The Pharma Innovation Journal 2019; 8(7): 272-278

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(7): 272-278

www.thepharmajournal.com
Received: 16-05-2019
Accepted: 18-06-2019

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Computational, *in vitro* and *in vivo* studies to evaluate anti-cancer activity of benzisothiazole derivative

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Abstract
Cancer is a leading cause of death all over the world. Though there are several effective medications available for the treatment, the search for the development of target-specific drugs with reduced side effect and cost-effective cancer medication is still ongoing. Moreover, the strategy to design synthetic compound and prediction of potential drugs is a state-of-the-art technology which is comparatively cheaper and less time consuming than conventional drugs production method. The prediction for drug-likeness and its suitability even before synthesis was made possible by computational drug development and design. The objective of this work was a docking approach to predict the affinity between benzisothiazole derivative and oncoproteins, and its *in vivo* and *in vitro* effect. Using *in silico* method, the binding site of the target proteins in 3D structure can be predicted. Research Collaboratory for Structural Bioinformatics (RCSB) study performed on the binding site of the important oncoproteins (Ras, Raf, Nfkb, and VEGF) had been used for the identification of respective active sites. Molecular docking study between the compound-g and proteins by keeping the center of the docking grid in the active site was performed and minimum binding energy was calculated. Anti-proliferative and anti-cancer activities were studied in MDA-MB231 cells and EAT cells upon treatment with compound g. The study of *in silico* based investigation determines the existence of a hydrogen bond between compound-g and the proteins in the vicinity of a binding site. Both *in vivo* and *in vitro* assays showed anti-proliferative and anti-cancer activity upon treatment with compound g. The compound g showed significant anticancer properties based on our *in silico, in vivo* and *in vitro* studies, therefore further studies are required to generate insights into the anticancer mechanism of action.

Keywords: Benzisothiazole derivative, molecular docking, anti-proliferation, survivability study, anticancer, synthetic compound

1. Introduction
Cancer is one of the leading causes of death and is a major public health problem worldwide. Development in cancer therapy has undergone constant and intense evolution over the past four decades. Current standard treatments are chemotherapy, radiotherapy, surgery, hormone, and immunotherapy depending on suitability and progression of cancer. Chemotherapy is the most powerful tools for the treatment of cancer, and the FDA had approved many chemotherapeutic drugs such as olaparib, ivosidenib, apalutamide, and larotrectinib, etc. had been approved in 2018 for the treatment of breast, leukemia, prostate, unresectable or metastatic NTRK gene fusion-positive solid tumors respectively [1]. Drug discovery begins with the identification of potential biological targets for the disease and its lead compound. Recent advancement in the field of genomics, proteomics, bioinformatics, and x-ray crystallography gives the possibility to predict three-dimensional interactions between target proteins and novel candidate drugs. Molecular docking is one such tool which computes the binding site of the macromolecules. The computer-aided drug designing were employed in the development of aliskiren and oseltamivir drug for hypertension and anti-influenza respectively [2]. These techniques have assisted in predicting the efficacy of the drug, even before its synthesis. Therefore, we aimed to study the *in silico* interaction between a novel benzisothiazole derivative on oncoprotein and its *in vitro* and *in vivo* effect. Benzisothiazole derivatives are known to have pharmacological significance such as anti-bacterial [3], kinase inhibitors [4], aldose reductase, antihyperglycemic agents [5], anti-inflammatory agents [6], etc. In our earlier study, we have synthesized and evaluated the anti-cancer activity of nine benzisothiazole derivatives [7]. Three out of nine derivatives, especially compound-g (Figure 1: 3-(4-(2-(3-(4-chlorophenyl)-4, 5-dihydroisoxazol-5-yl)methyl) piperazin-1-yl) benzo[d] isothiazole) had shown consistency efficacy against cancer cell lines. In this study, molecular docking was used to evaluate binding affinity and binding site of the compound with some of

