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Synthesis, cytotoxicity and antiviral activity of *N*,*N*′-bis-5-nitropyrimidyl derivatives of dispirotripiperazine

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Abstract

During the search for new antivirals, various *N*,*N*′-bis-5-pyrimidyl derivatives of 3,12-diaza-6,9-diazonia(5,2,5,2)dispirohexadecane dichloride (dispirotripiperazine) were synthesized. To reveal relationships between chemical structure and antiviral activity, the compounds were characterized by fast atom bombardment mass, nuclear magnetic resonance, infra red spectroscopy, and elemental analysis and examined for cytotoxicity, inhibition of cell growth and antiviral activity under in vitro conditions. The results of this study demonstrate an excellent compatibility of the test compounds for confluent as well as proliferating cells and a potent structure-dependent inhibition of herpes simplex virus type 1 replication when added during viral adsorption. Functional group analysis revealed that both the dispirotripiperazine as well as the pyrimidine ring with a nitro group in the 5 position are necessary for activity. A reduction of electron density in the terminal pyrimidine rings enhanced the antiviral activity whereas electron donor substitutions reduced it. Introduction of a methyl group in position 2 of the pyrimidine had no influence on cytotoxicity or antiviral activity.

Keywords: Dispirotripiperazine; 5-Nitropyrimidine; Herpes simplex virus type 1; Antiviral

1. Introduction

During the past three decades great successes were achieved in the therapy of human immunodeficiency virus (HIV), herpes virus, influenza virus, and hepatitis B virus infections (De Clercq, 1998; Molla et al., 1998; Schmidt et al., 2001; Zoulim, 1999). Nevertheless, effective treatment is not available for most other viral infections e.g. hepatitis virus C, respiratory syncytial virus, and picornaviruses (Rotbart et al., 1998; Wyde, 1998). Moreover, new antivirals are needed to overcome drug resistance (Andrei et al., 1995) and toxic side effects of existing drugs (Wutzler and Thust, 2001).

Searching for new potent antiviral agents, we have synthesized various *N*,*N*′-bis-5-pyrimidyl derivatives of 3,12-diaza-6,9-diazonia(5,2,5,2)dispirohexadecane dichloride (dispirotripiperazine).

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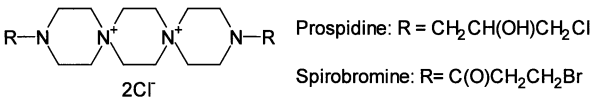

Fig. 1. Structure of prospidine and spirobromine.

Originally, derivatives of nitrogen-containing dispiro systems like prospidine and spirobromine (Fig. 1) were synthesized as antitumor drugs (Chernov et al., 1984; Grohn et al., 1984; Lavrik et al., 1993). Both drugs exerted also anti-inflammatory effects (Siubaev et al., 1990; Benenson and Timina, 1994). In addition, anti-herpes simplex virus type 1 (HSV-1) activity of spirobromine was described (Levkovskaya et al., 1989).

To investigate the relationship between structure and antiviral activity, the derivatives of dispirotripiperazine were structurally characterized, and examined for cytotoxic as well as antiviral activity under in vitro conditions during this study.

