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RESEARCH PAPER

The ω3-polyunsaturated fatty acid derivatives AVX001 and AVX002 directly inhibit cytosolic phospholipase A_2 and suppress PGE_2 formation in mesangial cells

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BACKGROUND AND PURPOSE

ω3-polyunsaturated fatty acids (ω3-PUFAs) are known to exert anti-inflammatory effects in various disease models although their direct targets are only poorly characterized.

EXPERIMENTAL APPROACH

Here we report on two new $cPLA_2$ inhibitors, the ω3-derivatives AVX001 and AVX002, and their effects on inflammatory PGE_2 production in cultures of renal mesangial cells.

KEY RESULTS

AVX001 and AVX002 dose-dependently inhibited the group IVA cytosolic phospholipase A_2 ($cPLA_2$) in an *in vitro* activity assay with similar IC_{50} values for AVX001 and AVX002, whereas the known $cPLA_2$ inhibitor $AACOCF_3$ was less potent and docosahexaenoic acid (DHA) was inactive. In renal mesangial cells, AVX001 and AVX002 suppressed IL-1β-induced PGE_2 synthesis. Mechanistically, this effect occurred by a down-regulation of IL-1β-induced group IIA-$sPLA_2$ protein expression, mRNA expression and promoter activity. A similar but less potent effect was seen with $AACOCF_3$ and no effect was seen with DHA. As gene expression of $sPLA_2$ is known to be regulated by the transcription factor NF-κB, we further investigated NF-κB activation. Both compounds prevented NF-κB activation by blocking degradation of the inhibitor of κB.

CONCLUSIONS AND IMPLICATIONS

These data show for the first time that the novel $cPLA_2$ inhibitors AVX001 and AVX002 exert an anti-inflammatory effect in cultures of renal mesangial cells and reduce the pro-inflammatory mediator PGE_2 through an inhibitory effect on NF-κB activation. Therefore, these compounds may represent promising novel drugs for the treatment of inflammatory disorders.

Abbreviations

$AACOCF_3$, ATK, arachidonyl-trifluoromethyl ketone; AVX001, 1-octadeca-2,6,9,12,15-pentaenylsulfanyl-propan-2-one; AVX002, 1-octadeca-3,6,9,12,15-pentaenylsulfanyl-propan-2-one; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IκB, inhibitor of κB; MAFP, methyl-arachidonyl fluorophosphonate; PAF-AH, PAF acetylhydrolase; PUFA, polyunsaturated fatty acid; RT-PCR, reverse transcriptase-PCR

Introduction

Mesangial cells are specialized smooth muscle-like cells located in the renal glomerulus and are not only involved in the regulation of the glomerular filtration rate and in the preservation of the structural integrity of the glomerulus, but also play a central role in most pathological processes of the renal glomerulus (Kashgarian and Sterzel, 1992; Pfeilschifter, 1994; Gómez-Guerrero *et al.*, 2005). Upon activation by a variety of pro-inflammatory cytokines, mesangial cells respond with three prominent reactions which are all hallmarks of many forms of glomerulonephritis: (i) increased proliferation; (ii) increased mediator production, including cytokines, chemokines, NO, superoxide radicals and PGs; and (iii) increased extracellular matrix production (Kashgarian and Sterzel, 1992; Pfeilschifter, 1994). The detailed mechanisms underlying these cellular responses are still not completely understood.

One important event in the inflammatory reaction and the rate-limiting step in the generation of PGs is the activation of a PLA_2, which hydrolyzes the *sn*-2 ester bond of substrate phospholipids and thereby generates arachidonic acid and lysophospholipids (Schaloske and Dennis, 2006). Arachidonic acid is then further converted by either COXs or lipoxygenases and downstream enzymes to the eicosanoids including PGs, TXs and LTs.

So far, 15 groups and many subgroups of PLA_2s have been identified which include five distinct types of enzymes, that is, secreted PLA_2s ($sPLA_2$), the cytosolic PLA_2s ($cPLA_2$), the Ca^{2+}-independent PLA_2s ($iPLA_2$), the PAF acetylhydrolases (AHs) and the lysosomal PLA_2s (Schaloske and Dennis, 2006).

As $cPLA_2$ preferentially hydrolyzes arachidonic acid-containing phospholipids at the *sn*-2 position, this enzyme is thought to be one key enzyme in inflammatory eicosanoid formation.