the important proteins responsible for the development of cancer. Nfκβ protein is a transcription factor and its activation regulates many genes which are responsible for vital cellular events, namely extracellular matrix digestion, adhesion, migration, adaptation to the hypoxia and invasion [8]. Similarly, RAS, RAF proteins signaling pathway plays an important role in the initiation of kinases in the ERK cascade [9]. Intense activation of ERK promotes growth and maintenance of various cancer cells. Tumor growth, survival, proliferation, and metastasis require oxygen and nutrients supply, which is facilitated by forming new blood vessels from an existing one, a process called angiogenesis [10]. VEGF is one of the primary growth factors that stimulate angiogenesis and are reported to be overexpressed in many tumor types. Thus, the proteins mentioned above are few of the main targets to prevent cancer growth, invasion and survival. Hence, we performed molecular docking of these biological targets with the compound-g and its *invivo* survivability assay, tumor growth in Ehrlich ascites tumor (EAT) cells bearing mice along with invitro invasion and proliferation studies on MDA-MB231 cells.

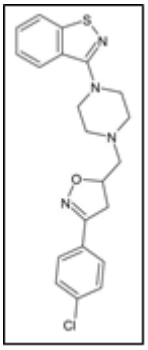

Fig 1: 3-(4-(2-(3-(4-chlorophenyl)-4,5-dihydroisoxazol-5-yl)methyl)piperazin-1-yl)benzo[d]isothiazole (compound-g).

2. Materials and Methods

2.1 Cell culture/Animal model: The MDAMB-231 and EAT cell lines were issued from NCCS (National Centre for Cell Science), Pune, India. Cells were cultured in RPMI-1640 media with 10%FBS, penicillin/streptomycin incubated at 37ºC in 5% CO2 incubator. Female Swiss albino mice (6-8weeks old, 35 ± 2g) were issued from the Central Animal Facility, University of Mysore. All the animal experiments performed according to the regulations of institutional animal care and use committee, the University of Mysore (No 122-1999/CPCSEA), approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2 Chemicals: RPMI-1640, FBS, penicillin/streptomycin and trypsin were procured from Gibco laboratories. All other chemicals are of analytical grade from Merck/Sigma. The benzo[d]isothiazole derivate was synthesized as previously mentioned[7] and dissolved in an appropriate solvent (0.1% DMSO in PBS).

2.3 Molecular Docking tools: ChemSketch and Open Babel software were used for conversion of chemical structure into a .pdb file, Swiss-PdbVierwer tools were used for energy minimization of the proteins, Auto dock 4.0 was installed for molecular docking, Cygwin software for running the algorithm, UCSF CHIMERA for the analysis of hydrogen bond.

2.4 Computational Molecular Docking AutoDock 4.0: To study the affinity of the compound-g on target proteins in silico docking approach was performed as described elsewhere [11]. The structure of compound-g was sketched and saved as MDL Molfiles (.mol file) using ChemSketch 1.1 software. This .mol file was converted to .pdb format by OpenBabel 2.4.1 tool to be readable in AutoDock software. The crystal structure of target proteins in .pdb files was acquired from the RCSB website. The compound g was then docked by selecting grid box in the vicinity of the active site of transcription factors Nfκβp50-p65 (PDB: 1VKX), MEK proteins Raf (5CSX), Ras (1LFD), angiogenic protein VEGF (3QTK) using a Lamarckian genetic algorithm method in AutoDock 4. In docking setup, polar hydrogen's were added on compound-g using hydrogen's module and Kollman united atom partial charges were assigned. Ten independent docking runs performed for each biological target and the least binding energy and inhibition constant (Ki) value were obtained from the RMSD table prepared using Cygwin Terminal program. The compound-g Lipinski's rule of 5 was carried out using molinspiration tools.

2.5 Scratch assay: MDA-MB231 cells (2 x 10^5) were grown in 6-well plate containing complete RPMI media and incubated at 37° C in 5% CO_2 incubator. The protocol was followed as reported elsewhere [12] with minor modification. Briefly, MDA-MB231 cells at 80% confluent were scratched using sterile pipette tips. The detached cells from scratching were removed by washing with PBS twice. The cells were treated with 1, 10, 50 and 100µM of compound-g and photographed at 0, 24 and 48hrs to study the cell migration. The cells migrated were measured by closure area and analyzed by ImageJ software (n=3, SEM p≥0.001).

