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Piroxicam inhibits herpes simplex virus type 1 infection *in vitro*

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Piroxicam is a potent, nonsteroidal, anti-inflammatory agent (NSAID) which also exhibits antipyretic activity. The antiviral effect of piroxicam against herpes simplex virus type 1 (HSV-1) was examined *in vitro* on RC-37 monkey kidney cells using a plaque reduction assay. Piroxicam was dissolved in ethanol or dimethylsulfoxide (DMSO) and the 50% inhibitory concentration (IC_{50}) was determined at 4 μg/ml and 75 μg/ml, respectively. The IC_{50} for the standard antiherpetic drug acyclovir was determined at 1.6 μM. At non-cytotoxic concentrations of these piroxicam solutions, plaque formation was significantly reduced by 62.4% for ethanolic piroxicam and 72.8% for piroxicam in DMSO. The mode of antiviral action of these drugs was assessed by time-on-addition assays. No antiviral effect was observed when cells were incubated with piroxicam prior to infection with HSV-1 or when HSV-1 infected cells were treated with dissolved piroxicam. Herpesvirus infection was, however, significantly inhibited when HSV-1 was incubated with piroxicam prior to the infection of cells. These results indicate that piroxicam affected the virus before adsorption, but not after penetration into the host cell, suggesting that piroxicam exerts a direct antiviral effect on HSV-1. Free herpesvirus was sensitive to piroxicam in a concentration-dependent manner and the inhibition of HSV-1 appears to occur before entering the cell but not after penetration of the virus into the cell. Considering the lipophilic nature of piroxicam, which enables it to penetrate the skin, it might be suitable for topical treatment of herpetic infections.

1. Introduction

Piroxicam is a potent anti-inflammatory and analgesic agent for the treatment of arthritis and other inflammatory diseases. This effective and well-tolerated nonsteroidal anti-inflammatory drug (NSAID) has been available for oral and parenteral administration for many years. A systematic review of topical NSAIDs concluded that these products are effective in relieving pain (Moore et al. 1998). Piroxicam relieves pain and reduces swelling of the affected area (Abdulkarim et al. 2010). The introduction of a piroxicam 0.5% gel provided a convenient alternative for patients with painful and inflammatory musculoskeletal conditions amenable to topical drug therapy. Multiple topical application of this piroxicam gel is well tolerated (Fourtillan and Girault 1983, 1992). Extensive clinical trials have demonstrated the high efficacy and excellent toleration of piroxicam in rheumatoid arthritis, osteoarthritis and various muscoskeletal disorders. Advantages of local application of piroxicam include targeting the site of action while minimising systemic blood concentrations and potential adverse effects. Pharmacokinetic data indicates that topically applied piroxicam is absorbed percutaneously from gel preparations. In various animal models, piroxicam inhibited cell migration into an inflamed site. *In vitro*, piroxicam inhibits both superoxide anion production and lysosomal enzyme release from human neutrophils (Andono and Lombardino 1983).

Labial or perioral outbreaks of vesicular herpetic lesions affect approximately 20-40% of the population (Spruance and Kriesel 2002). Cold sores usually affect the lips, they present in most cases with a clinical sequence of events including primary infection followed by a latency period and recurrences, the frequency of which is influenced by internal or external triggers (Griffiths et al. 2013). A small proportion of patients have outbreaks that occur monthly or even more frequently. The vast majority of cases are caused by herpes simplex virus type 1 (HSV-1). In a classic case of herpes labialis recurrence, six stages are defined: prodrome, erythema, papule or oedema, ulcer, crusting and healing (Spruance and Kriesel 2002). Lesions resolve spontaneously, usually within 1-2 weeks, but are characterized by functional symptoms. They commonly include pain, burning, itching and tingling sensations, which can result in significant discomfort for patients. Functional symptoms are the main concern of most patients during the active period of the infectious flare (Khemis et al. 2012). The latent virus is reactivated spontaneously or its reactivation is induced by a variety of stimuli. During the reactivation process, the virus is transported through the nerve cells axons to the original peripheral infection site, where HSV-1 replication occurs (Whitley and Roizman 2001). Antiviral agents licensed currently for the treatment of herpesvirus infections include acyclovir and its derivatives, which are nucleosides analogues that function as DNA chain terminators, ultimately preventing elongation of viral DNA (Tyring et al. 2002). Some of these antiviral agents might produce toxic

