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Identification, Biological Evaluation & Retrometabolic Drug Designing of Active Metabolites of 5-Amino-4-(3, 4-Dimethoxy Phenyl Sulfonamido)-5-Oxopentanoic Acid (SM-1) As Antimultiple Myeloma and Antiangiogenic Agents

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Abstract
A candidate drug, SM-1, with promising anticancer and antiangiogenic activity, was subjected to *in vivo* metabolite study in rabbits to identify if any of its metabolites is more active andless toxic than the parent candidate drug. The probable metabolites were predicted using MetaSite software. SM-1 and the predicted metabolites were docked,*in-silico,*at the active site of VEGFR-2 using the LibDock protocol of Discovery studio. VEGFR-2 is a well-known pro-angiogenic molecular target. Predicted metabolites having comparable binding affinity to the receptor with that of SM-1 were chosen for identification. Following oral administration of SM-1 to the rabbits, the collected blood samples were analyzed using RP-HPLC, and three metabolites, M-1, M-3 & M-4, were identified with the help of synthetic standards. The anticancer and antiangiogenic properties of the metabolites were evaluated on RPMI-8226 (Multiple Myeloma) and HUVEC cell lines, respectively. The cytotoxic effect on VERO cells was checked to study their toxic effect on normal epithelial cells. M-3 exhibited more activity than SM-1 and was found to be less toxic than SM-1. The structure of M-3 was explored as a lead for further drug design, suggesting six new molecules which could be more active than M-3.

Keywords: Antiangiogenic, VEGFR-2, RPMI-8226, HUVEC, Metabolite Study, RP-HPLC

Introduction

5-amino-4-(3, 4-dimethoxy phenyl sulfonamido)-5-oxopentanoic acid (SM-1), designed as a candidate anticancer drug, found to have promising anticancer and antiangiogenic properties as reported in our earlier investigation (Sen et al., 2011). The compound showed 86.34% inhibition of Ehrlich Ascites Carcinoma in Swiss Albino mice as calculated against Mitomycin C, the standard compoundused for this study (De & Ghosh, 1976; De& Pal, 1977). The result of the cytotoxicity study of SM-1 on VERO, a normal epithelial cell line, reveals that it is not toxic to normal cells (IC_{50} is 51µM, in comparison to 0.5µM for doxorubicin, the standard compound). However, on another normal cell line, HUVEC, the compound shows prominent cytotoxicity (IC_{50} is 1 µM, in comparison to 0.5 for doxorubicin, the standard compound). This selective toxicity towards HUVEC indicates that it has antiangiogenic activity, which might be through inhibition of VEGFR-2 receptor expressed in the HUVECs, a key player in the development of angiogenesis (Ferrara et al., 2003; Suarez et al., 2006; Sakurai & Kudo, 2011; Lian et al., 2019). Inhibition of the VEGFR-2 signaling pathway is regarded as an attractive therapeutic target for inhibition of tumor angiogenesis and subsequent tumor growth (Tugues et al., 2011; Aziz et al., 2016). In fact, HUVECs are used to evaluatethe antiangiogenic activity of compounds described in severalpieces of literature (Park et al., 2006; Goodwin, 2007; Staton, 2009). With this rationale, the *in silico*interaction (docking)of SM-1and its metabolites with the VEGFR-2 receptor (Asthana et al., 2015; Kang et al., 2018; Rampogu et al., 2019) is establishedherein. Further, authors tried to establish the anticancer activity of SM-1and the identified metabolites in RPMI-8226 cell line,a human multiple myeloma cell line (Koerber et al., 2015; Malacrida et al., 2019). Multiple myeloma (MM) is a malignant B-cell neoplasm characterized by the monoclonal plasma cells proliferation in the bone marrow (Rajkumar, 2020) and is a prevalent hematological malignancy affecting people globally with a survival rate of 52% (Costello et al., 2019; IARC/WHO, 2019; NCI, 2020). The antiangiogenic activity is studied extensively through *in vitro* enzyme kinase assay (Guo et al., 2015; Reddy et al., 2019) and VEGFR-2 Phosphorylation inhibition assay using western blotting technique (Shibuya, 2011; Arao et al., 2011; Yu et al., 2015: Rathinavelu et al., 2017).

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In the first place, an investigation is due to find if any metabolite of SM-1 is more potent than SM-1. Consequently, an attempt is made to characterize the metabolites of SM-1 and perform cytotoxicity study of the metabolites followed by retro-metabolic drug designing, in quest of new candidate drugs with better activity.

In the wake of characterizing the metabolites, a qualitative study is designed using RP-HPLC (Yeh et al., 1999; Nikolin et al., 2004), where detectability of the metabolites is augmented by increasing the concentration of the compounds in the samples to be injected in HPLC by pooling the blood samples from several rabbits, concentrating the extract from serum through evaporation in an inert atmosphere, and increasing the volume of injection in HPLC.

Experimental

1. Materials

SM-1 and the synthetic standards of identified metabolites were synthesized, purified, and characterized at A.P.C. Ray Memorial Cancer Chemotherapeutic Research Unit, College of Pharmaceutical Sciences, Berhampur, Odisha. Methanol, acetonitrile, and water (HPLC grade) were purchased from Merck (India) Ltd., Mumbai, India. The rest of the chemicals of analytical grade used in the experiments were purchased from different commercial sources. UV-VIS Spectrophotometer (UV-2450, Shimadzu, Japan) was used to determine λ_{max} of the SM-1 and its metabolites. For separation of SM-1 and its metabolites, isocratic HPLC (Waters India) with Waters 510 pump and UV detector was used.

2. Methods

2.1 Anticancer & Antiangiogenic activity of the candidate drug

Cell-based inhibition study on RPMI-8226 cell line

Cytotoxicity study of the compound SM-1 was carried out on RPMI-8226 cell line by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assaymethod (Malich et al., 1997; Ghosh et al., 2021).

