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Phospho-ibuprofen (MDC-917) is a novel agent against colon cancer: Efficacy, metabolism and pharmacokinetics in mouse models

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Running title: Efficacy, Metabolism and PK of phospho-ibuprofen

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ABBREVIATIONS: PI, phospho-ibuprofen; RLM, rat liver microsomes; HLM, human liver microsomes; PK, pharmacokinetcs; PD, pharmacodynamics; HPLC, high-performance liquid chromatography; CYP, Cytochrome P450; UGTs, UDP-glucuronosyltransferases; MS, mass spectrometry

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ABSTRACT

We have recently developed a novel chemical modification of conventional NSAIDs in order to reduce their toxicity and enhance their efficacy. Phospho-ibuprofen (PI; MDC-917), a novel derivative of ibuprofen strongly inhibited the growth of human colon cancer cells in vitro and of SW480 human colon cancer xenografts in nude mice. PI was metabolized minimally by cultured cells, but extensively by liver microsomes and mice, undergoing regioselective oxidation to produce 1-OH-PI and carboxy-PI, which can be hydrolyzed to 1-OH-ibuprofen and carboxy-ibuprofen, respectively. PI also can be hydrolyzed to release ibuprofen, which can generate 2-OH-ibuprofen, carboxy-ibuprofen and ibuprofen glucuronide. Following a single oral administration of PI 400 mg/kg, ibuprofen and ibuprofen glucuronide are the main plasma metabolites of PI; they have, respectively, C_{max} 530 and 215 μM, T_{max} 1 and 2 h, elimination $t_{1/2}$ 7.7 and 5.3 h, and $AUC_{0\text{-}24h}$ 1,816 and 832 μM*h. Intact PI was detected in several tissues but not in plasma; at a higher PI dose (1,200 mg/kg), PI plasma levels were 12.4 μM. PI generated the same metabolites in mouse plasma as conventional ibuprofen, but with much lower levels, perhaps accounting for the enhanced safety of PI. The antitumor effect of PI was significantly associated with plasma ibuprofen levels ($p=0.016$) but not with xenograft ibuprofen levels ($p=0.08$), suggesting a complex anticancer effect. These results provide a pharmacological basis to explain, at least in part, the anticancer efficacy and safety of this promising compound, and indicate that PI merits further evaluation as an anticancer agent.

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INTRODUCTION

Inflammation is a critical component of tumor progression, and nonsteroidal anti-inflammatory drugs (NSAIDs) are efficacious in inhibiting early neoplastic progression and malignant conversion (Coussens and Werb, 2002). For example, the NSAID ibuprofen is effective in reducing the risk of various human cancers including those of the colon (Meier *et al.*, 2002), breast (Harris *et al.*, 1996), lung (Marnett, 1995) and prostate (Harris *et al.*, 2005). Indeed, NSAIDs inhibit cancer through cyclooxygenase (COX)-dependent and COX-independent mechanisms, and their COX-independent targets include NF-κB, PPAR, lipoxygenase and apoptosis signaling (Ulrich *et al.*, 2006).

Conventional NSAIDs are associated with significant toxicity, which makes their long-term use for cancer prevention problematic (Piazza *et al.*, 2009). For example, significant side effects are seen with ibuprofen, including those of the central nervous system (McElwee *et al.*, 1990), gastrointestinal tract (Lanza, 1984), liver (Riley and Smith, 1998) and respiratory tract (Antonicelli and Tagliabracci, 1995). On the other hand, little has been accomplished over the same period of time for the treatment of advanced colon cancer, with its mortality remaining essentially unchanged for at least three decades [American Chemical Society, (2008)]. These considerations make compelling the need to develop new effective agents for the control of cancer.

Recently, we developed a novel chemical modification of conventional NSAIDs in order to reduce their toxicity and enhance their efficacy (Mackenzie *et al.*, 2010). One such modified NSAID is phospho-ibuprofen (PI), a derivative of ibuprofen, consisting of ibuprofen covalently attached to the diethylphosphate group via a spacer moiety (Fig. 1 inset). PI has strong anti-inflammatory properties both in vitro and in animal models of arthritis, while it was shown to have far better gastrointestinal safety than conventional ibuprofen (Huang *et al.*, 2010). PI inhibited cancer cell growth 16-23 times more potently than its parent drug, ibuprofen. PI also inhibited the growth of human colon cancer xenografts in nude mice, while manifesting minimal or no animal toxicity.

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Given the promise of PI, we studied its metabolism, pharmacokinetics and pharmacodynamics in mice. Here, our results establish the metabolic pathways of PI in vitro and in vivo, and provide an insight into the interplay between its metabolic transformations and pharmacological effects.

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MATERIALS AND METHODS

Reagents

PI was provided by Medicon, Inc (Stony Brook, NY). Ibuprofen and acetonitrile of HPLC grade, and porcine liver esterase were purchased from Sigma-Aldrich (St. Louis, MO). 1-OH-ibuprofen, 2-OH-ibuprofen, ibuprofen glucuronide were purchased from US Biological (Swampscott, MA). RPMI 1640 and antibiotics were obtained from Fisher-Mediatech (Herndon, VA). Human colon cancer cell lines were purchased from American Type Culture Collection (ATCC), Manassas, VA. Rat and human liver microsomes and NADPH regenerating solution were purchased from BD Biosciences, San Jose, CA.

Cell culture

Human colon cancer cells were grown as monolayers as recommended by ATTC. Cell growth was determined using the MTT assay (Promega, Madison, WI) following the manufacturer's instructions. To study PI metabolism in cultured cells, HCT-15 human colon cancer cells were seeded in 6 cm plates at a density of 10^5 cells/cm^2 in 4 ml of culture media and allowed to attach for 24 hr. The cells were treated with PI 100 µM for 6 hr, and were subsequently washed three times with cold PBS and lysed by sonication. Acetonitrile was added to extract PI and its metabolites from cells and the extracts were submitted for HPLC and LC-MS/MS analysis.

HPLC analysis

The HPLC system consists of a Waters Alliance 2695 Separations Module equipped with a Waters 2998 photodiode array detector (220 nm) and a Thermo BDS Hypersil C18 column (150 x 4.6 mm, particle size 3 µm). The mobile phase follows a gradient between buffer A {formic acid, acetonitrile, H_2O (95:4.9:0.1 v/v/v)} and buffer B (acetonitrile).

LC-MS/MS analysis

The LC-MS/MS system consists of Thermo TSQ Quantum Access (Thermo-Fisher) triple quadrupole mass spectrometer interfaced by an electrospray ionization probe with an Ultimate 3000 HPLC system

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(Dionex Corporation, Sunnyvale, CA). Chromatographic separations were achieved on a Luna C18 column (150 x 2 mm), and the mobile Phase consists of a gradient from 10% to 95% acetonitrile.

Metabolism of PI by liver microsomes

PI (120 µM) was preincubated at 37°C for 5 min with NADPH-regenerating solution (1.3 mM NADP, 3.3 mM D-glucose 6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/ml glucose-6-phosphate dehydrogenase) in 0.1 M potassium phosphate buffer (pH 7.4). Reaction was initiated by the addition of liver microsomes (protein concentration 0.5 mg/ml) and samples were maintained at 37°C for a given time. At the end of each of the incubations, 0.3-ml aliquots were mixed with 0.6 ml of CH_3CN containing 1% phosphoric acid 2 M, vortexed, and then centrifuged for 10 min at 5000 x g. The supernatants were subjected to HPLC analysis.

Hydrolysis of phospho-NSAIDs by liver esterases

To release their NSAID moieties, phospho-NSAIDs, namely PI, 1-OH-PI or carboxyl-PI, were treated with porcine liver esterase (10 mg/ml) at 37^{o}C for 30 min in 0.1 M potassium phosphate buffer (pH 7.4). Following this, 0.3-ml aliquots were mixed with 0.6 ml of CH_3CN, vortexed, and then centrifuged for 10 min at 5000 x g. The supernatants were subjected to HPLC analysis.

