TITLE: Pim-1: A Molecular Target to Modulate Cellular Resistance to Therapy in Prostate Cancer

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The contract supports studies to define the role of the PIM1 kinase in acquired resistance to chemotherapy by prostate cancer cells. Data to date for specific aim #1 define a signaling pathway induced by docetaxel, involving sequential steps of JAK1/2 activation, STAT3 phosphorylation, expression of PIM1, and activation of NFkB signaling. Blockade of this pathway by expression of dominant negative PIM1 proteins blocks drug-induced upregulation of NFkB activity, and sensitizes cells to docetaxel. Other studies (specific aim #2) focus on identifying a mechanism through which PIM1 activates NFkB. We have unambiguously identified S937 as the major PIM1 phosphorylation site on the NFKB1/p105 precursor protein, through use of LCM/MS/MS analysis. Other kinases that can phosphorylate this site include AKT and PKA. Additional data (specific aim #3) have been published to describe a small molecule inhibitor of PIM1. This molecule can sensitize prostate cancer cells to the cytotoxic effects of docetaxel in an additive or synergistic manner.
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INTRODUCTION
Studies under this funded activity are focused on characterizing the role of the PIM1 gene in acquired resistance to chemotherapy drugs, by prostate cancer cells. The proposal included three specific aims: 1) to define a novel signal transduction pathway activated by docetaxel, 2) to characterize the mechanism through which PIM1 activates and regulates NFkB signaling, and 3) to explore genetic and pharmacologic means of inhibiting PIM1 activity or expression to enhance the sensitivity of prostate cancer cells to docetaxel and other chemotherapy drugs. Substantial progress has been made in each of these areas during the 01 year of support.

BODY
We will outline our progress through reference to the specific aims described above. The first specific aim was to outline a signal transduction pathway activated by docetaxel and involving upregulation of PIM1 expression. This pathway has been substantially defined. Using RWPE2 prostate cells, we noted that docetaxel treatment rapidly leads to an increase in expression of the PIM1 serine/threonine kinase. Expression becomes apparent at 3hrs after drug addition, peaks at 9-12hrs, and returns to baseline by 24hrs (Fig. 1). This increase in expression is accompanied by an increase in pim-1 mRNA, as shown by real time-PCR analysis (Fig. 2). Thus the effects of docetaxel are primarily transcriptional or post-transcriptional.

We next wanted to define mechanisms through which pim-1 could be transcriptionally upregulated. Transcription of pim-1 is known to be activated by STAT transcription factors and by NFkB transcription factors. We examined the time course of STAT3 activation after docetaxel treatment (Fig. 1), and noted that it paralleled the course of pim-1 expression. We therefore suspected that docetaxel increased pim-1 expression in a STAT3-dependent manner. This was directly demonstrated by use of decoy oligonucleotides (Fig. 3). Double-stranded DNA oligonucleotides matching a known STAT3 binding site blocked the drug-induced upregulation of pim-1 expression, while a decoy based on a mutated (non-binding) STAT3 site did not. These data therefore establish a linear relationship among the following events: docetaxel treatment $\rightarrow$ STAT3 activation $\rightarrow$ pim-1 expression.

We hypothesized that NFkB transcriptional activation would be a downstream event in this signal transduction pathway, because many chemotherapy drugs and other stressors are known to activate NFkB. We engineered RWPE2 cells to constitutively express a NFkB-dependent promoter/luciferase plasmid, and...
found that docetaxel treatment increased NFκB transcriptional activity. We then transiently infected these cells with a pim-1-encoding retrovirus. Pim-1 expression also consistently increased NFκB transcriptional activity (Fig. 4). To determine if the drug-induced increase in NFκB activity occurred in a pim-1-dependent manner, we then infected the reporter cell line with a retrovirus encoding a dominant-negative form of pim-1, pimNT81. The dominant negative pim-1 cDNA completely blocked the drug-induced upregulation of NFκB activity, demonstrating that pim-1 expression is a necessary upstream step in the drug-induced activation of NFκB (Fig. 5). In aggregate these studies establish a signal transduction pathway triggered by docetaxel treatment of RWPE2 prostate cancer cells.

To determine if this pathway modified drug toxicity, we examined the effects of enforced expression of wild-type or NT81 pim-1 cDNAs of docetaxel cell kill (Fig. 6). Docetaxel produced dose-dependent cell kill in RWPE1, 2 cells. Enforced expression of wild-type pim-1 cDNA markedly reduced cell death. In contrast, expression of the dominant negative NT81 cDNA enhanced cell death after docetaxel treatment. These data demonstrate that pim-1 expression can modulate drug-induced cell death, and demonstrate that the survival pathway described above is a legitimate target for pharmacologic intervention. These data will be presented at the 2006 AACR meeting in poster form (1).
The goal of specific aim #2 was to define pathways through which the PIM1 kinase could activate NFkB transcriptional activity. We had hypothesized that PIM1 would phosphorylate the NFkB1/p105 precursor protein on serine-937, leading to proteolytic cleavage of the protein with release of active p50 protein as well as other sequestered NFkB components and the TPL2 kinase. To demonstrate the site of phosphorylation we used mass spectroscopy of trypsin-digested fragments of p105 that had been phosphorylated in vitro. We had previously demonstrated that PIM1-dependent phosphorylation happens exclusively on serine. Fragments were separated by LC/MS/MS analysis and mass/charge ratios were determined. The predicted peptide fragment that would result from phosphorylation at serine-937 was recovered, with a mass of 1016 (Fig. 7). Since there are several potential phosphorylation sites within this peptide, we proceeded to sequence the peptide with mass spectroscopy. Only the fragment corresponding to phosphoserine-937 was not recovered. These data unambiguously demonstrate that the major phosphorylation site of PIM1 on p105 is serine-937.