2.6 Resazurin assay: Cell viability study was performed using resazurin assay as described earlier [13]. MDA-MB231 cell lines were seeded into 96-well plate at $1X10^4$ cells/well. Novel synthetic compound g was added at a series of concentrations (0.1, 0.3, 1.0, 3.0, 10, 30, 100 and 300µM respectively, untreated wells served as vehicle control. After 24 hours of treatment at 37° C, 100µl resazurin (1x) added to the culture media and incubated for 4 hours (One well without resazurin dye served as a blank). Four hours incubation with resazurin allows oxidation-reduction reaction to take place with the metabolic active cells. The intensity of change in color from blue (oxidized form) to red (reduced form) is directly proportional to the number of live cells. The color intensity was read at O.D. 570nm using multi-reader TECAN system.

2.7 *Invivo* EAT cells bearing mice survivability, tumor development, and angiogenesis study: The study on mice survival analysis was performed using EAT cells transplanted mice as reported [14]. The 20 animals were issued and divided into two groups (untreated or treated with compound g) with 10 mice in each group. EAT cells (1 x 10^6) were transplanted intraperitoneally into 20 mice and body weight was recorded daily till the end of the experiment (day 33). Mice were treated with 5ng of compound-g (10µM/ml) at day 8 post-transplantation and Kaplan Meier survivability graph was plotted after the end of the experiment. On day 23, treated and untreated mice were sacrificed and the angiogenesis in the peritoneum tissues were photographed, microvessel density (MVD) was counted using ImageJ software.

3. Results

3.1 Molecular Docking: the molecular docking was performed to the affinity between the compound-g and target proteins by autodock 4.0. The compound-g had shown promising candidate drug following the Lipinski's rule of 5. The drug likeliness score of the compound-g was 1.51 with PSA value of 40.22, logP 4.44, logS value -5.77, number of hydrogen bond acceptors 5 and hydrogen bond donor 0, number of rotatable bond 4 predicted by Molinspiration and Molsoft. The docking of the compound g on the target proteins reveals the hydrogen bonding with one or more amino acids. The sulphur atom of the benzisothiazole in compound-g formed a hydrogen bond with the threonine 176 residue of the Nfkβp50-p65 protein with minimum binding energy -7.95 kcal/mol and inhibition constant, Ki value = 1.48µM (Figure 2). The amino acid residue lysine 690 of MAP kinase protein RAF had formed a hydrogen bond with the sulphur atom in the benzisothiazole group with a binding energy of -9.88 kcal/mol, Ki value = 57.66 nM (Figure 3.A). Similarly, Ras protein of MAP kinase pathway showed affinity to compound-g by forming five hydrogen bonds with arginine-241 (2 H-bonds), glycine 28, serine 338 and valine 25 amino acid residues with a binding energy of -9.03 kcal/mol, Ki value = 238.96 nM (figure 3.B-E). The angiogenic protein VEGF was docked against compound-g and hydrogen bonding was observed between the nitrogen of dihydroisoxazole and amino acid residue isoleucine 91 in the vicinity of the active site (phenylalanine 17) with a binding energy of -7.66 kcal/mol, Ki value = 2.42µM (Figure 4).

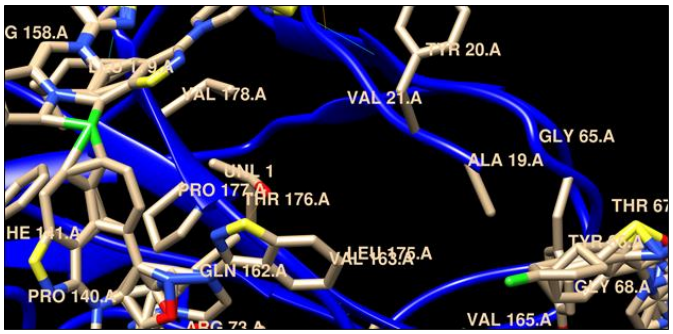

Fig 2: Autodock 4.0 Nfkb: The compound g forming a hydrogen bond at threonine 176 amino acid residue of transcription factors Nfκβp50p65 with binding energy -7.95 kcal/mol and estimated inhibition constant, Ki = 1.48µM. (Color code: yellow = sulphur, blue = nitrogen, red = oxygen, hydrogen bond=orange line).

