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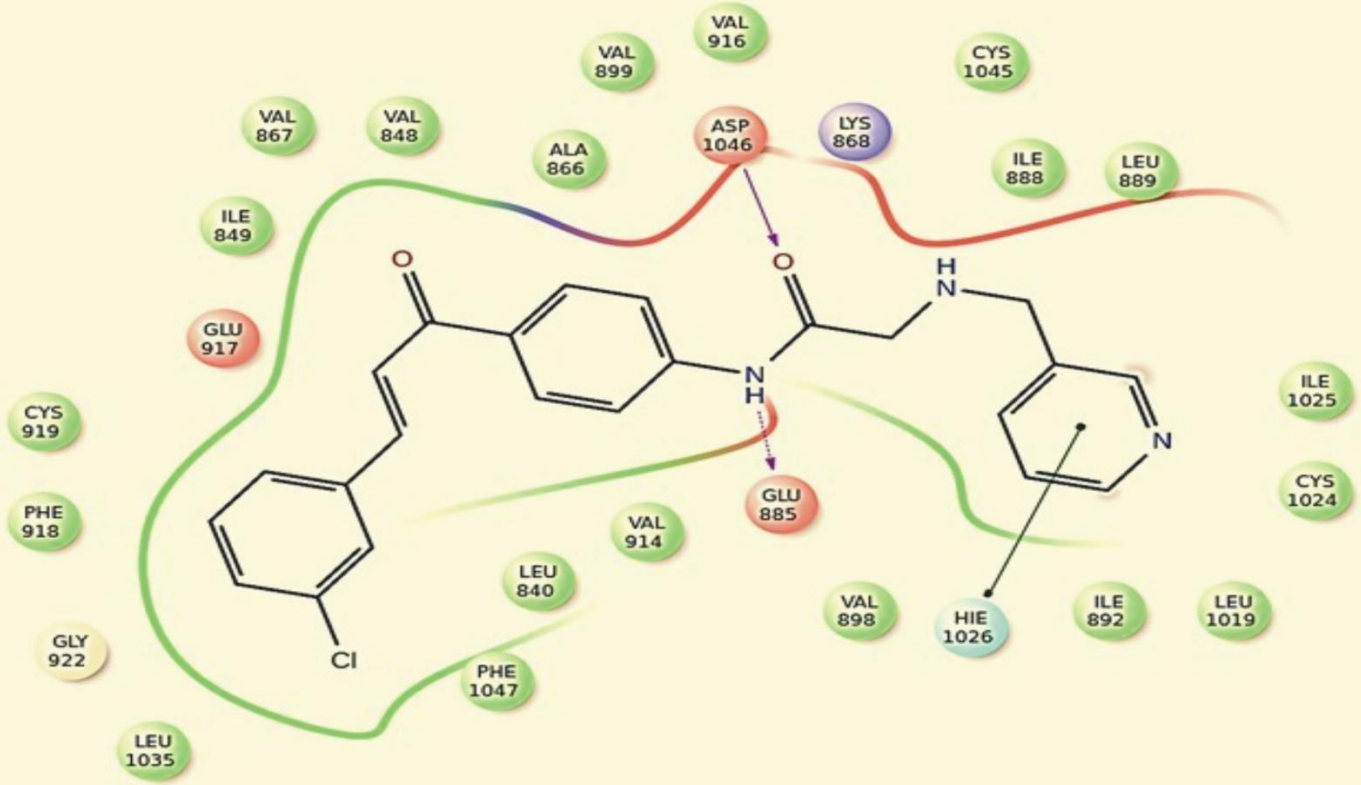

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Preface

Cancer is a fatal disease, which modulates many molecular targets to grow and metastases. In search of potent anticancer drug, researchers are screening many phytochemicals, having a potential to cure the disease via targeting multiple pathways involve in it. The active compound from plant extract is routinely identified, and modified for increasing its potency and specificity. The *in vitro* or *in vivo* based initial screening of these modified chemical derivative for its drug-likeliness is very time consuming and tedious, it also escalates the cost of the new drug. The *in silico-based* evaluation of multiple derivatives, for its potential targets, its pharmacokinetics, Absorption, Distribution, Excretion, Metabolism and Toxicity is easy, less time consuming, cost effective and do not require a biological expertise. This *in silico*-based study help the researcher to get insight, at very initial, about the potential modifications of the compound, its drug-likeliness, its molecular targets, its biological reactiveness etc. Here in this, I have evaluated the potential of hybrid chalcone derivative for its anticancer drug likeliness. Hence, with this book, my aim is to enlighten the new researcher, working with chalcones, with the basic knowledge, about the *in-silico* based techniques, the molecular targets and modifications which they can do to further increase the lacking of chalcone based anticancer derivative.

Dr. Shweta Umar

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About the Book

The book embraces the *in silico*-based evaluation of anticancer property of 3-aminomethyl pyridine derivative of 3-chloro 4'amino chalconeusing Schrödinger software. The anticancer study of 3-AMC-4C includes drug-likeliness, prediction of molecular targets and biological reactiveness. The 3-AMC-4C is a chemical analogues and hybrid of chalcone and pyridine. The threshold level expression of these molecular targets is crucial for homeostatic balance of proliferation and cell death. Any deviation from threshold lead to deadly disease like cancer. These molecular targets include p53, BAX, caspase 3, SNAIL, VEGF- α, VEGFR, and PTEN, involve in proliferation, metastasis and angiogenesis of tumour.The derivative and target interaction may modulate the expression of target molecules, and subsequently prevent the further proliferation of tumour cell as well as its spread. In previous *in vitro* studies, derivative showed a very good anticancer property against MCF-7 and A549 cell line without affecting the normal non-cancerous cell line. Hence, its drug likeliness is predicted here, using the QikProp module (ADME; Adsorption, Distribution, Metabolism, Excretion) inbuilt in the Schrödinger software suite by considering the Lipinski's rule of five or criteria of four. The biological reactiveness is also evaluated using DFT calculations and MESP map is generated. On the basis of methodology, result and discussion, the conclusion of this book includes that 3-AMP-4C is excellent hybrids of chalcone and pyridine and has all the potential to be a good future drug or can be used as a lead molecule for further anticancer drug development.

INTRODUCTION

Cancer is a multifactorial disease of rapid proliferating mass of cell, responsible for millions of deaths each year. The genesis as well as progression of cancer is very complex. It initiates with transformation and ends with acquisition of malignant property in cells. In the process of transformation, a normal cell acquires an immense proliferation efficiency by gain of function mutation in the gene/s involve in cell cycle progression called "oncogene" or by loss of functional mutation in gene/s involve in programmed cell death called "tumor suppressor" gene/s (Weinberg, 1994). This uncontrol growth of transformed cell, leads to formation of a mass of cell *in-situ*, called tumor (Lowe et al., 2004). However, till this stage it can be cured via surgery, though, some cancers are exception. Once tumor cell gains the ability to invade the surrounding tissues, it is called 'Cancer' and the process is known as 'Metastasis'. These metastatic tumors are the most dangerous, which accounts for a large percentage (approximately 90%) of cancer deaths (Sporn, 1996).

According to WHO report, new cases are expected to rise by 70% over next two decades (WHO, 2017). Approximately 19.3 million recent cases and nearly 10 million deaths took place because of some sort of cancer in the year 2020. Out of these, lung cancer topped with 18% (1.8 million) deaths and 6.9% deaths were due to female breast cancers, respectively(Sung et al., 2021). A global rise of 47% in new cancer cases are anticipated in 2040 with respect to the year 2020 (Sung et al., 2021). These data highlighting the need for new therapeutic agents to treat the disease. Since, available treatments, are associated with serious and severe side-effect as well as complications including multi-drug resistant (Qi et al., 2010) which limits their benefits (Safarzadeh et al., 2014). Therefore, novel medications are always approached to give maximum benefits to the cancer patient.

Phytochemicals are readily used for synthesis of novel derivatives as they are inexpensive, readily available, relatively nontoxic and can have broad biological spectrum which can target multiple molecular targets at a time. Thus, scientists are using these Phyto-component as a base for the synthesize new, more powerful and effective therapeutic drugs.

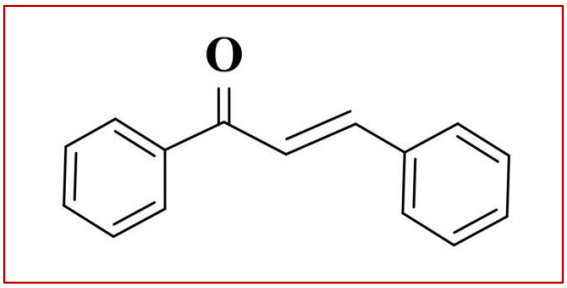

Figure 1: General structure of Chalcone.

Chalcone

A chalcone α, β-unsaturated ketones are the leading class of flavonoid compound found across the entire plant kingdom.The term chalcone is originated from the Greek name 'chalcos' which means 'bronze'. They basically exist as colors of petal and furthermore have been established in the heartwood, leaf, bark, fruit, vegetables and root of a range of plants, spices and teas (Schroder, 1999; Karthikeyan, 2015). Chalcone family members have received considerable attention because of their convent synthesis and scope of their biological activities, including anticancer (Gao et al., 2020), anti-inflammatory (Mahapatra et al., 2017), antidiabetic (Rocha et al., 2020), cancer chemo-preventive (Xu et al., 2015), antioxidant (Lin et al., 2019), antimicrobial (Henry et al., 2020), antileishmanial (de Mello et al., 2018) and antimalarial activities (Cheng et al., 2020). More importantly, several chalcone compounds have been already approved for market and clinical use for various other health conditionse.g., metochalcone for choleretic/diuretics (Figure 2-**1**); sofalcone-based drug for anti-ulcer/muco-protectives (Figure 2-**2**); and hesperidin methyl chalcone for vascular protectives (Figure 2-**3**), exemplifying the clinical potential of chalcones.

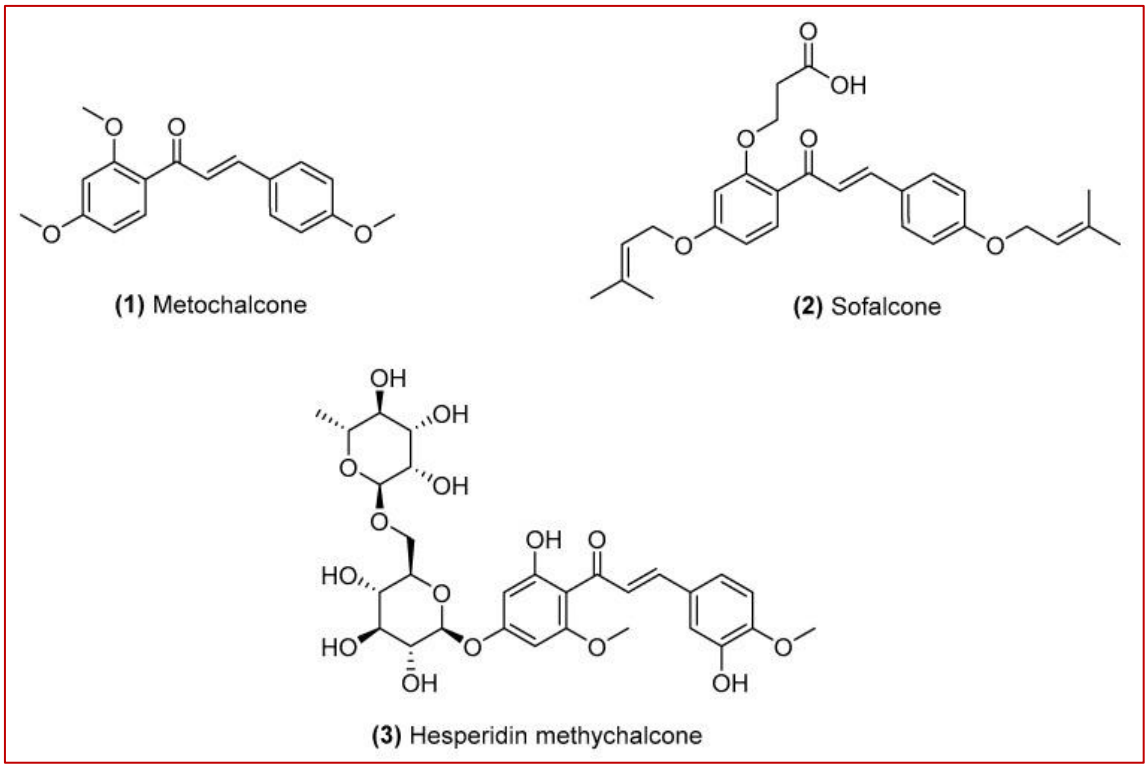

Figure 2. Chemical structure of approved and clinically tested chalcones. (Ouyang 2021)

Chalcones in cancer

Many natural chalcone derivatives occur in monomeric form and exhibit potential *in-vitro* and *in-vivo* activity against many cancers including colon cancer, gastrointestinal cancer, lung cancer, ovarian cancer, leukemia, and melanoma (Wang et al.,2021). Anticancer mechanisms of chalcones include cell cycle disruption, autophagy regulation, apoptosis induction, angiogenesis, invasion and migration inhibition or combination of these mechanism, which are the key steps responsible for the genesis as well as progression of cancer (Ouyang et al., 2021; Jandial et.al 2014; Xiang S., et al., 2021). Examples includes isoliquiritigenin, butein, and isobavachalcone, a natural chalcones with anticancer activity. The significant use of natural chalcones as potential anticancer agent directed towards the synthesis of novel synthetic chalcone derivatives, with improved biological as well physiochemical properties. Since, in natural chalcones, bioavailability, specificity, prolonged effect, transport to the target of the action and level of toxicity is a critical challenge (Roma-Rodrigues et al., 2019). Synthetic hybrid molecules have ability to overcome these challenges as they have different pharmacophores with multiple

mechanisms of action. Hence, hybridization of molecules to develop therapeutic agents, which are more effective than parent molecule is attractive area of pharmacology specially in the case of multifaceted disease like cancer (Cheng et al., 2020).

