Novel Pyrazole Derivatives as Anticancer and Radiosensitizing Agents

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Abstract

The present article describes the synthesis of some novel pyrrole, pyrazolo[4,3-*d*]oxazole, pyrrolo[2,3-*b*]pyridine, 1,2,3-triazole and oxoazetidin derivatives incorporating pyrazole moiety, the structures of which were confirmed by elemental analyses and spectral data. All the target compounds were subjected to in-vitro antitumor activity against liver and colon human tumor cell lines (HEPG2 and HCT), furthermore, the most potent compounds were evaluated for their ability to enhance the cell killing effect of γ-radiation (radiosensitizing evaluation). The results of in-vitro anticancer evaluation showed that compounds **3** and **16a** were the most potent compounds on HEPG2 (IC_{50} = 2.6 and 4.2 µg/ml) and compounds **2** and **10** were the most potent on HCT (IC_{50} = 2.7 and 3.9 µg/ml) compared to vinblastine (IC_{50} = 4.6 on HEPG2 and 2.6 µg/ml on HCT), while, the activity of the most potent compounds increased after combination with γ-radiation and they showed no toxicity on normal hepatocytes and colon cells at their effective concentrations.

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Introduction

Pyrazole showed promising anticancer effects and in the 1960s, it was evaluated in phase I studies as an antitumor agent in man, but, even in doses of 0.15 mmol/kg/day it proved too toxic for human use because of development of signs of hepato-toxicity [1]. Trying to overcome this toxicity, 1-carboxamidopyrazole and 1-thiocarbamoylpyrazole were synthesized and they showed significant anticancer effects on animal experiments, but failed to pass the clinical evaluation [2, 3]. In search for better antitumor treatment, a large series of pyrazole derivatives were synthesized and tested over the years, the use of this powerful pharmacophore being very popular and modern [4–6]. In the last decade several pyrazole derivatives proved to have potent anticancer action by the inhibition of the cyclin-dependent kinases (CDKs). CDKs are members of the large family of protein kinases and are responsible for the eukariotic cell cycle regulation; they are intensively studied for their cancer implication [7–11]. Based on the model of 1-carboxamidopyrazole, a series of pyrazole derivatives containing an urea scaffold proved to have antiproliferative effects by inhibiting Aurora kinase activity [12, 13]. Some other protein kinases are targets of pyrazole based derivatives, some aryl- and heteroaryl-substituted pyrazole act as inhibitors of the transforming growth factor beta type I receptor kinase domain [14]. The anticancer effects of some pyrazoleamide derivatives are mediated by inhibition of microtubule depolymerization [15]. Based on the above informations and due to our interest in pyrazole as a biologically active pharmacophore [16–21], we synthesized novel pyrazole derivatives containing oxazole, pyridine, triazole, oxoazetidin starting from 4-aminoantipyrine to explore their anticancer and radiosensiting activities (**Fig. 1**).

Materials and Methods

Chemistry

Melting points (°C, uncorrected) were determined in open capillaries on a Gallenkemp melting point apparatus (Sanyo Gallenkemp, Southborough, UK). Precoated silica gel plates (silica gel 0.25 mm, 60 G F 254; Merck, Germany) were used for thin layer chromatography, dichloromethane/methanol (9.5:0.5 ml) mixture was used as a developing solvent system at room

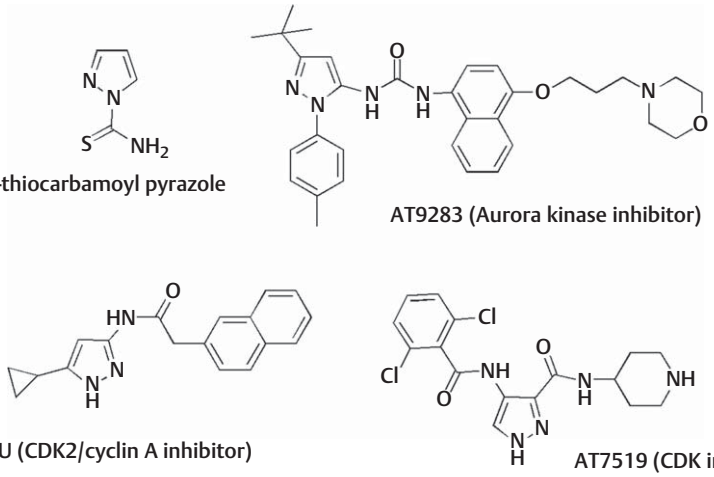

Fig. 1 Structures of antitumor pyrazoles.

temperature and the spots were visualized by ultraviolet light and/or iodine. Infrared spectra were recorded in KBr discs using IR-470 Shimadzu spectrometer (Shimadzu, Tokyo, Japan). ^{1}H NMR spectra (in DMSO-d_6) were recorded on Bruker Ac-300 ultra shield NMR spectrometer (Bruker, Flawil, Switzerland, ppm) at 300 MHz, using TMS as internal standard. Electron impact Mass Spectra were recorded on Shimadzu GCMS-QP-1000EX mass spectrometers at 70 eV. (Shimadzu, Tokyo, Japan). Elemental analyses were performed on Carlo Erba 1108 Elemental Analyzer (Heraeus, Hanau, Germany). All compounds were within ± 0.4% of the theoretical values.

