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RESEARCH PAPER

Phospho-ibuprofen (MDC-917) incorporated in nanocarriers: anti-cancer activity *in vitro* and *in vivo*

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BACKGROUND AND PURPOSE

Phospho-ibuprofen (P-I; MDC-917) inhibits the growth of colon cancer in mice. Here, we investigated the use of nanocarriers to improve its pharmacokinetics (PKs) and anti tumour efficacy.

EXPERIMENTAL APPROACH

The cellular uptake and cytotoxicity of P-I encapsulated into liposomes and micelles, and its *in vitro* metabolic stability, were determined in cultures of human colon adenocarcinoma cells. The performance of liposomal P-I was further evaluated in PK studies in mice, and in a model of colon cancer xenografts in nude mice.

KEY RESULTS

Liposomal P-I and micellar P-I showed significantly enhanced cellular uptake in the colon cancer cells. Liposomal P-I also demonstrated increased cytotoxicity *in vitro*. Free P-I was metabolized rapidly to ibuprofen in the presence of purified esterases. In contrast, liposomal P-I, and to a lesser extent micellar P-I, was resistant to esterase-mediated hydrolysis. In mice, liposomal P-I partially protected P-I from hydrolysis in the circulation, and improved the biodistribution of intact P-I and its metabolites compared to free P-I. Liposomal P-I was more effective at inhibiting the growth of human colon cancer xenografts in mice, which may be explained on the basis of its improved PK profile compared to free P-I.

CONCLUSIONS AND IMPLICATIONS

Liposome encapsulation of P-I partially protected P-I from esterase-mediated hydrolysis in mice, enhanced the cytotoxicity and bioavailability of P-I and increased its efficacy at inhibiting the growth of human colon cancer xenografts. These results indicate that liposomes are suitable nanocarriers for the delivery of P-I, and that the anti-tumour potential of liposomal P-I merits further evaluation.

Abbreviations

DSPE-PEG, methoxy-poly(ethylene glycol)2000-distearoyl phosphatidylethanolamine; NSAID, non-steroidal anti-inflammatory drug; PEG, polyethylene glycol; PEO-*b*-PLA, poly(ethylene oxide)-*b*-poly(lactic acid); P-I, phospho-ibuprofen; soy-PC, soy-phosphatidylcholine; TEM, transmission electron microscopy

Introduction

Colon cancer is the third leading cause of cancer-related deaths in the United States (Jemal *et al.*, 2010). Chronic inflammation is an important risk factor in the promotion of colon cancer (Terzic *et al.*, 2010), and consistent with this notion, regular non-steroidal anti-inflammatory drug (NSAID) use decreases the risk of death from colorectal cancer (Clevers, 2006; Harris *et al.*, 2008; Zell *et al.*, 2009). Ibuprofen is effective at suppressing colon cancer growth in preclinical models (Yao *et al.*, 2005; Janssen *et al.*, 2006, 2008), and protects against colon polyp formation in humans (Johnson *et al.*, 2010). However, long-term use of ibuprofen is problematic due to gastrointestinal and cardiovascular toxicities (Wolfe *et al.*, 1999; Hippisley-Cox and Coupland, 2005). This prompted us to develop phospho-ibuprofen (P-I; MDC-917),

a novel ibuprofen derivative, by conjugation of a diethylphospho-butanol moiety to its carboxylic acid group via an ester link. P-I inhibited cancer cell growth 16 to 23 times more potently than ibuprofen and was more effective in suppressing the growth of colon cancer xenografts in mice (Xie *et al.*, 2011) while exhibiting minimal toxicity (Huang *et al.*, 2011). P-I was also much more potent at inducing oxidative stress-mediated apoptosis compared to chemotherapeutic drugs (Sun *et al.*, 2011). Despite these promising results, our recent pharmacokinetic (PK) study (Xie *et al.*, 2011) revealed that the bioavailability of intact P-I is low in mice (<5%), due in part to poor solubility in aqueous solutions, rapid hydrolysis by non-specific esterases and fast clearance from the systemic circulation, which potentially compromise its efficacy *in vivo*.

P-I has unique chemical and physical properties compared with the parent compound: (i) esterification of –COOH groups prevents the possibility of drug ionization which increase its hydrophobicity; and (ii) the diethylphospho-butanol moiety in P-I, which has structural similarities to phospholipid, increases its lipophilicity. Thus, this chemical modification facilitates P-I's compatibility with various nanocarriers. Nanocarriers are emerging technologies that hold great promise for overcoming the limitations of many traditional anti-cancer drugs (Peer *et al.*, 2007; Alexis *et al.*, 2010), including, but not limited to, poor water solubility, rapid breakdown of drugs *in vivo*, lack of selectivity towards cancer cells and poor delivery to the tumour site.

Nanocarriers such as liposomes and micelles have achieved clinical success in improving traditional chemotherapy, as exemplified by liposome-encapsulated doxorubicin (Doxil®) and daunorubicin (Daunoxome®) that are already clinically approved for treating various types of cancer (Allen and Cullis, 2004; Cukierman and Khan, 2010). Liposomes have a vesicle structure composed of a self-assembled lipid bilayer. The drug could either be incorporated into the lipid bilayer or encapsulated inside the vesicle. Polymeric micelles, with their hydrophobic cores, are particularly suitable for the delivery of drugs that are barely water-soluble drugs (Torchilin, 2007). Polyethylene glycol (PEG)-stabilized nanocarriers have gained popularity as they are water soluble, biocompatible and have prolonged circulation times *in vivo* (Gabizon, 2001). These lipid-based nanocarriers accumulate preferentially in solid tumours via the enhanced permeability and retention effect (Maeda *et al.*, 2000), whereas low-molecular-weight drugs tend to distribute non-specifically. In the present study we investigated the PK and pharmacodynamic properties of P-I incorporated into liposomes or polymeric micelles.

Here, we demonstrated the successful encapsulation of P-I into poly(ethylene oxide)-b-poly(lactic acid) (PEO-*b*-PLA) copolymer micelles, and liposomes composed of soy-phosphatidylcholine (soy-PC) and sterically stabilized by a PEGylated lipid. We systematically evaluated the stability, cellular uptake and cytotoxicity of P-I in polymeric micelles or liposomes. Liposomal P-I exhibited a marked resistance to hydrolysis by esterases *in vitro* and *in vivo*. In addition, encapsulation of P-I in liposomes increased the cytotoxicity and bioavailability of P-I, which in turn led to enhanced efficacy in a colon cancer xenograft model in mice.

