Antiviral Activity of 2-Phenoxy-[1,2,4]Triazolo[1,5-*a*]Quinazoline Derivatives

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Abstract: A series of 2-phenoxy-triazoloquinazoline derivatives have been evaluated against herpes simplex viruses *i.e.* HSV-1 and HSV-2. The antiviral activity of the titled compounds against HSV-1 and HSV-2 evaluated using Vero cells (derived from the kidney of a normal African green monkey). In terms of EC_{50}, compounds **14, 16**, **17, 25**, **26** and **27** have shown remarkable significant activity against HSV-1 with respect to the reference drug (Acyclovir, EC_{50}= 1.8 µg/mL), whereas **14, 17, 21, 25** and **26** were found to exihibit a valuable activity against HSV-2. Many of the tested compounds have demonstrated lower EC_{50} values than the parents **1** and **2**, but compounds **14, 16**, **17, 25**, **26** and **27** are the most set populated antiviral active compounds throughout this study. The cytotoxicity (CC_{50}), antiviral effective concentration (EC_{50}), and the selectivity index (SI) were determined.
[Rashad Al-Salahi, Ibrahim Al-Swaidan, Mohamed Al-Omar And Mohamed Marzouk. **Antiviral Activity of 2-Phenoxy-[1,2,4]Triazolo[1,5-*a*]Quinazoline Derivatives.** *Life Sci J* 2013; 10(4): 2164-2169]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 289

Key words: 1,2,4-Triazoloquinazoline**s;** Antiviral; Acyclovir; EC_{50}; HSV-1, HSV-2

1. Introduction

Herpes simplex viruses (HSV), which belong to the *Herpes viridae* are group of large DNA viruses divided into three subfamilies known as α-, β- and γ-herpesvirinae. The human α-types are HSV-1, HSV-2 and varicella zoster virus (VZV), whereas, β-types included human cytomegalovirus (CMV) and human herpes viruses 6 and 7, while the Epstein-Barr virus (EBV) and human herpesvirus 8 are typical γ-herpes viruses. HSV are a common human pathogen that cause *herpes labiles, herpes genitalis*, keratitis and encephalitis. The HSV infection caused by HSV-1 and HSV-2 is mainly transmitted by close personal contact, and the virus can establishes lifelong latent infection in sensory neurons with recurrent lesions (Barton, 2005). *Herpes genitalis*, usually caused by HSV-2, spread silently through sex, wreaks emotional and huge financial damage due to its silent epidemic potential. Life threatening infection in immunocompromised people and neonates can be occurred, as well (Fatahzadeh & Schwartz, 2007). Moreover, HSV-2 is a high risk factor for acquisition of HIV infection (Cowan et al., 2003; Safrin et al., 1991, White et al., 2006) and there is a synergistic relationship between HIV and HSV (Wald, 2004; Mbopi-Kéou et al., 2000; Nagot et al., 2007). A recent researchs revealed that HSV-suppressive therapy greatly reduced genital and plasma HIV-1 RNA levels in co-infected patients (Al-Salahi et al., 2011a). Hence, the risk of acquiring or transmitting HIV infection can be greatly decreased by reducing the spread of genital herpes.

Triazoloquinazolines are 'privileged medicinal scaffolds, which played an important role for the designing and development of bioactive compounds for several uses. Compounds with this motif show a wide range of pharmacological activities such as adenosine antagonist, antifungal, antimicrobial, and antiviral (Al-Salahi et al., 2013a; Al-Salahi et al., 2013b; Pandey, et al., 2002; Al-Salahi & Geffken, 2010a). Considering the above reports, designing of new and simple synthetic route for elaborating a new target molecules involving the triazoloquinazoline moeity is therefore an interesting point of research. In this study we are aiming at the evaluation of antiviral activity for the synthesized compounds **(1-36)** against HSV-1 and HSV-2 in comparison with Acyclovir drug.

2. Experimental

2.1. General procedure for synthesis of 3-22

The methodology for preparation of all target molecules was given in our previous study (Al-Salahi, 2013c).

2.2. Antiviral methods

2.2.1. Mammalian cell line

Vero cells (derived from the kidney of a normal African green monkey) were obtained from the American Type Culture Collection (ATCC). The vero cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37ºC in a humidified

atmosphere with 5% CO_2 and were subcultured two times a week.

