Reactions of some pyrazole-3-carboxylic acids with various \( N,N' \)-binucleophiles and investigation of their antiproliferative activities

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Synthesis, characterization, and antiproliferative activity evaluation of a series of novel pyrazole bis-carboxamide derivatives (8–12) have been described. Synthesized molecules have been screened to evaluate their antiproliferative activities against HeLa (human uterus carcinoma), Vero (green monkey kidney), and C6 (Rat Brain tumor) cells as in vitro. The tests are carried out as dose-dependent assay starting from 100 \( \mu \)g/mL to 500 \( \mu \)g/mL. Generally the compounds have been shown to possess weak antiproliferative activity against all cell lines as compared with Cisplatin and 5-Fluorouracil. Only compound 8 shows moderate antiproliferative activity against Hela cancer cells.

Keywords: Pyrazole carboxylic acid, \( N,N' \)-binucleophiles, bis-carboxamide, antiproliferative, cancer cells

Cancer is one of the leading causes of death worldwide (accounting for 7.6 million deaths in 2008) and deaths from cancer are projected to continue rising, with an estimated 13.1 million deaths in 2030 (Ref 1-6). Cancer is a group of diseases characterized by loss of cellular growth control that can affect any part of the body. The non-selectivity of the currently available drugs in differentiating between tumor cells and normal tissue leads to severe host toxicity. For this reason, there is a need for novel antitumor agents that selectively act on the target, with high potency and less toxicity.

In the last 140 years, interest in pyrazole chemistry has significantly increased due to the discovery of the interesting biological properties exhibited by a great number of pyrazole derivatives. Rimonabant, Celecoxib and Pyrazofurin are recently discovered pyrazole containing therapeutics for obesity, inflammation and cancer, respectively. Rimonabant (anorectic antiobesity drug) and Celecoxib (non-steroidal anti-inflammatory drug) are commonly used and recent studies show that celecoxib possesses strong chemopreventive activity against mammary carcinogenesis. Also Pyrazofurin is a nucleoside analog, and inhibits DNA synthesis and cell replication. In addition pyrazoles have considerable therapeutic utility as phosphodiesterase inhibitors and c-Met inhibitors as novel targets in cancer therapy. Common usage of the pyrazoles especially in the synthesis of antitumor agents stimulated us to design and synthesize novel pyrazole bis-carboxamides, and test their antiproliferative activities against HeLa (human uterus carcinoma), Vero (green monkey kidney), and C6 (Rat Brain tumor) cells as in vitro.

Results and Discussion

Chemistry

The furandiones are excellent starting material for the synthesis of pyrazole-3-carboxylic acids via the reaction of furandiones with hydrazone under heating in a solventless media (Scheme I). Herein this one-step method for the synthesis of the pyrazole carboxylic acids was used according to our previous studies. Pyrazole carboxylic acids were converted to their acid chlorides and reacted with excess SOCl₂ in a solventless media again. This method is easy to perform and gives the product in high yield (90-95%). Compound 6 and 7 have been utilized as starting materials for the synthesis of target pyrazole bis-carbox-amides, thus acid chlorides reacted with \( N,N' \)-binucleo-philis in the presence of catalytic amount of pyridine to afford 8–12 in 72–82% yield (Scheme II).

The structures of 8–12 were confirmed from elemental and spectroscopic data. The IR spectrum of 8–12 revealed the appearance of amide carbonyl (C=O) band at 1670–1690 cm⁻¹. Also characteristic NH stretching bands observed at 3361–3380 cm⁻¹ region. The IR bands of C=O and C=N appear as a region rather than single sharp peaks. The absorption bands associated
with other functional groups appeared in the expected regions and the absorption values were consistent with our previous reports and literature\textsuperscript{24-26}.

The $^1$H NMR results also confirm the successful synthesis of the molecules. The signals of the aromatic protons appear between $\delta \sim 7.25$ and 8.50 in the $^1$H NMR spectra. The peaks belonging to NH groups are observed in the region between $\delta \sim 10.41$ and 11.11. The other $^1$H NMR peaks appeared at the expected chemical shifts and integral values. $^{13}$C NMR studies have also been carried out. The peaks of C=O for benzoyl groups are observed around $\delta \sim 191.00$ while the peaks of O=C-OR and O=C-NH are around $\delta \sim 162.00$ and $\delta \sim 160.00$, respectively. Other $^{13}$C resonance signals were observed in the expected regions. In summary, all the synthesized compounds exhibited satisfactory analytical and spectral data consistent with their structures (see Experimental Section).

