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Inhibition of Chikungunya Virus-Induced Cell Death by Salicylate-Derived Bryostatin Analogues Provides Additional Evidence for a PKC-Independent Pathway

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Abstract

Chikungunya virus (CHIKV) has been spreading rapidly, with over one million confirmed or suspected cases in the Americas since late 2013. Infection with CHIKV causes devastating arthritic and arthralgic symptoms. Currently, there is no therapy to treat this disease, and the only medications focus on relief of symptoms. Recently, protein kinase C (PKC) modulators have been reported to inhibit CHIKV-induced cell death in cell assays. The salicylate-derived bryostatin analogues described here are structurally simplified PKC modulators that are more synthetically accessible than the natural product bryostatin 1, a PKC modulator and clinical lead for the treatment of cancer, Alzheimer's disease, and HIV eradication. Evaluation of the anti-CHIKV activity of these salicylate-derived bryostatin analogues in cell culture indicates that they are among the most potent cell-protective agents reported to date. Given that they are more accessible and significantly more active than the parent natural product, they represent new therapeutic leads for controlling CHIKV infection. Significantly, these analogues also provide evidence for the involvement of a PKC-independent pathway. This adds a fundamentally distinct aspect to the importance or involvement of PKC modulation in inhibition of chikungunya virus replication, a topic of recent and growing interest.

Graphical abstract

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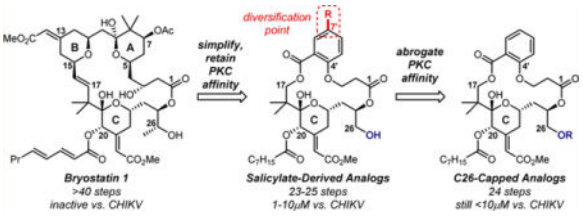
CHIKV CPE Reduction Assay. Assay protocol is described in the preceding article in this issue.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01017. Reproduction of Figure 2 with standard deviations included; characterization data and methods of prepara tion for all novel compounds (PDF)

Notes

The authors declare no competing financial interest.



Chikungunya virus (CHIKV) is an arbovirus of the *Alphavirus* genus (Togaviridae family) that has recently become an imminent threat in the Americas.[1] This mosquito-borne disease was limited for years to minor outbreaks in West Africa, but several recent epidemics have heightened the awareness surrounding CHIKV (e.g., India/Pacific Ocean, 2006–2007, estimated 1.4–6.5 million cases; Malaysia, 2009, 3000–42 000 cases). The mortality rate is estimated to be 1:1000, with most deaths occurring in neonates, the elderly, and those weakened by other health issues. Notably, the risk of these outbreaks is no longer geographically limited, as CHIKV has recently mutated to adopt a more ubiquitous mosquito vector.[1] Symptoms of the acute infection (onset 4–7 days postinfection) are debilitating arthritis and arthralgia, typically coupled with high fever, vomiting, and myalgia. This period generally lasts for 1–10 days, but the chronic phase of the disease, primarily consisting of arthralgia, can persist for months or even years.[1e] While over-the-counter anti-inflammatory drugs can be used to alleviate the symptoms, unfortunately, there is currently no antiviral therapy available to treat the infection itself. Several vaccination strategies have performed well in phase I and II clinical trials, offering promise for the prevention of infection and curtailment of future outbreaks.[2] With the recent spread of CHIKV into the Americas[3] (sporadic cases prior to 2013, over one million cases by the end of 2014), there is an increasing interest in the development of agents and strategies for the prevention and treatment of this disease.[1]

Recently, several protein kinase C (PKC) modulators based on tigliane[4] and aplysiatoxin[5] scaffolds were shown to inhibit virus-induced cell death when administered to buffalo green monkey (BGM) cells infected with CHIKV. In the preceding article in this issue, analogues of the bryostatin 1 (henceforth bryostatin) scaffold developed in the Wender group were also shown to be effective inhibitors, with one agent being the most potent compound studied to date in such assays. Intriguingly, bryostatin itself, a potent *pan*-conventional and novel PKC isoform modulator, when tested in the same assay, was inactive. For some time, the Wender group has been interested in bryostatin, specifically in generating more accessible and efficacious analogues, given that the natural product itself is neither evolved nor optimized for therapeutic use.[6] Bryostatin provides a superb starting point for developing such agents through synthesis-informed design.[7,8] In 2014, the Wender group disclosed a new class of designed analogues in which the complex A/B-ring system of bryostatin is replaced with simple salicylate-derived fragments[9] (see Figure 1). This enabled the synthesis of compounds that retained high affinity (nanomolar) for PKC isoforms, yet required >10 fewer synthetic operations to prepare than any of the reported total syntheses of natural bryostatins. We report here the evaluation of these new analogues against CHIKV.

