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Biological evaluation of 10-(diphenylmethylene)-4-azatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione derivatives

Research Article

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Abstract: Antibacterial and antifungal activity of 10-(diphenylmethylene)-4-azatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione derivatives were examined by the disc-diffusion method (growth inhibition zone diameter in agar medium). The MIC's for the most active agents were determined. Title compounds were also evaluated *in vitro* against representatives of different virus classes. Most of the tested compounds exhibit activity against CVB-2 virus.

Keywords: *10-(diphenylmethylene)-4-azatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione derivatives • Antibacterial • Antifungal and antiviral activity*

1. Introduction

Aminoalkanol derivatives show a broad spectrum of biological activities. Numerous compounds containing the 3-amino-2-hydroxypropyl/propoxy fragment express cardiostimulant, antiarrhytmic and hypotensive activity, acting at β- or α-adrenargic receptors [1-4]. Inserting selected piperazine moieties into the oxypropanolamine side chain increases the antagonistic α_1-adrenergic properties [5,6]. Arylpiperazine derivatives of this group also confer intraocular pressure lowering effects [7] and display considerable affinity for the serotonergic 5-HT receptors [8,9]. It was suggested that the isopropanolamine unit present in peptidic drugs (e.g. saquinavir, nelfinavir, amprenavir) could be responsible for their binding properties. Derivatives possessing this fragment were found to act as anti-HIV-1 protease inhibitors agents [10]. Furthermore, the piperazine nucleus is often found in the structure of agents exhibiting antioxidative [11] and antitumour properties [12-14].

It is known that compounds bearing short-amine fragments possess antibacterial and antifungal activity [15]. The introduction of an aryl group to the piperazine moiety shifted the activity from antibacterial [16] to antiviral, with a specific action against HIV [17-19]. To further define these effects, we decided to investigate derivatives, generally differently substituted

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10-(diphenylmethylene)-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-diones. Their activities against bacteria, fungi and viruses were examined.

2. Experimental Procedures

2.1 Chemicals

All chemicals were of analytical grade (Aldrich) and were used without further purification. The list of 10-(diphenylmethylene)-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5-dione and its derivatives that were studied is shown in Figure 1. Compounds used in this work were synthesized previously [20]. The starting compound was obtained in Diels-Alder reaction of 6,6-diphenylfulvene with maleimide and later subjected to the reaction with 2-(chloromethyl)oxirane in anhydrous medium. Later the resultant oxirane was reacted with appropriate amines giving aminoalkanol derivatives. For biological studies free bases were converted into corresponding hydrochloride salts.

2.2 Microorganisms

2.2.1 Bacterial strains

The antibacterial activity of compounds was tested against a series of Gram-positive bacteria: *Staphylococcus aureus* ATCC 4163, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Enterococcus hirae* ATCC 10541, *Micrococcus luteus* ATCC 9341, *Micrococcus luteus* ATCC 10240 and Gram-negative rods: *Escherichia coli* ATCC 10538, *Escherichia coli* ATCC 25922, *Escherichia coli* NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* NCTC 6749, *Pseudomonas aeruginosa* ATCC 27853, *Bordetella bronchiseptica* ATCC 4617. Antifungal activity was tested against yeasts: *Candida albicans* ATCC 10231, *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 220191. Microorganisms used in this study were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

2.2.2 Viruses

As reported in Table 2, title compounds were evaluated in cell-based assays against viruses representative for two from the three genera of the Flaviviridae family, *i.e.* Flaviviruses (Yellow Fever Virus, YFV) and Pestiviruses (Bovine Viral Diarrhoea Virus, BVDV), as Hepaciviruses can hardly be used in routine cell-based assays. Title compounds were also tested against representatives of other virus families. Among ssRNA+ group there was a retrovirus (Human Immunodeficiency Virus Type 1, HIV-1) and two Picornaviruses (Coxsackie Virus Type B2, CVB-2 and Polio virus type-1, Sabin strain, Sb-1); among ssRNA- viruses there was a Rhabdoviridae (Vesicular Stomatitis Virus, VSV) representative. Among double-stranded RNA (dsRNA) viruses it 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).

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.

