Chapter

4

Experimental Work

4. EXPERIMENTAL

All the chemicals and solvents were of analytical grade or purified by standard methods prior to use [Perin *et. al*., 1966]. These were purchased from Merck (Germany), M.P. bio medicals, S.D.Fine (Mumbai) and Sigma Aldrich (U.S.A.). Plasmid DNA (pUC19) was purchased from Invitrogen. Fetal bovine serum (FBS), DMEM, penicillin–streptomycin (PS) and glutamine were purchased from GIBCO life technologies (Grand Island, NY, USA). Trypsin was obtained from HiMedia laboratories. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, $DiSC_3(5)$ and Propidium Iodide (PI) were purchased from Sigma Aldrich. RNase A was obtained from Merck millipore, USA. Apoptosis detection kit, AnnexinV-AlexaFlor488 was procured from Invitrogen, USA. Phosphorylated EGFR (PY20) and anti-Actin antibodies (I-19) were purchased from Santa Cruz Biotechnology (Mumbai).

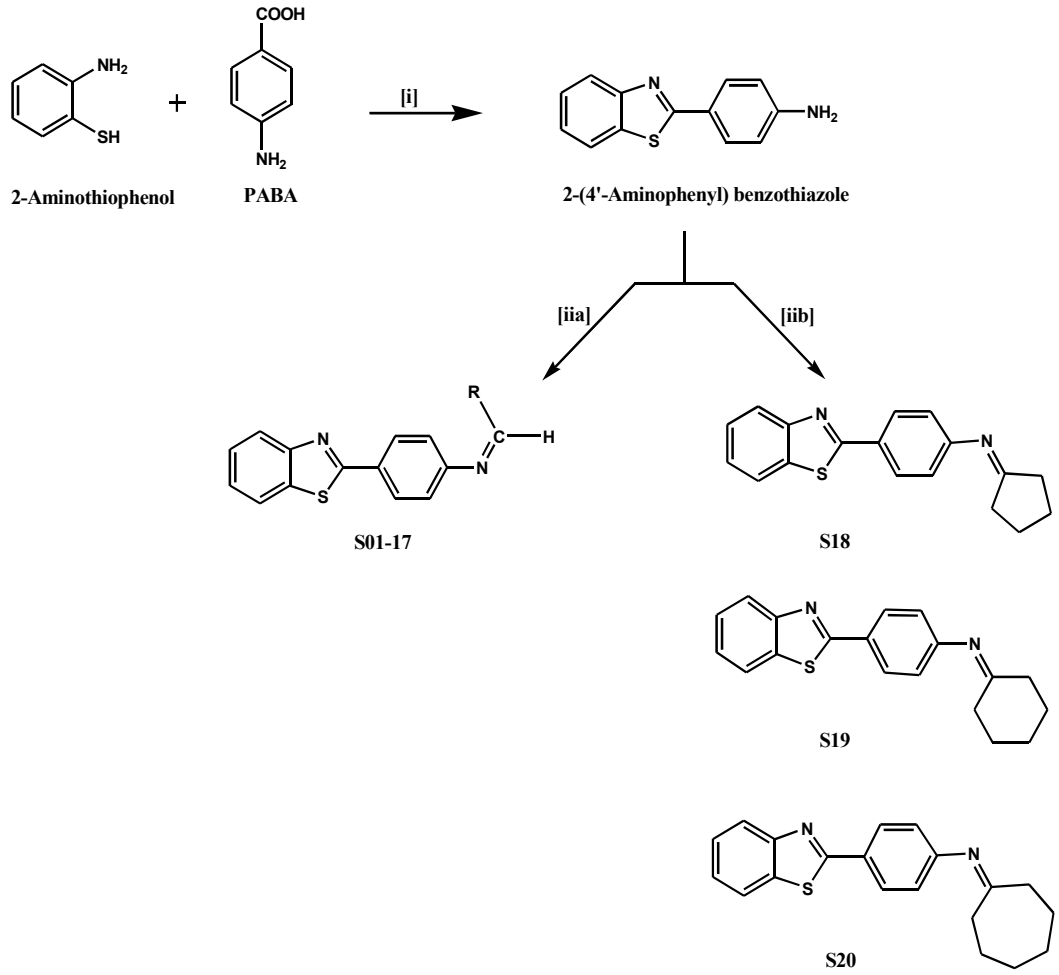
4.1. GENERAL PROCEDURE FOR THE SYNTHESIS OF SCHIFF BASE BENZOTHIAZOLE HYBRIDS

4.1.1. General procedure for the synthesis of Schiff bases from aromatic aldehydes [N-(arylidene)-4-(benzo[d] thiazol-2-yl)benzenamines ***(S01-17)****]*

A reaction mixture coupled with 2-aminothiophenol (0.053 M) and para amino benzoic acid (PABA) (0.05 M) in polyphosphoric acid (PPA; 85 g) was refluxed with stirring at 220°C for 4 h, yielded 2-(4'-aminophenyl) benzothiazole. The solution was cooled, and poured into ice-cold 10% aqueous sodium carbonate. The solid product was collected, washed with water and purified with aq. methanol in satisfactory yield, as described previously [Shi *et al.*, 1996]. Equimolar quantities of 2-(4'-aminophenyl) benzothiazole (0.01mol) in ethanol (20mL) and the appropriate substituted aldehyde/ketone (0.01mol) in ethanol/methanol (20mL) were set to reflux for 4-6 h in presence of few drops of glacial acetic acid. After completion of the reaction, as monitored by TLC, the reaction mixture was cooled, poured into ice cold water (five times its volume), solid thus separated was filtered and dried. Finally, the desired products obtained were recrystallized with ethanol-ethylacetate (1:1) or ethanol-dichloromethane (1:2) mixture (Scheme 4.1).

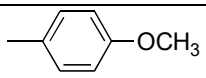
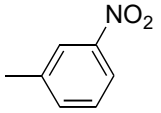
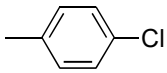
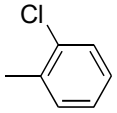
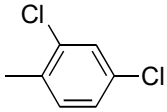
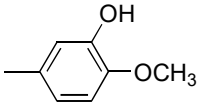
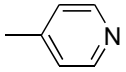
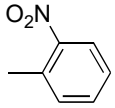
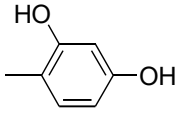
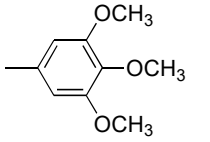
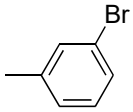
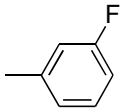
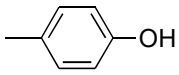
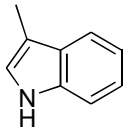
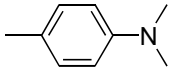
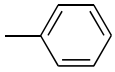
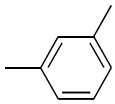
4.1.2. General procedure for the synthesis of Schiff bases from cyclic ketones [4-(benzo[d]thiazol-2-yl)-N-cyclopentylidene/cyclohexylidene/cycloheptylidene benzenamines ***(S18-20)****]*

To the equimolar ethanolic solutions of 2-(4'-aminophenyl) benzothiazole (0.01 mol) and of respective cyclic ketones (0.01 mol), few drops of conc. H_2SO_4 was added as catalyst (Scheme 4.1). The reaction mixture was refluxed for 6-10 h and after cooling the solution was poured on to the crushed ice and neutralized with K_2CO_3. Separated solid was filtered and dried. Finally, the product thus obtained was recrystallized from ethyl acetate and ethanol in graded proportions depending on the nature of the compound.



