Chemotherapeutic Properties of Phospho-Nonsteroidal Anti-Inflammatory Drugs, a New Class of Anticancer Compounds

Liqun Huang1, Gerardo G. Mackenzie1, Yu Sun1, Nengtai Ouyang1, Gang Xie1, Kvetoslava Vrankova1, Despina Komninou2, and Basil Rigas1

Abstract

Nonsteroidal anti-inflammatory drugs (NSAID) exhibit antineoplastic properties, but conventional NSAIDs do not fully meet safety and efficacy criteria for use as anticancer agents. In this study, we evaluated the chemotherapeutic efficacy of 5 novel phospho-NSAIDs, each of which includes in addition to the NSAID moiety a diethylphosphate linked through a butane moiety. All 5 compounds inhibited the growth of human breast, colon, and pancreatic cancer cell lines with micromolar potency. In vivo investigations confirmed the antitumor activity of phospho-aspirin (PA) and phospho-sulindac (PS) in inhibiting tumor growth in established human xenograft models, in which cell proliferation was suppressed and apoptosis enhanced in the absence of detectable animal toxicity. Notably, all of the phospho-NSAIDs tested induced reactive oxygen and nitrogen species in cultured cells, with PA and PS inducing detectable levels of oxidative stress in vivo that were associated positively with apoptosis and negatively with proliferation. Potentially explaining these effects, all of the phospho-NSAIDs tested also inhibited the thioredoxin system and the redox sensitive transcription factor NF-κB. Taken together, our findings show the strong anticancer efficacy and promising safety of phospho-NSAIDs in preclinical models of breast, colon, and pancreatic cancer, suggesting further evaluation as anticancer agents. Cancer Res 71(24): 7617–7. ©2011 AACR.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) are the most widely used anti-inflammatory compounds, with aspirin, the prototypical NSAID, being one of the oldest and still most extensively used medications in the world (1, 2). NSAIDs have a significant antineoplastic effect, which is currently viewed in the context of the recently appreciated role of inflammation in cancer (3). Intervventional studies have established aspirin and sulindac as chemopreventive agents against colon cancer (4, 5). For the remaining NSAIDs, the evidence of their chemopreventive properties, strong as it is, is mainly based on epidemiologic studies (4, 6, 7). For example, a meta-analysis of 91 epidemiologic studies showed a significant exponential decline with increasing intake of NSAIDs in the risk for 7 to 10 malignancies including the 4 major types: colon, breast, lung, and prostate cancer (8, 9). However, there is no chemotherapeutic application for aspirin or other NSAIDs in humans or animals. Only sulindac when combined with other anticancer agents is reported to have a chemotherapeutic effect in mice (10).

NSAIDs prevent cancer likely through pleiotropic effects (reviewed in refs. 11–13). Although their best recognized molecular target is the enzyme COX, there is considerable evidence that such an effect may not be required for their anticancer actions (14, 15). Regardless of mechanistic issues, it is clear that conventional NSAIDs do not meet the criteria of safety and efficacy for their application as anticancer agents. NSAIDs are associated with considerable side effects, and their chemoprevention efficacy is rather limited, at best not exceeding 50% (16). Thus, there is a need to develop compounds with improved efficacy and safety.

Prompted by these considerations, we synthesized a series of compounds based on 4 representative NSAIDs: aspirin, sulindac (2 derivatives, differing in the structure of the sulindac moiety), ibuprofen, and flurbiprofen (Supplementary Fig. S1). Each one of these NSAIDs is chemopreventive against at least one major type of cancer based either on preclinical or epidemiologic data (7, 8, 17, 18). Structurally, these 5 compounds belong to a broader class of novel compounds that we have synthesized, which conform to the general chemical formula A-aliphatic linker-DEP, where A can be any compound, the linker can vary in size and/or structure as long as it is aliphatic, and DEP = diethylphosphate. Here, we report on compounds where A = NSAID moiety and the linker is a moiety derived from 1,4-butane diol. Of note, the enhanced safety of the phospho-NSAIDs is attributed in part to this chemical modification, because the carboxylic group, present in nearly...
all NSAIDs, mediates much of their gastrointestinal toxicity (19).

