4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Cell line

HEp-2, a human laryngeal cancer cell line was used as an experimental system. It was procured from National Centre for Cell Science (NCCS) (Pune, India). HEp-2 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone GE healthcare, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Thermo Fischer Scientific, USA) and 1X Antibiotic/Antimycotic (Hyclone GE healthcare, USA). Cells were grown at 37℃ in a humidified incubator with 5% CO_2. HEp-2, being an adherent cell line, was sub-cultured using 0.25% w/v Trypsin (Lonza, Switzerland) using conventional methods. Cells, grown within 15 passages, were utilized for the experiments.

4.1.2 Antibodies and reagents

(i) Antibodies: Anti-CD44 PE, anti-BST-2 PE, anti-Ki-67 FITC, anti-Vimentin PE, anti-Nanog DyLight 488, anti-E-cadherin APC, anti-ALDH1 and anti-p-IRAK-1 were purchased from Thermo Fischer scientific, USA. Anti-IRAK-1, anti-IRAK-4, anti-p-IRAK-4 and anti-rabbit IgG (H+L) F(ab')2 Fragment-PE were purchased from Cell Signaling Technology (CST), USA. Anti-GAPDH and Goat anti-rabbit IgG HRP were purchased from ImmunoTag, USA. Goat anti-mouse IgG HRP was purchased from Bangalore Genei, India.

(ii) Chemo-drugs: Docetaxel, cisplatin and 5-FU, conventional chemo-drugs used for the management of HNSCC were utilized in the study to evaluate the efficacy of chemotherapy on HNSCC cells. Docetaxel and 5-FU were purchased from Zydus Cadila Pharmaceuticals, India. Cisplatin was purchased from Celplat, India. The structure and mode of action of the chemo-drugs are presented in ***Section 2.5.3, Chemotherapy***.

(iii) IRAK-1 &-4 dual inhibitor: To inhibit the TLR signaling in HNSCC cells, a small molecule based IRAK-1 &-4 dual inhibitor, that inhibits the activity of IRAK-1 and IRAK-4, downstream kinases in TLR signaling pathway, was used in the study. IRAK-1 &-4 dual inhibitor solution (Cat# 407602) with DMSO as solvent was purchased from Sigma-Aldrich, St. Louis, MO, USA. The structure **(Figure 2.19)** and mode of action of the inhibitor is presented in ***Section 2.9.2.1 Small molecule inhibitors of IRAK-1 and IRAK-4***. The inhibitor specifically inhibits the TLR signaling. In the study, on one hand, it has been used to know the

impact of TLR signaling on various pro-oncogenic attributes, while on the other hand it has been tested as therapeutic modality in combination with chemo-drugs.

(iv) Other reagents: Resazurin-dye and protease and phosphatase inhibitor cocktail were procured from Sigma-Aldrich, St. Louis, MO, USA. Primers for all the *TLRs*, *Bcl-2*, *Bcl-xL*, *MMP-2* and *GAPDH* were purchased from IDT, USA. TRIzol reagent, Pierce™ 16% Formaldehyde and eBioscience™ Foxp3/Transcription Factor Staining Buffer Set were procured from Thermo Fischer Scientific, USA. PrimeScript 1st strand cDNA synthesis kit and TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) were procured from Takara Bio Inc., Japan. Radioimmunoprecipitation assay **(**RIPA**)** buffer and Tween-20 was purchased from Himedia, India. SureCast™ Gel Handcast Bundle and Human IL-6 Antibody Pair Kit were procured from Invitrogen, USA. PVDF membranes were purchased from Amersham, UK. Enhanced chemiluminescence (ECL) substrate was procured from BioRad Laboratories, Inc, USA.

4.2 Methods

4.2.1 Drug sensitivity assay

The cytotoxic potential of drugs was calculated in terms of IC_{50} i.e., the concentration at which 50% of the cell death is observed with respect to untreated cells.

