

**CONCOMITANT DELIVERY OF BCL-2 PROTEIN
FAMILY INHIBITORS AND CHEMOTHERAPEUTIC
DRUGS FOR CANCER THERAPY**

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APRIL 2022**

Concomitant Delivery of BCL-2 Protein Family Inhibitors and Chemotherapeutic Drugs for Cancer Therapy

by

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Submitted

in fulfilment of requirements of the degree of Doctor of Philosophy

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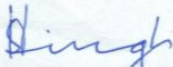


**Indian Institute of Technology Delhi
APRIL 2022**

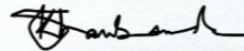
*Dedicated to the pursuit of
knowledge...*

CERTIFICATE

This is to certify that the thesis entitled "**Concomitant Delivery of BCL-2 Protein Family Inhibitors and Chemotherapeutic Drugs for Cancer Therapy**" submitted by **Ms. Neha Mehrotra** to **Indian Institute of Technology Delhi** for the award of degree of Doctor of Philosophy in Biomedical Engineering is a record of bona fide research work carried out by her. Ms. Mehrotra has worked under our supervision and has fulfilled the requirements for submission of this thesis. The results contained in this thesis are original and have not been submitted in any form to any other university or institute for the award of a degree or diploma.



Prof. Harpal Singh
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Dr. Surender Kharbanda
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Acknowledgements

I would like to express my deepest gratitude to my thesis supervisor Prof. Harpal Singh for his constant support and guidance. His mentorship, broad minded visionary approach to research and gentle constructive criticism have been instrumental to me during my dissertation. I will be forever grateful for his open-door policy towards students and the freedom it gave me to explore and hone my research skills.

I am equally grateful to my thesis co-supervisor, Dr. Surender Kharbanda for his invaluable guidance and inputs throughout my research work. His disciplined attitude and zeal towards scientific research were a great inspiration for me

I have been extremely fortunate to have supervisors who have treated me like family and their kindness and support has made this journey beautiful.

I would like to extend my sincere thanks to Indian Institute of Technology Delhi for financial support and provision of research facilities necessary to carry out this work. I would also like to acknowledge and thank the various staff members of Central Animal Facility at All India Institute of Medical Sciences, Delhi for their support in conducting animal experiments and the instrument operators at Central Research Facility, IIT Delhi for all their help in material characterization.

I would like to thank my lab members, Dr. Sruti Chattopadhyay, Mr. Mohd. Anees and Mr. Sachchidanand Tiwari for their support in this pursuit. The countless scientific and life discussions we've had through the years have been a very important part of my journey. I would also like to thank Mr. Anil Pandey for his indispensable help with all the animal experiments and Mr. Rajesh and Mr. Sitaram for their help in setting up experiments and day to day lab activities. I would like to extend my affectionate thanks to my juniors Ms. Priya Gupta and Ms. Harshdeep Kaur and wish them well for their Ph.D. journey. I would also like

to acknowledge support from my fellow Ph.D. scholars at the Centre for Biomedical Engineering, especially Ms. Shreemoyee De for all her help with the flow cytometry experiments.

Furthermore, I would like to acknowledge my previous mentors, Dr. K Selvaraj and Prof. Joachim Raedler. My bachelor's dissertation under Dr. Selvaraj's guidance was my first experience of research work in a laboratory, and it is an experience that I will cherish forever. I am deeply thankful to Prof. Raedler for giving me my first exposure to an international research lab. My stay at his lab showed me the true meaning of collaborative interdisciplinary research and helped me get a better understanding of the role of diversity in scientific community.

To my partner in life and crime- we did it! Being a fellow Ph.D. student, you know exactly how arduous and frustrating this journey can be. Your silent and unconditional support means a lot. Here's to setting and achieving many more life goals together.

To my parents – everything I am today, is because of you. Words can never be enough to express my gratitude to you both. To my father, you may not have understood half of what I did during my thesis, but your constant encouragement meant the world. You may not have contributed towards the scientific aspect of this journey, but your advice and guidance on the human side of research life has been invaluable. Your faith and confidence in me is something that I am thankful for everyday and has shaped me into the person I am today.

My mother, my first and forever mentor, this day would never have come without you. This thesis belongs as much to you as it does to me. You have been my teacher, my friend and sounding board for my entire life and I hope that never changes.

Abstract

Cancer is a major global health issue with approximately 19.3 million cases diagnosed and 10 million deaths in the year 2020. Current standards of cancer treatment – surgery, chemotherapy, radiation therapy, hormone therapy and immunotherapy have increased overall survival rates in most cancer types. However, their response has not been optimal as cancer therapy is an intricate process which needs to cover all facets of tumor growth. Newer approaches that combine two therapeutic modalities such as chemotherapy with radiation, targeted therapy, hormone or immune therapy have emerged to overcome the limitations of the current clinical practices. Combination therapy approaches, especially those based on two or more chemo drugs or chemotherapeutics with biological molecules have shown remarkable potential by being able to target multiple pathways simultaneously and therefore reducing the chances of resistance and relapse. However, these approaches are based on systemic delivery of free drugs which still suffers from issues such as low aqueous solubility, poor stability and bioavailability in systemic circulation, complicated dosage regimes and sub-optimal biodistribution profile. In recent years, nanoparticle-based drug delivery systems for combination therapies have gained a lot of momentum. Nanodelivery vehicles can overcome poor solubility, bioavailability and stability issues which would in turn improve biodistribution and tumor accumulation of the combination drugs, making nanomaterial-based delivery systems promising candidates for improved combination cancer therapy.