In vivo inhibition of colon cancer growth in xenograft mouse model

The animal study was approved by our Institutional Animal care and Use Committee. SW480 human colon cancer cells ($2x10^6$ cells suspended in 100 µl PBS) were injected s.c. in the left and right flanks of 5-6 weeks female NCr nude mice (Taconic, Hudson, NY). After the average tumor volume reached ~100 mm^3, four groups of animals (n=9/group) started receiving the following treatments intraperitoneally once daily five times a week (5 days continuously with a 2-day break) for 32 days: *group 1* received vehicle; *group 2* received 125 mg/kg/day ibuprofen; *group 3* received 225 mg/kg/day PI; and *group 4* received 400 mg/kg/day PI. The volume of each drug or vehicle dose was ~100 µl. Tumor volume was measured every 3 or 4 days as specified, and was calculated by measuring with a caliper its length (*L*) and width

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(*W*) and using the formula, *L*x*W*x(*L*+*W*/2)x0.56. At the endpoint, animals were euthanized following a standard protocol.

Pharmacokinetic (PK) studies in mice

PI or ibuprofen dissolved in corn oil was administered to female BALB/c mice as a single equimolar dose by gastric gavage. Mice were sacrificed at various time points after drug administration, and the blood was collected and immediately centrifuged. The resulting plasma was deproteinized by immediately mixing it with 2-fold volume of acetonitrile. PI and its metabolites were analyzed by HPLC as described above.

PK parameters in plasma were determined by noncompartmental PK data analysis using PK Solutions 2.0 software (Summit Research Services, Montrose, CO). The PK parameters determined include maximal plasma concentration (C_{max}), time to reach maximal plasma concentration (T_{max}), half life ($t_{1/2}$), and the area under the concentration–time curve (AUC).

Tissue distribution in mice

One hour following PI administration, various tissues of the sacrificed mice were collected, washed three times with 50% DMSO, homogenized and sonicated in PBS. PI and its metabolites were extracted by adding a two-fold volume of acetonitrile. After centrifugation for 10 min at 5000 x *g*, the supernatants were subjected to HPLC analysis.

Statistical analyses

Results, obtained from at least three independent experiments, were expressed as mean ± SEM and analyzed by one-factor analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. $P < 0.05$ was considered statistically significant.

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RESULTS

PI inhibits the growth of SW480 colon cancer cells in vitro and in vivo

To evaluate the effect of PI on cell growth, we determined the 24-hour IC_{50} values of PI and ibuprofen in HT-29, SW480 and HCT-15 human colon cancer cell lines (Fig. 1), the potency of PI is dramatically enhanced (about 16 to 23 times) compared to conventional ibuprofen.

We next evaluated the chemotherapeutic potential of PI and compared it with ibuprofen in a subcutaneous model of SW480 cell xenografts in nude mice. PI was administered intraperitoneally at two doses, 225 mg/kg and 400 mg/kg, the former being equimolar to the dose of ibuprofen (125 mg/kg). As shown in Fig. 1, both PI and ibuprofen inhibited tumor growth throughout the observation period with the efficacy being PI 400 mg/kg > PI 225 mg/kg > ibuprofen 125 mg/kg. At sacrifice, the tumor volume of each study group was as follows: control = 530±197 (mean±SEM for this and subsequent values), ibuprofen = 297±117 (no significantly different from control), PI 225mg/kg =196±29 ($p=0.05$ compared to contrl), and PI 400mg/kg =171± 42 mm^3 ($P<0.05$ compared to control). This indicates a reduction in tumor volume of 44%, 63% and 68% by ibuprofen, PI 225mg/kg and PI 400mg/kg respectively. Whereas both PI doses were more effective than ibuprofen, the differences between either one of them and the effect of ibuprofen did not reach statistical significance. Of note, these treatments produced no apparent adverse effects in mice during 32 days of administration.

The metabolism of PI by liver microsomes

Given the efficacy of PI, we explored its metabolism by rat liver microsomes (RLM), an enriched source of major metabolic enzymes (Bajrami *et al.*, 2009). These enzymes including oxidative enzymes, hydrolases and transferases, function to make xenobiotic compounds more polar and more water-soluble, thus facilitating their elimination from the body.

Three major metabolites of PI, namely ibuprofen, carboxy-PI and 1-OH-PI, were readily detected in the reaction mixture of PI and RLM (Fig. 2A). Ibuprofen was identified by comparing its HPLC retention time

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and UV spectrum to that of authentic compound; the other two metabolites were identified by LC-MS/MS analysis. The mass spectrum of carboxy-PI showed a $[M-H]^+$ ion at m/z 443.0, which was further fragmented at m/z 153, 197 and 219 (Fig. 2B). The mass spectrum of 1-OH-PI showed a $[M+Na]^+$ ion at m/z 453.0, which was further fragmented at m/z 176.8. (Fig. 2C). To determine the position of the –OH group, 1-OH-PI was treated with porcine liver esterase as described in Methods, and it was completely hydrolyzed to release 1-OH-ibuprofen, indicating that the -OH group was at the 1-position of the isobutyl group. Thus PI undergoes hydrolysis and oxidation in RLM, and its oxidation regioselectively occurs at the 1- and 3- positions of the isobutyl group (Fig. 2D). Like 1-OH-PI, PI and carboxy-PI were also hydrolyzed by the same esterase to release ibuprofen and carboxy-ibuprofen, respectively, indicating that the carboxyester bond of the phospho-NSAIDs is susceptible to hydrolysis by liver esterase. These results also support the identification of carboxy-PI and 1-OH-PI.

As shown in Fig. 3A, the level of PI in the reaction mixture continuously decreased during the entire period of observation, while, at the same time, the level of its hydrolysis product, ibuprofen, continuously increased. The levels of 1-OH-PI and carboxy PI increased in the first 30 min but slightly dropped afterwards because they can also be hydrolyzed by RLM. The level of 1-OH-PI was much lower and decreased earlier than that of carboxy-PI, suggesting that PI was oxidized preferentially at 3- position over the 1-position of its isobutyl group.

In addition, three minor metabolites, namely 1-OH-ibuprofen, 2-OH-ibuprofen and carboxy-ibuprofen, were also detected. They are derived from the oxidation of ibuprofen and the hydrolysis of hydroxyl-PI and carboxy-PI, and their levels are generally <10% of the major metabolites. These three minor metabolites shared similar kinetic behaviors in that their levels all were negligible in the first 10 min, after which they continued to increase until the end of the observation period.

Drug-metabolizing enzymes differ between humans and animals with respect to isoform composition, expression and catalytic activities (Martignoni *et al.*, 2006). In addition, testing drug metabolism by human liver microsomes (HLM) is required for the prediction of human pharmacokinetics of the drug (Liu *et al.*,

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2010). Therefore, we next explored the metabolism of PI by HLM. As was the case of RLM, three major metabolites, ibuprofen, hydroxyl-PI and carboxy-PI, as well as the three minor metabolites, 1-OH-ibuprofen, 2-OH-ibuprofen and carboxy-ibuprofen, were also detected in the reaction mixture of PI and HLM; this finding suggested that the two species share the same metabolic pathways in the metabolism of PI by liver microsomes.

The level of PI decreased rapidly and was cleared by 82% within the first 30 min of its reaction with HLM (Fig. 3B). Consistently, the levels of 1-OH-PI and carboxy-PI increased rapidly during the initial reaction, suggesting that in the beginning, the formation of these two metabolites was much faster than their hydrolysis due to the initial high concentration of PI. With the PI level decreasing rapidly, the formation rate of 1-OH-PI and carboxy-PI decreased and their consumption became dominant, resulting in the steady decrease of their levels during the later phase of the reaction. As was the case with RLM, the level of 1-OH-PI was much lower and decreased earlier than that of carboxy-PI.

The level of Ibuprofen increased initially and reached a plateau at 30 min, suggesting equilibrium between its formation from PI and oxidation to other metabolites. Similar to the case with RLM, all of the three minor metabolites, namely 1-OH-ibuprofen, 2-OH-ibuprofen and carboxy-ibuprofen, continued to increase during the entire period of observation.

Even though PI followed the same metabolic pathway in RLM and HLM, the kinetics of metabolite formation in the two species was distinct. PI appears to be more stable in RLM than in HLM. The $t_{1/2}$ of PI in RLM was 4.5 times longer than that in HLM (28.5 min *vs.* 6.4 min). PI was hydrolyzed more rapidly in HLM than RLM, as the rate constant of the formation of ibuprofen was 3.8 times higher in HLM that in RLM (0.088 min^{-1} *vs.* 0.023 min^{-1}). Thus, PI was metabolized more extensively and rapidly in HLM than in RLM. Because of the slow clearance of PI in RLM, 1-OH-PI and carboxy-PI were formed more rapidly in RLM than in HLM, and the observed highest concentration of 1-OH-PI and carboxy-PI in RLM was 2.9 and 2.0 times higher than those in HLM, respectively.

