Synthesis and *in vitro* Study of Antiviral and Virucidal Activity of Novel 2-[(4-Methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide Derivatives

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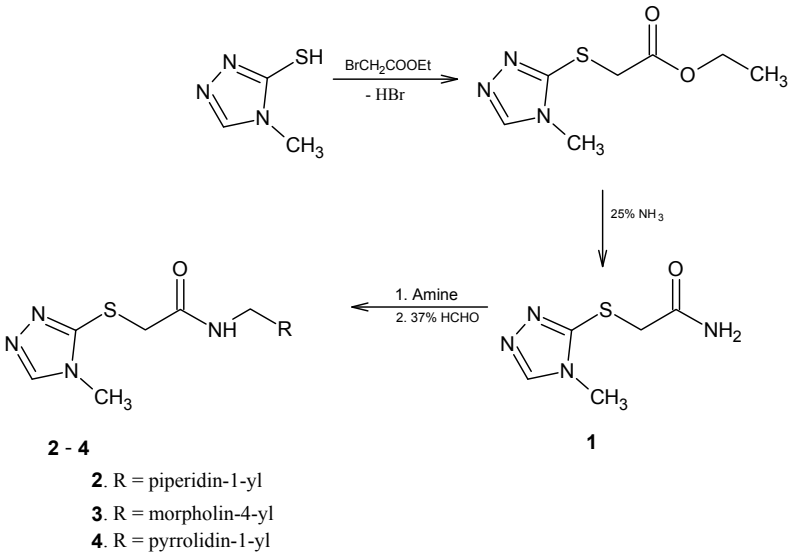
2-[(4-Methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide derivatives were synthesized and their structures were confirmed by ^{1}H NMR, IR, and elemental analysis. Cytotoxicity of the compounds towards HEK-293 and GMK cells was evaluated. Moreover, the antiviral and virucidal activities of these compounds against human adenovirus type 5 and ECHO-9 virus were assessed. Some of the newly synthesized derivatives have the potential to reduce the viral replication of both tested viruses.

Key words: Antiviral Agents, ECHO-9 Virus, Adenovirus-5

Introduction

The significant step in antiviral therapy development was the introduction of ribavirin [1-(β-D-ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide] into medical practice. This small and simple, from a structural point of view, molecule appeared to have an extremely wide antiviral activity spectrum, with respect to both DNA viruses (Sidwell *et al.*, 1972; Markland *et al.*, 2000) and RNA viruses (Hruska *et al.*, 1980; Neyts *et al.*, 1996; Jordan *et al.*, 2000; Crotty *et al.*, 2000). It is known, that ribavirin inhibits the activities of many enzymes, like inosine monophosphate dehydrogenases and viral RNA polymerases (Crotty *et al.*, 2002; Parker, 2005). The inhibition of these enzymes causes a block in viral replication. The 1,2,4-triazole scaffold, contained in the molecule, is essential for the pharmacological properties of ribavirin. Thus, further research concentrated on the triazole structure in other antiviral drugs (Zhu *et al.*, 2008; Kirschberg *et al.*, 2008; Küçükgüzel *et al.*, 2008). Smith and coworkers (1997) at the *Glaxo Wellcome* company introduced the triazole structure into zanamivir (a drug of the neuraminidase inhibitor group) and obtained new derivatives having promising antiviral effects on influenza viruses. Then scientists at the *Valeant* company, working on a relatively new group of drugs that are non-nucleoside reverse transcriptase inhibitors (NNRTIs) by virtue of the triazole moiety, synthesized a set of compounds having pharmacological activity similar to efavirenz (used in AIDS treatment and considered to be the current gold standard for NNRTIs) (De La Rosa *et al.*, 2006). The last two years have brought another breakthrough in antiviral therapy development. Food and Drug Administration (FDA) authorized the first drug from the group of HIV integrase inhibitors – raltegravir (Evering and Markowitz, 2008; Emery and Winston, 2009). The immense hopes related to this group of drugs stimulated present research on the use of the 1,2,4-triazole scaffold as a part of HIV integrase inhibitors (Johns *et al.*, 2009). Taking into consideration the pharmacological usefulness of the 1,2,4-triazole moiety for antiviral activity, we decided to investigate the activities of our newly synthesized compounds in relation to DNA virus (adenovirus type 5) and RNA virus (ECHO-9 virus). ECHO-9 virus is considered to be an etiological factor of aseptic meningitis as well as of infections of the alimentary tract and respiratory system. Adenovirus type 5 is one of the causes of life-threatening respiratory infections of infants and children under 5 years of age (WHO, 1989). Although other respiratory viruses



