Structure-activity relationship study of novel anticancer aspirin-based compounds

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Abstract. We performed a structure-activity relationship (SAR) study of a novel aspirin (ASA) derivative, which shows strong anticancer activity *in vitro* and *in vivo*. A series of ASA-based benzyl esters (ABEs) were synthesized and their inhibitory activity against human colon (HT-29 and SW480) and pancreatic (BxPC-3 and MIA PaCa-2) cancer cell lines was evaluated. The ABEs that we studied largely comprise organic benzyl esters bearing an ASA or acyloxy group (X) at the *meta* or *para* position of the benzyl ring and one of four different leaving groups. The nature of the salicyloyl/acyloxy function, the leaving group, and the additional substituents affecting the electron density of the benzyl ring, all were influential determinants of the inhibitory activity on cancer cell growth for each ABE. Positional isomerism also played a significant role in this effect. The mechanism of action of these compounds appears consistent with the notion that they generate either a quinone methide or an *m-oxybenzyl* zwitterion (or an *m-hydroxybenzyl* cation), which then reacts with a nucleophile, mediating their biological effect. Our SAR study provides an insight into the biological properties of this novel class of compounds and underscores their potential as anticancer agents.

Introduction

Accounting for nearly one quarter of the deaths in the US, cancer remains one of the major medical challenges of our time. Despite concerted efforts, the US death rate from cancer has remained unchanged in recent decades, in contrast to that of heart disease, which has decreased by >60% (1). Much of the progress in cardiology stems from the development of a wide variety of novel agents aimed at both the treatment and prevention of cardiovascular diseases. The oncology field, although it has witnessed some remarkable advances, is still in considerable need of new and effective agents.

We recently reported our findings on the anti-cancer activity of a novel benzyl ester-based derivative of acetylsalicylic acid (ASA) or aspirin, provisionally named phosphoaspirin (Fig. 1, compound **1a**). This compound exhibited two potentially significant properties: i) anticancer efficacy in a murine model of cancer, achieving over 60% reduction in tumor volume of xenografted HT-29 human colon cancer cells, and ii) limited toxicity, evidenced, among other parameters, by no reduction in body weight during treatment and no organ damage on necropsy (2). The mode of action of this compound includes, at the cytokinetic level, brisk induction of apoptosis and to a lesser degree, suppression of proliferation. Of note, similar cytokinetic effects were observed in cultured HT-29 cells, in which the 24-h IC_{50} for the compound **1a** was 276 μM (2). The *para* positional isomer of phosphoaspirin (compound **1b**) inhibited the growth of 10 human cancer cell lines originating from the colon, lung, liver, pancreas and breast, with an 18- to 144-fold greater potency than conventional ASA (3).

Prompted by these encouraging results, we developed a new series of ASA-benzyl esters, based on conventional ASA, which consist of benzyl derivatives having an ASA or an acyloxy substituent (Fig. 1). We have pursued the study of ASA-based benzyl esters (ABEs), primarily for their anti-cancer effects. In addition to synthesizing positional isomers of compound **1a**, we undertook a structure-activity relationship (SAR) study to ascertain those features of the molecule that contribute to its pharmacological activity. It is worthy of mention that compound **1a** is much more potent than conventional ASA of which it can be considered a derivative.

The potential importance of ASA and other NSAID (nonsteroidal anti-inflammatory drugs) derivatives in cancer control originates from their well-established efficacy in the prevention of human cancer and the accompanying understanding of their mechanisms of action, one of which centers on the role of cyclooxygenase-2 (COX-2) overexpression in carcinogenesis; inhibition of COX enzymes is the best recognized mode of

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action of NSAIDs. For example, epidemiological studies demonstrated that NSAID use prevents cancer of the colon by up to 50%, whereas interventional studies showed that ASA prevents 21-30% of colon polyp recurrence in humans (4-6). Combined with a vast amount of preclinical data, such findings underscore the potential of NSAIDs for cancer control (7).

Here, we report the synthesis of several congeners of compound **1a** and a SAR study with respect to their ability to inhibit cancer cell growth, a parameter that defines whether a drug can suppress cancer. Our findings provide a mechanistic understanding of the novel ABEs in terms of their chemistry.

Materials and methods

Synthesis of ABEs. Compounds **1c**, **1d**, **1e**, **1f**, **1g** and **2d** were prepared according to a published method (15). For the preparation of the diethyl phosphate esters, a general method is described illustrated by the synthesis of **1b**, which in this and all other cases began with the corresponding alcohol.

Reagents and relevant methods. All reagents and solvents were of commercial grade and used as such unless otherwise specified. ^{1}H NMR spectra were recorded on a Varian 300 spectrometer. Samples prepared for NMR analysis were dissolved in $CDCl_3$. Chemical shifts are reported in ppm relative to TMS. Electron ionization mass spectra were recorded on a Thermo Scientific DSQ (II) mass spectrometer. Thin-layer chromatography (TLC) was performed on silica gel sheets (Tiedel-deHaën, Sleeze, Germany) containing a fluorescent indicator. Flash column chromatographic separations were carried out on 60 Å (230-400 mesh) silica gel (TSI Chemical Company, Cambridge, MA). All experiments dealing with moisture- or air-sensitive compounds were conducted under dry nitrogen. The starting materials and reagents, unless otherwise specified, were the commercially best grade available (Aldrich, Fluka) and were used without further purification. All new products showed a single spot on TLC analysis in two different solvent systems, after purification.