RESULTS AND DISCUSSION

Notwithstanding current clinical interest in bryostatin, its supply is uncertain and limited by its low natural availability and environmental issues associated with massive harvesting of coastal marine organisms. The GMP production of bryostatin in the early 1990s produced only 18 g from 14 tons of source organism.[13] Aquaculture has failed[14] and biosynthesis is still a work in progress.[15] Syntheses of natural bryostatins are improving but have yet to positively impact the clinical supply or further clinical advancement. Using a function-oriented design and synthesis approach,[7] the Wender group has designed bryostatin analogues (bryologs) that are synthetically more accessible, superior to bryostatin in cell culture, animals, and primary human cells, and better tolerated.[8,16] This preliminary evaluation of the anti-CHIKV activity of the newest bryologs[9] indicates that they represent promising leads, on par with the best leads reported to date (EC_{50} of ca. 1 μM; see Figure 1).

While the parent analogue (**1**) showed little selectivity with nearly equal EC_{50} and CC_{50} values (measures of the cell-protective effect and compound-induced adverse effects on the host cells, respectively), essentially all of the remaining analogues showed low single-digit micromolar EC_{50} values with no observ able toxicity within the tested concentration range. As analogues with electron-rich arene substituents could exhibit toxicity issues upon moving to *in vivo* screens, these are not considered ideal lead compounds but were included to explore structure–activity relationships in this evaluation. The positioning of the alkoxy moieties in analogues **4**–**9** did not affect either the cell-protective activity or the cytotoxicity. Gratifyingly, some of the more hydro-philic analogues containing electron-deficient arene moieties also demonstrated high potency and low toxicity, such as isopropyl benzoate analogue **14** and sulfonamide analogues **16** and **17**. Curiously, methyl benzoate analogue **13** and diethyl benzamide analogue **15** were as potent as the slightly more lipophilic isopropyl benzoate analogue **14**, yet were significantly more toxic. By far the most intriguing result, however, was the performance of C-7′-(5-indolyl) analogue **11**. This compound exhibited very low affinity for PKC (qualitatively ~1 μM), yet is essentially as efficacious as any of the analogues that exhibit single-digit nanomolar affinities for PKC. Given the fact that the observed protective effects are occurring (at best) in the single-digit micromolar range, it is not unreasonable that even a low-affinity ligand such as indole analogue **11** could be acting through a PKC-mediated mechanism. However, the similar levels of performance across the entire salicylate-derived panel of analogues (despite varied PKC affinities) and the complete lack of activity seen with bryostatin itself (a high-affinity PKC modulator) suggest that these compounds might operate in part, if not predominantly, through a PKC-independent pathway.

Previous work on the tigliane scaffold might support the possibility of a PKC-independent mechanism as well. The most recent report in the field[4c] details the antiviral activity of several tigliane-, ingenane-, and daphnane-based scaffolds, all of which either are or resemble known PKC C1 domain binding ligands, leading the authors to reasonably implicate PKC as a potential target controlling this activity. However, several systems are acylated at the C-20 position (see Figure 3).[18] The C-20 hydroxy of tiglianes and related ligands or the C-26 hydroxy of bryostatin-based scaffolds is required for PKC activity,[1] and acylation has previously been shown to fully abrogate affinity for PKC.[19] In fact, the

addition of a homovanillate ester at C-20 (as is seen between the daphnanes resiniferinol and resiniferatoxin) is known to change the intracellular target from PKC to TRPV1, an enzyme involved in the nociceptive sensation of heat and pain. It is plausible that these C-20 acyl species are behaving as pro-drugs given that the assay involves a 5-day incubation period. However, subsequent data would suggest that the critical intra cellular interaction is indeed PKC-independent (*vide infra*).

