METHOD FOR IDENTIFYING COMPOUNDS WITH ANTI-HERPES ACTIVITY

The invention relates to a method for identifying compounds with antiviral activity, characterized in that those compounds are selected in the presence of which resistant viral mutants can be selected, wherein at least one selected viral mutant carries at least one mutation in the primase gene and another selected viral mutant carries at least one mutation in the helicase gene or a selected viral mutant carries at least one mutation in the helicase and at least one mutation in the primase gene.
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
Method for identifying compounds with anti-herpes activity

This invention relates to a method for identifying compounds with anti-herpes activity and to medicaments for the treatment of herpes infections in a mammal by inhibiting the herpes helicase-primase enzyme complex.

The goal of inventions in the field of the pharmaceutical area is to supply medications or therapies to treat a patient in a tolerable way that a desired therapeutic effect is achieved.

Numerous medications and therapies were invented and more recently, since mono-therapy fails in some cases to show a profound therapeutic benefit for the patient, these methods are often combined to increase the therapeutic efficacy. However, in many cases of combination therapy not only the desired potency of treatment is amplified, but also more side-effects occur and thus, the tolerability is decreased. A classic example is the combination therapy in the field of AIDS treatment.

In the field of anti-infectives, for example, a compound that inhibits two essential targets in the life cycle of a given pathogen simultaneously may be more active due to cumulative inhibitory effects as demonstrated by the analogous case of combination therapy in the past which is often superior to a (single compound chemo) mono-therapy where the drug targets only one relevant binding pocket on a single target protein or on one subunit of a complex. In the case where the compound binds at the interface of two targets a stronger binding may also be observed based on avidity effects. The better binding characteristics and or the cumulative inhibitory effects may result in a superior therapeutic treatment ideally accompanied by a better tolerability which may result from a lower dosage or pill burden during treatment.

Herpesviridae evolved over millions of years and are highly disseminated in nature. Members of the family were identified in humans, non-human primates and most other mammals and vertebrates (Virology, 1996, Fields et al. Lippincott-Raven
Publishers, Philadelphia, PA 19106, USA). Herpesviruses are enveloped double stranded DNA viruses which infect cells that carry negative charged structures such as heparansulfate and or glycosaminoglycans in addition to a herpes viral entry mediator on their surface. One key feature of these viruses is their ability to remain latent in their host for life after primary infection and to reactivate more or less frequently from a pool of latent infected cells upon diverse internal and external stimuli.

Eight human herpesviruses (HHV1 to HHV8) have been identified so far and their genomes (~ 125-230 kbp in size) were sequenced and published in the internet (GeneBank, EMBL database etc.). High sequence homologies were identified between the genomes especially in the case of the helicase and primase coding genes, thus, the replication machinery is conserved among the herpes viridae but is clearly different from eukaryotic DNA replication. The genomes code for more than 50 genes which are essential for the viral replication cycle in vitro and or in vivo and many publications discussed the more or less relevance of these targets for antiviral therapy. The function of the helicase-primase complex in the viral life cycle and its qualification as a target for antiviral (chemo) therapy of an infected host are published (Herpes Simplex Virus Replication, 1997, Annual review of Biochemistry, Boehmer PE & Lehmann IR, 66, 347-384; The Structure and function of the HSV DNA replication proteins: defining novel antiviral targets, 1993, Antiviral Research, Matthews JT, Terry BJ, Field AK, 20, 89-114).

Based on their similarity in biological properties the viruses were grouped in 3 sub-families, namely α-(HHV1 to 3), β-(HHV5 to 7), and γ-(HHV4 and HHV8) herpesviruses. Common names were derived from the clinical symptoms they cause or historical reasons e.g. Herpes simplex virus 1 or 2 (HSV-1 or –2 the cause of herpes labialis and genitalis) is used for HHV1 or 2, the Varicella-zoster virus (VZV causing chickenpox or zoster) is synonym to HHV3 and Epstein-Barr virus (EBV) and Cytomegalovirus (HCMV) are synonyms for HHV4 and 5, respectively.
Incidence and prevalence data range from below 10 to more than 90% of the population being infected with one or more herpesviruses depending on the HHV involved and the age, sex, social status and geographical aspects of the host. Since some herpesviruses are ubiquitous human pathogens causing a variety of disease ranging from benign illness interfering with normal day activities to life or sight threatening disease especially in immunocompromised patients (keratitis, disseminated disease or retinitis in AIDS patients), pregnancy (abortion, deafness) and newborns (hepatitis, encephalitis), there is a strong medical need for a safe and efficient treatment and much effort has been expended in the search for effective antiviral agents.