Phospho-sulindac (PS; OXT-328), phospho-aspirin (PA; MDC-118), and phospho-ibuprofen (PI; MDC-917) are highly effective against inflammation, as shown in a rat arthritis model (20). All 3 had a favorable safety profile, especially with regard to gastrointestinal toxicity (20, 21). Recently, PS was found in preclinical models of colon cancer to be strongly chemopreventive and chemotherapeutic. For example, in combination with difluoromethylornithine, PS prevented 91% of intestinal tumors in Min mice (21) and PS alone inhibited the growth of human colon xenografts in nude mice by 70% (22).

In this study, we evaluated the chemotherapeutic potential of these 5 compounds against breast, colon, and pancreatic cancer using human cancer xenografts. All displayed a significant anticancer effect, which was mediated, at least to some extent, by a redox effect that likely affected major downstream signaling pathways.

Materials and Methods

Reagents

Phospho-NSAIDs, synthesized following the methodology of Penning and colleagues (23), were provided by Medicon Pharmaceuticals, Inc. The lipids for the preparation of liposomes were from Avanti Phospholipids. For each compound, we prepared a 100-mmol/L stock solution in dimethyl sulfoxide (DMSO). In all cell culture media, the final DMSO concentration was adjusted to 1%. All general solvents and reagents were of high-performance liquid chromatography (HPLC) grade or of the highest grade commercially available.

Cell culture

Human breast (MCF-7 and MDA-MB-231), colon (HT-29 and SW480), and pancreatic (Mia PaCa-2 and BxPC-3) cell lines were obtained from American Type Culture Collection (ATCC) and were grown in the specific medium and conditions suggested by ATCC. All the cell lines were passaged in our laboratory for less than 6 months after receipt.

Cell viability assay

Following treatment with various concentrations of phospho-NSAIDs for 24 hours, the reduction of MTT dye was determined according to the manufacturer’s protocol (Promega).

Cell kinetics analysis

5-Bromo-2′-deoxyuridine (BrdU) incorporation into newly synthesized cellular DNA was used to determine cell proliferation as described (24). For apoptosis, 1.0 × 10⁶ cells per well were treated with phospho-NSAIDs for 24 hours, trypsinized, stained with Annexin V–fluorescein isothiocyanate (× 100 dilution; Invitrogen) and propidium iodide (0.5 μg/mL; Sigma), and analyzed by FACScaliber (BD Bioscience). Cell-cycle progression was analyzed by flow cytometry as described (24).

Determination of reactive oxygen and nitrogen species

After treatment, cells were collected by trypsinization, resuspended in 10 μmol/L of 5-(and-6)-carboxy-2′,7′-dichlorofluoresce diacetate (DCFDA; Invitrogen), incubated at 37°C for 30 minutes in the dark, and their fluorescence intensity was determined by flow cytometry (Beckman Coulter Inc.). We analyzed a minimum of 10,000 events with the WinMDI software and expressed the data as the fluorescent intensity versus events.

Determination of thioredoxin reductase activity

After treatment, cells were lysed and thioredoxin reductase (TrxR) activity was determined in the protein lysate by a commercially available kit, following the instructions of the manufacturer (Cayman Chemical). In this assay, TrxR uses NADPH to reduce 5,5′-dithiobis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid.

Protein extraction from whole-cell lysates

After treatment, cells were scraped on ice, washed with ice-cold PBS, and lysed in radioimmunoprecipitation assay lysis buffer (Sigma). Protein concentration was determined by the Bradford method (Bio-Rad).

Electrophoretic mobility shift assay

Nuclear fractions were isolated from 2 × 10⁶ cells (treated and controls) and subjected to electrophoretic mobility shift assay (EMSA) as previously described (24).

Liposome–PI

PI was loaded onto polyethylene glycolylated liposomes, following standard protocols (25, 26). Briefly, 50 mg of phosphatidylcholine, 19 mg of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N′[(methoxy(polyethylene glycol)-2000], and 40 mg of PI were dissolved in chloroform. The free drug was removed by dialyzing the liposomal solution against PBS, and PI concentration was determined by HPLC.