The present research work is focused on the development of polylactic acid-based block copolymeric nanoparticles for concomitant delivery of BCL-2 family inhibitors with chemotherapeutic drugs. PLA based block copolymers with different molecular weights and hydrophobic/hydrophilic block ratios were synthesized and evaluated for preparation of anticancer agent loaded nanoparticles. A hybrid block copolymer, synthesized via ring opening polymerization using L-Lactide, with methoxy polyethylene glycol 5000 and Pluronic L-61 as

macroinitiators was chosen as the final polymer. The synthesized block copolymer was characterized using GPC, proton NMR and FTIR spectroscopy. Block copolymer was found to have an average molecular weight of ~25kDa. Proton NMR spectrum showed characteristics peaks of PLA at 5.2 and 1.6 ppm while peaks at 3.6 ppm and 1.2 ppm confirmed the incorporation of PEG and PPG blocks into the hybrid block copolymers. The synthesized copolymer was used to prepare MS1 peptide and Navitoclax loaded single and dual nanoparticles with a high encapsulation efficiency, spherical morphology, and uniform dispersion. The use of PLA based copolymer for concomitant delivery of MS1/Paclitaxel and Navitoclax/Decitabine resulted in sustained release with rapid cellular internalization and localization at target intracellular site. Biological activity of MS1 NPs was evaluated in five different breast cancer cell lines and a moderate inhibition of cellular proliferation was observed with IC₅₀ ranging from 142–612nM for different cell lines (MCF-7, MDA-MB-468, SUM-149, ZR-75-1, SK-BR-3). The synergistic potential of MS1 with Paclitaxel, a widely used drug for breast cancer treatment, was evaluated for breast cancer therapy. However, out of the five breast cancer cell lines chosen for the study, synergistic effect of MS1/PTX dual NPs was observed only in MCF-7 cell line, which is a hormone-dependent breast cancer. No significant improvement in cytotoxicity was observed in the other cell lines.

Navitoclax/Decitabine dual NPs with a NAV/DCB molar ratio of 1:1 exhibited significant improvement in IC₅₀ over single NPs in breast cancer and AML cell lines. Treatment with single drug loaded NPs led to IC₅₀ values in the range of 0.055-7μM for NAV NPs and 0.092-5.5μM for DCB NPs in breast cancer and AML cell lines. Exposure to NAV/DCB dual NPs led to synergistic cytotoxicity and caused a reduction of 4 to 13-fold in IC₅₀ values depending on the cell line. *In vivo* biological activity of NAV/DCB NPs evaluated in AML xenograft model in NOD-SCID mice demonstrated 43.6% reduction in tumor on treatment with dual NPs as compared to control group. Pharmacokinetics and biodistribution studies of NAV/DCB NPs

conducted in EAC tumor bearing BALB/c mice confirmed prolonged blood circulation and significant accumulation in tumor tissue. The dual NPs showed good cytocompatibility and hemocompatibility with reduced *in vivo* platelet toxicity. Tumor inhibition studies in murine syngeneic breast cancer model with dual NPs resulted in 94% reduction in tumor size as compared to control while treatment with single NAV NPs and DCB NPs resulted in 45% and 13.5% reduction respectively. Concomitant delivery of Navitoclax and Decitabine using PLA based hybrid block copolymeric nanoparticles presents a novel, safe and effective nanoformulation for combination cancer therapy especially for breast cancer.

सार

कैंसर एक प्रमुख वैश्विक स्वास्थ्य समस्या है। वर्ष 2020 में लगभग 19.3 मिलियन मामलों का निदान किया गया और कैंसर के कारण 10 मिलियन मौतें हुईं। कैंसर के उपचार के वर्तमान मानकों - सर्जरी, कीमोथेरेपी, विकिरण चिकित्सा, हार्मोन थेरेपी और इम्यूनोथेरेपी ने अधिकांश कैंसर प्रकारों में समग्र जीवित रहने की दर में वृद्धि की है। हालांकि, उनकी प्रतिक्रिया इष्टतम नहीं रही है क्योंकि कैंसर चिकित्सा एक जटिल प्रक्रिया है जिसमें ट्यूमर के विकास के सभी पहलुओं को शामिल करने की आवश्यकता होती है। नए दृष्टिकोण जो दो चिकित्सीय तौर-तरीकों जैसे कि कीमोथेरेपी को विकिरण, लक्षित चिकित्सा, हार्मोन या प्रतिरक्षा चिकित्सा के साथ जोड़ते हैं, वर्तमान नैदानिक प्रथाओं की सीमाओं को दूर करने के लिए उभरे हैं। संयोजन चिकित्सा दृष्टिकोण ने, विशेष रूप से दो या दो से अधिक कीमोथेरेपी दवाओं या जैविक अणुओं के साथ कीमोथेराप्यूटिक्स पर आधारित, एक साथ कई मार्गों को लक्षित करने में सक्षम होने और इसलिए प्रतिरोध और रिलेप्स की संभावना को कम करने में उल्लेखनीय क्षमता दिखाई है। हालांकि, ये दृष्टिकोण वाहक मुक्त दवाओं के प्रणालीगत वितरण पर आधारित हैं जो अभी भी कम जलीय घुलनशीलता, खराब स्थिरता और प्रणालीगत परिसंचरण में जैव उपलब्धता, जटिल खुराक व्यवस्था और उप-इष्टतम जैव वितरण प्रोफाइल जैसे मुद्दों से ग्रस्त हैं। हाल के वर्षों में, संयोजन चिकित्सा के लिए नैनोपार्टिकल-आधारित दवा वितरण प्रणाली ने बहुत गति प्राप्त की है। नैनोडिलीवरी वाहन खराब घुलनशीलता, जैवउपलब्धता और स्थिरता के मुद्दों को दूर कर सकते हैं जो बदले में संयोजन दवाओं के जैव वितरण और ट्यूमर संचय में सुधार करेंगे, जिससे नैनोमटेरियल-आधारित डिलीवरी सिस्टम बेहतर संयोजन कैंसर चिकित्सा के लिए आशाजनक उम्मीदवार बनेंगे। वर्तमान शोध

