IN VITRO EVALUATION OF ANTIVIRAL EFFECT OF BAUER-7-EN-3b-YL ACETATE, THE MAJOR CONSTITUENT OF A SAMPLE OF BRAZILIAN PROPOLIS, AGAINST ALPHAHERPESVIRUSES[1]

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ABSTRACT: There is an increasing need for substances with antiviral activity since the treatment of viral infections with most antivirals is often unsatisfactory due to the problem of, amongst other things, viral latency and the likelihood of new viral agents arising. Previously we isolated bauer-7-en-3β-yl acetate (BA), a pentacyclic triterpenoid obtained from the chloroform extract of an sample of propolis from southeast Brazil (TEIXEIRA, *et al*., 2006). Here we investigated the antiviral activity of BA against the alphaherpesviruses bovine herpesvirus 1 (BoHV-1) and pseudorabiesvirus (SuHV-1, suid herpesvirus) during infection of Madin-Darby bovine kidney (MDBK) and African green monkey kidney (VERO) cells cultures, respectively. In short, BA was tested for its cytotoxic properties and antiviral effect through virus yield reduction and virucidal activity in both cells. Results showed that BA 20μg mL^{-1} and 15μg mL^{-1} were the maximal non-cytotoxic concentration (MNCC) to VERO and MDBK cells, respectively. BA reduced significantly SuHV-1 titrers in both antiviral tests ($p<0.05$) while inhibition was not observed against BoHV-1. However, how BA interferes on the virus multiplication still need to be elucidated.

Key words: antiviral effect, Bauer-7-en-3β-yl acetate, propolis.

AVALIAÇÃO "IN VITRO" DO EFEITO ANTIVIRAL DO ACETATO DE BAUER-7-EN-3β -ILA, O PRINCIPAL CONSTITUINTE DE UMA AMOSTRA DE PRÓPOLIS BRASILEIRA, CONTRA O ALPHAHERPESVIRUS

RESUMO: O crescente interesse na busca por substâncias que apresentem atividade antiviral se deve, entre outros fatores, à dificuldade de tratamento de infecções virais, tanto em virtude da característica de latência viral quanto pelo surgimento de novos vírus. O triterpenóide acetato de bauer-7-en-3β-ila (BA) foi previamente isolado do extrato clorofórmico de uma amostra de própolis coletada em Minas Gerais, região sudeste do Brasil (TEIXEIRA *et al*., 2006). A atividade antiviral desta substância contra o herpesvírus bovino tipo 1 (BoHV-1) e o herpesvírus suíno tipo 1 (SuHV-1) foi investigada através da infecção de culturas de células de rim bovino (MDBK) e de células de rim de macaco verde africano (VERO). O efeito citotóxico do triterpenóide foi previa-

mente avaliado e as concentrações máximas não tóxicas foram 20μg mL^{-1} e 15μg mL^{-1} para células VERO e MDBK, respectivamente. Os resultados mostraram atividade antiviral contra SuHV-1 ($p<0,05$), porém não contra BoHV-1. Todavia, a forma como o BA interfere na multiplicação do SuHV-1 ainda precisa ser elucidada.

Palavras-chave: acetato de bauer-7-en-3β-ila, atividade antiviral, própolis.

INTRODUCTION

Works on antiviral compounds date back to the 1950s, but for several reasons, only a couple of drugs were approved for clinical use about a decade later. To date, many antiviral drugs have been developed after extensive research and demanding trials, some of them with selective mechanisms against viral replication. Although the primary focus has been on synthetic products, the number of natural compounds with antiviral action for different DNA and RNA viruses that are being studied is increasing (Felipe *et al*., 2006).