Scheme 1. Synthetic route to compounds **1**–**4**.

are more frequently isolated, adenovirus is possibly the most aggressive viral agent and is responsible for many deaths from pneumonia (Abzug *et al.*, 1990).

Results and Discussion

The route of the synthesis of compounds **1**–**4** is depicted in Scheme 1. The 2-[(4-methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide derivatives **1**–**4** were obtained using a three-step reaction in which 4-methyl-4*H*-1,2,4-triazole-3-thiol was used as a starting reagent. The first step involves the nucleophilic attack of the thiol group of 4-methyl-4*H*-1,2,4-triazole-3-thiol to the bromine-bearing C atom of ethyl bromoacetate. Next, the ethyl [(4-methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl] acetate was treated with ammonia solution to obtain compound **1**. Finally, the key intermediate **1** was converted into derivatives **2**–**4** by the treatment of **1** with various amines in the presence of 37% formaldehyde solution. Compounds **1**–**4** were characterized by their spectral and physical data. The IR and ^{1}H NMR spectral data are consistent with the proposed structures. The IR spectrum of compound **1** showed the presence of sharp absorption peaks at 3306 cm^{-1} and 3154 cm^{-1} (amide NH_2) and at 1684 cm^{-1} (amide C=O). On the contrary, single bands at 3196 cm^{-1} (for **4**), 3294 cm^{-1} (for **2**), and at 3282 cm^{-1} (for **3**) for the N-H bond were observed due to conversion of **1** into the respective N-substituted amides. In the ^{1}H NMR spectra, the formation of the title compounds was confirmed by the appearance of two singlets at 7.20 ppm and 7.64 ppm for two protons of the –$CONH_2$ group in derivative **1** and a triplet in the range of 8.39–8.49 ppm for one proton of the –NH-CH_2– group in the other described compounds.

After 72 h of incubation, the effect of the title compounds on cell cultures (HEK-293 and GMK) was evaluated (Tables I and II). Compound **2** at a concentration range of 2.5–100 μg/mL, compounds **1** and **3** at concentrations of 2.5–200 μg/mL, and compound **4** at the concentration of 2.5–50 μg/mL were non-toxic towards HEK-293 cells. Compounds **1**–**3** were non-toxic against GMK cells in the concentration range of 2.5–200 μg/mL, and compound **4** was non-toxic at 2.5–100 μg/mL.

The results presented in Tables I and II clearly indicate that the cytotoxicity of compound **4** exceeds that of the others. Antiviral and virucidal activities of the title compounds were examined only at concentrations non-toxic to HEK-293 and GMK cells.

Evaluation of the virucidal activity showed that only compound **1** was totally inactive against human adenovirus type 5 (Ad-5) at all concentrations tested (Table III). The other three compounds caused a decrease in the titer of viruses

Table I. The effect of compounds **1**–**4** on the viability of HEK-293 cells. The results are presented as the percentage of cell viability of HEK-293 cells in comparison to the control. Exposure time was 72 h.

Concentration[a] [μg/mL]	Cell viability (%)[b]			
	1	**2**	**3**	**4**
100	100.00 ± 0.00	92.25 ± 5.24	100.00 ± 0.00	67.85 ± 2.46
200	93.45 ± 5.25	45.65 ± 4.85	95.00 ± 4.60	49.70 ± 3.76
500	12.25 ± 2.70	16.00 ± 2.27	29.25 ± 3.50	12.60 ± 2.01

[a] Up to 50 μg/mL cell viability was affected by none of the compounds.
[b] Given as mean ± SD of three independent experiments.