2.3 Susceptibility testing procedures

Antimicrobial activity was examined by the disc diffusion and MIC method under standard conditions, using Mueller–Hinton II agar medium (Becton Dickinson) for bacteria and RPMI agar with 2% glucose (Sigma) for yeasts, according to CLSI (previously NCCLS) guidelines [21,22]. Solutions containing the tested agents were prepared in methanol or DMSO. For the disc diffusion method, sterile paper discs (9 mm diameter, Whatman No. 3 chromatography filter paper) were dripped with the compound solutions tested to obtain 400 µg of substance per disc. Dry discs were placed on the surface of an appropriate agar medium. The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35°C. Minimal Inhibitory Concentration (MIC) were examined by the twofold serial agar dilution technique [22]. Concentrations of the tested compounds in solid medium ranged from 3.125 to 400 µg/mL. The final inoculum of studied organisms was 10^4 CFU/mL (colony forming units per mL), except the final inoculum for E. hirae ATCC 10541, which was 10^5 CFU/mL. Minimal inhibitory concentrations were read off after 18 h (for bacteria) and 24 h (for yeasts) of incubation at 35°C.

2.4 Cytotoxicity and antiviral activity assays

2.4.1 Compounds

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

2.4.2 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.

2.4.3 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 $6x10^5$, $1x10^6$ and $5x10^5$ cells/mL, respectively, in maintenance medium, with or without serial dilutions of tested compounds. Cell viability was determined after 48-120 hours 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.

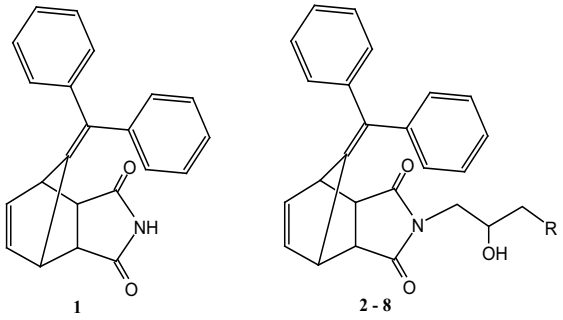
For cytotoxicity evaluations, exponentially growing cells derived from human haematological tumors [$CD4^+$ human T-cells containing an integrated HTLV-1 genome (MT-4)] were seeded at an initial density of $1x10^5$ cells/mL in 96 well plates in RPMI-1640 medium, supplemented with 10% fetal calf serum (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 presence or absence 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 [23].

2.4.4 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 $1x10^4$ MT-4 were added to each well of flat-bottom microtitre trays containing 50 µL of RPMI, with or without 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 $5x10^4$ and $3x10^4$ 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



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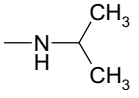
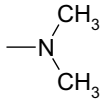
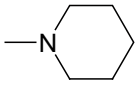
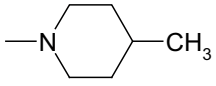
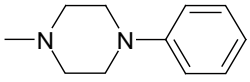
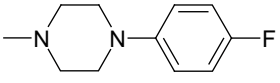
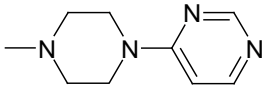
2	 isopropylamine
3	 dimethylamine
4	 piperidine
5	 4-methylpiperidine
6	 1-phenylpiperazine
7	 1-(4-fluorophenyl)piperazine
8	 1-pyridin-2-ylpiperazine

Figure 1. Chemical structures of 10-(diphenylmethylene)-4-azatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione and its derivatives.

medium) to give an m.o.i = 0.01. One hour later, 50 µL of MEM Earle's medium, supplemented with inactivated foetal calf serum (FCS), 1% final concentration, with or without 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 $2x10^5$ 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, with or without 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) days 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.

AZT (3'-azido-thymidine), NM 108 (2'-β-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.

3. Results and Discussion

In the present study 10-(diphenylmethylene)-4-azatricyclo-[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione and its derivatives (Figure 1) were tested *in vitro* against bacteria, yeast-like organisms and viruses.

Preliminary antimicrobial tests by the disc-diffusion method showed activity of compounds *2-5* against Gram-positive bacteria and yeasts. Gram-negative rods were resistant to all tested agents. Compound *1* (basic) and *6-8* were inactive. The active compounds in this assay were tested in order to determine their minimal inhibitory concentration (MIC) values. The results are summarized in Table 1.

