Targeting PI3K/mTOR Pathway in Hepatocellular Carcinoma

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Erklärung zur Dissertation:

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel „ Targeting PI3K/mTOR Pathway in Hepatocellular Carcinoma“ selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe. Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, 2011 Deepika Pothiraju

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Summary

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third most lethal cancer worldwide. It is commonly associated with viral hepatitis, chronic exposure to toxins and hereditary liver diseases. The prognosis of patients with HCC is dismal and the mortality rates are almost the same as the incidence rates. Surgical resection or local ablation of the tumor provides the best results for patients with limited disease. Therapeutic options for patients with advanced disease are still sobering and even combinations of cytotoxic drugs such as doxorubicin, cisplatin, irinotecan and/ or 5-Fu produce only modest anti-tumor activity. The multikinase inhibitor Sorafenib has shown survival benefits in some patients with advanced HCC proving that molecular therapies can be effective in this devastating disease. These results have triggered the search for additional molecular agents to further expand patient survival.

Recent studies revealed that the PI3K/mTOR kinase pathway is frequently deregulated in HCCs suggesting that it might play a pivotal role in hepatocarcinogenesis. PI3K/mTOR kinases are mainly involved in the regulation of cell proliferation, metabolism and survival. Although preclinical studies have shown that rapamycin and its analogous are potent inhibitors of tumor cell proliferation, the roles of specific down-stream pathways of mTOR have been poorly defined. Additionally, it is important to note that broad-based use of mTOR inhibitors has not been as effective as initially anticipated, although many trials are still underway. One reason for this might be due to the fact that multiple mTORC1 dependent feedback loops exist to regulate the activation of upstream components of the network. The aim of this study was therefore to identify new strategies to improve mTOR directed therapies.

In the first part of this study, the effect of RAD001 (mTOR inhibitor) an analogue of rapamycin, NVP-BKM120 (PI3K inhibitor) and NVP-BEZ235 (PI3K/mTOR inhibitor) alone and in combination with each other was studied. In addition, combination treatment of the above mentioned PI3K/mTOR inhibitors with the chemotherapeutic drugs doxorubicin, cisplatin, 5FU and irinotecan was analyzed in Huh7 cells in vitro and in xenograft mouse model. Inhibition of PI3K by novel drug NVP-BKM120 led to a more

potent tumor growth inhibition compared to RAD001 and NVP-BEZ235. Furthermore, dual PI3K/mTOR inhibition with NVP-BEZ235 is more effective as compared to RAD001 treatment alone. Next, the effect of the molecular drugs in combination with doxorubicin, cisplatin, 5-FU or irinotecan on cancer cell viability was tested. Interestingly, the combination of RAD001, NVP-BEZ235 and NVP-BKM120 with irinotecan or cisplatin was particular useful to decrease survival of Huh7 cells. These data provide evidence that mTOR inhibition is an effective strategy to increase sensitivity of hepatoma cells to conventional chemotherapeutic drugs suggesting that combination therapies with PI3K/mTOR and chemotherapeutic drugs could be a feasible option for non-cirrhotic HCC patients with good liver function.

Though mTOR inhibitors and chemotherapeutic drugs are tested as therapeutic agents in HCC, the development of resistance to these drugs remains an important clinical problem. Therefore it is of great interest to understand the key oncogenic markers and their mechanisms that drive resistance in HCC. In the second part of this study, an *in vitro* system was therefore established to perform a genome-wide shRNA screen to identify genes that mediate resistance or sensitivity to mTOR directed therapies. In order to validate the screening approach, the effect of a shRNA-mediated knockdown of FKBP12 was analysed on the anti-tumor effects of RAD001. RAD001 potently reduced phoshorylation of P-S6, which was almost completely prevented in cells pretreated with a shRNA directed against FKBP12. Furthermore, knockdown of FKBP12 by RNA interference abolished the RAD001 induced growth arrest *in vitro* indicating that shRNA-mediated knockdown of functionally important target genes can be used to identify molecular mechanisms underlying RAD001 sensitivity or resistance.

Key words:

HCC - Hepatocellular carcinoma
mTOR - mammalian Target Of Rapamycin
PI3K - Phospho Inositide 3 Kinase

Zusammenfassung

Das „Hepatozelluläre Karzinom“ (HCC) ist die fünft häufigste Krebserkrankung und die dritt häufigste krebsbedingte Todesursachen der Welt. In vielen Fällen tritt es zum Beispiel assoziiert mit einer viralen Hepatitis, im Zusammenhang mit chronischer Toxin-Exposition oder bedingt durch hereditären Lebererkrankungen auf. Die Prognose für HCC Patienten ist dabei gemeinhin sehr bedrückend und wird von einer sehr schlechten Mortalitätsrate und Inzidenz begleitet. Die chirurgische Resektion oder lokale Ablation des Tumors bietet in diesem Zusammenhang die besten Resultate für Patienten mit einer limitierten Erkrankung. Die therapeutischen Möglichkeiten für Patienten im fortgeschrittenen Stadium der Erkrankung sind bisher leider noch ernüchternd und selbst Kombinationen von Zytostatika wie Doxorubicin, Cisplatin, Irinotecan und/ oder 5-FU haben lediglich eine geringe Anti-Tumor-Aktivität. Sorafenib, ein Multi-Kinase-Inhibitor, war allerdings im Stande das Überleben einiger Patienten mit einem fortgeschrittenen Stadium des HCC zu erhöhen. Dies zeigt, dass molekulare Therapien, auch in Bezug auf diese verheerende Krankheit, Wirkung zeigen können. Diese Ergebnisse haben die Suche nach zusätzlichen molekularen Wirkstoffen, die das Überleben der Patienten fördern, weiter angeheizt.

Jüngste Studien konnten zeigen, dass es im Rahmen des HCCs oft zu Fehlregulationen des PI3K/mTOR Kinase Signalwegs kommt. Dies deutet darauf hin, dass dieser Mechanismus vielleicht eine entscheidende Rolle für die Hepatokarzinogenese spielt. Die PI3K/mTOR Kinasen sind vor allem an der Regulation der Zellproliferation, des Stoffwechsels und des Überlebens beteiligt. Obwohl präklinische Studien gezeigt haben, dass Rapamycin und analoge Substanzen potente Inhibitoren der Proliferation von Tumorzellen sind, wurde die Rolle spezifischer down-stream Wege von mTOR bisher schlecht definiert. Darüber hinaus ist es allerdings auch wichtig zu beachten, dass breit angelegte Studien von mTOR-Inhibitoren bisher nicht so effektiv waren wie ursprünglich erwartet. Nichtsdestotrotz sind aber auch noch weitere aktuelle Studien zu diesem Thema im Gange. Ein Grund dafür könnte die Tatsache sein, dass mehrere mTORC1 abhängig

Feedback-Schleifen die Aktivität vorgelagerter Komponenten des Netzwerks regeln. Das Ziel der hier vorliegenden Studie war es daher, neue Strategien zur Verbesserung einer mTOR gerichteten Therapien zu finden.

Im ersten Teil dieser Studie wurde die Wirkung von RAD001 (mTOR-Inhibitor), einem Analogon von Rapamycin, NVP-BKM120 (PI3K-Inhibitor) und NVP-BEZ235 (PI3K/mTOR-Inhibitor) allein und in Kombination untersucht. Darüber hinaus wurde eine Kombinationsbehandlung der oben genannten PI3K/mTOR Inhibitoren mit dem Chemotherapeutikum Doxorubicin, Cisplatin, 5FU und Irinotecan in Huh7 Zellen in vitro und in einem Xenotransplantat Mausmodell untersucht. Die Hemmung der PI3K durch das neuartige Medikament NVP-BKM120, führte zu einer deutlich höheren Inhibition des Tumorwachstums im Vergleich zu RAD001 und NVP-BEZ235. Des Weiteren konnte gezeigt werden, dass eine duale Hemmung durch PI3K/mTOR und NVP-BEZ235 effektiver ist als die RAD001 Behandlung allein. Im nächsten Schritt wurde die Wirkung der molekularen Medikamente in Kombination mit Doxorubicin, Cisplatin, 5-FU oder Irinotecan auf die Viabilität der Krebszelle getestet. Interessanterweise wurde dabei deutlich, dass die Kombination von RAD001, NVP-BEZ235 und NVP-BKM120 mit Irinotecan oder Cisplatin das Überleben insbesondere von Huh7-Zellen verringert. Diese Resultate sind einen Hinweis darauf, dass die Hemmung von mTOR eine wirksame Methode ist, um die Empfindlichkeit von Leberzellen gegenüber herkömmlichen Chemotherapeutika zu erhöhen. Eine Kombinationstherapie mit PI3K/mTOR und Chemotherapeutika könnte somit eine realisierbare Möglichkeit für Nicht-Zirrhose HCC Patienten mit guter Leberfunktion sein.

Obwohl mTOR-Inhibitoren und Chemotherapeutika als therapeutische Wirkstoffe gegen HCC getestet werden, bleibt die Entwicklung von Resistenzen gegen diese Medikamente ein wichtiges klinisches Problem. Daher ist es von großem Interesse die wichtigsten Onkogene und entsprechende Mechanismen zu verstehen, die die Resistenz der Zellen im HCC steuern. Im zweiten Teil dieser Studie wurde ein *in vitro* System etabliert, um über ein genomweites shRNA-Screening, die Gene zu identifizieren, die die Resistenz oder Sensitivität gegenüber einer mTOR gerichteten Therapie vermitteln. Um die Screening-Ansätze zu validieren, wurde die Wirkung einer

shRNA-vermittelte Reduktion von FKBP12 auf die Anti-Tumor-Wirkung von RAD001 analysiert. Die Phosphorylierung von P-S6 wurde durch RAD001 deutlich reduziert, konnte jedoch in Zellen, die mit shRNA gegen FKBP12 vorbehandelt wurden, fast vollständig verhindert werden. Darüber hinaus konnte ein Knockdown von FKBP12 durch RNA-Interferenz die RAD001 induzierte Wachstumshemmung *in vitro* aufheben. Dies zeigt, dass eine shRNA-vermittelte Reduktion von funktionell wichtigen Genen eingesetzt werden kann, um die grundlegenden molekularen Mechanismen der RAD001 Sensitivität oder Resistenz zu identifizieren.

Schlüsselbegrilfe:

HCC – Hepatozelluläres Karzinom
mTOR – Säuger Ziel gen für Rapamycin
PI3K – Phophoinositol 3 Kinase

Introduction

1.1 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most lethal, prevalent cancer and it represents a major health problem worldwide (Llovet et al. 2003). HCC is the fifth most common cancer and ranks as the third lethal cancer worldwide (Srivatanakul et al. 1991; Szymanska et al. 2009). It is commonly associated with hepatitis B and C virus infection, chronic exposure to toxins, alcohol abuse and hereditary liver diseases (Thorgeirsson and Grisham 2002). In contrast to other cancers, its incidence increases annually by 1.75% in the US and in Germany. HCC incidence by hepatitis B and C infections (~75%), and less frequently associated with chronic exposure to toxins or hereditary liver diseases (El-Serag et al. 2008).The prognosis of patients with HCC is dismal and the mortality rates are almost the same as the incidence rates. Surgical resection or local ablation of the tumor provides the best results for patients with limited disease. Therapeutic options for patients with advanced disease are still sobering and even combinations of cytotoxic drugs such as doxorubicin, cisplatin and/ or 5-FU produce only modest anti-tumor activity. Recently, the multikinase inhibitor sorafenib has shown survival benefits in some patients with advanced HCC proving that molecular therapies can be effective in this devastating disease. These results have triggered the search for additional molecular agents to further expand patient survival. Furthermore aberrant activation various signaling pathways, both in cirrhotic tissue and in overt HCC has been implicated in hapatocarcinogenesis, which was discussed in several previous studies (Villanueva et al. 2008; Zender et al. 2010) and it is well demonstrated in Figure 1.

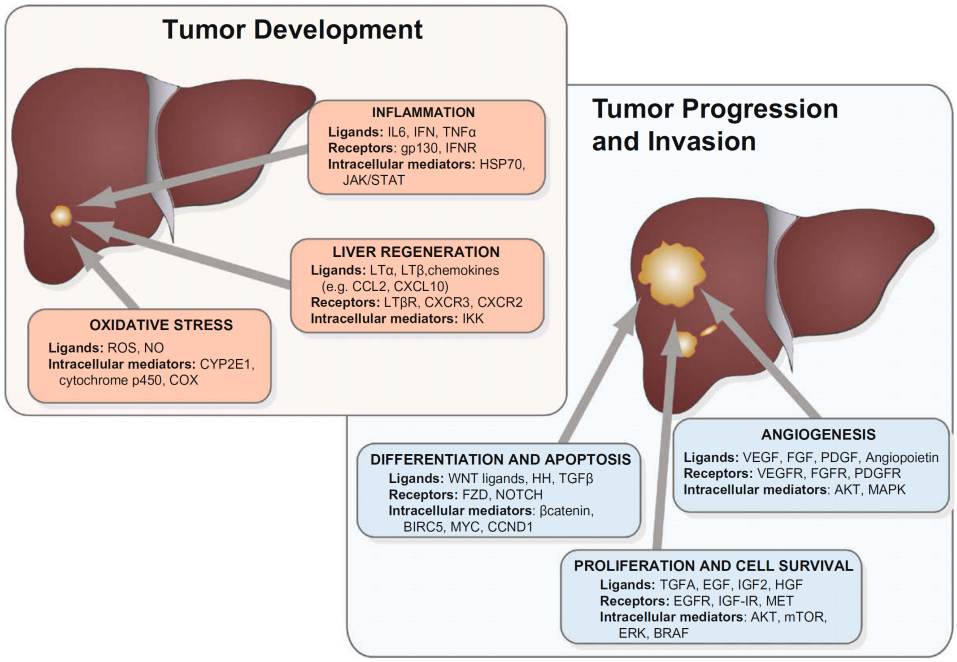

Figure 1: Signalling pathways frequently dysregulated in hepatocellular carcinoma

Pathways are clustered according to the biological process they have been mostly implicated in HCC development and progression, including cell proliferation/survival, differentiation, inflammation, oxidative stress and angiogenesis. Adapted from Zender *et al.,* (Zender et al. 2010)

1.2 Targeted therapies in HCC – positive and negative predictive factors

One major factor limiting the effective treatment of HCC is the heterogeneity of the disease with several genomic alterations; the identical treatment can produce remarkably different responses ranging from complete response to progressive disease in patients with indistinguishable tumors at the macromolecular level (Thorgeirsson and Grisham 2002).

Currently the gold standard to prognostication is the clinicopathological staging, which separates patients into groups with distinct outcomes, but offers little information about

response to treatment in individual patients. Understanding the factors that predict the benefits derived from systemic therapy will be very useful for the development of a more personalized and thereby more effective approach to cancer treatment. Furthermore, treatment can be withheld from patients that are unlikely to respond, avoiding not only unnecessary toxicity but also costs. Finally, understanding the mechanisms of resistance may uncover strategies to overcome resistance. In this regard, *K-ras* is an important predictive biomarker in colon cancer: mutations are limited to hot spots in the gene that are easily detected. The negative predictive value appeared to be high, because most patients with mutated *K-ras* do not respond to EGFR inhibition. Importantly however, more recent studies (COIN, NORDIC VII) have challenged this observation suggesting that a rigorous validation of biomarkers is critically important. Another very recent example of a prognostic and predictive biomarker is *HER2* in gastric cancer. *HER2* over-expression occurs in about 20% of patients with gastric cancer and has not only been correlated to poor outcomes and a more aggressive disease, but it has also become an important positive predictive biomarker for identifying a small subset of patients who respond to HER2 targeting therapy using the fully humanized monoclonal antibody trastuzumab.

1.3 The PI3K/mTOR pathway

The activated PI3K/mTOR pathway plays a critical role in cell proliferation, survival and has only recently emerged as a pivotal contributor to HCC development. PI3K and its downstream molecule AKT are commonly deregulated in many human cancers (Figure 2) (Engelman et al. 2006). PI3K (Phosphatidylinosital-3-Kinase) pathway plays a role in several cellular processes and contribute to malignant phenotype (Markman et al. 2010). PI3Kinase pathway begins with activation of receptor tyrosine kinase (RTK) in presence of growth factors, and then stimulates the production of phosphatidylinositol 3, 4, 5-triphosphate (PIP3) from PIP2 and subsequently the activation of (Phosphoinositide-Dependent kinase-1) PDK1/ AKT and mTOR. mTOR (mammalian target of rapamycin) is a serine/ threonine kinase that integrates mitogenic and nutrient signalling to regulate proliferation, cell cycle progression, mRNA translation, cytoskeletal organization and survival (Guertin and Sabatini 2007).

Interest in the regulation of mTOR has increased substantially in recent years largely because of an apparent link between deregulation of translation and cancer cell survival (Shaw and Cantley 2006). The first mTOR proteins were identified in a genetic screen for mutations conferring resistance to rapamycin. In a complex with FK506-binding protein 12 (FKBP12) rapamycin binds to and inhibits mTOR. mTOR forms two physically and functionally distinct complexes: a rapamycin sensitive complex, mTORC1, and a second rapamycin insensitive complex, TORC2. The best characterized downstream effectors of mTOR as part of TORC1 complex are proteins of the 4E-BP1 family and the P S6 protein kinases, which act in parallel to control mRNA translation (Bjornsti and Houghton 2004). Hypophosphorylated 4E-BP1 binds to and inhibits the translation initiation factor eIF4E. Upon phosphorylation by mTOR, 4E-BP1 releases eIF-4E, which binds the cap at the 5' ends of mRNA transcripts and assembles with other translation initiation factors to initiate cap-dependent translation. The eIF-4E complex is thought to particularly enhance translation of transcripts possessing complex 5'-untranslated regions, which encode various growth factors, cell cycle regulators, survival proteins and angiogenic factors.

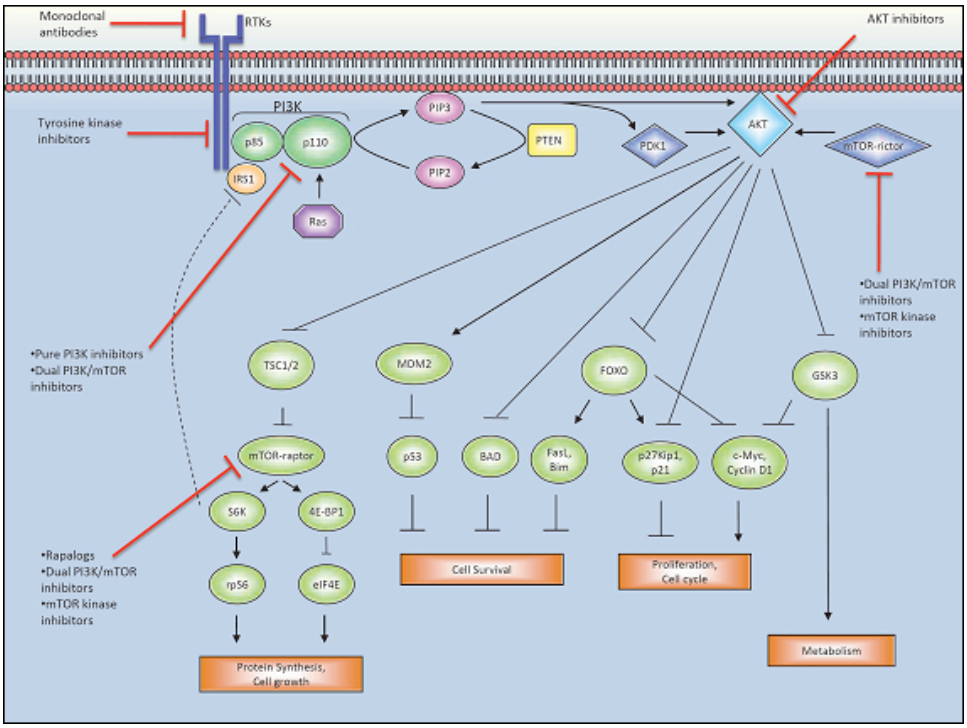

Figure 2: The PI3K/AKT/mTOR signaling pathway and associated inhibitors.

A ligand engaged RTK binds PI3K, either directly or indirectly via adaptor molecules such as IRS1, removing the inhibitory action of the p85 regulatory subunit on the catalytic p110 subunit. The active kinase generates PIP3 at the lipid membrane. PIP3 facilitates the phosphorylation of AKT by PDK1, while the mTOR-rictor complex contributes a second phosphate residue to AKT. As the central effector of the PI3K pathway, AKT transmits signal to a host of downstream substrates, thus influencing a variety of key cellular functions. Pathway activity is negatively regulated by PTEN and the S6K-IRS1 feedback loop. Pharmacological inhibition of the pathway is achieved through a variety of compounds in clinical use at various points along the pathway that are indicated by the red line. Adapted from Markman *et al.,* (Markman et al. 2010).

1.4 mTOR inhibitors role in cancer

The immunosuppressive drugs sirolimus (rapamycin), temsirolimus (CCI-779) and everolimus (RAD001) are currently used as pharmaceutical inhibitors of mTOR.

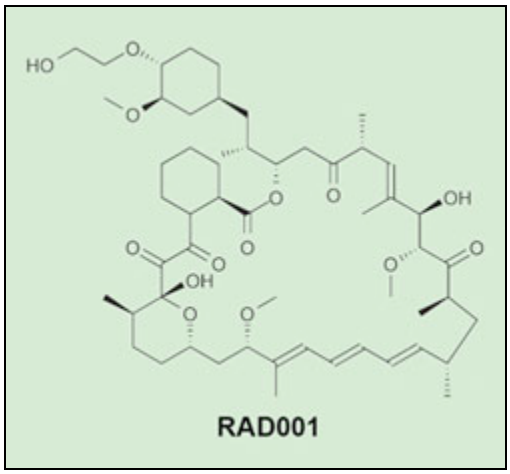

Figure 3: RAD001 chemical structure

The networks that normally signal to mTOR comprise numerous oncogenes and tumor suppressors. It is therefore not surprising that dysregulation of this pathway through genetic mutations, amplifications or loss of heterozygosity has been detected in a large percentage of the most common human cancers. These findings have spurred interest in developing and testing mTOR inhibitors to evaluate their potential as anticancer agents. In most mammalian cells treatment with rapamycin blocks or at least delays G1-cell cycle progression and increases cell death when used in combination with other therapeutic agents (Beuvink et al. 2005). *In vivo*, rapamycin impairs growth of primary and metastatic tumors. Inhibition of tumor growth was linked to a decreased production of VEGF and markedly reduced response of endothelial cells to stimulation by VEGF (Guba et al. 2002). The proof of principle that mTOR inhibitors can improve cancer patient survival has been obtained from clinical trials in

advanced renal carcinoma, but also in mantle cell lymphoma and endometrial cancer. This has started extensive preclinical and clinical programs to further evaluate mTOR inhibitors in nearly all major forms of cancer (Faivre et al. 2006).

Although preclinical studies have shown that rapamycin and its analogous are potent inhibitors of tumor cell proliferation, the roles of specific down-stream pathways of mTOR have been poorly defined. Additionally, it is important to note that broad-based use of mTOR inhibitors has not been as effective as initially anticipated, although many trials are still underway. One reason for this might be due to the fact that multiple mTORC1 dependent feedback loops exist to regulate the activation of upstream components of the network. For instance, a loss of the upstream tumor suppressors TSC1 and 2 results not only in the increased levels of mTORC1 signaling but also in a disease with a very low malignant potential. Loss of AKT activation due to S6K-mediated phosphorylation of IRS-1 most likely accounts for the very slow-growing nature of these tumors. Thus, targeting this pathway affects multiple cellular processes that are difficult to predict and may not only inhibit tumor development and progression, but can also result in exacerbation of tumor growth due to activation of feedback mechanisms.