2. Materials and methods

2.1. Compounds

The following compounds were synthesized and investigated for cytotoxic and antiviral activity: (**1**) 3,12-diaza-6,9-diazonia(5,2,5,2)dispirohexadecane (DDDHD) dichloride (diCl), $C_{12}H_{26}Cl_2N_4$; (**2**) *N*,*N*′-bisamino-DDDHD diCl, $C_{12}H_{28}Cl_2N_6$; (**3**) *N*,*N*′-bischloromethyl-DDDHD diCl, $C_{14}H_{28}Cl_4N_4$; (**4**) *N*,*N*′-bis(2-chloroethyl)carboxamide-DDDHD diCl, $C_{18}H_{34}Cl_4N_6O_2$; (**5**) *N*,*N*′-bis(4-phenylbutanone-1)-DDDHD diCl, $C_{32}H_{46}Cl_2N_4O_2$; (**6**) *N*,*N*′-bis(3-chloropropanamide)-DDDHD diCl, $C_{12}H_{34}Cl_4N_6O_2$; (**7**) 3,13-bis(2-oxiranylmethyl)-3,13-diaza-7,10-diazoniadispiro[6.2.6.2]octadecane dibromide, $C_{20}H_{38}Br_2N_4O_2$; (**8**) *N*,*N*′-bis(4-chloro-5-nitropyrimidyl-6)-DDDHD diCl trihydrate, $C_{20}H_{26}Cl_4N_{10}O_4{\cdot}3H_2O$; (**9**) *N*,*N*′-bis(2-methyl-4-chloro-5-nitropyrimidyl-6)-DDDHD diCl trihydrate, $C_{22}H_{30}Cl_4N_{10}O_4{\cdot}3H_2O$; (**10**) *N*,*N*′-bis(4-methoxy-5-nitropyrimidyl-6)-DDDHD diCl trihydrate, $C_{22}H_{32}Cl_2N_{10}O_6{\cdot}3H_2O$; (**11**) *N*,*N*′-bis(2-methylmercapto-4-chloro-5-nitropyrimidyl-6)-DDDHD diCl trihydrate, $C_{22}H_{30}Cl_4N_{10}O_4S_2{\cdot}3H_2O$; (**12**) *N*,*N*′-bis(4-(*N*′-diethylaminocarbamo-yl)piperazine)-5-nitropyrimidyl-6)-DDDHD diCl tetrahydrate, $C_{38}H_{68}Cl_2N_{16}O_6{\cdot}4H_2O$; (**13**) *N*,*N*′-bis(4-diethyldithiocarbamoyl-5-nitropyrimidyl-6)-DDDHD diCl trihydrate, $C_{30}H_{56}Cl_2N_{12}O_4S_4{\cdot}3H_2O$; (**14**) *N*,*N*′-bis(2-trifluoromethyl-4-chloro-5-nitropyrimidyl-6)-DDDHD diCl hydrate, $C_{22}H_{24}Cl_4F_6N_{10}O_4{\cdot}H_2O$; (**15**) *N*,*N*′-bis(4-pyrrolidino-5-nitropyrimidyl-6)-DDDHD diCl, $C_{28}H_{42}Cl_2N_{12}O_4$; (**16**) *N*,*N*′-bis(2-methyl-4-pyrrolidino-5-nitropyrimidyl-6)-DDDHD diCl, $C_{30}H_{46}Cl_2N_{12}O_4$; (**17**) *N*,*N*′-bis(4-hexamethylenimino-5-nitropyrimidyl-6)-DDDHD diCl, $C_{32}H_{50}Cl_2N_{12}O_4$; (**18**) *N*,*N*′-bis(2-methyl-4-hexamethylenimino-5-nitropyrimidyl-6)-DDDHD diCl, $C_{34}H_{54}Cl_2N_{12}O_4$; (**19**) *N*,*N*′-bis(4-hydroxy-5-nitropyrimidyl-6)-DDDHD diCl, $C_{20}H_{28}Cl_2N_{10}O_6$; (**20**) *N*,*N*′-bis(2-methyl-4-hydroxy-5-nitropyrimidyl-6)-DDDHD diCl, $C_{22}H_{32}Cl_2N_{10}O_6$; (**21**) *N*,*N*′-bis(2-amino-4-hydroxy-5-nitropyrimidyl-6)-DDDHD diCl, $C_{20}H_{30}Cl_2N_{12}O_6$; (**22**) *N*,*N*′-bis(4-chloro-5-aminopyrimidyl-6)-DDDHD diCl trihydrate, $C_{20}H_{30}Cl_4N_{10}{\cdot}3H_2O$; (**23**) *N*,*N*′-bis(2-methylmercapto-4-chloro-5-formylpyrimidyl-6)-DDDHD diCl trihydrate, $C_{24}H_{32}Cl_4N_8O_2S_2{\cdot}3H_2O$; (**24**) *N*,*N*′-bis(2-methylmercapto-5-ethoxycarbonylpyrimidyl-6)-DDDHD diCl, $C_{28}H_{42}Cl_2N_8O_4S_2$; (**25**) *N*,*N*′-bis(4-chloro-5-nitropyrimidyl-6)amino-DDDHD diCl, $C_{20}H_{28}Cl_4N_{12}O_4$; (**26**) *N*,*N*′-bis(4-chloro-2-methylthio-5-nitropyrimidyl-6)-3,13-diaza-7,10-diazonia(6,2,6,2)dispirooctadecane dibromide, $C_{24}H_{34}Br_2Cl_2N_{10}O_4S_2$; (**27**) *N*,*N*′-bis(1-oxido[1,2,5]oxadiazolo[3,4-D]pyrimidin-7-yl)-DDDHD diCl, $C_{20}H_{26}Cl_2N_{12}O_4$; (**28**) *N*,*N*′-bis(5-methyl-1-oxido-[1,2,5]oxadiazolo[3,4-D]pyrimidin-7-yl)-DDDHD diCl, $C_{22}H_{30}Cl_2N_{12}O_4$; (**29**) *N*,*N*′-bis(1-oxido[1,2,5]oxadiazolo[3,4-D]pyrimidin-7-yl)-3,13-diaza-7,10-diazonia(6,2,6,2)dispirooctadecane dibromide, $C_{22}H_{30}Br_2N_{12}O_4$; (**30**) *N*,*N*′-bis(3-nitropyridyl-2)-DDDHD diCl, $C_{22}H_{30}Cl_2N_8O_4$; (**31**) *N*,*N*′-bis(3-nitro-4-chloro-6-methylpyridyl-2)-DDDHD diCl, $C_{24}H_{32}Cl_4N_8O_4$; (**32**) *N*,*N*′-bis(2,4-dinitrobenzyl-1)-DDDHD diCl, $C_{24}H_{30}Cl_2N_8O_8$; (**33**) *N*,*N*′-bis(2-nitro-4-cyanobenzyl-1)-DDDHD diCl, $C_{26}H_{30}Cl_2N_8O_4$; (**34**) *N*,*N*′-bis(2-nitro-4-trifluoromethylbenzyl-1)-DDDHD diCl, $C_{26}H_{30}Cl_2F_6N_6O_4$; (**35**) *N*,*N*′-bis[(2-amino-3-nitro-7-oxo-4,7-dihydro-

pyrazolo[1,5-*a*]pyrimidin-5-yl)methyl]-DDDHD diCl, $C_{26}H_{36}Cl_2N_{14}O_6$; (**36**) *N*,*N*′-bis(7-nitro-2,1,3-benzoxadiazol-4-yl)-DDDHD diCl, $C_{24}H_{28}Cl_2N_{10}O_6$; (**37**) *N*,*N*′-(1-amino-2-nitro-2-cyanoethyl-en-1)-DDDHD diCl tetrahydrate, $C_{18}H_{28}Cl_2N_{10}O_9{\cdot}5H_2O$; (**38**) 4,6-di(DDDHD)-5-nitropyrimidine tetrachloride dihydrochloride tetrahydrate, $C_{28}H_{51}Cl_4N_{11}O_2{\cdot}2HCl{\cdot}4H_2O$; (**39**) 2-methyl-4,6-di(DDDHD)-5-nitropyrimidine tetrachloride dihydrochloride tetrahydrate, $C_{29}H_{53}Cl_4N_{11}O_2{\cdot}2HCl{\cdot}4H_2O$; (**40**) *N*,*N*′-bis(4-DDDHD-5-nitropyrimidyl-6)-DDDHD hexachloride dihydrochloride nanohydrate, $C_{44}H_{76}Cl_6N_{18}O_4{\cdot}2HCl{\cdot}9H_2O$; (**41**) 4-(6-chloro-5-nitro-4-pyrimidinyl)-1,1-dimethylpiperazinium iodide, $C_{10}H_{15}ClIN_5O_2$; (**42**) 4-(2-methyl-6-chloro-5-nitro-4-pyrimidinyl)-1,1-dimethylpiperazinium iodide, $C_{11}H_{17}ClIN_5O_2$; (**43**) 4,6-di[4,4-dimethylpiperazin-4-ium-1-yl]-5-nitro-4-pyrimidine diiodide, $C_{16}H_{29}I_2N_7O_2$; (**44**) 4,6-di[4,4-dimethylpiperazin-4-ium-1-yl]-2-methyl-5-nitro-4-pyrimidine diiodide, $C_{17}H_{31}I_2N_7O_2$; (**45**) 1,2-bis[4-(6-chloro-5-nitro-4-pyrimidinyl)-1-methylpiperazin-1-ium]-ethylen diiodide, $C_{20}H_{28}Cl_2I_2N_{10}O_4$; (**46**) 1,2-bis[4-(2-methyl-6-chloro-5-nitro-4-pyrimidinyl)-1-methylpiperazin-1-ium]-ethylen diiodide, $C_{22}H_{32}Cl_2I_2N_{10}O_4$.