Scheme 4.1. Reagents and conditions: (i) Polyphosphoric acid (PPA), 220^{o}C, 1-2 h reflux with stirring (iia) Aromatic aldehydes, CH_3OH/C_2H_5OH, AcOH, 4-6 h reflux (iib) Cyclic ketones, CH_3OH/C_2H_5OH, conc. H_2SO_4, 6-10 h reflux.

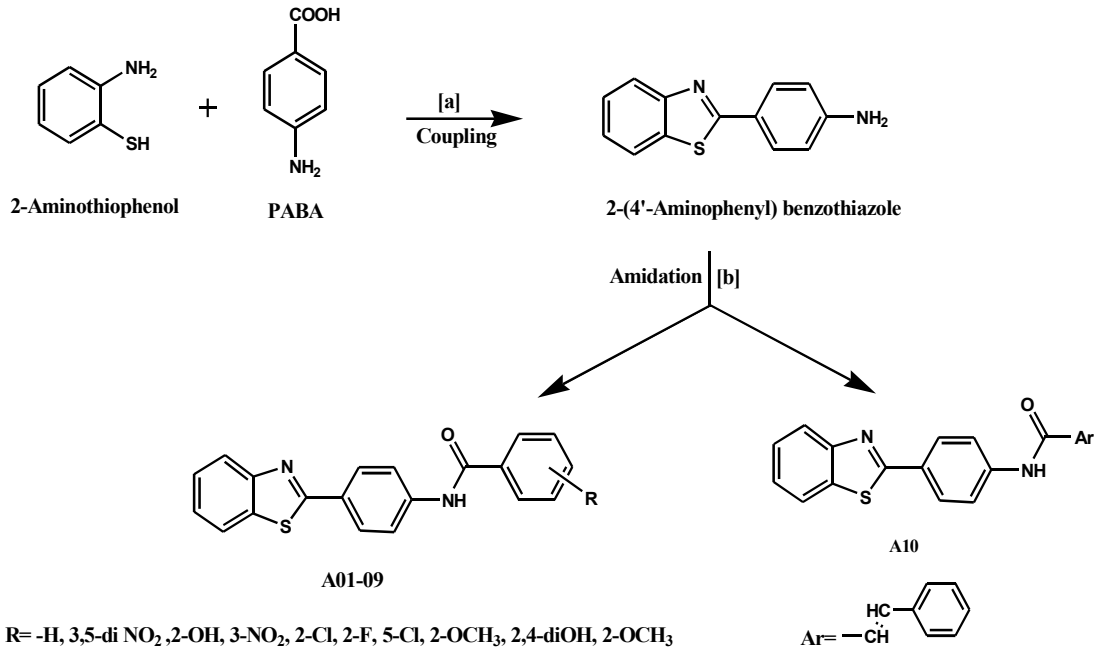
Table 4.1 Different Substitutions on aryl ring of synthesized compounds **(S01-20)**

Compound code	R	Molecular formula	Compound code	R	Molecular formula
S01		$C_{21}H_{16}N_2OS$	S11		$C_{20}H_{13}N_3O_2S$
S02		$C_{20}H_{13}ClN_2S$	S12		$C_{20}H_{13}ClN_2S$
S03		$C_{20}H_{12}Cl_2N_2S$	S13		$C_{21}H_{16}N_2O_2S$
S04		$C_{19}H_{13}N_3S$	S14		$C_{20}H_{13}N_3O_2S$
S05		$C_{20}H_{14}N_2O_2S$	S15		$C_{23}H_{20}N_2O_3S$
S06		$C_{20}H_{13}BrN_2S$	S16		$C_{20}H_{13}FN_2S$
S07		$C_{20}H_{14}N_2OS$	S17		$C_{22}H_{15}N_3S$
S08		$C_{22}H_{19}N_3S$	S18	-	$C_{18}H_{16}N_2S$
S09		$C_{20}H_{14}N_2S$	S19	-	$C_{19}H_{18}N_2S$
S10		$C_{21}H_{16}N_2S$	S20	-	$C_{20}H_{20}N_2S$

4.2. GENERAL PROCEDURE FOR THE SYNTHESIS OF BENZOTHIAZOLE AMIDE DERIVATIVES (A01-10)

A reaction mixture in equimolar quantities of 2-(4'-aminophenyl) benzothiazole (0.01mol) and the appropriate substituted aromatic acids (0.01mol) in acetonitrile (ACN, 20mL) was added together with DCC (2 equiv.) (Scheme 4.2). The mixture was refluxed under stirring for 4-10 hr. The completion of reaction was monitored by TLC at appropriate time

intervals. Following completion, the reaction mixture was cooled to room temperature. The reaction mixture was filtered through a medium frit. The solution was then given aqueous wash several times, solid thus separated was filtered and dried and the rest was flashed away. The residue was crystallized from ethanol to obtain the target compounds.



Scheme 4.2. Reagents and conditions: (a) Polyphosphoric acid (PPA), 220^{o}C, 1-2 h reflux with stirring (b) ArCOOH, Acetonitrile, DCC, reflux 4–10 h

Table 4.2 Different Substitutions on aryl ring of synthesized compounds **(A01-A10)**

Compound code	R, Ar	Molecular formula	Compound code	R, Ar	Molecular formula
A01	H	$C_{20}H_{14}N_2OS$	A06	2-F	$C_{20}H_{13}FN_2OS$
A02	3,5-di NO_2	$C_{20}H_{12}N_4O_5S$	A07	5-Cl, 2-OCH_3	$C_{21}H_{15}ClN_2OS$
A03	2-OH	$C_{20}H_{14}N_2O_2S$	A08	2,4-diOH	$C_{20}H_{14}N_2O_3S$
A04	3-NO_2	$C_{20}H_{13}N_3O_3S$	A09	2-OCH_3	$C_{21}H_{16}N_2O_2S$
A05	2-Cl	$C_{20}H_{13}ClN_2OS$	A10	-Styrene	$C_{22}H_{16}N_2OS$