Consequently, major efforts have been undertaken to identify surrogate markers for assessing the efficacy of mTOR inhibitors and to identify those patients who would benefit the most from mTOR inhibition. As mTOR is ubiquitously expressed, the sensitivity to mTOR inhibitors cannot be predicted on the basis of its tissue-specific expression. Most clinical trials have investigated expression of PTEN and activation/ phosphorylation of AKT, mTOR and P S6K1. Based on these studies one would predict that patients with PTEN inactivation and/ or high levels of phosphorylated and active AKT, mTOR and P S6K1 display increased sensitivity to mTOR inhibitors. However, other studies have shown that the effect on P S6 phosphorylation does not reliably predict the anti-cancer effects of these drugs. Therefore additional studies elucidating the mechanisms behind increased or decreased sensitivity to mTOR inhibitors are urgently needed to identify patients who will most likely benefit from these drugs.

The activated PI3K/mTOR pathway has only recently emerged as a pivotal contributor to HCC development. Aberrant mTOR signaling has been identified in up to 50-60% of HCC cases, associated with insulin-like growth factor pathway activation, epidermal growth factor up-regulation, and PTEN dysregulation (Sahin et al. 2004; Villanueva et al. 2008). Of note, loss of PTEN appears to occur at a rate second only to p53 and is reduced or absent in almost half of HCC patients (Horie et al. 2004).

In vitro as well as preclinical *in vivo* studies revealed that mTOR inhibition by rapamycin and its derivatives significantly reduces HCC growth and improves survival (Semela et al. 2007; Buitrago-Molina et al. 2009). mTOR inhibitor RAD001 specifically impaired cell cycle progression in non-transformed hepatocytes with DNA damage in vivo. Long-term treatment with RAD001 markedly delayed DNA damage-induced liver tumor development indicating that mTOR activation has a substantial effect on hepatocarcinogenesis (Buitrago-Molina et al. 2009). Recent data suggest that activation of AKT via mTORC2 in response to mTORC1 inhibition with rapamycin contributes to drug resistance (Carracedo et al. 2008).

Moreover, mTOR inhibition in combination with conventional chemotherapy or VEGF and EGFR inhibitors has been shown to enhance the anti-neoplastic potency of the respective monotherapeutic drugs (Sieghart et al. 2007; Huynh et al. 2008; Piguet et al. 2008). Several clinical trials have therefore been initiated to evaluate the effect of mTOR inhibitors for the treatment of advanced HCC (Rizell et al. 2005; Rizell et al. 2008).

Although some studies have explored the mechanisms by which rapamycin exerts its anti-tumor effects on HCCs, studies on rapamycin resistance are lacking. Only very recently a panel of 13 tumorigenic and non-tumorigenic hepatic cell lines has been profiled for rapamycin-induced growth arrest (Jimenez et al. 2009). Rapamycin potently inhibited phosphorylation of P S6K in all cases, however the cell lines showed a wide range of sensitivity/ resistance to the growth inhibitory effects of the drug implying a level of complexity that present challenges to identify HCC patient that will respond to mTOR inhibitors. Even microarray analysis did not disclose an unifying

mechanism for rapamycin resistance implying a level of complexity that present challenges to identify HCC patient that will respond to mTOR inhibitors.

1.5 PI3Kinase inhibitors in cancer

Previously, wortmannin (Powis et al. 1994) and LY294002 (Vlahos et al. 1994) were used as a PI3K inhibitors. Wortmannin is fungal metabolite and was isolated from *Pencillium wortmannin* in 1957. LY294002 is a synthetic compound derived from quercetin.

Both agents achieved significant growth inhibition in a broad spectrum of cancer cell lines especially in circumstances of excess PI3K activity. However, either Wortmannin or LY294002 have not progressed to clinical trials due to unfavorable pharmacokinetic properties, poor selectivity, cross talk inhibition of other lipid and protein kinases and toxicity concerns (Stein 2001). Regardless, their use has led to a better understanding of the PI3K pathway and has spawned a new generation of inhibitors that overcome some of the failings of these compounds. Of these new drugs, NVP-BKM120 and NVP-BEZ235 are more potent inhibitors of PI3K. In PI3K pathway, AKT activation requires phosphorylation of two critical residues Thr308 and Ser473. PDK1 subunit of PI3K is responsible for T308 phosphorylation and PDK2 for S473 phosphorylation.

1.5.1 NVP-BKM120 in cancer

Orally available, NVP-BKM120 is a PI3K inhibitor obtained after an extensive medicinal chemistry effort to modulate the biological activity and pharmaceutical properties of imidazo quinoline-based compounds. Recent study explained that NVP-BKM is a potentially useful drug to delay or prevent the development of resistance to Smo antagonists and may inform treatment strategies for medulloblastoma. Medulloblastoma is a malignant brain cancer which is characterized by mutations in Hedgehog (Hh) signaling pathway genes, which lead to constitutive activation of the G protein (heterotrimeric guanosine triphosphate–binding protein)–coupled receptor

Smoothened (Smo). The Smo antagonist NVP-LDE225 inhibits Hh signaling and induces tumor regression in animal models of medulloblastoma. However, resistance was exhibited by the tumors during the course of treatment. Molecular analysis of resistant tumors revealed an up-regulation of phosphatidylinositol 3-kinase (PI3K) signalling in resistant tumors, as another potential mechanism of resistance. Addition of the PI3K inhibitor NVP-BKM120 to the initial treatment with the Smo antagonist markedly delayed the development of resistance (Buonamici et al. 2010).

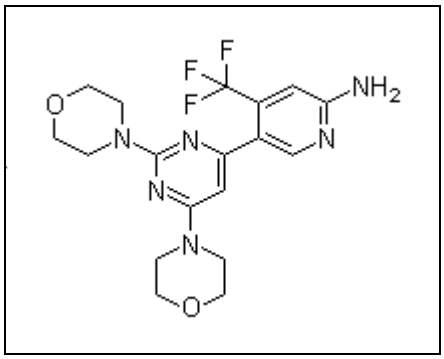

Figure 4: NVP-BKM120 chemical structure

1.5.2 NVP-BEZ235 in cancer

Orally available, NVP-BEZ235 is a dual PI3K/mTOR inhibitor obtained after an extensive medicinal chemistry effort to modulate the biological activity and pharmaceutical properties of imidazo-quinoline scaffold based compounds. NVP-BEZ235 effectively blocks the dysfunctional activation of the PI3K pathway in cellular and *in vivo* settings. The compound displayed disease stasis in relevant *in vivo* models of human cancer when given orally and also enhanced the antitumor activity of other cancer agents. NVP-BEZ235 displays all the features required for clinical development and has entered phase I clinical trials in cancer patients. NVP-BEZ235 compound is

an ATP competitive inhibitor that directly targets the PI3K and mTOR catalytic domains (Figure 6). NVP-BEZ235 reduces the proliferation rate and induces an important apoptotic response in acute myeloid leukemia (AML) cells without affecting normal CD34+ survival. These results clearly showed the antileukemic efficiency of the NVP-BEZ235 compound, therefore represents a promising option for future AML therapies (Chapuis et al. 2010).

In primary effusion lymphoma (PEL), PI3K/mTOR pathway was activated. PELs rely heavily on PI3K/mTOR signaling, are dependent on autocrine and paracrine growth factors. NVP-BEZ235, that dually inhibits both PI3K and mTOR kinase was significantly more efficacious in culture and in a PEL xenograft tumor model. NVP-BEZ235 was effective at low nanomolar concentrations and had a novel mechanism involving the suppression of multiple autocrine and paracrine growth factors required for lymphoma survival. This study showed a broad applicability for the treatment of cytokine-dependent tumors with PI3K/mTOR dual inhibitors (Bhatt et al. 2010). In another study, NVP-BEZ235 inhibited waldenstrom macroglobulinemia (WM) which had a constitutive activation PI3K/mTOR pathway. It was noticed that both rictor and raptor were inhibted by NVP-BEZ235 and thus abrogating the rictor-induced AKT phosphorylation. Additionally, NVP-BEZ235 targeted WM cells in the context of bone marrow microenvironment, leading to significant inhibition of migration, adhesion in vitro, and homing in vivo. Therefore, this study showed the dual targeting of the PI3K/mTOR pathway is a better modality of targeted therapy for tumors (Roccaro et al. 2010).

Multiple myeloma (MM) cells also showed antitumor sensitiveness towards NVP-BEZ235 with lower concentrations. Combinations of NVP-BEZ235 with conventional (e.g., dexamethasone and doxorubicin) or novel (e.g., bortezomib) anti-MM agents showed lack of antagonism. These results strongly support the NVP-BEZ235 efficacy in clinical testing, alone and in combination with other agents (McMillin et al. 2009).

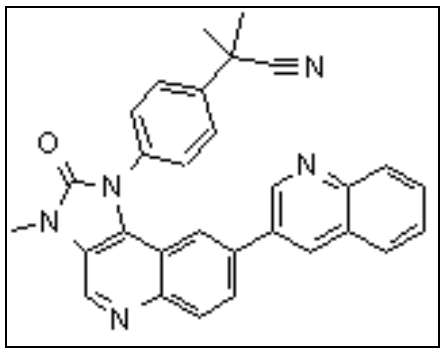

Figure 5: NVP-BEZ235 chemical structure

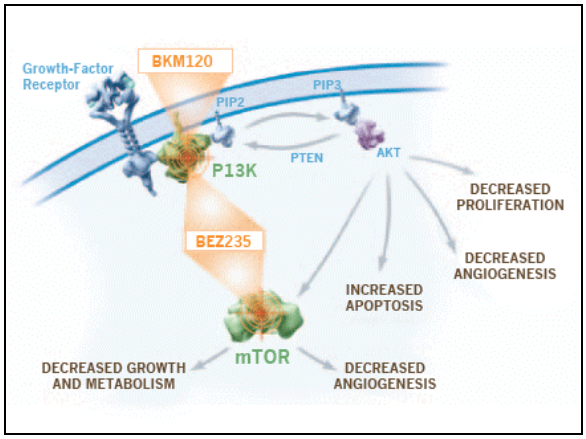

Figure 6: NVP-BKM120 and NVP-BEZ235 mechanism of action

NVP-BKM120 and NVP-BEZ235 inhibits tumor proliferation and survival by blocking the phosphatidylinositol-3-kinase (PI3K) pathway. Adapted from www.novartis.org

1.6 Chemotherapeutic drugs in cancer

Chemotherapeutic drugs are anticancer drugs that mainly disrupt the cancer cell growth. Chemotherapy is generally used to kill the cancer before surgery or sometimes after surgery to completely eliminate the cancer cells from the body. There are several chemotherapeutic drugs which are mainly used to treat the cancer.

1.6.1 Doxorubicin

Doxorubicin is an anthra cycline antibiotic, mainly used to treat cancer. It was isolated from soil bacterium *Streptomyces peucetius*, in 1950. Italian research company, Farmitalia Research Laboratory, found out the doxorubicin from soil microbes. This soil sample was isolated from the area of Castel del Monte, in Italy. This bacterial strain of *Streptomyces peucetius*, produced a red pigment, called as doxorubicin, that was found to have good activity against murine tumors. As this drug was in red colour, so it is called as a doxorubicin. French word for *rubis*, describing the color. It intercalates with DNA and therefore blocks the DNA replication. Based on clinical trials in 1960s, the drug proved to be successful in treating acute leukemia and lymphoma. However, it could produce fatal cardiac toxicity. In one study it was showed that, 14 patients with hepatocellular carcinoma were treated with adriamycin (trade name of doxorubicin) administered intravenously at a dose of 75 mg/m^2 every 3 weeks. 11 evaluable patients responded to the treatment, with 3 exhibiting complete tumor regressions after two, three, and five courses of adriamycin respectively. The median survival of the evaluable patients is 8 months (range 1-13 months). The side effects encountered include myelosuppression, anorexia, nausea, vomiting, and alopecia. Adriamycin seems to be an effective agent in hepatocellular carcinoma. Further more studies were carried out to test efficacy of doxorubicin both by single and combination with other drugs in controlling the tumor progression (Olweny et al. 1975). Combination with rapamycin had showed appreciable promise in treating the lymphomas in mice (Wendel et al. 2004). Additional study of combining doxorubicin and paclitaxel resulted mainly in arresting the cells in G2/M phase of the cell cycle. *In vivo* anti-tumor activity

assay also showed that the combination of the two drugs resulted in more significant tumor regression, compared to the single treatment(Jin et al. 2010).

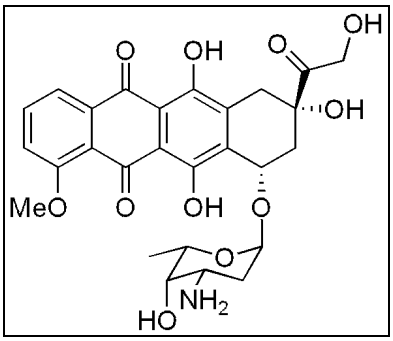

Figure 7: Doxorubicin chemical structure

1.6.2 Cisplatin

Cisplatin was discovered by Dr. Rosenberg in 1965 while he was examining the effect of electromagnetic field on bacterial cell growth (Rosenberg et al. 1965; Rosenberg 1985). Since the active principle that inhibited bacterial cell division was identified as cisplatin, he anticipated that it would also inhibit the proliferation of rapidly dividing cancer cells. Cisplatin was indeed demonstrated to possess antitumor activity in a mouse model (Rosenberg et al. 1969) and was first used in the clinical trial almost 30 years ago. Since its approval by the Food and Drug administration in 1978, cisplatin continues to be one of the most effective anticancer drugs used in the treatment of solid tumors.

Cisplatin is a platinum based drug which is used to treat many cancers. Basically, it intercalates with DNA and prevents DNA replication. In a mechanistic way, platinum binds to guanine base on DNA and leads to DNA damage, finally leading to apoptosis. In previous study, it was showed that S phase arrest occurred due to the cisplatin treatment. Endoreduplication as a result of cisplatin treatment, might be a valuable procedure to evaluate the risk of developing secondary leukaemia and other malignancies as well (Cantero et al. 2006).

Cisplatin treatment had several side effects like headache, nausea, sore throat; Infection chances are higer and one can experience tiredness and breathlessness due to low number of white blood cells and red blood cells. In one study, cisplatin was shown to be useful in the intra-arterial treatment of hepatocellular carcinoma with favourable prognostic factors in patients for whom surgical treatment is not suitable (Kajanti M, 1986). In a phase II study conducted in 28 previously untreated patients with unresectable hepatocellular carcinoma, cisplatin treatment resulted in partial responses lasting for > 3 months in 4 out of 26 patients evaluated, while no patient achieved a complete response. Out of 22 patients whose serum levels of alpha-fetoprotein (AFP) were high (> 400 ng/ml) before treatment, 6 (27.3%) showed a > 50% reduction in serum AFP levels after cisplatin treatment. This indicates that

cisplatin is an anticancer agent worthy of further testing in patients with this disease (Okada et al. 1993).

Recently, it was showed that HCC cell lines exposed to RAD001 in combination with cisplatin resulted in a significant increase in the number of apoptotic cells, downregulated the expression of pro-survival molecules, Bcl-2, survivin and cyclinD1, and increased the cleavage of PARP, compared to RAD001 or cisplatin alone. The suppression of HCC tumor growth *in vivo* was enhanced by RAD001 combined with cisplatin, accompanied by a significant increase in the number of apoptotic cells in tumor tissues (Tam et al. 2009). In another study, by combining adenovirus harboring ING4 with cisplatin enhanced antitumor activity in hepatocarcinoma cells (Xie et al. 2010).

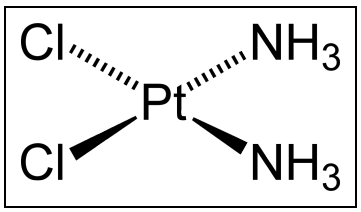

Figure 8: Cisplatin chemical structure

1.6.3 5FU (5 Fluro Uracil)

5FU is a pyramidine analogue which has been in use for the past 40 years in cancer treatment. It acts as a thymidylate synthase inhibitor. When pyrimidine thymidine is not available for DNA replication, cancerous cells undergo cell death via thymidineless death. Chemotherapy is commonly used for the treatment of human malignancies. Continuous infusion of 5FU to the patients is a potentially efficacious palliative therapy in the management of pancreas cancer (Hansen et al. 1988). Albeit, single chemotherapeutic agent is not having enough effectiveness in suppressing HCC, it suggested that combination of several chemotherapeutic agents may be effective against advanced HCC. Combination therapy with 5-fluorouracil (5-FU) and IFN-α in colon cancer and hepatocellular carcinoma rendered better effects than single drug treatments. IFN-α significantly enhanced the growth inhibitory effect of 5-FU in PLC/PRF/5 and delay in the progression of G0/G1 to S phase and down-regulation of cyclinD1 was observed (Eguchi et al. 2000). In another study it was prominent that HepG2 cells radiosensitivity is low, thereby combining a chemotherapeutic drug with a radiotherapeutic agent improves the radiosensitivity in a synergistic fashion. This combination is thus able to strengthen the therapeutic effect of internal radiation therapy in different malignancies, particularly in HCC (Chenoufi et al. 1998).

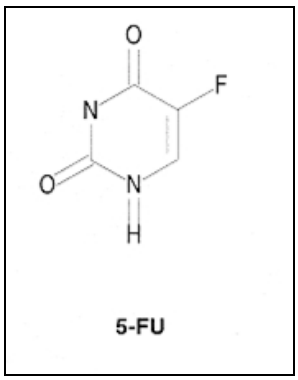

Figure 9: 5FU chemical structure

1.6.4 Irinotecan

Irinotecan is a topoisomerase 1 inhibitor, which prevents DNA from unwinding. Many studies proved that irinotecan and its active metabolite SN-38 are very effectively inhibiting the cancer cell proliferation, leading to cell death (Ueno et al. 2002). SN-38 (active metabolite of irinotecan) led to significant decrease in cell proliferation and induced apoptosis in Huh7 cells and HepG2 cells. SN-38 treatment significantly increased the expression of p53 protein and its phosphorylation at Ser15 in the nucleus and apoptosis-inducing proteins Bax, caspase-9, and caspase-3, while it significantly decreased the antiapoptotic protein Bcl-xL in Huh7 cells. An anti-tumor effect of SN-38 harbors the mechanism of the mitochondria-apoptotic pathway inducing p53 activation and thereby is a good strategy to control HCC (Takeba et al. 2007; Takeba et al. 2007). In another study, it was showed that the expression of P-glycoprotein induced by short-term culture of HCC with SN-38 is associated with the inhibition of apoptosis *via* p53- mitochondrial pathway and thus *p53* gene downregulation affects the chemosensitivity to irinotecan. Thereby, the inhibition of apoptosis in HCC is due to acquired resistance with SN-38 accumulation (Takeba et al. 2007).

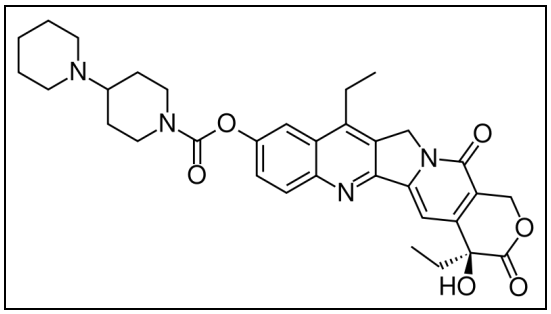

Figure 10: Irinotecan chemical structure

1.7 Genome wide shRNA screening using RNA interference

RNA interference (RNAi) has recently emerged as a powerful tool to perform genetic loss-of-function studies. RNA interference mechanism facilitates loss-of-function genetics in mammalian cells and has been used to explore various aspects of cancer biology, like function of oncogenes and tumor-suppressor genes.

Moreover, the availability of genome-wide libraries of shRNAs capable of stably repressing gene expression has enabled genetic screens for determinants of oncogenic transformation as well as potential therapeutic targets (Westbrook et al. 2005; Berns et al. 2007).

Libraries of DNA-based vectors encoding shRNAs capable of targeting most genes in the human genome have been produced, which enable forward genetic screens in mammalian cells. Previous studies have shown that RNAi based genetic screens can be highly efficient in identifying genes that control the cellular response to chemotherapy. Importantly, loss and gain of function screens can be carried out in a high throughput manner and can lead to the identification of causal factors due to the functional nature of the approach. Several reagents have been developed to trigger RNA interference and several delivery systems including Moloney virus-based, lentiviral and adenoviral vectors can be used for plasmid derived shRNAs (Zender et al. 2008).

shRNA is a sequence of RNA that makes a tight hairpin turn which then silences gene expression through RNA interference. shRNA introduced through lenti/retro viral vectors into cells utilizes the U6 promoter present in the vector and is expressed constitutively. This vector is usually passed on to the daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by the DICER, an endoribonuclease, into siRNA (small interefering RNA), which is then bound to the RNA-induced silencing complex (RISC). This complex binds to the complementary mRNA and cleaves the mRNAs (Figure 11).

shRNA screens may be particularly useful to identify genes that modulate the response to anti-proliferative drugs. A unique barcode of each shRNA vector can be used to identify the genes that confer resistance or sensitivity (Berns et al. 2004). Importantly, this approach may allow the identification of unknown components of targeted pathways and thereby of new druggable therapeutic targets (Brummelkamp et al. 2006; Tyner et al. 2009).

Several shRNA-based screens have been successfully performed to identify drug resistance genes. PTEN for example was identified to confer resistance to the HER2 antibody trastuzumab (Herceptin). Importantly, correlation of this *in vitro* finding with the outcome of breast cancer patients revealed that patients with an activated PI3-kinase pathway have poor prognosis after trastuzumab therapy (Berns et al. 2007).