General procedures

Synthesis of 2-acetoxy benzoic acid 4-(diethoxy phosphoryl oxymethyl) phenyl ester (**1b**): Diethylchlorophosphate (2.5 ml, 17.26 mmole) was added drop-wise to a solution of alcohol (**1e**, 1.9 g, 6.64 mmole) in methylene chloride (10 ml) containing diisopropylethylamine (2.2 ml, 13.28 mmole), followed by 4-(dimethylamino)pyridine (25 mg) as a solid. The reaction mixture was heated under reflux overnight. The reaction solution was washed with water (2x25 ml), dried over anhydrous sodium sulfate, filtered and concentrated. The crude residue was purified by column chromatography using hexane:ethyl acetate (60:40) as the eluant. The pure fractions were combined and evaporated to give a solid which was triturated with hot hexane several times to give pure title compound **1b** as a solid (690 mg, 25%). ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 2.3 (s, 3H), 4.05 (m, 4H), 5.05 (d, 2H), 7.2 (m, 3H), 7.4 (t, 1H), 7.45 (d, 2H), 7.62 (t, 1H), 8.20 (d, 1H). MS: 422 (M+).

Using the above general procedure, the following compounds were made, all of which were isolated as viscous oils:

2-Acetoxy benzoic acid 3-(diethoxy phosphoryloxymethyl) phenyl ester (**1a**): Yield 29%, ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 2.32 (s, 3H), 4.1 (m, 4H), 5.08 (d, 2H), 7.2 (m, 3H), 7.28 (d, 1H), 7.4 (m, 2H), 7.65 (t, 1H), 8.20 (d, 1H). MS: 422 (M+).

2-Acetoxy benzoic acid 3-(diethoxy phosphoryloxymethyl)-4-methoxy phenyl ester (**1h**): Yield 29%, ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 2.3 (s, 3H), 3.8 (s, 3H), 4.15 (m, 4H), 5.1 (d, 2H), 6.9 (d, 1H), 7.1 (d, 1H), 7.2 (m, 2H), 7.4 (t, 1H), 7.6 (t, 1H), 8.20 (d, 1H). MS: 452 (M+).

2-Acetoxy benzoic acid 4-(diethoxy phosphoryloxymethyl)-3-methoxy phenyl ester (**1i**): Yield 26%, ^{1}H NMR ($CDCl_3$): δ 1.4 (m, 6H), 2.4 (s, 3H), 3.9 (s, 3H), 4.2 (m, 4H), 5.2 (d, 2H), 6.8 (s, 1H), 6.83 (d, 1H), 7.22 (d, 1H), 7.5 (m, 2H), 7.75 (t, 1H) 8.3 (d, 1H). MS: 452 (M+).

2-Acetoxy benzoic acid 3-chloro-4-(diethoxy phosphoryloxymethyl) phenyl ester (**1j**): Yield 24%, ^{1}H NMR ($CDCl_3$): δ 1.39 (m, 6H), 2.3 (s, 3H), 4.15 (m, 4H), 5.18 (d, 2H), 7.18 (m, 2H), 7.2 4(d, 1H), 7.4 (t, 1H), 7.6 (d, 1H), 7.62 (t, 1H), 8.2 (d, 1H). MS: 456 (M+).

Acetic acid, 2-(diethoxy phosphoryloxymethyl) phenyl ester (**2a**): Yield 28%, ^{1}H NMR ($CDCl_3$): δ 1.38 (m, 6H), 2.05 (s, 3H), 4.22 (m, 4H), 5.2 (s, 2H), 7.17 (t, 1H), 7.32 (t, 1H), 7.4 (m, 2H). MS: 302 (M+).

Acetic acid, 3-(diethoxy phosphoryloxymethyl) phenyl ester (**2b**): Yield 29%, ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 2.3 (s, 3H), 4.05 (m, 4H), 5.02 (d, 2H), 7.02 (d, 1H), 7.1 (s, 1H), 7.24 (d, 1H), 7.4 (t, 1H). MS: 302 (M+).

Acetic acid, 4-(diethoxy phosphoryloxymethyl) phenyl ester (**2c**): Yield 30%, ^{1}H NMR ($CDCl_3$): δ 1.38 (m, 6H), 2.4 (s, 3H), 4.1 (m, 4H), 5.05 (d, 2H), 7.18 (d, 2H), 7.5 (d, 2H). MS: 302 (M+).

Acetic acid, 3-chloro-4-(diethoxy phosphoryloxymethyl) phenyl ester (**2d**): Yield 24%, ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 2.3 (s, 3H), 4.1 (m, 4H), 5.12 (d, 2H), 7.0 (d, 1H), 7.18 (s, 1H), 7.55 (d, 1H). MS: 336 (M+).

Benzoic acid, 3-(diethoxy phosphoryloxymethyl) phenyl ester (**3**): Yield 24%, ^{1}H NMR ($CDCl_3$): δ 1.33 (m, 6H), 4.11 (m, 4H), 5.09 (d, 2H), 7.20 (d, 1H), 7.26 (m, 2H), 7.5 (m, 3H), 7.62 (m, 1H), 8.20 (d, 2H). MS: 364 (M+).

Phosphoric acid, 4-(diethoxy phosphoryloxymethyl) phenyl diethyl ester (**4**): Yield 27%, ^{1}H NMR ($CDCl_3$): δ 1.34 (m, 12H), 4.12 (m, 4H), 4,22 (m, 4H), 4.66 (s, 2H), 7.2 (d, 2H), 7.32 (d, 2H). MS: 398 (M+).

Phosphoric acid, diethyl 4-fluoro benzyl ester (**5**): Yield 26%, ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 4.05 (m, 4H), 5.0 (d, 2H), 7.02 (m, 2H), 7.4 (m, 2H). MS: 262 (M+).

Phosphoric acid, benzyl diethyl ester (**6**): Yield 29%, ^{1}H NMR ($CDCl_3$): δ 1.3 (m, 6H), 4.05 (m, 4H), 5.02 (d, 2H), 7.4 (m, 5H). MS: 244 (M+).