To further characterize phosphorylation on this site we prepared an antibody specific for phosphoNFkB1/p105(S937). This antibody reacts quite specifically with its antigen in whole cell lysates and immunoprecipitates. Fig. 8 demonstrates that recombinant GST-PIM1 protein phosphorylates NFkB1/p105 on serine 937 in vitro. Furthermore wild-type PIM1 phosphorylates p105 in vivo, while a dominant-negative PIM1 (NT81) does not.

**In vitro**  
**In vivo**  
(RWPE2 cells)
The third specific aim proposed to use small molecule inhibitors of the PIM1 kinase as molecular probes to determine their effect on docetaxel sensitivity. A report describing one such molecule, the flavonol quercetagetin, has been accepted for publication and will appear in the January, 2007 issue of *Molecular Cancer Therapeutics* (see appendix A and Fig. 9). We have demonstrated that quercetagetin in a moderately potent (IC$_{50}$ = 340nM, specific, and cell-permeable inhibitor of PIM1 activity in prostate cancer cells. Key data include the demonstration that quercetagetin in competitive with ATP. A crystal structure of PIM1 in complex with quercetagetin, or with three other flavonoids, has been determined. We have also shown that quercetagetin is able to inhibit the activity of the PIM1 kinase in prostate cancer cells at an IC$_{50}$ of about 5.5μM. Interestingly the activity of the AKT kinase is not inhibited at all under these conditions (Fig. 9).

We have recently obtained, and begun characterizing, novel small molecule inhibitors of PIM1 from Excelixis Corporation. These molecules show additive, or at some concentrations synergistic, cell growth inhibition in combination with docetaxel. These studies confirm the central hypothesis of this overall project, that PIM1 kinase acts to inhibit cell death caused by the cytotoxic drug docetaxel, and that blocking the activity of PIM can potentiate cell kill and overcome cytotoxic drug resistance.

**KEY RESEARCH ACCOMPLISHMENTS**

- Definition of a novel survival pathway activated by docetaxel treatment, and involving sequential activation or expression of JAK2, STAT3, PIM1, and NFkB components.
- Identification of serine-937 as the major phosphorylation site for PIM1 on the p105/NFKB1 precursor protein
- Identification of quercetagetin as a moderately potent and specific, cell-permeable PIM1 kinase inhibitor
- Demonstration that XL-1075 and XL-1154 can show additive or synergistic cell kill in prostate cancer cells treated with docetaxel
- Abstract presented at the annual AACR meeting, Washington DC, April, 2006
- Manuscript accepted for publication in *Molecular Cancer Therapeutics*

**REPORTABLE OUTCOMES**

None in 02 year

**CONCLUSIONS**

Our data demonstrate that PIM1 is a critical component of a survival/stress pathway activated by docetaxel treatment of prostate cancer cells. This pathway leads to activation of NFkB-dependent transcription, possibly by phosphorylation of p105/NFKB1 by PIM1 at serine-937.
Targeting PIM1 kinase activity with quercetagetin, or other PIM1 kinase inhibitors, leads to additive or synergistic cell kill following docetaxel treatment.

REFERENCES

1. Zemskova M, Sahakian E, Lilly M: The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death (abstract #2777), presented at 97th Annual Meeting of AACR, Washington, DC, April 2006.

APPENDIX
Research data are presented throughout the body of this report. The appendix contains three items:

1. AACR abstract #2777, approved for presentation at the 97th Annual Meeting, April, 2006, entitled “The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death” by M. Zemskova, E. Sahakian, M. Lilly.
2. Galley proofs of manuscript accepted for Molecular Cancer Therapeutics
3. Curriculum vitae for Michael Lilly, MD
Characterization of a potent and selective small-molecule inhibitor of the PIM1 kinase

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Abstract

The pim-1 kinase is a true oncogene that has been implicated in the development of leukemias, lymphomas, and prostate cancer, and is the target of drug development programs. We have used experimental approaches to identify a selective, cell-permeable, small-molecule inhibitor of the pim-1 kinase to foster basic and translational studies of the enzyme. We used a ELISA-based kinase assay to screen a diversity library of potential kinase inhibitors. The flavonoid quercetin (3,3',4',5,7,3',4'-heptahydroxyflavone) was identified as a moderately potent, ATP-competitive inhibitor (IC50, 0.34 μM). Resolution of the crystal structure of complex with quercetin or two other flavonoids revealed a spectrum of binding poses and hydrogen-bonding patterns in spite of strong similarity of the ligands. Quercetin was a highly selective inhibitor of PIM1 compared with PIM2 and seven other serine-threonine kinases. Quercetin was able to inhibit PIM1 activity in intact RWPE2 prostate cancer cells in a dose-dependent manner (ED50, 5.5 μM). RWPE2 cells treated with quercetin showed pronounced growth inhibition at inhibitor concentrations that blocked pim-1 kinase activity. Furthermore, the ability of quercetin to inhibit the growth of other prostate epithelial cell lines varied in proportion to their dependence on pim-1 kinase. Quercetin can function as a moderately potent and selective, cell-permeable inhibitor of the pim-1 kinase, and may be useful for proof-of-concept studies to support the development of clinically useful PIM1 inhibitors.

Introduction

The pim family of serine-threonine kinases is composed of three highly homologous genes, pim-1, pim-2, and pim-3. These enzymes are increasingly being recognized as important mediators of survival signals in cancer, stress response, and neural development (1–6). In addition, these genes are constitutively expressed in some tumors and function as true oncogenes. Thus, they are of significant interest as targets for therapeutic intervention.