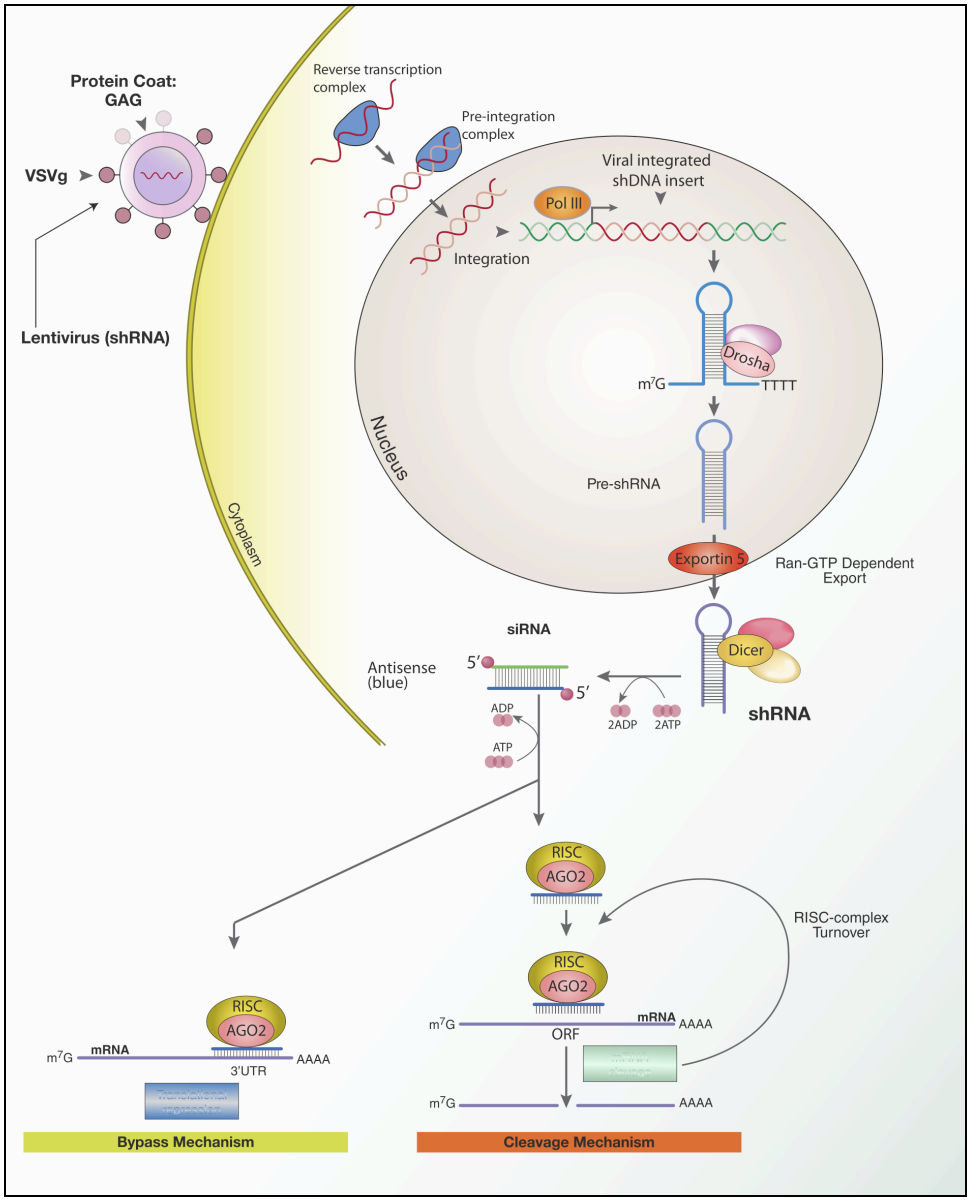

Figure 11: Lentiviral delivery of shRNAs and mechanism of RNA interference in mammalian cells.

(Adapted from wikipidea)

1.7.1 FKBP12

FKBP12 is a cytosolic ubiquitously expressed protein having the 12-KDa size. It mainly serves as a receptor for the immunosuppressant drug. FK506 binds tightly to intracellular calcium release channels and to the transforming growth factor β (TGF-β) type I receptor. Apart from working as a receptor for immunosuppressive drug, it also works as a receptor for mTOR inhibitors such as rapalogs like rapamycin, RAD001 etc. The schematic diagram clearly explains the link between FKBP12 action and RAD001 treatment (Figure 12). In the case of FKBP12 silencing, RAD001 mediated mTOR inhibition is lost there by the downstream mTOR targets will be activated leading to cell proliferation and metabolism.

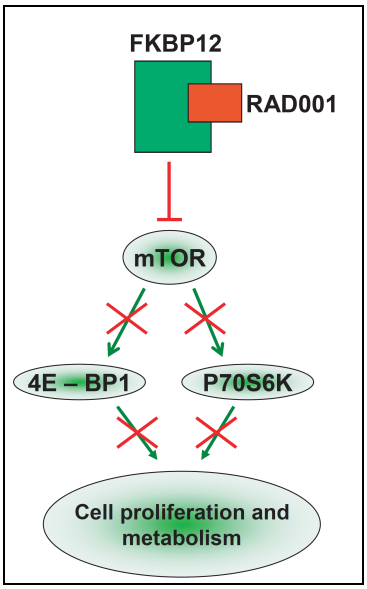

Figure 12: Schematic representation of mTOR pathway after using RAD001

FKBP12 come into the action in the presence of RAD001 and it forms a complex and binds to mTOR, thereby it prevents the mTOR phosphorylation and leads to dephosphorylation of 4E-BP1 and P70S6K. Eventually it drives the inhibition of cell proliferation and metabolism.

2 Aim of the study

The aim of the study was to determine the role of PI3K/mTOR inhibitors alone and in combination with chemotherapeutic drugs in HCC treatment and also to establish an in *vitro* system, to perform the genome wide shRNA screening in order to identify genes that mediate resistance or sensitivity to mTOR directed therapies.

3 Materials and Methods

3.1 Cell lines

3.1.1 Huh7 cell line

Huh7, is a well differentiated human hepatocellular carcinoma cell line that was originally taken from a liver tumor in a 57-year-old Japanese male in 1982, produces high levels of alpha-fetoprotein and having a point mutation of p53 (codon 220).

3.1.2 HepG2 cell line:

HepG2 is a hepatocellular carcinoma cell line which was derived from the liver tissue of a 15 year old Caucasian American male with a well differentiated hepatocellular carcinoma. These cells are epithelial in morphology. This cell line has been proven to be a useful model of the human liver cell as there is high proportion of liver-specific proteins identified in the medium, for which there are antisera commercially available (Liu et al. 1985). Thus, the studies encourage a wider application of this HepG2 cell line to biological problems that relate specifically to the role of the liver (Javitt 1990).

3.1.3 Pheonix cells

The Phoenix Retroviral Expression System offers the most time-tested method for producing infectious, replication-incompetent retrovirus that can efficiently transfer genes into a variety of mammalian cell types in vitro or in vivo. It is based on Moloney Murine Leukemia Virus (MMULV) and allows for delivery of genes to most dividing

mammalian cell types, including many difficult-to-transfect cells. The main features of Pheonix cells are, fast and high-titre virus production, production of helper free ecotropic and amphotropic retroviruses and are highly transfectable with either calcium phosphate mediated transfection or lipid-based transfection protocols.

3.1.4 HEK 293T cells

HEK 293 (Human Embryonic Kidney) T is a derivative of 293 cells that stably express the large T-antigen of SV40. Plasmids containing the SV40 origin are replicated to a copy number of between 400 - 1000 plasmids per cell within 293T, and therefore express at a higher level. The HEK293T cell line is for use in facilitating optimal lentivirus production.

3.1.5 Culture medium

Except HepG2 all cells were maintained in DMEM Glutamax medium (GIBCO) supplemented with 10% fetal calf serum (PAA) and penicillin 100 U\ml, streptomycin 100µg\ml antibiotics (Biochrom AG) at 37°C in 5% CO_2 in humidified chamber but HepG2 cells were maintained in RPMI Glutamax medium (GIBCO).

3.2 Nude mice

Nude mouse is a strain with a genetic mutation which leads to the lack of thymus. The genetic basis of the nude mouse mutation is a disruption of the FOXN1 gene resulting in an inhibited immune system due to a greatly reduced number of T cells. The phenotype of the mouse is a lack of body hair, which gives it as the "nude" nickname. The nude mouse is valuable to research because it can receive many different types of tissue and tumor grafts, as it mounts no rejection response. Therefore, nude mouse entry came into cancer research field with major importance.

Nude male mice (nu\nu) of 8-10 weeks old were used as an *in vivo* model of this study and purchased from Animal Centre of Hannover Medical School. Mice were housed in rooms with constant temperature, humidity and 12hrs light/dark cycles. Food and water were available *ad libitum*. Animal care and experiments were all in accordance with the guidelines of the department of animal care at Hannover Medical School, Hannover, Germany.

3.3 Chemotherapeutic drugs

Chemotherapeutic drugs Doxorubicin (Sigma), Cisplatin, 5FU (5Fluro Uracil) and Irinotecan were supplied by Hannover Medical School therapeutic drug centre.

3.4 PI3K/mTOR inhibitors

3.4.1 RAD001 (Everolimus)

RAD001, an orally available drug and is a rapamycin analogue, was provided by Novartis Pharma AG. RAD001 was formulated at 2% (w/v) in a micro emulsion vehicle, which was diluted in DMSO for *in vitro* studies and in water for *in vivo* studies. Animals were treated with 10mg/Kg body weight.

3.4.2 NVP-BKM120

NVP-BKM120 is an orally available drug and was provided by Novartis Pharma AG. For *in vitro* studies, it was diluted in DMSO and for *in vivo* studies it was dissolved in 1 volume of NMP (1-methyl-2-pyrrolidone: #Fluka) and 9 volumes of PEG300 (Fluka). Animals were treated with this solution in the next 30 minutes/1hr with 60mg/Kg body weight in a volume of 7.5ml/Kg bodyweight.

3.4.3 NVP-BEZ 235

NVP-BEZ235 is an orally available drug and was provided by Novartis Pharma AG. For *in vitro* studies, it was diluted in DMSO and for *in vivo* studies it was dissolved in 1 volume of NMP (1-methyl-2-pyrrolidone #Fluka) and 9 volumes of PEG300 (Fluka). Animals were treated with this solution in the next 30 minutes/1hr with 45mg/Kg body weight in a volume of 7.5ml/Kg bodyweight.

3.5 MTT assay

HCC cells were seeded in 96 well plates at a density of $1X10^4$ cells –Huh7 and $2X10^4$ cells–HepG2 per well (200µl) and were grown for 16 hrs. Then the cells were treated with respective PI3K/mTOR inhibitors and chemotherapeutic drugs alone and combination for 48hrs. After treatment period, 20µl of MTT solution (Promega) was added to each well and absorbance was measured at 490nm.

3.6 Crystal violet staining

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well (200µl) and were grown for 16 hrs. Then the cells were treated with respective PI3K/mTOR inhibitors and chemotherapeutic drugs alone and combination for 48hrs. Next, wash the cells with 200ul of PBS, fix the cells with 1% Glutaraldehyde (PAA) solution 100ul per well for 20 min at RT. A fresh stock of 25% glutaraldehyde was made each time. Staining of the cells was done with 0.02% crystal violet (made up in 10%EtOH) (Sigma) by adding 100ul per well at RT for 30 min. Cells were washed with 200ul of sterile water 2 X 15 minutes at room temperature. Then crystal violet was dissolved with 180ul of 70% ethanol per well. Keep it on shaker for 3-4 hrs at 400rpm. Make dilutions with sterile water and measure the absorbance at 590 nm.

3.7 Immunoblotting experiments

For *In vitro* studies, cells were seeded at a density of $1.5x10^6$ cells in 10cm dish and incubated for 16hrs at 37°C with 5% CO_2. Cells were treated with RAD001, NVP-BKM120, NVP-BEZ235, Doxorubicin, Cisplatin, 5FU, Irinotecan alone and in combination with each other for 48hrs. Cells were then lysed in 1x lysis buffer (Cell Signalling) containing 1X protease Inhibitor mixture (Roche, Mannheim, Germany), centrifuged for 10min at 15000 rpm. Then supernatant was collected.

For *In vivo* studies, tumour tissue was lysed in 1x lysis buffer (Cell Signalling) containing 1X protease Inhibitor mixture (Roche, Mannheim, Germany), centrifuged for 10min at 15000 rpm. Then supernatant was collected. Protein quantification was done from the supernatant by adding Bio-Rad Protein Assay Dye Reagent (80µl of diluted protein extract/distilled water to 20µl reagent) and measured OD at 595nm in a spectrophotometer. Then, 100µg protein extracts containing an electrophoresis sample buffer (4x ESB: 0,5M Tris Base, 40% glycerol, 12% SDS, 0,08% w/v bromophenol blue, 20% β-mercaptoethanol) were resolved in SDS-PAGE with 20mA and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a western transfer apparatus (Bio-Rad Mini-Protean) with a transfer buffer (25mM Tris base, pH 8.3, 192mM glycine) for 1h at 100V. After blotting, membranes were blocked with 5% dry milk in TBS-Tween (50mM Tris base, pH 7.5, 150mM NaCl, 0.1% Tween) for 30min at RT. Then membranes were incubated with the primary antibody solution (5% dry milk in TBS-Tween for unphosphorylated antibodies and 5% BSA in TBS-Tween for phosphorylated antibodies) overnight at 4°C. Afterwards, membranes were washed with TBS-Tween and incubated with the respective secondary antibody (5% dry milk in TBS-Tween) for 1h at RT and washed with TBS Tween. Detection of protein band was done using the chemiluminescence kit (Western Lightning®–ECL, Enhanced Chemiluminescence Substrate, and Perkin Elmer) and enhanced chemiluminescence films (HyperfilmTM ECL, Amersham/GE Healthcare). Ponceau red staining was performed with each membrane to demonstrate equal protein loading of the samples.

3.8 Primary Antibodies

Name	Company
4E-BP1	Cell Signalling
c-jun	Cell Signalling
cyclin A	Santa Cruz Biotechnologies
cyclin B1	Santa Cruz Biotechnologies
cyclin D1	Santa Cruz Biotechnologies
Phospho-Akt (Ser473)	Cell Signalling
Phospho-S6 ribosomal protein (Ser240/244)	Cell Signalling
p21	Santa Cruz Biotechnologies
p38	Cell Signaling
p-ERK	Cell Signalling

3.9 Secondary Antibodies

Name	Company
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnologies
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnologies
Donkey anti-goat IgG-HRP	Santa Cruz Biotechnologies

3.10 FACS experiments

3.10.1 Cell cycle analysis with PI (Propidium Iodide)

Huh7 cells were grown to 100% confluency in 6cm dish and allowed to grow 24 hrs extra to make tight contact inhibition. Then cells were splitted and seeded into new plates where cells are treated with all PI3K/mTOR inhibitors and chemotherapeutic drugs for 24hrs. Afterwards, cells were trypsinized and fixed in 70-75% ethanol. Then cells are centrifuged and suspended in 1ml solution containing 250µg of RNase (Sigma) and 20µg of propidim iodide (Sigma) with 1x PBS. Cells were kept at 37°C for 30 min. Cells were acquired with flow-cytometer (FACS Caliber, BD) and data was analysed with FlowJo software.

3.10.2 Apoptosis ananlysis with AnnexinV/PI staining.

Huh7 cells were seeded in 6 well plates at a density of $0.25X10^6$ cells per well and allowed to grow for 16hrs. Then cells were treated with all PI3K/mTOR inhibitors and chemotherapeutic drugs for 48hrs. Afterwards, supernatant was collected and rest adherent cells were trypsinized centrifuged along with supernatant cells at 1200rpm for 5min at room temperature. Then pellet was washed with 1xPBS and centifuged again at 1200rpm for 5min at room temperature. Rest of the protocol was performed according to manufacturer's protocol (BD). Cells were acquired with flow-cytometer (FACS Calibur, BD) and data was analysed with FlowJo software.

3.11 In vivo antitumor activity

Eight to ten week old male nude mice (nu\nu) were purchased from Animal Centre of Hannover Medical School and maintained under aseptic conditions as per MHH animal Care Centre. Healthy growing $9x10^6$ Huh7 cells were implanted into mice by subcutaneous injection in right or left rear dorsal flank region. Treatment was started when mice developed tumours at size of 5mm in any direction, using the higher

concentration of each molecular target which was recommended by manufacturer. Mice were treated by oral gavage (RAD001 20mg/kg, NVP-BKM 60mg/kg and NVP-BEZ 45mg/kg body weight) and cisplatin (i.p, 3mg/kg body weight) alone and combination of molecular target inhibitors with cisplatin. Mice were monitored daily. After 2 weeks of treatment, mice were sacrificed; size of the tumour was measured by calliper, body weight was measured, 0.5ml of blood from the orbital sinus or portal vein was collected and tumours were harvested. Tumour volume was calculated using the following formula.

Tumour volume $V = [(\pi/6) \times (\text{Length}) \times (\text{Width}^2)]$

3.12 Mouse blood collection

Mice blood was taken from the orbital sinus using a glass capillary tube or from portal vein and collected in tubes containing heparin (Li-heparin LH/1.3 ml, Sarstedt) and processed as per manufacturer instructions.

3.13 Mouse tumour collection

After sacrificing the mouse, tumour was separarted from the mouse and got rid of the skin and divided into four pieces. First and second pieces were frozen in liquid nitrogen and kept at -80°C for DNA, RNA and protein analysis. Third piece of the tumour was fixed in formalin and embedded in paraffin. The fourth piece was embedded in OCT and kept at -20°C.

3.14 Histology

3.14.1 Hematoxylin and Eosin (H&E) staining

Paraffinized tissue was sectioned into 5µm size and then deparaffinized in xylene and rehydrated in 3 different concentrations of ethanol solutions. Next slides were washed with distilled water and nucleus was stained with hematoxylin solution (Merck) for 15-30sec. Sections were washed with warm tap water to allow the stain to develop. After rinsing with distilled water, cytoplasm was stained with acidified eosin solution (Sigma-Aldrich) for 2min. Sections were rehydrated, cleared and mounted with a xylene-based mounting medium.

3.14.2 Ki67 staining

Proliferation of cancer cells (Huh7) was determined by the presence of Ki67 cell cycle-related nuclear protein expressed in all phases of the cell cycle. Tissue sections of 5µm were deparaffinized and rehydrated. Then washed with distilled water and antigen unmasking was performed by incubating sections in EDTA retrieval buffer (1mM EDTA,10mM Tris Base, 0.05% Tween) in a water bath at 95°C for 45min. After cooling down and washing with distiller water, endogenous peroxidases were blocked with 3% H_2O_2 for 10min. Endogenous avidin/biotin were blocked with Avidin/Biotin solutions (Avidin/Biotin Blocking Kit, Vector laboratories) for 15min each at RT. To reduce unspecific binding of antibodies, sections were blocked with 5% normal goat serum for 1h at RT. Then, sections were incubated with anti-Ki67 antibody (1:1000, 2% normal goat serum, Vector laboratories) overnight at 4°C. The next day, sections were incubated with broad-spectrum plus antibody (Vector laboratories) for 30min at RT. After washing, sections were incubated with HRP-Streptavidin Plus (Vector laboratories) for 30min at RT. Then, detection was done by adding AEC solution (Vector laboratories) until the desired stain intensity developed. Sections were counterstained with hematoxylin and mounted with a water-based mounting media (Vector laboratories).

3.14.3 pHis3 staining

Proliferation of cancer cells (Huh7) was determined by the presence of pHis3 cell cycle-related nuclear protein expressed in cell cycle. Tissue sections of 5µm were deparaffinized and rehydrated. Then washed with distilled water and antigen unmasking was performed by incubating sections in EDTA retrieval buffer (1mM EDTA,10mM Tris Base, 0.05% Tween) in a water bath at 95°C for 15min. After cooling down and washing with distiller water, endogenous peroxidases were blocked with 3% H_2O_2 for 10min. To reduce unspecific binding of antibodies, sections were blocked with 5% normal horse serum for 1h at RT. Then, sections were incubated with anti-pHis3 antibody (1:100, 5% normal horse serum, cell signalling #9701) overnight at 4°C. The next day, sectiones were washed and incubated with secondary antibody (1:100, goat anti-rabbit, Invitrogen) for 30 min at room temperature. After washing, sections were incubated with HRP-Streptavidin Plus (Vector laboratories) for 30min at RT. Then, detection was done by adding AEC solution (Vector laboratories) until the desired stain intensity developed. Sections were counterstained with hematoxylin and mounted with a water-based mounting media (Vector laboratories).

3.14.4 TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay

Detection of DNA strand breaks in apoptotic cells was confirmed with an *in situ* cell death detection kit (Roche). Five micrometer sections were deparaffinazed and rehydrated. Then washed with distilled water, endogenous peroxidases were blocked with 3% H2O2 for 10min. Antigens were unmasked by cooking sections in a microwave for 15min using citric acid retrieval buffer (10mM Tris Base, 0.05% Tween). Tissue permeabilization was done by incubating sections in a 20µg/ml proteinase K solution. Then, sections were fixed with 4% paraformaldehyde for 1h. After a second permeabilization with 0.1% Triton/0.1% sodium citrate, TUNEL analysis was performed by a fluorescein detection system, using reagents from a Roche kit. Sections were mounted with a DAPI-containing medium. Positive cells were counted using microscope.

3.14.5 CD31 and αSMA staining

Double staining for CD31 (green) and α-SMA (red) of cryosectioned tumor samples treated either with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks. CD31-positive tumor vessels are partially covered by α-SMA-positive pericytes (arrows) and contain α-SMA-positive fibrotic areas (arrow heads). This experiment was performed as per Skuli N 2009.

3.15 shRNA oligos for FKBP12 gene

shRNAs were designed against FKBP12 gene and each number denotes the position of designing shRNA on FKBP12 gene.

791
TGCTGTTGACAGTGAGCGCTTCTATGTAGTTTATAAATTATAGTGAAGCCACAGATGTATAA
TTTATAAACTACATAGAAATGCCTACTGCCTCGGA

764
TGCTGTTGACAGTGAGCGAATGGAGATGGCAGCTGTTTAATAGTGAAGCCACAGATGTAT
TAAACAGCTGCCATCTCCATGTGCCTACTGCCTCGGA

740
TGCTGTTGACAGTGAGCGAATGTGTATGTTCCCAGTTCAATAGTGAAGCCACAGATGTATT
GAACTGGGAACATACACATGTGCCTACTGCCTCGGA

689
TGCTGTTGACAGTGAGCGCTAGTGTATGCTTTGAATGTAATAGTGAAGCCACAGATGTATT
ACATTCAAAGCATACACTAATGCCTACTGCCTCGGA

472
TGCT
GTTGACAGTGAGCGATTCGATGTGGAGCTTCTAAAATAGTGAAGCCACAGATGTATTTTAG
AAGCTCCACATCGAAGTGCCTACTGCCTCGGA

262
TGCTGTTGACAGTGAGCGAATGCTTGAAGATGGAAAGAAATAGTGAAGCCACAGATGTATT
TCTTTCCATCTTCAAGCATCTGCCTACTGCCTCGGA

3.16 Amplification and purification of shRNA oligos

shRNAs are amplified by below protocol

PCR Program		
Initial denaturation	94°C	5 min
Cycle start		
Denaturation	94°C	20 sec
Annealing	52°C	20 sec
Elongation	68°C	25 sec
Cycle end		
Final elongation	68°C	5 min
End	4°C	∞

	Total volume 50µl	End concentration
Template (5ng/ µl)	2µl	10ng
Water	37µl	-
10x PCR buffer	5µl	1x
50mM $MgSO_4$	1µl	1mM
10mM each	1.5µl	0.3mM each
5' miR-Xho 0,1µM	1.5µl	3nM
3' miR-Eco	1.5µl	3nM
Pfx DNA Pol(Invitrogen #11708-013	0.5µl	1.25 Units

After amplification PCR products were confirmed on 2% agarose gel (Invitrogen) and then purified with PCR purification columns (Qiagen) according to the manufactures protocol.

3.17 Restriction digestion of amplified products (inserts) and vector MLP (Moloney murine leukemia vector with puromycin selection)

Amplified products were subjected to restriction digestion.

Insert	
	60µl total
Cleaned up template (0,2 – 1µg)	50µl
Buffer 4	6µl
100µg/ml BSA	0.6µl
XhoI (NEB #R0146S)	1µl
Water	1.5µl
EcoRI (NEB #R311S)	1.0µl
2 hr restriction at 37°C	

Vector	
	30µl total
vector	10µl
Buffer 4	3µl
100µg/ml BSA	0.3µl
XhoI (NEB #R0146S)	2.5µl
water	11.7µl
EcoRI (NEB #R311S)	2.5µl
2 hr restriction at 37°C	

3.18 Ligation and clonning

After restriction digestion, vector was treated with Cip Phosphatase (NEB #M0290S) and incubates for 1.5 hr at 37°C. Then vector was purified with PCR purification columns (Qiagen) according to the manufactures protocol.