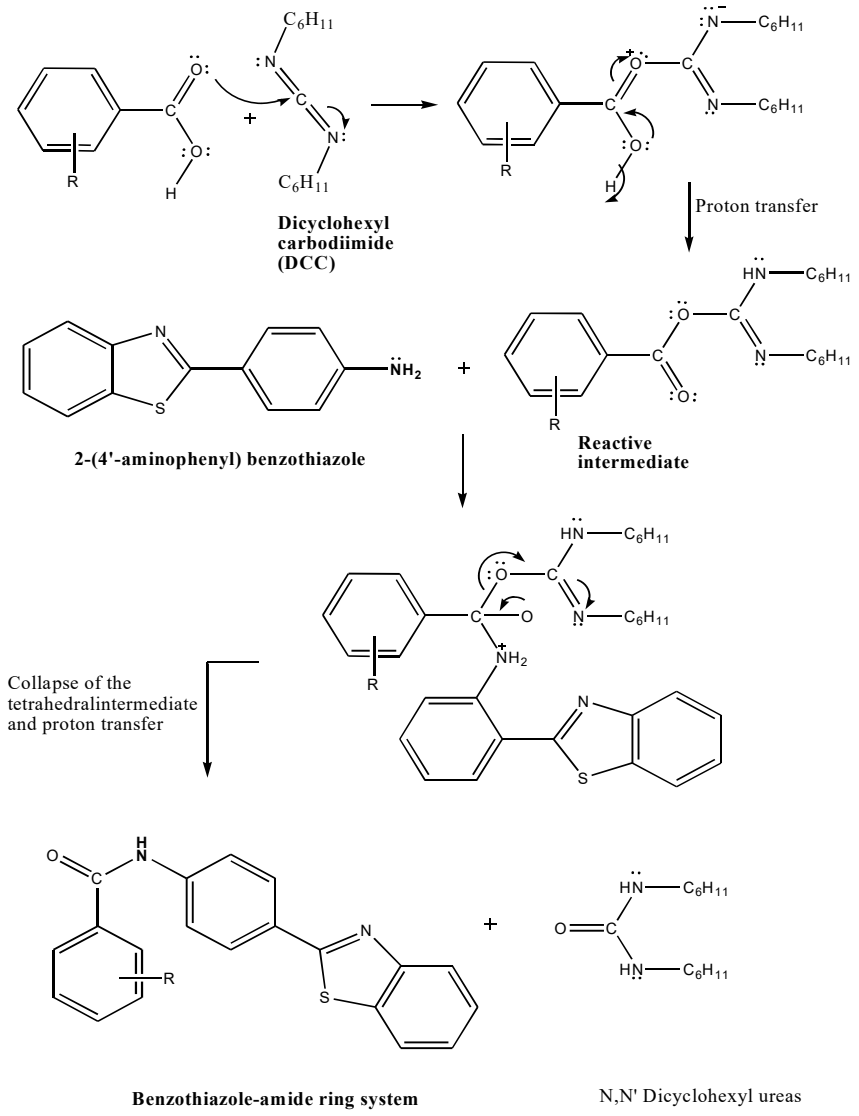
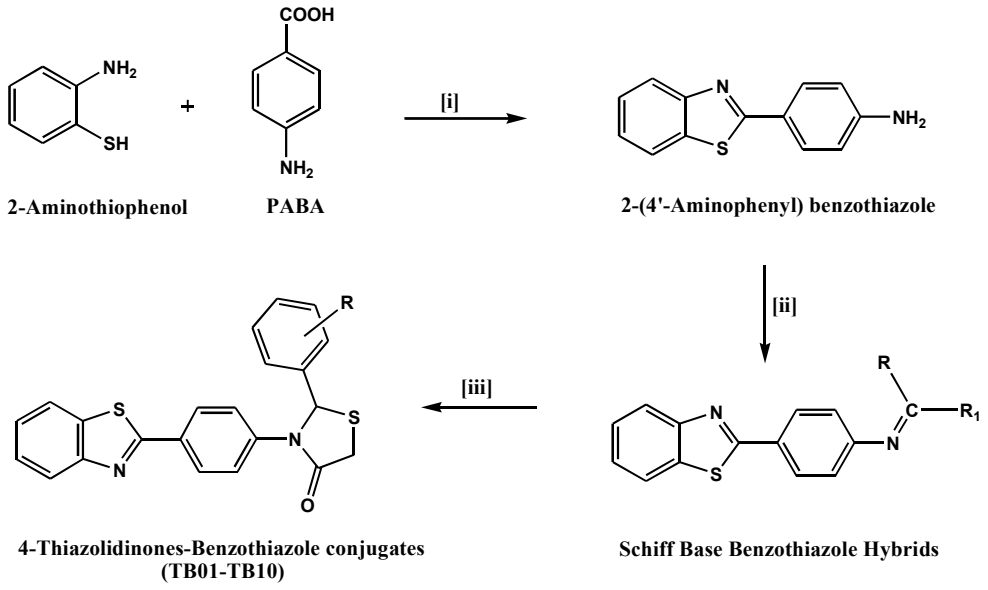


Fig. 4.1 Proposed reaction mechanism of formation of benzothiazole-amide ring system

4.3. GENERAL PROCEDURE FOR THE SYNTHESIS OF BENZOTHIAZOLE 4-THIAZOLIDINONES DREIVATIVES (TB01-10)

To the equimolar solution of respective Schiff bases of benzothiazole (0.01 mol) in DMF and of thioglycolic acid solution (0.01 mol), certain amount of anhyd. $ZnCl_2$ was added as catalyst (Scheme 4.3). The reaction mixture was refluxed on a water bath for 10-12 h. After cooling it to room temperature, the reaction mixture was poured on to the crushed ice, neutralized with K_2CO_3 to get the desired product. The solid that separated was filtered and dried. It was further recrystallized by ethanol/methanol.



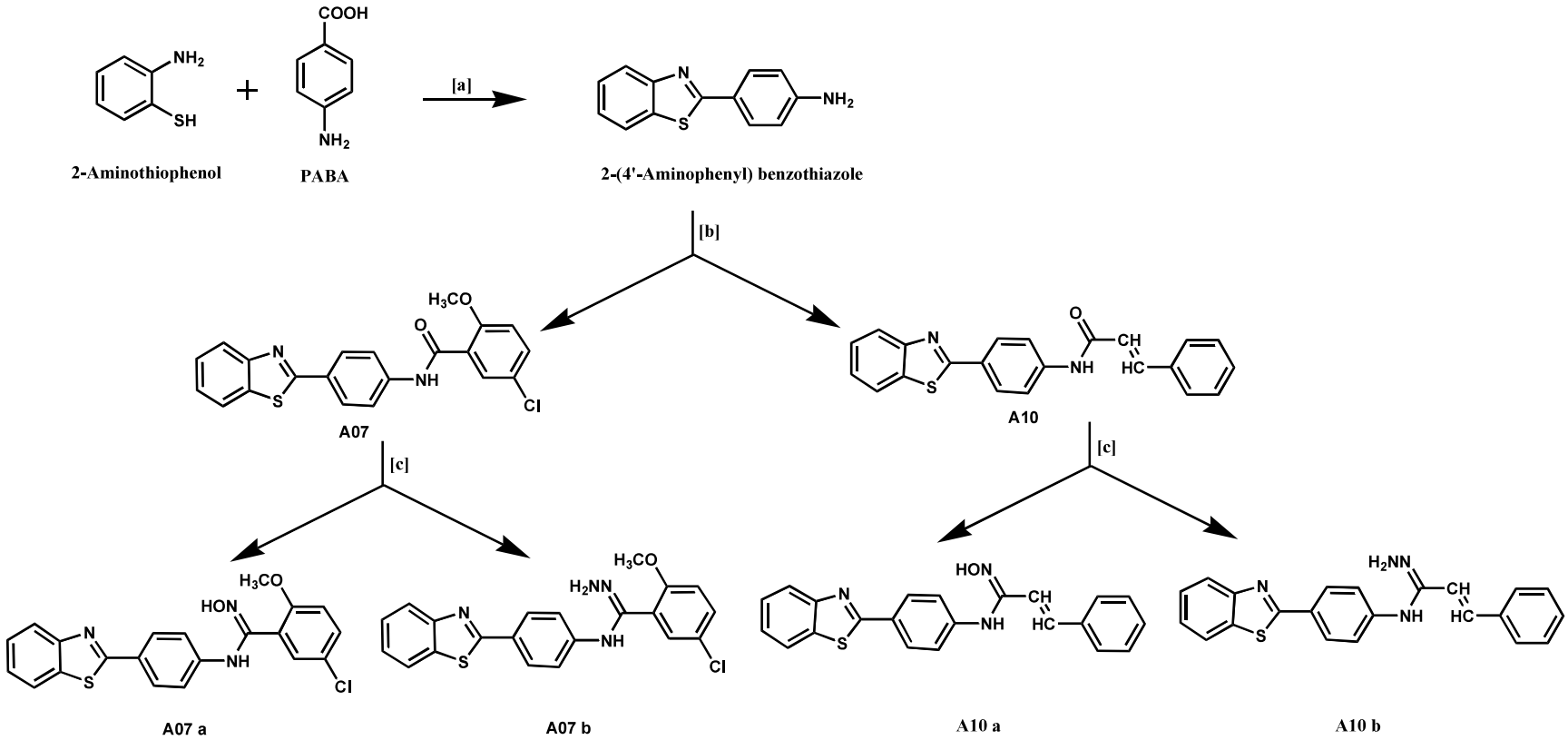
Scheme 4.3. Reagents and conditions: (i) Polyphosphoric acid (PPA), 220^{o}C, 1-2 h reflux with stirring (ii) Aromatic aldehydes, CH_3OH/C_2H_5OH, AcOH, 4–6 h reflux (iii) Thioglycolic acid, DMF, anhyd. $ZnCl_2$, 8-10 h reflux.

