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Synthesis and anticancer activity of substituted pyrazole derivatives

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ABSTRACT

Malonitrile was coupled with substituted aromatic amines to afford compounds **(1-3)**. Compounds **(4-5)** were prepared through the reaction of compounds **(1-3)** with phenyl hydrazine but the reaction of substituted phenyl hydrazono-malononitrile compounds **(1-3)** with hydrazine hydrate afforded diamino compounds **(7-9)**. The diaminocompounds **(7-9)** were condensed with different substituted aromatic aldehydes to give compounds **(10-18)**. The structure of newly synthesized compounds was confirmed by IR, ^{1}H NMR, MS spectral data and elemental analysis. All the compounds **(4-6)** and **(10-18)** were screened for their anticancer activities against breast carcinoma (T47D)and human hepatocarcinoma cell lines (Huh-7) compared with Doxorubicin positive control. The detailed synthesis, spectroscopic data and anticancer activities of the synthesized compounds were reported.

KEYWORDS

Anticancer;
Benzothiazole;
Benzimidazole;
Benzoxazole;
Pyrazole.

INTRODUCTION

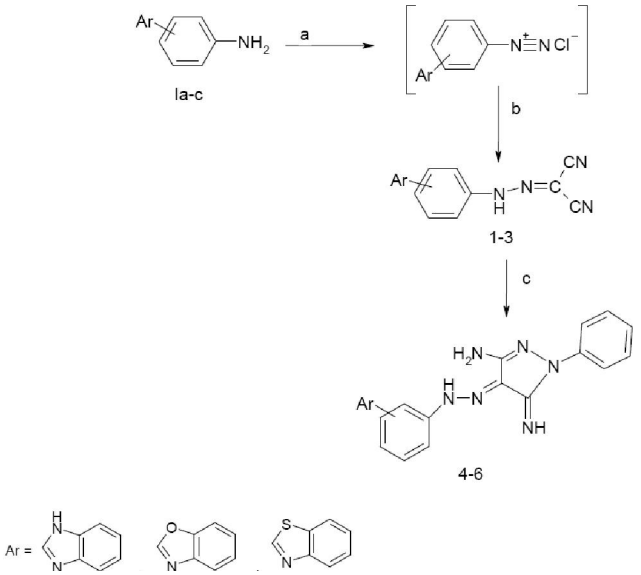
Cancer still remains a threat to men's health, representing the second leading cause of death worldwide[1]. It is estimated that 12 million people will die from cancer in 2030[2]. Recently, many efforts have been made to develop for finding safe and effective ways of treating this disease which include an increased in the understanding of the biological process involved in cancer cell survival and also the search for novel chemotherapeutic agents[1]. In this context, the major challenge is the development of more effective and safe drugs for the treatment of cancer. Through searching in the literature,it was found thatbenzothiazole derivatives play an important role on designing of new drugs, since they present an interesting pharmacological profile[3], including antiallergic[4], anti-inflammatory[5], antitumor[6], analgesic[7], antimicrobial[8,9], anthelmintic[10], antileishmanial[11], anticonvulsant[12] activity also with considerable efficacy as kinase, topoisomerase I/II and trans-retinoic acid metabolism inhibitors[1]. Pyrazole nucleus is important pharmacophore with a wide range of therapeutical activities such as antitumor[13], antibacterial, anti-inflammatory[14], hypotensive[14] and ligands for benzodiazepine receptors[15]. Also, benzoxazole or benzimidazole have variable pharmacological activities like antitubercullosis[16] antioxidant, anthelmintic[17] antimicrobial[18] anticancer[19]. Keeping this in mind, it was aimed in this work to synthesize a new series of heterocyclic compounds bearing pyrazole nucleus linked tobenzothiazole, benzoxazole or benzimidazole nucleus.

RESULTS AND DISCUSSION

Chemistry

The synthesis of the target compounds was sum-

marized in schemes 1 and 2.The substituted phenyl hydrazono-malononitriles**(1-3)** were synthesized by diazotization of different substituted aromatic amines (I a-c) followed by coupling theirdiazonium salt with malononitrile in the presence of sodium acetate (Scheme 1).The structure of compounds **(1-3)** was confirmed with the aid of spectroscopic data and element analysis, their IR spectra showed the appearance of cyano group at 2221-2224 cm^{-1}.



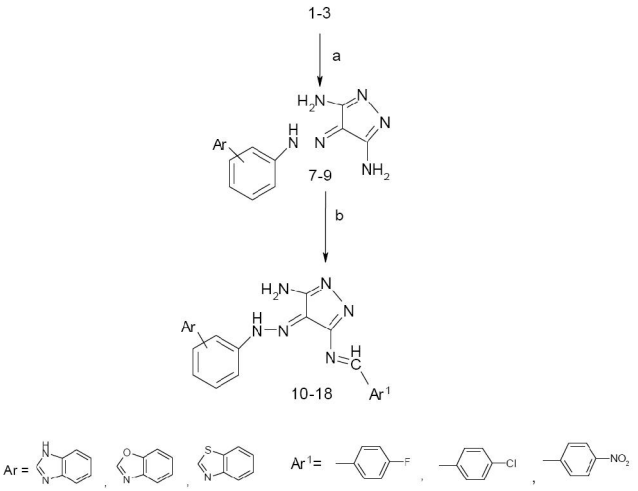
Scheme 1 : Reagent and conditions a) HCl, $NaNO_2$, b) $CH_2(CN)_2$, Na acetate, aqueous ethanol, stirring at 0 °C, c) Phenyl hydrazine, drops of glacial acetic acid, DMF, reflux for 10 h

Condensation of substituted phenyl hydrazono-malononitrile**(1-3)** with phenyl hydrazine afforded compounds **(4-6)** For the preparation of the diaminopyrazole derivatives **(7-9)**; the respective substituted phenyl hydrazonomalononitrile **(1-3)** was heated under reflux with hydrazine hydrate (Scheme 2). The structure of compounds 4-9 was confirmed by the disappearance of CN group peak in IR spectra of them.

