The anticancer effect of phospho-tyrosol-indomethacin (MPI-621), a novel phosphoderivative of indomethacin: *in vitro* and *in vivo* studies

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We have synthesized a novel derivative of indomethacin, phospho-tyrosol-indomethacin (PTI; MPI-621), and evaluated its anticancer efficacy *in vitro* and *in vivo*. PTI inhibited the growth of human colon, breast and lung cancer cell lines 6–30-fold more potently than indomethacin. *In vivo*, in contrast to indomethacin that was unable to inhibit colon cancer xenograft growth, PTI inhibited the growth of colon (69% at 10 mg/kg/day, $P < 0.01$) and lung (91% at 15 mg/kg/day, $P < 0.01$) subcutaneous cancer xenografts in immunodeficient mice, suppressing cell proliferation by 33% and inducing apoptosis by 75% ($P < 0.05$, for both). Regarding its pharmacokinetics in mice, after a single intraperitoneal injection of PTI, its plasma levels reached the maximum concentration ($C_{\max} = 46 \mu\text{M}$) at 2 h ($T_{\max}$) and became undetectable at 4 h. Indomethacin is the major metabolite of PTI, with plasma $C_{\max} = 378 \mu\text{M}$ and $T_{\max} = 2.5$ h; it became undetectable 24 h post-administration. The cellular uptake of PTI (50–200 μM) at 6 h was about 200-fold greater than that of indomethacin. Regarding its safety, PTI had no significant genotoxicity, showed less gastrointestinal toxicity than indomethacin and presented no cardiac toxicity. Mechanistically, PTI suppressed prostaglandin E_2 production in A549 human lung cancer cells and strongly inhibited nuclear factor- κB activation in A549 xenografts. These findings indicate that PTI merits further evaluation as an anticancer agent.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), widely used in the treatment of inflammatory diseases, possess antineoplastic properties predominantly attributed to their suppressive effects on inflammation (1,2). In 1980, Waddell *et al.* (3–5) reported that treatment with indomethacin, a conventional NSAID, led to the regression of colorectal polyps in patients with familial adenomatous polyposis. Since then, preclinical studies have established that indomethacin exhibits substantial efficacy against colon (6,7), lung (8–10) and breast (11) cancers. However, a major concern with indomethacin is its gastrointestinal toxicity. Chronic indomethacin administration leads to gastrointestinal ulcers, bleeding and perforation (12). In addition to its gastrointestinal toxicity, chronic indomethacin administration results in neurological side effects such as headache, dizziness and renal toxicity (13).

The potentially serious side effects of indomethacin and NSAIDs in general have prompted us to develop safer novel phosphoderivatives of these drugs. Phosphomodification of NSAIDs, such as aspirin, sulindac and ibuprofen, leads to their enhanced efficacy and reduced gastrointestinal toxicity (14–18). Here, we report on the synthesis and preclinical evaluation of phospho-tyrosol-indomethacin (PTI), a novel

derivative of indomethacin. PTI consists of a tyrosol linker, a natural phenolic antioxidant, between indomethacin and diethyl-phosphate (Supplementary Figure 1, available at *Carcinogenesis* Online). The esterification of the free carboxylic moiety of indomethacin, a major culprit for its gastrointestinal toxicity (19), could potentially contribute to the superior safety of PTI compared with the parent compound.

In this study, we document the enhanced potency of PTI over conventional indomethacin in suppressing the growth of cancer cell lines *in vitro*, its anticancer efficacy in lung and colon cancer animal tumor models and its improved safety.

Materials and methods

Materials

Chemicals and reagents were obtained from Sigma–Aldrich, unless otherwise noted. ¹H and ¹³C NMR spectra were recorded on a Varian 400 spectrometer. Samples for NMR were dissolved in CDCl₃. Electron spray ionization mass spectra were obtained on a Thermo Scientific DSQ (II) mass spectrometer. Thin-layer chromatography was performed on silica gel sheets (Tiedel-deHaën, Slezee, Germany). Flash column chromatographic separations were carried out on 60 Å (230–400 mesh) silica gel (TSI Chemical Company, Cambridge, MA).

Synthesis of PTI

PTI was synthesized in a stepwise manner (Supplementary Figure 1, available at *Carcinogenesis* Online). To make it simple, key compounds were numbered as 1–4, and these numbers were used instead of their long chemical names as customary, to describe the various synthesis steps. Because both hydroxyl group of tyrosol can react with the carboxyl group of indomethacin, we protect one of them prior to the esterification reaction [Supplementary Figure 1, step (i), available at *Carcinogenesis* Online]. At room temperature, tyrosol (6.9 g, 50 mmol) was reacted with *tert*-butyldimethyl-silyl chloride (8.25 g, 55 mmol) in imidazole (25 ml) to give *tert*-butyldimethyl-silyl protected tyrosol, alcohol 1, as a white solid with 90% yield. Alcohol 1 was used in the next step.

