2. REVIEW OF LITERATURE

2.1. Head and neck squamous cell carcinomas (HNSCCs)

2.1.1 General description

Head and neck cancers are a group of cancers anatomically located in the oral cavity, nasal cavity, paranasal sinus, oropharynx, nasopharynx, hypopharynx and larynx. A schematic illustration of regions of head and neck is presented in **Figure 2.1**. Cancers that originate in the squamous cells lining the mucosal surface of the head and neck regions are termed as head and neck squamous cell carcinomas (HNSCCs) and account for 90-95% of total head and neck cancers. The incidence rate is 600,000 cases per year with a 5-year patient survival rate of 40-50%. (Argiris *et al*., 2008, Leemans *et al*., 2011).

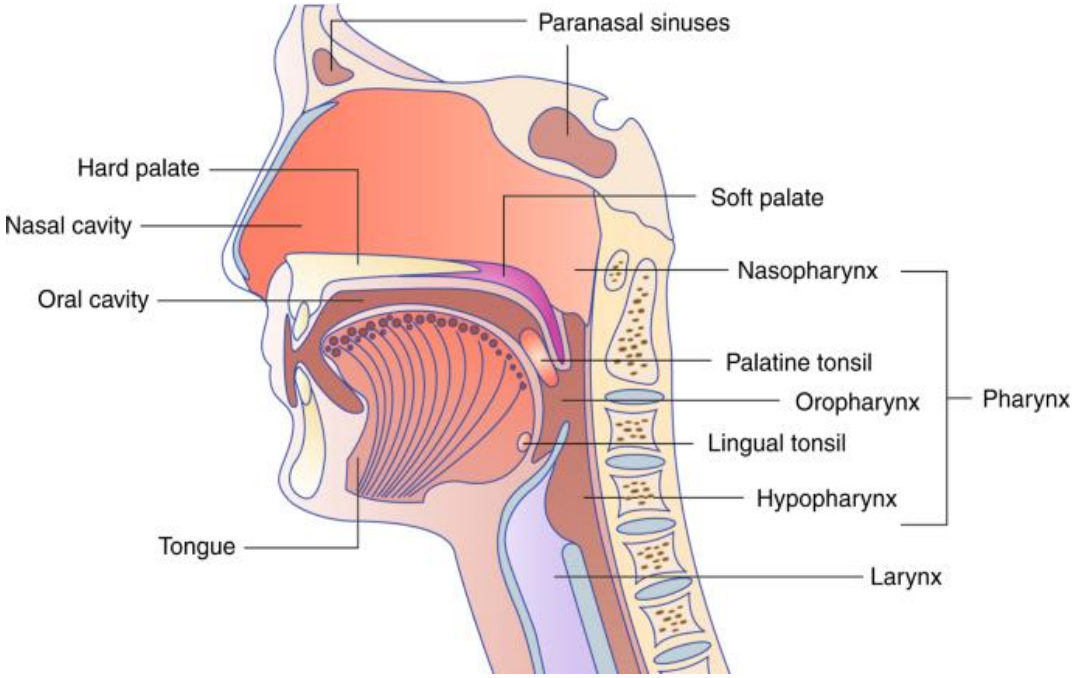

Figure 2.1: Regions of head and neck. (Source: Sabatini and Chiocca, 2020)

2.1.2 Epidemiology

Head and neck cancers are the seventh most common type of cancers worldwide. According to the Surveillance Epidemiology and End Results (SEER) data, the 5-year survival rate for head and neck cancers is 60%. According to the GLOBOCAN database, approximately 932,000 new cases and 467,000 deaths were registered worldwide in 2020 (Sung *et al.*, 2021). Head and neck cancers are highly frequent in Melanesia, South Central Asia (India, Pakistan, Bangladesh and Sri Lanka), Australia/New Zealand, Eastern and Western Europe. Prevalence is higher in men than in women (Argiris *et al.*, 2008). The Age-standardized incident rate (ASR) of head and neck cancers for USA is 8.2, with 58,864 new cases and 13,738 deaths in 2020. In India, they account for the second most common type of cancers and are a leading cause of cancer death in men with an ASR of 17.0 for both the sexes combined. Estimated new cases and deaths in India, in 2020, are 233,269 and 130,371 respectively (Sung *et al.*, 2021). A graphical representation for the number of cases in terms of incidence and mortality of various types of head and neck cancers in India is represented in **Figure 2.2**. India alone accounts for one-third of oral cancer burden in the world. In India, the ASR of head and neck cancers are highest in the North eastern regions mainly due to the intake of tobacco, pan masala and gutkha (Kulkarni 2013; Singh *et al.*, 2020).

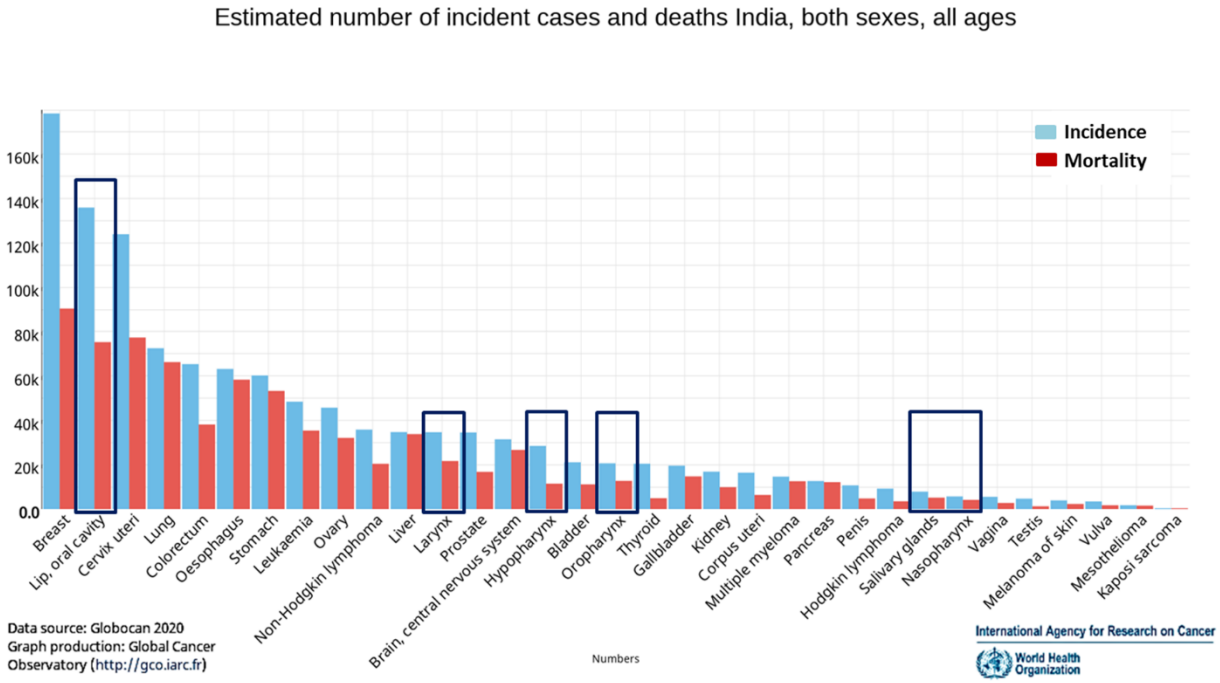

Figure 2.2: Statistics demonstrating incident cases and deaths of HNSCCs in India. Highest incidence rate of cancer occurs in the lip and oral cavity followed by larynx and hypopharynx. (Source: Global cancer observatory)

2.2 Classification of HNSCC

2.2.1 Classification based on anatomy

Based on the regions of development of tumor, HNSCC are classified into:

1. Oral cancer: tumors occurring in the lining of lips and cheeks, anterior tongue, roof and floor of the mouth (hard palate).
2. Sino-nasal cancer: tumors occurring in the nasal cavity and sinuses.
3. Oropharyngeal cancer: sites include the base of tongue, the mucosal membranes of tonsils, throat and soft palate.
4. Nasopharyngeal cancer: regions of the upper part of the throat, behind the nose and at the base of the skull.
5. Hypopharyngeal cancer: regions in the lower part of the throat
6. Laryngeal cancer: cancers in the regions of vocal cords, glottis, supra-glottis and sub-glottis.

(Cramer *et al.*, 2019; Jamal & Anjum, 2022)

2.2.2 Classification based on tumor staging

For the management of disease prognosis, HNSCCs are clinically categorized according to the TNM (tumor, node, metastasis) staging system. T describes the size and location of the primary tumor; N describes the extent of regional lymph node involvement and M describes the presence of metastasis in the distant regions. The TNM staging system allows to determine the spread of cancer in the head and neck regions and choose the type of therapy to be provided to the patient (Deschler & Day, 2008).

The TNM is determined after the pathological assessment of the excised tumor by the physician. Staging of the tumor sample is then allotted. The TNM classification and the staging classification for HNSCC according to the American Joint Committee on Cancer (AJCC) guidelines is presented in **Table 2.1** and **Table 2.2**. Grading of the tumor based on tissue differentiation as well into moderately or poorly differentiated tumors is also provided. This system determines the severity of the tumor. Stage I tumors have 90% survival rate, whereas stage II tumors have 70% survival rate. Well differentiated tumors resemble of a morphology similar to normal tissues whereas poorly differentiated tumors lose the normal tissue architecture (Lydiatt *et al.*,2017).

Table 2.1: TNM classification as per AJCC guidelines

Primary tumor (T)	
Tx	Primary tumor cannot be determined
T0	No evidence of primary tumor
Tis	Pre-cancerous stage/ in-situ carcinoma
T1	Tumor dimension is ≤ 2cm
T2	Tumor dimension is > 2cm but < 4cm
T3	Tumor dimension is > 4cm
T4a	Moderately advanced local tumor (invasion into face, skin, sinus, bone and muscle)
T4b	Very advanced local tumor (invasion into pterygoid plates, or skull base, masticator space and/or encases internal carotid artery
Regional lymph node (N)	
Nx	Involvement of regional lymph nodes cannot be determined
N1	Regional lymph nodes are not involved
N2a	Tumor metastasis in single ipsilateral lymph node, <3cm and >6cm
N2b	Tumor metastasis in multiple ipsilateral lymph node, >6cm
N2c	Tumor metastasis in bilateral/contralateral ipsilateral lymph nodes >6cm
N3	Tumor metastasis in lymph nodes, <6cm
Distant metastasis (M)	
Mx	Distant metastasis cannot be determined
M0	No distant metastasis
M1	Distant metastasis

Table 2.2: Classification for staging

	T1	T2	T3	T4a	T4b
N1	Stage I	Stage II	Stage III	Stage IV	Stage IV
N2	Stage III	Stage III	Stage III	Stage IV	Stage IV
N3	Stage IV	Stage IV	Stage IV	Stage IV	Stage IV
N4	Stage IV	Stage IV	Stage IV	Stage IV	Stage IV

2.3 Major risk factors

The major risk factors for the development of HNSCC are tobacco, alcohol consumption and infection by HPV virus. Genetic factors have also been studied vastly as risk factors of HNSCC.

2.3.1 Consumption of tobacco and alcohol

Tobacco and alcohol consumption are the dominant risk factors involved in 75% of HNSCC cases worldwide (Hashibe *et al.*, 2007).

Tobacco consumption- More than 70 carcinogens have been found in cigarette smoke. Tobacco contains genotoxic chemicals like tobacco specific nitrosamines (TSNA) and polycyclic aromatic hydrocarbons (PAH) which are regarded as carcinogenic. TSNA are formed during the processing of tobacco and are found in both combustible and smokeless forms of tobacco. TSNA such as are N'nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are potent and well-studied carcinogens (Jethwa & Khariwala, 2017).

Smokeless tobacco is also consumed along with betel quid (a mixture of betel leaf, areca nut-the carcinogenic source, and slaked lime) and is associated with a high-risk of oral cavity cancers in Asia-pacific population. Widespread use of smokeless tobacco is a main reason why India accounts for 74% of total HNSCC burden (Siddiqi *et al.*, 2015). Apart from this, India also accounts for 80% of the smokeless tobacco consumers and the consumption of smokeless tobacco has increased the risk of oral cancer by 1.8-5.8 fold in India (Jain *et al.*, 2021). A survey conducted among pan-cancer patients of rural Indian regions reported consumption of smokeless tobacco among 44% of the patients (230/517). Among these, smokeless tobacco was consumed by approximately two-third (70%) oral cancer patients (Pandey *et al.*, 2020). According to the Global Youth Tobacco Survey, tobacco consumption

is highly prevalent in Bihar and North Eastern states whereas its usage is low in Haryana, Delhi, Himachal Pradesh, Andhra Pradesh, Karnataka, Tamil Nadu, Goa and Punjab (Pandey *et al.*, 2020; Jain *et al.*, 2021).

