4. Chemistry

4.1. Instrumentation, Chemicals and Solvents

The chemicals, solvents and other reagents were obtained from various commercially accessible suppliers like TCI Chemicals India Pvt Ltd., Hi Media Laboratories Pvt. Ltd., Mumbai, India, Sisco Research Laboratories Pvt. Ltd., SD fine India Pvt. Ltd., Sigma Aldrich (India), India and Merck India Pvt. Ltd. The drug Imatinib was procured as gift samples from Ranbaxy Pvt. Ltd. H.P. All the solvents were properly distilled prior to use as per the standard procedures and dried using desiccants like anhydrous sodium sulphate, molecular sieves.

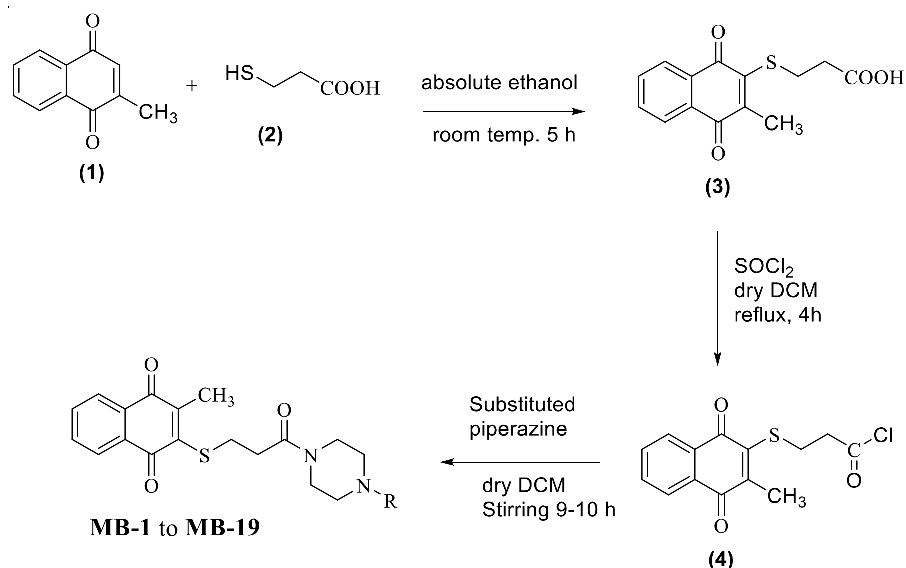

Figure 4.1: Scheme I for the synthesis of compound MB-1 to MB-19

4.2. Method for the Synthesis of 3-((3-methyl-1, 4-dioxo-1, 4-dihydro naphthalen-2-yl) thio) propanoic acid (3)

Initially condensation of 2-methylnaphthalene-1, 4-dione (1) (4.3 g, 25 mmol) and 3-mercaptopropionic acid (2) (2.6 g, 25 mmol) was carried out in absolute ethanol (50 mL) at room temperature for 5 h. The completion of the reaction was monitored by TLC. After completion of the reaction the slurry was filtered to yield a residue which

was recrystallized from ethanol to afford compound 3-((3-methyl-1, 4-dioxo-1, 4-dihydro naphthalen-2-yl) thio) propanoic acid (**3**) (Tandon *et al.,* 2004).

4.3. Method for the Synthesis of Compounds MB-1 to **MB-19 (Series I)**

Compound **3** (10mmol) was dissolved in dry dichloromethane (DCM). Subsequently thionyl chloride was added drop wise to the reaction mixture and the reaction mixture was reflux for 4 h. Formation of acid chloride was confirmed by TLC. As the acid chloride was too unstable the product was further precede for next step without isolation. In the next step acid chloride was stirred for 9-10 hours with different substituted piperazines (10mmol) in dry DCM to yield final compound **MB-1** to **MB-19 (Figure 4.1)**. Synthesis for compounds with different substituted piperazines is mentioned in **Table 4.1**.