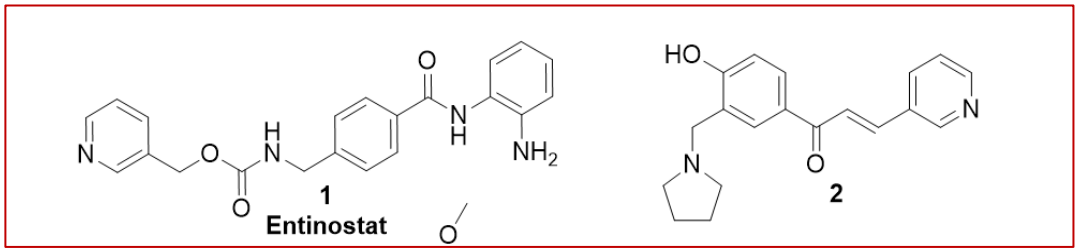

Figure 3. Anticancer derivatives of pyridine (Durgapal et al., 2018)

A series of the hybrids of chalcone were synthesized by many, and evaluated for their anticancer activity; some of them were found to have remarkable activity in both *in-vivo* and *in-vitro* condition (Gao et al., 2020; Ouyang et al., 2021). Similarly, the derivatives of pyridine are also reported to have very good anticancer activity specially when substituted at 3rd position. One such derivative is Entinostat(Figure 3) which is in phase II clinical trials for treatment of advanced stage of breast nevertheless showed anticancer activity against various other types of cancers including hormone receptor-positive breast cancer, Hodgkin lymphoma, and non-small-cell lung cancer(Suzuki et al., 1999). However, the combining effect of both pharmacophores *viz.*,chalcone and pyridine was found to be excellent, one of the compounds (compound **2)** (Figure 3) of Inci Gul*et al.*,is a hybrid of chalcone and pyridine moieties and it exerted an excellent tumor selective cytotoxicity(Bilginer et al., 2013).

Hence, for more efficient formulation, we have also synthesized a series of hybrid of coumarin and pyridine with addition of different functional group on both the moiety. These prepared novel chalcone hybrids were evaluated for their anticancer activity in human breast adenocarcinoma (MCF-7) and human lung adenocarcinoma (A549) cell lines (Durgapal et al., 2018). Among all, 3-aminomethyl pyridine derivatives of chalcones showed excellent activity against A549 and MCF-7 cell line. The derivative ***N-{4-[(2E)-3-(3-Chlorophenyl)prop-2-enoyl]phenyl}-2-{[(pyridin-3-oyl)methyl]amino} acetamide (3-AMP-4C)*** (Figure 4) which a hybrid of 3-aminomethyl pyridine and 4-amino chalcone

showed lowest IC_{50} concentration *viz.*,0.245µM and 0.0067µM in A549 and MCF-7 cell line respectively.

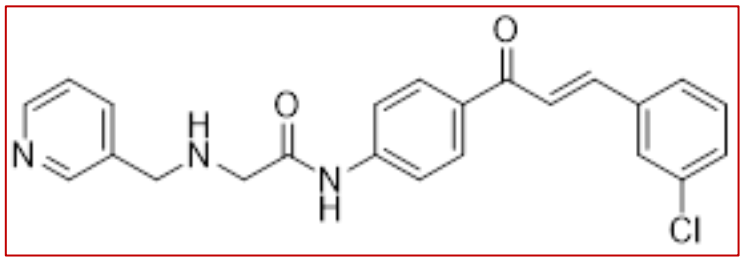

Figure 4: ***N-{4-[(2E)-3-(3-Chlorophenyl)prop-2-enoyl]phenyl}-2-{[(pyridin-3-yl)methyl]amino} acetamide***

The derivative***N-{4-[(2E)-3-(3-Chlorophenyl)prop-2-enoyl]phenyl}-2-{[(pyridin-3-yl)methyl]amino} acetamide*** (3-AMP-4C) not only had a lowest maximal inhibitory concentration but also exerted cytotoxicity via most acceptable pathway of cell death *viz.*, 'Apoptosis' as confirmed by EtBr/AO, and LDH assay done previously (Durgapal et al., 2018). The derivative also showed a negligible side effect on normal non-cancer fibroblast cell line NIH/3T3 atIC_{50} concentration, with all, the derivative can be a future drug candidate for cancer treatment. Hence, herein, in this book, we are going to emphasize the use of *in-silico* tools and techniques for analysis of the drug-likeliness, toxicity, biological reactiveness and physiochemical property of 3-AMP-4CusingADME, Lipinski's rule of five, and HOMO-LUMO. This physiochemical analysis, at an early stage will help in avoiding the late-stage failure of derivative in clinical trial. The computer based 'Molecular docking' is also done here, to understand the key molecular targets and pathway behind its anticancer property using Schrödinger, LLC, New York, NY. These key molecules are p53, Bax, Caspase 3 involve in apoptotic death, Snail, responsible for EMT, VEGF and VEGFR key to angiogenesis process and PTEN- negative regulator of PI3K/Akt pathway. These molecular targets are the common molecules involve in both pyridine and chalcone derivative's anticancer activity.

In-silico technique

The term *"in-silico"* stands for *"within a computer"* shows analogy with *in-vivo*, *in-vitro*, and *in-situ.* It is basically a computer-aided high throughput method of drug discovery and development which has overcome the tedious, time consuming, and expensive process of traditional drug discovery and became an essential part of every aspect of drug discovery (Le et al., 2015). Similarly, "drug-likeliness" of the any novel compound, can be analyzed using *in-silico* tools like ADME and Rule of Five develop by Lipinski (Lipinski et al., 1997). ADME stands for Absorption, Distribution, Metabolism, and Excretion, and applied to check the oral bioavailability of compound, hepatotoxicity, and human intestinal absorption of compounds. Similarly, rule of five (clogP≤5, molecular weight≤500, the number of hydrogen bond acceptors≤10 and donor≤5), is also used to check the drug-likeliness of the compound in terms of lipophilicity, critical molecular weight and chemical structure, required for crossing the blood-brain barrier (BBB). The compound violating more than one of these rules will be poor in terms of bioavailability and not be orally active, hence,cannot be taken further (Sakkiah and Lee, 2012).However, these rules are not appropriate for complicated natural products as they are deduced from relatively simpler, small molecules.

Another *in-silico* based calculation *viz.,* DFT (Density Functional Theory) calculations is used herein, for the prediction of the electronic molecular features of the compound, which defines the chemical, physical, and biological reactivity of the compound. The DFT calculations include the molecular electrostatic potential (MESP) map generation, which is a three-dimensional mapping of a compound that visualizes the charge distribution of the compound and helps in the prediction of reactive polarity and behavior of compound. The other remarkable guideline to predict the electronic property and reactivity of any compound which based on the DFT calculation is the frontier molecular orbital density (FMOs) field, i.e., HOMO-LUMO. HOMO stands for the highest occupied molecular orbital and correlated with the electron-donating ability of the molecule. In contrast, LUMO stands for the lowest unoccupied molecular orbital and correlated with the electron-accepting ability. The frontier molecular orbital energies are used to narrate the electron density cloud around the molecule. High LUMO energy is the favorite site for the nucleophilic attack, and high HOMO energy is predictive of high electrophilic attack. A large HOMO-LUMO gap is related to high kinetic stability and low chemical reactivity and vice versa (Lee et al., 1988). Based on the HOMO and LUMO energies and their energy gap, chemical as well physical

nature of the compound can also be analyzed, including hardness, softness, ionization potential, electron affinity, electronegativity and chemical potential of the compound. Thus, in modern drug designing and development, molecular docking and DFT study of *in-silico* are routinely used to evaluate drug-target interactions, the position of the reactive site, and biological reactivity of the novel compounds.

Molecular docking is an automated computer algorithm that determines how a compound will bind at the active site of the target macromolecule. Docking algorithm puts the ligand in many different orientations in the active site of the target macromolecule and predicts the pose with the lowest energy and then computes a "score" for each pose. The score is based on their van der Walls interactions, electrostatic interactions, solvation effects, and entropic effects between ligand and target (Gohlke and Klebe, 2002). Since target and protein both are flexible, it works as a "hand and gloves" phenomenon where both adjust themselves to achieve the "best fit" orientation as well as confirmation. Molecular Docking, therefore, aims to achieve an optimized confirmation and orientation so that the free energy of the system gets minimized (Jorgensen, 1991).

In standard Virtual Docking, the ligands are docked into the binding site of the receptor where the receptor held rigidly, and the ligand is free to move, but this is not as true since many of the protein undergo side chain or back-bone movement during binding of ligands. These changes allow the receptor to undergo alterations in their binding site shape and binding mode so that it can accommodate ligand more precisely. This mode of docking is known as "induced fit" in which the van der Walls radii is reduced, and the coulomb-vdW cut-off was increased with temporary deletion of the highly flexible side chain of the receptor (Schrodinger, 2015).

For molecular docking some key proteins (PDBs) are considered, which play important role in cancer induction and progression. These PDBs are p53 (5LGY), BAX (2K7W), caspase 3 (4QTX), SNAIL (4QLI), VEGF-α (5T89), VEGFR (5EW3) and PTEN (5BZX).

Cancer

According to Hanahan and Weinberg (2000), there are six hallmarks of cancer *viz.*, self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis.

The normal cell requires mitogenic growth signals, hormones, and other growth regulators, which act as a signaling molecule and cell assist these signals to grow and divide. However, in cancer, these cells modulate the signaling mechanism in such a way that it can receive a constitutive growth signal for achieving a limitless cell proliferation and growth. For this, most cancer cells either mutate the growth factor receptors or abolish the signals that prevent the growth of tumors include tumor suppressors. Sometimes these cancer cells covert most of the signaling pathway to autocrine, i.e., they start synthesizing their own growth regulator to evoke their receptor and do not depend on other cells to generate regulators and act on the paracrine manner (Hanahan and Weinberg, 2000). They modulate all the major pathways responsible for growth and proliferation. One such pathway includes PI3K/AKT signaling pathway, which is central to the growth, proliferation, and survival of normal as well as the tumor cells. In most of the cancer, the pathway was utilized for their growth and prevention of death through mutation of the PTEN (loss of function), AKT (gain of function), or receptor modulations (Akca et al., 2011). Since, PTEN and AKT act as key regulator of cancer progression. Hence, for predictive evaluation of molecular target for 3-AMP-4C here, PTEN is used for molecular docking with derivative.

Cancer cells acquire the capability to become resistant to the anti-growth signals, i.e., cell cycle arrest signals. Insensitivity to anti-growth signals and loss of tumor suppressors is the central hallmark for gaining oncogenic property. Duringnormal cellular growth and proliferation, check-point proteins and tumor suppressor proteins such as p53, PTEN etc. ensure that the cell is ready for division, as well as progression to the next phase of the cell cycle. Any abnormality like DNA damage, flaws during DNA replication, or segregation process, when sensed by the tumor suppressor proteins they halt the cell cycle and activate the repair mechanism. However, when damage is very intense, these tumor suppressor protein forces the cell to abort the cell cycle and activates the mechanism of programmed cell death, i.e., apoptosis. In this way, these tumor suppressor proteins block the transformation of a normal cell to cancerous cells. Through mutating their functions, neoplastic cells attain the unlimited replicative potential and evade the cell cycle arrest, growth arrest, and senescence by tumor suppressors. Dozens of tumor suppressor genes get activated to inhibit the proliferation of damaged/mutated cells through arresting cell cycle progression and inducing programmed cell death, hence the evasion of these tumor

suppressors is critical for carcinogenesis. p53 and Rb are typical tumor suppressor genes (Kohno and Yokota, 1999). Here in the study, the tumor suppressor protein p53 is focused.