Alcohol consumption- Alcohol acts as a solvent and increases the cellular uptake of the carcinogenic elements by exposing the mucosal surfaces to them. The acetaldehyde in alcohol interferes with DNA synthesis and repair mechanisms by forming adducts in the DNA (Feller *et al.*, 2013; Mizumoto *et al.*, 2017). Statistics related to alcohol associated HNSCC incidence is largely unreported, although alcohol consumption is reported to increase the risk of cancers of pharynx, oral cavity, oesophagus and larynx (Eashwar *et al.*, 2020). **Figure 2.3** illustrates the regions of India demonstrating HNSCC burden due to tobacco, alcohol consumption and other agents.

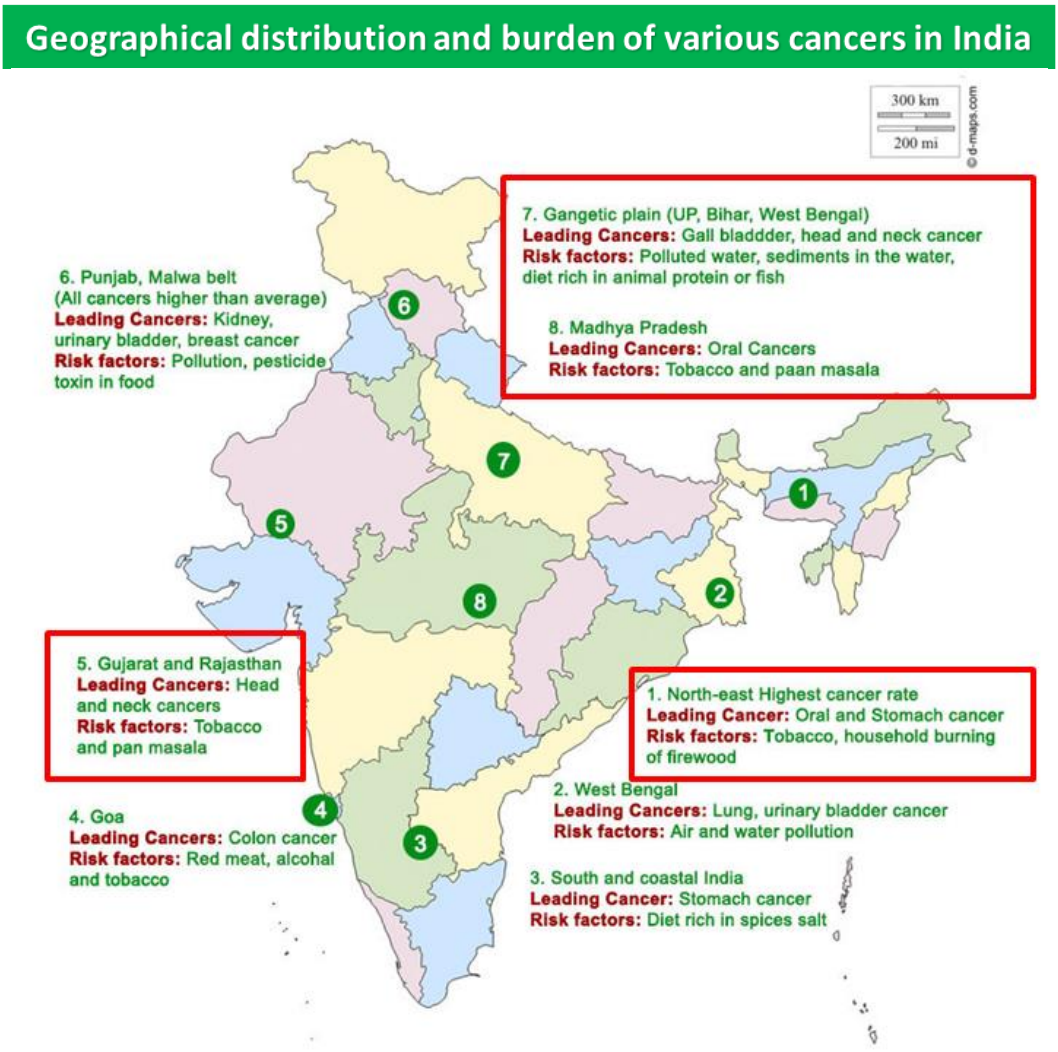

Figure 2.3: Region wise burden of various cancers and their major cause in India. Regions with high HNSCC prevalence and related with tobacco consumption as highlighted in boxes. (Source: cancerindia.org)

2.3.2 Infection by Human papillomavirus (HPV)

Human papillomavirus (HPV) is a double-stranded DNA virus, causing sexually transmitted viral infections. It mainly infects skin and mucous membranes. Infection by high-risk oncogenic types HPV 16 and HPV 18 are major causal factor of HNSCC. HPV 16 accounts for 90% of HPV DNA-positive HNSCCs. HPV E6 protein inactivates tumor suppresser protein p53 mediated apoptosis pathway. HPV E7 inactivates phosphorylated Rb (retinoblastoma) releasing E2F leading to cell cycle progression. The mode of action of E6 and E7 in the development of a malignant phenotype is shown in **Figure 2.4.** Techniques such as *P16* immunohistochemistry, FISH, genetic analysis of the gene from biopsy and histopathological samples have been used to detect HPV infection in HNSCC (Kobayashi *et al.*, 2018). HPV infection mediated HNSCC is frequently implicated in non-smokers, non-drinkers and immuno-suppressed individuals. Oropharyngeal regions such as the back of the throat, including the base of the tongue and tonsils, are the common sites for the development of HPV-associated HNSCC. HPV-associated HNSCC have a better prognosis and response to surgery and radiotherapy than tobacco and alcohol-associated HNSCC (Argiris *et al.*, 2008).

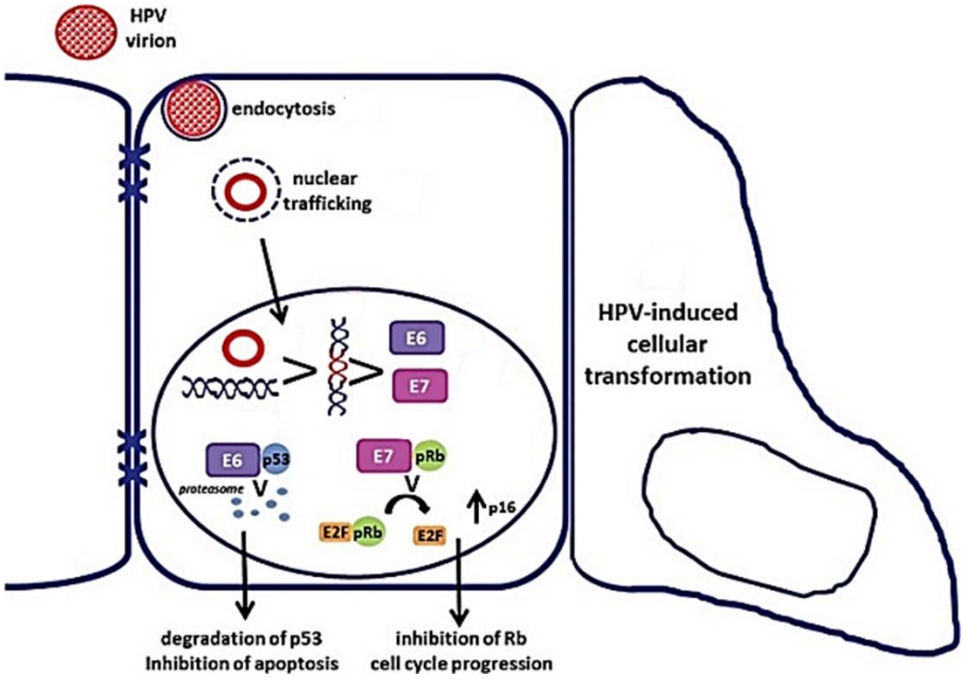

Figure 2.4: Mode of action of HPV E6 and E7 viral oncoproteins in malignant phenotype development. E6 targets tumor suppressor protein p53. E7 targets tumor suppressor protein pRb. (Source: Miller *et al.*, 2012)

In India, HPV infection is prevalent in 28.5% head and neck cancers. A majority of these are oral region cancer patients (Nandi *et al.*, 2021). In 2017, Yete *et al.*, reported the prevalence of HPV in 36.6% of oral squamous cell cancer (OSCC) patients. This statistic was more than the global average of 24.4%. In the same year, Murthy *et al.*, reported that HPV

prevalence was highest in south Indian OSCC patients (40%). Prevalence of HPV in laryngeal and hypopharyngeal origin cancers is comparatively low i.e., 5-20% (Nair *et al.*, 2018). In general, the statistics indicates a high rising prevalence of HPV positive HNSCC cases in India.

2.3.3 Genetic determinants

Genetic instability involving genetic and epigenetic alterations as well as dysregulation in cellular signaling pathways have been associated with the development of HNSCC.

TP53, CDKN2A, FAT1, NOTCH1, KMT2D, NSD1 and *TGFBR2* are among the most frequently mutated genes (Stransky *et al*., 2011). According to the TCGA cohort, the top 2 mutated or altered genes are *TP53* (72% mutations or 87% alterations) and *CDKN2A* (22% mutations or 58% alterations), observed mostly in HPV-negative tumors. These genes fall under the categories of cell proliferation genes and cell-cycle control genes, respectively. Apart from these two genes, few other mutated genes falling under the category of cell proliferation genes are *HRAS, EGFR,* and *PIK3CA,* and under the category of cell-cycle control gene is *CCND1*. *PIK3CA* mutations are observed in 14% of HNSCC, whereas *HRAS* mutations are infrequent, as observed in 4% of tumors only. Most mutations in genes *PIK3CA, PTEN* and *FBXW7* were observed in HPV-positive tumors than HPV-negative ones (Cho *et al.*, 2018, Jhonson *et al.*, 2020).

2.4 Pro-oncogenic factors for HNSCC

Various proteins play role in modulating stemness, enhancing survival and proliferation, triggering epithelial to mesenchymal transition (EMT) and metastasis in HNSCC. Due to their pro-oncogenic role and strong linkage with specific cancer promoting traits, they can also serve as biomarkers. Several biomarkers have been used for the purpose of diagnosis and studying prognosis of HNSCC. In this study, the below listed biomarkers were used as read-outs to assess HNSCC progression as well as to predict drug efficacy.

2.4.1 Survival and proliferation

Pro-survival proteins Bcl-2 (B-cell lymphoma-2) and Bcl-xL (B-cell lymphoma-extra-large) belong to the Bcl-2 family. These proteins regulate the other members of Bcl-2 family (Pro-apoptotic proteins Bax, Bak and Bok) that forms pores in the outer mitochondrial membrane, triggering membrane permeability and releasing cytochrome c. This leads to activation of caspases and initiates apoptosis (Dai *et al.*, 2022). They are largely over-expressed in almost all cancers and suppresses cell death induced by cytotoxic anticancer agents (Yip *et al.*, 2008). Bcl-2 proteins are over-expressed in HNSCC contributing to cancer development, progression

and chemotherapy resistance (Arumugam *et al.*, 2017; Li *et al.*, 2017). Bcl-xL is also found over-expressed in HNSCC contributing to development and pathogenesis of tongue carcinoma (Zhang *et al.*, 2014).

Ki-67 is a nuclear DNA binding protein widely used as proliferation marker for grading tumors (Sobecki *et al.*, 2017). Presence of Ki-67 has been reported in G1, S and G2 phases of the cell cycle, but not in the resting/G_0 phase (Urruticoechea *et al.*, 2005). Ki-67 binds to the peri-chromosomal layer during mitosis, maintains the mitotic spindle and hinders collapsing of chromosomes into a single chromatin mass after the disassembly of nuclear envelop. This allows the smooth motility of independent chromosomes and their efficient interactions with the mitotic spindle (Zhang *et al.*, 2021). High Ki-67 expression has been observed in HNSCC and is associated with poor prognosis of patients (Meyer *et al.*, 2019; Ahmed *et al.*, 2016).

