In vitro antiviral and immunomodulatory activity of arbidol and structurally related derivatives in herpes simplex virus type 1-infected human keratinocytes (HaCat)

Brunella Perfetto,1 Rosanna Filosa,2 Vincenza De Gregorio,1 Antonella Peduto,3 Annalisa La Gatta,2 Paolo de Caprariis,3 Maria Antonietta Tufano1 and Giovanna Donnarumma1

Correspondence
Maria Antonietta Tufano
mariaan.tufano@unina2.it

1Department of Experimental Medicine, Section of Microbiology, Second University of Naples, Italy
2Department of Experimental Medicine, Section of Biotechnology and Molecular Biology, Second University of Naples, Italy
3Department of Pharmaceutical and Biomedical Science, University of Salerno, Fisciano, Italy

Arbidol (ARB) is an antiviral drug that has broad-spectrum activity against a number of viral infections. To date, there are no specific data regarding its effects against a herpesvirus. Here, the in vitro antiviral effect of ARB and structurally related derivatives were evaluated in HaCat cells on different steps of herpes simplex virus type 1 replication: adsorption, entry and post-entry. The simplified pyrrolidine analogue, 9a2, showed the best antiviral activity in vitro by reducing the plaque numbers by about 50 % instead of 42 % obtained with ARB at the same concentration. Furthermore, we have reported that all tested compounds evaluated for their immunomodulatory activity showed the ability to reduce the viral proteins VP16 and ICP27 and to modify the virus-induced cytokine expression, allowing the host cell a more efficient antiviral response.

INTRODUCTION
Herpes simplex virus type 1 (HSV-1) is a large, enveloped, double-stranded DNA virus that usually causes infections of the skin or mucosal surfaces, but also immunological and neurological disorders (Roizman & Knipe, 2001; Us et al., 2011; Jubelt et al., 2011; Nicola et al., 2005). During cellular infection, once the nucleocapsid gains entry to the host cell cytoplasm, the process of uncoating occurs, a step related to tegumental viral protein (VP)16 release. Immediately following uncoating, the viral synthesis stage begins (Schelhaas et al., 2003), consisting of a sequential expression of three gene classes: the immediate–early, delayed–early and late genes (Knipe et al., 2001). In addition to the transactivating activity of the virion VP16 protein-induced complex, infected cell protein (ICP) 0 expression can be modulated by a variety of host-transactivating factors, including the nuclear factor-κB (Amici et al., 2006). This activation determines the transcription of pro-inflammatory cytokine genes (Melchjorsen et al., 2003; Li et al., 2006; Keadle et al., 2000). An efficient elimination of the viral infection requires a pro-inflammatory host response and development of T helper (Th)1-type immunity (Arena et al., 2010; Lucey et al., 1996; Romagnani, 1997). Mogensen et al. (2004) demonstrated that after early induction of the pro-inflammatory host response, HSV-1 down-regulates the pro-inflammatory cytokine production through the accumulation of two VPs, ICP4 and ICP27, whose transcription is induced by the release of tegumental protein VP16. By destabilizing the mRNAs of pro-inflammatory genes, these VPs delay cytokine production to an extent that allows the virus to replicate. Moreover, viral transactivating proteins ICP0 and VP16 induce the expression of the IL-10 and transforming growth factor (TGF)-β immunosuppressive cytokines. The up-regulation in the expression of IL-10 and TGF-β induced by HSV-1 in keratinocytes can be considered part of the viral strategy to avoid local immune defence mechanisms in the skin and to promote the establishment and maintenance of infection (Zak-Prelich et al., 2001).

Arbidol (ARB), an indole drug already licensed in Russia and China, is known to have anti-influenza activity with immunomodulatory properties. It inhibits influenza virus-induced membrane fusion and may have the capacity to induce interferon release in the host peripheral blood (Leneva et al., 2009). Recent studies extended its inhibitory