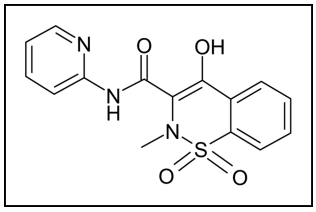

Fig. 1: Structural formula of piroxicam.

side-effects. In addition, the emergence of virus strains resistant to commonly used anti-herpesvirus drugs is a growing problem, particularly in immunocompromised patients (Chakrabarti et al. 2000; Chen et al. 2000). The most commonly used topical treatments reduce the duration of herpes recurrences by 10-15%, but not the functional symptoms (Evans et al. 2002). Topical therapies with acyclovir and penciclovir are limited by several factors, including low efficacy on signs and symptoms and formation of scab during the healing process. A number of essential oils and plant extracts have been shown to provide an antiherpetic effect, acting before virus penetration and by a mechanism that differs from the commonly used synthetic antiviral drugs. While natural products represent promising antiviral agents for a topical therapeutic application (Koch et al. 2008; Schnitzler et al. 2008a, 2008b; Reichling et al. 2008, 2009), there still remains a requirement for new antiviral agents with different or combined modes of antiviral action.

Repeated reports of patients applying piroxicam cream for the treatment of labial herpetic infections prompted us to examine a possible antiviral effect against HSV-1 *in vitro*. In the present study we have analysed the antiviral property of piroxicam against HSV-1. The infectivity of HSV-1 was significantly reduced, and the mode of antiviral action was analysed at different steps in the viral infection cycle.

2. Investigations and results

Piroxicam was dissolved in ethanol or DMSO and was serially diluted with cell culture medium to examine the effect on the growth and viability of RC-37 tissue culture cells, always resulting in an ethanol or DMSO concentration below 1%, which had no effect on cells and viruses. The structural formula of piroxicam is shown in Fig. 1. The concentration range tested for ethanolic piroxicam and piroxicam dissolved in DMSO was 0.1 – 10 μg/ml and 50 – 500 μg/ml, respectively. The maximum non-cytotoxic concentrations of this drug were > 10 μg/ml (ethanolic piroxicam) and 100 μg/ml (DMSO). CC_{50} values were > 10 μg/ml for piroxicam in ethanol, 350 μg/ml piroxicam dissolved in DMSO (Fig. 2) and > 1 mM for acyclovir.

For antiviral assays, cell monolayers were infected with 100 plaque forming units (pfu)/well of drug-pretreated HSV-1. The highest concentration of these drugs was always the maximum non-cytotoxic concentration. After incubation for 3 days at 37 °C, monolayers were fixed and plaques were counted. The concentration of drugs required to reduce plaques by 50% (IC_{50}) was calculated by regression analysis of the dose-response curves. IC_{50} for piroxicam in ethanol or DMSO was determined to be 4 μg/ml and 75 μg/ml, respectively (Fig. 3). IC_{50} for acyclovir was determined at 1.6 μM. Selectivity indices for piroxicam in ethanol, piroxicam in DMSO and acyclovir were > 1.4, 4.6 and > 625, respectively.

