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SYNTHESIS AND CYTOTOXIC ACTIVITY OF SOME NEW BIPYRAZOLE DERIVATIVES

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Abstract – A new series of bipyrazole derivatives were prepared and characterized via the reaction of 5-hydrazino-1,3-dimethyl-4-nitro-1*H*-pyrazole with various 1,3-dicarbonyl compounds. The synthetic pathway was based on the classical Knorr pyrazole synthesis. *In vitro* cytotoxic activity of the fully characterized bipyrazole derivatives was determined and their IC_{50} values were also reported.

Pyrazoles are one of the most studied heterocycles in the azole family. Since the beginning, the first pyrazole that was prepared in 1883 showed an interesting bioactivity as antipyretic.[1] Nowadays, a lot of reports appeared in the literature refer to the proven biological activities of the pyrazole family,[2,3] which include but not limited to antidepressant,[4] anti-inflammatory,[5,6] and analgesics.[7] Figure 1 shows some pharmaceutical drugs that contain a pyrazole moiety. Pyrazoles were synthesized for the first time by Knorr,[1] through a well-known Knorr pyrazole synthesis reaction, which includes the condensation of 1,3-dicarbonyl compound with the hydrazine, despite its age, the classical Knorr pyrazole synthesis still constitutes an attractive and easy method to prepare pyrazoles.[8,9] Among pyrazoles, bipyrazoles[10] were stated to have remarkable pharmacological activities such as antimicrobial,[11] anti-inflammatory,[12,13] and anticancer agents.[14] The first bipyrazole was synthesized in 1893,[10] and since then, many members of this class of compounds were prepared and tested for any possible bioactivity. The favorable biological activities of this class of compounds, urges scientists to continue exploring this field. Within this context,

driven by the great attractiveness of bipyrazoles, we decided to develop and synthesis of a new series of bipyrazole and exploring its cytotoxic activity.

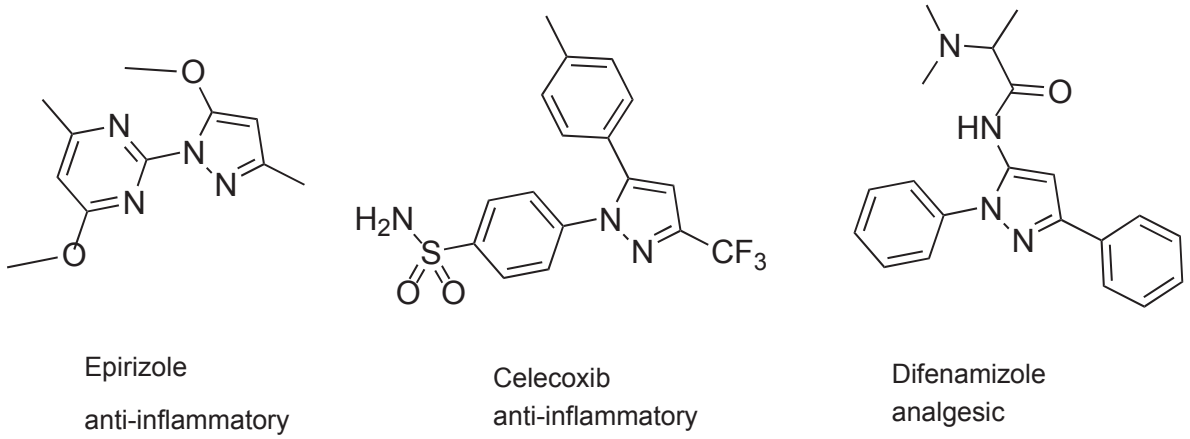
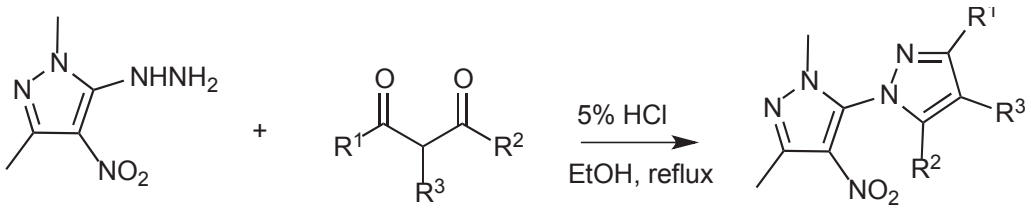