4.2.1.1 Estimation of cytotoxic potential of drugs

HEp-2 cells were seeded at a density of 1000 cells per well in a 96 well plate and incubated overnight. Further, cells were treated with chemo-drugs namely, Docetaxel, Cisplatin and 5-FU, and IRAK-1 &-4 dual inhibitor solution, separately for 72 h. The range of drug doses tested is as follows- Docetaxel: 0.1nM-10000nM, Cisplatin: 3.3µM-300µM, 5-FU: 0.03mM-10mM, and IRAK-1 &-4 dual inhibitor solution: 1µM-300µM.

For IC_{50} calculation, the viable cell count was determined by the resazurin-dye-based assay. Post-treatment, cells were incubated with resazurin dye solution at a final concentration of 10µg/mL for 4 h. Fluorescence of supernatant was measured at 530nm excitation/590nm emission in Synergy HT microplate reader (BioTek Instruments, USA). Percent change in cell viability at each dose of the drug was determined by the following equation:

$$\frac{Average\ fluorescence\ of\ treated\ cells}{Average\ fluorescence\ of\ control\ cells} \times 100$$

Dose-response curves were generated using GraphPad Prism 8.0 software and the IC_{50} of the specific chemo-drugs and IRAK-1 &-4 dual inhibitor were derived.

4.2.1.2 Development of triple chemo-resistant cell line

A triple chemo-resistant cell line was developed as a prototype experimental system to study the role of TLR signaling in HNSCC chemoresistance as well as assessment of therapeutic efficacy of combination therapy. It was developed by exposing parent HEp-2 cells to a combination of Docetaxel, Cisplatin and 5-FU in a dose incremental approach using the method described by Govindan *et al.,* (2015) in principle with minor modifications. From the dose response curves of the chemo-drugs, $IC_{3.125}$ and $IC_{6.25}$ (the concentrations at which 3.125% and 6.25% cell death is observed upon treatment with respect to untreated cells) of individual chemo-drugs **(Table 5.2)** were determined. Cells were treated with escalating dose of chemo-drugs in the subsequent cycles. In the first cycle, cells were exposed to a combination of $IC_{3.125}$ of the chemo-drugs for 72 h and the surviving cells were cultured in chemo-drug free medium for the next 72 h. In the second cycle, these remaining live cells were exposed to a combination of $IC_{6.25}$ of the chemo-drugs for 72 h and further cultured in chemo-drug free medium for the next 72 h. The generated cell line was further maintained in the chemo-drug free medium **(Figure 4.1-4.2)**. The IC_{50} of individual chemo-drugs on the developed chemo-resistant cell line was re-estimated by same resazurin-assay. The shift in IC_{50} of individual chemo-drugs on the chemo-resistant cell line with respect to the parent cell line was calculated to validate the acquisition of chemo-resistance on the newly developed chemo-resistant cell line.