Ligation	
	Total volume 20µl
Restricted vector	200ng
Restricted insert	30ng
Elution Buffer (from MN Kit)	5µl
T4 Ligase Buffer (Rapid kit)	10µl
T4 DNA Ligase	1µl
1hr at Room temperature	

After ligation, 5µl ligation mixture was added to prechilled 50µl DH5α (*E. coli*) competent cells and kept on ice for 30 min. Then heat shock was performed at 42°C for 30 seconds. Then kept on ice for 10 min and added 500µl of LB medium and plated 250µl on LB agar ampicillin50 plate (50µg/ml medium).

LB medium 1000 ml	
NaCl (Sigma)	5g
Trypton (Fluka)	10g
Yeast extract (Sigma)	5g
water	1000ml
Autoclaving	

LB plates (500ml)	
NaCl (Sigma)	2.5g
Trypton (Fluka)	5g
Yeast extract (Sigma)	2.5g
water	500ml
Agar	5g
Autoclave, cool down to warm temperature and add antibiotics	

3.19 Plasmid isolation, restriction digestion and sequencing

Four colonies were picked from each plate and inoculated into LB amp100 medium. Incubated the culture overnight and plasmid isolation was performed according to manufatureer kit protocol (Qigen). Restriction digestion was performed using EcoR1 and Xho1 enzymes inorder to confirm the clones.

Restiction digestion	
	Total volume 20µl
DNA	5µl
Buffer 4	2µl
100µg/ml BSA	0.2µl
XhoI (NEB #R0146S)	0.5µl
water	11.8µl
EcoRI (NEB #R311S)	0.5µl
2 hrs restriction at 37°C	

Correct clones were sequenced using mir30 primers. After confirmation of correct clone with sequencing, again transformation was performed with correct clone and midiprep (Qiagen) was carried out to get enough amount of plasmid DNA.

3.20 Preparation of large amount of helper plasmid DNA for retroviral and lentiviral transfection

Helper plasmid DNAs were transformed into DH5α competent cells (which was explained in previous experiments) and midi prep was performed according to manufacturer's protocol (Qiagen).

3.21 Retroviral transfection

Pheonix (packaging cells) cells were seeded in 6cm Petri dish (2 plates) at a density of $2X10^6$ cells and were grown for 16 hrs.

For each plate:

Solution A: plasmid DNA 10µg, helper DNA 5 µg, 31.25µl from 2M $CaCl_2$ (Fluka) and makeup the volume with sterile water to 250µl.

Solution B: 250µl of 2x HBS - 280nM Nacl (Sigma), 50mM HEPES (sigma), 1.5mN Na_2HPO_4 (Fluka), 12mM glucose (Sigma) and 10mM KCl (Roth) with pH 7.

Mix drop wise solution A to solution B. Use a pipette aid as a means to blow bubbles through solution B. Use a Pasteur pipette for drop by drop adding solution A to solution B while the pipette aid blows bubbles. Then leave the mixture at room temperature for 15 min. In the mean time remove the growth medium from the packaging cells and replace with 3.5ml of freshly made chloroquine 25mM (Sigma) containing DMEM medium. Add the mixture to the cells and leave for 10-12 hrs. Change the media after 12 hrs, and collect the virus every 24 hrs for 3 days. Virus can be stored in 4 degrees for up to one week. Virus was filtered through 0.45µm filter and polybrene 8µg/ml (Sigma) was added and the mixture was added to target cells (Huh7) with 20% confluency. Huh7 cells were transduced twice a day with 4ml virus for 4hrs and again 4ml of virus for 4hrs. Then remove the virus and replace with fresh DMEM medium to

regenerate the cells for over night. This mode of treatment was performed for 3 days. Then cells were selected under puromycin selection with 10µg/ml.

3.22 GFP enrichment assay

Cell population was cultured as 1:1000 ratios of FKBP12 silenced and normal Huh7 cells. These cells were subjected to RAD001 treatment for 3 days, 5 days and 10 days. GFP population was acquired by FACS and analysed by flowjo software.

3.23 Electroporation of shRNA pools

2µg of plasmid DNA was taken from each pool (contains 1000 shRNAs), and a total of 5 pools were mixed. From the mixture, 1µl was taken and added to 20µl of elecltrocompetent cells (DH10 beta mega invitogen). This comlete mixture was transferred into a cuvette and subjected to 2kv and 200 pulse controller (Bio rad gene pulser cuvette. 0,1cm electrode). Then 1ml recovery medium was added to the mixture and incubated at 37° C for 40 minutes. 1µl from this mixture was added to 1ml LB medium (1:1000). Rest of the mixture was spreaded on to two ampicillin 50 petridishes. In order to confirm the transformation efficiency, 50µl was taken from the diluted one (1:1000) and was spreaded on to a new petridish. This is to confirm the transformation of all hairpins by counting the colonies. Theoretically, 1ml main stock contains $5X10^6$ colonies and the diluted medium (1:1000) contains $5X10^3$ colonies. Therefore 50µl of the culture should theoretically give rise to 250 colonies. When colonies were between 200-250, experiment was continued further. All colonies were collected by scraper and added to 40 ml LB amp50 medium and were incubated at 37°C for 8hrs on a shaker and then transferred to 400ml culture and incubated overnight at 37°C on an orbital shaker at 220rpm. Culture was harvested and plasmid DNA isolation was carried out by Qiagen maxiprep kit.

3.24 lentiviral transfection

293T (packaging cells) cells were seeded in 10cm Petri dish at a density of $2X10^6$ cells and were grown for a 70% confluency.

For one plate:

Solution A: Plasmid DNA 20µg, pMDLg 10µg, pRSV10µg, pMD2G-10ug and 62.5µl from 2M $CaCl_2$ (Fluka) and makeup the volume with sterile water to 500µl.

Solution B: 500µl of 2x HBS - 280nM Nacl (Sigma), 50mM HEPES (sigma), 1.5mN Na_2HPO_4 (Fluka), 12mM glucose (Sigma) and 10mM KCl (Roth) with pH 7.

Mix drop wise solution A to solution B. Use a pipette aid as a means to blow bubbles through solution B. Use a Pasteur pipette for drop by drop adding solution A to solution B while the pipette aid blows bubbles. Then leave the mixture at room temperature for 15 min. In the mean time remove the growth medium from the packaging cells and replace with 9ml of freshly made chloroquine 25mM (Sigma) containing DMEM medium. Add the mixture to the cells and leave for 10-12 hrs. Change the media after 12 hrs, and collect the virus every 24 hrs for 3 days. Virus can be stored in 4 degrees up to one week. Huh7 cells which were seeded at a density of $2.75X10^6$ cells in $25cm^2$ T flask and allowed to grow for overnight. Virus was filtered through 0.45µm filter and polybrene 8µg/ml (Sigma) was added and then, cells were transduced with virus to estimate 10-15% MOI (Multiplicity of Infection). After 24hrs of transduction, cells were trypsinized and cells were acquired with flow-cytometer (FACS Calibur, BD) and data was analysed with FlowJo software. As transduced cells express GFP, it was easy to find out 10-15% MOI. Virus was filtered through 0.45µm filter and polybrene 8µg/ml (Sigma) was added and put it on $20X10^6$ target cells (Huh7) cells which are in $175cm^2$ T flask. After 24hrs of transduction, Huh7 cells were regenerated with fresh DMEM medium for 24hrs. Then cells were selected under puromycin selection. Selected cells are subjected to RAD001 and Placebo treatment for 10 days with one splitting on 5^{th} day and genomic DNA was isolated (pure gene kit, Qiagen)

3.25 Statistical analysis

Data was represented as the mean ± standard deviation. Two-way analysis of variance or Student *t*-test was measured to determine the significance.

4. Results

4.1: Therapeutic approaches for Hepatocellular Carcinoma using PI3K/mTOR inhibitors and combination with cytotoxic drugs.

4.1.1: PI3K/mTOR molecular target inhibitors have efficient inhibition on Huh7 cell proliferation

In order to find out the effect of PI3K/mTOR molecular target inhibitors – RAD001, NVP-BKM120 and NVP-BEZ235 on cell viability and also 50% inhibitory concentration (IC_{50}), MTT colorimetric assay was performed in Huh7 cells. The cells were treated with the drugs for 48hrs. RAD001 showed cytostatic effect on cell proliferation from 10nM to higher concentrations (Figure 13) and an IC_{50} concentration of 50nM was used for further studies. NVP-BKM120 treatment was efficient in inhibiting the cell proliferation by dose dependent manner (Figure 14) and an IC_{50} concentration of 1000nM was used for further studies. NVP-BEZ235 had a very sensitive effect on Huh7 cells lines even at lower concentration (Figure 15) compared to NVP-BKM120 and an IC_{50} concentration of 10nM was used for further studies.

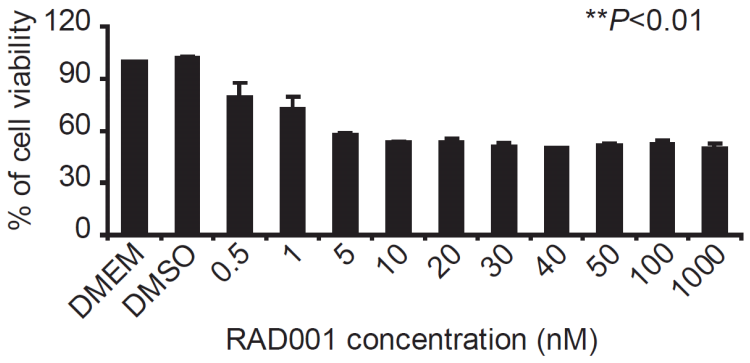

Figure 13: Effect of mTOR inhibitor (RAD001) on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of RAD001 for 48hrs and proliferation was measured by MTT assay. **P<0.01 value represents the treated vs DMSO values.

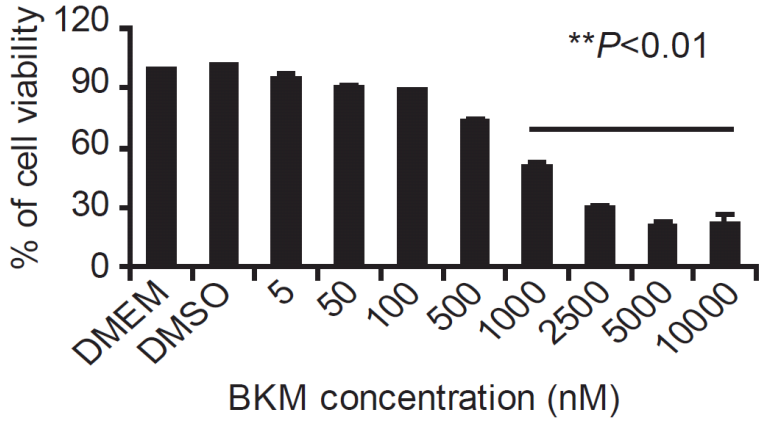

Figure 14: Effect of PI3K inhibitor (NVP-BKM120) on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of (NVP-BKM120) for 48hrs and proliferation was measured by MTT assay. **P<0.01 value represents the treated (represented with line) vs DMSO values.

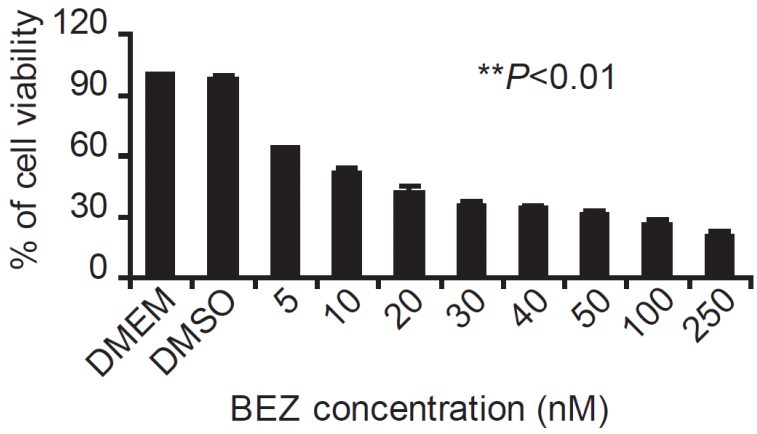

Figure 15: Effect of dual PI3K and mTOR inhibitor (NVP-BEZ235) on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of NVP-BEZ235 for 48hrs and proliferation was measured by MTT assay. **P<0.01 value represents the treated vs DMSO values

4.1.2: mTOR inhibitor RAD001 has efficient inhibition on HepG2 cell proliferation

In order to find out the effect of mTOR inhibitor– RAD001 on cell viability and also 50% inhibitory concentration (IC_{50}), MTT colorimetric assay was performed in another hepatocellular carcinoma cell line, HepG2. After 48hrs treatment with RAD001, HepG2 cells showed approximately 35μM of IC_{50} concentration (Figure 16).

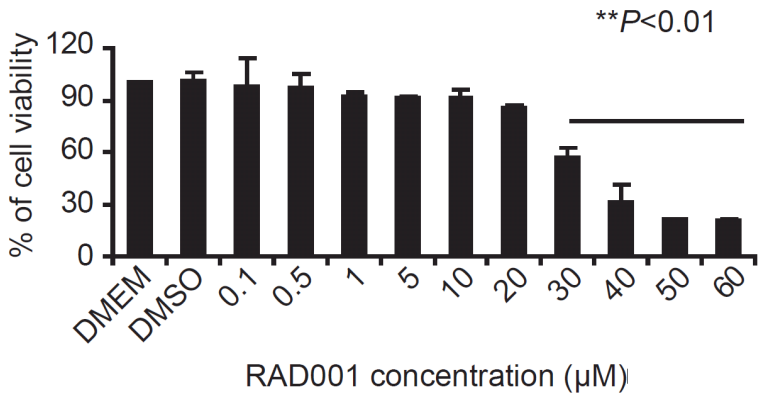

Figure 16: Effect of mTOR inhibitor (RAD001) on cell proliferation of HepG2 cell line

HepG2 cells were seeded in 96 well plates at a density of $2X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of RAD001 for 48hrs and proliferation was measured by MTT assay. **P<0.01 value represents the treated (represented with line) vs DMSO values.

4.1.3 Chemotherapeutic drugs have efficient inhibition on Huh7 cell proliferation

For chemotherapeutic drugs (Doxorubicin, Cisplatin, 5FU and Irinotecan) the dose dependency effect on cell proliferation and IC_{50} concentartion were determined by using MTT assay. The results had shown a dose dependent growth inhibition in Huh7 cells after 48hrs treatment for the respective drugs (Figure 17, 18, 19 and 20). Based on these results, we determined the IC_{50} concentration for each drug for further studies as follows, doxorubicin – 170nM, cisplatin - 8μM, 5FU – 10μM and irinotecan - 50μM concentrations. In addition to MTT assays to measure cell proliferation, we also used crystal violet staining assay using IC_{50} concentration of doxorubicin, cisplatin, 5FU and irinotecan. The results from this assay clearly supported the data from MTT assay (Figure 21).

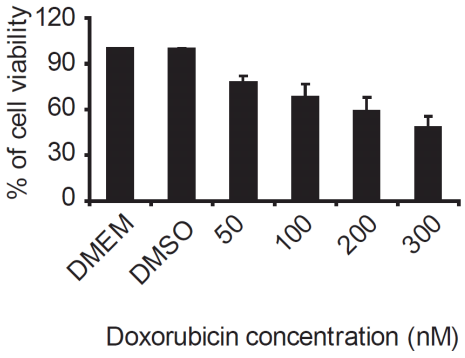

Figure 17: Effect of doxorubicin on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of doxorubicin for 48hrs and proliferation was measured by MTT assay. t-test value was $P<0.01$ between the treated vs DMSO values.

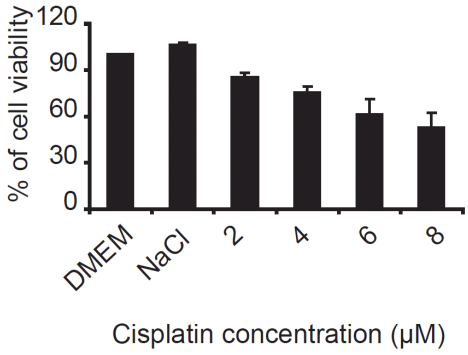

Figure 18: Effect of cisplatin on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of cisplatin for 48hrs and proliferation was measured by MTT assay. T-test value was $P<0.01$ between the treated vs NaCl values.

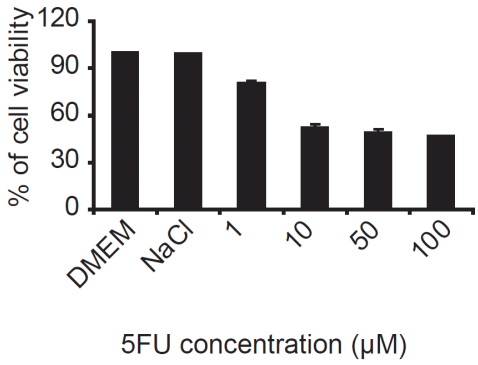

Figure 19: Effect of 5FU on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of 5FU for 48hrs and proliferation was measured by MTT assay. T-test value was $P<0.01$ between the treated vs NaCl values.

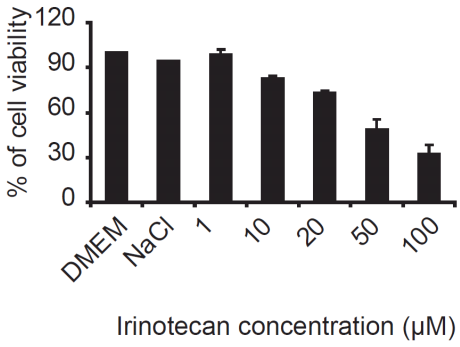

Figure 20: Effect of irinotecan on cell proliferation of Huh7 cell line

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated concentrations of Irinotecan for 48hrs and proliferation was measured by MTT assay. T-test value was $P<0.01$ between the treated (20μM-100μM) vs NaCl values.

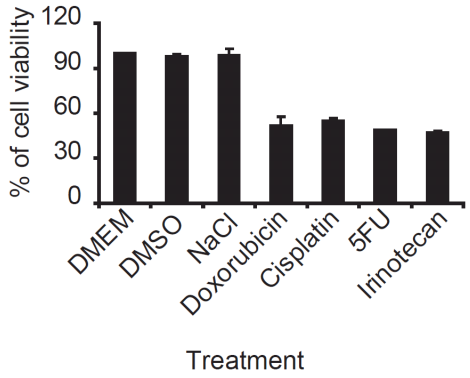

Figure 21: Assessment of chemotherapeutic drugs efficacy on cell proliferation of Huh7 cell line by crystal violet (CV) staining method.

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with indicated chemotherapeutic drugs with IC_{50} concentrations for 48hrs and proliferation was measured by CV staining method. t-test value was $P<0.01$ between the treated vs DMSO/NaCl values.

4.1.4: PI3K/mTOR molecular target inhibitors treatment resulted in the inhibition of respective signalling cascades in Huh7 cells

To assess the molecular mechanism of these PI3K/mTOR inhibitors in Huh7 cells, investigation of well known downstream mTOR targets, 4E-BP1 and P-S6 ribosomal proteins, were analzyed. Additionally, phosphorylation status of AKT, as an upstream target of mTOR was analzyed. RAD001 down regulated the expression of the P-S6 ribosomal protein completely and partially dephosphorylated 4E-BP1 upon mTOR inhibition. Contrary, phosphorylation of AKT was slightly induced. NVP-BKM120 treatment downregulated the P-S6 ribosomal protein, partially dephosphorylated 4E-BP1 and significantly reduced phosphorylation of AKT. Similarly to RAD001, NVP-BEZ235 downregulated the P-S6 ribosomal protein expression, partially dephosphorylated 4E-BP1 and slightly induced phosphorylation of AKT (Figure 22). However, AKT phosphorylation was repressed with higher concentration of NVP-BEZ235 (Figure 23).

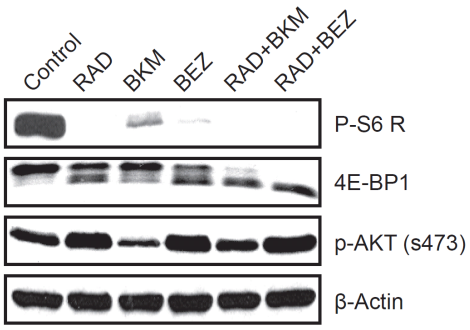

Figure 22: Analysis of P-S6R, 4E-BP1 and pAKT expression in Huh7 cells after treating with RAD001, NVP-BKM120, NVP-BEZ235 alone and in combination.

Analysis of the expression of P-S6R, 4E-BP1 and pAKT for 48hrs was performed with western blotting. β actin was used as an internal loading control.

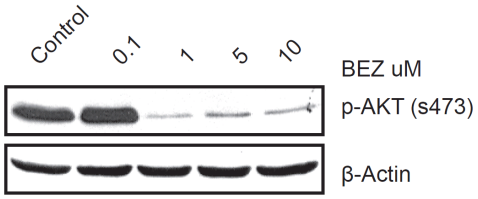

Figure 23: Analysis of pAKT expression in Huh7 cells after treating with higher doses of NVP-BEZ235.

Analyse the expression of pAKT in higher concentrations of NVP-BEZ235 for 48hrs was performed with western blotting. β actin was used as an internal loading control.

4.1.5: Synergistic effect of combination treatment using PI3K/mTOR molecular target inhibitors on cell proliferation inhibition

Cytostatic effect of RAD001 treatment on Huh7 cells, could lead the cells resistant to RAD001 treatment, as is the case in hepatocellular carcinoma patients (Ref). Therefore, the effect of combinatorial treatment of drugs that target either PI3K pathway alone (NVP-BKM120) or PI3K/mTOR pathway (NVP-BEZ235) with RAD001 was further investigated. Combination treatment of RAD001 with either NVP-BKM120 or NVP-BEZ235 significantly showed a synergistic effect on cell proliferation inhibition (Figure 24). Combination treatment led to almost 50% more inhibition on cell proliferation when compared to the single treatment. Thereby, combination treatment led to the Huh7 cell demise.

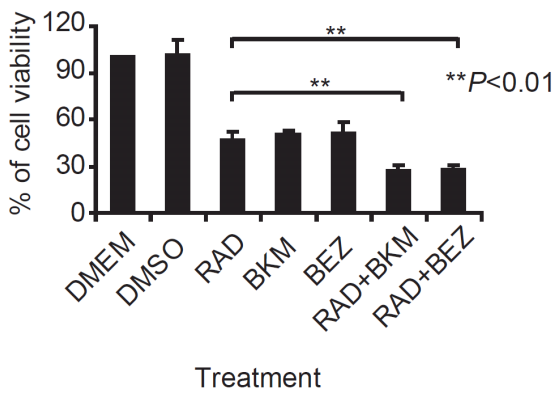

Figure 24: Effect of combination treatment of PI3K/mTOR inhibitors on cell proliferation of Huh7 cells.

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with using IC_{50} of RAD001, NVP-BKM120 and NVP-BEZ235 for 48hrs and cell proliferation was measured by MTT assay.

4.1.6: Synergistic effect of combination treatment using PI3K/mTOR molecular target inhibitors with chemotherapeutic drugs on cell proliferation inhibition

HCC patients were also treated with chemotherapeutic drugs, which were shown to have a moderate effect on controlling HCC. So, the next aim was to analyse the synergistic effect of PI3K/mTOR molecular target inhibitors in combination with classical chemotherapeutic drugs like doxorubicin, cisplatin, 5FU and irinotecan on Huh7 cell proliferation. Huh7 cells were treated with either RAD001 or NVP-BKM120 or NVP-BEZ235 in combination with chemotherapeutic drugs. MTT assay results clearly indicate that cisplatin and irinotecan combination with RAD001 had a significant influence on inhibiting cell proliferation compared to 5FU and doxorubicin combination (Figure 25, 26 and 27). The combination of RAD001 with cisplatin/irinotecan had an extra 50% inhibition compared to cisplatin/irinotecan single treatments.

