Identification of CDKs as Novel Targets of Aspirin and Its Metabolites: A Potential Role in Cancer Prevention

Rakesh Dachineni
South Dakota State University
IDENTIFICATION OF CDKS AS NOVEL TARGETS OF ASPIRIN AND ITS
METABOLITES: A POTENTIAL ROLE IN CANCER PREVENTION

BY

RAKESH DACHINENI

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Pharmaceutical Sciences

South Dakota State University

2017
IDENTIFICATION OF CDKS AS NOVEL TARGETS OF ASPIRIN AND ITS
METABOLITES: A POTENTIAL ROLE IN CANCER PREVENTION

RAKESH DACHINENI

This dissertation is approved as a creditable and independent investigation by a
candidate for the Doctor of Philosophy in Pharmaceutical Sciences degree and is
acceptable for meeting the dissertation requirements for this degree. Acceptance of this
does not imply that the conclusions reached by the candidates are necessarily the
conclusions of the major department.

G. Jayarama Bhat, Ph.D. Date

Dissertation Advisor

Omathanu Perumal, Ph.D. Date

Head, Department of Pharmaceutical Science

Dean, Graduate School Date
It is with deepest gratitude and affection, I would like to dedicate this thesis to my late Grandfather Dachineni Anjaneyulu for making me who I am today.
ACKNOWLEDGMENTS

This dissertation would not be completed without the support of my advisor, my family and friends. I would to thank everyone who has been in this journey with me over the last 4 years.

Primarily, I would like to thank my advisor Dr. Jayarama B Gunaje for giving me the opportunity to join his lab and work with him towards my Ph.D. on this exciting field of chemoprevention. I am lucky to have Dr. Jay as my advisor; he cared so much about my work and professional progress. He is the hardest working person I have met in my life. His ethical standards in scientific research and flexibility to change hypothesis based on the results and ability to dissect out the complicated phenomenon in simple experiments are remarkable. I consider him as an excellent example as a researcher, mentor, teacher and role model. He has always been a positive influence on me both in my work and personal life.

Besides my advisor, I would like to thank Dr. Hemachand Tummala for his support and guidance in training me towards drug delivery research. I got an opportunity to work with him independently and learn a lot more about the field of drug delivery. He also influenced my way of looking at and analyzing things in science and life. Dr. Jay and Dr. Tummala in their own kind way have helped me understand research.

I would like to thank my graduate advisory committee members Dr. Michael Hildreth, Dr. Jai Rohila and Dr. Michael Wimberly for their valuable advice and their encouragement. I also would like to thank Dr. D. Ramesh Kumar (University of Kentucky), Dr. Teresa Seefeldt, and Dr. Eduardo Callegari (USD Proteomics facility) for their support towards my research project.
I would like to thank my lab mates Dr. Guoqiang Ai and Ms. Ranjini Sankaranarayanan for their support and help in my research project. I also would like to thank Mr. Metab Alharbi, Mr. Siddharth Kesharwani, Mr. Somshuvra Bhattacharya and Mr. Shengang Wang for their support over the last 4 years. They have influenced, motivated and supported me both in my research and personal life. I also would like to thank other students in the department for their assistance, stimulating scientific discussions, and friendship.

I would like to thank the families of Dr. Ranjith Kumar Averineni, Dr. Satya Sai Sadhu and Dr. Kaushal Dave for their support and making me feel at home. I also would like to thank my friends Mr. Sundeep Vadlamudi, Mr. Chaitanya Krishna Valiveti, Mr. Keasava Sarath Chandra Lakamsani, Mr. Mahesh Donapati and Mr. Bhīma Sasikanth Reddy for being there for me through thick and thin over the last 8 years.

I would like express my sincere thanks to Dr. Om Perumal the chair and the Department of Pharmaceutical Sciences for providing all the facilities and resources. Support from the Translational Cancer Research Seed Grant, funded as 2010 Research Initiative Center by the State of South Dakota, Faculty Excellence Fund from South Dakota State University and from NIH (5RO3CA133061-02) to GJB is also gratefully acknowledged.

Finally, I would like to thank my family: my grandparents, my parents, my aunts and uncles, and my brothers and sisters for their unconditional love and support during my Ph.D. study. I would like to dedicate this thesis and my research work to my late grandfather Mr. Dachineni Anjaneyulu. I am deeply indebted to the support and
encouragement he had provided me throughout my educational career and also for instilling in me the importance of education.
# Table of Contents

**ABBREVIATIONS** ............................................................................................................. xiv

**LIST OF FIGURES** ........................................................................................................ xvii

**LIST OF TABLES** ........................................................................................................... xx

**ABSTRACT** ...................................................................................................................... xxi

**Chapter 1: Introduction** ................................................................................................ 1

1.1 Cancer as a genetic disease ..................................................................................... 1

1.2 Risk factors ............................................................................................................... 2

1.2.1 Genomic instability in colon tumorigenesis ....................................................... 2

1.3 Mutational inactivation of tumor suppressor genes ............................................ 3

1.3.1 APC ................................................................................................................... 3

1.3.2 p53 ..................................................................................................................... 4

1.3.3 TGF-β ......................................................................................................... 4

1.4 Mutational activation of oncogenes ...................................................................... 4

1.5 DNA Repair defects ............................................................................................... 5

1.6 Aberrant DNA methylation ................................................................................. 6

1.7 Inherited syndromes (FAP and HNPCC) ............................................................. 7

1.8 Acquired gene mutations ...................................................................................... 7

1.9 Tumor development: Transformation of a normal cell into a tumor cell .......... 8

1.10 Hallmarks of cancer and therapeutic targets .................................................... 11
1.10.1 Sustaining proliferative signaling ................................................................. 13
1.10.2 Evading growth suppressors ........................................................................... 13
1.10.3 Resisting Cell Death ....................................................................................... 13
1.10.4 Inducing Angiogenesis ................................................................................... 14
1.10.5 Activating Invasion and Metastasis ................................................................. 14
1.10.6 Enabling replicative immortality .................................................................... 15
1.10.7 Emerging Hallmarks ....................................................................................... 15
1.11 Cell cycle ........................................................................................................... 15
  1.11.1 Activation of CDKs ....................................................................................... 18
  1.11.2 Cyclins ......................................................................................................... 19
  1.11.3 CKIs ............................................................................................................ 19
  1.11.4 Role of Rb phosphorylation in cell cycle progression ................................. 20
  1.11.5 Cell cycle and cancer ................................................................................... 21
1.12 CDK inhibitors as anti-cancer drugs ................................................................. 22
  1.12.1 First-generation CDK inhibitors ................................................................. 22
  1.12.2 Second-generation CDK inhibitors ............................................................ 23
1.13 Salicylic acid and the discovery of aspirin .......................................................... 24
1.14 Aspirin as a drug to prevent cardiovascular disease .......................................... 28
1.15 Aspirin’s recommended dose, absorption, metabolism and side effects ........... 28
Chapter 1: Aspirin and Cancer

1.16 Aspirin and cancer ................................................................. 31

1.16.1 Mechanisms of chemoprevention by aspirin .................... 31

1.16.2 COX-dependent mechanism .............................................. 31

1.16.3 COX-Independent mechanisms .......................................... 34

1.16.4 Salicylic acid Binding proteins (SABP) ................................. 35

1.17 Rationale for studies on Cyclins and CDKs, and hypothesis ........ 39

Chapter 2: Materials and Methods ................................................. 41

2.1 Materials .................................................................................. 41

2.2 Buffers ..................................................................................... 44

2.3 Cell lines, culture media and other reagents ............................ 45

2.4 Recombinant proteins, enzymes, plasmid DNA and antibodies ..... 46

2.5 Cell Culture ............................................................................. 46

2.6 Cell proliferation ....................................................................... 47

2.7 Total cell lysate preparation and Western Blotting .................... 47

2.8 Protein estimation Using Bradford’s Reagent ............................ 48

2.9 Immunoprecipitation ................................................................. 48

2.10 Agar Plates .............................................................................. 48

2.11 Transformation of E. coli with plasmid DNA ............................ 49

2.12 Isolation of plasmid DNA and their insert ............................... 49

2.13 RNA isolation and Northern blot analysis ............................... 49
2.14 RT PCR ................................................................. 50
Table 2.1 List of CDK1, cyclin B1 and GAPDH primers used for RT-PCR ........ 51
2.15 Expression of recombinant DDK-tagged proteins ......................... 51
2.16 In-vitro CDK assay ................................................................... 51
2.17 Molecular docking studies .......................................................... 52
2.18 CDK2/ANS fluorescence assay .................................................. 53
2.19 Mass Spectrometry ..................................................................... 54
2.19 MTT Assay ................................................................................ 55
2.20 Statistical analysis ....................................................................... 55

Chapter 3: Cyclin A2 and CDK2 as Novel Targets of Aspirin and Salicylic acid ...... 56

3.1 Abstract ..................................................................................... 56
3.2 Background and Hypothesis .......................................................... 57
3.3 Results ....................................................................................... 57
  3.3.1 Aspirin and salicylic acid decrease cell proliferation in HT-29, SK-MEL-28, and MDA-MB-231 cells ................................................................. 57
  3.3.2 Effect of aspirin and salicylic acid on cell cycle regulatory proteins .... 59
  3.3.3 Aspirin and salicylic acid decrease cyclin A2 levels in multiple cell lines ...... 64
  3.3.4 Lactacystin completely prevents aspirin and salicylic acid-mediated down-regulation of cyclin A2 levels ............................................................... 64
3.3.5 Aspirin and salicylic acid decrease exogenously expressed, DDK-tagged, cyclin A2 protein levels ................................................................. 65

3.3.6 Aspirin and salicylic acid decrease cyclin A2 mRNA levels ............... 67

3.3.7 Aspirin and salicylic acid down-regulate CDK2 protein and mRNA levels in HT-29 cells ........................................................................................................ 67

3.3.8 Salicylic acid decreases CDK2 activity in HT-29 and SK-MEL-28 cells .. 70

3.3.9 Inclusion of salicylic acid during immunoprecipitation enhances the ability of anti-CDK2 antibody to immunoprecipitate CDK2 in HT-29 naïve total cell lysates 70

3.3.10 Inclusion of salicylic acid during in-vitro kinase assay does not affect the CDK2 activity................................................................................................. 73

3.3.11 Salicylic acid increases the ability of anti-CDK2 antibodies to bind to the purified recombinant CDK2 protein.......................................................... 74

3.3.12 Pre-incubation of salicylic acid with CDK2 decreases fluorescence due to ANS .............................................................................................................. 77

3.3.13 Molecular docking studies show potential interactions of salicylic acid with CDK2 and cyclin A2 ...................................................................................... 79

3.4 Additional Studies .................................................................................. 81

3.4.1 Aspirin and salicylic acid decrease nuclear cyclin B1 and CDK 1 protein levels in HT-29 cells ......................................................................................... 81
3.4.2 Effect of aspirin and salicylic acid on exogenously expressed, DDK-tagged, CDK1 and cyclin B1 levels ................................................................. 82

3.4.3 Lactacystin completely prevents aspirin and salicylic acid-mediated down-regulation of cyclin B1 levels ........................................................................ 85

3.4.3 Aspirin and salicylic acid decrease cyclin B1 and CDK1 mRNA levels in HT-29 cells ........................................................................................................... 87

3.5 Discussion ........................................................................................................ 89

Chapter 4: Salicylic Acid Metabolites and Derivatives Inhibit CDK Activity .......... 96

4.1 Abstract ........................................................................................................... 96

4.2 Background and Hypothesis ........................................................................ 97

4.3 Results ............................................................................................................ 98

4.3.1 Effect of aspirin, salicylic acid and salicylic acid derivatives on CDK1 kinase activity *in-vitro* .................................................................................................. 98

4.3.2 Dose dependent inhibition of CDK1 enzyme activity by 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA ..................................................................................... 99

4.3.3 Molecular docking studies show potential interactions of aspirin, salicylic acid, salicylic acid metabolites and derivatives with CDK1 and cyclin B1 .......... 102

4.3.4 Aspirin acetylates recombinant CDK1, and pre-incubation with salicylic acid, 2,3-DHBA, 2,6-DHBA or 2,4,6-THBA inhibits aspirin’s ability to acetylate CDK1 .............................................................................................................. 109

4.3.5 Aspirin acetylates cellular CDK1 .................................................................. 110
4.3.6 Effect of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA on
CDK2, CDK4 and CDK6 enzyme activity ................................................................. 112

4.3.7 Identification of aspirin-acetylated lysine residues on CDK1 ................. 116

4.4 Discussion ........................................................................................................... 119

Chapter 6: Summary, Conclusions, Significance, Limitations and Future Directions.....
........................................................................................................................................ 128

6.1 Summary and Conclusions ................................................................................. 128

6.2 Limitations and Future directions ..................................................................... 132

Chapter 7: Bibliography .......................................................................................... 134
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate activated Protein Kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspirin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Bak</td>
<td>BCL2-Antagonist/Killer</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2-Associated X Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaMKKβ</td>
<td>Ca2+/Calmodulin-dependent protein kinase kinase beta</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin Dependent Kinase 1</td>
</tr>
<tr>
<td>CDK2</td>
<td>Cyclin Dependent Kinase 2</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin Dependent Kinase 4</td>
</tr>
<tr>
<td>CDK6</td>
<td>Cyclin Dependent Kinase 6</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal Cancer</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>DHBA</td>
<td>Dihydroxy Benzoic Acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastro-Intestinal Tract</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary Non-Polyposis Colon Cancer</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INF-β</td>
<td>Interferon-β</td>
</tr>
<tr>
<td>Lac</td>
<td>Lactacystin</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver Kinase B1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MLH1</td>
<td>MutL Homolog 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MSH2</td>
<td>MutS Protein Homolog 2</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>MTT</td>
<td>Thiazolyl Blue Tetrazolinium Bromide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drug</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Sal</td>
<td>Salicylic Acid;</td>
</tr>
<tr>
<td>Sp</td>
<td>Specificity protein</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription Factor</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>Transforming Growth Factor β-Receptor II</td>
</tr>
<tr>
<td>THBA</td>
<td>Trihydroxy Benzoic Acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TXA2</td>
<td>Thromboxane A2</td>
</tr>
<tr>
<td>USPSTF</td>
<td>United State Preventive Services Task Force</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1.1 Transformation of a normal cell into a cancerous cell ................................. 10
Figure 1.2: Hallmarks of cancer and the potential therapeutic targets ............................. 12
Figure 1.3: Mammalian cell cycle ............................................................................. 17
Figure 1.4: Aspirin inhibits cyclooxygenases (COX), an enzyme in arachidonic acid metabolism. ............................................................................................................. 27
Figure 1.5: A model depicting how low dose aspirin inactivates COX-1 and contributes to the prevention of colorectal cancers ......................................................... 30
Figure 1.6: A model depicting how low dose aspirin inactivates COX-1 and contributes to the prevention of colorectal cancers. ................................................................. 33
Figure 1.7: Aspirin’s primary metabolite salicylic acid directly binds to cellular proteins NF-κB and IKK-β kinase (A) and AMP kinase (B) to modulate their activity... 38
Figure 3.1: Dose-dependent effect of aspirin and salicylic acid on the growth rates in HT-29, SK-MEL-28, and MDA-MB-231 cells. ................................................................. 58
Figure 3.2: Aspirin and salicylic acid down-regulate cyclin A2 protein levels in multiple cell lines. ................................................................................................................. 60
Figure 3.3: Dose-dependent effect of aspirin and salicylic acid on cyclins B1, D3 and E1 in HT-29 cells. ................................................................................................................. 61
Figure 3.4: Dose-dependent effect of aspirin and salicylic acid on CDKs 1, 4 and 6 in HT-29 cells: ................................................................................................................. 62
Figure 3.5: Dose-dependent effect of aspirin and salicylic acid on CDK inhibitors p-21 and p-27 in HT-29 cells. ......................................................................................................... 63
Figure 3.6: Down-regulation of cyclin A2 by aspirin and salicylic acid is mediated by 26S proteasomal pathway. ................................................................. 66

Figure 3.7: Down regulation of cyclin A2 mRNA by aspirin and salicylic acid in HT-29 cells ................................................................................................. 68

Figure 3.8: Aspirin/salicylic acid down-regulate CDK2 protein/mRNA levels and activity 69

Figure 3.9: Schematic representation of the steps involved in immunoprecipitation described for Fig. 3.10 A and B................................................................. 74

Figure 3.10: CDK2 as a Salicylic acid binding protein: ............................................................................. 76

Figure 3.11: ANS-CDK2 assay and a model showing potential salicylic acid binding: . 78

Figure 3.12: Molecular docking studies on aspirin and salicylic acid with CDK2, cyclin A2, and CDK2/cyclin A2 complex ................................................................................. 80

Figure 3.13: Aspirin and salicylic acid decreased exogenously expressed, DDK-tagged, CDK1 and cyclin B1 levels: ................................................................. 84

Figure 3.14: Down-regulation of cyclin B1, but not CDK1, by aspirin and salicylic acid is mediated by 26S proteasomal pathway................................................................. 86

Figure 3.15: Down regulation of cyclin B1 and CDK1 mRNA by aspirin and salicylic acid in HT-29 cells: ................................................................................................. 88

Figure 4.1: *In-vitro* kinase assays showing the effect of aspirin, salicylic acid metabolites and derivatives on CDK1 enzyme activity. ...................................................... 100

Figure 4.2: Molecular docking studies (space-filling model) showing the potential binding pockets in CDK1;................................................................................................. 103

Figure 4.3: Molecular docking studies showing the potential interactions of CDK1.... 104
Figure 4.4: Molecular docking studies showing the potential interactions of cyclin B1 ......................................................... 105

Figure 4.5: Molecular docking studies showing the potential interactions of CDK1/cyclin B1 complex ................................................................................................................. 106

Figure 4.6: Aspirin acetylates recombinant CDK1 in-vitro and cellular CDK1 in HCT116 cells. ........................................................................................................................................ 111

Figure 4.7: Effect of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA (at 0.5 mM) on CDK2, 4 and 6 enzyme activity................................................................. 113

Figure 4.8: In-vitro acetylation of recombinant CDK1 by aspirin at 2 different concentrations for mass spectrometry analysis:......................................................... 117

Figure 4.9: MS/MS fragmentation spectra showing acetyl modification on lysine 34 by aspirin .................................................................................................................. 118

Figure 4.10: MS/MS fragmentation spectra showing acetyl modification on lysine 296 by aspirin .................................................................................................................. 118

Figure 4.11: A model depicting how aspirin may preferentially act on colonic tissue to protect against CRC. .......................................................................................................... 126
LIST OF TABLES

Table 2.1: List of CDK1, cyclin B1 and GAPDH primers used for RT-PCR……50

Table 3.1: Free energy binding values and hydrogen bond lengths for the interaction of
salicylic acid and aspirin with CDK2, cyclin A2 and CDK2/cyclin-A2 complex……80

Table 4.1: Free energy binding values and hydrogen bond lengths for the interaction of
aspirin, salicylic acid, 2,3-DHBA, 2,5-DHBA, 2,6-DHBA and 2,4,6-THBA with CDK1,
cyclin B1 and CDK1/cyclin B1 complex………………………………………………107

Table 4.2: Shows the inhibitory effect of aspirin, salicylic acid 2,3-DHBA, 2,6-DHBA,
2,4,6-THBA and 3,4,5-THBA on CDK-1, 2, 4 and 6……………………………………114

Table 4.3: Shows IC_{50} for CDK1 inhibition in-vitro and cytotoxicity assays in HCT-116
cells…………………………………………………………………………………………114

Table 4.4: Acetylated sites varied depending upon the concentrations of aspirin used.
Aspirin acetylated 4 lysine residues at 0.5 mM; and 5 lysine residues at 2.5 mM……115
ABSTRACT
IDENTIFICATION OF CDKS AS NOVEL TARGETS OF ASPIRIN AND ITS METABOLITES: A POTENTIAL ROLE IN CANCER PREVENTION
RAKESH DACHINENI
2017

Background:

The pursuit of drugs that inhibit cyclin-dependent kinases (CDKs) has been an intense area of research for more than 15 years. Till date, although multiple CDK inhibitors have been identified and few are undergoing clinical trials, only two synthetic drugs have been approved by Food and Drug Administration (FDA) for use in the treatment of cancer. These two drugs are mainly used for the treatment of metastatic breast cancer in combination with other drugs; however they have toxicity associated with their use and extends patients life not more than 24 months. Therefore, there is an urgent need for developing newer drugs that are more safe and efficacious.

Uncontrolled cell proliferation is a hallmark of cancer. In mammalian cells, cell cycle is controlled by the sequential activation of cyclin dependent kinases (CDKs). Four CDKs (CDKs 1, 2, 4 and 6) and their activating cyclins (A, B, D and E), play key roles in cell cycle progression. It has been established that CDK4,6/cyclin D and CDK2/cyclin E/A promote the passage through G1 and S phases, whereas CDK1/cyclin B regulates the transition through the late G2 and mitosis. In addition, specific proteins classified as CDK inhibitors capable of binding to cyclin/CDK complexes to inhibit their enzyme activity also play a significant role in regulating cell cycle. While, expression and activity of cyclins
and CDKs are tightly regulated in normal cells, they are often deregulated in cancer cells through frequent overexpression and frequent inactivation.

Studies carried in the past 2 decades have clearly established that regular aspirin use for 5 – 10 years decreases the cancers of the epithelial tissues particularly the cancers of the colon. This evidence came from numerous epidemiological studies, clinical trials, in-vitro cell culture experiments as well as experiments in animal models. Despite its potential role in cancer prevention, it is not clear precisely how aspirin exerts its chemopreventative effects in epithelial tissues. In this context, multiple targets and signaling pathways have been identified; however a unifying mechanism has not been identified till date. The objective of this dissertation is to investigate the novel mechanisms by which aspirin prevents the occurrences of cancer and discover newer protein targets that maybe responsible for mediating its chemopreventative actions.

Understanding aspirin-mediated chemopreventive mechanism and pinpointing its direct cellular targets is of high value, if it is to be used as a prophylactic drug. We hypothesized that aspirin and/or its primary metabolite salicylic acid may target cell cycle regulatory proteins modulating their level as well as functions. To address this, numerous biochemical, molecular biological studies were carried out in multiple cancer cell lines along with molecular docking studies to determine the interactions between aspirin/salicylic acid with CDKs and cyclins. The studies carried out during the course of this dissertation work have established that aspirin, salicylic acid and salicylic acid metabolites and derivatives target all more 4 members of CDK family namely CDKs 1, 2, 4 and 6, the major findings of which are detailed below.
Results:

Major finding 1: Our studies demonstrate that both aspirin and its primary metabolite, salicylic acid, decreased cyclin A2, B1, D3, CDKs 1, 2, 4 and 6 protein levels in a diverse panel of cancer cell lines. The decrease in cyclin A2 and cyclin B1 levels as well as CDK1 and CDK2 protein levels were associated with a corresponding decrease in the levels of messenger RNAs, suggesting that both aspirin salicylic acid regulate their expression at both transcriptional and post translational levels. Aspirin and salicylic acid also increased the levels of CDK inhibitors namely p21 and p27. The decrease in cyclin A2 and cyclin B1 protein levels appears to be mediated through 26S proteasomes.

Major Finding 2: Through biochemical and molecular modeling studies we showed that salicylic acid directly binds to CDK2. Molecular docking studies identified Asp145 and Lys33 as the potential sites of salicylic acid interactions with CDK2. Extension of these studies showed that salicylic acid also binds to CDK1 using Asp146 and Lys33. Despite salicylic acid interacting with CDK 1 and 2 via interactions using amino acids in the active site of the enzyme, inhibition of the enzyme activity was not observed.

Major Finding 3: We investigated the ability of salicylic acid metabolites 2,3-dihydroxy benzoic acid (2,3-DHBA) and 2,5-dihydroxy benzoic acid (2,5-DHBA) known to be generated by cytochrome p450 metabolism to CDK enzyme activity. In-vitro CDK assays showed that both metabolites inhibited CDK1 enzyme activity. Interestingly several derivatives 2,4-dihydroxybenzoic acid (2,4, DHBA), 2,6-dihydroxybenzoic acid (2,6-DHBA) and 2,4,6-trihydroxybenzoic acid (2,4,6-THBA) also inhibited CDK1 enzyme activity. 2,3-DHBA and 2,6-DHBA did not inhibit CDK2 and 4; however, both inhibited CDK-6 activity. Interestingly, 2,4,6-THBA was highly effective in inhibiting CDK1, 2, 4
and 6 activity. Molecular docking showed that these compounds potentially interact with CDK1. Immunoblotting experiments showed that aspirin acetylated CDK1, and pre-incubation with salicylic acid and its derivatives prevented aspirin-mediated CDK1 acetylation, which supports the data obtained from molecular docking studies.

**Conclusion:**

We identified CDK1 and 2 as salicylic acid binding proteins. In addition, we have demonstrated the interactions of salicylic acid metabolites and derivatives with CDKs. We suggest that intracellularly generated salicylic acid metabolites through CYP450 enzymes within the colonic epithelial cells may be responsible to the preferential chemopreventive effect of aspirin against CRC through inhibition of CDKs. This novel hypothesis and mechanism of action in aspirin’s chemopreventive effects opens a new area for future research. In addition, structural modification to salicylic acid derivatives may prove useful in the development of a novel CDK inhibitors in cancer prevention and treatment.
Chapter 1: Introduction

1.1 Cancer as a genetic disease:

Cancer is increasingly a global health issue and affects millions of people of all ages around the world. In 2012, nearly 14.1 million people were diagnosed with cancer, of which 8.2 million people died. The world health organization projects that by the year 2035, 24 million people may get affected with cancer of which 14 million people may die. More than 1 million new cases of colorectal cancer (CRC) are diagnosed worldwide each year [1]. In the US alone, 160,000 cases of CRC are diagnosed and 57,000 patients die of this disease every year, making it the second leading cause of death from cancer among adults [2]. Making sustained progress against cancer requires advances across vast areas from biology of cancer cells to developing drugs, to studies of large populations on incidences and causes of cancers.

Cancer is a genetic disease, it arises due to genetic alterations/mutations in proto-oncogenes and/or tumor suppressor genes [3]. Proto-oncogenes are genes that help to regulate cell growth and differentiation, and their controlled expression is required for normal healthy cells. When they are mutated, they can become oncogenes leading to their unregulated expression in a normal cell transforming them into a cancerous cell [4]. Tumor suppressor genes code for proteins that deter cancer development through inhibition of inappropriate cell growth and proliferation [5]. They inhibit multiple steps in signal transduction pathways or modulate transcription of cell cycle regulatory proteins; thus contributing to the inhibition of cell proliferation. In cancer, mutation in proto-oncogenes cause their activation, and mutations in tumor suppressor genes cause their inactivation, both favor uncontrolled cell growth [6, 7].
1.2 Risk factors:

Both inherited gene mutations as well as acquired somatic mutations are linked to the development of cancer [8]. Several life style related factors such as diet, weight, and exercise are also some of the strongest factors that can influence CRC risk. Being overweight and obese, physical inactivity, a diet high in red and processed meat, smoking, heavy alcohol use, and being older are some of the risk factors for CRCs [9]. A personal history of colorectal polyps or CRC, inflammatory bowel disease, a family history of CRC or adenomatous polyps also increases the risk [10, 11].

1.2.1 Genomic instability in colon tumorigenesis: As seen with other cancers, one of the major reasons of colon tumorigenesis is loss of genomic stability and control over cell division. Genomic instability drives the development of CRC by facilitating the acquisition of multiple mutations in genes. The most common type of genomic instability in CRC is chromosomal instability, which causes numerous changes in chromosomal copy number and structure [8, 12]. Chromosomal instability causes physical loss or inactivation by mutation in several tumor suppressor genes such as adenomatous polyposis coli (APC), p53, transforming growth factor-β (TGF-β) and SMAD family member 4 (SMAD4) proteins. Under normal conditions, functions of these genes are important for maintaining chromosomal stability during DNA replication. In contrast to some cancers, CRC does not commonly involve amplification of gene copy number or gene rearrangement [2]. Mutations in proto-oncogenes such as Ras, BRAF and epidermal growth factor receptor (EGFR) are also commonly observed in colon tumorigenesis [8, 13].
1.3 Mutational inactivation of tumor suppressor genes:

1.3.1 APC: During the development of CRCs, cells acquire many genetic changes; however, certain signaling pathways are more often targeted as key initiators. One of the first event that is known to be altered in CRC is the activation of the Wnt signaling pathway. During the activation of Wnt signaling, the β-catenin, an oncoprotein that binds to other nuclear proteins to form a transcription factor complex inducing the expression of genes involved in cell proliferation. In normal cells, β-catenin is degraded efficiently by the β-catenin degradation complex, which contains the tumor suppressor protein APC. By forming complex with β-catenin, APC prevent its nuclear translocation, suppressing gene expression. Thus activation of Wnt signaling via abnormal expression of β-catenin and its activity is one of the hallmarks of CRC [14-16].

Mutations in APC gene, which causes its inactivation, is the most common genetic alteration observed in CRC. When APC gene is mutated, the β-catenin-Wnt signaling pathway is abnormally activated. In familial adenomatous polyposis (FAP), an inherited familial cancer, germ-line mutations in APC are commonly observed providing a link between APC mutation and cancer development. In these individuals, the presence of mutations in APC increases the risk of CRC almost 100% by the age 40. In case of most sporadic colorectal adenomas and cancers, somatic mutations and deletions inactivate both the copies of APC gene [14, 16]. In a small subgroup of cancer patients, mutations in β-catenin is observed although they may have wild type APC, which makes β-catenin resistant to APC mediated degradation and leading to constitutive activation of Wnt signaling pathway [14, 16-18].
1.3.2 p53: p53 is a tumor suppressor protein, its major function is to arrest cell cycle in DNA damaged cells by inducing the transcription of p21 and later assist in DNA repair process. If DNA is irreparable, p53 can induce the expression of Bax (a pro-apoptotic protein) and initiate cell apoptosis [19]. The inactivation of p53 function by mutation is the second key genetic alteration observed in CRC. In most tumors, the two p53 alleles are inactivated, usually by a combination of missense mutation that inactivates the transcriptional activity of p53 and a 17p chromosomal deletion that eliminates the second p53 allele [14, 20]. The inactivation of p53 is often associated with the transition of large adenomas into invasive carcinomas.

1.3.3 TGF-β: Mutational inactivation of TGF-β signaling is the third important step in the progression to CRC [21]. In about 30% of CRCs, somatic mutations inactivate TGFBR2 (TGF-β receptor2). Mutations that inactivate TGF-β pathway coincide with the transition from adenoma to dysplasia or carcinoma and therefore contributes to the malignant phenotype [21].

1.4 Mutational activation of oncogenes:

Several oncogenes play key roles in promoting CRC. Oncogenic mutations of Ras and B-RAF, which activate the mitogen activated protein-kinase (MAPK) signaling pathway, occur in 37% and 13% of CRCs respectively. Ras mutations, particularly K-RAS, activate GTPase activity that signals directly to RAF [13, 22]. B-RAF mutation activates serine-threonine kinase activity, which further drives the MAPK signaling cascade [23, 24]. B-RAF mutations are detectable even in small polyps, and as compared
with Ras mutations they are more common in hyperplastic polyps and proximal colon cancers.

It is also important to note that nearly 30% of CRCs possess mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA), which encodes the catalytic subunit of PI3K [25], leading to constitutive activation of the PI3K pathway. Occasionally mutations in other genes are also observed, for example, mutations in phosphatase and tensin homolog (PTEN), an initiator of PI3K signaling and insulin receptor substrate 2 (IRS2), an upstream activator of PI3K signaling are also observed. In addition, co-amplification of AKT and PAK4 (p21-activated kinase 4) (downstream mediators of PI3K signaling) are also observed [26].

1.5 DNA Repair defects:

In some CRC patients, DNA repair genes appear to be mutated and inactivated. Among these, the most common are mutations in mismatch-repair genes mutL homolog 1 and mutS homolog 2 (MLH1 and MSH2). In hereditary nonpolyposis colon cancer (HNPCC), also known as Lynch syndrome, the mutations are often inherited, and having the defects increases the risk of CRC by 80% by the age of 45. Germ-line mutations of another mismatch repair gene, MSH6, and germ-line inactivation of a base excision repair gene, mutY homologue (MUTYH or MYH) is also observed in some CRC patients [27]. The MYH protein removes 8-oxoguanine (an oxidative product of guanine, generated by the presence of oxygen free radicals) from the DNA and assists in repairing DNA [27, 28]. It was reported that the cancer characterized by mismatch repair deficiency is more commonly observed in the proximal colon [29].
1.6 Aberrant DNA methylation:

In humans, DNA methylation primarily occurs by the covalent modification of cytosine residues in CpG dinucleotides [30]. CpG dinucleotides are not evenly distributed across the human genome and are instead concentrated in short CpG-rich DNA stretches called “CpG islands”. While, most of the CpG sites in the genome are methylated, the majority of the CpG islands in the promoter regions of actively transcribing genes are unmethylated. The methylation of the cytosine is carried out by DNA methylases within the CpG dinucleotides. DNA methylation can lead to gene silencing (epigenetic silencing) by either preventing or promoting the recruitment of regulatory proteins to DNA. For example, it can inhibit the transcriptional activation by blocking transcription factors from accessing target-binding site. Alternatively, it can provide binding sites for methyl-binding domain proteins, which can mediate gene repression [31, 32].

A cancer epigenome is marked by genome wide hypomethylation and site-specific CpG island promoter hypermethylation. Global hypomethylation plays a significant role in tumorigenesis. In some CRC patients, the inactivation of mismatch repair genes arises due to methylation associated silencing of mismatch repair genes [33, 34]. By comparison, in CRC genome there is a depletion of cytosine methylation in the genome, but significant increase of aberrant methylation in the CpG islands within the promoter region of certain genes, such MLH1 gene, which causes epigenetic silencing [35]. Silencing of DNA repair genes enables cells to accumulate additional genetic alterations leading to the rapid progression of cancer.
1.7 Inherited syndromes (FAP and HNPCC):

About 5 - 10% of people who develop CRC have inherited gene defects. The most common syndromes that are linked with CRC are familial adenomatous polyposis (FAP) and Lynch syndrome (Hereditary non-polyposis colorectal cancer, or HNPCC). It is estimated that about 1% of all CRC are due to FAP, which arises due to inherited mutations in APC. FAP is characterized by the presence of hundreds or thousands of polyps in the colon and rectum, and this is usually observed during teen years or early adulthood. Cancer usually develops in one or more of these polyps as early as age 20. By the age 40, almost all people with this disorder will have developed colon cancer. People with FAP are also at increased risk for cancers of the stomach and small intestines. Lynch syndrome accounts for about 2 - 4% of all CRCs. In most cases, this disorder is caused by an inherited defect in either the MLH1 or MLH2 gene, but changes in other genes such as TGF-β receptor subtype-2 can also cause Lynch syndrome. People with Lynch syndrome, can develop cancers when they are young, but they tend to have fewer polyps unlike FAP patients. The lifetime risk of CRC in people with MLH1 and MLH2 mutations is very high (~80%) [12, 36-38].

1.8 Acquired gene mutations:

In most cases of CRC, DNA mutations that lead to cancer are acquired during a person’s life time rather than being inherited. As discussed earlier in many cases, the first mutation occurs in the APC gene. Further mutations may then occur in genes such as KRAS, p53, and SMAD4. These accumulation of mutations can lead cells to grow and spread uncontrollably [12].
1.9 Tumor development: Transformation of a normal cell into a tumor cell:

The development of cancer is a multi-step process involving mutations and selection for cells with progressively increasing capacity for proliferation, survival, invasion and metastasis [39]. These alterations occur in stages- initiation, promotion and progression, all these events lead to uncontrolled cell proliferation. A single mutation in a somatic cell may be a trigger for initiation of abnormal proliferation. With cytogenetic alterations and additional mutations, normal cell becomes transformed into cancer cells [40, 41].

During initiation, mutations caused by UV, carcinogens or defective DNA repair, activates a proto-oncogene (e.g., EGF-R). With this mutation, cell attains the capability to continuously divide and begins to grow rapidly to form a small collection of abnormally growing cells called tumor. Tumor cells multiply more frequently than normal cells. The altered cell and its descendants grow and divide too often, a condition called hyperplasia. Subsequently, at some point one of these cells experiences another mutation that further increases its tendency to divide, leading to its entry into the next state [41, 42].

During promotion, because of the genetic instability, a second and a third mutation occurs (e.g. first p53, BRAF). Tumor promoters such as growth factors, or hormones, epigenetic changes that occur due to hypomethylation of the genome, or hyper-methylation of the CpG islands in the promoter of genes, etc., may promote excessive cell growth. A primary tumor appears to contain cells that are very similar. At this stage, enhanced cell proliferation and altered cell behavior are often observed. The cell’s descendants excessively divide, which look abnormal and this condition is called dysplasia [43-45].
During progression, more genetic changes occur in the tumor cells, such as additional mutations in the second p53 gene and gene amplification (e.g., HER2, c-Myc). Tumor tissue acquires polymorphism, (consists of different cellular clones that differ from each other), adapts to the influence of regulatory (immune) system, increases its rate of growth and evolves into a self-sustaining growth stage [46]. Cells in the tumor invade neighboring tissues and shed cells to the blood or lymph. At this stage the tumor is said to be malignant. The escaped cells may establish new tumors (metastases) at other locations in the body. It is not clearly known the exact number of mutations required for a normal cell to become a cancer cell, but the number is predicted to be between 5 and 10 [47].

Most CRCs begin as a growth on the inner lining of the colon or rectum forming a small polyp. Some types of polyps can change into cancer cells over the course of several years, but not all polyps can become cancer. CRC begins as an adenomatous polyp, which develops into an advanced adenoma with characteristic dysplasia, and then progress into an invasive cancer. Invasive cancers that are confined within the wall of the colon (stage I and II) are curable, but if untreated, they can spread to the regional lymph nodes (stage III) and then metastasize to distant sites (stage IV). State I and II tumors are curable by surgical excision, and up to 73% of cases of stage III tumors are curable by surgery combined with adjuvant chemotherapy. Advances in recent years in chemotherapy have improved the survival rate, but state IV disease is usually incurable [2].
Figure 1.1  Transformation of a normal cell into a cancerous cell

Transformation of a normal cell to a cancer cell is often initiated following mutations in one of the several proto-oncogenes or tumor suppressor genes. With additional genetic alterations, such as point mutations, deletions and chromosomal exchanges or gene amplification, cells completely lose their ability to regulate cell division, thus change to cancerous phenotype. These events are described to occur in 3 distinct stages, involving initiation, promotion and progression culminating in the final stage metastasis. Among the 3 stages of cancer development, the initiation and promotion stages are generally considered to be reversible. Figure is modified from Siddiqui et al [48].
1.10 **Hallmarks of cancer and therapeutic targets:**

All cancer cells during a transformation from normal to malignant stage acquire common characteristics, which are referred to as “Hallmarks of Cancer”. This was initially proposed by Douglas Hanahan and Robert Weinberg in 2001 and later updated in 2010 [6, 7]. Acquiring these traits enable a healthy normal cell to transform into a tumorigenic and ultimately a malignant cell. A total of six hallmarks of cancer have been proposed, which are common to all cancer cells and these include: 1) Sustaining proliferative signaling; 2) Evading growth suppressors; 3) Resisting cell death; 4) Enabling replicative immortality; 5) Inducing angiogenesis; and 6) activating invasion and metastasis. Understanding of these hallmarks will enable to develop strategies to prevent, diagnose and to treat cancer.

**Enabling Characteristic of the Hallmarks of Cancer:** The hallmarks of cancer acquired by the cancer cells allows them to survive, proliferate, and disseminate. Acquisition of these capabilities are made possible by 2 enabling characteristics. Genomic instability is one of the most prominent development in cancer cells, which creates mutations and genetic alterations contributing to different hallmarks of cancer. A second enabling characteristic is that immune cells located in neoplastic lesions provides an environment that supports tumor development by providing with necessary growth factors, cytokines, proangiogenic factors, extracellular matrix (ECM) modifying enzymes, along with invasion and metastasis signals. These lead to the activation of epithelial mesenchymal transition (EMT) required for the spread of the cancer cells locally as well as to the distant organs.
Figure 1.2:  Hallmarks of cancer and the potential therapeutic targets

Drugs targeting at multiple steps during cancer cell growth and progression have been developed as a strategy to effectively treat cancer. These drugs target any of the six hallmarks of cancer, immune cells that provides the tumor with growth factors and cytokines, and angiogenic factors to prevent tumor growth, invasion and metastasis. Figure Modified from Hallmarks of Cancer [6].
1.10.1 Sustaining proliferative signaling: It is one of the most fundamental hallmark of a cancer cell and is required for the sustained proliferation. Proliferative signaling is required to regulate cell growth and division. It occurs when a growth promoting ligand binds to its cell surface receptor, transmitting the signal intracellularly (mostly tyrosine kinase domains) that regulates cell cycle, cell growth and its survival. This process is tightly controlled in a normal tissue to maintain the proper cell number and its function. But this process is deregulated in a cancer cell, causing uncontrolled cell division and growth. Constitutive activation of signal transduction pathway are observed upon mutations in EGFR, RAS, B-RAF, and PI3Kinase, which enables the cells to grow unrestrained.

1.10.2 Evading growth suppressors: Along with sustaining proliferating signals, cancer cells have to evade the function of tumor suppressor proteins that negatively control cell proliferation. Dozens of tumor suppressor proteins that limit cell growth and proliferation have been known to be mutated and inactivated in cancer cells. Two of the common tumor suppressors are p53 and retinoblastoma (RB). RB protein integrates extracellular and intracellular signals, and decides whether a cell should proceed through its division cycle or not. Cancers with RB mutation (loss of function) loses its regulatory function over cell cycle progression, leading to persistent cell proliferation. p53 responds to stress signals (UV, genotoxic agents etc.,) and can cause cell cycle arrest until normal conditions are favorable. Mutations in these proteins leading to their inactivation are commonly observed in many cancers.

1.10.3 Resisting Cell Death: Tumor cells have evolved elaborate mechanisms to overcome the process of apoptosis. For example, one of the major protein
involved in apoptosis is Bax, a mitochondrial protein which regulates the membrane permeability and affects the release of cytochrome c into the cytoplasm, a step critical in apoptosis. Bax is often induced by the tumor suppressor protein p53. Nearly 50% of all tumors have mutated p53, and this leads to the decreased expression of Bax and a negative effect on apoptosis and resistance towards cell death [19, 49]. Cancer cells overcome cell death by either loss of p53 function, decreased expression of pro-apoptotic protein (BAX) or increased expression of anti-apoptotic protein (Bcl-2) [50].

1.10.4 Inducing Angiogenesis: Angiogenesis is the process by which new blood vessels are formed from preexisting vessels. It is prevalent during embryogenesis, but also occurs during the course of life. It plays an important role in wound healing and reproductive cycling. But during tumorigenesis, angiogenesis is always active and generates new vessels to transport required nutrients and oxygen for sustenance as well as an ability to evacuate metabolic wastes and carbon dioxide present in the tumor environment. Tumor cells have the ability to synthesize and secrete pro-angiogenic factors such as vascular endothelial growth factors (VEGF), which can promote angiogenesis.

1.10.5 Activating Invasion and Metastasis: Invasion of cancer cells locally or to other distant tissues often involves alterations in their shape as well as their ability to attach to other cells and also to the extracellular matrix (ECM). To ensure this event, cancer cells downregulate adhesion molecules such as E-cadherin. E-cadherin is one of the best characterized adhesion molecule in cancers of epithelium (carcinomas), a key cell-to-cell adhesion protein, which helps to form adherent junctions with adjacent epithelial cells to maintain a quiescence of these cells within these sheets. E-cadherin is significantly down-regulated in many carcinomas or it is inactivated through mutation,
helping cancer cells to have an opportunity to detach from the site of origin (primary tumor) and translocate to a new site. Cancer cells also upregulate adhesion molecules (N-cadherin), which are normally associated with cell migration.

1.10.6 Enabling replicative immortality: In order to grow continuously, cancer cells require unlimited replicative potential. In normal cells, the replication potential is limited due to shortening of the telomeres at the ends of chromosomes as telomerase activity decreases with successive cell divisions. Cancer cells overcome this problem by abnormally expressing high levels of telomerase enzyme, which enables them to keep chromosome length intact during successive cell division.

1.10.7 Emerging Hallmarks: Reprograming energy metabolism and evading immune destruction are the other distinct features of cancer cells, which are proposed to be functionally required for tumor development. Cancer cells have the ability to modify or reprogram the cellular metabolism in order to support neoplastic proliferation and cell growth. An example of this is the increased utilization of glucose in cancer cells as compared to normal cells. It is estimated that cancer cells oxidize glucose 200 times greater than a normal cell [51]. This is required as many of the intermediates of the glycolysis serve as building blocks for the synthesis of amino acids, sugars and biomolecules. Cancer cells also have to actively evade immune system, particularly T cells, B cells, macrophages and natural killer cells to enhance tumor progression and development [52].

1.11 Cell cycle:

Cell cycle is the series of events that takes place in a cell leading to duplication of the DNA and cell division resulting in the production of two daughter cells. The eukaryotic
cell cycle contains three distinct stages: interphase, mitotic phase and cytokinesis. The interphase is further divided into three distinct stages, G1, S and G2 phases. After the G2 phase, the cell enters into mitosis and then divides. During G1 phase, the cell doubles in size, accumulates proteins and enzymes required for the S-phase. During the S phase, DNA is duplicated, histones are synthesized and cell moves to the G2 phase. During G2 phase, protein synthesis continues, microtubules are assembled, any errors in DNA replication are rectified and the cell then enters mitosis followed by cell division [53].
Figure 1.3: Mammalian cell cycle

The mammalian cell cycle is controlled by a subfamily of cyclin-dependent kinases (CDKs), the activity of which is modulated by several activators (cyclins), and inhibitors (Ink4, and Cip and kip inhibitors). Although human cells contain multiple cyclins and CDKs, only a certain subset of CDK-cyclin complexes is directly involved in driving the cell cycle. They include 3 interphase CDKs, (CDK2, CDK4 and CDK6) and a mitotic CDK (CDK1), and ten cyclins that belong to four different classes (the A, B, D and E types). The activity of cell cycle CDKs is deregulated in cancer cells owing to genetic or epigenetic changes in either CDKs, their regulators, or upstream mitogenic pathways. 

Figure Modified from Schafer et al [53].
1.11.1 **Activation of CDKs:** Cyclin dependent kinases (CDK) are activated by binding to their partners, cyclins. CDK protein levels remain constant throughout the cell cycle, whereas levels of cyclins rise and fall depending upon the stage of the cell cycle, and this results in the periodical activation of CDKs. Four CDKs are mainly involved in cell cycle regulation in animal cells, they are CDK1, 2, 4 and 6. Another family member CDK7 contributes indirectly by acting as a CDK activating kinase (CAK) that phosphorylates CDKs [54]. CDKs (monomeric) have the same two lobed structures as other protein kinases. They contain a large flexible loop called the T-loop or activation loop, which rises from the carboxy-terminal lobe to block the binding of protein substrate at the entrance of the active-site cleft. In the inactive CDKs, several important amino acids at the active site are incorrectly positioned, so that phosphates of ATP are not ideally oriented for the kinase reaction. CDK activation therefore requires extensive structural/conformational changes in the CDK active site [55].

The activation of monomeric CDK subunit involves its binding to a cyclin partner, which confers basal activity to the CDK/cyclin complex. This enables subsequent phosphorylation of the CDK on a conserved threonine in the activation loop (for e.g. Thr160 in Cdk2) by CAK, thereby converting the complex into a fully active form [56]. Among different CDKs, activation of CDK2 is best studied [57]. The determination of the structure of the unphosphorylated and phosphorylated CDK2/cyclin A complexes has revealed that cyclin binding induces conformational changes within the CDK2 that are important for its activation. The most significant feature is the reconfiguration of the ATP binding site into a conformation that favors its nucleophilic attack by the substrate and brings Glu51 together with Lys33 and Asp145 for catalysis. In addition, cyclin binding
induces a positional switch of the T loop by 20 Angstrom, which opens the catalytic cleft, affects the orientation of the putative substrate binding site of CDK2, and leads to appropriate exposure of Thr160 for phosphorylation. Subsequent phosphorylation of Thr160 by CDK7/cyclin H (CAK) induces further conformational changes in the T-loop and in the C-terminal lobe of CDK2 and stabilizes the substrate binding site, enabling it to be a fully functional enzyme.

In addition to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. For example, full activation of CDK1 requires phosphorylation of threonine 161 (threonine 172 in CDK4 and threonine 160 in CDK2), by CAK. These phosphorylations induce conformational changes and enhance the binding of cyclins [58]. The Wee1 and Myt1 kinase phosphorylate CDK1 at tyrosine-15 and/or threonine -14, thereby inactivating the kinase. Dephosphorylation at these sites by the enzyme Cdc25 is necessary for activation of CDK1 and further progression through the cell cycle [59].

1.11.2 Cyclins: Cyclins A, B, D and E are involved in the regulation of CDKs during cell cycle progression in human cells. The D-cyclins (D1, D2 and D3) are synthesized in response to growth factors, binds to CDK4 and CDK6, and are important for entry into G1 phase. Cyclin E associates with CDK2 to regulate progression from G1 into S phase. Cyclin A binds with CDK2 and this complex is required during S phase. In late G2 and early M phases, cyclin A complexes with CDK1 to promote entry into M phase. Mitosis is further regulated by cyclin B in complex with CDK1 [60].

1.11.3 CKIs: CDK activity can be regulated by cell cycle inhibitory proteins, called CDK inhibitors (CKI), which bind to CDK alone or to the CDK-cyclin complex.
Two distinct families of CDK inhibitors have been discovered, the INK (Inhibitors of CDK4) family and the Cip/Kip (CDK interacting protein/Kinase inhibitory protein) family. The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), p19 (INK4d), which specifically inactivate CDK4 and CDK6 [61, 62]. These CKI form stable complexes with the CDK enzyme before cyclin binding, preventing the association with cyclin D. The second family of inhibitors, the Cip/Kip family, includes p21 (Waf1, Cip1), p27 (Cip2), p57 (Kip2), which inactivate all CDK-cyclin complexes [61, 63].

1.11.4 Role of Rb phosphorylation in cell cycle progression: Activated CDKs have the ability to phosphorylate the target proteins at a conserved consensus sites, resulting in changes that are physiologically relevant for cell cycle progression. The most well studied target is the substrate of CDK4/6-cyclinD, Rb protein (retinoblastoma, a tumor suppressor gene). In the resting state, hypophosphorylated Rb binds to E2F and keeps the E2F transcription factor in its inactive state. CDK4/cyclin D or CDK6/cyclin D phosphorylates Rb, releasing the E2F required for the synthesis of proteins for G1 to S phase transition, these includes cyclin A, cyclin E and Cdc25. pRb remains in the hyperphosphorylated state for the remainder of the cell cycle and CDK2-cyclin E participates in maintaining this hyperphosphorylated state [54, 64]. It is also reported that CDK2/cyclin E phosphorylates H1 histone and this activity may be important for chromosomal condensation, which is required during DNA replication. Histone H1 is also a substrate for CDK1/cyclin B [65]. Other CDK substrates include CDK's own regulators, Wee1 and Cdc 25, and some cytoskeletal proteins such as nuclear lamins, microtubules and vimentin which are required for mitosis [66].
1.11.5 Cell cycle and cancer: In cancer there are fundamental alterations in the genetic control of cell division that results in unconstrained cell proliferation. As mentioned earlier mutations in proto-oncogenes and tumor suppressor genes promote tumor growth. Inactivation of tumor suppressor genes such as, Rb and p53 results in the dysfunction of proteins that normally inhibit cell cycle progression [67-69]. One of the consequences for inactivation of tumor suppressor genes and activation of proto-oncogenes is the dysregulation of cell cycle regulatory proteins. CDK activity is increased in proliferative diseases, such as cancer, owing to the frequent over expression of positive regulators (cyclins) and frequent inactivation of CDK inhibitors [70]. CDK4 overexpression, which occurs as a result of gene amplification has been identified in cell lines such as melanoma, sarcoma, and glioma [71]. Mutations in CDK4 and CDK6 genes resulting in loss of CKI binding have also been identified [72]. CDK1 and CDK2 have been reported to be overexpressed in a subset of colon adenomas, a greater over expression was seen in focal carcinomas in adenomatous tissue [73, 74].

Cyclin D1 gene amplification has been reported in breast, esophageal, bladder, lung and squamous cell carcinomas [75]. Cyclin D2 and cyclin D3 have also been reported to be overexpressed in some tumors. Cyclin E has been found to be amplified or overexpressed in some breast and colon cancers [54]. In addition, aberrant activation of CAKs is also observed in some cancers. Cdc25B, a phosphatase, is overexpressed in 32% of primary breast cancers. Transcription of Cdc25A and Cdc25B gene is activated by c-myc, an oncogene which is frequently mutated in human cancers [76].

The inhibitory activity of CKI results in growth suppression through activation of pRb, reflecting the tumor suppressor function of CKI. The p16 gene which is a CKI, is
inactivated in a variety of human tumors through deletion, point mutation and hypermethylation [77]. The p16 is a specific inhibitor of CDK-cyclin D, preventing phosphorylation of the Rb protein and arresting cells at the G1 phase. Cells with altered p16 will lose their ability to cause CDK inhibition leading to unrestrained progression through G1. Deletion of p16 have been reported in approximately 50% of gliomas and mesotheliomas, 40-60% of nasopharyngeal, pancreatic and bilary tract tumors and 20-30% of acute lymphoblastic leukemia [75]. Loss of p27 expression has been reported for a number of human tumor types (lung, breast, bladder), and has been correlated with poor prognosis and tumor aggressiveness [54].

1.12 CDK inhibitors as anti-cancer drugs:

1.12.1 First-generation CDK inhibitors: Due to the importance of CDKs and their regulators in cell cycle progression, they have become attractive targets to arrest cancer cell growth. Over the past 20 years, several CDK inhibitors have been developed as potential cancer therapeutics and were tested in numerous trails and several tumor types. The first generation CDK inhibitors developed were relatively non-specific and this included flavopiridol, olomucine and roscovitine [67]. Among these, flavopiridol, a semisynthetic flavonoid was shown to inhibit CDK1, 2, 4, 6, 7 and 9, is the most extensively studied CDK inhibitor. This compound was tested in more than 60 clinical trials in the past two decades [78, 79]. It is reported to induce cell cycle arrest in G1 and G2 phases; interestingly, it can also induce cytotoxic response as result of CDK7 and CDK9 inhibition leading to suppression of transcription. However, it showed poor clinical response in phase 2 studies and therefore, did not qualify for therapeutic purpose in cancer treatment.
Roscovitine, a purine based inhibitor, also went through clinical trials (Phase I), but did not find success due to poor clinical response. In another phase II trial, roscovitine was tested in patients with advanced non-small cell lung cancer, however, it again failed to improve the progression free survival [67].

1.12.2 Second-generation CDK inhibitors:

Based on the studies on flavopiridol and roscovitine, new investigation focused on developing more selective CDK inhibitors with increased selectivity towards CDK1 and CDK2. Although many CDK inhibitors appeared to be promising in preclinical studies, only few progressed past Phase I clinical trials. Among the second-generation CDK inhibitors, dinaciclib was the most extensively studied. It is a potent inhibitor of CDK1, 2, 5 and 9 (1-4 nM), with less activity towards CDK4, 6 and 7 (60-100 nM). Dinaciclib exhibited superior activity against phosphorylation of Rb, and inhibited cell cycle on a number of different cancer cell lines [80]. It also caused regression of tumors in mouse models. Following this, phase I studies provided encouraging results; however, phase II studies failed to support its use in clinic. Other CDK inhibitors that were developed and tested include AT7519, a pyrazole 3-carboxyamide compound that acts as an inhibitor of CDK1, 2, 4, 6 and 9; R547, an inhibitor of CDK1, 2 and 4; SNS-032, an inhibitor of CDK2, 7 and 9; AZD5438, an inhibitor of CDK1, 2 and 9. However, all these failed to achieve the desired clinical outcome [67, 81].

Crystal structure of CDK4 and 6 provided opportunities to develop a novel class of inhibitors through a combination of chemical screening and optimization. PD-0332991 (palbociclib) was developed, which had high degree of selectivity towards CDK4 and 6. Palbociclib was very effective in inhibiting Rb phosphorylation leading to cell cycle arrest
at G1 phase. It binds to an unique ATP binding site causing inhibition of CDK4 and CDK6 enzyme activity [67, 82]. It was approved by FDA in 2015 to be used together with hormone therapy, letrozole, as a first line of treatment for post-menopausal women with ER-positive, HER2 negative metastatic breast cancer. Another drug that targets CDK4/6 is ribociclib and was approved in 2017 by FDA for women with hormone receptor positive, HER2 negative advanced breast cancer [83].

1.13 Salicylic acid and the discovery of aspirin:

The therapeutic properties of the willow tree bark extract was known for the last 2400 years with the famous Greek physician, Hippocrates prescribing it for inflammation, pain and common headaches. Pain, fever and inflammation in the 19th century were treated with various plant extracts containing salicylic acid and its derivatives. For example, leaves of myrtle, bark of willow, bark of poplar and meadowsweet were used for treating inflammation related symptoms. Salicylic acid was first isolated from the bark of the willow tree in 1763 by Edward Stone, at the University of Oxford. In 1859, structure of salicylic acid was first identified (Ortho-hydroxybenzoic acid), then chemically synthesized and used for therapy by Freidrich Kolbe. However, when taken orally, salicylic acid had bitter taste and caused gastric irritation. In 1897, Felix Hoffman, a young chemist at Bayer synthesized acetyl derivative of salicylic acid, mainly to reduce the observed side effects such as gastric irritation. He synthesized acetylsalicylic acid by acetylating the hydroxyl group of the salicylic acid with acetyl chloride. This new derivative was named as aspirin by Arthur Eichengrun, another chemist at Bayer, Germany in 1899 [84]. Although Felix Hoffmann is credited with the synthesis of aspirin, whether this was his own initiative or under the direction of Arthur Eichengrun is controversial.
“A” in aspirin denotes acetyl chloride (or acetylation) the “spir” from *Spiraea Ulmaria* (meadows sweet, the plant from which the salicylic acid was derived) and the “in” which denotes the familiar name ending for medicines. Aspirin was marketed as a drug in January 1899. It is one of the drugs, whose invention occurred by serendipity, because acetylation of salicylic acid was done to reduce the side effects of salicylic acid; however, as we know today, the acetyl group actually causes acetylation and inactivation of cyclooxygenases, thus inhibiting prostaglandin synthesis through a different mechanism as compared to the parent compound, salicylic acid [84].

In early part of the 20th century, aspirin was extensively used for its anti-pyretic, analgesic and anti-inflammatory properties. Aspirin’s actual mechanism of action was not known until Sir John Vane in England made a crucial discovery in 1971. He showed that aspirin inhibits a key enzyme in prostaglandin synthesis, cyclooxygenase (COX), by acetylation. Prostaglandins are implicated in inflammation, fever, and pain, and also shown to play an important role in gastro-protective and platelet aggregation. This discovery helped in starting a whole new class of compounds namely non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin is a member of NSAIDs, but differs from most of the other members in its mechanism of action. The salicylates also have similar effects (anti-pyretic, anti-inflammatory and analgesic) as other NSAIDs, and inhibit the same COX enzymes, but aspirin’s inhibition COX is irreversible, and unlike others, it affects COX-1 more than COX-2 variant [85, 86].

Cyclooxygenase or prostaglandin H2 synthase or prostaglandin-endoperoxide synthase (PTGS) is an enzyme that catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. PGH2 is the central prostaglandin, which is later converted into other
primary prostanoids, based on the cell types. For example, injured cells synthesize and release PGE2 which binds to the PGE2 receptor on nerve endings relaying signals to the brain causing pain. The same PGE2 secreted by the intestinal/stomach epithelial cells acts as a gastro-protective agent [87].

Three isoforms of COX has been reported that are encoded by 2 separate genes. COX-1 and -3 are encoded by PTGS1, and COX2 is encoded by PTGS2 genes. COX-3 is the alternatively spliced form of COX-1, with COX-3 retaining an intron. COX-1 is constitutively expressed and COX-2 is inducible depending upon the condition. COX-2 has very similar structure and catalytic active site as that of COX-1; however, the active site in COX-2 is much larger. Therefore, when aspirin occupies the active site of these enzymes, the observed inhibitory effect is greater for COX-1 compared to COX-2 (~170 fold higher than COX-2). Aspirin acetylates COX-1 at Serine 530 and COX-2 at Serine 516 in the active site of the enzymes. Aspirin’s IC\textsubscript{50} for COX-1 is ~1.67 µM and for COX-2 is ~278 µM [86, 88, 89].
Figure 1.4: Aspirin inhibits cyclooxygenases (COX), an enzyme in arachidonic acid metabolism.

The arachidonic acid is released from phospholipids present in the membrane through the action of phospholipases. Cyclooxygenases convert arachidonic acid into prostaglandin G2, which then depending on the type of cell, is converted to various prostanoids. Figure modified from Dovizio et al [89].
1.14 **Aspirin as a drug to prevent cardiovascular disease:**

Prostanoids in cardiovascular system have been shown to modulate the pathogenesis of thrombosis and atherosclerosis via a variety of processes including platelet aggregation, vasodilation and vasoconstriction. Prostaglandin I2 (PGI2), also called prostacyclin, and thromboxane A2 (TXA2) are the 2 major prostanoids with opposing functions. In physiological conditions, PGI2 is synthesized from vascular smooth muscles and endothelial cells, whereas majority of TXA2 is synthesized from blood platelets. PGI2 functions as a potent vasodilator and also inhibits platelet activation, whereas TXA2 is a potent vasoconstrictor and induces platelet aggregation upon its activation. COX-1 is the major isoform expressed in platelets contributing to TXA2 production, whereas COX-1 and -2 both are expressed in vascular endothelial cells, contributing to PGI2 synthesis. Inhibition of COX-1 by aspirin inactivates the enzyme and causes decreased production of TXA2 leading to its anti-thrombotic effects. The required minimum dose for the anti-platelet effect of aspirin in humans is between 75 to 81 mg per day [90, 91].

1.15 **Aspirin’s recommended dose, absorption, metabolism and side effects:**

Depending on the conditions being treated, aspirin is used at various doses ranging from 75-81 mg (antiplatelet) to 325–600 mg (analgesic) to 1.2 g (anti-inflammatory) [92]. Aspirin is mainly absorbed in the acidic environment of the stomach and upper intestine. Rate of absorption is influenced by many factors including pH of the gastrointestinal lumen, gastric emptying time, and intestinal transit times. The gastric absorption of aspirin is limited, despite high proportion of the un-ionized aspirin present, its absorption being restricted by the surface area of the mucosa.
The systemic bioavailability of aspirin is 40-50% [93] and it undergoes hydrolysis in the intestine, liver, and plasma by esterases [89, 94]. Various specific hydrolases in different tissues also play a role in this hydrolysis. For example, human carboxyl esterase 1 and 2 (CES 1 and 2) are the major contributors to this activity in the liver and intestine respectively. In plasma, aspirin is rapidly hydrolyzed by butyrylcholine esterases. In contrast to the short half-life of intact aspirin (20 min), salicylic acid depending upon the dose has a half-life varying between 4 to 6h, and remains much longer in the plasma.

The maximum concentration of intact aspirin detected in the plasma with low dose aspirin is ~7 µM; however, analgesic and anti-inflammatory doses can yield plasma concentrations ranging from 30 to 150 µM. The plasma salicylate concentrations obtained from the hydrolysis of low-dose aspirin is estimated to be ~15 µM, whereas the analgesic and anti-inflammatory doses can yield concentrations ranging from 500 to 2500 µM [89].

Salicylic acid can be directly excreted, without undergoing modification (1-31%). In the liver, salicylic acid undergoes further metabolism through glucuronide formation to produce ester and ether glucuronides (1-42%), or conjugation with glycine to produce salicyluric acid (20-65%). In addition, the cytochrome P450 (CYP450) enzymes in the liver metabolize salicylic acid to 2,5-dihydroxybenzoic acid (2,5-DHBA, gentisic acid) and 2,3-dihydroxybenzoic acid (2,3-DHBA). The CYP-450 mediated oxidation of salicylic acid has been characterized as a minor pathway, with gentisic acid usually accounting for less than 5% of the elimination of the drug [84]. The 2,5-DHBA can undergo conjugation with glycine to form gentisuric acid. These metabolites are eliminated from the body either through kidneys or bile [95, 96].
Figure 1.5: A model depicting how low dose aspirin inactivates COX-1 and contributes to the prevention of colorectal cancers

Aspirin undergoes hydrolysis by esterases present in liver, intestine and plasma to salicylic acid (SA), which may further undergo conjugation (phase II) reactions with glucuronide and glycine to give salicylacyl glucuronide or salicylphenol glucuronide and salicyluric acid. It can further undergo hydroxylation reactions by cytochrome p450 to generate dihydroxy benzoic acid (2,3-DHBA or 2,5-DHBA). Figure is modified from Bojic et al. [96]
1.16 Aspirin and cancer:

Evidence from epidemiological studies have demonstrated a significant correlation between regular aspirin use and reduced cancer incidence and mortality. The association between low dose (75-325 mg) aspirin consumption and a significant (20-40%) reduction in cancer incidence was shown for colorectal cancer and the development of nine different non-gastrointestinal cancers [97]. This inverse correlation is now established for the cancers of the colon [98-103], breast, prostate, lung and skin [104-106]. One interesting observation that emerged from these studies is that aspirin is more effective in preventing the colorectal cancer as compared to the cancers of the distal tissues. Animal and human studies also support a role for aspirin in chemoprevention. Aspirin suppresses aberrant crypt foci formation in colorectal cancer patients [107]. Its use after the colorectal and breast cancer diagnosis was associated with a decreased risk and increased patient survival [101, 108].

1.16.1 Mechanisms of chemoprevention by aspirin:

The evidence that aspirin prevents cancer is compelling, however, the underlying mechanisms leading to its anticancer effect is enigmatic as numerous protein targets and pathways have been suggested. Both COX-dependent and COX-independent mechanisms have been proposed.

1.16.2 COX-dependent mechanism:

One of the hypothesis proposed for Aspirin’s anti-cancer effects involves acetylation-mediated inactivation of COX. Transcriptional upregulation of COX-2 gene has been observed in nearly 80-90 % of CRC; in contrast, the expression of COX-1 is
unaffected [109]. Since the IC$_{50}$ of COX-2 is ~278 µM [86], low dose aspirin (81 mg) taken daily, cannot achieve sustained inhibition of COX-2 in nucleated cells [99, 110]. If COX-2 is the direct target, then higher doses of aspirin (650 mg /three to four daily) are required to achieve complete inhibition of COX-2. One widely discussed theory is that aspirin-mediated inhibition of platelet COX-1 plays a role in preventing CRC [99]. The observation that low dose aspirin, which is used for cardio protective effect is also effective in the prevention of CRC, led to the hypothesis that a common pathway involving COX-1 inhibition in platelets may be central to aspirin’s chemopreventive effects [111]. If this is true, aspirin’s effect is likely to be indirect and may occur through multiple steps involving both COX-1 and COX-2. Activated platelets have been implicated in the early phases of colon tumorigenesis through the release of growth and angiogenic factors, as well as lipid mediators. It is suggested that the release of these factors may trigger COX-2 expression in colon epithelial cells leading to enhanced cellular proliferation and accumulation of mutations, triggering tumorigenesis [92, 99]. In this model, both COX-1 and COX-2 are important players; but, the two pathways operate sequentially. It is argued that the low dose aspirin when taken daily, will directly inhibit COX-1 activity in platelets preventing their activation, and this has an unending secondary effect on COX-2 expression, preventing tumorigenesis. While this is a tenable hypothesis, it is not yet confirmed.
Figure 1.6: A model depicting how low dose aspirin inactivates COX-1 and contributes to the prevention of colorectal cancers

According to this model, aspirin’s chemopreventative effect is believed to occur primarily through inhibition of the activity of COX-1 and secondarily through inhibition of the upregulation of COX-2. In the absence of inhibition of platelet activation it is envisioned that the activated platelets may release cytokines (IL-1β) as well as growth and angiogenic factors, which can act locally in the colonic tissue causing upregulation of COX-2. COX-2 in turn may cause the upregulation of PGE2 as well as growth factors leading to increased cell proliferation and genomic instability. This is supported by the report that COX-2 overexpression is often observed in colon tumorigenesis and inhibition of the COX-2 reverses the tumor growth [112]. Figure modified from Dovizio et al. [111].
1.16.3 COX-Independent mechanisms:

Interestingly, several COX-independent pathways have been proposed [89, 113]. We and others have demonstrated that aspirin acetylates multiple cellular proteins, which further potentially effects multiple cellular pathways [114-119]. We reported that aspirin acetylated both the wild type and mutant tumor suppressor protein p53 in several breast and colorectal cancer cell lines, and this was associated with an increase in the induction of p53 target genes [115, 120]. Demonstration of the ability of aspirin to acetylate mutant p53 is a significant observation as this tumor suppressor protein is mutated and inactivated in nearly 50% of all cancers and its reactivation via acetylation may restore the lost wild type function to the mutant forms [43, 121]. The ability of a derivative of aspirin (phospho aspirin) to acetylate mutant p53 and induce the expression of p53 target genes have been demonstrated by other investigators [122], suggesting that acetylation of mutant p53, indeed may be an important mechanism by which it exerts its anti-cancer effects.

One of the major pathway activated in colon cancer cell is the Wnt/β-catenin signaling pathway, and in fact this is observed in most sporadic and colorectal cancers [14, 16-18]. Aspirin has been shown to inhibit Wnt/β-catenin pathway through inhibition of PP2A phosphatase activity, providing a possible mechanism for its effects against cancer [123]. Din et al., showed that aspirin inhibited mTOR signaling in colorectal cancer cells, and this was associated with the activation of AMP-activated protein kinase and induction of autophagy [124]. Ai et al., showed that exposure of colon cancer cells to aspirin and salicylic acid down-regulates c-Myc protein and mRNA levels [125]. c-Myc regulates nearly 15% of all genes in the cells [126] and it is often activated through mutation or amplification in many cancers. Since c-myc is considered as a central driver of
tumorigenesis [127, 128], it is possible that the anti-cancer effect of aspirin may involve down-regulation of c-Myc. Aspirin was shown to decrease the levels of epidermal growth factor receptor (EGFR) during colon tumorigenesis [129]. Aspirin and salicylic acid, both caused down-regulation of Sp1, Sp3 and Sp4 transcription factors and caused decreased expression of Sp-regulated gene products including bcl-2, survivin, VEGF, VEGFR1, cyclin D1, c-MET, p65 (NFκB) and β-catenin [130].

1.16.4 Salicylic acid Binding proteins (SABP):

In addition, salicylic acid (the hydrolytic product of aspirin) has also been implicated in aspirin’s chemopreventive effects. Salicylic acid was shown to bind to a number of cellular proteins such as IκB kinase (IKK), a component of the NF-κB complex, AMP activated protein kinase [131], High Mobility Group (HMG) Box 1 proteins and GAPDH [132], and CDK 2 [133], affecting their levels and/or functional activities. Thus, aspirin affects multiple pathways rather than one single target; thus, the broadly-specific nature of its action may be the key to its chemopreventive properties. A summary of the different salicylic acid binding proteins identified till date and the potential modulation of their activity is described below.

**NF-κB:** NF-κB is a transcription factor that regulates the expression of genes involved in inflammatory process. Aspirin and its primary metabolite salicylic acid, have shown to inhibit NF-κB pathway. Patients treated with sodium salicylate (1 – 5 mM) showed decreased expression of NF-κB-inducing kinase (NIK) and TNF-α. Under normal conditions, NF-κB is localized to cytoplasm through the formation of a complex with the inhibitory protein, IκB. IκB, upon phosphorylation by IκB kinase (IKK), undergoes
degradation releasing NF-κB. Free NF-κB then translocates to the nucleus and induces gene expression, particularly that of TNF-α and other inflammatory mediators like IL-1, IL-6, IL-8, and interferon-β (IFN-β). Aspirin and salicylic acid have also shown to inhibit DNA binding capacity of NF-κB and IKK-β kinase activity in both *in-vitro* and *in-vivo* [134, 135].

**AMPK:** Adenosine monophosphate-activated protein kinase (AMPK) is a sensor that is conserved throughout eukaryotes to detect cellular energy levels. It is a heterotrimeric enzyme composed of catalytic α subunit and regulatory β and γ subunits. In response to metabolic stress, AMPK mediates the phosphorylation of targets that can lead to decrease ATP consumption and increase ATP generation. Its enzyme activity increased >100 fold upon phosphorylation at Thr172 on α subunit by LKB1 (Liver Kinase B1), a tumor suppressor protein, and CaMKKβ (Ca²⁺/Calmodulin-dependent protein kinase kinase beta), a calcium-dependent kinase. But, its enzyme activity increased >1000 fold when AMP (but not ADP) binds at an allosteric site on γ subunit, altering the conformation thereby preventing dephosphorylation of Thr172. Salicylic acid, (not aspirin), binds to the same allosteric site and inhibits dephosphorylation of Thr172 on α subunit, thus effecting its function by increasing autophagy. Salicylic acid mediated activation of AMPK has shown to suppress a serine/threonine protein kinase mTOR (mechanistic target of rampamycin) signaling, which controls cell growth, cell proliferation and cell survival by regulating transcription of proteins, metabolism and autophagy [124, 136]

**HMGB1:** High mobility box-1 (HMGB1) is chromatin-associated protein that is present in all animal and plant cells. It has multiple functions; inside the nucleus it helps
in nucleosome formation and in transcription factor binding to DNA, and when released into extracellular spaces (stressed cell) it acts as a DAMP (damage associated molecular patterns) molecule. It also functions as chemoattractant and modulate chemokine action. It can bind to several extracellular receptors such as TLR2, TLR4 and CXCR4 leading to the activation of inflammatory pathways resulting in the induction of proinflammatory cytokines. Salicylic acid was shown to bind to HMGB1, suppressing its proinflammatory function and expression of COX-2 and cytokines such as IL6 and TNF [137].

Figure 1.7: Figure legend continued in next page
Figure 1.7: Aspirin’s primary metabolite salicylic acid directly binds to cellular proteins NF-κB and IKK–β kinase (A) and AMP kinase (B) to modulate their activity.

A, Inactive form of NF-κB is localized to cytoplasm by forming a complex with IκB. In response to inflammatory signals such as lipopolysaccharide (LPS) and double stranded RNA, IκB is phosphorylated by IKK-β kinase releasing NF-κB, which then translocates to nucleus, leading to cytokine gene expression (IL-1, IL-6, IL-8, IFN-β) and TNF-α). Salicylic acid (SA) has been shown to inhibit NF-κB function (DNA binding) through direct interaction [134]. It is also known to inhibit IKK-β kinase, which is important in activation of NF-κB [135].

B, Adenosine monophosphate-activated protein kinase (AMPK) is an energy sensor, following its activation it increases ATP generation and decreases ATP consumption triggering autophagy. It is also known to inhibit mTOR signaling, a key pathway that contributes to cell proliferation. Salicylic acid (SA) has been shown to directly bind to γ-subunit of AMPK, altering its confirmation keeping the enzyme in its active state. This contributes to inhibition of cell growth and proliferation.
1.17 Rationale for studies on Cyclins and CDKs, and hypothesis:

As discussed earlier, numerous observational and randomized studies have demonstrated an association between aspirin intake and cancer risk and mortality. It was observed that daily aspirin intake for 5 years or longer reduces the risk of cancers of the colorectal, lung, prostate, breast and skin. Thus, the evidence that has emerged in recent years on aspirin’s ability to decrease cancers has ignited a renewed interest in its research to understand the mechanism of chemoprevention, as well as its potential to use as a prophylactic drug to prevent cancer. Despite extensive studies, aspirin’s mode of action in cancer prevention, as well as how it primarily prevents CRC as compared to distal cancers, has not been established. Although multiple targets and signaling pathways have been proposed, a unifying mechanism has not been identified till date, suggesting that different mechanisms may be responsible in different cancers or that the actual mechanism is yet to be discovered.

Since dysregulation of cell cycle is an important hallmark of cancer, we hypothesized that aspirin or its primary metabolite may target proteins involved in the regulation of cell cycle to exert its chemopreventive effects. The important CDKs in mammalian cell cycle regulation are: CDK1, 2, 4 and 6. CDK4 and 6, through binding to cyclin D, facilitate the progression through G1, whereas, CDK2 via binding to cyclin E helps to transition from G1 to S phase. CDK1 and 2 get activated via binding to cyclins A and B, which facilitate the progression through G2 and M phases. We hypothesized that aspirin or its metabolite salicylic acid may affect the levels or functional activity of one or more of these cell cycle regulatory proteins. The levels of various cell cycle proteins (CDK1, 2, 4 and 6; cyclins A, B, D and E) in cells treated with aspirin or salicylic acid
were initially determined by Western blot analysis to gain insight into the potential targets of aspirin and salicylic acid. Initial studies were focused on using a panel of human cancer cells (colon, breast, lung, prostate, ovary and skin), however subsequent detailed studies were performed using HT-29 and HCT-116 colon cancer cells.

In this dissertation, we performed a detailed study on how aspirin and salicylic acid affect the levels of cyclin A2 and CDK2, as well as the ability of these drugs to bind and interact with CDK2. Studies were also performed to determine how exposure of cancer cells to aspirin and salicylic acid regulates cyclin A2 and CDK2 mRNA levels. *In-vitro* studies were carried out to determine the effect of aspirin, salicylic acid, salicylic acid metabolites and derivatives on CDK enzyme activity to gain insight into the potential mechanisms by which these drugs may arrest cancer cell growth. Reports in literature has suggested that aspirin and salicylic acid has distinct targets to which they bind to alter their functional activity, however no studies have reported on the cellular targets for salicylic acid metabolites. It is important to emphasize that the investigations carried out in this project is the first attempt to identify cellular targets for salicylic acid metabolites and its derivatives.

Our goal in this project was to delineate, the novel pathways by which aspirin exerts its anticancer effects via formation of salicylic acid and its metabolites in colon cancer cells through the modulation of cell cycle regulatory proteins.
Chapter 2: Materials and Methods

2.1 Materials:

100 BP DNA ladder (Invitrogen)

2,3-DHBA (Thermo Fisher)

2,4,6-THBA (Sigma)

2,4-DHBA (Thermo Fisher)

2,5-DHBA (Thermo Fisher)

2,6-DHBA (Thermo Fisher)

3,4,5-THBA (Sigma)

3,4-DHBA (Thermo Fisher)

3,5-DHBA (Thermo Fisher)

6X DNA loading dye (Thermo Fisher)

$^{32}$P α-dCTP (MP Biochemical)

$^{32}$P γ-ATP (MP Biochemical)

AccessQuick™ RT-PCR (Promega)

Agarose (Thermo Fisher)

Ammonium persulfate (Bio-Rad)

Aspirin (Sigma)

Benzoic acid (Thermo Fisher)
β-mercaptoethanol (Thermo Fisher)

Bovine serum albumin (BSA) Heat Shock Fraction (Sigma)

Bradford’s reagent (Bio-Rad)

Bromophenol blue (Thermo Fisher)

Chloroform (Thermo Fisher)

Coomassie strain (Thermo Fisher)

Dimethyl Sulfoxide (DMSO) (Thermo Fisher)

Dithiothreitol (DTT) (Thermo Fisher)

Ethanol (Thermo Fisher)

Ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher)

FuGENE HD Transfection Reagent (Promega)

GeneJET Gel Extraction Kit (Life Technologies)

Glycerol (Thermo Fisher)

Glycine (Thermo Fisher)

H1 Histones (EMD Millipore)

Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher)

Immobilon (EMD Millipore)

Isopropyl alcohol (Thermo Fisher)
Lactacystin (EMD Millipore)

LB Agar (Thermo Fisher)

LB Broth (Thermo Fisher)

Magnesium Chloride (Thermo Fisher)

Methanol (Thermo Fisher)

Mlu I and EcoR I restriction enzymes (Life Technologies)

MOPS (3-(N-morpholino) propanesulfonic acid) (Thermo Fisher)

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Thermo Fisher)

Paraformaldehyde (Thermo Fisher)

Phenylmethylsulfonyl fluoride (PMSF)

Phosphate buffer saline (PBS) pH-7.4 (Sigma)

Protease inhibitor tablets (Thermo Fisher)

Protein G agarose (Life Technologies)

Random Primer DNA labelling Kit (Clontech)

Salicylic acid (Sigma)

Salmon Sperm DNA (Thermo Fisher)

Sodium acetate (Thermo Fisher)

Sodium chloride (NaCl) (Thermo Fisher)
Sodium citrate (Thermo Fisher)

Sodium dodecyl sulfate (SDS)

Sodium fluoride (Thermo Fisher)

Sodium Hydroxide (NaOH) (Thermo Fisher)

Sodium orthovanadate (Thermo Fisher)

SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher)

TRIzol reagent (Invitrogen)

Trypan blue (Thermo Fisher)

Trypsin-EDTA (Sigma)

Tris base (Thermo Fisher)

Zeta probe blotting membranes (Bio-Rad)

2.2 Buffers:

4X protein Dye: 20% SDS 4 ml; 2 M Tris (pH 6.8); Glycerol 8 ml; Bromophenol blue 40 mg and β-mercaptoethanol 200 µl/ml

10X Running Buffer: Tris base 30 g; Glycine 144 g; SDS 10 g and water up to 1 liter.

10X MOPS: 0.2 M MOPS; 50 mM sodium acetate; 1 mM EDTA and water

20X SSC: 3 M NaCl; 0.35 mM sodium citrate; pH adjusted to 7.0 with 10 N NaOH and 1 liter water
Extraction Buffer: 10 mM Tris (pH 7.4); 25% glycerol; 0.4 M NaCl; 1 mM EDTA and water

Hypotonic Buffer: 10 mM Tris (pH 7.4); 1.5 mM MgCl2; 10 mM KCl; 0.5 mM PMSF; 0.5 mM DTT; 1 mM Sodium orthovanadate and water

Lysis buffer: 1 M Tris-HCl (pH 7.4) – 2 ml; 4 M NaCl – 3.75 ml; Glycerol – 15 ml; Triton-X-100 1 ml and water up to 100 ml.

Northern blot prehybridization buffer: 6X SSC; 5X Denhardt’s solution; 0.5% SDS; 100 µg/ml of salmon sperm DNA.

Protein transfer buffer: Tris base – 11.6 g; Glycine – 5.85 g; Methanol – 500 ml and Water – 1482.55 ml

Washing buffer: 1 M Tris-HCl (pH 7.4) – 20 ml; NaCl – 11.5 g; Water up to 2 liters and Tween 20 – 1 ml

2.3 Cell lines, culture media and other reagents:

HCT 116, HT-29, SW480 (Human colon cancer cells) from ATCC

SK-MEL-28, SK-MEL-5 (Human skin melanoma cells) from ATCC

MDA-MB-231, MCF7 (Human Breast cancer cells) from ATCC

NCI-H226 (Human lung cancer cells) from ATCC

OVCAR-3 (Human Ovarian cancer cells) from ATCC

PC-3 (Human prostate cancer cells) from ATCC

B16-F10 (Mouse Melanoma cells) from ATCC
Trypsin-EDTA Solution from Sigma

Fetal bovine serum (FBS) from Invitrogen

Eagle’s minimum essential medium (ATCC)

McCoy 5A modified medium (Invitrogen)

2.4 **Recombinant proteins, enzymes, plasmid DNA and antibodies:**

Recombinant CDK1, CDK2, cyclin A2 and cyclin B1 from Prospec, CDK1/Cyclin B1 active enzyme from New England Biolabs (NEB), CDK1/Cyclin B1, CDK2/Cyclin A2, CDK4/Cyclin D1, CDK6/Cyclin D1 and Retinoblastoma (C-term) were purchased from SignalChem. Myc-DDK-tagged human cyclin A2, cyclin B1, CDK1, CDK2 and CDK4 plasmids, and anti-DDK antibody were obtained from OriGene; Anti-cyclin A2, anti-cyclin B1, anti-CDK1 and anti-β actin antibodies, and CDK, cyclin, cell cycle regulation and Retinoblastoma phosphorylation kits were purchased from Cell Signaling Technology; anti-Cyclin A2 antibody was from Abcam, Anti- CDK2 antibody for immunoprecipitation from EMD Millipore; goat anti-rabbit and goat anti-mouse antibodies were obtained from Bio-Rad.

2.5 **Cell Culture:**

The cell lines obtained from ATCC were cultured at 37°C and 5% CO₂, using recommended medium containing 10% FBS for 24-48 h before adding aspirin or salicylic acid for indicated times. Authentication of cell lines was done by ATCC through their DNA-STR profile.
2.6 Cell proliferation:

To determine the effect of aspirin and salicylic acid on cell proliferation, approximately 100,000 cells were seeded per 100 mm plates containing 10% FBS and grown overnight. Aspirin or salicylic acid were added at various concentrations and incubated for 48 h. The floating cells (if any) were collected from the conditioned media, and pooled with the trypsinized adhered cells, and counted in the Nexcelom Cellometer Auto T4 cell counter. The viability of the cells was determined by adding trypan blue staining.

2.7 Total cell lysate preparation and Western Blotting:

Cells were seeded at 50% confluency, left overnight and treated with aspirin or salicylic acid at different concentrations for the indicated time points. Cells were washed with phosphate buffered saline (PBS) and scraped in lysis buffer (10mM Tris-Cl pH 7.4, 150 mM NaCl, 15% glycerol, 1% Triton X-100 with protease inhibitors) and collected into an Eppendorf tube, vortexed every 5 mins while incubating on ice for 30 mins. Tubes were centrifuged at 14,000 RPM and supernatant was collected. Protein amount was estimated using Broadford’s reagent. Samples containing 50µg of proteins were separated by an 8 or 10% polyacrylamide gel electrophoresis (PAGE) unit, transferred onto an immobilon membrane and immunoblotted with respective antibodies. Immunoreactive bands were visualized using chemiluminescence (ECL) and they were detected using Epi Chemi II Darkrom (UVP). The intensities of bands were quantified using NIH ImageJ software.
2.8 Protein estimation Using Bradford’s Reagent:

Bovine serum albumin (BSA) (1 mg/ml) in water was used for standard curve. Protein was estimated using Bradford reagent following supplier’s instructions (Bio-Rad). Following color development, optical density (OD) were measured at 595 nm using Eppendorf BioPhotometer®. A graph of absorbance vs concentration was plotted to determine the concentration of the samples.

2.9 Immunoprecipitation:

For immunoprecipitations, 500 µg of the total proteins isolated from cells or 300 ng of the recombinant protein were diluted to 1 ml of lysis buffer, immunoprecipitated with respective antibodies overnight at 4ºC. The immune complexes were captured by adding 35 µls of protein G agarose and incubated at 4ºC for 3h. The immunocomplexes were spun down at 3000 RPM for 10 mins and the pellets were washed 2 times with lysis buffer and washed once with lysis buffer containing no triton X-100. The pellet was collected and suspended in SDS-sample buffer, boiled for 10 mins and loaded onto the SDS-PAGE. Alternatively the immune complexes were washed with kinase buffer 2 times and then used for in-vitro CDK kinase assay.

2.10 Agar Plates:

LB agar media was prepared and sterilized by autoclaving for 20 mins. The media was cooled to 55ºC, antibiotic was added (kanamycin 25 µg/ml) and poured onto agar plates.
2.11 Transformation of *E. coli* with plasmid DNA:

Transformation of *E. coli* with plasmid DNA was performed by using heat shock method [138]. Briefly, 100 µl of a suspension of DH5-α *E. coli* cells were incubated with 10 ng of DNA in an eppendorf tube and kept on ice for 30 mins. Tubes were then incubated at 42°C for 30 sec, immediately chilled in ice for 2 mins. The cells were then spread on an agar plate and incubated in a 37°C incubator overnight. Following the appearance of colonies, a single colony was picked for plasmid isolation.

2.12 Isolation of plasmid DNA and their insert:

A conical flask containing 200 ml of autoclaved LB broth containing 200 µg/µl ampicillin was inoculated with transformed cells (pCMV6-Entry vector carrying full-length CDK1, CDK2, cyclin A2 or cyclin B1) and placed on a shaker at 200 RPM (37°C) overnight. The next day, the cells were centrifuged at 4000 RPM (4°C) and the supernatant discarded. The recombinant plasmid DNA was then isolated using the (QIAGEN) QIAPERP Spin Maxiprep Kit protocol and the concentration of DNA was estimated at 260 nm in distilled water. The plasmid DNA was digested with MluI and EcoRI restriction enzyme to release the cDNA insert, the digested DNA was run in an agarose gel, DNA purified using GeneJET Gel Extraction Kit (Thermo Scientific).

2.13 RNA isolation and Northern blot analysis:

RNA was isolated using TRIzol method. For this, cells were seeded at 50% confluency overnight in 100 mm plates and treated with aspirin or salicylic acid for indicated time points. The plates were washed with PBS (3 times), lysed with 5ml of TRIzol reagent and incubated at room temperature for 5 mins. The lysates were collected
into a 50 ml tube, 1 ml of chloroform was added and shaken vigorously for 15 seconds. The samples were then centrifuged at 12,000 × g for 15 mins at 4°C. The aqueous phase containing RNA (DNA and protein in organic and intermediate phase) is transferred into a fresh tube, and 2.5 ml of isopropyl alcohol was added to precipitate RNA. After incubation at room temperature for 10 mins, RNA was collected by centrifugation at 12,000 × g for 10 mins at 4°C. The RNA pellet was twice washed with 75% ethanol and once with 100% ethanol, pellet was air dried and dissolved in DNase/RNase free water.

The pCMV6-Entry vector carrying full-length cyclin A2 and CDK2 plasmid DNA was digested with MluI and EcoRI to release the cDNA insert, run on a 1% agarose gel. The cDNA insert was purified using Gene gel extraction kit (Thermo Scientific). Total RNA (20 µg) was separated on a 1.25% agarose-formaldehyde gel and transferred onto a Zeta probe blotting membranes using 20x SSC. The blot was prehybridized using prehybridization buffer at 42°C in a water bath for 2 h, followed by hybridization with 32P α-dCTP labelled cDNA in the hybridization buffer overnight. The blots were washed with 0.1X SSC buffer containing 0.1% SDS for 1h at 65°C, dried and exposed to an X-ray film.

2.14 RT PCR:

RT-PCR was performed according to manufacturer’s instructions (Promega). Briefly, mRNA isolated from salicylic acid untreated or treated HT-29 cells was reverse transcribed to cDNA using AMV Reverse Transcriptase (AMV RT) followed by amplification of the cDNA product. Reverse transcription is done for 45 mins at 45 °C. This cDNA template is denatured at 94 °C for 2 min followed by the 20/30/40 cycles, with each cycle having denaturation (94 °C for 30 s), annealing (60 °C for 60 s) and extension (60 °C for 120 s). A final extension was performed at 68 °C for 5 mins and hold at 4 °C.
Samples are collected and ran on a 1% agarose gel containing ethidium bromide. Intensities of the bands were quantified using NIH ImageJ software.

| CDK1 (700 BP)       | F-GGTTCCTAGTACTGCAATTTCG  |
|                     | R- TTTGCCAGAAATTCTGTTTGG   |
| Cyclin B1 (191 BP)  | F- CAGTCAGACAAAAATACCTACTGGGT |
|                     | R- ACACCAACCAGCTGCAGCATCTTTT |
| GAPDH (90 BP)       | F-CCACTCCTCCACCTTGTGAC     |
|                     | R- ACCCTGTTGCTGTAAGCCA     |

**Table 2.1 List of CDK1, cyclin B1 and GAPDH primers used for RT-PCR**

### 2.15 Expression of recombinant DDK-tagged proteins:

Cells were seeded onto a 100 mm plate at 50% confluency overnight to perform transfection experiments according to the manufacturer’s instructions (FuGENE reagent; Promega). Briefly, 3 µg of recombinant cDNA plasmids were incubated with FuGENE HD Transfection Reagent for 15 mins, then added to the cells. The cells were incubated for 24 h-48 h to allow for the expression of recombinant proteins and treated with respective drugs for another 24 h. Cell lysates were prepared and immunoblotted with either anti-DDK antibodies, or respective proteins.

### 2.16 *In-vitro* CDK assay:

*In-vitro* CDK assay from cell lysates was performed according to the previously published method [139]. In brief, 500 µg of the protein from cell lysates were diluted with 1 ml of the lysis buffer and immunoprecipitated using anti-CDK2 antibody overnight.
followed by the addition of protein G agarose for 4 h. Samples were collected and spun at 6000 RPM for 10 mins followed by washing three times with lysis buffer, twice with lysis buffer containing no Triton X-100 and once with kinase buffer (40 mM Tris-HCl pH 8.0, 5mM MgCl2, and 5% glycerol). The final pellet was suspended in kinase buffer containing 20 µM ATP, 2 µci of $\gamma^{32}$P ATP, 5µg of H1 Histone, 0.5X phosphatase inhibitor, and incubated for 30mins at 37°C. The reaction was stopped by the addition of SDS-sample buffer, and loaded on a 10% SDS-PAGE, gel stained with Coomassie brilliant R strain, dried and exposed to X-ray film.

In-vitro CDK assays with purified commercial enzymes were performed as described by the protocols from NEB and SignalChem. Briefly, purified kinase was aliquoted into the reaction buffer provided by the respective kits and incubated with indicated compounds at various concentrations for 10 mins at room temperature. Kinase reactions were performed by incubating the enzyme with a kinase buffer containing 15 µM ATP, 2 µCi of $[\gamma^{32}]$P ATP, 5µg of H1 Histone or retinoblastoma, at 30°C for 20 mins. The final volume of the reaction was 50 µl. The reactions were stopped by adding EDTA to a final concentration of 20 mM and 4X loading buffer. The samples were boiled for 10 mins and loaded on 10% SDS-PAGE. The gel was stained using Coomassie Brilliant blue (R), dried and exposed to X-ray film. Intensities of the bands were quantified using NIH ImageJ software.

2.17 Molecular docking studies:

An in-silico approach was adopted to identify potential target inhibitors through molecular docking studies. In an attempt to understand the ligand-protein interactions in terms of binding affinity, aspirin, salicylic acid, DHBAs and THBAs were subjected to
docking with CDKs using AutoDockVina. The small-molecule topology generator Dundee PRODRG2 server was used for ligand optimization [140]. The crystallographic three-dimensional structures of selected target proteins were retrieved from the Protein Data Bank (PDB) http://www.pdb.org. The human Cyclin A2 (PDB ID: 1FIN B chain), CDK2 (PDB ID: 1AQ1), cyclin A2/CDK2 complex (PDB ID: 1FIN A, B chain), CDK1 (4y72 A chain) and cyclin B1 (PDB ID: 2b9r) and CDK1/cyclin B1 (4y72 A chain and B chain), protein molecules were selected for energy minimization using Gromacs 3.3.1 package with the GROMOS96 force field [141]. These molecules were used as the receptor for virtual small molecule docking with the ligand aspirin and salicylic acid using AutoDockVina. Python molecular viewer with AutoDock Tools were used for conversion to pdbqt format, required by AutoDockVina.

2.18 CDK2/ANS fluorescence assay:

The CDK2/ANS assay is based on the fluorescence emitted from the interaction of ANS within the allosteric pocket of CDK2 [142]. For the assays, the previously recommended concentrations of ANS and CDK2 at 50 µM and 1.6 µM (0.5mg/ml) respectively, was used. Commercially obtained recombinant CDK2 protein was mixed with ANS in a total volume of 50 µls in a 96 well plate, and the fluorescence was measured at excitation and emission wavelengths of 405 and 460 nm using a Spectramax M2 spectrophotometer. Alternatively, recombinant CDK2 was first pre-incubated with salicylic acid at different concentrations before the addition of ANS, and then the fluorescence was measured.
2.19 Mass Spectrometry:

After the CDK-1 was incubated with and without (control) aspirin for 12 h, the pH of the solution was adjusted to pH=8 and reduced with the reaction buffer containing 50 mM of Tris-HCl and 50 mM DTT (Sigma) at 37°C for 30 mins, and followed by alkylation using Iodoacetamide to a final concentration of 15 mM (Sigma) at room temperature. The resultant solution was digested using sequencing grade trypsin/Lys-C (Promega, Madison WI) overnight at 37°C, and the reaction was terminated using 0.5% v/v of trifluoroacetic acid (TFA). The samples were frozen in dry ice and concentrated in a SpeedVac centrifuge (Thermo Savant). The trypsin-digested peptides were later dissolved in 100 mM ammonium formate (pH10), and separated through 2D-nanoLC with dilution using a 2D-nanoAcquity UPLC (Waters, Milford, MA). The eluted ions were analyzed in Q-TOF Synapt G1 HDMS mass spectrometer (Waters, Milford, MA) equipped with MassLynx 4.1 (Waters, Milford, MA) software, selecting one full precursor MS scan (400-2000 m/z) followed by four MS/MS scans of the most abundant ions detected in the precursor MS scan while operating under dynamic exclusion or direct data acquisition system [143, 144]. Mascot server v2.5 and Mascot Daemon Toolbox v2.5 (www.matrix-science.com, UK) in MS/MS ion search mode were applied to conduct peptide matches (peptide masses and sequence tags) and protein searches against human CDK-1 individual protein database assembled in house (gi#4502709 from NCBInr). The following parameters were set for the search: carbamidomethyl (C) on cysteine was set as fixed; variable modifications included asparagine and glutamine deamidation, methionine oxidation and acetyl lysine (K). Also, the analysis was controlled through a second search using Error Tolerant option. Two missed cleavages were allowed; monoisotopic masses were counted; the precursor
peptide mass tolerance was set at 2 Da; fragment mass tolerance was 0.3 Da and the ion score or expected cut-off was set at 5 Da. The MS/MS spectra were searched with MASCOT using a 95% confidence interval (C.I. %) threshold (p<0.05), with which minimum score of 3 was used for peptide identification.

2.19 MTT Assay:

Cytotoxicity was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, HCT-116 cells were seeded in 24 well plates overnight at a density of 20,000 cells/well and treated with various compounds for 72 h. MTT assays were carried out as previously described [120].

2.20 Statistical analysis:

All experiments were repeated 3-6 times independently of each other. One-way ANOVA followed by Tukey’s range tests were adopted to compare group differences to control and significance was defined as P<0.05. Graph pad Prism is used for statistical analysis.
Chapter 3: Cyclin A2 and CDK2 as Novel Targets of Aspirin and Salicylic acid

3.1 Abstract:

Data emerging from the past 10 years have consolidated the rationale for investigating the use of aspirin as a chemopreventive agent; however, the mechanisms leading to its anti-cancer effects are still being elucidated. We hypothesized that aspirin’s chemopreventive actions may involve cell cycle regulation through modulation of the levels or activity of cyclin A2/cyclin dependent kinase-2 (CDK2). In this study, HT-29 and other diverse panel of cancer cells were used to demonstrate that both aspirin and its primary metabolite, salicylic acid, decreased cyclin A2 (CCNA2) and CDK2 protein and mRNA levels. The down regulatory effect of either drugs on cyclin A2 levels was prevented by pretreatment with lactacystin, an inhibitor of proteasomes, suggesting the involvement of 26S proteasomes. In-vitro kinase assays showed that lysates from cells treated with salicylic acid had lower levels of CDK2 activity. Importantly, three independent experiments revealed that salicylic acid directly binds to CDK2. Firstly, inclusion of salicylic acid in naïve cell lysates, or in recombinant CDK2 preparations, increased the ability of the anti-CDK2 antibody to immunoprecipitate CDK2, suggesting that salicylic acid may directly bind and alter its conformation. Secondly, in 8-anilino-1-naphthalene-sulfonate (ANS)-CDK2 fluorescence assays, pre-incubation of CDK2 with salicylic acid, dose-dependently quenched the fluorescence due to ANS. Thirdly, computational analysis using molecular docking studies identified Asp145 and Lys33 as the potential sites of salicylic acid interactions with CDK2. These results demonstrate that aspirin and salicylic acid down-regulate cyclin A2/CDK2 proteins in multiple cancer cell lines, suggesting a novel target and mechanism of action in chemoprevention.
3.2 **Background and Hypothesis:** Previous reports show that aspirin inhibits cell proliferation in multiple cancer cell lines [130, 145, 146]. We hypothesized that the anti-cancer effects of aspirin may involve down-regulation of cell cycle regulatory proteins comprising of cyclins, CDKs and CKIs. A detailed study on cyclin A2 and its binding partner CDK2 was carried out because of its pivotal role in cell cycle regulation, as they are involved in regulation of DNA synthesis during the S phase and important for inactivation of E2F. Both cyclin A2 and CDK2 are upregulated in breast, liver and lung cancers, therefore these have been identified as therapeutic targets in cancer treatment [147-152]. Our goal in this section was to study the effect of aspirin and salicylic acid on cyclin A2/CDK2 in multiple cancer cell lines representing cancers of various tissues such as colon, breast, lung, skin, prostate and ovary, which would also establish the universality of the observation.

3.3 **Results:**

3.3.1 **Aspirin and salicylic acid decrease cell proliferation in HT-29, SK-MEL-28, and MDA-MB-231 cells:**

Previous studies have shown that treatment of cells with aspirin and salicylic acid, at concentrations ranging from 2.5 mM to 10 mM, induced a profound reduction in cell proliferation in HT-29 and other cancer cells [130, 145, 146]; but, a comparison at lower concentrations were not reported. In this experiment, we chose HT-29 (colon), SK-MEL-
28 (skin) and MDA-MB-231 (breast) cancer cells to evaluate the effect of aspirin and salicylic acid on proliferation rate at the concentrations ranging from 0.25 to 2.5 mM. Cells were treated separately with both drugs for 48h, trypsinized and counted. We observed that aspirin and salicylic acid progressively reduced the cell number particularly from 0.5 mM to 2.5 mM (Figure 3.1). The cell viability was unaffected at all concentrations of the drugs tested. These results show that both drugs are effective in reducing the cell proliferation rate upon exposure for 48h, without affecting the viability.

**Figure 3.1:** Dose-dependent effect of aspirin and salicylic acid on the growth rates in HT-29, SK-MEL-28, and MDA-MB-231 cells

Cells were seeded at 50% confluency and treated with different concentrations of aspirin and salicylic acid for 48 h, trypsinized and cells were counted, viability determined as described. P-value < 0.001‡, <0.01†, < 0.05*. 
3.3.2 Effect of aspirin and salicylic acid on cell cycle regulatory proteins:

We hypothesized that aspirin and salicylic acid may exert their anti-proliferative effects through modulation of cell cycle regulatory proteins. Therefore, we sought to determine whether these drugs would affect the levels of cyclins A, B, D and E; CDKs 1, 2, 4 and 6; and CDK inhibitors p16, p21 and p27. To address this, HT-29 cells were left untreated or treated with aspirin or salicylic acid at various concentrations for 24h. Cell lysates were prepared and immunoblotted with various anti-cyclin, anti-CDK and anti-CDK inhibitor antibodies. We observed that both aspirin and salicylic acid down regulated the levels of cyclins A2, B1 and D3; and CDKs 1, 2, 4 and 6. Interestingly, both drugs up-regulated the levels of cyclin E1 as well as CDK inhibitors, p27 and p21. The levels of p16 were not detected in these experiments possibly reflecting the lower expression. The data on the effect of aspirin and salicylic acid on cyclin A2 in HT-29 cells is shown in Fig. 3.2A and B. The results obtained on CDK2 is discussed elsewhere in Fig. 4 (see below). The data on cyclins B1, E1 and D3 are shown in supplemental Fig. 3.3; CDKs 1, 4 and 6 in supplemental Fig. 3.4 and CDK inhibitors p21 and p27 in supplemental Fig. 3.5. It is clear from these results that aspirin exposure to HT-29 cells causes differential regulation of cell cycle regulatory proteins. Among these identified protein targets, we focused mainly on cyclin A2 and CDK2 in the present study because: a) they play an important role in the regulation of DNA synthesis during cell cycle progression; b) they are de-regulated or up-regulated in several cancers such as breast, liver and lung [136-141]. We hypothesized that aspirin and salicylic acid may primarily target cyclin A2/CDK2 to cause the cell cycle arrest, which has been previously reported by other investigators [130, 145, 146].
Figure 3.2: Aspirin and salicylic acid down-regulate cyclin A2 protein levels in multiple cell lines

A and B respectively represent the effect of aspirin and salicylic acid on cyclin A2 protein levels in HT-29 cells. C and D represent comparison of the effect of aspirin and salicylic acid in multiple cancer cell lines. For C and D, the intensity of bands in various western blots were quantified and expressed as percentage of control. P-value < 0.001‡, <0.01†
Figure 3.3: Dose-dependent effect of aspirin and salicylic acid on cyclins B1, D3 and E1 in HT-29 cells

A and B respectively represent the effect of aspirin and salicylic acid on cyclins B1, D3 and E1 in HT-29 cells. Cells were treated with aspirin and salicylic acid for 24 h, lysate immunoblotted with respective antibodies.
Figure 3.4:  Dose-dependent effect of aspirin and salicylic acid on CDKs 1, 4 and 6 in HT-29 cells

A and B respectively represent the effect of aspirin and salicylic acid on CDKs 1, 4 and 6 in HT-29 cells. Cells were treated with aspirin and salicylic acid for 24 h, lysate immunoblotted with respective antibodies.
Figure 3.5: Dose-dependent effect of aspirin and salicylic acid on CDK inhibitors p-21 and p-27 in HT-29 cells

A and B respectively represent the effect of aspirin and salicylic acid on CDKs inhibitors p21 and p27 in HT-29 cells. Cells were treated with aspirin and salicylic acid for 24 hours, lysate immunoblotted with respective antibodies.
3.3.3 Aspirin and salicylic acid decrease cyclin A2 levels in multiple cell lines:

We compared the ability of aspirin and salicylic acid at 3 different concentrations (0.5, 1.5 and 2.5 mM) to down-regulate cyclin A2 levels in 11 different cancer cell lines representing human colon (HCT 116, HT-29, SW480); breast (MDA-MB-231, MCF7), skin (SK-MEL-28, SK-MEL-5); Lung (NCI-H226); prostate (PC-3); OVCAR-3 (ovary), and also in mouse skin melanoma (B16-F10) cells. Figure 1C and 1D respectively demonstrates the comparison of the down-regulatory effect of aspirin and salicylic acid on cyclin A2 levels in these cell lines. It is clear from Figs. 3.2C and D that the decrease in cyclin A2 was greater at higher concentrations of the drugs, although the sensitivity of cells towards drug treatment differed. Most dramatic down-regulation was observed in HCT 116, MCF7, SK-MEL-5 and OVCAR-3 cells at all drug concentrations tested.

3.3.4 Lactacystin completely prevents aspirin and salicylic acid-mediated down-regulation of cyclin A2 levels:

Cyclin A2 naturally undergoes degradation via ubiquitin-proteasomal pathway [153]. During pro-metaphase, it is ubiquitinated by the APC/C and this tags cyclin A2 for degradation by the 26S proteasomes [142]. To determine a role for the proteasomal pathway, we tested the ability of lactacystin, a 26S proteasomal inhibitor [154], to prevent aspirin and salicylic acid-mediated decrease in cyclin A2 levels. Cells were left untreated or first treated with lactacystin (10 µM) for 1h, then aspirin or salicylic acid (2.5 mM) were added for 24h. Cell lysates were prepared and immunoblotted with the anti-cyclin A2 antibody. Figure 3.6A demonstrates that lactacystin pretreatment completely prevented the degradation of cyclin A2 caused by aspirin and salicylic acid. Quantification of these bands showed that aspirin and salicylic acid decreased the cyclin A2 levels by 47% and
52% respectively (Fig. 3.6B). Lactacystin treatment alone stabilized the cyclin A2 protein levels; it was 2.3-fold higher compared to untreated control. However, in the presence of lactacystin, both drugs failed to cause the down-regulation of the cyclin A2. This suggests that, 26S proteasomes are involved in aspirin and salicylic acid-mediated down-regulatory effects.

3.3.5 **Aspirin and salicylic acid decrease exogenously expressed, DDK-tagged, cyclin A2 protein levels:**

To investigate whether aspirin and salicylic acid could decrease the exogenously expressed DDK-tagged cyclin A2 protein levels, HT-29 cells were left untransfected or transfected with DDK-tagged full-length cyclin A2 cDNA cloned in the pCMV6 vector. After transfections, cells were incubated for 24 h to allow for the expression DDK-tagged cyclin A2. Following this, cells were either left untreated or treated with drugs at a concentrations of 2.5 mM for 24 h. Cell lysates were prepared and immunoblotted with anti-DDK or anti-cyclin A2 antibodies. Figure 3.6C demonstrates that anti-DDK antibody detected the expression of the DDK-tagged cyclin A2 protein in transfected cells (lane 4). Interestingly, both drugs decreased the DDK-tagged cyclin A2 (lanes 5 and 6). When the samples were immunoblotted with anti-cyclin A2 antibody, the levels of exogenously expressed, DDK tagged cyclin A2, as well as the endogenous cyclin A2 protein, were decreased following aspirin or salicylic acid treatment (Fig. 3.6D). Reprobing the blot of Figure 3.6C with β-actin antibody showed equal amounts of the protein in all lanes. Thus, both aspirin and salicylic acid caused a decrease in the endogenous as well as exogenous cyclin A2 protein levels.
Figure 3.6.  Down-regulation of cyclin A2 by aspirin and salicylic acid is mediated by 26S proteasomal pathway

A, effect of lactacystin on the ability of aspirin and salicylic acid to decrease cyclin A2 protein levels in HT-29 cells. B, represents the quantification of the bands in blot A. C and D, aspirin and salicylic acid down-regulate exogenously expressed DDK-tagged cyclin A2 protein. C and D, respectively represent immunoblots probed with anti-DDK tagged antibody and anti-cyclin A2 antibody. Positions of the exogenous and endogenous cyclin A2 were shown by arrows.
3.3.6 **Aspirin and salicylic acid decrease cyclin A2 mRNA levels:**

To investigate if aspirin and salicylic acid regulate cyclin A2 expression at the transcriptional/post-transcriptional level, we measured cyclin A2 mRNA levels in HT-29 cells treated with drugs following 24 h treatment. Cells were left untreated or treated with aspirin or salicylic acid at different concentrations for 24h; total RNA was prepared and analyzed for cyclin A2 mRNA in Northern blots. Figure 3.7A and C demonstrates that in untreated control cells (lane 1), abundant cyclin A2 mRNA was detected; both aspirin and salicylic acid respectively caused a significant decrease in cyclin A2 mRNA levels at 1.5 mM and 2.5 mM concentrations. Figure 3.7B and D shows the ethidium bromide stained pattern of the ribosomal 28S and 18S RNA, representing the blot in Fig. 3.7A and C, which shows equal RNA loading in all lanes. These results show that the observed decrease in cyclin A2 protein levels following treatment with drugs is at least in part due to decreased presence of cyclin A2 mRNA levels.

3.3.7 **Aspirin and salicylic acid down-regulate CDK2 protein and mRNA levels in HT-29 cells:**

We then determined whether exposure of cells to aspirin and salicylic acid modulates CDK2 protein and mRNA levels. For this, cells were left untreated or treated with aspirin or salicylic acid at various concentrations, total lysates or mRNAs were prepared and analyzed by Western blots and Northern blots respectively. Figures 3.8A and B, demonstrates that aspirin and salicylic acid decreased the CDK2 protein levels at all concentrations tested. Figure 3.8C demonstrates that salicylic acid caused a reduction in CDK2 mRNA levels. Similar results were obtained in SK-MEL-28 and other cancer cell lines (data not shown).
Figure 3.7. Down regulation of cyclin A2 mRNA by aspirin and salicylic acid in HT-29 cells

Aspirin (A) and salicylic acid (C) decrease cyclin A2 mRNA levels in a concentration dependent fashion. B and D respectively represents, the ethidium bromide stained ribosomal RNA pattern of blots in A and C.
Figure 3.8. Aspirin/salicylic acid down-regulate CDK2 protein/mRNA levels and activity

A, aspirin and salicylic acid down-regulate CDK2 protein in HT-29 cells. B, quantification of the band in blot A, expressed as percentage control. C, Northern blot analysis of CDK2 in response to salicylic acid treatment in HT-29 cells. D, ethidium bromide stained ribosomal RNA pattern of blot C. E and F, respectively represent CDK2 activity at two different concentrations (0.5 mM and 1.5 mM) in HT-29 and SK-MEL-28 cells, numbers on the blot represent intensities expressed as percentage of control. The lower panel shows the Coomassie blue stained histones following electrophoresis.
3.3.8 **Salicylic acid decreases CDK2 activity in HT-29 and SK-MEL-28 cells:**

It is clear from the results described above that both aspirin and salicylic acid decrease cellular cyclin A2 (Fig. 2) and CDK2 (Fig. 3.8A) levels. To investigate if this is associated with a corresponding reduction in the CDK2 activity, we carried out an *in-vitro* kinase assay to measure the CDK2 activity using anti-CDK2 immunoprecipitates isolated from cells treated with salicylic acid. Samples representing Fig. 3.8A (control, 0.5 and 1.5 mM) were immunoprecipitated with anti-CDK2 antibodies, and the immunocomplexes subjected to an *in-vitro* kinase assays using radiolabeled γ-32P ATP and H1-histones as substrates. Figure 3.8E and F demonstrates that CDK2 kinase activity is significantly reduced in cells treated with salicylic acid as measured by the ability of CDK2 to phosphorylate H1 histones. The amount of histones were similar in all lanes (Fig. 3.8E and F, lower panel).

3.3.9 **Inclusion of salicylic acid during immunoprecipitation enhances the ability of anti-CDK2 antibody to immunoprecipitate CDK2 in HT-29 naïve total cell lysates:**

It has been shown that cyclin A2 binds to CDK2 to form a hetero-dimer, and regulates CDK2 activity. The cell culture (*in-vivo*) experiments described in Fig. 2 and Fig. 3.8A respectively demonstrate that exposure of cells to aspirin / salicylic acid down-regulates cyclin A2 and CDK2 protein levels. In order to gain insight into the mechanisms of down-regulation, we hypothesized that salicylic acid may directly bind to CDK2 causing a conformation change. Such a scenario is also supported by reports in literature which suggest that salicylic acid indeed interacts with cellular proteins [134, 136, 137]. We further hypothesized that, salicylic acid bound CDK2 may still associate with cyclin A2 to form a triad of CDK2/salicylic acid/cyclin A2 complex, the formation of this un-natural
complex may lead to degradation of these proteins by the 26S proteasomes. To address this, we prepared lysates from HT-29 cells that were not treated with aspirin or salicylic acid (naïve cell lysates). We pre-incubated these naïve cell lysates with different concentration of salicylic acid and then tested the ability of anti-CDK2 antibodies to bind and immunoprecipitate CDK2 protein. We reasoned that salicylic acid-induced changes in CDK2 conformation may affect the ability of anti-CDK2 antibody to bind (due to changes in the accessibility of the epitope) and immunoprecipitate CDK2. Therefore, measuring the levels of CDK2 and cyclin A2 (cyclinA2 naturally associates with CDK2) in the anti-CDK2 antibody immunoprecipitates would suggest how salicylic acid affects CDK2 protein recognition by anti-CDK2 antibody. This approach is described in Fig. 3.9 (flow chart).

The association of cyclin A2 with CDK2 was determined by first immunoprecipitating the samples with the anti-CDK2 antibody (mouse monoclonal), followed by reprobing the blots with anti-cyclin A2 antibody (rabbit monoclonal). This approach was used to avoid detection of immunoglobulin heavy chain (Ig-H) in anti-cyclin A2 immunoblots of the anti-CDK2 immunoprecipitates, as Ig-H and cyclin A2 have a similar molecular weight of 54-56 kDa. The untreated control cell lysate was divided into 4 aliquots, each containing 500 µg of protein in a volume of 1 ml immunoprecipitation buffer. One aliquot was left untreated, and to the other three aliquots, salicylic acid was added at different concentrations (0.5, 1.5 and 2.5 mM) for 1 hour at RT. Pre-incubation of lysates with salicylic acid was performed to allow for the potential binding (if any) of salicylic acid to CDK2. The CDK2 protein was immunoprecipitated by adding monoclonal anti-CDK2 antibody, immunocomplexes were immunoblotted with rabbit anti-cyclin A2 antibody
(Fig. 3.9). Consistent with the literature, anti-CDK2 antibody immunoprecipitates from untreated control lysates (no incubation with salicylic acid), contained cyclin A2 protein, suggesting that it naturally associates with CDK2 (Figure 3.10A, lane 1). We observed that, with increasing salicylic acid concentration (pre-incubated samples), greater amount of cyclin A2 was detected in the anti-CDK2 immunoprecipitates (Fig. 3.10A lanes 2-4). Reprobing the blot in Fig. 3.10A with anti-CDK2 antibody showed that, in samples pre-incubated with salicylic acid, greater amount of CDK2 protein (33 kDa) was also immunoprecipitated by anti-CDK2 antibodies (Fig. 3.10B). There was no significant change in the pH of the immunoprecipitation buffer before and after the addition of salicylic acid and therefore, increased CDK2 immunoprecipitation does not appear to be due to changes in the buffer pH. It was also not due to a non-specific adsorption to protein G agarose (data not shown). The Ig heavy chain (Ig-H) and light chain (Ig-L) levels remained the same in Fig. 3.10B, confirming equal amount of anti-CDK2 antibody addition to the immunoprecipitation reactions. These results provided the first clues on the ability of salicylic acid to bind to CDK2, and possibly alter its conformation.

Intrigued with this observation, we repeated the immunoprecipitations described in Fig. 3.10A and B several times, and in lysates isolated from multiple cell lines (data not shown) to ensure reproducibility. We next determined whether the CDK2 present in the immunoprecipitated samples of Fig. 3.10A is catalytically active. For this, the experiment was performed similar to the one described in Fig.3.10A, wherein 3 different concentrations of salicylic acid was added to the naïve cell lysates for 1 h before immunoprecipitation with the anti-CDK2 antibody. Following immunoprecipitation, the immunocomplexes were subjected to an in-vitro kinase assay using radiolabeled 32P-γ-
ATP and H1 histone as a substrate, as described for Fig. 3.8E. Figure 3.10C shows a correlation between the presence of increasing concentration of salicylic acid during immunoprecipitation reactions and increased phosphorylation of H1 histones in the \textit{in-vitro} kinase assay. H1 Histone phosphorylation progressively increased when salicylic acid was included in the immunoprecipitation reactions at 0.5, 1.5 and 2.5 mM (lanes 2, 3 and 4). The lower panel in Fig. 3.10C shows that all lanes contained similar amounts of H1 histones. These results suggest that the increased amount of H1 phosphorylation observed in kinase assay in Fig. 3.10C probably reflects the greater amount of CDK2/cyclin A2 protein present in the anti-CDK2 immunoprecipitates, and that CDK2 in the immunoprecipitate is catalytically active.

\textbf{3.3.10 Inclusion of salicylic acid during \textit{in-vitro} kinase assay does not affect the CDK2 activity}

The experiments described in Fig.3.10C did not contain any salicylic acid added during the kinase assay, and therefore, it was of interest to determine if inclusion of salicylic acid during kinase assay reaction would affect the H1 histone phosphorylation. For this, naive cell lysates were immunoprecipitated with anti-CDK2 antibody (without pre-incubation with salicylic acid), immunoprecipitates were subjected to an \textit{in-vitro} kinase assays in the absence or presence of different concentrations of salicylic acid (0.5, 1.5 and 2.5 mM). Figure 3.10D demonstrates that inclusion of salicylic acid during the kinase assay had no effect on the ability of CDK2 to phosphorylate H1 histones at all concentrations tested.
Figure 3.9: Schematic representation of the steps involved in immunoprecipitation described for Fig. 3.10 A and B.

3.3.11 Salicylic acid increases the ability of anti-CDK2 antibodies to bind to the purified recombinant CDK2 protein:

In order to determine if salicylic acid can increase the ability of the anti-CDK2 antibody to recognize/bind directly to CDK2, the experiments performed in Figure 3.10A was repeated except that, commercially obtained purified CDK2 was used instead of total cell lysates. Three hundred ng of the recombinant CDK2 protein was mixed in 1 ml of immunoprecipitation buffer and incubated in the absence or presence of salicylic acid at different concentrations (0.5, 1.5 and 2.5mM) for 1h at RT. Following this, the anti-CDK2 antibody was added overnight, antigen-antibody complexes collected, and immunoblotted...
with the anti-CDK2 antibody. As shown in Fig. 3.10E, salicylic acid dose dependently increased the ability of anti-CDK2 antibody to bind and immunoprecipitate recombinant CDK2. The amount of the Ig-H chain and Ig-L chains were equal in all lanes. The increased CDK2 immunoprecipitation observed was not due to a change in the pH of the immunoprecipitation reaction as addition of 0.5 mM salicylic acid, for example, to 1 ml of the immunoprecipitation buffer caused only a marginal decrease in the pH (7.4 Vs 7.18). The isoelectric pH of the unmodified CDK2 is 8.8, and therefore, the increased immunoprecipitation of CDK2 observed in Fig. 3.10E in the presence of salicylic acid is not due to non-specific protein precipitation related to the isoelectric point.

Figure 3.10: Figure legend continued in next page
Figure 3.10  CDK2 as a Salicylic acid binding protein:

The inclusion of salicylic acid increases the ability of anti-CDK2 antibody to immunoprecipitate CDK2 from naïve cell lysates and recombinant CDK2 protein. A, anti-cyclin A2 antibody (cat. Number ab32386; Abcam) immunoblots of anti-CDK2 immunoprecipitates (cat. Number-05-596, EMD Millipore), showing the presence of increased levels of cyclin A2 with increased concentration of salicylic acid. In this blot, the area that has cyclin A2 bands are only shown. B, anti-CDK2 antibody immunoblots of the anti-CDK2 immunoprecipitates, shows the presence of increased levels of CDK2 with increased concentrations of salicylic acid. C, shows the results of the in-vitro kinase assay performed on anti-CDK2 immunoprecipitates in an experiment similar to figure 5A. For the experiment in 5C, salicylic acid was pre-incubated with lysate before immunoprecipitation, but not included in the kinase assay. D, shows the results of in-vitro kinase assay performed on anti-CDK2 immunoprecipitates. For the experiments in Fig. 5D, lysates were not pre-incubated with salicylic acid before immunoprecipitation, but was included during the kinase assay. E, anti-CDK2 immunoblot of anti-CDK2 immunoprecipitate of recombinant CDK2, immunoprecipitation was carried out in the presence of increasing concentration of salicylic acid. Ig-H, immunoglobulin heavy chain, Ig-L immunoglobulin light chain.
3.3.12 Pre-incubation of salicylic acid with CDK2 decreases fluorescence due to ANS:

8-anilino-1-naphthalene sulfonate (ANS) is an extrinsic fluorophore demonstrated to interact with CDK2 at an allosteric site, leading to a change in the conformation and also increase in fluorescence [142, 155]. Based on the results obtained in the immunoprecipitation experiments (Fig. 3.10B and E), we hypothesized that salicylic acid may physically interact with CDK2, causing a conformational change, this would affect the binding of ANS to CDK2 leading to decreased fluorescence. To address this, ANS (50 µM) was added to recombinant CDK2 (1.6 µM), or CDK2 (1.6 µM) which was pre-incubated with salicylic acid at different concentrations, and the fluorescence was measured. Figure 3.11A demonstrates that pre-incubation of CDK2 with salicylic acid dose-dependently quenched the fluorescence due to ANS. This suggests that salicylic acid is likely to bind to CDK2 protein, supporting the results obtained in immunoprecipitation reactions (Figs. 3.10A, B and E).
Figure 3.11. ANS-CDK2 assay and a model showing potential salicylic acid binding

A, effect of pre-incubation of salicylic acid with CDK2 on fluorescence due to ANS. CDK2 (1.6 µM) was incubated with ANS (50 µM) alone or with salicylic acid at different concentrations, fluorescence measured as described in the text. Salicylic acid mediated decrease in fluorescence was compared with fluorescence due to ANS/CDK2. The decrease in fluorescence was expressed as a percentage of control; B, is the molecular docking studies showing interactions of salicylic acid with CDK2; C, a model showing potential salicylic acid binding to CDK2. We predict that salicylic acid binds to an allosteric site on CDK2, similar to a site described for ANS binding to CDK2. Binding of salicylic acid to CDK2 changes the conformation; increases the ability of anti-CDK2
antibody to immunoprecipitate CDK2 due to a better exposure of the epitope. Binding of salicylic acid to CDK2 would also quench the fluorescence due to ANS.

3.3.13 Molecular docking studies show potential interactions of salicylic acid with CDK2 and cyclin A2

Molecular docking is used to predict binding modes and free energy calculations between the ligand and the receptor [156]. We used AutoDockVina to understand the interactions between aspirin/salicylic acid with CDK2/cyclin A2. The binding free energy and hydrogen bond lengths were determined to check the ability of aspirin and salicylic acid to dock separately with CDK2, cyclin A2 or with CDK2/cyclin A2 complex. The results of the docking studies are shown in Table-3.1 and Figs 3.12A-E. The free binding energy values for the interactions between aspirin or salicylic acid with CDK2 were similar (-5.8 Kcal/mol). The energy value was much greater when salicylic acid interacted with cyclin A2 monomer (-6.8 Kcal/mol), or with cyclin A2/CDK2 complex (-6.1 Kcal/mol), as compared to aspirin’s interactions with cyclin A2 monomer (-6.2 Kcal/mol), or with the complex (-5.2 Kcal/mol). Since negative energy values indicate a more favorable binding of ligands with receptor molecules, our data suggests that salicylic acid has a better binding affinity to cyclin A2 than aspirin. Among the potential interactions shown in Table-1 (also see Fig. 3.12), salicylic acid interactions with CDK2 through Asp 145 and Lys 33 is a very significant one (Fig. 3.11B), as it corroborates the results obtained in the immunoprecipitation experiments (Fig. 3.10A, B, E) and ANS-CDK2 fluorescence assay (Fig. 3.11A), which independently suggest that salicylic acid binds to CDK2.
Figure 3.12: Molecular docking studies on aspirin and salicylic acid with CDK2, cyclin A2, and CDK2/cyclin A2 complex

A, aspirin interaction with CDK2; B, aspirin interaction with cyclin A2; C, salicylic acid interaction with cyclin A2; D, aspirin interaction with CDK2/Cyclin A2 complex; E, salicylic acid interaction with CDK2/Cyclin A2 complex.
Table 3.1. Free energy binding values and hydrogen bond lengths for the interaction of salicylic acid and aspirin with CDK2, cyclin A2 and CDK2/cyclin-A2 complex

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ligand</th>
<th>Binding Affinity Kcal/mol.</th>
<th>Amino acids</th>
<th>Bond length (Å)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK2</td>
<td>Aspirin</td>
<td>-5.8</td>
<td>LYS33</td>
<td>3.2</td>
<td>Interacts with –COOH</td>
</tr>
<tr>
<td></td>
<td>Salicylic acid</td>
<td>-5.8</td>
<td>ASP145, LYS33</td>
<td>2.4, 2.6</td>
<td>Interacts with –OH and –COOH</td>
</tr>
<tr>
<td>Cyclin A2</td>
<td>Aspirin</td>
<td>-6.2</td>
<td>LYS194</td>
<td>3.2</td>
<td>Interacts with –COOH</td>
</tr>
<tr>
<td></td>
<td>Salicylic acid</td>
<td>-6.8</td>
<td>ASN237, ASP240, LYS194</td>
<td>2.1, 2.1, 3.1</td>
<td>Interacts with –OH and –COOH</td>
</tr>
<tr>
<td>CDK2/Cyclin A2 Complex</td>
<td>Aspirin</td>
<td>-5.2</td>
<td>LYS194 (B chain)</td>
<td>3.2</td>
<td>Interacts with –COOH to cyclin A2 only</td>
</tr>
<tr>
<td></td>
<td>Salicylic acid</td>
<td>-6.1</td>
<td>ASN237, ASP240, LYS194 (B chain)</td>
<td>2.0, 2.4, 2.6</td>
<td>Interacts with –OH and –COOH to cyclin A2 only</td>
</tr>
</tbody>
</table>

3.4 Additional Studies

3.4.1 Aspirin and salicylic acid decrease nuclear cyclin B1 and CDK 1 protein levels in HT-29 cells:

Earlier we demonstrated that exposure of HT-29 colon cancer cells to aspirin and salicylic acid decreased the levels of cyclin B1 and CDK1 proteins (Figs. 3.3 and 3.4). In order to investigate the mechanisms involved in their down-regulation more thoroughly, we performed experiments to determine the levels of cyclin B1 and CDK1 in the nucleus. For this, nuclear extracts prepared from aspirin and salicylic acid treated HT-29 cells were immunoblotted with anti-cyclin B1 or anti-CDK1 antibodies. Figure 3.13 A demonstrates that aspirin and salicylic acid, both, decreased the levels of cyclin B1 and CDK1 in the nuclear extracts, and this was observed in a concentration dependent fashion. Figure 3.13A
demonstrates that nuclear extracts used these blots contained equal amounts of PCNA antigen (loading control).

3.4.2 Effect of aspirin and salicylic acid on exogenously expressed, DDK-tagged, CDK1 and cyclin B1 levels:

To investigate whether the down regulatory effect of aspirin and salicylic acid occurs independent of transcriptional regulation, we determined the ability of both drugs to down regulate the exogenously expressed DDK-tagged cyclin B1 and CDK1 in HT-29 cells. For this, cells were left untransfected or transfected with DDK-tagged full-length cyclin B1 cDNA or CDK1 cDNA cloned in the pCMV6 vector. After transfections, cells were incubated for 24 h to allow for the expression DDK-tagged cyclin B1 and CDK1. Following this, cells were either left untreated or treated with drugs at a concentration of 2.5 mM for 24 h. Cell lysates were prepared and immunoblotted with anti-DDK antibody. Figure 3.13 B demonstrates that anti-DDK antibody detected the expression of the DDK-tagged cyclin B1 in transfected cells (lane 4). Interestingly, both drugs decreased the DDK-tagged cyclin B1 (lanes 5 and 6). When the samples were immunoblotted with anti-cyclin B1 antibody, the levels of endogenously expressed cyclin B protein appeared decreased following treatment with aspirin or salicylic acid (Fig. 3.13B, lanes 2, 3, 5 and 6). In the anti-cyclin B1 antibody immunoblots, the levels of exogenously expressed cyclin B1 protein was more clearly downregulated in cells treated with salicylic acid (Fig. 3.13 B, lane 3). Reprobing the blot of Figure 3.13 B with β-actin antibody showed equal amounts of the protein in all lanes (Fig. 3.13 B). These results collectively show that aspirin and
salicylic acid were able to decrease the levels of both endogenous as well as exogenous cyclin B1 protein levels in HT-29 cells.

Figures 3.13C demonstrates the results obtained from transfection experiments performed in cells with DDK tagged CDK1 cDNA. The results obtained from anti-DDK antibody immunoblots are shown in Fig. 3.13C. We observed that both aspirin and salicylic acid marginally decreased the exogenously expressed CDK1 protein (Fig. 3.13 BIV, compare lanes 5 and 6 with lane 4). It is important to note that we were able to detect the exogenously expressed CDK1 in Fig. 3.13C only after longer exposure of the immunoblots, and this resulted in an overexposure of the signals representing the endogenous CDK1 levels. However, a shorter exposure of the immunoblot of Fig. 3.13C showed that both aspirin and salicylic acid decreased the levels of endogenous CDK1 levels (compare lanes 5 and 6 with lane 4). These results show that while aspirin and salicylic acid were able to decrease the levels of endogenous CDK1, they were not very effective in decreasing the levels of exogenously expressed CDK1. The inability of aspirin and salicylic acid to decrease the exogenous CDK1 is not clear at this stage; however, it may be related to altered conformation of the DDK tagged recombinant CDK1, which probably are not recognized by proteases. Reprobing the blot of Figure 3.13C with β-actin antibody showed equal amounts of the protein in all lanes (Fig. 3.13C).
Figure 3.13: Aspirin and salicylic acid decreased exogenously expressed, DDK-tagged, CDK1 and cyclin B1 levels

A, effect of aspirin and salicylic acid to decrease nuclear cyclin B1 and CDK1 protein levels in HT-29 cells. B, aspirin and salicylic acid decreased exogenously expressed DDK-tagged cyclin B1 proteins. C, aspirin and salicylic acid decreased exogenously expressed DDK-tagged CDK1 protein. Positions of the exogenous and endogenous cyclin B1 and CDK1 were shown by arrows.
3.4.3 Lactacystin completely prevents aspirin and salicylic acid-mediated down-regulation of cyclin B1 levels:

Cyclin B1 naturally undergoes degradation via ubiquitin-proteasomal pathway. During pro-metaphase, it is ubiquitinated by the APC/C and this tags cyclin B1 for degradation by the 26S proteasomes. To determine a role for the proteasomal pathway, we tested the ability of lactacystin, a 26S proteasomal inhibitor [154], to prevent aspirin and salicylic acid-mediated decrease in cyclin B1 levels. Cells were left untreated or first treated with lactacystin (10 µM) for 1h, then aspirin or salicylic acid (2.5 mM) were added for 24h. Cell lysates were prepared and immunoblotted with the anti-cyclin B1 antibody. Figure 3.14 A demonstrates that lactacystin pretreatment completely prevented the degradation of cyclin B1 caused by aspirin and salicylic acid. Quantification of these bands showed that aspirin and salicylic acid decreased the cyclin B1 levels by 80% and 87% respectively (Fig. 3.14 B). Lactacystin treatment alone stabilized the cyclin B1 protein levels; it was 2.3-fold higher compared to untreated control. However, in the presence of lactacystin, both drugs failed to cause the down-regulation of the cyclin B1. This suggests that, 26S proteasomes are involved in aspirin and salicylic acid-mediated down-regulatory effects. When similar experiments were performed with CDK1, both aspirin and salicylic acid down regulated the CDK1 protein levels (Fig. 3.14 C, lanes 2 and3). Lactacystin, itself had a down regulatory effect on CDK1 levels, therefore, we could not access the role of proteases in aspirin and salicylic acid mediated decrease in CDK1 levels (Fig. 3.14 C, lanes 4-6).
Figure 3.14. Down-regulation of cyclin B1, but not CDK1, by aspirin and salicylic acid is mediated by 26S proteasomal pathway

A, effect of lactacystin on the ability of aspirin and salicylic acid to decrease cyclin B1 protein levels in HT-29 cells. B, represents the quantification of the bands in blot A. C, effect of lactacystin on the ability of aspirin and salicylic acid to decrease CDK1 protein levels in HT-29 cells. B, represents the quantification of the bands in blot A.
3.4.3 Aspirin and salicylic acid decrease cyclin B1 and CDK1 mRNA levels in HT-29 cells:

To investigate if aspirin and salicylic acid regulate cyclin B1 and CDK1 expression at the transcriptional / post-transcriptional level, we measured their mRNA levels in HT-29 cells following treatment with drugs. Cells were left untreated or treated with aspirin or salicylic acid at different concentrations for 24h; total RNA was prepared and analyzed for cyclin B1 and CDK1 mRNA by RT-PCR using CDK1 and cyclin B1 specific primers. As an internal control, primers specific for GAPDH was also used. Figures 3.15 A demonstrates that in untreated control cells (lane 1), abundant cyclin B1 and CDK1 mRNA was detected; both aspirin and salicylic acid caused a significant decrease in cyclin B1 and CDK1 mRNA levels. The levels of GAPDH mRNA were unaffected following treatment with aspirin and salicylic acid. Quantification of the data suggested that both aspirin and salicylic acid caused a more pronounced decrease in CDK1 mRNA as compared to cyclin B1 mRNA (Fig. 3.15 B and C). These results show that the observed decrease in cyclin B1 and CDK1 protein levels following treatment with drugs is at least in part due to decreased presence of cyclin B1 and CDK1 mRNA levels.
Aspirin and salicylic acid decreased cyclin B1 and CDK1 mRNA levels at 0.5 mM concentration (A). GAPDH is used as a control for this experiment. B and C respectively represents, the quantification of cyclin B1 and CDK1 blots in A.
3.5 Discussion:

Aspirin has attracted considerable attention as a potential drug in the chemoprevention of epithelial cancers. However, there is an extensive debate regarding the molecular pathways by which it exerts its anti-cancer effects. Aspirin contains acetyl and salicylate groups both of which have their own targets, and are believed to contribute to its chemopreventive actions. Studies from our laboratory [113, 115, 116, 120], and others [117, 118] showed that, besides COX, it can acetylate numerous other proteins. While the identification of the aspirin-mediated acetylation targets has recently gained momentum [117, 118] after our initial reports [116, 120], identification of direct binding targets for salicylic acid has been under-explored [113]. Till date, salicylic acid has been shown to bind directly and interact with three cellular proteins in human cells: IκB kinase (IKK) β, a component of the NF-κB complex [134], AMP-activated protein kinase [136] and High Mobility Group Box1 proteins [137]. We hypothesized that, salicylic acid being a small molecule with hydroxyl (-OH) and carboxyl (-COOH) functional groups, potentially could directly bind/interact with additional cellular proteins and affect their functions.

In the present study, we report several novel observations including a mechanism by which aspirin and salicylic acid may exert their anti-cancer effects in epithelial cell types. We report the identification of cyclin A2/CDK2 as novel targets of aspirin and salicylic acid in multiple cancer cell lines. We demonstrated that both drugs decrease cyclin A2 and CDK2 protein as well as their mRNA, in a concentration-dependent fashion. The down-regulatory effect of both drugs on cyclin A2 protein was sensitive to pretreatment with lactacystin, suggesting that 26S proteasomal enzymes are involved. It is
to be noted that cyclin A2 protein naturally undergoes degradation mediated by 26S proteasomal pathway [153] and our observation, therefore, is consistent with the known pathway of cyclin A2 degradation. The decrease in cyclin A2/CDK2 levels in aspirin/salicylic acid treated cells was associated with a corresponding decrease in CDK2 activity, which suggest that the cellular CDK2 activity is likely to be reduced upon drug exposure.

Our findings show that aspirin and salicylic acid regulate cyclin A2 expression at two levels, transcriptional/post-transcriptional, and post-translational levels. At the post-translational level, a lactacystin sensitive cysteine protease activated in response to aspirin or salicylic acid within the cells may cause the direct degradation of cyclin A2 protein. Alternatively, the observed decrease in the levels of cyclin A2 mRNAs in aspirin and salicylic treated cells may be also due to the degradation of a transcription factor(s) (TFs) (mediated by a lactacystin-sensitive protease) involved in cyclin A2 gene transcription. In this context, it is important to note that several TFs, such as c-Myc [157], CREB (cyclic AMP response element binding protein) and CREM (cyclic AMP response element modulators) [158] have been implicated in the transcription of cyclin A2 gene; and which of these TFs are affected by these two drugs requires additional study. In fact, in a recent study we reported the ability of aspirin and salicylic acid to down-regulate c-Myc protein and mRNA in cancer cells [125]. Therefore, it is likely that the decreased levels of cyclin A2 mRNA or CDK2 mRNAs observed in aspirin and salicylic acid treated cells is not a non-specific effect due to a general cell cycle arrest, but most likely due to the down-regulations of TFs.
Results obtained from three independent experiments strongly suggest that salicylic acid interacts with CDK2 possibly at an allosteric site leading to a change in its conformation. First clues for the binding of salicylic acid to CDK2 came from immunoprecipitation studies. We observed the increased ability of anti-CDK2 antibody to immunoprecipitate CDK2 protein in naïve cell lysates when they were pre-incubated with salicylic acid (Figs. 3.10A and B). The inclusion of salicylic acid also dose-dependently enhanced the ability of anti-CDK2 antibodies to immunoprecipitate purified recombinant CDK2 (Fig. 3.10E). Secondly, our molecular docking studies suggest that salicylic acid potentially interacts with Asp145 and Lys 33 in CDK2 (Table-3.1 and Fig. 3.11B), both of which have been previously identified as being present in its active site [57, 159]. Thirdly, pre-incubation of CDK2 with salicylic acid, dose-dependently quenched the fluorescence due to ANS (Fig. 3.11A). Interaction of ANS with CDK2 is well characterized and occurs at an allosteric pocket near the ATP binding site, leading to a large conformational change in CDK2 [155], and it has been shown to interact with Asp145 and Lys33 [142, 155]. It is interesting to note that, both ANS and salicylic acid share common amino acid residues Asp 145 and Lys 33, for interactions with CDK2, therefore, it is not surprising that pre-incubation of salicylic acid with CDK2, quenched the fluorescence due to ANS. It has been shown that CDK2 displays significant conformational flexibility and accommodates the binding of highly diverse small molecule ligands [142]. We predict that, similar to ANS, binding of salicylic acid to CDK2 occurs at an allosteric site causing a conformational change, and this would explain greater recognition and immunoprecipitation of CDK2 protein by anti-CDK2 antibody (Figs 3.10B and E; also see
Further confirmation of Asp145 and Lys33 as salicylic acid binding sites on CDK2 requires mutational and protein crystallization studies.

Endogenous cyclin A2 within cells can exist in the monomeric or dimeric state, bound to CDK2. Our molecular docking studies (Table-3.1) suggest that in addition to CDK2, aspirin and salicylic acid can potentially interact with cyclin A2 monomeric forms at specific amino acid residues (Fig 3.12B and 3.12C). However, if cyclin A2/CDK2 dimer has already been formed, it appears that aspirin and salicylic acid can interact only with cyclin A2, but not with CDK2 (Fig 3.12D and 3.12E). The standard hydrogen bond length between donor and the acceptor atoms is in the order of 2.6 to 3.5 Å, with optimum at 2.8 Å [160]. Based on the hydrogen bond length and the associated negative free energy, salicylic acid showed stronger interaction with binding pockets in CDK2 monomer, cyclin A2 monomer or with cyclin A2 in the cyclin A2/CDK2 hetero-dimer than aspirin. Additional studies involving biochemical and protein crystallization are required to confirm the direct binding of aspirin and salicylic acid with cyclin A2.

The *in-vitro* experiment described in Fig. 3.10B shows that pre-incubation of naïve cell lysates with salicylic acid increased the ability of anti-CDK2 antibody to bind and immunoprecipitate the CDK2 protein. These anti-CDK2 immunoprecipitates from salicylic acid pre-incubated lysates also contained greater levels of cyclin A2, as cyclin A2 is a natural binding partner with CDK2 (co-precipitation) (Fig. 3.10A), and showed increased CDK2 activity as measured by H1 histone phosphorylation assay (Fig. 3.10C). In these kinase experiments, salicylic acid was not included during the kinase reaction. Therefore, the increased CDK2 activity observed in anti-CDK2 immunoprecipitates from salicylic acid pre-incubated samples (Fig. 3.10C), reflects the greater amounts of cyclin
A2/CDK2 protein levels; and thus, not due a stabilization effect of salicylic acid. Interestingly, the inclusion of increasing amounts of salicylic acid during the *in-vitro* kinase assay performed on the anti-CDK2 immunoprecipitates from naïve cell lysates, had no effect on H1 histone phosphorylation (Fig. 3.10D). Taken together, these results suggest that binding of salicylic acid to CDK2 most likely changes the conformation leading to increased CDK2 immunoprecipitation; but the occupancy does not affect the CDK2 kinase activity. Efficient phosphorylation of H1 histones in the presence of salicylic acid (Fig. 3.10D), also suggest that the ATP binding site in CDK2 is unaffected due to interactions with salicylic acid.

Although our *in-vitro* experiments (Figs. 3.10A/B, 3.11A) and the molecular docking studies (Fig. 3.11B; table 3.1) suggest that salicylic acid binds to CDK2, it is not clear how within the cellular milieu, binding of salicylic acid to CDK2, causes subsequent degradation of cyclin A2 and CDK2 proteins. It is certain that proteasomal pathway is involved (Fig. 3.6). Inside the cellular milieu, the triad of CDK2/salicylic acid/cyclin A2 complex may be recognized by proteasomal enzymes as an un-natural complex, leading to degradation of both cyclin A2 and CDK2. Alternatively, the triad of CDK2/salicylic-acid/cyclinA2 complex, although still catalytically active, may have an altered substrate specificity. For example, salicylic acid bound CDK2 with an altered conformation and substrate specificity may phosphorylate and activate unique targets / proteasomal enzymes specific for the degradation of cyclin A2/CDK2. This view is supported by reports in literature that conformational changes in flexible parts of the protein have been indeed shown to alter substrate specificity [161]. Investigations into the pathways leading to
degradation of cyclin A2/CDK2 proteins following salicylic acid occupancy represent an important extension of this study.

Aspirin’s ability to inhibit cell proliferation or induce cell cycle arrest (G0/G1) has been documented in the literature in many cancer cell lines [145, 146, 162, 163]. In our study, aspirin and salicylic acid down-regulated cyclin A2/CDK2 in 11 different cancer cells representing the cancers of various epithelial tissues (colon, lung, prostate, ovary and skin), which suggest that this is a universal phenomenon and applicable to most cancer cells. Extension of these observations performed in HT-29 cells show that exposure of cells to aspirin and salicylic acid caused down-regulation of cyclins B1 and D3; CDKs 1, 4 and 6; and up-regulation of CDK inhibitors p21 and p27. Down-regulation of many of the important cyclins and CDKs, and up-regulation of CDK inhibitors, would tip the balance strongly towards cell cycle arrest, and will explain the documented ability of aspirin and salicylic acid to cause cell cycle arrest in literature.

In many cancers, CDK2 activity is deregulated, and cyclin A2 is over-expressed [147-152]. Therefore, attention is increasingly being focused on cell cycle, as a potential target for therapeutic intervention [152, 164, 165]. In this context, our finding that aspirin and salicylic acid down-regulate cyclin A2/CDK2 protein and mRNAs in multiple cancer cell lines should initiate new thinking and research on these age-old drugs in cancer treatment. The answer for an effective drug for chemoprevention may lie in revisiting salicylic acid, an ancient drug known for over two millennia in plants for its therapeutic properties. The observation that salicylic acid binds to CDK2 at an allosteric site can be exploited to develop novel anti-cancer drugs, for example, derivatives of salicylic acid can be screened for inhibition of CDK2 activity, or disruption of the cyclin A2/cdk2 complex.
Salicylic acid is abundantly present in many plants where it has been shown to protect the cells from infection through induction of cell death [166]. It will be also interesting to determine if salicylic acid-induced cell death in infected leaves involves down-regulation of cyclin A2/CDK2, or other related proteins.
Chapter 4: Salicylic Acid Metabolites and Derivatives Inhibit CDK Activity

4.1 Abstract:

Aspirin’s potential as a drug continues to be evaluated for the prevention of colorectal cancer (CRC). Although multiple targets for aspirin and its metabolite, salicylic acid, have been identified, no unifying mechanism has been proposed to clearly explain its chemopreventive effects. Our goal here was to investigate the ability of salicylic acid metabolites, known to be generated through cytochrome P450 (CYP450) enzymes, and its derivatives as cyclin dependent kinase (CDK) inhibitors to gain new insights into aspirin’s chemopreventive actions. Using in-vitro kinase assays, for the first time, we demonstrate that salicylic acid metabolites, 2, 3-dihydroxybenzoic acid (2, 3-DHBA) and 2, 5-dihydroxybenzoic acid (2,5-DHBA), as well as derivatives 2,4-dihydroxybenzoic acid (2,4-DHBA), 2,6-dihydroxybenzoic acid (2,6-DHBA), inhibited CDK1 enzyme activity. 2,3-DHBA and 2,6-DHBA did not inhibit CDK2 and 4; however, both inhibited CDK-6 activity. Interestingly, another derivative, 2,4,6-trihydroxybenzoic acid (2,4,6-THBA) was highly effective in inhibiting CDK1, 2, 4 and 6 activity. Molecular docking studies showed that these compounds potentially interact with CDK1. Immunoblotting experiments showed that aspirin acetylated CDK1, and pre-incubation with salicylic acid and its derivatives prevented aspirin-mediated CDK1 acetylation, which supported the data obtained from molecular docking studies. We suggest that intracellularly generated salicylic acid metabolites through CYP450 enzymes within the colonic epithelial cells, or the salicylic acid metabolites generated by gut microflora may significantly contribute to the preferential chemopreventive effect of aspirin against CRC through inhibition of CDKs. This novel hypothesis and mechanism of action in aspirin’s chemopreventive
effects opens a new area for future research. In addition, structural modification to salicylic acid derivatives may prove useful in the development of a novel CDK inhibitors in cancer prevention and treatment

4.2 Background and Hypothesis:

Previous reports have identified specific cellular targets for both aspirin and salicylic acid. For example, aspirin’s well studied acetylation targets include COX-1 and 2 [167, 168], p53 [120] and G6PD [114]. Aspirin is also known to acetylate numerous other cellular target, functional significance of these post translational modification are yet to be identified. In recent times, several targets for aspirin’s primary metabolite salicylic acid have been identified such as, IкB kinase (IKK), a component of the NF-κB complex [134], AMP activated protein kinase [136], High Mobility Group Box 1 proteins [137] and GAPDH [132], although the importance of these interactions towards aspirin’s chemopreventive effects is yet to be understood. Salicylic acid generated in the body is metabolized in the liver by cytochrome p450 systems to produce 2,3 dihydroxy benzoic acid (2,3-DHBA) and 2,5-DHBA as indicated in introduction. We hypothesized that the salicylic acid metabolites (2,3-DHBA and 2,5-DHBA) may inhibit on CDK enzyme activity and potentially contribute to aspirin’s anti-cancer effects. As an extension of this study, we also investigated the effect of salicylic acid derivatives 2,4-DHBA, 2,6-DHBA and 2,4,6-trihydroxybenzoic acid on CDK enzyme activity. Detailed studies were also carried out to identify the potential binding sites of aspirin, salicylic acid, 2,3-DHBA, 2,5DHBA and 2,4,6-DHBA on CDK1 using molecular docking as well as biochemical experiments.
4.3 Results:

4.3.1 Effect of aspirin, salicylic acid and salicylic acid derivatives on CDK1 kinase activity in-vitro:

In a previous report we demonstrated that aspirin potentially binds to CDK2 through Lys33, and salicylic acid via Asp145 and Lys33. Despite the strong evidence of interactions of these drugs with CDK2, both failed to inhibit CDK2 enzyme activity in in-vitro kinase assays [133]. In the current study, we initially extended these observations to determine the effect of aspirin and salicylic acid on CDK1 enzyme activity in-vitro. This was tested using commercially obtained CDK1/cyclin B1 assay kits, and by providing [γ-32P] ATP and H1 histones as substrates. As a positive control, we used flavopiridol, a known inhibitor of CDK1 [79]. Figure 4.1A demonstrates that neither aspirin nor salicylic acid inhibited CDK1 activity as measured by the ability to phosphorylate H1 histones; however, flavopiridol dose-dependently inhibited CDK1 activity (upper panel). These samples contained similar levels of H1 histones (lower panel, Fig. 4.1A). Quantification of the bands in Fig. 4.1A (upper panel) are shown in Fig. 4.1B. These results show that aspirin and salicylic acid do not inhibit CDK1, consistent with the results previously obtained with CDK2 [133].

We next determined the ability of salicylic acid metabolites (2,3-DHBA and 2,5-DHBA) as we all as salicylic acid derivatives (2,4-DHBA, 2,6-DHBA and 3,4-DHBA and 5-aminosalicylic acid) and benzoic acid to inhibit CDK-1 enzyme activity. We observed that, of the seven different compounds tested, all DHBA compounds with a common -OH group at the 2nd carbon inhibited CDK1 enzyme activity to varying degrees (Fig. 4.1C, upper panel). It is interesting to note that the salicylic acid metabolites, 2,3-DHBA and
2,5-DHBA, both showed inhibitory effects on CDK1 activity. Quantification of the intensities of the bands showed that, four compounds showed varied levels of inhibition (2,6-DHBA, 65%; 2,5-DHBA, 22%; 2,4-DHBA, 25% and 2,3-DHBA, 55%) (Fig.4.1D). Three compounds, 3,4-DHBA, 5-aminosalicylic acid and benzoic acid did not show any inhibition. In addition, 3,5-DHBA failed to inhibit the CDK1 enzyme activity (data not shown).

4.3.2 Dose dependent inhibition of CDK1 enzyme activity by 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA:

In experiments described in Fig. 4.1C, the salicylic acid metabolite 2,3-DHBA, and the salicylic acid derivative 2, 6-DHBA, showed greater inhibition on CDK1 activity as compared to other compounds tested. Therefore, we focused initially on these two compounds to study their dose dependent effects on CDK1 activity. Figures 4.1E and F show that both 2,3-DHBA and 2,6-DHBA dose dependently inhibit CDK1 activity with IC$_{50}$ 386 µM and 365 µM, respectively. The ability of 2,3-DHBA and 2,6-DHBA to inhibit CDK1 enzyme activity suggested that the hydroxyl group at the 2nd and 6th carbon in these compounds may be important, and that a compound having these two -OH groups may more potently inhibit CDK1 activity. Therefore, in in-vitro kinase assays, we determined the effect of 2,4,6-THBA on CDK1 activity at various concentrations. Figure 4.1G (upper panel) demonstrates that 2,4,6-THBA inhibited CDK1 activity with an IC$_{50}$ of 226 µM. Quantification of the bands are shown in Fig. 4.1H. Interestingly, 3,4,5-THBA (Gallic acid) did not show any inhibitory effect (data not shown). This again suggests that the -
OH group at the 2nd and 6th carbon may contribute to greater inhibition of CDK1 enzyme activity.

Figure 4.1. *In-vitro* kinase assays showing the effect of aspirin, salicylic acid metabolites and derivatives on CDK1 enzyme activity

A, Effect of aspirin (Asp), salicylic acid (Sal) and flavopiridol on CDK1 enzyme activity. B, Quantification of the blot in A. C, Effect of salicylic acid metabolites 2,3-DHBA, 2,5-DHBA and derivatives 2,4-DHBA, 2,6-DHBA, 3,4-DHBA, 5-amino salicylic acid (5-amino Sal), and benzoic acid (BA) on CDK1 enzyme activity. The lower panels below A and C shows coomassie stained H1 histones. D, quantification of the blot in C. The intensities of bands in various blots were quantified and expressed as percentage of control. P-value <0.05 *, <0.01 **, <0.001†, < 0.001‡.
Continued Figure 4.1.  *In-vitro* kinase assays showing the effect of aspirin, salicylic acid metabolites and derivatives on CDK1 enzyme activity

_E_ and _F_, respectively shows the dose-dependent effect of 2, 3-DHBA and 2,6-DHBA on CDK1 enzyme activity.  _G_, Dose-dependent effect of 2,4,6-THBA on CDK1. The lower panel of _G_, shows coomassie stained pattern of histone H1.  _H_, Quantification of the blot in _G_. The intensities of bands in various blots were quantified and expressed as percentage of control. P-value <0.05 *, <0.01 **, <0.001†, < 0.001‡.
4.3.3 Molecular docking studies show potential interactions of aspirin, salicylic acid, salicylic acid metabolites and derivatives with CDK1 and cyclin B1

We used AutoDockVina to understand the interactions between aspirin, salicylic acid, 2,3-DHBA, 2,5-DHBA 2,6-DHBA and 2,4,6-THBA with CDK1 alone, cyclin B1 alone, and the CDK1/cyclinB1 complex. The binding free energy and hydrogen bond lengths were also determined. The results of the docking studies are shown in Table-4.1, Figs.4.2A-F (space-filling model) and Figs. 4.3 – 4.5 (ball-and-stick model). Among the potential interactions shown in Table-4.1, salicylic acid interactions with CDK1 through Asp146 and Lys33 is significant, as these two amino acids are part of the active site of the enzyme and are conserved among different CDK family members [169]. Interestingly, salicylic acid also interacts with CDK1 through Asp146 and Lys33 when it is present as part of the cyclin B1/CDK1 complex (table 4.1). Docking studies also revealed that aspirin can potentially interact with Tyr 15 in the CDK1 monomer, or in the CDK1/cyclin B1 dimer. It is interesting to note that Tyr15 is conserved in various CDKs, and its phosphorylation by Wee1/Mik1 kinase family inactivates the enzyme activity [57]. Despite these predicted interactions of aspirin and salicylic acid with key amino acid residues in CDK1, both failed to inhibit CDK1 enzyme activity (Fig.4.1).

Table 4.1 also shows that all three DHBA compounds (2,3-DHBA, 2,5-DHBA and 2,6-DHBA) showed potential interactions with CDK1 monomer through Asp 146, whereas 2,4,6-THBA interacted via Arg123, Arg151 and Gly154. Figures 4.2A-F (space-filling model) shows that 2,3-DHBA, 2,5-DHBA and 2,6-DHBA bind to the same pocket in CDK1 (same as aspirin and salicylic acid); however, 2,4,6-THBA binds at a different site.
Interestingly, all four compounds (DHBAs/THBA) inhibited CDK1 enzyme activity (Fig. 4.1C-H).

**Figure 4.2.** Molecular docking studies (space-filling model) showing the potential binding pockets in CDK1; for aspirin (A), salicylic acid (B), 2,3-DHBA (C), 2,5-DHBA (D), 2,6-DHBA (E) and 2,4,6-THBA (F).
Figure 4.3: Molecular docking studies showing the potential interactions of CDK1 with aspirin (A), salicylic acid (B), 2,3-DHBA (C), 2,5-DHBA (D), 2,6-DHBA (E) and 2,4,6-THBA (F).
Figure 4.4: Molecular docking studies showing the potential interactions of cyclin B1 with aspirin (A), salicylic acid (B), 2,3-DHBA (C), 2,5-DHBA (D), 2,6-DHBA (E) and 2,4,6-THBA (F) with cyclin B1.
**Figure 4.5:** Molecular docking studies showing the potential interactions of CDK1/cyclin B1 complex with aspirin (A), salicylic acid (B), 2,3-DHBA (C), 2,5-DHBA (D), 2,6-DHBA (E) and 2,4,6-THBA (F).
<table>
<thead>
<tr>
<th>S. No</th>
<th>Ligand</th>
<th>Receptor</th>
<th>PDB ID</th>
<th>Interacting amino acids</th>
<th>No; H-bonds</th>
<th>Measurement (Å)</th>
<th>E.V (kcal/mol)</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aspirin</td>
<td>CDK1</td>
<td>4y72 (A Chain)</td>
<td>Asn133, Gln132 and Tyr15</td>
<td>3</td>
<td>2.7, 2.1 and 2.6</td>
<td>-7.4</td>
<td>-COOH – Asn133, Gln132 and Tyr15</td>
</tr>
<tr>
<td>2</td>
<td>Salicylic acid</td>
<td>CDK1</td>
<td>4y72 (A Chain)</td>
<td>Asp146 and Lys33</td>
<td>2</td>
<td>1.8 and 2.5</td>
<td>-8.6</td>
<td>-OH – Lys33; -COOH – Asp146</td>
</tr>
<tr>
<td>3</td>
<td>2,3 DHB A</td>
<td>CDK1</td>
<td>4y72 (A Chain)</td>
<td>Asp146</td>
<td>1</td>
<td>2.0</td>
<td>-6.8</td>
<td>-COOH – Asp146</td>
</tr>
<tr>
<td>4</td>
<td>2,5 DHB A</td>
<td>CDK1</td>
<td>4y72 (A Chain)</td>
<td>Asp146</td>
<td>1</td>
<td>2.1</td>
<td>-6.8</td>
<td>-COOH – Asp146</td>
</tr>
<tr>
<td>5</td>
<td>2,6 DHB A</td>
<td>CDK1</td>
<td>4y72 (A Chain)</td>
<td>Asp146</td>
<td>1</td>
<td>2.0</td>
<td>-7.1</td>
<td>6th -OH – Asp146</td>
</tr>
<tr>
<td>6</td>
<td>2,4,6 THB A</td>
<td>CDK1</td>
<td>4y72 (A Chain)</td>
<td>Arg123, Arg151 and Gly154</td>
<td>3</td>
<td>2.5, 2.0 and 2.4</td>
<td>-5.8</td>
<td>2nd -OH – Arg151 and Gly154; -COOH – Arg123</td>
</tr>
<tr>
<td>7</td>
<td>Aspirin</td>
<td>Cyclin B1</td>
<td>2b9r</td>
<td>Arg135</td>
<td>2</td>
<td>1.9 and 2.5</td>
<td>-6.8</td>
<td>-COOH – Arg135</td>
</tr>
<tr>
<td>8</td>
<td>Salicylic acid</td>
<td>Cyclin B1</td>
<td>2b9r</td>
<td>Arg135</td>
<td>4</td>
<td>2.2, 2.4, 2.7 and 2.3 A</td>
<td>-7.4</td>
<td>OH- Arg135; -OOH – Tyr60, Arg135</td>
</tr>
<tr>
<td>9</td>
<td>2,3 DHB A</td>
<td>Cyclin B1</td>
<td>2b9r</td>
<td>Arg135, Arg38 and Asp67</td>
<td>6</td>
<td>2.1, 2.2, 2.2, 2.4 and 2.3</td>
<td>-6.8</td>
<td>2nd -OH – Asp67; 3rd -OH – Arg38; COOH – Arg135</td>
</tr>
<tr>
<td>10</td>
<td>2,5 DHB A</td>
<td>Cyclin B1</td>
<td>2b9r</td>
<td>Arg135</td>
<td>2</td>
<td>2.0 and 2.4</td>
<td>-6.7</td>
<td>2nd -OH – Arg135</td>
</tr>
<tr>
<td>11</td>
<td>2, 6 DHB A</td>
<td>Cyclin B1</td>
<td>2b9r</td>
<td>Arg135</td>
<td>2</td>
<td>2.1 and 2.5</td>
<td>-5.4</td>
<td>6th -OH – Arg135</td>
</tr>
<tr>
<td>12</td>
<td>2,4,6 THB A</td>
<td>Cyclin B1</td>
<td>2b9r</td>
<td>Arg135, Phe175, Asp67, Arg38, Thr166</td>
<td>6</td>
<td>2.2, 2.4, 2.2, 2.2, 2.3 and 2.1</td>
<td>-6.8</td>
<td>2nd -OH – Asp67; 4th -OH – Arg38, Thr166; 6th -OH - Asp67; -</td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>Complex</td>
<td>Residue</td>
<td>Hydrogen Bond Lengths</td>
<td>Free Energy Binding Value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-------------</td>
<td>------------</td>
<td>---------</td>
<td>-----------------------</td>
<td>---------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Aspirin</td>
<td>CDK1_ Cyclin B1</td>
<td>4y72 (A, B chain)</td>
<td>Tyr15 (CDK1)</td>
<td>1</td>
<td>2.5</td>
<td>-7.0</td>
<td>COOH – Arg135</td>
</tr>
<tr>
<td>14</td>
<td>Salicylic acid</td>
<td>CDK1_ Cyclin B1</td>
<td>4y72 (A, B chain)</td>
<td>Asp146 and Lys33 (CDK1)</td>
<td>3</td>
<td>1.9, 2.5 and 2.6</td>
<td>-9.6</td>
<td>OH – Asp146; COOH – Lys33, Asp146 (CDK1)</td>
</tr>
<tr>
<td>15</td>
<td>2,3 DHB A</td>
<td>CDK1_ Cyclin B1</td>
<td>4y72 (A, B chain)</td>
<td>Thr329, Val336, Phe338 (Cyclin B1)</td>
<td>2</td>
<td>2.4, 2.1 and 2.4</td>
<td>-6.1</td>
<td>2nd and 3rd - OH – Phe338; 3rd - OH – Val336; COOH – Thr329 (cyclin B1)</td>
</tr>
<tr>
<td>16</td>
<td>2,5 DHB A</td>
<td>CDK1_ Cyclin B1</td>
<td>4y72 (A, B chain)</td>
<td>Arg123 (3), Leu125, and Arg151 (CDK1)</td>
<td>5</td>
<td>2.2, 2.2, 2.4, 2.4 and 2.4</td>
<td>-6.0</td>
<td>5th - OH – Leu125 and Arg151; COOH – Arg123 (CDK1)</td>
</tr>
<tr>
<td>17</td>
<td>2,6 DHB A</td>
<td>CDK1_ Cyclin B1</td>
<td>4y72 (A, B chain)</td>
<td>Val336 (Cyclin B1)</td>
<td>1</td>
<td>2.6</td>
<td>-5.6</td>
<td>6th - OH – Val336 (cyclin B1)</td>
</tr>
<tr>
<td>18</td>
<td>2,4,6 THBA</td>
<td>CDK1_ Cyclin B1</td>
<td>4y72 (A, B chain)</td>
<td>Tyr223, Asp 230, and Arg201 (Cyclin B1)</td>
<td>2</td>
<td>2.3, 2.5 and 2.5</td>
<td>-5.4</td>
<td>2nd - OH – Asp230; 4th - OH – Tyr223; COOH – Arg201 (cyclin B1)</td>
</tr>
</tbody>
</table>

Table 4.1. Free energy binding values and hydrogen bond lengths for the interaction of aspirin, salicylic acid, 2,3-DHBA, 2,5-DHBA, 2,6-DHBA and 2,4,6-THBA with CDK1, cyclin B1 and CDK1/cyclin B1 complex.
4.3.4 Aspirin acetylates recombinant CDK1, and pre-incubation with salicylic acid, 2,3-DHBA, 2,6-DHBA or 2,4,6-THBA inhibits aspirin’s ability to acetylate CDK1:

We performed experiments to provide a second line of evidence for the physical interactions of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA with CDK1. We hypothesized that if aspirin binds to CDK1, it should be able to acetylate CDK1 and this could be detected by immunoblotting with anti-acetyl lysine antibody. If this is true, then pre-incubation of CDK1 with salicylic acid, 2,3-DHBA, 2,6-DHBA or 2,4,6-THBA should prevent aspirin-induced CDK1 acetylation. Therefore, first, we tested the ability of aspirin at various concentrations to acetylate recombinant CDK1 (Prospec) in-vitro. For this, recombinant CDK1 (300 ng) was incubated with aspirin at various concentrations for 12 h, and then samples were immunoblotted with anti-acetyl lysine antibody. Figure 4.6A demonstrates that aspirin dose-dependently acetylated recombinant CDK1 beginning at 0.25 mM (upper panel). Stripping and reprobing the blot showed similar amounts of CDK1 protein in all lanes (Fig. 4.6A, lower panel). The ability of aspirin to acetylate CDK1 as shown in Fig. 4.6A, supports the results obtained from molecular docking studies which suggested that aspirin potentially binds to CDK1 (Table 4.1). Since salicylic acid also potentially binds to CDK1, we next determined if pre-incubation of CDK1 with salicylic acid would prevent aspirin’s ability to acetylate CDK1. For this, recombinant CDK1 was left untreated, or treated with aspirin alone (1.5 mM), or first pre-incubated with different concentrations of salicylic acid for 10 mins (0.01 mM to 0.25 mM) and then treated with aspirin (1.5 mM) for 12h. The samples were then immunoblotted with anti-acetyl lysine antibody. Figure 4.6B demonstrates that aspirin caused acetylation of recombinant CDK1; however, this was dose-dependently prevented by pre-incubation with salicylic acid (upper
Reprobing the blot in Fig. 4.6B showed that all lanes contained equal amounts of CDK1 protein (lower panel).

In similar experiments as mentioned above, we next determined whether pre-incubation of recombinant CDK1 with 2,3-DHBA and 2,6-DHBA would prevent aspirin-mediated acetylation of CDK1. Recombinant CDK1 was pre-incubated with 0.5 mM 2,3-DHBA and 2,6-DHBA for 10 mins; then 1.5 mM, aspirin was added and the samples were incubated for 12 h at room temperature. The samples were analyzed by immunoblotting with anti-acetyl lysine antibodies. Figure 4.6D (upper panel), demonstrates that pre-incubation of CDK1 with 2,3-DHBA or 2,6-DHBA significantly prevented aspirin-mediated acetylation of CDK1. In separate experiments, we also observed that pre-incubation of recombinant CDK1 with 2,4,6-THBA prevented aspirin-mediated acetylation of CDK1 (Fig. 4.6E, upper panel). These results collectively demonstrate that aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA can all directly bind to CDK1, and support the data obtained from molecular docking studies (Table 4.1).

4.3.5 Aspirin acetylates cellular CDK1:

We also performed experiments to determine if exposure of HCT-116 cells to aspirin would acetylate cellular CDK1. Cells were treated with aspirin at various concentrations (0.25 mM-2.5 mM) for 12 h, and lysates prepared and immunoprecipitated with anti-acetyl lysine antibody agarose conjugates and immunoblotted with anti-CDK-1 antibody. Figure 4.6F, upper panel, demonstrates that CDK1 was acetylated in aspirin treated samples in a dose-dependent fashion. We also analyzed equal amounts of proteins representing these samples by immunoblotting with anti-CDK1 antibody. Figure 4.6F, lower panel, shows that these samples contained similar amounts of CDK1 protein.
Figure 4.6: Aspirin acetylates recombinant CDK1 \textit{in-vitro} and cellular CDK1 in HCT116 cells

A, Upper panel, Dose dependent effect of aspirin on recombinant CDK1 acetylation. B, Salicylic acid pre-incubation prevents aspirin-induced acetylation (upper panel). C, shows quantification of blot in Fig. 4.6B, the decrease in CDK1 acetylation is expressed as a percentage of the control. D, Pre-incubation of recombinant CDK1 with 2,3-DHBA and 2,6-DHBA at 0.5 mM prevents aspirin induced acetylation of CDK1 (upper panel). E, Pre-incubation of recombinant CDK1 with 2,4,6-THBA prevents aspirin induced acetylation of CDK1 (upper panel). F, Aspirin acetylates cellular CDK1 in HCT-116 cells (upper panel). For 3F lower panel, equal amounts of proteins was immunoblotted with anti-CDK1 antibody. The lower panels in all other blots (A, B, D and E), the upper panel blots were stripped and reprobed with anti-CDK1 antibody. The intensities of bands in various blots were quantified and expressed as percentage of control. P-value <0.05 *, <0.01 **, <0.001†, < 0.001‡.
4.3.6 Effect of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA on CDK2, CDK4 and CDK6 enzyme activity:

Having demonstrated the ability of 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA to inhibit CDK1 enzyme activity *in-vitro*, we sought to determine if these compounds would also inhibit CDK2, 4 and 6 enzyme activity. Commercially available CDK2 enzymes were pre-incubated with these compounds for 10 mins, and then *in-vitro* kinase assays were carried out by providing H1 histones as substrates. Figures 4.7A and B demonstrates that aspirin, salicylic acid, 2,3-DHBA and 2,6-DHBA did not inhibit CDK2 activity. However, 2,4,6-THBA showed dose-dependent inhibition of CDK2 enzyme activity (Fig. 4.7C and D). The IC$_{50}$ for the inhibition of CDK2 by 2,4,6-THBA was ~300 µM. We next performed experiments to determine the effect of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-DHBA on CDK4 and CDK6 enzyme activity in *in-vitro* kinase assays similar to the experiments performed in Fig. 4.7A except that retinoblastoma (Rb) protein was provided as substrate for phosphorylation. Figure 4E and F demonstrates that aspirin, salicylic acid, 2,3 DHBA and 2,6 DHBA, all failed to inhibit CDK4; however, 2,4,6-DHBA inhibited CDK-4 activity (45%). Figure 4.7G and H demonstrates that aspirin and salicylic acid did not affect CDK6 activity; interestingly, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA significantly inhibited the CDK6 activity by 28%, 37% and 40% respectively.
Figure 4.7: Effect of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA (at 0.5 mM) on CDK2, 4 and 6 enzyme activity.

A, Upper panel represents in-vitro kinase assays showing the effect of aspirin, salicylic acid, 2,3-DHBA and 2,6-DHBA on CDK2 activity. Figure 4.7A lower panel shows coomassie stained H1 histones. B, Quantification of the blot in blot A. C, Upper panel represents in-vitro kinase assays showing the dose-dependent effect of 2,4,6-THBA on CDK2 activity. Figure 4.7C lower panel shows coomassie stained H1 histones. D, Quantification of the blot in blot 4.7C. The intensities of bands in various blots were quantified and expressed as percentage of control. P-value <0.05 *, <0.01 **, <0.001†, <0.001‡.
Continued Figure 4.7:  Effect of aspirin, salicylic acid, 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA (at 0.5 mM) on CDK2, 4 and 6 enzyme activity.

E, Upper panel represents the effect of aspirin, salicylic acid, 2,3-DHBA and 2,6-DHBA and 2,4,6-THBA on CDK4 activity. Figure 4.7E lower panel shows coomassie stained retinoblastoma protein.  F, Quantification of the blot in blot 4.7E.  G, Upper panel represents the effect of aspirin, salicylic acid, 2,3-DHBA and 2,6-DHBA (0.5 mM) and 2,4,6-THBA on CDK6 enzyme activity. Figure 4.7G lower panel shows coomassie stained retinoblastoma protein.  H, Quantification of the blot in blot 4.7G. The intensities of bands in various blots were quantified and expressed as percentage of control. P-value <0.05 *, <0.01 **, <0.001†, < 0.001‡.
Table 4.2: Shows the inhibitory effect of aspirin, salicylic acid 2,3-DHBA, 2,6-DHBA, 2,4,6-THBA and 3,4,5-THBA on CDK-1, 2, 4 and 6. Inhibitory effects are shown by plus sign; lack of inhibitory effect is shown by minus sign.

<table>
<thead>
<tr>
<th>CDKs</th>
<th>Aspirin</th>
<th>Salicylic Acid</th>
<th>2,3-DHBA</th>
<th>2,6-DHBA</th>
<th>2,4,6-THBA</th>
<th>3,4,5-THBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.3: Shows IC$_{50}$ for CDK1 inhibition in-vitro and cytotoxicity assays in HCT-116 cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CDK1 IC$_{50}$ (µM)</th>
<th>MTT assay HCT-116 (µM) 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-DHBA</td>
<td>386.1</td>
<td>1778.28</td>
</tr>
<tr>
<td>2,6-DHBA</td>
<td>365.2</td>
<td>1949.84</td>
</tr>
<tr>
<td>2,4,6-THBA</td>
<td>226.0</td>
<td>785.236</td>
</tr>
</tbody>
</table>
4.3.7 Identification of aspirin-acetylated lysine residues on CDK1:

We attempted to determine aspirin acetylated sites on recombinant CDK1 using LC-MS/MS. For this, 10 ug of purified recombinant CDK1 was acetylated by incubating protein with aspirin at 0.5 mM and 2.5 mM concentrations. The acetylation was confirmed by immunoblotting the samples with anti-acetyl lysine and anti-CDK1 antibodies (FIG 4.8). The remaining samples were subjected to LC-MS/MS. The spectra obtained for peptides containing lysine 34 and 296 are shown in fig. 4.9 and 4.10. It is interesting to note that the number of acetylated sites varied depending upon the concentrations of aspirin used with 4 sites detected at 0.5 mM; and 5 sites detected at 2.5 mM aspirin (Table 4.4).

<table>
<thead>
<tr>
<th>Lysine residues acetylated by Aspirin 0.5 mM</th>
<th>Lysine residues acetylated by Aspirin 2.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>6</td>
</tr>
<tr>
<td>106</td>
<td>34</td>
</tr>
<tr>
<td>274</td>
<td>245</td>
</tr>
<tr>
<td>296</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td>296</td>
</tr>
</tbody>
</table>

Table 4.4: Acetylated sites varied depending upon the concentrations of aspirin used. Aspirin acetylated 4 lysine residues at 0.5 mM; and 5 lysine residues at 2.5 mM.
Figure 4.8: *In-vitro* acetylation of recombinant CDK1 by aspirin at 2 different concentrations for mass spectrometry analysis:

Acetylation of CDK1 by aspirin was confirmed by immunoblotting the samples with anti-acetyl lysine and anti-CDK1 antibodies. Figure 4.8A and C, represents blots probed with anti- acetyl lysine antibody confirming aspirin induced acetylation with 0.5 mM and 2.5 mM respectively. Figure 4.8B and D, represent blots probed with anti- CDK1 antibody confirming equal amount of CDK1.
Figure 4.9: MS/MS fragmentation spectra showing acetyl modification on lysine 34 by aspirin

Figure 4.10: MS/MS fragmentation spectra showing acetyl modification on lysine 296 by aspirin
4.4 Discussion:

Since the discovery of aspirin in 1897, many of its therapeutic properties and mechanisms of actions have been identified. It is used for a variety of illnesses including inflammation, fever, pain, and for its anti-platelet properties. These actions of aspirin have been attributed mainly to acetylation-mediated inhibition of cyclooxygenases (COX). There are two COX enzymes: COX-1 and COX-2. Aspirin’s anti-platelet effect occurs through inhibition of COX-1 (IC$_{50}$ - 1.67 μM); whereas, its analgesic and anti-inflammatory effects occur primarily through inhibition of COX-2 (IC$_{50}$ - 278 μM) [86].

In recent years, evidence has emerged establishing aspirin’s ability to decrease cancer incidence, igniting a renewed interest in its research and use. Despite extensive studies, aspirin’s mode of action in cancer prevention, as well as how it primarily prevents CRC as compared to distal cancers, is not clear. Although multiple targets and signaling pathways have been proposed, a unifying mechanism has not been identified to date, suggesting that different mechanisms may be responsible in different cancers or that the actual mechanism is yet to be discovered.

One of the hypotheses proposed for aspirin’s anti-cancer effects is acetylation-mediated inactivation of COX, as upregulation of COX-2 is observed in nearly 80-90 % of CRC; in contrast, COX-1 expression is unaffected [109]. The observation that low dose aspirin, used for its cardio-protective effect, is also effective in the prevention of CRC, led to the hypothesis that a common pathway involving COX-1 inhibition in platelets may be central to both effects [99, 111]. Since low dose aspirin is insufficient to achieve complete inhibition of COX-2, it is argued that aspirin-mediated inhibition of platelet COX-1 plays a role in the prevention of CRC, and that aspirin’s effects may occur through sequential
steps involving COX-1 and COX-2. It is hypothesized that low dose aspirin by inhibiting COX-1 in platelets prevents their activation and the release of growth factors and lipid mediators, required for COX-2 expression and tumorigenesis in the colonic tissue. While this is a tenable hypothesis, it is not yet confirmed, although a recent study demonstrated the acetylation of COX-1 and inhibition of PGE2 in human intestinal mucosa. In addition, salicylic acid, the hydrolytic product of aspirin, has also been implicated in aspirin’s chemopreventive effects. Salicylic acid was shown to bind to a number of cellular proteins such as IκB kinase (IKK), a component of the NF-κB complex [134], AMP activated protein kinase [136] (Hawley et al, 2012), High Mobility Group Box 1 proteins [132, 137] and GAPDH [132] and CDK 2 [133], affecting their levels and/or functional activity.

Although specific protein targets have been identified for both aspirin and salicylic acid, no studies have reported on the cellular targets for salicylic acid metabolites. One of the most important findings of this study is the ability of salicylic acid metabolites, 2,3-DHBA and 2,5 DHBA, as well as other derivatives such as 2,4-DHBA, and 2,6-DHBA to inhibit CDK1 enzyme activity in-vitro (Fig. 4.1). Among these, 2,3-DHBA (IC$_{50}$ 386 μM) and 2,6-DHBA (IC$_{50}$ 365 μM) showed a greater degree of inhibition as compared to 2,4-DHBA and 2,5-DHBA. The ability of 2,3-DHBA and 2,5-DHBA to inhibit CDK1 activity is highly significant, because they are generated from salicylic acid metabolism through CYP450-mediated reactions [96]. Additionally, in this study, we also screened two THBA derivatives, 3,4,5-THBA and 2,4,6-THBA, for their ability to inhibit CDK1. We observed that 3,4,5-THBA failed to inhibit; however, 2,4,6-THBA (IC$_{50}$ 226 μM) significantly inhibited CDK1 activity in a dose-dependent fashion. In fact, the degree of inhibition observed with 2,4,6-THBA was greater than that of 2,3-DHBA and 2,6-DHBA (Fig. 4.1).
Not all compounds tested inhibited CDK1 *in-vitro*. For example, aspirin, salicylic acid, benzoic acid, 3,4-DHBA 3,5-DHBA, and 5-amino-salicylic acid, failed to inhibit CDK1 activity. These results collectively suggest that the DHBAs or THBAs with a common -OH group at the 2nd carbon is likely important for the inhibitory effect on CDK1. We also performed limited studies to determine the effect of 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA on CDK2, 4 and 6 enzyme activity. We observed that 2,3-DHBA and 2,6-DHBA were not effective in inhibiting CDK2 and 4 (at 500 µM) (Fig. 4.7A and 4.7E); however, both inhibited CDK6 activity (Fig. 4.7G). 2,4,6-THBA was highly effective and inhibited all CDKs examined (CDK1, 2, 4 and 6) (Fig. 4.7C, E and G). This suggests that although CDK members share significant homology, the binding pockets for these compounds in CDKs may differ, perhaps accounting for their differential effects. A compilation of the inhibitory effect of 2,3-DHBA, 2,6-DHBA and 2,4,6-THBA on CDK-1, 2, 4 and 6 are shown in table 4.2. Cytotoxicity assays in HCT-116 cells showed that the IC$_{50}$ for these compounds differ. For 2,3-DHBA, the IC$_{50}$ was ~1.8 mM; for 2,6-DHBA, it was 1.9 mM and for 2,4,6-THBA, it was 0.8 mM (see table 4.3). The IC$_{50}$ for compound’s cytotoxicity in HCT-116 cells is significantly higher than observed for *in-vitro* CDK1 inhibition (e.g., Fig. 4.1E-H); this may be related to poor uptake of these hydrophilic compounds by cells or differential sensitivity towards cellular targets. Our pilot studies using HPLC showed that the cellular uptake of dihydroxy and trihydroxy-benzoic acid are lower as compared to the mono-hydroxy benzoic acid (salicylic acid) (data not shown), possibly reflecting the presence of multiple OH groups making them more hydrophilic and less absorbable. The poor uptake of HBAs may also be related to the significantly lower expression of monocarboxylate transporters (MCTs, e.g., SLC5A8 and SLC5A12) in metastatic cancer.
cells [170]. Therefore, the effect of these compounds on cellular functions, such as cell cycle regulation, can be better studied after formulation with compounds that can enhance cellular uptake, or in cell lines expressing MCTs. Further work is required to determine, how DHBAs and THBAs affect cell cycle regulation and functions.

Molecular docking studies revealed that aspirin, salicylic acid, 2,3-DHBA, 2,5-DHBA and 2,6-DHBA potentially bind at the same pocket in CDK1; in contrast, 2,4,6-THBA appears to bind to a different site (Fig. 4.2). Despite the common binding pocket, aspirin and salicylic acid failed to inhibit CDK1; however, 2,3-DHBA, 2,5-DHBA and 2,6-DHBA inhibited CDK1 activity (Fig. 4.1A-D). Inhibition was also observed with 2,4,6-THBA (Fig. 4.1G and H). The reason for the inability of aspirin and salicylic acid to inhibit CDK1 activity is not clear and requires further investigations. All DHBAs potentially showed interaction with Asp146 in CDK1, either through -COOH or -OH groups. 2,3-DHBA and 2,5-DHBA use –COOH group; while, 2,6-DHBA uses the -OH group at the 6th carbon to interact with CDK1. Salicylic acid, which did not show any inhibition of CDK1 appears to interact with Asp146 via the -COOH group, and with Lys33 via the -OH group at the 2nd carbon. It is also interesting to note that 2,4,6-THBA which showed significant inhibition of CDK1 appears to interact with Arg123 via the -COOH group, and with Arg151 and Gly154 via the -OH group at the 6th carbon; this interaction occurs at the surface (Table 4.1, Fig. 4.2). These data suggest that the ability of these compounds to inhibit CDK1 is likely determined by the interacting functional groups, as well as their orientation at the binding pocket (Fig. 4.2), which may affect CDK1 conformation and activity.
In this study, using immunoblotting, we also demonstrated that incubation of recombinant CDK1 (Prospect Inc) monomer with aspirin for 12 h dose-dependently acetylated CDK1 (Fig. 4.6A), which was also observed in HCT-116 cells (Fig. 4.6F). An initial mass spectrometry analysis of aspirin-acetylated CDK1 showed that Lys34 is targeted for acetylation. It is not clear at this stage whether this modification affects CDK1 activity; however, the Lys34 is adjacent to the amino acid Lys33 (in the active site). Our in-vitro kinase assays using CDK1/cyclin B1 (NEN Biolabs) were designed to address how the binding of aspirin affects CDK1 activity by preincubating with aspirin for 10 min, before assaying for H1 histone phosphorylation. The effect of aspirin-mediated acetylation on CDK1 activity could not be assessed, as the short incubation time (10 mins) of CDK1/cyclin B1 complex with aspirin was insufficient to cause acetylation; longer incubation (12h) caused degradation (unpublished data). The ability of aspirin to acetylate recombinant CDK1 monomer (Prospect) was taken advantage of to demonstrate the binding of salicylic acid, and its derivatives 2,3-DHBA, 2,6-DHBA and 2,4,6-TBHA to CDK1, in competition experiments. Pre-incubation with all three compounds prevented aspirin-mediated CDK1 acetylation (Fig.4.6A-E), which supported the data from molecular docking studies that all compounds potentially bind to CDK1 (table 4.1).

It is well established that aspirin is more effective in preventing CRC as compared to distal cancers, but a clear explanation for its preferential protective effect in colorectal tissues has not emerged in any reports. Our observation that salicylic acid metabolites 2,3-DHBA and 2,5-DHBA inhibit CDK1 activity provides an important insight into aspirin’s chemopreventive actions. This is particularly relevant to the prevention of colon tumorigenesis, as expression of several CYP450 members, including CYP3A4/A5
implicated in aspirin/salicylic acid metabolism in the liver, are also reported to be present in healthy and cancerous colonic tissue [171-174]. Although the stomach and upper intestine are the major sites of aspirin absorption, it is likely that some aspirin and its hydrolytic product salicylic acid, is passed on to the lower intestine and colon. Also, availability of aspirin in the intestine and colon will be much higher after ingestion of enteric coated tablets, as they are designed to resist dissolution in the acidic environment of the stomach. Aspirin and salicylic acid absorbed by the intestinal and colonic epithelial cells may be metabolized by local CYP450s to produce 2,3-DHBA and 2,5-DHBA. Although 2,3-DHBA and 2,5-DHBA are the minor metabolites generated by CYP450 metabolism in the liver, the extent to which they are generated in epithelial cells of the GI are unknown, and it is possible that these metabolites may be produced at much higher levels in GI epithelial cells. These intracellularly produced hydroxyl-derivatives of salicylic acid are probably too hydrophilic to cross the basolateral side of the epithelial cell membrane, and within these cells, they may accumulate to pharmacologically relevant concentrations sufficient to inhibit CDKs and act locally to exert anti-cancer effects.

Another source of salicylic acid derivatives for epithelial cell uptake may be generated through GI microflora. The epithelial cells of the intestine/colon are likely to be exposed to higher concentrations of the salicylic acid metabolites and derivatives (HBAs) as opposed to other tissues resulting in the inhibition of CDKs. This additional layer of protection against tumor development may be unique to the colorectal tissue as compared to the distal tissues, allowing aspirin to preferentially act against CRC. A model depicting this new insight into aspirin’s effect against CRC is shown in Fig. 4.11.
Salicylic acid metabolites 2,3-DHBA and 2,5-DHBA were also reported to be present in the plasma of individuals even when there has been no intake of aspirin, indicating a dietary source [175]. It is suggested that diet containing fruits and vegetables may also provide a rich source of HBAs. 2,3-DHBA is present in avocados, batoko plum and in medicinal herbs such as Madagascar rosy periwinkle. 2,5-DHBA is also widely present in fruits including kiwi fruit, aloe vera, mushrooms, apple, bitter melon, blackberries, grapes and pears; it is also present in wine [176]. It is important to also note that 2,4,6-THBA was demonstrated as one of the in-vitro degradation product of catechins by the intestinal bacterium, Acinetobacter calcoaceticus [177]. 2,4-DHBA and 2,4,6-THBA was demonstrated to be produced through pH-dependent degradation of anthocyanins [178]. The exposure of the colonic epithelial cells to these pharmacologically active CDK inhibitors may provide a link between diet rich in salicylic acid/DHBAs/catechins/anthocyanins and decreased occurrences of CRC [179-181].
Figure 4.11: A model depicting how aspirin may preferentially act on colonic tissue to protect against CRC.

Figure 8. A model depicting how aspirin may preferentially act on colonic tissue to protect against CRC. We suggest that unabsorbed aspirin/salicylic acid in the stomach and upper intestine is passed on to the colon, taken up by the colonic epithelial cells, and metabolized by CYP450s to produce 2,3-DHBA and 2,5-DHBAs. These hydrophilic DHBAs may not easily cross the basolateral membrane of the epithelial cells and within these cells, they may accumulate to pharmacologically relevant concentrations, leading to the inhibition of CDK1 and CDK6, and exert anticancer effects. Colonic epithelial cells may also get exposed to salicylic acid metabolites generated by the gut microflora, and uptake of these HBAs by cells may also cause CDK inhibition. This additional layer of protection against tumor development may be unique to the colonic tissue allowing aspirin as a more effective drug against CRC.
We believe that our observations have opened a new frontier in aspirin research by identifying CDKs as novel cellular targets for salicylic acid metabolites and derivatives. The potential contributions of locally generated 2,3-DHBA and 2,5-DHBA through CYP450 metabolism within the colonic epithelial cells to aspirin’s chemopreventive actions against CRC via CDK inhibition represent an important area for future research.

Against the backdrop of our observations, several questions can be raised. Are there other cellular protein targets for salicylic acid metabolites besides CDKs? Will daily supplements of DHBAs/THBAs capable of inhibiting CDKs provide protection against CRCs? IC\textsubscript{50} for the inhibition of CDKs by DHBAs and THBAs are although in micromolar range (300 μM), it could be argued that these concentrations are still physiologically relevant. Given the abundance of the occurrences of DHBAs and THBAs in fruits and vegetables, it is possible that CDKs have been evolved to be less sensitive to inhibition for these natural compounds avoiding cytotoxicity. In recent times, attention is increasingly focused on developing CDK inhibitors to arrest the cell cycle as a therapeutic intervention to treat cancer. Although several potential CDK inhibitors are undergoing clinical trials, palbociclib and ribociclib are the only FDA approved drugs used in cancer treatment. We are hopeful that structural modification to DHBAs and THBAs may potentially lead to development of novel class of CDK inhibitors for cancer prevention and treatment.
Chapter 6: Summary, Conclusions, Significance, Limitations and Future Directions:

6.1 Summary and Conclusions:

Over the past several decades billions of dollars have been spent in cancer research for identifying drugs that can be used in cancer treatment and also its prevention. These investments have been successful; several potent drugs are now available for cancer treatment. In fact, although the global incidence of cancer is on raise, the mortality rate associated with cancers particularly the CRC has decreased, due to the discovery of many chemotherapeutic and immunotherapeutic drugs as well as, better screening procedures. Effective cancer eradication from the population also requires effective chemoprevention strategies. In this regard, except for two drugs tamoxifen and raloxifene, no other drugs exist or recommended for the prevention of cancers. Tamoxifen and raloxifene are recommended only in women who are at higher risk of developing breast cancers, however, they also show some degree of toxicity. Drugs to prevent other cancers such as colon, prostate or lung, do not exist or approved by FDA. The evidence that aspirin prevents cancer has attracted the attention of scientists, physicians and public alike to understand its chemopreventive mechanisms as well as its use for the prophylaxis of cancer. In view of this, United States Preventive Services Task Force (USPSTF) in February 2016, has recommended low dose aspirin’s use for the prevention of CRC within the age group 50 – 59.

Cancer is a deadly disease; NIH has strong research focus for developing drugs that can prevent cancer. According to the American cancer society, nearly 1.6 million people were diagnosed with cancer in the USA every year, of which nearly half a million die from
this disease. Thus there is an urgent need to identify and develop novel drugs that can be used in cancer prevention. The evidence that aspirin prevents cancer is now well accepted, however the precise mechanism by which it protects epithelial tissues against the cancer development is not elucidated. We believe that the studies carried out in this project provides new insight on its preferential protection against CRC through modulation of cell cycle regulatory proteins as well as the ability of salicylic acid metabolites to inhibit CDK enzyme activity. This observation although requires additional research, is far closer to its mechanism of action towards cancer prevention than those which have been proposed previously in the published literature.

The major findings from this study are as follows:

- Aspirin and its metabolite salicylic acid, downregulated cyclins A2, B1 and D3; CDKs 1, 2, 4 and 6 in multiple cancer cell lines representing cancers of various tissues. p21 and p27, which are the CDK inhibitors, were upregulated by both these drugs. It is possible that down-regulation of these cyclins and CDKs, and up-regulation of CDK inhibitors, would be contributing towards the observed effect of aspirin and salicylic acid on cell cycle arrest reported in the literature.

- The downregulation of cyclin A2 and cyclin B1 is reversible with lactacystin treatment, which suggested that 26S proteosomal pathway is involved. The involvement of 26S proteosomal pathway in the degradation of these cyclins is also in line with the reported pathway of degradation under normal conditions.
• For the first time we identified CDK2 as a salicylic acid binding protein (SABP). We also identified the potential binding site of salicylic acid with CDK2 using molecular docking studies. Our studies reveal that salicylic acid interacts with CDK2 via Asp145 and Lys33, which are present in the active site of enzyme. Despite the potential binding of salicylic acid to the CDK2, it did not inhibit the enzyme activity (cellular CDK2). We propose that binding of salicylic acid to CDK2 may trigger its degradation through proteosomal pathway.

• Aspirin and salicylic acid, both failed to inhibit recombinant CDK1, 2, 4 and 6 enzyme activity in *in-vitro* experiments. However, salicylic acid metabolites 2,3-DHBA and 2,5-DHBA, which are produced through CYP 450 catalyzed reactions inhibited the CDK1 and CDK6 enzyme activity. This is the first demonstration of the ability of salicylic acid metabolites to inhibit the CDK enzyme activity suggesting a potential role for these metabolites in aspirin’s chemopreventive actions.

• We believe that the ability of salicylic acid metabolites 2,3-DHBA and 2,5-DHBA to inhibit CDK enzyme activity is a very significant observation, as these metabolites are also generated in the intestinal and colonic tissues, in addition to the liver as both tissue types express CYP 450 capable of metabolizing salicylic acid.

• We believe that the ability of salicylic acid metabolites 2,3-DHBA and 2,5-DHBA to inhibit CDK enzyme activity is a very significant observation, as these metabolites are also generated in the intestinal and colonic tissues, in
addition to the liver as both tissue types express CYP 450 capable of metabolizing salicylic acid. We suggest that unabsorbed aspirin and salicylic acid will be taken up by colonic epithelial cells and metabolized to 2,3-DHBA and 2,5 DHBA by the CYP 450 enzymes. Generation of these metabolites within the colonic epithelial cells may lead to the inhibition of CDKs and exert a local inhibitory effect against the development of CRC. This may provide an added advantage for colonic epithelial cells against tumor development compared to the distal tissues, allowing aspirin to preferentially act against CRC. Although additional studies are required to establish the role of 2,3-DHBA and 2,5-DHBA in aspirin’s chemopreventive effect, the mere suggestion that metabolites may play a role in its actions is novel and provides a basis for future study.

- Derivatives of salicylic acid (2,4-DHBA, 2,6-DHBA and 2,4,6-THBA) also exhibited inhibitory effect. While, 2,4-DHBA and 2,6-DHBA was somewhat selective in its ability to inhibit CDK members, 2,4,6-THBA inhibited all CDK members studied. This provides an opportunity to further extend this study to introduce structural modifications to 2,4,6-THBA to increase its binding potential and specificity. Development of novel CDK inhibitors belonging to DHBA and THBA represents an extension of this study.

- 2,3-DHBA and 2,5-DHBA are also present in fruits and vegetables as part of normal constituent compounds. It will be interesting to determine the
observed link between consumption of diet rich in fruits and decrease incidence of cancer is mediated these DHBAs.

6.2 Limitations and Future directions:

- While we have demonstrated the ability of salicylic acid and its derivatives to bind to CDKs *in-vitro*, this has not been shown in *in-vivo* experiments. Also demonstration of the inhibition of CDKs with the administration of 2,3-DHBA and 2,5-DHBA *in-vivo* is required to validate the *in-vitro* observation.

- Estimation of the uptake of 2,3-DHBA and 2,5-DHBA metabolites by cells as well as the estimation of the concentration of 2,3-DHBA and 2,5-DHBA generated within the cells from salicylic acid through CYP 450 reactions has not been performed. These experiments need to be performed to further validate our hypothesis, and determine their potential contribution to aspirin’s anti-cancer effects.

- We have demonstrated the potential interaction between aspirin, salicylic acid, salicylic acid metabolites/derivatives with CDKs. Additional studies involving protein crystallization is required to further confirm the results obtained from molecular docking studies.

- The poor uptake of HBAs may also be related to the significantly lower expression of monocarboxylate transporters (MCTs, e.g., SLC5A8 and SLC5A12) in metastatic cancer cells. Therefore, the effect of these compounds on cellular functions, such as cell cycle regulation, can be better studied after formulation with compounds that can enhance cellular uptake, or in cell lines expressing MCTs
Incubation of CDK1 with aspirin showed dose-dependent acetylation of lysine residues. Similar experiment need to be performed with other CDK members to determine how aspirin effects the acetylation as well as how it modulates the enzyme activity.
Chapter 7: Bibliography


32. Ehrlich, M., *DNA methylation in cancer: too much, but also too little.* Oncogene,


129. Li, H., et al., *Aspirin prevents colorectal cancer by normalizing EGFR expression*.


147. Bukholm, I.R., G. Bukholm, and J.M. Nesland, Over-expression of cyclin a is highly associated with early relapse and reduced survival in patients with primary


165. Deng, Y., et al., *Modulating the interaction between CDK2 and cyclin A with a*


175. Paterson, J.R., et al., The identification of salicylates as normal constituents of