Table 4.1: Synthesis of compound with different substituted piperazines

Compound	R	Compound	R
MB-1	H	**MB-11**	4-FC_6H_5-
MB-2	C_6H_5-	**MB-12**	4-$CF_3C_6H_5$-
MB-3	$(C_6H_5)_2CH_2$-	**MB-13**	3-FC_6H_5-
MB-4	$C_6H_5CH_2$-	**MB-14**	4-$OCH_3C_6H_5$-
MB-5	C_5H_4N-	**MB-15**	2-$OCH_3C_6H_5$-
MB-6	2-$CH_3C_6H_5$-	**MB-16**	4-$NO_2C_6H_5$-
MB-7	2-ClC_6H_5-	**MB-17**	2,3-$(CH_3)_2C_6H_5$-
MB-8	2,3-ClC_6H_5-	**MB-18**	2,3-$(OCH_3)_2$-4-BrC_6H_5-
MB-9	4-ClC_6H_5-	**MB-19**	2-$NO_2C_6H_5$-
MB-10	2-FC_6H_5-		

4.4. Method for the Synthesis of Compounds MB-20 to **MB-33 (Series II)**

Compound **3** (10mmol) was dissolved in dry dichloromethane (DCM). Subsequently thionyl chloride was added to the reaction mixture drop by drop further the reaction mixture was reflux for 4 h. Formation of acid chloride was confirmed by TLC. As the acid chloride was too unstable the product was further preceded for next step without

isolation. In the next step acid chloride was stirred for 9-10 hours with various substituted oxadiazole (10 mmol) in dry DCM to yield final compound **MB-20** to **MB-33 (Figure 4.2)**. Synthesis of compounds with different substituted ozadiazoles is depicted in **Table 4.2.**

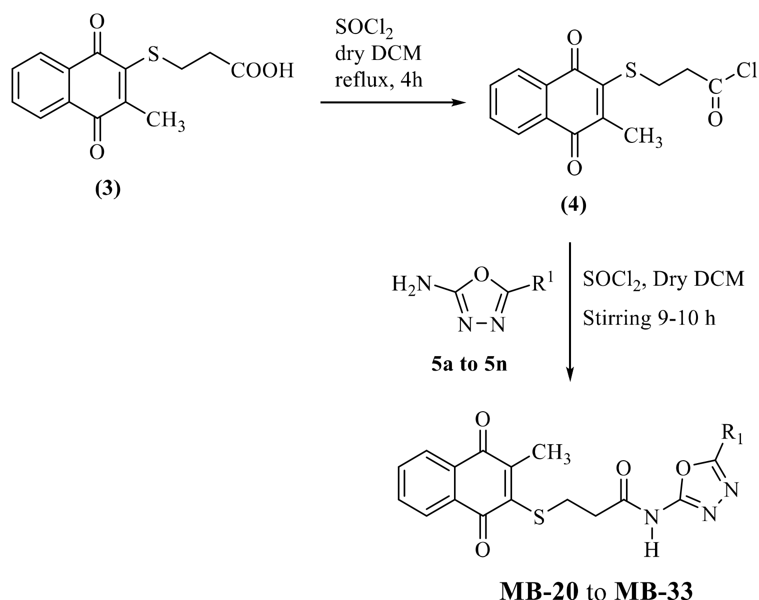

Figure 4.2: Scheme II for the synthesis of compound MB-20 to MB-33

Table 4.2: Synthesis of compounds with different substituted oxadiazoles

Compound code	R	Compound code	R
MB-20	CH_3-	**MB-27**	2,4-$(OCH_3)_2C_6H_5$-
MB-21	CH_2CH_3-	**MB-28**	4-Cl- C_6H_5-
MB-22	$CH_2CH_2CH_3$-	**MB-29**	2-ClC C_6H_5-
MB-23	C_6H_5-	**MB-30**	2,3-$(Cl)_2C_6H_5$-
MB-24	4-$OCH_3C_6H_5$-	**MB-31**	4-CH_3 C_6H_5-
MB-25	2-$OCH_3C_6H_5$-	**MB-32**	2,5-$(CH_3)_2C_6H_5$-
MB-26	3-$OCH_3C_6H_5$-	**MB-33**	4-OH-3-$CH_3C_6H_5$-

4.5. Characterization of Synthesized Compounds

The identification as well as characterization of newly synthesized compounds was carried out using various analytical methods to establish the newly synthesized compounds

4.5.1.Physicochemical Characterization

The physicochemical characterizations of synthesized compounds were carried out as mentioned below method:

- **Melting point determination**

Melting point determination is a preliminary criteria for the identification of the compound. The melting point of compounds was recorded using Veego melting point apparatus.

- **Solubility determination**

The solubility of compounds was determined using various solvent such as polar, semi polar and non polar.

- **Thin-layer chromatography (TLC) analysis**

Thin-layer chromatography is key techniques that give the information of about the progress of reaction. And it also give provide the information of the purity of the compound. For the TLC silica gel 60-F_{254} plates, were utilized to examine the progress of the reaction.