P53 a tumor suppressor protein

The P53 is the 'guardian gene' of the cell that activates the DNA repair as well as the death process in response to DNA damage (Strachan and Read, 1999) or stress, like hypoxia, DNA damage, and loss of normal cell contacts (Fridman and Lowe, 2003), it induces the senescence, cell cycle arrest or apoptosis. The importance of p53 can be understood by this that in more than 50% of human cancer functional inactivation of p53 protein, a product of the p53 gene is reported, which leads to the desensitization of DNA damage sensors that induces apoptotic cascades (Harris, 1996). The tumor suppressor TP53 regulates several processes, e.g., cell cycle arrest, repair, senescence, and apoptosis by transactivating the genes involved; these genes are *p21, MDM2, PCNA, cyclin D1, TGF-α, cyclin G, BAX, BCL-XL, Fas1, FasL DR-5,* and *Igf-BP3* (Beckerman and Prives, 2010). It is activated by DNA damage, oncogenic as well as hyperproliferative stimuli (Ras, Myc, and E1A), nucleotide deprivation, or in the presence of chemotherapeutics (Levin, 1997). When these stimuli are cue by p53, it arrests the cell at the G1 phase of the cell cycle by activating the expression of p21 protein (CKI-CDK inhibitor), which consequently decreases the cyclin D1/CDKs complex. In these circumstances, the phosphorylation of pRb was checked, which prevent the progression of cells from G1 to S phase. It also prevents the re-replication of the DNA when spindles fibers are damage and inhibits the entry of cells into the S phase of the cell cycle (Di-Leonardo et al., 1997) along with cytostatic activity it also activates the apoptosis in the presence of hypoxia, DNA damage, growth factor withdrawal and in the presence of Myc or E1A (Levin, 1997). During apoptosis induction, p53 transactivates its downstream targets BAX, DR-5, and Fas to activate the apoptosis. Fas is a cell surface receptor, upon binding of FasL (Fas ligand), it triggers the extrinsic pathway of apoptosis (Owen-Schaub et al., 1995). In the presence of DNA damage, p53 also transactivates the DR-5 (similar to Fas) to stimulate caspase-dependent apoptosis response in the cell upon binding with the ligand TRAIL (Wu et al., 1997). The choice to arrest cell cycle or induce apoptosis depends on the cell type and the intensity of the damage. If the cell is null mutant to p21 or the cross-talk between the pRb and p53 is impaired as well as if the damage is beyond repair, then

p53 induces apoptosis(Chiou et al., 1994, Polyak et al., 1996). Hence p53 is also taken for molecular docking study with 3-AMP-4C.

Apoptosis

The expansion of a tumor does not only depend on the rate of cell proliferation but also the rate of cell attrition. Apoptosis is a programmed cell death in which cells die in a mechanism programmed in their genetics. It is a major source of cell death in an organism that required for the maintenance of the homeostatic balance of the body. Once cell get a physiological response for cell death, the mechanism starts in a well-choreographed manner. It includes cellular membrane disruption, nuclear and cytoplasmic skeleton breakdown, extrusion of cytosol, the nucleus fragmentation, DNA damage and lastly shriveled cells were engulfed by the nearby phagocytic cells without any damage to the nearby healthy cells (Wyllie et al., 1980).

The apoptotic machinery is divided into two portions; first is 'Sensor', which senses the extracellular, as well as intracellular environmental changes and, takes the decision over a die or survive. Second is 'Effector' it includes all the signaling receptor that binds to the survival or the death factors, The extracellular death signal conveyed by Fas ligand via binding to Fas receptor or by TNF-α binding to TNF-R1 (Ashkenazi and Dixit, 1999). The intracellular sensors monitor the intracellular abnormalities, including DNA damage, oncogenic provocation of signaling imbalance, survival factor deficiency, or hypoxic condition, and activates the death pathway (Evan and Littlewood, 1998). This pathway also called an intrinsic pathway of apoptosis and govern by mitochondria, which releases cytochrome c in response to the death signal (Green and Reed, 1998). In response to cytochrome c release, the BCL-2 family of pro-apoptotic proteins (BAX, BAD, Bid, BAK) or anti-apoptotic proteins (BCL-2, BCL-2W, BCL-2XL) get activated to perform their part.

In response to DNA damage p53 -a tumor suppressor gene elicits the apoptosis by upregulating the BAX- a pro-apoptotic member of the BCL-2 family, which in turn contributes to the cytochrome c release from the mitochondria. The released cytochrome c activates the ultimate effector of apoptosis, which is an array of proteases termed 'Caspases.' There are two types of caspases, 'gatekeeper caspases' and 'effector caspases.' Gatekeeper caspases are caspase 8 and caspase 9, invoked by death receptor Fas and cytochrome c of mitochondria, respectively. These proximal caspases (gatekeeper caspases) activate dozens

of effector caspases that execute the death program through selective disruption of cellular, subcellular organelles, and genome (Thornberry and Lazebnik, 1998).

In 1972, the first time, it was noticed that apoptosis could serve as a barrier in the cancer growth when Kerr, Willie, and Currie observed a massive cell death (apoptosis) on withdrawal of hormone in the hormone-dependent tumor (Kerr et al., 1972). The mutation in BCL-2 oncogene via chromosomal translocation in follicular lymphoma, c-Myc overexpression along with BCL-2 disrupt the Fas death circuit (Korsmeyer, 1992, Hueber et al., 1997) confirmed that cancer cell takes advantage of these oncogenes to evade apoptosis. Resistance to apoptosis is also acquired by cells through inactivation or loss of tumor suppressor genes function along with overactivation of oncogenes. The most commonly reported loss of pro-apoptotic regulator through mutation involves the loss of tumor suppressor gene p53. Not only DNA damage but abnormalities induced by hypoxia as well as due to oncogenic hyperactivation are also funnel a part via p53 to the apoptotic machinery, the loss of p53 function impaired the apoptotic mechanism to elicit the response (Levin, 1997). The PI3K/AKT signaling pathway also transmits the anti-apoptotic signals for survival and involve in the mitigation of apoptosis in many human cancers. This survival signal can be activated by extracellular signals, eg.IL-3, IGF-1/2, or intracellular emanating from Ras or due to loss of PTEN a tumor suppressor phospholipid phosphatase and a negative regulator of AKT's survival signal (Cantlay and Neel, 1999).

As *in-vitro* based EtBr/AO and trypan blue assay revealed that derivative 3-AMP-4C exert its effect via cytotoxic as well as cytostatic pathway and induce cytotoxicity via apoptosis. All these findings indicate p53 involvement in derivative induce anticancer activity, since p53 activation induce the p21 mediated cell cycle arrest (cytostatic) as well as pathway of apoptosis (Durgapal et al., 2018). Hence, P53, BAX, caspase 3 and PTEN is consider for the molecular docking with 3-AMP-4C.

Angiogenesis and Metastasis

For growth and proliferation, nutrient and oxygen supply are crucial, for that every cell present in the close proximity within 100µm to the blood vessel. Because of this dependency, every cell within the tissue has an intrinsic ability to encourage vasculature growth. To attain larger size (beyond 25mm), neoplasm needs extensive angiogenesis

(Folkman, 1997), for that it itself secretes a large quantity of chemokines and proteins, and also attract immune cells, fibroblast cells, Endothelial cells, platelets, smooth muscle cells, and inflammatory cells (Ucuzian and Gassman, 2010) to stimulate angiogenesis. The process of tumor-associated sprouting of neo vessels from existing blood vessels is referred as a 'tumor angiogenesis' (Folkman, 1971). These angiogenic factors attract the endothelial cell toward the tumor mass, and these endothelial cells start migrating toward the stimuli and the cells behind, start proliferating and align themselves in the form of a lumen of the newly formed vessel to which blood flow (Bielenberg and Zetter, 2015). These new blood vessels not only provide nourishment to the tumor cells but also act as a site from which the tumor cell enters into the circulation.

VEGFs a potent mitogen whose overactivation act as initial angiogenic signals that bind to the tyrosine kinase receptor displayed by the endothelial cell (Veikkola and Alitalo, 1999) and promote initial angiogenesis. The promoter region of VEGF contains hypoxia-responsive elements indicating that hypoxia (poor oxygen) act as a major mediator of the angiogenesis via VEGF (Levy et al.,1995). The tumor suppressor gene p53 in some cell type positively regulate the negative inhibitor of angiogenesis, i.e., thrombospondin-1. Since in most of the cancer there is a loss of p53 expression, causes fall of thrombospondin-1 level and liberate endothelial cell from its inhibitory effect (Rak et al., 1995, Maxwell et al., 1999). Hence, for predicting anti-angiogenic potency of derivative 3-AMP-4C the VEGF-α and VEGFR are used for molecular docking study.

During the tumor progression sooner or later, the mass of cells invades adjacent tissue and travel to distant sites and settle in another niche. This distant settlement of tumor cells is known as metastasis and a major cause of cancer-related death worldwide (Spurn, 1996). For attaining invasive and metastasis ability first cancer cell changes their phenotype as well as plasticity through Epithelial to mesenchymal transition (EMT) in which cell loses its epithelial characteristic and attain a migratory mesenchymal characteristic and start separating from tumor mass to invade nearby cells. Once cells get the mesenchymal characteristic, it upregulates the extracellular proteases which enable the remodeling of ECM (Extracellular matrix) to invade the nearby cells. Molecularly, EMT is characterized by loss of epithelial characteristics and the concomitant gain of mesenchymal characteristics through gene expression alteration. Molecular hallmarks of EMT are the loss of cell polarity, the loss of epithelial markers, such as E-cadherin and ZO-1, the gain of the expression of

mesenchymal markers, such as N-cadherin, vimentin, and fibronectin, a dramatic cytoskeletal reorganization accompanied by the change from an epithelial, differentiated morphology to a fibroblast-like, motile, and invasive cell behavior (Polayak, et al.,1996Kalluri and Weinberg., 2009). Several proteins got modulated to attain the epithelial to mesenchymal transitions, cell-cell adhesion molecules; CAM is one such protein, includes members of immunoglobulins, and calcium-dependent cadherins both mediate cell to cell interaction and integrins which enable the cell to matrix attachment. All these adhesion acts as signals to the cells (Aplin et al.,1998). The measure alteration observed in the cell environment was a change in E-cadherin. In most of the epithelial cancer, E-cadherin is lost either due to mutational inactivation of E-cadherin or β-catenin. Cristofori and Semb (1999) reported that when E-cadherin was forced expressed in the mouse model of carcinogenesis, there is impaired invasion and metastasis capability. Thus E-cadherin function serves as a widely acting suppressor for invasion and metastasis, and its functional elimination helps in the acquisition of this capability. Downregulation of E-cadherin by the transcriptional repressors Snail/SNAI1, Slug/SNAIL2, SIP1/ZEB2, or Twist leads to the disassembly of adherence junctions and help the cell to gain mesenchymal characteristic by upregulating the mesenchymal markers such as vimentin and neuronal (N) cadherin a biomarker for the mesenchymal cells (Lehembre et al., 2008,Yilmaz and Christofori, 2010). Hence, herein SNAIL a transcription factor responsible for EMT is considered for molecular docking to predict the effect of derivative on EMT process.

All together these key molecule/transcription factors are chosen to check the effect of derivative 3-AMP-4C on the major pathways of cancer *viz.*, apoptosis, angiogenesis and metastasis.

METHODOLOGY

Density Functional Theory analysis (HOMO-LUMO and MESP)

DFT calculations which includes HOMO-LUMO analysis and molecular electrostatic map (MESP), signify the electronic molecular features of the compound. Frontier Molecular Orbital (FMOs) density field is also used to predict the molecular properties and biological activity of the compound. MESP maps (MESP isoenergy contours generated at -30.0kcal/mol) and HOMO and LUMO are computed using Jaguar (Schrödinger), an *ab initio* quantum chemical program, version 9.8 Schrödinger, installed on Intel core i7-2600 CPU @ 3.40GHZ by applying Becke's three-parameter exchange potential and the Lee-Yang-Parr correlation functional (B3LYP), using a basis set 631G**++ level (Gill et al., 1992). For stimulating physiological conditions, energy calculations are performed in an aqueous environment using "Poisson Boltzmann Finite" (PBF) solvation. For a better understanding of the structure, reactivity, and dynamics of any novel molecule, electronic calculations play a significant role. Herein, using Density Functional Theory (DFT) calculations the molecular electrostatic property and reactivity of the derivativeis evaluated.