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The metabolism of PI by cultured cancer cells

Given its potency in inhibiting cancer cell growth, we also explored the metabolism of PI by cultured HCT-15 human colon cancer cells. Cell lysates were prepared as described in Methods, and were analyzed using HPLC and mass spectrometry (MS) analysis. HPLC analysis using UV detection showed that PI primarily remained intact 6 h after treatment (Fig.4 inset). Consistent with this result, LC-MS analysis showed that the dominant ion of the highest intensity was at m/z 415, which further generated fragment ions at m/z 155, 161, and 261 (Fig. 4). This spectrum was identical to that of authentic PI, confirming that the majority of PI taken up by the cells stays intact. This result also confirmed the chemical structure of PI.

Multiple minor metabolites of PI were detected and identified in cultured cells using LC-MS/MS analysis. The mass spectrum of the hydroxy-PI showed a $[M+H]^+$ ion at m/z 431, which was further fragmented at m/z 155, 177 and 277 (Fig. 5A). Butanediol phosphate, the carboxyester hydrolysis product of PI, gave an $[M+H]^+$ ion at m/z 227 in the MS spectrum, which further generated fragment ions at 99,127 and 155 (Fig. 5B). Desethyl-PI, the phosphate ester hydrolysis product of PI, showed an $[M+H]^+$ ion at m/z 387, which was further fragmented to 161 and 261 (Fig. 5C). Thus PI undergoes hydrolysis and oxidation transformations in cultured cells (Fig. 5D), which are generally similar to its metabolism by liver microsomes.

We assessed the pharmacological activity of the various metabolites of PI in cultured cells. As shown in Table 1, each of the three structural components of PI (plain ibuprofen, spacer and diethyl phosphate) tested individually for its cell growth inhibitory effect was essentially inactive compared to PI. The same applied to three additional metabolites of PI, 2-OH-Ibuprofen, carboxy-Ibuprofen and ibuprofen glucuronide, all of which had IC_{50} values in the mM range. These results suggested that the limited metabolism of PI by cells may be important for its growth inhibitory effect.

Pharmacokinetics of PI in the mouse

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We studied the PK properties of PI following its single oral administration to mice. The two major metabolites in plasma were ibuprofen and ibuprofen glucuronide. As shown in Fig 6A, the plasma levels of ibuprofen increased rapidly and reached the maximum concentration of 530 μM 1 h after oral gavage, suggesting that PI was rapidly absorbed, metabolized, and distributed to the blood. The level of ibuprofen decreased substantially 4 h after drug administration and became negligible at 8 h. The plasma levels of ibuprofen glucuronide followed a similar pattern, but the peak value was much smaller.

As was the case with PI metabolism by liver microsomes, the three minor metabolites, namely 1-OH-ibuprofen, 2-OH-ibuprofen and carboxy-ibuprofen, were detected in mouse plasma following PI administration. These three metabolites shared similar kinetic behavior because they are derived from ibuprofen. The levels of these metabolites reached their peak values at 2 h after PI administration, but decreased dramatically afterwards. The T_{max} of each of these metabolites is 1 h later than that of ibuprofen (Table 2), which is reasonable because they are derived from ibuprofen.

When PI was administered at the dose of 400 mg/kg, intact PI was detected in mouse plasma by the MS detector (data not shown), but undetectable by the UV detector, suggesting high carboxyesterase activity in mouse plasma, as has been shown previously (Li *et al.*, 2005). However, PI at the dose of 1,200 mg/kg generated a significant plasma level of intact PI (12.4 μM). These results indicated that PI can indeed survive in the blood.

For purposes of comparison, we performed side-by-side PK studies of conventional ibuprofen and PI administered at equimolar doses. As shown in Fig. 6B, ibuprofen was metabolized to produce ibuprofen glucuronide, 1-OH-ibuprofen, 2-OH-ibuprofen and carboxy-ibuprofen in mouse plasma; this metabolic profile was the same as that following PI administration. The plasma levels of Ibuprofen and its metabolites increased rapidly and reached their peak values within ~0.5 h after ibuprofen administration (Fig. 6B), indicating that ibuprofen was rapidly absorbed, metabolized, and distributed to the blood. Compared to the plasma levels of 2-OH-ibuprofen and carboxy-ibuprofen, those of 1-OH-ibuprofen were negligible (Fig 6B).

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Compared to PI, ibuprofen generated higher levels of metabolites with the $AUC_{0\text{-}24h}$ of "total metabolites" (the sum of the five metabolites) being 2.2-fold higher (Table 2); this likely reflects the higher hydrophobicity of PI and hence lower solubility in plasma. The T_{max} of each metabolite following ibuprofen administration is much shorter than those following PI administration (Table 2). This finding suggests that the absorption and distribution of PI are slower than those of ibuprofen, also likely due to its higher hydrophobicity.

Biodistribution of PI in mouse model

We also measured the levels of PI and its metabolites in liver, heart, kidney and gastrointestinal tissues of mice following its oral administration (Table 3). Significant levels of intact PI were detected in all the tissues examined, confirming that PI can partially survive in mouse plasma, and therefore be distributed to other tissues. High levels of intact PI were detected in gastrointestinal tissues, whereas its liver concentration was the lowest, likely due to the high metabolic capacity of this organ.

As expected, the five metabolites detected in mouse plasma (ibuprofen, ibuprofen glucuronide, 1-OH-Ibuprofen, 2-OH-ibuprofen and carboxy-ibuprofen) were also detected in these tissues. High levels of ibuprofen glucuronide were detected in the liver and kidney, suggesting that most of the glucuronidation of ibuprofen occurs in these organs (Rudy *et al.*, 1991). Similar to PI metabolism by liver microsomes, the level of 1-OH-PI in liver tissue was much lower than that of carboxy-PI.

Blood and xenograft levels of PI metabolites in mice: Correlations with tumor size

To gain an insight into the interplay between its metabolic transformations and pharmacological effects, we measured the levels of PI metabolites in mouse blood and tumor tissue from the efficacy study. The major metabolite detected was ibuprofen, and there is a statistically significant association between the tumor volume and ibuprofen level in plasma (P=0.02), underscoring the relative importance of the metabolites of PI in cancer control. However, the association between the anticancer efficacy and tumor

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levels of ibuprofen is statistically significant only for the trend (P=0.08), suggesting that metabolite-independent mechanisms are also involved in inhibiting cancer growth.

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DISCUSSION

Our work demonstrated the preclinical anticancer efficacy of PI, a novel derivative of ibuprofen, established its metabolism in vitro and in vivo, defined its PK in mice and explored its PD in tumor xenografts. Collectively, these findings provide strong support for the anticancer potential of this agent and set the stage for its further pharmacological evaluation.

PI inhibited the growth of colon cancer cell lines much more potently than ibuprofen and also inhibited the growth of xenografts of one of these cell lines in nude mice. These effects are quantitatively significant and, considering the efficacy of PI on pancreatic and breast cancer from our unpublished data, constituted a firm justification to study its metabolism and PK/PD. Compared to ibuprofen, the two PI doses inhibited tumor growth about 1.5 times more effectively. The differences in efficacy between the two are statistically not significant; such lack of significance is probably due to lack of power. Nonetheless we deem these differences as clinically relevant, especially when the greater safety of PI is factored in [current data and (Huang *et al.*, 2011)].

As summarized in the metabolic diagram of Fig. 8, PI undergoes extensive biotransformations leading to at least 9 products. Three distinct pathways of PI metabolism are readily recognizable: a) *oxidation at the 1- position of the isobutyl group* to form hydroxyl-PI, which can be hydrolyzed to form 1-OH-ibuprofen; b) *oxidation at the 3- position of the isobutyl group* to form carboxyl-PI, likely through 3-OH-PI; and c) *carboxy-ester hydrolysis* to form ibuprofen, which, in turn, can follow three metabolic routes: i) conjugation with glucuronic acid to form ibuprofen glucuronide; or ii) oxidation to form 2-OH-ibuprofen; or iii) oxidation at the 3- position of the isobutyl group to form carboxyl-ibuprofen, likely through 3-OH-ibuprofen. In general, hydrolysis, oxidation and glucuronidation of drugs represent mechanisms employed by an organism to inactivate and/or eliminate them (Ulrich *et al.*, 2006). Given that all PI metabolites are much weaker than PI at inhibiting cancer cell growth and their aqueous solubility is enhanced (evidenced by their retention times on reverse-phase HPLC), it appears that PI follows the general fate of compounds that are inactivated and eliminated by the mice.

