Chapter 3

Material and Methods

3.1. MOLECULAR MODELING

Computer-assisted drug designing (CADD) in context of drug design and discovery involves generation, representation and utilization of 3D structures of therapeutic agents to calculate associated properties to interpret SAR with respect to their respective biological targets. Most CADD exercises are focused on estimating the conformation, binding affinity and fit value of ligands or drug like molecules in the binding pockets of biological targets. Some common CADD exercises include molecular docking, molecular dynamics, quantum mechanics, binding affinity calculations, pharmacophore and other drug property calculation processes. In the present study, a systematic virtual screening protocol was utilized (**Figure 3.1.**) whereby ligand-based drug designing approach was clubbed in association with structure-based drug design approach in order to explore the essential structural and chemical features required for the inhibition of ERK1/2. First scaffold hopping, also known as lead hopping strategy was utilized to find out the structurally new compounds by using Ulixertinib as standard known ERK2 inhibitor. Then Fragment Based Drug Discovery (FBDD) approach was employed to discover the leads. Molecular docking was performed in each approach to validate the results. Further molecular dynamic study was performed to study the stability of compounds. The MM-GBSA calculation, DFT study and ADMET study were also performed.

3.1.1. Softwares

Various softwares (commercial and open access) are used for performing molecular modeling studies. For the present study, AUTODOCK, MOE, SCHRÖDINGER and AMBER software were used for different *in-silico* exercises such as MD simulations, molecular docking analysis, MM-GBSA calculations and DFT. The details of the steps involved in the study are mentioned below (**Table 3.1.**).

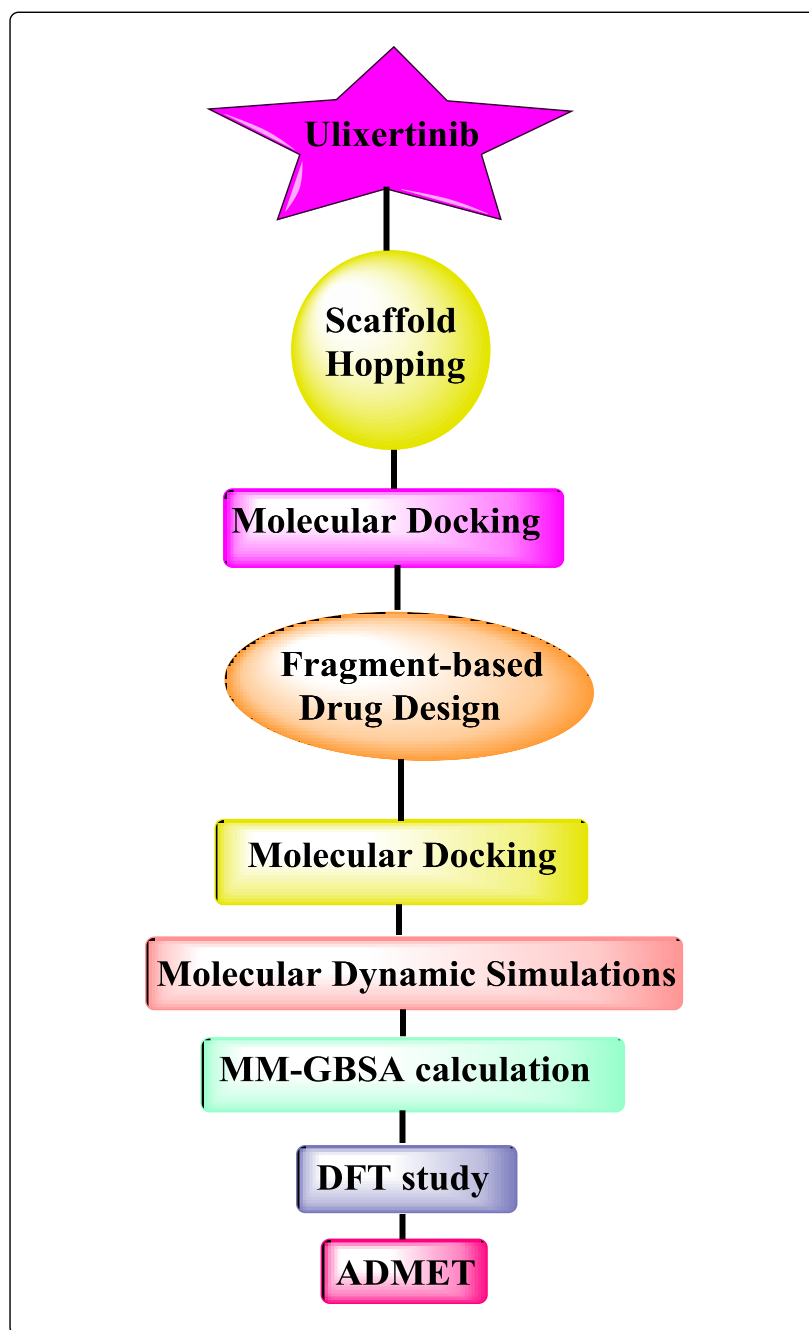


Figure 3.1. Virtual screening protocol utilized to screen putative hits against ERK2.

Table 3.1. List of softwares/modules and their role in the study.

S.No.	Name	Role
1.	*ChemMapper*	Scaffold Hopping
2.	Autodock 4.2	Molecular Docking
3.	ACFIS	FBDD
4.	MOE	Molecular docking and Dynamics
5.	AMBER	Molecular Dynamics and MM-GBSA
6.	Schrödinger’s Glide	Molecular docking
7.	Schrödinger’s Prime	MM-GBSA
8.	Desmond	Molecular dynamics
9.	Schrödinger’s Jaguar	DFT
10.	Schrödinger’s Qikprop	ADMET

3.1.2. Selection of ligand and protein

The structure of ERK2 protein was retrieved from the RCSB protein data bank considering type of organism (Homo sapiens). A cut-off value of 2.00 Å was employed to filter out high resolution structures, which resulted in 14 X-ray crystallography solved structures. Then, cross docking experiment was performed to select the most optimum PDB. Cross docking methodology involves putting all the ligands into all the protein structures, one by one and then calculating RSMD from the co-crystallized pose to determine docking sensitivity of a specific PDB structure (Mukherjee, Balius et al. 2010). All the ligands used in this study were sketched in Chem draw ultra-12. Ulixertinib was used as standard ligand in molecular modeling study.

3.1.3. Scaffold hopping

Scaffold hopping is defined as the process of identification of iso-functional central cores with diverse backbones and in the process improving or maintaining the

pharmacological and pharmacokinetic profiles. This term was given by Gisbert Schneider, former researcher at Hoffmann-La Roche. Scaffold hopping is performed to speed-up the drug discovery process. The concept of scaffold hopping is based on the principle of similarity, which means that compounds that are structurally similar show similar pharmacological profile (Silakari and Singh 2020). It helps in replacing scaffolds with similar ones on the basis of molecular 3D similarity calculation. In the current study, we used *ChemMapper*¸ a freely accessible webserver (http://www.lilab-ecust.cn/chemmapper/), to perform scaffold hopping (Gong, Cai et al. 2013). It works *via* identification of hits using SHAFTS, a method developed by Gong *et al* (Gong, Cai et al. 2013)), in which chemical feature matching and molecular shape superposition are combined. The *SHAFTS* method implements a hybrid similarity metric which include both pharmacophoric features and molecular shape resulting in the determination of similarity in 3D space, followed by ranking, thereby integrating the advantages of both pharmacophore matching and volumetric overlay methods.