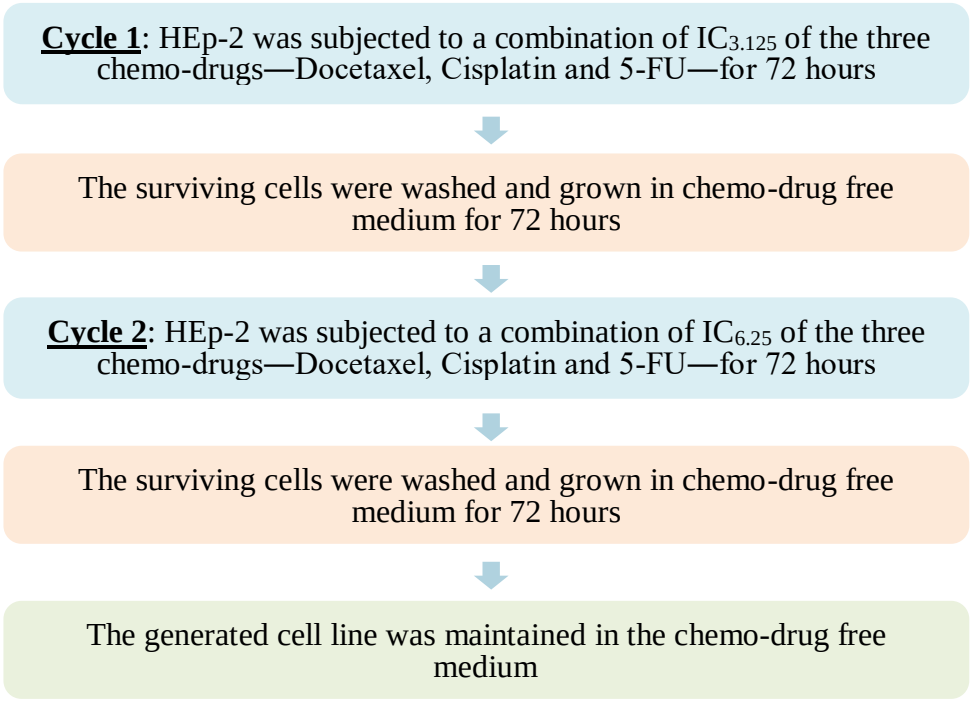

Figure 4.1: Schematic protocol for the development of triple chemo-resistant cell line

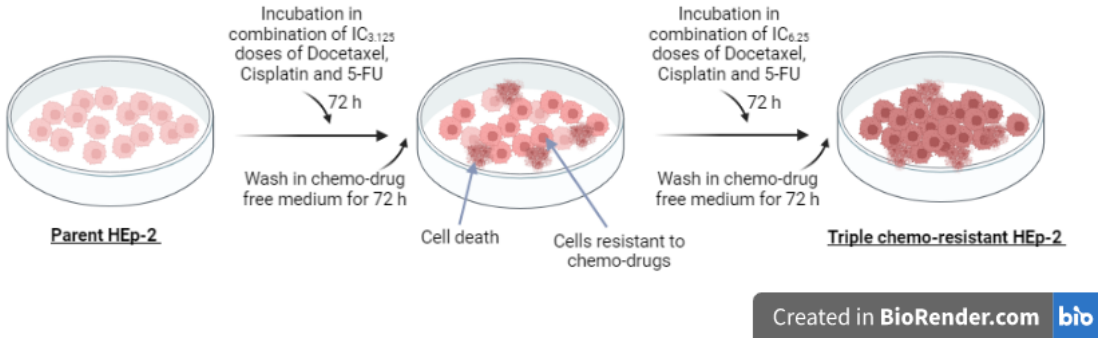

Figure 4.2: **Schematic illustration for the development of triple chemo-resistant cell line**

4.2.1.3 Determination of the efficacy of combination treatment

HEp-2 cells were seeded at a density of 1000 cells per well in a 96 well plate and incubated overnight. To evaluate the effect of combination treatment of the chemo-drugs and IRAK-1 &-4 dual inhibitor on cell proliferation, cells were incubated with increasing concentrations of individual chemo-drugs (in the dose-range mentioned in ***Section 4.2.1.1 Estimation of cytotoxic potential of drugs*)** in combination with single constant dose of IC_{25} (the concentration at which 25% cell death is observed upon treatment with respect to untreated cells) of IRAK-1 &-4 dual inhibitor **(Table 5.1 and *Section 5.4.6 Impact of inhibition of TLR signaling pathway on chemo-resistant cell line*)**. Cell viability was determined by resazurin-

dye-based assay as described previously. The dose-response curves were generated for both parent and chemo-resistant HEp-2 and the IC_{50} of the combination treatment were derived.

4.2.2 mRNA-based expression analysis using quantitative PCR

Expression of TLRs 1-10, Bcl-2, Bcl-xL and MMP-2 was determined at mRNA level. RNA was isolated from cells followed by cDNA synthesis and qPCR.

(i) RNA isolation

For RNA extraction, 1 x 10^6 cells were seeded in a 60mm culture dish and incubated overnight. Cells were harvested through scraping and washed in cold PBS. Total RNA was isolated using TRIzol reagent according to the manufacturer's instruction. RNA concentration was determined using NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, USA) and RNA purity was checked by evaluating A260/280 ratio, considering A260/A280 ratio ~ 2.0 as pure RNA. 1µg of RNA was converted to cDNA using PrimeScript 1st strand cDNA synthesis kit according to the manufacturer's instructions.