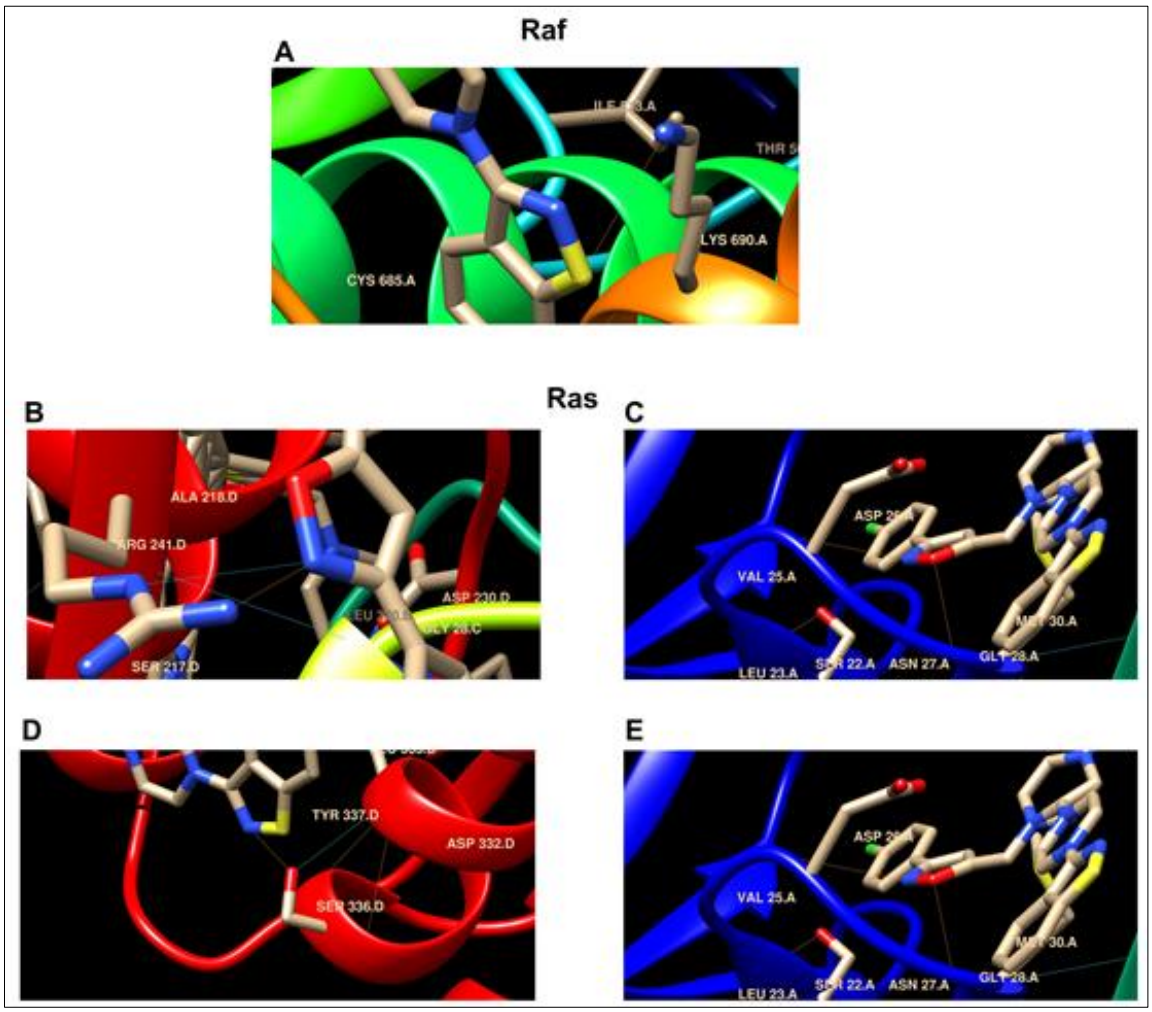

Fig 3: Raf, Ras docking: The compound g targetting the upstream of ERK map kinase protein RAF by forming a hydrogen bond with compound g at amino acid residue lysine 690 [A] of binding energy -9.88 kcal/mol and Ki value = 57.66 nM. Ras the upstream molecule in ERK, PI3K/Akt pathway binding to ligand compound g at arginine 241 (2 H bonds), glycine 28, serine 336 and valine 25 [B- E] with binding energy -9.03 kcal/mol, Ki= 238.96 nM