Table II. The effect of compounds **1**–**4** on the viability of GMK cells. The results are presented as the percentage of cell viability of GMK cells in comparison to the control. Exposure time was 72 h.

Concentration[a] [μg/mL]	Cell viability (%)[b]			
	1	**2**	**3**	**4**
100	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	96.65 ± 2.09
200	100.00 ± 0.00	98.00 ± 2.78	97.30 ± 2.04	56.00 ± 4.62
500	76.00 ± 3.58	13.30 ± 2.86	61.20 ± 8.28	9.00 ± 2.62

[a] Up to 50 μg/mL cell viability was affected by none of the compounds.
[b] Given as mean ± SD of three independent experiments.

by 0.23–1.38 log (9–53%). The Ad-5 virus was sensitive to compound **2** at the concentration of 100 μg/mL, with 29.14% reduction in the viral titer (that is by 0.463 log). The level of inhibition of the Ad-5 virus titer for all concentrations of derivative **3** was found to be ~36% (0.765 log), while for derivative **4** it was 53.26% (1.379 log) at a concentration of 50 μg/mL. All tested triazole derivatives affirmed virucidal activity against the ECHO-9 virus. Compound **2**, at the concentration of 200 μg/mL, was found to reduce the viral titer by 58.53% (1.589 log). Compounds **1** and **3** reduced the ECHO-9 virus titer by 34.34% (0.589 log). Compound **4** had moderate virucidal activity against the ECHO-9 virus (18.68% ≙ 0.278 log reduction in viral titer at a concentration of 100 μg/mL). Antiviral activities are shown in Table IV. Compounds **3** and **4** were found to reduce the replication of human Ad-5 by 47.04% (1.0 log), at all applied concentrations (10–50 μg/mL for **4**, 100–200 μg/mL for **3**).

The ECHO-9 virus was moderately susceptible to the four derivatives. Compound **2** at a concentration of 200 μg/mL, **1** at a concentration of 50 μg/mL, and **4** at a concentration of 100 μg/mL caused a decrease in the titer by 22.4%, 14.35%, and 11.09%, respectively (0.495 log, 0.228 log, 0.151 log). Compound **3** had no antiviral activity against ECHO-9 virus.

Conclusions

The aim of our study was to synthesize some new triazoles as potential antiviral and virucidal agents. The results suggest that substitution of the carboxamide nitrogen atom has significant impact on the antiviral and virucidal activities of triazoles. Compound **1**, without substituents at the amide nitrogen atom, was inactive. The N-substituted compound most active towards the Ad-5 virus was 2-[(4-methyl-4*H*-1,2,4-triazol-3-yl) sulfanyl]-*N*-(pyrrolidin-1-ylmethyl)acetamide (**4**). It has the five-membered pyrrolidine ring at the carboxamide nitrogen atom while compounds **2** and **3** have six-membered rings. It is possible that the size of a ring and chemical properties of the nitrogen atom of the amide group are an important factor for the virucidal and antiviral activity of this class of derivatives. These results motivate us to conduct further studies towards the under-

Table III. Virucidal activity of compounds **1**–**4**.

Concentration [μg/mL]	Inhibition of viral titers[a]							
	Ad-5				ECHO-9			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
0 (control)	1.589 ± 0.244	1.589 ± 0.244	2.126 ± 0.266	2.589 ± 0.244	1.715 ± 0.259	2.715 ± 0.255	1.715 ± 0.259	1.488 ± 0.239
10	-[b]	-	-	2.362 ± 0.241	-	-	-	-
25	-	1.361 ± 0.241	-	2.362 ± 0.241	-	-	-	1.488 ± 0.239
50	1.589 ± 0.244	1.361 ± 0.241	1.361 ± 0.241	1.21 ± 0.255	1.126 ± 0.266	2.362 ± 0.241	1.126 ± 0.266	1.488 ± 0.239
100	1.589 ± 0.244	1.126 ± 0.266	1.361 ± 0.241	-	1.126 ± 0.266	2.715 ± 0.255	1.488 ± 0.239	1.21 ± 0.255
200	1.589 ± 0.244	-	1.361 ± 0.241	-	1.361 ± 0.241	1.126 ± 0.266	1.488 ± 0.239	-

[a] The virus titers are shown as log ± SD. [b] – Not determined.

