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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Biological evaluation of novel 1,4-dithiine derivatives as potential antimicrobial agents

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Abstract The preparation of twelve aminoalkanol derivatives of 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione was described. Newly obtained compounds, as well as their propyl and butyl analogues, were evaluated in vitro against selected viruses. Selected derivatives were tested for their antibacterial and antifungal activity. Compounds **3h**, **3j**, **4b** and **5a–d** showed moderate to significant protections against CVB-2, HSV-1 and YFV viruses. The molecular structures of **4a**, **5c** and **5g** were determined by an X-ray analysis.

Keywords 1,4-Dithiine · X-ray analysis · Antiviral activity · Conformations

Introduction

Literature survey shows that sulphur play significant role in enhancement of biological activity (Tandon *et al.*, 2005, 2006). Sulphur-containing heterocyclic rings, such as thiazole, isothiazole, thiadiazole, thiazepine, tiophene, or benzothiazole are nuclei of antiviral (Vicini *et al.*, 2003; Venkatachalam *et al.*, 2004; Wang *et al.*, 2006; Mercorelli *et al.*, 2009; Struga *et al.*, 2009), antibacterial (Foroumadi *et al.*, 2005; Bozdag-Dündar *et al.*, 2007; Prasad and Kishore, 2007; Stefańska *et al.*, 2009; Tandon *et al.*, 2009) and antifungal (Stefańska *et al.*, 2009) agents. Recently, derivatives of other heterocycle, 1,4-dithiine, were found as the new class of antimicrobial agents with activity against broad spectrum of Gram-positive and Gram-negative pathogens and fungal strains (Zentz *et al.*, 2005). Sulphur atom could be also incorporated in nonaromatic part of the drug molecule, forming sulfanyl (Nawrozkij *et al.*, 2008) or thiol (Hadizadeh and Mehrparvar, 2004; Ahn *et al.*, 2006) groups. There is an increasing concern on thioureas as some of them also have been reported as antiviral agents, e.g. against HIV-1 (Venkatachalam *et al.*, 2004; Ranise *et al.*, 2003; Küçükgüzel *et al.*, 2008).

The anti-HIV-1 agent in a group of reverse transcriptase inhibitors (RTIs), delavirdine, as well as protease inhibitor nelfinavir, are sulphonoamido and phenylthio derivatives, respectively. Their activity is also connected with the presence of pyridinylpiperazine or 2-hydroxypropyl fragments (Fig. 1). Literature survey revealed that modifications of their structures lead to new potential antiviral drugs (Wang *et al.*, 2006; Pinna *et al.*, 2001; Di Santo *et al.*, 2002; Ragno *et al.*, 2004).

N-(4-Arylpiperazin-1-yl-alkyl) substituted derivatives of cyclic imides are the largest group of ligands of serotonergic 5-HT_{1A} receptors. According to pharmacophore model of 5-HT_{1A} receptor proposed by Chilmonczyk *et al.* (1997) and Bronowska's group calculations (Bronowska *et al.*, 2006), a folded conformer of a ligand promotes high affinity for the 5-HT_{1A} receptor. In the solid state buspirone, the free base as well as its hydrochloride, exist as entended conformers (Chilmonczyk *et al.*, 1995; Kozioł *et al.*, 2006). Theoretical calculations indicated that the fully extended conformations are those of minimum energy

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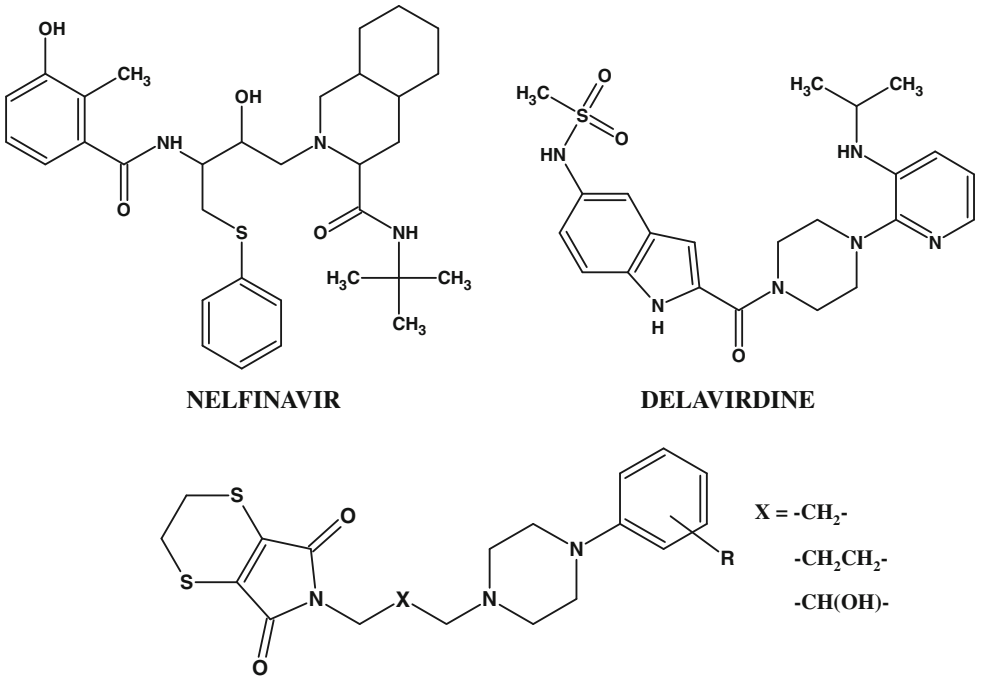


Fig. 1 Chemical structures of nelfinavir, delaviridine and 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione derivatives considered in this study

(Chilmonczyk *et al.*, 1995). The conformationally flexible *n*-butoxyl moiety of other neuroleptic aripiprazole adopts either folded or extended molecular conformation. Both structures are equally stable (Tessler and Goldberg, 2006).