**Antiproliferative Activity Studies**

Antiproliferation activities of 8-12 were determined against HeLa, Vero, and C6 cells using BrdU cell proliferation ELISA assay\textsuperscript{27-29}. Cisplatin and 5-FU were used as standards. The activities of samples and standards were investigated on three concentrations (100, 250 and 500 µg/ml). The IC\textsubscript{50} and IC\textsubscript{75} values of the most effective compounds against HeLa, Vero and C6 were given at (Table I).
Table 1 — IC$_{50}$ and IC$_{75}$ values of compound 8-12

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<th>Compd</th>
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<th>C6 cell IC$_{75}$</th>
<th>Vero cell IC$_{50}$</th>
<th>Vero cell IC$_{75}$</th>
<th>HeLa cell IC$_{50}$</th>
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Antiproliferative activities of compounds 8–12 against HeLa cancer cell lines

The activity studies established that compounds 8-12 demonstrate antiproliferative activity against HeLa cell lines in a concentration dependent manner (Figure 1). In addition, compound 8 have better antiproliferative activity than the other compounds (except 5-FU and Cisplatin). The potency of inhibition for HeLa cells at 500 µg/mL concentration is in the order of; 5-FU > Cisplatin > 8 > 11 > 12 > 10 > 9.

The antiproliferative activities of the compounds 8, 9 and standard compounds showed the following order at 500 µg/ml concentration against HeLa cell line: 5-FU > Cisplatin > 8 > 9. The pyrazole-3-carboxamide compound 8 has been found to show the highest antiproliferative activity against HeLa cell line amongst the compounds prepared here, yet show much less antiproliferative activity against HeLa cell line than 5-FU and Cis-platin. The molecular structures of the compounds 8 and 9 are very similar, differing only in the position of the substituents attached to the benzene ring. This shows that the position of the substituents have an effect on the antiproliferative activities. At compound 8, the substituents attached to the benzene ring were at ortho position, however, the same substituents were at para position for 9 (Figure 1 and Scheme II).

Antiproliferative activities of compounds 8–12 against Vero cell lines

The antiproliferative activities of compounds 8–12 against Vero cell lines were generally obtained in a concentration dependent manner (Figure 2). Compounds 8, 11 and 12 show higher antiproliferative activity than other compounds (except 5-FU and Cisplatin). In addition compounds 9 and 10 did not show any antiproliferative activity against Vero cell lines. The potency of inhibition: for Vero cells at 500 µg/mL concentration is in the order of; Cisplatin > 5-FU > 11 ~ 12 > 8 > 10 > 9.

Antiproliferative activities of compounds 8-12 against C6 cancer cell lines

According to the antiproliferative activity results, compounds 8–12 did not show considerable activity against C6 cell lines when compared with Cisplatin and 5-FU (Figure 3).

The potency of inhibition: for C6 cells at 500 µg/mL concentration is in the order of; Cisplatin > 5-FU > 11 > 8 ~ 12 > 10 > 9.
Materials and Methods

Unless otherwise noted, all materials were obtained commercially (Aldrich, Fluka and Merck) and used without further purification. Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under a dry nitrogen atmosphere immediately prior to use. Thin-layer chromatography was carried out on precoated, 0.25-mm silica gel plates (Kieselgel 60F 254 of E. Merck, Darmstadt, Germany). Compounds were visualized by Camag TLC devices UV (254 and 366 nm). Melting points were determined in capillary tubes using the Electrothermal 9200 melting point apparatus and remain uncorrected. Infrared (IR) spectra were recorded on Bruker Optics, Vertex 70 FT-IR Spectrometer with using of KBr pellets. ¹H and ¹³C NMR spectra were determined with a Varian Gemini-400 (400 MHz) spectrometer at 400 and 100 MHz, respectively. Chemical shifts were reported in parts per million (ppm, ² units). Elemental analyses (CHN) were obtained from LECO CHNS-932.

Experimental Section

General procedure for the synthesis of bis-carboxamide derivatives, 8–12

Appropriate amounts of pyrazole-3-carboxylic acid chloride 6 or 7 (2 mmol) that can be easily obtained from furan-2,3-diones 1 or 2 and 3-nitrophenylhydrazine 3 after some reaction steps as given in literature²⁴,²⁵ (Scheme 1) and the corresponding diamine (1 mmol) was dissolved in freshly distilled THF and refluxed together with catalytic amounts of pyridine for 2–4 hr. After cooling, the solution was acidified by adding diluted hydrochloric acid to give crude products 8–12 which were purified by recrystallization from the suitable alcohol.

N,N’-(1,2-phenylene)bis(4-benzoyl-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3-carboxamide) 8. This compound was obtained by the general procedure with a reflux time of 3 hr (o-phenylenediamine) and the crude product was crystallized from ethanol. Yield: 79%, m.p. 208–10°C; IR (KBr): 3365 (NH), 1594–1432 (C=N and C=C), 1356 cm⁻¹ (N=O str.); ¹H NMR (400 MHz, DMSO): δ 11.11 (s, 2H, CONH), 8.50–7.33 (m, 32H, ArH); ¹³C NMR (100 MHz, DMSO): δ 190.77 (C=O, benzoyl), 160.37 (C=O, amide), 148.34 (C-NO₂), 146.18 (pyrazole C-3), 144.52 (pyrazole C-5), 121.36 (pyrazole C-4), 144.03, 139.73, 139.36, 138.12, 133.93, 132.42, 131.07, 130.37, 130.16, 129.57, 129.17, 129.05, 128.94, 127.80, 127.72, 125.52, 124.87, 123.96, 122.76, 121.51; Anal. Calcd. for C₃₂H₂₈N₄O₈: C, 69.48; H, 3.81; N, 12.47. Found: C, 69.63; H, 3.86; N, 12.61%.

N,N’-(1,4-phenylene)bis(4-benzoyl-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3-carboxamide) 9. This compound was obtained by the general procedure with a reflux time of 4 hr (p-phenylenediamine) and the crude product was crystallized from methanol. Yield: 73%, m.p. 275–77°C; IR (KBr): 3361 (NH), 3063 and 3026 (Ar CH), 1685 (C=O), 1585–1490 (C=N and C=C), 1356 cm⁻¹ (N=O str.); ¹H NMR (400 MHz, DMSO): δ 10.44 (s, 2H, CONH), 8.38–7.25 (m, 32H, ArH); ¹³C NMR (100 MHz, DMSO): δ 191.14 (C=O, benzoyl), 159.29 (C=O, amide), 148.50 (C-NO₂), 146.96 (pyrazole C-3), 144.31 (pyrazole C-5), 121.26 (pyrazole C-4), 139.95, 138.30, 134.93, 134.04, 132.65, 131.21, 130.46, 130.30, 129.73, 129.34, 129.20, 128.01, 124.04, 122.88, 121.52; Anal. Calcd. for C₃₄H₂₈N₄O₈: C, 69.48; H, 3.81; N, 12.47. Found: C, 69.71; H, 3.90; N, 12.69%.

Diethyl 3,3’-((1,4-phenylenebis(azanediyl))bis(carbonyl)-bis(1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-4-carboxylate) 10. This compound was obtained by the general procedure with a reflux time of 2 hr (p-phenylenediamine) and the crude product was crystallized from ethanol. Yield: 77%, m.p. 260–62°C; IR (KBr): 3366 (NH), 3010 (aliph. CH), 2973 (aliph. CH), 1739 and 1674 (C=O), 1604–1447 (C=N and C=C), 1348 (N-O str.); ¹H NMR (400 MHz, DMSO): δ 10.68 (s, 2H, CONH), 8.24–7.37 (m, 22H, ArH); 4.08 (q, J = 6.9 Hz, 4H, 2 OCH₂), 1.00 (t, J = 7.3 Hz, 6H, 2 CH₃); ¹³C NMR (100 MHz, DMSO): δ 162.55 (C=O, ester), 160.23 (C=O, amide), 149.28 (C-NO₂), 148.49 (pyrazole C-3), 146.04 (pyrazole C-5), 120.93 (pyrazole C-4), 61.10 (OCH₂), 14.33 (CH₃), 139.74, 135.38, 132.33, 131.31, 130.92, 130.47, 129.16, 128.18, 123.96, 121.08, 114.31; Anal. Calcd. for C₅₅H₄₈N₄O₁₀: C, 63.31; H, 4.11; N, 13.42. Found: C, 63.52; H, 4.19; N, 13.51%.

N,N’-((1,1’-biphenyl)-4,4’-dийl)bis(4-benzoyl-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3-carboxamide) 11. This compound was obtained by the general procedure with a reflux time of 4 hr (benzidine) and the crude product was crystallized from methanol. Yield: 72%, m.p. 258–60°C; IR (KBr): 3380 (NH), 3062 and 3023 (Ar CH), 1671 (C=O), 1594–1432 (C=N and C=C), 1356 cm⁻¹ (N=O str.); ¹H NMR (400 MHz, DMSO): δ 10.44 (s, 2H, CONH), 8.38–7.25 (m, 32H, ArH); ¹³C NMR (100 MHz, DMSO): δ 191.14 (C=O, benzoyl), 159.50 (C=O, amide), 148.50 (C-NO₂), 146.96 (pyrazole C-3), 144.41 (pyrazole C-5), 121.38 (pyrazole C-4), 139.94, 138.30, 138.16, 135.75, 134.06, 132.74, 131.23, 130.48,
Diethyl 3,3’-(((1,1'-biphenyl)-4,4’-diyldi(azanediyl))bis(carbonyl))bis(1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-4-carboxylate) 12. This compound was obtained by the general procedure with a reflux time of 3 hr (benzidine) and the crude product was crystallized from ethanol. Yield: 82%, m.p. 256–58°C; IR (KBr): 3461 (NH), 3061 and 3026 (Ar CH), 2972 (aliph. CH), 1740 and 1689 (C=O), 1608–1440 (C=N and C=C), 1349 (N-O str.), 1212 and 1086 cm\(^{-1}\) (C-O-C asym. and sym.); \(^1\)H NMR (400 MHz, DMSO): \(\delta\) 10.77 (s, 2H, CONH), 8.24–7.39 (m, 26H, ArH); 4.09 (q, \(J = 6.6\) Hz, 4H, 2 OCH\(_3\)), 1.00 (t, \(J = 6.9\) Hz, 6H, 2 CH\(_3\))\(^3\)C NMR (100 MHz, DMSO): \(\delta\) 162.54 (C=O, ester), 160.43 (C=O, amide), 149.26 (C-NO\(_2\)), 148.49 (pyrazole C-3), 146.10 (pyrazole C-5), 120.89 (pyrazole C-4), 61.11 (OCH\(_3\)), 139.72, 138.60, 135.77, 132.37, 131.33, 130.93, 130.49, 129.17, 128.16, 127.29, 124.00, 121.11, 114.33; Anal. Calcd. for \(\text{C}_{55}\text{H}_{38}\text{N}_8\text{O}_{10}\): C, 71.45; H, 4.23; N, 11.53. Found: C, 71.63; H, 4.23; N, 11.53%.

Bioassays

Cell proliferation ELISA, BrdU (colorimetric) kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany). The anti-tumor drug 5-Florouracil and Cisplatin were provided from Sigma. Other of antiproliferative chemicals used were in analytical grade and obtained from Sigma–Aldrich, Merck and Roche.

Preparation of the stock solutions

The stock solution of compounds 8–12, 5-FU and Cisplatin were prepared in DMSO and diluted with Dulbecco’s modified eagle medium (DMEM). DMSO final concentration is below 1% in all tests.

Cell lines and cell culture

HeLa, Vero and C6 cell lines were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% penicillin streptomycin. The medium was changed twice a week.

Cell proliferation assay

Antiproliferative effects of the compounds were investigated on HeLa, Vero and C6 cell lines using proliferation BrdU ELISA assay\(^{27-29}\). Cisplatin and 5-Fluorouracil (5-FU) were used as positive controls. Cultured cells were grown in 96-well plates (COSTAR, Corning, USA) at a density of 3 \times 10^4 cells/well. In each experimental set, cells were plated in triplicates and replicated twice. The cell lines were exposed to three concentrations of compounds 8–12, Cisplatin and 5-FU for 24 hr at 37°C in a humidified atmosphere of 5% CO\(_2\). Cells were then incubated overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Germany) according to the manufacturer’s procedure. The amount of cell proliferation was assessed by determining the A450 nm of the culture media after addition of the substrate solution by using a microplate reader (Ryto, China). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. All assays were repeated at least twice using against HeLa, Vero and C6 cells. Percentage of inhibition of cell proliferation was calculated as follows: \([1-(A_{treatments}/A_{vehicle\ control})] \times 100\).

Statistical analysis

The results of investigation in vitro are means ± SD of nine measurements. Differences between groups were tested by ANOVA. \(p\) values of < 0.01 were considered significant.

Determination of \(\text{IC}_{50}\) and \(\text{IC}_{75}\) values

The half maximal inhibitory concentration (IC\(_{50}\)) is a measure of the effectiveness of a compound in inhibiting biological function. In this paper, IC\(_{50}\) and IC\(_{75}\) values were determined using ED50 plus v1.0.

Conclusions

A series of novel pyrazole bis-carboxamide derivatives 8–12 has been synthesized readily in one-pot from the pyrazole-3-carboxyl acid chlorides 6 and 7 and evaluated their antiproliferative activities against some cancer cell lines. Although the compounds 8–12 have weak activities against all cell lines when compared with the standards, compound 8 has a moderate antiproliferative activity against HeLa cell lines.

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