Table 4.3 Different Substitutions on aryl ring of synthesized compounds **(TB01-10)**

Compound code	R	Molecular formula	Compound code	R	Molecular formula
TB01	4-OCH_3	$C_{23}H_{18}N_2O_2S_2$	TB06	4-OH	$C_{22}H_{16}N_2O_2S_2$
TB02	4-Cl	$C_{22}H_{15}ClN_2OS_2$	TB07	3-CH_3	$C_{23}H_{18}N_2OS_2$
TB03	2, 4-Cl	$C_{22}H_{14}Cl_2N_2OS_2$	TB08	3-NO_2	$C_{22}H_{15}N_3O_3S_2$
TB04	2,4-diOH	$C_{22}H_{16}N_2O_3S_2$	TB09	3-F	$C_{22}H_{15}FN_2OS_2$
TB05	3-Br	$C_{22}H_{15}BrN_2OS_2$	TB10	-H	$C_{22}H_{16}N_2OS_2$

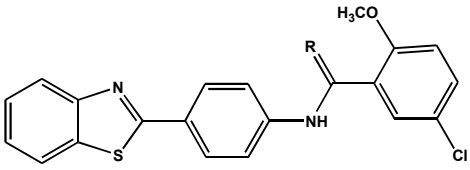
4.4 GENERAL PROCEDURE FOR SYNTHESIS OF BENZOTHIAZOLE OXIME AND HYRDRAZONE DERIVATIVES (A07a & A07b) AND (A10a & A10b)

To the suspension of benzothiazole bearing amides (**A07**, 1.0 mmol) or (**A10**, 1.0 mmol) in absolute ethanol (50 mL), 10.0 mmol of $NH_2OH.HCl/NH_2NH_2$ in water (10 mL) and 0.1 mmol HOAc were added. The mixtures were refluxed for 6–8 h (Scheme 4.4). The progress of reaction was monitored by TLC. Ethanol was distilled off and 50 mL cold water was poured into the reaction mixture. The precipitate formed was filtered and washed with ice cold water, and finally dried to get the desired product. The product thus obtained was recrystallized from ethanol to obtain the pure compounds.

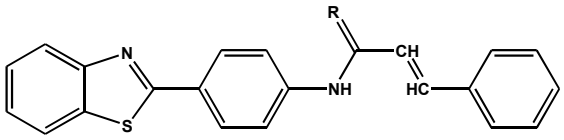


Scheme 4.4. Reagents and conditions: (a) Polyphosphoric acid (PPA), 220^{o}C, 1-2 h reflux with stirring (b) ArCOOH, Acetonitrile, DCC, NaOAc, reflux 4–10 h (c) Reflux 6-8 hr, R= NH_2OH, NH_2NH_2, HOAc.

Table 4.4 Different Substitutions on aryl ring of synthesized compounds **(A07a, A07b, A10a** and **A10b)**