ADME and Drug- Likeliness prediction

The derivative 3-AMC-4C is evaluated for its drug-likeliness using ADME properties and Lipinski's rule of five or criteria of four using the QikProp module (ADME; Adsorption, Distribution, Metabolism, Excretion predictions, version 4.4, Schrodinger, LLC, New York, 2015) inbuilt in the Schrödinger software suite. This software helps in predicting pharmacokinetics and pharmacodynamics of compounds and shows its pharmaceutical relevance as a drug candidate. The candidate molecules are assessed based on the biological properties such as the predicted octanol/water partition coefficient (QPlogPo/w), octanol/gas partition coefficient (QPlogPoct), water/gas partition coefficient (QPlogPw), brain/blood partition coefficient (QPlogBB), aqueous solubility (QPlogS), apparent Caco-2 cell permeability in nm/sec (QPPCaco) and apparent MDCK cell permeability in nm/sec (QPPMDCK) the overall CNS activity, the human serum albumin binding (QPlogKhsa), the skin permeability (QPlogKp), and the cardiac activity (QPlogHERG). Herein, the drug-likeliness of derivative is predicted using *in-silico* based ADME study (Table 1). Evaluation of drug-likeness of a derivative is based on the number of violations of Lipinski's rule of five, which is critical for rational drug design. It includes the molecular weight of the

compound, number of H-bond donors, number of H-bond acceptors and lipophilicity (Lipinski et al., 1997). The Lipinski's rule of five 1997 is the original and most well-known rule-based filter for drug-likeness of any active compound and assess whether the molecule will orally be absorbed well or not.

MOLECULAR DOCKING

All computational studies are carried out using Workstations from Supermicro with configurations of Intel® core™ I7p-2600 CPU @ 3.40 GHz, Operating System CentOS 6.3, Desktop from Lenovo with configurations of Intel ® Core ™ 2 Duo CPU E7300@2.66GHZ.

Ligand preparation

The structure of the derivative ***N-{4-[(2E)-3-(3-Chlorophenyl)prop-2-enoyl]phenyl}-2-{[(pyridin-3-yl)methyl]amino} acetamide***(3-AMP-4C) is generated using ChemDraw ultra 2012. The sketched ligand structure is prepared using LigPrep, an application of Maestro (LigPrep, version 3.4, Schrödinger, LLC, New York, NY, 2015). The database ligands are prepared, and different ionization states are generated at pH 7.0±2.0 using an epik state; a enumerates ligand protonation states and tautomers in biological conditions, version 3.2 (Schrödinger, LLC, New York, NY, 2015) and the substantial penalties for high energy ionization of tautomer states is removed. Geometry, and charges (partial atomic charges) are optimized using the optimized potentials for liquid simulations-2005 (OPLS-2005) force field. In the default constraint parameters, as a scaling van der Walls radius of 1.0 Å for the protein with a partial atomic charge set at less than 0.25 Å. LigPrep is a utility in the Schrödinger software suite that ensures optimization of ligand geometry, minimizes the energy of 3D structure, and gives the best tautomeric and steric isomer with minimized energy for the docking.

Protein preparation

X-ray crystallographic structure of target proteins, i.e., p53, BAX, caspase 3, VEGF-α, VEGFR, SNAIL and PTEN are retrieved from Protein Data Bank (PDB). The respective PDB IDs 5LGY, 2K7W, 4QTX, 5EW3, 5T89, 4QLI, and 5BZX, are prepared for docking using protein preparation wizard (Schrödinger Suite 2015 Protein Preparation Wizard Schrödinger, LLC, New York, 2014) in Maestro, (Schrödinger, LLC, New York, NY, 2015).

Protein preparation is a multistep process in which wizard adds missing bond as well as side chains, assigns correct bond order and charges, removes any crystallographic water whereas, hydrogens remain unstrained, and geometry of the input protein remains unaltered; instead, it checks for any problems in the protein structure and corrects them. In order to determine and optimize the atomic charges, the OPLS-force field of the Schrödinger software is used, which contains different parameters for treating different atoms. Additionally, the energy is minimized until the average root mean square deviation of non-hydrogen atoms reached 0.30 Å, it will help any structural deviations.

Active site prediction

The active site residues of PDB IDs: p53 (Vainer et al., 2016), BAX (Gavathiotis et al., 2008), caspase 8 (Shen et al., 2015), caspase 3 (Prashanth et al., 2019), VEGFR (Bold et al., 2016) VEGF-α (Markovic-Mueller et al., 2017), SNAIL (Prokop et al., 2013) and PTEN (Lee et al., 2015) are obtained from the literature. Once the structures are optimized, the receptor grid is generated, which encloses the active site of amino acids. For generating the receptor-grid, the scaling van der Walls radii is set at 1.0 Å and enclosed in the bounding box set at 20 Å.

Induced Fit Docking

An additional whole set of docking protocol is run *viz.*, "Induced Fit Docking" (mixed molecular docking/molecular dynamics protocol) to confirm the above result. It allows receptors to kept flexible, along with ligand (Singh et al. 2012). The same default parameters, as mentioned in the standard Glide XP docking method, is used. Ligand and protein are docked with Schrödinger's induced fit protocol by using Glide software to exhaustively consider possible binding modes and the associated conformational changes within the receptor's active sites. This unique procedure allows the prediction of active site geometries with minimal expense yet quickly. The graphical user interface for the Schrödinger software, Maestro, is used for the execution of induced fit simulations and interpretation of the result (Schrödinger internet). Once the receptor grid is generated, the derivativeis docked to the receptor using Glide (version 6.7) docking protocol with default parameters under the Extra precision (XP) model (Jayaraman and Jamil, 2014).

RESULT

DENSITY FUNCTIONAL THEORY CALCULATIONS

Frontier Molecular Orbitals (FMOs) density field of 3-AMP-4C

HOMO and LUMO orbitals play a significant role in prediction of the chemical nature, reactivity, bioactivity, and stability of any novel derivative. The DFT calculations is herein, done using Jaguar version 9.8 of the Schrödinger software suite and observed that in chalcone derivative the HOMO orbitals are located around benzene ring attached to amino group and LUMO orbital is around chalcone group (ketone and double bond in conjugation between two benzene rings (Figure 5). The calculated HOMO, LUMO, and HOMO-LUMO energy gap for derivative are -0.2473eV, -0.1046eV, and -0.1427eV, respectively (Table 1). These energies also characterize the electrophilic or nucleophilic nature of a compound (Eroglu and Türkmen, 2007). Since the E_{LUMO} and E_{HOMO} are responsible for charge transfer in a chemical reaction, the compound with lower gap energy is generally associated with more polarizable nature along with high chemical reactivity and low kinetic stability. The molecules which are associated with such properties are termed as a soft molecule (Powell et al., 2004). Based on the Koopman's theorem for closed-shell molecules, the global reactivity descriptors were also analyzed by using HOMO and LUMO energies of the compound (Pearson, 1986). These quantum chemical descriptors are Ionization Potential (I), Electron Affinity (A), Softness (S), Chemical Hardness (H), Chemical Potential (μ), Electronegativity (X) and Electrophilic Index (ω). As presented in Table 1.1, the Ionization potential, electron affinity, softness, chemical hardness, chemical potential, electronegativity, and electrophilic index for derivative is found to be 0.2473eV, 0.1046eV, 7.0eV, 0.07135eV, -0.175eV, 0.175eV and 0.215eV respectively.

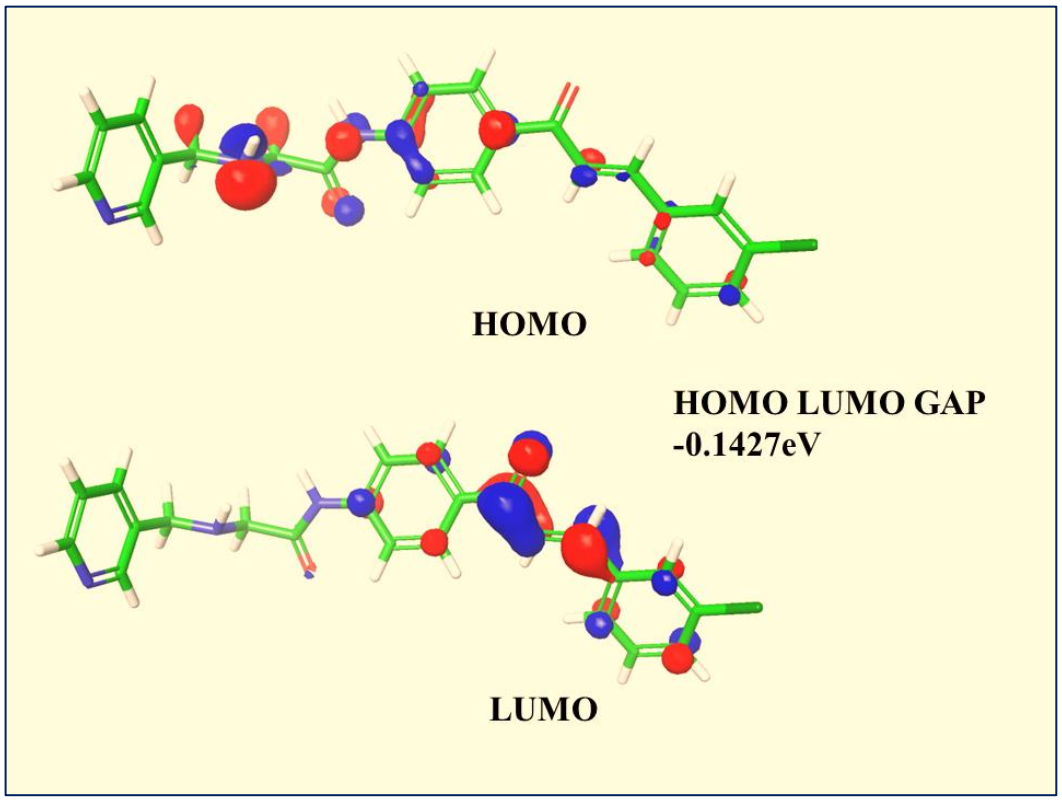

Figure 5. Frontier Molecular Orbital (HOMO-LUMO) distribution of derivative (3-AMP-4C) and their corresponding energies; HOMO orbital is distributed on the Benzene ring attached to amino group, and LUMO orbital on the chalcone ring of derivative and their corresponding energies are E_{HOMO} = -0.2473eV, E_{LUMO} = -0.1046eV and ΔE= -0.1427eV respectively.

DFT calculations (Frontier Molecular Orbital density field)	
Energy$_{HOMO}$	-0.2473eV
Energy$_{LUMO}$	-0.1046eV
HOMO-LUMO Energy Gap (ΔE)	-0.1427eV
Salvation Energy	-1663.2kcal/mol

Table 1: Frontier Molecular Orbital (HOMO and LUMO) and related transition energy (HOMO-LUMO energy gap) of 3-AMP-4C calculated using Jaguar version 9.8 (Schrödinger).

Reactive descriptors	Equations	3-AMP-4C
Ionization Potential (I)	$-E_{HOMO}$	0.2473eV
Electron Affinity (A)	$-E_{LUMO}$	0.1046eV
Chemical Hardness (η)	$\eta = (I - A)/2$	0.07135eV
Chemical Softness (S)	$S = 1/2\eta$	7.0eV
Chemical Potential (μ)	$\mu = -(I + A)/2$	-0.175eV
Electronegativity (χ)	$\chi = (I + A)/2$	0.174eV
Electrophilic Index (ω)	$\omega = \mu^2/2\eta$	0.215eV

Table 1.1. Frontier Molecular Orbital based quantum chemical descriptors of ***N-{4-[(2E)-3-(3-Chlorophenyl)prop-2-enoyl]phenyl}-2-{[(pyridin-3-yl)methyl]amino} acetamide***. The parameters are calculated from the equations based on the Koopman's theorem for closed-shell molecules.

Molecular Electrostatic Potential (MESP) of derivative 3-AMP-4C

The different values of the electrostatic potential in MESP are represented by different colors. It is widely accepted that the negative (red) and the positive (blue) potential regions in a mapped MESP represent regions susceptible to approach by electrophiles and nucleophiles, respectively. The color code of these maps ranges between -192.2281 a.u (deepest red) and -23.1788 a.u. (deepest blue). Most positive electro potential region colored deepest blue (most nucleophilic site) and the most negative electro potential region colored deepest red (most electrophilic site) (Govindrajan et al., 2012). In the MESP map of derivative ***N-{4-[(2E)-3-(3-Chlorophenyl)prop-2-enoyl]phenyl}-2-{[(pyridin-3-yl)methyl]amino} acetamide***(3-AMP-4C)(Figure 6), the region having the most negative potentials are over the nitrogen atom and oxygen atom of the pyridine and chalcone moisty, and the most positive region is present over the Hydrogen atom and the Methyl group.

