CELECOXIB: ITS NON-COX-2 TARGETS AND ITS ANTI-CANCER EFFECTS

DISSERTATION

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* * * * *

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ABSTRACT

Our previous work showed that celecoxib (Celebrex®), a nonsteroidal anti-inflammatory drug (NSAID, cyclooxygenase-2 inhibitor) was unique among other cyclooxygenase-2 (COX-2) inhibitors in its superior ability to induce prostate cancer cell death, indicating the involvement of non-COX-2 components in the mode of action of celecoxib. To test this hypothesis, we investigated the effect of COX-2 depletion on apoptosis by tetracycline controllable COX-2 antisense constructs and performed a structure-activity analysis of the COX-2 inhibitor celecoxib in PC-3 cells. Both strategies came out with the same conclusion, which is the unique apoptotic inducing activity of celecoxib comes from interfering with other important signaling pathways. We then focused on one of the most promising pathways, PI3K/PDK-1/Akt pathway, which was previously demonstrated in our laboratory to be severely interrupted by celecoxib. Further target identification showed that PDK-1 is one of major targets. Later, we learned that inhibition of PI3K/PDK-1/Akt pathway alone can only induce apoptotic cell death in LNCap cells but not PC3 cells, and further elucidated that the significantly higher level of Bcl-xL in PC3 cells is attributable to this discrepancy. However, the observations of quick apoptotic death in PC3 cells triggered by celecoxib indicate that other yet unidentified targets are also involved. By studying growth inhibitory effects in Human Umbilical Vein Endothelial Cells (HUVECs), we were able to identify cyclin dependent
kinases (CDKs), are important targets in addition to PDK-1 for celecoxib. In conclusion, celecoxib trigger its anti-cancer effect through interfering with multiple signaling pathways. This study was used as a fundamental basis in our laboratory for developing novel anti-cancer agents, targeting pathways important for cancer cell survival and growth, based on structural modifications starting from celecoxib.
Dedicated to my grandparents, parents, my sister and my wife
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FIELDS OF STUDY

Major Field: Pharmacy
# TABLE OF CONTENTS

Abstract..............................................................................................................ii

Acknowledgement..........................................................................................v

Vita....................................................................................................................vi

Table of Content...............................................................................................viii

List of Tables..................................................................................................xi

List of Figures................................................................................................xii

Abbreviations.................................................................................................xviii

Chapter 1 Introduction.........................................................................................1
  1.1 Deregulations of Apoptosis and Proliferation Cancer .........................1
    1.1.1 Apoptosis ..............................................................................................1
    1.1.2 The Regulations of Cell Cycle...............................................................2
  1.2 Cyclooxygenase 2 (COX-2)......................................................................5
    1.2.1 Cyclooxygenase ..................................................................................5
    1.2.2 COX-2 and Cancer .............................................................................5
    1.2.3 Celecoxib: COX-2 Inhibitor as an Anti-cancer Agent .....................6
  1.3 The Involvement of Non-Cox-2 Components in Celecoxib Mediated Anti-
    Cancer Effects ............................................................................................6
  1.4 PI3K/PDK-1/Akt Signaling Pathway..........................................................7
  1.5 Cancer Models...........................................................................................10
    1.5.1 Prostate Cancer ..................................................................................10
    1.5.2 Model of Anti-Angiogenesis ...............................................................11

Chapter 2 Celecoxib Induced Apoptosis in PC3 cell is Cox-2 Independent ........19
  2.1 Isolation and Characterization of Tet-On Antisense COX-2 Clones ....19
  2.2 COX-2 Ablation and Consequent Effect on Apoptosis in Prostate Cancer
    Cells..............................................................................................................20
  2.3 Structure-Activity Analysis Reveals no Correlation between COX-2
    Inhibition and Apoptotic Inducing Effect ...............................................21

Chapter 3 PDK1 acts as an Important molecular Target for Celecoxib ..........31
  3.1 Research on the Cause of Phospho-Akt Down Regulation ..................31
  3.2 PDK1 is One of the Major Targets .........................................................32
Chapter 4 Is PDK1 the only One Non-Cox-2 Target for Celecoxib?...............................37
  4.1 LNCaP and PC-3 cells Display Differential Susceptibility to PI3K Inhibition-Induced Apoptosis. ...............................37
  4.2 Bcl-xL Overexpression and Apoptosis Resistance in PC-3 Cells..............................38
  4.3 Ectopic Bcl-xL Expression Protects LNCaP cells from LY-294002-Induced Apoptosis..................................................39
  4.4 Antisense Downregulation of Bcl-xL Reduces the Threshold of LY294002-Mediated Apoptosis in PC-3 Cells ........................................40

Chapter 5 Inhibition of CDKs is Responsible for Celecoxib Induced Cell Cycle Arrest .................................................................51
  5.1 HUVEC Growth is Inhibited by Celecoxib and DMC...............................52
  5.2 Celecoxib and DMC Inhibit HUVEC Growth by Causing G1 Arrest ..................52
  5.3 Celecoxib and DMC Mediate G1 Arrest through the Inhibition of Multiple Signaling Targets ........................................53

Chapter 6 Anti-Angiogenesis Effect of Celecoxib AND DMC........................................62
  6.1 CAM assay ..............................................................................62

Chapter 7 Discussions and Conclusions..............................................................65
  7.1 In Vivo Effect of Celecoxib and DMC on PC-3 Xenograft Tumor Growth ..................................................65
  7.2 Conclusions..............................................................................65

Chapter 8 Materials AND Methods.....................................................................69
  8.1 Cells and reagents ..........................69
    8.1.1 Cells and Culturing Conditions ..................69
    8.1.2 Reagents.................................................................70
    8.1.3 Antibodies and Recombinant Proteins ............70
  8.2 Analysis for Apoptosis ........................................................................71
    8.2.1 Phosphatidylserine Externalization .................71
    8.2.2 4',6-Diamidino-2-phenylindole (DAPI) Staining of Nuclei ..............71
    8.2.3 Apoptosis Detection by an Enzyme-Linked Immunosorbent Assay.....72
    8.2.4 Western Blot Analysis of PARP Cleavage .............................73
    8.2.5 Western Blot Analysis of Cytochrome c Release into the Cytoplasm.....73
  8.3 Development of PC-3 Tet-On Antisense COX-2 Clones .....................74
  8.4 Preparation of Bcl-xL-Overexpressing LNCaP Transfectants ..............75
  8.5 Antisense Experiments .........................................................................75
  8.6 Cell viability.................................................................................76
  8.7 Prostaglandin E2 (PGE2) immunoassay ........................................77
  8.8 Growth Inhibition Assay ...................................................................78
  8.9 Flow Cytometry for Cell Cycle Analysis ........................................78
  8.10 Akt Kinase Assay ........................................................................79
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.11</td>
<td>Transient Transfection in HUVECs by Calcium Phosphate Coprecipitation Method</td>
<td>80</td>
</tr>
<tr>
<td>8.12</td>
<td>CDK1/Cyclin B Kinase Assay</td>
<td>80</td>
</tr>
<tr>
<td>8.13</td>
<td>CDK2/Cyclin E Kinase Assay</td>
<td>81</td>
</tr>
<tr>
<td>8.14</td>
<td>Immunoprecipitated Cyclin E Kinase Assay</td>
<td>82</td>
</tr>
<tr>
<td>8.15</td>
<td>CDK4 Kinase Assay</td>
<td>82</td>
</tr>
<tr>
<td>8.16</td>
<td>Quantitative Determination of pRbT821 and Total Rb in HUVECs</td>
<td>83</td>
</tr>
<tr>
<td>8.17</td>
<td>Chicken Chorioallantoic Membrane (CAM) Assay</td>
<td>84</td>
</tr>
<tr>
<td>8.18</td>
<td>Western Blot</td>
<td>85</td>
</tr>
</tbody>
</table>

Bibliography.................................................................................................................86
LIST OF TABLES

Table 5.1 Cell cycle phase distribution of HUVECs treated with celecoxib or DMC at the indicated concentrations in the same medium conditions listed in the legend of Fig. 5.1. for 48 h. Control cells received DMSO vehicle. Each tabulated percentage represents the average of three independent experiments. Student t test reveals that all values presenting G0/G1 phase in celecoxib or DMC treatment group are statistically different from that of DMSO control (P< 0.05). Intra group comparison in celebrex treatment group shows the value of 25 µM (69.3 ± 3.8) is statistically different (P=0.045) from the one of 10 µM (62.55 ± 5.7). In DMC group, the value of 25 µM statically differs from 10 µM (P=0.0034) and 15 µM (P=0.0066), respectively…………………………………………………………….....57
<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Cell cycle distribution</td>
<td>12</td>
</tr>
<tr>
<td>1.2</td>
<td>Illustration of G1/S transition and the key regulatory components</td>
<td>13</td>
</tr>
<tr>
<td>1.3</td>
<td>Simple illustration to Cyclooxygenase</td>
<td>14</td>
</tr>
<tr>
<td>1.4</td>
<td>PC-3 and LNCaP cells are susceptible to the induction of apoptosis by celecoxib in a dose- and time-dependent manner. The incubations were carried out in serum-starved RPMI 1640 medium. Cell death was determined by using trypan blue exclusion.</td>
<td>15</td>
</tr>
<tr>
<td>1.5</td>
<td>Celecoxib down regulates phospho-Akt. LNCaP and PC-3 cells were treated with DMSO vehicle (control) (A) or celecoxib (50 µM) (B) in serum-free medium for the indicated time. The supernatants were probed by Western blot with rabbit anti-Akt and anti-P-473Ser Akt antibodies.</td>
<td>16</td>
</tr>
<tr>
<td>1.6</td>
<td>Activated Akt protects PC-3 cells from celecoxib-induced cell death. A, Transient expression of myristoylated Akt (MyrAkt) in PC-3. The transfectant showed highest level of MyrAkt expression at 48 h, and were collected for the cell viability analysis. B, Viability of PC-3 cells overexpressing MyrAkt in the absence of celecoxib (a), PC-3 cells overexpressing MyrAkt in the presence of 50 µM celecoxib (b), and PC-3 cells transfected with the control pCMV vector in the presence of 50 µM celecoxib (c).</td>
<td>17</td>
</tr>
<tr>
<td>1.7</td>
<td>PI3K/PDK-1/Akt signaling pathway</td>
<td>18</td>
</tr>
<tr>
<td>2.1</td>
<td>Characterization of antisense cyclooxygenase-2 (COX-2) PC-3 transfectants. (A) Western blot analysis showing the time course of COX-2 depletion in the representative antisense COX-2 clone 3D9 in response to doxycycline. (Results from other clones were similar.) Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence of doxycycline at 2 µg/mL as indicated. (B) Western blot analysis showing the COX-2 protein levels in parental PC-3 cells and four independent antisense COX-2 clones in the presence (+) or absence (-) of doxycycline (Dox; 2 µg/mL) for 10 days. (C) Prostaglandin E2 (PGE2) production in parental PC-3 cells and four antisense COX-2 clones without (-) or with (+) doxycycline pretreatment as indicated. Data are expressed as means ± 95% confidence intervals (error bars) (n = 3). Western blots are representatives of three experiments, all with similar results.</td>
<td>23</td>
</tr>
</tbody>
</table>
Fig. Susceptibility of PC3 cells to celecoxib-induced apoptosis is independent of cyclooxygenase-2 (COX-2) expression levels. (A, left panel) Effect of 50 µM celecoxib on the viability of parental PC-3 cells and the COX-2-deficient clone 2F6. (Right panel) Effect of 50 µM celecoxib on the viability of the COX-2 antisense clone 7D9 with (+) or without (-) a doxycycline (Dox) pretreatment (2 µg/mL). Data represent means ± 95% confidence intervals (error bars) (n = 3). (B) Effect of celecoxib on the phosphorylation status of Akt in the COX-2 antisense clone 7D9 with or without doxycycline treatment (2 µg/mL) as indicated. Western blots are representatives of three independent experiments. These data indicate that the mechanism underlying celecoxib-induced apoptotic death in the 7D9 cells remained unaltered after COX-2 depletion.………………………..……………..25

Fig. Structures and characteristics of celecoxib and its structurally related compounds. COX-2 = cyclooxygenase-2; IC50 = concentration inhibiting COX-2 activity by 50%; T1/2 = time required for 50% cell death. ………………….………………..27

Fig. (A) Dose- and time-dependent effect of compound DMC on the cell viability of parental PC-3 cells. Data are the means ± 95% confidential intervals (error bars) (n = 3 to 6). (B) Effect of compound DMC on the morphology and membrane composition of PC-3 cells. PC-3 cells were grown on coverslips and treated with dimethyl sulfoxide (DMSO) vehicles (upper panels) or 50 µM compound DMC (lower panels) for 1 - 2 h as indicated below. (Left panel) Phase-contrast micrograph of PC-3 cells 2 h after treatment. (Center panel) Nuclear fragmentation viewed after DNA staining with 4’,6-diamidino-2-phenylindole (DAPI) and using fluorescence microscopy 2 h after treatment. Condensed and fragmented nuclei (arrow) were observed in drug-treated cells. (Right panel) Detection of annexin V binding to the surface of apoptotic cells by fluorescence microscopy 1 hour after treatment. Note that some blebbing cells show strong annexin V labeling of their surface. (C) Time course of the formation of nucleosomal DNA in PC-3 cells treated with DMSO vehicles or compound DMC (50 µM). The formation of nucleosomes was quantitatively measured by Cell Death Detection ELISA (enzyme-linked immunosorbent assay) with lysates equivalent to 10^4 cells for each assay. Data are the means ± 95% confidence intervals (error bars) (n = 3). (D) Induction of poly(ADP-ribose) polymerase (PARP) cleavage by compound DMC in PC-3 cells. PC-3 cells were treated with 50 µM compound DMC as indicated. PARP proteolysis of the 116-kDa native enzyme to the apoptosis-specific 85-kDa fragment was monitored by Western blotting…………………………….............28

Fig. Time-dependent effect of compound DMC on the phosphorylation status of Akt in PC-3 cells. The Western blots are representatives of three independent experiments, all with similar results…………………………………………………………………………30

Fig. Simple scheme shows how phospo-Akt levels can be regulated. PDK1 activates Akt by adding phosphoryl group on threonine residue, and Akt is subsequently
phosphorylated by PDK2 and get fully activated. The phosphatase can decrease Akt activity by removing the phosphoryl group from it. ........................................34

**Fig. 3.2** Scheme illustrates how the inhibitor of phosphatase may rescue the apoptosis promoting effect of Celecoxib ..........................................................35

**Fig. 3.3** Western blot showing the p-Akt levels on PC3 with calyculin A treatment alone as well as with compound DMC pretreated for 1 to 5 hours .........................36

**Fig. 4.1** LY294002 (25 μM)-induced Akt dephosphorylation leads to apoptosis in LNCaP cells. A, Western blot analysis of the phosphorylation state of Akt. LNCaP cells were treated with DMSO vehicle (left panel) or 25 μM LY294002 (right panel) in serum-free RPMI 1640 medium for the indicated times. B, change of morphology in LNCaP cells after 24 h treatment. The cells became shrunken, round, and detached from the flask after treated with LY294002. C, time course of the formation of nucleosomal DNA in LNCaP cells treated with DMSO (open bars) or 25 μM LY294002 (gray bars). The formation of nucleosomes was quantitatively measured by Cell Death Detection ELISA with lysates equivalent to 5 x 105 cells for each assay. Data are the means ± S.D. (n = 3). D, a time-dependent effect of LY294002 on cytochrome c release analyzed by Western blot in LNCaP cells. Cytosolic-specific, mitochondria-free lysates were prepared. An equivalent amount of protein (50 μg) was electrophoresed, and probed with anti-cytochrome c antibodies.................................................................41

**Fig. 4.2** PC-3 cells are susceptible to LY294002(25 μM)-induced Akt dephosphorylation, but resist the induction of apoptosis by preventing cytochrome c release. The experimental conditions were identical to that described under Fig. 4.1, except that PC-3 cells were used. A, Western blot analysis of the phosphorylation state of Akt. B, morphology of PC-3 cells treated or untreated. C, time course of the formation of nucleosomal DNA in PC-3 cells treated with DMSO (open bars) or 25 μM LY294002 (gray bars). D, a time-dependent effect of LY294002 on cytochrome c release in PC-3 cells.................................................................43

**Fig. 4.3** LY294002 treatment facilitates the dephosphorylation and targeting of BAD to mitochondria in LNCaP and PC-3 cells. Cells were treated with DMSO vehicle (-) or 25 μM LY294002 (+) in serum-free RPMI 1640 medium for 12 h, and lysed. The cytoplasmic and mitochondrial fractions were isolated, electrophoresed, and probed by Western blot with rabbit anti-phospho-BAD and anti-BAD antibodies, respectively. Actin and cytochrome c oxidase were used as internal reference proteins for the cytoplasm and mitochondria, respectively.................................45

**Fig. 4.4** A. Comparison of the basal expression levels of Bcl-xL, Bcl-2, and BAD between PC-3 and LNCaP cells by Western blot analysis. B. Ascending expression levels of ectopic Bcl-xL in B11, B1, and B3 clones. The band for ectopic Bcl-xL contained a Flag tag (eight amino acids long) from the construct, thus migrating slower than endogenous Bcl-xL.........................................................46
Ectopic Bcl-xL protects LNCaP cells from LY294002-induced apoptosis by attenuating cytochrome c release in an expression level-dependent manner. A. Formation of nucleosomal DNA in LNCaP (LN) cells and B11, B1, and B3 clones treated with DMSO (open bars) or 25 µM LY294002 (gray bars) at 12 h (left panel) and 24 h (right panel). The formation of nucleosomes was quantitatively measured by Cell Death Detection ELISA with lysates equivalent to 5 x 10^5 cells for each assay. Data are the means ± S.D. (n = 3). B. Effect of LY294002 on cytochrome c release in LNCaP cells and the three Bcl-xL overexpressing clones at 24 h. Cells were treated with DMSO vehicle (-) or 25 µM LY294002 (+) for 24 h. Cytosolic-specific, mitochondria-free lysates were prepared. An equivalent amount of protein (50 µg) from individual lysates was electrophoresed, and probed by Western blot with anti-cytochrome c antibodies.

Antisense downregulation of Bcl-xL sensitizes PC-3 cells to LY-294002-induced apoptosis by facilitating cytochrome c release. A. Treatment of PC-3 cells with different concentrations of the antisense oligonucleotide caused dose-dependent downregulation of Bcl-xL protein. No change in Bcl-xL expression was noted with the mismatch oligonucleotide at 1 µM. B. Reduced Bcl-xL expression by the antisense oligonucleotide enhanced the susceptibility of PC-3 cells to the induction of apoptosis, while the mismatch oligonucleotide has no effect on the cell death. C. Effect of the mismatch and antisense oligonucleotides on LY294002-induced cytochrome c release in PC-3 cells at 2 h and/or 4 h. Oligonucleotide-treated PC-3 cells were treated with DMSO vehicle (-) or 25 µM LY294002 (+) for 24 h. Cytosolic-specific, mitochondria-free lysates were prepared. An equivalent amount of protein (50 µg) from individual lysates was electrophoresed, and probed by Western blot with anti-cytochrome c antibodies.

Dose dependency of the growth inhibitory effect of celecoxib, DMC and rofecoxib in HUVECs. Cells were seeded into 96-well plates (1,500 cells/well) in six replicates in in 2% FBS-supplemented medium containing 2% FBS, 1µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin. After 24-h incubation, the test agents were added at the indicated concentrations. After 72-h drug exposure, cells were stained with crystal violet, and cell viability was determined. Cell growth over the treatment period was expressed as a percentage of that in the vehicle (DMSO)-treated group. Data point represents means ± S.D. Experiments were performed at least 3 times.

Dose-dependent effects of celecoxib and DMC on HUVEC apoptosis. HUVECs were exposed to varying concentrations of celecoxib or DMC for 24 h. Upper panel, quantitative measurement of the formation of nucleosomes by the Cell Death Detection ELISA. O.D. = optical density. Values are means ± S.D. (n = 3). Lower panel, induction of PARP cleavage by celecoxib. PARP proteolysis to the apoptosis-specific 85-kDa fragment was monitored by Western blotting.
Fig. 5.3 Dose-dependent effects of celecoxib and DMC on the kinase activity of Akt in drug-treated HUVECs. Cells were treated with the test reagent at the indicated concentrations for 2 hr. Akt kinase activity in the cell lysates was analyzed as described under the “Materials and Methods.” Values are means ± S.D. (n = 3).  

Fig. 5.4 Effects of celecoxib and DMC on Rb phosphorylation and CDK2 kinase activity of immunoprecipitated cyclin E complexes in HUVECs. (A) Time-dependent inhibition of RbT821 phosphorylation. HUVECs were treated with 20 µM celecoxib (Cele) or DMC for 24 and 72 hr, and were harvested for pRbT821 and total Rb determinations by ELISA, respectively. The pRbT281/total Rb ratio was determined, and expressed as a percentage of the ratio in the vehicle (DMSO)-treated group. (B) Dose-dependent inhibition of the CDK2 kinase activity of immunoprecipitated cyclin E complexes. Cell lysates of HUVECs were treated with cyclin E antibodies, and the CDK2 kinase activity of the immune complex was analyzed. Data point represent means ± S.D. (n = 3). (C) Celecoxib, at 10 – 30 µM, does not affect Thr160 phosphorylation levels of CDK2, excluding the involvement of the CDK-activating kinase (CAK) in inhibiting CDK2 kinase activity.  

Fig. 5.5 (A) Celecoxib at 10 – 30 µM does not affect the expression levels of cyclins, cyclin-dependent inhibitors, and CDKs. HUVECs were treated with 10 – 30 µM celecoxib for 72 h followed by Western blot determination for indicated cell-cycle regulatory proteins. β-Actin served as a loading control for each treatment. (B) Celecoxib and DMC, at 10 – 30 µM, have no major impact on the phosphorylation status of ERKs. HUVECs were treated with both agents at the indicated concentrations for 24 h. Western blot analyses for the respective phosphorylated and total proteins were performed.  

Fig. 6.1 Effect of celecoxib and DMC on neovascularization in the CAM assay. The CAMs of fertile 8-day old white Leghorn chicken eggs were treated celecoxib or DMC at the indicated doses. After 72 h, vascular densities in the CAMs were determined as described under the “Materials and Methods.” Vascular densities in the CAMs were expressed as a percentage of that determined in the vehicle-treated control group. Data points represent the mean ± S.D. for 8 replicates.  

Fig. 6.2 Images of representative CAMs after treatment with 15 nmol of celecoxib or DMC for 72 h.  

Fig. 7.1 Effect of celecoxib and DMC on the growth of established PC-3 tumors in nude mice [1]. Each mouse was inoculated s.c. in the dorsal flank with 5 x 10^5 PC-3 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel. When the tumor reached a volume of approximately 80 mm^3, mice received single daily oral treatments of celecoxib or DMC at 100 and 200 mg/kg body weight/day for the duration of the study. Controls received vehicle consisting of 0.5% methylcellulose.
and 0.1% polysorbate 80 in sterile water. Values are means ± SE ($n = 6$)
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<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<td>PDK-1</td>
<td>3-Phosphoinositol Dependent Kinase-1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 Kinase</td>
</tr>
<tr>
<td>PI45P2</td>
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</tr>
<tr>
<td>PP2A</td>
<td>Protein Phosphatase 2A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
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<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homologue Deleted on Chromosome Ten</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma Protein</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
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CHAPTER 1

INTRODUCTION

1.1 Deregulations of Apoptosis and Proliferation in Cancer

Although cancers are diverse and heterogeneous in their nature, most cancers have at least two aspects in common, suppressed cell death and deregulated cell proliferation, which make interfering of cell survival or proliferation as two important anti-cancer strategies [2-4]. Therefore, cellular components involved in the functions of either keeping cell alive or regulating cell divisions are being extensively investigated. Below is a brief introduction about how cells regulate their survival and proliferation.

1.1.1 Apoptosis

It has been known that survival of all somatic cells requires the continuous signals to suppress apoptosis. Apoptosis, so called program cell death, acts as a central mechanism for a multicellular organism to destroy cells that represent a threat to the integrity of the organism [5-7]. In cancer management, to induce cancerous cells undergoing apoptosis has become a very important strategy [4, 8]. Cells undergoing apoptosis share some basic features, including shrinkage, developing bubble-like blebs on their surface, degraded chromatin in nucleus, releasing of cytochrome c from mitochondria, and
exposing of phosphatidylserine on cell surface [5, 9], which can be identified by modern techniques.

Apoptosis can be triggered by either intrinsic (mitochondria) or extrinsic (death receptor) pathway. Both pathways share the same central effectors, caspases, which carry out the cleavage of both structural and functional elements of the cell. Caspases are generally inactive, but the cascade of their activations can be initiated through the cleavage of either caspase 9 (intrinsic pathway) or caspase 8 (extrinsic pathway) upon formation of apoptosome or activation of death receptors, respectively [5-7, 9, 10].

For extrinsic pathway, activation of death receptors can be triggered upon binding of their complementary ligands, for example, binding of Fas ligand to Fas [6]. On the other hand, formation of apoptosome is required for the cleavage of caspase 9 as well as its subsequent activation, and it is constituted by three components, cytochrome c, protease activating factor-1 (Apaf-1), and caspase 9 [6, 7, 11]. Research suggests that release of cytochrome c from mitochondria is critical for the formation of apoptosome. Further, so-called anti-apoptotic proteins, including Bcl-2 and Bcl-xL can block the release of cytochrome c and abort the apoptotic response, and pro-apoptotic proteins, like BAD, is known to antagonize this mode of action [6, 11].

1.1.2 Regulations of Cell Cycle

Cell proliferation can be viewed as a continuous progression of cell cycle [12, 13]. It has been well known that loss of cell cycle control can lead to undesired cell divisions and eventually tumorigenesis [4, 14-16]. Progress of cell cycle can usually be described as interphase and mitosis phase (M phase). During interphase, the cell focuses on RNA and
protein synthesis and growing in size. Basically, this is the time period for a cell to prepare all the required material for cell division. Interphase can be divided into 4 steps: Gap 0 (G0), Gap 1 (G1), S (synthesis) phase, Gap 2 (G2). They are briefly described below [12, 13, 17, 18]:

G0: This phase indicates a cell leave the cell cycle and quit dividing. It can be a temporary or permanent period. In neuronal system, most cells enter this phase when they reach the end stage of development.

G1 phase: At this stage, a cell increases in size, synthesizes RNA and protein to ensure enough material for entering S phase. There is an important cell cycle control mechanism, so called G1 checkpoint at this stage. This surveillance mechanism ensures that everything is ready for DNA synthesis, which is the major task in S phase.

S Phase: A cell has to duplicate the entire genomic DNA at this stage in order to produce two similar daughter cells at M phase.

G2 phase: After DNA synthesis is done, a cell enters this stage, continues to grow, and produces new proteins in preparation of cell division. Another checkpoint control (G2 checkpoint), which is to determine whether the cell is ready to enter M phase, is found at the end of this stage.

M Phase: A cell stops to grow at this phase and focuses on the cell division. Once completed, two similar daughter cells are formed. There is also a checkpoint (metaphase checkpoint), which ensures that the cell is ready for cell division, found at this phase.

From mechanistic point of view, cell cycle progression is governed by the oscillation of activities of cyclin-dependent kinase (CDKs), which in turn are tightly controlled by synthesis and degradation of their regulatory partners, cyclins and CDK inhibitors.
(CDKIs) [17, 19]. In general, when cyclins are degraded through ubiquitin-proteasome system, the corresponding CDKs are inhibited. In contrast to cyclins, CDKs are active when CDKIs are decomposed through ubiquitin-proteasome system or dissociate from cyclin-CDK complex [20, 21]. Precise regulation of CDK activities is one of the central mechanisms for checkpoint control, which can manage uni-directional transitions from one cell cycle stage to the next stage (Fig.1.1). Activation of CDKs also requires a phosphorylation step at conserved threonine residue at position 160, which is carried out by CDK7/cyclin H complex [12, 18, 19].

G1 checkpoint is briefly introduced below, since it was involved extensively in part of my studies (Fig. 1.2). Upon stimulation by growth factors, D type cyclins (cyclin D) are synthesized and are responsible for regulation of the first checkpoint at G1/S [12, 15, 18]. CDK4 is the main partner for these cyclins. Activated CDK4/cyclin D complex can phosphorylate Rb protein. This step together with subsequent activation of CDK2 can cause hyperphosphorylation of Rb, which is responsible for releasing of E2F transcription factor. Activation of E2F allows the cell to activate genes necessary for DNA synthesis. Cyclin E, which is expressed towards the end of G1, is believed to act after cyclin D. Cyclin E is rapidly degraded after the cell enters S phase and CDK2 is released to form complex with cyclin A [12, 15, 18].

CDKIs are known to inhibit CDKs activity and therefore play an important role in checkpoint control. CDKIs can be divided to two different classes [14, 15, 21]. The first class comprises p21, p27 and p57 which preferentially bind to CDKs in G1 and S phase. They inhibit CDKs through blocking of ATP binding site. The second class of CDKs, referred to as the INK4 family, includes p15, p16, p18 and p19. These inhibitors act on
CDK4/cyclin D complex, and inhibit CDK4 through competition with cyclin D [14, 15, 21].

1.2 Cyclooxygenase 2 (COX-2)

1.2.1 Cyclooxygenase

The cyclooxygenases (COX) utilize arachidonic acid (AA) as their substrate and convert it to prostaglandin H2 (PGH2) (Fig.1.3)[22]. AA is generated by phospholipase A2 and is further oxidized by COX. The downstream isomerases can convert PGH2 to prostacycline, prostaglandins, or thromboxane A2, which play a vital role in multiple physiologic and pathologic processes. Two isoforms, COX-1 and COX-2, are well known. COX-1 is believed to constitutively express in most tissues and is important in physiologic processes such as gastric and renal protection and platelet function. COX-2 is thought to be inducible and known to be up-regulated in inflammation, angiogenesis and neoplasia [22-24]. The third isoform, COX-3 is recently identified as splice variant derived from COX-1[25].

1.2.2 COX-2 and Cancer

A substantial body of research has attributed an important role for the enzyme, COX-2, and its products in cancer development, cancer cell growth and cancer cell survival. This research has included genetic studies in mice that implicate the involvement of COX-2 in carcinogenesis [26]. It also has been demonstrated that prostaglandins and other COX-2-generated downstream mediators promote tumor cell proliferation and survival in an autocrine and/or paracrine manner [27-31]. Furthermore, knockout of the
COX-2 gene suppressed tumorigenesis in mice with a genetic predisposition for polyp formation [26]. The role of COX-2 in enhancing angiogenesis also has been reported. Over-expression of COX-2 induces production of VEGF, PDGF, bFGF and TGF-β, which subsequently promotes vascular permeability, endothelial cell proliferation and migration[32-35].

1.2.3 Celecoxib: COX-2 Inhibitor as an Anti-cancer Agent

Epidemiological and clinical studies indicating that the use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs), which are known to inhibit COX enzymes, reduces the risk of colorectal cancer by 40–50%[36]. As a result, drugs that selectively inhibit COX-2 and were originally developed as anti-inflammatory agents and for the treatment of arthritis, such as Celebrex® and Vioxx®, have been investigated for possible anti-cancer activities. Celecoxib, the active entity in Celebrex®, has been most successful in this regard and is approved by the FDA for use as a preventive agent in patients with a genetic predisposition for colon cancer [37-39].

1.3 The Involvements of Non-COX-2 Components in Celecoxib Mediated Anti-Cancer Effects

The signaling mechanism utilized by COX-2 inhibitors to mediate apoptotic death in cancer cells has been the focus of many investigations [40-47]. However, several reports support that a COX-2-independent mechanism may be involved in the antitumor effect of COX-2 inhibitors. For instance, sulindac metabolites, which do not inhibit COX activity, induced apoptosis in prostate cancer cells with high potency[48]. In contrast to
gastrointestinal cells and PC-12 cells, COX-2 overexpression in immortalized human umbilical vein endothelial, HEK-293, COX-7, and NIH 3T3 cells led to increased cell death and/or cell cycle arrest[49, 50]. Moreover, malignant transformation in embryonic fibroblasts was reported to be independent of the status of COX expression [47].

Our initial studies on the mechanism by which celecoxib induces apoptosis in prostate cancer cells led us to hypothesize that celecoxib’s effects are independent of COX-2[51], and involve the inhibition of cell signaling pathways that are critically important to cancer cell survival and proliferation. In addition, the nature of rapid induction of apoptosis was unique to celecoxib because the potency of inducing apoptosis of other COX-2 inhibitors, including rofecoxib (Vioxx®), NS398, and DuP697, was much lower than that of celecoxib [52]. This discrepancy again indicates differences in the mechanisms by which these COX-2 inhibitors mediate apoptosis in prostate cancer cells. The objectives of my research were to elucidate these novel mechanisms and to facilitate the development of novel anti-cancer agents that are optimized to inhibit these newly identified targets.

1.4 PI3K/PDK-1/Akt Signaling Pathway

Previously, we demonstrated that celecoxib induced cell death in prostate cancer cells (Fig.1.4) accompanied with interruption of multiple signaling targets including Akt, ERK2, and endoplasmic reticulum Ca^{2+}-ATPases[51, 52]. Interfering with Akt signaling pathway (Fig. 1.5) is particularly noteworthy among them, since we have demonstrated that transient over-expression of activated Akt can partially protect PC3 cells from celecoxib induced apoptosis (Fig. 1.6)[51].
PI3K/PDK-1/Akt signaling pathway has been investigated extensively for its roles in tumorigenic transformation [53-55]. Early studies supported that this pathway plays a key role in prevention of apoptosis. However, many lines of evidence have also suggested a correlation between this pathway and regulation of cell cycle progression [53]. The PI3K/PDK-1/Akt signaling is one of the major pathways downstream of receptor tyrosine kinases (RTKs) (Fig. 1.7) [56]. Upon ligand binding to extracellular domain of RTKs, RTKs become dimerized, phosphorylated and activated. Class IA PI3K p85 regulatory unit binds to phosphorylated tyrosine, and PI3K becomes fully activated. PI3K utilizes phosphatidylinositol 4, 5-bisphosphate (PI45P2) as substrate and produces phosphatidylinositol 3, 4, 5-trisphosphate (PI345P3), which in turn serves as membrane tethers for proteins with pleckstrin homology (PH) regions, such as PDK-1 and Akt. PDK-1 is the direct upstream kinase for Akt. Binding to membrane PI345P3 provides a close contact between PDK-1 and Akt, which eventually leads to Akt phosphorylation at Thr308. Akt is further phosphorylated at Ser473 by a yet to be defined kinase, Also called PDK-2, and gets fully activated.

Akt regulates its downstream proteins, many of which are involved in apoptosis and cell proliferation, through adding phosphoryl group on them (Fig. 1.7)[56-58]. For example, Akt phosphorylates apoptotic protein BAD, which leads to dissociation of BAD from Bcl-2 and Bcl-xL and consequently restores the anti-apoptotic function of Bcl-2 and Bcl-xL. Akt inactivates forkhead family of transcription factors (FKHR) by phosphorylating and consequently sequestering them in cytoplasm as a result of binding to 14-3-3 proteins. Since FKHR is a nuclear transcription factor that stimulates the transcription of apoptotic proteins, such as Fas ligand, the apoptotic potential of a cell can

8
therefore be lowered down by Akt. Transcriptional factor NF-κB, which can transcribe anti-apoptotic proteins including Bcl-xL and FLIP, a caspase-8 inhibitor, is usually sequestered in cytoplasm by binding to inhibitory protein IκB. Akt regulates NF-κB activity through phosphorylating IKKα/β, which thereafter dissociates IκB from NFκB and allows NFκB to enter the nucleus for positively regulating anti-apoptotic proteins transcription.

Akt can positively regulate cell cycle progression through multiple mechanisms [56-58]. For instance, Akt can stabilize cyclin D1 protein levels through negatively regulating glycogen synthase kinase 3(GSK3). GSK3 phosphorylates cyclin D1, which is a critical step for cyclin D1 degradation via ubiquitin-proteosomal degradation system, and therefore facilitates decomposition of cyclin D1. Akt can modulate the function of p27 through either direct phosphorylation, which reduces nucleus p27 levels by sequestering it in cytosol, or indirect transcriptional down regulation through inhibiting FKHR. Akt can also phosphorylate mTOR, which in turn is activated and subsequently stimulates the translation of proteins important for cell cycle progression, such as cyclin D1.

Several phosphatases, including phosphatase and tensin homologue deleted on chromosome ten (PTEN), SH2 domain-containing phosphatase (SHIP1/2) and protein phosphatase 2A (PP2A), can negatively regulate PI3K/PDK-1/Akt signaling[59-61]. In brief, PTEN counters the action of PI3K by dephosphorylating PI345P3 at the 3-position. SHIP attenuates PI3K signaling by converting PI(3,4,5)P3 to PI(3,4)P2 or PI(4,5)P2 to PI4P. PP2A directly dephosphorylates Akt and as a result, causes Akt inhibition.
1.5 Cancer Models

1.5.1 Prostate Cancer

Prostate cancer (PCa) is the most common type of cancer for men in America. It is also the second leading cause of cancer death in men [62]. The American Cancer Society estimates that there would be about 230,900 new cases of PCa in the United States in 2004 (www.cancer.org). About 29,900 men would die of this disease. Treatment of PCa depends very much on the stage of disease. For PCa localized in the prostate, treatment options can be surgery, radiation therapy and cryotherapy [63-67]. As PCa metastasizes, the use of hormonal therapy options has gained widespread currency [68-71]. Hormone therapy may involve surgical castration (bilateral orchiectomy) or medical castration with luteinizing hormone-releasing hormone analogs (LHRH analogs). LHRH analogs have also been used in combination with androgen receptor (AR) antagonists, which is called combined androgen blockade (CAB). However, for patients who do not respond to or eventually become resistant to androgen deprivation treatment, second-line hormonal interventions, including withdrawal of anti-androgens, second anti-androgen and suppression of adrenal androgen, are applied [72-74]. But all of them have lower response rates, shorter durations of effect and greater toxicity than the first-line therapies. Chemotherapy has not been recognized as an effective treatment until very recently [75, 76]. Unfortunately, these options are solely palliative. Therefore, it is very important to develop therapeutic agents with novel mechanisms.

In order to find out a novel therapy for prostate cancer, we previously have used human prostate cancer cell lines, PC3 and LNCap cells, as our cell culture models [51, 52]. In this study, both cell lines were still used extensively.
1.5.2 Model of Anti-Angiogenesis

The delineation of the malignant epithelium as a cellular target for COX-2 inhibition is based on the expression of COX-2 in a wide variety of human epithelial malignancies. COX-2, however, is also expressed in the tumor microvasculature, indicating an angiogenic role for COX-2-derived prostaglandins in the support of tumor growth, and suggesting the tumor stroma as a target for COX-2 inhibition [77, 78]. To test whether the action of celecoxib involves modification of angiogenic activity, human umbilical vein endothelial cells (HUVECs) were selected for in vitro model based on NCI’s suggestion (NCI Angiogenesis Resources Center: http://dtp.nci.nih.gov/aa-resources/aa_index.html). To evaluate results obtained from HUVECs, chicken chorioallantoic membrane (CAM) assay was performed for testing the in vivo efficacy.
Fig. 1.1 Cell cycle distribution [53].

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Fig.1.2 Illustration of G1/S transition and the key regulatory components. “↓” indicates stimulation, and “─” indicates inhibition.
Fig. 1.3 Synthesis of prostanoids by cyclooxygenase [22].
Fig. 1.4 PC3 and LNCaP cells are susceptible to the induction of apoptosis by celecoxib in a dose- and time-dependent manner. The incubations were carried out in serum-starved RPMI 1640 medium. Cell death was determined by using trypan blue exclusion [51].
Fig. 1.5 Celecoxib down regulates phospho-Akt.

LNCaP and PC3 cells were treated with DMSO vehicle (control) (A) or celecoxib (50 µM) (B) in serum-free medium for the indicated time. The supernatants were probed by Western blot with rabbit anti-Akt and anti-P-473Ser Akt antibodies [51].
Fig. 1.6 Activated Akt protects PC3 cells from celecoxib-induced cell death. A, Transient expression of myristoylated Akt (MyrAkt) in PC3. The transfectant showed highest level of MyrAkt expression at 48 h, and were collected for the cell viability analysis. B, Viability of PC3 cells overexpressing MyrAkt in the absence of celecoxib (a), PC3 cells overexpressing MyrAkt in the presence of 50 μM celecoxib (b), and PC3 cells transfected with the control pCMV vector in the presence of 50 μM celecoxib (c)[51].
Fig. 1.7 PI3K/PDK-1/Akt signaling pathway [56]
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CHAPTER 2

CELECOXIB INDUCED APOPTOSIS IN PC3 CELL IS COX-2 INDEPENDENT

My first task was to determine what the role of COX-2 is in celecoxib induced apoptosis in prostate cancer cell lines. I used androgen independent prostate cancer cell line, PC3, for this study. Since PC3 endogenously expresses high levels of COX-2, it is possible to significantly alter COX-2 expression levels through genetic approach. In this case, I transfected antisense COX-2 cDNA construct under the control of a tetracycline-inducible promoter to parental PC3 cells, and stable clones were saved for further testing their sensitivity to celecoxib.

2.1 Isolation and Characterization of Tet-On Antisense COX-2 Clones

Four clones, 2F6, 1F2, 3D9, and 7D9, with differential COX-2 basal levels were chosen (Fig. 2.1B). The COX-2 level of clone 2F6 was undetectable by Western blot. Clone 1F2 showed similar levels of COX-2 in comparison with parental cells. Clone 3D9 and 7D9 remarkably possess higher COX-2 levels. Upon supplement of doxycycline (2 µg/mL) to cell culture medium, COX-2 protein levels appeared to reduce by 1 day, and
were virtually depleted by 4 days after the addition of doxycycline (Fig. 2.1A and B). It is notable that COX-1 expression was undetectable by Western blot analysis in both parental cell lines and Tet-On subclones (data not shown), and COX-2 was considered the major producer of prostaglandins. The levels of PGE₂ were carefully examined by ELISA kit (R&D), and they perfectly reflected the levels of COX-2 in each clone (Fig. 2.1C).

### 2.2 COX-2 Ablation dose not Alter the Effect on Apoptosis Triggered by Celecoxib in PC3 Cells

We first tested the apoptotic inducing activities of celecoxib on these four clones. Although COX-2 levels varied in these clones, we found that they were equally susceptible to apoptosis induced by celecoxib (Fig. 2.2). Importantly, this susceptibility was not altered after COX-2 depletion induced by doxycycline. Further, unlike celecoxib treated clones, all clones remained viable after doxycycline induced COX-2 depletion. As shown in Fig. 2.2A, the time course of cell death in the presence of 50 µM celecoxib in PC3 cells, COX-2-deficient 2F6 cells, and COX-2-overexpressing 7D9 cells with and without COX-2 depletion, the time required for 50% cell death (T₁/₂) of all the clones incubated with 50 µM celecoxib was approximately 2 hours. Similar results were observed with 1F2 and 3D9 clones. The evidence strongly suggests that the effect of celecoxib on apoptosis was independent of their COX-2-inhibitory activity.

Previously, we reported that celecoxib induced rapid apoptotic death in both LNCaP and PC3 prostate cancer cells by interfering the Akt signaling pathways[51]. In this study,
the apoptotic death induced in all four clones was also associated with decreased phosphorylation of Akt, as observed in parental PC3 cells, and the time course for the dephosphorylation of Akt in 7D9 cells (Fig. 2.2B) was consistent with that for cell death. Similar results were obtained with the three other clones. This result again highlights the significance of Akt signaling, in contrast to COX-2, in celecoxib induced apoptosis.

2.3 Structure-Activity Analysis Reveals no Correlation between COX-2 Inhibition and Apoptotic Inducing Effect

We further performed structural modifications of celecoxib to dissociate COX-2 inhibition and the induction of apoptosis. Dr. Xueqin Song synthesized a series of celecoxib derivatives with different substituents at the terminal phenyl ring and examined the apoptosis-inducing potency of each. Fig. 2.3 summarizes the structures, the COX-2 inhibitory activity ($IC_{50}$ = concentration of drug inhibiting COX-2 activity by 50%), and the apoptosis-inducing activity ($T_{1/2}$) of celecoxib and seven representative analogues. The structure-activity analysis found no correlation between the COX-2 inhibitory and apoptosis-inducing activities.

Importantly, compound 6 and DMC appeared to show high apoptotic activities ($T_{1/2} \leq 2\ h$) but had no COX-2 inhibitory activity ($IC_{50} > 100 \ \mu M$). Compound DMC is particularly interesting to us. With only slightly structural modification from celecoxib, it loses COX-2 inhibitory activity, but possesses slightly higher apoptotic activity than celecoxib. The time- and dose-dependent effect of compound DMC on cell viability is shown in Fig. 2.4A. PC3 cells treated with 50 $\mu M$ compound DMC resulted in a 50% decrease in cell viability within an hour, compared to 2 hours with celecoxib. We further
demonstrated PC3 cells treated with compound DMC showed significant apoptotic features, which are reminiscent to its derivative, celecoxib. Fig. 2.4B shows pronounced alterations in morphology and membrane compositions after treatment with compound DMC. As shown by phase-contrast microscopy, treated cells shrunken, rounded, and detached from the dish, and bleb formation was evident 1 hour after compound DMC was added (left panel). Morphologic evidence of apoptosis was assessed as nuclear fragmentation detected by staining cells with DAPI, and cells treated with compound DMC were found to have condensed and fragmented nuclei (center panel). Another test for apoptosis induced by compound DMC was the externalization of phosphatidylserine as detected by FITC-conjugated annexin V and fluorescence microscopy (right panel). Among other parameters, degradation of DNA to nucleosomal fragments and cleavage of PARP to the apoptosis-specific 85-kDa fragment are also well characterized events of apoptotic cell death (Fig. 2.4 C and D).

It is noteworthy that the mechanism used by compound DMC to facilitate apoptosis was the same mechanism used by the parent compound celecoxib, i.e., concurrent dephosphorylation of Akt. Fig. 2.6 illustrates the effect of compound DMC on the phosphorylation status of Akt in PC3 cells, which was reminiscent of that observed with celecoxib.
Fig. 2.1 Characterization of antisense COX-2 PC3 transfectants. (A) Western blot analysis showing the time course of COX-2 depletion in the representative antisense COX-2 clone 3D9 in response to doxycycline. (Results from other clones were similar.) Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum in the presence of doxycycline at 2 µg/mL as indicated. (B) Western blot analysis showing the COX-2 protein levels in parental PC3 cells and four independent antisense COX-2 clones in the presence (+) or absence (-) of doxycycline (Dox; 2 µg/mL) for 10 days. (C) Prostaglandin E₂ (PGE₂) production in parental PC3 cells and four antisense COX-2 clones without (-) or with (+) doxycycline pretreatment as indicated. Data are expressed as means ± 95% confidence intervals (error bars) (n = 3). Western blots are representatives of three experiments, all with similar results.
Fig. 2.1
Fig. 2.2 Susceptibility of PC3 cells to celecoxib-induced apoptosis is independent of COX-2 expression levels. (A, left panel) Effect of 50 µM celecoxib on the viability of parental PC3 cells and the COX-2-deficient clone 2F6. (Right panel) Effect of 50 µM celecoxib on the viability of the COX-2 antisense clone 7D9 with (+) or without (-) a doxycycline (Dox) pretreatment (2 µg/mL). Data represent means ± 95% confidence intervals (error bars) (n = 3). (B) Effect of celecoxib on the phosphorylation status of Akt in the COX-2 antisense clone 7D9 with or without doxycycline treatment (2 µg/mL) as indicated. Western blots are representatives of three independent experiments. These data indicate that the mechanism underlying celecoxib-induced apoptotic death in the 7D9 cells remained unaltered after COX-2 depletion.
Fig. 2.2

A

Cell Viability (%)

Time (h)

PC-3

2F6

7D9

+ Dox

- Dox

B

Without doxycycline

With doxycycline

<table>
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Fig. 2.2
Fig. 2.3 Structures and characteristics of celecoxib and its structurally related compounds.

COX-2 = cyclooxygenase-2; IC$_{50}$ = concentration inhibiting COX-2 activity by 50%; T$_{1/2}$ = time required for 50% cell death. IC$_{50}$ values were from ref. [79].

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Fig. 2.4 (A) Dose- and time-dependent effect of compound DMC on the cell viability of parental PC3 cells. Data are the means ± 95% confidential intervals (error bars) (n = 3 to 6). (B) Effect of compound DMC on the morphology and membrane composition of PC3 cells. PC3 cells were grown on coverslips and treated with DMSO vehicles (upper panels) or 50 µM compound DMC (lower panels) for 1 - 2 h as indicated below. (Left panel) Phase-contrast micrograph of PC3 cells 2 h after treatment. (Center panel) Nuclear fragmentation viewed after DNA staining with 4’,6-diamidino-2-phenylindole (DAPI) and using fluorescence microscopy 2 h after treatment. Condensed and fragmented nuclei (arrow) were observed in drug-treated cells. (Right panel) Detection of annexin V binding to the surface of apoptotic cells by fluorescence microscopy 1 hour after treatment. Note that some blebbing cells show strong annexin V labeling of their surface. (C) Time course of the formation of nucleosomal DNA in PC3 cells treated with DMSO vehicles or compound DMC (50 µM). The formation of nucleosomes was quantitatively measured by Cell Death Detection ELISA with lysates equivalent to 10^4 cells for each assay. Data are the means ± 95% confidence intervals (error bars) (n = 3). (D) Induction of poly(ADP-ribose) polymerase (PARP) cleavage by compound DMC in PC3 cells. PC3 cells were treated with 50 µM compound DMC as indicated. PARP proteolysis of the 116-kDa native enzyme to the apoptosis-specific 85-kDa fragment was monitored by Western blotting.
Fig. 2.4
Fig. 2.5 Time-dependent effect of compound DMC on the phosphorylation status of Akt in PC3 cells. The Western blots are representatives of three independent experiments, all with similar results.
CHAPTER 3

PDK-1 ACTS AS AN IMPORTANT MOLECULAR TARGET FOR CELECOXIB

3.1 Research on the Cause of Phospho-Akt Downregulation

After demonstrating COX-2 is not required in inducing excessive apoptosis in PC3 cells, one of the most crucial tasks at that time was to find out what the molecular target is for celecoxib. According to the previous work in our laboratory [51, 52], the disruption of Akt signaling plays an important role in celecoxib induced apoptosis. Therefore, I focused on examining molecular mechanisms involved in such event, phospho-Akt downregulation. At the time, that PI3K activity was not affected by celecoxib was previously reported in our laboratory, and phospho-ERK was demonstrated to be down regulated simultaneously with phospho-Akt reduction [51]. Therefore, first, I tested the possibility that celecoxib continuously promotes an unidentified phosphatase activity (Fig. 3.1).

A simple pharmacological approach was performed to explore this probability. I tried to apply a couple of commercially available phosphatase inhibitors to PC3 cells before or after they had been treated with celecoxib. Ideally, if an inhibitor, which can increase the phospho-Akt level, could be found, it probably can antagonize the action of celecoxib.
and therefore rescue PC3 cells from apoptosis induced by celecoxib. One inhibitor, calyculin A, was found to significantly increase phospho-Akt levels. Calyculin A is a phosphatase inhibitor which inhibits Protein Phosphatase Type 2A (PP2A) and Protein Phosphatase 1 (PP1) with IC50 of 0.5 - 1.0 nM and 2 nM, respectively. Unfortunately, this reagent itself killed PC3 cells, and makes it impossible to perform rescue experiments.

However, one experiment was performed to examine whether the upstream kinase activity of Akt is affected by celecoxib. PC3 were pretreated with compound DMC for different time durations and followed by applying calyculin A to the cells for an hour. The cells were harvested, and phospho-Akt level was determined by Western blot analysis (Fig. 3.3). The result indicated that the upstream kinase activity of Akt was rapidly diminished after challenging by DMC regardless of blocking phosphatase activity by calyculin A. Since PI3K activity is not affected, PDK-1 becomes a promising target for celecoxib.

### 3.2 PDK-1 is One of the Major Targets

This hypothesis was confirmed by Arico et. al. in 2002[80]. The authors presented that celecoxib induced apoptosis in colon cancer HT-29 cells accompanied with reduction of Phospho-GSK-3β and phospho-Akt levels. They were able to demonstrate that celecoxib suppressed PDK-1 kinase activity in in vitro kinase assay. Furthermore, over expression of constitutively active PDK-1 reduced 65% of celecoxib-induced apoptosis compared to empty vector. We therefore performed in vitro PDK-1 kinase assay, and confirmed that celecoxib inhibited PDK-1 with IC50 of 48 µM [81]. These results
together with the data published by Arico et al. reveal that PDK-1 is an important target for celecoxib.
Fig. 3.1 Simple scheme shows how phospho-Akt level can be regulated. PDK-1 activates Akt by adding phosphoryl group on threonine residue, and Akt is subsequently phosphorylated by yet unidentified PDK-2 and get fully activated. The phosphatase can decrease Akt activity by removing the phosphoryl group from it.
Fig. 3.2 Scheme illustrates how the inhibitor of phosphatase may rescue the apoptosis promoting effect of celecoxib.
Fig. 3.3 Western blot showing the phospho-Akt level in PC3 cells with calyculin A treatment alone as well as with compound DMC pretreated for 1 to 5 hours, followed with exposure of calyculin A for 1 additional hour.
CHAPTER 4

IS PDK-1 THE ONLY NON-COX-2 TARGET FOR CELECOXIB?

Even though, PDK-1 is an important target for celecoxib[80]. PDK-1 may not be only important non-COX-2 target of celecoxib. This question was raised with the observation that different cellular outcomes were obtained between LNCaP and PC3 cells when treated with PI3K inhibitor LY294002. When these cells were treated with LY294002 under serum-free condition, only LNCaP cells underwent significant apoptosis. In order to resolve the underlying molecular mechanisms, we performed a couple of experiments and demonstrated that this discrepancy, at least in part, is due to different basal levels of Bcl-xL between the two cell lines. The results are listed below. This part of work was mostly done by Chih-Cheng Yang and myself. I am grateful of Chih-Cheng’s hard work, which made this work possible.

4.1 LNCaP and PC3 cells Display Differential Susceptibility to PI3K Inhibition-Induced Apoptosis.

Exposure of LNCaP cells to LY294002 (25 μM) in serum-free medium resulted in rapid loss of phospho-Akt (Fig. 4.1A). Apoptosis in LNCaP cells is proved by multiple
lines of evidence, including morphological changes, cell viability, DNA fragmentation, and cytochrome c release from mitochondria (Fig. 4.1B-E). In PC3 cells, the effect of LY294002 on phospho-Akt was similar to that observed in LNCaP cells (Fig. 4.2A). However, these cells did not undergo apoptosis as no apparent change was detected in all apoptotic assays performed (Fig. 4.2E).

This discrepancy not only emphasizes differences in the regulation of apoptosis between LNCaP and PC3 cells, but suggests the existence of a survival mechanism that is independent of PI3K/PDK-1/Akt signaling in PC3 cells. To understand molecular mechanisms behind it, we focused our efforts on BAD, one of the major effectors of PI3K/PDK-1/Akt signaling. As mentioned in introduction, Akt can maintain mitochondrial integrity by phosphorylating BAD, and consequently induces dissociation of BAD from Bcl-2 and Bcl-xL, which are localized on mitochondria membranes [10, 56]. As shown in Fig. 4.3, Western blot analysis indicates that exposure to the PI3K inhibitor in both cell lines led to a decrease in phosphorylated BAD, accompanied by significant change of subcellular localization of BAD, from cytosol to mitochondria. These findings imply that PC3 cells were able to avoid PI3K/Akt inhibition-mediated apoptosis via a yet unresolved mechanism.

4.2 Bcl-xL Overexpression and Apoptosis Resistance in PC3 Cells.

We further examined BAD’s interacting counterparts, Bcl-2 and Bcl-xL in both cell lines in order to understand the mechanism underlying this differential response to BAD activation. Western blot analysis reveals that the ratio of Bcl-xL to BAD in PC3 cells was much higher than that in LNCaP cells (Fig. 4.4A), which is consistent with the finding
reported in the literature [82]. In contrast, the expression level of Bcl-2 was moderately lower in PC3 cells than in LNCaP cells. We therefore hypothesized that Bcl-xL overexpression in PC3 cells protected against apoptotic signals generated from PI3K inhibition.

4.3 Ectopic Bcl-xL Expression Protects LNCaP cells from LY-294002-Induced Apoptosis.

To test our hypothesis, LNCaP cells were transfected with the G418-selectable Bcl-xL expression construct pSFFV-Neo/Bcl-xL-Flag. Three transfected clones (B11, B1, and B3), which exhibited ascending expression levels of ectopic Bcl-xL protein (Fig. 4.4B), were isolated and further tested for the potential impact of Bcl-xL expression level on cellular vulnerability to LY294002. Among these three clones, B3 cells presented lessened endogenous Bcl-2 expression, while that in the other two clones remained relatively unaltered in comparison to untransfected LNCaP cells (Fig. 4.4B). As demonstrated in Fig. 4.5A, the extent of cytoprotection against LY294002 correlated very well with the Bcl-xL expression level among the three Bcl-xL clones. In line with the data obtained with PC3 cells, this differential resistance was attributable to the ability of Bcl-xL to suppress cytochrome c release into the cytoplasm (Fig. 4.5B). As demonstrated in B3 cells, the high level of ectopic Bcl-xL expression completely blocked the release of cytochrome c following LY294002 treatment, thereby rendering the antiapoptotic phenotype.
4.4 Antisense Downregulation of Bcl-xL Reduces the Threshold of LY294002-Mediated Apoptosis in PC3 Cells.

To test whether Bcl-xL is, on the other hand, vital for cytoprotection against LY294002 in PC3 cells, we used antisense oligonucleotide technology to downregulate Bcl-xL in PC3 cells. A phosphothioate oligonucleotide which had been reported with excellent ability to attenuate the expression of Bcl-xL as well as the one with a mismatched sequence [83] were used for transfection in PC3 cells. As presented in Fig. 4.6A, this antisense oligonucleotide reduced the level of Bcl-xL expression significantly in a dose-dependent manner. At high doses (> 2 µM), this antisense oligonucleotide was cytotoxic, perhaps due to the result of Bcl-xL ablation. Nevertheless, at 1 µM, it could sufficiently reduce the Bcl-xL expression to a level comparable to that of LNCaP cells without causing significant cell death. Treatment with 1 µM of antisense oligonucleotide rendered PC3 cells susceptible to LY294002, which induced DNA fragmentation within 2 hours of treatment (Fig. 4.6B). This difference in response to LY294002 was correlated with the respective abilities to maintain the mitochondrial integrity (Fig. 4.6C). As shown, exposure of PC3 cells transfected with the antisense oligonucleotide to LY294002 led to increased cytochrome c release, while no increase in cytosolic cytochrome c was noticed with the mismatch oligonucleotide-treated cells.

Together, these data strongly suggest that basal Bcl-xL expression could explain the discrepancy between LNCaP and PC3 cells in their sensitivity to the apoptotic effect caused by PI3K inhibition.
Fig. 4.1. LY294002 (25 µM)-induced Akt dephosphorylation leads to apoptosis in LNCaP cells. (A) Western blot analysis of the phosphorylation state of Akt. LNCaP cells were treated with DMSO vehicle (left panel) or 25 µM LY294002 (right panel) in serum-free RPMI 1640 medium for the indicated times. (B) change of morphology in LNCaP cells after 24 h treatment. The cells became shrunken, round, and detached from the flask after treated with LY294002. (C) time course of the formation of nucleosomal DNA in LNCaP cells treated with DMSO (open bars) or 25 µM LY294002 (gray bars). The formation of nucleosomes was quantitatively measured by Cell Death Detection ELISA with lysates equivalent to 5 x 10^5 cells for each assay. Data are the means ± S.D. (n = 3). (D) a time-dependent effect of LY294002 on cytochrome c release analyzed by Western blot in LNCaP cells. Cytosolic-specific, mitochondria-free lysates were prepared. An equivalent amount of protein (50 µg) was electrophoresed, and probed with anti-cytochrome c antibodies.
Fig. 4.1
Fig. 4.2. PC3 cells are susceptible to LY294002 (25 µM)-induced Akt dephosphorylation, but resist the induction of apoptosis by preventing cytochrome c release. The experimental conditions were identical to that described in legend of Fig. 4.1, except that PC3 cells were used. (A) Western blot analysis of the phosphorylation state of Akt. (B) morphology of PC3 cells treated or untreated. (C) time course of the formation of nucleosomal DNA in PC3 cells treated with DMSO (open bars) or 25 µM LY294002 (gray bars). (D) a time-dependent effect of LY294002 on cytochrome c release in PC3 cells.
Fig. 4.2
Fig. 4.3. LY294002 treatment facilitates the dephosphorylation and targeting of BAD to mitochondria in LNCaP and PC3 cells. Cells were treated with DMSO vehicle (-) or 25 µM LY294002 (+) in serum-free RPMI 1640 medium for 12 h, and lysed. The cytoplasmic and mitochondrial fractions were isolated, electrophoresed, and probed by Western blot with rabbit anti-phospho-BAD and anti-BAD antibodies, respectively. Actin and cytochrome c oxidase were used as internal reference proteins for the cytoplasm and mitochondria, respectively.
Fig. 4.4 (A) Comparison of the basal expression levels of Bcl-xL, Bcl-2, and BAD between PC3 and LNCaP cells by Western blot analysis. (B) Ascending expression levels of ectopic Bcl-xL in B11, B1, and B3 clones. The band for ectopic Bcl-xL contained a Flag tag (eight amino acids long) from the construct, thus migrating slower than endogenous Bcl-xL.
Fig. 4.5. Ectopic Bcl-xL protects LNCaP cells from LY294002-induced apoptosis by attenuating cytochrome c release in an expression level-dependent manner. (A) Formation of nucleosomal DNA in LNCaP (LN) cells and B11, B1, and B3 clones treated with DMSO (open bars) or 25 µM LY294002 (gray bars) at 12 h (left panel) and 24 h (right panel). The formation of nucleosomes was quantitatively measured by Cell Death Detection ELISA with lysates equivalent to 5 x 10^5 cells for each assay. Data are the means ± S.D. (n = 3). (B) Effect of LY294002 on cytochrome c release in LNCaP cells and the three Bcl-xL overexpressing clones at 24 h. Cells were treated with DMSO vehicle (-) or 25 µM LY294002 (+) for 24 h. Cytosolic-specific, mitochondria-free lysates were prepared. An equivalent amount of protein (50 µg) from individual lysates was electrophoresed, and probed by Western blot with anti-cytochrome c antibodies.
Fig. 4.5

**A**

Nucleosome Formation (OD at 405 nm)

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Treated with DMSO vehicle  Treated with LY294002  Treated with DMSO vehicle  Treated with LY294002

**B**

LY294002

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Cyt. c

[Image of a blot with bands labeled LN, B11, B1, and B3 with and without LY294002]
Fig. 4.6. Antisense downregulation of Bcl-xL sensitizes PC3 cells to LY-294002-induced apoptosis by facilitating cytochrome c release. (A) Treatment of PC3 cells with different concentrations of the antisense oligonucleotide caused dose-dependent downregulation of Bcl-xL protein. No change in Bcl-xL expression was noted with the mismatch oligonucleotide at 1 µM. (B) Reduced Bcl-xL expression by the antisense oligonucleotide enhanced the susceptibility of PC3 cells to the induction of apoptosis, while the mismatch oligonucleotide has no effect on the cell death. (C) Effect of the mismatch and antisense oligonucleotides on LY294002-induced cytochrome c release in PC3 cells at 2 h and/or 4 h. Oligonucleotide-treated PC3 cells were treated with DMSO vehicle (-) or 25 µM LY294002 (+) for 24 h. Cytosolic-specific, mitochondria-free lysates were prepared. An equivalent amount of protein (50 µg) from individual lysates was electrophoresed, and probed by Western blot with anti-cytochrome c antibodies.
Fig. 4.6
CHAPTER 5

INHIBITION OF CDKS IS RESPONSIBLE FOR CELECOXIB INDUCED CELL CYCLE ARREST

In chapter 4, I have discussed that the basal level of Bcl-xL is responsible for different cellular response induced by PI3K inhibition in LNCap and PC3 cells. However, the doubt questioning the specificity of celecoxib raised in the beginning of previous chapter remains unresolved. Since PC3 cells possess high level of Bcl-xL, the cells should theoretically be resistant to the inhibition of PI3K/PDK-1/Akt pathway. However, celecoxib, in contrast to LY294002, induces rapid apoptosis in both cell lines regardless the level of Bcl-xL, indicating that PDK-1 is not the only targets responsible for celecoxib induced apoptotic cell death.

My work using HUVECs as a second modeling system further strengthens this hypothesis. In this part of work, I focused on celecoxib induced cell cycle arrest instead of apoptosis, since the former phenomenon was not only found to occur at pharmacologically attainable concentrations (10 to 20 µM) but also to happen before the occurrence of apoptosis [1, 84]. From a mechanistic perspective, the genomic integrity of the HUVEC system also presents a vastly different intracellular context to examine how celecoxib acts to induce growth inhibition.
5.1 **HUVEC Growth is Inhibited by Celecoxib and DMC.**

We assessed the growth inhibitory effects of celecoxib vis-à-vis DMC and rofecoxib (Vioxx®) on HUVECs according to an NCI standard procedure (Fig. 5.1). Among these three agents, celecoxib and DMC exhibited moderate potency in suppressing HUVEC growth, with IC$_{50}$ of 18 and 15 µM, respectively. The concentration required to attain complete growth inhibition was approximately 30 µM for both agents. In contrast, rofecoxib could only cause partial inhibition of HUVEC growth at concentrations exceeding 50 µM. These data suggest that the in vitro growth inhibitory effects of celecoxib on HUVECs involved COX-2-independent mechanisms.

5.2 **Celecoxib and DMC Inhibit HUVEC Growth by Causing G$_1$ Arrest.**

Both celecoxib and DMC exhibited significant inhibitory effects on the growth of HUVECs between 10 - 20 µM. We obtained two lines of evidence that this growth inhibition was attributable to G$_1$ cell cycle arrest in lieu of apoptosis. First, examinations of DNA fragmentation and PARP cleavage in drug-treated HUVECs indicate that neither agent triggered apoptotic death at 20 µM (Fig. 5.2). Second, cell cycle analyses show a significant accumulation of cells in the G$_0$/G$_1$ phase (Table 1). Relatively, DMC exhibited higher potency than celecoxib in causing G$_1$ arrest, which is consistent with that observed in cell growth.
5.3 Celecoxib and DMC Mediate G1 Arrest through the Inhibition of Multiple Signaling Targets.

Since celecoxib and DMC inhibit PDK-1 activity with IC\textsubscript{50} values of 48 and 38 \( \mu \)M, respectively [1], we first examined the Akt kinase activity from HUVECs treated with celecoxib or DMC over a concentration range of 10 – 50 \( \mu \)M (Fig. 5.3). In line with our observation in PC3 cells [1], celecoxib or DMC treatment of HUVECs reduced intracellular Akt activity in a dose-dependent manner, with IC\textsubscript{50} values of 28 \( \mu \)M and 20 \( \mu \)M, respectively.

To examine whether Akt played an obligatory role in celecoxib-mediated G\textsubscript{1} arrest, we transiently transfected HUVECs with varying levels of a plasmid encoding constitutively active Akt (Akt\textsuperscript{T308D/S473D}). This transient transfection resulted in a good correlation between the Akt kinase activity and the dose of plasmid DNA, resulting in 36% and 75% increases in Akt activity with 7.5 and 15 \( \mu \)g DNA/10\textsuperscript{6} cells, respectively, as compared to mock transfection. However, cell cycle distribution in response to the treatment of 20 \( \mu \)M celecoxib in transiently transfected cells was the same as that of the mock-transfected counterparts (data not shown), suggesting the existence of other cellular target(s) for celecoxib in HUVECs.

As celecoxib inhibits PDK-1 activity through ATP competition [81], a crucial issue that could shed light on alternative targets is the specificity of its kinase inhibition, particularly with respect to CDKs in light of their direct involvement in cell cycle progression. As CDKs phosphorylate Rb to enable cells to progress from G\textsubscript{1} into S phase [85, 86], we analyzed the phosphorylation status of Rb as an indicator of CDK activity in drug-treated HUVECs. Specifically, we evaluated the phosphorylation level of Thr\textsuperscript{821}, a
preferential phosphorylation site for CDK2 [87], by ELISA following exposure of HUVECs to 20 µM celecoxib or DMC for 24 and 72 h. As shown in Fig. 5.4A, a time-dependent decrease in the ratio of pRb<sup>Thr821</sup> to total Rb was observed in both treatment groups. As intracellular CDK2 forms complexes with cyclin E and a number of cell-cycle regulators such as p21 and p27 [88], we examined the effect of celecoxib and DMC on the CDK2 kinase activity of immunoprecipitated cyclin E from HUVECs (Fig. 5.4B). Both agents exhibited significant inhibitory activity on the cyclin E immune-complexes with IC<sub>50</sub> of approximately 10 µM.

This IC<sub>50</sub>, however, was significantly lower than that determined with recombinant CDK2/cyclin E (IC<sub>50</sub>: celecoxib, 26 µM; DMC, 24 µM). This discrepancy might be attributable to the presence of co-regulators in the immune-complex. Alternatively, CDK2 activity might also be modulated through Thr<sup>160</sup> phosphorylation by CDK-activating kinase (CAK) [89]. Thus, we examined the Thr<sup>160</sup> phosphorylation status of CDK2 in HUVECs treated with 10 – 30 µM celecoxib. As shown in Fig. 5.4C, celecoxib at these doses had no appreciable effect on the Thr<sup>160</sup> phosphorylation, excluding the involvement of CAK in celecoxib-mediated CDK2 inhibition.

In addition to CDK2, celecoxib and DMC also inhibited recombinant CDK1/cyclin B1 and CDK4 immune complexes. The estimated IC<sub>50</sub> values for celecoxib and DMC were, respectively, 34 and 22 µM for recombinant CDK1, and 16 and 14 µM for the CDK4 immune complex.

Earlier reports indicate that celecoxib mediated growth arrest by altering the expression levels of various cyclins or CDK inhibitors in different cancer cell systems [90-93]. In light of this transcriptional regulation, we examined the effect of celecoxib at
10, 20, and 30 μM on the expression levels of various cell-cycle regulatory proteins in HUVECs after 72-h exposure, which included cyclins A, B1, D1, and E, p21, p27, CDKs 1, 2, 4, and 6 (Fig. 5A). However, celecoxib treatment did not alter the expression of any of these cell cycle-regulatory proteins, which suggests possible differences in signaling mechanisms governing celecoxib-mediated cell growth inhibition between cancer cells and HUVECs.

To examine whether celecoxib affected protein kinases other than PDK-1 and CDKs, it was submitted to a commercial kinase profiling service (Upstate) to assess its specificity in a panel of kinases, including Akt, CDK7/cyclin H (CAK), IKKβ, PKA, p70S6K, PKCγ, MEK1, MAPK2, PDGFRα, c-RAF, and cSrc. However, none of these kinases was inhibited by 30 μM celecoxib (data not shown).

We previously reported that treatment of PC3 cells with 50 μM celecoxib or DMC under serum-free conditions resulted in rapid dephosphorylation of ERK [51]. However, within the growth inhibitory range of 10 – 30 μM in medium containing 2% serum (as recommended by the HUVEC supplier, Cascade Biologics Inc.), neither agent caused an appreciable change in the level of phospho-ERKs in HUVECs (Fig. 5B). This finding suggests that ERK inhibition does not play a role in celecoxib-induced G1 arrest under these conditions.

In conclusion, the overall data suggests that celecoxib arrested the growth of HUVECs by acting on multiple non-COX-2 targets, including PDK-1 and CDKs, involved in the regulation of cell proliferation.
Fig. 5.1 Dose dependency of the growth inhibitory effect of celecoxib, DMC and rofecoxib in HUVECs. Cells were seeded into 96-well plates (1,500 cells/well) in six replicates in 2% FBS-supplemented medium containing 2% FBS, 1µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 µg/ml heparin. After 24-h incubation, the test agents were added at the indicated concentrations. After 72-h drug exposure, cells were stained with crystal violet, and cell viability was determined. Cell growth over the treatment period was expressed as a percentage of that in the vehicle (DMSO)-treated group. Data point represents means ± S.D. Experiments were performed at least 3 times.
Table 5.1 Cell cycle phase distribution of HUVECs treated with celecoxib or DMC at the indicated concentrations in the same medium conditions listed in the legend of Fig. 5.1 for 48 h. Control cells received DMSO vehicle. Each tabulated percentage represents the average of three independent experiments. Student t test reveals that all values presenting $G_0/G_1$ phase in celecoxib or DMC treatment group are statistically different from that of DMSO control ($P<0.05$). Intra group comparison in celebrex treatment group shows the value of 25 $\mu$M ($69.3 \pm 3.8$) is statistically different ($P=0.045$) from the one of 10 $\mu$M ($62.55 \pm 5.7$). In DMC group, the value of 25 $\mu$M statically differs from 10 $\mu$M ($P=0.0034$) and 15 $\mu$M ($P=0.0066$), respectively.

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Fig. 5.2 Dose-dependent effects of celecoxib and DMC on HUVEC apoptosis.

HUVECs were exposed to varying concentrations of celecoxib or DMC for 24 h. (A) Quantitative measurement of the formation of nucleosomes by the Cell Death Detection ELISA. O.D. = optical density. Values are means ± S.D. (n = 3). (B) Induction of PARP cleavage by celecoxib. PARP proteolysis to the apoptosis-specific 85-kDa fragment was monitored by Western blotting.
Fig. 5.3 Dose-dependent effects of celecoxib and DMC on the kinase activity of Akt in drug-treated HUVECs. Cells were treated with the test reagent at the indicated concentrations for 2 hr. Akt kinase activity in the cell lysates was analyzed as described under the “Materials and Methods.” Values are means ± S.D. (n = 3).
Fig. 5.4 Effects of celecoxib and DMC on Rb phosphorylation and CDK2 kinase activity of immunoprecipitated cyclin E complexes in HUVECs.  (A) Time-dependent inhibition of Rb\textsuperscript{T821} phosphorylation. HUVECs were treated with 20 µM celecoxib (Cele) or DMC for 24 and 72 hr, and were harvested for pRb\textsuperscript{T821} and total Rb determinations by ELISA, respectively. The pRb\textsuperscript{T821}/total Rb ratio was determined, and expressed as a percentage of the ratio in the vehicle (DMSO)-treated group.  (B) Dose-dependent inhibition of the CDK2 kinase activity of immunoprecipitated cyclin E complexes. Cell lysates of HUVECs were treated with cyclin E antibodies, and the CDK2 kinase activity of the immune complex was analyzed. Data point represent means ± S.D. (n = 3).  (C) Celecoxib, at 10 – 30 µM, does not affect Thr\textsuperscript{160} phosphorylation levels of CDK2, excluding the involvement of the CDK-activating kinase (CAK) in inhibiting CDK2 kinase activity.
Fig. 5.5 (A) Celecoxib at 10 – 30 µM does not affect the expression levels of cyclins, cyclin-dependent inhibitors, and CDKs. HUVECs were treated with 10 – 30 µM celecoxib for 72 h followed by Western blot determination for indicated cell-cycle regulatory proteins. β-Actin served as a loading control for each treatment. (B) Celecoxib and DMC, at 10 – 30 µM, have no major impact on the phosphorylation status of ERKs. HUVECs were treated with both agents at the indicated concentrations for 24 h. Western blot analyses for the respective phosphorylated and total proteins were performed.
CHAPTER 6

ANTI-ANGIOGENESIS EFFECT OF CELECOXIB AND DMC

Since *in vitro* data suggested the involvement of COX-2 independent mechanisms in celecoxib mediated growth inhibitory effect, we further tested the *in vivo* efficacy of celecoxib and DMC in the inhibition of neovascularization by using a chicken chorioallantoic membrane (CAM) assay.

6.1 CAM assay

The CAMs of fertile 8-day old white Leghorn chicken eggs were treated *in ovo* with 0.15, 1.5 or 15 nmol of celecoxib or DMC, suspended in 15 µl of PBS. Fig. 6.1 shows that, in comparison to the untreated group, DMC reduced vascular densities in the CAMs at all levels tested, while celecoxib caused a significant reduction at the highest dose only ($p < 0.05; n = 8$ for all treatment groups). Fig. 6.2 shows images of representative CAMs after treatment with 15 nmol of celecoxib or DMC. Celecoxib and DMC were effective in inhibiting neovascularization in the CAM assay, though with different potency. The *in vivo* efficacy of DMC appeared to be higher than that of celecoxib, which might be due to its slightly higher potency in inhibiting non-COX-2 targets. The results of CAM in our

62
study do suggest that the non-cox-2 targets of celecoxib might play a role in the process of anti-angiogenesis.
Fig. 6.1 Effect of celecoxib and DMC on neovascularization in the CAM assay. The CAMs of fertile 8-day old white Leghorn chicken eggs were treated celecoxib or DMC at the indicated doses. After 72 h, vascular densities in the CAMs were determined as described under the “Materials and Methods.” Vascular densities in the CAMs were expressed as a percentage of that determined in the vehicle-treated control group. Data points represent the means ± S.D. for 8 replicates.
Fig. 6.2 Images of representative CAMs after treatment with 15 nmol of celecoxib or DMC for 72 h.
CHAPTER 7

Conclusions

To make information more comprehensive, the *in vivo* effects of celecoxib and DMC on PC3 xenograft model which was elegantly demonstrated by my colleagues [1] were briefly discussed here.

7.1 *In Vivo* Effect of Celecoxib and DMC on PC3 Xenograft Tumor Growth.

Oral celecoxib and DMC at two different doses, 100 and 200 mg/kg, were administered daily to nude mice bearing established PC3 xenograft tumors. The effects of individual treatments on tumor growth were assessed (Fig. 7.1). Although, among the four treatments, only the group receiving 200 mg/kg/day DMC displayed a significant effect on the PC3 tumor growth ($P < 0.1$), this result dose emphasize that targeting non-COX-2 components for anti-cancer therapy could be a valid and promising approach.

7.2 Conclusions

In this study, we explored the anti-cancer effect of celecoxib by utilizing prostate cancer cell lines and HUVECs. We concluded that celecoxib exerts its anti-cancer effect in cell culture condition through its ability to interfere with multiple non-COX-2 components, including PDK-1 and CDKs. Most importantly, this unique property of celecoxib has been extensively explored in our laboratory. By utilizing celecoxib as a
lead compound, several compounds structurally similar to celecoxib, including OSU 02067 and OSU 03212, have been synthesized and proved to be very effective in anti-cancer models both in vitro and in vivo [81 and unpublished results], validating the approach of utilizing celecoxib as a platform to develop a novel class of anti-cancer agent.
Fig. 7.1. Effect of celecoxib and DMC on the growth of established PC3 tumors in nude mice [1]. Each mouse was inoculated s.c. in the dorsal flank with $5 \times 10^5$ PC3 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel. When the tumor reached a volume of approximately 80 mm$^3$, mice received single daily oral treatments of celecoxib or DMC at 100 and 200 mg/kg body weight/day for the duration of the study. Controls received vehicle consisting of 0.5% methylcellulose and 0.1% polysorbate 80 in sterile water. Values are means ± SE ($n = 6$).
Fig. 7.1
CHAPTER 8

MATERIALS AND METHODS

8.1 Cells and reagents

8.1.1 Cells and Culturing Conditions

LNCaP, PC3, and Jurkat T cells, used as the source for preparing the CDK4 immune-complex, were purchased from American Type Tissue Collection (Manassas, VA). All cells above were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in a humidified CO₂ incubator. HUVECs were purchased from Glycotech Inc. (Gaithersburg, MD). HUVEC cells were seeded on 0.1% gelatin coated T-75, and were cultured in Medium 200 (Cascade Biologics, Portland, OR) supplemented with Low Serum Growth Supplement (Cascade Biologics) at 37°C in a humidified CO₂ incubator. Final concentrations of the following components in the medium were: fetal bovine serum, 2%; hydrocortisone, 1 µg/ml; human epidermal growth factor, 10ng/ml; basic fibroblast growth factor, 3ng/ml; and heparin, 10 µg/ml. All the experiments were performed with cells between passages 3 and 7. For the drug treatment of HUVECs, test compounds were added in the presence of the Low Serum Growth Supplement.
8.1.2 Reagents

The antisense COX-2 construct was a gift from Drs. Rebecca Chinery and Jason Morrow (Vanderbilt University Medical School). It contained an almost complete human COX-2 insert (1.93 kilobases) that was cloned into the Xbal/EcoRV sites in the TRE (tetracycline response element)-response plasmid pUHD.2neo[94]. This tetracycline-inducible antisense COX-2 construct has been used in colorectal cancer cells to assess the role of prostaglandins in cell proliferation [94]. Celecoxib and rofecoxib were obtained from commercial Celebrex® and Vioxx® capsules, respectively, by solvent extraction followed by recrystallization. Compound 1 to 6 as well as compound DMC were synthesized by Dr. Xueqin Song according to published procedures [79]. LY294002 and calyculin A were purchased from Calbiochem (San Diego, CA).

8.1.3 Antibodies and Recombinant Proteins

Rabbit anti-COX-2 antibodies were obtained from Cayman Chemical Co. (Ann Arbor, MI). Mouse anti-actin monoclonal antibody was from ICN (Costa Mesa, CA). Goat anti-rabbit and anti-mouse IgG-horseradish peroxidase conjugates were purchased from Jackson ImmunoResearch (West Groove, PA). Rabbit anti-poly(ADP-ribose) polymerase (PARP) antibodies were from PharMingen (San Diego, CA). Rabbit anti-Bcl-xL, anti-Akt, anti-phospho-Ser473-Akt, anti-ERK, anti-phospho-ERK, and phospho-Thr160 CDK2 were purchased from Cell Signaling Technology Inc. (Beverly, MA). Primary antibodies against BAD, Bcl-xL, Bcl-2, cyclin A, cyclin B1, cyclin D1, cyclin E, CDK2, CDK4, CDK6, p21, and p27 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz,
CA). Mouse monoclonal anti-cytochrome c was from BD Pharmingen (San Diego, CA). Rabbit anti-CDK1 was obtained from Transduction Laboratories (Lexington, KY).

Recombinant CDK1/cyclin B and CDK2/cyclin E complexes were purchased from Upstate USA, Inc. (Charlottesville, VA). Histone H1 was obtained from Sigma-Aldrich, Corp. (St. Louis, MO), and Rb substrate peptide was purchased from Panvera Inc. (Madison, WI).

8.2 Analysis for Apoptosis

8.2.1 Phosphatidylserine Externalization (results shown in chapter 2)

Approximately 2.5 x 10^5 PC3 cells were grown on glass coverslips for 24 hours. At various times after drug treatment, cells were washed gently with PBS and then exposed to 0.5 mL of binding buffer (10 mM HEPES [pH 7.4], containing 150 mM NaCl, 2.5 mM CaCl₂, 1 mM MgCl₂, and 4% bovine serum albumin), followed by 0.6 mL of annexin V-fluorescein isothiocynate (FITC) (200 µg/mL) for 30 minutes. After washing with binding buffer, apoptotic cells were identified directly as cells with annexin V-FITC on their outer membrane under a fluorescence microscope. In a set of controls, cells received medium containing DMSO vehicle in lieu of the test agent.

8.2.2 4’,6-Diamidino-2-phenylindole (DAPI) Staining of Nuclei (results shown in chapter 2)

At various times after treatment with different test agents, morphologic changes were detected in nuclei of apoptotic cells by staining with the DNA binding fluorochrome
For adherent PC3 cells, cells were grown on glass coverslips until approximately 70% confluent and exposed to the test agent at 50 µM for various times. Supernatants then were carefully removed, adherent cells were washed with PBS, DAPI (0.5 µg/mL) was added in a fixation solution (4% paraformaldehyde, 2 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid], and 13.7% sucrose in PBS), and the mixture was incubated at room temperature for 20 minutes in the dark. Cells were then washed for two 20-minute periods with PBS. Floating PC3 cells were examined by a modification of the above method. PC3 cells were cultured in T-25 flasks and treated with the test agent. Floating cells then were collected, washed, and stained with DAPI as described above. Cells were allowed to attach to poly-L-lysine-coated coverslips and viewed by microscopy at a magnification of 400X.

### 8.2.3 Apoptosis Detection by an Enzyme-Linked Immunosorbent Assay

Induction of apoptosis was also assessed by using a “Cell Death Detection ELISA” assay (Boehringer-Mannheim) by following the manufacturer’s instructions. This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mono- and oligonucleosomes after induced apoptotic death. In brief, cells were cultured in a T-75 or T-25 flask 24 hours before the experiment. Cells were washed twice in 5 mL of serum-free medium and then treated with a test agent or the DMSO vehicle as indicated. Both floating and adherent cells were collected, and cell lysates equivalent to 10^4 cells were used in the ELISA.
8.2.4 Western Blot Analysis of PARP Cleavage.

Drug-treated cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer [20 mM Tris-HCl (pH 8), 137 mM NaCl, 1 mM CaCl₂, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 100 µM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin at 10 µg/mL, and aprotinin at 10 µg/mL]. Soluble cell lysates were collected after centrifugation at 1,500 x g for 5 minutes. Equivalent amounts of protein (50 – 100 µg) from each lysate were resolved in 10% SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-PARP antibodies, as described at section 8.18.

8.2.5 Western Blot Analysis of Cytochrome c Release into the Cytoplasm (results shown in chapter 4)

Cytosolic-specific, mitochondria-free lysates were prepared according to a reported procedure [10]. After individual treatments, both the incubation medium and adherent cells in T-25 flasks were collected, and centrifuged at 200 x g for 5 min. The pellet fraction was recovered, placed on ice, and triturated with 300 µl of a chilled hypotonic lysis solution [220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 1 mM DTT, and a mixture of protease inhibitors consisting of 100 µM AEBSF, 80 nM aprotinin, 5 µM Bestatin, 1.5 µM E-64 Protease Inhibitor, 2 µM Leupeptin, and 1 µM Pepstatin A]. After a 45 min-incubation on ice, the mixture was centrifuge at 200 x g for 10 min. The supernatant was collected in a
microcentrifuge tube, and centrifuged at 14,000 rpm for 30 min. An equivalent amount of protein (50 µg) from each supernatant was resolved in 10% SDS-polyacrylamide gel. Bands were transferred to nitrocellulose membranes, and analyzed by immunoblotting with anti-cytochrome c antibodies, as described at section 8.18.

8.3 Development of PC3 Tet-On Antisense COX-2 Clones

PC3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in T-25 flasks at 37 °C in a humidified CO₂ incubator to 80% confluency. Each flask was washed with 6 ml of serum-free Opti-MEM (Invitrogen Life Technologies; Carlsbad, CA), and then 3 ml of serum-free Opti-MEM was added. Aliquots containing 0.12 µg of the Tet-On regulator plasmid pTet-On (Clontech; Palo Alto, CA) and 0.12 µg of the antisense COX-2 construct in 150 µL of serum-free OPTI-MEM medium were preincubated with 3 µL of the Plus reagent from the LipofectAMINE Plus Reagent kit (Invitrogen Life Technologies) at 25 °C for 15 minutes, followed by 12 µL of the LipofectAMINE reagent in 150 µL of Opti-MEM medium. The resulting mixture was incubated at 25 °C for 15 minutes and then added to each flask with gentle mixing. After 5 hours at 37 °C, the transfection medium was replaced with 5 mL of RPMI 1640 medium containing 10% Tet-System-approved FBS (Clontech). After 48 hours, cells were cultured in fresh medium containing G418 at 100 µg/mL to select for transfected clones. The G418-supplemented medium was changed every 4 days. After 3 weeks, G418-resistant cells were subcloned into 96-well plates by limiting dilution with a final cell density of about 0.5 cell per well. After 12 days with a change of G418-
containing medium every 4 days, viable clones were further subcloned into 12-well plates. After 4 or 5 days, cells in each well were divided into three T-25 flasks. The level of COX-2 expression was determined 120 hours after cells were exposed to doxycycline (2 \( \mu \text{g/mL} \)) by Western blot analysis. By this procedure, the following four independent clones (2F6, 1F2, 3D9, and 7D9) were selected for analyses: 2F6 was a COX-2-deficient clone, and 1F2, 3D9, and 7D9 expressed different levels of COX-2 in the absence of doxycycline.

8.4 Preparation of Bcl-xL-Overexpressing LNCaP Transfectants

The pSFFV-Neo/Bcl-xL-Flag expression plasmid containing the human Bcl-xL cDNA subcloned upstream of the constitutive splenic focus forming virus (SFFV) promoter was kindly provided by Gabriel Nunez (University of Michigan, Ann Arbor, MI). LNCaP cells were culture in 10% FBS-supplemented RPMI 1640 medium in T-25 flasks. At 50% confluence, each flask was washed twice with 4 ml of serum-free Opti-MEM (Invitrogen Life Technologies, Carlsbad, CA), and then added 1.6 ml of Opti-MEM. Meanwhile, the Bcl-xL expression construct (0.1 \( \mu \text{g} \)) in 350 \( \mu \text{l} \) Opti-MEM was preincubated with 3\( \mu \text{l} \) of the Plus reagent form the LipofectAMINE Plus Reagent kit (invitrogen) at room temperature for 15 min, and 6 \( \mu \text{l} \) of the LipofectAMINE reagent in 150 \( \mu \text{l} \) of Opti-MEM was added to the mixture. The resulting mixture was incubated at room temperature for 15 min, and added to each flask with gentle mixing. After 5 h at 37 °C, the transfection medium was replaced with 5 ml of 10% FBS-supplemented RPMI 1640 medium. After 48 h, the transfected cells were transferred into 96-well plates with a
density of approximately 10,000 cells per well, and cultured in the same medium containing 300 µg/ml G418 (San Diego, CA). The G418-containing medium was changed every 4 days. At 80% confluence, they were subcloned into 24-well plates, and grown in the presence of G418. The subcloning procedure was repeated at 80% confluence with 12-well plates, followed by T-25 flasks, from which three independent clones (B1, B3, and B11) expressing differential basal levels of Bcl-xL-Flag were selected.

8.5 Antisense Experiments (results shown in chapter 4)

To attenuate Bcl-xL expression in PC3 cells, we obtained an antisense 2’-O-methyl phosphorothioate “gap-mer” [83] from Integrated DNA Technologies (Coralville, IA): CT*G*CGAt*c*cgac*t*cAC*C*A*A*T, where bases with 2’-O-methyl-modified sugar moieties are given in capital letters, C5-methyl-modified cytosine is represented by small c, and * stands for a phosphorothioate internucleotide bond. PC3 cells were transfected with this antisense oligonucleotide with the Oligofectamine reagent (Invitrogen). A 250 µM-stock solution of the antisense oligonucleotide was prepared in distilled water. Varying amounts of the antisense stock solution, diluted in a final volume of 350 µl with Opti-MEM, was mixed with a solution containing 6 µl of the Oligofectamine and 34 µl of Opti-MEM, and incubated at room temperature for 20 min. The combined mixture was added to a T-25 flask, in which 8 x 10^5 PC3 cells were cultured in 1.6 ml of Opti-MEM. After incubating the cells at 37 °C for 5 h, an equal volume of Opti-MEM medium supplemented with 20% FBS was added to the flask without removing the transfection
medium. The transfected cells were incubated with the medium for another two days before LY294002 treatment.

8.6 Cell viability (Results shown in chapter2)

Parental or transfected PC3 cells with or without doxycycline (2 µg/mL) pretreatment were plated in 12-well plates and cultured in RPMI 1640 medium supplemented with 10% FBS in the absence or presence of 2 µg/ml doxycycline for 48 hours. Various concentrations of compounds dissolved in dimethyl sulfoxide (DMSO; final concentration, 0.1%) were then added to the cells in serum-starved RPMI 1640 medium. Control cells received DMSO vehicle at the same concentration. During treatment, the percentage of floating cells increased over time. At the end of the treatment, adherent cells were harvested by trypsinization, and floating cells were recovered by centrifugation at 3,200 x g for 5 minutes. Cell morphology was assessed with a light microscope at x400. Both adherent and floating cells were combined, and cell viability was assessed by trypan blue dye exclusion.

8.7 Prostaglandin E₂ (PGE₂) immunoassay

Parental and transfected PC3 cells with or without a doxycycline (2 µg/mL) pretreatment were grown to 10 x 10⁶ cells in T-75 flasks in 10% FBS-supplemented RPMI 1640 medium with or without a doxycycline. Culture medium was changed, and 24 hours later conditioned medium was collected to assay PGE₂. Conditioned medium was centrifuged to remove particulate material, and then cells were collected by scraping
to determine the protein concentration. PGE$_2$ was assayed in 100 µL medium in triplicate according to the manufacturer’s instruction (R&D Systems, Inc.; Minneapolis, MN). PGE$_2$ data were normalized to protein content.

8.8 Growth Inhibition Assay (results shown in chapter 5)

The assay was carried out according to a slight modification of the procedure suggested by the NCI Angiogenesis Resources Center (http://dtp.nci.nih.gov/aa-resources/aa_index.html). HUVECs were plated into 96-well plates at 1.5 x 10$^3$ cells/well in 100 µl of the aforementioned medium. After 24 h (day 0), the test compound in 100 µl of the same medium was added to each well at 2X the desired concentration. On day 0, one plate was stained with 0.5% crystal violet in 20% methanol for 10 minutes, rinsed with water, and air-dried. The remaining plates were incubated at 37°C for 72 h, then stained with 0.5% crystal violet in 20% methanol, rinsed with water, and air-dried. The crystal violet stain was eluted with a mixture of ethanol-0.1 M sodium citrate (1 : 1), and absorbance at 540 nm was measured with an ELISA reader (Dynatech Laboratories). Day 0 absorbance was subtracted from the absorbance values of the 72 h samples, and data were plotted as percentage of control proliferation (vehicle-treated cells) to calculate IC$_{50}$ values (drug concentrations that cause 50% inhibition).

8.9 Flow Cytometry for Cell Cycle Analysis

A detergent-trypsin method was used for the preparation of nuclei for flow cytometric DNA analysis [95]. In brief, HUVECs were treated with DMSO or the test agent at the indicated concentration for 24 h. The harvested cells (1 x 10$^6$) were suspended in 500 µl
of 40 mM citrate buffer, pH 7.6, containing 250 mM sucrose and 10% DMSO, and stored at –80 °C until analysis. The cells were centrifuged, resuspended in 500 µl of solution A (3.4 mM trisodium citrate, 0.5 mM Tris, 0.1% NP 40, and 1.5 mM spermine tetrahydrochloride with final pH of 7.4) containing 15 µg/ml trypsin and 10 µg/ml EDTA. After incubating at 37°C for 30 min, 500 µl of solution A containing 0.5 mg/ml trypsin inhibitor and 0.1 mg/ml DNase-free RNase A was added. After another incubation at 37°C for 30 min, 500 µl of solution A containing 0.05 mg/ml propidium iodide and 1.2 mg/ml spermine tetrahydrochloride was added, and incubated on ice for 1 h. Cell cycle phase distributions were determined on a FACScan flow cytometer (Beckman-Coulter, Mountain View, CA).

8.10 Akt Kinase Assay

Akt kinase assay was carried out according to a modified published procedure [96]. Briefly, HUVECs were treated with DMSO vehicle or the test agents at the indicated concentrations for 12 h, then lysed and homogenized in lysis buffer (50mMTris HCl, pH 7.5, 120 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, and 1 µg/ml each of aprotinin, pepstatin, and leupeptin). Cell lysates were centrifuged at 13,000g for 10 min, and the supernatants were collected for the kinase assay. Protein concentrations in the supernatants were determined by the Bradford method (Bio-Rad, Hercules, CA). The kinase assay was performed by adding 10 µl of substrate peptide (RPRAATF; about 80 µM final assay concentration) and 10 µl [γ-32P]ATP (1 µCi/µl) to equivalent amounts of supernatant (20 µg of total protein). After
incubation for 30 min at 30°C, 25 µl of each reaction mixture was slowly spotted onto P81 phosphocellulose paper. After three washes with 0.75% phosphoric acid, the papers were transferred to scintillation vials containing 5 ml liquid scintillation cocktail. Radioactivity was measured in a scintillation counter.

8.11 Transient Transfection in HUVECs by Calcium Phosphate Coprecipitation Method

The constitutively active Akt construct HA-PKB-T308D/S473D was kindly provided by Dr. Brian Hemmings (Friedrich Miescher Institute, Basel). HUVECs were seeded into T-75 flasks (1 x10⁶/flask). Various amounts of the plasmid were added to 450 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.2) followed by the addition of 50 µl 2.5 M CaCl₂ solution (10 mM HEPES pH7.2). After briefly mixing the DNA - CaCl₂ solution, it was added dropwise to 500 µl 2X HBS (280 mM NaCl, 10 mM KCl, 12 mM dextrose, 50 mM HEPES, and 1.5 mM Na₂HPO₄). After 30 min incubation, the mixture was applied to HUVECs directly. The calcium phosphate-containing medium was replaced by normal medium after 6 h incubation, and the transfected cells were harvested after additional 3 days for experiments.

8.12 CDK1/Cyclin B Kinase Assay

The reagents used in this assay were prepared according to the manufacturer’s instructions (Upstate, 14-450). Briefly, for each assay, 10 µl of assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium
orthovanadate, and 1 mM dithiothreitol) containing the test agent at varying concentrations, 10 µl of inhibitor cocktail (20 µM PKC inhibitor peptide, 2 µM PKA inhibitor peptide, and 20 µM compound R24571) and 10 µl of active CDK1/cyclin B (20 ng) were mixed and incubated for 15 min. Then, 10 µl histone H1 (0.5 mg/ml) and 10 µl of diluted [γ-32P] ATP solution were added to the mixture. After reacting at 30°C for 30 min, 25 µl of the mixture was transferred to P81 paper. The paper was washed with 0.75% phosphoric acid three times and acetone once, and put into 5 ml scintillation cocktail. The radioactivity was measured by the scintillation counter.

8.13 CDK2/Cyclin E Kinase Assay

The reagents used in this assay were prepared according to the manufacturer’s instructions (Upstate, 14-475). Briefly, for each assay, 10 µl of reaction buffer (50 mM MOPS, pH 7.0, and 2.5 mM EDTA) containing the test agents at varying concentrations, and 2.5 µl of active CDK2/cyclin E (20 ng) were mixed and incubated for 15 min. Then, 2.5 µl of histone H1 (1 mg/ml) and 10 µl of diluted [γ-32P] ATP solution were added into the mixture. After reacting at 30°C for 30 min, 20 µl of the mixture was transferred to P81 paper. The paper was washed with 0.75% phosphoric acid three times and acetone once, and transferred to 5 ml scintillation cocktail. The radioactivity was measured by the scintillation counter.
8.14 Immunoprecipitated Cyclin E Kinase Assay

Cyclin E immunoprecipitates were prepared from the lysates of HUVECs as follows. HUVECs were collected by scraping from T75 flasks at 80% confluence and resuspended in lysis buffer (50 mM Tris HCl, pH 7.5, 120 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM NaF, 40 mM \(\beta\)-glycerophosphate, and 1 \(\mu\)g/ml each of aprotinin, pepstatin, and leupeptin). After homogenization, the lysates were centrifuged at 13,000 x g for 10 min. The supernatants were collected and diluted with the same buffer at a 1 to 500 ratio. The diluted lysates were then subjected to immunoprecipitation at 4 °C for 2 hr with antibodies (1:100) against cyclin E (Santa Cruz). Protein A-agarose beads were then added followed by an additional 1 hr incubation at the same temperature. The beads with captured immuno-complexes were washed 3 times with lysis buffer and used for the CDK2 kinase assay. The assay was performed as described above for the CDK2/cyclin E kinase assay except for the use of the cyclin E immunoprecipitates instead of the recombinant enzymes and the inclusion of a short centrifugation step to pellet the beads before transfer of supernatants to P81 paper.

8.15 CDK4 Kinase Assay

CDK4 immunoprecipitates were prepared from the lysates of Jurkat T cells as follows. Jurkat T cells (8 x 10^8 cell) were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM NaF, 40 mM \(\beta\)-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 1 \(\mu\)g/ml each of aprotinin, pepstatin, and leupeptin) followed by homogenization and centrifugation at 13,000 g for 10 minutes. The supernatant was incubated with anti-CDK4 antibodies (Santa Cruz) at
4°C for 2 h, followed by addition of protein A-agarose beads for 1 h. The beads with captured immune-complexes were washed 3 times by lysis buffer, 2 times by CDK4 assay buffer (50 mM MOPS, pH 7.0, and 2.5 mM EDTA) and subjected to CDK4 kinase assay. In brief, 5 μl of assay buffer with test agent at the desired concentration and 25 μl of the same buffer containing CDK4 immunoprecipitates were incubated together for 15 min. Then, 10 μl of assay buffer containing Rb substrate peptide (2.5 ng) and 10 μl of the diluted [γ-32P] ATP solution were added into the mixture. The reaction mixture was incubated at 30°C for 1.5 h, followed by brief centrifugation. Thirty μl of the supernatant was carefully transferred to P81 paper. The paper was washed three times with 0.75% phosphoric acid followed by one wash with acetone, and transferred to 5 ml of scintillation cocktail. Radioactivity was measured by the scintillation counter.

8.16 Quantitative Determination of pRbT821 and Total Rb in HUVECs

The levels of phosphorylated Rb and total Rb in cell lysates were determined using Human Rb [pT821] ELISA Kit and Human Total Rb ELISA Kit, respectively (both from Biosource International, Camarillo, CA). HUVEC cells were seeded on 0.1% gelatin coated T-75 flasks one day before treatments. The cells were treated with 20 μM celecoxib or DMC, or DMSO vehicle for 24 or 72 h. For the 24-hr treatment, cells were plated at a density of 1.5 x 10⁶ cells/flask with twelve flasks per treatment group. For the 72-hr treatment, cells were plated at a density of 8 x 10⁵ cell/flask to avoid over confluency with twenty flasks per treatment group. Cells were harvested by scraping, and treated with lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet P-40
(v/v), 1 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of aprotinin, pepstatin, and leupeptin), briefly sonicated, and then centrifuged at 13,000 g for 25 min. The supernatants were collected for immediate analysis of pRb T821 and total Rb contents by the immunoassays. The ELISAs were performed according to the manufacturer’s instructions.

8.17 Chicken Chorioallantoic Membrane (CAM) Assay

The CAM assay was performed in accordance with procedures described by Marks et al. [97]. Eight-day old fertile white Leghorn chicken eggs were obtained from The Ohio State University Department of Animal Sciences. Eggs were candled to ensure fertility and viability of the embryos prior to inclusion in the experiment. A small hole (~1 cm diameter) was made in the shell over the air sac through which treatment solutions were directly pipetted onto the surface of the CAM. Treatments included celecoxib and DMC which were prepared as suspensions in PBS and applied to the CAMs in a total volume of 15 µl containing 0.15, 1.5 or 15 nmol of drug. Controls received 15 µl of PBS only. Each treatment group contained 8 eggs. The holes were then sealed with Micropore surgical tape (3M Health Care, St. Paul, MN) and the eggs were incubated in a humidified egg incubator (Murray McMurray Hatcheries, Webster City, Iowa) at 37°C for 72 hours. After treatment, the area of each CAM to which the treatment agent had been applied was quickly excised and fixed in 4% paraformaldehyde (w/v in PBS). Images of the CAMs were acquired with a digital camera (Nikon Coolpix 990) and visualized for quantitation of vascularity in Adobe Photoshop 6.0 software. Each digital CAM image was overlaid with a 1 x 1-cm grid and the number of blood vessel branchpoints within the grid was counted. Vascular densities in the CAMs were
expressed as a percentage of the blood vessel branchpoints in the vehicle–treated control CAMs.

### 8.18 Western Blot

Drug-treated cells were collected, washed with PBS, resuspended in SDS gel-loading buffer (100 mM Tris–HCl [pH 6.8], 4% [w/v] SDS, 0.2% [w/v] bromophenol blue, 20% [v/v] glycerol, and 200 mM dithiothreitol), sonicated for 5 s, and boiled for 5 min. After brief centrifugation, equivalent amounts of soluble proteins, as determined by the Bradford method, were resolved in 8 to 15% SDS–polyacrylamide minigels, depending on the size of desired protein, and transferred to nitrocellulose membranes with the use of a semidry transfer cell (Bio-Rad). The membranes were washed twice with TBS (0.3% [w/v] Tris, 0.8% [w/v] NaCl, and 0.02% [w/v] KCl) containing 0.05% Tween 20 (TBST) and then incubated with TBS containing 5% nonfat dry milk for 20 minutes to block nonspecific antibody binding. Each membrane was then incubated at 4°C for 12 hours with a primary antibody which was diluted 1 : 1000 in TBS containing 1% nonfat dry milk. The membranes were washed twice with TBST and then incubated at room temperature for 1 hour with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG) diluted 1 : 5000 in TBS containing 1% nonfat dry milk. The membranes were washed twice with TBST, and bound antibody was visualized by enhanced chemiluminescence using ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech, Little Chalfont, U.K.).
Bibliography


89


