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Design, synthesis and *in vitro* antitumor activity of novel phthalazin-1,4-dione/chalcone hybrids and phthalazin-1,4-dione/pyrazoline hybrids

Said A.H. El-Feky[a,b], Hanan A. Abd El-Fattah[b], Nermine A. Osman[b], Mohd. Imran[a] and Mohamed N. Zedan[c*]

[a]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Northern Border University, Rafha, Saudi Arabia*
[b]*Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt*
[c]*Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Nahda University, Beni-suef, Egypt*

ABSTRACT

*In continuation of our study of phthalazinones with antitumor activity, several new phthalazin-1,4-diones based on the structure of vatalanib (PTK787) were designed and synthesized. The cytotoxicity of the final compounds was tested in vitro on HCT-116 colon cancer and MCF-7 breast cancer. Compound **4f** exerted the highest cytotoxic activity against both colon cancer and breast cancer with IC_{50}values equal to 2.21and 1.0µM respectively. Besides, **4f** demonstrated the highest binding profiles into VEGF-2 generated by MOE docking.*

Keywords: Phthalazine-1,4-diones, chalcones, pyrazolines, cytotoxic activity, VEGF-2.

INTRODUCTION

Cancer is now considered as one of the most serious health problems and leading cause of death over the world. Although a significant proportion of cancers can be cured by surgery combined with radiotherapy or chemotherapy especially if they are detected early, but disseminated cancer can't be 100% effectively treated [1].

Therefore, there is an urgent need to give attention to update and modify drug leads for cancer treatment from the point of view of medicinal chemistry and drug design to identify more potent and effective therapies.

In the past few years, a large number of phthalazine derivatives have been prepared and studied for their antitumor activities [2-6]. For example, phthalazin-1,4-diones have been reported as potent type II IMP dehydrogenase inhibitors[7] and as effective anti-proliferative agents against different human and murine tumor cells[8-9], particularly against hepatocellular carcinoma[10]. In addition, 1,4-disubstitutedphthalazines have been emerged as promising and attractive antitumor agents. For example, 1-piperazinyl-4-substitutedphthalazines have been reported as active cytotoxic agent against A549, HT-29and MDA-MB-231[11-12], whereas 1-anilino-phthalazines derivatives showed as interesting cytotoxic activity against Bel-7402 and HT-1080[13-14]. Moreover, a series of N-(aryl)-2-(1-oxo-4-phenylphthalazin-2(1*H*)-yl)acetamides have been reported as potent cytotoxic agents in human hepatocellular carcinoma cells[15].

From the view point of molecular design, the combination of two active molecules or pharmacophores with a linker is a well-known approach for the buildup of drug like molecule which allows medicinal chemists to find more potent agents. In our ongoing medicinal chemistry research program[16], we have reported the synthesis and anticancer activity of a number of phthalazinone derivatives as bicyclic heteroaromatic systems of fused or linked types containing pyridazinone fragment. Since some phthalazinone containing acetamide linker, such as compound **I**(Fig. 1) exhibited a potent anticancer activity, it promoted us to modify the established structures on the basis of isosteric replacements[17]. The bioisoster concept is an oversimplification of the role of scaffolds for activity, unless it plays

a pivotal role for function or interaction such as for β-lactam in penicillin[18]. Moreover, bioisosterism revealed as a useful strategy for the lead optimization process and molecular modification for rational drug design[19]. On the basis of these results, we summarize that replacement of acetamide linker from 4-position in compound **I**to 2-position and maintaining the free carbonyl group at 1 and 4 position could give compounds **II** with improved anticancer activity (Fig. 1).

Vatalanib (PTK787) **III** (Fig. 1) inhibits both VEGFR-1 and VEGFR-2 with IC_{50} of 380 and 20 nM, respectively[20]. Vatalanib **III** is well absorbed orally and shows in vivo antitumor activity against a panel of human tumor xenograft models, however, vatalanib **III**is currently in phase III clinical trials for the treatment of colorectal cancer[21-22].In addition, many anilino-phthalazines have been reported as potent inhibitors of VEGFR-2 as AAC789**V** and IM-023911 **VI** with IC_{50}= 20 and 48 nM, respectively (Fig. I)[23-28].

Interestingly, a novel class of potent, selective and orally bioavailable inhibitors of aurora-A kinase based upon a 4-(pyrazole-3-ylamino)phenyl-2H-phthalazin-1-one scaffold have been reported. Compound **IV** inhibits aurora-A with IC_{50} of 71 nM, also, it showed in vitro cytotoxic activity against HCT116 colon cell line with IC_{50} of 5.44 mM[29].
On the other hand, chalcones are reported in many researches as potent anticancer agents. Naturally occurring and synthetic chalcone derivatives are of current interest as cytotoxic agents[30-31]. Chalcones had been reported to inhibit cancer cell proliferation, induce apoptosis in various cell types and exhibit remarkable effect against skin carcinogenesis[32]. Several mechanisms have been reported for the cytotoxic action of chalcone derivatives including; inhibiting tubulin polymerization[33], inhibition of angiogenesis, induction of apoptosis, anti-estrogenic activity and reversal of multidrug resistance or combination of these mechanisms[34]. A recent report showed that the 2-phenylquinoline/chalcone hybrid is highly active against the growth of MDA-MB-231 cells with IC_{50} less than 0.10 µM in addition to inhibition of H1299SKBR-3, MCF-7, and SKBR-3 cells with $IC_{50}$of 0.71, 0.91, and 0.52 µM respectively[35].

In view of the facts mentioned above, the majority of the reported studies were concerned with C-1 or C-4 substituted phthalazines whereas a little attention was given to investigate SAR of N-2 substituted phthalazines. This has inspired the present study to design a series of new N-2 substituted phthalazines in an attempt to obtain a potent anticancer agent. The strategy adopted included moving the acetamido moiety of compounds **I** from C-1 to N-2 of phthalazine moiety as compounds **II**. The flexible acetyl linker (-CH_2CO-) was selected to link the anilino moiety to the phthalazine core. In the target phthalazines, the novel phthalazinones (4-6) were synthesized to evaluate their anti-tumor activity against two cell lines namelyHCT-116 colon cancer and MCF-7 breast cancer.

EXPERIMENTAL SECTION

2.1. General methods:

Melting points were determined on a Gallen Kamp melting point apparatus and are uncorrected. Infrared spectra were recorded on a Pye Unicam SP 1000 Infrared Spectrophotometer and Shimadzu Infrared Spectrophotometer using potassium bromide disc and are expressed in wave number (cm^{-1}). ^{1}H-NMR spectra and ^{13}C-NMR were recorded on a Bruker 400 MHz Spectrophotometer in DMSO-d_6. Chemical shifts were expressed in part per million (ppm) with tetramethylsilane (TMS) as an internal standard. D_2O exchange was carried out for N-H and O-H protons in ^{1}H-NMR. MS spectra were measured with an HP5995 instrument and Hewlett Packard 5988 Spectrometer at Micro Analytical Centre, Egypt. Elemental analyses (C, H, and N) were performed on VARIO El Elementer Apparatus at the Regional Center for Mycology and Biotechonology, Al-Azhar University, Cairo, Egypt. All the compounds were routinely checked by thin layer chromatography (TLC) on aluminium-backed silica gel plates. All solvents were dried by standard methods. Chalcones **1a-h** were synthesized according to reported procedure [40-41].