This method involved two steps i) preparation of query molecule ii) uploading the query molecule in SMILES format on the server. The hit molecules were then obtained through SHAFT by calculating the 3D similarities *i.e*. shape similarity and feature matching amongst the query and molecules of a specific database (NCI database), followed by ranking was done on the basis of 3D similarity score (scaled to 0-2). The score closer to 2 represent the highest pharmacological association among the hits. Five threshold values (0.8, 1.0, 1.2, 1.5, and 1.8) were set to limit the output and all the compounds below these threshold values were not considered. Overall, total 100 hits were selected for the job.

3.1.4. Molecular Docking

Molecular docking analysis is one of the conventional and most reliable method which predicts the interaction behavior nature of drug-like molecules with their respective targets (Singh, Chaudhari et al. 2019). Thus, molecules obtained as result

of scaffold hopping and further after FBDD were subjected to molecular docking using Autodock 4.2 (Forli, Halliday et al. 2012). The selected co-crystallized protein was subjected to protein preparation by removing water molecules and addition of polar hydrogens. The missing side chain amino acid residues in the loop of 6GDQ were filled using the Modeller package in Chimera (version 1.12) (Fiser and Do 2000). Charges for protein complex were attuned by means of Gasteiger charges employed in AutoDock 4.2. Molecular docking was performed using genetic algorithm protocol (Boraei, Singh et al. 2019). Docking grid generation was performed considering the co-ordinates of co-crystallized ulixertinib in the pdb file with 50 grid points along every axis, with spacing of 0.375 Å *via* AutoGrid module. The key amino acid residues which form the catalytic domain of ERK2 include Gly34, Ala35, Tyr36, Gly37, Lys54, Ile56, Asp167, Leu107 and Met108. The obtained docked poses were analyzed in the light of interaction diagram of ulixertinib in complex with ERK2. Binding conformation and affinity of the screened hits were also studied. The complexes with essential required interactions were scrutinized.

3.1.5. Fragment based drug discovery

Fragment based drug discovery (FBDD) is an approach of breaking down the binding pocket into smaller sites and identifying sub-structures which have higher affinity for these smaller regions in the binding pocket. FBDD was carried out using ACFIS (Auto Core Fragment In silico Screening) server, based on three modules including PARA_GEN, CORE_GEN and CAND_GEN (Hao, Jiang et al. 2016). The PARA_GEN tool comprises of parameters of molecular force field as required for molecular dynamic simulation studies (http://chemyang.ccnu.edu.cn/ccb/server/ACFIS/). The CORE_GEN (Kolb and Caflisch 2006) is used to identify the pharmacophores structure from a bioactive molecule *via* deconstruction of fragment. It can also help in the optimizing pharmacologically active molecules in such a manner that ligand efficiency also improves. The CORE_GEN provides an ideal fragment which can selected as core for

the CAND_GEN job. CAND_GEN tool is used to identify the hit candidates by fragment linking approach. For the PARA_GEN analysis, total formal charge was auto-calculated and partial charge calculation was done *via* Gasteiger charge method. For the CORE_GEN analysis, protein-hit complex prepared *via* PARA_GEN was utilized. Finally, for the CAND_GEN analysis, ΔG (MM/PBSA) was chosen as a method to sort the hit candidates. Core FDA Drug Fragment database with 290 fragments was chosen as the fragment database for screening. Time for MD simulation was kept 5ps for each ligand and total 10 hits were obtained following this protocol.

3.1.6. Molecular Dynamic Simulations

Molecular dynamic (MD) simulations is still developing field of computational drug discovery field. It was initially developed to study solid spheres in 1950s by Alder and Wainwright. Later on it was improved to study liquids like water and finally it entered the field of drug discovery with the simulation of trypsin inhibitor in 1977 (Alder and Wainwright 1957; Alder and Wainwright 1959; Singh and Silakari 2018). Out of all the *in-silico* methodologies, MD simulations is the closest to the wet lab experiments. Therefore, this method is extensively utilized to study biological systems and their real-life parameters on a time dependent manner. The top binding poses in the catalytic domain obtained after molecular docking were selected as initial coordinates for the MD simulation analysis. The prime reason to perform MD simulation analysis was to validate the binding mode of the designed molecules and to evaluate the stability of hit-protein complexes *via* review of the 3D interaction plots and RSMD plots for the time period of production run. In the current study, MD simulation analysis was performed using MOE and AMBER software (ChemicalComputingGroup 2008; Zhou, Negi et al. 2019) with AMBER99 force field. The protocol involves calculation of the partial charges, followed by performing energy minimizations of the complex. The methodology of MD simulations followed solvating hit-protein complexes in a SPC water molecules using periodic boundary, in

a spherical box. The production step of MD simulations was run for 100 ns. The simulations were carried out using the NPT ensemble, with the fixed temperature via Nose–Hoover method as the thermostat at 310K and fixed pressure using Berendsen barostat at 1 bar. The equations of motion were solved at the time step of 2 fs during the whole simulation run and the data of coordinates of all atoms in the complexes were stored in the database. Finally, RMSD value for the ligand molecule and protein backbone was calculated after the run of 100 ns.

3.1.7. MM-GBSA calculation

MM-GBSA (Molecular Mechanics-Generalized Born Surface Area) calculations in combination with MD simulations provide one of the most prominent methodologies to estimate free binding energies of hit molecules in complex with target proteins. This free binding energy ΔGbind represents the binding affinity of the ligand-protein complexes. The various components of binding energy include polar and non-polar solvation energies, potential energy, etc. Therefore, in the current study, MM-GBSA calculations were performed by extracting MD scripts. MM-GBSA program of Amber was used for the MM-GBSA calculations and analysis (Wang, Wang et al. 2019). Following equation summarizes the methodology to perform these binding energy calculations:

$$\Delta G_{bind} = G_{complex} - (G_{receptor} + G_{ligand})$$

$\Delta G_{complex}$: free energy of the complex, $G_{receptor:}$ free energy of unbounded receptor, and $G_{ligand:}$ free energy of unbounded ligand.

The calculations were performed based on receptor ligand complexes obtained after molecular dynamic simulations.

3.1.8. Density functional theory (DFT) analysis

DFT (Density functional theory) calculations represents the quantum mechanical calculations which provides information regarding electronic properties of the designed molecules. It gives insight into the structural behavior and orientation of

the top hits and effect of electronic properties on the pharmacological activeness of the hits (Ahmad, Jadhav et al. 2021). In the current study, DFT based single-point energies were calculated to determine energy states, structure and electronics of each atom in all the hit compounds. Orbital energy values i.e., HOMO (Highest Occupied Molecular Orbital) and LUMO (Lowest Unoccupied Molecular Orbital Energy) along with the energy gap of the top screened hits were also computed. As per frontier molecular orbital theory, HOMO has the priority to donate electrons, while LUMO is responsible to accept electrons and this ability of transferring electrons in any compound dictates its interaction within the binding pocket of target protein and thereby influencing its bioactivity (Asati, Thakur et al. 2018). Jaguar module (Schrödinger version 2020-3) was used to perform all the DFT calculations (Bochevarov, Harder et al. 2013). Becke three-parameter exchange potential and B3LYP (Lee-Yang-Parr correlation functional) along with 6-31G** basis set were employed for geometry optimization (Gill, Johnson et al. 1992) (Lee, Yang et al. 1988).