In renal mesangial cells, four PLA_2 subtypes are expressed either constitutively or inducibly. These include the $cPLA_2$, the $iPLA_2$, and the group IIA and V $sPLA_2$ (Schalkwijk *et al.*, 1992; Gronich *et al.*, 1994; Akiba *et al.*, 1998; van der Helm *et al.*, 2000). In previous studies on mesangial cells, it was shown that the drastic increase of cytokine-triggered PGE_2 formation involved both IIA-$sPLA_2$ and $cPLA_2$ activation (Pfeilschifter *et al.*, 1993). By using the specific $sPLA_2$ inhibitor CGP43187 or by using a neutralizing antibody against IIA-$sPLA_2$, about 80% of the cytokine-triggered PGE_2 formation was depleted, suggesting that the remaining small amount of approx. 20% of PGE_2 derives from cytokine-stimulated $cPLA_2$ activity (Pfeilschifter *et al.*, 1993). Indeed, the $cPLA_2$ is not only acutely activated by IL-1 (Gronich *et al.*, 1994), but also up-regulated upon prolonged IL-1 treatment by a transcriptional mechanism (Lin *et al.*, 1992; Schalkwijk *et al.*, 1993). Furthermore, a cross-communication exists between the different PLA_2s. Thus, in mesangial cells, we previously showed that the IIA-$sPLA_2$, acting from the outside of cells, is able to activate the $cPLA_2$ intracellularly via a PKC and MAPK-dependent mechanism (Huwiler *et al.*, 1997). In mouse P388D1 macrophages, it was shown that $cPLA_2$ also contributes to V-$sPLA_2$ activation (Balsinde *et al.*, 1998). All these data suggest that the different PLA_2s regulate each other and are critically participating in pro-inflammatory PGE_2 formation.

In this study, we have identified and characterized two novel direct $cPLA_2$ inhibitors, the ω3-polyunsaturated fatty acid (PUFA) derivatives AVX001 and AVX002. We showed that in cultures of renal mesangial cells, AVX001 and AVX002 down-regulated cytokine-stimulated PGE_2 formation through a mechanism that involved the blocking of cytokine-triggered and $cPLA_2$-dependent NF-κB activation and subsequent gene transcription of $sPLA_2$. These data suggest that AVX001 and AVX002 could serve as novel anti-inflammatory drugs.

Methods

AVX001 and AVX002

Both compounds were synthesized and characterized according to Holmeide and Skattebol (2000) and kindly provided by Dr Inger Reidun Aukrust and Dr Marcel Sandberg (Synthetica AS, Norway). The chemical structures are indicated in Figure 1. Both compounds were analysed by HPLC for purity (Figure 2). For this, all peaks in the chromatogram were taken for area integration using an integrated HPLC software. The purities were determined as 97% for AVX001 and 92.7% for AVX002. Both compounds were stored at –80°C at a 20 mM stock solution in DMSO under argon gas to minimize oxidation.

Cell culture

Rat renal mesangial cells were isolated, characterized and cultured as previously described (Pfeilschifter *et al.*, 1984). For the experiments performed in this study, cells between passages 8–30 were used.

In vitro $cPLA_2$ activity assay

Recombinant human $cPLA_2$ enzyme was used for an *in vitro* activity assay as described by Wijkander and Sundler (1991), with some modifications according to Lucas and Dennis (2005). Enzyme with inhibitor (in DMSO, final concentration 1%) or solvent alone was pre-incubated in assay buffer (1 mM EDTA, 80 mM KCl and 10 mM HEPES (pH 7.4) containing 1.56 mM $CaCl_2$, and 2.36 mM dithiothreitol for 80 s at 37°C and 10 min at 25°C. Lipid vesicles were prepared by drying 4.2 nmol of L-α-1-palmitoyl-2-arachidonyl-[arachidonyl-1-

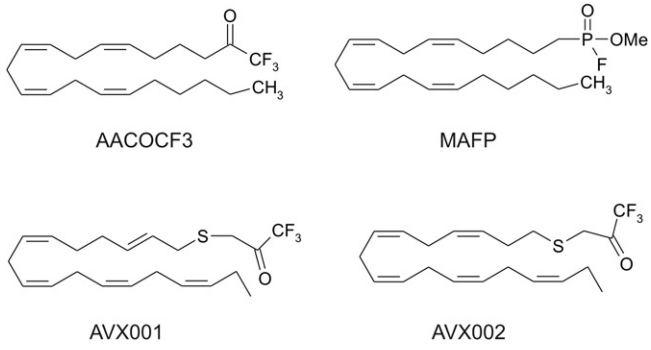

Figure 1
Chemical structure of various $cPLA_2$ inhibitors.

