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Antimicrobial and antineoplastic activities of new 4-diazopyrazole derivatives

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Abstract – Several new 4-diazopyrazole derivatives were prepared by the reaction of 3-methyl-5(substituted-benzamido)pyrazoles with an excess of nitrous acid in acetic acid solution. The compounds were tested for antiretroviral activity in HIV-1 infected MT-4 cells and antiproliferative effects against a panel of human leukemia, lymphoma and solid tumor cell lines. They were also tested for activity against representative gram-negative (*Shigella, Salmonella*) and gram-positive (*S. aureus, D group Streptococcus*) bacteria as well as fungi (*C. albicans, C. paratropicalis, C. neoformans* and *A. fumigatus*). Compounds were devoid of anti HIV-1 and antimicotic activities, whereas they were active against tumor cell lines, with inhibitory activity (IC_{50}) in the range 2.4–20 μM and bacteria. The highest microbial susceptibility was shown by gram-positive bacteria, with minimum inhibitory concentrations in the range 0.8–12.5 μM. © Elsevier, Paris

4-diazopyrazole / antiproliferative activity / antibacterial activity

1. Introduction

Besides being useful precursors for the synthesis of antineoplastic triazenes (e.g. dacarbazina) [1], diazoazoles represent a class of biologically active substances, primarly endowed with antimicrobial and antineoplastic activities [2]. Baraldi et al. reported in vitro activity as well as QSAR studies for several 4-diazopyrazoles bearing an N-heterocycle substituted carbamoyl group [3–5]. The bioactivity of this class of compounds is probably due to reactivity towards nucleophylic groups of essential biomolecules such as enzymes and structural proteins.

Recently some of us have reported a facile synthesis of 1-substituted 5-benzamido-4-diazopyrazoles which showed antimicrobial activity against several gram-positive bacteria, yeasts and a mycelial fungus. The highest microbial susceptibility was shown by gram-positive bacteria, with minimum inhibitory concentrations (MIC) in the range 0.5–12.5 μg/mL. Moreover, some compounds were active in the range 2–8 μg/mL against methicillin-resistant clinical isolates of *S. aureus* [6].

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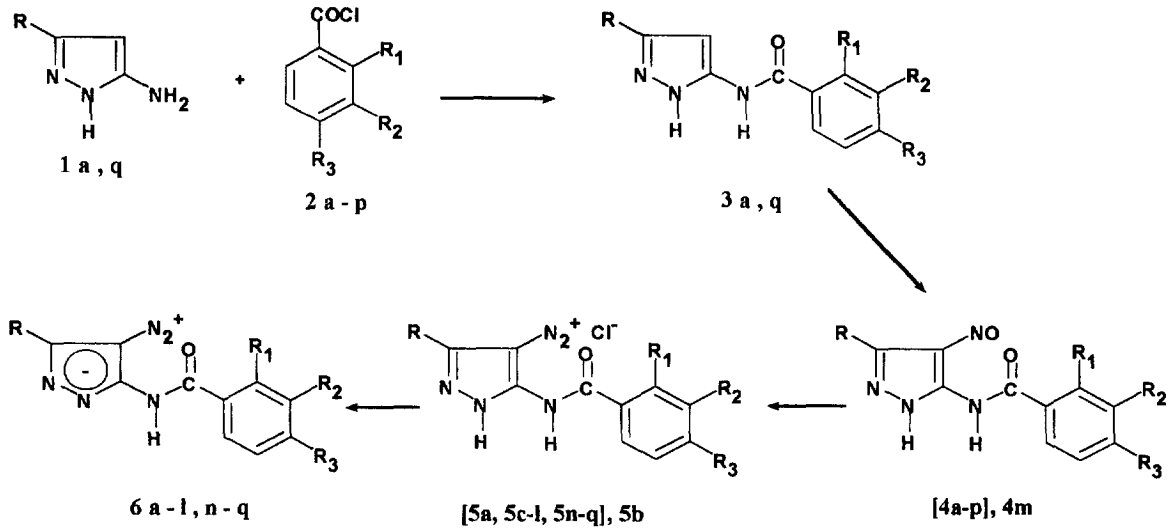
As an extension of this research, in order to gain more insight in the structure–activity relationships of 5-benzamido-4-diazopyrazoles, we prepared a series of 1-unsubstituted 5-benzamido-4-diazopyrazoles bearing a number of different groups on the benzamide moiety which give a good discrimination between hydrophobic, electronic and steric effects. The new compounds were tested against representative gram-negative and gram-positive bacteria, as well as fungi. Moreover, it was thought of interest to test these compounds for their potential antineoplastic and anti-HIV activities.