Acetic (diethyl phosphoric) anhydride (**7**): Yield 28%, ^{1}H NMR ($CDCl_3$): δ 1.4 (m, 6H), 2.2 (s, 3H), 4.3 (m, 4H). MS: 196 (M+).

Cell culture studies

Cell lines. HT-29 and SW480 human colon adenocarcinoma cell lines, and BxPC-3 and MIA PaCa-2 human pancreatic adenocarcinoma cell lines (American Type Culture Collection, Manassas, VA) were grown as monolayers in either McCoy 5A medium (HT-29), RPMI-1640 (SW480 and BxPC-3) or DMEM (MIA PaCa-2). Media were supplemented with 10% fetal bovine serum (FBS; Mediatech, Herndon, VA) (except for DMEM that was supplemented with 2.5% horse serum); penicillin (50 U/ml); and streptomycin (50 mg/

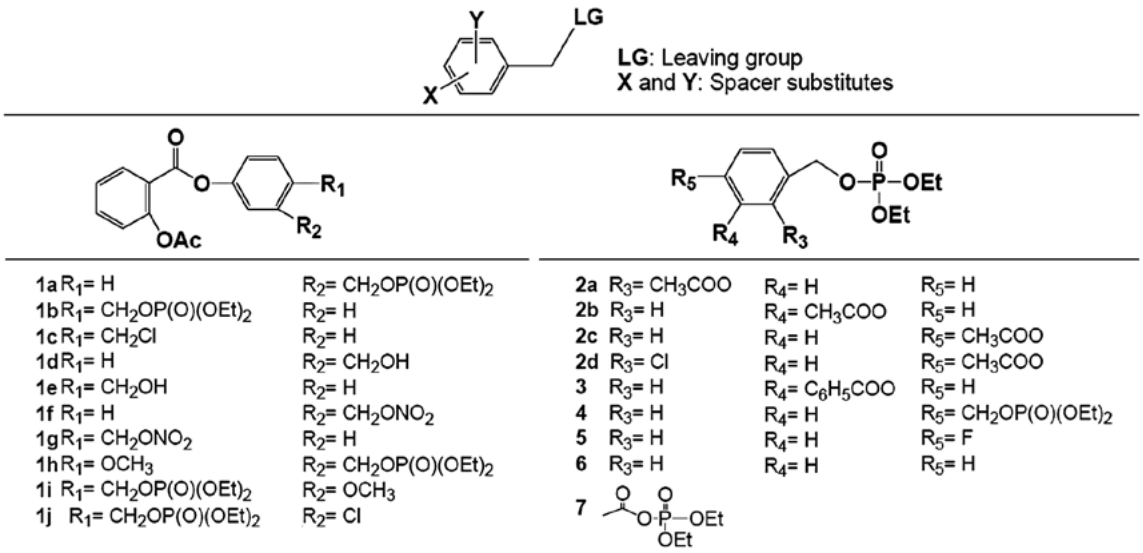


Figure 1. The chemical structure of the ABEs. X represents the salicyloyl/acyloxy group; the benzyl group is regarded as the spacer; LG represents the leaving group; the X group is at the *meta* or *para* position with respect to the benzylic methylene and the Y group is a second substituent on the benzyl ring.

ml) (Life Technologies, Inc., Grand Island, NY). Cells were seeded at a density of 1.5-3x10^4 cells/cm^2 in a culture dish and incubated at 37˚C in 5% CO_2 at 90% relative humidity. Single-cell suspensions were obtained by trypsinization (0.05% trypsin/EDTA), and cells were counted using a hemacytometer. Viability was determined by the trypan blue dye exclusion method.

Determination of IC_{50}. Cells, plated into 96-well plates (424 cells/mm^2), were grown overnight and then treated with various concentrations of each test compound for 24 h. Test compound stock (200 or 100 mM) solutions were prepared in DMSO (Fisher Scientific, Fair Lawn, NJ). The final DMSO concentrations were adjusted accordingly, but never exceeded 1%. After treatment, viable cells were assayed using the 'MTT cell proliferation assay' according to the manufacturer's instructions (Sigma). The plates were read at 595 nm, and the data were handled with SoftmaxPro Version 3.1.1 (Molecular Devices, Sunnyvale, CA). For each compound and each cell line, IC_{50} values were determined in triplicate.

Cell kinetic assay. Cells were seeded at a density of 5x10^4 cells/cm^2 and allowed to attach for 24 h, when various treatments were applied. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay following the protocol of the manufacturer (Roche Diagnostics, Indianapolis, IN). For cell proliferation assay, HT-29 cells, treated with **1a** and **1b**, were pulse-labeled with 10 mM bromodeoxyuridine (BrdU) (BD Bioscience, San Jose, CA) 30 min prior to harvesting and analyzed by flow cytometry. To measure cell death, cells were treated with **1a** or **1b** for 24 h, harvested by trypsinization, stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI) according to the manufacturer's protocol and analyzed by flow cytometry. For cell cycle analysis, cells were fixed using cold 70% ethanol and stained with PI following standard protocols prior to flow cytometric analysis.

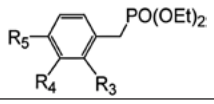
Results

We synthesized the compounds listed in Fig. 1 and used their ability to inhibit the growth of cancer cells *in vitro* as a measure of their anticancer pharmacological effect. For each compound, we determined its 24-h IC_{50}, i.e. the concentration of the test compound that inhibits cell growth 50% at 24 h. Under our experimental conditions, IC_{50} values >500 μM could not be determined accurately. We employed four human cancer cell lines, two derived from colon (HT-29 and SW480) and two from pancreatic cancer (BxPC-3 and MIA PaCa-2). In each pair, one cell line expresses COX-2 (HT-29 and BxPC-3) whereas the other does not. This is of potential mechanistic value, since our compounds are structurally related to ASA, whose best-recognized molecular target is COX, which is thought to play a role in carcinogenesis (8,9).