In our previous studies (Stefańska *et al.*, 2009; Stefańska *et al.*, 2010), we presented strongly hydrophobic aromatic compounds with nitrogen-containing imide part that have shown good antimicrobial activity. This study reports biological activity of compounds purposely designed to combine the heterocyclic sulphur containing ring of 1,4-dithiine with aminoalkanol or alkyl linker bearing different substituents. Cytotoxicicty as well as activity of selected compounds against virus, bacteria and fungi were examined.

Results and discussion

Chemistry

Series of aminoalkanol and alkyl derivatives of 1,4-dithiine was designed and synthesized, in order to check the impact of different substituents at N-4 on antimicrobial activity. The starting material for the synthesis was 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione (**2**), prepared from its anhydride. It was subjected to the reaction with 2-(chloromethyl)oxirane in anhydrous medium to give oxirane 3. Next the semiproduct was reacted with short-chain or cyclic amines, as well as with piperazine derivatives giving amino alcohol derivatives **3a**–**3k** (Fig. 2). The second route of the synthesis was alkylation of imide **2** with 1,3-dibromopropane or 1,4-dibromobutane, in which the respective bromoalkyl derivatives were obtained. Next, they were condensed with appropriate arylpiperazines to give derivatives **4a**–**4d** and **5a**–**5g** (Table 1). The structure of all newly synthesized compounds have been established on the basis of elemental analysis, ^{1}H-NMR and MS. For biochemical studies free bases were converted into their hydrochlorides.

Molecular structure

The crystal and molecular structure of **4a**, **5c** and **5g** have been determined (Fig. 3). The torsion angle values describing the alkyl chain conformation indicate that in the solid state the *n*-alkyl linker of **4a** and **5g** adopts the folded conformation, whereas the compound **5c** exists as an extended conformer. Rigid fragments of the molecules, i.e. the imide, piperazine, and aryl groups have varying relative orientations due to linker flexibility (Fig. 4). In the crystal structures of **4a**, **5c** and **5g** different intermolecular bonding motifs are observed depending on the number of proton donor/acceptor groups. The strongest intermolecular interactions are formed in the crystal **5g** where the hydroxyl groups participate as donors in the O–H···$N_{piperazine}$ hydrogen bonds. In the crystal of **5c**, the molecules form layers via intermolecular C–H···X (X = O, N, S) weak hydrogen bonds. Molecules of **4a** are connected by C–H···π interactions between piperazine and pyrimidine fragments to give centrosymmetric dimers.

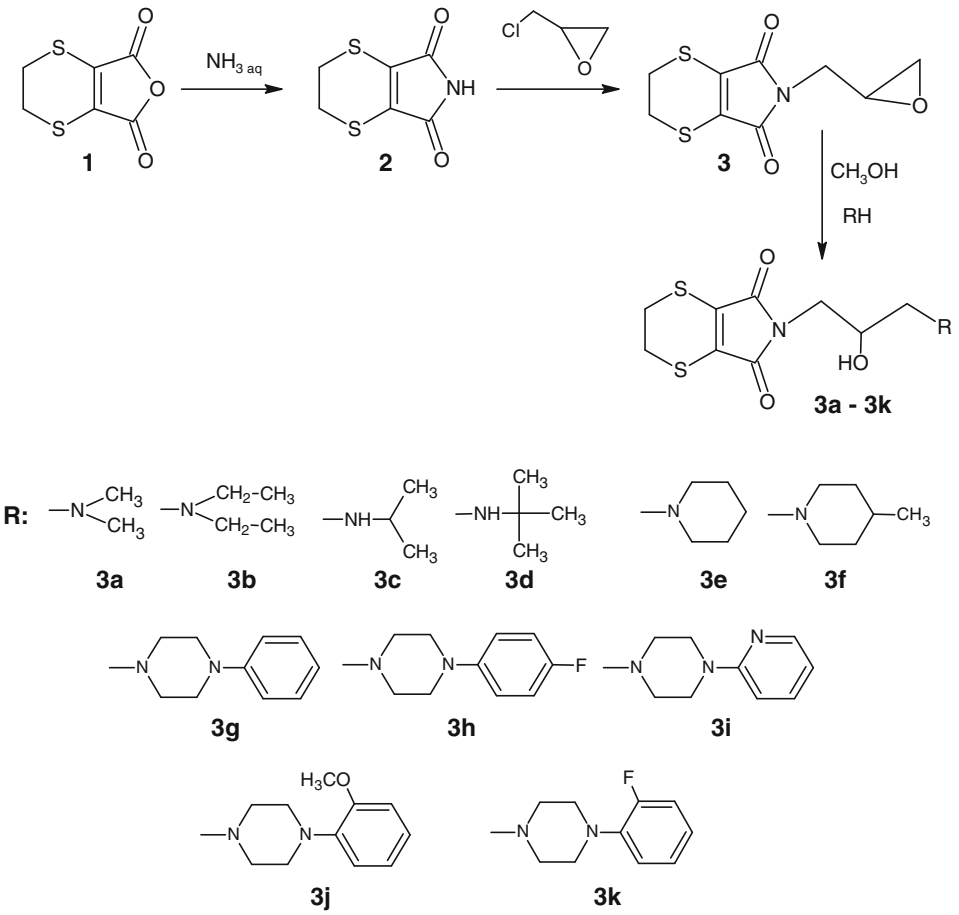


Fig. 2 Method of preparation of compounds **1**, **2**, **3**, **3a**–**3k**

Antimicrobial activity

Twenty two compounds, derivatives of 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione, were evaluated in vitro against viruses, and 17 of them were tested against bacteria and fungi.