In Vitro VEGFR-2 tyrosine enzyme kinase inhibition assay

The *in vitro* kinase inhibition assay against VEGFR-2 (Jia et al., 2007; Wang et al., 2017) was detected using the ADP-Glo™ kinase assay kit (Promega, Madison). The VEGFR-2 tyrosine kinase (0.6 ng/mL) were incubated with substrate (0.2 mg/mL), SM-1 (1μM) and ATP (50 μM) in a final buffer of Tris 40 mM, $MgCl_2$ 10 mM, BSA 0.1 mg/mL, DTT 1 mM in 96-well plate with the total volume of 5 μL. The assay plate was incubated at 30 °C for 1 h. After the plate was cooled at room temperature for 5 min, 5 μL of ADP-Glo reagents was added into each well to stop the reaction and consume the remaining ADP within 40 min. In the end, 10 μL of kinase detection reagent was added into the well and incubated for 30 min to produce a luminescence signal. VICTOR-X multi-label plate reader read the luminescence. The signal was correlated with the amount of ATP present in the reaction and was inversely correlated with the kinase activity.

VEGFR-2 Phosphorylation inhibition assay using western blotting technique

Inhibition of phosphorylation at Tyr-1175 residue of VEGFR-2 was checked using the immunoblotting method (Shibuya, 2011; Arao et al., 2011; Yu et al., 2015: Rathinavelu et al., 2017). HUVEC grown in 12 well plates were serum-starved overnight and treated with test sample for 2 hours followed by treatment with 50ng/ml of VEGF for 15 minutes. Cells were washed twice with cold PBS, lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS) supplemented with Complete R protease inhibitors (Roche), Samples were supplemented with 5% β- mercaptoethanol, heated (95°C; 12 minutes); size-fractionated on a 9% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked for 45 minutes in PBS-T-milk (0.05% Tween, 5% dried fat-free milk); incubated with primary antibody (3 hours; RT; diluted in PBS-T milk), washed (PBS-T), and incubated with secondary antibodies (1hour; RT; 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).

2.2 Prediction of the metabolic fate

Prediction of major metabolites of SM-1 was made by the software MetaSite 6.0 (Molecular Discovery Ltd., Middlesex, UK) using the P450 liver model. The structure of SM-1 was drawn using Chem Bio Draw Ultra 12.0 and submitted to MetaSite in .mol format using default parameters with all CYP models, CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. All CYP models were ran using as input the whole conformer population obtained from both Hybrid Monte Carlo (HMC) module and the conformational search algorithm implemented in MetaSite (Caron et al., 2007; Trunzer et al., 2009).

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2.3 Molecular Docking with VEGFR-2

The binding affinities of the candidate drug and the predicted major metabolites with the VEGFR-2 receptor were studied using 3-D QSAR software (Discovery studio, Dassault Systèmes BIOVIA). The molecular docking study was initiated to explore the extent of the interaction of SM-1 and the predicted major metabolites with the amino acid residues at the allosteric site of VEGFR-2. The 3D Crystal structure of VEGFR-2 was taken from Research Collaboratory for Structural Bioinformatics-Protein Data Bank (RCSB-PDB),with PDB accession code, 3VHE (Oguro, 2010). The 3D structure of the receptor was imported to the LibDock protocol of Discovery Studio 4.1 (Diller &Merz, 2001; Rao et al., 2007).LibDock is a hotspot-based docking method for screening large libraries using catConf (Smellie et al., 1995) and LibDock algorithms (Diller & Li, 2003). The receptor was made free of the bound inhibitor and water molecules. The protein was prepared by inserting the missing atoms to incomplete residues (optimizing side-chain conformations), remodelling missing loop regions, deleting all alternate conformations, standardizing the atom names, and protonating the titratable residues using predicted pKs. Polar hydrogens were added. The binding site was defined as per PDB site records. 3Dstructure of SM-1 and the predicted major metabolites were drawn in Chem 3D ultra 12.0 software and imported to Dock Ligands tools. These ligands, SM-1, and its predicted metabolites were prepared by setting standard formal charges on common functional groups. During the refinement procedure, the conformation of the VEGFR-2 receptor was kept rigid, allowing the ligands to flex. From the initial structure of the ligand, random conformations were generated through molecular dynamics, which were refined by simulated annealing and full force field minimization.

2.4 Animals

Six healthy rabbits (New Zealander strain) of either sex (3 male and 3 female) weighing 1.8 kg (±100g) were selected and housed for a week under standard conditions, following the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). Animals were deprived of food for 12hrs before administration of SM-1, while water was allowed *ad libitum*. Each rabbit was administered a dose of 0.6 mmol/kg bodyweight of SM-1 by oral gavage in the form of an aqueous suspension. For conducting this experiment, permission was taken from the Institutional Animal Ethics Committee, College of Pharmaceutical Sciences, Berhampur, Odisha, India (Regn. No. 1170/P0/Re/S/08/CPCSEA).

2.5 Biological Sample preparation

For qualitative estimation of metabolite in rabbits, after oral administration of SM-1, the blood samples were collected and storedat different intervals fora period of 12hrs and pooled together. The basic steps for the preparation of biological sample for analysis by HPLC like centrifugation, protein precipitation, and liquid-liquid extraction wereperformedfollowing the protocol reported in ourearlier investigation (Laha et al., 2015). The pooled blood sample was concentrated using a nitrogen evaporator to increase the sensitivity of HPLC method. After complete evaporation to dryness, it was reconstituted with the mobile phase solvent used in HPLC.

2.6 Chromatographic condition

For separation of SM-1 and its metabolites present in rabbit's plasma, an isocratic HPLC system (Waters India) equipped with Millennium-32 software, a single pump (Waters 510), a UV Detector (Waters 486 tunable absorbance detector), and an RP-C_{18} column (Bondapak C_{18}, 250 × 4.6 mm, 5 µm) were used. Separation of SM-1 and its metabolites was achieved by taking Methanol-Acetonitrile-Phosphate buffer (pH 7.4; 50 mM) = 6:3:1 (v/v/v) as the mobile phase with a flow rate of 1.5 mL/min. Detection of the analytes was carried out by setting the wavelength at 265 nm. To increase the sensitivity of the machine, an injection loop of 200 µL capacity was used.

