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Virtual screening identified compounds that bind to cyclin dependent kinase 2 and prevent herpes simplex virus type 1 replication and reactivation in neurons

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ABSTRACT

HSV-1 is one of the most prevalent viruses worldwide, and due to the limited therapies mainly with acyclovir and analogues and the emergence of acyclovir (ACV) resistant strains, the search for new drugs with different modes of action is needed. This study identified compounds that bind *in silico* to cyclin dependent kinase 2 (CDK2), a cellular enzyme required for efficient HSV-1 replication, and have anti-HSV-1 activity. Compounds obtained from virtual screening by Pharmit were filtered in FAF-Drugs4 for good pharmacokinetic and toxicological profiles and submitted to molecular docking on CDK2 using Autodock Vina. The six most promising compounds were evaluated for inhibiting lytic replication of HSV-1 wild-type and ACV-resistant strains on human fibroblasts. The compounds were also assayed for cytotoxicity. Compounds **1**, **2** and **3** showed antiviral activity with EC_{50} (50% of effective drug concentration) of 32, 29 and 64 μM and CC_{50} (50% of cytotoxic concentration) of 159, 1410 and 2044 μM, respectively. Compounds **1** and **2** were also active against ACV resistant strains and compound **3** inhibited the reactivation of HSV-1 in neurons, which is an important finding to guide drug design of new anti-HSV-1 antivirals with different modes of action. These compounds are promising candidates for optimization into more potent agents to treat HSV-1 infections and recurrences.

1. Introduction

Herpes simplex virus 1 (HSV-1) is a double-stranded DNA virus with an icosahedral capsid, covered by the tegument and envelope, the latter of which is composed of host membrane components and viral glycoproteins (Roizman et al., 1974). HSV-1 infections affect a large part of the human population, invading the epithelial cells with the formation of cold sores or lesions on the eyelid, though the majority of infections are asymptomatic (Fatahzadeh and Schwartz, 2007). HSV-1 also causes keratitis, which can consequently lead to severe visual impairment or blindness (Farooq and Shukla, 2012). Less frequently, HSV-1 is associated with infection of the CNS resulting in encephalitis (Alsweed et al., 2018; Kawada et al., 2018). According to Kawada (Kawada et al., 2018), HSV encephalitis is the main cause of death by encephalitis worldwide.

After infecting the host mucosal tissue, HSV-1 causes an acute infection in epithelial cells with subsequent infection of sensory nerve cells, where the virus remains latent for the life of the host (Wysocka and Herr, 2003). Periodically the virus reactivates from the neurons and travels to the original site of infection where it causes recrudescence of clinical symptoms or sheds asymptomatically. The molecular processes that regulate HSV-1 neurotropism and reactivation have not yet been fully elucidated (Shipley et al., 2017). Our study exploits a recently described *in vitro* model of HSV-1 latency and reactivation (Edwards and Bloom, 2019) that is comprised of differentiated Lund human mesencephalic neurons (LUHMES), which exhibit phenotypic

Abbreviations: ACV, acyclovir; CC_{50}, 50% of cytotoxic concentration; CDK2, cyclin-dependent kinase 2; DMSO, dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle's medium; h, hour; EC_{50}, 50% effective drug concentration; hpi, hours post-infection; HSE, herpes simplex encephalitis; HSV-1, Herpes simplex virus 1; IgG, Immunoglobulin G; LBVS, ligand based virtual screening; Log P, octanol-water partition coefficient; LUHMES, Lund human mesencephalic; MEM, Minimum Essential Medium Eagle; MOI, multiplicity of infection; PAINS, Pan Assays Interferences Compounds; PBS, phosphate buffered saline; PDB, Protein Databank; PFU, plaque-forming units; PI3K, phosphatidylinositol-3-kinase; qPCR, Polymerase chain reaction quantitative real time; RS, Rabbit skin; RMSD, root mean square deviation; SBVS, structure-based virtual screening; SI, selective index

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characteristics of post-mitotic human neurons and are considered a robust and reliable *in vitro* model for biological studies on neurons (Scholz et al., 2011).

Currently, the standard treatment for HSV-1 infection is acyclovir (ACV), which targets the viral thymidine kinase and viral DNA polymerase (Elion, 1993). However, because of its low oral bioavailability (10%–20%), ACV is usually administered as a prodrug, valacyclovir, which improves its pharmacokinetic properties and is rapidly converted to ACV (Tyring et al., 2002). Other anti-HSV drugs also target DNA replication, which is a cause of concern because most ACV-resistant HSV isolates might also be resistant to these drugs (Jiang et al., 2016). Thus, the discovery of new anti-HSV agents is important, especially new drugs with distinct molecular targets than acyclovir and its analogues.

Cellular proteins are potential targets for antiviral therapy since most viruses rely on specific cellular proteins for replication. In addition, mutations in cellular genes may accumulate more slowly compared to mutations in viral genes, making resistance to drugs directed against these targets less likely. Additionally, antivirals to these targets may have broad-spectrum activity against viruses (Schang et al., 2006). Cyclin-dependent kinases (CDKs) are potential cellular targets, involved in transcription and replication of viral genomes (Schang, 2003). CDK inhibitors have been investigated as antivirals because they have tolerable adverse effects in clinical studies for the treatment of cancer and other diseases (Kouroukis et al., 2003; Stadler et al., 2000; Cyclacel Pharmaceutical, 2018).

