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Design, Synthesis and Anticancer Screening of Novel Pyrazole Derivatives Linking to Benzimidazole, Benzoxazole and Benzothiazole

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Abstract

Substituted aromatic amines were diazotized followed by coupling with malononitrile to afford substituted phenyl hydrazono-malononitrile compounds 2a-c. Compounds 3a-c were prepared through the reaction of substituted phenyl hydrazono-malononitriles 2a-c with phenyl hydrazine, while the reaction of compounds 2a-c with hydrazine hydrate afforded diamino compounds 4a-c. The diamino compounds 4a-c was condensed with different substituted aromatic aldehydes to yield *N*-benzylidine pyrazole diamines 5a-i. The structure of the newly synthesized compounds was confirmed by IR, ^{1}H NMR, MS spectral data and elemental analysis. Twelve novel compounds 3a-c and 5a-i were screened for their anticancer activities against breast carcinoma (T47D) and human hepatocarcinoma cell lines (Huh-7) compared with Doxorubicin. The detailed synthesis, spectroscopic data and anticancer activities of the synthesized compounds were reported.

Keywords: Anticancer effects; Benzothiazoles; Benzimidazoles; Benzoxazoles; Pyrazoles

Introduction

Cancer still remains a mean threat to human health, representing the second leading cause of death worldwide [1]. It is estimated that 13.1 million people will die from cancer in 2030 [2]. Recently, many efforts have been made to develop safe and effective ways of treating this disease and to search for novel chemotherapeutic agents with minimal side effects [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 that benzothiazole derivatives play an important role on designing of new drugs, since they present an interesting pharmacological profile [3], including anti-allergic [4], anti-inflammatory [5], antitumor [6], analgesic [7], antimicrobial [8,9], anthelmintic [10], antileishmanial [11], anticonvulsant [12] activities, also with considerable efficacy as kinase, topoisomerase I/II and trans-retinoic acid metabolism inhibitors [1]. In addition, pyrazole nucleus is an important pharmacophore with a wide range of therapeutically activities such as antitumor [13], antibacterial, anti-inflammatory [14] and hypotensive [14] efficacies and also acts as ligands for benzodiazepine receptors [15]. In addition, benzoxazole or benzimidazole have variable pharmacological activities like antituberculosis [16], antioxidant, anthelmintic [17], antimicrobial [18] and anticancer [19] potentials. Keeping this in mind, and in continuation of our previous work on the synthesis of new anticancer agents [20-22], it was aimed in this work to synthesize a new series of heterocyclic compounds bearing pyrazole nucleus linked to benzothiazole, benzoxazole or benzimidazole nucleus and to assess their anticarcinogenic effects against breast carcinoma (T47D) and human hepatocarcinoma cell lines (Huh-7).

Materials and Methods

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 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 1a-c were prepared according to reported procedures [23,24].

General method for preparation of compounds 2a-c

To an ice cooled solution of the corresponding amino compounds 1a-c (0.01 mol) in hydrochloric acid (2.5 mL) and distilled water (5 mL), a solution of sodium nitrite (0.013 mol) in distilled water (5 mL) was added portion-wise to a well-stirred cold solution of malononitrile (0.01 mol) in 50% aqueous ethanol (10 mL) containing sodium acetate (0.9 g, 0.011 mol). The reaction mixture was kept in ice for 2 h and then filtered. The obtained solid was dried and crystallized from ethanol. Physical and spectroscopic data for 2a-c are listed below.

2-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]hydrazono} malononitrile (2a): Yellow color solid; yield, 80%; mp 180-182°C; IR (KBr, cm^{-1}): 3408, 3218 (2NH), 3055 (CH aromatic), 2221 (CN), 1610 (N=C); ^{1}H-NMR (DMSO-*d6*) δ: 4.44 (s, 1H, NH, D_2O exchangeable); 7.53 (d, 2H, Ar H, J_{value}=9 Hz); 7.71(s,1H, NH, D_2O exchangeable); 7.74-7.83 (m, 4H, Ar H); 8.34 (d, 2H, Ar H, 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%.

