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RESEARCH ARTICLE

3-[(6-Arylamino)pyridazinylamino]benzoic acids: design, synthesis and in vitro evaluation of anticancer activity

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Abstract A series of novel substituted 3,6-disubstituted pyridazines based on the structure of vatalanib (PTK787) were designed and synthesized. The cytotoxicity of the final compounds was tested in vitro on HT-29 colon cancer cell line. Compounds **2a** and **2b** with 4-chlorophenylamino moiety, exerted the highest cytotoxic activity with IC_{50} values equal to 15.3 and 3.9 μM respectively. The most promising compound, **2b**, was found to be about fivefold more active than vatalanib against HT-29 colon cancer cell line.

Keywords Pyridazine · Colon cancer · Vatalanib · Cytotoxic

Introduction

Cancer represents the second leading cause of human mortality after cardiovascular diseases (Eckhardt 2002; Lee et al. 2002). Although chemotherapy is the mainstay of cancer therapy, the use of available chemotherapeutics still suffers from major limitations, such as lack of selectivity for cancer tissues, bringing about unwanted side effects and the acquisition by cancer cells of multiple-drug resistance (Rojo et al. 2008). Therefore, the search for new drugs, which can selectively target the tumor cells is today's goal of cancer therapy. In the recent years, inhibition of the VEGFR-2 (vascular endothelial growth factor receptor) signalling pathway, approved to have an antiangiogenic effect on human cancers (Dvorak 2002). In addition, the small-molecule VEGFR-2 kinase inhibitors, sorafenib (Ahman and Eisen 2004) and pazopanib (Podar et al. 2004) (GW786034) have been approved for the treatment of advanced renal cell carcinoma (Morabito et al. 2006). Vatalanib (PTK-787), a 1,4-disubstituted phthalazine VEGFR inhibitor, is currently in phase III clinical trials for metastatic colorectal cancer (Scott et al. 2007) (Fig. 1).

Introduction of appropriate heterocycles into a lead compound is a common strategy in the drug discovery process. A wide range of heterocyclic ring systems have been studied for the development of novel chemical entities as lead molecules.

Over the past years, pyridazine derivatives have been extensively studied and have shown a wide range of pharmacological activities such as antidepressant (Coelho et al. 2003), antihypertensive (Siddiqui et al. 2010), anticonvulsant (Rubat et al. 1990) cardiotonic (Sircar et al. 1987), antibacterial (Kandile et al. 2009), hepatoprotective (Kwon and Moon 2005), anti-HIV (Livermone et al. 1993), COX-2 inhibitor (Harris et al. 2004), and anticancer (Ahmad et al. 2010; Abd El-Ghaffar et al. 2011) activities. The encouraging anticancer activity observed by pyridazine derivatives prompted us to investigate new analogues involving modification of the structure of vatalanib. The introduction of new substituents or functional groups at various positions on aromatic or heteroaromatic fragments of a potential drug might lead to changes in its molecular shape that allows optimum binding to the receptor, as well as its physicochemical properties that affect drug distribution and metabolism (Piatnitski et al. 2005). Our strategy was directed toward designing a variety of ligands with diverse chemical properties hypothesized that the potency of the molecule

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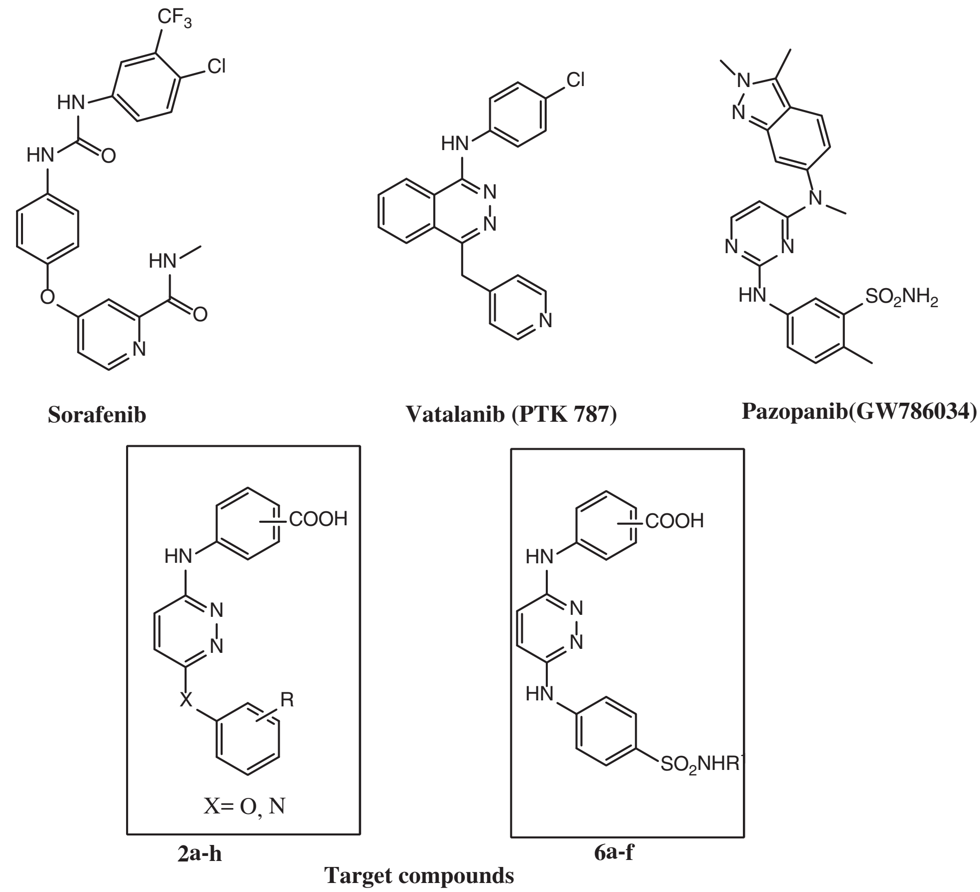
Fig. 1 Structures of VEGFR kinase inhibitors under clinical investigation and the designed compounds



Sorafenib **Vatalanib (PTK 787)** **Pazopanib(GW786034)**

2a-h **6a-f**

Target compounds

might be enhanced by adding alternative binding group such as carboxylic acid, amide, or sulphonamide group in the aniline moiety.

