STUDY OF NEW CURCUMIN ANALOGS FOR THE TREATMENT OF ERα NEGATIVE BREAST CANCERS

BABASAHEB D. YADAV

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
AT THE UNIVERSITY OF OTAGO, DUNEDIN, NEW ZEALAND

4TH OF JANUARY 2012
Abstract:

Breast cancer is the most prevalent form of cancer diagnosed in women, and there continues to be limited drug treatment options for the \( \sim 30\% \) of patients with estrogen receptor (ER\( \alpha \))-negative breast cancers. In the search for effective drugs for ER\( \alpha \) negative breast cancer, several lead compounds from natural products such as curcumin (diferuloylmethane), the primary bioactive compound isolated from the rhizome of turmeric (Curcuma longa Linn.), have emerged. Though curcumin showed promising anticancer activity in various \textit{in vitro} and \textit{in vivo} models of breast cancer, its clinical application was limited due to its low bioavailability and low stability in physiological media. Therefore, research groups have concentrated on the synthesis and characterization of curcumin analogs. Our lab previous developed cyclohexanone curcumin analogs and showed enhanced anticancer activity towards ER\( \alpha \) negative breast cancer cells compared to curcumin. To search for more potent compounds, this study was designed to develop second generation heterocyclic cyclohexanone curcumin analogs and also examine their anticancer activity in various \textit{in vitro} and \textit{in vivo} models of ER\( \alpha \)-negative breast cancer. This work demonstrated that among 20 heterocyclic curcumin analogs screened, 3,5-bis(3,4,5- trimethoxybenzylidene)-1-methylpiperidine-4-one (RL71) and 1-methyl-3,5-bis[(E)-(4-pyridyl) methylidene]-4-piperidone (RL66) showed the most potent anticancer activity with IC\textsubscript{50} values in submicromolar range (<1µM) in MDA-MB-231, MDA-MB-468 and SKBr3 breast cancer cells. Further \textit{in vitro} mechanism of action studies of RL71 and RL66 demonstrated a cell cycle arrest in G2/M phase or S/G2/M phase and induction of apoptosis in all the three ER\( \alpha \) negative breast cancer cells in a concentration-, cell line- and time-dependent manner. Moreover, the effect on various cell signaling proteins involved in cell proliferation and cell death was also examined by Western blotting. Both compounds showed a similar type of result. RL71 and RL66 significantly increased the phosphorylation of stress activated protein kinases, p38 and JNK1/2 in a concentration and time-dependent manner. Moreover, both the compounds modulated the PI3K/Akt/mTOR pathway and showed activation of p27kip1 and cleaved caspase-3 in a time- and cell-line dependent manner. In HER2 overexpressing SKBr3 cells, RL71 and RL66 significantly down regulated phosphorylation of HER2.

The study also involved assessment of anti-angiogenic activity of both the compounds \textit{in vitro}. The results showed that at 1 µM, RL71 and RL66 inhibited HUVEC cell invasion by 46% and 48% respectively compared to control, along with the ability of
these cells to form tube like networks and thus showed anti-angiogenic potential in vitro. In addition, RL71 (1 µM) and RL66 (2 µM) inhibited migration of MDA-MB-231 cells in vitro.

Both the drugs were further tested for their bioavailability in mice. The results showed that RL71 and RL66 were bioavailable after an oral dose of 8.5 mg/kg dose. The next study was designed to study the anticancer potential of both the compounds in vivo. For this, female athymic nude mice were subcutaneously inoculated with MDA-MB-468 (8 x 10^6) breast cancer cells and were treated with either vehicle (water) or RL71 or RL66 at 0.85 mg/kg or 8.5 mg/kg dose for 10 weeks. The results showed that RL71 did not significantly reduce the tumor volume compared to vehicle treated mice. However, RL66, at a dose of 8.5 mg/kg significantly reduced the tumor volume by 48% compared to the vehicle group. Both the compounds did not produce any toxicity after 10 weeks treatment as the total body weight remained unchanged. Moreover, RL66 treatment showed no significant change in major organ weight and gave normal plasma ALT values. The mechanism of tumor reduction by RL66 was further studied by examining its effect on various cell signaling proteins by Western blotting. The results showed that the tumors treated with 8.5 mg/kg of RL66 had a marginally reduced expression of EGFR whereas the expression of NF-κB and the phosphorylation of Akt were considerably increased compared to vehicle treatment. Moreover, the expression of p27kip1 was up regulated and the phosphorylation of mTOR was down regulated compared to vehicle treatment. However, none of the effects were significantly different compared to control. Further mechanistic studies by immunohistochemistry showed a significant reduction in the microvessel density of tumors as seen by a 59% reduction in CD105 protein in tumors from mice treated with 8.5 mg/kg of RL66 compared to vehicle treatment. In conclusion we have shown that RL71 and RL66 have potent anticancer and anti-angiogenic properties in vitro. However, RL71 needs further modification to enhance its anticancer effect in vivo, whereas RL66 has potential for development into novel treatments for ERα negative breast cancer.
Acknowledgement

I express my deep sense of gratitude to Asst. Prof. Rhonda Rosengren for giving me an opportunity to do PhD under her guidance. I appreciate all her contributions of time, ideas, and funding to make my PhD experience productive and stimulating. I am thankful to her for support and motivation and help for my thesis writing.

I thank Dr. Lesley Larsen for providing the compounds and explaining the ideas. My special thanks to Dr. Sebastian Taurin for his incredible help and patience during my PhD. He has contributed immensely for the technical and mental support during all the tough times. I am grateful to him for the suggestions and for explaining the concepts.

Thanks to Mhairi for helping me during my finishing days of PhD. I thank all lab mates Kirstie, Andrew, Khanh, Julian and Ingrid for their help and fun time.

I would like to acknowledge the staff of Department of Pharmacology and Toxicology for the help and suggestions throughout my PhD. I thank my PhD committee members Prof. Paul Smith, Dr. Ivan Sammut and Asst. Prof. Steve Kerr for their guidance and support.

I thank Michele Wilson, Department of microbiology for assisting me for FACS analysis and Dr. Paul Hessian, Department of physiology for teaching me apoptosis analysis on FACS.

I thank the University of Otago for providing me PhD scholarship. I thank the Maurice and Phyllis Paykel Trust and the Genesis Oncology Trust for funding my travel for the Stockholm cancer conference.

Thanks to all postgrad colleagues Yimin, Simran, Sangeeta, Prasanta, Sweta, Phil, Jack, Lucy, Emily, Irene, John, Morgyen, Oliver and Mimi for their company, help and all the fun I had during last 3 years.

I specially thank Punam and Shweta for their immense support during my initial time of PhD. Thanks for the unconditional friendship and fun we had and for teaching me how to cook yummy Indian food.

I am grateful for time spent with flatmates Ricky and John and our memorable trips around New Zealand, having dinner together and watching movies.

Lastly, I would like to thank my family for all their love and encouragement. For my parents who supported me in all my pursuits. Thanks for being there all the time I needed. It is to them this thesis is dedicated.
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List of Abbreviations

ADH  atypical ductal hyperplasia
Akt  serine/threonine-specific protein kinase family, also known as protein kinase B
ALH  atypical lobular hyperplasia
ALT  alanine amino transferase
APS  ammonium persulfate
ATM  ataxia telangiectasia mutated
BCA  4, 4’-dicarboxy-2,2’-biquinoline acid solution (bicinchoninic acid solution)
Bcl-2  B-cell lymphoma 2
Bcl-XL  B-cell lymphoma-extra large
BDHPC  2, 6-bis((3,4-dihydroxyphenyl)methylene)-cyclohexanone
BDMP  1, 5 bis(3,5 dimethoxy phenyl) 1,4 pentadiene-3-one
bFGF  basic fibroblast growth factor
BLBC  basal-like breast cancer
BMHPC  2, 6-bis((3-methoxy-4-hydroxyphenyl)methylene)-cyclohexanone
BSA  body surface area
BUN  blood urea nitrogen
CDK  cyclin dependent kinase
CDKI  cyclin dependent kinase inhibitor
CI  combination index
CK  cytokeratin
Cmax  maximum concentration obtained in the plasma
Compound 14  3, 5-bis(2-fluorobenzylidene)-piperidin-4-one, acetic acid salt
COX-2  cyclooxygenase-2
CRC-FNPs  curcumin loaded fibrinogen nanoparticles
Curc-OEG  curcumin oligo ethylene glycol nanoparticles
DAB  diaminobenzidine
DCIS  ductal carcinoma in situ
dd H2O  double distilled water
DHA  decosahexaenoic acid
DMBA  7, 12 dimethylbenzantracene
DMC  dimethoxycurcumin
DMEM/HamF12  dulbecco’s modified eagle’s media/nutrient mixture F-12 Ham
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
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<td>DPX</td>
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<td>EGM</td>
<td>endothelial growth media</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescein-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FEA</td>
<td>flat epithelial atypica</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GFP</td>
<td>green florescence positivity</td>
</tr>
<tr>
<td>GI50</td>
<td>concentration at which cell growth is inhibited by 50%</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HC</td>
<td>hydrazinocurcumin</td>
</tr>
<tr>
<td>HEPES</td>
<td>hydroxyethyl piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor-2</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>haematoxylin and eosin staining</td>
</tr>
<tr>
<td>HIF-1</td>
<td>hypoxia-inducible factor-1</td>
</tr>
<tr>
<td>HMBME</td>
<td>4-hydroxy-3-methoxybenzoic acid methyl ester</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horse-radish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IC50</td>
<td>concentration which kills 50% of cells.</td>
</tr>
<tr>
<td>IGFR</td>
<td>insulin-like growth factor receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IκB</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κB kinase</td>
</tr>
<tr>
<td>IOA</td>
<td>isoobtusilactone</td>
</tr>
<tr>
<td>IOP</td>
<td>inhibition of apoptosis protein</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal (route of administration)</td>
</tr>
<tr>
<td>IU/L</td>
<td>international units per litre</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>Kd</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug-resistant</td>
</tr>
<tr>
<td>MeHPLA</td>
<td>methyl-p-hydroxyphenyllactate</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>M1G</td>
<td>malondialdehyde-DNA</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3, 5-dimehtylthiazole-2-yl-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mammalian target of rapamycin complex 2</td>
</tr>
<tr>
<td>MVD</td>
<td>microvessel density</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>Nano-CUR</td>
<td>curcumin-PLGA nanoparticle</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature compound</td>
</tr>
<tr>
<td>ORR</td>
<td>overall response rate</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>Estrogen</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>PAC</td>
<td>5-bis (4-hydroxy-3-methoxybenzylidnen)-N-methyl-4-piperidone</td>
</tr>
<tr>
<td>PAkt</td>
<td>phosphorylated Akt</td>
</tr>
</tbody>
</table>
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP</td>
<td>poly ADP-ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidyl choline</td>
</tr>
<tr>
<td>pCR</td>
<td>complete pathological response</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>P-Akt</td>
<td>phosphorylated Akt</td>
</tr>
<tr>
<td>P-4E-BP1</td>
<td>phosphorylated 4E-BP1</td>
</tr>
<tr>
<td>PEGFR</td>
<td>phosphorylated EGFR</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>P-gp</td>
<td>p glycoprotein</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PHER2</td>
<td>phosphorylated HER2</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol-4, 5-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol-3, 4, 5-triphosphate</td>
</tr>
<tr>
<td>P-JNK</td>
<td>phosphorylated JNK</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>P-mTOR</td>
<td>phosphorylated mTOR</td>
</tr>
<tr>
<td>PO</td>
<td>per oral</td>
</tr>
<tr>
<td>P-P38</td>
<td>phosphorylated P-38</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>pRB</td>
<td>phosphorylated retinoblastoma protein</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PS6K</td>
<td>phosphorylated S6K</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RHEB</td>
<td>ras homologue enriched in brain</td>
</tr>
<tr>
<td>RICTOR</td>
<td>rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>RL90</td>
<td>2, 6-bis (3-pyridinylmethylene)-cyclohexanone</td>
</tr>
<tr>
<td>RL91</td>
<td>2, 6-bis (4-pyridinylmethylene)-cyclohexanone</td>
</tr>
<tr>
<td>RL92</td>
<td>2, 6-bis ((3-methoxy-4-(2-(4-morpholinyl) ethoxy)-phenyl) methylene)-cyclohexanone</td>
</tr>
</tbody>
</table>
List of abbreviations

**RL10** 2, 6-Bis ((1-methyl-1H-pyrrol-2-yl) methylene)-cyclohexanone  
**RL11** (2E, 6E)-2, 6-Bis ((1-methyl-1H-imidazol-5-yl) methylene) cyclohexanone  
**RL7** (2E, 6E)-2, 6-Bis ((1-methyl-1H-indol-3-yl) methylene) cyclohexanone  
**RL8** (2E, 6E)-2, 6-Bis ((1-methyl-1H-imidazol-2-yl) methylene) cyclohexanone  
**RL12** (3E, 5E)-3, 5-Bis (2, 5-dimethoxybenzyldiene)-1-methylpiperidin-4-one  
**RL54** (2E, 6E)-2, 6-Bis ((2-fluoropyridine-3-yl) methylene) cyclohexanone  
**RL55** (2E, 6E)-2, 6-Bis ((2-fluoropyridine-4-yl) methylene) cyclohexanone  
**RL66** 1-Methyl-3, 5-bis [(E)-(4-pyridyl) methyldiene]-4-piperidone  
**RL62** 1-methyl-3, 5-bis [(E)-(3-pyridyl) methyldiene]-4-piperidone  
**RL71** (3E, 5E)-3, 5-Bis (3, 4, 5-trimethoxybenzyldiene)-1- methylpiperidin-4-one  
**RL6** (3E, 5E)-3, 5-Bis (2-fluoro-4, 5-dimethoxybenzyldiene)-1-methylpiperidin-4-one  
**RL9** (3E, 5E)-3, 5-Bis (2, 5-dimethoxybenzyldiene)-1-methylpiperidin-4-one  
**RL26** 1-methyl-3, 5-bis [(E)-(2-thienyl) methyldiene]-4-piperidone  
**RL27** (3E, 5E)-1-Methyl-3, 5-bis((1-methyl-1H-imidazol-2-yl)methylene) piperidin-4-one  
**RL53** (2E, 4E)-8-Methyl-2,4-bis((pyridine-4-yl)methylene)-8-aza-cyclo[3.2.1]octan-3-one  
**RL60** (2E,4E)-8-Methyl-2,4-bis((pyridine-3-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one  
**RL65** (2E,4E)-2,4-Bis (3,4,5-trimethoxybenzyldiene)-8-methyl-8-aza- bicycle [3.2.1] octan-3-one  
**RL63** 2, 5-bis (3-pyridylmethylene)-cyclopentanone  
**RL75** tert-butyl 4-oxo-3,5-bis((pyridin-3-yl)methylene)piperidine-1-carboxylate  
**RL197** tert-butyl 3,5-bis(2,5-dimethoxybenzyldiene)-4-oxopiperidine-1-carboxylate  
**ROS** reactive oxygen species  
**RT** room temperature  
**RTK** receptor tyrosine kinase  
**SAPK** stress activated protein kinase  
**SAR** structure activity relationship  
**s.c.** subcutaneous (route of administration)  
**SCID** severely compromised immunodeficiency  
**SDS-PAGE** sodium dodecylsulfate-polyacrylamide gel electrophoresis  
**SEM** standard error of the mean
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SERM</td>
<td>selective oestrogen receptor modulator</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2</td>
</tr>
<tr>
<td>S6K</td>
<td>S6 kinase 1</td>
</tr>
<tr>
<td>SMEDDS</td>
<td>self-microemulsifying drug delivery system</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulphorhodamine B</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tween Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethlenediamine</td>
</tr>
<tr>
<td>TF</td>
<td>tissue factor</td>
</tr>
<tr>
<td>Tf-C-SLN</td>
<td>transferrin-mediated solid lipid nanoparticles of curcumin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNBCs</td>
<td>triple negative breast cancers</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>tumor node metastasis</td>
</tr>
<tr>
<td>TPCPD</td>
<td>tetrahydrocurcumin</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>VECs</td>
<td>vascular endothelial cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
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</table>
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1.1 Breast cancer

Breast cancer is a leading cause of cancer related mortality in women exceeded only by lung cancer (Jemal et al., 2011; Wu et al., 2005). The World Health Organization estimates that there are 519,000 breast cancer deaths every year worldwide. Furthermore, the New Zealand Breast Cancer Foundation estimates that over 2500 women are diagnosed with breast cancer every year in New Zealand and approximately 600-700 women die from the disease each year. Moreover, NZ women have a life time risk of 11% of being diagnosed of breast cancer. Maori women are reported to have 66% higher mortality rate compared to non-Maori women. Overall, the incidence rates of breast cancer are higher in the Western world, Northern Europe, Australia/New Zealand, and North America; intermediate in South America, the Caribbean, and Northern Africa; and low in sub-Saharan Africa and Asia (Jemal et al., 2011). This variability in the incidence rates is mainly due to the reproductive and hormonal factors and the availability of early detection services (Jemal et al., 2011). In addition, several epidemiological studies have mentioned other factors that increase the risk of breast cancer; for example, age, socioeconomic status, family history, life style, diet, alcohol, body mass index, height and exposure to radiation (Adami et al., 1990).

The National Cancer Institute, USA, defines breast cancer as the cancer that forms in tissues of the breast, usually the ducts (tubes that carry milk to the nipple) and lobules (glands that make milk). It develops due to the uncontrolled growth of breast cells which occurs as a result of mutations, or abnormal changes, in the genes responsible for regulating the growth of cells (Elenbaas et al., 2001). Breast cancer is an important disease because of its heterogeneity in terms of morphology, biological characters, clinical behavior and different prognostic and therapeutic implications (Rakha et al., 2009a; Tavassoli et al., 2003). Hanahan and Weinberg (2000) described various characteristics of cancer and termed them ‘hallmarks of cancer’. They include; self-sufficiency in growth signals, insensitivity to growth inhibitory and apoptotic signals, unlimited replicative potential, sustained angiogenesis and motility, tissue invasion and metastasis (Hanahan et al., 2000).
1.1.1 Classification of breast cancer

Breast cancers are classified in different ways which help physicians in planning the proper treatment and determining prognosis. This classification is based mainly on histopathological type, grade, stage, hormone receptor status, and the molecular type as determined by gene expression profiling (Andre et al., 2006; Rakha et al., 2010). On the pathological level, breast cancer is divided into two types, namely ductal carcinoma and lobular carcinoma. Two models have been proposed to describe the progression of ductal carcinoma. According to Wellings and colleagues, ductal carcinoma develops from the normal terminal duct lobular unit (TDLU) and further undergoes various stages over a long period of time (Wellings et al., 1973; Wellings et al., 1975). The key stages in its progression include flat epithelial atypical (FEA), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). The second model by Page and colleagues proposed introduction of usual epithelial ductal hyperplasia (UDH) as an intermediate stage of progression between FEA and DCIS (Dupont et al., 1985; Page et al., 1985). The different steps in the progression of ductal carcinoma are shown in Fig. 1.1. In the case of lobular carcinoma, the progression takes place through atypical lobular hyperplasia (ALH), lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC) (Hanby et al., 2008; Lakhani et al., 2006).

![Figure 1.1 Progression of breast cancer](image)

**Figure 1.1 Progression of breast cancer**: This is a hypothetical model of breast cancer progression which shows different key stages of cancer development in ducts of the normal breast. Adapted from Lopez-Garcia et al. (2010).

The in situ and invasive cancers are further classified by histological tumor grade, which encompasses factors such as the tumor’s differentiation and proliferative potential.
A low-intermediate grade (I-II) represents a well, or moderately, differentiated tumor with a low proliferative potential, while a high grade (III) represents a poorly differentiated tumor with a high proliferative potential (Fabbri et al., 2008). Clinically, high- and low-grade tumors are associated with the highest and lowest rates of recurrence and the shortest and longest recurrence times respectively, while intermediate-grade breast cancers display phenotypic and clinical behavioral characteristics that lie in between low- and high-grade tumors (Sgroi, 2010). The staging system of classification is another type and is called the tumor-node-metastasis (TNM) system. It includes the tumor size, involvement of lymph node and metastasis status of tumor (Fabbri et al., 2008).

The traditional and well known classification of breast cancer includes determination of hormone receptor status of the tumor by immunohistochemistry techniques. It is mainly based on assessing estrogen receptor (ER) and human epidermal growth factor receptor-2 (HER2) status of the tumor (Bast et al., 2001). Unfortunately this system does not give accurate measurement of ER and HER2 and shows lack of reproducibility and substantial variability. Therefore, gene expression profiling techniques were introduced for better understanding of the heterogeneity of breast cancer at molecular level and for accurate measurement of ER, HER2 and several other genes involved in its progression and spread. Based on this, various studies reported the existence of five main molecular subtypes. They differ from each other based on the presence or absence of three receptors: ERα, progesterone receptor (PR) and HER2. They include luminal A, luminal B, basal-like, HER2 and normal breast-like subtypes (Fig. 1.2). In addition, luminal C and interferon-regulated subtypes have also been described (Hu et al., 2006; Perou et al., 2000; Sorlie et al., 2003; Sorlie et al., 2001; Sotiriou et al., 2003; Weigelt et al., 2010a). It is reported that these five subtypes differ from each other with respect to clinical presentation, sites of relapse, histological features, response to chemotherapy and outcome (Carey et al., 2006; Livasy et al., 2006; Parker et al., 2009; Smid et al., 2008; Weigelt et al., 2010a). Luminal cancers which are ERα positive originate from luminal epithelial cells and have a low- to intermediate-grade tumor. Luminal A shows greater expression of ER-related genes and lower expression of proliferative genes than luminal B (Brenton et al., 2005; Venetia R et al., 2011; Voduc et al., 2010). ERα negative breast cancers which are divided into HER2 positive and basal-like cancers are of high grade (Sheikh et al., 1994) and have poor clinical outcome compared to ERα positive breast cancers (Hu et al., 2006; Sotiriou et al., 2003). In addition, triple negative breast cancers (TNBCs) have been recognized as a subgroup of ERα negative breast cancers and have been shown to resemble
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the profile of basal-like cancers (Badve et al., 2011). Despite the controversial definition of TNBCs, they brought attention due to poor prognosis and highly aggressive nature.

**Figure 1.2 Molecular classification of breast cancer:** Breast tumors are classified on molecular level by gene expression profiling. This classification gives a better idea about the heterogeneity and prognosis of breast tumors and accordingly helps to plan an appropriate treatment strategy in the clinic. Adapted from (Perou et al., 2000; Sorlie et al., 2003; Sorlie et al., 2001; Sotiriou et al., 2003).

The molecular subtype classification system of breast cancer by using microarray has been widely accepted and it is believed to be associated with the clinical subgroups of breast cancer (Mackay et al., 2011). However, some studies have confirmed that there are large scale gene expression differences between ERα positive and ERα negative breast cancers and suggested that further molecular subsets may exist within or in addition to these groups (Pusztai et al., 2003; Rouzier et al., 2005; Sorlie et al., 2001). Moreover, Weigelt and colleagues demonstrated that the molecular breast cancer subtypes (luminal A, luminal B and HER2), with the exception of the basal-like subtype, are not highly reproducible and the slight variation in the classification methodologies have a significant impact on classification assignment for individual breast cancer patients (Weigelt et al., 2010b). Therefore, a standardization of microarray-based breast cancer classification is warranted before it is implemented in the clinical practice (Colombo et al., 2011).

As mentioned before, each molecular subtype has a different prognosis and therefore they have different sensitivity towards treatment. The selection of appropriate breast cancer therapy relies on tumor characteristics such as size, histopathology, estrogen and progesterone receptor status, the level of HER2 expression, and lymph node infiltration. Accordingly, the various therapies includes surgery (lumpectomy, mastectomy), radiotherapy, hormonal therapy, chemotherapy and immunotherapy (Wiechec et al., 2009).

### 1.1.2 Evolution of different types of breast cancer

Before studying various breast cancer sub-types and their specific treatment options in detail, it is necessary to understand the theory behind their evolution and progression. Various theories have been proposed to elucidate the mechanism behind heterogeneity and
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evolution of breast cancer. In particular, the basic evolution of breast tumorigenesis has been explained by the two models, the clonal evolution model and the cancer stem cell model (Fig. 1.3) (Campbell et al., 2007; Hsiao et al., 2010; Kakarala et al., 2008; Nowell, 1976; Reya et al., 2001; Wicha et al., 2006). According to the clonal evolution model, any breast epithelial cells (stem cells, progenitor or differentiated cells) undergo random multiple mutations which provide them a selective growth advantage over adjacent normal cells. Later over time additional advantageous genetic and epigenetic changes lead to tumor proliferation and cellular heterogeneity.

Figure 1.3 Hypothetical models of origin of human breast cancer: A) Stem cell model B) Clonal evolution model. This is a hypothetical model of breast cancer progression which shows different key stages of cancer development in ducts of normal breast. Adapted from Sgroi, et al. (2010).

The cancer stem cell model states that the breast cancer originates from normal stem cells or progenitor cells. Their ability for self-renewal and differentiation leads to the generation of different sub types of breast cancer cells with phenotypic heterogeneity. Also, tumor progression occurs due to the metastatic spread of cancer stem cells, and cancer recurrence is caused by their resistance to therapy. In support of the stem cell theory, Lim et al. (2009) demonstrated that progenitor cells within the luminal compartment of the human breast cells represent a cancer initiating population for BRCA1 carrying breast tissues and basal-like tumors (Lim et al., 2009). Similarly, Molyneux et al. (2010) stated that basal-like tumors with BRCA1 mutations originate from luminal epithelial progenitors and not from basal stem cells. Thus these studies suggest another
possible mechanism of breast tumorigenesis and state that it is not the ‘cell of origin’ alone but the inherent plasticity of stem and progenitor cells that decides the final phenotype of the tumor during tumorigenesis.

1.2 ERα negative breast cancer

It is reported that approximately 60% of total breast cancer patients are ERα positive and remaining 30-40% are ERα negative (Carey et al., 2006). ERα positive breast cancer relies on estrogen binding to the ERα which leads to dimerization of the receptor-ligand complex and its binding to the estrogen response element (ERE) (Pietras et al., 2007). Consequently, estrogen promotes cell growth and proliferation in an estrogen-dependent fashion (Pietras et al., 2007). On the other hand, ERα negative breast cancer does not grow in an estrogen-dependent fashion (Pietras et al., 2007). It is poorly differentiated, of higher histological grade, and associated with a higher recurrence rate and a decreased overall survival (Rakha et al., 2007b). Moreover, two thirds of breast cancers diagnosed in pre-menopausal women are ERα negative (Dew et al., 2002; Habel et al., 1993). The exact mechanism of progression of ERα negative breast cancer is not fully understood. ERα negative invasive breast cancers are often considered to develop from ERα positive breast cancers by genetic alteration (gene instability, loss of heterozygosity exon deletion and so on), epigenetic alteration such as promoter methylation or ER protein degradation in proteasome after hypoxia in non-vascularized tumor (Rochefort et al., 2003). However, some invasive human breast cancers may be directly ERα negative via a hormone-independent pathway as shown by the gene knock out studies in mice (Hewitt et al., 2002; Korach, 1994). In the absence of ER, ERα negative breast cancers rely on other growth stimulating factors such as the epidermal growth factor (EGF) (Biswas et al., 2000) and the vascular endothelial growth factor (VEGF) for their progression (Somanath et al., 2006). Knowing this fact, targeted therapy is considered to be the best option for the treatment of ERα negative breast cancers. However, no specific drug therapy has been found to be beneficial so far for ERα negative breast cancer patients. Therefore, there is an urgent need for the development of a safe and efficacious drug therapy for the treatment of ERα negative breast cancers.

1.2.1 Triple negative breast cancer

TNBCs, which are subgroup of ERα negative breast cancers, account for about 10-17% of all breast cancers (Reis-Filho et al., 2008). They are characterized by the lack of expression of ER, PR and HER2. Epidemiological studies have suggested that they are
more prevalent among young African and African-American women (Lin et al., 2009b; Reis-Filho et al., 2008). Moreover, TNBC patients are associated with younger age at first birth, increased parity, decreased breastfeeding, higher BMI, and lower socioeconomic status (Ayca et al., 2011; Trivers et al., 2009). TNBCs are poorly differentiated, highly malignant and aggressive and have a poor outcome (Chen et al., 2009). They are characterized by a high rate of early recurrence and visceral metastasis to the brain and lung (Haffty et al., 2006; Zhang et al., 2011). Moreover, it is reported that the peak risk of recurrence occurs within three years of diagnosis and the mortality rates are increased for five years after diagnosis (Dent et al., 2007; Kwan et al., 2009). A recent clinical study showed that TNBCs are more malignant and have a poorer disease free survival compared with HER2 overexpressing breast cancers (Wang et al., 2009). At the histological level, the majority of TNBCs are of grade III (Dent et al., 2007). Although they have a phenotype similar to basal-like cancers which also do not express ER, PR and HER2, TNBCs are more heterogeneous compared to basal-like cancers (Bertucci et al., 2008; Kreike et al., 2007). It is established that there is an overlap between molecular features and clinical/pathological features of TNBCs and basal-like cancers.

Various immunohistochemical studies have demonstrated the molecular features associated with TNBCs. They mainly include $p53$ mutation, high Ki67, epidermal growth factor receptor (EGFR) over-expression, and dysfunction in the BRCA1 pathway (Rowe et al., 2009). It is estimated that the EGFR is expressed in 60% of TNBCs (Arslan et al., 2009). Also, TNBCs are associated with over expression of cytokeratins 5, 6, 14, and 17, smooth muscle actin, P-cadherin and c-kit. These characteristics are also similar to basal-like breast cancers.

### 1.2.1.1 Treatment for triple negative breast cancer

TNBCs have limited treatment options due to the lack of a specific therapeutic target (Arslan et al., 2009). Also, they are considered to have diverse biology and treatment sensitivity. Therefore, the standard approach to systemic adjuvant therapy is not desirable for TNBCs. However, chemotherapy is the most standard approach for the treatment of TNBCs (Arslan et al., 2009). Furthermore, they do not respond to currently available hormonal therapies such as selective estrogen receptor modulators (SERM) and HER2 based therapies (trastuzumab) (Cheang et al., 2008). Therefore, there is an urgent need of novel therapeutic agents for management of TNBCs.
Various studies have demonstrated the benefits of chemotherapy in neoadjuvant, adjuvant and metastatic treatment of TNBCs (Isakoff, 2010). For example, there is evidence that conventional cytotoxic agents such as anthracyclins and taxanes have proven to be effective in the treatment of TNBCs in a neoadjuvant setting (Kang et al., 2008). Recently, a retrospective clinical trial was carried out in 1118 patients with stage I-III breast cancer. The patients received neoadjuvant chemotherapy with anthracyclin and taxane. The results showed that the patients with TNBCs had higher pCR (complete pathological response) rates compared to non-TNBCs (22% vs 11%). However, TNBC patients showed lower 3 year progression free survival (PFS) (63% vs 76%) and 3 years overall survival (OS) rates (74% vs 89%) compared to non TNBCs (Liedtke et al., 2008). Nevertheless, despite the higher initial response rates (RR), they were found to be associated with poor long term survival. A meta-analysis of four studies comparing anthracyclin based regimens with cyclophosphamide, methotrexate and 5 fluorouracil (CMF) indicated that TNBC patients had a 23% reduction in disease relapse from anthracyclins relative to CMF treated patients (Di Leo et al., 2009).

The use of specific cytotoxic agents is currently being investigated in TNBCs. For example, DNA damaging drugs such as platinum derivatives have been found to achieve increased response rate towards TNBCs (Sirohi et al., 2008). A preoperative phase II study of cisplatin showed promising results in women with stage-2 or 3 TNBC (Silver et al., 2010). The patients were treated with four cycles of cisplatin at 75 mg/m\(^2\) every 21 days. 22% of patients achieved pCR including two patients with BRCA1 mutations while 50% of patients showed good pathological responses. Platinum drugs have also been effective in combination with other agents in neoadjuvant settings. A phase II study of 74 patients with triple negative breast cancer showed 65% pCR after the administration of eight weekly cycles of cisplatin (30 mg/m\(^2\)), epirubicin (50 mg/m\(^2\)) and paclitaxel (120 mg/m\(^2\)) (Frasci et al., 2009).

Epothilones are another novel class of microtubule stabilizing agents which have been tested among TNBC patients. Among epothilones, ixabepilone is one of the currently approved agents used for the treatment of taxane resistant metastatic breast cancer (Conte et al., 2009). In a phase III clinical trial of ixabepilone, the combination of ixabepilone and capecitabrin in advanced breast cancer showed a significant overall response rate (ORR) and PFS in TNBC patients compared to capecitabrin alone (Arslan et al., 2009). Another phase III clinical trial demonstrated therapeutic effectiveness of ixabepilone in patients with ER\(\alpha\) negative or ER/PR/HER2-negative metastatic breast cancer (Pivot et al., 2009).
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It is postulated that multi-targeted therapies might be the most promising strategies in the management of TNBCs. The novel targets are those molecules which are important in the growth and progression of cancer. Based on the biology of TNBCs, some of the potential targets include EGFR targeted agents (cetuximab, erlotinib), angiogenesis inhibitors (bevacizumab, sunitinib) and PARP inhibitors (AZD2281, BSI-201) and protein kinase components of the mitogen activated protein (MAP)-kinase pathway and phosphatidylinositol-3 kinase (PI3K) pathway whose downstream targets include Akt and mammalian target of rapamycin (mTOR) (everolimus) (Table 1.1) (Anders et al., 2008; Cleator et al., 2007; Dawson et al., 2009; Irvin et al., 2008; Shiu et al., 2008).

Several clinical trials of drugs targeting EGFR, PARP and VEGF are currently being conducted as a single agents or in combination for the treatment of TNBCs (Venkitaraman, 2010). The EGFR targeting monoclonal antibody, cetuximab in combination with carboplatin showed a modest response rate of 17% in a pre-treated population of triple negative patients (Carey, 2010; Carey et al., 2007). In addition, PARP inhibitors showed sensitivity towards triple negative tumors which carry BRCA1 mutations. A randomized Phase II study of the PARP inhibitor, BSI-201 was conducted in patients with metastatic TNBC (O'Shaughnessy et al., 2009). The patients administered with BSI-201 in combination with carboplatin and gemcitabine showed better responses and significant improvement in survival. Similarly, oral administration of the PARP inhibitor olaparib (400 mg) showed a 41% response rate in chemotherapy-refractory BRCA-deficient breast cancer patients, 50% of whom had triple negative tumors (Tutt et al., 2010). Furthermore, anti-angiogenic agents targeting VEGF have been found to be beneficial for the treatment of TNBCs. Also, the combination of anti-VEGF monoclonal antibody bevacizumab and paclitaxel resulted in higher progression free survival compared with paclitaxel alone in patients with metastatic breast cancer including a triple negative subset (Miller et al., 2007). However, many other specific targets are currently under investigation for treatment of TNBCs.
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Table 1.1: Therapeutic targets and drugs for triple negative breast cancer: Adapted from Cleator et al. (2007), Dawson et al. (2009) and Irvin et al. (2008).

<table>
<thead>
<tr>
<th>Targeted strategy</th>
<th>Drug</th>
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<tr>
<td>Targeting aberrant DNA repair</td>
<td>PARP inhibitors AZD2281 (olaparib); BSI-201</td>
</tr>
<tr>
<td></td>
<td>Trabectedin (DNA transcription inhibitor)</td>
</tr>
<tr>
<td></td>
<td>Platinatum agents (Cisplatin, carboplatin)</td>
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<tr>
<td>Anti-angiogenic agents</td>
<td>Bevacizumab, sunitinib</td>
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<tr>
<td>EGFR targeting</td>
<td>Cetuximab, erlotinib, gefitinib</td>
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<tr>
<td>Epigenetic modifications</td>
<td>Trichostatin A, butyrate, vorinostat</td>
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<tr>
<td>c-Kit targeted</td>
<td>Imatinib, sunitinib</td>
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<tr>
<td>Src inhibitor</td>
<td>Dasatinib</td>
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<tr>
<td>mTOR inhibitor</td>
<td>Everolimus</td>
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1.2.2 Basal-like breast cancer

Basal-like breast cancers (BLBCs) are characterized by expression of genes found in basal/myoepithelial cells of the normal breast (Rakha et al., 2009b). They represent 15% of all breast cancers depending on the way they are defined and they also contribute to a high proportion (~ 80%) of the overall triple negative subgroup (Kilburn, 2008). Many authors stated that both TNBC and BLBC share a number of molecular and morphological features with 60%-90% overlap but they are not identical. It has been established that not all TNBCs have basal-like phenotype and not all BLBCs are TN (Chen et al., 2009; Dawson et al., 2009). It is stated that 50%-80% of TNBC tumors express basal markers and are associated with poor outcome (Rakha et al., 2009b). TNBCs are designated on the
basis of clinical assays for ER, PR and HER2, whereas gene expression profiling is supposed to be the gold standard for the identification of basal-like breast cancers (Dawson et al., 2009) but some studies have also used immunohistochemistry. Some authors have used basal cytokeratins alone (e.g. CK5/6, CK17 and CK14) to define BLBCs irrespective of presence of other markers (Banerjee et al., 2006; Foulkes et al., 2004; Rakha et al., 2007a) while some authors used EGFR expression along with basal cytokeratins (CK5/6, CK14, CK17) to define BLBCs by immunohistochemical analysis in ER and HER2 negative tumors (Dawson et al., 2009; Rakha et al., 2009b). This definition is one of the widely used definitions of BLBCs. In addition, some authors included PR negativity of tumors to define BLBCs as triple negative tumors that express CK5/6 and or EGFR (Carey et al., 2006; Cheang et al., 2008). Perou et al. (2000) studied the gene expression pattern in a set of 65 surgical specimens of human breast tumors. They showed that the basal-like gene expression cluster consisted of CK5, CK17, integrin β4 and laminin. In addition, other markers such as c-kit, P-cadherin, caveolins 1 and 2, nestin, osteonectin, vimentin and laminin have also been identified to define BLBCs (Rakha et al., 2009a; Viale et al., 2009). However, there is no consensus on the definition of BLBCs and there are some shortcomings. Some authors argued that identification of BLBCs on the basis of gene expression profiling is misleading and requires more understanding of breast cancer biology (Gusterson, 2009). A recent study demonstrated a lack of expression of pRB and p16 along with p53 mutation in approximately 30% of BLBCs (Subhawong et al., 2008). In addition, BLBCs displayed alterations in various molecular pathways such as the MAPK pathway, the Akt pathway, and the poly ADP-ribose polymerase 1 (PARP1) pathway (Petrelli et al., 2009).

BLBCs are more aggressive (Brenton et al., 2005), have poor clinical output (Dawson et al., 2009) and according to some studies the poor prognosis experienced by patients is likely due to the lack of availability of an effective treatment (Brenton et al., 2005). The majority of basal-like tumors are invasive ductal cancers with high histological grade. The histological features include a pushing non-infiltrative border of invasion, larger zones of geographic or comedo-type necrosis, stromal lymphocytic infiltrate, scant stromal content, lack of tubule formation, marked cellular pleomorphism, high nuclear-cytoplasmic ratio, vesicular chromatin, prominent nuclei, high mitotic index and frequent apoptotic cells (Livasy et al., 2006; Rakha et al., 2009a). The clinical and pathological profile of BLBCs are nearly similar to the tumors of BRCA1 carriers (Seal et al., 2010).
1.2.2.1 Treatment options for basal-like cancers

Similar to TNBCs, BLBCs also do not respond to SERM and trastuzumab. Therefore, there are no standard adjuvant treatment options for basal-like breast cancer. However, it was reported that basal-like tumors may have a better response than non basal-like tumors to neoadjuvant adriamycin and cyclophosphamide as well as paclitaxel, doxorubicin, 5-fluorouracil and cyclophosphamide therapy (Rakha et al., 2009a). One study showed a complete pathological response to neoadjuvant paclitaxel and doxorubicin chemotherapy in 45% of basal-like cancers, 45% of HER2-positive cancers and 6% of luminal cancers (Cleator et al., 2007). Currently, various targets have been identified in BLBCs. For example, surface receptors such as EGFR, HER3 and HER4, c-kit, MAPK pathway, Akt pathway and DNA signaling kinase, ataxia telangiectasia mutated (ATM) (Rakha et al., 2009a). However, the current treatment options available for BLBCs appear to be similar to TNBCs. Nevertheless, genetic instability has been the greatest hurdle in the treatment of basal-like cancers due to its potential to develop resistance (Cleator et al., 2007).

1.2.3 HER2 Subtype

It is estimated that 25% of all breast cancers over express HER2 (Dean-Colomb et al., 2008). In post-menopausal women, it was reported that an early age at menarche was associated with HER2+ breast cancer (Trivers et al., 2009). Currently HER2 status is evaluated by immunohistochemistry or gene expression via fluorescent in situ hybridization (FISH) (Dean-Colomb et al., 2008). Recently, in order to avoid unreliable results, the recommendations for HER2 testing have been published. HER2 positive status is defined as an IHC staining score of 3+ and a FISH score of more than six HER2 gene copies/nucleus or a FISH ratio (HER2 gene signal to chromosome 17 signal) greater than 2.2 (Wolff et al., 2007). HER2+ breast cancers are more aggressive and the patients experience significantly shorter disease free periods and overall survival (Kulkarni et al., 2008). In addition, HER2 positive cancers have poor prognostic factors and unfavorable pathologic tumor characters including large tumor size, higher nuclear grade, S phase fraction, aneuploidy, positive lymph node, a higher proliferative index and high histological grade (Kulkarni et al., 2008; Nielsen et al., 2009; Skarlos et al., 2011). It is reported that VEGF is up regulated in HER2 over expressing cancer which contributes to its aggressive phenotype (Dean-Colomb et al., 2008). Moreover, the tumors of this subtype have a high proportion (40% to 80%) of TP53 mutations (Brenton et al., 2005). The overall
summary of several immunohistochemical and pathological features of breast cancer subgroups are mentioned in Table 1.2.

1.2.3.1 HER2 treatment

Trastuzumab, a recombinant humanized monoclonal antibody, has been approved as a first-line treatment for patients with HER2 positive metastatic breast cancer (Nielsen et al., 2009). Clinically, trastuzumab was found to potentiate the effectiveness of conventional chemotherapies. Therefore, trastuzumab along with an adjuvant therapy containing doxorubicin, cyclophosphamide, and paclitaxel has also been approved (Kulkarni et al., 2008). Furthermore, combination with taxanes and vinorelbine is also established (Nielsen et al., 2009). In metastatic breast cancer patients, who were previously treated with trastuzumab, the use of lapatinib in combination with capecitabine is established as a second line treatment (Nielsen et al., 2009). The median duration of response with trastuzumab was less than one year and its mechanism of action involved HER2 downregulation, selective inhibition of HER2-HER3 heterodimerization, prevention of HER2 extracellular domain proteolytic cleavage, and activation of an immune response including antibody-dependent cellular cytotoxicity (Shabaya et al., 2011). However, there is evidence that HER2 tumors develop resistance to anticancer therapies including trastuzumab (Kulkarni et al., 2008). As a single agent, trastuzumab achieved an ORR of a median duration of about nine months (Nielsen et al., 2009). In HER2 patients, the low response rate indicates primary resistance to trastuzumab, whereas a short duration of response indicates rapid development of acquired resistance. The main mechanism behind trastuzumab resistance includes up regulation of PI3K signaling and increased activity of mTOR, Src, IGF-IR and cdk2 (Shabaya et al., 2011). In addition, trastuzumab develops cardiotoxicity in a dose independent manner leaving several questions unanswered such as patient selection for anti-HER2 treatment of metastatic disease based on HER2 testing, dose scheduling of trastuzumab, duration and tolerability of therapy, role of alternative agents like lapatinib, and clinical importance of trastuzumab resistance and efficacy (Sengupta et al., 2008). Therefore, there is an urgent need for the development of novel agents for the treatment of HER2+ cancers.
<table>
<thead>
<tr>
<th>BC subgroups</th>
<th>Properties</th>
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<tr>
<td></td>
<td><strong>ER, PR, HER2 status</strong></td>
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<tr>
<td>Luminal A (LA)</td>
<td>ER+/PR+, HER2−</td>
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<tr>
<td>Luminal B (LB)</td>
<td>ER+, PR+, HER2+</td>
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<tr>
<td>Triple negative/Basal-like breast cancer (TNBC)/(BLBC)</td>
<td>ER−, PR−, HER2−,</td>
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<tr>
<td>HER2-positive</td>
<td>HER2+, ER−, PR−</td>
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Table 1.2 Immunohistochemical and pathological features of breast cancer subgroups. 
1.3 Key Signaling in ERα negative breast cancers

It is known that estrogen regulates the proliferation of ERα positive breast cancer cells through the regulation of various gene networks and signaling pathways. However, ERα negative breast cancer cells proliferate independently of estrogen signaling. Stimulation of various signaling pathways leads to the growth and proliferation of ERα negative breast cancer cells. Among them, EGFR, downstream PI3K/Akt/mTOR and NF-κB pathways as well as stress kinase pathway will be discussed in detail.

1.3.1 EGFR structure

The epidermal growth factor receptor belongs to subclass I of the receptor tyrosine kinase super-family and comprises four members: EGFR/ERBB1, ERBB2/HER2, ERBB3, and ERBB4 (Hynes et al., 2005). All the members have an extracellular ligand-binding region, transmembrane region and an intracellular tyrosine kinase domain (El-Rayes et al., 2004; Hynes et al., 2005). Under normal physiological conditions, ERBB receptors are activated by different ligands such as EGF and transforming growth factor-α (TGF-α) (Yarden, 2001). These ligands bind to the extracellular domain of the receptor and activate the tyrosine kinase domain by inducing the formation of receptor homodimers or heterodimers (Yarden, 2001). Consequently, the specific tyrosine kinase residues within the cytoplasmic region are auto-phosphorylated resulting in the stimulation of various signaling cascades (Fig. 1.4) (Olayioye et al., 2000).

The ERBB receptors are involved in the development of various types of cancers including breast cancer (Hynes et al., 2005). In particular, overexpression of EGFR is recently described in 60% of breast cancers (Bo et al., 2008). In human tumors, ERBB receptors are activated by various mechanisms such as, 1) overexpression of the receptor 2) mutation in the receptor which results in ligand-independent activation 3) autocrine activation by overproduction of ligand or 4) ligand-independent activation through another receptor system such as the urokinase plasminogen receptor (El-Rayes et al., 2004).

1.3.1.1 Mechanism of ERBB receptor signaling

Various groups have proposed a ligand-induced dimerization mechanism for EGFR activation. The structural studies on the extracellular region of the EGFR family members showed that the ligand induces a dramatic conformational change in the receptor which promotes dimerization and activation of EGFR (Bose et al., 2009).
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Figure 1.4 EGFR signaling: Upon activation by ligand in the extracellular region, EGFR undergoes dimerization which leads to autophosphorylation of the intracellular tyrosine kinase domain and the downstream signal cascade. Adapted from Lemmon et al. (2010) and Schlessinger et al. (2002)

The extracellular region of each ERBB receptor consists of the four domains I, II, III and IV (Fig. 1.4). Domain I and III are ligand binding domains, each ~ 160 amino acids in length and comprise B helix LRR-like solenoid domains. Domain II and IV are cysteine-rich domains consisting of ~ 150 amino acids each (Lemmon et al., 2010; Schlessinger, 2002). The extracellular region of EGFR, ERBB3 and ERBB4 exists in two conformations: tethered/closed conformation and untethered/extended conformation (Riese et al., 2007). In closed conformation, EGFR remains in an inactive state where the domain II interacts with the domain IV via a critical sequence of amino acids at position 242–259 in the domain II and further inhibits ligand binding and receptor dimerization (Gan et al., 2007; Lemmon et al., 2010). In untethered conformation, domain I and domain IV undergo a 130° rotation so that the dimerization arm is no longer in contact with domain IV but is available to facilitate dimerization with a second EGFR molecule (Gan et al., 2007). Moreover, in
untethered conformation, domain I and III form a ligand binding pocket that allows the interaction between a single ligand molecule and domain I and III (Riese et al., 2007). In contrast to other receptors, the ERBB2 receptor does not bind ligands. It has a structurally different extracellular region from the others (Hynes et al., 2005). The domain II-IV interaction is absent and the dimerization loop in domain II is absent (Hynes et al., 2005). It is established that ERBB2 is the preferred partner for the other ligand activated ERBBs. ERBB2 possesses a unique subdomain I-III interaction that blocks the site of interaction making the ligand binding impossible (Hynes et al., 2005).

The dimerization of the extracellular region facilitates receptor signaling by activating the intracellular kinase domain. Furthermore, one or more tyrosine residues in the activation loop of the tyrosine kinase domain undergoes trans autophosphorylation (Schlessinger, 2002). The activation loop is located between the N-terminal lobe consisting of residues 685-769 and the C-terminal lobe, consisting of residues 773-953 of the kinase domain. The activation loop and the α C helix in the kinase N-lobe adopt a specific configuration in the activated tyrosine kinase domain that is required for catalysis of phosphotransfer from adenosine triphosphate (ATP) molecule to the tyrosine residue of the non-catalytic region of EGFR (Lemmon et al., 2010). However, some studies reported that the EGFR/ERBB family do not require trans phosphorylation of their activation loops for activation and instead follow an allosteric mechanism of activation (Lemmon et al., 2010). This mechanism states that the EGFR tyrosine kinase domain forms an asymmetric dimer where the C-lobe of one tyrosine kinase domain, called the “Activator” makes a strong connection with the N lobe of the second tyrosine kinase domain, called the “Receiver”. Consequently, the N lobe of the receiver kinase undergoes conformational changes activating EGFR without phosphorylation of its activation loop (Lemmon et al., 2010). The phosphorylation of the C-terminus of the EGFR provides specific docking sites for the Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domains of intracellular signal transducers and adaptors leading to the intracellular signal transduction (Jorissen et al., 2003). The activated EGFR mediates a number of signaling cascades including the PI3K/Akt/ mTOR pathway and MAPK pathway (Jorissen et al., 2003).

1.3.1.2 HER2 receptor

HER2/Neu is a member of the epidermal growth factor receptor family and is localized to chromosome 17q (Ross et al., 2004). It is found to be overexpressed or amplified in 10-34% of invasive breast cancers (Schechter et al., 1984). It is associated
with increased cell proliferation, cell motility, tumor invasiveness, metastasis and angiogenesis in breast cancer (Moasser, 2007). As mentioned before, HER2 does not bind to its own ligand and it is activated via heterodimerization with other members of the EGFR receptor family by their respective ligands (Graus-Porta et al., 1997). It is also reported to homodimerize with itself when expressed at high levels (Di Fiore et al., 1987; Hudziak et al., 1987). However, HER2 heterodimers are more potent than the homodimers. In addition, heterodimers containing HER2 have a particularly high ligand binding and signaling potency compared with non-HER2 containing dimers resulting in prolonged and enhanced activation of signaling pathways (Karunagaran et al., 1996; Sliwkowski et al., 1994). Upon activation, specific tyrosine sites in the carboxyl tail of HER2 are autophosphorylated (Akiyama et al., 1991; Margolis et al., 1989; Segatto et al., 1990). This further provides docking sites for the adaptor proteins grb2 and/or Shc which bind to the carboxyl tail through Src homology 2 (SH2) domain and activate the ras/MAPK pathway (Egan et al., 1993; Pelicci et al., 1992; Yarden et al., 2001). In addition, HER2 also activates the PI3K/Akt pathway by forming a heterodimer with ERBB3 (Holbro et al., 2003). Specific inhibition of HER2 by use of a humanized monoclonal antibody or small molecule tyrosine kinase inhibitors has gained clinical importance for patients overexpressing HER2.

1.3.2 PI3K/Akt/mTOR signaling pathway

PI3K is a major signaling component downstream of EGFR. It is implicated in various normal cellular processes such as cell proliferation, survival, growth, and motility (Luo et al., 2003). It is reported that PI3K signaling is dysregulated in various types of cancers including breast cancer (Bose et al., 2006; Castaneda et al., 2010). PI3K belongs to a large family of PI3K-related kinases or PIKK (Hennessy et al., 2005). Class I A type of PI3Ks are heterodimers composed of a catalytic subunit (p110) and a regulatory subunit (p85) (Vara et al., 2004). Three isoforms each of catalytic subunit (p110), p110α, p110β, p110δ, and a regulatory subunit (p85), p85α, p85β, p55γ, have been identified (Foster et al., 2003). The phosphorylated receptor tyrosine kinase such as ERBB receptor binds to p85 which further relieves the inhibition of p110 and recruits the dimer to the plasma membrane (Hennessy et al., 2005). The activated PI3K converts the plasma membrane lipid PIP2 (3, 4) to PIP3 (3, 4, 5). PIP3 mediates its cellular effects through specific binding to the protein lipid binding domains, namely the FYVE and pleckstrin homology (PH) domains (Osaki et al., 2004). PIP3 is subsequently metabolized to produce PIP2 by various phosphatases such as SHIP-1 and -2 and the phosphatase and tensin homologue on
chromosome 10 (PTEN) which dephosphorylates the 3’OH group phosphorylated by PI3K and thus controls PI3K signaling (Fig. 1.5) (Hennessy et al., 2005). Activated PIP3 acts as a second messenger and recruits the PH domain containing proteins such as serine/threonine kinase 3’-phosphoinositide-dependent kinase1 (PDK1) and Akt/PKB and activates downstream signaling (Castaneda et al., 2010).

1.3.2.1 Akt

Akt is a serine/threonine kinase that belongs to the family of protein kinase B (PKB) or the related to protein kinase A and C (RAC) family (Castaneda et al., 2010; Osaki et al., 2004; Testa et al., 2005). The PKB family of Akt consists of three closely related, highly conserved cellular homologues, Akt1, Akt2 and Akt3 which are also referred to as PKBα, PKBβ and PKBγ respectively (Castaneda et al., 2010; Testa et al., 2005). Akt is implicated in diverse signaling cascades that regulate cell proliferation and survival, cell growth and metabolism, invasiveness, genome stability and angiogenesis (Testa et al., 2005). Each Akt gene is composed of a PH domain on the N-terminus, a central kinase domain and a C-terminal regulatory domain (Osaki et al., 2004). Upon activation of PI3K, the 3’-phosphoinositides interact with the PH domain in the N-terminal region of Akt and recruits it to the plasma membrane (Hennessy et al., 2005). Consequently, Akt is activated by phosphorylation at two sites, Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain (Martelli et al., 2005). The phosphorylation at Thr308 and Ser473 is brought about by the enzymes phosphoinositide-dependent kinase-1 (PDK1) and phosphoinositide-dependent kinase-2 (PDK2) leading to the full activation of Akt (Carnero, 2010). Finally, Akt is translocated to the cytoplasm and nucleus where it phosphorylates and activates various target proteins (Fig. 1.5) (Jiang et al., 2008).

The PI3K/Akt signaling pathway modulates various events related to cell cycle and apoptosis. It regulates cell cycle progression through G1-S phase transition by phosphorylating the CDKIs p21 and p27 and blocking their nuclear translocation (Jiang et al., 2008). Moreover, Akt can indirectly stabilize the cell cycle proteins c-myc and cyclin D1 (Vara et al., 2004). Akt activates mTOR-raptor kinase complex (mTORC-1), which in turn leads to the activation of p70S6K1 and phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and enhances protein synthesis (Engelman, 2009; Osaki et al., 2004).
Akt can increase glycogen synthesis and cell metabolism through the inactivation of the forkhead (FOXO) family of transcription factors and glycogen synthase kinase 3 (GSK3) (Engelman, 2009). The inhibition of GSK3 by Akt further stimulates the activity of β-catenin which is a transcriptional factor involved in various cellular processes (Osaki et al., 2004). Importantly, Akt enhances cell survival through the inhibition of pro-apoptotic proteins such as FasL, Bim, Bad and BAX and stimulation of anti-apoptotic genes, BcL2 and NFκB (Dillon et al., 2007; Wickenden et al., 2010). Akt inactivates several upstream stress activated protein kinases (SAPKs) via phosphorylation and leads to an increased apoptosis via terminal kinases c-Jun N-terminal kinase (JNK) and p38 (Liao et al., 2003; Uzgare et al., 2004). It is reported that the PI3K/Akt pathway is up regulated in about 70% of breast cancer patients and is associated with aggressive behavior and poor clinical outcome (Castaneda et al., 2010; Lopez-Knowles et al., 2010). In particular, Akt is constitutively activated in HER2 positive and TNBCs where it leads to cell survival and cell proliferation (Umemura et al., 2007; Zhou et al., 2000). Also, its activation is associated with the development of multi-drug resistance in breast cancer (Knuefermann et al., 2003). It has been shown that inhibition of Akt leads to the inhibition of ERα negative

**Figure 1.5 PI3K/Akt signaling:** Upon activation of PI3K by ligand, PIP2 is converted to PIP3. Akt is activated by PDK1 and binds to PIP3 through the SH2 domain. Akt further phosphorylates multiple downstream proteins involved in cell proliferation and cell death. Adapted from Jiang et al. (2008) and Castaneda et al. (2010).
breast tumors both \textit{in vitro} and \textit{in vivo} (Weng \textit{et al.}, 2008). Thus Akt inhibition has a therapeutic relevance in treatment of ERα negative breast cancer.

\subsection*{1.3.2.2 \textit{mTOR}}

\textit{mTOR} belongs to a group of serine-threonine protein kinases of the PI3K superfamily, referred to as class IV PI3Ks (Liu \textit{et al.}, 2009a). It plays an important role in regulation of protein synthesis, cell cycle progression, cellular proliferation and growth, autophagy and angiogenesis (Ciuffreda \textit{et al.}, 2010). \textit{mTOR} is a 289kD protein and consists of a catalytic kinase domain, a FKBP12–rapamycin binding (FRB) domain, a putative auto-inhibitory domain (‘repressor domain’) near the C-terminus and up to 20 tandemly repeated HEAT (Huntingtin, EF3, A subunit of PP2A and TOR) motifs at the N-terminus, as well as FRAP–ATM–TRRAP (FAT) and FAT C-terminus domains (Huang \textit{et al.}, 2003a). HEAT motifs serve as protein–protein interaction units, whereas FAT and FAT C terminus domains participate in modulation of the catalytic kinase activity of \textit{mTOR}.

\textit{mTOR} exists in two distinct complexes; \textit{mTORC1} and \textit{mTORC2} (Gibbons \textit{et al.}, 2009). The \textit{mTORC1} complex is composed of a complex of the \textit{mTOR} catalytic subunit, the regulatory associated protein of \textit{mTOR} (RAPTOR), the proline-rich Akt substrate 40 kDa (PRAS40) and the protein mlST8 (Liu \textit{et al.}, 2009a). \textit{mTORC2} is composed of \textit{mTOR}, rapamycin-insensitive companion of \textit{mTOR} (RICTOR), mammalian stress-activated protein kinase interacting protein 1 (mSIN1) and mlST8 (Liu \textit{et al.}, 2009a). Akt can activate \textit{mTOR} by phosphorylating both PRAS40 and tuberous sclerosis 2 protein (TSC2; also known as tuberin) (Li \textit{et al.}, 2004). \textit{mTOR} further phosphorylates and activates two translational regulatory proteins: ribosomal protein S6 kinase 1 (S6K1; also known as p70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Li \textit{et al.}, 2004). S6K1 phosphorylates the 40S ribosomal protein S6, which is involved in the translation of mRNAs with repressive 5′-terminal oligopolypyrimidine (5′TOP) tracts (Yang \textit{et al.}, 2008; Zoncu \textit{et al.}, 2011). As the role of \textit{mTORC2} in breast cancer progression is not clear, only \textit{mTORC1} signaling is discussed here. The activity of \textit{mTORC1} is controlled by the small GTPase Ras homologue enriched in brain (Rheb) and the tuberous sclerosis complex (\textbf{Fig. 1.6}) (Garami \textit{et al.}, 2003; Saucedo \textit{et al.}, 2003). TSC acts as a GTPase-activating protein that inactivates the Rheb and thereby further activates \textit{mTORC1} kinase activity (Tee \textit{et al.}, 2003; Zhang \textit{et al.}, 2003). A molecular link between \textit{mTOR} and cancer came from the evidence from mutations in TSC-Rheb-\textit{mTORC1} circuit (Zoncu \textit{et al.}, 2011). The role of \textit{mTOR} in cancer progression has been demonstrated in
various human cancers including lung, gastric, hepatocellular, colorectal, renal, ovarian, breast, prostate, and pancreatic cancer (Advani, 2010; Bjornsti et al., 2004; Huang et al., 2003b; Neshat et al., 2001; Yu et al., 2001). In particular, in breast cancer mTOR is constitutively activated due to the signaling defects in various signaling pathways such as overexpression of HER2 and/or EGFR, loss of PTEN, mutations in the PI3K and overexpression of Akt (Hynes et al., 2006). mTOR promotes breast cancer progression by regulating nutrient uptake, cell metabolism, and angiogenesis (Hay et al., 2004). Pre-

![Diagram of mTORC1 signaling](image)

**Figure 1.6 mTORC1 signaling:** Akt phosphorylates and inhibits the complex of TSC-1/2. Upon phosphorylation, the TSC complex acts as a GTPase activating protein (GAP) and turns down the activity of RHEB resulting in the mTORC1 activation. mTORC1 can further phosphorylate 4E-BP1 and S6K1 and thereby promote cell growth, proliferation and survival. Adapted from Ciuffreda et al. (2010) and Li et al. (2004).

clinical studies have demonstrated that the inhibition of mTOR leads to the inhibition of HER2 positive (Lu et al., 2007) and TNBCs (Umemura et al., 2007). In addition, the clinical trials of an oral mTOR inhibitor everolimus have demonstrated promising antitumor activity in metastatic breast cancer patients (Ellard et al., 2009; Tabernero et al., 2008).
1.3.2.3 4E-BP1

4E-BP1 (also known as PHAS) is a eukaryotic translation initiation factor 4E (eIF-4E) binding protein that plays an important role in mRNA translation initiation and progression and thus controls the rate of protein synthesis and cell proliferation (Heesom *et al.*, 2001; Pause *et al.*, 1994). 4E-BP1 is activated via phosphorylation by mTORC1 which leads to the dissociation of eIF-4E from the 4E-BP1-eIF-4E complex, thereby providing free eIF-4E-mediated cap-dependent translation (Yang *et al.*, 2008; Zoncu *et al.*, 2011). mTOR is the main phosphorylation pathway of 4E-BP1 although other kinases such as CDK1, PI3K-Akt, ERK1/2 and ataxia-telangiectasia (ATM) have also been reported to be involved (Petroulakis *et al.*, 2006). mTOR phosphorylates 4E-BP1 at four main sites, Thr37, Thr46, Thr70 and Ser65 (Mothe-Satney *et al.*, 2000a; Mothe-Satney *et al.*, 2000b). Under dephosphorylated conditions, 4E-BP1 remains bound to eIF-4E, which impairs further protein translation. 4E-BP1 has been reported to be dysregulated in various cancers including the breast (Kerekatte *et al.*, 1995). A clinical trial conducted in breast cancer patients demonstrated that the overexpression of 4E-BP1 was associated with increased tumor size, poor differentiation, lymph node metastasis and disease recurrence (Rojo *et al.*, 2007).

It is established that the expression of 4E-BP1 and eIF-4E are positively associated with each other in breast tumors and 4E-BP1 regulates the influence of eIF-4E on cancer progression (Coleman *et al.*, 2009). Also, the ratios of p4E-BP1 to total 4E-BP1 and of eIF-4E to 4E-BP1 have been shown to correlate with high tumor grade and lymph node metastasis (Armengol *et al.*, 2007). eIF-4E functions by binding to the cap at 7-mehtylguanosine in the 5´ untranslated regions (5´ UTRs) of mRNAs (Gingras *et al.*, 2001; Mamane *et al.*, 2004; Zimmer *et al.*, 2000). Subsequently, mRNA is recruited to the eIF-4E complex leading to the unwinding of the secondary structure of 5´ UTRs and thus revealing the translation initiation codon necessary for ribosomal binding. The overexpression of eIF-4E, either by amplified transcription or gene amplification, has been reported in metastatic human breast cancers (Li *et al.*, 1997) and its overexpression has been associated with a high rate of cancer recurrence and poor prognosis (Li *et al.*, 1998). Moreover, a recent clinical trial in node negative breast cancer patients showed that patients with higher expression of eIF-4E had a higher rate of cancer recurrence and cancer related deaths (Holm *et al.*, 2008). It is reported that eIF-4E dependent translation generates specific oncogenic gene products such as VEGF, cyclin D1, c-myc and fibroblast growth factor (FGF-2) and thus contributes to cancer progression (Culjkovic *et al.*, 2007).
Consequently, eIF-4E has been established as a target for cancer therapy (Graff et al., 2008). Recent preclinical and clinical studies have shown the therapeutic benefits of the targeted inhibition of eIF-4E (Assouline et al., 2009; Graff et al., 2007).

1.3.3 NFκB

Nuclear factor κB (NFκB) is a transcriptional factor which plays an important role in promoting cell survival, inflammation, differentiation and cell growth (Sethi et al., 2008). NFκB belongs to the Rel family of five distinct proteins: RelA (p65), RelB, c-Rel, NFκB1 (p50/p105) and NFκB2 (p52/p100) (Ghosh et al., 1998; Wu et al., 2005). These structurally-related proteins consist of an approximately 300 amino acid sequence called the Rel homology domain (RHD) (Grimm et al., 1993). The N-terminal domain of RHD contains sequences important for DNA binding, dimerization and inhibitor (IκB) binding (Rayet et al., 1999). The C-terminal regions of RelA, RelB and c-Rel contain transcriptional activation domains, whereas the C-terminal regions of p105 and p100 contain inhibitory domains (Rayet et al., 1999). Close to the C-terminal end of the RHD, there is the nuclear localization signal (NLS) which is essential for the transport of active NF-kB complexes into the nucleus (Siebenlist et al., 1994). Upon activation, the Rel proteins can form homodimers or heterodimers which bind to DNA and regulate the expression of various genes (Sethi et al., 2008).

A commonly activated NFκB consists of p65 and p50 heterodimers (Sarkar et al., 2008). In an inactive state, NFκB exists in the cytoplasm in association with the member of the inhibitor of κB (IκB) family (Garg et al., 2002). The IκB family consists of structurally related proteins, IκB-α, IκB-β, IκB-γ, IκB-ε and IκB-ζ, BCL-3 and the precursor proteins p100 and p105 although IκB-α is the most common protein (Gilmore et al., 2006; Lee et al., 2007). The IκBs contain multiple copies of a 30-33 amino acid sequence, called ankyrin repeats which makes a connection between IκB and NF-κB dimers (Gilmore et al., 2006). The ankyrin repeats interact with a region in the RHD of the NF-κB proteins and prevents their nuclear translocation by masking NLS (Manavalan et al., 2010; Siebenlist et al., 1994). There are two well-known activation pathways of NFκB: The canonical pathway and atypical pathway (Gilmore et al., 2006; Scheidereit, 2006; Tergaonkar, 2006). Both the pathways involve a common regulatory step of an IκB kinase (IKK) complex comprising of catalytic kinase subunits (IKKα and/or IKKβ) and the regulatory non-enzymatic scaffold protein NEMO (NF-kappa B essential modulator also known as IKKγ).
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The canonical pathway is activated by the binding of TNF-α or cytokines to the TNF receptor or cytokine receptor respectively leading to recruitment of IkB kinase (IKK) which is a multisubunit complex containing two catalytic subunits (IKKa & IKKβ) and a regulatory subunit, IKKγ (Wu et al., 2005). The phosphorylation of IkB at two critical serine residues (Ser32 and Ser36 in IkBa, Ser19 and Ser23 in IkBβ) in their N terminal regulatory domain by the IKK complex further triggers their polyubiquitination and subsequent degradation by the 26S proteosome (Lee et al., 2007). Consequently, NFκB is released from IkB and translocated to the nucleus where it binds to DNA at a specific κB site (Ghosh et al., 1998).

The non-canonical or atypical pathway is activated through other receptors which are a subset of TNFR superfamily members such as lymphotoxin β-receptor (LTβR), B-cell-activating factor belonging to TNF family receptor (BAFFR), receptor activator for

Figure 1.7 NFκB signaling: Upon activation by Akt, IKK is phosphorylated leading to subsequent phosphorylation of IkBa. This results in dissociation of NFκB and its translocation to the nucleus. Adapted from Gilmore et al. (2006), Scheidereit et al. (2006), Tergaonkar et al. (2006).
nuclear factor κB (RANK), TNFR2 and CD40L (Sun, 2011). In addition, receptor tyrosine kinases such as EGFR may also induce the atypical pathway. Akt has been implicated in the activation of NF-κB via phosphorylation of IKKβ that subsequently causes phosphorylation of IκBα (Fig. 1.7) (Gustin et al., 2006). Activated NF-κB modulates the activity of the proteins involved in cell cycle and apoptosis which lead to the cell proliferation and survival (Dolcet et al., 2005).

It has been shown that NFκB is constitutively activated in ERα negative breast cancer cells and primary tumors (Biswas et al., 2000; Nakshatri et al., 1997; Romieu-Mourez et al., 2001; Sovak et al., 1997). In ERα negative breast cancer, it is activated through EGFR and the HER2 signaling (Biswas et al., 2006; Merkhofer et al., 2010). It’s activity is associated with an invasive and drug resistant breast cancer (Weldon et al., 2001). In addition, NFκB activation causes bone metastasis in breast cancer (Park et al., 2007). It is evident that inhibition of NFκB leads to the inhibition of growth and development of ERα negative breast tumors in vitro and in vivo (Biswas et al., 2003; Ciucci et al., 2006). Therefore, NFκB is an attractive therapeutic target for ERα negative breast cancers.

1.3.4 MAPK/SAPK

Mitogen-activated protein kinases (MAPKs) are components of kinase signaling cascades that regulate normal cell proliferation, survival and differentiation (Roberts et al., 2007; Zhang et al., 2002). Mammalian systems are comprised of three distinct subgroups of the MAP Kinase family: ERKs (extracellular signal-regulated kinases), JNKs (c-Jun N-terminal kinases) and p38 MAPKs (Benhar et al., 2002). Specifically, JNK and p38 are called stress activated protein kinases (SAPK) which are activated by environmental stresses, as well as by mitogens, inflammatory cytokines, oncogenes, and inducers of cell differentiation (Zhang et al., 2002). In addition, they play an important role in inducing programmed cell death (Davis, 2000; Kyriakis et al., 2001; Lewis et al., 1998). It is reported that the SAPK signaling is dysregulated in many cancers such as pancreas, lung, colon, breast and prostate (Demuth et al., 2007; Esteva et al., 2004; Greenberg et al., 2002; Hui et al., 2008; Iyoda et al., 2003; Juntila et al., 2007; Sakurai et al., 2006; Vivanco et al., 2007). The SAPK signaling cascade is activated by the phosphorylation of threonine and tyrosine residues located in the activation loop of the kinase domain. This phosphorylation is mediated via the MAPK kinases (MAPKK), which are in turn activated by the MAPKK kinase (MAPKKK) (Fig. 1.8).
The P38 kinase family was identified with four isoforms, namely p38 α, p38 β, p38 γ and p38 δ (Zhang et al., 2002). In response to a variety of extracellular stimuli such as UV light, heat, osmotic shock, inflammatory cytokines (TNF-α & IL-1) and growth factors (CSF-1), p38 isoforms undergo dual phosphorylation by M KK3 and M KK6 leading to their activation (Zarubin et al., 2005). In addition, p38 α can also be phosphorylated by M KK4. Activated p38 further activated various downstream substrates. For example, p38 can phosphorylate various transcriptional factors including ATF-2, Sap-1a and GADD153 (Wang et al., 1996). In addition, it controls NF-κB dependent transcription. p38 also regulates various events related to cell cycle and apoptosis, cell differentiation and tumor suppression (Takenaka et al., 1998; Zarubin et al., 2005). However, these effects are found to be cell type dependent and activator dependent (Nebreda et al., 2000).

Another SAPK member, JNK, consists of three isoforms, JNK1, 2 and 3, (Manning et al., 2002) also known as SAPKγ, SAPKα and SAPKβ respectively (Kyriakis et al., 1994). JNK 1 and 2 are ubiquitously expressed whereas JNK 3 expression is found only in

<table>
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<tr>
<th>Activators</th>
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<th>Stress, Cytokines, Growth factors, Ceramides</th>
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<td>MEKK 1-4, MLKs, ASK, TAK1</td>
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<td>MAP</td>
<td>P38/SAPK</td>
<td>JNK/SAPK</td>
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**Figure 1.8  SAPK signaling:** p38 and JNK SAPK are activated by various extracellular stimuli. Upon activation, they further activate various downstream substrates and play an important role in cell proliferation, differentiation and apoptosis. Adapted from Zhang et al. (2002)
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the brain (Yang et al., 1997). JNK is activated by phosphorylation at two amino acid residues in the activation loop by MKK4/SEK1 and MKK7 (Fleming et al., 2000). Upon activation, it binds to various substrates such as c-Jun, ATF-2 (activating transcription factor 2), Elk-1, p53, DPC4, Sap-1a and NFAT4 (Widmann et al., 1999). JNK can bind the NH2-terminal activation domain of c-Jun and phosphorylate c-Jun on Ser-63 and Ser-73 (Kunz et al., 2001). The transactivation of c-Jun causes AP-1 dependent transcription and various physiological responses such as inflammation, cell proliferation, survival and apoptosis (Dhanasekaran et al., 2008). The c-Jun/AP1-dependent apoptosis is also called a nuclear mechanism of JNK induced apoptosis. JNK can also induce apoptosis in a mitochondrial dependent manner, in which activated JNK translocates to mitochondria and phosphorylates the BH3-only family of Bcl2 proteins to antagonize the antiapoptotic activity of Bcl2 or Bcl-XL. This further leads to the release of cytochrome c and activation of the caspase-9-dependent caspase cascade (Dhanasekaran et al., 2008). In addition, JNK can also directly phosphorylate Bad and promote apoptosis (Dhanasekaran et al., 2008). The anti-apoptotic or pro-apoptotic function of JNK is reported to be dependent on the cell type and the activators (Liu et al., 2005).

In breast cancer cells, increased activation of both JNK and p38 has been reported to induce an anti-apoptotic effect which consequently leads to the increased cell proliferation and tumor growth (Santen et al., 2002; Whyte et al., 2009). It is reported that phosphorylated p38 is present in 17–20% of breast carcinomas (Esteva et al., 2004). Also, in breast invasive ductal carcinoma, increased phosphorylation of JNK1/2 and P38 were related to each other and were associated with poor overall survival (Yeh et al., 2006). It is suggested that activation of both JNK and p38 may increase tumor angiogenesis and growth, thus leading to a poor outcome in breast cancer (Yeh et al., 2006). Importantly, targeting p38 and JNK pathway is dependent mainly on the tumor cell type and tumor stage. Accordingly, certain cytotoxic agents have been reported to increase the activation of p38 and JNK in cancer cells in vitro and thus induce their anticancer effect via cell cycle arrest and apoptosis (Collett et al., 2004; Kuo et al., 2007; Liu et al., 2010a; Mansouri et al., 2003; Weir et al., 2007). In contrast, various clinical trial studies have been investigating the therapeutic potential of inhibitors of JNK and p38 for certain types of tumors (Wagner et al., 2009).
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1.3.5 Angiogenesis

Angiogenesis is the process of formation of new blood vessels from the pre-existing ones (Munoz-Chapuli et al., 2004). Angiogenesis plays an important role in the formation of vascularization during normal physiological processes including embryonic development, growth, regeneration and wound healing (Hoeben et al., 2004). Abnormal angiogenesis is implicated in several pathological processes such as tumor growth, metastasis, rheumatoid arthritis and diabetic retinopathy (Hoeben et al., 2004). Tumors beyond 1-2 mm³ in size rely on angiogenesis for the adequate supply of oxygen and nutrients (Folkman, 1971). It is established that the tumor cells release various signaling molecules to the surrounding host tissue for the initiation and formation of a new blood supply (Kalluri, 2003; Makrilia et al., 2009). The extracellular signals which stimulate angiogenesis mainly involve the transmembrane receptors: tyrosine kinase receptors, G-

Figure 1.9 Developmental steps of angiogenesis: The tumor cells release growth factors and MMPs towards the vascular basement membrane (VBM). In response, the VBM undergoes various derivative and structural changes such as endothelial cell proliferation, migration, tube formation and loop formation finally leading to the formation of new mature VBM. Subsequently, the pericytes along with the new VBM form a blood vessel. Adapted from www.angio.org and Kalluri, 2003.

protein-coupled receptors, tyrosine-kinase-associated receptors and serine-threonine kinase receptors (Munoz-Chapuli et al., 2004).
The process of angiogenesis is highly organized and involves a cascade of events such as the proliferation of endothelial cells, breakdown of extracellular matrix and migration of endothelial cells (Fig. 1.9) (Woodhouse et al., 1997). The angiogenic process is initiated with the production and release of angiogenic growth factors by tumor cells which mainly include the heparin-binding growth factor or fibroblast growth factor family, transforming growth factor-α, angiopoietins (Ang-1, -2, -3 and -4), vascular permeability growth factor (VPF) and VEGF (Carmeliet et al., 2000; Makrilia et al., 2009). The angiogenic growth factors bind to the specific receptors located on the endothelial cells of nearby pre-existing or parent blood vessels and activate the signaling cascade in the endothelial cells leading to their proliferation (Pandya et al., 2006). In addition, various key enzymes such as proteases and matrix metalloproteinase, are secreted and these play an important role in the cleavage of the basement membrane and extracellular matrix of parent blood vessels (Eichhorn et al., 2007; Jones et al., 2006). Subsequently, the activated endothelial cells migrate through the dissolved basement membrane towards the tumor cells and adhere to the extracellular matrix of the parent blood vessel by means of specialized molecules called adhesion molecules or integrins; for example, αvβ3, αvβ5 (Pandya et al., 2006). The integrins help the sprouting blood vessels grow forward which consequently leads to the formation of a blood vessel tube (Jones et al., 2006; Pandya et al., 2006). Tips of neighboring blood vessel tubes unite to form a loop through which blood begins to flow (Jones et al., 2006). Finally, the newly formed blood vessel is stabilized by smooth muscle cells and pericytes which further regulate the blood flow (Bergers et al., 2005).

Angiogenesis in the tumor vasculature is assessed in terms of microvessel density (MVD) (Hlatky et al., 2002). The higher vascular density is related to an increased risk of breast cancer. For example, Weidner et al. (1991) demonstrated that higher microvessel density correlated with the presence of metastatic disease (Weidner et al., 1991). In addition, in node negative breast cancers, microvessel density was an independent prognostic factor for survival (Weidner et al., 1992). VEGF is reported to be another prognostic factor for the relapse free and overall survival in patients with node-positive and node-negative breast cancers (Gasparini et al., 1997; Linderholm et al., 2003). VEGF has been reported to be an important factor in the development of breast tumor angiogenesis (Relf et al., 1997).

Angiogenesis inhibition is one of the important therapeutic strategies for breast cancer. Accordingly, various chemotherapeutic agents either alone or in combination with
other drugs have been developed for the inhibition of angiogenesis in breast tumors. In particular, the VEGF targeting agent bevacizumab (Gray et al., 2009; Miles et al.) has shown promising effects in clinical trials. In addition, other agents such as sunitinib, sorafenib, PTK787, AZD2171 and ZD6474 are undergoing clinical trials in advanced breast cancer patients (Burstein et al., 2008; Stöger et al., 2008).
1.4 Curcumin for the treatment of breast cancer

Curcumin (diferuloylmethane), a yellow colored polyphenol is an active component obtained from the roots and rhizomes of the perennial plant *Curcuma longa* (*Hatcher et al.*, 2008). It is the major component in the spice turmeric and is principally cultivated in India, Southeast Asia, China, and other Asian and tropical countries. Turmeric contains 3%-5% of curcuminoides. Also, commercial curcumin contains curcumin (curcumin I, 77%), demethoxycurcumin (curcumin II, ~17%), and bisdemethoxycurcumin (curcumin III, ~3%) (*Goel et al.*, 2008) (Fig. 1.10). Epidemiological studies have suggested a relationship between dietary consumption of curcumin and its chemopreventive effects (*Aggarwal et al.*, 2003). Curcumin exerts a myriad of biological activities such as antioxidant, anti-inflammatory, antiseptic, analgesic, antimalarial, neuroprotective and cardioprotective effects (*Goel et al.*, 2008). In addition, curcumin is reported to inhibit cancer cell survival, proliferation, invasion, angiogenesis and metastasis (*Kunnumakkara et al.*, 2008). The anticancer effect of curcumin is attributed to its ability to interact with multiple cell signaling proteins (*Kunnumakkara et al.*, 2008).

![Chemical structure of curcumin](image)

*Figure 1.10: Chemical structure of curcumin.* Adapted from *Kunnumakkara et al.* (2008)
1.4.1 Anti-breast cancer effects of curcumin *in vitro*

Curcumin exerts its anti-breast cancer properties through either ER dependent or independent pathways by inhibiting the proliferation of both ERα positive and ERα negative breast cancer cells (Verma *et al.*, 1997). Many studies have shown an anti-proliferative effect of curcumin in hormone-independent (SKBr3, MDA-MB-231, MDA-MB-468, BT-483), hormone-dependent (MCF-7, T-47D) and multi drug resistant (MCF-7 ADR, MCF-7/TH, MCF-7R, BT-20 TNF) breast cancer cells (Chiu *et al.*, 2009; Korutla *et al.*, 1995; Labbozzetta *et al.*, 2009; Lai *et al.*, 2012; Mehta *et al.*, 1997). For example, curcumin at a concentration of 10 μM completely inhibited the proliferation of both MDA-MB-468 and BT-483 cell lines with a median inhibitory concentration (**IC**$_{50}$) between 1 and 5 μM (Squires *et al.*, 2003) whereas in MDA-MB-231 cells the **IC**$_{50}$ value was reported to be 44.11 μM (Chiu *et al.*, 2009). Mehta *et al.*, (1997) measured curcumin mediated changes in the cell proliferation of various breast cancer cells by using the thymidine incorporation assay. Curcumin (27 μM) reduced cell viability to 1%, 8%, 6%, 9%, 15%, 13% and 26% in BT-20, BT-20TNF, SKBr3, MCF-7, MCF-7 ADR, T-47 D and ZR-75-1 cells, respectively, compared to control. Furthermore, Labbozzetta *et al.* (2009) reported the cytotoxicity of curcumin in MCF-7 and MCF-7R cells by the MTT assay. The **IC**$_{50}$ of curcumin was of 29 μM in MCF-7 cells and of 26 μM in MCF-7R cells (Labbozzetta *et al.*, 2009). Similarly, Shao *et al.* (2002) demonstrated that 50 μM curcumin completely suppressed the growth of MCF-7 cells stimulated by 17β-estradiol whereas in ERα negative MDA-MB-231 cells, the antiproliferative effect of curcumin was independent of estrogen. Recently, Lai *et al.* (2011) reported the cytotoxicity of curcumin in HER2 positive SKBr3 and BT-474 cells. They demonstrated that 27.15 μM of curcumin decreased the cell proliferation of both SKBr3 and BT-474 cells to 65% and 20% respectively, compared to control (Lai *et al.*, 2012).

1.4.2 The mechanism of anti-breast cancer effects of curcumin *in vitro*

Several studies have examined the mechanism of cytotoxicity of curcumin in various breast cancer cells. Specifically, in ER positive MCF-7 cells, curcumin inhibited the expression of ER transcripts in a dose-dependent manner in the presence of estrogen. This suggested that the inhibitory effect of curcumin may be occurring due to its interaction with estrogen at a receptor level. This was further supported by evidence that curcumin inhibited estrogen response element (ERE)-CAT activities in breast cancer cells (Shao *et al.*, 2002) in addition to the inhibition of the genes downstream of the ER
including pS2 and TGF-α (Shao et al., 2002). The anti-proliferative effects of curcumin have been mainly reported to occur via cell cycle arrest, induction of apoptosis and modulation of various cell signaling proteins involved in cell survival, proliferation and death.

Cell cycle studies demonstrated that curcumin induced cell cycle arrest at the G2/M phase in MDA-MB-231, SKBr3, BT474 and MCF-7 cells (Chiu et al., 2009; Fang et al., 2011; Lai et al., 2012; Mehta et al., 1997). Specifically, at 24 h curcumin (20 µM) increased the proportion of MDA-MB-231 cells in G2/M phase by 164%, whereas 47 µM curcumin increased the proportion of MCF-7 cells in S phase from 44% to 55% after 48 h with a concomitant increase of G2/M cells from 5% to 10% (Chiu et al., 2009; Fang et al., 2011). Conversely, some studies demonstrated that curcumin induced cell cycle arrest in G0/G1 phase in MCF-7 (Choudhuri et al., 2002) and MDA-MB-231 cells (Huang et al., 2011). Squires et al. (2003) reported a S/G2/M phase arrest in the MDA-MB-468 breast cancer cell line following curcumin treatment. Curcumin (20 µM) increased the proportion of MDA-MB-468 cells in the S/G2/M phase by 143% at 48 h (Squires et al., 2003). Recently, Lai et al. (2011) reported a G2/M phase cell cycle arrest in HER2 positive BT-474 and SKBr3 cells after curcumin treatment. In BT474 cells, curcumin decreased the S phase from 19% to 9% and increased the G2/M phase from 7% to 20% whereas in SKBr3 cells, after 48 h there was a decrease in the G0/G1 phase from 65% to 37% and an increase in the S phase from 28% to 37% and G2/M phase from 6% to 25% (Lai et al., 2012). The studies conducted by Poma et al. (2007b) reported that curcumin induced G2/M phase cell cycle arrest and apoptosis in the human multi-drug resistant breast cancer cell lines (MCF-7/TH, MCF-7R).

Various mechanisms have been suggested to explain the cell cycle arrest induced by curcumin. In particular, Holy et al. (2002) demonstrated that in MCF-7 cells, curcumin (10-20 µM) disrupted the mitotic spindle structure and induced micronucleation. This led to G2/M phase arrest and further halted DNA synthesis. Moreover, they also observed that the arrested mitotic cells exhibited monopolar spindles and chromosomes did not undergo normal anaphase movements. These observations suggested that curcumin-induced G2/M arrest is due to the assembly of aberrant, monopolar mitotic spindles that are impaired in their ability to segregate chromosomes (Holy, 2002).

Another mechanism by which curcumin produces cell cycle inhibition is via its effect on various cell cycle regulatory proteins. The cell cycle is promoted by activation of
cyclin dependent kinases (CDK), which are positively regulated by cyclins and negatively by CDK inhibitors (Malumbres et al., 2009). Cyclin D1 regulates cell cycle progression through G1-phase of the cell cycle by activating CDK4 and CDK6. It is established that cyclin D1 is a proto-oncogene which is overexpressed in ERα positive and ERα negative breast cancers and predicts poor prognosis (Umekita et al., 2002), while cyclin E along with CDK2 regulates the entry of cells from late G1 to S phase. Cyclin E overexpression is associated with poor prognosis and high proliferation in ERα negative breast cancer patients (Potemski et al., 2006). CDKIs, p21 and p27 belong to Cip/Kip family of proteins and their decreased expression has been correlated with poor prognosis in patients with breast cancer (Catzavelos et al., 1997; Pellikainen et al., 2003). It is reported that altered expression of proteins regulating the cell cycle make TNBCs more sensitive to cytotoxic therapy (Rouzier et al., 2005).

Studies have demonstrated that curcumin modulates the expression of cyclins, CDKs and CDKIs (p21, p27) in breast cancer cells. Curcumin (0.1 µM) decreased the expression of cyclin D1 and induced levels of p21 expression in MDA-MB-231 cells (Liu et al., 2009b) further leading to apoptotic cell death. Recently, Huang et al. (2011) demonstrated that in MDA-MB-231 cells, curcumin increased the expression of p27 along with the modulation of another cell cycle regulatory protein, Skp2 which is the F-box protein S phase kinase-associated protein. The Skp2 mainly acts through targeting p27 for degradation (Huang et al., 2011). A study conducted by Liu Q et al. (2009) reported that curcumin (5 µg/ml) down regulated the expression of CDK4 in BT-483 cells. In MCF-7 breast cancer cells, curcumin (50 µM) inhibited cyclin D1 and cyclin E expression, increased levels of CDK inhibitors p21 and p27 and up regulated tumor suppression gene p53 (Aggarwal et al., 2007).

It is evident that a strong relationship exists between the cell cycle arrest and the induction of apoptosis. Curcumin induces apoptosis in most, if not all, breast cancer cell lines. Apoptosis is regulated by a series of complex biochemical events and distinct morphologic alterations such as cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and the formation of apoptotic bodies (Ravindran et al., 2009). Apoptosis assessment using Annexin V and propidium iodide staining demonstrated that curcumin (20 µM) caused 47% of MDA-MB-468 cells to undergo apoptosis at 48 h (Squires et al., 2003). Similarly, in MCF-7 cells curcumin (47.4 µM) increased the proportion of apoptotic cells (both early and late phase apoptotic cells) by 26% compared with the control group at 48 h (Fang et al., 2011).
Various mechanisms have been reported to explain the apoptotic effect of curcumin in breast cancer cells. Curcumin induces apoptosis in breast tumor cells mainly via the mitochondrial dependent pathway (Karunagaran et al., 2005), which is characterized by subsequent events including a loss of mitochondrial membrane potential, opening of the transition pore, release of cytochrome c, caspase-9 activation, caspase-3 activation and cleavage of PARP (Aggarwal et al., 2003; Ravindran et al., 2009). Also, down regulation of antiapoptotic proteins (Bcl-2 and Bcl-XL) and up regulation of proapoptotic proteins (Bad and Bax) lead to curcumin-induced apoptosis in breast cancer cells (Ravindran et al., 2009). The activity of these proteins is in turn regulated by curcumin via both p53 dependent or independent pathways, which ultimately lead to apoptosis.

Accordingly, Chiu et al. (2009) showed that curcumin induces apoptosis in MDA-MB-231 breast cancer cells by increasing the protein expression of Bax and decreasing the expression of Bcl-2 protein independently of p53. In contrast, in MCF-7 cells curcumin induced apoptosis via a p53 dependent pathway involving the activation of Bax (Choudhuri et al., 2002). In order to assess apoptotic genes regulated by curcumin in MCF-7 cells, microarray analysis was used by Ramachandran et al. (2005). Of the 214 apoptosis-associated genes, the expression of 104 genes was significantly altered after curcumin exposure. In MCF-7 cells, curcumin altered the gene expression up to 14-fold as compared to 1.5-fold in MCF-10A cells. Curcumin up regulated (>3 fold) 22 genes and down regulated (<3-fold) 17 genes at both 0.7 µM and 1.4 µM concentrations in MCF-7 cells. The up regulated genes included HIAP1, CRAF1, TRAF6, CASP1, CASP2, CASP3, CASP4, HPRT, GADD45, MCL-1, NIP1, BCL2L2, TRAP3, GSTP1, DAXX, PIG11, UBC, PIG3, PCNA, CDC10, JNK1 and RBP2. The down regulated genes were TRAIL, TNFR, AP13, IGFBP3, SARP3, PKB, IGFBP, CASP7, CASP9, TNFSF6, TRICK2A, CAS, TRAIL-R2, RATS1, hTRIP, TNFb and TNFRSF5 (Ramachandran et al., 2005). In another study, Kim et al. (2001a) demonstrated curcumin induced apoptosis in human breast epithelial cells (H-ras MCF10A) by down regulation of Bcl-2 and up regulation of Bax. They also showed the role of caspase-3 in curcumin-induced apoptosis.

The role of curcumin in apoptosis induction via inhibition of reactive oxygen species (ROS) has also been suggested. ROS regulate intracellular signaling pathways in various cancer cells including breast cancer (Waris et al., 2006). Higher production of ROS and glutathione depletion cause oxidative stress, loss of cell function and ultimately leads to apoptosis. Curcumin causes rapid depletion of glutathione (GSH) which results in an increase in the production of ROS and induction of apoptosis (Shehzad et al., 2010). Syng
et al. (2004) reported that curcumin induced apoptosis in MCF-7 and MDA-MB cells through the generation of ROS originating from glutathione depletion by buthioninesulfoximine thereby further sensitizing the tumor cells to curcumin. In another study, Kim et al. (2001b) suggested redox signaling as a possible mechanism for curcumin-induced apoptosis in H-ras MCF10A cells.

Various morphological changes in breast cancer cells following curcumin-induced apoptosis are also reported. Accordingly, fluorescent microscopy demonstrated that curcumin induces apoptosis in MCF-7 cells by formation of characteristic apoptotic features such as chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (Fang et al., 2011; Roy et al., 2002). Curcumin induced apoptosis in multidrug-resistant (MDR) tumor cells has also been documented. The development of multidrug resistance is one of the major clinical hurdles for many chemotherapeutic agents (Bradley et al., 1988). Poma et al. (2007) demonstrated that curcumin induced cytotoxicity in the MCF-7R cell line which is a MDR variant of MCF-7 breast cancer cells. MCF-7R lacks aromatase and ERα and overexpresses the multidrug transporter p glycoprotein (P-gp) and the products of different genes implicated in cell proliferation and survival, such as c-IAP-1, NAIP, survivin, and COX-2. The RT-PCR studies demonstrated that the anticancer effect of curcumin was mediated via the reduction in the expression of genes such as Bcl-2 and Bcl-XL in MCF-7 cells while MCF-7R cells showed the reduction in the inhibition of apoptosis proteins (IAPs) and COX-2. They also suggested that the anticancer effect of curcumin was not altered by the presence of P-gp (Poma et al., 2007).

The effects of curcumin in normal mammary epithelial cells have also been reported. Curcumin was found to be less effective in normal mammary epithelial cells. Also, compared with breast cancer cells, curcumin-induced apoptosis was significantly diminished in normal mammary epithelial cells. Furthermore, curcumin caused down regulation of p21 mRNA and up regulation of Bax mRNA expression in normal breast epithelial cells (MCF-10A) (Ramachandran et al., 1999). A study conducted by Choudhuri et al. (2005) showed that curcumin arrested normal mammary epithelial cells at the G0 phase of cell cycle without further induction of apoptosis. This effect was observed by the down regulation of cyclin D1 expression and its association with CDK4/CDK6 as well as the inhibition of phosphorylation and inactivation of retinoblastoma protein (Choudhuri et al., 2005).
The induction of apoptosis and the modulation of the cell cycle result from the effect of curcumin on various intracellular pathways. Curcumin inhibits EGF stimulated phosphorylation of the EGFR and further inhibits downstream ERK1/2, JNK, and Akt activity in MDA-MB-468 cells (Squires et al., 2003). In HER2 positive BT-474 and SKBr3 cells, curcumin inhibited the expression of HER2 in a concentration-dependent manner (Lai et al., 2012). Moreover, it decreased the expression of Akt and MAPK, the downstream signaling of HER2. Curcumin also inhibited the expression of NFκB, a downstream target of Akt, in ERα negative breast cancer cells. Specifically, 0.1 µM Curcumin reduced the expression of nuclear NFκB in MDA-MB-231 cells and BT-483 cells (Liu et al., 2009b). Also, curcumin abolished paclitaxel induced NFκB activation in MDA-MB-435 breast cancer cells (Aggarwal et al., 2005), whereas in MCF-7 cells curcumin blocked TNF-α induced NFκB activation (Yoon et al., 2007). Similarly, in HER2 positive BT-474 and SKBr3 cells, curcumin decreased the expression of NFκB in a concentration-dependent manner (Lai et al., 2012).

Various other mechanisms have also been postulated to explain the anti-proliferative effects of curcumin in breast cancer cells. For example, curcumin inhibits the proliferation of MCF-7 cells by depolymerizing and perturbing mitotic microtubules (Gupta et al., 2006). Dynamic microtubules are essential elements in mitotic spindle formation and they organize chromosome distribution during the cell division. Curcumin strongly suppressed the dynamic instability of individual microtubules and thus inhibited proliferation of MCF-7 cells. Furthermore, curcumin exerted additive effects when combined with vinblastine, a microtubule depolymerizing drug, whereas the combination of curcumin with paclitaxel, a microtubule-stabilizing drug, produced an antagonistic effect on the inhibition of MCF-7 cell proliferation (Banerjee et al., 2010).

Inhibition of telomerase activity is another mechanism of anti-breast cancer activity of curcumin. Telomerase is activated in more than 90% of breast carcinomas and has diagnostic and therapeutic potential (Herbert et al., 2001). Ramachandran et al. (2002) reported that curcumin inhibited telomerase activity in MCF-7 cells in a concentration dependent manner through human telomerase reverse-transcriptase (hTER); telomerase activity was 6.9 times higher when compared with MCF-10A cells. Furthermore, curcumin (100 µM) inhibited telomerase activity by 93% and this was independent of the c-myc pathway (Ramachandran et al., 2002).
Modulation of the Wnt/β-catenin pathway by curcumin has been shown to play a role in the inhibition of cell proliferation and induction of apoptosis in MCF-7 and MDA-MB-231 breast cancer cells (Prasad et al., 2009). The Wnt/β-catenin pathway is an important pathway as it is associated with worse overall survival in basal-like breast cancers and is a target for this aggressive breast cancer subtype (Khramtsov et al., 2010).

TNBCs are associated with mutations in the BRCA1 tumor suppressor gene (Foulkes et al., 2003). Curcumin is reported to induce DNA damage by phosphorylation, increased expression, and cytoplasmic retention of the BRCA1 protein. Modulation of the BRCA1 by curcumin also leads to induction of apoptosis and inhibition of migration in TNBCs (Rowe et al., 2009). Recently, Fang et al. (2011) reported a new anticancer mechanism of curcumin in MCF-7 cells. They identified 12 differentially expressed proteins which contributed to multiple functional activities such as DNA transcription, mRNA splicing and translation, amino acid synthesis, protein synthesis, folding and degradation, lipid metabolism, glycolysis and cell motility. The proteomic studies
demonstrated that down regulations of TDP-43, SF2/ASF and eIF3i, as well as up regulation of 3-PGDH, ERP29 and platelet-activating factor acetylhydrolase IB subunit beta positively contributed to the anticancer activity of curcumin in MCF-7 cells (Fang et al., 2011). Thus overall, curcumin modulates a variety of cell signaling pathways that culminate in a strong apoptotic response (Fig. 1.11).

1.4.3 Anti-angiogenic and anti-metastatic effect

Curcumin is a potent inhibitor of angiogenesis (Shao et al., 2002) which is stimulated by pro-angiogenic factors such as VEGF, b-FGF and HIF (Schneider et al., 2005). VEGF-targeting anti-angiogenic agents such as bevacizumab have been proven beneficial in treating TNBC patients (Carey et al., 2010). Curcumin (50 µM) suppressed the transcription levels of VEGF and b-FGF in MDA-MB-231 cells (Shao et al., 2002). In addition, curcumin down regulated both HIF-1α and HIF-1β at post-transcriptional level in MDA-MB-231 cells in a concentration-dependent manner (Thomas et al., 2008).

Invasion and migration are prerequisites for growth and metastasis of solid tumors. Matrix metalloproteinases (MMPs) play an important role in the progression of invasive and metastatic breast cancer (Schneider et al., 2005). Therefore, targeting MMPs and various other markers that inhibit the rapid growth and metastasis has emerged as one of the strategies for treating highly proliferative TNBCs (Greenberg et al., 2010). Curcumin is reported to inhibit invasion and migration in breast cancer cells by various mechanisms. For example, curcumin inhibited the invasive potential of MDA-MB-231 cells by down regulation of MMP-2, MMP-3 and MMP-9 and up regulation of tissue inhibitor metalloproteinase (TIMP-1, 2) which potentially regulates tumor cell invasion (Boonrao et al., 2010; Shao et al., 2002). In another study, the anti-invasive properties of curcumin were mediated through inhibition of recepteur d'origine nantais (RON) tyrosine kinase receptor (Narasimhan et al., 2008). Curcumin also inhibited integrin α6β4, a laminin adhesion receptor in MDA-MB-231 cells and thus inhibited cell motility and invasion (Kim et al., 2008). Prasad et al. (2010) reported that curcumin induced up regulation of maspin, a serine protease inhibitor and thus inhibited invasion of MDA-MB-231 and MCF-7 cells. They also suggested maspin mediated apoptosis in MCF-7 cells by up regulation of p53 protein and down regulation of Bcl-2.

A study conducted by Chiu et al. (2009) demonstrated that curcumin inhibited the migration of MDA-MB-231 cells via decreasing the expression of NFκB. Similarly, Zong, et al. (2011) reported that curcumin inhibited adhesion, invasion and the migration in
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MCF-7 breast cancer cells through suppression of activity of urokinase type plasminogen activator whose activity was in turn regulated by NFκB. Bachmeier et al. (2008) suggested a novel mechanism for the anti-metastatic activity of curcumin in MDA-MB-231 cells. They demonstrated that curcumin reduced the expression of the two prometastatic cytokines, CXCL1 and CXCL2, which in turn reduced the expression of the chemotactic receptor CXCR4 along with other metastasis-promoting genes (Bachmeier et al., 2008).

1.4.4 Anti-breast cancer effects of curcumin in vivo

Several studies have suggested chemopreventive as well as chemotherapeutic effects of curcumin against in vivo breast cancer models. The first animal study reported that curcumin inhibited DMBA-induced mammary tumors and the formation of mammary DMBA-DNA adducts in female rats (Singletary et al., 1996). Curcumin at a dose of 100 and 200 mg/kg (i.p.) significantly reduced the number of palpable mammary tumors and mammary adenocarcinomas. Also, in curcumin (200 mg/kg) treated animals, mammary tumor incidence was 20% less than control animals, while 100 mg/kg and 200 mg/kg showed reduction in the number of adenocarcinomas/rat by 58% and 68% respectively, compared to control. However, the same study showed that diets containing 1% curcumin fed to animals had no effect on DMBA-induced mammary tumors (Singletary et al., 1996).

In another study, the effects of turmeric, ethanolic turmeric extract and curcumin free aqueous turmeric extract were studied on the initiation or post initiation phases of DMBA-induced mammary tumorigenesis in female Sprague-Dawley rats (Deshpande et al., 1998). The study showed that there was a 47% reduction in tumor multiplicity, 80% reduction in tumor burden and 50% reduction in tumor incidence of mammary tumorigenesis after dietary administration of 1% turmeric, 2 weeks before, on the day of DMBA treatment (day 55) and 2 weeks after a single dose (15 mg/animal) of DMBA (during the initiation period). In addition, administration of 0.05% ethanolic turmeric extract during the initiation period caused a 78% reduction in tumor multiplicity, a 97% reduction in tumor burden and a 50% reduction in tumor incidence (Deshpande et al., 1998).

Other investigators used Sencar mice to demonstrate that feeding 1% dibenzoylemethane (DBM), a derivative of curcumin, in the AIN 76A diet during both the initiation and the post-initiation periods inhibited both the multiplicity and incidence of DMBA-induced mammary tumor by 97% (Huang et al., 1998). Lin et al. (2001) further studied inhibitory action of DBM in Sencar mice. The authors showed that feeding a 1%
DBM diet inhibited formation of DMBA–DNA adducts in mammary glands and lowered the development of mammary tumors (Lin et al., 2001). Similarly, dietary administration of 1% curcumin to pregnant rats reduced the gamma radiation-induced mammary tumors by 28% (Inano et al., 1999). The authors also demonstrated that curcumin decreased the multiplicity and Iball’s index of mammary tumors. Moreover, rats fed a curcumin diet showed a reduced incidence of both mammary adenocarcinoma and ER(+) PgR(+) tumors in comparison to the control group. They further showed that whole mounts of the mammary glands of rats treated with curcumin yielded morphologically indistinguishable proliferation and differentiation compared with the glands of the control rats (Inano et al., 1999).

In contrast to all these studies, Somasundaram et al. (2002) suggested that dietary curcumin may interfere with the ability of chemotherapy to kill cancer cells through apoptosis. The authors demonstrated that curcumin inhibited camptothecin-, mechloethamine-, and doxorubicin-induced apoptosis of MCF-7, MDA-MB-231, and BT-474 human breast cancer cells by up to 70%. This inhibition of programmed cell death was time- and concentration-dependent, but occurred after relatively brief 3 h exposures, or at curcumin concentrations of 1 µM. Moreover, studies in MCF-7 mouse xenografts showed that dietary supplementation of curcumin (25 g/kg) significantly inhibited cyclophosphamide induced tumor regression (Somasundaram et al., 2002). This was confirmed by the decrease in apoptosis induced by cyclophosphamide as well as decreased JNK activation. Moreover, the tumors of the mice receiving the curcumin diet showed 1.6-fold less apoptosis than the cyclophosphamide treated group.

Recently, the effect of curcumin was investigated on DMBA induced medroxyprogesterone acetate (MPA)-accelerated tumors in Sprague-Dawley rats. Curcumin (200 mg/kg) delayed the first appearance, decreased incidence and reduced multiplicity of progestin-accelerated tumors in a rat model. The MPA-treated rats showed appearance of tumors on day 35 after DMBA treatment, whereas curcumin delayed the appearance of tumors until day 42. Moreover, curcumin reduced the incidence of tumor formation and tumor multiplicity by about 70% and 50% respectively, compared to MPA receiving rats. In addition, curcumin prevented the appearance of gross morphological abnormalities in the mammary glands (Carroll et al., 2009).

The anti-metastatic effect of curcumin has also been studied in various in vivo models. Dietary administration of curcumin (2%) significantly decreased the incidence of breast
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Table 1.3: Summary of the mechanism of anticancer activity of curcumin in TNBC cells.
cancer metastasis to the lung in a human breast cancer xenograft model. The authors also observed that curcumin significantly suppressed the expression of NFκB, COX2 and MMP-9 (Aggarwal et al., 2005). In another study, Bachmeier et al. (2007) showed the effect of curcumin on lung metastasis in a mouse xenograft model. MDA-MB-231 cells were inoculated into nude mice by inter-cardiac injection and the treatment group was fed with 1% dietary curcumin. After 5 weeks of treatment, 21% of animals from the treatment group were found to be metastasis free compared with the control group who all had metastases (Bachmeier et al., 2007). The overall mechanism of anticancer activity of curcumin in various in vitro and in vivo models of TNBCs is summarized in Table 1.3.

1.4.5 Anti-breast cancer effects of combination of curcumin with other anticancer agents

Various combination studies with curcumin have been conducted in order to enhance its anticancer effect. Verma et al. (1997) reported that curcumin in combination with genistein showed a synergistic effect with respect to the inhibition of proliferation of MCF-7 breast cancer cells induced by estrogen and environmental pesticides. Treatment of MCF-7 cells with 10 µM curcumin and 25 µM genistein completely inhibited the estrogen induced cell proliferation. A similar effect was also produced in the presence of the mixture of environmental pesticides such as endosulfane, chlordane and DDT where the combination of curcumin and genistein complexly inhibited the growth of MCF-7 cells (Verma et al., 1997).

Our lab previously demonstrated that curcumin and EGCG in combination is effective in both in vitro and in vivo models of ERα negative breast cancer. A combination of curcumin (200 mg/kg/day, po) and EGCG (25 mg/kg/day, ip) reduced the tumor volume by 49% compared to vehicle control mice (5 ml/kg/day, po) after 10 weeks of treatment (Somers-Edgar et al., 2008). This was in part driven by a 78% decrease in the levels of VEGFR-1 protein expression in tumors. The study conducted by Cheah et al. (2009) demonstrated that curcumin administered in combination with xanthorrhizol elicited synergistic growth inhibitory activity in MDA-MB-231 human breast cancer cells. The combination treatment induced apoptotic cell death which was evidenced by alteration of mitochondrial membrane potential, DNA condensation, cell shrinkage and DNA fragmentation (Cheah et al., 2009). Another study conducted by Kakarala et al. (2010) revealed that the combination of curcumin and piperine worked synergistically to inhibit breast cancer stem cell self-renewal without affecting normal cells. This effect was
mediated by the inhibition of mammosphere formation and the Wnt signaling pathway. The addition of 5 µM curcumin and 10 µM piperine to primary mammospheres inhibited the ability of cells to form secondary mammospheres by more than 50% compared to control. In addition, the inhibition of Wnt signaling was reported in MCF-7 cells by using the TCF-Lef reporter assay. Accordingly, treatment of MCF-7 cells with 10 µM curcumin for 12 h reduced green florescence positivity (GFP) expression by 93% compared to control while 10 µM piperine showed 82% inhibition in GFP expression. The combination of curcumin and piperine caused a further inhibition of GFP expression to 96% (Kakarala et al., 2010).

In a xenograft model of breast cancer using MDA-MB-231 cells, a 78% reduction in tumor growth was reported in nude mice receiving both curcumin (100 mg/kg) and paclitaxel (7 mg/kg) compared to control. However, the treatment with either agent alone showed no significant tumor suppression (Kang et al., 2009). The mechanistic studies of tumor suppression demonstrated that the combination treatment decreased the tumor proliferative protein PCNA by 84-fold and increased the TUNEL-positive apoptotic cells by 4-fold compared to control whereas single treatment of curcumin or paclitaxel did not show any significant change. In addition, expression of MMP-9 was also significantly decreased following the combination treatment. Interestingly, the dose of paclitaxel was much lower (7 mg/kg, i.p.) than previously used in breast cancer mouse xenograft models (Kang et al., 2009). In another study, Lou et al. (2010) demonstrated that the transition metal Cu (II) significantly potentiated the cytotoxicity of curcumin in MCF-7 cells. Treatment of MCF-7 cells with 10 µM CuCl₂ and 10 or 30 µM curcumin for 72 h caused significant cytotoxicity compared to control (Lou et al., 2010).

Recently, Altenburg et al. (2011) reported that curcumin in combination with decosahexaenoic acid (DHA) which belongs to omega-3 fatty acid family produced synergistic cytotoxicity in HER2 positive SKBr3 cells. The combination of curcumin and DHA (2:3) below 50 µM exerted a synergistic cytotoxicity as indicated by a combination index (CI) value of less than 1. However, in MDA-MB-231, MDA-MB-361, MCF-7 and MCF10AT cells, sub-additive to additive effects were observed following the administration of curcumin and DHA. This effect was mediated through up regulation of various genes involved in cell cycle arrest, apoptosis and inhibition of metastasis and cell adhesion. Moreover, treatment of SKBr3 cells with 30 µM curcumin and DHA combination (2:3) caused up regulation of apoptosis related proteins, NLRP1, DUSP13 and UCHL1, tumor suppressor genes, HTRA3, VWA5A, GPX3, PANX2 and GDF15 and anti-
metastatic gene MAP2 by 10-fold whereas SERPINB5 (tumor suppressor gene) was up-regulated by 19-fold. Another important protein CYP1B1 which promotes the anti-proliferative activity of dietary anticancer compounds was up-regulated by 7.4-fold by the combination treatment. Similar treatment also increased the expression of p53 and PPARγ. However, this effect was not seen by the treatment with either drug alone. Interestingly, DHA increased the cellular uptake of curcumin in SKBr3 cells and thus enhanced its cytotoxic effects (Altenburg et al., 2011).

Similarly, Lai et al. (2011) demonstrated a combination cytotoxic effect of curcumin and herceptin towards another HER2 positive breast cancer cell line, BT-474. The authors used a xenograft model of BT-474 cells and showed that the combination of herceptin and curcumin had a greater antitumor effect than curcumin alone (88% versus 77%) but a similar effect to that of herceptin (88% versus 87%). Although this combination was not better than herceptin alone, another combination of curcumin with taxol showed a comparable antitumor effect to taxol (84% versus 79%) and herceptin alone (84% versus 87%) (Lai et al., 2012).

1.4.6 Pharmacokinetics and bioavailability studies of curcumin

A number of studies have demonstrated the pharmacokinetic properties of curcumin in rodent models. In the first study, which was conducted in 1978, the uptake, distribution and excretion of curcumin was examined in rats. The study showed that oral administration of curcumin at a dose of 1 g/kg resulted in poor bioavailability. Approximately 75% of the ingested curcumin was excreted in the feces and a negligible amount of curcumin appeared in the urine (Wahlstrom et al., 1978). In another study, tritium labeled curcumin was administered orally and intraperitoneally to rats and the results showed detectable amounts of curcumin in the blood with doses ranging from 10 to 400 mg of curcumin per animal. However, most of curcumin was excreted in the feces (Ravindranath et al., 1981). Similarly, Pan et al. (1999) investigated the pharmacokinetic properties of curcumin administered either orally or intraperitoneally in female BALB/c mice (Pan et al., 1999). The study showed that an oral administration of 1 g/kg of curcumin produced low plasma levels of 0.1 μg/ml after 15 min, while a maximum plasma level of 0.2 μg/ml was obtained after 1 h. However, the authors could not detect any curcumin 6 h after the drug administration. In contrast, i.p. administration of 0.1 g/kg of curcumin produced a peak plasma concentration of 2.3 μg/ml within first 15 min of administration which declined rapidly within 1 h (Pan et al., 1999).
An oral bioavailability study conducted by Maiti et al. (2007) reported that after an oral administration of 1 g/kg of curcumin to male Wistar rats, the mean peak serum concentration was 0.5 μg/ml after 1 h which further dropped to below the detectable limit after 6 h. The bioavailability study conducted by Yang et al. (2007) showed that an intravenous administration of 10 mg/kg of curcumin in rats produced a maximum serum concentration level of 0.36 μg/ml, while orally administered curcumin at a 50-fold higher dose produced a maximum serum level of only 0.06 μg/ml in rats. In another study, a high dose of curcumin (2% in the diet, or ~1.2 g curcumin/kg body weight) was orally administered in F344 rats for 14 days. The authors reported low nanomolar levels of curcumin in the plasma whereas the concentrations in the liver and colon mucosal tissue were in the range from 0.1 to 1.8 nM/g tissue (Sharma et al., 2001).

Overall, all the animal studies have reported low bioavailability of curcumin. Various factors have been considered to contribute to the low bioavailability of curcumin and some of them include limited intestinal absorption and first pass hepatic metabolism (Anand et al., 2007). Ireson et al. (2002) studied the metabolism of curcumin in rat and human intestine for the first time. The authors showed that in humans and rats, curcumin was extensively metabolized to curcumin glucuronide which was identified in intestinal and hepatic microsomes, and other curcumin metabolites such as curcumin sulfate, tetrahydrocurcumin, and hexahydrocurcumin were found in intestinal and hepatic cytosol. The authors also reported that the extent of conjugation and reduction was higher in human intestinal tissue than in rats (Ireson et al., 2002). In another study, Suresh et al. (2007) demonstrated that incubation of 50-1000 μg of curcumin with everted sacs of rat intestines in 10 ml medium produced a negligible amount of curcumin in the serosal fluid.

As compared to rodents, the pharmacokinetic profile of curcumin was found to be different in humans although the bioavailability of curcumin was still low. For example, Sharma et al. (2001b) showed that daily ingestion of a dose of 180 mg of curcumin failed to produce detectable amounts of curcumin and its metabolites in plasma or urine for up to 29 days. Also, after administering higher doses of 4, 6 and 8 g of curcumin the average peak serum concentrations were 0.5 μM, 0.6 μM and 1.8 μM respectively. However, curcumin was still undetected in the urine (Cheng et al., 2001). In another human study, Garceae et al. (2004) showed that oral consumption of up to 3.6 g curcumin led to a concentration of 10 nM/g tissue in human colorectal mucosa. A single dose pharmacokinetics study conducted by Vareed et al. (2008) showed that oral administration of 10 and 12 g of curcumin in healthy human volunteers could detect free curcumin in only
one subject. The area under the curve for the 10 and 12 g doses was estimated to be 35 and 27 μg/ml x h respectively, whereas $C_{\text{max}}$ was 2.3 and 1.7 μg/ml. The $T_{\text{max}}$ and $t_{1/2}$ were estimated to be 3.3 and 6.8 h. The study also reported that consumption of 10 or 12 g of curcumin lead to the generation of two curcumin metabolites namely, curcumin glucuronide and curcumin sulfate (Vareed et al., 2008). All these studies concluded that curcumin has limited bioavailability due to its poor absorption in gastrointestinal tract, extensive metabolism and rapid elimination from the body.

1.4.7 Clinical trials of curcumin

There has been a number of curcumin clinical trials involving patients with different types of cancer including oral, skin, liver, colorectal, bladder, prostate and cervical cancer (Kunnumakkara et al., 2008). Various clinical trials have addressed the pharmacokinetics, safety and efficacy of curcumin. So far curcumin has shown promising effects in patients with chronic anterior uveitis, post-operative inflammation, idiopathic inflammatory orbital pseudo tumors, extended cancer lesions and pancreatic cancers (Shehzad et al., 2010). However, clinical trials of curcumin in breast cancer are still in their early phases of investigation.

The first clinical trial of curcumin in breast cancer patients investigated the feasibility and tolerability of the combination of docetaxel and curcumin in an open label phase I study in patients with advanced and metastatic breast cancer. In this study, 14 patients were administered docetaxel (100 mg / m$^2$) as a 1 h i.v. infusion every 3 weeks on day 1 for six cycles. Curcumin (500 mg/d) was given for seven consecutive days by cycle (from d-4 to d+2) and escalated until dose-limiting toxicity occurred. The authors concluded that curcumin in combination with docetaxel was safe, feasible and well tolerated even up to 8 g/day (Bayet-Robert et al., 2010). In addition, five patients showed a partial tumor response and three patients had a stable disease with the combination therapy. However, additional clinical trial studies in a higher number of locally advanced and metastatic breast cancer patients are required to more precisely establish the toxicity and anticancer activity of the combination of curcumin with docetaxel versus curcumin or docetaxel alone. Based on the results from previous studies, phase I/II/III clinical trials of curcumin alone or in combination with other chemotherapeutic agents are currently ongoing in patients with different types of cancers including breast cancer.
1.4.8 New strategies for enhancing the bioactivity of curcumin

It is apparent that curcumin has potential chemopreventive and chemotherapeutic activity. However, its clinical application is limited due to low bioavailability and instability in physiological media. Therefore, numerous improvements such as novel drug delivery systems and synthetic modification of curcumin have been carried out in order to develop a molecule with greater stability, bioactivity and ultimate efficacy.

1.4.8.1 Enhancing bioactivity- Novel drug delivery of curcumin

Over the last few years, various novel drug delivery systems such as nanoparticles, liposomes, micelles, adjuvants and phospholipid complexes have been developed in order to improve the chemotherapeutic effect of anticancer agents (Anand et al., 2008). Nanoparticles can provide an advantage of better penetration to membrane barriers because of their small size and thereby improve biodistribution of anticancer drugs (Cho et al., 2008). Several studies have reported the development of different types of curcumin nanoparticles and demonstrated their enhanced bioavailability compared to curcumin. For example, Suresh et al. (2007) prepared a formulation of curcumin using polymeric micelles. They demonstrated that the absorption of curcumin was increased in vitro in an everted intestinal sac from 47% to 56% when prepared in micelles. Similarly, Ma et al. (2007) prepared a nano formulation of curcumin by loading it into copolymeric micelles of poly(ethylene oxide)-b-poly(epsilon-caprolactone) (PEO-PCL) and showed a 162-fold increase in its biological half-life in rats compared to a solubilized curcumin. Liu A et al. (2006) showed an increase in curcumin bioavailability due to curcumin-phospholipid complex formulation. The study demonstrated that oral administration of 100 mg/kg of curcumin-phospholipid complex in rats produced a maximum plasma concentration of 600 ng/ml after 2.3 h compared to that of curcumin which showed a maximum plasma concentration of 267 ng/ml after 1.6 h. In addition, the half-life of the curcumin-phospholipid complex was increased by 1.5-fold over free curcumin (Liu et al., 2006a) In another study, Cui et al. (2009) showed an enhanced bioavailability of curcumin by using the formulation of a curcumin-loaded self-microemulsifying drug delivery system (SMEDDS). Curcumin suspension and curcumin-loaded SMEDDS (50 mg/kg of body weight) were given to mice by intragastric administration and the mice were sacrificed at 2, 4, 6, 8, 10, 12 and 24 h after administration. The study showed that curcumin-loaded SMEDDS had 3.9 times higher absorption percentage than curcumin suspension after 24 h (Cui et al., 2009).
Nanoparticles are beneficial for anticancer therapy as they are able to act as carriers of anticancer drugs by selectively using the unique pathophysiology of tumors, such as their enhanced permeability and retention effect, the tumor microenvironment and extensive angiogenesis (Cho et al., 2008). In addition, they help in improving the selective and sustained delivery of anticancer agents in cancer cells. Therefore, curcumin nanoparticles have been further explored for their application in cancer therapeutics. In particular, use of curcumin nanoparticles for anti-breast cancer activity is discussed here.

Gupta et al. (2009) prepared silk fibroin-derived curcumin nanoparticles (< 100 nm) and demonstrated their higher uptake, intracellular residence time and efficacy against MCF-7 cells and HER2 positive MDA-MB-453 breast cancer cells. Exposure of 0.1% w/v silk fibroin (SF) and 10% w/v SF nanocurcumin to MCF-7 and MDA-MB-453 cells significantly decreased the number of viable cells compared to control. Similarly, curcumin-PLGA nanoparticle (nano-CUR) formulation showed higher stability, enhanced cellular uptake and retention as well as a sustained release of curcumin in MDA-MB-231 breast cancer cells. Nano-CUR showed enhanced inhibitory effect on the growth of MDA-MB-231 cells compared with curcumin alone (Yallapu et al., 2010). The nano-CUR6 formulation had an IC$_{50}$ of 9.1 µM compared to curcumin which showed an IC$_{50}$ of 16.4 µM in MDA-MB-231 cells. Moreover, nano-CUR6 at 6 and 8 µM showed a superior inhibitory effect on colony formation compared to curcumin in MDA-MB-231 cells. The apoptosis studies in MDA-MB-231 cells showed that nano-CUR6 increased the proportion of apoptotic cells by 8-fold compared to curcumin at 48 h. This effect was also confirmed by increased PARP cleavage shown by nano-CUR6 compared to curcumin. In another study, Anand et al. (2010) formulated curcumin-PLGA-PEG nanoparticles and reported their concentration dependent antiproliferative effect on MDA-MB-231 cells. Moreover, curcumin nanoparticle formulation had higher bioavailability and longer half-life in rats compared to curcumin. After intravenous administration of curcumin or curcumin (NP) (2.5 mg/kg), the serum levels of curcumin were almost twice as high in the case of curcumin (NP) administered rats as it was in curcumin administered rats (Anand et al., 2010).

In a recent study, Mulik, et al. (2010) formulated transferring-mediated solid-lipid nanoparticles of curcumin (Tf-C-SLN) for targeted delivery to breast cancer cells. To indicate the targeted effect of Tf-C-SLN, the curcumin solubilized surfactant solution (CSSS) and curcumin-loaded solid-lipid nanoparticles (C-SLN) were used for comparison. The authors showed that Tf-C-SLN produced greater cytotoxicity and cellular uptake in
MCF-7 breast cancer cells compared to CSSS and C-SLN. Tf-C-SLN (81 µM) produced a cell viability of 4% compared to CSSS and C-SLN which showed a cell viability of 29% and 23% respectively (Mulik et al., 2010). Moreover, Tf-C-SLN was more efficient in inducing apoptosis compared to CSSS and C-SLN. At 24 h, the proportion of early and late apoptotic cells produced with 10 µM of CSSS, C-SLN and Tf-C-SLN were 6% and 34%, 35% and 32%, 42% and 45%, respectively. Similarly, cell cycle studies demonstrated that 10 µM of CSSS, C-SLN and Tf-C-SLN significantly increased the proportion of MCF-7 cells in the subG1 phase by 42%, 61% and 88%, respectively.

Tang et al. (2010) produced another novel curcumin nanoparticle formulation (Curc-OEG) by conjugating curcumin with two short oligo (ethylene glycol) chains via beta-thioester bonds that are labile in the presence of intracellular glutathione and esterase and studied their anticancer effect in both in vitro and in vivo models of breast cancer. Curc-OEG formed stable nanoparticles in aqueous conditions and acted as an anticancer prodrug and a drug carrier. The authors demonstrated that Curc-OEG nanoparticles had broad in vitro antitumor activity against several human cancer cells with IC50 values of 7.8 µg/ml and 1.4 µg/ml in MCF-7 and MDA-MB-468 breast cancer cells respectively. Moreover, a 50% reduction in the tumor growth compared to control was observed after intravenous administration of 25 mg/kg of Curc-OEG nanoparticles to the mice bearing MDA-MB-468 subcutaneous xenografts for 48 h. Also, the mice did not show any acute or subchronic systemic toxicity. In addition, Curc-OEG nanoparticles carried other anticancer drugs such as doxorubicin and camptothecin and transported them into multidrug resistant MCF-7/ADR cells. Co-administration of Curc-OEG nanoparticles and doxorubicin showed synergistic cytotoxicity towards MCF-7/ADR cells (Tang et al., 2010). When treated alone in MCF-7 ADR cells, Curc-OEG (100 µg/ml) and doxorubicin (5 µg/ml) produced cell viability of 45% and 56% respectively, whereas the combination of the Curc-OEG and doxorubicin produced ~2% cell viability.

Shahani et al. (2010) formulated injectable sustained release microparticles of curcumin for breast cancer chemoprevention. The curcumin microparticles exhibited enhanced bioavailability compared to curcumin in mice. A single dose of subcutaneously injected microparticles sustained curcumin levels in the blood for a month whereas single or multiple i.p. injections of curcumin resulted in a shorter half-life. Curcumin concentration was 10 to 30-fold higher in the lungs and brain than in blood. The in vivo studies demonstrated that curcumin microparticles inhibited the growth of tumors in a MDA-MB-231 mouse xenograft model by 49% compared to the mice treated with blank
microparticles. The anticancer effect of curcumin microparticles was attributed to the down regulation of the markers of angiogenesis, metastasis and proliferation. The curcumin microparticle treated group showed smaller and less well developed CD31 positive microvessels compared to curcumin treatment. Furthermore, curcumin microparticles decreased the relative VEGF expression in tumors by 78% whereas curcumin showed 48% reduction in VEGF expression. In addition, there were 57% and 11% reductions in the relative MMP-9 expression in tumors from curcumin-microparticle and curcumin-treated groups respectively. Also, curcumin microparticles reduced the relative expression of Ki-67 and cyclin D1 by 45% and 52%, respectively, compared with blank microsphere treatment. Curcumin microparticle also resulted in a 2.5-fold increase in the number of apoptotic cells relative to that with blank microparticle treatment. However, repeated systemic dosing of curcumin had no effect on tumor cell proliferation, apoptosis, or the relative cyclin D1 expression compared with vehicle treatment. There was a 1.5-fold decrease in the relative COX-2 expression in tumors from curcumin-microparticle and curcumin-treated groups compared with the respective controls. The study concluded that sustained release microparticles of curcumin are more effective than repeated systemic injections of curcumin for breast cancer chemoprevention.

Recently, Sanoj Rejinold et al. (2011) produced curcumin loaded fibrinogen nanoparticles (CRC-FNPs) for cancer therapy. They reported enhanced internalization and retention of curcumin nanoparticles inside MCF-7 breast cancer cells. Also, the nanoparticles induced apoptosis in MCF-7 cells compared to L929 mouse fibroblast cells. (Sanoj Rejinold et al., 2011). Treatment with 1 mg/ml of CRC-FNPs produced 40% apoptotic cells in MCF-7 at 24 h.

1.4.8.2 Enhancing bioactivity - Synthetic curcumin analogs

Development of curcumin analogs has emerged as a novel strategy to enhance bioavailability and selectivity towards cancer cells. Modification of the aromatic rings and ß-diketone moiety of curcumin has led to different curcumin analogs with improved activities (Mosley et al., 2007). The first generations of curcumin derivatives were the cyclohexanones and these exhibited enhanced activity and stability in biological medium compared to curcumin. For example, the cyclohexanone-containing curcumin derivatives 2,6-bis ((3- methoxy-4-hydroxyphenyl) methylene)-cyclohexanone (BMHPC) and 2,6-bis ((3,4-dihydroxyphenyl) methylene)-cyclohexanone (BDHPC) demonstrated cytotoxicity towards human breast cancer cells (Markaverich et al., 1992). Treatment of MCF-7 cells
<table>
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with BDHPC produced a IC\textsubscript{50} of 4 \( \mu \)M. Moreover, in female BALB/c mice bearing transplantable mammary tumors, BDHPC (5 mg/kg/d) significantly reduced tumor volume by 78\% compared to vehicle treated mice after 12 days (Markaverich \textit{et al.}, 1992). Also, in MDA-MB-231 cells, 5 \( \mu \)M BDHPC significantly reduced cell proliferation by 60\% after 4 days. Similarly, BMHPC was cytotoxic toward MDA-MB-231 cells (IC\textsubscript{50} of 5.0 \( \mu \)M) (\textit{Table 1.4}) and also displayed anti-angiogenic effects in human and murine endothelial cell lines (Adams \textit{et al.}, 2004). These results led the authors to further synthesize several fluorinated derivatives, one of which, 3,5-bis(flurobenzylidene) piperidin-4-one (EF-24), has shown potent cytotoxicity toward MDA-MB-231 cells (IC\textsubscript{50} of 0.8 \( \mu \)M) (\textit{Table 1.4}) (Adams \textit{et al.}, 2005). Moreover, EF-24 induced breast tumor regression in athymic nude mice. The solid breast tumors were grown in the flanks of female athymic nude mice and the drug was administered subcutaneously after three weeks of tumor growth. A dose-dependent decrease in tumor weight was observed. The average tumor weight following 20 mg/kg was decreased by 70\% compared to control, whereas the 100 mg/kg reduced tumor weight by 55\%. Interestingly, no toxicity was observed at a dose of 100 mg/kg, which was well below the maximum tolerated dose of 200 mg/kg.

The mechanism of the anticancer effect of EF-24 was also studied in MDA-MB-231 breast cancer cells. EF-24 (10 \( \mu \)M) inhibited cell proliferation by 70%-80\% and 20 \( \mu \)M of EF-24 induced a cell cycle arrest in the G2/M phase. Additionally, EF-24 (20 \( \mu \)M)
increased the percentage of early apoptotic and late apoptotic cells to 25% and 46%, respectively, after 72 h. Apoptosis was observed by signs of cell death such as mitochondrial membrane depolarization, activation of caspase 3, externalization of PS and DNA fragmentation. In addition, in MDA-MB-231 cells, EF-24 increased intracellular ROS levels by 55% at 48 h (Adams et al., 2005). Thomas et al. (2008) further studied the mechanism of anticancer activity of EF-24 and demonstrated that EF-24 inhibited pro-angiogenic transcription factor HIF-1α at a posttranscriptional level by a VHL-dependent but proteasome-independent mechanism in MDA-MB-231 cells. In addition, EF-24 disrupted the microtubule cytoskeleton without directly binding to tubulin in MCF-7 cells (Thomas et al., 2008).

The therapeutic potential of EF-24 was further explored by using coagulation factor VIIa (fVIIa) as a carrier for targeted delivery of EF-24 to the tissue factor expressed in tumor cells and vascular endothelial cells (Shoji et al., 2008). The studies demonstrated that EF-24-FFRck-fVIIa conjugate significantly decreased the viability of the TF-expressing MDA-MB-231 and HUVEC cells in a concentration-dependent manner. Furthermore, the administration of 5 intravenous injections of the EF-24-FFRck-fVIIa conjugate (containing 50 µM of EF-24) for two week significantly reduced the tumor size in luciferase-positive MDA-MB-231 breast cancer xenografts. Moreover, the tumor cells showed activation of caspase-3 as a marker of apoptosis (Shoji et al., 2008). The anticancer activity of the EF24–FFRck–fVIIa conjugate was also seen by its anti-angiogenic activity. The authors demonstrated that the conjugate inhibits VEGF-A induced angiogenesis in both the rabbit cornea model and the Matrigel model in female athymic nude mice.

Liang et al. (2009) designed a series of mono-carbonyl analogs of curcumin using three different 5-carbon linkers; cyclopentanone, acetone and cyclohexanone, with various substituents on aryl rings. They reported that all the analogs had enhanced stability in vitro and improved pharmacokinetic profile in vivo. Following an oral administration of 500 mg/kg of compound B02 and B33 in rats, the peak plasma concentrations were 0.82 and 4.1 µg/ml respectively compared to curcumin (0.091 µg/ml). In addition, both the compounds showed decreased plasma clearance values (B02 (125.4 l/kg/h) and B33 (38.98 l/kg/h)) compared to curcumin (835.2 l/kg/h). Furthermore, the half-life of B02 was increased by two-fold over curcumin and absorption of B33 was rapid. The other analogs with acetone or cyclohexanone spacer groups showed increased cytotoxicity against several tumor cell lines (Liang et al., 2009).
Another set of curcumin analogs (FLLL 11 and FLLL 12) produced by exchanging the \( \beta \)-diketone moiety for an \( \alpha \beta \) unsaturated ketone, exhibited more potent antitumor activity than curcumin in various ER positive and ER\( \alpha \) negative human breast cancer cells (Table 1.4) (Lin et al., 2009a). The IC\textsubscript{50} values for FLLL11 and FLLL12 ranged from 9 to 48-fold lower than curcumin. Furthermore, both the analogs at 10 \( \mu \)M inhibited STAT3, Akt and HER2/Neu pathways and induced apoptosis. The apoptosis was mediated via activation of cleaved PARP and caspase-3. These analogs in combination with doxorubicin exhibited a synergistic anti-proliferative effect in MDA-MB-231 breast cancer cells as seen by the combination index (CI) values below 1 for both the compounds. In addition, the compounds inhibited anchorage independent growth and cell migration in MDA-MB-231 cells (Lin et al., 2009a). At 5 \( \mu \)M, both FLLL11, and FLLL12 decreased the colony formation in soft agar by 95% and 80% respectively in MDA-MB-231 and SKBr3 cells whereas at the same concentration curcumin showed 60% reduction in the colony formation. In another study, Fuchs et al. (2009) synthesized two series of monoketone curcumin analogs, namely heptadienone and pentadienone series and investigated their anti-breast cancer properties \textit{in vitro}. Among 24 compounds synthesized, compound 23 was the most potent analogue with IC\textsubscript{50} values in the sub-micromolar range in MCF-7 and MDA-MB-231 cells.

Another potent isoxazole curcumin analogue, MR39, was effective in inducing cytotoxicity in both MCF-7 and the multi-drug resistant and hormone independent MCF-7R cells. MR39 produced IC\textsubscript{50} values of 13 \( \mu \)M and 12 \( \mu \)M, respectively, in MCF-7 and MCF-7R cells compared to curcumin which showed IC\textsubscript{50} values of 29 and 26 \( \mu \)M respectively. MR39 induced G2/M phase cell cycle arrest in both MCF-7 and MCF-7R cells. Moreover, RT-PCR studies demonstrated that MR39 reduced the mRNA expression of number of genes such as c-IAP-1, XIAP, NAIP, survivin and COX-2 in MCF-7R cells whereas in MCF-7 cells, MR39 decreased the expression of Bcl-2 and Bcl-xL genes. Importantly, MR39 retained its antiproliferative effect in the presence of the drug transporter protein, P-gp (Poma et al., 2007).

Another study on a series of curcumin analogs led to the design of various 1,5-diarylpentadienon containing curcumin analogs with an alkoxy substitution on aromatic rings at each of the positions 3 and 5 (Ohori et al., 2006). One of the analogs, GO-YO30 showed substantially higher cytotoxicity and anchorage independency compared to curcumin in MDA-MB-231 cells. GO-YO30 showed an IC\textsubscript{50} value of 1.2 \( \mu \)M whereas
curcumin showed an IC$_{50}$ value of 19.3 µM in MDA-MB-231 cells (Table 1.4). Furthermore, in MDA-MB-231 cells GO-YO30 inhibited STAT activity at a concentration 3-times lower than curcumin (5 verses 15 µM, respectively). Interestingly, GO-YO30 showed apoptosis induction with PARP cleavage at 2.5 and 5 µM in MDA-MB-231 whereas curcumin required 4 to 8 times higher concentration (20 µM) to elicit a comparable effect (Hutzen et al., 2009).

The monoketo curcumin analogs with the piperidine ring have a rigid confirmation leading to a broad spectrum antitumor activity. Two such compounds bearing n-alkyl piperidine groups are compound 8 (3,5-bis(4-hydroxy-3-ethoxy-5-methylcinnamyl)-N-methylpiperidone) and 18 (3,5-bis(4-hydroxy-3-methoxy-5-methylcinnamyl)-N-ethylpiperidone), which showed potent cytotoxic effects towards various breast cancer cells including MCF7, DR-RES, MDA-MB-331/ATCC, HS-578T, MDA-MB-435, BT-549 and T-47D (Youssef et al., 2005). Compound 8 showed high IC$_{50}$ values of 2.3, 4.9, 17.9, 5.4, 6.8, 32.8, and 15.14 µM, respectively whereas compound 18 showed IC$_{50}$ values of 3.3, 5.5, 2.8, 3.8, 3.7, 2.6, and 2.7 µM, respectively (Table 1.4). This structure was recently further modified by replacing the methylene groups and the two carbonyl groups in curcumin by N-methyl-4 piperidone. The resulting compound (5-bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4-piperidone) (PAC) was 5 times more effective than curcumin in inducing apoptosis in ER$\alpha$ negative breast cancer cells (MDA-MB-231, Bec114). Also, its pro-apoptotic effect was 10 times higher against ER$\alpha$ negative breast cancer cells than against ER$\alpha$ positive cells (MCF-7, T-47D). The mechanistic studies revealed that in MDA-MB-231 cells, PAC (10 µM) increased the proportion of G2/M cells by 185% at 16 h. Furthermore, at 10 µM, PAC induced apoptosis in 55% of MDA-MB-231 cells. In MCF-7 and T-47D cells, PAC (40 µM) increased the proportion of apoptotic cells by 35% and 70%, respectively compared to 20% apoptosis induced by curcumin. The cytotoxic effect of PAC was mediated through down regulation of the expression of NFkB, survivin and its downstream effectors cyclin D1 and Bcl-2 and subsequently showed up regulation of p21$^{WAF1}$ expression both in vitro and in vivo. Interestingly, PAC (100 mg/kg/day, i.p.) suppressed the growth of MDA-MB-231 breast cancer xenografts in nude mice after 2 weeks of treatment. Interestingly, PAC showed higher water solubility and stability in blood thus showing better pharmacokinetics and tissue bio-distribution (Al-Hujaily et al., 2010).
Second generation curcumin analogs have been synthesized by replacing the phenyl group of cyclohexanone curcumin derivatives with heterocyclic rings. Two analogs, RL-90 2,6-bis(pyridin-3-ylmethylene)-cyclohexanone and RL91 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone showed potent cytotoxicity towards MDA-MB-231, SKBr3 breast cancer cells compared to curcumin. RL90 and RL91 elicited IC₅₀ values of 1.54 and 1.10 µM respectively in MDA-MB-231 cells and IC₅₀ values of 0.51 and 0.23 in SKBr3 cells. All other new compounds were less potent than curcumin, which elicited IC₅₀ values of 7.6 and 2.4 µM in MDA-MB-231 and SKBr3 cells respectively (Table 1.4). This cytotoxic effect was seen by the modulation of the expression of a variety of cell signaling proteins such as EGFR, Akt, HER2, β catenin and NFκB. RL90 and RL91 also showed activation of stress kinases evidenced by phosphorylation of both JNK1/2 and p38 MAPK. Furthermore, RL90 and RL91 produced cell cycle arrest at G2/M phase in MDA-MB-231 and SKBr3 cells. In MDA-MB-231 cells, RL90 (3 µM for 30 h) or RL91 (2.5 µM for 24 h) significantly increased the proportion of cells in G2/M phase by 52% and 49% compared to control respectively. A similar but earlier G2/M phase arrest was also observed in SKBr3 cells following treatment of 2 µM of RL90 or RL91. After 18 h RL90 and RL91 increased the proportion of cells in the G2/M phase cells by 39% and 25% above control respectively. RL90 and RL91 induced apoptosis in MDA-MB-231 and SKBr3 cells. In MDA-MB-231 cells, RL90 (3 µM) or RL91 (2.5 µM) significantly increased the proportion of apoptotic cells by 164% and 406% of control respectively at 36 h. Similarly, treatment of SKBr3 cells with 2 µM of RL90 or RL91 for 30 h increased the proportion of cells undergoing apoptosis by 331% and 483% of control respectively (Somers-Edgar et al., 2011).

Recently, Anand et al. (2011) studied the anti-breast cancer activity of various curcumin analogs containing an αω-bisaryl alkanone unit namely DBM (dibenzoylmethane), DBA (dibenzoylacetone) and DBP (1, 3,-dibenzoylpropane) in vitro. The authors demonstrated that curcumin and DBA were more active than DBM in producing cytotoxicity in MDA-MB-231 cells. At 25 and 50 µM both curcumin and DBA had similar cytotoxicity towards MDA-MB-231 cells. However, DBM and DBP were found to be much less potent than curcumin and DBA. Also, these analogs were much less potent than any other analogs studied to date.
1.5 AIM OF THE PROJECT

This project was designed to develop new heterocyclic cyclohexanone analogs of curcumin for treatment of ERα negative breast cancers. The study was mainly focused on nitrogen heterocycles since these analogs have an advantage of their ability to exist in both a protonated and neutral form, allowing both higher solubility in aqueous media as well as the potential to cross cellular membranes. Thus, they are a group of compounds worthy of study in order to potentially develop new drugs. The study was divided into the following specific aims.

1) To screen a variety of cyclohexanone analogs of curcumin for their anticancer activity in ERα negative breast cancer cells (MDA-MB-231, MDA-MB-468 and SKBr3) and study their structure activity relationship for anti-breast cancer activity.

2) To assess the most potent compounds obtained in aim 1 for anticancer mechanisms in vitro, including the effect on cell cycle progression, induction of apoptosis and various cell signaling proteins.

3) To study anti-angiogenic and anti-migratory potential in vitro.

4) To determine oral bioavailability of the most potent compounds from aim 1 and 2.

5) To further examine the most promising compounds from aim 4 for safety and efficacy in a mouse xenograft model of ERα negative breast cancer.

6) To examine potential mechanisms for the tumor suppression.
CHAPTER 2: METHODS

2.1 MATERIALS

MDA-MB-231, MDA-MB-468 and SKBr3 breast cancer cells as well as human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection (Manassas, VA). Primary antibodies to EGFR, NFκB (p65), p38, P-p38, JNK, P-JNK (Tyr183/Thr185), cleaved caspase-3, 4E-BP1, P-4E-BP1 (Thr70), p27, mTOR, P-mTOR (Ser2448), HER2, P-HER2 (Tyr1221/1222) and β actin were purchased from Cell Signaling Technology (Danvers, MA). Akt and P-Akt (Ser473) primary antibodies were purchased from BD Biosciences (Auckland, New Zealand). Rat anti-mouse CD-105 primary antibody, BD BioCoat invasion chambers, diamino benzadine (DAB) kit, Geltrex Matrigel plates and Matrigel were purchased from BD Biosciences (San Diego, CA, U.S.A.). Molecular weight (MW) markers, horse radish peroxidase (HRP) conjugated goat anti-mouse, goat anti-rabbit and streptavidin-biotin goat anti-rat IgG secondary antibodies and sodium dodecylsulfate (SDS) were purchased from BioRad Laboratories (Hercules, CA, U.S.A.). Minimum essential medium α modification, Dulbecco’s modified eagle’s medium nutrient mixture F-12 Ham, glutamine, sulfparhodamine B salt, propidium iodide, ammonium persulfate, trypan blue, copper sulphate (CuSO₄), potassium chloride (KCl), sodium bicarbonate (NaHCO₃), sodium chloride (NaCl), sodium orthovanadate (Na₃VO₄), sodium pyrophosphate (Na₄P₂O₇), sodium fluoride (NAF), hydrochloric acid (HCl), sodium hydroxide (NaOH), trizma hydrochloride (Tris HCl), triton-X 100, trypsin, Mayer’s Haematoxylin, methanol, ponceau red stain, soyabein trypsin inhibitor, TEMED, trizma base, glycine for electrophoresis, antibiotic-antimycotic solution, bicinechinonic acid, dimethyl sulfoxide (DMSO) and HEPES were purchased from Sigma-Aldrich (St Louis, MO). Endothelial growth media (EGM) was purchased from Lonza (U.S.A.). Acrylamide, bisacrylamide, sodium dodecylsulfate and PVDF membrane were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Complete mini EDTA-free protease inhibitor cocktail and annexin-V-FLUOS were purchased from Roche Diagnostics Corporation (Mannheim, Germany). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific (Rockford, Illinois, U.S.A). Plasma alanine aminotransferase (ALT) reagent was purchased from ThermoFisher (U.S.A.). DiffQuick staining solutions A, B and C were purchased from IMEM Inc. (San Marcos, CA). Acetic acid (CH₃COOH), disodium hydrogen orthophosphate anhydrous (Na₂HPO₄), ethylene-
diaminetetra-acetic acid (EDTA), DPX mounting medium, propan-2-ol, xylene, potassium dihydrogen orthophosphate (KH₂PO₄), D-glucose, sodium citrate, glycerol, calcium chloride (CaCl₂), sodium carbonate and Tween-20 were purchased from BDH Laboratory Supplies (Poole, England). RNase A, bovine serum albumin (BSA) and trypsin were purchased from Invitrogen (Auckland, New Zealand). Ethanol (96%) was purchased from Scharlau, Spain. Trichloro acetic acid (TCA) was purchased from (Merck, Germany). All synthetic curcumin analogs were synthesized by Dr. Lesley Larsen from New Zealand Institute for Plant and Food Research Ltd., Dunedin, New Zealand. For animal studies, the hydrochloride salt form of RL71 and RL66 were prepared and the compounds were dissolved in dd water to make appropriate doses.

2.2 METHODS

2.2.1 IN VITRO STUDIES

2.2.1.1 Cell maintenance

MDA-MB-231, MDA-MB-468 and SKBr3 cells were maintained in modified Eagle’s medium alpha-modification medium (MEM) (pH 7.4) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B and 0.2% NaHCO₃. HUVEC cells were maintained in EGM supplemented with 2% FBS, hydrocortisone, hEGF, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamicin/amphotericin-B. Cells were cultured in 75cm² flasks and incubated in 5% CO₂/95% humidified air at 37°C. Once the cells reached 90% confluency, flasks containing MDA-MB-231, MDA-MB-468, SKBr3 or HUVEC cells were passaged under sterile conditions. The cells were washed with 5 ml of phosphate buffered saline solution (PBS) and then incubated for 2 min in trypsin solution (2.69 mM EDTA, 1g/l trypsin, 0.14 M NaCl, 76.78 mM Tris HCl; pH 8.0) at 37°C to allow cells to detach from the bottom of the flask. An equal volume of complete growth media was added and the cell suspension was transferred into a 50 ml conical tube. Cells were then centrifuged at 1200 rpm for 3 min at 4°C. The supernatant was discarded and the cell pellet resuspended in fresh supplemented growth media. Cells were then counted under the microscope on a haemocytometer and used as required.

2.2.1.2 Cell cytotoxicity study by the sulforhodamine B (SRB) assay

Human breast cancer cells were seeded in 12-well plates (70,000 cells/ well) in 1 ml DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml
streptomycin, 25 ng/ml amphotericin B and 2.2 g/l NaHCO$_3$, and incubated for 24 h at 37°C. For dose–response assays, cells were treated with a range of concentrations (0-30 µM) of curcumin analogs for 5 days. Vehicle control cells were treated with DMSO (0.1%). Cell number in each well was determined using the SRB assay. The concentration of each compound required to decrease the cell number by 50% of control (IC$_{50}$) was determined by nonlinear regression using Prism software.

For cytotoxicity time course studies, breast cancer cells were treated with curcumin analogs (RL71 or RL66) at 1, 1.5 or 2 µM for a treatment period of 6, 12, 24, 36, 46 and 72 h. DMSO (0.1%) was used as control. Cell number was determined by the SRB assay.

**SRB assay**

The SRB assay was carried out as described (Skehan et al., 1990). At the end of treatment, DMEM media was aspirated off the plate and the cells were fixed using 0.25 ml or 0.5 ml (12- and 6-well plates, respectively) of 10% TCA solution. The plates were incubated at 4°C for 30 min and rinsed under a light stream of tap water. Then the plates were left overnight to air-dry at room temperature. The cells were stained by adding 0.5 ml or 1 ml (12- and 6-well plates, respectively) of SRB stain (0.4% SRB in 1% CH$_3$COOH) and incubated at room temperature for 10 min. The unbound SRB stain was then rinsed off five times with 1% CH$_3$COOH and the plates were left overnight in the dark to dry. After drying, 1 ml or 2 ml (12- and 6-well plates, respectively) of 10 mM Tris base (pH 10.5) was added to each well and left for 10 min on a shaking platform to solubalize the SRB dye. Three aliquots of 100 µl each were transferred into a 96-well plate and the absorbance was read at 490 nm using a Spectramax Plus plate reader. Data was obtained using SoftMax Pro software (version 4.6).

**2.2.1.3 Cell cycle analysis**

Flow cytometry was used to analyze DNA content in order to determine the changes in the various phases of cell cycle. To assess the effect of RL71 or RL66 on cell cycle progression, MDA-MB-231 (200,000 cells per well), MDA-MB-468 and SKBr3 (250,000 cells per well) cells were plated in 6-well plates in 2 ml DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l NaHCO$_3$, and incubated in 5% CO$_2$ / 95% humidified air for 24 h at 37°C. The cells were treated with RL71 (1 µM) or RL66 (1.5 or 2 µM) using 0.1% DMSO as control for 6-48 h. The cell cycle analysis was carried out as described (Nicoletti
et al., 1991). After the treatment, the cells were washed with 400 µl of isotonic PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$; pH: 7.4) maintained at 4°C followed by addition of 300 µl of trypsin. Then plates were incubated at 37°C for 3 min, allowing cells to detach from the base of the wells. Plates were immediately placed on ice and the cell suspension was transferred into a fresh 15 ml tube. The tubes were centrifuged at 1200 rpm for 3 min at 4°C. The supernatant was removed from each tube and the cell pellet was resuspended in 500 µl isotonic PBS (4°C). The centrifugation step was repeated as above. Finally, the supernatant was removed from each tube and the pellet was resuspended in 0.3 ml PBS solution. The cell suspension was transferred to new tubes and fixed by adding 600 µl cold ethanol (70%) and vortexing gently. The tubes were sealed with paraffin and stored at 4°C till all the times points were finished.

On the day of cell cycle analysis, the tubes containing the cell suspensions were centrifuged at 1200 rpm for 3 min at 4°C and the supernatant was aspirated. The cells were washed two times with cold PBS. Then the cell pellet was resuspended in 300 µl of sample buffer (PBS with 0.1 g/l d-glucose) and incubated at 37°C for 5 min. The cells were then treated with 3 µl of RNase A and incubated at 37°C for 5 min. Finally, the cells were stained with 15 µl of PI solution (0.1% sodium citrate, 0.1% Triton-X, 50 μg/ml PI) and incubated in the dark at 4°C for 30 min. The samples were analyzed via flow cytometry using a Becton Dickson FACScalibur flow cytometer. Data was acquired and analyzed using the CellQuest Pro software (version 5.1.1). Data is expressed as the mean proportion of cells in each phase ± SEM.

### 2.2.1.4 Apoptosis studies by flow cytometry

To examine the effect of RL71 or RL66 on apoptosis induction, MDA-MB-231 (200,000 cells per well), MDA-MB-468 and SKBr3 (250,000 cells per well) cells were seeded in 6-well culture plates in 2 ml of DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l NaHCO$_3$ and incubated in 5% CO$_2$ / 95% humidified air for 24 h at 37°C. The cells were treated with RL71 (1 µM) or RL66 (1.5 or 2 µM) using 0.1% DMSO as control for 12-48 h. Vehicle control cells were treated with 0.1% DMSO. The apoptosis analysis was carried out as described (Somers-Edgar et al., 2008). After treatment, the cells were washed with 400 µl of PBS maintained at 4°C followed by addition of 300 µl of trypsin. Then plates were incubated at 37°C for 3 min, allowing cells to detach from the base of the wells. Plates were immediately placed on ice and the cell suspension was transferred into a fresh
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tube. The tubes were centrifuged at 1200 rpm for 3 min at 4°C. The supernatant was removed from each tube and the cell pellet was resuspended in 500 µl isotonic PBS (4°C). The cell suspension was transferred to the tubes and the centrifugation step was repeated as above. Finally, the supernatant was removed from each tube and the cell pellet was resuspended in 0.1 ml staining solution containing 0.5 µl Annexin-V-FLUOS + 1 µl of PI (50 µg/ml) + 100 µl of binding buffer (10 mM HEPES, 10 mM NaOH, pH 7.4; 140 mM NaCl, 5 mM CaCl₂). The cells were incubated in the dark at room temperature for 15 min and 300 µl of binding buffer was added to each tube before analysis on a Becton Dickson FACSCalibur, where Annexin-V-FLUOS and PI were detected in the FL-1 and FL-2 channels, respectively. Data was acquired and analyzed using the CellQuest Pro software (version 5.1.1). The representative apoptosis dot plots were obtained from FACS analysis. The lower left quadrant represented the viable cells without any Annexin V and PI staining. The lower right quadrant represented early apoptotic cells that stain only Annexin V. The upper right quadrant represented late apoptotic cells that stain both Annexin V and PI and the upper left quadrant represented necrotic cells that stain PI. The early apoptotic cells stained with only Annexin V were mainly reported. Values are expressed as the number of apoptotic cells as a % of the total number of cells ± SEM from three independent experiments conducted in triplicate.

2.2.1.5 Effect of RL71 and RL66 on protein expression

The study of anticancer mechanisms following curcumin analog treatment was a part of the aim of this project. Therefore, Western blotting was performed in order to examine the changes in the expression of various cell signaling proteins involved in the progression of ERα negative breast cancer. Specifically, the changes in the expression levels of EGFR, HER2, P-HER2, Akt, P-Akt, mTOR, P-mTOR, 4E-BP1, P-4E-BP1, p27, NFkB, p38, P-p38, JNK1/2, P-JNK1/2 and cleaved caspase 3 were examined.

2.2.1.5.1 Seeding cells for cell lysates preparation

MDA-MB-231, MDA-MB-468 and SKBr3 cells were seeded in 10 cm cell culture dishes at 2.5×10⁶ cells per well in 10 ml of DMEM/HamF12 supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B and 2.2 g/l NaHCO₃ and incubated in 5% CO₂ / 95% humidified air for 24 h at 37°C. The cells were treated with RL71 (1 µM) or RL66 (1.5 or 2 µM) or vehicle control (0.1% DMSO) for variable time points (0-36 h).
2.2.1.5.2 Preparation of whole cell lysates

At the end of treatment, each dish was placed on ice and the media was aspirated. Cells were then washed twice with 5 ml of isotonic PBS maintained at 4°C followed by addition of 400 µl of lysing buffer (50 mM Tris base, 1% NP-40, 0.25% sodium deoxycolate, 100 mM NaCl, 1 mM EDTA, sodium orthovanadate 1 mM, sodium pyrophosphate 2.5 mM, sodium flouride 10 mM and complete protease inhibitor cocktail tablets). The cells were detached using a cell scraper and transferred to corresponding eppendorf tubes. Before protein estimation, cell lysates were sonicated for 3 X 7 sec followed by further centrifugation at 12,500 rpm for 8 min, at 4°C. The supernatant was transferred into new tubes and protein concentration was determined using the BCA method (Smith et al., 1985).

2.2.1.5.3 Bicinchoninic Acid (BCA) Method

2 µl of protein lysate of each sample was added to a 96-well plate in triplicate followed by addition of 18 µl of 1% of Triton X-100. The 4, 4’-dicarboxy-2, 2’-biquinoline (BCA) solution was prepared by combining both solution one BCA and solution two (4% CuSO$_4$.5H$_2$O, (w/v)) in a 50:1 ratio. 200 µl of BCA solution was added to each sample and a standard curve was obtained using BSA as a standard with a range of concentration of 0-500 µg/ml. The plate was then incubated for 30 min at 37°C. Absorbance was determined using a Biorad Benchmark Plus microplate reader at 595 nm with data being obtained using Microplate Manager Software (version 5.2.1). The standard curve was used to determine protein concentration of the samples. The samples were diluted with required volumes of lysing buffer in order to obtain 40 µg/µl of protein. Finally, 4 X sample buffer was added to all samples and the samples were boiled for 5 min, and cooled onto ice for 5 min. Samples were then stored at -20°C until required for gel electrophoresis.

2.2.1.5.4 Western immunoblotting

Sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) electrophoresis was used to separate proteins based on molecular weight (MW), as described by Laemmli, 1970. Each gel consisted of a 10% or 15% resolving gel (5 ml acrylamide/bisacrylamide solution, 3.75 lower Tris buffer, 500 µl of 50% glycerol, 75 µl APS, 5.75 ml dH$_2$O, 10 µl TEMED) and a 4% acrylamide stacking gel (0.85 ml acrylamide/bisacrylamide solution, 1.25 ml upper Tris buffer, 50 µl APS, 2.9 ml dH$_2$O, 2.5 µl TEMED). The percent of the acrylamide/bisacrylamide in the resolving gel was determined according to the MW of the
target protein. 40 μg of protein was loaded into each well of the polyacrylamide gels. A MW marker was loaded into the first well to identify protein size. Gels were run at 100 V in running buffer (25 mM Tris-base, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS) using a Bio-Rad Mini-Protean III apparatus until the dye had migrated to the end of the stacking gel. The voltage was then increased to 130 V and run until the dye front had reached the end of the resolving gel.

2.2.1.5.5 Transfer and development

After resolving the proteins, they were transferred to a PVDF membrane in transfer buffer (25 mM Tris-base, pH 8.3, 0.192 M glycine and 10% methanol). Prior to transfer, the PVDF membrane was activated in methanol for 2 min, washed with water and incubated in transfer buffer. Proteins were transferred for 1.5 h (100 V) using a Bio-Rad wet transfer system. Following transfer, membranes were washed in dd H$_2$O and stained with Ponceau red stain for 1 min. Membranes were then rinsed again in dd H$_2$O and incubated in blocking buffer (5% BSA, 1% sodium azide) for 1 h at room temperature. Membranes were then washed with TBS (0.025 mM Tris-base, 0.1 M NaCl, pH 7.4) and incubated with the appropriate anti-human primary antibody (diluted in 5% BSA) overnight at 4°C. The concentration of various primary antibodies used was as follows, EGFR (1:2000), HER2 (1:1000), P-HER2 (1:1000), Akt (1:2000), P-Akt (1:1500), NFkB (1:2000), JNK (1:1000), P-JNK (1:1000), p38 (1:2000), P-p38 (1:2000), p27 (1:2000), cleaved caspase-3 (1:1000), mTOR (1:2000), P-mTOR (1:1000), 4E-BP1 (1:1000), P-4E-BP1 (1:1000) and β actin (1:5000). Following incubation, each membrane was washed with TBST (0.05% Tween 20, 0.025 mM Tris-base, 0.1 M NaCl, pH 7.4) 5 times for 5 min each. Membranes were then incubated in 5% non-fat milk/TBS with horseradish-peroxidase conjugated goat anti-mouse or goat anti-rabbit secondary antibody at room temperature for 1 h. Membranes were then washed again in TBST 5 times for 5 min each wash. Protein bands were visualized by incubating membranes in SuperSignal West Pico Chemiluminescent Substrate. The digital chemiluminescence images were taken using a Versadoc (BioRad) imaging system and ALL-PRO imaging system and quantified using Quantity One software (BioRad).

2.2.1.6 Scratch assay

The scratch assay was performed in order to study the migration potential of MDA-MB-231 breast cancer cells in vitro. This assay mimics the cell migration process in vivo (Rodriguez et al., 2005). As migration is a crucial step for development of metastasis, the
effect of RL71 or RL66 on inhibition of cell migration was of particular importance. The scratch assay was performed as described by (Liang et al., 2007). MDA-MB-231 cells were grown to confluence in a 6 well plate (500,000 cells/well) supplemented with 2 ml DMEM and incubated in 5% CO₂/95% humidified air at 37°C. A scratch was made on the cell monolayer using a 200 µl pipette tip and the cell debris was removed by washing the cells with 1ml DMEM. The cells were treated with 0.1% DMSO, RL71 (1 µM) or RL66 (2 µM) and photos were taken at time zero. Then the cells were incubated for 24 h and photos were taken (200X magnification, Canon XTI camera) to observe the cell migration across the scratch.

2.2.1.7 Anti-angiogenesis assay

An anti-angiogenic potential of RL71 and RL66 was evaluated using the endothelial tube formation assay as described by Arnaoutova et al., 2010 and the Transwell migration assay as described by the instruction manual from BD Biosciences (Bedford, MA).

2.2.1.7.1 Endothelial tube formation assay

The day before performing the tube formation assay, the Matrigel matrix was removed from -20°C freezer and incubated on ice at 4°C overnight. On the day of the assay, 125 µl Geltrex Matrigel was transferred to 12 wells of a 24-well plate. The plate was incubated at 37°C, 5% CO₂ for 30 min. During the incubation time, endothelial cells were washed with PBS, trypsinized and suspended in 8 ml EGM medium. The HUVEC cells (50,000 per well) were seeded into each well, followed by addition of 50 µl DMSO (0.1%), RL71 (1 µM) or RL66 (1 µM) diluted in EGM media. The plate was incubated at 37°C, 5% CO₂ for 18 h and photos (200X Canon XTI camera) of endothelial tube formation were taken.

2.2.1.7.2 Transwell Migration assay

The migration assay was performed using 24 well plate containing 12 cell culture inserts with 8 µM pore size (BD BioCoat™ Matrigel™ Invasion Chambers; BD Biosciences, Bedford, MA). Control plates with no Matrigel were also purchased. Prior to the assay, in order to rehydrate Matrigel, 500 µl of EGM serum free media (maintained at 37°C) was added in the plate and into the well inserts and incubated for 2 h. The HUVEC cells were washed with PBS and trypsinized to detach from the surface. Trypsin was inactivated using trypsin inhibitor and mixed with serum free EGM. HUVEC cells (25,000 per well) were plated on rehydrated Matrigel coated culture inserts. The bottom chamber of
the plate contained 500 µl of EGM media supplemented with 5% serum. The cells were treated with 0.1% DMEM or RL71 (1 µM) or RL66 (1 µM) and incubated for 18 h at 37°C in a humidified 5% CO₂ incubator. After incubation, all contents from well inserts were aspirated and non-migrated cells were removed with a cotton swab. Migrated cells on the bottom of the filters were stained with DiffQuick solutions A, B and C for 1 min and excess stain was washed with water and dried. Membranes were cut out and removed from the bottom of the insert, air-dried and placed on a clean microscope slide. Cells on the filters were counted using a Zeiss Axioplan camera and compared to the control well insert that contained no Matrigel. The slides were blinded and analyzed by two independent examiners.

2.2.2 IN VIVO STUDIES

After studying the anticancer potential of curcumin analogs towards ERα negative breast cancer cells in vitro, the next aim of this study was to examine their safety and efficacy in vivo using a mouse xenograft model. Accordingly, the in vivo studies mainly involved the use of female CD-1 mice for evaluation of bioavailability and female athymic nude mice for evaluation of safety and efficacy of selective curcumin analogs. The various techniques and experimental studies performed are mentioned below.

2.2.2.1 Animal housing and care

Female athymic nude mice (5-6 weeks old) were purchased from the Hercus Taieri Resource Unit (Dunedin, NZ). All procedures were approved by the University of Otago (AEC# 91/07). Mice were housed in pathogen-free conditions with sterile woodchip bedding with access to sterile food (Reliance rodent diet, Dunedin, NZ) and water ad libitum. Mice were housed in a 21-24°C environment on a scheduled 12 h light/dark cycle.

2.2.2.2 Oral bioavailability studies

Female CD-1 mice (6 weeks old, 3/group) were orally gavaged with RL71 or RL66 and blood samples were collected following a time course (5 min, 10 min, 15 min, 30 min, 1 h, 1.5 h and 2 h). The blood was centrifuged at 5000 rpm and the plasma was collected and stored at -20°C. The samples for analysis were prepared by addition of methanol to precipitate the proteins, followed by sonication and filtration. The samples were analyzed by HPLC with UV-DAD detection.
2.2.2.2.1 **HPLC method**

HPLC analysis was performed using an Agilent HP1100 system at 25°C on a C\textsubscript{18} column (Phenomenex Gemini-NX) 3µ (110A, 150 X 2 mm) with a 2 X 4 mm C\textsubscript{18} guard column. Peaks were detected at 390 nm and 305 nm for RL71 and RL66, respectively. The mobile phase for RL71 was acetonitrile in water, both with 0.1% formic acid: t\textsubscript{0} =30%, t\textsubscript{10} =70%, t\textsubscript{15} =100%, t\textsubscript{17} =30%, t\textsubscript{20} =30%. Whereas for RL66, the gradient was t\textsubscript{0} =20%, t\textsubscript{10} =50%, t\textsubscript{15} =100%, t\textsubscript{17} =20%, t\textsubscript{20} =20%. The flow rate was 0.3 ml/min, with an injection volume of 5 µl. The limit of detection was 0.1 µg/ml.

2.2.2.3 **MDA-MB-468 mouse xenograft model**

The use of a mouse xenograft model for assessment of the anti-cancer potential of drugs *in vivo* is well established. For this study, a mouse xenograft model was developed using MDA-MB-468 breast cancer cells. Some research groups have also shown use of other ER\textalpha negative breast cancer cells such as MDA-MB-231 and SKBr3 for xenograft development. But the MDA-MB-468 xenograft model was advantageous as MDA-MB-468 cells can easily form solid tumors in immunocompromised mice and grow at a reasonable rate for extended time.

To generate the MDA-MB-468 mouse xenograft model, a cell suspension of MDA-MB-468 (8 x 10\textsuperscript{6} cells/ml) in DMEM growth media and Matrigel (1:1 ratio) was prepared. After three days acclimatization, mice were inoculated with 200 µl of the cell suspension into the lower right flank. The tumors were allowed to grow for 14 days (~150 mm\textsuperscript{3}). Mice were randomly assigned into treatment and control groups with 10 mice in each group and dosed daily by oral gavage with either vehicle (water) or RL71 or RL66 (8.5 mg/kg or 0.85 mg/kg) for 10 weeks. Mice were weighed daily and monitored for weight gain and general animal health. Tumor volume (length x width x height) was measured weekly with electronic callipers by the two independent examiners. The tumor volume was expressed as X ± SEM in mm\textsuperscript{3}. The mortality rate of mice for RL71 study was 2, 2 and 0 in the vehicle, 0.85 mg/kg and 8.5 mg/kg group respectively, whereas RL66 showed mortality rate of 5, 1 and 1 in respective groups.

2.2.2.4 **Tissue and Blood Collection**

Animals were euthanized after 10 weeks treatment by CO\textsubscript{2} inhalation. Blood was drawn immediately from the inferior vena cava using a 20-gauge needle and heparinised syringe and immediately placed on ice. Major organs (liver, spleen, kidneys and uterus)
and tumors were removed, weighed and immediately frozen in liquid nitrogen or OCT compound (in N-hexane) and stored at -80°C until further required. Each organ weight was expressed as a percentage of total body weight.

2.2.2.5 Assessment of ALT levels

At the time of necropsy, blood was collected as described above. Blood samples were centrifuged (5000 rpm, 4°C) for 5 min (Eppendorf 5810R centrifuge) and plasma was transferred into corresponding eppendorf tubes. Plasma ALT was measured using a commercially available kit and used as an indicator of hepatotoxicity. To estimate plasma ALT activity, 100 μl of plasma was transferred to a plastic cuvette, followed by 1 ml of ALT reagent reconstituted and warmed to 37°C. Absorbance was measured at 340 nm on a spectrophotometer (BioRad Benchmark Plus Microplate Spectrophotometer) each minute for 3 min. ALT activity, expressed in international units per litre (IU/L) was determined by the equation: Δ absorbance x (TV X 1000) / (6.3 x SV x P), where Δ absorbance = (Abs0min – Abs3min), TV = total volume in the cuvette (1.1 ml), SV = volume of sample added (0.1 ml) and P = path length (1 cm).

2.2.2.6 Effect of curcumin analogs on tumor protein expression

To study the tumor suppression mechanism following the curcumin analog treatment, Western blotting of the tumor samples was carried out. Mainly, the changes in the expression of various cell signaling proteins involved in the progression of ERα negative breast tumors such as EGFR, Akt, m-TOR, p27 and NFκB were studied.

2.2.2.6.1 Preparation of tumor protein extracts

The tumor tissues were ground into thin powder before protein extraction followed by addition of 250 μl of lysing buffer (20 μM Tris HCl pH 8, 1% NP-40, 10% glycerol, 0.5% sodium deoxycolate, 137 mM NaCl, 2 mM EDTA and complete protease inhibitor cocktail tablets at 4°C. The tumor tissue was then homogenized using a mechanical eppendorf probe at 1500 rpm for 10 X 3 sec. The samples were further sonicated for 3 X 7 sec followed by centrifugation at 12,500 rpm for 8 min, at 4°C. The supernatant was transferred into new tubes and protein concentration was determined using the BCA method. Western blotting was then performed to examine the changes in the expression of a variety of proteins.
2.2.2.7 Immunohistochemistry

This method was used to determine microvessel density in the tumor. At the time of necropsy, tumors were frozen as previously described and stored at -80°C until required. Before slicing, the blade and glass plate were cooled at -20°C. Using a microtome (Leica), 10 micrometre thick sagital slices were cut at -20°C and placed on poly-L-lysine-coated microscope slides. The thickness of the slices was chosen based on findings from previous literature (Weidner 1995). The slices were dried under a fan for 30 min at room temperature and stored at -20°C until required. Slides were then fixed and stained over a two-day process as described below.

Part 1

Slices were thawed for 30 min at room temperature and then washed in 1X PBS twice for 5 min each wash. PBS was aspirated and the tumor sections were fixed by applying 4% acetone for 10 min at room temperature. The excess acetone was aspirated and the slides were allowed to dry. The sections were marked with the DAKO pen and allowed to dry for a further 10 min. 0.3% hydrogen peroxide in methanol was then applied for 20 min at room temperature and sections then washed again in 1X PBS three times for 2 min each wash. Blocking solution (1.5% goat serum and 0.2% BSA diluted in PBS) containing 0.001% avidin D solution was applied for 1 h at room temperature in a humidified chamber. The sections were rinsed with 1X PBS for 5 min. The primary antibody, rat anti-mouse CD-105 which binds preferentially to proliferating endothelial cells, (Weidner 1995) was then applied at a concentration of 1:100 and left to incubate overnight (22 h) at 4°C.

Part 2: Staining

The sections were removed and washed four times with 1X PBS for 5 min. This was followed by application of goat anti-rat IgG secondary antibody at a 1:200 dilution and an incubation time of 30 min at room temperature. Slices were then washed in antibody washing buffer twice, 5 min each wash. The streptavidin biotin solution was applied for 30 min at room temperature. This was followed by three washes of 1X PBS for 5 min each wash. Slices were incubated with diamin benzadine (DAB) in the dark for 20 min and then washed three times with dd H₂O for 2 min each wash. Slices were then stained with Mayer’s haematoxylin for 15 sec and then rinsed thoroughly in tap water. Slices were then washed with 0.1% sodium bicarbonate solution twice for 10 sec each wash, followed by a
rinse in dd H₂O for 30 sec. They were then dehydrated in 70% ethanol and 90% ethanol for 5 min wash and 95% ethanol for 10 min. Slices were then soaked in xylene twice for 5 min each wash. DPX mounting medium was then applied, followed by cover slips. Pictures were then taken (400 x magnification, Zeiss Axioplan camera) and treatment groups compared. The slides were blinded and analyzed by two independent examiners.

2.3 STATISTICAL ANALYSIS

Time course cytotoxicity, cell cycle progression and apoptosis induction data were analyzed by two-way ANOVA coupled with a Bonferroni post-hoc test. To assess tumor growth in vivo, two-way repeated measures ANOVA with Bonferroni post-hoc test was performed. To assess tumor weight, mouse weight, individual organ weight, plasma ALT levels, the differences in protein density from Western immunoblotting, and the number of CD105 positive cells in tumors, a one-way ANOVA with a Bonferroni post-hoc test was performed. A Student t-test was used to assess the number of migrated cells in the Transwell migration assay. All the experimental data are expressed as mean and SEM and the test of significance confirmed at p<0.05.
CHAPTER 3: RESULTS

3.1 CURCUMIN ANALOGS

3.1.1 Aim

The aim of this study was to screen various heterocyclic cyclohexanone analogs of curcumin for their cytotoxicity towards ERα negative breast cancer cells and further explore the structural requirements for anti-breast cancer activity in particular in relation to other heterocyclic analogs with differing electronic effects.

3.1.2 Synthesis of curcumin analogs

The analogs designed in this study were based on cyclic ketone structures which were produced by reducing the keto-enol moiety and seven carbon linker group of curcumin (between the two aromatic benzene rings) (Fig. 3.1.1). Accordingly, the structure of the analogs contained a core cyclic ketone group, with aromatic groups linked to the core via two methyldiene groups. The aromatic groups were further substituted with or without heterocyclic groups.

![Figure 3.1.1 General structure of curcumin analogs: The keto-enol moiety of curcumin is reduced to give more stable cyclic ketone analogs.](image-url)
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A series of heterocyclic analogs of curcumin were prepared: Series A contains a cyclohexanone core; series B, an N-methylpiperidone core; series C, a tropinone core, series D, a cyclopentanone core unit; and series E, a t butyl carboxy core unit (Fig. 3.1.2). The aromatic groups included fluorine substituted pyridines, since there are examples of fluorine substitution of aromatics giving enhanced activity (Adams et al., 2004). The electron rich five-membered aromatic units of pyrrole, imidazole and indole, as well as electron rich trimethoxyphenyl and two dimethoxyphenyl groups were also included because there has been some evidence that electron rich aromatics can have enhanced activity (Fig. 3.1.2) (Amolins et al., 2009).

Figure 3.1.2: Structures of the heterocyclic curcumin analogs.
3.1.3 Dose-response cytotoxicity assessments of curcumin analogs

In order to assess the cytotoxic potency of these curcumin analogs, MDA-MB-231 breast cancer cells were treated with a range of concentrations (0.01 - 30 µM) of each compound for 5 days and the cell number was determined using the SRB assay.

3.1.3.1 Cytotoxicity of series A cyclohexanone core compounds

Seven different series A cyclohexanone analogs of curcumin were screened for their toxicity towards MDA-MB-231 cells: RL10 2,6-bis ((1-methyl-1H-pyrrol-2-yl) methylene)-cyclohexanone, RL11 (2E,6E)-2,6-bis ((1-methyl-1H-imidazol-5-yl) methylene) cyclohexanone, RL7 (2E,6E)-2,6-bis ((1-methyl-1H-indol-3-yl) methylene) cyclohexanone, RL8 (2E,6E)-2,6-bis ((1-methyl-1H-imidazol-2-yl) methylene) cyclohexanone, RL12 (3E,5E)-3,5-bis (2,5-dimethoxybenzylidene)-1-methylpiperidin-4-one, RL54 (2E,6E)-2,6-bis ((2-fluoropyridine-3-yl) methylene) cyclohexanone, and RL55 (2E,6E)-2,6-bis ((2-fluoropyridine-4-yl) methylene) cyclohexanone. Amongst them, RL54, RL55 and RL12 showed potent cytotoxicity. The corresponding IC$_{50}$ values

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)
Figure 3.1.3: Cytotoxicity of series A cyclohexanone curcumin analogs towards MDA-MB-231 cells: MDA-MB-231 cells were seeded in 12 well plates at 7X10^4 cells per well and incubated for 24 h at 37º C. Cells were treated with 0.1 to 30 µM of RL10 (A), RL11 (B), RL7 (C), RL8 (D), RL12 (E), RL55 (F) and RL54 (G) for five days. The vehicle control cells were treated with 0.1% DMSO. At the end of the treatment, the cell number was determined by the SRB assay. Results are expressed as cell number ± SEM obtained from 3 independent experiments conducted in triplicate. The dose response curves were obtained using non-linear regression.

obtained with these compounds were 3.2 µM (95% CI, 2.7 to 3.9), 3.3 µM (95% CI, 3.1 to 3.5) and 1.9 µM (95% CI, 1.4 to 2.4) respectively (Fig. 3.1.3). RL7 and RL8 showed IC_{50} values greater than 10 µM whereas IC_{50} values for RL10 and RL11 could not be determined over the range of concentrations tested (Fig. 3.1.3). In this group of compounds, RL12 showed the greatest cytotoxicity whereas RL54 and RL55 showed similar potency.
3.1.3.2  Cytotoxicity of series B an N-methylpiperidone core

Seven different series B N-methylpiperidone analogs of curcumin were screened for their toxicity towards MDA-MB-231 cells: RL66 1-methyl-3,5-bis[(E)-(4-pyridyl) methylidene]-4-piperidone, RL62 1-methyl-3,5-bis[(E)-(3-pyridyl) methylidene]-4-piperidone, RL71 (3E,5E)-3,5-bis(3,4,5-trimethoxybenzylidene)-1-methylpiperidin-4-one, RL6 (3E,5E)-3,5-bis(2-fluoro-4,5-dimethoxybenzylidene)-1-methylpiperidin-4-one, RL26 1-methyl-3,5-bis[(E)-(2-thienyl)methylidene]-4-piperidone and RL27 (3E,5E)-1-methyl-3,5-bis((1-methyl-1H-imidazol-2-yl)methylene)piperidin-4-one. Amongst them, five analogs namely

A) RL66

B) RL62

C) RL71

D) RL6

E) RL9
RL66, RL62, RL71, RL6 and RL9 showed potent cytotoxicity. The corresponding IC\textsubscript{50} values obtained with these compounds were 0.8 \text{µM} (95\% CI, 0.78 to 0.88), 2.1 \text{µM} (95\% CI, 2.0 to 2.2), 0.27 \text{µM} (95\% CI, 0.25 to 0.28), 3.8 \text{µM} (95\% CI, 1.2 to 11.8) and 1.1 \text{µM} (95\% CI, 0.9 to 1.3) respectively (Fig. 3.1.4). The IC\textsubscript{50} values for RL26 and RL27 could not be determined over the range of concentrations tested (Fig. 3.1.4). Thus, in this group of compounds, RL71 showed the greatest cytotoxicity followed by RL66, RL9, RL62 and RL6.

3.1.3.3 Cytotoxicity of series C tropinone core

Three series C tropinone analogs of curcumin were screened for their toxicity towards MDA-MB-231 cells: RL53, (2\text{E},4\text{E})-8-methyl-2,4-bis((pyridine-4-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one, RL60 (2\text{E},4\text{E})-8-methyl-2,4-bis((pyridine-3-yl)methylene)-8-aza-bicyclo[3.2.1]octan-3-one and RL65 (2\text{E},4\text{E})-2,4-bis(3,4,5-trimethoxybenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one. Of these, RL53 showed potent cytotoxicity whereas RL60 showed moderate cytotoxicity. The IC\textsubscript{50} values obtained with these compounds were 1.1 \text{µM} (95\% CI, 0.9 to 1.4) and 12.9 \text{µM} (95\% CI, 6.4 to 18.2) respectively (Fig. 3.1.5). However for RL65, the IC\textsubscript{50} value could not be determined over the range of concentrations tested (Fig. 3.1.5).
Figure 3.1.5: Cytotoxicity of series C tropinone curcumin analogs towards MDA-MB-231 cells: MDA-MB-231 cells were seeded in 12 well plates at 7X10^4 cells per well and incubated for 24 h at 37º C. Cells were treated with 0.01 to 30 µM of RL53 (A), RL60 (B) and RL65 (C) for five days. The vehicle control cells were treated with 0.1% DMSO. At the end of the treatment, the cell number was determined by the SRB assay. Results are expressed as cell number ± SEM obtained from 3 independent experiments conducted in triplicate. The dose response curves were obtained using non-linear regression.
3.1.3.4 Cytotoxicity of series D cyclopentanone core

The cyclopentanone analog RL63, 2,5-bis(3-pyridylmethylene)-cyclopentanone showed moderate cytotoxicity against MDA-MB-231 cells. The corresponding IC\textsubscript{50} value obtained was 8.6 µM (95% CI, 7.8 to 9.6) (Fig. 3.1.6).

Figure 3.1.6: Cytotoxicity of series D cyclopentanone curcumin analog towards MDA-MB-231 cells: MDA-MB-231 cells were seeded in 12 well plates at 7X10\textsuperscript{4} cells per well and incubated for 24 h at 37\degree C. Cells were treated with 0.1 to 30 µM of RL63 for five days. The vehicle control cells were treated with 0.1% DMSO. At the end of the treatment, the cell number was determined by the SRB assay. Results are expressed as cell number ± SEM obtained from 3 independent experiments conducted in triplicate. The dose response curves were obtained using non-linear regression.
3.1.3.5  Cytotoxicity of series E tert butyl carboxy group core

The tert butyl carboxy analogs RL175 tert-butyl 4-oxo-3,5-bis((pyridin-3-yl)methylene)piperidine-1-carboxylate and RL197 tert-butyl 3,5-bis(2,5-dimethoxybenzylidene)-4-oxopiperidine-1-carboxylate showed potent cytotoxicity against MDA-MB-231 cells. The corresponding IC\textsubscript{50} values obtained with these compounds were 1.1 µM (95% CI, 0.6 to 2.1) and 0.5 µM (95% CI, 0.43 to 0.53) respectively (Fig. 3.1.7). RL197 was found to be more potent compared to RL175. A summary of all compounds screened and their resulting IC\textsubscript{50} values are shown in Table 3.1.1.

Figure 3.1.7: Cytotoxicity of series E tert butyl carboxy curcumin analogs towards MDA-MB-231 cells: MDA-MB-231 cells were seeded in 12 well plates at 7X10\textsuperscript{4} cells per well and incubated for 24 h at 37\textdegree C. Cells were treated with 0.01 to 10 µM of RL175 (A) and RL197 (B) for five days. The vehicle control cells were treated with 0.1% DMSO. At the end of the treatment, the cell number was determined by the SRB assay. Results are expressed as cell number ± SEM obtained from 3 independent experiments conducted in triplicate. The dose response curves were obtained using non-linear regression.
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**Table 3.1.1:** Summary of IC<sub>50</sub> values of curcumin analogs in MDA-MB-231 cells. Dash (-): IC<sub>50</sub> values could not be determined over the range of concentrations tested (1-30 µM).
3.1.4.6 Cytotoxicity of potent curcumin analogs in MDA-MB-468 and SKBr3 cells

The analogs which showed potent cytotoxicity (IC$_{50}$ values of ~1 µM) in MDA-MB-231 cells were further examined for their ability to elicit cytotoxicity in other ERα negative cell lines, namely MDA-MB-468 and SKBr3. B1 (RL66), B10 (RL71), B12 (RL9), and C1 (RL53) were examined in cell lines that both contained (SKBr3) and lacked (MDA-MB-468) HER2. All four compounds produced significant cytotoxicity towards these two breast cancer cell lines.

**Figure 3.1.8:** Cytotoxicity of curcumin analogs RL66, RL71, RL9 and RL53 towards MDA-MB-468 cells: MDA-MB-468 cells were seeded in 12 well plates at 7X10$^4$ cells per well and incubated for 24 h at 37º C. Cells were treated with 0.01 to 10 µM of RL66 (A), RL71(B), RL9 (C) and RL53(D) for five days. The vehicle control cells were treated with 0.1% DMSO. At the end of the treatment, the cell number was determined by the SRB assay. Results are expressed as cell number ± SEM obtained from 3 independent experiments conducted in triplicate. The dose response curves were obtained using non-linear regression.
5 days treatment of MDA-MB-468 cells with RL66, RL71, RL9 and RL53 produced IC$_{50}$ values of 0.52 μM (95% CI, 0.47 to 0.58), 0.3 μM (95% CI, 0.28 to 0.4), 1.1 μM (95% CI, 1.02 to 1.2) and 0.6 μM (95% CI, 0.5 to 0.8) respectively (Fig. 3.1.8). The compound RL71 showed the greatest cytotoxicity followed by RL66, RL53 and RL9.

In SKBr3 cells, the corresponding IC$_{50}$ values obtained were 0.62 μM (95% CI, 0.57 to 0.66), 0.37 μM (95% CI, 0.33 to 0.38), 1.1 μM (95% CI, 0.9 to 1.3) and 0.74 μM (95% CI, 0.69 to 0.8) for RL66, RL71, RL53 and RL9 respectively (Fig. 3.1.9). Thus RL71 showed greatest cytotoxic potency towards SKBr3 cells. A summary of the IC$_{50}$ values for all four compounds and curcumin in three breast cancer cell lines is shown in Table 3.1.2.
Table 3.1.2: IC₅₀ values of potent curcumin analogs in all three ERα negative human breast cancer cell lines.

3.1.4 Structure activity relationship analysis (SAR)

Previously our lab demonstrated that the two pyridine analogs, 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone, RL₉₁ and 2,6-bis(pyridin-3-ylmethylene)-cyclohexanone, RL₉₀ were cytotoxic towards MDA-MB-231 cells (Somers-Edgar et al., 2011). In an extension of this work, the structural requirements for anticancer activity were explored in particular in relation to other heterocyclic analogs with differing electronic effects. Previous studies have reported that the six-membered cyclohexanone ring system is in general superior to the five-membered cyclopentanone system for inhibitory activity in a group of seven cancer cell lines (Liang et al., 2009). Evidence that this was also the case for MDA-MB-231 cells was found when the five-membered cyclopentanone derivative RL₆₃ was compared with cyclohexanone RL₉₀ and showed a five-fold decrease in activity. Therefore, further investigation was restricted only to the six-membered cyclohexanone group.

Concentrating initially on the cyclohexanone core A series, the fluorine substituted pyridine rings were then examined. Two examples RL₅₅ and RL₅₄ were found to be 2–3 times less active than the parent pyridine compounds RL₉₁ and RL₉₀. In the next approach, the pyridine ring was replaced with different heterocyclic rings. Thiophene RL₉₄, N-methylpyrrole RL₁₀, N-methylindole RL₁₁ and 4-substituted N-methylimidazole RL₇ exhibited no activity (IC₅₀ >30 μM). 2-substituted N-methylimidazole RL₁₂ showed good cytotoxicity towards MBA-MB-231 cells. The pyridyl groups were also replaced with electron rich trimethoxyphenyl and dimethoxyphenyl to give analogs RL₇₂ and RL₈ but neither analog showed any activity in MDA-MB-231 cells.
For the N-methylpiperidone core series B analogs, the heteroaromatic analogs generally had similar or slightly increased activity over cyclohexanone core, series A analogs. Thus 4-pyridyl RL66 showed some increased activity over RL91, the 3-pyridyl analog RL62 slightly less activity than the corresponding RL90, and thiophene RL26 retained the lack of activity of RL94. Surprisingly, N-methylimidaz-2-yl analog RL27 showed no activity compared to RL12 which showed good activity. In contrast the tri- and dimethoxyphenyl B series derivatives showed good activity compared to the A series where little activity was observed. In fact the trimethoxyphenyl analog RL71 exhibited a level of activity about three times greater than any other compound examined. The dimethoxyphenyl analogs were also active, with RL6 being less active than RL9, further indicating that the fluorine group conferred no improvement in activity in these compounds.

For the tropinone core series C analogs which have a more rigid structure, as well as being more sterically hindered, there was either no change in activity, as in the case of the 4-pyridyl compound RL53, or else reduced activity, as seen for compounds RL60 or RL65.

In another series of compounds, series E, the methyl group on the piperidone of series B compounds was replaced by a t-butylcarboxy group. The idea was to be able to produce a compound where the group on the piperidone nitrogen can be removed and replaced with a marker or tumor targeting carrier. The results showed that RL197 was more potent than RL9 and was the second most potent compound after RL71. However, because of the bulky group attached, RL197 was not able to form a salt and thus was not readily soluble. Also, it was observed that attachment of any group at piperidone of RL197 leads to its deactivation further making it less likely to cross biomembrane. Another compound from the same group, RL175, also had good cytotoxicity and was more potent compared to RL90, RL62, RL60 and RL63.

In summary, it was found that pyridine heteroaromatic substituted bismethylene cyclohexanones were the most active of the heteroaromatic analogs tested to date, and that the five-membered heteroaromatics in general have no activity in MDA-MB-231 breast cancer cells. Replacement of the cyclohexanone core with N-methylpiperidone can give some improvement in activity for the heteroaromatic analogs, although this varied. In contrast, the polymethoxyphenylmethylene N-methylpiperidone derivatives had good activity compared to the cyclohexanone core derivatives which had no activity. The use
of the more sterically hindered tropinone led to equal to or reduced activity in all cases. Additionally, fluorine substituents on the aromatic groups do not increase the activity of these analogs. These results suggest that activity in this cell line is not determined by the electronic effects of the aromatic groups. It also appears that a nitrogen heteroatom in either the aromatic group or in the core cyclic ketone provides analogs with good activity. Accordingly, methyl piperidone analog RL71 was found to be the most potent towards all three ERα negative breast cancer cells. Therefore, further study was designed to comprehensively investigate the in vitro and in vivo mechanism of action of this lead compound in order to determine its potential for further drug development for ERα negative breast cancer.

### 3.2 ANTICANCER ACTIVITY OF RL71

#### 3.2.1 Aim

As RL71 (Fig. 3.2.1) was the most potent compound obtained during the initial screening, its mechanism of the anticancer activity was examined in both in vitro and in vivo models of ERα negative breast cancer. Specifically, the time-course of cytotoxicity and further effects on cell cycle progression and induction of apoptosis were investigated. Moreover, the effect on various cell signaling proteins that lead to cell death was investigated by Western blotting. It was also an aim to assess an anti-angiogenic potential of RL71 in vitro in addition to the study of migration potential in MDA-MB-231 cells. Finally, by using an athymic nude mice model of MDA-MB-468 cells, the effect of RL71 on tumor suppression was examined in vivo.

![Chemical Structure of RL71](image)

**Figure 3.2.1: Chemical Structure of RL71**
3.2.2 Effect of RL71 in vitro

3.2.2.1 Time-course cytotoxicity studies

To examine the cytotoxicity of RL71 over a time-course, various ERα negative breast cancer cells (MDA-MB-231, MDA-MB-468 and SKBr3) were treated with RL71 (1 µM) for 6, 12, 24, 36, 48 and 72 h and the cell number was determined by the SRB assay. The concentration of RL71 that reduced cell number by approximately 80% was chosen for further assessment of cell cytotoxicity. The results showed that RL71 (1 µM) elicited time-dependent and cell-line dependent cytotoxicity. In particular, time-dependent cytotoxicity was elicited in SKBr3 cells with significantly increased cytotoxicity at 72 h compared with all other time points (Fig. 3.2.2C). However, in the two TNBC cell lines no further cytotoxicity was elicited after 24 h (Fig. 3.2.2A, B). Thus, RL71 showed potent cytotoxicity toward SKBr3 cells compared to a cytostatic effect in TNBC cells. Also, 72 h following the treatment, cell number was decreased to 29 ± 1, 29 ± 0.1% and 12 ± 0.4% of control, respectively for MDA-MB-231, MDA-MB-468 and SKBr3 cells.

3.2.2.2 Cell cycle progression

To further examine whether the cytotoxicity of RL71 was due to the cell cycle arrest, MDA-MB-231, MDA-MB-468 and SKBr3 cells were treated with 1 µM of RL71 for 6 to 48 h and the proportion cells in various phases of cell cycle was examined by cell cycle analysis. The results showed that RL71 produced G2/M phase arrest in all three cell lines (Fig. 3.2.3). 1 µM of RL71 induced a significant increase in the proportion of breast cancer cells in the G2/M phase. Specifically, at 48 h, RL71 caused an 62 ± 1% increase in the proportion of MDA-MB-231 cells in G2/M phase compared to control whereas in MDA-MB-468 cells, the proportion of cells in G2/M phase increased by 40 ± 7% compared to control at 36 h. This effect was accompanied by a significant decrease in the proportion of cells in G0/G1 phase by 27 ± 1% and 15 ± 5% respectively, compared to control. However, there was no significant change in the proportion of cells in S phase. In SKBr3 cells, after 12 h, the proportion of cells in the S and G2/M phases was significantly increased by 28 ± 4% and 53 ± 4% compared to control respectively. This effect was accompanied by a significant decrease in the proportion of cells in G0/G1 phase by 18 ± 2% of control. Moreover, there was a significant reduction in the proportion of cells in S phase after 24, 36 and 48 h as well as G2/M phase after 36 and 48 h. SKBr3 cells were the only cell type to show an increase in subG1 cells. The effect in
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MDA-MB-231 cells was time-dependent as the number of cells undergoing G2/M phase arrest was significantly increased at 48 h compared to all other time points. Also, it was observed that MDA-MB-231 and SKBr3 cells were more sensitive to the cell cycle inhibitory effects of RL71 as G2/M-phase arrest peaked earlier in both cells.

**A. MDA-MB-231**

![Graph showing time-course cytotoxicity of RL71 on MDA-MB-231 cells](image)

**B. MDA-MB-468**

![Graph showing time-course cytotoxicity of RL71 on MDA-MB-468 cells](image)

**C. SKBr3**

![Graph showing time-course cytotoxicity of RL71 on SKBr3 cells](image)

**Figure 3.2.2: Time-course cytotoxicity of RL71 following the treatment of ERα negative breast cancer cells.** (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells were treated with either RL71 (1 μM) or DMSO (0.1%) for 6–72 h. Cell number was determined using the SRB assay. Each point represents the mean ± SEM of three independent experiments performed in triplicate. The data was analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. *significantly different from control (p<0.001). # indicates a statistically significant difference compared with all previous time points, p<0.01.
Figure 3.2.3: Effect on cell cycle progression following treatment of ERα negative breast cancer cells with RL71. (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells were treated with RL71 (1µM) for 6, 12, 24, 36 and 48 h. Vehicle control cells were treated with 0.1% DMSO. Propidium iodide staining and flow cytometry were used to determine the proportion of cells in the various cell cycle phases. Values are expressed as the mean proportion of cells in various phases of cell cycle (% of total) ± SEM of 3 independent experiments conducted in triplicate. Data was analyzed with a two-way ANOVA coupled with a Bonferroni post-hoc test. *significantly different from control \( p<0.001 \). # indicates a statistically significant difference compared with all previous time points, \( p<0.01 \).
3.2.2.3 **Induction of apoptosis**

To determine if cell cycle arrest drives apoptosis, time-dependent changes in apoptosis were examined in MDA-MB-231, MDA-MB-468 and SKBr3 cells. The cells were treated with 1 µM of RL71 for 12 to 48 h and at the end of the treatment the proportion of early apoptotic cells stained with Annexin V-FLUOS was reported using flow cytometry. The results showed that RL71 significantly increased the proportion of early apoptotic cells in all of the ERα negative breast cancer cell lines (Fig. 3.2.4). In MDA-MB-231 cells, RL71 (1 µM) produced 43 ± 3% and 47 ± 3% early apoptotic cells at 18 h and 24 h, compared to 4 ± 0.3% and 4 ± 0.2% in vehicle treated cells respectively. Similarly, RL71 induced apoptosis in MDA-MB-468 and SKBr3 cells. The effect in SKBr3 was time-dependent as 33 ± 3% of SKBr3 cells were apoptotic after 48 h compared to 7 ± 0.4% of vehicle treated cells. Also, the proportion of early apoptotic cells was significantly elevated at all other time points. In contrast, 14-18% of MDA-MB-468 cells underwent apoptosis and this effect was maintained from 12-48 h indicating the lack of a time-dependent effect. Specifically, 19 ± 2% of MDA-MB-468 cells were apoptotic after 18 h compared to 7 ± 1% of control cells. Also, it was observed that G2/M arrest did not drive apoptosis in MDA-MB-468 cells, as the apoptosis was increased at 12 h, which was prior to the increase in G2/M phase arrest. However, the early appearance of G2/M phase arrest at 12 h in SKBr3 cells is a likely reason why these cells show a strong apoptotic response over time. Additionally, amongst all cell types, the strongest apoptotic effect was observed in MDA-MB-231 cells from 18-36 h. Thus, the time-dependent increase in G2/M phase arrest is followed by the sustained apoptotic effect.
Figure 3.2.4: Apoptosis induction following treatment of ERα negative breast cancer cells with RL71. (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells were treated with RL71 (1 μM) for 12, 18, 24, 36 and 48 h. Vehicle control cells were treated with 0.1% DMSO. Values are expressed as mean % apoptotic cells ± SEM from three independent experiments conducted in triplicate. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. *significantly different from control (p<0.001). # indicates a statistically significant difference compared with all previous time points, p<0.01
3.2.2.4 Expression of key cell signaling proteins

As RL71 caused cell cycle arrest and induction of apoptosis in all the three ERα negative breast cancer cells, the molecular mechanism underlying this effect was further investigated. The changes in the expression of various key cell signaling proteins were assessed in a time course by Western blotting. The cells were treated with 1 µM concentration of RL71 for 0.5-36 h. After treatment, the cell lysates were prepared and the level of expression of various cell signaling proteins was determined.

The results showed that RL71 significantly decreased the expression of the EGFR in MDA-MB-231 cells but not in MDA-MB-468 cells. Treatment of MDA-MB-231 cells with 1 µM of RL71 for 36 h significantly decreased the EGFR expression by 51 ± 11% of control, (Fig. 3.2.5A). However, there was no change in the phosphorylation of EGFR in both the cell lines. Next, effect of RL71 on the expression of Akt was studied in MDA-MB-231 and MDA-MB-468 cells. RL71 significantly decreased the phosphorylation of Akt at Ser 473 in both the cell lines in a time-dependent manner. Treatment of MDA-MB-231 cells with 1 µM of RL71 for 6, 12 and 24 h significantly decreased the phosphorylation of Akt by 68 ± 4%, 83 ± 6% and 75 ± 8% of control, respectively (Fig. 3.2.5B) while in MDA-MB-468 cells, the phosphorylation of Akt was significantly decreased by 49 ± 10% of control after 36 h (Fig. 3.2.6B).

The effect of RL71 on various downstream targets of Akt was also investigated. 4E-BP1 is a downstream target of mTOR, which in turn is a downstream target of Akt. RL71 significantly decreased the phosphorylation of 4E-BP1 and mTOR in MDA-MB-231 cells and MDA-MB-468 cells respectively. Treatment of MDA-MB-231 cells with 1 µM of RL71 for 6 to 36 h decreased phosphorylation of 4E-BP1 from 53 ± 3% to 47 ± 2% of control (Fig. 3.2.5D). However, no significant change in the phosphorylation of mTOR was observed in MDA-MB-231 cells. In contrast, in MDA-MB-468 cells RL71 treatment after 36 h decreased phosphorylation of mTOR by 69 ± 10% of control (Fig. 3.2.6D). However, no significant change in the phosphorylation of 4E-BP1 was observed in MDA-MB-468 cells.

NFκB is another downstream target of Akt. In MDA-MB-231 cells, RL71 (1 µM) significantly decreased the expression of NFκB after 24 and 36 h by 52 ± 9% and 49 ± 11% of control, respectively (Fig. 3.2.5E). However, there was no significant change in NFκB expression in MDA-MB-468 cells.
Figure 3.2.5: Effect of RL71 on cell signaling proteins in MDA-MB-231 cells. Cells were seeded in 10 cm culture dishes at 2.5×10⁶ cells per well. The cells were treated with RL71 (1 μM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of EGFR (A), P-Akt/Akt (B), P-mTOR/mTOR (C), P-4E-BP1/4E-BP1 (D), NFκB (E), P-JNK/JNK (F), P-p38/p38 (G) and cleaved caspase-3 (H) was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control * p < 0.05.
The effect of RL71 treatment on stress kinases (p38 and JNK1/2) was also investigated. In both MDA-MB-231 and MDA-MB-468 cells, RL71 (1 µM) produced a time-dependent activation of p38 and JNK1/2. The treatment did not change the total expression of p38 and JNK and only the phosphorylation state of both the proteins was transiently increased. Specifically, 1 µM of RL71 treatment in MDA-MB-231 cells for 2, 3, 6 and 24 h increased the phosphorylation of p38 by 239 ± 64%, 256 ± 65%, 232 ± 47% and 216 ± 64% of control, respectively (Fig. 3.2.5G) whereas treatment of MDA-MB-468 cells for 6 and 12 h increased the phosphorylation by 10 and 11-fold of control, respectively (Fig. 3.2.7C). A similar effect was also observed in case of JNK1/2.

Figure 3.2.6: Effect of RL71 on Akt signaling in MDA-MB-468 cells. Cells were seeded in 10 cm culture dishes at \(2.5 \times 10^6\) cells per well. The cells were treated with RL71 (1 µM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of EGFR (A), P-Akt/Akt (B), NFκB (C), P-mTOR/mTOR (C), P-4E-BP1/4E-BP1 (D), was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control \(p<0.05\).
Figure 3.2.7: Effect of RL71 on stress kinases, p27 and cleaved caspase 3 in MDA-MB-468 cells. Cells were seeded in 10 cm culture dishes at 2.5×10^6 cells per well. The cells were treated with RL71 (1 μM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of p27 (A), P-JNK/JNK (B), P-p38/p38 (C) and cleaved caspase-3 (D) was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control p < 0.05.

1 μM of RL71 treatment in MDA-MB-231 and MDA-MB-468 cells for 12 and 24 h significantly increased the phosphorylation of JNK 1/2 by 7.6, 7.5 and 140, 244-fold compared to control, respectively (Fig. 3.2.5F, 3.2.7B).

In addition to MDA-MB-231 and MDA-MB-468 cells, the effect of RL71 on cell signaling was also examined in HER2 positive SKBr3 cells. Treatment of SKBr3 cells with 1 μM of RL71 significantly decreased the phosphorylation of HER2 in a time-dependent manner with an almost complete inhibition after 6 h (Fig. 3.2.8A). Furthermore, the effect of RL71 on the proteins related to the cell cycle and apoptosis was investigated. Treatment of MDA-MB-468 with 1 μM of RL71 for 12 and 24 h significantly increased the expression of p27 by 6 and 5-fold compared to control (Fig. 3.2.7A) whereas RL71 treatment of SKBr3 cells for 12, 24 and 36 h significantly...
increased the expression of p27 by 4, 6 and 4-fold compared to control (Fig. 3.2.8B). The changes in p27 were not studied in MDA-MB-231 cells, as it is expressed at very low levels in these cells.

Importantly, RL71 showed activation of caspase 3 in a time-dependent manner in all the three ERα negative breast cancer cells. The treatment of MDA-MB-231 and SKBr3 cells with 1 µM of RL71 for 24 and 36 h significantly increased the expression of cleaved caspase 3 by 10, 6 and 19, 25-fold compared to control, respectively (Fig. 3.2.5H, 3.2.8C). Whereas in MDA-MB-468 cells, the treatment with 1 µM of RL71 for 6, 12, 24 and 36 h significantly increased the expression of cleaved caspase 3 by 11, 12, 17 and 11-fold of control (Fig. 3.2.7D). Thus the strongest apoptotic effect was observed in MDA-MB-468 cells.

![Figure 3.2.8: Effect of RL71 on cell signaling proteins in SKBr3 cells.](image)

Cells were seeded in 10 cm culture dishes at 2.5×10⁶ cells per well. The cells were treated with RL71 (1 µM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of P-HER2/HER2 (A), p27 (B) and cleaved caspase-3 (C) was analysed. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control p<0.05.
3.2.2.5 **Anti-angiogenic potential of RL71 in vitro**

To determine if RL71 could modulate angiogenesis, *in vitro* assays using HUVEC cells were performed, as the ability of these cells to migrate through Matrigel and form tube-like networks are hallmarks of angiogenesis. Both quantifiable and visual assays were used to form a more complete *in vitro* picture.

3.2.2.5.1 **Effect of RL71 on endothelial tube formation**

To study the anti-angiogenic potential of RL71 *in vitro*, the endothelial tube formation assay was performed. The HUVEC cells were grown on the surface of Matrigel and were allowed to form a capillary-like tube network. The cells were then treated with 1 μM of RL71 for 18 h. As shown in (Fig. 3.2.8A), tube formation by HUVEC cells on Matrigel was completely inhibited by 1 μM of RL71.

![Figure 3.2.9: Anti-angiogenic effect of RL71 in HUVEC cells. (A) Effect of RL71 on endothelial tubes formation by HUVEC cells. Endothelial cells (50,000/well) were seeded and treated with DMSO (0.1%) or RL71 (1 μM) for 18 h. Photographs were taken after this time point to compare endothelial tube formation. **(B) Effect of RL71 on HUVEC cell invasion.** HUVEC cells (25,000) were seeded and treated with either DMSO (0.1 %) or RL71 (1 μM) for 18 h. Migrated cells were stained and counted manually. RL71 (1μM) significantly reduced the number of migrated cells compared to control. Bars represent mean ± SEM for three replicates per treatment group. **Significantly different compared to control p<0.01, using a Student t test.**](image)
3.2.2.5.2 Effect of RL71 on HUVEC cell invasion

Since invasion and migration of endothelial cells is crucial for angiogenesis in vivo (Nguyen et al., 2001), the effect RL71 on the endothelial cell invasion was performed using the Matrigel invasion assay. This assay reflects the invasive potential of cells in vivo. The results showed that RL71 (1 μM) significantly reduced cell invasion by 46% compared to control (Fig. 3.2.8B).

3.2.2.6 Migration potential of RL71 in MDA-MB-231 cells

Migration is a crucial step in metastasis. In order to determine the effect of RL71 on cell migration in vitro, the scratch assay was performed. MDA-MB-231 breast cancer cells (500,000/well) were plated in a 6 well plate and a scratch was made to the cell monolayer. The cells were treated with either DMSO (0.1%) or RL71 (1 μM) for 24 h. Photographs were taken at 0 and 24 h to compare cell migration. The results showed that the wound was completely closed in control cells after 24 h whereas RL71 inhibited the wound closure suggesting the ability of the compound to inhibit cell migration (Fig.3.2.9).

![Figure 3.2.10: Effect of RL71 on cell migration. MDA-MB-231 breast cancer cells (500,000 cells/ml) were plated and treated with either DMSO (0.1%) or RL71. Shown are representative photos of ‘scratches’ taken at the 0 h and 24 h time points for cells treated with DMSO (0.1%) or RL71.](image)
3.2.3 **Effect of RL71 in vivo**

3.2.3.1 **Oral bioavailability of RL71**

After studying the mechanism of anticancer activity of RL71 *in vitro*, the next aim of the study was to examine its oral bioavailability in mice before testing its effect *in vivo*. CD1 mice were orally administered 8.5 mg/kg of RL71 and the blood was collected after 5 min, 10 min, 15 min, 30 min, 1 h, 1.5 h and 2 h and the plasma samples were analyzed by HPLC. The results showed a peak plasma concentration of 0.405 µg/ml, 5 min after administration of the compound. The plasma concentration of RL71 further decreased in a time-dependent manner and the compound was under the limit of detection after 2 h (Fig. 3.2.10). Thus the study provided evidence that RL71 was orally bioavailable at a dose of 8.5 mg/kg.

![Figure 3.2.11: Oral bioavailability of RL71.](image)

**Figure 3.2.11: Oral bioavailability of RL71.** Female CD-1 mice (6 weeks old, n=3) were orally gavaged with RL71 (8.5 mg/kg). The blood was collected at different time intervals, plasma was separated and the concentration of RL71 was determined by HPLC analysis.

3.2.3.2 **Effect of RL71 in vivo in a mouse xenograft model of MDA-MB-468**

After it was confirmed that RL71 was orally bioavailable, its effect on ERα negative breast tumor growth *in vivo* was determined. Female athymic nude mice were implanted subcutaneously with MDA-MB-468 xenografts and the tumors were allowed to grow to a size of approximately 150 mm³ for two weeks. The animals were then...
randomized into treatment groups of 10 mice each and administered 8.5 or 0.85 mg/kg of RL71 or vehicle (water) daily by oral gavage for 10 weeks. Tumor volumes were measured weekly and animal weight was assessed daily. The study showed no significant difference in the tumor volumes of mice treated with 8.5 or 0.85 mg/kg of RL71 compared to vehicle treated mice after 10 weeks (Fig. 3.2.11). RL71 also did not alter body weight in both the treatment groups compared to vehicle treated group.

Figure 3.2.12: *In vivo* effect of RL71 in nude mice bearing MDA-MB-468 xenograft tumors. Female athymic nude mice were inoculated subcutaneously with MDA-MB-468 human breast cancer cells ($8 \times 10^6$ cells per 100 µl Matrigel). Following implantation, tumors were given time to grow to a size of 150 mm$^3$ for 2 weeks. The mice were assigned into three treatment groups. Animals were dosed orally with 8.5 mg/kg or 0.85 mg/kg of RL71 (n=10) or vehicle (water) (n=10) daily for 10 weeks. Tumors were measured weekly and weight changes were assessed daily. Data displayed are mean ± SEM.
3.3 Anticancer activity of RL66

3.3.1 Aim

As RL71 failed to suppress tumor growth in ERα negative breast cancer, the anticancer potential of RL66 (Fig. 3.3.1) was examined, as it was the second most potent compound obtained during initial screening. The aim of this work was to study the mechanism of the anticancer activity of RL66 in various *in vitro* and *in vivo* models of ERα negative breast cancer.

![Chemical structure of RL66](image)

**Figure 3.3.1**: Chemical structure of RL66

3.3.2 Effect of RL66 *in vitro*

3.3.2.1 *Time-course cytotoxicity studies*

To study the time-dependent cytotoxicity of RL66 on various ERα negative breast cancer cells, MDA-MB-231, MDA-MB-468 and SKBr3 cells were treated with RL66 for 6, 12, 24, 36, 48 and 72 h. The concentration that inhibited cell number by approximately 80% was selected for cytotoxicity time course studies. Accordingly, MDA-MB-231 and SKBr3 cells were treated with 2 µM and MDA-MB-468 cells were treated with 1.5 µM of RL66. The results showed that RL66 significantly decreased the cell number in all the three ERα negative breast cancer cells in a time, concentration and cell line-dependent manner. Specifically, treatment with 2 µM of RL66 elicited time-dependent cytotoxicity in MDA-MB-231 and SKBr3 cells from 24 to 48 h with no further decrease in cell number after 48 h (Fig. 3.3.2 A, C). In contrast, the treatment of MDA-MB-468 cells with 1.5 µM of RL66 showed no further cytotoxicity after 24 h (Fig. 3.3.2B). Thus RL66 showed potent cytotoxicity towards MDA-MB-231 and SKBr3 cells compared to cytostatic effect in MDA-MB-468 cells. Again 72 h following the treatment of MDA-MB-231 and SKBr3 cells with 2 µM of RL66, the cell number had significantly decreased by 82 ± 3% and 78 ± 2% of control, respectively, while treatment with 1.5 µM RL66 decreased MDA-MB-468 cell number by 71 ± 2% of control after 72 h.
Figure 3.3.2: Time-course cytotoxicity of RL66 following the treatment of ERα negative breast cancer cells. (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells were treated with either RL66 (1.5 or 2 μM) or DMSO (0.1%) for 6–72 h. Cell number was determined using the SRB assay. Each point represents the mean ± SEM of three independent experiments performed in triplicate. The data was analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. *significantly different from control (p<0.001). # indicates a statistically significant difference compared to all previous time points, p<0.01.
3.3.2.2 Cell cycle progression

To further examine whether the cytotoxicity of RL66 was due to cell cycle arrest, MDA-MB-231 and SKBr3 cells were treated with 2 µM of RL66 while MDA-MB-468 cells were treated with 1.5 µM of RL66 for 6 to 48 h. The results showed that RL66 produced G2/M phase arrest in MDA-MB-231 cells whereas MDA-MB-468 cells and SKBr3 cells showed a cell cycle arrest at the S/G2/M phase. Treatment of MDA-MB-231 cells for 24 h significantly increased the proportion of cells in G2/M phase by 34 ± 2% compared to control (Fig. 3.3.3 A) and there was a concomitant decrease in the proportion of G0/G1 phase by 16 ± 1% over control. The treatment of SKBr3 cells for 6 h significantly increased the proportion of cells in G2/M phase and S phase by 36 ± 9% and 23 ± 1% compared to control (Fig. 3.3.3 C) with a concomitant 11 ± 3% decrease in the proportion of G0/G1 phase cells. After 24 h this effect was reversed with a significant increase in the proportion of cells in G0/G1 phase by 10 ± 1% and a concomitant decrease in the proportion of cells in G2/M phase and S phase by 20 ± 4% and 41 ± 6%, respectively, compared to control. Similarly, treatment of MDA-MB-468 cells for 24 h significantly increased the proportion of cells in G2/M phase and S phase by 41 ± 7% and 22 ± 6% over control (Fig. 3.3.3 B) with a concomitant 15 ± 3% decrease in the proportion of G0/G1 phase cells. After 48 h this effect was reversed with a significant increase in the proportion of cells in G0/G1 phase by 11 ± 1% and a concomitant decrease in the proportion of cells in G2/M phase and S phase by 25 ± 5% and 9 ± 3%, respectively, compared to control.

3.3.2.3 Induction of apoptosis

To determine if cell cycle arrest drives apoptosis, time-dependent changes in apoptosis were examined in all three ERα negative breast cancer cells. MDA-MB-231 and SKBr3 cells were treated with 2 µM of RL66 while MDA-MB-468 cells were treated with 1.5 µM of RL66 for 12 to 36 h and at the end of the treatment the proportion of early apoptotic cells stained with Annexin V-FLUOS was reported using flow cytometry. The results showed that RL66 significantly increased the proportion of early apoptotic cells in all the ERα negative breast cancer cells compared to control (Fig. 3.3.4). Specifically in MDA-MB-231 cells, treatment of RL66 for 12 h produced 16 ± 0.8% early apoptotic cells compared to 5 ± 0.5% in vehicle treated cells (Fig. 3.3.4 A). Furthermore, there was a decrease in apoptosis from 18-36 h. Similarly, treatment of MDA-MB-468 cells with RL66 for 12 h significantly increased the proportion of apoptotic cells by 363% over
Figure 3.3.3: Effect on cell cycle progression following treatment of ERα negative breast cancer cells with RL66. (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells were treated with RL66 (1.5 or 2 µM) for 6, 12, 24, 36 and 48 h. Vehicle control cells were treated with 0.1% DMSO. Propidium iodide staining and flow cytometry was used to determine the proportion of cells in the various cell cycle phases. Values are expressed as the mean proportion of cells in various phases of cell cycle (% of total) ± SEM of 3 independent experiments conducted in triplicate. Data was analyzed with a two-way ANOVA coupled with a Bonferroni post-hoc test. *significantly different from control ($p<0.05$).
control (Fig. 3.3.4B). This effect was further decreased from 18-36 h. It was observed that the cell cycle arrest in MDA-MB-231 and MDA-MB-468 cells did not drive apoptosis as apoptosis was increased at 12 h, which was prior to the increase in S/G2/M phase arrest. In contrast, in SKBr3 cells, the early S/G2/M arrest at 6 h was followed by a strong apoptotic response over time. A time-dependent apoptotic effect was observed in SKBr3 cells with 41% of cells undergoing apoptosis compared to 5 ± 0.1% of vehicle treated cells at 36 h and this effect was significantly greater compared to all other time points (Fig. 3.3.4C). Thus it is evident that RL66 displayed a more potent cytotoxic effect in SKBr3 cells.

3.3.2.4 Effect of RL66 on the expression of cell signaling proteins

As RL66 caused cell cycle arrest and induction of apoptosis in all the three ERα negative breast cancer cells, the molecular mechanism underlying this effect was further investigated. The changes in the expression of various key cell signaling proteins were assessed in a time course by Western blotting. The cells were treated with 2 or 3 µM of RL66 for 0.5-36 h. After treatment, the cell lysates were prepared and the level of expression of various cell signaling proteins was determined.

The result showed that RL66 treatment did not alter the expression of EGFR in MDA-MB-231 and MDA-MB-468 cells. However, RL66 significantly decreased the phosphorylation of Akt at Ser 473 in both the cell lines in a time-dependent manner. Treatment of MDA-MB-231 cells with 2 µM of RL66 for 6, 12, 24 and 36 h significantly decreased the phosphorylation of Akt by 46 ± 12%, 64 ± 0.3%, 82 ± 3% and 84 ± 1% of control, respectively, (Fig. 3.3.5B) while in MDA-MB-468 cells, the phosphorylation of Akt was significantly decreased by 49 ± 7% of control after 36 h treatment with 3 µM of RL66 (Fig. 3.3.6B). Furthermore, the effect of RL66 on various downstream targets of Akt was investigated. RL66 significantly decreased the phosphorylation of both mTOR and 4E-BP1 in MDA-MB-231 cells whereas in MDA-MB-468 cells only mTOR expression was significantly changed. Treatment of MDA-MB-231 cells with 2 µM of RL66 significantly decreased phosphorylation of mTOR by 49 ± 7% and 92 ± 2% of control. (Fig. 3.3.5C) at 24 and 36 h, respectively, while the phosphorylation of 4E-BP1 was decreased by 65 ± 8%, 34 ± 2%, 75 ± 8% and 87 ± 4% of control (Fig. 3.3.5D) at 6, 12, 24, and 36 h, respectively. In contrast, the treatment of MDA-MB-468 cells with 3 µM of RL66 did not change the phosphorylation of 4E- BP1 but phosphorylation of mTOR was
Figure 3.3.4: Apoptosis induction following treatment of ERα negative breast cancer cells with RL66. (A) MDA-MB-231, (B) MDA-MB-468 and (C) SKBr3 cells were treated with RL66 (1.5 or 2 μM) for 12, 18, 24 and 36 h. Vehicle control cells were treated with 0.1% DMSO. Values are expressed as mean % apoptotic cells ± SEM from three independent experiments conducted in triplicate. Data were analyzed using a two-way ANOVA coupled with a Bonferroni post-hoc test. * significantly different from control (p<0.001). # indicates a statistically significant difference compared with all previous time points, p<0.01
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Figure 3.3.5: Effect of RL66 on cell signaling proteins in MDA-MB-231 cells. Cells were seeded in 10 cm culture dishes at $2.5 \times 10^5$ cells per well. The cells were treated with RL66 (2 μM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of EGFR (A), P-Akt/Akt (B), P-mTOR/mTOR (C), P-4EBP1/4E-BP1 (D), NFκB (E) P-JNK/JNK (F), P-p38/p38 (G) and cleaved caspase-3 (H) was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. *Significantly different from control, p<0.05.
Figure 3.3.6: Effect of RL66 on Akt signaling in MDA-MB-468 cells. Cells were seeded in 10 cm culture dishes at 2.5×10⁶ cells per well. The cells were treated with RL66 (3 μM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of EGFR (A), P-Akt/Akt (B), P-mTOR/mTOR (C), P-4E-BP1/4E-BP1 (D) and NFκB (E) was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control, p<0.05.

significantly decreased by 81 ± 7% of control (Fig. 3.3.6C) at 36 h.

The effect of RL66 on NFκB was also studied. RL66 (2 μM) significantly decreased the expression of NFκB in MDA-MB-231 cells after 12, 24 and 36 h by 62 ± 13%, 60 ± 11% and 58 ± 10% of control, respectively (Fig. 3.3.5E). However, the expression of NFκB was not changed in MDA-MB-468 cells.
Figure 3.3.7: Effect of RL66 on stress kinases, p27 and cleaved caspase-3 in MDA-MB-468 cells. Cells were seeded in 10 cm culture dishes at $2.5 \times 10^6$ cells per well. The cells were treated with RL66 (3 µM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of P-JNK/JNK (A), P-p38/p38 (B), p27 (C) and cleaved caspase-3 (D) was examined. β-actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control, $p < 0.05$.

The effect of RL66 on stress kinases (p38 and JNK1/2 MAPK) was also investigated. In both MDA-MB-231 and MDA-MB-468 cells, RL66 produced a concentration and time-dependent activation of p38 and JNK1/2 MAPK. The treatment did not change the total expression of p38 and JNK and only the phosphorylation state of both the proteins was significantly increased. Specifically, treatment of MDA-MB-231 cells for 1, 2, 3 and 6 h increased the phosphorylation of p38 by $745 \pm 278\%$, $761 \pm 286\%$, $715 \pm 269\%$ and $800 \pm 241\%$, respectively, (Fig. 3.3.5G) whereas treatment of MDA-MB-468 cells with 3 µM of RL66 for 1 and 3 h increased the phosphorylation of p38 by $940 \pm 99\%$ and $650 \pm 88\%$, respectively (Fig. 3.3.7B). A similar effect was also observed in the case of JNK1/2. Treatment of MDA-MB-231 cells for 1 and 2 h increased the phosphorylation of JNK1/2 by $325 \pm 28\%$ and $268 \pm 7\%$ of control, respectively, (Fig. 3.3.5F) whereas 3 µM
of RL66 treatment of MDA-MB-468 cells for 30 min and 1 h increased the phosphorylation of JNK1/2 by 40-fold and 236-fold compared to control, respectively, (Fig. 3.3.7A).

In addition to TNBCs, the effect of RL66 on cell signaling was also examined in HER2 positive SKBr3 cells. The results showed that treatment of SKBr3 cells with 2 μM of RL66 significantly decreased the ratio of P-HER2/HER2 in a time-dependent manner.

**Figure 3.3.8: Effect of RL66 on HER2 signaling proteins in SKBr3 cells.** Cells were seeded in 10 cm culture dishes at 2.5×10^6 cells per well. The cells were treated with RL66 (2 μM) or control (0.1% DMSO) for indicated time. At the end of treatment, whole cell lysates were prepared and protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of HER2/p-HER2 (A), EGFR (B), P-Akt/Akt (C), P-mTOR/mTOR (D), P-4E-BP1/4E-BP1 (E) and NFκB (F) was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. *Significantly different from control p<0.05.
with an almost complete inhibition after 1 h (Fig. 3.3.8A). RL66 treatment also decreased EGFR expression in a time-dependent manner. The treatment of SKBr3 cells with 2 μM of RL66 for 24 and 36 h significantly decreased the expression of EGFR by 83 ± 6% and 85 ± 4% of control, respectively (Fig. 3.3.8B). However, the phosphorylation of EGFR was not changed. A significant time-dependent decrease in Akt, mTOR, 4E-BP1 and NFκB levels was also observed in SKBr3 cells. The phosphorylation of Akt was almost completely inhibited after 1 h (Fig. 3.3.8C) leading to almost complete inhibition of phosphorylation of mTOR and 4E-BP1 after 6 h (Fig. 3.3.8D and E).

Figure 3.3.9: Effect of RL66 on stress kinases, p27 and cleaved caspase-3 expression in SKBr3 cells. Cells were seeded in 10 cm culture dishes at 2.5×10⁶ cells per well. The cells were treated with RL66 (2 μM) or control (0.1% DMSO) for the indicated time. At the end of treatment, whole cell lysates were prepared and the protein concentration of the lysates was determined. Cell lysates were separated by SDS-PAGE and the expression of P-JNK/JNK (A), P-p38/p38 (B), p27 (C) and cleaved caspase-3 (D) was examined. β actin was used as a loading control. Results are shown as mean ± SEM of three independent experiments. Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. * Significantly different from control p<0.05.
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The expression of NFκB was significantly inhibited by $49 \pm 3\%$ and $37 \pm 6\%$ of control, at 24 and 36 h, respectively (Fig. 3.3.8F). Moreover, a transient increase in the stress kinases (p38 and JNK1/2) was observed in a time-dependent manner. 2 µM of RL66 treatment of SKBr3 cells for 30 min, 1, 2, 3 and 6 h increased the phosphorylation of p38 by 114, 159, 133, 89, and 60-fold (Fig. 3.3.9B) whereas phosphorylation of JNK was increased by 374, 626, 548 and 342-fold compared to control, following 30 min, 1, 2 and 3 h, respectively (Fig. 3.3.9A).

The effect of RL66 on the proteins related to the cell cycle and apoptosis was also investigated. Treatment of MDA-MB-468 cells with 3 µM of RL66 for 12 h significantly increased the expression of p27 by $205 \pm 10\%$ of control (Fig. 3.3.7C) whereas RL66 (2 µM) treatment of SKBr3 cells for 12 and 36 h significantly increased the expression of p27 by $248 \pm 40\%$ and $231 \pm 52\%$ of control (Fig. 3.3.9C). Importantly, RL66 showed activation of cleaved caspase-3 in a time-dependent manner in all the three ERα negative breast cancer cell lines. The treatment of MDA-MB-231 cells with 2 µM of RL66 and MDA-MB-468 cells with 3 µM of RL66 for 12 and 24 h significantly increased the expression of cleaved caspase-3 by 3.5 and 3.7-fold (Fig. 3.3.5H) as well as 18 and 9-fold compared to control (Fig. 3.3.7D), respectively. In SKBr3 cells, the treatment with 2 µM of RL66 for 12, 24 and 36 h significantly increased the expression of cleaved caspase 3 by 258, 455 and 475-fold compared to control (Fig. 3.3.9D).
3.3.2.5 Anti-angiogenic potential of RL66 in vitro

3.3.2.5.1 Effect of RL66 on endothelial tube formation

To study the anti-angiogenic potential of RL66 in vitro, the endothelial tube formation assay was performed. The HUVEC cells were grown on the surface of Matrigel and were allowed to form a capillary-like tube network. Later, the cells were treated with 1 μM of RL66 for 18 h. As shown in (Fig. 3.3.10 A), tube formation by HUVECs on Matrigel was completely inhibited by 1 μM of RL66.

3.3.2.5.2 Effect of RL66 on HUVEC cell invasion

To study the effect RL66 on the endothelial cell invasion, the Matrigel invasion assay was performed. The results showed that RL66 (1 μM) significantly reduced cell invasion by 48% compared to control (Fig. 3.3.10 B).

Figure 3.3.10: Anti-angiogenic effect of RL66 in HUVEC cells. (A) Effect of RL66 on endothelial tube formation by HUVEC cells. Endothelial cells (50,000/well) were seeded and treated with DMSO (0.1%) or RL66 (1 μM) for 18 h. Photographs were taken after this time point to compare endothelial tube formation. (B) Effect of RL66 on HUVEC cell invasion. HUVEC cells (25,000) were seeded and treated with either DMSO (0.1 %) or RL66 (1 μM) for 18 h. Migrated cells were stained and counted. RL66 (1μM) significantly reduced the number of migrated cells compared to control. Bars represent mean ± SEM for three replicates per treatment group. * Significantly different compared to control p<0.01, using a Student t test.
3.3.2.6 Migration potential of RL66 in MDA-MB-231 cells

In order to determine the effect of RL66 on cell migration, the scratch assay was performed. MDA-MB-231 breast cancer cells (500,000/well) were plated in a 6 well plate and a scratch was made to the cell monolayer. The cells were treated with either DMSO (0.1%) or RL66 (2 µM) for 24 h. Photographs were taken at 0 and 24 h to compare cell migration. The results showed that the wound was completely closed in controls after 24 h whereas RL66 inhibited the wound closure suggesting the ability of the compound to inhibit cell migration (Fig. 3.3.11).

**Figure 3.3.11: Effect of RL66 on cell migration.** MDA-MB-231 breast cancer cells (500,000 cells/ml) were plated and treated with either DMSO (0.1%) or RL66 (2 µM). Shown are representative photos of ‘scratches’ taken at the 0 h and 24 h time points for cells treated with DMSO (0.1%) or RL66.
3.3.3  **Effect of RL66 in vivo**

3.3.3.1  **Oral bioavailability of RL66**

To determine the oral bioavailability of RL66, CD1 mice were orally administered with 8.5 mg/kg of RL66 and the blood samples were withdrawn after 5 min, 10 min, 15 min, 30 min, 1 h, 1.5 h and 2 h and the samples were analyzed by HPLC. RL66 was orally bioavailable at a dose of 8.5 mg/kg and showed a peak plasma concentration of 0.056 µg/ml after 10 min. However, at all further time points the plasma concentration of RL66 was under the limit of detection (Fig. 3.3.12).

![Oral bioavailability of RL66](image)

**Figure 3.3.12: Oral bioavailability of RL66.** Female CD-1 mice (6 weeks old, 3/group) were orally gavaged with RL66 (8.5 mg/kg). The blood was collected at different time intervals, plasma was separated and the compound concentration was determined by HPLC analysis.

3.3.3.2  **Effect of RL66 on tumor growth in MDA-MB-468 mouse xenograft model.**

After the confirmation that RL66 was orally bioavailable, the compound was further studied in vivo. To study the anti-breast cancer effect of RL66 in vivo, a mouse xenograft model was created by subcutaneously injecting (8 x 10^6) MDA-MB-468 cells in female athymic nude mice. When tumors reached a size of ~150 mm^3, mice were randomized into the various treatment groups of 10 mice each. Mice were then gavaged orally with 8.5 or 0.85 mg/kg of RL66 or vehicle (water) daily for 10 weeks. The results showed that there was a 48% reduction in the average tumor volume in mice treated with 8.5 mg/kg dose compared to control (Fig. 3.3.14 A). However, the 0.85 mg/kg dose did
not show any reduction in tumor volume. Moreover, the treatment with 8.5 mg/kg of RL66 reduced the mean tumor weight by 49% compared to control (Fig. 3.3.14 B). However, this was not significantly different compared with the control.

Figure 3.3.13: *In vivo* effect of RL66 in nude mice bearing MDA-MB-468 xenograft tumors. MDA-MB-468 breast cancer cells (8x10^6 cells per 100 µl Matrigel) were subcutaneously injected into the mice and tumors were allowed to grow for 2 weeks (~ 150 mm^3). The mice were assigned into three treatment groups. Animals were dosed daily orally with 8.5 mg/kg (n=9) or 0.85 mg/kg (n=9) of RL66 or vehicle (water) (n=5) for 10 weeks. (A) Tumor volume was measured weekly and (B) tumor weight changes were assessed daily and average tumor volume and weights were recorded at the end of the study. Bars represent mean ± SEM from tumors (n ≥ 5). Data were analyzed using (A) a two-way repeated measures ANOVA with Bonferroni *post-hoc* test or (B) a one-way ANOVA with Bonferroni *post-hoc* test. *Significantly different from control p<0.05.
3.3.3.3 Toxicity profile of RL66

In order to assess the toxicity profile of RL66, animal weight was recorded and the weight of major organs (liver, kidney, spleen and uterus) as a percentage of body weight was evaluated at necropsy (Table 3.3.1). No significant change in the body weight of mice treated with either group of RL66 was observed throughout the 10 weeks. Moreover, the study showed no significant change in the mean weights of liver, kidney, spleen, or uterus. In addition, plasma ALT levels were recorded at the end of the study as a measure of liver toxicity. All the mice had plasma ALT value, in the normal range and below the 200 IU/L threshold that represents liver damage (Goldring et al., 2004).

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>RL66 (8.5 mg/kg)</th>
<th>RL66 (0.85 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean weight (g)</td>
<td>27.2 ± 1.4</td>
<td>26.9 ± 1.1</td>
<td>27.2 ± 0.4</td>
</tr>
<tr>
<td>Mean Plasma ALT (IU/L)</td>
<td>23.4 ± 11.5</td>
<td>85.0 ± 21.3</td>
<td>69.7 ± 10.3</td>
</tr>
<tr>
<td>Liver weight (% of body weight)</td>
<td>5.7 ± 0.2</td>
<td>5.5 ± 0.1</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>Kidney weight (% of body weight)</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.03</td>
<td>1.4 ± 0.04</td>
</tr>
<tr>
<td>Spleen weight (% of body weight)</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>Uterus weight (% of body weight)</td>
<td>0.35 ± 0.04</td>
<td>0.29 ± 0.02</td>
<td>0.25 ± 0.03</td>
</tr>
</tbody>
</table>

Table 3.3.1: Effect of RL66 treatment on weight and plasma ALT levels in mice. Female athymic nude mice bearing MDA-MB-468 xenografts were treated daily orally with 8.5 or 0.85 mg/kg of RL66 or vehicle (water) for 10 weeks. Data displayed are mean ± SEM. Data were analyzed using a one-way ANOVA with Bonferroni post-hoc test.
3.3.3.4  Effect of RL66 treatment on various cell signaling proteins.

As RL66 showed moderate tumor suppression in a mouse xenograft model and a limited number of tumor samples were available for analysis at the end of the study, a comprehensive mechanism of tumor suppression of RL66 could not be determined. However, to investigate the trend of the mechanism of the anticancer effect of RL66 in vivo, tumor protein extracts were prepared and the expression of key cell signaling proteins were examined by Western blotting. The proteins previously analyzed in vitro were then examined for their changes in vivo following 0.85 or 8.5 mg/kg of RL66.

Figure 3.3.14: Effect of RL66 treatment on EGFR, Akt, p-Akt, mTOR and P-mTOR in MDA-MB-468 mouse xenograft tumors. Tumor lysates were extracted from the mice treated with RL66 (0.85 or 8.5 mg/kg) or vehicle (water). The expression of (A) EGFR, (B) P-Akt/Akt and (C) P-mTOR/mTOR was examined using Western blotting and β actin was used as a loading control. Bars represent the mean optical density ± SEM from tumor extracts (n ≥ 5). Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. *Significantly different from control p<0.05.
Figure 3.3.15: Effect of RL66 treatment on NFκB and p27 in MDA-MB-468 mouse xenograft tumors. Tumor lysates were extracted from the mice treated with RL66 (0.85 or 8.5 mg/kg) or vehicle (water). The expression of (A) NFκB and (B) p27 was examined using Western blotting and β actin was used as a loading control. Bars represent the mean optical density ± SEM from tumor extracts (n ≥ 5). Data were analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test. *Significantly different from control p<0.05

The results showed that EGFR expression was decreased by 13% after RL66 treatment (8.5 mg/kg) compared to vehicle-treated mice (Fig. 3.3.14A). Moreover, the downstream signaling proteins of EGFR were also investigated. Tumors of mice treated with 8.5 mg/kg showed increased pAkt/Akt expression by 520% compared to vehicle treated mice (Fig. 3.3.14B). Moreover, the expression of NFκB, a downstream target of Akt was increased by 244% (Fig. 3.3.15A). However, the expression of mTOR, which is another downstream target of Akt, was down regulated by 41% after RL66 (8.5 mg/kg) treatment compared to vehicle treated mice (Fig. 3.3.14C). In addition, the expression of p27kip1 was increased by 145% in the tumors from mice treated with 8.5 mg/kg compared to vehicle control (Fig. 3.3.15B). However, none of these changes were statistically significant.
3.3.3.5 Effect of RL66 treatment on microvessel density (MVD)

The potential mechanism of tumor suppression of RL66 was further studied by using immunohistochemistry to examine the changes in microvessel density in the tumors from treated mice. The results showed a significant reduction in CD105 positive tumor blood vessels to 59% of control in mice treated with 8.5 mg/kg of RL66 (Fig. 3.3.16A).

**Figure 3.3.16:** Effect of RL66 treatment on microvessel density of MDA-MB-468 mouse xenograft tumors. (A) Tumors treated with RL66 (0.85 or 8.5 mg/kg) or vehicle (water) were sectioned and stained for CD105 by immunohistochemistry. Results are shown as the number of CD105 positive cells ± SEM from (n=5 tumors). Data was analyzed using a one-way ANOVA coupled with a Bonferroni post-hoc test (B) Representative photos obtained from tumors stained with CD105. *Significantly different from control p<0.05.
CHAPTER 4: DISCUSSION & CONCLUSION

4.1 EVALUATION OF STUDY DESIGN

The aim of this study was to find synthetic compounds that had potential as new treatments for ERα negative breast cancer. The study involved the screening of a variety of heterocyclic curcumin analogs for their cytotoxicity towards ERα negative breast cancer cells. The two most potent curcumin analogs obtained during screening were further examined for their anticancer potential by using various in vitro and in vivo models of ERα negative breast cancer. In order to correctly interpret the results obtained during this study, a discussion of the aptness and relevance of various methods and models is required. In addition, the advantages and drawbacks of these methods will be discussed in the context of the literature.

4.1.1 Cell lines as experimental tools

For development of breast cancer therapies, in vitro testing of human breast cancer cell lines is an early step that involves assessment of cytotoxicity, proliferation and apoptosis. Cell line-based testing has several advantages. They are simple to handle and inexpensive. They provide an unlimited self-replicating source of cancer cells that grow in infinite quantities and which closely resemble the genotype and phenotype of the human tumor cells from which they are derived (Burdall et al., 2003). This type of resemblance is particularly reported in breast cancer cells (Gazdar et al., 1998; Wistuba et al., 1998). Another advantage is their relatively high degree of homogeneity which avoids inter-species differences that exist in animal models of cancer. However, current cell-based studies have shortcomings in terms of reliability and variability. A number of factors affect the drug response in cell lines which may in turn affect its predicted in vivo response. Some of these factors include incubation time, cell cycle time, rate of drug uptake, efflux and metabolism (Baguley et al., 2002). In addition, differences in various cell culture media and media constituents (e.g. serum, growth factors, pH, and oxygen) can differentially alter drug concentration and stability thereby affecting cell proliferation, differentiation, migration and death by modulating intracellular signal transduction (Finlay et al., 1986).

There is a discrepancy between the anticancer activity of drugs in a cell based environment in vitro and in human patients. This is mainly due to the differences
observed between cell lines and tumor-derived cells from patients. Cell lines consist of rapidly dividing identical cells whereas tumor derived cells are generally heterogeneous and grow slowly. In addition, cell lines may lose or modify their characteristics due to prolonged sub-culturing and changes in the environment (Bhadriraju et al., 2002). Consequently, the sensitivity of cancer cell lines towards anticancer agents is changed and the cell-based assays can give different results than in vivo responses. Therefore, in order to improve the predictability of the in vitro efficacy, an appropriate microenvironment that closely resembles the in vivo cues must be selected. Accordingly, use of the three dimensional cell culture systems which mimic the cell-cell interaction and pathophysiological situation in human tumor tissues is warranted to predict the clinical response to drugs (Kunz-Schughart et al., 2004). However, three dimensional models show variability in creating an in vivo tissue environment and are devoid of vasculature and normal transport of small molecules, host immune responses, and other cell-cell interactions (Yamada et al., 2007).

Despite all the pitfalls mentioned above, cancer cell lines have been an indispensable tool for anticancer drug screening due to their ease of use and availability. In particular, the translational potential of breast cancer cell lines in predicting the response to anticancer chemotherapy has been extensively explored (Burdall et al., 2003). For the current study MDA-MB-231, MDA-MB-468 and SKBr3 breast cancer cells were used. These cells have different origins and properties and are considered to be heterogeneous. Each cell line represents a particular subtype of breast cancer and is therefore relevant for the development of an anticancer therapy for that specific subtype. For example, MDA-MB-231 and MDA-MB-468 cells are used for development of breast cancer therapies against triple negative breast cancer while SKBr3 cells are used for ERα negative and HER2 positive breast cancer therapies. The MDA-MB-231 cell line was derived from pleural effusion of a Caucasian patient diagnosed with breast adenocarcinoma in 1973 at the M. D. Anderson Cancer Centre. Morphologically, they are mesenchymal-like cells with a spindle shape phenotype (Jiratchariyakul et al., 2011) MDA-MB-231 cells express the mesenchymal marker vimentin and possess the epithelial to mesenchymal transition (EMT) phenotype that offers high motility, proliferation, invasiveness and elevated resistance to apoptosis (Liu et al., 2010b; Sommers et al., 1992). Moreover, they can form xenograft tumors in immune compromised mice in vivo. The MDA-MB-468 cell line was derived from a pleural effusion of an African American female patient diagnosed with metastatic breast adenocarcinoma. These cells are weakly
invasive in vitro. However, they are tumorigenic and form xenograft tumors in immune compromised mice. Both the MDA-MB-231 and MDA-MB-468 cells are negative for ER, PR and HER2 and overexpress EGFR (deFazio et al., 1997). In addition, they express the basal-like gene profile and have mutant p53 and BRCA1 genes (Harkes et al., 2003). All these molecular features make them a suitable in vitro breast cancer model. In addition, MDA-MB-468 cells show a mutation in PTEN that leads to a very high expression of Akt (Marty et al., 2008). The SKBr3 cell line was derived from the pleural effusion of a Caucasian patient diagnosed with invasive ductal carcinoma. It is an adherent cell line with luminal-like morphology (Jiratchariyakul et al., 2011). Ultra structures of cells show microvilli and desmosomes, glycogen granules, large lysosomes and bundles of cytoplasmic fibrils (Jiratchariyakul et al., 2011). In nude mice these cells form poorly differentiated adenocarcinoma. SKBr3 cells show absence of ER and PR and overexpression of HER2 and the leptin receptor (Lacroix et al., 2004).

**Cytotoxicity assay**

Cytotoxic activity against cancer cell lines is routinely investigated by using several methods which are based on alterations of plasma membrane permeability, radioisotope incorporation and colorimetric detection. The membrane permeability based dye exclusion assay includes counting cells under microscope or in an automated cell counter with or without staining with dye such as trypan blue (Jiratchariyakul et al., 2011). The dye does not cross an intact plasma membrane and thus only labels dead cells. However, this assay does not give a correct interpretation of dead cells as some cytotoxic agents cause cytotoxicity by intracellular damage keeping the plasma membrane intact. Also, it is a time consuming and laborious method. Therefore, a number of other indirect methods that involve quantification of live or dead cells have been established. These methods are based on the fact that live cells modulate certain proteins or even nucleic acids that can indicate cytotoxicity (Haslam et al., 2000). The radioactive methods such as measurement of \(^{51}\)Cr-labeled proteins or \(^{3}\)H-thymidine incorporation have also been employed (Wagner et al., 1999). Though these methods are very specific and efficient, they suffer from some limitations such as having lengthy sample preparation procedures, inherent danger and high cost (Jiratchariyakul et al., 2011). Also, they are methods of cell proliferation not cytotoxicity. Therefore, other non-radioactive methods such as colorimetric methods are preferred over radioactive methods. The colorimetric assays use tetrazolium dyes such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-
2H-tetrazolium (XTT), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, (WST-1) or protein-binding dyes such as SRB (Nedel et al., 2011). The modern colorimetric assay using a microtitre plate is a well established way of determining cytotoxicity. It allows rapid and simultaneous analysis of a large number of compounds by using dyes that either stain cells directly or are reduced via cellular metabolic activity (Weyermann et al., 2005). However, colorimetric assays require multiple washing steps which can be practically difficult for suspension cultures or poorly adherent cell cultures. Moreover, they can not differentiate between cytotoxic and anti-proliferative effects (Kepp et al., 2011). Despite of this, colorimetric assays have been widely accepted for quantitative estimation of cytotoxicity within 24-96 h of cell culture.

For the current studies, the cytotoxicity of the curcumin analogs was examined by the SRB assay. The assay is based on the ability of a negatively charged bright-pink aminoxanthene dye SRB, to bind to the basic amino acids of the cells under mild acidic conditions (Vichai et al., 2006). SRB has two sulfonic groups which bind with the basic amino acids. The amount of dye taken up by cells after fixing is directly proportional to the cell mass (Houghton et al., 2007). Though other dyes such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) are popular for detection of cell viability, MTT has some disadvantages such as poor linearity with cell number, sensitivity to the environmental conditions and its ability to get reduced to an insoluble formazan by cell metabolism which may produce false positive results (Funk et al., 2007; Marques-Gallego et al., 2010). The SRB assay offers several advantages over the MTT assay. For example, the SRB assay is sensitive, simple, quick and reproducible. It gives better linearity and a good signal to noise ratio (Houghton et al., 2007). Also, the SRB assay provides a colorimetric end point which is stable and visible to the naked eye (Skehan et al., 1990). Importantly, the SRB staining is not affected by any interference of the test compounds, is independent of cell metabolic activity and requires few steps to optimize the assay conditions (Vichai et al., 2006). Importantly, SRB is the assay of choice by the National Cancer Institute. Therefore, the cytotoxicity of various curcumin analogs was determined by using the SRB assay.

4.1.2 Use of flow cytometry

The flow cytometry technique has been widely used to study cell cycle distribution and apoptotic changes in cells. It is based on the principle of sorting a cell population
based on the size and granularity and structural complexity by using a beam of light (Muirhead et al., 1985). As the cells intercept the light, they scatter the light which is detected by a detector to produce a spectrum. It has several advantages. It is a quick, efficient, and cost effective method to get accurate, reliable and reproducible results. It collects the data for every single cell and thus gives an idea regarding the heterogeneity of the cell population. It is very sensitive and gives quantitative results. It allows the study of various cell parameters such as size, protein content, DNA content, lipid content, enzyme activity, antigenic properties and so on and thus gives multidimensional representation of cells (Davey, 2002).

The identification of cell cycle distribution is commonly based on the measurement of relative cellular DNA content (Nunez, 2001). When cellular DNA is stained with fluorescent dye, the fluorescence can then be measured by flow cytometry (Darzynkiewicz et al., 2001). The intensity of fluorescence is directly proportional to the DNA content and thus can be used to determine the cell cycle stage (Darzynkiewicz et al., 2001). Propidium iodide, which is a nucleic acid dye, is commonly used for DNA staining (Nunez, 2001). However, some precautions need to be taken in order to avoid false interpretation during analysis. For example, ethanol is used to fix and permeabilize cells so that they can be accessible to propidium iodide. The fixation is done in order to store or transport the samples before analysis (Darzynkiewicz et al., 2001). In addition, RNase A is added to increase the specificity of DNA staining and to avoid the uptake of PI by double stranded sections of RNA which can interfere with the staining (Darzynkiewicz et al., 2001). Overall, use of flow cytometry and PI is a well-established and reliable method for detection of the various phases of cell cycle progression.

Apoptosis can also be quantified using Annexin V/PI staining and flow cytometry. Among various events, the exposure of phosphatidylserine (PS) on the outer leaflet of plasma membrane is one of the most widely used markers for detection of apoptotic cells (Brumatti et al., 2008). Annexin V is a Ca\(^{2+}\) dependent anticoagulant protein with a high affinity to PS and hence it is applicable for detection of apoptotic cells by flow cytometry (Pozarowski et al., 2003). The externalization of PS also occurs in other forms of cell death such as necrosis which is characterized by the complete loss of membrane integrity. In contrast, the cell membrane remains intact during the early events of apoptosis (Vermes et al., 1995). Therefore by staining the cells with the combination of Annexin V and PI, it is possible to differentiate between apoptosis and necrosis (Pozarowski et al., 2003). The Annexin V assay has many advantages over other fluorocytometric assays.
For example, it is rapid and does not require any fixation. In addition, it is specific for the detection of an early event in the executioner phase of apoptosis (Galluzzi et al., 2009).

### 4.1.3 In vitro anti-angiogenic assays

Tumor metastasis is a complex process that includes cell adhesion, proteolytic degradation of the extracellular membrane, cell migration to basement membranes to reach the circulatory system, and remigration and growth of the tumor at the metastatic site (Chambers et al., 2002). In the present study, cell migration was studied in vitro by using the wound healing or scratch assay. MDA-MB-231 cells were preferred for this assay as they have high motility and invasive power as mentioned before. The assay is based on making a scratch on the confluent cell monolayer with an object such as a pipette tip and taking photographs at the beginning and at regular intervals during which the cells migrate to completely recover the monolayer and close the scratch. The images are compared to determine the cell migration.

This assay offers various advantages. It is simple, economic and quick to perform. It allows the study of the effect of cell-matrix and cell-cell interaction on cell migration as upon making the scratch on the confluent cell monolayer, the cells on the edge of the newly created gap migrate toward the other end to close the scratch until new cell–cell contacts are established. It mimics the cell migration in vivo and is suitable for imaging of live cells during cell migration. Moreover, this method can be modified and used for studying intracellular signaling events during cell migration by transfecting the gene of interest and using fluorescent microscopy. Moreover, the migration path of an individual cell can be tracked by using the time-lapse microscopy and image analysis software (Liang et al., 2007).

However, the scratch assay suffers from certain disadvantages such as the variability in scratch size between the experiments which in turn affects reproducibility and consistency. In addition, the cancer cells in real metastatic situations migrate in response to MMPs, integrins, cytokines and growth factors from the extracellular matrix (Bozzuto et al., 2010), which is unlikely to be mimicked in a wounded cell monolayer. Thus the assay cannot examine the chemo attractant effects and is non-quantitative.

To overcome the problems with manual scratch making, alternative sophisticated methods such as laser photoablation and electrical wounding have been developed to remove cells in a way that controls shape, size and position of the scratch and thus offers
consistent and reproducible results (Keese et al., 2004; Tamada et al., 2007). However, these commercially available methods are expensive and still give some confounding results arising from cell injury at the border. The current scratch assay still remains the preferred method to study cell migration in vitro as it is the simplest and feasible method to perform.

The in vitro anti-angiogenic potential was assessed by using the endothelial tube formation assay and Transwell migration assay in HUVEC cells. Both are models of neovascularization and are widely used to study the response of endothelial cells to angiogenic inhibitors, as they represent multiple steps during angiogenesis (Garrido et al., 1995). The Transwell migration apparatus offers a chemo-gradient and the Matrigel contains various components such as laminin, collagen type IV, heparin sulfate, proteoglycan, entactin and growth factors that mimic physiological conditions. Moreover, the membrane is of suitable pore size to fit different cell types.

The main disadvantage of this method is that the commercial kit is very expensive. Also, the chemotactic gradient is non-linear with no control on the rate of migration of cells. Moreover, only 15-20% of cells migrate in 18-24 h. In order to avoid this problem an alternative assay was developed which uses transepithelial electrical resistance to indicate the invasiveness of the cancer cells (Mandic et al., 2004). Another disadvantage is that the endothelial cells cannot be visualized and photographed during the experiment. Care must be taken during preparation of the cell suspension for the migration assay as under-trypsinization can cause cell clumping while over-trypsinization can damage certain adhesion molecules required for migration (Eccles et al., 2005). An uneven distribution and staining of cells can lead to non-consistent results. The use of automated imaging and software can solve these problems. However, automated software sometimes cannot distinguish between pores in the filters and cells and therefore can give false results. Overall, Transwell migration assay is still popular and widely used because it is easy, reliable, and flexible and can be applied to any cell type to study invasion ability.

4.1.4 Western blotting

Western blotting is the most commonly utilized and well established technique for the analysis of changes in the protein expression after anticancer drug treatment in both in vitro cell culture and in vivo tumor samples. It is a multistep process that involves separation of proteins by gel electrophoresis, transfer of proteins on PVDF or nitrocellulose membrane, blocking to avoid non-specific binding and detection by the
addition of primary antibody. This is followed by the addition of labeled secondary antibody that amplifies the signal. It has many advantages such as being an easy, reliable and reproducible method allowing detection of multiple proteins from a single blot. The non-specific detection of proteins is overcome by using a standard molecular weight marker that provides an accurate detection of the proteins of interest. However, it has some drawbacks such as it is time consuming and can lead to variability due to the involvement of multiple steps. Moreover, it is not feasible for high throughput analysis. A large amount of sample is required for analysis and the results obtained are qualitative or semi-quantitative. Considering these limitations other applications such as ProteinChip proteomics has been developed. ProteinChip technology incorporates surface-enhanced laser desorption/ionization with mass spectrometry to allow the rapid profiling of protein expression in a given sample (Fung, 2001). The protein sample of interest is loaded on an appropriate ProteinChip array followed by a short incubation time and washing of excess proteins or contaminants. Subsequently, a solution of energy-absorbing molecules is applied and the samples are analyzed in a ProteinChip reader (Wiesner, 2004). The greatest advantage of ProteinChip proteomics is that it eliminates the interference of salts and detergents and gives an enhanced signal (Seibert et al., 2004). It has a low sample requirement. It is highly sensitive and can detect proteins with low concentration including the detection of post translational modifications (Seibert et al., 2004). It allows quantification of proteins in a short time and can be used for high throughput screening. It has a wide application in cancer diagnosis, screening and prognosis (Wiesner, 2004). However, it still needs to be improvised for sensitivity and specificity of detection of proteins. Overall, Western blotting is still a widely used method for detection of expression of proteins in lab research due to its reliability and ease of use.

4.1.5 Use of mouse xenograft model for in vivo studies

Though in vitro cell culture systems enable the study of efficacy and mechanism of anticancer agents, they do not give any information regarding the complex relationship between the tumor and its microenvironment, such as local blood supply and angiogenesis, interactions between tumor cells and the organ where the tumor resides, and the influence of hormones, growth factors and cytokines on tumor growth and survival. Therefore, mouse xenograft models have been widely used for the comprehensive investigation of anticancer activity in vivo. Mouse xenograft models involve transplantation of the human cancer cells into immunocompromised mice either subcutaneously or injected into the organ type in which the tumor originated (orthotopic
tumor model). The xenografts are readily accepted by athymic nude mice, severely compromised immunodeficient (SCID) mice, or other immunocompromised mice as immunocompromised mice do not reject human tumor cells. According to the number of cells injected, the desired tumor size is achieved over a certain time (from 1-10 weeks) and the anticancer activity of the appropriate therapeutic regimen can be studied in vivo. The mouse xenograft model offers several advantages such as being easy to perform and needing a shorter time to evaluate the response of a therapeutic regime. Tumors are localized externally so they can be measured easily by a caliper. As one can actually transplant human tumor cells which feature the complexity of genetic and epigenetic abnormalities of human tumors, the study provides realistic heterogeneity of human tumors. Also, it allows the study of different types of therapeutic responses such as the effect on tumor progression, regression and survival.

However, xenograft studies using human cancer cells often do not correlate with the clinical activity in patients (Kerbel, 2003). Also, subcutaneously injected tumor cells are not representative of a primary tumor site and they rarely have metastatic disease. Another thing is that xenograft models using athymic nude or SCID mice lack the lymphocyte mediated immune response to the tumor. Moreover, xenografts contain different stromal environment than human tumors, resulting in a chimeric tumor that has unpredictable growth, differentiation or metastatic properties (Hahn et al., 2002).

Some of these problems can be resolved by using alternative models. For example, the orthotopic tumor model offers rapid growth of local tumors and also their metastasis. For development of an orthotopic human breast cancer model, the human breast cancer cells are implanted into mammary fat pad (Hovey et al., 1999). However, the orthotopic models have limited application due to the requirement of high levels of technical skills.

Another model, the ‘humanized mouse model’ enables a direct inoculation of the human stem cells or lymphocytes into immunodeficient mice and thus bridges the gap between preclinical and clinical research (Chang et al., 2006; Legrand et al., 2006). For this model, SCID mice are preferred over nude mice which have a poorly developed reproductive and hormonal system. The humanized mouse model mimics the human tumor microenvironment and also offers complete reconstitution of immune responses to the tumor whereas in nude/SCID mice xenografts there is lack of immune response against the tumor cells (Richmond et al., 2008).
The use of genetically engineered mouse model (GEM) can also be another alternative. The GEM model is widely used for studying human cancer initiation, progression and metastasis. It is produced by the alteration of certain genes that are involved in malignancy by mutation or deletion or overexpression (Richmond et al., 2008). GEM models have several advantages, 1) they create a similar tumor microenvironment in mice as seen in human tumors and grow in the presence of an intact immune system and are thus considered to be superior to xenograft models, 2) they allow study of a specific type of cancer by altering specific genes, 3) the various stages of tumor progression can be studied over the time thus allowing development of several therapeutic approaches at various stages of tumor progression, and 4) genetic models are also useful in humanized mice, where human genes, such as the cytochrome P450 genes or human tumor antigens, are inserted in mice to study drug metabolism or immunological responses to the tumor (Talmadge et al., 2007). However their role in drug discovery has been uncertain so far. The disadvantages of GEM are that it is expensive, time consuming, has intellectual property limitations and species-specific differences. Also, the heterogeneity of the human tumor cannot be reliably mimicked and therefore it is hard to predict the therapeutic response.

In spite of all its limitations, the xenograft model has been routinely used to successfully predict clinical response to anticancer therapy. For example, Herceptin was shown to increase the anticancer potential of paclitaxel and doxorubicin against HER2 overexpressing breast cancer (Baselga et al., 1998). This was further successfully tested in clinical trials and was subsequently approved by the FDA (Sporn et al., 1999). VEGFR2 targeting blocking antibodies in combination with paclitaxel was shown to be effective in inhibiting tumor growth and inhibiting metastatic spread in an orthotopic xenograft model (Davis et al., 2004). This study was followed by development of bevacizumab, a humanized monoclonal antibody that targets VEGF-A as an anti-angiogenic therapy. Bevacizumab was effective in Phase III clinical trials for colorectal and renal carcinoma and received FDA approval in 2004 (Hurwitz et al., 2004; Yang et al., 2003). Moreover, mouse xenograft models are useful for anticipating ADME and toxicity in response to anticancer therapies. Thus, for many types of human tumors, the information gained from mouse xenograft studies using human tumors has led to the translational development into successful clinical trials. Importantly, it is also recommended by drug regulatory agencies for new drug approval (Liu et al., 2011).
For the current study, the MDA-MB-468 subcutaneous mouse xenograft model was used. This model is frequently used to investigate new drugs therapies for breast cancer. It readily forms tumors in immunocompromised mice allowing them to grow at a reasonable rate for short or long term studies (Zhang et al., 1991). Also, it was found to be better than our previously used MDA-MB-231 invasive breast cancer model which can have a low success or uptake rate (Mehta et al., 1993). The tumor volumes were measured by using an electronic caliper which is the standard noninvasive method for accurate measurement of subcutaneous tumors in mice. The possible human error associated with this measurement was reduced by two independent measurements for each tumor. The tumor measurement by caliper is often considered to be subjective and it is affected by certain factors such as variability in tumor shape, skin thickness and subcutaneous fat layer thickness. Therefore for more accurate, reproducible and reliable measurement of tumors, modern methods such as computed tomography (CT) and positron emission tomography (PET) are widely used clinically (Weber et al., 2006). Preclinically, imaging with ultrasonography (Cheung et al., 2005), magnetic resonance imaging (Mazurchuk et al., 1997), microCT and microPET (Jensen et al., 2008) have been developed. However, these types of instruments are expensive and therefore manual measurement of tumor volume using caliper remains the most preferred method.

4.1.6 Immunohistochemistry

This technique is widely used both clinically and preclinically for assessment of receptor status of breast tumors, as well as for detection of markers of cell proliferation, apoptosis, angiogenesis and metastasis. Immunohistochemistry has a number of advantages such as use of routine microscopic techniques, relatively low cost, and better conservation of slides for further analysis. However, a number of technical variables may affect sensitivity or specificity towards various markers. They include storage, selectivity and quality of the antibodies employed, incubation times and tissue fixation, incubation/washing steps, as well as the characteristics of the positive and negative controls used (Press et al., 1994). Despite these concerns, immunohistochemistry has been a routine technique for detection of tumor markers in breast cancer.

4.1.6.1 CD105 marker for detection of micro vessel density of tumors

The present study mainly involved the application of immunohistochemistry for detection of the angiogenesis marker CD105 in tumors. Clinically, the extent of angiogenesis is measured in terms of microvessel density which is in turn assessed by
using various markers such as factor VIII, CD31, CD34, CD146, and CD105 (Duff et al., 2003). Amongst all of these CD105, also known as endoglin, is more specific for malignant angiogenesis (Dallas et al., 2008; Kumar et al., 1999). CD105 is a member of TGF-receptor family and is strongly expressed by the tumor endothelial cells (Duff et al., 2003). Also, enhanced levels of CD105 is well correlated with micro vessel density and tumor metastasis and has been found to be an independent prognostic marker (Duff et al., 2003). In breast cancer patients, CD105 expression in tumor endothelial cells was correlated with overall survival and disease free survival (Kumar et al., 1999). Vo et al. (2010) reported that elevated CD105 levels in plasma of metastatic breast cancer patients predicted a decreased clinical benefit and shorter overall survival. Recently, Gluz et al. (2011) reported that among the various subtypes of breast cancer, higher CD105 expression was associated with aggressive basal-like and HER2 subtype which leads to increased angiogenesis and chemoresistance ultimately resulting in decreased event free survival and overall survival. This suggests the aptness of CD105 as a target for anti-angiogenic therapies.

Various molecular imaging techniques have been developed for accurate detection of CD105. They include molecular magnetic resonance imaging (Zhang et al., 2009), ultrasound (Korpanty et al., 2007), single photon emission computed tomography (Costello et al., 2004), near-infrared fluorescence (Yang et al., 2011) and positron emission tomography (Hong et al., 2011). In addition, a radioimmunotherapy application involves use of a 177Lu-labeled anti-CD105 antibody (Lee et al., 2009). All of these techniques are based on conjugating various imaging or therapeutic labels (e.g., radioisotopes such as 111In/99mTc/125I/177Lu/64Cu, Gd-diethylenetriaminepentaacetic acid liposomes, or microbubbles) to anti-CD105 monoclonal antibodies (e.g., MAEND3, E9, J7/18, and TRC105). Although these techniques are accurate, sensitive and reliable they are expensive and need proper handling skills.
4.2 INTERPRETATION OF RESULTS

4.2.1 Cytotoxicity study

The cytotoxicity study demonstrated that 14 curcumin analogs were more cytotoxic towards MDA-MB-231 cells than curcumin. Amongst these, four analogs (RL71, RL66, RL9, and RL53) showed potent cytotoxicity (IC$_{50}$ ~ 1 µM) compared to curcumin. These potent analogs were further tested in other ERα negative breast cancer cells, namely MDA-MB-468 and SKBr3. All four compounds were found to be more potent than curcumin towards these cell lines, as all elicited IC$_{50}$ values that were 2 to 30-fold lower than that for curcumin.

Overall, RL71 and RL66 were superior to our previous analogs and the ones reported by other researchers. In MDA-MB-231 breast cancer cells, RL71 and RL66 showed more potent cytotoxicity (IC$_{50}$ values of 0.3 and 0.8 µM, respectively) as compared to RL90, RL91 and BMHPC (IC$_{50}$ values of 1.5, 1.1 and 2.6 µM, respectively) (Somers-Edgar et al., 2011). In SKBr3 cells, both RL71 and RL66 showed similar potency compared to RL90 and RL91. However, RL90 and RL91 were not tested in MDA-MB-468 cells (Somers-Edgar et al., 2011). RL71 and RL66 also showed more potent cytotoxicity compared to other cyclohexanone curcumin analogs in ERα negative breast cancer cells. Both the compounds had superior cytotoxicity compared with EF24 (Adams et al., 2004), PAC (Al-Hujaily et al., 2011) and GO-Y030 (Hutzen et al., 2009) in MDA-MB-231 cells. The corresponding IC$_{50}$ values reported with EF24 and GO-Y030 were 1.2 and 1 µM, respectively (Adams et al., 2004; Hutzen et al., 2009). The curcumin analogs FLLL11 and FLLL12 were as potent as RL71 and RL66 in MDA-MB-468 cells with similar IC$_{50}$ values (Lin L et al., 2009). However, this effect did not translate to other breast cancer cell types as these analogs had IC$_{50}$ of 2-5 µM in MDA-MB-231 and SkBr3 cells. Thus, the current study reports that RL71 and RL66 showed the most potent cytotoxicity towards MDA-MB-231, MDA-MB-468, and SkBr3 cell lines.

4.2.2 Mechanism of anticancer activity of RL71 and RL66 in vitro

4.2.2.1 Effect on cell cycle progression

Cell cycle studies revealed that both RL71 and RL66 produced cell cycle arrest in a cell line, time, and concentration-dependent manner. RL71 (1 µM) produced G2/M-phase cell cycle arrest in all the three ERα negative breast cancer cells whereas RL66 (1.5 or 2 µM) showed a cell cycle arrest in G2/M phase in MDA-MB-231 cells and in S/G2/M
phase in MDA-MB-468 and SKBr3 cells. These findings were similar to our previous studies that showed a G2/M phase cell cycle arrest produced by RL90 and RL91 in MDA-MB-231 and SKBr3 cells (Somers-Edgar et al., 2011). In MDA-MB-231 cells, RL90 (3 μM for 30 h) or RL91 (2.5 μM for 24 h) significantly increased the proportion of cells in G2/M phase by 152% and 149% over control, respectively. A similar but earlier G2/M phase arrest was also observed in SKBr3 cells following treatment with 2 μM of RL90 or RL91. Specifically, after 18 h RL90 and RL91 increased the proportion of cells in the G2/M phase cells by 139% and 125% above control, respectively. Thus, RL71 was more potent than RL90 and RL91 at producing a cell cycle arrest in MDA-MB-231 and SKBr3 cells as the concentration of RL71 required was 2 to 3-times lower, whereas RL66 was slightly more potent than RL90 and RL91 in MDA-MB-231 cells. In SKBr3 cells, RL66 produced a similar effect as that of RL90 and RL91. However, RL66 produced cell cycle arrest earlier at 6 and 12 h. Both RL90 and RL91 were not studied in MDA-MB-468 cells for their effect on cell cycle progression.

Curcumin is also reported to induce a G2/M arrest in MDA-MB-231 and SKBr3 cells whereas in MDA-MB-468 cells it causes a cell cycle arrest in S/G2/M phase. Treatment of MDA-MB-231 cells with 20 μM of curcumin increased the proportion of cells in G2/M phase by 164% at 24 h (Chiu et al., 2009) while treatment of SKBr3 cells with 135 μM of curcumin for 48 h caused an increase in the S and G2/M phase by 132% and 417%, respectively (Lai et al., 2012). In MDA-MB-468 cells, 20 μM curcumin increased the proportion of S/G2/M phase by 143% at 48 h (Squires et al., 2003). Thus, both RL71 and RL66 were 10 to 20-times more potent than curcumin at producing a cell cycle arrest in all the three ERα negative breast cancer cells.

A similar effect was also shown by a synthetic curcumin analog BMHPC that caused a significant increase in the G2/M and S phase in PC-3 and LNCaP prostate cancer cells (Markaverich et al., 1998). Moreover, other curcumin analogs, EF24 and PAC, showed the G2/M arrest in MDA-MB-231 cells. However, both the compounds showed this effect at 10 μM concentration which is 5-10 times higher than the concentration of RL71 and RL66. Thus, these studies showed that RL71 and RL66 were more potent than curcumin and other curcumin analogs in inducing cell cycle arrest.

4.2.2.2 Effect on apoptosis induction

Similar to cell cycle studies, the apoptosis studies showed that the treatment of MDA-MB-231, MDA-MB-468 and SKBr3 cells with RL71 and RL66 significantly
increased the proportion of Annexin V positive early apoptotic cells in a cell line, time, and concentration-dependent manner. In particular, in MDA-MB-231 cells, RL71 (1 µM) caused 43% of cells to be apoptotic after 18 h. This effect was more potent at eliciting apoptosis than our previously studied cyclohexanone curcumin derivatives RL90 and RL91 which at concentration of 2–3 µM caused less than 20% of MDA-MB-231 cells to undergo apoptosis following 18 h of treatment (Somers-Edgar et al., 2011). However, RL91 is a stronger inducer of apoptosis than RL66, as RL91 exhibited a peak apoptotic induction of ~40% of cells after 36 h, compared to both RL90 and RL66 which showed less than 20% of cells undergoing apoptosis at this time-point. Moreover, only RL71 was more potent at eliciting apoptosis than curcumin which required a 20 to 40-fold higher concentration to produce similar effect in breast cancer cells (Fang et al., 2011; Squires et al., 2003). Similarly, superior activity was also found compared to other synthetic curcumin analogs. For example, PAC caused 55% of cells to undergo apoptosis at 16 h in MDA-MB-231 cells at a concentration 10-time higher than the concentration of RL71 (Al-Hujaily et al., 2011). Additionally, 25 µM of the analog 4- hydroxy-3-methoxybenzoic acid methyl ester (HM-BME) was required to cause 37% of LNCaP prostate cancer cells to undergo apoptosis after 24 h (Kumar et al., 2003). Another curcumin analog, EF-24 (20 µM) increased the percentage of early apoptotic cells to 25% after 72 h in MDA-MB-231 (Adams et al., 2005). Similarly, in SKBr3 cells, both RL71 and RL66 produced ~ 40% apoptosis after 36 h. This effect was found to be similar to RL90 and RL91. Thus, only RL71 was more potent at eliciting apoptosis than curcumin, and other previously studied cyclohexanone curcumin derivatives.

4.2.2.3 Effect of curcumin analogs on cell signaling in vitro

The effect of RL71 and RL66 on various cell signaling proteins involved in cell proliferation and death was studied in MDA-MB-231, MDA-MB-468 and SKBr3 cells by Western blotting. The results showed that RL71 and RL66 mainly modulated the stress response MAPK pathway, the HER2 signaling pathway and the Akt-dependent pathway.

4.2.2.3.1 Effect on stress kinase pathway

Various cytotoxic agents induce apoptotic cell death via activation of stress kinase signaling (Kuo et al., 2007; Liu et al., 2010a; Wada et al., 2004). Activation of p38 and JNK MAPK is associated with DNA fragmentation and caspase activation induced by anticancer agents (Deschesnes et al., 2001; Liu et al., 2010a). Their role in apoptosis has been demonstrated in various studies by using specific pharmacological inhibitors or
siRNA. For example, treatment of MDA-MB-231 cells with 2.5 µM of benzyl isothiocyanate significantly increased the phosphorylation of JNK and p38 resulting in ROS and Bax-dependent apoptosis (Xiao et al., 2008). The role of stress kinases in apoptosis induction was further demonstrated by the use of pharmacological inhibitors of JNK and p38, namely SP600125 and SB202190. The treatment of MDA-MB-231 cells with 20 µM of SP600125 and SB202190 for 18 h caused a 3-fold decrease in apoptosis in both treatments (Xiao et al., 2008). Similarly, isoobtusilactone (IOA) at a concentration of 4 µM caused sustained activation of p38 and JNK after 1 h in MDA-MB-231 cells. Only JNK activation resulted in induction of apoptosis. However, treatment with both p38 and JNK siRNA significantly decreased apoptosis induction in MDA-MB-231 cells (Kuo et al., 2007).

A study conducted by Park et al. (2010) demonstrated that treatment of MDA-MB-231 cells with 40 µM of γ-tocotrienol (γ-T3) caused increased phosphorylation of p38 and JNK from 12 to 16 h resulting in increased apoptosis. Specifically, at 16 h the phosphorylation of p38 and JNK was increased by 3-fold and 6-fold respectively, compared to control. Moreover, siRNA knockdown of p38 and JNK and further treatment with 30 µM of γ-T3 resulted in partial decrease of apoptosis in MDA-MB-231 cells (Park et al., 2010).

Recently, Park et al. (2011) demonstrated that treatment of MDA-MB-231 cells with 10 µM of 2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone (RH1) resulted in increased phosphorylation of p38 and JNK from 0.5 h to 24 h. Further, pharmacological inhibition of JNK using SP600125 (10 µM) and treatment with 10 µM RH1 for 36 h, resulted in a 2.5-fold decrease in apoptosis in MDA-MB-231 cells. Similar results were also obtained when JNK expression was inhibited using siRNA. However, inhibition of p38 had no effect on apoptosis (Park et al., 2011). Another study conducted by Liu B et al. (2010) demonstrated that treatment of MDA-MB-231 with 20 µM of the acetylbritannilactone (ABL) derivative 5-(5-(ethylperoxy)pentan-2-yl)-6-methyl-3-methylene-2-oxo-2,3,3a,4,7,7a-hexahydrobenzofuran-4-yl 2-(6-methoxynaphthalen-2-yl) propanoate (ABL-N) for 0.5 to 24 h caused a time-dependent increase in phosphorylation of p38 and JNK. Furthermore, co-treatment of MDA-MB-231 cells with JNK inhibitor, SP600125 (30 µM) and 20 µM of ABL-N decreased the cell viability whereas p38 inhibitor, SB203580 had no effect. Also, JNK siRNA (25 nM) blocked ABL-N induced loss of cell viability. Moreover, both SP600125 (30 µM) and JNK siRNA (25 nM) caused reduction in caspase-3 activity by 2.5-fold each compared to ABL-N-induced apoptosis in MDA-MB-231 cells (Liu et al., 2010a).
The apoptotic events were also reported in other ERα negative breast cancer cells. McLean (2008) reported that in MDA-MB-468 cells, the aryl hydrocarbon receptor (AhR) agonists induced cytotoxicity via activation of stress kinases. The treatment of MDA-MB-468 cells with 1 µM each of aminoflavone (AF) or [(5-amino-2,3-fluorophenyl)-6 (5F 203)] for 6 and 12 h caused a significant increase in the activation of p38 and JNK resulting in increased generation of ROS. Furthermore, combined treatment of MDA-MB-468 cells with AF or 5F 203 and MAPK inhibitors SP600125 or SB202129 for 6 h significantly reduced ROS generation induced by the drug treatment (McLean, 2008). Thus, these studies suggest that stress kinases play an important role in inducing apoptosis in TNBC cells.

In SKBr3 cells, ligand-induced apoptosis was found to be dependent on activation of stress kinases. Tikhomirov et al. (2004) demonstrated that in EGFR transfected SKBr3 cells (SKBr3/EGFR), the EGF-induced apoptosis was dependent on p38 MAPK. The treatment of SKBr3/EGFR cells with p38 specific inhibitors SB203580 or SB202190 (10 µM) completely prevented cell death induced by EGF (100 ng/ml) after 8 days. Similarly, heregulin or Neu differentiation factor (NDF)-induced apoptosis in SKBr3 cells was significantly inhibited by SB203580. The treatment of SKBr3 cells NDF (1 nM) for 5 h and subsequent addition of SB203580 (4 µM) after 90 min, resulted in a 3-fold decrease in apoptosis compared to NDF treatment alone suggesting that p38 MAPK is required for NDF-induced apoptosis (Daly et al., 1999).

Curcumin was reported to induce apoptotic cell death via activation of p38, JNK1/2 and caspase-3 (Collett et al., 2004; Weir et al., 2007). The treatment of HCT116 colon cancer cells with 35 µM of curcumin caused sustained activation of JNK and p38 from 4 to 24 h. Moreover, JNK activation led to 2.7-fold increase in AP-1 transcriptional activity. The co-treatment of curcumin (35 µM) with specific JNK inhibitor SP600125 (30 µM) or p38 inhibitor SB205380 (10 µM) for 24 h caused inhibition of curcumin-induced activation of JNK and p38. JNK inhibition resulted in a significant decrease in cell viability along with a 40% decrease in PARP cleavage. However, p38 inhibition had no effect on cell viability (Collett et al., 2004).

