Rigid amphipathic fusion inhibitors demonstrate antiviral activity against African swine fever virus

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Abstract

Rigid amphipathic fusion inhibitors (RAFIs) are a family of nucleoside derivatives that inhibit the infectivity of several enveloped viruses by interacting with virion envelope lipids and inhibiting fusion between viral and cellular membranes. Here we tested the antiviral activity of two RAFIs, 5-(Perylen-3-ylethynyl)-arabino-uridine (aUY11) and 5-(Perylen-3-ylethynyl) uracil-1-acetic acid (cm1UY11) against African swine fever virus (ASFV), for which no effective vaccine is available. Both compounds displayed a potent, dose-dependent inhibitory effect on ASFV infection in Vero cells. The major antiviral effect was observed when aUY11 and cm1UY11 were added at early stages of infection and maintained during the complete viral cycle. Furthermore, virucidal assay revealed a significant extracellular anti-ASFV activity for both compounds. We also found decrease in the synthesis of early and late viral proteins in Vero cells treated with cm1UY11. Finally, the inhibitory effect of aUY11 and cm1UY11 on ASFV infection in porcine alveolar macrophages was confirmed. Overall, our study has identified novel anti-ASFV compounds with potential for future therapeutic developments.

INTRODUCTION

Due to its devastating impact on pig farming, African swine fever (ASF) is one of the most important viral diseases of domestic pigs, causing huge socio-economic losses in affected countries. Depending on host characteristics and the circulating viral isolate, clinical signs may vary from the highly lethal form with 100% mortality to sub-clinical and aclinical forms [1,2]. In the highly lethal form, clinical signs may include high fever, severe depression, anorexia, bloody diarrhea, cyanosis and haemorrhagic lesions. Clinical signs are accompanied by severe thrombocytopenia and lymphopenia [3,4]. Animals usually die within 10–14 days of infection. Although ASF is widespread in sub-Saharan Africa, it also circulates on the European continent following a single introduction into Georgia in the Caucasus [5]. To date, there is no effective vaccine for ASF and the control of this disease is totally reliant on early diagnosis and the application of quarantine, including slaughter of affected pigs [6,7]. Therefore, ASF poses a serious and constant risk for all European countries.

The causative agent of ASF is African swine fever virus (ASFV), a large, enveloped, double-stranded DNA virus of approximately 190 kbp. It is the sole member of the Asfarviridae family and the only known DNA arbovirus infecting different species of soft ticks (Ornithodoros spp.). In infected pigs, ASFV replicates in cells of the mononuclear phagocyte system, mostly in monocytes and macrophages, although other cell types can also be infected in later stages of the disease [8,9]. The ASFV life cycle starts with viral attachment and entry into the cells. ASFV enters the host cells by exploiting different entry mechanisms such as clathrin-mediated endocytosis and micropinocytosis [10,11]. The decapsidation and disassembly of virions occur at late endosomal compartments. Once decapsidated, ASFV particles expose the inner envelope which allows the fusion of this viral membrane with the membrane of the endosomes, and naked cores can be released into cytosol in order to start replication [12,13]. Viral DNA replication involves the nuclear phase, when relatively small DNA fragments are synthesized, and the cyttoplasmic phase, occurring in perinuclear cytoplasmic viral factories [14]. In these factories newly synthesized virions are...
assembled and then transported to the plasma membrane, where they are released by budding [15]. ASFV completes its life cycle within 18–24 h post-infection (hpi).

In the absence of vaccines, antiviral compounds attacking ASFV at any stage of its life cycle can be of great interest. In this study, we aimed to assess the effect of rigid amphipathic fusion inhibitors (RAFIs), 5-(Perylen-3-ylethynyl)-arabinouridine (aUY11) and 5-(Perylen-3-ylethynyl)uracil-1-acetic acid (cm1UY11) (Fig. 1a), on the replication of ASFV in vitro. RAFIs are a novel family of nucleoside derivatives that prevent the fusion of viral and cellular membranes [16]. They target envelope lipids to prevent the curvature changes required for the fusion process during viral entry. Although the antiviral effect of aUY11 on other virus infections has been previously studied [17], nothing is known about its antiviral activity against ASFV. Furthermore, the present work also includes, for the first time, cm1UY11, which has an acetic acid group in place of the arabinose of aUY11. Both perylene compounds are highly lipophilic, thus further supporting their high affinity to the lipid bi-layer.

