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ORIGINAL ARTICLE

Antiviral effect of theaflavins against caliciviruses

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Caliciviruses are contagious pathogens of humans and various animals. They are the most common cause of viral gastroenteritis in humans, and can cause lethal diseases in domestic animals such as cats, rabbits and immunocompromised mice. In this study, we conducted cytopathic effect-based screening of 2080 selected compounds from our in-house library to find antiviral compounds against three culturable caliciviruses: feline calicivirus, murine norovirus (MNV) and porcine sapovirus (PoSaV). We identified active six compounds, of which two compounds, both related to theaflavins, showed broad antiviral activities against all three caliciviruses; three compounds (abamectin, a mixture of avermectin B1a and B1b; avermectin B1a; and (−)-epigallocatechin gallate hydrate) were effective against PoSaV only; and a heterocyclic carboxamide derivative (BFTC) specifically inhibited MNV infectivity in cell cultures. Further studies of the antiviral mechanism and structure-activity relationship of theaflavins suggested the following: (1) theaflavins worked before the viral entry step; (2) the effect of theaflavins was time- and concentration-dependent; and (3) the hydroxyl groups of the benzocycloheptenone ring were probably important for the anti-calicivirus activity of theaflavins. Theaflavins could be used for the calicivirus research, and as potential disinfectants and antiviral reagents to prevent and control calicivirus infections in animals and humans.
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INTRODUCTION

Caliciviruses are non-enveloped viruses that have a single-stranded RNA genome. They are widely found in animals and humans. The family Caliciviridae is currently divided into five genera: *Vesivirus*, *Lagovirus*, *Norovirus*, *Sapovirus*, and *Nebovirus*.[1–4] Infections with noroviruses (NoVs) and sapoviruses (SaVs) are a global public health concern because they cause acute gastroenteritis outbreaks in people of all ages, and infections are difficult to prevent and control.[5,6] Efficient cell culture systems for human NoVs and SaVs have not been established. Instead, feline calicivirus (FCV; genus *Vesivirus*), murine norovirus (MNV; genus *Norovirus*) and porcine sapovirus (PoSaV; genus *Sapovirus*) have been used to investigate disinfection and survival in environmental studies because they grow well in cultured cells.[7] In this study, we conducted cytopathic effect (CPE)-based screening of an in-house library to identify anti-calicivirus compounds, and we found that theaflavins showed potent antiviral activities against three culturable caliciviruses, FCV, MNV and PoSaV. Further evaluation of theaflavins using FCV indicated their antiviral mechanism and structure-activity relationship.

MATERIALS AND METHODS

Materials

Chemical compounds. From our in-house chemical compound library, 2080 compounds were randomly selected. Theaflavin monogallates and theaflavin digallate for screening were purchased from MicroSource (Ann Arbor, MI, USA). Abamectin and avermectin B1a were purchased from Wako Pure Chemical Industries (Osaka, Japan). (−)-Epigallocatechin gallate (EGCG) hydrate and tannic acid were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. 5-Bromo-*N*-(6-fluorobenzo[*d*]thiazol-2-yl)thiophene-2-carboxamide (BFTC) was synthesized as described previously.[8] For further experiments, theaflavin (TF1), theaflavin-3-*O*-gallate (TF2A), theaflavin-3′-*O*-gallate (TF2B) and theaflavin-3, 3′-*O*, *O*-digallate (TF3) were purchased from Nagara Science (Gifu, Japan). Tea extracts containing 40% theaflavins and containing 90% theaflavins (obtained by removing caffeine and catechins from the tea extract containing 40% theaflavins) were purchased from Yaizu Suisankagaku Industry (Shizuoka, Japan). Stock solutions of each compound were prepared in DMSO and kept at −20 °C. Appropriate dilutions were freshly prepared just prior to each assay.