Small-molecule inhibitors are important molecular probes for studying protein kinases. In addition, they may serve as prototype therapeutic agents for treating diseases resulting from unregulated kinase activity. Three prior reports have shown that known, promiscuous kinase inhibitors can inhibit PIM1 function in vitro. Jacobs et al. (7) showed that several staurosporine and bisindolylmaleimide analogues, as well as the morpholino-substituted chromone LY294002, were able to inhibit PIM1 activity in vitro. Subsequently, Fabian et al. (8) presented an interaction map involving 113 kinases and 20 small-molecule kinase inhibitors now under clinical study. Only three inhibitors had detectable binding to (and presumably inhibitory activity against) PIM1—two staurosporine analogues and flavopiridol, a flavonoid undergoing evaluation as an inhibitor of cyclin-dependent kinases. A recent report (9) confirmed the activity of bisindolylmaleimide derivatives as well as some flavonoids in vitro. All of the identified inhibitors either lacked specificity for PIM1 or were only modestly active at low micromolar concentrations, or both. Furthermore, none of these reports showed that the test agents could selectively inhibit PIM1 activity in intact cells.

To further our basic and translational studies of the pim-1 kinase, we have sought to identify small-molecule inhibitors of PIM1. We have reported that the flavonoid quercetin is a selective PIM1 inhibitor with nanomolar potency and can differentially inhibit the kinase in cell-based assays.

Materials and Methods

Cell Lines and Culture Methods

The prostate epithelial cell lines RWPE1, RWPE2, LNCaP, and PC3 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in the recommended medium. We produced additional pools of RWPE2 prostate cells that overexpressed pim-1 through retroviral transduction. The coding region for the human pim-1 gene was cloned into the pLNCX retroviral vector. Infectious viruses were produced in the GP-293 packaging cell line by cotransfection with retroviral backbone plasmids (pLNCX or pLNCX/pim-1) and with pVSV-G, a plasmid that expresses the envelope glycoprotein from
vesicular stomatitis virus. Forty-eight hours after transfection, the medium was collected and the virus particles were concentrated as described in the manufacturer’s protocol (Clontech). RWF2E cells were plated at $1 \times 10^6$ per 60-mm plate 16 to 18 h before infection. Cells were infected with $5 \times 10^5$ viral particles in the presence of 8 µg/ml polybrene. After 6 h of incubation, the virus-containing medium was replaced with fresh medium, and on the next day G418 (400 µg/ml) was added to select infected cells. After 10 days of selection, stable cell pools were established and PIM1 expression was verified by immunoblotting.

For growth-inhibition experiments, cells were plated onto 24-well plates and fixed with formaldehyde at intervals. Cell number was quantified by crystal violet staining (10).

Recombinant pim Kinases and Kinase Assays
We prepared recombinant PIM1 and PIM2 as glutathione S-transferase (GST) fusions in Escherichia coli, as described (11). For the inhibitor screening assays, a solid-phase kinase assay was developed based on our demonstration that PIM1 is a potent kinase for phosphorylating BAD on Ser112 (11, 12). Ninety-six-well flat-bottomed plates (Corning) were coated overnight at 4°C with recombinant GST-BAD [1 µg/well in HEPES buffer: 1.36 mM NaCl, 2.6 mM KCl, 20 mM HEPES (pH 7.5)]. The plates were then blocked for 1 h at room temperature with 10 mg/ml bovine serum albumin in HEPES buffer. The blocking solution was replaced and 5 µl of each inhibitor, dissolved in 50% DMSO, were added to each well. Then, 100 µl of kinase buffer [20 mM/L, MOPS (pH 7.0), 1.25 mM/L, MgCl2, 1 mM/L, MnCl2, 1 mM/L, EGTA, 150 mM/L, NaCl, 10 µM/L ATP, 1 mM/L, DTT, and 5 µM/L, p-glycerophosphate] containing 25 ng recombinant GST-PIM1 kinase were added to each well. The final concentration of each inhibitor was $-10$ µM/L. The plate was placed on a gel slab dryer proximal to 30°C, and the kinase reaction was allowed to proceed. The reaction was stopped after 60 min by removal of the reaction buffer, followed by the addition of 100 µl of HEPES buffer containing 20 mM/L EDTA at each well. Phosphorylated GST-BAD was detected by an ELISA reaction, using as a first antibody a monoclonal anti- phospho-BAD (Ser112) antibody (Cell Signalling), a secondary goat anti-mouse IgG-peroxidase conjugated antibody (Pierce), and TMB peroxidase substrate (Pierce). The level of phosphorylated GST-BAD present was proportional to the absorbance at 450 nm.

For quantitative and kinetic studies of inhibitors against various BAD(S112) kinase, a solution phase assay was used. A biotinylated peptide based on the PIM1 phosphorylation site of human BAD was synthesized (GGGGA-VEIKSRHSEYPAGTE) and used as the assay substrate. Recombinant GST-PIM1 (25 µg/reaction) was preincubated with various concentrations of inhibitors in the previous kinase buffer (final volume 100 µl). The reaction proceeded by addition of substrate peptide, followed by incubation for 5 min in a 30°C water bath. The reaction was terminated by transferring the mixture to a streptavidin-coated 96-well plate (Pierce) containing 100 µl/well of 40 mM/L EDTA. The biotinylated peptide substrate was allowed to bind to the plate at room temperature for 10 min. The level of phosphorylation was then determined by ELSA as described above. Curve fitting and enzyme analyses were done using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). For the additional BAD(S112) kinases (PIM2, BSK2 (ribosomal S6 kinase 2), and PKA (cyclic AMP-dependent protein kinase)), reaction components were as described above. As with the PIM1 assays, an ATP concentration of 10 µM/L was used. Furthermore, with each kinase, linear reaction velocities for the duration of the reaction were confirmed (data not shown).

To further assess the specificity of quercetin as a PIM1 inhibitor, its activity against a panel of serine-threonine kinases was also studied through a commercial kinase inhibitor profiling service (KinaseProfiler; Upstate Biotechnology). All KinaseProfiler assays were conducted using 10 µM/L ATP concentrations.

Small-Molecule Library Screening
We obtained a library of 1,200 compounds that had structural affinity to known kinase inhibitors (Tocris, Inc.). The entire library was screened once with our solid-phase ELISA kinase assay, with each compound at $\leq 10$ µM/L concentration. Positive hits were re-screened at the same concentration. Compounds that had reproducible activity at 10 µM/L were then screened at a range of concentrations from 0.01 to 300 µM/L. Additional flavonoids were purchased from Indolene Chemicals and were tested in a similar protocol.

Measurement of PIM1 Kinase Activity in Cells
RWF2E cell pools, stably infected with empty retrovirus or pim-L-encoding retrovirus, were seeded in six-well plates at $5 \times 10^5$ cells per well. After 18 h, the normal supplemented keratinocyte medium was removed and replaced with supplement-free keratinocyte medium. Cells were then incubated for an additional 20 h. Quercetin, or an equivalent volume of DMSO, was added to the cells 3 h before the end of the starvation period. At the conclusion of the starvation period, the cells were washed twice with PBS and subsequently lysed in a denaturing buffer with protease, phosphatase inhibitors. The lysates were normalized by total protein content (BCA protein assay, Pierce), then analyzed by immunoblotting with the following antibodies: monoclonal anti-PIM1 (Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal anti-p-actin (Sigma); monoclonal anti-BAD (Transduction Laboratories); and monoclonal anti-phospho-BAD(S112), polyclonal anti-phospho-AKT(S473), and anti-AKT (all from Cell Signalling).

Cloning, Expression, Purification, and Crystallization of PIM1
The production, purification, and characterization of recombinant His-tagged PIM1 proteins for crystallography have been described previously (13). To obtain cocrystals of complexes of the protein with ligands, the protein solution was initially mixed with the compound (dissolved in DMSO) at a final compound concentration of 1 µM/L and then set up for crystallization. The protein was
crystallized by a sitting-drop, vapor-diffusion experiment in which equal volumes of protein (10–15 mg/mL concentration) and reservoir solution [0.4–0.9 mol/L sodium acetate, 0.1 mol/L imidazole (pH 6.5)] were mixed and allowed to equilibrate against the reservoir at 4 °C. The crystals routinely grew to a size of 200 × 200 × 300 μm in ~2 to 3 days.

Structure Determination

X-ray diffraction data were collected at Advanced Light Source (Berkeley, CA). All data were processed and reduced with MOSFLM and scaled with SCALA of the CCP4 suite of programs using the software ELSIE. The sacre group of all crystals was determined to be P63, with the cell axes being approximately 99, 99, and 80, and one protein monomer being present in the asymmetric unit. All structures were determined by molecular replacement using the apo PIM1 structure (1YVF; ref. 13) as a model, and refined by CNS and REFMAC5. Crystallographic statistics are reported in Supplementary Table S1. The coordinates and structure factors for the structures have been deposited with the RCSB Protein Data Bank (accession codes).

Results

Screening of a Chemical Library with Structural Affinity to Known Kinase Inhibitors

As an initial approach to the identification of PIM inhibitors, we screened a library of small molecules whose structures were similar to those of known kinase inhibitors. Of the seven compounds that had reproducible inhibitory activity at 10 μmol/L, six were flavonoids (quercetin, luteolin, kaempferol, 7-hydroxyflavone, (S)-5,7-dihydroxy-3-(3-methylbutyl)-2-methylflavone, and (R)-5,7-dihydroxyflavone). These compounds exhibited a range of inhibitory potencies (as IC50) from 1.1 to 60 μmol/L. Thirty-seven other flavonoids failed to show detectable inhibitory activity at 10 μmol/L. These inactive compounds were characterized in most cases by bulky (charged or uncharged) groups at the 3, 3', 4, or 7 positions, lack of at least two hydroxyl bond donors on the A or C rings present of glycoside linkages or failure of all rings to adopt a planar conformation.

The most active compound in the chemical library was the flavonol quercetin (IC50, 1.1 μmol/L), a known inhibitor of kinases and many other enzymes (14–19). Furthermore, six of the seven compounds with reproducible activity at 10 μmol/L were flavonoids. Hence, we screened additional flavonoids to identify molecules with inhibitory activity against the PIM1 kinase (Fig. 1). The most active molecule was the flavonol quercetin (IC50, 0.34 μmol/L). The four flavonoids with the highest inhibitory activity were characterized by the presence of five to six –OH groups distributed between the A and B rings. In comparison, the hydroxyl groups on the B ring seemed to be more critical for the activity of the compounds than those on the A ring, as compounds with an unsubstituted B ring showed greatly reduced activity. Finally, a hydrophobic substituent at the 8 position was tolerated.

Quercetatin is a Selective Potent Inhibitor of PIM1 In Vitro

To assess the selectivity of quercetatin for PIM1, we determined its IC50 value toward the alternative BADs (S112) kinase RSK2, PKA, and PIM2 (Table 1). The IC50 of quercetatin for PIM1 kinase was 0.34 μmol/L, whereas the corresponding values for the other kinases were 9- to 70-fold higher.

To further characterize the specificity of quercetatin, its inhibitory activity was examined at 1 or 10 μmol/L against additional serine-threonine kinases (c-Jun-NH2-kinase 1, PKA, Aurora-A, c-Raf, and FAKC, Fig. 2). At the lower concentration, the selectivity of quercetatin was most apparent. In the presence of 1 μmol/L inhibitor, PIM1 activity was inhibited by 9%. In contrast, the activity of the other kinases was inhibited by only 0% to 41%. In aggregate, these studies established that quercetatin was a sevenfold more potent inhibitor for pim-1 kinase than for seven other serine-threonine kinases. In addition, quercetatin was completely inactive against the α-totyrosine kinase when tested at the 200 μmol/L concentration (data not shown).

Crystallographic Analysis of Quercetatin in Complex with PIM1

Recently, several crystal structures of the pim-1 kinase have been solved and presented, including apo forms and the enzyme complex with a variety of ligands (7, 9, 13, 20, 21). Because the PIM1 protein has several unique structural features around its ATP-binding pocket, including the lack of the canonical hydrogen bond donor from the hinge region typically used by kinases to bind ATP-like ligands, we determined the crystal structure for the kinase in complex with three flavonoid inhibitors: quercetatin, myricetin, and 5,7,3',4',5'-pentahydroxyflavone (Fig. 3).

The three flavonoids inhibitors show two distinct binding poses, denoted here as orientations I and II, respectively. Quercetatin, the compound with two hydroxyl groups on the B ring, adopts orientation I, whereas the compounds with a tri-substituted B ring (myricetin and 5,7,3',4',5'-pentahydroxyflavone) adopt orientation II.

The binding pose of quercetatin in PIM1 (Fig. 3A) closely resembles that of quercetin in phosphatidylinositol 3-kinase γ (1BS; ref. 22) and that of fisetin in CDK6 (DXC2; ref. 23), designated here as orientation I. As seen in the two earlier structures (Fig. 3D and E), the 3-OH of the quercetatin (Fig. 3A) makes a canonical hydrogen bond with backbone carbonyl oxygen of the hinge residue arg52. In addition, the B ring of quercetatin binds deep inside the PIM1 ATP-binding pocket, with the 4-hydroxyl group hydrogen-bonded to the side chains of two highly conserved residues, Lys87 and Gln151. However, significant difference was also observed between the current structure and the two reported structures. In both 1BS and DXC2, the 4-keto group of the chromone core of the compound
formed a hydrogen bond with the same hinge amide nitrogen (Val\textsuperscript{182} in phosphatidylinositol 3-kinase-\beta (PI3K) and Val\textsuperscript{184} in CDK6 (Fig. 3E)). However, there is no direct interaction between the 4-keto group of quercetagetin and the amide nitrogen of the corresponding residues Pro\textsuperscript{180} in PIM1 because proline is incapable of acting as a hydrogen bond donor. Instead, the 4-keto group of quercetagetin makes close contact with the backbone C\textsubscript{\textalpha} of Arg\textsuperscript{182} (3.4 Å). It is not clear whether this interaction makes a positive contribution to the binding of quercetagetin to PIM1.

The B ring of quercetagetin binds deep inside the PIM1 ATP-binding pocket. The 4'-hydroxy group forms hydrogen bonds with both Lys\textsuperscript{\beta} and Glu\textsuperscript{\alpha}, two of the most conserved residues in kinases. As has been noted, satisfying the hydrogen bonding requirements at this region is one of the determining features of binding of compounds to PIM1 (13).

When compared with quercetagetin, the chromone core of myricetin (Fig. 3B) and 5,7,3',4',5'-pentahydroxyflavone (Fig. 3C) have flipped 180° in PIM1 such that the B ring is now oriented toward the entrance of the ATP pocket. A possible explanation for adopting this orientation is that the interior of the ATP pocket cannot accommodate the B ring with these hydroxyl substitutions. Although they bind in the same orientation, there are important differences between the binding poses of the two compounds, which can be attributed to the presence or absence of the 3-hydroxy group. The 3-hydroxy group in myricetin still makes a hydrogen bond with the carbonyl oxygen of Glu\textsuperscript{\alpha}, despite the difference in binding orientation. Because of the adjacent 4-keto group, the 3-hydroxy is likely to be most acidic of all the hydroxyl groups in the compound, and, as a result, it dictates the overall positioning of the compound. Another interaction that may contribute to the observed binding pose is a hydrogen bond between the 3'-hydroxy group of myricetin and the carbonyl oxygen of Pro\textsuperscript{182} (Fig. 3B). The importance of the 3-hydroxy group is evident. The second compound, 5,7,3',4',5'-pentahydroxyflavone, lacking such a group, makes no direct interaction with the hinge region.

Quercetagetin Inhibits PIM1 Kinase Activity in Intact Cells

To determine if quercetagetin could act as a cell-permeable PIM1 inhibitor, we examined the activity of the flavonol in RWPE2 prostate cancer cells. We studied the phosphorylation of endogenous BAD with staining under conditions of growth factor starvation, as an indicator of intracellular PIM1 activity (Fig. 4).

RWPE2 cells infected with a non-expressing adenovirus showed little phospho-BAD (SI 12) when cultured overnight.
Table 1. Quercetin is a selective inhibitor of the PIM1 kinase over other BAD (S112) kinases

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50 (μM/L)</th>
<th>Log IC50 (μM/L)</th>
<th>SE of log IC50</th>
<th>K^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIM1</td>
<td>0.34</td>
<td>-0.46</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>PIM2</td>
<td>3.45</td>
<td>0.28</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>PKA</td>
<td>212</td>
<td>1.33</td>
<td>0.25</td>
<td>0.09</td>
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<tr>
<td>BSK2</td>
<td>202</td>
<td>0.48</td>
<td>0.09</td>
<td>0.09</td>
</tr>
</tbody>
</table>

NOTE: All data were derived from nonlinear regression analysis using a three-parameter logistic that assumes a Hill coefficient of -1.

in basal serum-free medium. However, cells with enforced expression of pim-1 kinase had a 4-fold higher amount of phospho-BAD, reflecting the ability of the PIM1 protein to phosphorylate the endogenous BAD protein. When pim-1 expressing cells were treated with quercetin, phospho-BAD (S112) levels were markedly reduced in proportion to the concentration of the inhibitor. Half-maximal inhibition occurred at 5.5 μM/L extracellular concentration. Quercetin did not inhibit the activity of the AKT kinase under these conditions, as indicated by persistent phosphorylation of AKT on Ser473. These data indicate that quercetin was able to selectively block the ability of PIM1 to phosphorylate BAD in intact cells.

