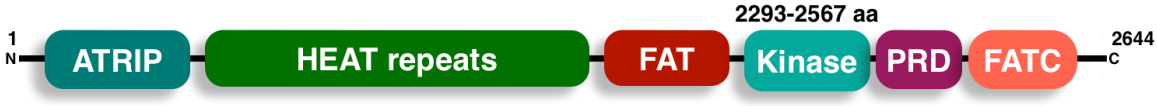
Synopsis

The genome of our body goes through a number of cell cycle events to ensure proper growth, replication, and cell division. As the cell proceeds through different phases of the cell cycle, various checkpoints become activated in response to any kind of genotoxic insult [like replication stress, ultraviolet (UV) light, ionizing radiation (IR), and reactive oxygen species] to the cell. DNA damage response (DDR) pathways form a part of complex network signalling at checkpoints to monitor the integrity of our genome by initiating repair. An understanding of the DDR components of cancer cells has shed light on the regulators of such pathways to be considered for cancer cells selective sensitization. In this thesis, we aimed to study two important proteins involved in the single- and double-strand break DNA damage repair pathway, which have shown promising results in achieving sensitization of cancer cells in response to radio- or chemo-therapy.

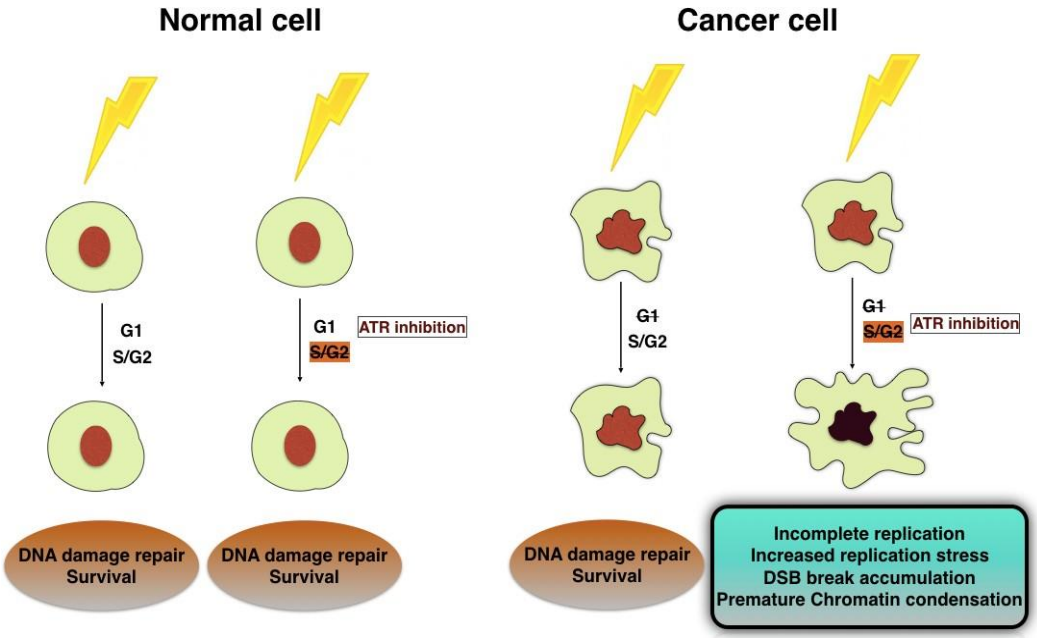
Part I: Cloning, expression, purification and characterization of the human ATR kinase domain



ATR (ataxia telangiectasia and Rad-3 related kinase) is one important apical kinase intricately involved in the DNA damage response to counter stalled replication forks or single-strand breaks. It belongs to the large phosphatidylinositol-3-kinase-related kinase (PIKK) family of serine/threonine protein kinases, displaying a high level of sequence homology in their kinase, FAT (FRAP-ATM-TRRAP), and FATC (FAT carboxyl-terminal) domains. Additionally, inhibition of ATR using specific ATR kinase inhibitors can prove to be useful in achieving selective sensitization of ATM- or p53-deficient cancer cells. However, one of the major drawbacks in the field of ATR kinase drug discovery is the lack of specific ATR kinase inhibitors, which can be attributed to a number of factors including the inability to obtain active protein to facilitate design of novel agents, challenges with compound screening because of the large size of the kinase, lack of standardized assays for high throughput screening of compounds and high degree of sequence homology present between the PIKK family members. Because the kinase

domain (amino acids 2293–2567) is an essential aspect for assessing ATR activity, we focused on its expression in a bacterial system. The characterization of ATR kinase domain can provide insights into its active site required for considering structure-based drug design of domain specific inhibitors. We were able to successfully clone, express, purify and characterize the ATR kinase domain (as a fusion protein) using p53 Ser-15 phosphorylation. Our results indicated that the overexpressed recombinant ATR kinase domain was catalytically active and can be potentially used for characterization of kinase domain specific inhibitors.