2-Bromo-N-[4-[3-acryloyl]phenylacetamides **2a-h** were prepared according to reported procedures[36-37]. 2-Phenyl-2,3H-phthalazin-1,4-dione **3** was prepared by cyclocondensation of phthalic anhydride with phenyl hydrazine according to the reported procedure[29].

2.2. General procedure for the synthesis of the target compounds (4a-h)

To a stirred mixture of compound **3** (2 mmol), anhydrous potassium carbonate (4 mmol) and catalytic amount of KI in dry acetone, a solution of appropriate chalcone derivative **2a-h** in acetone was added. The mixture was heated under reflux for 30 h and the precipitate formed was filtered while hot, washed with water several times and crystallized from ethanol to give compounds**4a-h**.

2.2.1.N-(4-(3-(4-chlorophenyl))acryloyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydrophthalazin-2(1H)-yl)acetamide (4a)
White crystals; yield 67%; m.p. 259-260^{o}C; IR (KBr, cm^{-1}): 3271 (N-H), 1699, 1658, 1626 (C=O), 1606 (CH=CH and C=N); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 5.03 (s, 2H, -CH$_2$-), 7.29-8.17 (m, 19H, Ar-H + 2H of -CH=CH-), 8.34 (s, 1H, NH, D$_2$O exchangeable); MS (m/z) [%]: 538 (M^{+}+2) [20], 536 [60], 477 [60], 280 [80], 238 [100]; Elemental Analysis ($C_{31}H_{22}ClN_3O_4$), Found % (Calculated %): C, 69.54 (69.47); H, 4.18 (4.14); N, 7.91 (7.84).

2.2.2. N-(4-(3-(4-methoxyphenyl))acryloyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydrophthalazin-2(1H)-yl)acetamide (4b)
White crystals; yield 72%; m.p. 271-273^{o}C; IR (KBr, cm^{-1}): 3273 (N-H), 1716, 1699, 1645 (C=O), 1627 (C=N), 1600 (CH=CH); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 3.82 (s, 3H, -OCH$_3$), 5.06 (s, 2H, -CH$_2$-), 7.01-8.37 (m, 19H, Ar-H + 2H of -CH=CH-), 10.64 (s, 1H, NH, D$_2$O exchangeable); ^{13}C-NMR (100 MHz, DMSOd$_6$) δ ppm: 39.55, 39.75, 39.96, 40.17, 40.38, 40.59, 55.85, 66.05, 114.87, 119.19, 119.87, 124.10, 124.14, 125.45, 127.40, 127.68, 127.88, 128.75, 129.41, 130.32, 131.20, 133.34, 133.49, 134.51, 141.98, 143.25, 143.94, 149.06, 157.86, 161.76, 167.08, 187.88; MS (m/z) [%]: 532 (M^{+}+1) [100], 416 [30], 238 [70]; Elemental Analysis ($C_{32}H_{25}N_3O_5$), Found % (Calculated %): C, 72.48 (72.31); H, 4.79 (4.74); N, 8.01 (7.91).

2.2.3. N-(4-Cinnamoylphenyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)acetamide (4c)
White crystals; yield 72%; m.p. 252-253^{o}C; IR (KBr, cm^{-1}): 3265 (N-H), 1710, 1658, 1641 (C=O), 1597 (CH=CH and C=N); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 5.06 (s, 2H, -CH$_2$-), 7.30-8.37 (m, 18H, Ar-H + 2H of -CH=CH- + 11H of NH; NH appeared at δ 8.12 and was D2O exchangeable); ^{13}C-NMR (100 MHz, DMSOd$_6$) δ ppm: 39.54, 39.74, 39.95, 40.16, 40.37, 40.58, 66.05, 119.22, 122.40, 124.10, 124.13, 125.45, 127.41, 127.68, 128.76, 128.89, 129.31, 129.39, 130.48, 131.01, 133.06, 133.50, 134.51, 135.23, 141.97, 143.46, 143.95, 149.06, 157.86, 167.13, 188.03; MS (m/z) [%]: 501 [20], 444 [20], 279 [50], 221 [80]; Elemental Analysis ($C_{31}H_{23}N_3O_4$), Found % (Calculated %): C, 74.31 (74.24); H, 4.69 (4.62); N, 8.45 (8.38).

2.2.4. 2-(1,4-Dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)-N-(4-(3-(2-methoxy phenyl)acryloyl)acetamide (4d)
White crystals; yield 68%; m.p. 246-248^{o}C; IR (KBr, cm^{-1}): 3275 (N-H), 1697, 1651, 1625 (C=O), 1598 (CH=CH and C=N); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 3.90 (s, 1H, -OCH$_3$), 5.01 (s, 2H, -CH$_2$-), 7.03-8.37 (m, 19H, Ar-H + 2H of -CH=CH-), 10.52 (s, 1H, NH, D$_2$O exchangeable); MS (m/z) [%]: 531 [10], 500 [20], 446 [20], 413 [30], 279 [40], 238 [80]; Elemental Analysis ($C_{32}H_{25}N_3O_5$), Found % (Calculated %): C, 72.46 (72.31); H, 4.79 (4.74); N, 8.02 (7.91).

2.2.5. N-(4-(3-(4-Nitrophenyl)-acryloyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)acetamide (4e)
White crystals; yield 66%; m.p. 295-297^{o}C; IR (KBr, cm^{-1}): 3414 (N-H), 1705, 1660, 1650 (C=O), 1597 (C=N and CH=CH), 1521, 1342 (-NO$_2$); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 5.06 (s, 2H, -CH$_2$-), 7.29-8.29 (m, 19H, Ar-H + 2H of -CH=CH-), 8.35 (s, 1H, NH, D$_2$O exchangeable); MS (m/z) [%]: 547 [20], 520 [20], 502 [10], 478 [10], 463 [30], 405 [40], 383 [100]; Elemental Analysis ($C_{31}H_{22}N_4O_6$), Found % (Calculated %): C, 68.13 (68.20); H, 4.06 (4.09); N, 10.36 (10.25).

2.2.6. N-(4-(3-(2,4-Dichlorophenyl)acryloyl)phenyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydro phthalazin-2(*1H*)-yl) acetamide (4f)
White crystals; yield 65%; m.p. 293-295^{o}C; IR (KBr, cm^{-1}): 3265 (N-H), 1699, 1658, 1645 (C=O), 1597 (CH=CH and C=N); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 5.06 (s, 2H, -CH$_2$-), 7.29-8.34 (m, 18H, Ar-H + 2H of -CH=CH-), 10.94 (s, 1H, NH, D$_2$O exchangeable); MS (m/z) [%]: 568 (M^{+}) [10], 566 [30], 549 [30], 510 [30]; Elemental Analysis ($C_{31}H_{21}Cl_2N_3O_4$), Found % (Calculated %): C, 65.49 (65.27); H, 3.69 (3.71); N, 7.45 (7.37).