Many of these natural products have been used for thousands of years in folk medicine for several purposes. Among them, propolis has attracted increased interest due to its antimicrobial activity against a wide range of pathogenic microorganisms (Koo *et al*., 2000) such as viruses, fungi and bacteria (reviewed by Burdock, 1998, Kujumgiev *et al*., 1999). Propolis is a hive product, formed by a complex mixture of beewax, plant exudates collected by *Apis mellifera* and a suite of other substances (Banskota *et al*., 1998). The precise composition of raw propolis varies with the source (Burdock, 1998) and more than 200 compounds have been identified (Banskota *et al*., 2001). Some interesting points emerge from limited work that has been carried out on the constituents of propolis because the largest group of compounds isolated are flavonoid pigments, which are ubiquitous in the plant kingdom (Burdok, 1998) for propolis and its constituents. Many pentacyclic triterpenoids have been obtained from chloroform extracts of Brazilian propolis (Negri *et al*., 2003A; Negri *et al*., 2003B). And recently we isolated a triterpenoid compound from propolis, from the municipality of Paula Candido, Minas Gerais, southeast Brazil, denominated bauer-7-en-3β-yl acetate (BA) (Teixeira *et al*., 2006).

Hespesviruses are widespread, enveloped and double-stranded DNA agents which cause various infections in humans and animals, especially in immunocompromised individuals (Felipe *et al*., 2006). All herpesviruses share common virion morphology with a T=16 (triangulation number) icosahedral capsid, a cell-derived lipid envelope containing virally encoded membrane proteins and a tegument, which links capsid and envelope like the matrix in RNA viruses (Mettenteiter *et al*., 2006). Viral gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E) or late (L) and it is generally agreed that tissue-specific factors mediate latency and/or pathogenesis by influencing viral gene expression (Pagnini *et al*., 2006). Herpesvirus assembly is a multistep process involving many protein-protein, as well as protein-DNA interactions which occurs in at least two distinct subcellular compartments. While capsid assembly and DNA packing take place in the nucleus of infected cells, the addition of most of the tegument, as well as final envelopment, proceed in the cytoplasm (Klupp *et al*., 2006).

Bovine herpesvirus-1 (BoHV-1), a member of the *Herpesviridae* family, *Alphaherpesvirinae* sub-family, is an important pathogen in cattle. It is responsible for a variety of clinical signs and especially for a respiratory disease called infectious bovine rhinotracheitis (IBR) (Del Médico Zajac *et al*., 2006; Patel, 2005A), causing an economic loss in the cattle industry worldwide. The common illness due to IBR virus infection is febrile rhinotracheitis and the virus, less frequently, has been associated with abortions, still births, and reproductive diseases, central nervous and alimentary tract systems (Ackermann and Engels, 2006; Takiuchi *et al*., 2005). Conventional attenuated vaccines have contributed to disease regression (Felipe *et al*., 2006). However, none of the currently available BoHV-1 vaccines are completely effective in preventing virus shedding and the febrile respiratory disease due to BoHV-1 infection (Patel, 2005B). Moreover, the disease control is still difficult due to the establishment of life-long latency after primary infection or after vaccination with attenuated viruses (Felipe *et al*., 2006).

Suid herpesvirus 1 (SuHV-1), usually named Aujeszky's disease virus or pseudorabies virus (PrV)

also belongs to the *Alphaherpervirinae* subfamily and is an important viral pathogen that causes severe economic losses in the swine industry (DEL MÉDICO ZAJAC *et al*., 2006; MARCACCINI *et al*., 2006; MÜLLER *et al*., 2005; VENGUST *et al*., 2005). Like other alphaherpesviruses, SuHV-1 is highly neurotropic and after primary replication in the nasal and pharyngeal mucosa invades the central nervous system (CNS) through several nervous pathways. Infection of the CNS by SuHV-1 causes a nonsuppurative meningoencephalomyelitis and ganglioneuritis that is often fatal in piglets. Older pigs can recover from the primary infection, although a lifelong latent infection is established by the virus in neuronal and nonneuronal cells of its host (MARCACCINI *et al*., 2006; BRUKMAN and Enquist, 2006; BOUMA, 2005; FUCHS *et al*., 2005). Reactivation of latent SuHV-1 leads to release of viral particles into the environment and subsequent transmission to new uninfected hosts. Furthermore, the currently used attenuated vaccines do not completely prevent the establishment of latent infection. Thus, latency and reactivation of SuHV-1 is considered to be a major obstacle in the efforts to control and eradicate this disease (POMERANZ *et al*., 2005; YOON *et al*., 2006).