Table IV. Antiviral activity of compounds **1**–**4**.

Concentration [μg/mL]	Inhibition of viral titers[a]							
	Ad-5				ECHO-9			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
0 (control)	1.21 ± 0.255	1.21 ± 0.255	2.126 ± 0.266	2.126 ± 0.266	1.589 ± 0.244	2.21 ± 0.255	1.589 ± 0.244	1.361 ± 0.241
10	-[b]	-	-	1.126 ± 0.266	-	-	-	-
25	-	1.488 ± 0.239	-	1.126 ± 0.266	-	-	-	1.488 ± 0.239
50	1.21 ± 0.255	1.21 ± 0.255	1.589 ± 0.244	1.126 ± 0.266	1.361 ± 0.241	2.21 ± 0.255	1.589 ± 0.244	1.361 ± 0.241
100	1.21 ± 0.255	1.361 ± 0.241	1.126 ± 0.266	-	1.589 ± 0.244	2.21 ± 0.255	1.589 ± 0.244	1.21 ± 0.255
200	1.488 ± 0.239	-	1.126 ± 0.266	-	1.589 ± 0.244	1.715 ± 0.259	1.589 ± 0.244	-

[a] The virus titers are shown as log ± SD. [b] – Not determined.

standing of the mode of action of our newly synthesized compounds.

Experimental

General

All reagents were purchased from Lancaster (Ward Hill, USA) and Merck Co. (Darmstadt, Germany) and used without further purification. Melting points were determined in a Fisher-Johns block (Fisher Scientific, Schwerte, Germany) and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance 250 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) in DMSO-d_6 with TMS as an internal standard. Chemical shifts are given in ppm (δ scale). The IR spectra were recorded in KBr using a Specord IR-75 spectrophotometer (Carl Zeiss, Jena, Germany). Purity of all compounds was checked by TLC on plates precoated with silica gel Si 60 F_{254} (Merck Co.) eluted with CH_3Cl/C_2H_5OH (10:1, v/v) as solvent system. The spots were detected by exposure to a UV lamp at 254 nm. Elemental analyses were performed on an AMZ 851 CHX analyser (PG, Gdańsk, Poland), and the results were within ± 0.4% of the theoretical value.

*Preparation of 2-[(4-methyl-4H-1,2,4-triazol-3-yl)-sulfanyl]acetamide (**1**)*

To the suspension of anhydrous potassium carbonate (9 g) and 4-methyl-4*H*-1,2,4-triazole-3-thiol (3.45 g, 0.03 mol) in 50 mL of anhydrous acetone, ethyl bromoacetate (3.4 mL, 0.03 mol) was added. The mixture was refluxed for 24 h. Next, inorganic residues were filtered off, and the filtrate was distilled under reduced pressure to give the appropriate ethyl ester. The obtained product was treated with 25% aqueous ammonia solution (10 mL), and the mixture was left at room temperature for 2 d.

The resulting solid was filtered off, washed with diethyl ether, and recrystallized from 95% ethanol.

Yield 82%. – M.p. 178–180 °C. – ^{1}H NMR (250 MHz, DMSO-d_6): δ = 3.59 (s, 3H, CH_3), 3.81 (s, 2H, CH_2), 7.20, 7.64 (2 s, 2H, NH_2, exchangeable with D_2O), 8.54 (s, 1H, CH). – IR (KBr): ν = 3306, 3154 (NH_2), 1684 (C=O), 1524 (C=N), 2933, 1408, 696 cm^{-1} (CH aliphatic). – $C_5H_8N_4OS$ (172.21): calcd. C 34.87, H 4.68, N 32.53; found C 34.99, H 4.51, N 32.39.

