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Design, Synthesis, Molecular Docking and *in vitro* Evaluation of *N*-(4-Ethoxyphenylsulfonyl)pyrrolidine-2-carboxylic Acid Analogues as Antiangiogenic and Anticancer Agents on Multiple Myeloma

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In present work, *N*-(4-ethoxyphenylsulfonyl)pyrrolidine-2-carboxylic acid analogs were designed, synthesized and biologically evaluated as an antiangiogenic and anticancer agent on multiple myeloma. Compounds **3i**, **3k** and **3m** exhibited the cytotoxic action on human multiple myeloma cell line RPMI8226 with IC_{50} (μM) value 3.72, 3.89 2.28, respectively. These compounds possessed the antiangiogenic property and are selectively cytotoxic to cancer cells, as observed from the *in vitro* study of human umbilical vein endothelial cell (HUVEC) and African green monkey epithelial cell (VERO), respectively. Antiproliferative assay of the compounds on HUVECs was carried out using the dye exclusion method with trypan blue. Molecular docking study of compound **3m** with vascular endothelial growth factor receptor-2 (VEGFR-2) showed possible interaction with a binding energy -62.27 kcal/mol.

Keywords: Anti-angiogenesis, VEGFR-2, Multiple myeloma, Vero cell, *N*-(4-Ethoxyphenylsulfonyl)pyrrolidine-2-carboxylic acid.

INTRODUCTION

Multiple myeloma, infrequently referred to as Kahler's disease, is a neoplasm of the plasma cell that originates in the bone marrow and develops a tumor in many bones of the body [1,2]. Plasma B cells, derived from the bone marrow, act as the body's defense by producing antibodies when invaded with antigens [3,4]. In multiple myeloma, bone marrow produces abnormal plasma cells, which rapidly proliferates and secretes abnormal antibodies rather than the normal antibodies [5]. Myeloma cells resist their destruction, overproduce these abnormal proteins, thicken the blood, damage the kidney and produce bone and soft tissues' tumors [6]. The disease is termed as plasmacytoma when there is only one tumor and with multiple tumors, it is called multiple myeloma [4]. Plasma cells derived from the bone marrow of multiple myeloma patients were found to be highly angiogenic [7]. Tumor angiogenesis is a survival strategy opted by the tumor cells to proliferation through a network of blood vessels that continuously supply the oxygen and nutrient-rich supportive microenvironment to sustain optimal growth [8]. Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are crucial angiogenic factors responsible for multiple myeloma associated angiogenesis [9]. HGF/cMET enhances the expression of VEGF/VEGFR-2 in multiple myeloma endothelial cells [10,11]. VEGFR-2 (KDR/Flk-1) primarily responds to the VEGF signal and regulates endothelial migration and proliferation [12]. Inhibition of angiogenesis in multiple myeloma is a crucial step towards the development of new anticancer agents. There are numerous regimens to treat multiple myeloma in patients following risk stratification.

The significant classes include monoclonal antibodies (MAB's) like daratumumab and isatuximab that targets CD38 receptor; elotuzumab, targeting the SLAMF7 antigen and are instrumental in the of multiple myeloma; alkylating agents (melphalan, cyclophosphamide) corticosteroids (dexamethasone, prednisone) drugs (thalidomide, lenalidomide, pomalidomide), proteasome inhibitors (bortezomib, carfilzomib, ixazomib), histone deacetylase inhibitor (HDAC) panobinostat and selinexor, an inhibitor of exportin-1 (XPO1) [13-15]. Some multidrug treatment regimens for aggressive or refractory multiple myeloma include doxorubicin and liposomal doxorubicin along with the drugs as mentioned earlier [16]. Most of the drugs or MAB's evokes severe adverse effects like toxicity to normal cells, drug

resistance and even anaphylactic shocks. The modern-day approach is to discover cancer cell-specific drugs or drug delivery systems.

As described [17] about the design, synthesis and anti-cancer evaluation of a series of conformationally restricted glutamic acid derivatives, *i.e.*, *N*-(4-methoxyphenylsulfonyl)-pyrrolidine-2-carboxylic acid analogs, the design was guided by the bioisosteric modification of a major metabolite of thalidomide; *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid (Fig. 1). The compounds showed selective cytotoxicity to the multiple myeloma cell line (RPMI8226) and had primary antiangiogenic action on the HUVEC cell line. The outcome of the study led us to design a new series of compounds following ligand-receptor interactions.

In continuation to our work [17], herein, a design is extended where *N*-(4-methoxyphenylsulfonyl)pyrrolidine-2-carboxylic acid analogs were designed as bioisosteres of the major metabolite of thalidomide. The active compounds' primary antiangiogenic property imparts an idea to dock the active molecules with the VEGFR-2 receptor. The ligand-receptor interaction studies infer that the molecules could be VEGFR-2 type-II inhibitors as they occupy the same allosteric site and extends to the ATP binding site of the VEGFR-2 hinge region. The methoxy group, which was found to interact with CYS919 residue in the 'DFG' hydrophobic motif of the active site, was extended by one methylene (-CH_2-) group (ethoxy) to enhance the lipophilicity of the molecule to optimize its interaction at the hydrophobic pocket (Fig. 2A).

EXPERIMENTAL

All the chemicals were purchased from Himedia (India), Merck (India) and Loba Chemie (India) and used without further purification unless stated. Reaction steps were monitored by analytical thin-layer chromatography (TLC). Melting points were determined in open capillary tubes and are uncorrected. ^{1}H & ^{13}C NMR spectra were recorded on JEOL 400 MHz spectrometer. Elemental analysis of the synthesized compounds was carried out on Carlo-Erba 1108 analyzer. Mass spectral analysis was carried out with Shimadzu LC-MS, LC-2010EV Mass spectrometer in ESI probes. Compounds were purified by Grace Reveleris flash column chromatography by using silica gel cartridge (230-400 mesh, 40 µm silica). The 2D-structure of the compound was drawn in Chem 3D ultra 12.0 [18].