Compounds behave differently against different strains of the same bacterial species because standard bacterial strains have a different sensivity to antibacterial compounds (antibiotics and disinfectants). Compounds which inhibited growth of microorganisms in the disc diffusion method were defined as active. Growth of inhibition zones above 15 mm and MIC values of at least 100 µg/mL were indicative of compounds with interesting antimicrobial activity.

The data may serve as a good subject for discussion of the influence of a compound's molecular structure on its biological activity. It seems that antibacterial activity is determined by the nature of the substituent of the hydroxypropyl branch. Depending on the amine's type an increase or a decrease of antimicrobial properties can be observed. Derivatives containing short-amine or cyclic-amine fragments show activity, whereas those with piperazine moieties appear inactive. The

Compound / Strain	2 mm (µg/mL)	3 mm (µg/mL)	4 mm (µg/mL)	5 mm (µg/mL)
Staphylococcus aureus ATCC 4163	23 (100)	19 (200)	20 (100)	16 (200)
Staphylococcus aureus ATCC 25923	24 (200)	17 (200)	17 (200)	14 (200)
Staphylococcus aureus ATCC 6538	24 (100)	17 (200)	19 (100)	16 (100)
Staphylococcus aureus ATCC 29213	25 (100)	17 (200)	18 (100)	15 (100)
Staphylococcus epidermidis ATCC 12228	25 (100)	19 (200)	18 (100)	17 (100)
Bacillus subtilis ATCC 6633	27 (100)	23 (200)	23 (100)	18 (100)
Bacillus cereus ATCC 11778	21 (200)	20 (200)	20 (100)	16 (200)
Enterococcus hirae ATCC 10541	18 (200)	16 (200)	13 (200)	11 (400)
Micrococcus luteus ATCC 9341	28 (50)	24 (200)	26 (50)	19 (100)
Micrococcus luteus ATCC 10240	28 (100)	25 (200)	24 (50)	19 (100)
Candida albicans ATCC 10231	20 (200)	14 (200)	14 (200)	17 (200)
Candida albicans ATCC 90028	16 (200)	13 (200)	15 (200)	14 (200)
Candida parapsilosis ATCC 220191	18 (200)	12 (200)	12 (100)	14 (200)

Table 1. Antibacterial and antifungal activities of 10-(diphenylmethylene)-4-azatricyclo- [5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione and its derivatives - diameter of the growth inhibition zone [mm] and Minimal Inhibitory Concentration (MIC in parentheses) [µg/mL].

Compounds not listed above were completely inactive in concentration up to 400 µg per disc.

Compounds	[a]MT-4 CC_{50} [μM]	[b]HIV-1 EC_{50} [μM]	[c]MDBK CC_{50} [μM]	[d]BVDV EC_{50} [μM]	[e]BHK-21 CC_{50} [μM]	[f]YFV EC_{50} [μM]	[f]Reo-1 EC_{50} [μM]	[g]Vero-76 CC_{50} [μM]	[h]HSV-1 EC_{50} [μM]	[h]VV EC_{50} [μM]	[h]VSV EC_{50} [μM]	[h]CVB-2 EC_{50} [μM]	[h]Sb-1 EC_{50} [μM]
1	51	>51	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2	17	>17	30	>30	17	>17	>17	60	>60	>60	>60	12	>60
3	20	>20	50	>50	35	12	>22	60	>60	>60	>60	6	>60
4	20	>20	45	>45	10	>10	>10	60	>60	>60	>60	3	>60
5	21	>21	17	>17	9	>9	>9	100	>100	>100	>100	>100	>100
6	>100	>100	>100	>100	>100	>100	>100	92	>92	>92	>92	10	>92
7	25	>25	13	>13	9	>9	>9	50	>50	>50	>50	4	>50
8	22	>22	18	>18	11	>11	>11	14	>14	>14	>14	3	>14
Reference compounds													
AZT[§]	50	0.01											
NM 108^				1.8		2.5							
NM 176*												23	18
M 5255**										1.8			
ACG***									3				

Table 2. Cytotoxicity and antiviral activity of 10-(diphenylmethylene)-4-azatricyclo[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione and its derivatives.

