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RESEARCH PAPER

Differential inhibition of tumour cell-induced platelet aggregation by the nicotinate aspirin prodrug (ST0702) and aspirin

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BACKGROUND AND PURPOSE

Tumour cell-induced platelet aggregation (TCIPA) facilitates cancer cell invasion, angiogenesis and the formation of metastatic foci. TCIPA can be modulated by pharmacological inhibitors of MMP-2 and ADP; however, the COX inhibitor aspirin did not prevent TCIPA. In this study, we have tested the pharmacological effects of a new group of isosorbide-based aspirin prodrugs on TCIPA.

EXPERIMENTAL APPROACH

TCIPA was induced in human platelets by mixing with human adenocarcinoma or fibrosarcoma cells under no flow and flow conditions. The release of gelatinases and P-selectin expression during TCIPA were studied by zymography and flow cytometry respectively.

KEY RESULTS

Tumour cells caused platelet aggregation. This aggregation resulted in the release of MMP-2 and a significant up-regulation of P-selectin on platelets, indicative of platelet activation. Pharmacological modulation of TCIPA revealed that ST0702, one of the aspirin prodrugs, down-regulated TCIPA while aspirin was ineffective. The deacetylated metabolite of ST0702, 5-nicotinate salicylate (ST0702 salicylate), down-regulated both ADP-stimulated platelet aggregation and TCIPA.

CONCLUSIONS AND IMPLICATIONS

Our results show that ST0702 was an effective inhibitor of TCIPA *in vitro*. Its deacetylated metabolite may contribute to the effects of ST0702 by inhibiting ADP-mediated TCIPA.

Abbreviations

GPIb, glycoprotein Ib; GPIIbIIIa, glycoprotein IIbIIIa; HRMS, high-resolution mass spectrometry; ISAS, isosorbide-2-aspirinate-5-salicylate MEM, minimum essential medium; TCIPA, tumour cell induced-platelet aggregation; VWF, von Willebrand factor; WP, washed platelets

Introduction

It has been known for over a century that interactions between tumour cells and platelets are crucial for the haematogenous spread of cancer (Trousseau, 1865), but these interactions are only recently being studied more carefully (Jurasz *et al.*, 2004). Growing evidence suggests that successful metastatic spread may depend on the ability of tumour cells to undergo extensive interactions with platelets (Gupta and Massague, 2004; Jurasz *et al.*, 2004). Cancer cells have the ability to induce platelet aggregation and this ability confers several advantages to the tumour cells in terms of their survival and subsequent successful metastasis (Honn and Tang, 1992; Rickles *et al.*, 2001). Moreover, this ability to aggregate platelets, termed tumour cell-induced platelet aggregation (TCIPA), correlates with the metastatic potential of tumour cells (Radomski *et al.*, 1991). Therefore, inhibition of TCIPA may attenuate the rate of tumour progression and metastasis.

There is evidence that tumour cells can stimulate the release of platelet granules leading to the liberation of several pro-aggregatory agents, such as thromboxane-A_2 (TXA_2), ADP and MMP-2 (Bastida *et al.*, 1986; Heinmoller *et al.*, 1996; Alonso-Escolano *et al.*, 2004). In addition, the involvement of platelet receptors GPIb, GPIIb/IIIa and P-selectin in TCIPA has been also reported (Oleksowicz and Dutcher, 1995; Alonso-Escolano *et al.*, 2004; Medina *et al.*, 2006). GPIb, the von Willebrand factor-binding subunit of the GPIb/V/IX, mainly mediates platelet adhesion (Andrews and Berndt, 2004); GPIIb/IIIa plays an important role in platelet aggregation (Shattil *et al.*, 1998); whereas P-selectin mediates platelet-leucocyte aggregation (Andrews and Berndt, 2004).

In platelets, one of the most important mediators regulating haemostasis is TXA_2. Indeed, TXA_2 promotes thrombosis by promoting platelet aggregation and constricting blood vessels. However, clinical trials of the antiplatelet agent aspirin, which inhibits platelet COX and thereby TXA_2 production, have proven inconclusive in cancer patients. Indeed, some clinical studies have found that treatment with high doses of aspirin did not protect patients from metastasis (Lipton *et al.*, 1982; Lebeau *et al.*, 1993). These observations are consistent with the failure of aspirin to prevent TCIPA *in vitro* with different tumour cell lines (Jurasz *et al.*, 2001; Medina *et al.*, 2006). Nevertheless, aspirin use has been strongly associated with reduced risk of colorectal adenoma and to a lesser extent with reduction in cancers of the upper intestine, oesophagus, breast, lung and ovary (Sandler *et al.*, 2003; Bosetti *et al.*, 2009).

We have recently developed a new group of isosorbide-based aspirin prodrugs that are potent inhibitors of ADP and collagen-induced platelet aggregation (Jones *et al.*, 2009). Indeed, several prodrugs were more potent inhibitors of aggregation than the unmodified aspirin. The aim of the present study was to determine if the observed increase in potency in platelet aggregation assays translated into an effect on TCIPA. Accordingly, several aspirin prodrugs (Figure 1) were evaluated as inhibitors of TCIPA under conditions where aspirin itself is ineffective. Some of these compounds are designed to release simultaneously a second pharmacologically active moiety, such as NO, that might make them more suitable for interrupting processes such as TCIPA that operate along multiple activation pathways. One of the test compounds, an aspirin-nicotinic acid codrug (ST0702), under development as a dual anti-platelet and lipid modifying agent, markedly inhibited TCIPA under both no-flow and flow conditions. We have been able to determine the mode of action of ST0702 as an inhibitor of TCIPA by monitoring its activation during platelet–tumour cell interactions.

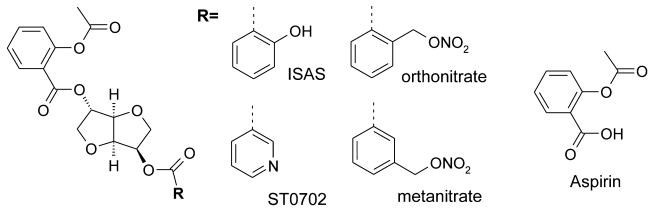

Figure 1

Structural formulae for aspirin and isosorbide-based prodrugs. These are metabolized by human plasma and platelet esterases liberating aspirin along with NO (from the isomeric ortho and metanitrate compounds), nicotinic acid (ST0702) and salicylic acid (ISAS).

Methods

Tumour cell culture

Three human tumour cell lines, 59 M ovarian adenocarcinoma, Caco2 colon adenocarcinoma and HT1080 fibrocarcinoma cells, were obtained from the European Cell Culture Collection. Cell lines were cultured as monolayers in 75 mL culture flasks at 37°C in a humidified atmosphere with 5% CO_2. 59 M cells and HT1080 were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum (FBS) and gentamycin (0.05 mg·mL^{-1}), penicillin (0.06 mg·mL^{-1}) and streptomycin (0.01 mg·mL^{-1}). The Caco2 cell line was cultured under similar conditions but supplemented with 20% FBS. The cells were supplied with fresh medium and subcultured thrice weekly.

Preparation of human platelets

Blood was collected from healthy volunteers who had not taken any drugs known to affect platelet function for at least 14 days prior to the study. Platelet-rich plasma (PRP) and washed platelet (WP) suspensions (2.5×10^8·cells mL^{-1}) were prepared from blood as described by Radomski and Moncada, (1983).

Platelet aggregation under no-flow conditions

The interactions between platelets and tumour cells were measured by light aggregometry (Radomski *et al.*, 1991; Jurasz *et al.*, 2001; Alonso-Escolano *et al.*, 2004). Briefly, WP samples (2.5×10^8· cells mL^{-1}) were placed in an eight channel PAP 8 aggregometer (Bio/Data Corporation, Horsham, PA, USA) and incubated for 10 min at 37°C, with stirring at 900 r.p.m., before the addition of aggregating agents (collagen 2 μg·mL^{-1}, or cancer cells). PRP (2.5×10^8· cells mL^{-1}) was used for the study of ADP-stimulated platelet aggregation (ADP 10 μM). Following addition of aggregating agents, aggregation was

measured for at least 30 min using Aggro-Link software (Labmedics, Stockport, UK). For experiments using inhibitors, aggregation was initiated after 10 min pre-incubation with test compounds.