Synthesis of 4-ethoxybenzene-1-sulfonyl chloride (2a): Ethoxybenzene (6.25 mL, 50 mmol) was dissolved in 50 mL of anhydrous chloroform and cooled to 0 ºC; chlorosulphonic acid (8.5 mL, 125 mmol) was added dropwise, with stirring,

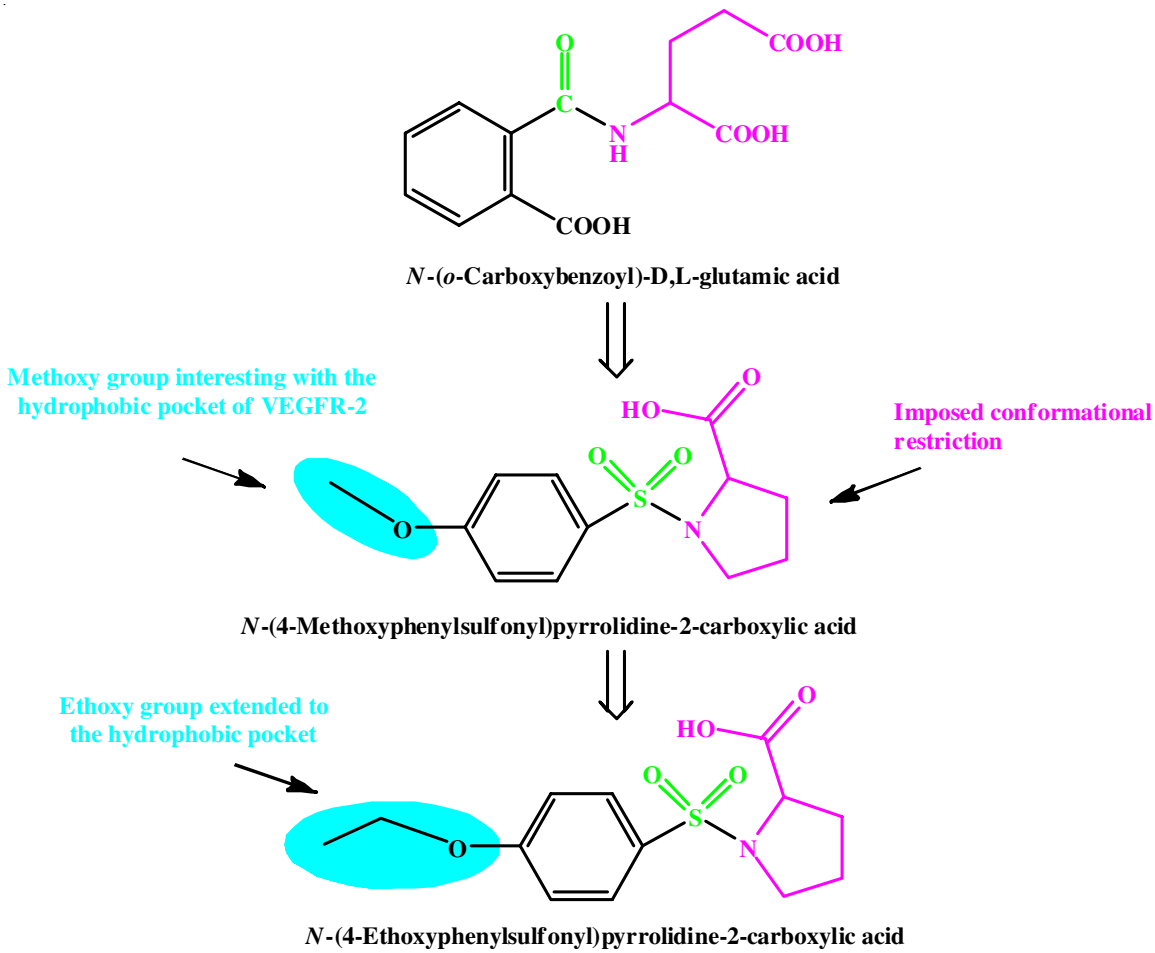


Fig. 1. Colour coded structures of the major metabolite of thalidomide and the designed series. Ethoxy substitution at the para position of the phenyl ring in N-(4-Ethoxyphenylsulfonyl) pyrrolidine-2-carboxylic acid analogs enhances the binding of the ligands at the active site of VEGFR-2

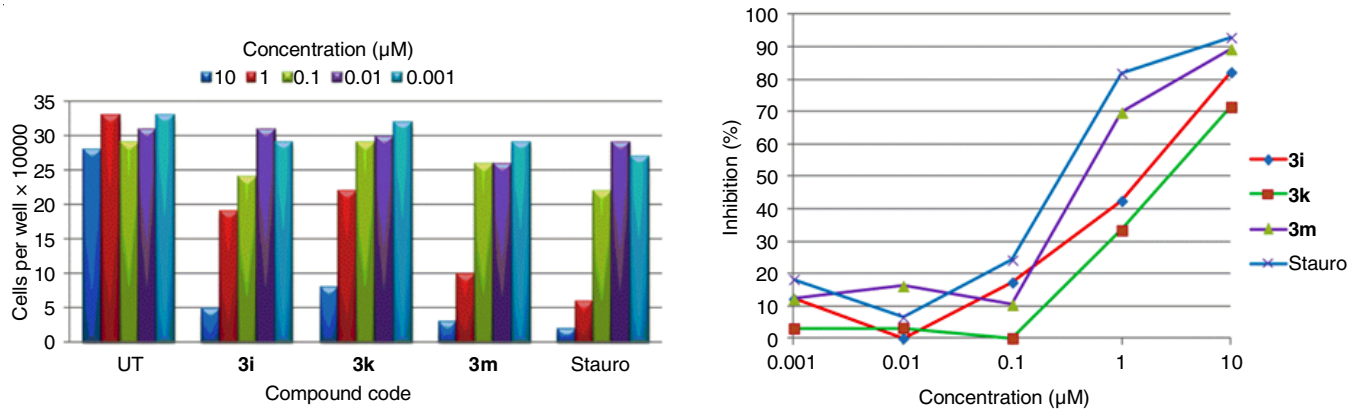
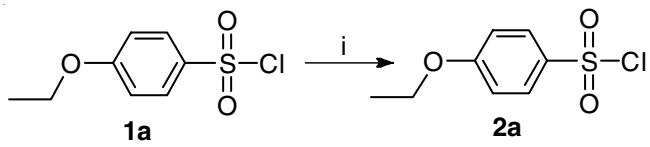