Abbreviations: ARB, arbidol; FCS, fetal calf serum; GAPDH, glycer- aldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HSV-1, herpes simplex virus type 1; ICP, infected cell protein; TGF, transforming growth factor; VP, viral protein.
activity to other human viruses such as the respiratory syncytial virus, parainfluenza virus 3, rhinovirus 14 and hepatitis B virus (Brooks et al., 2004; Boriskin et al., 2006; Haid et al., 2009). Pécheur et al. (2007) recently demonstrated that it inhibits hepatitis C virus (HCV) infection in vitro. It has been proved that ARB shows a dose-dependent inhibition of HCV membrane fusion and a reduction in HCV growth in cell culture (Pécheur et al., 2007; Boriskin et al., 2008). The anti-HCV effect appears to be due to ARB-induced membrane alterations. ARB directly interacts with the lipid membrane–water interface and is able to bind to aromatic residues present in HCV glycoproteins in their membrane-associated form (Teissier et al., 2011). The wide range of activity against a number of RNA, DNA, enveloped and non-enveloped viruses suggests that ARB may target common critical steps in the virus–cell interaction. Recent data showed that ARB incorporates into cellular membranes, leading to perturbed membrane structures and inhibition of virus replication (Sellitto et al., 2010; Delogu et al., 2011).

In previous work, some of us reported on the synthesis and evaluation of new ARB derivatives which prevented the entry and replication of HCV in HuH-7.5 hepatoma cells. All compounds inhibited HCV entry and replication in the low micromolar range. Two of them, 9a2 (Ethyl 1-methyl-2-((phenylsulfonyl)methyl)-5-((pyrrolidin-1-yl)methyl)-1H-indole-3-carboxylate) and 9b1 (Ethyl 5-((dimethylamino)-methyl)-1,2-(dimethyl)-1H-indole-3-carboxylate), showed a better therapeutic index compared to that obtained with ARB treatment (Sellitto et al., 2010).

Encouraged by these preliminary results, we aimed at identifying novel compounds active against emergent human infectious diseases (Peduto et al., 2011; Brancato et al., 2013) and to gain a better understanding of the structural features of ARB important for its (broader) antiviral activity, we investigated the effect of ARB and its derivatives on HaCat cells at the different steps of HSV-1 replication, and their immunomodulatory effects by assessing cytokine release. Our results reveal that the ARB analogues tested show a clear ability to influence HCV replication through reduction of viral expression and modulation of the cytokine pathway.

**METHODS**

**Cell culture conditions and stimuli preparation.** The spontaneously immortalized human keratinocyte HaCat cells were cultured as a monolayer using Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. Vero cells (African green monkey kidney) were grown in Roswell Park Memorial Institute (RPMI; Gibco) 1640 medium supplemented with 1% glutamine, 1% penicillin/streptomycin and 10% heat-inactivated FCS at 37 °C in a 5% CO₂ incubator. Cells were grown to confluence with a medium change every 2 days, and then trypsinized and seeded in microplates (tissue culture grade, 6, 12 and 96 wells, flat bottomed) for each experiment in a suitable medium (2% FCS or serum-free medium). For structure-activity relationship studies, several analogues of ARB were synthesized as described by Sellitto et al. (2010). The derivatives included: ethyl 5-bromo 1H-indole-3-carboxylates (5a-b and 6a-b), in which the hydroxy group in position 5 and the amino group in position 4 were removed and bromine was shifted from position 6 to position 5; compounds 5b and 6b, where phenylthiomethyl was replaced by a methyl group. Other modifications were mainly focused on a second series of compounds (9a1–9a3, 9b1–9b2) in which the hydroxy and bromo groups at positions 5 and 6 of the indole ring were removed and the amino group was introduced at position 5 instead of position 4. The phenylthiomethyl substituent was replaced by a phenylsulfonylmethyl or methyl group (Table 1). Free-base powders of ARB and its derivatives were dissolved in DMSO followed by dilution in sterile, distilled water to a final concentration of 0.25 M. The stocks were used to prepare the different concentrations required to perform the experiments; all the dilutions prepared contained a non-cytotoxic concentration of DMSO (<0.001%).