To further explore whether activity might arise in part from a PKC-independent pathway, a number of analogues that lack the PKC binding features were prepared. It was assumed that any productive performance by these compounds within the assay would provide evidence for the existence of a PKC-independent pathway that is involved in the antiviral effect of these compounds.

The first class of analogues borrows from the tigliane C-20-capping strategy, placing different functional groups on C-26 of the parent salicylate-derived analogue 1 (Figure 4A), thereby blocking the hydrogen-bond donor role of the C-26-OH. The acetate (**19**) was made for comparison to the trigocherrins[18] and other C-20-capped scaffolds, but this still carries some potential to behave as a pro-drug, as the acetate could be converted to the hydroxy group by hydrolysis or esterases. The C-26 benzyl carbamate and C-26 methoxy analogues (**20** and **21**) are obviously not as susceptible to these modes of degradation and thus should not release any free active C-26 hydroxy compound (**1**) under the assay conditions. The parent system was chosen, as this would allow one to see any beneficial effects on toxicity as well as efficacy. The capping strategy significantly reduced affinity for PKC (see Figure 4A), as the K_i values for analogues **19**–**21** were ~2 orders of magnitude higher than parent analogue **1**; relative to lead analogues from Figure 2 or from the preceding article, the discrepancy in binding efficiency approaches or even exceeds 3 orders of magnitude. Significantly, not only did these analogues perform well in the cell protective assay, they outperformed the parent C-26-hydroxy analogue by both improving potency and reducing toxicity. As with the C-7′-(5-indolyl) analogue **11**, the demonstration that capped analogues **19**–**21** provide cell-protective effects competitive with the best leads to date while exhibiting low affinity to PKC strongly suggests that a PKC-independent mechanism for evading CHIKV-mediated cell death exists and is at least partially accessible with these particular compounds. Despite the fact that our studies were originally directed at PKC modulators, the suggestion of an alternative pathway could prove highly beneficial for the development of new antiviral leads with new modes of action.

Beyond the C-26-capped full analogues, a number of northern-fragment-only analogues were also studied. It was considered plausible that the biphenyl subunit itself might be contributing to the observed activity. Thus, analogues **22**–**24** (see Figure 4B) were prepared to explore that possibility. However, these compounds were completely inactive in the anti-CHIKV assay, indicating that some feature of the macrocycle (be it the substitution pattern or perhaps merely the increased rigidity) is required for the observed activity.

The analogues disclosed here are among the most potent compounds reported thus far in the CHIKV cell protective assay. The activity observed with C-26-capped analogues implies that PKC might not be involved in the observed cell-protective effect, though our findings do not

rule out PKC as a target for other scaffolds (e.g., tiglianes, aplysiatoxins) or even for the salicylate-derived compounds with a free C-26 hydroxy. These results do, however, suggest that a PKC-independent pathway might significantly contribute to anti-CHIKV activity. The existence of such an alternative biological effector route is itself of high interest and could figure in the development of more effective agents. Further biochemical analyses of the mode of action of these new agents are under way and will be reported in due course. These efforts lay the foundation for studies on animal models of disease and ultimately contribute to the design of superior leads for the treatment of CHIKV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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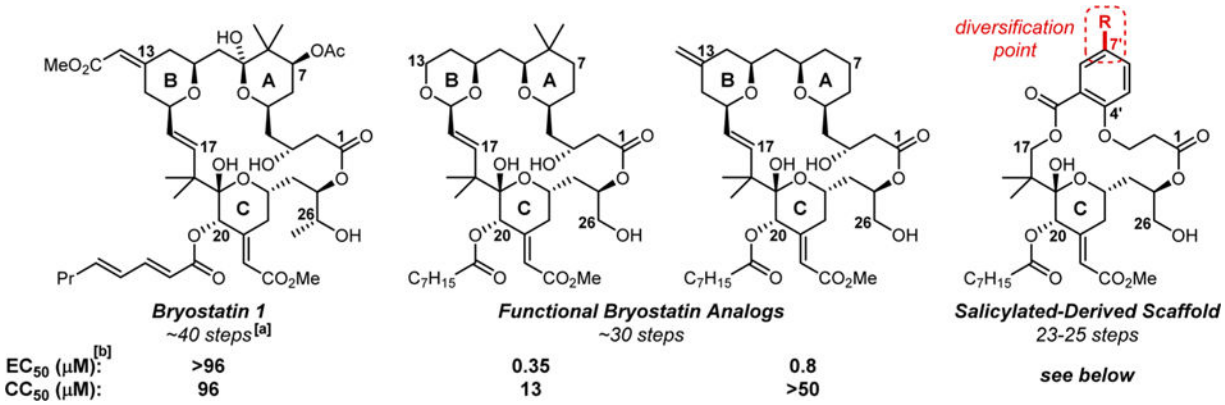

Figure 1.