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 CC50 (Cytocidal Concentration – the amount of a drug (μM) at which 50% of cells become dead). .
a - Compound concentration (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 MTT method.
b - Compound concentration (μM) required to achieve 50% protection of MT-4 cells from the HIV-1-induced cytopathogenicity, as determined by the MTT method.
c - Compound concentration (μM) required to achieve 50% protection of MDBK cells from the BVDV (Bovine Viral Diarrhea Virus) induced cytopathogenicity, as determined by the MTT method.
d - Compound concentration (μM) required to achieve 50% protection of BHK cells (Kidney fibroblast) from the YFV (Yellow Fever Virus) induced cytopathogenicity, as determined by the MTT method.
e - Compound concentration (μM) required to reduce the plaque number of CVB-2 (Coxsackievirus B2), Sb-1(Poliovirus 1), RSV (Respiratory Syncytial Virus), VV (Vaccinia Virus) and VSV (Vesicular Stomatitis Virus) by 50% in VERO-76 monolayers (Monkey normal kidney).
f - Compound concentration (mM) required to achieve 50% protection of BHK cells from the YFV and Reo (Reovirus 1) – induced cytopathogenicity, as determined by the MTT method.
g - Compound concentration (μM) required to reduce the viability of mock-infected VERO-76 monolayers by 50%, as determined by the MTT method.
h - Compound concentration (μM) required to reduce the plaque number of HSV-1 (Herpesvirus 1), VV, VSV, CVB-2, Sb-1 and RSV by 50% in VERO-76 monolayers.
§3'-azido-thymidine
^2'-ß-methyl-guanosine
**2'-ethynyl-D-citidine*
***mycophenolic acid*
****acycloGuanosine*
N.D. - not determined

best results were found for compound *2*, in which an isopropyl branch is present. It was active mainly against various *Staphylococcus aureus* and *Micrococcus luteus* strains, as well as *Bacillus subtilis* ATCC 6633 and *Bacillus cereus* ATCC 11778. Compounds *4, 3* and *5* (with dimethylamine, piperidine and 4-methylpiperidine units) were also characterized by strong and broad antibacterial activities. Mentioned derivatives were active against all tested Gram-positive bacteria and showed antifungal activity against selected *C. albicans* and *C. parapsilosis* strains. The MIC values are in the range of 50 μg/mL to 400 μg/mL. The presence of phenyl and/or piperazine rings could be the reason for inactivity of compounds *6, 7* and *8*.

Surprisingly all tested derivatives, apart from *5*, presented antiviral activity (Table 2). As far as the antiviral activity is concerned, compounds *2-4* and *6-8* turned out to be inhibitors of CVB-2 (EC_{50} range = 3-12 μM). Compound *3* was also active against YFV (EC_{50} = 12 μM). In contrast to antibacterial tests, the representative viruses tested were susceptible to both short-chain, cyclic and arylpiperazine containing derivatives. The most active compounds are derivatives of 1-pyridin-2-ylpiperazine (*8*), 1-(4-fluorophenyl)-piperazine (*7*),

dimethylamine (*3*) and piperidine (*4*). The antiviral profile of 10-(diphenylmethylene)-4-azatricyclo-[5.2.1.$0^{2,6}$] dec-8-ene-3,5-dione derivatives could be related to the volume of its imide part, high lipophilicity and aromaticity of the compound.

None of the title compounds, however, turned out to be as active against HIV-1, BVDV or representatives of ssRNA-, dsRNA or DNA viruses.

4. Conclusions

In light of the above-mentioned results, we conclude that derivatives of bicyclic imides with aliphatic secondary amines attached to a hydroxypropyl linker are active against a broad spectrum of microorganisms. Imide derivatives with arylpiperazine units were inactive. This dependence was not seen in tests of the compounds against viruses. All tested derivatives, apart from *5*, presented antiviral activity. In particular, in cell-based assays the compounds *2-4* and *6-8* were found to be inhibitors of CVB-2. However compound *3* was most potent against YFV.

In conclusion we can affirm that derivatives of 10-(diphenylmethylene)-4-azatricyclo-[5.2.1.$0^{2,6}$]dec-8-ene-3,5-dione can act as antimicrobial agents.

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