Synthesis of [1-(4-chloro-benzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-acetic acid 4-(2-hydroxy-ethyl)-phenyl ester (3). The synthesis of compound 3, the precursor of PTI, is depicted in Supplementary Figure 1, steps (ii) and (iii), available at *Carcinogenesis* Online. Under nitrogen, indomethacin (1.3 g, 3.6 mmol), *N,N*-dicyclohexylcarbodiimide (0.9 g, 4.3 mmol), *N*-hydroxybenzotriazole (0.6 g, 3.9 mmol) and CH₂Cl₂ (20 ml) were added to a flask and stirred at room temperature for 1 h. Then, a solution of alcohol 1 (0.9 g, 3.2 mmol) and 4-(dimethylamino) pyridine (60 mg) in CH₂Cl₂ (10 ml) was added and stirred at room temperature overnight. The resulting solid residue was dissolved in ethyl acetate; washed sequentially with 2% NaCO₃, distilled water and brine; and dried over NaSO₄. After removing the solvent, the crude product was purified by flash column chromatography, which yielded compound 2 as a pale yellow oil at 90%. Compound 2 (7 mmol) was then dissolved in tetrahydrofuran (40 ml) and reacted with tetra-*n*-butylammonium fluoride (7.8 mmol) and acetic acid (7 ml) at room temperature for 5 h. Alcohol 3 was obtained as a pale yellow solid at 88% yield.

Synthesis of [1-(4-chloro-benzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-acetic acid 4-[2-(diethoxy-phosphoryloxy)-ethyl]-phenyl ester (4). As shown in Supplementary Figure 1, step (iv), available at *Carcinogenesis* Online, PTI was synthesized as follows: diethylchlorophosphate (1.2 ml, 8.0 mmol) was added drop-wise to a solution of alcohol 3 (1.7 g, 3.6 mmol) in methylene chloride (10 ml) containing diisopropylethylamine (2.6 ml, 8.0 mmol). This was followed by the addition of 4-(dimethylamino) pyridine (25 mg) as a solid. The reaction mixture was heated under reflux overnight. The solution was washed with water (2 × 25 ml), dried over anhydrous sodium sulfate, filtered and concentrated. The resulting crude residue was purified by column chromatography using hexane:ethyl acetate (40:60) as the eluant. The pure fractions were combined and evaporated to produce a viscous yellowish oil at 90% yield.

Cell culture

Human lung [A549 (*p53* wild-type; *Kras* mutant) and H358 (*p53* null; *Kras* mutant)], colon [SW480 (*p53* mutant; *Kras* mutant) and HT29 (*p53* mutant; *Kras* wild-type)] and breast [MDA-MB-231 (*p53* mutant; *Kras* mutant)] cancer cell lines (American Type Culture Collection, Manassas, VA) were grown in the media recommended by American Type Culture Collection. F-12K medium

Abbreviations: NF- κB , nuclear factor- κB ; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate-buffered saline; PGE₂, prostaglandin E₂; PTI, phospho-tyrosol-indomethacin.

was purchased from American Type Culture Collection. McCoy's 5a medium, RPMI 1640, L-15 and antibiotics were purchased from Mediatech (Manassas, VA). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following the protocol of the manufacturer (Roche Diagnostics, Indianapolis, IN).

Determination of apoptosis by annexin V and propidium iodide staining

After the A549 cells were treated with the test drug in 6-well plates for 72 h, all cell populations (suspended and attached) were collected and stained with annexin V–fluorescein isothiocyanate and propidium iodide (PI) (Invitrogen, Carlsbad, CA) for 15 min. Annexin V–fluorescein isothiocyanate and PI fluorescence intensities were analyzed by flow cytometry with a FACScalibur. Annexin V(+)/PI(–) cells are early apoptotic cells, annexin V(+)/PI(+) cells are late apoptotic cells and annexin V(–)/PI(+) cells are necrotic cells.

Determination of cell cycle by PI staining

SW480 cells were seeded in 60 mm plates and treated with the test drug for 24 h. The adherent cells were harvested and fixed with 70% ethanol for at least 30 min, washed with phosphate-buffered saline (PBS), resuspended in 0.5 ml PBS containing ribonuclease (50 µg/ml) and incubated at 37°C for 30 min. PI was then added to the solution to a final concentration of 40 µg/ml. The fluorescence intensities were analyzed by flow cytometry with a FACScalibur.