The design of our ligands was based on the replacement of the phthalazine scaffold in vatalanib by pyridazine ring, which was connected with the aryl system via a heteroatom, as well as replacement of the chlorine atom in vatalanib by an electron withdrawing carboxylic function with the hope of attaining superior biological activity, Furthermore, a number of novel sulfonamide derivatives have recently been reported to show substantial antitumor activity both in vitro and/or in vivo (Kamal et al. 2011; Rathish et al. 2012). In this direction, we introduced larger moiety at 4 position of aniline such as sulphonamido or heterocyclylsulphonamido, so that the bulky group could be oriented deep in the binding site, and makes predominantly hydrophobic interactions with the protein (Fig. 1).

Materials and methods

Chemistry

All chemicals and reagents were obtained from Aldrich (Sigma-Aldrich), and were used without further purification. Reactions were monitored by TLC, performed on silica gel glass plates containing 60 GF-254, and visualization on TLC was achieved by UV light or iodine indicator. IR spectra were determined on Shimadzu IR 435 spectrophotometer (KBr, cm^{-1}). ^{1}HNMR spectra were carried out using a Mercury 300-BB 300 MHz using TMS as internal standard. Chemical shifts (δ) are recorded in ppm on δ scale, Micro analytical Center, Cairo University, Egypt. ^{13}CNMR spectra were carried out using a Mercury 300-BB 75 MHz using TMS as internal standard. Chemical shifts (δ) are recorded in ppm on δ scale, Micro analytical Center, Cairo University, Egypt. Mass spectra were recorded on Shimadzu Qp-2010 plus spectrometer, Micro analytical Center, Cairo University, Egypt. Elemental analyses were carried out at the Micro analytical Center, Cairo University, Egypt. Melting points were determined with Stuart apparatus and are uncorrected.

The starting material 3,6-dichloropyridazine is commertially available. 6-Chloro-3-substituted-aminopyridazines (Abouzid et al. 2012) **1a–h**, 2-(6-chloropyridazine-3-yloxy)benzamide (Abouzid et al. 2012) (**3**) and 4-(6-chloropyridazin-3-ylamino) benzenesulphonamide (Mariño et al. 1980) (**5a**) were prepared as reported.

All chemicals were obtained from Aldrich, Fluka, or Merck chemicals.

General procedure for 2**a**–**h**, 4**a**, **b** and 6**a**–**f**

A mixture of an equimolar amounts of the appropriate **1a**–**h**, **3** or **5a**–**c** and an appropriate amino benzoic acid (0.01 mol each) in isopropanol (30 mL) was heated under reflux for 4–5 h. The solution was concentrated under reduced pressure to half its volume and cooled. The separated solid was filtered, washed with sodium carbonate solution (20 mL), dried and crystallized from isopropanol.

*3-{[6-[(4-Chlorophenyl)amino]pyridazin-3-yl]amino}benzoic acid (**2a**)*

Yield: 70 %; mp 141–142 °C; IR (cm^{-1}): 3421–2553 (N–H and O–H), 1685 (C=O), 1631 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.73 (d, 1H, pyridazine), 6.76 (d, 1H, pyridazine), 7.05–5.07 (m, 4H, Ar–H), 7.12 (d, 2H, Ar–H), 7.16 (d, 2H, Ar–H), 8.05 (s, 1H, NH D_2O exchangeable), 9.83 (s, 1H, NH D_2O exchangeable); MS (EI) *m*/*z* (% rel. Int.): 342 (M+2, 0.57), 340 (M$^+$, 5.00), 261 (23.18), 204 (6.96), 135 (9.22). Anal. Calcd for $C_{17}H_{13}ClN_4O_2$: C, 59.92; H, 3.85; N, 16.44; Found C, 59.78; H, 3.98; N, 16.56.

*4-{[6-(4-Chlorophenyl)amino]pyridazin-3-ylamino}benzoic acid (**2b**)*

Yield: 65 %; mp 129–130 °C; IR (cm^{-1}): 3471–2546 (N–H and O–H), 1678 (C=O), 1624 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.54 (d, 1H, pyridazine), 6.57 (d, 1H, pyridazine), 6.88 (dd, 2H, Ar–H), 7.22 (dd, 2H, Ar–H), 7.58–7.78 (m, 4H, Ar–H), 8.04 (s, 1H, NH D_2O exchangeable), 9.88 (s, 1H, NH D_2O exchangeable); MS (EI) *m*/*z* (% rel. Int.): 342 (M+2, 0.01), 340 (M$^+$, 0.03), 137 (100). Anal. Calcd for $C_{17}H_{13}ClN_4O_2$: C, 59.92; H, 3.85; N, 16.44; Found C, 59.88; H, 3.65; N, 16.15.

*3-{[6-(2,4-Dichlorophenyl)amino]pyridazin-3-ylamino}benzoic acid (**2c**)*

Yield: 60 %; mp 209–210 °C; IR (cm^{-1}): 3421-2561 (N–H and O–H), 1685 (C=O), 1627 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 7.26 (d, 1H, pyridazine), 7.30 (d, 1H, pyridazine), 7.30–7.69 (m, 5H, Ar–H), 7.95 (d, 1H, Ar–H), 8.03 (s, 1H, NH D_2O exchangeable), 8.39 (s, 1H, Ar–H), 9.87 (s, 1H, NH D_2O exchangeable); EIMS (% rel. abundance): 376 (M+2, 0.00), 374 (M$^+$, 0.00), 161 (19.84), 137 (100), 120 (39.29), 92 (40.30). Anal. Calcd for $C_{17}H_{12}Cl_2N_4O_2$: C, 54.42; H, 3.22; N, 14.93; Found C, 54.15; H, 3.35; N, 14.82.