Efficacy in nude mouse breast, colon, and pancreatic xenografts

Female Balb/c nude mice (Charles River Laboratories) were inoculated s.c. into each of their flanks with 2.5 × 10⁶ MDA-MB-231 human breast cancer cells in Matrigel (BD Biosciences) or 1.5 × 10⁶ HT-29 colon cancer cells or 2 × 10⁶ BxPC-3 human pancreatic cancer cells in PBS (final volume 100 μL). Once the tumor reached approximately 100 to 150 mm³, animals were randomized into the control group, which received corn oil, and the treatment groups, which received PA 110 mg/kg or PS 160 mg/kg in corn oil or PI or liposome–PI (Lipo-PI) 300 mg/kg (n = 10 per group). The treatment was administered once daily for 5 days a week by oral gavage for PA and PS or intraperitoneally for PI and Lipo-PI. Tumor volume was calculated as [length × width × (length + width)/2] × 0.56. Tumor growth inhibition was calculated by dividing the percentage of increase from baseline of the tumor volume of the treated group over the corresponding percentage of volume increase of the control group. At the end of treatment, animals were sacrificed and tumors were removed and weighed.

Immunohistochemistry

Immunohistochemical staining for Ki-67, TrxR, and phospho–NF-κB (activated form of NF-κB) was carried out on
human breast, colon, and pancreatic xenograft tissue samples as previously described (27). Apoptosis was determined by the terminal deoxynucleotidyl transferase–mediated DUTP nick end labeling (TUNEL) assay (28). The positive control was treatment of samples with DNase I. At least 10 fields per sample (at magnification ×200) were scored independently by investigators blinded to their identity. Cells with a blue nucleus (or blue cytoplasm in the case of TrxR) were considered unlabelled, whereas those with a brown nucleus (or brown cytoplasm in the case of TrxR) were considered labeled. We calculated the percentage of proliferating and apoptotic cells by dividing the number of labeled cells by the number of cells in each field and multiplying by 100.

Statistical analyses
Results are expressed as mean ± SEM. Differences between groups were determined by 1-factor ANOVA followed by the Tukey test for multiple comparisons and the association between data sets by correlation analysis and computing the correlation coefficient (r). P < 0.05 was considered statistically significant.

Results
Phospho-NSAIDs inhibit the growth of human breast, colon, and pancreatic cancer in vitro and in xenografts
Initially, we evaluated the growth inhibitory effect of the 5 phospho-NSAIDs in 6 human cancer cell lines, 2 each from breast, colon, and pancreatic cancers. The members of each pair differ significantly from each other. Table 1 shows key genotypic features of each cell line and the 24-hour IC50 of these compounds, which range from 17 to 303 μmol/L. It seems that the growth inhibition potency of these compounds was independent of the tissue of origin, receptor status, or COX expression of these cell lines.

We then determined the effect of PA, PS, and PI on the growth of breast, colon, and pancreatic human cancer xenografts in athymic nude mice (Fig. 1). Our results are as follows: In MDA-MB-231 human breast cancer xenografts, PA (110 mg/kg) and PS (160 mg/kg) were both given orally daily, 5 days a week, starting when the tumor volume reached approximately 100 mm3 and were continued to day 37 when mice were euthanized. Compared with controls, both drugs inhibited tumor growth to a statistically significant extent between day 12 and the end of the study. On day 37, the tumor volume of each study group was as follows: control = 250 ± 48 mm3 (mean ± SEM for this and all subsequent values), PA = 119 ± 50 mm3, and PS = 87 ± 32 mm3, representing growth inhibition of 87.3% (tumor stasis) by PA and 108.6% (tumor regression) by PS (P < 0.01–0.05 for both). For HT-29 human colon cancer xenografts, PS 160 mg/kg was given orally daily, 5 days a week, starting when the tumor volume was approximately 150 mm3. Tumor growth inhibition became statistically significant starting on treatment day 9. After 17 days of treatment, the tumor volume of the control group was 1,020 ± 130 mm3, and that of the PS-treated group was 554 ± 96 mm3 (mean ± SEM for both groups), representing a 54.0% tumor growth inhibition by PS (P < 0.05). For BxPC-3 human pancreatic cancer xenografts, PI 300 mg/kg, plain or incorporated in Lipo-PI, was given intraperitoneally daily, 5 days a week for 20 days, starting when the tumor volume was approximately 100 mm3. At sacrifice, the tumor volume of each study group was as follows: control = 246 ± 40 mm3 (mean ± SEM for this and all subsequent values), PI = 157 ± 20 mm3, and Lipo-PI = 90 ± 23 mm3. After 20 days of treatment, PI reduced tumor growth by 57% (not statistically significant), whereas Lipo-PI completely prevented tumor growth (100% inhibition, P < 0.01–0.05), maintaining tumor stasis throughout the treatment period. Underscoring the significance of the drug delivery method, the mouse plasma level of PI was 3.5-fold higher in Lipo-PI than in PI (data not shown). Of note, the liposomal formulation of PI had a minimal effect on its in vitro IC50 (Supplementary Fig. S2).