4-Amino-1,5-dimethyl-2-phenyl-1,2-dihydropyrazole-3-thione (2)

A mixture of compound **1** (2.03 g, 0.01 mol), and P_2S_5 (3 g, 0.01 mol) in pyridine (50 ml) was heated under reflux for 10 h. The solvent was removed under reduced pressure and the obtained residue was washed with 2*N* HCl, and recrystallized from dioxane to give gray crystals **2.** (69% yield), m.p. 210–212 °C; IR(KBr, cm^{-1}): 3398, 3338 (NH_2), 3155 (CH arom.), 2967 (CH aliph.), 1555 (C=S), MS(m/z): 219 (M^+-1, 5.86%), 63(100% base peak), ^{1}HNMR (DMSO-d_6) δ: 1.9 (s, 3H, CH_3), 3.4 (s, 3H, N-CH_3), 7.2–7.5 (m, 5H, Ar-H), 9.0 ppm (s, 2H, NH_2). Anal. Calculated for $C_{11}H_{13}N_3S$: C, 60.24; H, 5.97; N, 19.16; S, 14.62; Found: C, 60.04; H, 5.57; N, 19.06; S, 14.42.

2,3-Dimethyl-1-phenyl-1,2-dihydro-4H-pyrazolo[4,3-d] oxazole-5(6 aH)-thione (3)

To a warmed ethanolic potassium hydroxide solution [prepared by dissolving (0.40 g, 10 mmol) potassium hydroxide in ethanol (50 mL)] of compound **1** (2.03 g, 0.01 mol) excess carbon disulphide (10 mL) was added. The mixture was heated on a water bath at 80 °C under reflux for 7 h, then allowed to cool to r.t. poured into water (100 mL), and neutralized by dilute HCl; the formed precipitate was filtered off and dried. The product was crystallized from benzene to give white crystals **3.** (70% yield), m.p. > 280 °C; IR(KBr, cm^{-1}): 3328 (NH), 3102 (CH arom.), 2917(CH aliph.), 1145 (C=S), MS (m/z): 247 (M, 3.59%), 55 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 1.9 (s, 3H, CH_3), 3.4 (s, 3H, N-CH_3), 7.3–7.5 (m, 5H, Ar-H), 9.88 ppm (s, H, NH). Anal. calculated for $C_{12}H_{13}N_3OS$: C, 58.28; H, 5.30; N, 16.99; S, 12.97; Found: C, 58.08; H, 5.10; N, 16.69; S, 12.57.

2,3,5,8-Tetramethyl-1-phenyl-1,2,3,5,8,9-hexahydropyrazolo[4,3 : 4`,5`] imidazolo[2,1: 3`,4`] [1,2,4]triazolo[4,3-b][1,2,4]triazole (6)

A mixture of **3** (2.47 g, 0.01 mol) and hydrazine hydrate (0.02 mol, 80%) in acetic acid (20 ml) was refluxed for 5 h. The H_2S liberated during the reaction was detected on a lead acetate paper. The reaction mixture was allowed to cool and the obtained solid was filtered off, washed with water, dried and recrystallized from acetic acid to give **6** as pale yellow crystals (88% yield), m.p. 138–139 °C; IR (KBr, cm^{-1}): 3287 (NH), 3191 (CH arom.), 2954 (CH aliph.), MS (m/z): 311 (M^++1, 12.66%), 55 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 1.7, 1.9, 2.0 (s, 9H, $3CH_3$), 2.8 (s, 3H, N-CH_3), 4.2(s, 1H, NH), 7.2–7.5 ppm (m, 8H, Ar-H). Anal. Calculated for $C_{16}H_{21}N_7$: C, 61.72; H, 6.80; N, 31.49; Found: C, 61.42; H, 6.50; N, 31.29.

Ethyl-2-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-ylamino) acetate (7)

A mixture of **1** (2.03 g, 0.01 mol), anhydrous potassium carbonate (2.7 g, 0.02 mol) and ethyl bromoacetate (1.85 g, 0.015 mol) in dry acetone (50 ml) was refluxed for 24 h. The excess solvent was removed and the solid product was filtered off, and recrystallized from benzene to give **7** as colorless crystals; (74% yield), m.p. 167–169 °C; IR (KBr, cm^{-1}): 3210 (NH), 3156 (CH arom.), 2950, 2848(CH aliph.), 1728, 1668 (2C=O), ^{1}HNMR (DMSO-d_6) δ: 1.2 (s, 3H, CH_3), 1.4 (t, 3H, CH_2CH_3), 2.4 (s, 2H, NH), 3.3 (s, 3H, N-CH_3), 4.0 (q, 2H, CH_2CH_3), 4.6 (s, 2H, CH_2CO), 7.2–7.5 ppm (m, 5H, Ar-H). Anal. Calculated for $C_{15}H_{19}N_3O_3$: C, 62.27; H, 6.62; N, 14.52; Found: C, 62.07; H, 6.32; N, 14.32.

2-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-ylamino)acetic acid (9)

A mixture of **1** (2.03 g, 0.01 mol), chloroacetatic acid (0.5 g, 0.005 mol) and anhydrous sodium acetate (2 g, 0.02 mol) in acetic acid (10 ml)/acetic anhydride (5 ml) was refluxed for 6 h. The reaction mixture was allowed to cool and then poured onto water, stirred for 1 h and left overnight. The precipitated solid was filtered off, dried and recrystallized from petroleum ether to give **9** as white crystals (61% yield), m.p. 150–152 °C; IR (KBr, cm^{-1}): 3440, 3284 (br, OH/NH), 3025 (CH arom.), 2950, 2880 (CH aliph.), 1710, 1690 (2C=O), ^{1}HNMR (DMSO-d_6) δ: 1.8 (s, 3H, CH_3), 3.3 (s, 3H, N-CH_3), 4.3(s, 2H, CH_2CO), 7.2–7.7 (m, 6H,

Ar-H+NH), 9.4 ppm (s, 1H, OH). Anal. Calculated for $C_{13}H_{15}N_3O_3$: C, 59.76; H, 5.79; N, 16.08; Found: C, 59.46; H, 5.69; N, 15.78.