Some studies have shown that CDK2, in particular, plays an important role in viral infections. One of the first studies correlating CDK2 to viral replication was in 1998, which demonstrated that inhibitors of this enzyme, roscovitine and olomoucine, decreased viral infection *in vitro* (Schang et al., 1998). In 2002, Schang et al. (2002) showed that roscovitine also blocks the reactivation of HSV-1 in neurons *ex vivo*. Recently, the study by Zhou et al. (2016) showed that administration of olomoucine improved neurological function and increased survival of mice with herpes simplex encephalitis (HSE). Based on this evidence, the present study selected CDK2 as a therapeutic target of interest with the aim to identify potential compounds with antiviral activity against HSV-1.

2. Material and methods

2.1. In silico evaluation

2.1.1. Virtual screening of potential CDK2 inhibitors

The three-dimensional structure of CDK2 (PDB: 2A4L) was obtained from Protein Data Bank (https://www.rcsb.org/) and a pharmacophoric model was constructed based on the pose and binding mode of roscovitine at its active site. This model was the basis for the virtual screening of CDK2 inhibitors using the Pharmit server (http://pharmit.csb.pitt.edu/) (Sunseri and Koes, 2016) and its databases: Molport (6,525,901 molecules), Mcule (999,989 molecules), Drugbank approved (1995 drugs) and in the Zinc Natural Products database (137,972 molecules) found on the ZINCPharmer server (http://zincpharmer.csb.pitt.edu/) (Koes and Camacho, 2012). In order to select molecules with promising pharmacokinetic profiles (Lipinski et al., 2012; Veber et al., 2002), the following filters were applied at this step: molecular mass ≤ 500 Da and number of rotatable bonds (nRot) ≤ 10. In addition, the search was directed to select only one orientation for each conformation of the molecules (Max hits per Mol = 1).

2.1.2. Pharmacokinetic and toxicological properties filters

Compounds obtained were submitted to a filtering step using the FAF-Drugs4 server (fafdrugs4.mti.univ-paris-diderot.fr/), in which duplicate compounds, covalent inhibitors, toxicophores and Pan Assay Interference Compounds (PAINS) were identified and removed. The Lipinski Rule of five was also applied.

2.1.3. Docking of potential inhibitors in cyclin-dependent kinase 2 (CDK2)

Initially, the compounds obtained from the virtual screening were prepared for the docking studies by the addition of hydrogens, and Gasteiger charges using the Autodock Tools 1.5.7 program (Morris et al., 2009). All rotatable bonds were kept flexible. The three-dimensional structure of CDK2 was obtained from protein Databank (PDB 2A4L) and prepared using the Autodock Tools 1.5.7. Polar hydrogens and Gasteiger charges were added.

Subsequently, molecular docking studies were performed using the Autodock Vina 1.1.2 program (Trott and Olson, 2009). To validate the method, the redocking of roscovitine was carried out. The grid box for the docking calculation was centered on the active site of the enzyme (coordinates: x = 101, y = 101, z = 79) with the dimensions 22 × 20 × 20 Å, encompassing the residues important for binding. After docking, the lower binding ligands were chosen for visual inspection in order to analyze the binding mode and the interactions with CDK2 using the Swiss PDB Viewer (SPDBV) programs (Guex and Peitsch, 1997; Guex et al., 2009) and Discovery Studio (Dassault Systèmes BIOVIA).

2.2. In vitro assays

2.2.1. Compounds

The most promising compounds according to the molecular modeling studies were purchased from Enamine Ltd. The purity of compounds was determined by means of Liquid chromatography-mass spectrometry (LCMS) and the results were registered in their certificate of analysis issued by provider. All active compounds were obtained with purity higher than 95%.

These compounds were diluted at the concentration of 50 mM in dimethylsulfoxide (DMSO) and stored at −20 °C. We tested acyclovir (Fluka Analytical®) as a reference antiviral drug and roscovitine (ApexBio), a CDK2 inhibitor, to compare the results.

2.2.2. HSV-1 strains

The wild-type strain HSV-1 17syn+ from the Bloom Lab, Department of Molecular Genetics and Microbiology (University of Florida) was used in all assays. We also tested the compounds against the clinical strain HO-1 (kindly provided by D. Phelan, unpublished data) and the mutant strain PAA^{r}5 (Coen and Schaffer, 1980) (kindly provided by D. Coen), both resistant to ACV. Stock cultures of viruses were prepared from lysates of infected cells and stored at −80 °C until use.

2.2.3. Cell lines

Human fibroblast (HFL1 ATCC® CCL-153™) cells were maintained in Dulbecco's Modified of Eagle medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum and 1% of L-Glutamine–Penicillin-Streptomycin solution (Sigma).

