SYNOPSIS

Cancer is a disease, causes due to failure of control of growth and cell proliferation mechanisms. There are genetic control systems, regulate the balance between cell birth and death in response to growth signals, growth-inhibiting signals, and death signals in normal conditions. So, the losses of cellular regulation and corresponding genetic damage give rise to cancer. The ultimate goal of cancer therapy is to destroy cancer cells selectively without affecting the normal tissues of patients. But, most of the currently available anticancer agents (as well as radiation therapy) do not possess higher selectivity towards cancer cells and destroy rapidly dividing normal cells including vital tissues such as lympho-hematopoetic tissues, gonad, hair follicles and the lining epithelium of gastrointestinal tract and mouth. So, adverse side effect of anticancer agents limits the doses applied to cancer patients causing incomplete killing of cancer cells. As a result, prolong treatment is needed and that makes cancer cells 'un-sensitized' towards drugs. Recurrence of cancer is also an unavoidable outcome of this incomplete killing.

A lot of developmental researches across the globe are going on to overcome these limitations with alternative approaches towards cancer therapy. Targeted cancer therapy using lipid-based delivery systems and subsequent gene delivery is an important strategy for selective cancer

eradication. It is also possible to reduce the drug dose using suitable adjuvant to overcome the adverse effect of drugs on normal cells and make cells sensitized towards drug molecules.

In targeted cancer therapy, receptors are targeted using suitable delivery systems. Certain receptors are over-expressed in various cancers but not in normal cells. So, by targeting those receptors, cancer cell specific delivery of anticancer agents is possible. On the other hand, some receptors behave differently in cancer and normal cells. Those receptors can also be targeted to kill cancer cells selectively leaving the normal tissues unaffected. The development of a receptor-targeted delivery of anticancer agent is described in the second chapter of the present thesis.

Chapter 1A:

In This chapter, the development of synthetic bivalent inhibitors of N-end rule ubiquitin pathway and their in-vitro and in-vivo biological effect of inhibition is described. N-end rule pathway is a subset of ubiquitin-dependent proteolysis pathway. The N-end rule is a relation between the metabolic stability and fate of a protein and the identity of its N-terminal residue, which is called N-degron. So, the in-vivo half life of a protein depends upon its N-terminal residue. Proteins contain some specific N-terminal amino acid residues like Arg, His, Phe, Lys, Leu, Ile, called destabilizing residue are degraded rapidly. There are also some

stabilizing N-terminal residues (e.g. Met, Val) are not degraded in this pathway and are stable. The proteins bearing basic N-terminal residues (e.g. Arg, Lys, His) are type 1 substrates and the proteins bearing bulky hydrophobic N-terminal residues (e.g. Phe, Leu, Ile) are type 2 substrates of the N-end rule pathway. There is one more type of substrate, available in mammals, called type 3. The proteins bearing internal amino acids like Ala, Ser, Thr at a specific position, also recognized by N-end rule pathway as a type 3 substrates. The protein degradation pathway consists of two successive steps: (a) covalent attachment of multiple ubiquitin molecules to the target protein (ubiquitination) through recognition by a class of proteins E3, called N-recognin and (b) degradation of the ubiquitin-tagged protein by the 26S proteasome. N-recognins are the E3 ubiquitin ligases involved in N-end rule pathway that recognize and bind to the target proteins depending on the presence of N-terminal destabilizing residues of proteins. Then they attach poly-ubiquitin chains to target proteins. So, N-recognins are worthwhile to be targeted for selective inhibition of the N-end rule pathway of protein degradation.