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2-[(4-Benzoxazol-2-yl-phenyl)hydrazono]malononitrile (2b): Yellow color solid; yield; 70%; mp 183-185°C; IR (KBr, cm^{-1}): 3425 (NH), 3045 (CH aromatic), 2224 (CN), 1608 (N=C); ^{1}H-NMR (DMSO-*d6*) δ: 4.99 (s, 1H, 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 (2c): Yellow color solid; yield; 46%; mp 186-188°C; IR (KBr, cm^{-1}): 3444 (NH), 3050 (CH aromatic), 2221 (CN), 1599 (N=C); ^{1}H-NMR (DMSO-*d6*): δ 7.37-7.57 (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}=7.8 Hz), 15.15 (s, 1H, NH, D_2O exchangeable); 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 3a-C

A mixture of the corresponding phenyl hydrazonomalononitriles 2a-c (0.01 mol) and phenyl hydrazine (0.011 mol) in DMF (20 mL) 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. Physical and spectroscopic data for 3a-c are listed below.

4-{[4-(1*H*-Benzoimidazol-2-yl)phenyl]hydrazono}-5-imino-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-ylamine (3a): Red color solid; yield; 75%; mp > 300°C;, IR (KBr, cm^{-1}): 3395,3344 (NH_2), 3207, 3167 (2 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, 4 ArH and 2 NH, D_2O exchangeable), 7.47-7.60 (m, 6H (5H, ArH and 1H, D_2O exchangeable), 7.93 (d, 2H, J_{value}=9 Hz), 8.24 (d, 2H, ArH protons, J_{value}=9 Hz); 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 (3b): Red colour solid; yield; 75%; mp > 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, 4ArH and 2H, D_2O exchangeable); 7.50-7.60 (m,4H, ArH); 7.93 (d, 2H, J_{value}=8.7 Hz); 8.05(d,1H, ArH, J_{value}=8.7 Hz); 8.13 (d, 2H, ArH protons, J_{value}=8.4 Hz); 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 (3c): Red color solid; yield; 65%; mp >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, 3ArH and 2H, D_2O exchangeable); 7.59 (d, 2H, ArH, J_{value}=8.4 Hz); 7.93 (d, 2H, J_{value}=8.7 Hz); 8.03-8.11(m, 4H, ArH); 8.13 (d, 2H, ArH, J_{value} =7.5 Hz);, 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 4a-c

A mixture of the corresponding phenyl hydrazonomalononitrile 2a-c (0.01 mol) and 99% hydrazine hydrate (20 mL) 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). Physical and spectroscopic data for 4a-c are listed below.

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]-hydrazono}-4*H*-pyrazole-3,5-diamine (4a): Red color solid; yield; 85%; mp > 300°C; IR (KBr, cm^{-1}): 3393, 3297 (NH_2), 3182 (NH) 1617 (C=N); ^{1}H-NMR (DMSO-*d6*) δ: 3.34 (s, 1H, NH, D_2O exchangeable), 6.33 (br. s, 4H, $2NH_2$, D_2O exchangeable), 6.69 (s, 1H, NH, D_2O exchangeable), 7.20 (d, 2H, ArH, J_{value}=8.7 Hz), 7.51-7.60 (m, 2H, ArH), 7.61-7.66 (m, 2H, ArH), 8.20 (d, 2H, ArH, J_{value}=8.7 Hz); 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 (4b): 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 (s, 1H, NH, D_2O exchangeable), 6.30(br. s, 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); 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 (4c): Red color solid; yield; 70%; mp > 300°C; IR (KBr, cm^{-1}): 3330, 3210 (NH_2); ^{1}H-NMR (DMSO-*d6*): δ 6.40 (br. s, 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}=7.2 Hz), 10.80 (s, 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 5a-i

A mixture of the corresponding pyrazole derivative 4a-c (0.005 mol) and the appropriate aromatic aldehyde (0.005 mol) in DMF (25 mL) was treated with glacial acetic acid (2 drops) and heated under reflux for 10 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 5a-i. Physical and spectroscopic data for 5a-i are listed below.