4.5.2. Spectral characterization and elemental analysis

- **Fourier Transform Infrared Spectroscopy (FT-IR)**

The FT-IR spectra of the synthesized compound were obtained from Spectrum II FT-IR spectrometer (Perkin Elmer, USA) with potassium bromide (KBr) pellets. FT-IR spectra of compound (~ 2 mg in 20 mg of KBr) were measured over the range of 4000-400 cm^{-1}. The data analysis was carried out with the help of spectrum RX 1 software.

- **^{1}H NMR and ^{13}C NMR Spectroscopy**

^{1}H NMR and ^{13}C NMR spectra of synthesized compounds were recorded in solution. ^{1}H NMR and ^{13}C NMR spectra of synthesized compounds were recorded in solution $CDCl_3$ by utilizing Bruker Advance II 400 NMR spectrometer at

sophisticated analytical instrumentation facility, Panjab University Chandigarh. The signals were recorded in parts per million (δ, ppm), using tetramethylsilane (Me_4Si) standard. The spin multiplicities are designated s (singlet), d (doublet), t (triplet), and m (multiplet) symbols.

- **Mass Spectroscopy**

Mass spectra were recorded using Waters, Q-TOF micromass, TOF MS ESI^+ at NIPER Mohali SAS Nagar, Panjab.

- **Elemental Analysis**

The elemental analysis of the synthesized compound were obtained using Thermo Scientific (FLASH 2000) instrument at sophisticated analytical instrumentation facility, Panjab University Chandigarh.

4.6. Computer Aided Drug Design (CADD) Studies

The computer aided studies were aimed to identify and visualize the important structural feature requirements of the compounds in the rational design of potential epidermal growth factor receptor inhibitors. The CADD studies were carried out using vLife MDS 4.6 software. The structures of the molecules were built using structure drawing tool in the ChemDraw Ultra 12.0 software.

4.6.1. Molecular Docking Studies

The molecular docking study is used as an important tool in drug discovery to better understand the interactions between the ligands and receptors. Therefore docking is frequently used to predict binding orientation of small molecule drug candidates to their protein targets in order to predict the affinity and activity of the small molecule. Molecular docking correlating the chemical structures with their biological activities compounds. Moreover, it could afford dynamic pharmacokinetic data, for example, absorption, distribution, metabolism and excretion (ADME), which is vital factor in the

design of new drug molecule. Hence docking plays an important role in the rational design of potential epidermal growth factor receptor inhibitors (EGFR). Docking study was done of the all the synthesized compounds (**MB-1** to **MB-33)** by GRIP batch docking method with the help of vLife MDS (version 4.6) software.

- **Ligand preparation**

The 2D structures of the synthesized compounds were drawn using Chem Draw Ultra 12 and converted to 3D conformations. The conformers thus obtained, were optimized till they reached a root mean square (rms) gradient of 0.01 kcal/mol.A° using Merck molecular force field (MMFF). The lowest energy conformations were selected for further studies.

- **Protein preparation**

The crystal structure of epidermal growth factor receptor (EGFR), (2GS6) was obtained from the RSCB Protein Data Bank (PDB). All bound water molecules and ligands were removed from the proteins and polar hydrogens were added. The protein structure was energy minimized using MMFF with distance dielectric function and energy gradient 0.01 kcal/mol, with 10000 numbers of cycles.

4.6.2. *In-Silico* Pharmacokinetic Studies

- **ADME prediction**

Number of the drugs under clinical trials could not see the clinics due to failure at the stage of pharmacokinetic evaluation. Initial screening of hits and leads before their clinical testing will not only decrease the rate of failure, but it reduces the cost of drugs discovery program. Taking into consideration, a preliminary predictive *in-silico* pharmacokinetic study of the synthesized compounds was undertaken using QikProp analysis of Schrodinger Maestro. Incorporation of such tools as a part of the drug design process can screen molecules that are more likely to exhibit satisfactory

absorption, distribution, metabolism, excretion (ADME) properties and indicating their potential to act as ‘drug-like’ molecules.

- **Drug likeliness prediction**

The low success rate of converting lead compounds into drugs owing to unfavorable pharmacokinetic parameters has evoked a renewed interest in understanding more clearly what makes a compound drug-like. Different authors have tried to define and differentiate between drug and non-drug molecules covering parameters like functional groups/ physical properties/ ADME/ fragments/ spacer/linkers. Number of computational techniques for identifying drug-like molecules, ranging from simple counting schemes to sophisticated machine learning techniques such as neural networks is becoming widespread. In the present study, amongst the currently available drug like and non-drug like databases, tools like Lipinski’s rule (Rule of five), rule have been used to predict the drug-likeliness of newly synthesized compounds.