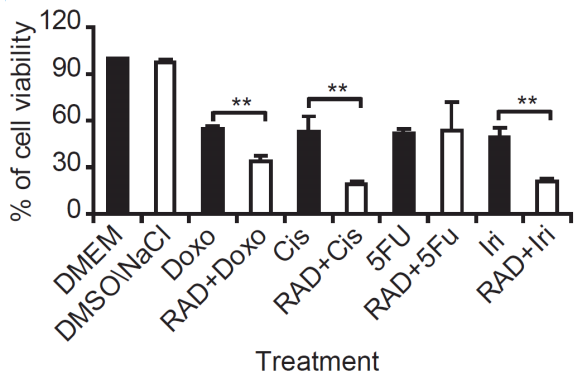

Figure 25: Effect of combination treatment of mTOR inhibitor (RAD001) and chemotherapeutic drugs on cell proliferation of Huh7 cell lines.

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with using IC_{50} of RAD001 and combination of chemotherapeutic drugs for 48hrs and cell proliferation was measured by MTT assay. ** indicates $P<0.01$ between single drug treatment vs combination treatment.

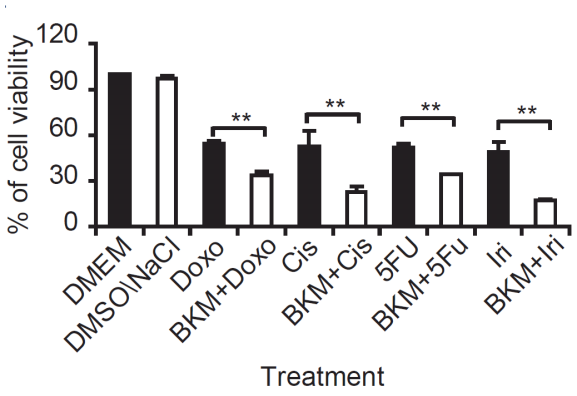

Figure 26: Effect of combination treatment of PI3Kinhibitor (NVP-BKM120) and chemotherapeutic drugs on cell proliferation of Huh7 cell lines.

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with using IC_{50} of NVP-BKM120 and combination of chemotherapeutic drugs for 48hrs and cell proliferation was measured by MTT assay. ** indicates P<0.01 between single drug treatment vs combination treatment.

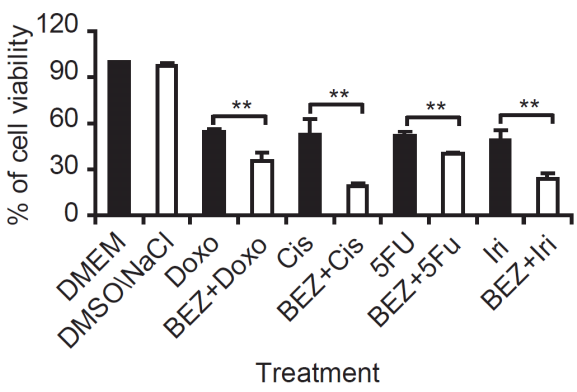

Figure 27: Effect of combination treatment of PI3K\mTOR inhibitor (NVP-BEZ235) and chemotherapeutic drugs on cell proliferation of Huh7 cell lines.

Huh7 cells were seeded in 96 well plates at a density of $1X10^4$ cells per well and were grown for 16 hrs. Then the cells were treated with using IC_{50} of NVP-BEZ235 and combination of chemotherapeutic drugs for 48hrs and cell proliferation was measured by MTT assay. ** indicates P<0.01 between single drug treatment vs combination treatment.

4.1.7: PI3K/mTOR molecular target inhibitors alone and in combination with chemotherapeutic drugs enhanced the inhibition of respective signalling cascades in Huh7 cells

The results from the cell proliferation assays clearly showed that combination treatment had a potential effect in inhibiting Huh7 cell proliferation, therefore molecular mechanisms involved in this phenomenon were elucidated. Major downstream targets of PI3K/mTOR pathway, P-S6R, 4E-BP1 and upstream signalling protein AKT phosphorylations were analyzed after the treatment of Huh7 cells with PI3k/mTOR inhibitors in combination with chemotherapeutic drugs. Combination of chemotherapeutic drugs with either RAD001 (Figure 28) or NVP-BEZ235 (Figure 30) dephosphorylated 4E-BP1 and P-S6 ribosomal protein expression, whereas in the case of AKT, phosphorylation of AKT was upregulated due to the inhibition on mTOR negative feedback loop mechanism. In Huh7 cells treated with NVP-BKM120 and chemotherapeutic drugs combination, 4E-BP1 and P-S6 ribosomal protein dephosphorylation was enhanced whereas phosphorylation of AKT was decreased (Figure 29).

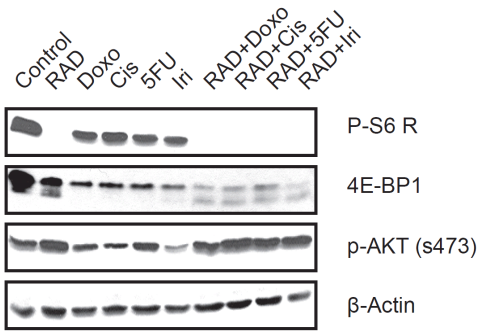

Figure 28: Analysis of P-S6R, 4E-BP1 and pAKT expression in Huh7 cells after treating with combination of mTOR inhibitor RAD001 and chemotherapeutic drugs.

Huh7 cells were seeded in 10cm petridish at a density of $1.5X10^6$ cells and were grown for 16 hrs. Then the cells were treated with using IC_{50} of RAD001 and combination of chemotherapeutic drugs for 48hrs, cells were harvested and western blotting was performed for indicated markers. β actin was used as an internal control.

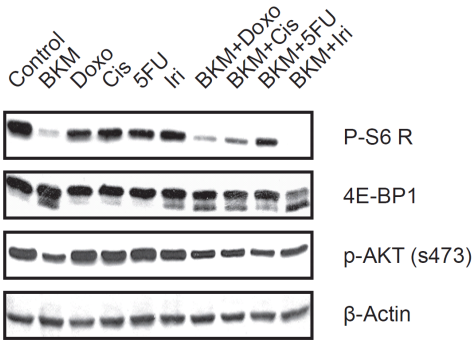

Figure 29: Analysis of P-S6R, 4E-BP1 and pAKT expression in Huh7 cells after treating with combination of PI3K inhibitor NVP-BKM120 and chemotherapeutic drugs.

Huh7 cells were seeded in 10cm petridish at a density of $1.5X10^6$ cells and were grown for 16 hrs. Then the cells were treated with using IC_{50} of NVP-BKM120 and combination of chemotherapeutic drugs for 48hrs, cells were harvested and western blotting was performed for indicated markers. β actin was used as an internal control.

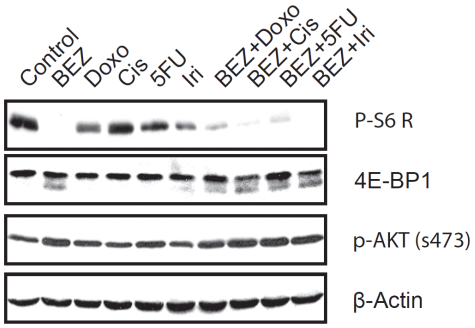

Figure 30: Analysis of P-S6R, 4E-BP1 and pAKT expression in Huh7 cells after treating with combination of PI3K/mTOR inhibitor NVP-BEZ235 and chemotherapeutic drugs.

Huh7 cells were seeded in 10cm petridish at a density of $1.5X10^6$ cells and were grown for 16 hrs. Then the cells were treated with using IC_{50} of NVP-BEZ235 and combination of chemotherapeutic drugs for 48hrs, cells were harvested and western blotting was performed for indicated markers. β actin was used as an internal control.

4.1.8: PI3K/mTOR molecular target inhibitors alone and combination with cisplatin led to cell cycle arrest in Huh7 cells

As per the cell proliferation assay results cisplatin and irinotecan showed significant synergistic effect in combination with PI3K/mTOR molecular target inhibitors in inhibiting Huh7 cell proliferation. As cisplatin is in use of clinical trials further studies were carried with cisplatin. To evaluate the antiproliferative activity and the effect on cell cycle by the PI3K/mTOR molecular target inhibitors alone and in combination with cisplatin in Huh7 cells, flow cytometric analysis was performed. Treatment of Huh7 cells with PI3K/mTOR molecular target inhibitors resulted in a higher accumulation of cells in G1 phase, with RAD001 arresting 53.6% of cells, NVP-BKM 120 and NVP-BEZ235, arresting 54.8% and 50.65% of cells in G1 phase (Figure 31). Cisplatin treatment led to accumulation of cells in S phase (56.9%) when compared to control (42.1%). Furthermore, in combination treatment cell cycle was arrested between G1 and S phase (Figure 32).

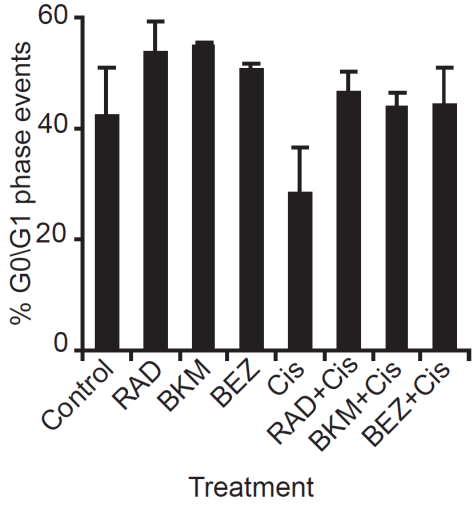

Figure 31: Assessment of cell cycle arrest by combination of PI3K/mTOR inhibitors and cisplatin.

Huh7 cells were synchronized by tight contact inhibition among them, then splitted and treated with indicated drugs for 24hrs and cell cycle experiment was performed by FACS calibre. % of G0\G1 phase population was analyzed with flowJo software.

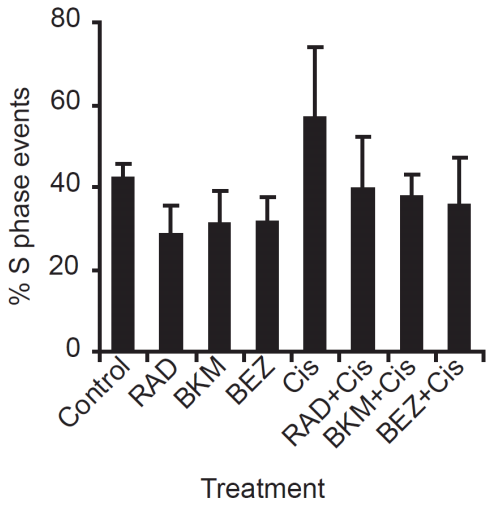

Figure 32: Assessment of cell cycle arrest by combination of PI3K/mTOR inhibitors and cisplatin.

Huh7 cells were syncronized by tight contact inhibition among them, then splitted and treated with indicated drugs for 24hrs and cell cycle experiment was performed by FACS calibre. % of S phase population was analyzed with flowJo software.

4.1.9: PI3K/mTOR molecular target inhibitors alone and in combination with cisplatin led to apoptosis induction in Huh7 cells

As cell proliferation inhibition could be due to cell cycle arrest/induction of apoptosis/ induction of autophagy/ necrosis, induction of apoptosis was studies in Huh7 cells after the drug treatment by annexin V/PI staining (Figure 33). Qauntification of apoptosis identified more apoptotic cells in RAD001 (5.3%), NVP-BKM120 (5.8%), NVP-BEZ235 (4.9%) and cisplatin (6.9%) treatments compared to control (3.74%). Combination of cisplatin with RAD001 (12.8%), NVP-BKM120 (9.51%) and NVP-BEZ235 (13.7%) showed increased apoptotic cells compared to single treatment (Fig 34).

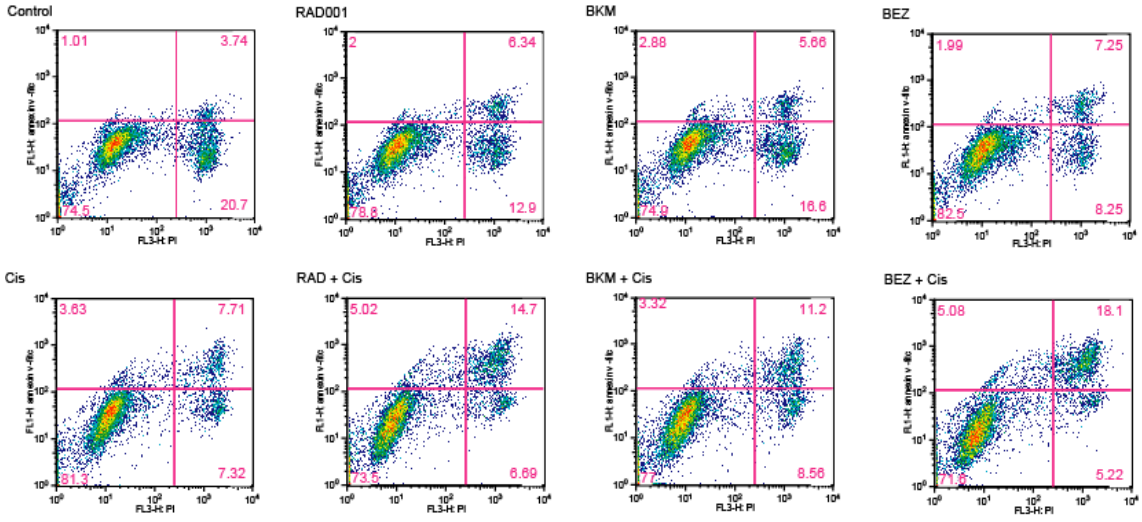

Figure 33: Assessment of apoptosis induction by combination of PI3K/mTOR inhibitors and chemotherapeutic drugs.

Huh7 cells were seeded in 6 well plate at a density of 0.25X10^6 cells per well and were grown for 16 hrs. Then the cells were treated with using IC_{50} of indicated drugs for 48hrs, cells are harvested, stained with AnnexinV\PI and apoptosis in cells was analyzed by FACS calibre. Data was analyzed with flowJo software. Data represents one out of two experiments done.

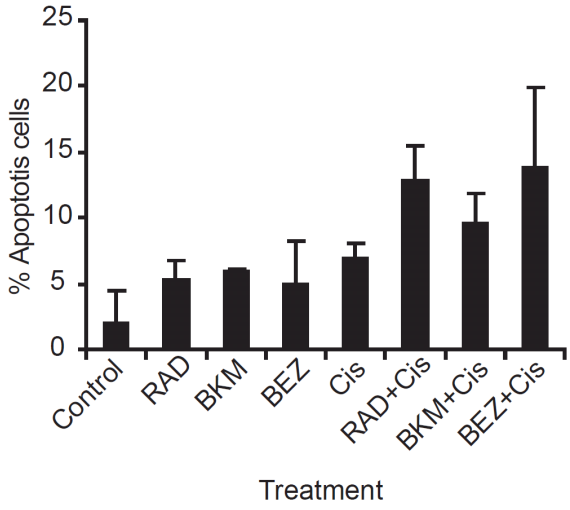

Figure 34: Quantification of apoptosis induction by combination of PI3K/mTOR inhibitors and cisplatin.

After analyzing the cells on FACS calibre (n=2) data was quantified with flowJo software.

4.1.10: PI3K/mTOR inhibitors induced tumor regression in xenograft model

In vitro data in Huh7 cells clearly showed that the combination of PI3K/mTOR molecular target inhibitors in combination with cisplatin plays significant role in inhibiting cell proliferation compared to their respective single treatments. Further studies were carried out to check the efficacy of these drugs *in vivo* by using nude mouse xenograft model. Nude mice were injected at the flank region with Huh7 cells and followed for tumor development. When tumor size reached 5mm size in any direction, then the mice were treated with respective drugs. Anti-tumor activity was observed in xenograft model after treatment with PI3K/mTOR molecular target inhibitors (Figure 35A). Tumor volume was effectively reduced in RAD001 (408 mm^3), NVP-BKM120 (69.9 mm^3) and NVP-BEZ235 (276.3 mm^3) treated mice compared to control (1485 mm^3) mice (Figure 35B). NVP-BKM120 had a prolific influence on tumor

regression followed by NVP-BEZ235 and RAD001. However, NVP-BKM120 treated mice were less healthy compared to other treated mice.

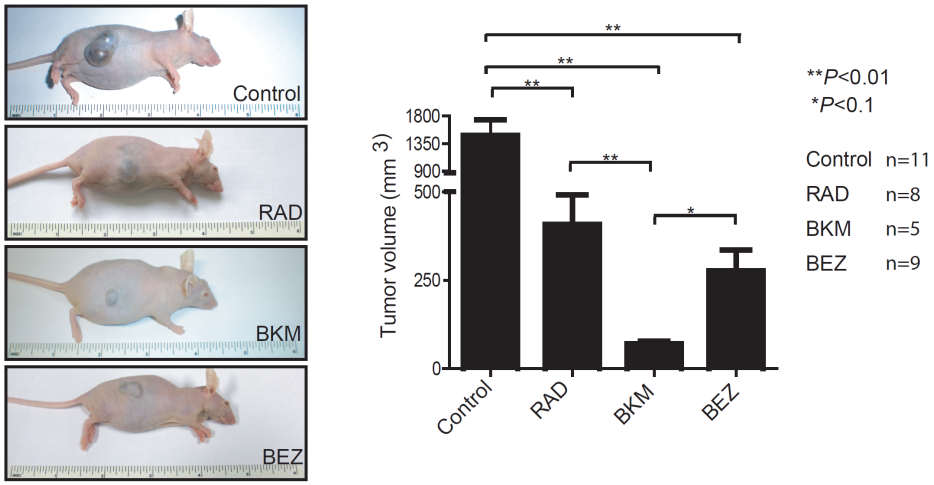

Figure 35: Analysis of tumor growth of Huh7 xenograft by PI3K/mTOR inhibitors RAD001, NVP-BKM120 and NVP-BEZ235.

A) Huh7 cells were subcutaneously injected into the nude mouse in a flank region on one side and when tumors are 5mm in size in any direction, treatment was started with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks. Representative pictures of tumor bearing mice after treatment period are shown here. B) Statistical representation of tumor volume of treated and control xenograft mice.

Tumor tissues from treated mice revealed great differences in morphology demonstrated by HE staining. Control mice and RAD001 treated mice showed more necrosis when compared to NVP-BKM120 and NVP-BEZ235 treated mice. Among treated tumor tissues, RAD001 treated tumors had more necrosis compared to all (Figure 36).

Immunohistochemistry results using Ki67 marker illustrated more population of proliferating cells in control tumor samples compared to treated mice (Figure 26).

pHis3 marker is one of the most prominent cell proliferation markers used to distinguish the proliferating cell population from non proliferating cell population. Immunohistochemistry results clearly showed the difference between treated and control tumor samples. Treated tissue samples have less pHis3 cells compared to control tissues. TUNEL staining results illustrated that there are a few more apoptotic cells in treated tissues compared to control (Figure 36). Quantification of the stainings showed that among the three drugs NVP-BKM120 and NVP-BEZ235 have a potential effect on tumor regression compared to RAD001 (Figure 37).

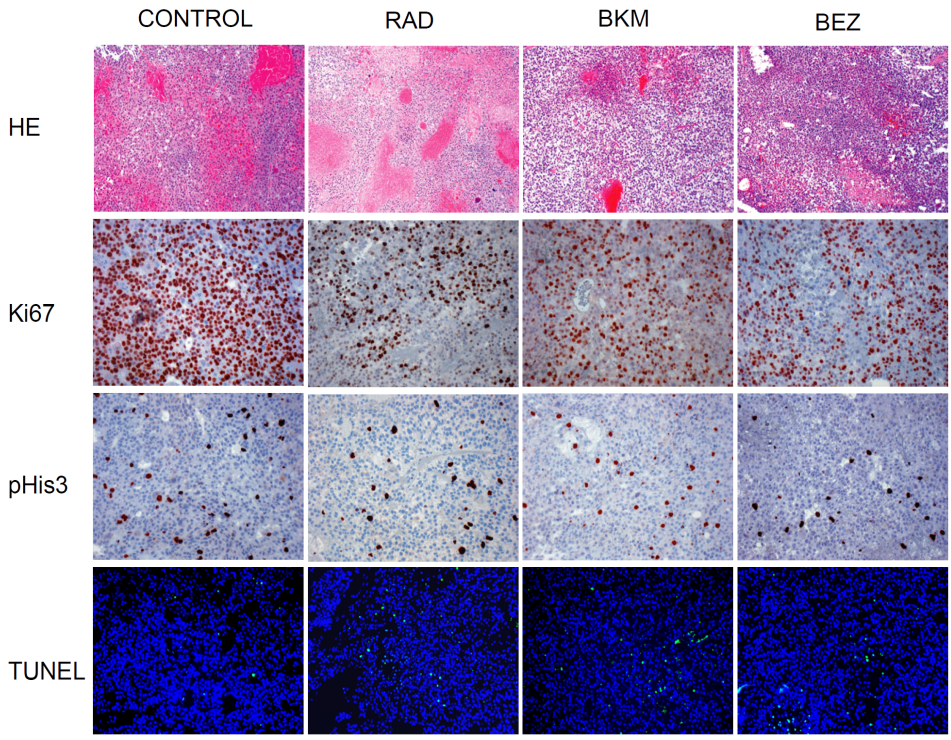

Figure 36: Histopathological analysis of Huh7 xenograft tumor tissue treated with PI3K/mTOR inhibitors RAD001, NVP-BKM120 and NVP-BEZ235.

Pathological analysis of tumor tissues treated with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks using H&E, Ki67, pHis3 and Tunel staining.

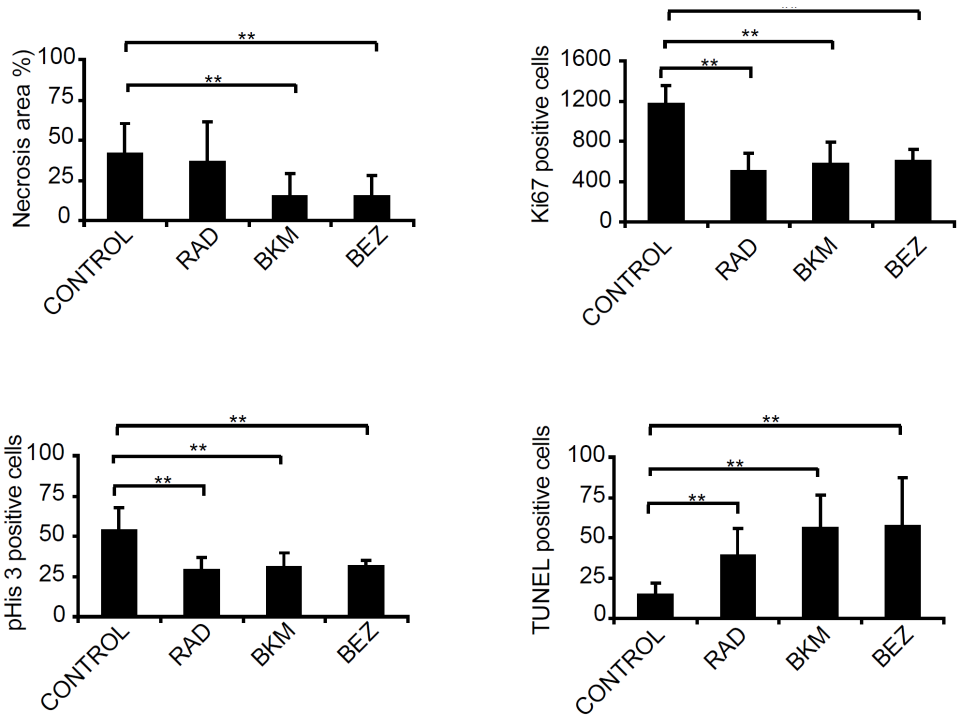

Figure 37: Quantification of histopathological data in Huh7 xenograft tumor tissue treated with PI3K/mTOR inhibitors RAD001, NVP-BKM120 and NVP-BEZ235.