Determination of cell proliferation by BrdU staining

Cells were seeded in 60 mm plates and treated with the test drugs for 16 h. Bromodeoxyuridine (BrdU; BD Biosciences, San Jose, CA) was added

directly to the culture medium to a final concentration of 10 µM and incubated in the CO₂ incubator for 30 min at 37°C, harvested and fixed in 70% ethanol for 30 min on ice. DNA was denatured by incubating the cells with 2 N HCl/Triton X-100 for 30 min, followed by neutralization in 0.1 M Na₂B₄O₇ (pH 8.5). Ten million cells were incubated with 20 µl of anti-BrdU–fluorescein isothiocyanate (BD Biosciences, San Jose, CA) for 30 min, cells were washed and resuspended in PBS containing 5 µg/ml PI. Cell fluorescence intensity was analyzed by flow cytometry with a FACScalibur.

Cellular uptake of PTI and indomethacin

A549 cells were seeded in 100 mm² plates and allowed to grow as a monolayer. Upon reaching 80% confluence, different concentrations of indomethacin or PTI were added and the cells were incubated at 37°C for 2, 6 or 16 h. The incubation was terminated by washing the cell monolayer with complete medium and PBS. The cells were harvested by scraping, extracted by 2-fold volume of acetonitrile and centrifuged at 13 000 r.p.m. for 5 min. Drug levels were determined by high-performance liquid chromatography (20).

Salmonella plate incorporation mutagenicity assay

The genetic toxicology assay was performed by BioReliance Corporation (Rockville, MD). PTI concentrations of 5000, 1500, 500, 150, 50, 15, 5.0 and 1.5 µg/plate were evaluated with tester strain TA98 with and without metabolic activation in duplicate plates using the plate incorporation method of treatment. Dimethyl sulfoxide was used as the vehicle. PTI is soluble at all dose levels.

Fig. 1. The cytokinetic effect of PTI. **(A)** The growth inhibitory effect of PTI. Values are the average of at least duplicates; individual values were within 12% of each other. **(B)** BrdU cell proliferation assay in A549 cells following treatment of PTI for 16 h. A549 cells were grown overnight and treated with various concentrations of PTI or indomethacin (Indo) as shown. The number in the right upper box indicates the percentage of cells in S phase. **(C)** Cell cycle profiles generated from fluorescence-activated cell sorting of PI-stained cells after treatment with PTI or indomethacin for 24 h. PTI blocked the G₁-to-S transition. **(D)** The percentages of A549 apoptotic cells determined by flow cytometry using dual staining (annexin V and PI) at 72 h of PTI treatment. The fold increase over control at 1 × IC₅₀, 1.5 × IC₅₀ and 2 × IC₅₀ is shown.

COX-1 and COX-2 assay

The COX-1 and COX-2 inhibitory activities of indomethacin and PTI were determined with the COX fluorescent inhibitor screening assay kit (Cayman Chemical Co., Ann Arbor, MI) following the manufacturer's instructions.

Determination of prostaglandin E₂

Prostaglandin E₂ (PGE₂) levels in the cell culture media were determined with the immunoassay kit purchased from Cayman Chemical Co. according to the manufacturer's instructions.

Efficacy in lung and colon cancer xenografts in mice

All animal studies were approved by our Institutional Animal Care and Use Committee.

Lung cancer treatment protocol. A549 cells (1.5×10^6) suspended in 100 μ l of PBS (25% hydrogel) were injected subcutaneously into both the left and right flanks of 5–6-week-old female NOD SCID mice (Taconic Farms, Germantown, NY). When the average tumor volume reached 100 mm³, the mice were divided into three groups: vehicle, PTI 10 mg/kg/day and PTI

15 mg/kg/day ($n = 10$ /group); treatment lasted 2 weeks. At the end of the treatment, animals were euthanized and the xenografts were harvested.

Colon cancer prevention protocol. Six-week-old female athymic nude mice (Taconic Farms, Germantown, NY; $n = 6$ per group) were pretreated by oral gavage with corn oil (vehicle) indomethacin 2 mg/kg/day or PTI 10 mg/kg/day for 5 days, and then 1.2×10^6 SW480 colon cancer cells suspended in 100 μ l of PBS were inoculated subcutaneously to each flank. These two doses represent 50% of the respective maximum tolerated dose for these animals, as calculated by us. The treatment was continued for another 38 days. Tumor size was monitored by measuring the length (L) and width (W) with a digital caliper and the volume was calculated according to the formula, $L \times W \times (L + W/2) \times 0.56$.

Immunohistochemistry

Staining for proliferating cell nuclear antigen and phospho-p65 (Ser276) was performed as described (21). Apoptosis was determined immunohistochemically by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling assay (17).

Fig. 2. Time- and concentration-dependent cell uptake of PTI and indomethacin in A549 cells. (A) Concentration-dependent uptake of PTI and indomethacin (Indo) determined at 6 h and (B) time-dependent uptake of PTI and Indo studies were carried out as described in Materials and methods. Data are mean $\pm$ SEM of at least three separate experiments. (C) Stability of PTI in porcine liver esterase. PTI was incubated with complete culture medium containing 10% fetal bovine serum for up to 48 h (upper) or with porcine liver esterase in PBS for up to 3 h (middle and bottom) at 37°C. The percentage of the intact PTI was determined at the indicated time points. Data are mean $\pm$ SEM of three separate experiments.