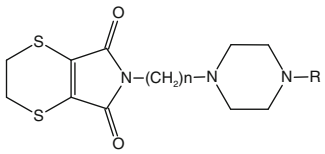
Title compounds were evaluated for antiviral activity against viruses representative of two of the three genera of Flaviviridae family, that is, *Flaviviruses* (*Yellow Fever Virus*, YFV) and *Pestiviruses* (*Bovine Viral Diarrhoea Virus*, BVDV), as *Hepaciviruses* can hardly be used in routine cell-based assays. Compounds were also tested against representatives of other virus families. Among ssRNA+ were a retrovirus (*human immunodeficiency virus type 1*, HIV-1) and two *Picornaviruses* (*Coxsackie Virus* type B2, CVB-2 and *Poliovirus type-1*, Sabin strain, Sb-1); among ssRNA− were a Rhabdoviridae (*Vesicular Stomatitis Virus*, VSV) representative. Among double-stranded RNA (dsRNA) viruses was a Reoviridae representative (Reo-1). Two representatives of DNA virus families were also included: *Herpes Simplex type-1*, HSV-1 (Herpesviridae), and *Vaccinia Virus*, VV (Poxviridae).

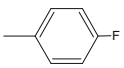
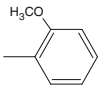
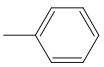
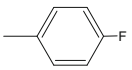
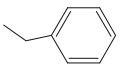
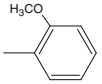
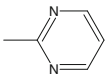
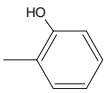
In addition to the antiviral activity, compounds were evaluated in vitro against representative strains of Gram-positive and Gram-negative bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*), yeasts and moulds (*Candida albicans* and *Aspergillus niger)*.

AZT (3′-azido-thymidine), NM 108 (2′-ß-methyl-guanosine), NM 176 (2′-ethynyl-D-citidine), M 5255 (mycophenolic acid) and ACG (acycloGuanosine) were used as reference inhibitors of ssRNA+, ssRNA− and DNA viruses, respectively.

Seven of tested derivatives presented antiviral activity (Tables 2, 3), which varied from moderate (compounds **3h**, **3j**, **5c**, **5d**) to significant (**4b**, **5a**, **5b**). The data may serve as a good subject for discussion of the influence of molecular structure of a compound on its biological activity. It seems that antiviral activity depends on the type of the substituent of the hydrocarbon branch and the linker itself. The representative viruses tested were susceptible to unsubstituted or *para*-substituted long-chain arylpiperazine derivatives. The most active compounds were alkyl derivatives of 1-(4-fluorophenyl)-piperazine (**4b, 5b**) and 1-phenylpiperazine (**5a**). Introduction of any substituent in *ortho*-position as well as inserting of short-amine or cyclic moiety to the molecule dramatically decreases antimicrobial activity of the compound. Derivatives **4b** and **5b** were most potent against YFV, at a concentration that was at

Table 1 Structures of (4-aryl-piperazin-1-ylalkyl) derivatives of 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione



Compound	*n*	R
4a	3	
4b	3	
4c	3	
4d	3	
5a	4	
5b	4	
5c	4	
5d	4	
5e	4	
5f	4	
5g	4	

least 4.8-fold lower than the cytotoxic concentration. The 50% cytotoxic concentration (CC_{50}) of **5a** and **5d** in Vero-76 cells was higher than 100 μM, resulting in a selectivity index ($SI = CC_{50}/EC_{50}$) of above 5 and 3.5, respectively. The compound **5b** was found as the most selective and potent anti-CVB-2 agent. Compounds **4b** and **5a** expressed the broadest antiviral activity, viz against *Flaviviruses*, DNA and ssRNA viruses. For 1-(4-fluorophenyl)-piperazine derivatives **4b** and **5b** the observed EC_{50} value in anti-CVB tests was lower than for the standard 2′-ethenyl-D-cytidine (NM 176). In a group of aminoalkanol derivatives of 1,4-dithiine, only **3h** and **3j** revealed moderate activity against YFV. The activity of presented compounds decreased when three-unit propyl or 2-hydroxypropyl branch is inserted, instead of the butyl one.

Gram-negative rods as well as gram-positive strains and fungal organisms were resistant to all tested agents. Their minimal inhibitory concentration (MIC) values for all compounds were above 100 μM (Table 4). Moreover, none of title compounds turned out to be active against HIV-1, BVDV or representatives of ssRNA− and dsRNA viruses.

Experimental

Chemistry

All chemicals and solvents were purchased from Aldrich (Vienna, Austria). Melting points were determined on Electrothermal Digital Melting Point Apparatus (Essex, UK) and are uncorrected. The ^{1}H-NMR spectra were recorded on a Bruker (Rheinstetten, Germany) spectrometer, operating at 400 or 300 MHz. The chemical shift values are expressed in ppm relative to TMS as an internal standard. Elemental analyses were recorded on a CHN model 2400 Perkin-Elmer (Hitachi, Tokyo, Japan). Mass spectra were performed on MARINER PE Biosystems instrument (Foster City, USA) with TOF detector. Methanol was used as a solvent. The spectra were performed in the positive ion mode with a declustering potential 140–300 V. TLC was carried out using silica gel 60 F254, layer thickness 0.25 mm (E. Merck, Darmstadt, Germany) and the results were visualized using UV lamp at 254 nm. Column chromatography was carried out using silica gel 60 (200–400 mesh, Merck).

X-ray crystallography

Diffraction data for **4a**, **5g** and **5c** were measured at 293 K on a KM4 diffractometer using graphite monochromated CuKα radiation ($\lambda = 1.54178$ Å) and variable scan speed in the $\omega - 2\theta$ scan mode. The structures were solved by direct methods using the SHELXS-97 (Sheldrick, 1997) and refinement was performed by the full-matrix least-squares method on F^2 using the SHELXL-97 (Sheldrick, 1997) program. The non-hydrogen atoms were refined with anisotropic displacement parameters. H-atom positions were calculated from the geometry and were given isotropic factors of 1.2 U_{eq} of the bonded non-H atoms.