**Virus preparation.** The HSV-1 HF strain obtained from the American Type Culture Collection was grown and titred in Vero cells. To produce the virus for the experiments, the cells were incubated in RPMI medium supplemented with 1% glutamine, 1% penicillin/streptomycin and 2% heat-inactivated FCS in a 75 cm² flask, then rinsed with serum-free medium and infected with HSV-1 at a low m.o.i. of 1.0 p.f.u. per cell. One hour post-infection at 37 °C, 15–20 ml RPMI with 2% FCS was added and the cells were cultured for approximately 3 days. Culture supernatants were harvested at 72 h after virus challenge; after three cycles of freezing at −80 °C and defrosting, they were clarified by centrifugation at 800 g for 10 min and stored in small volumes at −80 °C. A virus stock solution containing approximately 10⁹ p.f.u. per cell was used in all the experiments. Prior to use, the virus was thawed and used for the infections. To obtain a virus batch for a mock infection, the virus was inactivated by exposure to UV-C germicidal light (30 W for 15 min at a distance of 20 cm (Mogensen et al., 2004; Pollara et al., 2004; Scherbik et al., 2010).

**Cytotoxicity assay: MTT.** 2 × 10³ HaCat cells, treated or not treated with ARB, its 11 structural analogues (1, 3, 6, 12 and 24 m) and DMSO at 0.001%, were grown in Falcon 96-well, flat-bottomed microplates of tissue culture grade in a final volume of 200 μl 2% serum DMEM in each well, at 37 °C and 5% CO₂ for 72 h. Untreated cells grown in 2% serum conditions were used as a negative control, while untreated cells grown in the presence of 10% FCS were considered a positive control of proliferation. The assays were carried out using an MTT proliferation kit (Roche Molecular Biochemicals). The coloured solution obtained was spectrophotometrically measured.

**Table 1. Structures of ARB derivatives**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>Hydrogen</td>
<td>Phenylthio</td>
<td>Bromine</td>
</tr>
<tr>
<td>6a</td>
<td>Methyl</td>
<td>Phenylthio</td>
<td>Bromine</td>
</tr>
<tr>
<td>5b</td>
<td>Hydrogen</td>
<td>Hydrogen</td>
<td>Bromine</td>
</tr>
<tr>
<td>6b</td>
<td>Methyl</td>
<td>Hydrogen</td>
<td>Bromine</td>
</tr>
<tr>
<td>9a₁</td>
<td>Methyl</td>
<td>Phenylsulfonyl</td>
<td>Dimethylamino</td>
</tr>
<tr>
<td>9a₂</td>
<td>Methyl</td>
<td>Phenylsulfonyl</td>
<td>Pyrrolidinyl</td>
</tr>
<tr>
<td>9a₃</td>
<td>Methyl</td>
<td>Phenylsulfonyl</td>
<td>4-Methyl piperazinyl</td>
</tr>
<tr>
<td>9a₄</td>
<td>Methyl</td>
<td>Phenylsulfonyl</td>
<td>Morpholinol</td>
</tr>
<tr>
<td>9a₅</td>
<td>Methyl</td>
<td>Phenylsulfonyl</td>
<td>Homopiperazinyl</td>
</tr>
<tr>
<td>9a₆</td>
<td>Methyl</td>
<td>Phenylsulfonyl</td>
<td>Imidazolyl</td>
</tr>
<tr>
<td>9b₁</td>
<td>Methyl</td>
<td>Hydrogen</td>
<td>Dimethylamino</td>
</tr>
<tr>
<td>9b₂</td>
<td>Methyl</td>
<td>Hydrogen</td>
<td>Pyrrolidinyl</td>
</tr>
</tbody>
</table>
at 570/655 nm wavelength. The absorbance of the samples was compared with that obtained from untreated cells grown in 2% serum medium. Three independent experiments were performed.

**Plaque assays for ARB.** HaCat cells seeded in multi-wells (six wells) at 0.5 x 10^6 cells per well in suitable medium at 2% FCS were infected with HSV-1 at an m.o.i. of 0.0001 p.f.u. per cell, in the absence or presence of increasing concentrations of ARB (1, 3, 6, 12 and 24 μM). ARB was added to cells at increasing concentrations but using similar volumes, during HSV-1 infection or before and during HSV-1 infection. After HSV-1 entry (1 h at 37 °C), the cell culture medium was removed and replaced by fresh medium containing 2% FCS with carboxymethyl cellulose (ratio 1:1); infection was assessed for 72 h. After 72 h of incubation, the cells were fixed and stained with 1% crystal violet dissolved in a 10% formalin solution. Plaques were counted and the IC50 value (concentration of compound required to reduce the number or size of plaques by 50%) was calculated. Three independent experiments were performed. The m.o.i. used was chosen as the most appropriate viral concentration in preliminary experiments (data not shown).