2.4.2 Stemness

Stemness is defined by the molecular process regulating stem cell properties like self-renewal and differentiation into daughter cells. Adult stem cells are resistant to DNA-damage induced cell death, which may help in protecting against apoptosis and increasing the survival of tissues (Liu *et al.*, 2014; Abdelwahid *et al*., 2016). Dysregulation in these processes may initiate and promote cell transformation. A small subset of cells in the tumor micro-environment with stem-cell like phenotype, regarded as cancer stem cells (CSCs), promote tumor growth, mediate drug-resistance and are responsible for recurrence after treatment (Mushtaq *et al.*, 2020; Zhao *et al.*, 2017; Phi *et al.*, 2018). These CSCs were quantitated using several markers including CD44, Nanog, ALDH1 and others:

(i) CD44 is a type 1 transmembrane protein, generally expressed in adult hematopoietic stem cells and fetal cells, that binds to hyaluronic acid (HA) in the extracellular matrix. HA binding to CD44 can activate the cytoskeleton and signaling pathways that regulates cell division, migration and adhesion (Senbanjo & Chellaiah, 2017, Zhao *et al.*, 2017, Chen *et al.*, 2018). CD44 expression was observed greater in HNSCC compared to normal tissues. Approximately, 80-97% CD44+ cells were observed in HNSCC cell lines (Ludwig *et al.*, 2019). Expression of CD44 is more in high grade tumors compared to primary HNSCC tissues. Its expression is also reported to stimulate HNSCC tumor growth (Chukka *et al.*, 2021).

(ii) Nanog is a homeobox protein and a transcription factor that regulates pluripotency and self-renewal of cells (Zhang *et al.*, 2016). Nanog with transcription factors OCT-4 and SOX-2 maintains the embryonic stem cells in an undifferentiated state. It is overexpressed in CSCs

repressing apoptosis and inducing metastasis, self-renewal, tumorigenesis, tumor relapse, and drug-resistance. (Gawlik-Rzemieniewska & Bednarek, 2016; Najafzadeh *et al.*, 2021). Nanog is over-expressed in HNSCC. Study from Vicente *et al.*, observed its overexpression in 72% HNSCC cases and even more frequent in patients with lymph node metastasis (de Vicente *et al.*, 2019). Its expression also correlates with poor prognosis, poor differentiation and chemoresistance in HNSCC (Mishra *et al.*, 2020).

(iii) Aldehyde dehydrogenase 1 (ALDH1) is a cytosolic enzyme which detoxifies endogenous and exogenous aldehydes (Tomita *et al.*, 2016). It is functionally active in the mitochondria and cytoplasm. Its expression is high in normal hematopoietic progenitor cells (Marchitti *et al.*, 2008). ALDH1 expression also mediates drug-resistance and metastasis in tumors (Wei *et al.*, 2022). It is a widely recognized CSCs marker in various cancers including HNSCC (Dong *et al.*, 2017). Its expression is high in primary HNSCC tumors and is associated with poor tissue differentiation and poor prognosis of patients (Zhou & Sun, 2014). In HNSCC, ALDH1+ CSCs exhibit tumorigenic capacity and correlates with EMT (Götz *et al.*, 2018). It further holds a prognostic value for locally advanced and metastatic HNSCC patients (Qian *et al.*, 2014).

2.4.3 EMT

E-cadherin and vimentin are potential indicators of EMT, found colocalized in the same tumor cells (Yamashita *et al.*, 2018).

E-cadherin is a cell-cell adhesion molecule mediating adhesion between cells and tissue. It regulates cytoskeleton and controls cell polarity. It also affects activation of cellular signaling pathways, and is also regarded as a tumor suppressor gene (Tunggal *et al.*, 2005; Theodoraki *et al.*, 2018). Vimentin is an intermediate filament in cells. It maintains cellular integrity and stabilizes interactions of cytoskeleton. It also provides resistance against stress (Satelli & Li, 2011).

Loss of E-cadherin and gain of vimentin is associated with epithelial-to-mesenchymal transition, essential for invasion and migration of tumors (Yeung *et al.*, 2017). In a study from Fan *et al.*, E-cadherin was found downregulated in 63% of HNSCC patients (Fan *et al.*, 2013). Further low expression correlated with metastasis, poor tissue differentiation, tumor recurrence and poor overall survival (López-Verdín *et al.*, 2019). Its expression also correlates negatively with tumor size and lymph node metastasis status (Mehendiratta *et al.*, 2014). Liu *et al* have shown that Vimentin was found over-expressed in 53% of oral cancer tissues that gradually developed tumor recurrence (Liu *et al.*, 2010). Simultaneous decreased expression of E-

cadherin and increased expression of vimentin has been observed in recurrent HNSCC and is regarded as a useful prognostic marker of the cancer (Liu *et al.,* 2010; Nijkamp *et al.,* 2011).

2.4.4 Metastasis

Matrix metalloprotein-2 (MMP-2) also known as Gelatinase-A degrades extracellular matrix components to initiate cellular invasion. It is ubiquitously expressed in many cells and is associated with inflammation, angiogenesis and tissue repair. MMP-2 activity in tumors is associated with metastasis and invasion of cancer cells (Turpeenniemi-Hujanen 2005; Ma *et al.*, 2015; Cui *et al.*, 2017). In a study by Liu *et al.*, Higher expression of MMP-2 is observed in more than 50% of HNSCC patients especially those who develop local recurrence or metastasis (Liu *et al.*, 2005). It was also significantly higher in patients with lymph node metastasis than without lymph node metastasis and its expression correlates with poor survival of HNSCC patients. It is widely identified as a diagnostic biomarker for the cancer (Aparna *et al.*, 2015; Rosenthal *et al.*, 2006; Ruokolainen *et al.*, 2006).

IL-6 is a pro-inflammatory cytokine secreted by cells reportedly involved in immune responses, inflammation and haematopoiesis. It signals through JAK/STAT and MAPK pathways (Hirano, 2021). IL-6 levels were significantly high in HNSCC patients compared to healthy individuals and predicted tumor recurrence, metastasis and poor survival of the cancer (Choudhary *et al.*, 2016). It also promotes metastasis of HNSCC by inducing EMT via activating the JAK-STAT3-SNAIL signaling pathway (Yadav *et al.*, 2011). It is an established and widely used diagnostic biomarker for metastatic HNSCC (Choudhary *et al.*, 2016).

Bone marrow stromal cell antigen 2 (BST-2) also called tetherin/CD317 is an anti-viral protein situated in cell surface or vesicular compartments (Mahauad-Fernandez *et al.*, 2015). It tethers viruses like HIV, herpesvirus and retrovirus (Van *et al*., 2008; Douglas *et al.*, 2010; Neil *et al.*, 2008). BST-2 crosslinking leads to the activation of NF-κB and production of cytokines such as CXCL10 and IL-6 (Mahauad-Fernandez *et al.*, 2015). It has been found over-expressed in various cancers like breast, lung, HNSCC and glioblastoma (Cai *et al.*, 2009; Wang *et al.*, 2009; Yang *et al.*, 2018; Wainwright *et al.*, 2011). It has been associated with invasion, migration, progression and chemoresistance in breast cancer (Mahauad-Fernandez *et al.*, 2018; Yi *et al.*, 2013). In HNSCC, it confers resistance to chemotherapies like cisplatin and gefitinib (Kuang *et al.*, 2017; Jin *et al.*, 2019). Overall, it is associated with poor survival in patients of HNSCC and breast cancer.

2.5 Treatment

Treatment modalities for HNSCCs are surgery, radiotherapy and chemotherapy. Early stage (stage I/II) cancer is usually treated by surgery or radiotherapy alone. For locally advanced (stage III/IV) resectable or unresectable cancer, chemotherapy or a multidisciplinary model treatment approach including combination of chemotherapy with surgery or radiotherapy is the preferred regimen (Marur & Forastiere, 2016).

2.5.1 Surgery

Surgery is used for the treatment of small and transorally accessible cancers of oral cavity, pharynx and larynx. Advances in surgical techniques, over the years, include robotic techniques or laser micro-surgery and has become cost saving and technically feasible than radiotherapy (Werner *et al.*, 2002; Hans S *et al.*, 2012). Laser treatment has many advantages like lower chances of infection, reduced bleeding, swelling and healing time. Transoral laser micro-resection is performed using an endoscopic approach. The endoscope is used to visualize the tumor and the lasers are used to resect it (Rubinstein & Armstrong, 2010). Transoral robotic techniques uses an endoscope to visualize the three-dimensional optics of the mucosal surfaces of head and neck regions and a da-Vinci surgical system to assist the surgeon for performing the surgery (Loevner *et al.*, 2013). Surgery is generally the standard approach for the treatment of HNSCC but has limited use in many cases due to the less efficient anatomical accessibility of the tumors and low rates in achievement of functional preservation of the organs involved.

2.5.2 Radiotherapy

Radiotherapy is used as a primary treatment for early stage HNSCC and as a post-operative treatment for most of the locally advanced HNSCC. It is also combined with chemotherapy for the treatment of locally advanced unresectable HNSCC to increase tumor control and to preserve organ integrity (Shyh-An Yeh, 2010). The standard radiotherapy methods are intensity modulated radiotherapy (IMRT) and volumetric modulated arc therapy (VMAT). IMRT uses photon and proton beams to deliver precise radiation doses to specified tumor areas. VMAT is an advanced form of IMRT in which the machine rotates around the patient while the treatment is ongoing. The advantage of VMAT is improved delivery efficiency and treatment time. By these techniques, dosage of radiation and risk of toxicity to organs is minimized (Teoh *et al.*, 2011; Figen *et al.*, 2020). The radiation dose is determined by the size and location of the tumor. Hyper-fractionated radiations, with ≥2Gy daily, are scheduled to employ 50Gy for the treatment of low-risk nodal tumors and 60-66Gy in post-operative radiotherapy. However,

doses exceeding 55Gy leads to the increase in the risk of long-term toxicity to salivary glands, pharyngeal muscles and thyroid glands (Shyh-An Yeh, 2010). Despite various advances in radiotherapy strategies, the five-year survival rates of patients treated with radiation ranges from 40-60%. A local relapse of tumor is observed in 25% of the patients treated with radiotherapy (Hutchinson *et al.*, 2020).

2.5.3 Chemotherapy

Chemotherapy plays a central role in the treatment of locally advanced HNSCC, and is well investigated in both induction and adjuvant settings. Various taxanes, platinum compounds and anti-metabolites have demonstrated activity against HNSCC. Commonly used agents are paclitaxel, docetaxel, cisplatin, carboplatin, 5-Florouracil (5-FU) and methotrexate (Cognetti *et al.*, 2008; Burchhardt & Sukari, 2016). Cisplatin **(Figure 2.5)** cross links with the purines in the DNA, interfering with the DNA repair mechanisms, subsequently causing apoptosis. (Dasari & Tchounwou, 2014). It is the standard treatment regimen for recurrent and metastatic HNSCC, used in combination with other agents and radiotherapy (Helfenstein *et al*., 2019). 5-FU (**Figure 2.6)** inhibits thymidylates synthesis and incorporates in the DNA, causing cell death (Ghafouri-fard *et al.*, 2021). 5-FU in combination with cisplatin (also known PF, Platinol-fluorouracil) as induction therapy have shown tumor shrinkage, decreased nodal burden and better response than radiotherapy. This was evident from an improved 5-year survival and larynx preservation, however decrease in malignancy was insignificant and patients developed mucositis, thrombocytopenia, nausea, vomiting, stomatitis, and hearing loss (Haddad *et al.*, 2018). Docetaxel (**Figure 2.7**) inhibits the depolymerization of microtubules in cells (Mollinedo *et al.*, 2003). Induction chemotherapy combining docetaxel with cisplatin and 5-FU (also known as TPF, taxotere-platinol-fluorouracil or DCF, docetaxel-cisplatin-fluorouracil) have shown improved progression free survival, overall survival, better larynx preservation and reduced drug-mediated toxicity than PF and single agents. This was confirmed by the two clinical trials: TAX323 and TAX324 that made TPF a standard of care treatment for HNSCC (Kim *et al.*, 2016, Zhong *et al.* 2013; Haddad *et al.*, 2018; Karabajakian *et al.*, 2018). TPF is the FDA approved treatment for gastric cancer. In 2006, FDA approved TPF for the treatment of HNSCC (Vermorken *et al*. 2007, Lorch *et al.* 2011).