(ii) Primer designing and sequences

Primers for gene amplification were designed from DNA sequences retrieved from GenBank DNA Database using National Centre for Biotechnology Information's (NCBI) Primer-Basic Local Alignment Search Tool (BLAST). Sequences of primers used with corresponding annealing temperature are presented in **Table 4.1.**

Table 4.1: Sequences of primers used in the study

Gene	Sequence	Annealing temperature (°C)
TLR-1	F- GCCCAAGGAAAAGAGCAAAC R- AAGCAGCAATATCAACAGGAG	52
TLR-2	F- TCTCCCATTTCCGTCTTTTT R- GGTCTTGGTGTTCATTATCTTC	55
TLR-3	F- TAAACTGAACCATGCACTCT R- TATGACGAAAGGCACCTATC	47
TLR-4	F- GAAGCTGGTGGCTGTGGA R- GATGTAGAACCCGCAAG	64

TLR-5	F- TTGCTCAAACACCTGGACAC R- CTGCTCACAAGACAAACGAT	53
TLR-6	F- GTGCCATTACGAACTCTA R- TTGTTGGGAATGCTGTT	47
TLR-7	F- CTGACCACTGTCCCTGAG R- AACCCACCAGACAAACCA	50
TLR-8	F- AACATCAGCAAGACCCAT R- GACTCCTTCATTCTCCCT	50
TLR-9	F- CGCCAACGCCCTCAAGACA R- GGCGCTTACATCTAGTATTTGC	55
TLR-10	F- CTCCCAACTTTGTCCAGAAT R- GGTGGGAATGCAATAGAAT	45
Bcl-2	F- ACGAGTGGGATGGGGGAGATGTG R- GCGGTAGCGGCGGGAGAAGTC	60
Bcl-xL	F- CTGAATCGGAGATGGAGACC R- TGGGATGTCAGGTCACTGAA	60
MMP-2	F- TGGACTTAGACCGCTTGGC R- CTCCTTGGGGCAGCCATAGA	61
GAPDH	F- AATGGGCAGCCGTTAGGAAA R- GCGCCCAATACGACCAAATC	60

(iii) qPCR cycle

qPCR was performed with 10ng of cDNA for each reaction using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), a 2X qPCR master mix containing TaKaRa Ex Taq HS (Taq polymerase), dNTP mixture, Mg2+, Tli RNaseH (a heat resistant RNase H), TB Green, and ROX Reference Dye. qPCR for *TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, TLR-7, TLR-8, TLR-9, TLR-10, Bcl-2, Bcl-xL* and *MMP-2* was performed on CFX-96 Touch^{TM} Real-time PCR detection system (BioRad Laboratories, Inc, USA). *GAPDH* was used as an internal control. The cycling conditions are mentioned in **Table 4.2.**

Table 4.2: Cycling conditions of qPCR

Step	Temperature	Time	
Initial denaturation	95°C	5 min	
Denaturation	95°C	5 min	**44 cycles**
Annealing	Variable	45 sec	
Extension	72°C	45 sec	
Plate read			
Final Extension	72°C	10 min	
Melt curve	65°C-95°C (with increment 0.5°C)	5 sec for each temperature increment	

C_t values of less than 35 was taken into consideration for the presence of the mRNA. Specific experiment were set up in triplicate. For parent HEp-2's TLR expression analysis, only C_t values were used. The mean C_t values of the target genes were normalized with the mean C_t values of *GAPDH* as house-keeping genes referred as ΔC_t.

The fold difference in mRNA expression of the related genes between two samples was calculated by the Comparative C_t or $2^{-\Delta\Delta C}{}_t$ method (Livak and Schmittgen, 2001) using the below mentioned formula:

$$\Delta C_t = C_t \text{(target gene)} - C_t \text{(housekeeping gene)}$$

$$\Delta\Delta C_t = \Delta C_t \text{ (experimental sample)} - \Delta C_t \text{ (control sample)}$$

$$\text{Fold change} = 2^{-\Delta\Delta C}{}_t$$

Data is presented for three independent experiments, each set in triplicates.