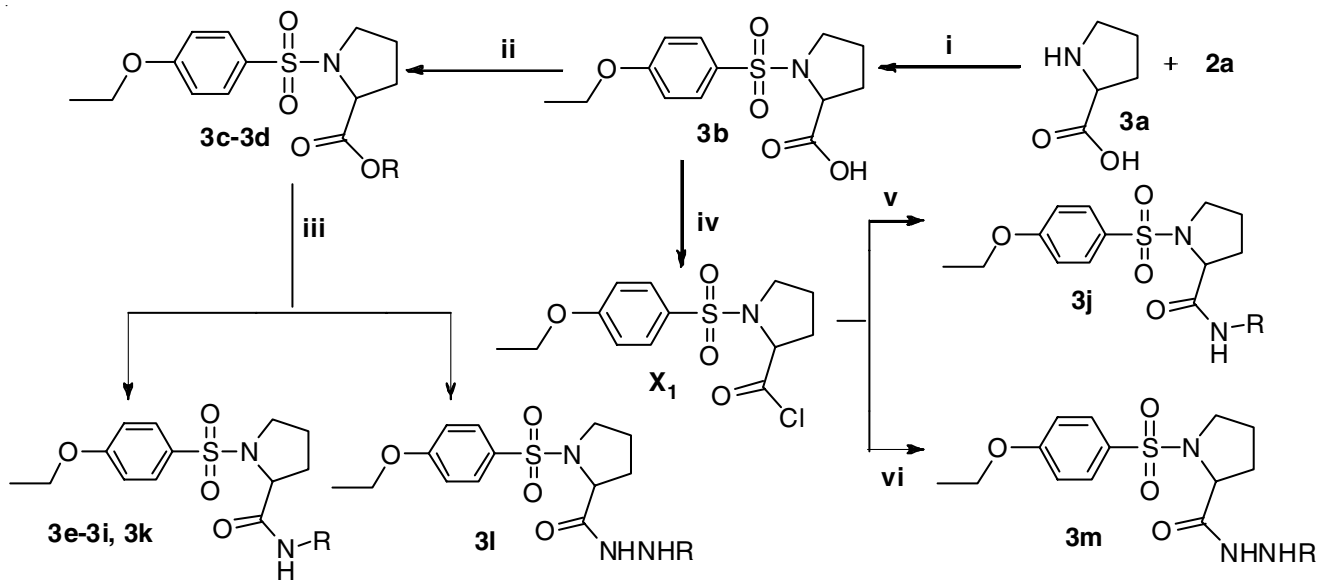
Fig. 2. Antiproliferative assay of test compounds on HUVECs. UT: Untreated; Sample codes: **3i, 3K** and **3m**; std: standard, staurosporine

within 30 min. After complete addition, the reaction mixture was stirred for another 45 min at room temperature. The excess chlorosulphonic acid was neutralized using 100 g of crushed ice. It was extracted with chloroform (3 × 25 mL) and dried over anhydrous sodium sulphate. The chloroform layer was distilled under vacuum to afford compound **2a** as a light brown coloured liquid with a 68% yield (**Scheme-I**).



Scheme-I: Synthetic route of intermediate **2a**. Reagents and conditions: (i) $ClSO_3H$, $CHCl_3$, Stirred at 0-5 °C for 0.5 h

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)pyrrolidine-2-carboxylic acid (3b): To a stirred aqueous solution of sodium carbonate (8 g, 85 mmol) and L-proline (**3a**) (5.75 g, 50 mmol), 4-ethoxy benzene-1-sulfonyl chloride (**2a**) (11 g, 50 mmol) was added dropwise within 30 min. After complete addition, the reaction mixture was stirred at room temperature for 24 h. The reaction was carried out at pH 9-10 by occasional addition of sodium carbonate. The solution was filtered and acidified to pH 2-3 by addition of conc. HCl. After cooling, the compound was separated, filtered and dried under vacuum. It was purified by flash chromatography using ethyl acetate and benzene in a ratio of 9:1 to afford compound **3b** as a white solid (**Scheme-II**) with a 79% yield; m.p.: 88-90 ºC. ^{1}H NMR: (400 MHz, DMSO-d_6): δ = 12.70 (1H, s, -COOH), 7.75 (2H, m, Ar-H), 7.11 (2H, m, Ar-H), 4.12 (2H, q, *J* = 7 Hz, -OCH_2-CH_3), 4.04 (1H, m, -SO_2NCHCH_2-), 3.32 (1H, m, -SO_2NCH-HCH_2-), 3.13 (1H, m, -SO_2NCHHCH_2-), 1.84 (3H, m, -SO_2N-CH_2CH-, SO_2NCHCH_2-), 1.56 (1H, s, -SO_2NCH_2CH-), 1.35 (3H, t, *J* = 7 Hz, -OCH_2CH_3). ^{13}C NMR: (100 MHz, DMSO-d_6): δ 173.3 (C18), 162.0 (C1), 129.4 (C4), 128.9 (C3, C5), 114.9 (C2, C6), 63.8 (C14), 60.5 (C9), 48.5 (C17), 30.5 (C15), 24.3 (C16), 14.5 (C10). MS/ESI *m/z* (pos): 300 (M+H), 322 (M+Na). MS/ESI *m/z* (neg): 298 (M-H). Anal. calcd. (found) % for $C_{13}H_{17}NO_5S$ (*m.w.* 299.34): C, 52.16 (51.89); H, 5.72 (5.78); N, 4.68 (4.82).