2. Results and discussion

2.1. Chemistry

The 5-benzamidopyrazoles **3a–q**, used as starting compounds, were obtained by reacting 3(5)-*R*-5(3)-aminopyrazoles **1a,q** with substituted benzoyl chloride **2a–p**.

The 4-diazopyrazole derivatives **6** were prepared, as outlined in *figure 1*, by reaction of nitrous acid with the amides **3** in acetic acid solution. The structure of all the new compounds were assigned on the basis of their satisfactory analytical and spectroscopic data. In



	a	b	c	d	e	f	g	h	i	l	m	n	o	p	q
R	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	C_6H_5
R_1	H	NO_2	Cl	H	H	H	H	H	H	H	H	H	H	H	NO_2
R_2	H	H	H	H	Cl	Cl	H	H	H	H	H	H	H	H	H
R_3	H	H	H	Cl	H	Cl	CH_3	OCH_3	CF_3	$C(CH_3)_3$	NO_2	I	Br	F	H

Figure 1.

fact, the IR spectra of diazo derivatives **6** showed the diazo and carbonyl stretching bands in the 2180–2130 cm^{-1} and 1680–1655 cm^{-1} region respectively. The marked difference in the carbonyl absorption of the 1-unsubstituted 5-benzamido-4-diazopyrazoles **6** respect on the 1-substituted ones **7** [6–8] (ν (CO): 1620–1600 cm^{-1}) is indicative of a different charge distribution in these two classes of diazopyrazoles (see *figure 2*). Moreover, because of the presence in the IR spectra of compounds **6** of NH bands in the 3240–2780 cm^{-1} range, it is reasonable to hypothesize that diazopyrazoles might bind to produce dimers of type **8**, at least in the solid state (see *figure 3*).

The nitrous acid/amide molar ratio influenced significatively the reaction products formation. In fact, when a seven fold excess of nitrous acid than the amide **3** was used, diazopyrazoles were obtained as the unique reaction product when the amide bears, in the benzamide moiety, the 2-NO_2 and 2-Cl substituents (derivatives **3b,c**), whereas for the analogous 4-NO_2 substituted amide **3m**, only the 4-nitrosopyrazole derivative **4m** was isolated. Green mixtures of 4-nitroso and 4-diazoderivatives were obtained for all the other amides.

At this point we doubled the nitrous acid/amide molar ratio and recovered again the nitrosopyrazole derivative **4m** from the amide **3m**, and the mixture nitroso/diazo from the 3,4-dichloro derivative **3f**. Finally, all the other amides gave only 4-diazopyrazoles.

It is reported that 4-diazopyrazoles take place through intermediates nitrosopyrazoles and pyrazole-diazonium salts which are yielded when pyazoles are reacted with excess nitrous acid [9]. On the basis of

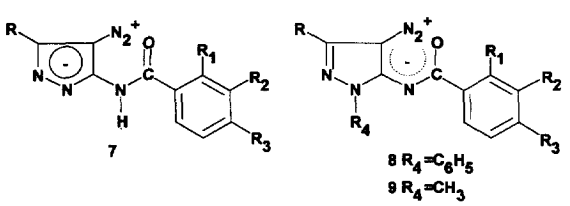


Figure 2. Charge distribution for substituted and unsubstituted 5-benzamido-4-diazopyrazoles. For identification of substituents see *figure 1*.

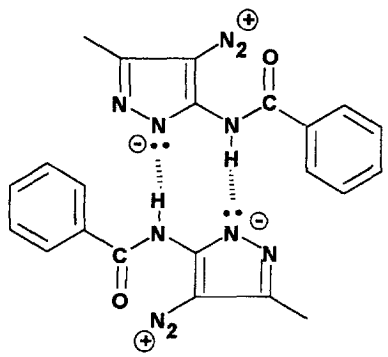


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Figure 3.

these observations, as well as of our experimental data, it seems that the intermediates 4-nitrosopyrazoles **4** require different amounts of nitrous acid to afford the related diazonium salts **5** which, in turn, on pH adjustment transform in the diazopyrazoles.

The different chemical beaviour of the 4-nitrosopyrazole derivatives can be perhaps justified comparing the isomers **4b** and **4m** (see *figure 4*). The only apparent difference for the two nitroso compounds is the solubility in the reaction medium. In fact, the solubility in acetic acid of the 2-NO_2 isomer **4b** [10] is 0.3920 g/100 mL, and for the 4-NO_2 one **4m** 0.012 g/100 mL. At this point we realized that the 4-NO_2 isomer, in view of the low concentration into the acetic acid medium, afford the diazonium salt very slowly and, as a consequence, the nitrosopyrazole is recovered after 24 h practically unreacted. No attempt was carried out in order to transform **4m** by using a greater quantity of nitrous acid.

The intermediate pyrazole-4-diazonium salts 5 were converted at pH = 5 into 4-diazopyrazole derivatives **6**. Only the diazonium salt **5b**, was isolated and identified.

2.2. Biological results

The 4-diazopyrazole derivatives **6** were evaluated for antiretroviral activity in MT-4 cells acutely infected with HIV-1. None of them, however, was capable of protecting the cells from the cytophatic effect induced by the virus (data not shown).

Although devoid of anti-HIV-1 activity, a number of derivatives inhibited the proliferation of uninfected MT-4 cells at micromolar concentrations, thus showing a potential antiproliferative activity (*table III*).

In order to confirm the antiproliferative activity of the title compounds and to obtain some insights into their spectrum of activity, **6a,c,d,f, g,n,o** were further tested against a panel of human leukemia, lymphoma and solid tumor cell lines. Doxorubicin was used as reference drug (*table III*). Despite the good discrimination between hydrophobic, electronic and steric effects of substituents, the 50% inhibitory activity (range 2.4–20 μM) was poorly influenced by the substitution pattern of the benzamide moiety. Probably this is due to small electronic effects of substituents, because they are fairly removed from the diazo group which is the active centre of the molecule.

Test compounds were evaluated in vitro also against bacteria, such as *Staphylococcus aureus*, *D*

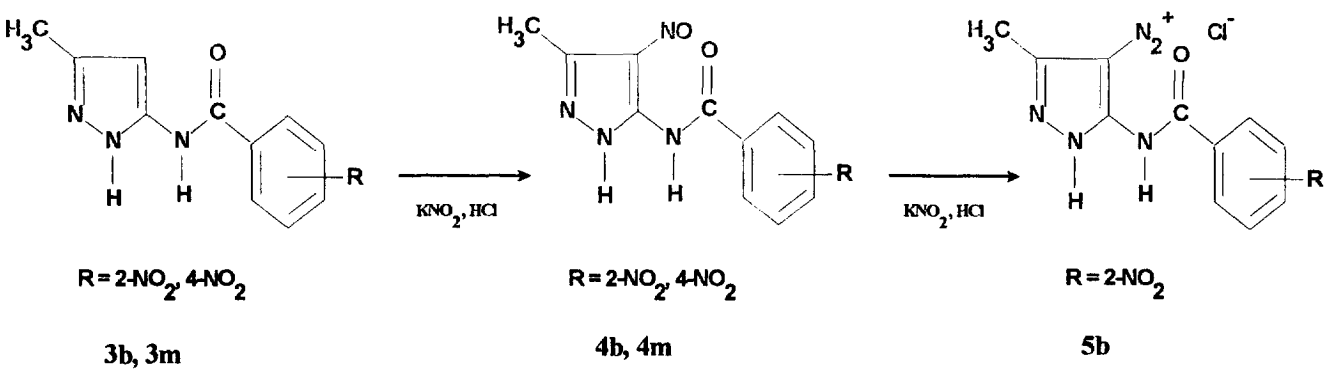

Figure 4.

group Streptococcus, *Salmonella sp.*, *Shigella sp.* and against a range of human pathogenic fungi, such as *C. albicans*, *C. parapsilosis*, *C. paratropicalis*, *Criptococcus neoformans*, and *Aspergillus fumigatus*. Miconazole (fungi) and Streptomycin (bacteria) were used as reference drugs.

The compounds showed inhibitory activity against gram-positive bacteria at concentrations between 0.8 and 12.5 μM (*table IV*). Among them, some (**6d–h,p,q**) were active against one or both the microorganisms at concentrations equal or slightly inferior to that of streptomycin. Moreover, the above compounds generally were bactericidal at doses equivalent to the MIC. It is worth noting that the compounds resulted substantially selective, i.e. active at concentrations lower than those cytotoxic for CD_4 lymphocytes (MT-4).The most selective derivative was **6i**, with a selective index (SI = CC_{50}/MIC) of 39 and 19.5 when evaluated against *D. Streptococcus* and *Staphylococcus aureus* respectively. As for the antiproliferative activity, the different effects of substituents poorly influenced the microbial susceptibility of the two microorganisms.

