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Design, Synthesis and *in vitro* Evaluation of 2-(Quinoline-8-sulfonamido)pentanedioic Acid Analogues as Antiangiogenic and Antitumor Agents on Multiple Myeloma

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Thalidomide is presently approved as antiangiogenic and anticancer drug in multiple myeloma. The authors present a number of analogue-based designs of *N*-(*o*-carboxybenzoyl)-DL-glutamic acid, a major thalidomide metabolite. The compounds were synthesized and biologically tested in multiple myeloma as anticancer agents. Three compounds inhibited HUVEC proliferation at low micromolar concentrations, indicating that they are antiangiogenic and cytotoxic to human multiple myeloma RPMI8226. The active compounds were tested for antiproliferative activity on HUVECs using the dye exclusion method with trypan blue. Dimethyl-2-(quinoline-8-sulfonamido)pentanedioate (**2c**), in particular, inhibits VEGFR-2 phosphorylation at the Tyr-1175 residue, as determined by SDS PAGE. The binding mode of (**2c**) was predicted *in silico* in order to better understand how it interacts with essential amino acid residues in the VEGFR-2 active site. The binding energy was calculated as -161.41kcal/mol. *in vitro* Study of the compounds on the Vero cell line shows less toxicity towards the normal endothelial cells than the cancer cells.

Keywords: Bioisostere, Multiple myeloma, Angiogenesis, HUVEC, Molecular docking, Western blot.

INTRODUCTION

Thalidomide, a repurposed drug, is effective against leprosy, myelodysplastic syndrome and a chemotherapeutic agent against multiple myeloma [1]. It has multifaceted mechanisms like inhibition of VEGF, TNF-α, basic fibroblast growth factor (bFGF), augmentation of apoptosis, the proliferation of NK cells and T cells stimulation [2]. Exhaustive metabolite studies on thalidomide inferred the presence of *N*-(*o*-carboxybenzoyl)-DL-glutamic acid as a major metabolite. Some glutamine and glutamic acid are also the end products of the hydrolytic cleavage of thalidomide [3,4]. Glutamine is a highly essential substrate for malignant cells to carry out unusual aerobic glycolysis for ATP production [5]. Glutamine is converted by the kidney derived glutaminase (GLS1) into glutamate to perform the TCA cycle in mitochondria [6]. With the aid of glutamine synthetase, L-glutamate converts to L-glutamine and provides a ceaseless cache of L-glutamine for the malignant cells [7]. The role of glutamine and glutamic acid in the biosynthesis of deoxy-ribonucleotides and ribonucleotides through 3- and 9-nitrogen atoms the purine base, 2-amino group of guanine, 3-nitrogen atom and the 4-amino group of cytosine are well known [8,9]. L-glutamine takes part in numerous cellular signaling pathways by activating mTORC1. This activation of the protein complex helps the malignant cells evade autophagy, support their unrestrictive proliferation and cause hypoxia in the cells, resulting in the release of different growth factors [10,11].

The work of Faigel & Kemper established *N*-(*o*-carboxybenzoyl)-DL-glutamic acid as a major metabolite of thalidomide [3,4]. Kemper observed some striking similarities between folic acid and the metabolite, suggesting its future use as an anti-folate. Streptovitacin A and cyclohexidine, the antineoplastic antibiotics, are the structural variants of thalidomide and share a glutarimide moiety in common [12-14]. Azaserine & DON are established as glutamine antagonists [15].

Angiogenesis is a general physiological process for the growth and differentiation of normal endothelial cells. It occurs through the formation of new blood vessels from the existing ones. Abnormal changes in metabolic conditions trigger the upregulation of angiogenesis for malignant cell survival.

Angiogenic inhibitors are crucial for arresting the formation of new blood vessels in malignant tissues [16]. VEGFR-2, a cell surface-based tyrosine kinase receptor, plays a pivotal role in the regulation of angiogenesis. It is a primary responder to the VEGF signal [17]. Multiple myeloma is a type of cancer specific to the plasma cells where a group of plasma cells become malignant and accumulate in the bone marrow, thereby replacing the healthy normal blood cells [18].

Design: Considering the structural similarities between folic acid and the metabolite, three significant modifications were made in the design (Fig. 1). As reported in our previous work [19-22], the metabolite's carbonyl moiety was replaced with a stronger electron-withdrawing sulfonyl moiety (-SO_2-). This substitution led to a shift of electron cloud from the adjacent –NH- moiety towards the sulfonyl, hence favouring the participation of –NH- hydrogen with the neighboring receptor residues. The -NH- moiety at the *para*-position of the PABA fragment is modified by replacing it with a pyridine nucleus, *i.e.* forming a quinoline ring. The carboxylic groups were replaced with esters, amides and hydrazide.

The designed compounds were synthesized and characterized through ^{1}H & ^{13}C NMR and mass spectroscopy. Molecular docking studies of the synthesized compounds were performed to know the probable binding mode with VEGFR-2. Cytotoxicity study (determination of IC_{50}) of the synthesized molecules was carried out on RPMI 8226 and HUVEC to assess the anticancer and antiangiogenic activity. Analysis of the Vero cell line was performed to determine the compounds' selective toxicity for cancer cells.

EXPERIMENTAL

Chemicals for organic synthesis were procured from Merck India, Loba Chemie. The reagents used for cytotoxicity study were purchased from Sigma-Aldrich, BD biosciences, Himedia and Vector laboratories. Cell lines were procured from American Type Culture Collection (ATCC). Melting points were determined in an open capillary tube and are uncorrected. The compounds were characterized by different analytical tools like ^{1}H & ^{13}C NMR and mass spectroscopy. ^{1}H NMR (300/400/600 MHz) spectra and ^{13}C NMR (150/75 MHz) spectra were recorded on a Bruker DPX spectrometer using $CDCl_3$, DMSO-d_6 and CD_3OD as the solvent. The chemical shift values were noted as δ ppm using tetramethyl silane (TMS) as the internal standard. Elemental analysis of the synthesized compounds was carried out on Carlo Erba 1108 analyzer. Mass spectral analysis was carried out with Waters MICROMASS Q-Tof micro™. The compounds were purified by flash column chromatography using 230-400 mesh silica gel (Sigma- Aldrich), ethyl acetate, acetone and benzene (at different ratio).