2.2.2. Evaluation of the antiviral activity using cytopathic effect inhibition assay

The screening antiviral assay system was established using cytopathic effect inhibition assay at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University. This assay was selected to show specific inhibition of a biologic function, *i.e.,* cytopathic effect (CPE) in susceptible mammalian cells (Hu & Hsiung, 1989). In brief, monolayers of 10,000 vero cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24h at 37ºC in a humidified incubator with 5%CO_2. The plates were washed with fresh DMEM and challenged with 10^4 herpes simplex type 1 or 2 virus (HSV-1 or HSV-2) doses and simultaneously the cultures were treated with two-fold serial dilutions of tested compound in fresh maintenance medium and incubated at 37ºC for 3 days. An infection control as well as untreated vero cells control was made in the absence of tested compound. Six wells were used for each concentration of the tested compound. Every 24 h the observation under the inverted microscope was made until the virus in the control wells showed complete viral-induce cytopathic effects (CPE). Antiviral activity was determined by the inhibition of cytopathic effect compared to control, *i.e*., the protection offered by the tested compound to the cells was scored (Vijayan et al., 2004).

Three independent experiments were assessed each containing four replicates per treatment. Acyclovir, which is clinically used for the treatment of herpetic viral disease, was used as a positive control under this assay system (Dargan, 1989).

After the incubation period, the media was aspirated, and then the cells were stained with a 0.1% crystal violet solution for 4 h. The stain was removed and the plates rinsed using tap water until all excess stain was removed. The plates were allowed to dry for 24 h and then glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), at 620 nm. Viral inhibition rate was calculated as follows:

$$[(ODtv-ODcv)/(ODcd-ODcv)] \times 100\%$$

Where ODtv, ODcv and ODcd indicate the absorbance of the tested compounds with virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively.

From these data, the dose that inhibited viral infection by 50% (EC_{50}) was estimated with respect to virus control from the graphic plots using STATA modeling software. EC_{50}, the effective concentration needed to restrain 50% virus infection compared to untreated infected cells, was determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples. To determine if each compound has sufficient antiviral activity that exceeds its level of toxicity, a selectivity index (SI) was calculated. The selectivity index (SI) was measured from the ratio of CC_{50}/EC_{50} (Zandi et al., 2007). This index, also referred to as a therapeutic index, was used to determine if a compound warrant further study. Compounds that had an SI $\geq$ 2 are considered active and $\geq$ 10 are very active.

2.2.3. Cytotoxicity evaluation using viability assay

For cytotoxicity assay, the vero cell lines were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100 µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After the end of incubation period, the viable cells yield was determined by a colorimetric method. In brief, the media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 590 nm. The absorbance is proportional to the number of surviving cells in the culture plate. All the results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated (Mosmann, 1983; Wilson, 2000).

2.3. Data and statistical analysis

The percentage cell viability was calculated using the Microsoft Excel®. Percentage cell viability was calculated as follows:

% Cell viability = (Mean Abs Control – Mean Abs test metabolite/Mean Abs control) X 100

Where: Abs: absorbance at 590 nm.

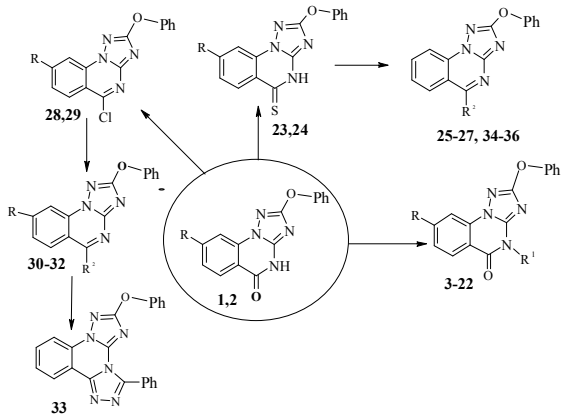
The 50% cell cytotoxic concentration (CC_{50}), the concentration required to kill or cause visible changes in 50% of intact mammalian cells, was estimated from graphic plots. STATA statistical analysis package was used for the dose response curve drawing in order to calculate CC_{50}.

The selective index (SI), a marker of antiviral activity, was determined as the ratio of CC_{50} to EC_{50}. The statistically different effects of molecules **1-36** and acyclovir on the inhibition of HSV viruses were compared, using Anova one way test.

3. Results and discussion

3.1. Chemistry

In continuation of our research (Al-Salahi & Geffken, 2010b; Al-Salahi & Geffken, 2011b; Al-Salahi & Geffken, 2011c; Al-Salahi et al., 2013c), that aimed at preparation of bioactive 2-phenoxy-4H-[1,2,4]triazolo[1,5-a]quinazoline derivatives, thirty four compounds were obtained through three main reactions (Scheme 1 & Table 1). First, treatment of **1** and **2** with alkyl and hetero halides in a molar ratio of 1:1.5 in dry dimethyl formamide at room temperature in the presence of K_2CO_3, led to the formation of **3-22**. Second, an equimolar reaction of **1** and **2** with P_2S_5 in dry pyridine produced **23** and **24;** then, reaction of **23** with different alkyl halides in aqueous NaOH afforded thioethers **25-27.** Whereas, its reaction with various secondary amines that followed by treatment with hydrogen peroxide produced compounds **34-36**. Third, **1** and **2** were transformed into their corresponding 5-chloro derivatives (**28,29**) by the reaction with $POCl_3$ in boiling benzene. Thereafter, 5-chloroderivative (**28**) was converted into the corresponding 5-ethoxytriazoloquinazolines (**30,31**) through the reaction with NaOEt. Finally, the reaction of **28** with phenylhydrazide afforded the amidrazones **32**, which subsequently treated with $POCl_3$ to produce the tetracyclic system **33.**