2.7 Synthesis and characterization of the metabolites

The route for synthesizing the three predicted metabolites, M-1, M-3, and M-4,with comparable binding affinity to VEGFR-2 with that of SM-1 is depicted in Fig. 1.

Synthesis of 3, 4-dimethoxyphenylsulfonyl chloride (B):

Veratrole (A) (13.8 g, 0.1 mole) was added dropwise to a solution of chlorosulfonic acid (11.6 g, 0.1 mole) in 25 mL chloroform maintained at 0°C with stirring, within 30 minutes, followed by stirring for 45 minutes at room temperature. Later, the content was poured on crushed ice (100 g) and subsequently extracted with chloroform (3x25 ml). The extract was made water-free by keeping overnight with a small amount of magnesium sulfate. The compound was distilled, dried using a pistol dryer, and purified by recrystallization with ethanol. Solid with light pink color, Yield 89.93%, 21.26 g; m.p. 66-69°C.

Synthesis of 5-amino-4-(3, 4-dimethoxy phenylsulfonamido)-5-oxopentanoic acid (M-4):

To a stirred aqueous solution of glutamic acid, C (7.35g, 0.05 mole) in alkaline medium, 3, 4-Dimethoxy

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benzene-1-sulfonyl chloride, B (11.83g, 0.05 mole) was added dropwise within 30 minutes. Following this, 4-Dimethyl aminopyridine (20 mg) was added to this reaction medium as a catalyst and stirred for another 30 minutes. Throughout the reaction, the pH of the medium was maintained at 10 by the gradual addition of sodium carbonate. The solution was filtered and acidified to pH 2-3 and subsequently extracted thrice with ethyl acetate. The extract was dried with anhydrous $MgSO_4$ overnight. The compound collected after distillation was bone dried and purified by flash chromatography using ethyl acetate and benzene in a ratio of 9:1. Further, this was recrystallized using hydroalcoholic solution—white solid, Yield 74.94%, 13.01 g; m.p. 214-216°C.

^{1}H NMR (300 MHz, DMSO-d_6): δ 12.248 (br, 2H, -COOH), 8.109 (s, 1H, -SO_2NH-), 7.319 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.268 (s, 1H, Ar- H), 7.069 (d, 1H, *J* = 8.4Hz, Ar-H), 3.923 (s, 3H,-OCH_3), 3.895 (s, 3H,-OCH_3), 3.843 (m, 1H, -NHC**H**-), 2.230 (m, 2H, -NHCHCH_2C**H_2**-), 1.754 (m, 2H, -NHCHC**H_2**CH_2-). **^{13}C-NMR (DMSO-d_6)**: δ 25.13, 30.04, 55.31, 58.20, 112.23, 115.63, 118.41, 133.42, 150.12, 153.02, 174.73, 178.43. **Mass (*m/z*) ESI TOF**: 348.06 (M+H), Anal. Calcd. for $C_{13}H_{17}NO_8S$ (347.34): C, 44.95; H, 4.93; N, 4.03%. Found: C, 45.10; H, 4.96; N, 4.37%.

Synthesis of 1-(3,4-dimethoxyphenylsulfonyl)-5-oxopyrrolidine-2-carboxylic acid (D):

To 7 g (~0.02 mole) of the compound, M-4, 10 mL of acetyl chloride was added and refluxed for 2 hrs. The content was poured in crushed ice and kept overnight. The compound was filtered and dried. The final product, D, was purified by recrystallization using propyl alcohol. White crystalline solid, Yield 92.5%, 6.14 g; m.p. 128-131°C.

Synthesis of 5-amino-4-(3,4-dimethoxyphenylsulfonamido)-5-oxopentanoic acid (SM-1):

To a suspension of D (6.6 g, 0.02 mole, in 30 mL water), excess liquor ammonia (0.06 mole) was added, shaken, and kept overnight in a closed glass container. The excess amine was evaporated out on a steam bath; 40 mL water was added, cooled, and acidified with HCl (2N) to congored paper. The precipitate was washed with water, filtered and dried. After complete drying of the final product, SM-1,it was recrystallized using ethanol. White solid, Yield 75.94%, 5.27 g; m.p. 183-185°C.

Synthesis of 5-amino-2-(3,4-dihydroxyphenylsulfonamido)-5-oxopentanoic acid (M-3):

Boron tribromide (15 gm, 0.06 mole) was added slowly to SM-1 (7 g, ~0.02 mole) taken in dichloromethane, maintaining the temperature between -10 to -15°C. After complete addition of the reagent, the temperature was increased gradually within a period of 30 minutes to reach room temperature (Vickery et al., 1979). Completion of the reaction was monitored by TLC. The final product, M-3, was purified by flash chromatography using ethyl acetate and dichloromethane in a ratio of 6:4 and recrystallized using ethanol. White solid, Yield 76.29%, 4.91 g; m.p. 145-152°C.

^{1}H NMR (300 MHz, DMSO-d_6): δ 7.927 (s, 1H, -SO_2NH-), 7.278 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.241 (s, 1H, Ar- H), 7.041 (d, 1H, *J* = 8.4Hz, Ar-H), 4.542 (s, 1H,-OH), 4.037 (s, 2H, -NH_2), 3.766 (m, 1H, -NHC**H**-), 2.280 (m, 2H, -NHCHCH_2C**H_2**-), 1.784 (m, 2H, -NHCHC**H_2**CH_2-). **^{13}C-NMR (DMSO-d_6)**: δ 25.70, 30.24, 56.10, 112.22, 115.61, 118.48, 133.44, 150.22, 153.12, 171.52, 173.13. **Mass (*m/z*) ESI TOF**: 319.10 (M+H), Anal. Calcd. for $C_{11}H_{14}N_2O_7S$ (318.30): C, 41.51; H, 4.43; N, 8.80%. Found: C, 41.62; H, 4.55; N, 8.73%.