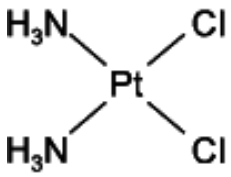

Figure 2.5: **Chemical structure of cisplatin** (Source: Han Sei Jun *et al.*, 2009)

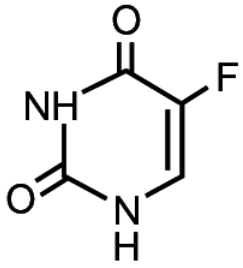

Figure 2.6: **Chemical structure of 5-FU** (Source: da Costa & Moraes, 2003)

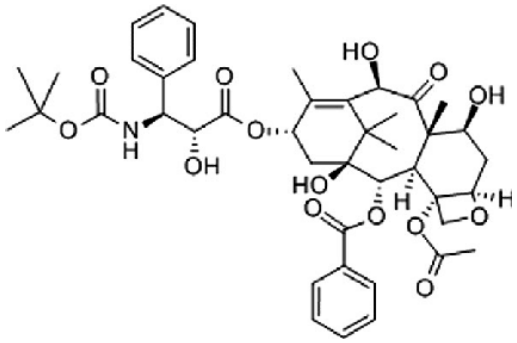

Figure 2.7: **Chemical structure of docetaxel** (Source.: Siddiqui *et al.*, 2013)

A major proportion of stage III and IV HNSCC patients require treatment with chemotherapy or chemo-radiotherapy. However, resistance to chemo-therapy is still an obstacle in the treatment with 5-year survival rate of patients remaining not more than 50%. Response to TPF treatment in HNSCC patients is around 50-60%. Patients gradually show high toxicity and side effects such as febrile neutropenia, stomatitis and diarrhea. This remains a critical issue as it is widely observed in advanced stage and elderly patients. (Ilie *et al*., 2012; Guigay *et al*., 2019). Resistance to chemo-therapy not only limits the effectiveness of the treatment but also leads to tumor relapse, mortality and poor prognosis (Mansoori *et al.*, 2017; Wang *et al.*, 2019). HNSCC cells exposed to cisplatin, docetaxel and 5-FU generally acquire resistance through mechanisms such as DNA/RNA damage repair, inhibition of apoptosis, drug efflux and activation of EGFR pathway. Nucleotide excision repair (NER) based DNA repair is the primary mechanism through which the cisplatin-induced DNA damage is repaired to acquire resistance to cisplatin (Kanno *et al.*, 2021). Resistance to 5-FU and docetaxel is commonly mediated through drug efflux and apoptosis inhibition strategy in HNSCC cells

(Nagata *et al.*, 2011; Kanno *et al.*, 2021). Multidrug cross-resistance mechanism observed in HNSCC cells resistant to a combination of docetaxel, cisplatin and 5-FU can suggest that cells undergo DNA damage repair, apoptosis inhibition and drug efflux altogether. This is reflected in higher resistance to chemo drugs and increased expression of molecules associated with drug efflux and apoptosis inhibition in triple drug resistant HNSCC cells (Govindan *et al.*, 2015; Kanno *et al.*, 2021).

2.5.4 Targeted therapy and immunotherapy

(i) Anti-EGFR therapy- The Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that signals through PI3K/AKT/mTOR, STAT and RAS/RAF/ERK pathways (**Figure 2.8**). These pathways regulate angiogenesis, cell proliferation, invasion and migration (Huang & Fu, 2015). EGFR was found overexpressed in 90% of HNSCC tumors (Kalyankrishna *et al.*, 2006). It is an also extensively studied biomarker in HNSCC. EGFR overexpression in HNSCC is also associated with poor overall survival of patients (Martinez-Useros *et al.*, 2015; Qin *et al.*, 2021; Fasano *et al.*, 2021). In 2006, FDA approved the use of cetuximab (EGFR antibody) for the treatment of HNSCC (Cassell *et al.*, 2010). Cetuximab with cisplatin and 5-FU as combination therapy was approved by FDA for recurrent/metastatic (R/M) HNSCC in 2011 (Cohen *et al*., 2013). It is used as a standard of care first-line of therapy for patients with R/M HNSCC, as per the guidelines of the Chinese Society of Clinical Oncology (CSCO) and National Comprehensive Cancer Network (NCCN) but is used limitedly due to high price (Lang & Dong, 2020).

Based on different anatomical locations, HNSCCs are categorized as both “hot” and “cold” tumors. This suggests a high heterogeneity in the immune landscape of HNSCC. HNSCCs generally have high tumor-infiltrating cells (TILs) in different locations. Such HNSCC subtypes are regarded as “hot” tumors and are associated with improved prognosis as these become better targets for immunotherapy treatments. The immune infiltrates are low in tumors caused by smoking, which are generally regarded as “cold” tumors (Duijvenvoorde *et al.*, 2022). Targeted therapy against molecules overexpressed in HNSCC and immune checkpoint inhibitors have offered new methods for HNSCC treatment.

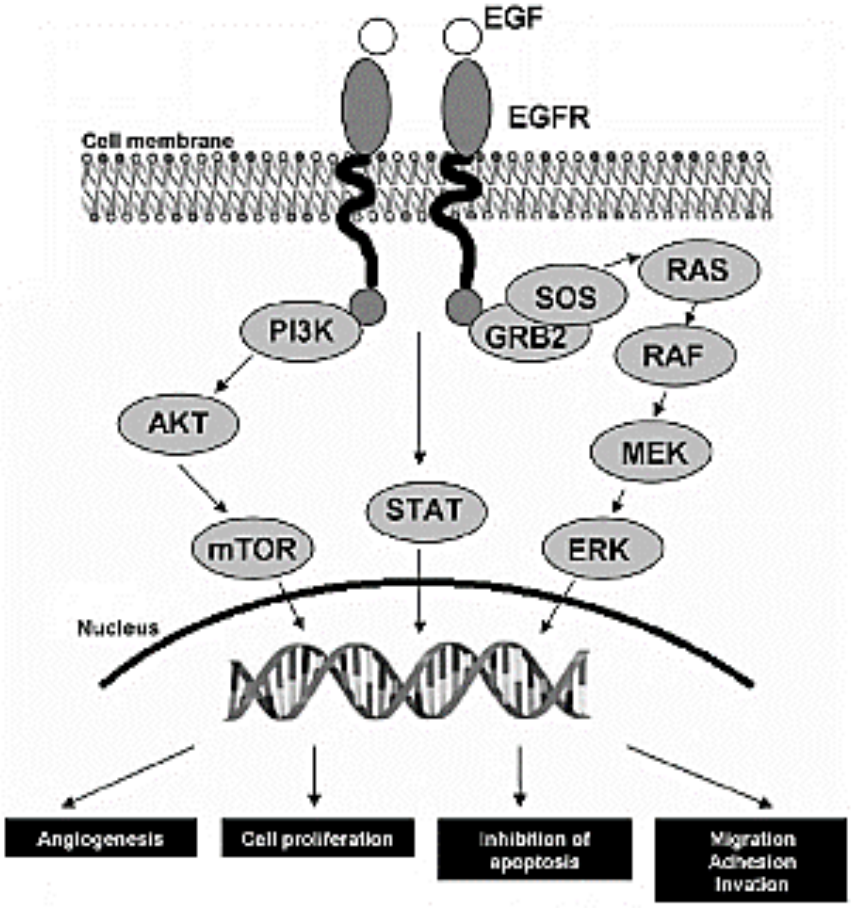

Figure 2.8: EGFR signaling pathway. Activation of EGFR initiates downstream signaling which induces angiogenesis, cell proliferation, migration and invasion in cells (Source: Martinez-Useros *et al.*, 2015)

(ii) Immune check point inhibitors (ICIs) - The immune checkpoint pathways in a tumor microenvironment regulates immune activation and T-cell responses. Commonly targeted checkpoints in HNSCC are programmed death ligand-1 (PD-L1) on cancer cells, programmed cell death protein-1 (PD-1) and cytotoxic T-lymphocyte associated protein-4 (CTLA-4) present on T-cells **(Figure 2.9)**. PD-L1 binding to PD-1 causes inactivation of the T-cell. Similarly, CTLA-4 on T-cells binds with CD80/CD86 that inactivates the T-cells. Check point inhibitors, monoclonal antibodies binding to the immune checkpoints that inhibits binding of T-cells with cancer cells/antigen presenting cells (APCs) are used as immunotherapy for the treatment of cancer. This association restores the normal functioning of the T-cells i.e., mediating of T-cell responses against tumor antigens (Neal *et al.*, 2019; Mei *et al.*, 2020; Poulose *et al.*, 2022). Expression of CTLA-4 was found higher in HNSCC tissues compared to normal adjacent tissues (Yu *et al.*, 2016). PD-L1 expression was found in 60% of HNSCC tumors, especially in HPV+ HNSCC samples. Higher expression of PD-1 in the infiltrating T-cells and peripheral Tregs in HNSCC tumors was found mediating immunosuppression (Veigas *et al.*, 2021).

In 2019, for the treatment of recurrent and metastatic HNSCC, FDA approved the use of two PD-1 antibodies, nivolumab and pembrolizumab. Nivolumab was approved as a second line monotherapy for patients exposed to cisplatin treatment. Pembrolizumab was approved as a first-line monotherapy, for PD-L1 positive tumors and in combination with cisplatin and 5-FU in PD-L1 high and low expressing HNSCC (Cramer *et al.*, 2019; Cohen *et al.*, 2019).

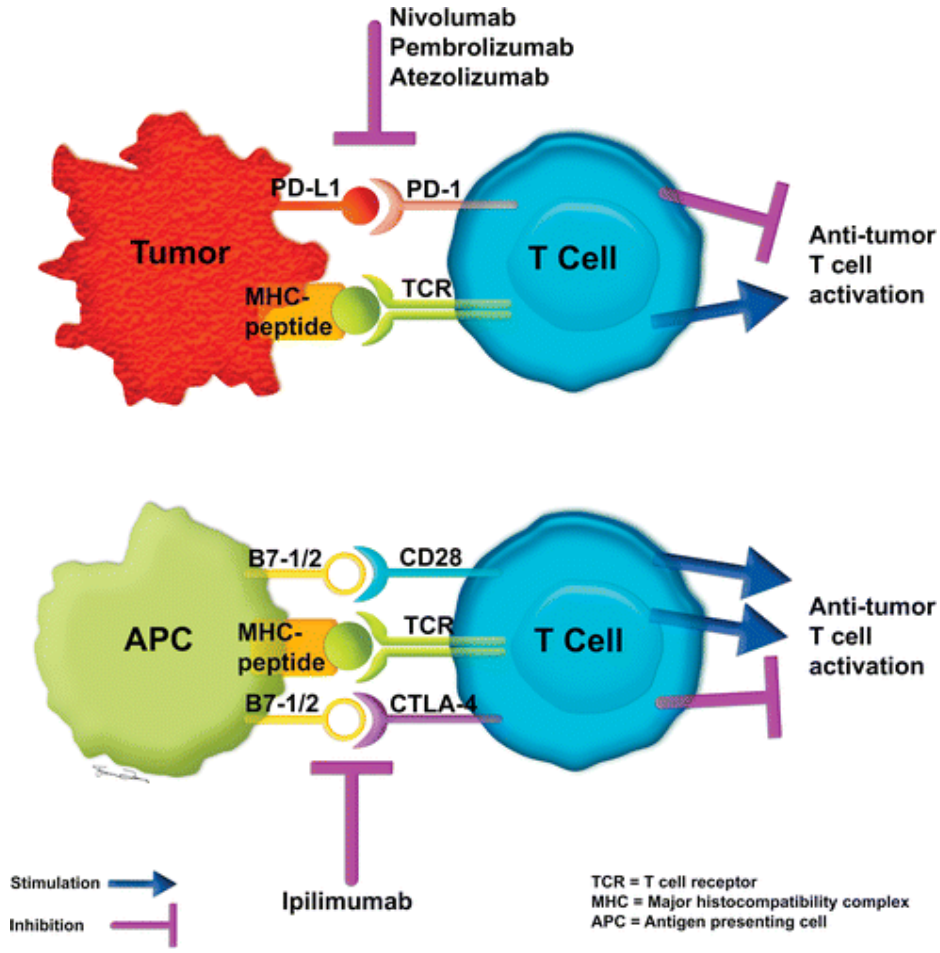

Figure 2.9: Immune checkpoint pathways and mechanism of action of checkpoint inhibitors (Source: Wang *et al.*, 2017)

2.6 Toll-Like Receptors (TLRs)

Mammalian PRRs are classified majorly into the following five types: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic-acid inducible gene-I (RIG-I) like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma (AIM)-like receptors (ALRs) (Li & Wu, 2021). TLRs were the first studied and best characterized PRRs with an extensive spectrum of pathogen recognition (Akira & Kawai, 2011). The TLR-1 in mammals was first identified in 1994 and the role of TLRs in the generation of immune response was established in 1998 (Beutler 2009). TLRs are employed by the innate immune system for the detection of conserved molecular signatures from the

surface of pathogens and damaged cells. They recognize microbe specific structures known as pathogen-associated molecular patterns (PAMPs) and molecules released from damaged cells known as damage-associated molecular patterns (DAMPs). The recognition and binding of ligands to TLRs leads to the recruitment of adaptor molecules and initiation of the downstream signaling pathways. The downstream signaling in the immune cells activates the transcription factors and leads to the generation of immuno-protective effects, such as anti-infection and anti-tumor effects (Lee & Kim, 2007; Kawasaki & Kawai, 2014).

