Abstract. We have synthesized a series of novel phenylester compounds and present our assessment of such a derivative of aspirin, 3-((diethoxyphosphoryloxy)methyl)phenyl 2-acetoxybenzoate, provisionally named phosphoaspirin. We determined its anticancer activity both in vitro and in vivo. Phosphoaspirin inhibited the growth of HT-29 human colon adenocarcinoma cells \( IC_{50} = 276.6\pm12.3 \text{ μM (mean ± SEM)} \) through a combined antiproliferative and mainly proapoptotic effect. Phosphoaspirin (100 mg/kg body weight intraperitoneally daily for 21 days) also inhibited the growth of HT-29 tumors grown as xenografts in nude mice. The size of the tumors decreased progressively in the phosphoaspirin treated group, compared to controls, being reduced by 57% \((p<0.001)\) on day 21. Phosphoaspirin achieved this effect by modulating cell kinetics; the proliferation index of cancer cells was reduced by 18.13% compared to controls \((p<0.001)\) and the apoptosis index was increased by 94.6% \((p<0.003)\). There was no apparent toxicity from phosphoaspirin. We conclude that phosphoaspirin is a promising agent for the control of cancer that deserves further evaluation.

Materials and methods

Reagents and cell culture. Phosphoaspirin (3-((diethoxyphosphoryloxy)methyl)phenyl 2-acetoxybenzoate) was synthesized as described (3). Stock (100 mM) solutions were prepared in DMSO (Fisher Scientific, Fair Lawn, NJ); the final DMSO concentration was adjusted in all media to 1%. HT-29 human colon adenocarcinoma cells were from American Type Tissue Collection, Rockville, MD. Cells were grown in 5% CO₂ at 37°C in McCoy 5A medium supplemented with 10% fetal calf serum, 10,000 IU/ml penicillin, and 10 mg/ml streptomycin.

Growth of HT-29 cells in immunosuppressed mice. Ten million HT-29 cells suspended in PBS were mixed 1:1 (v/v) with matrigel matrix (BD Biosciences, San Jose, CA) and injected subcutaneously into the flank of nude mice; the total volume was adjusted to 100 μl. The size of the xenografts was recorded using digital calipers and calculated with the formula \( V = L \times W \times L + W / 2 \times 0.56 \) \((V = \text{volume}, L = \text{length}, W = \text{width})\). When animals were sacrificed at the end of the experiment, tumors were weighed and there was an excellent correlation between tumor volume based on weight and pre-sacrifice measurements. When the tumors grew to an easily measurable size, two groups were selected having tumors within a rather limited range of sizes and were divided into two groups of 11 animals each, based on tumor volumes. The tumor volume of the control group was 194.00±18.13 mm³ and of the treatment group 202.91±21.61 mm³ \((\text{mean ± SEM; groups statistically not different})\). Animals were started on phosphoaspirin...
100 mg/kg body weight or vehicle, injected intraperitoneally daily for 21 days, when they were euthanized and the xenotransplants were excised and immediately weighed.

**Determination of proliferation and apoptosis**. Proliferation and apoptosis of cancer cells were determined histochemically, by staining for proliferating cell nuclear antigen (PCNA) and by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) assay, respectively, as described (4).

**PCNA staining**. Tumor specimens fixed in formalin were embedded in paraffin and 4-μm sections were mounted on microscope slides, deparaffinized, rinsed with PBS, and incubated with 1% hydrogen peroxide for 10 min to block the endogenous peroxidase activity. They were then heated in a microwave oven for 3 min covered with 0.01 M citrate buffer (pH 6.0). The anti-PCNA antibody (FL-261, Santa Cruz Biotechnology, Santa Cruz, CA) was applied at 1:100 dilution at 4°C overnight. The slides then received three 5-min washes with PBS, followed by incubation with the biotinylated secondary antibody using the IHC Select kit (Chemicon, Temecula, CA) was applied at 1:100 dilution at 4°C overnight. The slides then received three 5-min washes with PBS, followed by incubation with the biotinylated secondary antibody using the IHC Select kit (Chemicon, Temecula, CA) following the instructions of the manufacturer. The chromogen was 3,3′-diaminobenzidine (DAB) (Sigma) and slides were counterstaining with hematoxylin. Positive control; treatment of samples with DNase I.

**TUNEL staining**. TUNEL staining was performed in formalin-fixed, paraffin-embedded tissues that were cut 4-μm thick and deparaffinized. We used the In Situ Cell Death Detection Kit, POD (Roche) and followed the instructions of the manufacturer. Briefly, nuclear protein was hydrolyzed using proteinase K, biotinylated d-UTP was applied and incorporated into DNA strand breaks using the TdT enzyme. The chromogen was DAB and counterstaining was with hematoxylin. Positive control; treatment of samples with DNase I.