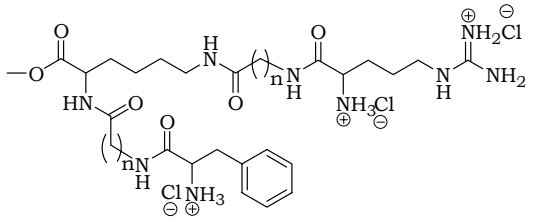
Recent biochemical and proteomic studies identified many components of the mammalian N-end rule pathway. The studies in various species revealed its essential role in various vital physiological processes, ranging from cardiovascular development and mitosis/meiosis to the pathogenesis of human genetic diseases. Studies indicated that

the increasing rates of ubiquitin conjugation via the activation of N-end rule pathway activation enhanced the rapid proteins degradation in skeletal muscles. The rapid loss of muscles accompanies many diseases like cancer, sepsis, diabetes etc. N-end rule pathway plays an important role in cardiogenesis. Recent studies showed its involvement in the regulation of apoptosis. So, the N-end rule inhibitor is gaining importance in recent days.

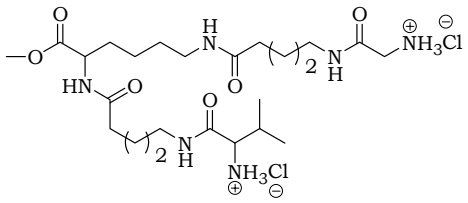
The structural characterization of N-recognins reveals the presence of at least two spatially proximal binding sites: type 1 site binds to basic N-terminal residues (e.g. Arg, Lys, His) and the type 2 site binds to bulky hydrophobic N-terminal residues (e.g. Phe, Leu, Trp, Ile). RF-C11 was synthesized by our group as one of the model compounds, L_1L_2-Cn, which are composed of three replaceable components: ligand (L_1L_2), linker (Cn), and core (lysine). In RF-C11, two C11 hydrocarbon chains, attached with core lysine, were conjugated to the type-1 substrate Arg (R) and the type-2 substrate Phe (F). If the off-target interaction of the linker and ligand with themselves and other cellular macromolecules is ignored, then the N-end rule inhibitory effect of RF-C11 hetero-bivalent inhibitor can be mainly featured to the linker length.

To this end a series of molecules (RF-Cn) have been synthesized with varying chain length. Arg and Phe were conjugated to hydrocarbon linkers (n= 1, 2, 4, 7, 8, and 14), which in turn were linked to the core

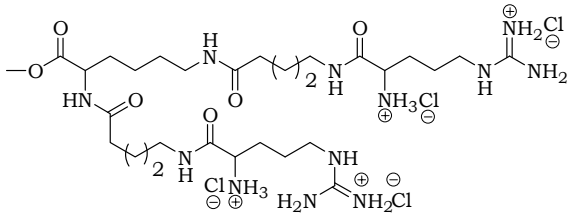
Lys moiety by its ε- and α- amine group respectively. The carboxylic acid group of Lys is kept esterified to prevent further reactions. We got a series of compounds namely, RF-C2, RF-C3, RF-C5, RF-C8, RF-C9, and RF-C15 respectively. Two homo-bivalent inhibitors, RR-C5 (where, n=4, & bearing Arg at its both termini) and FF-C5 (where, n=4, & bearing Phe at its both termini), were also synthesized to observe the effects of homo-bivalent interactions compared to hetero-bivalent interaction. A structural control, GV-C5, bearing two stabilizing amino acid residues Gly and Val at its termini, had been synthesized to look at the non-selective interaction with N-recognins. The structures of the synthesized hetero-bivalent inhibitor molecules are represented in scheme 1.



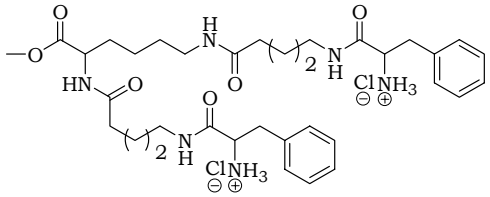
RF-Cn (where n = 1, 2, 4, 7, 8 & 14)



GV-C5



RR-C5



FF-C5

Scheme 1: Structures of synthesized bivalent inhibitors.