Scheme-II: Synthetic route of compounds **3b-3m**. Reagents and conditions: (i) Na_2CO_3, 24 h, RT; (ii) Super dry R-OH saturated with dry hydrogen chloride gas, reflux, 4 h; (iii) R-NH_2 (alkyl amines), 72 h stirring, RT; $NH_2NH_2{\cdot}H_2O$, ethanol, 3 h reflux; (iv) $SOCl_2$, 4 h, RT; (v) CH_2Cl_2, 15% NaOH, *n*-pentylamine; (vi) phenyl hydrazine

Synthesis of ethyl 1-((4-ethoxyphenyl)sulfonyl)pyrrolidine-2-carboxylate (3c): Esterification of compound **3b** was carried out by Fischer-Speier method [19]. Compound **3b** (3 g, 10 mmol) was dissolved in 50 mL of super dried ethanol saturated with dry HCl gas. The product was filtered and purified by flash chromatography using ethyl acetate and petroleum ether in a ratio of 2:1 to get compound **3c** as an off white solid with 66% yield; m.p.: 60-62 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.75 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.11 (5H, m, -OC$\underline{H}_2$CH$_3$, -COOC$\underline{H}_2$CH$_3$, -SO$_2$NC$\underline{H}$CH$_2$-), 3.34 (1H, m, -SO$_2$-NCH$\underline{H}$CH$_2$-), 3.14 (1H, m, -SO$_2$NC$\underline{H}$HCH$_2$-), 1.94 (1H, m, -SO$_2$NCHCH$\underline{H}$), 1.82 (2H, m, -SO$_2$NCH$_2$CH$\underline{H}$-, -SO$_2$NCH-C$\underline{H}$H-), 1.60 (1H, s, -SO$_2$NCH$_2$C$\underline{H}$H-), 1.35 (3H, m, -OCH$_2$-C$\underline{H}_3$), 1.19 (3H, t, *J* = 7.2 Hz, -COOCH$_2$C$\underline{H}_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.6 (C6), 162.01 (C15), 129.4 (C12), 128.7 (C13, C17), 114.9 (C14, C16), 63.7 (C19), 60.70 (C2), 60.2 (C21), 48.4 (C5), 30.4 (C3), 24.2 (C4), 14.4 (C20), 13.9 (C22). MS/ESI *m/z* (pos): 328 (M+H), 350 (M+Na). MS/ESI *m/z* (neg): 326 (M-H). Anal. calcd. (found) % for $C_{15}H_{21}NO_5S$ (*m.w.* 327.4): C, 52.16 (55.28); H, 6.47 (6.33); N, 4.28 (4.37).