The objective of this study was to investigate the *in vitro* antiviral activity of BA against both SuHV-1 and BHV-1 viruses.

MATERIALS AND METHODS

Propolis sample

Propolis produced by africanized *Apis mellifera* were collected in the dry season (August) at the municipality of Paula Candido, Minas Gerais, southeast Brazil (TEIXEIRA *et al*., 2006). Briefly, a process of extraction and isolation of triterpenoids from propolis sample followed by gas chromatography revealed that the substance obtained was a single compound, which was identified as bauer-7-en-3β-yl acetate (BA) by EIMS, IR, ^{1}H and ^{13}C NMR spectroscopy. It was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10mg mL^{-1} and stored at 4°C previous to dilution with medium at the appropriate concentrations.

Cell cultures and media

Permanent cell lines from African green monkey kidney (VERO, ATTC CCL-81) and Madin Darby bovine kidney (MDBK, ATTC CCL-22) were cultivated in Eagle's minimal essential medium (MEM-Gibco BRL, USA), supplemented with 10% fetal bovine serum (FBS-Gibco BRL, USA) for VERO cells or 5% for MDBK cells, with the addition of antibiotics Penicillin 500IU mL^{-1} and Streptomycin sulfate 100mg mL^{-1} (Sigma Chem. Co., USA). Amphotericine B at 0.1% (Fungizon® Bristol) and 0.25% sodium bicarbonate (growth medium) were added to the medium, at 37°C in the presence of humidified CO_2 atmosphere (5% CO_2) until the formation of confluent monolayers. Virus-infected MDBK or VERO cells were maintained in MEM supplemented with 2.5 and 5% FBS, respectively (maintenance medium).

Viruses

Bovine herpesvirus type 1 (BoHV-1, ATCCVR-2106) and pseudorabies virus (SuHV-1), kindly provided by Laboratory of Virology, UFRS, were propagated in MDBK and VERO cells to obtain stocks, respectively. Viruses stocks were prepared as follows: nearly confluent monolayer cultures were infected at low multiplicities, incubated for 3 days, then frozen and thawed 3 times before clearing of the preparations by centrifugation at low speed to remove cell debris. The resulting supernatant fluids were stored in liquid nitrogen until use. The viruses titer were estimated from cytopathogeneticity and expressed as 50% tissue culture infectious doses per mL ($TCID_{50}$), described by REED and MUENCH (1938). Viruses stocks titers obtained were $10^{6.8}$ and $10^{7.6}$ $TCID_{50}$ mL^{-1} to BoHV-1 and SuHV-1, respectively. The experiments with viruses were performed in the Federal University of Viçosa, Animal Virology Laboratory.

Cytotoxicity assay

To test for cytotoxicity, MDBK and VERO cultures were prepared in 96-well microtiter plates ($2x10^4$ cells per well) and incubated at 37°C in a humidified CO_2 atmosphere (5% CO_2) incubator. When the cell cultures became confluent (approximately 18 hours), culture medium was removed from each well and replenished with 0.1mL of maintenance medium. For the cytotoxicity test, 0.1mL of maintenance medium containing different concentrations of BA (ranging from 0.5 to 150µg mL^{-1}) was added to the wells. For the cell control, 0.1mL maintenance medium without BA was added. All cultures were incubated at 37°C for 3-4 days.

The morphology of the cells was inspected daily and observed for microscopically detectable alterations, e.g. loss of monolayer, rounding, cell shrinkage, granulation and cytoplasm vacuolization. The maximal non-cytotoxic concentration (MNCC) was determined as the maximal concentration of the BA that did not exert a toxic effect detected microscopic monitoring (Serkedjieva and Ivancheva, 1999). Cytotoxicity was also determined by counting cells in treated and untreated cultures with BA by Trypan exclusion after trypsinization (Amoros *et al.*, 1992).