*4-{[6-(2,4-Dichlorophenyl)amino]pyridazin-3-ylamino}benzoic acid (**2d**)*

Yield: 75 %; mp 239–240 °C; IR (cm^{-1}): 3294-2546 (O–H and N–H), 1666 (C=O), 1612 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.68 (d, 1H, pyridazine), 6.71 (d, 1H, pyridazine), 7.27–7.34 (m, 3H, Ar–H), 7.62–7.93 (m, 4H, Ar–H), 8.03 (s, 1H, NH D_2O exchangeable), 9.95 (s, 1H, NH D_2O exchangeable); ^{13}CNMR (75 MHz, DMSO) δ ppm: 114.50 (2C, Ar), 117.40 (1C, Ar), 117.50 (1C, Ar), 120.97 (2C, pyridazine), 123.10 (1C, Ar), 129.53 (1C, Ar), 130.49 (2C, Ar), 131.06 (2C, Ar), 144.26 (1C, Ar), 148.12 (1C, Ar), 156.42 (2C, pyridazine), 167.19 (1C, CO); EIMS (% rel. abundance): 378 (M+4, 2.67), 376 (M+2, 1.59), 144 (13.18). Anal. Calcd for $C_{17}H_{12}Cl_2N_4O_2$: C, 54.42; H, 3.22; N, 14.93; Found C, 54.35; H, 3.10; N, 14.77.

*3-{[6-(2,6-Dichlorophenyl)amino]pyridazin-3-ylamino}benzoic acid (**2e**)*

Yield: 62 %; mp 197–198 °C; IR (cm^{-1}): 3294–2557 (O–H and N–H), 1685 (C=O), 1627 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 7.20 (d, 1H, pyridazine), 7.23 (d, 1H, pyridazine), 7.40–7.97 (m, 6H, ArH), 8.39 (s, 1H, ArH), 8.03 (s, 1H, N–H D_2O exchangeable), 9.81 (s, 1H, N–H D_2O exchangeable); ^{13}CNMR (75 MHz, DMSO) δ ppm: 119.17 (1C, Ar), 120.55 (2C, pyridazine), 122.39 (1C, Ar), 122.61 (1C, Ar), 128.97 (1C, Ar), 129.37 (2C, Ar), 129.47 (1C, Ar), 131.22 (2C, Ar), 131.63 (1C, Ar), 140.36 (1C, Ar), 147.58 (1C, Ar), 156.54 (2C, pyridazine), 167.43 (1C, CO); EIMS (% rel. abundance): 374 (M$^+$, 0.00), 161 (13.34), 137 (100), 120 (76.40), 92 (71.66). Anal. Calcd for $C_{17}H_{12}Cl_2N_4O_2$: C, 54.42; H, 3.22; N, 14.93; Found C, 54.35; H, 3.45; N, 14.88.

*4-{[6-(2,6-Dichlorophenyl)amino]pyridazin-3-ylamino}benzoic acid (**2f**)*

Yield: 78 %; mp 144–145 °C; IR (cm^{-1}): 3460–2549 (O–H and N–H), 1666 (C=O), 1624 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.57 (d, 1H, pyridazine), 6.60 (d, 1H, pyridazine), 7.62–7.65 (m, 3H, ArH), 7.82 (dd, 2H, Ar–H), 7.91 (dd, 2H, Ar–H), 8.02 (s, 1H, N–H D_2O exchangeable), 9.94 (s, 1H, N–H D_2O exchangeable); EIMS (% rel. abundance): 377 (M+2+H, 1.23), 161 (10.33), 144 (20.78), 137 (44.36), 92 (21.35). Anal. Calcd for $C_{17}H_{12}Cl_2N_4O_2$: C, 54.42; H, 3.22; N, 14.93; Found C, 54.24; H, 3.63; N, 15.20.

*3-{[6-(2-Chloropyridin-3-yl)amino]pyridazin-3-ylamino}benzoic acid (**2g**)*

Yield: 64 %; mp 126–127 °C; IR (cm^{-1}): 3425–2561 (O–H and N–H) 1685 (C=O), 1624 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.55 (s, 2H, N–H D_2O exchangeable), 6.76 (d, 1H, pyridazine), 6.80 (d, 1H, pyridazine), 6.95–7.18 (m, 6H, ArH), 7.52 (s, 1H, ArH); EIMS (% rel. abundance): 341 (M^+, 0,00), 137 (100), 120 (49.68), 112 (5.44), 92 (66.97). Anal. Calcd for $C_{16}H_{12}ClN_5O_2$: C, 56.23; H, 3.54; N, 20.49; Found C, 56.44; H, 3.86; N, 20.65.

*4-{[6-(2-Chloropyridin-3-yl)amino]pyridazin-3-ylamino}benzoic acid (**2h**)*

Yield: 65 %; mp 139–140 °C; IR (cm^{-1}): 3460–2549 (O–H and N–H), 1670 (C=O), 1615 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.86 (s, 2H, N–H D_2O exchangeable), 6.52 (d, 1H, pyridazine), 6.55 (d, 1H, pyridazine), 7.59–8.07 (m, 7H, ArH); EIMS (% rel. abundance): 341 (M^+, 0.05), 137 (89.47), 120 (100), 92 (48.93). Anal. Calcd for $C_{16}H_{12}ClN_5O_2$: C, 56.23; H, 3.54; N, 20.49; Found C, 56.12; H, 3.66; N, 20.45.

