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RESEARCH PAPER

# Inhibition of cyclooxygenases 1 and 2 by the phospholipase-blocker, arachidonyl trifluoromethyl ketone

HJ Leis and W Windischhofer

*Medicinal University of Graz, University Children's Hospital, Research Unit of Osteology and Analytical Mass Spectrometry, Auenbruggerplatz 30, Graz, Austria*

**Background and purpose:** Arachidonyl trifluoromethyl ketone (ATK) is widely used as an inhibitor of cytosolic group IV phospholipase $A_2$ (cPLA$_2$) and calcium-independent group VI phospholipase $A_2$ (iPLA$_2$). ATK thus reduces arachidonic acid (AA) substrate for cyclooxygenase (COX; also known as prostaglandin H synthase) and attenuates prostaglandin (PG) synthesis. It has been shown previously, that ATK blocks thromboxane $B_2$ production induced by exogenous AA in human platelets. It remains, however, unknown whether ATK also directly modulates the activity of cyclooxygenase (COX).
**Experimental approach:** Time courses for inhibition of COX by ATK was obtained using osteoblast-like MC3T3-E1 cells, with exogenous AA as substrate and the pure enzymes COX-1 and COX-2. $PGE_2$ was measured by GC-MS.
**Key results:** ATK was a potent inhibitor of COX-1 and COX-2 with $IC_{50}$ values of 0.5 and 0.1 μM in MC3T3-E1 cells and of 1.7 and 2.6 μM using the pure enzymes. Inhibition was reversible, with slow- and tight-binding characteristics. The arachidonyl carbon chain was essential, as the saturated palmitoyl analogue had no effect.
**Conclusions and implications:** Attenuation of PG synthesis by ATK is taken to be the consequence of PLA$_2$ inhibition and the findings of many studies are interpreted on that basis. If there are, however, alternative routes for AA liberation (such as phospholipase C/diacyl glycerol lipase or phospholipase D), this interpretation can lead to false conclusions. As ATK is a widely used and important pharmacological tool in eicosanoid research, knowledge of its interactions with other major enzymes of the cascade is of considerable importance.
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**Keywords:** arachidonyl trifluoromethyl ketone; COX; prostaglandin; phospholipase; cyclooxygenase; MC3T3-E1; osteoblast

**Abbreviations:** α-MEM, α-minimum essential medium; AA, arachidonic acid; ATK, arachidonyl trifluoromethyl ketone; COX, cyclooxygenase; DAG, diacyl glycerol; FCS, fetal calf serum; GC-NICI-MS, gas chromatography-negative ion chemical ionization mass spectrometry; PG, prostaglandin; PL, phospholipase; PLA$_2$, phospholipase $A_2$; PLC, phospholipase C; PLD, phospholipase D; PTK, palmitoyl methyltrifluoro ketone

## Introduction

Phospholipase $A_2$ (PLA$_2$) enzymes hydrolyze the fatty acid ester bond at the *sn*-2 position in phospholipids (Dennis, 1997). PLA$_2$ activity results in the formation of lysophospholipids and the eicosanoid precursor arachidonic acid (AA). Alternatively, PLC and PLD enzymes can indirectly provide free AA. PLCs metabolize phosphatidylinositol (PI) and phosphatidylinositol phosphates to inositol phosphates and diacylglycerol (DAG) (Rebecchi and Pentyala, 2000). DAG is further metabolized sequentially by DAG lipase to 2-arachidonylglycerol and monoacylglycerol lipase or fatty acid amidohydrolase to release free AA (Chau and Tai, 1981), as found in platelets (Bell *et al.*, 1979) and bovine pulmonary artery endothelial cells (Whatley *et al.*, 1993). PLD hydrolyzes phospholipids, such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol to form phosphatidic acid, which can be further metabolized to DAG and lysolphosphatidic acid (Exton, 1990). DAG can provide AA through the route described above.

Free AA is subsequently metabolized to prostaglandins (PGs) by COX enzymes. Two isoforms exist, the constitutively expressed COX-1 and the inducible COX-2. In MC3T3-E1 osteoblastic cells both enzymes lead to the formation of

Correspondence: Professor HJ Leis, Research Unit of Osteology and Analytical Mass Spectrometry, Medical University of Graz, University Children's Hospital, Auenbruggerplatz 30, Graz A-8036, Austria.
E-mail: hans.leis@meduni-graz.at
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$PGE_2$, which is considered to be a powerful modulator of bone cell function. As in osteoblasts, prostanoids are ubiquitously produced by many cell types and serve as autocrine and paracrine mediators of numerous cellular functions. Inhibition of COX by non-steroidal anti-inflammatory drugs is only one example of the extensive potential of pharmacological intervention at the site of prostanoid synthesis.