**Scoring**. Using a light microscope at magnification x400, at least 10 fields per sample were scored independently by two investigators blinded to the identity of the samples. Cells with a blue nucleus were considered unlabeled, while those with a brown nucleus were considered labeled. For apoptosis, to exclude the possibility that necrosis produced TUNEL-positive cells (instead of apoptosis), we carefully examined each field, applying the criteria established by Ben-Sasson et al (5). In our study, there was no evidence of necrosis. We calculated the proliferation index (PI) and apoptotic index (AI) by dividing the number of labeled cells by the number of cells in each field and multiplying by 100.

**Statistical analysis**. Data were analyzed using the t-test; p<0.05 was considered statistically significant.

**Results**

**Phosphoaspirin inhibits the growth of HT-29 cells**. The IC₅₀ for cell growth by phosphoaspirin at 24 h was 276.6±12.3 μM (mean ± SEM for this and all subsequent values). It is clear that phosphoaspirin has an in vitro potency similar to that observed with the corresponding nitroaspirin (meta positional isomers, based on the position of the phosphor- or nitro-group on the benzene ring, with respect to the ester bond between aspirin and the spacer moiety linking the two) (6). At its IC₅₀ concentration, phosphoaspirin inhibited proliferation by 19% compared to controls whereas the apoptotic cells were 38% of the total compared to 4% in untreated controls.

**Phosphoaspirin inhibits the growth of HT-29 xenotransplants**. Phosphoaspirin induced no apparent adverse effects on the mice during its 3 weeks of administration, as described above. The body weights of mice administered phosphoaspirin were comparable to those of the control group throughout the study (data not shown); at sacrifice the treated group weighed 23.14±1.04 g vs. 23.58±0.66 g. We also noted no gastrointestinal erosions or other signs of toxicity nor any gross changes indicative of toxicity in several organs that we examined.

The size of the tumors showed a progressive decrease in the phosphoaspirin treated group, compared to controls as shown in Fig. 2. At sacrifice, the weight of the tumors, the most unambiguous measure of their size, was 1.05±0.12 g in controls vs. 0.45±0.04 g in treated mice (p<0.001), representing a 57% reduction.

Phosphoaspirin achieved this effect by modulating cell kinetics (Fig. 3). Proliferation of the cancer cells was reduced by 18.13% compared to controls; the proliferation index was 75.99±2.28 in controls vs. 62.21±2.82 in treated mice (p<0.001). Phosphoaspirin also induced apoptosis briskly; the apoptosis index was essentially doubled from 6.87±0.70 in controls to 13.37±1.52 in treated mice (increase of 94.6%);
Discussion

Our findings establish the anticancer effect of this novel compound both *in vitro* and *in vivo*. The growth of human HT-29 cancer cells, a widely used cell line to study colon cancer, was inhibited by phosphoaspirin. The animal study clearly demonstrated the anticancer properties of phosphoaspirin. Xenograft models represent a useful system to study human cell lines *in vivo* and they are used extensively for the preclinical evaluation of novel compounds. The changes observed in cultured cells and in the animal model are in agreement with each other and quantitatively significant. It is of interest that the reduction in tumor size increased progressively over the period of observation showing a steadily downward slope.

To a first approximation the anticancer effect of phosphoaspirin is based on a strong and favorable cytokinetic effect. Although proliferation is inhibited significantly, the dominant effect appears to be the induction of apoptosis. Again, the findings in cultured cells and the animal study are congruent, suggesting that this is likely the mechanism of action of phosphoaspirin. The molecular details underlying this effect remain to be determined.
Mechanistically, this study is significant, as it indicates that the phenylester derivatives of at least aspirin possess significant anticancer properties and introduce the possibility that they may be clinically useful. There are three reasons to consider that a new extensive class of compounds may emerge. First, the synthesis of such compounds is relatively facile and simple, lending itself to large-scale methodologies. Second, many of the currently available anticancer compounds possess the appropriate structural features for such modification. And, third, there are several additional groups that can substitute the diethyl phosphate group (e.g., Cl, tosylate).

These findings bear indirectly on the question of whether the NO released from nitroaspirin plays a role on its anticancer properties. Our data cannot provide an answer to so complex a question. What is apparent is that a molecule identical to nitroaspirin, save for the substitution of the NO-donating moiety by diethyl phosphate, did inhibit the growth of cancer cells in vitro and the growth of their xenograft. This effect is similar to that reported previously by us for nitroaspirin, although the animal models were not the same (7,8). In fact, no direct in vivo comparisons between nitro- and phospho-aspirin exist to generate an unambiguous conclusion. It appears, however, likely that the role of NO in the effect of nitroaspirin may not be as critical as initially thought. It is, however, important to keep in mind that NO-donating compounds that have an entirely different spacer have been effective in the prevention of colon cancer in an animal model (8).

In conclusion, our studies have demonstrated that phospho-aspirin is an effective anticancer agent in vitro and in vivo in an animal tumor model. The basis of this effect is a strong cytokinetic effect, with the induction of apoptosis being its most important component. Our findings suggest that a new class of phenylester-centered anticancer compounds is a realistic possibility and their role in cancer control should be explored further.

References