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The metabolic fate of PI is largely context dependent. Cultured colon cancer cells preserve PI essentially intact, with only a minor fraction (<10%) metabolized to mostly biologically inactive metabolites. Of these, the most active metabolite of PI is ibuprofen, which is 22-fold less potent than PI in inhibiting cell growth; all others are >67-fold less potent than PI. Liver microsomes generate a different profile of metabolites; at 90 min the four most abundant metabolites are (in descending order) ibuprofen, carboxy-PI, PI, and 1-OH-PI. Human and rat liver microsomes generate the same metabolic profile, although the ratio between the four most abundant metabolites is different between the two. Finally, a markedly different metabolite profile is obtained in mice. AUC quantification shows that ibuprofen and ibuprofen glucuronide make up 90% of the total plasma metabolites, while PI is conspicuous by its absence.

Both liver and mice microsomes oxidized PI at the 1- and 3- positions of its isobutyl group to form 1-OH-PI and 3-OH-PI, respectively, with the latter further oxidized to carboxy-PI. In contrast, ibuprofen, generated as a metabolite of PI, was oxidized at the 2- and 3- positions of its isobutyl group, generating 2-OH-ibuprofen and 3-OH-ibuprofen, the latter leading to carboxy-ibuprofen. The metabolism of ibuprofen generated from PI is identical to that of conventional ibuprofen (Hamman et al., 1997; Chang et al., 2008). The differential regioselectivity of the oxidation of ibuprofen and PI is reflected in our finding that PI generated 15.6 times more 1-OH-ibuprofen in murine plasma than ibuprofen (2.5% *vs.* 0.16%).

The CYP superfamily, and particularly CYP2C9, plays a major role in the regioselective oxidation of ibuprofen (Hamman et al., 1997; Chang et al., 2008). The molecular basis of regioselectivity of CYP2C9 has been examined in the case of the anticoagulant warfarin using molecular dynamics simulations (Seifert *et al.*, 2006). Warfarin binds to the active site of CYP and is preferentially oxidized at its C-H bond close to the active oxygen of the heme moiety of CYP (Seifert *et al.*, 2006). By analogy, we propose that the phosphate moiety of PI may affect its binding to CYP, resulting in geometric preference of position 1 over position 2 for its oxidation by CYP.

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Glucuronidation reactions are an important part of the detoxification process (Zheng *et al.*, 2002). The only glucuronide derivative that we detected was ibuprofen glucuronide. Such a transformation is well-known for conventional ibuprofen, whose carboxylic acid group is conjugated with the hydroxyl group of UDP-glucuronic acid; this reaction is catalyzed by UDP-glucuronosyltransferases (UGTs), especially UGT2B7 and UGT1A9 (Kepp *et al.*, 1997; Kuehl *et al.*, 2005). Glucuronidation of PI is unlikely to occur due to the lack of a free carboxylic acid group. Of note, ibuprofen glucuronide can undergo spontaneous chemical reactions, resulting in acyl migration and irreversible binding to endogenous proteins, which can have toxicological consequences (Johnson *et al.*, 2007)

HLM and RLM metabolize PI in much the same manner, generating an identical array of metabolites. The principal differences between them concern the rate and extent of each metabolic step, with HLM metabolizing PI faster and generating more ibuprofen and less carboxy-PI than RLM. The faster metabolism of PI by HLM (evidenced by the much faster formation of ibuprofen by HLM) is likely due to differences in esterase activity between HLM and RLM. Differential metabolic kinetics in RLM and HLM has been reported for other drugs. For example, p-nitrophenylacetate was hydrolyzed more rapidly by HLM than RLM, whereas the opposite was observed for isocarboxazid (Hosokawa *et al.*, 1990; Imai *et al.*, 2003). Taken together, these findings indicate significant differences in esterase activity between animals and humans (Kremers *et al.*, 1981).

We determined the basic PK parameters of PI in mice and also assessed its biodistribution. PI is rapidly absorbed, metabolized and distributed to the blood and other tissues. PI, administered at the dose of 400 mg/kg, was not detected in plasma, whereas the C_{max} of its main metabolites was in the high µM range**.** All PI metabolites reached T_{max} within 2 h and their $t_{1/2}$ was relatively short (<8h), indicating an efficient elimination process; the high degree of glucuronidation likely accounts to a large extent for this rapid elimination. Even though PI was not detectable in plasma, remarkably, it was found in all other tissues that we evaluated, albeit at low concentrations; its liver concentration was the lowest, likely reflecting the high metabolic capacity of this organ. The simplest interpretation of this apparent paradox is that PI partially and briefly escapes blood esterases and that once taken up by other tissues its degradation

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becomes much slower. This is supported by the finding that PI at a 3-fold higher dose generated significant plasma levels of PI (12.4 μM). These results indicated that PI can indeed survive in the blood.

As a corollary, it is conceivable that protection of PI from plasma esterases would lead to higher levels of intact PI in target tissues and higher anticancer efficacy, since PI is much more potent against cancer cells than any of its metabolites, including ibuprofen. Nonspecific carboxylic ester hydrolases, the suspected culprit in the hydrolysis of PI in plasma, are grouped into carboxyesterase, arylesterase and acetylesterase, based on their substrate specificity (Xie *et al.*, 2008); carboxyesterase can efficiently catalyze the hydrolysis of carboxyester drugs to release free acids (Imai *et al.*, 2003). It is of great interest that in human plasma carboxyesterase activity is either undetectable or very low, compared to mice and rats (Li *et al.*, 2005). Thus, it is reasonable to predict that in humans, who have very low plasma carboxyesterase activity, the plasma and tissue levels of PI will be high. If this proves to be the case, PI should have a stronger anticancer efficacy in humans. In agreement with this notion, irinotecan was reported to undergo much less hydrolysis by carboxyesterase in human plasma than in mouse plasma (Mathijssen *et al.*, 2002).

The side-by-side comparison of PI to conventional ibuprofen in which these compounds were administered at equimolar doses, corresponding to the lower dose of PI was of interest: a) both compounds generated the same metabolites in plasma; b) the relative abundance of the two major metabolites of PI, ibuprofen and ibuprofen glucuronide, was similar between the two (89.4% vs. 86.1%) ; and c) of the quantitatively minor metabolites, the most striking difference concerned 1-OH ibuprofen and to a lesser extent carboxy-ibuprofen; PI generated 15.6-fold more 1-OH ibuprofen and 3-fold less carboxy-ibuprofen than ibuprofen. As discussed above, the difference in 1-OH ibuprofen was expected. Perhaps the mechanistically most interesting observation was that PI, even though equimolar to ibuprofen, generated lower plasma $AUC_{0\text{-}24h}$ of ibuprofen (2.3-fold) and less "total metabolites" (2.2-fold)**.** Characteristically, the C_{max} value of ibuprofen derived from PI is about half that of ibuprofen derived from conventional ibuprofen administered at the equimolar dose. The explanation of these differences is not

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readily apparent. However, it is conceivable that these differences account, at least partially, for the greater safety of PI compared to ibuprofen.

Pharmacodynamically, there was a statistically significant association between plasma levels of ibuprofen and the antitumor effect of PI, determined as the volume of the xenograft. The association between ibuprofen levels in the xenografts and the antitumor effect of PI, however, did not reach statistical significance ($p=0.08$). Whether this is due to our small sample size or to an effect by additional PI metabolites and/or effects triggered by intact PI is yet to be determined.

In conclusion, our work established the metabolic pathway of PI in vitro and in vivo, defined its PK and biodistribution in mice, and explored its PD in tumor xenografts. These results provide a pharmacological basis to explain the anticancer efficacy and safety of this promising compound, suggest areas of potential improvements in terms of drug formulation and administration, and set the stage for its further evaluation.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Xie, Sun and Rigas

Conducted experiments: Xie, Sun, Nie, Mackenzie and Huang

Contributed new agents: Komninou and Kopelovich

Performed data analysis: Xie, Sun, Nie and Rigas

Wrote the manuscript: Xie, Komninou, Kopelovich and Rigas

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Effect of phospho-ibuprofen on colon cancer cell growth in vitro and in vivo. *Top*: IC_{50} values for colon cancer cells treated with PI or ibuprofen for 24 h. ***Bottom:*** PI inhibits the growth of SW480 human colon cancer xenografts in nude mice. The study was performed as described in Methods. The doses of ibuprofen 125 mg/kg and PI 225 mg/kg are equimolar. The structure of PI is also shown; the shaded part of the molecule corresponds to the conventional ibuprofen. Values are *mean*±*SEM*, *p≤0.05 compared to the corresponding control value.