Synthesis of 1-((3, 4-dimethoxy phenyl) sulfonyl)-4-hydroxy-5-oxopyrrolidine-2- carboxylic acid (F):

To a stirred aqueous Na_2CO_3 and 4-hydroxy-5-oxopyrrolidine-2-carboxylic acid solution, **E** (7.25 gm, 0.05 mole), 3,4-dimethoxybenzene-1-sulfonyl chloride, **B** (11.8 gm, 0.05 mole) was added dropwise within 30 minutes. After complete addition, DMAP (50 mg, 0.41 mmol) was added as a catalyst and stirred for 4 hours. The reaction was carried out maintaining a basic medium (pH 9-10) by gradual addition of sodium carbonate. The basic reaction mixture was filtered andsubsequently acidified to a pH 2-3 by addition of hydrochloric acid. The reaction mixture was then 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 dichloromethane in a ratio of 8:2. White solid, yield 77%; m.p. 155-160°C.

Synthesis of 5-amino-2-(3,4-dimethoxyphenylsulfonamido)-4-hydroxy-5-oxopentanoic acid (M-1):

To a suspension of **F** (6.9 g, 0.02 mole, in 30 mL water), excess liquor ammonia (0.06 mole) was added, shaken, and kept overnight in a closed glass container. The excess amine was evaporated out on a steam bath; 40 mL water was added, cooled, and acidified with HCl (2N) to congored paper. The precipitate was washed with water, filtered, and dried. Completion of the reaction was checked by TLC. The final product, **M-1,** was purified by flash chromatography using dichloromethane and ethanol in a ratio of 4:6 and recrystallized using ethanol. White solid, Yield 71.25%, 5.16 g; m.p. 158-162°C.

^{1}H NMR (300 MHz, DMSO-d_6): δ 7.933 (s, 1H, -SO_2NH-), 7.323 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.243 (s, 1H,

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Ar- H), 7.046 (d, 1H, *J* = 8.4Hz, Ar-H), 4.723 (s, 2H, -NH_2), 4.163 (s, 1H, -CHO**H**), 3.893-3.846 (o, 7H, -NHC**H**-, -OCH_3, -OCH_3), 2.286 (m, 2H, -C**H**OH), 1.784 (m, 2H, -NHCHC$\mathbf{H_2}$-). **^{13}C-NMR (DMSO-d_6)**: δ 31.84, 51.73, 56.15, 69.28, 112.18, 115.56, 118.51, 133.24, 150.02, 153.24, 172.17, 174.39. **Mass (*m/z*) ESI TOF**: 363.10 (M+H), Anal. Calcd. for $C_{13}H_{18}N_2O_8S$ (362.36): C, 43.09; H, 5.01; N, 7.73 %. Found: C, 43.12; H, 4.95; N, 7.66 %.

2.8 Identification of the metabolites

The solutions of SM-1 and the metabolites, M-1, M-3 & M-4, (Synthetic standards) were prepared separately with a 50 µg/mL concentrationusing the mobile phase solvent. These solutions were analyzed by HPLC separately and their retention time (R_t) values were recorded. The candidate drug and the three metabolites present in the rabbits' plasma were identified by matching their R_t values observed from the plasma sample earlier, with the pure candidate drug and the synthetic standards of the metabolites, respectively.

2.9 Biological evaluation of the metabolites

The cell-based inhibition study of the metabolites was performed in a very similar manner as was done for SM-1 to get to a position where a comparison of biological activity of the metabolites can be made with SM-1. The cytotoxicity study of the metabolites was performed on HUVEC and VERO cell lines as per the protocol reported in our earlier investigation(Sen et al., 2011) and on RPMI-8226 following the protocol described in the literature (Malich et al., 1997; Ghosh et al., 2021). *In Vitro* VEGFR-2 tyrosine enzyme kinase inhibition assay (Jia et al., 2007; Wang et al., 2017) and Inhibition of phosphorylation at Tyr-1175 residue of VEGFR-2 was also checked through western blotting technique (Shibuya, 2011; Arao et al., 2011; Yu et al., 2015: Rathinavelu et al., 2017).

2.9 Receptor based drug design of the active metabolite as a Lead molecule

We resort to scaffold hopping to design and identify novel isofunctional molecules with different scaffolds of the lead compound, M-3 (the active metabolite of SM-1), and better physicochemical properties. The Replace Fragment protocol present in Discovery Studio 4.1 was used, which performs scaffold hopping by replacing part of the scaffold structure while maintaining the favorable binding between the receptor and the ligand. The default libraries (1,495,478 fragments) were used for the work, and parity between chemotype diversity and isofunctional similarity with the original lead compound was ensured. The 3, 4-Dihydroxy phenyl nucleus of M-3 was selected for scaffold hopping as the remaining part of M-3 helped make meaningful interaction with the amino acid residues present at the active site of the receptor. Different linear and cyclic fragments were used to get the best scaffolds.

The novel ligands are scored using some scoring functions, which is a method to rapidly estimate the binding affinity of a ligandbased on candidate ligand pose geometry docked into a target receptor structure.

Results and Discussion

The candidate drug, SM-1, with established anticancer and antiangiogenic effect, was further subjected to a cell-based inhibition study on the RPMI-8226 cell line. The IC_{50} value of SM-1 was found to be 0.9 µM which is comparable to doxorubicin, the standard compound used in this cell-based inhibition assay with an IC_{50} value of 0.5 µM (Table-1). The antiangiogenic activity was further tested extensively by *in vitro*VEGFR-2 enzyme kinase inhibition assay, where the enzyme inhibition potency is expressed as IC_{50} value against sorafenib as a positive control. The IC_{50} value of SM-1 was 1.13 nM, compared to 0.77nM for sorafenib (Table-2).