Fig. 3. Pharmacokinetic study of PTI and indomethacin. A single equimolar dose of PTI (100 mg/kg, upper) or indomethacin (58 mg/kg, lower) was administered to mice by intraperitoneal injection. The concentration of intact PTI and indomethacin in plasma at the indicated time points was determined. The AUC_{total} of PTI plus is about 3.5 times higher than that of indomethacin. All values are mean $\pm$ SEM ($n = 3$).

Gastrointestinal toxicity

The gastrointestinal toxicity of PTI was determined in rats following a standard protocol (22). Six week-old Sprague–Dawley rats ($n = 5$ per group) were administered by gavage for 4 days vehicle indomethacin 4.75 mg/kg/day (positive control) or PTI 10 mg/kg/day. On day 5, the animals were killed and gastric toxicity was evaluated by hematoxylin and eosin staining and light microscopy.

Statistical analyses

Results were expressed as mean $\pm$ SEM. $P < 0.05$ was considered statistically significant. Data were analyzed using descriptive statistics and graphical displays. Tumor volumes were compared among the treatment groups using repeated-measures analysis of variance. Differences were analyzed using Pearson's modification of the χ^2 test.

Results

PTI inhibits the growth of human cancer cell lines

We evaluated the growth inhibitory effect of PTI on human cancer cell lines originating from colon (SW480, HT29), lung (A549, H358) and breast (MDA-MB-231). Their IC₅₀ was measured after 72 h of indomethacin or PTI treatment. As shown in Figure 1A, the range of 72 h IC₅₀ for PTI was from 23 μ M (MDA-MB-231) to 87 μ M (HT29), suggesting that breast cancer cell lines were more

sensitive to PTI, whereas colon cancer cell lines (HT29) were the most resistant. Compared with indomethacin, PTI was more potent in all five cell lines, with the potency enhancement ranging from 6- to 30-fold.

Cell kinetic effect of PTI on human cancer cell lines

The cytokinetic effect of PTI was measured in order to assess its mechanism of cell growth inhibition. Cell proliferation was evaluated by BrdU incorporation. As shown in Figure 1B, PTI reduced BrdU incorporation in A549 cells in a concentration-dependent manner. At 60 μ M, PTI decreased the proportion of BrdU positive cells by 96%. In contrast, equimolar amounts of indomethacin only reduced BrdU positive cells by 15%.

Cell cycle analysis showed that treatment of cells with $1 \times$ IC₅₀ PTI induced a significant G₁-to-S block, with the proportion of cells in G₀/G₁ phase increasing from 56.8 to 69.7% (Figure 1C). The percentage of cells in G₀/G₁ phase is much higher than that following treatment with indomethacin at equimolar concentration (G₀/G₁: 57.7%). Of note, this effect became prominent only at 72 h, with a trend toward significant changes in cell cycle phase distribution being present at 24 and 48 h. Thus, our findings suggest that PTI blocks G₁-to-S transition more potently than indomethacin.

Fig. 4. Toxicity study of PTI. (A) Gastrointestinal toxicity was evaluated by hematoxylin and eosin staining. Images of tissue sections from rat (5 per group) stomachs treated with vehicle or indomethacin (Indo) or PTI for 5 days. On day 5, rats with at least one gastric ulcer were scored as positive for GI toxicity. PTI-treated rats showed limited gastrointestinal toxicity compared with indomethacin. (B) PTI exhibited no genotoxicity. The average number of revertants of TA98, the *Salmonella typhimurium* strain used in the study, following PTI treatment or 2-nitrofluorene (positive control) is shown.

Annexin V–PI staining showed that PTI induced concentration-dependent apoptosis in A549 cancer cells *in vitro*. Both early and late apoptosis were present, but the former predominated. At 72 h, in A549 cells, the annexin V+ cells increased from 7.5% in control to 12.3% at PTI 1.5× IC₅₀ and to 71.4% at 2× IC₅₀ (Figure 1D). These results indicate that the *in vitro* cytotoxic effect of PTI encompasses cell proliferation, cell cycle and apoptosis.

Cellular uptake

In order to compare the cellular penetration of PTI and indomethacin, we assessed their uptake by A549 cells. Cellular uptake of PTI was dose and time dependent (Figure 2A and B). After 6 h of incubation, the cellular uptake of PTI (50–200 μM) was about 200-fold greater than that of indomethacin. After 16 h, the cellular uptake of PTI was about 230-fold greater than that of indomethacin. These findings suggest that PTI has a markedly greater ability to penetrate cancer cells compared with its parental compound.