**Temporal characterization of ARB antiviral activity.** In another set of experiments, 3 μM ARB was the best concentration in plaque assays and was used to identify which phase of HSV-1 replication (binding, entry and post-entry) was being targeted by the drug. A time-course in synchronized assays was performed: cells seeded in multi-wells (six wells) with 0.5 x 10^6 cells per well in a suitable medium with 2% FCS were inoculated with HSV-1 at an m.o.i. of 0.001 p.f.u. per cell for 5 h at 4 °C, a temperature allowing viral binding but not entry. The unbound virus was removed by washing the cells three times with PBS, and the cultures were shifted to 37 °C for 30 min to permit penetration. After inactivating any non-penetrated virus by treating the monolayer with a pH 3.0 citrate buffer and washing three times with PBS, fresh medium containing 2% serum was added and plaques were counted at 72 h post-infection, after fixing and staining with 1% crystal violet dissolved in a 10% formalin solution. ARB was added: (1) together with HSV-1, with a binding period at 4 °C for 5 h, (2) at the time of the temperature shift (entry), (3) post-citrate treatment (post-entry), and (4) 3 h before HSV-1 infection and again at the time of the temperature shift. This assay permits experimentally to differentiate the binding step (4 °C) from the penetration/entry phase which occurs after the shift to 37 °C and from the post-entry phase which occurs when a low pH buffer is used. The m.o.i. used was chosen as the most appropriate viral concentration in preliminary experiments (data not shown). Three independent experiments were performed.

**Plaque and extracellular viral titres.** HaCat cells seeded in 6-well tissue culture plates (0.5 x 10^6 cells per well in a suitable medium with 2% FCS) were treated for 3 h with ARB or its 11 derivatives (all used at the same concentration as ARB [3 μM] which was chosen as the best concentration in the experiments reported above). After incubation, the cells were washed and infected with HSV-1 at an m.o.i. of 2 p.f.u. per cell and with the addition of ARB or its derivatives. After the adsorption period, the virus was removed and fresh medium with 2% FCS was added. The supernatants of the co-culture were collected after 24 and 48 h of incubation. All supernatants from infected cells were treated for 3 h with ARB or its derivatives (all used at 3 μM) for 3 h and then infected with HSV-1 (at an m.o.i. of 2 p.f.u. per cell) with a re-addition of ARB or its derivatives for 3 and 6 h, was retro-transcribed by reverse transcriptase (Expand Reverse Transcriptase, Roche Diagnostics) at 42 °C for 45 min according to the manufacturer's instructions. Aliquots of 2 μL cDNA were amplified in a reaction mixture containing, in a final volume of 50 μL, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 200 μM dNTP and 2.5 U of Taq DNA polymerase (Roche Diagnostics) and 0.5 μM primers for TGF-β and TGF-α. For the co-amplification, 0.05 μM primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin were used. Table 2 shows the primer sequences, PCR conditions and size of products. The reactions were carried out in a DNA thermal cycler (Perkin Elmer-Cetus Instruments). All PCRs were performed in the exponential phase of amplification and started with a 3 min denaturation step at 95 °C. The PCR products were analysed by electrophoresis on 1.6% agarose gels in Tris/boric acid/EDTA buffer. The identity of the amplification products was confirmed by comparing their size to the size expected from the known gene sequences. For densitometry analysis, the intensity of the bands was measured using Scion Image alpha 4.0.3.2. software and normalized with β-tubulin intensity. Three independent experiments were performed.

**Reverse transcription-PCR for IL-6, TGF-β and TNF-α.** Total RNA, isolated using a High Pure RNA Isolation Kit (Roche Diagnostics) from human keratinocyte cells treated or not treated with ARB, 9b2, 9b3, 9a2 or 6a (3 μM) for 3 h and then infected with HSV-1 (at an m.o.i. of 2 p.f.u. per cell) with a re-addition of ARB or its derivatives for 3 and 6 h, was reverse-transcribed by reverse transcriptase (Expand Reverse Transcriptase, Roche Diagnostics) at 42 °C for 45 min according to the manufacturer’s instructions. Aliquots of 2 μL cDNA were amplified in a reaction mixture containing, in a final volume of 50 μL, 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 200 μM dNTP and 2.5 U of Taq DNA polymerase (Roche Diagnostics) and 0.5 μM primers for TGF-β. The co-amplification, 0.05 μM primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin were used. The PCR products were analysed by electrophoresis on 1.6% agarose gels in Tris/boric acid/EDTA buffer. The identity of the amplification products was confirmed by comparing their size to the size expected from the known gene sequences. For densitometry analysis, the intensity of the bands was measured using Scion Image alpha 4.0.3.2. software and normalized with GAPDH or β-actin intensity. Three independent experiments were performed.

