dilution series of compound 1 (X-axis) and **A**) compound 2 or **B**) compound 3 (Y-axis) were compiled and added to the cells, after which they were immediately infected with CHIKV (MOI=0.001). After 5 days of incubation, a time at which untreated, infected cells show massive signs of virus-induced cytopathic effects, the antiviral effect of treatment was quantified by the MTS/PMS method (Promega). Data were analyzed at the 95% confidence level with the MacSynergy II template based on the method of Prichard and Shipman [20,21]. Values under the zero plane indicate antagonistic activity, values in the zero plane indicate additive activity, and values above the zero plane indicate synergistic activity. Data are mean values of three independent experiments.

Table 1: Effect of bryostatin analogs on the replication of selected alphaviruses in Vero A cells

Compound	*CC ₅₀ ± SD (μM)	*EC ₅₀ ± SD (μM)		
		CHIKV-899	SINV	SFV
1	> 50 ^a	4.0 ± 0.4 ^a		
		2.1 ± 0.1 ^b	32 ± 10 ^a	> 50 ^a
		2.0 ± 0.4 ^c		
2	> 50 ^a	8.0 ± 0.4 ^a		
		4.6 ± 0.8 ^b	43 ± 1.0 ^a	> 50 ^a
		4.5 ± 1.0 ^c		
3	> 50 ^a	7.5 ± 2 ^a		
		6.0 ± 0.4 ^b	> 50 ^a	> 50 ^a
		4.1 ± 0.2 ^c		

*Data are obtained from at least three independent experiments.
Values were determined by **a)** CPE reduction assay (MTS readout), **b)** qRT-PCR or **c)** end-point titration assay

Table 2: Binding affinity of salicylate-derived bryostatin analog 1 and capped analogs 2 and 3 to conventional and novel PKC isoforms

PKC isoform		PKC binding affinity, K_i^* μ M		
		1	2	3
conventional	PKC α	0.0098	6.4	3.5
	PKC β I	0.0049	8.2	8.5
	PKC γ	0.039	3.1	7.3
	PKC δ	0.0013	1.2	1.0
novel	PKC ϵ	0.0049	9.4	5.6
	PKC η	0.123	1.7	0.96
	PKC θ	0.0039	1.1	10.0

* K_i values from a heterogeneous competitive binding assay against [3 H]-phorbol dibutyrate

Figure

Figure

Figure

Figure

Figure

A

B