Virus yield reduction test

To screen for antiviral activity, monolayers of cells described earlier were grown in 96-well plastic plates as in the 2.4 item. After the cell cultures became confluent, culture medium was removed from the monolayer cells. BoHV-1 stock suspension was serially diluted (10^{-3}-10^{-8}) in MEM without FBS and 0.05mL of each dilution were mixed with equal volume of medium at 10% FBS containing the MNCC of BA (15µg mL^{-1}) and the mixture was added on confluent monolayers of MDBK cells (maintenance medium). SuHV-1 stock suspension also was diluted (10^{-3}-10^{-8}) and 0.05mL from it were mixed with equal volume of medium (5% FBS) containing the MNCC of BA (20µg mL^{-1}) and then added on VERO cells confluent monolayers. As the viruses control, 0.05mL of BoHV-1 or SuHV-1 from each dilution (10^{-3}-10^{-8}) and 0.05mL of maintenance medium without BA also were added on cells monolayers. The plates were incubated at 37ºC in a humidified CO_2 atmosphere (5% CO_2), for 3-4 days. After that, CPE was observed under inverted microscope. The reduction of viruses multiplication was estimated based on viruses control and viral titers were determined by the method of Reed and Muench (1938). All antiviral tests were performed in quadruplicate and repeated three times. The significance of the difference in infectious titers was estimated (Student's t-test).

Virucidal activity

The direct virus inactivating effect of the bauerenyl acetate was tested by direct contact assay. Fifty microliters of the BoHV-1 ($10^{5.5}$ $TCID_{50}$) and SuHV-1 ($10^{6.3}$ $TCID_{50}$) stocks were incubated with 0.05mL of MEM without FBS, besides 2.5mL 1 M Hepes (*n*-[2-hydroxyethyl]piperazine-*n'*-[2-ethane sulfonic acid]) (Sigma-Aldrich), containing 40 to 300mg of BA (final concentrations on viruses from 20 to 150mg mL^{-1}) or with 0.05mL of MEM without BA for 1 hour at 37ºC. After that, BoHV-1 or SuHV-1 suspensions were serially diluted 10-fold and assayed for remaining viruses in MDBK and VERO, respectively. Differences in the biological activities of treated and control viruses were determined based on infectivity. Surviving infectious virus titers were determined using a CPE assay (Reed and Muench, 1938).

RESULTS

To assay for possible cytotoxic effects of BA (Figure 1), VERO and MDBK cells were incubated with increasing amounts of the BA for 3-4 days. The cytotoxicity was determined by microscopic examination of cell death and integrity after an incubation time of 72h. BA at concentrations up to 20mg mL^{-1} was well tolerated by VERO cells over 72 hour exposure. On the other hand the MNCC of BA to MDBK cells was 15µg mL^{-1}. These observations were confirmed by Trypan Blue dye exclusion.

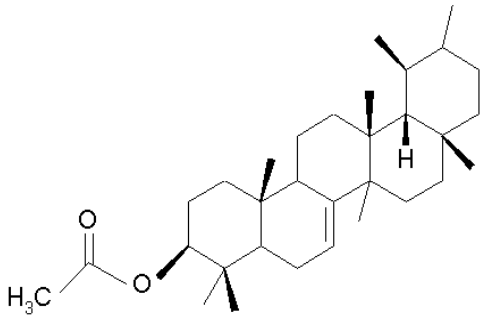

Figure 1. Chemical structure of bauer-7-en 3β -yl acetate (BA)

As shown in Figure 2, BA significantly reduced SuHV-1 multiplication (p= 0.0002) after treatment of VERO cells with MNCC immediately post-infection. However when MDBK cells were infected with BoHV-1 and immediately treated with BA at MNCC, there was no significant difference on BoHV-1 titre in BA presence when compared with the control (Figure 3).