**Statistical analysis.** The data are the mean ± standard deviation (SD) of three independent experiments. A difference in mean values was deemed significant if the P values were <0.05. Calculation of the statistical significance was done using a one-way analysis of variance (ANOVA) and Student’s t-test.
RESULTS

Cytotoxic analysis

The cytotoxic effect of ARB and its derivatives was evaluated by the MTT assay. ARB and all 11 derivatives at the concentrations of 1, 3, 6, 12 and 24 μM and the DMSO (0.001 %) tested did not reveal any cytotoxic effects on the keratinocyte viability (data not shown).

Table 2. Oligonucleotides and related amplification programmes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Conditions</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>5’-ATGAACTCCTTCTCCACAAAGGC-3’</td>
<td>33 cycles 95 °C for 30 s</td>
<td>628</td>
</tr>
<tr>
<td></td>
<td>5’-GAAGAGCCCTACAGCTGGACCTG-3’</td>
<td>58 °C for 1 min 11 s, 72 °C for 2 min 22 s</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’-AAAGTAGATCTGCCAGACTCGG-3’</td>
<td>35 cycles 95 °C for 30 s</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td>5’-GAGCACTGAAAGCATGATCCG-3’</td>
<td>57 °C for 45 s, 72 °C for 30 s</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5’-CCGACTAATGCAAGACTCCA-3’</td>
<td>32 cycles 94 °C for 1 min</td>
<td>439</td>
</tr>
<tr>
<td></td>
<td>5’-AGGGCCGTTTACATGGAATGGTG-3’</td>
<td>60 °C for 1 min, 72 °C for 2 min</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’-TGACGGGCTTCACCCACACTTGCGCCATCTA-3’</td>
<td>33 cycles 94 °C for 30 s</td>
<td>661</td>
</tr>
<tr>
<td></td>
<td>5’-CTAGAAGCATTGCGGTGGAGATGAGC-3’</td>
<td>63 °C for 30 s, 72 °C for 30 s</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-GGGACTACACTACGCCAAGGACCTC-3’</td>
<td>32 cycles 94 °C for 30 s</td>
<td>439</td>
</tr>
<tr>
<td></td>
<td>5’- AGGCCGGGTTCATGCCATGAATGGTG-3’</td>
<td>60 °C for 1 min, 72 °C for 2 min</td>
<td></td>
</tr>
</tbody>
</table>

Plaque assays for ARB

When ARB was present during the course of HSV-1 infection, a decrease in infectivity was observed and the inhibitory effect was proportional to the ARB concentration with an IC50 of 12 μM.

When HaCat cells were preincubated with ARB before infection, with subsequent continuous ARB presence during infection, a dose-dependent inhibition of infection was again observed with an IC50 of 3 μM (data not shown).

Fig. 1. Assay binding at 4 °C. Temporal characterization of antiviral activity of ARB (3 μM) treatment during different steps of HSV-1 replication (binding, entry and post-entry, and pre-treatment + addition during HSV-1 infection). The graph shows the percentage of infectivity after 72 h of infection (at an m.o.i. of 0.0001 p.f.u. per cell). The results are mean percentages ± SD of three experiments, each run in duplicate. *, the decrease in virus production detected with treatments was statistically significant (P <0.05) against HSV-1; Mock, mock-infected cells which were treated with virus inactivated by exposure to UV light for 15 min.
Temporal characterization of ARB antiviral activity

In order to understand at which level of the viral replication cycle ARB exerts its inhibitory activity, synchronized infectivity assays were developed. No significant difference was observed in the HSV-1 infectivity when ARB (3 μM) was added to the culture medium during the post-entry phase. On the other hand, ARB (3 μM) addition during the adsorption period or during the entry period gave a reduction in infectivity of about 25%. ARB exerted its main effect at the virus–cell interface when it was added before and during viral infection (50% reduction, Fig. 1).