Heating equimolaramounts of the pyrazole derivatives **(7-9)** with the corresponding aromatic aldehyde in dimethyl formamide containing catalytic amount of glacial acetic acid yielded*N*-benzylidinepyrazolediamine **10**-(Scheme 2).The structure of the synthesized compounds was confirmed using microanalyses and spectral data. ^{1}H NMR spectra of compounds **(10-18)** indicated the presence of azomethine proton as a singlet signal.

Anti-tumor cytotoxicity

For the evaluation of the anti-tumor cytotoxicity **12x** of the synthesized compounds, two different human cancer cell lines were used: Huh-7 (liver carcinoma cell line), T47D (breast carcinoma cell line). Cytotoxicity of pyrazol-3-ylamine derivatives **(4-6)** and pyrazole-3,5-diamine derivat **(10-18)** against Huh-7 and T47D is shown in figures 1 and 2 respectively. The IC_{50} values are found associated with each figure.



Scheme 2 : Reagent and conditions a) Excess hydrazine hydrate 99%, reflux for 6h, b) substituted aromatic aldehydes, DMF, drops of glacial acetic acid, reflux for 12h

Based on the data obtained, all the tested pyrazol-3-ylamine and pyrazole-3,5-diamine derivatives have anti-proliferative potentials on Huh-7 cell lines to various degrees. In general, pyrazole-3,5-diamine derivatives **(10-18)**. seemed to be more effective in decreasing the survival percent than pyrazol-3-ylamine derivatives **(4-6)**. Based on their cytotoxic efficacy and IC_{50}, the compounds are arranged in a descending order as follows: compounds **(11)** (IC_{50}= 16.22 µg/ml), **(10)** (29.85 µg/ml), **(12)** (34.20 µg/ml), **(13)** (34.87 µg/ml), **(16)** (38,57 µg/ml), **(17)** (53.71 µg/ml), **(18)** (87.70 µg/ml), **(4)** (96.00 µg/ml), **(15)** (100.78 µg/ml), **(14)** (100.90 µg/ml), **(5)** (120.22 µg/ml) and **(6)** (IC_{50}= 127.17 µg/ml) (Figure 1).

Figure 2 revealed that compound 4 exhibited the most potent effect in decreasing the survival percent of T47D breast carcinoma cell lines (IC_{50}= 37.02µg/ml). The other tested derivatives are arranged, based on their cytotoxic potencies against T47D and on the obtained IC_{50}, in the following order: derivatives **(14)**