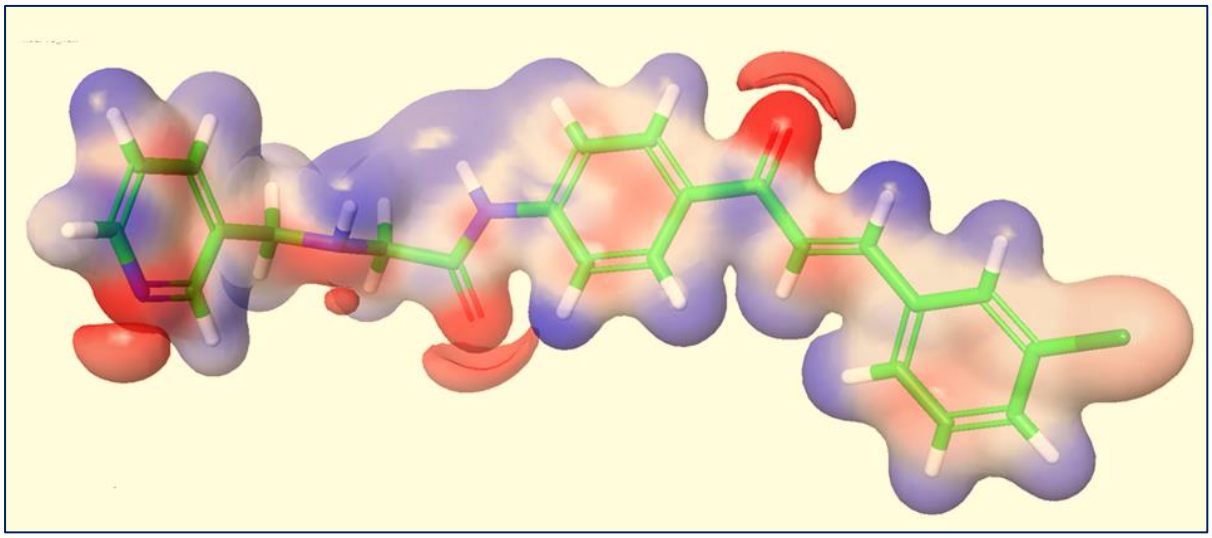

Figure 6. MESP Map of the **3-AMP-4C**; the calculated electrostatic potential surface on the compound color code -192.2281 a.u (deepest red) and -23.1788 a.u. (deepest blue). The most positive electrostatic potential region is deep blue, and the most negative electrostatic potential region is deep red in color. B3LYP functional and 631G**++ level basic set.

Drug-Likeliness and ADME Prediction of 3-AMP-4C

The *in-silico* based ADME prediction of any active compound is an important experimental assessment for its the physiochemical properties, its competency and its *in-vivo* behavior as a biopharmaceutical to enter into an advanced stage of the drug development process to become a future drug candidate for the treatment of the disease. With this aim, the Drug-likeliness properties such as molecular weight <500, clogP not greater than 5 with <5 hydrogen bond donors, and <10 hydrogen bond acceptors are assessed using the criteria set by Lipinski's rule of five (Lipinski et al., 1997). The result revealed that, 3-AMP-4C has a molecular weight of 405.9, hydrogen bond donor 2, hydrogen bond acceptor 7.5 and QPlogPo/w octanol-water partition coefficient, 2.7 (Table 2). All the properties are under the acceptable range and satisfy the criteria set for drug-likeliness with Lipinski's rule of five.

The octanol/water partition coefficient and water solubility are the crucial parameters for the estimation of absorption and distribution of active compound within the body. The brain-blood partition coefficient is too, an important criterion of absorption and distribution for predicting the ability of the compound to penetrate the blood-brain barrier. The result

revealed that QPlogPo/w, QPlogS and QPlogBBof 3-AMP-4C, are in the accepted range, i.e., 3.7, -5.23, and -1.02(Table 2). Similarly, the Caco-2 cell permeability, whichis an essential factor in regulating the drug metabolism and its transport across membranes, is found to be 130.09 nm/sec. The Caco-2 cells are the *in-vitro* monolayer cell model shows a morphological and functional similarity with human enterocyte used for intestinal permeability/gut-blood barrier of drug, give an early indication of the likelihood of compound being absorbed in the gastrointestinal tract (GI). The MDCK cells are considered a good mimic of the blood-brain barrier and the compound that has permeability less than 500nm/sec considered to have a good drug-likeness. The MDCK cell permeability of 3-AMP-4C is found to be 148.92 nm/sec. The predicted IC_{50} value for the blockage of the HERG K^+(Human ether-a-go-go-related gene potassium) channel is-8.5(above -5 hence, slightly lower than the accepted range). HERG or potassium channel gives an early indication of potential adverse cardiac toxicity it is best known for electrical activity of the heart that coordinates the heart's beating, appears to be the molecular target responsible for the cardiac toxicity of a wide range of therapeutic drugs (Vandenberg 2001). Thus, HERG K+ channel blockers are potentially toxic and the predicted IC_{50} values often provide reasonable predictions for cardiac toxicity of drugs in the early stages of drug discovery (Aronov AM., 2005). The percentage of human oral absorption are computed as 86.41%. The other parameters, such as #rtvFG (number of the reactive functional group), SASA (Total solvent accessible surface area), FOSA (a hydrophobic component of the SASA), FISA (a hydrophilic component of the SASA),and PSA (a component of the SASA, Polar nitrogen and oxygen van der Waals surface area) were also predicted and found to be under the acceptable range except for the QPlogKhsa (human serum albumin binding efficiency) and QPlogKp (the skin permeability),which is 0.313 and -3.5 higher than the acceptable range (Table 2).

The compound which satisfies all the criteria of ADME, and Lipinski's rule of five, considered as a drug-like molecule. The derivative, 3-AMP-4Cis satisfying all the criteria of Lipinski's rule and most of ADME descriptors, hence, it has drug like property for human usage and can be taken further.

Descriptors	**Range of 3-AMP-4C**	**Acceptable range** (range is for 95% of known drugs)
Molecular weight	405.883	130-725
No of H-bond Donors	2	0- 6
No of H-bond acceptors	7.5	2- 20
QPloPo/w	3.703	-2 - 6.5
QPlogPoct	22.212	8-35
QPlogPw	14.14	4-45
QPlogS	-5.239	-6.5- 0.5
QPlogBB	-1.02	-3-1.2
QPlogHERG	-8.56	above -5
QPPMDCK	148.92	<25 is low; >500 is great
QPPCaco	130.09	<25 is low; >500 is great
Human Oral Absorption	3	1-low, 2-medium, 3-high
Percentage Human Oral Absorption	86.46%	>80% is high; <25% is low
Violation of Rule of Five	0	Maximum 4
Overall CNS activity	0	-2 (inactive) to +2 (active)

Descriptors	**Range of 3-AMP-4C**	**Acceptable range** (range is for 95% of known drugs)
QPlogKhsa	0.313	-1.5- 1.5
QPlogKp	-3.50	-0.8 - -1.0
#rtvFG	1	0-2
SASA	779	300-1000
FOSA	91.16	0-750
FISA	134.8	7-330
PSA	90.1	7-200

Table 2. Pharmacokinetics prediction of 3-AMP-4C using QuikProp 4.4; Table representing the ADME properties of the derivative, and drug likeliness prediction using Lipinski's Rule of Five. QPlogPo/w, QPlogPoct, QPlogPw, QPlogS, QPlogBB; predicted partition coefficient of octanol/water, octanal/gas, water/ gas, aqueous solubility, and brain/blood, respectively. QPPMDCK and QPPCaco; predicted apparent cell permeability in MDCK and Caco-2 cell nm/sec. QPlogHERG; The predicted IC_{50} value for the blockage of HERG K^+ channel. Human Oral Absorption; qualitative human oral absorption based on a knowledge-based set of rules, Percentage Human Oral Absorption; quantitative prediction based on multiple linear regression model. QPlogKhsa, QPlogKp; predicted human serum albumin binding and skin permeability. #rtvFG; no of the reactive functional group. SASA, FOSA, FISA, PSA; Total solvent accessible surface area, a hydrophobic component of the SASA, hydrophilic component of the SASA, Polar nitrogen, and oxygen van der Waals surface area.

Molecular Docking

Herein, 3-AMP-4Cis docked against all the PDB IDs; 5LGY (p53), 2K7W (BAX), 4QTX (caspase 3), 5T89 (VEGF-α), 5EW3 (VEGFR), 4QLI (SNAIL) and 5BZX (PTEN) and their affinities are evaluated. The details of this analysis are presented below.

p53 (5LGY)

Any active compound which has capability to induce cellular stress by mean of DNA damage, the guardian gene P53 activates the pathway of self-destruction in the cells. However, this is highly regulated mechanism, in normal cell the low cellular level is maintain by many regulators. MDM2 is one such regulator, which act as negative regulator of p53. It physically interacts with p53 induces ubiquitination and degradation of p53 through proteosome (Momand et al., 1998). If a drug occupies the interactive site of p53 with mdm2, it can able to overcome the negative regulation of mdm2 and induce the p53 mediated pathways responsible for anticancer activity.

The 3-AMP-4C is docked efficiently with p53 (5LGY) at the active site and generated a docking score of -6.89kcal/mol, Glide energy of -41.5kcal/mol, Glide emodel of -59.28kJ/mol and IFD score of -423.861kcal/mol(Figure 7, Table 3). Ligand interacts with the targeted protein, p53 by 3 hydrogens (H-bond) interactions, *viz.,* Arg280 NH of protein interact with the oxygen atom of acetaldehyde at a distance of 1.77 Å. Leu137 NH form H-bond with the pyridine ring of ligand (3-AMP-4C) at a distance of 2.45 Å and Methyl -pyridin-2-ylmethyl-amine (NH) of ligand form H- bond with the oxygen atom of Asn239 at a distance of 2.35 Å. (Figure 7, Table 3.1). The negative value of the docking score and interactions with the derivative 3-AMP-4C may be responsible for the binding site competition with Mdm2 and activation of the p53 to perform its function However, *in-vitro* based molecular level experimentation is needed for further confirmation.

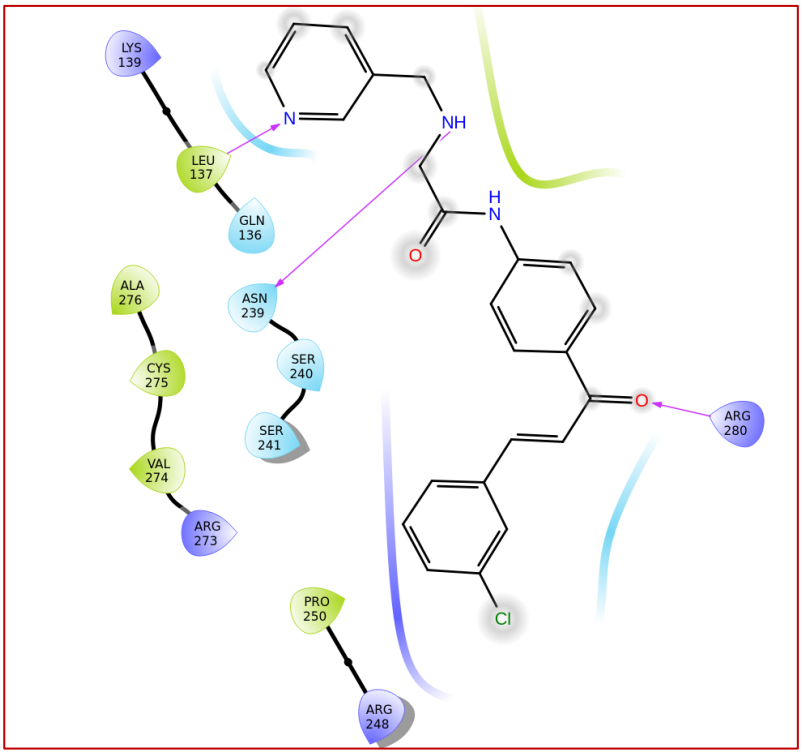

Figure 7: Molecular Docking of p53 (5LGY): 2D interaction diagram of 3-AMP-4C with p53 displaying hydrogen bond with magenta line.

