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Graphical Abstract

**Anti-HSV activity and mode of action study of α-pyrone carboxamides**

Srinivas Karampuri, Durbadal Ojha, Paromita Bag, Harapriya Chakravarty, Chandralata Bal, Debprasad Chattopadhyay Ashoke Sharon

Potential anti-HSV lead candidate 3d (EC_{50} = 9.8 µg/ml) and its possible binding mode to utilize cavity-A and cavity-B of viral enzyme HSV polymerase.
Anti-HSV activity and mode of action study of α-pyrone carboxamides

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Abstract

The clinical management of herpes virus diseases is limited due to ineffective clearance of virus particles and frequent emergence of drug-resistant viruses, particularly in immunocompromised patients, pregnant women and neonates. In our continued quest for new antiviral lead, α-pyrone carboxamide propanol derivatives were synthesized and evaluated in HSV infected Vero cells. The compound 3d showed potent antiviral activity against HSV-IF (EC_{50} = 9.8 µg/ml and EC_{99} = 18.0 µg/ml) and HSV-2G (EC_{50} = 12.4 µg/ml and EC_{99} = 24.0 µg/ml) at 4-6 h post-infection. The mode of action studies demonstrated that 3d did not interfere in viral attachment or penetration, however, reduced the expression of ICP4 and ICP27 (immediate-early gene products) as well as the HSV DNA polymerase.
1. Introduction

The *Herpesviridae* are a large family of DNA viruses that include Herpes Simplex Virus type 1 and type 2 (HSV-1 and HSV-2). HSV-1 and HSV-2 are responsible for the cold sores and genital herpes that have plagued humanity for centuries. The DNA polymerase is one of the most important early proteins that play a key role during replication of these viruses. The HSV and human DNA polymerase belong to the family of type B DNA polymerase and share six to seven highly conserved domains including domain C.\(^1\) We are focused to discover new non-nucleoside analogs (NNA) capable of doing selective inhibition of viral DNA polymerase. This may lead to achieve desirable inhibition with lesser side effects and toxicity. PNU-183792 (I, Figure 1) having broad spectrum anti-HSV activity with high specificity for viral polymerases compared to human polymerases is a 4-oxo-dihydroquinoline-3-carboxamide (DHQ) based derivative.\(^2\) The earlier report shown that DHQ molecules were active in plaque reduction assays against various isolates of HSV and the average IC\(_{50}\)s were found in the range of 14 ± 4.0 µM in comparison to reference drug acyclovir (ACV) of 8.6 ± 5.4 µM.\(^2\) Further, the studies on promising analog I shown comparable drug like properties with respect to licensed drugs acyclovir, ganciclovir, and cidofovir.\(^3\)

![Chemical structure of potential compound PNU-183792 (I), preliminary lead (II) and new prototype molecules (III).](image)

**Figure 1** Chemical structure of potential compound PNU-183792 (I), preliminary lead (II) and new prototype molecules (III).

ACV, a synthetic guanine nucleoside analog is a prodrug that phosphorylates to ACV monophosphate preferentially by thymidine kinase of herpes virus, which then further phosphorylated to the di and tri-phosphate forms by cellular enzymes. The pharmacologically active ACV-triphosphate then inhibits the viral DNA polymerase along with chain termination. Despite the availability of ACV and its analogues the HSV can cause life-threatening infections such as herpes encephalitis. Neither acyclovir nor its analogues can eliminate the virus in the latent state\(^4\); while their extensive clinical use leads to the
emergence of drug-resistant viruses\textsuperscript{5}. Consequently, continuation of the HSV pandemic is anticipated and efforts to develop vaccines and novel therapies aimed at eliminating latent virus are still warranted.\textsuperscript{6,7} However, a recent study showed that anti-HSV therapy significantly reduces HIV-1 RNA load in co-infected patients.\textsuperscript{8} Therefore, alternative treatments to minimize the development of resistance, its side-effects with better efficacy are urgently needed. Till date no antiviral compound has been able to completely inhibit the replication of any virus and a proportion of viral particles always seem to be able to circumvent the drug-induced blockade.

In our recent report, we described inclusion of pharmacophoric motif (red) of I for designing new molecules based on $\alpha$-pyrone carboxamide and identified II (Figure 1) as our preliminary lead candidate with EC\textsubscript{50} of 12.4 $\mu$g/ml and CC\textsubscript{50} of 52.4 $\mu$g/ml.\textsuperscript{9} The identification of pharmcophoric motif were further followed by modeling studies and the preliminary lead compound (II) showed the possibility to fit into the allosteric site in palm region of HSV DNA polymerase. In addition, the two cavities were identified in the allosteric active site of polymerase, which were utilizing by piperdine and alcoholic mofit of II. Thus, to explore the possibility of our hypothesis, we extended our synthesis in this direction, so that target compounds can utilize these cavities more significantly to enhance anti-HSV activity. Therefore, new analogs (III, Figure 1) were selected with one more carbon (alcoholic motif) for synthesis, which may allow the molecule to utilize the remaining space of identified cavity to enhance anti-HSV activity. We had also carried out the modeling studies of III on understanding the molecular basis of compound binding in the allosteric site of viral DNA polymerase. If allosteric binding phenomenon exists, it opens a scope to evaluate the synergistic role of this compound (nonnucleoside inhibitor) with nucleoside inhibitor acyclovir. Thus, the mode of action studies including possible synergism was also carried out for one of the molecules with licensed drug ACV to test our hypothesis.