Consistent with our previous findings (20, 21), none of the animals showed any signs of toxicity from the phospho-NSAIDs, including weight loss or distress. In addition, at autopsy at sacrifice there were no signs of gastrointestinal bleeding or gastrointestinal, pancreatic, renal, or liver damage.

Table 1. The effect of phospho-NSAIDs on the growth of human cancer cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>Breast</th>
<th>Colon</th>
<th>Pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF-7</td>
<td>MDA-MB-231</td>
<td>HT-29</td>
</tr>
<tr>
<td></td>
<td>ER+, PR+</td>
<td>ER+, PR+, HER2/Neu</td>
<td>COX-2+</td>
</tr>
<tr>
<td>PS</td>
<td>62</td>
<td>17</td>
<td>65</td>
</tr>
<tr>
<td>PDS</td>
<td>38</td>
<td>18</td>
<td>70</td>
</tr>
<tr>
<td>PF</td>
<td>65</td>
<td>17</td>
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<tr>
<td>PI</td>
<td>79</td>
<td>28</td>
<td>82</td>
</tr>
<tr>
<td>PA</td>
<td>32</td>
<td>199</td>
<td>54</td>
</tr>
</tbody>
</table>

NOTE: Values are representative of at least 3 independent experiments carried out in quintuplicate; results were within approximately 15%. Abbreviations: ER, estrogen receptor; PDS, phospho-desoxy-sulindac; PF, phospho-flurbiprofen; PR, progesterone receptor; WT, wild type.
The cytokinetic effect of phospho-NSAIDs

Inhibition of cell proliferation.

At their IC50, the 5 phospho-NSAIDs inhibited the proliferation of MDA-MB-231 breast cancer cells by 37% to 87% compared with controls, with PS being the most and PA the least potent. Similar results were obtained in BxPC-3 human pancreatic cells. The phospho-NSAIDs also inhibited cell proliferation in xenografts from these cell lines by 29% to 59%, except for plain PI, for which the effect (13% reduction) was not statistically significant and contrasted with that of Lipo-PI. These results are shown in Table 2, Fig. 2A, and Supplementary Table S1 and Fig. S3.

Induction of apoptosis.

We distinguished 2 stages of apoptosis, discernible by flow cytometry, namely "early apoptosis," in which the cells are viable (they exclude propidium iodide) and "late apoptosis," known also as secondary necrosis, in which the cells cannot exclude propidium iodide. The main findings in MDA-MB-231 cells are as follows: (i) late apoptosis predominates; (ii) PI had the strongest and PA the weakest proapoptotic effect; and (iii) despite their minimal structural differences, phospho-desoxy-sulindac (PDS) was more potent than PS. Of note, PI evaluated in BxPC-3 cells had similar effects. In breast, colon, and pancreatic xenografts PA, PS, and PI induced apoptosis significantly, ranging between 71% and 171% over controls. These results are shown in Table 2, Fig. 2B, and Supplementary Table S1 and Fig. S4.

Induction of necrosis.

All 5 compounds induced necrosis modestly, not exceeding 7.2% of the total number of MDA-MB-231 cells (Supplementary Table S1). As with apoptosis, PI was the most potent inducer of cell necrosis, generating similar results in BxPC-3 cells (Supplementary Fig. S4A).