Schem 1. Synthetic routes of compounds **1–36**

3.2. Antiviral activity

The antiviral activity of prepared compounds **1-36** against HSV-1 and HSV-2 was evaluated by cytopathic effect inhibition assay (Hu & Hsiung, 1989). The *in vitro* results of the investigated compounds are summarized in Tables 2. From the obtained results, it can be noticed that the target molecules were found to posses weak, moderate and high anti-HSV activity.

The effect of tested samples on both HSV-1 and HSV-2 was recorded in table 2, where target compounds **14, 16, 17, 25**, **26** and **27** have shown remarkable significant activity against HSV-1, with EC_{50} values of 24, 21, 32 10.9, 6.8 and 18.2 µg/mL, respectively with respect to Acyclovir (1.8 µg/mL). Whereas **14, 16, 17, 25** and **26** exihibited a good activity against HSV-2, giving EC_{50} values of 67.3, 72.4, 64.2, 36.8, and 63.4 µg/mL, respectively with respect to Acyclovir (3.4 µg/mL).

Structure modifications on the lead compounds **1** and **2** have afforded derivatives with a different cytotoxicity effects, such as alkylation of lactam moiety furnished *N*-alkylated products **3-22**, that have shown remarkable activity in regard to their parents. However, **8**, **9**, **21** and **22**can be considered inactive structures against both viruses. Furthermore, variation in the substituted alkyl(heteroalkyl) groups have demonstrated various activity, such as compounds **14-20** were found to display the good activity against HSV-1 and HSV-2 in regards to the parents **1** and **2**. Among all alkylated products, the highest activity against both viruses for **14-17** could be attributed to the presence of morphilino- and benzoimidazole moieties. Thionation of **1** and **2** into **23** and **24** resulted in increasing the cytotoxicity that indicated from decrease of EC_{50} into 58.4 and 63.2 from 314 and 186 µg/mL, respectively in case of HSV-1. Thereafter, conversion of **23** into the corresponding *S*-alkylated products (**25-27**) created the highest active compounds in the cytotoxicity profile and set the most populated among these compounds. This indicated by their lowest EC_{50}-values (10.9, 6.8 and 8.2 µg/mL) in comparison with Acyclovir as the reference drug (1.8 µg/mL).

A comparable antiviral effect against HSV-2 was recorded with previous mentioned compounds. Replacement of thiocarbonyl in **23** by secondary amines (**34-36**) does not offer any advantageous in the term of activity. Chlorination of **2** into imodyl chloride (**29**) led to slight increasing in the activity. On the other hand the imodyl chloride product (**28**) was less potent than its parent **1**. Moreover, replacement of chlorine atom in **28** by different nucleophiles *i.e.* ethoxy and phenylhydrazide, resulted in **30-32**, which have shown higher activity. Finally, chemical transformation of **32** into **33** decreased the cytotoxic

effect. However, these compounds have emerged almost the same behavior, but in low extent, in terms of activity in case of HSV-2 (Tables 1 and 2).

Table 1. Substitution pattern in compounds **1-36**

Cpd	R	R^1	R^2
1	H	-	-
2	CH_3	-	-
3	H	-CH_2CH_3	-
4	CH_3	-CH_2CH_3	-
5	H	-$CH_2CH=CH_2$	-
6	CH_3	-$CH_2CH=CH_2$	-
7	H	*p*-Nitrobenzyl	-
8	CH_3	*p*-Nitrobenzyl	
9	H	-Benzyl	-
10	H	2-Pr-isoindole-1,3-dione	-
11	CH_3	2-Pr-isoindole-1,3-dione	-
12	CH_3	Et-piperidinyl	-
13	H	Et-piperidinyl	-
14	CH_3	Et-morphlinyl	-
15	H	Et-morphlinyl	-
16	H	2-Me-benzoimidazole	-
17	CH_3	2-Me-benzoimidazole	-
18	CH_3	4-Me-Benzonitile	-
19	H	4-Me-Benzonitile	-
20	H	3-Me-Benzonitile	-
21	CH_3	*p*-Chlorobenzyl	-
22	H	*p*-Chlorobenzyl	-
23	H	-	-
24	CH_3	-	-
25	H	-	*S*-allyl
26	H	-	*S*-phenyl
27	H	-	*S*-ethyl
28	H	-	-
29	CH_3	-	-
30	H	-	*O*-ethyl
31	CH_3		*O*-ethyl
32	H	-	Phenylhydrazide
33	H	-	-
34	H	-	Morphlino
35	H	-	Dimethylamino
36	H	-	Piperidino