Herpesvirus replication is characterized by a complex sequence of different steps that provide targets for antiviral agents. To identify the step at which the virus is affected by piroxicam, HSV-1 was preincubated with the active compound before infection of RC-37 cells, or RC-37 cells were treated with piroxicam

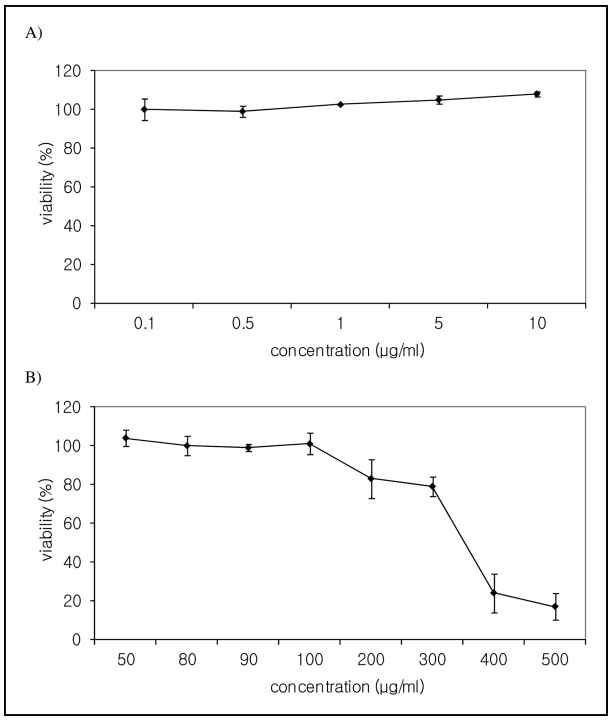

Fig. 2: Cytotoxicity of piroxicam dissolved in ethanol (A) and DMSO (B) for RC-37 cells. Viability of the drug-treated cells was determined in a standard neutral red assay.

either before or after viral infection. In all experiments, virus-infected cells were also incubated with 1% ethanol or 1% DMSO in the absence of the active compound to control for effects of these solvents on viral replication. The reduction of plaque formation was calculated relative to the amount of plaques counted in the absence of drugs. The standard antiherpetic drug acyclovir inhibited HSV infection completely when this drug was added during the intracellular replication period of HSV infec-

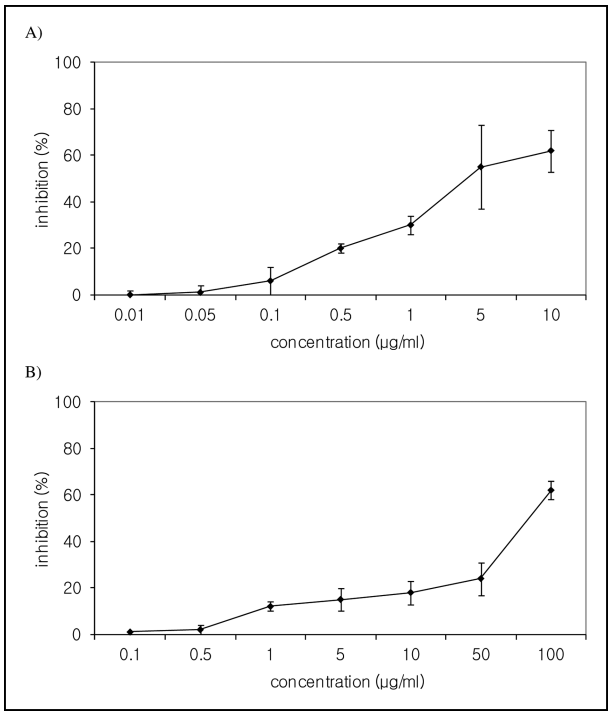

Fig. 3: Determination of the 50% inhibitory concentration (IC_{50}) of piroxicam dissolved in ethanol (A) and DMSO (B) against HSV-1. Viruses were incubated for 1 hour at room temperature with different concentrations of piroxicam and immediately tested in a plaque reduction assay.