*3-{[6-(2-Carbamoylphenoxy)pyridazin-3-yl]amino}benzoic acid (**4a**)*

Yield: 68 %; mp 114–115 °C; IR (cm^{-1}): 3394–2500 (O–H and N–H), 1678, 1660 (2C=O), 1631 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.30 (br s, 2H, NH_2, D_2O exchangeable), 6.74 (d, 1H, pyridazine), 6.76 (d, 1H, pyridazine), 6.85–7.85 (m, 7H, ArH), 8.03 (s, 1H, ArH), 8.40 (s, 1H, N–H D_2O exchangeable), 13.02 (s, 1H, OH D_2O exchangeable); EIMS (% rel. abundance): 352 (M+2H, 22.48), 120 (100), 92 (82.91). Anal. Calcd for $C_{18}H_{14}N_4O_4$: C, 61.71; H, 4.03; N, 15.99; Found C, 61.45; H, 4.32; N, 16.18.

*4-{[6-(2-Carbamoylphenoxy)pyridazin-3-yl]amino}benzoic acid (**4b**)*

Yield: 70 %; mp 99–100 °C; IR (cm^{-1}): 3437–2542 (O–H and N–H), 1680, 1666 (2C=O), 1624 (C=N); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.70 (br s, 2H, NH_2, D_2O exchangeable), 6.52 (d, 1H, pyridazine), 6.56 (d, 1H, pyridazine), 6.82–6.88 (m, 2H, ArH), 7.36–7.42 (m, 2H, ArH), 7.59–7.62 (m, 2H, ArH), 7.81–7.85 (m, 1H, ArH), 8.02 (d, 1H, ArH), 8.35 (s, 1H, N–H D_2O exchangeable), 12.97 (br s, 1H, OH D_2O exchangeable); EIMS (% rel. abundance): 350 (M^+, 14.71), 137 (76.05), 120 (100), 92 (50.70). Anal. Calcd for $C_{18}H_{14}N_4O_4$: C, 61.71; H, 4.03; N, 15.99; Found C, 61.55; H, 4.15; N, 15.78.

General procedure for 5**a**–**c**

An equimolar mixture of 3,6-dichloropyridazine and the appropriate substituted suphonamide (0.01 mol each) in isopropanol (30 mL) was heated under reflux for 4 h. The reaction mixture was concentrated under reduced pressure to half its volume and cooled. The separated solid was filtered, washed with sodium carbonate solution (20 mL), dried then crystallized from isopropanol.

*N-Carbamimidoyl-4-[(6-chloropyridazin-3-yl)amino]benzenesulphonamide (**5b**)*

Yield: 65 %; mp 129–130 °C; IR (cm^{-1}): 3498, 3441, 3394 (N–H and NH_2), 1620 (C=N), 1384 and 1126 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.60 (br s, 2H, NH_2 D_2O exchangeable), 6.51–6.54 (m, 3H, 2ArH and 1H pyridazine), 7.35–7.40 (m, 2H, ArH), 8.04 (d, 1H, pyridazine); EIMS (% rel. abundance): 324 (M−2, 0.06), 214 (96.43), 108 (100), 92 (75.20). Anal.Calcd for $C_{11}H_{11}ClN_6O_2S$: C, 40.43; H, 3.39; N, 25.72; Found C, 40.28; H, 3.13; N, 25.84.

*4-[(6-Chloropyridazin-3-yl)amino]-N-(pyrimidin-2-yl)benzenesulphonamide (**5c**)*

Yield: 55 %; mp 209–210 °C; IR (cm^{-1}): 3468, 3375 (2N–H), 1627 (C=N), 1323 and 1153 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.01 (s, 2H, 2NH D_2O exchangeable), 6.54 (d, 1H, pyridazine), 7.00 (t, 1H, pyrimidine), 6.78–6.81 (d, 2H, ArH), 7.58–7.72 (m, 2H, ArH), 8.05 (d, 1H, pyridazine), 8.46 (d, 2H, pyrimidine); EIMS (% rel. abundance): 364 (M+2, 0.05), 362 (M^+, 0.07), 185 (100), 108 (26.08), 92 (50.24). EIMS (% rel. abundance): Anal. Calcd for $C_{14}H_{11}ClN_6O_2S$: C, 46.35; H, 3.06; N, 23.16; Found C, 46.56; H, 2.89; N, 23.36.

*3-{[6-[(4-Sulphamoylphenyl)amino]pyridazin-3-yl]amino}benzoic acid (**6a**)*

Yield: 70 %; mp 119–120 °C; IR (cm^{-1}): 3460, 3375, 3340, 3244 (N–H and NH_2), 1678 (C=O), 1627 (C=N), 1388 and 1145 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.30 (s, 1H, NH D_2O exchangeable), 5.80 (s, 2H, NH_2 D_2O exchangeable), 6.56 (d, 1H, pyridazine), 6.59 (d, 1H, pyridazine), 6.73–6.77 (m, 1H, ArH), 6.89 (s, 1H, NH D_2O exchangeable), 7.05–7.17 (m, 5H, ArH), 7.45 (d, 2H, ArH); EIMS (% rel. abundance): 385 (M^+, 0.00), 172 (100), 156 (84.02), 108 (67.31), 92 (85.17), 80 (23.64). Anal. Calcd for $C_{17}H_{15}N_5O_4S$: C, 52.98; H, 3.92; N, 18.17; Found C, 53.05; H, 3.88; N, 18.23.

*4-{[6-[(4-Sulphamoylphenyl)amino]pyridazin-3-yl]amino}benzoic acid (**6b**)*

Yield: 72 %; mp 144–145 °C; IR (cm^{-1}): 3475, 3375, 3267, 3217 (N–H and NH_2), 1680 (C=O), 1627 (C=N), 1315 and 1145 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.40 (s, 1H, NH D_2O exchangeable), 5.80 (s, 2H, NH_2 D_2O exchangeable), 6.56–6.78 (m, 6H, 4 ArH and 2H pyridazine), 6.89 (s, 1H, NH D_2O exchangeable), 7.43–7.63 (m, 4H, ArH); ^{13}CNMR (75 MHz, DMSO) δ ppm: 112.31 (2C, Ar), 112.44 (2C, Ar), 116.68 (2C, Ar), 127.32 (2C, pyridazine), 129.82 (2C, Ar), 131.14 (2C, Ar), 151.80 (2C, Ar), 153.03 (2C, pyridazine), 167.43 (1C, CO); EIMS (% rel. abundance): 384 ($M^{+}-1$, 0.04), 172 (100), 156 (78.20), 108 (58.57), 92 (74.43), 80 (17.80). Anal. Calcd for $C_{17}H_{15}N_5O_4S$: C, 52.98; H, 3.92; N, 18.17; Found C, 53.15; H, 3.74; N, 18.35.