Effect of esterases on the stability of PTI *in vitro*

Stability of PTI is critical for its pharmacological activity. Hydrolysis of the intact drug by esterases leads to significant attenuation of its cytotoxicity *in vitro* (23,24). This hydrolysis markedly depends on esterase concentration. In complete media containing 10% serum, PTI is slowly hydrolyzed starting at 1 h of incubation, with 40% hydrolyzed after 24 h (Figure 2C). To determine its half-life, PTI was incubated *in vitro* with purified porcine liver esterases at 2 and 4 IU/ml. As shown in Figure 2C, the breakdown of PTI in the presence of 4 IU/ml esterase (half-life: 2 min) was more rapid compared with the 2 IU/ml esterase (half-life: 5 min). In the presence of 4 IU/ml of porcine liver esterase, we observed approximately 20% intact PTI remaining after 20 min of incubation. Although the stability and integrity of PTI were affected by esterase, PTI is to some degree stable in the presence of pure liver esterase.

Pharmacokinetics

We evaluated the pharmacokinetics of PTI and indomethacin in mice. As shown in Figure 3 (upper panel), after a single intraperitoneal

Fig. 5. PTI inhibits human cancer xenograft growth. (A) Tumor volume (% baseline) of A549 human non-small cell lung cancer cell xenografts treatment model (upper) and SW480 human colon cancer cell xenograft prevention model (lower). (B) The cytokinetic effect of PTI. PTI inhibited the proliferation and increased apoptosis in the A549 xenografts tumors. The xenograft tissue sections were stained for proliferating cell nuclear antigen (a proliferation marker) or terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling for apoptosis. Magnification $\times 40$. Values, mean $\pm$ SEM; * $P < 0.02$ versus vehicle-treated controls.

injection of PTI, its plasma levels reached the maximum concentration ($C_{\max} = 46 \mu\text{M}$) at 2 h and became undetectable at 4 h. Indomethacin is the major metabolite of PTI, reaching a maximum concentration of $378 \mu\text{M}$ at 2.5 h, and could be detected in blood 24 h postadministration. Compared with PTI, a single intraperitoneal administration of an equimolar dose of indomethacin resulted in a peak plasma level of $127 \mu\text{M}$ at 1 h and became negligible at 24 h postadministration (Figure 3, lower panel). The $\text{AUC}_{0-24\text{h}}$ of PTI plus its metabolite was $1700 \mu\text{M}\cdot\text{h}$, whereas that of indomethacin was $500 \mu\text{M}\cdot\text{h}$. Our results show that the bioavailability of PTI is significantly higher (3.5-fold) compared with that of indomethacin.

PTI shows less gastrointestinal toxicity and no cardiotoxicity or genotoxicity in rats

We evaluated the safety of PTI by examining its gastrointestinal toxicity, cardiotoxicity and genotoxicity and compared this with conventional indomethacin.

Gastrointestinal toxicity

Rats were administered vehicle, indomethacin (4.75 mg/kg/day) and PTI (10 mg/kg/day) by gavage for 4 days (Figure 4A). At killing on day 5, 100% of the rats treated with indomethacin developed ulcers compared with 40% of the PTI-treated rats, as shown in Figure 4A, representing a 60% reduction in gastrointestinal toxicity ($P < 0.01$).

Cardiotoxicity

Heart tissue sections from mice treated with PTI for 1.5 months were examined and scored histologically, following hematoxylin and eosin staining and light microscopy for tissue damage and for the presence of inflammatory cells. No differences in cardiotoxicity were observed between PTI-treated and control mice.

Genotoxicity

The genotoxicity of PTI was evaluated by measuring its ability to induce reverse mutations of two bacterial strains of *Salmonella typhimurium* (TA98 and TA100) in the presence and absence of metabolic activation (rat liver S9). In the tested concentration range ($1.5\text{--}5000 \mu\text{g/plate}$), with or without rat liver S9, PTI showed a frequency of revertants close to that of the vehicle, but far less than that of the positive control (Figure 4B). These studies indicate that PTI has no significant genotoxicity.

In vivo efficacy

The ability of PTI to inhibit the growth of A549 and SW480 human cancer cell xenografts was investigated. We conducted two studies: a lung cancer treatment study and a colon cancer prevention study. In the lung cancer treatment study, A549 human non-small cell cancer cells were injected subcutaneously to SCID mice. When the tumors reached 100 mm^3 , mice were treated with PTI at 10 or 15 mg/kg/day

Fig. 6. Signaling effects of PTI. (A) COX-inhibitory activity of indomethacin (Indo) and PTI. The IC₅₀ values were determined with purified COX-1 and COX-2 isozymes, as in Materials and methods. (B) The effect of Indo and PTI on PGE₂. A549 cells were pretreated with 2 $\times$ IC₅₀ of Indo or PTI for 30 min, followed by treatment with the calcium ionophore A23187 10 μ M for 3 h, and PGE₂ levels in the culture medium were measured by enzyme-linked immunosorbent assay. (C) PTI inhibits NF- κ B activation in A549 lung tumor xenografts in mice. Tumor tissue sections were immunohistochemically stained with phospho-NF- κ B p65 (Ser276) antibody. Representative images of phospho-p65 for the various groups. The number of phospho-p65 positive cells was counted and expressed as percentage of the total number of cells in the field (immunohistochemical staining, $\times 10$).

for 2 weeks. As shown in Figure 5A, PTI suppressed tumor growth and the effect became statistically significant ($P < 0.05$) starting 11 days after the initiation of treatment. The antitumor effect of PTI was dose dependent. At the end of the study, PTI 10 and 15 mg/kg/day reduced tumor volume by 68 and 91%, respectively, compared with the control group.

In the prevention study, SW480 colon cancer cells were inoculated subcutaneously into nude mice following pretreatment for 5 days with vehicle, PTI or indomethacin, which were each given by oral gavage. As shown in Figure 5A, compared with control, PTI 10 mg/kg/day reduced tumor growth by 69% at the end of the study, whereas indomethacin had no significant effect compared with control. The effect became statistically significant starting on day 20 of treatment.

PTI inhibited the growth of cancer xenografts via a potent cytokinetic effect. Sections from A549 xenografts were stained for proliferating cell nuclear antigen expression (proliferation marker) or by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (apoptosis marker). Cell proliferation in PTI-treated tumors ($40.2 \pm 4.5\%$) was reduced by one-third compared with controls ($59.7 \pm 7.5\%$). Interestingly, apoptosis index was almost doubled from $3.3 \pm 0.3\%$ in control to $5.8 \pm 1.2\%$ in PTI-treated mice, representing an increase of 75.8% (Figure 5B).

Signaling effects of PTI

By analogy to indomethacin, a strong COX inhibitor, we investigated the effects of PTI on the COX pathway, examining COX activity, PGE₂ production and nuclear factor- κ B (NF- κ B) activation. COX assays with purified COX-1 and COX-2 showed that PTI inhibits COX-2 more potently than COX-1 (Figure 6A), with a 12.6-fold selectivity for COX-2 (IC₅₀: 71.7 μ M) over COX-1 (IC₅₀: 905 μ M). Indomethacin was a strong inhibitor for both COX-1 (IC₅₀: 0.38 μ M) and COX-2 (IC₅₀: 18.2 μ M) under the same assay conditions. Figure 6B shows the effect of PTI and indomethacin on PGE₂ production by A549 cells. Indomethacin at 1.1 mM (2 $\times$ IC₅₀) was more potent than PTI at 50 μ M (2 $\times$ IC₅₀) in reducing the basal PGE₂ production in A549 cells. Additionally, both PTI and indomethacin prevented the increase in PGE₂ levels induced by the calcium ionophore A23187. The inhibitory activity of PTI and indomethacin may be a result of COX-2 inhibition.

To further explore the mechanism of action of PTI, we investigated the activation of NF- κ B in the A549 lung tumor xenografts from the control and PTI-treated groups, by determining Ser276 phosphorylation of the p65 subunit of NF- κ B. As shown in Figure 6C, compared with controls, PTI decreased the levels of phospho-p65 Ser276 in tumor xenografts by 90.0% ($P = 0.004$). These results establish that PTI down regulates COX and NF- κ B signaling in lung cancer cells.

Discussion

Our study indicates that PTI, a novel derivative of indomethacin, possesses potent anticancer efficacy in preclinical models of human colon and lung cancer and that it lacks the significant gastrointestinal toxicity associated with conventional indomethacin. PTI exerts its effect through a strong cytostatic effect on cancer cells and demonstrates improved cellular penetration and a favorable pharmacokinetic profile, both of which contribute to its superior efficacy compared with indomethacin.

Efficacy and safety are two critical factors that should be taken into consideration when evaluating a new anticancer agent. PTI was synthesized by conjugation of three moieties: indomethacin, a diethyl-phosphate group and a linker. The aromatic linker molecule, known as tyrosol, is non-toxic and has antioxidant activity (25). *In vitro*, PTI was 6–30-fold more potent than indomethacin in inhibiting the growth of cancer cells. PTI, given at 50% of its maximum tolerated dose, was also highly efficacious in inhibiting the growth of human cancer xenografts in mice. In the lung cancer treatment study, with PTI treatment for 2 weeks, we observed complete inhibition of the growth of A549 xenografts, achieving tumor stasis. In the colon cancer prevention study, PTI inhibited tumor growth by 69% compared with controls and was significantly more effective than its parent compound indomethacin. Moreover, PTI lacks significant gastrointestinal toxicity, cardiotoxicity and genotoxicity. These results indicate that PTI is efficacious and safe in preclinical models. Given the predictive value of xenograft models (26), PTI is a promising anticancer drug candidate for further evaluation.