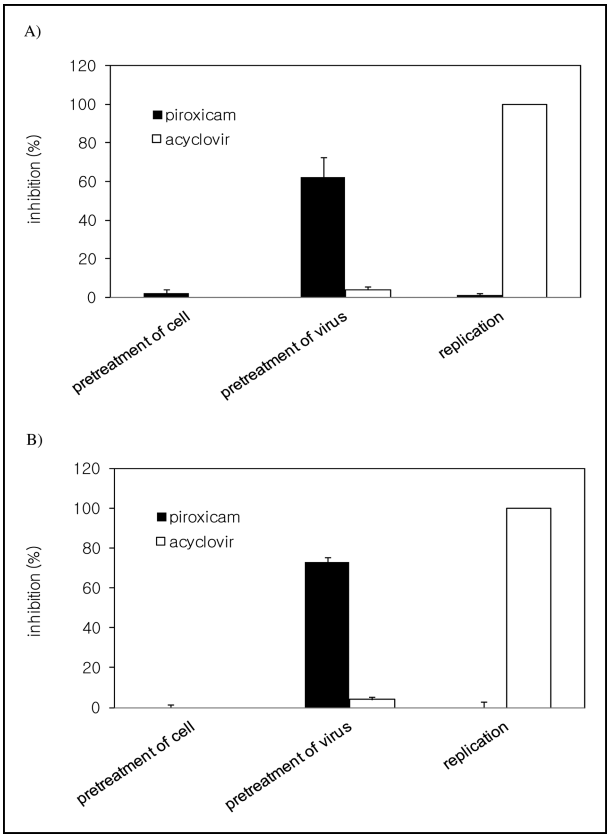

Fig. 4: Effect of piroxicam dissolved in ethanol (A) or DMSO (B) and acyclovir on herpes simplex virus-induced plaques on a monolayer of RC-37 cells. Piroxicam and acyclovir were added to the cell culture either before (pretreatment of cell) or after (replication) or the virus was pretreated with piroxicam and acyclovir prior to infection of the cells (pretreatment of virus).

tion (Fig. 4A and Fig. 4B). Pretreatment of HSV-1 (pretreatment virus) with piroxicam dissolved in ethanol or DMSO prior to infection caused a significant decline in the amount of plaques. At maximum non-cytotoxic concentrations of the tested drug, infectivity was reduced by 62.4% and 72.8% for the ethanolic and DMSO solution, respectively. In contrast, when the active compound was added to the host cells before viral infection (pretreatment cells) or after viral penetration of the host cells (replication), plaque formation was not reduced (Fig. 4A and Fig. 4B).

These results suggest that piroxicam interferes with the virions directly. It may interfere with its envelope and mask viral structures that are necessary for entry into host cells. Free herpesvirus is sensitive to piroxicam in a concentration-dependent manner (Fig. 3) and the inhibition of HSV-1 appears to occur before entering the cell but not after penetration of the virus into the cell. In conclusion, piroxicam affected the virus before penetration into the host cell and thus reveals a different mode of action than the classical drug acyclovir.

3. Discussion

The pharmaceutical industry is increasingly targeting synthetic and natural products focusing particularly on suitable additional antiviral agents (Khan et al. 2005). Several drugs are currently available for the management of HSV infections such as acyclovir and penciclovir. Acyclovir and related synthetic nucleosides interfere with viral DNA replication through activation by viral thymidine kinase (Brady and Bernstein 2004; De Clercq 2004). The efficacy of penciclovir is greater than that of acyclovir cream and acyclovir cream is more effective against herpes labialis than acyclovir ointment (McKeough and Spruance 2001). Acyclovir does not prevent the development of classical lesions, i.e. progression to vesicles, ulcers and crusts and its efficacy is higher when applied in the early prodrome or erythema lesion stage (Spruance et al. 2002). Acyclovir was shown to penetrate human skin more effectively in a cream than in an ointment formulation (Freeman et al. 1986). A new class of synthetic anti-lipopolysaccharide peptides that bind to heparin sulphate moieties on the cell surface inhibit infection with a variety of enveloped viruses, including HSV (Krepstakies et al. 2012).The incidence and severity of disease produced by herpes simplex virus are increasing in recent years, especially in the immunocompromised host where viral resistance to acyclovir represents a particular problem (Stranska et al. 2005). Drug-resistant viruses might be treated by humanized antibody therapy, as recently reported (Krawczyk et al. 2013).