2. Results and Discussion

2.1 Synthesis of $\alpha$-pyrone carboxamide derivatives

The $\alpha$-pyrone-3-carboxylic acid derivatives (1a-c) were synthesized from appropriate acetophenone derivatives (Scheme 1).\textsuperscript{9} The carboxyl group of 1a-c was coupled with propanolamine using HATU as the coupling reagent to generate respective amides (2a-c).
The methylthio group at C-4 of 2a-c was replaced with secondary amines by heating in dioxane to yield 3a-i.

Scheme 1  Reagents and conditions: i) propanolamine, HATU, DMF, rt, 6h ii) appropriate amine, dioxane, 70°C, 4h.

All the intermediates and final compounds were adequately characterized by spectroscopic analysis, and one of the potential lead compounds 3d was further confirmed by a single crystal X-ray analysis.\(^{10}\)

Figure 2. ORTEP diagram of 3d showing the X-ray molecular structure in 30% probability level. The piperidine motif at C4 was found disordered due to conformational flipping in chair form of piperidine in the crystal structure. (CCDC # 970219)

The ellipsoidal plot as ORTEP diagram was shown with 30 % probability in Figure 2. The piperidine motif was found disordered, seen by two position of N7 atom at N7A and N7B and it may be
associated due to conformational flipping in chair conformation of piperidine ring. The complete X-ray structure was solved and refined with good resolution.

2.2 Biological Evaluation

2.2.1 Assessments of cytotoxicity and anti-HSV efficacy

The antiviral effects of 3a-i were evaluated and results are presented in Table 1. Surprisingly out of nine only three compounds (3a, 3d and 3g) showed anti-HSV activity below their CC₅₀ concentrations. Compounds 3a and 3g were considered inactive as their EC₅₀ values were above 30 µg/ml while 3d with EC₅₀ of 9.8 µg/ml was considered active. The only difference between the preliminary lead (II, Figure 1) and 3d is in the hydroxyalkyl side chain and they seem to be structurally very close. Although, the anti-HSV activity (EC₅₀) was not improved much, the cytotoxicity of 3d (CC₅₀ = 151.4 µg/ml) has improved almost three times in comparison to II (CC₅₀ = 52.4 µg/ml). As a result the selectivity index (SI) of 3d (15.4), which is a measure of the preferential antiviral activity of a drug in relation to its cytotoxicity (CC₅₀/EC₅₀) was also enhanced.

Table 1 Evaluation of Cytotoxicity and Anti-HSV activity of 3a-i

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC₅₀ (µg/ml)</th>
<th>EC₅₀ HSV-1F (µg/ml)</th>
<th>EC₅₀ HSV-2G (µg/ml)</th>
<th>SI (CC₅₀/EC₅₀)</th>
<th>EC₉⁹ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>52.4</td>
<td>12.4</td>
<td>13.1</td>
<td>4.23</td>
<td>4.0</td>
</tr>
<tr>
<td>3a</td>
<td>92.5</td>
<td>37.2</td>
<td>46.2</td>
<td>2.49</td>
<td>2.0</td>
</tr>
<tr>
<td>3b</td>
<td>39.4</td>
<td>36.4</td>
<td>&gt;39</td>
<td>1.08</td>
<td>ND</td>
</tr>
<tr>
<td>3c</td>
<td>98.2</td>
<td>95</td>
<td>&gt;98</td>
<td>1.03</td>
<td>ND</td>
</tr>
<tr>
<td>3d</td>
<td>151.4</td>
<td>9.8</td>
<td>12.4</td>
<td>15.4</td>
<td>12.2</td>
</tr>
<tr>
<td>3e</td>
<td>29.2</td>
<td>12</td>
<td>15</td>
<td>2.43</td>
<td>1.94</td>
</tr>
<tr>
<td>3f</td>
<td>43.6</td>
<td>40</td>
<td>&gt;43</td>
<td>1.09</td>
<td>ND</td>
</tr>
<tr>
<td>3g</td>
<td>76.8</td>
<td>33.1</td>
<td>72</td>
<td>2.32</td>
<td>1.07</td>
</tr>
<tr>
<td>3h</td>
<td>117.5</td>
<td>112.5</td>
<td>&gt;117</td>
<td>1.04</td>
<td>ND</td>
</tr>
<tr>
<td>3i</td>
<td>37.8</td>
<td>36.5</td>
<td>&gt;37</td>
<td>1.03</td>
<td>ND</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>136.8</td>
<td>2.4</td>
<td>2.8</td>
<td>57.0</td>
<td>48.8</td>
</tr>
</tbody>
</table>

The 50% cytotoxic concentration for Vero cells (CCL 81) in µg/ml.
Concentration of compound (µg/ml) producing 50% inhibition of virus induced CPE of three separate experiments.
Selectivity index (SI) = CC₅₀/EC₅₀ [SI Index below 4 is insignificant]
ND, Not detected, as the anti-HSV activity was either equal to or above the CC₅₀ concentrations. The EC₉⁹ was above their CC₅₀ concentrations, for most of the compounds.

2.2.2 Dose response assay
Vero cells were infected with HSV-1F or HSV-2G and exposed to the various concentrations of 3d and acyclovir. The dose-dependent antiviral activity was evaluated by MTT assay. The results (Figure 3) revealed that 3d inhibited HSV-1 and HSV-2 in a dose-dependent manner and maximum (> 99%) inhibition was observed at 18 and 24 µg/ml respectively. Acyclovir achieved > 99% inhibition at 5 µg/ml for both HSV-1 and HSV-2.

