Molecular Pharmacology Fast Forward. Published on December 3, 2008 as doi:10.1124/mol.108.053066
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Pharmacological Targeting of the Integrated AKT, PTEN and TGF-β Pathways in Prostate Cancer.

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Running Title Page: Pharmacological targeting of the AKT/PTEN/TGF-β pathways

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Pages: 38

Tables: 0

Figures: 4

References: 117

Words in Abstract: 161

Manuscript Word Count: 4389

Non-Standard Abbreviations: AA, arachidonic acid; cdk, cyclin-dependent kinase; COX, cyclooxygenase; $cPLA_2$-α, cytosolic phospholipase A2-α; DFO, desferrioxamine; Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; FOXO3a, forkhead transcription factor; GSK3B, glycogen synthase kinase 3β; HIF-1α, hypoxia inducible factor-1α; LOX, lipooxygenase; mTOR, mammalian target of rapamycin; NDRG1, N-myc downstream regulated gene-1; PDKs, 3-phosphoinositide-dependent kinases; PIP_3, phosphatidylinositol-3,4,5-triphosphate; PIK3CA, phosphoinositide 3-kinase class 2 alpha polypeptide; PI3-K/AKT, phosphoinositide 3-kinase/protein kinase B; PLA_2-α, phospholipase A2-α; pRb, retinoblastoma protein; PSA, prostate specific antigen; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RR, ribonucleotide reductase; Tf, transferrin; TfR1, transferrin receptor 1; TGF-β, transforming growth factor-β; TGF-βRI, transforming growth factor-β receptor I; TGF-βRII, transforming growth factor-β receptor II; VEGF1, vascular endothelial growth factor 1.

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Abstract

Prostate cancer is a highly heterogenous disease where a patient tailored care program is much sought after. Central to this goal is the development of novel targeted pharmacological interventions. In order to develop these treatment strategies, an understanding of the integration of cellular pathways involved in both tumorigenesis and tumor suppression is crucial. Of further interest are the events elicited by drug treatments that exploit the underlying molecular pathology in cancer. This review will briefly describe the evidence that suggests integration of three established pathways: the tumorigenic phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway, the tumor suppressive phosphatase and tensin homolog deleted on chromosome 10 pathway and the tumor suppressive transforming growth factor β pathway. More importantly, it will discuss novel pharmaceutical agents that target key points of integration in these three pathways. These new therapeutic strategies include: **(1)** the use of agents that target iron to inhibit proliferation *via* multiple mechanisms and; **(2)** suppression of AKT by cytosolic phospholipase A_2-α inhibitors.

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1. Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous cancer in men and is the second leading cause of death (Assinder and Nicholson, 2007). It is estimated that about 186,000 new cases of prostate cancer will be diagnosed in the USA during 2008, accounting for approximately 22,000 deaths (American Cancer Society, http://www.cancer.org).

Radical prostatectomy (surgical removal) is the most common treatment strategy for organ-confined tumors (Steineck et al., 2002). While this procedure has a ten-year survival of 60%, surgery has a 2% mortality rate, 70% of patients develop erectile dysfunction, 50% have urine leakage with 2-5% of patients being left incontinent (Steineck et al., 2002). The most common treatment option for advanced metastatic prostate cancer is androgen deprivation either by surgical or chemical castration. However, in the majority of patients, the cancer becomes insensitive to androgen-deprivation, leading to relapse and they inevitably die from androgen-independent metastatic prostate cancer (Hussain and Dawson, 2000). Thus, improved treatment options are desperately needed.

Key to developing improved pharmacological interventions for prostate cancer is an understanding of the integration of pathways known to be involved in the pathophysiology of prostate cancer. This review will: **(1)** briefly describe the tumorigenic phosphoinositide 3-kinase/protein kinase B (P13K/AKT) pathway, the phosphatase and tensin homologue deleted on chromosome ten (PTEN) and transforming growth factor beta (TGF-β) tumor suppressive pathways; **(2)** examine current evidence for the integration at key points of these pathways; and **(3)** discuss novel pharmaceutical agents that target these key points.

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2. Biochemical Pathways Known to Play a Role in Prostate Cancer

2.1 The Tumorigenic Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT) Pathway

A high level of activated (phosphorylated) AKT (pAKT) is correlated with poor prognosis of prostate cancer, whereas in normal prostate tissue, pAKT is undetectable (Wegiel et al., 2008). It is estimated that 30-40% of solid tumors have constitutively activated pAKT (Samuels and Ericson, 2006).

Activation of a tyrosine kinase receptor, such as the insulin-like growth factor receptor, activates phosphatidylinositol-3 kinase (PI3K) (Figure 1A). Subsequently, PI3K catalyses phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to form phosphatidylinositol-3,4,5-triphosphate (PIP_3; Manning and Cantley, 2007). PIP_3 acts as a platform and brings AKT in close proximity to 3-phosphoinositide-dependent kinases (PDKs) that subsequently phosphorylate and activate AKT. Activated AKT results in signaling cascades involving various downstream signaling proteins (Gao et al., 2003; Figure 1A).

Activated AKT affects many targets and can inhibit glycogen synthase kinase 3β (GSK3B; Figure 1A), which normally prevents up-regulation of cellular proliferation due to increased cyclin D1 degradation and suppression of expression, possibly *via* cytosolic (free) β-catenin (de la Taille et al., 2003; Lee et al., 2007). An excessive rate of cyclin D1 production promotes cell cycle progression (Chen et al., 1998) due to increased levels of cyclin D1/cyclin-dependent kinase 4 (Cdk4) complexes that promote G_1/S progression. On reaching appropriate levels, these complexes phosphorylate retinoblastoma protein (Rb) and cause the dissociation of the E2F transcription factor, which heralds progression of the cell cycle from G_1 to S phase (Yu et al., 2007).

Another key effector downstream of pAKT is the serine/threonine kinase known as the mammalian target of rapamycin (mTOR; Figure 1A; reviewed in Vignot et al., 2005). It plays a central role in

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protein translation, cell proliferation and evasion of apoptosis (Vignot et al., 2005). Activation of AKT also helps to evade apoptosis directly by phosphorylation and inactivation of the pro-apoptotic protein, Bad (Grünwald et al., 2002).

2.2 The Tumor Suppressive Phosphatase and Tensin Homologue Deleted on Chromosome Ten (PTEN) Pathway

Fifty percent of prostate cancers display loss of PTEN (Facher and Law, 1998). Re-expression of normal PTEN in prostate cancer cell lines causes apoptosis (Sharrard and Maitland, 2000; Davies et al., 1999), Furthermore, *Pten* null mice have increased numbers of tentative prostate stem cells and develop prostate cancer with pathological changes similar to human disease (Wang et al., 2006). The major tumor suppressive activity of PTEN is *via* antagonism of the AKT pathway by dephosphorylation of PIP_3, converting it back to PIP_2 (Cantley and Neel, 1999). PTEN thus modulates AKT signaling, with opposite effects from PI3K on cell proliferation and survival (Figure 1B).

Interaction of PTEN with p53 also plays a significant role in tumor suppression (Figure 1B; Stambolic et al. 2001). PTEN suppresses expression of the p53 repressor MDM2 (Mayo et al., 2002). Furthermore, nuclear PTEN interacts directly with p53 to enhance p53-mediated cell cycle arrest and apoptosis in prostate cancer cells (Mayo et al., 2002; Chang et al., 2008; Figure 1B).

2.3 The Tumor Suppressive Transforming Growth Factor (TGF-β) Pathway

TGF-β acts on normal prostate epithelial cells and some prostate cancer cell lines to inhibit proliferation and induce apoptosis (Sutkowski et al., 1992; Wilding, 1991). In prostate cancer specimens, it is common to find down-regulation of TGF-β receptors (Shariat et al., 2004). In particular, down-regulation of the type II receptor is associated with aggressive tumors (Shariat et al., 2004).

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TGF-β signaling is activated by binding to its receptor TGF-βRII and subsequent recruitment and phosphorylation of TGF-βRI (Shi and Massagué, 2003; Figure 1C). This activates a SMAD signaling cascade (Ten Dijke et al., 2002) resulting in the up-regulation of cyclin-dependent kinase inhibitors (CDKIs) (Guo and Kyprianou, 1998; Li et al., 2006) and down-regulation of c-Myc (Massagué et al., 2000), leading to suppressed cellular proliferation (Figure 1C). Apoptosis is promoted by TGF-β-induced expression of pro-apoptotic Bax, down-regulation of anti-apoptotic Bcl-2 and increased expression and activation of the effector caspases (Guo and Kyprianou, 1999). Additional apoptotic cross talk with the androgen receptor also occurs depending on the mutational status of this protein (Zhu et al., 2008; Shariat et al., 2004).

3. Interactions of the PI3K/AKT, PTEN and TGF-β Pathways: Implications for Pharmacological Targeting.

3.1 TGF-β and PTEN Integration Act to Oppose AKT

pAKT suppresses TGF-β activated SMAD-signaling (Figure 1), while PTEN inhibits AKT suppression (Song et al., 2003). In turn, TGF-β induces PTEN expression through its classical SMAD-dependent pathway (Figure 1C) and stimulates a tumor suppressive response (Chow et al., 2007). Considering this, it is known that iron chelation up-regulates TGF-β (Yoon et al., 2002) and this may potentially increase PTEN levels, although further studies are needed to investigate this. As mentioned above, SMAD activation suppresses oncogenic c-Myc (Massagué et al., 2000) and Bcl-2 expression (Guo and Kyprianou, 1999), but increases expression of the cyclin-dependent kinase inhibitors p15, p21, and p27 (Figure 1) (Guo and Kyprianou, 1998; Li et al., 2006). However, AKT activation has the opposite effect (Vignot et al., 2005; Figure 1).

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3.2 The Metastasis Suppressor NDRG1: A Possible Common Point of AKT, PTEN and TGF-β Integration.

It has been shown that expression of both the N-myc downstream regulated gene-1 (NDRG1) and PTEN are repressed in prostate tumors (Bandyopadhyay et al., 2004b). Forced expression of PTEN up-regulates *NDRG1* expression in the prostate cancer cell lines PC-3 and DU145 *via* suppression of the AKT pathway (Bandyopadhyay et al., 2004a; Figure 2). It is therefore hypothesised that AKT activation acts to inhibit *NDRG1* expression, while PTEN antagonises this effect (Figure 2).

Up-regulation of *NDRG1* expression following hypoxia or cellular Fe-depletion is driven, at least in part, by hypoxia inducible factor-1α (HIF-1α; Le and Richardson, 2004), which stimulates *NDRG1* expression (Kovacevic and Richardson, 2006). Interestingly, raised HIF-1α levels during hypoxia correlate with increased TGF-β expression (Berger et al., 2003), mediating further potential integration between the AKT, PTEN and TGF-β pathways. In fact, as already discussed, there is up-regulation of TGF-β after Fe chelation that may act to increase PTEN which subsequently could up-regulate NDRG1 expression. PI3K/AKT and PTEN have completely opposite effects on the activity of HIF-1α (Emerling et al., 2008). PTEN inhibits the transcriptional activity of *HIF-1α*, while AKT induces *HIF-1α* transcriptional activity *via* suppression of the forkhead transcription factor FOXO3a (Emerling et al., 2008).

This effect of tumor suppressive PTEN inhibiting HIF-1α activity can be seen as both logical, but also counter intuitive. For instance, the PTEN-mediated decrease in HIF-1α would lead to the appropriate decrease in expression of one of its classical targets, *VEGF1*, that promotes angiogenesis and tumor progression. However, paradoxically, PTEN would also potentially lead to decreased expression of the metastasis suppressor, NDRG1, which can be up-regulated by a HIF-1α-dependent mechanism (Le and Richardson, 2004). However, NDRG1 can also be up-regulated by a HIF-1α independent process (Le and Richardson, 2004) and it is probable that the regulation of

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this metastasis suppressor is complex. Moreover, it may be that HIF-1α serves more than one function and this needs to be examined in detail. Indeed, over-expression of HIF-1α has been found in various cancers and down-regulation of this molecule is currently evaluated as a therapeutic target for cancer treatment (Greenberger et al., 2008; Groot et al., 2008; Yasui et al., 2008).

4. The Arachidonic Acid and Eicosanoid Signaling Pathway: A Possible Regulator of AKT and PTEN.

Arachidonic acid (AA) is an omega-6 polyunsaturated essential fatty acid (Burr and Burr, 1929). As such, it cannot be synthesized by cells *de novo*. Rather, it is obtained from the diet or synthesized from its precursor linoleic acid (Brenner, 1974). In resting cells, AA is stored within the phospholipid bilayer of the cell membrane (Hughes-Fulford et al., 2001). Stimulation of cells by a number of diverse agonists leads to the activation of intracellular phospholipases, namely cytosolic phospholipase A_2-α (cPLA_2-α) that then releases AA from the cell membrane (Clark et al., 1995; Niknami M et al., 2008). When AA is released it can either become reincorporated into the membrane phospholipid bilayer, diffuse out of the cell or be metabolised *via* the lipooxygenase (LOX) or cyclooxygenase (COX) enzymes to eicosanoids such as prostaglandin E2 (PGE2; Figure 2; Niknami et al., 2008).

Previous studies have demonstrated that the eicosanoid synthetic rates from labeled AA are significantly higher in malignant than in benign prostate tissue, suggesting an increased AA flux through the COX and LOX pathways (Chaudry et al., 1994; Faas et al., 1996). Eicosanoids can contribute to cancer progression by promoting cell proliferation, motility, invasion and angiogenesis (Nie et al., 1998; Nie et al., 2001; Pidgeon et al., 2002; Porkka et al., 2004). Other evidence also supports a role for eicosanoids in prostate carcinogenesis. For example, eicosanoid inhibitors effectively reduce the size of prostate cancer xenografts (Hsu et al., 2000). Treatment of the androgen-independent human prostate carcinoma cell line, PC-3, with linoleic acid stimulates

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growth (Tjandrawinata et al., 1997). Furthermore, a study by Hughes-Fulford and associated (2001) has demonstrated that linoleic acid, arachidonic acid and PGE2 all stimulate prostate tumor growth and alter gene expression in PC-3 cells. In mice, diets high in corn oil (hence rich in the AA precursor linoleic acid), markedly stimulate the growth of human prostate cancer xenografts (Wang et al., 1995). Collectively, these studies suggest a stimulatory effect of dietary omega-6 fatty acid on prostate tumor cell growth which may be critical for the development and progression of cancer.

In humans, it was shown that a rise in serum PSA following prostatectomy or radiotherapy was slowed in patients treated with COX-2 inhibitors (Pruthi et al., 2006; Smith et al., 2006). However, an increased risk of thrombosis was found in patients treated with COX-2 inhibitors due to the sparing effect on COX-1 (Marcus et al., 2002). In addition, COX-2 inhibitors inhibit production of the vasodilator prostacyclin with no effect on the synthesis of the vasoconstrictive thromboxane A_2, thus potentially tipping the balance toward vasoconstriction and thrombosis (reviewed in Funk and FitzGerald, 2007).

In the past few years, it has become clear that AA and/or eicosanoids can also lead to increased AKT activation. In pancreatic cancer cells, AA and/or eicosanoids can augment AKT activation indirectly by increasing inactivation of PTEN by oxidation (Covey et al., 2007; Figure 2). Alteration of PLA2 can directly regulate PI3K/AKT in the prostate (Hsu et al., 2000; Patel et al., 2005; Hughes-Fulford et al., 2006) and vascular smooth muscle cells (Li and Malik, 2005a,b; Wildrout, 2006). Inhibition of COX-2 by celecoxib has been shown to induce apoptosis in both androgen-responsive LNCaP and androgen-unresponsive PC-3 cells by blocking AKT phosphorylation (Hsu et al., 2000) and by down-regulating cyclin D1 (Patel et al., 2005). The potential biological significance of the regulation between $cPLA_2$-α and PI3K has been highlighted in a recent review (Wymann and Schneiter, 2008).

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5. Pharmacological Targeting of the Integrated AKT, PTEN and TGF-β Pathways

Prostate cancer accounts for significant morbidity and mortality with current therapies being far from adequate. Therefore, new therapeutic approaches are essential. Since the tumorigenic AKT and tumor suppressive PTEN and TGF-β pathways have potential and established points of integration, this indicates the possibility of specifically targeting these pathways (Figure 2). In this section, we will describe novel pharmacological agents that have been shown to do this. First, we will describe the development of iron chelators that act on a number of molecular targets including NDRG1, cyclin D1 and TGF-β. Second, we will examine the suppression of AKT by cytosolic phospholipase A_2-α inhibitors.

5.1 Targeting Iron for Inhibition of Tumor Growth

Iron is vital for life as it is an essential component of many proteins and enzymes that are involved in cell growth and replication (Bohnsack and Hirshi, 2004; Dunn et al., 2007). For example, iron is involved in the rate-limiting step of DNA synthesis catalysed by ribonucleotide reductase (RR) (reviewed by Jordan and Reichard, 1998). Also, without iron cells cannot proceed from the G_1 phase to the S phase of the cell cycle (Yu et al., 2007). Depleting cells of iron by chelators, such as desferrioxamine (DFO), results in cell cycle arrest (Buss et al., 2003; Kalinowski and Richardson, 2005).

Iron is transported by transferrin (Tf), which binds to the transferrin receptor 1 (TfR1) and donates its iron to cells (reviewed by Richardson and Ponka, 1997). TfR1 is expressed at high levels in tumor cells, reflecting their greater need for iron than normal cells (Larrick and Cresswell, 1979; Sutherland et al., 1981; Richardson and Baker, 1990). The importance of TfR1 and iron uptake in growth is shown by the ability of the anti-TfR1 monoclonal antibody 42/6 to inhibit cancer cell growth by blocking Tf-binding to TfR1 and preventing iron uptake (Trowbridge and Lopez, 1982).

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Furthermore, tumor cells express higher levels of the iron-containing enzyme, RR, which is a crucial target for chelators (Cooper et al., 1996; Nyholm et al., 1993). The greater level of RR in cancer cells explains, at least in part, their greater sensitivity to iron chelators than normal cells (Elford et al., 1970; Richardson, 2002). Indeed, the cytotoxic agent, hydroxyurea (HU), acts on RR *via* a mechanism independent of Fe chelation, showing its utility as a useful target. However, HU has limited potency due to its short half-life, low affinity for RR and the fact that tumor cells develop resistance to it (Green et al., 2001; Richardson, 2002).

5.1.1 Iron Chelators: A Novel Class of Anti-Tumor Agents

Many *in vitro* and *in vivo* investigations and clinical trials have clearly shown that iron chelators are effective anti-tumor agents (reviews: Buss et al., 2004; Kalinowski and Richardson, 2005). The classical iron chelator, DFO (Figure 3A), that is used for treating iron overload, is the best studied in terms of its anti-tumor efficacy (reviewed in: Kalinowski and Richardson, 2005; Richardson et al., 2008). However, due to its limited membrane permeability and short half-life, DFO has shown modest anti-tumor activity (Richardson et al., 1994; Kalinowski and Richardson, 2005). There has been continuing efforts to improve the efficacy of iron chelators as cancer treatments. Indeed, the potential of iron chelators to act as anti-tumor agents was confirmed by the entrance of the chelator, Triapine®, into clinical trials (Yu et al., 2006; Figure 3A).

5.1.2 DpT and BpT Chelators Possess Marked and Selective Anti-Tumor Activity

The development of novel aroylhydrazone chelators such as 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311; Figure 3A) have resulted in compounds that show far greater anti-tumor and iron chelation efficacy than DFO (Richardson et al., 1995; Richardson and Milnes, 1997; Darnell and Richardson, 1999). More recently, novel chelators of the DpT class have been generated (Figure 3A; Lovejoy and Richardson, 2002; Becker et al., 2003; Yuan et al., 2004; Richardson et al., 2006; Whitnall et al., 2006). Some of these chelators are far more effective than

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DFO at entering cells, inducing cellular iron efflux and preventing iron uptake from Tf (Yuan et al., 2004). A control DpT analogue known as Dp2mT (Figure 3A) that does not bind iron, has also been synthesized and demonstrates that the mechanism of activity of these compounds is due to their ability to bind metal ions (Yuan et al., 2004).

Studies *in vivo* showed that Dp44mT significantly decreased tumor weight in mice bearing the chemotherapy-resistant lung carcinoma to 47% of the control after only 5 days (Yuan et al., 2004). Significantly, no changes in animal weight or hematological indices were found, demonstrating that the chelator acts selectively to inhibit tumor growth (Yuan et al., 2004). In addition, Dp44mT has been shown to be highly effective *in vivo* using a panel of human tumor xenografts and a wide range of cultured tumor cells *in vitro* (Whitnall et al., 2006). Of importance, little or no toxicity was seen in normal tissues at optimal doses.

More recent studies have resulted in the development of the BpT class of chelators (Figure 3B), which show a general increase in anti-tumor activity relative to the DpT group of ligands (Kalinowski et al., 2007). In these compounds, the non-coordinating 2-pyridyl group of the DpT ligands is replaced with a phenyl ring (Kalinowski et al., 2007), which increases the lipophilicity of the molecule that probably allows greater access to the tumor micro-environment (Tredan et al. 2007). These chelators show selectivity against tumor cells being far less active in normal fibroblasts (IC_{50}: 1.86 to >6.25 μM; Kalinowski et al., 2007). In addition, relative to the DpT ligands, the BpT series shows a marked increase in redox activity that is vital for anti-tumor activity (Kalinowski et al., 2007).

5.1.3 Iron Chelators Affect Multiple Molecular Targets

Studies have revealed that the successful anti-tumor activity of iron chelators is due to their effects on multiple molecular targets (Le and Richardson, 2004; Fu and Richardson, 2007; Nurtjahaja et

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al., 2007). These include the classical target, RR (Le and Richardson, 2002; Richardson, 2005), as well as their ability to: **(1)** up-regulate the iron-regulated metastasis suppressor, NDRG1 (Le and Richardson, 2004; Kovacevic et al., 2008); **(2)** to prevent iron uptake from transferrin *in vitro* (Yuan et al., 2004); **(3)** to increase iron efflux from cells (Yuan et al., 2004); and **(4)** to affect the expression of molecules involved in cell cycle progression that can inhibit proliferation and lead to apoptosis (*eg*., p53, cyclin D1, $p21^{WAF1/Cip1}$) (Liang et al., 2003; Yuan et al., 2004; Fu and Richardson, 2007). Notably, the novel mechanism of action of these drugs means that they overcome resistance to established chemotherapeutics (Whitnall et al., 2006).

5.1.4 Iron Chelators Up-Regulate the Growth and Metastasis Suppressor, NDRG1

As described above, NDRG1 expression is up-regulated by hypoxia (Le and Richardson, 2004). Several studies have also shown that *NDRG1* is up-regulated by iron-depletion (Dong et al., 2005; Le and Richardson, 2004). The transcription factor, HIF-1α, is expressed in response to hypoxia and Fe-depletion and has been found to up-regulate NDRG1 (Le and Richardson, 2004; Cangul, 2004). However, the up-regulation of NDRG1 by iron-depletion is mediated by both HIF-1α-independent and -dependent mechanisms (Le and Richardson, 2004).

Classically, Fe-depletion using chelators has been shown to stabilise HIF-1α by inhibiting the activity of prolyl hydroxylase that acts to degrade this transcription factor (Ivan et al., 2001; Jaakkola et al., 2001). More recently, in cancer cell types, it has been suggested that DFO up-regulates COX-2 through an ERK signaling mechanism (Tanji et al., 2001; Woo et al., 2006). The elevated activity of COX-2 is thought to increase HIF-1α (Woo et al., 2006). It is of interest that TGF-β1 increases HIF-1α protein stability by decreasing HIF-1α-associated prolyl hydroxylase through the SMAD signaling pathway (McMahon et al., 2006). This observation follows the intriguing finding by others that TGF-β1 is up-regulated by DFO (Yoon et al., 2002) and this may lead to increased PTEN that inhibits the AKT pathway. As described above, PTEN and PI3K/AKT

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have completely opposite effects on the activity of HIF-1α, and hence, probably also NDRG1 expression (Emerling et al., 2008).

Interestingly, DFO has been shown to inhibit pAKT in the pre-malignant keratinocyte cell line HaCaT (Faurschou and Gniadecki, 2008). In contrast, DFO was recently shown to increase the phosphorylation status of AKT and its targets FoxO1 and GSK3B in HepG2 hepatoma cells (Dongiovanni et al., 2008). It remains to be determined if the up-regulation of NDRG1 by iron chelation in cancer cells is influenced by inhibition of pAKT. It has also been shown that the early growth response gene (EGR-1) transcription factor mediates expression of NDRG1 in response to hypoxia and iron-depletion in some cells through direct binding to the *NDRG1* promoter (Zhang et al., 2007). In addition, it was recently found that EGR-1 regulates the *HIF-1α* gene during hypoxia (Sperandio et al., 2008), suggesting another mechanism that may modulate NDRG1 expression. There appears to be many points of integration between pathways that act to regulate NDRG1 and its metastasis suppressor function. However, increased expression of EGR-1 is found in prostate cancer cells (Thigpen et al., 2006) suggesting an alteration of the coupling mechanism between EGR-1 and NDRG1. Thus, the role of EGR-1 in the up-regulation of NDRG1 and HIF-1α and the link between the three proteins clearly need to be investigated further particularly in the context of prostate cancer. Since many studies have shown that the up-regulation of NDRG1 plays a vital role in preventing metastatic spread in cancer (Bandyopadhyay et al., 2003, 2004a, b), the up-regulation of NDRG1 by iron chelators is of significance. In summary, novel iron chelators show great promise as future anti-cancer agents through their effect on multiple targets such as the metastasis suppressor, NDRG1.

5.2 Targeting Arachidonic Acid and Eicosanoid Formation for Suppression of Tumor Growth.

The phospholipases can be classified into four classes on the basis of their nucleotide and amino acid sequence homology (Patel et al., 2008a). These enzymes are differentially expressed in a

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tissue-, species- and/or genotype-specific manner (Patel et al., 2008a). Of these proteins, $cPLA_2$-α is the most important, as it is the only family member that cleaves AA selectively from membrane phospholipids (Ghosh et al. 2006). Importantly, in prostate cancer there is a clear loss of the cellular $cPLA_2$ inhibitors, Annexin A1 (ANX1) and A2 (ANX2) (Paweletz et al., 2000; Chetcuti et al., 2001), suggesting that increased activity of these enzymes may be important in neoplasia.

5.2.1 Rationale for Targeting cPLA2-α

Inhibition of AA metabolism has become an attractive new target for treating cancer (Cummings, 2007). As the cleavage of AA by $cPLA_2$-α could be the rate-limiting step in eicosanoid synthesis, $cPLA_2$-α is a strong candidate to target in the treatment of prostate cancer. Considering the role of AA signaling in promoting mutagenesis, mitosis, angiogenesis and metastasis, it can be reasoned that a better outcome may be achieved with $cPLA_2$-α inhibitor(s) than COX or LOX inhibitors alone (Figure 4). This is because the latter approaches only suppress the production of COX and LOX metabolites (Figures 2 and 4). In contrast, blockade of the $cPLA_2$-α enzyme will decrease the supply of substrate to all eicosanoid-producing enzymes (Figure 4). For this same reason, inhibition of cPLA2-α should not induce the side effects of COX-2 selective inhibitors such as thrombosis.

5.2.2 The Efficacy of Targeting cPLA2-α in Prostate Cancer Cells In Vitro and In Vivo

In an effort to assess the potential of $cPLA_2$-α as a therapeutic target in prostate cancer, the expression levels of $cPLA_2$-α has been examined in prostate cancer cell lines (Patel et al., 2008b). In these studies, androgen-sensitive LNCaP cells expressed less $cPLA_2$-α mRNA and protein than the androgen-independent PC-3 cell line. Comparison was made examining the immuno-staining of phospho-$cPLA_2$-α (Ser^{505}) in androgen-sensitive prostate cancer with samples from the same patient once they had reached androgen-independent status. This latter investigation demonstrated a clear further increase in phospho-$cPLA_2$-α staining intensity in cells that displayed androgen-independence. When $cPLA_2$-α mRNA was silenced with siRNA, there was a decrease in cell

Molecular Pharmacology Fast Forward. Published on December 3, 2008 as DOI: 10.1124/mol.108.053066
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proliferation and increase in apoptosis in LNCaP and PC-3 cells. Similar to the results obtained using siRNA, the cPLA$_2$-α inhibitor Efipladib (also referred to as Wyeth-1; Ni et al., 2006; Figure 4), decreased p-cPLA$_2$-α and mitochondria-active cell numbers in a dose-dependent manner. An accumulation of cells in G_0/G_1 and a corresponding decrease in S phase were also observed and this decreased tumor cell proliferation was also found *in vivo*. In fact, Wyeth-1 reduced PC-3 xenograft growth by approximately 33% within 2 weeks (Patel et al., 2008b).

5.2.3 cPLA$_2$-α and its Effect on AKT and p53

To determine if the AKT pathway could be affected by inhibition of cPLA$_2$-α, a recent study examined LNCaP and PC-3 cells treated with cPLA$_2$-α siRNA (Patel et al., 2008b). Compared to control cells transfected with scrambled siRNA, cPLA$_2$-α siRNA treated cells showed a reduction in pAKT and cyclin D1. Increasing concentrations of Wyeth-1 for 72 h resulted in a reduction in pAKT with a simultaneous decrease in cyclin D1 expression (Patel et al., 2008b). This work demonstrated a significant role of cPLA$_2$-α in prostate cancer cell proliferation.

A recent study by Sun and colleagues (2008) has demonstrated that treatment of the LNCaP cell line (wild type p53) with the cPLA2-α selective inhibitor, bromoenol lactone, effectively blocked EGF-induced cellular proliferation. This was associated with G_o/G_1 stage cell cycle arrest proceeded by increased p53 and p21 and decreased expression of the p53 antagonist, MDM2 (Sun et al., 2008). Bromoenol lactone also inhibited proliferation of the PC-3 (mutant p53) cell type, indicating that both p53-dependent and independent pathways can be influenced by cPLA2. These data support our hypothesis that targeting AKT *via* cPLA2 inhibitors can influence important sites of cross-talk between cellular pathways that regulate proliferation.

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6. Conclusions

This review has demonstrated that there are many established and potential key points of integration between the TGF-β, PI3/AKT and PTEN pathways in tumor cell biology. It has highlighted the potential of two new pharmacological approaches of iron chelation and $cPLA_2$-α inhibition. These agents could exploit our suggested points of cross-talk in cell pathways that are commonly disrupted in prostate tumors and many other cancers. Further analysis and verification of these points of integration and their roles in cancer development will undoubtedly result in the development of new pharmaceutical agents for the treatment of prostate cancer and, possibly, other tumors.

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Molecular Pharmacology Fast Forward. Published on December 3, 2008 as DOI: 10.1124/mol.108.053066
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Footnote to the Title:

The work was supported by a fellowship and grants from the National Health and Medical Research Council (NHMRC) of Australia and Australian Research Council (to D.R.R.). H.M. is the recipient of a NHMRC Australian Based Biomedical Post-Doctoral Fellowship.

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Figure Legends

Figure 1: Overview of the AKT, PTEN and TGF-β pathways illustrating key points of cross-talk that influence tumorigenic and tumor-suppressive cell responses. The integration of these pathways is important for understanding the development of novel therapeutics.

(A) The PI3K/AKT pathway is stimulated by activation of phosphatidylinositol-3 kinase (PI3K) upon ligand binding to a tyrosine kinase receptor (TKR). Phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) allows for 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylation of AKT to active pAKT. Activated AKT elicits tumorigenic effects through stimulation of its downstream effectors, mTOR (mammalian target of rapamycin) and MDM2, as well as suppression of glycogen synthase kinase 3β (GSK3-β) and the cyclin-dependent kinase inhibitors (CDKIs) p15, p21 and p27.

(B) The phosphatase and tensin homolog deleted on chromosome ten (PTEN) promotes tumor suppression through antagonism of AKT by dephosphorylation of PIP3, stimulation of p53 activity and suppression of MDM2.

(C) Transforming growth factor β (TGF-β) tumor suppression is mediated by TGF-β-induced formation of its receptor complex (TGF-βRII/RI) and subsequent activation of the SMAD signaling cascade. SMAD signaling induces expression of tumor suppressive PTEN and the cyclin dependent kinase inhibitors p15, p21 and p27, but suppresses tumorigenic c-Myc and anti-apoptotic *Bcl2* expression. Activation of mTOR by pAKT suppresses TGF-β activated SMAD signaling.

Figure 2: The metastasis suppressor NDRG1, and the potentially tumorigenic fatty acid, arachidonic acid (AA), provide targets for pharmacological modulation of cancer cell proliferation. Cytosolic phospholipase ($cPLA_2$-α) releases arachidonic acid (AA) into the cytosol from membrane phospholipids. AA is converted to eicosanoids (*e.g.*, prostaglandin E2; PGE2) by lipooxygenases and cyclooxygenases (COX and LOX, respectively) that indirectly stimulates phosphorylation of AKT (pAKT). Alternatively, AA can directly stimulate formation of pAKT,

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leading to increased cellular proliferation. Pharmacological inhibitors of cPLA2-α, COX or LOX would prevent this activity. Furthermore, AA can reduce the activity of PTEN, blocking its antagonism of the AKT pathway and may also reduce expression of NDRG1. As PTEN is down-regulated in many cancers, stimulation by pharmacological intervention (*e.g.*, iron chelators) can lead to increased expression of the metastasis suppressor NDRG1 and subsequently the inhibition of proliferation and metastasis.

Figure 3. Structures of iron chelators discussed in this review. Desferrioxamine (DFO) and Triapine are both chelators examined in clinical trials for their anti-tumor activity (Kalinowski and Richardson 2005; Kalinowski et al., 2007). Development of novel chelators that improve upon the efficacy of DFO led to the aroylhydrazone, 2-hydroxy-1-napthylaldehyde isonicotinoyl hydrazone (311). Subsequently, chelators of the DpT series were generated including dipyridylketone 4,4-dimethylthiosemicarbazone (Dp44mT) that markedly inhibits tumor growth and the control chelator, dipyridylketone 2-methylthiosemicarbazone (Dp2mT), that does not bind iron. Further development of these chelators resulted in synthesis of the effective 2-benzoylpyridine thiosemicarbazone (BpT) series of ligands (Kalinowski et al., 2007).

Figure 4. Inhibitors of key enzymes of the arachadonic acid (AA) pathway. The enzyme cytosolic phospholipase A_2-α (cPLA_2-α) liberates AA from the plasma membrane. The release of AA can be tumorigenic leading to activation of AKT. However, this enzyme can be inhibited by Efipladib (see insert for structure) (Ni et al., 2006). The AA can also be metabolised further by cyclooxygenase 1 and 2 (COX-1 and -2) leading to prostaglandins (*e.g.*, PGE2). COX-1 and 2 inhibitors are well known and include Celecoxib. Liberated AA can also be acted upon by 5-lipooxygenase (5-LOX) or 12-lipooxygenase (12-LOX) leading in turn to the hydroxyeicosatetraenoic acids 5-HETE and 12-HETE. The inhibitors, MK886 and Baicalein, prevent the activity of 5-LOX and 12-LOX, respectively.

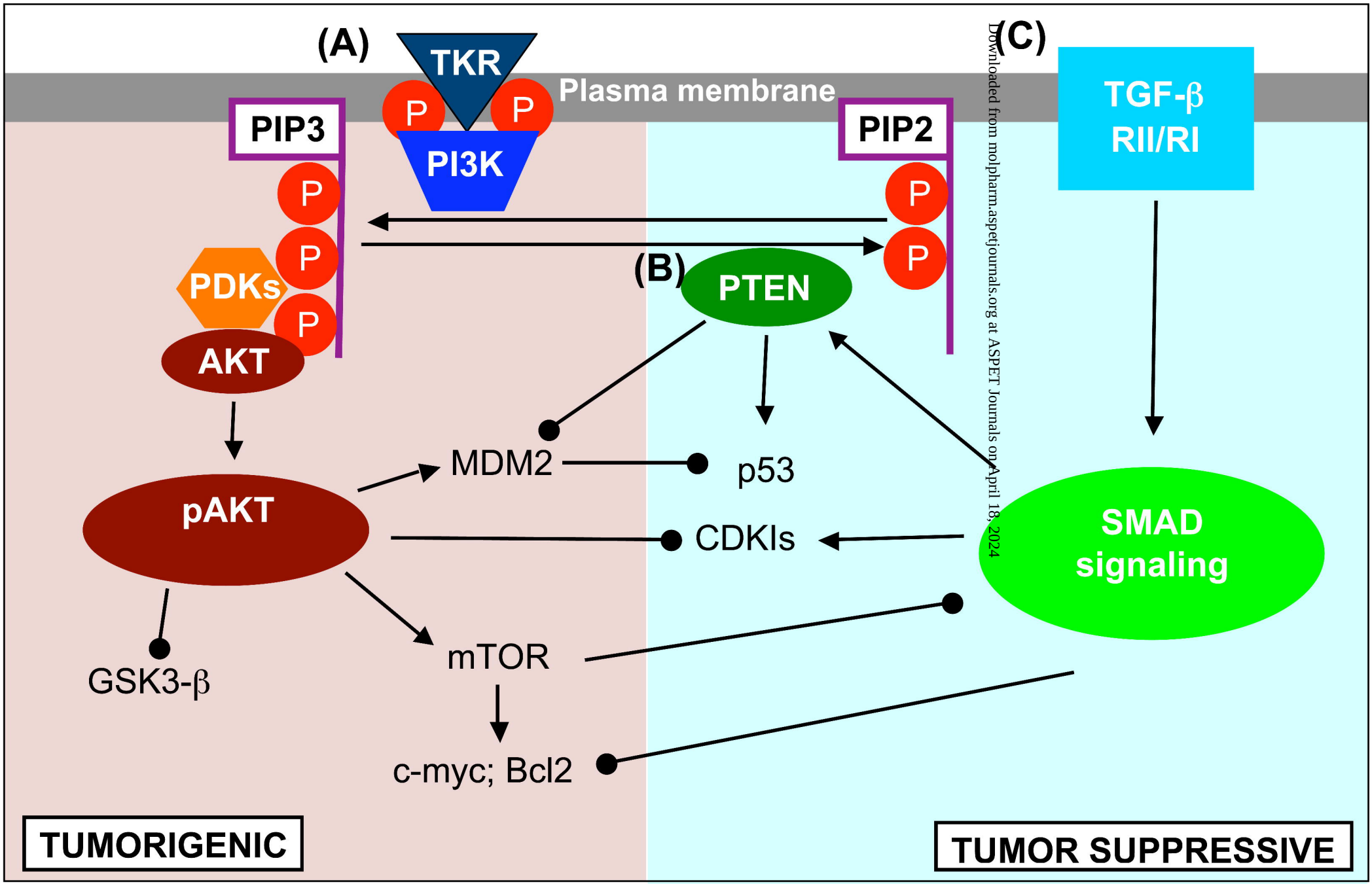

FIGURE 1

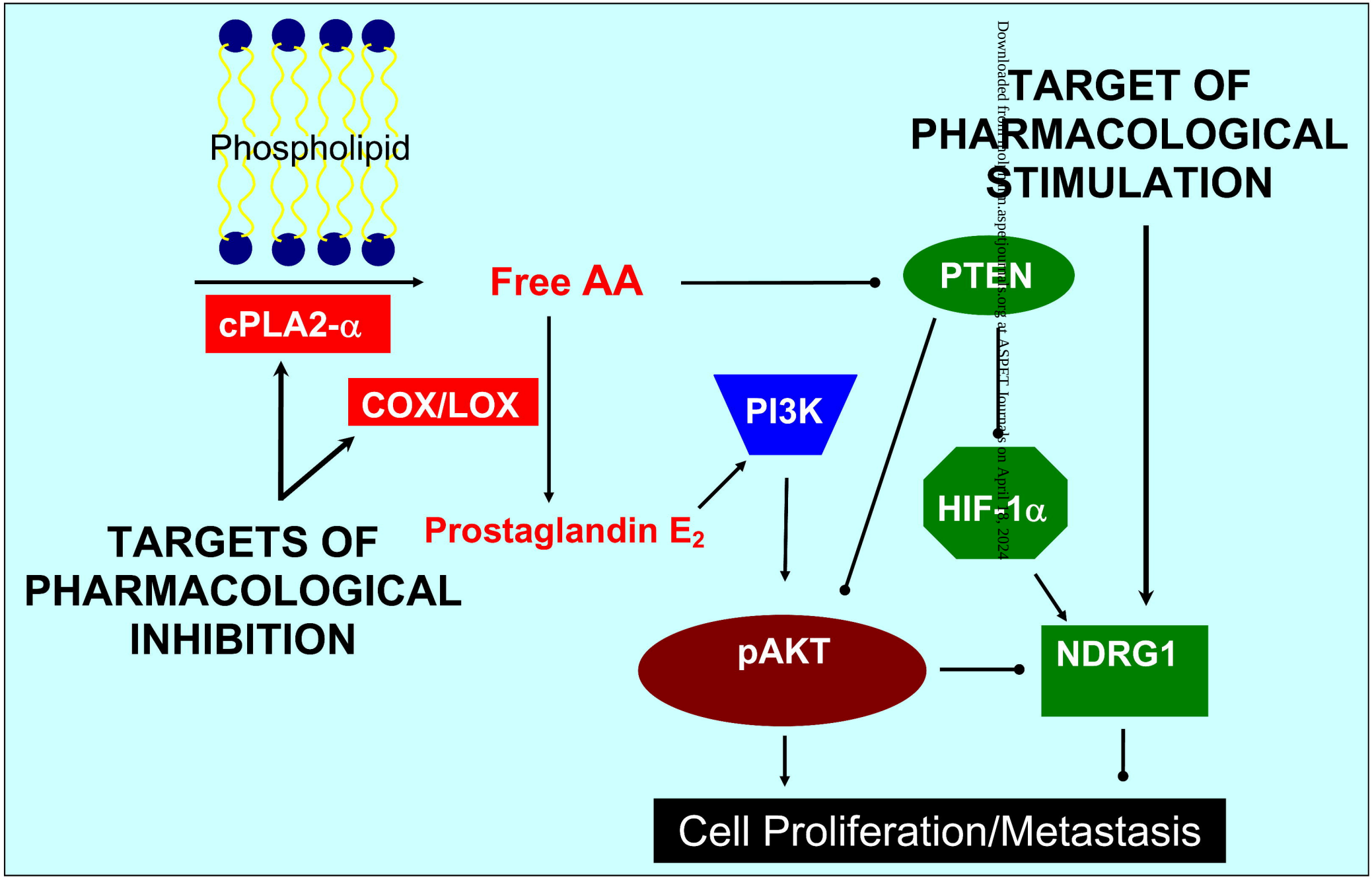

Figure 2

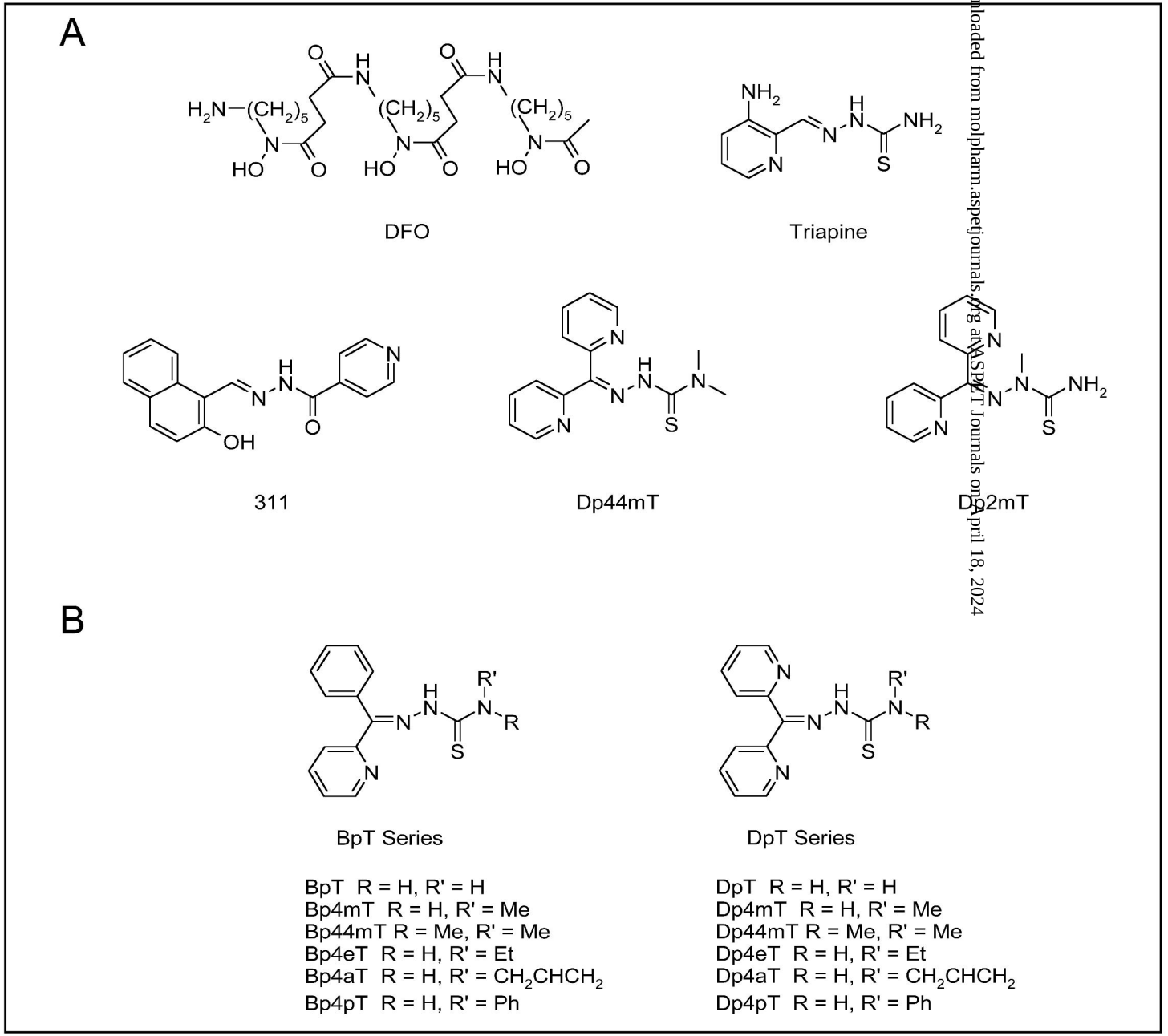

Figure 3

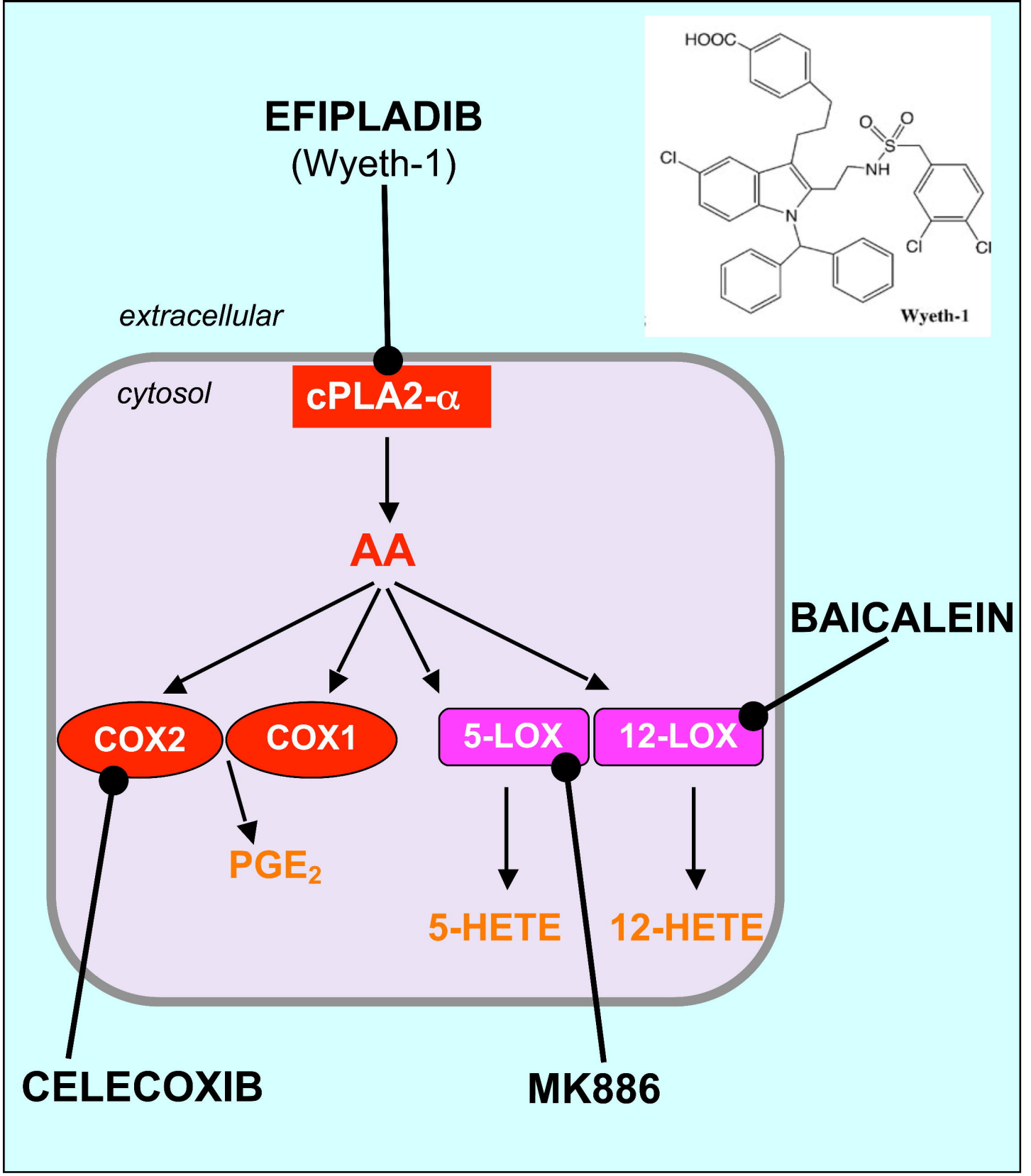
EFIPLADIB
(Wyeth-1)
HOOC
Cl
O
O
S
NH
N
Cl
Cl
Wyeth-1
extracellular
cytosol
cPLA2-α
AA
BAICALEIN
COX2
COX1
5-LOX
12-LOX
PGE2
5-HETE
12-HETE
CELECOXIB
MK886

Figure 4