PTI achieves its growth inhibitory effect by altering cytokinetics. The *in vitro* antiproliferative effect of PTI is mainly attributed to the suppression of cell proliferation, cell cycle arrest at G₁/S phase transition and the induction of apoptosis. In agreement with the *in vitro* findings, PTI strongly induced cell growth arrest and apoptosis in the cancer xenografts. Our previous studies have shown that modification of several NSAIDs has a strong triple cytokinetic effect as well (15–18).

An important factor contributing to the superior efficacy of PTI compared with indomethacin is the altered physiochemical properties of the molecule introduced by the phosphomodification. The phosphomodification significantly enhances the hydrophobicity of PTI, which leads to improved cell membrane permeability. As a consequence, cellular uptake studies showed that PTI penetrated A549 lung cancer cell line much more (>200-fold) efficiently than indomethacin.

Increased hydrophobicity also leads to higher bioavailability of PTI compared with its parental NSAID. Indomethacin is the major metabolite of PTI *in vivo*; it results from the hydrolysis of PTI by carboxylesterases (24). The integrity of the drug is critical to its anticancer efficacy. PTI can be hydrolyzed at the carboxyl ester linkage by carboxylesterases 1 and 2, leading to a significant attenuation of its cytotoxicity. Nevertheless, intact PTI was detected in the blood of mice, which may have contributed to its potent activity *in vivo*. Other factors that probably contributed to this outcome are its improved cellular penetration and its favorable pharmacokinetic profile.

Our work has also unraveled key elements of the mechanisms of action of PTI. Increased levels of COX-2 expression and its metabolite PGE₂ have been reported in many malignancies, including lung cancer; COX-2/PGE₂ are considered to play key roles in promoting and maintaining tumorigenesis (27,28). NSAIDs, such as indomethacin, exert their antineoplastic effect partly through the inhibition of COX-2 and PGE₂, although COX-independent effects are well documented (29). PTI exhibits virtually no activity toward COX-1 and only weakly inhibits COX-2 compared with indomethacin. This sparing of COX-1 may be responsible for PTI's improved gastrointestinal toxicity profile (30). Interestingly, PTI is as effective as indomethacin in inhibiting calcium ionophore-induced PGE₂ production in cancer cells, suggesting that PTI is a potent COX-2 inhibitor *in situ*. In addition, PTI suppresses NF-κB activity, whose aberrant activation is linked to inflammation and cancer (31). Inhibition of COX-2 and NF-κB, consistent with the antitumor activity of PTI, may mediate part of its growth inhibitory activity.

In summary, our study demonstrates that PTI is a promising agent that can effectively inhibit cancer cell lines *in vitro* and human cancer xenografts *in vivo*. It has higher potency than indomethacin and is apparently safe. Its effectiveness and safety in the prevention and treatment of cancer suggest that this compound merits further evaluation.