Effect of the X group. To assess the effect of the salicyloyl group (X group in Fig. 1) on the anticancer potency of the drug, we studied a series of compounds containing the benzyl spacer and diethylphosphate as the leaving group and as the X moiety of any one of the following groups: a salicyloyl group (**1b**), an acetoxy group (AcO-, **2a**, **2b** and **2c**), a benzoyloxy group (C_6H_5COO-, **3**), a diethylphosphate group [-OP(O)$(OEt)_2$, **4**], a fluorine atom (-F, **5**), or hydrogen (-H, **6**) (Table I). Most of these compounds are *p*-isomers.

It is clear from the results summarized in Table I and Fig. 3, that the X group plays a role in determining the anticancer potency of ABEs. We studied six different X groups in compounds sharing the same benzyl spacer and leaving groups. Of these, only the salicyloyl, acetoxy and benzoyloxy showed appreciable (<500 μM, i.e. measurable) potency in inhibiting cancer cell growth. All three have an enzyme cleavable ester bond. A convincing demonstration of the importance of the ester bond for biological activity was obtained when the acyloxy group was replaced by -OP(O)$(OEt)_2$ (**4**), F (**5**) or H (**6**). In all three cases, this change prevented the formation of drug

Table I. Effect of the acyloxy group on the IC_{50} for cancer cell growth.

Compound				IC_{50}, μM			
				BxPC-3	MIA PaCa-2	HT-29	SW480
1b	R_3 = H	R_4 = H	R_5 = ASA	57.1±1.8	90.8±0.9	39.3±2.9	90.3±2.8
2a	R_3 = CH_3COO	R_4 = H	R_5 = H	>500	>500	>500	>500
2b	R_3 = H	R_4 = CH_3COO	R_5 = H	>500	>500	>500	>500
2c	R_3 = H	R_4 = H	R_5 = CH_3COO	4.2±0.7	17.3±2.2	8.2±1.2	4.5±0.5
3	R_3 = H	R_4 = C_6H_5COO	R_5 = H	91.2±0.5	>500	447±14.3	347±23.5
4	R_3 = H	R_4 = H	R_5 = $P(O)(OEt)_2$	>500	>500	>500	>500
5	R_3 = H	R_4 = H	R_5 = F	>500	>500	>500	>500
6	R_3 = H	R_4 = H	R_5 = H	>500	>500	>500	>500

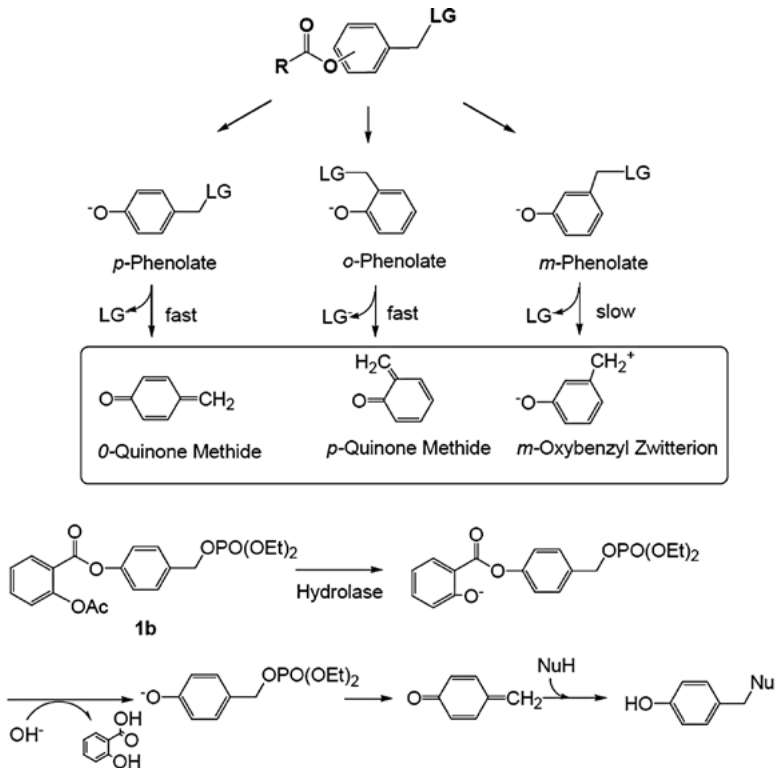


Figure 2. Proposed mechanism for the generation of ABE drug intermediates. Upper panel: most ABEs generate one of the three intermediates, *para* or *ortho* quinone methide or a methylphenol zwitterion, as discussed in the text. Lower panel: the transformation of *para* phosphoaspirin (**1b**). These reactions follow the mechanism shown in the upper panel.

intermediates and led to complete loss of biological activity. Of further interest, the IC_{50} of the *p*-acetoxy-containing compound (**2c**) was 5 to 20-fold lower than that of the *p*-ASA-containing compound (**1b**). The proposed mechanisms for compounds **1b** and **2c** are shown in Fig. 2, where compound **1b** needs one more step to generate quinone methide, thus leading to a lower reaction rate and lower pharmacological potency. It is, however, possible that the differential biological activity of **1b** and **2c** is due to different rates of hydrolysis of the ester bond (k_1>>k_1').

Requirement of the spacer for biological activity. The importance of the spacer for the biological activity of ABEs was directly assessed by omitting it from the most potent compound **2c**. This shortened version (compound **7**) exhibited virtually no activity against any of the cell lines (data not shown). If we consider compounds **3**, **5**, **6** as not containing a spacer molecule (which is reasonable), then the requirement for the spacer is underscored further; none of them had any detectable biological activity as determined in our assays.