2.6.1 Expression of TLRs

Conventionally, TLRs are expressed on immune cells, particularly APCs such as monocytes, macrophages and dendritic cells. They are also observed on neutrophils, mast cells and Natural killer (NK) cells. TLR expression has also been observed on B cells and T cells, and play a role in the functional state of T cell subtypes **(Figure 2.10**). Apart from their expression on immune cells, they are also found expressed on non-immune cells such as fibroblasts and epithelial cells (Vijay 2018; Sun *et al.*, 2019, Nouri *et al.*, 2021).

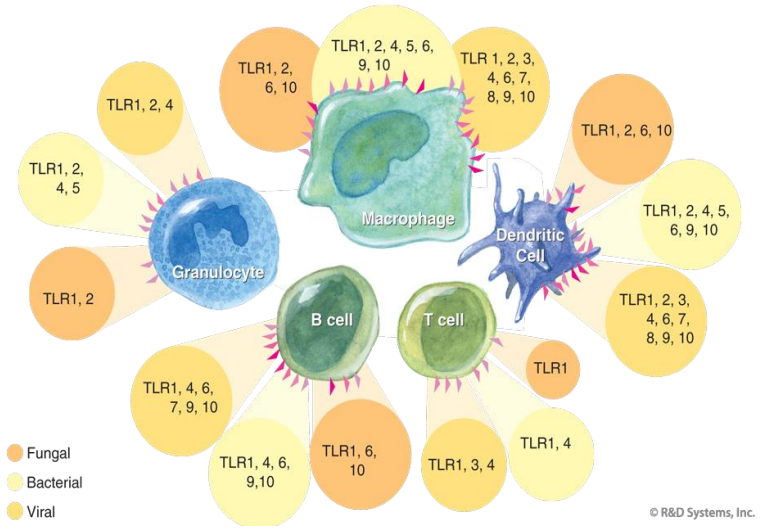

Figure 2.10: TLRs expression on different types of immune cells. TLRs on different immune cells and the type of ligands they recognize (Source: R & D systems, Inc).

2.6.2 Structure of TLRs

TLRs are type 1 membrane glycoproteins consisting of an ecto-domain of 20-27 leucine-rich repeats (LRR) responsible for the detection of ligands, a transmembrane domain, and an endo-domain known as Toll/IL-1R (TIR) domain for initiating downstream signaling (**Figure 2.11**). A total of 13 TLRs are detected in human and mice combined, TLR 1-10 in humans and TLR 1-9,11-13 in mice. TLR 10 is non-functional in mice (EL-Zayat *et al.*, 2019). TLR 11

sequences are present in human genome however, it exists as a pseudogene due to the presence of various stop codons in the open reading frame (ORF), hence cannot code for a functional protein (Gonzalez *et al.*, 2014).

Ligand binding induces homodimerization or heterodimerization of TLRs. All TLRs are capable of existing as homodimers and heterodimers (such as TLR1/2 or TLR2/6) (Ghosh *et al.*, 2020) except TLR 3 and 5 which exists only in homodimer forms (Anwar *et al.*, 2019). Depending on the cellular localization and type of ligands sensed, TLRs are divided into two categories: Cell surface TLRs comprising of TLRs 1, 2, 4, 5, 6 and 10, and endosomal surface TLRs comprising of TLRs 3, 7, 8, and 9.

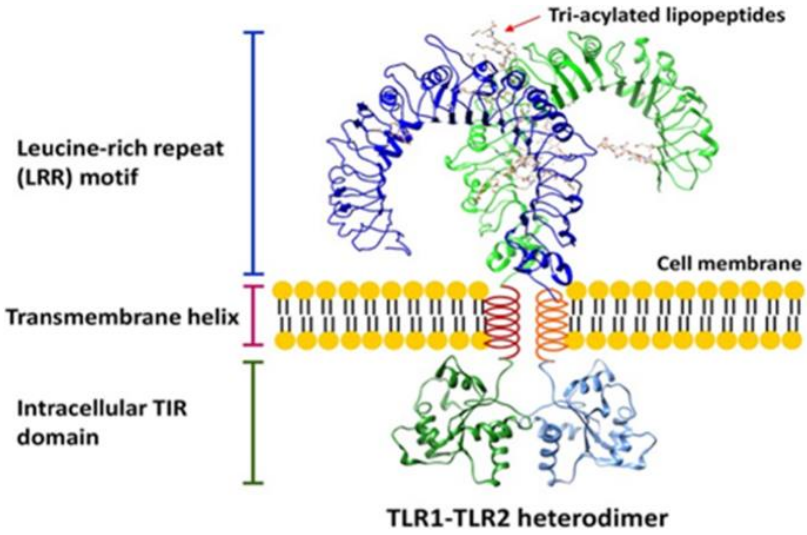

Figure 2.11: Structure of a TLR. (Source: Gao *et al.*, 2017)

2.6.3 TLR Ligands

The TLRs recognizes ligands that are classified into two categories: Pathogen associated molecular patterns (PAMPs) and Damage associated molecular patterns (DAMPs). PAMPS or exogenous ligands are non-self, highly expressed molecular motifs which are parts of microbes, such as Lipopolysaccharide (LPS), a component of gram-negative bacterial cell membrane. The TLRs are capable of recognizing these motifs, which are not present in humans (Kumar *et al.*, 2011; Sun *et al.*, 2021).

DAMPs or endogenous ligands are intracellular molecules of cells that have physiological functions such as regulating the transcription, controlling differentiation and proliferation of cells. These molecules are released upon stress/cell death. Upon binding to their respective TLRs, they mediate host defense by stimulating downstream signaling. DAMPs such as S100, HMGB1, histones and mitochondrial DNA also participate in recruitment and activation of immune cells such as macrophages and neutrophils. DAMPS are

also often regarded as alarmins acting as warning signals for the immune system (Yu *et al.*, 2010; Sun *et al.*, 2021). A list of TLRs with corresponding PAMPs and DAMPs is presented in **Table 2.3**.

Table 2.3: TLRs and associated PAMPs and DAMPs

TLRs	PAMP ligands	DAMP ligands
TLR 1	Tri-acylated lipopeptides, soluble factors	β – defensin-3
TLR 2	Lipopeptides, Peptidoglycan, Lipoteichoic acid, Glycolipids, Porins, Zymosan, Lipopolysaccharides	HSP60, HSP70, HMGB1, Hyaluronic acid, S100 proteins, Histones, β – defensin-3
TLR 3	dsRNA	dsRNA, mRNA
TLR 4	Lipopolysaccharides, Fusion protein, Envelope protein, Taxol	HSP22, HSP60, HSP70, HSP96, HMGB1, Fibronectin, Hyaluronic acid, Heparan sulfate, Fibrinogen, Syndicans, Glypicans, S100 proteins, β – defensin-2
TLR 5	Flagellin	Unknown
TLR 6	Di-acylated lipopeptides, Lipoteichoic acid, Zymosan, Heat labile soluble factors	Unknown
TLR 7	ssRNA	Endogenous RNA
TLR 8	ssRNA	Endogenous RNA
TLR 9	Unmethylated CpG DNA	HMGB1, Nuclear and mitochondrial DNA, IgG-chromatin complexes
TLR 10	Unknown	Unknown

(Correa *et al.*, 2014 Schaefer *et al.*, 2014, Piccinini & Midwood, 2010)

2.6.4 TLR signaling pathways

Ligand sensing leads to homo/hetero-dimerization of TLRs and initiation of downstream signaling via the Toll-Interleukin-1R (TIR) domain. The activation from TIR domain induces signaling from two downstream pathways: the MyD88 dependent and MyD88-independent pathway (**Figure 2.12**). All TLRs, except TLR 3 signal through the MyD88-dependent pathway. The MyD88-independent pathway is utilized by TLR 3 completely, and TLR 4 which uses both of the pathways for signaling (Takeda & Akira, 2004).

MyD88-dependent pathway- Upon activation of the TLR signaling pathway, the TIR domain interacts with the adaptor myeloid differentiation primary response 88 protein (MyD88). The MyD88 protein interacts with the family of Interleukin-1 receptor associated kinases (IRAKs) to form a myddosome complex which induces the activation of active kinases IRAK-4 and IRAK-1. IRAK-1 further activates downstream molecule tumor necrosis factor receptor-associated factor (TRAF-6), a RING-domain E3 ubiquitin ligase. TRAF-6 leads to K-63 linked ubiquitination of itself and transforming growth factor-beta activated kinase (TAK1), a member of mitogen-activated protein kinase (MAPK) family. TAK1 also interacts with TAK1 binding protein (TAB) family of proteins-TAB1, TAB2 and TAB3. TAK1 further activates MAPK-pathway and inhibitor of kappaB kinase (IKK) complex-nuclear factor-κB (NF-κB) pathway. TAK1 complexes with IKK complex, phosphorylating IKKβ, which activates it. The IKK complex phosphorylates IKβa, an NF-κB inhibitory protein, leading to its proteasomal degradation. This allows the translocation of NF-κB into the nucleus and induction of pro-inflammatory gene expression (Kawasaki & Kawai, 2014; Kawai & Akira, 2006).

MyD88-independent pathway- The MyD88-independent pathway or the TRIF-dependent pathway, utilized by TLR 3 and TLR 4, leads to the production of Interferon-β and is essential for inducing an anti-viral state in the cells (Yamamoto *et al.*, 2003). The cytosolic adaptor molecule TIR-domain-containing adaptor-inducing interferon-β (TRIF) is recruited to the TIR domain upon induction of TLRs. TRIF further recruits TRAF-3 and TRAF-6 (Gohda *et al.*, 2004). TLR 4 also utilizes an additional adaptor protein TRIF-related adaptor molecule (TRAM) to induce signaling to TRIF (Yamamoto *et al.*, 2003). TRAF-3 recruits TANK-binding kinase 1 (TBK1) and IKKε for phosphorylation of IRF3. IRF3 dimerizes and translocates to the nucleus to induce type 1 IFN gene expression (Kawasaki & Kawai, 2014).

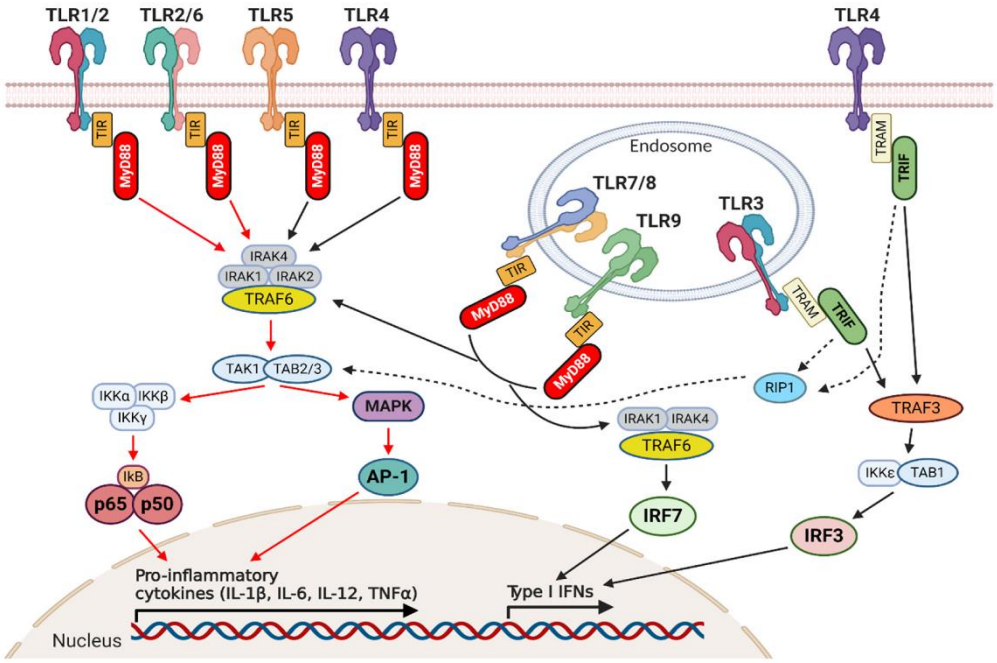

Figure 2.12: TLR signaling pathways. TLRs signal through the MyD88-dependent and MyD88-independent pathways. TLR 3 and TLR 4 utilizes the MyD88-independent pathway. (Source: Lorenzo *et al.*, 2020)

2.7 Interleukin-1 receptor associated kinases (IRAKs)

2.7.1 Members and structure of IRAK proteins

Interleukin-1 receptor associated kinases (IRAKs) are downstream kinases in the MyD88-dependent signaling pathway and the key signal transducers in the TLR and IL-1R signaling pathway. The family of IRAKs comprises of 4 members: IRAK-1, IRAK-2, IRAK-3/M and IRAK-4. IRAK-1, IRAK-2 and IRAK-4 are expressed in all immune cells, whereas IRAK-M is expressed in monocytes and macrophages only (Janssen & Beyaert, 2003; Flannery & Bowie, 2010).