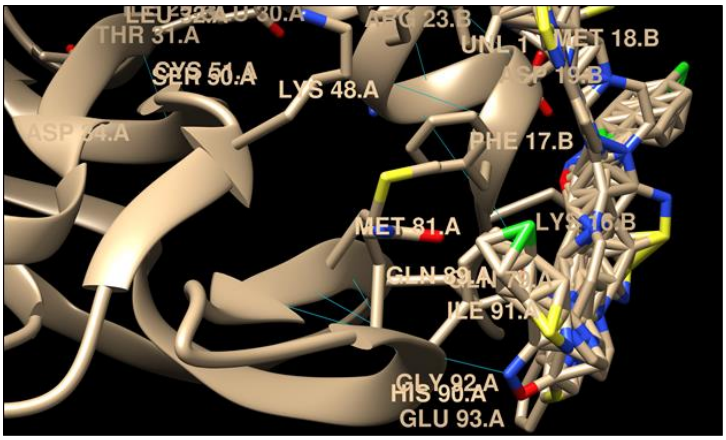

Fig 4: VEGF docking: VEGF protein binding with compound g at isoleucine 91 amino acid residue with binding energy -7.66, Ki value = 2.42µM

3.2 Scratch assay: The effect of compound-g on cell proliferation and migration was investigated using scratch assay and reported at 24 and 48 hours. Migration of MDA-MB231 cells was inhibited by 27.42 % and 58.08 % at concentrations 1 and 100µM of compound-g treated wells after 24 hr incubation time compared to the untreated group (n=3, SEM p≥0.001). Similarly, a significant inhibition in cell migration was observed after 48 hours of treatment with the compound –g.

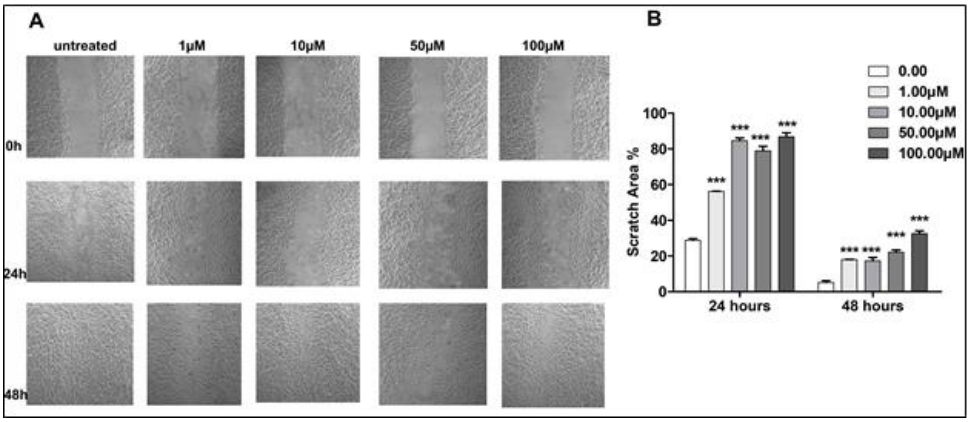

Fig 5: Cell Migration Assay: *Invitro* scratch assay to study the effect of compound g on MDA-MB231 cells migration at different time points paneled vertically image and dosage paneled horizontally [A]. Graphical representation of scratch area (%), considering zero hour as 100 % by using graphpad and ImageJ analysis tool

3.3 Resazurin assay: MDA-MB 231 breast cancer cell lines treated with compound g showed inhibition of proliferation in a dose-dependent manner. Cells treated with 0.3 µM concentration showed 5.94 % inhibition in proliferation and 63.4 % at 300µM concentration compared to untreated cells. After 24 hours of treatment with compound g, significant cytotoxicity observed from 10µM. Compound g showed significant antiproliferative activity in a short period of treatment (24 hours). The absorbance from the color developed in treated cells was normalized with untreated and proliferation percentage graph was prepared (Figure 6 B).