Compound **4a** crystallizes as monoclinic, in the space group $P2_1/n$, $a = 12.512(3)$ Å, $b = 6.181(1)$ Å, $c = 24.210(5)$ Å, $\beta = 94.25(3)°$, $V = 1867.2(7)$ Å^3, $Z = 4$,

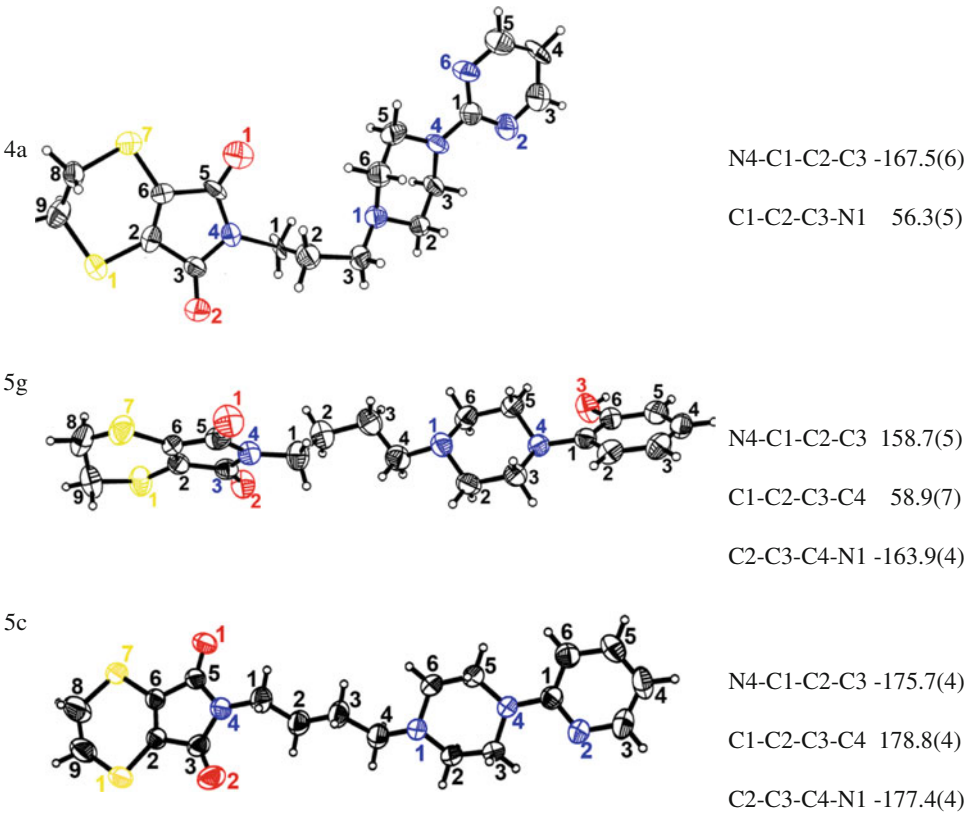


Fig. 3 Perspective view of molecules **4a**, **5g** and **5c** with atom numbering scheme and values of torsion angles [°] within the *N*-alkyl linker

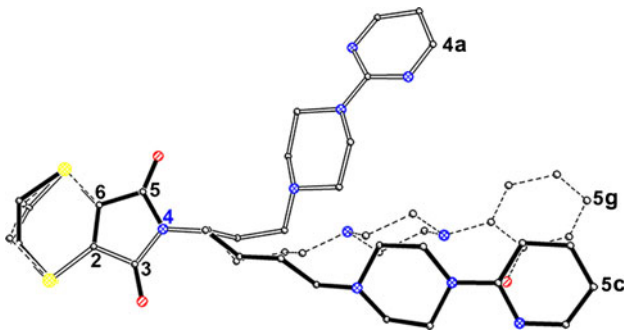


Fig. 4 Fit of molecules **4a**, **5c** and **5g** through the atoms of imide ring

d_{calc} = 1.393 g cm^{-3}, $F(000)$ = 824, μ(Cu$K\alpha$) = 2.775 cm^{-1}. In the θ range 6.76–72.11°, 3,575 reflections were collected and 1,883 unique reflections were used in the refinement. For 231 parameters refined the final discrepancy factors are: $R1 = 0.032$, $wR2 = 0.043$ for reflections with $I > 2\sigma(I)$ and $R1 = 0.1932$, $wR2 = 0.0602$ for all data. Extinction coefficient equals 0.0043(1).

Compound **5g** crystallizes in the orthorhombic system, space group $P\ 2_12_12_1$, $a = 7.440(1)$ Å, $b = 9.429(2)$ Å, $c = 28.552(6)$ Å, $V = 2003.0(7)$ Å^3, $Z = 4$, d_{calc} = 1.391 g cm^{-3}, $F(000) = 888$, μ(CuKα) = 2.633 cm^{-1}. In the (range 4.94–72.22°, 4,163 reflections were collected and 3,876 unique reflections were used in the refinement. For 253 parameters refined the final discrepancy factors are: $R1 = 0.0389$, $wR2 = 0.0973$ for reflections with $I > 2\sigma(I)$ and $R1 = 0.1968$, $wR2 = 0.1373$ for all data.

Compound **5c** crystallizes in the monoclinic system, space group $P2_1/n$, $a = 14.295(3)$ Å, $b = 9.013(2)$ Å, $c = 16.325(3)$ Å, $\beta = 107.81(3)$, $V = 2002.5(7)$ Å^3, $Z = 4$, $d_{calc} = 1.342$, $F(000) = 856$, μ(Cu$K\alpha$) = 2.589 cm^{-1}. In the θ range 4.93–72.14°, 3,248 reflections were collected and 3,051 unique reflections were used in the refinement. For 245 parameters refined the final discrepancy factors are: $R1 = 0.0455$, $wR2 = 0.124$ for reflections with $I > 2\sigma(I)$ and $R1 = 0.1485$, $wR2 = 0.1604$ for all data. Extinction coefficient equals 0.0014(3).

