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Original article

Synthesis and antiviral activity of new pyrazole and thiazole derivatives

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ABSTRACT

New *N*-acetyl (**5**–**8**) and *N*-thiocarbamoyl (**9**–**12**) derivatives of 4,5-dihydropyrazole were synthesized starting from α,β-unsaturated ketones under the effect of hydrazine hydrate and thiosemicarbazide, respectively. *N*-Thiocarbamoylpyrazole derivatives (**9**–**12**) were cyclized using either ethyl bromoacetate or phenacyl bromides to afford the novel pyrazolothiazol-4(5*H*)-ones (**13**–**16**) or pyrazolothiazoles (**17**–**24**). The antiviral activity for such novel compounds against a broad panel of viruses in different cell cultures revealed that *N*-acetyl 4,5-dihydropyrazole **7** was the only active one at subtoxic concentrations against vaccinia virus (Lederle strain) in HEL cell cultures with a 50% effective concentration (EC_{50}) value of 7 μg/ml.

1. Introduction

Many diseases are indeed caused by the different members of DNA- and RNA-containing viruses. Amongst DNA-containing viruses, the herpes group of viruses, especially herpes simplex virus-1 (HSV-1) primarily causes stomatitis, encephalitis, ocular infections and herpes simplex virus-2 (HSV-2) primarily causes genital lesions or skin eruptions, cytomegalovirus (CMV) is associated with severe morbidity and mortality in patients at risk for disease because of immune system disabilities and varicella-zoster virus (VZV) is the ethiological agent of chickenpox and shingles [1].

Vesicular stomatitis virus (VSV), alphaviruses (e.g. sindbis virus), parainfluenza-3 virus, respiratory syncytial virus (RSV) and Influenza viruses (INF) are examples of enveloped single-stranded RNA-containing viruses. Vesicular stomatitis virus causes an economically important disease in cattle and horses [2]. Both parainfluenza-3 virus and respiratory syncytial virus (RSV) are an important cause of respiratory tract infections [3,4]. Human immunodeficiency virus (HIV) is a lentivirus causing the AIDS pandemic. This virus contains an RNA genome, but goes through a DNA intermediate during its infection cycle.

The treatment of viral infectious diseases still remains an important challenge because of the emergence of drug-resistant strains due to the rapid mutability of the virus [5,6].

Previous antiviral research has primarily focused on the development of nucleoside analogues but recently, nonnucleoside derivatives [7–9] have also received considerable attention. Among the nonnucleoside analogues, some novel pyrazoles; A [10,11], thiazolones; B [12] and thiazole derivatives especially BILS 179 BS; C [13] were reported to exhibit a high antiviral activity against hepatitis A virus, hepatitis C virus (HCV) and HSV, respectively.

Therefore, it is tempting to develop new nonnucleoside antiviral agents, however, pyrazole or pyrazolothiazolone derivatives which are structurally related to the aforementioned ones, in addition to a hybrid molecules containing both pyrazole and thiazole moieties with the aim to explore their potential antiviral activity for the first time against a broad panel of viruses.

2. Results and discussion

2.1. Chemistry

The reaction sequences employed for synthesis of the target pyrazole and thiazole derivatives are illustrated in Schemes 1 and 2.

The key chalcone intermediates (**1**–**4**) were synthesized through condensation of equimolar amounts of acetophenone derivatives and 4-benzyloxybenzaldehyde under basic catalyzed reaction through stirring the reactants in aqueous ethanolic solution containing 20% NaOH at room temperature for 24 h in accordance with the method described in the literature [14,15].

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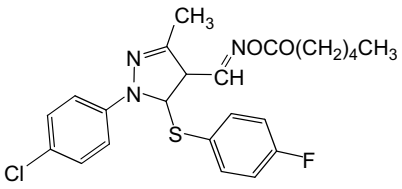
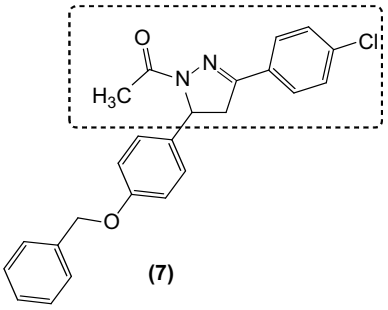


(A)



(7)

In the present work, two types of pyrazole derivatives were prepared utilizing different reaction conditions. In acidic media, the novel *N*-acetyl pyrazole derivatives (**5**–**8**) were prepared by heating at reflux the corresponding chalcone (**1**–**4**) with hydrazine hydrate in acetic acid for 4 h. *N*-Acetyl pyrazoles were obtained rather than the non-acetylated ones due to heating in glacial acetic acid for long periods of time (Scheme 1).

The presence of *N*-acetyl group was established using IR and ^{1}H NMR spectra whereas IR showed a strong absorption band at ν 1649 cm^{-1} for the carbonyl group, meanwhile, ^{1}H NMR spectra revealed the presence of singlet signal integrating three protons around $\delta = 2.40$ ppm due to the methyl group confirming its existence.

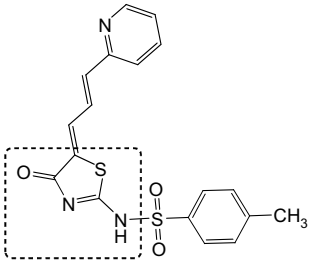
On the other side, upon using basic media, the novel 1-thiocarbamoyl pyrazole derivatives (**9**–**12**) were obtained by heating at reflux equimolar amounts of thiosemicarbazide and the corresponding α,β-unsaturated ketones (**1**–**4**) in hot ethanolic NaOH solution for 8 h (Scheme 1). These 1-thiocarbamoyl pyrazole derivatives were characterized using IR and ^{13}C NMR spectra whereas IR showed strong absorption bands at ν 3464–3303 cm^{-1} due to NH_2 group. Furthermore, ^{13}C NMR spectra displayed a signal at 176.55–176.90 ppm assignable to thiocarbamoyl carbon (C=S).

Moreover, the aforementioned 1-thiocarbamoyl pyrazole derivatives (**13**–**16**) were cyclized to either pyrazolothiazol-4(5*H*)-ones (**13**–**16**) or pyrazolothiazole derivatives (**17**–**24**) through their reaction with ethyl bromoacetate or phenacyl bromide derivatives [16] in hot ethanol for 1 h. The structures of the new thiazol-4(5*H*)-ones (**13**–**16**) were confirmed using IR spectra which showed strong absorption bands at ν 1696 cm^{-1} due to carbonyl group. In addition, ^{1}H NMR revealed the appearance of peaks (two doublets) around $\delta = 3.89$–3.99 ppm integrating two protons due to C_5–H of the thiazolone ring. ^{13}C NMR confirmed the proposed structure due to the appearance of signal at $\delta = 187.62$–187.67 ppm due to carbonyl carbon as well as the appearance of signal around $\delta = 39.04$–39.07 ppm assignable to C_5 of the thiazolone ring.