Symmetric *N*,*N*′-bisheteroaryl derivatives of DDDHD were synthesized by nucleophilic substitution of dispirotripiperazine with 2 equivalents of 4-R-6-chloro-5-nitropyrimidines under alkali conditions. The synthesis of compounds **1**–**7** has been performed as described by Safanova et al. (1983) and Fomina et al. (1989). The compounds **8**, **12**, **13**, **15**, **17**, and **37** were synthesized according to the publications of Makarov et al. (1994) and Makarov et al. (1996), respectively. Compounds **9**, **11**, **14**, **25**, **26**, **29**–**36** and **41**–**44** were obtained analogously (Schmidtke et al., 2000).

The 3,12-bis(4-methoxy-5-nitropyrimidyl-6)-3,12-diaza-6,9-diazoniadispiro(5,2,5,2)hexadecane dichloride (**10**), $C_{22}H_{32}Cl_2N_{10}O_6$ (Mw. 603.46) was synthesized by adding the solution of 1.5 g (5.23 mmol) of 3,12-diaza-6,9-diazoniadispiro(5,2,5,2)-hexadecane dichloride in 10 ml water to the suspension of 2.15 g (11.37 mmol) of 4-methoxy-5-nitro-6-chloropyrimidine (Kimura et al., 1993) in 50 ml ethanol. Afterwards, 1.5 ml (10.58 mmol) of Et_3N were added to the reaction mixture (temperature < 25 °C). After 4 h, 50 ml of acetone were added. The resulting white precipitate was filtered and recrystallized (water/ethanol). The final product was analyzed (yield 2.1 g, 65%) as described in Section 2 and showed melting points (mp.) 253–256 °C with decomposition; MS fast atom bombardment (FAB) (3NBA) $[M + H]^+$: m/z = gef. 532.2; IR (oil): 1561, 1240, 1001, 720/cm; ^{1}H NMR (DMSO-D_6): δ (ppm): 8.18 (2H, s, 2 CH-pyrimidine), 4.24 (6H, s, 2 CH_3), 4.08 (br), and 3.94–4.27 (24H, m, CH_2–CH_2 piperazine). Compounds **16**, **18**–**24**, **38**–**40**, **45**, and **46** were prepared analogously.

The 3,12-bis(5-methyloxadiazolo[4,3-D]pyrimid-7-yl-1-oxid)-3,12-diaza-6,9-diazonia-dispiro-(5,2,5,2)hexadecane dichloride (**28**), $C_{21}H_{28}Cl_2N_{12}O_4$ (Mw. 583.41) was synthesized by treating the suspension of 1.6 g (238 mmol) 3,12-bis(2-methyl-4-chloro-5-nitropyrimid-yl-6)-3,12-diaza-6,9-diazonia-dispiro(5,2,5,2)hexadecane dichloride (Makarov et al., 1994) in 50 ml of methanol with a solution of 0.3 g (4.6 mmol) of sodium azide in 5 ml water for 3 h. Then, 50 ml of acetone were added. The resulting white precipitate was filtered and recrystallized from water. The final product (yield: 1.1 g; 64%) was analyzed as described in Section 2 and showed mp. 271–273 °C with decomposition; MS FAB (3NBA) $[M + H]^+$: m/z = gef. 514.4; IR (oil): 1555, 1218, 980, 827, 720/cm;UV λ_{max}(lg): 222 (4.38), 250 (shoulder), 320–360 nm (3,96); ^{1}H NMR (DMSO-D_6): δ (ppm): 2.45 (6H, s, 2 CH_3), 4.08 (br), and 4.24 (24H, m, CH_2–CH_2 piperazine). Compound (**27**) was obtained analogously.

All starting chemicals were obtained from Lancaster Synthesis Ltd. (Lancashire, UK). The chemical structure of these compounds (Fig. 2) was determined by FAB mass, nuclear magnetic resonance (NMR), and infra red (IR) spectrometry, elemental analysis and by UV-spectrometry if necessary. Mass spectra were obtained on a Finnigan SSQ-700 spectrometer. NMR spectra were recorded on an Oxford Unity spectrometer at 400 MHz (Varian) with tetramethylsilane as the reference. IR spectra were recorded on a Perkin–Elmer 2000 FT-IR unit. Elemental analyses were performed on a Carlo–Erba model 5500 elemental analyser. Melting points were obtained on an