Bryostatin, prior bryostatin-inspired leads, and general structure of salicylate-derived scaffolds to be evaluated for anti-CHIKV activity. [a]While the current total synthesis of bryostatin 1 requires ca. 55 steps,[10] the groups of Wender[11] and Krische[12] have provided syntheses of natural, highly active bryostatins, differing from bryostatin 1 only by ester variations, that require only ~40 steps and could likely be adapted to afford bryostatin 1; [b]EC_{50} and CC_{50} values taken from the preceding article: EC_{50} = concentration of compound that reduces the CHIKV-induced cytopathogenic effect by 50%; CC_{50} = concentration at which cell viability is 50% relative to untreated cells as a result of treatment with compound alone.

Compound	Bryo 1	1	2	3	4	5	6	7	8	9
R[a]	-	H	Br		OMe	OMe	MeO OMe	OiPr	OiPr	iPrO OiPr
PKCδ[b] K_i (nM)	1.1	18	28	9.1	4.0	5.1	1.3	2.7	3.4	1.9
CHIKV EC_{50} (μM)[c]	>50	6.7	5	2.0	3.7	2.0	1.4	2.8	2.0	3.5
CHIKV CC_{50} (μM)[c]	>50	12	26	12.4	>50	>50	>50	>50	>50	>50

Compound	10	11	12	13	14	15	16	17	18
R	C_6H_{13}	HN	H N	CO_2Me	CO_2iPr	O NEt_2	O O NEt_2	O O NEt_2	O–N
PKCδ K_i (nM)	4.4	>250	12	3.3	7.6	7.9	5.9	3.6	2.8
CHIKV EC_{50} (μM)	4.	3.7	3.2	2.	3.4	>5.9[d]	2.2	3.1	2.3
CHIKV CC_{50} (μM)	>50	41	9.5	16	>50	5.9	>50	>50	8.6

Figure 2.

In vitro data for salicylate-derived bryostatin analogues against CHIKV. Summary of cell-protective activities seen with salicylate-derived bryostatin analogues as compared to PKC affinity. [a]R represents functional group cross-coupled onto diversifiable C7′-Br scaffold 2; see Figure 1 for full structure. [b]Affinity for human PKC*δ* (obtained from Life Technologies) generated via heterogeneous competitive binding assay with [^{3}H]-PDBu.[16,17] cSee Figure 1 for description of EC_{50} and CC_{50} values, standard deviations for all values can be found in the Supporting Information. [d]Early onset of cytotoxicity precluded determination of EC_{50} value.

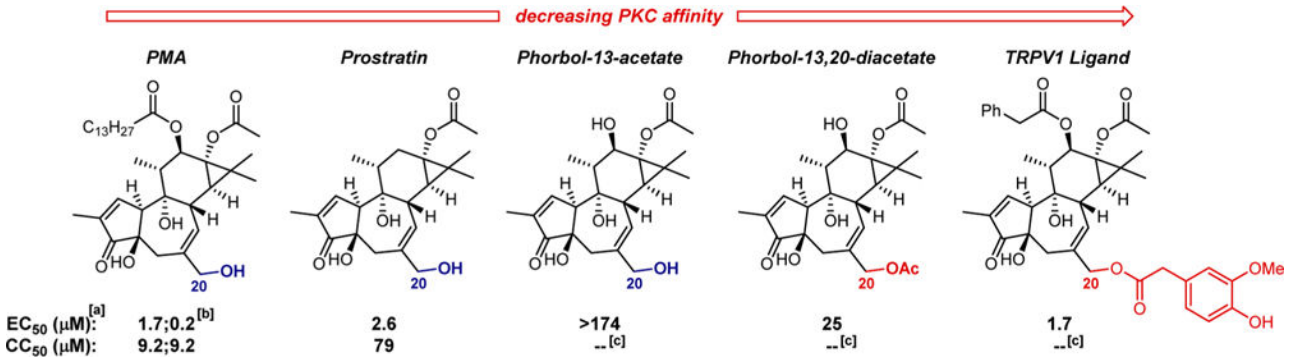

Figure 3.