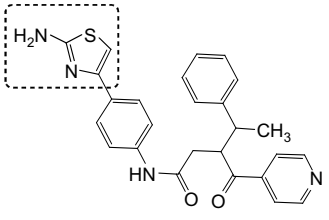
The structures of the new thiazole derivatives (**17**–**24**) were characterized using ^{1}H NMR spectra which revealed the presence of singlet peak at $\delta = 6.76$–6.99 ppm assignable to 5-thiazole H as well as ^{13}C NMR confirmed the proposed structure due to the appearance of characteristic peaks around $\delta = 178.0$ (azomethine carbon), 164.0 (C_2 of thiazole), 149.21–151.58 (C_4 of thiazole) and 102.39–105.15 ppm indicating the presence of thiazole ring.

Further evidence for the formation of 4,5-dihydropyrazole ring was obtained from ^{1}H and ^{13}C NMR spectra which provide diagnostic tools for the positional elucidation of the protons. The geminal pyrazoline protons at C_4 appeared in the region of 3.06–3.43 and 3.62–4.04 ppm as doublet of doublets in all compounds. The CH proton at C_5 also appears as doublet of doublets in the region of 5.51–6.05 ppm due to vicinal coupling with two non-magnetically equivalent geminal protons of C_4 carbon. The signals obtained from ^{13}C NMR spectra further confirmed the proposed structures; the C_4 and C_5 carbons of the pyrazoline ring resonate at 42.94–43.41 and 62.93–64.4 ppm, respectively. All compounds showed signals at 150.57–158.84 ppm, which was assignable to azomethine carbon of pyrazoline ring.

All the target compounds were characterized by using thin layer chromatography and melting point techniques. Both analytical and spectral data of all the compounds are in full agreement with the proposed structures. Moreover, comparison of the spectroscopic data of the new compounds with those of the previously reported analogues further confirmed the above structures.



(B)



(C)

2.2. Antiviral activity

The novel compounds **5**–**24** were evaluated against a broad panel of viruses in different types of cell cultures using cytopathicity (CPE) assay [17,18] and their activities were compared with the reference antiviral drugs (brivudin, ribavirin, cidofovir, ganciclovir, (*S*)-DHPA, DS-5000 and nevirapine).

HEL cell culture was used to evaluate compounds **5**–**24** against herpes simplex virus type 1 (KOS) [HSV-1 KOS], herpes simplex virus type 2 (G) [HSV-2G], vaccinia virus [VV], vesicular stomatitis virus [VSV] and thymidine kinase-deficient herpes simplex virus type 1 TK$^-$ KOS ACV^r [HSV-1 TK-KOS ACV^r] (Table 1).

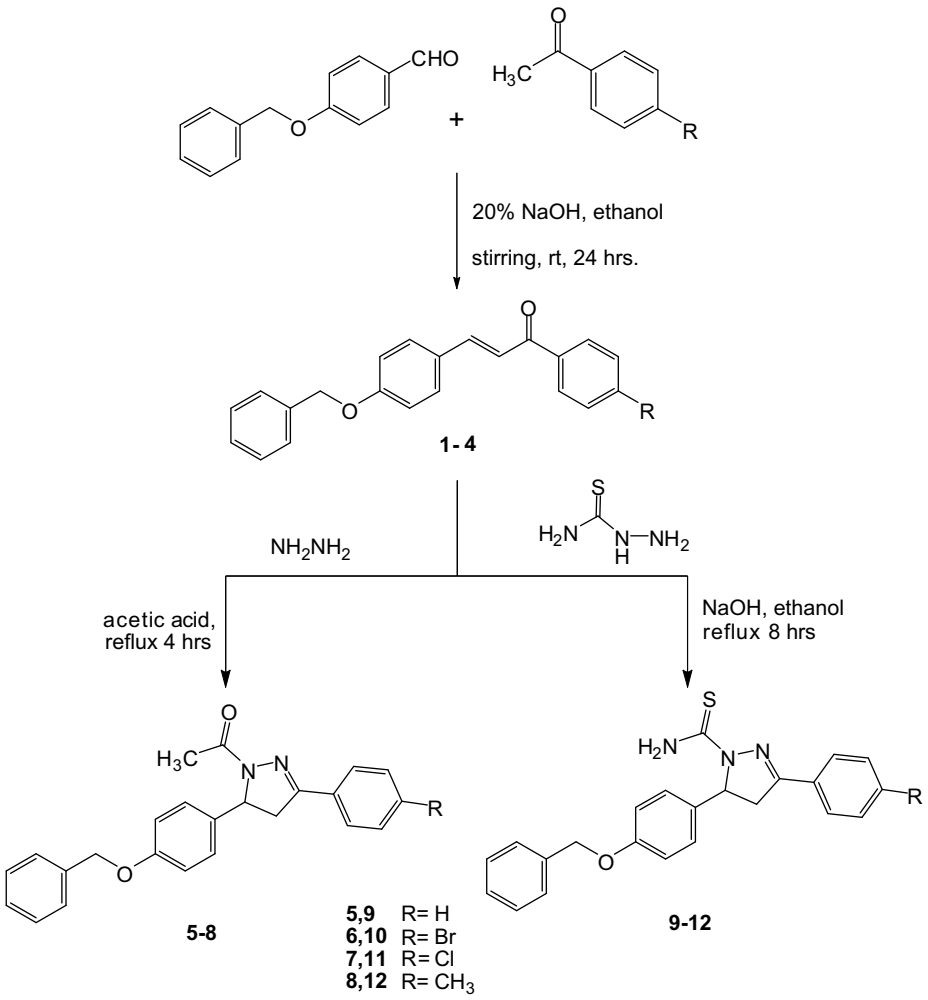
Moreover, the HeLa cell culture was utilized to test such compounds against vesicular stomatitis virus (VSV), coxsackie virus B4 [CV-B4] and respiratory syncytial virus [RSV] (Table 2).

Furthermore, the novel compounds **5**–**24** were evaluated in Vero cell cultures against parainfluenza-3 virus [PI-3V], reovirus-1 [RV-1], sindbis virus [SV], coxsackie virus B4 [CV-B4] and punta toro virus [PTV] (Table 3).

Finally, the same compounds were tested also against human immunodeficiency virus type 1 (HIV-1)(III_B) and HIV-2 (ROD) in MT-4 cell cultures.