Here we showed that the tested compounds were able to effectively inhibit ASFV infection through targeting early stages of the ASFV life cycle, including virus attachment and internalization.

RESULTS

Lipophilicity of compounds
Both compounds, aUY11 and cm1UY11, when distributed between water and 1-octanol showed partition ratio P>1000 (P=Co rg/C waq). No more precise determinations were done. Cytotoxicity of compounds
We tested different concentrations at which aUY11 and cm1UY11 might exhibit cytotoxic effects on Vero cells. After treatment for 96 h, which is the same duration that was used for antiviral assays, the percentage of viable cells was determined using MTT assay. Both compounds demonstrated a potent cytotoxic effect on Vero cells at 125 and 250 µM (Fig. 1b). However, the cytotoxic effect of cm1UY11 was lower than that of aUY11. Indeed, the calculation of CC50 showed that aUY11 with CC50=126.2±8.5 µM is more toxic than cm1UY11 with CC50=160.5±10.1 µM. The organic solvent DMSO had no effect on cells (data not shown). In antiviral studies, aUY11 and cm1UY11 were used at a concentration of 25 µM, which is lower than the maximum non-toxic dose (30 µM for both compounds). The cytotoxic effect of aUY11 and cm1UY11 on macrophages was less than on Vero cells, with CC50=185.6±10 µM and 214.1±5.5 µM, respectively (data not shown).

Effect of compounds on viral yield in Vero cells
To establish whether aUY11 and cm1UY11 have antiviral activity against ASFV, we first examined the antiviral effect of the tested compounds at various concentrations using CPE-based assay. As shown in Fig. 2(a), ASFV infection was inhibited by the tested compounds. The first compound, aUY11, decreased viral titres from 6.2±0.5 to 4.0±0.1 log TCID50/ml (P<0.05), reducing the ASFV yield by more than 99 % (IC50=1.6 µM; SI=78.8). This inhibition was in a dose-dependent manner. Similarly, cm1UY11 also showed antiviral activity against ASFV infection in a dose-dependent manner (Fig. 2a). It reduced the viral yield by 1.6 log (IC50 =2.3 µM; SI=69.7).

We then analysed their antiviral effect by quantifying the viral DNA in treated cells using real-time PCR. The presence of aUY11 and cm1UY11 at a concentration of 25 µM reduced viral DNA quantity by about 3.7-fold (P<0.05) and 2.4-fold (P<0.05), respectively (Fig. 2b). As expected, these results coincided with viral titres. Finally, we used a monoclonal antibody against major virus capsid protein p72 to study the number of viral factories in treated and untreated cells (Fig. 2c). Cells treated with aUY11 and cm1UY11 at a concentration of 25 µM showed a marked decrease in the number of viral factories, by 97 and 94 %, respectively (Fig. 2d). Taken together, these data demonstrated that RAFIs exert an antiviral effect on ASFV infection in vitro. Therefore, further studies were conducted.

Effect of compounds on entry of ASFV into Vero cells
To further explore the mechanism of the inhibition of ASFV by aUY11 and cm1UY11, we next determined which steps in the ASFV life cycle were inhibited by the tested compounds.
To define whether aUY11 and cm1UY11 can inhibit ASFV attachment to Vero cells, RAFIs were added at 4 °C, a condition in which virus binds to but does not enter cells. Treatment with aUY11 reduced viral titres from 6.7±0.5 to 5.1 ±0.2 log TCID_{50}/ml (P<0.05) (Fig. 3). A more significant anti-binding effect was observed when the cells were treated with cm1UY11. The ASFV titres were decreased by approximately 3.5 log (Fig. 3).

To determine whether RAFIs act at the internalization stage of infection, these compounds were added immediately after a temperature shift from 4 to 37 °C and removed after 1 h of treatment, in order to exclude their effect on other stages of infection. The results indicated that both compounds demonstrated potent effects when they were added during virus internalization. The titres were reduced from 6.5±0.1 to 3.0 ±0.8 log TCID_{50}/ml (P<0.02) and 2.7±0.1 log TCID_{50}/ml (P<0.001) upon treatment with aUY11 and cm1UY11, respectively (Fig. 3). These results suggest that the tested compounds are able to interfere with ASFV entry by inhibiting the attachment and internalization stages.