All the synthesized bivalent inhibitors were examined for their efficiency in inhibiting the N-end rule pathway mediated protein degradation *in vitro* and *in vivo.* The structure-activity relationship study was done with synthesized RF-Cn molecules in a time- and dose-dependent manner. Among the inhibitors the maximum protein degradation inhibiting activity was observed with RF-C5 and RF-C9 both in type 1 and type 2 models for longer time. All the hetero-bivalent inhibitors were more effective in inhibiting the degradation of both type 1 and type 2 N-end rule substrates than that of di-peptides bearing N-terminal destabilizing residues, used as control molecule. As RF-C5 was one of the most efficient inhibitor, corresponding homo-bivalent control molecules RR-C5 and FF-C5 were synthesized. RR-C5 showed very limited inhibition of degradation of type 1 substrates only, though at higher concentrations in comparison to RF-C5. Similarly, FF-C5, at higher concentrations, inhibited the degradation of type 2 substrates only. Structural control molecule GV-C5 showed no inhibition of degradation of both type 1 & type 2 substrates.

Chapter 1B:

This chapter describes the development of hetero-bivalent N-end rule pathway inhibitor as an adjuvant for drug molecules in various cancer cells. As discussed earlier, N-end rule pathway has a great importance in animal physiology. It had been shown that N-end rule

regulates apoptosis, a process of programmed cell death in multicellular organism. Multiple apoptosis and cell cycle-related factors are ubiquitinated by N-end rule pathway. Caspases cleave various target proteins to give protein fragments of destabilizing N-degron residues, which are recognized and degraded via N-end rule pathway.

On the other hand, the destructive potential of caspases is restrained by Inhibitors of Apoptosis Proteins (IAPs). IAPs exhibit self-Ub ligase activity which is important for its inhibitor of apoptosis function as it complexes with equimolar amount of caspases for the ubiquitination and degradation. During apoptosis IAPs are cut by activated caspases and the fragmented parts of IAPs are degraded through N-end rule pathway. Incidentally, XIAP, the mammalian counterpart of IAP, is fragmented into a Type 3 N-end rule substrate by caspases. However, our hypothesis was to decipher this pathway's role in functional regulation of XIAP in cancer.

In cancer, XIAP has another critical role to play. PTEN, which regulates Akt/PKB kinase pathway, is ubiquitinated and degraded by XIAP. This Akt/PKB kinase pathway regulates phosphorylation of Mdm2 and hence, regulates the recognition and ubiquitination of nuclear wild type p53 present in 50% of all cancer cells.

Additionally, N-end rule pathway also maintains the chromosomal stability by degrading a cohesin complex sub-unit (SCC1/RAD21, SCC1 counterpart in mammals) cleaved by separase at the metaphase to

anaphase transition. This also clues to the fact that antagonizing N-end rule pathway might lead to chromosomal instability in normal functioning cells. This chromosomal instability makes cells 'sensitized' towards drug molecules.

The present study was designed to see the sensitizing effect of our novel, synthetic hetero-bivalent N-end rule pathway inhibitor RF-C11, previously designed and characterized in our lab. RF-C11 was co-treated with existing anticancer drugs on cells. We observed that the co-treatment significantly enhanced drug sensitivity in cancer cells through simultaneous reduction of XIAP and *in vivo* stabilization of RAD21. But, there was no such effect on normal cells. Wild type p53 up-regulation was observed due to the down-regulation of XIAP via N-end rule pathway. Co-treatment group induced apoptosis in cancer cells and also cell cycle arrest at G2/M phase. So, altogether we got significant cytotoxicity effect of co-treatment in cancer cells but not in normal cells. These data have given new insights into how N-end rule pathway may be manipulated for the development of anticancer therapeutics.

Chapter 2:

This chapter of present thesis consists of the targeted liposomal delivery of micro-RNA precursor in plasmid form into cancer cells. Micro-RNAs (miRNAs) are 19-24 nucleotide long non-coding RNAs, regulate gene expression by binding with mRNAs imperfectly or perfectly. miRNAs are encoded by the specific genes and sometimes derived from the

introns of pre-mRNAs. If miRNAs have nearly perfect complementarities to mRNA target sequences positioned in either coding or 3' untranslated (3'UTR) regions (in plants), then the perfect base pairing triggers mRNA degradation through a mechanism similar to that of RNA interference (RNAi). But, in animals, with very few exceptions, miRNAs do not posses perfect base pairing like plants. In animals, they regulate gene expression by imperfect base pairing to the 3' UTR of target mRNAs and inhibit protein synthesis or causing mRNA degradation. miRNAs have important role in physiological processes, as they regulate gene expression.