Table-1 IC_{50} values of the candidate drug and its metabolites in different cell lines

Compound codes	IC_{50} (µM)		
	RPMI-8226	HUVEC	VERO
SM-1	0.9	1	51
M-1	5.6	19	38
M-3	0.6	0.7	62
M-4	23	42	74
Standard (Doxorubicin)	0.5	0.5	0.5

Table-2 Result of the in vitro enzyme kinase inhibition assay

Compound codes	IC_{50} (nM)
SM-1	1.13
M-1	34.84

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M-3	1.29
M-4	52.28
Standard (Sorafenib)	0.77

Further, it was established that this enzyme inhibitory activity of SM-1 is because of inhibition of phosphorylation of Tyr-1175 residue of VEGFR-2 as revealed by the western blotting technique (Fig. 2).
The major metabolites of SM-1 predicted by MetaSite are 5-amino-2-(3,4-dimethoxy phenylsulfonamido)-4-hydroxy-5-oxo-pentanoic acid (M-1), 5-amino-2-(3,4-dimethoxyphenyl sulfonamido)-4,5-dioxo-pentanoic acid (M-2), 5-amino-2-(3,4-dihydroxy phenylsulfonamido) -5-oxo-pentanoic acid (M-3), 2-(3,4-dimethoxyphenylsulfonamido) pentanedioic acid (M-4), 5-amino-2-(N-hydroxy-3,4-dimethoxyphenylsulfonamido)-5-oxo-pentanoic acid (M-5) and 3,4,5-trihydroxy-6-oxotetrahydro-2H-pyran-2-yl-5-amino-2-(3,4-dimethoxyphenylsulfonamido)-5-oxo-pentanoate (M-6).(Fig. 3)
Each of these predicted metabolites was subjected to 3D QSAR study to determine the binding affinity withthe VEGFR-2 receptor. Three of the predicted metabolites such as; M-1, M-3 and M-4 show binding affinity (expressed as Dock Score and Binding Energy) comparable with SM-1 (Table-3). Thus, an attempt was taken to identify these three metabolites only, with theidea that they might show antiangiogenic activity comparable with SM-1.

Table-3 Result of molecular docking of SM-1 & its metabolites with VEGFR-2

Compounds	Total dock score	Binding energy
SM-1	387.53	-170.465
M-1	384.62	-152.705
M-2	328.46	-124.724
M-3	376.03	-151.978
M-4	364.12	-169.499
M-5	308.13	-138.59
M-6	339.9	-128.868

The three predicted metabolites, M-1, M-3, and M-4, were synthesized, purified, and characterized by MS, ^{1}H &^{13}C NMR to use them as markers or standards for identifying metabolites present in rabbit's plasma. The retention time values (R_t) of these metabolites (standards) were used to identify the metabolites present in the rabbit's plasma. The chromatogram of the plasma sample showed eight peaks with R_t values as 1.252, 1.632, 2.043, 4.257, 6.331, 6.879, 8.849, and 12.087 mins (Fig. 4). The R_t values of the standards, SM-1, M-1, M-3 & M-4, were recorded individually as 8.851 (Fig. 5), 4.261 (Fig. 6), 6.353 (Fig. 7), and 12.063 (Fig. 8), respectively, maintaining the same chromatographic condition as that of for the plasma sample. The R_t values of the peaks found in the chromatogram of the plasma sample were compared with the standard R_t values, and the peak with R_t of 8.849 mins was identified to be SM-1. Similarly, the peaks with R_t 4.257, 6.331, and 12.087 mins were identified to be M-1, M-3 & M-4, respectively.
The three metabolites identified in rabbits' plasma were evaluated for their cytotoxicity activity on RPMI-8226, VERO, and HUVEC cell lines. One of the three metabolites, M-3, was more antiangiogenic and anticancer and less toxic than the parent candidate drug, SM-1 (Table-1). M-3 was further studied for its *In-Vitro* VEGFR-2 tyrosine enzyme kinase inhibition activity (Table-2)and Inhibition of phosphorylation at Tyr-1175 residue of VEGFR-2 through western blotting technique (Fig. 2). Both these studies suggested that the metabolite M-3 is a better drug candidate than SM-1, and possibly the anticancer activity shown by them is due to their antiangiogenic effect.
The structure of M-3 was subjected to further lead modification and optimization to design new molecules with better interaction with the VEGFR-2 receptor, which could show better biological activity than M-3. Using the scaffold hopping protocol of 3D QSAR studies, ninety-five newly designed molecules were enumerated. Six molecules were sorted using Lipinski's RO5 filter, binding energy LibDock score, total scoring functions, synthetic feasibility, and PAINS alert. These six newly designed molecules are, 5-amino-2-(6-fluoro-4-hydroxypyridine-2-sulfonamido)-5-oxopentanoic acid **(D-1)**, 5-amino-2-(3-(aminomethyl)furan-2-sulfonamido)-5-oxopentanoic acid **(D-2)**, 5-amino-2-(2-fluoro-6-hydroxypyridine-3-sulfonamido)-5-oxopentanoic acid **(D-3)**, 5-amino-2-(6-fluoro-3-hydroxypyridine-2-sulfonamido)-5-oxopentanoic acid **(D-4)**, 5-amino-2-(2-(aminomethyl)furan-3-sulfonamido)-5-oxopentanoic acid **(D-5)** and 5-amino-2-(5-(aminomethyl)furan-3-sulfonamido)-5-oxopentanoic acid **(D-6)** as depicted in Fig. 9.
Interaction of these designed compounds with the amino acid residues of VEGFR-2 was noted. The isomeric

forms of the compounds were also taken into account. The results of the docking parameters reveal that the designed molecules have better binding energy, LibDock score, and better estimated binding affinity scores than the lead compound M-3. While investigating the ligand interactions, it is evident that the lead molecule occupies some part of the ATP binding site and the allosteric site of VEGFR-2; hence, it can be considered type-II inhibitors. The molecules form hydrogen bonds with the crucial amino acids like GLU885, ASP1046, CYS919, CYS1045, LYS868. The hydrophobic interaction of the ligands with PHE1047, CYS919, LEU840, ALA866, VAL848 amino acid residues confirm that the compounds can bind with the active site of the receptor with the desired orientation and hence can probably induce a conformational change in the allosteric site and ATP binding site. The present investigation calls for the Synthesis and extensive biological evaluation of the newly designed molecules in future investigations.