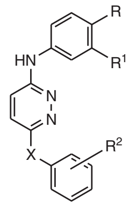
*3-{[6-[(4-(N-Carbamimidoylsulphamoyl)phenyl)-amino]pyridazin-3-yl]amino} benzoic acid (**6c**)*

Yield: 68 %; mp 139–140 °C; IR (cm^{-1}): 3433–2600 (N–H and O–H), 1654 (C=O), 1631 (C=N), 1307 and 1185 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 4.12 (br s, 2H, NH_2 D_2O exchangeable), 6.58 (d, 1H, pyridazine), 6.62 (d, 1H, pyridazine), 6.66 (s, 2H, 2NH D_2O exchangeable), 7.23–8.04 (m, 8H, ArH); EIMS (% rel. abundance): 429 (M+2H, 3.55), 137 (57.17), 92 (45.72), 79 (9.60). Anal. Calcd for $C_{18}H_{17}N_7O_4S$: C, 50.58; H, 4.01; N, 22.94; Found C, 50.33; H, 4.34; N, 22.70.

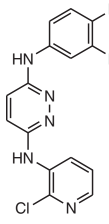
*4-{[6-[(4-(N-Carbamimidoylsulphamoyl)phenyl)-amino]pyridazin-3-yl]amino} benzoic acid (**6d**)*

Yield: 72 %; mp 124–125 °C; IR (cm^{-1}): 3448–2700 (N–H and O–H), 1650 (C=O), 1627 (C=N), 1327 and 1138 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 5.80 (br s, 2H, NH_2 D_2O exchangeable), 6.51–6.55 (m, 6H, 4 ArH and 2H pyridazine), 6.70 (s, 2H, 2NH D_2O exchangeable), 7.36 (d, 2H, ArH), 7.58 (d, 2H, ArH); EIMS (% rel. abundance):

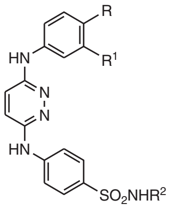
Table 1 Cytotoxicity of the tested compounds against HT-29 colon cell line in vitro



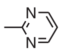
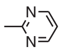
2a-f, 4a,b



2g,h



6a-f

Compound No.	X	R	R^1	R^2	HT-29 (IC_{50})^{a,b} μM
2a	NH	H	COOH	4-Cl	15.3
2b	NH	COOH	H	4-Cl	3.9
2c	NH	H	COOH	2,4-Cl_2	31.4
2d	NH	COOH	H	2,4-Cl_2	NA^c
2e	NH	H	COOH	2,6-Cl_2	103.8
2f	NH	COOH	H	2,6-Cl_2	119.5
2g	–	H	COOH	–	87.0
2h	–	COOH	H	–	69.2
4a	O	H	COOH	2-$CONH_2$	126.4
4b	O	COOH	H	2-$CONH_2$	50.53
6a	–	H	COOH	H	NA^c
6b	–	COOH	H	H	198.1
6c	–	H	COOH	$C(NH)NH_2$	104.4
6d	–	COOH	H	$C(NH)NH_2$	NA^c
6e	–	H	COOH		NA^c
6f	–	COOH	H		NA^c
Vatalanib^d					22.11

[a] IC_{50}: dose of the compound which inhibit tumor cell proliferation by 50 %

[b] Values are means of three experiments

[c] NA: compounds having IC_{50} value > 200 μM

[d] Used as positive control

428 (M+H, 0.01), 92 (0.25), 108 (0.37), 80 (100). Anal. Calcd for $C_{18}H_{17}N_7O_4S$: C, 50.58; H, 4.01; N, 22.94; Found C, 50.25; H, 4.14; N, 23.18.

*3-{[6-[(4-(N-(Pyrimidin-2-yl)sulphamoyl)phenyl)amino]-pyridazin-3-yl]amino} benzoic acid (**6e**)*

Yield: 65 %; mp 237–238 °C; IR (cm^{-1}): 3425–2519 (N–H and O–H), 1651 (C=O), 1600 (C=N), 1323 and 1157 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.01 (br s, 3H, 3NH D_2O exchangeable), 6.53–6.56 (m, 6H, 4ArH and 2H pyridazine), 7.00 (t, 1H, pyrimidine), 7.58–7.61 (m, 4H, ArH), 8.46 (d, 2H, pyrimidine); EIMS (% rel. abundance): 463 (M$^+$, 11.52), 385 (12.83), 326 (12.07), 136 (13.48), 95 (100), 80 (86.63). Anal. Calcd for $C_{21}H_{17}N_7O_4S$: C, 54.42; H, 3.70; N, 21.15; Found C, 54.13; H, 3.66; N, 21.22.

*4-{[6-[(4-(N-(Pyrimidin-2-yl)sulphamoyl)phenyl)amino]-pyridazin-3-yl]amino} benzoic acid (**6f**)*