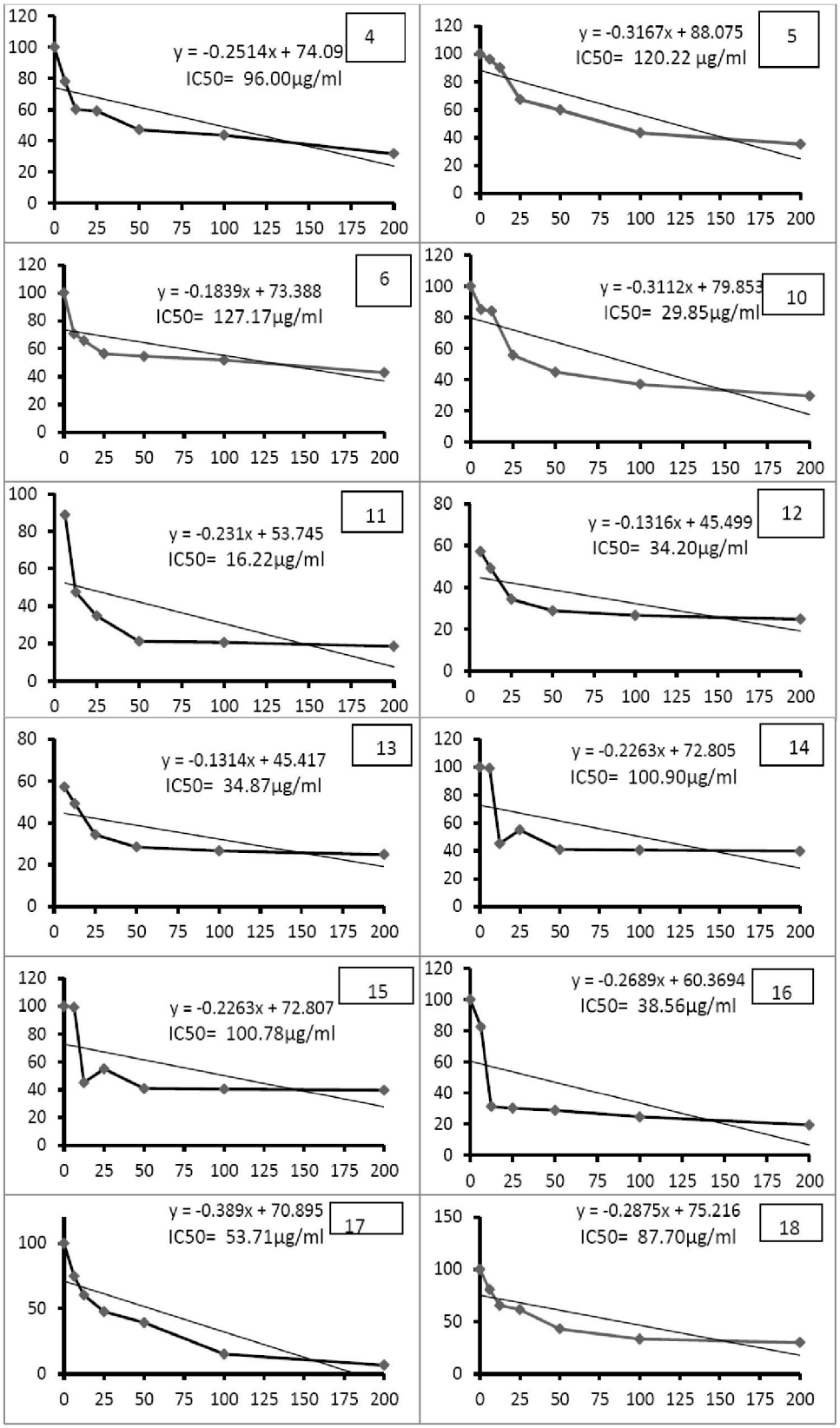


Figure 1 : Anti-tumor cytotoxicity of different concentrations (µg/ml) of pyrazol-3-ylamine and pyrazole-3,5-diamine derivatives against human hepatocarcinoma cell lines (Huh-7) *in vitro*.

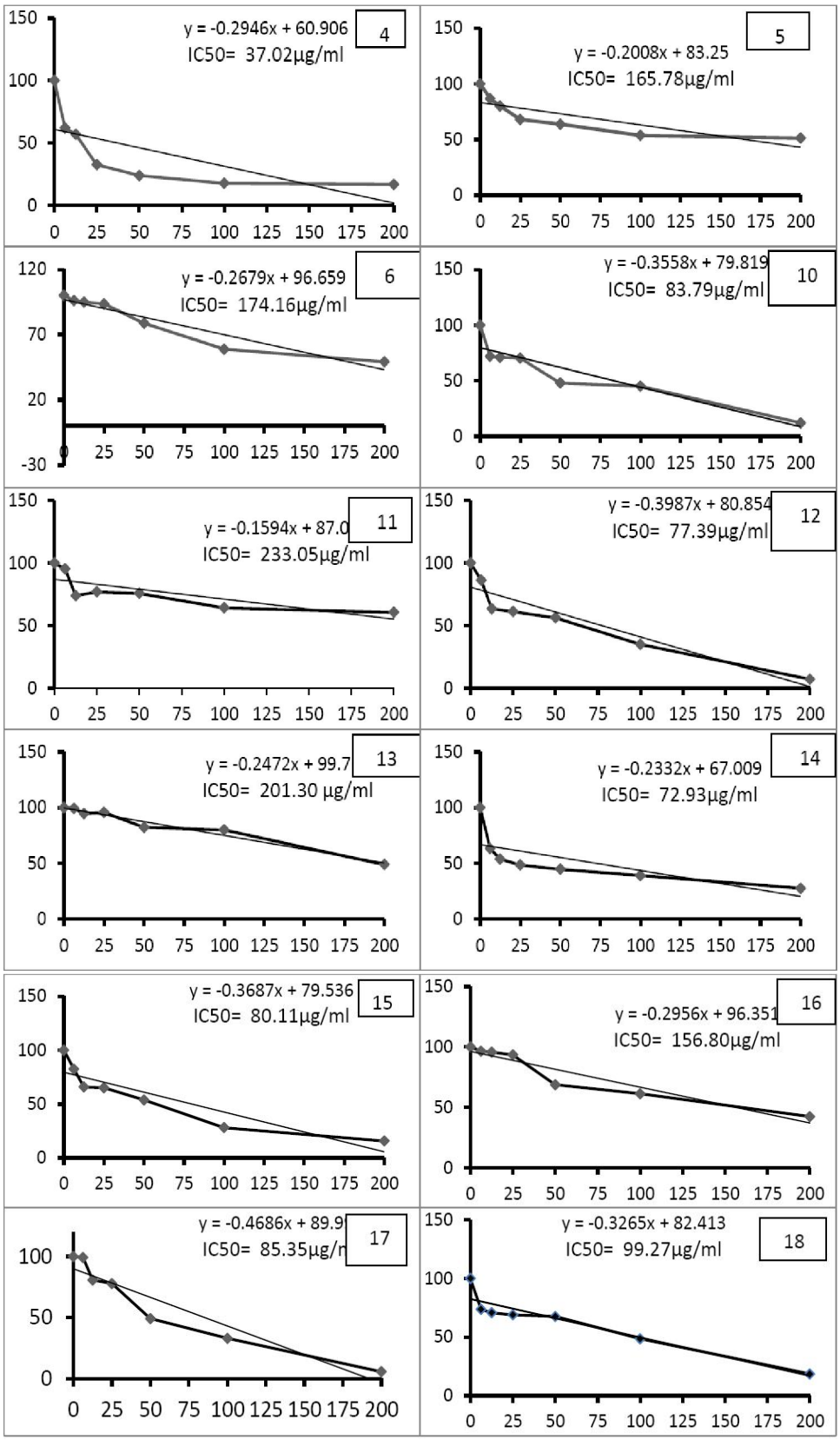


Figure 2 : Anti-tumor cytotoxicity of different concentrations (µg/ml) of pyrazol-3-ylamine and pyrazole-3,5-diamine derivatives against human breast cell lines (T47D) *in vitro*

(IC_{50}= 72.93 µg/ml), **(12)** (77.39 µg/ml), **(15)** (80.11 µg/ml), 10 (83.79 µg/ml), **(17)** (85.35 µg/ml), **(18)** (99.27 µg/ml), **(16)** (156.80 µg/ml), **(5)** (165.78 µg/ml), **(6)** (174.16 µg/ml), **(13)** (201.30 µg/ml) and **(11)** (IC_{50}= 233.05 µg/ml). Thus, derivative 4 followed by **(14)** to **(12)** seemed to have the most cytotoxic effects against breast carcinoma cell lines (Figure 2).