The ability of BA to produce a direct viral inactivating effect was studied using a contact assay. Exposure of SuHV-1 to BA at concentrations up 150mg mL^{-1} for 1 hour resulted in a loss of infectivity (Figure 4). This was significantly reduced within 1

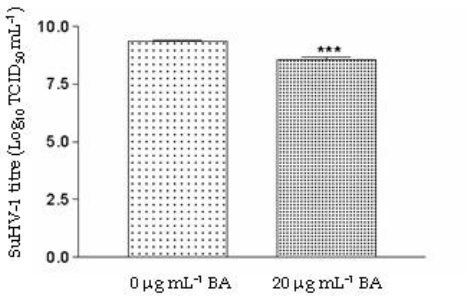

Figure 2. Susceptibility of SuHV-1 to BA determined by virus yield reduction assay in VERO cells. Virus titers are presented as log_{10} 50% tissue culture infectious doses mL^{-1}. Data are expressed as mean ± sd (n = 3). ***p = 0.0002, no treatment vs. BA (20µg mL^{-1})

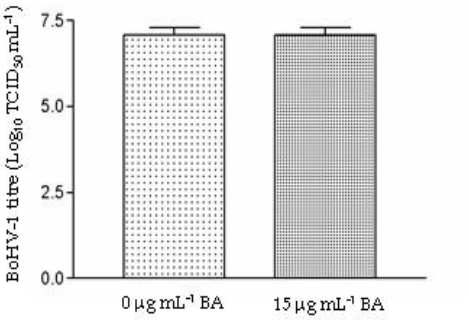

Figure 3. Susceptibility of BoHV-1 to BA determined by virus yield reduction assay in MDBK cells. Virus titres are presented as log_{10} 50% tissue culture infectious doses mL^{-1}. Data are expressed as mean ± sd (n = 3), no treatment vs. BA (15µg mL^{-1})

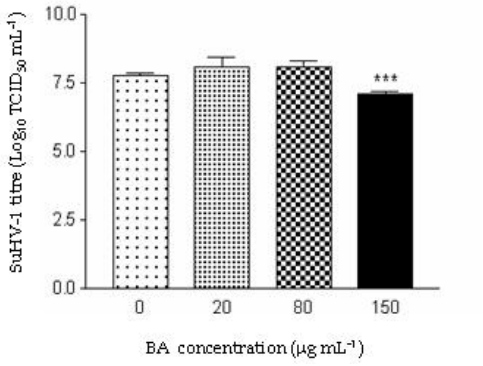

Figure 4. SuHV-1 inactivating effect of BA in VERO cells, determined by contact assay. Virus titres are presented as log_{10} 50% tissue culture infectious doses mL^{-1}. Data are expressed as mean ± sd (n = 3). ***p = 0.0007, no treatment vs. different concentrations of BA

hour by a dose of 150µg mL^{-1} BA (p= 0.0007), whereas BoHV-1 was not susceptible (Figure 5).

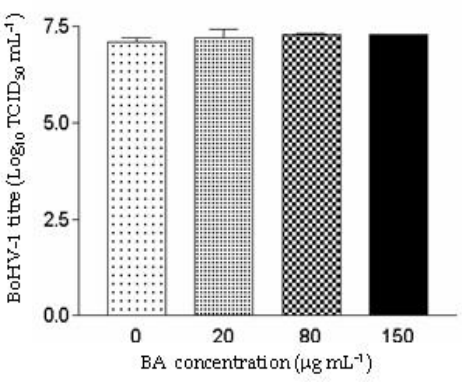

Figure 5. BoHV-1 inactivating effect of BA in MDBK cells, determined by contact assay. Virus titres are presented as log_{10} 50% tissue culture infectious doses mL^{-1}. Data are expressed as mean ± sd (n = 3), no treatment vs. different concentrations of BA

DISCUSSION AND CONCLUSIONS

Many efforts have been made to evaluate the antiviral activity of a wide array of natural products in order to isolate and characterize novel compounds which could inhibit virus replication and/or treat viral infection, or even serve as models for new molecules (SCHIMITT *et al.*, 2001). Propolis is a resinous hive product collected by *Apis mellifera* bees from tree buds or exsudate and mixed with secreted beeswax. In addition there is substantial evidence indicating that propolis has antiseptic, antifungal, antibacterical, antiviral, anti-inflammatory and antioxidant properties (PIETTA *et al.*, 2002).