The structure of an IRAK protein comprises of an N-terminal death domain, a Proline-Serine-Threonine (ProST) domain, a kinase domain and a C-terminal domain (absent in IRAK-4). The N-terminal domain is essential for binding with the MyD88 protein and dimerization with other IRAK proteins. The ProST domain plays a regulatory function, i.e., the auto-phosphorylation of IRAK-1 takes plays in its ProST region. The kinase domain contains an invariant lysine, which is required for the binding of ATP and catalytic functions. IRAK-1 and IRAK-4 contain an active kinase domain, whereas IRAK-2 and IRAK-3/M contain an in-active/pseudokinase domain. The C-terminal domain, present in IRAK-1, IRAK-2 and IRAK-

M, consists of Pro-X-Glu-X-X-(Ar/Ac) motifs which are required for binding with TRAF-6. The IRAK proteins contain a Tyr262 residue also called the "kinase gatekeeper residue", exclusively found in the IRAK proteins. This gatekeeper residue blocks the hydrophilic pocket at the back of ATP binding site, where small molecule inhibitors bind (Flannery & Bowie, 2010; Jain *et al.*, 2014; Rhyasen & Starczynowski, 2015). The structure of IRAK proteins with size, location and expression in cells is presented in **Figure 2.13**.

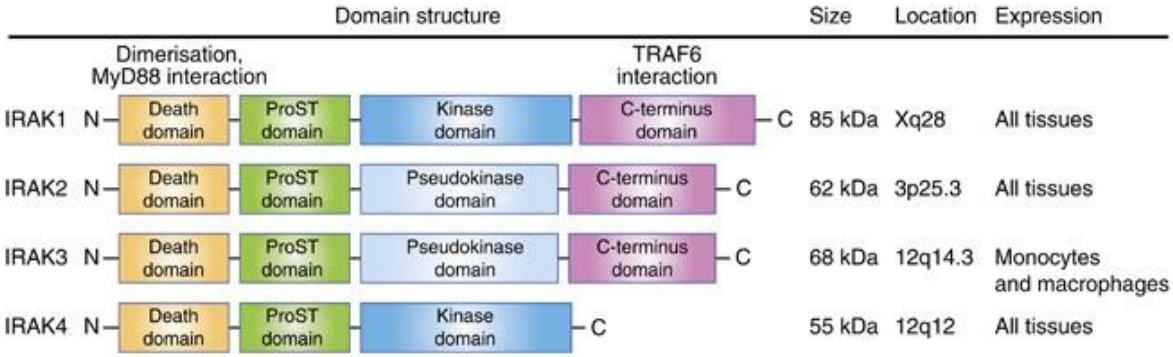

Figure 2.13: **Domain structure of Human IRAK proteins with size, location and expression in cells** (Source: Rhyasen & Starczynowski, 2015)

2.7.2 The Myddosome complex formation and downstream signaling

Induction of TLRs activates MyD88 which further recruits IRAK proteins, forming the myddosome complex. The myddosome complex is a left-handed helical oligomer comprising of 6 molecules of MyD88, 4 molecules of IRAK-4 and 4 molecules of IRAK-2. MyD88 assembles with IRAK-4, the most proximal kinase in the pathway. This is an essential interaction for the Myddosome formation and for the trans-autophosphorylation of IRAK-4 in its kinase domain. Activated IRAK-4 phosphorylates downstream kinases IRAK-1 and IRAK-2 (Lin *et al.*, 2010). The Structure of Myddosome is presented in **Figure 2.14**.

IRAK-4 mediated phosphorylation of IRAK-1 leads to auto-phosphorylation of IRAK-1 initially at Thr209, followed by Thr387 and finally in the ProST domain multiple times. Auto-phosphorylation at Thr209 cause a conformational change in IRAK-1 and at Thr387 leads to the complete activation of IRAK-1. Auto-phosphorylation at multiple sites in the ProST domain facilitates dissociation of IRAK-1 from the Myddosome complex while remaining bonded with TRAF-6. IRAK-1 complexed with TRAF-6 binds to TAB-1, TAK-1 and TAB-2 and the ubiquitin-mediated-degradation of IRAK-1 takes place. The remaining complex associates with ubiquitin ligase in the cytosol which mediates degradation of TRAF-6, eliciting activation of TAK-1 and phosphorylation of MAPKs and IKKs. This enables nuclear translocation of transcription factors AP-1 and NF-κB leading to gene transcription and

production of inflammatory mediators (Jain *et al.*, 2014; Flannery & Bowie, 2010; Muroi & Tanamoto, 2007).

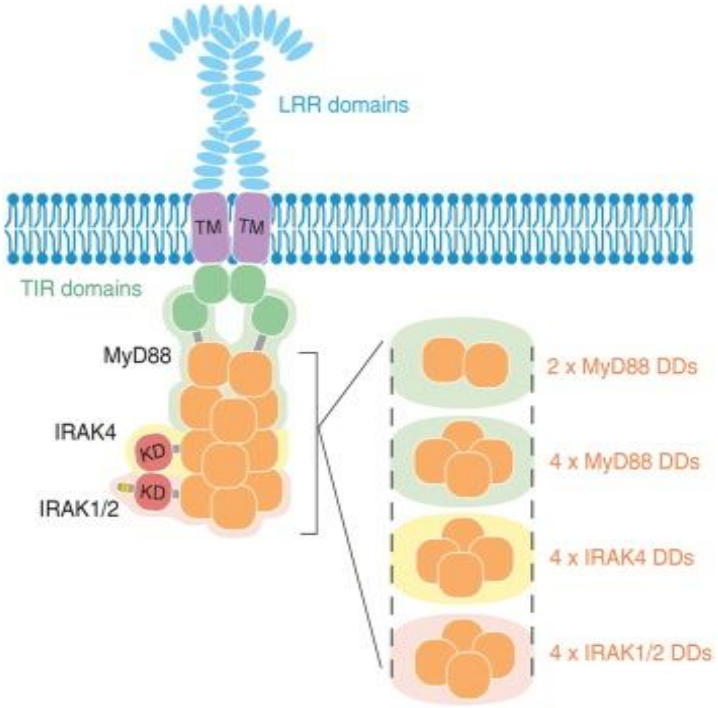

Figure 2.14: Formation of Myddosome complex. 4 molecules of IRAK-4 and IRAK-1/2 recruits to 6 molecules of MyD88. (Source: Balka & Nardo, 2018)

2.7.3 IRAK-2 and IRAK-3: Pseudokinases

IRAK-2 is considered as an inactive kinase as it cannot auto-phosphorylate. This is because of presence of an asparagine in place of an aspartate, a critical residue for the catalytic activity of IRAK, in its kinase domain (Jain *et al.*, 2014). IRAK-3/M, whose expression is largely limited to monocytes and macrophages, is also considered an inactive-kinase and is implicated in the negative regulation of the TLR signaling pathway (Kobayashi *et al.*, 2002).

2.8 Dysregulation in TLR signaling pathway

Dysregulation in the TLR signaling pathway is associated with a wide range of diseases. A suppression of TLR signaling in immune cells can lead to downregulation of immune responses, making the system susceptible to infections. An overactivated TLR signaling can elicit aberrant release of cytokines, chemokines and inflammatory mediators which can lead to the development of cancer (Keshavarz *et al.*, 2021), autoimmune diseases such as RA and SLE (Li *et al.*, 2009), chronic inflammatory diseases such as obesity, diabetes and sepsis (Alkanani *et al.*, 2012; Jialal *et al.*, 2014; Vijay K, 2020), and cardiovascular diseases such as atherosclerosis and hypertension (Hadi *et al.*, 2021; Lazaridis *et al.*, 2021).

2.8.1 Role of TLRs in Cancer

Recent studies indicate that apart from conventional immune cells, TLRs are also expressed on cancer cells. Expression of TLRs on cancer cells can promote cancer progression in the tumor micro-environment. This is regulated by NF-κB activity in cancer cells that can lead to proliferation and tumor progression, and also in release of cytokines and chemokines thereby recruiting immune cells in the tumor micro-environment. Release of pro-inflammatory cytokines, growth factors and proangiogenic factors dampens the anti-tumor activity of immune cells such as APCs and effector T cells (Sato *et al.*, 2009). Expression of TLRs have been reported in gastric, colorectal, ovarian, cervical, lung, prostrate, melanoma and many other cancers. A list of cancer types with corresponding TLRs and their functions in tumorigenesis is presented in **Table 2.4**.

Table 2.4: Role of TLRs in progression of various cancers

Cancer	TLRs	Functions
Gastric cancer	TLR 2, TLR 4, TLR 5, TLR 9	Induces growth of cells (West *et al.*, 2017), Production of pro-inflammatory cytokines and chemokines mediated by H-pylori infection (Schmaußer *et al.*, 2005)
Colorectal cancer	TLR 2, TLR 3, TLR 4, TLR 7, TLR 8, TLR 9	Tumor cell proliferation (Meng *et al.*, 2020) (Grimmig *et al.*, 2015), increased ROS and RNS production (Lucas and Maes, 2013), Cell survival (Nojiri *et al.*, 2013)
Melanoma	TLR 2, TLR 3, TLR 4	Increases migration and production of pro-inflammatory cytokines and chemokines (Goto *et al.*, 2008)
Breast cancer	TLR 2, TLR 3, TLR 4, TLR 9	Survival, proliferation, migration, invasion, angiogenesis and aggressiveness of tumor (Xie *et al.*, 2009; Merrell *et al.*, 2006; Ahmed *et al.*, 2013)
Hepatocellular cancer	TLR 2, TLR 4, TLR 9	Invasion, metastasis, production of inflammatory cytokine production, survival, cell proliferation, angiogenesis (Yang *et al.*, 2015; Tanaka *et al.*, 2010; Mohamed *et al.*, 2020)
Brain cancer	TLR 2, TLR 4	Invasion (Wang *et al.*, 2015) and proliferation (Litak *et al.*, 2020)

HNSCC cancer	TLR 2, TLR 3, TLR 4, TLR 9	Proliferation, invasion, increased production of IL-6, IL-8, VEGF and GM-CSF (Makinen *et al.*, 2014; Szczepanski *et al.*, 2009; Chuang *et al.*, 2018)
Lung cancer	TLR 2, TLR 4, TLR 9	Proliferation, production of immunosuppressive cytokines and pro-angiogenic chemokines and metastasis (Gergen *et al.*, 2020; He *et al.*, 2007; Ren *et al.*, 2007)

2.8.2 Role of IRAKs in Cancer

TLR mediated IRAK signaling in immune cells is a crucial event in order to harbour an immune response via production of inflammatory cytokines, chemokines. Contrastingly, in a tumor micro-environment, inflammation leads to the dampening of immune response, enhanced tumor progression, metastasis and chemo-resistance. The role of IRAK-1 and IRAK-4, direct and indirect, in human malignancies, in this context is extensively investigated.

2.8.3 Expression status and role of IRAKs in haematological malignancies

Myelodysplastic syndrome (MDS) and Acute myeloid leukemia (AML) – IRAK-1 overexpression and activation are observed in MDS and AML. Along with IRAK-1, TLR 1, TLR 2, TLR 4 and TLR 6 were also over-expressed in MDS patients (Rhyasen *et al*., 2013; Maratheftis *et al.*, 2007). Genetic inhibition of IRAK-1 induced apoptosis and impaired progenitor cell functions in MDS and AML. IRAK inhibitor, in addition to exhibiting these effects also showed a concentration-dependent inhibition in cell growth of MDS cells. However, IRAK inhibitor did not show any effect in the AML cells (Rhyasen *et al.*, 2013). Expression of IRAK-4 is associated with poor prognosis and aggressiveness of AML patients. AML cell lines expressing IRAK-4 treated with IRAK inhibitor exhibited suppressed leukemic functions (Smith *et al.*, 2019).