Figure 3 Dose dependent effects of 3d and acyclovir against HSV-1F and HSV-2G. No inhibition was noticed with 0.1% DMSO, used as solvent (data not shown).

2.2.3 Time response assay
To investigate the activity of 3d on viral infection cycle, we performed the time-of-addition assay. Addition of 3d (18 µg/ml) to HSV-1F infected Vero cells at different time points revealed that it was effective at 4-6 h post-infection (hpi). No inhibition was found when the virus particles were exposed to 3d before infection (pre-infection) or at the time of infection (co-infection). These findings suggested that the antiviral activity of 3d was not due to the
inhibition of viral adsorption but due to the inhibition or blocking of early replication of HSV (Figure 4).

![Figure 4](image)

**Figure 4** Time of addition assay of 3d and acyclovir against HSV-1F.

2.2.4 Attachment and penetration assay

The time of addition assay revealed that HSV-1F was inhibited by 3d at 4-6 hpi which indicates that the action was during early event of viral life cycle including attachment and penetration. Therefore, we investigated these steps separately. The result of attachment and penetration assays showed that 3d had no effect either on viral attachment or penetration into Vero cells (Figure 5) like acyclovir, indicating that 3d was not inhibiting viral entry to the host cell.

![Figure 5](image)
**Figure 5** Effect of 3d and Acyclovir on HSV-1F attachment and penetration respectively.

### 2.2.5 Drug combination assay

We then examined whether the efficacy of 3d could be increased in combination with ACV by the drug combination assay. For this we evaluated the antiviral activity of various combinations of 3d and ACV, alone and in combination, using the isobologram method and assessed by the fractional inhibitory concentration (FIC) index, which represents the sum of the FICs of each drug tested. The FIC for each drug was determined by dividing the MIC of each drug in combination by the MIC of each drug used alone. Based on the Loewe additivity zero-interaction theory a drug cannot interact with itself and the effect of a combination will be additive when FIC index is 1.0. An FIC index smaller or higher than 1 indicates synergy or antagonism.\(^{11-13}\) Our results showed that FIC index in combination of 3d and acyclovir was 0.389, indicative of strong synergism (Table 2), as found with amphotericin B or voriconazole plus caspofungin (FIC indices 0.5-1.0) that prolonged the survival with reduced fungal burden in aspergillosis;\(^{11}\) as well as with trifluorothymidine and ganciclovir against HSV-1 at concentrations less toxic than these antivirals alone.\(^{14}\) Interestingly a minor synergy can augmented the pharmacokinetic and other factors *in vivo*.\(^{15}\)

**Table 2** Inhibitory effects of 3d in combination with Acyclovir\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC(_{50}) HSV-1F</th>
<th>FIC(<em>{compound}) + FIC(</em>{ACV}) HSV-1F</th>
<th>Inhibitory effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>3d alone</td>
<td>9.8 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acyclovir alone</td>
<td>2.4 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acyclovir + 3d</td>
<td>0.75 ± 0.1</td>
<td>0.75/9.8 + 0.75/2.4 = 0.389</td>
<td>Synergism</td>
</tr>
</tbody>
</table>

\(^a\)The interaction between 3d and ACV was interpreted according to the combined FIC index [FIC\(_{compound}\) + FIC\(_{ACV}\)] as synergy (≤0.5), no interaction (0.5 - 4) or antagonism (>4) (32).

### 2.2.6 Effect of compound 3d on HSV early gene expression

It is known that five infected cell proteins (ICPs) ICP0, ICP4, ICP22, ICP27, and ICP47 are the products of immediate early (IE) gene, essential to support the lytic infection of HSV.\(^{16}\) Among these, ICP4 (product of RS1 gene) is required for the subsequent expression of both early (E) and late (L) viral genes.\(^{17}\) This protein probably interacts with the basal transcription factors (a part of the RNA polymerase transcription complex) and transactivates the viral gene transcription.\(^{18}\) On the other hand, the ICP27, encoded by UL54 plays an essential role in viral replication\(^{17}\) by suppressing host mRNA synthesis through repression of
primary transcription\textsuperscript{19} and pre-mRNA splicing.\textsuperscript{20} Therefore, to determine the effect of 3d on HSV early gene expression we measured two essential IE proteins ICP4 and ICP27 by quantitative real-time PCR. The results demonstrated a significant decrease in both IE proteins ICP4 and ICP27 (Figure 6) at 2-6 hpi in 3d treated cells (18 µg/ml), while both were expressed in untreated HSV-1F infected cells.

![Figure 6](image)

**Figure 6** Effect of 3d on IE gene expression of HSV-1F by Quantitative real time PCR.

2.2.7 Effect of 3d on DNA polymerase expression of HSV-1F

Further, it is known that the DNA polymerase of HSV is encoded by \textit{UL30} gene\textsuperscript{21} and exists as a heterodimer in association with UL42 phosphoprotein.\textsuperscript{22,23} Moreover, the DNA polymerase is tethered to DNA by the double-strand DNA binding activity of the UL42 protein.\textsuperscript{24,25} Therefore, we monitored the expression of viral DNA polymerase in HSV-1F infected cells as well as infected cells treated with the test compound, by immunoblotting. The whole cell extract from HSV-1F infected Vero cells treated with 3d (9.8 and 18 µg/ml) at 12 hpi was processed for the detection of DNA polymerase. The result (Figure 7) demonstrated depletion in HSV DNA polymerase expression level in presence of 3d compared to the house keeping gene β-actin, indicates 3d interferes with the HSV DNA polymerase expression.
2.3 Molecular Modeling Studies

In our previous report, we suggested that the binding cleft for pyranone class of molecules may be positioned in the palm sub-domain of HSV DNA polymerase (Figure 8a). When the preliminary lead compound (II) was docked into the active site, cavity A was partially occupied by the ethanol motif and the cavity B was fully occupied by piperidine motif (Figure 8b).