1,5-Dimethyl-4-(2-oxo-2-phenylethylamino)-2-phenyl-1,2-dihydropyrazol-3-one (10)

A mixture of **1** (2.03 g, 0.01 mol) and phenacyl bromide (1.99 g, 0.01 mol) in ethanol (50 mL) was refluxed for 3 h. After cooling the formed precipitate was filtered, washed with absolute ethanol and dried to give the final product as white crystals (80% yield), m.p. 138–139 °C; IR (KBr, cm^{-1}): 3292 (NH), 3058 (CH arom.), 2923(CH aliph.), 1699, 1668 (2C=O), ^{1}HNMR (DMSO-d_6) δ: 1.7 (s, 3H, CH_3), 3.3 (s, 3H, N-CH_3), 3.9 (s, 2H, CH_2CO), 6.5 (s, 1H, NH) 7.0–7.7 ppm (m, 10H, Ar-H). Anal. Calculated for $C_{19}H_{19}N_3O_2$: C, 71.01; H, 5.96; N, 13.08; Found: C, 71.31; H, 6.26; N, 13.38.

4,6-Diamino-1-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-3-phenyl-1H-pyrrolo[2,3-b]pyridine-5-carbonitrile (11)

A mixture of compound **10** (3.21 g, 0.01 mol) and malononitrile (1.4 g, 0.02 mol) was refluxed in ethanol (50 mL) containing sodium ethoxide (0.5 g, 0.01 mol) was refluxed for 5 h. The reaction mixture was cooled and acidified with diluted HCl. The separated crystals were recrystallized from petroleum ether to give brown crystals **11**: (85% yield), m.p. 126–127 °C; IR(KBr, cm^{-1}): 3331, 3287 (br,2NH_2), 3056 (CH arom.), 2924, 2858 (CH aliph.), 2201 (C≡N),1668 (C=O), MS(m/z): 435 (M^+, 2.36%), 77 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 1.5 (s,3H, CH_3), 3.4 (s, 3H, N-CH_3), 6.3, 6.5 (2s, 2H, 2NH_2), 7.0–7.5 ppm (m, 10H, Ar-H). Anal. Calculated for $C_{25}H_{21}N_7O$: C, 68.95; H, 4.86; N, 22.51; Found: C, 68.65; H, 4.56; N, 22.31.

General procedure for diazodisation and Coupling of antipyrine 1

To a mixture of **1** (0.5 g, 0.002 mol), water (7 ml) and conc. HCl (3 ml), $NaNO_2$-solution (0.20 g in 1 ml water, 0.002 mol) was added dropwise at 0 °C. After 30 min in an ice bath, the mixture was filtered and the diazonium salt solution was added dropwise to cold solution of the corresponding nucleophile (sodium azide and ethyl cyanoacetate). The reaction mixture was stirred for 30 min, extracted with Et_2O, dried with Na_2SO_4 and evaporated. The obtained products were purified by column chromatography to give **13** and **15,** respectively.

4-Azido-1,5-dimethyl-2-phenyl-1,2-dihydropyrazol-3-one (13)

Yellow crystals: (84% yield), m.p. >280 °C; IR(KBr, cm^{-1}): 3066 (CH arom.), 2927 (CH-aliphatic), 2112 (N3), 1700 (C=O), MS(m/z): 229 (M^+-1, 1.47%), 55 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 2.3 (s,3H, CH_3), 3.2 (s, 3H, N-CH_3), 7.0–7.5 ppm (m, 5H, Ar-H). Anal. Calculated for $C_{11}H_{11}N_5O$: C, 57.63; H, 4.84; N, 30.55; Found: C, 57.93; H, 4.94; N, 30.75.

Ethyl-2-cyano-2-((1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)diazenyl)acetate (15)

Brown crystals: (88% yield), m.p. 162–164 °C; IR(KBr, cm^{-1}): 3207 (NH), 3180 (CH arom.), 2931(CH aliph.), 2221 (C≡N),1774,1681 (2C=O), ^{1}HNMR (DMSO-d_6) δ: 1.4 (t, 3H, CH_2CH_3),1.9 (s,3H, CH_3), 2.2 (s, 3H, N-CH_3), 4.0 (q, 2H, CH_2CH_3), 6.4(s, H, NH), 7.0–7.8 ppm (m, 5H, Ar-H). Anal. Calculated for $C_{16}H_{17}N_5O_3$: C, 58.71; H, 5.23; N, 21.39; Found: C, 58.51; H, 5.03; N, 21.19.

5-Amino-1-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-1H-1,2,3-triazole-4-carbonitrile (14)

A mixture of compound **13** (2.99 g, 0.01 mol) and malononitrile (1.4 g, 0.02 mol) was refluxed in ethanol (50 mL) containing sodium ethoxide (0.5 g, 0.01 mol) for 5 h. The reaction mixture was cooled and acidified with diluted HCl. The separated crystals were recrystallized from petroleum ether to give brown crystals **14**: (95% yield), m.p.>280 °C; IR (KBr, cm^{-1}): 3331, 3245 (NH_2), 3056 (CH arom.), 2980, 2854 (CH aliph.), 2220 (C≡N),1668 (C=O), MS(m/z): 295 (M+1, 3.82%), 261 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 1.8 (s,3H, CH_3), 2.8 (s, 3H, N-CH_3), 6.5 (s, 2H, NH_2), 7.0–7.8 ppm (m, 5H, Ar-H). Anal. calculated for $C_{14}H_{13}N_7O$: C, 56.94; H, 4.44; N, 33.20; Found: C, 56.64; H, 4.14; N, 33.00.

General procedure for the synthesis of schiff base derivatives (16a,b)

A mixture of compound **1** (2.03 g, 0.01 mol) and 4-nitro and/or 4-florobenzaldehyde (0.02 mol) in the presence of anhydrous K_2CO_3 (0.5 g) was refluxed in dioxane (50 mL) containing triethylamine (0.01 mol) for 5 h. The reaction mixture was cooled and acidified with diluted HCl. The separated crystals were recrystallized from ethanol to give yellow crystals **16a,b,** respectively.