Fig. 2. Identification of the metabolites of PI by RLM. A: HPLC chromatogram of an extract of RLM incubated with PI for 30 min; PI and two of its metabolites are identified. PI and its metabolites were extracted and fractionated by HPLC as described in Methods. **B:** MS and MS/MS spectrum of hydroxyl-PI fraction collected from HPLC. **C:** MS and MS/MS spectrum of carboxyl-PI fraction collected from HPLC. **D:** Metabolic transformations of PI by liver microsomes.

Fig. 3. Kinetics of PI and its metabolites by rat and human liver microsomes. PI was incubated with rat **(A)** or human **(B)** liver microsomes at 37^{o}C for up to 90 min. PI and its metabolites were extracted at the designated time points and assayed as described in Methods.

Fig. 4. PI metabolism by cultured colon cancer cells. PI and its metabolites were extracted from the PI-treated HCT-15 cell lysates and analyzed using HPLC and LC/MS/MS as described in Methods. ***Top:*** MS spectrum of PI obtained from LC separation. *Inset:* HPLC chromatogram of extract from HCT-15 cells treated with PI, only PI was detected by UV detector. ***Bottom:*** MS/MS spectrum of fragment ions of the PI.

Fig. 5. Identification of the metabolites from PI-treated cancer cells. The metabolites of PI were extracted from the PI-treated HCT-15 cells and analyzed using LC/MS/MS as described in Methods. **A.**

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MS/MS spectrum of fragment ions of hydroxyl-PI. **B.** MS/MS spectrum of fragment ions of butanediol phosphate. **C.** MS/MS spectrum of fragment ions of desmethyl-PI. **D.** Proposed metabolic transformations of PI by liver microsomes.

Fig. 6. Comparison of pharmacokinetics of PI and ibuprofen in mice. Equimolar doses (1 mmol/kg) of PI **(A)** and ibuprofen **(B)** dissolved in corn oil were administered to mice as a single dose by gastric gavage. Plasma levels of PI metabolites at the indicated time points were determined as described in Methods.

Fig. 7. Correlation of PI metabolite levels with tumor volume. Mice with SW480 colon cancer xenografts were sacrificed 1 hr after the last dosing, and Ibuprofen levels in mouse blood and tumors were determined as described in Methods. A significant correlation (P=0.016) between plasma levels of ibuprofen and tumor volume ***(Top)***, and a trend between tumor levels of ibuprofen and tumor volume ***(Bottom)*** were observed.

Fig. 8. PI metabolism in vitro and in vivo. PI can be metabolized through three distinct pathways: a) oxidation to form hydroxyl-PI; b) oxidation to form carboxyl-PI, or c) hydrolysis to form ibuprofen, which in turn, can be metabolized through: i) glucuronidation to form IBPG; or ii) oxidation to form 2-OH-ibuprofen; or iii) oxidation to form carboxyl-ibuprofen.

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TABLES

Table 1. IC_{50} values of Phospho-ibuprofen and its components/metabolites in cultured HT-29 cancer cells

Compound	IC_{50}, μM
Phospho-ibuprofen	67
Ibuprofen	1,516
1,4-butanediol (spacer)	>10,000
diethyl phosphate	10,229
2-OH-Ibuprofen	>4,500
Carboxy-Ibuprofen	>4,500
Ibuprofen glucuronide	>4,500

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Table 2. Pharmacokinetic parameters of phospho-ibuprofen (400 mg/kg) and ibuprofen (200 mg/kg) in mice*

Drug administered	Measurement	C_{max} μM	T_{max} hr	Elimination $t_{1/2}$ hr	AUC_{0-24h} μM*hr	Sum of AUC_{0-24h} (μM*hr)
Phospho-ibuprofen	Ibuprofen	530	1.0	7.7	1,816	2,963
	1-OH ibuprofen	28	2.0	ND**	74	
	2-OH ibuprofen	77	2.0	ND	193	
	Carboxy-ibuprofen	16	2.0	ND	48	
	Ibuprofen glucuronide	215	2.0	5.3	832	
Ibuprofen	Ibuprofen	932	0.5	12.8	4,146	6,437
	1-OH ibuprofen	5	0.5	ND	11	
	2-OH ibuprofen	83	2.0	ND	572	
	Carboxy-ibuprofen	81	0.5	10.0	311	
	Ibuprofen glucuronide	274	0.5	6.3	1,398	

**The doses of PI and ibuprofen are equimolar*

***ND: Not Determined*

Experiments were done independently three times; values were generally within 15%

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Table 3. PI metabolite levels in mouse tissues 1 hr after oral administration of PI 400 mg/kg

	PI	1-OH-PI	Carboxy -PI	Ibuprofen	1-OH-ibuprofen	2-OH-ibuprofen	Carboxy-ibuprofen	Ibuprofen glucuronide
				pmol/mg protein				
Liver	82	22	1,013	739	19	33	13	408
Heart	101	*ND	ND	2,409	34	96	ND	55
Kidney	121	ND	ND	1,946	75	220	104	322
Stomach	421	ND	ND	462	ND	ND	ND	ND
Small intestine	521	ND	36	913	ND	ND	ND	50
Colon	414	ND	20	431	ND	ND	ND	64
Blood	ND	ND	ND	530	24	60	10	121

** ND: Not Detected*

***Metabolite levels in the blood were expressed as µM in plasma*

Experiments were done independently three times; values were generally within 15%

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Fig. 1

	IC_{50}, μM		
Cell line	**Phospho-ibuprofen**	**Ibuprofen**	**Potency enhancement**
HT-29	67.4	1,516	**23**
SW480	59.1	1,057	**18**
HCT-15	81.6	1,305	**16**