Induction of cell-cycle arrest.

All compounds inhibited the G1 to S cell-cycle phase transition in MDA-MB-231 cells, except for PS, which arrested the G2–M phase transition (Supplementary Fig. S5A and S5B). Similar effects were obtained in BxPC-3 cells treated with PI (Supplementary Fig. S5C).

Induction of reactive oxygen and nitrogen species: correlation with cytokinetic changes

Reactive oxygen and nitrogen species (RONS) play a crucial role in the mechanism of action of several anticancer agents (29–31). We determined the effect of phospho-NSAIDs on the intracellular levels of RONS by the general RONS probe, DCFDA. Compared with control by 1 hour and at 1.5 × IC50, RONS levels were enhanced 3.3-fold by PI (BxPC-3 cells), 3-fold by PA (MDA-MB-231 cells), and 1.9-fold by PS (MDA-MB-231 cells). These results are shown in Table 2, Fig. 2A, and Supplementary Table S1 and Fig. S3.

### Table 2. The effect of phospho-NSAIDs on cytokinetics in xenografts

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-231</th>
<th>HT-29</th>
<th>BxPC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>Inhibition of proliferation, % over control</td>
<td>32</td>
<td>59</td>
</tr>
<tr>
<td>PS</td>
<td>P &lt; 0.05</td>
<td>NS</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>PI</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.005</td>
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</tbody>
</table>

NOTE: Each statistical comparison is to the respective control group.
Abbreviation: NS, not significant.
by PS (HT-29 cells); this effect was concentration dependent (MDA-MB-231 cells; Fig. 3A). Interestingly, pretreatment of the cells with 10 mmol/L N-acetyl-cysteine (NAC) abrogated RONS induction (completely in MDA-MB-231 cells and by 59% in BxPC-3 cells treated with PI; Fig. 3A and Supplementary Fig. S6A).

A similar effect was documented in vivo, by measuring the urinary levels of F2-isoprostane in mice with MDA-MB-231 and HT-29 xenografts by between 53% and 338% compared with the corresponding controls (all changes were statistically significant). We then assessed whether the induction of oxidative stress by phospho-NSAIDs correlated with the cytokinetic changes induced by these compounds in the xenografts (Fig. 3B). In both MDA-MB-231 and HT-29 xenografts, the urinary F2-isoprostane levels were positively associated with the percentage of apoptotic cells ($R = 0.824, P < 0.01$ and $R = 0.720, P < 0.02$, respectively) and negatively associated with the percentage of proliferating cells ($R = 0.757, P < 0.01$ and $R = 0.781, P < 0.05$, respectively) by PS (Fig. 3B and Table 3).

**Figure 2.** The cytokinetic effect of phospho-NSAIDs on MDA-MB-231 cells in vitro and in vivo. A, top, cell proliferation assay based on BrdU incorporation into DNA during the S-phase of the cell cycle. MDA-MB-231 cells were grown overnight and treated with various phospho-NSAIDs as shown. The percentage of BrdU-positive cells after treatment with PA is shown in the right upper corner of each panel. The bar graph summarizes the finding with each phospho-NSAID. The study was repeated at least once generating results within 11%. Bottom, cell proliferation was determined by Ki-67 expression in tissue sections of MDA-MB-231 xenografts harvested at sacrifice. The bar graph shows the percentage of Ki-67-positive cells. Values are mean ± SEM; * P < 0.01 compared with control. Three representative tissue sections are shown; magnification ×200. B, top, flow cytometric analysis of cells stained with propidium iodide and Annexin V (A). A(−)/PI(−) cells are viable cells, A(−)/PI(+) are early apoptotic, A(+)PI(−) are late apoptotic, and A(+)PI(+) are necrotic. The numbers inside each panel represent the percentage of cells in each category. The bar graph summarizes the finding with each phospho-NSAID. The study was repeated at least once generating results within 10%. Bottom, apoptosis was determined by the TUNEL assay in tissue sections of MDA-MB-231 xenografts harvested at sacrifice. The bar graph shows the mean ± SEM values of each group; * P < 0.01 compared with control. Three representative tissue sections are shown; magnification ×200. FITC, fluorescein isothiocyanate.
cells ($R = -0.78$, $P < 0.01$ and $R = -0.60$, $P < 0.05$, respectively; data not shown). These findings suggest a potential etiologic connection between oxidative stress and the cytokinetic changes in tumor xenografts that underlie the antitumor effect of the phospho-NSAIDs.

