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Evaluation of Some Pyrazoloquinolines as Inhibitors of Herpes Simplex Virus Type 1 Replication

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Three structurally related aminopyrazoloquinoline derivatives were evaluated for their antiviral activity against Herpes Simplex virus type 1. These compounds were examined for their in vitro antiviral activity by two different bioassays, namely; crystal violet staining and tetrazolium dye (MTS) measurement. The antiviral role of these compounds was confirmed by enumerating the infectious particles with plaque assay. The acute toxicity values of the biologically active compounds were determined prior to their screening as antiviral agents.

Keywords: Pyrazoloquinolines; Herpes Simplex virus type 1; Acute toxicity

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Introduction

Herpes simplex virus (HSV) infection is an ancient disease, with descriptions of orolabial herpes appearing in records from the fifth century BC [1]. HSV remains a common cause of ulcerative mucocutaneous diseases today [2−4]. Due to the high prevalence of HSV infection worldwide, most persons infected with HIV are also infected with one or both HSV types [5] Initial infection results in painful ulcerative mucosal and cutaneous lesions. Herpes simplex virus (HSV) type 1 (HSV-1) has the ability to become latent in sensory ganglia and to induce recurrent infections following reactivation [6]. The frequencies of recurrent herpetic infections in the U.S. population are estimated to be 50 to 70% for HSV-1 and 23% for HSV-2 [7]. Mucosal or skin surfaces are the usual sites of primary infection. Recurrent herpes labialis and herpes genitalis represent the most common clinical manifestations associated with HSV-1 and HSV-2 infections, respectively. Most recurrences are asymptomatic infections, and the shedding of herpes virus under these conditions represents the most common form of transmission of this disease. Recurrences are associated with physical or emotional stress, fever, exposure to UV light, tissue damage, and immune suppression. The frequency of recurrences has also been correlated with the severity and duration of the initial infection [7]. Although herpes is usually a mild disease in immunocompetent individuals, mucocutaneous herpetic infections are troublesome, especially for patients with frequent episodes. Moreover, immunocompromised (HIV or cancer) patients have an increased risk of developing severe and more frequent herpetic infections.

During the past several decades, acyclovir has been the drug of choice for the treatment of herpetic infections. However, the emergence of acyclovir-resistant HSV isolates has been reported for immunocompromised patients [8] as well as for organ and bone marrow recipients [9, 10]. Recurrent acyclovir-resistant genital herpes has also been described for an immunocompetent host [11]. Foscarnet (trisodium phosphonoformate) has a broad antiviral spectrum and in vitro activity against all human viruses of the herpes virus family, including cytomegalovirus, HSV, and varicella-zoster virus [12, 13]. This drug is also effective against acyclovir-resistant HSV and varicella-zoster virus [14-18]. Moreover, acyclovir-resistant HSV strains that become resistant to foscarnet may once again be susceptible to acyclovir [19]. Because the intravenous administration of foscarnet is limited by the occurrence of nephrotoxic reactions, the development of topical formulations represents an attractive approach for the treatment of mucocutaneous herpetic infections, especially for those caused by acyclovir-resistant strains.

Topical formulations currently available for the treatment of mucocutaneous herpetic infections include 5% acyclovir ointment (Zovirax) and penciclovir cream formulation (Vec-

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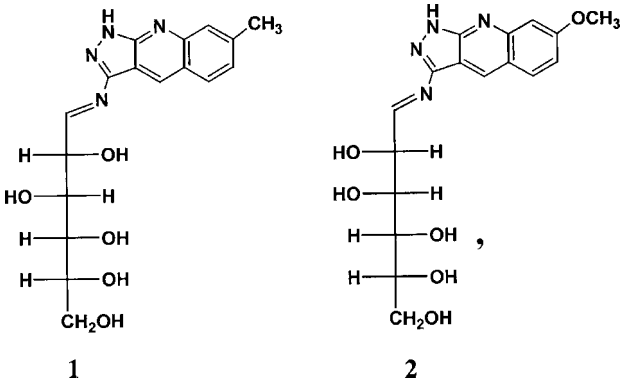


Figure 1. Structures of aldehydo-sugar pyrazoloquinoline derivatives.

tavir cold sore cream or Denavir cream in the United States). The currently available treatment, either topical or systemic, has only limited efficacy, particularly against symptomatic recurrent herpes. Treatment of recurrent herpes with topical acyclovir demonstrated no or only limited clinical benefit [20–23]. Wallin et al. demonstrated a limited but significant effect of topical foscarnet cream on time to healing for recurrent genital herpes [24]. Conversely, no significant improvements in time to healing or loss of symptoms were observed for recurrent genital herpes in two other clinical trials [25, 26]. Patients who received treatment in the prevesicular stage had a slightly reduced number of days with lesions [27]. Treatment of herpes labialis in immunocompetent patients with penciclovir cream was reported beneficial for treatment started in the prodrome and erythema stages as well as in the papule and vesicle lesion stages [28].