Herpesviruses are widespread, enveloped and double-stranded DNA agents which cause various infections in human and animals, especially in immunocompromised individuals (SUMMERFIELD *et al.*, 1997; FELIPE *et al.*, 2006). SuHV-1 and BoHV-1 are members of *Herpesviridae* family, *Alphaherpesvirinae* subfamily and important pathogens in cattle (ACKERMANN and ENGELS, 2006) and pigs (VENGUST *et al.*, 2005). To both viruses, disease control caused by them is difficult due to the establishment of life-long latency in various nervous tissues of the natural host. Furthermore, the currently used attenuated vaccines do not completely prevent the establishment of latent infection (FELIPE *et al.*, 2006, YOON *et al.*, 2006).

The triterpenoid bauerenyl acetate (BA) was investigated for its antiviral effect on BoHV-1 and SuHV-1 by two separate assay methods. In virus yield reduction assay, BA showed activity statistically significant against SuHV-1 in VERO cells culture but did not show activity against BoHV-1 in MDBK cells. The antiviral effect detected in this assay could have taken place in many different steps of SuHV-1 multiplication cycle. According to PRUSOFF *et al.* (1986) effective antiviral agents can inhibit the viral infection by affecting the virus particle itself, by interfering with the virus entry across the cell membrane and subsequent uncoating or with the integrity and transport of viral genome, or even by interfering with replication of the viral genome or other early steps of intracellular viral replication, including assembly and release of virions. While BA shows antiviral activity against SuHV-1, specific steps in the SuHV-1 multiplication cycle that BA could interfere, still need to be studied in more detail.

In the virucidal assay, BA in low concentrations (20 and 80µg mL^{-1}), could not inactivate the viral particles but 150µg mL^{-1}, BA decreased significantly SuHV-1 infectivity, while there was not significant alterations in BoHV-1 titers. This assay is useful because it allows high concentrations of compound to be tested. Toxicity to cells is not a problem as infectivity titers are assessed at below the toxic concentration of the compound (SELWAY, 1986). The direct interaction with virus capsid proteins could be at least one of the modes of the inhibitory effect (SERKEDJIEVA and IVANCHEVA, 1999). Many natural products have been shown to act either in the early stage of adsorption or on virus surface structures thereby inhibiting penetration into susceptible cells.

In addition to other biocidal properties, propolis and its extracts clearly have virucidal properties as well. AMOROS *et al.* (1992) investigated the *in vitro* effect of propolis on several DNA and RNA viruses including herpes simplex type I (HSV-1), herpes simplex type 2 (HSV-2), adenovirus type 2, vesicular stomatitis virus and poliovirus type 2. Propolis inhibited clearly poliovirus propagation, reduced the titers of herpesviruses, whereas vesicular stomatitis and adenovirus were less susceptible. Also, propolis was found to exert a virucidal action on the enveloped viruses HSV and VSV. MAKSIMOVA-TODOROVA *et al.* (1985) reported that various fractions of propolis affected the replication of influenza viruses A and B, *Vaccinia virus* and Newcastle disease virus. Moreover substances isolated from propolis have also been examined for antiviral activity. SERKEDJIEVA *et al.* (1992) showed that isopentyl ferulate inhibited the infectious activity of influenza virus a/Hong Kong (H_3N_2). As most constituents of propolis are flavonoids, DEBIAGGI *et al.* (1990) examined several of them from propolis, using a battery of viruses. Two of the flavonoids studied were highly active in inhibiting the replication of several herpes viruses, adenoviruses and a rotavirus.

The results presented here provide evidence that bauer-7-en-3β-yl acetate is active *in vitro* against SuHV-1 whereas BoHV-1 is not susceptible. At the present, the true nature of this antiviral principle has not yet been established.

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