To study the ability of the aspirin prodrugs to inhibit TCIPA in WP, the cancer cell lines such as HT1080 cells (2×10^5·cells mL^{-1}), Caco2 cells (1.5×10^3·cells mL^{-1}) or 59 M cells (1×10^3·cells mL^{-1}) were used in the presence or absence of the compounds shown in Figure 1 (ST0702, isosorbide-2-aspirinate-5-nicotinate; ISAS, isosorbide-2-aspirinate-5-salicylate; orthonitrate, isosorbide-2-aspirinate-5-(2-nitroxymethylbenzoate); metanitrate, isosorbide-2-aspirinate-(3-nitroxymethylbenzoate) at 300 and 500 μM. ST0702 is hydrolyzed by plasma esterases along two pathways liberating both aspirin and the deacetylated metabolite, 5-nicotinate salicylate (ST0702 salicylate) (Figure 1). The latter is further hydrolysed to isosorbide-5-nicotinate and eventually to nicotinic acid. The orthonitrate and metanitrate compounds liberate aspirin and NO. ISAS, meanwhile, is hydrolyzed to aspirin and salicylic acid. Aspirin (300 and 500 μM) was used in control experiments. Results were expressed in percent changes in maximal light transmission, with 100% representing light transmission of platelet medium alone. Further experiments were performed with ST0702 salicylate (500 μM). This was obtained by flash chromatography as a hydrolysis product of ST0702 during isolation of the parent compound. Its purity and identity was confirmed by NMR, HPLC and high-resolution mass spectrometry.

Effect of physostigmine (eserine) on platelet inhibitory activity of ST0702 salicylate

Eserine (10 μM) was incubated with PRP for 5 min in the aggregometer, stirring at 900 r.p.m., before the addition of test compound ST0702 salicylate (500 μM), which was incubated in PRP for a further 10 min before the addition of the agonist ADP (10 μM) to induce platelet aggregation.

Flow cytometry

In order to analyse receptor expression on the surface of individual platelets and to minimize platelet activation caused by sample preparation procedures, no stirring or vortexing steps were used. The abundance of P-selectin on the surface of platelets in the presence and absence of inhibitors was measured by flow cytometry. Platelet samples were first activated with 59 M cells (1×10^3·mL^{-1}). When platelet aggregation reached 50% maximal light transmission, the reaction was terminated by 10-fold dilution with physiological saline. Resting platelets were used as control. In most of the experiments, platelets were pre-incubated with inhibitors for 10 min prior to the addition of 59 M cells (1×10^3·mL^{-1}). Platelet samples were then incubated in the dark without stirring for 5 min at room temperature in the presence of saturating concentrations (10 μg·mL^{-1}) of P-selectin (CD62P-APC). Following incubation, samples were diluted in FACS flow fluid and analysed within 5 min using a BD FACSArray (BD Biosciences). Flow cytometry was performed on single stained platelet samples as described before (Radomski *et al.*, 2005). The instrument was set up to measure the size (forward scatter), granularity (side scatter) and cell fluorescence. A two-dimensional analysis gate of forward and side scatter was drawn in order to include single platelets and exclude platelet aggregates and microparticles. Antibody binding was measured by analysing individual platelets for fluorescence. The mean fluorescence intensity was determined after correction for cell autofluorescence. For each sample, the fluorescence was analysed using a logarithmic scale. Fluorescence histograms were obtained for 10 000 individual events. Data were analysed using BD FACS Array software.

Microscopy of TCIPA

The structure of platelet–tumour cell aggregates was studied using phase-contrast microscopy (Jurasz *et al.*, 2001; Alonso-Escolano *et al.*, 2004). Briefly, 59 M cells (1×10^3·mL^{-1}) were added to the platelet suspension (2.5×10^8·mL^{-1}) in the presence or absence of aspirin or prodrugs, and aggregation was terminated at 50% maximal aggregation, as determined using the aggregometer. The samples were then fixed by adding 2% paraformaldehyde in Tyrode's solution, pH 7.4, and then incubated for 30 min at room temperature. Aliquots of each sample were then mounted on slides using a cytospin and taken for phase-contrast microscopy examination using an Olympus CKX41 microscope (Olympus America Inc., Melville, NY, USA). Photomicrographs were captured using a digital camera and MicroFire (Olympus America Inc.) software (Jurasz *et al.*, 2001; Alonso-Escolano *et al.*, 2004).

Sample preparation for zymography

Sample collection was carried out as previously described (Jurasz *et al.*, 2001; Medina *et al.*, 2006). Briefly, platelets at a concentration of 2.5×10^8·mL^{-1} were placed into a lumi-aggregometer and tumour cells (59 M) were added to yield 1×10^3·cells mL^{-1}. When platelet aggregation reached 50% maximal light transmission, the reaction was terminated and samples were collected. The samples were then centrifuged at 900× g at room temperature for 10 min. After centrifugation, platelet supernatants were collected and stored at –80°C until assayed for the presence of MMP activity by zymography.

Zymography

Gelatin zymography was used to detect the activity of MMP-2 in the platelet supernatants as previously described (Jurasz *et al.*, 2001; Alonso-Escolano *et al.*, 2004; Medina *et al.*, 2006). Briefly, samples were subjected to 10% SDS-PAGE with copolymerized gelatin (0.2%; Sigma Chemical Co., St Louis, MO, USA) incorporated as a substrate for gelatinolytic proteases. After electrophoresis, the gels were washed with 2.5% Triton X-100 (three times, 20 min each), and then incubated for 48 h at 37°C in enzyme assay buffer (25 mM Tris HCl, 0.9% NaCl, 5 mM $CaCl_2$ and 0.05% Na_3N, pH = 7.5). The conditioned medium of HT-1080 human fibrosarcoma cells (that contains high amounts of proMMP-2, MMP-2, proMMP-9 and MMP-9) was used as control. After 72 h development, gels were fixed and stained in 40% methanol, 10% acetic acid and 0.1% (wt/v) Coomassie Blue R-250 (Sigma Chemical Co.) for 1 h and then de-stained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie blue-stained gelatin. The intensities of the separate bands were analysed and quantified using ChemiDoc XRS System (Bio-Rad, Her-

cules, CA, USA). The gelatinolytic activity of each band was expressed as arbitrary units of density mg protein^{-1}.

Flow-mediated TCIPA using an ultrasound standing wave trap

The ultrasound trap had three essential features: a transducer (Ferroperm, Kvistgard, Denmark) in a housing of radial symmetry, an aqueous phase and a reflector that provided optical access from above as previously described (Bazou *et al.*, 2011). The trap was driven with a function generator (Hewlett Packard, Bristol, UK). Aggregation was followed with a fast, high-resolution XM10 camera (Soft Imaging System, SIS, GmbH, Münster, Germany) mounted on an Olympus BX51M reflection epi-fluorescence microscope. Images were captured by a standard PC equipped with the Cell-D image acquisition software (Soft Imaging System).

The experimental procedure was carried out as previously published (Bazou *et al.*, 2011) with minor modifications: 59 M cells were introduced into the trap, the acoustic field (2.13 MHz, 0.85 MPa) was initiated and cell clusters were allowed to form. Clusters remained levitated in suspension for 10 min. Washed platelets (2.5×10^8·mL^{-1}) were introduced into the trap at a flow rate of 3 μL·min^{-1}. Perfusion of washed platelets around the cluster proceeded following initial adhesion of platelets to the periphery of the cluster.