कार्य कीमोथेराप्यूटिक दवाओं के साथ बीसीएल-2 परिवार अवरोधकों के सहवर्ती वितरण के लिए पॉलीलैक्टिक एसिड-आधारित ब्लॉक कोपोलिमरिक नैनोकणों के विकास पर केंद्रित है। विभिन्न आणविक भार और हाइड्रोफोबिक/हाइड्रोफिलिक ब्लॉक अनुपात वाले पीएलए आधारित ब्लॉक कॉपोलिमर को एंटीकैंसर एजेंट लोडेड नैनोकणों की तैयारी के लिए संश्लेषित और मूल्यांकन किया गया था। एल-लैक्टाइड का उपयोग करके रिंग ओपनिंग पोलिमराइजेशन के माध्यम से संश्लेषित एक हाइब्रिड ब्लॉक कॉपोलीमर, मेथाक्सी पॉलीइथाइलीन ग्लाइकॉल 5000 और प्लुरोनिक एल -61 के साथ मैक्रोइनिटिएटर के रूप में अंतिम बहुलक के रूप में चुना गया था। संश्लेषित ब्लॉक कॉपोलीमर को जीपीसी, प्रोटॉन एनएमआर और एफटीआईआर स्पेक्ट्रोस्कोपी का उपयोग करने की विशेषता थी। ब्लॉक कॉपोलीमर का औसत आणविक भार $\sim 25\text{kDa}$ पाया गया। प्रोटॉन एनएमआर स्पेक्ट्रम ने पीएलए की विशेषताओं को 5.2 और 1.6 पीपीएम पर दिखाया, जबकि 3.6 पीपीएम और 1.2 पीपीएम पर चोटी ने हाइब्रिड ब्लॉक कॉपोलिमर में पीईजी और पीपीजी ब्लॉकों को शामिल करने की पुष्टि की। संश्लेषित कॉपोलीमर का उपयोग MS1 पेप्टाइड और Navitoclax लोडेड सिंगल और डुअल नैनोपार्टिकल्स को उच्च एनकैप्सुलेशन दक्षता, गोलाकार आकारिकी और समान फैलाव के साथ तैयार करने के लिए किया गया था। MS1/Paclitaxel और Navitoclax/Decitabine के सहवर्ती वितरण के लिए PLA आधारित कॉपोलीमर के उपयोग के परिणामस्वरूप लक्ष्य इंद्रासेल्युलर साइट पर तेजी से सेलुलर आंतरिककरण और स्थानीयकरण के साथ निरंतर रिलीज हुई। MS1 NPs की जैविक गतिविधि का मूल्यांकन पांच अलग-अलग स्तन कैंसर सेल लाइनों में किया गया था और विभिन्न सेल लाइनों (MCF-7, MDA-MB-468, SUM-149, ZR) के लिए $142\text{-}612\text{nM}$ से लेकर IC_{50} के साथ सेलुलर प्रसार का एक मध्यम निषेध देखा गया था। -75-1, एसके-बीआर-3)। स्तन कैंसर के इलाज के लिए व्यापक रूप से इस्तेमाल की

जाने वाली दवा पैक्लिटैक्सेल के साथ MS1 की सहक्रियात्मक क्षमता का मूल्यांकन स्तन कैंसर चिकित्सा के लिए किया गया था। हालांकि, अध्ययन के लिए चुनी गई पांच स्तन कैंसर सेल लाइनों में से, MS1 / PTX दोहरे NPs का सहक्रियात्मक प्रभाव केवल MCF-7 सेल लाइन में देखा गया, जो एक हार्मोन-निर्भर स्तन कैंसर है। अन्य सेल लाइनों में साइटोटोक्सिसिटी में कोई महत्वपूर्ण सुधार नहीं देखा गया। 1:1 के NAV/DCB मोलर अनुपात के साथ Navitoclax/Decitabine दोहरे NPs ने स्तन कैंसर और AML सेल लाइनों में एकल NPs पर IC50 में महत्वपूर्ण सुधार प्रदर्शित किया। सिंगल ड्रग लोड एनपी के साथ उपचार ने एनएवी एनपी और 0.092- के लिए 0.055-7 μ M की सीमा में IC50 मान का नेतृत्व किया। स्तन कैंसर और एएमएल सेल लाइनों में डीसीबी एनपी के लिए 5.5 μ M। NAV/DCB दोहरे NPs के संपर्क में आने से सहक्रियात्मक साइटोटोक्सिसिटी हुई और सेल लाइन के आधार पर IC50 मानों में 4 से 13 गुना की कमी आई। एनओडी-एससीआईडी चूहों में एएमएल ज़ेनोग्राफ्ट मॉडल में मूल्यांकन किए गए एनएवी / डीसीबी एनपी की विवो जैविक गतिविधि में नियंत्रण समूह की तुलना में दोहरे एनपी के साथ उपचार पर ट्यूमर में 43.6% की कमी का प्रदर्शन किया। ईएसी ट्यूमर असर बीएएलबी/सी चूहों में किए गए एनएवी/डीसीबी एनपी के फार्माकोकाइनेटिक्स और बायोडिस्ट्रीब्यूशन अध्ययनों ने लंबे समय तक रक्त परिसंचरण और ट्यूमर के ऊतकों में महत्वपूर्ण संचय की पुष्टि की। दोहरी एनपी ने विवो प्लेटलेट विषाक्तता में कमी के साथ अच्छी साइटोकम्पैटिबिलिटी और हेमोकंपैटिबिलिटी दिखाई। दोहरी एनपी के साथ म्यूरिन सिनजेनिक स्तन कैंसर मॉडल में ट्यूमर निषेध अध्ययन के परिणामस्वरूप ट्यूमर के आकार में नियंत्रण की तुलना में 94% की कमी आई, जबकि एकल एनएवी एनपी और डीसीबी एनपी के साथ उपचार के परिणामस्वरूप क्रमशः 45% और 13.5% की कमी आई। पीएलए आधारित हाइब्रिड ब्लॉक कोपोलिमरिक नैनोपार्टिकल्स का उपयोग करते हुए

नेवीटोक्लैक्स और डेसिटाबाइन का सहवर्ती वितरण विशेष रूप से स्तन कैंसर के लिए संयोजन कैंसर चिकित्सा के लिए एक उपन्यास, सुरक्षित और प्रभावी नैनोफॉर्म्यूलेशन प्रस्तुत करता है।

Table of Contents

<u>Chapter 1: Introduction and literature review</u>	1-52
1.1 Introduction	3
1.2 Cancer and its hallmarks	4
1.3 Strategies for cancer therapy	8
1.3.1 Surgery	9
1.3.2 Radiation therapy	9
1.3.3 Chemotherapy	9
1.3.4 Hormone therapy	10
1.3.5 Immunotherapy	10
1.3.6 Biomolecules as anti-cancer agents	10
1.3.7 Combination therapy	11
1.4 Combination therapy for cancer – advantages and limitations	12
1.5 Nanomaterials for cancer therapy	18
1.5.1 Inorganic nanomaterials	19
1.5.2 Organic nanomaterials	19
1.6 Strategies for designing nanocarriers for combination therapy	24
1.7 BCL-2 family of proteins and their role in cancer therapy	27
1.7.1 BCL-2 family of proteins in cancer	29
1.7.2 BCL-2 protein family inhibitors for cancer therapy	32
1.8 Rationale and objective of the work	38
References	43
<u>Chapter 2: Synthesis and characterization of PLA based block copolymers for development of MS1 peptide loaded nanoparticles</u>	53-74
2.1 Introduction	55

2.2	Materials and Methods	56
2.2.1	Synthesis of PLA based block copolymers	57
2.2.2	Characterization of PLA based block copolymers	59
2.2.3	Preparation of MS1 loaded copolymeric nanoparticles	59
2.2.4	Characterization of MS1 peptide loaded nanoparticles	60
2.3	Results and discussion	61
2.3.1	Synthesis of PLA based block copolymers	61
2.3.2	Characterization of PLA based block copolymers	62
2.3.3	Preparation and characterization of MS1 loaded copolymeric NPs	67
2.4	Conclusion	71
	References	73
<u>Chapter 3: Concomitant delivery of MS1 peptide and Paclitaxel for evaluation of synergistic potential in breast cancer model</u>		75-99
3.1	Introduction	77
3.2	Materials and Methods	78
3.2.1	Preparation of MS1/PTX loaded copolymeric nanoparticles	78
3.2.2	Characterization of MS1/PTX loaded copolymeric nanoparticles	79
3.2.3	<i>In vitro</i> evaluation of biological activity of MS1 loaded NPs	82
3.2.4	<i>In vitro</i> evaluation of synergistic potential of MS1/PTX dual loaded polymeric NPs for breast cancer therapy	83
3.3	Results and Discussion	84
3.3.1	Preparation and characterization of MS1/PTX loaded polymeric NPs	84
3.3.2	<i>In vitro</i> evaluation of biological activity of MS1 loaded NPs	90
3.3.3	<i>In vitro</i> evaluation of synergistic potential of MS1/PTX dual loaded polymeric NPs for breast cancer therapy	92

3.4	Conclusion	96
	References	97
<u>Chapter 4: Concomitant delivery of Navitoclax with Decitabine for synergistic combination therapy in acute myeloid leukemia and breast cancer model</u>		100-157
4.1	Introduction	104
PART-I Preparation and Characterization of Navitoclax/Decitabine Loaded Polymeric Nanoparticles		
4.2	Materials and Methods	105
4.2.1	Synthesis of navitoclax single and dual loaded copolymeric nanoparticles	106
4.2.2	Characterization of NAV/DCB single and dual loaded nanoparticles	107
4.2.3	<i>In vitro</i> release and stability studies of NAV/DCB loaded polymeric nanoparticles	108
4.2.4	<i>In vitro</i> cellular uptake and localization studies for dye tagged dual loaded NPs	110
4.2.5	Biocompatibility studies of polymeric NPs	110
4.3	Results and discussions	111
4.3.1	Synthesis and characterization of NAV/DCB loaded polymeric NPs	111
4.3.2	<i>In vitro</i> release and stability studies for polymeric NPs	115
4.3.3	<i>In vitro</i> cellular uptake and localization studies of dye tagged dual loaded NPs	119
4.3.4	Biocompatibility studies of polymeric NPs	121
PART-II Navitoclax/Decitabine Dual Loaded Polymeric NPs for Acute Myeloid Leukemia		
4.4	Methods	123
4.4.1	<i>In vitro</i> cytotoxicity assay of NAV/DCB single and dual NPs	123

4.4.2	Effect of different molar ratios on therapeutic efficacy	124
4.4.3	Time dependent inhibition of proliferation assay for NAV/DCB NPs	124
4.4.4	<i>In vivo</i> dose tolerability study of NAV/DCB dual loaded polymeric NPs	124
4.4.5	<i>In vivo</i> evaluation of tumor inhibition efficacy of NAV/DCB dual loaded NPs in AML xenograft model	125
4.5	Results and Discussion	125
4.5.1	<i>In vitro</i> cytotoxicity assay of NAV/DCB single and dual NPs	125
4.5.2	Effect of different molar ratios on therapeutic efficacy	127
4.5.3	Time dependent inhibition of proliferation assay for NAV/DCB NPs	127
4.5.4	<i>In vivo</i> dose tolerability study for NAV/DCB dual loaded NPs	129
4.5.5	<i>In vivo</i> evaluation of tumor inhibition efficacy of NAV/DCB dual NPs in AML xenograft model	130

PART-III Navitoclax/Decitabine Dual Loaded Polymeric NPs for breast cancer

4.6	Methods	132
4.6.1	<i>In vitro</i> cytotoxicity assay of NAV/DCB single and dual NPs	132
4.6.2	Effect of dual NPs with different molar ratios on therapeutic efficacy	132
4.6.3	Time dependent inhibition of proliferation assay of NAV/DCB NPs	133
4.6.4	<i>In vitro</i> analysis of mitochondrial depolarization	133
4.6.5	Apoptosis assay with Annexin V/PI staining	133
4.6.6	Determination of <i>in vivo</i> platelet toxicity of polymeric NPs	134
4.6.7	Pharmacokinetics and biodistribution studies of NAV/DCB dual loaded polymeric NPs	135
4.6.8	<i>In vivo</i> evaluation of tumor inhibition efficacy of NAV/DCB NPs in syngeneic breast cancer model	136

4.7	Results and discussion	137
4.7.1	<i>In vitro</i> evaluation of synergistic potential of NAV/DCB polymeric NPs	137
4.7.2	Effect of dual NPs with different molar ratios on synergistic efficacy	140
4.7.3	Time dependent inhibition of proliferation assay of NAV/DCB NPs	141
4.7.4	<i>In vitro</i> analysis of mitochondrial depolarization	141
4.7.5	Apoptosis assay with Annexin V/PI staining	142
4.7.6	Determination of <i>in vivo</i> platelet toxicity for polymeric NPs	143
4.7.7	Pharmacokinetic and biodistribution studies of NAV/DCB dual loaded polymeric NPs	145
4.7.8	<i>In vivo</i> evaluation of drug loaded NPs for tumor inhibition efficacy and survival study in syngeneic breast cancer model	147
4.8	Conclusion	153
	References	155
	<u>Chapter V: Summary and scope for future work</u>	158-164
5.1	Summary and Conclusion	160
5.2	Future scope of the work	164

List of Figures

Chapter 1: Introduction and literature review

- Figure 1.1** Therapeutic approaches for targeting hallmarks of cancer.
- Figure 1.2** Schematic representation of different categories of nanomaterials applied in cancer drug delivery.
- Figure 1.3** Expression of different anti-apoptotic members of BCL-2 protein family a) BCL-2, b) MCL-1, c) BCL-W and d) BCL-x in cancer. Bar charts depict percentage of patient samples with positive expression of the various proteins detected by immunohistochemistry in different tumor histotypes.
- Figure 1.4** Schematic representation of the mitochondrial apoptotic pathway and its regulation by BCL-2 family of proteins. Activation of BAX/BAK pro-apoptotic proteins in response to cellular stress leads to mitochondrial outer membrane permeabilization and cytochrome c release into the cytosol. Activation or oligomerization of BAX/BAK is induced by BH3 only proteins. Anti-apoptotic proteins can inhibit this oligomerization by directly interacting with BAK/BAX or by blocking the BH3 only proteins.
- Figure 1.5** Schematic representation of different models of mitochondria outer membrane potential regulation by members of the BCL-2 family of proteins a) direct activation, b) displacement, c) embedded together and d) unified model. (↑) Activation; (⊥) inhibition; (⊥↑) mutual recruitment/sequestration.
- Figure 1.6** Schematic representation of research work carried out in the present thesis

Chapter 2: Synthesis and characterization of PLA based block copolymers for development of MS1 peptide loaded nanoparticles

- Figure 2.1** a) Schematic representation of synthesis of PLA72kDa-PEG-PPG-PEG block copolymer via Steglich esterification of PLA72kDa with Pluronic F-127 and characterization of the block copolymer using b) ¹H NMR (a-5.18ppm, b-3.66ppm, c-1.78ppm and d-1.27ppm) and c) FTIR spectroscopy
- Figure 2.2** a) Schematic representation of polycondensation of L-Lactic acid for synthesis of PLA17kDa and its subsequent conjugation with Pluronic F-127, characterization of PLA17kDa, PEG-PPG-PEG and PLA17kDa-PEG-PPG-PEG using b) ¹H NMR (a-5.18ppm, b-3.66ppm, c-1.78ppm and d-1.27ppm) and c) FTIR spectroscopy
- Figure 2.3** a) Schematic representation of ring opening polymerization of L-Lactide with Pluronic L-61 and m-PEG 5000 as initiators for synthesis of PLA-mPEG/PLA-L-61-PLA hybrid block copolymer and its characterization using b) ¹H NMR (a-

5.18ppm, b- 4.36ppm, c-3.6ppm, d-1.58ppm and e-1.28ppm) and c) FTIR spectroscopy

Figure 2.4 Comparison of hydrodynamic diameters of a) blank and b) MS1 loaded NPs prepared by solvent evaporation method using PLA72kDa-PEG-PPG-PEG, PLA17kDa-PEG-PPG-PEG and PLA-mPEG/PLA-L-61-PLA block copolymers

Figure 2.5 *In vitro* release kinetics of MS1 NPs prepared using PLA72kDa-PEG-PPG-PEG, PLA17kDa-PEG-PPG-PEG and PLA-mPEG/PLA-L-61-PLA block copolymers (mean $\pm$ SD, n=3). MS1 NPs were dispersed in PBS at pH 7.4 at a loaded peptide concentration of 1mg/mL and kept on orbital shaking with 150rpm. Released MS1 peptide was quantified using BCA assay.

Chapter 3: Concomitant delivery of MS1 peptide and Paclitaxel for evaluation of synergistic potential in breast cancer model

Figure 3.1 Field Emission-Scanning electron microscopy images of a) MS1 NPs, b) PTX NPs, c) MS/PTX 3:1 NPs, d) MS/PTX 1:1 NPs, e) MS/PTX 1:3 NPs. All copolymeric nanoparticles were sputter coated with gold under argon atmosphere prior to imaging at 10kV

Figure 3.2 High Resolution-Transmission electron microscopy images of a) MS1 NPs, b) PTX NPs, c) MS/PTX 3:1 NPs, d) MS/PTX 1:1 NPs, e) MS/PTX 1:3 NPs. NPs were drop casted on carbon coated copper grids and stained with uranyl acetate prior to imaging.

Figure 3.3 *In vitro* release profile for a) MS1 NPs, b) PTX NPs and c) MS1/PTX NPs in PBS at pH7.4. The NPs were dispersed at loaded peptide/drug concentration of 1mg/mL at 37 $\pm$ 2°C with orbital shaking at 150 rpm.

Figure 3.4 Percentage hemolysis of murine RBCs on exposure to prepared blank, MS1, PTX and MS1/PTX dual loaded nanoparticles for 2 hours at 37 $\pm$ 2°C. Positive control – double distilled water

Figure 3.5 Cellular uptake -Confocal laser scanning microscopy images of MCF-7 cells incubated with FITC-PTX and Rhodamine B-MS1 dual loaded NPs for 2 hours at 37 $\pm$ 2°C. (Blue: DAPI, Green: FITC-PTX and Red: Rhodamine B-MS1).

Figure 3.6 Intracellular localization of MS1 peptide: Confocal laser scanning microscopy images of MCF-7 cells incubated with RhoB-MS1 NPs for 2 hours at 37 $\pm$ 2°C and cross stained with MitoTracker Green dye. Co-localization of MS1 with mitochondria evaluated using Pearson's coefficient (Pearson's coefficient=1 represents 100% colocalization).

Figure 3.7 Cytotoxicity of MS1 NPs on SK-BR-3, ZR-75-1, MCF-7, MDA-MB-468 and SUM-149 cells breast cancer cell lines as determined by MTT assay. 5 $\times$ 10³ cells/well were exposed to a range of MS1 NPs (0.1-10000nM) for 72 hours.

- Figure 3.8** Cytotoxicity of MS1 NPs on SK-BR-3, ZR-75-1, MCF-7, MDA-MB-468 and SUM-149 cells breast cancer cell lines as determined by MTT assay. 5×10^3 cells/well were exposed to a range of MS1 NPs (0.1-10000nM) for 72 hours.
- Figure 3.9** Cytotoxicity of MS1 NPs, PTX NPs and MS1/PTX dual NPs on a) SUM-149, b) MDA-MB-468 c) SK-BR-3 and d) EAC breast cancer cell lines. For SUM-149, MDA-MB-468 and SK-BR-3, 5×10^3 cells/well and for EAC 2.5×10^3 cells/well were exposed to MS1/PTX single and dual NPs for 72 hours in a concentration range 0.1-10000nM and cytotoxicity was determined using MTT assay.

Chapter 4: Concomitant delivery of Navitoclax with Decitabine for synergistic combination therapy in acute myeloid leukemia and breast cancer model

- Figure 4.1** Field emission-scanning electron microscopy images of a) NAV NPs, b) DCB NPs, c) NAV/DCB dual NPs. Copolymeric nanoparticles were sputter coated with gold under argon atmosphere prior to imaging at 10kV.
- Figure 4.2** High resolution-transmission microscopy images of a) DCB NPs, b) NAV NPs, c) NAV/DCB dual NPs. NPs were drop casted on carbon coated copper grids and stained with osmium tetroxide prior to imaging.
- Figure 4.3** Serum release profile of navitoclax/decitabine single and dual NPs: In vitro drug release from DCB NPs, NAV NPs and DCB/NAV NPs at 24, 48 and 72 hours in PBS containing 10% fetal bovine serum. NPs were incubated with release buffer at a loaded drug concentration of 1mg/mL and kept at $37 \pm 2^\circ\text{C}$ under orbital shaking at 150 rpm.
- Figure 4.4** Aqueous stability of a) Navitoclax NPs, b) Decitabine NPs, c) Navitoclax/Decitabine dual NPs in PBS 7.4 at $37 \pm 2^\circ\text{C}$.
- Figure 4.5** Serum stability of Navitoclax/Decitabine single and dual NPs as observed in PBS 7.4 with 10% fetal bovine serum at $37 \pm 2^\circ\text{C}$
- Figure 4.6** In vitro protein adsorption quantification for blank, Navitoclax/Decitabine single and dual loaded NPs after 6 and 24 hours of incubation with bovine serum albumin (1mg/mL).
- Figure 4.7** Cellular uptake analysis of dye tagged NAV/DCB dual loaded NPs in EAC cells post 2 hours treatment by a) confocal laser scanning microscopy (Red: RhoB-DCB Green: FITC-Navitoclax Blue: DAPI) and b) flow cytometry (95.5% positive cells in red channel representing RhoB-DCB uptake and 99.9% positive cells in green channel representing FITC-NAV uptake)
- Figure 4.8** Intracellular localization of Navitoclax: Confocal laser scanning microscopy images of EAC cells incubated with FITC-NAV NPs for 2 hours at $37 \pm 2^\circ\text{C}$ and cross stained with MitoTracker dye. Co-localization of navitoclax with mitochondria evaluated using Pearson's coefficient (Pearson's coefficient=1 represents 100% colocalization)

- Figure 4.9** Cytocompatibility of prepared blank, NAV/DCB single and dual loaded nanoparticles on noncancerous human embryonic kidney HEK-293 cells as determined by MTT assay after 72 hours of NPs incubation.
- Figure 4.10** Percentage hemolysis of murine RBCs on exposure to prepared blank, DCB, NAV and DCB/NAV dual loaded nanoparticles for 2 hours at $37\pm 2^{\circ}\text{C}$. Positive control – double distilled water
- Figure 4.11** Cytotoxicity of navitoclax/decitabine single and dual NPs against a) THP-1 and b) WEHI-3B cells as determined by CCK-8 assay. 10^4 cells/well were exposed to NAV/DCB single and dual NPs for 72 hours at a concentration range 0.1-1000nM
- Figure 4.12** Comparison of therapeutic efficacy of NAV/DCB dual NPs loaded with different molar ratios of NAV and DCB in a) THP-1 and b) WEHI-3B cells as determined by CCK-8 assay. 10^4 cells/well were exposed to NAV/DCB dual NPs for 72 hours at a concentration range 0.1-1000nM
- Figure 4.13** Time dependent cytotoxicity of NAV/DCB single and dual NPs in THP-1 cells as assessed by CCK-8 assay at 24, 48, 72 and 96 hours. 10^4 cells/well were exposed to NAV, DCB and NAV/DCB NPs at $1\mu\text{M}$ concentration
- Figure 4.14** In vivo dose tolerability determination for NAV/DCB dual NPs: Dose tolerability was evaluated in healthy BALB/c mice using intravenous injections of NAV/DCB NPs via tail vein. Three dosing schedules – 7.5mg/Kg twice a week (green), 10mg/Kg once a week (maroon) and 10mg/Kg twice a week (black) were evaluated. Body weight was measured every three days.
- Figure 4.15** Tumor inhibition efficacy of NAV/DCB NPs in AML xenograft model. Tumor bearing mice were divided into two groups: Control and NAV/DCB NPs treated. Treated mice were given twice weekly I.V. doses of dual NPs via tail vein at total drug dose of 7.5mg/Kg -100 μL for three weeks
- Figure 4.16** Cytotoxicity of NAV/DCB single and dual NPs on a) MCF-7, b) MDA-MB-468 and c) EAC breast cancer cells as determined by MTT assay. 5×10^3 cells/well were exposed to a drug concentration range of (0.1-10000nM) for 72 hours.
- Figure 4.17** Comparison of therapeutic efficacy of NAV/DCB dual NPs loaded with different molar ratios of NAV and DCB in a) MDA-MB-468 and b) EAC cells as determined by MTT assay. 5×10^3 cells/well for MDA-MB-468 and 2.5×10^3 cells/well for EAC were exposed to NAV/DCB dual NPs for 72 hours at a concentration range 0.1-1000nM
- Figure 4.18** Time dependent cytotoxicity of NAV/DCB single and dual NPs in EAC cells as assessed by CCK-8 assay at 24, 48, 72 and 96 hours. 2.5×10^3 cells/well were exposed to NAV, DCB and NAV/DCB NPs at $1\mu\text{M}$ concentration
- Figure 4.19** Analysis of mitochondrial membrane potential in EAC cells after treatment with NAV/DCB single and dual NPs. 10^5 cells/well were exposed to 100nM DCB, NAV

and NAV/DCB NPs for 48 hours and change in mitochondrial membrane potential was assayed using flow cytometry via Rhodamine123 dye

- Figure 4.20** Analysis of apoptosis using confocal laser scanning microscopy and flow cytometry in Annexin V/PI dual stained EAC cells after 48 hours of incubation with 100nM NAV/DCB single and dual NPs
- Figure 4.21** Nanoencapsulation of navitoclax ameliorates thrombocytopenia: platelet count in healthy BALB/c mice 24 hours after intravenous injection of 10mg/Kg Navitoclax, NAV NPs and NAV/DCB NPs.
- Figure 4.22** (a) Pharmacokinetics and (b, c) biodistribution of NAV/DCB dual NPs in EAC syngeneic tumor bearing BALB/c mice. Mice were administered intravenous injection of NAV/DCB NPs via tail vein at a single combined dose of 7.5mg/Kg
- Figure 4.23** Tumor inhibition and survival study: a) EAC tumor growth curve after inoculation in BALB/c mice and serial I.V. treatment with NAV NPs (6.15mg/Kg), DCB NPs(1.35mg/Kg) and NAV/DCB NPs (7.5mg/Kg) on days 1,4,8,11,15 and 18. b) Kaplan-Meier survival curves of EAC syngeneic tumor bearing mice post treatment with NAV/DCB single and dual NPs
- Figure 4.24** Microscopic images of tissues stained with H&E after isolation from a) control, b) DCB NPs, c) NAV NPs and d) NAV/DCB NPs treated mice. First panel – Control- normal heart, kidney and spleen while liver shows mild sinusoidal congestion (SC) and lung shows mild alveolar congestion (AC). Section from tumor shows focal tumor necrosis (N) and vascular proliferation (VP). Second panel – DCB NPs – Heart, lung and spleen are normal while kidney shows edema around the glomeruli (E) while the liver shows portal triaditis (PT) and sinusoidal congestion (SC). Section from tumor shows focal tumor necrosis (N). Third panel – NAV NPs – Heart, kidney and spleen are normal while liver shows mild sinusoidal congestion (SC). Section from tumor shows moderate necrosis. Fourth panel – NAV/DCB NPs – Heart, spleen and kidney are normal while liver shows mild sinusoidal congestion (SC). Section from tumor shows extensive tumor necrosis (N)
- Figure 5.1** Schematic representation of chapter-wise accomplishments as concluded in this thesis

List of Tables

Chapter I: Introduction and literature review

Table 1.1 FDA approved combination therapies comprising of two or more biological agents

Table 1.2 FDA approved combination therapies comprising of two or more cytotoxic agents

Table 1.3 FDA approved combination cancer therapies comprising of cytotoxic and biological agents

Table 1.4 Nanoformulations approved by the FDA or in clinical trial phase for cancer therapy

Table 1.5 Nanoformulations in clinical trial or approved by the FDA for combination cancer therapy

Table 1.6 Inhibitors of BCL-2 family of proteins currently reported in literature

Table 1.7 Combination therapy approaches using BCL-2 family inhibitors in active clinical trial or approved for cancer therapy

Chapter 2: Synthesis and characterization of PLA based block copolymers for development of MS1 peptide loaded nanoparticles

Table 2.1 Gel permeation chromatography data for the different synthesized block copolymers

Table 2.2 Physicochemical properties of blank and MS1 peptide loaded nanoparticles prepared using the different synthesized PLA based block copolymers

Chapter 3: Concomitant delivery of MS1 peptide and Paclitaxel for evaluation of synergistic potential in breast cancer model

Table 3.1 Physiochemical properties of MS1/PTX single and dual loaded NPs

Table 3.2 Encapsulation efficiency for MS1/PTX single and dual loaded NPs

Table 3.3 Comparison of IC₅₀ values of MS1 NPs, PTX NPs and MS1/PTX NPs on different breast cancer cell lines.