Regarding the gram-negative bacteria, they essentially showed a lower microbial susceptibility when compared to gram-positive bacteria; this might be related to the complex structure of their cell wall. Moreover, the substitution pattern on the benzamide moiety was more incisive on the biological activity, being the 2-nitro substituent the most effective of the series. Activity fell dramatically with the bulky *t*-butyl substitution.

Lastly, when the methyl group at the 3-position of the pyrazole nucleus in compounds **6b** was substituted with a phenyl one, activity encreased only against *D group Streptococcus*. The phenyl derivative showed high potency (MIC = 0.8 μM) but a low selectivity index due to its high cytotoxicity (SI = 12.5).

With respect to antifungal activity, compounds **6a–q** were found devoid of activity at the tested concentration (200 μM).

3. Experimental protocols

3.1. General procedure

All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected; IR spectra were recorded with a Jasco IR-810 spectrophotometer as nujol or hexachlorobutadiene mulls supported on NaCl disks; ^{1}H-NMR spectra were obtained in DMSO-d_6 using a Brüker AC-E 250 MHz spectrometer (using tetramethylsilane as internal standard). Microanalyses (C, H, N) performed in the laboratories of the Institut de Chimie Pharmaceutique, Université de Genève, Switzerland, were within ±0.4% of the theoretical values.

3.2. Syntheses

3.2.1. 3-R-5-(R_1,R_2,R_3-substituted)benzamidopyrazoles, ***3a–p***

Equimolar amounts (50 mmol) of 3(5)-*R*-5(3)-amino-pyrazole **1a** [11] and the substituted benzoyl chloride **2a–p** (commercially avalaible) in dry chloroform (125 mL) were reacted under reflux for 5 h. After the first hour, triethylamine was added in four portions of 3.4, 1.7, 0.9 and 0.9 mL respectively, at intervals of 1 h. The solution was evaporated under vacuum and the residue was first washed with H_2O and then stirred with a 20% aqueous sodium hydroxide solution for 1 h at room temperature. The suspension thus obtained was filtered and the solid material was washed throughly with water, air dried and crystallized from the appropriate solvent to give **3a,d–p**; yields 23–52 % (see *table I*). The alkaline mother liquors, when treated with a saturated ammonium chloride solution, gave a solid product which, by crystallization from ethanol, afforded **3b** [10], **3c** (yields 45–10% respectively) (see *table I*).

3.2.2. 3-R-5-(R_1,R_2,R_3-substituted)benzamido-4-diazopyrazoles ***6a–l,n–q***

Aqueous 37% hydrochloric acid solution and then potassium nitrite were added at room temperature to a magnetically stirred solution of **3a,b** [10], **3c–p,q** [12] (12.4 mmol) in acetic acid (120 mL). For amides **3b,c** were used 9 mL of hydrochloric acid solution, and 7.38 g (86.8 mmol) of potassium nitrite dissolved in water (3.8 mL). For all the remaining amides **3**, the above quantities were doubled. The mixture was then stirred for 24 h, and the suspension thus obtained was filtered. At this point the procedure depended on the compound. For diazopyrazoles **6b,e,n,o,q** the solid separated was suspended in water (250 mL) and the pH adjusted to 5 with sodium hydrogen carbonate. In the case of **6b**, the mixture was extracted with ethyl acetate (3 x 80 mL), and the combined extracts were dried (Na_2SO_4), and concentrated under reduced pressure. The solid residue was crystallized (see *table II*). Instead, in the case of **6e,n,o,q**, the solid product which separated out was filtered and crystallized (see *table II*).

For the other diazopyrazoles, as well as for **6o,q**, the acetic mother liquors were poured into crushed ice (80 g) and the pH was adjusted to 5 with 40% aqueous potassium hydroxide. The solid material that separated was filtered, washed with cold water and then crystallized (see *table II*).

3.2.3. 3(5)-methyl-5(3)-(2-nitrobenzamido)pyrazolediazonium chloride ***5b***

The solid that separated from the acetic acid medium for the reaction of the amide **3b** with nitrous acid (see the above procedure) was washed with cold water and then crystallized from acetic acid.

Yield 44%; m.p. 260 °C; IR (cm^{-1}) (hexachlorobutadiene): 3150–2590 (NH); 2230, 2180 (w)(N_2^+); 1695 (CO); ^{1}H-NMR (δ): 2.69 (3H, s, CH_3); 7.83–8.21 (4H, a set of signals, C_6H_4).

3.3. Biological assays

3.3.1. Compounds

Test compounds were dissolved in DMSO at an initial concentration of 200 μM and then were serially diluted in culture medium.