Acyclovir, a selective and specific inhibitor of viral replication, was a true milestone in the development of antiviral drugs in the late 1970s. Newer nucleosidic drugs that are similar to acyclovir such as penciclovir or the more convenient pharmaco-kinetically optimized pro-drugs like valacyclovir and famciclovir were launched in the late 1990s. Nucleosides indeed became the treatment of choice in the herpes indication, however, nucleosides are pro-drugs and have to be phosphorylated by the viral thymidine kinase (TK) and subsequently by cellular kinases for activity upon the viral DNA-polymerase. If the virus does not express a functional TK e.g. a resistant HHV1 strain or TK negative viruses such as HCMV, or the DNA-Pol has not the optimal primary structure, the potency of the drug diminishes, the selectivity index is significantly smaller, higher doses have to be administrated and adverse effects are more likely to be associated with treatment. Since nucleosides are obligate or non-obligate chain terminators of DNA-polymerization they are potentially mutagenic which is well documented for ganciclovir. Thus, broad spectrum anti-herpesvirus activity, efficacy especially upon delayed treatment, safety and resistance are goals for the next generation of drugs directed against novel antiviral targets.

WO 97/24343 describes a method to identify inhibitors with anti-herpes properties by selecting compounds which, upon binding to the DNA-helicase-primase complex, stabilize the latter. A disadvantage of this method is that often compounds are identi-
fied that have not a suitable selectivity index in vitro or the necessary tolerability in vivo, and thus, are often not suitable for treatment of herpes infections due to side effects.

WO 99/42455 and Spector, F.C., *Journal of Virology* 1998, pages 6979-6987 describe compounds that exclusively bind to the UL5 subunit of the complex, which is extensively confirmed by analysis of at least 25 different UL5 mutants.

The goal of this invention is therefore to provide a method to identify alternative or more active compounds which have a better selectivity index and/or tolerability.

In another aspect the goal of the present invention is to provide a method for identifying compounds which are active against acyclovir-resistant viral mutants.

The present invention solves the abovementioned problems by providing a method for identifying compounds with antiviral activity, characterized in that those compounds are selected in the presence of which resistant viral mutants can be selected, wherein at least one selected viral mutant carries at least one mutation in the primase gene (e.g. UL52 in the case of HSV) and another selected viral mutant carries at least one mutation in the helicase gene (e.g. UL5 in the case of HSV) or a selected viral mutant carries at least one mutation in the helicase and at least one mutation in the primase gene.

In a preferred embodiment, the method is further characterized in that the primase and helicase are at least 50% homologue to the primase and helicase of herpes simplex virus 1 based on the nucleotide sequence or the amino acid sequence, whichever is higher.

In another preferred embodiment, the method is further characterized in that the virus is a herpes virus.
In another preferred embodiment, the method is further characterized in that before
determining the generation of resistant viral mutants in the presence of said
compounds,

a) the compounds are tested for their ability to inhibit the helicase and
b) the compounds are tested for their ability to inhibit the primase
c) such compounds are selected, that inhibit the helicase and the primase

In more preferred embodiment, the method is further characterized in that such
compounds are selected, that bind simultaneously to the helicase and primase
subunits of the helicase-primase complex. Without being bound by theory, it is
believed that compounds bind at the interface of the helicase and primase subunits of
the helicase-primase complex of a herpes simplex virus (and homologous regions of
other herpes virus helicase primase enzymes) and thereby cause the inhibition of
essential enzymatic activities of the helicase-primase complex (either the minimal
heterodimeric complex UL5/UL52 or the native heterotrimeric complex UL5/UL8
and UL52 in the case of a herpes simplex virus) possibly by blocking the ATP
binding site competitively. The compound may either be bound in a ternary complex
involving residues of the helicase and the primase subunits and possibly stabilized by
avidity effects or the compounds bind independently to at least one site on the
helicase and the primase subunits. "To bind simultaneously" here describes a state,
where the very same molecule is bound to both UL5 and UL52 gene products at the
same time.

In another more preferred embodiment, the method is further characterized in that the
compounds inhibit the DNA-dependent NTPase activity of a herpes helicase-
primase. One additional assay that may be run determines the ability of test
compounds to inhibit helicase-primase-associated DNA-independent NTPase
activity. The compounds useful in this invention do not inhibit this activity, in the
way that competitively-acting nucleoside analogues inhibit this activity.
In another preferred embodiment, the method is further characterized in that the compounds inhibit the replication of a herpes virus.

In another more preferred embodiment, the method is further characterized in that such compounds are selected, that have the ability to inhibit replication of a herpes virus in cell culture by at least 50 % at a concentration of less than about 10 μM.

In another more preferred embodiment, the method is further characterized in that such compounds are selected, that have the ability to inhibit replication of a herpes virus in cell culture by at least 50 % at a concentration of less than about 500 nM.

In another more preferred embodiment, the method is further characterized in that the herpes virus is HSV.

In another more preferred embodiment, the method is further characterized in that the herpes virus is PRV or BHV.

In another embodiment, the invention relates to a compound identified by the abovementioned method.

In a more preferred embodiment, the invention relates to a compound which is not a nucleoside or nucleotide.

The present invention also relates to the use of such a compound for the preparation of a medicament for the treatment and prevention of herpes infections and to a pharmaceutical composition comprising such a compound.

The present invention also relates to a method for treating herpes infection in a mammal comprising the step of administering to a mammal in need of such treatment a therapeutically effective amount of the abovementioned pharmaceutical composition.
The present invention also relates to a method for identifying compounds, characterized in that

1. a) the compounds are tested for their ability to bind to at least two therapeutically relevant targets simultaneously and
b) the compounds are tested for their ability to inhibit and/or activate these targets upon binding
c) the compounds are tested for reduction of their ability to inhibit and/or activate these targets in the presence of at least one mutation in one target and at least one mutation in the other target or at least one mutation in either target.