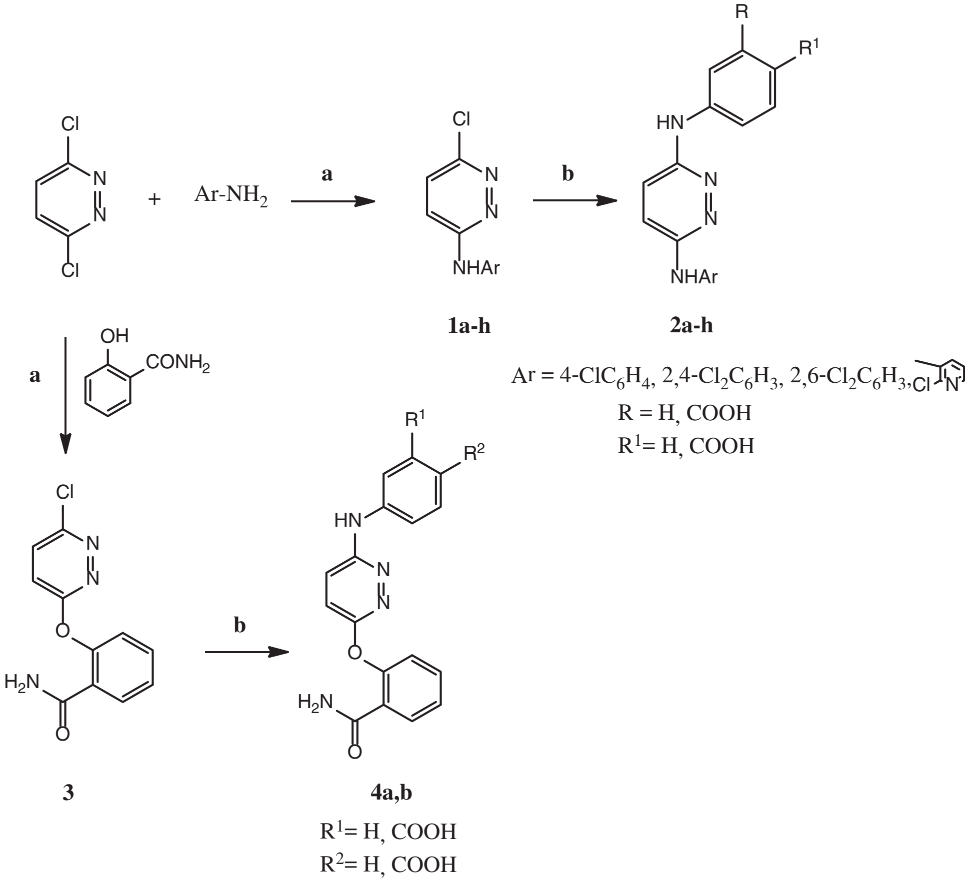
Yield: 74 %; mp 221–222 °C; IR (cm^{-1}): 3425–2661 (N–H and O–H), 1651 (C=O), 1615 (C=N), 1323 and 1157 (SO_2); ^{1}HNMR (300 MHz, DMSO-d_6) δ ppm: 6.01 (br s, 3H, 3NH D_2O exchangeable), 6.53-6.58 (m, 6H, 4ArH and 2H pyridazine), 7.00 (t, 1H, pyrimidine), 7.57–7.62 (m, 4H, ArH), 8.45 (d, 2H, pyrimidine); EIMS (% rel. abundance): 462 (M−1, 6.07), 419 (7.31), 136 (13.48), 95 (94.28), 81 (12.74). Anal. Calcd for $C_{21}H_{17}N_7O_4S$: C, 54.42; H, 3.70; N, 21.15; Found C, 54.72; H, 3.55; N, 21.05.

Biological studies

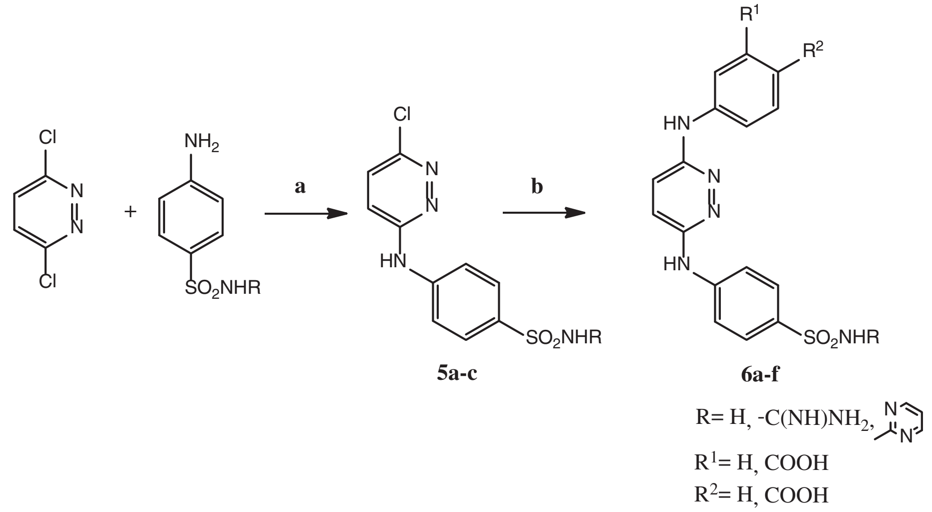
In vitro cytotoxicity assay

The cytotoxic activity was measured in vitro using the sulphorhodamine B (SRB) colorimetric assay using the method of Skehan et al. (1990). Cells were inoculated in 96-well microtiter plate (104 cells/well) for 24 h before treatment with the compound(s) to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the compound under test (0.1, 2.5, 5, and 10 mmol/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 h at 37 °C and in atmosphere of 5 % CO_2. After 48 h, cells were fixed, washed, and stained for 30 min with 0.4 % (w/v) SRB dissolved in 1 % acetic acid. Unbound dye was removed by four washes with 1 % acetic acid, and attached stain was recovered with Tris–EDTA buffer.