Nevertheless, a high medical need exists for improved antiherpetic drugs for the treatment of severe disease. Encephalitis in newborns, for example, results in 15% mortality, and only 29% of survivors develop normally after acyclovir therapy [29]. Also, for patients with less severe disease, an agent that will achieve a better reduction of lesion duration with episodic treatment beyond the 1 to 2 days' reduction achieved with current medications is urgently required [30]. Furthermore, a drug which continues to show profound efficacy when given at later stages of herpetic disease would be a new and highly desired standard in the treatment of herpes [31]. We have already reported the synthesis and herpes simplex virus type 1 replication inhibitory activity of aldehydo-sugar derivatives of pyrazoloquinoline [32]. The study resulted in two aldopentose derivatives 1 and 2 possess inhibitory activity against virus growth, Figure 1. In the present work, the activity of animopyrazoloquinoline precursors of the already reported aldehydo-sugar derivatives were evaluated for their inhibitory activity against herpes simplex type 1 replication.

Results and discussion

Chemistry

The tested aminopyrazoloquinoline derivatives were synthesised according our reported procedure [33, 34] as shown in Scheme 1.

Biological screening

The plaque assays repeatedly demonstrated that the compound **5** had the maximum antiviral activity against HSV. Proper controls included the diluent and the well-known anti-HSV guanosine analog, Acyclovir. Previously reported compounds **1** and **2** were also included in this study and shown to have antiviral activity by the plaque assay. Without the sugar moiety, compounds **3** and **4** were restricting the replication of HSV while compound **5** clearly had similar or more anti-HSV activity than Acyclovir as shown in Figure 2.

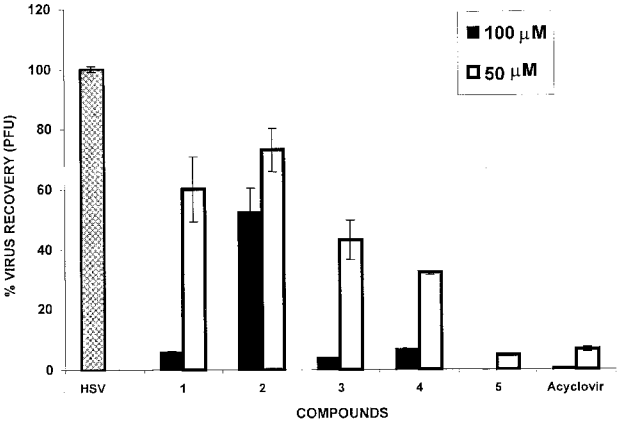
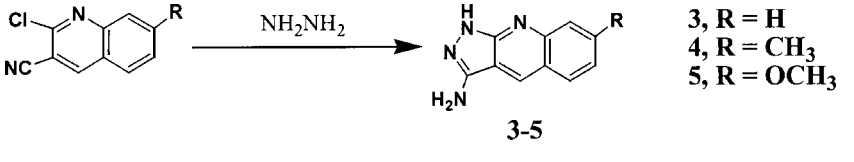


Figure 2. Effect of Pyrazoloquinolines on HSV replication.



Scheme 1. Synthesis of the aminopyrazoloquinoline derivatives that were tested in this study.

Full Paper

The MTT and crystal violet assays are based on the integrity of the eukaryotic cells either at the presence of intact cytoplasmic membrane or the cellular enzymatic level. In certain cases where the reagents interfere with the spectrophotometric analysis, the interpretation of result becomes difficult. With the previously reported result, this interference was negligible and a proper correlation was obtained when the antiviral effect of compound **1** and **2** was reported [32]. With these pyrazoloquinolines, the spectrophotometric observation was difficult and, therefore, the plaque assay was used in this study.