Methods

Materials

Soy-PC and methoxy-poly(ethylene glycol)2000-distearoyl phosphatidylethanolamine (DSPE-PEG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). PEO-*b*-PLA copolymer (M_w/M_n = 1.04) was obtained from Polymer Source, Inc. (Quebec, Canada). The block lengths were 114 and 81 repeating units for PEO (5000 g·mol^{-1}) and PLA (4700 g·mol^{-1}), respectively. P-I was a gift from Medicon Pharmaceuticals, Inc. Stony Brook, NY, USA. All other reagents were from Sigma-Aldrich, St Louis, MO, USA. The drugs were dissolved in DMSO as 100 mM stock solution and diluted to their final concentration in cell culture media.

Preparation of liposome-encapsulated P-I

Liposomes were prepared by the thin lipid film hydration method. Soy-PC, DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] ammonium salt) and P-I were dissolved in chloroform evaporated to a thin film in rotary evaporator, rehydrated with PBS and gradually extruded through double polycarbonate membranes of 0.08 or 0.02 μm pore size (Waters, Milford, MA, USA), using an extruder device (Lipex Biomembranes, Vancouver, BC, Canada). The final concentration of each component was 37 mg·mL^{-1} Soy-PC, 7.4 mg·mL^{-1} DSPE-PEG and 20 mg·mL^{-1} P-I. The non-encapsulated drug was separated from liposomes by extensive dialysis against saline. An identical process that omitted P-I was used to prepare empty liposomes. Liposomes were stored under argon at 4°C and tested within 1 month after preparation. The amount of P-I incorporated into liposomes was determined by HPLC. The molar ratios between soy PC, DSPE-PEG and P-I were determined by 1H NMR analysis of the freeze-dried liposomal P-I.

Preparation of micelle-encapsulated P-I

For micelles, we added dropwise 1 mL of a dilute acetone solution of P-I and PEO-*b*-PLA block copolymer into 10 mL of PBS under stirring. Acetone was removed under reduced pressure. The non-encapsulated P-I and trace amounts of organic solvent were removed by extensive dialysis against saline solution. The final polymer concentration was 10 mg·mL^{-1}, with 6.3 mg·mL^{-1} P-I. An identical process that omitted P-I was used to prepare empty PEO-*b*-PLA micelles. Micelles were stored under argon at 4°C and tested within 1 month after preparation. The molar ratio between PEO-*b*-PLA copolymer and P-I were determined by 1H NMR analysis of the freeze-dried micellar P-I.

Characterization of nanoparticles

Examination of particle morphology. The morphology of P-I-containing nanoparticles was examined by transmission electron microscopy (TEM) with negative staining. Briefly, the samples were prepared by wetting a carbon-coated grid with a small drop of diluted liposome or micelle solutions. Upon drying, they were stained with 1% uranyl acetate and 2% phosphotungstic acid, air-dried at room temperature and viewed with a FEI BioTwinG2 electron microscope (FEI, Hillsboro, OR, USA).

Determination of particle size. The particle size distribution was measured by dynamic light scattering, using Zetaplus with the BI-MAS option (Brookhaven Instruments Co., Holtsville, NY, USA). All measurements were performed at 25°C at a measurement angle of 90°, in triplicate.

Drug content determination. P-I content was determined by HPLC, as described previously (Xie *et al.*, 2011). The HPLC system consisted of a Waters alliance 2695 Separations Module equipped with a Waters 2998 photodiode array detector and a Thermo Hypersil BDS C18 column (150 × 4.6 mm, particle size 3 µm). 1H NMR spectra were recorded on a Varian 300 spectrometer. Samples prepared for NMR analysis were dissolved in $CDCl_3$. Chemical shifts were reported in p.p.m. relative to tetramethylsilane (TMS). Drug loading capacity was calculated with the following formula: Drug loading capacity (%) = the content of drug encapsulated in nanoparticle/the quantity of the detected solution of nanoparticles × 100%.

Evaluation of P-I stability in nanocarriers

P-I stability in cell culture medium. A 0.1 mL aliquot of 100 µM solution of each compound in DMSO was diluted to 1 mL of RPMI-1640 containing 10% fetal bovine serum (FBS). The resulting solution was maintained at 37 ± 0.5°C, and at appropriate time intervals, the P-I concentration was analysed by RP-HPLC. Experiments were performed in triplicate.

P-I stability in the presence of purified esterase. Solutions containing 500 µM of P-I or P-I in nanocarriers were incubated at 37°C with 3.0 IU·mL^{-1} carboxyl esterase (from porcine liver) in PBS solution. Samples were taken at predetermined time points to mix with two volumes of acetonitrile. The samples were vortexed to immediately arrest enzymatic activity. The samples were then centrifuged for 5 min at 14 000× *g*. The supernatant was injected into the HPLC for determination of P-I and ibuprofen. Experiments were performed in triplicate.

Cell culture

HT-29, HCT116 and SW480 human colon adenocarcinoma cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured in either McCoy 5A medium (HT-29 and HCT116) or RPMI-1640 (SW480) complete medium as recommended by American Type Culture Collection.

Cell viability. Cell viability was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol. Briefly, 96-well microtitre plates were seeded with 10 000 cells per well and allowed to adhere for 8 h before the assay. Cells were exposed to various doses (0–200 µM on P-I basis) of P-I alone, nanocarriers alone or P-I-loaded nanocarriers for 24 h at 37°C.

Cellular uptake in human colon cancer cells. Quantitative studies of the cell uptake of P-I-containing nanoparticles were performed as follows. SW480 cells were seeded in 100 cm^2 plates (BD Biosciences, San Jose, CA, USA) and cultured until they formed a confluent monolayer. Upon reaching confluence, the culture medium was replaced by transport buffer (Hank's balanced salt solution, pH 7.4) and incubated at 37°C for 30 min. After equilibration, cell uptake of nanoparticles was initiated by adding 10 µL of specified P-I or P-I containing nanoparticle suspension and incubating the cells at 37°C. The incubation was terminated by washing the cell monolayer three times with PBS (pH 7.4) to remove excess particles not entrapped by the cells. The cells were scraped off the dishes and extracted twice with twofold volumes of acetonitrile and centrifuged at 13 000× *g* for 5 min. The P-I concentration was determined by HPLC. Experiments were performed in triplicate.