4-{[4-(1*H*-Benzoimidazol-2-yl)-phenyl]hydrazono}-*N*(4-fluoro-benzylidine)-4*H*-pyrazole-3,5-diamine (5a): Red color solid; yield; 45%; mp > 300°C; IR (KBr, cm^{-1}): 3418 ((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}=7.2 Hz), 7.44 (d, 2H, ArH, J_{value}=6.9 Hz); 7.74-7.94 (m, 4H, ArH); 7.99 (d, 2H, ArH, J_{value}=7.2 Hz); 8.20 (d, 2H, ArH, J_{value}=6.9 Hz), 8.95 (s, 2H, NH_2, D_2O exchangeable), 9.99 (s, 1H, N=CH), 10.65 (s, 1H, NH, D_2O exchangeable); 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 (5b): Red color solid; yield; 50%; mp > 300°C; IR (KBr, cm^{-1}): 3408 (NH, NH_2), 1605 (C=N); ^{1}H-NMR (DMSO-*d6*): δ 3.40 (s, 1H, NH, D_2O exchangeable), 7.41 (d, 2H, ArH, J_{value}=6.9 Hz), 7.44 (d, 2H, ArH, J_{value}=6.6 Hz), 7.74-7.94 (m, 4H, ArH), 7.99 (d, 2H, ArH, J_{value}=6.9 Hz), 8.20 (d, 2H, ArH, J_{value}=6.6 Hz); 8.90 (s, 2H, NH_2, D_2O exchangeable), 9.96 (s, 1H, N=CH); 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 (5c): Red color solid; yield; 40%; mp > 300°C; IR (KBr, cm^{-1}): 3276 (NH, NH_2), 1596 (C=N); ^{1}H-NMR (DMSO-*d6*): δ 7.47 (d, 2H, ArH, J_{value}=7.2 Hz), 7.53 (d, 2H, ArH, J_{value}=6.9 Hz); 7.81-8.00 (m, 4H, ArH), 8.09 (d, 2H, ArH, J_{value}=7.2

Hz), 8.17 (d, 2H, ArH, J_{value}=6.9 Hz), 9.10(s, 1H, N=CH), 10.40 (s, 2H, NH_2, D_2O exchangeable),11.60 (s, 1H, NH, D_2O exchangeable); 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 (5d): Red color solid; yield; 40%; mp > 300°C; IR (KBr, cm^{-1}): 3309 (broad band, NH, NH_2), 1585 (C=N); ^{1}H-NMR (DMSO-*d6*): δ 3.88 (s, 2H, NH_2, D_2O exchangeable); 4.40(s, 1H, D_2O exchangeable); 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, 1H, 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 (5e): Red color solid; yield; 45%; mp > 300°C; IR (KBr, cm^{-1}): 3438, 3268 (NH, NH_2), 1592 (C=N); ^{1}H-NMR (DMSO-*d6*): δ 4.38 (s, 2H, NH_2, D_2O exchangeable), 7.41 (d, 2H, ArH, J_{value}=5.7 Hz), 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}=7.2 Hz), 9.00 (s, 1H, N=CH), 10.40 (s, 1H, NH, D_2O exchangeable), 11.60 (s,1H,NH, D_2O exchangeable); 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 (5f): Red color solid; yield; 45%; mp > 300°C; IR (KBr, cm^{-1}): 3283 (NH, NH_2), 1598 (C=N), ^{1}H-NMR (DMSO-*d6*): δ 7.44 (d, 2H, ArH, J_{value}=6.6 Hz), 7.53 (d, 2H, ArH, J_{value}=8.7 Hz), 8.04-8.11 (m, 4H, ArH); 8.13 (d, 2H, ArH, J_{value}=8.7 Hz), 8.16 (d, 2H, ArH, J_{value}=6.6 Hz), 8.98 (s, H, N=CH); 10.50 (s, 2H, NH, D_2O exchangeable), 11.65 (s, 1H, NH D_2O exchangeable); 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 (5g): Red color solid; yield; 45%; mp > 300°C; IR (KBr, cm^{-1}): 3410 (NH, NH_2), 1628 (C=N); ^{1}H-NMR (DMSO-*d6*): δ 3.53 (s, 1H, NH, D_2O exchangeable), 7.22 (d, 2H, ArH, J_{value}=5.4 Hz), 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, 1H, N=CH), 10.30 (s, 2H, NH_2, D_2O exchangeable), 11.40 (s, 1H, NH, D_2O exchangeable); 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 (5h): Red color solid; yield; 55%; mp > 300°C; IR (KBr, cm^{-1}): 3408 (NH, NH_2), 1610 (C=N); ^{1}H-NMR (DMSO-*d6*): δ 7.39 (d, 2H, ArH, J_{value}=8.7 Hz), 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}=6.9 Hz), 9 (s, 1H, N=CH), 10.60 (s, 2H, NH_2, D_2O exchangeable), 11.60 (s, 1H, NH, D_2O exchangeable); 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 (5i): Red color solid; yield; 50%; mp > 300°C IR (KBr, cm^{-1}): 3398 (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}=8.4 Hz), 8.14 (d, 2H, ArH, J_{value}=8.7 Hz), 8.34 (d, 2H, ArH, J_{value}=8.7 Hz); 8.90 (s, 1H, N=CH), 10.20 (s, 2H, NH_2, D_2O exchangeable), 11.30 (s, 1H, NH D_2O exchangeable); 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%.