Performance of other known or potential PKC C1 domain binding ligands in an anti-CHIKV *in vitro* assay. C-20 functionality is highlighted and must be an alcohol (blue) to retain affinity for PKC. [a]See Figure 1 for description of EC_{50} and CC_{50} values, with all values being taken from ref[4a] (prostratin) or[4c]; TRPV1 ligand (12-*O*-phenylacetyl-13-*O*-acetylphorbol-20-homovanillate). [b]PMA is reported at 2.9 nM in Vero A cells,[4a] but when including PMA as an internal control in the present study, which was performed in BGM cells, the above values were obtained. [c]Ref[4c] does not supply CC_{50} values but provides selectivity indices instead (SI = CC_{50}/EC_{50}), and these are N/A, 1.7, and 14, respectively.

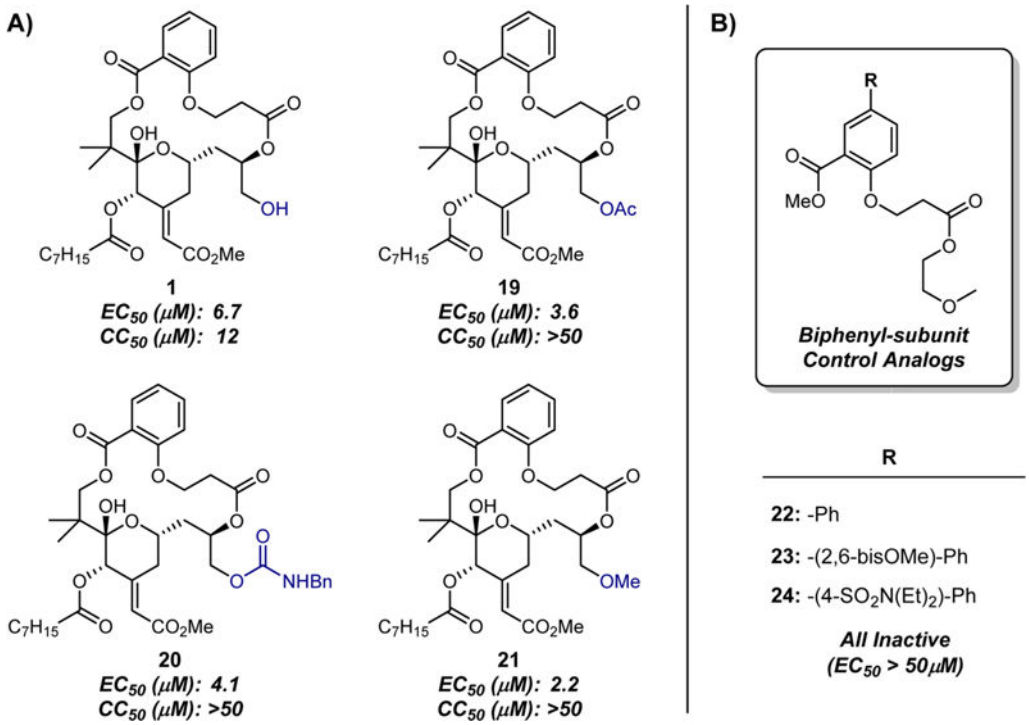

Figure 4.

PKC-independent salicylate-derived analogues. Two classes of control analogues (those with no affinity for PKC) were prepared and evaluated in the CHIKV cell protective assay: (A) C-26-capped variations on the original salicylate-derived scaffold **1** (while C-26-OAc analogue **19** could putatively behave as a prodrug, the C-26 benzyl carbamate **20** and C-26-methoxy **21** analogues are not susceptible to hydrolysis under the assay conditions; K_i values for PKCδ: **19** = 1.2 μM, **20** = 0.88 μM, **21** = 1.0 μM. (B) Northern-fragment-only analogues to determine the importance of the macrocycle; see text and Figure 1 for description of EC_{50} and CC_{50} values. Standard deviations for all values can be found in the Supporting Information.