Activated B cell–like (ABC) subtype of diffuse large B cell lymphoma (ABC-DLBCL) – Approximately 29% of (ABC-DLBCL) bear L265P single amino-acid substitution in the TIR-MyD88 domain. As discovered by co-immunoprecipitation, L265P mutants exhibited strong association of MyD88 with IRAK-1 and IRAK-4. This association promotes cell survival of ABC-DLBCL by activation of IRAK-4, phosphorylation of IRAK-1 and activation of downstream NF-κB and JAK-STAT signaling. Genetic and pharmacologic inhibition of IRAK-1 and IRAK-4 reduced cell proliferation and cytokine secretion verifying dependency of ABC-DLBCL oncogenesis on IRAK signaling (Ngo *et al.*, 2011).

Waldenstrom's Macroglobulinemia (WM) - The L265P mutation is widely observed in waldenstrom's macroglobulinemia patients (Treon *et al.*, 2012). In WM, the mutant MyD88 also complexes with BTK. Hence therapeutic drug testing of BTK inhibitors have been carried out. Combination of IRAK inhibitor with ibrutinib (BTK inhibitor) inhibited NF-κB signaling and enhanced killing of WM cells (Yang *et al.*, 2012). Use of a more potent and specific IRAK inhibitor suppressed the BTK-IRAK-NF-κB signaling and enhanced the killing of WM cells (Rhyasen & Starczynowski, 2022).

T-cell acute lymphoblastic leukaemia (T-ALL)-IRAK-1 overexpression is observed in T-ALL types. Genetic inhibition of IRAK-1 induces apoptosis and interrupts cell-cycle. In 2015, Dussiau *et al.*, concluded that these functions are kinase independent as the IRAK inhibitor treatment was partially effective compared to the genetic IRAK-1 inhibition. However, in the same year, Li *et al.*, showed that IRAK inhibitor reduced the growth of T-ALL cells and also sensitized the effect of chemo-therapies in T-ALL.

2.8.4 Expression status and role of IRAKs in solid tumors

Expression of IRAK proteins have been detected in various solid tumors as represented in **Figure 2.15**. Expression and activation of IRAKs is also associated with the development and progression of a large number of solid tumors.

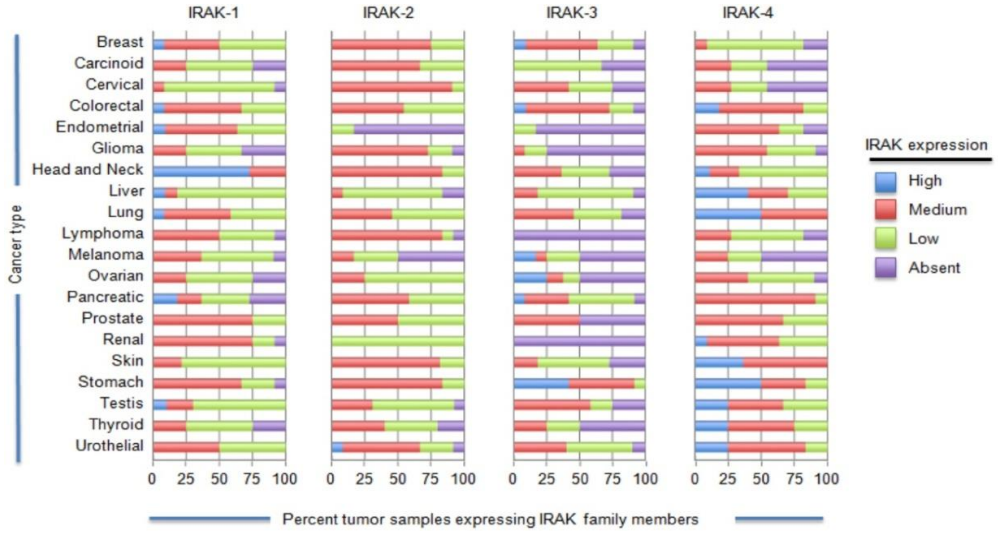

Figure 2.15: Expression of IRAKs in tumors cells according to the data by protein atlas (Source: Jain *et al.*, 2014)

Non-small cell lung carcinoma (NSCLC)- Over-expression of IRAK-1 was observed in NSCLC patient samples (Behrens *et al.*, 2010). Expression of IRAK-1 also promoted growth, proliferation and survival of NSCLC (Chen *et al.*, 2013).

Breast cancer- According to TCGA, IRAK-1 was found overexpressed in TNBC. IRAK-1 activation regulated the production of pro-inflammatory cytokines IL-6, IL-8 and CXCL-1 that were essential for mammosphere growth. Inhibition of IRAK-1 suppressed tumor growth and metastasis *in vitro* and *in vivo*. Paclitaxel treatment increased the phosphorylation of IRAK-1 and production of IL-6, IL-8, CXCL-1 and cancer stem cells (CSCs) indicating that resistance to chemotherapy contributes to IRAK signaling in tumor (Wee *et al.,* 2015).

Hepatocellular carcinoma (HCC)- Expression of IRAK-1 were upregulated in HCC (Ye *et al*., 2017). Sorafenib (a multi-kinase inhibitor) treatment induced IRAK-4 phosphorylation indicating the contribution of IRAK signaling in chemoresistance. IRAK-1 also regulated the expression of downstream protein AKR1B10, overexpressed in various cancers and associated with chemoresistance. A reduction of AP-1 promoter activity was observed at the promoter binding site of AKR1B10 upon IRAK inhibition. A small molecule IRAK-1 & -4 inhibitor reduced tumor growth, metastasis and self-renewal in HCC. IRAK-1 & -4 inhibitor combined with sorafenib induced apoptosis-mediated cell death synergistically (Cheng BY *et al.*, 2018; Cheng YL *et al.*, 2016).

Melanoma- Srivastava *et al.* in 2012, reported the over-expression of IRAK-4 in various melanoma cell lines. Few of them also over-expressed IRAK-1. A constitutive expression of phosphorylated IRAK-1 and IRAK-4 was also observed. Phosphorylated IRAK-4 expression was also observed in melanoma patient samples. Inhibition of IRAK-1 and 4 suppressed the downstream signaling and cytokine expression and enhanced the effect of vinblastine treatment *in vitro* and *in vivo*.

Colorectal cancer (CRC)- Expression of phosphorylated IRAK-4 was observed in CRC patient samples, benign tumor clumps, and in tumor around normal colon samples indicating a potential role of IRAK-4 activity in neoplastic CRC progression. IRAK-4 expression is correlated with poor prognosis and survival of CRC. Chemotherapy with 5-FU and oxaliplatin induced TLR9 mediated-IRAK-4 activation and downstream NF-κB signaling. Treatment with IRAK-4 inhibitors enhanced the effect of chemotherapy on cell death synergistically *in vitro* and reduced tumor size and volume *in vivo* (Li *et al.*, 2019).

Head and neck squamous cell carcinoma (HNSCC)- According to the TCGA data analysis, IRAK-1 is overexpressed significantly in head and neck squamous cell carcinomas (HNSCCs) patients. Expression of IRAK-1 reportedly regulated survival of oral cancer cells. IRAK-1 was regulated by DEK, a nuclear protein found up-regulated in a most of the HNSCCs and an important transcriptional regulator of HNSCCs growth. Genetic and pharmacological

inhibition of IRAK-1 increased apoptosis of cells. Co-targeting of IRAK-1 and DEK induced cell death synergistically compared to targeting of individual protein (Adams *et al.*, 2015). IRAK-1 regulated the metastasis of nasopharyngeal carcinoma (NPC) cells. Inhibition of IRAK-1 suppressed metastasis of NPC cells *in vitro* and *in vivo* (Meng *et al.*, 2020). In paclitaxel-resistant cells, phosphorylated IRAK-1 levels were high and inhibition of IRAK-1 re-sensitized NPC cells to paclitaxel (Liu *et al.*, 2021).

2.9 TLR based therapeutic approaches

As many of the inflammatory conditions, autoimmune diseases and cancer involves aberrant signaling by the TLRs, the pathway acts as an attractive therapeutic target. Therapeutic strategies using TLR agonists or antagonists comprise of antibodies, oligonucleotides, micro-RNA and small molecule inhibitors. They have been tested in various diseases like rheumatoid arthritis, SLE and cancers (Gao *et al.*, 2017; Farooq *et al.*, 2021).

2.9.1 TLR agonists

TLR agonists mediated anti-cancer responses work by activating the patient immune cells and initiating signaling cascades that facilitate the recruitment of cyototoxic T lymphocytes, helper T cells and natural killer cells. These leukocytes release cytotoxic mediators and IFN-γ which mediates the immune mediated killing of tumor cells. In this view, various agonists of TLRs are being discovered and synthetically developed. More commonly, bacterial cellular components are used as adjuvants (Farooq *et al.*, 2021). **Table 2.5** summarizes the TLR agonists approved by the FDA or in clinical trials for various cancers.

Table 2.5: **List of agonists of TLRs used in cancer treatment**

Target TLR(s)	Compound	Cancer model	Clinical trial reference
TLR 2/4	BCG	Bladder cancer (FDA approved)	
TLR 3	Poly IC12U Poly ICLC	Melanoma, Prostrate cancer, Colorectal cancer, Non-Hodgkin's lymphoma, Breast cancer, Head and neck squamous cell carcinoma, Melanoma, Mesothelioma, Prostrate cancer	NCT03403634 (Phase II) NCT04093323 (Phase II), NCT03899987 (Phase II) NCT03789097 (Phase I/II), NCT03617328 (Phase I) NCT03835533 (Phase I)
TLR 4	MPLA	HPV-induced cervical cancer (FDA approved), Melanoma, Ovarian cancer, Lung cancer	NCT01584115 (Phase I/II)
TLR 5	Entolimod	Advanced or Metastatic solid tumors	NCT01527136 (Phase I)
TLR 7	Imiquimod	Basal cell carcinoma (FDA approved), Malignant melanoma, High risk melanoma	NCT00142454 (Phase I), NCT00273910 (Phase II)
TLR 7/8	Resiquimod	Melanoma	NCT00470379 (Phase I)
TLR 8	VTX-2337	Metastatic HNSCC	NCT01836029 (Phase II)
TLR 9	MGN1703	Metastatic colorectal carcinoma	NCT02077868 (Phase III)
	SD-101	Non-Hodgkin lymphoma	NCT03410901 (Phase I)

2.9.2 TLR antagonists

TLR antagonists or inhibitors fall into the categories of small molecule inhibitors, antibodies, micro-RNAs, oligonucleotides and Lipid A analogs (Gao *et al.*, 2017; O Neill *et al.*, 2009). TLR antagonists block the TLRs signaling at various steps in the pathway (Keshavarz *et al.*, 2021). A schematic illustration showing potential drug targets acting at various steps in the TLR signaling pathway is presented in **Figure 2.16**.