**Phospho-NSAIDs inhibit the thioredoxin system**

A critical redox modulator in the cells is the thioredoxin (Trx) system (34). We have shown that the thioredoxin system could mediate redox-induced cell death in response to various anticancer agents (31). Trx-1, overexpressed in human cancers, has been linked to aggressive tumor growth, inhibition of apoptosis, and decreased patient survival (35). In cultured MDA-MB-231 cells, the phospho-

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### Table 3. The effect of phospho-NSAIDs on urinary F2-isoprostane levels in mice

<table>
<thead>
<tr>
<th>Xenograft</th>
<th>Control</th>
<th>PA</th>
<th>PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>4.2 ± 1.3</td>
<td>9.4 ± 2.2 (124%)</td>
<td>18.4 ± 3.7 (338%)</td>
</tr>
<tr>
<td>HT-29</td>
<td>18.7 ± 1.3</td>
<td>ND</td>
<td>28.6 ± 4.2 (53%)</td>
</tr>
</tbody>
</table>

NOTE: Each statistical comparison is to the respective control group. Abbreviation: ND, not determined.

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Figure 3. The effect of phospho-NSAIDs on RONS levels in breast, colon, and pancreatic cancer cells and xenografts. A, top, MDA-MB-231, BxPC-3, and HT-29 cells were preloaded with DCFDA, a general probe for RONS, and treated with PA, PI, or PS for 1 hour as shown. The values in parentheses are the geometric mean of the respective histograms. Bottom, left, RONS levels, measured with DCFDA, were quantified in MDA-MB-231 cells after treating them with 1 × or 2 × IC50 phospho-NSAIDs for 1 hour. Bottom, right, RONS levels, detected by DCFDA flow cytometry, were decreased following pretreatment with NAC. B, the levels of F2-isopostane were determined by an ELISA kit, as described in Materials and Methods, in 24-hour urine of nude mice bearing MDA-MB-231 or HT-29 xenografts. Values are mean ± SEM; *, $P < 0.05$ and **, $P < 0.01$ compared with control. Right, the association between apoptosis (TUNEL+) cells in xenografts and urinary F2-isoprostane levels.
NSAIDs decreased the expression of Trx-1 (Fig. 4A). The effect of phospho-NSAIDs on Trx-1 expression in xenografts was variable. In BxPC-3 xenografts, Lipo-PI decreased Trx-1 expression levels by 61.6% \( (P < 0.001) \) but plain PI failed to do so, in keeping with its lack of antitumor effect (Supplementary Fig. S6B). PA and PS also decreased Trx-1 expression in MDA-MB-231 xenografts by 75% and 63%, respectively \( (P < 0.05 \text{ for both}; \text{Fig. 4A}) \). However, in HT-29 xenografts, PS did not decrease Trx-1 levels, determined both by immunoblotting of tissue extracts and immunohistochemistry (Supplementary Fig. S6C).

TrxR, overexpressed in many cancers and associated with drug resistance, represents a key drug target (36). Thus, we determined the activity of TrxR in cultured breast, colon, and pancreatic cancer cells treated with our phospho-NSAIDs. As shown in Fig. 4B, in MDA-MB-231, BxPC-3, and HT-29 cells, our 5 phospho-NSAIDs reduced TrxR activity by 53% to 84%, compared with controls (statistically significant for all). PA and PS also decreased the expression of TrxR in MDA-MB-231 xenografts by 43% and 51% \( (P < 0.03 \text{ and } P < 0.02, \text{ respectively}; \text{Fig. 4A}) \). Furthermore, in HT-29 xenografts, PS reduced TrxR activity by 40% \( (P < 0.02) \) and TrxR levels determined immunohistochemically by 55% \( (P < 0.01; \text{Fig. 4C}) \).