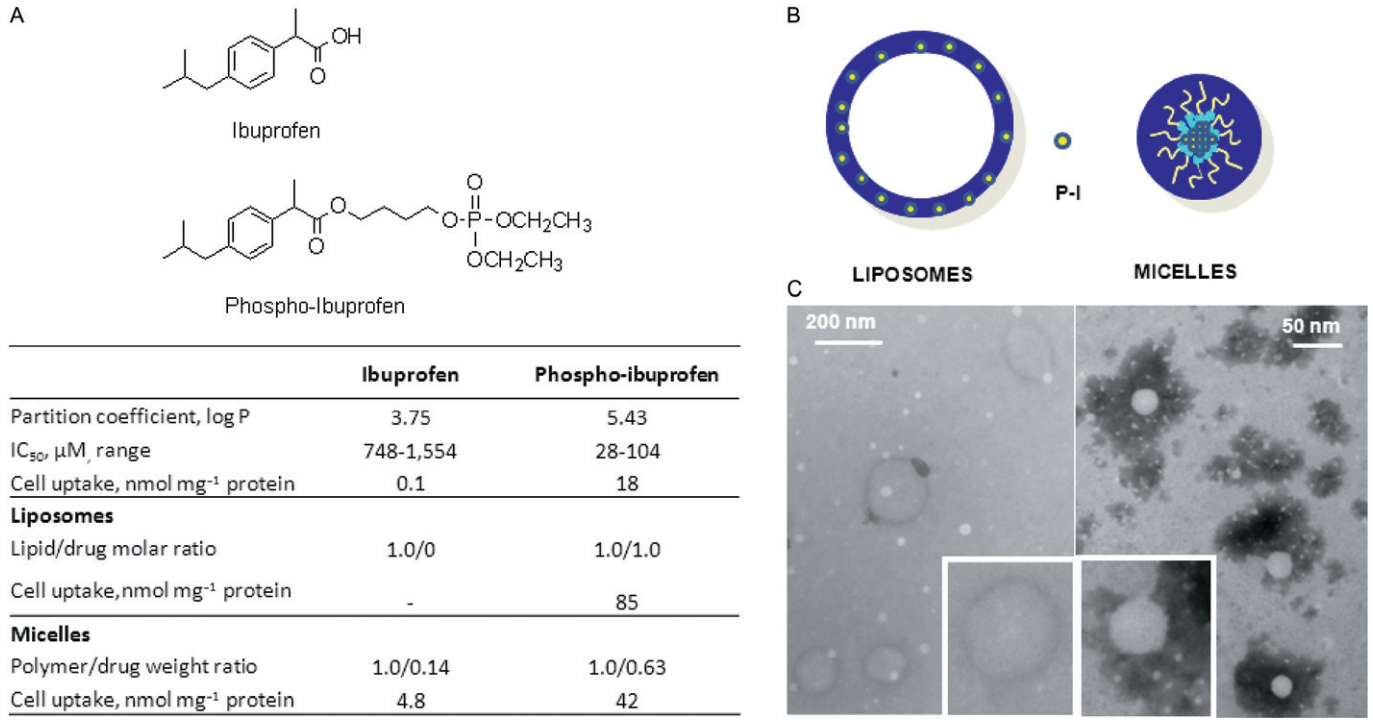
Animal studies

All animal care and experimental procedures complied with NIH guidelines and were approved by the Institute's Animal Care and Use Committee.

Liposome enhanced P-I stability in serum and tissues in mice. P-I or liposomal P-I was administered by i.p. injection to female BALB/c mice at a dose of 400 mg·kg^{-1}. After 15 min, mice were killed by CO_2 inhalation, blood was collected and organs were excised. The blood samples were centrifuged to obtain the plasma. The organs were rinsed three times with 50% DMSO, snap-frozen in liquid nitrogen and stored at –80°C until analysis. For analysis of drug levels, acetonitrile (2× the volume of plasma) was added to the plasma samples, mixed and centrifuged at 13 000× *g* for 15 min, and the supernatants were analysed by HPLC. The organs were homogenized in PBS (pH 7.4), sonicated and extracted with acetonitrile. Subsequent steps for drug level determination were the same as those applied to the plasma samples.

PK studies in mice. P-I or liposomal P-I was administered to mice as a single 100 mg·kg^{-1} dose i.p. Mice were killed by CO_2 inhalation at designated time points after drug administration, and blood was collected and immediately centrifuged. The resulting plasma was deproteinized by immediately mixing it with a twofold volume of acetonitrile. PI and its metabolites were analysed by HPLC as described above.

In vivo *anti-cancer efficacy.* Female Ncr nude mice (5–6 weeks old) were purchased from Taconic (Taconic Farms, Germantown, NY, USA). After an acclimatization period of 1 week, the animals were anaesthetized with isoflurane administered via inhalation, at 4-5% for induction and 1.5-2% for maintenance,before they were inoculated s.c. in their right and left flanks, each with 2.0 × 10^6 SW-480 colon cancer cells suspended in 100 µL of PBS. When the average tumour size reached 200 mm^3, the animals were divided into three groups (six per group) which were given the following treatments, respectively: (i) vehicle (PBS); (ii) P-I (100 mg·kg^{-1}·day^{-1} in PBS, i.p.); and (iii) liposomal P-I (100 mg·kg^{-1}·day^{-1}, i.p.). Tumours were measured twice a week with a digital microcaliper by an observer unaware of the identity of the mice, and tumour volumes were calculated using the following formula: tumour volume = [length × width × (length + width/2) × 0.56]. After treatment for 3 weeks, the animals were killed by CO_2 inhalation and their tumours were removed and weighed.



	Ibuprofen	Phospho-ibuprofen
Partition coefficient, log P	3.75	5.43
IC_{50}, μM, range	748-1,554	28-104
Cell uptake, nmol mg^{-1} protein	0.1	18
Liposomes		
Lipid/drug molar ratio	1.0/0	1.0/1.0
Cell uptake, nmol mg^{-1} protein	-	85
Micelles		
Polymer/drug weight ratio	1.0/0.14	1.0/0.63
Cell uptake, nmol mg^{-1} protein	4.8	42

Figure 1

P-I, ibuprofen and their incorporation in nanocarriers. Left panel (A): The structure and physical properties of ibuprofen and P-I. The partition coefficient in *n*-octanol/water (log P) was obtained using ChemDraw 7.1. IC_{50}s were determined in HT29, HCT116 and SW480 colon cancer cell lines; we show the range of values in these three cell lines. Drug compatibility with each nanocarrier and cell uptake was studied in SW480 cells incubated for 3 h with 50 μM P-I or P-I-containing nanoparticles. Right panel: Schematic illustration (B) and TEM images (C) of P-I-containing liposomes and micelles. Liposomes were composed of L-α-lysophosphatidylcholine (Soy PC) and DSPE-PEG. Micelles were self-assembled PEO-*b*-PLA diblock copolymers. Insets: enlarged images of a single liposome and a single micelle.

Statistical analyses

Statistical analyses were performed using one-way ANOVA with Tukey's HSD *post hoc* tests; *P*-values ≤0.05 were considered statistically significant.