3.3.2. Cells

Cell lines were from American Type Culture Collection (ATCC); bacterial and fungal strains were either clinical isolates (obtained from Clinica Dermosifilopatica, University

Table I.

Compound	R	R_1	R_2	R_3	M.p. (°C)	Crystallization solvent	Formula	IR (Nujol, cm^{-1})	
								ν (CO)	ν (NH)
3a	CH_3	H	H	H	220–222	Methanol	$C_{11}H_{11}N_3O$	1650	3300–3120
3c	CH_3	Cl	H	H	184–185	Ethyl acetate	$C_{11}H_{10}N_3OCl$	1690–1660	3320–3100
3d	CH_3	H	H	Cl	215–216	Ethanol	$C_{11}H_{10}N_3OCl$	1665	3320–3140
3e	CH_3	H	Cl	H	205–206	Ethanol	$C_{11}H_{10}N_3OCl$	1650	3320–3140
3f	CH_3	H	Cl	Cl	236–238	Ethanol	$C_{11}H_9N_3OCl_2$	1655	3280–3100
3g	CH_3	H	H	CH_3	202–204	Ethanol	$C_{12}H_{13}N_3O$	1655	3360–3140
3h	CH_3	H	H	OCH_3	200	Ethanol	$C_{12}H_{13}N_3O_2$	1650	3360–3140
3i	CH_3	H	H	CF_3	225–227	Ethanol	$C_{11}H_{10}N_3OCl$	1660	3300–3120
3l	CH_3	H	H	$C(CH_3)_3$	> 310	Ethanol	$C_{15}H_{19}N_3O$	1670	3440–3040
3m	CH_3	H	H	NO_2	255	Methanol	$C_{11}H_{10}N_4O_3$	1680–1640	3370–3100
3n	CH_3	H	H	I	230–232	Ethanol	$C_{11}H_{10}N_3OI$	1655	3300–3240
3o	CH_3	H	H	Br	222–224	Ethanol	$C_{11}H_{10}N_3OBr$	1660, 1650	3340–3200
3p	CH_3	H	H	F	202–204	Ethanol	$C_{11}H_{10}N_3OF$	1655	3360–3160

^1H NMR (δ): **3a** 2.23 (3H, s, CH_3); 6.41 (1H, s, pyrazole H-4); 7.44–8.01 (5H, a set of signals, C_6H_5); 10.68 (1H, s, exchangeable, NH); 12.12 (1H, s, exchangeable, NH). **3c** 2.22 (3H, s, CH_3); 6.37 (1H, s, pyrazole H-4); 7.36–7.47 (4H, a set of signals, C_6H_4); 10.75 (1H, s, exchangeable, NH); 12.06 (1H, s, exchangeable, NH). **3d** 2.22 (3H, s, CH_3); 6.38 (1H, s, pyrazole H-4); 7.53–8.02 (4H, a set of signals, C_6H_4); 10.79 (1H, br, exchangeable, NH); 12.14 (1H, br, exchangeable, NH). **3e** 2.25 (3H, s, CH_3); 6.43 (1H, s, pyrazole H-4); 7.49–8.06 (4H, a set of signals, C_6H_4); 10.85 (1H, s, exchangeable, NH); 12.16 (1H, s, exchangeable, NH). **3f** 2.24 (3H, s, CH_3); 6.40 (1H, s, pyrazole H-4); 7.75–8.24 (3H, a set of signals, C_6H_3); 10.91 (1H, s, exchangeable, NH); 12.17 (1H, br, exchangeable, NH). **3g** 2.23 (3H, s, CH_3); 2.35 (3H, s, CH_3); 6.40 (1H, s, pyrazole H-4); 7.26–7.93 (4H, a set of signals, C_6H_4); 10.59 (1H, br, exchangeable, NH); 12.01 (1H, br, exchangeable, NH). **3h** 2.23 (3H, s, CH_3); 3.82 (3H, s, CH_3); 6.40 (1H, s, pyrazole H-4); 6.99–8.18 (4H, a set of signals, C_6H_4); 10.52 (1H, s, exchangeable, NH); 12.08 (1H, s, exchangeable, NH). **3i** 2.25 (3H, s, CH_3); 6.45 (1H, s, pyrazole H-4); 7.84–8.20 (4H, a set of signals, C_6H_4); 10.98 (1H, s, exchangeable, NH); 12.19 (1H, s, exchangeable, NH). **3l** 1.30 (9H, s, ter-but); 2.09 (3H, s, CH_3); 6.37 (1H, s, pyrazole H-4); 7.27–8.00 (4H, a set of signals, C_6H_4); 10.65 (1H, br, exchangeable, NH); 12.15 (1H, br, exchangeable, NH). **3m** 2.24 (3H, s, CH_3); 6.43 (1H, s, pyrazole H-4); 8.18–8.33 (4H, a set of signals, C_6H_4); 11.08 (1H, s, exchangeable, NH); 12.20 (1H, s, exchangeable, NH). **3n** 2.23 (3H, s, CH_3); 6.40 (1H, s, pyrazole H-4); 7.75–7.88 (4H, a set of signals, C_6H_4); 10.75 (1H, s, exchangeable, NH); 12.12(1H, s, exchangeable, NH). **3o** 2.24 (3H, s, CH_3); 6.41 (1H, s, pyrazole H-4); 7.68–7.96 (4H, a set of signals, C_6H_4); 10.81 (1H, s, exchangeable, NH); 12.15 (1H, s, exchangeable, NH). **3p** 2.23 (3H, s, CH_3); 6.40 (1H, s, pyrazole H-4); 7.27–8.10 (4H, a set of signals, C_6H_4); 10.71 (1H, s, exchangeable, NH); 12.11 (1H, s, exchangeable, NH).