Lund human mesencephalic (LUHMES) cells obtained from the ATCC (catalog no. CRL-2927) were cultivated and differentiated in neurons according to Scholz et al. (2011) with slight modifications described by Edwards and Bloom (2019). Briefly, flasks and plates were pre-coated with 50 µg/mL poly-1-ornithine (Sigma-Aldrich, St. Louis, MO, USA) and 1 µg/mL fibronectin (Sigma-Aldrich, St. Louis, MO, USA) in water for 3 h. After removal of the coating solution, flasks and plates were washed once with water and air-dried as preparation for cell seeding. The proliferation medium consisted of Advanced DMEM/F12, 1· N-2 supplement (Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine (Gibco, Rockville, MD, USA) and 40 ng/mL of recombinant basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA). The differentiation medium consisted of Advanced DMEM/F12, 1· N-2 supplement, 2 mM of L-glutamine, 1 mM of dibutyryl cAMP (Sigma-Aldrich), 1 ng/mL of tetracycline (Sigma-Aldrich) and 2 ng/mL of recombinant human GDNF (R&D Systems).

Rabbit skin (RS) cells were maintained in Minimum Essential Medium Eagle (MEM), with Earle's salts and L-glutamine, supplemented with 5% heat-inactivated bovine serum and 1% of L-Glutamine–Penicillin–Streptomycin solution (Sigma). All cell lines were cultivated at 37 °C and 5% CO_2 until sub-confluency, after which they were seeded into 24 or 96-multiwell plates according to the assay.

2.2.4. Determination of cytotoxicity

HFL1 and LUHMES cells were cultivated in 96-multiwell plates (5×10^4 cell/well) and incubated at 37 °C, 5% CO_2 for 24 h. Then, the cells were treated with the active compounds in different concentrations (50, 250, 500, and 1000 μM) diluted in DMEM, and the plates were incubated at 37 °C/24 h and 5% CO_2. Cell viability was determined by using the CellTiter-Glo® luminescent cell viability assay (PROMEGA) according to the product protocol. The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression analysis of the dose-response curves.

2.2.5. Analysis of antiviral activity

To test the effects of the compounds during acute HSV-1 infection, confluent HFL1 cells in 24-multiwell plates were infected with HSV-1 strain 17syn + at a multiplicity of infection (MOI) of 1.0 for 1 h at 37 °C and 5% CO_2. MOI of 1.0 was applied because it is well-tolerated by the cells and most become infected. Infection spreads to all cells in the culture during the assay. Then, the viral inoculum was removed, cells were washed with phosphate-buffered saline (PBS) and the medium was added with the compounds at 50 μM. DMEM without any compound was used as a control for HSV-1 infection. We also tested the influence of DMSO on the HSV-1 infection as vehicle control. As positive controls, infected cells were treated with ACV at 50 μM or roscovitine. 24 h post-infection (hpi) at 37 °C and 5% CO_2, cells were lysed with 3 cycles of freezing and thawing and the content was collected.

2.2.6. Plaque assay

The viral titer was determined by standard plaque assay. Confluent RS cells, maintained in 24-multiwell plates were infected with several dilutions (1:10) of the HSV-1 lysates from the acute infection assay, for 1 h at 37 °C and 5% CO_2. The monolayers were washed with PBS and overlayed with MEM with 0.3% of Immunoglobulin G (IgG) from human serum (Sigma) for 48 h. Human IgG neutralizes HSV-1 released from the infected cells and prevents secondary infection (Sarfo et al., 2017). Following this incubation, the monolayers were fixed and stained with crystal violet 0.5% and 20% methanol. The viral titers were determined according to the number of viral plaque-forming units per mL (PFU/mL).

2.2.7. Determination of EC_{50}

In order to determine the effective concentration required to reduce the number of plaques by 50% (EC_{50}), confluent HFL1 cells in 24-multiwell plates were infected with HSV-1 17syn + MOI of 1.0 for 1 h at 37 °C and 5% CO_2. Then, the viral inoculum was removed, cells were washed and overlayed with medium containing the compounds at different concentrations (6.25, 12.5, 25 and 50 μM). After 24 h, the cells were lysed and harvested for titration using the plaque assay described above. The EC_{50} value was determined by linear regression from the dose-response curve. The selective index (S.I.) was calculated by the ratio of CC_{50} for HFL-1 cells and EC_{50} for HSV-1 17syn + (CC_{50}/EC_{50}).

2.2.8. Antiviral activity against acyclovir-resistant HSV-1 strains

The active compounds were tested on acyclovir-resistant HSV-1 strains: HO-1 and PAA^r5 (Coen and Schaffer, 1980). Confluent monolayers of HFL1 cells in 24-multiwell plates were infected with HO-1 or PAA^r5 (MOI of 0.1) for 1 h at 37 °C. This MOI was used because these clinical resistant strains are more virulent to the cells compared to the wild-type laboratory strain. The cells were washed and treated with the compound at 50 μM for 24 h at 37 °C and 5% CO_2. The samples were titered by the plaque assay.

2.2.9. Effects on HSV-1 reactivation

The HSV-1 reactivation assay was conducted according to Edwards and Bloom (2019). LUHMES cells were seeded (5×10^4 cells/well) into pre-treated 24-well plates (see section Cell lines), covered with 500 μL of the proliferation medium and incubated at 37 °C, 5% CO_2 for 48 h. The proliferation medium was then replaced by the differentiation medium. After 5 days, when neuronal characteristics were observed, the cells were infected with HSV-1 17syn + (MOI of 3.0) for 1 h. The inoculum was removed, the monolayers washed with PBS, and the differentiation medium with ACV at 50 μM was added. Every 2 days, the medium was changed (without the addition of ACV) and after 6 days the neurons were treated with 50 μM of compound **3** or roscovitine for 48 h. Then, the stimulation of reactivation was performed by the addition of 1 μM of Wortmannin (Sigma, catalog no. W1628), an inhibitor of phosphatidylinositol-3-kinase (PI3K) (Liu and Cohen, 2015). After 48 h, the cells were lysed for the plaque assay and DNA was extracted with 500 μl of TRIzol® reagent (Ambion-USA) and quantified by qPCR assay.