Thus in the present manuscript, the cavity A was explored by prototypes III in which the ethanol motif was replaced with a propanol motif. The surface diagram for one of the
prototypes (3d, Figure 8c) reveals similar binding mode as II inside the palm region. However, the propanol motif occupied more space in the cavity A. The binding scores of II and prototypes III summarized in Table 3 indicate increase in overall binding affinity of 3d compared II which may be the possible reason for enhanced activity. Thus, the present study supports lead optimization to enhance the anti-HSV activity using structure-based approach.

Table 3 The molecular docking studies and scoring for synthesized analogs

<table>
<thead>
<tr>
<th>Comp</th>
<th>G-Scorea</th>
<th>Emodelb</th>
<th>DG Bind</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>-4.8</td>
<td>-64.9</td>
<td>-59.9</td>
</tr>
<tr>
<td>3b</td>
<td>-6.1</td>
<td>-63.8</td>
<td>-68.4</td>
</tr>
<tr>
<td>3c</td>
<td>-5.6</td>
<td>-62.1</td>
<td>-69.5</td>
</tr>
<tr>
<td>3d</td>
<td>-5.7</td>
<td>-67.1</td>
<td>-72.3</td>
</tr>
<tr>
<td>3e</td>
<td>-5.6</td>
<td>-69.1</td>
<td>-73.1</td>
</tr>
<tr>
<td>3f</td>
<td>-5.1</td>
<td>-61.6</td>
<td>-76.8</td>
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<tr>
<td>3g</td>
<td>-5.2</td>
<td>-60.7</td>
<td>-62.3</td>
</tr>
<tr>
<td>3h</td>
<td>-5.6</td>
<td>-65.4</td>
<td>-60.7</td>
</tr>
<tr>
<td>3i</td>
<td>-5.1</td>
<td>-63.6</td>
<td>-60.7</td>
</tr>
<tr>
<td>II</td>
<td>-4.9</td>
<td>-64.9</td>
<td>-68.7</td>
</tr>
</tbody>
</table>

aG-Score (Glide Score): The minimized poses are rescored using Schrödinger’s proprietary Glide Score scoring function to get G-Score. 
bEmodel score: The binding affinity can be estimated by Glide Emodel Score, which is the combination of G-Score and non-bonded interaction energy. 
cMM-GBSA score: Ligand binding energies calculated for all compounds with a single receptor using protein flexibility option (defined: 7 Å from docked ligand). Solvation model: VSGB, The binding energy is calculated according to the equation: DG bind = E_complex (minimized) - E_ligand (minimized) - E_receptor (minimized)

3. Materials and Methods

3.1 Chemistry

3.1.1 General All reactions were carried out in oven-dried glassware under nitrogen atmosphere. The chemicals and solvents were purchased from Spectrochem, Across, Rankem or Sigma-Aldrich. Melting points were recorded on Veego melting point apparatus. Analytical thin layer chromatography (TLC) was performed on precoated plates (silica gel 60 F-254) purchased from Merck Inc. Purification by gravity column chromatography was carried out on silica gel (100-200 mesh). Elico UV/Vis spectrophotometer was used for recording the UV spectra. ¹H/¹³C NMR were obtained from a Varian (400 MHz) spectrometer or Bruker spectrometer using CDCl₃ or DMSO-d₆, as solvents. Peaks are recorded with the
following abbreviations: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; J, coupling constant (hertz).

3.1.2 Method for synthesis of 2a–c
To a solution of appropriate 2H-pyran-4-methylthio-3-carboxylic acid (1a–c, 2 mmol) in 15 ml DMF, HATU (2.1 mmol) and diisopropylethylamine (8 mmol) was added and stirred for 15 min. Propanolamine (2.1 mmol) was added and stirred for 5 h at room temperature. The reaction was monitored by TLC for completion. The reaction mixture was poured onto ice water with stirring. The organic layer was separated and the water layer was extracted with DCM. The combined organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The residue was recrystallized with methanol which was used for next reaction.

3.1.3 Method for synthesis of 3a–i
To a solution of an appropriate 2a–c (1 mmol) in dioxane, appropriate amine (1.5 mmol) was added and refluxed for 6 h and monitored by TLC for completion. The solvent was removed under vacuum; ice cold water was added and extracted with DCM. The organic layer was dried over Na₂SO₄, concentrated under reduced pressure and column purified with 1% methanol in DCM to obtain pure compound.