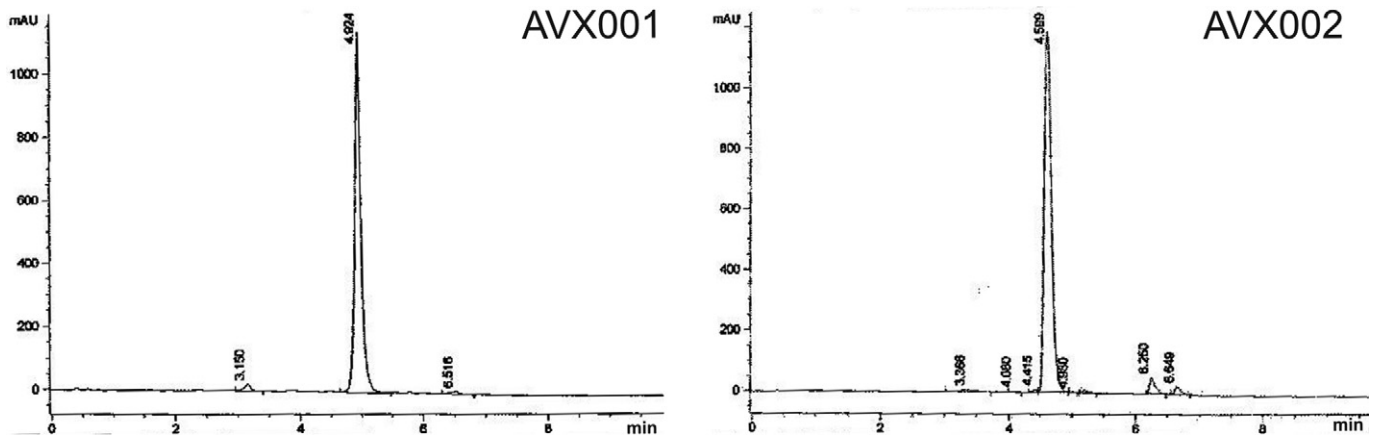

Figure 2

HPLC profiles of AVX001 and AVX002. The peak at 4.924 min (left panel) corresponds to AVX001. The peak at 4.599 min (right panel) corresponds to AVX002.

^{14}C]-phosphatidylcholine under a stream of nitrogen. Dried lipids were resuspended in 2 mL assay buffer and sonicated twice for 7 min (setting: output 3.5 and 50% duty cycles) in a Branson Sonifier 250 (Branson Ultrasonic Corporation, Danbury, CT, USA). Sonicated lipid vesicles were added to the reaction to a final concentration of 0.2 μM. The reaction mixture was incubated for 1 h at 37°C and stopped by addition of 1.7 mL chloroform/methanol/37% KCl/ 0.45 M BHT/ 0.33 M AA (2:1:0.01:0.015:0.005, by vol). After phase separation, the lower phase was transferred to a glass tube, dried under nitrogen, and resuspended in chloroform/methanol (9:1, v v^{-1}), and applied to a silicagel TLC. Free [1-^{14}C]-arachidonic acid and L-α-1-palmitoyl-2-arachidonyl-[arachidonyl-1-^{14}C]-phosphatidylcholine (Perkin Elmer, Waltham, MA, USA) were separated and analysed as described by Anthonsen *et al*. (2001).

PGE$_2$ determination

Confluent mesangial cells in 24-well plates were pretreated for 90 min in the presence or absence of the inhibitors before stimulation for 24 h in a volume of 0.5 mL with IL-1β (1 nM) to induce PGE$_2$ formation. Thereafter, equal volumes of supernatants were subjected to a PGE$_2$-ELISA according to the manufacturer's instructions. Data were calculated as pg of PGE$_2$ per 1.3×10^5 cells which was the cell number per well.