The most interesting results from Table 1 are as below

- *N*-Acetyl pyrazole **7** was the most active one against vaccinia virus in HEL cell cultures with EC_{50} values in the range of 7 μg/ml and the selective index (SI, ratio of minimal cytotoxic



Scheme 1. Synthetic pathways to the novel *N*-acetyl (**5**–**8**) and *N*-thiocarbamoyl (**9**–**12**) pyrazoles.

concentration (MCC) to EC_{50} for virus replication) in the range of 3.6 to 14 depending on the cell line evaluated for toxicity.

- Regarding vesicular stomatitis virus, it was found that *N*-acetyl pyrazole **8** and the thiazolone **16** which bearing methyl substituent showed moderate activity with EC_{50} values in the range of 4 μg/ml and SI values in the range of 5 while all the references drugs displayed no selectivities because of their high cytotoxicity as shown in Table 1.
- The remainder of compounds exhibited the same activity with $EC_{50} = 20$ μg/ml against all the viruses in HEL cell culture (Table 1) except compounds **11**, **18**, **19**, **21** and the reference drug ribavirin whereas their antiviral activity levels were usually equal to their cytotoxic activity, thus, it is considered inactive.

Through analysis of Table 2, it was observed that *N*-acetyl pyrazole **8** ($EC_{50} = 4$ μg/ml) and *N*-thiocarbamoyl pyrazole **12** ($EC_{50} = 0.8$ μg/ml) exhibited moderate activity against vesicular stomatitis virus, coxsackie virus B4, respiratory syncytial virus in HeLa cell cultures. Compounds **5**, **7**, **9**, **11**, **14**, **18**, **19**, **21**–**24** and (*S*)-DHPA were active at a concentration which caused microscopically detectable alteration of normal cell morphology i.e. thus, these compounds were totally inactive because of their high cytotoxicity and poor selectivity as shown in Table 2.

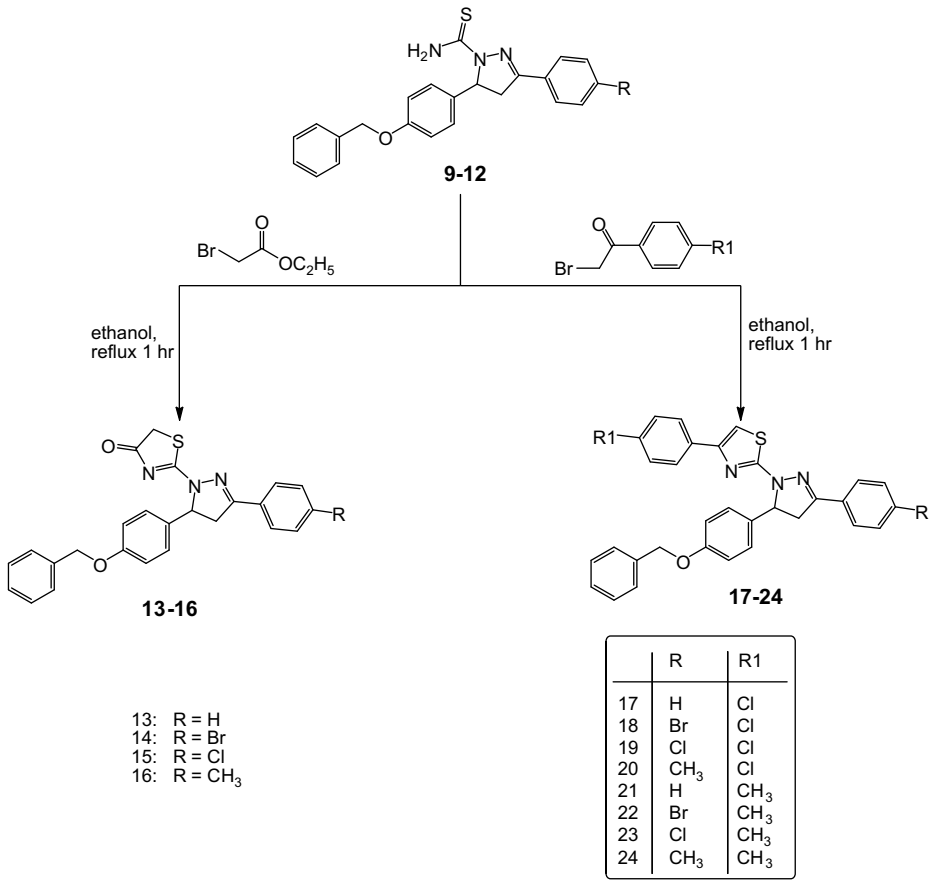
Moreover, compounds **8** and **12** with their EC_{50} values in the range of 0.8–4 μg/ml and SI equal to 5 were more active than other derivatives (Table 2) against coxsackie virus B4. Apparently, the higher activity for such compounds (**8**, **12**) may be due to they meet the structural requirements of the 'butterfly like' conformation with a central polar part and two lateral hydrophobic wings which seems particularly important in other known NNRTIs e.g. nevirapine and thiazolidones [7,19].

Table 3 revealed that *N*-acetyl pyrazoles **7**, **8** ($EC_{50} = 4$ μg/ml; $SI = 5$) were the most active ones against reovirus-1, sindbis virus, coxsackie virus B4 and punta toro virus in Vero cell cultures. Compounds **11**, **18**, **24**, DS-5000 and (*S*)-DHPA were considered inactive due to their antiviral activity levels were usually equal to the cytotoxic activity.

Based on the observed promising activity of compound **7** ($EC_{50} = 7$ μg/ml; $SI = 14$) against vaccinia virus in HEL cell cultures, we further evaluated this compound against several other poxviruses, including vaccinia virus strains Lister, Western Reserve and Copenhagen. Also, cowpox (Brighton strain) was included in the study. Unfortunately, none of these investigated poxviruses were found to be sensitive to the inhibitory activity of the 4,5-dihydropyrazole **7** at subtoxic concentrations.

3. Conclusion

In the search for new potentially antiviral agents, not only a series of 4,5-dihydropyrazole derivatives bearing either acetyl or thiocarbamoyl moieties but also a hybrid combining 4,5-dihydropyrazole with thiazolones or thiazoles have been synthesized



	R	R1
17	H	Cl
18	Br	Cl
19	Cl	Cl
20	CH_3	Cl
21	H	CH_3
22	Br	CH_3
23	Cl	CH_3
24	CH_3	CH_3

Scheme 2. Synthetic pathways to the novel pyrazolothiazol-4(5*H*)-ones (**13**–**16**) and pyrazolothiazoles (**17**–**24**).

and evaluated against a broad panel of viruses in different cell cultures. It was found that:

- Among the tested compounds, *N*-acetyl 4,5-dihydropyrazole **7** ($EC_{50} = 7$ μg/ml; SI = 3.6 to14) has shown the best antiviral activity against vaccinia virus in HEL cell cultures but unfortunately not active against other poxvirus strains including cowpox at subtoxic concentrations. Moreover, *N*-acetyl pyrazole **8** and the thiazolone **16** ($EC_{50} = 4$ μg/ml and SI = 5) showed a moderate activity against vesicular stomatitis virus in comparison with the used reference drugs (brivudin, ribavirin, cidofovir and ganciclovir; Table 1).
- In HeLa cell cultures, *N*-acetyl pyrazole **8** and *N*-thiocarbamoyl pyrazole **12** with their EC_{50} values in the range of 0.8–4 μg/ml and SI equal to 5 were more active than other derivatives against coxsackie virus B4 (Table 2).
- In Vero cell cultures, *N*-acetyl pyrazoles **7**, **8** ($EC_{50} = 4$ μg/ml, SI = 5) were the most active ones against reovirus-1, sindbis virus, coxsackie virus B4 and punta toro virus than the other derivatives (Table 3).