Synthesis of quinoline-8-sulphonyl chloride (1b): Quinoline-8-sulphonyl chloride (**1b**) was synthesized by chlorosulphonation at 145 ºC by following the method described in the literature [23]. The white crystalline quinoline-8- sulphonyl chloride was dried for 30 min. Yield 35 %, m.p.: 124-126 ºC.

Synthesis of 2-(quinoline-8-sulfonamido)pentanedioic acid (2b): To a stirred aqueous solution of 3.6 g (24.47 mmol) of L-glutamic acid (**2a**) in Na_2CO_3 (10 g), 5.6 g (24.6 mmol) quinoline-8-sulphonyl chloride (**1b**) was added in portions over

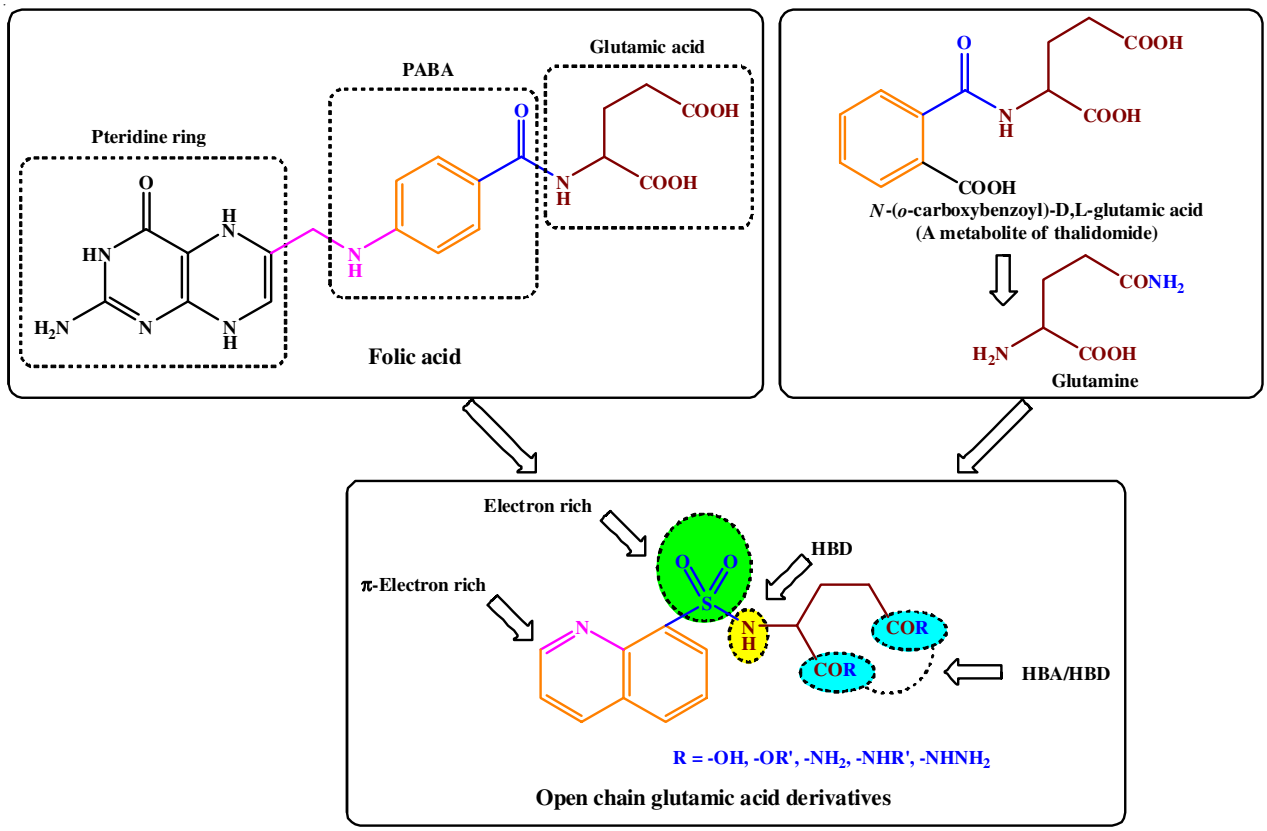


Fig. 1. Schematic diagram of 3-point bioisosteric replacement of the thalidomide metabolite