Cell stimulation and Western blot analysis

Confluent mesangial cells were stimulated as indicated in the figure legends in DMEM supplemented with 0.1 mg·mL^{-1} of fatty acid-free BSA and 10 mM HEPES. After stimulation, the supernatant was taken for detection of secreted IIA-sPLA$_2$. The cell monolayers were homogenized in lysis buffer and processed exactly as previously described (Xin *et al*., 2004). Cell lysates were taken for protein determination. Lysates containing 60 μg of protein, were separated by SDS-PAGE, transferred to nitrocellulose membranes and subjected for Western blot analysis using antibodies as indicated in the figure legends. Bands were detected by enhanced chemiluminescence method as recommended by the manufacturer.

Detection of secreted IIA-sPLA$_2$

Equal volumes of supernatants derived from the same number of cells were taken for protein precipitation using 7% (w v^{-1}) of trichloroacetic acid. Precipitated proteins were redissolved in SDS-Laemmli buffer without dithiothreitol and subjected to SDS-PAGE (15% acrylamide gel), transferred to nitrocellulose membranes and immunostained by using a monoclonal antibody against rat IIA-sPLA$_2$ at a dilution of 1:60 in 0.01% milk powder containing PBS as previously described (Petry *et al*., 2004). GAPDH was stained and densitometrically evaluated in the corresponding cell lysates and used for normalization by calculating the ratio between secreted sPLA$_2$ and cytosolic GAPDH for each sample.

Quantitative PCR analysis

Total RNA (2 μg) was used for reverse transcriptase (RT)-PCR (first-strand cDNA synthesis kit, MBI Fermentas, St.Leon-Rot, Germany) using random hexamer primers for amplification. mRNA levels were determined by quantitative real-time PCR. The following primers were used: rat sPLA$_2$-IIA: forward: GCC AAA TCT CCT GCT CTA CAA ACC, reverse: ACT GGG CGT CTT CCC TTT GC; 18S RNA (forward: CGA TTC CGT GGG TGG TGG TG, reverse: CAT GCC AGA GTC TCG TTC GTT ATC); iQ™ SYBR® Green Supermix was from Bio-Rad (Hercules, CA, USA). The cycling conditions were as follows: initial activation step (95°C for 3 min), followed by 40 cycles of denaturation (95°C for 15 s) and annealing (58°C for 1 min). PCR products were detected by monitoring the increase in fluorescence with iCycler, iQ™5 Multicolor Real-Time PCR Detection System, Bio-Rad. The Bio-Rad iQ5 Standard Edition Optical System Software Version 2.0 was used to analyse real-time and end point fluorescence.

Cell transfection and luciferase reporter gene assay

A 2.67 kb fragment of the rat sPLA$_2$-IIA promoter was cloned according to a previous report (Scholz-Pedretti *et al*., 2002) and fused to a luciferase reporter gene. Cells were cultured in 12-well plates and transfected with 0.3 μg of the

promoter-containing plasmid plus 0.03 μg of a plasmid containing the *renilla luciferase* gene by using Effectene Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Thereafter, the transfection medium was removed and cells were stimulated as indicated. Values for the relative gene promoter activities were calculated from the ratio of firefly/*renilla luciferase* activities.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's *post hoc* test for multiple comparisons (GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, USA).

Chemicals

IL-1β was from Cell Concept GmbH (Umkirch, Deutschland); hyperfilm MP and horseradish-coupled secondary antibodies were from GE Healthcare Systems (Freiburg, Germany); $AACOCF_3$, docosahexaenoic acid (DHA) and the PPARγ antagonist G3335 were from Merck Biosciences (Schwalbach, Germany); the monoclonal antibody against rat IIA-$sPLA_2$ was generated and characterized as previously described (Aarsman *et al.*, 1989); the IκB-α antibody was from Cell Signalling (Frankfurt am Main, Germany); the PGE_2 ELISA was from Assay Designs, BIOTREND Chemikalien GmbH (Köln, Germany); all cell culture nutrients were from Invitrogen/Life Technologies (Karlsruhe, Germany).