3.1.9. Improving the top hit: Library generation and screening of derivatives of the top hit

Upon identifying top hits from the virtual screening exercise, *in-silico* hit optimization of the top hit was performed to improve its binding affinity for ERK2. Different modules available in *Schrödinger* molecular modelling suite were utilized for this step. A library of derivatives of hits and their different protonation states at pH 7.4 ± 0.5 were build and optimized using the *Ligprep* module of *Schrödinger* 2021-1 molecular modelling platform (Schrödinger Inc, USA) with OPLS4 force field (Harder, Damm et al. 2016). To perform molecular docking analysis against ERK2, the co-crystallized ERK2 protein (PDB: 6GDQ) (Heightman, Berdini et al. 2018) was extracted from protein data bank. *Protein Preparation Wizard* module of *Schrödinger* 2021-1 was used to prepare protein, adding polar hydrogens and partial charges. Bond orders were also thoroughly reviewed. Crystal

waters were removed, and energy was minimized using OPLS4 force field. XP (extra precision) mode in Glide module of *Schrödinger* 2021-1 (Halgren, Murphy et al. 2004), was employed for docking analysis (Friesner, Murphy et al. 2006). The grid coordinates were generated using co-crystallized ligands in the selected PDB. Post-docking minimization was utilized to optimize the ligand geometries. The top scoring docked poses were reviewed for essential interactions within the binding pocket.

Hits maintaining the essential interactions within the binding pocket of ERK2 during docking analysis were subjected for the molecular dynamic simulations using OPLS4 force field. Desmond was utilized to perform MD simulations because of its easy-to-use graphical user interface (Shaw 2005). MD simulation analysis was employed to study the stable conformation of the designed compounds in complex with ERK2. The interaction network obtained after production run of MD simulations is considered more reliable. Briefly, in the current protocol MD simulations was initiated with solvating the ligand-protein complexes using TIP3P solvent model in an orthorhombic box (Kumar Singh and Silakari 2019). Na^+ ions were added to adjust the pH with salt concentration of 0.15 M. Followed by running production simulation for the time period of 100ns with a time step of 10.0fs, temperature at 310K, and pressure at 1.01325bars.

The free binding energy, $\Delta G_{binding}$ which represents the binding affinity of the designed molecules in the ATP-binding pocket of ERK2 was calculated using MM-GBSA method (Jacobson, Pincus et al. 2004; Genheden and Ryde 2015; Singh and Silakari 2017). Briefly, free binding energy, estimated using an implicit solvent model with molecular mechanics force field (Generalized Born Surface Area), is the difference between the free energy of the complex and sum of individual free energy of unbound ligand and unbound receptor. MM-GBSA experiments are faster and able method to predict relative binding energies in correlation with experimental data (Parenti & Rastelli, 2012). Following equation represents methodology to perform the binding energy calculations:

$$\Delta G_{binding} = G_{complex} - (G_{receptor} + G_{ligand})$$

$\Delta G_{complex}$: free energy of the complex, $G_{receptor:}$ free energy of unbounded receptor, and $G_{ligand:}$ free energy of unbounded ligand.

Pharmacokinetic properties of the designed molecules can be determined in terms of different physicochemical parameters such as molecular weight (MW), QP logPo/w, QP PCaco, hydrogen bond donor, hydrogen bond acceptor, QP logKhsa, percent oral absorption and HERG toxicity prediction. These parameters are crucial in determining the drug-like capability of the designed compounds and their availability at the site of action, to attain clinically active concentration. Therefore, all the designed molecules were subjected to Qikprop module of *Schrödinger* 2021-1 to predict the drug-like and ADMET properties.

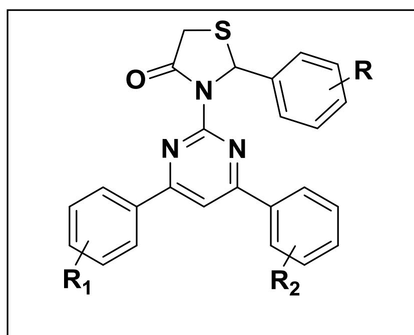
3.2. SYNTHESIS

3.2.1. Chemistry

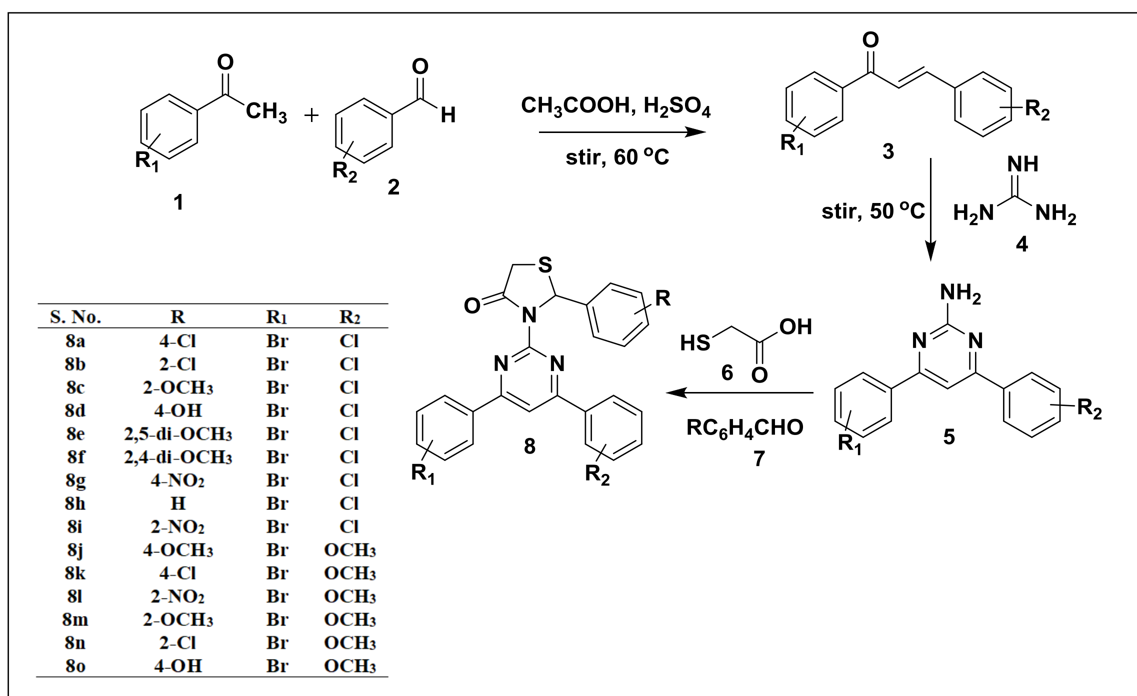
All the solvents and reagents employed in the synthetic experiments were procured from commercial suppliers such as Sigma-Aldrich and TCI. Standard procedures were followed to dry all the solvents. All the reagents were used without further purification unless indicated otherwise. Commercially procured pre-coated TLC plates were used to monitor reaction mixtures. The compounds were purified using column chromatography with 100-200 mesh silica gel and combination of ethyl acetate and hexane as mobile phase. Open capillary melting point apparatus was used to determine melting points, uncorrected. FT-IR spectrophotometer, using KBr pellet, was used to record Infrared (IR) spectra. Bruker Avance II 400 MHz NMR Spectrometer was used to record ^{1}H-NMR and ^{13}C-NMR spectra (400 MHz and 100 MHz, respectively) using $CDCl_3$. Tetramethylsilane (TMS) was used as internal standard, chemical shift (δ) values were recorded as parts per million (ppm) and their multiplicities were observed as singlet (s), doublet (d), triplet (t), quartet (q) and multiplet (m). The coupling constants have been expressed in hertz (Hz). Waters Q-TOF micro ESI-MS spectrometer was employed to record Mass spectra, at positive ionization mode by electro-spray ionization (ESI^{+}).

3.2.2. General procedures for the synthesis of compounds pertaining to Formula-I

Following synthetic route (**Scheme 3.1.**) was utilized for the synthesis of designed molecules pertaining to formula-I (thiazolidinone-pyrimidines).



Formula-I

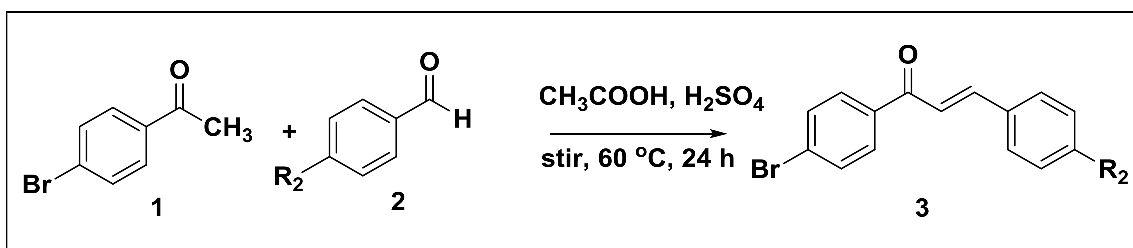


S. No.	R	R1	R2
8a	4-Cl	Br	Cl
8b	2-Cl	Br	Cl
8c	2-OCH3	Br	Cl
8d	4-OH	Br	Cl
8e	2,5-di-OCH3	Br	Cl
8f	2,4-di-OCH3	Br	Cl
8g	4-NO2	Br	Cl
8h	H	Br	Cl
8i	2-NO2	Br	Cl
8j	4-OCH3	Br	OCH3
8k	4-Cl	Br	OCH3
8l	2-NO2	Br	OCH3
8m	2-OCH3	Br	OCH3
8n	2-Cl	Br	OCH3
8o	4-OH	Br	OCH3

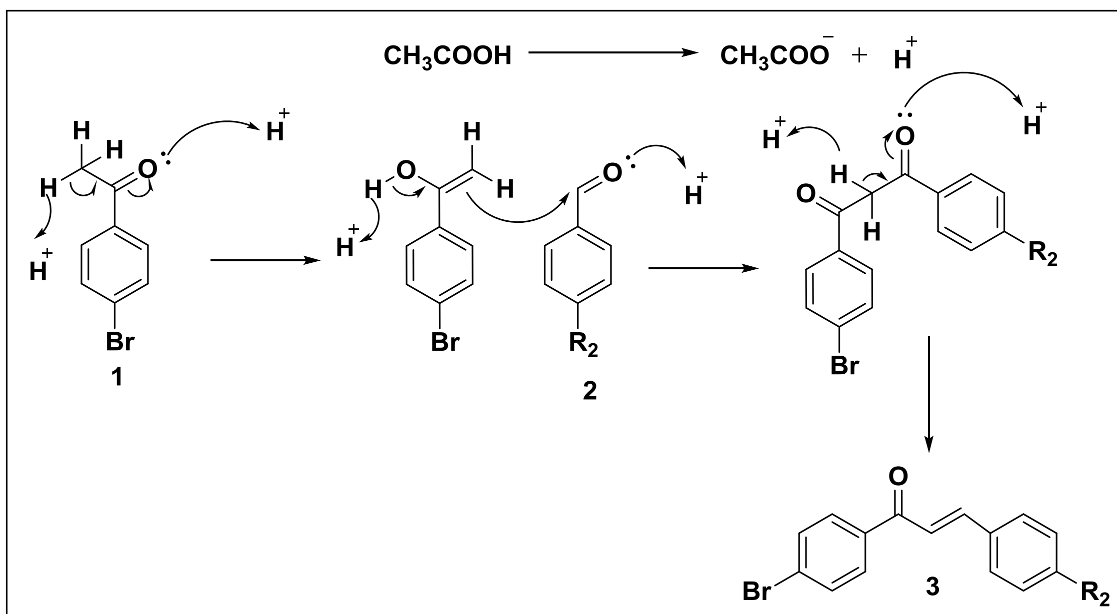
Scheme 3.1. Synthetic scheme of designed thiazolidinone -pyrimidine analogues (Formula-I).

3.2.2.1. Synthesis of (*E*)-1-(4-bromophenyl)-3-(4-substitutedphenyl)prop-2-en-1-one (3)

To the solution of 4' -bromoacetophenone (**1**) (1 gm, 0.005 mol) and 4-substituted benzaldehyde (**2**) (1 eq., 700 mg) in glacial acetic acid (10 ml), catalytic amount of conc. H_2SO_4 was added and left for stirring at 60°C, for 24 h. Exhaustion of reactants was monitored using thin layer chromatography. Cold water was added to quench the reaction upon completion. The freshly formed precipitates were filtered to obtain the crude product. Recrystallization in ethanol was done to obtain pure chalcone as product (3) (**Scheme 3.2.**).



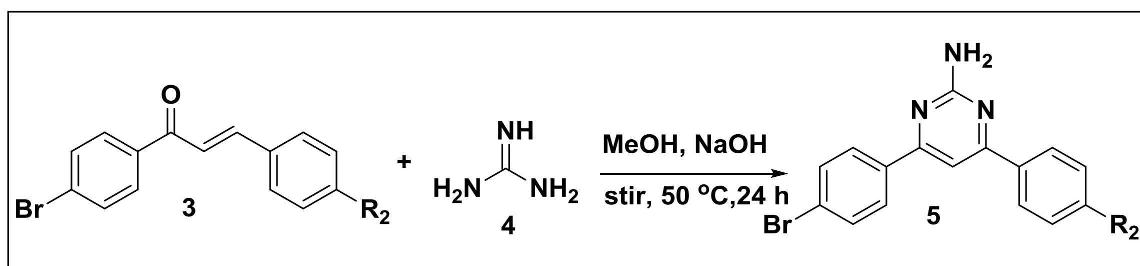
Scheme 3.2. Synthesis of (*E*)-1-(4-bromophenyl)-3-(4-substitutedphenyl)prop-2-en-1-one (**3**).



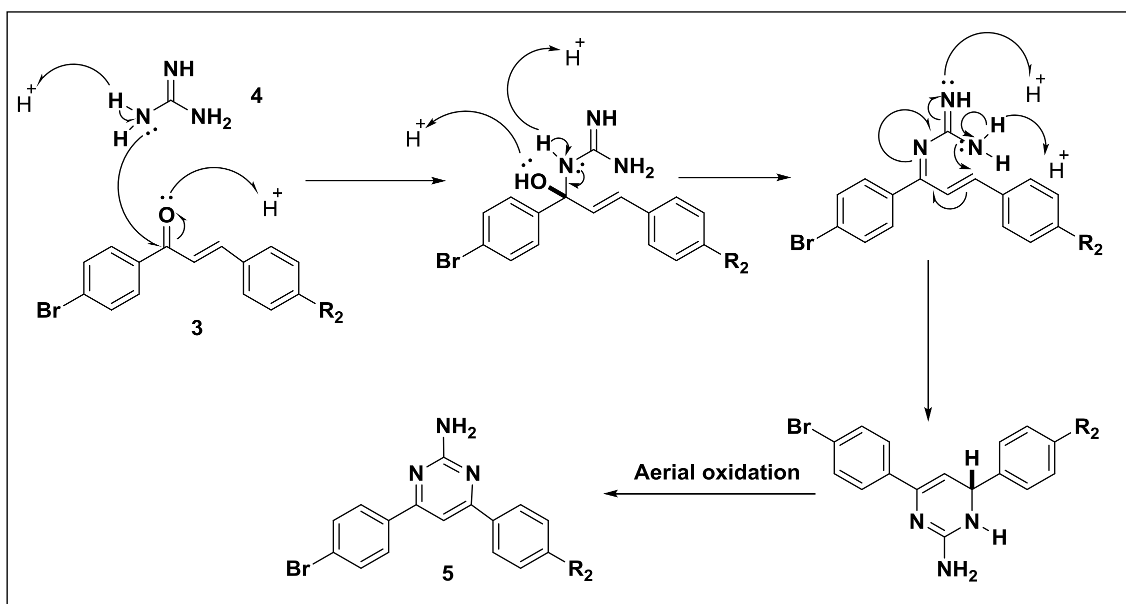
Scheme 3.3. Proposed mechanism for the synthesis of (*E*)-1-(4-bromophenyl)-3-(4-chlorophenyl)prop-2-en-1-one (**3**).

3.2.2.2. Synthesis of 4-(4-bromophenyl)-6-(4-substitutedphenyl)pyrimidin-2-amine (5)

In 10 ml of methanol, 3 (640 mg, 0.002 mol) and NaOH (2 eq.) were dissolved. Upon forming clear solution, 1 eq. guanidine hydrochloride (4) was added and the reaction mixture was stirred for 24 h, at 50°C. TLC was done to monitor the completion of reaction and, upon completion, 5% solution of HCl was added to quench excess base and stop the reaction. The freshly formed precipitates were filtered to obtained crude product. Recrystallization in ethanol was done to obtain pure 4,6-biaryl pyrimidine as product (5) (**Scheme 3.4.**).



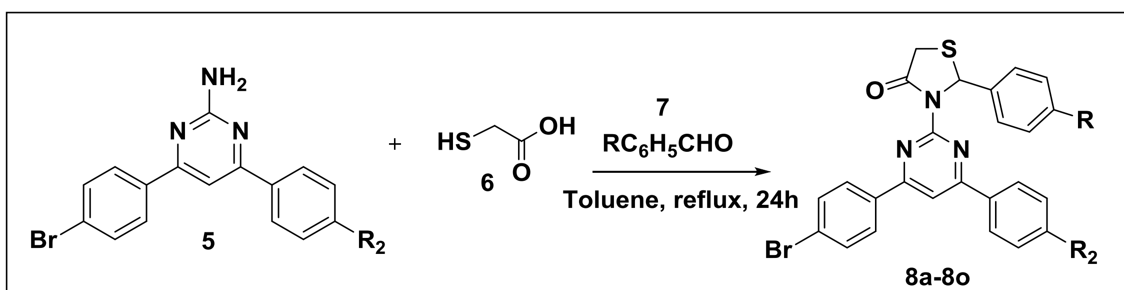
Scheme 3.4. Synthesis of 4-(4-bromophenyl)-6-(4 substitutedphenyl)pyrimidin-2-amine (**5**).



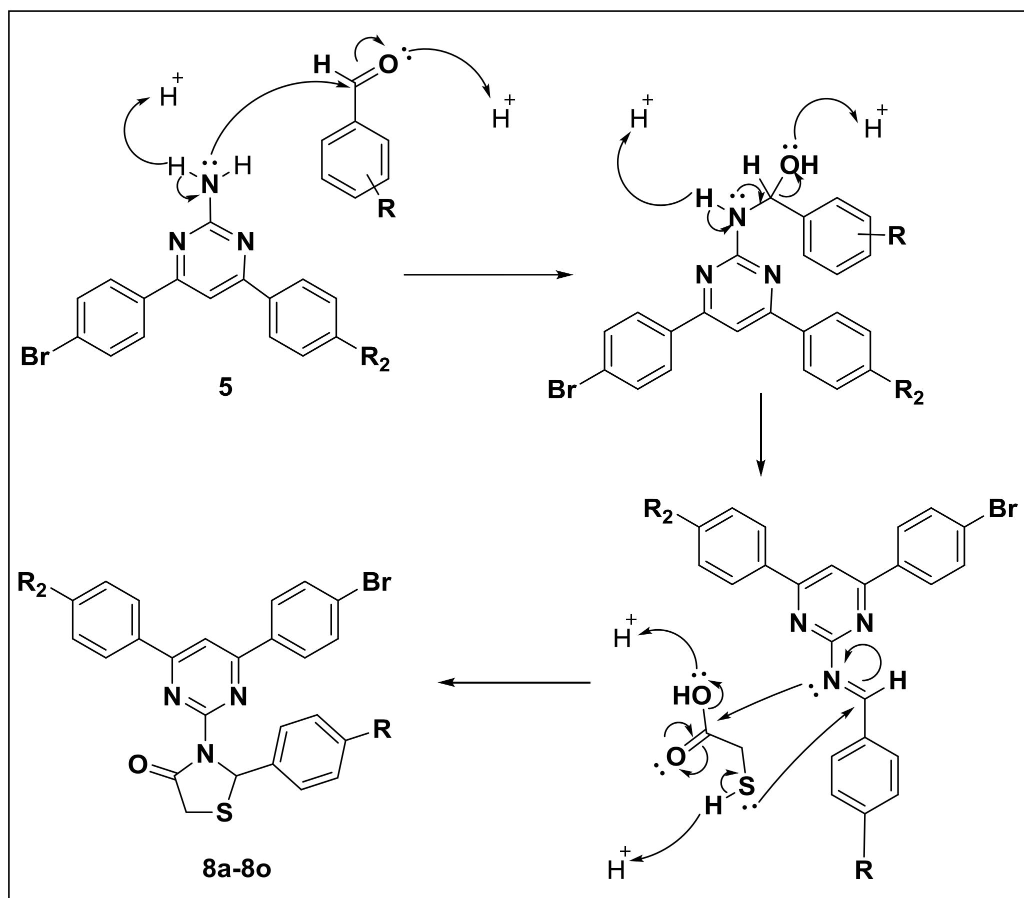
Scheme 3.5. Proposed mechanism for the synthesis of 4-(4-bromophenyl)-6-(4-chlorophenyl)pyrimidin-2-amine (**5**).

3.2.2.3. Synthesis of final compounds pertaining to Formula-I (8a-8o)

In 8 ml toluene, 360 mg of compound **5** (0.001 mol), 1 eq. of substituted benzaldehydes (7) and catalytic amount of acetic acid were added. Reaction mixture was then refluxed for 24 h. Afterwards, thioglycolic acid (6) (2 eq) was added and reflux continued for another 24 h (**Scheme 3.6.**). Upon completion, the reaction mixture was concentrated under vacuum and the obtained crude product was extracted with chloroform (3×15 ml) and dried over sodium sulfate. The filtrate was concentrated under vacuum and the crude product was column chromatographed using 20% ethyl acetate in hexane over 100-200 mesh silica to afford pure compounds (**8a-8o**).



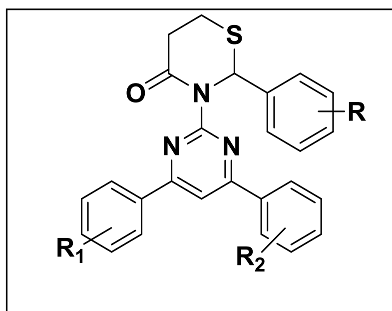
Scheme 3.6. Synthesis of final compounds pertaining to Formula-I (**8a-8o**).



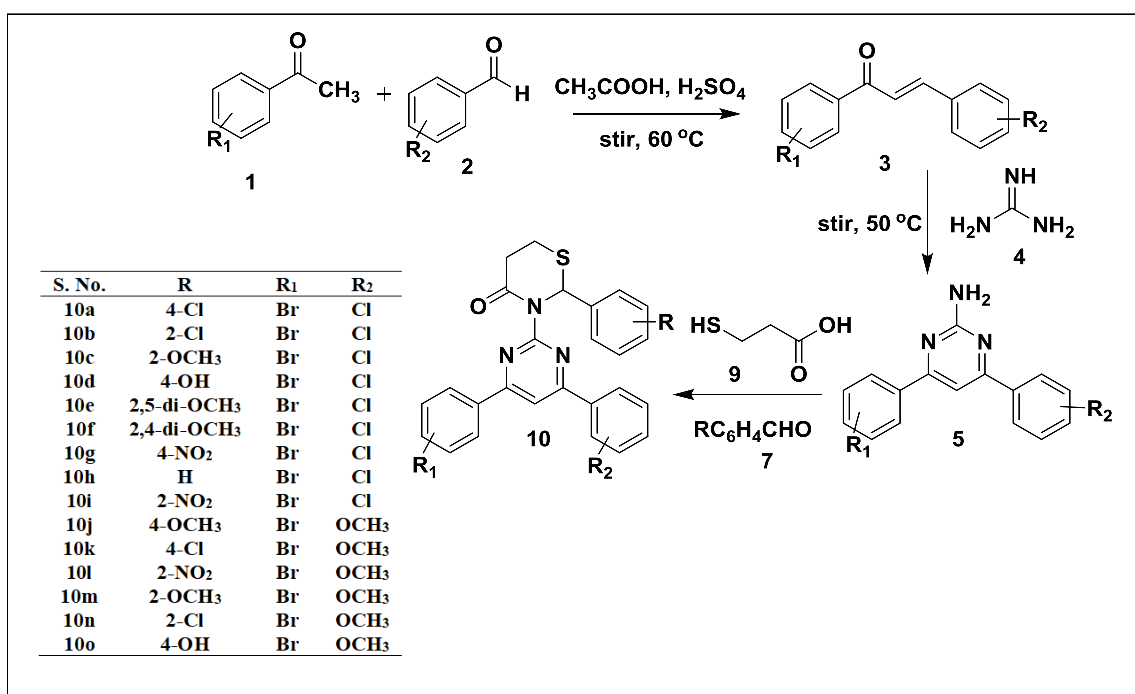
Scheme 3.7. Proposed mechanism for the synthesis of final compounds pertaining to Formula-I (**8a**-**8o**).

3.2.3. General procedures for the synthesis of compound pertaining to Formula-II

Following synthetic route (**Scheme 3.8.**) was utilized for the synthesis of designed molecules pertaining to Formula-II (thiazinanone-pyrimidine).



Formula II



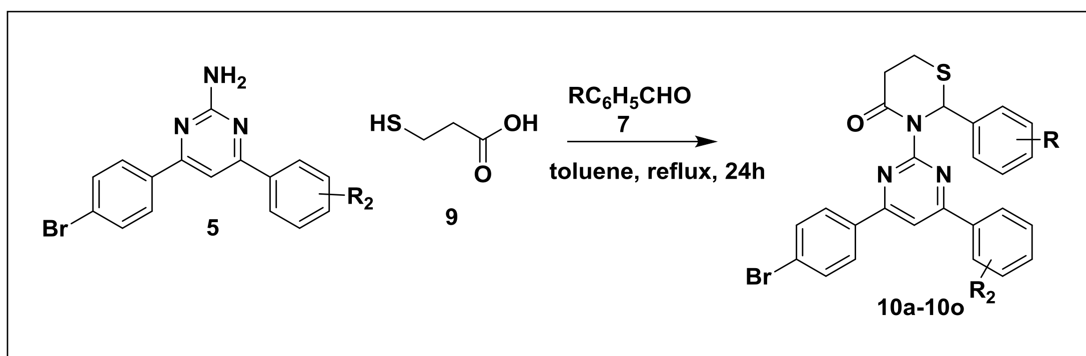
S. No.	R	R_1	R_2
10a	4-Cl	Br	Cl
10b	2-Cl	Br	Cl
10c	2-OCH_3	Br	Cl
10d	4-OH	Br	Cl
10e	2,5-di-OCH_3	Br	Cl
10f	2,4-di-OCH_3	Br	Cl
10g	4-NO_2	Br	Cl
10h	H	Br	Cl
10i	2-NO_2	Br	Cl
10j	4-OCH_3	Br	OCH_3
10k	4-Cl	Br	OCH_3
10l	2-NO_2	Br	OCH_3
10m	2-OCH_3	Br	OCH_3
10n	2-Cl	Br	OCH_3
10o	4-OH	Br	OCH_3

Scheme 3.8. Synthetic scheme of designed thiazinanone-pyrimidine analogues (**Formula-II**).

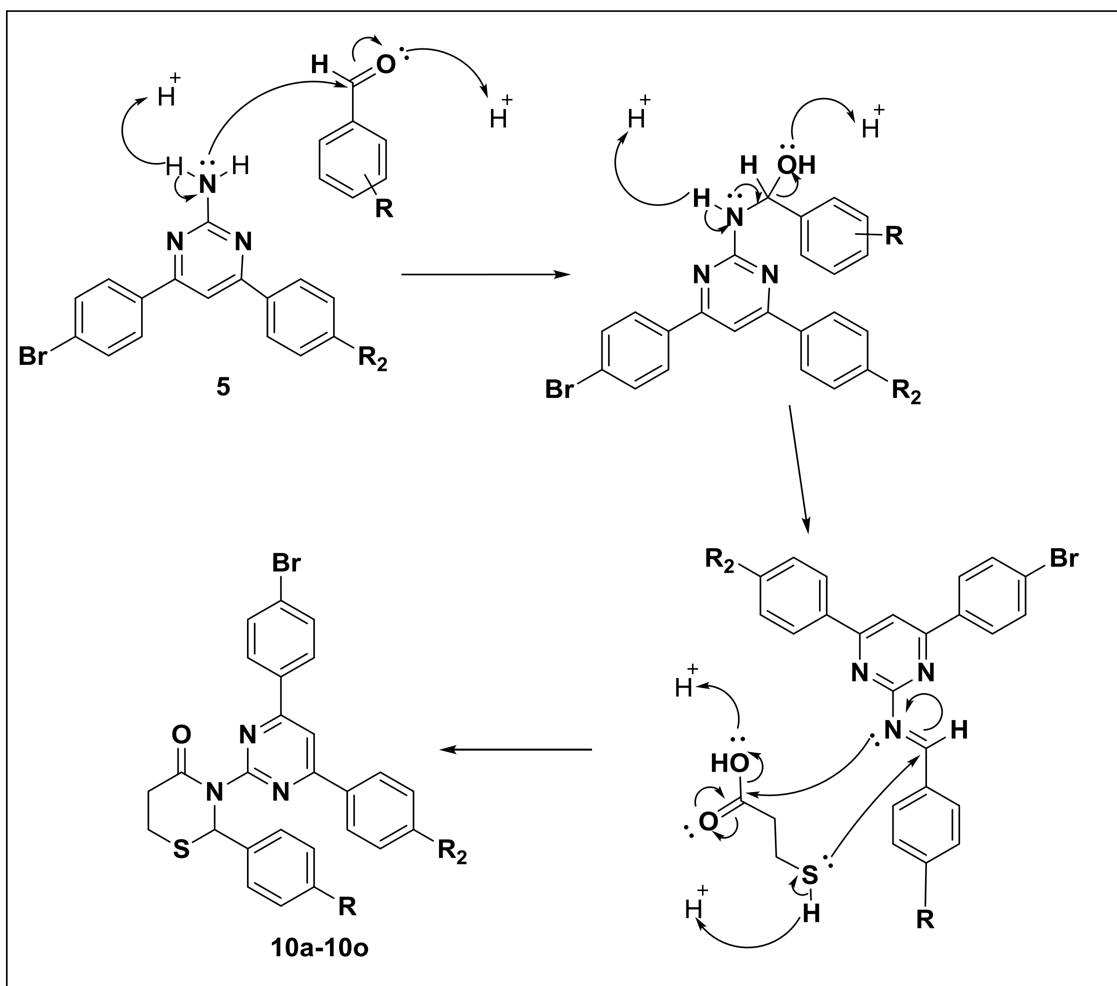
3.2.3.1. Synthesis of final compounds pertaining to Formula-II (10a-10o)

Initially, the same synthetic protocol was followed for the preparation of compound 3 and 5 as depicted in **3.2.2.1.** and **3.2.2.2**. After that, in 8 ml toluene, 360 mg of compound **5** (0.001 mol), 1 eq. of substituted benzaldehydes and catalytic amount of acetic acid were added and refluxed for 24 h. Afterwards, 3-mercaptopropionic acid (2 eq) was added and reflux continued for another 24 h (**Scheme 3.9.**). Upon

completion, the reaction mixture was concentrated under vacuum and the obtained crude product was extracted with chloroform (3×15 ml) and dried over sodium sulfate. The filtrate was concentrated under vacuum and the crude product was column chromatographed using 20% ethyl acetate in hexane over 100-200 mesh silica to afford pure compounds (**10a-10o**).



Scheme 3.9. Synthesis of final compounds pertaining to Formula-II (**10a-10o**).



Scheme 3.10. Proposed mechanism for the synthesis of final compounds pertaining to Formula-II (**10a-10o**).

3.3 BIOLOGICAL EVALUATION

3.3.1. Enzymatic assay

To evaluate the ERK2 inhibitory activity of the designed compounds, *in-vitro* enzymatic assay, using Z'-LYTE® kinase assay, against ERK2 was performed. In principle, Z′-LYTE® kinase assay is a fluorescence-based assay. It utilizes a peptide with FRET pair which emits fluorescence. This assay explores different sensitivities of peptides (non-phosphorylated and phosphorylated) towards enzymatic cleavage. Specifically, in this assay two fluorophores are coupled together to form a FRET pair

(**Figure 3.2.**). In the experiment, first, test compounds are incubated with kinase and labelled peptide for an hour. Afterwards, the fraction of peptides which remains unphosphorylated are cleaved, unsettling the FRET pair. The activity of the test compounds is quantified on the basis of ratio of emissions between cleaved vs. intact peptides. The excitation, prior to emission, for this assay is done at 400 nm (Jia, Quinn et al. 2008; Ma, Deacon et al. 2008).

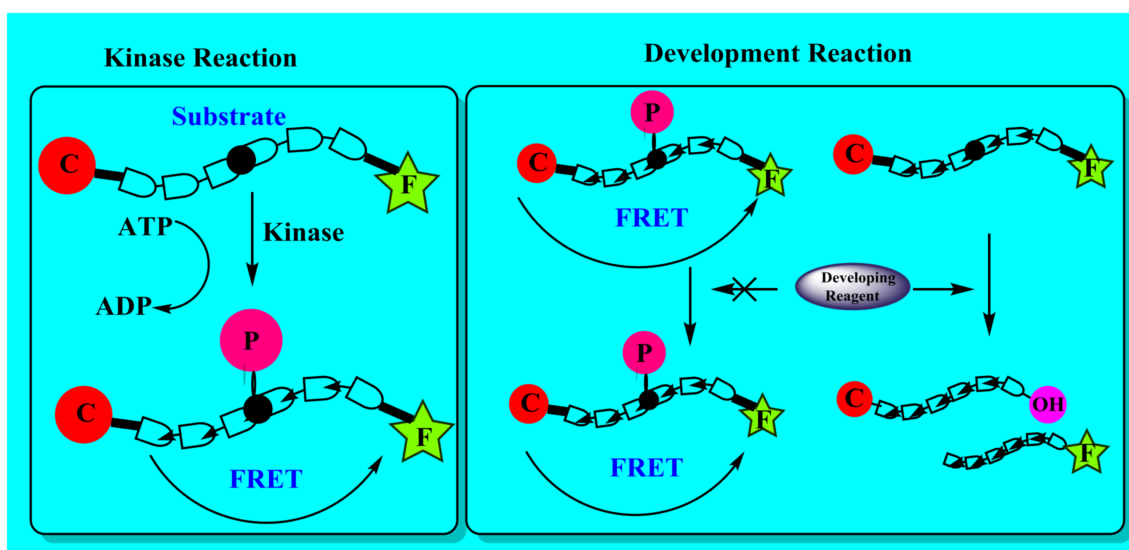


Figure 3.2. Schematic diagram of Z'-LYTE® kinase assay method.

3.3.1.1. Procedure for enzymatic assay

Briefly, 2X MAPK1 (ERK2)/Ser/Thr 03 mixture was prepared in 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.01% BRIJ-35, 1 mM EGTA. The final 10μL Kinase Reaction consisted of 25 ng ERK2 and 2 μM Ser/Thr 03 in 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 0.01% BRIJ-35, 1 mM EGTA. Kinase reaction was incubated for 1 h, followed by addition of 5 μL of a 1:1024 dilution of Development Reagent A. Thereafter, 5 μL of Stop reagent was added to quench the kinase reaction and emitted fluorescence signals were measured for both donor and acceptor emission at 445 nm and 520 nm, respectively.

3.3.1.2. Analysis of data

Fluorescence emission signals of both fluorescein at 520 nm and coumarin at 445 nm were used to calculate the emission Ratio for each well on the assay plate using the following formula:

$$Emission\ Ratio = Coumarin\ Emission\ (445\ nm) / Fluorescein\ Emission\ (520\ nm)$$

Thereafter, % Phosphorylation in each well was calculated by determining 0% and 100% Phosphorylation control wells. Following formula was used:

$$\%\ Phosphorylation = \left\{1 - \frac{\{(Emission\ Ratio\ x\ F100\%) - C100\%\}}{\{(C0\% - C100\%) + [Emission\ Ratio\ x\ (F100\% - F0\%)]\}}\right\} * 100$$

Where, $C_{0\%}$ = Average coumarin emission signal of the 0% Phos. Control; $C_{100\%}$ = Average coumarin emission signal of the 100% Phos. Control; $F_{0\%}$ = Average fluorescein emission signal of the 0% Phos. Control; $F_{100\%}$ = Average fluorescein emission signal of the 100% Phos. Control;

$$\%\ Inhibition = \left\{1 - \frac{\%\ Phos\ sample}{\%\ Phos\ 0\%\ Inhibition\ Ctl}\right\} * 100$$

3.3.2. Anti-proliferative assay

3.3.2.1. Cell lines under study

Three cancer cell lines, MCF-7, MDA-MB 231 and A549 were employed to evaluate the anti-cancer activity of the designed compounds. While HBL-100 cell line was used to evaluate normal cell toxicity.

MCF-7

This human mammary cancer cell line is most commonly used in research laboratories. MCF-7 cell line was derived from the 69-year-old Caucasian women's breast tissue in 1970 at Michigan Cancer Foundation-7 institute. This cell line is noninvasive and shows the expression of wild type and variant estrogen receptor and progesterone receptor. The cell line possesses characteristics of mammary epithelium like synthesis of estradiol and domes formation. MCF cells can be used for detecting MAPK and PI3K involvement, along with easy detection of ERK and Akt phosphorylation.

MDA-MB-231

This human breast cancer cell line is used to evaluate the anti-cancer potential of the compounds against TNBC. MDA-MB-231 was derived from the 51-year-old Caucasian women's pleural fluid. This invasive cell line does not show overexpression of ER, PR and HER and thus represents a good model for TNBC. The cell line possesses unique metastatic properties with the basal subtype of breast cancer. Later it was identified as claudin-low molecular subtype due to evident downregulation of claudin-3 and claudinin-4.

A549

A549 is a human lung cancer line was first developed by D. J. Giard and was first isolated from 58-year-old Caucasian male in 1972 having an adenocarcinoma. This cell line consists of hypotriploid alveolar basal epithelial cells. A549 cells are squamous in nature and have been used to model the alveolar Type II pulmonary epithelium. These cells contain a high percentage of unsaturated fatty acids and have the ability to synthesize lecithin.

HBL-100 (Normal cell toxicity)

HBL-100 cell line represents normal breast cell and it is commonly used to evaluate toxicity profile of the compounds. It was derived from the milk of a nursing mother after 3 days of delivery by E.V. Gaffney and associates. These cells have the ability to synthesize lactose and produce increased amounts of casein in response to prolactin or estrogen. The recovered cells of this cell line were abnormal even though there was no breast lesion or family history of breast cancer in the host.

3.3.2.2. Culturing of the cell lines

All the cell lines under study (MCF-7, A549, MDA-MB-231 and HBL-100) were cultivated at 37°C with 5% CO_2 in the culture flasks (75 cm^2). The growth medium for the cell line consisted of RPMI 1640 with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin and it was replaced on every other day. Trypsinization of the cultured cells was performed using 0.25% trypsin-EDTA solution, after reaching 90% confluency. In a 96-well cell culture plate, 10,000 cells/well were seeded for the MTT assay.

3.3.2.3. Cytotoxicity assay (MTT)

The cytotoxicity of the designed compounds against different cancer cell lines and normal breast cell line was determined by performing MTT assay, following the standard protocol (Kushwah, Agrawal et al. 2017; Kushwah, Katiyar et al. 2018; Singh, Chaudhari et al. 2019). MTT assay measure cell proliferation via a colorimetric estimation method (Mosmann, 1983). It was specified by Mosmann and is also known as the cell viability assay. Briefly, yellow color tetrazolium dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) is reduced by mitrochondrial enzymes to a purple-colored insoluble formazan indicating metabolic activity in the cells as evidence of their viability (**Figure 3.3.**). During the assay, the formazan product is solubilized in DMSO and estimated spectrophotometrically.

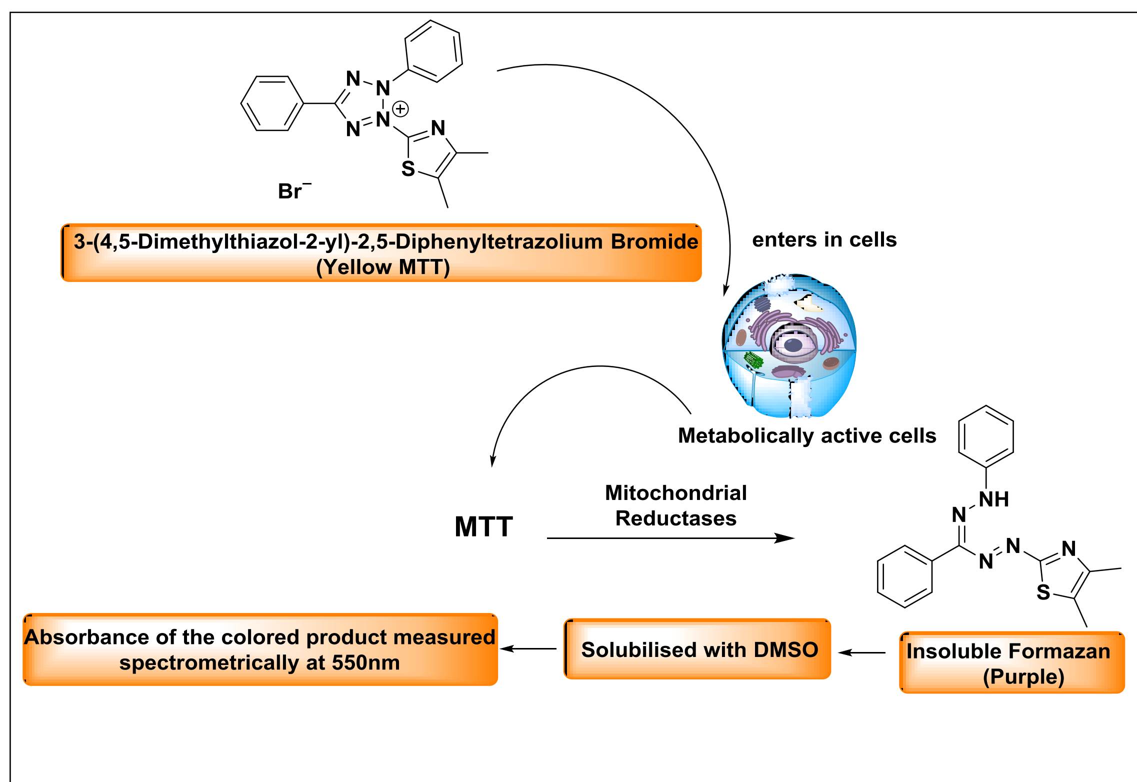


Figure 3.3. Basic principle of MTT assay.

3.3.2.4. Procedure

During evaluating each cell line, cultured cells were seeded in each well of a 96-well plate, with a total volume of 200 µL of RPMI media, and for attachment it was kept overnight. Following cell attachment, fresh media (200 µL) containing different compounds were added, replacing previous media and incubated for 24 h. Afterwards, media containing compounds was carefully aspirated and for 3 h with MTT (0.5 mg/mL; 0.2 mL), cells were incubated again. Finally, the crystals of formazan were solubilized in DMSO and OD (optical density) of the dissolved formazan was measured *via* an ELISA plate reader at 550 nm. Determination of the cell viability was done using the following equation:

Relative cell viability = Absorbance of Sample/Absorbance of Control

The IC_{50} values were then determine by Graph Pad Prism tool *via* median-effect plot.