EXPERIMENTAL

Chemistry

Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel sheets that precoated with UV fluorescent silica (MERCK 60 F 254) and spots were developed using I_2vapour / UV light as visualizing agents. Solvent system was chloroform: methanol (in different ratio). ^{1}H NMR spectra were determined in $CDCl_3$, or DMSO-d_6 solvent with Varian Gemini 300 MHZ Spectrometer. Peak positions were given in parts per million (δ) downfield the tetramethylsilane as internal standard. IR spectra were recorded on a Shimadzu 435 Spectrometer, using KBr discs and values were represented in cm^{-1}. GC Mass spectra were run on Shimadzu QP-2010 spectrometer and Mass spectra were run on Hewlett Packard 5988 spectrometer at the Microanalytical Center, Cairo University, Egypt. Melting points were determined on a Griffin instrument and are uncorrected. All reported products showed ^{1}H NMR spectra in agreement with the assigned structures. Elemental analysis was performed at the Micro-analytical Center, Cairo University, Egypt. Compounds Ia-c were prepared according to reported methods[20-21].

General methods for preparation of compounds (1-3)

To an ice cooled solution of the corresponding amino compounds Ia-c (0.01mol) in hydrochloric acid (2.5ml) and distilled water (5ml), a solution of sodium nitrite (0.013mol) in distilled water (5ml) was added portion-wise to a well-stirred cold solution of malononitrile (0.01mol) in 50% aqueous ethanol (10ml) containing sodium acetate (0.9g, 0.011mol). The reaction mixture was kept in ice for 2 h and then filtered. The product was driedand crystallized from ethanol.

2-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]-hydrazono}-malononitrile (1)

Yellow color solid; yield, 80%; m p 180-182°C; IR (KBr, cm^{-1}): 3408, 3218(2NH), 3055 (CH aromatic), 2221 (CN),1610(C=N);^{1}H-NMR (DMSO-d6) δ: 4.44 (s, H, NH, D_2O exchangeable); 7.53 (d, 2H, ArH protons,J_{value} = 9 Hz); 7.71(s,1H, NH, D_2O exchangeable); 7.74-7.83 (m, 4H, Ar H);8.34 (d, 2H, ArH protons, J_{value} = 9Hz); MS (m/z): 286 ($M^{+\cdot}$, 80%) 208(100%); Anal. Found: C, 67.10%; H, 3.60%; N, 29.30% ;$C_{16}H_{10}N_6$Calcd. C, 67.12%; H, 3.52,%; N, 29.35%.

2-[(4-Benzoxazol-2-yl-phenyl)-hydrazono]malononitrile (2)

Yellow color solid; yield; 70%; m.p. 183-185°C; IR (KBr, cm^{-1}): 3425 (NH),3045 (CH aromatic), 2224 (CN),1608 (C=N); ^{1}H-NMR (DMSO-d6) δ: 4.99 (s, H, NH, D_2O exchangeable); 7.43 (d, 2H, ArH protons, J_{value} = 8.7 Hz); 7.62-7.78 (m, 4H, Ar H); 8.25 (d, 2H, ArH protons, J_{value} = 8.7Hz); MS (m/z): 287 ($M^{+\cdot}$, 68%),195 (100%); Anal. Found: C, 66.90%; H, 3.40%; N, 24.30% ; $C_{16}H_9N_5O$Calcd. C, 66.89%; H, 3.16,%; N, 24.38%

2-[2-(Benzothiazol-2-yl-phenyl)-hydrazono]-malononitrile (3)

Yellow color solid; yield; 46%; m.p. 186-188 °C; IR (KBr, cm^{-1}): 3444 (NH),3050 (CH aromatic), 2221 (CN), 1599(C=N);^{1}H-NMR (DMSO-d6): δ7.37-7.57(m, 4H, Ar H);7.60(d, 1H, ArH,J_{value} = 6.9Hz); 7.77(d, 1H, ArH, J_{value} = 8.4Hz), 8-8.08(m, 1H,ArH);8.17(d, 1H, ArH,J_{value} = 7.8Hz),15.15 (s,H, NH, D_2O exchangeable)ppm; MS (m/z): 303 ($M^{+\cdot}$, 100%); Anal.Calcd.for $C_{16}H_9N_5S$; C, 63.35%; H, 2.99%; N, 23.09%.; Found: C, 63.40%; H, 3.10%; N, 23.20%.

General method for preparation of compounds

A mixture of compound **(1-3)** (0.01mol) and phenyl hydrazine (0.011mol) in DMF (20ml) was refluxed for 6 h. The reaction mixture was evaporated under reduced pressure. The residue was washed with water, dried and crystallized from DMF/H_2O mixture.

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]-hydrazono}-5-imino-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-ylamine (4)

Red color solid; yield; 75%; m.p. more than 300

°C;, IR (KBr, cm^{-1}): 3395,3344 (NH_2), 32073167 (NH), 1614(C=N);^{1}H-NMR (DMSO-d6) δ: 3.30 (s, 2H, NH_2, D_2O exchangeable), 7.19-7.35 (m, 6H (4H, ArH protons and 2H, D_2O exchangeable), 7.47-7.60(m,6H (5H,Arh and 1H, D_2O exchangeable), 7.93 (d, 2H, J_{value} = 9Hz), 8.24 (d, 2H, ArH protons, J_{value} = 9Hz) ; MS (m/z): 394 ($M^{+\cdot}$, 80%), 77(100%); Anal. Calcd.for$C_{22}H_{18}N_8$: C, 66.99%; H, 4.60%; N, 28.41% ; Found: C, 67.20%; H, 4.70%; N, 28.20%;