4. Experimental

4.1. Chemistry

4.1.1. General

Melting points were determined with a Gallenkamp melting point apparatus and are uncorrected. IR spectra (KBr, cm^{-1}) were recorded on Bruker or Testscan Shimadzu FT 8000 spectrometer. ^1H NMR (200 and 500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker AC 200 and Avance 500 MHz spectrometers in DMSO-d_6 or $CDCl_3$ as a solvent and Tetramethylsilane (TMS) as an internal standard (chemical shift in δ, ppm). Mass spectra were determined using a GC/MS Mat 112 S or GCMS-QP1000EX-*SHIMADZU* spectrometer with ionization energy 70 eV. Elemental analyses were determined using Heraeus and Vario EL-III (Elemntar), CHNS analyzer (Germany) at Microanalytical Center, Faculty of Science, University of Cairo, Egypt. All the results of elemental analyses corresponded to the calculated values within experimental error. TLC was performed on silica gel G (*Fluka*) and spots were visualized by iodine vapors or irradiation with UV light (254 nm). All the chemicals were purchased from *Sigma–Aldrich*. The chalcone intermediates (**1**–**4**) were synthesized in accordance with the method described in the literature [14,15]. Phenacyl bromide derivatives were prepared according to the reported method [16].

4.1.2. General procedure for synthesis of compounds *5*–*8*

The corresponding chalcones **1**–**4** (0.01 mol) were refluxed in acetic acid (20 ml) with hydrazine hydrate (0.04 mol) for 4 h. The mixture was cooled, poured into crushed ice and then neutralized by ammonia. The obtained product was washed by cold water and crystallized from an appropriate solvent.

*4.1.2.1. 1-Acetyl-5-(4-(benzyloxy)phenyl)-3-phenyl-4,5-dihydro-(1H)-pyrazole (**5**).* Yield: 60%; m.p.: 129–130 °C; crystallized from ethanol/H_2O; ^1H NMR (200 MHz, $CDCl_3$) δ = 2.41 (s, 3H, CH_3), 3.10–3.21 (dd, 1H, C_4–H of pyrazole), 3.65–3.80 (dd, 1H, C_4–H of pyrazole), 5.02 (s, 2H, benzylic H), 5.51–5.59 (dd, 1H, C_5–H of pyrazole), 6.89–7.75 (m, 14H, ArH) ppm. Analysis for $C_{24}H_{22}N_2O_2$, Calcd: C, 77.81; H, 5.99; N, 7.56; Found: C, 78.09; H, 6.04; N, 7.32.

Table 1
Cytotoxicity and antiviral activity of compounds **5**–**24** in HEL cell cultures.

Compound	Minimum cytotoxic concentration[a] (μg/ml)	EC_{50}[b] (μg/ml)				
		Herpes simplex virus-1 (KOS)	Herpes simplex virus-2 (G)	Vaccinia virus	Vesicular stomatitis virus	Herpes simplex virus-1 TK^- KOS ACV^r
5	100	>20	>20	>20	>20	>20
6	100	>20	>20	>20	>20	>20
7	100	>20	>20	7 ± 3	>20	>20
8	20	>4	>4	>4	>4	>4
9	100	>20	>20	>20	>20	>20
10	100	>20	>20	>20	>20	>20
11	>100	>100	>100	>100	>100	>100
12	100	>20	>20	>20	>20	>20
13	100	>20	>20	>20	>20	>20
14	100	>20	>20	>20	>20	>20
15	100	>20	>20	>20	>20	>20
16	20	>4	>4	>4	>4	>4
17	100	>20	>20	>20	>20	>20
18	>100	>100	>100	>100	>100	>100
19	>100	>100	>100	>100	>100	>100
20	100	>20	>20	>20	>20	>20
21	>100	>100	>100	>100	>100	>100
22	100	>20	>20	>20	>20	>20
23	100	>20	>20	>20	>20	>20
24	100	>20	>20	>20	>20	>20
Brivudin (μM)	>250	0.08	126	10	>250	>250
Ribavirin (μM)	>250	>250	>250	146	>250	>250
Cidofovir (μM)	>250	3	5	10	>250	5
Ganciclovir (μM)	>100	0.08	0.08	>100	>100	6

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
[b] Required to reduce virus-induced cytopathogenicity by 50%.

Table 2
Cytotoxicity and antiviral activity of compounds **5**–**24** in HeLa cell cultures.

Compound	Minimum cytotoxic concentration[a] (μg/ml)	EC_{50}[b] (μg/ml)		
		Vesicular stomatitis virus	Coxsackie virus B4	Respiratory syncytial virus
5	>100	>100	>100	>100
6	100	>20	>20	>20
7	≥20	>20	>20	>20
8	20	>4	>4	>4
9	≥20	>20	>20	>20
10	100	>20	>20	>20
11	>100	>100	>100	>100
12	4	>0.8	>0.8	>0.8
13	100	>20	>20	>20
14	>100	>100	>100	>100
15	100	>20	>20	>20
16	100	>20	>20	>20
17	100	>20	>20	>20
18	>100	>100	>100	>100
19	>100	45	>100	>100
20	100	>20	>20	>20
21	>100	>100	>100	>100
22	>100	>100	>100	>100
23	>100	>100	>100	>100
24	>100	>100	>100	>100
DS-5000	>100	>100	9	0.8
(*S*)-DHPA (μM)	>250	>250	>250	>250
Ribavirin (μM)	>250	29	146	10

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
[b] Required to reduce virus-induced cytopathogenicity by 50%.

Table 3
Cytotoxicity and antiviral activity of compounds **5**–**24** in Vero cell cultures.