The present invention also relates to a compound with antiviral activity, characterized in that those compounds are selected in the presence of which resistant viral mutants can be selected, wherein at least one selected viral mutant carries at least one mutation in the primase gene and another selected viral mutant carries at least one mutation in the helicase gene or a selected mutant carries at least one mutation in the helicase and at least one mutation in the primase gene.

The present invention also relates to a compound capable of binding simultaneously to the helicase and primase subunits of the helicase-primase complex.

Compounds with antiviral activity are described in PCT/00/01498. They are not preferred in the context of the present invention.

The invention described herein overcomes the above-mentioned limitations and satisfies the medical needs by providing a method for identifying non-nucleosidic inhibitors that act directly by interfering with the essential enzymatic activity helicase-primase in a novel way and consequently inhibits herpes virus DNA replication and herpes virus replication \textit{in vitro} and \textit{in vivo}. Furthermore, since the herpes virus helicase-primase enzyme is conserved across the herpes viridae, the inventors
could demonstrate broad anti-herpes virus spectrum activity (see also table 1). The selective action of these inhibitors against herpes viruses and especially against acyclovir-resistant herpes viruses, combined with a wide margin of safety, renders the compounds as desirable agents for combating herpes infections.

The term "herpes" as used herein refers to any virus in the herpes family of viruses and particularly, to those herpes viruses that encode a herpes helicase-primase homologous to the herpes helicase-primase of HSV-1. The herpes family of viruses includes, but is not limited to HHV-1 to HHV-8, EHV, BHV, PRV etc. Preferred are herpes simplex viruses type 1 and 2. Herpes simplex virus 1 and 2 refers to a virus that is characterized by specific monoclonal antibodies (serotype 1 or 2).

The term helicase-primase refers to the helicase-primase complex involved in DNA replication. In the case of the herpes simplex virus it is composed of the UL5, UL8 and the UL52 or the least (necessary or essential) UL5 and UL52 gene products, in the case of other herpes viruses of the corresponding homologues.

The term helicase refers to the helicase subunit of the helicase-primase complex involved in DNA replication. In the case of the herpes simplex virus it is the UL5 gene product, in the case of other herpes viruses it is the corresponding homologue.

The term primase refers to the primase subunit of the helicase-primase complex involved in DNA replication. In the case of the herpes simplex virus it is the UL52 gene product, in the case of other herpes viruses it is the corresponding homologue.

The term selectivity index refers to the quotient of the concentration were the compound reduces the viability of the cell by 50 % and the EC<sub>50</sub> or IC<sub>50</sub> (CC<sub>50</sub>/IC<sub>50</sub>). The term therapeutic index (tolerability) refers to the quotient of the dose (lethal dose) were 50 % of the animals die and the dose (effective dose) were 50 % of the animals survive the infection (LD<sub>50</sub>/ED<sub>50</sub>).
The term pharmaceutically acceptable carrier or veterinarily acceptable as used herein means a non-toxic, generally inert vehicle for the active ingredient which does not adversely affect the ingredient.

The term inhibit, when used in connection with enzymatic activity, refers generally to inhibiting the enzymatic activity by at least about 50% at a concentration of about IC_{50} = 100 \mu M or less and preferably at a concentration as low as possible in a conventional in vitro assay for enzymatic inhibition.

The term inhibit, when used in connection with in an in vitro cell-based viral replication assay, refers generally to inhibiting viral replication by at least about 50% at a concentration of about IC_{50} = 10 \mu M or less and preferably at a concentration as low as possible in the in vitro cell-based viral replication assay presented here.

The term target describes a gene product involved in the manifestation of diseases.

The term mutation in one target describes a mutation in a gene that leads to an altered amino acid sequence of the respective protein.

The term reduction describes a loss of activating or inhibiting activity in the order of at least a factor of two, preferably of one order of magnitude.

The compounds of this patent are new and can be clearly differentiated structurally from WO 97/24343, WO 99/42455 and any other publication to date.

Compounds useful for inhibiting a herpes helicase-primase according to the above mechanism may either be identified by assaying a test compound's ability to bind to the helicase (subunit) and the primase (subunit) of the helicase-primase complex or more preferably to inhibit enzyme-associated single stranded DNA-dependent NTPase activity of a herpes helicase-primase (such as the helicase-primase of herpes
simplex viruses) advantageously in a high throughput (HTS) assay or by assaying a
test compound’s ability to inhibit viral replication in a cell-based viral replication
assay followed by selecting resistant viruses in the presence of compound and
sequencing the helicase-primase genes of the selected mutants.

Numerous assays are described to directly determine binding of compounds to
proteins, however, several of these assays require purified or at least partially purified
protein. In addition to that, not all binders interfere with the function of the enzyme
or protein.

The enzymatic assay is easy to perform, but the enzyme has to be purified from
herpes infected cell culture or the genes have to be cloned, expressed and produced
and purified using an expression system and protein purification techniques.