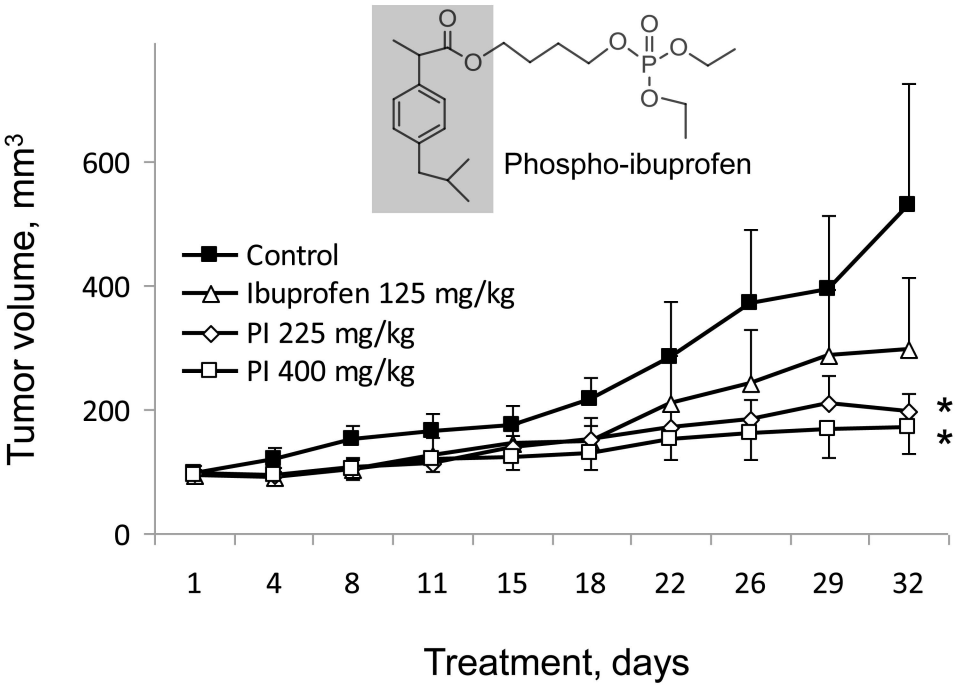

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Fig. 2

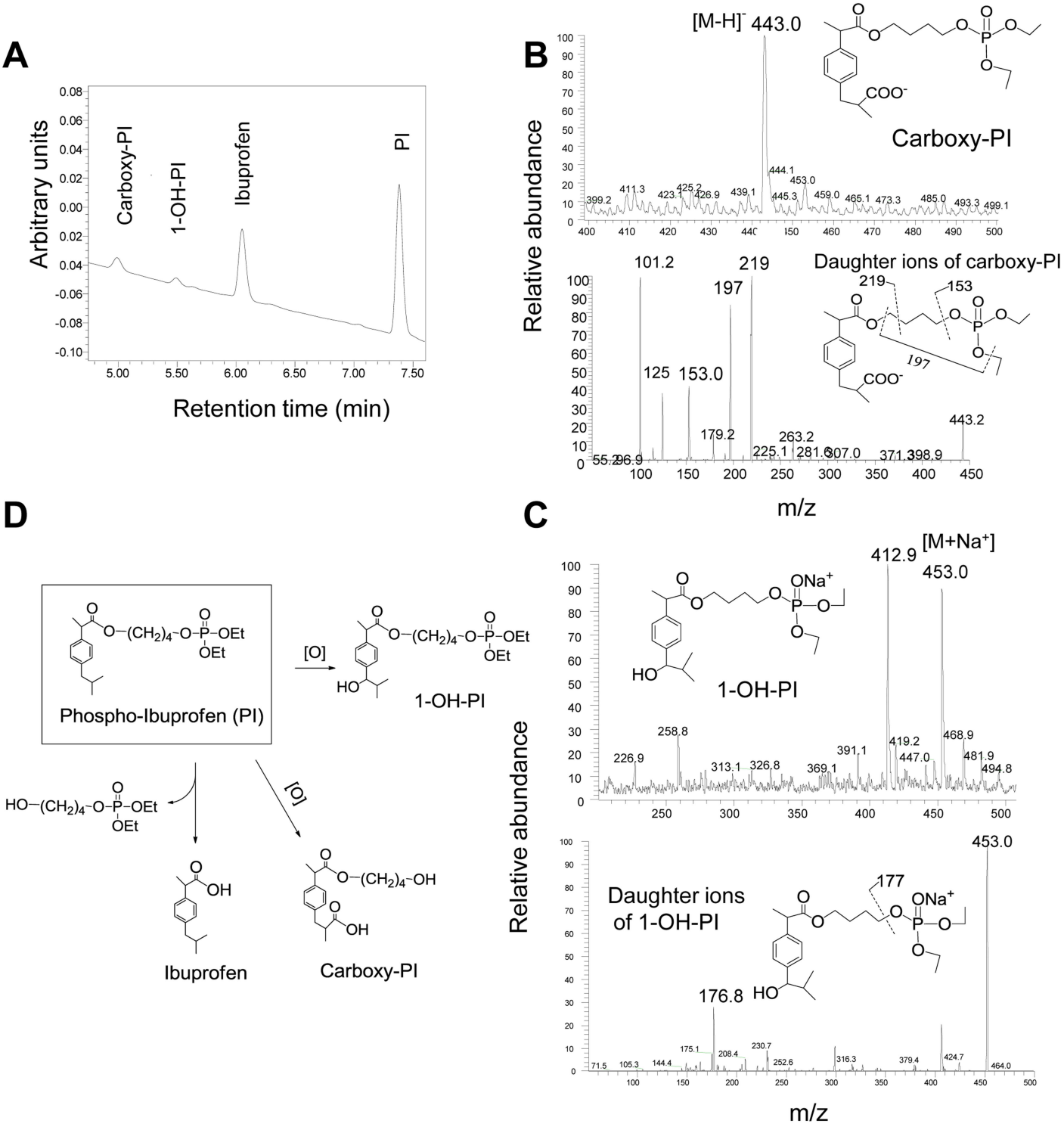

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Fig. 3

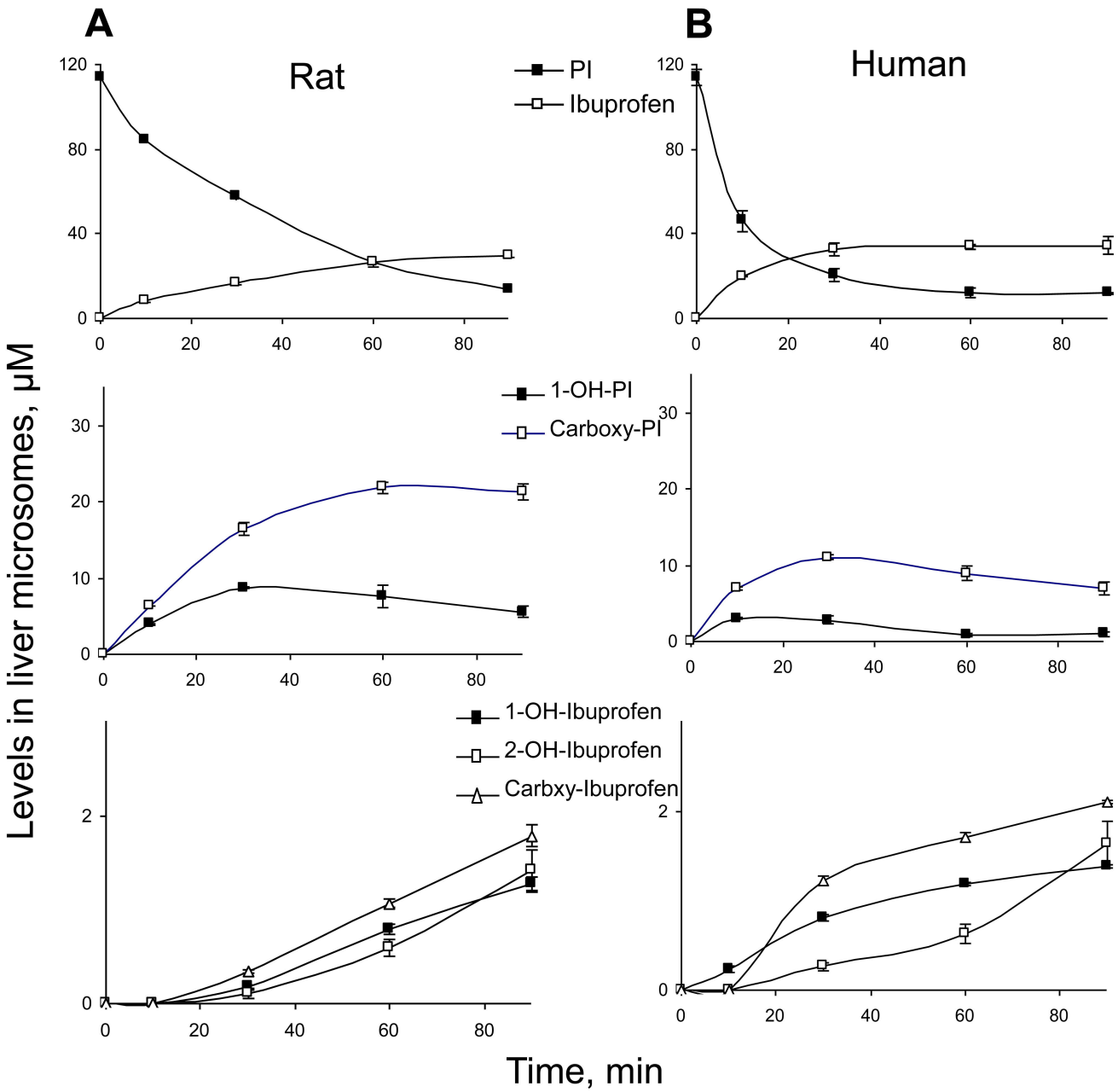