4-[(4-Benzoxazol-2-yl-phenyl)hydrazono]-5-imino-1-phenyl-4,5-dihydro-*1H*-pyrazol-3-ylamine (5)

Red colour solid; yield; 75%; m.p. more than 300 °C; IR (KBr, cm^{-1}): 3424, 3346 (NH_2),3209, 3168 (NH),1608(C=N), ^{1}H-NMR (DMSO-d6) δ: 6.25 (s, 2H, NH_2, D_2O exchangeable); 7.29-7.47 (m, 6H (4H, ArH protons and 2H, D_2O exchangeable); 7.50-7.60 (m,4H, ArH); 7.93 (d, 2H, J_{value} = 8.7Hz); 8.05(d,1H, ArH, J_{value} = 8.7Hz);8.13 (d, 2H, ArH protons, J_{value} = 8.4Hz); MS (m/z): 395 ($M^{+\cdot}$, 100%); Anal.Calcd.for $C_{22}H_{17}N_7O$; C, 66.82%; H, 4.33%; N, 24.80%; Found: C, 67.10%; H, 4.50%; N, 24.70%.

4-[(2-Benazothiazol-2-yl-phenyl)-hydrazono]-5-imino-1-phenyl-4,5-dihydro-*1H*-pyrazol-3-ylamine (6)

Red color solid; yield; 65%; m.p. more than 300 °C; IR (KBr, cm^{-1}): 3463, 3280(NH_2), 3222, 3169 (2NH),1600(C=N);^{1}H-NMR (DMSO-d6): δ 6.65 (s, 2H, NH_2, D_2O exchangeable); 7.31-7.54 (m, 5H (3H, ArH and 2H, D_2O exchangeable); 7.59 (d, 2H, ArH,J_{value} = 8.4); 7.93 (d, 2H, J_{value} = 8.7Hz); 8.03-8.11(m,4H, ArH); 8.13 (d, 2H, ArH protons, J_{value} =7.5);, MS (m/z): 411 ($M^{+\cdot}$, 73%), 77(100%); Anal.Calcd.for $C_{22}H_{17}N_7S$: C, 64.22%; H, 4.16%; N, 23.83% ; Found: C, 64.20%; H, 4.30%; N, 23.90%.

General method for preparation of compounds (7-9)

A mixture of compound **(1-3)** (0.01mol) and 99% hydrazine hydrate (20mL) was heated under reflux for 6 h. The reaction mixture was cooled and the formed solid was filtered, washed with water, dried and crystallized from DMF/ methanol (1:1).

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]-hydrazono}-4*H*-pyrazole-3,5-diamine (7)

Red color solid; yield; 85%; m.p. more than 300 °C; IR (KBr, cm^{-1}): 3393, 3297 (NH_2), 3182(NH) 1617 (C=N);^{1}H-NMR (DMSO-d6) δ: 3.34(1H,NH,D_2O, exchangeable);6.33(4H, 2NH_2, D_2O exchangeable); 6.69(1H, NH, D_2O, exchangeable); 7.20 (d, 2H, ArH, J_{value} = 8.7Hz), 7.51-7.60 (m, 2H, ArH), 7.61-7.66(m,2H,ArH), 8.20(d,2H,ArH,J_{value} = 8.7Hz);MS (m/z): 318 ($M^{+\cdot}$, 26%),129(100%);Anal.Calcd.for $C_{16}H_{14}N_8$:C, 60.37%; H, 4.43%; N, 35.20% ; Found: C, 60.20%; H, 4.30%; N, 35.10%.

4-[(4-Benzoxazol-2-yl-phenyl)-hydrazono]-4*H*-pyrazole-3,5-diamine (8)

Red color solid; yield; 65%; m.p. more than 300 °C;IR (KBr, cm^{-1}): 3394, 3297 (NH_2),1617 (C=N);^{1}H-NMR (DMSO-d6) δ: 3.34(1H,NH,D_2O exchangeable); 6.30(4H, 2NH_2, D_2O exchangeable);7.40 (d, 2H, ArH, J_{value} = 6.6Hz);7.74-7.87 (m, 4H, ArH); 8.20(d,2H,ArH,J_{value} = 6.6 Hz);10.80 (1H, NH, D_2O exchangeable);MS (m/z): 319 ($M^{+\cdot}$, 5,45%),55 (100%), Anal. Calcd.for $C_{16}H_{13}N_7O$: C, 60.18%; H, 4.10%; N, 30.70% ; Found: C, 60.10%; H, 4.20%; N, 30.60%.

4-[(2-Benzothiazol-2-yl-phenyl)-hydrazono]-4*H*-pyrazole-3,5-diamine (9)

Red color solid; yield; 70%; m.p. more than 300 °C; IR (KBr, cm^{-1}): 3330, 3210 (NH_2);^{1}H-NMR (DMSO-d6): δ6.40(4H, 2NH_2, D_2O exchangeable);7.42 (d, 2H, ArH, J_{value} = 7.2 Hz);7.50-7.58 (m, 2H, ArH); 7.81-8.05 (m, 2H, ArH); 8.12 (d,2H,ArH,J_{value} = 7.2Hz);10.80 (1H,NH,D_2O exchangeable); MS (m/z): 335 ($M^{+\cdot}$, 45%),225(100%); Anal. Calcd.for $C_{16}H_{13}N_7S$: C, 57.30%; H, 3.91%; N, 29.23%; Found: C, 57.10%; H, 4.10%; N, 29.50%.

General procedures for synthesis of compounds (10-18).

A mixture of compound 6-9 (0.005mol) and the appropriate aromatic aldehyde (0.005 mol) in DMF (25 mL) was treated with glacial acetic acid (2-3 drops) and heated under reflux for 8-12 h. The reaction mixture was poured into ice-cooled water. The formed precipitate was filtered, dried and crystallized from DMF/ethanol (1:1) to give compounds **(10-18)**.