Compound	Minimum cytotoxic concentration[a] (μg/ml)	EC_{50}[b] (μg/ml)				
		Parainfluenza-3 virus	Reovirus-1	Sindbis virus	Coxsackie virus B4	Punta toro virus
5	100	>20	>20	>20	>20	>20
6	100	>20	>20	>20	>20	>20
7	20	>4	>4	>4	>4	>4
8	20	>4	>4	>4	>4	>4
9	100	>20	>20	>20	>20	>20
10	100	>20	>20	>20	>20	>20
11	>100	>100	>100	>100	>100	>100
12	100	>20	>20	>20	>20	>20
13	100	>20	>20	>20	>20	>20
14	100	>20	>20	>20	>20	>20
15	100	>20	>20	>20	>20	>20
16	100	>20	>20	>20	>20	>20
17	100	>20	>20	>20	>20	>20
18	>100	>100	>100	>100	>100	>100
19	100	>20	>20	>20	>20	>20
20	100	>20	>20	>20	>20	>20
21	100	>20	>20	>20	>20	>20
22	100	>20	>20	>20	>20	>20
23	100	>20	>20	>20	>20	>20
24	>100	>100	>100	>100	>100	>100
DS-5000	>100	>100	>100	59	>100	100
(*S*)-DHPA (μM)	>250	>250	>250	>250	>250	>250
Ribavirin (μM)	>250	45	>250	>250	>250	146

[a] Required to cause a microscopically detectable alteration of normal cell morphology.
[b] Required to reduce virus-induced cytopathogenicity by 50%.

*4.1.2.2. 1-Acetyl-5-(4-(benzyloxy)phenyl)-3-(4-bromophenyl)-4,5-dihydro-(1*H*)-pyrazole (**6**).* Yield: 70%; m.p.: 124–125 °C; crystallized from benzene/pet.-ether (60–80); ^{1}H NMR (200 MHz, $CDCl_3$) $\delta = 2.40$ (s, 3H, CH_3), 3.06–3.17 (dd, 1H, C_4–H of pyrazole), 3.62–3.77 (dd, 1H, C_4–H of pyrazole), 5.03 (s, 2H, benzylic H), 5.51–5.60 (dd, 1H, C_5–H of pyrazole), 6.90–7.63 (m, 13H, ArH) ppm. Analysis for $C_{24}H_{21}BrN_2O_2$, Calcd: C, 64.15; H, 4.71; N, 6.23; Found: C, 64.33; H, 4.52; N, 6.00.

*4.1.2.3. 1-Acetyl-5-(4-(benzyloxy)phenyl)-3-(4-chlorophenyl)-4,5-dihydro-(1*H*)-pyrazole (**7**).* Yield: 57%; m.p.: 131–132 °C; crystallized from ethanol; IR: $\nu = 3040$ (CH, aromatic), 2919 (CH, aliphatic), 1649 (C═O), 1585 (C═N), 1512 (C═C) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) $\delta = 2.37$ (s, 3H, CH_3), 3.06–3.17 (dd, 1H, C_4–H of pyrazole), 3.61–3.76 (dd, 1H, C_4–H of pyrazole), 5.02 (s, 2H, benzylic H), 5.49–5.55 (dd, 1H, C_5–H of pyrazole), 6.88–7.70 (m, 13H, ArH) ppm; MS: *m*/*z* (rel. int.) = 406 ($M^+ + 2$, 2.2), 405 ($M^+ + 1$, 2.4), 404 (M^+, 5.1), 362 (5.8), 267 (11.7), 91 (100.0). Analysis for $C_{24}H_{21}ClN_2O_2$, Calcd: C, 71.19; H, 5.23; N, 6.92; Found: C, 71.39; H, 5.22; N, 6.59.

*4.1.2.4. 1-Acetyl-5-(4-(benzyloxy)phenyl)-3-(4-methylphenyl)-4,5-dihydro-(1*H*)-pyrazole (**8**).* Yield: 75%; m.p.: 94–95 °C; crystallized from pet.-ether (60–80); ^{1}H NMR (200 MHz, $CDCl_3$) $\delta = 2.16$ (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.06–3.17 (dd, 1H, C_4–H of pyrazole), 3.61–3.76 (dd, 1H, C_4–H of pyrazole), 5.00 (s, 2H, benzylic H), 5.48–5.56 (dd, 1H, C_5–H of pyrazole), 6.87–7.75 (m, 13H, ArH) ppm; MS: *m*/*z* (rel. int.) = 384 (M^+, 20.5), 343 (19.8), 251 (14.8), 91 (100.0). Analysis for $C_{25}H_{24}N_2O_2$, Calcd: C, 78.10; H, 6.29; N, 7.29; Found: C, 78.39; H, 6.01; N, 6.94.

*4.1.3. General procedure for synthesis of compounds **9**–**12***

To a suspension of chalcones **1**–**4** (0.01 mol) and sodium hydroxide (0.025 mol) in ethanol (50 ml), thiosemicarbazide (0.01 mol) was added. The mixture was refluxed for 8 h. The products were poured into crushed ice and the solid mass which separated out was filtered, dried and crystallized from an appropriate solvent.

*4.1.3.1. 5-(4-(Benzyloxy)phenyl)-3-phenyl-1-thiocarbamoyl-4,5-dihydro-1H-pyrazole (**9**).* Yield: 40%; m.p.: 205–206 °C; crystallized from toluene; IR: $\nu = 3440$, 3280 (NH_2), 3064 (CH, aromatic), 2931 (CH, aliphatic), 1579 (C=N), 1513 (C=C), 1334 (C=S) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): $\delta = 3.20–3.30$ (dd, 1H, C_4–H of pyrazole), 3.80–3.95 (dd, 1H, C_4–H of pyrazole), 5.05 (s, 2H, benzylic H), 5.95–6.05 (dd, 1H, C_5–H of pyrazole), 6.95–7.80 (m, 16H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 176.76$ (C=S), 158.30 (C–O), 156.04 (C=N), 136.99–115.14 (phenyl-C), 70.06 (OCH_2), 63.02 (C_5 of pyrazole), 43.11 (C_4 of pyrazole) ppm. Analysis for $C_{23}H_{21}N_3OS$, Calcd: C, 71.29; H, 5.46; N, 10.84; Found: C, 71.56; H, 5.30; N, 10.68.

*4.1.3.2. 5-(4-(Benzyloxy)phenyl)-3-(4-bromophenyl)-1-thiocarbamoyl-4,5-dihydro-1H-pyrazole (**10**).* Yield: 50%; m.p.: 226–227 °C; crystallized from toluene; IR: $\nu = 3463$, 3301 (NH_2), 3031 (CH, aromatic), 2900 (CH, aliphatic), 1577 (C=N), 1511 (C=C), 1336 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.16–3.21$ (dd, 1H, C_4–H of pyrazole), 3.78–3.84 (dd, 1H, C_4–H of pyrazole), 5.05 (s, 2H, benzylic H), 6.01–6.04 (dd, 1H, C_5–H of pyrazole), 6.94–7.62 (m, 15H, ArH + NH_2) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 176.90$ (C=S), 158 (C–O), 154.90 (C=N), 136.93–115.18 (phenyl-C), 70.07 (OCH_2), 63.16 (C_5 of pyrazole), 42.94 (C_4 of pyrazole) ppm. MS: m/z (rel. int.) = 466 (M^+, 0.00), 465 ($M^+ - 1$, 15.6), 432 (10.5), 283 (21.1), 193 (26.1), 91 (100.0). Analysis for $C_{23}H_{20}BrN_3OS$, Calcd: C, 59.23; H, 4.32; N, 9.01; Found: C, 59.50; H, 4.49; N, 8.88.