Results

Preparation and characterization of liposomal and micellar P-I

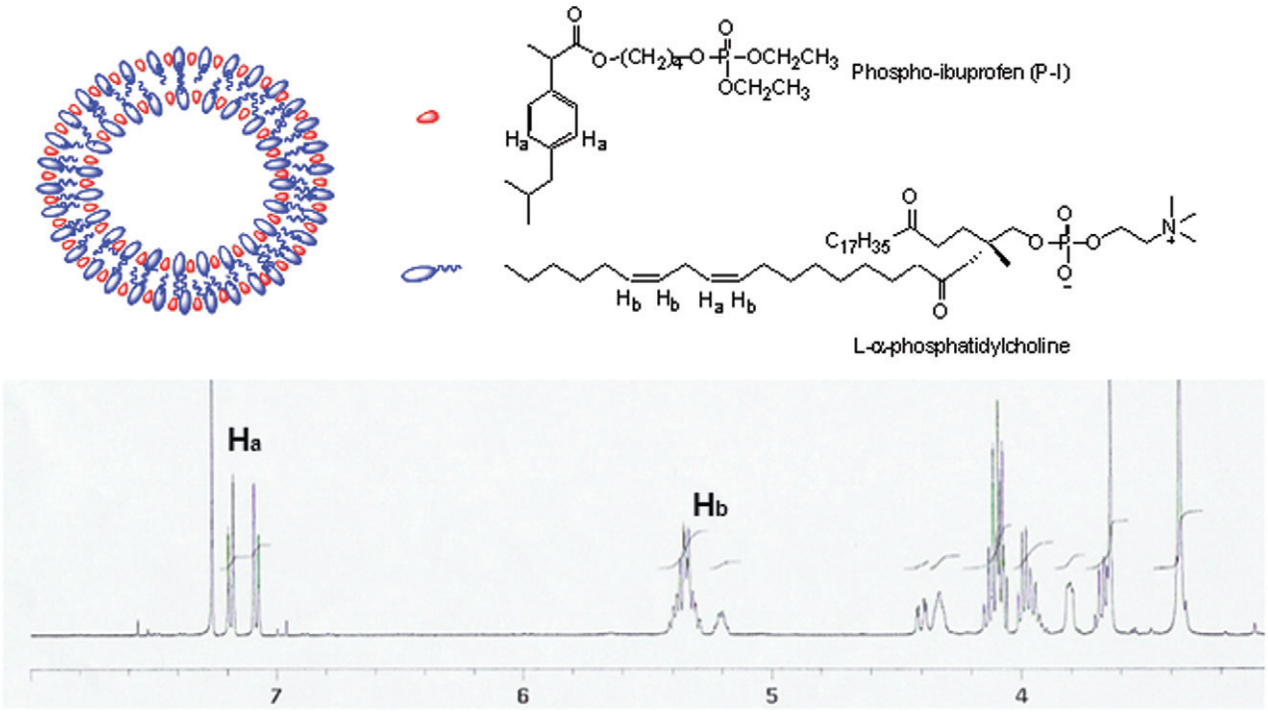
P-I (Figure 1A) was generated by an esterification between the carboxylic acid group from ibuprofen and diethylphosphobutanol. The partition coefficient of P-I was much higher than that of ibuprofen (5.43 vs. 3.75), indicating higher hydrophobicity of the former. This may partly explain the much better incorporation of P-I into liposomes and micelles than ibuprofen. Figure 1B illustrates the distribution of P-I in the lipid bilayer of liposomes, as well as in the core of polymer micelles. TEM (Figure 1C) revealed that P-I-containing liposomes maintained a lipid bilayer structure with an average hydrodynamic radius of 209 nm. These liposomes carried a negative charge with a zeta potential of –15.5 ± 2.6 mV. The P-I-containing polymer micelles have a condensed structure with an average size of 78 nm, and more neutral charge with a zeta potential of –6.2 ± 0.6 mV.

To further study the stability of P-I in liposomes, the integration of H_a from the P-I benzene ring (7.0–7.2 p.p.m.) and H_b from the PC unsaturated bond (5.2–5.4 p.p.m.) were analysed by 1H NMR. The precise value 1.0/1.0 of PC/P-I molar ratio suggested that each molecule of P-I and PC were assembled with an alternating pattern into a vesicle structure, as illustrated in Figure 2. However, ibuprofen was almost unable to be loaded in nanocarriers, liposomes in particular, with no detectable ibuprofen in the lipid bilayer (lipid/ibuprofen molar ratio is 1.0/0). This is in accordance with its significantly lower drug cell uptake compared with P-I, as liposomes structurally mimic the cell membrane. Both the micelle and liposome formulation further increased the P-I cell uptake 2- to 5-fold.

Cellular uptake

To evaluate the effect of liposome and micelle encapsulation on the cellular penetration of P-I, we assessed the uptake of P-I, liposomal P-I and micellar P-I into the SW480 human colon cancer cell line. Cellular uptake of P-I was found to be dose- and time-dependent (Figure 3). Under the experimental conditions examined, the cellular uptake of liposomal P-I and micellar P-I was greater than that of free P-I. The enhancement of cellular uptake was particularly marked with regard to liposomal P-I. The cellular uptake of liposomal P-I was 5- to

A



B

	P-I/nanocarrier molar ratio	R_h, nm	PDI	Zeta potential, mV
Micellar P-I	1.0/0.63	78 ± 8	0.14± 0.03	-6.2± 0.6
Plain micelles	1.0/0	62 ± 6	0.13± 0.02	-6.9± 0.6
Liposomal P-I	1.0/1.0	209 ± 16	0.17± 0.06	-15.5± 2.6
Plain liposomes	1.0/0	181 ± 12	0.18± 0.08	-15.3± 2.9

Figure 2

Physical characteristics of P-I-containing liposomes and micelles. (A) Liposomal P-I structure analysis. The P-I/lipid molar ratio was determined by 1H NMR. H_a (7.0–7.2 p.p.m.) represents the P-I benzene ring and H_b (5.2–5.4 p.p.m.) the PC unsaturated bond. This arrangement is shown in the schematic illustration. The PC/P-I molar ratio of 1/1 suggests that P-I and PC are assembled in an alternating pattern in the vesicle structure. (B) The hydrodynamic radius is the average of three independent measurements performed using dynamic light scattering. The zeta potential was obtained from electrophoretic mobility measured at pH 7.4. PDI, polydispersity index.

6-fold greater than that of P-I over the range tested (50–100 μM), whereas micellar P-I increased cellular uptake by less than 2-fold. A similar trend was also observed when the incubation time was extended to 3 h. Liposomal P-I uptake was about twice as much as P-I at all the time intervals examined. On the other hand, cellular uptake of micellar P-I was similar to that of free P-I after an extended incubation period (3 h). These data suggest that nanocarriers, in particular liposomes, have the ability to enhance P-I penetration into cancer cells.