Synthesis of methyl 1-((4-ethoxyphenyl)sulfonyl)pyrrolidine-2-carboxylate (3d): It was synthesized according to the general procedure adopted from compound **3c**, by using compound **3b** (3 g, 10 mmol) with 50 mL of super dried methanol saturated with dry HCl gas. The final product was purified by flash chromatography using ethyl acetate and benzene in a ratio of 3:2 to afford compound **3d** as an offwhite solid with 74% yield; m.p.: 48-52 ºC. ^{1}H NMR: (400 MHz, DMSO-d_6): δ 7.75 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.14 (3H, m, -OC$\underline{H}_2$CH$_3$, -SO$_2$NC$\underline{H}$CH$_2$-), 3.65 (3H, s, -COOC$\underline{H}_3$), 3.34 (1H, m, -SO$_2$NCH$\underline{H}$CH$_2$-), 3.14 (1H, m, -SO$_2$NC$\underline{H}$HCH$_2$-), 1.87 (3H, m, -SO$_2$NCH$_2$CH$\underline{H}$-, SO$_2$NCHC$\underline{H}_2$-), 1.59 (1H, s, -SO$_2$N-CH$_2$-C$\underline{H}$H-), 1.35 (3H, t, *J* = 7 Hz, -OCH$_2$C$\underline{H}_3$). ^{13}C NMR: (100 MHz, DMSO-d_6): δ = 172.2 (C6), 162.0 (C15), 129.4 (C12), 128.6 (C13, C17), 114.9 (C14, C16), 63.7 (C19), 60.1 (C2), 52.1 (C5), 48.4 (C21), 30.3 (C3), 24.2 (C4), 14.4 (C20). MS/ESI *m/z* (pos): 314 (M+H), 336 (M+Na). MS/ESI *m/z* (neg): 312 (M-H). Anal. calcd. (found) % for $C_{14}H_{19}NO_5S$ (*m.w.* 313.37): C, 53.66 (53.28); H, 6.11 (5.94); N, 4.47 (4.36).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)pyrrolidine-2-carboxamide (3e): Compound **3e** was synthesized by stirring a mixture of compound **3c** (2 g, 6.1 mmol) and 5 mL of 30% NH_4OH at room temperature for 72 h. The excess amine was evaporated under vacuum to get the product and purified by flash chromatography using ethyl acetate and benzene in a ratio of 3:2 to achieve compound **3e** as a grayish white solid with 73% yield; m.p.: 62-64 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.75 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.11 (5H, m, -OC$\underline{H}_2$CH$_3$, -CONH$_2$, -SO$_2$NC$\underline{H}$CH$_2$-), 3.35 (1H, m, -SO$_2$N-CH$\underline{H}$-), 3.14 (1H, m, -SO$_2$NC$\underline{H}$H-), 1.93 (1H, m, -SO$_2$NCH$_2$-CH$\underline{H}$-), 1.83 (2H, m, -SO$_2$NCHC$\underline{H}_2$-), 1.59 (1H, m, -SO$_2$NCH$_2$-C$\underline{H}$H-), 1.35 (3H, t, *J* = 7.2 Hz, -OCH$_2$C$\underline{H}_3$). ^{13}C NMR: (100 MHz, DMSO-d_6): δ 171.7 (C6), 162.0 (C15), 129.4 (C12), 128.7 (C13, C17), 114.9 (C14, C16), 63.7 (C2), 60.7 (C19), 48.4 (C5), 30.4 (C3), 24.2 (C4), 14.4 (C20). MS/ESI *m/z* (pos): 299 (M+H), 322 (M+Na). MS/ESI *m/z* (neg): 298 (M-H). Anal calcd. (found) % for $C_{13}H_{18}N_2O_4S$ (*m.w.* 298.36): C, 52.33 (52.31); H, 6.08 (6.29); N, 9.39 (9.17).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-*N*-methylpyrrolidine-2-carboxamide (3f): Compound **3f** was synthesized according to the general procedure adopted from compound **3e** with 5 mL of 40% methylamine (w/w) and purified by flash chromatography using acetonitrile and benzene in a ratio of 4:1 to achieve compound **3f** as a white fluffy solid with 56% yield; m.p.: 154-156 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.89 (m, 1H, -CONH-), 7.77 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.13 (2H, q, *J* = 7, -OC$\underline{H}_2$CH$_3$), 3.92 (1H, m, -SO$_2$NC$\underline{H}$-CH$_2$-), 3.41 (1H, m, -SO$_2$NCH$\underline{H}$CH$_2$-), 3.12 (1H, m, -SO$_2$NC$\underline{H}$-HCH$_2$-), 2.61 (m, 3H, -CONHC$\underline{H}_3$), 1.72 (2H, m, SO$_2$NCH-C$\underline{H}_2$-), 1.60 (1H, m, -SO$_2$NCH$_2$CH$\underline{H}$-), 1.45 (1H, m, -SO$_2$NCH$_2$-C$\underline{H}$H-), 1.35 (3H, t, *J* = 7 Hz, -OCH$_2$C$\underline{H}_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.5 (C6), 162.1 (C16), 128.6 (C13), 127.9 (C14, C18), 114.9 (C15, C17), 63.8 (C20), 61.7 (C2), 49.1 (C5), 30.6 (C3), 25.8 (C9), 24.0 (C4), 14.4 (C21). MS/ESI *m/z* (pos): 313 (M+H), 335 (M+Na). MS/ESI *m/z* (neg): 311 (M-H). Anal. calcd. (found) % for $C_{14}H_{20}N_2O_4S$ (*m.w.* 312.38): C, 53.83 (53.64); H, 6.45 (6.42); N, 8.97 (9.13).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-*N*-ethylpyrrolidine-2-carboxamide (3g): The method described in compound **3e** was repeated by the addition of 5 mL of 70% ethylamine (w/w) and purified by flash chromatography using acetonitrile and benzene in a ratio of 2: 1 to afford compound **3g** as a beige white solid with 42% yield; m.p.: 106-108 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.91 (t, 1H, -CONH-), 7.77 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.13 (2H, q, *J* = 7 Hz, -OC$\underline{H}_2$-CH$_3$), 3.92 (1H, m, -SO$_2$NC$\underline{H}$CH$_2$-), 3.41 (1H, m, -SO$_2$N- CH$\underline{H}$-CH$_2$-), 3.10 (3H, m, -SO$_2$NC$\underline{H}$HCH$_2$-, -CONHC$\underline{H}_2$), 1.73 (2H, m, SO$_2$NCHC$\underline{H}_2$-), 1.60 (1H, m, -SO$_2$NCH$_2$CH$\underline{H}$-), 1.47 (1H, m, -SO$_2$NCH$_2$C$\underline{H}$H-), 1.35 (3H, t, *J* = 6.9 Hz, -OCH$_2$C$\underline{H}_3$), 1.02 (3H, t, *J* = 7.2 Hz, -NHCH$_2$C$\underline{H}_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.8 (C6), 162.0 (C17), 129.6 (C14), 128.1 (C15, C19), 114.8 (C16, C18), 63.8 (C21), 61.7 (C2), 49.1 (C5), 33.5 (C9), 30.6 (C3), 24.0 (C4), 14.7 (C10), 14.4 (C22). MS/ESI *m/z* (pos): 327 (M+H), 349 (M+Na). MS/ESI *m/z* (neg): 325 (M-H). Anal. calcd. (found) % for $C_{15}H_{22}N_2O_4S$ (326.41): C, 55.19 (54.96); H, 6.79 (6.57); N, 8.58 (8.35).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-*N*-propylpyrrolidine-2-carboxamide (3h): It was synthesized according to the general procedure of compound **3e** by the addition of *n*-propylamine (2 mL, 24 mmol) and was purified by flash chromatography using ethyl acetate and benzene in a ratio of 3:2 to obtain compound **3h** as a white solid with 79% yield; m.p.: 112-114 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.89 (1H, t, *J* = 5.8 Hz, -CONH-), 7.77 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.13 (2H, q, *J* = 7 Hz, -OC$\underline{H}_2$CH$_3$), 3.93 (1H, dd, J_1 = 8.5 Hz, J_2 = 3.5 Hz, -SO$_2$NC$\underline{H}$CH$_2$-), 3.41 (1H, m, -SO$_2$NCH$\underline{H}$CH$_2$), 3.12 (1H, m, -SO$_2$NCH$\underline{H}$CH$_2$-), 3.03 (2H, m, -CONHC$\underline{H}_2$-), 1.73 (2H, m, -SO$_2$NCHC$\underline{H}_2$-), 1.62 (1H, m, -SO$_2$NCH$_2$C$\underline{H}$H-), 1.44 (m, 3H, -NHCH$_2$C$\underline{H}_2$CH$_3$, -SO$_2$NCH$_2$CH$\underline{H}$-), 1.35 (3H, t, *J* = 7 Hz, -OCH$_2$C$\underline{H}_3$), 0.84 (3H, t, *J* = 7.4 Hz, -NHCH$_2$CH$_2$C$\underline{H}_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.9 (C6), 162.0 (C18), 129.6 (C15), 128.1 (C16, C20), 114.8 (C17, C19), 63.8 (C22), 61.7 (C2), 49.1 (C5), 40.3 (C9), 30.7 (C3), 24.1 (C4), 22.3 (C10), 14.4 (C23), 11.2 (C11). MS/ESI *m/z* (pos): 341 (M+H), 363 (M+Na). MS/ESI *m/z* (neg): 339 (M-H). Anal. calcd.