Antiproliferative 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 test 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 200 µg/mL in culture medium were used.

Chemicals: Dimethylsulphoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), trypan blue, Fetal Bovine Serum, Penicillin/ Streptomycin antibiotic and Trypsin- EDTA Sigma Aldrich Chemical Co., St. Louis, Mo, U.S.A. Tris buffer was obtained from Applichem, Germany. All chemicals and reagents used in this study are of highest analytical grade.

Methods:

Reagents and buffers: 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% CO_2.

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

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

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.

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

Procedures:

Maintenance of the human cancer cell lines in the laboratory: A cryotube containing frozen cells was taken out of the liquid nitrogen container and then thawed in a water bath at 37°C. 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. The tube was incubated for 2 hours then centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded and the cell pellet was suspended and Seeded in 5 mL supplemented medium in T25 Nunclon sterile tissue culture flasks. The cell suspension was incubated and followed up daily the supplemented medium was replaced every 2- 3 days. 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.

Collection of cells by trypsinization: First, the medium was

discarded. The monolayer cell was washed twice with 5 mL phosphate buffered saline. 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.

Determination and counting of viable cells: Fifty µL of 0.05% trypan blue solution was added to 50 µL of the single cell suspension. The cells were examined under the inverted microscope using the haemocytometer. Non stained (viable) cells were counted (Viable cells /mL=(number of cells in 4 quarters x 2 (dilution factor) x 10^4)/ 4). The cells were then diluted to give the required cell number for each experiment.

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

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

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

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

Determination of potential cytotoxicity of drug on human cancer cell line

Principle: The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay following the method reported by Vichai and Kirtikara, (2006) [25]. 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.

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

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

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

10 mM tris 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.

Procedure: Cells will be seeded in 96 well microtiter plates at a concentration of 1000-2000 cells/well, 100 µL/well. After 24 h, cells will be incubated for 72 h with various concentrations of drugs (0, 6.25, 12.5, 25, 50, 100 and 200 µg/mL). For each derivative concentration and doxorubicin, 3 wells were used. The plates were incubated for 72 hours. The medium is discarded. The cells were fixed with 150 µl cold trichloroacetic acid 10% final concentration for 1 hour at 4°C. 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. The plates were washed with 1% acetic acid to remove unbound dye and air-dried (24 h). The dye was solubilized with 150 µl/well of 10 mM tris base (PH 7.4) for 5 min on a shaker at 1600 rpm. 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 linear trendline equation.