In vitro *cytotoxicity*

Given that the nanocarriers enhanced the cellular penetration of P-I in cancer cells, we proposed that micelle or liposome encapsulation may enhance the cytotoxicity of P-I. The 24 h IC_{50} values of P-I, liposomal P-I and micellar P-I were determined in SW480, HCT116 and HT-29 human colon cancer cells (Figure 4). Consistent with our hypothesis, liposomal P-I was more cytotoxic than free P-I in these cell lines. This effect was not the result of the nanocarrier components,

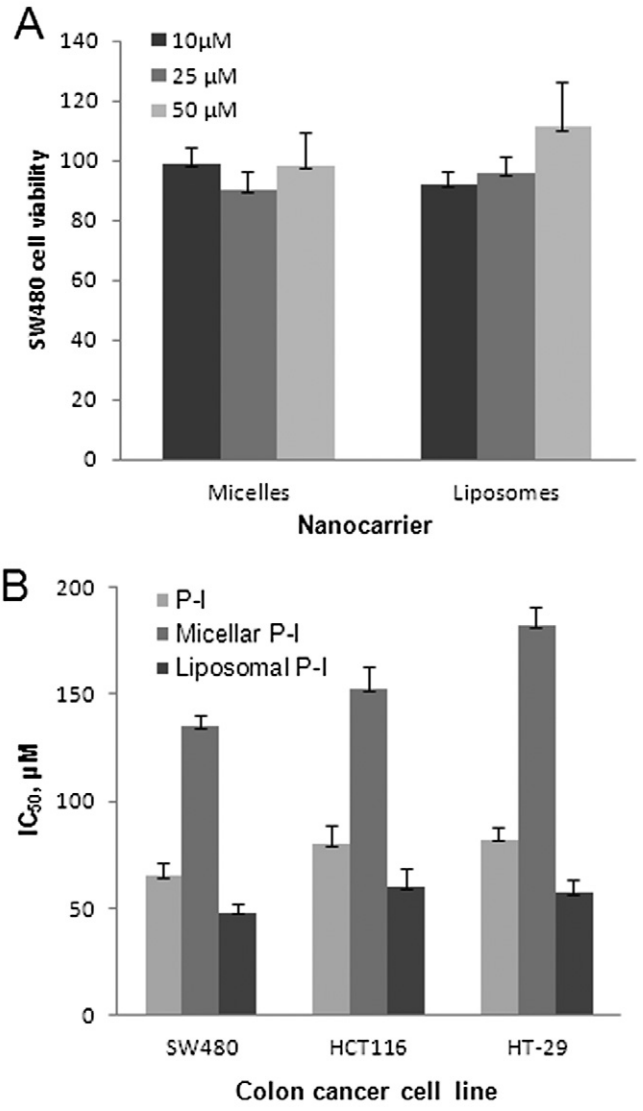


Figure 3

Incorporating P-I in liposomes increases its cytotoxicity. (A) SW480 human colon cancer cells were treated with various concentrations of empty liposomes or empty micelles for 24 h (concentrations shown correspond to the amount of nanocarriers required to achieve the indicated concentrations of P-I, had it been incorporated in each of them). Empty micelles and liposomes were nontoxic to SW480 cells. Values are mean ± SD of % control values. (B) The three human colon cancer cell lines shown here were treated with various concentrations of P-I free or incorporated in micelles or in liposomes and the 24 h IC_{50} values were determined as in the Methods section. Values are mean ± SD of at least three independent assays. There are statistically significant differences between free P-I and P-I in both liposomes and micelles ($P < 0.05$) for all cell lines.

since blank liposomes or micelles were not cytotoxic to the colon cancer cell lines. Interestingly, we observed a significantly attenuated cytotoxicity of liposomal P-I (165 μM) in normal NCM480 cells compared to P-I (103 μM). This suggests that liposomal P-I may preferentially target cancer cells. On the other hand, the cytotoxic potency was decreased in the colon cell lines when P-I was incorporated into polymeric

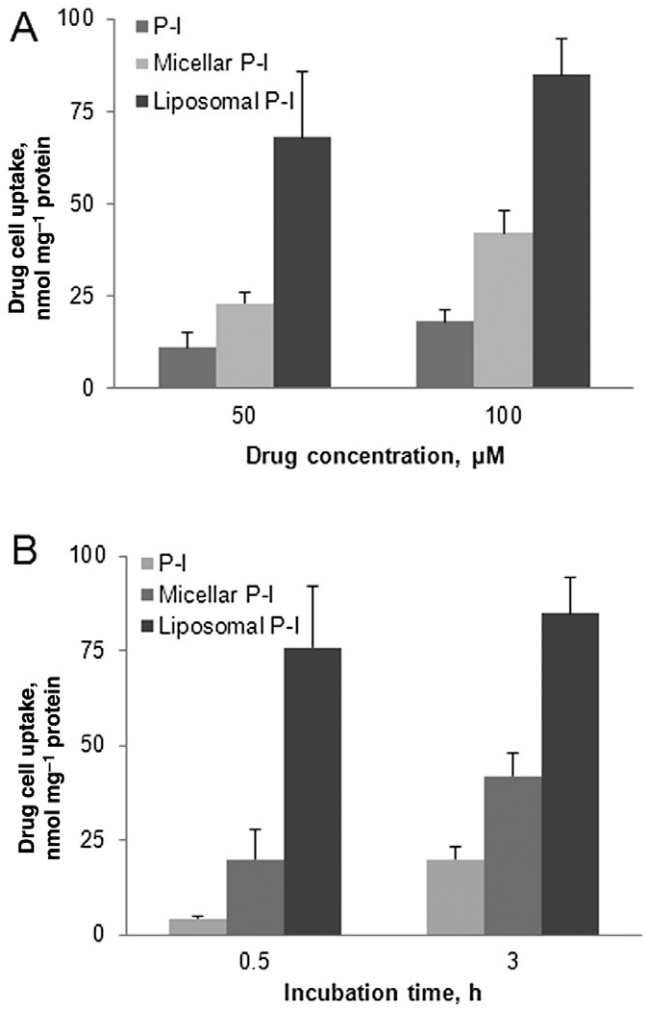


Figure 4

Incorporating P-I in nanocarriers increases its uptake. Concentration- (A) and time-(B) dependence of the uptake by SW480 of P-I unincorporated and incorporated in micelles or liposomes cells, as in the Methods section. In (A), cells were treated for 3 h. Values are mean ± SD of at least three independent experiments. All differences are statistically significant.

micelles. This may be attributed to the strong hydrophobic interaction between P-I and the hydrophobic core of the micelles (Greish *et al.*, 2004), which delays intracellular release of free drug.