Preparation of acetylated theaflavins. Pyridine was added to the solution of tea extract containing 90% theaflavins (10 mg) in acetic anhydride and the solution was stirred for 3 h at room temperature. MeOH was added to decompose excess acetic anhydride and the solution was concentrated. The residue was

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purified by silica-gel column chromatography (eluent: EtOAc/n-hexane = 1:1) to give acetylated theaflavins (12 mg) as light-yellow powder. The product was characterized by LC/MS analysis on a system consisting of a sample manager (2767, Waters, Milford, MA, USA), photodiode array detector (2996, Waters) and controller (600, Waters). ESI/MS spectra were recorded at 60 eV on a mass spectrometer (ZQ2000, Waters). A Cosmosil 5C18-ARII column (2.0 × 50 mm, Nacalai Tesque) was used with a linear gradient of CH_3CN containing 0.05% (v/v) trifluoroacetic acid (TFA) at a flow rate of 1 ml min^{-1}, and the eluting products were detected by UV at 254 nm. The product was a mixture of multiple acetylated theaflavins. The nonaacetylated theaflavin monogallate LC/MS spectrum suggested that the nine hydroxyl groups of the main product were acetylated. ESI/MS *m/z* 1118 $[M+H+Na]^{2+}$.

Viruses and cells. FCV strains (strain FCV-2280 and strain F-9, ATCC cat. code; VR-2057 and VR-782, respectively, Manassas, VA, USA) were propagated in CRFK cells (ATCC cat. code; CCL-94) cultured in MEM supplemented with 5% fetal bovine serum (FBS; heat-inactivated at 56 °C for 30 min), antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B), 1000 mg l^{-1} D-glucose, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid and 10 mg l^{-1} phenol red at 37 °C in a humidified atmosphere of 5% CO_2. The virus was titrated in CRFK cells with a conventional assay as described previously.[9]

MNV (strain S-7, kindly provided by Professor Yukinobu Tohya, Department of Veterinary Medicine, Nihon University, Kanagawa, Japan) was propagated in RAW264.7 cells (ATCC cat. code; TIB-71) cultured in DMEM supplemented with 10% FBS (heat-inactivated at 60 °C for 1 h), antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B), 4500 mg l^{-1} D-glucose, 4 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate and 15 mg l^{-1} phenol red at 37 °C in a humidified atmosphere of 5% CO_2. The virus was titrated in RAW264.7 cells with a conventional assay as described previously.[10]

PoSaV (strain Cowden) were propagated in LLC-PK cells (ATCC cat. code; CL-101) cultured in MEM supplemented with 5% FBS without heat inactivation, antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B), 0.1 mM non-essential amino acid and 10 mg l^{-1} phenol red at 37 °C in a humidified atmosphere of 5% CO_2. The virus was titrated in LLC-PK cells as described previously.[7]

Measurement of antiviral activity

Method A: CPE-based screening. Test compound solution (50 μM, 120 μl) diluted in FBS-free cell culture maintenance medium (MM) was mixed with FCV strain FCV-2280 or MNV (120 μl) corresponding to ~100 $TCID_{50}$ (50% tissue culture infectious dose) per 50 μl and incubated for 30 min in 96-well plates at 37 °C in 5% CO_2. Cells were exposed to 100 μl of the mixture. After incubation for 1 h, the mixture was removed and replaced with MM for CRFK or 10% FBS including growth medium as described above for RAW264.7 cells. The mixture was incubated for 3–7 days. For the anti-PoSaV assay, a 50 μM test compound solution (120 μl) diluted in MM was mixed with 120 μl of PoSaV corresponding to ~500 $TCID_{50}$ per 50 μl and incubated for 30 min at 37 °C in 5% CO_2. Prior to exposing the cells to the compound and virus mixture, confluent LLC-PK cells cultured in 96-well plates were washed with MM twice and incubated with MM for 2 h, and then the MM was replaced with MM containing 100 μM bile acids (100 μl). The virus/compound mixtures (100 μl) were inoculated onto LLC-PK cells in 96-well plates and incubated for 1 h at 37 °C in 5% CO_2. After inoculation, the mixtures were removed and MM (150 μl) containing 50 μM bile acids was added. The LLC-PK cells were cultured for ~7 days.

Inhibition of virus infection and growth was evaluated by examining CPE with an optical microscope. Complete CPE was observed in infected untreated cells (eight wells) under the conditions used, and compounds that completely prevented CPE were considered hits. Each assay was performed in duplicate and at least twice, and the hit compounds were evaluated further in two more experiments to confirm their reproducibility. The minimal effective concentrations of the hit compounds were determined by using a dilution series of compounds (6.4–100 μM) and their cytotoxicities were evaluated by alamarBlue (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions.