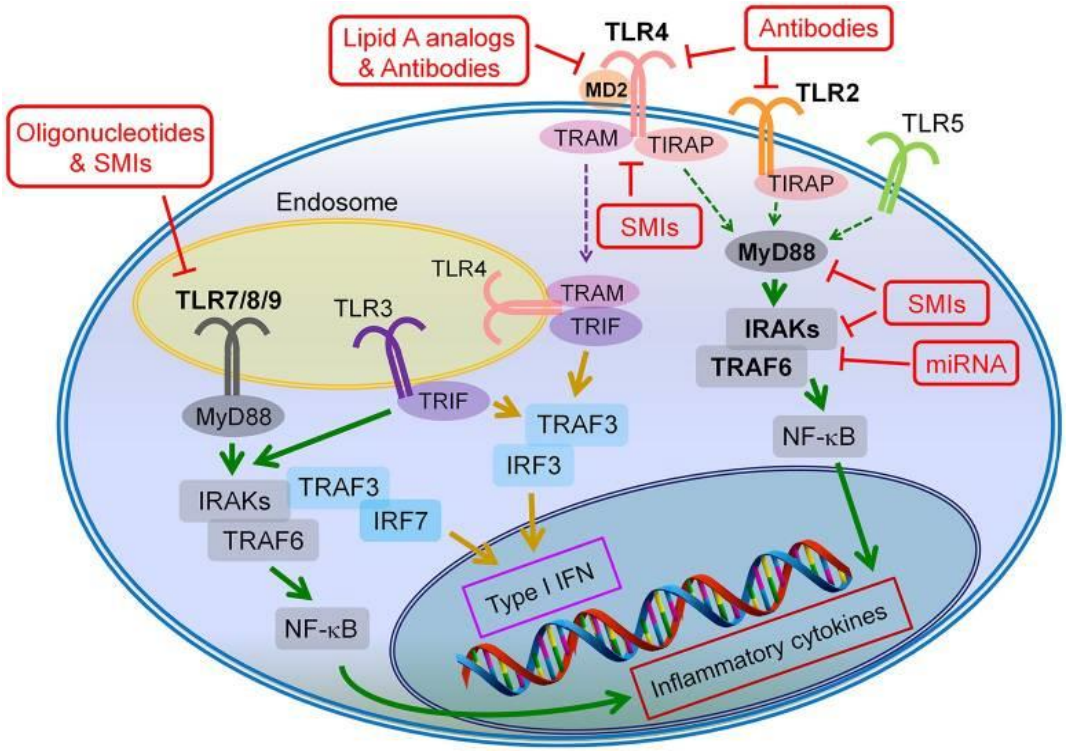

Figure 2.16: Drug targets of the TLR signaling pathway and therapeutic strategies to inhibit the signaling (Source: Gao *et al.*, 2017)

Antibodies blocking TLR can hinder with the binding of PAMPs and DAMPs binding to TLRs. Also, the antibodies have a higher specificity to drug targets compared to any other drug candidate but their high manufacturing cost and poor cell penetration, still is a limitation. Antibodies targeting TLR-2 such as OPN-305 and T2.5 are being tested for the treatment of delayed graft rejection, septic shock and ischemia. NI-0101, an antibody against TLR 4 is being tested for the treatment of RA (Gao *et al.*, 2017).

Endosomal TLRs generally recognizes nucleic acid sequences like CpG-DNA, dsRNA and ssRNA. Oligonucleotides binding to these endosomal TLRs can interfere with the binding of nucleic acid to the TLRs and inhibit their activation. Oligonucleotides such as IRS-954 and DV-1179 (targeting TLR 7/9) are being tested for the treatment of SLE. IMO-8400 against

TLR 7/8/9 are being tested for the treatment of dermatomyositis and plaque psoriasis (Gao *et al.*, 2017).

miRNAs are small non-coding RNAs that bind to the 3' untranslated region of the messenger RNA (mRNA) further degrading them and inhibiting their translation. Till date, about 20 miRNA have been identified that inhibit the TLR signaling pathway (He *et al.*, 2014). miRNAs miR-146a and miR-21 (targeting TLR 4) are being tested for the treatment of SLE and inflammation (Gao *et al.*, 2017).

For the treatment of cancer, a small number of TLR antagonists have been tested. **Table 2.6** summarizes these TLR antagonists in clinical trials for various cancers.

Table 2.6: List of antagonists of TLRs used in cancer treatment

Category of compound	Target TLR(s)	Compound	Cancer model	Clinical trial reference
Antibodies	TLR 2	OPN-305	MDS	NCT03337451(Phase I/II) NCT02363491(Phase I/II)
Lipid A analog	TLR 4	CX-01	AML, MDS	NCT02873338 (Phase II), NCT02995655 (Phase I)
Oligonucleotides	TLR 7/8/9	IM-8400	WM, Diffuse large B cell lymphoma	NCT02363439 (Phase I/II), NCT02252146 (Phase I/II)

(Anwar *et al.*, 2018; Urban-Wojciuk *et al.*, 2019)

Presently, small molecule inhibitors targeting specific adaptor molecules of TLR signaling pathway are emerging as modulators of TLR signaling and also aid in unraveling the role of the TLR pathway. These are generally synthetic molecules that are easily manufactured possesing amphipathic properties and can pass easily through cell membranes. These features also make them suitable drug candidates. Common anti-malarial drugs, hydroxychloroquine

sulphate (HCQ) and chloroquine (CQ) are also small molecule inhibitors of TLR 7/8/9 used for the treatment of arthritis and SLE (Gao *et al*., 2017). These molecules change the chemical environment of the endosomes, where these TLRs are present, and mask the ligand-binding epitopes of these TLRs (Kuznik *et al.*, 2011). Other small molecule inhibitors like TAK242, targeting TLR 4 and CpG-52364 targeting TLR 7/8/9 are in phase III and phase I, respectively for the treatment of septic shock and auto-immune diseases (Gao *et al*., 2017). Small molecule MyD88 inhibitors have been tested pre-clinically for the treatment of SLE and breast cancer (Loiarro *et al*., 2007; Liu *et al.*, 2020). In the past decade, small molecule inhibitors of IRAK-1 and IRAK-4 have emerged as drug candidates for the treatment of large number of diseases including cancers.

2.9.2.1 Small molecule inhibitors of IRAK-1 and IRAK-4

Numerous studies indicate a link between dysregulation of downstream kinases of TLR pathway- IRAK-1 and IRAK-4, and development of diseases such as cancer. Since most of the TLRs converge at the MyD88 dependent signaling pathway, targeting IRAK-1 and IRAK-4 is of a greater therapeutic advantage than targeting of individual TLRs. A large number of kinase inhibitors of IRAK-1 and IRAK-4 have been developed and studied preclinically and clinically.

Mode of action of IRAK inhibitor: **Figure 2.17** shows the structure of IRAK-4 kinase domain. The structure comprises of 4 distinct regions: the activation loop, the substrate binding region, the ATP binding region and the inhibitor binding region. The ATP binding pocket is located between the N-terminal β-sheets and C-terminal α-helices of the IRAK-4 kinase domain. The inhibitor binds in the region overlapping the ATP binding site further preventing the phosphorylation of Ser346 and Thr345 residues in the activation loop of the kinase (Patra *et al.*, 2016).

Several classes of the inhibitors with chemically diverse structures that target IRAK-1 and IRAK-4 directly and indirectly that include amino-benzimidazole, thiazole/pyridine amides, imidazo[1,2-a] pyridines, imidazo[1,2-b] pyridazine, benzimidazole-indazoles and 5Z-7-oxozeaenol have been designed. Apart from that, nitrogen bisphosphonates (NBPs) and several plant-derived compounds such as Protopanaxatrial ginsenoside and 1,3,5- Trihydroxy-4-prenylxanthone (TH-4-PH) have also been reported to induce IRAK inhibition (Jain *et al.*, 2014).

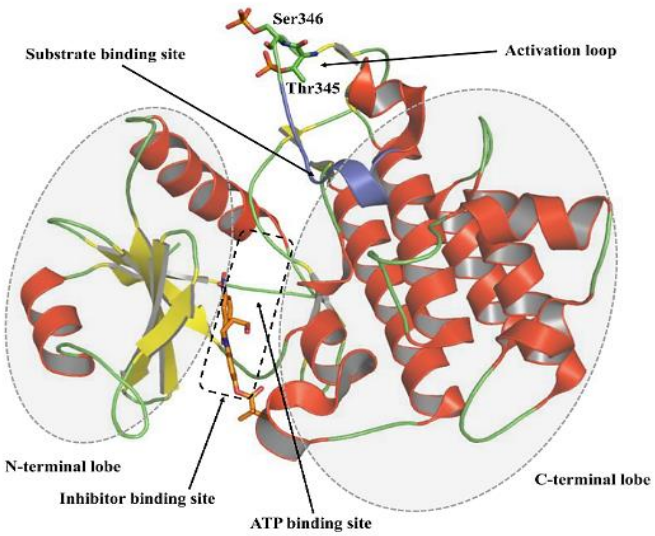

Figure 2.17: Structure of IRAK-4 kinase demonstrating the inhibitor binding site (Source: Patra *et al.*, 2016)

Numerous pharmaceutical companies have reported discovery and development of small molecule IRAK-1 and IRAK-4 inhibitors. Some of these are Aurigene discovery technology limited, Curis, Bayer Pharma, Biogen MA Inc., Bristol-Meyers Squibb (BMS) company, Genentech, Merck and Pfizer (McElroy, 2019). Of these companies, the chemical structure of the inhibitors reported in clinic are presented in **Figure 2.18**.

Small molecule IRAK-4 inhibitor from Pfizer (PF-06650833) has completed Phase II studies for Rheumatoid Arthritis (NCT04413617, NCT02996500) demonstrating good safety and efficacy. Clinical studies on IRAK-1 &-4 inhibitor for the treatment of cancer have not been reported by companies till date, except by Curis and Aurigene that have initiated the clinical evaluation of IRAK-4 inhibitor (CA-4948) for the treatment of Acute myeloid leukemia (AML), Myelodysplastic syndrome (MDS) and Haematological malignancies (NCT04278768, NCT03328078) (McElroy 2019). Currently, NCT04278768 is in Phase I/II recruiting AML and MDS patients to examine escalating doses of CA-4948 monotherapy and combination therapy with Azacitidine and Venetoclax. NCT03328078 is also in Phase I/II recruiting patients with relapsed Haematological malignancies to examine escalating doses of CA-4948 as monotherapy and in combination with ibrutinib.

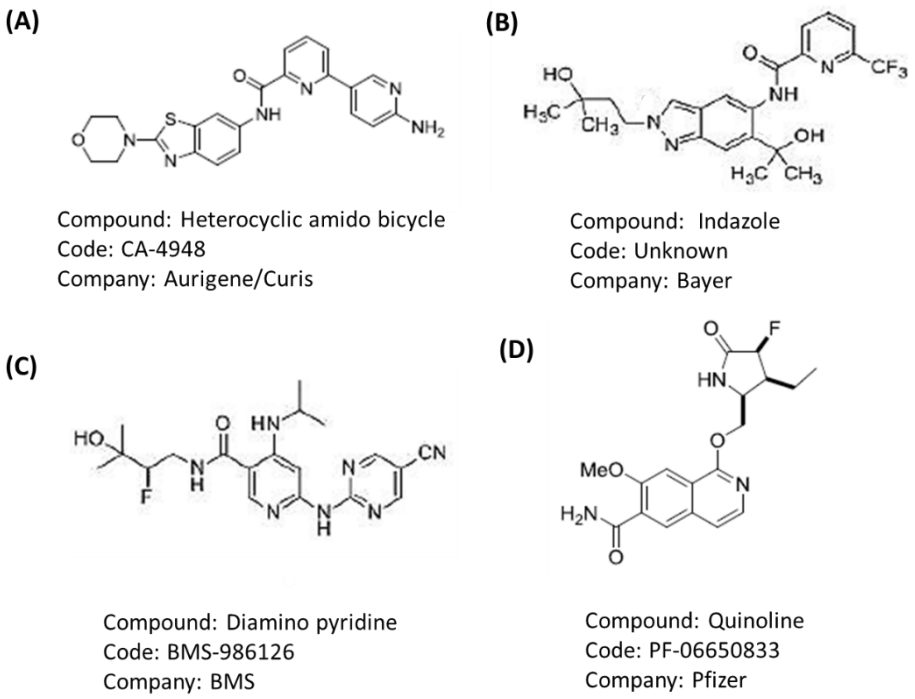

Figure 2.18: Chemical structures of IRAK inhibitors in clinic (Source: McElroy, 2019)

In this study, to target the TLR signaling in the cells, we have used a cell permeable benzimidazole based IRAK-1 &-4 dual inhibitor, chemically known as N-(2-Morpholinylethyl)-2-(3-nitrobenoylamido)-benzimidazole. The compound has shown efficacy in mice models of cancers like melanoma (Srivastava. *et al.*, 2012) and HCC (Li *et al.*, 2016) by suppressing the diseased state with reportedly no sign of drug mediated toxicity or adverse effects. The chemical structure of the compound is presented in **Figure 2.19**.

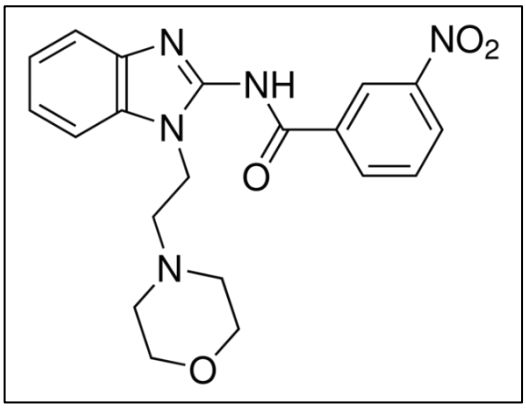

Figure 2.19: Chemical structure of IRAK-1 &-4 dual inhibitor used in this study (Source: Sigmaaldrich.com)