Quercetin Treatment Reproduces a Known pim-1 Knockdown Phenotype

If quercetin acts as a true PIM1 inhibitor, then it should reproduce a pim-1-dependent phenotype in the target cells. We have shown that PIM1 inhibition by genetic means (i.e., all interfering RNA) inhibits the proliferation of RWPE1 and RWPE2 cells (Supplementary Fig. S1). We therefore determined if quercetin could reproduce this phenotype. RWPE2 cells were treated with quercetin for up to 72 h (Fig. 5A). Marked dose-dependent growth inhibition was apparent by 24 h, leading to persistent growth arrest thereafter. Quercetin reproduced this pim-1-dependent phenotype at a drug concentration that inhibited the enzyme in cells (ED50, 3.9 μM/L, Fig. 5B). Similar results were seen in RWPE1 cells (data not shown).

Apoptotic cells, showing cytoplasmic blebbing and detachment, were rare, but dividing cells virtually disappeared in cultures treated with quercetin at 6.25 μM/L or higher concentrations (data not shown). DNA histograms obtained at 24 h after the addition of quercetin (6.25 μM/L) or DMSO vehicle were very similar (Fig. 5C). Neither showed a >2n population suggestive of a polyploid. There was a slight increase in the proportion of cycling cells (S + G2-M) in the drug-treated samples.

A PIM1 inhibitor would be predicted to inhibit the growth of cells that express the molecular target, more than cells with little or no pim-1 expression. We examined the effects of quercetin on the growth of prostate cell lines that express a spectrum of PIM1 levels. RWPE2 cells expressed the highest amount of PIM1 protein; PC3 had an intermediate level; and LNCaP cells showed the lowest amount of kinase protein (Fig. 6A). Treatment of the cells with various concentrations of quercetin for 72 h resulted in inhibition of cell growth (Fig. 6B). At all concentrations, RWPE2 cells were inhibited the most, being significantly more sensitive to quercetin growth inhibition than the other prostate cancer cell lines. PC3 cells showed intermediate growth suppression and were also significantly more sensitive than were LNCaP cells at quercetin concentrations of 1.25 μM/L. Thus, the ability of the flavonoid to inhibit proliferation was proportional to the amount of PIM1 protein in the target cells, particularly at lower drug concentrations. Although other interpretations are possible, these data support our observation that quercetin is an effective inhibitor of pim-1.

Discussion

The development of clinically useful small-molecule kinase inhibitors has been a seminal event in the world of oncology. Flavonoids were among the early scaffolds structures identified as potential kinase inhibitors. However, although many flavones, isoflavones, and flavonoids have been shown to regulate the activity of kinases in cell-based assays, fewer data exist to show that these molecules can directly bind and inhibit kinase targets in vitro and in cells. It is clear that some flavonoids are ATP-competitive ligands for both tyrosine and serine-threonine kinases, as well as other ATP-binding enzymes. The flavonoid quercetin is one such ligand, and its ability to directly bind to ATP-binding enzymes has been well shown. At low-micromolar concentrations, it directly binds and inhibits such diverse enzymes as the phosphatidylinositol 3-kinase (14), the epidermal growth factor receptor tyrosine kinase (15), reoviral serine phosphatases (16), DNA gyrase (17), phosphodiesterases (18), and thiopeptide reductase (19). Other direct flavonoid inhibitors have been described for BSK2 kine (24), mitogen-activated protein/extra-cellular signal-regulated kinase 1 (25), and several cyclin-dependent kinases (23, 26–29). One such ligand, flavopiridol, has already entered clinical trials for the treatment of cancer. Others, such as FD8019, are familiar laboratory reagents for inhibition of kinase pathways. We now show, by means of crystallography, that quercetin is a direct ligand for the ATP-binding pocket of PIM1 kinase (Fig. 3).

![Figure 2. Quercetin is a selective inhibitor of PIM1 kinase.](image-url)
Specificity is always a concern with ATP pocket ligands. There are probably no absolutely selective inhibitors for a kinase but rather ligands that show a spectrum of affinities for their various targets. We have shown that quercetagetin is several-fold more active against PIM1 than against eight other serine-threonine kinases and a tyrosine kinase, either with in vitro assays or in cell cultures. Interestingly, quercetagetin showed 10-fold more selectivity for PIM1 than for the homologous PIM2 kinase (sequence identity 56%). The ATP-binding pockets of these two kinases are identical with the exception of three residues along the edge of the PIM1 ATP-binding pocket—Ser\(^{14}\) (Ala\(^{14}\) in PIM2), Glu\(^{134}\) (Leu\(^{126}\) in PIM2), and Val\(^{135}\) (Ala\(^{128}\) in PIM2). Val\(^{135}\) of PIM1 makes direct van der Waal’s contact with the A ring of quercetagetin (Fig. 3A). Loss of such a contact due to the Val-to-Ala substitution is likely a contributing factor to the reduced activity of the compound in PIM2. The other residues are located close to the hinge Arg\(^{23}\) (Arg\(^{118}\) in PIM2). The polar side chains of Ser\(^{14}\) and Glu\(^{134}\) can form hydrogen bonds with Arg\(^{23}\), thus affecting its conformation. Substitutions of these residues to hydrophobic amino acids in PIM2 will change the local environment (Fig. 3A).

