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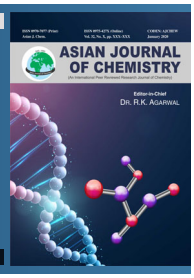

Design, Synthesis, Molecular Docking and *in vitro* Evaluation of *N*-(4-Methoxyphenylsulfonyl)pyrrolidine-2-carboxylic Acid Analogues as Antiangiogenic and Anticancer Agents on Multiple Myeloma

K. Sarker, A. Ghosh, S. Mishra, A. Saha and S. Sen*

A.P.C. Ray Memorial Cancer Chemotherapeutic Research Unit, College of Pharmaceutical Sciences, Mohuda, Berhampur-760002, India

*Corresponding author: Email: prof.dr.subratasen@gmail.com

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A series of *N*-(4-methoxyphenylsulfonyl)pyrrolidine-2-carboxylic acid analogs were designed as bioisosteres of a major metabolite of thalidomide, *i.e*., *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid. Compounds **2b**, **2d**, **2f**, **2i** and **2k** exhibited anticancer activity on multiple myeloma (RPMI 8226) by MTS assay and were tested for primary antiangiogenic activity on HUVEC cell line by MTT assay. Compound **2f** was excluded from further study as it was found to be cytotoxic to normal epithelial cells. **2b**, **2d**, **2i** and **2k** were found to have primary antiangiogenic activity along with low cytotoxicity on normal vero cells in MTT assay indicating selective cytotoxicity towards highly angiogenic multiple myeloma. Antiproliferative assay of compounds **2b**, **2d**, **2i** and **2k** on HUVECs was carried out using the dye exclusion method with trypan blue. Molecular docking study of compound **2b** calculated the binding energy -89.78 kcal/mol and displayed five hydrogen bonds with critical amino acid residues. The compounds are potential candidate drugs for advanced investigations.

Keywords: Thalidomide, Angiogenesis, Multiple myeloma, Vero, RPMI8226, Molecular docking.

INTRODUCTION

Plasma B cells, which originates from the bone marrow, are responsible for the release of antibodies in response to an invasion of antigen in the body [1]. Multiple myeloma (MM) is a type of blood cell cancer that affects the immune system when the plasma cells turn to become malignant [2]. During the disease progression, bone marrow′s microvessel density increases rapidly, and this abrupt change in the bone marrow (BM) microenvironment triggers the release of cytokines, mitogen, and other growth factors, which induce proliferation of the cancerous cells by angiogenesis and protect the cells from surviving [3-5]. Angiogenesis is the phenomenon of the formation of blood vessels from the existing one and is one of the ten vital hallmarks of cancer [6]. Vascular endothelial growth factor (VEGF) is the prime mitogen that interacts with its complementary receptor, *i.e*. vascular endothelial growth factor receptor-2 (VEGFR-2) and help progress angiogenesis in tumors [7]. VEGFR-2 Tyr-1175 is the primary autophosphorylation site that controls the proangiogenic responses [8]. Inhibition of autophosphorylation will restrict angiogenesis in the tumors and may be a better option to treat patients suffering from multiple myeloma (MM) as it is highly angiogenic [9].

Several classes of drugs are approved for treatment of multiple myeloma (MM) like (i) anti-myeloma monoclonal antibodies targeting CS1, CD38, CD138, BCMA and immune checkpoints, (ii) DNA damaging agents such as melflufen, bendamustine, (iii) Inhibitor of BCL-2 family proteins venetoclax; (iv) Epigenetic inhibitors-panobinostat, a pan-HDAC inhibitor and tazemetostat an EZH2 inhibitors; (v) MEPK pathway inhibitors-trametinib and afuresertib; (vi) Inhibitors of nuclear-cytoplasmic transport receptor: XPO1 inhibitor-selinexor; (vii) IMiDs-thalidomide, lenalidomide and pomalidomide and h) Kinesin spindle protein inhibitor-Filanesib [10, 11].

However, there is a paucity of small molecules with selective antiangiogenic and antitumor activity along with lesser cytotoxicity on normal cells, which are most sought after as the different classes of approved drugs evoke a wide range of adverse effects like toxicity, resistance, and anaphylactic shock (especially antibody therapeutics). Therefore, it is pertinent to discover newer drugs without these shortcomings.

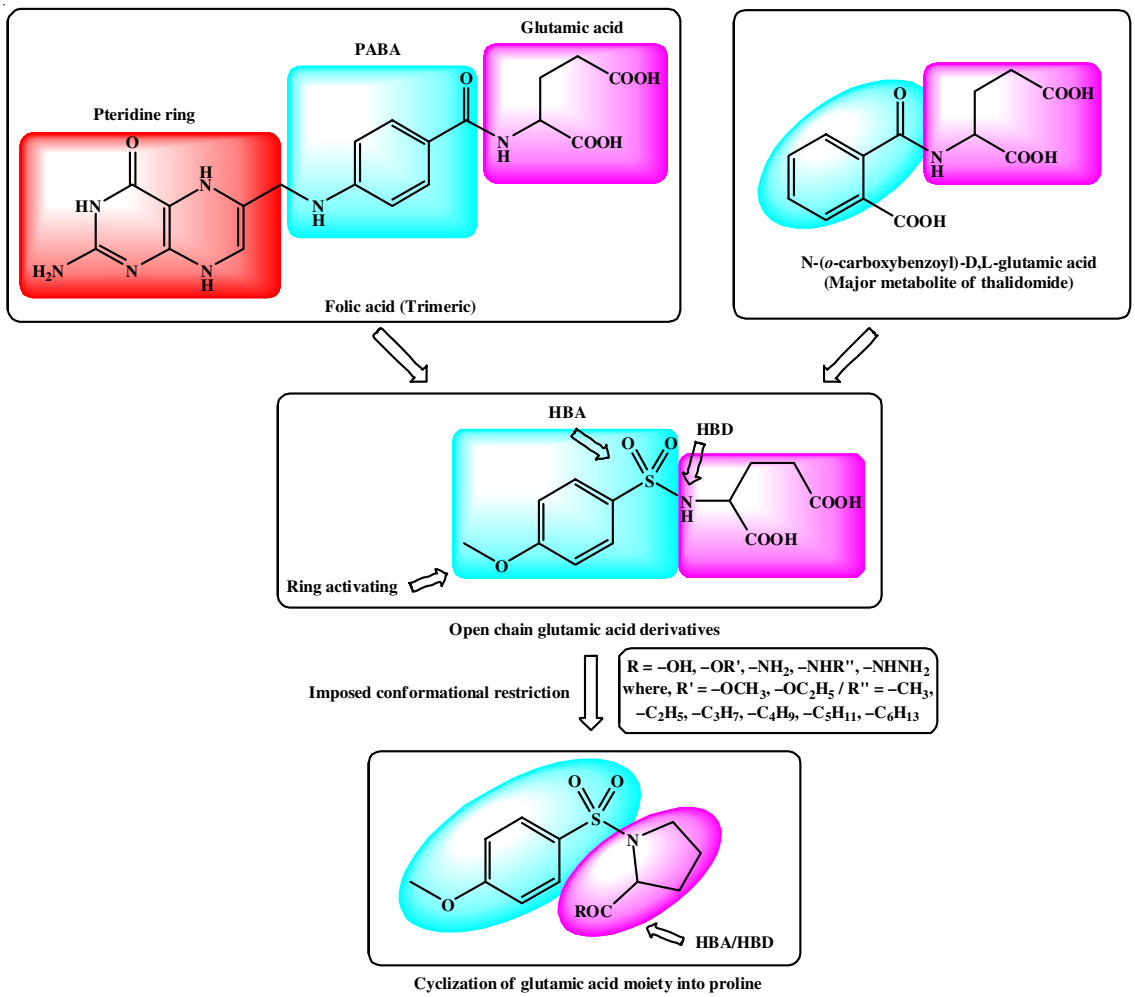
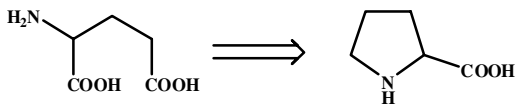