1,5-Dimethyl-4-(4-nitrobenzylideneamino)-2-phenyl-1,2-dihydro-pyrazol-3-one (16a)

Yellow crystals: (90% yield), m.p 156–158 °C; IR(KBr, cm^{-1}): 3071(CH arom.), 2932(CH-aliphatic), 1663 (C=O), 1615 (C=N), MS(m/z): 336 (M^++1, 45.8%), 56 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 2.4 (s,3H, CH_3), 3.3 (s, 3H, N-CH_3), 7.3,7.4 (2d, 9H, Ar-H AB system), 8.3 ppm (s, 1H, N=CH). Anal. Calculated for $C_{18}H_{16}N_4O_3$: C, 64.28; H, 4.79; N, 16.66; Found: C, 64.58; H, 4.99; N, 16.86.

4-(4-Fluorobenzylideneamino)-1,5-dimethyl-2-phenyl-1,2-dihydropyrazol-3-one (16b)

Brown crystals: (70% yield), m.p 90–92 °C; IR(KBr, cm^{-1}): 3051(CH arom.), 2927 (CH-aliphatic), 1665 (C=O), MS(m/z): 309 (M^+, 4.62%), 56 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 2.4 (s,3H, CH_3), 3.2 (s, 3H, N-CH_3), 7.5,7.6 (2d, 9H, Ar-H AB system), 8.1 ppm (s, 1H, N=CH). Anal. Calculated for $C_{18}H_{16}FN_3O$: C, 69.89; H, 5.21; N, 13.58; Found: C, 69.59; H, 5.01; N, 13.38.

4-(3-Chloro-2-(4-nitrophenyl)-4-oxoazetidin-1-yl)-1,5-dimethyl-2-phenyl-1,2-dihydropyrazol-3-one (17)

The mixture of 1,5-dimethyl-4-(4-nitrobenzylideneamino)-2-phenyl-1,2-dihydro pyrazol-3-one **16a** (0.01 mol) and chloroacetyl chloride (0.01 mmol) was dissolved in ethanol (50 ml) containing piperidine (0.01 mol). The reaction mixture was then stirred for 3 h and left at room temperature for 48 h. The product obtained was purified by column chromatography using 30% ethyl acetate: 70% benzene as an eluent to give 2-azetidinones **17** as yellow crystals: (87% yield), m.p 160–162 °C; IR(KBr, cm^{-1}): 3075(CH-arom.), 2928(CH-aliphatic), 1764 (C=O of β-lactam), 1665 (C=O), MS(m/z): 412 (M^+, 4.2%), 56 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 2.3 (s,3H, CH_3), 3.3(s, 3H, N-CH_3), 5.3 (s, 1H, N-CH), 5.3(s,1H, CH–Cl), 7.9,8.0 ppm (2d, 9H, Ar-H AB system) Anal. Calculated for $C_{20}H_{17}ClN_4O_4$: C, 58.19; H, 4.15; N, 13.57; Found: C, 58.39; H, 4.45; N, 13.77.

4-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-ylamino)-4-oxobutanoic acid (18)

A mixture of compound **1** (2.03 g, 0.01 mol) and succinic anhydride (1 g, 0.01 mol) was refluxed in ethanol (50 mL) for 5 h, the solid obtained was poured onto cold water and acidified with diluted HCl. The separated crystals were recrystallized from dioxane to give white crystals **18**: (66% yield), m.p. 184–186 °C; IR (KBr, cm^{-1}): 3410, 3352 (br, OH/NH), 3062 (CH arom.), 2936 (CH aliph.), 1764,1741,1664 (3C=O), ^{1}HNMR (DMSO-d_6) δ: 1.8 (s,3H, CH_3), 2.4, 3.3 (2t, 4H, $2CH_2$), 3.1 (s, 3H, N-CH_3), 7.3–7.5 ppm (m, 5H, Ar-H), 9.1(s,1H,NH), 12.4(s,1H,OH). Anal. Calculated for $C_{15}H_{17}N_3O_4$: C, 59.40; H, 5.65; N, 13.85; Found: C, 59.70; H, 5.85; N, 13.95.

1-(1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-3-methylenepyrrolidine-2,5-dione(19)

A mixture of compound **1**(2.03 g, 0.01 mol) and 3-methylenedihydrofuran-2,5-dione (1.12 g, 0.01 mmol) was fused in an oil bath at 250 °C for 15 min, the product was triturated in dimethyl formamide and poured onto cold water, the solid obtained was recrystallized from ethanol to give black crystals **19**: (74% yield), m.p. 162–164 °C; IR (KBr, cm^{-1}): 3144 (CH arom.), 2951 (CH aliph.), 1744,1706,1669 (3C=O), MS(m/z): 297 (M+1, 1.66%), 50 (100% base peak), ^{1}HNMR (DMSO-d_6) δ: 2.3 (s,3H, CH_3), 2.7 (s, 2H, CO-CH_2-C), 3.3 (s, 3H, N-CH_3), 5.5(d,2H,CH_2=C), 7.3–7.8 ppm (m, 5H, Ar-H). Anal. Calculated for $C_{16}H_{15}N_3O_3$: C, 64.64; H, 5.09; N, 14.13; Found: C, 64.34; H, 2.9; N, 14.03.

In-vitro anticancer evaluation

Materials and methods

The human tumor cell lines (HEPG2 and HCT) were available at the regional center for mycology & biotechnology at Al-Azhar University, Cairo, Egypt. Mammalian cell lines: HEPG2 and HCT cells (human cell line of a well differentiated hepatocellular and colon carcinoma isolated from a liver colon biopsy of a male Caucasian aged 15 years) were obtained from the American Type Culture Collection (ATCC). Chemicals Used: Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza. Crystal violet stain (1%): It composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O and filtered through a Whatmann No.1 filter paper.