Experiments to assess the cytotoxicity of piroxicam indicated relatively low toxicity in cell cultures according to Halle and Göres (1987). Selectivity indices for piroxicam in ethanol, piroxicam in DMSO and acyclovir were > 1.4, 4.6 and > 625,

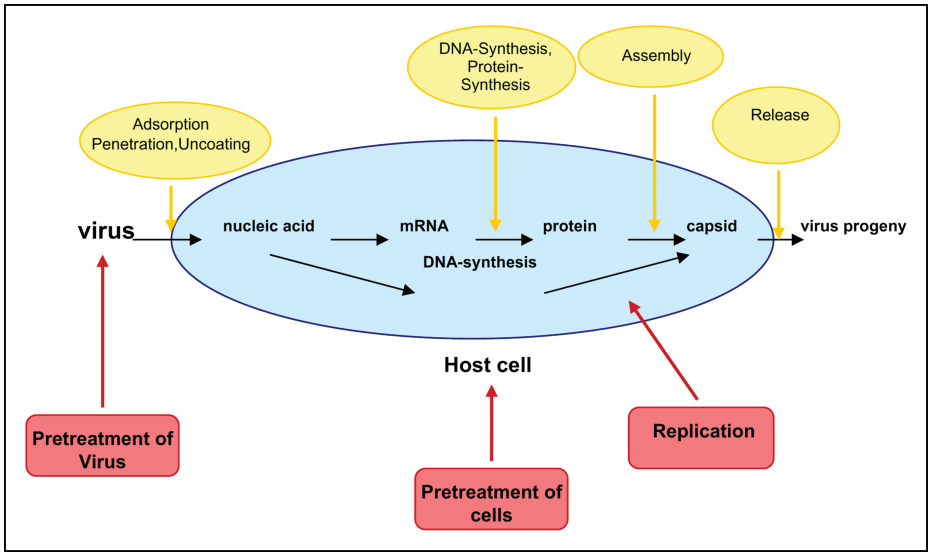

Fig. 5: Replication cycle of HSV-1 and possible interactions with piroxicam are shown in red. Illustration of time-on-addition experiments during the herpes simplex virus replication cycle. Cells were pretreated with piroxicam prior to virus infection (pretreatment of cells), viruses were pretreated with the drug prior to infection (pretreatment of virus), or piroxicam was added after penetration of the viruses into cells (replication).

respectively. We analysed the inhibitory effect of piroxicam on HSV-1 *in vitro* using plaque reduction assays. At maximum non-cytotoxic concentration of dissolved piroxicam, plaque formation of herpesvirus was significantly reduced.