Figure 1. Some pharmaceutical drugs that contain pyrazole moieties

Chemistry

A new series of bipyrazoles have been synthesized and identified. We started our synthesis taking advantage of the hydrazino pyrazole **1** that was prepared in our lab[15] from the commercially available chloropyrazole by the displacement of the chlorine atom with hydrazine. Afterward, we started a classical Knorr pyrazole synthesis by the reaction of **1** with several symmetrical and unsymmetrical 1,3-dicarbonyl compounds **2a-g** in the presence of 5% HCl as catalyst[1] to provide the new bipyrazole derivatives **3a-g** (Scheme 1 and Table 1).

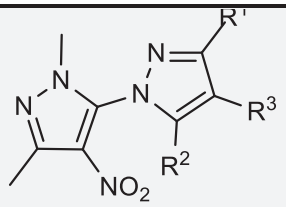


1 **2a-g** **3a-g**

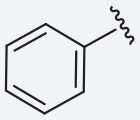
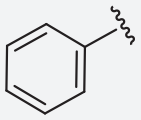
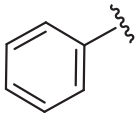
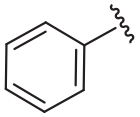
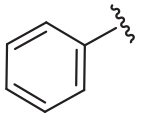
Scheme 1

While the reaction of our hydrazine **1** with symmetrical 1,3-dicarbonyl compounds **2a,b** gave, as expected, one product, most of its reactions with unsymmetrical dicarbonyl compounds were regioselective, and only one regioisomer was isolated and identified in most cases in consistence with the previous finding.[16] In only one case, the reaction of **1** with the dicarbonyl compound **2c**, the minor bipyrazole regioisomer **3d** could also be isolated and identified.

Table 1. Derivatives of the bipyrazoles **3a-g**



3a-g

Comp No#	a	b	c	d	e	f	g
R^1	Me		Me		F_3C	Me	Me
R^2	Me			Me			
R^3	H	H	H	H	H		
Yield%	70	90	79	21	39	73	78

The structures of the bipyrazoles were characterized and identified by IR, ^{1}H NMR, ^{13}C NMR, mass spectra, and elemental analysis in assistance with DEPT, HMQC, and HMBC experiments, thus, their IR spectra showed the disappearance of the NH_2 bands in addition to the appearance of aliphatic CH bands in the range of 2920 and 2860 cm^{-1} corresponding to different methyl groups in the structure. ^{1}H NMR and ^{13}C NMR spectra of the bipyrazoles **3a-g** showed the appearance of new protons and carbons corresponding to the methyl, aliphatic and phenyl groups of the newly formed pyrazole ring in addition to the methyl groups that were originally attached to the first pyrazole. Spectra of compounds **3f** and **3g** showed four and three signals respectively, typical to methylene protons and carbons. In all compounds C3' resonated as the most downfield carbon around δ 155 ppm, in agreement with the proposed structures.

Biology

The cytotoxic activities of the corresponding compounds **3a-g** were evaluated against human K562 and MCF-7 cells. The results are shown in Table 2 in terms of IC_{50} values (the concentration needed to inhibit 50% of cellular proliferation). For comparison purposes, the cytotoxicity of doxorubicin, a standard antitumor drug, was tested under the same conditions.

The results shown in Figures 2 and 3 and Table 2 indicate a better activity for compound **3b** against K562 leukemia cells (IC_{50} = 114.1 μM, 48 h) and MCF-7 breast cancer cells (IC_{50} = 86.4 uM, 24 h), as compared to doxorubicin (IC_{50} = 6.9 μM at 72 h and 48 μM at 72 h, respectively) under similar condition.

As for compound **3d**, the results indicate that it had a better activity against MCF-7 when compared to doxorubicin (IC_{50} = 35.9 μM at 48 h and 48 μM at 72 h, respectively) but not against K562 cells (IC_{50} = 57.2 μM at 72 h and 6.9 μM at 72 h, respectively). However, the other compounds did not show any cytotoxicity even after 72 h of treatment. From the above results, it is clear that when R^1 is phenyl the compound exhibit a good cytotoxic activity, this open the door for future study on structure modeling and structure-activity relationship.