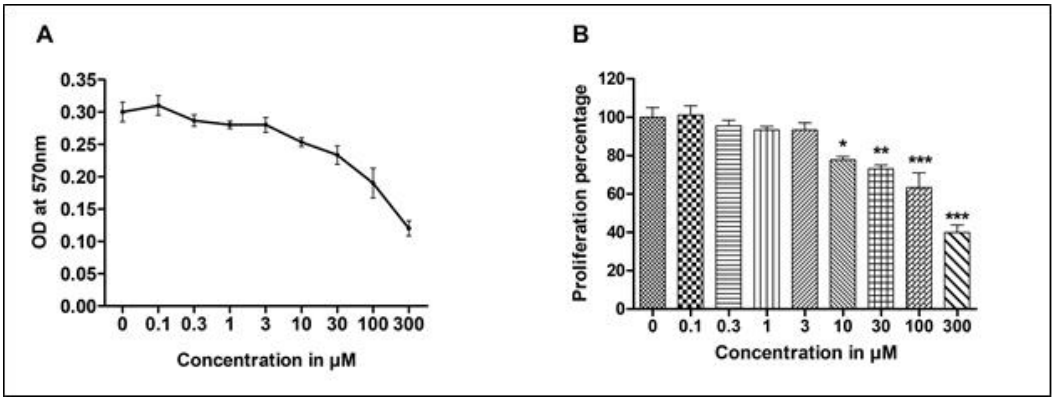

Fig 6: Cell proliferation assay: MDA-MB 231 breast cancer cell lines were treated with compound g and proliferation activity estimated using Resazurin proliferation assay by absorbance readings of colour change at 570 nm (A). Percentage of proliferation of treated cells normalised with untreated and inhibition of proliferation was increasing at dose dependent manner (B). n=3, mean=SEM, p≤0.05

3.4 EAT cells bearing mice survivability, tumor growth, and angiogenesis study: Swiss albino mice were transplanted with EAT cells intraperitoneally to study its survival time and tumor growth in both treated and untreated group. Upon treatment with compound g (0.1mg/kg body weight), there was a significant increase in survival days upto 32 days (mean 26 days) compared to the untreated group with maximum survival upto 24 days only (20 days mean survival, n=10, Log-rank test (Mantel-Cox) p≤0.05). Tumor growth was reduced after day 3 of treatment in the treated group compared to untreated animals (Figure 7.B). Peritoneal angiogenesis observation showed 50% inhibition of MVD upon treatment with compound-g (Figure 8).