Thus, similar to all these studies, the present study showed that RL71 and RL66 induced phosphorylation of JNK and p38 MAPK in MDA-MB-231, MDA-MB-468 and SKBr3 cells in a time- and concentration-dependent manner. These results were consistent with our previously reported curcumin analogs RL90 and RL91 which showed
sustained activation of both p38 and JNK in a time-dependent manner as described in Somers-Edgar et al., 2011. Similarly, the curcumin analog compound 19 [(1E,4E)-1,5-bis(2,3-dimethoxyphenyl) penta-1,4-dien-3-one], at a concentration of 20 µM, induced c-Jun phosphorylation in non-small cell lung cancer H420 cells at 12 h. Moreover, compound 19 (20 µM) caused caspase-3 activation after 24 h (Wang et al., 2011). So far no other curcumin analogs have been reported to induce apoptosis via stress kinase activation. Overall, RL71 and RL66 were more potent than curcumin and other curcumin analogs at producing activation of stress kinase. Thus, the present study suggests that the apoptosis and cytotoxicity induced by RL71 and RL66 could be occurring via activation of stress kinases. Use of specific inhibitors of p38 and JNK will further elucidate the role of stress kinases in RL71 and RL66-induced apoptotic cell death.

The activation of stress kinases has been reported to regulate the activity of other signaling pathways such as Akt (Ho et al., 2009; Levresse et al., 2000; Park et al., 2002a). In MCF-7 breast cancer cells and PC-3 prostate cancer cells, activation of JNK repressed the activity of Akt (Ho et al., 2009). The stress kinase-induced apoptotic effects of cisplatin in A2780S ovarian cancer cells were reported to occur via Akt2 activation (Yuan et al., 2003). Moreover, in our previous studies with RL90 and RL91 the increased activation of p38 and JNK was correlated with decreased phosphorylation of Akt. In the current study, the activation of p38 and JNK was observed prior to the decreased phosphorylation of Akt in all the three ERα negative breast cancer cells. Though both the events did not coincide, the activation of stress kinases at early time-points could be responsible for the decreased activity of Akt at later time-points.

**Role of stress kinases in regulating cell cycle**

The stress kinases (JNK and p38) have also been shown to induce cytotoxicity via regulation of the cell cycle (Santen et al., 2002). In particular, the role of the stress kinases in inducing a cell cycle arrest in breast cancer cells has been elucidated. For example, IOA at a concentration of 4 µM caused sustained activation of p38 after 1 h in MDA-MB-231 cells resulting in G2/M cell cycle arrest. The inhibition of p38 by using siRNA caused inhibition of IOA-induced G2-M arrest. However, JNK activation had no effect on the cell cycle (Kuo et al., 2007). The treatment of MCF-7 and MDA-MB-231 breast cancer cells with 10 µM of asiatic acid for 1, 3, 6 and 12 h resulted in sustained activation of p38 but not JNK (Hsu et al., 2005). Furthermore, inhibition of p38 using SB203580 resulted in a decrease in the asiatic acid-induced S/G2/M cell cycle arrest. The
co-treatment of MDA-MB-231 and MCF-7 cells with 10 μM of asiatic acid and 20 μM SB203580 for 12 h showed 12% and 12% of cells in S phase and 33% and 20% of cells in G2/M phase, respectively. In contrast, treatment with asiatic acid alone showed 14% and 14% of cells in S phase and 44% and 38% of cells in G2/M phase, respectively. Moreover, the same treatment also inhibited Cdc25C degradation by increasing phospho-Cdc25 in both MDA-MB-231 and MCF-7 cells. Consequently, apoptosis was delayed until 48 h suggesting that the S/G2/M arrested breast cancer cells were sensitive to apoptosis induced by asiatic acid treatment (Hsu et al., 2005). Similarly, in the present study, the activation of both p38 and JNK MAPK was observed prior or during the cell cycle arrest in MDA-MB-231, MDA-MB-468 cells and SKBr3 cells. Thus our studies suggest that the cell cycle inhibitory effects of RL71 and RL66 could be occurring via activation of stress kinases.

The cell cycle regulatory effects of stress kinases are in turn reported to occur via various cell cycles regulatory proteins such as p27 Kip1. It has been demonstrated that the MAPK pathway may up-regulate p27Kip1 and thus induce cell cycle arrest and apoptosis (Eto, 2006). Also, some studies have demonstrated that p27Kip1 is an important regulator of cell cycle progression through the G2/M phase and of the induction of apoptosis (Hsieh et al., 2006; Hsu et al., 2011). Treatment of MCF-7 cells with 10 μg/ml of gallic acid for 24 h caused a 1.7-fold increase in the expression of p27Kip1 compared to control. This effect was responsible for G2/M arrest in MCF-7 cells. To further study the role of p27Kip1, the expression of p27Kip1 was knocked down by siRNA. The authors demonstrated that gallic acid (10 μg/ml) increased the proportion of MCF-7 cells in G2/M phase to 23% which was significantly decreased to 15% after treatment with siRNA of p27Kip1. Similarly, in MDA-MB-231 cells, physalis angulata induced G2/M arrest by significantly increasing the expression of p27Kip1 by approximately 75% at concentrations of 150, 300 and 450 μg/ml for 24 h (Hsieh et al., 2006). In hepatoma HepG2 cells, methanolic extract of mulberry leaves induced G2/M arrest via an increased expression of p27Kip1. The expression of p27Kip1 was increased by 12% and 36% at 13 and 33 μg/ml respectively, compared to control (Naowaratwattana et al., 2010). Similarly, curcumin induced G2/M arrest in a p27-dependent manner in immortalized human umbilical vein endothelial (ECV304) cells (Park et al., 2002b), melanoma cells (Zheng et al., 2004), Bcr-Abl-expressing chronic myeloid leukaemia cells (Wolanin et al., 2006) and HCT-116 colon cancer cells (Giri et al., 2009).
Our results demonstrated that in MDA-MB-468 and SKBr3 cells, RL71 and RL66 treatment enhanced the expression of p27Kip1 which might explain the G2/M cell cycle arrest. Overall, the cell cycle arrest induced by RL71 and RL66 could be occurring via activation of stress kinases and/or activation of p27Kip1. Use of specific inhibitors of p27, p38 and JNK will further elucidate the role of stress kinases and p27kip1 in RL71 and RL66-induced cell cycle arrest.

4.2.2.3.2 Effect on PI3K/Akt/mTOR pathway

The effect of RL71 and RL66 was further studied on the PI3K/Akt/mTOR pathway. Akt is constitutively active in breast cancer cells and has been implicated in a myriad of regulatory mechanisms involving protein synthesis, cell cycle progression and inhibition of apoptosis (Dillon et al., 2007; Vivanco et al., 2002). The role of Akt in inducing apoptosis has been demonstrated by using its specific inhibitors in breast cancer cells. For example, Weng et al. (2008) reported that inhibition of Akt using a specific PDK-1 inhibitor, OSU-03012 resulted in the increased sensitization of MDA-MB-231 cells to tamoxifen. Treatment of MDA-MB-231 cells for 72 h showed 3-fold decrease in IC$_{50}$ value for OSU-03012 (IC$_{50}$ 4 µM) compared to tamoxifen (IC$_{50}$ 13 µM). Moreover, treatment of MDA-MB-231 cells with 5 µM of OSU-03012 for 24 h resulted in 6-fold increase in apoptosis induction compared to control (Weng et al., 2008). It was also demonstrated that HER2 positive breast cancer cells are sensitive to the inhibition of Akt. In BT474 cells, 1 µM of Akt1/2 inhibitor (AKTi-1/2) completely inhibited the phosphorylation of Akt at Ser473 and Thr308 from 1 h to 24 h (She et al., 2008). Moreover, the treatment caused down-regulation of the downstream targets of Akt namely, Foxo1, GSK3α, p70S6k, S6 and 4E-BP1 and cyclin D1 and induction of p27 and PARP cleavage. In addition, 1 µM of AKTi-1/2 caused G1 arrest with concomitant loss of S phase. This was seen by an increase in the proportion of G1 phase from 69% in control to 92% in AKTi1/2 treated cells with concomitant decrease in S phase from 25% to 5% at 24 h. Further studies in BT474 mice xenografts showed complete reduction in the tumor volumes in the mice receiving AKTi1/2 (100 mg/kg) for 4-6 weeks (She et al., 2008).

Inhibition of Akt via use of an inhibitor of its upstream target PI3K, LY294002 also led to a decrease in breast cancer progression. In MDA-MB-231 cells, 2, 5 and 10 µg/ml of LY294002 significantly reduced cell number by 32%, 59% and 66% respectively, compared to control at 72 h (Weng et al., 2008). In MDA-MB-468 cells, LY294002 did
not produce apoptosis on its own but it potentiated the apoptotic effects of another anticancer agent, cerulenin. Co-treatment of MDA-MB-468 cells with 20 µM of LY294002 and 2.5, 5 or 10 µg/ml cerulenin for 24 h induced 20%, 36% and 33% apoptosis (Liu et al., 2006b). Thus overall, these studies suggest that Akt is an important target for ERα negative breast cancer therapy.

The present results showed that RL71 and RL66 decreased the phosphorylation of Akt on Ser473 in a cell line- and time-dependent manner. In MDA-MB-468 cells, RL71 decreased Akt phosphorylation fully whereas in MDA-MB-231 cells the phosphorylation of Akt was partially decreased. The partial decrease observed in phosphorylation of Akt by RL71 in MDA-MB-231 cells was similar to that previously reported (Serra et al., 2008). They demonstrated that treatment of SKBr3, BT474 and MDA-MB-468 cells with a dual PI3K/mTOR inhibitor (NVP-BEZ235) at concentrations of 100 and 500 nM for up to 48 h led to an initial decrease in the phosphorylation of Akt which was recovered after 24 to 48 h (Serra et al., 2008). Moreover, NVP-BEZ235 completely down regulated the phosphorylation of 4E-BP1, which was also observed in the case of RL71.

RL71 and RL66 were more potent than curcumin and other curcumin analogs at inhibiting Akt. Our previously reported analogs RL90 and RL91 did not reduce the ratio of P-Akt/Akt in MDA-MB-231 cells even at 4 µM (Somers-Edgar et al., 2011). Also, no other curcumin analogs have been reported to inhibit Akt in TNBCs. Moreover, curcumin required a very high concentration of 40 and 80 µM for reduction of P-Akt in MDA-MB-468 cells (Squires et al., 2003).

4.2.2.3.3 mTOR and 4E-BP1 inhibition

The effects of RL71 and RL66 were further studied on mTOR and 4E-BP1 which are the downstream targets of Akt. The results demonstrated that the decreased activity of Akt led to the decreased activation of its substrate mTOR in MDA-MB-468 cells by both RL71 and RL66. In contrast, in MDA-MB-231 cells, RL71 did not change the expression of mTOR but significantly down regulated the expression of 4E-BP1 which is downstream of mTOR. This suggests that RL71 directly targets the downstream events of PI3K/Akt signaling in MDA-MB-231 cells whereas RL66 decreased 4E-BP1 expression via Akt. However, the expression of 4E-BP1 was not reduced in MDA-MB-468 cells by either of the compounds.
Various studies have reported the therapeutic role of mTOR and/or 4E-BP1 inhibitors in breast cancer treatment. For example, Yu et al. (2001) reported that treatment with the cell cycle inhibitor-779 (CCI-779), which is a rapamycin analog that inhibits mTOR function, leads to inhibition of MDA-MB-468 cells with an IC$_{50}$ value of 0.7 nM. However, MDA-MB-435 and MDA-MB-231 cells were resistant to the treatment with CCI-779. The authors further demonstrated that MDA-MB-468 cells were sensitive to treatment with CCI-779 both in vitro and in vivo. Treatment of MDA-MB-468 cells with 50 nM of CCI-779 for 16 h decreased the phosphorylation of p70 S6K and 4E-BP1 (Yu et al., 2001). Also, in vivo treatment of mice bearing MDA-MB-468 tumors with CCI-779 at 20 or 40 mg/kg for 5 days resulted in complete regression of tumors whereas 10 mg/kg extended the delay in growth of tumors by 10 days beyond the last dose given. In addition, the inhibition of mTOR by CCI-779 resulted in an increase in p27kip1 levels in MDA-MB-468 cells. The study suggested that the growth of MDA-MB-468 cells which have PTEN loss is dependent on mTOR and the expression of p27kip1 is regulated by PTEN in an mTOR dependent manner (Yu et al., 2001). In another study, Albert et al. (2006) utilized the rapamycin derivative, RAD001, to inhibit radiation-induced mTOR in MDA-MB-231 cells. Treatment of irradiated MDA-MB-231 cells with 20 nM of RAD001 significantly decreased cell survival. Moreover, combination of RAD001 with 3 Gy radiation increased apoptosis by 3.7-fold compared to RAD001 alone. The cell cycle study demonstrated that RAD001 alone had no effect on cell cycle progression whereas radiation (5 Gy) caused a 223% increase in the G2/M phase compared to control at 24 h. The combination of RAD001 and radiation caused a 464% increase in the G2/M phase compared to control at 24 h (Albert et al., 2006). Thus these studies suggest that mTOR is an important target for breast cancer therapy.

Curcumin is reported to inhibit phosphorylation of mTOR and 4E-BP1 in Rh1 and Rh30 human rhabdomyosarcoma cells. Treatment of serum starved Rh 30 cells with 2.5-40 µM of curcumin for 2 h significantly reduced phosphorylation of mTOR which further reduced phosphorylation of 4E-BP1 with 5-40 µM of curcumin (Beevers et al., 2006). Similar effects were also observed in DU145 human prostate cancer cells, MCF-7 human breast cancer cells and HeLa human cervical cancer cells. Treatment of DU1465 and MCF-7 cells with 40 µM of curcumin for 2 h significantly reduced phosphorylation of 4E-BP1 whereas in HeLa cells, 2.5 µM of curcumin was enough to completely inhibit phosphorylation of 4E-BP1 after 1 h (Beevers et al., 2006). However, the levels of Akt remained unchanged until the concentration of curcumin reached 40 µM which suggested
that the inhibition of 4E-BP1 occurred independently of Akt inhibition (Beevers et al., 2006). Based on the above evidence, inhibition of Akt, mTOR and 4E-BP1 could be a potential mechanism of cytotoxicity by RL71 and RL66 in triple negative breast cancer cells.

4.2.2.3.4 NFκB inhibition

It is reported that Akt contributes to the activity of NFκB by controlling its translocation to the nucleus (Burow et al., 2000) and a decrease in Akt activity may affect the stability and level of NFκB (Gong et al., 2003). Therefore, further studies were carried out to study the effect of RL71 and RL66 on NFκB in ERα negative breast cancer cells. NFκB belongs to a family of transcription factors which has been associated with inhibition of apoptosis by promoting the expression of antiapoptotic proteins such as Bcl-xL, c-Myc and caspase inhibitors (Barkett et al., 1999; Lauder et al., 2001). The present study demonstrated that RL71 and RL66 down regulated the expression of NFκB protein in MDA-MB-231 cells. However, in MDA-MB-468, RL71 and RL66 had no effect on NFκB expression.

Similar to our results, curcumin and other synthetic analogs have been shown to interfere with the functions of Akt and further inhibit its downstream target NFκB (Dhandapani et al., 2007; Shehzad et al., 2010). Treatment of gliomas cells T98G, U87MG and T67 with 25 or 50 μM of curcumin for 3 to 6 h, significantly reduced NFκB-DNA binding and further decreased its transcriptional activity. Moreover, inhibition of Akt with the PI3K inhibitor LY294002 in T98G and U87MG cells reduced the constitutive NFκB transcriptional activity (Dhandapani et al., 2007). Similarly, a synthetic curcumin analog, 4-hydroxy-3-methoxybenzoic acid methyl ester (HMBME, 25 μM) reduced Akt and P-Akt to approximately 80% and 75% of control respectively, resulting in a complete inhibition of NFκB signaling in LNCaP prostate cancer cell lines in vitro after 24 h (Kumar et al., 2003).

Various other anticancer agents have also been reported to inhibit Akt-dependent activation of NFκB in breast cancer cells. For example, treatment of MDA-MB-231 cells with *Ganoderma lucidum* (1 mg/ml) for 96 h significantly reduced the phosphorylation of Akt at Ser473 resulting in further decrease in NFκB activity (Jiang et al., 2004). Similarly, genistein inhibited NFκB via Akt inhibition. When pLNCX-Akt transfected MDA-MB-231 cells were treated with 50 μM of genistein for 36 h, the luciferase activity was inhibited by approximately 60% (Gong et al., 2003). Genistein also modulated EGF-
induced luciferase activity. Moreover, genistein inhibited NF-kB DNA-binding activity in pLNCX-Akt-transfected MDA-MB-231 cells with or without EGF stimulation (Gong et al., 2003). A similar activity was also shown by LY294002. A study conducted by Biswas et al (2000) demonstrated that co-treatment of MDA-MB-231 cells with LY294002 and EGF reduced the NFkB DNA-binding activity by 50% and 4% at 2 and 4 h, respectively (Biswas et al., 2000).

Masuda et al. (2002) reported that suppression of Akt by EGCG results in the inhibition of constitutively active and TNF-α-induced NFκB in a reporter gene assay. When MDA-MB-231 cells transfected with an NF-kB element-driven luciferase reporter plasmid were treated with 30 µg/ml of EGCG for 24 h, the luciferase activity was completely inhibited (Masuda et al., 2002). In another study, Pianette et al. (2002) reported that treatment of NF mouse mammary carcinoma cells transfected with NF-kB element-driven luciferase reporter plasmid with 40 µg/ml of EGCG for 24 h, the luciferase activity was inhibited by 76% compared to control. Compared to all these studies RL71 and RL66 were more potent at inhibiting NFκB expression in MDA-MB-231 cells. However, further studies with RL71 and RL66 are required to confirm the Akt-dependent down-regulation of NFκB in triple negative breast cancer cells.

4.2.2.3.5 Mechanism in HER2 positive cells

Along with the mechanistic studies in triple negative breast cancer cells, the signaling events of RL71 and RL66 were also studied in HER2 positive SKBr3 breast cancer cells. The present study showed that RL71 and RL66 almost completely inhibited HER2 expression in SKBr3 cells in a time- and concentration-dependent manner. HER2 overexpression further activates the PI3K/Akt/mTOR pathway leading to breast tumor progression (Holbro et al., 2003). Also, it is reported that aberrant activation of this pathway promotes a resistance to anti-HER2 and other anti-cancer agents (Shabaya et al., 2011). Therefore, co-targeting HER2 and the PI3K/Akt/mTOR pathway has a strong rationale for treatment of HER2 positive breast cancer.

Various studies have reported enhanced cytotoxicity of anticancer agents in combination with mTOR inhibitors towards HER2 positive breast cancer cells. For example, twice a week treatment of mice bearing MMTV/HER2 tumors with trastuzumab (30 mg/kg) or rapamycin (1 mg/kg) showed complete tumor regression in only 3 out of 10 and 1 out of 10 cases, respectively, after 4 weeks. In contrast, the combination treatment caused a complete tumor regression in all 10 cases in 3.5 weeks. Thus the
combination was more effective at eliciting tumor regression than either single treatment (Miller et al., 2009). Moreover, in SKBr3 and BT-474 cells, the combination of the rapamycin analog RAD001 and trastuzumab produced greater cytotoxicity than the single agent treatment (Miller et al., 2009). Also, trastuzumab partially decreased PI3K activity (as indicated by p-Akt) but not mTOR activity (as indicated by S6K) whereas the combination treatment showed simultaneous inhibition of P-Akt and mTOR (Miller et al., 2009). Another rapamycin analog CCI-779 induced a strong cytotoxicity in HER2 positive breast cancer cells as shown by the IC₅₀ values of <10 nM, 0.7 nM and <10 nM, in BT-549, SKBr3 and BT-474 cells, respectively (Yu et al., 2001).

In another study, Zhou et al. (2004) demonstrated that activation of the PI3K/Akt/mTOR pathway was associated with up regulation of 4E-BP1 in HER2 positive breast tumors. In addition, increased phosphorylation of Akt/mTOR/4E-BP1 was responsible for shorter disease-free survival in HER2 positive breast cancer patients (Zhou et al., 2004). The expression of EIF-4E which regulates the activity of 4E-BP1 has also been reported to be a marker of resistance to anti-HER2 therapies. A study conducted by Zindy et al. (2011) demonstrated that HER2 overexpressing invasive breast tumors from patients who received trastuzumab neoadjuvent therapy had high expression of EIF-4E. Moreover, 42% of patients with tumors over expressing EIF-4E had an incomplete pathologic response. Thus in addition to Akt and mTOR, 4E-BP1 is also a potential target in HER2 positive breast cancer.

The PI3K/Akt pathway also activates another downstream substrate, NFκB, leading to progression of HER2 positive breast cancer (Zhou et al., 2000). In addition, activity of cell cycle regulatory protein p27kip1 has also been associated with proliferation of HER2 positive cells as well as resistance to certain HER2 targeting agents. Nahta et al. (2004) reported that trastuzumab resistant cells were associated with reduced expression of p27kip1 and increased cell proliferation whereas tranfection of wild-type p27kip1 increased the sensitivity of trastuzumab sensitivity in these cells. In another study, Cardoso et al. (2006) reported that proteosomal inhibitor bortezomib induced p27kip1 and increased the efficacy of trastuzumab in HER2 overexpressing breast cancer cells. Treatment of SKBr3 cells with the combination of bortezomib (10⁻⁶ µM) and trastuzumab (20 µg/ml) caused a 10-fold increase in the nuclear concentration of p27kip1 compared to control whereas trastuzumab did not increase the presence of p27kip1 (Cardoso et al., 2006). The present study demonstrated that both RL71 and RL66 induced p27kip1 and
cleaved caspase 3 expression in SKBr3 cells. In addition, RL66 down regulated NFκB in a time-dependent manner.

Overall, these studies reported that both RL71 and RL66 down regulated HER2 signaling in SKBr3 cells. Further, RL66 down regulated EGFR, Akt, mTOR, 4E-BP1 and NFκB and induced expression of p38 and JNK1/2 in a time-dependent manner. Both RL71 and RL66 were more potent than our previously reported curcumin analogs, RL90 and RL91, at down regulating the expression of HER2 (Somers-Edgar et al., 2011). Moreover, RL90 and RL91 down regulated Akt and NFκB and up regulated p38 and JNK1/2 at a concentration double that of RL66. Also, other curcumin analogs FLLL11 and FLLL12 down regulated HER2 in BT-474 and SKBr3 cells, but at a concentration of 10 µM (Lai et al., 2012). In addition, FLLL11 and FLLL12 increased caspase-3 activation in SKBr3 and MDA-MB-453 breast cancer cells and PC3 prostate cancer cells at 10 µM. The same study also showed that FLLL12 (10 µM) down regulated HER2 and P-Akt in MDA-MB-453 cells. Thus, RL71 and RL66 were more potent than FLLL11 and FLLL12. Curcumin has also been reported to inhibit HER2-over expressing and/or herceptin-resistant breast cancer cells via suppression of HER2, Akt, MAPK and NFκB (Lai et al., 2012). Treatment of HER2 positive BT-474 and herceptin resistant SKBr3 cells with curcumin (27 µM) decreased HER2 by 50% whereas P-Akt was decreased by 70% compared to control. Moreover, 27 µM of curcumin also caused 50% reduction both in P-MAPK and NFκB in BT-474 and SKBr3 cells, respectively. However, a higher concentration of 68 µM of curcumin was required for 50% reduction of P-MAPK in SKBr3 and 90% reduction of NFκB in BT-474 cells. The combination of curcumin (27 µM) and herceptin (10 µg/ml) decreased HER2 by 50% in both the cell lines whereas P-Akt was decreased by 90% in BT-474 cells and by 50% in SKBr3 cells. NFκB levels were almost completely inhibited in BT-474 cells while SKBr3 cells showed 50% reduction (Lai et al., 2012). Thus overall, RL71 and RL66 were more potent than curcumin and synthetic curcumin analogs at inhibiting the growth of HER2 positive breast cancer cells. As RL66 showed almost complete inhibition of HER2/Akt/mTOR signaling and a strong activation of stress kinases at just 1 h, this could be the reason behind increased sensitivity of RL66 towards cell cycle arrest and induction of apoptosis in SKBr3 cells.
4.2.2.4  In vitro migration and angiogenesis studies

It is reported that the PI3K/Akt/mTOR pathway, the stress kinase pathway, the HER2 pathway as well as NFκB contribute to a number of processes such as metastasis and angiogenesis in various human solid tumors including the breast (Phung et al., 2006; Tsutsui et al., 2010; Wen et al., 2006). Therefore, the effect of RL71 and RL66 on breast cancer cell migration and angiogenesis was examined in vitro.

The studies showed that both RL71 and RL66 inhibited the migration potential of MDA-MB-231 cells at 1 and 2 µM, respectively. These studies were consistent with other findings that showed anti-migratory potential of curcumin and its synthetic analogs. For example, curcumin (3 µM) was reported to reduce the cell motility (wound closure) in MDA-MB-231 cells by 61% compared to control. Similarly, another curcuminoid, demethoxycurcumin, inhibited the motility of MDA-MB-231 cells at 7.5 and 15 µM (Yodkeeree et al., 2010). The curcumin analogs FLLL11 and FLLL12 significantly inhibited the wound healing ability in MDA-MB-231 cells compared to control at 5 and 10 µM (Yodkeeree et al., 2010). Thus, RL71 and RL66 were more potent than curcumin and other curcumin analogs at producing anti-migratory effects. However, in addition to this assay, a study with the Transwell migration assay would have created a more complete picture of the anti-metastatic potential of RL71 and RL66 in vitro.

RL71 and RL66 also inhibited angiogenesis in vitro by inhibiting endothelial tube formation and the migration of these cells through Matrigel in a concentration-dependent manner. A similar result was also shown by curcumin and its analogs. For example, curcumin (5 µM) and its two cyclohexanone-containing synthetic analogs with methoxy and hydroxyl substituents (5 µM) reduced endothelial cell migration by 75%, 35% and 55%, respectively, compared to untreated cells (Hahm et al., 2004). Moreover, at 5 µM, curcumin and both the analogs inhibited Matrigel-induced network formation of HUVEC cells, producing less extensive, broken, foreshortened, and much thinner vessels when compared with the control. Another synthetic curcumin analog, hydrazinocurcumin (100-300 nM) inhibited endothelial tube formation by >50% compared to control, without affecting the viability of endothelial cells (Shim et al., 2002). Similarly, EF24 showed potent anti-angiogenic activity compared to curcumin where the IC50 values obtained for endothelial cell tube formation and migration for curcumin and EF24 were >10, 1.8 and 1.5, 0.8 µM, respectively (Adams et al., 2004). A study conducted by Shankar et al (2007) reported that curcumin (20, 40 and 60 µM) significantly inhibited the endothelial
tube formation and the migration of HUVEC cells in a dose-dependent manner. Moreover, pre-treatment of HUVEC cells with an ERK inhibitor (10 µM) followed by curcumin (40 µM) for 24 h potentiated the inhibitory effects of curcumin on tube formation and migration (Shankar et al., 2007). Various other models have also been used to report the anti-angiogenic potential of curcumin and curcumin analogs. For example, Arbiser et al. (1998) demonstrated that curcumin and its naturally occurring analogs demethoxycurcumin and bisdemethoxycurcumin inhibited basic fibroblast growth factor (bFGF) and inhibited proliferation of HUVEC cells in vitro as well as angiogenesis in mouse model. Treatment of primary capillary endothelial cells with 5-10 µM of curcumin caused a sharp decrease in endothelial cell number in the presence or absence of 1 ng/ml of bFGF after 72 h. Moreover, both curcumin and its analogs significantly inhibited bFGF-induced corneal neovascularization in mice as shown by inhibition of corneal blood vessel length and clock hours which measure an area of corneal neovascularization (Arbiser et al., 1998).

Taken together, these findings suggest that RL71 and RL66 have anti-angiogenic properties in vitro. In order to study the mechanism of this effect, future experiments need to be undertaken for various markers of angiogenesis such as VEGF, MMP9 and so on.

4.2.3 Oral bioavailability and tumor suppression in vivo

The bioavailability studies of RL71 demonstrated that a single oral dose of 8.5 mg/kg produced plasma concentrations of 0.405 µg/ml and 0.283 µg/ml, after 5 and 15 min, respectively. The plasma concentration then decreased in a time-dependent manner. However, the daily oral treatment of mice bearing MDA-MB-468 xenograft with RL71 (8.5 or 0.85 mg/kg) did not significantly reduce the tumor volume compared to vehicle treated mice. One of the reasons behind this could be the instability or low potency of RL71 at this dose. Therefore, when we further increased the dose of RL71 by 5 times (42.5 mg/kg) and 10 times (85 mg/kg) the preliminary data from one mouse and a single time point of 15 min showed an increase in the plasma concentrations by 3 and 3.8-fold, respectively. Moreover, when the route of administration of RL71 was changed and a single dose of 8.5 mg/kg of RL71 was administered intraperitoneally, the plasma concentrations were increased by almost 2-fold after 15 min. These studies showed that it would be desirable to either increase the oral dose of RL71 or change its route of administration. However, increasing the dose might also increase toxicity. Another approach would be to use a novel drug delivery system to increase the bioavailability of
RL71 and selectively target it to the tumor. As mentioned previously (section 1.8.1.1) various novel drug delivery systems for curcumin have been successfully developed which showed an enhanced anticancer activity in mouse breast cancer models. Accordingly, our lab recently incorporated RL71 into a miceller drug delivery system and the preliminary studies showed that RL71 micelles released the compound at a sustained rate and retained the cytotoxic activity of RL71 in vitro towards various ERα negative breast cancer cells. The future study of these micelles in a mouse xenograft model would demonstrate the potency of RL71 in ERα negative breast cancer.

For RL66, bioavailability studies demonstrated that administration of a single oral dose of 8.5 mg/kg produced a peak plasma concentration of 0.056 µg/ml after 10 min. The plasma concentration then declined rapidly to the limit of detection. Furthermore, the daily oral treatment of nude mice bearing MDA-MB-468 cells with 8.5 mg/kg of RL66 resulted in a significant reduction in the tumor volume by 48% compared to vehicle treated group after 10 weeks. However, this effect did not coincide with the bioavailability of RL66. It is possible that the detection method used was not sensitive enough to analyze RL66 in plasma. Also, RL66 could be producing an active metabolite which is responsible for the tumor suppression. Moreover, it would be desirable to analyze RL66 in the tumor or other tissues. Therefore, comprehensive pharmacokinetic studies using sophisticated instruments such as LCMS or LCMS/MS would give better idea about the concentration, localization, clearance and metabolism of RL66 in vivo.

Although the treatment with RL66 caused an apparent decrease in the tumor weight, this effect was not statistically significant. Importantly, the studies of RL66 did not show any change in the animal body weight throughout 10 weeks. In addition, the treatment with RL66 did not significantly alter the major organ weights (including spleen, kidney, liver and uterus) and the plasma ALT values were in the normal range. These observations indicate that RL66 treatment is not overtly toxic to mice and warrants further investigation. Thus, RL66 has potential for further development as a treatment for ERα negative breast cancer.

These results were consistent with the tumor suppressive effects shown by curcumin and other curcumin analogs in a mouse xenograft model of ERα negative breast cancer. For example, our previous studies demonstrated that combination of epigallocatechin gallate (EGCG, 25 mg/kg/day, i.p.) and curcumin (200 mg/kg/day, p.o.) significantly suppressed the growth of MDA-MB-231 mouse xenograft tumors by 49%
compared to control mice (Somers-Edgar et al., 2008). The tumor reduction by RL66 (8.5 mg/kg/day, p.o.) also showed similar tumor suppressive effects at a dose 24-fold lower than that of curcumin. Similarly, a synthetic curcumin analog, BDHPC, also showed significant reduction in the growth of estrogen-independent mouse mammary tumors. Syngeneic BALB/c female mice bearing transplanted mammary tumors were treated daily from day zero with 160 mg/kg of BDHPC subcutaneously. The study demonstrated that BDHPC caused significant reduction in mammary tumor growth compared to control. The authors also suggested the role of nuclear type II receptor for binding of BDHPC in breast cancer cells (Markaverich et al., 1992). Another curcumin analog, EF24, caused regression of MDA-MB-231 breast tumors in athymic nude mice. MDA-MB-231 breast cancer xenografts were grown in female mice for 3 weeks and the mice were treated subcutaneously with 2, 20 or 100 mg/kg of EF24 for 2 weeks. A dose-dependent decrease in tumor weight was observed following the drug treatment. The average tumor weight in the 20 mg/kg group was decreased by 70% compared to vehicle treated group (Adams et al., 2004). Moreover, no toxicity was reported at up to 100 mg/kg of EF24. Similarly, another curcumin analog PAC suppressed the growth of MDA-MB-231 breast cancer xenografts in nude mice. Female nude mice were subcutaneously injected with MDA-MB-231 cells and subsequently treated intraperitoneally with 100 mg/kg/day of PAC for 2 weeks. PAC decreased the average tumor volume by 50% during the first 5 days of treatment compared to vehicle treated mice and continued to significantly suppress the tumor growth after 2 weeks treatment. Moreover, PAC treatment was not associated with any sign of toxicity.

RL66 was found to be superior to BDHPC, EF24 and PAC at inducing tumor suppressive effects, as the concentration of RL66 required was 19, 2.4 and 12-fold lower than BDHPC, EF24 and PAC, respectively. Another advantage of RL66 over these compounds was its oral route of administration which can offer better patient compliance. However, both EF24 and PAC were studied in a MDA-MB-231 mouse xenograft model whereas RL66 was studied in a MDA-MB-468 mouse xenograft model. As the in vitro studies of RL66 have shown potent cytotoxicity towards MDA-MB-231 cells, it is predicted that RL66 could also induce tumor suppressive effects in an in vivo model of MDA-MB-231 cells.
4.2.3.1 Mechanism of tumor suppression of RL66

4.2.3.1.1 Mechanism by Western blotting

As RL66 showed a significant reduction in the tumor volume after 10 weeks of treatment, the effect on key cell signaling proteins was determined by Western blotting. The proteins that were examined in vitro were studied in the subsequent in vivo study. Western blotting data demonstrated that the tumors from mice treated with 8.5 mg/kg of RL66 had a marginal decrease in the expression of EGFR. Moreover, RL66 treatment caused an increase in the phosphorylation of Akt in the tumor which did not correlate with the in vitro results that showed significant decrease in phosphorylation of Akt at a later time point (36 h). In addition, the expression of NFκB was increased in tumors treated with 8.5 mg/kg of RL66 whereas in the in vitro study the expression of NFκB was not changed compared to control. Furthermore, RL66 treatment decreased the phosphorylation of mTOR and increased the tumor expression of p27 which was similar to the results obtained in the in vitro studies. However, these changes in the tumor were not statistically significant compared to control. This could be due to the variability and small sample size. A greater sample size could have resulted in significantly different changes in the expression of these proteins. Thus, these results suggest that RL66 could be directly targeting mTOR and an increase in the phosphorylation of Akt could be due to a feedback loop mechanism which has been previously reported. For example, the tumors of the patients treated with the rapamycin analog RAD001 for 4 weeks showed elevated levels of P-Akt (O’Reilly et al., 2006). Similarly another clinical study involving the treatment of patients with advanced solid tumors with 20, 50, and 70 mg weekly or 5 and 10 mg daily of RAD001 showed elevation of P-Akt in 50% of the patients (Tabernero et al., 2008). Also, it is reported that breast cancer cells that express high levels of activated Akt were highly sensitive to rapamycin (Noh et al., 2004). It was also found that in rapamycin-sensitive cells, p27kip1 levels were up-regulated. Moreover, treatment of MDA-MB-468 cells with the mTOR inhibitor, CCI-779 (50 nM) caused an increase in p27kip1 levels at 16 h suggesting that the expression of p27kip1 is regulated by PTEN in an mTOR dependent manner (Yu et al., 2001). Thus, this supports our finding that inhibition of mTOR could be associated with increased activity of p27 in RL66 treated MDA-MB-468 tumors. This result is also consistent with our previous findings where nude mice bearing MDA-MB-231 xenografts treated daily orally with RL92 showed decreased expression of mTOR and increased expression of p-Akt. However, p27kip1 levels were not changed (data not published).
4.2.3.1.2 Role of CD105 in tumors treated with RL66

Various studies have reported the therapeutic role of CD105 reduction in tumor related angiogenesis in a mouse xenograft model. For example, Minhajat et al. (2009) reported that treatment of SCID mice bearing WiDr human colon cancer xenografts with anti-CD105 antibody (50 μg/every 2 days) intraperitoneally caused 27% reduction in the tumor volume compared to vehicle control group after 4 weeks. Moreover, the treatment caused 70% reduction in CD105 expression compared to vehicle group (Minhajat et al., 2009). Similarly, systemic administration of unconjugated anti-CD105 monoclonal antibody SN6j (0.6 or 1.8 µg/g) in BALB/c mice bearing colon-26 or 4T1 tumors showed significant tumor suppression and increased overall survival compared to control (Tsujie et al., 2006). In another study, the recombinant human/mouse chimeric anti-CD105 antibody, c-SN6j showed promising pharmacokinetic and toxicity data in monkeys and its phase I clinical trial studies are on-going in patients with metastatic solid tumors (Shiozaki et al., 2006).

Similar to these studies, tumors from mice treated with 8.5 mg/kg of RL66 showed significant reduction in the number of CD105 positive blood vessels by 59% compared to control. These results correlated well with the in vitro anti-angiogenic data in HUVEC cells and suggest that inhibition of angiogenesis could be one of the mechanisms of anticancer activity of RL66. Curcumin has also been reported to inhibit angiogenesis by reducing the expression of CD105 in tumors. Daily treatment of mice bearing U-87 human glioma tumor xenografts with curcumin (60 mg/kg, i.p.) for 29 days caused 55% reduction in CD105 mRNA (Perry et al., 2010). However, no curcumin analog has been reported to inhibit CD105 expression in a mouse cancer model. It would be desirable to further explore the anti-angiogenic property of RL66 by studying some more markers such as VEGF and MMP9 in tumor samples. Metastasis to other sites can also be accurately studied by in vivo imaging of metastatic breast cancer cells lines injected into the mammary fat pad. Thus, these types of experiments would shed more light on the role of RL66 as an inhibitor of metastasis.
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Chapter six: List of publications


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6.1 JOURNAL ARTICLES:


Babasaheb Yadav, Sebastien Taurin, Lesley Larsen, Rhonda J. Rosengren. RL71, a second generation curcumin analog induces apoptosis and down-regulates Akt in ER negative breast cancer cells (manuscript accepted in International Journal of Oncology)

Babasaheb Yadav, Sebastien Taurin, Lesley Larsen, Rhonda J. Rosengren RJ. New curcumin analogue RL66 showed promising anticancer properties towards estrogen receptor negative breast cancer cells both in vitro and in vivo (manuscript in preparation)

Book Chapter:


Review article:


6.2 Abstracts


6.3 Achievements:

- Received prize for the best poster presentation at 3rd QMB Cell Signaling meeting held at Queenstown, New Zealand, 28-29th August 2011.

- Received prize for the best poster presentation at PhD colloquium session organized by Otago School of Medical Sciences at Dunedin, New Zealand, 2011.

- Received travel grant from division of health sciences, University of Otago to attend 101st American association of cancer research (AACR) conference held at Washington DC in April 2010.

- Received travel grant from Maurice and Phyllis Paykel Trust, New Zealand to attend The 2011 European Multidisciplinary Cancer Congress held at Stockholm, Sweden in September 2011.

- Received Professional Development Award from Genesis Oncology Trust, New Zealand to attend The 2011 European Multidisciplinary Cancer Congress held at Stockholm, Sweden in September 2011.

- Received PhD scholarship from University of Otago.