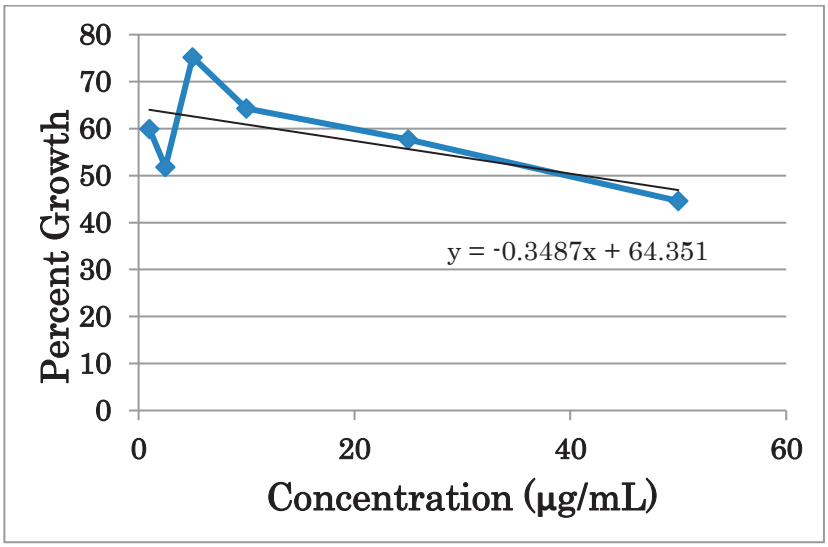


Figure 2. MTT assay results of drug **3b** against K562 cells after 48 h of treatment

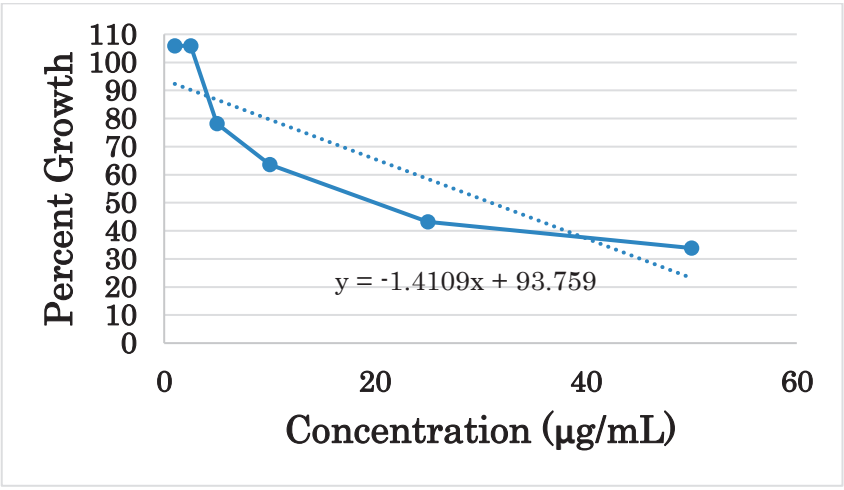


Figure 3. MTT assay results of drug **3b** against MCF-7 cells after 24 h of treatment

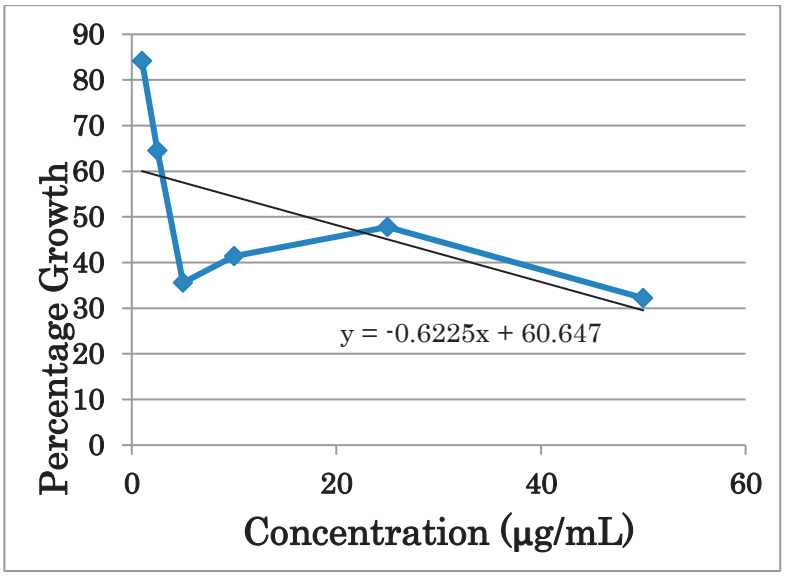


Figure 4. MTT assay results of drug **3d** against K562 cells after 72 h of treatment

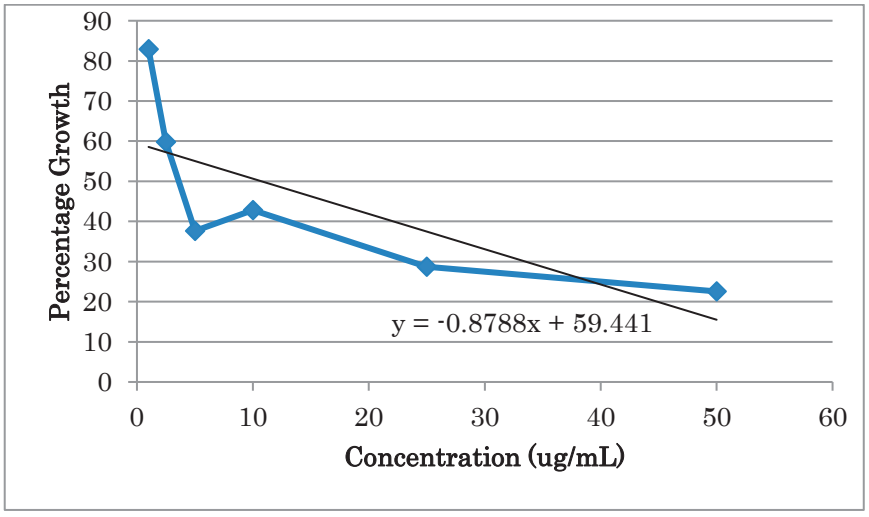


Figure 5. MTT assay results of drug **3d** against MCF-7 cells after 48 h of treatment

Table 2. The MTT (3-(4,5-dimethylthiazol-2-yl)−2,5 diphenyltetrazolium bromide) assay results

Drug	IC_{50} K562	Time of Response	IC_{50} MCF-7	Time of Response
Doxorubicin	**3.77 µg/mL 6.9 µM**	**72 h**	**26 µg/mL 48 µM**	**72 h**
3a	X	no effect after 72 h even with 50 µg/mL	X	no effect after 72 h even with 50 µg/mL
3b	**41 µg/mL 114.1 µM**	**48 h**	**31 µg/mL 86 µM**	**24 h**

3c	X	no effect after 72 h even with 50 µg/mL	X	no effect after 72 h even with 50 µg/mL
3d	**17.1 µg/mL 57.2 µM**	**72 h**	**10.7 µg/mL 35.9 µM**	**48 h**
3e	X	no effect after 72 h even with 50 µg/mL	X	no effect after 72 h even with 50 µg/mL
3f	X	no effect after 72 h even with 50 µg/mL	X	no effect after 72 h even with 50 µg/mL
3g	X	no effect after 72 h even with 50 µg/mL	X	no effect after 72 h even with 50 µg/mL

EXPERIMENTAL

Chemistry

Materials and Instrumentation

Reagent grade chemicals were used as received unless otherwise stated. Melting points (uncorrected) were determined using a Gallenkamp melting point apparatus in one-end open glass capillaries. NMR spectra were recorded on AVANCE-III 400 MHz. IR spectra were measured on Brüker Vertex 70 (Germany). Mass spectra were recorded on Brüker_PC apex-IV Spectrometer. Elemental analyses were performed using EA3000 Eurovector (Italy).

Synthesis of 5-hydrazino-1,3-dimethyl-4-nitro-*1H*-pyrazole (1)

To a solution of 5-chloro-1,3-dimethyl-4-nitropyrazole (0.88 g, 5 mmol) in absolute EtOH (30 mL), hydrazine hydrate (85%) (8 mL, 0.16 mol) was added dropwise. The yellow solution was stirred for 15 min at the ambient temperature, then refluxed for two additional hours. EtOH was removed under vacuum, and the solid product was recrystallized from EtOH to afford yellow crystals. The yield was 0.59 g (69%), mp 191-192 °C.[15]

General procedure for the synthesis of substituted 4'-nitro-1,5'-bi-1*H*-pyrazole (3a-g)

To a solution of **1** (0.17 g, 1 mmol) in 20 mL EtOH at rt, 1.1 mmol of the appropriate 1,3-dicarbonyl compounds **2a-g** was added in the presence of 5% HCl as a catalyst. The reaction mixture was stirred for 15 min, then refluxed for 2 h. The solvent was removed under vacuum and the residue was treated with water, the precipitate was filtered off and washed with cold water. Preparative TLC was used to obtain the desired products **3a-g** in their pure forms.

The following compounds were prepared according to the above general procedure.

1',3,3',5-Tetramethyl-1,5'-bi-1*H*-pyrazole (3a):

According to the standard procedure, the reaction of **1** (0.17 g, 1 mmol) with acetylacetone (0.11 g, 1.1 mmol), gave **3a**, 0.17 g (70%), mp 102.1-103.1 °C. IR: ῡ = 1572(C=N), 1518, 1397 (NO_2) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.16 (s, CH_3), δ 2.61 (s, CH_3), δ 3.32 (s, CH_3), 3.67 (s, CH_3), 6.11 (s, CH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.0 (C_5-$\underline{C}H_3$), 13.7 ($C_{5'}$-$\underline{C}H_3$), 14.4 (C_3-$\underline{C}H_3$), 36.5 (N-CH_3), 107.5 (C_4-H), 134.9 (C_5), 143.6 ($C_{5'}$), 145.9 (C_3), 152.7 ($C_{3'}$); HRMS (ESI) *m/z*: calculated for $C_{10}H_{13}N_5NaO_2$ $[M+ Na]^+$ =258.09615, found 258.09623

Anal. Calcd for $C_{10}H_{13}N_5O_2$: C, 51.06; H, 5.57; N, 29.77. Found: C, 50.89; H, 5.60; N, 29.92.

2',5'-Dimethyl-4'-nitro-3,5-diphenyl-2'*H*-1,3'-bipyrazole (3b):

According to the standard procedure, the reaction of **1** (0.17 g, 1 mmol) with 1,3-diphenyl-1,3-propanedione (0.25 g, 1.1 mmol), gave **3b**, 0.32 g (90%), mp 93.1-96.1 °C. IR: ῡ =1586 (C =N), 1557, 1390 (NO_2) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.57 (s, CH_3), 3.76 (s, CH_3), 6.98 (s, 1H), 7.41-7.31 (m, 2H), 7.42 (m, 3H), 7.43 (d, *J*= 7.2, 1H), 7.48 (t, *J*= 7.3, 2H), 7.92 (d, *J*= 7.3, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3 ($C_{5'}$-CH_3), 36.7 (N-CH_3), 105.0 (C_4-H), 126.1 ($C_{2''+6''}$), 127.4 ($C_{4''}$), 128.6 ($C_{4'''}$), 128.8 ($C_{3''+5''}$), 128.9 ($C_{3'''+5'''}$), 129.0 ($C_{2'''+6'''}$), 135.6 (C_5), 145.9 ($C_{5'}$), 148.5 ($C_{3'}$), 155.3 (C_3); HRMS (ESI) *m/z*: calculated for $C_{20}H_{17}N_5NaO_2$ $[M+ Na]^+$ = 382.12745, found 382.12690.

Anal. Calcd for $C_{20}H_{17}N_5O_2$: C, 66.84; H, 4.77; N, 19.49. Found: C, 66.84; H, 4.94; N, 19.49.

2',3,5'-Trimethyl-4'-nitro-5-phenyl-2'*H*-1,3'-bipyrazole (3c) and 2',5,5'-trimethyl-4'-nitro-3-phenyl-2'*H*-1,3'-bipyrazole (3d):

According to the standard procedure, the reaction of **1** (0.17 g, 1 mmol) with 1-phenyl-1,3-butanedione (0.18 g, 1.1 mmol), gave **3c**, 0.22 g (79%), and **3d** 0.063 g (21%).

Data for 3c, mp 92.0-94.0 °C, IR: ῡ = 1592 (C =N), 1495, 1440 (NO_2) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.42 (s, $C\underline{H}_3$), 2.54 (s, $C\underline{H}_3$), 3.69 (s, $C\underline{H}_3$), 6.47 (s, 1H), 7.21-7.13 (m, 2H), 7.40-7.29 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.8 (C_5-$\underline{C}H_3$), 14.3 ($C_{5'}$-CH_3), 36.5 (N-CH_3), 107.7 (C_4-H), 127.3 ($C_{2'',6''}$), 128.8 ($C_{4'}$), 128.9 ($C_{4''}$), 129.2 ($C_{3'',5''}$), 135.8 ($C_{1''}$), 145.9 (C_4), 148.0 ($C_{5'}$), 153.2 ($C_{3'}$). HRMS (ESI) *m/z*: calculated for $C_{15}H_{16}N_5NaO_2$ $[M+ Na]^+$= 320.11180, found 320.10966.

Anal. Calcd for $C_{15}H_{15}N_5O_2$: C, 60.60; H, 5.09; N, 23.56. Found: C, 60.45; H, 5.39; N, 22.38.

Data for 3d: mp 92.0-94.0 °C. IR: ῡ = 1590 (C =N), 1513, 1377 (NO_2) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.27 (s, 3H, CH_3), 2.64 (s, 3H, CH_3), 3.73 (s, 3H, CH_3), 7.41 (m, 3H) , 7.84 (d, *J*= 7.9, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 11.2 ($C_{5''}$-CH_3), 14.4 (N-CH_3), 36.7 ($C_{5'}$-$\underline{C}H_3$), 104.8 (C_4-H), 128.7 ($C_{4''}$), 128.7 ($C_{3''+5''}$), 128.7 ($C_{2''+6''}$), 132.2 ($C_{1''}$), 134.7 (C_5), 144.3 ($C_{5'}$), 146.0 ($C_{3'}$), 154.9 (C_3).

Anal. Calcd for $C_{15}H_{15}N_5O_2$: C, 60.60; H, 5.09; N, 23.56. Found: C, 60.72; H, 5.21; N, 23.01.

2',5'-Dimethyl-4'-nitro-3-phenyl-5-(trifluoromethyl)-2'*H*-1,3'-bipyrazole (3e):

According to the standard procedure, the reaction of **1** (0.17 g, 1 mmol) with 4,4,4-trifluoro-1-phenyl-1,3-butanedione (0.24 g, 1.1 mmol), gave **3e**, 0.14 g (39%). mp 124.9-125.1 °C; IR: $\bar{\upsilon}$ = 1596 (C=N), 1512, 1370 (NO_2) cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 2.55 (s, CH_3), 3.72 (s, CH_3), 6.90 (s, 1H), 7.25-7.17 (m, 2H), 7.47-7.34 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2($C_{5'}$-CH_3), 37.7 (N-CH_3), 105.3 ($\underline{C}_4$-H), 120.6 ($\underline{C}F_3$), 124.6 ($C_{4''}$), 127.5 ($C_{2''+6''}$), 129.2 ($C_{3''+5''}$), 130.3 ($C_{1''}$), 134.1 (C_5), 146.0 ($C_{5'}$), 146.4 ($C_{3'}$), 148.0 (C_3);

Anal. Calcd for $C_{15}H_{12}F_3N_5O_2$: C, 51.29; H, 3.44; N, 19.94. Found: C, 51.45; H, 3.54; N, 20.02.

4,5,6,7-Tetrahydro-3-methyl-1-(1,3-dimethyl-4-nitro-1*H*-pyrazol-5-yl)- 1*H*-indazole (3f):

According to the standard procedure, the reaction of **1** (0.17 g, 1 mmol) with 2-acetylcyclohexanone (0.15 g, 1.1 mmol), gave **3f**, 0.20 g (73%), mp 162.2-165.2 °C. IR: $\bar{\upsilon}$ = 1588 (C=N), 1518, 1396 (NO_2) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 1.90-1.80 (m, 4H), 2.07 (s, CH_3), 2.61 (s, $C\underline{H}_3$), 2.81-2.66 (m, 2H), 2.53-2.50 (m, 2H), 3.67 (s, N-$C\underline{H}_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 9.5 (C_2-CH_3), 14.4 ($C_{5'}$-CH_3), 20.3 (C_4), 23.0 (C_5), 23.0 (C_6), 23.5 (C_7), 36.6 (N-CH_3), 116.0 (C_3), 135.4 (C_2), 138.4 ($C_{5'}$), 145.8 ($C_{3'}$), 153.5 (C_8); HRMS (ESI) *m/z*: calculated for $C_{13}H_{17}N_5NaO_2$ $[M+ Na]^+$ = 296.12745, found 296.12657.

Anal. Calcd for $C_{13}H_{17}N_5O_2$: C, 56.71; H, 6.22; N, 25.44. Found: C, 56.53; H, 6.27; N, 25.27.

1-(1,3-Dimethyl-4-nitro-1*H*-pyrazol-5-yl)-3-methyl-1,4,5,6-tetrahydrocyclopenta[*c*]pyrazole (3g):

According to the standard procedure, the reaction of **1** (0.17 g, 1 mmol) with 2-acetylcyclopentanone (0.14 g, 1.1 mmol), gave **3g**, 0.20 g (78%), mp 115.1-118.1 °C; IR: $\bar{\upsilon}$ = 1591 (C=N), 1521, 1372 (NO_2) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 2.26 (s, CH_3), 2.58 (s, CH_3), 3.77 (s, N-CH_3), 2.63-2.60 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 12.8 (C_3-$\underline{C}H_3$), 14.4 ($C_{5'}$-CH_3), 37.1 (N-CH_3), 128.6 (C_{3a}), 135.7 (C_5), 145.7($C_{5'}$), 147.5($C_{3'}$), 154.4(C_{6a}); HRMS (ESI) *m/z*: calculated for $C_{12}H_{15}N_5NaO_2$ $[M+ Na]^+$ =284.11180, found 282.12985.

Anal. Calcd for $C_{12}H_{15}N_5O_2$: C, 55.16; H, 5.79; N, 26.80. Found: C, 55.02; H, 5.86; N, 26.62.

Biological Screening

Materials and Methods

Cell Culture.

Human K562 chronic myelogenous leukemic cells and human MCF-7 breast cancer cells were cultured in RPMI-1640 medium (Euroclone, Italy) supplemented with 10% fetal bovine serum (FBS) (Euroclone,

Italy). Trypsin-EDTA (Lonza, Switzerland) was routinely used for subcultures. Cell growth was accomplished at 37 °C in a 5% carbon dioxide atmosphere.

In Vitro Cytotoxicity (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT Test.

Cytotoxicity of the various compounds on K562 and MCF-7 cells was evaluated by means of MTT (tetrazolium salt reduction) test.[17,18] Briefly, 5×10^4 viable cells were added to each well of a 96-well tissue culture plate containing growth media supplemented with FBS.[19] Cells were kept in a humified 5% CO_2 incubator at 37 °C for 24 h. The compounds were tested and for each compounds six concentrations were prepared in growth media: 0.1, 0.5, 2.5,5, 25, and 50 ug/mL. The compounds were solubilized in 10% DMSO. The next morning, the different concentrations were added, and the cells were incubated for 24, 48, and 72 h. Freshly prepared MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL) was added to each well to give a final concentration of 0.5 μg/mL. The plates were incubated for 4 h and the formation of formazan crystals was checked using an inverted microscope. Equal volume of 1:1 (200 μL) DMSO and isopropanol mixture was added to each well and incubated for 30-45 min. The inhibition of cell growth induced by the various compounds was detected by measuring the absorbance of each well at 570 nm using a microplate reader (Synergy HTX Multi-Mode Reader, USA). For comparison purposes, the cytotoxicity of doxorubicin was evaluated under the same experimental conditions. Percent growth was calculated according to the following formula: Growth (%) = OD treated/OD vehicle-treated control×100%. The concentration-percent growth curve was used to calculate the concentration which caused 50% growth inhibition (IC_{50}) by linear interpolation from a semi-log plot of a dose-response curve. The experiment was performed three times in triplicates.

ACKNOWLEDGEMENTS

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