Procedure

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were subcultured 2 times a week. Cell toxicity was monitored by determining the effect of the test samples on cell morphology and cell viability.

Cytotoxicity evaluation using viability assay [22, 23]**:** For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100 µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial 2-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for a period of 48 h. 3 wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) has no effect on the cell line. After incubation of the cells for 24 h at 37 °C, various concentrations of sample (50, 25, 12.5, 6.25, 3.125 & 1.56µg) were added, and the incubation was continued for 48 h and viable cells yield was determined by a colorimetric method. In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 min. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The relation between surviving fraction and drug concentration was plotted to get the survival curve of each tumor cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and compared with the reference drug vinblastine and the results are given in ◉ **Table 1**.

Radiosensitizing evaluation

The most potent compounds resulted from the previous experiment; compounds **3, 16a, 2** and **10**, were selected to be evaluated again for their in vitro anticancer activity alone and in combination with γ-radiation. This study was conducted to evaluate the ability of these compounds to enhance the cell killing effect of γ-radiation and to prove the efficacy of combining radiotherapy with chemotherapy to reduce the dosage and toxicity of both. Cells were subjected to a single dose of γ-radiation at a dose level of 8 Gy with a dose rate of 2 Gy/min. Irradition was performed in the National Cancer Institute, Cairo University, using Gamma cell-40 (^{60}CO) source. The surviving fractions were expressed as means ± standard error and were analysed

Table 1 In-vitro anticancer evaluation against human tumor liver and colon cell lines (HEPG2 and HCT).

	IC50 [a,b] (µg/ml)	
Cpd. No.	HEPG2	HCT
2	8.8	2.7
3	2.6	9.4
6	12.6	8.2
8	36.4	24.8
9	20.6	6.4
10	12.5	3.9
11	43.8	7.4
13	9.8	9.5
14	23.5	12.6
15	30.2	25.9
16a	4.2	9.4
16b	16.8	9.6
17	13.2	13.8
18	32.4	24.6
19	22.9	19.6
Vinblastine	4.6	2.6

[a] IC50, compound concentration required to inhibit tumor cell proliferation by 50%

[b] Values are means of 3 experiments

Table 2 In-vitro anticancer evaluation of compounds **3, 16a** against human liver cell line (HEPG2) and compounds **2**, **10** against human colon cell line (HCT) after subjection to radiation.

Cpd.No	Cell line	Irradiation (8 Gy)	Cpd.Concentration (µg/ml)+ irradiation(8 Gy)				IC_{50}(µg/ml)	IC_{50}(µg/ml) (normal cells)
			0.5	1	1.5	3		
			Survival fraction[b] ± standard error					
3	HEPG2	0.927 ± 0.02*	0.126 ± 0.01*	0.092 ± 0.01*	0.084 ± 0.02*	0.052 ± 0.03*	0.12	47.7
16a		0.927 ± 0.02*	0.097 ± 0.01*	0.076 ± 0.03*	0.055 ± 0.04*	0.029 ± 0.03*	0.13	NA[c]
2	HCT	0.927 ± 0.02*	0.134 ± 0.02	0.096 ± 0.03*	0.077 ± 0.05*	0.058 ± 0.02*	0.14	35.9
10		0.927 ± 0.02*	0.099 ± 0.04*	0.081 ± 0.01*	0.067 ± 0.01*	0.036 ± 0.01*	0.12	NA[c]

[a] Each value is the mean of 3 experiments ± standard error

[b] Each value is the mean of 3 experiments

[c] No activity

* Significant difference from control group at $p < 0.05$

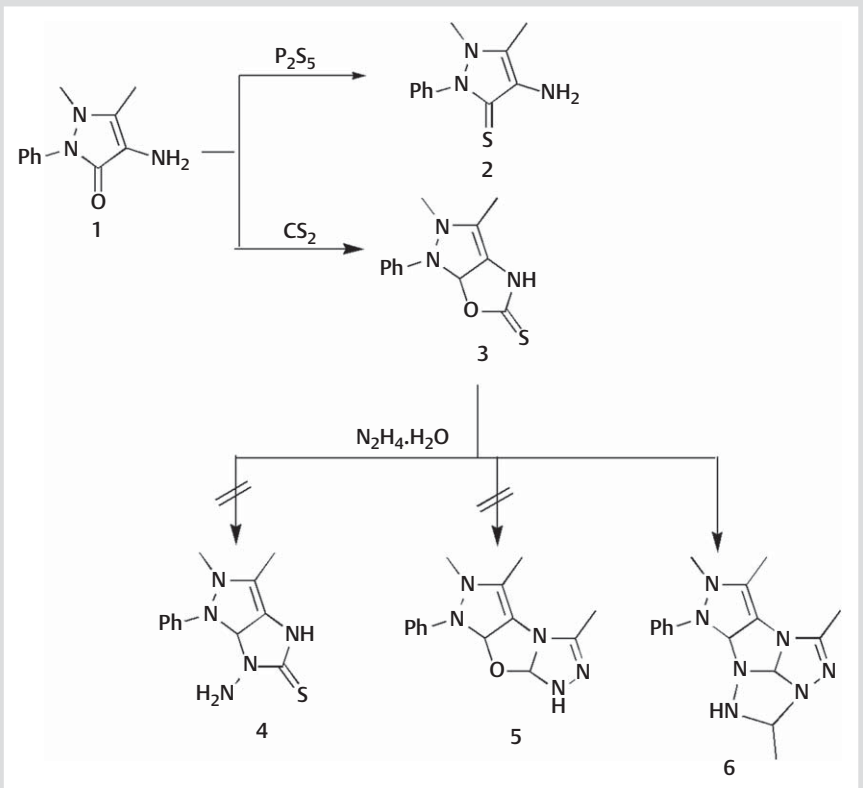

Fig. 2 Synthetic pathways for compounds 2, 3 and 6.

using 1-way ANOVA test. The results are given in **Table 2**. For better predicting the selectivity of these compounds to cancer cells rather than normal cell, the 4 compounds were tested on normal hepatocytes (cpd. **3** and **16a**) and colon cells (cpd. **2** and **10**) and their IC_{50} was calculated and is shown in **Table 2**.