Pr= propyl; Et= Ethyl; Me= Methyl

Based on statistical analyses and in terms of selective index SI, as a marker for antiviral activity, it can be concluded that compounds which had an SI-value of 2 or more can be labeled as active, while those of SI equals to 10 or greater are considered to be very active. Taken together of these documents and information in table 2, it can be sorting all investigated compounds as active or very active except for **1**, **8**, **9**, **21**, **22** and **31** that were considered as inactive compounds against HSV-1. In addition, six compounds are very active in the order of **26> 25> 27> 17> 16> 14**. The remaining products are considered to be active in the order of **15> 18> 19= 20> 12> 33> 3> 6= 13> 5> 4> 36> 32> 12> 10= 11> 2= 24> 23> 30> 34> 29> 7**. In case of the activity against HSV-2 virus and based on the same facts of sorting, eighteen compounds *i.e.* **6-10**, **13**, **21-23** and **28-36** could be considered as inactive, while the remaining eighteen compounds lie in the range of active ones in the order of **17> 25> 18> 14> 16> 20> 19> 11> 27= 1> 2> 15> 26> 3> 4= 5= 12> 25**.

According to cytopathic effect inhibition assay, the loss of infectivity on HSV-1 and HSV-2 viruses could be attributed to effect of the target samples on viral genome (DNA or RNA) or effect on the capsid protein of the tested viruses. Treatment with tested materials may lead to loss of virus infectivity i.e. the viral particles are not intact and some damages have been occurred to either viral genome or capsid proteins.

Table 2. Antiviral activity of **1-36** in terms of **CC_{50}, EC_{50}** and **SI** against **HSV-1** and **HSV-2** viruses

Cpd Nr.	CC_{50}	HSV-1		HSV-2	
		EC_{50}	SI	EC_{50}	SI
1	580	314	1.8	172	3.4
2	512	186	2.8	154	3.3
3	376	93	4.0	162	2.3
4	248	68	3.6	117	2.1
5	176	47.8	3.7	82.3	2.1
6	284	73.2	3.9	198	1.4
7	404	198	2.0	312	1.3
8	412	>500	-	>500	-
9	376	212	1.8	>500	-
10	314	108	2.9	176	1.8
11	286	97	2.9	82.3	3.5
12	362	68.4	5.3	176	2.1
13	416	108	3.9	214	1.9
14	324	24.6	13.2	67.3	4.8
15	286	31.8	9.0	93.7	3.1
16	302	21.8	13.9	72.4	4.2
17	508	32.8	15.5	64.2	7.9
18	436	51.4	8.5	82.6	5.3
19	386	70.8	5.5	103.2	3.7
20	344	62.4	5.5	90.3	3.8
21	375	>500	-	>500	-
22	397	>500	-	>500	-
23	156	58.4	2.7	96.2	1.6
24	178	63.2	2.8	88.4	2.0
25	284	10.9	26.1	36.8	7.7
26	186	6.8	27.4	63.4	2.9
27	378	18.2	20.8	112	3.4
28	324	>500	-	>500	-
29	332	156	2.1	>500	-
30	403	178	2.3	>500	-
31	316	184	1.7	>500	-
32	286	88.3	3.2	242	1.2
33	512	126	4.1	324	1.6
34	384	176	2.2	218	1.8
35	392	>500	-	>500	-
36	284	86.4	3.3	172	1.7
Acvr	600	1.8	333.3	3.4	176.5

Acvr= Acyclovir (reference drug), SI= CC_{50}/EC_{50}

Conclusion

Finally, in terms of SI-values six compounds are very active in the order of **26> 25> 27> 17> 16> 14** against HSV-1 relative to acyclovir as a reference drug. In addition, the highest active products against HSV-2 were found to be in the order of **17> 25> 18> 14> 16> 20> 19> 11> 27= 1> 2> 15> 26> 3> 4= 5= 12> 25.** Accordingly, it will be of importance and interest to pay more attention for further antiviral investigation of the first group of compounds (5-*S*-alkyl derivatives, **25-27**) and 4-*N*-alkylmorphlinyl and benzoimidazole derivatives (**14-17**). In addition, our group will pay strong attention to synthesis of new triazoloquina-zolines based on thio ethers and N-alkyls functional groups at 5- and 4-positions.

Acknowledgment

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group no **RGP- VPP-291**.

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9/16/2013