The cell-based viral replication assay is easy to perform and possibly more sensitive
than the enzymatic assay, since it mimics the real situation of a viral infection more
closely, but mutant viruses have to be generated and analyzed subsequently by
sequencing or by a complementation analysis to confirm the viral target helicase
primase.

Compounds effective in either the enzymatic assay or the cell-based viral replication
assay may be further assayed to determine their herpes helicase-primase binding
specificity or compounds effective in a binding assay have to be evaluated in either
the enzymatic assay or the cell-based viral replication assay. Although the following
assays are described in one particular sequence, it should be understood that not all of
these assays need to be performed for successful identification of herpes helicase-
primase inhibitors. In addition, the exact order of assay may be altered, if desired.
These and other procedural options can be considered by those of ordinary skill in
the art.
Other assays measure a test compound’s ability to inhibit enzyme-mediated RNA primer biosynthesis or a test compound’s ability to inhibit helicase activity.

Any DNA but preferably single stranded DNA with a primase consensus site for the primase assay or any DNA substrate designed to model a replication fork-like structure may be used for the helicase assay. Several options are outlined in the cited review by Boehmer PE & Lehmann IR 1997 or more recently in the paper entitled High-Throughput Screening Assay for Helicase Enzymes, 1998, Analytical Biochemistry, M Sivaraja, H Giordano, MG Peterson, 265, 22-27.

Compounds useful in this invention do not intercalate into, nor otherwise bind directly, double stranded DNA and are not positive in a cytogenetic or Ames Test.

In all of the above-methods, the non-nucleoside compound is preferably further characterized by an ability to inhibit herpes helicase-primase mediated RNA primer biosynthesis or helicase activity. In addition, preferred non-nucleoside inhibitors of this invention are further characterized by an ability to inhibit replication of a herpes virus in cell culture by at least about 50 % at a concentration of less than about 10 μM more preferably at a concentration as low as possible e.g. in the nM range or even less.

It is important to recognize that the compounds, compositions and methods of this invention may be used against nucleoside non-responsive and nucleoside resistant herpes infections.

This aspect of the invention involves a method for treating acyclovir-resistant herpes infections in a mammal which comprises administering to the mammal an anti-acyclovir-resistant herpes effective amount of a compound as defined herein, or a therapeutically acceptable acid addition salt thereof.
Using the above noted screening methodologies, several compounds were identified as inhibitors of herpes helicase-primase. The selective action of these compounds against these viruses, combined with a wide margin of safety, renders the compounds as desirable agents for combating herpes infections.

Anti-herpes Activity

The antiviral activity of the compounds can be demonstrated by biochemical, microbiological and biological procedures showing the inhibitory effect of the compounds on the replication of herpes viruses e.g. HSV-1, HSV-2, BHV etc.

A biochemical procedure for demonstrating anti-herpes activity for compounds is described below under Biochemical assay. This ATPase assay is one among numerous possible assays that are described for instance in High-Throughput Screening Assay for Helicase Enzymes, 1998, M. Sivaraja, H. Giordano and M.G. Peterson, Analytical Biochemistry, 265, 22-27. Other assays cited there measure the helicase or the primase activity of the helicase-primase complex with diverse assay configuration or methods.

Methods for demonstrating the inhibitory effect of compounds on herpes viral replication involving in vitro and cell culture techniques are described in cell based viral replication assay.

The therapeutic effect of the compounds can be demonstrated in vivo in a lethal challenge model as shown in section lethal challenge in vivo animal model.
Description of the Table

Table 1 summarizes the IC$_{50}$ or EC$_{50}$ of wild type or resistant HSV strains, the ED$_{50}$ of wild type, the relevant mutations and cross resistance patterns.

A direct comparison of the *in vitro* cell culture IC$_{50}$ values shows that some of the example compounds are at least one order of magnitude more potent than Acyclovir/Zovirax. The *in vivo* data demonstrate that this antiviral activity translates to a superior potency and efficacy in animal models. Example compounds inhibit acyclovir resistant HSV-1 (F) mutants and wild type HSV-1 (F) with nearly identical IC$_{50}$ values. Broad Spectrum activity is exemplified by examples 1, 4, 5, 6 and 7, which inhibit as different viruses as the human herpes simplex virus as well as porcine (PRV) and bovine (BHV) animal viruses. These compounds are also active on all clinical isolates tested so far (39 clinical isolates (HSV-1) and 19 clinical isolates (HSV-2)). It demonstrates for the first time that compounds that qualify the above mentioned characteristics outperform the antiviral activity of the state of the art by at least one order of magnitude.
**Assay**

HSV-1 DNA-dependent ATPase assay (*in vitro* assay based on the inhibition of HSV-1 helicase-primase).

a) Preparation of the enzyme

HSV-1 helicase-primase heterodimer was produced in doubly infected Sf9 (Spodoptera frugiperda) cells using recombinant baculoviruses expressing the UL5 and the UL52 helicase-primase subunits.