Results and Discussion

Chemistry

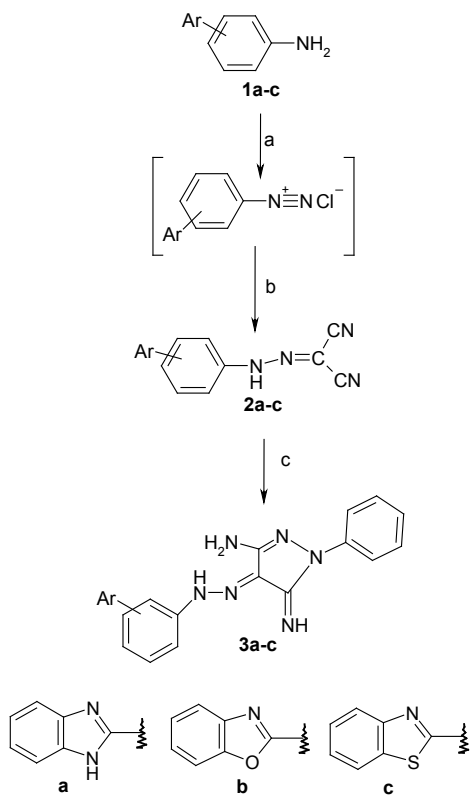
The synthesis of the target compounds was summarized in schemes 1 and 2. The substituted phenyl hydrazono-malononitriles 2a-c were synthesized by diazotization of different substituted aromatic amines 1 a-c followed by coupling their diazonium salt with malononitrile in the presence of sodium acetate.

The structure of compounds 2a-c was confirmed through ^{1}Hnmr which showed the appearance on single new D_2O exchangeable peak at δ 4.44, 4.99 and 15.15 ppm corresponding to NH proton. The IR spectrum showed a pronounced peak of CN group at 2224 to 2221 cm^{-1}. Also in mass spectrum the molecular ion peaks of 2a-c were appeared.

Condensation of substituted phenyl hydrazono-malononitriles 2a-c with phenyl hydrazine afforded compounds 3a-c (Scheme 1).

^{1}HNMR of compounds 3a-c showed single signals at δ 3.30, 6.65 and 6.25 ppm which corresponding to NH_2 protons and also D_2O peaks in aromatic region.

In IR spectrum the peaks appeared from 3463 to 3167 cm^{-1}which



Scheme 1: Reagent and conditions: a) HCl, $NaNO_2$; b) $CH_2(CN)_2$, Na acetate, aqueous ethanol, 0 °C, 2 h; c) Phenyl hydrazine, glacial acetic acid, DMF, reflux, 10 h.

indicated NH or NH_2, in the same time CN peak disappeared. Also the mass spectrum of compound 3a-c showed the molecular ion peaks.

The respective substituted phenyl hydrazono malononitrile 2a-c was heated under reflux with hydrazine hydrate to yield the diamino pyrazole derivatives 4a-c.

The structure of 4a-c was confirmed by ^{1}HNMR, IR, mass spectroscopy and elemental analysis.

^{1}HNMR of compounds 4a-c showed broad D_2O exchangeable peak at δ 6.33, 6.30 and 6.40 ppm which corresponding to 2 NH_2 protons.

Also in IR spectrum the forked peaks of NH_2 groups were appeared at 3394 to 3210 cm^{-1} in the same time disappeared of CN peak. The molecular ion peaks of 4a-c appeared in suitable relative intensity.

Heating equimolar amounts of the pyrazole derivatives 4a-c with the corresponding aromatic aldehyde in dimethyl formamide containing catalytic amount of glacial acetic acid yielded *N*-benzylidine pyrazole diamine 5a-i (Scheme 2) The structure elucidation of compounds 5a-i was made using HNMR mass, IR and elemental analysis.

The broad D_2O exchangeable peak of compounds 4a-c disappeared and azomethane peak appeared at δ 8.83 to 9.99 in ^{1}HNMR spectrum. The relative intensities of molecular ions of 5a-i were showed in mass spectrum.