*4.1.3.3. 5-(4-(Benzyloxy)phenyl)-3-(4-chlorophenyl)-1-thiocarbamoyl-4,5-dihydro-1H-pyrazole (**11**).* Yield: 45%; m.p.: >300 °C; crystallized from toluene; IR: $\nu = 3463$, 3301 (NH_2), 3050 (CH, aromatic), 2900 (CH, aliphatic), 1577 (C=N), 1511 (C=C), 1336 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.35–3.39$ (dd, 1H, C_4–H of pyrazole), 3.91–4.00 (dd, 1H, C_4–H of pyrazole), 5.07 (s, 2H, benzylic H), 5.75–5.78 (dd, 1H, C_5–H of pyrazole), 6.93–7.71 (m, 15H, ArH + NH_2) ppm. Analysis for $C_{23}H_{20}ClN_3OS$, Calcd: C, 65.47; H, 4.78; N, 9.96; Found: C, 65.82; H, 4.79; N, 10.38.

*4.1.3.4. 5-(4-(Benzyloxy)phenyl)-3-(4-methylphenyl)-1-thiocarbamoyl-4,5-dihydro-1H-pyrazole (**12**).* Yield: 56%; m.p.: 171–172 °C; crystallized from toluene; IR: $\nu = 3464$, 3303 (NH_2), 3049 (CH, aromatic), 2969 (CH, aliphatic), 1577 (C=N), 1511 (C=C), 1335 (C=S) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 2.42$ (s, 3H, CH_3), 3.18–3.22 (dd, 1H, C_4–H of pyrazole), 3.78–3.84 (dd, 1H, C_4–H of pyrazole), 5.04 (s, 2H, benzylic H), 5.99–6.02 (dd, 1H, C_5–H of pyrazole), 6.94–7.40 (m, 15H, ArH + NH_2) ppm. ^{13}C NMR ($CDCl_3$) $\delta = 176.55$ (C=S), 158.27 (C–O), 156.19 (C=N), 141.57–115.11 (phenyl-C), 70.06 (OCH_2), 62.93 (C_5 of pyrazole), 43.14 (C_4 of pyrazole), 21.56 (CH_3) ppm. Analysis for $C_{24}H_{23}N_3OS$, Calcd: C, 71.79; H, 5.77; N, 10.47; Found: C, 72.02; H, 5.74; N, 10.21.

*4.1.4. General procedure for the synthesis of compounds **13**–**17***

To a suspension of compounds **9**–**12** (0.01 mol) in ethanol (20 ml), ethyl bromoacetate (0.01 mol) was added and heated at reflux for 1 h. After cooling, the separated product was filtered and washed. The product was crystallized from appropriate solvent.

*4.1.4.1. 2-(5-(4-(Benzyloxy)phenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)thiazol-4(5H)-one (**13**).* Yield: 50%; m.p.: 193–194 °C; crystallized from ethanol/H_2O; IR: $\nu = 3060$ (CH, aromatic), 2915 (CH, aliphatic), 1704 (C=O), 1569 (C=N), 1538 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.39–3.43$ (dd, 1H, C_4–H of pyrazole), 3.86–3.97 (dd, 1H, C_4–H of pyrazole), 3.91–3.96 (two d, 2H, C_5–H of thiazolone), 5.05 (s, 2H, benzylic H), 5.75–5.78 (dd, 1H, C_5–H of pyrazole), 6.92–7.84 (m, 14H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 187.67$ (C=O), 178.29 (C=N, thiazolone), 159.63 (C–O), 158.84 (C=N, pyrazole), 136.78–115.32 (phenyl-C), 70.10 (OCH_2), 63.57 (C_5 of pyrazole), 43.30 (C_4 of pyrazole), 39.04 (C_5 of thiazolone) ppm. Analysis for $C_{25}H_{21}N_3O_2S$, Calcd: C, 70.24; H, 4.95; N, 9.83; Found: C, 70.55; H, 5.26; N, 9.64.

*4.1.4.2. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)thiazol-4(5H)-one (**14**).* Yield: 57%; m.p.: 225–226 °C; crystallized from toluene; IR: $\nu = 3051$ (CH, aromatic), 2912 (CH, aliphatic), 1696 (C=O), 1590 (C=N), 1524 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.34–3.38$ (dd, 1H, C_4–H of pyrazole), 3.86–3.88 (dd, 1H, C_4–H of pyrazole), 3.90–3.99 (two d, 2H, C_5–H of thiazolone), 5.04 (s, 2H, benzylic H), 5.75–5.78 (dd, 1H, C_5–H of pyrazole), 6.93–7.69 (m, 13H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 187.62$ (C=O), 178.41 (C=N, thiazolone), 158.89 (C–O), 158.56 (C=N, pyrazole), 136.74–115.36 (phenyl-C), 70.11 (OCH_2), 63.72 (C_5 of pyrazole), 43.17 (C_4 of pyrazole), 39.07 (C_5 of thiazolone) ppm. Analysis for $C_{25}H_{20}BrN_3O_2S$, Calcd: C, 59.29; H, 3.98; N, 8.30; Found: C, 59.28; H, 4.00; N, 7.85.

*4.1.4.3. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)thiazol-4(5H)-one (**15**).* Yield: 51%; m.p.: 235–236 °C; crystallized from ethanol; IR: $\nu = 3052$ (CH, aromatic), 2913 (CH, aliphatic), 1695 (C=O), 1590 (C=N), 1525 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.34–3.38$ (dd, 1H, C_4–H of pyrazole), 3.85–3.86 (dd, 1H, C_4–H of pyrazole), 3.89–3.94 (two d, 2H, C_5–H of thiazolone), 5.04 (s, 2H, benzylic H), 5.75–5.78 (dd, 1H, C_5–H of pyrazole), 6.93–7.76 (m, 13H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 187.62$ (C=O), 178.38 (C=N, thiazolone), 158.88 (C–O), 158.48 (C=N, pyrazole), 137.74–115.16 (phenyl-C), 70.10 (OCH_2), 63.72 (C_5 of pyrazole), 43.23 (C_4 of pyrazole), 39.07 (C_5 of thiazolone) ppm. Analysis for $C_{25}H_{20}ClN_3O_2S$, Calcd: C, 65.00; H, 4.36; N, 9.10; Found: C, 65.32; H, 4.49; N, 9.36.