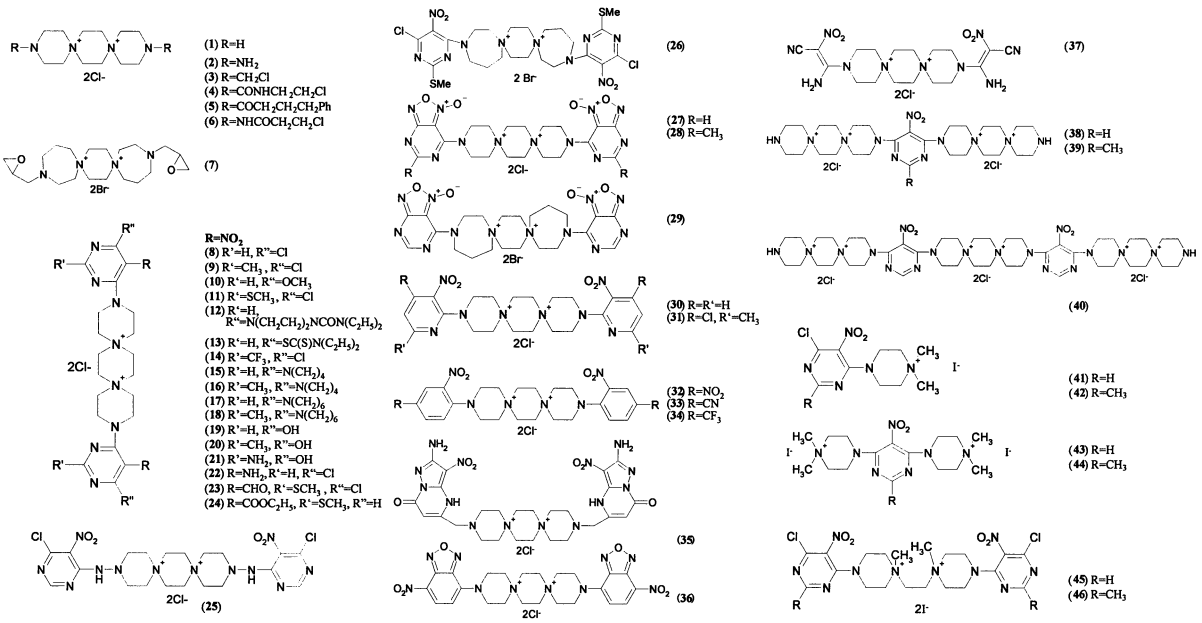

Fig. 2. Structure of the dispirotripiperazine derivatives.

electrothermal 9100 (UK) melting point apparatus and are uncorrected.

Guanidine-HCl (SIGMA, N: G-4505), amantadine (SIGMA, No: A-1260), and phosphonoformic acid (PFA: SIGMA, No: P-4669) were used as positive control compounds in antiviral assays with coxsackievirus B3 (CVB3), influenza virus A, and HSV-1, respectively. In addition, prospidine and spirobromine were used as reference compounds in antiviral assays with HSV-1. Stock solution of the compounds in water or DMSO (10 mg/ml) were stored at -20 °C. Further dilutions on the day of use were done with complete test medium.

2.2. Cells and viruses

HeLa Ohio (human cervix carcinoma; ATCC No: CCL-2), GMK (green monkey kidney cells; Schaper & Brümmer, Salzgitter, Germany), and MDCK cells (Madin-Darby canine kidney, Institute for influenza viruses, St. Petersburg, Russia) were used for cytotoxic and antiviral studies. The HeLa Ohio cells were grown in Eagle's minimal essential medium (MEM/E SIGMA No: M-0643) and the GMK and MDCK cells in Dulbecco's modified MEM/E (SIGMA No: D-7777) supplemented with 10% neonatal calf serum (NCS: HeLa Ohio, Greiner No: 758010, Germany) or 10% fetal bovine serum (FBS: GMK and MDCK, Greiner No: 758075, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin. The test medium contains only 2% of the appropriate serum (CVB3 and HSV-1) or not at all (influenza virus A). In addition, the human chronic myeloid leukaemic cell line K-562 (DSMZ, Heidelberg, Germany) and the mouse fibroblast cell line L-929 (DSMZ, Heidelberg, Germany) were used in antiproliferative assays. Both cell lines were cultured in RPMI 1640 medium (GIBCO BRL 42402-016), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat inactivated FBS (GIBCO BRL 10500-064), and L-glutamate (GIBCO BRL 25030-024).

Virus stocks of the CVB3 strain Nancy (Institute of Poliomyelitis and Virus Encephalitides, Moscow, Russia), the influenza virus A strain Hong Kong/68 (H3N2; Schaper & Brümmer, Salzgitter, Germany), and the HSV-1 strain Kupka (Institute of Antiviral Chemotherapy,

FSU Jena, Germany) were prepared in HeLa Ohio, MDCK, and GMK cells, respectively. Aliquots of the virus stock were stored at −70 °C until use.

2.3. Cytotoxicity assay

The cytotoxicity of the test compounds was determined on two day old confluent HeLa Ohio, MDCK, and GMK cell monolayers grown in flat-bottomed microtiter plates (Falcon 3075) as described previously (Schmidtke et al., 2001). Briefly, after removing the growth medium, nine twofold dilutions of the compounds in 100 µl of test medium were added. Following a 72 h incubation (37 °C, 5% CO_2), the supernatant was aspirated, the cell monolayers were washed, fixed, and stained with a crystal violet/methanol solution using the Dynex Immuno Assay System (DIAS, Guernsey, GB). After extraction of the dye the optical density of individual wells was quantified spectrophotometrically at 570/630 nm with the DIAS. Cell viability was evaluated as the percentage of the mean value of optical density resulting from six mock-treated cell controls which was set 100%. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration reducing the viability of untreated cell cultures by 50%.