The genes were amplified by PCR from HSV-1 (F) (American Tissue Culture Collection ATCC VR-733) and cloned into the baculovirus expression system (UL5 -> pFASTBAC1; UL52 -> pFASTHTb) according to the Instruction Manual BAC-TO-BAC Baculovirus Expression Systems, Life-Technologies. The heterodimeric enzyme was purified by IMAC-Chromatography as described in “Inhibition of Herpes Simplex Virus Replication by a 2-Amino Thiazole via interactions with the Helicase Component of the UL-5-UL8-UL52 Complex”, 1998, F.C. Spector, L. Liang, H. Giordano, M. Sivaraja and M.G. Peterson.

b) ATPase Assay:

Purified heterodimeric helicase-primase complex (200 ng) was incubated with 1 µg DNA (Sigma D3287 or D8681) in 20 mM HEPES (pH 7.6; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5 mM MgCl₂, 0.5-5 mM ATP, 100 µg BSA (bovine serum albumin) per ml, 10% glycerol, 1 mM DTT (DL-dithiothreitol) and for 60 min at 37 °C. The released inorganic phosphate was detected colorimetrically as described previously in “An improved assay for nanomole amounts of inorganic phosphate”, 1979, P.A. Lanzetta, I.J. Alvarez, P.S. Reinach and O.A. Candia, Analytical Biochemistry, 100:95-97.
DNA-dependent ATPase activity was calculated from the net absorbance change in the presence and absence of inhibition.

Figure 1 shows a dose dependent inhibition/titration of the enzymatic ATPase activity of the HSV-1 helicase-primase with representative example 7. For their purpose, ATPase activity of the helicase-primase heterodimer (release of inorganic phosphate (Pi)) was measured in the presence of saturated DNA concentrations and varying ATP and inhibitor concentrations as described under ATPase Assay. Inhibition of the ATPase activity by compound example 7 is dose dependent and in addition dependent on the ATP concentration (e.g. 50% inhibition of Pi release at 5 mM ATP and ~10 μM of the compound of example 7 versus 50% inhibition of Pi release at ~100 nM of the compound of example 7 (ATP control : without enzyme and without DNA).

**Cell based viral replication assay**

a) Growth of HSV and preparation of virus stocks

Herpes simplex viruses (HSV-1 Walki, HSV-1F or HSV-2G) were routinely propagated on African green monkey kidney cells (Vero cells; ATCC CCL-81), however, many tissues-culture lines can be used for the growth and quantification of HSV (Herpes Simplex Virus Protocols, 1998, Ed. S.M. Brown & A.R. MacLean, Humana Press, Totowa, New Jersey). These cells were grown in M199 media supplemented with 5% foetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin in cell culture flasks (e.g. 175 cm² / 25-50 ml) at 37°C in the presence of 5% CO₂. Cells were split 1:4 twice a week (cell viability >95%). For virus infection the medium was removed, the cells were washed with Hank's solution, trypsinized with 0.05% trypsin, 0.02% EDTA (Seromed, L2143) and seeded at a density of 4x10⁵ cells per ml in cell culture flasks and incubated for 24 hours at 37°C in the presence of 5% CO₂. The medium was removed and virus suspension was added at a multiplicity of infection (m.o.i) of < 0.05 in a volume of
2 ml per 175 cm² surface area. The flask was allowed to stand 1 hour at 37°C in the
presence of 5 % CO₂ with occasional agitation to distribute the virus evenly over the
cells. Then medium was added to a final volume of 50 ml per 175 cm² tissue culture
flask. The cultures were incubated until all cells showed signs of infection
(approximately 2-3 days). To harvest the virus, flasks were frozen at -80°C and
thawed at 37°C (in a water bath) two times. Cell debris was removed by centri-
fugation (300 g, 10 min, 4°C) and the supernatant was stored in aliquots at -80°C.

The titer of the virus stock was determined in a plaque assay. Briefly, Vero cells were
seeded at a density of 4x10⁵ cells per well of a 24 well tissue culture plate. After an
incubation period of 24 hours (37°C, 5% CO₂) cells were infected with dilutions of
the virus stock ranging from 10⁻² to 10⁻⁶. Infection volume was 100 μl per well. It
was removed after 1 hour incubation at 37°C, 5 % CO₂ and the cells were gently
covered with 1 ml overlay medium (0.5% methyl cellulose, 0.225 % sodium
bicarbonate, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5%
FCS in MEME (MEM-Eagle with Earle's salt)) and allowed to stand for 3 days at
37°C in the presence of 5 % CO₂. Cells were fixed with 4% formaline for at least 30
minutes, washed with water, stained with giemsa solution (Merck, Darmstadt) for 30
minutes and washed again. After drying the plates, plaques were counted and the titer
of the virus stock was calculated. Virus stocks used in the experiments described here
were HSV-1 F (ATCC VR-733, stock 5x10⁷ PFU/ml) and HSV-2 G (ATCC VR-
734; stock 3.5x10⁶ PFU/ml).

b) Measurement of antiviral activity of test compounds in vitro

Antiviral activity was measured with a microtiter plate screening test system
employing cells of diverse origin such as neuronal, lymphoid and epithelial lineages
e.g. Vero cells (african green monkey kidney cells), MEF (murine embryonic
fibroblasts), HELF (human embryonic lung Fibroblasts), NT2 (human neuronal cells)
or Jurkat (humane lymphoid T-cell line). The inhibition of the compounds on the
growth, spread and resulting cytopathic effect of the viruses was analyzed in direct
comparison to uninfected cells, uninfected but treated cells, infected but untreated
cells and infected cells in the presence of example compounds or the reference compound Acyclovir-Natrium (Zovirax®), a generic nucleosidic anti-Herpes-drug.