Scheme 1 Reagents and conditions: **a** K_2CO_3, isopropanol, 4 h, **b** 3- or 4-aminobenzoic acid, isopropanol, 4–5 h



Scheme 2 Reagents and conditions: **a** K_2CO_3, isopropanol, 4 h, **b** 3- or 4-aminobenzoic acid, isopropanol, 4–5 h



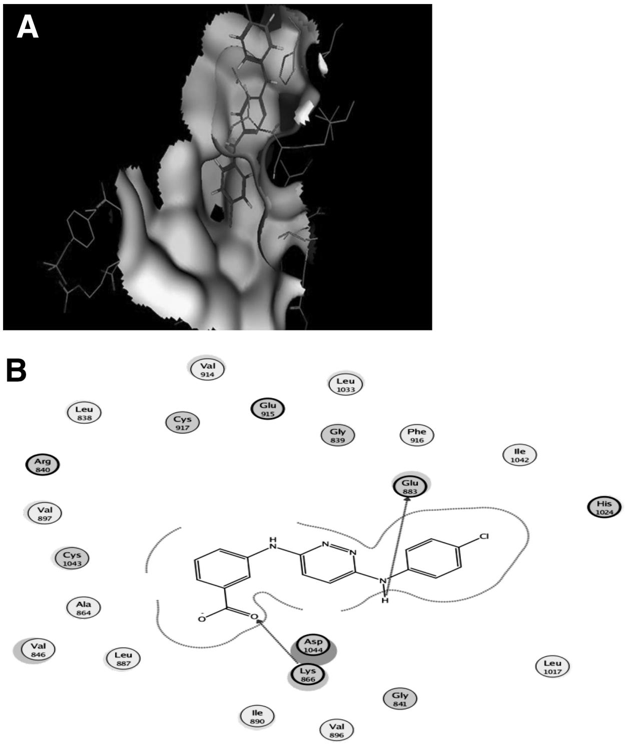


Fig. 2 **a** Docked pose of **2a** in VEGFR-2 binding site generated by MOE docking. **b** Simplified 2D ligand interaction of **2a**. The most important amino acids are shown together with their respective numbers. Compound **2a** forms a hydrogen bond with Glu883 through its anilino nitrogen, and other hydrogen bond with Lys866 through the carboxylate oxygen

Colour intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for tumor cell line after the specified time. The concentration required for 50 % inhibition of cell viability (IC_{50}) was calculated (Table 1).

Results and discussion

The synthetic route for target compounds **2a–h** and **4a**, **b** is outlined in Scheme 1. The intermediates **1a–h** and **3** were prepared from the commercially available 3,6-dichloropyridazine and different primary amino compounds or salicylamide as a starting materials, according to the reported procedure (Abouzid et al. 2012). The target anilino substituted pyridazine benzoic acids **2a–h** and the corresponding 2-aminocarbonylphenoxy analogues **4a**, **b** were obtained in 60–78 % yields by heating the precursors **1a–h** or **3** with either 3-aminobenzoic acid or 4-aminobenzoic acid in refluxing isopropanol for 4–5 h. The newly synthesised compounds were characterized by IR, ^{1}HNMR, ^{13}CNMR and mass spectral data as well as elemental analyses.

Support for the structure of the compounds **2a–h** was evidenced by the presence of prominent bands in the IR spectra for the carboxylic acid C=O str. (1,678–1,666 cm^{-1}). The structures were further established by ^{1}HNMR spectral data. In the compounds **2a–f**, signals due to the two NH (controlled with D_2O) were resonated at (8.02–8.04) and (8.81–9.95) ppm, while the signals due to OH group did not appear. In the carboxamide derivatives **4a**, **b**, IR spectra showed two bands for the carboxylic acid and the amide C=O str. In their ^{1}HNMR spectra, the broad NH_2 signals (controlled with D_2O) were observed at 5.20–5.80 ppm.

In Scheme 2, using methodology previously described (Abouzid et al. 2012), the intermediates **5a–c** were synthesized from a commercially available 3,6-dichloropyridazine and either sulphanilamide, sulphadiazine or sulphadimidine. Thereafter, reaction of **5a–c** with either 3-aminobenzoic acid or 4-aminobenzoic acid afforded **6a–f** in 65-74 % yields.

The two new intermediates **5b**, **c** and the final targets **6a–f** displayed IR, ^{1}HNMR, ^{13}CNMR, mass spectral data and elemental analyses consistent with the assigned structures.

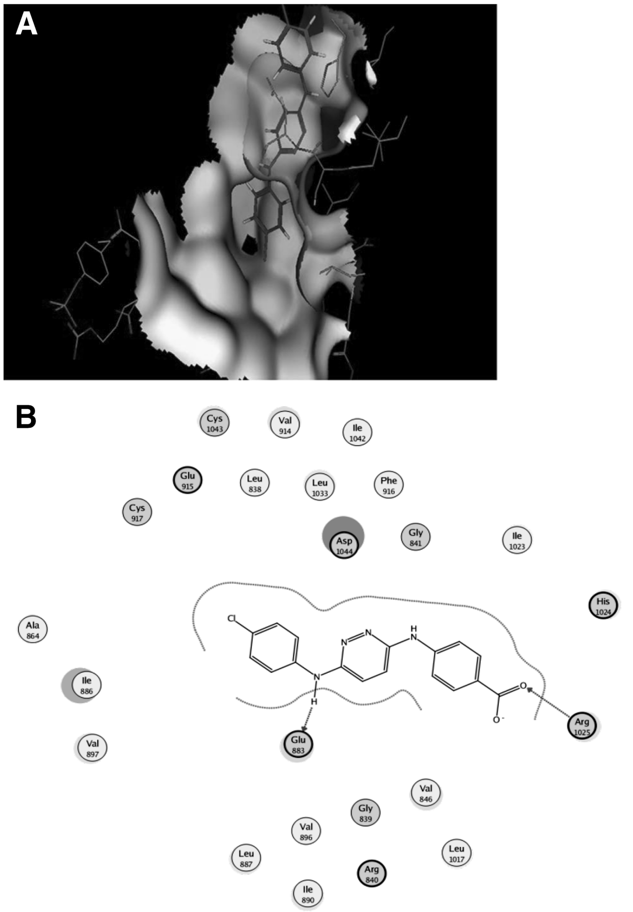


Fig. 3 **a** Docked pose of **2b** in VEGFR-2 binding site generated by MOE docking. **b** Simplified 2D ligand interaction of **2b.** The most important amino acids are shown together with their respective numbers. Compound **2b** forms a hydrogen bond with Glu883 through its anilino nitrogen, and other hydrogen bond with Arg1025 through the carboxylate oxygen