To study the effect of aspirin and aspirin prodrugs in TCIPA, WP samples were pre-incubated for 10 min at room temperature with the inhibitors before their introduction into the ultrasound trap. Those aspirin prodrugs which were found to have an inhibitory effect under static conditions were tested; ST0702, ISAS, orthonitrate and metanitrate (500 μM); aspirin (500 μM) was used as control. The cell cluster-platelet aggregate remained under microscopic observation for further 10 min (the upper time limit established in the current study) under continuous flow conditions. Further experiments were performed with ST0702 salicylate (500 μM).

Profiling the breakdown of ST0702 during the TCIPA experiment

To study the hydrolysis of the prodrug ST0702 in TCIPA, HPLC analysis of supernatants and lysates was performed. Briefly, WPs (2.5×10^8·cells mL^{-1}) were placed in an eight channel PAP 8 Aggregometer. ST0702 (500 μM) was incubated with the platelets, with stirring for 10 min before the addition of cancer cells, HT1080 (2×10^5·mL^{-1}) or Caco2 (1.5×10^3·mL^{-1}) to induce aggregation. TCIPA was monitored for 30 min; supernatants/lysates were collected as soon as control (HT1080 or Caco2 alone) and test samples were plateaued and reached maximal aggregation. For collection of supernatant, after the control plateaued and reached maximal aggregation, 100 μM PMSF was added and the WP/ST0702 suspension was centrifuged at 13 000× g for 5 min at 4°C; the supernatant was removed and stored at –20°C until analysis by HPLC. For collection of lysate after the control plateaued and reached maximal aggregation, ice-cold 10× RIPA lysis buffer [20 mM Tris pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 20 mM pyrophosphate, 1 mM Na_3VO_4, 10% Triton-X, 10 mM PMSF and 10× protease inhibitor cocktail (5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1.5 mM aprotinin, 10μM E-64 protease inhibitor, 10μM leupeptin hemisulfate)] was added to WP/ST0702 suspensions. Samples were stored on ice for 1 h with frequent vortexing to promote lysis. The samples were centrifuged at 13 000× g for 5 min at 4°C and the supernatant lysates were stored at –20°C until analysis by HPLC. HPLC was performed as described previously but with detection at 260 nm (Jones *et al.*, 2009).

Statistics

The data were analysed using one-way analysis of variance (GraphPad Prism 5, San Diego, CA, USA). The results were expressed as mean ± SEM of at least three independent experiments. Tukey–Kramer multiple comparisons test, and paired and unpaired Student's *t*-tests were performed, where appropriate. Statistical significance was considered when $P < 0.05$.

Materials

All reagents were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise indicated. Collagen and ADP were obtained from Chronolog (Havertown, PA, USA). Allophycocyanin (APC)-conjugated monoclonal antibody against human platelet P selectin (CD62P) was purchased from BD Biosciences (Oxford, UK). The aspirin prodrugs (Figure 1) were synthesized as reported (Moriarty, 2008; Jones *et al.*, 2009).

The compounds were dissolved in DMSO, then diluted in platelet-rich plasma (PRP) or washed platelets (WPs) to give a final concentration not more than 0.25% DMSO, which pilot studies had shown not to affect platelet aggregation (Harmon *et al.*, 2012). No precipitation of any drug was observed following dilution.

Results

TCIPA

Caco2, HT1080 and 59 M cells were tested for their ability to induce platelet aggregation. When platelets were incubated in the aggregometer for 30 min at 37°C without the addition of tumour cells, no platelet aggregation was detected. However, all cell lines were able to induce platelet aggregation (Figure 2). As the concentration of 2×10^5·cells mL^{-1} (HT1080 cells), 1.5×10^3 cells mL^{-1} (Caco2 cells) and 1×10^3 cells mL^{-1} (59 M cells) were sufficient to induce platelet aggregation, all remaining experiments were performed at these cell densities.

Effects of ISAS, ST0702, ortho- and metanitrate or aspirin on TCIPA as measured by aggregometry

ST0702 consistently inhibited TCIPA in response to all three cell lines, as shown by aggregometry (Figure 3) and phase contrast microscopy (Figure 4). Only ST0702 and the NO-releasing orthonitrate inhibited platelet aggregation induced by HT1080 cells (Figure 3). In addition, the ISAS, orthonitrate and metanitrate compounds inhibited platelet aggregation induced by Caco2 and 59 M cells (Figures 3 and

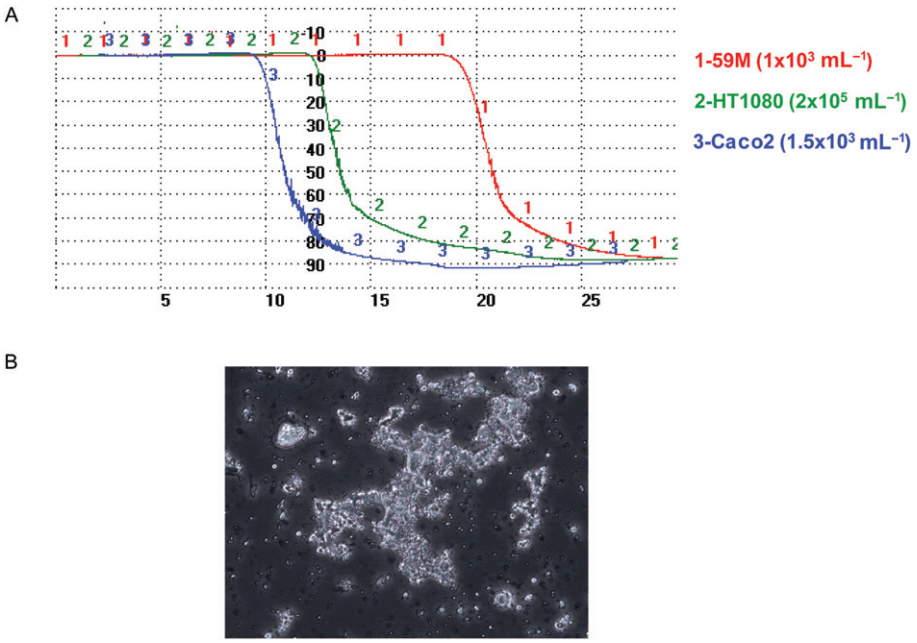

Figure 2

HT1080, Caco2 and 59 M cells induced platelet aggregation. (A) Representative aggregometer traces showing TCIPA (representative of four experiments). TCIPA was induced by HT1080 cells ($2 \times 10^5 \cdot mL^{-1}$), Caco2 cells ($1.5 \times 10^3 \cdot mL^{-1}$) and 59 M cells ($1 \times 10^3 \cdot mL^{-1}$). (B) Representative phase-contrast microscopy of 59 M-induced TCIPA.