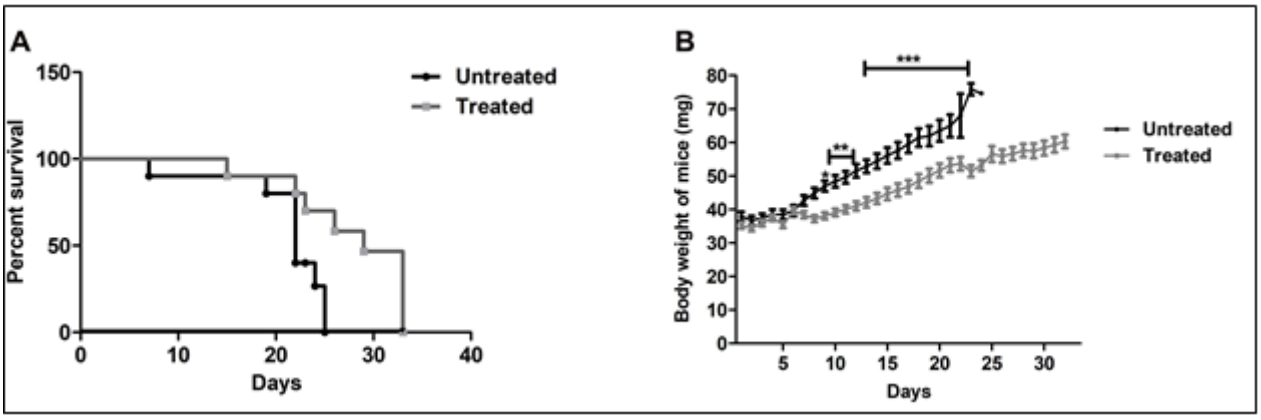

Fig 7: *In vivo* survivability and tumor growth: The Kaplan Meier survivability graph of *invivo* EAT cell bearing mice (n=10) with or without treatment of compound g (IP administration, 0.1mg/kg body weight of mice every alternate days after 6th day EAT cells transplantation). All the untreated animals died after 24th day of EAT transplantation while animals treated upon treatment survived till 32 days. Log-rank test (Mantel-Cox) revealed significant survivability of mice upon treatment with p-value ≥ 0.01 (A). Body weight/tumor development in EAT cells transplanted mice with/without treatment of compound-g. SEM± p≥0.05 after third day of compound g treatment (B)

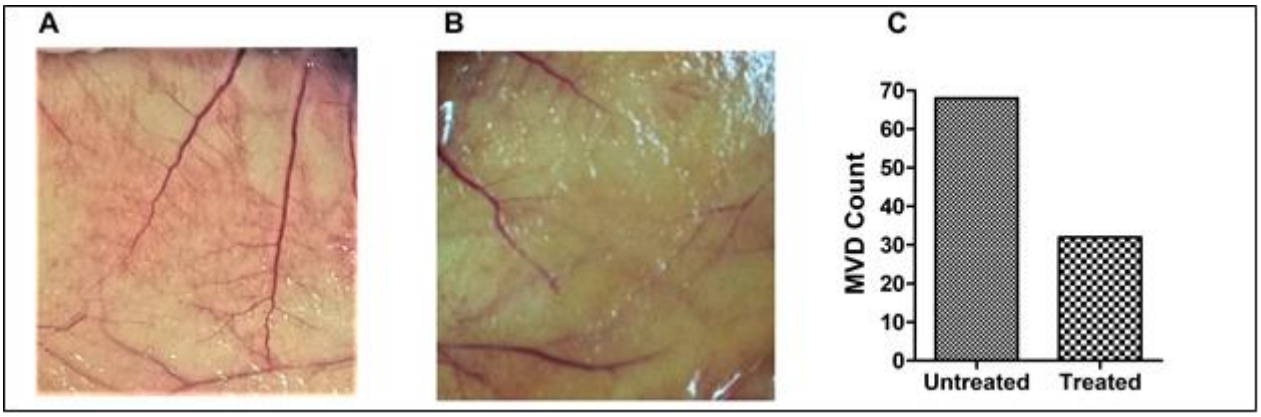

Fig 8: Angiogenesis study: Peritoneum tissues showing neovasculaturization in untreated (A) and treated (B) group on day 23. Micro vessel density (MVD) of the two group was counted using Image J software and its graphical representation was plotted (C)