*Preparation of 2-[(4-methyl-4H-1,2,4-triazol-3-yl)-sulfanyl]-N-(piperidin-1-ylmethyl)acetamide (**2**)*

To the flask containing 0.005 mol of 2-[(4-methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide and 0.01 mol of piperidine in 10 mL methanol, 0.015 mol of 37% formaldehyde solution was added. The reaction mixture was kept at room temperature for 14 d. Next, the solvent was evaporated under reduced pressure. The residue was washed with hexane and diethyl ether. The product was purified by crystallization from ethanol.

Yield 87%. – M.p. 88–89 °C. – ^{1}H NMR (250 MHz, DMSO-d_6): δ = 1.31 – 1.47 (m, 6H, 3 x CH_2, piperidine), 2.31 (t, 4H, 2 x CH_2, J = 5.72 Hz, piperidine), 3.59 (s, 3H, CH_3), 3.81 (s, 2H, CH_2), 3.86 (d, 2H, CH_2, J = 6.1 Hz), 8.39 (t, 1H, NH, J = 5.6 Hz, exchangeable with D_2O), 8.54 (s, 1H, CH). – IR (KBr): ν = 3294 (NH), 1687 (C=O), 1514, 1552 (C=N), 2931, 1421, 696 cm^{-1} (CH aliphatic). – $C_{11}H_{19}N_5OS$ (269.37): calcd. C 49.05, H 7.11, N 26.00; found C 49.17, H 7.24, N 25.91.

*Preparation of 2-[(4-methyl-4H-1,2,4-triazol-3-yl)-sulfanyl]-N-(morpholin-4-ylmethyl)acetamide (**3**)*

To a flask containing 0.005 mol of 2-[(4-methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide and 0.01 mol of morpholine in 10 mL methanol, 0.015 mol of 37% formaldehyde solution was added. The reaction mixture was refluxed for 3 h. After cooling, the solvent was evaporated under reduced pressure. The residue was washed with acetonitrile and hexane, and the formed product was crystallized from ethanol.

Yield 77%. – M.p. 129–130 °C. – ^{1}H NMR (250 MHz, DMSO-d_6): δ = 2.35 [t, 4H, $(CH_2)_2N$, J = 4.61 Hz, morpholine], 3.52 [t, 4H, $(CH_2)_2O$, J = 4.63 Hz, morpholine], 3.59 (s, 3H, CH_3), 3.80 (s, 2H, CH_2), 3.88 (d, 2H, CH_2, J = 6.0 Hz), 8.49 (t, 1H, NH, J = 5.6 Hz, exchangeable with D_2O), 8.54 (s, 1H, CH). – IR (KBr): ν = 3282 (NH), 1673 (C=O), 1519, 1540 (C=N), 2958, 1407, 698 cm^{-1} (CH aliphatic). – $C_{10}H_{17}N_5O_2S$ (271.34): calcd. C 44.26, H 6.31, N 25.81; found C 44.42, H 6.45, N 25.65.

*Preparation of 2-[(4-methyl-4H-1,2,4-triazol-3-yl)-sulfanyl]-N-(pyrrolidin-1-ylmethyl)acetamide (**4**)*

To a flask containing 0.005 mol of 2-[(4-methyl-4*H*-1,2,4-triazol-3-yl)sulfanyl]acetamide and 0.01 mol of pyrrolidine in 10 mL methanol, 0.015

mol of 37% formaldehyde solution was added. The reaction mixture was refluxed for 3 h. After cooling, the solvent was evaporated under reduced pressure. The residue was washed with acetonitrile and the formed product was crystallized from ethanol.