Compounds were dissolved in DMSO (Dimethyl Sulfoxide) to a stock concentration of 50 mM and analyzed on microtiter plates (e.g. 96-Well MTP or higher formats such as 384 or 1536 well plates) at final concentration of 250 - 0.5 µM (micro molar) in duplicates (e.g. 4 compounds/96 well MTP). In the case of potent compounds the serial 1:2 dilutions were continued over several plates to the pM (pico molar) range. Precipitation of compounds at higher concentrations as well as toxic and cytostatic side effects were recorded in parallel by microscopic evaluation. After the serial dilution (1:2) of compounds in media on the MTP a cell suspension was added (1x10^4 cells/well of a 96 well MTP) e.g. Vero-cells in M199 (Medium 199) complemented with 5% FCS, 2 mM L-glutamine and optional 100 IU/ml penicillin and 100 µg/ml streptomycin or MEF-cells in EMEM (Eagle's Minimum Essential Medium) complemented with 10% FCS, 2 mM L-glutamine and optional 100 IU/ml penicillin and 100 µg/ml streptomycin, or HELF-cells in EMEM complemented with 10% FCS, 2 mM L-glutamine and optional 100 IU/ml penicillin and 100 µg/ml Streptomycin, or NT2- and Jurkat-cells in DMEM (4.5 mg/l Glucose plus Pyridoxine) complemented with 10% FCS, 2 mM L-glutamine, 1 mM Natrium-Pyruvate, non essential amino acids and optional 100 IU/ml penicillin and 100 µg/ml streptomycin to each well. The cells in the relevant wells were then infected with the virus under investigation as follows: HSV-1 F or HSV-2 G with an m.o.i (multiplicity of infection) of 0.0025 for HELF, Vero and MEF-cells and an m.o.i. of 0.1 for NT2- and Jurkat-cells). The plates were then incubated at 37 °C in a CO2-incubator (5% CO2) for several days (preferably 5 days). After the incubation period the cells in compound free wells, starting from 25 infectious centers, are completely lysed or destroyed by the cytopathic effect of replicating Herpes viruses (100 % CPE). The plates were first briefly analyzed under a microscope and then the read out was generated using a quenched fluorescent dye which is cleaved by the enzymatic esterase activity of viable cells. The cell culture supernatant in the MTP was aspirated, the remaining cells or debris were washed once with 200 µl PBS (phosphate buffered saline) and 200 µl Fluorescein-diacetate dye containing solution were added
(10 μg/ml in PBS). After an incubation of 30-90 min at room temperature the fluorescence of the MTP was recorded in a Fluorescent Ascent (Labsystems) at 485 nm excitation and 538 nm emission wavelength.

At an IC<sub>50</sub> concentration the viral replication is inhibited by 50 % as compared to the non infected cell control or the read out of the fluorescence based assay reaches 50 % of the signal as compared to the cell control.

The relevant data and results are summarized in Table 1.

c) Generation and sequencing of resistant viral mutants

Naturally occurring, resistant viral mutants were selected in the presence of 1 μM example 7 or of at least 100 times the IC<sub>50</sub> concentration as listed in table 1 using the cell based viral replication assay. 10 000 Vero cell were seeded in 96 well MTPs as described above and incubated over night. Compound was added to the final concentration and the cells were infected with 1000 plaque forming units (m.o.i. 0,1) per well. Mutants were identified at a frequency of 1-5 per million HSV-1 F under a microscope after 3-5 days or by storing replica samples of 10 μl of each well and analyzing the 20-40 MTP with the fluorescence dye fluorescein diacetate as described above. If a resistant virus is present in an individual well, the fluorescence read out decreases at least by a factor of 3 as compared to mutant free wells. Mutant positive supernatants or stored samples were used to produce stocks, the titer was determined, the DNA prepared by the method found in "Herpes Simplex Virus Protocols, 1998, Ed. S.M. Brown & A.R. MacLean, Humana Press, Totowa, New Jersey" and sequenced according to "DNA sequencing with chain-terminating inhibitors, 1977, Sanger F, Nicklen S, Coulson AR, PNAS, 74, 5463-5467" using the ABI PRISM Big Dye Terminator Kit from Applied Biosystems or the DYEnamic ET Terminator Cycle Sequencing Kit from Amersham (apbiotech).