Results

AVX001 and AVX002 inhibit cPLA$_2$ activity in vitro *and reduce cytokine-stimulated PGE$_2$ formation in rat renal mesangial cells*

Previously, it was shown that the ω6-PUFA derivatives arachidonyl-trifluoromethyl ketone ($AACOCF_3$, ATK; Figure 1) (Street *et al.*, 1993) and methyl-arachidonyl fluorophosphonate (MAFP; Figure 1) (Lio *et al.*, 1996) are direct inhibitors of the group IVA $cPLA_2$ *in vitro*. In the present study, we tested the ability of AVX001 and AVX002 to inhibit $cPLA_2$ activity. In these compounds the methylene group β to the carbonyl group of the ketone in $AACOCF_3$ was replaced by a sulphur atom. Holmeide and Skattebol, (2000) speculated that this would make the carbonyl carbon more electrophilic and consequently the molecule a more potent inhibitor of $cPLA_2$. *In vitro* activity assays revealed that AVX001 blocked $cPLA_2$ activity in a concentration-dependent manner (Figure 3). Multiple experiments were taken for the determination of the IC_{50} value, which was calculated as 120 ± 58 nM (n = 28). The structurally similar compound AVX002 also blocked $cPLA_2$ activity *in vitro* (Figure 3). The IC_{50} value was analysed from several experiments to be 126 ± 37 nM (n = 14). By comparison, $AACOCF_3$ was a less potent inhibitor; it showed 30% inhibition at 0.3 μM and 72% inhibition at 1 μM. In contrast, the ω3-fatty acid DHA had no direct inhibitory effect on $cPLA_2$ (Figure 3). These data suggest that not only ω6-PUFA derivatives, but also certain ω3-PUFA derivatives, such as AVX001 and AVX002, but not DHA, are effective direct $cPLA_2$ inhibitors.

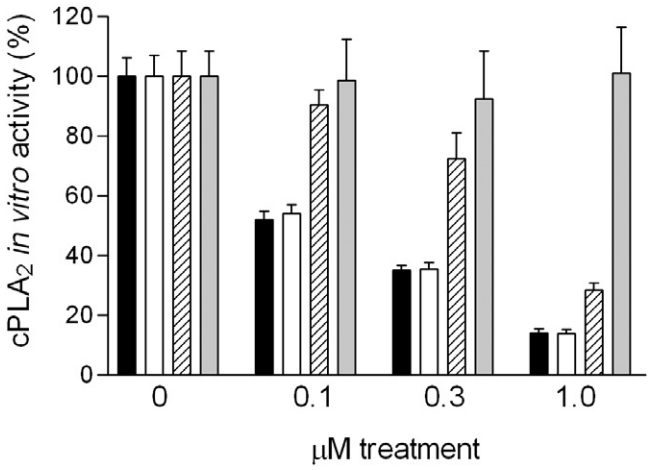

Figure 3

Direct inhibitory effect of AVX001, AVX002, $AACOCF_3$ and DHA on $cPLA_2$ activity *in vitro*. Recombinant $cPLA_2$ enzyme was incubated with either solvent (DMSO, final concentration 1%, 0) or the indicated concentrations of AVX001 (solid columns) or AVX002 (open columns), $AACOCF_3$ (hatched columns), and DHA (grey columns), and taken for an *in vitro* activity assay as described in the Methods section. $cPLA_2$ enzyme activity is given as % of control value (activity in the absence of inhibitors). The results shown are representative of at least three independent experiments, and data represent mean of duplicate determinations.

To further test the effectiveness of AVX001 and AVX002 in a cell culture system, rat renal mesangial cells were used, as they represent a good model system to investigate molecular inflammatory mechanisms (Pfeilschifter, 1994; Gómez-Guerrero *et al.*, 2005).

Treatment of mesangial cells with IL-1β led to a high production of PGE_2 confirming many previous reports of our group (Pfeilschifter *et al.*, 1989; 1990; 1993). The average amount of PGE_2 in different experiments varied from 0–55 pg per 1.3×10^5 cells in unstimulated cells, to 2359–3751 pg per 1.3×10^5 cells in IL-1β-stimulated cells. Due to this variability, which seemed to depend on the cell passages, the maximal IL-1β-stimulated values were always set to 100%. In the presence of AVX001, PGE_2 formation was concentration-dependently reduced with a 90% inhibition at 10 μM (Figure 4). A similar inhibitory effect was also seen for AVX002 (Figure 4). $AACOCF_3$ also reduced PGE_2 levels but was less potent, and DHA even at 20 μM had no significant effect (Figure 4).