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Fig. 4

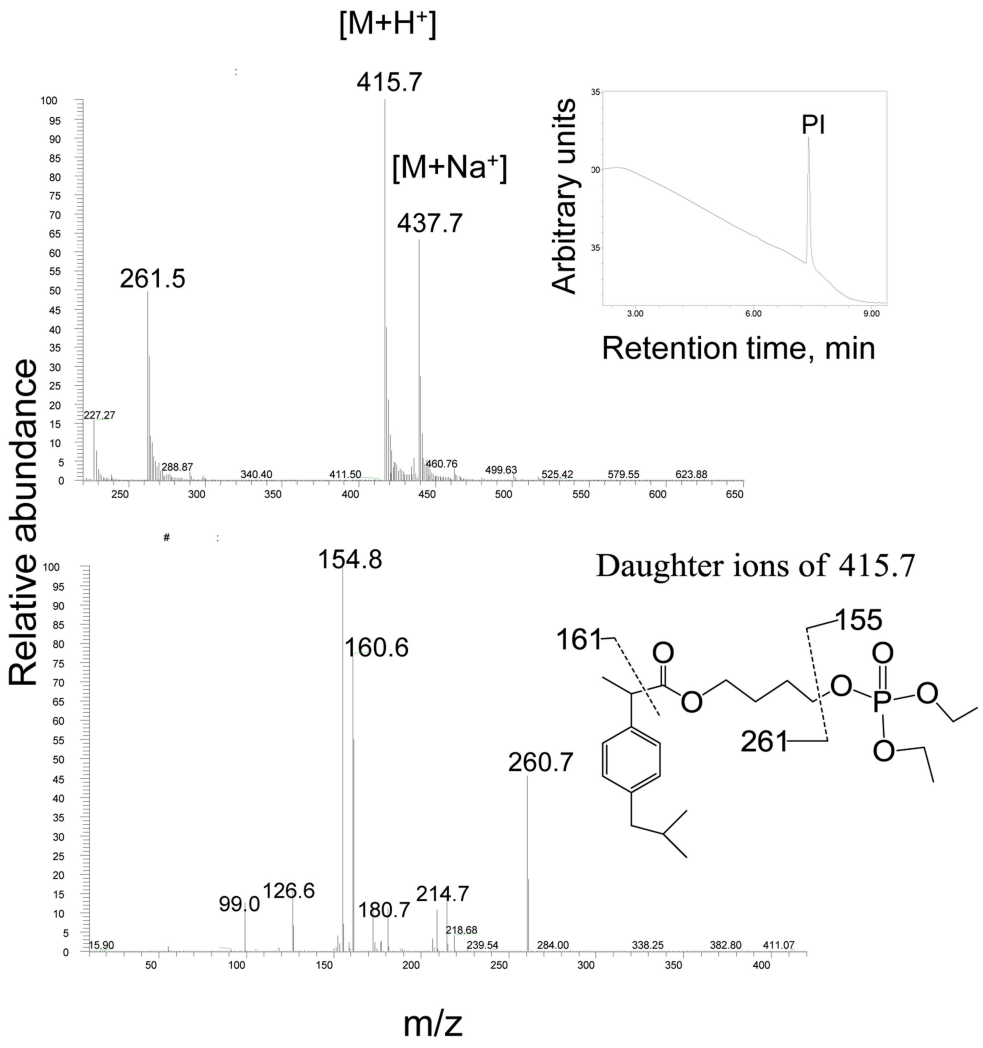

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Fig. 5

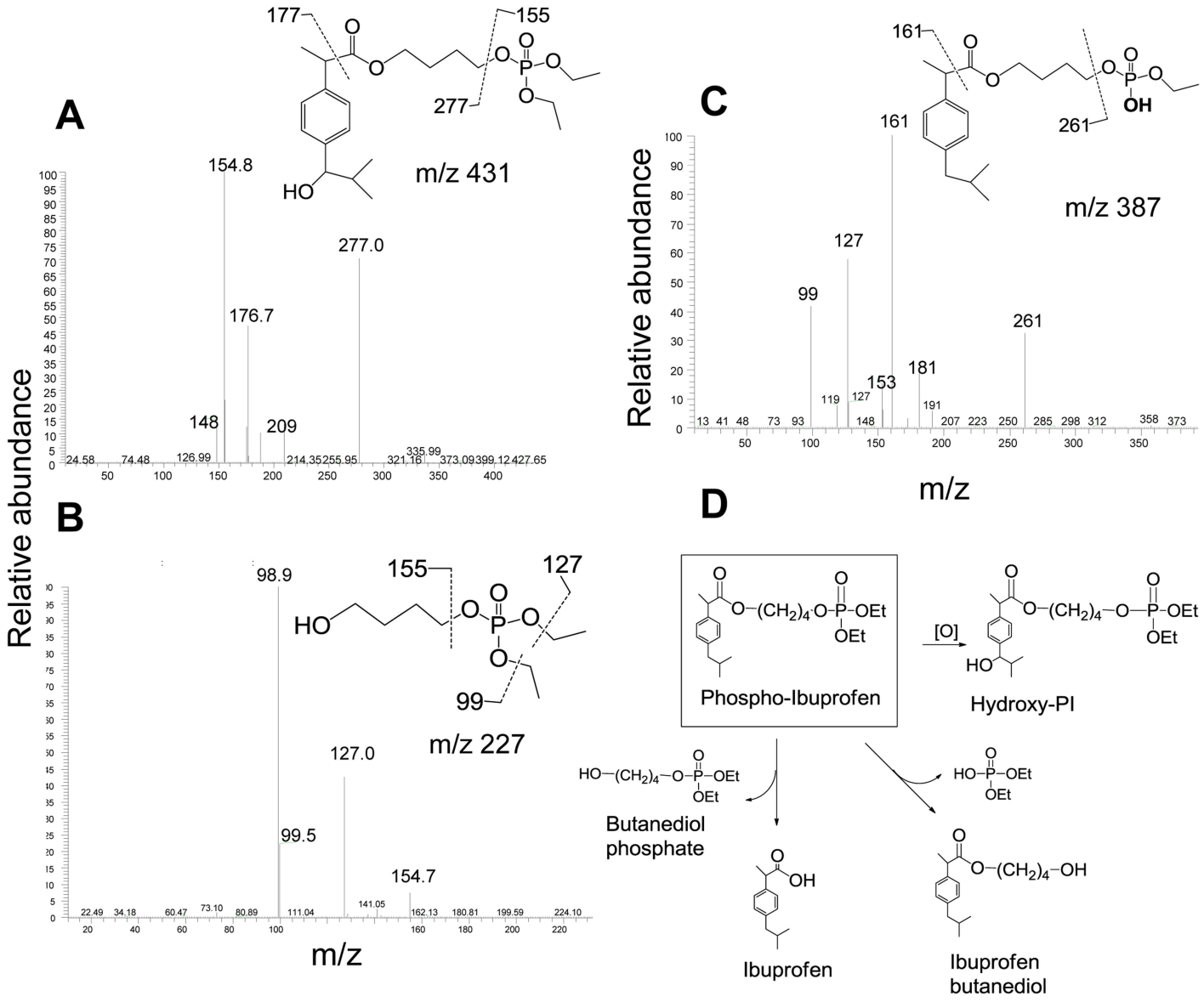

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Fig. 6

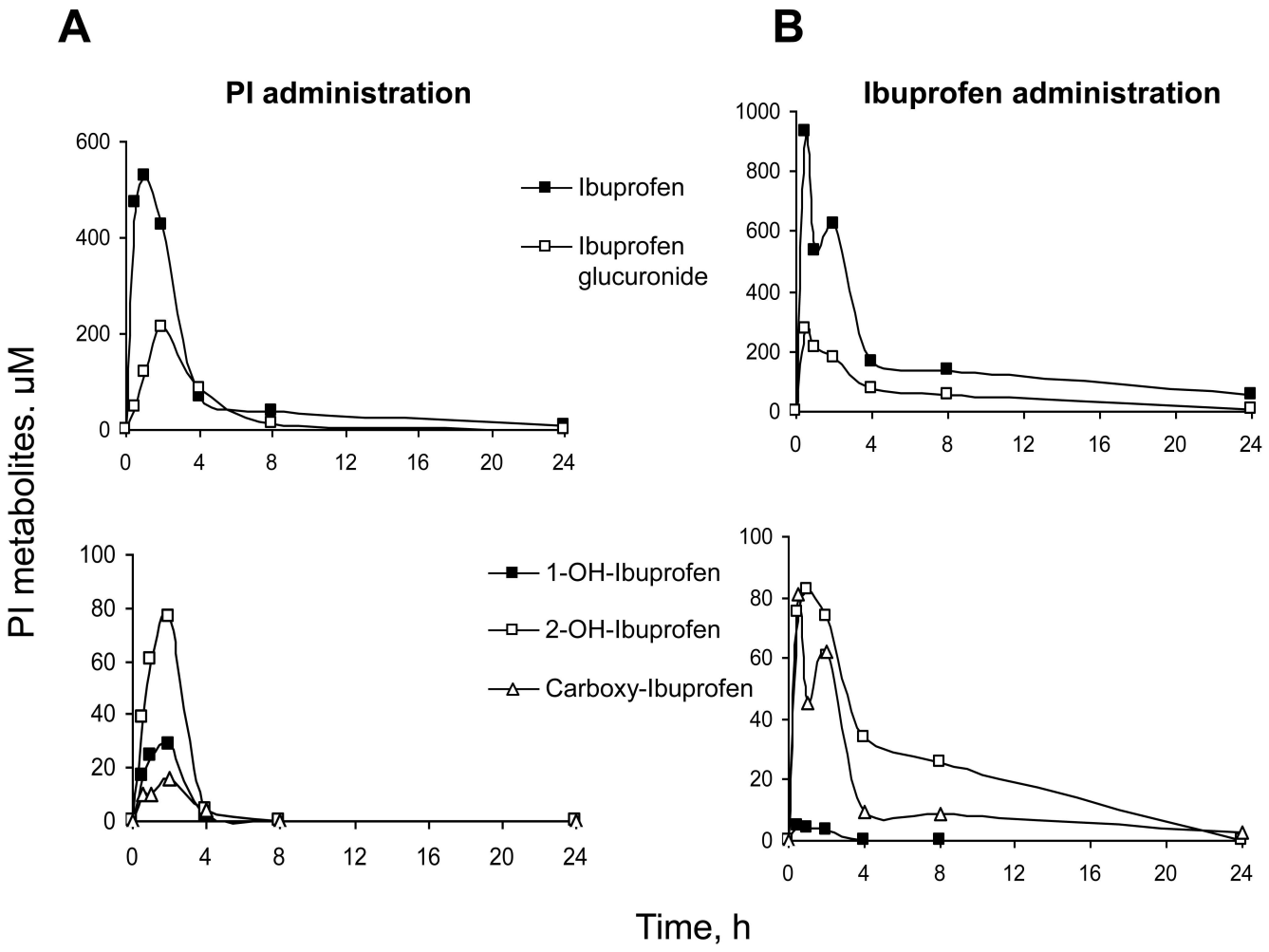