**Phospho-NSAIDs inhibit the activation of NF-κB**

To further assess the biologic relevance of the induction of oxidative stress by phospho-NSAIDs, we explored their effect on an intracellular redox-dependent signaling pathway. Thus, we analyzed their effect on NF-κB, in which transcriptional
activity is sensitive to redox changes (37). NF-κB modulates tumor cell growth, with the outcome depending on biologic context (38).

In vitro, all 5 compounds inhibited NF-κB activation determined by the EMSA in MDA-MB-231 breast cancer cells (Fig. 5A). A similar inhibitory effect was observed in vivo; we assessed NF-κB activation in xenografts by determining immunohistochemically the levels of p-p65 (Fig. 5B). As shown in Table 4, in MDA-MB-231, HT-29, and BxPC-3 xenografts, PA and PS, PI and Lipo-PI reduced the percentage of p-p65-positive cells by 21% to 70% (all statistically significant). Of note the effect of the more effective Lipo-PI was nearly double that of plain PI.

Discussion

Our data show in preclinical models of breast, colon, and pancreatic cancer the significant anticancer properties of phospho-NSAIDs, representing a broader class of compounds. Phospho-NSAIDs exert their anticancer effect by inducing a state of oxidative stress in tumors that then modulates intracellular signaling pathways, such as NF-κB.

Table 4. The effect of phospho-NSAIDs on NF-κB activation in xenografts

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-231</th>
<th>HT-29</th>
<th>BxPC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>44%</td>
<td>56%</td>
<td>35%</td>
</tr>
<tr>
<td>PS</td>
<td>64%</td>
<td>36%</td>
<td>43%</td>
</tr>
<tr>
<td>PI</td>
<td>21%</td>
<td>33%</td>
<td>38%</td>
</tr>
<tr>
<td>Lipo-PI</td>
<td>70%</td>
<td>57%</td>
<td>59%</td>
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</table>

Inhibition of NF-κB, % over control

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-231</th>
<th>HT-29</th>
<th>BxPC-3</th>
</tr>
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<tbody>
<tr>
<td>PA</td>
<td>0.04</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>PS</td>
<td>0.03</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>PI</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Lipo-PI</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
</tr>
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</table>

NOTE: Each statistical comparison is to the respective control group.
an effect that culminates in enhanced apoptosis and suppressed cancer cell renewal.

The inhibition of cancer growth by phospho-NSAIDs has 5 features. First, it is quantitatively significant, as evidenced by the effect of the 3 compounds tested in xenograft tumor models. Second, the degree of tumor inhibition differs between compounds, with PS being the most efficacious. Third, the response of tumors to a given compound depends on the origin of the tumor, as shown with PS, which caused tumor stasis in breast cancer xenografts but only modest growth inhibition in colon cancer xenografts. Fourth, the mode of drug delivery seems to influence drug efficacy substantially, as shown in the case of PI, in which efficacy was significantly increased by its incorporation into liposomes. Finally, in contrast to conventional NSAIDs, the phospho-NSAIDs tested in mice appear to be safe, a finding consistent with our previous results (20, 21).

An interesting observation concerns the relatively high IC50 values of our compounds in vitro that contrast with their in vivo efficacy. Although counterintuitive, there are numerous examples of such discordant behavior, including difluoromethylornithine (DFMO), aspirin, and valproic acid, that have IC50 values in the millimolar range but are quite effective in animals and humans (5, 39, 40). What underlies this difference is not readily apparent, but the differential complexity of the 2 systems affects the ability of in vitro IC50 values to predict in vivo responses.

As might be anticipated, tumor growth inhibition by these compounds is associated with a cytokinetic effect. All phospho-NSAIDs induced apoptosis and inhibited proliferation to a quantitatively significant but varying degree; neither effect, however, seemed dominant. Of interest, our cell culture studies mirror to some extent the in vivo cytokinetic effect of these compounds.