2.2.10. DNA quantification - qPCR assay

After DNA extraction with TRIzol® reagent, 100 μL of chloroform was added to each tube and after 3 min they were centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was discarded, 100 μL of ethanol PA was added and after 3 min the tubes were centrifuged at 2000 × *g* for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 100 μL 0.1 M sodium citrate in 10% ethanol (pH 8.5) for 30 min. The samples were centrifuged at 2000 × *g* for 5 min at 4 °C. This step was repeated once. The pellet was resuspended in 75% ethanol for 20 min and centrifuged again at 2000 × *g* for 5 min at 4 °C. The supernatant was discarded and the DNA pellet resuspended in 8 mM NaOH. qPCR was conducted using 20 ng input DNA, TaqMan® Fast Universal PCR 2X Master Mix (Applied Biosystems) with TaqMan® probes (Applied Biosystems) and HSV-1 polymerase. Real-time PCR of the samples was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems).

2.3. Statistical analysis

The results were analyzed using the software GraphPad Prism v.5.02 (GraphPad Software, La Jolla California USA). The experimental and control groups were compared using the ANOVA method, complemented by the Tukey test. The linear regression analysis for determination of EC_{50} and CC_{50} were performed using Microsoft Office Excel 14.0 (2010).

3. Results and discussion

3.1. Virtual screening in search of potential inhibitors

CDK2, a cellular enzyme, was chosen as a target of interest for the search for new antivirals, since previous studies have described CDKs involved in viral replication and showed that some CDK2 inhibitors have anti-HSV-1 activity (Schang, 2003; Schang et al., 1998; Davido et al., 2002). Additionally, reactivation occurs in neurons expressing CDK2, suggesting that the level of this protein is involved in the reactivation of HSV-1 from the latency (Schang et al., 2002).

The initial strategy for the identification of potential inhibitors of CDK2 was the ligand-based virtual screening (LBVS), which considers that inhibitor-like structures are more likely to have similar biological activity (Hamza et al., 2012). Thus, a pharmacophoric model (Fig. 1A) was constructed based on the structure of roscovitine and its binding mode with CDK2 (Azevedo et al., 1997). We also evaluated the interactions of other CDK2 inhibitors to construct the model (Ayaz et al., 2016; Chen et al., 2016; Blake et al., 2016; Hylsová et al., 2017). For

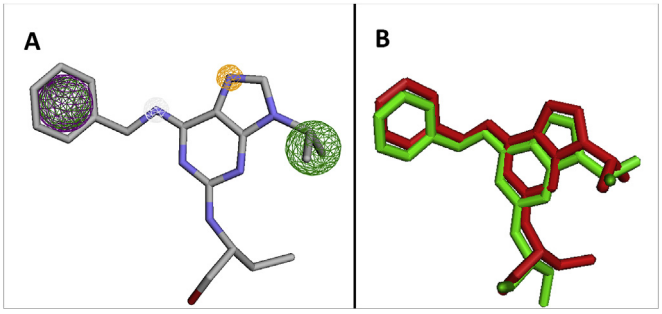

Fig. 1. Initial strategies applied for virtual screening (**A**) Pharmacophore model constructed on roscovitine structure based on its mode of binding and interaction and of other CDK2 inhibitors. (**B**) Redocking of roscovitine on the crystallographic structure of CDK2 (PDB: 2A4L). In red, the crystallographic conformation of roscovitine and, in green, the pose obtained from the docking.

this screening, the following filters were applied: molecular mass less than 500 Da and number of rotatable bonds less than 10. Since the Pharmit server presented many molecules in the outcome, the number of results was limited to ten thousand based on the root mean square deviation (RMSD) compared to the pharmacophore for each database. Thus, Pharmit's results were: 10,000 molecules (RMSD 0.06 to 0.22 Å) in the Molport database; 10,000 molecules (RMSD 0.06 to 0.343 Å) in the Mcule database; and 16 molecules (RMSD 0.556 to 0.643 Å) in the Drugbank Approved database. The screening result of the Zinc Natural Products database was 448 molecules (RMSD 0.140 to 0.805 Å). Total of possible CDK2 ligands was 20,424 molecules.

Using the FAF-Drugs4 server, these molecules were filtered with the parameters of Rule of five: molecular mass ≤500 Da; number of hydrogen bonding donors ≤5, number of hydrogen bond acceptors ≤10 and octanol-water partition coefficient (log P) ≤ 5 (Lipinski et al., 2012). According to this rule, compounds fulfilling these parameters, or at least three of them, are more likely to have good oral availability (Lipinski et al., 2012). The outcome excluded covalent inhibitors and Pan Assays Interferences Compounds (PAINS) as well. The result of this filter identified 2093 duplicated molecules, 425 PAINS, 1575 covalent inhibitors and 3580 rejected, 7755 intermediate and 7132 accepted compounds. Accepted and intermediate compounds (14,887) were selected for the structure-based virtual screening (SBVS).