(found) % for $C_{16}H_{24}N_2O_4S$ (*m.w*. 340.44): C, 56.45 (56.89); H, 7.11 (6.93); N, 8.23 (8.17).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-*N*-butylpyrrolidine-2-carboxamide (3i): The method described in compound **3e** was repeated by the addition of *n*-butylamine (2 mL, 20 mmol). It was purified by flash chromatography using ethyl acetate and benzene in a ratio of 2:1 to acquire compound **3i** as a white fluffy solid with 56% yield; m.p.: 196-198 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.85 (1H, m, -CONH-), 7.77 (2H, d, J = 7, Ar-H), 7.12 (2H, dd, J_1 = 6, J_2 = 2, Ar-H), 4.14 (2H, q, J = 7 Hz, -OCH$_2$CH$_3$), 3.94 (1H, m, -SO$_2$NCHCH$_2$-), 3.39 (1H, m, -SO$_2$NCHHCH$_2$-), 3.12 (1H, m, -SO$_2$NCHHCH$_2$-), 3.07 (2H, m, CONHCH$_2$-), 1.75 (2H, m, -SO$_2$NCHCH$_2$-), 1.63 (1H, m, -SO$_2$NCH$_2$CHH-), 1.47 (1H, m, -SO$_2$NCH$_2$CHH-), 1.39 (2H, m, -CH$_2$CH$_2$CH$_3$), 1.31 (5H, m, -OCH$_2$CH$_3$, -CH$_2$CH$_3$), 0.87 (3H, t, J = 6.8 Hz, -CH$_2$CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.9 (C6), 162.7 (C19), 129.6 (C16), 128.4 (C17, C21), 114.5 (C18, C20), 63.8 (C23), 61.7 (C2), 49.1 (C5), 38.7 (C9), 31.1 (C10), 30.7 (C3), 24.0 (C4), 22.1 (C11), 14.8 (C24), 13.7 (C12). MS/ESI *m/z* (pos): 355 (M+H), 377 (M+Na). MS/ESI *m/z* (neg): 353 (M-H). Anal. calcd. (found) % for $C_{17}H_{26}N_2O_4S$ (*m.w.* 354.46): C, 57.60 (57.43); H, 7.39 (7.42); N, 7.90 (8.08).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)pyrrolidine-2-carbonyl chloride (X_1): Moisture free compound **3b** (3 g, 9 mmol) and 10 mL of purified thionyl chloride were stirred at room temperature in an inert atmosphere for 4 h. The excess of thionyl chloride was removed by distillation with benzene (4 × 20 mL) under vacuum to achieve the intermediate compound **X$_1$**, a yellowish brown semisolid (2.7 g, 84%), which was carried to the next step without further purification.

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-*N*-pentylpyrrolidine-2-carboxamide (3j): Compound **3j** was synthesized by Schotten-Baumann method, where **X$_1$** (2 g, 6.3 mmol) was dissolved in 5 mL of dry dichloromethane and triturate vigorously with dropwise addition of 15% NaOH (to maintain the reaction alkaline) and *n*-pentyl amine (1 mL, 9.2 mmol). Yellowish white solid was separated out and washed with dil. hydrochloric acid to remove excess amine and finally with ice cold water. Filtered under vacuum and was purified by flash chromatography using ethyl acetate and benzene in a ratio of 3:2 to obtain compound **3j** as a grayish white solid with 57% yield; 154-156 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.85 (1H, m, -CONH-), 7.77 (2H, dd, J_1 = 6.8 Hz, J_2 = 2 Hz,), 7.18 (2H, dd, J_1 = 6 Hz, J_2 = 2 Hz, Ar-H), 4.14 (2H, q, J = 7.2 Hz, -OCH$_2$-CH$_3$), 3.94 (1H, m, SO$_2$NCHCH$_2$-), 3.41 (m, 1H, -SO$_2$NCH-HCH$_2$-), 3.12 (1H, m, -SO$_2$NCHHCH$_2$-), 3.07 (2H, m, -CONH-CH$_2$-), 1.77 (2H, m, -SO$_2$NCHCH$_2$-), 1.75 (1H, m, -SO$_2$NCH$_2$-CHH-), 1.63 (1H, m, -SO$_2$NCH$_2$CHH-), 1.46 (2H, m, -CONH-CH$_2$CH$_2$-), 1.37 (3H, t, J = 7.2 Hz, -OCH$_2$CH$_3$), 1.26 (4H, m, -CH$_2$CH$_2$CH$_3$), 0.86 (3H, t, J = 6.8 Hz, -CH$_2$CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.1 (C6), 163.5 (C20), 129.9 (C17), 127.1 (C18, C22), 114.5 (C19, C21), 63.8 (C24), 61.6 (C2), 49.9 (C5), 38.6 (C9), 30.0 (C10), 29.2 (C3), 27.1 (C11), 24.3 (C4), 22.7 (C12), 14.8 (C25), 13.7 (C13). MS/ESI *m/z* (pos): 369 (M+H). MS/ESI *m/z* (neg): 367 (M-H). Anal. calcd. (found) % for $C_{18}H_{28}N_2O_4S$ (368.49): C, 58.67 (58.39); H, 7.66 (7.84); N, 7.60 (7.26).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-*N*-hexylpyrrolidine-2-carboxamide (3k): Compound **3k** was synthesized according to the general procedure adopted from compound **3e** with addition of *n*-hexylamine (2 mL, 15 mmol). It was purified by flash chromatography using ethyl acetate and benzene in a ratio of 1:1 to afford compound **3k** as a pale yellowish liquid with 49% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.84 (1H, t, J = 5.7 Hz, -CONH-), 7.77 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.13 (2H, m, -OCH$_2$CH$_3$), 3.94 (1H, dd, J_1 = 8.4 Hz, J_2 = 3.4 Hz, -SO$_2$NCHCH$_2$-), 3.41(1H, m, -SO$_2$N-CHHCH$_2$-), 3.13 (1H, m, -SO$_2$NCHHCH$_2$-), 3.05 (2H, m, -CO-NHCH$_2$-), 1.73 (2H, m, SO$_2$NCHCH$_2$-), 1.62 (1H, m, -SO$_2$N-CH$_2$CHH-), 1.48 (m, 1H, -SO$_2$NCH$_2$CHH-), 1.41 (2H, m, -CO-NHCH$_2$CH$_2$-), 1.35 (3H, m, -OCH$_2$CH$_3$), 1.29 (6H, m, -CH$_2$CH$_2$-CH$_2$CH$_3$), 0.86 (3H, t, J = 6.8 Hz, -CH$_2$CH$_2$CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.9 (C6), 162.0 (C21), 129.5 (C18), 128.2 (C19, C23), 114.8 (C20, C22), 63.7 (C25), 61.65 (C2), 49.0 (C5), 38.5 (C9), 30.9 (C10), 30.6 (C12), 28.9 (C3), 25.9 (C11), 24.0 (C4), 22.0 (C13), 14.3 (C26), 13.8 (C14). MS/ESI *m/z* (pos): 383 (M+H), 322 (M+Na). MS/ESI *m/z* (neg): 381 (M-H). Anal. calcd. (found) % for $C_{19}H_{30}N_2O_4S$ (*m.w.* 382.52): C, 59.66 (59.20); H, 7.91 (7.66); N, 7.32 (7.48).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)pyrrolidine-2-carbohydrazide (3l): Compound **3c** (2 g, 6.1 mmol) was dissolved in 15 mL of ethanol and to it 80% hydrazine hydrate (0.5 mL, 12.5 mmol) was added in a portion and refluxed gently for 3 h and distilled to dryness under vacuum. It was purified by flash chromatography using ethyl acetate and benzene in a ratio of 4:1 to get compound **3l** as a white solid with 60% yield; m.p.: 180-182 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.13 (1H, s, -NHNH$_2$), 7.77 (2H, m, Ar-H), 7.12 (2H, m, Ar-H), 4.26 (2H, q, J_1 = 7 Hz, -NHNH$_2$), 4.13 (2H, s, -OCH$_2$CH$_3$), 3.96 (1H, dd, J_1 = 8.5, J_2 = 3.9, -SO$_2$NCHCH$_2$-), 3.39 (m, 1H, -SO$_2$NCHHCH$_2$), 3.11 (1H, m, -SO$_2$NCHHCH$_2$-), 1.77 (2H, m, -SO$_2$NCHCH$_2$-), 1.63 (1H, m, -SO$_2$NCH$_2$CHH-), 1.47 (1H, m, -SO$_2$NCH$_2$CHH-), 1.36 (3H, m, -OCH$_2$CH$_3$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.3 (C6), 162.1 (C16), 129.6 (C13), 128.2 (C14, C18), 114.8 (C15, C17), 60.2 (C20), 49.1 (C5), 30.6 (C3), 14.4 (C21). MS/ESI *m/z* (pos): 314 (M+H), 336 (M+Na). MS/ESI *m/z* (neg): 312 (M-H). Anal. calcd. (found) % for $C_{13}H_{19}N_3O_4S$ (*m.w.* 368.49): C, 49.83 (49.89); H, 6.11 (6.17); N, 13.41 (13.27).