Crystallographic data haves been deposited with the Cambridge Crystallographic Data Center as CCDC Nos 758466, 758465 and 758464 for compounds **4a**, **5g**, and **5c**, respectively. Copies of the data can be obtained on application to the Director, CCDC Union Road, Cambridge CB2 1EZ U.K. (Fax: +44-1223-336-033; e-mail: deposite@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

2,3-Dihydro-5H-[1,4]dithiino[2,3-c]pyrrole-5,7(6H)-dione (**2**) was obtained according to the method described previously (Schweizer, 1969) from commercially available anhydride 1.

6-(Oxiran-2-ylmethyl)-2,3-dihydro-5H-[1,4]dithiino[2,3-c]pyrrole-5,7(6H)-dione (**3**) was prepared by dissolving of 2 g (0.011 mol) of the starting imide 2 in 29 cm^3 of

Table 2 Cytotoxicity and antiviral activity of compounds **3h**, **3j**, **4b** and **5a–5d**

Compds.	MT-4[a]	HIV-1[b]	MDBK[c]	BVDV[d]	BHK-21[e]	YFV[f]	SI[1]	Reo-1[f]	Vero-76[g]	HSV-1[h]	*SI*[2]	VV[h]	VSV	CVB-2[h]	*SI*[3]	Sb-1[h]
	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)		EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)		EC_{50} (μM)		EC_{50} (μM)		EC_{50} (μM)
3h	>100	>100	>100	>100	>100	80	>1.25	>100	>100	>100	–	>100	>100	>100	–	>100
3j	>100	>100	>100	>100	>100	75	>1.33	>100	>100	>100	–	>100	>100	>100	–	>100
4b	>100	>100	>100	>100	>100	52	>1.92	>100	96	>96	–	>96	>96	20	>4.8	>96
5a	>100	>100	>100	>100	>100	88	>1.14	>100	>100	19	>5.26	>100	>100	66	>1.51	>100
5b	>100	>100	>100	>100	>100	>100	–	>100	>100	>100	–	>100	>100	21	>4.76	>100
5c	>100	>100	>100	>100	>100	>100	–	>100	>100	>100	–	>100	>100	32	>3.12	>100
5d	>100	>100	>100	>100	>100	>100	–	>100	>100	29	>3.45	>100	>100	>100	–	>100
Ref. compds.																
AZT[i]	50	0.01														
NM 108[j]				1.8		2.5										
NM 176[k]														23		18
M 5255[l]												1.8				
ACG[m]										3						

Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%

Antiviral activity is given as EC_{50} (median effective concentration—the concentration of a drug (μM) required to induce a 50% effect), and cytotoxicity is given as CC_{50} (cytotoxic concentration—the amount of a drug (μM) at which 50% of cells become dead)

SI (selectivity index) was determined as the ratio between CC_{50} and EC_{50} for YFV (SI^1), HSV-1 (SI^2) and CVB-2 (SI^3)

[a] Compd. concn. (μM) required to reduce the viability of mock-infected MT-4 ($CD4^+$ Human T-cells containing an integrated HTLV-1 genome) cells by 50%, as determined by the colorimetric MTT method

[b] Compd. concentration (μM) required to achieve 50% protection of MT-4 cells from the HIV-1-induced cytopathogenicity, as determined by the MTT method

[c] Compd. concn. (μM) required to reduce the viability of mock-infected MDBK (Bovine normal kidney) cells by 50%, as determined by the MTT method

[d] Compd. concn. (μM) required to achieve 50% protection of MDBK cells from the BVDV (*Bovine Viral Diarrhoea Virus*)-induced cytopathogenicity, as determined by the MTT method

[e] Compd. concn. (μM) required to reduce the viability of mock-infected BHK (Hamster normal kidney fibroblast) monolayers by 50%, as determined by the MTT method

[f] Compd. concentration (μM) required to achieve 50% protection of BHK (Kidney fibroblast) cells from the YFV (*Yellow Fever Virus*) and Reo (*Reovirus 1*)-induced cytopathogenicity, as determined by the MTT method

[g] Compd. concn. (μM) required to reduce the viability of mock-infected VERO-76 (Monkey normal kidney) monolayers by 50%, as determined by the MTT method

[h] Compd. concn. (μM) required to reduce the plaque number of HSV-1 (*Herpesvirus 1*), VV (*Vaccinia virus*), VSV (*Vesicular Stomatitis virus*), CVB-2 (*Coxsackievirus B2*), Sb-1 (*Poliovirus 1*) and RSV (*Respiratory Syncytial Virus*) by 50% in VERO-76 monolayers

[i] Azido-thymidine

[j] 2′-β-methyl-guanosine

[k] 2′-Ethynyl-D-citidine

[l] Mycophenolic acid

[m] AcycloGuanosine

2-(chloromethyl)oxirane, to which 2 g (0.014 mol) of anhydrous potassium carbonate was added. The mixture was stirred under reflux condenser at 40°C for 24 h. The crude product was filtered off, the solvent was evaporated and the oily residue was purified by column chromatography using chloroform as eluent.

Yield 87%, oil, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 4.08 (2H, m, N–CH_2), 3.33 (4H, s, $(CH_2)_2$–S), 3.15 (1H, m, OCH), 2.79 (1H, dd, $J_1 = 4.4$ Hz, $J_2 = 8.4$ Hz, CH_2–O), 2.63 (1H, m, CH_2–O); ESI MS for $C_9H_9NO_3S_2$: m/z [%]: 265.9 $[M + Na]^+$ 100, 243.9 $[M + H]^+$ 39, 509.0 $[2\ M + Na]^+$ 23.