*4.1.4.4. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-methylphenyl)-4,5-dihydro-1H-pyrazol-1-yl)thiazol-4(5H)-one (**16**).* Yield: 52%; m.p.: 151–152 °C; crystallized from ethanol; IR: $\nu = 3050$ (CH, aromatic), 2917 (CH, aliphatic), 1693 (C=O), 1590 (C=N), 1526 (C=C) cm^{-1}; ^1H NMR (200 MHz, DMSO-d_6): $\delta = 2.43$ (s, 3H, CH_3), 3.48, 3.97 (two s, 2H, C_4–H of pyrazole), 4.04–4.18 (two d, 2H, C_5–H of thiazolone), 5.12 (s, 2H, benzylic H), 5.75–5.80 (d, 1H, C_5–H of pyrazole), 7.03–7.82 (m, 13H, ArH) ppm; MS: m/z (rel. int.) = 442 ($M^+ + 1$, 38.1), 441 (M^+, 66.7), 296 (19.1), 91 (100.0). Analysis for $C_{26}H_{23}N_3O_2S$, Calcd: C, 70.72; H, 5.25; N, 9.52; Found: C, 70.79; H, 5.23; N, 9.43.

*4.1.5. General procedures for the synthesis of compounds **17**–**24***

To a suspension of compounds **9**–**12** (0.01 mol) in ethanol (20 ml), phenacyl bromide derivatives (0.01 mol) were added and heated at reflux for 1 h. After cooling, the obtained product was collected by filtration and crystallized from an appropriate solvent.

*4.1.5.1. 2-(5-(4-(Benzyloxy)phenyl)-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-chlorophenyl)thiazole (**17**).* Yield: 60%; m.p.: 141–142 °C; crystallized from dioxane/H_2O; IR: $\nu = 3032$ (CH, aromatic), 2926 (CH, aliphatic), 1585 (C=N), 1510 (C=C) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.33–3.38$ (dd, 1H, C_4–H of pyrazole), 3.87–3.93 (dd, 1H, C_4–H of pyrazole), 5.06 (s, 2H, benzylic H), 5.61–5.65 (dd, 1H, C_5–H of pyrazole), 6.80 (s, 1H, C_5–H of thiazole), 6.97–7.80 (m, 18H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 165.08$ (C_2 of thiazole), 158.39 (C–O), 151.73 (C=N, pyrazole), 150.42 (C_4 of thiazole), 136.93–114.97 (phenyl-C), 103.67 (C_5 of thiazole), 70.06 (OCH_2), 64.23 (C_5 of pyrazole), 43.41 (C_4 of pyrazole) ppm. Analysis for $C_{31}H_{24}ClN_3OS$, Calcd: C, 71.32; H, 4.63; N, 8.05; Found: C, 71.39; H, 4.67; N, 7.77.

*4.1.5.2. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-chlorophenyl)thiazole (**18**).* Yield: 72%; m.p.: 191–192 °C; crystallized from dioxane/H_2O; ^1H NMR (500 MHz,

$CDCl_3$): $\delta = 3.29–3.34$ (dd, 1H, C_4–H of pyrazole), 3.85–3.89 (dd, 1H, C_4–H of pyrazole), 5.06 (s, 2H, benzylic H), 5.62–5.65 (dd, 1H, C_5–H of pyrazole), 6.81 (s, 1H, C_5–H of thiazole), 6.96–7.65 (m, 17H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 164.81$ (C_2 of thiazole), 158.45 (C–O), 150.57 (C═N, pyrazole), 150.47 (C_4 of thiazole), 136.89–115.01 (phenyl-C), 103.81 (C_5 of thiazole), 70.06 (OCH_2), 64.4 (C_5 of pyrazole), 43.19 (C_4 of pyrazole) ppm. Analysis for $C_{31}H_{23}BrClN_3OS$, Calcd: C, 61.96; H, 3.86; N, 6.99; Found: C, 62.20; H, 4.08; N, 7.04.

*4.1.5.3. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-chlorophenyl)thiazole (**19**).* Yield: 71%; m.p.: 194–195 °C; crystallized from dioxane/H_2O; ^{1}H NMR (500 MHz, DMSO-d_6): $\delta = 3.34–3.39$ (dd, 1H, C_4–H of pyrazole), 3.98–4.04 (dd, 1H, C_4–H of pyrazole), 5.06 (s, 2H, benzylic H), 5.63–5.67 (dd, 1H, C_5–H of pyrazole), 6.99–7.81 (m, 18H, ArH + C_5–H of thiazole) ppm; ^{13}C NMR (DMSO) $\delta = 164.21$ (C_2 of thiazole), 157.78 (C–O), 151.93 (C═N, pyrazole), 149.21 (C_4 of thiazole), 136.99–114.70 (phenyl-C), 105.15 (C_5 of thiazole), 69.17 (OCH_2), 63.88 (C_5 of pyrazole), 42.77 (C_4 of pyrazole) ppm. Analysis for $C_{31}H_{23}Cl_2N_3OS$, Calcd: C, 66.91; H, 4.17; N, 7.55; Found: C, 67.19; H, 4.15; N, 7.37.

*4.1.5.4. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-methylphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-chlorophenyl)thiazole (**20**).* Yield: 71%; m.p.: 168–169 °C; crystallized from ethyl acetate/petroleum ether (3:2); ^{1}H NMR (500 MHz, DMSO-d_6): $\delta = 2.36$ (s, 3H, CH_3), 3.34–3.42 (dd, 1H, C_4–H of pyrazole), 3.97–4.03 (dd, 1H, C_4–H of pyrazole), 5.06 (s, 2H, benzylic H), 5.60–5.64 (dd, 1H, C_5–H of pyrazole), 6.99–7.76 (m, 18H, ArH + C_5–H of thiazole) ppm; ^{13}C NMR (DMSO) $\delta = 164.0$ (C_2 of thiazole), 157.0 (C–O), 152.5 (C═N, pyrazole), 151.0 (C_4 of thiazole), 137.0–114.69 (phenyl-C), 104.88 (C_5 of thiazole), 69.17 (OCH_2), 63.55 (C_5 of pyrazole), 43.00 (C_4 of pyrazole), 21.01 (CH_3)ppm. MS: *m/z* (rel. int.) = 537 ($M^+ + 1$, 42.2), 536 (M^+, 32.9), 535 (90.4), 444 (16.7), 327 (11.1), 236 (13.7), 173 (10.2), 91 (100.0). Analysis for $C_{32}H_{26}ClN_3OS$, Calcd: C, 71.69; H, 4.89; N, 7.84; Found: C, 71.75; H, 4.75; N, 7.55.