Chapter 4: Concomitant delivery of Navitoclax with Decitabine for synergistic combination therapy in acute myeloid leukemia and breast cancer model

Table 4.1 Physico-chemical characterization of navitoclax/decitabine single and dual NPs

Table 4.2 Comparison of CI values for NAV/DCB NPs with different molar ratios

Table 4.3 Treatment groups for tumor inhibition study in murine breast cancer syngeneic model

Table 4.4 IC₅₀ value comparison for breast different cell lines treated with NAV/DCB single and dual polymeric NPs

Table 4.5 Hematology analysis of blood isolated from control, DCB NPs, NAV NPs and NAV/DCB NPs treated tumor bearing mice

Table 4.6 Serum biochemistry analysis of blood isolated from control, DCB NPs, NAV NPs and NAV/DCB NPs treated tumor bearing mice

ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microliter
5-FU	5-Flourouracil
Abx	Abraxane
ACN	Acetonitrile
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AML	Acute Myeloid Leukemia
ASO	Anti-sense Oligonucleotide
AST	Aspartate Amino Transferase
ATP	Adenosine Triphosphate
ATR-FTIR	Attenuated Total Reflection-Fourier transform Infrared Spectroscopy
BAD,	BCL-2 Associated Death Promoter
BAK	BCL-2 Homologous Antagonist Killer
BAX	BCL-2 Associated X Protein
BCA	Bicinchonic Assay
BCL-2	B-cell Lymphoma 2
BFL-1/BCL2A1	BCL-2 Related Protein A1
BH3	BCL-2 Homology 3
BH4	BCL-2 Homology 4
BID	BH-3 Interacting Domain Death Agonist

BIK	BCL-2 Interacting Killer
BIM	BCL-2 like Protein 11
BMF	BCL-2 Modifying Factor
BOK	BCL-2 Related Ovarian Killer
BRAF	V-RAF Murine Sarcoma Viral Oncogene Homolog B1
BUN	Blood Urea Nitrogen
°C	Degree Centigrade
CAR	Chimeric Antigen Receptor
CBC	Complete Blood Count
CEBPA	CCAAT Enhancer Binding Protein Alpha
CI	Combination Index
CLL	Chronic Lymphocytic Leukemia
CLSM	Confocal Laser Scanning microscope
CTLA-4	Cytotoxic T-Lymphocyte–Associated Antigen 4
DAPI	4',6-diamidino-2-phenylindole
DCB	Decitabine
DCB NPs	Decitabine loaded copolymeric nanoparticles
DCC	N, N-dicyclohexuylcarbodiimide
DMAP	4-dimethylaminopyridine
DLS	Dynamic Light Scattering
DMEM	Dulbecco's modified eagle's media
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid

EAC	Ehrlich Ascites Carcinoma
EDTA	Ethylene-diamine-tetra acetic acid
EE%	Encapsulation Efficiency %
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FE-SEM	Field emission – scanning electron microscopy
FITC	Fluorescein isothiocyanate
FLT3	FMS-Like Tyrosine Kinase 3
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
gm	Gram
GPC	Gel Permeation Chromatography
HEK 293	Human Embryonic Kidney 293
HER2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
HIF 1	Hypoxia Inducible Factor 1
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRK	Harakiri, BCL-2 Interacting Protein
HR-TEM	High Resolution- Transmission Electron Microscopy
hrs	Hour
H & E	Hematoxylin and Eosin stain
IAEC	Institutional Animal Ethics Committee

IC ₅₀	50% Inhibitory Concentration
INF	Interferon
IP	Intraperitoneal
ITD	Internal Tandem Duplication
IV	Intravenous
IU	International Unit
K	Kelvin
kDa	Kilo Dalton
Kg	Kilogram
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
Kv	Kilo Volt
MCL-1	Myeloid Cell Leukemia-1
MCF-7	Michigan Cancer Foundation-7
MDA-MB-468	M.D. Anderson Metastatic Breast Cancer 468
MDR	Multi drug resistance
mg	Milligram
mm	Millimeter
mM	Millimole
MM	Multiple Myeloma
Mn	Number Average Molecular Weight
MOMP	Mitochondrial Outer Membrane Potential
MS1 NPs	MS1 Peptide Loaded Copolymeric Nanoparticles
Mw	Weight Average Molecular Weight
MWCO	Molecular Weight Cut-Off

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide
MUC-1	Mucin-1
MYC	Human Homolog of Avian Viral Myelocytomatosis Gene
NAV	Navitoclax
NAV NPs	Navitoclax loaded copolymeric nanoparticles
NC	Negative Control
NCT	National Clinical Trial
nm	Nanometer
nM	Nano mole
NMR	Nuclear Magnetic Resonance
NOD	Non-Obese Diabetic
NPs	Nanoparticles
NSCLC	Non-Small Cell Lung Cancer
NuBCP-9	Nur 77 Derived BCL-2 Converting Peptide-9
OD	Optical Density
PARP	Poly Adenosine Diphosphate-Ribose Polymerase
PBS	Phosphate Buffer Saline
PC	Positive Control
PCL	Polycaprolactone
PD	Pharmacodynamics
PD-L1	Programmed Death -Ligand 1
PDI	Polydispersity Index
PEG	Polyethylene glycol
PGA	Polyglycolic Acid

P-gp 1	Poly-glycoprotein 1
pH	Logarithmic value of hydrogen
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PK	Pharmacokinetics
PLA	Polylactic acid
PLGA	Poly (lactic-co-glycolic acid)
PPG	Polypropylene glycol
ppm	Parts per Million
PROTAC	Proteolysis Targeting Chimera
PS	Phosphatidylserine
p-TSA	p-Toluenesulfonic acid
PTX	Paclitaxel
PTX NPs	Paclitaxel loaded copolymeric nanoparticles
PUMA	p53 Upregulated Modulator of Apoptosis
RBC	Red Blood Cells
RhoB	Rhodamine B
RNA	Ribodeoxynucleic Acid
ROP	Ring Opening Polymerization
RPM	Rotation per minute
RT	Room Temperature
SAHB	Stabilized α -Helices of BCL-2 Domains
SC	Subcutaneous
SCID	Severe Combined Immune Deficient

SCLC	Small Cell Lung Cancer
SD	Standard Deviation
SGOT,	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
TFA	Trifluoroacetic Acid
TKI	Tyrosine Kinase Inhibitor
TME	Tumor Microenvironment
TNBC	Triple Negative Breast Cancer
TOR	Target of Rapamycin
TUSC2	Tumor Suppressor Candidate 2
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cells
WHO	World Health Organization