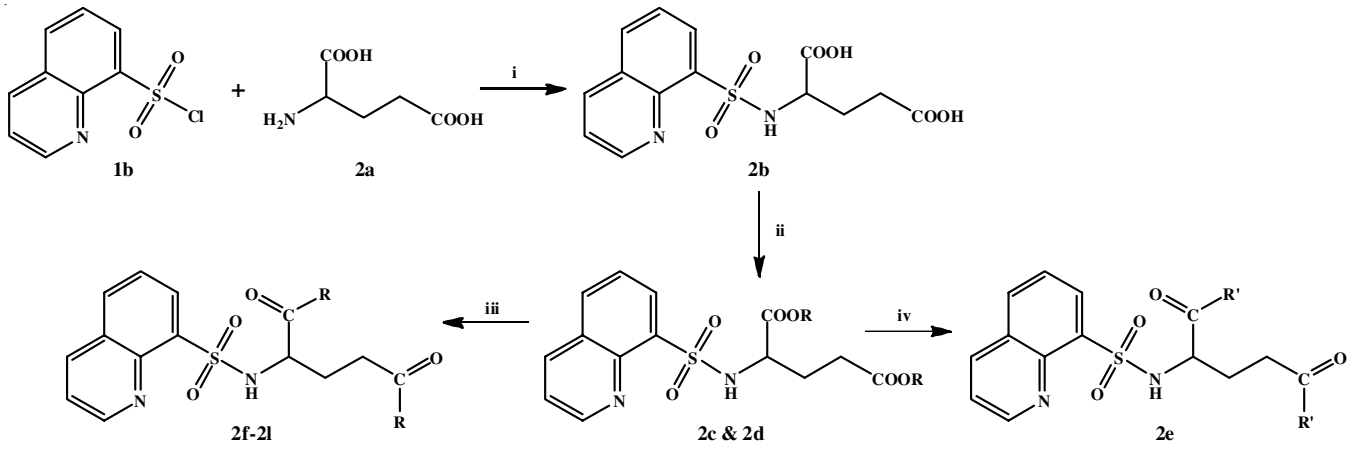
1 h period and stirred for 2.5 h at room temperature in alkaline condition The reaction was catalyzed by 50 mg (0.4092 mmol) of 4-dimethylaminopyridine. After 2.5 h, the pH of the reaction mixture was adjusted to 3, saturated with NaCl and extracted with ethyl acetate; the organic layer was washed twice with brine followed by drying over anhydrous sodium sulfate overnight. The organic layer was distilled to get **2b** as a white solid which was purified by flash chromatography using ethyl acetate and benzene in a ratio of 8:2. Yield 55.14%, m.p. 114-116 ºC. ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 1.757 (m, 1H), 1.927 (m, 1H), 2.277 (m, 2H), 4.256 (m, 1H), 7.708 (o, 3H), 8.281 (o, 2H), 8.515 (d, *J* = 8.1 Hz, 1H), 9.032 (s, 1H), 12.274 (br, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 27.78, 29.71, 55.69, 122.50, 125.64, 128.58, 130.23, 133.64, 136.99, 137.77, 142.90, 151.15, 172.95, 173.73. Mass (*m/z*) ESI TOF: 339.03 $(M+H)^+$. Anal calcd. (found) % for $C_{14}H_{14}N_2O_6S$: C, 49.70 (49.64); H, 4.17 (4.32); N, 8.28 (8.41).

Synthesis of dimethyl 2-(quinoline-8-sulfonamido)-pentanedioate (2c): Esterification of **2b** was carried out by Fischer-Speier method [24]. 2-(Quinoline-8-sulfonamido)pentanedioic acid (**2b**, 2 g, 6 mmol) was dissolved in 50 mL of super dried methanol saturated with dry hydrogen chloride gas and refluxed for 4 h in inert atmosphere. The final product was purified by flash chromatography using ethyl acetate and petroleum ether in a ratio of 3:2. Yield 86.17 %, m.p.: 88-90 ºC. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.88 (m, 2H), 2.32 (m, 2H), 3.212 (s, 3H), 3.489 (s, 3H), 4.385 (m, 1H), 7.7-7.748 (o, 1H), 8.002 (d, *J* = 7.8 Hz, 1H), 8.253-8.323 (*o*, 2H), 8.524 (d, *J* = 7.8 Hz, 1H), 9.009 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 27.19, 29.19, 51.29, 51.56, 55.51, 122.35, 125.58, 128.52, 130.34, 133.56, 136.89, 137.79, 142.83, 151.02, 171.71, 172.47. Mass (*m/z*) ESI TOF: 366.7970 $(M+H)^+$. Anal. calcd. (found) % for $C_{16}H_{18}N_2O_6S$: C, 52.45 (52.72); H, 4.95 (5.24); N, 7.65 (7.28).

Synthesis of diethyl-2-(quinoline-8-sulfonamido)-pentanedioate (2d): The procedure of **2c** was repeated to get compound **2d**. The product was purified by flash chromatography using ethyl acetate and benzene in 3:2 ratio. Yield 88.14 %, m.p.: 45-47 ºC. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.049 (t, *J* = 6 Hz, 3H), 1.148 (t, *J* = 6.3 Hz, 3H), 1.746 (m, 1H), 1.929 (m, 1H), 2.347 (m, 2H), 3.467 (s, 2H), 3.539 (s, 2H), 4.069 (m, 1H), 7.645 (m, 1H), 7.878 (d, *J* = 7.8 Hz, 1H), 8.063 (t, *J* = 7.8 Hz, 1H), 8.526 (d, *J* = 8.1 Hz), 8.669 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 14.13, 14.58, 27.19, 29.19, 51.27, 51.55, 55.54, 122.13, 125.28, 128.55, 130.28, 133.50, 136.88, 137.82, 142.75, 151.48, 171.65, 172.54. Mass (*m/z*) ESI TOF: 395.10 $(M+H)^+$. Anal. calcd. (found) % for $C_{18}H_{22}N_2O_6S$: C, 54.81 (54.39); H, 5.62 (5.84); N, 7.10 (6.97).

Synthesis of 2-(quinoline-8-sulfonamido)pentanedihydrazide (2e): Compound **2c** (2 g, 5.46 mmol) was dissolved in 15 mL of ethanol; to it 80% hydrazine hydrate (1.43 mL, 1.39 mol) was added in portion and refluxed gently for 3 h and distilled to dryness under vacuum to get white solid which was purified by flash chromatography using acetone, ethyl acetate and benzene in a ratio of 5:3:2. Yield: 81.12 %, m.p.: 174-176 ºC; ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm: 1.709 (br, 2H), 1.952 (m, 2H), 3.945 (m, 1H), 4.353 (br, 4H), 7.434 (br, 1H), 7.685-7.761 (o, 2H), 8.277 (d, *J* = 7.5 Hz, 2H), 8.528 (d, *J* = 8.1 Hz, 1H), 8.922 (S, 1H), 8.999 (s, 1H), 9.06 (d, *J* = 2.4, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 29.44, 29.65, 55.27, 122.63, 125.69, 128.55, 130.24, 133.82, 136.84, 137.11, 142.69, 151.38, 169.39, 170.94. Mass (*m/z*) ESI TOF: 367.02 $(M + H)^+$. Anal. calcd. (found) % for $C_{14}H_{18}N_6O_4S$: C, 45.89 (45.62); H, 4.95 (5.24); N, 22.94 (22.55).