of Cagliari) or collection strains from ATCC. H9/III_B, MT-4 and C8166 cells (grown in RPMI 1640 containing 10% foetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin were used for anti-HIV assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

3.3.3. Viruses

Human immunodeficiency virus type-1 (HIV-1, III_B strain) was obtained from supernatants of persistently infected H9/III_B cells. HIV-1 stock solutions had a titre of 6 x 10^6 cell culture infectious dose fifty ($CCID_{50}$)/mL.

3.3.4. Antiviral assays

Activity of compounds against the HIV-1 multiplication in acutely infected cells MT-4 cells was based on inhibition of virus-induced cytopathogenicity. Briefly, 50 μL of RPMI 10% FCS containing 1 x 10^4 cells were added to each well of flat-bottomed microtiter trays containing 50 μL of medium with or without various concentrations of test compounds. 20 μL of an HIV-1 suspension containing 100 $CCID_{50}$ were added. After a 4 day incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [13, 14]. The cytotoxicity of compounds was evaluated in parallel with their antiviral acti-

Table II.

Compound	R	R_1	R_2	R_3	M.p. (°C)	Crystallization solvent	Formula	IR (hexachlorobutadiene, cm^{-1})		
								ν (CO)	ν (N_2^+)	ν (NH)
6a	CH_3	H	H	H	180–181	Ethyl ether	$C_{11}H_9N_5O$	1665	2140	3240–2950
6b	CH_3	NO_2	H	H	185–186	Ethanol	$C_{11}H_8N_6O_3$	1680	2160	3150–2800
6c	CH_3	Cl	H	H	205	Ethanol	$C_{11}H_8N_5OCl$	1675	2160	3180–2800
6d	CH_3	H	H	Cl	228–230	Ethanol	$C_{11}H_8N_5OCl$	1675	2160	3180–2800
6e	CH_3	H	Cl	H	178–179	Ethanol	$C_{11}H_8N_5OCl$	1660	2130	3150–2780
6f	CH_3	H	Cl	Cl	215–216	Ethanol	$C_{11}H_7N_5OCl_2$	1670	2150	3200–2800
6g	CH_3	H	H	CH_3	210	Ethanol	$C_{12}H_{11}N_5O$	1655	2130	3220–3000
6h	CH_3	H	H	OCH_3	203–204	Ethanol	$C_{12}H_{11}N_5O_2$	1650	2120	3150–2830
6i	CH_3	H	H	CF_3	218–220	Ethanol	$C_{12}H_8N_5OF_3$	1665	2150	3180–2920
6l	CH_3	H	H	$C(CH_3)_3$	204–205	Ethanol	$C_{15}H_{17}N_5O$	1665	2140	3160–2850
6n	CH_3	H	H	I	231	Acetic acid	$C_{11}H_8N_5OI$	1670	2150	3200–2840
6o	CH_3	H	H	Br	233	Ethanol	$C_{11}H_8N_5OBr$	1670	2150	3220–2840
6p	CH_3	H	H	F	203	Ethanol	$C_{11}H_8N_5OF$	1670	2160	3240–2800
6q	C_6H_5	NO_2	H	H	234	Methanol	$C_{16}H_{10}N_6O_3$	1690, 1680	2140	3000–2800

