Effect of lysine antifibrinolytics and cyclooxygenase inhibitors on the proteolytic profile of breast cancer cells interacting with macrophages or endothelial cells

B. Afsharimani1, P. J. Cabot1 and M.-O. Parat1,2*

1 School of Pharmacy, University of Queensland, Brisbane, Australia
2 Outcomes Research Consortium, Cleveland, OH, USA
* Corresponding author: School of Pharmacy, University of Queensland, PACE, 20 Cornwall Street, Woolloongabba, QLD 4102, Australia.
E-mail: m.parat@uq.edu.au

Editor’s key points

- Extracellular matrix (ECM) proteases can affect cancer cell migration and invasion and hence their ability to metastasize.
- This in vitro study evaluated the effect of antifibrinolytic agents and cyclooxygenase (COX) inhibitors, which are commonly used perioperatively, on ECM proteases released by breast cancer cells alone or when cocultured with endothelial cells or macrophages.
- Antifibrinolytics and COX inhibitors have mixed effects on ECM proteases and their endogenous inhibitors, thus functional analyses are warranted to determine the outcome of their administration on cancer cell invasion.

Background. Extracellular matrix (ECM) proteases play a key role in the regulation of tumour invasion, growth, and transendothelial migration. The expression of ECM proteases and their endogenous inhibitors by cancer cells is regulated by stromal cells. We investigated the effect of commonly used perioperative medications on this regulation.

Methods. Breast cancer cells (4T1) were cultured alone or with endothelial cells (H5V) or macrophages (RAW264.7). Cell grown alone or in cocultures were treated with clinically relevant concentrations of cyclooxygenase (COX) inhibitors, aspirin (ASA), ketorolac, celecoxib, or lysine antifibrinolytics, ε-aminocaproic acid (EACA) and tranexamic acid (TXA). We determined the level of the ECM proteases urokinase-like plasminogen activator (uPA), matrix metalloproteinase (MMP)-2 and MMP-9, and endogenous MMP inhibitors, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in the conditioned media.

Results. Antifibrinolytics and COX inhibitors exerted a complex effect on cells grown alone and in cocultures. EACA increased the activity of MMP-9 and TIMP-1 in cocultures of 4T1 and RAW264.7. TXA increased TIMP-1 in the coculture without affecting MMP-9. EACA and TXA both attenuated MMP-2 detected in 4T1 and H5V cocultures. ASA and ketorolac both decreased the activity of MMP-2, MMP-9, and uPA. Celecoxib increased the activity of TIMP-1 in cocultures of 4T1 with both macrophages and endothelial cells.

Conclusions. Antifibrinolytics and COX inhibitors can affect the proteolytic profile of the tumour microenvironment. Animal and clinical investigations are warranted to assess the effect of these proteolytic changes on the outcome of cancer surgery.

Keywords: endothelial cells; macrophages; matrix metalloproteinase-9; urokinase plasminogen activator

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Postoperative tumour recurrence and metastasis are a major challenge in cancer treatment. Extensive neuroendocrine and immunological changes during and after surgery provide pro-metastatic conditions in favour of the localization or growth of tumour cells in secondary organs. Extracellular matrix (ECM) proteases are key players in tissue remodelling that allow tumour growth, invasion, and angiogenesis. In the tumour microenvironment, the expression and activity of proteases and their endogenous inhibitors are finely regulated by the interaction between tumour cells and stromal cells such as tumour-associated fibroblasts and inflammatory cells. It is important to determine whether the growth of micrometastases existing at the time of surgery or disseminated tumour cells can be promoted by perioperative factors.

Synthetic derivatives of the amino acid lysine, ε-aminocaproic acid (EACA) and tranexamic acid (TXA), are used as antifibrinolytic drugs to control excessive bleeding during and after surgery. They inhibit the activation of plasminogen into plasmin by the plasminogen-activating enzymes, tissue plasminogen activator (tPA), and urokinase-like plasminogen activator (uPA). In vitro, lysine antifibrinolytics are reported to have anti-invasive effects, but the in vivo data on the outcome of their administration on tumour metastasis are inconclusive.
As inexpensive drugs with a favourable adverse effect profile, non-steroidal anti-inflammatory drugs (NSAIDs) are a substantial component of the postoperative pain management.\textsuperscript{12} NSAIDs produce their analgesic effects by inhibiting the conversion of arachidonic acid to prostaglandins by cyclooxygenases (COXs) in peripheral tissues.\textsuperscript{13} The role of COXs and their products, mainly PGE\textsubscript{2}, in tumour angiogenesis and cell apoptosis is well established.\textsuperscript{14 15} COXs and COX-derived prostanoids are important mediators of the crosstalk between tumour cells and their microenvironment.\textsuperscript{16} We tested whether the proteolytic changes induced by interaction between breast tumour cells and macrophages or endothelial cells could be affected by members of two commonly used perioperative drug classes, namely antifibrinolytics and COX inhibitor analgesics.

**Methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), DMEM/F12, RPMI-1640, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen (Life Technologies, Mulgrave, VIC, Australia). The following reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): Thiazolyl Blue Tetrazolium Blue, dimethyl sulfoxide (DMSO), EACA, bovine skin gelatine type B, bovine milk casein, and bovine plasma plasminogen. Aspirin (ASA) was from Enzo Life Sciences (Farmingdale, NY, USA), ketorolac tromethamine from Cyman Chemical Company (Ann Arbor, MI, USA), celecoxib from Tocris Biosciences (Bristol, UK), and TXA from LKT laboratories (St Paul, MN, USA).

**Cell culture**

4T1 murine breast cancer cells were grown in RPMI-1640 medium supplemented with 5% (v/v) FBS, penicillin (100 units ml\textsuperscript{-1}), streptomycin (100 \mu g ml\textsuperscript{-1}), and 1% (v/v) sodium pyruvate. RAW264.7 murine macrophages were maintained in DMEM with 10% FBS, 100 units ml\textsuperscript{-1} penicillin, and 100 \mu g ml\textsuperscript{-1} streptomycin. H5V murine endothelial cells were grown in DMEM-F12 medium supplemented with 5% FBS, penicillin (100 units ml\textsuperscript{-1}), and streptomycin (100 \mu g ml\textsuperscript{-1}). All cells were kept in a humidified atmosphere containing 5% CO\textsubscript{2}.

**Conditioned media preparation**

To prepare conditioned media, 80% confluent cells were rinsed with serum-free medium and placed in serum-free medium for 48 h. The cell-conditioned media were then collected and centrifuged at 1100\textsuperscript{g} for 5 min. Supernatants were collected and kept at −20°C until analysed.

**Cocultures**

4T1 and RAW264.7 murine macrophages were cultured alone or together in equal numbers (8 × 10\textsuperscript{4}) in 24-well plates. Similarly, 4T1 cells and H5V endothelial cells were cultured alone or together in equal numbers (5 × 10\textsuperscript{4}) in 24-well plates. Cells were seeded and maintained in a medium consisting of a 1:1 mixture of the medium of each cell line for 24 h. Cells were washed twice and incubated in a 1:1 mixture of the same medium without serum, added with different concentrations of drugs or the same volume of buffer as control. The 48 h conditioned media were collected, measured for protein content and analysed by gelatine, reverse gelatine, and casein–plasminogen zymography. We have previously established that coculturing 4T1 with either H5V or RAW264.7 cells resulted in major alterations of the proteolytic profile in the conditioned media.\textsuperscript{17}

**Drug treatment of cells**

4T1, RAW264.7, and H5V cells and also the cocultures of 4T1 cells with either RAW264.7 or H5V cells were treated with drugs or equal volume of buffer for 48 h. The concentrations of ASA (0.05, 0.5, 5 mM) were chosen based on the plasma concentrations of ASA and its main metabolite salicylate achieved after oral administration\textsuperscript{16} adjusted with the commonly used concentrations of ASA in \emph{in vitro} studies.\textsuperscript{19–22} The range of concentrations for ketorolac used in our experiments (0.1, 1, 10 \mu M) was chosen to best cover the plasma concentrations achieved after administration of different dosages and modes of administration\textsuperscript{23} and was adjusted with the concentrations commonly used in \emph{in vitro} studies.\textsuperscript{24 25} The concentration range for celecoxib (0.05, 0.5, 5 \mu M) was chosen based on the plasma concentrations achieved after oral administration of single or multiple dosages of celecoxib from 1 to 5 \mu M\textsuperscript{18 26} and adjusted with the concentrations commonly used in \emph{in vitro} studies.\textsuperscript{27–29} The concentrations of EACA (0.1, 1, 10 mM) and TXA (0.06, 0.6, 6 mM) were chosen according to therapeutic plasma concentrations\textsuperscript{30 31} and were matched with those used in \emph{in vitro} experiments.\textsuperscript{6 8}

**Gelatine, reverse gelatine, and casein–plasminogen zymography**

Conditioned media were analysed for MMPs and uPA content using gelatine and casein – plasminogen zymography, respectively. An 11% polyacrylamide gel containing 1% (w/v) gelatine was used to measure the level of gelatinases (MMP-2 and MMP-9). To measure uPA, 1.5% (w/v) casein and 0.02 units ml\textsuperscript{-1} plasminogen were added to the gel. In order to measure TIMPs, NIH3T3-conditioned medium was added to a 12% polyacrylamide gel as a source of gelatinase along with 1% (w/v) gelatine.

Equal amounts of protein were separated by SDS–PAGE. The gels were incubated in a renaturing solution [50 mM Tris, 5 mM CaCl\textsubscript{2}, and 2.5% (v/v) Triton X-100] overnight. The gels were rinsed and incubated at 35°C in a solution containing 50 mM Tris–HCl and 5 mM CaCl\textsubscript{2} for 3 h. Gels were stained with a solution of Coomassie Blue R-250 [0.25% (w/v) in methanol 45% (v/v) and glacial acetic acid 10% (v/v)] and destained with a solution of methanol 25% (v/v), acetic acid 10% (v/v) until the proteolytic bands appeared clear on a dark background, or the proteolysis inhibition appeared as dark areas against a pale background. The gels were scanned and the intensity of the bands was measured with NIH Image J software.
Cell viability assay
Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as previously described. Briefly, 4T1, H5V, and RAW264.7 cells were cultured separately and together under the same conditions used in the conditioned media preparation for zymography. Forty-eight hours after treatments, conditioned media were replaced with the medium containing 0.5 mg ml⁻¹ MTT for 3 h. The medium was then replaced with DMSO to dissolve formazan formed by viable cells. Absorbances were recorded at 595 nm presented as the percentage of control cells treated with buffer.

Statistical analysis
Results are presented as the mean values (SEM) of three independent experiments. Statistical analysis was performed using Graphpad Prism. Groups were compared using two-way analysis of variance (ANOVA) with the Bonferroni post-test. A P-value of <0.05 was considered of statistical significance.

Results
The non-selective COX inhibitors ASA and ketorolac differentially modulate the activity of uPA, gelatinases and their endogenous inhibitors produced by 4T1, H5V, or RAW264.7 cells or their cocultures
The coculture of 4T1 breast cancer cells with H5V endothelial cells resulted in increased production of uPA in the conditioned media compared with either cell type cultured alone (Fig. 1C and D). Similarly, MMP-2 and -9 and TIMP-1 and -2 were increased when cells were cocultured (Fig. 2A and B). Moreover, treatment with ASA and ketorolac significantly decreased the level of uPA in the conditioned media of 4T1, H5V, and 4T1 and H5V cocultures (Fig. 1). MMP-2, TIMP-1, and TIMP-2 were all decreased in 4T1 cells after treatment with 5 mM ASA (Fig. 2). There was a decrease in MMP-9, MMP-2, and TIMP-1 in the cocultures of breast cancer cells with endothelial cells after treatment with ASA (Fig. 2). The inhibitory effect of ketorolac on the production of proteases was significant only...
Fig 2 Effect of aspirin and ketorolac on gelatinases and TIMPs in the conditioned media of 4T1, H5V cells, and their cocultures. Cells were treated with different concentrations of aspirin, ketorolac, or the same volume of buffer as control for 48 h. (a and b) Conditioned media were tested for MMP-2 and MMP-9 by gelatine zymography and for TIMP-1 and TIMP-2 by reverse gelatine zymography. For each sample, an equal amount of protein was loaded on gels. (c–j) The intensity of the bands on gels was quantified by densitometry using NIH Image J. The density of each band is expressed as a percentage of control in each group. The mean (SEM) of percentage values of three independent assays is presented. *P<0.05, **P<0.01, ***P<0.001 (two-way ANOVA). n.d., not detectable.
in the cocultures of 4T1 cells with H5V cells while having little or no effect on cells grown alone \((P < 0.05)\) (Fig. 2).

The COX-2 inhibitor celecoxib increases TIMP-1 in the cocultures

Treatment with celecoxib did not alter the level of uPA, MMP-9, MMP-2, or TIMP-2 in any of the cells grown alone or in cocultures (data not shown). The level of TIMP-1 was unchanged in cells grown alone but significantly increased in cocultures of 4T1 cells with RAW264.7 cells \((P < 0.05)\) or H5V cells \((P < 0.01)\) after treatment with 5 \(\mu\)M celecoxib (Fig. 3).

Aminocaproic acid and TXA do not affect the activity of uPA produced by 4T1, H5V, or RAW264.7 cells grown individually or in cocultures

EACA and TXA at the concentrations used did not have any statistically significant effect on the level of uPA in the conditioned media of individual cells or their cocultures (Fig. 4A–D).

Aminocaproic acid and TXA differentially modulate the activity of gelatinases and their endogenous inhibitor TIMP-1 produced by 4T1, H5V, or RAW264.7 cells or their cocultures

EACA treatment significantly increased MMP-9 in the conditioned media of RAW264.7 cells and in the cocultures of 4T1 and RAW264.7 cells at 10 \(\mu\)M \((P < 0.01)\) (Fig. 5A) and TIMP-1 in 4T1 and RAW264.7 cocultures at 1 and 10 \(\mu\)M \((P < 0.05\) and \(< 0.01\), respectively) (Fig. 5A). TXA at 6 \(\mu\)M increased MMP-9 in RAW264.7 cells (although this was not statistically significant) and increased TIMP-1 at 0.6 \(\mu\)M \((P < 0.05)\) and 6 \(\mu\)M \((P < 0.01)\) (Fig. 5A). The MMP-9 to TIMP-1 ratio was unchanged with EACA and slightly decreased with TXA (Fig. 5B and i). EACA and TXA both attenuated the amount of MMP-2 detected in the cocultures of 4T1 and H5V cells \((P < 0.05)\) for EACA 10 \(\mu\)M and TXA 0.6 \(\mu\)M, \(P < 0.01\) for TXA 6 \(\mu\)M (Fig. 6).

Effect of EACA, TXA, ASA, ketorolac, and celecoxib on the viability of 4T1, H5V, or RAW264.7 cells grown individually or in cocultures

Using the MTT assay, we tested whether the selected concentrations of antifibrinolytics or COX inhibitors affect the viability of cells cultured alone or in cocultures. 4T1, H5V, and RAW264.7 cells were cultured separately or together under the same conditions used in the conditioned media preparation for zymography. The effect of drugs was tested on 4T1 cells at two different confluencies matching the numbers seeded for 4T1 and RAW264.7 and 4T1 and H5V cocultures, respectively. TXA, ketorolac, and celecoxib did not have any effect on the viability of individual cells or cocultures. EACA decreased the viability of cells at the highest concentration (10 \(\mu\)M) which was statistically significant in RAW264.7 cells (data not shown). Moreover, the number of viable cells was slightly lower in almost all culture types after treatment with the highest concentration of ASA (5 \(\mu\)M). However, this effect of ASA was not statistically significant.

Discussion

Non-malignant cells in the tumour microenvironment play a key role in the regulation of ECM proteases. In in vitro coculture models, including our own, macrophages and endothelial cells induce the production of gelatinases, TIMPs, and the plasminogen activator, uPA by cancer cells. These interactions are necessary for local invasion and systemic dissemination of tumours. Accordingly, our current results show a remodelling of the proteolytic profile when breast cancer cells are cultured together with accessory cells. Our results further show that the non-selective COX inhibitors, ASA, and ketorolac significantly
decrease uPA in breast cancer cells, endothelial cells, and their cocultures. In line with previous findings, we observed a decrease in MMPs with ASA and ketorolac. ASA further caused a decrease in TIMPs. This might be explained by the unique ability of ASA to acetylate COX, which leads to the production of antitumourigenic mediators; however, we cannot rule out that it is the result of cytotoxicity of ASA at the highest concentration we used, 10 mM. The decrease in gelatinases in the cocultures of 4T1 and H5V cells after treatment with ASA and ketorolac might be a result of the reduction in uPA produced by H5V cells, leading to the suppression of plasminogen-mediated activation of MMPs or growth factors and cytokines that mediate the up-regulation of MMPs. Alternatively, NSAIDs might interfere with a PGE2-mediated crosstalk between cancer cells and endothelial cells.

Celecoxib had no effect on individual cells but selectively caused an increase in TIMP-1 in cocultures of breast cancer cells with endothelial cells or macrophages. Others have documented that the inhibition of the COX-2/PGE2 system reduced TIMPs and MMPs in different cell types. The suppression of COX-2 is known to result in the up-regulation of COX-1 which in turn promotes the expression of COX-2 and other pro-tumorigenic factors and therefore a combination of COX-1 and -2 inhibition is considered by some investigators to be superior in chemo-protection to COX-2 inhibition. The celecoxib-induced increase in TIMP-1 that we unveiled and is specific to coculture conditions might participate in a protective effect of this drug in vivo via altering the tumour microenvironment.

Antifibrinolytic drugs did not affect the level of uPA in cells grown individually or in coculture. Despite the increase in MMP-9, there was a tendency to a decrease in the proteolytic potential of the cocultured cells, resulting from decreased MMP-2 (which is elevated as a result of tumour–endothelial cells interaction) and increased TIMP-1. EACA and TXA can deplete cell surface-bound plasminogen and plasmin, and thus could potentially interfere with ECM degradation and activation of MMPs and also several growth factors and cytokines that mediate the crosstalk between tumour cells and their surroundings.

Although plasmin is known to activate MMPs by proteolytic cleavage, in our model, MMP-9 and TIMP-1 activities were increased by both EACA and TXA. An increase in TIMP-1 gene expression by plasmin inhibitors including TXA has previously been reported. A possible explanation for the increase in MMP-9 is a compensatory increase in MMP-9 activity in response to diminished proteolytic function of plasmin, as
previously observed in *in vivo* models.45 46 However, since both MMP-9 and TIMP-1 were increased with EACA, the net resulting effect on the balance between MMP-9 and TIMP-1 (which is important in maintaining ECM homeostasis)47 was null. On the other hand, a decrease in the MMP-9/TIMP-1 ratio was observed with TXA, resulting from increased TIMP-1 and unaltered MMP-9.

It is interesting to note that several of the effects of perioperative drugs on cancer and accessory cells that we report in this study are seen in cocultures of accessory cells with breast cancer cells but not in cells grown individually (Table 1). This is highly relevant to the tumour microenvironment where the behaviour of multiple cell types is interrelated, and the crosstalk between tumour cells and elements of the
tumour microenvironment determines the level of proteases in the tumour tissue.48 49

Our study is not without limitations. First, our model uses murine breast cancer cells, because it was designed to relate in vitro coculture data with preclinical data using murine breast cancer cells in mice.17 Murine endothelial cells and murine macrophages were then chosen to maximize the compatibility of soluble factors and cytokines exchanged by the different cell types when placed in coculture. Whether the same results would be obtained from human cell cocultures treated with antifibrinolytics and COX inhibitors, which can commonly be used perioperatively in cancer surgery patients, alter the proteolytic profile of interacting cells of the tumour microenvironment in vitro; however, whether this translates into protection towards tumour recurrence or metastasis remains to be demonstrated in vivo. Recent work has indicated that celecoxib did not, when used alone, improve survival of tumour-bearing mice,50 and that another COX-2 inhibitor, etodolac, was only able to prevent spontaneous postoperative metastasis in a mouse model when used in combination with the β-adrenergic inhibitor propranolol.51 The latter study used a single s.c. injection of the drugs in oil 30 min before the resection.

Although previous findings have indicated potential antitumour effects for plasminogen activator inhibitor antifibrinolytics, their simplicity in that in a tumour in vitro, the environment is more complex. Lastly, our in vitro results indicate that antifibrinolytics and COX inhibitors, which can commonly be used perioperatively in cancer surgery patients, alter the proteolytic profile of interacting cells of the tumour microenvironment in vitro; however, whether this translates into protection towards tumour recurrence or metastasis remains to be demonstrated in vivo. Recent work has indicated that celecoxib did not, when used alone, improve survival of tumour-bearing mice, and that another COX-2 inhibitor, etodolac, was only able to prevent spontaneous postoperative metastasis in a mouse model when used in combination with the β-adrenergic inhibitor propranolol. The latter study used a single s.c. injection of the drugs in oil 30 min before the resection.

Table 1 Summary of the effect of drugs on the level of proteases in mono- and cocultures. COX inhibitors and antifibrinolytics differentially modulate the level of gelatinases, TIMPs, and uPA in cells grown alone and cocultures. \textbackslash, decrease; \textbar, increase. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\) (two-way ANOVA)

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and COX inhibitors, the underlying mechanisms involved in these effects are not clearly understood. Elucidating the targets of these drugs at cellular and molecular levels can lead to a more effective targeting of these systems for better therapeu-tic outcome. Further studies to determine the mechanism by which these drugs influence the proteolytic profile of tumour cells and their microenvironment and whether their systemic administration could have similar effects in vivo are warranted to shed more light on the potential effect of these drugs on the postoperative outcome of cancer surgery.

Authors’ contributions
B.A.: design, data acquisition, data analysis, drafting of the article, and critical revision. P.J.C.: study design and analysis and interpretation of the data, and critical revision of the manuscript. M.-O.P.: study conception and design, analysis and interpretation of the results, and revision of the manuscript.

Declaration of interest
None declared.

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