Nanocarriers stabilize P-I in vitro

Since intact P-I is much more potent than its hydrolysed products at inhibiting the growth of colon cancer cells (Xie *et al.*, 2011), the stability of P-I would be critical for optimal efficacy. Both liposomes and micelles stabilized P-I under physiological conditions. In complete media containing 10% serum, free P-I was slowly hydrolysed starting at 8 h of incubation, with 33% hydrolysed after 24 h. P-I in nanocarriers was much more stable with less than 5% hydrolysed after 24 h (Figure 5A). We next determined whether nanocarriers can protect P-I against hydrolysis by non-specific esterases

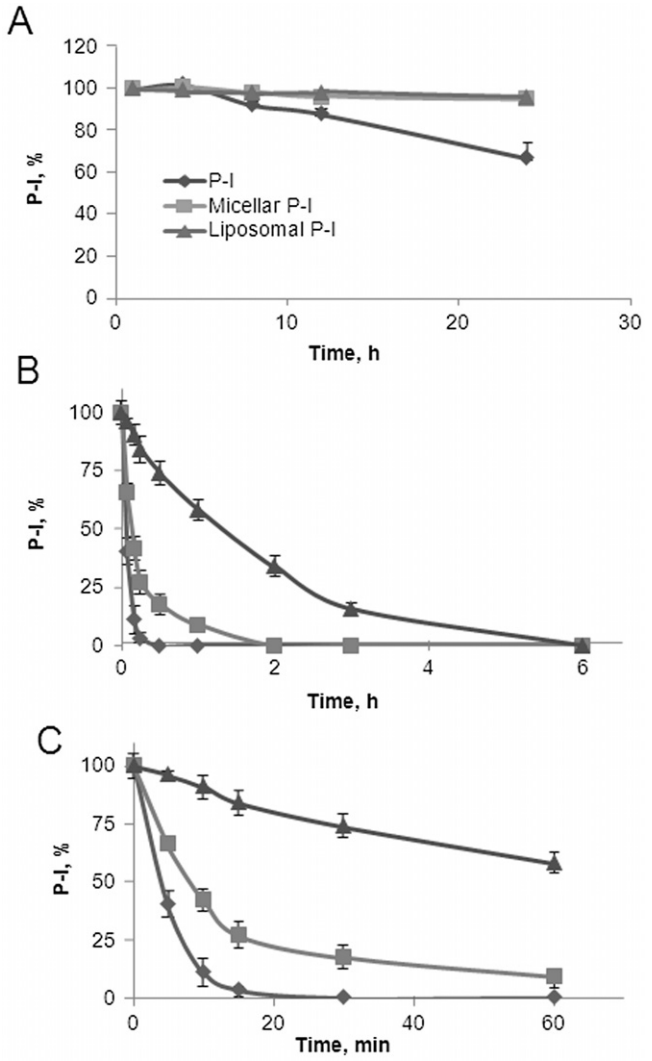


Figure 5

The stability of P-I is enhanced by its incorporation into nanocarriers. P-I was incubated at 37°C for up to 6 h with cell culture medium containing 10% fetal bovine serum (A) or porcine liver esterase (B and C) as described in the Methods section. The percentage of input P-I that remained intact was determined at the indicated time points. Values are mean ± SD of at least three independent experiments.

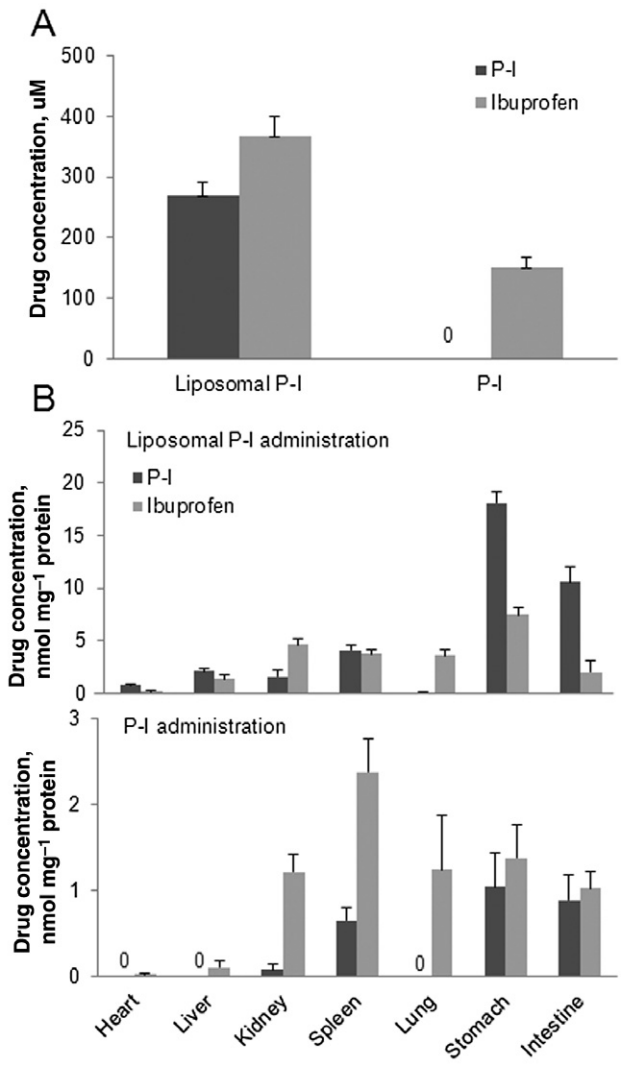


Figure 6

Liposomal formulation of P-I improves its biodistribution. The biodistribution of liposomal P-I and free P-I was determined in mice as described in the Methods section. The concentration of P-I and its main metabolite, ibuprofen, was determined in serum (A) and in the major organs (B) of mice (n = 3), 15 min after i.p. administration of P-I 400 mg·kg^{-1}.

from liver, a major metabolic organ with high expression of esterases (Xie *et al.*, 2002). P-I, liposomal P-I and micellar P-I were incubated with purified esterases *in vitro*, and the half-life of P-I was determined. As shown in Figure 5B and C, the breakdown of free P-I in the presence of esterases was very rapid, resulting in a short half-life of 4 min. Micellar P-I had slightly greater stability, with a half-life of 8 min. Liposomal P-I, on the other hand, showed remarkable stability in the presence of esterases, with the majority of the drug remaining intact even after an extended period of incubation (half-life: 1.2 h). Hence, the stability of P-I was significantly enhanced by incorporation into micelles or liposomes, especially the latter, indicating that nanocarriers can significantly improve the metabolic stability of P-I.