2.4. Inhibition of cell growth

1×10^4 K-562 or L-929 cells, each in 100 µl culture medium, were added to ten compound dilutions (each concentration in triplicate, dilution factor 2) in microplates (NUNC 163320). After an incubation time of 72 h at 37 °C in a humidified atmosphere with 5% CO_2, suspension cultures of K-562 cells in microplates were analysed by an electronic cell analyser system (CASY 1, SCHÄRFE, Reutlingen, Germany) as described recently (Schmidtke et al., 2001). From the dose–response curves the concentration which inhibited cell growth by 50% (GI_{50} values) was calculated with the software for data evaluation CASYSTAT (SCHÄRFE, Reutlingen, Germany). In parallel, the adherent L-929 cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue. After gently washing the stain was eluted, and the optical densities were measured at 630 nm in a Dynatech MR 7000 microplate reader (Guernsey, GB).

2.5. Cytopathic effect inhibitory assay

The cytopathic effect (CPE) inhibitory assays have been described in detail (Schmidtke et al., 2001). Briefly, the CPE inhibitory tests were carried out in two-day-old confluent cell monolayers growing in 96-well flat-bottomed microtiter plates (Falcon 3075). 50 µl of drug solution and a constant amount of virus in a volume of 50 µl (1, 0.1, and 0.1 multiplicity of infection for CVB3, influenza virus A, and HSV-1, respectively) were added successively to the HeLa, MDCK, and GMK cells, respectively. Six wells of noninfected and six wells of infected cells without the test compound served as cell and virus control, respectively, on each plate. In addition, the 50 and 100% plaque inhibitory concentrations of antiviral compounds (each 3 wells) were included as positive controls in each microtiter plate. Using the crystal violet uptake assay described for cytotoxic investigations, the inhibition of the virus-induced CPE was scored 24 h (CVB3 and influenza virus A) or 48 h (HSV-1) post infection when untreated infected control cells showed maximum CPE and the positive control compound-treated wells a 50 or 100% protection. The IC_{50} values of antiviral active compounds were determined from the mean dose–response curves (2 parallels per concentration) of 3 separate experiments.

2.6. Plaque reduction assay

The plaque reduction assay was performed as described previously (Schmidtke et al., 2001). Briefly, after removal of the cell growth medium, confluent 2-day-old GMK cell monolayer in 6-well plates (FALCON 3046) were inoculated with approximately 100 plaque forming units (pfu) of HSV-1 in test medium in the absence or presence of serial twofold dilutions of the compound **27**. After adsorption at 37 °C for 1 h, the inoculum was aspirated and 2 ml of the respective test medium containing 0.4% agar and the appropriate

concentrations of the drug were added. Three untreated virus controls and one uninfected untreated cell control were included in all assays. Each compound concentration was tested in duplicate. The tests were incubated at 72 h until plaques appeared and then fixed and stained with a solution of 0.4% crystal violet in a mixture of formalin (3% v/v) and ethanol (1.67% v/v) in water overnight. Plaques were counted over a light box after removal of the agar overlay.

3. Results

3.1. Cytotoxicity and cell growth inhibition

To exclude non-specific antiviral activities, spirobromine, prospidine, and the derivatives of dispirotripiperazines were tested for cytotoxicity on confluent monolayers of HeLa Ohio, MDCK, and GMK cells as well as for the inhibition of cell growth on L-929 and K-562 cells. Compounds lacking any antiviral activity in the parallel performed CPE inhibitory assays were tested once in each cell line. The cytotoxicity of antiviral compounds was determined in three separate assays in GMK cells each with two parallels per concentration. The 50% cytotoxic concentrations were calculated from the single or the mean dose–response curve of three separate assays, respectively. Tables 1 and 2 summarize the 50% cytotoxic and the 50% growth inhibitory concentrations of the test compounds in GMK cells and L-929 or K-562 cells, respectively. Comparable cytotoxicity data were obtained in HeLa and MDCK cells (data not shown). The results demonstrate an excellent compatibility of the dispirotripiperazine derivatives for the tested cell lines. Most of the compounds were not cytotoxic and did not inhibit the cell growth in the tested dose range. A moderate cytotoxicity was determined for compound **2** ($CC_{50} = 207.1$ μM). Remarkable differences between cytotoxicity and growth inhibition were only detected for compounds **7**, **8**, and **19** (factor 3 to 10).

3.2. Antiviral activity

An automated CPE-inhibitory assay was used to determine the antiviral activity of the test compounds against CVB3, influenza virus A, and HSV-1 (Schmidtke et al., 2001) in the primary screening. None of the compounds inhibited the CVB3- or influenza virus A-induced CPE in HeLa Ohio or MDCK cells, respectively (results not shown). In contrast, highly active compounds were discovered in the HSV-1/GMK cell system (Table 3). The IC_{50} values were in the range from approximately 1 μM (compounds **8** and **19**) to 71.9 μM (compound **2**). Therapeutic indices $\geqslant 100$ were determined for compounds **8**, **9**, **11**, **12**, **19**, **25**, **27**, **38**, **39**, and **40**. The dose–response curves from the compounds inhibiting the HSV-1-induced CPE (Fig. 3) demonstrate in more detail the potential antiviral activity of dispirotripiperazine derivatives. Three wells treated with 416.7 μM and three wells treated with 41.67 μM of PFA were included as positive control in each microtiter plate. Scoring the inhibition of the virus-induced CPE, these control wells showed a 80.12–87.74% and 45.74–65.88% protection, respectively.

To provide information whether the compounds prevent viral binding or fusion or whether they inhibit an intracellular event in the replication cycle, plaque reduction assays were performed with one selective compound. The compound **27** was added before and after virus adsorption. Plaque reduction could not be observed when the compound was first added after viral adsorption.