Results and Discussion

Chemistry

The synthetic procedures adopted to obtain the target new compounds are showed in Schemes **1–4**. The 4-amino-1,5-dimethyl-2-phenyl-1,2-dihydropyrazol-3-one **1** was obtained from Sigma Company and used without further purification. 4-aminophenazone **1** was reacted successfully with phosphorus pentasulfide to afford the 4-amino-1,5-dimethyl-2-phenyl-1,2-dihydropyrazole-3-thione **2**. (Scheme **1**). On the other hand, pyrazolo[4,3–d]oxazole derivative **3** was prepared by treatment of compound **1** with bifunctional one carbon donor cyclizing agents. Thus, when 4-aminoantipyrine **1** heating with carbon disulphide in ethanolic potassium hydroxide gave pyrazolo[4,3-d]oxazole derivative **3**. Moreover, on reacting pyrazolo[4,3-d] oxazole derivative **3** with hydrazine hydrate in boiling ethanol was recovered unchanged. When the reaction was repeated in boiling acetic acid yielded the corresponding pyrazole derivative **6** and the other expected pyrrolo[3,2-c]pyrazoluredo derivative **4** and **5** were ruled out on the basis of analytical and spectral data. Structure of compound **6** was confirmed on the basis of elemental analysis and spectral data (**Fig. 2**).

The reactivity of amino group in the 4-position for antipyrine **1** towards halogenated compounds have been investigated. Thus, the treatment of antipyrine **1** with ethyl bromoacetate affords the *N*-alkylated product **7** and attempts to cyclizing the pyrazol-4-yl-aminoacetate derivative **7** by refluxing in ethanol containing piperidine and/ or pyridine to afford 2,3-dimethyl-1-phenyl-1,2-dihydropyrazolo[3,4-b] [1,4]oxazin-6-one **8** was unsuccessful on the basis of analytical and spectral data. The structure of compound **7** was confirmed by their IR, ^{1}HNMR and Mass spec-

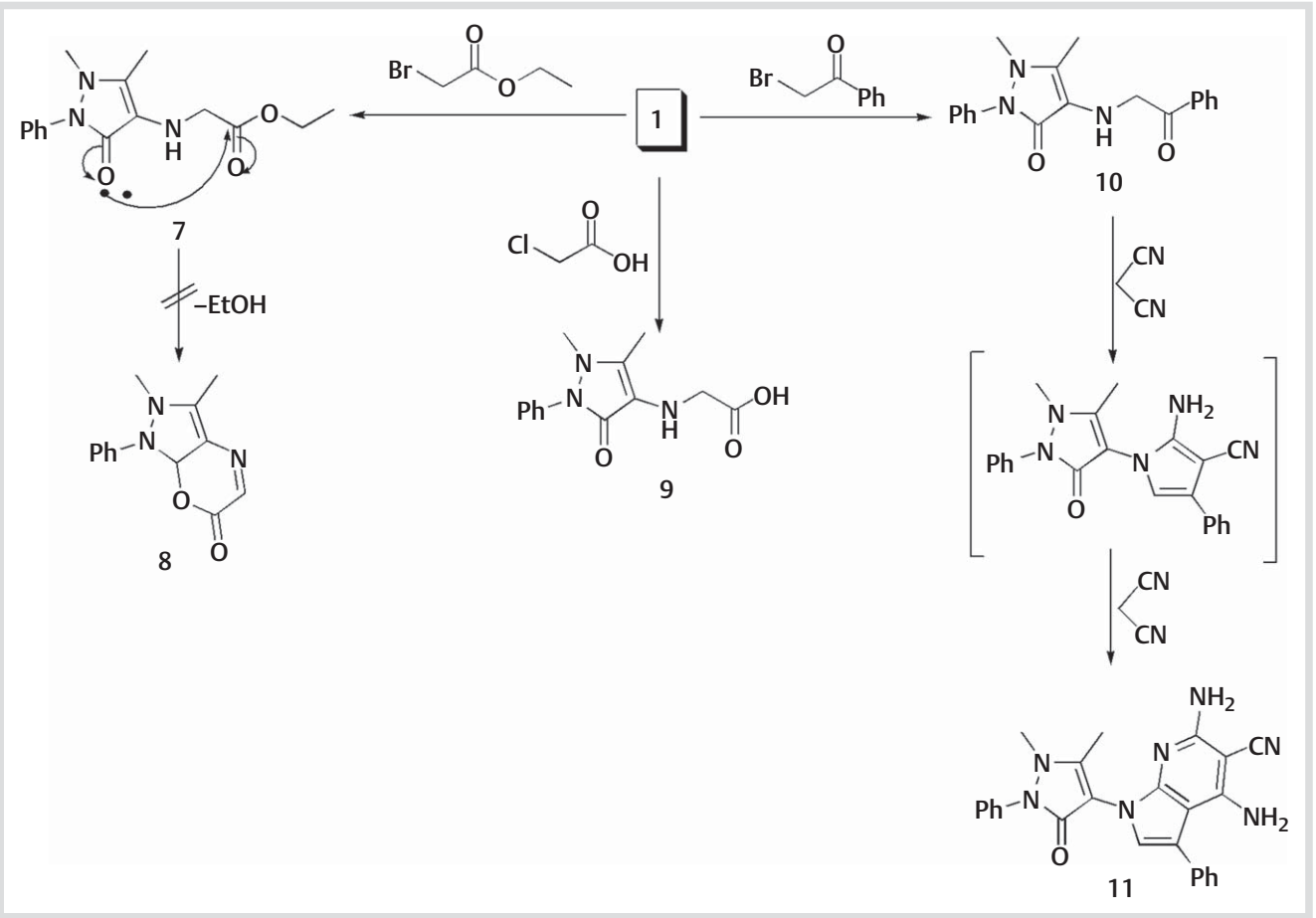

Fig. 3 Synthetic pathways for compounds 7, 9–11.