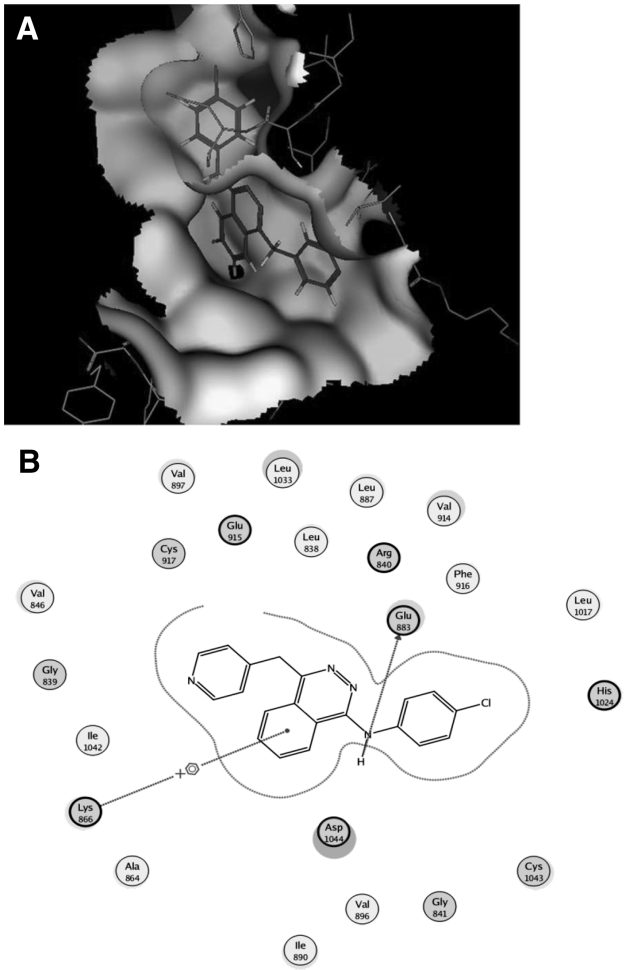


Fig. 4 **a** Docked poses of **vatalanib** in VEGFR-2 binding site generated by MOE docking. **b** Simplified 2D ligand interaction of vatalanib. The most important amino acids are shown together with their respective numbers. Vatalanib forms one hydrogen bond with Glu883 through its anilino nitrogen and π-cation with Lys866 amino acid

IR spectra of these compounds showed two bands for SO_2 in the range of (1,384–1,307 cm^{-1} and 1,185–1,126 cm^{-1}). The structures were further established by ^1H-NMR spectral data. All compounds exhibited additional D_2O exchangeable signals due to sulphonamido protons.

In vitro antitumor evaluation

Compounds **2a**–**h**, **4a**, **b** and **6a**–**f** were screened for their antiproliferative activity on colon carcinoma (HT-29) cell line using SRB colorimetric assay, in comparison to PTK787 as a reference drug. The cytotoxic activities are expressed by median growth inhibitory concentration (IC_{50}) and provided in Table 1.

Six of the tested compounds revealed cytotoxic activity with IC_{50} range from 3.9 to 87.0 μM. Other compounds were found to exhibit poor activity. From the results, it is evident that the cytotoxicities of **2a**–**h** and the oxygen-linked carboxamides **4a**, **b** were more potent than the sulphonamide derivatives **6a**–**f**. The 4-chloro derivatives **2a** and **2b** displayed the most potent cytotoxic activity, $IC_{50} = 15.3$ and 3.9 μM respectively. The presence of COOH group in the position 4 of the phenyl ring, compound **2b**, seems to increase the cytotoxic activity fourfolds than the corresponding isomer, **2a**. It was also noticed that the activity has been enhanced in compounds **2h** and **4b**, which include COOH group in the 4 position of the aryl moiety, compared to the corresponding 3-substituted analogues **2g** and **4a** respectively. However, the dichloro derivative, **2c** has shown higher cytotoxicity than its isomer **2d** containing COOH group at 4-position.

Molecular docking studies of the synthesized compounds were performed in order to rationalize the obtained biological results as well as to help us in understanding the various interactions between the ligands and enzyme active site.

The X-ray crystallographic structure of VEGFR-2 complexed with vatalanib was utilized in our docking studies (PDB ID: 1YWN). All water molecules in the experimental structure were removed and hydrogen atoms were added and the protonation states of the amino acid residues were assigned using the Protonate3D algorithm. Ligand molecules were modelled using MOE builder, and the structures were energy minimized using the MMFF94x force field. Validation of the function implemented in MOE was done by docking of the native ligand into its binding site. The docked results were compared to the crystal structure of the bound ligand–protein complex.

Next, we performed docking studies to our synthesized compounds, and the final docked complexes of ligand–enzyme were selected according to the criteria of interaction energy combined with geometrical matching quality.

In summary, the biological results revealed that, small lipophilic electron withdrawing substituents on the benzene ring enhances the cytotoxic effect. This was evident from the appreciable activity observed by the 4-chloro derivatives **2a** and **2b**. This result may be substantiated on the basis of various reported SAR studies (Virsodia et al. 2009; Zhang et al. 2010), which demonstrates the importance of chloro substituent in the position 3 or 4 of the benzene ring.

On the other hand, introduction of a second substituent to the benzene ring results in decline of activity, as shown by 2,4- and 2,6-dichloro derivatives, **2c**, **2d**, **2e** and **2f**. Besides, the presence of bulky sulphonamido substituent abolishes the activity, suggesting a relatively restricted binding area.

These preliminary encouraging results of biological screening of some of the tested compounds could offer an excellent framework in this field, that may lead to discover novel leads for future drug development.

Docking studies revealed that the compounds **2a** and **2b** showed similar binding mode to vatalanib with binding energy scores equal to −11.0079, −11.8305 and −14.2118 respectively, which may explain their potential anticancer activity (Figs. 2, 3, 4).

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