AA release is considered as the rate-limiting step in PG synthesis and many studies have been performed to clarify the function of various PL enzymes in this event. Classifying and assigning PL activity relies largely on the use of pharmacological modulators of these enzymes. Thus, Lucas and Dennis (2005) have reviewed how to distinguish $PLA_2$ types in biological samples using group-specific assays and inhibitors. One of the most widely used inhibitors is arachidonyl trifluoromethyl ketone (ATK). It is considered to be a specific, slow- and tight-binding inhibitor of group IV $cPLA_2$ (Street *et al*., 1993) and group VI $iPLA_2$ (Ackermann *et al*., 1995; Ghomashchi *et al*., 1999). It has been used in numerous studies to link PG synthesis to the activity of certain PLA types (Kurusu *et al*., 1997; Kuwata *et al*., 1998; Saunders *et al*., 1999; D'Orazi *et al*., 2006). Attenuation of PG synthesis was thereby attributed to the inhibitory effect of ATK on the above-mentioned $PLA_2$s. Inhibition of PLAs certainly blunts PG formation, and the conclusions of such inhibition experiments may be correct if there are no other effects of ATK on the PG synthesizing system. It has been previously shown that ATK blocks AA-induced thromboxane $B_2$ production in human platelets, but direct effects of this compound on COX enzymes have not been investigated so far (Riendeau *et al*., 1994). This study was therefore conducted to investigate such effects. Our results showed that ATK potently inhibited both COX-1 and COX-2.

## Materials and methods

*Cell culture*

MC3T3-E1 cells (passage number 10–20, kindly provided by) were cultured routinely in α-minimum essential medium (α-MEM) containing 5% fetal calf serum (FCS), gentamycin sulphate ($83.4\,mg\,L^{-1}$), $50\,\mu g\,mL^{-1}$ ascorbate and L-glutamine ($0.584\,g\,L^{-1}$) in a humidified atmosphere of 5% $CO_2$ in $80\,cm^2$ flasks (initial plating density $2\times10^4$ cells $cm^{-2}$) and transferred to $4\,cm^2$ 12-well culture dishes before experiments. Experiments were carried out at confluency (day 7 of culture). For serum induction of COX-2, cells were cultured under starving conditions (0.2% FCS in α-MEM) for 24 h. Serum induction was accomplished with 5% FCS in α-MEM (Pilbeam *et al*., 1993).

*COX-1 and COX-2 enzyme assays*

Enzyme inhibitor potencies were measured in Tris–HCl buffer (30 mM, pH 8.0) containing glutathione (0.49 mM), adrenaline (1 mM) and haematin (1 μM). One unit of COX and 1 μM sodium arachidonate were used. Pre-incubation with ATK was carried out for 30 min at room temperature. Incubation with sodium arachidonate was performed for 30 min at 37 °C. The mixture was then cooled in an ice bath and the reaction terminated by the addition of ice-cold formic acid (0.2%). Assay for $PGE_2$ was then performed as described below.

*$PGE_2$ analysis*

For assessment of short-term $PGE_2$ production, medium (1 mL) was removed and the cell monolayer was incubated in 1 mL of HEPES-buffered (20 mM) Hank's balanced salt solution without calcium. Incubations with AA, test compounds or vehicle were carried out for 30 min. Pre-incubations with ATK were carried out for 30 min. For determination of long-term $PGE_2$ synthesis, COX-2 was induced by serum in the presence of ATK for 3 h (Pilbeam *et al*., 1993). Subsequent stimulation with AA was carried out for 30 min. The incubation buffer was removed and $PGE_2$ measured by GC-NICI-MS. Briefly, $PGE_2$ was converted to its pentafluorobenzyl ester-trimethylsilyl ether-O-methyloxime derivative. Quantitation was carried out by the use of tetradeuterated $PGE_2$ (Leis *et al*., 1987). A TACE GC-MS system (Thermo) was used. GC was performed on a 15 m DB-5MS fused silica capillary column (Thermo). The temperature of the splitless Grob injector was kept at 290 °C, initial column temperature was 160 °C for 1 min, followed by an increase of $40^\circ\,min^{-1}$ to 310 °C. Negative ion chemical ionization was carried out in the single ion recording mode with methane as a moderating gas.