Liposome stabilizes P-I in vivo

With enhanced P-I stability and cell cytotoxicity, we next compared the biodistribution of liposomal P-I and free P-I in serum and in the major organs in mice, including the heart, liver, lungs, intestine and the kidneys (Figure 6B). After i.p. administration of liposomal P-I, intact P-I could be detected in all the organs evaluated. This suggests that P-I can be efficiently distributed despite its relatively short half-life. Despite the high esterase activity in serum, 40% of intact P-I (270 μM) was found after liposomal P-I administration, while P-I was not detectable after free P-I administration (Figure 6A). A similar trend was observed in P-I tissue levels (Figure 6B). After liposomal P-I injection, gastrointestinal tissues have the highest levels of intact P-I, while the lungs

have the lowest levels. Ibuprofen was also detected in these tissues, but generally at levels much lower than those of P-I. Intact P-I was also detected in the gastrointestinal tissues, the kidneys and spleen following administration of free P-I, but at much lower levels (5- to 10-fold) compared to liposomal P-I. Ibuprofen was the major metabolite found in all the organs analysed, reflecting the limited bioavailability of intact P-I in the absence of protection by liposomes.

PKs

Having shown that liposomal P-I improves cellular uptake and confers resistance to esterase-mediated hydrolysis, we next compared the bioavailability of liposomal P-I and free P-I *in vivo*. PK properties of liposomal P-I and free P-I were studied after a single i.p. administration to mice. As shown in Figure 7, ibuprofen, but not intact P-I, was found in whole blood 30 min after the administration of free P-I. Hence, free P-I did not survive in mouse blood due to the high esterase activity in blood (Morton *et al.*, 2000). On the other hand, high levels of intact P-I (>200 μM) were detected following the administration of liposomal P-I. Liposome encapsulation thus protects against esterase-mediated hydrolysis *in vivo*. The plasma levels of intact P-I, however, decreased rapidly and became undetectable 30 min post-administration. The plasma levels of P-I metabolites, ibuprofen, hydroxyl-ibuprofen and ibuprofen glucuronide, were also much greater after the administration of liposomal P-I compared to free P-I. As shown in Figure 7A, plasma ibuprofen increased rapidly and reached the maximum concentration of over 1106 μM 1 h after injection of liposomal P-I. Free P-I was more slowly absorbed (T_{max} = 2 h), and the peak level of ibuprofen was considerably lower (418 μM). Ibuprofen levels decreased rapidly thereafter and became negligible 8 h after drug administration. As with the case for ibuprofen, peak levels of ibuprofen glucuronide and hydroxy ibuprofen were much higher (C_{max} > threefold) following the administration of liposomal P-I compared to free P-I (Figure 7B and C). The area under curve (AUC0-12h) of total metabolites for liposomal P-I and free P-I were 5398 and 1210 μM × h, respectively, with a 4.4-fold increase in the total AUC. Our results suggest that liposome encapsulation improves the bioavailability of P-I, and partially protects P-I from esterase activity *in vivo*.

In vivo *efficacy*

Finally, we investigated the anti-tumour efficacy of P-I and liposomal P-I in s.c. xenografts of SW480 human colon cancer cells in nude mice. When the tumours reached 200 mm^3, the mice were treated with free P-I or liposomal P-I (100 $mg \cdot kg^{-1} \cdot day^{-1}$ for both) for 21 days. As shown in Figure 8, liposomal P-I suppressed tumour growth, and the effect was statistically significant beginning 5 days after the initiation of treatment until the end of the study (day 5 to 10, $P < 0.03$; day 15 to 21, $P < 0.01$). Compared with the control, liposomal P-I reduced tumour volume by 51%. Free P-I reduced the tumour volume by 23%, but the inhibition did not reach statistical significance. Liposomal P-I and free P-I also reduced the tumour weight by 45% and 73% ($P < 0.01$), respectively (Figure 8B). In this study, both the liposomal P-I and free P-I were well tolerated with no weight loss during treatment. The superior efficacy of liposomal P-I over free P-I is probably due to the enhanced bioavailability and biodistribution of the former.

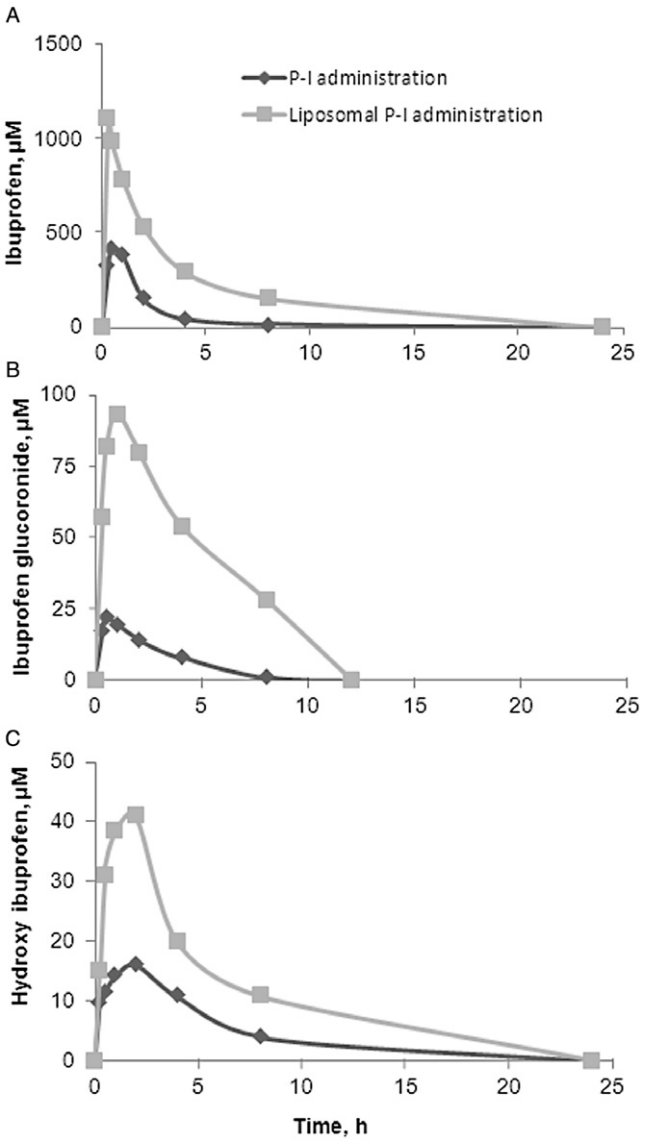


Figure 7

Pharmacokinetic study of liposomal P-I; 100 $mg \cdot kg^{-1}$ of liposomal P-I or free P-I were administered to mice as a single i.p. dose, and blood samples were collected at the indicated time points starting 30 min post-injection. Plasma levels of the P-I metabolites ibuprofen (A), ibuprofen glucuronide (B) and hydroxy ibuprofen (C) were determined. Values are the average of duplicate samples (all within 12% of each other).