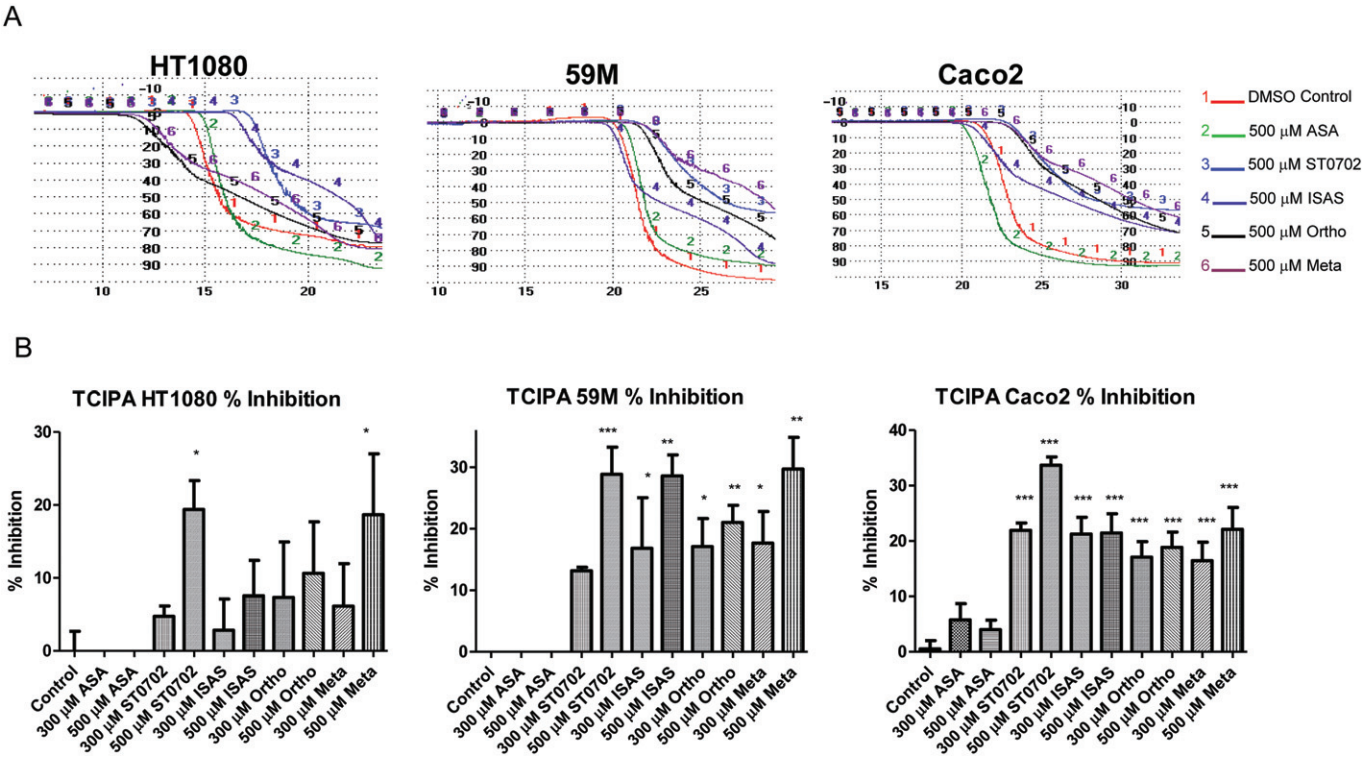

Figure 3

Pharmacological effect of asprin prodrugs on TCIPA. Representative traces (A) and the statistical analysis (B) showing the effects of ASA, ST0702, ISAS, orthonitrate and metanitrate at 300 and 500 µM on TCIPA. TCIPA was induced by HT1080 cells ($2 \times 10^5 \cdot mL^{-1}$), CaCo2 cells ($1.5 \times 10^3 \cdot mL^{-1}$) and 59 M cells ($1 \times 10^3 \cdot mL^{-1}$). Aggregated platelets with tumour cells in the absence of inhibitors were used as controls. Bars are mean ± SEM from four separate experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, treatments versus control.

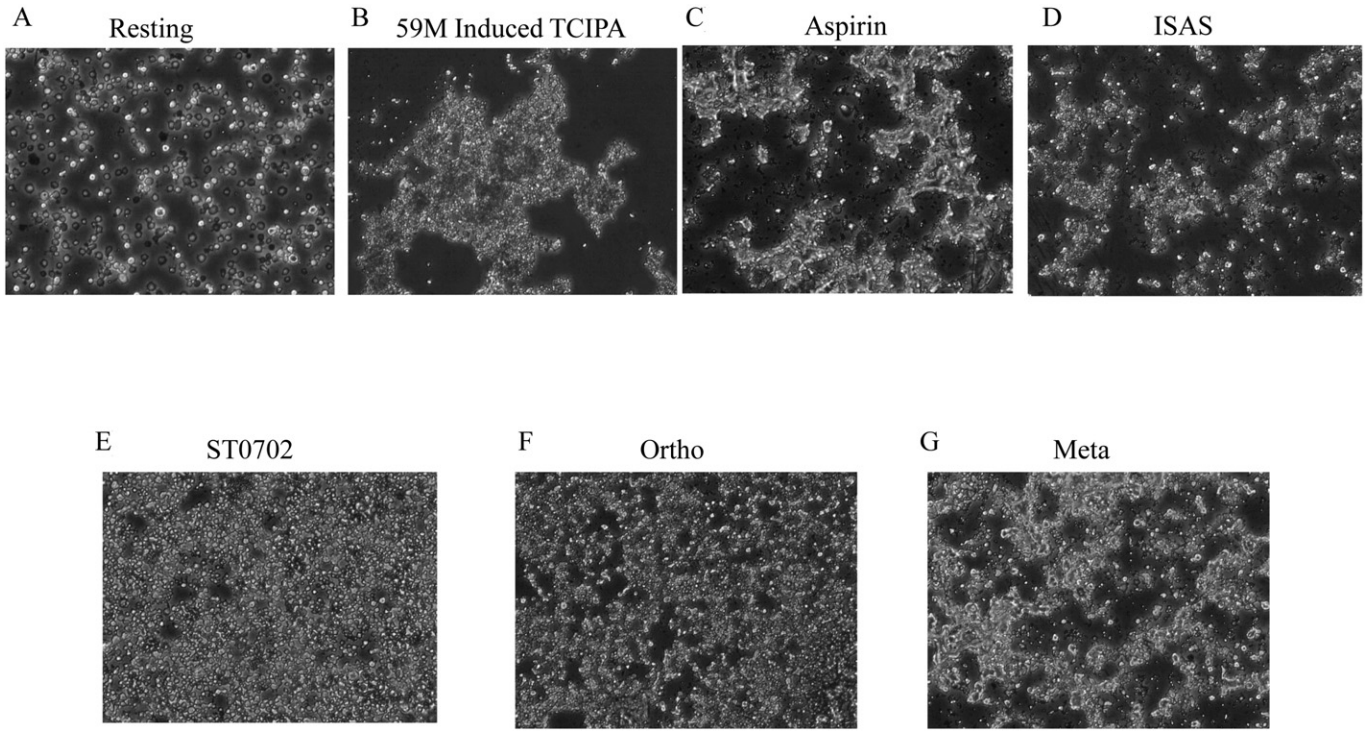

Figure 4

Phase-contrast microscopy of TCIPA in the presence of aspirin prodrugs. Phase contrast microscopy of un-aggregated platelets (A), TCIPA induced by 59 M cells at 50% of aggregation, with the presence of large platelet aggregates in the absence (B) or presence of aspirin (C), ISAS, (D) ST0702 (E), orthonitrate (F) and metanitrate (G) with less formation of large aggregates. Scale bar, 20 μm.

4). In contrast, aspirin (300–500 μM) did not exert a significant effect on TCIPA (Figure 3). However, as expected, aspirin (300 μM) inhibited collagen-induced aggregation by 56 ± 7% (n = 4). The general pattern of TCIPA inhibition was consistent with the observations of platelet-cancer cell masses detected by microscopy following treatment with aspirin or the prodrugs (Figure 4).

MMP-2 release measured by zymography

As MMP-2 is released during TCIPA, zymographic analysis was conducted to study whether or not MMP-2 was involved in our observations. We have previously shown that MMP-2 is the major gelatinase detected during platelet aggregation induced by both HT1080 and Caco2 cells (Jurasz *et al.*, 2001; Medina *et al.*, 2006). Therefore, we studied the release of MMP-2 in platelet aggregation induced by 59 M cells. We found that MMP-2 was also released during TCIPA, as shown by zymography (Figure 5). However, aspirin and all prodrugs failed to prevent the release of MMP-2 during platelet aggregation induced by 59 M cells (P > 0.05; n = 4; Figure 5).