Fig. 1. Bioisosteric modification of *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid keeping similarity with folic acid fragments

Thalidomide (2,6-dioxo-3-phthalimidopiperidine), a veritable phoenix, was a popular drug among the pregnant as well as for the treatment of morning sickness due to its non-addictive sedative action [12]. Later, thalidomide was withdrawn from the market as causes phocomelia, a congenital disability and found to be teratogenic [13]. After some decades, thalidomide was repurposed as antiangiogenic agent for the treatment of multiple myeloma in combination with dexamethasone, and in the treatment of erythema nodosum leprosum (ENL), a complication of lepromatous leprosy [14].

A detailed metabolite study of thalidomide was carried out independently by Faigel *et al*. [15] and Kemper [16]. It was found that the metabolite shares two common residues with folic acid which is considered as a trimer of pteridine ring, *p*-aminobenzoic acid (PABA) and glutamic acid residues [16]. It was concluded that *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid is the major metabolite of thalidomide and may be responsible for its teratogenic activity by means of antiangiogenesis [13].

With the notion that *N*-(*o*-carboxybenzoyl)-D,L-glutamic acid, plays a crucial role in antiangiogenesis, the structure was modified based on bioisosterism to develop some more potent inhibitors of angiogenesis and hence multiple myeloma (MM) (Fig. 1). Proline was supplanted instead of glutamic acid to impose a conformational restriction on glutamic acid. Comparison of physico-chemical properties of glutamic acid and proline is given in Fig. 2. On top, the carbonyl group of carboxamide moiety was replaced with a sulphonyl group (-SO_2-), which acts as an electron-rich hydrogen bond acceptor (HBA). The *para*- position in the aromatic ring was substituted with -OCH_3 group to keep resemblance with the -NH- of PABA residue.



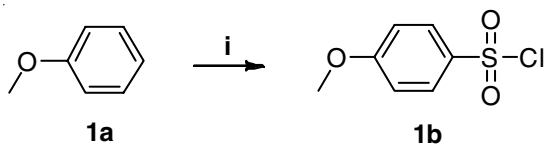
Physico-chemical properties	Glutamic acid	Pyrrolidine-2-carboxylic acid
log P	-1.68	-0.92
MW	147.13	115.13
HBD	3	2
HBA	5	3
Number of rotatable bonds	4	1

Fig. 2. A comparison of featured physicochemical properties of glutamic acid and pyrrolidine-2-carboxylic acid. The values are calculated from web tool SwissADME [17]. MW- Molecular weight; HBD-Hydrogen Bond Donor; HBA- Hydrogen Bond Acceptor (Note the decrease in number of rotatable bonds)

EXPERIMENTAL

All the chemicals were purchased from Himedia (India), Merck (India) and Loba Chemie (India) and used without further purification unless it is 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 500 and 400 MHz spectrometer (500 MHz, 400 MHz and 125 MHz for 1H NMR and 13C NMR, respectively). 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).

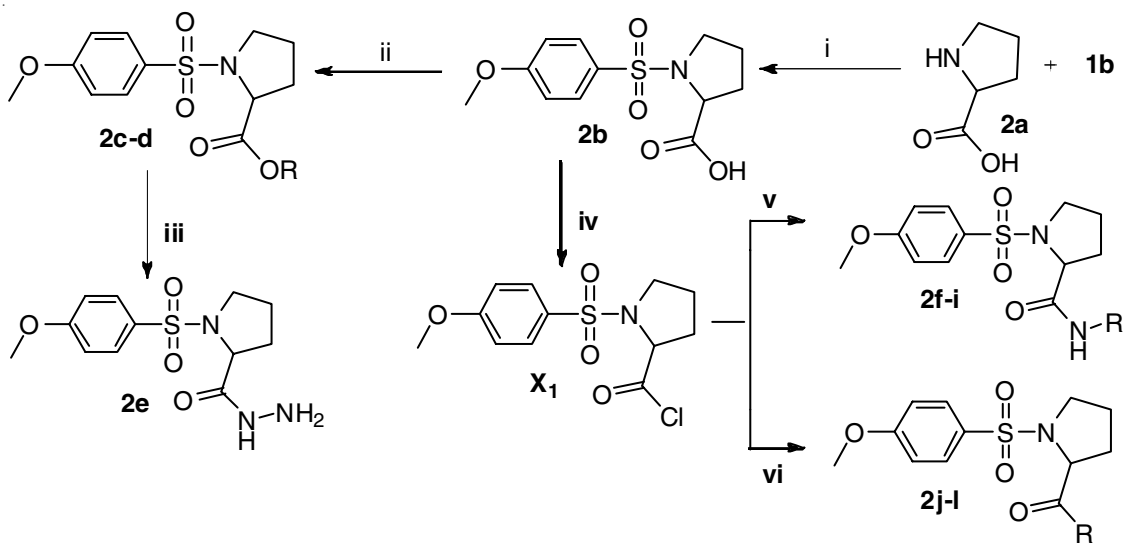
Synthesis of 4-methoxybenzene-1-sulfonyl chloride (1b): Methoxybenzene (**1a**) (5 ml, 47 mmol) was dissolved in 50 mL of anhydrous chloroform and cooled to 0 ºC, where chlorosulphonic acid (8 mL, 121 mmol) was added dropwise, while stirring, within 30 min. After complete addition, the reaction mixture was stirred for another 45 min at room temperature. The excess chlorosulphonic acid was destroyed using 100 g of crushed ice. It was extracted with chloroform (3 × 25 mL) and the organic layer was washed with ice-cold water and dried over anhydrous sodium sulphate overnight [18]. The chloroform layer was distilled under vacuum to afford compound **1b** as a grayish white solid (68%, 8 g, m.p. 36-38 ºC) (**Scheme-I**) [18].