BAX (2K7W)

BAX is a pro-apoptotic member of a BCL-2 gene family whose ratio to BCL-2 determines the fate of the cell, to die, or to survive. The increased expression ratio of BAX to BCL-2 or pro-apoptotic to anti-apoptotic pushes the cell toward apoptotic death (Raisova et al., 2001). To permeabilize the mitochondrial membrane, BAX binds to the outer membrane of the mitochondria as a monomeric unit, and within seconds it oligomerizes and forms pore, which helps in the release of cytochrome c to mediate apoptosis. However, when BCL-2 is present in higher concentrations than BAX, it dissociates the BAX oligomers from the mitochondrial outer membrane and pushes cells toward survival (Subburaj et al., 2015). Therefore, a strong binding or interaction of derivative (3-AMP-4C) with the protein (PDB ID; 2K7W) can increase or decrease the oligomerization process and thereby activation of the BAX to act on cells. 3-AMP-4C derivative interact with 2K7W at the active site amino acid residue and generated a docking score of -13.8kcal/mol, Glide energy of -59.79kcal/mol, Glide emodel of -91.13kJ/mol and IFD score of -434.721kcal/mol (Figure 8, Table 3).

The protein interacts with the 3-AMP-4C through one hydrogen bond (H-bond), one pi-pi stacking; and one pi- cation interaction. Oxygen group of the Gly166 of ligand form H-Bond with the amine group of the protein at a distance of 1.83 Å,(Figure 8, Table 3.1). One pi-pi stacking and one pi-cation interaction observed with pyridine ring of ligand and Phe 165 and Arg 65 of protein at a distance of 4.00 Å and 4.29 Å respectively. Since, increased expression of BAXis responsible for the induction of apoptosis, the interaction of BAX with 3-AMP-4C induces significant conformation changes, which leads to the oligomerization and activation of BAX to perform its apoptotic function.

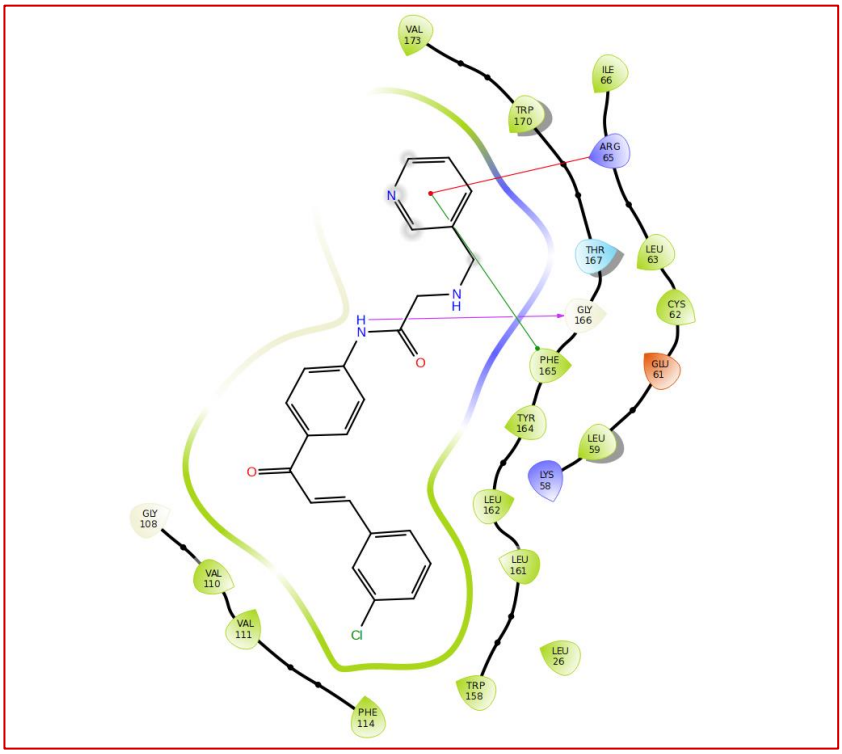

Figure 8: Molecular Docking of BAX (2K7W): 2D interaction diagram of 3-AMP-4C with BAX, displaying hydrogen bond (magenta line), π-π stacking (green line) and π-cation (red line).

Caspase 3 (4QTX)

Caspase 3 is the effector caspase of the apoptotic pathway, which initiates the process of apoptosis upon receiving upstream activation signals. The PDB ID: 4QTX was interacting through two hydrogen bonds, one pi-pi interaction and one pi-cation interaction with the derivative 3-AMP-4C (Figure 9, Table 3.1).Gln161 form H- bond with nitrogen atom of pyridine moiety of ligand (NH..N) at a distance of 2.7 Å and Ser 209 (OH)form H-bond with

Pen-1-en-3-one at a distance of 2.87 Å. Special interaction, salt bridge observed between Arg 207 and pyridine ring of ligand. The pi-pi interaction also occurs between Phe 252 and 3-chloro phenyl ring of chalcone with pi-pi interaction. These interactions result in the docking score of -3.46 kcal/mol, Glide energy of -41.86 kcal/mol, Glide emodel of -61.154 kJ/moland IFD score of 401.2kcal/mol(Table 3). This direct interaction between 3-AMP-4C and caspase 3 deduce the active involvement of caspase 3 in derivative induced apoptosis.

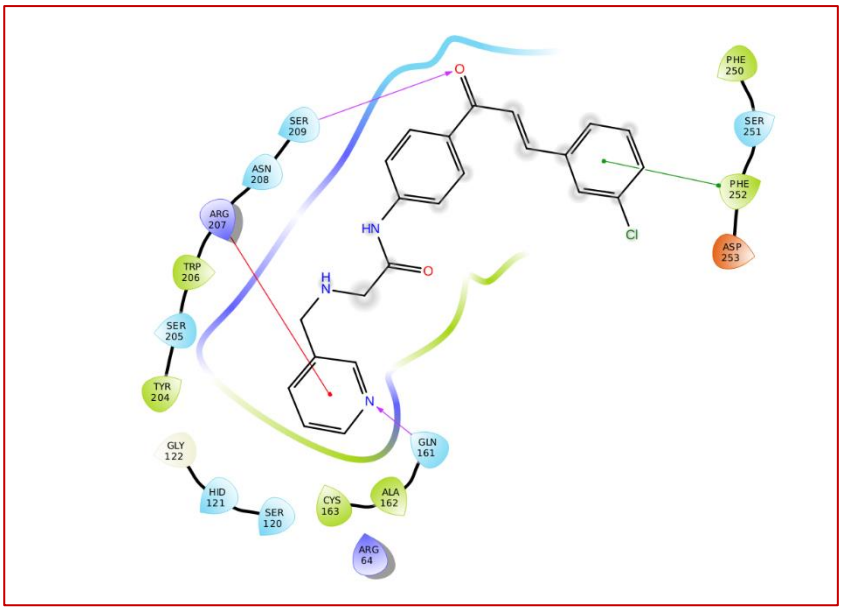

Figure 9: Molecular Docking of caspase 3 (4QTX): 2D interaction diagram of 3-AMP-4C with 4QTX, displaying hydrogen bond (magenta line), π-π stacking (green line) and π-cation (red line) interactions.

SNAIL (4QLI)

The transcription factor Snail, plays an important role in the downregulation of E-cadherin - an epithelial cell marker and upregulation of mesenchymal markers like vimentin and N-cadherin which promote EMT during cancer metastasis. Herein, we are predicting the interaction of Snail with the derivative 3-AMP-4C using molecular docking. When 3-AMP-4C is docked with the PDB ID, 4QLI, it generates a docking score of -3.58 kcal/mol, Glide energy of -39.93kcal/mol,Glide emodel of -57.58kJ/moland IFD score of kcal/mol(Table 3). It interacts with the ligand (3-AMP-4C) through three hydrogen bonds (Figure 10, Table 3.1) in which Arg 129 and Arg 56 -NH group interact with the oxygen atom of chalcone at a distance of 1.43 Å and 2.89 Å respectively followed by Lys 49interact with double bond

O atom at a distance of 2.13 Å. Some special interaction, pi-cation interaction is also observed with two amino acid residue Lys49 and Lys122 with benzene moieties.

The interaction between 3-AMP-4C and SNAIL may be negative and block the binding of other TFs responsible for the transcription of E-cadherin to maintain the epithelial characteristics.

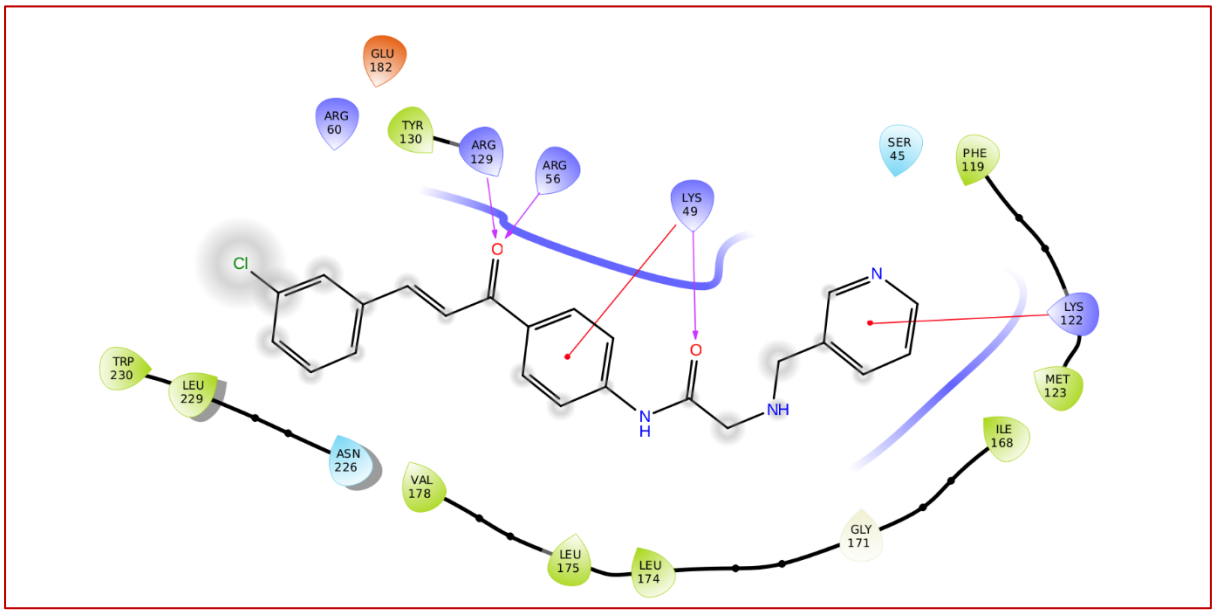

Figure 10: Molecular Docking of SNAIL (4QLI)): 2D interaction diagram of 3-AMP-4C with 4QLI, displaying hydrogen bond (magenta line),) and π-cation (red line) interactions.

VEGF-α (5T89)

Vascular Endothelial growth factor- α, is a potent pro-angiogenesis factor and found to be elevated in most of the tumor (Senger et al.,1983). The binding of VEGF to its receptor VEGFR initiates the process of vessel morphogenesis. This ligand -receptor binding is crucial for angiogenesis process. Any active compound which can bind efficiently at the active site of either VEGF-α, VEGFR or to the complex of both, can stop the whole process of angiogenesis. With this, the molecular docking is performed with the derivative 3-AMP-4C and PDB 5T89. The VEGF-α is efficiently docked with derivative and generated the docking score of -6.39kcal/mol, Glide energy of -53.43kcal/mol, Glide emodel of -84.43kJ/mol and IFD score of -1105.99kcal/mol (Figure 11, Table 3). This interaction occurs via three hydrogen bonds between Ser 95 (NH), Arg 261(NH) and Tyr 39 (NH) of protein

and Oxygen atom of chalcone, Nitrogen atom of pyridine ring and amine group of 3-AMP-4C with the distance of 1.79 Å, 2.65 Å and 2.20 Å respectively. A pi-pi interaction is observed between Tyr 39 and amine group of 3-AMP-4C, however, Arg 82 form special bond of pi-cation and pi-pi interaction with both benzene ring of the chalcone at a distance of 3.06 Å (Figure 11, Table 3.1). These data suggest a very strong interaction between derivative and protein.