2.2.7. N-(4-(3-(2,4-Chlorophenyl)acryloyl)phenyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydro phthalazin-2(*1H*)-yl) acetamide (4g)
White crystals; yield 61%; m.p. 335-337^{o}C; IR (KBr, cm^{-1}): 3311-3260 (N-H), 1705, 1660, 1651 (C=O), 1597 (CH=CH and C=N); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 5.06 (s, 2H, -CH$_2$-), 7.29-8.37 (m, 20H, Ar-H + 2H of -CH=CH- + 1H of NH); MS (m/z) [%]: 537 (M^{+}+2), 535 [5], 477 [5], 413 [5], 296 [5], 279 [30], 248 [40], 238 [100]; Elemental Analysis ($C_{31}H_{22}ClN_3O_4$), Found % (Calculated %): C, 69.68 (69.47); H, 4.22 (4.14); N, 8.03 (7.84).

2.2.8.2-(1,4-Dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)-N-(4-(3-(furan-2-yl)acryloyl) phenyl)acetamide (4h)
White crystals; yield 66%; m.p. 222-224^{o}C; IR (KBr, cm^{-1}): 3277 (N-H), 1697, 1658, 1640 (C=O), 1597 (CH=CH and C=N); ^{1}H-NMR (400 MHz, DMSOd$_6$) δ ppm: 5.06 (s, 2H, -CH$_2$-),6.96 (t, 1H, H-4 of 2-furyl), 7.01-8.35 (m, 16H, Ar-H + 2H of -CH=CH- + 1H of H-3 of 2-furyl), 8.37 (d, 1H, H-5 of 2-furyl), 10.70 (s, 1H, NH, D$_2$O

exchangeable); MS (m/z) [%]: 491 [30], 358 [35], 311 [25], 296 [15], 279 [40]; Elemental Analysis ($C_{29}H_{21}N_3O_5$), Found % (Calculated %): C, 71.13 (70.87); H, 4.37 (4.31); N, 8.76 (8.55).

2.3. General procedure for the preparation of the target compounds (5a-d)

To a mixture of appropriate chalcone **(4)** (0.02 mol) and hydrazine hydrate (99%), 10 ml of acetic acid was added. The mixture was refluxed for 5 h, a catalytic amount of hydrochloric acid (4-5 drops) was added and mixture was again refluxed for 1 h. The resulting mixture was cooled for 30 minutes, water was added, and the resulting precipitate was filtered and washed with water several times. The solid product was collected and crystallized from ethanol to give compounds **5a-d**.

2.3.1. N-(4-(1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl)-3,4-dihydrophthalazin-2(*1H*)-yl)acetamide (5a)

White crystals; yield 64%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 3305 (N-H), 1688, 1686, 1645 (C=O), 1600 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.34 (s, 3H, -CH_3), 3.35 (dd, J = 11.7 & 18 Hz, 1H, C-4H of pyrazoline), 3.98 (m, 1H, C-4H of pyrazoline), 5.02 (s, 2H, -CH_2-), 5.88 (m, 1H, C-5 of pyrazoline), 7.08-8.37 (m, 17H, Ar-H), 9.66 (s, 1H, NH, D_2O exchangeable); MS (m/z) [%]: 537 (M^++2), 415 [20], 371 [20]; Elemental Analysis ($C_{33}H_{26}ClN_5O_4$), Found % (Calculated %): C, 67.12 (66.95); H, 4.49 (4.43); N, 12.01 (11.83).

2.3.2. N-(4-(1-Acetyl-5-phenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl)-3,4-dihydro -phthalazin-2(*1H*)-yl)acetamide (5b)

White crystals; yield 66%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 3444-3408 (N-H), 1700-1624 (C=O), 1598 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.31 (s, 3H, -CH_3), 3.13 (dd, J = 11.7 & 18.0 Hz, 1H, C-4H of pyrazoline), 3.88 (m, 1H, C-4H of pyrazoline), 5.02 (s, 2H, -CH_2-), 5.55 (m, 1H, C-5H of pyrazoline), 7.17-8.36 (m, 18H, Ar-H), 9.75 (s, 1H, NH, D_2O exchangeable); MS (m/z) [%]: 557; Elemental Analysis ($C_{33}H_{27}N_5O_4$), Found % (Calculated %): C, 71.2(71.08); H, 4.90 (4.88); 12.6 (12.56).

2.3.3. N-(4-(1-Acetyl-5-(4-nitrophenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl)-3,4-dihydrophthalazin-2(*1H*)-yl)acetamide (5c)

White crystals; yield 60%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 1684-1624 (C=O), 1598 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.50 (s, 3H, -CH_3), 3.61 (dd, J = 11.78 & 18 Hz, 1H, C-4H of pyrazoline), 4.11 (m, 1H, C-4H of pyrazoline), 5.00 (s, 2H, -CH_2-), 5.81 (m, 1H, C-5H of pyrazoline), 7.30-8.35 (m, 18H, Ar-H + 1H of NH); MS (m/z) [%]: 602 [2.2%]; Elemental Analysis ($C_{33}H_{26}N_6O_6$), Found % (Calculated %): C, 65.90 (65.77); H, 4.32 (4.35); N, 14.19 (13.95).

2.3.4. N-(4-(1-Acetyl-5-(2-chlorophenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl)-3,4-dihydrophthalazin-2(*1H*)-yl)acetamide (5d)

White crystals; yield 65%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 3377-3305 (N-H), 168-1645 (C=O), 1600 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.51 (s, 3H, -CH_3), 3.21 (dd, J = 11.78 & 18 Hz, 1H, C-4H of pyrazoline), 4.10 (m, 1H, C-4H of pyrazoline), 5.02 (s, 2H, -CH_2-), 5.75 (m, 1H, C-5H of pyrazoline), 7.08-8.37 (m, 17H, Ar-H), 9.81 (s, 1H, NH, D_2O exchangeable); MS (m/z) [%]: 593 (M^++2), 415 [20], 371 [20], 162 [60]; Elemental Analysis ($C_{33}H_{26}ClN_5O_4$), Found % (Calculated %): C, 67.12 (66.95); H, 4.49 (4.43); N, 12.01 (11.83).

2.4. General procedure for the preparation of the target compounds (6a-d)

To a mixture of appropriate chalcone **(4)** (0.06 mol) and hydrazine hydrate (99%), 10 ml of ethanol was added. The mixture was refluxed for 5 h and cooled. The precipitate was filtered, washed with ethanol and diethyl ether. The solid product was collected and crystallized from ethanol to give compounds **6a-d**.