Scheme-I: Synthetic route of intermediate **1b**. Reagents and conditions: (i) $ClSO_3H$, $CHCl_3$, Stirred at 0-5 °C for 30 min

Synthesis of 1-((4-methoxyphenyl)sulfonyl)pyrrolidine-2-carboxylic (2b): Compound **1b** (5 g, 24 mmol) was added portion wise to a stirred aqueous solution of Na_2CO_3 and L-proline (**2a**) (2.8 g, 24 mmol) within 30 min. After complete addition, dimethylaminopyridine (DMAP) (50 mg, 0.41 mmol) was added as catalyst and stirred for another 3 h. The reaction was maintained at pH 9-10 by adding sodium carbonate. The solution was filtered, acidified to pH 2-3 with HCl, extracted with ethyl acetate and dried over anhydrous $MgSO_4$ overnight. It was distilled under vacuum and purified by flash chromatography using ethyl acetate and benzene in 4:1 ratio followed by recrystallization using hydro-alcoholic solution to afford compound **2b** as a white solid (**Scheme-II**) (76%, 5.3 g, 80-82 ºC). Elemental analysis of $C_{12}H_{15}NO_5S$ calcd. (found) %: C, 50.52 (50.27); H, 5.30 (5.43); N, 4.91 (4.79). ^{1}H NMR: (500 MHz, $CDCl_3$): δ 1.77 (1H, m, -SO_2NCH_2CHH-), 1.94 (2H, m, -SO_2N-CH_2-CH**H**-, -$SO_2NCHCHH$-), 2.19 (1H, m, -SO_2NCH-C**H**H-), 3.25 (1H, q, *J* = 8.3 Hz, -NHC**H**HCH_2-), 3.52 (1H, m, -NC**H**H-CH_2-), 3.89 (3H, s, -O**CH_3**), 4.25 (1H, m, -N-C**H**-CH_2), 7.02 (2H, d, *J* =9 Hz, Ar-H), 7.83 (2H, d, *J* = 9 Hz, Ar-H). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 24.59, 30.64, 48.66, 55.59, 60.27, 114.31, 128.91, 129.63, 163.20, 176.84. ESI+MS (*m/z*): 170.94 ($C_7H_7O_3S$), 286.08 (M+H), 287.12 (M+2H) , 308.08 (M+Na); ESI-MS(*m/z*): 284.10 (M-H).

Methyl 1-((4-methoxyphenyl)sulfonyl)pyrrolidine-2-carboxylate (2c): Esterification of compound **2b** was carried out by Fischer-Speier method, where compound **2b** (3 g, 10.5 mmol) was dissolved in 60 mL of super dried methanol saturated with dry HCl gas and was refluxed in inert atmosphere for 4 h. The content was added in 100 mL of ice-cold water and neutralized with sodium bicarbonate while a off white colored cloudy mass appeared. It was kept overnight under distilled water for maturation and filtered under vacuum. The final product was purified by flash chromatography using ethyl acetate and n-hexane in 3:2 ratio and recrystallized using hydro-alcoholic



Scheme-II: Synthetic route of compounds **2b-2l**. Reagents and conditions: (i) Na_2CO_3, 4-dimethylaminopyridine (DMAP), 3.5 h stirring, room temperature; (ii) Super dry R-OH saturated with dry hydrogen chloride gas, reflux, 4 h; (iii) NH_2NH_2. H_2O, ethanol 3 h reflux (iv) $SOCl_2$, 4 h stirring, room temperature (v) CH_2Cl_2, 15% NaOH, alkyl amine (vi) *N*,*N*′-dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), triethyl amine, alkyl amines, 2 h stirring, room temperature

solution. Compound **2c** was obtained as an off white solid (68%, 2.1 g ; m.p. 84-86 ºC) (**Scheme-II**). Elemental analysis of $C_{13}H_{17}NO_5S$ calcd. (found) %: C, 52.16 (51.98); H, 5.72 (5.66); N, 4.68 (4.81). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.77 (1H, td, J_1 = 5 Hz, J_2 = 3 Hz, -SO_2NCH_2CH**H**-), 1.99 (3H, m, -SO_2NCH_2CH**H**-, -SO_2N-CHC**H**$_2$-), 3.31 (1H, m, -SO_2NCH**H**-), 3.47 (1H, m, -SO_2NCH**H**-), 3.72 (3H, s, -COOC**H**$_3$-), 3.87 (3H, s, -OC**H**$_3$), 4.30 (1H, dt, J_1 = 6.5 Hz, J_2 = 2.5 Hz, -SO_2N-C**H**CH_2-), 6.99 (2H, d, J = 9, Ar-H), 7.82 (2H, m, Ar-H). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 24.49, 30.75, 48.28, 52.21, 55.49, 60.21, 114.06, 129.45, 129.69, 162.92, 172.49. ESI+MS (*m/z*): 170.86 ($C_7H_7O_3S$), 239.93 ($C_{11}H_{14}NO_3S$), 299.98 (M+H), 322.10 (M+Na).

