

Leflunomide Synergizes with Gemcitabine in Growth Inhibition of PC Cells and Impairs c-Myc Signaling through PIM Kinase Targeting

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The immunosuppressive agent leflunomide has been used in the treatment of over 300,000 patients with rheumatoid arthritis. Its active metabolite, teriflunomide (Ter), directly inhibits dihydroorotate dehydrogenase (DHODH), an enzyme involved in nucleoside synthesis. We report that Ter not only shows *in vitro* anti-proliferative activity in pancreatic cancer (PC) cells as a single agent but also synergizes with the chemotherapeutic gemcitabine (Gem) in growth inhibition of PC cells. The growth-inhibitory effects of Ter are not solely caused by inhibition of DHODH. Through a kinase screening approach, we identified the PIM-3 serine-threonine kinase as a novel direct target. Subsequent dose-response kinase assays showed that Ter directly inhibited all three PIM family members, with the highest activities against PIM-3 and -1. The PIM-3 kinase was the PIM family member most often associated with PC oncogenesis and was also the kinase inhibited the most by Ter among more than 600 kinases investigated. Ter in PC cells induced changes in phosphorylation and expression of PIM downstream targets, consistent with the effects achieved by overexpression or downregulation of PIM-3. Finally, pharmacological inhibition of PIM proteins not only diminished PC cell proliferation, but also small-molecule pan-PIM and PIM-3 inhibitors synergized with Gem in growth inhibition of PC cells.

INTRODUCTION

Pancreatic cancer (PC) is poised to become the second leading cause of cancer death in the United States within the next 10 years.^{1,2} At present, the overall average 5-year survival is only 8%. The majority of patients present with metastatic disease and are offered systemic genotoxic chemotherapy. Patients with excellent performance status are offered the FOLFIRINOX (fluorouracil, leucovorin, irinotecan, and oxaliplatin) regimen, with an estimated median survival of 11 months.³ However, many patients are not thought to be fit for such a regimen and are offered the alternative of gemcitabine (Gem) and *nab*-paclitaxel or Gem alone—treatments that have median survivals of 8 and 6 months, respectively.⁴ Patients initially respond with decreases in the tumor marker CA19-9; however,

over time, patients develop resistance and progression. Therefore, there is an urgent need for improved therapy.

The mechanisms of resistance of PC are diverse and include changes in the tumor environment, drug metabolism, and drug efflux.^{5,6} Metabolic reprogramming, a known mechanism of chemoresistance leading to cancer cell proliferation and survival, can arise in response to genotoxic stress. Genotoxic chemotherapy agents can induce the *de novo* pyrimidine synthesis pathway to increase the availability of the nucleotides essential for DNA repair.⁷ Inhibition of the *de novo* pyrimidine synthesis pathway can sensitize cancer cells to genotoxic chemotherapy agents.⁷ Leflunomide (Lef), an agent with a long history of safety and efficacy in the treatment and prevention of autoimmune disorders and allograft rejection, targets *de novo* pyrimidine synthesis via inhibition of dihydroorotate dehydrogenase (DHODH).⁸ Lef (original brand name, Arava) is a commercially available agent that was approved by the US Food and Drug Administration (FDA) in 1998 for the treatment of rheumatoid arthritis and, in 2004, for the treatment of psoriatic arthritis. Lef is rapidly metabolized in the gut wall, plasma, and liver into its active ingredient, teriflunomide (Ter).⁹ Ter directly inhibits DHODH *in vitro* at sub-micromolar concentrations.^{8,10} Inhibition of DHODH leads to decreased ribonucleotide uridine monophosphate (rUMP) levels and thus to decreased DNA and RNA synthesis and inhibition of proliferation in susceptible cells. DHODH is the rate-limiting enzyme in the synthesis chain of uridine and is a critical enzyme in this pathway.

The immunosuppressive role of Lef and/or Ter has been attributed primarily to anti-proliferative and anti-inflammatory actions on T lymphocytes, in part by inhibition of DHODH.¹¹ Activated

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lymphocytes require an 8-fold increase in rUMP and other pyrimidine nucleotides to progress from the G1 to the S phase of the cell cycle and to proliferate and depend on both *de novo* pyrimidine synthesis and pyrimidine salvage pathways, whereas normal cells and resting lymphocytes can utilize pyrimidine salvage pathways to satisfy their requirements for nucleotide synthesis.¹¹ Thus, Ter-mediated inhibition of DHODH leads to anti-proliferative effects in activated lymphocytes. However, in cancer cells, the anti-proliferative effects of Ter have been shown not to be caused solely by inhibition of DHODH.^{10,12} Pre-clinical data show that Ter has potent anti-neoplastic effects in multiple myeloma (MM), oral squamous cell carcinoma, renal cell carcinoma, melanoma, and non-small cell carcinoma, through a variety of mechanisms.^{12–16}

The PIM family of serine-threonine kinases (PIMs), which consist of PIM-1, PIM-2, and PIM-3, have been associated with the regulation of cell survival pathways, chemotherapy resistance, and cell migration.^{17,18} PIM family members are overexpressed and implicated in multiple types of human hematologic and solid tumor malignancies of epithelial origin.^{19,20} In PC, overexpression of PIM-3 protein is associated with a more advanced stage and worse survival.²¹ PIM-3 can interact with a variety of target molecules, thereby regulating biologic pathways including apoptosis, cell cycle, protein synthesis, and transcription.²² PIMs have been shown to promote cell cycle progression via upregulation of phosphorylated p27, p21, Cdc25A, Cdc25C, and C-TAK1.^{22,23} Protein synthesis is induced by PIMs via upregulation of peroxisome-proliferation-activated receptor γ co-activator 1 α (PGC-1 α) and AMP-dependent protein kinase (AMPK).²⁴ PIM-3 expression is associated with upregulation of the survival genes p-Bad and Bcl-2.^{25,26} In addition, PIM expression is associated with increased endothelial cell migration and increased levels of p-Stat3 and c-Myc transcription factors.^{27,28} It has been shown that PIMs phosphorylate, stabilize, and enhance c-Myc and that c-Myc activity is necessary for PIMs to induce oncogenesis.^{17,29}

c-Myc is a master regulator of many cellular processes, and its expression is associated with increased DNA replication, cell growth and survival, protein synthesis, and tumor cell metabolism.^{30–33} c-Myc overexpression is associated with poor prognosis in many cancers, including PC.^{34–36} Targeting the c-Myc signaling pathway in PC represents a promising but challenging therapeutic strategy. c-Myc inhibition may lead to increased toxicity because of interference with the pleiotropic effects of c-Myc in normal homeostasis; additionally, lack of a druggable site in the c-Myc protein makes development of c-Myc inhibitors difficult.³⁷ On the other hand, inhibition of c-Myc by a dominant-negative Myc mutant demonstrated tumor regression in a pre-clinical mouse model of lung cancer and was also surprisingly well tolerated by the animals.³⁸

RESULTS

Ter Inhibits Proliferation and Induces G0 and/or G1 Cell Cycle Arrest in PC Cells

Because *Lef in vivo* is rapidly converted into its active ingredient Ter, we used Ter for all *in vitro* cell culture studies. We first assessed the

anti-proliferative properties of Ter in four representative PC cell lines: MIA PaCA-2, PANC-1, AsPC-1, and BxPC-3. Our data show that *in vitro* growth of PC cells was inhibited at clinically achievable concentrations of Ter (Figure 1A), which, based on preliminary pharmacokinetic results from a single agent phase 1 clinical trial in patients with MM (ClinicalTrials.gov: NCT02509052; M. Rosenzweig, 2017, Am. Soc. Hematol., abstract), were 100–500 μ M (R. Buettner, 2017, Am. Soc. Hematol., abstract). In the PC cell lines, Ter half-maximal inhibitory concentrations (IC₅₀) ranged from approximately 32 to 123 μ M after 72 h of incubation (Figure 1A). Interestingly, although significant cell growth inhibition was observed at Ter concentrations well below 200 μ M, apoptosis was not observed with Ter doses up to 200 μ M (data not shown). Cell cycle analysis demonstrated that Ter induced G0 and/or G1 cell cycle arrest in all four PC cell lines investigated (Figure 1B) and that Ter decreased the expression of proteins involved in cell cycle regulation, including cyclins D1, A2, and E; phosphatase Cdc25a; and cyclin-dependent kinase CDK2, and it increased expression of the cyclin-dependent kinase inhibitor p21 (Figure 1C).

Ter Synergizes with Gem in Growth Inhibition of PC Cells

Many PC patients are offered genotoxic chemotherapy agents to treat their disease; however, the cells may become resistant over time, at least in part because these agents may induce the *de novo* pyrimidine synthesis pathway to increase the availability of nucleotides that are essential for DNA repair. Since the *de novo* pyrimidine synthesis pathway is directly inhibited by Ter-mediated DHODH inhibition, we reasoned that combination treatment of PC cells with Gem plus Ter would synergize in growth inhibition of PC cells. As shown in Figure 2A, compared with either drug alone, combination treatment of PC cells with Ter plus Gem demonstrated a synergistic anti-proliferative effect on PC cells, as shown by combination index (CI) values <1 at all effective doses (EDs) investigated.

The Growth-Inhibitory Effect of Ter in PC Cells Can Be Explained Only Partially through Inhibition of DHODH

DHODH is the rate-limiting enzyme in the *de novo* biosynthesis of uridine and is directly inhibited by Ter, at sub-micromolar concentrations, *in vitro*. The anti-proliferative effect on PC cells seen with the combination of Gem plus Ter may suggest that these agents synergize, at least in part, through the combined effects of induction of DNA damage and withdrawal of the nucleoside precursor uridine required for DNA damage repair. We first asked whether PC cells express DHODH. As shown by western blotting, all four PC cell lines investigated expressed the DHODH protein (Figure 2B, left). We then hypothesized that the effect of Ter on PC proliferation would be reversed through the external addition of a surplus of uridine, as evaluated previously in other cancers.^{10,12} We therefore incubated MIA PaCA-2 and PANC-1 cells for 72 h with increasing concentrations of Ter, in the presence or absence of 100 μ M uridine, and determined cell proliferation via incubation with MTS reagent. As shown in Figure 2B, Ter-induced growth inhibition of MIA PaCA-2 cells was almost completely reversed by 100 μ M uridine at Ter concentrations of 25 and 50 μ M, whereas only partial rescue was observed at 100 and

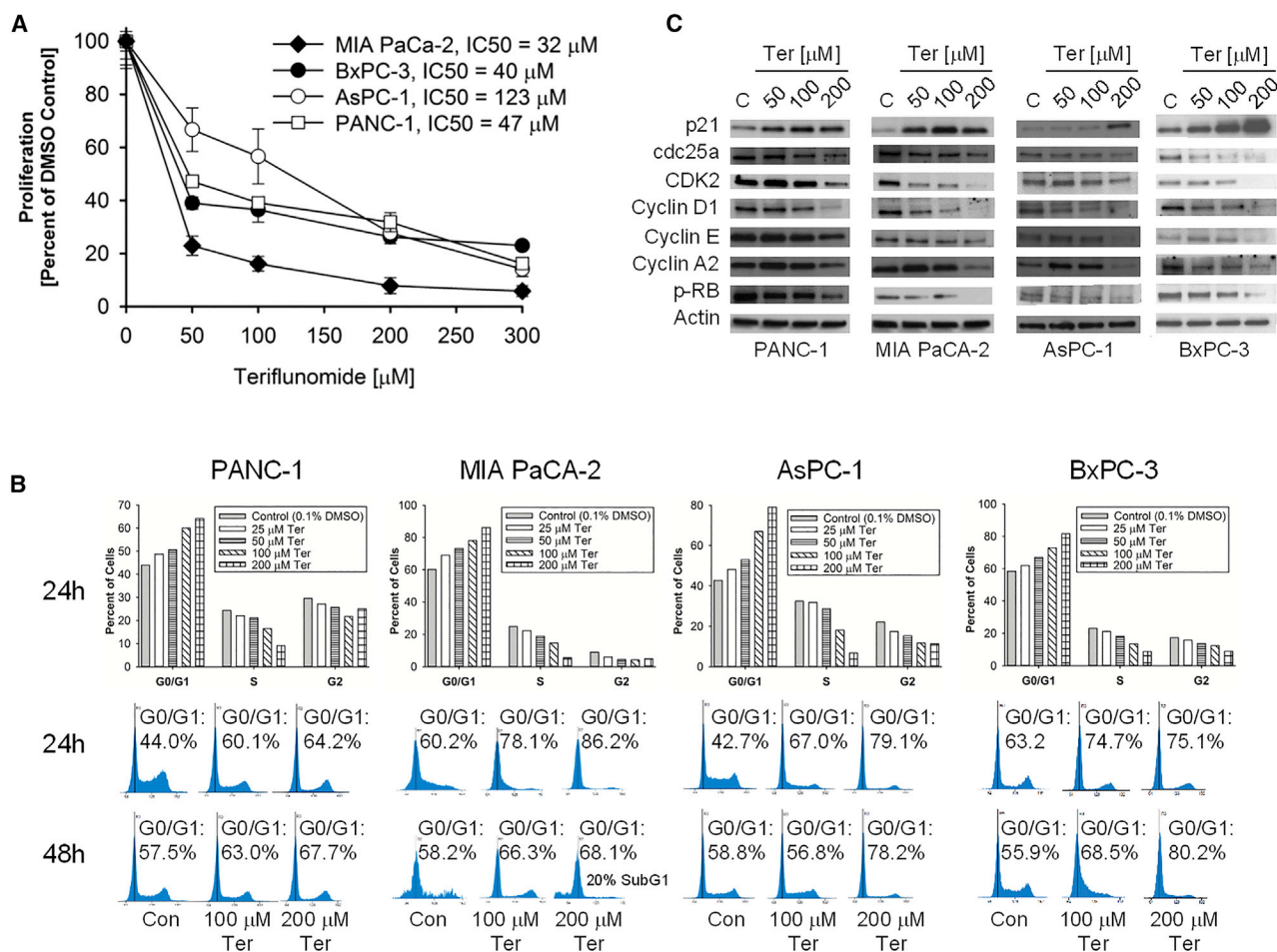


Figure 1. Teriflunomide Inhibits Proliferation and Induces G0 and/or G1 Cell Cycle Arrest in PC Cells

Cells from the PC cell lines PANC-1, AsPC-1, MIA PaCA-2, and BxPC-3 cells were treated with 50–300 μ M Ter for 24 to 72 h, as indicated. Cell growth was measured by the MTS assay. Cell cycle status was measured by propidium iodide staining followed by flow cytometry and western blotting. (A) Cells were treated with increasing concentrations of Ter for 72 h, and cell growth was measured, using the MTS assay. Results from one representative experiment are presented as means \pm SD, with quadruplicate determinations. (B) Top panel: flow cytometry results for cell cycle distribution at $t = 24$ h for 25–200 μ M Ter are presented as bar graphs; bottom panel: flow cytometry results for cell cycle distribution at $t = 24$ h and $t = 48$ h for 100 and 200 μ M Ter are shown as histograms. (C) Cells were treated for 48 h at increasing Ter concentrations (50–200 μ M) prior to cell lysis and immunoblotting with the indicated cell-cycle-related antibodies.

200 μ M Ter. For PANC-1 cells, only partial rescue was observed at all concentrations tested, with less rescue observed at the higher concentrations of Ter (100 and 200 μ M Ter). Our results demonstrate that the effect of Ter on PC cell growth cannot be explained solely by its inhibitory effect on DHODH, suggesting that other molecular targets of Ter, aside from DHODH, may exist in PC cells that may also contribute to the regulation of PC cell growth, similar to observations from studies with Ter and uridine in MM cells.¹²

PIM Serine-Threonine Kinases Participate in PC Cell Growth and Are Novel Direct Molecular Targets of Ter

To identify potential novel molecular targets of Ter that may contribute to PC cell growth, in addition to DHODH, we performed *in vitro* kinase screening by testing 200 μ M Ter, in duplicate, against

the activity of >600 known recombinant kinases. Of these, PIM-3 serine-threonine kinase activity was inhibited the most by a single dose of Ter (\sim 90% inhibition at 200 μ M Ter; data not shown). Importantly, among the three PIM kinase family members—PIM-1, PIM-2, and PIM-3—the PIM kinase most frequently associated with PC is PIM-3. Indeed, in contrast to PIM-1 and PIM-2, PIM-3 is frequently overexpressed in many PC tumor tissues and has been established as a valid molecular target in PC. However, all PIM family members have been linked to regulation of cancer cell survival pathways and are known to modulate c-Myc stability by various mechanisms, including phosphorylation events. We subsequently performed *in vitro* dose-response kinase assays with all three PIM family members. Our results not only confirmed PIM-3 as a direct molecular target of Ter, but also identified PIM-1 and, to a lesser extent PIM-2, as novel

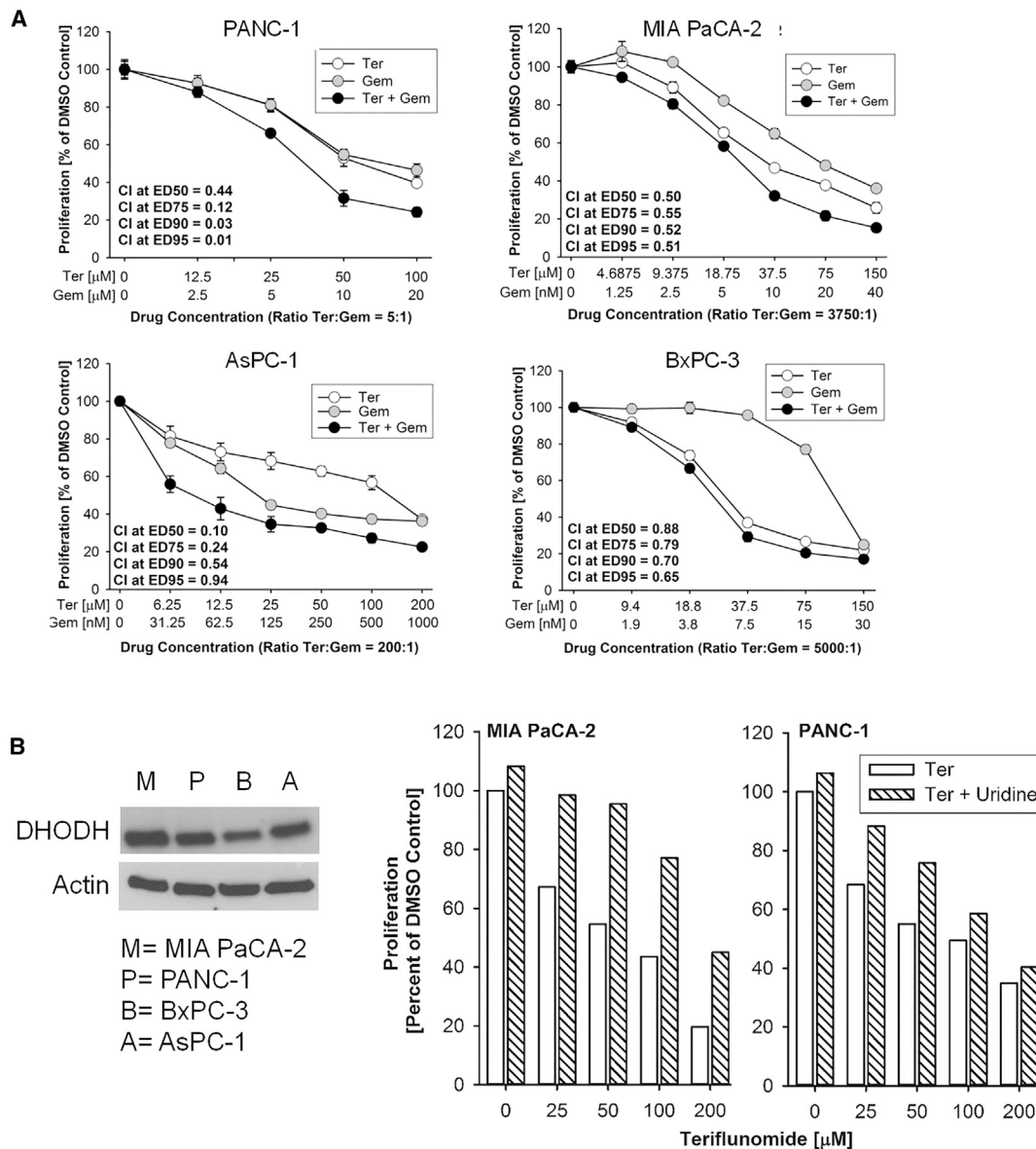


Figure 2. Teriflunomide Synergizes with Gemcitabine in Growth Inhibition of PC Cells

The growth-inhibitory effect of teriflunomide in PC cells cannot be fully explained by inhibition of DHODH. (A) For two-drug combination experiments, PANC-1, MIA PaCA-2, AsPC-1, and BxPC-3 cells were treated with Ter and Gem for 72 h, as single agents and in combination, at constant ratios, on the basis of the previously calculated IC_{50} values for each drug. Quantitative analysis of dose-effect relationships was determined after measurement of cell growth using the MTS assay. Potential synergistic or additive effects were calculated using CompuSyn software (Cambridge, UK). Isobolograms and combination index (CI) plots (not shown) were created, and CI values were calculated. Drug synergism, addition, and antagonism effects were defined by CI values of <1.0 , 1.0 , and >1.0 , respectively. CI values for effective doses 50 (ED_{50}), 75 (ED_{75}), 90 (ED_{90}), and 95 (ED_{95}) are shown. Results from one representative experiment are presented as means \pm SD, with triplicate determinations. (B) Expression of DHODH in untreated PC cell lines (western blot, left). Effect of uridine on Ter-mediated growth inhibition of PC cell lines (bar graphs, right). The PC cell lines MIA PaCA-2 and PANC-1 cells were treated for 48 h with 25, 50, 100, or 200 μ M Ter in the presence or absence of 100 μ M uridine prior to measurement of proliferation using an MTS assay.

targets of Ter (Figure 3A). IC_{50} values for *in vitro* inhibition of PIM kinase activity were 28.7, 255, and 20.6 μ M, for PIM-1, PIM-2 and PIM-3, respectively. Western blot analysis of PIM protein expression demonstrated that all three PIM family members are expressed in PC cells (Figure 3B). Our *in silico* molecular modeling studies demon-

strated that Ter may dock at the ATP-binding site of PIM-3, suggesting that Ter may act as an ATP-competitive inhibitor of PIMs (Figure 3C). Because PIMs in cancer in general and PIM-3 in PC in particular play pivotal roles in cancer oncogenesis, we asked whether pharmacological inhibition of PIMs, including PIM-3, would be

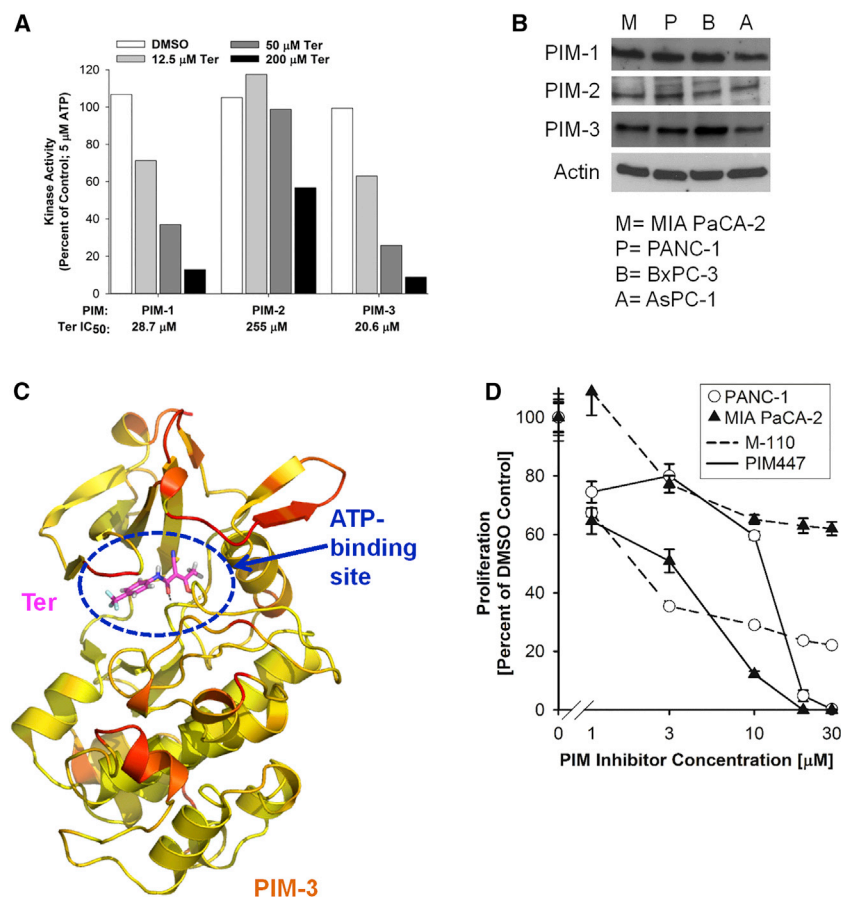


Figure 3. PIM Serine-Threonine Kinases Participate in PC Cell Growth and Are Novel Direct Molecular Targets of Teriflunomide

PIM-3, a member of the PIM family of serine-threonine kinases was identified as the most inhibited direct target of Ter in an *in vitro* kinase screening assay (not shown). (A) An *in vitro* Ter dose-response kinase assay showing the effects of Ter on the kinase activity of PIMs (10 μM ATP). (B) Expression of PIM proteins in untreated PC cell lines (western blotting) (C) Proposed binding mode of Ter on PIM-3 protein. The displayed binding pose of Ter at the PIM-3 ATP-binding site resulted from 190 ns molecular dynamics simulation. The kinase backbone is displayed as a yellow ribbon, with red regions denoting the most flexible residues during simulation. Teriflunomide forms two H-bonds with G105 and I107, together with a water-bridge interaction with D189. (D) The effect of the pan-PIM inhibitor PIM447 and the PIM-3-selective inhibitor M-110 on growth of PC cell lines. MIA PaCA-2 and PANC-1 cells were treated with 1 to 30 μM of PIM inhibitor for 72 h before measurement of cell growth with the MTS assay. Results from one representative experiment are presented as means ± SD, with quadruplicate determinations.

Accordingly, treatment of these PC cells with small interfering RNA (siRNA) against PIM-3 diminished phosphorylation levels of Bad, MDM2, and Stat3 proteins and decreased protein expression levels of c-Myc and Bcl-xL (Figure 4B). Because we hypothesized that Ter alters phosphorylation and expression of these PIM downstream target proteins, we next treated MIA PaCA-2 and

sufficient to inhibit PC cell growth. As shown in Figure 3D, treatment of the PC cell lines PANC-1 and MIA PaCA-2 with the pan-PIM inhibitor PIM447 and the PIM-3-selective inhibitor M-110 dose dependently inhibited growth of these cells. Although the PIM-3-selective inhibitor caused about 35% and 70% growth inhibition in MIA PaCA-2 and PANC-1 cells, respectively, at concentrations of 10 μM, the pan-PIM inhibitor demonstrated a similar percentage of growth inhibition at concentrations up to 10 μM, but completely abolished growth at concentrations above 10 μM, further suggesting that PIMs, including PIM-3, are involved in growth regulation of PC cells.

Ter Inhibits PIM Downstream Signaling in PC Cells

We next evaluated the effects of Ter on PIM signaling and PIM-mediated regulation of PC cell growth. We first evaluated the effect of lentivirus-mediated PIM-3 overexpression on signaling events in PC cells. PIM proteins are known to directly phosphorylate Bad and MDM2 proteins at serine residues and to impair Stat3 tyrosine phosphorylation and c-Myc stability, all of which are critical proteins involved in cell growth and survival. As shown in Figure 4A, overexpression of PIM-3 in MIA PaCA-2 and PANC-1 cells caused increases in phosphorylated Bad (Ser112), MDM2 (S166), and Stat3 (Y705) proteins and increased the expression levels of c-Myc and Bcl-xL proteins.

PANC-1 cells with increasing concentrations of Ter prior to cell lysis and western blotting. In line with the results obtained by overexpression of PIM-3 and small siRNA-mediated knockdown of PIM-3, treatment of the cells with 50 to 200 μM Ter caused downregulation of Bad, MDM2, and Stat3 phosphorylation and a decrease in total expression of c-Myc and Bcl-xL proteins (Figure 4C), further suggesting that Ter exerts its anti-proliferative activity in PC cells, at least in part, through inhibition of PIMs. Because pharmacological PIM-3 inhibition caused significant, but not complete, growth inhibition in both PC cell lines (Figure 3D) and because PIM-3 is the PIM kinase most often associated with PC cell oncogenesis, we asked whether overexpression of PIM-3 protein would partially rescue PC cells from growth inhibition mediated by Ter. As shown in Figure 4D, overexpression of PIM-3 protein partially rescued the PC cell lines MIA PaCA-2 and PANC-1 from Ter-mediated growth inhibition, further suggesting that Ter-induced growth inhibition of PC cells is mediated partially by inhibition of PIM-3.

Gem Synergizes with Pharmacological PIM Inhibitors in Growth Inhibition of PC Cells

Our data suggest that Ter exerts its activity in PC cells, at least to some extent, through inhibition of both DHODH and PIM kinase activity. Because we showed that Ter-directed growth inhibition of PC cells is

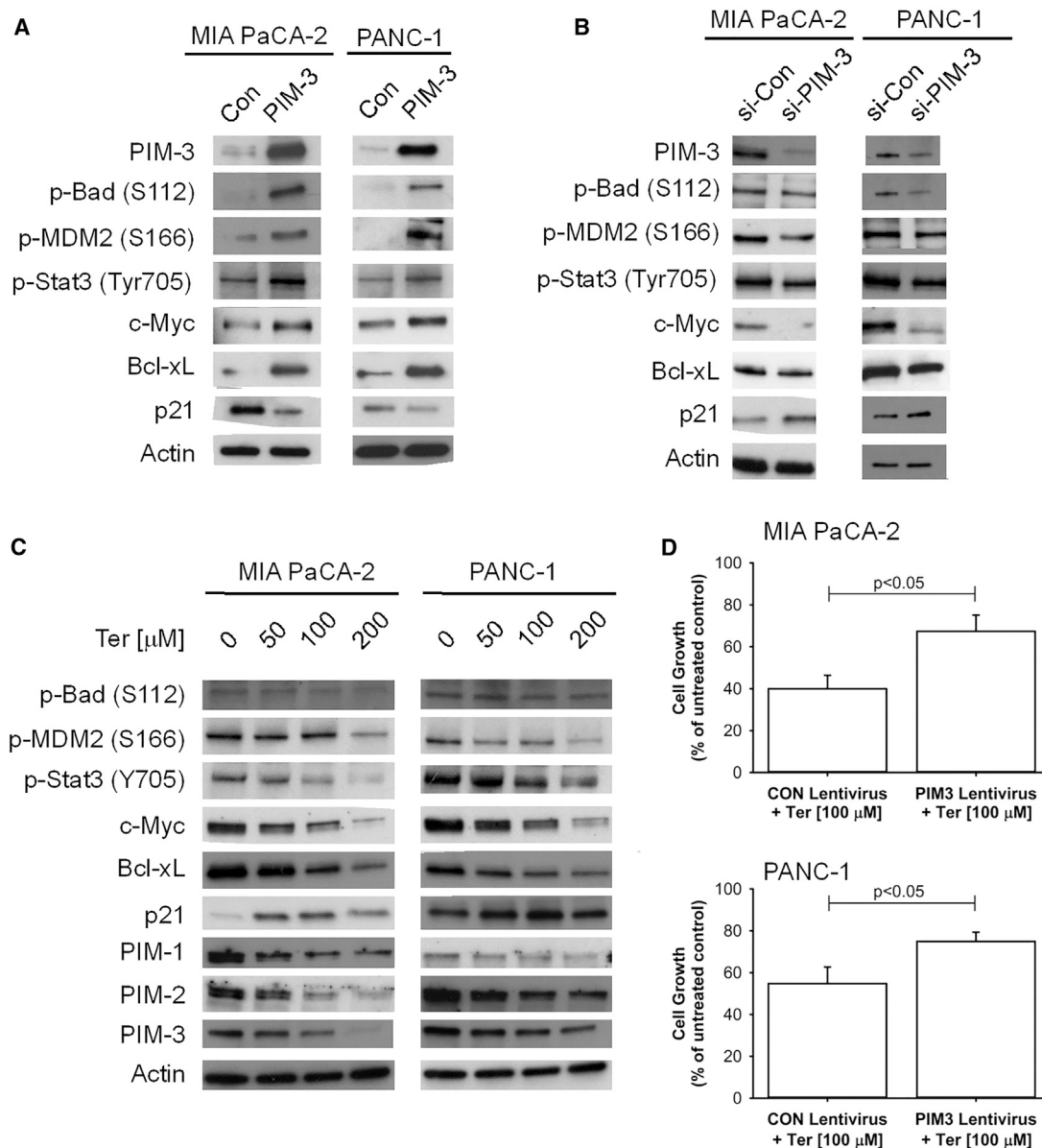
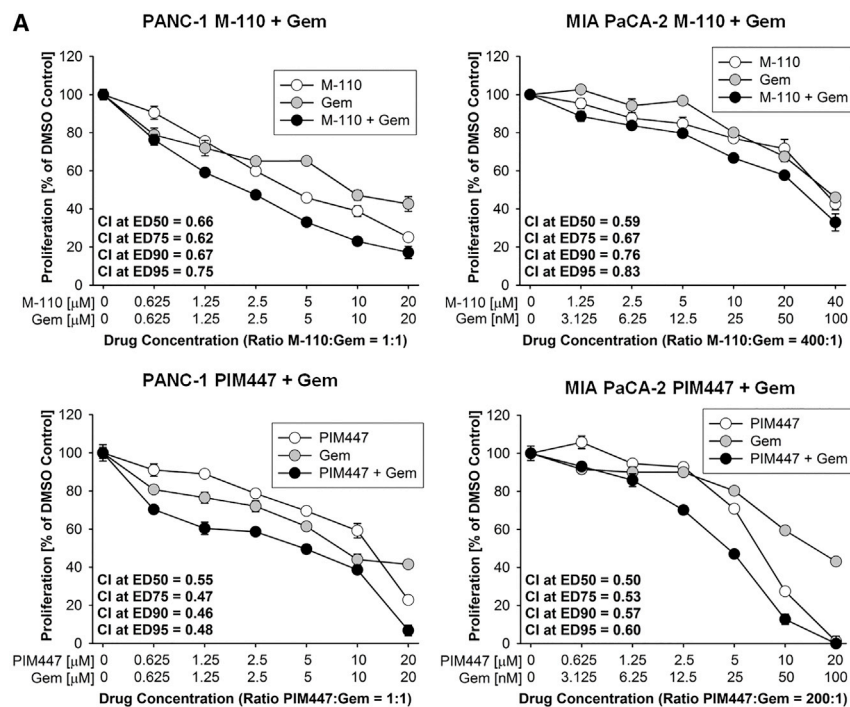


Figure 4. Teriflunomide Inhibits PIM Downstream Signaling in PC Cells

(A) MIA PaCA-2 and PANC-1 cells transfected with lentiviral particles for overexpression of PIM-3 or control lentivirus particles were subjected to western blotting with the indicated antibodies. (B) MIA PaCA-2 and PANC-1 cells transfected with PIM-3 or control siRNA for 48 h were subjected to western blotting with the indicated antibodies. (C) MIA PaCA-2 and PANC-1 cells were treated with 50 to 200 μ M of Ter for 48 h and subjected to western blotting with the indicated antibodies. (D) MIA PaCA-2 and PANC-1 cells overexpressing PIM-3 or control lentivirus particles were treated with or without 100 μ M Ter for 48 h, followed by measurement of cell growth with an MTS assay. Results from one representative experiment are presented as means \pm SD, with quadruplicate determinations.

mediated at least in part through inhibition of PIMs and that Ter synergized with Gem in *in vitro* growth inhibition of PC cells, we reasoned that Gem may also synergize with pharmacological inhibitors of PIM in growth inhibition of PC cells. Similar to the Gem/Ter experiments shown in Figure 2A, we treated PC cell lines with Gem in combination with the pan-PIM inhibitor PIM447 and the PIM-3-selective inhibitor M-110. As shown in Figure 5A, compared to either

drug alone, combination treatment of PC cells with Gem plus PIM inhibitors demonstrated synergistic anti-PC effects (CI <1) at all EDs investigated. Figure 5B shows a summary of the CI values at four different EDs for combination treatments of the PC cell lines MIA PaCA-2 and PANC-1 with Gem in combination with Ter, M-110 (PIM-3 inhibitor), or PIM-447 (pan-PIM inhibitor). We further showed that Gem synergized with all three compounds



B

Combination Index at Effective Dose (CI@ED)	MIA PaCA-2			PANC-1		
	Gemcitabine w/:			Gemcitabine w/:		
	Ter	M-110	PIM447	Ter	M-110	PIM447
CI@ED ₅₀	0.50	0.59	0.50	0.44	0.66	0.55
CI@ED ₇₅	0.55	0.67	0.53	0.12	0.62	0.47
CI@ED ₉₀	0.52	0.76	0.57	0.03	0.67	0.46
CI@ED ₉₅	0.51	0.83	0.60	0.01	0.75	0.48

(Ter, M-110, and PIM-447) at all EDs (CI <1). Moreover, the data suggest that the Gem/Ter combination has lower ED₉₀ and ED₉₅ CI values than those of the Gem/PIM inhibitor combinations. In summary, Ter synergizes with the genotoxic drug Gem in inhibition of PC cell growth, at least in part through direct inhibition of PIM-3 protein activity.

DISCUSSION

We demonstrate that the active metabolite of the drug Lef, Ter, both inhibited PC cell growth alone and synergized with Gem. A possible explanation for the synergistic effect seen with these two compounds may be that, whereas Gem sensitizes PC to genotoxic stress and leads to increased *de novo* pyrimidine synthesis, Ter counteracts nucleoside synthesis and DNA repair mechanisms by blocking *de novo* pyrimidine synthesis. Although our findings suggest that PC cells require high amounts of uridine, the effects of Ter on PC cell growth cannot be explained solely through Ter's inhibitory effect on DHODH. Our observations are consistent with previous findings reported for Ter in

Figure 5. Gemcitabine Synergizes with Pharmacological PIM Inhibitors in Growth Inhibition of PC Cells

(A) PANC-1 and MIA PaCA-2 cells were treated with M-110 (PIM-3) and PIM447 (pan-PIM inhibitor) for 72 h as single agents and in combination with gemcitabine, at constant ratios, on the basis of the previously calculated IC₅₀ for each drug. Quantitative analysis of the dose-effect relationships was performed after measurement of cell growth by using the MTS assay. Potential synergistic or additive effects were calculated with CompuSyn software (Cambridge, UK). Isobolograms and combination index (CI) plots (not shown) were created, and CI values were calculated. Drug synergism, addition, and antagonism effects are defined by CI values of <1.0, 1.0, and >1.0, respectively. CI values for effective dose (ED) 50 (ED₅₀), 75 (ED₇₅), 90 (ED₉₀), and 95 (ED₉₅) are shown. Results from one representative experiment are presented as means ± SD, with quadruplicate determinations. (B) Summary view of CI values at EDs 50, 75, 90, and 95 for the PC cell lines MIA PaCA-2 and PANC-1, each treated with three different gemcitabine drug combinations for 72 h.

MM. In MM, apoptosis induced by Ter is completely reversed by external addition of 100 μM uridine in the NCI-H929 cell line and is almost completely reversed in the RPMI-8226 cell line; however, at 200 μM Ter, uridine does not reverse induction of apoptosis.¹² These findings imply that the mechanism of Ter-mediated growth inhibition is not limited to the inhibitory effects on DHODH.

In addition to inhibition of DHODH enzyme activity and thus *de novo* pyrimidine biosynthesis, our results suggest that Ter mediates its activity

in PC cells, to some degree through inhibition of the PIM-c-Myc signaling pathway. We showed that Ter inhibited PIM-3 activity and downstream signaling in PC cells, including inhibition of c-Myc protein expression. PIM-3 is important in cell proliferation, survival, and protein synthesis of PC.²² Moreover, PIM-3 activity can prevent Bad-mediated apoptosis.²⁵ We demonstrate that inhibition of PIM-3 with a specific inhibitor and with Ter all lead to growth inhibition of PC cells. PIM-3 silencing can lead to apoptosis, mediated in part through the downstream inhibition of p-Bad by Ter. In addition to phosphorylation of Bad, PIM kinases can phosphorylate transcription factors such as Stat3 and c-Myc.^{27,28} c-Myc is a well-characterized master regulator of many vital biologic processes in normal and cancerous cells, including PC; however, development of c-Myc inhibitors remains a challenge. For example, c-Myc is lacking an enzymatic active site, thus challenging the development of direct c-Myc inhibitors.⁴¹ Therefore, many inhibitors of c-Myc have been developed to target signaling events upstream of c-Myc. Of note, many of these inhibitors have been associated with increased toxicity,

likely because of broad inhibition of the many pleiotropic functions of this transcription factor.⁴¹ On the other hand, inhibition of c-Myc by a dominant-negative mutant construct was well tolerated.⁴² We show here that Ter inhibited c-Myc signaling in PC cells, at least in part, through inhibition of the PIM family of kinases, including PIM-3. In PC, PIM-3 overexpression is associated with more advanced stage and worse survival, and targeting of PIM-3 with miR-377, a micro-RNA that binds to the 3' UTR of PIM-3, thereby suppressing PIM-3 expression, has been shown to inhibit PC cell growth.²¹ PIM-3 kinase can interact with various biologic pathways, including apoptosis, cell cycle, protein synthesis, and transcription. We thus investigated the respective downstream products of these pathways.²² Ter downregulated markers of survival, such as p-Bad and Bcl-xL. The transcription factors c-Myc and p-Stat3 were also downregulated with Ter. With PIM-3 overexpression, these findings were reversed. Overexpression of PIM-3 kinase also partially rescued cells from Ter-induced growth inhibition, again implicating PIM-3 kinase as an important mechanism of action of Ter.

Because Lef is approved by the FDA, has a long-standing history of biosafety, is orally bioavailable, can be combined with other drugs, and is highly affordable, it represents a promising new option for PC treatment regimens, such as in combination with established genotoxic chemotherapy. *In vivo* experiments have demonstrated synergy of Gem with Lef in athymic nude mice to induce growth inhibition of PC cells.⁴³ What remains to be determined is the efficacy of Lef with genotoxic chemotherapy in immunocompetent models of PC and the impact on anti-tumor immunity. We have determined that Lef inhibits growth of KPC cells *in vitro* (data not shown). KPC cells are derived from the genetically engineered mouse model of PC *LSL-Kras^{G12D/+}; Trp53^{R172H/+}; Pdx1-Cre*, and future studies will evaluate the efficacy of Lef with Gem in this cell line in a syngeneic mouse model.⁴⁴

In conclusion, Ter, the metabolically active component of the agent Lef, alone and in synergy with Gem, inhibited the growth of human PC cell lines. The mechanism of action was mediated in part via inhibition of *de novo* pyrimidine synthesis and inhibition of the PIM-3 kinase pathway. Future work in *in vivo* immunocompetent animal models will demonstrate the efficacy of this agent for translation into clinical trials.

MATERIALS AND METHODS

Cell Lines and Generation of PIM-3-Overexpressing Cell Lines and Reagents

Cells of the PC lines PANC-1, MIA PaCA-2, BxPC-3, and AsPC-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), cultured in RPMI-1640 or DMEM, per the provider's instructions, and supplemented with 10% FBS and 1× antibiotics at 37°C with 5% CO₂. Cell lines purchased from ATCC more than 6 months before submission of this manuscript and not frozen at an early passage were authenticated using ATCC's human short tandem repeat (STR) DNA profiling authentication service. The morphology of the cell lines was monitored

routinely, and the cell lines were routinely subjected to mycoplasma detection with a mycoplasma detection kit (Roche, Germany). Lentivirus particles for overexpression of PIM-3 kinase or control lentivirus were purchased from System Biosciences (SBI, Palo Alto, CA, USA). Briefly, PIM sequences from the NCBI nucleotide database were synthesized and subcloned into the CD710B-1 (MSCV-MCS-EF1a-Puro) cloning vector to avoid the possibility of modification of the PIM protein sequence due to the addition of 17 aa at the C terminus of the genes. Third-generation, biosafe (replication-incompetent), transduction-ready pseudoviral particles were generated, by using the pPACKH1 HIV packaging kit (SBI). Non-competency (absence of replication-competent lentivirus [RCL]) was determined by HIV p24 ELISA. All lentivirus work was conducted in designated biosafety level II areas. PC cells stably overexpressing PIM proteins were generated by transduction of the cells with the PIM/control pseudoviral particles at MOI 30 in the presence of 8 µg/mL polybrene and centrifugation for 60 min at 2,300 rpm (700 g) at 37°C. Stable cell lines were generated by puromycin selection (3 µg/mL). Ter and the pan-PIM inhibitor PIM447 were purchased from Selleckchem (Houston, TX, USA). The PIM-3 inhibitor M-110 was purchased from Millipore-Sigma (Burlington, MA, USA).