A07a, A07b

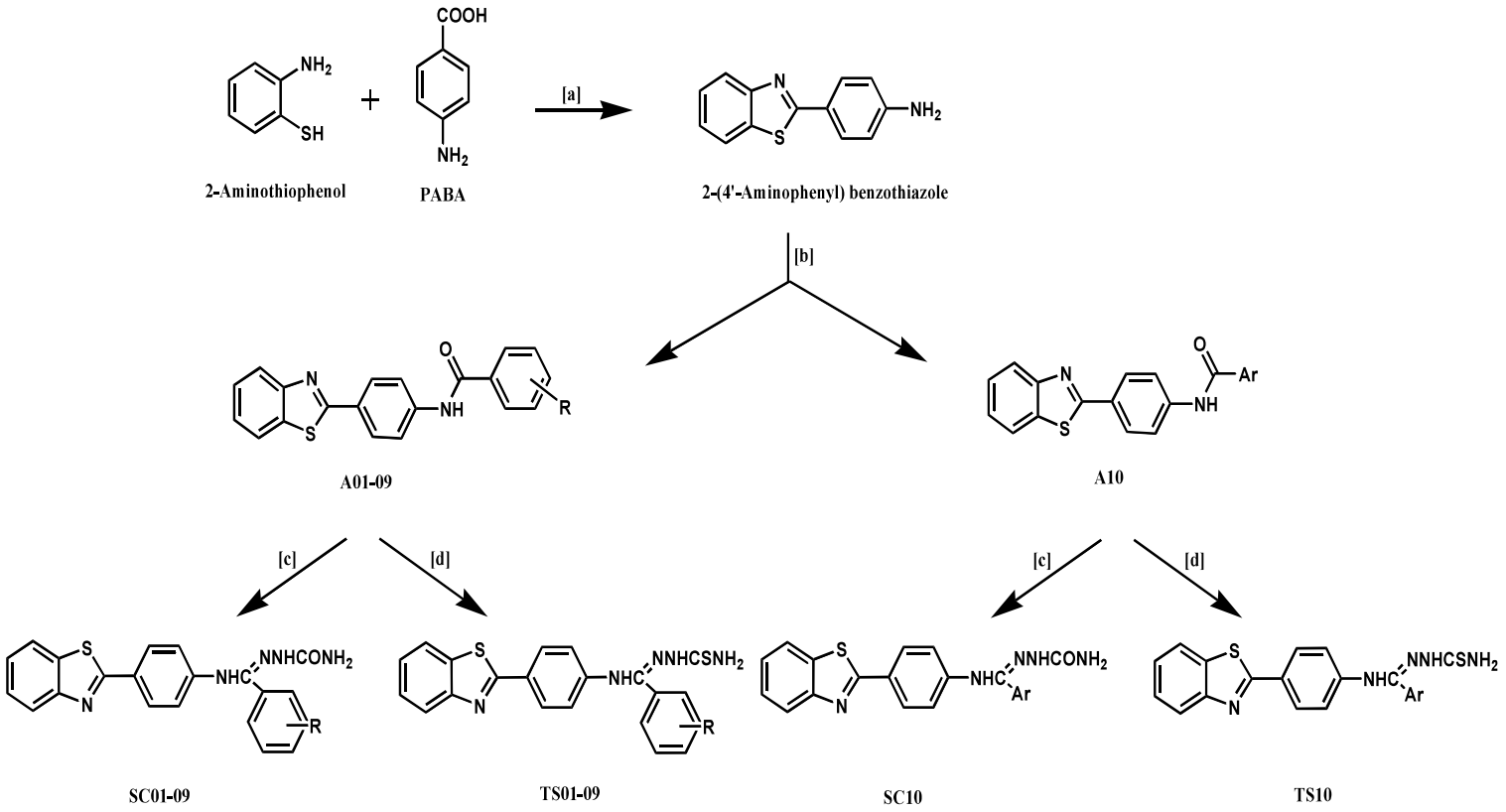


A10a, A10b

Compound code	R	Molecular Formula
A07a	=NOH	$C_{21}H_{16}ClN_3O_2S$
A07b	$=NNH_2$	$C_{21}H_{17}ClN_4OS$
A10a	=NOH	$C_{22}H_{17}N_3OS$
A10b	$=NNH_2$	$C_{22}H_{18}N_4S$

4.5. GENERAL PROCEDURE FOR THE SYNTHESIS OF BENZOTHIAZOLE SEMICARBAZONE AND THIOSEMICARBAZONE DERIVATIVES (SC01-10) AND (TS01-10)

Semicarbazide/ Thiosemicarbazide hydrochloride (1.0 mM) dissolved in 5 mL hot distilled water and catalyst NaOAc were added to a suspension of N-(4-(benzo[d]thiazol-2-yl)phenyl)-substituted benzamides A01-10 (1.0 mM) in 50.0 mL ethanol. The reaction mixture was refluxed on a water bath for 4–10 h (Scheme 4.5). The progress of the reaction was monitored by TLC at appropriate time intervals. After completion of the reaction, the reaction mixture was cooled to room temperature. The precipitate formed was filtered, washed several times with warm ethanol and dried. Compounds were purified by either recrystallization or column chromatography as required.



Scheme 4.5 Reagents and conditions: (a) Polyphosphoric acid (PPA), 220^{o}C, 1-2 h reflux with stirring (b) ArCOOH, Acetonitrile, DCC, reflux 4–10 h (c) Semicarbazide hydrochloride, CH_3OH/C_2H_5OH, NaOAc, reflux 6–8 h (d) Thiosemicarbazide hydrochloride, CH_3OH/C_2H_5OH, NaOAc, reflux 4–8 h.

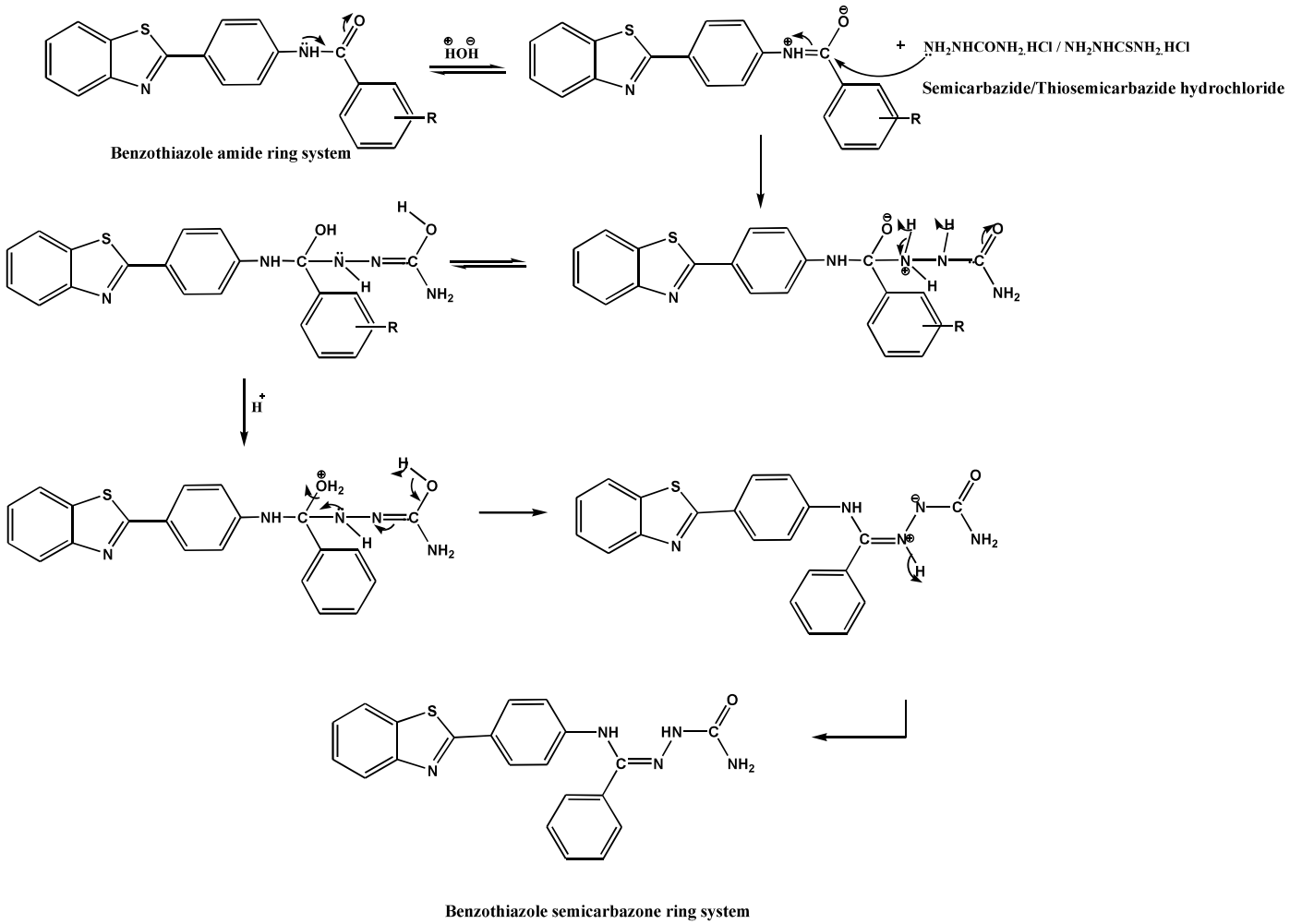


Fig. 4.2 Proposed reaction mechanism of formation of benzothiazole-semicarbazone/ thiosemicarbazone ring system.