4. Discussion

The present study describes the synthesis, cytotoxicity, and antiviral activity of DDDHD derivatives, also named dispirotripiperazine derivatives. These compounds are interesting polycationic and polycyclic saturated systems used in organic chemistry based on piperazine cycle condensation. Dispirotripiperazines contain two free positions on the terminal nitrogen atoms. Therefore, these systems can be used for condensation in the presence of alkali with compounds containing active halogen atoms. Usually, the products of this process have symmetrical structure, white or slight yellow color, are soluble in water and insoluble in organic solvents. They decompose under heating above

260 °C. From physico–chemical properties of studied compounds it is necessary to indicate the strong peak at 900/cm in the IR spectrum, which is a special property of all dispirotripiperazine compounds. Some features of the ^{1}H NMR spectra of the studied compounds have proven to be unexpected. Most of the synthesized compounds have a water of crystallization. These molecules of water were detected in the proton spectra as an independent peak (δ (ppm) 4.17–4.29) in the presence of the peak of water as solvent. This independent peak of water (5–10%) was seen in the ^{1}H NMR spectra whether deuterium oxide or DMSO-d_6 with admixture of water was used as solvent.

An excellent compatibility of dispirotripiperazine derivatives has been demonstrated for confluent HeLa, MDCK, and GMK cells as well as proliferating L-929 and K-562 cells (Tables 1 and 2). The results of this study confirm those from Traganos et al. (1980) who examined the effect of prospidine on cell cycle kinetics of a variety of cell lines. In this study, prospidine was cytostatic only for Friend leukemia and L1210 cells at a concentration of 10 mg/ml. A slightly enhanced but also low cytotoxicity and/or growth inhibitory activity were found for the compounds **2**, **7**, **8** and **19**. The enhanced cytotoxicity in proliferating cells was comparable with that observed for PFA, used as positive control, and acyclovir (Schmidtke et al., 2001).

Table 1
Cytotoxicity and growth inhibitory activity if dispirotripiperazines **1**–**22**

Compound no.	MW	CC_{50} (μM) in GMK cells	GI_{50} (μM) in growing	
			L-929 cells	K-562 cells
Prospidine	481.3	>1038.8[a]	>415.5[a]	>415.5[a]
Spirobromine	566.2	>883.1	>353.2	>353.2
1	297.3	>672.7	>672.7	>672.7
2	327.3	207.1[a,b]	113.7[a]	187.0[a]
3	394.2	>507.4	437.3	305.4
4	508.3	>90.4	>90.4	>90.4
5	589.6	>339.2	>339.2	235.4
6	508.3	>393.5[a,b]	>393.5[a]	>393.5[a]
7	526.4	826.9[a]	312.9[a]	141.1[a]
8	666.3	708.8[a]	67.2[a]	111.5[a]
9	694.4	>720.0[a]	>288.0[a]	>288.0[a]
10	657.5	>760.4[a]	>304.2[a]	>304.2[a]
11	758.5	>659.2[a]	>263.7[a]	>263.7[a]
12	982.0	>509.2[a]	>203.7[a]	>203.7[a]
13	892.0	>560.5[a]	>224.2[a]	>224.2[a]
14	766.3	>652.5[a]	>261.0[a]	>261.0[a]
15	681.6	>733.6[a]	>293.4[a]	>293.4[a]
16	709.7	>70.7	>281.8	>281.8
17	737.7	>67.8	>271.1	>271.1
18	765.8	>65.3	>261.2	>261.2
19	575.4	>869[a]	132.1[a]	150.0[a]
20	603.5	>331.4	>331.4	>331.4
21	605.4	>330.9	not tested	not tested
22	606.4	>659.6	>82.4	>82.4

[a] Values of anti-HSV-1-active compounds.
[b] Values calculated from one test only.

Table 2
Cytotoxicity and growth inhibitory activity if dispirotripiperazines **23**–**46**

Compound no.	MW	CC_{50} (μM) in GMK cells	GI_{50} (μM) in growing	
			L-929 cells	K-562 cells
23	724.6	>552.0	>55.2	>55.2
24	689.7	>290.0	>72.5	>72.5
25	642.3	715.1[a]	>311.4[a]	>311.4[a]
26	821.4	>243.5	>243.5	>243.5
27	569.4	>1756.2[a]	>351.2[a]	>351.2[a]
28	597.3	>837.1[a]	>334.8[a]	>334.8[a]
29	686.4	>291.4	>291.4	>291.4
30	541.4	>369.4	>369.4	>369.4
31	638.4	>313.3	>313.3	>313.3
32	629.5	>317.7	Not tested	Not tested
33	589.5	>339.3	Not tested	Not tested
34	675.4	>296.1	Not tested	Not tested
35	711.6	>70.3	>70.3	>70.3
36	623.4	>320.8	Not tested	Not tested
37	609.5	>820.3	>328.1	>328.1
38	860.6	>581.0[a]	>232.4[a]	>232.4[a]
39	874.6	>571.8[a]	>228.7[a]	>228.7[a]
40	1369.0	>365.2[a]	>146.1[a]	>146.1[a]
41	399.6	>500.5	>500.5	>500.5
42	413.6	>483.6	>483.6	>483.6
43	605.3	>330.4	>330.4	>330.4
44	619.3	>322.9	>322.9	>322.9
45	797.2	>627.2	Not tested	Not tested
46	825.3	>242.3	Not tested	Not tested

[a] Values of anti-HSV-1-active compounds.

Whereas no antiviral activity was detected against CVB3 and influenza virus A, various test compounds inhibited the HSV-1-induced CPE in GMK cells very effectively. In order to investigate the mechanism of activity, one selected compound was tested in plaque reduction assays. It was found that, the compound did not remain active when first added after viral adsorption. Therefore, we believe that the antiviral effect of the compound is based on the inhibition of viral binding or fusion. Certain differences in the anti-HSV-1-activity were observed between different compounds depending on their structure. In contrast to previous studies (Levkovskaya et al., 1989), spirobromine, used as reference compound in this study, did not exhibit any antiviral activity. Prospidine, the second reference compound used in this study, and its analogues **2**, **6** and **7** inhibited the replication of HSV-1 in GMK cells (Table 3). Interestingly, most of the bis-heteryl derivatives of dispirotripiperazine (**8**–**36**), synthesized with derivatives of pyrimidine, pyridine or benzene with active chlorine atoms exhibited a more potent antiviral effect than prospidine. The 50% inhibitory concentration of some compounds was lower than or comparable to that of the anti-HSV-1 drug PFA used as positive control in each microtiter plate. However, only 5-nitropyrimidine derivatives possessed antiviral activity indicating that the nitrogroup in the 5 position of the pyrimidine ring is necessary for activity (compounds **8**–**20**, **25**). Compounds with furoxanopyrimidine parts can be regarded as direct analogs of the 5-nitroderivatives **8** and **9**. These compounds exhibited an excellent antiviral activity

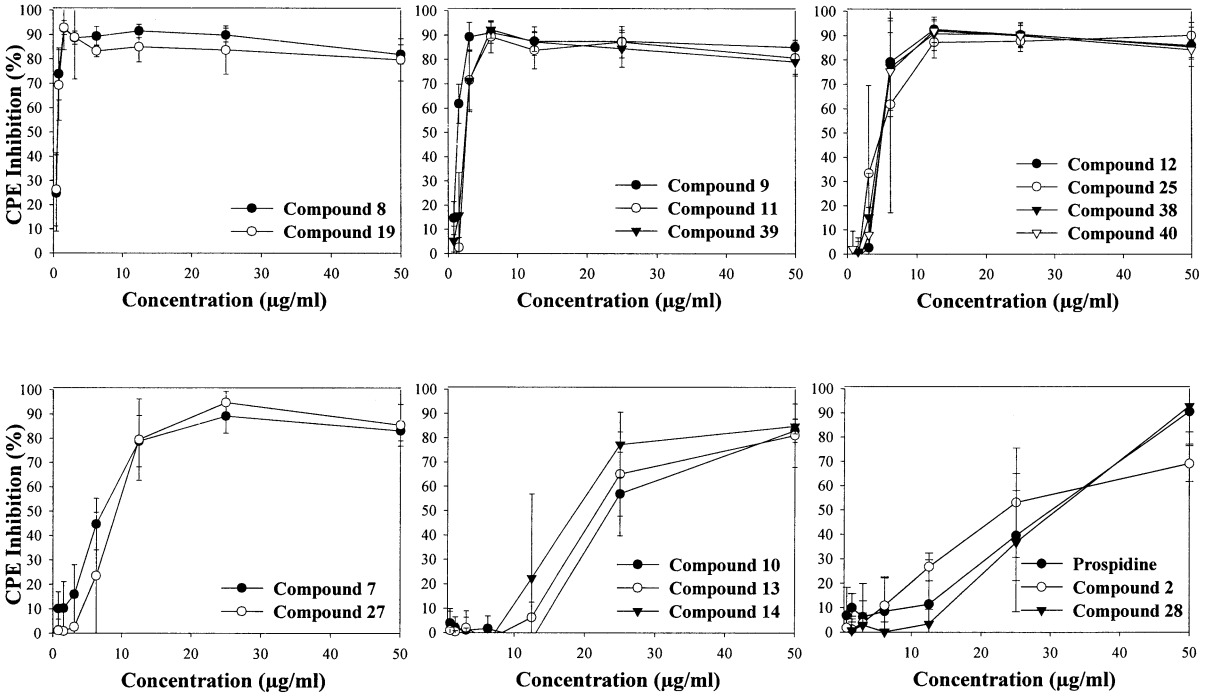

Fig. 3. Dose–response curves of anti-HSV-1 active dispirotripiperazines in GMK cells. GMK cells were treated with test compounds and immediately afterwards infected with HSV-1. 48 h post-infection the inhibition of CPE was scored as described in Section 2. Values are the mean ± standard deviation of three separate experiments.

which was only slightly reduced in comparison to derivatives **8** and **9**. After the introduction of electron donating substituents in the 4 position of pyrimidine (**15**–**18**) this activity disappeared. The compounds **8**, **9** and the derivative **19** (compounds **20** is insoluble in water) showed nearly the same antiviral effect (Fig. 3). Since the chlorine substituent may hydrolize quickly, it is likely that in these cases the anti-HSV-1-activity of hydroxy/oxo derivatives was determined. Taken together, these facts demonstrate that the lowering of electron density of the terminal pyrimidine rings increases the antiviral activity whereas electron donating substituents (for example, different amino groups) reduce it. Introduction of a methyl group (**10**) in position 2 of the pyrimidine ring resulted in a slight decrease of antiviral activity. In addition, the replacement of one or both nitrogen atoms of the pyrimidine ring by carbon (**30**–**36**) or the use of other derivatives derived from dispirotripiperazine (**26**, **29**, **41**–**46**) led to the disappearance of antiviral activity. The fact that derivatives either without a dispirotripiperazine moiety or with that moiety disrupted (**41**–**46**) did not inhibit the replication of HSV-1 demonstrate that this positively charged part of the test compounds is also absolutely necessary for antiviral activity. The impact of symmetric arrangement of heterocycles at both ends of the derivatives of dispirotripiperazine could not be finally demonstrated because the synthesis of asymmetrical compounds was unsuccessful. However, the results obtained with compounds **38**, **39**, and **40** suggest that symmetry of heterocycles is not absolutely necessary. The results of this study strongly suggest that dispirotripiperazines may be potential candidates for antiviral therapy. Therefore, further studies about the precise mechanism of activity, antiviral activity against other herpesviruses, toxicity and antiviral activity in vivo seemed warranted.

Table 3
IC_{50} and TI in the HSV-1/GMK cells system

Compound no.	IC_{50} against HSV-1 (μM)	TI (CC_{50}:IC_{50})
Prospidine	62.47	>16.63
2[a]	71.92	>2.88
6[a]	66.10	>5.95
7	14.02	58.98
8	0.90	787.6
9	2.02	>356.43
10	35.83	>21.22
11	3.49	>188.88
12	5.15	>98.87
13	24.44	>22.93
14	24.55	>26.58
19	1.08	>806.43
25	7.71	92.75
27	16.17	>108.61
28	51.77	>16.17
38	5.67	>102.47
39	1.48	>386.35
40	3.71	>98.44

[a] Calculated from the mean dose–response-curve of two assays.