Conclusions

SM-1, a candidate anticancer molecule, was subjected to *in vivo* metabolic profiling to develop newer drug candidates with better potentiality through ligand/receptor-based drug designing. Seven metabolites were detected in rabbit's plasma using HPLC, out of which only three metabolites, M-1, M-3, and M-4, were identified purposefully, with the help of the synthetic standards of these metabolites. They were evaluated on RPMI-8226, HUVEC, and VERO cell lines, and one of the metabolites, M-3, was found to have better activity than the parent candidate drug, SM-1. Thus M-3 may be regarded as the active metabolite of SM-1. The anticancer activity shown by SM-1 & M-3 may be because of their antiangiogenic property. Thus, the structure of M-3 was studied further through receptor-based drug designing, considering the structure of VEGFR-2 to develop better anticancer drug candidates. Six new molecules, such as D-1, D-2, D-3, D-4, D-5 & D-6, were suggested to show better antiangiogenic and anticancer activity than the active metabolite, M-3.

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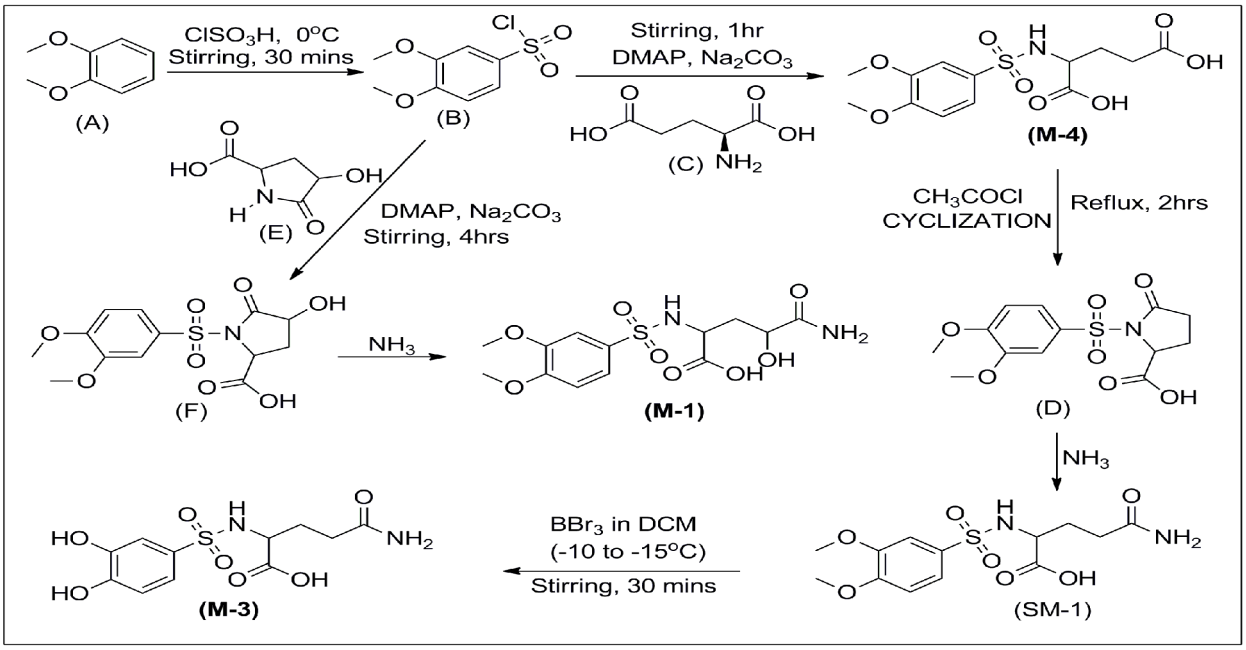

Fig. 1 Synthetic scheme of M1,M3 & M4

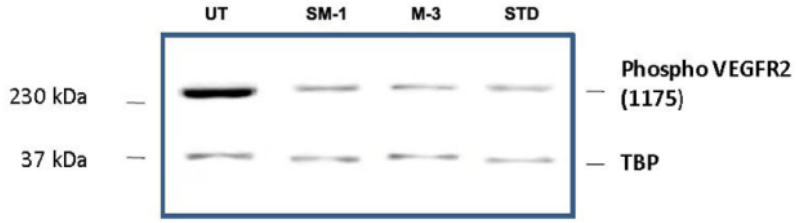

Fig. 2 Western blotting analysis showing inhibition of phosphorylation at Tyr-1175 of VEGFR-2 protein by SM-1 & M-3. UT-Untreated, STD-Standard (Staurosporine)