4.2.3 Flow cytometry

Flow cytometry was performed to assess the expression of the following surface and intracellular molecules in this study.

- Surface molecules: CD44, BST-2, E-cadherin and vimentin
- Intracellular molecules: IRAK-1, IRAK-4, p-IRAK-1, p-IRAK-4, Ki-67 and Nanog

1 X 10^5 cells were seeded per well in a 12-well culture plate and incubated overnight.

4.2.3.1 Staining procedure for surface markers

To preserve the structural integrity and expression of surface molecules, cells were harvested non-enzymatically with citric acid saline dissociation buffer (preparation in appendix) and washed in FACS buffer (PBS with 1% FBS). Cells were further incubated with fluorochrome-conjugated anti-CD44 (1:100 dilution), anti-BST-2 (1:100 dilution), anti-E-cadherin (1:50 dilution), anti-Vimentin (1:50 dilution) and corresponding isotype control antibodies for 30 mins at room temperature (RT). Post incubation, cells were washed to remove unbound antibodies and cell pellets were resuspended in FACS buffer.

4.2.3.2 Staining procedure for intracellular markers

(i) Intracellular staining by paraformaldehyde-methanol fixation method

Staining of Ki-67 and Nanog in cells was performed by paraformaldehyde-methanol fixation protocol. Cells were harvested and washed in FACS buffer. Cells were fixed with paraformaldehyde at a final concentration of 2% for 10 mins in dark at RT. Paraformaldehyde was prepared by diluting Pierce™ 16% Formaldehyde with PBS. Cells were washed with FACS buffer. Permeabilization was carried out by adding 1 mL of 100% cold methanol dropwise to cells. Cells were incubated in methanol for 30 mins at 4°C followed by a wash in FACS buffer. Cells were further incubated with fluorochrome-conjugated anti-Ki-67 (1:100 dilution), anti-Nanog (1:100 dilution) and appropriate isotype control antibodies for 30 mins at 4°C. Cells were washed to remove unbound antibodies and cell pellets were resuspended in FACS buffer.

(ii) Intracellular staining using Foxp3/Transcription factor staining buffer set

Staining of IRAK-1, IRAK-4, p-IRAK-1 and p-IRAK-4 were carried out using commercially available eBioscience™ Foxp3/Transcription factor staining buffer set following the

manufacturer's instruction. Cells were incubated in primary antibodies anti-IRAK-1 (1:800 dilution), anti-IRAK-4 (1:1000 dilution), anti-p-IRAK-1(1:500 dilution), anti-p-IRAK-4 (1:400 dilution) and secondary antibody anti-rabbit IgG (H+L) F(ab')2 Fragment-PE (1:500 dilution) for 30 mins each at RT. Cells were washed to remove unbound antibodies and cell pellets were resuspended in FACS buffer.

4.2.3.3 Acquisition and analysis of flow cytometry results

10,000 cells were acquired on FACS Calibur flow cytometer (BD Biosciences, USA) within the live cells gate based on the Forward scatter (FSC) vs. Side scatter (SSC). CellQuest Pro software (BD Biosciences, USA) was used as acquisition software.

Flowing Software version 2.5.1 (Turku bioscience, Finland) was used for analyzing the data, generation of dot plots and histograms. Data was collected in terms of both % positive cells and mean fluorescence intensity (MFI) units. Isotype antibody-based fluorescence signals were used as staining negative controls.

4.2.4 Western blotting

Western blotting was performed to estimate the expression of cancer stem cells marker ALDH1. GAPDH was used as an internal control. 1 x 10^6 cells were seeded in a 60mm culture dish and incubated overnight.