**Time-of-addition studies**

We conducted time-of-addition studies to investigate whether RAFIs are able to inhibit ASFV when they are used prior to (pre-treatment) or after (post-treatment) infection. The compounds were added at 2 and 1 h before infection and 0, 2, 4, 8 and 12 h p.i. As shown in Fig. 4, when aUY11 was added during the early stage of infection, no later than 4 h p.i., it significantly inhibited ASFV replication in Vero cells (P<0.05). The addition of aUY11 later than 4 h p.i. had no statistically significant effect on viral yield. The same results were observed for cm1UY11 (Fig. 4). These findings indicate that aUY11 and cm1UY11 may act in the early stages of ASFV infection.

**Virucidal activity of compounds**

To investigate whether aUY11 and cm1UY11 possess direct virucidal activity against ASFV, we pre-incubated the tested
compounds with the virus suspension and then diluted (20-fold) them to sub-therapeutic concentrations before adding them to Vero cells. Our results showed that both compounds exhibited a significant activity against extracellular virus (Fig. 5). The viral titres were decreased from 5.4±0.2 to 3.6±0.3 log TCID₅₀/ml and 4.3±0.2 log TCID₅₀/ml for aUY11 and cm1UY11, respectively (P<0.05).

**Effect of compounds on viral protein synthesis**

We performed immunoblotting analysis to study the synthesis of the early and late viral proteins, p30 and p72, respectively, in the presence of aUY11 and cm1UY11 (Fig. 6a). As shown in Fig. 6 (b), the amounts of early and late proteins in cells treated with aUY11 were not statistically different from untreated cells (P>0.1). In contrast, the presence of cm1UY11 during the infection had an effect on the synthesis of both p30 and p72.

**Effect of compounds on infection of ASFV in porcine alveolar macrophages**

Since both compounds demonstrated potent antiviral activity against strain ASFV BA71V in Vero cells, we conducted additional experiments to define the inhibitory activity of aUY11 and cm1UY11 against the virulent ASFV strain in macrophages, the natural host cells. As shown in Fig. 7(a), viral titres were decreased from 6±0.2 to 3.7±0.2 log HADU₅₀/ml and 4±0.2 log HADU₅₀/ml in the presence of aUY11 and cm1UY11, respectively (P<0.01). This inhibition was in a dose-dependent manner. The IC₅₀ of aUY11 and cm1UY11 was 5.8 and 3.6 µM, respectively.

We also tested whether aUY11 and cm1UY11 are able to block ASFV attachment to or internalization into porcine macrophages. In contrast to the results from Vero cells, aUY11 and cm1UY11 did not exhibit inhibitory effect on ASFV attachment to macrophages (Fig. 7b). However, when cells were exposed to the test compounds at the internalization stage of ASFV, the viral yields were reduced from 6.7±0.6 log HADU₅₀/ml to 3.2±0.3 log HADU₅₀/ml and 3.7±0.3 log HADU₅₀/ml for aUY11 and cm1UY11,
respectively \((P<0.05)\). Taken together, these data suggest that RAFIs act as potent inhibitors against ASFV in the natural host cells.

DISCUSSION

Since all attempts to develop vaccines against ASFV have failed to induce effective protection, it is reasonable to evaluate antiviral agents against this virus. It has previously been shown that certain compounds may inhibit ASFV infection \textit{in vitro}. These compounds include nucleoside analogues \cite{18}, sulfated polysaccharides \cite{19}, microalgae \cite{20}, lauryl gallate \cite{21}, small peptide inhibitors \cite{22}, resveratrol and oxyresveratrol \cite{23}, fluoroquinolones \cite{24}, apigenin \cite{25} and polyvalent 2D entry inhibitors \cite{26}. Our current study aimed to investigate the antiviral activity of two compounds from a novel family of nucleoside analogues, called RAFI, against ASFV infection \textit{in vitro}.

It has previously been reported that RAFIs inhibit the infectivity of unrelated enveloped viruses by targeting the envelope lipids to prevent the curvature changes required for the fusion of viral and cellular membranes during viral entry \cite{16, 17}. The antiviral activity of aUY11 was demonstrated against different enveloped viruses including HSV-1, HSV-2, HCV, influenza virus A and Sindbis \cite{17} and tick-borne encephalitis virus \cite{27}, but its antiviral effect on ASFV remained unknown. Furthermore, cm1UY11, which has an
acetic acid group in place of the arabinose of aUY11, has not been studied to date. However, both perylene compounds are highly lipophilic, thus further supporting their high affinity to the lipid bi-layer, including viral envelopes.