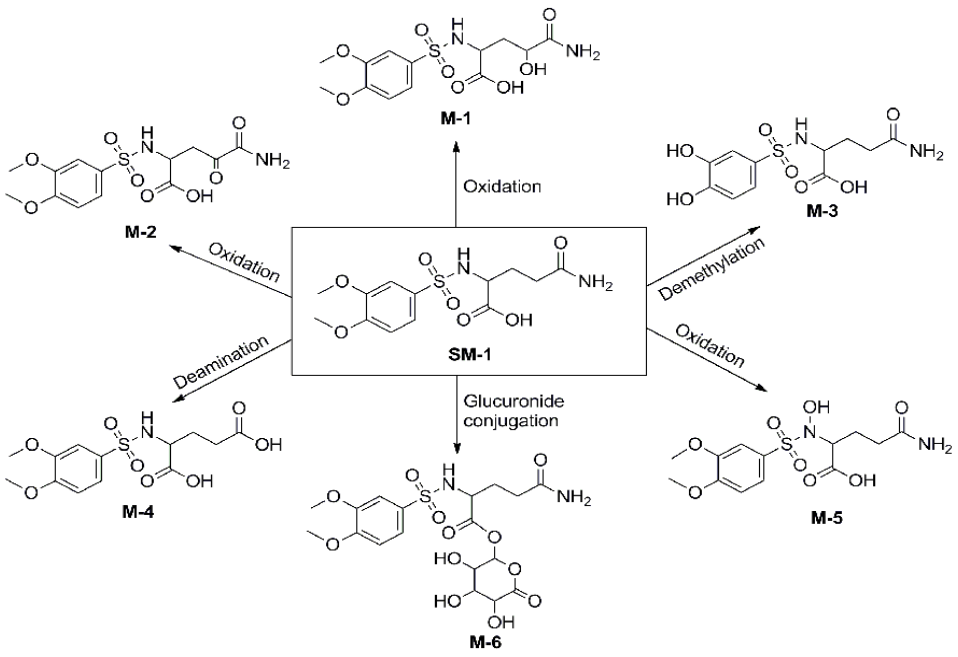

Fig. 3 Prediction of primary metabolites of SM-1 by MetaSite

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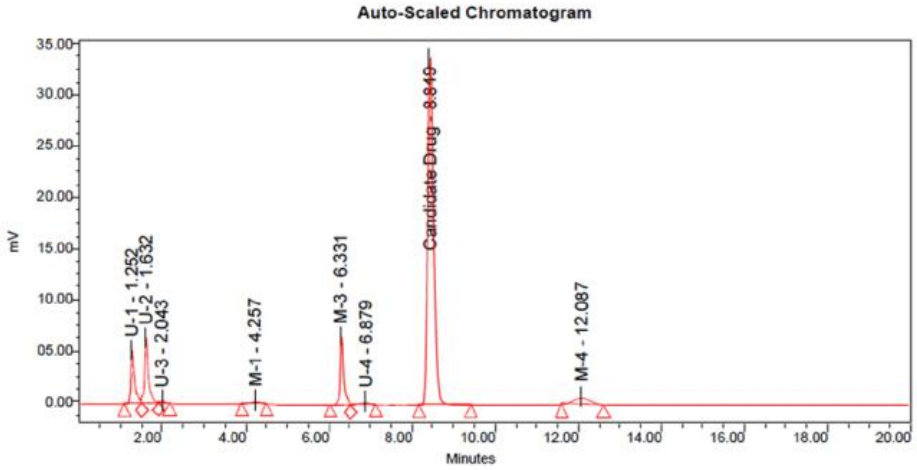

Fig. 4 HPLC Chromatogram of sample extracted from rabbit's plasma

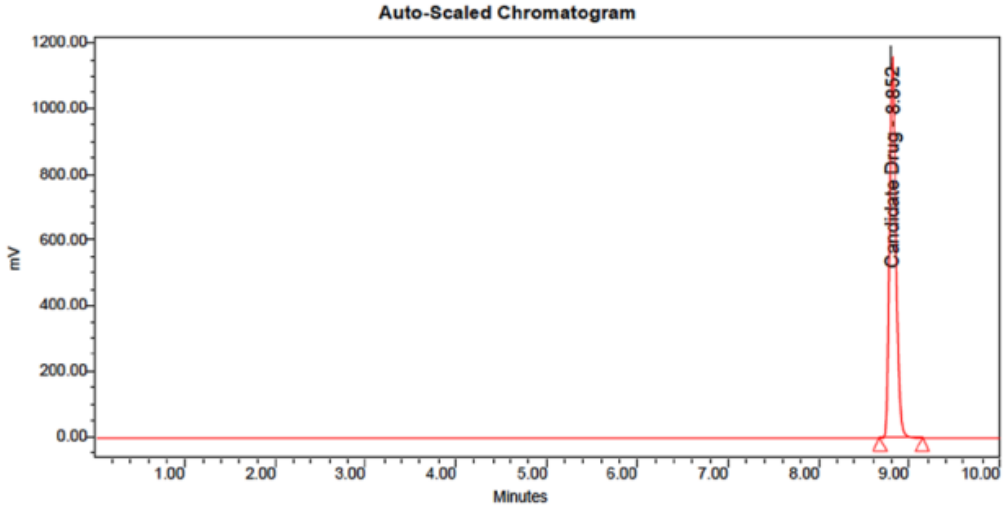

Fig. 5 HPLC Chromatogram of candidate drug (SM-1)

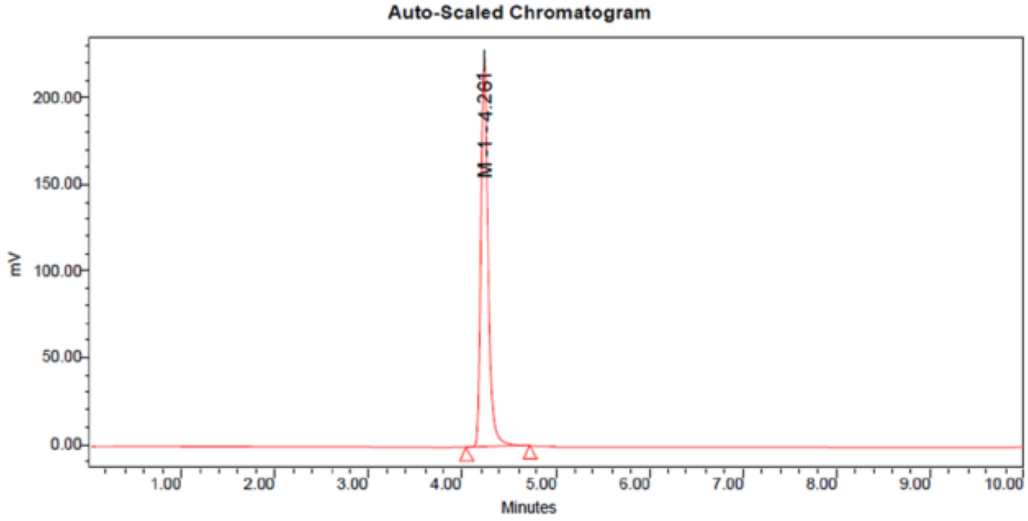

Fig. 6 HPLC Chromatogram of M-1 (Synthetic Standard)