Ethyl 1-((4-methoxyphenyl)sulfonyl)pyrrolidine-2-carboxylate (2d): The compound was synthesized according to the general procedure from compound **2c**, by using compound **2b** (3 g, 10.5 mmol) and dry HCl gas saturated super dried ethanol. The final product was purified by flash chromatography using ethyl acetate and benzene in 4:1 ratio and recrystallized using hydro-alcoholic solution to get compound **2d** as an off white solid (73%, 2.4 g; m.p. 102-104 ºC) (**Scheme-II**). Elemental analysis of $C_{14}H_{19}NO_5S$. calcd. (found) %: C, 53.66 (53.72); H, 6.11 (6.05); N, 4.47 (4.34). ^{1}H NMR: (500 MHz, $CDCl_3$): δ 1.27 (3H, t, J_1 = 6.9 Hz, -$COOCH_2CH_3$), 1.78 (1H, q, J_1 = 3.9 Hz, -SO_2NCH_2-C**H**H-), 2.01 (3H, m, -SO_2NCH_2CH**H**-, -SO_2N-CHC**H**$_2$-), 3.32 (1H, m, -SO_2NCH**H**-), 3.46 (1H, m, -SO_2N-CH**H**-), 3.87 (3H, s, -OC**H**$_3$) , 4.18 (2H, s, -COOC**H**$_2$-), 4.29 (1H, q, J_1 = 3.9 Hz, -SO_2NCH**H**CH_2-), 6.98 (2H, d, J = 9, Ar-H), 7.83 (2H, d, J = 9, Ar-H). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 14.09, 24.63, 30.92, 48.33, 55.56, 60.43, 61.29, 114.11, 129.63, 130.13, 162.97, 172.16. ESI+MS (*m/z*): 170.89 ($C_7H_7O_3S$), 239.88 ($C_{11}H_{14}NO_3S$), 314.08 (M+H), 336.08 (M+Na), 377.12 (M+2H+Na+K).

1-((4-Methoxyphenyl)sulfonyl)pyrrolidine-2-carbohydrazide (2e): Compound **2c** (3 g, 10 mmol) was dissolved in 15 ml of ethanol, to it excess , 80% hydrazine hydrate (1 mL, 25 mmol) was added in portion and refluxed gently for 3 h and distilled to dryness under vacuum to get the solid product which was purified by flash chromatography using ethyl acetate and *n*-hexane in a ratio of 5:2, and recrystallized from absolute ethanol to acquire compound **2e** as a grayish white solid (**Scheme-II**) (53%, 1.6 g; m.p. 142-144 ºC). Elemental analysis of $C_{12}H_{17}N_3O_4S$ calcd. (found) %: C, 48.15 (48.36); H, 5.72 (5.59); N, 14.04 (13.92). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.62 (2H, m, -$SO_2NCH_2CH_2$-), 1.76 (1H, m, -SO_2NCH_2CH**H**-) , 2.18 (1H, m, -SO_2NCHCH**H**-), 3.15 (1H, td, J_1 = 9.6 Hz, J_2 = 6.2 Hz, -SO_2N-CH**H**-), 3.54 (1H, m, -SO_2NCH**H**-) , 3.91 (5H, s, -CONHN**H**$_2$, -OC**H**$_3$), 4.12 (1H, m, -SO_2NCH**H**CH2-), 7.02 (2H, d, J = 9 Hz, Ar-H), 7.78 (2H, d, J = 9 Hz, Ar-H), 8.08 (1H, s, -CONH-). **^{13}C NMR** (125 MHz, $CDCl_3$): δ 14.43, 24.15, 30.62, 49.02, 55.76, 60.19, 114.82, 128.22, 129.56, 162.06, 170.31. ESI+MS (*m/z*): 170.98 ($C_7H_7O_3S$), 239.99 ($C_{11}H_{14}NO_3S$), 300.01 (M+H), 322.13 (M+Na) 363.14 (M+2H+Na+K). ESI-MS (*m/z*): 298.12 (M-H).

1-((4-Methoxyphenyl)sulfonyl)pyrrolidine-2-carbonyl chloride (X): Fully dried compound **2b** (3 g, 10.5 mmol) was dissolved with 10 mL of $SOCl_2$ and then stirred at room temperature for 4 h in an inert atmosphere (argon). The excess $SOCl_2$ was removed by repeated distillation with benzene (4 × 20 mL) under vacuum. The intermediate compound, a yellowish brown semisolid, was carried to the next step without further purification (2.6 g; 82%) (**Scheme-II**).

1-((4-Methoxy phenyl)sulfonyl)pyrrolidine-2-carboxamide (2f): Compound **2f** was synthesized by Schotten-Baumann method (**Scheme-II**), where **X$_1$** (2 g, 5.4 mmol) was dissolved in 5 mL dry dichloromethane and triturate vigorously with dropwise addition of 15% NaOH (to maintain the reaction alkaline) and 30% NH_4OH solution (1 mL, 25 mmol) where grayish white solid was separated and washed with dil. HCl to remove excess amine and finally with ice cold water. Purification was carried out by flash chromatography using acetone, ethyl acetate and *n*-hexane in 2:2:1 ratio and recrystallization from ethanol to fetch compound **2f** as a pure white solid (57%, 1.1 g; m.p. 114-116 ºC). Elemental analysis of $C_{12}H_{16}N_2O_4S$ calcd. (found) %: C, 50.69 (50.48); H, 5.67 (5.80); N, 9.85 (9.89). ^{1}H NMR: (400 MHz, $CDCl_3$): δ 1.82 (1H, m, -SO_2NCH_2CH**H**-), 1.84 (2H, m, -SO_2NCHCH_2-), 1.93 (1H, m, -SO_2NCH_2CH**H**-), 3.14 (1H, m, -SO_2NCH**H**HCH_2-), 3.35 (1H, m, -SO_2NCH**H**CH_2-), 4.12 (6H, m, -OC**H**$_3$, -$CONH_2$, -SO_2NCH-), 7.12 (2H, m, Ar-H), 7.75 (2H, m, Ar-H). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 24.09, 29.99, 49.40, 55.42, 61.47, 114.26, 127.10, 129.63, 163.19, 171.33. ESI-MS (*m/z*): 283.36 (M-H), 307.22 (M+Na).