**N-(3-hydroxypropyl)-2-oxo-6-phenyl-4-(pyrrolidin-1-yl)-2H-pyran-3-carboxamide (3a)**
Yield 55%, mp: 144-145 °C, MS-ESI (m/z): 342.9 [M⁺+1]; IR (KBr) (ν, cm⁻¹): 3360.00 (NH), 3290 (OH), 1655 and 1624 (C=O); ¹H NMR (400 MHz, DMSO-d₆): δ 1.59-1.62 (t, J = 6.8 Hz, 2H), 1.88 (s, 4H), 3.18-3.21 (t, J = 6.8 Hz, 2H), 3.44-3.47 (t, J = 6 Hz, 2H), 3.53 (s, 4H), 4.41-4.44 (t, J = 5.2 Hz, 1H), 6.79 (s, 1H), 7.51-7.53 (m, 3H), 7.89-7.90 (m, 2H), 8.06-8.09 (t, J = 5.6 Hz, 1H).

**6-(4-fluorophenyl)-N-(3-hydroxypropyl)-2-oxo-4-(pyrrolidin-1-yl)-2H-pyran-3-carboxamide (3b)**
Yield 52%, mp: 188-189 °C, MS-ESI (m/z): 360.9 [M⁺+1]; IR (KBr) (ν, cm⁻¹): 3365 (NH), 3333 (OH), 1670 and 1639 (C=O); ¹H NMR (400 MHz, DMSO-d₆): δ 1.59-1.62 (t, J = 6.8 Hz, 2H), 1.88 (brs, 2H), 3.19-3.22 (t, J = 6.4 Hz, 2H), 3.43-3.48 (q, J = 6 Hz, 2H), 3.52 (brs, 4H), 4.41-4.44 (t, J = 5.2 Hz, 1H, OH), 6.78 (s, 1H), 7.34-7.38 (t, J = 8 Hz, 2H), 7.78-7.80 (d, J = 6Hz, 1H), 8.05-8.08 (t, J = 6Hz, 1H, NH).

**N-(3-hydroxypropyl)-2-oxo-4-(pyrrolidin-1-yl)-6-(p-tolyl)-2H-pyran-3-carboxamide (3c)**
Yield 55%, mp: 135-136 °C, MS-ESI (m/z): 357.1 [M⁺+1]; IR (KBr) (ν, cm⁻¹): 3362 (NH), 3298 (OH), 1661 and 1628 (C=O); ¹H NMR (400 MHz, DMSO-d₆): δ 1.59-1.62 (t, J = 6.4 Hz, 2H), 1.87 (s, 4H), 2.36 (s,3H), 3.17-3.21 (t, J = 6.4 Hz, 2H), 3.45-3.48 (t, J = 6 Hz, 2H), 3.52 (s, 4H), 4.40-4.42 (t, J = 5.6 Hz,1H, OH), 6.73 (s,1H), 7.31-7.33 (d, J = 8 Hz, 2H), 7.78-7.80 (d, J = 8.4 Hz, 2H), 8.02-8.05 (t, J =5.6 Hz,1H, NH).
N-(3-hydroxypropyl)-2-oxo-6-phenyl-4-(piperidin-1-yl)-2H-pyran-3-carboxamide (3d)
Yield 59%, mp: 172-173 °C, MS-ESI (m/z): 356.9 [M^-1+]; IR (KBr) (v, cm^-1): 3362 (NH), 3289 (OH), 1656 and 1627 (C=O); 1H NMR (400 MHz, DMSO-d6): δ 1.59-1.62 (m, 8H), 3.18-3.23 (t, J = 6.8 Hz, 2H), 3.39-3.46 (m, 6H), 4.42-4.45 (t, J = 5.2 Hz, 1H, OH), 6.98 (s, 1H), 7.51-7.52 (t, J = 3.2 Hz, 3H), 7.91-7.92 (d, J = 2.8 Hz, 2H), 8.15 (s, 1H, NH).

6-(4-fluorophenyl)-N-(3-hydroxypropyl)-2-oxo-4-(piperidin-1-yl)-2H-pyran-3-carboxamide (3e)
Yield 59%, mp: 166-167 °C, MS-ESI (m/z): 374.9 [M^-1+]; IR (KBr) (v, cm^-1): 3367 (NH), 3306 (OH), 1666 and 1628 (C=O); 1H NMR (400 MHz, DMSO-d6): δ 1.59-1.62 (m, 8H), 3.18-3.23 (q, J = 6.8 Hz, 2H), 3.43-3.47 (m, 6H), 4.42-4.45 (t, J = 5.2 Hz, 1H, OH), 6.97 (s, 1H), 7.34-7.38 (t, J = 8.8 Hz, 2H), 7.98-8.02 (m, 2H), 8.13-8.16 (t, J = 5.6 Hz, 1H, NH).

N-(3-hydroxypropyl)-2-oxo-4-(piperidin-1-yl)-6-(p-tolyl)-2H-pyran-3-carboxamide (3f)
Yield 55%, mp: 153-154 °C, MS-ESI (m/z): 371.1 [M^-1+]; IR (KBr) (v, cm^-1): 3359 (NH), 3292 (OH), 1661 and 1631 (C=O); 1H NMR (400 MHz, DMSO-d6): δ 1.59-1.64 (m, 8H), 2.37 (s, 3H), 3.18-3.21 (t, J = 6.4 Hz, 2H), 3.43-3.47 (m, 6H), 4.41-4.43 (t, J = 5.6 Hz, 1H, OH), 6.92 (s, 1H), 7.31-7.33 (d, J = 8 Hz, 2H), 7.81-7.83 (d, J = 8.4 Hz, 2H), 8.12-8.15 (t, J = 5.6 Hz, 1H, NH).