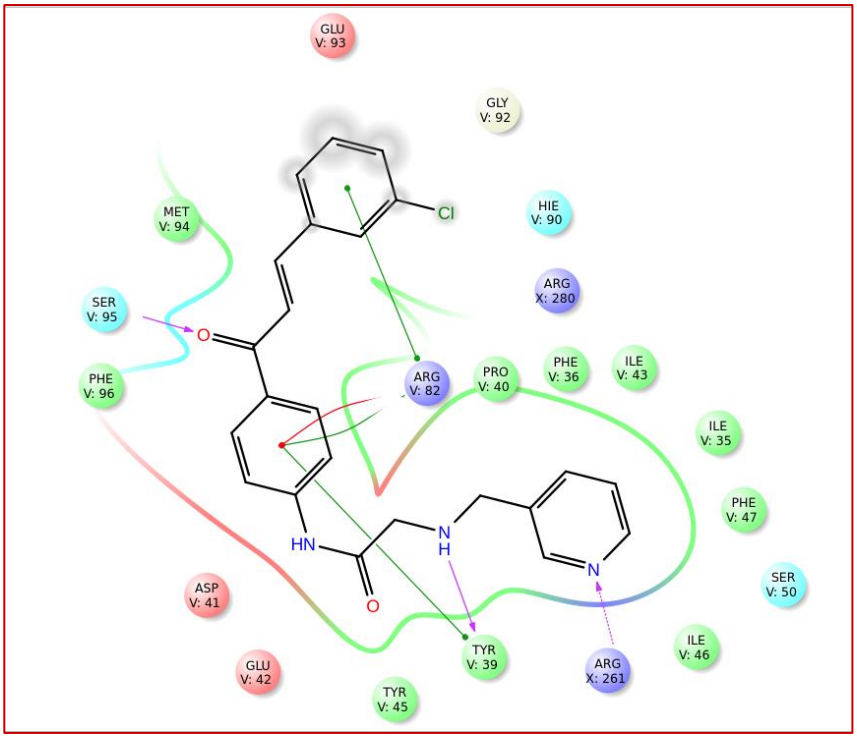

Figure 11: Molecular Docking of VEGF-α (5T89)): 2D interaction diagram of 3-AMP-4C with 5T89, displaying hydrogen bond (magenta line),) and π-cation (red line) interactions and π-π interaction (green line).

VEGFR (5EW3)

Since derivative 3-AMP-4C is showing an excellent docking score with VEGF-α, it's binding efficiency with its receptor *viz.*, VEGFR is analyzed herein. The derivative is efficiently docked into the active site of VEGFR (5EW3) with a docking score of -10.48kcal/mol, Glide energy of -62.84kcal/mol, Glide emodel of -101.445kJ/mol and IFD score of -559.03kcal/mol (Figure 12, Table 3). The interaction with ligand includes two

hydrogen bonds and one pi-pi stacking, Glu885 -hydrogen bond between the NH group of the ligand at the distance of 2.1 Å, Asp1046 form hydrogen-bond with the oxygen atom of the ligand at the distance of 2.8 Å. His 1026 form pi-pi stacking with the pyridine ring of the 3-AMP-4C (Figure 12, Table 3.1). The interaction of 3-AMP-4C may be strong enough to block the binding of VEGF to VEGF-receptor and hamper the process of the neovascularization process, which need to be confirmed by *in -vitro* based molecular study.

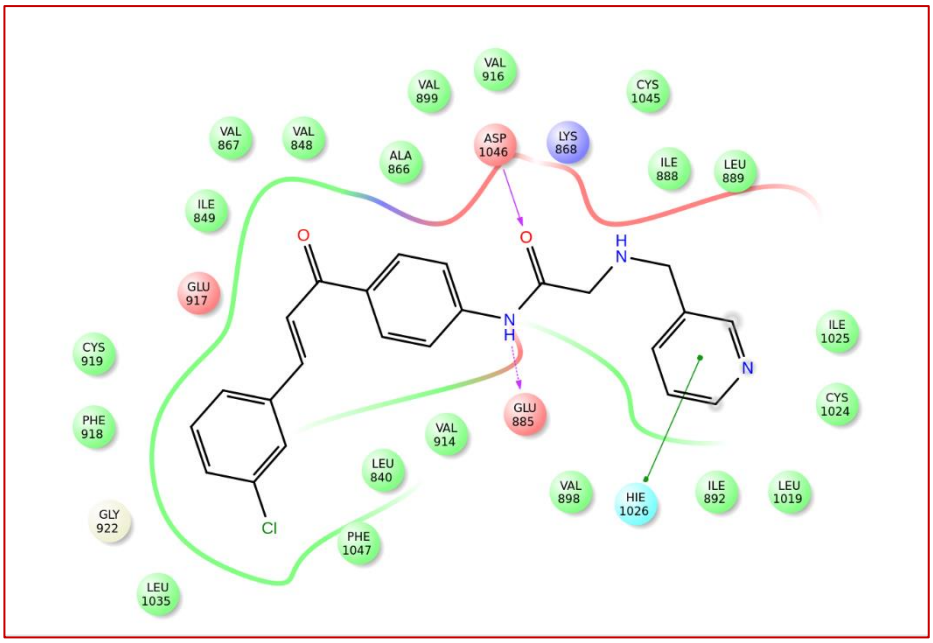

Figure 12: Molecular Docking of VEGFR (5EW3)): 2D interaction diagram of 3-AMP-4C with 5EW3, displaying hydrogen bond (magenta line),) and π-π interaction (green line).

PTEN (5BZX)

PI3K/Akt pathway is found to be involve in most of the cancer type. The hyperactivation of the pathway leads to various hallmarks of cancer, namely uncontrolled tumor cell proliferation, inhibition of apoptosis, sustained angiogenesis, increased invasion, enhanced migration, and adhesion-independent tumor growth and metastasis (Slomovitz and Coleman, 2012, Wu and Hu, 2012). PTEN, act as a negative regulator of AKT, the downstream mediator of the PI3K signaling pathway. When PTEN gets activated, it hampers the activity of AKT and hence, the major pathway responsible for the proliferation and survival of cells. In cancer cells, the activity of AKT was increased either due to mutation

or other factors, activation of PTEN can help to minimize its effect on the cellular processes. Hence, this is considered a good target for anticancer therapy. The effect of derivative on PTEN is predicted here, using molecular docking. When 3-AMP-4Cis docked with the PTEN (PDB ID; 5BZX) it generates docking score of -8.404kcal/mol, Glide energy of -50.16kcal/mol, Glide emodel of -94.97kJ/mol and IFD score of -1050.01kcal/mol (Table 3). The interaction is made with four hydrogen bond (Figure 13, Table 3) that includes, Asp 24 -NH group and NH2 group ligand with the oxygen atom of amino acid at a distance of 1.43 Å and 1.28 Å respectively. Tyr16 -H bond between O atom of amide group with the distance of 2.80 Å and one pi- cation stacking with NH2 group of ligands (3-AMP-4C) and Tyr 46 -H bond with oxygen atom of chalcone at distance of 1.9 Å (Figure 13, Table 3.1). This interaction may be activating the PTEN, which leads to a substantial decrease in the level of AKT, and induction of cytotoxic and cytostatic activity of derivative as observed in EtBr/AO and trypan blue assay (Durgapal et al., 2018).

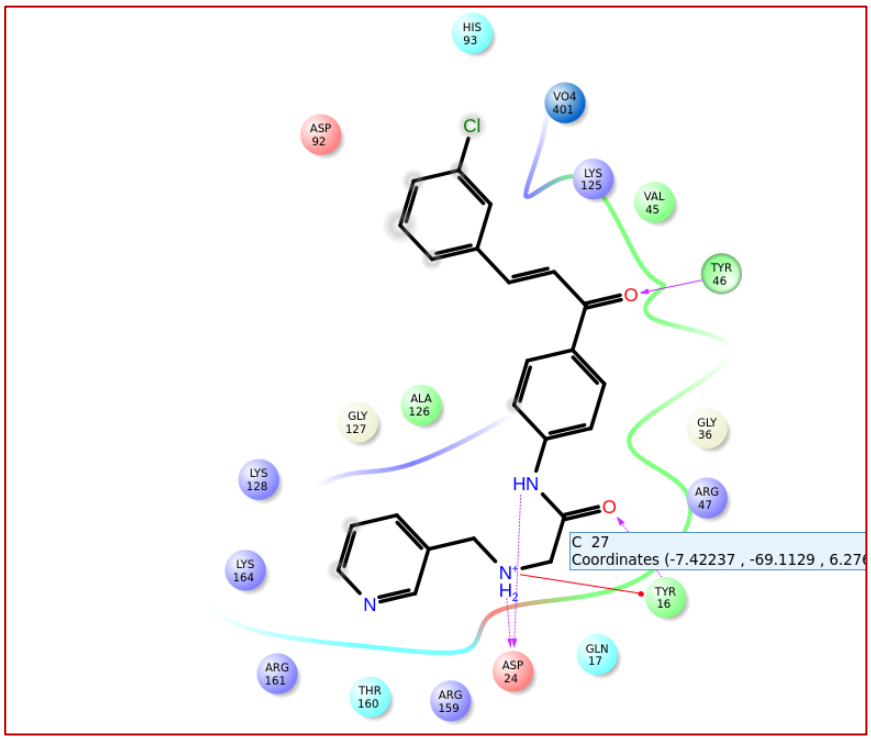

Figure 13: Molecular Docking of PTEN (5BZX): 2D interaction diagram of 3-AMP-4C with 5BZX, displaying hydrogen bond (magenta line), and π-cation (red line) interactions.

Protein (PDB ID)	Docking score (kcal/mol)	IFD score (kcal/mol)	Glide energy (kcal/mol)	Glide emodel (kJ/mol)
p53 (5LGY)	-6.89	-423.86	-41.53	-59.28
BAX (2K7W)	-13.83	-434.72	-59.7	-91.13
caspase 3 (4QTX)	-4.78	-401.2	-43.78	-51.847
SNAIL (4QLI)	-3.58	-383.62	-39.93	-57.58
VEGF-α (5T89)	-6.39	-1105.05	-53.43	-84.41
VEGFR (5EW3)	-10.48	-559.05	-62.84	-101.45
PTEN (5BZX)	-8.404	-1050.01	-50.16	-94.97

Table 3. Molecular Docking study of 3-AMP-4C with PDB IDs; p53, BAX, caspase 3, SNAIL, VEGF- α, VEGFR, and PTEN using Glide, 6.7, Schrödinger, LLC, New York, NY, 2015. Table representing the Docking score (kcal/mol), IFD score (kcal/mol), Glide Energy (kcal/mol) and Glide emodel (kJ/mol).

PDB ID	Bond	Amino Acid interaction
P53 (5LGY)	Three H-bond	Arg 280,Leu137, Asn 239
BAX (2K7W)	One H-bond, one pi-pi and one pi-cation interaction	Gly 166, Phe 165 and Arg 65
Caspase 3 (4QTX)	Two H-Bond, one pi-pi and one pi-cation interaction	Gln 161, Ser 209, Arg 207, and Phe 252
SNAIL (4QLI)	Three H-bond, two pi-cation interaction	Arg 129, Arg 56, Lys 49, Lys 49 and Lys122
VEGF- α (5T89)	Three H-bond, one pi-cation and one pi- pi interaction	Ser 95, Arg 261 and Tyr 39, Arg 82
VEGFR (5EW3)	Two H-bond and one pi-pi interaction	Glu 885, Asp 1046, His 1026,
PTEN (5BZX)	Four H-bond and one pi-cation stacking	Asp 24, Tyr16, Tyr 46

Table 3.1: Molecular Docking study of 3-AMP-4C with PDB IDs; p53, BAX, caspase 3, SNAIL, VEGF- α, VEGFR, and PTEN using Glide, 6.7, Schrödinger, LLC, New York, NY, 2015. Table representing the number and type of bond and amino acid interaction between ligand and protein.

DISCUSSION

The everyday rise of cancer and insufficiency of available medication, pin-pointed toward the necessity of new drug which designed with novel bioactive component, and has improved potential over current therapeutics. Phytochemicals always attracted the chemist for the novel therapeutic intervention, as they are the richest source of bio-actives, inexpensive and less toxic. However, lower specificity, lower availability, and tedious process of extraction leads toward the formation of chemical analogue of these pharmaceutical bioactive compounds. Chalcone is one of them, which has many potential therapeutic properties, including anticancer. To further increase its efficacy, bioavailability, specificity, prolonged effect, and level of toxicity, many chalcone hybrids were prepared by adding different active anticancer pharmacophore as well as by adding different functional group on hybrid molecule and evaluated for their anticancer activity. Pyridine is one such pharmacophore which has been extensively reported in anticancer drug development (Mohamed et al., 2021). It is a heterocyclic ring of six carbon and one nitrogen atom. The hybridization of these two active pharmacophores is reported to have excellent anticancer property hence, we had also attempted to synthesize a novel hybrid of these two active compounds with different function group addition on both moieties and evaluated for their anticancer activity (Durgapal et al., 2018). The derivative 3-AMP-4C showed very good anticancer activity against A549 and MCF-7 cell line. The respective IC_{50} concentration of 3-AMP-4C in A549 and MCF-7 cell line was 0.245μM and 0.0067μM, the IC_{50} concentration of derivative in normal non-cancerous mouse fibroblast cell line *viz.*, NIH/3T3 was 79.31μM much higher than the cancer cell line which shows that the derivative 3-AMP-4C is very potent and associated with low side-effect to nearby non-cancerous cells. The *in-vitro* based EtBr/AO assay and LDH assay was performed which spotlighted that the mechanism of cytotoxicity is apoptosis. The Trypan blue assay also confirmed that the derivative 3-AMP-4C exerts cytostatic property causing cell cycle arrest. Hence, for further analysis of molecular target, physiochemical property and drug-likeliness of 3-AMP-4C *in-silico* based molecular docking, DFT analysis and ADME prediction is done, herein.