Table 4.5 Different Substitutions on aryl ring of synthesized compounds **(SC01-10)** and **(TS01-10)**

Compound code	R, Ar	Molecular formula	Compound code	R, Ar	Molecular formula
SC01, TS01	H	$C_{21}H_{17}N_5OS$ / $C_{21}H_{17}N_5S_2$	SC06, TS06	2-F	$C_{21}H_{16}FN_5OS$ / $C_{21}H_{16}FN_5S_2$
SC02, TS02	3,5-di NO_2	$C_{21}H_{15}N_7O_5S$ / $C_{21}H_{15}N_7O_4S_2$	SC07, TS07	5-Cl, 2-OCH_3	$C_{22}H_{18}ClN_5O_2S$ / $C_{22}H_{18}ClN_5OS_2$
SC03, TS03	2-OH	$C_{21}H_{17}N_5O_2S$ / $C_{21}H_{17}N_5OS_2$	SC08, TS08	2,4-diOH	$C_{21}H_{17}N_5O_3S$ / $C_{21}H_{17}N_5O_2S_2$
SC04, TS04	3-NO_2	$C_{21}H_{16}N_6O_3S$ / $C_{21}H_{16}N_6O_2S_2$	SC09, TS09	2-OCH_3	$C_{22}H_{19}N_5O_2S$ / $C_{22}H_{19}N_5OS_2$
SC05, TS05	2-Cl	$C_{21}H_{16}ClN_5OS$ / $C_{21}H_{16}ClN_5S_2$	SC10, TS10	-Styrene	$C_{23}H_{19}N_5OS$ / $C_{23}H_{19}N_5S_2$

4.6. PHYSIOCHEMICAL AND SPECTROSCOPIC CHARACTERIZATION

Physicochemical characterization of synthesized product includes:

- **Melting point determination:**

  Melting point is a valuable criterion for the purity of the organic compound. The melting points were determined by open capillary method using BARNSTEAD/Electrothermal Stuart-SMP10 melting point apparatus.

- **Solubility determination:**

  The solubility of synthesized compounds was tested in different polar, semi polar and non-polar solvents.

- **TLC analysis (R_f value):**

  Thin Layer Chromatography is an important technique which provides information regarding progress of reaction and determines the purity of compounds. R_f value is the characteristic for each compound and calculated through TLC analysis by using the equation given below:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

For the R_f value determination, the pre coated aluminium plate (silica gel GF-254, 0.25 mm) was used. Chromatograms were developed by ascending technique. When solvent front travelled for appropriate distance, plates were taken out and dried. The locations of spots were detected by UV-cabinet and iodine chamber.

Spectral characterization was performed by using following instruments:

- **FT-IR Spectroscopy**

  IR absorption spectra were recorded on Shimadzu FTIR-8400s by using KBr pellets in the range of 4000–400 cm^{-1} at the Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi.

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

  ^{1}H and ^{13}C NMR spectra were recorded on the JEOL AL300 FTNMR spectrometer operating with the frequency of 300 MHz at the Department of Chemistry, Faculty of Science, (Banaras Hindu University), Varanasi. ^{1}H spectra were also recorded on the BRUKER 300 FTNMR spectrometer at Sophisticated Analytical Instrument Facility (SAIF), CSIR-CDRI, Lucknow. Tetramethylsilane (TMS) was used as an internal standard. The ^{1}H and ^{13}C NMR chemical shifts were reported as parts per million (ppm) downfield from TMS (Me_4Si). The splitting patterns are designated as follows: s, singlet; d, doublet; m, multiplet.

- **Mass Spectrometry**

  Mass spectra were obtained on Micromass Quattro II spectrometer on ESI positive mode and molecular ion peaks are reported as m/z ratio and recorded at SAIF, CSIR-CDRI, Lucknow. .

- **Elemental analysis**

  Elemental analysis has been performed with Exeter Analytical Inc., USA, CE-440 elemental analyser at the Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi. All the compounds gave C, H and N analysis within ± 0.3% of the theoretical values.

4.7. *IN-VITRO* ANTIMICROBIAL ACTIVITY EVALUATION

4.7.1. Agar Disc diffusion method

Antimicrobial activity of newly synthesized compounds was evaluated on different Gram-positive and Gram-negative human pathogens viz. *Staphylococcus aureus* (ATCC 25323), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27893), *Klebsiella pneumonia* (ATCC 31488), *Enterococcus faecalis* (clinical isolate) and *Salmonella typhi* (MTCC 3216) according to the guidelines of National Committee for Clinical Laboratory Standards [Wayne, 1997] using the agar disc diffusion method [Bharti *et al.,* 2010a]. Briefly, a 24/48 h-old culture of selected bacteria was mixed with sterile physiological saline (0.85%) and the turbidity was adjusted to the standard inoculums of Mac- Farland scale 0.5 [~106 colony forming units (CFU) per millilitre]. Petri plates containing 20 mL of Mueller Hinton Agar (MHA, Hi- Media) were used for all the bacteria tested. The inoculums was spread on the surface of the solidified media and Whatman no. 1 filter paper discs (6 mm in diameter) impregnated with the test compound (20 µL/disc) were placed on the plates. Ciprofloxacin (5µg/disc, Hi-Media) was used as positive control for bacteria. A paper disc impregnated with dimethylsulfoxide (DMSO) was used as negative control. Plates inoculated with the bacteria were incubated for 24 h at 37°C. The inhibition zone diameters were measured in millimeters. All the tests were performed in triplicate.

Determination of MIC

Minimum inhibitory concentration (MIC) of any compound is defined as the lowest concentration which completely inhibits visible growth (turbidity on liquid media). MIC values were determined by broth micro dilution method, according to NCCLS guidelines document M27-A [Wayne, 1997]. Equal volume of test compounds with different dilutions and nutrient broth were mixed in wells of micro titre plate, which were serially two fold diluted to determine the MIC. Specifically 0.1mL with approximately 5 x 10^5 CFU/mL of actively dividing bacterial cells was inoculated in each well. The standard antibiotic, ciprofloxacin (10µg/mL) for bacteria was used as positive controls and 100 µL of DMSO used as a negative control. All the inoculated plates were incubated at 37ºC and the results were evaluated by visible turbidity in each well after 24 h. All determinations were done in triplicates and the average was taken as final MIC value.

4.7.2. Mechanism of action study

4.7.2.1. Bactericidal kinetics

Overnight cultures of *S. aureus* ATCC 25323 and *E. coli* ATCC 35218 were diluted 10^{-2} times in fresh MHB and allowed to grow to exponential phase (optical density at 600 nm of 0.6). The time course killing activity of synthesized compounds was determined using the 96-well plates of plate reader as described previously [Joshi *et al.,* 2012]. The lead compounds were added at 4 times their MICs, and this suspension was incubated at 37°C at 200 rpm. After addition of the compounds, the absorbance of the plates was recorded at 600 nm at regular time intervals i.e. 0, 1, 2, 3, 4 and 5 h. The experiment was repeated on three different days and values are plotted as mean ± SD.

4.7.2.2. Membrane Depolarization assay

Compound-induced polarization of *S. aureus* ATCC 25323 and *E.coli* ATCC 35218 membrane measures the efficacy to dissipate the potential across these cell membranes and was determined using the membrane potential-sensitive cyanine dye DiSC3(5) described previously [Friedrich *et al.,* 2000]. Briefly, exponential-phase bacteria were harvested by centrifugation, washed and resuspended in 5 mM HEPES–20 mM glucose buffer (pH 7.2) to an optical density of 0.06. This cell suspension was incubated with 100 mM KCl (to equilibrate cytoplasmic and external K+ concentration) and DiSC3(5) at a concentration of 1µM for 1h at room temperature. When the fluorescence level (excitation at 622 nm and emission at 670 nm wavelength) of the bacterial suspension became stable (approximately 90% reduction in fluorescence due to DiSC3(5) uptake and quenching in the cell in response to an intact membrane potential), a 400µL aliquot of cell suspension and the 4 × MIC concentration of lead compounds were added in the cuvette in order to record the membrane hyperpolarization of bacterial cell membrane. All samples were then measured on Perkin Elmer Life Sciences LS 50-B spectrofluorimeter (Perkin-Elmer Corp., Norwalk, Conn.) in a 5 mm path length quartz cell at 25°C. Samples were stirred during the experiment at a constant temperature of 37°C. An increase in fluorescence due to partitioning of samples into the membrane was recorded as a function of time until no further increase in intensity was observed.