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Fig. 7

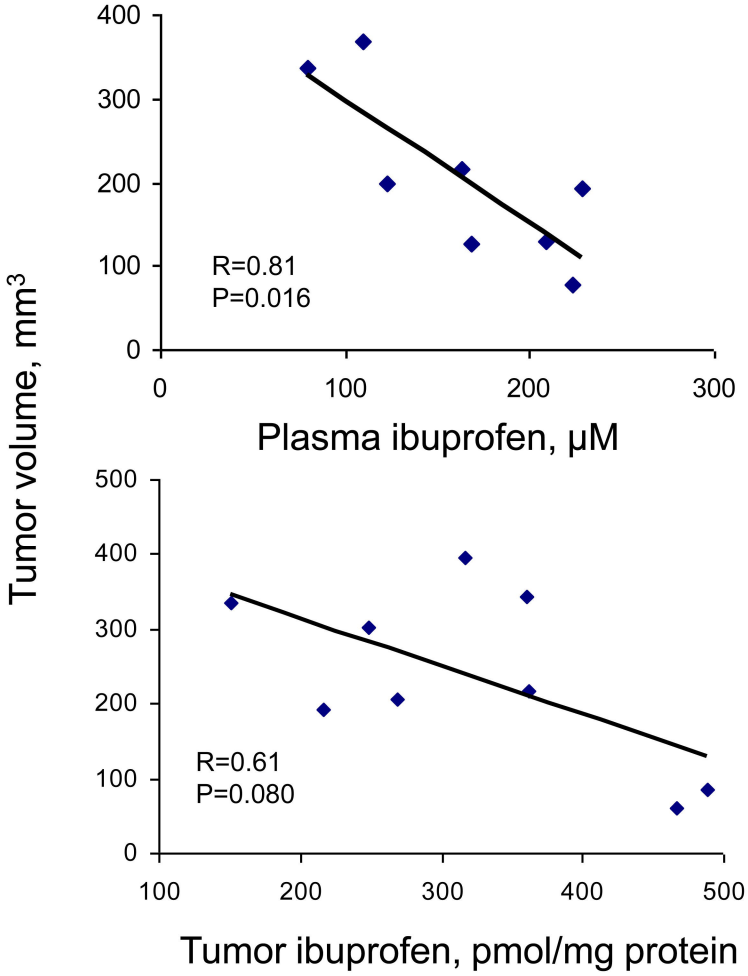

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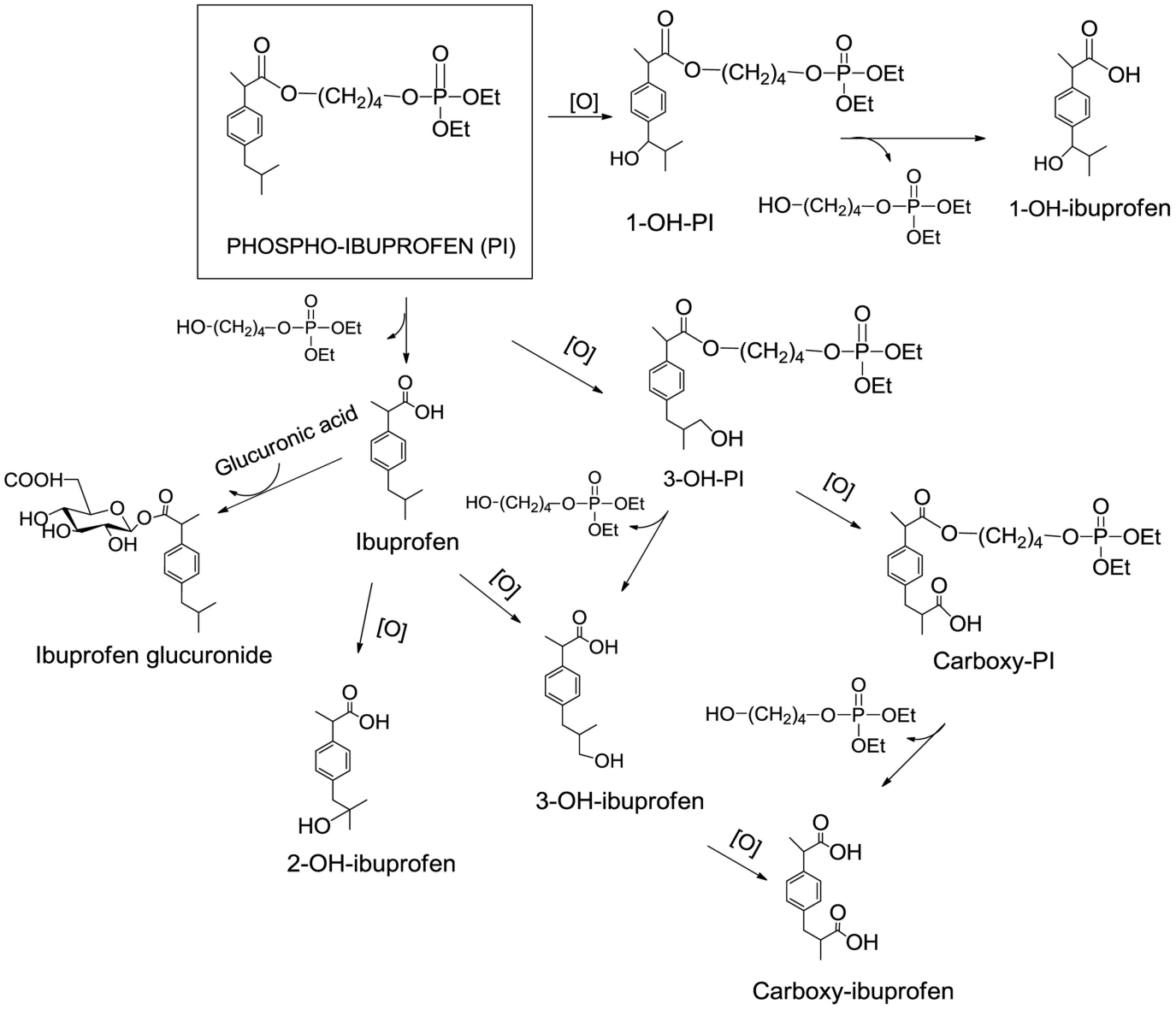
Fig. 8



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