Drug-likeliness has been predicted using tools like Lipinski’s rule (Rule of five. Lipinski's rule (Rule of five) is a refinement of drug-likeness and is used to predict whether a chemical compound will have pharmacological or biological activity as an orally active drug in humans. It covers the 4 parameters like partition coefficient (log P ≤ 5), molecular weight (≤ 500), no. of hydrogen bond donors ≤ 5 (The sum of OHs and NHs), no. of hydrogen bond acceptor ≤ 10 (The sum of Os and Ns).

4.7. Cell Culture

The three different human cancer cells, *i.e.* breast carcinoma (MCF-7), prostate carcinoma (HeLa) and liver carcinomas (HeGP2) cell lines were purchased from the National Center for Cell Sciences (NCCS), Pune-India. These cancer cells were preserved in Dulbecco’s modified Eagle medium (DMEM). The media containing 10% of fetal bovine serum (FBS), 1% L-glutamine, 1% Eagle’s nonessential amino acids,

and 1% penicillin-streptomycin with 10% (v/v) streptomycin (100 μg/mL), were used for the supplementation of cancer cells. Further, this media was monitored at 37 °C in a humidity controlled incubator with 5% carbon dioxide. Before subculture cells were washed with Phosphate Buffer Saline. The growth medium every 48 hours was changed and cells were removed through trypsinization by utilizing 0.05% trypsin and 0.02% EDTA (Gautam *et al.,* 2019; Senthilraja *et al.,* 2015).

4.7.1. *In-vitro* Cytotoxicity Screening

In-vitro anticancer activity was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded into 96-well plates along with the density $1x10^4$ cells in each well. Further it was allowed to attach for 24 hours in a humidified atmosphere that contain 5% CO_2 at 37°C. After that, the cells were treated *via* different concentrations (1-50 μM) of synthesized compounds **MB-1** to **MB-33** as well as standard drug imatinib and the plates were kept collectively for 24 h. Again in each well 100 μL of MTT solution was added and incubated for 4 h at 37°C. After formation of purple formazan crystals, it was dissolved with the addition 100 μL of dimethyl sulfoxide (DMSO) in the each well. The intensity of the purple product directly proportional to the number of living cells in the control groups as well as, treatment groups was quantitatively measured on ELISA plate reader at 590 nm (Markovic *et al.,* 2011; Gautam *et al.,* 2019). Each experiment was carried out in triplicate.

The IC_{50} was computed by employing graph Pad Prism Version 5. The cytotoxicity was calculated using the formula given below:

$$\% \text{ Cytotoxicity} = 1 - \frac{\text{Absorbance of test}}{\text{Absorbance of Control}} \times 100$$

$$\% \text{ cell viability} = \frac{\text{Absorbance of test}}{\text{Absorbance of Control}} \times 100$$

4.8. *In-vitro* EGFR Tyrosine Kinase Inhibitory Activity

The enzyme protein tyrosine kinase inhibitory activity was assessed with the help of enzyme-linked immunosorbent assay (ELISA) method. About, 20µg/mL Poly (Glu, Tyr) 4:1 (Sigma, St. Louis, MO) was precoated in 96-well ELISA plates as substrate. After adding 50 µL of 10 µmol/L ATP solution which was diluted in kinase reaction buffer (50mM HEPES pH 7.4, 20mM $MgCl_2$, 0.1mM $MnCl_2$, 0.2mM Na3VO4, 1mM DTT), the plate was treated with 1µL of indicated concentrations of compounds (**MB-1** to **MB-33** and reference standard imatinib were diluted in 4% DMSO) per well. Experiments at each concentration were performed in duplicate. Reaction was initiated by adding tyrosine kinase diluted in kinase reaction buffer. After incubation at 37 ºC for 1 h, the wells were washed three times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (TPBS). One hundred microliters of anti-phosphotyrosine (PY99) antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in T-PBS containing 5 mg/mL BSA was added and the plate was incubated at 37 ºC for 30 min. After the plate was washed three times, 100 µL horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000, Calbiochem, SanDiego, CA) was added and the plate was incubated at 37 ºC for 30min. Plate was read using spectrophotometer (Spectrum II, Perkin Elmer, USA) at 492 nm (Hu *et al.,* 2017).

$$\text{Inhibitory rate}(\%) = \left(1 - \frac{\text{treated groups}}{\text{control groups}}\right) \times 100\%$$

4.9. *In-vivo* Anticancer Activity

The compound having selective cytotoxicity against cancer cell line in comparison to normal cells were further evaluated *in-vivo* for anticancer activity using N-methyl-N-nitrosourea induced breast cancer model (Roomi *et. al.,* 2005). This model imitates the breast carcinoma in numerous regards. Therefore it has been utilized widely to assess

preventive and helpful impact of newly synthesized compounds in breast cancer. Thus the effect of compounds on mean survival time, tumour volume, haematological parameters and % increase life span were studied.