1-((4-Methoxyphenyl)sulfonyl)-*N*-methylpyrrolidine-2-carboxamide (2g): The above process was repeated with methylamine 40% (w/w) aq. solution (1 mL, 23 mmol), while yellowish white solid was appeared and washed with dil. HCl, filtered under vacuum pump and purified by flash chromatography using ethyl acetate and benzene in a ratio of 9:1, followed by recrystallization from hydro-alcoholic solution to get compound **2g** as an off white solid (51%, 1 g; m.p. 154-156 ºC). Elemental analysis of m.f.: $C_{13}H_{18}N_2O_4S$. calcd. (found) %: C, 52.33 (52.47); H, 6.08 (6.13); N, 9.39 (9.14). ^{1}H NMR: (500 MHz, $CDCl_3$): δ 1.47 (1H, m, -SO_2NCH_2CH**H**-), 1.60 (1H, m, -SO_2NCH_2CH**H**-), 1.72 (2H, m, -SO_2NCHCH_2-), 2.61 (3H, d, J = 5 Hz, -$CONHCH_3$-), 3.12 (1H, m, -SO_2NCH**H**HCH_2-), 3.41 (1H, m, -SO_2NCH**H**CH_2-), 3.85 (3H, s, -OC**H**$_3$) , 3.93 (1H, m, -SO_2NCH**H**-), 7.14 (2H, d, J = 9 Hz, Ar-H), 7.79 (2H, m, Ar-H), 7.87 (1H, m, J = 4 Hz, -CONH-). ^{13}C NMR (125 MHz, $CDCl_3$): δ 24.21, 30.03, 35.41, 36.59, 48.28, 55.60, 57.74, 114.35, 129.33, 162.44, 170.69. ESI+MS (*m/z*): 170.90 ($C_7H_7O_3S$), 239.99 ($C_{11}H_{14}NO_3S$), 299.15 (M+H) , 321.24 (M+Na) . ESI-MS (*m/z*): 297.18 (M-H), 359 (M-H+23+39).

N-Ethyl-1-((4-methoxyphenyl)sulfonyl)pyrrolidine-2-carboxamide (2h): As a cognate preparation, the method described in **2g** was repeated in the next steps with respective amine, 70% ethylamine (w/w) (1 mL, 15 mmol) was used to fetch the final compound and was purified by flash chromatography using acetonitrile and benzene in a ratio of 4:1, followed by recrystallization from hydro-alcoholic solution to get compound **2h** as a white fluffy solid (69%, 1.4 g; m.p. 122-124 ºC). Elemental analysis of $C_{14}H_{20}N_2O_4S$ calcd. (found) %: C, 53.83 (53.66); H, 6.45 (6.39); N, 8.97 (9.11). ^{1}H NMR: (500 MHz, $CDCl_3$): δ 1.02 (3H, t, J = 7.2 Hz, -$CONHCH_2CH_3$), 1.47 (1H, m, -SO_2NCH_2CH**H**-), 1.62 (1H, m, -SO_2NCH_2CH**H**-) , 1.73 (2H,

m, -SO_2NCHC**H**$_2$-), 3.11 (3H, m, -CONHC**H**$_2$$CH_3$-, -$SO_2$N-CH**H**-), 3.41 (1H, m, - SO_2NC**H**H -), 3.85 (3H, s, -OC**H**$_3$), 3.92 (1H, q, *J* = 3.9 Hz, -SO_2NC**H**CH_2-), 7.14 (2H, td, J_1 = 6 Hz, J_2 = 3.4 Hz, Ar-H), 7.79 (2H, td, J_1 = 6 Hz, J_2 = 3.7 Hz, Ar-H), 7.89 (1H, t, *J* = 5.5 Hz, -CON**H**-). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.65, 24.01, 30.60, 33.44, 49.05, 55.65, 61.64, 114.48, 128.35, 129.57, 162.70, 170.75. ESI+MS (*m/z*): 170.93 ($C_7H_7O_3S$), 239.99 ($C_{11}H_{14}NO_3S$), 313.14 (M+H) , 335.23 (M+Na) , 376.30 (M+2H+Na+K). ESI-MS (*m/z*): 311.18 (M-H).

1-((4-Methoxyphenyl)sulfonyl)-N-propylpyrrolidine-2-carboxamide (2i): The method described in **2h** was repeated by the addition of *n*-propylamine (1 mL, 12 mmol) to synthesize compound **2i**. It was purified by flash chromatography using ethyl acetate and benzene in a ratio of 3:2, followed by recrystallization from hydro-alcoholic solution to afford **2i** as a white solid (73%, 2.76 g; m.p. 90-92°C) (**Scheme-II**). m.f.: $C_{15}H_{22}N_2O_4S$. Elemental analysis calcd. (found) %: C, 55.19 (55.27); H, 6.79 (6.62); N, 8.58 (8.55). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.97 (3H, t, *J* = 7.3, -CH_2C**H**$_3$), 1.58 (4H, m, -C**H**$_2$$CH_3$, -$SO_2NCH_2$-C**H**$_2$-), 1.71(1H, m, -$SO_2$NCHC**H**H-), 2.18 (1H, m, -SO_2NCH-CH**H**-), 3.24 (3H, m, -CONHC**H**$_2$-, -SO_2NCH**H**-), 3.55 (1H, m, -SO_2NC**H**H-), 3.89 (3H, s, -OC**H**$_3$), 4.05 (1H, m, -SO_2N-C**H**-CH_2-), 6.95 (1H, m, -CON**H**-), 7.01 (2H, m , Ar-H), 7.78 (2H, m, Ar-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.23, 22.68, 24.27, 30.09, 41.23, 49.91, 55.61, 62.60, 114.45, 127.13, 129.89, 163.45, 171.05. ESI+MS (*m/z*): 170.87 ($C_7H_7O_3S$), 239.91 ($C_{11}H_{14}NO_3S$), 327.04 (M+H), 349.13 (M+Na).