Table 3 Cytotoxicity and antiviral activity of 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione derivatives

Compds.	MT-4[a] CC_{50} (μM)	HIV-1[b] EC_{50} (μM)	MDBK[c] CC_{50} (μM)	BVDV[d] EC_{50} (μM)	BHK-21[e] CC_{50} (μM)	YFV[f] Reo-1[f] EC_{50} (μM)	Vero-76[g] CC_{50} (μM)	HSV-1[h] VV[h] VSV[h] CVB-2[h] [h]Sb-1 EC_{50} (μM)
3a, 3b, 3d, 3e, 3f, 3g, 3i, 4a, 5	>100							
2, 4c, 4d, 5e, 5f, 5g	>100		ND					

ND not determined

Table 4 Antibacterial and antifungal activities of 2,3-dihydro-5*H*-[1,4]dithiino[2,3-*c*]pyrrole-5,7(6*H*)-dione derivatives

MIC[a] (μM)				
Compds[b]	*S.aureus* DSM 2569	*P. aeruginosa* DSM 1117	*C. albicans* DSM 1386	*A. niger* DSM 1988
1, 2, 3a, 3b, 3d, 3e, 3f, 3g, 3h, 3i, 3j, 4a, 4b, 5a, 5b, 5c, 5d	>100	>100	>100	>100
Ref. compds.				
Ciprofloxacin	4	0.8	–	–
Miconazole	–	–	0.8	20

Miconazole was solubilized in DMSO (0.1 M solution) and stored at 4° C overnight

Reference Compounds were diluted from 100 to 0.0013 μM

[a] The antimicrobial activity is given as minimum inhibitory concentration (MIC) corresponding to the lowest concentration of an antimicrobial compound that showed complete growth inhibition

[b] Ciprofloxacin was solubilized in water (0.1 M solution) according to the British Society for Antimicrobial Chemotherapy (BSAC) protocol and stored at 4°C overnight

General procedure for preparation of 6-(3-amino-2-hydroxypropyl)-2,3-dihydro-5H-[1,4]dithiino [2,3-c]pyrrole-5,7(6H)-diones (**3a**–**3k**)

To 0.3 g (0.0012 mol) of 6-(oxiran-2-ylmethyl)-2,3-dihydro-5H-[1,4]dithiino[2,3-c]pyrrole-5,7(6H)-dione (**3**) dissolved in 35 cm^3 of methanol:water mixture (33.9:1.1 vol), appropriate chain or cyclic amine (0.0024 mol) was added. The mixture was stirred at 60°C under reflux condenser. The reaction was monitored by TLC (silica gel, developing system: chloroform:methanol, 4.5:0.5). The liquid was distilled off and the oily residue was purified by column chromatography (chloroform:methanol, 9:1).

3a. Yield 65%, m.p. 240–242°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 4.25 (1H, m, CH–OH), 3.69 (1H, dd, $J_1 = 6.4$ Hz, $J_2 = 14.4$ Hz, N–CH_2), 3.44 (1H, dd, $J_1 = 4.8$ Hz, $J_2 = 14.0$, N–CH_2), 3.33 (4H, s, $(CH_2)_2$–S), 2.90 (2H, m, CH_2–N), 2.77 (6H, s, $N(CH_3)_2$). $C_{11}H_{17}ClN_2O_3S_2$: Calcd. C 40.67, H 5.27, N, 8.63; Found C 40.86, H 5.43, N, 8.63.

3b. Yield 60%, m.p. 91–93°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 4.30 (1H, m, CH–OH), 3.69 (1H, dd, $J_1 = 6.4$ Hz, $J_2 = 14.0$ Hz, N–CH_2), 3.57 (1H, dd, $J_1 = 5.2$ Hz, $J_2 = 14.4$ Hz, N-CH_2), 3.33 (4H, s, $(CH_2)_2$–S), 2.88 (6H, m, CH_2–N–$(CH_2)_2$), 1.29 (6H, m, $N(CH_3)_2$). $C_{13}H_{20}N_2O_3S_2$: Calcd. C 49.34, H 6.37, N 8.85. Found C 49.29, H 6.29, N 8.79.

3c. Yield 25%, oil, ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 4.33 (1H, m, CH–OH), 3.70 (2H, m, NCH_2), 3.32 (4H, s, $(CH_2)_2S$), 3.15 (1H, m, $CH(CH_3)_2$), 3.07 (1H, dd, $J_1 = 2.4$ Hz, $J_2 = 12.3$ Hz, CH_2N), 2.87 (1H, dd, $J_1 = 9.9$ Hz, $J_2 = 12.0$ Hz, CH_2N), 1.40 (6H, dd, $J_1 = 3.6$ Hz, $J_2 = 6.6$ Hz, $(CH_3)_2$); ESI MS for $C_{12}H_{18}N_2O_3S_2$: m/z [%]: 303,1 $[M + H]^+$ 100.

3d. Yield 50%, m.p. 156–158°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 4.00 (1H, m, CH–OH), 3.63 (2H, m, N–CH_2), 3.32 (4H, s, $(CH_2)_2$–S), 2.86 (1H, dd, $J_1 = 2.4$ Hz, $J_2 = 12.4$ Hz, CH_2–N), 2.60 (1H, dd, $J_1 = 8.0$ Hz, $J_2 = 12.0$ Hz, CH_2–N), 1.23 (9H, s, $(CH_3)_3$). $C_{13}H_{20}N_2O_3S_2 \cdot H_2O$: Calcd. C 46.68, H 6.63, N 8.38. Found C 47.07, H 6.35, N 8.18.