Analysis of statistical data of pathological analysis of tumor tissues treated with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks using H&E, Ki67, pHis3 and Tunel staining. ** indicates P< 0.01 between control vs treated mice.

4.1.11: Effect of PI3K/mTOR molecular target inhibitors on angiogenesis in xenograft tumor tissue

Angiogenesis markers were examined to assess the expression status of CD31 and αSMA. It was known that CD31 is mainly involved in endothelial cell to cell interaction and drives angiogenesis. αSMA is generally expressed in smooth muscle cells of blood vessels. Immunohistochemistry with anti-CD31 and anti- αSMA clearly showed no difference in treated versus control tissues (Figure 38).

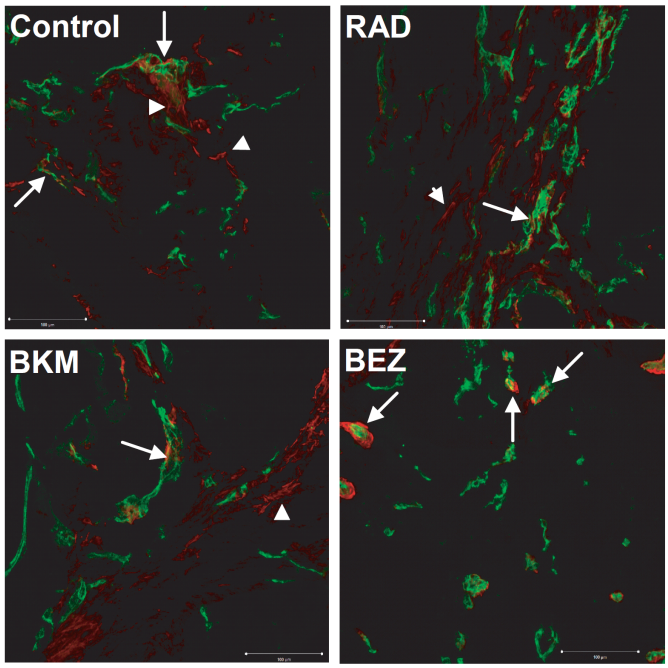

Figure 38: Angiogeneis analysis in xenograft tumor tissue upon treatment with PI3K/mTOR inhibitors RAD001, NVP-BKM120 and NVP-BEZ235.

Double staining for CD31 (green) and α-SMA (red) of cryosectioned tumor samples treated either with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks. CD31-positive tumor vessels are partially covered by α-SMA-positive pericytes (arrows) and contain α-SMA-positive fibrotic areas (arrow heads). Scale bar represents 100 µm.

4.1.12: Effect of PI3K/mTOR molecular target inhibitors on expression of molecular markers in mouse xenograft tissues

To investigate the phosphorylation of AKT, tumors were harvested 1hr post treatment with NVP-BEZ235 and NVP-BKM120. Western blots were performed for checking the phosphorylation status of P-S6R, 4E-BP1 and AKT in mouse xenografts. The results clearly showed a strong dephosphorylation of P-S6R, 4E-BP1 in treated tumors compared to control tumors and no difference in pAKT expression in treated and control samples (Figure 39).

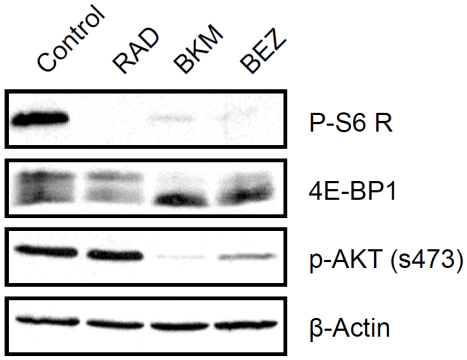

Figure 39: Analysis of mTOR markers and phospo-AKT expression in tumor tissues after treatment with RAD001, NVP-BKM120 and NVP-BEZ235.

Protein was isolated from tumor tissues treated with RAD001 10mg\Kg, BKM 60mg\kg and BEZ 45mg\Kg body weight on every day for 2 weeks and western blotting was performed for P-S6R, 4E-BP1, pAKT and βactin was an internal loading control.

4.1.13: Effect of PI3K/mTOR molecular target inhibitors on expression of other kinase pathways in mouse xenograft tissues

After checking the phosphorylation status of downstream markers of PI3K\mTOR pathway, we checked the expression status of p-JNK, c-JUN, p-P38, and p-ERK to assess the possible cross talk mechanism activation by RAD001, NVP-BKM120 and NVP-BEZ235. Western blot analysis clearly showed that there was no difference of p-JNK, c-JUN and p-P38 expression between treatment and control tissue samples. But pERK was showed activation in treated samples compared to control (Figure 40).

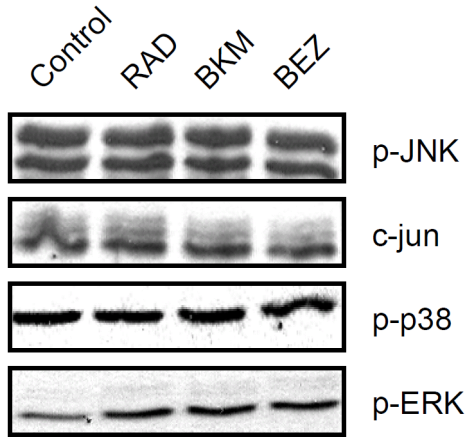

Figure 40: Analysis of JNK and MAPK pathway expression in tumor tissues after treating with RAD001, NVP-BKM120 and NVP-BEZ235.

Protein was isolated from tumor tissues treated with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks and western blotting was performed for p-JNK, c-jun, p-p38, p-ERK and βactin was used as an internal loading control.

4.1.14: Effect of PI3K/mTOR molecular target inhibitors on expression of cell cycle related proteins in mouse xenograft tissues

Cell cycle related proteins like cyclins expression was next analyzed. It was noticed that cyclin A and cyclin D1 were significantly down regulated in treated samples compared to control samples. It strongly confirms the G0/G1 phase arrest in cell cycle upon RAD001, NVP-BKM120 and NVP-BEZ235 treatment. CyclinB1 protein expression was also less in RAD001 treatment compared to control, whereas NVP-BKM120 and NVP-BEZ235 treatment had more down regulation than RAD001. In addition to these proteins p21 expression was analyzed. p21 protein expression was down regulated upon RAD001 treatment but not in NVP-BKM120 and NVP-BEZ235 treatment (Figure 41).

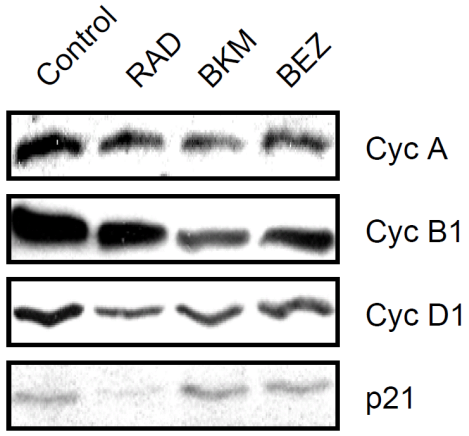

Figure 41: Analysis of cell cycle related proteins expression in tumor tissues after treating with RAD001, NVP-BKM120 and NVP-BEZ235.

Protein was isolated from tumor tissues treated with RAD001 10mg\Kg, NVP-BKM120 60mg\kg and NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks and western blotting was performed for cyclin A, cyclin B1, Cyclin D, p21 and βactin was used as an internal loading control.

4.1.15: Combination of PI3K/mTOR molecular target inhibitors with cisplatin induced tumor regression in xenograft model

Tumor regression was significantly enhanced in combination treatment of PI3K/mTOR inhibitors with cisplatin compared to single treatment (Figure 42A and B). Tumor volume was significantly reduced in combination of NVP-BKM120 with cisplatin (28.9mm^3) compared to NVP-BEZ235 with cisplain (42.3mm^3) and RAD001 with cisplatin (68.3mm^3) (Figure 42B).

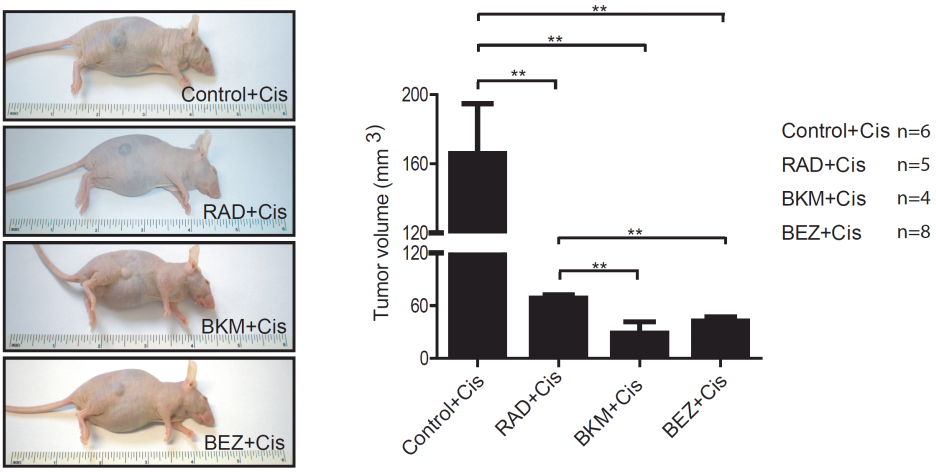

Figure 42: Analysis of tumor growth by combination of PI3K/mTOR inhibitors and cisplatin in Huh7 xenograft tissues.

A). Huh7 cells were subcutaneously injected into the nude mouse in a flank region on one side and when tumors are 5mm in size at any direction, treatment was started with the combination of RAD001 10mg\Kg, NVP-BKM120 60mg\kg, NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks and with cisplatin 3mg\Kg body weight once in a week for 2 weeks. Representative pictures are of tumor bearing mice after treatment period. B) Statistical representation of tumor volume of treated and control xenograft mice.

HE staining displayed larger necrotic areas in control and in mice treated with RAD001 in combination with cisplatin, compared to NVP-BKM120 and NVP-BEZ235

combination with cisplatin. Immunohistochemistry results explained the less population of proliferating cells in combination treated tumor samples by ki67 marker and pHis3 compared to single drug treated mice. TUNEL staining revealed more apoptotic bodies in combination treated samples compared to single drug treated tumor samples (Figure 43). Qunatification of the stainings showed that the combination treatment had more advantage compared to single cisplatin treatment (Fig 44).

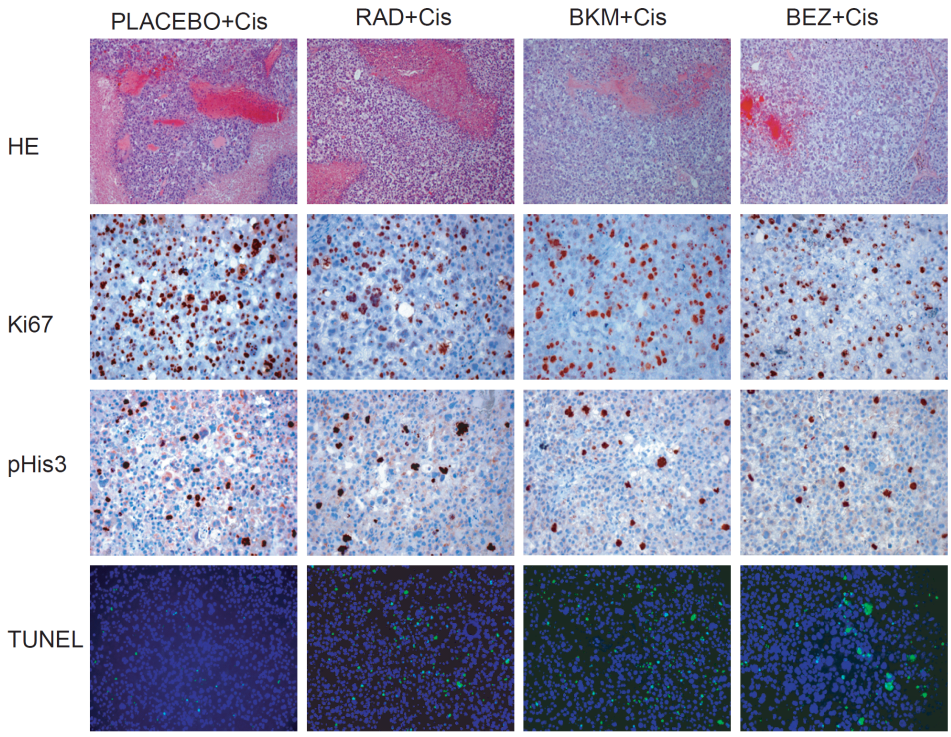

Figure 43: Histopathological analysis of Huh7 xenograft tumor tissue treated with combination of PI3K/mTOR inhibitors with cisplatin.

Pathological analysis of tumor tissues treated with RAD001 10mg\Kg, NVP-BKM120 60mg\kg, NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks and with cisplatin 3mg\Kg body weight once in a week for 2 weeks using H&E, Ki67, pHis3 and Tunel staining.

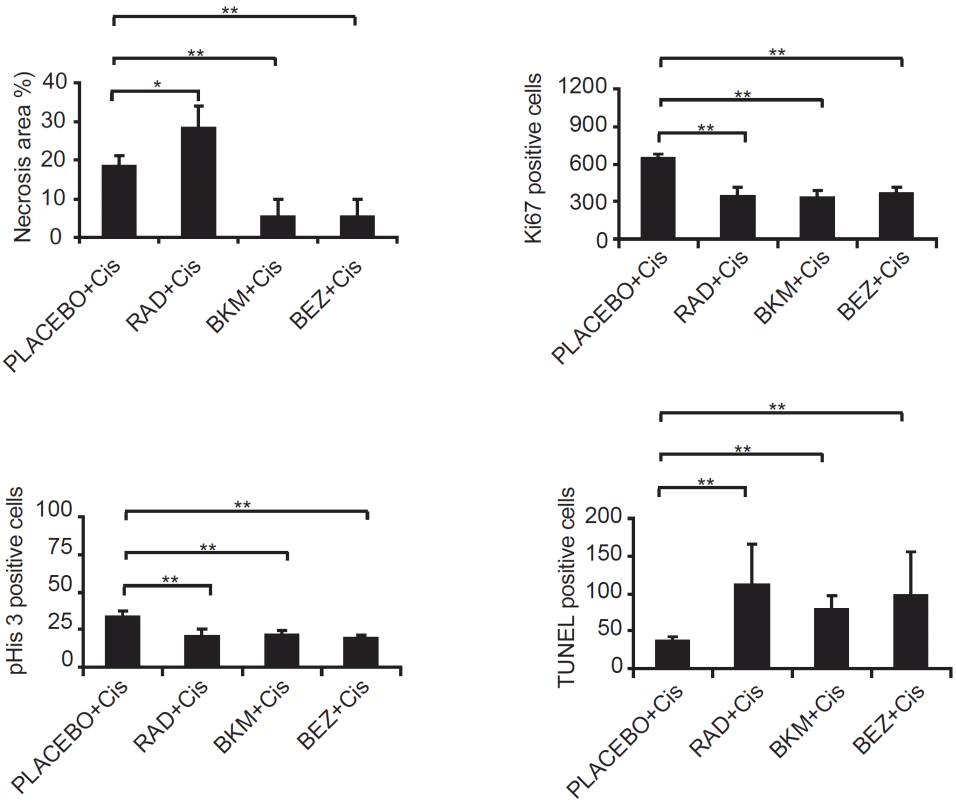

Figure 44: Histopathological analysis of Huh7 xenograft tumor tissue treated with combination of PI3K/mTOR inhibitors with cisplatin.

Analysis of statistical data of pathological analysis of tumor tissues treated with the combination of RAD001 10mg\Kg, NVP-BKM120 60mg\kg, NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks and with cisplatin 3mg\Kg body weight once in a week for 2 weeks using H&E, Ki67, pHis3 and Tunel staining.

** indicates P< 0.001 and * indicates P< 0.01 between control vs treated.

4.1.16: Molecular markers expression in tumor tissue treated with the combination of PI3K/mTOR molecular target inhibitors with cisplatin

P-S6 ribosomal protein was completely dephosphorylated in combination treated samples compared to cisplatin alone. 4E-BP1 dephosphorylation was observed more in combination treated samples compared to cisplatin alone (Figure 45).

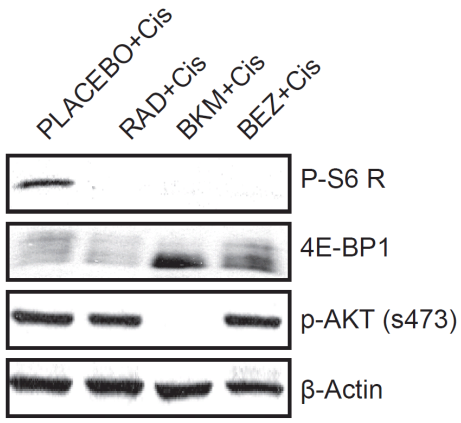

Figure 45: Analysis of mTOR markers and pAKT expression in tumor tissues treated with combination of PI3K/mTOR inhibitors with cisplatin.

Protein was isolated from tumor tissues treated with RAD001 10mg\Kg, NVP-BKM120 60mg\kg, NVP-BEZ235 45mg\Kg body weight on every day for 2 weeks and with cisplatin 3mg\Kg body weight once in a week for 2 weeks and western blotting was performed for P-S6R, 4E-BP1, pAKT and βactin was used as an internal loading control.

4.2: Therapeutic approaches of Hepato Cellular Carcinoma by identification of molecular targets of RAD001 sensitivity and resistance in HCC cell lines

In recent years, RNA interference (RNAi) studies became one of the most powerful tools to identify and characterize novel drug resistant and sensitive candidate genes. This drug resistance was noticed in many HCC patients and due to this poor treatment response there is a need of new gene targets to improve the treatment of HCC. Therefore, in this part of the study, screening of the broad range of human genome library pool for RAD001 resistant and sensitive genes was aimed. In principle, RAD001 forms a complex with FKBP12 and binds to mTOR and leads to dephosphorylation of 4E-BP1 and P70S6K and ultimately down regulation of cell proliferation and metabolism. A working method of shRNA screening was first established, in which FKBP12 was silenced using shRNA against FKBP12 gene.

4.2.1: Amplification of shRNAs by PCR

shRNA oligos targeted against FKBP12 gene were (791, 764, 472, 689, 262 and 740) first amplified with specially designed EcoR1 and Xho1 primers and amplified products were confirmed on agarose gel (Figure 46). Number of each oligo denotes the oligo construction based on starting position on the mRNA.

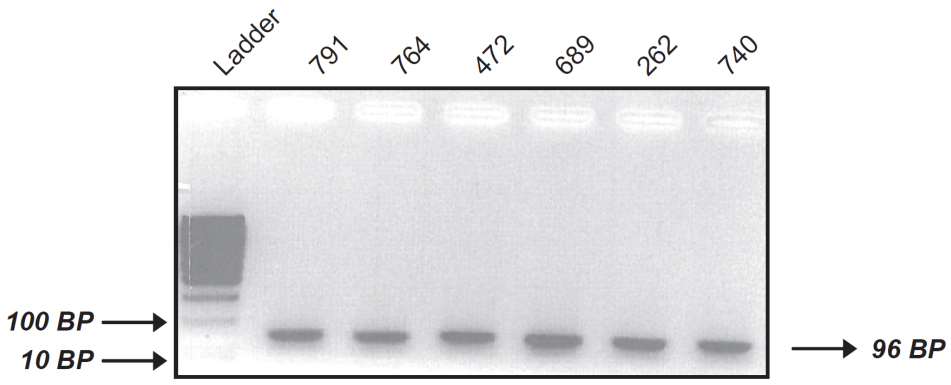

Figure 46: Amplification of FKBP12 shRNA oligoes by PCR .

shRNA oligos were amplified by specific restriction enzyme site primers which are EcoR1 and Xho1, amplified products were confirmed on 2% agarose gel.

4.2.2: Cloning of shRNA oligoes directed against FKBP12

FKBP12 shRNA oligos were cloned into MLP vector using the EcoR1 and Xho1 sites present in the multiple cloning site of the plasmid (moloney murine vector under puromycin selection). Restriction digestion of the clones, was performed with EcoR1 and Xho1 and the release of inserts were confirmed (Figure 47). Further confirmation was carried out by DNA sequencing using mir30 primers. Midiprep was performed in order to produce more amount of plasmid DNA for further retroviral transfection.

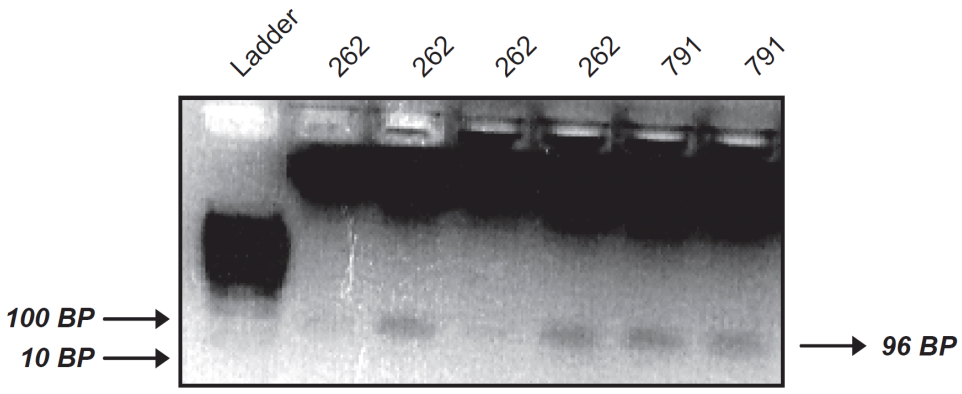

Figure 47: Confirmation of FKBP12 shRNA oligoes clones by restriction digestion.

FKBP12 shRNA oligos clones were restriction digested with EcoR1 and Xho1 for 2hr at 37°C and the insert release was confirmed on 2% agarose gel. The figure represents two out of 6 clones that showed insert release.

4.2.3: Retroviral transfection of shRNAs lead to silencing the FKBP12 gene

Once cloning of the FKBP12 shRNA was confirmed, retrovirus was prepared using phoenix cells as packaging cells. This virus was efficiently transduced into the Huh7 cells and transduced cells were successfully selected in the presence of puromycin. After transduction, the silencing effect of shRNAs on FKBP12 gene was confirmed with RAD001 treatment. In figure 48, it was clearly showed that 262 shRNA was able to silence FKBP12 in Huh7 cells among all shRNAs. When FKBP12 was silenced, RAD001 treatment was not able to inhibit the mTOR which leads to the activation of mTOR down stream targets like P-S6 proteins and thereby leading to normal cell proliferation and metabolism. Schematic picture in Figure 49 shows the mechanism which explains the strategy behind the confirmation of FKBP12 silencing by P-S6 protein expression after RAD001 treatment.

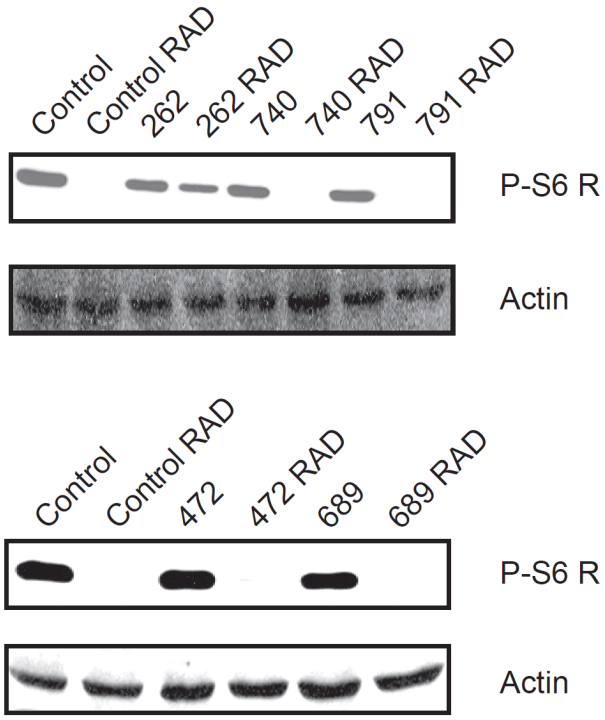

Figure 48: Confirmation of FKBP12 silencing by western blotting.

Transduced cells with shRNA oligos were treated with RAD001 for 24hrs, then protein was harvested and western blotting was performed for P-S6 protein to find out the FKBP12 silencing.

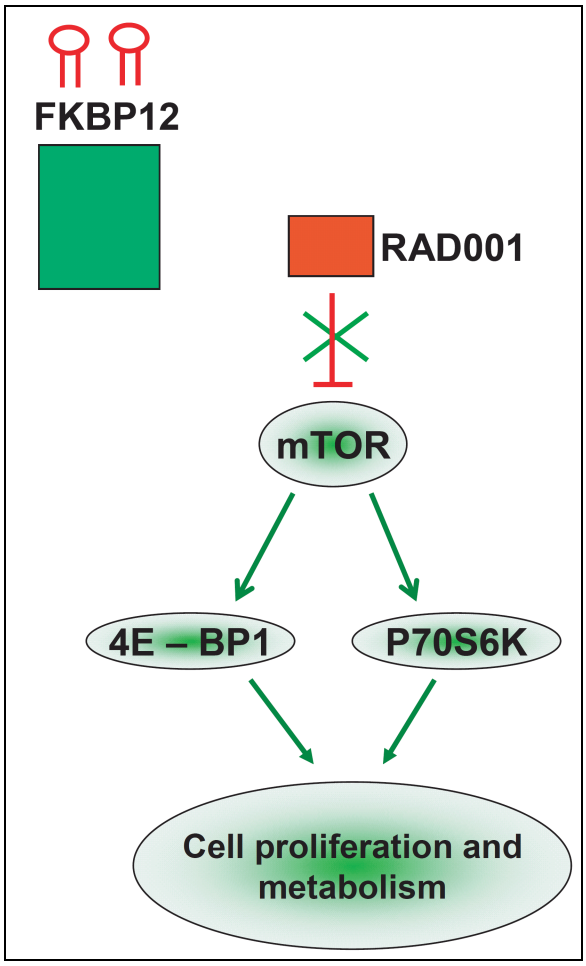

Figure 49: Schematic representation of mTOR pathway after using RAD001 when FKBP12 gene was silence.

In the case of FKBP12 silencing, RAD001 mediated mTOR inhibition is lost there by the downstream mTOR targets will be activated leading to cell proliferation and metabolism.

4.2.4: GFP enrichment of FKBP12 silenced (262 shRNA) Huh7 cells in presence of RAD001 treatemnt

In order to confirm, whether FKBP12 silencing in Huh7 cells might have any kind of regulation on cell proliferation after RAD001 treatment, cell population was maintained as 1:1000 ratios of FKBP12 silenced and normal Huh7 cells. 3 days after RAD001 treatment Huh7 cells showed 2.66% GFP population where as in control it was 2.13% (Figure 50 A). 5 days of RAD001 treatment also had shown increment in GFP population compared to control (3.93 % vs 2.36 %) (Figure 50 B) and it was more compared to 3 days treatment. Furthermore, GFP population was even increased in 10 days RAD001 treatment (8.37 %) compared to 3 days and 5 days RAD001 treatment (Figure 50 C).

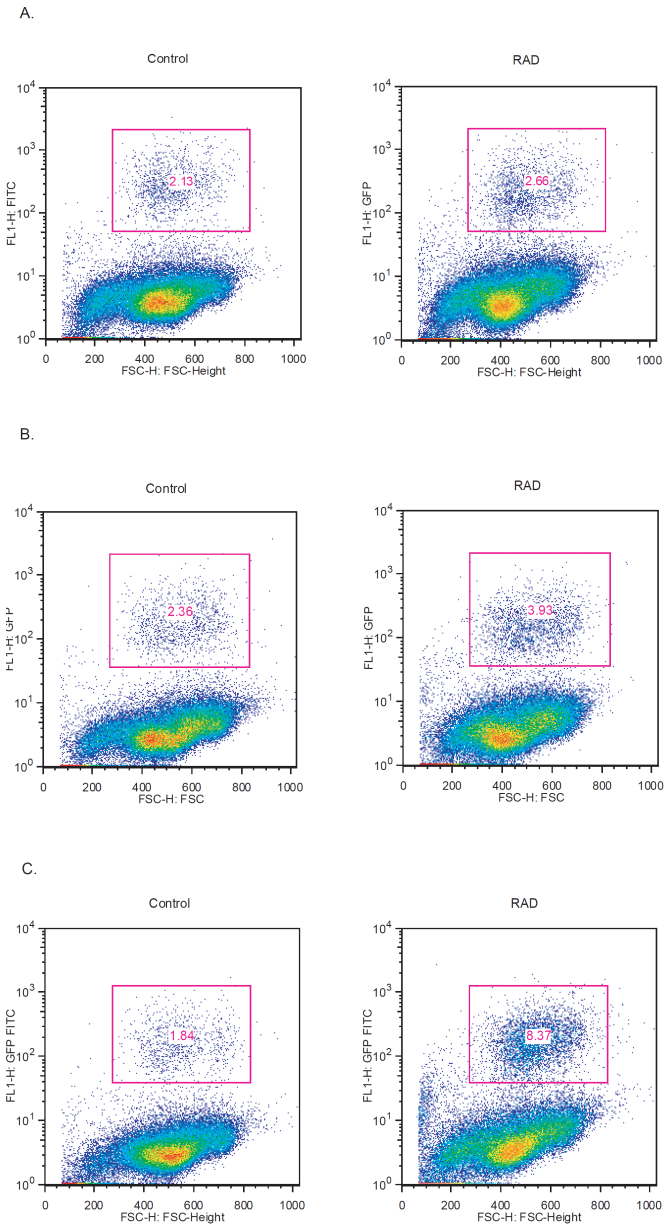

Figure 50: GFP enrichment in competition.

FKBP12 silenced Huh7 cells (262 shRNA) and normal Huh7 cells were mixed at a ratio of 1:1000 and co-cultured for A) 3days, B) 5 days and C) 10 days with RAD001 and assessed the GFP enrichment by flow cytometry.

4.2.5: RAD001, NVP-BKM120 and NVP-BEZ235 molecular target inhibitors effect on FKBP12 silenced (262 shRNA) Huh7 cell proliferation

Cell proliferation assay (MTT) was carried out using IC_{50} concentration of molecular target inhibitors on FKBP12 silenced Huh7 cells. As expected, RAD001 treatment did not have any effect on cell proliferation inhibition in FKBP12 silenced Huh7 cells. NVP-BEZ235 treatment had a partial inhibitory effect on proliferation. Contrary, NVP-BKM120 showed a significant effect on inhibiting cell proliferation (Figure 51).

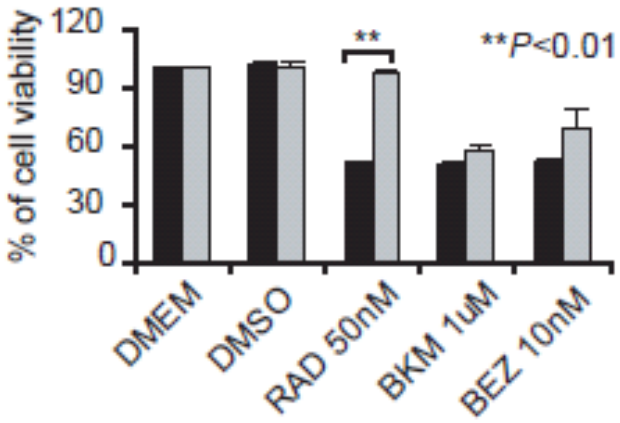

Figure 51: Comparison of cell proliferation between FKBP12 silenced Huh7 cells and normal Huh7 by MTT assay.

FKBP12 silenced cells and control cells were plated in 96 well plates at a density of 1×10^4 cells and grown for 16hrs. Then the cells were treated with respective IC_{50} concentrations of RAD001, NVP-BKM120 and NVP-BEZ235 for 48hrs and cell proliferation was measured by MTT assay. Black bar represents Huh7 cells and grey bar represents FKBP12 silenced Huh7 cells.

4.2.6: PI3K/mTOR molecular target inhibitors treatment resulted in the inhibition of respective signalling cascades in FKBP12 silenced Huh7 cells

As expected, RAD001 treatment was not able to suppress the P-S6 ribosomal protein expression in FKBP12 silenced Huh7 cells. Contrary, NVP-BKM120 and NVP-BEZ235 treatments led to dephosphorylation of P-S6 ribosomal protein expression (Figure 52).

Figure 52: Analysis of P-S6R expression in FKBP12 silenced Huh7 cells after treating with RAD001, NVP-BKM120and NVP-BEZ235.

FKBP12 silenced Huh7 cells were plated at a density of 1.5×10^6 cells and grown for 16 hrs. Then the cells were treated with respective IC_{50} concentrations of RAD001, NVP-BKM120 and NVP-BEZ235 for 48hrs, after treatment protein was isolated and western blotting was performed with P-S6R. β actin was used as an internal loading control.

4.2.7: Screening of genome wide library containing shRNAs in Huh7 cells in presence of RAD001

Pool of 5000 shRNAs was successfully transformed into electro competent *E. Coli* cells by electroporation. In order to confirm the transformation of all the 5000 shRNAs, transformed cells were plated as described in methods. Maxipreps were done to prepare large amount of plasmid DNA. Then using this pool of plasmids, lentivirus was produced using 293T cells. Lentiviral transfection was carried out in order to transduce the cells with shRNAs. As a positive control for transduction, 293T cells are also transduced along with Huh7 cells as they are known to be highly transducible cells. Standardisation of the MOI (Multiplicity of Infection) for virus was done in a way that transduced cells will receive a single virus particle which contains only one shRNA was achieved (Figure 53). Later on, transduced cells were selected under puromycin selection, subjected to RAD001 and placebo treatment for 10 days. Genomic DNA was isolated from 10 days RAD001 and Placebo treated Huh7 transduced cells. Genomic DNA will be subjected to solexa sequencing in order to find out novel resistant and sensitive target candidates towards mTOR inhibition by RAD001.

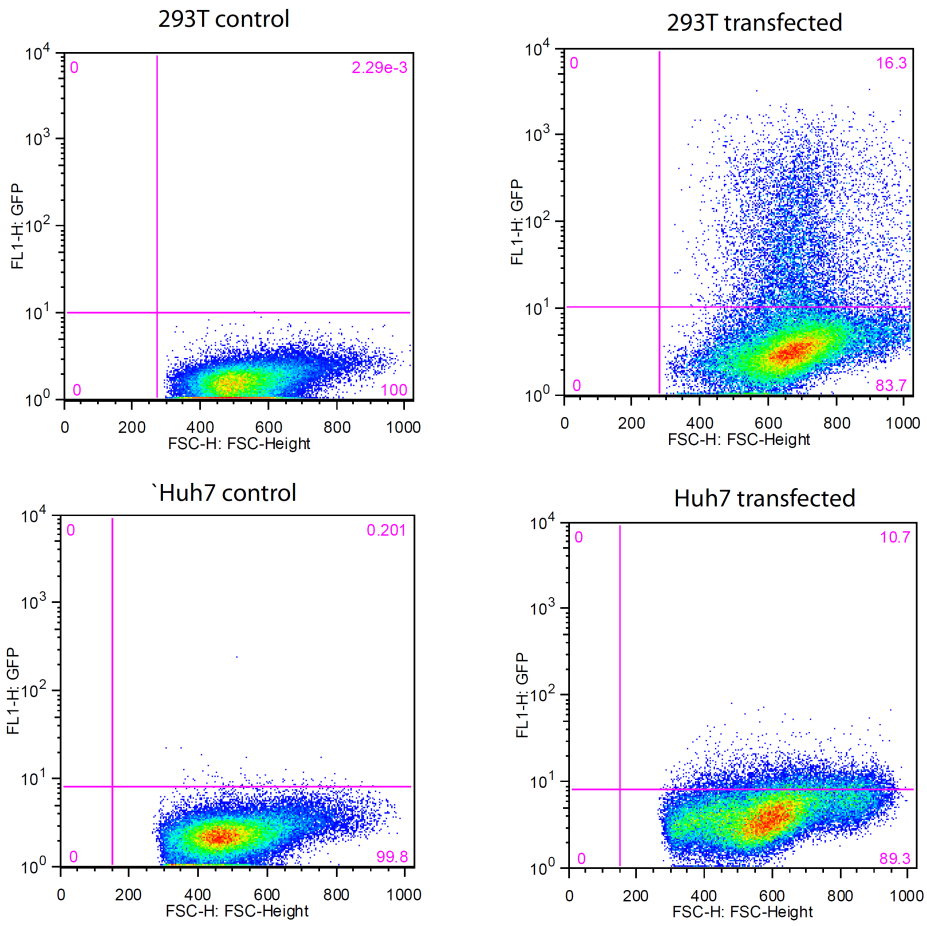

Figure 53: Assessment of 10-15% lenti virus MOI in Huh7 cells.

Huh7 cells were seeded in T25 flask at a density of 2.75 X10^6 cells and were grown for 16 hrs. Then the cells were treated with virus for 24hrs and then trypsinized, cell fluorescence intensity was measured by FACS calibre and analysis was performed with flowJo software.

5 Discussion

PI3K/mTOR pathway is one of the most pivotal signalling transduction cascade, involved in important physiological functions like cell metabolism, cell growth, cell proliferation, cell survival and angiogenesis (Cantley 2002). It was noticed that this pathway was either constitutively activated or deregulated in several cancers (Engelman et al. 2006; Yuan and Cantley 2008; Yuan et al. 2008). Since this pathway is involved in several signalling mechanisms, it was considered as a pathway that can be targeted for developing therapeutics in cancer treatment (Cantley 2002) and in fact, it was highly deregulated in HCC progression. The aim of this study was to target PI3K/mTOR pathway in HCC, by its specific kinase inhibitors and in combination with chemotherapeutic drugs. Inhibitors against PI3K/mTOR pathway provided appreciable growth arrest in Huh7 and HepG2 cells and were even more enhanced with combination of chemotherapeutic drugs. Combination of PI3K/mTOR inhibitors with cisplatin and irinotecan had a significant synergistic effect on HCC cell growth inhibition. In this study we also found that Huh7 cells when treated with RAD001 showed a cytostatic effect on cell proliferation, which shows that these cells are resistant to RAD001 treatment. In general, few HCC patients are found resistant to RAD001 treatment. Recent studies showed that functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer (Berns et al. 2007). We therefore aimed to unravel novel resistant genes for RAD001 treatment in HCC, by genome-wide shRNA screen in Huh7 cells.

5.1 Application of PI3K/mTOR inhibitors for HCC treatment

The major etiologies of HCC are hereditary genetic mutations, viral hepatitis, chronic exposure to toxins, excessive alcohol consumption and/or deregulation of kinase pathways. In the case of deregulated kinase pathways in HCC, PI3K/mTOR pathway is considered to be one of the pioneering pathways that is highly deregulated. A number of drugs have been evaluated in clinical trials for HCC, of which RAD001 (analogue of rapamycin) has emerged out with its specific efficiency and is still in phase trails. One of the most prominent features of advanced and metastatic HCC is

increased threshold level of proliferation. PI3K/mTOR pathway plays a pivotal role in cell proliferation, which makes this pathway as an ideal target in treating HCC. RAD001, a specific inhibitor of mTOR pathway, promotes the inhibition of HCC proliferation (Buitrago-Molina et al. 2009). But the drawback of using RAD001 is the negative feedback loop on AKT pathway, as it renders the phosphorylation of AKT and could not able to eliminate HCC progression completely (Guertin and Sabatini 2007). Guertin *et al*. showed that negative feedback loop inhibition occurs due to the connection between S6 kinase and insulin receptor substrate-1 (IRS1) and that activates AKT kinase following suppression of mTOR.

In this study we showed that treatment of Huh7 cells with RAD001 led to a cytostatic effect (from 5nM-1000nM) on cell proliferation (Figure 13). This cytostatic effect could be due to the genetic instability of this cell line or on the other hand it could be due to the RAD001 inhibition of negative feedback loop of AKT. Indeed the results from Huh7 cells clearly showed a significant increase in phosphorylated AKT expression after RAD001 treatment (Figure 22). In contrast to this, there was no difference in phosphorylated AKT levels between control and treated xenograft tissue samples (Figure 39). Treatment of RAD001 led to completely dephsophorylated P-S6 R and partial dephosphorylated 4E-BP1 in both *in vitro* and *in vivo* conditions (Figure 22 and 39). HepG2 cells treated with RAD001 showed no significant effect on cell proliferation in a concentration range of 0.1-20μM, where as a strong dose dependent inhibitory effect on cell proliferation was observed between 30-60μM range (Figure 16). This clearly explains the differential sensitivities of cells to RAD001 treatment. As cytostatic effect of RAD001 on Huh7 cell proliferation was evident, which is a mimic condition to the resistance against RAD001 in HCC patients, and also Huh7 cells sensitiveness to lower concentrations of RAD001 compared to HepG2 cells, further studies were continued in Huh7 cells only.

RAD001 is a well known mTOR inhibitor; therefore the downstream targets of mTOR in cells treated with RAD001 were analyzed. For this, P-S6 R and 4E-BP1, well charachaterized mTOR down stream proteins, were choosen and western blots for these proteins revealed complete dephosphorylation of P-S6 R and partial dephosphorylation of 4E-BP1 proteins (Figure 22), there by confirming the RAD001

effect on Huh7 cells. Studies done in HCC and other cancers showed that RAD001 treatment promotes the inhibition of cell proliferation (Boulay et al. 2004; Beuvink et al. 2005), which strongly supports the cell proliferation results in this study.

In a previous study, subcutaneous patient-derived HCC xenografts were used to study the effects of RAD001 on tumor growth, proliferation, apoptosis and angiogenesis, reported that oral administration of RAD001 to mice bearing patient-derived HCC xenografts resulted in a dose-dependent inhibition of tumor growth (Huynh et al. 2009). In order to check *in vitro* cell proliferation results with RAD001 treatment, in this study nude mouse xenograft models were used. Once the mice developed tumors, they were treated with RAD001 for 2 weeks and tumor growth was measured. RAD001 treatment of xenograft tumors resulted in significant inhibition of tumor growth (Figure 35), which supported *in vitro* cell proliferation data and is in accordance with previous studies. In addition to this other proliferation markers like Ki67 and p-His3 were analyzed (Figure 36). Quantification of positively stained cells showed that RAD001 treatement led to a decrease in Ki67 and p-His3 stainings revealing that cell proliferation was arrested by RAD001. Histology studies of tumor tissues from mice treated with RAD001 revealed that there was no marked difference in necrosis when compared to control mice.

Earlier studies done in cells showed that treatment with RAD001 resulted in increase of cells in G0/G1 phase of the cell cycle. Even at nanomolar concentrations of RAD001, there was a 10-70% increase in delaying progression at the early G0/G1 phase in cell cycle (Vignot et al. 2005). In this study, treatment of Huh7 cells with RAD001 (50nM) resulted in the increase of cells in G0/G1 phase (Figure 31). Although preclinical studies have shown that rapamycin and its analogous are potent inhibitors of tumor cell proliferation leading to the accumulation of cells in G1 phase (Wendel et al. 2004; Wanner et al. 2006), the roles of specific down-stream pathways of mTOR have been poorly defined. So, therefore important proteins that regulate G1 phase of cell cycle were analyzed in xenograft tissues.

In general, cyclinD1 is over expressed in several cancers and it plays a significant role in tumor progression. Previously, cyclinD1 was shown to regulate both growth and

proliferation downstream of mTOR in hepatocytes (Nelsen et al. 2003). In this study, RAD001 treatment resulted in down regulation of cyclinD1. In addition to this, cyclinA and the mitosis marker cyclinB1 were also down regulated upon RAD001 treatment. RAD001 treatment of Diffuse Large B-Cell Lymphoma (DLBCL) cells resulted in inducing G1phase arrest with an effect on cyclinD1 and other cyclin molecules like cyclinA during cell cycle regulation (Wanner et al. 2006). Down regulation of these key markers clearly support the *in vitro* data in this study where Huh7 cells were arrested in G1 phase of the cell cycle (Figure 41). In a previous study, in addition to the down regulation of cyclins, p21 protein down regulation was observed upon treatment with RAD001 in *Fah/p53* -/- mice (Buitrago-Molina et al. 2009). Analysis of p21 expression correlated well with the above study, showing a down regulation of p21 in xenograft tumor tissues treated with RAD001.

As the results from cell proliferation and cell cycle analysis revealed that RAD001 treatment leads to inhibition of cell proliferation and arrest of cells in G0/G1 phase, and also earlier studies had observed mTOR being a key regulator during apoptosis in cancer, RAD001 effect on apoptosis in Huh7 cells and xenograft tumor model was further analyzed. RAD001 treatment resulted in an increase of apoptotic cells in both Huh7 cells (Figure 33 and 34) and xenograft model (Figure 36 and 37). RAD001 treated Huh7 cells showed moderately increased apoptotic cells compared to control cells which correlated well with an earlier study done in rapamycin treatment showed moderate activity on apoptosis in cancer cells (Tam et al. 2009).

A cytostatic effect on Huh7 cell proliferation and a negative feedback loop activation of AKT after RAD001 treatment can be well correlated with the development of resistance against RAD001 in HCC patients. Therefore, new class of drugs were designed to target both PI3K and mTOR kinase proteins. So, thereby the dual inhibitor for PI3K and mTOR *i.e* NVP-BEZ235 and PI3K specific inhibitor *i.e*., NVP-BKM120 were used to find out their effect on Huh7 cell proliferation and xenograft tumor model. NVP-BKM120 is being used for the first time in the treatment of liver cancer. As far as now there is only one published study where it was demonstrated that addition of the PI3K inhibitor NVP-BKM120 or the dual PI3K/mTOR inhibitor NVP-BEZ235 to the initial treatment with the Smo antagonist markedly delayed the development of

resistance (Buonamici et al. 2010). In this study new PI3K inhibitors were used instead of conventional PI3K inhibitors like wortmannin and LY294002, as it was already shown earlier, that these conventional drugs had a cross talk inhibition of other protein kinases and were toxic to cells (Stein 2001).