N-Butyl-1-((4-methoxyphenyl)sulfonyl)pyrrolidine-2-carboxamide (2j): Compound **2b** (3 g, 10.5 mmol) was dissolved in 20 mL of ethyl acetate. *N*,*N*′-Dicyclohexylcarbodiimide (DCC) (1 g, 5 mmol) and hydroxybenzotriazole (HOBt) (0.7 g, 5 mmol) were added in the solution with continuous stirring to catalyze the reaction and maintain the temperature at about 0-5 ºC for 30 min. *n*-Butylamine (1.1 mL, 10.5 mmol) was added in the reaction mixture with continuous stirring for 2 h at room temperature. Filtered and washed the filtrate continuously with 4% HCl (3 times), $NaHCO_3$ solution (2 times) and at last with brine solution. The ethyl acetate layer was dried over anhydrous $MgSO_4$ for overnight and evaporate under vacuum to obtain compound **2j** as a white colour solid (64%, 2.3 g; m.p. 60-62 ºC). Elemental analysis of $C_{16}H_{24}N_2O_4S$ calcd. (found) %: C, 56.45 (56.59); H, 7.11 (7.03); N, 8.23 (8.37). ^{1}H NMR: (500 MHz, $CDCl_3$): δ 0.87 (3H, t, *J* = 7.2 Hz, -CH_2-C**H**$_3$), 1.28 (2H, q, *J* = 7.3 Hz, -CH_2C**H**$_2$$CH_3$-), 1.42 (3H, m, -C**H**$_2$$CH_2CH_3$, -$SO_2NCH_2$C**H**H) , 1.62 (1H, m, -SO_2NCH_2CH**H**), 1.73 (2H, m, -SO_2NCHC**H**$_2$-), 3.09 (3H, m, -NHC**H**$_2$$CH_2$-, -$SO_2$NCH**H**-), 3.40 (1H, m, - SO_2NC**H**H -), 3.85 (3H, s, -OC**H**$_3$), 3.94 (1H, m, -SO_2NC**H**CH_2-), 7.14 (2H, d , *J* = 9 Hz, Ar-H), 7.79 (2H, d , *J* = 8.3 Hz, Ar-H) , 7.85 (1H, m, -CON**H**-). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 13.66, 19.36, 24.63, 30.66, 31.13, 38.16, 49.05, 55.65, 61.64, 114.48, 128.35, 129.57, 162.70, 170.88. ESI+MS (*m/z*): 171.17 ($C_7H_7O_3S$), 240.19 ($C_{11}H_{14}NO_3S$), 341.27 (M+H), 363.33 (M+Na). ESI-MS (*m/z*): 339.24 (M-H), 283.31 ($C_{12}H_{15}N_2O_4S$).

1-((4-Methoxyphenyl)sulfonyl)-*N*-pentylpyrrolidine-2-carboxamide (2k): The method described in compound **2j** was repeated by the addition of *n*-pentyl amine (1.2 mL, 10.5 mmol) to synthesize compound **2k**. It was purified by flash chromatography using ethyl acetate and benzene in a ratio of 2:1, followed by recrystallization from hydro-alcoholic solution to afford compound **2k** as a grayish white solid (47%, 1.8 g, m.p.188-190 ºC (**Scheme-II**). Elemental analysis of $C_{17}H_{26}N_2O_4S$ calcd. (found) %: C, 57.60 (57.38); H, 7.39 (7.52); N, 7.90 (7.52). ^{1}H NMR: (400 MHz, DMSO-d_6): δ 0.86 (3H, t, *J* = 6.8 Hz, -CH_2C**H**$_3$), 1.26 (4H, m, -C**H**$_2$$CH_3$, -C**H**$_2$$CH_2CH_3$-), 1.36 (1H, m, -$SO_2NCH_2$C**H**H-), 1.39 (2H, m, -CONHCH_2C**H**$_2$-), 1.46 (1H, m, -SO_2NCH_2CH**H**-) , 1.76 (2H, m, -SO_2NCHC**H**$_2$-), 3.06 (2H, m, -CONHC**H**$_2$-), 3.13 (1H, m, -SO_2NC**H**HCH_2-), 3.41(1H, m, -SO_2NCH**H**-), 3.95 (4H, m, -OC**H**$_3$, -SO_2NC**H**CH_2-), 7.12 (2H, dd , J_1 = 6 Hz, J_2 = 2 Hz, Ar-H), 7.77 (2H, dd, J_1 = 6.8 Hz, J_2 = 2 Hz, Ar-H) , 7.85 (1H, t, *J* = 8 Hz, -CON**H**-). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 14.76, 22.68, 24.27, 27.14, 29.21, 30.04, 41.23, 51.95, 55.63, 62.61, 114.46, 127.14, 129.89, 163.45, 171.06. ESI+MS (*m/z*): 170.89 ($C_7H_7O_3S$), 239.98 ($C_{11}H_{14}NO_3S$), 355.15 (M+H) , 393.17 (M+K).

***N*-Hexyl-1-((4-methoxyphenyl)sulfonyl)pyrrolidine-2-carboxamide (2l):** The compound was synthesized according to the general procedure of compound **2k** with the respective *n*-hexylamine (1.4 ml, 10.5 mmol) to afford compound **2l**. The solid was purified by flash chromatography using ethyl acetate and benzene in a ratio of 2:1, followed by recrystallization from hydro-alcoholic solution to get compound **2l** as a white solid (52%, 2 g; m.p. 74-76 ºC) (**Scheme-II**). Elemental analysis of $C_{18}H_{28}N_2O_4S$. calcd. (found) %: C, 58.67 (58.43); H, 7.66 (7.79); N, 7.60 (7.48). ^{1}H NMR: (400 MHz, DMSO-d_6): δ 0.86 (3H, t, *J* = 6.8 Hz, -CH_2C**H**$_3$), 1.27 (6H, m, -C**H**$_2$$CH_3$-, -C**H**$_2$$CH_2$-$CH_3$, -C**H**$_2$$CH_2CH_2CH_3$-), 1.35 (2H, m, CONHC$H_2$C**H**$_2$-), 1.45 (1H, m, -$SO_2NCH_2$C**H**H-), 1.63 (1H, m, -SO_2NCH_2CH**H**-), 1.74 (2H, m, -SO_2NCHC**H**$_2$-), 3.06 (2H, m, -CONHC**H**$_2$-), 3.07 (1H, m, -SO_2NC**H**H-), 3.41 (1H, m, -SO_2NCH**H**-), 3.93 (4H, m, -OC**H**$_3$, -SO_2NC**H**CH_2-), 7.12 (2H, dd , J_1 = 9.2 Hz, J_2 = 2 Hz, Ar-H), 7.77 (2H, dd, J_1 = 6.8 Hz, J_2 = 2 Hz, Ar-H) , 7.82 (1H, m, -CO-N**H**-). ^{13}C NMR: (125 MHz, $CDCl_3$): δ 14.41, 19.69, 22.29, 24.87, 28.92, 30.70, 31.92, 40.27, 49.10, 61.67, 63.75, 114.82, 128.10, 129.59, 162.04, 170.98. ESI+MS (*m/z*): 170.86 ($C_7H_7O_3S$), 239.83 ($C_{11}H_{14}NO_3S$), 369.18 (M+H), 370.32 (M+2H).