3e. Yield 60%, m.p. 112–113°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 4.19 (1H, m, CH–OH), 3.66 (1H, dd, $J_1 = 6.4$ Hz, $J_2 = 14.0$ Hz, N-CH_2), 3.55 (1H, dd, $J_1 = 5.2$ Hz, $J_2 = 14.4$ Hz, N–CH_2), 3.32 (4H, s, $(CH_2)_2$–S), 2.64 (6H, m, CH_2–N, $H\alpha_{piper}$), 1.79 (4H, m, $H\beta_{piper}$),

1.41 (2H, m, Hγ_{piper}). $C_{14}H_{20}N_2O_3S_2$: Calcd. C 51.19, H 6.14, N 8.53. Found C 50.68, H 6.18, N 8.05.

3f. Yield 65%, m.p. 173–175°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 4.40 (1H, m, CH–OH), 3.69 (1H, dd, $J_1 = 6.0$ Hz, $J_2 = 14.0$ Hz, N–CH_2), 3.58 (1H, dd, $J_1 = 5.6$ Hz, $J_2 = 14.4$ Hz, N–CH_2), 3.33 (4H, s, $(CH_2)_2$–S), 2.87 (2H, m, CH_2–N), 2.59 (2H, m, Hα_{piper}), 1.81 (4H, br. s, Hβ_{piper}), 1.60 (2H, br. s, Hβ_{piper}), 1.02 (3H, d, $J = 6.2$ Hz, CH_3), 0.94 (1H, m, Hγ_{piper}). $C_{15}H_{23}ClN_2O_3S_2$: Calcd. C 47.54, H 6.12, N 7.39. Found C 47.56, H 6.10, N 7.29.

3g. Yield 62%, m.p. 168–170°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.88 (4H, m, H_{arom}), 4.15 (1H, m, CH–OH), 3.62 (2H, m, N–CH_2), 3.31 (4H, s, $(CH_2)_2$–S), 3.18 (4H, m, N$(CH_2)_{2piperazine}$–Ph), 2.98 (2H, m, CH_2–N$(CH_2)_{piperazine}$), 2.59 (2H, m, CH_2-N$(CH_2)_{piperazine}$), 2.44 (2H, m, CH_2–N). $C_{19}H_{24}ClN_3O_3S_2{\cdot}2H_2O$: Calcd. C 47.74, H 5.90, N 8.79. Found C 47.74, H 5.54, N 8.69.

3h. Yield 68%, m.p. 225–227°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.91 (4H, m, H_{arom}), 3.98 (1H, m, CH–OH), 3.63 (2H, m, N–CH_2), 3.32 (4H, s, $(CH_2)_2$–S), 3.11 (4H, m, N$(CH_2)_{2piperazine}$–Ph), 2.79 (2H, m, CH_2–N$(CH_2)_{piperazine}$), 2.59 (2H, m, CH_2–N$(CH_2)_{piperazine}$), 2.47 (2H, m, CH_2–N). $C_{19}H_{24}Cl_2FN_3O_3S_2$: Calcd. C 45.97, H 4.87, N 8.47. Found C 45.59, H 4.99, N 8.25.

3i. Yield 65%, m.p. 178–180°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 8.20 (1H, m, Hα_{arom}), 7.53 (m, 1H, Hγ_{arom}), 6.69 (2H, m, H$_{\beta arom}$), 4.32 (1H, m, CH–OH), 3.82 (4H, m, N$(CH_2)_{2piperazine}$–Ph), 3.65 (2H, m, N–CH_2), 3.32 (4H, s, $(CH_2)_2$–S), 3.00 (4H, m, CH_2–N$(CH_2)_{2piperazine}$), 2.79 (2H, m, CH_2–N). $C_{18}H_{24}Cl_2N_4O_3S_2{\cdot}2H_2O$: Calcd. C 41.94, H 5.48, N, 10.87. Found C 42.06, H 5.58, N 10.54.

3j. Yield 60%, m.p. 185–187°C, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.93 (4H, m, H_{arom}), 3.97 (1H, m, CH–OH), 3.86 (3H, s, OCH_3), 3.64 (2H, m, N–CH_2), 3.32 (4H, s, $(CH_2)_2$–S), 3.07 (4H, m, N$(CH_2)_{2piperazine}$–Ph), 2.82 (2H, m, CH_2–N$(CH_2)_{piperazine}$), 2.63 (2H, m, CH_2–N$(CH_2)_{piperazine}$), 2.49 (1H, m, CH_2–N), 2.41 (1H, m, CH_2–N). $C_{20}H_{27}Cl_2N_3O_4S_2{\cdot}1½H_2O$: Calcd. C 44.86, H 5.65, N 7.85. Found C 45.33, H 5.80, N 7.32.

3k. Yield 65%, m.p. 142–144°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 6,98 (4H, m, H_{arom}), 4,01 (1H, m, CH–OH), 3,62 (2H, m, NCH_2), 3,32 (4H, s, $(CH_2)_2$S), 3,11 (4H, m, N$(CH_2)_{2piperazine}$-Ph), 2,84 (2H, m, CH_2–N$(CH_2)_{2piperazine}$), 2,66 (2H, m, CH_2–N$(CH_2)_{2piperazine}$), 2,49 (2H, m, CH_2N); ESI MS for $C_{19}H_{22}FN_3O_3S_2$: m/z [%]: 425,11 $[M + 2H]^+$ 81,72.

(4-aryl/heteroaryl)piperazin-1-yl)alkyl]-2,3-dihydro-5H-[1,4]dithiino[2,3-c]-pyrrole-5,7- (6H)-diones (**4a–4d, 5a–5g**) were obtained according to the method published previously (Kossakowski *et al.*, 2007).

Microbiological assays

Compounds

Compounds were dissolved in DMSO at 100 mM and then diluted in culture medium.