The *in silico* analysis of pharmacokinetic and toxicity risks in the early stages of drug development reduces time, costs and attrition rates, and helps to prioritize better compounds for screening (Li, 2001; Khanna, 2012). Applying molecular docking, we evaluated the binding modes' theoretical affinities and the interactions of the ligands to the therapeutic target in order to select promising compounds to be evaluated experimentally (Smith, 1996; Shoichet, 2004; Krüger and Evers, 2010; Meng et al., 2011). To validate the molecular docking parameters, the redocking of roscovitine present in the crystallographic structure of CDK2 (PDB: 2A4L) was performed. In this technique, the co-crystallized ligand is removed from the crystal and replaced at the active site of the enzyme using molecular docking. By means of this procedure, the predictive capacity of the method used can be assessed (Morris et al., 2008). The root mean square deviation (RMSD) resulting from the overlapping of the pose from redocking with the pose in the crystallized structure was 1.53 Å (Fig. 1B). RMSD values below 2.0 Å indicate a good accuracy of the method (Kontoyianni et al., 2004; Leach et al., 2006; Gani, 2007). Thus, these parameters were used for the molecular docking studies of 14,887 hits with CDK2.

According to the theoretical affinity values calculated by Autodock Vina 1.1.2, 204 molecules from three databases were submitted to a detailed visual inspection, by evaluating the interactions with CDK2 and the conservation of the pharmacophoric model, whereby six compounds were selected to be tested *in vitro* (Fig. 2).

During the visual inspection, the binding mode and the interactions with important residues of the active site were considered, such as the hydrogen bond with Leu83, cation–π with Lys89 and sigma–π with Leu134, as found in roscovitine (Fig. 3) and other CDK2 inhibitors

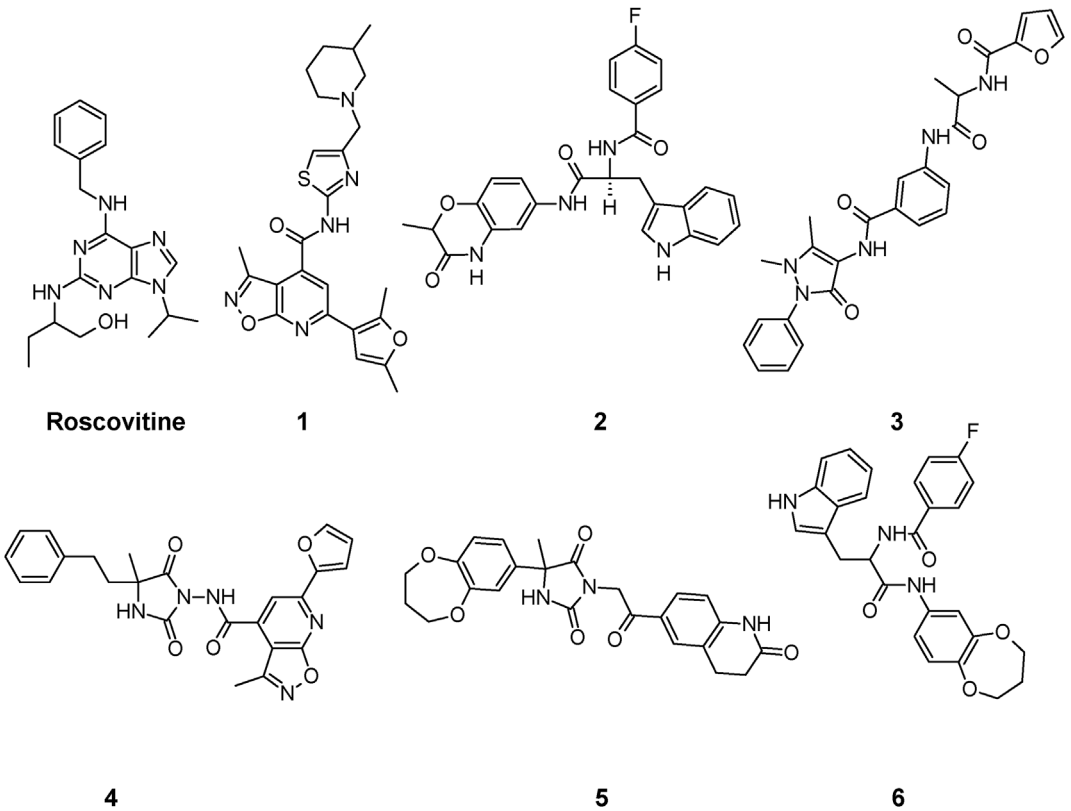

Fig. 2. Structure of the six compounds identified as possible inhibitors of CDK2 by virtual screening and selected for *in vitro* assays.