Discussion and conclusions

Our results established that incorporation of P-I into micelles and liposomes led to a significant improvement in its PK and pharmacodynamic properties. This effect was mediated via a triad of mechanisms: improved solubilization; enhanced cellular penetration; and protection from metabolic

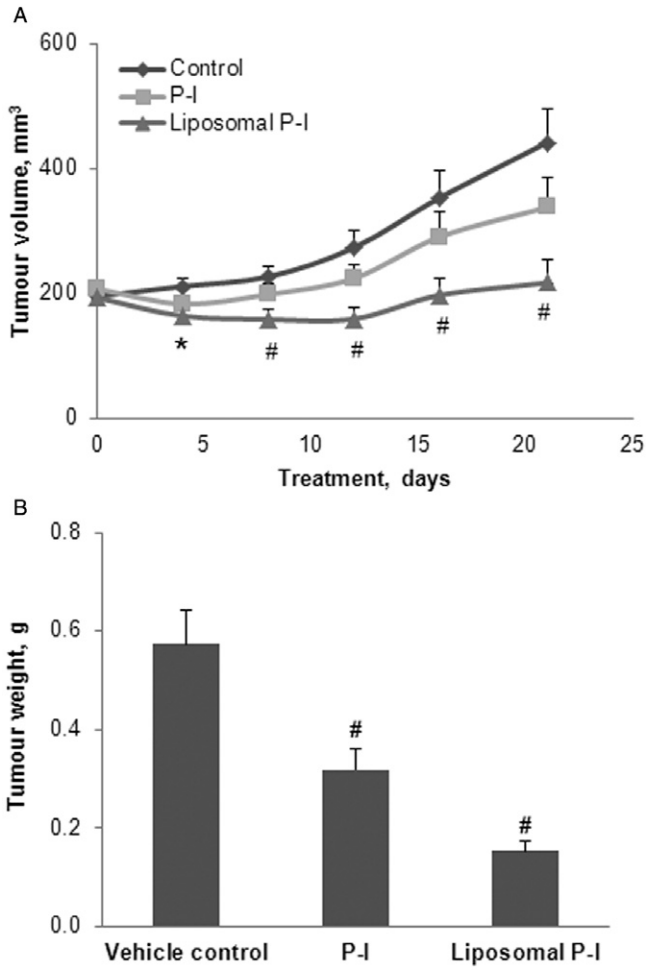


Figure 8

The effect of liposomal P-I on the growth of SW480 human colon xenografts. SW480 human colon carcinoma cells were implanted s.c. into nude mice, as described in the Methods section. Liposomal P-I or free P-I 100 mg·kg^{-1}·day^{-1} were administered i.p. to mice for 3 weeks, starting when tumours reached an average size of 200 mm^3. Controls received vehicle. The weight of tumours was determined at death. Values are mean ± SEM; $n = 20$ mice per group. *$P = 0.03$ and $^{\#}P = 0.01$, compared to control.

inactivation, all of which improve the delivery of intact P-I, ultimately enhancing anti tumour efficacy.

Phospho-NSAIDs are novel anti-cancer drugs that exhibit potent inhibition of colon cancer growth *in vitro* and *in vivo* (Mackenzie *et al.*, 2010, 2011; Xie *et al.*, 2011), and have greatly reduced gastrointestinal toxicity (Huang *et al.*, 2010). The phospho-modification has an important effect on the physicochemical properties of NSAIDs. P-I, as an example, is significantly more hydrophobic than ibuprofen and is poorly soluble in water. A high lipophilicity is a property shared by many anti-cancer agents, since this allows the drug to permeate the cell membrane to act on intracellular targets. However, the bioavailability of poorly soluble drugs is often limited (Pouton, 2006) and i.v. administration may not be an option due to the high risk of drug aggregation in the circulation (Fernandez *et al.*, 2001).

Nanocarriers, such as liposomes and polymeric micelles, are popular vehicles to facilitate the solubilization of hydrophobic drugs and improve their PK properties. Our results indicated that P-I could be readily incorporated into liposomes and polymeric micelles, which improves its cellular penetration *in vitro* and bioavailability *in vivo*. Liposomes accommodate hydrophobic drugs in the lipid bilayers. Polymeric micelles, with their hydrophobic cores, are thought to have higher loading capacity for hydrophobic drugs compared to liposomes (Kataoka and Nishiyama, 2006). However, we observed a high loading of P-I in liposomes, with a drug to lipid molar ratio of 1:1. The high P-I : phospholipid ratio suggests a unique arrangement of P-I in the lipid bilayers, in which P-I and the phospholipid molecule assemble with an alternating pattern into the vesicle structure. Hydrophobic drugs such as amphotericin B and chlorpromazine (Caron *et al.*, 1999; Abdiche and Myszka, 2004) have also been incorporated into liposomes bilayer in a similar manner. Comparison of cellular uptake and *in vitro* cytotoxicity of liposomal and micellar P-I revealed that liposomal P-I was more effective in the intracellular delivery of P-I, probably as a result of the more efficient loading of P-I in the latter.

Phospho-NSAIDs, with their carboxylic ester group, could be hydrolyzed by non-specific esterases present in the liver, intestine or plasma (Satoh and Hosokawa, 1998; Redinbo and Potter, 2005). Our previous investigations have shown that the intact phospho-NSAIDs are considerably more cytotoxic towards human cancer cells than the linker, diethylphosphate or the parent NSAIDs. Rodents, such as mice, have high expression of esterases in the plasma (Morton *et al.*, 2000), which may lead to rapid hydrolysis of phospho-NSAIDs. Indeed, in previous studies, we could not detect intact phospho-NSAIDs in mouse blood following the administration of P-I or phospho-sulindac (Xie *et al.*, 2011). Hence, protection from esterase-mediated degradation is an important criterion for the choice of nanocarriers.

The incorporation of P-I into liposomes significantly reduced the rate of its hydrolysis in the presence of liver esterases, by 18-fold over free P-I. Polymeric micelles provided limited protection, increasing the half-life of P-I by only 2-fold. Neutral, hydrophobic drugs encapsulated into liposomes and polymeric micelles are often released rapidly from the carriers (Allen and Cullis, 2004), as is probably the case for micellar P-I. If P-I is released rapidly, then the rate of hydrolysis by esterases would approach that of the free drug. The unusually high stability of liposomal P-I may be due to the strong interaction between P-I and the phospholipids, thereby limiting P-I release and its subsequent hydrolysis.

In the present study we also demonstrated that liposome encapsulation of P-I partially protected P-I from esterase-mediated hydrolysis in mice, leading to a significantly improved bioavailability and biodistribution of intact P-I as well as its metabolites. As a consequence, liposomal P-I was more effective than free P-I at inhibiting the growth of colon cancer xenografts (73% vs. 45%).

In summary, we have shown that nanocarrier-incorporated P-I displays superior PK properties, leading to potent anti-tumour efficacy *in vivo*. Our data indicate that the use of nanocarriers is a promising approach for the optimized delivery of P-I.

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Conflicts of interest

There are no conflicts of interest with the exception of BR, who has an equity position in Medicon Pharmaceuticals, Inc. and PPC, who is affiliated to this company.

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