2.4.1. 2-(1,4-Dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)-N-(4-(5-(4-methoxyphenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl acetamide (6a)

White crystals; yield 68%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 3307, 3260 (N-H), 1697, 1651, 1635 (C=O), 1598 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.67 (dd, J = 10.7 & 16.3, 1H, C-4H of pyrazoline), 3.38-3.40 (m, 1H, C-4H of pyrazoline), 3.82 (s, 3H, -OCH_3), 3.98 (t, 1H, C-5H of pyrazoline), 5.01 (s, 2H, -CH_2-), 6.92-8.32 (m, 17H, Ar-H + 1H of NH), 10.35 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (400 MHz, DMSO-d_6) δ ppm: 42.61, 51.11, 52.81, 55.81, 114.11, 114.11, 121.77, 121.77, 122.81, 123.92, 123.92, 126.61, 126.61, 127.31, 127.71, 129.22, 129.22, 129.44, 129.44, 128.71, 128.71, 132.01, 132.11, 132.11, 136.20, 136.55, 141.11, 151.11, 157.11, 158.12, 166.55, 172.51; MS (m/z) [%]: 545 [1], 267 [100]; Elemental Analysis ($C_{32}H_{27}N_5O_4$), Found % (Calculated %): C, 70.63 (70.45); H, 5.07 (4.99); N, 12.97 (12.84).

2.4.2. N-(4-(5-(4-Chlorophenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydro -phthalazin-2(*1H*)-yl)acetamide (6b)

White crystals; yield 70%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 3441-3417 (N-H), 3271 (N-H), 1700, 1660, 1645 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.81 (dd, J = 10.7 & 16.3, 1H, C-4H of pyrazoline), 3.38-3.40 (m, 1H, C-4H of pyrazoline), 3.58 (t, 1H, C-5H of pyrazoline), 5.00 (s, 2H, -CH_2-), 7.25-8.33 (m, 17H, Ar-H + 1H of NH), 9.78 (s, 1H, NH, D_2O exchangeable); MS (m/z) [%]: 551 (M^++2), 393 [20], 329 [100]; Elemental Analysis ($C_{31}H_{24}ClN_5O_3$), Found % (Calculated %): C, 67.94 (67.70); H, 4.46 (4.40); N, 12.98 (12.73).

2.4.3. 2-(1,4-Dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)-N-(4-(5-(phenyl)-4,5-dihydro-*1H*-pyrazol-3-yl) phenyl acetamide (6c)

White crystals; yield 71%; m.p. >300^{o}C; IR (KBr, cm^{-1}): 3317-3308 (N-H), 1700-1650 (C=O), 1598 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.84 (dd, J = 10.7 & 16.3, 1H, C-4H of pyrazoline), 3.48-3.50 (m, 1H, C-4H of pyrazoline), 4.01 (t, 1H, C-5H of pyrazoline), 5.06 (s, 2H, -CH_2-), 7.30-8.37 (m, 19H, Ar-H + 1H of NH), 9.75 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (400 MHz, DMSO-d_6) δ ppm: 41.16, 40.57, 40.36, 66.04, 64.12, 63.75, 113.96, 119.76, 124.13, 124.17, 125.46, 126.52, 127.11, 127.22, 127.38, 127.58, 127.64, 128.77, 128.86, 129.09, 129.39, 133.44, 134.46, 138.86, 141.99, 143.53, 148.89, 149.12, 157.87, 166.47; MS (m/z) [%]: 515; Elemental Analysis ($C_{31}H_{25}N_5O_3$), Found % (Calculated %): C, 72.51 (72.22); H, 4.94 (4.89); N, 13.61 (13.58).

2.4.4. 2-(1,4-Dioxo-3-phenyl-3,4-dihydrophthalazin-2(*1H*)-yl)-N-(4-(5-(2-methoxyphenyl)-4,5-dihydro-*1H*-pyrazol-3-yl)phenyl acetamide (6d)

White crystals; yield 66%; m.p. 295-297^{o}C; IR (KBr, cm^{-1}): 3323-3309 (N-H), 1674, 1658, 1630 (C=O), 1598 (C=N); ^{1}H-NMR (400 MHz, DMSO-d_6) δ ppm: 2.76 (dd, J = 10.7 & 16.3, 1H, C-4H of pyrazoline), 3.36-3.38 (m, 1H, C-4H of pyrazoline), 3.72 (s, 3H, -OCH_3), 4.77 (t, 1H, C-5H of pyrazoline), 5.00 (s, 2H, -CH_2-), 6.87-8.30 (m, 17H, Ar-H + 1H of NH), 9.50 (s, 1H, NH, D_2O exchangeable); MS (m/z) [%]: 546, 387 [10], 308 [50], 272 [80]; Elemental Analysis ($C_{32}H_{27}N_5O_4$), Found % (Calculated %): C, 70.67 (70.45); H, 5.08 (4.99); N, 12.97 (12.84).

2.5. Anticancer Activity

Cell line

Human colon carcinoma (HCT-116) and human breast carcinoma (MCF-7) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/ml gentamycin. The cells were maintained at 37^{o}C in a humidified atmosphere with 5% CO_2 and were sub cultured two to three times a week.

Evaluation of the antitumor activity using Viability assay

The antitumor activity was evaluated on tumor cells. The cells were grown as monolayers in growth RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/ml gentamycin. The monolayers of 10,000 cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h at 37^{o}C in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffered saline (0.01 M pH 7.2) and simultaneously the cells were treated with 100 µl from different dilutions of tested sample in fresh maintenance medium and incubated at 37^{o}C. A control of untreated cells was made in the absence of tested sample. A positive control containing Doxorubicin drug was also tested as reference drug for comparison. Six wells were used for each concentration of the test sample. Every 24 h the observation under the inverted microscope was made. The number of the surviving cells was determined by staining the cells with crystal violet[38-39] followed by cell lysing using 33% glacial acetic acid and read the absorbance at 590 nm using ELISA reader (SunRise, TECAN, Inc., USA) after well mixing. The absorbance values from untreated cells were considered as 100% proliferation.

The number of viable cells was determined using ELISA reader and the percentage of viability was calculated as [1 - (OD_t/OD_c)] x 100%, wherein OD_t is the mean optical density of wells treated with the tested sample and OD_c is the mean optical density of untreated cells. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from the plotted graph.

2.6. Molecular modelling:

Docking analysis was carried using the Molecular Operating Environment (MOE 2008.10) on the basis of high resolution crystal structures of VEGF2 complexes with Ligand (PDB 1YWN). Target proteins and ligands were energy-minimized using MOE, all water molecules were removed and hydrogen atoms were added and the protonation states of the amino acid residues were assigned using the Protonate3D algorithm. The following parameters were used for energy minimization; gradient: 0.01, force field: MMFF94X, chiral constraint: current geometry; total runs = 30. We compared newly synthesized active compounds to crystallographic reference inhibitors. First, crystallographic inhibitors were redocked to reproduce their structure and adjust docking parameters accordingly. Figure 2 and 3 showing docking of inhibitors vatalanib and Ligand inhibitors respectively.