Yield 81%. – M.p. 90–92 °C. – ^{1}H NMR (250 MHz, DMSO-d_6): δ = 1.51–1.60 (m, 4H, 2 x CH_2, pyrrolidine), 2.33–2.42 (m, 4H, 2 x CH_2, pyrrolidine), 3.58 (s, 3H, CH_3), 3.83 (s, 2H, CH_2), 3.99 (d, 2H, CH_2, J = 6.0 Hz), 8.46 (t, 1H, NH, J = 5.6 Hz, exchangeable with D_2O), 8.53 (s, 1H, CH). – IR (KBr): ν = 3196 (NH), 1677 (C=O), 1525, 1544 (C=N), 2928, 1397, 697 cm^{-1} (CH aliphatic). – $C_{10}H_{17}N_5OS$ (255.40): calcd. C 47.04, H 6.71, N 27.43; found C 47.28, H 6.81, N 27.59.

Cells and viruses

HEK-293 (human embryonic kidney) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) (ATCC CRL-1573) and GMK (green monkey kidney) cells from the Serum and Vaccine Production Plant Ltd. (Biomed, Lublin, Poland), respectively. HEK-293 cells were grown in Eagle's Minimal Essential Medium (MEM; Sigma, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Sigma) while for GMK cells the Eagle 1959 medium supplemented with 10% calf serum (Biomed) was used. 100 U/mL of penicillin (Polfa, Tarchomin, Poland) and 100 μg/mL of streptomycin (Polfa) were added to the media. The cell cultures were incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2. The strains of human adenovirus type 5 (Ad-5) (ATCC VR-1516) and ECHO-9 virus (ATCC VR-1050) were obtained from the American Type Culture Collection and were propagated in HEK-293 cells and GMK cells, respectively. Final concentration of the viruses' suspension was $2 \cdot 10^4$ $TCID_{50}$/mL for Ad-5 and $5 \cdot 10^5$ $TCID_{50}$/mL for ECHO-9, where $TCID_{50}$ is the median tissue culture infective dose. Viruses were stored at –70 °C until used.

Cytotoxicity assay

All investigated compounds were dissolved in dimethyl sulfoxide (10 mg/mL) and then diluted in cell culture media supplemented with 2% FBS. GMK and HEK-293 cells were placed into 96-well plastic plates (Nunc, Roskilde, Denmark) at a cell density of $2 \cdot 10^4$ cells per well. After 24 h of incubation at 37 °C, the media were removed and cells treated with the derivatives, diluted in media at final concentrations of 500, 200, 100, 50, 25, 10, 5, and 2.5 μg/mL. Cell cultures were incubated at 37 °C for 72 h. The cytotoxicity was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that is cleaved into a coloured formazan product by metabolically active cells, according to the assay previously described by Takenouchi and Munekata (1998). The quantity of the formazan product was measured at 540 nm and 620 nm in an automatic plate reader. The results were given as mean ± SD of three independent experiments.

Virucidal activity

For evaluation of the virucidal activity of the investigated compounds, concentrations non-toxic to the two cell cultures were applied. At the applied concentration, dimethyl sulfoxide, used as a solvent, was not toxic to any of the cell cultures or the viruses. Viral suspensions were mixed with solutions of the derivatives (1:1, v/v) which had been diluted in media without FBS to the appropriate final concentrations. Mixtures were incubated at 37 °C for 1 h, and then viruses were titrated in the appropriate cell cultures (Ad-5 in the HEK-293 line, ECHO-9 in the GMK line). The suspensions of Ad-5 and ECHO-9 with media but without a derivative, were used as control. The cytopathic effect (CPE) of each virus observed after 24 h of incubation was measured using a software package by Spouge (2010). The results were given as mean ± SD of three independent experiments.

Antiviral activity assay

GMK and HEK-293 cells were infected with 100 $TCID_{50}$ of virus (ECHO-9 and Ad-5, respectively). After incubation at 37 °C for 1 h, the inoculum was removed and replaced with medium supplemented with 2% FBS in either the absence (control group) or presence (at appropriate concentrations) of the tested compounds. Infected cells were incubated at 37 °C for 48 h and then frozen to disrupt them and release intracellular virions. Next, viruses were titrated and the CPE was detected by light microscopy and calculated using a software package by Spouge (2010). The results were given as mean ± SD of three independent experiments.

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