Supplementary material

Supplementary Figure 1 can be found at <http://carcin.oxfordjournals.org/>

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References

- Grivennikov, S.I. *et al.* (2010) Immunity, inflammation, and cancer. *Cell*, **140**, 883–899.
- Zhang, Z. *et al.* (2006) NF-κB, inflammation and pancreatic carcinogenesis: NF-κB as a chemoprevention target (review). *Int. J. Oncol.*, **29**, 185–192.
- Waddell, W.R. *et al.* (1980) Indomethacin and ascorbate inhibit desmoid tumors. *J. Surg. Oncol.*, **15**, 85–90.
- Waddell, W.R. *et al.* (1983) Nonsteroid antiinflammatory drugs and tamoxifen for desmoid tumors and carcinoma of the stomach. *J. Surg. Oncol.*, **22**, 197–211.
- Waddell, W.R. *et al.* (1983) Sulindac for polyposis of the colon. *J. Surg. Oncol.*, **24**, 83–87.
- Hirata, K. *et al.* (1994) Regression of rectal polyps by indomethacin suppository in familial adenomatous polyposis. Report of two cases. *Dis. Colon Rectum*, **37**, 943–946.
- Lundholm, K. *et al.* (1994) Anti-inflammatory treatment may prolong survival in undernourished patients with metastatic solid tumors. *Cancer Res.*, **54**, 5602–5606.
- Cai, J.B. *et al.* (2011) A study of ¹³¹Iodine labeling of indomethacin, its *in vivo* biological distribution in Lewis-bearing lung cancer, and its induction of apoptosis in lung cancer. *Saudi Med. J.*, **32**, 15–22.
- Kato, T. *et al.* (2011) Indomethacin induces cellular morphological change and migration via epithelial-mesenchymal transition in A549 human lung cancer cells: a novel cyclooxygenase-inhibition-independent effect. *Biochem. Pharmacol.*, **82**, 1781–1791.
- Brown, J.R. *et al.* (2004) Cyclooxygenase as a target in lung cancer. *Clin. Cancer Res.*, **10**, 4266s–4269s.
- Ackerstaff, E. *et al.* (2007) Anti-inflammatory agent indomethacin reduces invasion and alters metabolism in a human breast cancer cell line. *Neoplasia*, **9**, 222–235.
- Henry, D. *et al.* (1996) Variability in risk of gastrointestinal complications with individual non-steroidal anti-inflammatory drugs: results of a collaborative meta-analysis. *BMJ*, **312**, 1563–1566.
- Seidman, P. *et al.* (1991) Cerebral blood flow and indomethacin drug levels in subjects with and without central nervous side effects. *Br. J. Clin. Pharmacol.*, **31**, 429–432.
- Huang, L. *et al.* (2011) The novel phospho-non-steroidal anti-inflammatory drugs, OXT-328, MDC-22 and MDC-917, inhibit adjuvant-induced arthritis in rats. *Br. J. Pharmacol.*, **162**, 1521–1533.
- Huang, L. *et al.* (2011) Chemotherapeutic properties of phospho-nonsteroidal anti-inflammatory drugs, a new class of anticancer compounds. *Cancer Res.*, **71**, 7617–7627.
- Huang, L. *et al.* (2010) Phospho-sulindac (OXT-922) inhibits the growth of human colon cancer cell lines: a redox/polyamine-dependent effect. *Carcinogenesis*, **31**, 1982–1990.
- Mackenzie, G.G. *et al.* (2010) Phospho-sulindac (OXT-328), a novel sulindac derivative, is safe and effective in colon cancer prevention in mice. *Gastroenterology*, **139**, 1320–1332.

18. Sun, Y. *et al.* (2012) Phospho-ibuprofen (MDC-917) suppresses breast cancer growth: an effect controlled by the thioredoxin system. *Breast Cancer Res.*, **14**, R20.
19. Piazza, G.A. *et al.* (2009) A novel sulindac derivative that does not inhibit cyclooxygenases but potently inhibits colon tumor cell growth and induces apoptosis with antitumor activity. *Cancer Prev. Res. (Phila.)*, **2**, 572–580.
20. Xie, G. *et al.* (2011) Phospho-ibuprofen (MDC-917) is a novel agent against colon cancer: efficacy, metabolism, and pharmacokinetics in mouse models. *J. Pharmacol. Exp. Ther.*, **337**, 876–886.
21. Mackenzie, G.G. *et al.* (2011) Phospho-sulindac (OXT-328) combined with difluoromethylornithine prevents colon cancer in mice. *Cancer Prev. Res. (Phila.)*, **4**, 1052–1060.
22. Whiteley, P.E. *et al.* (2001) Models of inflammation: measuring gastrointestinal ulceration in the rat. *Curr. Protoc. Pharmacol.*, **Chapter 10**, Unit 10.2.
23. Xie, M. *et al.* (2002) Human and rodent carboxylesterases: immunorelatedness, overlapping substrate specificity, differential sensitivity to serine enzyme inhibitors, and tumor-related expression. *Drug Metab. Dispos.*, **30**, 541–547.
24. Wong, C.C. *et al.* (2012) Carboxylesterases 1 and 2 hydrolyze phospho-NSAIDs drugs: relevance to their pharmacological activity. *J. Pharmacol. Exp. Ther.*, **340**(2), 422–432.
25. Covas, M.I. *et al.* (2003) Bioavailability of tyrosol, an antioxidant phenolic compound present in wine and olive oil, in humans. *Drugs Exp. Clin. Res.*, **29**, 203–206.
26. Wong, C.C. *et al.* (2012) Preclinical predictors of anticancer drug efficacy: critical assessment with emphasis on whether nanomolar potency should be required of candidate agents. *J. Pharmacol. Exp. Ther.*, **341**, 572–578.
27. Krysan, K. *et al.* (2006) The potential and rationale for COX-2 inhibitors in lung cancer. *Anticancer Agents Med. Chem.*, **6**, 209–220.
28. Horn, L. *et al.* (2009) Targeting the eicosanoid pathway in non-small-cell lung cancer. *Expert Opin. Ther. Targets*, **13**, 675–688.
29. Shiff, S.J. *et al.* (1999) The role of cyclooxygenase inhibition in the anti-neoplastic effects of nonsteroidal antiinflammatory drugs (NSAIDs) [comment]. *J. Exp. Med.*, **190**, 445–450.
30. Warner, T.D. *et al.* (1999) Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. *Proc. Natl Acad. Sci. U.S.A.*, **96**, 7563–7568.
31. Ganchi, P.A. *et al.* (1992) I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. *Mol. Biol. Cell*, **3**, 1339–1352.

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