Effect of the leaving group. To determine the effect of the leaving group on the pharmacological activity of ABEs, we studied a series of analogues in which X = ASA or AcO, and the leaving group was one of four different moieties, namely diethylphosphate [$-OP(O)(OEt)_2$], chloro (-Cl), hydroxyl (-OH) and nitrate ($-ONO_2$) (Fig. 4). Since previous study with nitrate esters established the essential equivalence of the *ortho*- and

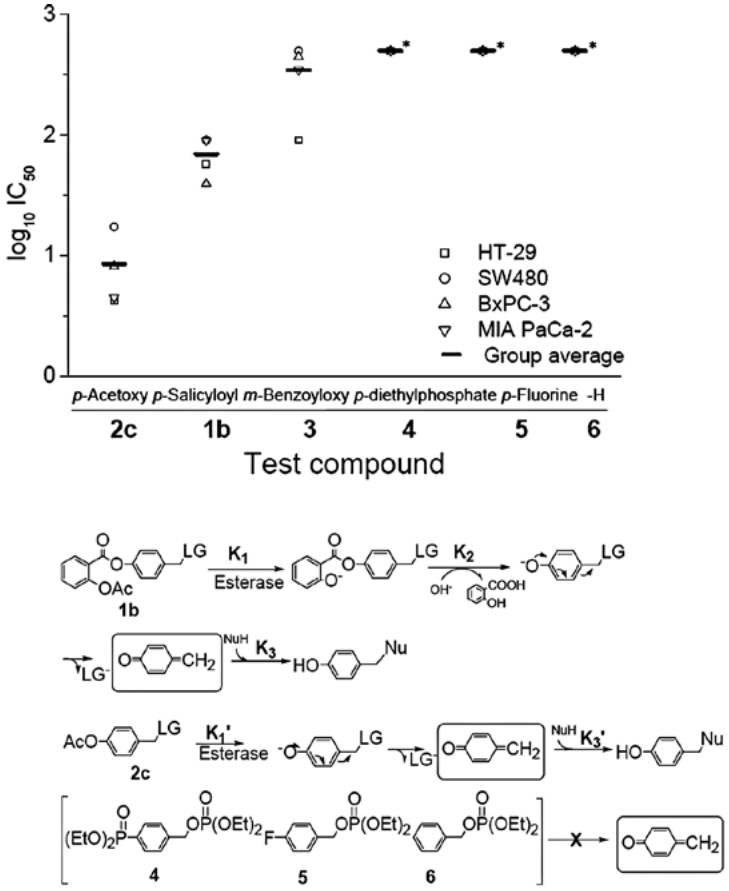
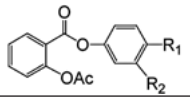


Figure 3. Effect of the X group on the IC_{50} for cell growth. Upper panel: the four different cell lines shown in the inset were treated with the compounds indicated in the abscissa for 24 h, and their IC_{50} for cell growth (ordinate) was determined as in Materials and methods. The average IC_{50} for each compound for all four cell lines is indicated by the horizontal line. *IC_{50} >500 μM; the results of several cell lines overlap and are not clearly delineated. Lower panel: metabolic transformations of representative compounds. Compounds **1b** and **2c** proceed through the general pathway outlined in Fig. 2 (the latter has one step less) to generate quinone methide (boxed) which reacts with a nucleophile giving the final product. Compounds **4**, **5** and **6** cannot generate quinone methides.

Table II. Effect of the leaving group on the IC_{50} for cancer cell growth.

Compound			IC_{50}, μM			
			BxPC-3	MIA PaCa-2	HT-29	SW480
1a	R_1 = H	R_2 = OP(O)(OEt)$_2$	236±5.0	>500	207±9.5	418±33
1b	R_1 = OP(O)(OEt)$_2$	R_2 = H	57.1±1.8	90.8±0.9	39.3±2.9	90.3±2.8
1c	R_1 = CH_2Cl	R_2 = H	33.6±0.4	>500	86.7±3.9	146±8.0
1d	R_1 = H	R_2 = CH_2OH	>500	>500	>500	>500
1e	R_1 = CH_2OH	R_2 = H	>500	>500	>500	>500
1f	R_1 = H	R_2 = CH_2ONO_2	269±2.0	>500	>500	346±24.3
1g	R_1 = CH_2ONO_2	R_2 = H	9.1±0.1	110±3.8	35.4±2.8	16.0±1.3

para-positional isomers (14), we restricted our study to the *meta*- and *para*-isomers.

The *para*-isomers are significantly more potent than the *meta*-isomers. The *m*-/*p*- potency ratios ranged between 4.1 and >5.5 for the diethylphosphate derivatives, and between >4.5 and 29.6 for the nitrate derivatives; it was not possible to calculate such ratios for the -OH derivatives since the IC_{50} values of both isomers for all four cell lines were >500 μM. Indeed, the -OH compounds (**1d** and **1e**) had such a low potency that their IC_{50} values for cell growth could not be reliably established in our experimental system. Of the remaining three, the nitrates (**1f** and **1g**) and diethylphosphates (**1a** and **1b**), roughly equipotent, were much more potent than the -OH compounds. The potency of the corresponding -Cl derivative (**1c**) was considerably less, which could be due to lower solubility or other factors. Individual variations in IC_{50}, based on the cell line. For example, in the case of the HT-29 colon cancer cells, the nitrate and phosphate compounds were equipotent, whereas