tra. Furthermore, treatment of **1** with chloroacetic acid in the presence acetic acid/acetic anhydride and anhydrous sodium acetate yielded pyrazol-4-ylaminoacetic acid derivative **9**, which showed evolution of CO_2 gas with aqueous sodium carbonate solution indicating the presence of carboxylic group. In a similar manner, the reaction of antipyrine **1** with equimolar amount of phenacyl bromide in refluxing ethanol afforded the corresponding phenylethylamine derivative **10**. Both of compounds **9** and **10** were elucidated by analytical and spectral data. Compound **10** was allowed to react with malononitrile in sodium ethoxide as a catalyst yielded the corresponding pyrrolo[2,3-b]pyridine derivative **11** (**Fig. 3**).

Diazodization of 4-aminoantipyrine **1** with sodium nitrite and hydrochloric acid at 5–10 °C followed by coupling with the corresponding nucleophile (such as sodium azide and ethyl cyanoacetate) furnished novel 4-azidopyrazole **13** [24] and hydrazone derivatives **15**, respectively. Interaction of compound **13** with malononitrile in sodium ethoxide as a catalyst yielded the corresponding 1,2,3-triazole derivative **14** [25] (**Fig. 4**).

Schiff base derivatives were reported to possess significant biological activities and new series have been tested for their antitumor, antimicrobial, and antiviral activities [26]. In the light of these facts, we report the synthesis of 1,5-dimethyl-4-(4-substituted-benzylideneamino)-2-phenyl-1,2-dihydropyrazol-3-one **16a,b** by the condensation reaction of compound **1** with 4-nitro and/or 4-florobenzaldehyde in presence of anhydrous K_2CO_3. The azomethine group in **16a** underwent cycloaddition with chloroacetyl chloride in dimethylformamide afforded 4-(3-chloro-2-(4-nitrophenyl)-4-oxoazetidin-1-yl)-1,5-dimethyl-2-phenyl-1,2-dihydropyrazol-3-one **17** (**Fig. 4**). The structure of compound **17** was confirmed by IR, ^{1}H NMR, MS spectra, and elemental analysis. To explain the outcome of this reaction, we postulated the reaction mechanism of azetidinone formation from chloroacetyl chloride and imines. We concluded that the atom carrying a free pair of electrons would form a loose bond with carbon and generate a 5 membered transition state (**I**). This would ease the formation of azetidinone (**II**) by bringing the appropriate carbon atoms C-3 and C-4 in (**II**) close enough to form a bond (**Fig. 5**) [27,28]. Finally, the behavior of aminoantipyrine towards the anhydride derivatives was also investigated. Thus, the reaction of antipyrine **1** with succinic anhydride in ethanol yielded the corresponding pyrazole derivative **18**. While, treatment of antipyrine **1** with 3-methylenedihydrofuran-2,5-dione under condition of fusion, gave the corresponding 1-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)-3-methylenepyrrolidine-2,5-dione **19** (**Fig. 4**).

In-vitro anticancer evaluation

The synthesized compounds were evaluated for their in vitro cytotoxicity against human liver and colon cancer cell lines (HEPG2 and HCT), vinblastine, which is one of the most potent anticancer agents, is the reference drug used in this study. From the results obtained in **Table 1**, concerning HEPG2, SAR of the tested compounds showed that the pyrazolooxazole **3** was the most active compound (IC_{50} = 2.6 µg/ml) and was found to be more active than the reference drug (IC_{50} = 4.6 µg/ml), while, its

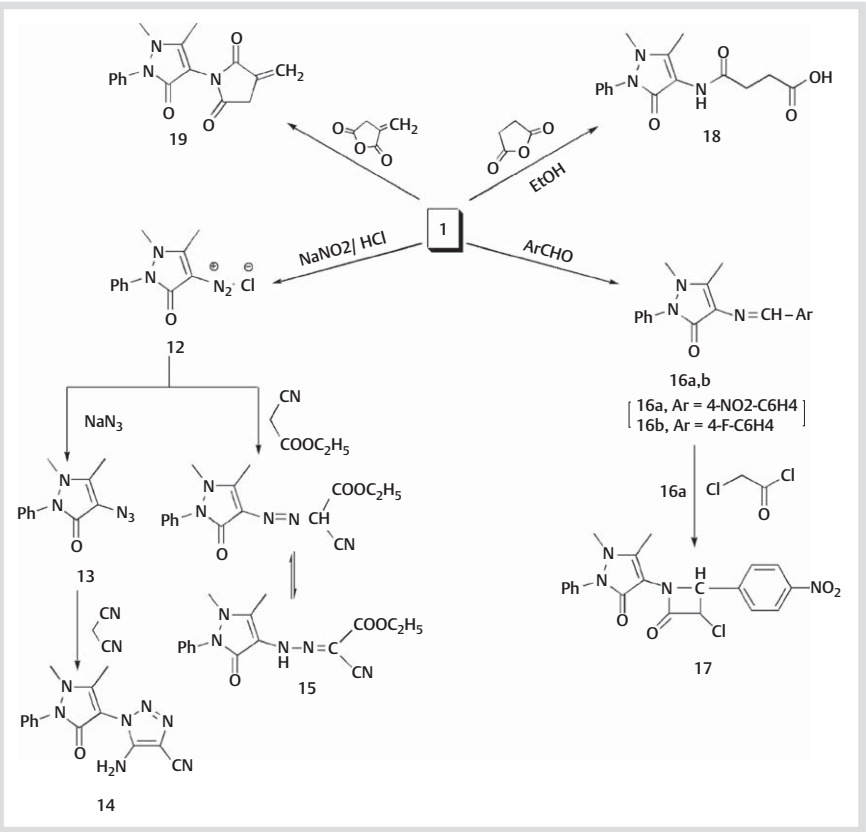

Fig. 4 Synthetic pathways for compounds 12–19.