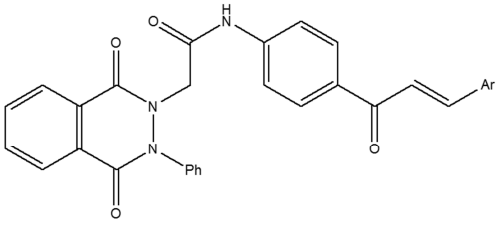
RESULTS

The chalcone derivatives, **1a-h** were synthesized by a base catalyzed claisen-schmidt condensation of 4-aminoacetophenones with different benzaldehyde derivatives[40-41]. Treatment of the chalcone derivative intermediates **1a-h** with bromoacetyl bromide in the presence of potassium carbonate afforded the corresponding 2-bromo-N-(4-(3-arylacryloyl)phenyl)acetamides, **2a-h**in high yields. Alkylation of **3** with the acylated chalcones **2a-h**in acetone using potassium carbonate as a base and traces of KI as a catalyst gave the target phthalazin-1,4-dione-chalcone hybrids, **4a-h**, in good yield (scheme 1).

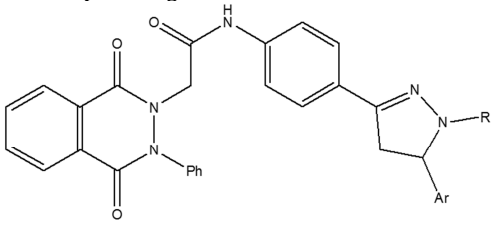
Anti-proliferative activity of the newly synthesized phthalazin-1,4-diones **4a-h**, **5a-d**, and **6a-d** was examined in two human cell lines, namely HCT-116 colon cancer and MCF-7 breast cancer using sulforhodamine B(SRB) colorimetric assay [42]. Doxorubicin was included in the experiment as a reference cyctotoxic compound for the two cell lines. The results were expressed as growth inhibitory concentration (IC_{50}) values which represent the compound concentration required to produce a 50% inhibition of cell growth after 72 h of incubation compared to untreated controls (Table I).Also, N-acetyl pyrazolines series showed higher activity than Pyrazolines series. Compound **4f** (IC_{50} = 1.00μM) was found to be the most potent derivative overall the tested compounds against MCF-7 as it was nearly equal to that of doxorubicin (IC_{50} = 0.72μM). Also the same compound **4f** (IC_{50} = 2.21μM) was found as the most potent derivative among the tested compounds against HCT-116 and it is slightly less than doxorubicin (IC_{50} = 0.81μM). Beside compounds **4b**, **4g**, **4h**, **5c**, and **5d** were moderately active against MCF-7 with IC_{50} of 2.50, 3.32, 6.76, 4.83, and 4.37 μM, respectively. Concerning activity against HCT-116, compounds **4g**, **4h**, and **5c** showed moderate activity with IC_{50} of 2.55, 5.47, and 3.77 μM, respectively. The remaining tested compounds displayed weak activity against both HCT-116 with IC_{50} ranging from 8.75 to 77.77 μM.

The newly synthesized compounds were docked into the active site of the VEGF-2. The compounds bind to the active sites in a similar fashion of the inhibitors. Compounds form the essential two hydrogen bonds with Glu 883 and Asp 1044 with good S score and small distance as illustrated in (Table II).Docking studies revealed that compounds **4f**, **5c**, and **6b** showed similar binding mode to vatalanib with binding energy score equal to -13.48, -12.30, and -16.01 respectively, which may explain their potential anticancer activity (Fig. 4-6).

Table I In vitro cytotoxic activities of the newly synthesized compounds against HCT-116 and MCF-7 cell lines



4a-h



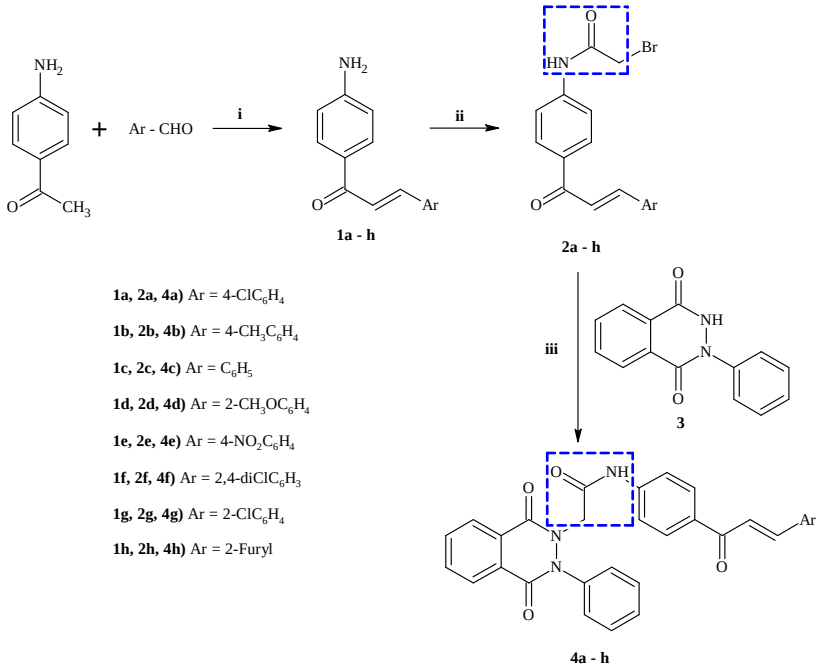
5a-d and 6a-d

Compound	Ar	R	HCT-116 (IC_{50})[a] μM	MCF-7 (IC_{50}) μM
4a	4-Cl-C_6H_4	-	9.29	7.73
4b	4-OCH_3-C_6H_4	-	8.81	2.50
4c	-C_6H_5	-	22.75	25.34
4d	2-OCH_3-C_6H_4	-	77.77	45.95
4e	4-NO_2-C_6H_4	-	10.75	9.94
4f	2,4-di-Cl-C_6H_3	-	2.21	1.00
4g	2-Cl-C_6H_4	-	2.55	3.32
4h	2-Furanyl	-	5.47	6.76
5a	4-Cl-C_6H_4	-$COCH_3$	14.71	10.33
5b	-C_6H_5	-$COCH_3$	15.90	48.11
5c	4-NO_2-C_6H_4	-$COCH_3$	3.77	4.83
5d	2-Cl-C_6H_4	-$COCH_3$	8.75	4.37
6a	4-OCH_3-C_6H_4	H	56.90	16.18
6b	4-Cl-C_6H_4	H	21.65	18.14
6c	-C_6H_5	H	32.23	22.52
6d	2-OCH_3-C_6H_4	H	16.09	40.18
Doxorubicin[b]	-	-	0.81	0.72

a :IC_{50} : Dose of the compound which inhibit tumor cell proliferation by 50 %
b : Used as positive control