Synthesis of 1-((4-ethoxyphenyl)sulfonyl)-N'-phenylpyrrolidine-2-carbohydrazide (3m): Compound **X$_1$** (2 g, 6.3 mmol) was dissolved in 5 mL dry dichloromethane. Phenyl hydrazine (1 mL, 10 mmol) was added to the reaction mixture with stirring to get compound **3m**. The product was purified by flash chromatography using acetonitrile and benzene in a ratio of 4:1 to afford compound **3m** as a yellowish white solid with 64% yield; m.p.: 176-178 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.65 (1H, s,-CONHNH-), 8.45 (1H, s, Ph-NHNH), 7.77 (2H, dd, J_1 = 6.8 Hz, J_2 = 2 Hz, Ar-H), 7.28 (2H, dd, J_1 = 8 Hz, J_2 = 2.4 Hz, Ar-H), 7.13 (2H, dd, J_1 = 6.4 Hz, J_2 = 2 Hz, Ar-H), 7.01 (2H m, Ar-H), 6.92 (1H, m, Ar-H), 4.13 (2H, q, J = 6.8 Hz, -OCH$_2$CH$_3$), 3.92 (1H, m, -SO$_2$NCHCH$_2$-), 3.42 (1H, m, -SO$_2$N-CHHCH$_2$), 3.12 (1H m, -SO$_2$NCHHCH$_2$-), 1.73 (2H, m, -SO$_2$N-CHCH$_2$-), 1.60 (1H, m, -SO$_2$NCH$_2$CHH-), 1.47 (1H, m, -SO$_2$N-CH$_2$CHH-), 1.36 (3H, t, J = 7.2 Hz, -OCH$_2$CH$_3$). ^{13}C NMR (100

MHz, DMSO-d_6): δ 173.3 (C6), 163.4 (C22), 148.9 (C10), 130.7 (C19), 129.0 (C12, C14), 127.3 (C20, C24), 125.0 (C13), 115.3 (C11, C15), 114.4 (C21, C23), 64.1 (C2), 60.3 (C26), 49.2 (C5), 30.6 (C3), 24.3 (C4), 14.4 (C27). MS/ESI *m/z* (pos): 390 (M+H), 322 (M+Na). MS/ESI *m/z* (neg): 388 (M-H). Anal. calcd. (found) % for $C_{19}H_{23}N_3O_4S$ (*m.w.* 389.47): C, 58.59 (58.73); H, 5.95 (5.86); N, 10.79 (10.46).

RESULTS AND DISCUSSION

Cell-based inhibition assay: Cytotoxicity study of the compounds **3b-3m** was carried out on HUVECs and normal Vero cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [20,21] and RPMI 8226 by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay [22]. All the cell lines were procured from American Type Culture Collection (ATCC). The study was intended to explore the potent anticancer compounds and their effect on normal endothelial cells and have an idea about their primary antiangiogenic features. The results of the cytotoxicity study are presented in Table-1. The IC_{50} values of the compounds with respect to the positive control Doxorubicin shows compound **3m** as the most active compound with IC_{50} = 2.28 μM (RPMI 8226) and IC_{50} = 3.12 μM (HUVECs). The compounds with IC_{50} values of 5 μM or less in RPMI 8226 were considered for further studies.

Proliferation assay: Proliferation assay of HUVECs were carried out by trypan blue method [23]. HUVECs were seeded with 1×10^4 cells/well in 96 well plates in DMEM medium supplemented with 20 ng/mL of VEGF (mitogen). After 48 h of incubation, the viable cells were counted in a hemocytometer following a dye exclusion method using trypan blue. The number of HUVECs in the untreated group proliferate 28×10^4 cells/well compared to compounds **3i**, **3k** and **3m** where the number proliferate to 5×10^4, 8×10^4 and 3×10^4 cells/well, respectively. From this comparison, it was found that compounds **3i, 3k** and **3m** reduced significant HUVECs proliferation by 82.1, 71.4 and 89.3%, respectively at 10 μM concentration. By contrast, compounds **3i** and **3m**, significantly reduced HUVEC proliferation at 1 μM with 42.4 and 69.7%. These results confirm the antiproliferative activity of the test compounds against endothelial cells, which causes for angiogenesis (Fig. 2).