Cells and viruses

Cell lines were purchased from American Type Culture Collection (ATCC). The absence of mycoplasma contamination was checked periodically by the Hoechst staining method. Cell lines supporting the multiplication of RNA viruses were the following: $CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4); Madin Darby Bovine Kidney (MDBK); Baby Hamster Kidney (BHK-21) and Monkey kidney (Vero 76) cells.

Bacterial strains

The antibacterial activity of compounds was tested against collection strains representative of Gram-positive bacteria (*S. aureus* DSM 2569) and Gram-negative bacteria (*P. aeruginosa* DSM 1117). Antifungal activity was tested against collection strains representative of yeasts (*C. albicans* DSM 1386) and moulds (*A. niger* DSM 1988).

Cytotoxicity assays

For cytotoxicity tests, run in parallel with antiviral assays, MDBK, BHK and Vero 76 cells were resuspended in 96-multiwell plates at an initial density of 6×10^5, 1×10^6 and 5×10^5 cells/ml, respectively, in maintenance medium, without or with serial dilutions of tested compounds. Cell viability was determined after 48–120 h at 37°C in a humidified CO_2 (5%) atmosphere by the MTT method. The cell number of Vero 76 monolayers was determined by staining with the crystal violet dye.

For cytotoxicity evaluations, exponentially growing cells derived from human haematological tumours [$CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4)] were seeded at an initial density of 1×10^5 cells/ml in 96 well plates in RPMI-1640 medium, supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin. Cell cultures were then incubated at 37°C in a humidified, 5% CO_2 atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 h at 37°C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Pauwels *et al.*, 1988).

Antiviral assay

Activity of compounds against Human Immunodeficiency virus type-1 (HIV-1) was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50 µl of RPMI containing 1×10^4 MT-4 were added to each well of flat-bottom microtitre trays containing 50 µl of RPMI, without or with serial dilutions of test compounds. Then, 20 µl of an HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4-day incubation, cell viability was determined by the MTT method.

Activity of compounds against *Yellow Fever Virus* (YFV) and *Reo virus type-1* (Reo-1) was based on inhibition of virus-induced cytopathogenicity in acutely infected BHK-21 cells. Activities against *Bovine Viral Diarrhoea Virus* (BVDV), in infected MDBK cells, were also based on inhibition of virus-induced cytopathogenicity.

BHK and MDBK cells were seeded in 96-well plates at a density of 5×10^4 and 3×10^4 cells/well, respectively, and were allowed to form confluent monolayers by incubating overnight in growth medium at 37°C in a humidified CO_2 (5%) atmosphere. Cell monolayers were then infected with 50 µl of a proper virus dilution (in serum-free medium) to give an m.o.i = 0.01. 1 h later, 50 µl of MEM Earle's medium, supplemented with inactivated foetal calf serum (FCS), 1% final concentration, without or with serial dilutions of test compounds, were added. After 3–4 days of incubation at 37°C, cell viability was determined by the MTT method.

Activity of compounds against *Coxsackie virus*, B-2 strain (CVB-2), *Polio virus type-1* (Polio-1), Sabin strain, *Vesicular Stomatitis Virus* (VSV), *Vaccinia Virus* (VV) and *Herpes Simplex Virus type-1* (HSV-1), in infected Vero 76 cells, was determined by plaque reduction assays in Vero 76 cell monolayers. To this end, Vero 76 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium at 37°C in a humidified CO_2 (5%) atmosphere. Then, monolayers were infected with 250 µl of proper virus dilutions to give 50–100 PFU/well. Following removal of unadsorbed virus, 500 µl of Dulbecco's modified Eagle's medium, supplemented with 1% inactivated FCS and 0.75% methyl cellulose, without or with serial dilutions of test compounds, were added. Cultures were incubated at 37°C for 2 (Sb-1 and VSV) or 3 (CVB-2, VV and HSV-1) and then fixed with PBS containing 50% ethanol and 0.8% crystal violet, washed and air-dried. Plaques were then counted. 50% effective concentrations (EC_{50}) were calculated by linear regression technique.

Antibacterial and antifungal assays

The antibacterial and antifungal activities were evaluated by determining the Minimum inhibitory concentration (MIC) by the broth microdilution procedure.

Bacterial strains were grown on Tryptic soy agar at 37°C for 1 day. Cell suspensions of these recent cultures were prepared in sterile 0.85% saline solution by 4–5 colonies. The turbidity of the suspensions was adjusted to the McFarland 0.5 standard. Suspensions were diluted in cation-supplemented Mueller–Hinton broth. For each microorganism, 100 µl of the fivefold serial dilutions of the compounds in cation-supplemented Mueller–Hinton broth and 100 µl of *inoculum* were added to each well of a microdilution plate (final titre 5×10^5 CFU/ml). The inoculated plates were incubated at 37°C in non-CO_2 incubator and humid atmosphere. The MICs were determined after 16–20 h (EUCAST, 2003).

Fungal strains were grown on Sabouraud's dextrose agar at 35°C for 1–5 days. Suspensions of these recent cultures were prepared in sterile saline solution (NaCl 0.85%). Suspensions were then diluted in Sabouraud's dextrose broth. 100 µl of the fivefold serial dilutions of the compounds in Sabouraud's dextrose broth and 100 µl of *inoculum* were added to each well of a microdilution plate (*C. albicans* 1×10^4 cell/ml; *A. niger* OD_{600} 0.05). The inoculated plates were incubated at 35°C in non-CO_2 incubator and humid atmosphere. The MICs were determined after 24 and 48 h.

The concentration of each *inoculum* was confirmed by viable counts on agar plates by plating the appropriate dilution of the growth control well, immediately after inoculation, and incubating until visible growth. MIC corresponded to the lowest concentration of an antimicrobial compound that showed complete growth inhibition.

Linear regression analysis

Viral and cell growth at each drug concentration was expressed as percentage of untreated controls and the concentrations resulting in 50% (EC_{50}, CC_{50}) growth inhibition were determined by linear regression analysis.

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