A key feature of the mechanism of action of phospho-NSAIDs was their ability to induce oxidative stress. This effect was noted in both cultured cells and animals. In cultured cells, the induction of oxidative stress levels was significant, concentration dependent, and inhibitable by the antioxidant NAC. Again, there were individual variations, depending on the cell line and the compound. In animals, oxidative stress was assayed by the urinary levels of F2-isoprostane. This metabolite of 15-isoprostane F2α (PGF2αt) provides a validated measure of oxidative stress in the animal over some period of time (usually hours). As we have shown recently, in the case of PI, the xenograft is indeed the source of the increased F2-isoprostane (30). Thus, we can extrapolate that the oxidative stress that we observed in these studies originated in the xenografts. The induction of oxidative stress by the phospho-NSAIDs was a consequential event. There is a clear association between oxidative stress and cytokinetic changes (enhanced apoptosis, suppressed proliferation) both in vitro and, importantly, in vivo. To a first approximation, these changes underlie the tumor-inhibitory effect of these compounds, making the redox homeostatic mechanism their prime molecular target.

Although cell signaling tends to be complex, the effect of the phospho-NSAIDs on the NF-κB pathway illustrates a possible link between drug-induced oxidative stress and cancer growth inhibition. It is conceivable that the anticancer effect of phospho-NSAIDs is related in part to their redox effect on NF-κB, a prototypical redox-sensitive signaling pathway with relevance to cancer. Constitutive NF-κB activation is a hallmark of various types of cancer, including breast, colon, and pancreatic (41–44). NF-κB, activated by oncogenes, carcinogens, and inflammatory stimuli, promotes carcinogenesis and consequently has become a therapeutic target in cancer. When the strategically located Cys62 of NF-κB is oxidized, the NF-κB dimer is rendered incapable of binding to DNA and thus loses its extensive transcriptional effects. Phosphono-NSAIDs inhibited NF-κB activation both in vitro and in vivo. This is a broader property of phospho-NSAIDs, which we recently showed to inhibit NF-κB activation in arthritis-associated inflammation (20). It is plausible that the effect of phospho-NSAIDs mediates part of their anticancer effect.

Equally complex is the mechanism by which these compounds induce oxidative stress. The central role of ROS as an apoptotic trigger in response to phospho-NSAIDs was documented recently (30). In this work, we focused on the thioredoxin system that consists of Trx, TrxR, and NADPH. A pivotal component of cellular redox homeostasis, the thioredoxin system, is involved in carcinogenesis and also in the response of tumors to chemotherapy (45–47). When RONS oxidize a cellular protein, thioredoxin reduces it, being itself oxidized in the process; TrxR restores the oxidized Trx to its normal status. Phospho-NSAIDs had a profound effect on the 2 protein members of this system, Trx-1 and TrxR. The expression of both was suppressed by phospho-NSAIDs and, in addition, the activity of TrxR (the only one of the 2 that was assayed for) was suppressed, generating a substantial cumulative effect. In only one case (HT-29 xenografts) the expression of Trx-1 was not suppressed by a phospho-NSAID (PS), but both the activity and expression of TrxR was suppressed, likely inactivating the thioredoxin system. Of note, PS did suppress Trx-1 expression in HT-29 xenografts treated under a different drug administration protocol (22). It is, therefore, clear, that based on the role of the thioredoxin system this effect contributes to the induction of the all-important state of oxidative stress with its direct repercussion on tumor mass (loss of cells through apoptosis and dampened cell renewal). Indeed, between the 2, TrxR was the one that was suppressed the most by the phospho-NSAIDs. Consistently, we have shown in an effective combination study of PS and DFMO that TrxR correlated with tumor growth, stressing the importance of the Trx system as a molecular target of these drugs (22).

In conclusion, our findings indicate that these 5 phospho-NSAIDs, belonging to the broader pharmacologic category of modified-NSAIDs, show strong anticancer efficacy and promising safety. Of particular interest is their shared ability to induce oxidative stress, an early event that seems to trigger cancer-inhibitory cascades. Taken together, our data indicate that phospho-NSAIDs merit further evaluation as anticancer agents.

Disclosure of Potential Conflicts of Interest

B. Rigas has an equity position in Medicon Pharmaceuticals, Inc., and D. Komninou is an employee of Medicon Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.
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Liqun Huang, Gerardo G. Mackenzie, Yu Sun, et al.

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