double cyclization to the 4-ringed **6** yielded in a decrease in activity, the thione **2** and azide **13** derivatives showed moderate activity (IC_{50} = 8.8 and 9.8 µg/ml), while, cyclization of the azide to the corresponding triazole derivative **14** resulted in a decrease in the activity. The Schiff base **16a** showed high activity similar to that of the reference drug (IC_{50} = 4.2 µg/ml), while, a decrease in activity was observed upon cyclization to the corresponding lactam **17**. Compound **10** showed moderate activity (IC_{50} = 12.6 µg/ml), while, its cyclization to give **11** yielded in a drop in activity. Compounds **5**, **8**, **9** **18** and **19** were the least potent compounds on HEPG2 with their IC_{50} ranging from 20.6–36.4 µg/ml concerning HCT cell line, most of the compounds showed better activity than on HEPG2, the thione **2** was the most active (IC_{50} = 2.7 µg/ml) then comes compounds **10** and **9** (IC_{50} = 3.9 and 6.4 µg/ml). On the contrary to their results on HEPG2, compound **6** showed higher activity (IC_{50} = 8.2 µg/ml) than its starting material **3**. On the other hand, the azide **13** and the Schiff bases **16a**,**b** showed moderate activities (IC_{50} = 9.5, 9.4, 9.6 µg/ml, respectively) and they were found to be more active than the triazole **14** and the lactam derivative **17**. Finally, the least potent compounds on HCT were compounds **5**, **8**, **18** and **19** with their IC_{50} ranging from 19.6–25.9 µg/ml.

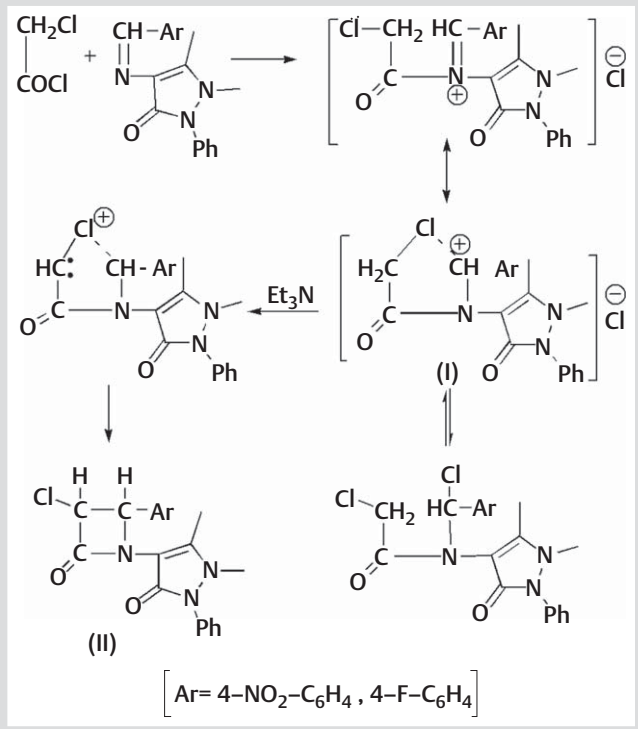

Fig. 5 Postulated mechanism for the formation of compound 17.

Radiosensitizing evaluation

The rationale for combining chemotherapy and radiotherapy is based mainly on 2 ideas, one being spatial cooperation, which is effective if chemotherapy is sufficiently active to eradicate subclinical metastases and if the primary local tumor is effectively treated by radiotherapy. In this regard, no interaction between radiotherapy and chemotherapy is required. The other idea is the enhancement of radiation effects. Cytotoxic agents can enhance radiation effects by direct enhancement of the initial radiation damage by incorporating drugs into DNA, inhibiting cellular repair, accumulating cells in a radiosensitive phase or eliminating radioresistant phase cells, eliminating hypoxic cells or inhibiting the accelerated repopulation of tumor cells [29, 30]. Consequently, the ability of the most active 4 compounds, compounds **3, 16a, 2** and **10**, to enhance the cell killing effect of γ-irradiation was studied. From the results obtained in

Table 2, we can observe that their IC_{50} synergistically decreased after irradiation. Concerning HEPG2, compounds **3** and **16a** showed IC_{50} of 2.6 and 4.2 µg/ml, while after irradiation decreased to be 0.12 and 0.13, respectively, similarly, on HCT cell line, compounds **2** and **10** their IC_{50} decreased from 2.7 and 3.9 µg/ml to become 0.14 and 0.12 µg/ml after irradiation. On the other hand, concerning the test of toxicity on normal cells, compound **3** showed IC_{50} = 47.7 µg/ml on normal hepatocytes, while, compounds **16a** showed no activity on the same cells. Compound **2** showed IC_{50} = 35.9 µg/ml on normal colon cells, while, compounds **10** showed no activity on the same cells which indicates their selectivity on cancer cells rather than normal cells when given at their effective dosages.

Conclusion

From the above results, we can conclude that administration of the tested compounds on human liver and colon (HEPG2 and HCT) cell lines showed promising cytotoxic activity especially on colon cell line, while, combining the most potent compounds with radiation at lower concentrations enhances their activity which demonstrates the importance of the combination therapy for the patients with cancer to decrease the side effects of both drugs and radiation. On the other hand, the test of toxicity showed that the tested compounds were safe on normal hepatocytes and colon cells which indicate their selectivity to cancer cells.

Conflict of Interest

The authors declare that they have no conflict of interest with respect to this paper.

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