Treatment of Huh7 cells with increasing doses of NVP-BEZ235 and NVP-BKM120 led to a dose dependent inhibition of cell proliferation (Figure 14 and 15). Among these two drugs NVP-BEZ235 was more effective at lower concentrations than NVP-BKM120. The dual inhibitor NVP-BEZ235 was shown consistently more potent in inhibiting the proliferation of different cancer cells at concentrations >10 nM compared to RAD001 treatment (Serra et al. 2008). The results from this study were also in accordance with the above study as RAD001 had cytostatic effect from 5nM concentration and NVP-BEZ235 had a strong dose dependent inhibition of cell proliferation from 5nM-250nM range. Recent studies also showed, NVP-BEZ235 anti-proliferative effect, on multiple myeloma cells (Baumann et al. 2009), melanoma cells (Marone et al. 2009) and in glioma cells (Liu et al. 2009). In the case of NVP-BKM120, this is the first study being conducted in HCC cells where we show that it inhibits Huh7 cell proliferation at higher concentrations compared to RAD001 and NVP-BEZ235.

In xenograft model, treatment with specified doses from the manufacturer resulted in increased tumor regression in the mice treated with NVP-BKM120 rather than NVP-BEZ235 (Figure 35). But the mice treated with NVP-BKM120 were less healthy compared to RAD001 and NVP-BEZ235 treated mice. Maira *et al*., had also previously showed that chronic treatment of PC3M tumor-bearing animals with NVP-BEZ235 twice a daily resulted in a statistically significant inhibition of tumor growth (Maira et al. 2008), which supports the results in this study. Additionally another group using primary pancreatic cancer xenografts grown orthotopically, observed that acute oral dosing with NVP-BEZ235 strongly suppressed the phosphorylation of PKB/AKT and the growth of the tumors (Cao et al. 2009). In contrast in the present study, treatment of Huh7 cells with NVP-BEZ235 resulted in an increase of AKT phosphorylation, whereas both P-S6 and 4E-BP1 were dephosphorylated. Phosphorylation of AKT in NVP-BEZ235 treatment was due to IC_{50} concentration which inhibited only mTOR but not p-AKT, as it was observed that higher concentrations of NVP-BEZ235 significantly

dephosphorylated AKT (Figure 22 and 23). In xenograft tumor tissue, treatment of NVP-BKM120 and NVP-BEZ235 led to complete dephsophorylation of P-S6R, partial dephosphorylation of 4E-BP1 and dephosphorylation of p-AKT in xenograft tumor model. NVP-BKM120 treatment led to complete dephosphorylation P-S6R, 4E-BP1 and pAKT in *in vitro* as well as in xenograft tumor tissues.

NVP-BEZ235 and NVP-BKM120 treatment also resulted in a decrease of Ki67 and p-His3 staining revealing that cell proliferation was arrested, which was comparable to that of RAD001 treatment. This shows that these drugs could be used alternative to RAD001 treatment, where RAD001 is not sensitive enough to control cancer progression. Histology studies of tumor tissues from mice treated with NVP-BEZ235 and NVP-BKM120 revealed that there was less necrosis when compared to control and RAD001 treated mice.

Treatment of Huh7 cells with NVP-BEZ235 and NVP-BKM120 resulted in the increase of cells in G0/G1 phase (Figure 31) whereas the percentage of cells in S phase were decreased (Figure 32). Initial studies done with NVP-BEZ235 using different types of cancer cells showed that upon NVP-BEZ235 treatment cells were arrested in G1 phase of the cell cycle (Maira et al. 2008). In case of NVP-BKM120, this is the first study showing its effect on G0/G1 phase arrest in cell cycle. NVP-BEZ235 and NVP-BKM120 treatment of mice carrying xenograft tumors resulted in down regulation of cyclinD1, cyclinA and cyclinB1 as was the case with RAD001. A very recent study showed that treatment of renal cell carcinoma cell line with NVP-BEZ235 resulted in complete suppression of cyclinD1 (Cho et al. 2010), which is in support to the above results. Down regulation of these key markers clearly support the *in vitro* data where Huh7 cells were arrested in G1 phase of the cell cycle (Figure 41). Analysis of p21 expression, showed no difference in regulation of p21 in xenograft tumor tissues treated with NVP-BEZ235 and NVP-BKM120. RAD001 showed a strong down regulation of p21 which shows that NVP-BEZ235 and NVP-BKM120 have different signalling mechanisms in arresting cell cycle, other than inhibiting cyclin proteins. In a recent study, RAD001 treatment of Fah/ p53-/- mice showed a strong down regulation of p21 expression in hepatocytes. Huh7 cells are well known p53 mutated cells, and the down regulation of p21 upon RAD001 treatment correlates well with the above

study and at the same time it also confirms that at least NVP-BEZ235 and NVP-BKM120 do not act through p53 signalling mechanism in arresting cells at G1 phase.

Previous study in breast cancer cell lines showed that NVP-BEZ235 treatment resulted in apoptosis of these cell lines (Serra et al. 2008). In this study, Huh7 cells after treatment with NVP-BEZ235 and NVP-BKM120 showed an increase in number of apoptotic cells compared to control cells (Figure 33 and 34). NVP-BEZ235 and NVP-BKM120 treated mice not only had more apoptotic cells than control mice but also more than RAD001 treated cells (Figure 36 and 37). This data further imposes the use of these drugs as an alternative to RAD001.

Furthermore, potential influence of RAD001, NVP-BKM120 and NVP-BEZ235 on angiogenesis did not achieve success rate in this study. There was no difference in CD31 staining and αSMA staining between treated and control samples (Figure 38). In contrast to this, it was shown that NVP-BEZ235 treatment resulted in a potential inhibition of VEGF-induced cell proliferation and survival *in vitro* and VEGF-induced angiogenesis *in vivo*. Moreover, NVP-BEZ235 strongly inhibited micro vessel permeability both in normal tissue and in BN472 mammary carcinoma grown orthotopically in syngeneic rats. Similarly, tumor interstitial fluid pressure, a phenomenon that is dependent of tumor permeability, was significantly reduced with NVP-BEZ235 treatment (Schnell et al. 2008). Additionally, another study also showed that treatment with RAD001 diminished the expression of vascular endothelial growth factor in tumor-derived cell lines and inhibited angiogenesis in vivo (Mabuchi et al. 2007).

In this study, mTOR inhibition by RAD001 did not show any crosstalk with molecular mechanism like p38 and JNK pathways, but p-ERK level was upregulated in xenograft tumor tissues (Figure 40). In human cancer patients and *in vivo* mouse models phosphorylation of ERK was observed upon RAD001 treatment (Carracedo et al. 2008). C-jun expression was unaltered in RAD001 treated xenograft tissues. Earlier studies showed that mTOR regulates c-Jun function during hepatocyte proliferation (Behrens et al. 2002; Eferl et al. 2003). Additionally, NVP-BKM120 and NVP-BEZ235 also did not have any influence on c-Jun/JNK and p38 expression, but upregulation of

pERK was noticed (Figure 40). Recent studies showed that p70S6K-mediated, cross-inhibitory regulation between the MEK/ERK and PI3K/mTOR pathways, in which each contribute to the maintenance of the self-renewal and tumorigenic capacity of glioblastoma cells (Sunayama et al. 2010).

5.2 HCC treatment improvisation by combinatorial treatment with PI3K/mTOR inhibitors.

Intrinsic sensitivity to rapamycin analogues between cell lines may vary by several orders of magnitude. Recent studies in multiple myeloma and other cancer cells using mTOR inhibitors showed an increase of basal AKT activation and phosphorylation, which is dependent on basal IGF-I/IGF-R signaling. IGF-I receptor inhibition prevented rapamycin-induced AKT activation and sensitized tumor cells to inhibition of mTOR (O'Reilly et al. 2006). As RAD001 treatment in Huh7 cells resulted in cytostatic effect on cell proliferation and also increase in basal AKT phosphorylation, we next aimed to find out the effect of RAD001 combination therapy with NVP-BKM120 and NVP-BEZ235 on Huh7 cell proliferation. RAD001 in combination with NVP-BKM120 and NVP-BEZ235 resulted in a strong inhibition of cell proliferation in Huh7cells, compared to control and single treatments (Figure 24). Here it strongly implies that NVP-BEZ235 had sensitized the RAD001 effect in Huh7 cells. These results were strongly supported in another study, where NVP-BEZ235 sensitized the lapatinib resistance (Eichhorn et al. 2008). The combination of RAD001 with NVP-BKM120 led to complete dephsophorylation of P-S6 R protein which could be due to the RAD001, as RAD001 alone had complete dephsophorylation of P-S6 R protein. But this combination had a synergistic effect on the dephsophorylation of 4E-BP1 and p-AKT (Figure 22). The combination of RAD001 and NVP-BEZ235 had synergistic effect on dephosphorylation of 4E-BP1, where they completely dephosphorylated 4E-BP1. However the combination of RAD001 and NVP-BEZ235 did not have any inhibitory effect on AKT phosphorylation. This could be due to the concentration of NVP-BEZ235 used for this study, as NVP-BEZ235 dephosphorylates AKT only at higher concentrations than its IC_{50} concentration (Figure 23).

5.3 HCC treatment improvisation by combination treatment of PI3K/mTOR inhibitors with classical chemotherapeutic drugs

The ability of cancer cells to become resistant to anticancer agents remains a significant impediment to successful chemotherapy. Various clinical trials investigated the role of single-agent chemotherapy in patients with unresectable HCC and reported response rates varying from 0% to 20%. Combination of drugs is always congenial for better treatment of the cancer. In this study, it was clearly explained the efficient inhibition of cell proliferation by kinase inhibitors. Several chemotherapeutic agents have been evaluated for the treatment of HCC in the recent past; however, no single or combination chemotherapy regimen is particularly effective. Previous studies have shown that mTOR inhibition can restore the sensitivity to some conventional cheomtherapeutic drugs (Mabuchi et al. 2007). Therefore the effect of RAD001, NVP-BKM120 and NVP-BEZ235 in combination with doxorubicin, cisplatin, 5FU or irinotecan on cancer cell viability was analyzed further.

Treatment of Huh7 cells with chemotherapeutic drugs resulted in a dose dependent loss of cell viability. Interestingly the combination of RAD001 with doxorubicin, irinotecan and cisplatin significantly decreased Huh7 viability compared to single drug treatment. Additionally, cisplatin and irinotecan combination with RAD001 had significant reduction in cell proliferation (Figure 25). These results add further evidence to previous studies (Mabuchi et al. 2007; Saunders et al. 2010) necessitating mTOR inhibition as an effective strategy to increase sensitivity of HCC to conventional chemotherapeutic drugs. In this study, the combination of NVP-BKM120 and NVP-BEZ235 with doxorubicin, cisplatin, 5FU and irinotecan significantly decreased Huh7 viability. Moreover, cisplatin and irinotecan combination with NVP-BEZ235 and/or NVP-BKM120 had a severe effect on inhibiting the cell proliferation (Figure 26 and 27). The best hallmark future of combination treatment in this study was the synergistic effect exhibited by PI3K/mTOR inhibitors and cisplatin/irinotecan, where combination treatment enhanced the loss of cell viability.

The combination of RAD001, NVP-BKM120 and NVP-BEZ235 with chemotherapeutic drugs led to complete or partial dephsophorylation of P-S6 R and 4E-BP1 (Figure 28, 29 and 30). However, the combination of RAD001 and NVP-BEZ235 with chemotherapeutic drugs did not have any inhibitory effect on AKT phosphorylation, whereas NVP-BKM120 in combination with chemotherapeutic drugs showed a synergistic effect in inhibiting phosphorylation of AKT.

RAD001 effect on the induction of apoptosis in cancer cells was shown to be moderately effect (Tam et al. 2009). In this study little apoptosis was observed after RAD001 treatment and it was further enhanced in combination with cisplatin (Figure 34). Treatment of ovarian cancer cells with rapamycin sensitized the cells to cisplatin by inducing apoptosis (Schlosshauer et al. 2009). RAD001 treatment sensitized the cisplatin induced apoptosis in an *in vivo* and *in vitro* model (Mabuchi et al. 2007) and in other study RAD001 treatment sensitized A549 cells (lung cancer cells) with wild type p53 to cisplatin induced apoptosis by inhibiting p21 translation in vitro (Beuvink et al. 2005). In contrast, in the same study it had been shown that rapamycin sensitizes cisplatin-induced apoptosis in wild-type p53, but not mutant p53 tumour cells (Beuvink et al. 2005). In this study, combination of NVP-BEZ235 and NVP-BKM120 with cisplatin resulted in an increase of apoptotic cells when compared to respective single treatments. NVP-BEZ235 has been evaluated in combination with conventional cytotoxic agents, thus showing promising efficacy with both doxorubicin and vincristine (Manara et al. 2010). These results further imply that a combination of PI3K/mTOR inhibitors with chemotherapeutic drugs will enhance the treatment of HCC.

5.4 Identification of predictive and prognostic factors to improve cancer treatment by RNA interference screening of genome wide shRNAs.

In vitro RNAi screening delineates new candidates for tumor suppression and oncogenesis. Cancer arises due to accumulation of many passenger and driver mutations as well as genetic instability and heterogenous nature. Today, there are no

effective drugs for curing liver cancer. Using integrative genomic and proteomic approaches in mouse models, new tumor supressors in liver cancer were identified (Lee et al. 2008; Zender et al. 2008). Another part of the present study dealt with unravelling novel oncogenes and tumor suppressors that are resistant to conventional RAD001 therapy in HCC, using genome wide shRNA screening by high throughput analysis.

To approach this study, first an *in vitro* model system was established to check the functional status of shRNAs, which were designed in such a way that they possess mir30 sequence and long terminal repeats. microRNA-based short hairpin RNA are more potent triggers of the RNAi machinery and can efficiently suppress gene expression (Dickins et al. 2005; Silva et al. 2005).

5.5 Functionally identifying the microRNA-Based FKBP12 short hairpin RNAs (FKBP12 shRNA) in HCC cell lines

The procedure for constructing FKBP12 clones was performed (Figure 46 and 47) and suppressing the expression of FKBP12 gene using a retroviral-mediated approach was performed and silencing was confirmed with RAD001 treatment. In the absence of FKBP12, RAD001 did not have any effect on cell proliferation inhibition, therefore it was confirmed with P-S6 R protein expression, where 262 silenced cells showed P-S6R protein expression (Figure 48 and 49). This kind of approach was well characterized in previous studies (Dickins et al. 2005; Silva et al. 2005; Liu et al. 2009).

Furthermore, evaluation of normal penetrance of the FKBP12 transduced cells was validated with GFP enrichment assay by pooling FKBP12 transduced cells and normal Huh7 cells with 1:1000 ratios under RAD001 treatment. Here it was noticed that GFP enrichment occurred day by day with the treatment of RAD001. After 3 days of RAD001 treatment showed 2.66% of GFP population, in 5 days treatment it was 3.93% and in 10 days treatment it was notified 8.37% of GFP population (Figure 50).

Additional results of FKBP12 transduced cells in respect to proliferation studies with PI3K/mTOR inhibitors resulted in variable differences in the proliferation rate (Figure 51). Here RAD001 treatment did not have any influence on inhibiting cell proliferation, where as NVP-BKM120 and NVP-BEZ235 had significant influence on inhibition of cell proliferation. Moreover, NVP-BKM120 and NVP-BEZ235 treatment resulted in complete dephosphorylation of P70S6 ribosomal protein but not in RAD001 treatment. These results additionally confirmed that there was no effect of RAD001 on FKBP12 transduced cells (Figure 52).

5.6 Genomic screen of shRNA libraries in Huh7 cells upon RAD001 treatment

Electroporation studies enabled 12 pools, each pool of 5000 shRNAs. shRNAs very efficiently suppress gene expression when expressed from a single genomic copy in the cell (Dickins et al. 2005; Silva et al. 2005). In order to achieve this, standardisation of the MOI (Multiplicity of Infection) for virus to get a transduction efficiency of 10-15% was performed using FACS analysis (Figure 53). Furthermore, transduced cells were selected by puromycin and then subjected to RAD001 and placebo treatment for 10 days. Genomic DNA was isolated from 10 days RAD001 and Placebo treated Huh7 transduced cells. Genomic DNA will be subjected to solexa sequencing in order to find out novel resistant and sensitive target candidates towards mTOR inhibition by RAD001 (Zender et al. 2008).

6 Conclusion

Though PI3K/mTOR inhibitors and chemotherapeutic drugs are tested as therapeutic agents in treating HCC, due to their effective role in controlling cancer progression, still they are not considered to be potent inhibitors of HCC progression because of the development of resistance to these drugs by HCC. Therefore it is of great interest to understand the key oncogenic markers and their mechanisms, which are involved in sensitizing the mechanisms of resistance in HCC. The first part of this study suggest that inhibition of PI3K by novel drug NVP-BKM120 led to potent tumor growth inhibition compared to RAD001 and NVP-BEZ235. NVP-BKM120 is one of its kinds of novel drug that is yet to be tested in liver cancers, which makes our study a significant one. Additionally, dual inhibition of PI3K/mTOR with NVP-BEZ235 is effective in preventing HCC proliferation and tumor growth as compared to RAD001 treatment alone. Combination of PI3K/mTOR inhibitors with cisplatin and irinotecan showed a strong synergistic activity indicating that combinatorial therapy using PI3K/mTOR and chemotherapeutic drugs could be a feasible option for non-cirrhotic HCC patients with good liver function. In the second part of this study, a sucessful *in vitro* system was established to perform a genome-wide shRNA screen to unravel the novel genes responsible for drug resistance or sensitiveness to mTOR directed therapies in HCC.

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8 Abbreviations

AEC	3 amino-9-ethyl carbazol
AMPK	AMP-Activated Protein Kinase
APS	Ammonium per sulphate
BSA	Bovine serum albumin
CDK	Cyclin-Dependent Kinases
DAPI	4’6-diamidino-2-phenyl indol
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
4E-BP1	4E-Binding Protein 1
eIF-4E	Eukaryotic Initiation Factor 4E
FACS	Fluorescence activated cell sorter
FAH	Fumarylacetoaccetate Hydrolase
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
H&E	Hematoxylin and Eosin staining
IGF	Insulin-like Growht Factor
IRS1	Insulin Receptor Substrate 1
LB medium	Luria Bertani medium
MAPKK	Mitogen-activated Protein Kinase Kinase
mRNA	Messenger ribonucleic acid
mTOR	mammalian Target of Rapamycin
mTORC1	mTOC Complex 1
mTORC2	mTOC Complex 2
PI3K	Phosphoinositide-3 Kinase
pten	Phosphatase and Tensin Homolog
PVDF	polyvinylidenfluoride
RNA	Ribonucleic acid
RNAi	Ribonucelic acid interference
RPMI	Roswell park memorial Institute
S6	Ribosomal Protein S6
S6K1	p70 Ribosomal Protien S6 Kinase 1
shRNA	Short hairpin RNA
TEMED	Tetramethyl ethylene di amin

9 Curriculum vitae

Deepika.Pothiraju

Personal Information

Date of Birth: 25.07.1979

Place of Birth: Nellore, A.P, India

Nationality: Indian

Educational Qualifications

2008-till to date: PhD in Gastroenterology Dept, Hannover Medical School, Germany.

2006-2008: PhD (Discontinued) in Cell and Developmental Biology Dept., Scuola Normale Superiore, University of Pisa, Italy.

2004-2006: Project assistant in Dr.A.J.Rao's Lab, Dept.of Biochemistry, Indian Institute of Science, Bangalore, India.

2001-2003: Masters in Life Sciences **(78.66%)** from Devi Ahilya University, Indore, India.

1998-2001: Bachelors Degree in Genetics, Botany and Chemistry **(86%)** from Nagarjuna University, Vijayawada, India.

1994-1996: Senior Secondary Education in Biology **(79%)** from Board of Intermediate education, Andhra pradesh, India.

1984-1994: Primary and secondary school, Andhra pradesh, India.

Research Experience

PhD: Since 2008

PhD in Hannover Medical School, Hannover, Department of Gastroenterology, Hepatology and Endocrinology.

Project Handling:

1. Role of PI3K/mTOR inhibitors alone and in combination with chemotherapeutic drugs hepatocellular carcinoma (HCC) treatment.

2. Human genome wide shRNAs screening to find out novel candidates for resistance and sensitiveness in HCC treatment.

PhD (Discontinued): October 2006 – November 2007

PhD under the International Postgraduate Program of Scuola Normale Superiore, University of Pisa, Italy

Project Handled:

Role of different isoforms of CIPP (Channel Interacting PDZ domain protein) and its interacting partner IRSp53 in retina development (Xenopus model system)

Project Assistant from May 2004-Febraury 2006

Department of Biochemistry, Indian Institute of Science, Bangalore, India.

Projects Handled:

1. Androgen and estrogen regulation of contraceptive target genes expression in epididymis of bonnet monkey.
2. Androgen and estrogen regulated target genes expression in epididymis of Rat.
3. Cloning and expression of WAP (Whey Acidic Protein) of bonnet monkey epididymis.
4. Demonstration of the presence of LHR (Lutenizing hormone receptor) in rat epididymal cauda.

Master's Thesis: January 2003-July 2003

Department of Microbiology and cell biology, Indian Institute of Science, Bangalore, India.

Thesis title: "Studies on interactions between rotavirus nonstructural proteins NSP5 and NSP6."

Summer Training: June 2002

Cloning and colony hybridization technique in the lab of Molecular Reproduction and Development Genetics [MRDG] Dept, Indian Institute of Science, Bangalore, India.

Conference Experience and poster presentations

1. "Genomics/Proteomics"sponsored by Department of Biotechnology, Government of India New Delhi held at Bioinformatics Sub-Centre, School of biotechnology DAVV, Indore, India. (14 to 15-1-2002).

2. “Computer in Biological Science” sponsored by Department of Biotechnology, Government of India, New Delhi held at Bioinformatics Sub Centre, School Of biotechnology DAVV, Indore, India. (21 to 22-8-2002).

3. Poster presented at EASL 2009, Copenhagen, Denmark.

Combinatorial effect of PI3K\AKT\mTOR inhibitors with cytotoxic drugs in treatment of hepatocellular carcinoma

4. Poster presented at Summer school 2010, conducted by MHH HBRS programme

PI3K\mTOR dual inhibitor alone or in combination with chemotherapy effectively inhibits hepatocellular carcinoma growth

5. Poster was accepted in APASL for 2011.

PI3K and mTOR inhibitors alone or in combination with chemotherapy effectively inhibits hepatocellular carcinoma growth

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Achievements

1. Secured 19th Rank in National level entrance Examination for M.Sc Life Sciences conducted by DAVV, Indore, India in July 2001.

2. Secured 16th Rank in National level entrance for M.Sc Human Genetics conducted by Andhra University, Vishakapatanam, India in June 2001.

3. Selected in Molecular and Human Genetics M.Sc program in B.H.U, Varanasi, India in July 2001.

4. Selected in Biotechnology M.Sc programme in Sri Venkateswara University, India in July 2001.

5. GRE score of 1030/ 1600 (2004).

6. Secured 1st Rank in Biology Section (8.8/10 pointer) in Scuola Normale Superiore International PhD programme in Italy, August 2006.

Publications

Buitrago-Molina LE, **Pothiraju D**, Lamlé J, Marhenke S, Kossatz U, Breuhahn K, Manns MP, Malek N, Vogel A. Rapamycin delays tumor development in murine livers by inhibiting proliferation of hepatocytes with DNA damage. Hepatology. 2009 Aug;50(2):500-9.

Pothiraju D, Buitrago-Molina LE, Marhenke S, Manns MP, Vogel A.

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