Table II Fit values of the investigated compounds 4a-h, 5a-c and 6b-d to the hypothetical model

Compound	S score	Distance in Å	
		Glu 883	Asp 1044
4a	-7.13	1.30	2.77
4b	-16.64	1.28	2.61
4c	-11.56	1.27	2.68
4d	-15.43	1.28	2.62
4e	-12.21	1.27	2.66
4f	-13.48	1.31	3.01
4g	-16.23	1.33	2.87
4h	-15.63	1.30	2.78
5a	-17.16	1.28	2.61
5b	-15.29	1.29	2.62
5c	-12.30	1.27	2.67
6b	-16.01	1.27	2.63
6c	-7.03	1.31	3.04
6d	-7.90	1.25	2.63



1a, 2a, 4a) Ar = 4-ClC_6H_4

1b, 2b, 4b) Ar = 4-$CH_3C_6H_4$

1c, 2c, 4c) Ar = C_6H_5

1d, 2d, 4d) Ar = 2-$CH_3OC_6H_4$

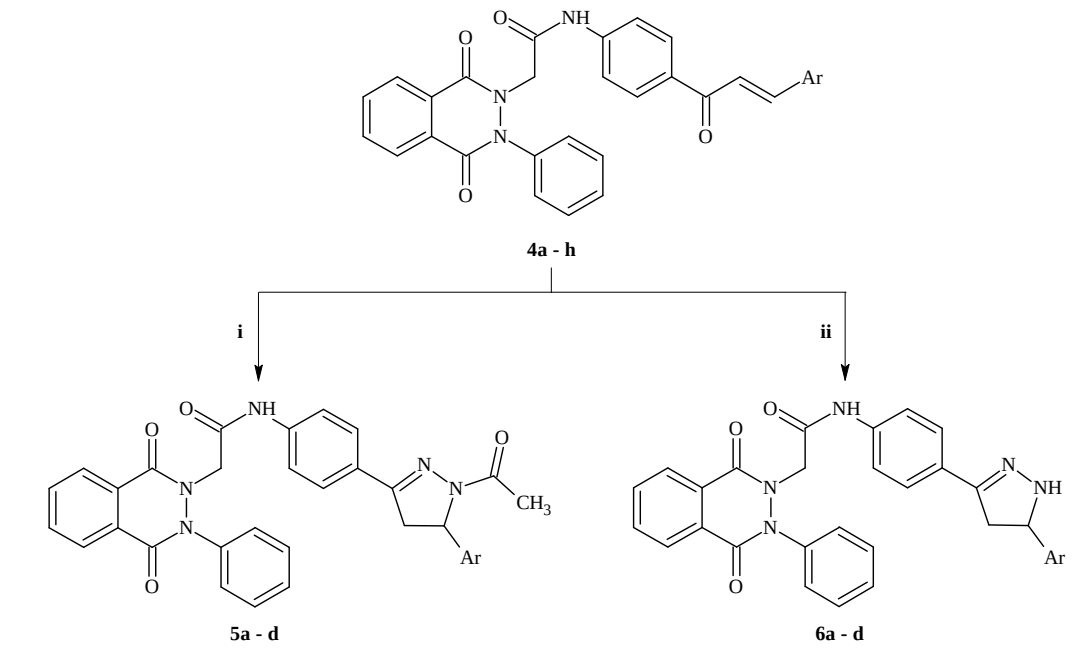
1e, 2e, 4e) Ar = 4-$NO_2C_6H_4$

1f, 2f, 4f) Ar = 2,4-diClC_6H_3

1g, 2g, 4g) Ar = 2-ClC_6H_4

1h, 2h, 4h) Ar = 2-Furyl

Scheme 1. Reagents and conditions: (i) 10% NaOH/EtOH(ii)$BrCH_2COBr$/K_2CO_3(iii)K_2CO_3/Acetone



5, **a** = 4-ClC_6H_4, **b** = C_6H_5, **c** = 4-$NO_2C_6H_4$, **d** = 2-ClC_6H_4

6, **a** = 4-$CH_3OC_6H_4$, **b** = 4-ClC_6H_4, **c** = C_6H_5, **d** = 2-$CH_3OC_6H_4$

Scheme 2. Reagents and conditions: (i) NH_2NH_2/CH_3COOH (ii) $NH_2NH_2/EtOH$

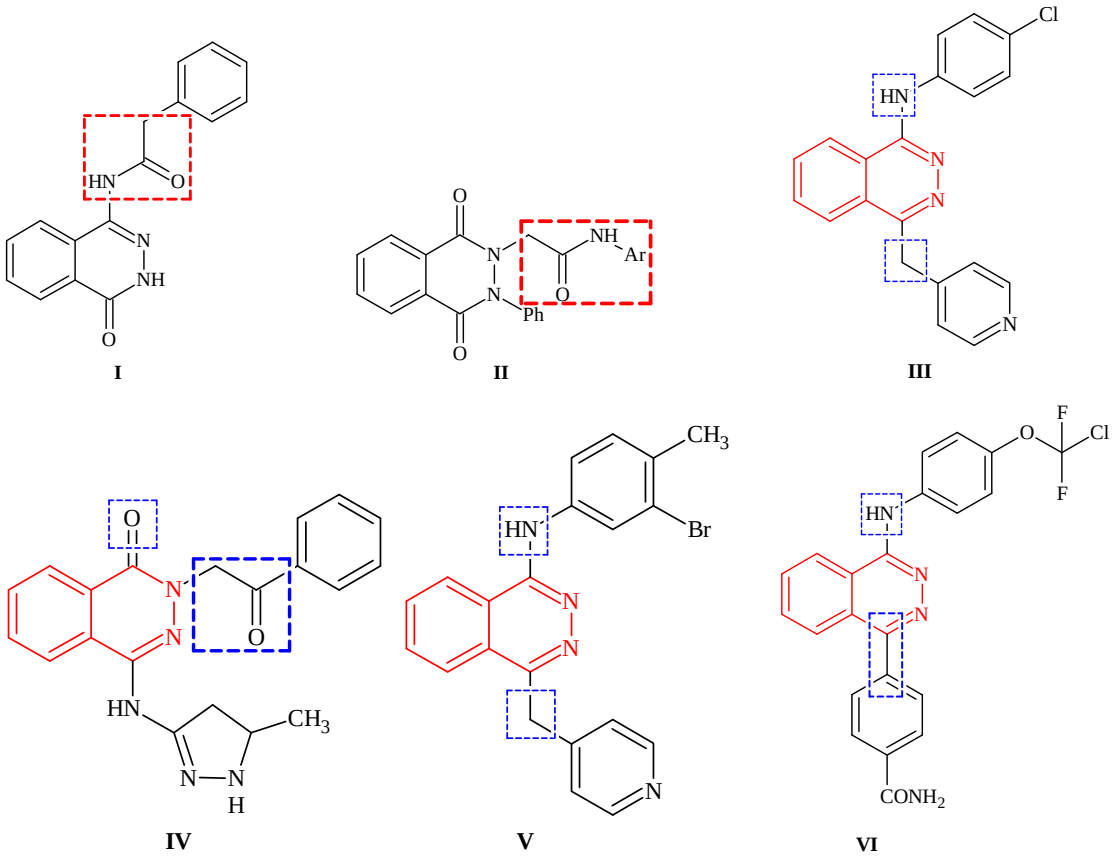


Fig. 1. Structure of the designed phthalazine derivatives I & II, and the lead anticancer phthalazine derivatives III-V