In order to determine the mode of antiviral action, time-on-addition experiments have been performed at different steps in the herpesvirus replication cycle. Pretreatment of host cells with piroxicam and addition of piroxicam after the penetration of the viruses into host cells revealed no or minor effects on viral replication. No antiviral effect was observed during the entry and attachment phase of HSV. However, a drastic decrease in viral infectivity was detected for HSV-1, when viruses were treated with piroxicam dissolved in either ethanol or DMSO prior to infection. The standard antiherpetic drug acyclovir inhibited HSV infection completely when this drug was added during the intracellular replication period of HSV infection. However, clinical trials are needed to analyse the effectiveness of piroxicam against herpetic infections in direct comparison to patients treated with acyclovir. The high antiviral activity of piroxicam may be due to direct drug-virus interaction detected. A similar mode of antiviral action has been reported for some natural products, e.g. peppermint oil (Schuhmacher et al. 2003) and eucalyptus oil (Astani et al. 2010, 2011). Some essential oils, e.g. from thyme, were shown to be effective even against acyclovir-resistant HSV (Schnitzler et al. 2007). Our results indicate that piroxicam affected viruses before adsorption and in a different manner than acyclovir. An inhibition of herpes simplex virus by piroxicam late in the replication cycle can be excluded. A similar antiviral mode of action against HSV had been reported for *Melissa* extract and its isolated phenolic components which did not abolish HSV-1 protein expression of ICP0 and gD (Astani et al. 2012) and for a birch bark extract and its major components betulin, lupeol and betulinic acid with no effect on immediate early (ICP27), early (UL42) and late (gB and gD) protein expression of HSV-1 (Heidary Navid et al. 2014). However both extracts showed high antiherpetic activity when herpesvirus was pretreated with these extracts prior to infection, but *Melissa* extract and the major component rosmarinic acid inhibited viral attachment to the cellular receptor in a dose-dependent manner (Astani et al. 2012). Chebulagic acid and punicalagin, two hydrolyzable tannins isolated from the dried fruits of *Terminalia chebula Retz.* (*Combretaceae*) inhibited HSV-1 entry by interacting with viral glycoproteins involved in attachment and membrane fusion (Lin et al. 2011). Some studies reported pentacyclic triterpene, betulinic acid, interacting with HIV-1 viral glycoproteins gp41 and gp120 and inhibition of viral fusion and entry (Cichewicz and Kouzi 2004; Lai et al. 2008). *Melissa* extract (Astani et al. 2012) and blackberry extract (Danaher et al. 2011) showed an effective virucidal activity against HSV-1 *in vitro*. Consequently, there are several possibilities to explain the antiviral activity of piroxicam, i.e. inhibition of virus attachment to cellular receptors, inhibition of virus penetration into cells or direct virucidal activity. It remains to be determined whether the inhibitory effect of piroxicam is due to binding of the drug to viral proteins involved in host cell attachment/penetration or is due to damage of the virions, possibly their envelopes, thereby impairing their ability to infect host cells.

The influence of penetration enhancers on the percutaneous absorption and transdermal transport of piroxicam has been investigated. Oleic acid was found to be a very efficient enhancer for piroxicam absorption; 5% oleic acid increased piroxicam flux values 8 – 9-fold (Santoyo et al. 1995). Various techniques have been used, including pretreatment of the skin with permeation enhancers (Hsu et al. 1991) and application of permeation enhancement (Shin et al. 2000). The correlation between the physicochemical properties of nonsteroidal anti-inflammatory agents and the extent of their absorption from the skin has been confirmed by many investigations (Beetge et al. 2000).

In conclusion, piroxicam demonstrated low cytotoxicity and significant antiherpetic activity *in vitro*. The mode of antiviral action is different to the widely used drug acyclovir. Piroxicam affects herpesvirus at an early stage during viral replication and inhibits infection of host cells, whereas acyclovir targets viral DNA synthesis late during viral replication. Considering the lipophilic nature of piroxicam, which enables it to penetrate the skin, it might be suitable for topical treatment of herpetic infections, especially for those patients who experience frequent recurrences.

4. Experimental

4.1. Piroxicam

Piroxicam was provided by HOV GmbH, Hanover, Germany, dissolved in ethanol or DMSO to a concentration of 10 μg/ml (in ethanol) and 500 μg/ml (in DMSO), respectively, and further diluted in cell culture medium for *in vitro* experiments. The ethanol and DMSO concentration in the experiments never exceeded 1%, which has no effect on cells or viruses.