Antiviral activity at 24 and 48 h in HaCat cells pre-treated for 3 h and after re-addition of the compounds after HSV-1 infection: measurements of virus yield in the supernatants of the treated cells

After HSV-1 infection, the virus yield in the supernatants of HaCat cells pre-treated for 3 h with ARB or its analogues (3 μM), and after their re-addition during HSV-1 infection (see Methods) is shown in Fig. 2. ARB, 9b2, 9b1, 9a2 and 6a at 3 μM were the only effective compounds able to reduced virus replication compared to the HSV-1-infected control. A significant reduction in the plaque numbers occurred between 24 and 48 h in the presence of ARB (42%) and 9a2 (49%). For 9b1 (35%), 9b2 (36%) and 6a (50%), a significant reduction was observed after only 24 h of infection. Only ARB and 9a2 maintained their effect up to 48 h. Structure-activity relationship evaluations regarding bromo derivatives (5a–b and 6a–b) indicate that the presence of N-methylation and phenylthiomethyl substituents is crucial for inhibitory activity. Among amine analogues (9a1–a6, 9b1–b2), the pyrrolidinyl substituent leads to the significant improvement in potency (9a2 and 9b2), while introduction of more sterically hindered amines results in loss of activity.

Evaluation of VP16 and ICP27 production in virus-infected HaCat cells

Cell lysates collected from HaCat cells at 3 and 6 h post-infection (Figs 3 and 4, respectively), in the presence or absence of ARB or its analogues, were tested for VP16 and ICP27 viral protein expression. ARB and its derivatives significantly reduced the levels of VP16 protein expression after 3 h of infection compared to HSV-1 alone, and the reduction was more marked after 6 h of treatment. This reduction was more intense with the analogues compared to that obtained with ARB treatment. In addition, the HSV-1-induced ICP27 protein expression was reduced by

![Fig. 2. Antiviral activity of ARB, 9b2, 9b1, 9a2 and 6a on virus yield. The compounds were used at a concentration of 3 μM, 3 h pre-HSV-1 adsorption period and during HSV-1 infection (at an m.o.i. of 2 p.f.u. per cell). Each bar represents the percentage of infectivity decrease compared to the HSV-1-infected controls. Virus production was assessed 24 h and 48 h after viral challenge. The results are mean percentages ± SD of three experiments, each run in duplicate. *, the decrease in virus production detected with ARB and its analogues was statistically significant (P < 0.05) against HSV-1; Mock, mock-infected cells which were treated with virus inactivated by exposure to UV light for 15 min.](image-url)
ARB and the analogues tested only after 6 h of infection. A small amount of constitutive VP16 protein was detected in the mock-infected cells at 3 h compared to untreated cells.

**The IL-6, TNF-α and TGF-β cytokine expression in HSV-1-infected HaCat cells**

IL-6 gene expression was evaluated in HaCat cells 6 h post-infection with HSV-1. This was compared with mock-infected cells and cells treated with ARB or the analogues alone at the same time. Treatment with ARB and its analogues modulated this gene expression to different degrees. Among the compounds tested, only ARB and 6a revealed an enhancement of this cytokine compared to the control cells (data not shown), which gave similar results to the mock-infected cells. In the presence of HSV-1 infection, ARB and each of its derivatives strongly enhanced the expression of this cytokine compared to infection alone and to the compounds alone (Fig. 5a).

TNF-α gene expression was evaluated in HaCat cells after 6 h of treatment with ARB or the analogues alone, HSV-1 alone and in the presence of ARB or its analogues during HSV-1 infection. The compounds did not give a statistically significant difference in the TNF-α gene expression compared to the control cells (data not shown), which gave similar results to the mock-infected cells. ARB and its analogues in the presence of HSV-1 infection showed a marked enhancement of TNF-α compared to the HSV-1-infected cells and to the compounds alone (Fig. 5b).

The virus alone and ARB alone showed a significant TGF-β gene expression enhancement after 6 h of treatment compared to the mock-infected cells. The ARB derivatives, but not ARB, reduced the HSV-1-induced TGF-β expression after 6 h of infection (Fig. 5c).