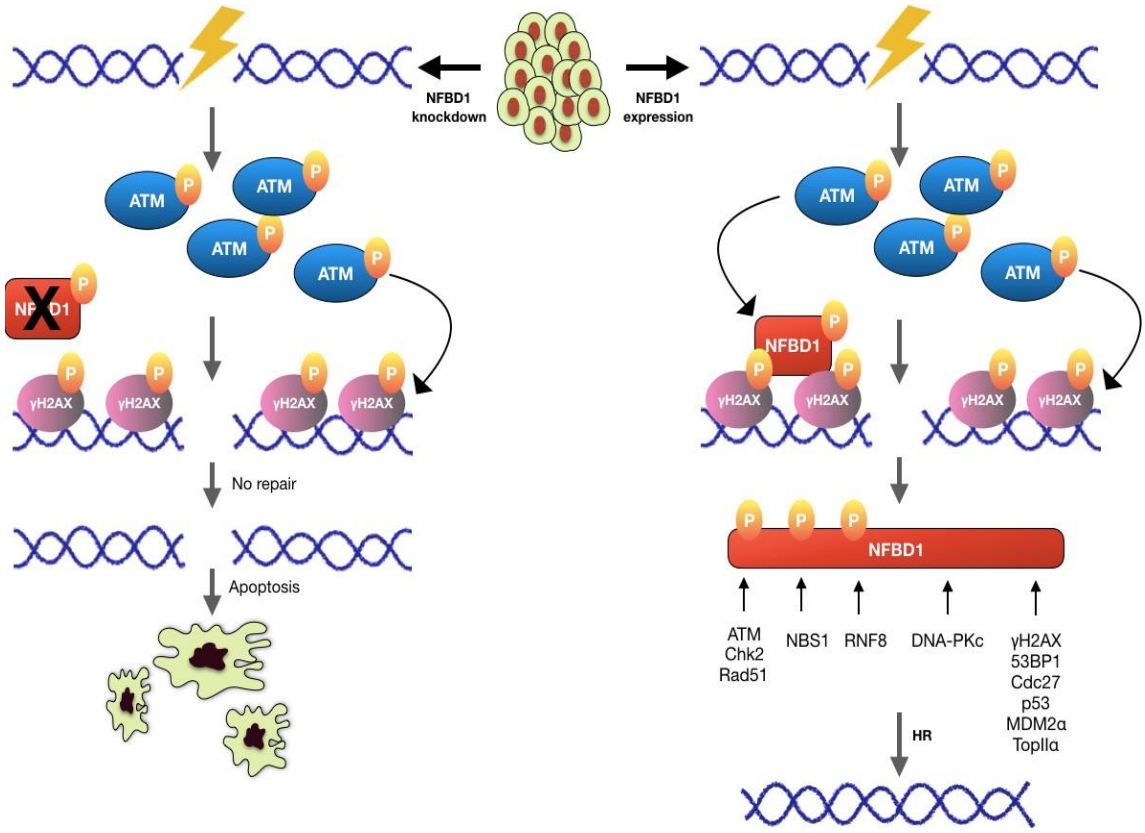
Part II: Characterization of the novel synthesized inhibitors for ATR kinase inhibition



Cancer cells deficient of the ATM/p53 signalling mostly rely on ATR-Chk1 pathway for the repair of DNA damage inflicted on them as a result of chemo- or radio-therapy. Following activation, the 301 kDa ATR kinase phosphorylates its immediate downstream target Chk1 (checkpoint kinase 1) at Ser-317 and Ser-345 residues to initiate cell cycle arrest and DNA repair. And, hence, inhibition of ATR-Chk1 pathway using ATR kinase inhibitors can be helpful in causing selective sensitization of such cancer cells. Accordingly, inhibition of ATR using specific kinase inhibitors has been gaining importance with ATP-competitive inhibitors constituting a major part of such an inhibition strategy. However, a high degree of sequence homology between the kinase domains of ATR and other PIKK family members poses a significant risk of selectivity while considering the design of such inhibitors. Torin2, a second

generation ATP competitive mTOR inhibitor, exhibits potent biochemical and cellular activity against ATR. Although Torin2 inhibits ATR and mTOR in sub-micromolar range, it needs optimization in terms of reducing the cross reactivity amongst PIKK family members. In this part of our work, we were able to design and optimize a number of assays (including cell viability, immunoblotting, immunofluorescence and mitotic spreads) for the characterization of novel in-house synthesized inhibitors to identify their selectivity towards ATR kinase inhibition. Our characterization studies revealed that SPK 98 (an ATP-competitive inhibitor) selectively sensitized cancer cells to UV induced DNA damage and caused them to die by premature condensation of their chromatin.

Part III: Studies on NFBD1 expression in response to cisplatin treatment in cervical cancer cells



The major players that participate in DDR have broadly been characterized as sensors, transducers and effectors molecules. Recently, a new class of proteins has been introduced to the concept of DDR i.e., mediators. These proteins do not have any enzymatic activity and work as a recruiting platform for the other proteins in the DDR pathway. NFBD1 (nuclear factor

with BRCT domains 1) is one such important mediator protein that works majorly in the ionizing radiation (IR) induced DDR pathway and helps in the amplification of ATM and gamma H2AX signal at the site of DNA damage to induce DNA repair. The role of NFBD1 as a tumour suppressor or oncogene is still debatable as various studies support multiple understanding of its function. Since the number of expression studies regarding NFBD1 expression and its relevant role in a variety of cancers is small, it has remained elusive whether overexpression or downregulation of NFBD1 can be related to carcinogenesis. Based on our understanding of the role of NFBD1 in DNA damage response and other pathways, we hypothesized that silencing NFBD1 expression can be helpful in sensitizing ATM proficient cervical cancer cells towards radio- or chemo-therapy as it will directly inhibit amplification of IR induced DNA damage signal at the site of DNA damage. In this part of our work, we generated stable cervical cancer cell lines for the overexpression and downregulation of NFBD1. Our preliminary results indicated that NFBD1 has a detrimental effect in inducing phosphorylated γH2AX and ChK2 foci formation as a part of the DDR and is anti-apoptotic in nature because of its indispensable role in modulation of p53 stabilization and activation in case of sustained DNA damage in cells knocked down for NFBD1. Thus, NFBD1 knockdown has the potential to regulate cisplatin induced ATM-Chk2 based DNA damage response pathway, which has further prospects to be utilized for the sensitization of HPV infected cervical cancer cells in combination with chemotherapeutic drugs such as cisplatin.