4.2. Cell culture and herpes simplex virus

RC-37 cells (African green monkey kidney cells) were grown in monolayer cultures with Dulbeccoś modified Eagleś medium (DMEM) supplemented with 5 % fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The monolayers were serially passaged whenever they became confluent and cells were plated into 96-well or 6-well culture plates for cytotoxicity and antiviral assays, respectively, and propagated at 37 °C in an atmosphere of 5 % CO_2. Herpes simplex virus type 1 (HSV-1) strain KOS was used for experiments (Schnitzler et al. 2008a). Viruses were routinely grown on RC-37 cells as described previously. Herpes simplex virus stock cultures were prepared from supernatants of infected cells and stored at – 80°C. Infectivity titers were determined by a standard plaque assay on confluent RC-37 cells. The replication cycle of HSV-1 and possible interaction with piroxicam is shown in Fig. 5. Acyclovir was purchased from GlaxoSmithKline (Bad Oldesloe, Germany), dissolved in sterile water and served as positive antiviral control at a concentration of 100 μM.

4.3. Cytotoxicity test and plaque inhibition assay

For cytotoxicity assays, cells were prepared into 96-well plates and incubated for 24 h at 37°C. The medium was removed and fresh DMEM containing the appropriate dilutions of piroxicam were added onto subconfluent cells in eight replicates for each concentration of the drug. Wells containing medium with 1 % ethanol or DMSO but no drug were also included on each plate as controls. After three days of incubation, the growth medium was removed and viability of the drug treated cells RC-37 was determined in a standard neutral red assay. The neutral red assay quantifies the number of viable cells after their exposure to toxicants by measuring the amount of neutral red dye taken up by the cells. Neutral red dye uptake was determined by measuring the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer. The mean OD of the cell-control wells was arbitrarily assigned a value of 100 %. Uptake and accumulation of the supravital dye had been shown to be linear with viable cell numbers. The cytotoxic concentration of the drug, which reduced viable cell number by 50 % (TC_{50}) was determined from dose-response curves. Additionally the maximum non-cytotoxic concentration of drug was determined (Schnitzler et al. 2008b).

Inhibition of HSV-1 replication was evaluated with plaque reduction assays. Usually 2×10^3 plaque forming units (pfu) were incubated with different concentrations of powder solutions for 1 h at room temperature, afterwards treated viruses were allowed to adsorb to RC-37 cells for 1 h at 37°C. The residual inoculum was then discarded and infected cells were overlaid with medium containing 0.5% methylcellulose. Each assay was performed in six replicates. After incubation for 3 days at 37°C, monolayers were fixed with 10% formalin. Treated cells were stained with 1% crystal violet and subsequently plaques were counted. By reference to the number of plaques observed in virus control monolayers (untreated cells), the concentration of test compound which inhibited plaque numbers by 50 % (IC_{50}) was determined from dose-response curves (Astani et al. 2010).

4.4. Mode of antiviral activity

Cells and viruses were incubated with piroxicam at different stages during the viral infection cycle in order to trace the mode of antiviral action.

Cells were pretreated with piroxicam before viral infection, viruses were incubated with this drug before infection or cells and viruses were incubated together with piroxicam after penetration of the virus into the host cells. Piroxicam solutions were always used at the maximum non-cytotoxic concentration. Cell monolayers were pretreated with the drug prior to inoculation with virus by adding the drug to the culture medium and subsequent incubation for 1 h at 37°C. After incubation, the medium containing the active component was aspirated and cells were washed immediately before the HSV-1 inocolum was added. For pretreatment of herpes simplex virus, the maximum non-cytotoxic concentration of the drug was added to viruses for 1 h at room temperature prior to infection of RC-37 cells. The effect of piroxicam solutions against HSV-1 was also tested during the replication period by adding powder solutions after adsorption to the overlay medium, as typically performed in antiviral susceptibility studies. Each assay was performed in five replicates, the number of plaques of drug-treated cells and viruses were compared to untreated controls to calculate the extent of plaque reduction (Koch et al. 2008).

4.5. Statistical analysis

All experiments were performed in triplicate and statistical analysis was performed by SPSS software (SPSS for Windows, 11.0, 2001, SPSS Chicago, Illinois). The means and standard errors were recorded.

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