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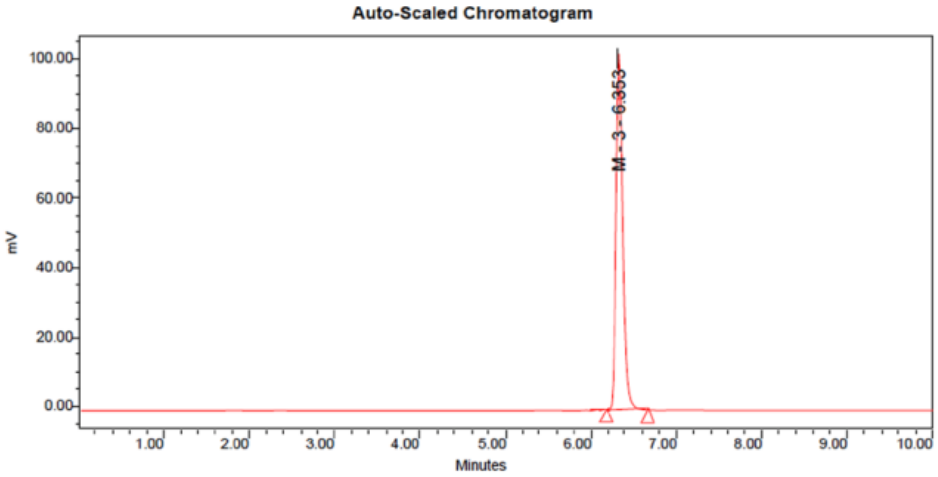

Fig. 7 HPLC Chromatogram of M-3 (Synthetic Standard)

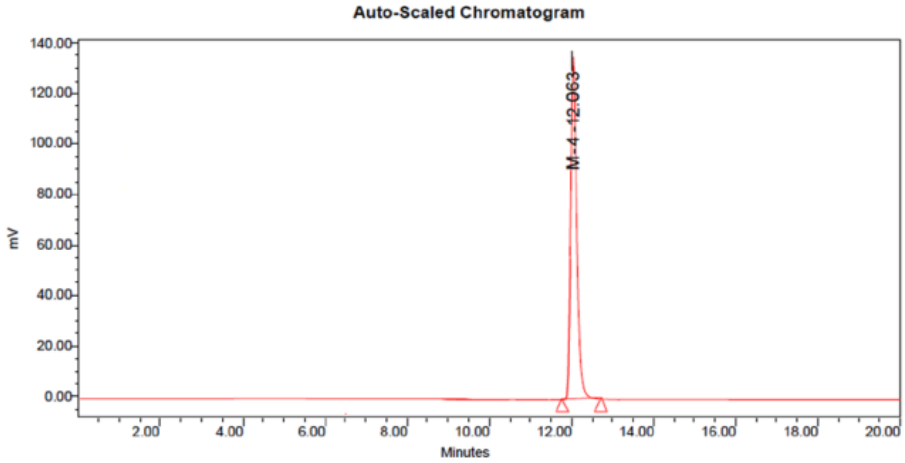

Fig. 8 HPLC Chromatogram of M-4 (Synthetic Standard)

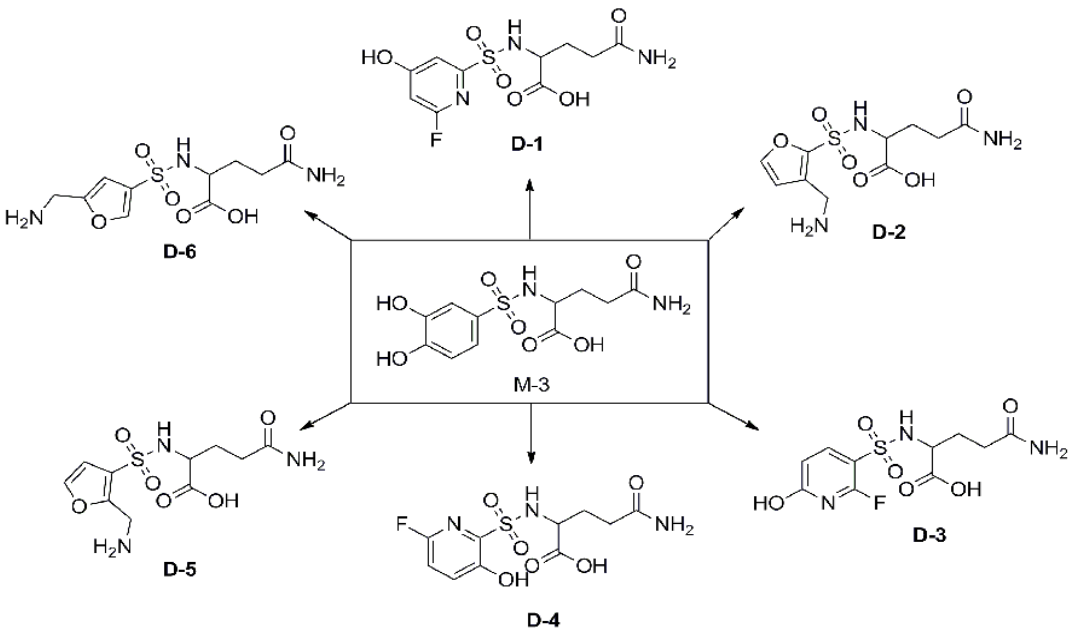

Fig. 9 Structures of new drug candidates designed from M-3