Biological evaluations

Cytotoxicity: Cytotoxicity study of the compounds **2b-l** was carried out on HUVECs and normal Vero cell by MTT assay [19,20] and RPMI 8226 by MTS assay [21]. The cells were procured from ATCC. The compounds were screened for anti-multiple myeloma activity and antiangiogenic properties along with their cytotoxicity towards normal epithelial cells.

Cytotoxicity analysis on HUVECs by MTT assay: The primary culture of HUVECs was done in endothelial growth medium-2 and cells between 2-5 passages were considered for the study. In each well of a 96-well microtiter plate, 1×10^4 HUVECs were seeded. Fresh DMEM with 10% FBS, penicillin, and streptomycin were added in each well. The culture was grown in a humidified atmosphere of 5% CO_2 at 37 ºC. The cells were then exposed to different concentrations of test compounds and standard drugs, in triplicate, in DMEM and incubated for 24 h. After 24 h, the cells were feed with 50 µL

of fresh medium containing 1 mg/mL of MTT. The plates were incubated for further 4 h in the dark at 37 ºC. The metabolically active cells reduced the MTT into water-insoluble MTT-formazan crystals, which were dissolved in DMSO and Sorensen's glycine buffer solution was added to adjust the pH (7.0-7.4). The absorbance was recorded in an ELISA plate reader at 540 nm. Doxorubicin was taken as standard.

Cytotoxicity Analysis of compounds against vero cells by MTT assay: VERO cells are normal, adherent, primary kidney cells from adult African green monkey (Vervet monkey) *Chlorocebus aethiops*. The culture medium was taken as DMEM with 20% FBS. The remaining protocol was followed as described above. The absorbance was recorded immediately in an ELISA plate reader at 570 nm. Doxorubicin was taken as standard.

Cytotoxicity analysis on RPMI 8226 (MM) by MTS assay: 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 (1×10^4/well) were placed on 96-well cell culture plates in fresh growth medium and grown to 70-80% confluency. Subsequently, a range of concentrations of test compounds and standard drugs were placed in RPMI 1640 base medium in serial dilutions (100 µL/well) in triplicate. The culture plate was incubated for 24 h. After incubation the plate was centrifuged (5 min at 200 g) to pellet the cells and the medium was removed by syringe 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 ºC 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. Doxorubicin was taken as standard.

Proliferation assay method: HUVECs were serum-starved overnight and seeded with 1×10^4 cells/well in 96 well plates 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 **2b**, **2d**, **2i** and **2k** in triplicate. Staurosporine was used as a standard. The HUVECs were challenged with 50 ng/mL of VEGF and incubated for 48 h. After incubation, the cells were trypsinized and the viable cells were counted in a hemocytometer following a dye exclusion method using trypan blue.

Docking methods: Molecular docking study of the most active compound **2b** was performed. 3D-crystal structure of VEGFR-2 (PDB code 3VHE) [22] complexed with an inhibitor was imported from RCSB PDB to LibDock protocol of DS 4.1 Accelrys [23]. The steps for docking were followed as described in LibDock protocol. The 2D-structure of the compound was drawn in Chem 3D ultra 12.0 [24] and was imported to Dock Ligand tools. LibDock [25] is a high throughput molecular docking algorithm. The binding mode of compound **2b** along with binding energy was calculated and interactions with different amino acid residues were studied.

RESULTS AND DISCUSSION

The designed compounds were synthesized and their structures were elucidated using ^{1}H & ^{13}C NMR and elemental analysis. The spectra of the synthesized compounds are well correlated. At the preliminary screening of the compounds with RPMI 8226 cell line, five compounds *viz.* **2b**, **2d**, **2f**, **2i** and **2k** were found to have anticancer activity on multiple myeloma (MM) (Table-1). Compound **2b** is the most active compound with IC_{50} = 2.02 µM on RPMI 8226 and IC_{50} = 3.37 µM on HUVECs showing both effective primary antiangiogenic and antitumor activities (Table-2). The compound is considered non-toxic to the primary epithelial cells, which is evident from the IC_{50} value (53.13 µM) obtained from cytotoxicity study on vero cell line. Compound **2f** was found to be equally cytotoxic to normal epithelial cells as on MM cells (IC_{50} = 3.14 µM on vero cells and IC_{50} = 3.14 µM on RPMI 8226 cells). Anti-proliferative study of the four compounds showed that the number of HUVECs in the untreated group rose to 32×10^4 cells compared to **2b**, **2d**, **2i** and **2k** where the number rose to 2×10^4, 9×10^4, 5×10^4 and 3×10^4, respectively. This result exhibits the antiproliferative action of the test compounds and compound **2b** is the most effective amongst all the four compounds (Fig. 3). Molecular docking simulation of comp-

TABLE-1
in vitro CYTOTOXIC ACTIVITIES OF TARGET COMPOUNDS **2b-2l** AND DOXORUBICIN AS A POSITIVE CONTROL OBTAINED IN 24 h INCUBATION

Compd.	R	IC_{50} (µM) ± SD		
		HUVEC	RPMI8226	Vero
2b		3.37 ± 0.21	2.02 ± 0.37	53.13 ± 0.25
2c	$-CH_3$	24.59 ± 0.76	17.44 ± 0.29	Nd
2d	$-C_2H_5$	4.38 ± 0.21	3.23 ± 0.29	33.07 ± 0.11
2e	$-NHNH_2$	29.41 ± 0.63	19.02 ± 1.13	Nd
2f	-H	4.17± 0.89	3.26 ± 0.30	3.14 ± 0.43
2g	$-CH_3$	25.89 ± 0.53	23.1 ± 1.09	Nd
2h	$-C_2H_5$	> 100	87.16 ± 2.11	Nd
2i	$-C_3H_7$	4.89 ± 0.07	3.12 ± 1.87	31.49 ± 0.16
2j	$-C_4H_9$	21.65 ± 0.26	20.87 ± 0.32	Nd
2k	$-C_5H_{11}$	3.11 ± 0.76	2.33 ± 0.42	20.49 ± 0.16
2l	$-C_6H_{13}$	37.89 ± 0.25	41.27 ± 0.16	Nd
Doxorubicin[b]		0.65 ± 0.12	0.14 ± 0.66	0.6 ± 0.11