4-(dimethylamino)-N-(3-hydroxypropyl)-2-oxo-6-phenyl-2H-pyran-3-carboxamide (3g)
Yield 45%, mp: 132-133 °C, MS-ESI (m/z): 316.9 [M^-1+]; IR (KBr) (v, cm^-1): 3359 (NH), 3286 (OH), 1654 and 1630 (C=O); 1H NMR (400 MHz, DMSO-d6): δ 1.61-1.64 (t, J = 6.4 Hz, 2H), 3.10 (s, 6H), 3.19-3.24 (q, J = 6 Hz, 2H), 3.43-3.48 (q, J = 6 Hz, 2H), 4.42-4.45(t, J = 5.6 Hz, 1H, OH), 6.91 (s, 1H), 7.47-7.53 (m, 3H), 7.90-7.93 (m, 2H), 8.09-8.12 (t, J = 5.6 Hz, 1H, NH).

4-(dimethylamino)-6-(4-fluorophenyl)-N-(3-hydroxypropyl)-2-oxo-2H-pyran-3-carboxamide (3h)
Yield 42%, mp: 112-113 °C, MS-ESI (m/z): 335.2 [M^-1+]; IR (KBr) (v, cm^-1): 3361 (NH), 3286 (OH), 1649 and 1624 (C=O); 1H NMR (400 MHz, DMSO-d6): δ 1.57-1.60 (t, J = 6.4 Hz, 2H), 3.12 (s, 6H), 3.18-3.23 (q, J = 6.8 Hz, 2H), 3.46-3.49 (t, J = 5.6 Hz, 2H), 4.46-4.49 (t, J = 5.2 Hz, 1H, OH), 6.89 (s, 1H), 7.31-7.34 (d, J = 8.6 Hz, 2H), 8.01-8.13 (m, 2H), 8.15-8.18 (t, J = 5.6 Hz, 1H, NH).

4-(dimethylamino)-6-(4-fluorophenyl)-N-(3-hydroxypropyl)-2-oxo-2H-pyran-3-carboxamide (3i)
Yield 45%, mp: 86-87 °C, MS-ESI (m/z): 331.1 [M^-1+]; IR(KBr) (v, cm^-1): 3355 (NH), 3291(OH), 1651 and 1632 (C=O); 1H NMR (400 MHz, DMSO-d6): δ 1.57-1.64 (m, 2H), 2.49 (s, 3H), 3.09 (s, 6H), 3.19-3.22 (q, J = 6.4 Hz, 2H), 3.43-3.48 (q, J = 6.4 Hz, 2H), 4.31-4.44 (t, J = 5.2 Hz, 1H,OH), 6.85 (s, 1H), 7.32-7.34 (d, J = 8.3 Hz, 2H), 7.80-7.82 (d, J = 8Hz, 2H), 8.07-8.10 (t, J = 5.2 Hz, 1H, NH).

3.2 Biological Evaluations
3.2.1 Cell and viruses:
Vero cells (African green monkey kidney cells; ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 5-10% fetal bovine serum (FBS;
Invitrogen, USA), 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C in 5% CO₂. The viral strains used were HSV-1F and HSV-2G (ATCC 734), purchased from the ATCC (Manassas, VA, USA). Virus stocks were prepared from infected culture at a multiplicity of infection (moi) of 0.5 for 1 h at 37°C. The residual viruses were then washed out with phosphate-buffered saline (PBS) and the cells were cultured for another 48-72 h. The cultured cells were lysed finally by three cycles of freezing and thawing, centrifuged at 1500 g at 4°C for 20 min and the collected supernatant was tittered by plaque assay, and stored at -80°C for further studies.

3.2.2 Cytotoxicity assay:
To determine the effect of the test compounds on uninfected cells, cultured Vero cells (10⁴ cells/well) in 96 well plates were exposed to various concentrations of the test drugs, in triplicate and incubated at 37°C in 5% CO₂, using ACV and dimethylsulfoxide (DMSO, 0.1%) as controls. After 48 h, 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was added to each well with DMEM, incubated for 3-4 h, and then mixed with 100 µl of MTT solubilising solution (Sigma), and the optical density (OD) was read by a plate reader at 570 nm with a reference wavelength of 690 nm. Data were calculated as the percentage of cell viability using the formula: [(sample absorbance-cell free sample blank)/mean media control absorbance)] X100%. The 50% cytotoxic concentration (CC₅₀) causing visible morphological changes in 50% of Vero cells with respect to cell control was determined from the concentration-response curves after viable cell count.⁹ ²⁶

3.2.3 Antiviral activity by Plaque reduction assay:
Plaque reduction assay was used to evaluate the antiviral efficacy of the test compound(s), using ACV and DMSO (0.1%) as positive and negative control respectively. Vero cell monolayer in six well plates, infected with HSV-1F and HSV-2G (100 pfu) were exposed to serial dilutions of test compound for 1-2 h at 37°C, then washed with PBS and overlaid with 1% methylcellulose (Fluka, USA) to form plaques. The plaques developed after 72 h of incubation were fixed with 4% paraformaldehyde and stained with methylene blue (0.03%) in 70% methanol. The virus titers were calculated by scoring the plaque-forming units (pfu). The effective concentration of test compound that inhibited the number of viral plaques by 50% (EC₅₀) was interpolated from the dose-response curves.²⁷ ²⁹