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]-hydrazono}-*N*(4-fluoro-benzylidine)-4*H*-pyrazole-3,5-diamine (10)

Red color solid; yield; 45%; m.p. more than 300 ^{o}C; IR (KBr, cm^{-1}): 3418 (broad band)(NH, NH_2),1629 (C=N);^{1}H-NMR (DMSO-d6): δ 3.60 (s, 1H, NH, D_2O exchangeable); 7.43 (d, 2H, ArH, J_{value} = 7.2Hz); 7.44 (d, 2H, ArH, J_{value} = 6.9Hz); 7.74-7.94 (m, 4H, ArH); 7.99 (d, 2H, ArH,J_{value} = 7.2Hz); 8.20 (d, 2H, ArH,J_{value} = 6.9Hz);8.95 (s, 2H, NH_2, D_2O exchangeable); 9.99(s, H, N=CH); 10.65 (s, 1H, NH, D_2O exchangeable) ppm; MS (m/z): 424 ($M^{+\cdot}$, 14%) 57(100%); Anal.Calcd.for $C_{23}H_{17}FN_8$: C, 65.09%; H, 4.04%; N, 26.40%; Found: C, 65.10%; H, 4.10%; N, 26.30%.

4-[(4-Benzoxazol-2-yl-phenyl)-hydrazono]-*N*(4-fluoro-benzylidine)-4*H*-pyrazole-3,5-diamine (11)

Red color solid; yield; 50%; m.p. more than 300 ^{o}C ; IR (KBr, cm^{-1}): 3408 (broad band, NH, NH_2), 1605 (C=N);^{1}H-NMR (DMSO-d6): δ3.40 (s, 1H,NH,D_2O exchangeable);7.41 (d, 2H, ArH, J_{value} = 6.9Hz);7.44(d, 2H, ArH, J_{value} = 6.6Hz);7.74-7.94(m, 4H,ArH); 7.99 (d,2H,ArH,J_{value} = 6.9Hz);8.20 (d, 2H, ArH,J_{value} = 6.6Hz);8.90 (s, 2H, NH_2, D_2Oexchangeable), 9.96(s, H, N=CH) ppm;MS (m/z): 425 ($M^{+\cdot}$, 18%), 125(100%); Anal. Calcd.for $C_{23}H_{16}FN_7O$: C, 64.94%; H, 3.79%; N, 23.05%; Found: C, 65.10%; H, 3.90%; N, 23.20%.

4-[(2-Benzothiazol-2-yl-phenyl)-hydrazono]-*N*-(4-fluoro-benzylidine)-4*H*-pyrazole-3,5-diamine (12)

Red color solid; yield; 40%; m.p. more than 300 ^{o}C; IR (KBr, cm^{-1}): 3276 (broad band, NH, NH_2), 1596 (C=N);^{1}H-NMR (DMSO-d6): δ7.47 (d, 2H, ArH, J_{value} = 7.2Hz);7.53 (d, 2H, ArH, J_{value} = 6.9Hz); 7.81-8.00 (m, 4H, ArH);8.09 (d, 2H, ArH,J_{value} = 7.2Hz); 8.17 (d, 2H, ArH,J_{value} = 6.9Hz);9.10(s, H, N=CH); 10.40 (s, 2H, NH_2, D_2O exchangeable); 11.60 (s, 1H, NH, D_2O exchangeable) ppm;MS (m/z): 441 ($M^{+\cdot}$, 11%) 60 (100%); Anal.Calcd. $C_{23}H_{16}FN_7S$: C, 62.57%; H, 3.65%; N, 22.21%;Found: C, 62.70%; H, 3.80%; N, 22.40%.

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]-hydrazono}-*N*-(4-chloro-benzylidine)-4*H*-pyrazole-3,5-diamine (13)

Red color solid; yield; 40%; m.p. more than 300 ^{o}C; IR (KBr, cm^{-1}): 3309 (broad band, NH, NH_2), 1585 (C=N);^{1}H-NMR (DMSO-d6): δ 3.88 (s, NH_2, D_2O exchangeable); 4.40(s,1H, D_2Oexchangeable); 7.31 (d, 2H, ArH, J_{value} = 7.8Hz); 7.67 (d, 2H, ArH, J_{value} = 7.2 Hz); 7.75-7.84 (m, 4H, ArH); 7.94 (d, 2H, ArH,J_{value} = 7.8Hz); 8.31 (d, 2H, ArH,J_{value} = 7.2Hz);8.65(s, 1H, NH, D_2O exchangeable);9.95 (s, H, N=CH)ppm; MS (m/z): 440 ($M^{+\cdot}$, 9%) 403(100%); Anal. Calcd for $C_{23}H_{17}ClN_8$; C, 62.66%; H, 3.89%; N, 25.42%; Found: C, 62.70%; H, 3.90%; N, 25.40%.

4-[(4-Benzoxazol-2-yl-phenyl)-hydrazono]-*N*-(4-chloro-benzylidine)-4*H*-pyrazole-3,5-diamine (14)

Red color solid; yield; 45%; m.p. more than 300 ^{o}C; IR (KBr, cm^{-1}): 3438,3268 (broad band, NH, NH_2), 1592 (C=N);^{1}H-NMR (DMSO-d6): δ 4.38 (s, NH_2, D_2O exchangeable); 7.41(d, 2H, ArH, J_{value} = 5.7Hz); 7.78 (d, 2H, ArH, J_{value} = 7.2 Hz); 7.94-7.99 (m, 4H, ArH); 8.22 (d, 2H, ArH,J_{value} = 5,7 Hz); 8.25 (d, 2H, ArH,J_{value} = 7.2Hz); 9 (s, H, N=CH); 10.40 (s, 1H, NH, D_2O exchangeable);11.60 (s,1H,NH,D_2O exchangeable) ppm; MS (m/z): 441 ($M^{+\cdot}$, 14%) 403(100%); Anal.Calcd.for $C_{23}H_{16}ClN_7O$; C, 62.52%; H, 3.65%; N,22.19%; Found: C, 62.40%; H, 3.70%; N, 22.20%.