**DISCUSSION**

In our on-going project to elucidate the structural requirements of natural compounds or licensed drugs (Filosa et al., 2007, 2013; Petronzi et al., 2011, 2013; Schaible et al., 2013) with anti-inflammatory and antiviral activity, we selected ARB for its broad antiviral activity, aiming to improve the ARB therapeutic index, or to identify novel lead
compounds active against HSV. This is the first research about the anti-HSV activities of ARB and structurally related compounds.

ARB is an antiviral drug which has proved efficient in reducing the duration of the illness during influenza A and B viral infection and its complications. It has demonstrated a broad-spectrum antiviral activity against a number of enveloped and non-enveloped viruses and, in addition, a possible immunomodulatory effect (Teissier et al., 2011). In this work, we studied the effects of ARB and its derivatives on HSV-1 infection in human epidermal keratinocytes, the first line of defence against HSV-1 viral attack that involves the activation of the innate immune response by recruitment of, e.g. natural killer cells, triggered by cytokine and chemokine production (Donnarumma et al., 2010).

Upon HSV-1 infection, VP16 tegumental protein released from the virions into the cell forms a complex with two mammalian proteins that is able to interact with the cis regulatory site in HSV-1 immediate/early promoters and is responsible for cell cycle progression (Lai & Herr, 1997). In our previous reports, VP16 and ICP27 expressions were used as markers to evaluate HSV-1 infection and replication (Ayala et al., 2008; Baroni et al., 2007).

In vivo, after the acute-stage productive infection, HSV undergoes a non-productive infection in neurons, resulting in latency. Periodic reactivation occurs, resulting in a productive infection and virus transport back to surface sites where further rounds of productive replication ensue (Flint et al., 2000).

It has been shown that during primary HSV-1 infection, the acute immune response is supported by the stimulation of several cytokine expressions after nuclear factor-κB activation. The secretion of IL-6 has been linked to the recruitment of neutrophils to the virus infection site. TNF-α was also a potent pro-inflammatory cytokine and had a function in the mediation of the acute-phase response, chemotaxis and the activation of inflammatory and antigen-presenting cells (Li et al., 2006). The secretion of TGF-β by HSV-1-stimulated HaCat cells may be important because of its ability to down-regulate the T-lymphocyte response against viruses. In fact, production of TGF-β1 by HaCat cells may be responsible in part for the enhancement of HSV-1 replication because this cytokine suppresses

**Fig. 4.** VP16 and ICP27 protein expression from HaCat cells treated or not treated with ARB, 9b2, 9b1, 9a2 and 6a (3 μM) and then infected with HSV-1 (at an m.o.i. of 2 p.f.u. per cell) plus the re-addition of ARB or derivatives. The cells were harvested after 6 h of infection. As a negative control mock-infected cells were used compared with untreated cells (data not shown). The results are mean percentages ± so of three experiments, each run in duplicate. *, the decrease was statistically significant (P < 0.05) against HSV-1; Mock, mock-infected cells which were treated with virus inactivated by exposure to UV light for 15 min.
the ability of the immune system to mount a strong antiviral host response (Méndez-Samperio et al., 2000).

In addition, ARB has been shown to be an entry inhibitor of influenza virus infection and was found to be active in vitro against HCV infection through the inhibition of the HCV glycoprotein conformation changes needed for the membrane fusion process (Leneva et al., 2009; Boriskin et al., 2006; Sellitto et al., 2010). Here, we report the finding that ARB and some related derivatives are able to reduce HSV-1 replication by acting on the viral binding/entry phases. The compound 9a showed the best antiviral activity in vitro both at 24 and 48 h of treatment compared to ARB. In addition, 9b2 and 6a showed an early antiviral effect after 24 h of infection but this was lost at 48 h. The mechanism of antiviral activity is related to a lesser release of VP16 after 3 and 6 h of infection and, consequently, a reduction in the ICP27 expression after 6 h of infection. In addition, ARB and its derivatives showed the ability to modulate virus-induced cytokine expression. The co-presence of HSV-1 with ARB or its derivatives determined a marked enhancement of IL-6 and TNF-α. Only the ARB analogues, and not ARB, in the presence of the virus drastically reduced the virus-induced TGF-β expression. The preliminary information emerging from these results suggests that the introduction of appropriate substitutions at positions 2 and 5 might be a promising approach for the development of new indole compounds able to reduce HSV-1 infectivity.
REFERENCES