3.2.4 Dose response assay
The Vero cell (10^4 cells/well) culture on 96-well plates were infected with HSV-1F and HSV-2G (1 moi) separately and then exposed to various concentrations of test compounds (0-50 µg/ml) and ACV, in triplicate, and incubated for 72 h at 37°C in 5% CO₂. The MTT assay was carried out, as described above and viral inhibition rate was calculated as: 
\[ \frac{(A_{tv}-A_{cv})}{(A_{cd}-A_{cv})} \times 100\% \], where \( A_{tv} \) indicates the absorbance of test compound with virus-infected cells. \( A_{cv} \) indicates the absorbance of virus control, and \( A_{cd} \) the absorbance of the cell control. The antiviral concentration of 50% effectiveness (EC₅₀) was defined as the concentration that achieved 50% inhibition of virus-induced cytopathic effects.³⁰

### 3.2.5 Time response assay:

Following three different approaches, Vero cells were exposed to the test compound (18.0 µg/ml) before infection, during infection or after infection with HSV-1F (100 pfu/well) at 0-12 h time intervals in triplicate, using DMSO (0.1%) and ACV (5.0 µg/ml) as controls. For pre-infection Vero cells were treated with the test compound either for 1 h or for 3 h, washed with PBS and then infected with the virus in DMEM containing 2% FBS at 37°C. For co-infection cells were subsequently infected and treated with the test compound and after 1 h of incubation the virus-drug mixture was removed, washed with PBS three times, and added with fresh media. While for post-infection (p.i) the cells were infected with HSV-1F first, allow adsorbing (1 h) and then treated with the test compound. Then the mixture was removed from the respective well(s) at different time intervals upto 12 h, washed with PBS and added with the fresh media to carry out the plaque reduction assay, as described above.²⁷, ²⁹, ³¹

### 3.2.6 Attachment and penetration assay:

To investigate whether the compound have any effect on viral adsorption or attachment, Vero cell monolayer were prechilled at 4°C for 1 h and subsequently challenged with HSV-1F (200 pfu/well) in the presence of test compound (9.8 µg/ml), DMSO (0.1%) or ACV (5.0 µg/ml) for 3 h at 4°C. After infection, the wells were washed twice with ice-cold PBS to remove unbound virus, and overlaid with 1% methylcellulose to allow plaque formation. The plaques developed after 72 h of incubation were stained and counted.³²

For viral penetration assay prechilled (at 4°C for 1 h) Vero cell monolayer were subsequently incubated with HSV-1F (300 pfu/well) for 3 h at 4°C to allow viral adsorption. The infected cells were then incubated with test compound (18.0 µg/ml), DMSO (0.1%) or ACV (5.0
µg/ml) for another 20 min at 37°C to facilitate viral penetration. At the end of the incubation period, extracellular non-penetrated virus was inactivated by citrate buffer (pH 3.0) for 1 min, and then the cells were washed with PBS and overlaid with overlay medium for plaque formation. The viral plaques developed after 48 h of incubation at 37°C were stained and counted.

### 3.2.7 Combined effect of test compound with Acyclovir

In order to analyze the combined effect of test compound (3d) and ACV on plaque formation, the EC₅₀ of these agents at various concentrations of the compounds against HSV-1F were tested. The combined effect of both the agents on HSV was examined by plaque assay. Duplicate culture of Vero cells were infected with 100 PFU/0.2 ml of HSV-1F for 1 h and the cells were overlaid with 5 ml of overlay medium containing 1% methylcellulose with various concentrations of the test compound and/or ACV and then incubated at 37 °C for 5 days. The cells were washed and then fixed (4% paraformaldehyde) and stained with methylene blue (0.03%) to count the numbers of plaques for the determination of 50% inhibitory concentration of the plaque number from a curve, while the combined treatment was analyzed by isobologram method. The EC₅₀ was used to calculate the fractional inhibitory concentration (FIC) of the agents in combination. The interaction between test compound and ACV was interpreted according to the combined FIC index (FIC\textsubscript{compound} + FIC\textsubscript{ACV}) as synergy (≤0.5), no interaction (0.5-4) or antagonism (>4).

### 3.2.8 Quantitative real-time PCR:

HSV-1F (5 moi) infected Vero cells were treated with the test compound (9.8 and 18.0 µg/ml) for 2 h, 4 h and 6 h post-infection and immediately, RNA was isolated using RNeasy Mini kit (QIAGEN) following the manufacturer’s protocol. Total RNA (0.1 mg/ml) in RNase-free water in 20 µl of RT mix (containing 5X VILO Reaction Mix, 10X SuperScript Enzyme Mix and DEPC treated water) was subjected to cDNA synthesis using the GeneAmp PCR System 9600 (Perkin Elmer Corp, USA). Then real-time PCR was performed with these products by using SYBR Green PCR Master Mix (Qiagen) following manufacturer protocol in a ABI Prism 7000 sequence detection system (Applied Biosystems, CA, USA). The PCRs were amplified at cycling conditions of: 95°C for 10 min and 40 cycles (15 s at 95°C, then 60 s at 60°C) in triplicate. The sequences of primers used were as follows: ICP4 (5'-GACGTTGTGGACTGGAAG-3' and 5'-ACTTAATCAGGTCGTTGCCG-3'); ICP27 (5'-CCTTTCCTCCAGTGCTACCTG-3' and 5'-GCCAGAATGACAAACACGAAG-3') and
GAPDH (5'-AAGGTCGGAGTCAACGGATT-3' and 5'-CTGGAAGATGGTGATGATGGGATT-3').