Acute toxicity

Compounds **3**, **4** and **5** were further evaluated for their oral acute toxicity in male mice using a literature method [35, 36]. The results indicated that the tested compounds proved to be non-toxic and well tolerated by the experimental animals up to 200 mg/kg, although no mortality was recorded at 350 mg/kg. The oral LD_{50} of compounds **3**, **4** and **5** were found to be 364, 378 and 356 mg/kg respectively. Moreover, these compounds were tested for their toxicity through parenteral route [37]. The results revealed that all the test compounds were non-toxic up to 80 mg/kg.

It can be safely concluded from repeated experiments, that compound **5** outperformed Acyclovir at equimolar ratio. We intend to continue this study at the molecular level to understand the mechanism of action of compounds **1**, **2** and **5**. Such compounds would represent a fruitful matrix for the development of a new class of antiherpetic agent with good safety margin.

Experimental

Chemistry

All chemicals were purchased from E. Merck (Darmstadt, Germany), Fluka AG (Buchs, Switzerland) and Aldrich (St. Louis, MO, USA) companies. Melting points were determined in open glass capillaries using a Thomas capillary melting point apparatus and are uncorrected. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-protected aluminum sheets (Type 60 F254, Merck) and the spots were detected by exposure to UV-lamp at 254 nm for few seconds.

3-amino-1H-pyrazolo[3,4-b]quinoline (**3**), 3-amino-7-methyl-1H-pyrazolo[3,4-b]quinoline (**4**) and 3-amino-7-methoxy-1H-pyrazolo[3,4-b]quinoline (**5**) were synthesised according to our previously reported procedure [33, 34]. The selected 2-chloroquinoline-3-carbonitrile (910 mmol) was heated with hydrazine hydrate (25 mL) for 5 h. The reaction mixture was cooled, then poured in ice-cold water. The separated solid product was filtered, washed with water and crystallised from ethanol. All spectral data and elemental analysis confirmed the structures of the synthesised compounds.

Biological screening

Antiviral assay

Reagents needed for the MTT cytotoxicity test, Crystal Violet staining and the compound dilutions are described in our previous communication [32]. Cell culture and the viral plaque assay reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acyclovir (for intravenous injection) was obtained from Faulding Pharmaceutical Co. (Paramus, N.J. USA).

The antiviral activity of these pyrazoloquinolines was determined as follows. Twelve-well cluster tissue culture plates (Nunc, Denmark) were seeded with African green monkey kidney (Vero) cells. After 24 h of incubation the medium was aspirated and the HSV inoculum was added at a multiplicity of infection of 10 and left for 90 min for adsorption at 37°C. Unadsorbed virus particles were removed with phosphate buffered saline (PBS) containing 2% fetal bovine serum. The stock solution of these chemical compounds were suspended in the maintenance medium (EMEM containing 2% fetal bovine serum [FBS]) and added wells in duplicate to give a working concentration of 100 and 50 μM**.** Cells were lysed with two cycles of freezing and thawing after 24−36 h of incubation. Lysed cell suspensions were collected in separate tubes and kept frozen at −80°C until used. Before conducting the plaque assay, the cell lysates were centrifuged at 1500*g* for 5 min, and log dilutions of the supernatant were made in PBS containing 2% FBS. The plaque assay for the enumeration of infectious HSV particle has been reported earlier [38]. Briefly, the 60-mm tissue culture dishes (Nunc, Denmark) were seeded with Vero cells and after 48 h of incubation medium was aspirated and virus dilutions were added in triplicate. After 90 min of adsorption at 37°C, 3 mL of PBS-FBS was added to wash away unadsorbed virus particle. Agar overlay in a volume of 5ml was added to each dish and the dishes were transferred to incubator after the agar was solidified. Dishes were incubated for 3-5 days, inoculated with 3 mL of neutral red solution (0.033%) made in PBS, and incubated for 4 h. Excess stain was decanted and the plaques were counted over a light box.

Acute toxicity

Compounds **3**, **4** and **5** were further investigated for their acute toxicity in male mice [35, 36]. Eight groups of mice each consisting of six animals, were used. The compounds were given orally in doses of 1, 10, 100, 200, 250, 500 mg/kg, respectively. Twenty-four hours later, the% mortality in each group and for each compound was recorded. The LD_{50} values were calculated using the method described by Litchfield and Wilcoxon [36].

Moreover, these compounds were tested for their toxicity through parenteral route [37]. Groups of mice each consisting of six animals were used. The compounds or their vehicle, propylene glycol (control) were given by intraperitoneal injection in doses of 10, 25, 50, 80, 100 mg/kg, respectively. Survival was followed up to 7 days.

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