Synthesis of 2f, 2g, 2h, 2i, 2j, 2k and 2l: Compounds **2f**, **2g**, **2h**, **2i**, **2j**, **2k** and **2l** were synthesized by stirring of 5.46 mmol of compound **2c** with ammonia & aliphatic amines for definite hours *viz.*, 24, 24, 72, 96, 96, 96 and 96, respectively at room temperature. The excess amine was distilled off and washed with dil. HCl (**Scheme-I**). The product was purified by flash chromatography using different solvent systems with definite ratio.



Designed & synthesized compounds
2c: R = -CH_3; 2d: R = C_2H_5; 2e: R' = -$NHNH_2$; 2f: R = -NH_2; 2g: R = -$NHCH_3$; 2h: R = NHC_3H_7 (iso); 2i: R = -NHC_4H_9; 2j: R = -NHC_5H_{11}; 2k: R = -NHC_6H_{13}; 2l: R = -NHC_6H_{13} (cyclo)

Scheme-I: Synthetic route of compounds **2b-l**. Reagents and conditions: (i) 4-dimethylaminopyridine (DMAP), Na_2CO_3, 3.5 h stirring, Room temperature (RT); (ii) R-OH (Super dry) saturated with HCl; reflux for 4 h; (iii) R-NH_2, stirred for definite hours; (iv) 80% NH_2-NH_2.H_2O, Reflux in ethyl alcohol for 3 h

2-(Quinoline-8-sulfonamido)pentanediamide (2f): The product was purified by flash chromatography using acetone, ethyl acetate and benzene in a ratio of 5:3:2. Yield 92.44%, m.p.: 182-186 ºC (melts with decomposition). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.744 (m, 2H), 2.0465 (m, 2H), 3.875 (m, 1H), 6.7 (s, 1H), 6.869 (s, 1H), 7.218 (s 1H), 7.262 (s, 1H), 7.341 (d, *J* = 6.8 Hz, 1H), 7.687-7.758 (m, 2H), 8.285 (m, 1H), 8.529 (dd, *J* = 8.4 Hz, 1.6 Hz, 1H), 9.059 (dd, *J* = 4.4 Hz, 1.6 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 27.77, 30.11, 55.48, 122.75, 125.69, 128.72, 130.42, 133.66, 137.11, 138.01, 143.13, 150.95, 172.09, 173.31. Mass (*m/z*) ESI TOF: 292.09 ($C_{13}H_{14}N_3O_3S$), 337.08 $(M+H)^+$. Anal. calcd. (found) % for $C_{14}H_{16}N_4O_4S$: C, 49.99 (49.68); H, 4.79 (4.97); N, 16.66 (16.13).

***N*^{1},*N*^{5}-Dimethyl-2-(quinoline-8-sulfonamido)pentanediamide (2g):** Compound **2g** was purified by flash chromatography using ethyl acetate and benzene in a ratio of 9:1. Yield: 89.55%, m.p.: 120-122 ºC; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 1.755 (m, 2H), 2.053 (m, 2H), 3.113 (s, 3H), 3.194 (s, 3H), 4.032 (dd, *J* = 12 Hz, 6.4 Hz, 1H), 6.849 (s, 1H), 7.265 (s, 1H), 7.373 (d, *J* = 9.2 Hz, 1H), 7.685-7.759 (m, 2H), 8.279 (m, 2H), 8.531 (dd, *J* = 8.4 Hz, 1.6 Hz, 1H), 9.068 (dd, *J* = 4.4 Hz, 1.6 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 26.65, 30.11, 56.04, 122.19, 126.23, 128.72, 130.43, 133.75, 137.21, 138.09, 142.99, 151.05, 171.75, 173.29. Mass (*m/z*) ESI TOF: 365.11 $(M+H)^+$. Anal. calcd. (found) % for $C_{16}H_{20}N_4O_4S$: C, 52.73 (52.46); H, 5.53 (5.89); N, 15.37 (15.11).

***N*^{1},*N*^{5}-Diisopropyl-2-(quinoline-8-sulfonamido)pentanediamide (2h):** Compound **2h** was purified by flash chromatography using ethyl acetate and benzene in a ratio of 8:2. Yield: 65%, m.p.: 184-186 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.492 (d, *J* = 6.2 Hz, 3H), 0.657 (d, *J* = 6.2 Hz, 3H), 1.005 (d, *J* = 6.3 Hz, 6H), 1.704 (m, 2H), 2.038 (m, 2H), 3.229 (m, 1H), 3.727-3.820 (o, 2H), 7.292 (d, *J* = 8.1 Hz, 1H), 7.477 (d, *J* = 7.2 Hz), 7.66-7.754 (o, 3H), 8.27 (d, *J* = 7.8 Hz, 2H), 8.52 (d, *J* = 8.1 Hz, 1H), 9.056 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm: 21.59, 21.65, 22.36, 29.80, 31.45, 44.55, 46.10, 56.50, 122.45, 125.52, 128.47, 130.14, 133.60, 136.53, 142.63, 151.18, 168.65, 170.11. Mass (*m/z*) ESI TOF: 421.1 $(M+H)^+$. Anal. calcd. (found) % for $C_{20}H_{28}N_4O_4S$: C, 57.12 (57.46); H, 6.71 (7.02); N, 13.32 (13.48).