4-[(2-Benzothiazol-2-yl-phenyl)-hydrazono]-*N*-(4-chloro-benzylidine)-4*H*-pyrazole-3,5-diamine (15)

Red color solid; yield; 45%; m.p. more than 300 ^{o}C; IR (KBr, cm^{-1}): 3283 (broad band, NH, NH_2), 1598 (C=N), ^{1}H-NMR (DMSO-d6):δ 7.44 (d, 2H, ArH, J_{value} = 6.6Hz); 7.53 (d, 2H, ArH, J_{value} = 8.7 Hz);8.04-8.11 (m, 4H, ArH); 8.13 (d, 2H, ArH,J_{value} = 8.7Hz);8.16 (d, 2H, ArH,J_{value} = 6.6Hz);8.98 (s, H, N=CH); 10.50 (s, 2H, NH, D_2O exchangeable); 11.65 (s, 1H, NH D_2O exchangeable) ppm; MS (m/z): 457 ($M^{+\cdot}$, 35%) 157(100%);Anal. Calcd.for $C_{23}H_{16}ClN_7S$; C, 60.32%; H, 3.52%; N, 21.41% ; Found: C, 60.50%; H, 3.60%; N, 21.40%.

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]hydrazono}-*N*-(4-nitro-benzylidine)-4*H*-pyrazole-3,5-diamine (16)

Red color solid; yield; 45%; m.p. more than 300 ^{o}C; IR (KBr, cm^{-1}): 3410 (broad band, NH, NH_2), 1628 (C=N);^{1}H-NMR (DMSO-d6): δ3.53 (s, NH, D_2Oexchangeable); 7.22 (d, 2H, ArH, J_{value} = 5.4Hz);

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7.61 (d, 2H, ArH, J_{value} = 5.4 Hz);7.85-7.94 (m, 4H, ArH); 8.14 (d, 2H, ArH,J_{value} = 6.6 Hz); 8.22 (d, 2H, ArH,J_{value} = 6.6Hz);8.83 (s, H, N=CH); 10.30 (s, 2H, NH_2, D_2O exchangeable); 11.40 (s,1H, NH D_2Oexchangeable) ppm;MS (m/z): 451 ($M^{+\cdot}$, 10%), 57(100%); Anal.Calcd.for $C_{23}H_{17}N_9O_2$: C, 61.19%;H, 3.80%; N, 27.92% ; Found: C, 61.30%; H, 3.90%; N, 27.80%.

4-[(4-Benzoxazol-2-yl-phenyl)-hydrazono]-*N*-(4-nitro-benzylidine)-4*H*-pyrazole-3,5-diamine (17)

Red color solid; yield; 55%; m.p. more than 300 °C; IR (KBr, cm^{-1}): 3408 (broad band, NH, NH_2), 1610 (C=N);^{1}H-NMR (DMSO-d6): δ 7.39 (d, 2H, ArH, J_{value} = 8.7Hz); 7.63 (d, 2H, ArH, J_{value} = 6.9 Hz); 7.65-7.96 (m, 4H, ArH); 8.17 (d, 2H, ArH,J_{value} = 8.7 Hz); 8.30 (d, 2H, ArH,J_{value} = 6.9Hz);9 (s, H, N=CH); 10.60 (s, 2H, NH_2, D_2O exchangeable); 11.60 (s, 1H, NH,D_2O exchangeable) ppm;MS (m/z): 452 ($M^{+\cdot}$, 13%), 55(100); Anal. Calcd.for $C_{23}H_{16}N_8O_{3:}C$, 61.06%; H, 3.56%; N, 24.77%; Found: C, 61.10%; H, 3.50%; N, 24.80%;

4-[(2-Benzothiazol-2-yl-phenyl)-hydrazono]-*N*-(4-nitro-benzylidine)-4*H*-pyrazole-3,5-diamine (18)

Red color solid; yield; 50%; m.p. more than 300 °C IR (KBr, cm^{-1}): 3398 (broad band, NH, NH_2), 1604 (C=N);^{1}H-NMR (DMSO-d6) δ: 7.44 (m, 4H, ArH); 7.75 (d, 2H, ArH, J_{value} = 8.4 Hz);8.06 (d, 2H, ArH, J_{value} = 8.4Hz); 8.14 (d, 2H, ArH,J_{value} = 8.7 Hz); 8.34 (d, 2H, ArH,J_{value} = 8.7Hz);8.90 (s, H, N=CH); 10.20 (s, 2H, NH_2, D_2O exchangeable); 11.30 (s, 1H, NH D_2O exchangeable) ppm;MS (m/z): 468 ($M^{+\cdot}$, 18%), 80(100); Anal. Calcd.for $C_{23}H_{16}N_8O_2S$; C, 58.97%; H, 3.44%; N, 23.92%; Found: C, 59.10%; H, 3.40%; N, 23.80%.

Anticancer activity

Materials

Human tumor cell lines

Human hepatocarcinoma cell lines (Huh-7) and breast carcinoma cell lines (T47D) used in this study were obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.) through the Tissue Culture Unit, the Egyptian Organization for Biological Products and Vaccines, Vacsera, 51 Wezaret EI Zeraa St., Agouza, Giza, Egypt. The tumor cell lines were maintained at Center for Genetic Engineering, Al-Azhar University, Cairo, Egypt by serial sub-culturing.

Preparation of newly synthesized compounds

The tested derivatives 1-10 and standard anticancer drug, doxorubicin were prepared by dissolving in dimethylsulfoxide (DMSO) and the prepared stock was stored at -20°C. Different concentrations of the compounds 0, 6.25, 12.5, 25, 50, 100 and 200µg/ml in culture medium were used.

Chemicals

The following chemicals are obtained from:

a) Sigma Aldrich Chemical Co., St. Louis, Mo, U.S.A., was the source of the following chemicals: Dimethylsulphoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), trypan blue, Fetal Bovine Serum, Penicillin/ Streptomycin antibiotic and Trypsin-EDTA.

b) Applichem, Germanywas the source of Tris buffer.

All chemicals and reagants used in this study are of highest analytical grade.

Methods

Cells and culture conditions

(i) Reagants and buffers

1 Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal calf serum, sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C and 5% CO2.

2 Trypan blue dye: 0.05 % of the dye was prepared and used for viability counting.

3 Fetal Bovine Serum (FBS): 10 % concentration was prepared and used for supplementation of Dulbecco's Modified Eagle Medium (DMEM) prior to use.

4 Penicillin/ Streptomycin: 100 units/ ml Penicillin/2 mg/ml Streptomycin were used for the supplementation of Dulbecco's Modified Eagle Medium (DMEM) prior to use.

5 Trypsin-EDTA: 0.25 % solution containing 2.5 g Porcine trypsin was used for the harvesting of cells.

(ii) Procedures

(a) Maintenance of the human cancer cell lines in the laboratory

1 A cryotube containing frozen cells was taken out of the liquid nitrogen container and then thawed in a

water bath at 37°C.

2 The cryotube was opened under strict aseptic conditions and its contents were supplied by 5 ml supplemented medium drop by drop in a 50 ml sterile falcon tubes.

3 The tube was incubated for 2 hours then centrifuged at 1200 rpm for 10 minutes and the supernatant was discarded.

4 The supernatant was discarded and the cell pellet was suspended and Seeded in 5 ml supplemented medium in T25 Nunclon sterile tissue culture flasks.

5 The cell suspension was incubated and followed up daily the supplemented medium was replaced every 2- 3 days.

6 Incubation was continued until a confluent growth was achieved and the cells were freshly subcultured before each experiment to be in the exponential phase of growth.

(b) Collection of cells by trypsinization

1 The medium was discarded.

2 The monolayer cell was washed twice with 5 ml phosphate buffered saline.

3 All the adherent cells were dispersed from their monolayer by the addition of 1 ml trypsin solution (0.25 % trypsin w/v) for 2 minutes.

(c) Determination and counting of viable cells

1 50 µl of 0.05 % trypan blue solution was added to 50 µl of the singlecell suspension.

2 The cells were examined under the inverted microscope usingthe haemocytometer.

3 Non stained (viable) cells were counted and the following equation was used to calculate the cell count /ml of cell suspension.

Viable cells /ml = (number of cells in 4 quarters x 2 (dilution factor) x 10^4)/ 4

4 The cells were then diluted to give the required cell number for each experiment.

(d) Cryopreservation of cells

To avoid the loss of the cell line, excess cells were preserved in liquid nitrogen as follows:

1 Equal parts of the cell suspension and freezing medium (10 % DMSO in supplemented medium) were dispersed to cryotubes.

2 The cryotubes were racked in appropriately labeled polystyrene boxes, gradually cooled till reaching -80 °C.

3 Then the cryotubes were stored in a liquid nitrogen (-180°C) till use.

Determination of potential cytotoxicity of drug on human cancer cell line

(i) Principle

The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara, (2006)[22]. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

(ii) Reagants and buffers

1 Glacial acetic acid: 1 % was used for dissolving the unbound SRB dye.

2 Sulphorhodamine-B (SRB): 0.4 % concentration was dissolved in 1 % acetic acid was used as a protein dye.

3 Trichloroacetic acid (TCA): 50 % stock solution was prepared, 10 % solution was used for protein precipitation.

4 10 mMtris base (PH 7.4) was used for SRB dye solubilization. It was prepared by dissolving 121.1 gm of tris base in 1000 ml distilled water and pH was adjusted by 2 M HCl.

(iii) Procedure

1 Cells will be seeded in 96 well microtiter plates at a concentration of 1000-2000 cells/well, 100 µl/well.

2 After 24 h, cells will be incubated for 72 h with various concentrations of drugs (0, 6.25, 12.5, 25, 50, 100 and 200µg/ml).

3 For each derivative concentration and doxorubicin, 3 wells were used. The plates were incubated for 72 hours.

4 The medium is discarded.

5 The cells were fixed with 150 µl cold trichloroacetic acid 10% final concentration for 1 hour at 4 °C.

6 The plates were washed with distilled water using (automatic washer Tecan, Germany) and stained with 50 µl 0.4 % SRB dissolved in 1 % acetic acid for 30 minutes at room temperature in dark.

7 The plates were washed with 1 % acetic acid to

remove unbound dye and air-dried [24 h].

8 The dye was solubilized with 150 µl/well of 10 mMtris base (PH 7.4) for 5 min on a shaker at 1600 rpm.

9 The optical density (OD) of each well will be measured spectrophotometrically at 490 nm with an ELISA microplate reader. The mean background absorbance was automatically subtracted and mean values of each derivative and doxorubicin concentration was calculated. The experiment was repeated 3 times.

Calculation

The percentage of cell survival was calculated as follows:

Surviving fraction = O.D. (treated cells)/ O.D. (control cells)

The IC_{50} values (the concentrations of derivatives and doxorubicin required to produce 50% inhibition of cell growth) were also calculated using sigmoidal concentration–response curve fitting models (SigmaPlot software).

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