Method B: $TCID_{50}$ assay with filtration for titration of polyphenols and theaflavins. A total volume of 50 μl of a mixture containing a compound in FBS-free MM and 5×10^3 to 8×10^3 $TCID_{50}$ of FCV strain F-9 was incubated at room temperature in a centrifugal filter tube (Amicon Ultra-0.5 (100 K), Merck Millipore, Darmstadt, Germany). The same amount of DMSO was used instead of compound solution as a control, resulting in 2.3% DMSO in the mixture. After 1 h, MM (450 μl) was added and the mixture was washed twice by centrifuging at 20 000 *g* for 1 min to eliminate the compound. FCV was recovered from the filter according to the manufacturer's instructions and was fivefold serially diluted with MM. CRFK cells in a 96-well plate were infected with the diluted virus solution. After 3 days, the cells were observed for CPE, and $TCID_{50}$ was calculated.

RESULTS AND DISCUSSION

Selection of compounds for antiviral screening

From our in-house library, 2080 small molecules, including natural products and synthetics were selected and screened with a qualitative antiviral activity assay. The classification of selected compounds is summarized in Supplementary Table 1.

Because several natural products have been reported as anti-calicivirus compounds,[11–13] 771 commercially available natural products were selected for screening. Of the antiviral agents usually effective for various viruses,[14–16] 312 known anti-RNA virus agents, such as anti-influenza agents and related compounds, were selected and evaluated. Twenty-nine alkylating agents were selected that were expected to inhibit virus multiplication by alkylating nucleic acids. Although the NoVs are generally resistant to detergents,[2] 30 typical detergents (for example, sodium cholate, decyl-β-D-maltopyranoside, 3-[(3-cholamidopropyl) dimethylammonio] −1-propanesulfonate) were evaluated for their anti-calicivirus activity. Furthermore, 784 synthetic compounds were selected for their structural diversity. Considering drug repurposing, 154 bioactive compounds, such as anti-inflammatory agents and anti-carcinogenic agents, were also selected.

Discovery of anti-calicivirus compounds

To obtain broad-spectrum antiviral agents, we explored compounds that inhibit CPE caused by three viruses from three genera by using a cell-based screening system (Figure 1). Each compound was mixed

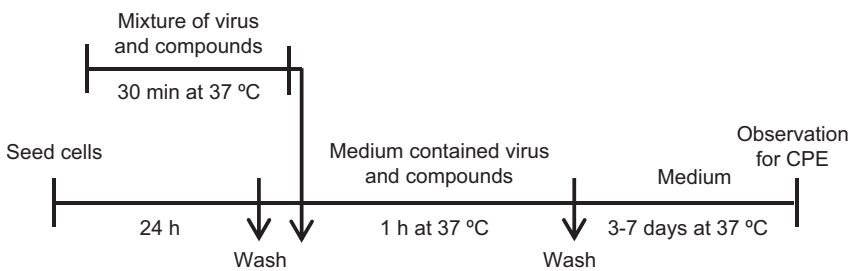


Figure 1 Protocol for cell-based screening system. CPE, cytopathic effect.

with the viruses (FCV, MNV or PoSaV) and the mixture was exposed to susceptible cells (CRFK, RAW264.7 and LLC-PK, respectively) for 1 h. After incubation for 3–7 days, cells were observed by optical microscopy to check whether CPE caused by the viruses was inhibited. Of the 2080 compounds, six compounds were identified as hits. The minimal effective concentrations of the hit compounds were determined by using a dilution series (6.4–100 μM; Table 1). Each compound showed no cytotoxicity at their minimal effective concentration (Supplementary Table 2). Theaflavins (theaflavin monogallates and theaflavin digallate), which are black tea polyphenols,[17] showed potent antiviral activity against FCV, MNV and PoSaV (Supplementary Figure 1). A synthetic heterocyclic carboxamide derivative, BFTC showed specific inhibition of CPE induced by MNV. Further structural optimization of BFTC resulted in identification of a 70-fold more potent derivative as previously reported.[8] PoSaV-induced CPE was specifically inhibited by EGCG hydrate, abamectin (a mixture of avermectin B1a and B1b) and avermectin B1a (Table 1). EGCG is the most abundant green tea catechin and shows many physiological activities, including antioxidant, antifungal, antibacterial, antiviral, anti-obesity and anti-inflammatory effects.[18,19] Although catechins and theaflavins have been reported to be equally effective antioxidants,[18] there was a difference in anti-calicivirus activity (Table 1). It was reported by Oh *et al.*[20] that a much higher concentration of EGCG (50% effective concentration (EC_{50}) = 12 mg ml^{-1}, about 26 mM calculated from the MW of EGCG) showed antiviral activity against FCV. Avermectins are a series of 16-membered macrocyclic lactones produced by fermentation of the microorganism *Streptomyces avermitilis* in the soil.[21] They are known as broad-spectrum antiparasitic agents, and are widely used in veterinary medicine and as pesticides in agriculture. The mechanism of the action of avermectins is modulating γ-aminobutyric acid-gated chloride channels.[21–23] Very recently, antiviral activities of avermectins against chikungunya and other alphaviruses were reported.[24] Our new findings suggest that avermectins also possess anti-PoSaV activity. Additional studies of avermectins are necessary to clarify their unknown antiviral mechanism. In this study, we evaluated the theaflavins further because of their potent activity against three representative caliciviruses.

Theaflavins act before the viral entry step

Theaflavins, which are polyphenols, are major active constituents of black tea, and they are produced by the enzymatic oxidation and dimerization of the green tea catechins, epicatechin, epigallocatechin, epicatechin gallate and EGCG.[17] The major components of the theaflavin series in black tea are TF1, TF2A, TF2B and TF3 (Figure 2). They have many physiological activities, such as antiviral, antioxidant and antibacterial activities.[17,18,25–27] To determine the antiviral mechanism of theaflavins, we examined antiviral activity when FCV was treated with theaflavin monogallates (mixture of TF2A and 2B) and TF3 before contact with the cells. A mixture of theaflavins and virus were filtered to remove the remaining compounds from the test solution, and then cells were infected with the treated virus. For this assay, the FCV strain F-9 was used instead of the strain FCV-2280. Tannic acid, reported to have antiviral activity against the FCV strain F-9, was used as a positive control.[28] The virus titers were determined by the $TCID_{50}$ assay. Theaflavins reduced FCV strain F-9 virus infectivity from 6.2 to 3.9 ($\log_{10}$ $TCID_{50}$ ml^{-1}) units (Table 2, entries 3 and 4 compared with entry 1). This result indicated that theaflavins inhibit virus infectivity before the FCV entry step. The antiviral activity of theaflavins was higher than that of tannic acid (Table 2).

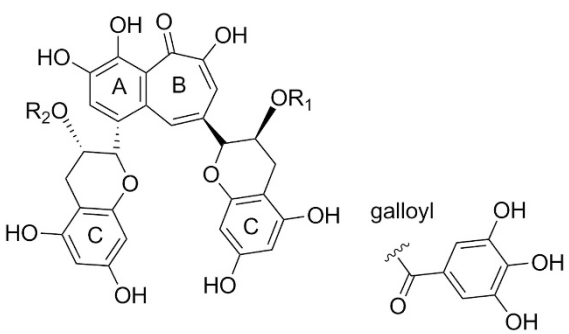
Table 1 Antiviral activity of hit compounds

	Minimal effective concentration (μM)		
Compound	*FCV*	*MNV*	*PoSaV*
Theaflavin monogallates (mixture)	100	50	50
Theaflavin digallate	25	50	25
BFTC	>100	25	>100
EGCG hydrate	>100	>100	25
Abamectin (avermectin B1a and B1b)	>100	>100	12.5
Avermectin B1a	>100	>100	25

Abbreviations: BFTC, 5-bromo-*N*-(6-fluorobenzo[*d*]thiazol-2-yl)thiophene-2-carboxamide; EGCG, epigallocatechin gallate; FCV, feline calicivirus; MNV, murine norovirus; PoSaV, porcine sapovirus.
Results from two independent experiments, data are expressed as the mean. FCV strain FCV-2280 was used.

Galloyl moiety of theaflavins is not necessary for their anti-calicivirus activity

We investigated the antiviral activity of the individual theaflavins listed in Table 2 further (entries 5–7) by conducting a $TCID_{50}$ assay with filtration. To evaluate the antiviral activity of black tea extract, a synthetic tea extract containing 40% theaflavins (mixture of TF1, 2A, 2B and 3) from green tea catechins and green tea leaf was used (entry 8). A second extract (entry 9), made by removing the caffeine and catechins from the first extract, contained 90% theaflavins. The $TCID_{50}$ assay indicated that all the theaflavins might act before the cellular entry step of FCV and showed potent anti-FCV activity at similar levels. The galloyl moiety bearing three hydroxyl groups on a phenyl ring, which is part of the structure of theaflavins, might not contribute to the antiviral activity because TF1, which lacks a galloyl moiety, showed similar antiviral activity to other theaflavins (Table 2 and Figure 2). The galloyl moiety itself did not show antiviral activity in the cell-based screening (Oka T, personal communication). This result also indicates that the galloyl moiety is not necessary for the antiviral activity of theaflavins. It has been reported that the presence of the galloyl group in TF3 is unnecessary for anti-influenza virus activity as well.[25]



TF1 (Theaflavin); $R_1 = R_2 = H$

TF2A (Theaflavin-3-O-gallate); R_1 = galloyl, $R_2 = H$

TF2B (Theaflavin-3'-O-gallate); $R_1 = H$, R_2 = galloyl

TF3 (Theaflavin-3,3'-O,O-digallate); $R_1 = R_2$ = galloyl

Figure 2 Structures of theaflavins.

Table 2 Anti-FCV activity of various theaflavins

Entry	*Compound*	*Virus infectivity (log_{10} $TCID_{50}$ ml^{-1})*
1	DMSO (control)	6.2 ± 0.4
2	Tannic acid	4.7 ± 0.1
3	TF2A and 2B (theaflavin monogallates: mixture)	3.9 ± 0.3
4	TF3 (theaflavin digallate)	3.9 ± 0.4
5	TF1	3.9 ± 0.3
6	TF2A	4.1 ± 0.4
7	TF2B	4.3 ± 0.6
8	Tea extract containing 40% theaflavins	4.0 ± 0.4
9	Tea extract containing 90% theaflavins (removal of caffeine and catechins from entry 8)	3.9 ± 0.4

Abbreviations: FCV, feline calicivirus; TCID, tissue culture infectious dose; TF1, theaflavin; TF2A, theaflavin-3-*O*-gallate; TF2B, theaflavin-3′-*O*-gallate; TF3, theaflavin-3, 3′-*O*, *O*-digallate. FCV strain F-9 was used. Results from three independent experiments, values are expressed as the mean ± s.d. Final concentration of compounds was 225 μm and virus exposure time was 60 min.

Table 3 Effect of virus exposure time on the anti-FCV activity of theaflavins

Time	*Virus infectivity (log_{10} $TCID_{50}$ ml^{-1})*
DMSO control (0 min)	5.3
5 min	3.8
30 min	3.8
60 min	3.2

Abbreviations: FCV, feline calicivirus; TCID, tissue culture infectious dose.
Final concentration of the mixture of theaflavins (Table 2, entry 9) was 1.6 mg ml^{-1} (about 2.25 mM calculated from the MW of TF2A). FCV strain F-9 was used. Results from two independent experiments, values are expressed as the mean.

Importance of hydroxyl groups for anti-calicivirus activity of theaflavins

To determine the importance of the hydroxyl groups of theaflavins, acetylated theaflavins were prepared and evaluated for anti-FCV activity (Supplementary Scheme 1). The purified mixture of theaflavins (entry 9 in Table 2) was mixed with acetic anhydride and pyridine, and a mixture of acetylated theaflavins was obtained. Although LC/MS analysis showed that the main product had nine *O*-acetylated groups, the acetylated product showed no detectable anti-FCV activity (Ohba M, personal communication). These results suggested that free hydroxyl groups of theaflavins are necessary for anti-FCV activity.

Time- and concentration-dependent antiviral effect of theaflavins

The effect of virus exposure time was evaluated by using the purified mixture of theaflavins (entry 9 in Table 2). Reduction of virus infectivity was greater when the virus was exposed for 60 min compared with 5 or 10 min (Table 3). We also examined the concentration-dependent activity by using the same mixture of theaflavins (entry 9). The virus infectivities were evaluated when the virus was exposed 0.016, 0.16 or 1.6 mg ml^{-1} of theaflavins. The effect of theaflavins was concentration-dependent (Table 4). The virus titer was reduced from 6.2 to 3.2 (log_{10} $TCID_{50}$ ml^{-1}) by 1.6 mg ml^{-1} of theaflavins.

Lack of antiviral activity in 37 other polyphenols

Polyphenols exhibit antimicrobial activity.[11] They are abundant in persimmon extract, grape seed extract and red wine, which were reported to show anti-NoV activity.[11–13] Because theaflavins are polyphenols, we investigated the activity of a panel of 37 polyphenols for potential antiviral activity against FCV strain F-9; however, they showed no inhibitory activity (Supplementary Table 3).

Table 4 Concentration-dependent activity of theaflavins against FCV

Concentration (mg ml^{-1})	*Virus infectivity (log_{10} $TCID_{50}$ ml^{-1})*
DMSO control	6.2
0.016	5.5
0.16	4.5
1.6	3.2

Abbreviations: FCV, feline calicivirus; TCID, tissue culture infectious dose.
FCV strain F-9 and mixture of theaflavins (Table 2, entry 9) were used. Results from two independent experiments, values are expressed as the mean. Virus exposure time was 60 min.

In this study, we identified theaflavins as anti-calicivirus agents acting before the viral entry step. They might affect the virus particle directly, as in our $TCID_{50}$ assay with filtration, theaflavins and virus were separated by filtration after they were mixed and the cells were not treated with theaflavins. Whereas, another hit BFTC (Table 1) inhibits intracellular MNV replication or the late stages of MNV infection.[8] Our screening system, therefore, may be useful to discover various types of antiviral compounds. Four theaflavins (TF1, 2A, 2B and 3) and their mixtures showed more potent antiviral activity than tannic acid, which has been reported as an anti-FCV agent.[28] The anti-FCV activities of the theaflavins increased proportionally with the exposure time and concentration. In this study, all of the assays were performed under the conditions of FBS-free medium. FCV infectivities without FBS and with 10% FBS were 4.5 log_{10} and 5.5 log_{10}, respectively (Supplementary Table 4). The anti-FCV activity of theaflavins was still observed when FBS-containing medium was used, although the antiviral effect was reduced. The hydroxyl groups on the benzocycloheptenone rings (A and B ring) might be related to the antiviral activity of theaflavins because of the following results: (1) the galloyl moiety is not essential (see the section 'Galloyl moiety of theaflavins is not necessary for their anti-calicivirus activity'); (2) free hydroxyl groups are necessary (see the section 'Importance of hydroxyl groups for anti-calicivirus activity of theaflavins'); (3) polyphenols with C ring-like structure (for example, equol and apigenin) had no antiviral activity (Supplementary Table 3). In contrast to our findings (summarized in Tables 1–4), Seo *et al.*[29] reported that TF and TF3 did not possess antiviral activity against FCV and MNV.[29] They also reported that kaempferol showed anti-FCV activity[29] that was ineffective in our study (Supplementary Table 3). The difference of antiviral activity may be partly explained by differences in the tested conditions (for example, virus titer, incubation time of compound and virus).

Theaflavins may be suitable as a broad-spectrum disinfectant owing to their antiviral mechanism through reduction of viral infectivity prior to the viral entry into host cells and their low bioavailability.[30] We are still investigating the details of the anti-calicivirus mechanism of theaflavins. Because theaflavins inactivated FCV (strains FCV-2280 and F-9), MNV (strain S-7) and PoSaV (strain Cowden) belonging to representative genera of *Caliciviridae*, it would be interesting to evaluate their efficacy on human caliciviruses (that is, NoV and SaV) when efficient culture systems become available in the future. Theaflavins may also be suitable for veterinary calicivirus infection control.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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