4. Discussion and conclusion:

The 90% of orally active drugs that have reached phase II clinical follow Lipinski's rule of five (RO5) [15]. The five rules are molecular weight ≤ 500, log P (lipophilicity) ≤ 5, H bond donor ≤ 5, H bond acceptors ≤ 10 and number of rotatable bond ≤ 10. These physicochemical parameters are related with suitable aqueous solubility, absorption, intestinal membrane permeability and binding to a receptor. Our synthesized compound-g satisfied the RO5 signifying that it is a drug-like compound. The computer-assisted docking is a powerful tool to study the structure-based interaction between protein and ligand. Here we predicted the binding of compound-g with important proteins involved in cancer progression. We have selected transcription nfκβ, MAP kinase proteins ras, raf and angiogenic protein VEGF for docking studies with compound-g. The transcription factor nfκβ protein is complexed with κβ inhibitor (κβI) in the cytosol. Inflammatory response induced removal of κβI from the complex which allows translocation of the protein into the nucleus and transcriptional regulations of κβ DNA-containing genes [8]. Several studies have reported the differential expression and higher DNA binding activity of nfκβ in various stages of several cancers [16]. So, blockage of interaction between the two is likely to inhibit tumor growth. It was reported that disrupting glycine-65 residue in nfκβp50/p65 inhibits binding to the κβ sequence in DNA [17]. In our *in silico* interaction studies, we have reported a favorable binding between nfκβp50/p65 (thr-176) and compound-g in the same beta sheet as that of gly-65 (Figure 2). Ras, Raf kinases were among the first oncoproteins to be reported [18]. These proteins are highly activated in various cancers and responsible for activation of ERK kinase pathways controlling cell proliferation and survival, targeting Ras/Raf/Erk signaling pathway have been recognized as important therapeutic targets for cancer therapy [19]. Using the autodock docking tool, we found H-bond between compound-g and ras, raf proteins. These changes in the conformation of protein after binding with compound-g might have resulted in the decreased activation of ERK kinase, which causes inhibition of cell proliferation (Figure 6.). VEGF is a major

angiogenic protein which binds to its receptor and promotes the formation of new blood vessels (angiogenesis) and enhances proliferation of cancer cells [20]. It was reported that 17-25 amino acid residues of VEGF are involved in VEGF receptor activation [21]. In our study, we found that compound-g was efficiently binding to the isoleucine 91 amino acid residue, which is around the proximity of the binding pocket (17-25 amino acid residue) of VEGF. This interaction might have blocked the binding of VEGF to its receptor and inhibiting VEGF mediated growth and angiogenesis (Figure 7.B, 8). This binding pocket could be a potent novel allosteric inhibitory site of VEGF binding to its receptor.

In our earlier *in vitro* study, compound-g demonstrated pro-apoptotic and cytotoxic activity in EAT cells and MDA-MB231 cell lines [7]. Here, we studied the effect of the compound on cell migration and proliferation using scratch assay and resazurin assay. Cell migration is one of the hallmarks of cancer invasion and metastasis. We found that upon a compound-g treatment, a significant ($p \leq 0.001$) inhibition of cell migration and proliferation compared to the untreated in a dose-dependent manner. Ras and Raf are involved in cell proliferation, migration, and metastasis. It was reported that targeting Ras/Raf and Nfκβ inhibited transcription of the MMP9 gene, which plays a crucial role in cell proliferation, growth and cancer cells migration [22, 23]. The affinity between the ras/raf and nfκβ with compound-g could result in a disruption of nfκβ mediated transcription of the MMP9 gene and may be further involved in the inhibition of MDA-MB231 cells migration and proliferation. Raf inhibitors vemurafenib and dabrafenib are FDA approved drugs which showed improved survival of patients with BRAF-V600E/K melanoma [24]. Our *insilico* and *invivo* studies reported similar results as compound-g was forming a bond with the Raf protein, therefore, a significant increase in survivability of EAT-bearing mice was observed.

Angiogenesis plays a critical role in tumor development and survival by facilitating the supply of oxygen and nutrients to the highly proliferating cancer cells [25]. In this study, we investigated the *in-silico* relation and the *in vivo* survivability assay. An angiogenic protein VEGF is associated with tumor growth and metastasis. VEGF docking with compound-g showed a hydrogen bonding at the vicinity of the binding pocket of VEGF, thus blocking the interaction between the VEGF and its receptor. The reduction in angiogenesis was also observed in peritoneal tissues where the compound-g inhibited the formation of neovascularization. The interruption of VEGF mediated angiogenesis might have inhibited EAT cells proliferation, thus decreasing the tumor growth which results in increasing the survivability of compound-g treated animals.

Acknowledgment: The author thanks DoE, Central Tibetan Administration, for fellowship, the financial support from University Grants Commission- (UGC-SAPDSA) Government of India No. 4-1/2013(SAP-II) [B.P.S], Institute of Excellence, Prof. Yajurvedy and Central Animal Facility, DoS in Zoology, the University of Mysore for the issue of animal models.

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