P-selectin translocation measured by flow cytometry

For these experiments, 59 M cells were used. The interactions of platelets with HT1080 and Caco2 cells have been previously characterized by our group (Jurasz *et al.*, 2001; Medina *et al.*, 2006). The interactions of platelets with 59 M cells induced a significant increase (P < 0.005, n = 4) in the

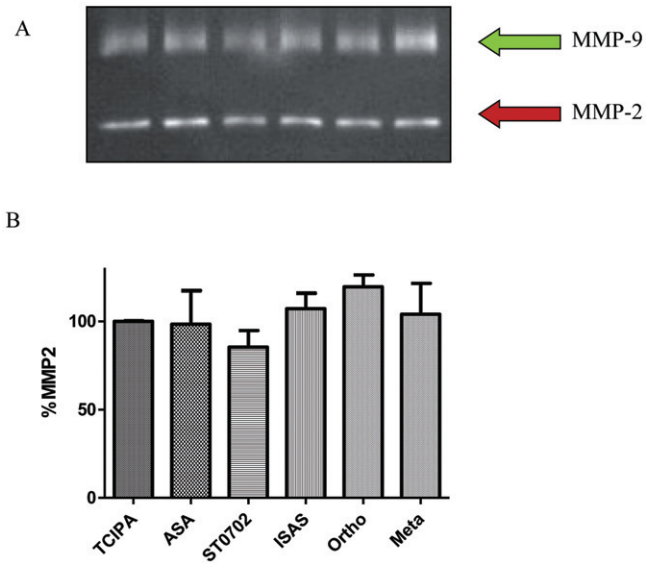

Figure 5

Zymography of supernatants of 59 M cells and platelets in the presence of aspirin prodrugs. Representative zymography (A) and the statistical analysis (B) showing the effects of ASA, ST0702, ISAS, orthonitrate and metanitrate (500 μM) on the release of MMP-2 during TCIPA. TCIPA was induced by 59 M cells ($1 \times 10^3 \cdot mL^{-1}$). Aggregated platelets with tumour cells in the absence of inhibitors were used as controls. Bars are mean ± SEM from four separate experiments. P > 0.05 versus TCIPA.

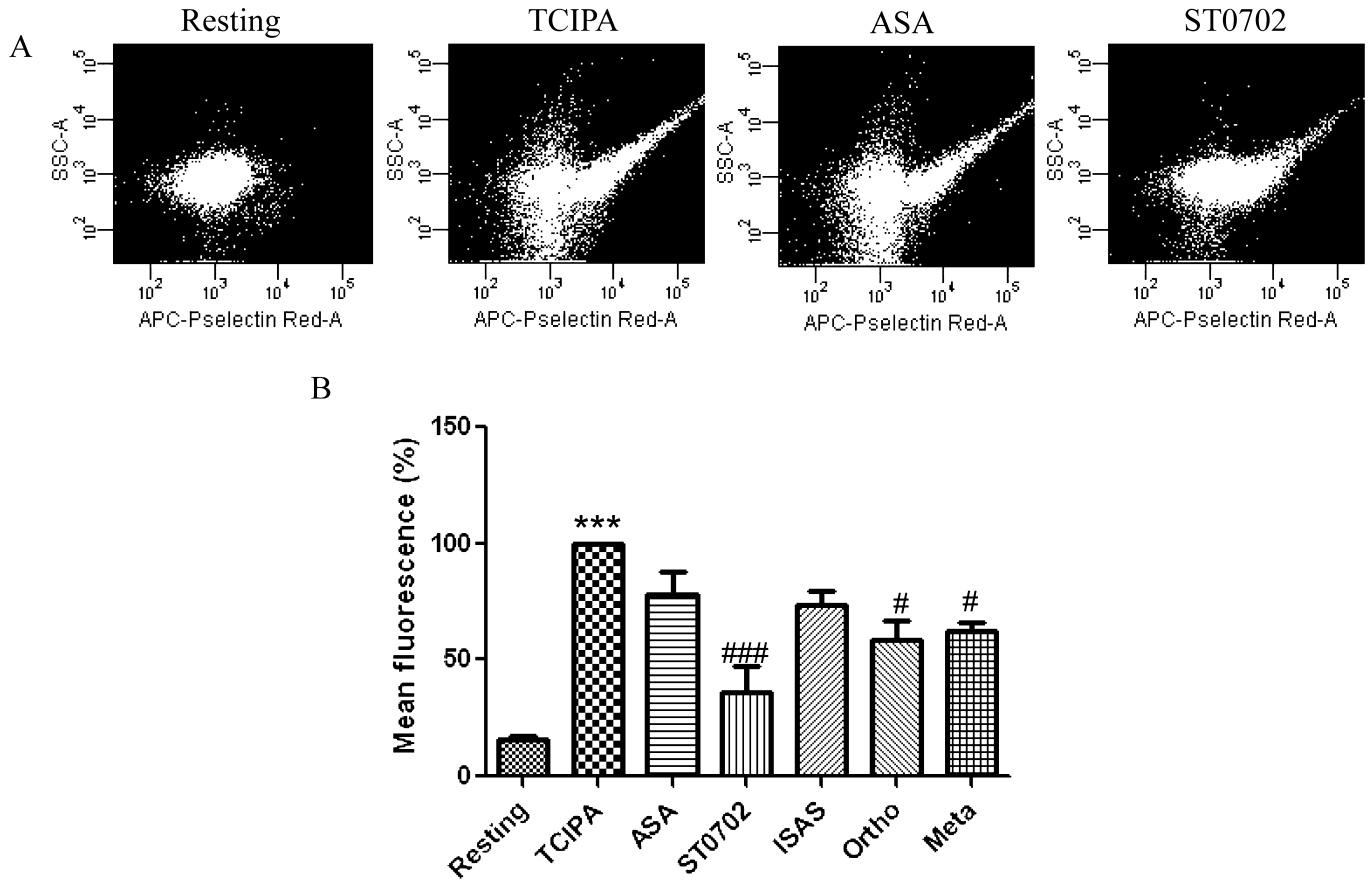

Figure 6

Flow cytometry analysis of P-selectin on platelets during TCIPA in the presence of different inhibitors. (A)Representative graphs of resting platelets (resting), TCIPA and the effects of aspirin and ST0702; (B) Statistical analysis showing the effects of ASA, ST0702, ISAS, orthonitrate and metanitrate (500 μM) on TCIPA. TCIPA was induced by 59 M cells ($1 \times 10^3 \cdot mL^{-1}$). Aggregated platelets with tumour cells in the absence of inhibitors (TCIPA) and resting platelets were used as controls. Bars are mean ± SEM from four separate experiments. ***$P < 0.01$, TCIPA versus resting; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.01$, treatments versus TCIPA.

number of copies of P-selectin on the platelet surface (Figure 6A).

ST0702 and the orthonitrate and metanitrate pro-drugs significantly ($P < 0.05$, $n = 4$) inhibited 59 M cell-mediated increase in total P-selectin (Figure 6A, B). In contrast, aspirin and ISAS failed to prevent the increase in platelet surface abundance of P-selectin ($P > 0.05$, $n = 4$) (Figure 6A,B).

ST0702 inhibits TCIPA under flow conditions

Following levitation of a 59 M cell cluster in the trap for 10 min (Figure 7A), platelet perfusion was initiated. Platelets approached the aggregate within 1 min and established contact with its periphery. Complete platelet 'encapsulation' of the aggregate was seen within 2 min (Figure 7B). Platelet activation (identified as a transition to a gel-like sheet around the cell cluster) occurred within 4 min of platelet-cell cluster contact and resulted in the disruption of the cancer cell aggregate (Figure 7C). ST0702 was the only compound that significantly arrested TCIPA ($P < 0.05$, $n = 4$) under these conditions (Figure 7D).

ST0702 releases small amounts of aspirin and its salicylate during the TCIPA experiment

Following the TCIPA experiments with HT1080 or Caco2 cells, supernatants were collected for further analysis along with the cellular pellets which were lysed in the presence of esterase/protease inhibitors. The supernatants and lysates were analysed by HPLC (Figure 8). This indicated that in the presence of cancer cell and platelet esterases, ST0702 was broken down to substantial amounts of its deacylated metabolite (isosorbide-2-salicylate-5-nicotinate; ST0702 salicylate; Figure 8) along with smaller amounts of aspirin, salicylic acid, nicotinic acid and isosorbide-5-nicotinate. There were no significant differences between the contents of the supernatants or lysates following stimulation with Caco2 or HT1080 cells.

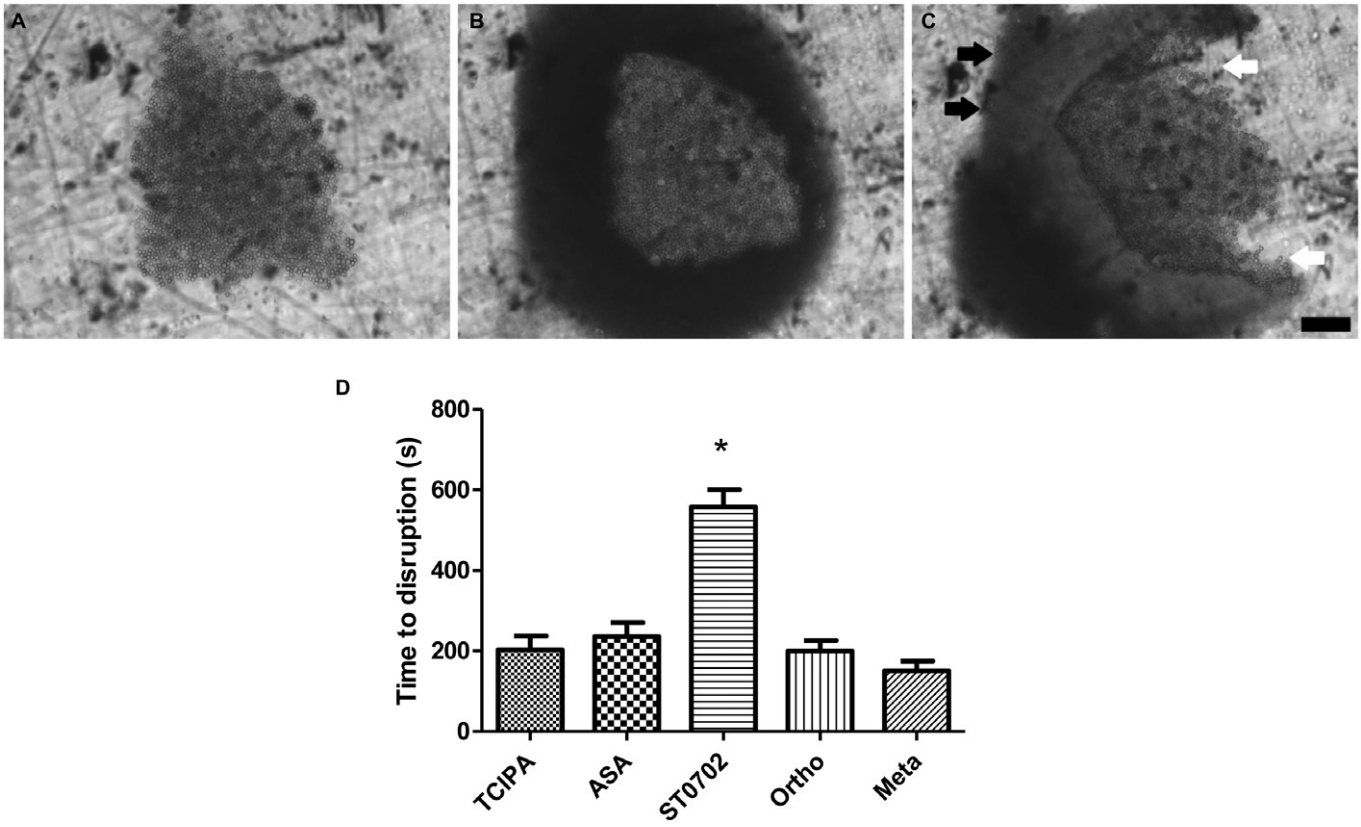

Figure 7

Effect of aspirin prodrugs on TCIPA under flow conditions. Microscopy of 59 M cell cluster in the trap (A), platelet encapsulation of the tumour cell aggregate (B) and platelet activation (black arrows) which resulted in disruption of the platelet-cancer cell aggregate (white arrows) (C). The effects of ASA, ST0702, orthonitrate and metanitrate (500 μM) on platelet-tumour cell disruption were evaluated. Aggregated platelets with tumour cells in the absence of inhibitors (TCIPA) were used as negative controls. Bars are mean ± SEM from four separate experiments. *$P < 0.05$, treatments versus TCIPA.

ST0702 salicylate inhibits TCIPA and ADP-induced platelet aggregation

In order to study the mechanism of action of ST0702 in TCIPA, more experiments were carried out with the metabolites produced *in vitro* and identified in the HPLC experiments: nicotinic acid, isosorbide-5-nicotinate and the ST0702 salicylate. This last deacetylated metabolite, ST0702 salicylate, inhibited TCIPA in response to HT1080 and Caco2 cells under no flow conditions (Figure 9A). However, unlike the parent ST0702, this deacetylated metabolite did not inhibit collagen-induced platelet aggregation (Figure 9B). The ST0702 salicylate showed a tendency to inhibit ADP-induced aggregation in PRP (Figure 9C), an effect that became significant in the presence of the esterase inhibitor eserine, which protected the ST0702 salicylate from further hydrolysis by esterases in PRP. Unlike ST0702, the salicylate metabolite did not inhibit TCIPA in the ultrasound trap model (Figure 9D), indicating that under flow conditions, its ADP inhibitory properties were insufficient to prevent TCIPA. The remaining fragments identified in cell lysates by HPLC (nicotinic acid and isosorbide-5-nicotinate) did not inhibit TCIPA at up to 3 mM.

Discussion and conclusions

The main function of platelets is the maintenance of vascular haemostasis. Platelets also play crucial roles in the pathogenesis of vascular thrombosis and disease. There is increasing evidence that platelet–cancer cell interactions participate in the complex multi-step process of carcinogenesis including blood-borne metastasis. When platelets are activated, the arachidonic acid cascade is initiated, leading to TXA_2 synthesis. This reaction is catalysed by a number of enzymes, the most important being COX which converts arachidonic acid to prostaglandin H_2 (PGH_2) and thromboxane synthase which converts PGH_2 to TXA_2. TXA_2 mediates one of major pathways of platelet aggregation by stimulating platelet thromboxane receptors leading to activation of platelet inositol phosphate pathways and an increase in intracellular Ca^{2+} (Reilly and Fitzgerald, 1993) and release of dense- and α-granules (Armstrong, 1996). Aspirin reduces the synthesis of TXA_2 by irreversibly inhibiting platelet COX, blocking PGG_2 production. Aspirin preferentially inhibits the COX-1 isoform of the enzyme, but its effects on COX-2 are an important part of the explanation for its anti-inflammatory and

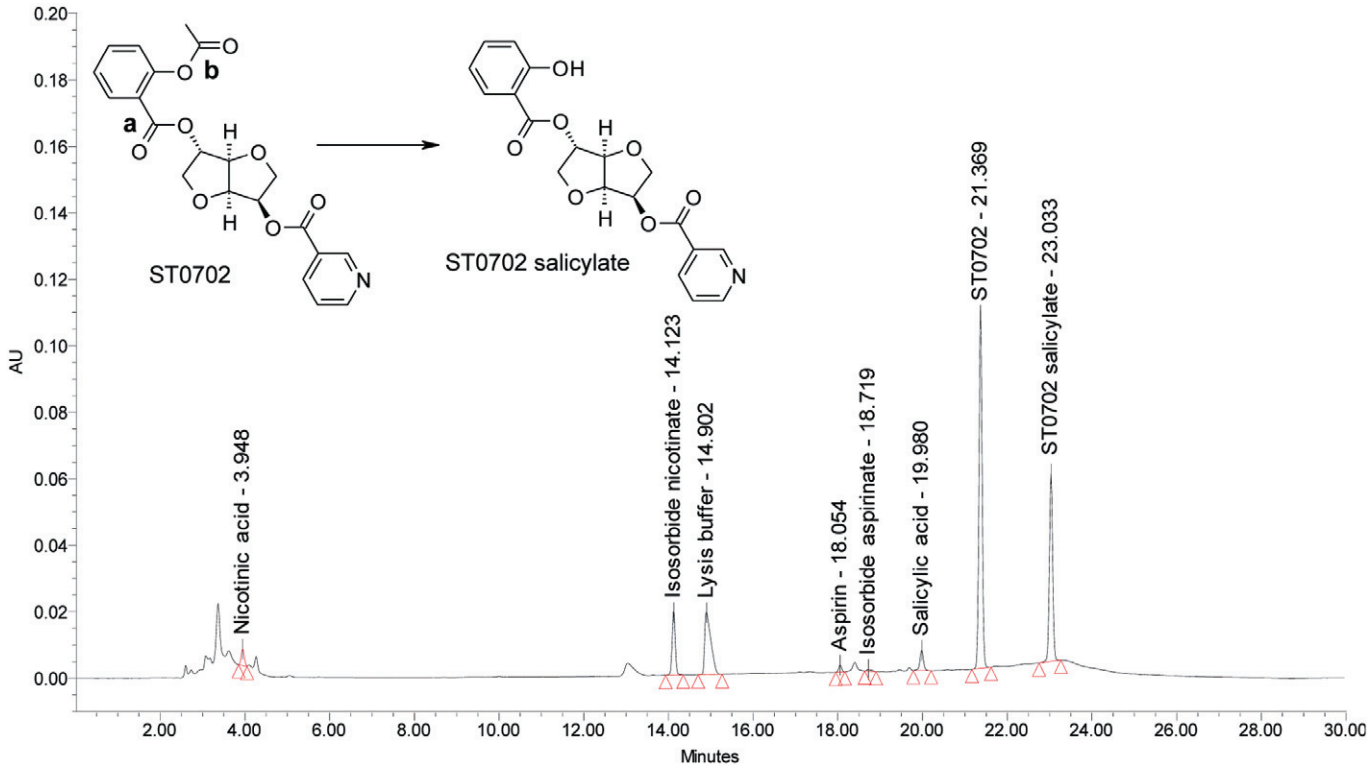

Figure 8

HPLC chromatogram of cell lysate following TCIPA inhibition with ST0702 (500 μM). At the termination of a TCIPA experiment, HPLC analysis indicated that the platelet-Caco2 cells contained a mixture of the unchanged ST0702 (retention time (RT), 21.36 min) along with 33.7% of its deacetylated, esterase-mediated, hydrolysis product, isosorbide-2-salicylate-5-nicotinate (ST0702 salicylate; RT, 23.03 min), along with smaller amounts of aspirin (RT, 18.05 min). Aspirin is deacylated to salicylic acid (RT, 18.71 min). Isosorbide-5-nicotinate (RT, 14.13 min) was also observed as a metabolite of ST0702.

putative anti-cancer effects (Cha and DuBois, 2007). Numerous studies have shown an inverse relationship between aspirin consumption and cancer incidence (Elwood *et al.*, 2009; Opie, 2011; Rothwell *et al.*, 2011). Epidemiological and randomized trial data indicate that aspirin-mediated cancer preventative effects are related to dose, duration of use and length of follow-up (Langley *et al.*, 2011). The strongest evidence for an anti-cancer effect of aspirin is from patients with COX-2 over-expressing tumours, suggesting the effect is dose-dependent considering aspirin's COX-1 selectivity. The evidence for a therapeutic effect of aspirin treatment in cancer patients is more equivocal. Two recent non-randomized trials have reported a reduction in colorectal- and breast cancer-specific mortality (Chan *et al.*, 2009; Holmes *et al.*, 2010); however, several older studies failed to detect an improvement in survival in patients on high-dose aspirin (Lipton *et al.*, 1982; Lebeau *et al.*, 1993). Consistent with its limited therapeutic efficacy, aspirin failed to inhibit TCIPA *in vitro* (Medina *et al.*, 2006). The implication of this is that, in terms of stimulating platelet activation and recruitment, cancer cells can surmount the COX-1 blockade resulting from pharmacologically relevant levels of aspirin. In this context, we were prompted to assess aspirin prodrugs as inhibitors of TCIPA because of their greater efficacy and potency in response to classical platelet stimuli such as collagen and ADP and ability to produce additional metabolites including NO (the ortho and metanitrate compunds), salicylic acid (ISAS) or nicotinic acid (ST0702).

The isosorbide-based aspirin prodrugs caused significant inhibition of TCIPA in response to HT1080, 59 M and Caco2 cell lines. Of the test compounds, the nicotinate aspirin prodrug ST0702 most consistently inhibited platelet aggregation under static conditions but all of the prodrugs exhibited some activity. It is worth mentioning that nicotinic acid has been shown to mildly inhibit platelet aggregation, an effect which differs from other anti-platelet drugs such as aspirin, suggesting potential opportunities for therapeutic combination in this field. However, in this study, nicotinic acid by itself did not inhibit TCIPA up to 3 mM (Serebruany *et al.*, 2010).

Evaluation of tumour cell-platelet interactions has usually been performed under static conditions. We have recently reported the development of a new method to study TCIPA under flow conditions using an ultrasound standing wave trap (Bazou *et al.*, 2011). The approach permits the study of TCIPA and assessment of inhibitors under more realistic (patho)physiological conditions where flow dynamics play a role in adhesion and tumour mass rupture. Initial studies with this method have shown that platelet recruitment and degranulation by tumour cells are followed by rupture of the tumour cell mass with consequent evolution of satellite

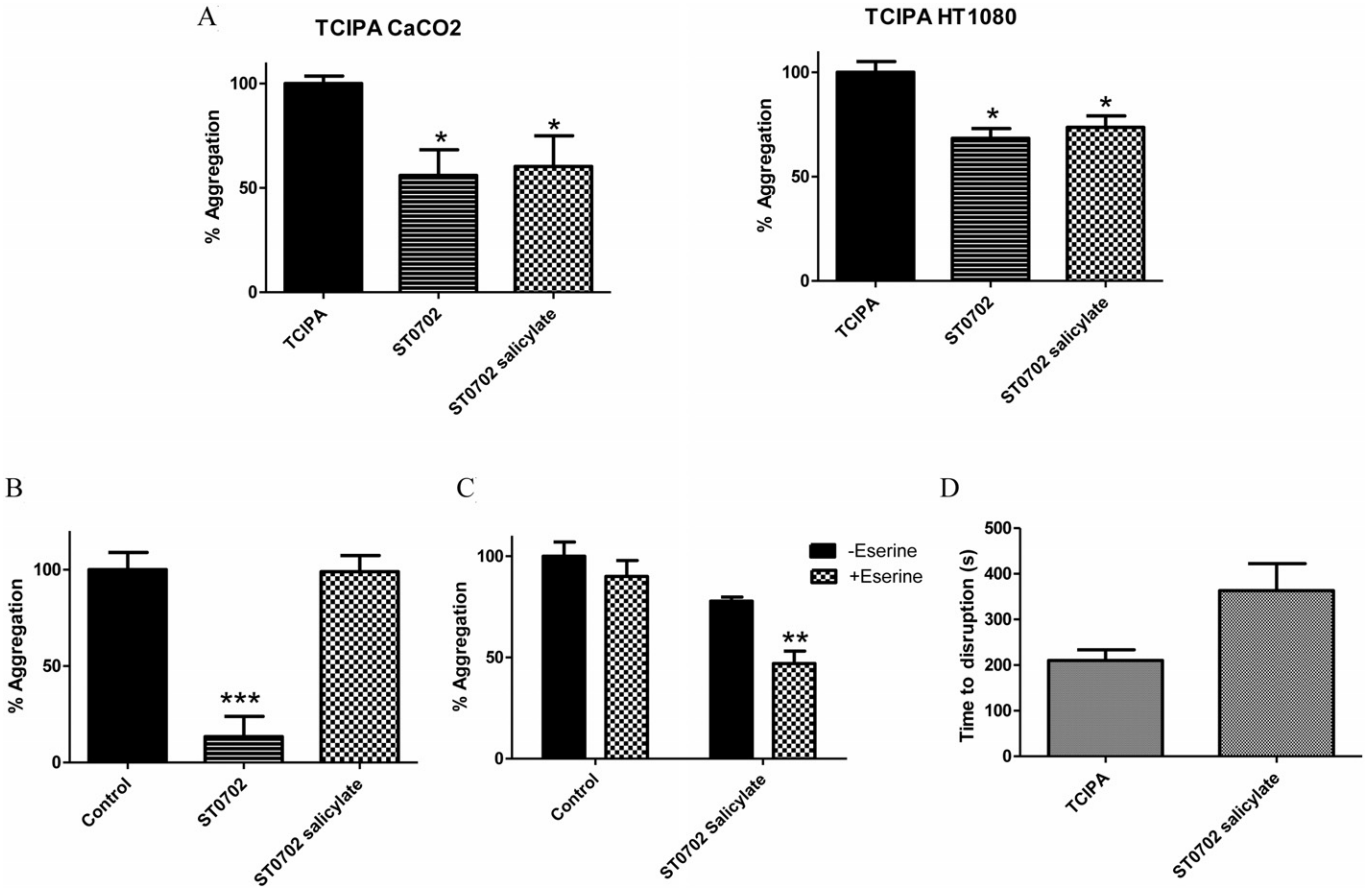

Figure 9

Effect of ST0702 on TCIPA and platelet aggregation. (A) Statistical analysis showing the effects of ST0702 and its metabolite ST0702 salicylate (500 μM) on TCIPA under static conditions. TCIPA was induced by HT1080 cells ($2 \times 10^5 \cdot mL^{-1}$) and Caco2 cells ($1.5 \times 10^3 \cdot mL^{-1}$). Aggregated platelets with tumour cells in the absence of inhibitors (TCIPA) were used as controls. *$P < 0.05$; treatments versus TCIPA. (B) Statistical analysis showing the effects of ST0702 and ST0702 salicylate on collagen-induced platelet aggregation. Aggregated platelets with collagen ($2\ \mu g \cdot mL^{-1}$) were used as control. ***$P < 0.001$; treatments versus control. (C) Effects of ST0702 salicylate on ADP-induced platelet aggregation. Aggregated platelets with ADP ($10\ \mu g \cdot mL^{-1}$) were used as controls. Both control and ST0702 salicylate-treated samples were incubated in the presence and absence of eserine (10 μM). **$P < 0.01$; treatments versus control. (D) The effects of ST0702 salicylate (500 μM) on disruption of platelet-tumour cell aggregates were evaluated. Aggregated platelets with tumour cells in the absence of the inhibitor (TCIPA) were used as negative controls.

aggregates. Although aspirin treatment fails to delay the rupture of the tumour cell-platelet aggregates (Bazou *et al.*, 2011), ST0702 was the only prodrug to effectively inhibit flow-induced TCIPA. Surprisingly, the NO-releasing prodrugs (ortho and metanitrate) did not interfere with TCIPA under flow conditions. Nitro-aspirins or NO-donating aspirin compounds, such as NCX4016, have been extensively evaluated *in vitro* and *in vivo* as chemopreventative agents but not in models of TCIPA (Rigas and Williams, 2008). Interpretation of the biochemical efficacy of NCX4016 is moreover complicated by its metabolic conversion to a quinone methide that irreversibly modifies cellular biomolecules leading to reduced viability (Dunlap *et al.*, 2008). Nevertheless, there is substantial evidence that NO release from nitro-aspirins can augment the anti-platelet effects of the aspirin component (Gresele and Momi, 2006). The ortho- and metanitrate prodrugs evaluated in the present study are among the first to release aspirin and NO. These inhibit ADP-induced aggregation in a manner that is sensitive to inhibition by the soluble guanylate cyclase inhibitor ODQ (Jones *et al.*, 2009). One interpretation of the present data is that NO amplification of aspirin effects may be insufficient to prevent key steps in the mutual activation of platelets and cancer cells.

One of the main pathways involved in TCIPA is the MMP-2 dependent pathway. We have previously shown the requirement for activated MMP-2 to induce the MMP-2 dependent pathway both in agonist and platelet aggregation induced by HT1080 and Caco2 cells (Jurasz *et al.*, 2001; Medina *et al.*, 2006). Phenanthroline, a synthetic broad spectrum MMP inhibitor, was able to reduce TCIPA and the abundance of receptors on platelet surface. Therefore, we studied the effect of aspirin prodrugs on MMP-2 release during TCIPA and found that none of the test drugs significantly reduced the release of MMP-2. These results clearly indicate that the effect of aspirin prodrugs on TCIPA was MMP-independent.

TCIPA is partly mediated by ADP (Alonso-Escolano *et al.*, 2004; Medina *et al.*, 2006). Although ADP is a weak agonist, it is essential for platelet function. The release of ADP from activated platelets and stimulation of P2Y2 purinergic receptors accounts for the non-TXA_2, non-MMP-2-mediated pathway of platelet aggregation (Gachet, 2001; 2006; 2008). In order to find out why ST0702 was a more efficacious inhibitor of TCIPA than its analogous prodrugs, we analysed platelet-tumour cell supernatants and lysates following TCIPA experiments using an HPLC method capable of separating and identifying potential metabolites of ST0702. The most prominent by-product of cellular hydrolysis of ST0702 was the corresponding salicylate ester resulting from esterase-mediated deacylation (ST0702 salicylate). Interestingly, ST0702 salicylate was able to inhibit ADP-stimulated platelet aggregation but not collagen-induced aggregation, which is more aspirin-sensitive. Furthermore, the ST0702 salicylate caused inhibition of TCIPA under no flow conditions, implicating ADP blockade in the mode of action of ST0702. Notably, the salicylate did not inhibit TCIPA under flow conditions, unlike ST0702, which can also release aspirin, suggesting that ADP inhibition by ST0702 is not sufficient for the inhibitory effects observed. The present results are consistent with our previous studies where scavenging ADP with potato and human apyrase decreased TCIPA (Jurasz *et al.*, 2003; Alonso-Escolano *et al.*, 2006; Medina *et al.*, 2006). Similar effects to apyrase could be demonstrated using selective inhibitors of the P2Y12 receptor such as 2-methylthio-AMP (Alonso-Escolano *et al.*, 2004).

As platelet receptors mediate TCIPA, we next studied the changes in the abundance of P-selectin on platelets induced by 59 M cells. In fact, P-selectin and its association with mucin is likely to mediate TCIPA in a variety of mucin-producing cancers (Kim *et al.*, 1999; Varki and Varki, 2002; Wahrenbrock *et al.*, 2003). In our study, we found that 59 M cells increased the number of copies of P-selectin, as measured by flow cytometry. These results are in agreement with our previous studies *in vitro* (Medina *et al.*, 2006). We next studied the effect of aspirin prodrugs on P-selectin in TCIPA. Indeed, we have previously shown that TCIPA inhibition is strongly associated with P-selectin down-regulation (Medina *et al.*, 2006). Our results showed that ST0702 again was the most efficacious inhibitor of P-selectin expression during TCIPA. Interestingly, the ortho- and metanitrate compounds, but not ISAS, significantly reduced P-selectin expression but to a lesser extent. This may be due to the fact that the ortho- and metanitrate compounds are able to produce NO and aspirin, unlike ISAS which is metabolised to aspirin.

In conclusion, isosorbide-based aspirin prodrugs, which are potent inhibitors of collagen and ADP-induced platelet aggregation, also inhibit TCIPA, whereas aspirin does not. The aspirin–nicotinic acid prodrug ST0702 inhibited TCIPA under no flow and flow conditions. The inhibitory effect of ST0702 appears to be due to its dual capacity to release aspirin as well as its blockade of ADP and P-selectin-mediated function, partly through its deacetylated salicylate metabolite. This effect distinguished ST0702 from aspirin and related NO-releasing compounds. The therapeutic potential of ST0702 as an aspirin prodrug and inhibitor of TCIPA in the prevention of blood-borne metastasis merits further attention.

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Conflict of interest

None

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