Lipinski's rule of five is hereby, used to analyze the physicochemical properties of the compound and its drug-likeliness. It includes physiochemical properties, which are imperative as a basic necessity to pass the blood-brain barrier, efficiently namely passive diffusion, includes molecular weight, lipophilicity, hydrogen bonding, and charge. When

there is a violation of more than one rule, it leads to poor bioavailability and ADME property. Any drug like molecule with molecular weight less than 500Da are easily transported, diffused, and absorbed as compared to larger molecules. The compound with high molecular weights is likely to have low solubility and have difficulty passing through the cell membrane (Srimai et al., 2013). Lipophilicity is an important parameter required by the drug candidate, which helps in crossing the hydrophobic phospholipid bilayer of the membrane. A balance of solubility and polar/hydrophobic properties is crucial for specific routes of absorption and crossing any biological barriers that a drug needs to penetrate to reach the desired site of action, in order to affect the specific event. For better oral bioavailability, lipophilicity (a ratio of the molecule's solubility in octanol to solubility in water) should be less than 5 (QlogPo/w≤5). When more than 5, H-bond donors, and more than 10 H-bond acceptors group exists, it further minimizes the bioavailability of the drug. In this study, we found that all the parameters are within the limit of Lipinski's Rule of Five (Table 2). The molecular weight of the compound is 405.8, comes within an acceptable range to have high solubility, and high probability of being able to enter a cell. H bond donor group is only 2≤5, and the H bond acceptor group in 7.5≤10, as well as clogP (QPlogPo/w), is 2.7, which again ≤5. With all these parameters, 3-AMP-4Cshows great drug-likeness.

Another important parameter for selecting a good drug candidate is its good pharmacokinetic property, i.e., ADME property. Most of the drug candidates fail in clinical trials due to poor ADME properties which lead to rapid escalation in the cost of drug development. The early-stage elimination of flawed drug candidates can reduce the wastage of time and resources and streamline the overall process of drug development. Therefore, ADME predictions at the early stage of the development process through lowering the cost and appraising the performance of candidate drug molecules during clinical trials. The pharmacokinetic property includes Blood-Brain Barrier penetration or absorption and distribution. Caco-2 cell permeability represents drug metabolism, transport, and percentage of oral absorption. All the parameters are under the acceptable limit, with 1.02 (QPlogBB), 130.9 (QPPCaco), and 86.46% oral absorption property (Table 2). No violation of Lipinski's rule of five has been observed and all together with these results,3-AMP-4C can be considered as drug-like candidate with lesser toxicity and good bioavailability.

For understanding the biological reactivity of any compound, the electron density is considered as a very important factor, especially the reactivity of electrophilic and nucleophilic sites as well as the interactions of hydrogen bonding and molecular electrostatic potential of the compound (Scrocco and Tomasi, 1979). In DFT calculation, Molecular electrostatic potential theory is used for the prediction and forecasting of all the optimized sites of the molecule's reactive sites for electrophilic and nucleophilic attack. Therefore, for predicting the reactivity of nucleophilic and electrophilic sites for 3-AMP-4C, MESP is simulated using the B3LYP level of the optimized geometry. The different colors (red, blue, and light blue) at the MESP surface (Figure 6) represent different values of the electrostatic potential as the regions of most negative, most positive, and zero electrostatic potential, respectively. The negative electrostatic potential at the MESP (red) indicates that this region is attractive to the proton by the aggregate electron density in the molecule and corresponds to electrophilic reactivity (regions of most electronegative electrostatic potential). In contrast, the positive electrostatic potential (blue) is the region that is responsible for the repulsion of the proton by the atomic nuclei and corresponds to nucleophilic reactivity (regions of the most positive electrostatic potential). In contrast, the light blue shade represents regions of zero potential. In 3-AMP-4C the nucleophilic sites are lying on the nitrogen and oxygen atom of the pyridine and chalcone moieties, which are the electronegative compound and have a tendency to attract nucleophiles. On the other side, electrophilic or positive potential sites are existing on hydrogen and methyl group of the compound, which again justifying the periodic property of hydrogen and methyl group in 3-AMP-4C. However, around the 3-AMP-4C nucleus, the electron density is equally distributed, making it electroneutral. Therefore, the MESP study helped in giving a clear vision about the electrostatic feature of the compound and with the help of this, we can presume that the derivative 3-AMP-4C will possess a high biological activity.

From the literature, it is known that the lower band-energy gap, which is the energy difference between two frontier orbitals ($\Delta E = E_{LUMO} - E_{HOMO}$), is critical for the chemical reactivity and kinetic stability of any molecule. In 3-AMP-4C, the highest occupied molecular orbital HOMO and LUMO energies were located on two distinct parts of the molecule. HOMO orbitals are located around benzene ring attached to amino group and LUMO orbital is around chalcone group (ketone and double bond in conjugation between two benzene rings) indicating the active participation of the compound during protein-ligand interactions (Figure 5). The calculated HOMO, LUMO, and HOMO-LUMO energy gap for

derivative 3-AMP-4C are -0.2473eV, -0.1046eV, and -0.1427eV, respectively (Table 1), the low and negative Eigenvalues for band energy gap indicate that both rapid electrons transfer, as well as the exchange, is equally possible, this low energy gap making the derivative 3-AMP-4C chemically very reactive. Together, these results depicted the potential reactive site and also confirmed the highly reactive nature of the 3-AMP-4C.

HOMO represents the tendency to give electron, hence ionization potential is directly related to the HOMO energy. LUMO, on the other hand, represents the electron-accepting potential of the compound therefore, LUMO energy is directly related to the electron affinity (Gece, 2008; Fukui, 1982). By using ionization potential and electron affinity, quantum chemical descriptors, such as Softness, Chemical Hardness, Chemical Potential, Electronegativity, and Electrophilic Index, were calculated (Table 1.1). The higher value of ionization potential is predictive of higher stability and chemical inertness of the molecule, while the opposite characteristics are the representative of lower ionization potential. The chemical hardness indicates the resistance towards the deformation or polarization of the electron cloud of molecules. The hard molecule has a large energy gap; however, a soft molecule has a small energy gap (Pearson, 1988). Soft molecules are more reactive than the hard ones because they can release electrons quickly. 3-AMP-4C is showing lower ionization potential of -0.247eVand higher value for chemical softness *viz.*, 7.02eV, indicating the lower stability and higher chemical reactivity of the compound. In contrast, the lower electrophilicity index ω=0.215eV (the tendency of chemical species to acquire electrons) and lower chemical potential μ=-0.175eV (an indicator of electrons escape; electrons escapes from high chemical potential to those have lower chemical potential) of3-AMP-4C signify it's potential to attract electron. Therefore, the overall global reactivity descriptors reconfirming the Frontier Molecular Orbital (HOMO and LUMO) based prediction of 3-AMP-4C.

Since derivative 3-AMP-4C is very reactive in nature, having a good ADME property, and causing cytotoxicity via apoptosis at very low IC_{50} concentration in both the cell line *viz.*, A549 and MCF-7 cell line with negligible effect on non-cancerous mouse fibroblast cell line NIH/3T3 as revealed in *in-vitro* study. Hence, major regulators*viz.*,p53, BAX, caspase 3, Snail, HIF-1α VEGF-α, VEGFR, and PTEN of key pathway i.e., apoptosis, metastasis and angiogenesis are analyzed here using in silico based molecular docking. From the results of *in-silico* study, we observe that the 3-AMP-4C is highly reactive in nature, and when docked with p53, it gives docking score of -6.89 kcal/mol and interacting with three hydrogen bond

and with amino acids, *viz*., Arg280,Leu137, Asn239, (Figure 7, Table 3.1). BAX, a pro-apoptotic protein of the BCL-2 family is critical for the outer mitochondrial permeabilization, release of cytochrome c, and initiation of apoptosis, which is directly activated by p53 and permeabilize the mitochondria (Chipuk et al., 2004). The *in-vitro* study revealed that there is

a loss of mitochondrial membrane potential and upregulation in BAX and the *in-silico* study also confirmed that 3-AMP-4C interact with BAX (Figure 8, Table 3.1), and generated docking score -13.83kcal/mol by one H-bond with amino acid Gly166, one pi-pi interactions with Phe165and pi-cation interaction with Arg 65 which may be responsible for BAX induced apoptosis (Table 3.1). Activated BAX also upregulated the caspase 8, which cleaves the caspase 3 into cleaved caspase 3 and activates the intrinsic pathway of apoptosis. The *in-silico* molecular docking strategy confirmed the interaction of 3-AMP-4C with caspase 3. Derivative 3-AMP-4C interacts with caspase 3 with two hydrogens, one pi-pi and one pi-cation interactions (Figure 9, Table 3.1)and generates docking score of -4.78kcal/mol which, signify that 3-AMP-4C induces caspase 3 and ultimately apoptosis.

3-AMP-4C interact with SNAIL to perform its anti-metastasis function via three hydrogen bonds (Arg 129, Arg 56 and Lys 49) and special interactions *viz.*,two pi-cation interactions with the Lys 49 and Lys 122. With this interaction, it generated a docking score of -3.58kcal/mol (Figure 10, Table 3, Table 3.1). The interaction is not very strong as compare to other PDBs. Similarly, VEGF-α is docked with derivative 3-AMP-4C along with VEGFR a receptor of VEGF. VEGF-α generated a docking score of -6.39kcal/mol and interacted with multiple amino acids i.e., Ser 95, Arg 261 and Tyr 39, Arg 82 via three hydrogen bond and one pi-pi and pi-cation interaction. VEGFR when docked with compound it showed a docking score of -10.48kcal/mol and interacted with two hydrogen and one pi-pi interaction at the active site of the amino acid *viz.*Glu 885, Asp 1046, His 1026, (Figure 12, Table 3.1). The docking score and interaction might have placed the ligand at the active pocket or near the site at which VEGF binds and may be inhibit their binding and minimize the process of angiogenesis in 3-AMP-4C treated cells however, interaction is positive or negative will need to be confirm by *in-vitro* based assay.

PTEN a negative regulator of the PI3K signaling pathway (PI3K/AKT pathway). When gets activated, it downregulates the intrinsic pathway of apoptosis by phosphorylating pro-apoptotic gene BAX and BAD activates MDM2, thereby downregulate the p53 and hampers

all the pathway regulated by p53 including that of metastasis. Herein, *In-silico* study revealed that 3-AMP-4C interact with active site amino acid Asp 24, Tyr16, Tyr 46by three H-bond and one pi-cation stacking (Figure 13, Table 3, Table 3.1) and gives docking score of -8.4kcal/mol which indicate involvement of PI3K/Akt pathway in 3-AMP-4C induced anticancer effect in cell lines. On the basis of molecular docking study, we can presume that 3-AMP-4C may exert its anticancer effect via p53 mediated apoptotic pathway in which BAX act as activator of intrinsic and extrinsic pathway of apoptosis and caspase 3 act as an effector caspase. Similarly, Snail VEGF, and VEGFR are the key molecules involve in 3-AMP-4C induced ant-metastasis and anti-angiogenic effect as well as PI3K/AKT as a major pathway behind its anticancer effect.

CONCLUSION

The overall finding demonstrates that *in-silico* molecular docking study is a powerful technique to study the action of many key molecules involves in potentiate the anticancer effect of any compound. Herein, an *in-silico* molecular docking study is performed to predict the molecular targets behind the anticancer effect of derivative 3-AMP-4C which need to be confirm by *in-vitro* based assays. However, the interaction of compound 3-AMP-4C with key molecules *viz.*, p53, BAX, caspase 3, SNAIL, VEGF- α, VEGFR, and PTEN, were analyzed. The result revealed that the 3-AMP-4C is directly interacting with these molecules and ensuing its effect. The corresponding sites of reactivity and highly reactive nature of the compound are uncovered in MESP and HOMO-LUMO studies. Drug-likeliness and ADME property of 3-AMP-4C revealed that compound has good drug-likeliness and ADME property with high oral absorption quality. Therefore, it can be taken further as a novel chemotherapeutic agent for future anticancer drug development.

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