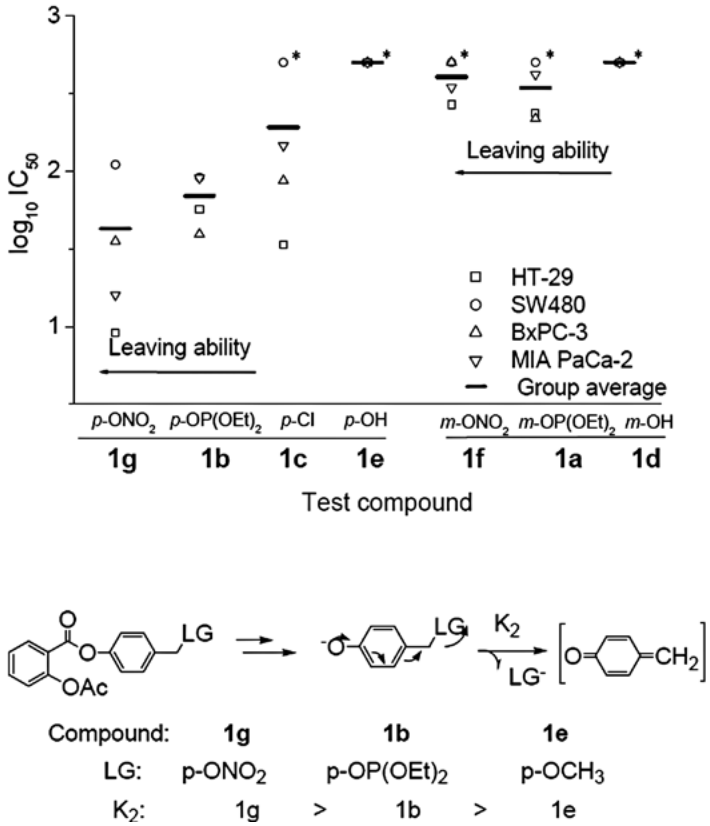


Figure 4. Effect of the leaving group on the IC_{50} for cell growth. Upper panel: the four different cell lines shown in the inset were treated with the compounds indicated in the abscissa for 24 h, and their IC_{50} for cell growth (ordinate) was determined as in Materials and methods. The compounds were organized according to their positional isomerism (*para* vs. *meta*) and the leaving ability of their leaving groups (in decreasing order). The average IC_{50} for each compound in all four cell lines is indicated by the horizontal line. *IC_{50} >500 μM; in some cases the results of several cell lines overlap and are without clear demarkation. Lower panel: metabolic transformations of *para* compounds generating quinone methide.

in the SW480 cell line the nitrate compound was 5.6-fold more potent than the diethylphosphate (**1b** and **1g**).

Thus, the leaving group had a clear effect on the potency of the ABEs. Both the -$OP(O)(OEt)_2$ and ONO_2 groups are absolutely critical to their biological activity; their function as leaving groups determines the formation of drug intermediates. Of the four leaving groups that we examined, -$OP(O)(OEt)_2$ and -ONO_2 where roughly equivalent, whereas the -Cl was slightly less effective. The -OH group, a non-leaving group, appeared to eliminate the ability of ABEs to inhibit cancer cell growth.

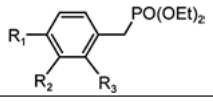
Effect of the electron density of the spacer. To assess the importance of the spacer group (the benzyl moiety) on the pharmacological effect of ABEs, we studied a series of compounds based on X = ASA or AcO and LG = diethyl phosphate in which the spacer was modified by a second substituent (Y in Fig. 1). This substituent was either an electron-donating methoxy group (-OMe, **1i**), or an electron-withdrawing chloro atom (-Cl, **1j**, **2c**) (Table III). Most of these spacer modifications were positioned *ortho* to the benzyl ester group.

As shown in Table III and Fig. 5, the electron density of the spacer had a profound effect on the potency of the ABE molecule. The -OMe group consistently and significantly reduced the potency of the compound (**1i** vs. **1b**). In contrast to the effect of the -OMe, the chloro group increased the potency of the compound, reducing its IC_{50} on average 3.3-fold (**1j** vs. **1b**; **2c** vs. **2d**). The biological effects of additional substituents on the benzylic ring (-Cl or -OMe) reflect either differences in accommodation at the active site or differences in the rates of elimination of the diethylphosphate groups. It is conceivable that the electron density of the spacer affects the formation of the drug intermediate. -OMe, an electron-donating group, stabilized a quinone methide; whereas -Cl, an electron-withdrawing group, increased the reactivity of the quinone methide.

Effect of positional isomerism in the relationship of X to the leaving group. Positional isomerism plays an important role in determining the generation of the drug intermediate and thus drug potency. In all instances where direct comparisons were possible, the *para*-isomers were significantly more potent than the *meta*-isomers (Tables II and III, Figs. 4 and 5). Overall, the *para*-isomers were between 5-100 times more potent than the *meta*-isomers in inhibiting the growth of pancreatic and colon cancer cells (**1a** vs. **1b** and **1f** vs. **1g**). The reason for this striking difference may be found in the general mechanism of action of these compounds, as outlined in Fig. 2. We previously studied in detail the effect of positional isomerism on the pharmacological behavior of compounds **1f** and **1g** and also of their *ortho*-isomer (11,14).

*Cytokinetic effect of meta and para phosphoaspirin (**1a** and **1b**).* The cytokinetic effect of the *meta* and *para* phosphoaspirin (**1a** and **1b**) were explored to access the mechanism

Table III. Effect of the electron density of the spacer on the IC_{50} for cancer cell growth.