The only large-scale examination of the specificity of flavonoid kinase inhibitors was reported recently by Fabian et al. (8). This investigation used a competitive binding assay to predict the inhibitor potency and specificity of the test agents. Flavopiridol was tested for binding affinity to 119 kinases. Twenty-three kinases bound flavopiridol under the test conditions, with binding constants ranging from 0.019 to 6.6 \(\mu M\). Interestingly, the tested cell-dependent
kinases bound flavopiridol less well than did calcium/calcmodulin-dependent protein kinase kinase 1. These data suggest that cyclin-dependent kinases may not be the only kinases inhibited in cells by flavopiridol. Both PIM1 and PIM2 were among the bound kinases, with binding constants of 0.52 and 0.65 μmol/L, respectively. Although there is no absolute correlation between binding constants and enzymatic activity, flavopiridol could conceivably inhibit the activity of both PIM1 and PIM2 in test systems. Because quercetin has not been tested against a large number of other kinases, we cannot predict what other enzymes would be perturbed by this flavonoid. It is likely, however, that the spectrum of selectivity will be substantially different from that of flavopiridol. Quercetin showed clear preference for inhibiting PIM1 over PIM2, whereas flavopiridol did not. Furthermore, quercetin inhibited the activity of the Aurora-A kinase (IC₅₀ ~ 4 μmol/L), a kinase that did not bind flavopiridol (8). The substantial

Figure 4. Quercetin inhibits PIM1 kinase activity in intact cells. A. RWPE-1neo or RWPE-1/ pM1L1 cells were cultured in unsupplemented keratinocyte medium overnight, then treated with quercetin (0–50 μmol/L) for 3h. Lysates were then prepared and examined by immunoblotting with the indicated antibodies. B. quantification of the pBAD512/actin ratio in immunoblots by using densitometry on the digital file. IC₅₀, 5.56 μmol/L.
homology between Aurora-A kinase and PIM1 kinase likely contributed to the low-level inhibitory activity of quercetin for the former; Aurora-A and PIM1 are 29% identical over their entire kinase domains; and the ATP binding pockets have 68% conserved amino acids.

An earlier, smaller-scale study looked at the effect of the flavonol quercetin on the in vitro kinase activity of 25 kinases, none of which were pim family kinases (29). At the tested concentration (20 μmol/L), quercetin inhibited the enzymatic activity of eight of the kinases. The propensity of this flavonol to form aggregates in aqueous solution has been advanced as an explanation for its widespread enzyme-inhibitory activity in vitro (30). We have not detected quercetin aggregates at concentrations of <10 μmol/L in aqueous solution, using a light-scattering assay (data not shown). Thus, we feel that this artifact does not account for the ability of this flavonol to inhibit PIM1 at nanomolar concentrations.

Because of the potential ambiguities that may accompany the use of small-molecule kinase inhibitors, a series of standards have been proposed for their use (29). To validate the results, it is desirable to show that the effects of an inhibitor disappear when a drug-resistant mutant of the protein kinase is overexpressed. Although convincing, this standard often fails due to the lack of an identified mutant with the desired properties. No such mutant has been identified for any of the pim kinases. Another potential standard is to show that the cellular effect of the drug occurs at the same concentrations that prevents the phosphorylation of an authentic physiologic substrate of the protein kinase. We have seen in these studies that half-maximal growth inhibition of prostate cancer cells occurred at a drug concentration (3.8 μmol/L) that approximated the IC50 for PIM1 enzyme inhibition in cells (5.5 μmol/L). Furthermore, the selectivity for prostate cancer growth inhibition, in proportion to endogenous PIM1 levels, was greatest at 6.25 μmol/L. Higher concentrations suppressed growth more, but the relationship to endogenous PIM1 levels was obscured. These data suggest that, at relatively low concentrations (perhaps 5–10 μmol/L), the growth-inhibitory effects of quercetin likely involve PIM1 antagonism. A third standard is to observe the same effect with at least two structurally unrelated inhibitors of the protein kinase.

Previously described inhibition of pim

Figure 5. Quercetin inhibits growth of prostate cancer cells at concentrations that inhibit PIM1 kinase activity. A., growth curve of RWPE2 cells with different concentrations of quercetin. Cell number is measured by crystal violet staining. Points are mean of triplicate determinations from one of four similar experiments. B., calculation of IC50 at 24, 48, and 72 h of drug exposure. Average IC50 from all curves is 3.8 μmol/L. C., DNA histograms from RWPE2 cells treated with vehicle or quercetin at 6.25 μmol/L for 24 h. Proportion of cells in G0/G1 or S + G2/M fractions. Columns, mean of triplicate determinations from three independent experiments; bars, SD. P-values show the probability of no difference by t test.
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Figure 8. Quercetatin inhibits the growth of prostate cancer cells in
proportion to their content of PIM1. A, measurement of intracellular PIM1
in PC3, LNCaP, and RWPE2 prostate cancer cells by immunoblotting. B,
growth inhibition by treatment of prostate cancer cells with quercetatin
for 72 h. Columns, mean of triplicate determinations from one of two
similar experiments; bars, SD. P values were calculated by paired t tests
and represent the probability that there is no difference between the
two compared populations.

kineses are either less active or less specific flavonoids
(7, 9), the same structural class as quercetatin, or
stauroporine analogues (8, 9, 21). We therefore used small
interfering RNA as a genetic means to identify a pim-1–
dependent phenotype. Proliferation of prostate cells was
suppressed with both the genetic and chemical inhibitors of
PIM1 activity. These data show that quercetatin is an
authentic small-molecule inhibitor of PIM1 kinase.