3.2.9 Western blot analysis of DNA polymerase:
For Western blot analysis, equal amounts of protein (40 µg/sample) from HSV-1F infected and untreated or treated cells with test compound (9.8 and 18.0 µg/ml) at 12 h p.i. for HSV DNA polymerase were immediately harvested in buffer (200 µl per well) containing 20 mM Tris (pH 7±0.5), 50 mM NaCl, 5% NP40, 0.05% DOC and separated by centrifugation at 16000 g with a microcentrifuge for 10 min at 4°C, subjected to SDS-PAGE and blotted to pre-equilibrated PVDF membrane (Thermo scientific, USA). Then the membrane was blocked in 5% NFDM in 1X TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween 20), rinsed and incubated with anti-HSV DNA polymerase or polyclonal anti-β-actin (Shanta Cruz Biotech Inc., USA) antibody in 5% BSA at 4°C overnight. Immunoblotting was performed with peroxidase-labeled anti-rabbit polyclonal antibodies and visualized by ECL Western blotting detection kit (Millipore, USA).34,35

3.3 Molecular Modeling

3.3.1 Receptor preparation
In continuation to our modeling studies, the previously prepared and reported structure of HSV DNA polymerase was used9 and optimized by protein preparation wizard (PPW) of Schrödinger Suite 2013.36

3.3.2 Ligands preparation
The 3D structure for synthesized compounds were built using Maestro interface of Schrödinger. The hydrogens were added, valences were satisfied, all bonds were corrected and formal charges were assigned. Finally all compounds were treated in LigPrep module37 of Schrödinger using OPLS force field 2005 followed by conformational search through MacroModel MMFFs force field to obtained the suitable conformations. The lowest energy conformer was selected for each compound to perform molecular docking studies. The reference compound II has been used as primary lead and prepared for docking studies.

3.3.3 Molecular docking
The receptor grid generation and molecular docking studies were conducted by Glide module37-38 of Schrodinger Suite 2013. The grid was generated around the catalytic triad of
aspartic acid residues (717, 886 and 888) which has been reported essential for the polymerase activity in HSV DNA-polymerase. Glide module of Schrodinger suite 2013 was used to perform docking analysis. All ligand compounds including reference compound II were docked at the predefined receptor grid using extra precision mode (XP) mode.

### 3.3.4. Embrace minimization

The top score (G-score, Table 3) docked pose of each compounds were subjected to eMBrAcE module of MacroModel for simulation to determine the binding energy differences. The minimization was performed using OPLS2005 force field utilizing GB/SA continuum water solvation model. During eMBrAcE minimization, all the residues were allowed to move freely. Minimization was conducted for 5000 steps or until the energy difference between subsequent conformations attains 0.05 kJ/mol. The energy difference results were obtained based on the calculation using $\Delta E = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{protein}})$.

### 3.3.5 Ligand free energy of binding energy calculation

The best two docked structures obtained after Glide docking were compared by minimizing with local optimization in Prime and the energies of the complex were calculated using OPLS2005 force field and Generalized-Born/Surface Area continuum solvent model. The Prime MMGBSA was used to calculate the binding free energy DG of ligands. Using equation $DG = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} + \Delta G_{\text{SA}}$, where $\Delta E_{\text{MM}}$ is the difference between the minimized energy of the Ligand-HSVcomplex and the sum of the minimized energies of HSV protein and ligands. $\Delta G_{\text{SA}}$ is the difference in surface area energies for the complex and the sum of the surface area energies for the individual molecules . $\Delta G_{\text{solv}}$ is the difference in the generalized-Born/surface area

### 4. Summary and Conclusions

The present study reveals the structure based optimization of preliminary lead compound II to a potential lead candidate 3d as possible anti-HSV agent. A modeling study suggests that 3d can efficiently utilized tubular cavity-A for binding with HSV DNA polymerase and opens a future scope for drug candidate development. The newly synthesized compound 3d demonstrated the promising anti-HSV activity and biological studies were conducted to understand possible mode of action. The MTT and plaque reduction assay revealed that 3d
has moderate antiviral activity against HSV-1F and HSV-2G with respect to its EC$_{50}$ and selectivity index, compared to ACV. The plaque reduction assay demonstrated that 3d inhibited HSV-1F and HSV-2G infection in a dose-dependent manner and >99% inhibition was achieved at 18.0 and 24.0 µg/ml. In order to understand the quantitative and temporal aspects of the antiviral activity of 3d, we conducted kinetic studies, which revealed that the addition of 3d to virus infected cells at 4-6 h post-infection is highly effective in killing the virus. Further study showed that 3d was unable to prevent the attachment or penetration of HSV, like ACV, suggesting that the mode of action of 3d is not the prevention of viral adsorption or penetration, but perhaps the interference of early events of HSV replication. Moreover, the drug combination assay (3d ± ACV) showed strong synergistic effects, suggesting that 3d may work through similar targets but at different time points. Next, to investigate the possible mode of action of 3d on early events of viral infection cycle we studied the effect of 3d on IE gene expression of HSV. To study whether 3d interferes any of the events of IE gene expression first we measure the two major end products, ICP4 and ICP27 of IE gene by quantitative real-time PCR analysis. The results demonstrated the reduced expression of both ICP4 and ICP27 in HSV infected 3d treated cells but not in untreated cells. Furthermore, the depletion of viral DNA polymerase expression, demonstrated by immunoblotting studies, in HSV-1F infected cells treated with 3d indicated that it can prevent HSV multiplication.

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