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

TABLE-2
PROLIFERATION ASSAY OF COMPOUNDS **2b**, **2d**, **2i**, **2k** WITH STAUROSPORINE AS A STANDARD ON HUVECs

Conc. (μM)	Cells/mL (X10000)					
	UT	**2b**	**2d**	**2i**	**2k**	Stauro
10	32	2	9	5	3	1
1	26	12	20	17	14	6
0.1	32	26	32	30	28	23
0.01	34	31	35	32	32	29
0.001	36	32	36	34	33	30

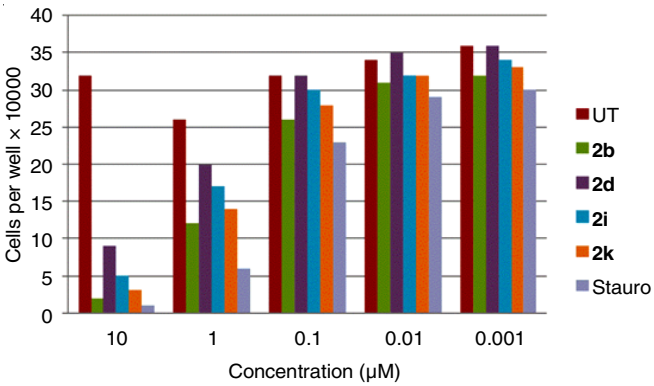


Fig. 3. Antiproliferative assay of test compounds on HUVECs. UT: Untreated; Sample codes: **2b**, **2d**, **2i**, **2k**, standard: *Staurosporine*

ound **2b** revealed that it adopted an orientation as observed with type-II VEGFR-2 inhibitors in the hinge region. The 3D binding pose of compound **2b** was generated with a LibDock score of 101.46 and the calculated binding energy is -89.78 kcal/mol. Essential interactions of compound **2b** with the amino acid residues were noted. The oxygen atoms of -SO_2- moiety forms hydrogen bonds with PHE1047 (2.49 Å), LYS868 (1.66 Å), ASP1046 (1.94 Å) and CYS1045 (2.23 Å) residues (Fig. 4). This region in the receptor active site is hydrophobic and deeply buried as it is the 'DFG' motif where PHE1047 induces 'DFG-out' conformation to the receptor. The carbonyl moiety extends to the back of the hydrophobic pocket and forms hydrogen bonds with ASP1046 (1.92 Å) and CYS1045 (2.51 Å). The hydrogen atom of the carboxyl group makes a hydrogen bond with the most critical GLU885 (1.95 Å) residue. The phenyl ring also shows favorable non-covalent interactions with the amino acid residues.

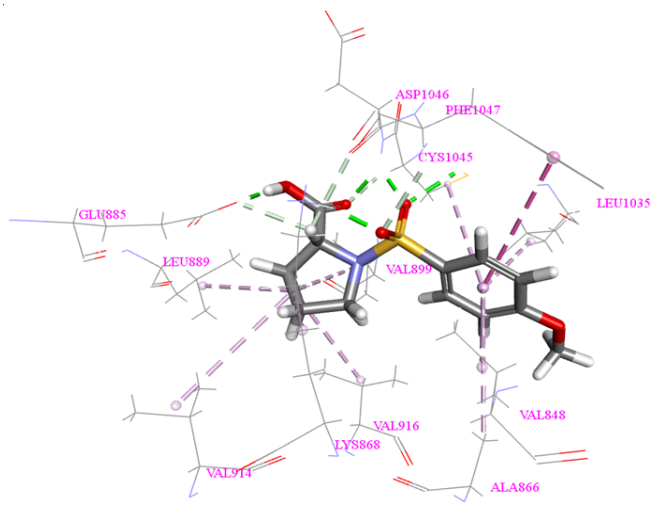


Fig. 4. Predicted binding mode of **2b** (stick model) at the active site of VEGFR-2 (PDB ID: 3VHE). Hydrogen bonds between the **2b** atoms and the amino acid residues (line representation) are shown as green dotted lines (------)

Pharmacokinetics and drug-likeness prediction by Swiss ADME: All the target compounds follow the Lipinski's rule of five and also exhibit good GI absorption values. Especially, compound **2b** shows better TPSA values with HBA properties (Table-3). Compound **2b** also exhibits good log P value, which enhances drug likeness of the molecule.

Conclusion

The present study infers that bioisosteric modification of the major metabolite of thalidomide along with the imposition of conformational restriction in the design is promising. It led to the identification of five compounds as potent inhibitors of MM cells (RPMI 8226). They are also antiangiogenic, as evident from the HUVEC results. Cytotoxicity study of the compounds on normal epithelial cells is significantly enough. It detects one compound **2f** as equally cytotoxic on VERO cells, *i.e.*, it is not safe on normal cells and not fit for further studies. The antiproliferative study helped us to confirm the cytostatic potential of the compounds on rapidly proliferating cells. Compound **2b** was found to be the most active molecule with anti-MM effect, antiangiogenic, comparatively non-toxic to normal epithelial cells, and a potent cytostatic agent against highly

TABLE-3
ENUMERATED PHARMACOKINETIC PROPERTIES, DRUG-LIKENESS PREDICTIONS AND ADME PARAMETERS OF COMPOUNDS BY SwissADME

Compd.	m.w.	cLog P	Lipinski rule of five violation	TPSA (Å^2)	HBD	HBA	Number of rotatable bonds	GI absorption
2b	285.32	0.95	0	92.29	1	6	4	High
2c	299.34	1.4	0	81.29	0	6	5	High
2d	313.37	1.71	0	81.29	0	6	6	High
2e	299.35	0.22	0	110.11	2	6	5	High
2f	284.33	0.65	0	98.08	1	5	4	High
2g	298.36	1.01	0	84.09	1	5	5	High
2h	312.38	1.29	0	84.09	1	5	6	High
2i	326.41	1.64	0	84.09	1	5	7	High
2j	340.44	1.96	0	84.09	1	5	8	High
2k	354.46	2.32	0	84.09	1	5	9	High
2l	368.49	2.63	0	84.09	1	5	10	High

proliferative cells. The docking study reveals that synthesized compounds share the position in the bioactive site of the receptors where type-II inhibitors of VEGFR-2 occupy the active site.

CONFLICT OF INTEREST

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

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