***N*^{1},*N*^{5}-Dibutyl-2-(quinoline-8-sulfonamido)pentanediamide (2i):** Compound **2i** was purified by flash chromatography using ethyl acetate and benzene in a ratio of 2:3. Yield: 87.77%, m.p.: 172-174 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.839 (o, 6H), 1.154-1.352 (m, 4H), 1.656 (m, 2H), 1.998 (m, 2H), 2.805 (m, 2H), 2.983 (m, 2H), 4.078 (m, 1H), 7.013 (d, *J* = 8.1 Hz, 1H), 7.318 (d, *J* = 4.2 Hz, 1H), 7.635-7.719 (o, 3H), 8.27 (o, 2H), 8.528 (d, *J* = 8.1 Hz, 1H), 9.037 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 13.69, 13.77, 19.56, 19.66, 29.27, 30.69, 31.00, 31.40, 56.26, 122.54, 125.61, 128.60, 130.31, 133.75, 136.56, 136.99, 142.72, 151.27, 169.64, 171.14. Mass (*m/z*) ESI TOF: 449.07 $(M+H)^+$. Anal. calcd. (found) % for $C_{22}H_{32}N_4O_4S$: C, 58.91 (58.96); H, 7.19 (7.28); N, 12.49 (12.62).

***N*^{1},N^5-Dipentyl-2-(quinoline-8-sulfonamido)pentanediamide (2j):** Compound **2j** was purified by flash chromatography using ethyl acetate and benzene in a ratio of 1:9. Yield: 82.82%, m.p.: 152-156 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.834 (t, *J* = 7.6 Hz, 3H), 0.899 (t, *J* = 7.2 Hz, 3H), 1.081-1.485 (o, 12H), 1.886 (m, 2H), 2.269 (m, 2H), 2.924 (m, 2H), 3.177 (m, 2H), 3.852 (m, 1H), 5.8 (s, 1H), 6.59 (s, 1H), 7.229 (s, 1H), 7.567 (m, 1H), 7.619 (m, 1H), 8.055 (dd, *J* = 8.2 Hz, 1.2 Hz, 1H), 8.3625 (dd, *J* = 7.2 Hz, 1.2 Hz, 1H), 9.0957 (dd, *J* = 4.2 Hz, 1.6 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 14.10, 14.21, 22.08, 22.34, 28.32, 28.53, 29.34, 29.62, 30.09, 31.52, 40.51, 40.95, 55.43, 122.85, 125.10, 128.52, 130.93, 133.62, 136.15, 137.01, 143.63, 151.18, 170.45, 171.61. Mass (*m/z*) ESI TOF: 477.27 $(M + H)^+$. Anal. calcd. (found) % for $C_{24}H_{36}N_4O_4S$: C, 60.48 (60.19); H, 7.61 (7.86); N, 11.75 (11.39).

***N*^{1},*N*^{5}-Dihexyl-2-(quinoline-8-sulfonamido)pentanediamide (2k):** The product was purified by flash chromatography using ethyl acetate and benzene in a ratio of 1:9. Yield: 73.2%, m.p.: 102-105 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.858 (m, 6H), 1.087 -1.449 (o, 16H), 1.888 (m, 2H), 2.273 (m, 2H), 2.897 (m, 2H), 3.163 (m, 2H), 3.888 (m, 1H), 6.023 (s, 1H), 6.704 (s, 1H), 7.276 (s, 1H), 7.546-7.633 (o, 2H), 8.05 (d, *J* = 8 Hz, 1H), 8.257 (d, *J* = 8 Hz, 1H), 8.36 (d, *J* = 7.2 Hz, 1H), 9.094 (d, *J* = 3.2 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ ppm: 14.33, 14.47, 21.51, 21.66, 26.13, 26.41, 29.31, 29.97, 30.09, 30.31, 31.05, 31.41, 41.26, 41.62, 56.52, 122.16, 125.27, 128.22, 130.03, 133.28, 136.19, 136.75, 142.65, 150.82, 170.54, 172.17. Mass (*m/z*) ESI TOF: 505.27 $(M+H)^+$. Anal. calcd. (found) % for $C_{26}H_{40}N_4O_4S$: C, 61.88 (61.63); H, 7.99 (7.55); N, 11.10 (10.87).

***N*^{1},*N*^{5}-Dicyclohexyl-2-(quinoline-8-sulfonamido)pentanediamide (2l):** The product was purified by flash chromatography using ethyl acetate and benzene in a ratio of 1:9. Yield: 71.5%, m.p.: 204-206 ºC. ^{1}H NMR (600 MHz, $CDCl_3$) δ ppm: 0.795-1.905 (o, 20H), 2.237 (m, 2H), 3.410 (m, 1H), 3.672 (m, 1H), 3.806 (m, 1H), 5.814 (s, 1H), 6.413 (d, *J* = 7.8 Hz, 1H), 7.571 (m, 1H), 7.618 (m, 1H), 8.056 (d, *J* = 7.8 Hz, 1H), 8.263 (d, *J* = 7.8 Hz, 1H), 8.362 (d, *J* = 7.2 Hz, 1H), 9.096 (m, 1H). ^{13}C NMR δ ppm: 24.58, 24.83, 25.32, 25.44, 29.95, 32.31, 32.40, 32.55, 32.95, 48.08, 48.29, 56.56, 76.79, 77.00, 77.21, 122.45, 125.38, 128.79, 130.85, 133.51, 135.88, 136.71, 143.18, 151.42, 169.25, 171.14**.** Mass (*m/z*) ESI TOF: 501.14 $(M+H)^+$. Anal. calcd. (found) % for $C_{26}H_{36}N_4O_4S$: C, 62.37 (62.59); H, 7.25 (7.40); N, 11.19 (11.42).