Compound				IC_{50}, μM			
				BxPC-3	MIA PaCa-2	HT-29	SW480
1b	R_1 = ASA	R_2 = H	R_3 = H	57.1±1.8	90.8±0.9	39.3±2.9	90.3±2.8
1i	R_1 = ASA	R_2 = H	R_3 = OCH_3	114±2.0	>500	>500	>500
1j	R_1 = ASA	R_2 = H	R_3 = Cl	6.9±1.8	47.2±3.5	19.3±0.5	109.3±0.8
2c	R_1 = OAc	R_2 = H	R_3 = H	4.2±0.7	17.3±2.2	8.2±1.2	4.5±0.5
2d	R_1 = OAc	R_2 = H	R_3 = Cl	7.9±0.5	30.1±4.5	12.4±1.7	22.8±3.5

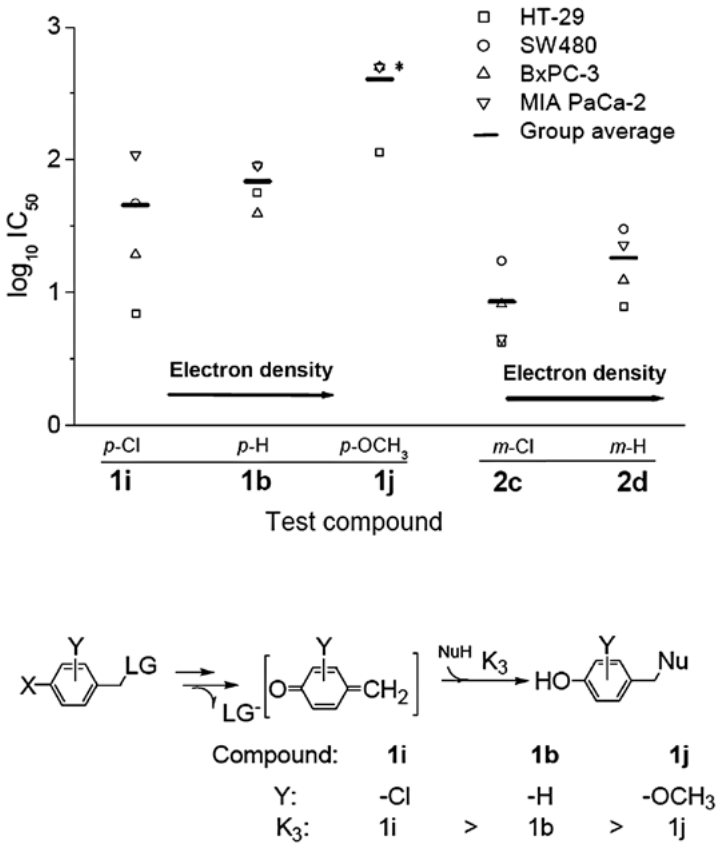


Figure 5. Effect of the benzyl ring electron density on the IC_{50} for cell growth. Upper panel: the four different cell lines shown in the inset were treated with the compounds indicated in the abscissa for 24 h, and their IC_{50} for cell growth (ordinate) was determined as in Materials and methods. Compounds have been ordered according to their positional isomerism (*para* vs. *meta*) and the electron density of their spacer (in increasing order). The average IC_{50} for each compound for all four cell lines is indicated by the horizontal line. *IC_{50} >500 μM; for **1j** the results of several cell lines overlap and are not clearly identified. Lower panel: metabolic transformations of representative *para* compounds generating quinone methide followed by the addition of a nucleophile.

of their growth inhibitory effect. Both *meta* and *para* phosphoaspirin induced apoptosis in HT29 and BxPC-3 (data not shown) cells after 24 h treatment with the drugs. Concurrent staining of the cells with PI distinguishes between viable, early apoptotic (they exclude PI), and necrotic or late apoptotic cells (they stain with PI). The proportion of apoptotic cells increased proportionally with the drug concentration (Fig. 6A). Late apoptotic cells increased even more. Cell proliferation was evaluated by BrdU incorporation method (Fig. 6B). At 1.0 x IC_{50} concentration, *meta* phosphoaspirin significantly decreased BrdU-positive cells from 34.1 to 5.2%, while there was no significant change in *para* phosphoaspirin (from 34.1 to 28.9%). After 24 h of incubation of *meta* phosphoaspirin, a G_1 to S arrest was observed; the proportion of G_1 phase increased from 60.2 to 81.0%, together with a reduction in cells in the S phase from 16.2 to 5.2%. As shown in Fig. 6C, *para* phosphoaspirin caused no G_1 arrest, with a limited G_1 phase change from 60.2 to 54.4%, and an S phase change from 16.2 to 23.3%.

Discussion

We examined the contribution to the pharmacological activity of ABEs by their three structural components shown in Fig. 1:

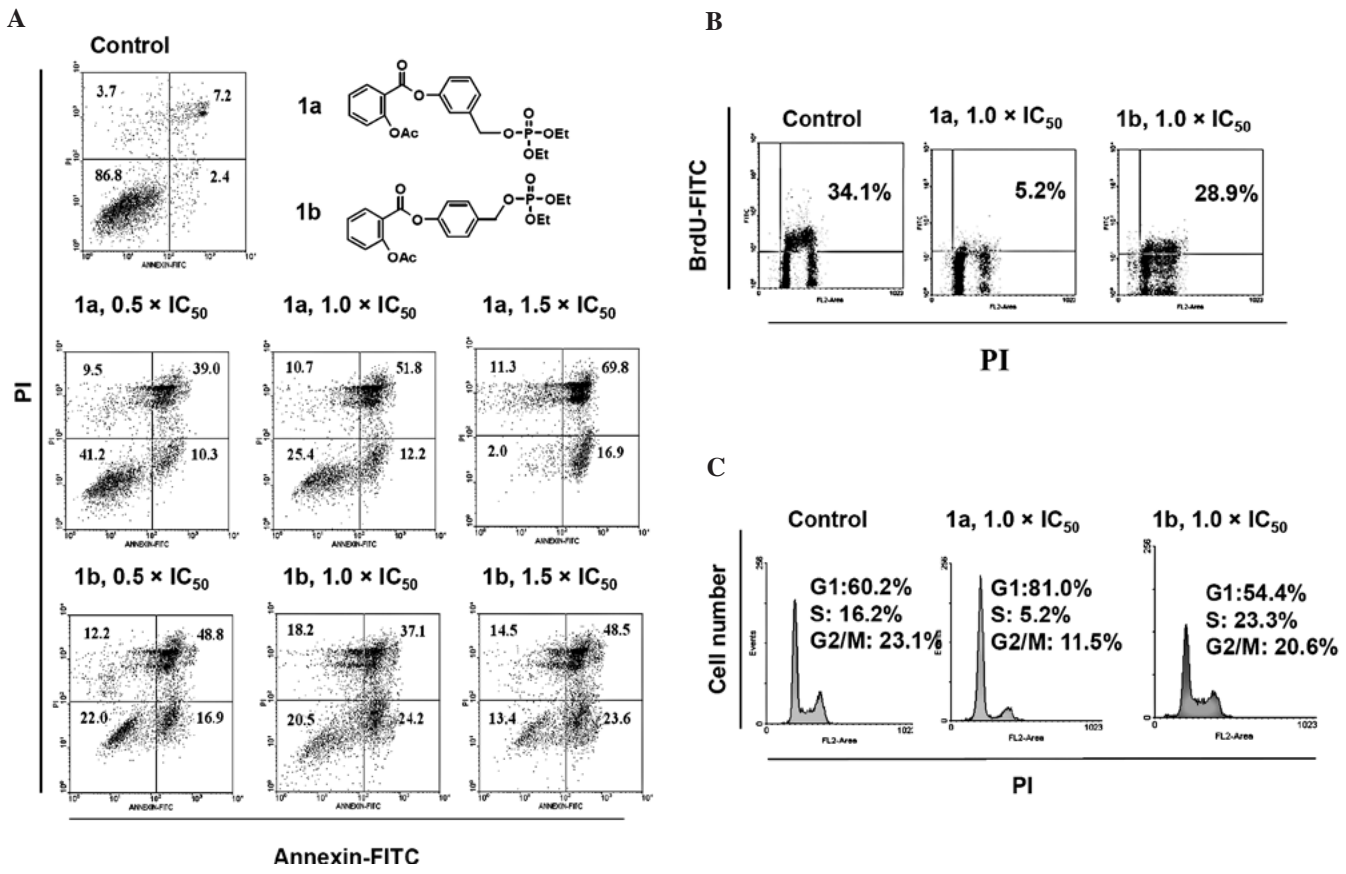


Figure 6. Cytokinetic effect of phosphoaspirin. (A) HT-29 cells treated with *meta* phosphoaspirin (**1a**) and *para* phosphoaspirin (**1b**) for 24 h were stained with PI and Annexin V and analyzed by flow cytometry. The numbers inside each box represent the percentage of cells in each category. (B) BrdU incorporation assay in HT-29 cells following *meta* phosphoaspirin (**1a**) and *para* phosphoaspirin (**1b**) treatment for 24 h. The number in the right upper box indicates the percentages of cells in the S phase. (C) Cell cycle profiles generated from fluorescence-activated cell sorting of PI-stained cells 24 h after *meta* phosphoaspirin (**1a**) and *para* phosphoaspirin (**1b**) treatment.

the leaving group (LG), usually ASA (X), and the spacer group linking the two. Our SAR findings can be viewed in the context of a mechanism of action recently proposed independently by Hulsmann *et al* (10) and by us (11) concerning ASA-based ABE compounds in which the leaving group is -ONO_2. The main feature of this mechanism is that it considers the spacer as the predominately active moiety (Fig. 2). Briefly, the spacer forms a reactive intermediate, either a quinone methide (from its *para* and *ortho* positional isomers) or a carbocation (from the *meta* isomer). This drug intermediate can react with a nucleophile (i.e. glutathione or a suitable group of an enzyme), accounting for most, if not all, of the compound's biological activity. The properties of both the LG and the X group affect the formation of the drug intermediate of each ABE, and thus its biological activity. Fig. 2 provides an illustrative example. Compound **1b**, the *para* derivative of ASA, undergoes such a series of reactions. The first step is catalyzed by hydrolase(s), e.g., acetylsalicylate O-acetylhydrolase (12,13). The next two steps form the drug intermediate quinone methide, which then reacts with a nucleophile to generate a biological functionality. It is clear that, in this formulation, positional isomerism plays an important role in determining drug intermediate activity and thus drug potency (we have studied in detail the effect of positional isomerism on the pharmacological behavior of compounds **1f**, **1g** and also of their *ortho*-isomer) (14). A significant difference exists between the *meta* and *para* phosphoaspirin (**1a** and **1b**) in regards to the anti-proliferative and apoptosis-inducing ability in colon cancer cells. *Para* phosphoaspirin formed a reactive intermediate *o*-Quinone methide, which activated the intrinsic cell apoptosis pathway. Indeed all *para* ABEs in this study did not cause colon cancer cell G_1 arrest (data not shown) while *meta* phosphoaspirin, with less reactive intermediate *m*-Oxybenzyl zwitterions, inhibited the *in vitro* growth of colon cancer cells by G_1 arrest.

In conclusion, we developed a series of novel aspirin derivatives (ABEs), some of which are active against several human cancer cell lines. We identified the elements in these compounds critical to their biological activity, namely the nature of the acyloxy/salicyloxy function, the leaving group, and the electron density of the benzylic spacer, provided by the secondary substituent. This SAR study also provides clear evidence that positional isomerism of ABEs is influential with regard to their inhibitory action on cancer cell growth *in vitro*. Our findings suggest that optimizing the structural elements of these novel ABEs may enhance their anticancer properties and provide a class of compounds useful in the prevention and/or treatment of cancer.

Acknowledgements

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