*4.1.5.5. 2-(5-(4-(Benzyloxy)phenyl)-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-methyl phenyl)thiazole (**21**).* Yield: 77%; m.p.: 167–168 °C; crystallized from dioxane/H_2O; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 2.36$ (s, 3H, CH_3), 3.33–3.38 (dd, 1H, C_4–H of pyrazole), 3.87–3.93 (dd, 1H, C_4–H of pyrazole), 5.05 (s, 2H, benzylic H), 5.64–5.68 (dd, 1H, C_5–H of pyrazole), 6.78 (s, 1H, C_5–H of thiazole), 6.96–7.80 (m, 18H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 164.88$ (C_2 of thiazole), 158.33 (C–O), 151.61 (C═N, pyrazole), 151.42 (C_4 of thiazole), 137.12–114.89 (phenyl-C), 102.53 (C_5 of thiazole), 70.04 (OCH_2), 64.17 (C_5 of pyrazole), 43.31 (C_4 of pyrazole), 21.27 (CH_3) ppm. Analysis for $C_{32}H_{27}N_3OS$, Calcd: C, 76.62; H, 5.43; N, 8.38; Found: C, 76.52; H, 5.41; N, 8.33.

*4.1.5.6. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-bromophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-methylphenyl)thiazole (**22**).* Yield: 80%; m.p.: 188–190 °C; crystallized from ethyl acetate; IR: $\nu = 3050$ (CH, aromatic), 2917 (CH, aliphatic), 1585 (C═N), 1510 (C═C) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 2.40$ (s, 3H, CH_3), 3.28–3.33 (dd, 1H, C_4–H of pyrazole), 3.82–3.88 (dd, 1H, C_4–H of pyrazole), 5.05 (s, 2H, benzylic H), 5.64–5.68 (dd, 1H, C_5–H of pyrazole), 6.78 (s, 1H, C_5–H of thiazole), 6.96–7.65 (m, 17H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 164.62$ (C_2 of thiazole), 158.41 (C–O), 151.69 (C═N, pyrazole), 150.25 (C_4 of thiazole), 137.18–114.97 (phenyl-C), 102.66 (C_5 of thiazole), 70.07 (OCH_2), 64.38 (C_5 of pyrazole), 43.08 (C_4 of pyrazole), 21.24 (CH_3)ppm. Analysis for $C_{32}H_{26}BrN_3OS$, Calcd: C, 66.20; H, 4.51; N, 7.24; Found: C, 66.30; H, 4.51; N, 7.40.

*4.1.5.7. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-chlorophenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-methylphenyl)thiazole (**23**).* Yield: 79%; m.p.: 183–184 °C; crystallized from ethyl acetate; ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 2.36$ (s, 3H, CH_3), 3.28–3.33 (dd, 1H, C_4–H of pyrazole), 3.82–3.88 (dd, 1H, C_4–H of pyrazole), 5.05 (s, 2H, benzylic H), 5.64–5.68 (dd, 1H, C_5–H of pyrazole), 6.79 (s, 1H, C_5–H of thiazole), 6.96–7.72 (m, 17H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 164.65$ (C_2 of thiazole), 158.41 (C–O), 151.68 (C═N, pyrazole), 150.21 (C_4 of thiazole), 137.17–114.96 (phenyl-C), 102.64 (C_5 of thiazole), 70.07 (OCH_2), 64.37 (C_5 of pyrazole), 43.15 (C_4 of pyrazole), 21.23 (CH_3) ppm. Analysis for $C_{32}H_{26}ClN_3OS$, Calcd: C, 71.69; H, 4.89; N, 7.84; Found: C, 71.52; H, 5.00; N, 7.25.

*4.1.5.8. 2-(5-(4-(Benzyloxy)phenyl)-3-(4-methylphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-4-(4-methylphenyl)thiazole (**24**).* Yield: 78%; m.p.: 177–178 °C; crystallized from ethyl acetate/petroleum ether (3:2); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 2.36$ (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 3.31–3.35 (dd, 1H, C_4–H of pyrazole), 3.84–3.90 (dd, 1H, C_4–H of pyrazole), 5.05 (s, 2H, benzylic H), 5.61–5.65 (dd, 1H, C_5–H of pyrazole), 6.76 (s, 1H, C_5–H of thiazole), 6.96–7.69 (m, 17H, ArH) ppm; ^{13}C NMR ($CDCl_3$) $\delta = 164.99$ (C_2 of thiazole), 158.32 (C–O), 151.62 (C═N, pyrazole), 151.58 (C_4 of thiazole), 139.93–114.91 (phenyl-C), 102.39 (C_5 of thiazole), 70.07 (OCH_2), 64.12 (C_5 of pyrazole), 43.40 (C_4 of pyrazole), 21.48 (CH_3), 21.23 (CH_3) ppm. Analysis for $C_{33}H_{29}N_3OS$, Calcd: C, 76.86; H, 5.67; N, 8.15; Found: C, 77.02; H, 5.64; N, 7.73.

4.2. Antiviral activity evaluation

4.2.1. Antiviral assays

The antiviral activity of the new compounds **5**–**24** was determined using cytopathicity (CPE) assay [17] against herpes simplex virus type 1 (KOS), herpes simplex virus type 2 (G), vaccinia virus, vesicular stomatitis virus, herpes simplex virus-1 TK^- KOS ACV^r in HEL cell cultures; vesicular stomatitis virus (VSV), coxsackie virus B4, respiratory syncytial virus in HeLa cell cultures; parainfluenza-3 virus, reovirus-1, sindbis virus, coxsackie virus B4, punta toro virus in Vero cell cultures. Stock solutions of the test compounds were prepared in DMSO at a concentration of 10 mg/ml. Cells, grown to confluency in 96-well plates, were infected with 100 $CCID_{50}$ of virus, one $CCID_{50}$ being the 50%-cell culture infective dose. After an adsorption period of 2 h at 37 °C, virus was removed and serial dilutions of the compounds were added. The cultures were further incubated at 37 °C for 3 days, until complete CPE was observed in the infected and untreated virus control. The determination of the anti-HIV activity of the compounds was based on virus-induced cytopathicity (destruction) of HIV-infected MT-4 cells, measured at day 5 post virus infection by the MTT colorimetric method [18]. The results were expressed as the 50% effective concentration (EC_{50}) as shown in Tables 1–3. The 50% effective antiviral concentration (EC_{50}) was defined as the compound concentration required to protect 50% of the virus-infected cells against viral cytopathogenicity. The symbol "$>$" is used to indicate the highest concentration at which the compounds were tested and found not to be active.

4.2.2. Cytotoxicity assays

The cytotoxicity of the compounds was evaluated in parallel with their antiviral activity in uninfected cell cultures, and is expressed as the minimum cytotoxic concentration (MCC) that causes a microscopically detectable alteration of normal cell morphology (HEL, HeLa and Vero cells).

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