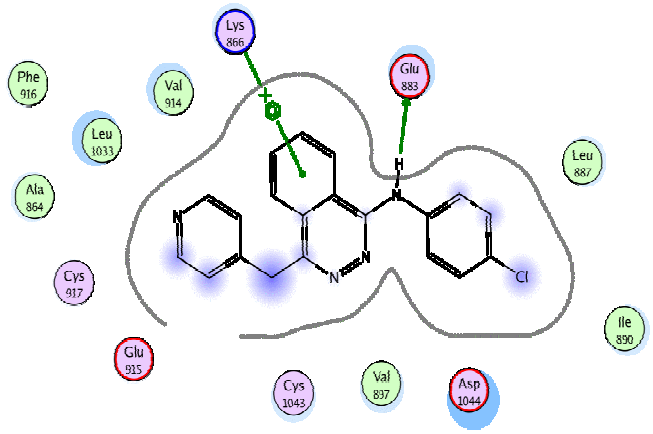


Fig. 2: Docked poses of vatalanib in VEGFR-2 binding site generated by MOE docking. Simplified 2D ligand interaction of vatalanib. The most important amino acids are shown together with their respective numbers. Vatalanib forms one hydrogen bond with Glu883 through its anilino nitrogen. Vatalanib also form π cation with lys 866. Also flanked by the amino acids ASP 1044 and Glu 915 and CYS 917. S score= -7.23

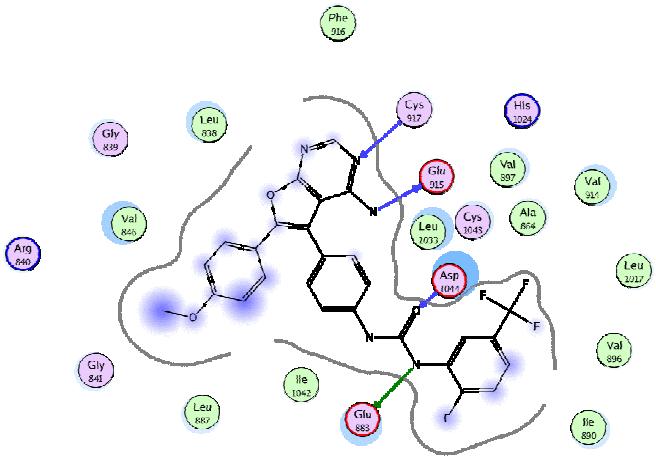


Fig. 3: Docked poses of inhibitor VEGF-2 generated by MOE docking. Simplified 2D ligand interaction of vatalanib. The most important amino acids are shown together with their respective numbers. The NH and CO motifs of the urea form interactions with the backbone of Asp1044 and the carboxylic acid residue of Glu883, respectively. Also ligand form hydrogen bond with Glu 915 and Cys 917. S score = -9.29

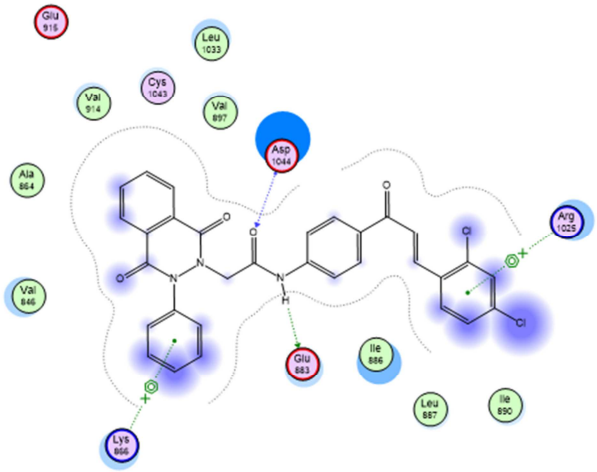


Fig. 4: Docked poses of Compound 4f in VEGFR-2 binding site generated by MOE docking.Simplified 2D ligand interaction of 4f. The most important amino acids are shown together with their respective numbers. Compound 4f form one hydrogen bond with Glu 883 through its anilino NH motif (Distance = 1.31 A) and another hydrogen bond with ASP 1044 through its CO motif(Distance= 3.01 A). It also form two π cation interactions with Lys 866 and Arg 1025. It also flanked by the amino acids Ile 886 , Glu 915 , Val 914, Cys 1043. S score = -13.48

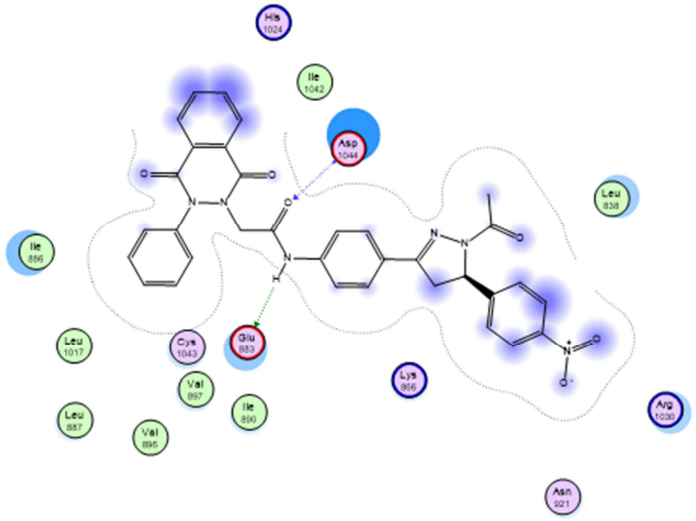


Fig. 5: Docked poses of Compound 5c in VEGFR-2 binding site generated by MOE docking.Simplified 2D ligand interaction of 5c. The most important amino acids are shown together with their respective numbers. Compound 5c form one hydrogen bond with Glu 883(distance=1.27 A) through its anilino NH motif and another hydrogen bond with ASP 1044(distance=2.67) through its CO motif. It also flanked by the amino acids LYS 866 , leu 838 , His 1024. S score = -12.30