Docking: A docking study with the most active compound **3m** was carried out employing LibDock protocol from Discovery studio, 4.1, Biovia [24,25]. LibDock uses Hotspots (protein site features) and the rigid ligand poses are placed into the active site and HotSpots are matched as triplets. After final optimization, poses are scored. The binding energy of compound **3m** was found to be -62.27 Kcal/mol with a LibDock score of 131.7. The docking score calculated for LibDock is Pairwise Linear Potential-1 and 2 (PLP). PLP has been shown to correlate well with protein-ligand binding affinities. In this study, the ligand attached to the co-crystal structure of 3VHE (42Q) was tagged as control ligand and the scores were compared with compound **3m**. The binding affinities were comparable. The control ligand's total score was found to be -289.91 and that of compound **3m** is -219.28.

From the result of the cytotoxicity study and proliferation assay of the compounds, it was proven that the compound **3m** is the most potent angiogenic inhibitor, which might be the cause of multiple myeloma activity. The non-bond interaction study revealed that the compound **3m** forms four hydrogen bonds with ASP1046, GLU885 and two bonds with LYS868 (Fig. 3). The -NH- hydrogen of phenyl hydrazide moiety forms one H Bond with ASP1046 (2.81 Å) and the other with GLU885 (2.69 Å). The carbonyl oxygen atom forms two H bonds with LYS868 (2.46 Å and 2.91 Å). Both the phenyl groups were found to interact with the amino acid residues in the pocket. The carbon atom of $–CH_3$ moiety of the ethoxy group interacts with PHE918 (4.81 Å) and forms π-alkyl interaction and with LEU840 (3.84 Å) and forms alkyl interaction.

Conclusion

The design, synthesis and anticancer activity of a series of conformationally restricted glutamic acid analogs with extended methylene chain in the ligand to better fit at the active site of the VEGFR-2 receptor was presented. The study revealed that the compounds **3i, 3k** and **3m** showed selective anticancer

TABLE-1
in vitro CYTOTOXICITIES OF TARGET COMPOUNDS **3b-m** AND DOXORUBICIN AS A POSITIVE CONTROL OBTAINED IN 24 h INCUBATION

Compd.	R	IC_{50} (μM) ± SD		Vero
		HUVEC	RPMI 8226	
3b	–	17.52 ± 0.22	14.89 ± 0.13	>100
3c	$-C_2H_5$	17.34 ± .0.31	23.58 ± 1.23	70.24 ± 0.71
3d	$-CH_3$	40.69 ± 2.16	29.03 ± 0.81	Nd
3e	-H	13.45 ± 0.58	18.71 ± 1.12	34.21 ± 0.18
3f	$-CH_3$	15.8 ± 0.17	18.29 ± 0.53	59.14 ± 0.26
3g	$-C_2H_5$	56.32 ± 1.21	34.73 ± 2.41	Nd
3h	$-C_3H_7$	> 100	70.18 ± 1.35	Nd
3i	$-C_4H_9$	3.81 ± 0.49	3.72 ± 0.51	32.76 ± 0.13
3j	$-C_5H_{11}$	13.48 ± 0.95	28.07 ± 0.21	17.49 ± 0.12
3k	$-C_6H_{13}$	4.63 ± 1.02	3.89 ± 0.31	34.83 ± 0.42
3l	-H	18.84 ± 0.63	25.37 ± 0.19	26.47 ± 0.25
3m	-Ph	3.12 ± 0.49	2.28 ± 0.22	43.11 ± 0.17
Doxorubicin[b]		0.53 ± 0.09	0.15 ± 0.01	0.5 ± 0.11

IC_{50} values are determined as the mean ± SD of three independent experiments performed; [a]Nd: Not determined. [b]Used as a positive control.

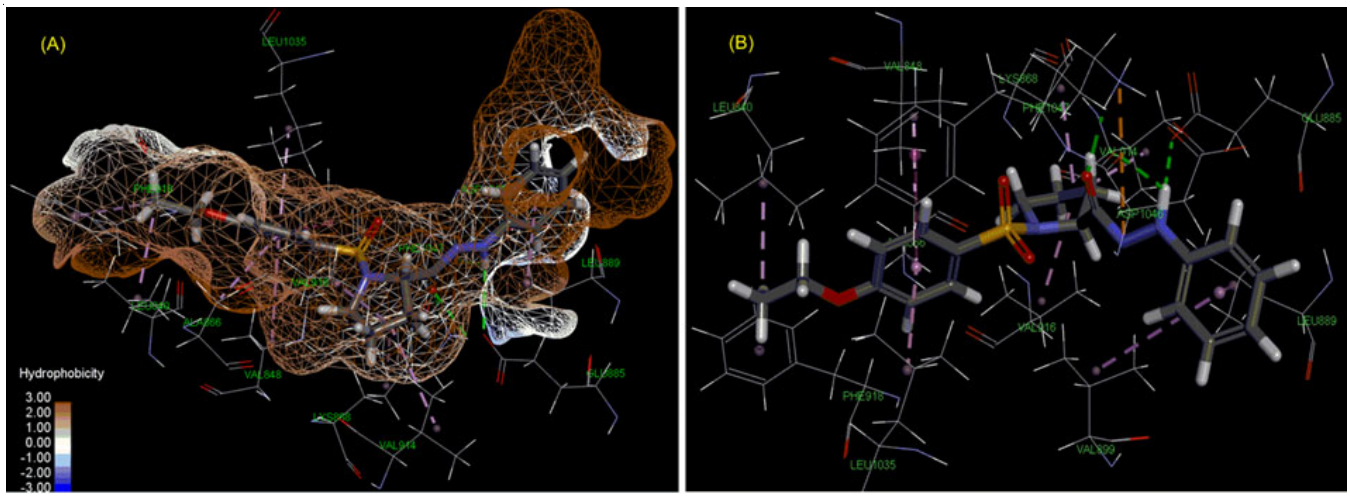


Fig. 3. (A) Hydrophobic interaction of the ethoxy hydrogens with the hydrophobic amino acid residues; (B) 3D-Interaction of the 3m ligand atoms with their complimentary amino acid residues at the active site of VEGFR-2 (PDB ID: 3VHE)

activity against multiple myeloma and possessed antiangiogenic features. It was also found that the active compounds are non-toxic to the normal VERO cells. Docking results provided an insight into the binding mode of compound **3m** at the catalytic cleft of VEGFR-2, which defines the necessity of the ethoxy group as a replacement of the methoxy group to support better binding with the VEGFR-2 tyrosine kinase enzyme.

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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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