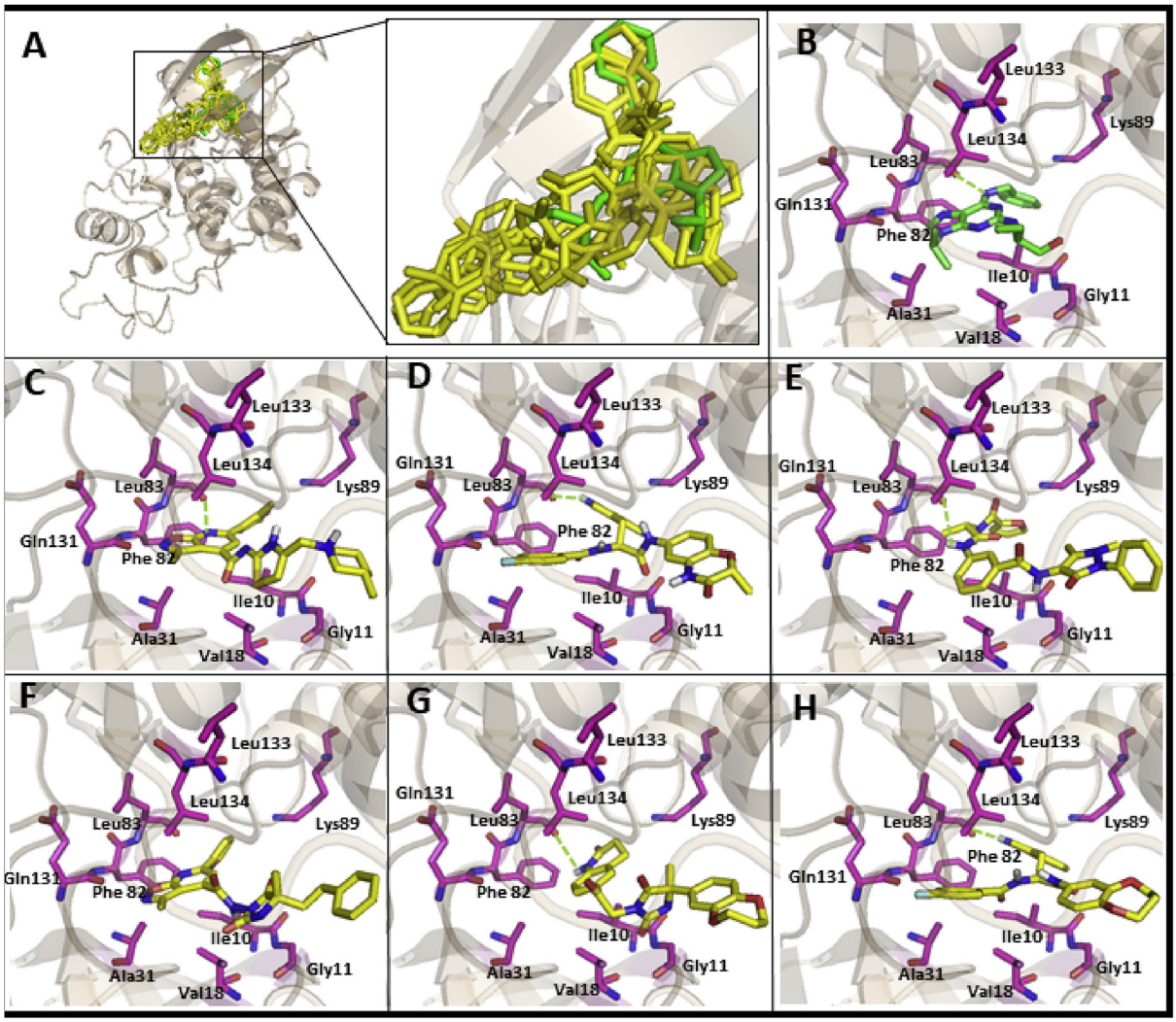

Fig. 3. Visual inspection of molecular dockings of ligand and selected compounds (**C**) Docking of the six compounds identified as possible inhibitors of CDK2 by virtual screening (yellow) in comparison to roscovitine (green). (**D-J**) Binding mode of the ligands with CDK2 predicted by the molecular docking (**D**) roscovitine (**E**) compound **1**, (**F**) compound **2**, (**G**) compound **3** (**G**) compound **4**, (**H**) compound **5**, (**I**) compound **6**.

(Azevedo et al., 1997; Ayaz et al., 2016; Chen et al., 2016; Blake et al., 2016; Hylsová et al., 2017). Compounds **1** and **5** showed a hydrogen bond with Leu83, as well as sigma–π with Leu134. Compound **2** presented a hydrogen bond with Leu83 and cation–π with Lys89. Compound **3** had a hydrogen bond with Leu83 and hydrophobic interaction with Ile10 and Val18, similar to roscovitine. Compound **4** showed sigma–π with Leu134 and compound **6** presented a hydrogen bond with Leu83, cation–π with Lys89 and sigma–π with Leu134 (Fig. 3). Therefore, these six compounds (Fig. 2) whose pose, binding mode and interactions were similar to roscovitine and other CDK2 inhibitors, were selected to proceed to the *in vitro* assay. All virtual screening steps and their outcomes are summarized in Fig. 4.

3.2. *In vitro* assays

Initially, the six compounds from the virtual screening were tested at 50 μM on HSV-1 17syn + infection in HFL1 cells for 24 h. Compounds **1, 2** and **3** had promising antiviral activity: The EC_{50} of compounds **1, 2** and **3** were 32, 29 and 64 μM, respectively (Table 1).

The cytotoxicity of the active compounds on the HFL-1 cells was also evaluated by cellular viability measured indirectly by the quantification of the luminescence proportional to the ATP quantification, which indicates the presence of metabolically active cells (PROMEGA). The CC_{50} of compounds **1, 2** and **3** was 159, 1410 and 2044 μM, respectively (Table 1).

