CYCLIC GMP PHOSPHODIESTERASE AS A NOVEL MOLECULAR TARGET FOR THE PREVENTION AND TREATMENT OF BREAST CANCER

by

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A DISSERTATION

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Breast cancer remains a major health concern, despite efforts to develop improved therapeutics. Chemoprevention is a promising strategy for reducing breast cancer-related morbidity and mortality. However, with the exception of the selective estrogen receptor modulators (SERMs), which have limited efficacy and severe toxicities, no drugs have been approved for breast cancer chemoprevention. Studies demonstrate that certain nonsteroidal anti-inflammatory drugs (NSAIDs) display promising chemopreventive efficacy. Unfortunately, the depletion of physiologically important prostaglandins due to inhibition of the cyclooxygenase (COX) enzymes results in potentially fatal toxicities, which exclude the use of NSAIDs and COX-2 selective inhibitors for chemoprevention. Ample data, however, suggest that a COX-independent target may be responsible for the anticancer activity of the NSAIDs. Inhibition of cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) and subsequent activation of cGMP signaling has been proposed as one COX-independent mechanism. While cGMP signaling can regulate growth in certain cell types and alterations within this pathway have been noted in human cancers, little is known about its role in human breast cells. Here we demonstrate that selective inhibition of the cGMP specific PDE5 isozyme was sufficient to selectively induce apoptosis of human breast tumor cells through a pathway involving activation of the cGMP-dependent protein kinase (PKG) and subsequent attenuation of oncogenic β-catenin transcriptional activity. Moreover, PDE5 was found to be overexpressed, while
the expression of other PDE isozymes was significantly reduced in breast tumor cells when compared to normal mammary epithelial cells. The NSAID sulindac sulfide (SS) was also found to preferentially inhibit the growth of breast tumor cells through a mechanism involving selective inhibition of PDE5, activation of PKG, and attenuation of β-catenin transcriptional activity. These effects occurred independently of COX inhibition, as novel sulindac derivatives that lacked COX-inhibitory activity demonstrated enhanced anticancer and PDE5-inhibitory activities. These data demonstrate that PDE5 may serve as a novel target for breast cancer chemoprevention. Furthermore, modifying the indene scaffold of sulindac to remove COX-inhibitory activity while enhancing PDE5 inhibitory activity could lead to the development of novel breast cancer chemopreventive agents that are potentially safer and more efficacious than the NSAIDs or the SERMs.
DEDICATION

This dissertation is dedicated to my loving husband, Bill, who is my constant source of encouragement, and to my amazing mother, Wanda, who is my biggest inspiration.

Without their unwavering love and support, none of this would have been possible.
ACKNOWLEDGEMENTS

First, I must acknowledge my mentor, Dr. Gary Piazza, for all of his guidance, support, and, most of all, patience throughout this process. He has shown me how to succeed in science and has provided me with the knowledge to do so.

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TABLE OF CONTENTS

ABSTRACT...........................................................................................................iii
DEDICATION........................................................................................................v
ACKNOWLEDGEMENTS..................................................................................vi
LIST OF TABLES.................................................................................................x
LIST OF FIGURES...............................................................................................xi
LIST OF ABBREVIATIONS..................................................................................xiii

BACKGROUND.................................................................................................1

Breast Cancer .....................................................................................................1
Anticancer Activities of the Nonsteroidal Anti-inflammatory Drugs......................2
Cyclic Nucleotide Signaling ..............................................................................5
  cAMP Signaling..............................................................................................6
  cGMP Signaling..............................................................................................9
  Crosstalk between Cyclic Nucleotide Pathways.............................................10
Cyclic Nucleotide Signaling in Malignant Disease ...........................................11
Cyclic GMP Phosphodiesterase as a Target for the Prevention and Treatment of Cancer .........................................................15

HYPOTHESIS AND AIMS..............................................................................21

MATERIALS AND METHODS........................................................................23

  Drugs and Reagents ......................................................................................23
  Cells and Cell Culture ....................................................................................24
  Growth Assay................................................................................................25
  Caspase Assay................................................................................................25
Introduction ............................................................................................................. 33
Results .................................................................................................................... 34
    cGMP is a proapoptotic and antiproliferative signal in human breast cells .......... 34
Selective inhibition of certain cGMP PDE isozymes selectively inhibits growth and induces apoptosis of breast tumor cells .......... 35
    cGMP PDE inhibition is associated with attenuation of Wnt/β-catenin mediated transcription .......... 38
Breast tumor cells rely heavily on PDE5 for cGMP hydrolysis ............................ 40
    PDE5 is necessary for breast tumor cell growth and survival .......... 41
Discussion ............................................................................................................. 43

SULINDAC SULFIDE SELECTIVELY INHIBITS GROWTH OF BREAST TUMOR CELLS THROUGH INHIBITION OF PDE5, ACTIVATION OF PKG, AND ATTENUATION OF WNT/β-CATENIN MEDIATED TRANSCRIPTION .......... 60

Introduction ............................................................................................................. 60
Results .................................................................................................................... 61
    The anticancer activity of certain NSAIDs is more closely associated with cGMP PDE inhibition than with COX-2 inhibition .......... 61
    SS selectively inhibits growth of breast tumor cells ...................... 61
    Induction of apoptosis by SS is associated with inhibition of cGMP PDE, elevation of intracellular cGMP levels, and activation of PKG .......... 62
    Induction of apoptosis by SS is associated with attenuation of Wnt/β-catenin signaling ...................... 65
Discussion ............................................................................................................. 70
SULINDAC CAN BE CHEMICALLY MODIFIED TO REMOVE COX-INHIBITORY ACTIVITY WHILE IMPROVING PDE5 INHIBITORY ACTIVITY AND ANTICANCER EFFICACY...............................................................83

Introduction...........................................................................................................................................83
Results.....................................................................................................................................................83
Carboxylic acid moiety of SS is necessary for COX-1 and COX-2 inhibitory activity........................................83
Amine derivatives of sulindac demonstrate improved potency for inhibition of PDE5 and enhanced anticancer activity...........85
Discussion...............................................................................................................................................88

CONCLUSIONS AND FUTURE DIRECTIONS......................................................98

APPENDIX A: CHARACTERIZATION OF RECOMBINANT PDE ISOZYMES…120
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Characterization of PDE superfamily.</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Observed alterations in cyclic nucleotide signaling pathways in different types of malignancies.</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Characterization of breast tumor cell lines used for experiments.</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>Sequences of oligonucleotide primers used for PCR amplification of cDNA.</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>PDE isozyme selectivity of trequinsin and MY5445.</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>cGMP PDE isozyme selectivity of SS.</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td>Effects of SS treatment on expression of Wnt related genes.</td>
<td>68</td>
</tr>
<tr>
<td>8</td>
<td>Structures and COX inhibitory activities of novel sulindac derivatives.</td>
<td>85</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Metabolism of the NSAID prodrug sulindac</td>
</tr>
<tr>
<td>2</td>
<td>Cyclic nucleotide signaling pathways</td>
</tr>
<tr>
<td>3</td>
<td>Effects of cAMP and cGMP signaling on breast cell growth</td>
</tr>
<tr>
<td>4</td>
<td>Growth inhibitory activity of PDE isozyme inhibitors in human breast cells</td>
</tr>
<tr>
<td>5</td>
<td>Effects of PDE inhibition on apoptosis and proliferation of human breast cells</td>
</tr>
<tr>
<td>6</td>
<td>Activation of cGMP signaling through PDE inhibition</td>
</tr>
<tr>
<td>7</td>
<td>Effects of PDE inhibition on Wnt/β-catenin mediated transcription</td>
</tr>
<tr>
<td>8</td>
<td>Effects of PDE inhibition of expression of Tcf/Lef regulated genes</td>
</tr>
<tr>
<td>9</td>
<td>Relative activities of cGMP PDE isozymes in human breast cells</td>
</tr>
<tr>
<td>10</td>
<td>Expression of cGMP PDE isozymes in human breast cells</td>
</tr>
<tr>
<td>11</td>
<td>Verification of PDE5 knockdown with siRNA</td>
</tr>
<tr>
<td>12</td>
<td>Effect of PDE5 knockdown on growth and survival of human breast cells</td>
</tr>
<tr>
<td>13</td>
<td>Effect of PDE5 knockdown on Wnt/β-catenin mediated transcription</td>
</tr>
<tr>
<td>14</td>
<td>Proposed mechanism of growth inhibition mediated by PDE5 inhibition</td>
</tr>
<tr>
<td>15</td>
<td>Association between growth inhibition and other molecular mechanisms for a panel of NSAIDs and COX-2 inhibitors</td>
</tr>
<tr>
<td>16</td>
<td>Anticancer activity of SS</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>17</td>
<td>PDE inhibitory activity of SS in breast cell lysates</td>
</tr>
<tr>
<td>18</td>
<td>Involvement of cGMP in the anticancer activity of SS</td>
</tr>
<tr>
<td>19</td>
<td>Activation of PKG by SS</td>
</tr>
<tr>
<td>20</td>
<td>PDE5 is a target of SS</td>
</tr>
<tr>
<td>21</td>
<td>Effects of SS on β-catenin expression and phosphorylation status</td>
</tr>
<tr>
<td>22</td>
<td>Effects of SS on β-catenin signaling</td>
</tr>
<tr>
<td>23</td>
<td>Effects of SS on cyclin D1 and survivin expression</td>
</tr>
<tr>
<td>24</td>
<td>SS docked into the catalytic site of COX-1</td>
</tr>
<tr>
<td>25</td>
<td>cGMP PDE inhibitory activity of sulindac derivatives</td>
</tr>
<tr>
<td>26</td>
<td>Molecular modeling studies of sulindac derivatives</td>
</tr>
<tr>
<td>27</td>
<td>Effects of sulindac derivatives on cGMP signaling and Wnt/β-catenin mediated transcription</td>
</tr>
<tr>
<td>28</td>
<td>Effects of SRI 21878 on breast cell growth, apoptosis, and proliferation</td>
</tr>
<tr>
<td>29</td>
<td>Titration of recombinant PDE isozymes</td>
</tr>
<tr>
<td>30</td>
<td>Sensitivity of recombinant PDE isozymes to known inhibitors</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-m-IBMX</td>
<td>8-methoxymethyl-3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNG</td>
<td>cyclic nucleotide gated ion channels</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol teraacetic acid</td>
</tr>
<tr>
<td>EHNA</td>
<td>Erythro-9-(2-hydroxy-3-nonyl)adenine</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme-linked immunoassay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<tr>
<td>EPAC</td>
<td>exchange factor directly activated by cAMP</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>Fl</td>
<td>fluorescein</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescence polarization</td>
</tr>
<tr>
<td>GAF</td>
<td>cGMP-activated PDEs, adenylyl cyclase, and Fh1A</td>
</tr>
<tr>
<td>GC</td>
<td>guanylyl cyclase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization-activated cyclic nucleotide gated ion channel</td>
</tr>
<tr>
<td>HMEC</td>
<td>human mammary epithelial cells</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration resulting in 50% inhibition</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>MY5445</td>
<td>1-(3-Chlorophenylamino)-4-phenylphthalazine</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOR-3</td>
<td>(+/-)-(E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide</td>
</tr>
<tr>
<td>NSAID</td>
<td>nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>pGC</td>
<td>particulate guanylyl cyclase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>p-VASP&lt;sub&gt;Ser239&lt;/sub&gt;</td>
<td>VASP phosphorylated at the serine 239 residue</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylyl cyclase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribose nucleic acid</td>
</tr>
<tr>
<td>TAMRA</td>
<td>tetramethylrhodamine</td>
</tr>
<tr>
<td>Tcf/Lef</td>
<td>T-cell factor and lymphoid enhancer factor transcription factors</td>
</tr>
<tr>
<td>VASP</td>
<td>vasoactivator stimulated phosphoprotein</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless-type mouse mammary tumor virus integration site family</td>
</tr>
</tbody>
</table>
BACKGROUND

Breast Cancer

Breast cancer is a major health problem with more than 200,000 new cases diagnosed in the United States each year (American Cancer Society 2009). While mortality rates attributed to the disease have declined slightly in recent years, it remains the most commonly diagnosed cancer and the second leading cause of cancer-related deaths for women (American Cancer Society 2009). Numerous environmental and lifestyle factors, such as obesity and exposure to certain chemicals, have been implicated as risks for breast cancer, but the most significant risk factor is a family history. Approximately 5-10% of all breast cancers occur in patients who have a close relative previously diagnosed with the disease (Mann, Thorne et al. 2006). Despite the introduction of novel therapeutics, mortality rates for breast cancer have declined less than 10% in the past decade. The lack of improvement in breast cancer therapy, known risk factors for disease development, and the length of time necessary for disease development and progression suggest that chemoprevention would be a promising strategy for significantly reducing breast cancer related mortalities. Chemoprevention involves pharmacologically treating a patient who is at increased risk of disease development or progression in order to prevent the undesired outcome. Because a significant portion of the patient population that is targeted for chemoprevention will not develop the disease, potential chemopreventive agents must have a large therapeutic window, meaning that the benefits must greatly outweigh the risks associated with it.
The selective estrogen receptor modulators (SERMs), tamoxifen and raloxifene, are currently the only drugs that are FDA approved for breast cancer chemoprevention. Initial prevention trials with tamoxifen showed a 38% decrease in overall breast cancer risk with a 48% reduction in the risk of developing estrogen receptor (ER) positive disease (Cuzick, Powles et al. 2003). However, tamoxifen did not alter the patients’ risk of developing ER negative disease, which accounts for about 25% of all newly diagnosed breast cancer cases and is considered a more invasive form of the disease with a poorer prognosis and higher mortality rate (Rochefort, Glondu et al. 2003). Additionally, adjuvant trials of women undergoing tamoxifen therapy for three or more years showed a more than three-fold increase in the risk of developing endometrial cancer and a two-fold increase in the risk of thrombotic events (Cuzick, Powles et al. 2003). Raloxifene has relatively similar efficacy and toxicity profiles to tamoxifen, with the exception of the increased risk of endometrial cancer (Bevers 2006). Furthermore, use of the SERMs for chemoprevention is often associated with poor patient compliance. For these reasons, the SERMs are generally only used for the prevention of contralateral ER positive tumors in post-menopausal women. As such, there is an unmet medical need for improved chemopreventive agents that have enhanced efficacy compared to the SERMs but minimal side effects.

Anticancer Activities of the Nonsteroidal Anti-inflammatory Drugs

Preclinical, clinical, and epidemiological studies have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase 2 (COX-2) selective inhibitors, display significant chemopreventive efficacy against many types of cancers,
including breast cancer (Chan 2002; Thun, Henley et al. 2002). The NSAIDs are a chemically diverse family of drugs commonly used to treat chronic inflammatory conditions such as arthritis. The pharmacological basis for their anti-inflammatory activity involves inhibition of the COX enzymes and the subsequent blockage of arachidonic acid conversion to prostaglandin H$_2$, a precursor to prostaglandins, prostacyclins, and thromboxanes, which play a role in physiological processes such as inflammation, renal function, clot formation, and GI protection (Vane and Botting 1998). Two isoforms of the COX enzyme are expressed in humans. COX-1 is a constitutively active form of the enzyme whereas COX-2 is an inducible form whose expression is often increased during pathophysiological conditions such as chronic inflammation. While COX inhibition is responsible for their anti-inflammatory activity, the depletion of prostaglandins through long term use of NSAIDs is associated with potentially fatal side effects that include gastrointestinal ulcers and bleeding, renal toxicity, and increased risk of heart attack (Vane, Bakhle et al. 1998). Consequently, these toxicities have precluded the use NSAIDs or COX-2 selective inhibitors for cancer chemoprevention.

Because inflammation is closely associated with tumorigenesis and COX-2 has been shown to be overexpressed in precancerous and malignant lesions (Dannenberg, Altorki et al. 2001; Brown and DuBois 2005), COX-2 inhibition and the suppression of eicosanoid biosynthesis is generally accepted as being the primary mechanism responsible for the anticancer activity of the NSAIDs (Alberts, Hixson et al. 1995; Chan 2002; Raz 2002; Kashfi and Rigas 2005). However, numerous studies suggest that a COX-2 independent mechanism, or off-target effect, may either contribute to or be fully responsible for the chemopreventive activity of NSAIDs. For instance, these compounds
have been found to inhibit the growth of tumor cells that completely lack the expression of the COX enzymes (Hanif, Pittas et al. 1996; Elder, Halton et al. 1997; Grosch, Tegeder et al. 2001). Supplementation with exogenous prostaglandins does not rescue cancer cells from the growth inhibitory activity of the NSAIDs (Piazza, Rahm et al. 1995; Kusuhara, Matsuyuki et al. 1998). The rank order of potency among the NSAIDs to inhibit tumor cell growth does not correlate with the potency to inhibit prostaglandin synthesis (de Mello, Bayer et al. 1980; Carter, Ip et al. 1989; Erickson, Longo et al. 1999), as much higher doses are required to inhibit tumor cell growth than those required for anti-inflammatory activity (Piazza, Rahm et al. 1997; Williams, Watson et al. 2000; Raz 2002). Additionally, certain NSAID analogs and metabolites have been identified that lack COX inhibitory activity but retain the anticancer properties of the parent compound.

The NSAID prodrug, sulindac, is a prime example. As depicted in Figure 1, sulindac is administered orally as a non-COX inhibitory prodrug in its sulfoxide form, which can be reversibly reduced by liver enzymes and colonic bacteria to its active, COX-1 and COX-2 inhibitory sulfide metabolite. A small portion of the parent compound can also be irreversibly oxidized to its sulfone metabolite, which, like the sulfoxide prodrug, does not inhibit COX-1 or COX-2. Interestingly, sulindac sulfone displays chemopreventive activity in animal models of colon, mammary, lung, and bladder carcinogenesis, despite its inability to inhibit the COX enzymes or affect prostaglandin production (Thompson, Briggs et al. 1995; Piazza, Alberts et al. 1997; Thompson, Jiang et al. 1997; Malkinson, Koski et al. 1998; Reddy, Kawamori et al. 1999; de Jong, Skinner et al. 2000; Piazza, Thompson et al. 2001). Sulindac sulfone
(exisulind) was clinically developed by Cell Pathways, Inc. (Horsham, PA) and shown to cause colon polyp regression in patients with either familial (Stoner, Budd et al. 1999) or sporadic adenomas (Arber, Kuwada et al. 2006), but did not receive FDA approval due to hepatotoxicity. Interestingly, sulindac sulfone was found to be more potent in experimental models of mammary tumorigenesis (Thompson, Briggs et al. 1995; Thompson, Jiang et al. 1997) compared with colon models (Piazza, Rahm et al. 1997) but was never developed clinically for breast cancer chemoprevention.

Several different mechanisms have been implicated in the COX-independent anticancer properties of sulindac sulfone as well as traditional NSAIDs or COX-2 selective inhibitors, including inhibition of the expression of epidermal growth factor receptor (EGFR) and activation of c-Jun NH2-terminal kinase 1 (JNK1) (Soh, Mao et al. 2000; Rice, Goldberg et al. 2001; Pangburn, Kraus et al. 2005; Pangburn, Ahnen et al. 2010). However, one of the best characterized mechanisms for sulindac sulfone is inhibition of cyclic guanosine monophosphate phosphodiesterse (cGMP PDE), which results in activation of cGMP dependent protein kinase (PKG) (Thompson, Piazza et al. 2000; Soh, Kazi et al. 2008), but this mechanism has not been studied with regards to COX inhibitors or in breast cancer models.

**Cyclic Nucleotide Signaling**

The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cGMP, have long been recognized as important intracellular signal transduction molecules, acting as second messengers between an extracellular signal such as a hormone, neurotransmitter, or cytokine and the elicited intracellular response. While the specific
function of a given signal varies according to the cell type, extracellular environment, stimulus activating the signal, localization of the signal, and the type of cyclic nucleotide formed, an extracellular signal will generally activate a cyclase enzyme, which catalyzes the formation of the cyclic nucleotide from its nucleotide triphosphate precursor, as depicted in Figure 2A. Once formed, the cyclic nucleotide will modulate the activity of downstream effector molecules including protein kinases, ion channels, transcription factors, and scaffolding proteins. Both the amplitude and duration of a cyclic nucleotide signal also varies and is largely dependent on the expression and activity levels of the PDE enzymes, which catalyze the hydrolytic breakdown of the cyclic nucleotides.

\textit{cAMP Signaling}

First described in the late 1950s, cAMP is the more well studied of the cyclic nucleotides. As reviewed previously (Rehmann, Wittinghofer et al. 2007) and depicted in Figure 2B, cAMP is produced from its precursor, adenosine triphosphate (ATP), through the catalytic activity of the adenylyl cyclases (ACs). Differing primarily in tissue distribution and subcellular localization, nine membrane-bound and one soluble AC have been identified in mammals (Taussig and Gilman 1995). ACs are indirectly activated by various stimuli including adrenergic agonists, which bind to G protein coupled receptors (GPCRs) on the cell membrane resulting in the activation of the GPCR and release of the \(G_s\) subunit that is subsequently responsible for binding to and activating AC (Hanoune and Defer 2001).

The effect of cAMP depends largely on the specific receptor(s) that is activated, which is most often dictated by the cell type and/or subcellular localization of the signal. Intracellular receptors for cAMP include the exchange factor directly activated by cAMP
(EPAC), the cAMP-dependent serine/threonine protein kinase (PKA), and ion channels such as the cyclic nucleotide gated (CNG) channels and the hyperpolarization-activated cyclic nucleotide gated (HCN) channels. EPAC is a guanine exchange factor that activates the small G protein Rap in order to mediate cell adhesion, junction formation, and secretion (Rehmann, Wittinghofer et al. 2007). PKA phosphorylates metabolic enzymes to mediate glucose metabolism and the transcription factor cAMP response element binding protein (CREB) to promote gene transcription (Beavo and Brunton 2002; Rehmann, Wittinghofer et al. 2007). Responsible for mediating heart rate and rhythm, HCN channels are regulated by the binding of cAMP, which shifts the membrane potential necessary for the channels to open (Beavo and Brunton 2002). CNG channels, which are important regulators of olfaction, are also regulated by the binding of cAMP but in a manner independent of membrane potential (Rehmann, Wittinghofer et al. 2007).

While inhibitory GPCR subunits modulate the activity of ACs in some cell types and PKA activity can act as a negative regulator of cAMP signaling by phosphorylating and inactivating ACs (Rehmann, Wittinghofer et al. 2007), the amplitude and duration of a cAMP signal within most cells is largely dependent on PDE enzyme activity, which is responsible for hydrolyzing cAMP to 5’AMP in order to terminate its signal (Omori and Kotera 2007). Eleven PDE families comprised of more than 20 distinct genes have been identified to date. Due to alternative splicing and post-translational modifications, each PDE gene is capable of producing multiple protein products within a single cell or tissue. Each PDE isoform displays unique biochemical properties including substrate specificity, regulatory processes, and/or pharmacological sensitivity, as summarized in Table 1 (Beavo 1995; Bender and Beavo 2006; Conti and Beavo 2007). Hydrolysis of cAMP is
Table 1. Characterization of PDE superfamily.

<table>
<thead>
<tr>
<th>Family</th>
<th>Number of Genes</th>
<th>Number of Isoforms</th>
<th>Substrate Specificity</th>
<th>Regulators</th>
<th>Inhibitors</th>
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<tbody>
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<td>1</td>
<td>3</td>
<td>21</td>
<td>dual</td>
<td>Ca^{2+} -CaM: ↑ PKA: ↓</td>
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</tr>
<tr>
<td>2</td>
<td>1</td>
<td>3</td>
<td>dual</td>
<td>cGMP: ↑</td>
<td>EHNA</td>
</tr>
<tr>
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<td>2</td>
<td>4</td>
<td>dual</td>
<td>cGMP: ↓ PKA: ↑</td>
<td>milrinone</td>
</tr>
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<td>4</td>
<td>31</td>
<td>cAMP</td>
<td>PKA: ↓</td>
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</tr>
<tr>
<td>5</td>
<td>1</td>
<td>3</td>
<td>cGMP</td>
<td>PKG, cGMP: ↑</td>
<td>sildenafil</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>3</td>
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<td>Transducin: ↑</td>
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<td>unknown</td>
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<tr>
<td>11</td>
<td>1</td>
<td>4</td>
<td>dual</td>
<td>unknown</td>
<td>dipyridamole</td>
</tr>
</tbody>
</table>

Note: Human PDE isoforms are divided into 11 families and differ according to substrate specificity, mechanisms of regulation, and sensitivity to inhibitors [6-8]. ↑ represents an increase in catalytic activity, whereas ↓ represents a decrease in activity.

primarily mediated by the PDE1, 2, 3, 4, 7, 8, 10, and 11 families, with PDE4, 7, and 8 being cAMP selective and therefore generally unable to hydrolyze cGMP under physiological conditions (Omori and Kotera 2007). Despite relatively equal levels of AC and cAMP PDE protein expression in most cell types, the rate of cAMP hydrolysis in virtually all human tissues far exceeds the rate of synthesis, making PDE enzymes an important determinant of intracellular cAMP levels, which, under basal conditions, are typically less than 5 pmol per mg of protein (Beavo and Brunton 2002).
cGMP Signaling

As depicted in Figure 2C, cGMP is formed through the activity of guanylyl cyclase (GC) enzymes from its precursor guanosine triphosphate (GTP). One distinct difference from cAMP signaling is that GC enzymes are more evenly dispersed between the membrane and the cytosol of cells and are directly activated by their stimuli (Rehmann, Wittinghofer et al. 2007). Seven particulate or membrane bound GCs (pGC) have been identified, each consisting of a single transmembrane region (Feil and Kemp-Harper 2006). As with the membrane-bound AC isoforms, pGC isoforms differ largely in their tissue distribution as well as in their sensitivity to ligands, which include natriuretic peptides, small paracrine peptide hormones such as guanylin, enterotoxins, and certain cytokines. Conversely, the heme-containing soluble GC (sGC) enzyme is restricted to the cytoplasm and is solely activated by nitric oxide (NO) under physiological conditions (Rehmann, Wittinghofer et al. 2007).

Similar to cAMP signaling, the specific effect of a cGMP signal depends largely on the receptor(s) that is activated. Under standard physiological concentrations, which for cGMP are typically ten-fold less than those observed for cAMP (Beavo and Brunton 2002), cGMP preferentially activates receptors distinctly different than those activated by cAMP, including certain PDE isozymes, PKG, and CNG ion channels (Rehmann, Wittinghofer et al. 2007). Modulation of CNG channel activity is a more common event in cGMP signaling compared to cAMP signaling and serves as an important step for mediating the effects of cGMP on phototransduction, natriuresis, and intestinal fluid and electrolyte secretion (Lincoln and Cornwell 1993). Similar to PKA and cAMP signaling, PKG mediates cGMP signaling by modulating the activity of downstream effector molecules through protein phosphorylation (Rehmann, Wittinghofer et al. 2007). For
example, PKG can phosphorylate and activate myosin phosphatase to promote
vasodilation and muscle relaxation (Feil and Kemp-Harper 2006). The regulation of PDE
activity is unique to cGMP signaling and serves multiple functions within cells such as
acting as negative feedback for cGMP signaling by activating cGMP specific PDE5 or
acting as crosstalk between cyclic nucleotide pathways by increasing or decreasing the
activity of non-selective PDE isozymes such as PDE2 or PDE3, respectively (Omori and
Kotera 2007).

Some GC isoforms serve as substrates for phosphorylation by PKG resulting in
decreased cGMP production. However, like cAMP, cGMP levels are predominantly
controlled by degradation via PDE enzymes. PDE1, 2, 3, 5, 6, 9, 10, and 11 families are
capable of hydrolyzing cGMP with PDE5, 6, and 9 being selective for cGMP (Omori and
Kotera 2007). In many tissues, PDE5 is the isoform thought to be predominantly
responsible for cGMP hydrolysis and subsequent termination of a cGMP signal. The
activity of the PDE5 enzyme is tightly controlled by cGMP signaling. In the presence of
a cGMP signal, cGMP will bind to the cGMP-activated PDEs, adenylyl cyclase, and
Fh1A (GAF A) domain of the N-terminal region of the PDE5 protein to promote its
phosphorylation at a separate N-terminal site by PKG, an event that produces a several-
fold increase in the activity of the enzyme while simultaneously increasing the affinity of
the catalytic site for cGMP (Beavo 1995).

*Crosstalk between Cyclic Nucleotide Pathways*

An additional level of regulation occurs between cAMP and cGMP signaling
pathways and is referred to as “crosstalk.” The most notable source of crosstalk between
the cAMP and cGMP pathways is found in the ability of cGMP to modulate the activity
of various PDEs, particularly PDE2 and PDE3, which can hydrolyze both cAMP and cGMP but have higher affinity for cAMP. For example, low nanomolar concentrations of cGMP are sufficient to produce more than a tenfold increase in cAMP hydrolysis by PDE2 and almost complete inhibition of cAMP hydrolysis by PDE3 (Beavo 1995). In fact, it is thought that the effects of atrial natriuretic peptide (ANP) on aldosterone secretion are at least partially due to a drop in cAMP levels mediated by cGMP signaling via activation of PDE2 (Omori and Kotera 2007).

Phosphorylation events mediated by PKA and PKG serve as another source of crosstalk between the cAMP and cGMP pathways. Both kinases share a number of substrates allowing for specific downstream events to be mediated by both cAMP and cGMP signaling (Beavo and Brunton 2002). For example, the cytoskeletal protein, vasoactivator stimulated phosphoprotein (VASP), is preferentially phosphorylated at its serine 157 residue by PKA and its serine 239 residue by PKG in order to modulate focal adhesions, cell shape, or platelet aggregation in response to a number of stimuli (Deguchi, Soh et al. 2002). Alternatively, PKA and PKG, while predominantly activated by their respective cyclic nucleotides, can be activated by the alternate cyclic nucleotide. For instance, high levels of cAMP have been found to activate PKG \textit{in vitro} in the absence of cGMP, but the validity of this effect is questionable as it has not been observed in intact cells or under physiological conditions (Lincoln and Cornwell 1993).

Cyclic Nucleotide Signaling in Malignant Disease

As described in Table 2, altered expression and/or activity of one or more cyclic nucleotide signaling mediators have been reported in various carcinomas (DeRubertis and
Craven 1976; Chawla, Nixon et al. 1979; Chawla, Shlaer et al. 1980; Heinonen and Metsa-Ketela 1988; Ciardiello, Pepe et al. 1993; Drees, Zimmermann et al. 1993; Carlson, Smithers et al. 1999; Piazza, Thompson et al. 2001; Whitehead, Earle et al. 2003; Zhu, Strada et al. 2005; Zhu, Vemavarapu et al. 2005; Zhu and Strada 2007; Pertuit, Barlier et al. 2009). Also, cAMP and cGMP signaling have been found to have negative effects on cell growth and survival, depending on the cell or tissue type (Jiang, Li et al. 1996; Piazza, Thompson et al. 2001; Whitehead, Earle et al. 2003; Ahn, Jung et al. 2005; Wharton, Strange et al. 2005; Zhu, Strada et al. 2005; Zhu, Vemavarapu et al. 2005; Lerner and Epstein 2006; Zhu and Strada 2007; Kloster, Hafte et al. 2008). These observations suggest that aberrant cyclic nucleotide signaling may play an important role in tumorigenesis.

One of the most well characterized alterations involves decreased expression of the peptide hormones guanylin and uroguanylin that is accompanied by increased expression of the guanylin receptor, guanylyl cyclase C (GC-C) in colorectal adenomas and adenocarcinomas (Waldman, Barber et al. 1998; Steinbrecher, Wowk et al. 2002; Birbe, Palazzo et al. 2005; Camici 2008). Consistent with this observation, colorectal cancer cells generally have decreased basal levels of cGMP signaling and are hypersensitive to cGMP signaling activation with GC-C ligands compared to the normal colonic mucosa (Steinbrecher, Wowk et al. 2002; Pitari, Zingman et al. 2003; Camici 2008).
Mammary tumors have also been found to possess altered cyclic nucleotide signaling, particularly in terms of hydrolytic capacity (Cohen, Straka et al. 1976; Singer, Sherwin et al. 1976; Kung, Bechtel et al. 1977; Whitfield, Boynton et al. 1980; Marko, Pahlke et al. 2000; Johansson, Yndestad et al. 2004). For example, faster growing and more invasive mammary tumors show an overall decreased ability to hydrolyze cyclic nucleotides (Cohen, Straka et al. 1976; Singer, Sherwin et al. 1976; Marko, Pahlke et al. 2000). While this may be due to alterations in PDE expression, there also appears to be

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Observed Alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>↑ PDE5 expression</td>
</tr>
<tr>
<td></td>
<td>↑ MRP5 expression</td>
</tr>
<tr>
<td>Breast</td>
<td>↑ PDE expression and activity</td>
</tr>
<tr>
<td></td>
<td>Altered PDE localization</td>
</tr>
<tr>
<td></td>
<td>↑ MRP5 expression</td>
</tr>
<tr>
<td>Colon</td>
<td>↑ GC-C expression; ↓ ligand expression</td>
</tr>
<tr>
<td></td>
<td>↓ PKG expression</td>
</tr>
<tr>
<td></td>
<td>↓ PKA expression</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>↑ basal levels of cAMP and cGMP</td>
</tr>
<tr>
<td>Leukemia</td>
<td>Altered PDE isozyme expression</td>
</tr>
<tr>
<td></td>
<td>↑ PDE activity</td>
</tr>
<tr>
<td>Lung</td>
<td>↑ PDE expression and activity</td>
</tr>
<tr>
<td></td>
<td>↑ MRP5 expression</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>↑ PDE activity</td>
</tr>
<tr>
<td></td>
<td>↓ basal levels of cAMP and cGMP</td>
</tr>
<tr>
<td>Ovarian</td>
<td>↓ basal levels of cAMP</td>
</tr>
<tr>
<td></td>
<td>↑ MRP5 expression</td>
</tr>
<tr>
<td>Pituitary</td>
<td>↓ AC activity</td>
</tr>
<tr>
<td></td>
<td>↑ PDE expression and activity</td>
</tr>
<tr>
<td>Prostate</td>
<td>↑ MRP5 expression</td>
</tr>
<tr>
<td>Skin</td>
<td>↑ PDE activity</td>
</tr>
</tbody>
</table>

Note: ↑ refers to increase, whereas ↓ refers to decrease.

Table 2. Observed alterations in cyclic nucleotide signaling pathways in different types of malignancies.
an alteration in compartmentalization of PDE isozymes (Singer, Sherwin et al. 1976). Alternatively, the multi-drug resistance protein 5 (MRP5), which exports cyclic nucleotides, particularly cGMP, from cells and serves as a mechanism in addition to the PDEs for termination of intracellular cGMP signaling, has been found to be overexpressed in a number of epithelial cancers including cancers of the lung, bladder, ovaries, prostate, breast, and colon (Kool, de Haas et al. 1997; Jedlitschky, Burchell et al. 2000).

The relevance of altered cyclic nucleotide signaling, particularly cGMP signaling, in carcinomas has been studied, although not extensively. These studies have found that cGMP signaling appears to play an important role in promoting apoptosis and inhibiting proliferation of epithelial cells (Pitari, Di Guglielmo et al. 2001; Steinbrecher, Wowk et al. 2002; Mann, Steinbrecher et al. 2005; Lugnier 2006; Li, Lin et al. 2007; Li, Schulz et al. 2007), suggesting that the decreased levels of cGMP signaling or the decreased responsiveness to cGMP signals that have been observed in carcinomas could instill a growth advantage and potentially promote tumorigenesis in these tissues. While some of the anti-proliferative and pro-apoptotic effects of cGMP signaling in epithelial cells have been attributed to intracellular ion fluxes via activation of CNGs (Clementi, Sciorati et al. 1995) and/or crosstalk with cAMP pathways via modulation of PDE activity (Lucas, Pitari et al. 2000), PKG is thought to be largely responsible for these effects (Idriss, Gudi et al. 1999; Pilz and Casteel 2003; Chen, Levine et al. 2008). In support of this idea, studies have shown that expressing constitutively active forms of PKG enzymes results in increased apoptosis and decreased cell viability in colon cancer cells (Deguchi, Thompson et al. 2004). In addition to its profound effects on intracellular epithelial cell
processes, cGMP signaling has also been implicated in extracellular remodeling, an important step in tumor invasion and metastasis. Namely, activation of GC-C in colon cancer cells has been shown to reduce secretion of matrix metalloproteinase 9 (MMP-9) in a cGMP-dependent manner (Lubbe, Zhou et al. 2006).

Cyclic GMP Phosphodiesterase as a Target for the Prevention and Treatment of Cancer

Because alterations in cyclic nucleotide signaling are common to a number of cancer types, appear to occur early in the tumorigenic process, and correlate with stage and prognosis, components of these pathways could potentially serve as novel targets for cancer chemoprevention and/or chemotherapy. However, as is the case with any signaling pathway, pharmacologically targeting the cyclic nucleotide pathways poses a number of challenges, particularly concerning toxicity due to lack of specificity. For this reason, targeted approaches for different pathway components, specifically the cyclases, kinases, and PDEs, have been investigated with varying degrees of success. Unfortunately, toxicity has severely limited the development of cyclase and kinase activators (Baumann, Felix et al. 1990; Christenson, Thulesius et al. 1995; Ding and Staudinger 2005). Conversely, PDE inhibitors, which would promote the accumulation of cyclic nucleotides within cells in order to amplify or mimic a cyclic nucleotide signal, have shown promising efficacy with low toxicity for both the prevention and the treatment of various types of carcinomas.

The large numbers of distinct protein products that comprise the PDE superfamily provide a level of specificity that makes inhibiting cyclic nucleotide degradation a promising target for the development of novel anticancer agents. Individual PDE
isozyms differ in tissue expression patterns, subcellular localization, regulatory properties, and sensitivity to inhibitors (Beavo 1995), suggesting the possibility for selective targeting of a single PDE isozyme in order to increase the specificity and reduce the toxicity of a given agent (Bischoff 2004). As such, numerous studies have found alterations in the activity and expression of specific PDE isozymes in various types of cancers. For example, studies have found increased expression of PDE5A in bladder cancer, PDE7B in leukemia, PDE1C in glioblastoma, and PDE5A in lung cancer compared to the normal tissue counterpart; additionally, many of these tumor types rely more heavily on these PDE enzymes for cyclic nucleotide hydrolysis than the normal tissues which typically express a greater variety of PDE isoforms (Marko, Pahlke et al. 2000; Piazza, Thompson et al. 2001; Zhang, Murray et al. 2008). This implies that selective inhibition of these respective PDE enzymes could result in selective growth inhibition of tumor cells compared to normal cells, suggesting both enhanced efficacy and reduced toxicity.

Additionally, mechanistic studies have suggested that one COX-independent mechanism responsible for the antineoplastic activity of the NSAIDs and COX-2 selective inhibitors may involve selective inhibition of cGMP PDE (Thompson, Piazza et al. 2000; Piazza, Thompson et al. 2001; Whitehead, Earle et al. 2003). Several of these drugs, including celecoxib, indomethacin, meclofenamic acid, and sulindac, have been shown to inhibit cGMP PDE, leading to an increase in intracellular cGMP levels and subsequent activation of PKG (Soh, Mao et al. 2000; Thompson, Piazza et al. 2000; Deguchi, Thompson et al. 2004; Soh, Kazi et al. 2008).
The effectors downstream of PKG that are responsible for the inhibition of growth are not well defined, but several studies have suggested that inhibition of canonical wingless-type mouse mammary tumor virus integration site family (Wnt) signaling at the level of β-catenin may play an important role. β-catenin has been shown in previous studies to be a substrate for PKG in vitro (Thompson, Piazza et al. 2000). Additionally, in colon cancer cells, activation of PKG by cGMP signaling has been shown to increase β-catenin degradation and decrease β-catenin-mediated gene transcription, ultimately leading to decreased expression of oncogenes such as \textit{c-myc}, \textit{cyclinD1}, and \textit{survivin} (Soh, Mao et al. 2000; Dihlmann, Siermann et al. 2001; Kim, Plescia et al. 2003; Boon, Keller et al. 2004; Deguchi, Thompson et al. 2004; Gardner, Hawcroft et al. 2004; Scheper, Nikitakis et al. 2007; Cen, Deguchi et al. 2008; Soh, Kazi et al. 2008; Kwon, Wang et al. 2010). Because Wnt/β-catenin signaling is thought to play an important role in the growth and survival of multiple types of cancers, including colon, prostate, and breast, inhibiting this pathways is a promising approach for the discovery of novel anticancer agents (Barker and Clevers 2000; Barker and Clevers 2006).

Another promising feature of targeting cGMP PDE for the prevention or treatment of cancer is the advanced stage of development of PDE as a pharmacological target due to its importance in other pathologies. PDE inhibitors have been developed as therapies for a number of conditions including heart failure, asthma, erectile dysfunction, and pulmonary hypertension (Lugnier 2006). As such, today’s researchers possess a diverse collection of PDE inhibitors with different biochemical and pharmacological properties including varying degrees of isozyme selectivity and elucidated toxicities, which, as reviewed previously (Marko, Pahlke et al. 2000; Hirsh, Dantes et al. 2004;
Lerner and Epstein 2006), has prompted the ongoing effort to identify PDE inhibitors with potential anticancer efficacy. This suggests that targeting PDE is a feasible pharmacological approach that, in light of the potential involvement of these enzymes in tumorigenesis, should be investigated for the prevention and treatment of cancer, particularly breast cancer.
Figure 1. Metabolism of the NSAID prodrug sulindac. Upon ingestion, the majority of the compound is reversibly reduced by liver enzymes and colonic bacteria to its active sulfide metabolite. A small portion of the compound is irreversibly oxidized by liver enzymes to its inactive sulfone metabolite. Sulindac sulfide is a nonselective COX-1 and COX-2 inhibitor, whereas neither the sulfoxide (parent compound) nor the sulfone metabolite inhibits either COX isozyme.
Figure 2. Cyclic nucleotide signaling pathways. A, generalized schematic of cyclic nucleotide signaling. B, cAMP signaling. C, cGMP signaling.
HYPOTHESIS AND AIMS

We and others have identified cGMP PDE isozymes as potential molecular targets responsible for the antineoplastic activity of certain NSAIDs. Furthermore, cyclic nucleotide signaling, particularly at the level of PDEs, has been shown to be dysregulated in tumor cells compared to their normal counterparts. However, little is known about the role of cyclic nucleotide signaling in breast cells, how cyclic nucleotide signaling is regulated in these cells, whether PDE isozyme expression changes during breast tumorigenesis, or whether inhibition of cGMP PDE may serve as an effective mechanism for the prevention and/or treatment of breast cancer. Based on the results of previous studies, we hypothesized that cGMP PDE could serve as a novel target for the prevention and treatment of breast cancer through a mechanism involving activation of PKG and attenuation of Wnt/β-catenin mediated transcription. We developed the following aims to experimentally test this hypothesis:

Aim 1: Characterize the role of cyclic nucleotide signaling in the growth and survival of human breast cells and determine the importance of cGMP PDE isozymes and Wnt/β-catenin signaling for mediating these effects.

Aim 2: Determine whether the anticancer activity of the NSAID, SS, in human breast tumor cells is mediated through a mechanism involving inhibition of cGMP PDE, activation of PKG, and attenuation of Wnt/β-catenin mediated signaling.

Aim 3: Determine whether chemical modifications to the indene scaffold of sulindac can yield better breast cancer chemopreventive agents with reduced COX-inhibitory activity and enhanced cGMP PDE-inhibitory and anticancer activities.
This project was designed to validate cGMP PDE as a target for breast cancer chemoprevention and to characterize the PDE-related mechanism responsible for the antiproliferative and proapoptotic effects of sulindac sulfide in breast cancer cells. Developing cGMP PDE as a COX-independent target of the NSAIDs could potentially yield safer and more efficacious new drugs for breast cancer chemoprevention and/or chemotherapy.
MATERIALS AND METHODS

Drugs and Reagents

(+/-)-(E)-4-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (NOR-3), Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), and 1-(3-Chlorophenylamino)-4-phenylphthalazine (MY5445) were purchased from BioMol (Plymouth Meeting, PA). LY83583 was purchased from Cayman Chemical (Ann Arbor, MI). Sildenafil was a generous gift from Pfizer. Vardenafil and tadalafil were extracted from the tablet excipients with dimethyl sulfoxide (DMSO) following pulverization. Recombinant PDE isozymes were purchased from BPS Biosciences (San Diego, CA). The family-specific anti-PDE antibodies were purchased from GeneTex (San Antonio, TX). The anti-VASP and anti-β-catenin antibodies were obtained from BD Transduction Laboratories (San Jose, CA). The anti-phospho-VASP-Ser239, anti-cyclin D1, anti-survivin, and anti-β-actin antibodies were all purchased from Cell Signaling Technologies (Beverly, MA). The isoform-specific anti-PKG and anti-RhoA antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were also obtained from Cell Signaling Technologies. Anti-rabbit Alexa Fluor 488-conjugated secondary antibody, Draq5, and Lipofectamine LTX were obtained from Invitrogen (Carlsbad, CA). PDE5A and negative control small interfering ribose nucleic acid (siRNA) constructs and SureFECT transfection reagents were purchased from SABiosciences (Frederick, MD). DMSO was
used as the vehicle for all compounds. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Cells and Cell Culture**

The human cell lines HT-29, MDA-MB-231, SK-BR-3, and ZR75-1, as described in Table 3 (Sutherland, Watts et al. 1999), were obtained from American type culture collection (ATCC) while human mammary epithelial cells (HMEC) were obtained from Lonza (Basil, Switzerland). All cells were grown under standard cell culture conditions at 37°C in a humidified atmosphere with 5% CO₂. MDA-MB-231, SK-BR-3, ZR75-1, and HT-29 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 cell culture medium containing 5% fetal bovine serum (FBS). Human mammary epithelial cells (HMEC) and MCF10A were grown in mammary epithelial cell complete growth medium (MEGM). Assays were performed using the same growth conditions. Cell counts and viability were determined by trypan blue exclusion followed by hemacytometry. Only cultures displaying >95% viability were used for experiments.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>RECEPTOR STATUS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ER</strong></td>
<td><strong>PR</strong></td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>-</td>
</tr>
<tr>
<td>SKBR3</td>
<td>-</td>
</tr>
<tr>
<td>ZR75-1</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: + indicates that the receptor is expressed, whereas - indicates that the receptor is not expressed (Sutherland, Watts et al. 1999).
Growth Assay

Tissue culture microtiter 96-well plates were seeded at a density of 5,000 cells per well. Cells were incubated for 18 to 24 hours, treated with the specified compound or vehicle control, and incubated an additional 72 hours. The inhibition of cell growth caused by treatment was determined using the luminescent Cell Titer Glo Assay by Promega (Madison, WI), which measures viable cells based on ATP content. The assay was performed according to the manufacturer’s specifications with a maximum DMSO concentration of 0.2%. Luminescence was measured using a VictorV (PerkinElmer) plate reader.

Caspase Assay

The induction of apoptosis caused by treatment was determined using the luminescent Caspase3/7 Glo Assay (Promega) which measures cleavage of a substrate for caspases 3 and 7. Tissue culture microtiter 96-well plates were seeded at a density of 10,000 cells per well. Cells were incubated for 18 to 24 hours, treated with the specified compound or vehicle control, and incubated an additional 6 hours. The assay was performed according to the manufacturer’s specifications with a maximum DMSO concentration of 0.2%. Luminescence was measured using a VictorV (PerkinElmer) plate reader.

Proliferation Assay

Cells were seeded in 10 cm tissue culture dishes at a density of 1x10^6 cells per dish. After 18-24 hours, the media on each dish was replaced with serum free media.
After an additional 18-24 hours, cells were treated with compound or vehicle control in serum-containing media. After 4 hours of treatment, 10µM of Invitrogen EdU, which is a bromodeoxyuridine (BrdU) derivative, dissolved in DMSO was added to each dish. After an additional 18 hours, cells were collected and stained for EdU incorporation using the Click-iT EdU Alexa Fluor 488 kit (Invitrogen). The assay was performed according to the manufacturer’s specifications. The percentage of BrdU-positive cells was quantified using a Guava EasyCyte Plus flow cytometer. A minimum of 10,000 events were collected for each treatment group with use of minimal electronic compensation. Data was analyzed using CytoSoft 5.0 software (Guava Technologies).

Cell Lysis

Cells were harvested and vortexed in ice cold lysis buffer, which consisted of 20mM tris acetate, 5mM magnesium acetate, 1mM ethylene glycol teraacetic acid (EGTA), 0.8% Triton X-100, 50mM sodium fluoride, 1.25mM sodium vanadate, and protease inhibitor cocktail at pH 7.4. Lysates were clarified by centrifugation at 10,000g for 10 minutes at 4°C. Protein content was determined using the Thermo/Pierce bicinchoninic acid (BCA) protein assay (Rockford, IL) following the manufacturer’s specifications.

PDE Activity Assay

PDE activity in cell lysates was measured using the IMAP fluorescence polarization (FP) PDE assay from Molecular Devices (Sunnyvale, CA) in which binding of hydrolyzed fluorescent cyclic nucleotide substrate to the IMAP reagent increases FP.
The assay was modified to use fluorescein (Fl)-cAMP and tetramethylrhodamine (TAMRA)-cGMP as substrates, allowing for simultaneous measurement of cAMP and cGMP hydrolysis. Each well of a 96-well non-binding plate contained 0.25 mg/mL of cell lysate or recombinant enzyme preparations. Enzymes were incubated with compound or vehicle control for 30 minutes at 30°C prior to the addition of a substrate mixture to a final concentration of 25 nM of each Fl-cAMP and TAMRA-cGMP. After 90 minutes of incubation at 30°C, the reaction was terminated by the addition of binding reagent. The maximum DMSO concentration for each experiment was 2%. FP was measured using a Synergy4 (BioTek) plate reader.

**cGMP Assay**

Cells were seeded at a density of 1x10^6 cells per 10 cm tissue culture dish, incubated for 48 hours, and treated with the specified compound or vehicle control. After 30 minutes of treatment, cells were lysed and assayed for cGMP content using the General Electric Biosciences cGMP Direct Biotrak Enzyme Linked Immunoassay (EIA) kit (Piscataway, NJ). The assay was performed according to the manufacturer’s specifications. Optical density was measured at 630 nm using a Synergy4 (BioTek) plate reader.

**Immunoblotting**

Cell lysates (15 µg protein) were separated by sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel followed by electrophoretic transfer to a nitrocellulose membrane. The membranes were blocked
with 5% milk (p-VASP\textsuperscript{Ser239} or RhoA antibodies) or 5% bovine serum albumin (BSA) in tris-buffered saline (TBS) containing 0.05% Tween-20. Membranes were incubated in primary antibodies overnight in blocking buffer followed by incubation in secondary antibody for 2 hours at room temperature. Protein bands were visualized on HyBlot CL autoradiography film using Super Signal West Pico Enhanced Chemiluminescence Reagent (Pierce).

**Molecular Modeling**

Molecular modeling was performed using the Schrödinger Suite 2008 (Schrödinger, LLC). The PDE5, COX-1, and COX-2 protein structures were obtained from the protein databank (PDB identification: 1UTD PDE5, 2OYE, 6COX, respectively). The induced fit docking (IFD) protocol, which takes into consideration of the ligand-induced receptor conformational change, was used for all docking studies. Residues within 5 Å from the ligand were allowed to be flexible. The docking results were scored using the extra-precision (XP) mode of Glide® version 4.5 (Schrödinger, LLC). The IDF docking protocol and parameters were first validated by docking sildenafil in the PDE5 catalytic site. The docking result excellently reproduced the sildenafil-PDE5 crystal complex conformation. The same protocol and parameters were then used to study the docking of other compounds to PDE5. SS was docked into the COX-1 and COX-2 structures using default parameters.
Wnt3A Conditioned Media

Wnt3A-secreting L cells and control L cells were obtained from ATCC and were cultured in Dulbecco’s modified eagle medium (DMEM) containing 10% FBS and 400 μg/mL geneticin (G418). Cells were cultured in 15 cm dishes in DMEM without G418. After the cells reached confluency, the media was replaced with fresh serum free DMEM. After an additional 48 hours of incubation, the media was collected, centrifuged to remove cell debris, and stored at -80°C until future use.

β-catenin Imaging Assay

Cells were seeded in 96-well optical bottom microtiter plates at a density of 10,000 cells per well. After 18-24 hours, the growth media was replaced with serum free media. After an additional 18-24 hours, cells were treated with compound or vehicle control for 30 minutes. β-catenin signaling was then stimulated with Wnt3A conditioned media or control media. After 4 hours, cells were fixed with 4% formalin and blocked with 5% BSA. Cells were incubated in β-catenin primary antibody for 2 hours followed by 1 hour incubation in secondary antibody. Nuclei were counterstained with Draq5. β-catenin expression was visualized using an Evotec Opera confocal microscope and nuclear localization was measured using Acapella image analysis software.

Gene Expression Studies (PCR)

Cells were seeded at a density of 1x10^6 cells per 10 cm dish and grown to 70-80% confluency. Cells were then lysed and RNA isolated using the Axygen AxyPrep Total RNA Miniprep kit (Union City, CA). RNA concentration was determined by measuring
absorbance at 260 nm and purity by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Only samples having an absorbance ratio of 1.7 to 2.0 were utilized for reverse transcription. Reverse transcription was performed with 1 µg of RNA using the Applied Biosciences High Capacity Complementary Deoxyribose Nucleic Acid (cDNA) Archive kit according to manufacturer’s specifications (Foster City, CA). The reaction was performed at 25°C for 10 minutes followed by 37°C for 2 hours.

Polymerase chain reaction (PCR) amplification of target cDNA was performed with 50 nM cDNA and 1 µM primers (Table 4) in a final volume of 25 µL using the Qiagen PCR Master Mix kit according to manufacturer's specifications. Thermocycler conditions included an initial denaturation step of 95°C for 5 minutes followed by 5 cycles of 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute; 5 cycles of 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute; 5 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute; 5 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute; 20 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and a final elongation step of 10 minutes at 72°C. 2 µL of PCR product was electrophoresed in a 1% agarose gel containing 1 µg/mL ethidium bromide at 100V for 20 minutes. DNA bands were visualized with 305 nm ultraviolet light. For the Wnt signaling array, MDA-MB-231 cells were seeded at a density of 1.5x10^6 cells in T-75 flasks. After 24 hours, media was replaced with serum free RPMI. After 18 hours of serum starvation, cells were treated with compound or vehicle control in serum free media. After 4 hours of incubation with compound, cells were lysed and RNA was extracted using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen) according to manufacturer’s protocol. RNA (500 ng) was then reverse transcribed to cDNA using the
RT² First Strand Synthesis kit (SA Biosciences) according to manufacturer’s specifications. The expression of Wnt related genes such as those encoding β-catenin and cyclin D1 was then determined using the Wnt Signaling PCR Array (SA Biosciences) in 96-well format. With the exception of performing 60 amplification cycles, the assay was performed according to manufacturer’s specifications using an Eppendorf Real Plex thermal cycler.

Table 4. Sequences of oligonucleotide primers used for PCR amplification of cDNA

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE5 Forward</td>
<td>GGTGTGCTGAGAGAGTTACCA</td>
</tr>
<tr>
<td>PDE5 Reverse</td>
<td>GAGCACTGGTCCCCCTCAT</td>
</tr>
<tr>
<td>Actin Forward</td>
<td>TCTACAATGAGCTGCTGTG</td>
</tr>
<tr>
<td>Actin Reverse</td>
<td>AATGTCACGCACGATTTCCC</td>
</tr>
</tbody>
</table>

siRNA

HMEC and ZR75-1 cells were transfected with OptiMEM media containing 0.5% SureFECT transfection reagent and 200 nM of either negative control or PDE5A siRNA constructs. Transfection was carried out under standard cell culture conditions for 96 hours if no drug treatment followed or 24 hours prior to drug treatment.

Tcf/Lef Reporter Assay

Activity of T-cell factor and lymphoid enhancer factor (Tcf/Lef) transcription factors was measured in ZR75-1 cells as follows. ZR-75-1 cells were plated into 24-well plates then were transiently transfected after 24 hours of incubation with 0.1 µg of the Super8XTOPFlash TOPFlash construct (kindly provided by Dr. Randall T. Moon,
University of Washington, Seattle, WA) and 0.1 µg of the β-galactosidase-expressing vector (Promega, Madison, WI). After 24 hours of transfection, cells were treated with the compound at the indicated concentrations. After 24 hours of treatment, cells were lysed and both luciferase and β-galactosidase activities were determined. The luciferase and β-galactosidase assay systems were purchased from Promega. The luciferase activity was normalized to the β-galactosidase activity.

Experimental Design and Data Analysis

Drug effects on cell growth and PDE activity were measured and the potency expressed as the concentration resulting in 50% inhibition when compared to the vehicle control (IC$_{50}$). For growth assays, the IC$_{50}$ value was determined by testing a range of 8 concentrations with a minimum of 4 replicates per dose. Dose response curves were constructed using Prism5 software (Graphpad), which calculates IC$_{50}$ values using a four parameter logistic equation. All experiments were repeated twice in order to determine the reproducibility of the results. All values represent a comparison between drug treatment at the specified concentration and vehicle treated controls. All error bars represent standard error of the mean (SEM). Calculation of p values was done by comparing the specified treatment group to vehicle treated controls using a student’s t test.
PDE5 ACTIVITY IS NECESSARY FOR THE GROWTH AND SURVIVAL OF HUMAN BREAST TUMOR CELLS THROUGH ITS TERMINATION OF GROWTH-INFRINGEMENT CYCLIC GMP SIGNALING AND SUBSEQUENT ATTENUATION OF WNT/β-CATENIN MEDIATED TRANSCRIPTION

Introduction

The cyclic nucleotides, cAMP and cGMP, serve as important second messengers responsible for mediating a number of physiological processes including cell adhesion, glucose metabolism, vasodilation, and gene transcription (Rehmann, Wittinghofer et al. 2007). Both cAMP and cGMP have been shown to have antiproliferative and proapoptotic effects, depending on the cell type, compartmentalization of the signal, and stimulus activating the signal (Suenobu, Shichiri et al. 1999; Martin, Dransfield et al. 2001; Pitari, Di Guglielmo et al. 2001; Zhou, Li et al. 2005; Castro, Verde et al. 2006). Furthermore, alterations in either the expression or the activity of cyclic nucleotide signaling components have been noted in various malignancies, including breast cancer (Cohen, Straka et al. 1976; DeRubertis and Craven 1976; Singer, Sherwin et al. 1976; Aleksijevic, Lang et al. 1983; Aleksijevic, Lugnier et al. 1987; Heinonen and Metsa-Ketela 1988; Ciardiello, Pepe et al. 1993; Drees, Zimmermann et al. 1993; Kool, de Haas et al. 1997; Carlson, Smithers et al. 1999; Marko, Pahlke et al. 2000; Piazza, Thompson et al. 2001; Deguchi, Thompson et al. 2004; Lerner and Epstein 2006; Li, Schulz et al. 2007; Zhang, Murray et al. 2008; Pertuit, Barlier et al. 2009; Kwon, Wang et al. 2010), suggesting that aberrant cyclic nucleotide signaling may play an important role in tumorigenesis. However, little is known about whether cyclic nucleotides regulate
proliferation and/or apoptosis of breast tumor cells or which cyclic nucleotide signaling mediators, particularly PDE isozymes, are responsible for signal transduction in these cells. Here we investigated the role of cyclic nucleotide signaling in the growth of human breast cells and whether activation of these pathways through stimulation of the cyclase enzymes or inhibition of the PDE enzymes is sufficient for inhibition of breast tumor cell growth.

Results

cGMP is a proapoptotic and antiproliferative signal in human breast cells

To determine whether cyclic nucleotide signaling serves as a growth-inhibitory signal in human breast cells, we evaluated the effects of the AC activator, forskolin, and the NO donor and GC activator, NOR-3, on growth of two breast tumor cell lines, MDA-MB-231 and ZR75-1, and primary mammary epithelial cells (HMEC) after 72 hours of treatment. As shown in Figure 3A, activation of cAMP signaling with forskolin did not significantly inhibit growth of any of the cell lines at concentrations up to 100 µM. Conversely, activation of cGMP signaling with NOR-3 inhibited growth of all three cell lines (Figure 3B). Interestingly, with an average IC$_{50}$ value of 77 µM in the tumor cells compared to 33 µM in the HMEC, normal breast cells were more sensitive to the growth inhibitory effects of GC activation than were tumor cells.

The growth inhibitory effects of NOR-3 were associated with both induction of apoptosis and inhibition of proliferation. As shown in Figure 3C, NOR-3 treatment resulted in a modest, but significant, increase in the activity of effector caspases 3 and 7 after 6 hours of treatment in all three cell lines. Consistent with the increased sensitivity of the HMEC to the growth inhibitory activity of NOR-3, HMEC were also most
sensitive to the proapoptotic effects of the compound. NOR-3 also caused a dose-
dependent reduction in the number of proliferating MDA-MB-231 breast tumor cells
after 24 hours as measured by BrdU incorporation (Figure 3D).

Selective inhibition of certain cGMP PDE isozymes selectively inhibits growth and
induces apoptosis of breast tumor cells

Although activation of cGMP signaling is sufficient to inhibit breast tumor cell
growth, nonselective activation of this pathway, such as that which is achieved through
GC activators, is not a plausible pharmacological approach due to the myriad of roles that
cGMP plays in normal physiology and the potential for adverse effects. For this reason,
we investigated different PDE isozyme-selective inhibitors as a potentially safer and
more effective strategy for targeting this pathway. First, we screened a series of PDE
inhibitors for inhibition of SKBR3 breast tumor cell growth, looking specifically at the
effects of the PDE1 inhibitor 8-methoxymethyl-3-isobutyl-1-methylxanthine (8-m-
IBMX), the PDE2 inhibitor EHNA, the PDE3 inhibitor milrinone, the PDE4 inhibitor
rolipram, the PDE5 inhibitor MY5445, the nonselective PDE inhibitors zaprinast and
dipyridamole, and the PDE2 and PDE3 inhibitor trequinsin, each of which were screened
at a concentration of 50 µM. The PDE5 selective inhibitor tadalafil was also used but at
a concentration of 1 µM. As shown in Figure 4A, only EHNA, MY5445, and trequinsin
significantly inhibited the growth of the SKBR3 cells.

Next, we evaluated the active compounds for growth inhibitory activity against
additional tumor cells lines, MDA-MB-231 and ZR75-1, and HMEC at multiple
concentrations in order to evaluate potency. The growth inhibitory activity of EHNA
appeared to be specific for SKBR3 cells, having only a modest effect on the growth of
MDA-MB-231 cells and no effect on the growth of HMEC or ZR75-1 cells (Figure 4B). Conversely, MY5445 (Figure 4C) and trequinsin (Figure 4D) inhibited the growth of all four cell lines. Interestingly, both compounds showed selectivity for the tumor cells compared to the HMEC in terms of growth inhibitory potency and/or efficacy. For example, MY5445 inhibited the growth of HMEC by about 40% compared with 60% for the ZR75-1 and SKBR3 cells and 80% for MDA-MB-231. On the other hand, trequinsin demonstrated similar efficacy in all of the cell lines, but, with an average IC$_{50}$ value of 35 µM in the breast tumor cells compared with almost 60 µM in the HMEC, trequinsin was significantly more potent for tumor cell growth inhibition.

Similar to NOR-3, growth inhibition by both MY5445 and trequinsin was associated with induction of apoptosis and inhibition of proliferation. Reflecting the tumor selective growth inhibitory activity of the compounds, MY5445 (Figure 5A) and trequinsin (Figure 5B) were more potent and efficacious for inducing apoptosis of the tumor cells compared to the HMEC when measured as activation of the effector caspases 3 and 7. Furthermore, apoptosis induction occurred at concentrations that also inhibited growth. Conversely, neither MY5445 (Figure 5C) nor trequinsin (Figure 5D) demonstrated tumor selectivity in terms of inhibition of proliferation, an effect that occurred at concentrations significantly lower than those necessary for overall inhibition of growth.

We next looked at the effects of MY5445 and trequinsin on cGMP signaling in the breast cells to verify that the growth inhibitory effects of these compounds were related to activation of cGMP signaling. As shown in Figure 6A, 100 µM MY5445 and 10 µM trequinsin significantly inhibited cGMP hydrolysis in the tumor cell lysates by an
average of 52% and 72%, respectively, but had little effect on cGMP hydrolysis in the HMEC lysate. Consistent with these results, 30 minutes of trequinsin treatment resulted in a two-fold increase in intracellular cGMP levels in SKBR3 tumor cells, but had no significant effect on cGMP levels in the HMEC (Figure 6B).

To determine whether these changes in cGMP levels are sufficient to activate downstream cGMP signaling, we measured the activation of PKG, an important cGMP signaling mediator. VASP is a ubiquitously expressed cytoskeletal component that is preferentially phosphorylated at its serine 239 residue by PKG, and measurement of VASP phosphorylation at this residue (p-VASP\textsuperscript{Ser239}) has been documented as an important intracellular marker of PKG activation (Deguchi, Soh et al. 2002). As shown in Figure 6C, 3 hours of MY5445 treatment caused a dose-dependent increase in the expression of p-VASP\textsuperscript{Ser239} in the MDA-MB-231 and ZR75-1 breast tumor cells despite no change in overall VASP expression. Conversely, MY5445 did not increase the expression of p-VASP\textsuperscript{Ser239} in the HMEC even though the levels of VASP protein expressed in these cells was comparable to the levels expressed in the tumor cells.

To determine which of the cGMP PDE isozymes are targeted by MY5445 and trequinsin, we evaluated the sensitivity of recombinant PDE1, 2, 3, 5, and 9 isozymes to inhibition by the compounds. As shown in Table 5, MY5445 was confirmed to be selective for PDE5, having no measurable effect on any of the other isozymes at concentrations up to 200 µM. On the other hand, trequinsin was found to be highly selective for PDE3, inhibiting both cAMP and cGMP hydrolysis by this enzyme at concentrations less than 1 nM, which was sufficiently lower than the 10 µM concentration necessary for inhibition of cGMP hydrolysis in the tumor cell lysates.
However, we found that trequinsin was also able to inhibit PDE2 and PDE5 with IC$_{50}$ values of 1.3 and 3.4 µM, respectively, which are much closer to the concentrations for inhibition of tumor cell cGMP PDE.

Table 5. PDE isozyme selectivity of trequinsin and MY5445.

<table>
<thead>
<tr>
<th>Recombinant PDE Isozyme</th>
<th>Trequinsin Sensitivity</th>
<th>MY5445 Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
</tr>
<tr>
<td>1A</td>
<td>Inactive</td>
<td>Inactive</td>
</tr>
<tr>
<td>2A</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>3A</td>
<td>0.00023</td>
<td>0.00067</td>
</tr>
<tr>
<td>5A</td>
<td>-</td>
<td>3.4</td>
</tr>
<tr>
<td>9A</td>
<td>-</td>
<td>Inactive</td>
</tr>
</tbody>
</table>

Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3336. Copyright 2009 by AACR. Adapted with permission.

Sensitivity values represent IC$_{50}$ values in µM. “Inactive” indicates that there was no appreciable inhibition of cyclic nucleotide hydrolysis at concentrations of up to 200 µM. - indicates that there was no enzyme activity for hydrolyzing the given nucleotide.

cGMP PDE inhibition is associated with attenuation of Wnt/β-catenin mediated transcription

Several mechanisms have been proposed for mediating the proapoptotic effects of cGMP signaling, including changes in intracellular calcium levels, activation of p21, and attenuation of Wnt/β-catenin mediated transcription (Feil and Kemp-Harper 2006; Cen, Deguchi et al. 2008; Kwon, Wang et al. 2010). Because of the potential importance of oncogenic Wnt/β-catenin signaling for growth and survival of breast tumor cells
(Smalley and Dale 2001), we chose to evaluate inhibition of Wnt/β-catenin signaling as a potential mechanism involved in the growth inhibitory activity of MY5445 and trequinsin. Canonical Wnt signaling requires translocation of β-catenin from the cytosol to the nucleus where it may interact with the Tcf/Lef family of transcription factors to mediate expression of growth promoting and apoptosis inhibiting proteins such as cyclin D1 and survivin (Smalley and Dale 2001). Because of its central role in mediating Wnt signaling, we first evaluated the effects of MY5445 treatment on the expression of the ctnnb1 gene, which is the gene that encodes the β-catenin protein, in MDA-MB-231 cells by semi-quantitative real time PCR. As shown in Figure 7A, 4 hours of 100 µM MY5445 treatment resulted in complete loss of detection of β-catenin mRNA.

We also developed an imaging assay to measure nuclear localization of β-catenin protein in response to Wnt stimulation, which is a hallmark of active canonical Wnt signaling. As shown in the representative images in Figure 7B, 4 hours of Wnt stimulation resulted in more than one-third of the cells demonstrating active Wnt signaling, which is defined as colocalization of Alexa Fluor 488 labeled β-catenin and the Draq5 labeled nuclei (arrows). As depicted in Figure 7B and quantified in Figures 7C and 7D, 2 hours of pretreatment with MY5445 or trequinsin prevented Wnt-stimulated β-catenin nuclear localization by as much as 50% or 30%, respectively, in both MDA-MB-231 and ZR75-1 breast tumor cells.

To determine whether the decrease in β-catenin expression and β-catenin nuclear localization that are mediated by MY5445 are sufficient to affect the expression of Tcf/Lef transcribed genes, we measured the expression of cyclin D1 and survivin after 3 hours of MY5445 treatment by immunoblotting. As shown in Figure 8, MY5445 caused
a dose-dependent decrease in cyclin D1 and survivin expression in both MDA-MB-231 and ZR75-1 breast tumor cells. The decrease in expression of these proteins was inversely proportional to the increase in p-VASP$^{Ser239}$ expression.

*Breast tumor cells rely heavily on PDE5 for cGMP hydrolysis*

The data with NOR-3, MY5445, and trequinsin suggest that, while activation of cGMP signaling is sufficient to inhibit breast cell growth, selective inhibition of cGMP PDE isozymes, particularly PDE5, may be an effective approach for targeting the cGMP signaling pathway for breast cancer chemoprevention due to its apparent tumor selectivity. We hypothesized that differential expression of cGMP PDE isozymes between normal and tumor cells may account for the differential sensitivity of these cells to growth inhibition by MY5445 and trequinsin. To study this possibility, we first determined the relative activity of the cGMP hydrolyzing PDE1, 2, 3, and 5 families in the breast cell lines by measuring the sensitivity of cell lysates to specific activators or inhibitors of the PDE isozymes. As shown in Figure 9A, the addition of calcium (Ca$^{2+}$) and calmodulin (CaM), positive regulators of PDE1, did not significantly affect cGMP hydrolysis in ZR75-1 or MDA-MB-231 tumor cell lysates, but they did cause a twelve-fold increase in cGMP PDE activity in the HMEC lysate. The PDE2 selective inhibitor EHNA (Figure 9B) and the PDE3 selective inhibitor milrinone (Figure 9C) had only minimal effects on cGMP hydrolysis in any of the cell lysates. The PDE5 selective inhibitor sildenafil, on the other hand, had little effect on cGMP hydrolysis in the HMEC lysate but caused greater than 40% inhibition in both tumor cell lysates (Figure 9D).

We also looked at the expression of cGMP PDE isozymes by immunoblotting in the aforementioned cell lines as well as an additional non-tumorigenic breast cell line,
MCF10A. As shown in Figure 10, normal and tumor cells displayed very different cGMP PDE isozyme expression patterns. For example, PDE1 and PDE9 did not appear to be expressed in any of the breast tumor cell lines. However, two isozymes of each PDE1 and PDE9 were expressed in HMEC and a single isozyme of each were expressed in MCF10A. Conversely, the tumor cells expressed very high levels of PDE5 which was not detected in either of the types of normal cells.

PDE5 is necessary for breast tumor cell growth and survival

To further investigate the importance of PDE5 for growth and survival of human breast cells, we utilized PDE5 siRNA to decrease expression of the protein in HMEC and ZR75-1 cells. As shown in Figure 11A, 96 hours of transfection resulted in a greater than 90% or 50% decrease in PDE5 mRNA detected in HMEC or ZR75-1 cells, respectively, when measured by reverse transcription and PCR. Furthermore, 96 hours of transfection resulted in a 50% or 80% reduction in PDE5 protein detected in HMEC or ZR75-1 cells, respectively, by immunoblotting (Figure 11B).

We also looked at the effects of PDE5 knockdown on cyclic nucleotide hydrolysis in the cell lysates. Consistent with PDE5 being cGMP specific, suppressing PDE5 expression did not significantly affect cAMP hydrolysis in either cell line (Figure 11C). Conversely, PDE5 knockdown caused a 40% reduction in cGMP hydrolysis in the ZR75-1 cells but had no effect on cGMP hydrolysis in the HMEC, which is similar to the effects of the PDE5 selective inhibitor sildenafil as described above.

To determine the importance of PDE5 for growth of human breast cells, we measured the effects of PDE5 knockdown on growth, apoptosis, and proliferation. As shown in Figure 12A, suppression of PDE5 with siRNA cause a 30% reduction in ZR75-
1 growth without affecting HMEC growth, which mirrors the effect of PDE5 suppression on cGMP PDE activity. This reduction in growth in the ZR75-1 cells was associated with an induction of apoptosis, as measured by a two-fold increase in the activity of caspases 3 and 7 (Figure 12B), with no change in proliferation, as measured by BrdU incorporation (Figure 12C). Consistent with the lack of an effect on growth, PDE5 siRNA did not affect apoptosis or proliferation of HMEC.

We also measured the effect of PDE5 knockdown on sensitivity of ZR75-1 cells to growth inhibition by MY5445. After 24 hours of transfection, cells were treated with MY5445, and growth was assayed after an additional 72 hours of incubation. As shown in Figure 12D, PDE5 knockdown sensitized the breast tumor cells to the growth inhibitory activity of MY5445 as evidenced by a 25% decrease in the IC$_{50}$ value.

We then evaluated the effect of PDE5 suppression with siRNA on Wnt/β-catenin mediated transcription. As shown in Figure 13A, PDE5 siRNA did not cause a significant change in the expression of cyclin D1 in either HMEC or ZR75-1, which is consistent with the lack of an effect on proliferation. Survivin, which is an oncogene that is primarily expressed in tumor cells, was not detected in the HMEC, which is consistent with the lack of tumorigenicity of these cells. However, survivin was expressed in ZR75-1 cells and PDE5 knockdown resulted in a significant reduction in its expression. We also measured the effects of PDE5 siRNA on Wnt stimulated β-catenin nuclear localization using the imaging assay described above. As shown in Figure 13B, PDE5 siRNA caused a modest, but significant, reduction in nuclear levels of β-catenin in ZR75-1 breast tumor cells by about 10%.
Discussion

Acting as second messengers between an extracellular signal and its elicited intracellular response, the cyclic nucleotides, cAMP and cGMP, serve as important signal transduction molecules, regulating a number of physiological processes (Rehmann, Wittinghofer et al. 2007). Both cAMP and cGMP signaling have been shown to have negative effects on cell growth and survival, depending on the cell type, extracellular environment, stimulus, and subcellular localization (Suenobu, Shichiri et al. 1999; Martin, Dransfield et al. 2001; Pitari, Di Guglielmo et al. 2001; Zhou, Li et al. 2005; Castro, Verde et al. 2006). Furthermore, dysregulation of cyclic nucleotide signaling has been reported in various malignancies, including breast cancer, suggesting that alteration of these pathways may play an important role in tumorigenesis (Cohen, Straka et al. 1976; DeRubertis and Craven 1976; Singer, Sherwin et al. 1976; Aleksijevic, Lang et al. 1983; Aleksijevic, Lugnier et al. 1987; Heinonen and Metsa-Ketela 1988; Ciardiello, Pepe et al. 1993; Drees, Zimmermann et al. 1993; Kool, de Haas et al. 1997; Carlson, Smithers et al. 1999; Marko, Pahlke et al. 2000; Piazza, Thompson et al. 2001; Deguchi, Thompson et al. 2004; Lerner and Epstein 2006; Li, Schulz et al. 2007; Zhang, Murray et al. 2008; Pertuit, Barlier et al. 2009; Kwon, Wang et al. 2010). However, little is known about the effects of cyclic nucleotide signaling in human breast cells or whether these pathways regulate growth and/or survival of such cells. Here we show that cGMP, but not cAMP, serves as a growth inhibitory signal in human breast cells and that selective inhibition of PDE5 could serve as a novel mechanism for chemoprevention by selectively activating pro-apoptotic cGMP signaling in breast tumor cells.
The growth inhibitory activity of cGMP signaling was first demonstrated through studies performed with the GC activator, NOR-3. This compound was shown to inhibit growth of both normal and tumor cells through a dual mechanism involving inhibition of proliferation and induction of apoptosis. As evidence that these effects are specific to the cGMP pathway and not related to cyclic nucleotide signaling in general, the AC activator, forskolin, had no effect on breast cell growth. While this is some of the first evidence suggesting negative effects of cyclic nucleotide signaling on breast cell growth, these findings are consistent with previous studies that have demonstrated an antiproliferative and proapoptotic role of cGMP signaling in certain epithelial cell types, particularly colon epithelial cells (Pitari, Di Guglielmo et al. 2001; Zhu, Vemavarapu et al. 2005; Fraser, Chan et al. 2006; Cen, Deguchi et al. 2008). Unfortunately, the lack of tumor selectivity obtained with GC activation suggests the potential for toxicity, excluding this as a viable mechanism for chemoprevention.

However, the number of protein products that comprise the PDE superfamily suggest that inhibiting cGMP hydrolysis may be a promising approach for chemoprevention. Targeting a specific PDE isozyme that is only lowly expressed outside of the tumor could potentially reduce toxicity while enhancing anticancer efficacy of a compound. This idea is supported by the studies presented here, which demonstrated that the vast majority of PDE inhibitors have little effect on either normal or tumor cell growth. Even the two compounds that did have an effect on breast tumor cell growth were less potent and/or efficacious for inhibiting the growth of the normal breast cells compared to the tumor cells. Furthermore, the growth inhibitory effect on the normal cells, which occurred only at high concentrations, appeared to be predominantly
antiproliferative, or cytostatic, rather than proapoptotic, suggesting that withdrawal of the compound could result in reversal of the effects on normal cells.

While the involvement of PDE2 and PDE3 inhibition cannot be excluded from mediating the growth inhibitory effects of trequinsin, the PDE5 selectivity of MY5445 suggests that inhibition of PDE5 is sufficient for inhibition of tumor cell growth. This was confirmed with PDE5 siRNA, which demonstrated that suppression of PDE5 in ZR75-1 cells caused proportional suppression in cell growth and inversely proportional activation of apoptosis. Surprisingly, PDE5 knockdown did not alter proliferation, whereas treatment with MY5445 did. While this difference suggests that additional mechanisms may be involved in the antiproliferative effects of MY5445, the sensitization of cells to the growth inhibitory activity of MY5445 through PDE5 knockdown demonstrates that PDE5 inhibition is responsible for at least a portion of the growth inhibitory activity of MY5445 treatment.

Interestingly, suppression of PDE5 with MY5445 or siRNA was highly selective for tumor cells, and the HMEC were relatively insensitive to both the cGMP PDE inhibitory and growth inhibitory effects. The most plausible explanation for this difference in sensitivity is the difference in PDE isozyme expression between normal and tumor cells. We found that PDE5 is the predominantly expressed cGMP degrading PDE isozyme in breast tumor cells and that it is largely responsible for cGMP hydrolysis in lysates from these cells. Conversely, normal breast cells have very low levels of PDE5 expression and appear to rely more heavily on PDE1 and/or PDE9 for cGMP hydrolysis. This is consistent with studies that have been performed in colon, bladder, and lung, which have described overexpression of PDE5 in tumor tissue when compared to its
normal counterpart (Thompson, Piazza et al. 2000; Piazza, Thompson et al. 2001; Whitehead, Earle et al. 2003). Because tumor cells are reliant for PDE5 hydrolysis of the growth inhibitory cGMP signal, we believe that selective inhibition of this enzyme results in apoptosis induction through a mechanism involving activation of PKG and attenuation of Wnt/β-catenin signaling as depicted in Figure 14A. However, the expression of alternate cGMP degrading PDE isozymes (e.g. PDE1 and PDE9) in normal cells likely acts as a compensatory mechanism, allowing for cGMP hydrolysis even in the presence of PDE5 inhibitors, thereby desensitizing these cells to the growth inhibitory effect of PDE5 inhibition (Figure 14B). The resulting tumor selectivity suggests that PDE5 may be a promising anticancer target.

Furthermore, the potential involvement of Wnt/β-catenin signaling in the growth inhibitory mechanism of PDE5 inhibition also suggests that this isozyme may be an interesting anticancer target. PDE5 inhibition was associated with attenuation of Wnt signaling as evidenced by decreased β-catenin mRNA, decreased nuclear localization of β-catenin protein, and decreased expression of Tcf/Lef-regulated genes. This is consistent with previous reports, which have demonstrated that activation of PKG can inhibit Wnt signaling at the level of β-catenin-mediated gene transcription (Thompson, Piazza et al. 2000; Kwon, Wang et al. 2010). While Wnt signaling plays an important role in lobular development during pregnancy, this pathway is largely quiescent in differentiated adult mammary cells. However, deregulation of this pathway resulting in amplified Wnt signaling has been noted in a substantial number of breast carcinomas and has been shown in animal models to promote breast tumorigenesis (Smalley and Dale 2001). For example, mouse mammary tumor virus (MMTV) proviral integration near the
*wnt1* gene leads to Wnt1 protein overexpression, which is sufficient to generate hyperplasias (Smalley and Dale 2001). Furthermore, activated Wnt/β-catenin signaling in breast cancer stem cells has been implicated as a mechanism of resistance to conventional chemotherapeutics (Ischenko, Seeliger et al. 2008). Consequently, targeting Wnt/β-catenin signaling has emerged as a leading approach for the development of novel anticancer agents. Moreover, because Wnt signaling is rarely active in normal, non-tumorigenic breast cells, the involvement of this pathway in the growth inhibitory mechanism of PDE5 inhibition could serve as an additional explanation of its tumor selectivity.

Taken together, these findings demonstrate the importance of PDE5 expression and activity for the growth and survival of human breast tumor cells and suggest that inhibition of PDE5, activation of cGMP signaling, and attenuation of Wnt/β-catenin mediated transcription is a highly selective mechanism for inducing tumor cell apoptosis. While more studies are necessary to elucidate the mechanism(s) through which cGMP signaling affects Wnt/β-catenin signaling, this work indicates the utility of PDE5 as a novel oncogenic target for future drug discovery efforts.
Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3336. Copyright 2009 by AACR. Adapted with permission.

Figure 3. Effects of cAMP and cGMP signaling on breast cell growth. A, lack of an effect of the AC activator, forskolin, on growth of HMEC, MDA-MB-231, and ZR75-1 cells after 72 hours of treatment. B, dose-dependent inhibition of HMEC, MDA-MB-231, and ZR75-1 growth after 72 hours of treatment with the GC activator, NOR-3. C, dose-dependent increase in caspases 3 and 7 after 6 hours of NOR-3 treatment. D, dose-dependent effect of NOR-3 treatment on BrdU incorporation in MDA-MB-231 breast tumor cells after 24 hours of treatment.
Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3336. Copyright 2009 by AACR. Adapted with permission.

Figure 4. Growth inhibitory activity of PDE isozyme inhibitors in human breast cells. A, single dose evaluation of PDE isozyme selective inhibitors in SKBR3 breast tumor cells. 8-m-IBMX (PDE1), EHNA (PDE2), milrinone (PDE3), rolipram (PDE4), MY5445 (PDE5), zaprinast (cGMP PDE), dipyridamole (cGMP PDE), and trequinsin (PDE2, 3) were each screened at 50 µM. Tadalafil (PDE5) was screened at 1 µM. B, growth inhibitory activity of EHNA. C, growth inhibitory activity of MY5445. D, growth inhibitory activity of trequinsin. Each growth assay was performed after 72 hours of treatment.
Figure 5. Effects of PDE inhibition on apoptosis and proliferation of human breast cells. 
A, dose-dependent effects of MY5445 on caspase activation in breast cells after 6 hours of treatment. 
B, dose-dependent effects of trequinsin on caspase activation after 6 hours of treatment. 
C, dose-dependent effects of MY5445 on breast cell proliferation as measured by BrdU incorporation after 24 hours of treatment. 
D, dose-dependent effects of trequinsin on breast cell proliferation as measured by BrdU incorporation after 24 hours of treatment.
Figure 6. Activation of cGMP signaling through PDE inhibition. A, inhibition of cGMP hydrolysis by 100 µM MY5445 or 10 µM trequinsin in lysates from SKBR3, MDA-MB-231, and ZR75-1 breast tumor cells compared to HMEC. B, dose-dependent effects of trequinsin on intracellular cGMP levels in SKBR3 breast tumor cells or HMEC after 30 minutes of treatment. C, dose-dependent effects of MY5445 treatment on PKG activation as measured by expression of p-VASP<sub>Ser239</sub> in MDA-MB-231 and ZR75-1 breast tumor cells and HMEC after 3 hours of treatment. Total VASP is shown for comparison. RhoA was used as a loading control.
Figure 7. Effects of PDE inhibition on Wnt/β-catenin mediated transcription. 

A, Effect of 100 µM MY5445 on β-catenin gene expression in MDA-MB-231 breast tumor cells after 4 hours of treatment as measured by semi-quantitative real time PCR.

B, representative images depicting induction of β-catenin nuclear localization with Wnt stimulation and inhibition of this nuclear localization with 1 hour of 100 µM MY5445 or 50 µM of trequinsin pretreatment in MDA-MB-231 breast tumor cells. Arrows point to cells considered to have active canonical Wnt signaling as measured by colocalization of Draq5 nuclear stain and Alexa Fluor 488 labeled β-catenin.

C, quantification of the dose-dependent effects of MY5445 on Wnt-stimulated β-catenin nuclear localization in MDA-MB-231 and ZR75-1 breast tumor cells.

D, quantification of the dose-dependent effects of trequinsin on Wnt-stimulated β-catenin nuclear localization in MDA-MB-231 and ZR75-1 breast tumor cells.
Figure 8. Effects of PDE inhibition of expression of Tcf/Lef regulated genes. Dose-dependent effects of MY5445 on the expression of cyclin D1 and survivin in MDA-MB-231 and ZR75-1 breast tumor cells after 3 hours of treatment as measured by immunoblotting. Activation of PKG as measured by expression of p-VASP$^{\text{Ser239}}$ is included for comparison. RhoA was used as a loading control.
Figure 9. Relative activities of cGMP PDE isozymes in human breast cells. 

A, effects of the PDE1 activators calcium (Ca\(^{2+}\)) and calmodulin (CaM) on cGMP hydrolysis in HMEC and MDA-MB-231 and ZR75-1 breast tumor cell lysates. B, effects of the PDE2 selective inhibitor EHNA on cGMP hydrolysis in the breast cell lysates. C, effects of the PDE3 selective inhibitor milrinone on cGMP hydrolysis in the breast cell lysates. D, effects of the PDE5 selective inhibitor sildenafil on cGMP hydrolysis in the breast cell lysates.
Figure 10. Expression of cGMP PDE isozymes in human breast cells. HMEC and MCF10A represent “normal,” or non-tumorigenic, breast cells. MDA-MB-231, SKBR3, and ZR75-1 represent breast tumor cells.
Figure 11. Verification of PDE5 knockdown with siRNA.  

A, effect of PDE5 siRNA on PDE5 mRNA levels in HMEC and ZR75-1 cells as measured by reverse transcription and PCR. Gel images are shown on top and densitometry on bottom. Actin was used as a PCR and loading control.  

B, effect of PDE5 siRNA on PDE5 protein levels in HMEC and ZR75-1 cells as measured by immunoblotting. Blots are shown on top and densitometry on bottom. RhoA was used as loading control.  

C, effect of PDE5 siRNA on cAMP and cGMP hydrolysis in HMEC and ZR75-1 cells. All experiments were performed using lysates from cells that had been transfected with 0.5% SureFECT transfection reagent and 200 nM siRNA for 96 hours.
Figure 12. Effect of PDE5 knockdown on growth and survival of human breast cells. 

A, effect of PDE5 siRNA on growth of HMEC and ZR75-1 cells after 96 hours of transfection. 

B, effect of PDE5 siRNA on caspase activation in HMEC and ZR75-1 cells. 

C, effect of PDE5 siRNA on proliferating cells as measured by BrdU incorporation in HMEC and ZR75-1 cells. 

D, effect of PDE5 siRNA on sensitivity of ZR75-1 cells to growth inhibition induced by MY5445.
Figure 13. Effect of PDE5 knockdown on Wnt/β-catenin mediated transcription.  

A, effect of PDE5 siRNA on the expression of cyclin D1 and survivin in HMEC and ZR75-1 cells as measured by immunoblotting. RhoA was used as a loading control.  

B, effect of PDE5 siRNA on nuclear translocation of β-catenin protein in ZR75-1 cells.
Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3338. Copyright 2009 by AACR. Adapted with permission.

Figure 14. Proposed mechanism of growth inhibition mediated by PDE5 inhibition. A, overexpression of and heavy reliance on PDE5 in breast tumor cells sensitizes these cells to the antiproliferative and proapoptotic effects of PDE5 inhibition. B, PDE5 is not important for cGMP hydrolysis in normal breast cells, which makes these cells less sensitive to the antiproliferative and proapoptotic effects of PDE5 inhibition.
SULINDAC SULFIDE SELECTIVELY INHIBITS GROWTH OF BREAST TUMOR CELLS THROUGH INHIBITION OF PDE5, ACTIVATION OF PKG, AND ATTENUATION OF WNT/β-CATENIN MEDIATED TRANSCRIPTION

Introduction

NSAIDs have shown promising activity for the prevention of breast cancer (Harris, Namboodiri et al. 1996; Harris, Chlebowski et al. 2003; Kwan, Habel et al. 2007), but their use for such indications is not practical because of gastrointestinal, renal, and cardiovascular toxicities that result from COX-1 and/or COX-2 inhibition and the depletion of physiologically important prostaglandins (Vane and Botting 1998; Mukherjee 2002). Previous studies have concluded that a COX-independent mechanism may be responsible for their tumor cell growth inhibitory and apoptosis inducing activities, which suggests the feasibility of developing safer and more efficacious drugs for cancer chemoprevention by targeting such mechanisms (Alberts, Hixson et al. 1995; Piazza, Rahm et al. 1995; Hanif, Pittas et al. 1996; Elder, Halton et al. 1997; Piazza, Rahm et al. 1997; Soh, Kazi et al. 2008). The non-COX inhibitory sulfone metabolite of the NSAID prodrug sulindac has been shown to inhibit cGMP PDE (Thompson, Piazza et al. 2000; Piazza, Thompson et al. 2001; Whitehead, Earle et al. 2003). However, the specific PDE isozyme responsible has not been identified, and this putative mechanism has yet to be studied for NSAIDs with more potent anticancer activity or in models of breast cancer. Here we investigated cGMP PDE inhibition as a potential COX-independent mechanism involved in the anticancer activity of the potent NSAID, SS, in various human breast cell lines.
Results

The anticancer activity of certain NSAIDs is more closely associated with cGMP PDE inhibition than with COX-2 inhibition

To evaluate whether cGMP PDE is a potential COX-independent target of the NSAIDs, particularly sulindac, we compared the growth inhibitory potency of a panel of chemically diverse NSAIDs and COX-2 inhibitors (Figure 15A) with their respective potencies for cGMP PDE inhibition in HT-29 colon tumor cell lysates and for inhibition of purified COX-2 enzyme. Among these anti-inflammatory drugs, a strong positive correlation was observed between potency for HT-29 cell growth inhibition and potency for inhibition of cGMP PDE activity with an $r^2$ value of 0.933 (Figure 15B). However, no correlation was observed by comparing potencies of these NSAIDs for COX-2 inhibition as previously reported (Warner, Giuliano et al. 1999) with potencies for HT-29 cell growth inhibition (Figure 15C). The salicylate family, which contains aspirin as well as non-carboxylate NSAIDs from the pyrazole (e.g. phenylbutazone) and oxicam (e.g. piroxicam) families displayed low potency for HT-29 growth inhibition and did not affect cGMP PDE activity.

SS selectively inhibits growth of breast tumor cells

To evaluate the anticancer activity of SS in breast tumor cells, we used two human breast tumor cell lines, the ER negative cell line MDA-MB-231 and the ER positive cell line ZR75-1. For comparison, we used a primary culture of normal human mammary epithelial cells (HMEC) as representative normal, non-tumorigenic breast cells. As shown in Figure 16A, SS inhibited the growth of each cell line as determined by measuring ATP content after 72 hours of treatment. However, with an average IC$_{50}$
value of 80 µM in the breast tumor cells compared to greater than 150 µM in the HMEC, SS demonstrated selectivity for inhibiting the growth of the tumor cells compared to the normal cells.

The inhibition of breast tumor cell growth by SS was associated with the induction of apoptosis as measured by the activation of effector caspases 3 and 7 after 6 hours of treatment. As shown in Figure 16B, treatment with SS resulted in a 2-12 fold increase in the activity of caspases 3 and 7 compared to vehicle treatment in the breast tumor cell lines at concentrations comparable to those that inhibited growth. Conversely, HMEC were resistant to SS-induced caspase activation despite undergoing apoptosis after treatment with 1 µM staurosporine, a non-selective apoptosis inducing agent. The selectivity observed for inducing tumor cell apoptosis mirrored the selectivity for inhibition of tumor cell growth.

SS treatment was also associated with an inhibition of proliferation. As shown in Figure 16C, 24 hours of SS treatment resulted in a dose-dependent decrease in proliferating cells in all three breast cell lines as measured by BrdU incorporation. This effect was surprisingly most pronounced in the HMEC, with as much as an 80% reduction in the number of proliferating cells. SS inhibited proliferation at concentrations as low as 25 µM, significantly lower than the concentrations necessary for the induction of apoptosis or inhibition of overall growth.

*Induction of apoptosis by SS is associated with inhibition of cGMP PDE, elevation of intracellular cGMP levels, and activation of PKG*

To determine the effects of SS on the activity of PDE enzymes, we developed a fluorescence polarization assay to simultaneously measure cGMP and cAMP hydrolysis
in cell lysates. As shown in Figure 17A, SS inhibited cGMP hydrolysis in lysates from ZR75-1 and MDA-MB-231 tumor cells with IC\textsubscript{50} values of 94 and 110 µM, respectively, but did not significantly affect cAMP hydrolysis (Figure 17B). Additionally, SS did not significantly affect cGMP or cAMP hydrolysis in lysates from HMEC.

Because intracellular cGMP levels reflect a balance between synthesis by GC and degradation by PDE, we measured the concentration of cGMP within cells after 45 minutes of SS treatment to determine whether inhibition of cGMP PDE by SS was sufficient to increase intracellular cGMP. As shown in Figure 18A, SS increased cGMP levels by 2-4 fold in MDA-MB-231 and ZR75-1 cells over a broad concentration range comparable to the concentration range resulting in maximum growth inhibition and caspase activation. Also, SS demonstrated selectivity for breast tumor cells and had little effect on cGMP levels in HMEC.

To determine whether the tumor cell growth inhibitory activity of SS is associated with the compound’s effects on cGMP levels, basal cGMP levels were modulated using GC activators and inhibitors prior to treatment with SS (Figure 18B). By pre-treating MDA-MB-231 cells with the GC inhibitor LY83583 for 1 hour to reduce basal cGMP levels, significantly higher concentrations of SS were required to suppress the growth of the cells as evidenced by a significant increase in the IC\textsubscript{50} value, from 97 µM with no pre-treatment to 148 µM with 1.25 µM LY83583. Conversely, pre-treatment of MDA-MB-231 cells with the NO donor and GC activator NOR-3 to increase basal cGMP levels significantly increased the sensitivity of breast tumor cells to the growth inhibitory activity of SS as evidenced by a reduction in the IC\textsubscript{50} value of SS, from 97 µM with no pre-treatment to 73 µM with 60 µM NOR-3.
To determine whether the increase in cGMP induced by SS is sufficient to result in PKG activation, we evaluated effects of SS on the expression of p-VASP\textsuperscript{Ser239} in MDA-MB-231, ZR75-1, and HMEC after 2 hours of treatment. As shown in Figure 19, SS increased the expression of p-VASP\textsuperscript{Ser239} in a dose-dependent manner in the MDA-MB-231 and ZR75-1 breast tumor cells, but had no effect on overall VASP expression. The concentrations of SS responsible for these effects are comparable to the concentrations necessary for growth inhibition and apoptosis induction. Conversely, SS had no effect on p-VASP\textsuperscript{Ser239} expression in the HMEC despite expression of VASP in these cells, which mirrors the lack of an effect on cGMP PDE activity, cGMP levels, and apoptosis, further demonstrating the tumor selectivity of SS.

To identify the PDE isozyme(s) target of SS, we evaluated the sensitivity of recombinant PDE isozymes to inhibition by SS. As shown in Table 6, only cGMP

<table>
<thead>
<tr>
<th>Recombinant PDE Isozyme</th>
<th>SS Sensitivity IC\textsubscript{50} (µM)</th>
<th>cAMP</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Inactive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A</td>
<td>Inactive</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>3A</td>
<td>Inactive</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>5A</td>
<td>-</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>6C</td>
<td>-</td>
<td>Inactive</td>
<td></td>
</tr>
<tr>
<td>9A</td>
<td>-</td>
<td>Inactive</td>
<td></td>
</tr>
<tr>
<td>10A</td>
<td>Inactive</td>
<td></td>
<td>Inactive</td>
</tr>
<tr>
<td>11A</td>
<td>Inactive</td>
<td></td>
<td>Inactive</td>
</tr>
</tbody>
</table>

Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3336. Copyright 2009 by AACR. Adapted with permission.

- indicates no detectable activity for hydrolysis of the given cyclic nucleotide. “Inactive” indicates that there was no appreciable inhibition of cyclic nucleotide hydrolysis at concentrations up to 200 µM.
hydrolysis by PDE 2, 3, and 5 was inhibited by SS. However, SS displayed selectivity for PDE5 and was about two-fold more potent for inhibition of this enzyme.

The ability of SS to inhibit PDE5 was further supported by molecular modeling studies using previously reported crystal structures of the catalytic site of PDE5. As shown in Figure 20A, silfenafil docked into the catalytic site of PDE5 with favorable binding kinetics. As previously reported, the compound formed salt bridges with the glutamine 817 (GLN817) residue, also known as the “glutamine switch,” which is important for preferential binding of cGMP to the active site of the enzyme (Zoraghi, Corbin et al. 2006). SS also docked into the catalytic site of PDE5 with favorable binding kinetics (Figure 20B). The positioning of the molecule resulted in salt bridges being formed with the GLN817 in a manner very similar to sildenafil. Additionally, the carboxylic acid of SS appeared to be positioned in close proximity to the metal ion of the catalytic site, which did not occur with sildenafil. Because the metal ion is important for catalysis, this feature of SS may provide for more efficient inhibition of the enzyme when compared to sildenafil (Zoraghi, Corbin et al. 2006).

As further evidence of the involvement of PDE5, we found that suppressing PDE5 expression with siRNA sensitized ZR75-1 breast tumor cells to the growth inhibitory activity of SS, as was evidenced by a significant reduction in the IC$_{50}$.

*Induction of apoptosis by SS is associated with attenuation of Wnt/β-catenin signaling*

Previous studies have suggested that inhibition of oncogenic Wnt/β-catenin signaling plays an important role in mediating the chemopreventive efficacy of some NSAIDs and their derivatives (Thompson, Piazza et al. 2000; Boon, Keller et al. 2004; Chang, Everley et al. 2005; Barker and Clevers 2006; Lu, Tinsley et al. 2009). Other
studies suggest that the anticancer activity of PKG activation also involves attenuation of Wnt/β-catenin mediated transcription (Thompson, Piazza et al. 2000; Kwon, Wang et al. 2010). To investigate whether the growth inhibitory activity of SS is associated with negative regulation of Wnt/β-catenin signaling, we first evaluated the effects of the compound on total and phosphorylated β-catenin expression in intact MDA-MB-231 breast tumor cells. As shown in Figure 21A, 2 hours of SS treatment resulted in a two-fold increase in the expression of β-catenin phosphorylated at the serine 33, serine 37, and/or threonine 41 residues (p-β-catenin\textsuperscript{Ser33/37/Thr41}), which are the phosphorylation sites known to promote ubiquitination and subsequent proteasomal degradation of the protein. Additionally, SS caused a two-thirds decrease in total β-catenin protein levels after 48 hours of treatment. Both of these effects occurred at concentrations necessary for growth suppression. Conversely, SS had no effect on β-catenin phosphorylation or total β-catenin expression in HMEC, which is consistent with the tumor selective activation of cGMP signaling as described above. We also measured the levels of β-catenin mRNA by quantitative real time PCR in cells treated with vehicle or 100 µM SS for 4 hours. As shown in Figure 21B, SS surprisingly resulted in complete loss of detection of β-catenin mRNA when compared to vehicle treated cells.

To determine whether the observed effects of SS on β-catenin phosphorylation and expression are sufficient to silence Wnt/β-catenin signaling, we evaluated the effects of SS on nuclear levels of β-catenin, a hallmark of oncogenic Wnt/β-catenin signaling activity. As shown in Figure 22A, representative images from these treated cultures depict a reduction in the nuclear localization of β-catenin after 5 total hours of SS treatment in the MDA-MB-231 cells compared with vehicle. These effects were quantified by automated image analysis using an Evotec Opera confocal microscope,
which demonstrated that SS caused a decrease in mean β-catenin nuclear fluorescence in MDA-MB-231 and ZR75-1 breast tumor cell lines by as much as 80% and 45%, respectively (Figure 22B).

When β-catenin translocates to the nucleus, it interacts with the Tcf/Lef family of transcription factors to activate transcription of growth promoting and apoptosis inhibiting proteins. We utilized a luciferase reporter assay to measure activity of the Tcf/Lef family of transcription factors after treatment of ZR75-1 breast tumor cells with SS. As shown in Figure 22C, SS treatment caused a 45% reduction in Tcf/Lef reporter activity at concentrations that were necessary for the effects on cGMP signaling activity.

SS treatment of MDA-MB-231 cells was also associated with a reduction in the expression of the cell cycle regulatory protein, cyclin D1, and the apoptosis inhibitor protein, survivin (Figure 23), both of which are known to be regulated Tcf/Lef transcriptional activity. The expression of p-VASP^{Ser239} peaked with 100 µM of SS treatment in the MDA-MB-231 cells after 2 hours of treatment, whereas cyclin D1 expression decreased significantly after just 30 minutes of treatment and survivin after 2 hours of treatment. The effects of SS on survivin expression were consistent with the timing necessary for the observed effects on activation of PKG, suggesting that these effects may be related. Conversely, the reduction in cyclin D1 expression occurred at a much earlier time point than that required for activation of PKG, suggesting that the effect on cyclin D1 may be independent of the effects on cGMP signaling. In support of this possibility, SS markedly suppressed the expression of cyclin D1 in HMEC despite being unable to activate PKG or attenuate β-catenin signaling in the normal cells.

While β-catenin serves as an important intracellular mediator within the canonical Wnt signaling pathway, more than 100 distinct proteins have also been identified as
important players within this pathway. To identify Wnt-related proteins other than β-
catenin that may be altered in response to SS treatment, we utilized a semiquantitative
real time PCR array comprised of primers specific for the cDNAs of 84 genes whose
proteins are potential mediators of the Wnt signaling pathway. As shown in Table 7, 25
of the 84 genes contained within the array were found to be expressed at the mRNA level
in vehicle treated MDA-MB-231 breast tumor cells. SS had no effect on the expression
of most of the genes including cyclin D1 and cyclin D2. However, SS caused a
significant decrease in the levels of mRNA for 1 gene (FBXW11) but increased the levels
of mRNA of 2 genes (FOSL1 and SFRP1). Interestingly, 6 genes (CSNK1G1, CTNNB1,
DKK1, FZD7, and WNT6) that were expressed in vehicle treated MDA-MB-231 cells
were not detected in the SS treated cells, whereas 5 genes (FZD6, FZD4, FRAT1, LRP6,
and WNT2B) were found to be expressed in SS but not vehicle treated cells.

Table 7. Effects of SS treatment on expression of Wnt related genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>CT&lt;sub&gt;DMSO&lt;/sub&gt;</th>
<th>CT&lt;sub&gt;SS&lt;/sub&gt;</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSNK1A1</td>
<td>Casein kinase 1, alpha 1</td>
<td>41.6</td>
<td>45.5</td>
<td>NC</td>
</tr>
<tr>
<td>CSNK1G1</td>
<td>Casein kinase 1, gamma 1</td>
<td>51.9</td>
<td>ND</td>
<td>L</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
<td>42.6</td>
<td>47.0</td>
<td>NC</td>
</tr>
<tr>
<td>BCL9</td>
<td>B-cell CLL/lymphoma 9</td>
<td>48.7</td>
<td>53.5</td>
<td>NC</td>
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<td>FZD5</td>
<td>Frizzled Homolog 5</td>
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<td>36.4</td>
<td>NC</td>
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<td>CCND1</td>
<td>Cyclin D1</td>
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<td>34.8</td>
<td>NC</td>
</tr>
<tr>
<td>CCND2</td>
<td>Cyclin D2</td>
<td>43.6</td>
<td>46.6</td>
<td>NC</td>
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<td>CTBP1</td>
<td>C-terminal binding protein 1</td>
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<td>NC</td>
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<tr>
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<td>Beta catenin</td>
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<td>38.3</td>
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<td>Hypoxanthine phosphoribosyltransferase 1 (Housekeeping)</td>
<td>38.2</td>
<td>38.8</td>
<td>NC</td>
</tr>
</tbody>
</table>
Note: + or - indicates a significant increase or decrease, respectively, of gene expression with treatment. “ND” represents that mRNA for the given gene was not detected, “G” represents a gain of gene expression, “L” represents a loss of gene expression, and “NC” represents no change in expression with treatment compared to vehicle control. MDA-MB-231 cells were treated with 0.1% DMSO or 100 µM SS for 4 hours prior to cell lysis and mRNA isolation. Hypoxanthine phosphoribosyltransferase 1 was used as a housekeeping gene to verify equal loading of cDNA.

Discussion

Despite demonstrated anticancer activity, the NSAIDs and COX-2 selective inhibitors are not utilized for the prevention or treatment of breast cancer due to potentially severe toxicities that are associated with their inhibition of COX, which is the primary mechanism responsible for their anti-inflammatory activity. Several lines of evidence, however, suggest that the mechanism responsible for the antineoplastic activity of these compounds may be COX-independent. For example, the NSAID metabolite sulindac sulfone lacks COX-inhibitory activity yet retains the tumor cell growth inhibitory activity of the active sulfide metabolite. Studies suggest that the anticancer properties of this compound are due to its inhibition of cGMP PDE and resulting elevation of cGMP, yet little is known about whether this mechanism is specific to sulindac sulfone, which PDE isozyme(s) is targeted, or what pathway is involved in mediating the growth inhibitory effects. The studies presented here demonstrate that sulindac sulfide (SS) also inhibits growth and induces apoptosis of breast tumor cells through a mechanism involving selective inhibition of cGMP PDE, particularly PDE5, accumulation of cGMP, activation of PKG, and attenuation of Wnt/β-catenin signaling.

Initial evidence for the involvement of cGMP PDE was suggested by experiments which showed a correlation between the growth inhibitory potency of a panel of chemically diverse NSAIDs and their potency for inhibition of cGMP PDE in colon
tumor cells. The most potent of these NSAIDs, SS, displayed a similar association for induction of apoptosis and inhibition of cGMP PDE in human breast cells. Consistent with the studies performed with sulindac sulfone, SS did not affect cAMP hydrolysis by breast cell lysates or recombinant PDE enzymes, whether cAMP specific (PDE4, 7, and 8) or of dual specificity (PDE1, 2, 3, 10, and 11).

The biological relevance of SS to inhibit cGMP PDE was confirmed by experiments showing that SS treatment increased intracellular cGMP levels in human breast tumor cells within the same concentration range as was required for cGMP PDE inhibition in cell lysates. Additionally, the magnitude of cGMP elevation by SS was sufficient to activate PKG, as measured by phosphorylation of VASP, a known PKG substrate. Moreover, disrupting cGMP synthesis by GC inhibition was found to desensitize breast tumor cells to the growth inhibitory activity of SS, while GC activation enhanced sensitivity to SS, demonstrating that the effects of SS on cGMP signaling in breast tumor cells is both necessary and sufficient for growth inhibitory activity.

Other data suggest that SS can directly and specifically inhibit PDE enzymes, particularly PDE5. For example, while able to inhibit recombinant PDE2 and PDE3, SS demonstrated two-fold greater potency for inhibition of PDE5 and this potency was comparable to its potency for both cGMP PDE inhibition in breast tumor cell lysates and induction of apoptosis in breast tumor cells. Additionally, molecular modeling studies demonstrated that SS theoretically binds to the catalytic domain of PDE5 in a manner similar to the highly potent and selective PDE5 inhibitor, sildenafil, with favorable binding kinetics.

The inability of SS to bind to the catalytic sites of other PDE isozymes in the molecular modeling studies despite identical IDF docking protocols also suggests PDE5
selectivity. For example, the docked SS-PDE1 complex showed a relatively unfavorable docking score of ~4 kcal/mol higher than that obtained for SS-PDE5, which is equivalent to ~800 fold higher dissociation constant. Previous studies have concluded that the closed catalytic pocket of PDE5 is responsible for the selectivity of sildenafil binding to PDE5 (Sung, Hwang et al. 2003). Therefore, the structural differences near the binding pockets of PDE isozymes might explain the selectivity of SS to bind PDE5. However, while SS is most selective for inhibition of PDE5, these data cannot disregard the potential involvement of additional cGMP degrading isozymes, such as PDE2 or PDE3, in mediating the anticancer activity of SS.

Although these studies suggest that the selective inhibition of tumor cell growth and induction of apoptosis by SS and potentially other NSAIDs is associated with inhibition of cGMP PDE, the pathway downstream of PKG remains unclear. Several mechanisms, including attenuation of β-catenin transcriptional activity and activation of JNK1, have been implicated as potential mediators of PKG-induced apoptosis in human cancer cells (Soh, Mao et al. 2000; Thompson, Piazza et al. 2000; Deguchi, Thompson et al. 2004; Kwon, Schoenlein et al. 2008). Because β-catenin transcriptional activity is known to be an important growth promoting signal in breast cancer (Smalley and Dale 2001), because its attenuation has also been implicated as an important anticancer mechanism of the NSAIDs (Barker and Clevers 2006), and because previous studies have shown that PKG can directly phosphorylate β-catenin to induce ubiquitination and subsequent proteasomal degradation (Thompson, Piazza et al. 2000), we investigated whether the induction of apoptosis by SS was associated with a decrease in Wnt/β-catenin signaling in the human breast tumor cells.
We found that SS reduced Wnt/β-catenin signaling as measured by β-catenin protein phosphorylation and expression, nuclear localization of β-catenin protein, Tcf/Lef transcriptional activity, expression of proteins that are transcriptionally regulated by β-catenin, and expression of genes thought to play important roles in regulating canonical Wnt signaling. The increase in β-catenin phosphorylation and decrease in total β-catenin protein levels are consistent with previous studies performed in colon cancer cells, which demonstrated that sulindac sulfone activates PKG to promote β-catenin proteasomal degradation and that this effect is necessary for the pro-apoptotic effects of cGMP signaling activation in these cells (Thompson, Piazza et al. 2000; Chang, Everley et al. 2005). However, the effects of SS on gene expression suggest that the attenuation of Wnt signaling by SS involves an additional mechanism whereby the transcription of β-catenin and other Wnt signaling regulators is inhibited. Importantly, the effects of SS on PDE5 inhibition and activation of cGMP signaling closely parallel the suppression of Wnt/β-catenin signaling and induction of apoptosis, suggesting that attenuation of Wnt/β-catenin signaling is an important mediator of cGMP-dependent apoptosis induction in breast cancer cells. Furthermore, these findings suggest another potential explanation for the tumor selectivity of SS because β-catenin signaling is rarely active in normal, non-tumorigenic breast cells (Smalley and Dale 2001). Nevertheless, the mechanism through which the cGMP and β-catenin signaling pathways converge remains unclear, and we cannot exclude the involvement of additional pathways.

From the data presented here, we conclude that SS inhibits breast tumor cell growth through a mechanism involving inhibition of PDE5, intracellular accumulation of cGMP, activation of PKG, and attenuation of Wnt/β-catenin mediated signaling. These findings further demonstrate the potential utility of PDE5 as a novel anticancer target.
Figure 15. Association between growth inhibition and other molecular mechanisms for a panel of NSAIDs and COX-2 inhibitors. A, structures of NSAIDs analyzed. B, positive association between the potencies for HT-29 growth inhibition and inhibition of cGMP hydrolysis. C, no association between the potencies for HT-29 growth inhibition and COX-2 inhibition.

Note: Adapted from "Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G" by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3334. Copyright 2009 by AACR. Adapted with permission.

Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3334. Copyright 2009 by AACR. Adapted with permission.

Figure 17. PDE inhibitory activity of SS in breast cell lysates. A, cGMP PDE inhibitory activity of SS. B, lack of cAMP PDE inhibitory activity of SS.

Figure 18. Involvement of cGMP in the anticancer activity of SS. *A*, dose-dependent increase in intracellular cGMP levels after 30 minutes of SS treatment. *B*, activation of GC by 1 hour of NOR-3 pretreatment sensitizes breast tumor cells to SS, whereas inhibition by 1 hour of LY83583 pretreatment desensitizes the cells.

Figure 19. Activation of PKG by SS. Phosphorylation of VASP at the serine 239 residue was measured by immunoblotting in lysates of cells treated with SS for 2 hours. Total VASP was included for comparison, and β-actin was utilized as a loading control.
Note: Adapted from “Sulindac sulfide selectively inhibits growth and induces apoptosis of human breast tumor cells by phosphodiesterase 5 inhibition, elevation of cyclic GMP, and activation of protein kinase G” by H.N. Tinsley, B.D. Gary, et al., 2009, Molecular Cancer Therapeutics, 8, p. 3338. Copyright 2009 by AACR. Adapted with permission.

Figure 20. PDE5 is a target of SS. A, molecular modeling studies depicting sildenafil (Viagra®) bound to the catalytic site of PDE5. B, SS bound to the catalytic site of PDE5. C, effect of PDE5 siRNA on sensitivity of ZR75-1 breast tumor cells to the growth inhibitory activity of SS. Cells were incubated with negative control or PDE5 siRNA for 24 hours prior to the addition of the compound.
Figure 21. Effects of SS on β-catenin expression and phosphorylation status. A, expression of total and phosphorylated β-catenin protein after 2 or 48 hours of SS treatment, respectively, in HMEC and MDA-MB-231 cells as measured by immunoblotting. B, expression of β-catenin mRNA by semi-quantitative real time PCR in MDA-MB-231 cells treated with vehicle or 100 µM SS for 4 hours.
Figure 22. Effects of SS on β-catenin signaling. A, SS reversal of β-catenin nuclear localization in MDA-MB-231 breast tumor cells. Arrows point to cells with active β-catenin signaling as measured by colocalization of Alexa Fluor 488 labeled β-catenin and Draq5 labeled nuclei. B, quantification of the dose-dependent reduction in β-catenin nuclear localization in breast tumor cells with SS treatment. Cells were pretreated with vehicle or compound for 1 hour prior to 4 hours of Wnt stimulation. C, effect of 24 hours of SS treatment on Tcf/Lef promoter activity in ZR75-1 breast tumor cells.
Figure 23. Effects of SS on cyclin D1 and survivin expression. A, dose-dependent effects of SS on p-VASP$^{\text{Ser239}}$, cyclin D1, or survivin expression in the breast cell lines after 2 hours of treatment as measured by immunoblotting. B, time-dependent effects of 100 µM SS on p-VASP$^{\text{Ser239}}$, cyclin D1, or survivin expression in the breast cell lines as measured by immunoblotting. RhoA was used as a loading control.
SULINDAC CAN BE CHEMICALLY MODIFIED TO REMOVE COX-INHIBITORY ACTIVITY WHILE IMPROVING PDE5 INHIBITORY ACTIVITY AND ANTICANCER EFFICACY

Introduction

Previous studies demonstrate that the anticancer activity of certain NSAIDs involves a COX-independent mechanism such as cGMP PDE inhibition, which we have shown to be mediated by PDE5. Although less potent than SS, sulindac sulfone has also been shown to inhibit growth of cancer cells through a mechanism involving cGMP PDE inhibition. However, sulindac sulfone lacks COX-inhibitory activity, which suggests that these two activities are independent of one another and that chemical modifications of the molecule can effectively separate COX-inhibitory activity from cGMP PDE inhibitory activity, yielding potentially safer chemopreventive agents. Unfortunately, sulindac sulfone is not very potent for either tumor cell growth or PDE inhibition and is associated with solubility and bioavailability problems, which have limited its development for breast cancer chemoprevention. Here we investigate different chemical modifications to the indene scaffold of sulindac and determine the implications of these modifications for COX inhibition, cGMP signaling activation, and anticancer activity.

Results

Carboxylic acid moiety of SS is necessary for COX-1 and COX-2 inhibitory activity

To identify which substituent(s) on SS is important for its binding to and inhibition of the COX enzymes, we performed molecular modeling studies using the
known structures for both COX-1 and COX-2. Figure 24 depicts SS docked in the catalytic site of COX-1. The conformation of SS is nearly identical whether bound to COX-1 (orange structure) or COX-2 (white structure). Interestingly, the carboxylic acid moiety of SS appears to form salt bridges between the arginine 120 and tyrosine 355 residues present in the catalytic sites of the enzymes, which are important residues for substrate binding. This suggests that the carboxylic acid may be important for stabilizing the interaction between SS and the COX enzymes.

To further evaluate the importance of the carboxylic acid for COX interactions, we synthesized a series of sulindac derivatives by replacing the carboxylic acid with a positively charged substituent through either an amide or amine linkage. Representative compounds from this group of derivatives are shown in Table 8. SRI 21009 is a dimethylethyl amide derivative of SS. SRI 21878 is a trimethoxy derivative of sulindac with a benzyl amine replacing the carboxylic acid. SRI 21882 is a benzyl amine derivative of sulindac sulfoxide. As predicted, modifying the carboxylic acid of sulindac to a positively charged substituent was sufficient to significantly reduce COX inhibitory activity. While SS potently inhibited COX-1 and COX-2 enzymes with IC$_{50}$ values of 3.3 and 9.68 µM, respectively, neither SRI 21878 nor SRI 21882 were able to inhibit either isozyme at concentrations up to their solubility limits, and SRI 21009 was only able to inhibit COX-1 with significantly reduced potency compared to SS.
Amine derivatives of sulindac demonstrate improved potency for inhibition of PDE5 and enhanced anticancer activity

To assess the implications of chemically modifying the carboxylic acid of sulindac for inhibition of cGMP PDE, we first evaluated its inhibitory activity against recombinant PDE5. As shown in Figure 25A, SRI 21009, the dimethylethyl amide derivative of SS, showed a significant reduction in both efficacy and potency for PDE5 inhibition compared to the parent compound. However, the amine derivatives, SRI 21878 and SRI 21882, were about five-fold more potent for inhibiting PDE5 compared to SS. Interestingly, modifications to the benzyl ring of the sulindac scaffold did not seem

<table>
<thead>
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<th>Compound</th>
<th>Structure</th>
<th>Notes</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>COX-1</th>
<th>COX-2</th>
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<td>Sulindac sulfide</td>
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<td></td>
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<td>SRI 21009</td>
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<td>81.6</td>
<td>&gt;200</td>
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<tr>
<td>SRI 21878</td>
<td>Trimethoxy Amine</td>
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<td>&gt;100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRI 21882</td>
<td>Amine</td>
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</tbody>
</table>
to be important determinants of PDE5 binding, as both the sulfoxide (SRI 21882) and trimethoxy (SRI 21878) derivatives had similar potency for PDE5 inhibition.

Consistent with an increase in potency for PDE5 inhibition, SRI 21878 and SRI 21882 also demonstrated increased potency for inhibition of cGMP hydrolysis in MDA-MB-231 breast tumor cell lysate with IC$_{50}$ values of 13.4 µM and 7.3 µM, respectively (Figure 25B), which were nearly identical to the IC$_{50}$ values for inhibition of recombinant PDE5. Interestingly, SRI 21878 and SRI 21882 also demonstrated improved potency for inhibition of cGMP hydrolysis in HMEC lysate compared to SS. However, the IC$_{50}$ values in HMEC lysate were around 25 µM, which is significantly higher than the IC$_{50}$ values obtained for either recombinant PDE5 or for MDA-MB-231 lysate (Figure 25C).

To characterize the isozyme selectivity of the active derivatives, we also evaluated their ability to inhibit cGMP hydrolysis by PDE1A, 1B, and 9A, the PDE isozymes that were found to be important for cGMP hydrolysis in the HMEC. As shown in Figure 25D, these isozymes were relatively insensitive to inhibition by SRI 21878 or SRI 21882 at concentrations up to 20 µM.

To investigate the observed differences in PDE5 inhibitory potency between SS, SRI 21878, and SRI 21882, we performed molecular modeling studies using the known structure of the PDE5 catalytic site. As shown in Figure 26A and mentioned previously, SS docked into the catalytic site of PDE5 with favorable binding kinetics. Positioning of the molecule resulted in the sulfide interacting with the glutamine switch and the carboxylic acid in proximity of the metal ion. Interestingly, SRI 21878 and SRI 21882 docked into the catalytic site of PDE5 in a flipped orientation. For SRI 21878, the amine formed salt bridges with the glutamine switch, whereas the trimthoxy was positioned
towards the metal ion (Figure 26B). Demonstrating that this binding conformation is likely due to the amine modification rather than the trimethoxy, SRI 21882 docked into the PDE5 catalytic site in a very similar conformation to SRI 21878 (Figure 26C). SRI 21009, which had an amide substituent replacing the carboxylic acid of SS, did not dock to PDE5 with favorable kinetics.

We also investigated the effects of the sulindac derivatives on cGMP and Wnt/β-catenin signaling activity in MDA-MB-231 breast tumor cells. First we measured the effects of four hours of treatment on the activity of PKG by measuring the expression of p-VASP$^{\text{Ser239}}$ by immunoblotting. As shown in Figure 27A, treatment with either SRI 21878 or SRI 21882 caused a dose-dependent increase in p-VASP$^{\text{Ser239}}$ expression at concentrations that also inhibited PDE5 activity and cGMP hydrolysis in the cell lysate.

Next, we investigated the effects of the derivatives on Wnt/β-catenin mediated transcription in MDA-MB-231 breast tumor cells. Both SRI 21878 and SRI 21882 caused a dose-dependent decrease in the expression of cyclin D1 and survivin, and these effects were inversely proportional to the effects on p-VASP$^{\text{Ser239}}$ expression (Figure 27A). We also found that SRI 21878 and SRI 21882 caused a significant reduction in nuclear localization of β-catenin in the MDA-MB-231 cells, reducing it by an average of 31% at 5 µM and 79% at 10 µM (Figure 27B).

As further evidence of the involvement of the Wnt/β-catenin signaling pathway in the activity of SRI 21878, we evaluated the effects of 24 hours of treatment on Tcf/Lef transcriptional activity in the ZR75-1 breast tumor cells. As shown in Figure 27C, SRI 21878 caused as much as a 68% reduction in the activity of Tcf/Lef transcription factors, when measured using a luciferase reporter assay.
Finally, we measured the effects of SRI 21878 on growth, apoptosis, and proliferation of the breast tumor cell lines and the HMEC. As shown in Figure 28A, SRI 21878 inhibited the growth of all three breast cell lines with a greater than 25-fold improvement in potency compared to SS. Furthermore, with IC₅₀ values around 3 µM in the tumor cells compared to over 6 µM in the HMEC, the derivative retained the two-fold tumor selectivity that we observed with SS. Interestingly, SRI 21878 caused only a modest, but significant, increase in the activity of caspases 3 and 7 in the HMEC and MDA-MB-231 cells, peaking at just under a two-fold increase in these cells (Figure 28B). However, the compound had a pronounced effect on apoptosis in the ZR75-1 cells, peaking at a five-fold increase in caspases 3 and 7 activities in these cells. Conversely, SRI 21878 inhibited proliferation in the MDA-MB-231 cells, while having a much less pronounced effect in HMEC and ZR75-1 cells (Figure 28C).

Discussion

Despite demonstrated anticancer activity, the NSAIDs cannot be recommended for cancer chemoprevention due to potentially fatal COX-associated toxicities such as intestinal perforation, ulcers, and increased risk of heart attack. However, numerous studies suggest that a COX-independent target is at least partially involved in the anticancer activity. For example, studies performed with sulindac and its metabolites have identified cGMP PDE, particularly PDE5, as one such COX-independent target (Soh, Mao et al. 2000; Thompson, Piazza et al. 2000; Piazza, Thompson et al. 2001; Piazza, Keeton et al. 2010). Furthermore, studies performed with the sulfone metabolite of sulindac show that this metabolite is able to inhibit cGMP PDE but not COX-1 or
COX-2, demonstrating that these activities can be effectively separated from one another while maintaining growth inhibitory activity (Thompson, Piazza et al. 2000). While sulindac sulfone was never developed clinically for breast cancer prevention, clinical trials for colon cancer prevention demonstrated modest efficacy (Stoner, Budd et al. 1999; Arber, Kuwada et al. 2006). Unfortunately, these trials also revealed some inherent problems with the compound, namely hepatotoxicity, which prompted a premature end to its development and precluded its FDA approval (Stoner, Budd et al. 1999; Arber, Kuwada et al. 2006).

Because of the demonstrated efficacy of sulindac in clinical trials for colon cancer and in animal models of breast cancer (Giardiello, Hamilton et al. 1993; Thompson, Briggs et al. 1995; Thompson, Jiang et al. 1997; Stoner, Budd et al. 1999; Arber, Kuwada et al. 2006), we decided to generate derivatives of sulindac with three important characteristics: reduced or eliminated COX-inhibitory activity, enhanced PDE5 inhibitory activity, and more potent anticancer activity when compared to the parent compound. To rationally design out the COX-inhibitory activity, we performed molecular modeling studies to identify which portion(s) of the sulindac molecule is important for its interactions with the COX active sites. These studies demonstrated that, by interacting with key active site amino acids, the carboxylic acid of sulindac is imperative for COX-binding and subsequent inhibition. This was confirmed when we generated novel sulindac derivatives, including SRI 21009, SRI 21878, and SRI 21882, with a positively charged amide or amine substituent in place of the carboxylic acid, which practically eliminated COX-1 and COX-2 inhibitory activity.
Although these novel derivatives lacked the ability to significantly inhibit either COX isozyme, a subset of these compounds demonstrated enhanced PDE5 inhibitory activity. Interestingly, the dimethylethyl amide derivative, SRI 21009, was only weakly able to inhibit cGMP hydrolysis by the recombinant PDE5 enzyme in the biochemical assay and was unable to dock with favorable kinetics into the PDE5 active site. This characteristic appeared to be common for most, although not all, of the amide derivatives that have been generated to date.

Unlike SRI 21009, SRI 21878 and SRI 21882 demonstrated significantly improved PDE5 inhibitory activity as well as improved potency for activation of cGMP signaling and attenuation of Wnt/β-catenin mediated transcription. While the enhanced affinity of these derivatives for PDE5 could be due to a number of factors, we believe that the altered conformation of the molecules within the PDE5 catalytic site results in more complete inhibition of the enzyme. SS effectively binds to the PDE5 active site through a single salt bridge with the glutamine 817 residue. This amino acid, known as the “glutamine switch,” is imperative for cGMP binding, so interaction with this residue is thought to be important for successful enzyme inhibition (Zoraghi, Corbin et al. 2006). The amine derivatives, on the other hand, dock into the PDE5 active site 180° opposite of SS, allowing for two salt bridges to form between the amine of the derivative and the glutamine switch. Because the amine groups that have replaced the carboxylic acid are bulkier, the strongly electronegative trimethoxy and sulfoxide groups of the derivatives are able to interact with the metal ion of the catalytic site as a result of their close proximity to one another. It is these additional interactions that we believe are
responsible for adding stability to the enzyme-drug complex and for enhancing the potency of the compounds.

Consistent with enhanced PDE5 inhibitory activity, SRI 21878 also demonstrated enhanced antitumor efficacy, resulting in a 25-fold shift in potency to inhibit breast tumor cell growth. This further demonstrates that COX inhibition is not necessary for the anticancer activity of the NSAIDs and is consistent with the studies of sulindac sulfone. While the improvement in growth inhibitory potency paralleled the improvement in PDE5 inhibitory activity, we saw no improvement in tumor selectivity with SRI 21878. We predict that this may be a result of decreased specificity of the new derivative for PDE5. While neither SRI 21878 nor SRI 21882 appeared to inhibit PDE1 or PDE9, they were only tested at concentrations at or below 20 µM. The ability of these compounds to inhibit cGMP hydrolysis in HMEC lysates, which lack expression of PDE5, indicates that they are able to inhibit other cGMP PDE isozymes at higher concentrations. However, more work is necessary to further characterize the isozyme selectivity of these compounds.

While additional studies, particularly evaluation of their in vivo efficacy, are needed to further characterize these novel sulindac derivatives and more effective compounds are still needed, these data clearly demonstrate that, at least for sulindac, COX-inhibitory and PDE5-inhibitory activities are independent of one another and slight modifications to the indene scaffold of sulindac can effectively remove the ability of the compound to bind to either COX-1 or COX-2 while improving PDE5 inhibitory potency and anticancer activity. Therefore, chemically modifying NSAIDs is a promising approach for future drug discovery efforts, which could yield novel agents for breast
cancer chemoprevention that are safer and potentially more efficacious than the traditional NSAIDs or the current standard of care, the SERMs.

Figure 24. SS docked into the catalytic site of COX-1. The orange structure represents the conformation of SS when bound to COX-1, whereas the white structure represents the conformation of SS when bound to COX-2.
Figure 25. cGMP PDE inhibitory activity of sulindac derivatives. A, effects of SS and derivatives on cGMP hydrolysis by PDE5. B, effects of SS and derivatives on cGMP hydrolysis in MDA-MB-231 lysate. C, effects of SS and derivatives on cGMP hydrolysis in HMEC lysate. D, effects of SS derivatives on cGMP hydrolysis by recombinant PDE1A, 1B, and 9A.
Figure 26. Molecular modeling studies of sulindac derivatives.  
A, SS docked into the catalytic site of PDE5.  
B, SRI 21878 docked into the catalytic site of PDE5.  
C, SRI 21882 docked into the catalytic site of PDE5.  
The space filling model that is shown is the glutamine 817 residue (glutamine switch).
Figure 27. Effects of sulindac derivatives on cGMP signaling and Wnt/β-catenin mediated transcription. A, effects of SRI 21878 and SRI 21882 on p-VASPSer239, cyclin D1, and survivin expression in MDA-MB-231 breast tumor cells after 4 hours of treatment. GAPDH was used as a loading control. B, effects of SRI 21878 and SRI 21882 on Wnt-stimulated nuclear localization of β-catenin signaling in MDA-MB-231 cells. Cells were pretreated with compound for 1 hour prior to the addition of Wnt3A conditioned media. The cells were fixed after 4 hours of Wnt stimulation. C, effects of SRI 21878 on Tcf/Lef transcriptional activity in ZR75-1 breast tumor cells after 24 hours of treatment.
Figure 28. Effects of SRI 21878 on breast cell growth, apoptosis, and proliferation.  

A, effects of SRI 21878 on HMEC, MDA-MB-231, and ZR75-1 growth after 72 hours of treatment.  

B, effects of SRI 21878 on apoptosis of HMEC, MDA-MB-231, and ZR75-1 cells as measured by caspases 3 and 7 activity after 6 hours of treatment.  

C, effects of SRI 21878 on proliferation in HMEC, MDA-MB-231, and ZR75-1 cells as measured by BrdU incorporation after 24 hours of treatment.
CONCLUSIONS AND FUTURE DIRECTIONS

The public health impact of breast cancer is substantial, with more than 200,000 diagnoses and 40,000 deaths estimated to occur each year in the United States (American Cancer Society 2009). Despite ongoing efforts of clinicians and researchers to develop better screening strategies and improved therapeutics, there have been only modest changes in the incidence, morbidity, or mortality associated with breast cancer in recent years. Because of the demonstrated benefits of early intervention, chemoprevention is widely accepted as a promising strategy for substantially reducing incidence, morbidity, and mortality from this deadly disease.

Tamoxifen and raloxifene are the only drugs that have been FDA approved specifically for breast cancer prevention. While these drugs cause as much as a 50% reduction in the risk of breast cancer recurrence, their efficacy is limited to preventing ER-positive tumors, with no effect on the more deadly ER-negative forms of the disease, and their long term use is associated with significant toxicity (Cuzick, Powles et al. 2003; Bevers 2006; Castrellon and Gluck 2008; Thomsen and Kolesar 2008; Virnig, Tuttle et al. 2010). For these reasons, use of these drugs is only recommended for the prevention of contralateral ER-positive cancer in postmenopausal women, which encompasses only a small subset of the population that is considered to be at high risk for breast cancer. As such, there is an urgent need for better chemopreventive agents.
In this report, we identified PDE5 as a novel molecular target for both the treatment and the prevention of breast cancer. Here we demonstrated that inhibition of the cGMP-specific PDE5 isozyme with known pharmacological inhibitors such as MY5445, novel pharmacological inhibitors such as sulindac sulfide and its derivatives, or through siRNA knockdown of PDE5 expression is sufficient to selectively inhibit breast tumor cell growth through a mechanism involving activation of PKG and attenuation of oncogenic β-catenin mediated transcription. The effects of PDE5 inhibition on tumor cell growth appeared to occur independent of receptor expression status because ER-positive and negative, PR-positive and negative, and HER2-positive and negative breast tumor cells were all sensitive to the growth inhibitory effects of PDE5 inhibition. Furthermore, overexpression of PDE5 and decreased expression of other cGMP-degrading PDE isozymes were traits common to all of the breast tumor cells, regardless of receptor expression. These findings strongly suggest the potential utility of PDE5 as a target for breast cancer chemoprevention in a wider range of high risk patients when compared to the SERMs.

In addition to enhanced efficacy, these studies also demonstrated the potentially low toxicity that may be associated with PDE5 inhibition. While cGMP signaling appears to serve as a growth inhibitory signal in breast cells regardless of tumorigenicity, only tumor cells are sensitive to the growth inhibitory effects of PDE5 inhibition. This difference in sensitivity is most likely a reflection of the increased reliance of breast tumor cells on PDE5 for cGMP hydrolysis. While PDE5 is a relatively ubiquitously expressed isozyme, breast tumor cells are only the second cell type that has been identified to date in which PDE5 is thought to be almost entirely responsible for cGMP...
hydrolysis, with corpus cavernosum smooth muscle cells being the other cell type (Beavo 1995). Like the primary mammary epithelial cells that were evaluated here, all other cells that have been characterized for PDE isozyme expression appear to rely on a minimum of two PDE isozymes for cGMP hydrolysis (Beavo 1995). This suggests that selectively targeting PDE5 would have a favorable toxicity profile because the most profound effects would be in the tumor cells whereas other cells would have an inherent compensatory mechanism. Moreover, clinical use of PDE5 inhibitors for erectile dysfunction and pulmonary hypertension has been shown to be well tolerated and has minimal side effects (Bischoff 2004).

The potential involvement of the Wnt/β-catenin signaling pathway in the mechanism of PDE5 inhibition also makes this a promising target for the treatment of breast cancer. Aberrant Wnt signaling has been observed in breast cancer, and activation of this pathway has been implicated in driving both tumorigenesis and chemotherapeutic resistance (Smalley and Dale 2001; Ischenko, Seeliger et al. 2008). As such, inhibiting this pathway has emerged as a leading strategy for the discovery of new breast cancer therapeutics. Unlike colon cancer in which canonical Wnt signaling is almost always activated by mutations in the Apc gene, Wnt signaling activation in breast cancer can occur through a number of different mechanisms, including overexpression of one or more of the 16 Wnt ligands or decreased expression of secreted frizzled protein or another of the signaling inhibitors (Smalley and Dale 2001). Not only does inhibition of PDE5 appear to attenuate oncogenic Wnt signaling in breast cancer cells, it appears to do so at the level of β-catenin, which is a point in the pathway that is a necessary component for activity no matter the mechanism of pathway activation. Therefore, targeting the Wnt
pathway through inhibition of PDE5 and activation of PKG is a novel approach that may be effective for treating a wide range of Wnt-reliant breast cancers.

The most promising outcome of these studies is the identification of a novel class of compounds that can be studied and optimized in future drug discovery efforts. While these studies have characterized the importance of cGMP signaling in vitro, we know little about this pathway in vivo. Unfortunately, the high dosages of MY5445, trequinsin, and SS that are needed to significantly impact cGMP signaling are not readily achieved in vivo. The novel sulindac derivatives that were characterized here, namely SRI 21878 and SRI 21882, demonstrate significantly enhanced potency for inhibition of PDE5, activation of cGMP signaling, and anticancer activity, and preliminary studies suggest that the concentrations required to see these effects in vitro are readily achievable in vivo. Therefore, we predict that these compounds will serve as important probes to characterize the involvement of this pathway in animal models of mammary tumorigenesis, to characterize the in vivo efficacy of PDE5 inhibition, and to identify potential biomarkers of the PDE5-PKG-Wnt/β-catenin pathway. Furthermore, the lack of COX-inhibitory activity makes these compounds ideal for studying this pathway as it will remove a confounding factor from the studies that have been performed with sulindac and eliminate the primary source of dose-limiting toxicity.

The studies presented here clearly identify PDE5 as a novel molecular target for the prevention and treatment of breast cancer. However, additional studies are necessary to more fully define the proapoptotic mechanism involved in PDE5 inhibition in breast tumor cells as well as to characterize the in vivo significance of our in vitro observations.
In terms of proapoptotic mechanism, our current data suggest that inhibition of PDE5 negatively regulates β-catenin transcriptional activity through a dual mechanism involving decreased levels of β-catenin mRNA and increased levels of phosphorylated, or inactive, β-catenin protein. However, the mechanism through which PDE5 inhibition reduces β-catenin mRNA levels and the importance of β-catenin phosphorylation for the proapoptotic effects of PDE5 inhibition remain undefined.

Decreased mRNA could result from one of two processes – decreased gene transcription or increased mRNA degradation. In order to understand the mechanism through which PDE5 inhibition results in decreased β-catenin mRNA, it is imperative to first determine which of these two processes is involved. To address whether altered gene transcription is involved, cells could be transfected with a plasmid containing the CTNNB1 gene driven by a constitutively active promoter. If treatment of transfected cells with PDE5 inhibitors no longer results in loss of β-catenin mRNA, then PDE5 inhibition is likely working at the level of β-catenin gene transcription. Conversely, a decrease of β-catenin mRNA in transfected cells would suggest altered gene transcription is not involved.

One of the most well understood mechanisms of altering mRNA stability involves microRNA induced mRNA degradation (Guo, Ingolia et al. 2010). Because processing of pre-microRNA by the nuclease dicer is necessary for the function of mature microRNA, knocking down the expression of dicer with siRNA would be a promising approach for determining whether the effects of PDE5 inhibition on β-catenin mRNA levels is related to altered mRNA stability. For example, if decreasing dicer expression
does not rescue cells from the effects of PDE5 inhibition on β-catenin mRNA levels, then microRNA is likely not involved in this activity.

Another question remaining from the present study is whether β-catenin phosphorylation induced by PDE5 inhibition results in proteasomal degradation of the protein and whether this effect is necessary for the proapoptotic effects of PDE5 inhibition. Proteasome inhibitors such as MG132 and lactacystin could be employed to address this question. Measuring the effects of the proteasome inhibitors on the drop in β-catenin protein expression and the increase in apoptosis induced by PDE5 inhibition would elucidate whether proteasomal degradation of β-catenin is induced by PDE5 inhibition and whether this effect is necessary for PKG-mediated apoptosis induction, respectively.

Our present data strongly suggest the potential chempreventive and chemotherapeutic efficacy of sulindac and its analogs. However, it will be important to verify this in in vivo models of breast cancer. The MNU model of chemical carcinogenesis is a well-characterized breast cancer model that has been used previously to show chemopreventive efficacy of sulindac and would, therefore, be a good model to evaluate the chemopreventive efficacy of novel sulindac analogs. Also, because of the importance of Wnt/β-catenin signaling activity in driving tumorigenesis in the MMTV breast cancer model, this too would be a good secondary model for evaluating the chemopreventive efficacy of sulindac and its analogs. In terms of chemotherapeutic efficacy, the MDA-MB-231 xenograft model would be useful because we have already characterized in vitro sensitivity of this cell line to sulindac and the sulindac analogs. These in vivo studies would also be beneficial for defining biomarkers of sensitivity and
resistance to treatment by analyzing changes in protein expression in tumors from both treated and untreated animals. Another important question concerning efficacy that remains unanswered is whether these drugs are beneficial when given in combination with conventional chemotherapeutics or targeted therapeutics. Using the isobologram method as previously described (Tallarida 2006), potential synergism between drugs could be assessed \textit{in vitro}. In \textit{vivo} combination efficacy could also be assessed in the above mentioned animal models.

Most importantly, clinical relevance of our \textit{in vitro} observations remains to be determined. The findings reported here suggest that decreased expression of non-PDE5 cGMP PDE isozymes and increased expression of PDE5 in human breast cells are associated with tumorigenicity and sensitivity to the proapoptotic effects of PDE5 inhibition. One method to evaluate this possibility clinically would be to measure the expression and activity of different cGMP degrading PDE isozymes in archival human breast biopsy samples representing various stages of disease including normal tissue, hyperplasia, dysplasia, carcinoma \textit{in situ}, nonmetastatic carcinoma, and metastatic carcinoma. Also, sulindac could be administered to women who are awaiting surgery in order to compare biomarkers of response to sulindac, such as VASP phosphorylation, \(\beta\)-catenin nuclear localization, and caspase activation, with cGMP PDE isozyme expression.

While additional studies are needed to validate PDE5 as a clinically relevant target and to determine the potential efficacy of the novel class of anticancer agents described here, the studies presented here serve to identify PDE5 as a novel molecular target for the prevention and treatment of breast cancer. We believe that safer and more
efficacious agents for breast cancer can be developed through selective targeting of PDE5, and such drug development efforts have the potential to make a significant impact in the fight against this deadly disease.
REFERENCES


APPENDIX A

CHARACTERIZATION OF RECOMBINANT PDE ISOZYMES
Figure 29. Titration of recombinant PDE isozymes. Each recombinant PDE isozyme was evaluated for cAMP and cGMP hydrolytic activity using a minimum of 8 concentrations spanning a range of at least 3 logarithmic units. The EC\textsubscript{80} value was calculated from these curves and used in subsequent inhibitor studies.
Figure 30. Sensitivity of recombinant PDE isozymes to known inhibitors. Each recombinant PDE isozyme was evaluated for sensitivity to a single concentration of known isozyme selective inhibitors.