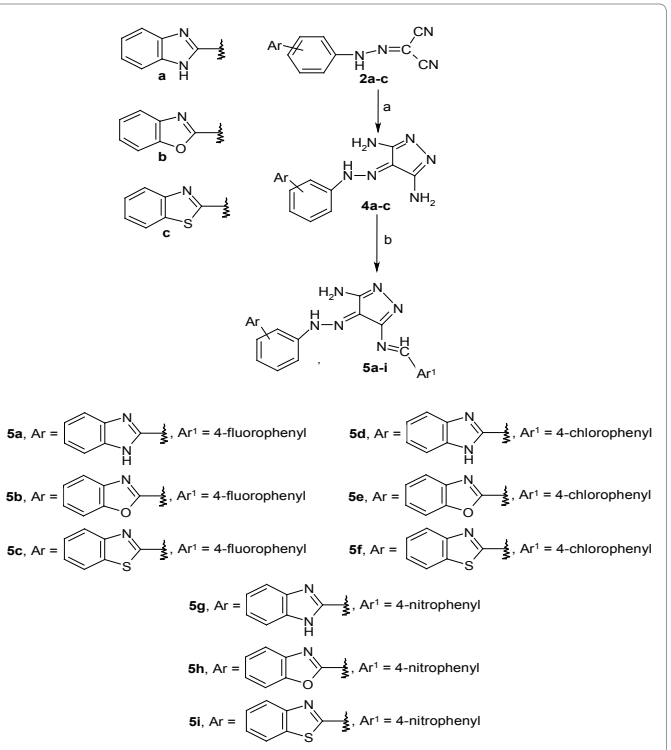
Antiproliferative activity

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

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 5a-i seemed to be more effective in decreasing the survival percent than pyrazol-3-ylamine derivatives 3a-c. Based on their cytotoxic efficacy and IC_{50}, the compounds are arranged in a descending order as follows: compounds 5b (IC_{50}= 16.22 µg/mL), 5a (29.85 µg/mL), 5c (34.20 µg/mL), 5d (34.87 µg/mL), 5g (38.57 µg/mL), 5h (53.71 µg/mL), 5i (87.70 µg/mL), 3a (96.00 µg/mL), 5f (100.78 µg/mL), 5e (100.90 µg/mL), 3b (120.22 µg/mL) and 3c (IC_{50}= 127.17 µg/mL) (Figure 1).

Figure 2 revealed that compound 3a 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 5e (IC_{50}= 72.93 µg/mL), 5c (77.39 µg/mL), 5f (80.11 µg/mL), 5a (83.79 µg/mL), 5h (85.35 µg/mL), 5i (99.27 µg/mL), 5g (156.80 µg/mL), 3b (165.78 µg/mL), 3c (174.16 µg/mL), 5d (201.30 µg/mL) and 5b (IC_{50}= 233.05 µg/mL). Thus, derivative 3a followed by 5e and 5c seemed to have the most cytotoxic effects against breast carcinoma cell lines (Figure 2).

The previous report [26] revealed that the benzimidazole derivatives and benzoxazole natural products against lung cancer cell line A549 and breast cancer cell line MCF-7. Benzothiazole derivatives were reported to show growth inhibition against a broad spectrum of human cancer cells and induced apoptosis of HepG2 cells in a dose-