Cytotoxicity for HFL1 cells and the effect on HSV-1 were compared using the selectivity index (S.I.), which consists of the ratio of CC_{50} for the cells and the EC_{50} for the virus. The S.I. of compounds **2** and **3** were higher than roscovitine, while compound **1** showed the smallest S.I., even though its CC_{50} was five times greater than its EC_{50}.

These compounds were also tested on acyclovir-resistant strains HO-1 and PAA^r5. Compounds **1** and **2** showed remarkable antiviral activity against HO-1, with reduction of 78% of PFU, whereas ACV reduced viral yields only 8.8%. Compounds **1** and **2** also showed activity against the PAA^r5 strain, with a reduction of 92% and 74% of PFU, respectively, while ACV inhibited yields by only 17%. Roscovitine was also tested and showed reduction greater than 99% for both strains (Table 2). The different sensitivity profiles of the strains to compounds **1**, **2,** and roscovitine in comparison to ACV suggests that they may have a different mode of action. In addition, these results reinforce the importance of searching for new compounds with an antiviral activity that might be an alternative for the treatment of resistant strains.

The compounds were then tested for their ability to inhibit HSV-1 reactivation. The reactivation assay was performed using LUHMES cells after differentiation into post-mitotic neurons, which is a robust model to study HSV-1 latency *in vitro* with similarities to the *in vivo* infection

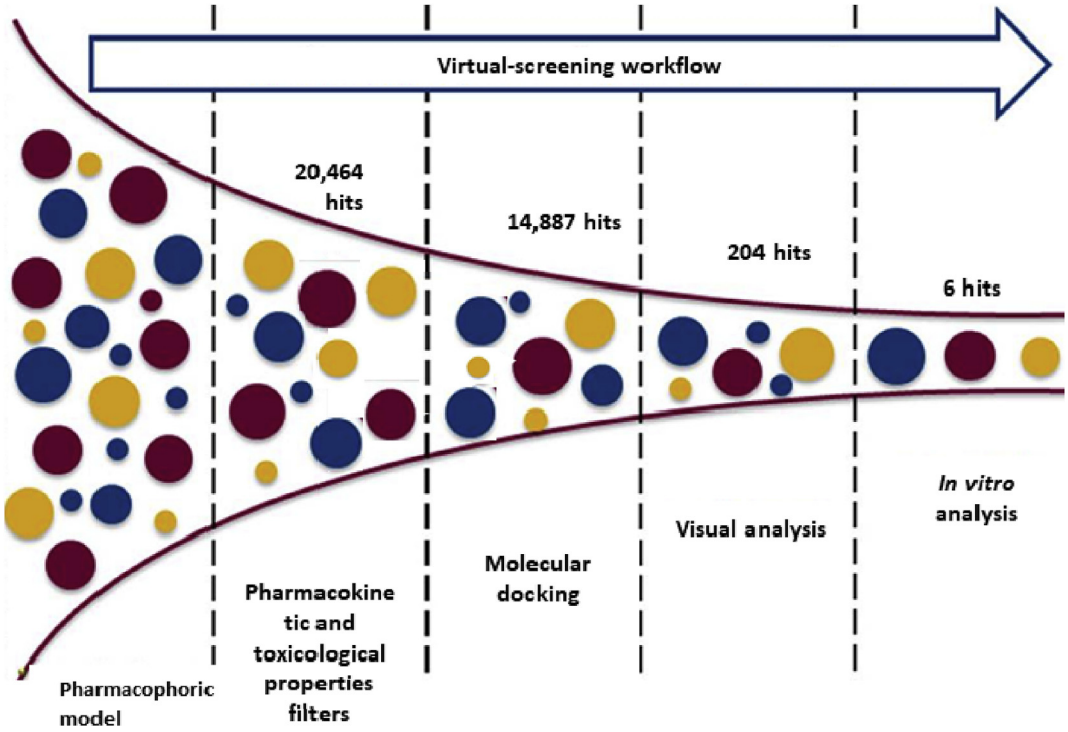

Fig. 4. Virtual-screening workflow used to identify CDK2 inhibitors with anti-HSV-1 activity.

Table 1
Cytotoxicity for HFL1 cells (CC_{50}), antiviral activity (EC_{50}) and selectivity index (S.I. = CC_{50}/EC_{50}) of active compounds, roscovitine (RCV) and acyclovir (ACV).

Compounds	CC_{50} (μM)	EC_{50} (μM)	S.I.
1	159 ± 28.2	32 ± 5	5
2	1410 ± 212.2	29 ± 4.3	48.6
3	2044 ± 457	64 ± 6.1	31.9
RCV	491 ± 18.9	21 ± 1.6	23.4
ACV	1287 ± 237	1.1 ± 0.7	1170

Table 2
Antiviral activity of compounds at 50 μM on 17syn + (wild-type strain) and HO-1 and PAA^r5 (acyclovir-resistant strains). Roscovitine (RCV). Acyclovir (ACV). PFU (Plaque-forming units).