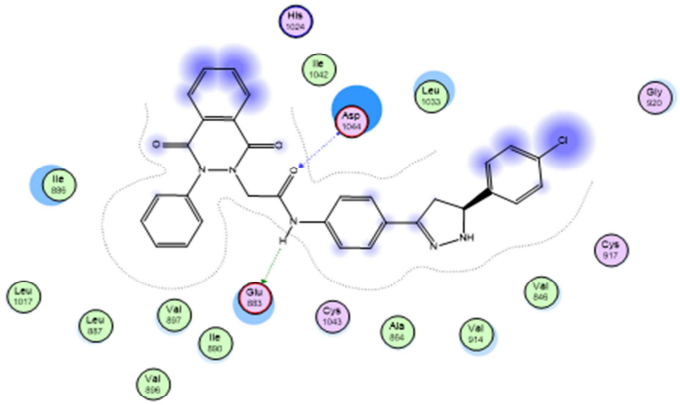


Fig. 6: Docked poses of Compound 6b in VEGFR-2 binding site generated by MOE docking. Simplified 2D ligand interaction of 6b. The most important amino acids are shown together with their respective numbers. Compound 6b form one hydrogen bond with Glu 883(distance=1.27 A) through its anilino NH motif and another hydrogen bond with ASP 1044(distance=2.63) through its CO motif. It also flanked by the amino acids LYS 866, leu 838, Cys 917. S score = -16.01

DISCUSSION

The newly synthesized compounds **4a-h** were identified by ^{1}H-NMR and mass spectrometry. The purity of these compounds was checked by elemental analysis. The ^{1}H-NMR data for compounds, **4a-h** showed the known characteristic pattern for both the parent phthalazine and chalcone derivatives in addition to the characteristic singlet of –NH-CH_2- moiety at δ 5.01-5.15 ppm.

The reaction between the appropriate chalcone **4** and hydrazine hydrate in refluxing acetic acid or in refluxing ethanol was monitored by TLC. After 5 hours all starting material was consumed. The condensation of **4** and hydrazine hydrate in acetic acid gave the corresponding N-(4-acetyl)-5-(aryl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydrophthalazin-2(1*H*)-yl)acetamide **5a-d**, while in ethanol gave the correspondingN-(4-(5-(aryl)-4,5-dihydro-1*H*-pyrazol-3-yl)phenyl)-2-(1,4-dioxo-3-phenyl-3,4-dihydrophthalazin-2(1*H*)-yl)acetamide **6a-d** (Scheme 2).

The spectra of the 5-membered heterocycles in compounds **5** and **6** changed significantly when $–COCH_3$ substituent attached in position 1.

In the ^{1}H-NMR spectra of compound **5** and **6**, the three hydrogen atoms attached to the C-4 and C-5 carbon atoms of the pyrazoline ring gave ABx spin system as reported[43]. In the acetylated pyrazolines **5a-d**, the H-5 proton deshielded dramatically to appear in the range of δ 5.08-5.75 ppm due to the high electronegativity of carbonyl group in acetyl radical. In the non-acetylated pyrazolines **6a-d**, measured chemical shift equivocally prove the 2-pyrazoline structure. H-4 (Trans) appeared as double doublets at the range of δ 2.67-2.84 ppm with *J* constant equal to 10.7 and 16.3 Hz. The peak of H-4 (Cis) of pyrazolines appeared as multiplet in the range of δ 3.38-3.36 ppm. The H-5 of pyrazoline appeared in the range of δ 3.87-4.1 ppm.

From the obtained results, it was explicated that most of the prepared compounds showed excellent to moderate growth inhibitory activity against the two tested cancer cells.

Generally, it is notable that chalcones series showed highest growth inhibitory activity among all synthesized series, and we observe that substitution on the phenyl ring of chalcones almost increase activity, introduction of chlorine atom in position number 2 and position number 4 enhance activity and in the two position maximize anticancer activity (compound **4f**),introduction of NO_2 group at position number 4 increase activity proving that introduction of electron-withdrawing groups enhance activity.

Introduction of methoxy group at position number 4 cause increasing in activity; however in position 2 lead to decrease in activity, replacement of phenyl ring with furan ring is well-tolerated.

Molecular docking studies of the synthesized compounds were performed in order to rationalize the obtained biological results as well as to help in understanding the various interactions between the ligands and enzyme active sites. It is obvious that aniline-moiety separated from certain pharmacophores with one or more atoms is essential for activity which appears in all docking results.

From all above results, we can see that aniline-moiety of phthalazin-derivatives is essential for activity. Also, substitution on the anilino-moiety affects the activity markedly.

And so, we aim to studying the effect of attaching other different pharmacophores to the anilio-moiety in the future.

Structure activity relationship (SAR)

The preliminary SAR study has focused on how the combination of two pharmacophores with a linker allows us to find more potent anticancer agents. Further investigation of the impact of the substitution pattern of the anilino moiety on the prepared phthalazin-1,4-diones. So, combination of $–CH_2$-CO- linker with aniline moiety and chalcone has very good role in inhibiting cancer cell proliferation and induce apoptosis in HCT-116 and MCF-7 cell lines. Compound **4f**has been identifies as the best example to represent that combination. Also, in **4f**the presence of two Cl-atoms on phenyl ring of chalcone has been found to be important for activity. This may be due to the high lipophilicity imparted by the two Cl-atoms as well as their role in increasing the ability of phenyl group to form two π cation interactions with Lys 866 and Arg 1025 (Fig. 4).

Moreover, docking studies revealed that compound **4f** showed similar binding mode to vatalanib with binding energy score equal to -13.48, which also explain its potential anticancer activity. Prime *et al.*have reported a novel class of phthalazine incorporated pyrazolines[32]. This allows us to cyclize the chalcones to give the corresponding pyrazolines **5a-d**and **6a-d.** Compound **5c**having acetylated pyrazoline ring linked with aniline and $–CH_2$-C=-linker showed moderate activity against HCT-116 and MCF-7 cell lines with IC_{50} of 2.27 and 2.91 μM, respectively. This is evident from docking study (Fig. 5).

Finally, it was noticed that the activity has been enhanced in compounds **5a-d** which include $–COCH_3$ group on pyrazoline, compared to the corresponding non acetylated pyrazoline derivatives **6a-d** analogues.

CONCLUSION

In the present paper we report the synthesis, spectral studies and antitumor evaluation of a novel series of phthalazin-1,4-dione incorporated chalcones and pyrazoline derivatives **4a-h**, **5a-d**, and **6a-d**, respectively. The structures proposed to all the newly synthesized compounds are well supported by spectroscopic data and elemental analysis. Compounds **4f**, **4b**, **4g**, **4h**, **5c**, and **5d** exhibited considerable antitumor activity against two tested tumor cell lines. Specifically, compound **4f** exhibited promising anti-proliferative activity with IC_{50} values equal to 2.21

and 1.00 μM in colon cancer and breast cancer in comparison to the standard drug doxorubicin (IC_{50} values equal to 0.81 μg/ml and 0.72 IC_{50} values). These preliminary encouraging results of biological screening of all the tested compounds could offer an excellent framework in this field that may lead to discover novel leads for future drug development.

Acknowledgement
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Appendix A. Supplementary data associated with this article can be found in the online version.

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