Cell based inhibition assays: Cytotoxicity assay of the synthesized compounds was performed on HUVEC, RPMI 8226 and normal Vero cell line. The study was intended to explore the potent anticancer compounds and their effect on normal endothelial cells and also to have an idea about their primary antiangiogenic features. The results of cytotoxicity study are furnished in Table-1.

Cytotoxicity study on HUVECs was carried out by MTT assay method: HUVEC cells were cultured in endothelial growth medium-2 and the cells between 2-5 passages were used in the present study. Subsequently, 10^4 cells were placed on each well of the gelatin-coated 96-well cell culture plates in fresh DMEM with 10% FBS, penicillin and streptomycin. The culture was grown lower than 70-80% confluency. Following a range of concentrations of test compounds and standard drug were placed in DMEM in serial dilution (100 µL/well) in triplicate, the plates were incubated for 24 h in

TABLE-1
IC_{50} VALUES OF SYNTHESIZED COMPOUNDS AGAINST RPMI 8226, HUVEC AND VERO CELL LINES

Compd. No.	IC_{50} (μM)[a] RPMI8226	IC_{50} (μM)[a] HUVEC	IC_{50} (μM)[a] VERO
2b	49 ± 1.63	45 ± 1.5	75.5 ± 1.26
2c	0.8 ± 0.56	1.5 ± 0.46	55.7 ± 1.4
2d	61.42 ± 1.25	15.5 ± 1	70 ± 1.19
2e	75.5 ± 1.6	70 ± 1.5	90.5 ± 1.3
2f	60 ± 1.35	85.3 ± 0.5	> 100
2g	1.2 ± 0.51	3.5 ± 1.1	50.5 ± 1.7
2h	> 100	95 ± 2	> 100
2i	85.4 ± 1.41	65 ± 1.32	70.4 ± 1.58
2j	> 100	85.6 ± 1.7	85 ± 1.6
2k	1.7 ± 0.67	4.2 ±0.69	66.4 ± 1.3
2l	> 100	80.46 ± 0.98	> 100
Doxorubicin	0.6	0.7	4.6
Thalidomide	> 100	> 100	Not done

[a]Results are the average of three separate experiments and represented as ± SD.

5% CO_2 and 95% air atmosphere at 37 ºC. After incubation, 50 μL of fresh medium containing 1 mg/mL of MTT was added in each well. The plates were incubated for a further 4 h in dark at 37 ºC. The resulting water insoluble MTT- formazan crystals were dissolved in DMSO, Sorensen's glycine buffer solution was added to adjust the pH (7.0-7.4) and the absorbance was recorded in an ELISA plate reader at 540 nm [25,26]. Doxorubicin was taken as standard.

Cytotoxicity analysis on RPMI 8226 (MM): RPMI 8226 cell line was cultured in RPMI 1640 base medium containing 10% FBS with 95% air and 5% CO_2 atmosphere at 37 ºC. RPMI 8226 cells were placed on 96-well cell culture plates in fresh growth medium and grown to 70-80% confluency. Following a range of concentrations of test compounds and standard drug were placed in RPMI 1640 base medium in serial dilution (100 μL/well) in triplicate, the culture plate was incubated for 94 h. At the end of the drug exposure period, the plate was centrifuged (5 min at 200 g) to pellet the cells. Then, the medium was removed by using a fine-gauge needle to prevent disturbance of the cell pellet and the cells were fed with 100 μL of fresh medium. To each well 40 μL of MTS/PMS solution was added. The plate was incubated for 4 h at 37 °C in a humidified, 5% CO_2 atmosphere. To measure the amount of soluble formazan produced by reduction of MTS, the absorbance was recorded immediately at 490 nm using ELISA plate reader [27]. Doxorubicin was taken as standard.

Cytotoxicity analysis of compounds against Vero cell line by MTT assay method: VERO cell lines are normal, adherent, primary kidney cells from adult African green monkey (Vervet monkey) *Chlorocebus aethiops*. The protocols are same as in HUVEC. The absorbance was recorded immediately in an ELISA plate reader at 570 nm. Doxorubicin was taken as standard.

Proliferation assay: Proliferation assay of HUVECs were carried out by trypan blue method [28]. HUVECs were subcultured as described before. HUVECs were serum-starved overnight and seeded with 1×10^4 cells/well in a 96 well plate in DMEM medium supplemented with 20 ng/mL of VEGF (mitogen). Cells were treated with test samples with five different concentrations (0.001, 0.01, 0.1, 1 and 10 μM) of the active compounds **2c, 2g** and **2k** in triplicate. Staurosporine was taken as standard. After 24 h of incubation, the cells were trypsinized and the viable cells were counted in a hemocytometer following a dye exclusion method using trypan blue. Results of proliferation assay are represented in Fig. 2.

Statistical analysis: Raw data were analyzed by one-way ANOVA method using STATISTICA [29]. Differences were considered statistically significant when $p < 0.05$.