4.7.2.3. Fluorescence assisted cell sorting (FACS) study

The membrane damage of *S. aureus* ATCC 25323 and *E.coli* ATCC 35218 was examined by flow cytometer after employing suitable fluorescent probes [Novo *et al.,* 2000]. Briefly, the cells at exponential phase were stained with propidium iodide after the treatment with

the lead compounds at 37°C for 1 h with constant shaking. The cells were centrifuged, washed two times with PBS and incubated further with propidium iodide (PI) at 4°C for 30 min, followed by removal of the unbound dye through washing with an excess of PBS and re-suspended in buffer. These cells were then analyzed by flow cytometry in the form of dots (excitation and emission wavelength set at 488 and 617nm, respectively for propidium iodide), plots with respect to the control without PI treated, with PI treated cells without preincubation with test compounds and with test compound treated cells.

4.7.2.4. DNA binding assay

Gel retardation experiments were performed on synthesised compounds by agarose gel electrophoresis as described previously [Bharti *et al.,* 2010b]. Briefly, 200 ng of plasmid DNA (pUC19) was mixed with increasing amounts of lead compounds in 20 µL of binding buffer (5% glycerol, 10 mM Tris- HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl, and 50 µg/mL bovine serum albumin). The reaction mixtures were incubated for 1h at 37°C. Consequently, loading buffer containing 25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol was added and then subjected to 1 % agarose gel electrophoresis at 60 V for 1h in 10 mM Tris-HCl buffer. The migration of DNA was detected by the fluorescence of ethidium bromide. The illuminated gel was photographed by Alpha Innotech Corporation Instrument. Besides, the reaction was also monitored upon addition of various groove binders-methyl green (MG) and DAPI.

4.8. *IN-VITRO* ANTICANCER ACTIVITY EVALUATION

Maintenance of cell lines

Human ovarian cancer cell lines, A2780 parent cells and their respective resistant counterpart were cultured in DMEM supplemented with 10% FBS. Similarly, cervical cancer cell lines SiHa & C-33A and, HEK-293 (normal epithelial human embryonic kidney cell line) were grown and maintained in DMEM media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1 % penicillin-streptomycin antibiotic cocktail. One of these cell lines (C-33A) is human papillomavirus (HPV) negative, while SiHa is HPV-16 positive. IOSE 364 (normal ovarian surface epithelial cell line) was cultured in MCDB-M199 medium supplemented with 15% FBS. SKOV3 cells were cultured in McCoy's medium supplemented with 10% FBS along with 1% glutamine and 50 mM/mL antibiotic solution. All the cell lines were obtained from ATCC and maintained at 37°C in a CO_2 incubator with a humidified atmosphere of 5% CO_2 and 95% air.

4.8.1. MTT assay

The MTT cell proliferation assay was based on the protocol described in [Prasad *et al.* 2015]. This method used to determine cytotoxic effect of synthesized compounds in ovarian and cervical cancer cell lines. Briefly, cells ($5x10^5$/well) were seeded in 96-well culture plates and incubated in a CO_2 incubator at 37°C for 24 hours to allow the cells to adhere and achieve 70%–80% confluency. After 24 hours, the medium was removed and the cells were incubated with different concentrations of compounds for 48 h. Cell viability was estimated before and after treatment with the compounds using an automated cell counter (TC-10 Biorad, USA) in conjunction with trypan blue exclusion staining. Cell proliferation was assayed using a MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye. After 48 h drug exposure, 20 µL MTT solutions (6mg/mL) was added to each well of a 96-well plate and the plates were incubated for 3h at 37°C, allowing viable cells to convert the yellow-colored MTT into dark-blue formazan crystals. The total amount of formazan crystals formed was solubilised with dimethyl sulfoxide (DMSO) and was examined by measuring the absorbance at 570 nm using microplate reader (Biorad, USA). The assay was carried out in triplicate. The IC_{50} value of each test compounds was obtained by plotting the percentage of inhibition versus the concentration of test compounds.

Morphological analysis

Cervical cancer cells SiHa and C33-A were grown in a 6-well culture plates at a density of 1×10^5 cells/well plate for 24 h using standard culture conditions. The test compounds were then added at their respective IC_{50} values and the cells were further incubated for 48h. After these morphological changes in the apoptotic bodies of the SiHa and C33-A cells were examined by inverted microscope (Olympus, BX53F) with fluorescent lamps and digital cameras. The data was acquired and analyzed by CellSens software.

4.8.2. Mechanism of action study

4.8.2.1. Immunoblot analysis

Western blot analysis was used to assess the phosphorylation pattern of EGFR. The assay was performed as described previously [Johnson *et al.,* 2000]. Briefly, the ovarian cancer cells were grown overnight in 60 cm^2 tissue culture dish until sub confluence, then were exposed with different chemical treatments at single concentration 100 µM and also without any chemical treatment. A single dose of 10 µM gefitinib was used as positive

control. After 48 hours, cells were pellet down and washed thrice in cold PBS and lysed using buffer [50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM DTT, 50 mM Na_3VO_4 and 0.5% NP-40] containing protease inhibitor cocktails (Roche Diagnostics, IN). Protein estimation of the cell lysates was determined using BCA assay kit (Thermo Scientific). 50 µg of proteins were resolved by 10% SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MA). After 1 hour, blot was blocked with 5% non-fat dry milk and 5% BSA in TBST at room temperature and further probed overnight at 4°C with desired phosphorylated EGFR antibody. To ensure equal loading, another blot containing actin was blocked in 5% non-fat dry milk and probed overnight with anti-actin antibody at 4°C. Membranes were then washed thrice with TBST, incubated with peroxidase conjugated secondary antibody for 1 hour at room temperature, and specific protein bands were detected by X-ray film (Kodak, Rochester, NY) using the enhanced chemiluminescence (ECL) detection system (GE Healthcare). The densitometry of the western blots was calculated by Image J (IJ1.46r).

4.8.2.2. Cell cycle study

Equal number of cells ($1.0x10^5$) were seeded in a 6-well culture plate, grown for 24 h and then treated with test compounds (A01-10) at their respective IC_{50} values for 48 h. Briefly, the cells were collected by trypsinization, centrifuged at 3000 rpm for 10 min, washed twice with chilled 1× PBS (pH 7.4) followed by centrifugation at 2000 rpm for 5 min. Pellet was re-suspended and fixed in chilled ethanol (70 %) for 30 min at 4 °C followed by centrifugation at 4000 rpm for 15 min, as described previously [Prasad *et al.,* 2015]. The cells were re-suspended in 1× PBS and incubated with 5 µL RNaseA (10 mg/mL) for 30 min at 37°C followed by staining with 10 µL propidium iodide (PI) (1mg/mL) for 30 min. The cell cycle distributions were analyzed using a FACS Calibur flow cytometer (BD, San Jose, CA, USA) in conjunction with PI staining using Cell Quest Pro software (Becton Dickinson, USA).