In this study, the cytotoxic effect of RAFIs on Vero cells and porcine alveolar macrophages was confirmed by quantifying cell viability with MTT assay. Our results demonstrated that cm1UY11 had a less cytotoxic effect on both cell types than aUY11, suggesting that arabinose substitution with acetic acid can result in a different biological activity. However, if cm1UY11 interacts with membrane lipids, it should demonstrate antiviral inhibition similar to aUY11. We performed the primary antiviral screen using strain ASFV BA71V, which is an excellent model for antiviral and molecular studies of ASFV infection since it has been adapted to grow in the Vero cell line [28]. Three different assays showed that both compounds significantly inhibited ASFV infection in Vero cells. Investigation into the mechanism of action revealed that the major anti-ASFV effect occurred when aUY11 and cm1UY11 were added at the internalization stage of ASFV infection. St. Vincent et al. [16] presented evidence that RAFIs inhibit virus–cell fusion by stabilizing the positive curvature of membranes by virtue of their molecular structure. Therefore, we can infer that aUY11 and cm1UY11 act on the events that are required for ASFV fusion and penetration at the endosomal membrane. Interestingly, although St. Vincent et al. [16] demonstrated that dUY11, another member of the RAFIs, had no effect on viral attachment, our experiments revealed an inhibitory effect on this step of strain ASFV BA71V infection, suggesting that intact envelope lipids are required for ASFV attachment to Vero cells.

Recently, Vignat et al. [29] showed that RAFIs may act like membrane-binding photosensitzers. These induce singlet oxygen-mediated lipid oxidation, causing changes in the biophysical properties of the viral envelope. Thus, RAFIs may exert a direct virucidal activity by causing oxidative damage to the extracellular viral particles. Indeed, the pre-incubation of strain ASFV BA71V with aUY11 and cm1UY11 resulted in a reduction in viral infectivity by more than 1 log.

We also found that the expression of early and late viral proteins was affected by cm1UY11. Early protein p30 is involved in specific interaction between ASFV and cellular receptors [30], whereas p72 is the major component of capsomeres and accounts for about one third of the protein mass of ASFV particles [31]. Thus, the antiviral activity of cm1UY11, but not aUY11, can be partially associated with a decrease in the expression of viral proteins p30 and p72 in Vero cells.

In vivo, monocytes and alveolar macrophages are the main targets for ASFV infection. Therefore, it was also important to ascertain the effectiveness of aUY11 and cm1UY11 in a relevant cell type. We used virulent strain ASFV Armenia/07 currently circulating in Eastern Europe to infect porcine alveolar macrophages. Our results showed that both compounds significantly reduced ASFV infection in macrophages at concentrations above 6.25 µM (Fig. 7a). The major inhibiting activity was observed when aUY11 and cm1UY11 were added at the internalization stage of viral infection. This finding is in complete agreement with the results for Vero cells. In contrast, no inhibitory effect on ASFV attachment to macrophages was found. Since strains ASFV BA71V and Armenia/07 have different cell tropism, this may explain the contradictory results observed in our experiments.

In conclusion, our work revealed two new nucleoside analogues that effectively inhibited ASFV infection in Vero cells and porcine alveolar macrophages. It is possible that aUY11 and cm1UY11 could serve as antiviral agents for interference with ASFV infection, because their antiviral effect likely relies on biophysical mechanisms. One factor that limits the usefulness of aUY11 and cm1UY11 is their low solubility in water. To overcome this limitation, the micellar form of perylene compounds, e.g. aUY11 and cm1UY11, in aqueous solutions containing a low percentage of DMSO, would be the first choice for future testing on pigs [32, 33]. Therefore, additional studies are needed to assess their in vivo efficacy against ASFV.

METHODS

Cells and viruses

Vero (African green monkey kidney) cells were maintained at 37 °C in Eagle’s minimum essential medium (EMEM) (Lonza, Belgium) or Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (all Sigma-Aldrich, Germany). Preparation of porcine alveolar macrophages was done as previously described [34]. Alveolar cells were cultured at 37 ºC in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. In experiments with Vero cells, the Vero-adapted strain ASFV BA71V was used. For strain ASFV BA71V, viral titration was performed by cytopathic effect (CPE-based) assay on Vero cells by 10-fold serial dilutions of supernatants. The titre was calculated by the Spearman–Kärber endpoint method and expressed as TCID⁵₀/ml. In experiments with porcine alveolar macrophages, the virulent strain ASFV Armenia/07 was used. For strain ASFV Armenia/07, viral titration was performed by haemadsorption (HAD) assay as previously described [32]. The titre was expressed as HADU⁵₀/ml.

Compounds

The aUY11 compound was prepared as previously described [16, 35], and the synthesis of cm1UY11 will be reported elsewhere (Chistov et al., in preparation). Both compounds were dissolved in dimethyl sulfoxide (DMSO) as 20 mM stock and resuspended in EMEM without FBS at the indicated concentrations for further use. At the time of
the experiments, dilutions in cell culture medium were performed with the final concentration of DMSO not exceeding 1 % (v/v).

**Lipophilicity assay**

Compounds aUY11 or cm1UY11 (2 mg) were shaken overnight at 20 °C with water-saturated 1-octanol (2.0 ml) and centrifuged. The supernatant was diluted appropriately with water-saturated 1-octanol and UV-Vis spectra were registered on a spectrophotometer. The saturated solution of a perylene compound in water-saturated 1-octanol (1.0 ml) was extracted 1, 5 and 10 times with 1-octanol-saturated water, then the residual concentration of a perylene compound in organic phase was determined as above.

**Cytotoxicity assay**

The cytotoxicity of aUY11 and cm1UY11 was evaluated in Vero cells and porcine alveolar macrophages by 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) assay. Cells in a 96-well cell culture plate (2×10^4 cell/well) were treated with increasing concentrations of the compounds ranging from 15.6 to 250 µM. Treated cells were incubated for 96 h at 37 °C in 5 % CO₂. After incubation, the medium was removed and MTT solution (Sigma-Aldrich, Germany) was added. The microplates were incubated at 37 °C for 4 h after adding MTT solution, followed by purple formazan extraction by MTT solvent. The colorimetric measurements were performed on a microplate reader at 570 nm. The percentage of viable cells was calculated for each concentration as [(OD_T/OD_C)×100], where OD_T and OD_C correspond to the absorbance of treated and control cells, respectively. The 50 % cell cytotoxicity (CC₅₀) was determined as the concentration of compounds causing 50 % cellular death. The cytotoxicity of the final concentration of DMSO was also measured.

**Yield reduction assay**

This assay was performed to quantify viable virus particles upon treatment with the tested compounds. Vero cells and porcine alveolar macrophages in a 24-well cell culture plate (2×10⁵ cell/well) were incubated with strains ASFV BA71V (0.5 TCID₅₀/cell) and ASFV Armenia/07 (0.5 HADU₅₀/ cell), respectively, and the tested compounds in decreasing concentrations. After 1 h, the virus inoculum was removed and the new medium with the tested compounds was added. Virus supernatant was collected at 96 h.p.i. and titrated by CPE-based assay and haemadsorption (HAD) assay.

**Attachment and internalization assays**

For attachment assay, Vero cells and porcine alveolar macrophages in a 24-well cell culture plate (2×10⁵ cell/well) were incubated with strains ASFV BA71V (0.5 TCID₅₀/cell) and ASFV Armenia/07 (0.5 HADU₅₀/ cell), respectively, and the tested compounds at 4 °C for 1 h, to permit binding but prevent viral internalization. Unbound virus and RAFs were then discarded and EMEM and DMEM containing 3 % FBS were added. The plate was then switched to 37 °C and incubated for 96 h. For internalization assay, Vero cells and porcine alveolar macrophages were incubated with ASFV at 4 °C for 1 h. Then, the unbound virus was discarded and the temperature was changed to 37 °C to allow virus entry to proceed. The tested compound was added at 0 h and removed at 1 h following temperature shift. The time point at which cells were changed to 37 °C was considered as 0 h. After 96 h, the virus titre was analysed by cytopathic effect assay.

**Time-of-addition assays**

For this assay, Vero cells in a 24-well cell culture plate (2×10⁵ cell/well) were tagged as –2,–1, 0, 2, 4, 8, 12 h, according to the time of ASFV infection. In pre-treatment assay, Vero cells were treated with compounds for 2 h and 1 h before infection with ASFV (0.5 TCID₅₀/ cell). In the co-treatment assay, Vero cells were treated with each compound at the same time with ASFV inoculation to the cells. In post-treatment assay, Vero cells were infected with ASFV, and the compounds were added at 2, 4, 8 and 12 h after infection. For virus control, ASFV was added to the respective wells at 0 h. The plate was then incubated at 37 °C in 5 % CO₂ for 96 h. The supernatant was collected and titrated by CPE-based assay.

**Virucidal assay**

The ASFV suspension containing 2×10⁵ TCID₅₀/well was incubated with an equal volume of each compound for 1 h at 37 °C. Then, Vero cells in a 96-well cell culture plate (2×10⁵ cell/well) were infected with the 20-fold diluted treated viral suspension to eliminate the potential effects of the tested compounds on viral entry. After 1 h adsorption at 37 °C, the cells were washed and EMEM containing 3 % FBS was added. After 96 h, the virus titre was analysed by CPE-based assay.

**Detection of viral factories**

Vero cells were grown on coverslips at 60 % confluency in DMEM and infected with ASFV (1 TCID₅₀/cell) in the absence or presence of aUY11 and cm1UY11 at a concentration of 25 µM. After 24 h.p.i., cells were fixed with 4 % paraformaldehyde in PBS for 12 min. After washing in PBS, cells were permeabilized for 15 min with PBS containing 0.1 % Triton X-100. Then, a monoclonal antibody against major virus capsid protein p72 (clone 1BC11 Ingenasa) at a working dilution of 1:1000 was used. Secondary antibody conjugated to Alexa fluor 594 was purchased from Molecular Probes, and cell nuclei were detected with Topro3 (Molecular Probes) following the manufacturer’s instructions. Coverslips were mounted onto glass slides using Pro-Long Gold (Invitrogen), examined under a TCS SPE confocal microscope (Leica) and data were analysed using Leica Confocal Software.

**Detection of ASFV DNA by real-time PCR**

Vero cells were infected with ASFV (1.5 TCID₅₀/cell) in the absence or presence of aUY11 (25 µM) and cm1UY11 (25 µM). DNA was purified from Vero cells at 16 h p.i. using the ‘Dneasy blood and tissue’ kit (Qiagen) and
following the manufacturer’s instructions. The PCR assay used fluorescent hybridization probes to amplify a region of the p72 viral gene, as described previously [36]. For amplification, 150 ng template DNA was added to the final reaction mixture of 20 µl comprising 1 µl oligonucleotide OE3F (50 pmol), 1 µl oligonucleotide OE4R (50 pmol), 10 µl PCR Premix Ex Taq™ (2X; Takara) probe and 1 µl TaqMan probe SE2 (5 pmol). Positive amplification controls were DNA extracted from mock-infected Vero cells. Amplifications were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 1 cycle at 94°C for 10 min, 45 cycles at 94°C for 15 s and 45 cycles at 72°C for 1 min.

Western blotting analysis
Vero cells seeded in 6-well plates were infected with ASFV (1.5 TCID50/cell) in the presence or absence of aUY11 (25 µM) and cm1UY11 (25 µM). After 24 h p.i., cells were dissociated in Laemmli buffer, boiled for 5 min at 95°C, electrophoresed in sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes (GE Healthcare). The membranes were incubated with mouse monoclonal antibodies anti-p30, anti-p72 or anti-tubulin (Sigma) diluted 1/1000, 1/2000 or 1/2000, respectively. Antibodies were detected with horseradish peroxidase (HRP) and conjugated secondary antibodies, and the bands obtained were detected with the ECL system (Amersham) diluted 1/1000, 1/2000 or 1/2000, respectively. Obtained were detected with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 1 cycle at 94°C for 10 min, 45 cycles at 94°C for 15 s and 45 cycles at 72°C for 1 min.

Statistics
Data are expressed as mean ±SD of three independent experiments. Data were analysed by one-way ANOVA using Graph Pad Prism 6 software. For multiple comparison, Bonferroni’s correction was applied. The CC50 was calculated by a linear regression analysis of dose–response curves generated from the data. The IC50 was calculated by a nonlinear regression analysis of dose–response curves generated from the data. P<0.05 was considered to be statistically significant.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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