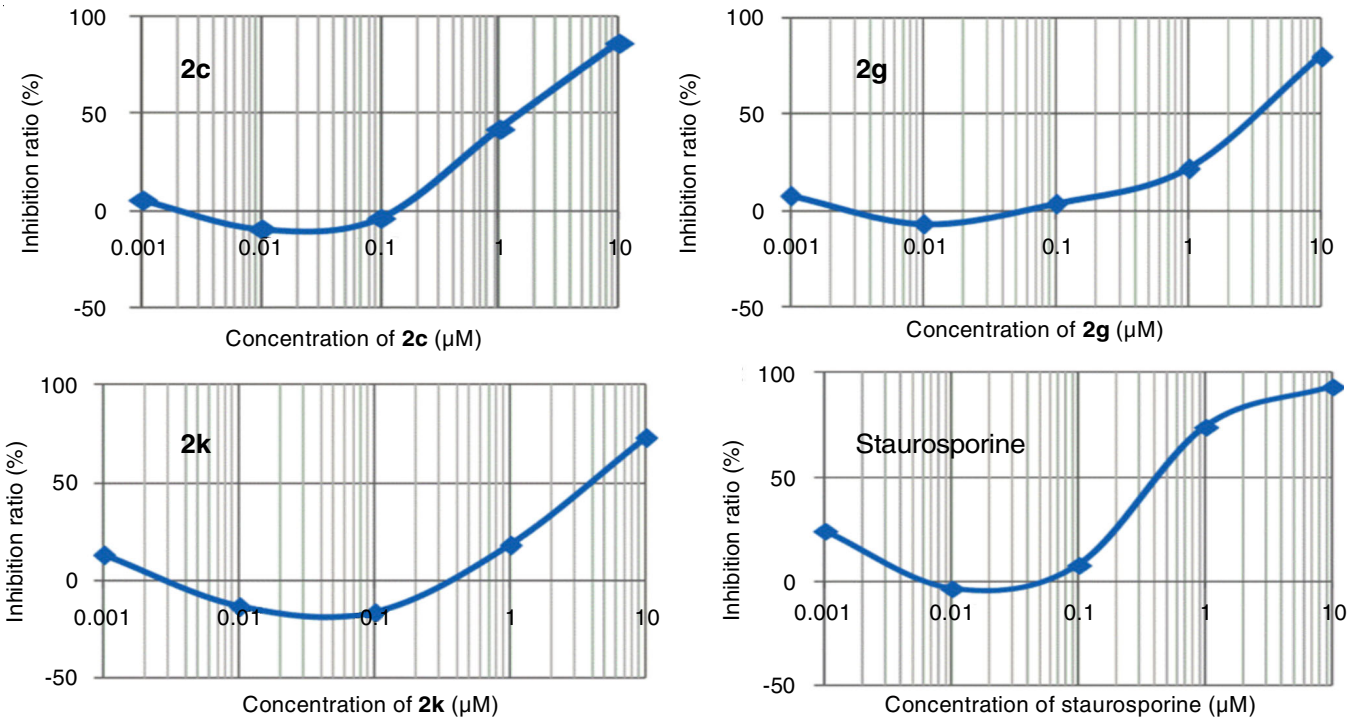


Fig. 2. Proliferation assay of compounds **2c**, **2g**, **2k** and staurosporine as a standard on HUVECs

VEGFR-2 Tyr-1175 Phosphorylation inhibitions SDS PAGE assay: HUVEC grown in 12 well plates was forced to starve overnight with the lack of serum. The next day, HUVEC were treated with test samples for 2 h. After 2 h, 50 ng/mL of VEGF was applied as long as 15 min. Cold PBS was used twice to wash the cells. The cells were lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS) and added with cOmplete™ protease inhibitors (Roche) [30]. α-Mercaptoethanol (5%) was added to the samples with equal protein quantity and heated at 95 ºC for 12 min. Size fractionation was carried out on a 9% SDS-PAGE gel and transferred to PVDF membranes. Membranes were clogged with PBS-T-milk for approximately 45 min. PBS-T-milk is composed of 0.05% of Tween and 5% of dried de-fatted milk. Cells were incubated with primary antibody for 3 h at room temperature and diluted with PBS-T milk. After washing with PBS-T, the cells were incubated with secondary antibodies for 1 h at room temperature and diluted in PBS-T milk. Anti Phospho-VEGF Receptor 2 (Tyr1175) (Santa Cruz sc-101819) was used at a dilution of 1:5000. Anti-TATA binding protein TBP antibody (Abcam 1TBP18) was used as the loading control at a dilution of 1:5000. After incubation with HRP conjugated secondary antibody, the signals were revealed using Super Signal West Femto maximum sensitivity substrate (Pierce) (Fig. 3).

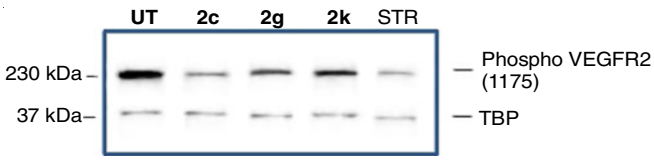


Fig. 3. Expression of VEGFR-2 following treatment with compounds **2c**, **2g**, **2k** and STR, staurosporine as standard for 2 h at the concentration 10 μM. UT as untreated

Molecular docking: Molecular docking study was initiated with the most active compound **2c** in order to explore the probable interaction of the ligand with amino acid residues at the ATP binding site of VEGFR-2 and to find the preferred conformation of the ligand (Fig. 4). 3D-Crystal structure of VEGFR-2 (3VHE.pdb) complexed with an inhibitor was imported from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), www.rcsb.org to CDOCKER protocol of DS 4.1 Accelrys [31]. The bound inhibitor and water molecules were removed from the protein. The protocol was followed as it is in CDOCKER protocol. 2D-structure of compound **2c** (ligand) was drawn in Chem 3D ultra 12.0 software [32] and was imported to Dock Ligands tools. CHARMm, a force field, is employed in CDOCKER, a grid-based method for molecular docking [33]. During refinement, the ligands are considered as flexible and the receptor as rigid body. High-temperature MD (molecular dynamics) generated random conformation of the ligands from the initial structure.