The mutations are summarized in Table 1.
<table>
<thead>
<tr>
<th></th>
<th>in vitro viral replication assay</th>
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<th>in vivo lethal challenge model</th>
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<tbody>
<tr>
<td></td>
<td>wild type HSV-1 (F) IC₅₀ [µM]</td>
<td>drug resistant HSV-1 (F) mutants IC₅₀ [µM]</td>
<td>Acyclovir resistant HSV-1 (F) mutant IC₅₀ [µM]</td>
<td>human HSV-2 G IC₅₀ [µM]</td>
<td>porcine PRV IC₅₀ [µM]</td>
</tr>
<tr>
<td>Example 1 Mutant 1</td>
<td>0.75 350 &gt;250 UL5 M355V UL5 V662</td>
<td>0.75 1.5 167</td>
<td>4 &gt;63</td>
<td>1.5 &gt;167</td>
<td>60 weak</td>
</tr>
<tr>
<td>Example 2</td>
<td>0.5 400 &gt;250 UL5 M355V UL5 V662</td>
<td>0.5 1.5 200</td>
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<td></td>
<td>26 weak</td>
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<td>Example 3</td>
<td>0.001 250000 &gt;250 UL5 K356N UL52 A897T</td>
<td>0.001 0.005 25000</td>
<td>0.005 0.05 5000</td>
<td>3 66</td>
<td>0.15 400</td>
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<td>Example 4 Mutant 2</td>
<td>0.0005 100000 &gt;250 UL5 K356N UL52 A897T</td>
<td>3.6-08 0.005 5000</td>
<td>8 12.5</td>
<td>0.05 5000</td>
<td>0.5 1.1</td>
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<td>Example 6 M1+M2</td>
<td>5.0-07 5.6-06 &gt;125 UL5 G352V</td>
<td>5.0-07 5.6-07 1.0-06</td>
<td>1.5 133</td>
<td>0.03 686</td>
<td>&lt;0.5 0.5</td>
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<td>Example 7 M1+M2</td>
<td>0.02 25000 &gt;8 UL5 G352V</td>
<td>0.02 0.02 3000</td>
<td>5 &gt;50</td>
<td>0.125 240</td>
<td>0.5 0.5</td>
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<td>Acyclovir</td>
<td>1 250 1 on all ex. mutants</td>
<td>125 4 125</td>
<td>22 16</td>
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<td>Veltrex</td>
<td>17 14</td>
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Preferred are compounds with an IC\textsubscript{50} (HSV-1 F/Vero) obtained with the above described in vitro cell based viral replication screening test system using the dye read out of about or less than 50 \(\mu\)M, more preferred with an IC\textsubscript{50} of about 25 \(\mu\)M or less and ideally with an IC\textsubscript{50} below 10 \(\mu\)M.

Compounds as herein provided can be used as drugs for treatment and prophylaxis of diseases caused by Herpes viruses, especially herpes simplex viruses.

The following indications are listed as examples:

1) Treatment and prophylaxis of herpes infections, especially Herpes simplex infections in patients with disease symptoms such as herpes labialis, herpes genitalis, and keratitis, encephalitis, pneumonia, hepatitis etc. caused by herpes viruses

2) Treatment and prophylaxis of herpes infections, especially herpes simplex infections in immunocompromized patients (e.g. AIDS patients, cancer patients, patients with genetically based immunodeficiency, transplant patients)

3) Treatment and prophylaxis of herpes infections, especially herpes simplex infections in newborns, babies, infants and children.

4) Treatment and prophylaxis of herpes infections, especially herpes simplex infections and Herpes-, especially herpes simplex positive patients for maintenance or suppression therapy of recurrent herpes disease

d) In vivo-Activity

6 (six) week old female mice strain BALB/cABom, purchased from Bomholtgard Breeding and Research Centre Ltd., were anaesthetized in a sealed glass vessel with diethylether (Merck). 50\(\mu\)l of a diluted virus stock solution (infectious dose 5x10^4
Pfu (plaque forming units)) were used for intranasal infection of the anaesthetized animals. This dose leads to the death of 90-100% of the infected animals within 5-8 days. The infected animals show symptoms of a generalized infection such as respiratory or central nervous system symptoms.

Treatment and Analysis of Potency and Efficacy:

6 hours after infection animals were treated with doses of 0.1-100 mg/kg body weight 3 times daily (7.00 h, 14.00 h and 19:00 h) over a period of 5 days. The compounds were dissolved in DMSO and resuspended in Tylose/PBS(Hoechst) (final concentration 1.5% DMSO, 0.5% Tylose in PBS).

After the final application animals were observed and the time of death was recorded.

Data are summarized in Table 1 e.g. an ED$_{50}$ of 0.7 mg/kg means that 50% of the infected animals survived at this dose.

The following test compounds Example 1 to 7 were synthesized according to the following general figures (figure 2 for thiazolyl amides and figure 3 for thiazolyl urea derivatives).
Claims

1. A method for identifying compounds with antiviral activity, characterized in that those compounds are selected in the presence of which resistant viral mutants can be selected, wherein at least one selected viral mutant carries at least one mutation in the primase gene and another selected viral mutant carries at least one mutation in the helicase gene or a selected viral mutant carries at least one mutation in the helicase and at least one mutation in the primase gene.

2. A method according to claim 1, characterized in that the primase and helicase are at least 50% homologue to the primase and helicase of herpes simplex virus 1 based on the nucleotide sequence or the amino acid sequence, whichever is higher.