^{1}H-NMR (δ): **6a** 2.38 (3H, s, CH_3); 7.45–8.10 (5H, a set of signals, C_6H_5); 12.31 (1H, br, exchangeable, NH). **6b** 2.38 (3H, s, CH_3); 7.66–7.97 (4H, a set of signals, C_6H_4); 12.63 (1H, s, exchangeable, NH). **6c** 2.38 (3H, s, CH_3); 7.38–7.64 (4H, a set of signals, C_6H_4); 12.27 (1H, br, exchangeable, NH). **6d** 2.24 (3H, s, CH_3); 7.51–8.10 (4H, a set of signals, C_6H_4); 12.56 (1H, br, exchangeable, NH). **6e** 2.37 (3H, s, CH_3); 7.47–8.09 (4H, a set of signals, C_6H_4); 12.65 (1H, br, exchangeable, NH). **6f** 2.37 (3H, s, CH_3); 7.71–8.24 (3H, a set of signals, C_6H_3); 12.21 (1H, br, exchangeable, NH). **6g** 2.37 (6H, s, 2 x CH_3); 7.27–8.00 (4H, a set of signals, C_6H_4); 12.19 (1H, br, exchangeable, NH). **6h** 2.37 (3H, s, CH_3); 3.83 (3H, s, CH_3); 7.00–8.08 (4H, a set of signals, C_6H_4); 12.05 (1H, br, exchangeable, NH). **6i** 2.38 (3H, s, CH_3); 7.81–8.29 (4H, a set of signals, C_6H_4); 12.16 (1H, br, exchangeable, NH). **6l** 1.30 (9H, s, 3 x CH_3); 2.37 (3H, s, CH_3); 7.48–8.03 (4H, a set of signals, C_6H_4); 12.16 (1H, br, exchangeable, NH). **6n** 2.37 (3H, s, CH_3); 7.85 (4H, s, C_6H_4); 12.50 (1H, br, exchangeable, NH). **6o** 2.36 (3H, s, CH_3); 7.85–8.02 (4H, a set of signals, C_6H_4); 12.55 (1H, br, exchangeable, NH). **6p** 2.38 (3H, s, CH_3); 7.24–8.17 (4H, a set of signals, C_6H_4); 12.45 (1H, br, exchangeable, NH). **6q** 7.52–8.28 (9H, a set of signals, C_6H_4 and C_6H_5); 12.77 (1H, br, exchangeable, NH).

vity. It was based on the viability of mock-infected cells, as monitored by the MTT method.

3.3.5. Antiproliferative assays

Exponentially growing leukemia and lymphoma cells were resuspended at a density of 1 x 10^5 cells/mL in growth medium containing serial dilutions of the drugs. 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. Activity against solid tumor-derived cells was evaluated in exponentially growing cultures seeded at 5 x 10^4 cells/mL and allowed to adhere for 16 h to culture plates before addition of the drugs. Cell viability was determined by the MTT method four days later. Cell growth at each drug concentration was expressed as percentage of untreated controls and the concentration resulting in 50% growth inhibition (IC_{50}) was determined by linear regression analysis.

3.3.6. Antibacterial assays

Staphylococcus aureus, group D Streptococcus, Shigella and Salmonella spp. were recent clinical isolates. Tests were carried out in nutrient broth, pH 7.2, with an inoculum of 10^3 cells/tube. MICs were determined after 18 h incubation at 37 °C in the presence of serial dilutions of the test compounds.

3.3.7. Antimycotic assays

Yeast blastospores were obtained from a 30 h old shaken culture incubated at 30 °C in Sabouraud dextrose broth. The dermatophyte inoculum was scraped aseptically with a spatula from a 7 day-old culture on agar and the macerate was finely suspended in Sabouraud dextrose broth using a glass homogenizer. Glycerol, final concentration 10%, was added as a cryoprotective agent to both yeast and dermatophyte suspensions, aliquots of which were then stored in liquid nitrogen. Test tubes were inoculated with 10^3 blastospores or colony

Table III. Antiproliferative activity of 4-diazopyrazole derivatives and Doxorubicin.