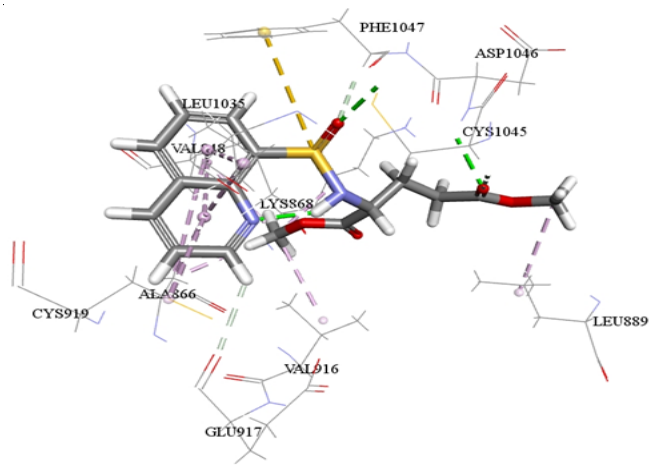


Fig. 4. Predicted binding mode of **2c** (stick model) at the active site of VEGFR 2 (PDB ID: 3VHE). Hydrogen bonding between the **2c** atoms and the amino acid residues (line representation) is shown as green dotted lines

RESULTS AND DISCUSSION

All the synthesized compounds were confirmed by elemental and spectral analysis. The compounds were evaluated against three cell lines and the IC_{50} values (Table-1) of compounds with respective to the positive control doxorubicin is reported. Dimethyl 2-(quinoline-8-sulfonamido)pentanedioate (**2c**) is the most active molecule with IC_{50} = 0.8 μM against RPMI 8226 and IC_{50} = 1.5 μM against HUVEC. Compounds **(2g)**, N^1,N^5-dimethyl-2-(quinoline-8-sulfonamido)pentanediamide (**2k**) and N^1,N^5-dihexyl-2-(quinoline-8-sulfonamido)-pentanediamide, are also promising inhibitors of both RPMI 8226 and HUVEC with IC_{50} = 1.2 μM and IC_{50} = 3.5 μM and IC_{50} = 1.7 μM and IC_{50} = 4.2 μM, respectively. VEGFR-2 Tyr-1175 phosphorylation inhibition assay of the three compounds showed promising results with compounds **2c** and **2g**, which confirmed the mechanism of angiogenesis. The bandwidth of compound **2k** suggests that it cannot inhibit the phosphorylation at Tyr-1175 of VEGFR-2 (Fig. 4). The mode of action of compound **2k** as antiangiogenic may be different.

All three compounds were poor inhibitors of VERO cells; hence, they can be considered nontoxic for normal epithelial cells. The IC_{50} value of Thalidomide was determined on HUVEC and RPMI 8226 but did not show a significant result. Compounds **2c**, **2g** and **2k** have reduced HUVEC proliferation significantly by 86.66%, 80% and 73.33%, respectively at the highest concentration (10 μM). Compound **2c** has significantly reduced HUVEC proliferation at 1 μM concentration by 42.13% compared with the vehicle control (DMSO). The proliferation assay result exhibits the antiproliferative action of the test compounds. Endothelial cell proliferation is one of the critical steps in angiogenesis. Therefore, the ability of compounds **2c**, **2g** and **2k** to inhibit the proliferation of endothelial cells was examined. Compounds **2c**, **2g** and **2k** have induced a marked inhibition of cell proliferation in a dose dependant manner. The IC_{50} values of compounds **2c**, **2g** and **2k** were 1.6 ± 0.09, 3.4 ± 0.13 and 3.9 ± 0.1, respectively, in the HUVEC cells after 24 h.

Docking: The 3D binding conformation of compound **2c** was generated with calculated CDOCKER energy as -43.738 kcal/mol and binding energy as -161.41 kcal/mol. Compound **2c** was found to form two intermolecular conventional hydrogen bonds with the amino acid residues and one intramolecular conventional hydrogen bond. One of the oxygen atoms of the

sulfonyl moiety (acceptor) interacts with -NH- (donor) of Cys1045 (2.30 Å). The furthest carbonyl oxygen of carboxamide interacts with -NH- of Asp1046 (2.24 Å) and forms the second H-bond; this region extends as the hydrophobic back pocket of the receptor. The hydrogen atom of -NH-(donor) of sulphonamide makes an intramolecular H-bond with the ring nitrogen atom of quinoline (acceptor). It forms a hydrogen bond with a bond distance of 2.02 Å. Three non-conventional hydrogen bonds are also seen, *i.e.* with Cys1045, Phe1047 and Glu917. This region is a deeply buried hydrophobic pocket created by the movement of PHE1047 residue of the "DFG" motif, which induces the "DFG-out" conformation of the receptor. One π-sulfur interaction of the sulfur atom of sulfonyl moiety is observed with Phe1047 (4.37 Å) residue. Other crucial amino acid residues interact with compound **2c**, forming π-alkyl hydrophobic interaction with the quinoline ring of compound **2c** and with the other atoms of **2c** as alkyl-alkyl interaction. These residues are Ala866 (3.8 Å, 5.25 Å, 3.79 Å), Leu889 (4.05 Å), Val 848 (2.85 Å), Lys868 (4.20 Å), Val916 (3.86 Å), Val848 (4.1 Å, 5.24 Å), Leu1035 (4.42 Å, 4.04 Å), Cys919 (4.9 Å) (Fig. 4).

Three compounds *viz.* **2c**, **2g** and **2k** were discovered as anticancer agents against multiple myeloma by *in vitro* cell culture technique. Compound **2c** showed a low micromolar inhibitory effect on the *in vitro* RPMI8226 study and had primary antiangiogenic property as revealed from the HUVEC data. Compound **2c** is non-toxic towards Vero cells; hence it can be considered a selective inhibitor of cancer cells. Docking results gave an insight into the binding mode of Compound **2c** and probable interaction with the active site's residues. Compound **2c** shares space in the receptor, which is generally occupied by the type-II VEGFR-2 inhibitors. Band intensity of Compound **2k** suggests that its anticancer activity is not due to inhibition of VEGFR-2, preferably through some other pathways.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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