3. A method according to claim 1 or 2, characterized in that the virus is a herpes virus.

4. A method according to claim 1, 2 or 3, characterized in that, before determining the generation of resistant viral mutants in the presence of said compounds,

   a) the compounds are tested for their ability to inhibit the helicase and
   b) the compounds are tested for their ability to inhibit the primase
   c) such compounds are selected, that inhibit the helicase and the primase

5. A method according to claim 4, characterized in that such compounds are selected, that bind simultaneously to the helicase and primase subunits of the helicase-primase complex.
6. A method according to any one of claims 1 to 5, characterized in that the compounds inhibit the DNA-dependent NTPase activity of a herpes helicase-primase.

7. A method according to any one of claims 1 to 6, characterized in that the compounds inhibit the replication of a herpes virus.

8. A method according to any one of claims 1 to 7, characterized in that such compounds are selected, that have the ability to inhibit replication of a herpes virus in cell culture by at least 50% at a concentration of less than about 10 μM.

9. A method according to claim 8, characterized in that such compounds are selected, that have the ability to inhibit replication of a herpes virus in cell culture by at least 50% at a concentration of less than about 500 nM.

10. A method according to any one of claims 1 to 9 wherein the herpes virus is HSV.

11. A method according to any one of claims 1 to 9 wherein the herpes virus is PRV or BHV.

12. A compound identified by the method according to any one of claims 1 to 11.

13. A compound according to claim 12, characterized in that the compound is not a nucleoside or nucleotide.

14. Use of a compound according to claim 12 or 13 for the preparation of a medicament for the treatment and prevention of herpes infections.
15. A pharmaceutical composition comprising a compound according to claim 12 or 13.

16. A method for treating herpes infection in a mammal comprising the step of administering to a mammal in need of such treatment a therapeutically effective amount of the pharmaceutical composition according to claim 15.

17. A method for identifying compounds, characterized in that

10 a) the compounds are tested for their ability to bind to at least two therapeutically relevant targets simultaneously and

b) the compounds are tested for their ability to inhibit and/or activate these targets upon binding

c) the compounds are tested for reduction of their ability to inhibit and/or activate these targets in the presence of at least one mutation in one target and at least one mutation in the other target or at least one mutation in either target.

18. A compound with antiviral activity, characterized in that those compounds are selected in the presence of which resistant viral mutants can be selected, wherein at least one selected viral mutant carries at least one mutation in the primase gene and another selected viral mutant carries at least one mutation in the helicase gene or a selected mutant carries at least one mutation in the helicase and at least one mutation in the primase gene.

19. A compound capable of binding simultaneously to the helicase and primase subunits of the helicase-primase complex.
Fig. 1

Pi generation of the UL5/UL52 ATPase Reaction in the Presence of Example 7 [μM]

- without ssDNA
- 0
- 100
- 20
- 4
- 0.8
- 0.16
- 0.032
- 0.0064
- 0.00128
- ATP control
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/569 G01N33/50 C07D227/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q C12N G01N C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category * Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.

X SPECTATOR F C ET AL: "Inhibition of herpes simplex virus replication by a 2-amino thiazole via interactions with the helicase component of the UL5–UL8–UL52 complex." JOURNAL OF VIROLOGY, vol. 72, no. 9, 1998, pages 6979–6987, XP002153596 ISSN: 0022–538X cited in the application the whole document 1–19

A WO 97 24343 A (BOEHRINGER INGELHEIM CA LTD ; BOEHRINGER INGELHEIM PHARMA (US)) 10 July 1997 (1997-07-10) cited in the application claim 30 1–19

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Date of the actual completion of the international search 5 December 2000

Date of mailing of the international search report 22/12/2000

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo md, Fax: (+31–70) 340–3016

Authorized officer Pellegrini, P

Form PCT/ISA/210 (second sheet) (July 1990)
**DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>WO 95 05453 A (SYSTEMIX INC ; BONYHADI MARK L (US); MCCUNE JOSEPH M (US); KANESHIM) 23 February 1995 (1995-02-23) the whole document</td>
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<td>WO 00 53951 A (PORSCHE AG ; SCHAIBLE WALTER (DE)) 14 September 2000 (2000-09-14) page 61 example 1 page 77</td>
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Continuation of Box I.2

Claims Nos.: 12-16,18-19

Claims 12-16 and 18 relate to compounds defined by reference to a desirable characteristic or property, namely being identified by the method of claims 1 to 11, to uses of these compounds, to pharmaceutical preparations containing these compounds, and to methods of treatment of herpes infection by administering these pharmaceutical compositions. Claim 19 relates to compounds defined by reference to a desirable characteristic or property, namely "being capable of binding simultaneously to the helicase and primase subunits of the helicase-primase complex".

The claims cover all compounds having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only those compounds which are present in Fig. 2 and Fig. 3.

In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the subject-matter for which protection is sought in claim 12-16 and 18-19 is impossible. However, a search has been performed on the above-mentioned compounds.

Furthermore, it is pointed out that claim 16 relates to a method of treatment of the human body by therapy.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.
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