Cell lines*	IC_{50}[a]							
	6a	**6c**	**6d**	**6f**	**6g**	**6n**	**6o**	Doxorubicin
Leukemia/Limphoma								
WIL2-NS	6.5	6.8	6.1	7.4	7.2	8.7	9.6	0.05
CCRF-SB	9.3	12	7.4	8.5	7.8	8.2	12.2	0.04
Raji	14	20	11	12	12.5	7.0	10.7	0.03
CCRF-CEM	12	18	19	10	9.5	6.5	5.3	0.05
MOLT-4	8.4	6.5	12.7	9.5	10.2	5.2	4.8	0.03
MT-4	4.5	5	3.7	4.1	6.6	2.4	4.0	0.02
C8166	5.2	5.5	6	4.7	7.5	3.2	4.0	0.03
Melanoma								
G361	14.3	10.3	11.8	12.7	20	15	13	0.2
Carcinoma								
HT-29	16.5	17.8	17.6	16.8	20	18.5	19	0.25
5637	6.2	7.5	7.4	8.0	12	6.3	15	0.02
KB	15	16.5	14.6	17.8	21	14	18	0.23

[a]Inhibitory concentration fifty: compound concentration required to reduce cell multiplication by 50% under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication.
*WIL2-NS, human splenic B-lymphoblastoid cells; CCRF-SB, human acute B-lymphoblastic leukemia; Raji, human Burkitt lymphoma; CCRF-CEM and MOLT-4, human acute T-lymphoblastic leukemia; MT-4 and C8166, human $CD4^+$ T-cells containing an integrated HTLV-1 genome; G361, human melanoma; HT-29, human colon adenocarcinoma; 5637, human bladder carcinoma; KB, human epidermoid carcinoma.

Table IV. Antibacterial activity and cytotoxicity of 4-diazopyrazole derivatives.

Compound	R	R_1	R_2	R_3	CC_{50}[a]	MIC[b]/MBC[c]					
						Salmonella	Shigella	Staphylococcus	*S.I.*[d]	Streptococcus	*S.I.*
6a	CH_3	H	H	H	4.5	12.5 / 12.5	6.2 / 25	6.2 / 12.5	–	6.2 / 25	–
6b	CH_3	NO_2	H	H	11.8	6.2 / 6.2	6.2 / 6.2	6.2 / 25	*1.9*	3.1 / 6.2	*3.8*
6c	CH_3	Cl	H	H	5	12.5 / 12.5	12.5 / 12.5	6.2 / 25	–	3.1 / 6.2	*1.6*
6d	CH_3	H	H	Cl	3.7	25 / 50	12.5 / 25	3.1 / 3.1	*1.2*	1.6 / 3.1	*2.3*
6e	CH_3	H	Cl	H	11.1	50 / 50	12.5 / 50	3.1 / 3.1	*3.6*	1.6 / 1.6	*6.9*
6f	CH_3	H	Cl	Cl	4.1	25 / 50	25 / 50	3.1 / 3.1	*1.3*	1.6 / 6.2	*2.5*
6g	CH_3	H	H	CH_3	6.6	12.5 / 12.5	6.2 / 12.5	1.6 / 3.1	*4.1*	1.6 / 1.6	*4.1*
6h	CH_3	H	H	OCH_3	8.3	12.5 / 12.5	6.2 / 25	3.1 / 3.1	*2.7*	1.6 / 1.6	*5.2*
6i	CH_3	H	H	CF_3	121	50 / 100	100 / 100	6.2 / 6.2	*19.5*	3.1 / 3.1	*39*
6l	CH_3	H	H	$C(CH_3)_3$	56	>200 / >200	>200 / >200	6.2 / 6.2	*9*	6.2 / 6.2	*9*
6n	CH_3	H	H	I	2.4	25 / >200	6.2 / 50	6.2 / 12.5	–	0.8 / 1.6	*3*
6o	CH_3	H	H	Br	4	50 / >200	25 / 100	12.5 / 25	–	3.1 / 6.1	*1.3*
6p	CH_3	H	H	F	12.5	6.2 / 25	6.2 / 25	6.2 / 6.2	*2*	1.6 / 3.1	*7.8*
6q	C_6H_5	NO_2	H	H	10	25 / 25	12.5 / 25	6.2 / 12.5	*1.6*	0.8 / 0.8	*12.5*
Streptom.	–	–	–	–	> 200	6.2 / 6.2	3.1 / 3.1	6.2 / 6.2	*32.2*	1.6 / 1.6	*125*

[a]Compound dose (μM) required to reduce the viability of mock-infected MT-4 cells by 50%; [b]minimum inhibitory concentration (μM); [c]minimum bactericidal concentration (μM); [d]selectivity index.

forming units (CFU)/tube. The minimal inhibitory concentration (MIC) was determined by serial dilutions using Sabouraud dextrose broth (pH 5.7) and incubating at 37 °C. The growth control for yeasts was read after 1 day and for dermatophytes after 3 days (5 days for Cryptococcus neoformans). The MIC was defined as the compound concentration at which no macroscopic signs of fungal growth were detected. The minimal germicidal concentration (MGC) was determined by subcultivating negative test tubes in Sabouraud dextrose agar.

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