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References

Andrei, G., Snoeck, R., De Clercq, E., 1995. Susceptibilities of several drug-resistant herpes simplex virus type 1 strains to alternative antiviral compounds. Antimicrob. Agents Chemother. 39, 1632–1635.

Benenson, E.V., Timina, O.B., 1994. Prospidine versus methotrexate pulse in highly active rheumatoid arthritis: a controlled 6-month clinical trial. Clin. Rheumatol. 13, 54–59.

Chernov, V.A., Safonova, T.S., Minakova, S.M., Dorokhova, M.I., 1984. Spirobromine: antitumoral preparations used in acute leucosis, cutaneous reticulosis, malignant lymphoma, sarcoma and larynx cancer. Pat. SU 1092774, C.A. 103:32292.

De Clercq, E., 1998. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. Antiviral Res. 38, 153–179.

Fomina, A.N., Nikolaeva, I.S., Pushkina, T.V., Krylova, Y.L., Levkovskaya, L.G., Deev, V.B., Safonova, T.S., 1989. Synthesis and antiviral activity of spirobromine and its analogs. Khim. Farm. Zh. 24, 307–310.

Grohn, P., Heinonen, E., Appelqvist, P., Hajba, A., Holopainen, E., Paavolainen, M., Palva, T., 1984. Prospidine chemotherapy in recurrent head and neck carcinoma: a phase II study. Cancer Treat Rep. 68, 915–917.

Kimura, T., Takase, Y., Hayashi, K., Tanaka, H., Ohtsuka, I., Saeki, T., Kogushi, M., Yamada, T., Fujimori, T., Saitou, I., Akasaka, K., 1993. Structure-activity relationship of *N*-[2-(dimethylamino)-6-[3-(5-methyl-4-phenyl-1H-imidazol-1-yl)propoxy]phenyl]-*N*′-pentylurea and analogues. Novel potent inhibitors of acyl-CoA: cholesterol *O*-acyltransferase with antiatherosclerotic activity. J. Med. Chem. 36, 1630–1640.

Lavrik, O.I., Kinzirskii, A.S., Shashkina, L.F., Ivanova, V.M., Kruglova, O.N., 1993. The testing of the carcinogenicity of dispirotripiperazine derivatives by combined administration with sodium nitrite. Eksp. Klin. Farmakol. 56, 54–57.

Levkovskaya, D.G., Mamaeva, I.E., Pushkina, T.V., Nikolaeva, I.S., Krylova, L.V., Fomina, A.N., 1989. Synthesis and antiviral activity of bisquaternary salts of 3,12-(3′-Halogenoacyl)3,12-diaza6,9-diazonia dispiro (5.2.5.2) hexadecane. Khim. Farm. Zh. 23, 307–310.

Makarov, V.A., Sedov, A.L., Anisimova, O.S., Granik, V.G., 1996. Highly polarised enamines. 2. Synthesis and study of transmission of α,α-diamino-β-nitro-β-cyanethylene derivatives. Khim. Geterocicl. Soed. 32, 669–707.

Makarov, V.A., Sedov, A.L., Nemeriuk, M.P., Safonova, T.S., 1994. Investigation of bisheteryl derivatives of piperazine and its analogs. 1. Synthesis and transformation of *N*,*N*′-bis(2-R-5-nitropyrimidyl-6)dispirotripiperazinium dichloride. Khim. Geterocicl. Soed. 7, 971–975.

Molla, A., Granneman, G.R., Sun, E., Kempf, D.J., 1998. Recent developments in HIV protease inhibitor therapy. Antiviral Res. 39, 1–23.

Rotbart, H.A., O'Connell, J.F., McKinlay, M.A., 1998. Treatment of human enterovirus infections. Antiviral Res. 38, 1–14.

Safanova, T.S., Chernov, V.A., Minakova, S.M., Dorokhova, M.I., Levkovskaya, L.G., Traven, N.I., Andreayanova, T.A., Serochkina, L.A., Mamaeva, I.E., 1983. New antitumor agent—spirobromine. Khim. Farm. Zh. 5, 626–629.

Schmidt, A.C., Couch, R.B., Galasso, G.J., Hayden, F.G., Mills, J., Murphy, B.R., Chanock, R.M., 2001. Current research on respiratory viral infections: third international symposium. Antiviral Res. 50, 157–196.

Schmidtke, M., Makarov, V., Werner, W., 2000. Verwendung von Diazadispiro-Heterozyklen zur Prophylaxe und Therapie von Virusinfektionen. Patent, Dt. 198 51 375.5.

Schmidtke, M., Schnittler, U., Jahn, B., Dahse, H., Stelzner, A., 2001. A rapid assay for evaluation of antiviral activity against coxsackie virus B3, influenza virus A, and herpes simplex virus type 1. J. Virol. Meth. 95, 133–143.

Siubaev, R.D., Stebaeva, L.F., Shvarts, G.I., Chernov, V.A., 1990. The effect of spirobromine and prospidine on acute and chronic inflammation of rats. Farmakol. Toksikol. 53, 58–60.

Traganos, F., Staiano-Coico, L., Darzynkiewicz, Z., Melamed, M.R., 1980. Effects of prospidine on survival and growth of mammalian cells in culture. J. Natl. Cancer Inst. 65, 993–999.

Wutzler, P., Thust, R., 2001. Genetic risks of antiviral nucleoside analogues—a survey. Antiviral Res. 49, 55–74.

Wyde, P.R., 1998. Respiratory syncytial virus (RSV) disease and prospects for its control. Antiviral Res. 39, 63–79.

Zoulim, F., 1999. Therapy of chronic hepatitis B virus infection: inhibition of the viral polymerase and other antiviral strategies. Antiviral Res. 44, 1–30.