4.9.1. Animals and maintenance

The *in-vivo* studies were conducted on female wistar rats with body weights ranging between 160-180 g, and age between 10-12 weeks. The animal were housed in polypropylene cages and kept under standard laboratory conditions *i.e.* at 25 ± 2°C with relative humidity of 55 ± 5%. The animals were free access to standard diet and water ad libitum. All the experiments protocols were carried out as per committee for the purpose of the control and supervision on experiments on animals (CPC-SEA) guidelines. The animal protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the Panjab University, Chandigarh, India, under the approval number **PU/IAEC/51/51**.

4.9.2. Estimation of acute toxicity and LD_{50} determination

The acute toxicity was performed according to the OECD guideline. Acute toxicity of drug can be determined by the calculation of LD_{50} and it is the dose at which 50% animal will kill of a particular species (Randhaw *et al.,* 1994). For determination of LD_{50} five different dosages (50, 100, 200, 400 and 800 mg/kg) of the compound were administered to rat by a single intraperitonial injection. At 2nd, 6th and 24th hour the animals were observed for any toxic symptom. After 24 hours the quantity of diseased rat were counted from each group then percentage of mortality were calculated. LD_{50} value was calculated followed by the log-probit method. The determination of maximum tolerate dose (MTD) was also performed to minimise the number of animal to be sacrificed in the experiment (Rathis *et al.,* 2012). Three different dosage 50,100,200 mg/kg of compounds were administered to rats by a single intraperitonial

injection and the rats were observed for 2 weeks. The animals were sacrificed; if they lose more than 20 % of their body weight or any other sign of significant toxicity.

4.9.3. Induction of mammary carcinomas

The anticancer activity was performed with different treatment of the compounds using N -methyl- N -nitrosourea (NMU) induced female Wistar rat breast tumor. At 9-10 weeks of age, all rat received a single dose 50 mg/kg of body weight of NMU intraperitonially. NMU was dissolved instantly before use in 0.9% NaCl and acidified with acetic acid (pH 4). After 3 week of first NMU administration they were palpated two times per week for the occurrence of any mammary tumor. The incidence of tumor formation was started from 4th week onward.

4.9.4. *In-vivo* antitumor efficacy

The antitumor effect of the compounds was assessed by measuring the average tumor volume, percentage increase in life span (%ILS), mean survival time (MST) as well as changes in hematological parameters (Chan *et al.,* 2005; Perse *et al.,*2009). The animal were divided in different groups (N=3). The normal control group did not receive MNU while remaining groups received NMU for tumor induction. The pharmacological treatments were started at 5th week onward because of the tumor appeared after 4th week of MNU administered. The day by day treatments were started, counted as day 1 and the selected compounds (MB-9, MB-18, MB-24 and MB-32) at dose of 5 mg /kg intraperitonial were administered once in a week to the treated groups (**Table 4.3)**. The reference standard drug imatinib at the dose of 5mg/kg (i.p.) was given to each animal of one group having tumor incidence. Normal control and MNU control received 0.9% sterile normal saline. The pharmacological treatment lasted after 21 days and tumor volume was measured.

Table 4.3: Animal groups for in-vivo antitumor activity studies

Group I	:	Normal control administered with normal saline
Group II	:	Disease control Group with cancer induced using MNU
Group III	:	MNU + Intraperitonial administration of the standard drug (Imatinib)
Group IV	:	MNU + Intraperitonial administration of MB-9
Group V	:	MNU + Intraperitonial administration of MB-18
Group VI	:	MNU + Intraperitonial administration of MB-24
Group VII	:	MNU + Intraperitonial administration of MB-32

At 21th day of treated groups the blood sample were withdrawn from each group and the histopathological parameter *viz.* WBC, RBC and Hb content of all rats were determined immediately after lasting pharmacological treatment. Further to study the dose dependent behavior of compound at three different doses 5, 10 and 20 mg/kg were administered intraperitonially once in a week to the treatment groups and the remaining procedure was the same as described above. MST (Mean survival Time) = (Day of first death + Day of last death)/2. % ILS (Percentage increase in life span) = [(mean survival time of treated group/ mean survival time of control group)] – 1] ×100. Tumor volume = $a \times b^2/2$, a= largest diameter, b= shortest diameter of tumor.

4.10. Statistical Analysis: The statistical analysis was run using Sigma Plot (version 11.1) by applying a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests.