Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159
Published OnlineFirst on August 3, 2010 as 10.1158/1535-7163.MCT-10-0159



Molecular Cancer Therapeutics

# Tyrosine Phosphatase Inhibitor-3 Sensitizes Melanoma and Colon Cancer to Biotherapeutics and Chemotherapeutics

Suman Kundu[1], Keke Fan[1], Mingli Cao[1], Daniel J. Lindner[1,2,3], Ralph Tuthill[4], Lili Liu[5], Stanton Gerson[5], Ernest Borden[1,2,3,5], and Taolin Yi[1,3,5]

## Abstract

Drug resistance is a major obstacle in cancer treatments and diminishes the clinical efficacy of biological, cytotoxic, or targeted therapeutics. Being an antiapoptotic mediator of chemoresistance in breast and lung cancer cells, MKP1 phosphatase might be targeted for overcoming chemoresistance and improving therapeutic efficacy. In this work, tyrosine phosphatase inhibitor-3 (TPI-3) was identified as a novel small molecule inhibitor of MKP1 and was capable of sensitizing tumors to bio- and chemotherapeutics in mice as a tolerated oral agent. Effective against recombinant MKP1, TPI-3 selectively increased MKP1 phosphosubstrates in Jurkat cells and induced cell death via apoptosis at nanomolar concentrations. TPI-3 also increased MKP1 phosphosubstrates in WM9 human melanoma cells and synergized with biotherapeutic IFNα2b in the growth inhibition of melanoma cells *in vitro* (combination index, <1). WM9 xenografts unresponsive to individual agents were significantly inhibited (62%, $P = 0.001$) in mice by a tolerated combination of oral TPI-3 (10 mg/kg, 5 d/wk) and IFNα2b. MKP1 expression was detected in human melanoma cell lines and tissue samples at levels up to six times higher than those in normal or nonmalignant melanocytes. TPI-3 also interacted positively with chemotherapeutics, 5-fluorouracil/leucovorin, against MC-26 colon cancer cells *in vitro* and in mice. Altogether, our data show the preclinical activities of TPI-3 in overcoming cancer resistance to bio- and chemotherapeutics, implicate MKP1 as a drug-resistant molecule in melanoma, and support the targeting of MKP1 for improving cancer therapeutic efficacy. *Mol Cancer Ther; 9(8); 2287–96. ©2010 AACR.*

## Introduction

Drug resistance is common in many types of cancers. It could be intrinsic but is often acquired following initial treatments. It is a major obstacle for clinical efficacy of biological, cytotoxic, or targeted therapeutics (1–4). Identification of the mediators of drug resistance and understanding their mechanism(s) of action could provide insights for overcoming resistance and lead to novel approaches for improving cancer treatments.

MKP1 (mitogen-activated protein kinase phosphatase-1, dual-specificity phosphatase 1, and CL-100) was identified recently as a mediator of resistance to several chemotherapeutics in breast and lung cancer cells (5–7). Transient or stable overexpression of MKP1 in breast cancer cells enhanced viability in the face of treatment with alkylating agents (mechlorethamine), anthracyclines (doxorubicin), and microtubule inhibitors (paclitaxel; ref. 5). Overexpression of MKP1 rendered lung cancer cells resistant to cisplatin, whereas knocking down MKP-1 expression with short interfering RNA sensitized lung cancer cells to cisplatin (6, 7). The clinical significance of these observations is indicated by the frequent overexpression of MKP1 in many cancer tissues including breast cancer, colon cancer, lung, prostate cancer, ovarian cancer, and pancreatic cancer (6, 8–12). The potential clinical effect is underscored by the association of MKP1 overexpression with early transformation and poor prognosis (9–11).

MKP1 mediates chemoresistance in a significant part via its antiapoptotic activity through dephosphorylating/inactivating c-*Jun*-$NH_2$-kinase (JNK), a proapoptoic signaling molecule (13). Overexpression of MKP1 in breast cancer cells reduced caspase activation induced by chemotherapeutics (5). Knocking down MKP1 expression in cancer cells increased apoptosis (12, 14). Among the MKP1 substrates (JNK, p38, and ERK1/2; refs. 15, 16), JNK was reported as a key molecule in MKP1 antiapoptotic action. Inducible MKP1 expression blocked JNK activation and inhibited apoptosis (17), whereas suppression of MKP1 expression potentiates JNK activation coincident with apoptosis (14). Interestingly, MKP-1

**Authors' Affiliations:** [1]Department of Cancer Biology of Lerner Research Institute, [2]Department of Translational Hematology and Oncology, [3]Taussig Cancer Institute, [4]Department of Pathology, The Cleveland Clinic, and [5]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio

**Note:** S. Kundu and K. Fan contributed equally to this work.

**Corresponding Author:** Taolin Yi, Department of Cancer Biology, Lerner Research Institute, The Cleveland Clinic, 9500 Euclid Avenue, NB4-67, Cleveland, OH 44195. Phone: 216-445-9656; Fax: 216-445-6269. E-mail: yit@ccf.org

**doi:** 10.1158/1535-7163.MCT-10-0159

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



is also a negative regulator of innate and adaptive immune responses (18).

Being an antiapoptotic mediator of chemoresistance in several types of cancers (6, 8–12, 19), MKP1 is a potential cancer therapeutic target. Indeed, MKP1-inhibitory compounds active at low micromolar ranges induced cancer cell death in culture and sensitized cancer cells to chemotherapeutics *in vitro* (20). However, the effectiveness and safety of targeting MKP1 as an anticancer strategy *in vivo* remain to be established. MKP1 inhibitors with preclinical antitumor activity *in vivo* have not been reported. It has not been clear whether MKP1 is also significant in cancer resistance to biotherapeutics.

In this work, a novel MKP1 inhibitor was identified and called tyrosine phosphatase inhibitor-3 (TPI-3). Being a small organic compound (274 Da) with defined structure (Fig. 1) and of little prior interest, TPI-3 has not been reported for biological activities. The compound was included in chemical collections for high-throughput screening and found inactive for the measles virus RNA polymerase or 14-3-3 molecule (PubChem). Herein, we provide preclinical evidence demonstrating that TPI-3 improved the efficacy of bio- and chemotherapeutics for melanoma and colon cancer in mice as a tolerated oral agent. Our data suggest that targeting MKP1 could be an effective and tolerated strategy for overcoming resistance to multiple types of anticancer agents and implicate TPI-3 as a promising lead compound for further development.

## Materials and Methods

### Cells, cell culture, and reagents

Cancer cell lines including Jurkat (21), WM9 (22), A375 (23), MCF-7 (24), DU145 (25), SW 620 (26), and MC-26 (27) were obtained from colleagues and cultured in RPMI 1640 or DMEM supplemented with 10% FCS. The effects of chemical compounds on cancer cell growth in culture were quantified by MTT assays following established procedures (28). Normal human melanocytes and human melanoma cell lines were from the institutional melanoma core.

Recombinant human IFNα2b (specific activity, $2 \times 10^8$ units/mg protein, Intron A, Schering-Plough), recombinant MKP1 and recombinant SHP-1 have been described previously (29). TPI-2 (L6, Chembridge), its analogues (a1-a6, Chembridge), 5-fluorouracil (5FU; Sigma), and leucovorin (LV; Sigma) were purchased from the indicated commercial sources. Antibodies to pERK1/2, ERK1/2, p-p38, pJNK, pLck-pY394, pZAP70, and actin were obtained from a commercial source (Cell Signaling).

### Chemical library, databases, and phosphatase assay

A library of drug-like chemical compound was from a commercial source (Chembridge). Upon identification of TPI-2 as a MKP1-inhibitory lead, we sought to derive improved compounds. However, the lack of crystal or solution structure of MKP1 has prevented the rational design for analogues. Moreover, the limited sequence homology (~50%) of the MKP1 protein to the other related phosphatases with resolved structures also hindered the development of a computer-assisted three-dimensional model. Accordingly, we initially focused on the identification and evaluation of analogues with chemically structural similarities to TPI-2. Analogues of TPI-2 were identified from chemical databases by computer-assisted structure analyses. Briefly, chemical structure of TPI-2 was compared with individual structures in commercial chemical databases (Chembridge and Asinex) of approximately

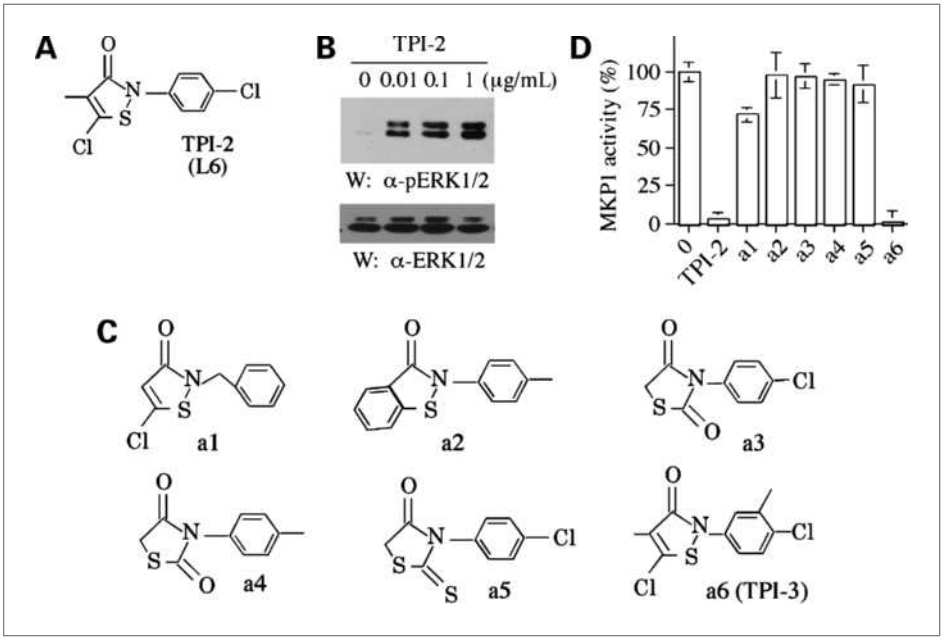


Figure 1. Identification of MKP1 inhibitors TPI-2 and TPI-3. A, chemical structure of TPI-2 (L6). B, Jurkat cells treated with TPI-2 for 30 min were analyzed by Western blotting to quantify pERK1/2 and ERK1/2 levels. C, chemical structures of TPI-2 analogues. D, activities of recombinant MKP1 in the presence of TPI-2 or its analogues (1 μg/mL) in phosphatase assays (mean ± SD of duplicates).

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



1 million small organic compounds, using the software at the commercial sites for calculating structural similarities with TPI-2. Six compounds with similarities ≥70% with TPI-2 were selected and purchased from a commercial source (Chembridge). Their effects on the phosphatase activity of recombinant MKP1 *in vitro* were evaluated following established procedures (29).

### Induction and detection of cellular phospho-proteins

Cells in culture medium were treated with agents for designated times and concentrations at 37°C and lysed on ice for 30 minutes in cold lysis buffer [1% NP40, 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 20 mmol/L NaF, and 0.2 mmol/L $Na_3VO_4$] containing a cocktail of proteinase inhibitors (10 mL/tablet; Sigma). The lysates were cleared by centrifuging (14,000 rpm, 10 minutes) in a microfuge at 4°C to remove insoluble parts, mixed with equal volumes of 2× SDS-PAGE sample buffer, boiled for 5 minutes, and analyzed by SDS-PAGE/Western blotting with commercial antibodies as described previously (28, 30, 31). Relative intensities of phosphoprotein bands were quantified through densitometry analysis.

### Induction and quantification of apoptotic cells

Jurkat cells were cultured for 16 hours in the presence of TPI-2, a1, a2, or TPI-3 (0, 0.1, and 1 μg/mL, respectively), washed thrice in PBS, and stained with the DNA dye 7-AAD (Invitrogen) and phycoerythrin-conjugated Annexin V antibody (BD Biosciences) in darkness at room temperature for 30 minutes. Following staining, the samples were washed thrice, resuspended in 200 μL of 1% paraformaldehyde solution, and analyzed (20,000 cells/sample) using a BD FACSCalibur cytometer as described previously (32, 33).

### Statistical analysis

Median effect analysis (34), which provides the most general form of studying the interactions between drugs, was used to analyze the interaction between TPI-3 and IFNα2β. Median effect plots were generated for IFNα2b alone, TPI-3 alone, and the combination in inhibiting the growth of WM9 melanoma cells in culture. The combination index (CI) was determined and plotted versus the fraction affected (fa). Data were analyzed in both modes, mutually exclusive and mutually nonexclusive. The interaction between two mutually nonexclusive drugs is described by the equation $CI = D_1/D_{x1} + D_2/D_{x2} + D_1D_2/D_{x1}D_{x2}$, where $D_{x1}$ and $D_{x2}$ are the doses of drug 1 and drug 2 that are required to inhibit growth x%. $D_1$ and $D_2$ in combination also inhibit growth x% (i.e., drug 1 and drug 2 are isoeffective). When $CI < 1$, drugs are synergistic, when $CI = 1$, drugs are additive, and when $CI > 1$, drugs are antagonistic. Student's *t* test was used to assess the significance of the effects of different treatments against tumors in mice.

### Evaluation of MKP1 expression in melanoma cell lines and melanoma tissues

The expression levels of MKP1 transcripts in normal human melanocytes and human melanoma cell lines were quantified by gene expression array analysis following established procedures (35). Briefly, RNA samples were harvested from cells (~80% confluence) lysed in Trizol (Invitrogen) and evaluated with the Illunima Sentrix Human-6_v2 Expression BeadChip technology. Data were analyzed with BeadStudio V3.2 software. Total cell lysates were also prepared (28) from cancer cell lines and endothelial cell line EA.hy926 (36). The protein levels of MKP1 in the lysates were quantified by SDS-PAGE/Western blotting (28) using a commercial antibody for the phosphatase (Santa Cruz Biotechnology).

MKP1 expression in human melanoma tissue samples was investigated via immunohistochemistry analysis of an institutional melanoma tissue microarray. The array consisted of formalin-fixed, paraffin-embedded tissues from human melanoma samples of radial growth phase (15) or vertical growth phase (11), and tissue samples of nonmalignant human nevus (5). Sections of the array were prepared and stained for immunohistochemistry (32) with a commercial MKP1 antibody (Santa Cruz Biotechnology) that was reported for immunohistochemical detection of the human molecule (37). The relative MKP1 staining levels in melanoma cells and adjacent benign melanocytes in the tissue samples were estimated via microscopy.

### Animal and animal studies

To evaluate the interactions of TPI-3 with IFNα2b against melanoma *in vivo*, athymic nude mice (*nu/nu*, NCR, female, 6 weeks old; Taconic Farms) were inoculated (s.c.) in the flanks with WM9 human melanoma cells ($2 \times 10^6$ cells/site). On day 4 postinoculation, the mice were subjected to treatment with vehicle control, TPI-3 (10 mg/kg, orally 5 d/wk) via oral gavage, IFNα2b (500,000 units/mouse, s.c., 5 d/wk), or the combination. The TPI-3 dose was chosen based on its tolerance in mice in a pilot experiment. The dose of IFNα2b used for treatment was comparable with those in previous studies (28, 38) but was given only 5 days per week instead of the previous daily treatment. For assessing interactions between TPI-3 and 5FU/LV, BALB/c mice (female, 6 weeks old; Taconic Farms) were inoculated with MC-26 colon cancer cells (s.c., $10^6$ cells/site). Starting on day 4 postinoculation, the mice were treated with vehicle control, TPI-3 (10 mg/kg, orally 5 d/wk), 5FU/LV (50 mg/kg–100 mg/kg, i.p., weekly), or the combination. The 5FU/LV treatment was similar to those reported previously (39). Tumor volume was measured and calculated using the formula for a prolate spheroid ($V = 4/3\ \pi\ a^2b$; ref. 40). Mouse viability and body weights were also recorded. Internal organs of the mice were inspected visually upon their termination at the end of the experiment. All studies involving mice were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



## Results

### Identification of MKP1 inhibitors TPI-2 and TPI-3

Given the role of MKP1 in cancer chemoresistance, we sought to identify small molecule inhibitors for the phosphatase. Lead six (L6; Fig. 1A), a compound from a chemical library, increased MKP1 phosphosubstrate pERK1/2 (41) in Jurkat leukemia cells (Fig. 1B) in which MKP1 and the substrate were expressed (42). This prompted us to investigate the MKP1 inhibitory activities of L6 and its analogues, six of which (L6a1-6; Fig. 1C) were identified based on structural similarities to the parental compound. L6 and analogue L6a6 reduced the activity of recombinant MKP1 *in vitro*, whereas the other analogues were less effective or not effective (Fig. 1D). These data indicated L6 and L6a6 as MKP1 inhibitors. They were named as TPI-2 and TPI-3 for further investigation.

The potency and selectivity of the compounds for cellular MKP1 were evaluated via quantification of cellular MKP1 phosphosubstrates levels. TPI-2 and TPI-3 increased all three of the MKP1 phosphosubstrates in Jurkat cells with effective concentrations at 0.1 to 1 μg/mL (Fig. 2A, left), suggesting a capacity to inhibit the cellular MKP1 (cellular $IC_{50}$, 0.1–1 μg/mL). Consistent with their lack of activity *in vitro* (Fig. 1D), analogue L6a1 only increased pERK1/2 but not the other MKP1 phosphosubstrates whereas L6a2 did not increase any of the three phosphosubstrates (Fig. 2A, left). Suggesting a selective action, TPI-2 at 0.001 to 1 μg/mL had no apparent affect on SHP-1 phosphosubstrates (pLck-pY394 and pZAP-70) in Jurkat cells (Fig. 2B and D), in which the expression of these molecules were reported (43), or on the general cellular phospho-proteins (Fig. 2C). Similarly, TPI-3 treatment also had little effect on the cellular SHP-1 substrates (Fig. 2D). In further support, TPI-2 and TPI-3 at 1 μg/mL or lower concentrations had little effect on the activity of recombinant SHP-1 (data not shown). Moreover, the durability of cellular MKP1 phosphosubstrates induced by TPI-3 was substantial at more than 4 hours (Fig. 2A, right), indicating a significant effect of TPI-3 on these substrates. These data together provided evidence that intracellular MKP1 was selectively inhibited by TPI-2 or TPI-3 at ~1 μg/mL for a duration of more than 4 hours.

### TPI-2 and TPI-3 induce cell death via apoptosis

Being an antiapoptotic molecule in cancer cells, MKP1 might be targeted with inhibitors to induce cell death via apoptosis. TPI-2, TPI-3, and the negative control analogues a1 and a2 were therefore evaluated for their capacities to induce Jurkat cell apoptosis and affect cancer cell viability *in vitro*.

Apoptotic cells, as indicated by surface Annexin V expression, were induced markedly by TPI-2 (52%) and TPI-3 (58%) in Jurkat cells cocultured with compounds at 1 μg/mL for 16 hours (Fig. 3A). The two leads at a lower dose (0.1 μg/mL) also induced apoptotic cells with less potency (~23–28%; Fig. 3A). Analogue a1 failed to induce cell apoptosis under comparable conditions, whereas a2 was modestly effective only at 1 μg/mL (Fig. 3A). Jurkat cells were completely dead when cultured in the presence of TPI-2 or TPI-3 at 0.3 μg/mL or higher levels for 6 days (Fig. 3B), but were only partially inhibited (<50%) by the other analogues (Fig. 3B).

TPI-2 and TPI-3 also induced cell death in a panel of cancer cell lines *in vitro* whereas the control a2 was not effective (Fig. 3C–H). The responsive cell lines include those of melanoma (WM9 and A539), breast cancer

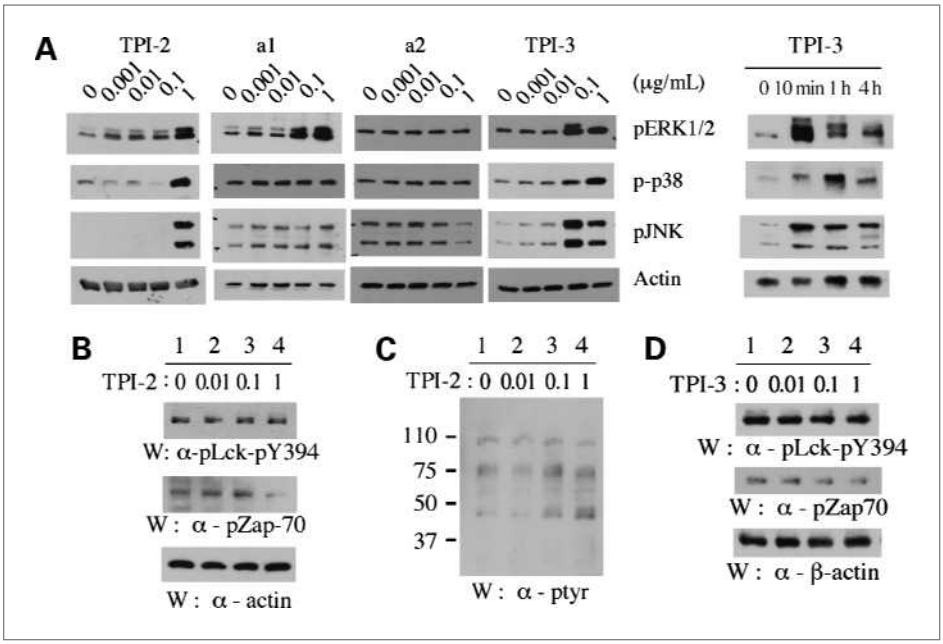


Figure 2. TPI-2 and TPI-3 selectively increase MKP1 phosphosubstrates in Jurkat cells *in vitro*. A, Jurkat cells were treated with TPI-2 or the analogues at different doses for 10 min. Total cell lysates were analyzed by Western blotting for MKP1 phosphosubstrates (left). Jurkat cells treated with TPI-3 (1 μg/mL) for 0 to 4 h were also analyzed similarly (right). B and C, Jurkat cells were treated with TPI-2 for 10 min and then analyzed by Western blotting with antibodies for SHP-1 phosphosubstrates (B) or total cellular phospho-tyrosine proteins (C). D, Jurkat cells treated with TPI-3 were analyzed by Western blotting for SHP-1 phosphosubstrates.

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



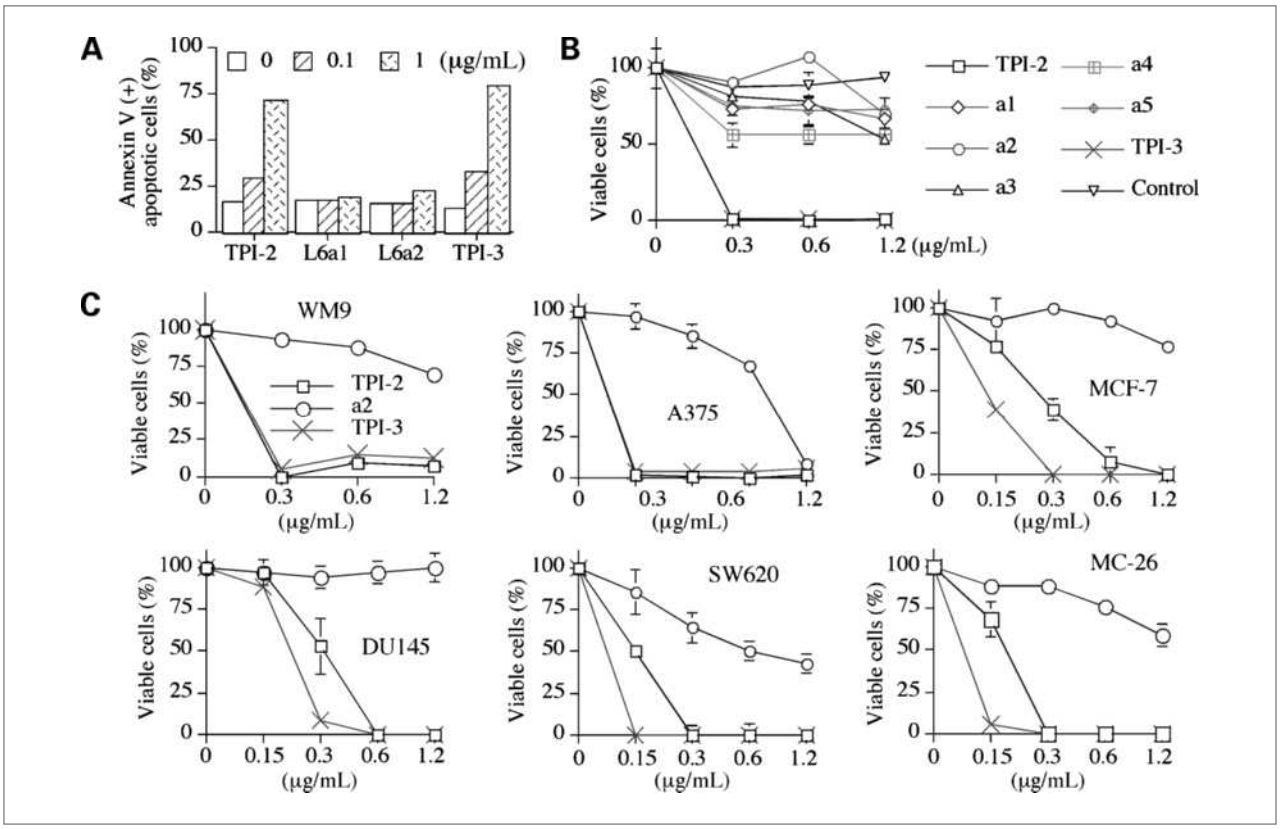


Figure 3. TPI-2 and TPI-3 induce apoptosis in Jurkat cells and cell death in multiple cancer cell lines. A, Jurkat cells cultured in the presence of vehicle control, TPI-2, or its analogues for 16 h were stained with the DNA dye 7-AAD and phycoerythrin-conjugated anti–Annexin V antibody and analyzed by FACS to quantify Annexin V–positive apoptotic cells. B, viable Jurkat cells cultured in the presence of vehicle control, TPI-2, or its analogues for 6 d as quantified by MTT assays. C, cell lines of different solid tumors were cultured for 4 d in the presence of TPI-2, TPI-3, or control analogue a2. Viable cells were then quantified by MTT assays (mean ± SD of triplicates).

(MCF-7), prostate cancer (DU-145), and colon cancer (SW620 and MC-26).

These results showed that TPI-2 and TPI-3 had proapoptotic activity in Jurkat cells (Fig. 3) at concentrations effective for MKP1 inhibition (Fig. 2), and suggested a similar action for the leads in various cancer cell lines *in vitro*.

### TPI-3 interacts positively with IFNα2b in growth inhibition of WM9 melanoma *in vitro* and in mice

Being an antiapoptotic mediator of chemoresistance, MKP1 might be targeted with inhibitors for increased responses to cancer therapeutics. Accordingly, TPI-3 was evaluated for its ability to overcome drug resistance in cancer cells. Because WM9 human melanoma cells were resistant to growth inhibition by melanoma therapeutic IFNα2b (28), the capacity of TPI-3 to sensitize melanoma cells to IFNα2b *in vitro* and *in vivo* was determined.

TPI-3 enhanced the growth inhibition of WM9 cells by IFNα2b in culture (Fig. 4A) in a synergistic manner (CI < 1.0) as defined by median effect analysis (40). The two agents also interacted positively against WM9 xenografts in nude mice (Fig. 4B). Their combination significantly inhibited WM9 tumor growth (62%, $P < 0.001$), in contrast to the limited effects from single agents (Fig. 4B). It remained effective beyond the point when the control mice had to be terminated due to large tumor burden and tumor ulceration (Fig. 4B). TPI-3 alone and its combination were tolerated: all the mice survived to the end of study with comparable body weights (Fig. 4C) and no obvious abnormalities in behaviors or gross anatomy (data not shown).

For mechanistic insights, the effect of TPI-3 on phosphosubstrates of MKP1 in WM9 cells in culture was determined. TPI-3 increased cellular pJNK levels and was effective starting at ~0.1 μg/mL (Fig. 4D). pJNK in WM9 cells was also increased by TPI-2 but not by control analogues a1 or a2 (Fig. 4D). Cellular pERK1/2 and pp38 were present at high basal levels in WM9 cells and was 10 times higher than those in Jurkat cells (data not shown). They were not increased further by TPI-3 treatment (Fig. 4D). The samples were further evaluated for pStat1 and pStat3 molecules. Cellular levels of pStat1 and pStat3 in WM9 cells were not significantly affected by treatments with TPI-3 (0.001–1 μg/mL) under these experimental conditions (data not shown).

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



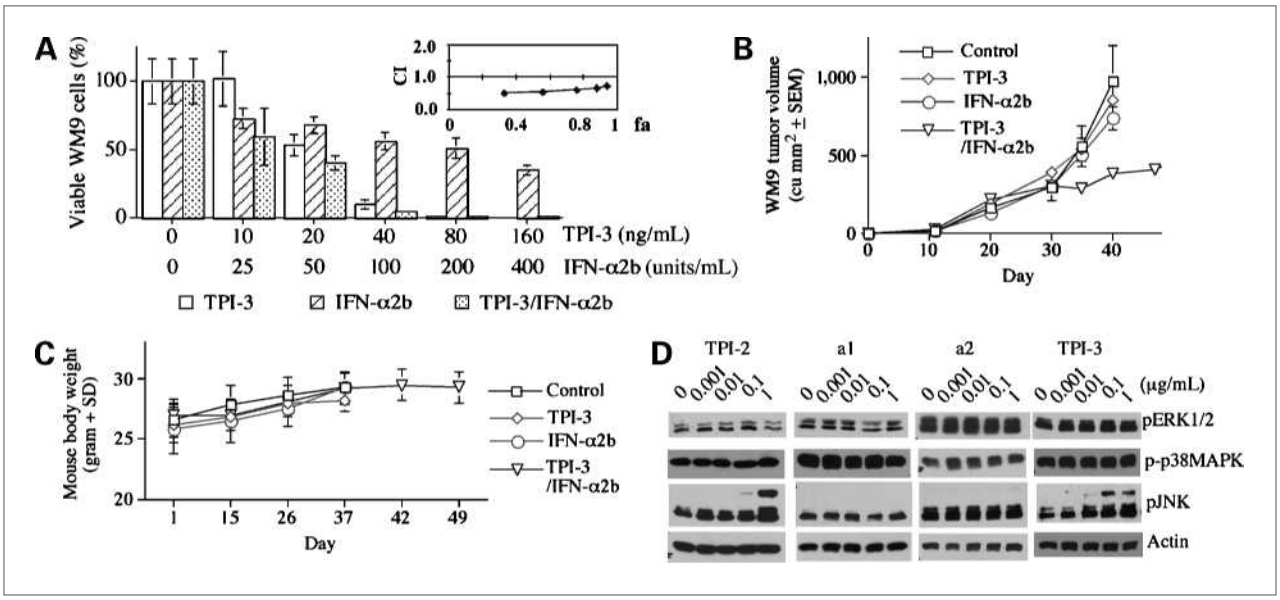


Figure 4. TPI-3 synergizes with IFNα2b in growth inhibition of WM9 melanoma cells *in vitro* and sensitized WM9 tumors to IFNαb2 therapy in mice. A, WM9 cells were cultured in the absence or presence of TPI-3, IFNα2b, or the combination for 6 d. Viable cells were quantified by MTT assays (mean ± SD of triplicates). Interactions of TPI-3 with IFNα2b in growth inhibition of WM9 cells in culture as evaluated by median effect analysis to calculate CI value (inset). B, nude mice bearing 4-d established WM9 tumors were treated with vehicle control, TPI-3 (10 mg/kg, orally daily Monday to Friday/wk), IFNα2b ($5 \times 10^5$ IU/mouse, s.c., daily Monday to Friday/wk), or the combination. Tumor volumes ($n = 8$) were recorded. C, body weights of the mice in B during the study. D, WM9 cells treated with TPI-2 and its analogues for 10 min in culture were analyzed by Western blotting with antibodies as indicated. The blots represent two replicates.

These results showed that TPI-3 sensitized IFNα2b-refractory WM9 melanoma to the cytokine *in vitro* and *in vivo*. Consistent with targeting MKP1, TPI-3 increased pJNK levels in melanoma cells that expressed the phosphatase. Its lack of effect on cellular pERKs and pp38 was likely due to high basal levels of the phosphoproteins, which were downstream events of the activating B-RafV600E mutation (44, 45) that was common in melanoma (46) and detected in WM9 cells (data not shown). Given the synergy between TPI-3 and IFNα2b (Fig. 4), additional studies of TPI-3 on IFNα2b signaling in WM9 cells might provide mechanistic insights.

### MKP1 is expressed at significant levels in human melanoma cell lines and melanoma tissue samples

The capacity of TPI-3 to sensitize WM9 melanoma to IFNα2b supported targeting MKP1 for improving melanoma treatments. However, it was unclear whether and at what levels MKP1 was expressed in human melanoma. We therefore assessed the expression of MKP1 in human melanoma cell lines and melanoma tissue samples.

MKP1 transcripts in human melanoma cell lines and normal human melanocytes cultured under comparable conditions were quantified by gene expression array analysis as described previously (35). The MKP1 transcripts were significantly higher (~1- to 6-fold) in the melanoma cell lines in comparison with normal human melanocytes (Fig. 5A). Expression of the MKP1 protein in human melanoma cell lines and several other cancer cell lines was verified (Fig. 5B and C).

The MKP1 protein was detected in tissue samples of advanced human melanoma by immunohistochemistry at levels approximately three times higher than those in benign melanocytes or melanocytic nevus cells (Table 1; Fig. 5D). MKP1 protein was present predominantly in the nuclei, as reported for other types of cancer cells (5, 7). Significant MKP1 staining (+++) was detected in ~90% of the tissue samples of radial or vertical growth melanoma in contrast with the weaker MKP1 staining in nonmalignant human melanocytes (0/+) or nevus cells (+) (Table 1; Fig. 5D).

MKP1 was therefore expressed in human melanoma cells and melanoma tissues and often at levels several folds higher than those in normal or nonmalignant melanocytes.

### TPI-3 interacted positively with 5FU/LV against MC-26 colon cancer tumors in mice

To extend the observed activity of TPI-3 to sensitize melanoma response to IFNα2b, the capacity of TPI-3 to sensitize other types of cancers to chemotherapeutics was investigated. For this, we determined the capacity of TPI-3 to sensitize MC-26 colon cancer tumors *in vivo* to 5FU/LV, a backbone of chemotherapy (47). Consistent with the reported expression of MKP1 in colon cancer tissues (8), MKP1 protein was detected in MC-26 cells (data not shown).

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



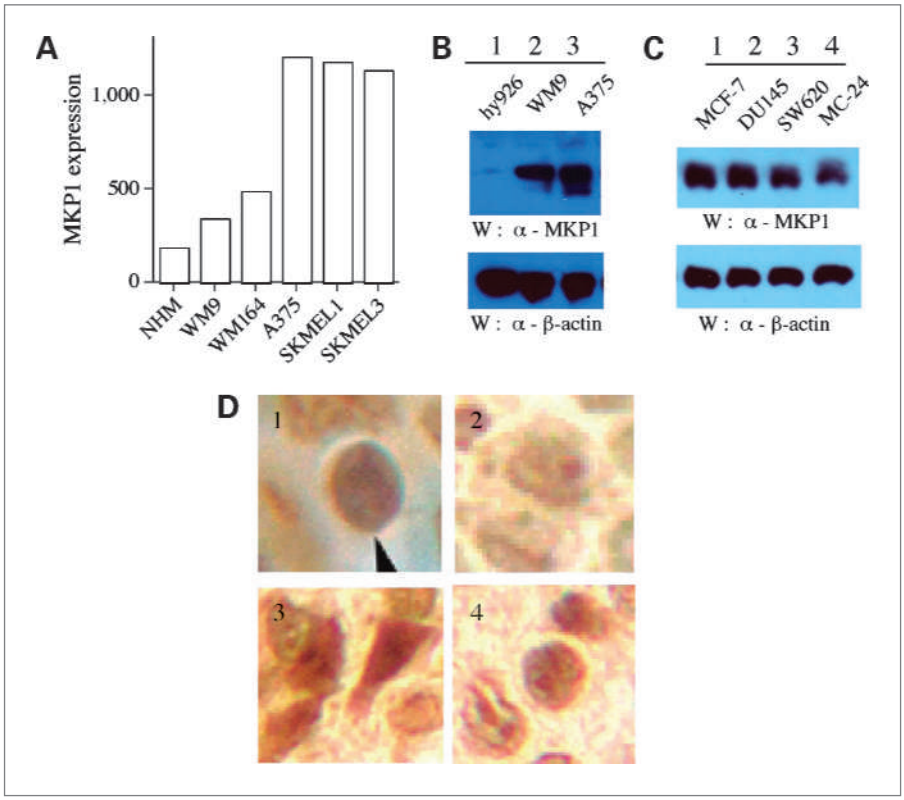


Figure 5. MKP1 is expressed at significant levels in human melanoma cell lines and tissue samples. A, MIP1 transcripts in normal human melanocytes (NHM) and human melanoma cell lines were quantified by array analysis (signals + SD). B, expression of MKP1 protein in human melanoma cell lines and hy926 endothelial cell line were quantified by Western blotting with antibodies as indicated. C, expression of MKP1 protein in cancer cell lines as quantified by Western blotting with antibodies as indicated. D, representative immunohistochemistry images (magnification, ×40): 1, the variably stained nuclei of benign melanocytes (arrow); 2, junctional nevus; 3, radial growth melanoma; and 4, advanced vertical growth melanoma. Immunohistochemistry results can be found in Table 1.

MC-26 tumor growth was inhibited 78% by the combination, significantly more than by 5FU/LV (54%, $P = 0.011$) or TPI-3 (18%, $P = 0.004$) as single agents (Fig. 6A). The increased inhibition of tumor growth by the combination suggested a positive interaction between TPI-3 and 5FU/LV. TPI-3 and 5FU also interacted positively in growth inhibition of MC-26 cells in culture (Fig. 6B). Supporting a mechanism of action targeting MKP1 for sensitization, TPI-3 increased MKP1 phosphosubstrates in MC-26 cells (Fig. 6C). The compound also had the capacity to interact with 5FU against human colon cancer cells (HT-29) *in vitro* (Fig. 6D).

**Table 1.** Relative MKP1 expression levels in samples of a human tissue microarray seen in Fig. 5D were quantified by immunohistochemistry

| Tissue type | MKP1 staining (no. of samples) | | | |
|---|---|---|---|---|
| | 0/+ | + | ++ | +++ |
| Melanocytes | 5 | | | |
| Nevus | | 5 | | |
| Melanoma of radial growth | | | | 15 |
| Vertical growth | | 1 | 1 | 9 |

## Discussion

In the current study, we provide evidence for the first time that MKP1 inhibitor TPI-3 sensitized cancer cells to IFNα2b and 5FU *in vitro* and in mice. TPI-3 sensitized WM9 human melanoma cells *in vitro* and WM9 xenografts to the biotherapeutic IFNα2b (Fig. 4). It also sensitized MC-26 colon cancer cells to colon cancer chemotherapeutic 5FU *in vitro* and in mice (Fig. 6). These actions of the compound were likely mediated via targeting MKP1 given that TPI-3 was an inhibitor of recombinant MKP1 (Fig. 1) and was capable of increasing MKP1 phosphosubstrates in both WM9 and MC-26 cells (Figs. 4 and 6). Our data thus indicate that targeting MKP1 could be an effective strategy for sensitizing differential cancers to both biotherapeutics and chemotherapeutics *in vitro* and *in vivo*. Moreover, the tolerance of TPI-3 and its combinations with cancer therapeutics suggests that the strategy is safe and supports further translational evaluation. Additional studies are clearly needed to define the role of MKP1 as the target of TPI-3. In particular, assessment of the responses of cancer cells with differential MKP1 expression levels to TPI-3 could be of significance and might provide mechanistic insights.

The effectiveness and tolerance of TPI-3 in sensitizing melanoma and colon cancer to therapeutics in mice also suggested the potential of the compound as a lead for developing MKP1-targeted agents to overcome drug

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



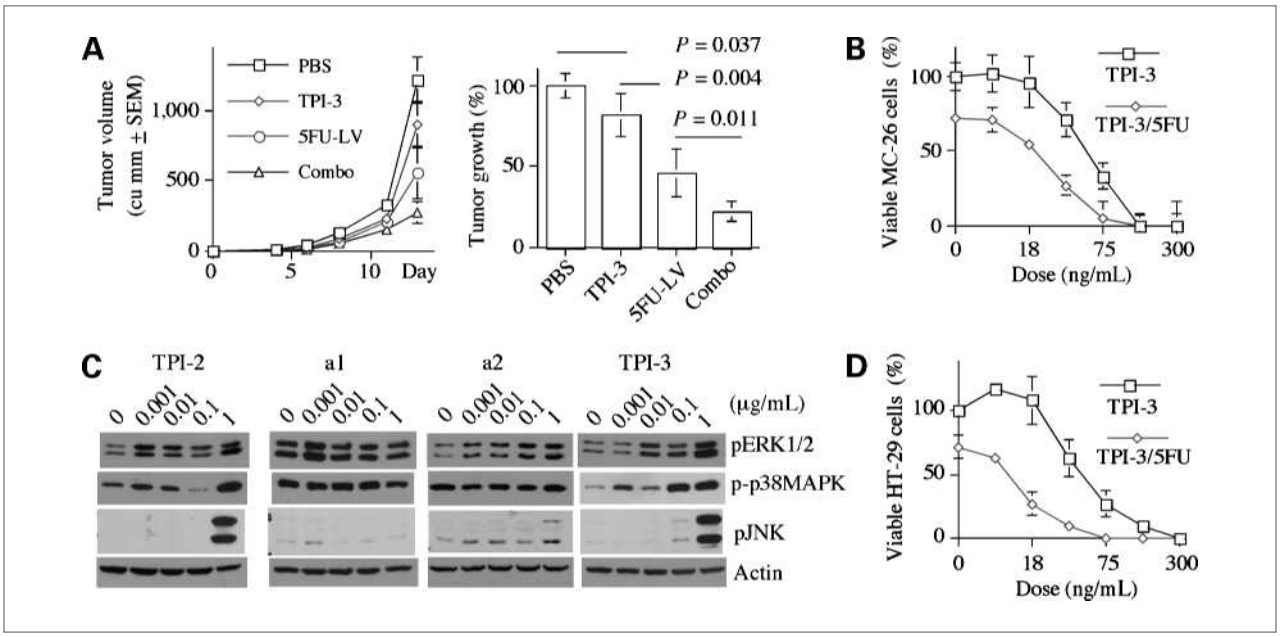


Figure 6. TPI-3 positively interacts with 5FU/LV against MC-26 colon cancer tumors in mice and increases MKP1 phosphosubstrates in MC-26 cells. A, BALB/c mice bearing 4-d established MC-26 tumors were treated with vehicle control, TPI-3 (10 mg/kg, orally M-F/wk), 5FU/LV (50–100 mg/kg, s.c., 5 d/wk), or the combination. Tumor volumes ($n$ = 5) were recorded (left). Tumor volumes at the end of the study were analyzed for assessing statistical significance (right). B, MC-26 cells were cultured with differential doses of TPI-3 in the absence or presence of 5FU (2 μmol/L) for 3 d. The cells were allowed to grow for 4 d after washing and evaluated for viable cells by MTT assays (mean ± SD of triplicates). C, MC-26 cells were cultured in the absence or presence of TPI-2, a1, a2, or TPI-3 for 10 min. Total cell lysates were harvested and analyzed by Western blotting with antibodies as indicated. The blots represent two replicates. D, HT-29 cells were cultured with differential doses of TPI-3 in the absence or presence of 5FU (2 μmol/L) for 3 d. The cells were allowed to grow for 4 d after washing and evaluated for viable cells by MTT assay (mean ± SD of triplicates).

resistance. The oral availability (Figs. 4 and 6) of TPI-3 further increases its attractiveness for translational development. In addition, its chemical characteristics (data not shown) show no violation of Lipinski's rule of five and the extensions (48), indicating good druggability. Moreover, the higher potency of TPI-3 in comparison with its structurally related TPI-2, L6a1, or L6a2, suggests potential and insights for improving potency via structural modifications. In addition, TPI-3 analogues may be exploited for developing more selective inhibitors. TPI-3 displayed selective actions for MKP1 as it was inactive for SHP-1 under these experimental conditions (Figs. 1 and 2). It is also encouraging that L6a1, although inactive for MKP1 (Figs. 1 and 2), selectively increased pERK1/2 (Fig. 2A), suggesting that it targets a pERK1/2-specific phosphatase (e.g., MKP3; ref. 18). The limited structural differences between TPI-3 and L6a1 (Fig. 1) implicate the feasibility of modulating target specificity via minor chemical variations. Whereas the full spectrum of TPI-3–targeted phosphatases remains to be established, it is worth noting that several inhibitors targeting multi-kinases have been approved as effective and tolerated cancer therapeutics (e.g., imatinib and sunitinib), suggesting translational potential for inhibitors with multi-phosphatase targets. Altogether, our data provide a basis for developing refined inhibitors against MKPs (or other phosphatases) and for biological evaluations of new candidate inhibitors.

Our results also provide the first evidence implicating MKP1 overexpression as a drug-resistant mechanism in melanomas. MKP1 overexpression was detected in human melanoma cell lines and advanced human melanoma tissues (Fig. 5). Its functional significance in therapeutic resistance was suggested by the sensitization of WM9 melanoma to IFNα2b *in vitro* and in mice (Fig. 4). Advanced melanoma responds poorly (~10%) to each of the three approved melanoma treatments (IFNα2b, interleukin-2, and dacarbazine; ref. 49). As indicated by TPI-3 activity in sensitizing WM9 melanoma to IFNα2b (Fig. 4), MKP1-targeted agents might be combined with melanoma therapeutics as more efficacious treatment options. Indeed, TPI-3 also near significantly increased WM9 tumor growth inhibition induced by the dacarbazine analogue temozolomide or its combination with methoxyamine (ref. 50; data not shown). Moreover, the capacity of TPI-3 to sensitize WM9 melanoma (Fig. 4), which harbors the B-RafV600E mutant[6] with heightened downstream pERK1/2 levels (Fig. 4) also suggests that TPI-3 and other MKP1 inhibitors might be complementary with B-Raf inhibitors in antitumor actions and could be exploited for combination treatments. Mutations of B-Raf, N-Ras, and c-kit have been reported in melanoma, leading to active oncogenic molecules. Because MKP1

[6] Our unpublished data.

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



substrates are signaling molecules downstream of the oncoproteins, targeting MKP1 with TPI-3 is expected to be effective in melanoma with each of the mutant genotypes. Comparative evaluation of TPI-3 responses by melanoma cells with differential genotypes will be informative in this regard and could have implications for MKP1-targeted agents. Interestingly, low MKP1 expression levels were detected in ~10% samples of the advanced melanoma tissues (Fig. 5) and may correlate with better clinical responses that might be exploited for preselecting potential responding cases.

Among the three MKP1 phosphosubstrates, pJNK was the only one that was consistently induced by TPI-3 in Jurkat, WM9, and MC-26 cells (Figs. 2, 4, and 6). Thus, pJNK was implicated as a key mediator for TPI-3 in proapoptotic action and antitumor action, and could be further evaluated to define the mechanism of action of the compound and its significance as a biomarker. Because MKP1 negatively regulates innate and adaptive immune responses (18), targeting the phosphatase might lead to immune cell activation. Indeed, TPI-2 and TPI-3 were capable of increasing mouse splenocyte IFNγ+ cells *in vitro*,[7] although immune cell activation was apparently not required for TPI-3 sensitization of WM9 melanoma to IFNα2b because the sensitization occurred *in vitro* in the absence of immune cells and in T cell–deficient nude mice (Fig. 4). Despite its effectiveness in inducing cancer cell death *in vitro* (Fig. 3), TPI-3 did not exhibit significant activity as a single agent against WM9 xenografts and had only a modest effect on MC-26 tumors (Figs. 4 and 6). This might be related to the lower doses (≤10 ng/mL) of TPI-3 required for sensitization in comparison with its death-induction doses (~100 ng/mL; Figs. 3 and 4). Taken together, our results provide a strong basis for elucidating the mechanism of action of MKP1 inhibition and for potential translation in future studies.

[7] Our unpublished data.

## Disclosure of Potential Conflicts of Interest

T. Yi has a patent pending for TPI-3 and analogs.

## Acknowledgments

The authors thank Becky Haney for excellent technical assistance.

## Grant Support

NIH grants CA096636 (T. Yi), CA095020 (D.J. Lindner), and CA0890344 (E. Borden).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 02/17/2010; revised 05/25/2010; accepted 06/14/2010; published OnlineFirst 08/03/2010.