4.2.4.1 Sample preparation and protein quantification

After the treatment, cells were washed with cold PBS and harvested by scraping. Cells were centrifuged at 1500 RPM for 5 mins at 4°C and the supernatant was discarded. The cell pellets were resuspended in 200 µL RIPA buffer supplemented with 1% (v/v) protease and phosphatase inhibitor cocktail and incubated on ice for 30 mins. After cell lysis, the samples were centrifuged for 10 mins at 12,000 RPM at 4°C and the supernatant were collected.

The concentration of protein was estimated using the folin-lowry method. Bovine Serum Albumin (BSA) standards were prepared in the range of 0.25 mg/mL-20 mg/mL to generate the protein standard curve. By correlating absorbance of the standards (plotted on *y-axis*) to the protein concentration (plotted on *x-axis*), the equation $y=mx+c$ was obtained from the linear graph in MS Excel. In the equation, *m* represents the slope and *c* represents the y-intercept. Using this equation, the protein concentration of the samples were derived. 50µg of

total protein from each sample were loaded after mixing with 2X laemmli buffer. Samples were denatured by incubating at 95°C for 5 mins, cooled and used for the SDS-PAGE procedure.

4.2.4.2 Gel electrophoresis, blotting and antibody-incubation

Stacking and resolving gels were prepared using SureCast™ Gel Handcast Bundle. Proteins were separated using 4% stacking gel and 10% resolving gel in 1X running buffer at 110V. The stacking and resolving gels were prepared using SureCast™ Handcast reagents as per manufacturer's instructions. Composition of buffers used for western blotting experiments are mentioned in the appendix. Proteins were transferred to PVDF membranes at 80V for 2.5 h at 4°C in 1X transfer buffer containing 20% fresh methanol. PVDF membranes were activated in methanol for 2-3 mins and soaked in 1X transfer buffer before use. Membranes were blocked in freshly prepared Tris Buffered Saline Tween-20 (TBST) containing 3% BSA for 1.5 h at RT at shaking conditions. Membranes were incubated in a mix of primary antibodies anti-ALDH1 (Protein band at 55kDa; 1:2000 dilution) and anti-GAPDH (Protein band at 37kDa; 1:30,000 dilution) overnight at 4°C at shaking conditions. Membranes were washed 3 times for 10 min each with TBST to remove unbound antibodies. Membranes were further incubated in secondary antibodies Goat anti-mouse IgG HRP (1:5000 dilution) and Goat anti-rabbit IgG HRP (1:5000 dilution) for 1.5 h at RT at shaking conditions and washed 3 times for 10 mins each with TBST to remove unbound antibodies. All antibodies were diluted in the blocking buffer.

4.2.4.3 Visualization and analysis

Membranes were soaked in ECL substrate, the chemiluminescence signals were captured in Chemidoc Touch™ (BioRad Laboratories, Inc, USA) and the images were procured.

Densitometric quantification of blot signals was performed using Image J software (NIH Bethesda, MD). Relative expression of ALDH1 in samples was estimated after normalization with GAPDH expression and presented in terms of fold changes.

4.2.5 ELISA

Cell culture supernatants were collected and stored at -80°C for the estimation of IL-6. Sandwich ELISA was performed using Human IL-6 Antibody Pair Kit as per the manufacturer's instructions. Known standards were prepared using the recombinant IL-6 provided in the kit within the range of 15 pg/mL to 1000 pg/mL. Absorbance was measured at

450nm (reference wavelength: 650nm) in Synergy HT microplate reader (BioTek Instruments, USA). Average absorbance from experimental wells was normalized with average absorbance from control wells. The standard curve was fitted by 4-parameter logistic (4PL) regression in GraphPad prism. The IL-6 concentrations in the samples were derived by extrapolation from the standard curve and expressed in pg/mL.

4.2.6 Statistical analysis

Experiments were performed ≥ 3 times independently. All data are expressed as mean values ± S.D. For graphical presentations of data sets and statistical analysis, GraphPad prism version 8.0 (GraphPad software Inc., California, USA) was used. Comparison between two data sets was evaluated by unpaired student's *t*-test. Comparison between multiple data sets was evaluated by analysis of variance (ANOVA). $p \leq 0.05$ was considered statistically significant.