MTS Proliferation Assay, Annexin-V Apoptosis Assay, and Cell Cycle Analysis

The CellTiter-96 Aqueous One Solution Cell Proliferation Assay (MTS assay) was used for colorimetric measurement of proliferation of PC cell lines, per the manufacturer's instructions (Promega, Madison, WI, USA). Annexin V and DAPI double staining was used to determine apoptosis. Briefly, cells were harvested and washed twice with Annexin V binding buffer (BD Biosciences, San Jose, CA, USA) and resuspended in 100 µL of the same buffer containing APC Annexin V (BD Biosciences). The cells were then incubated in the dark at room temperature for 15 min, washed again, and resuspended in 300 µL buffer. DAPI (Sigma-Aldrich) was added immediately before analysis by the LSR II flow cytometer (BD Biosciences). For cell cycle analysis, cells were harvested, washed twice in PBS, and resuspended and fixed in 70% ethanol for >30 min at 4°C. The cells were then washed twice in PBS and treated with 50 µL of a 100 µg/mL ribonuclease stock solution, before addition of 200 µL propidium iodide (PI; 50 µg/mL stock solution), and analyzed by the LSR II flow cytometer.

In Vitro Kinase Screening Assay for Identification of Novel Direct Targets of Ter

We used the largest kinase panel available for screening and profiling services (600 kinases; Reaction Biology, Malvern, PA, USA). Briefly, a final concentration of 200 µM Ter was tested against the >600 kinases of the full kinase panel in duplicate, in a radiometric assay based on conventional filter-binding assays, which directly measures kinase catalytic activity, as described on the manufacturer's web page. The kinase panel report provided by the company included the percentage of inhibition of kinase activity for Ter and the control compound. For PIMs, a subsequent dose-response assay with Ter and different ATP

concentrations was performed to determine the IC_{50} values for Ter against PIM proteins.

Molecular Modeling of Ter in the ATP-Binding Site of PIM Kinases

To explore the binding mechanism of Ter on PIMs, we carried out docking of the drug molecule to PIM-1, -2, and -3 by applying our in-house-developed all-around docking (AAD) method and the Induced Fit Docking (IFD) package (Schrödinger, San Diego, CA, USA). The homology model of PIM-3 was built by using the X-ray crystal structure of human PIM-1 as the template, whereas the structures of PIM-1 (PDB: 4xhk) and PIM-2 (PDB: 4x7a) were downloaded from the PDB. First, we used our AAD method to search the best binding pocket of Ter on protein, because the method can dock the small molecule on the whole surface of the protein and result in top binding pockets. The ATP-binding sites of the three PIM kinases were found to be the best binding pockets for Ter. Then, we used IFD software to perform fine docking at the ATP-binding site, as the IFD method allows the side chains of protein residues to be flexible, which can generate a more accurate binding pose and docking scores. Thereafter, 190 ns molecular dynamics simulation was performed by using Nanoscale Molecular Dynamics (NAMD) software (Intel, Santa Clara, CA, USA). Figure 3C displays the binding pose of Ter at the ATP-binding site of PIM-3. The drug molecule interacts with PIM-3 via two hydrogen bonds with G105 and I107, one water bridge with D189, and hydrophobic interactions with V54/L94/V106/L123/L177/I188/F190, together with other van der Waals interactions. The Schrödinger extra precision (XP) docking score is fairly high at -8.1 kcal/mol, showing strong binding affinity of Ter on PIM-3 kinase. Meanwhile, Ter binds PIM-2 and -1 with docking scores of -7.4 and -7.1 kcal/mol, respectively. The drug molecule forms only one hydrogen bond with PIM-2 D182 residue, together with a salt-bridge interaction with K61. The drug molecule also forms only one hydrogen bond with PIM-3 K67 residue, together with a π -stacking interaction with F49. Thus, our docking studies suggest that Ter may bind at the ATP-binding pockets of PIM kinases. It may have higher binding affinity for PIM-3 than for PIM-2 and -1, which is generally consistent with our experimental results.

Western Blot Analysis

For western blotting, cells were washed and harvested in ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer containing 10 mM protease inhibitor cocktail (Thermo Scientific, Lafayette, CO, USA). The cell lysates were then separated on NuPAGE 4%–12% gradient gels (Invitrogen, Carlsbad, CA, USA), proteins were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and incubated with primary antibodies overnight, followed by incubation with horseradish-peroxidase (HRP)-conjugated secondary antibodies and visualization with enhanced chemiluminescence reagent (Thermo Scientific, Lafayette, CO, USA). Antibodies from Cell Signaling (Danvers, MA, USA) were PIM-3 (clone D17C9), p-Bad S112 (clone S112), Bcl-xL (clone 54HS), p-Stat3 Y705 (clone D3A7), p-MDM2 S166 (cat. no. 3521S), p21 Waf/Cip1 (clone 12D1), Cdc25a (cat. no. 3652S), CDK2 (clone 78B2), cyclin

D1 (clone 92G2), cyclin E1 (clone HE12), cyclin A2 (clone BF683), and p-RB Ser795 (cat. no. 9301). Antibodies from Santa Cruz (Dallas, TX, USA) were DHODH (clone E-8), PIM-1 (clone 12H8), PIM-2 (clone 1D12), and c-Myc (clone 9E10). Actin (clone AC-15) was purchased from Millipore-Sigma (Burlington, MA, USA).

Knockdown of PIM Proteins Using siRNA

siGENOME SMARTpool for knockdown of PIM proteins and the control was purchased from Dharmacon (Lafayette, CO, USA). MIA PaCA-2 and PANC-1 cells were transfected with siRNA in Lipofectamine RNAiMAX reagent, per the manufacturer's instructions (Invivogen, San Diego, CA, USA), at a final concentration of 100 nM siRNA. Forty-eight hours after the start of transfection, the cells were harvested and processed for western blotting.

Drug Combination Experiments and Analysis

For two-drug combination experiments, PC cells were treated with the drugs for 72 h, as single agents or in combination, at constant ratios, on the basis of previously calculated IC_{50} values for each drug. A quantitative analysis of dose-effect relationships was performed after measurement of cell growth, using the MTS assay. Potential synergistic or additive effects were calculated with CompuSyn software (Cambridge, UK). Isobolograms and CI plots (not shown) were created, and CI values were calculated. Drug synergism, addition, and antagonism effects are defined by CI values of <1.0 , 1.0 , and >1.0 , respectively. ED_{50} , ED_{75} , ED_{90} , and ED_{95} are the EDs that describe the amount of drug(s) needed to produce a response in 50%, 75%, 90%, and 95% of the cells, respectively.

Statistical Analysis

Where indicated, to compare the means of two groups, results were generally compared by unpaired, two-tailed Student's *t* test, with values from at least three independent experiments. Data are presented as means \pm SD. $p < 0.05$ was considered statistically significant; ns indicated not significant. All statistical analyses were conducted using SigmaPlot 12.5 (Systat Software, Chicago, IL, USA). All statistical tests were two-sided.

AUTHOR CONTRIBUTIONS

C.M., R.B., X.W., and H.L. performed the experiments. R.B., L.G.M., and S.T.R. wrote the manuscript. J.F.S. edited and critically reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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