4.8.2.3. Apoptosis assay

The quantification of cell death was determined by staining the cells with the AnnexinV-AlexaFlor488 and PI (Vibrant apoptosis detection kit, Invitrogen, USA) according to the manufacturer's instructions (BD Pharmingen, BD Bioscience, USA) as described previously [Prasad *et al.,* 2014]. Briefly, 1×10^5 of the SiHa and C33-A cells were seeded into six-well culture plate and after 24 h incubation, treated with test compounds (A01-10)

at their respective IC_{50} values for 48 h. The cells were then washed twice with PBS and suspended in 100 µL of 1X annexinV binding buffer to which 5 µL of AlexaFluor488 annexin V and 1 µL PI was added. Cells were incubated for 15 min at room temperature. The samples were then analyzed using FACS calibur (BD Bioscience, USA) using CellQuest Pro analysis software (Becton Dickinson, USA).

4.8.2.4. DNA fragmentation

SiHa and C-33A cells (2×10^5 cells/60 mm dish) were treated with or without the compounds A01-A10 at their respective IC_{50}s for 48 h. Cells were transferred to 1.5-mL sterile microcentrifuge tubes, centrifuged at 2000 rpm for 5 minutes at 4°C and the supernatant was discarded. The cells were then washed twice with PBS and lysed in a buffer containing 50 mM Tris–HCl pH 8.0 and 0.5% SDS, incubated for 30 min at 37°C. Cell pellet was stirred with a wide-bore pipette tip to ensure uniform mixing. The pellet was incubated with 1 µL of DNAase free RNase (10 mg/mL) for 1 h at 37°C. Proteinase K (50 µg/mL) solution was further added and samples were incubated for 90 min at 50°C. The precipitated DNA was dissolved in minimum quantity of TE buffer and quantified spectrophotometrically as described previously [Kasibhatla *et al.,* 2006]. Equal concentration of DNA (10 µg) was resolved on 1% agarose gel at 50 V for 4 hr, viewed under UV light and documented using Alpha Innotech system.

4.9. MOLECULAR DOCKING STUDY

The molecular docking software, Autodock 4.0 was used to study the binding behaviour of the ligands onto receptor binding pockets. The association of EGFR family of tyrosine kinases in cancer proliferation suggests ligands which block the kinase activity of the entire EGFR family and may have profound therapeutic potential [Mendelsohn and Baselga, 2000]. With the exposition of the 3D-structures of kinase domains, ATP-binding pocket has been the spotlight of small molecular inhibitor design [Noble *et al.,* 2004, Traxler *et al.,* 2001]. Benzothiazoles act via challenging with ATP intended for binding at the catalytic domain of tyrosine kinase [Yates *et al.,* 1991]. Thus, we selected EGFR target to carry out the docking study of synthesized compounds and delineate their best possible mechanism of action against cancer cells.

The molecular docking study of benzothiazole compounds against antimicrobial target Glucosamine-6-phosphate synthase was performed using the SYBYL X 1.2 software from

Tripos Inc., St. Louis, MO, USA [Sybyl-X1.2, 2010; Ghate *et al.,* 2013]. All the compounds were sketched using SKETCH function of SYBYL X 1.2. Partial atomic charges were calculated by the Gasteiger Huckel method and energy minimizations were performed using the Tripos force field with a distance-dependent dielectric and the Powell conjugate gradient algorithm convergence criterion of 0.01 kcal/mol Å.

4.9.1. Glucosamine-6-phosphate synthase (Glc-6-PS)

Molecular Docking Studies was used to determine the orientation of inhibitors bound to the active site of GlcN-6-P synthase. Surflex-Dock module of Tripos Sybyl X1.2 was used for molecular docking. Receptor complexed with ligand [PDB: 1JXA] was taken from protein data bank (PDB) and modified for docking calculations. Co-crystalized ligand was removed from the structure, water molecules were removed, H atoms were added and side chains were fixed during protein preparation. Protein structure minimization was performed by applying Tripos force field and partial atomic charges were calculated by Gasteiger Huckel method. Surflex module docks ligands automatically into ligand binding site of receptor using a protomol based method and an empirically derived scoring function. The protomol is a unique and important factor of the docking algorithm and is a computational representation of assumed ligands that interact with the binding site of receptor. Surflex-Dock's scoring function contains hydrophobic, polar, repulsive, entropic, and solvation terms. Scoring function of Surflex can also be improved by matching algorithm which allows docking of a fragment of ligand to protein. The fragment is allowed to rotate from its original position during optimization of pose. Docking results contain three information: total score, crash value and polar value. Total score is the total Surflex Dock score expressed as -log (Kd) to represent binding affinities which include hydrophobic, polar, repulsive, entropic and solvation terms. Crash value is the degree of inappropriate penetration by the ligand into the protein and of interpenetration between ligand atoms (self-clash) that are separated by rotatable bonds. Crash scores close to 0 are favourable. Polar value is contribution of the hydrogen bonding and salt bridge interactions to the total score. The polar score may be useful for excluding docking results that make no hydrogen bonds [Balamurugan *et al.,* 2012]. Hits which scored comparable or more than marketed drugs were selected as final lead molecules.

4.9.2. Epidermal Growth Factor Receptor- Tyrosine Kinase (EGFR-TK)

Molecular docking was performed using the X-ray crystal structure of tyrosine kinase as a template retrieved from protein data bank (PDB ID: 2J5F) and further modified for docking calculations. Protein preparation was carried out by deleting the water molecules and adding the hydrogen atoms, where they were missing. Further, grids of default box size were defined using the ligand binding site of the crystal structure. The 3D structure of the ligands were built with the help of Arguslab server, energetically minimized upto 0.01 rms gradient using Merck Molecular Universal Force Field (MMUFF) [Casewit *et al.,* 1992] and Gasteigere-Marsili partial charges were assigned to the ligands for docking calculations. Furtherance, the Lamarckian genetic algorithm and the Pseudo-Solis as well as Wets methods were applied using default parameters for minimization. The receptor tyrosine kinase was docked with ligand having lowest energy conformation using the grid generated along its structure. Number of docking runs, genetic algorithm population, energy evaluations and the maximum number of iterations were 50, 250, 10000, and 100000 respectively. The results were interpreted on the basis of docking score and best binding pose was considered as the final output. The docking results also showed optimized binding energy, inhibition constant, intermolecular energy and subsequently analysed for other interactions like hydrogen bonding, hydrophobic and vander Waal's interaction.

4.10. *IN-SILICO* PHARMACOKINETIC PREDICTIONS

The *in silico* pharmacokinetic prediction study was performed on preADMET server for ligands that acquired good docking scores in order to remove compounds with poor pharmokinetic properties and hence minimises expensive and time consuming steps. This program simultaneously calculates physically important descriptors and pharmaceutically related properties to predict experimentally various human ADMET processes as well as other significant pharmacokinetic parameters such as oral absorption, bioavailability, skin penetration, clearance (C_L), volume of distribution (V_d), and metabolism [Clark, 2003; Didziapetris *et al.,* 2003].