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

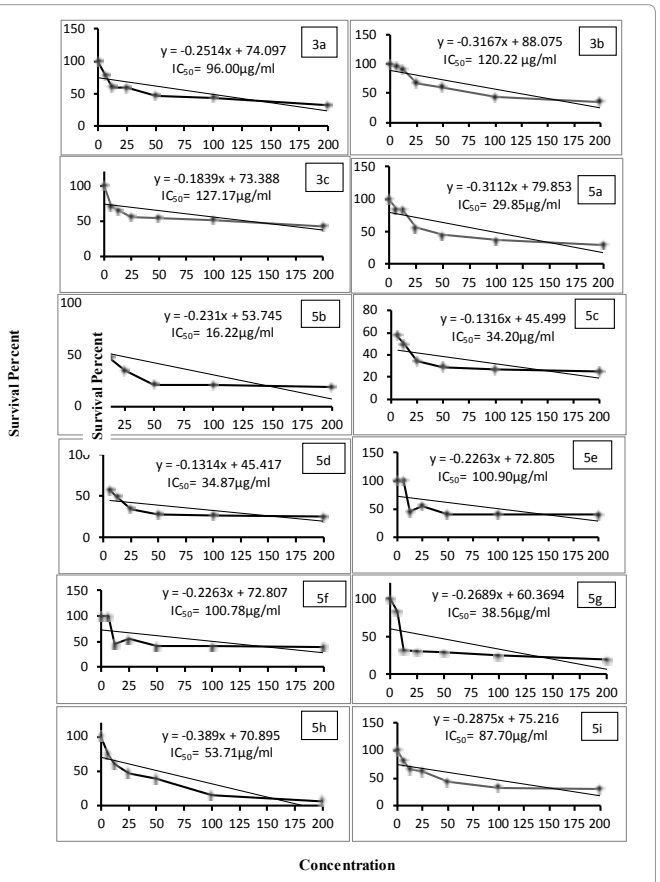


Figure 1: Anti-tumor cytotoxicity of different concentrations (µg/mL) of pyrazol-3-ylamine and pyrazole-3,5-diamine derivatives against human hepatocarcinoma cell lines (Huh-7) *in vitro*.

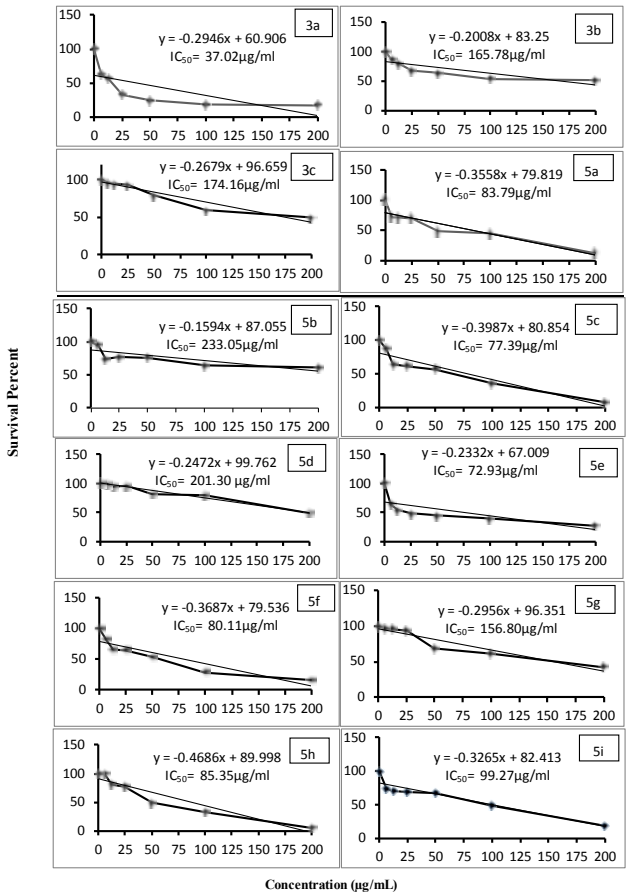


Figure 2: Anti-tumor cytotoxicity of different concentrations (µg/mL) of pyrazol-3-ylamine and pyrazole-3,5-diamine derivatives against human breast cell lines (T47D) *in vitro*.

and time-dependent manner [27,28]. In addition, pyrazole nucleus was revealed to have an important role in producing anti-tumor potencies [13,29]. Based on these reports, the linking pyrazole derivatives to benzimidazole, benzoxazole and benzothiazole in the present study may potentiate the anti-tumor efficacies of novel pyrazol-3-ylamine and pyrazole-3,5-diamine derivatives.

In conclusion, he preliminary biological evaluations revealed that the newly synthesized pyrazole derivatives 5b showed the potent *in vitro* antitumor activity against Huh-7 hepatocarcinoma cell lines (16.22 µg/mL), while derivative 3a was the most effective in decreasing the survival percent of T47D breast carcinoma cell lines (IC_{50}= 37.02µg/mL). However, further future studies are required to elucidate the mechanism of action of these antiproliferative derivatives both *in vitro* and *in vivo*.

The structure activity relationship of the synthesized compounds based upon the anticancer screening showed that, the activity of the substituted pyrazoles increases upon increasing electron negativity of halogen of phenyl ring as a result, the flouro derivatives (3a, 5b, 5c) and the chloro derivative (5e) showed the pronounced activity.

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