## References

1. Lage H. An overview of cancer multidrug resistance: a still unsolved problem. Cell Mol Life Sci 2008;65:3145–7.
2. Janne PA, Gray N, Settleman J. Factors underlying sensitivity of cancers to small-molecule kinase inhibitors. Nat Rev Drug Discov 2009;8:709–23.
3. Rini BI, Atkins MB. Resistance to targeted therapy in renal-cell carcinoma. Lancet Oncol 2009;10:992–1000.
4. Borden EC. Melanoma 2007: current state and preview of the future. Semin Oncol 2007;34:449–51.
5. Small GW, Shi YY, Higgins LS, Orlowski RZ. Mitogen-activated protein kinase phosphatase-1 is a mediator of breast cancer chemoresistance. Cancer Res 2007;67:4459–66.
6. Chattopadhyay S, Machado-Pinilla R, Manguan-Garcia C, et al. MKP1/CL100 controls tumor growth and sensitivity to cisplatin in non-small-cell lung cancer. Oncogene 2006;25:3335–45.
7. Wang Z, Xu J, Zhou JY, Liu Y, Wu GS. Mitogen-activated protein kinase phosphatase-1 is required for cisplatin resistance. Cancer Res 2006;66:8870–7.
8. Loda M, Capodieci P, Mishra R, et al. Expression of mitogen-activated protein kinase phosphatase-1 in the early phases of human epithelial carcinogenesis. Am J Pathol 1996;149:1553–64.
9. Magi-Galluzzi C, Mishra R, Fiorentino M, et al. Mitogen-activated protein kinase phosphatase 1 is overexpressed in prostate cancers and is inversely related to apoptosis. Lab Invest 1997;76:37–41.
10. Magi-Galluzzi C, Montironi R, Cangi MG, Wishnow K, Loda M. Mitogen-activated protein kinases and apoptosis in PIN. Virchows Arch 1998;432:407–13.
11. Denkert C, Schmitt WD, Berger S, et al. Expression of mitogen-activated protein kinase phosphatase-1 (MKP-1) in primary human ovarian carcinoma. Int J Cancer 2002;102:507–13.
12. Liao Q, Guo J, Kleeff J, et al. Down-regulation of the dual-specificity phosphatase MKP-1 suppresses tumorigenicity of pancreatic cancer cells. Gastroenterology 2003;124:1830–5.
13. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer 2009;9:537–9.
14. Mizuno R, Oya M, Shiomi T, Marumo K, Okada Y, Murai M. Inhibition of MKP-1 expression potentiates JNK related apoptosis in renal cancer cells. J Urol 2004;172:723–7.
15. Zhao Q, Wang X, Nelin LD, et al. MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. J Exp Med 2006;203:131–40.
16. Hammer M, Mages J, Dietrich H, et al. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. J Exp Med 2006;203:15–20.
17. Srikanth S, Franklin CC, Duke RC, Kraft RS. Human DU145 prostate cancer cells overexpressing mitogen-activated protein kinase phosphatase-1 are resistant to Fas ligand-induced mitochondrial perturbations and cellular apoptosis. Mol Cell Biochem 1999;199:169–78.
18. Liu Y, Shepherd EG, Nelin LD. MAPK phosphatases—regulating the immune response. Nat Rev Immunol 2007;7:202–12.
19. Wang HY, Cheng Z, Malbon CC. Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. Cancer Lett 2003;191:229–37.
20. Lazo JS, Skoko JJ, Werner S, et al. Structurally unique inhibitors of human mitogen-activated protein kinase phosphatase-1 identified in a pyrrole carboxamide library. J Pharmacol Exp Ther 2007;322:940–7.
21. Schneider U, Schwenk HU, Bornkamm G. Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer 1977;19:621–6.
22. Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M, Westermark B.

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc Natl Acad Sci U S A 1993;90:393–7.
23. Giard DJ, Aaronson SA, Todaro GJ, et al. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst 1973;51:1417–23.
24. Sugarman BJ, Aggarwal BB, Hass PE, Figari IS, Palladino MA, Jr., Shepard HM. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. Science 1985;230:943–5.
25. Mickey DD, Stone KR, Wunderli H, Mickey GH, Vollmer RT, Paulson DF. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. Cancer Res 1977;37:4049–58.
26. Leibovitz A, Stinson JC, McCombs WB III, McCoy CE, Mazur KC, Mabry ND. Classification of human colorectal adenocarcinoma cell lines. Cancer Res 1976;36:4562–9.
27. Singh P, Walker JP, Townsend CM, Jr., Thompson JC. Role of gastrin and gastrin receptors on the growth of a transplantable mouse colon carcinoma (MC-26) in BALB/c mice. Cancer Res 1986;46:1612–6.
28. Yi T, Pathak MK, Lindner DJ, Ketterer ME, Farver C, Borden EC. Anticancer activity of sodium stibogluconate in synergy with IFNs. J Immunol 2002;169:5978–85.
29. Pathak MK, Yi T. Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. J Immunol 2001;167:3391–7.
30. Yi T, Ihle JN. Association of hematopoietic cell phosphatase with c-Kit after stimulation with c-Kit ligand. Mol Cell Biol 1993;13:3350–8.
31. Yi T, Mui AL, Krystal G, Ihle JN. Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor β chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis. Mol Cell Biol 1993;13:7577–86.
32. Fan K, Zhou M, Pathak M, et al. Sodium stibogluconate interacts with IL-2 in anti-Renca tumor action via a T cell-dependent mechanism in connection with induction of tumor-infiltrating macrophages. J Immunol 2005;175:7003–8.
33. Fan K, Borden EC, Yi T. IFN-γ is induced in human peripheral blood immune cells *in vitro* by SSG/IL-2 combination and mediates its anti-tumor activity *in vivo*. J Interferon Cytokine Res 2009;29:451–60.
34. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984;22:27–55.
35. Leaman DW, Chawla-Sarkar M, Jacobs B, et al. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-β compared with IFN-α2. J Interferon Cytokine Res 2003;23:745–6.
36. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci U S A 1983;80:3734–7.
37. Vicent S, Garayoa M, Lopez-Picazo JM, et al. Mitogen-activated protein kinase phosphatase-1 is overexpressed in non-small cell lung cancer and is an independent predictor of outcome in patients. Clin Cancer Res 2004;10:3639–49.
38. Li J, Lindner DJ, Farver C, Borden EC, Yi T. Efficacy of SSG and SSG/IFNα2 against human prostate cancer xenograft tumors in mice: a role for direct growth inhibition in SSG anti-tumor action. Cancer Chemother Pharmacol 2007;60:341–9.
39. Yao M, Zhou W, Sangha S, et al. Effects of nonselective cyclooxygenase inhibition with low-dose ibuprofen on tumor growth, angiogenesis, metastasis, and survival in a mouse model of colorectal cancer. Clin Cancer Res 2005;11:1618–28.
40. Lindner DJ, Borden EC, Kalvakolanu DV. Synergistic antitumor effects of a combination of interferons and retinoic acid on human tumor cells *in vitro* and *in vivo*. Clin Cancer Res 1997;3:931–7.
41. Owens DM, Keyse SM. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene 2007;26:3203–13.
42. Acuna-Castillo C, Aravena M, Leiva-Salcedo E, et al. T-kininogen, a cystatin-like molecule, inhibits ERK-dependent lymphocyte proliferation. Mech Ageing Dev 2005;126:1284–91.
43. Mary F, Moon C, Venaille T, Thomas ML, Mary D, Bernard A. Modulation of TCR signaling by β1 integrins: role of the tyrosine phosphatase SHP-1. Eur J Immunol 1999;29:3887–97.
44. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855–7.
45. Estrada Y, Dong J, Ossowski L. Positive crosstalk between ERK and p38 in melanoma stimulates migration and *in vivo* proliferation. Pigment Cell Melanoma Res 2009;22:66–76.
46. Haluska F, Pemberton T, Ibrahim N, Kalinsky K. The RTK/RAS/BRAF/PI3K pathways in melanoma: biology, small molecule inhibitors, and potential applications. Semin Oncol 2007;34:546–4.
47. Wolpin BM, Mayer RJ. Systemic treatment of colorectal cancer. Gastroenterology 2008;134:1296–310.
48. Keller TH, Pichota A, Yin Z. A practical view of 'druggability'. Curr Opin Chem Biol 2006;10:357–61.
49. Tawbi HA, Kirkwood JM. Management of metastatic melanoma. Semin Oncol 2007;34:532–5.
50. Liu L, Gerson SL. Targeted modulation of MGMT: clinical implications. Clin Cancer Res 2006;12:328–31.

Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0159



Molecular Cancer Therapeutics

# Tyrosine Phosphatase Inhibitor-3 Sensitizes Melanoma and Colon Cancer to Biotherapeutics and Chemotherapeutics

Suman Kundu, Keke Fan, Mingli Cao, et al.

*Mol Cancer Ther* Published OnlineFirst August 3, 2010.