Here, we delivered artificial miRNA (amiR) against one of the most abundant and ubiquitously express cellular chaperon protein Hsp90 (amiR-Hsp90) in the form of plasmid. Hsp90 as chaperon maintains the structural integrity of many of its client proteins, which are involve in cellular growth, differentiation and apoptotic pathways. So, by targeting one protein expression it could be possible to target many other proteins involving in various cellular processes. Here, we used amiR-Hsp90 plasmid in-stead of small molecules which have some limitations like non-specific toxicity, bio-availibility, and solubility.

Cationic lipid-based delivery system, liposome, is a good choice to overcome the limitations created by small molecules. Dexamethasone associated lipoplex (liposome plasmid complex, DX) was used here as

delivery system, which acts via glucocorticoid receptor (GR). GR is also a client protein of Hsp90 and it maintains the structural integrity of ligand-binding domain (LBD) of GR. The LBD of GR allows only two molecules of synthetic GR-ligand, dexamethasone (Dex), as it is tightly regulated by Hsp90 in normal cells. Our group recently discovered that DX could gain GR-mediated nuclear access only in cancer cells and eventually expresses the delivered gene, though the size of DX is many folds bigger than that of LBD of GR. Similar GR-mediated nuclear delivery of DX lipoplex could not be observed in normal cells unless Hsp90 was inhibited. This proved that functional activity of Hsp90 must be somewhat compromised in cancer cells. GR helps nuclear delivery DX only in cancer cells using the compromised functional activity of Hsp90, though the cellular uptake of DX was same for both cancer and normal cells. This encouraged us to use this delivery system (DX) to deliver amiR-Hsp90 plasmid in cancer cells. The DX-mediated delivery to cancer cells & selective processing of plasmid in those cells is expected to be more effective in comparison to that in normal cells.

Artificial miRNAs (amiRs), in plasmid form, utilize the cellular machinery for expression, processing and targeting of mRNAs to repress their protein level expression. Artificial miRNA in plasmid form (pri-miRNA) has to be processed in nucleus by Drosha following the processing in cytoplasm by Dicer to form mature miRNA. Therefore, this targeted delivery system (DX) has been used to deliver genetic cargo like

amiR-Hsp90 into nucleus of cancer cells to get the repressor effect of miRNA against Hsp90 protein.

Unlike amiRs in plasmid form, mature miRNAs need not to be processed from nucleus. Cytoplasmic delivery of mature miRNAs is enough to get their effect. As far as cytoplasmic delivery is concerned, our delivery system DX cannot differentiate between cancer cells and normal cells. But DX can differentiate between cancer cells and normal cells as far as GR-mediated nuclear delivery of genetic cargo is concerned. On the basis of this important observation, we planned to use amiR-Hsp90 in plasmid form to target specific deliver and process it to down-regulate Hsp90 in cancer specific manner.

The plasmid bearing the artificial miRNA gene (amiR-Hsp90) was then complexed with GR-targeted cationic liposomal delivery system DX (DX-amiR-hsp90). The anticancer efficacy of lipid/DNA complex was first tested in cells *in vitro.* The down-regulation of Hsp90 at the mRNA level was observed by RT-PCR analysis. The comparison of the levels of Hsp90 and its cancer implicated client proteins was accomplished by western blot analysis. We observed down-regulation of Akt1 and corresponding phospho-Akts, anti-apoptotic protein bcl-2 and VEGF-R2 & up-regulation of apoptotic protein p53 and Caspases. Overall western blot data indicated the induction of apoptosis and repression of cells proliferation through the down-regulation of cancer related client

proteins of Hsp90. The in-vivo studies in two different tumor models (mouse melanoma & human lung adenocarcinoma) showed significant tumor regression. This, to our knowledge, is the first evidence of GR-assisted targeted gene therapy against Hsp90 for eliciting selective and highly efficient anticancer response.