Compounds	Reduction of PFU (%) at 50 μM		
	17syn +	HO-1	PAA^r5
1	80	78	92
2	82	78	74
3	60	4.5	1.9
RCV	99.6	99.1	99.9
ACV	99.9	8.8	17

(Edwards and Bloom, 2019). First, the cytotoxicity of compounds **1**, **2**, **3** and roscovitine was evaluated in these cells. Compounds **1** and **2** at 50 μM were cytotoxic to these cells, with viability of 32% and 22%, respectively. For this reason, they were not tested in the reactivation assay. Compound **3** and roscovitine did not compromise the viability of these cells, and at 50 μM the viability was 98% and 71%, respectively. Thus, the CC_{50} assay was carried out with values of 413 and 91 μM respectively, and then these two compounds were tested in the reactivation assay. The compounds were added to the medium of HSV-1 latently infected neurons 6 days post-infection. At this time, there is a loss of lytic gene transcription and an increase in numbers of neurons that express the latency-associated transcripts (LATs), which is consistent with latency (Edwards and Bloom, 2019). After 48 h, reactivation was induced by the addition of Wortmannin, an inhibitor of phosphatidylinositol-3-kinase (PI3K), which is a protein essential for the maintenance of HSV-1 latency in neurons. Treatment with inhibitors of this enzyme results in viral reactivation (Liu and Cohen, 2015).

Compound **3** reduced HSV-1 reactivation by 85%, while roscovitine completely blocked detectable viral reactivation (Fig. 5A). Although there was some viral genome replication, it was significantly less than in the untreated group. The reduction of DNA replication, however, was similar for both compounds (Fig. 5B). The efficiency of these compounds in blocking reactivation compared to lytic infection is significant and desirable for an anti-herpetic drug since periodic reactivation is responsible for much of the morbidity associated with HSV-1 infections and the potential subsequent infection of other hosts (Bloom et al., 2010).

The reactivation inhibition of these compounds in the LUHMES is consistent with the study of Schang et al. (2002), which demonstrated the inhibition of HSV-1 reactivation by roscovitine in the trigeminal ganglia explanted from previously infected rats. They found CDK2 and CDK4 expression increased, while the levels of the other CDKs remained low. As roscovitine inhibits CDK2 and has no effect on CDK4, the authors concluded that CDK2 was required for the reactivation process (Schang et al., 2002). Thus, the fact that compound **3** inhibited the reactivation of HSV-1 with a similar profile to roscovitine shows a possibility of this compound to act on CDK2, as predicted in the computational approach.

Herein, we explored virtual screening to find compounds with anti-HSV-1 activity, which is a relatively fast and less expensive strategy. Three compounds showed antiviral activity, two compounds were effective toward the ACV-resistant strains and one compound inhibited reactivation of latent HSV-1. Our *in silico* study using molecular docking showed the compounds had high affinities for CDK2, and future *in vitro* studies may confirm the hypothesis that they inhibit the CDK2 enzyme, whose interference with HSV-1 replication has been reported in previous studies (Schang et al., 1998, 2002, 2006). The compounds we identified are potential CDK2 inhibitors and may have a different

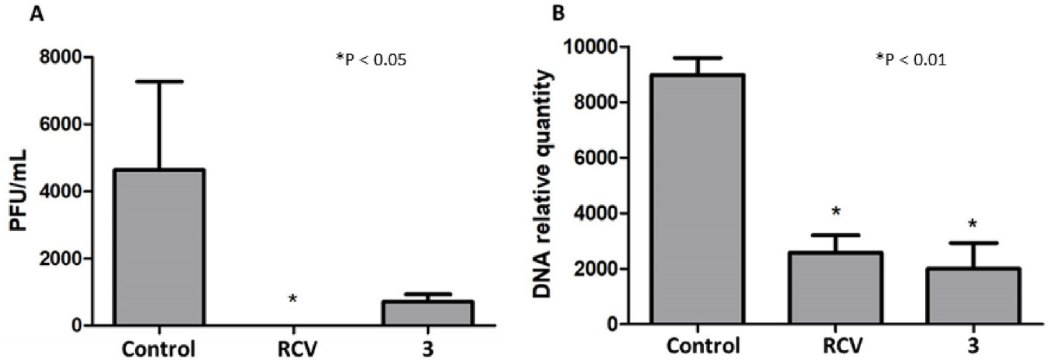

Fig. 5. The activity of roscovitine (RCV) and compound **3** on HSV-1 reactivation. Viral titers (**A**) and DNA quantification (**B**) 48 h after stimulation of HSV-1 reactivation by the PI3K inhibitor (PI3K-I). Asterisks indicate statistical significance (Tukey's Test).

antiviral mechanism of action than acyclovir. Further studies on the structure activity relationship (SAR) and molecular modifications will be necessary to progress from hits to leads in the drug development process.

4. Conclusions

Through virtual screening, six potential CDK2 inhibitors were selected out of thousands of compounds from databases. Three were identified with antiviral activity in the *in vitro* assays, showing the power of the method to find active compounds. These compounds also possess satisfactory pharmacokinetic and toxicological profiles *in silico*. Compounds **1** and **2** were effective even against ACV-resistant strains and compound **3** inhibited the reactivation of HSV-1 similarly to roscovitine. Taken together, our findings endorse the use of the cellular CDK2 as an interesting target for anti-HSV-1 and show that virtual screening is a useful strategy to prospect compounds. In conclusion, we present some novel anti-HSV-1 compounds, which are promising candidate hits for the development of new drugs for the treatment of HSV-1 infections with different modes of action than acyclovir and that may prevent virus reactivation.

Declaration of competing interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104621.

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