The crystal structures of PIM1 complexed with quercetatin,
myricetin, and 5,7,3′,4′,5′-pentahydroxylflavone show
that flavonoids bind to PIM1 in two distinct orientations. Although
interesting, this is not a surprising observation, as flavonoids have shown a variety of binding
modes in kinases (9, 22, 23, 26–28). An examination of the
intermolecular interactions of each flavonoid with PIM1
does not clearly reveal why one orientation was adopted over
the other. However, it is possible that the presence of
these hydroxyl groups on the B ring of myricetin and
5,7,3′,4′,5′-pentahydroxylflavone discourages these two
flavonoids from adopting the binding orientation observed
for quercetatin. The hydrophilic side chain of Leu340,
which extends into the ATP pocket in the same region
occupied by the B ring of quercetatin (Fig. 3A), may be
incompatible with the 5′ hydroxyl group of myricetin and
5,7,3′,4′,5′-pentahydroxylflavone.

Both pim-1 and pim-3 can phosphorylate 4EBP-1, a
regulator of protein translation (31, 32). Rapamycin was
unable to block this effect. These data suggest that pim
kineses may function in a parallel pathway to the
phosphatidylinositol 3-kinase/AKT/mammalian target of
rapamycin cascade to regulate and support protein
synthesis under stress conditions. Because AKT-1 and
PIM2 function cooperatively to induce lymphoma forma-
tion in transgenic mice (6), it may be necessary to target
both pathways for effective antitumor effects. Several
prototype AKT inhibitors have been described (33, 34).
Our identification of quercetatin as a PIM1 inhibitor
provides a tool for tissue culture studies to investigate this
hypothesis. Under the tested conditions, we found no
evidence that quercetatin inhibited the phosphorylation
of AKT on Ser^473. Thus, it may be possible to combine
inhibitors of these kinases to detect additive or synergistic
effects resulting from the blockade of the two kinase pathways.

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1987  Diagnostic Radiology
1988  Experimental Therapeutics
Member, site visit team for program project
Dr. George Hahn, PI; Stanford University
1988, 1989
Member, site visit team for program project
Dr. Bayard Clarkson, PI, Memorial-Sloan Kettering Inst., 1997

Special Local Responsibilities

Member, Scientific Review Subcommittee
Member, Research & Development Committee
SVAMC, 1994, 1995
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SVAMC, 1994, 1995
Board Development Committee, Leukemia & Lymphoma Society (Southern California Chapter), 2003

Consultant

Cetus Corporation (1986)
EncorePharma (2001-present)
Myriad Genetics (2002-present)
Exelixis Pharmaceuticals (2005-present)

GRANTS & CONTRACTS (PRINCIPAL INVESTIGATOR)  Note: This listing does not include multicenter clinical trials in which Dr. Lilly was the local principal investigator.

National Institutes of Health  F32CA27980 Hyperthermia of animal and human tumors; 7/80-6/82

National Institutes of Health  R01CA18138-11 Prediction of thermal tolerance by in vivo NMR spectroscopy; 7/82-6/83

National Institutes of Health  R01CA36790 Assessment of hyperthermia by in vivo \(^{31}\)P-NMR spectroscopy; 9/84-9/87

Cetus Corporation  Characterization of a human granulocyte CSF; 7/85-6/86

National Institutes of Health  R01CA45672 Cytokine signaling in myeloid leukemia; 9/87-10/98

VA Merit Review Award  Non-protein hematopoietic agents; 10/90-4/97

March of Dimes Birth Defects Foundation  Characterization of a 28kd protein related to G-CSF; 7/93-6/96

Lymphoma Research Foundation of America  Mechanism of action of the pim-1 oncogene; 7/95-7/96
Roche Pharmaceuticals  *Preclinical study of Roferon and bryostatin 1 in a melanoma model*; 1/98-12/99

Department of Defense, National Medical Technology Testbed #76-FY99: *Cell-permeable proteins for cell regulation.* 12/99 – 7/02

Leukemia Society of American Translational Award *Propionic Acid Analogues for CLL.* 9/1/01 – 8/31/05

Celgene Corporation, Phase I-II trial of combined GM-CSF (sargramostim) and thalidomide for hormone-refractory prostate cancer (5/02-5/04).

National Institutes of Health  R03CA107820  *Molecular Targets of NSAIDs in Prostate Cancer*; (5/1/04 – 4/30/07)

Department of Defense, CDMRP Prostate Cancer Program PC040635  *Pim-1: A Molecular Target to Modulate Cellular Resistance to Therapy in Prostate Cancer* (10/04 – 10/07)

Pharmion Corporation, *Use of azacytidine to reverse silencing of GST-p1 in early prostate cancer.* (10/05 – 10/07)

**GRANTS and CONTRACTS (Co-investigator)**

National Institutes of Health  R01CA097043  *Molecular pathology of 2-deoxy-5-azacytidine*; L. Sowers, PI; Michael Lilly, co-investigator (10% FTE). 7/1/03 – 6/30/08

**PUBLICATIONS IN PEER-REVIEWED JOURNALS**


BOOKS AND CHAPTERS:


RECENT ABSTRACTS:


Lilly M, Cooper JJ: Enforced expression of the human 33kd Pim-1 kinase prevents apoptosis-associated mitochondrial dysfunction and upregulates bcl-2 mRNA expression in murine myeloid cells. (oral presentation, ASH 12/97)


Chen CS, Lilly MB, Wang FS, Howard FD, Houwen B: Rapid monitoring of peripheral blood stem cells (PBSC) mobilization by using cell membrane phospholipid content correlates well with CD34+ measurements, successful harvest and engraftment (abstract #1642). Blood 96:380a (poster presentation, ASH 12/00)


Lilly MB, Wechter W, Puuvula L, Henry H: R-Flurbiprofen (RFB) a non-steroidal anti-inflammatory drug (NSAID) with anti-tumor activity, inhibits the expression of CYP24 in murine prostate carcinomas. (poster presentation at Biennial Vitamin D Conference “Vitamin D and Cancer Chemoprevention”, NIH, Bethesda, MD, November 2004)