*Western blot analysis of COX-2 induction*

MC3T3-E1 cells were treated with different concentrations of ATK and COX-2 expression was induced with serum. Cells were washed with chilled phosphate-buffered saline (pH 7.4) and lysed on ice for 15 min in 100 μL of lysis buffer (HEPES, 50 mM; NaCl, 150 mM; EDTA, 1 mM; $Na_4P_2O_7$, 10 mM; $Na_3VO_4$, 2 mM; NaF, 10 mM; Triton X-100, 1%, v/v; glycerol, 10%, v/v; protease inhibitor cocktail tablets and pH 7.4). Cell lysates were scraped off and cell debris was removed by centrifugation at $11\,000\times g$ at 4 °C for 10 min. Protein lysates (130 μg) were diluted in NuPAGE LDS sample buffer and NuPAGE sample reducing agent to a final volume of 30 μL. The samples were heated for 10 min at 70 °C and then subjected to electrophoresis on 4–12% NuPAGE Bis–Tris gels, 1.5 mm in NuPAGE MES SDS running buffer (80 min at 130 V). Proteins were transferred to nitrocellulose membranes (2 h, 0.3 A) and blocked with non-fat milk powder. The blots were incubated with goat polyclonal antibody COX-2 (dilution 1:200 in 3% bovine serum albumin) for 2 h at room temperature. The membranes were washed and then incubated with horseradish peroxidase-conjugated donkey anti-goat IgG (dilution 1:200 000) for 1 h at room temperature. After washing, immunoreactive signals were detected with SuperSignal West Pico chemiluminescent substrate and exposure to Hyperfilm MP. For loading controls, membranes were stripped and re-probed with a primary antibody recognizing β-actin and horseradish peroxidase-conjugated goat anti-mouse IgG was used as a secondary antibody.

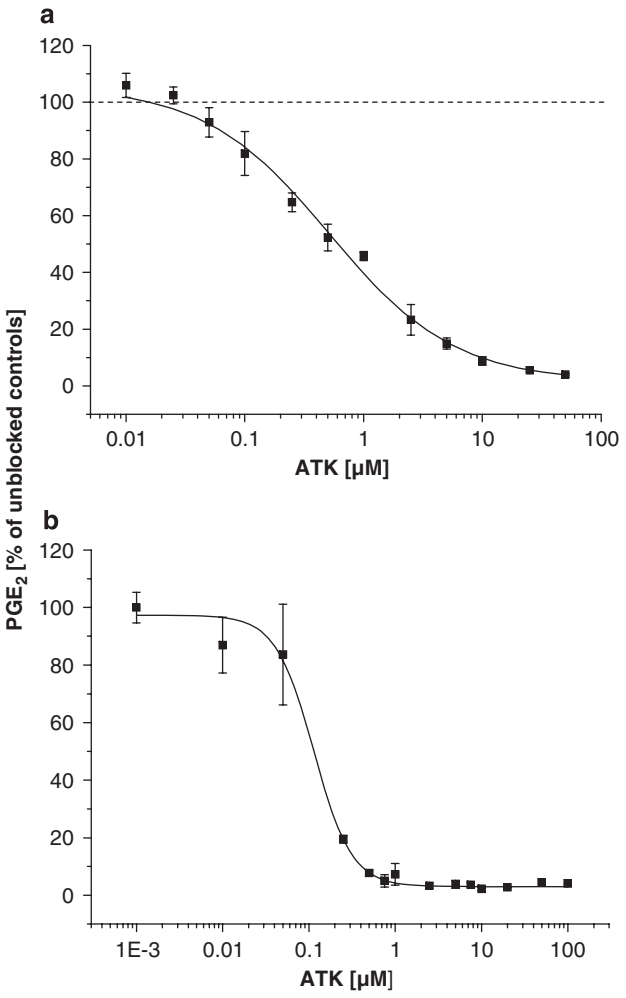


**Figure 1** Inhibition of immediate (**a**) and delayed (**b**) $PGE_2$ production by ATK in AA-stimulated MC3T3-E1 cells. Cells were cultured as described under methods. For short-term (COX-1 related) $PGE_2$ synthesis, medium was exchanged with HEPES buffer. Cells were pre-incubated with ATK for 30 min, followed by stimulation with AA (6 μM) for 30 min. For long-term (COX-2 related) $PGE_2$ synthesis, COX-2 was induced with serum in the presence of ATK for 3 h, followed by stimulation with AA (6 μM) for 30 min. $PGE_2$ was measured in buffer or medium as described under methods.

*Association and dissociation time course of ATK in MC3T3-E1 cells*

Experiments were performed with 10 μM ATK and 6 μM AA. For association time course, cells were pre-incubated with ATK for different times. At time 0, ATK and AA were added simultaneously. Dissociation behaviour was elaborated as follows: cells were pre-incubated with ATK for 30 min, medium was removed, cells were washed twice with incubation buffer and left for indicated periods of time before stimulating with AA for 30 min. Assay for $PGE_2$ was then performed as described above.

*Statistical methods and nomenclature*

Statistical analysis was performed with Student's *t*-test for paired samples, where appropriate. All data shown are representative of at least three independent experiments. Sigmoidal fits were calculated with ORIGIN software version 6.0 from Microcalc using pooled data from triplicate measurements.

Nomenclature used in this paper conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2008).

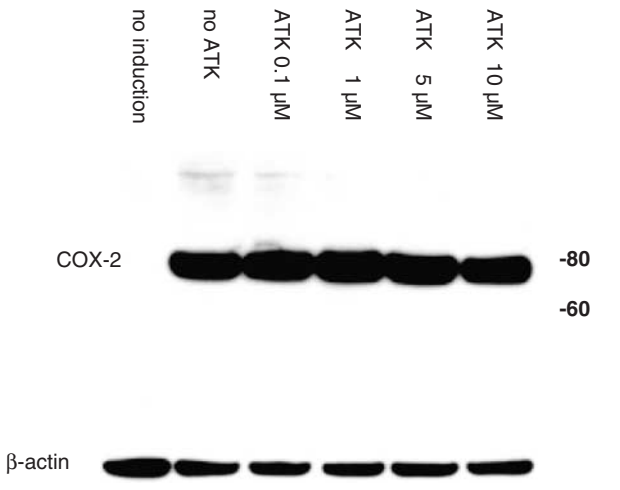


**Figure 2** Western blot analysis of COX-2 expression in MC3T3-E1 cells. COX-2 induction was accomplished with serum in the presence of the indicated concentrations of ATK. β-Actin was used as a loading control. Western blotting conditions are described in detail under methods.

*Reagents*

ATK and palmitoyl methyltrifluoro ketone (PTK) were purchased from Biomol, Hamburg, FRG. AA and HEPES buffer was from Sigma Chemical Co., Vienna, Austria, α-MEM and FCS were obtained from Sera-lab, Haywarth, UK. COX-1, COX-2 and $PGH_2$ were from Cayman Chemical, Ann Arbor, MI, USA. Goat polyclonal antibody COX-2 (C20) and β-actin antibody (C4) were from Santa Cruz Biotechnology via Szabo, Vienna, Austria. Horseradish peroxidase-conjugated donkey anti-goat IgG was from Jackson and horseradish peroxidase-conjugated goat anti-mouse IgG was obtained through Rockland via Biomol, Hamburg, FRG. The L-glutamine was from Serva, via AL-Labortechnik, Amstetten, Austria. Trypsin-EDTA was purchased from Böhringer, Vienna, Austria. Pentafluorobenzyl bromide, bis(N,O-trimethylsilyl)-trifluoroacetamide, silylation grade pyridine, acetonitrile and O-methoxyamine hydrochloride were from Pierce Chemical Co., Vienna, Austria. Culture dishes were from Falcon through Szabo, Vienna, Austria. MC3T3-E1 cells were kindly donated by Dr Klaushofer (Vienna). Deuterated $PGE_2$ was obtained through MSD Isotopes through IC Chemikalien GmbH, Vienna, Austria. All other chemicals and reagents were from Merck, Vienna, Austria.

## Results

*Inhibition of COX-1 and COX-2 by ATK in MC3T3-E1 cells*

Inhibitor potencies of ATK on short-term (COX-1 related) $PGE_2$ production in MC3T3-E1 cells are shown in Figure 1a. When stimulated with exogenous AA (6 μM), ATK blocked $PGE_2$ synthesis in a dose-dependant manner by 96% with a

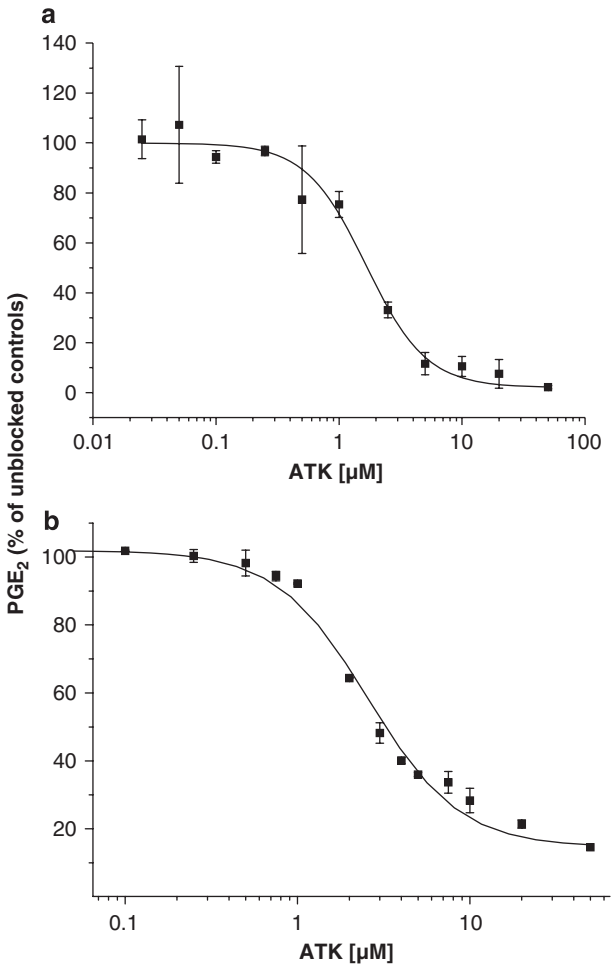


**Figure 3** Inhibition of $PGE_2$ production of pure COX-1 (**a**) and COX-2 (**b**) by ATK in AA-stimulated MC3T3-E1 cells. Enzyme inhibitor potencies were measured in Tris–HCl buffer (30 mM, pH 8.0) containing glutathione (0.49 mM), adrenaline (1 mM) and haematin (1 μM). One unit of COX and 1 μM sodium arachidonate were used. Pre-incubation with ATK was carried out for 30 min at room temperature. Incubation with sodium arachidonate was performed for 30 min at 37 °C. $PGE_2$ was measured as described under methods.

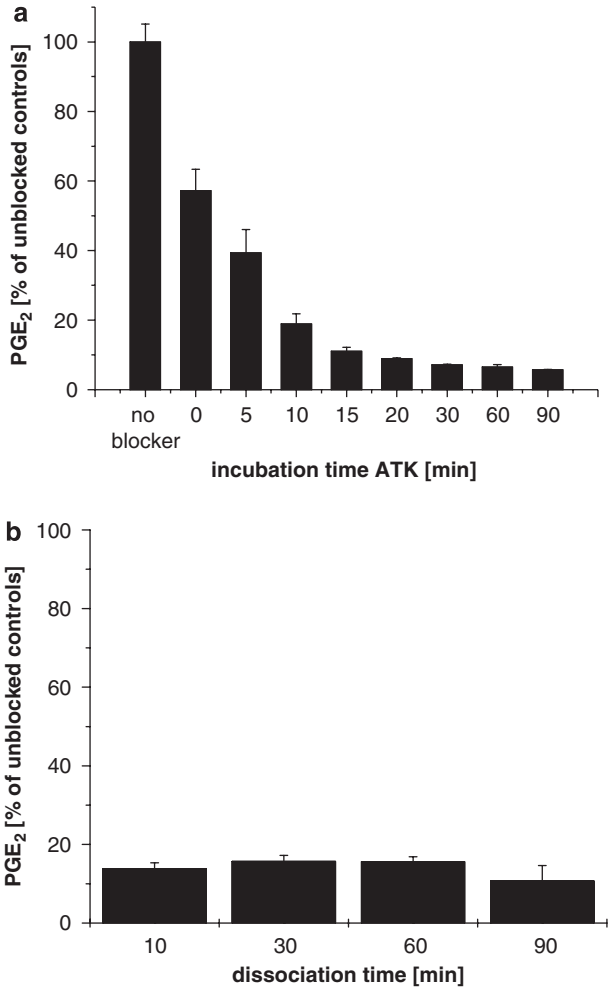


**Figure 4** Association (**a**) and dissociation time course (**b**) of ATK in MC3T3-E1 cells. Experiments were performed with 10 μM ATK and 6 μM AA as described under methods. At time 0 in graph (**a**), ATK and AA were added simultaneously. Assay for $PGE_2$ was then performed as described under methods.

calculated $IC_{50}$ concentration of $0.5 \pm 0.08$ μM (sigmoidal fit data: order $= 0.824 \pm 0.0981$; $\chi^2 = 1.67901$; $R^2 = 0.99522$). Calcium-free conditions and the use of exogenous AA substrate ensured uncoupling of the measured $PGE_2$ synthesis from the effects of ATK on PL activities. Figure 1b shows the inhibitory effect of ATK on long-term (COX-2 related) $PGE_2$ production in MC3T3-E1 cells. As induction of COX-2 was accomplished with serum for 3 h in the presence of ATK, the inhibitory effect seen in Figure 1b relates to the combined inhibition of both COX-1 and COX-2. Essentially, the same results were observed when medium was removed after induction of COX-2 (3 h) and stimulation with AA carried out in the buffer (results not shown). ATK blocked long-term $PGE_2$ synthesis almost completely with an $IC_{50}$ of $0.1 \pm 0.02$ μM (sigmoidal fit data: order $= 1.9707 \pm 0.303$; $\chi^2 = 2.07969$; $R^2 = 0.96661$). Western blot analysis of COX-2 confirmed the induction of the enzyme by serum and revealed no influence of ATK on COX-2 protein expression. The results are shown in Figure 2.

*Inhibition of pure COX-1 and COX-2 by ATK*

Inhibitor potencies of ATK on COX-1 and COX-2 are given in Figures 3a and b, respectively. Maximum inhibition caused by ATK was of 98% for COX-1 and 85% for COX-2. $IC_{50}$ were $1.7 \pm 0.1$ μM (sigmoidal fit data: order $= 1.7686 \pm 0.187$; $\chi^2 = 0.90592$; $R^2 = 0.99971$) for COX-1 and $2.6 \pm 0.2$ μM (sigmoidal fit data: order $= 1.63448 \pm 0.15527$; $\chi^2 = 15.26087$; $R^2 = 0.9951$) for COX-2.

*Effect of ATK on $PGH_2$ conversion to $PGE_2$*

The effect of ATK was measured under the experimental conditions described above for pure COX-1 and COX-2. $PGH_2$ at a concentration of 50 and 100 ng was used instead of COX enzymes. With 50 ng $PGH_2$, the following amounts of $PGE_2$ per experiment were measured: $8.8 \pm 0.6$ ng (no ATK), $9.9 \pm 1.5$ ng (10 μM ATK) and $8.6 \pm 0.1$ ng (25 μM ATK). With 100 ng $PGH_2$, the values for $PGE_2$ per experiment were $24.0 \pm 1.9$ ng (no ATK), $24.8 \pm 1.0$ ng (10 μM ATK) and $23.8 \pm 0.3$ ng (25 μM ATK).

*Association and dissociation time course for ATK in MC3T3-E1 cells*
Figures 4a and b show the association and dissociation time courses for ATK in MC3T3-E1 cells, respectively. As shown in Figure 4a, simultaneous addition of ATK and AA resulted in 43% inhibition of $PGE_2$ synthesis. Ninty-three percent inhibition was achieved after 30 min pre-incubation time. Longer exposure to ATK did not significantly ($P<0.05$) enhance the inhibitory effect. However, up to 30 min there was a sustained attenuation of $PGE_2$ production. Removing the inhibitor and equilibrating cells before AA stimulation for various periods of time yielded the dissociation time course displayed in Figure 4b. There was no significant recovery of $PGE_2$ synthesis even after 90 min equilibration time ($P<0.05$).

*Nature of ATK binding to COX*
To check the binding behaviour of ATK to COX, varying concentrations of AA were used for stimulation after pre-incubation with ATK at different concentrations. The results are given in Figures 5a and b. In Figure 5a both, ATK (0.5, 1 and 2 μM) and AA (1, 2, 4 and 6 μM) were used at concentrations below the $K_m$ of COX (apparent 8.3 μM for COX-1 from ram seminal vesicle, Johnson *et al*., 1995). Inhibition kinetics is strictly dependant on the concentrations of substrate and inhibitor, suggesting a reversible mechanism. At ATK and AA concentrations above the $K_m$ of COX (ATK: 0, 10 and 25 μM; AA: 0.5, 10, 20 and 40 μM) the reversible nature is still apparent, as shown in Figure 5b.

*Effect of ATK analogues*
A saturated analogue of ATK and PTK was used in this experiment. As shown in Figure 6, PTK has no effect on COX activity in MC3T3-E1 cells.

## Discussion and conclusions

In this study, we found that ATK is a specific and potent blocker of $cPLA_2$ and $iPLA_2$, inhibited COX-1 and COX-2 in osteoblast-like MC3T3-E1 cells and in an enzyme assay using purified COX. At least in the experiments with MC3T3-E1 cells, $PGE_2$ synthase must be considered as a potential target for ATK. However, as shown previously by another group, ATK-related inhibition of $PGE_2$ synthase occurs only to a minimal extent, reaching 20% inhibition with 10 μM ATK (Quraishi *et al*., 2002).

For ATK-dependant inhibition of macrophage $iPLA_2$, the $IC_{50}$ value was found to be 15 μM (Ackermann *et al*., 1995). Thus, ATK must be considered as a strikingly more potent inhibitor of COX-1 and COX-2 on a molecular basis. As shown in our experiments, these effects are not related to some molecular interaction between ATK and the enzymatic product of COX, $PGH_2$ and can thus be reasonably attributed to the inhibition of COX. Higher $IC_{50}$ values obtained in the enzyme assay are not unusual, as the cellular environment assures more adequate conditions for optimal activity. The results from association time measurements indicate a relatively slow onset of ATK inhibition of COX, reaching its maximum effect only after 30 min. Surprisingly, apparent competition with AA substrate is rapid, which could be because of different binding affinities at the catalytic site of

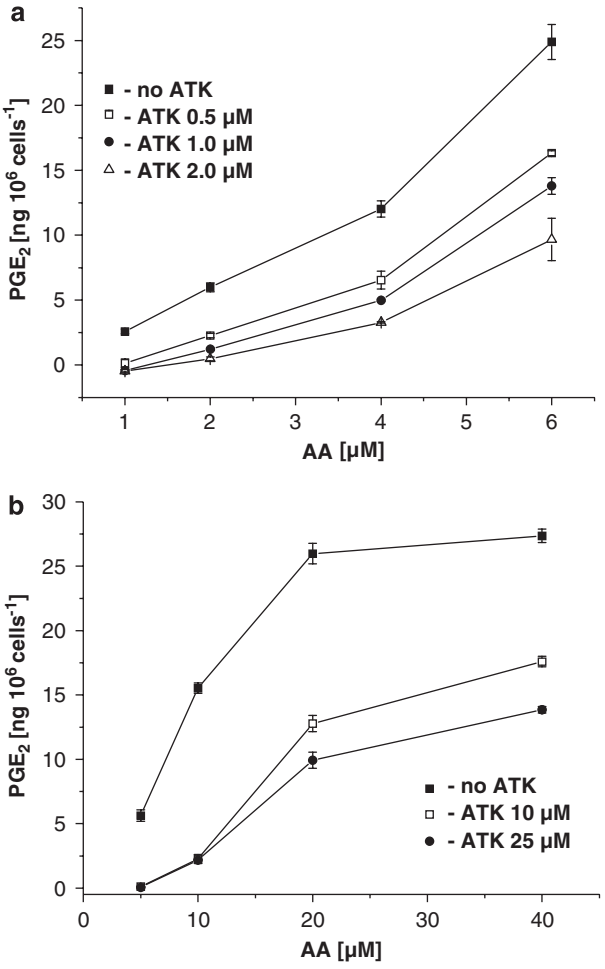


**Figure 5** Inhibitor potency of ATK in MC3T3-E1 cells.

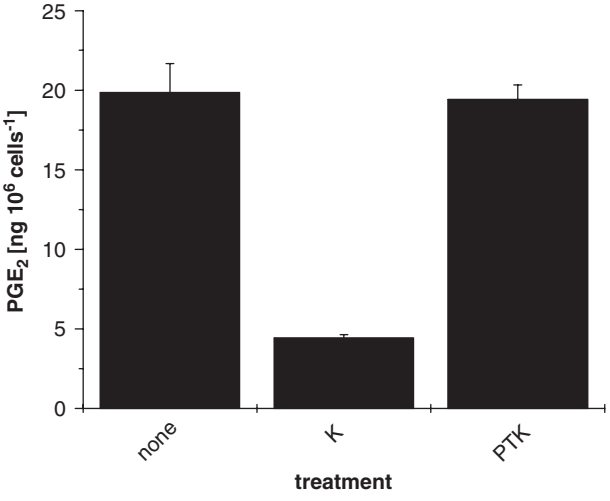


**Figure 6** Effects of PTK and ATK on $PGE_2$ production in AA-stimulated MC3T3-E1 cells. Pre-incubation with ATK or PTK was carried out for 30 min at room temperature. Incubation with AA was performed for 30 min. $PGE_2$ was measured as described under methods.

COX. Although we cannot infer what particular process is slow from our experiments, a slow binding velocity of ATK might reasonably explain the slow onset of inhibition.

Even more unique are the features of dissociation time courses. There was no noticeable reduction in blocking activity even after 90 min. At first glance this could be interpreted as an irreversible mode of action, but this can be ruled out for several reasons. First, AA competes dose-dependently with the inhibitor over a wide concentration range. Additionally, ATK shows slow- and tight-binding kinetics for human 85-kDa group IV $cPLA_2$, showing only 14% dissociation of the $Ca^{2+}$–$cPLA_2$–ATK complex after 5 h (Street *et al*., 1993). In contrast, no such effects were observed with group VI $iPLA_2$ (Ackermann *et al*., 1995). Nevertheless, such slow- and tight-binding could reasonably explain the findings of our dissociation experiments. Hypothetically, ATK could also act as a co-substrate of AA for COX-1 and COX-2. In this case, the inhibitor should be subjected to peroxidation and cyclooxygenation by COX, which would lead to the well-described suicide inhibition of the enzyme (Hemler and Lands, 1980; Wu *et al*., 1999). This would result in an irreversible loss of COX activity that could not be restored by AA. It is, however, clear that our dissociation and association data do not solely reflect substrate–enzyme binding rates. Membrane transport dynamics may significantly contribute to the time lag observed. In contrast, on the basis of similar polarity and structure to AA, ATK membrane transport should not be markedly different. In addition, our data suggest that inward membrane transport is effective enough to produce maximal inhibition after 30 min. Assuming a similar rate for the outward transport, some recovery of PG production should be observed after that time.

PTK, a saturated analogue of ATK, is a more potent inhibitor of group VI $iPLA_2$ than ATK, whereas it is inactive towards group IV $cPLA_2$ (Ackermann *et al*., 1995). In our study, PTK did not inhibit COX. This suggests that the unsaturated carbon backbone of ATK is essential for the binding and positioning of the inhibitor at the catalytic site of COX-1. In fact, there are more than 50 mostly hydrophobic interactions with 19 amino acid residues at this site, as described for AA (Thuresson *et al*., 2001).

Our results clearly demonstrate inhibitory effects of ATK on both COX-1 and COX-2. Under the conditions employed for measuring cellular COX-2 activity, however, there might be a concerted effect of ATK on enzyme inhibition and expression as well. Western blot analysis, however, clearly demonstrated that COX-2 protein expression was not influenced by ATK. Thus, the inhibitory effect of ATK is solely related to enzyme inhibition.

Inhibitors of COX enzymes have gained much attention and pharmacological applications are many (such as non-steroidal anti-inflammatory drugs). The main purpose of this study was not to define a potential function of ATK as another modulator of COX activity, but to prevent misinterpretation of experiments, where ATK-related attenuation of prostaglandin synthesis is accepted to be the direct consequence of $PLA_2$ inhibition (Kurusu *et al*., 1997; Kuwata *et al*., 1998; Saunders *et al*., 1999; D'Orazi *et al*., 2006). If, however, alternative routes for AA liberation are present, such as DAG metabolism preceded by the action of PLC or PLD, then conclusions drawn on the AA liberation pathway involved could be untenable. As there is widespread use of ATK, which relies on the acknowledged specificity of ATK, it is of particular interest to elucidate effects of this compound on other key enzymes of the AA cascade.

In conclusion, ATK is a potent inhibitor of COX-1 and COX-2. On a molar basis, it is more effective in blocking COX than $PLA_2$ enzymes. Inhibition by ATK is reversible and displays slow- and tight-binding characteristics.

## Conflict of interest

The authors state no conflict of interest.

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