As we previously showed that the cytokine-induced PGE_2 formation in mesangial cells involves both $sPLA_2$ and $cPLA_2$ activation (Pfeilschifter *et al.*, 1993), we next investigated the effect of AVX001 on $sPLA_2$ protein and mRNA expressions. As seen in Figure 5A, AVX001 and AVX002 both concentration-dependently down-regulated the $sPLA_2$ protein expression paralleling the reduced PGE_2 formation. To verify that supernatants derived from equal cell amounts, corresponding cell monolayers were lysed and stained for GAPDH protein expression (Figure 5A, inset, lower panels) and used for nor-

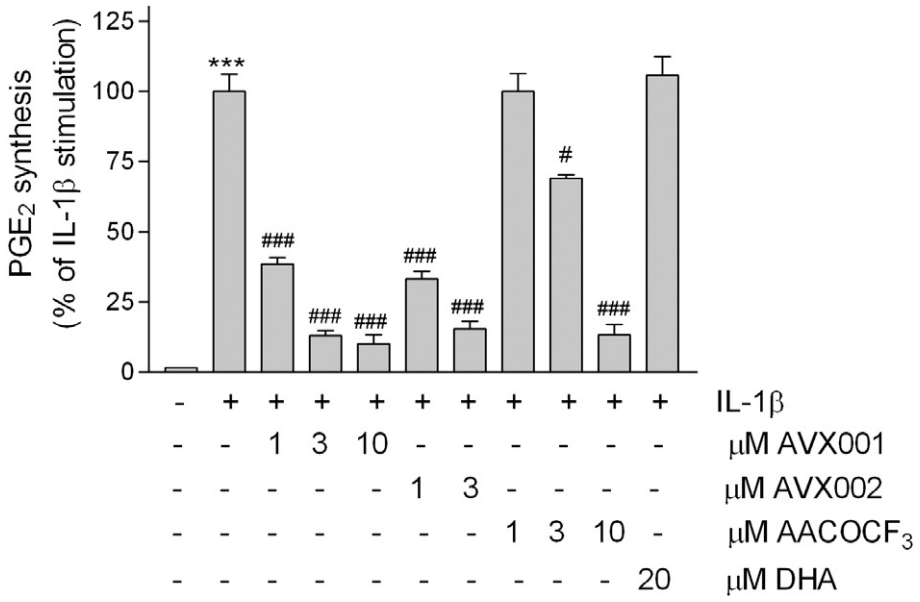

Figure 4

Effect of AVX001 and AVX002 on IL-1β-stimulated PGE_2 formation in mesangial cells. Quiescent cells were stimulated for 24 h with either DMEM (-), and IL-1β (1 nM) in the absence (-) or presence of the indicated concentrations (in μM) of AVX001, AVX002, $AACOCF_3$ and DHA. The inhibitors were all added as a pretreatment for 90 min before stimulation. Supernatants were collected and taken for PGE_2 quantification by using an ELISA. Data are expressed as % of maximal IL-1β-stimulated PGE_2 and are means ± SD (n = 3).***P < 0.001 considered statistically significant when compared with the control values; $^{\#}P$ < 0.05, $^{\#\#\#}P$ < 0.001 when compared with the IL-1β-stimulated values.

malization of secreted proteins. A similar reducing effect on $sPLA_2$ protein expression was also seen for $AACOCF_3$, whereas DHA had no significant effect (Figure 5A). IIA-$sPLA_2$ mRNA expression was also reduced by AVX001 and AVX002 (Figure 5B). Again, $AACOCF_3$ was less potent and DHA was ineffective at reducing IIA-$sPLA_2$ mRNA expression (Figure 5B). Notably, higher concentrations of AVX001 and AVX002 were needed to down-regulate the $sPLA_2$ protein and mRNA expressions compared with the reduction in PGE_2.

To see whether this effect on $sPLA_2$ protein and mRNA expression was due to a reducing effect on $sPLA_2$ promoter activation and subsequent gene transcription, luciferase reporter gene assays were performed. A 2.26 kb fragment of the rat IIA-$sPLA_2$ promoter was cloned according to Scholz-Pedretti *et al*. (2002) and ligated into a luciferase-containing vector (pGL3) and used to transfect mesangial cells. AVX001 and AVX002 at 20 μM significantly reduced IL-1β-stimulated promoter activity (Figure 5C), suggesting that $cPLA_2$-regulated transcription factors are essential for $sPLA_2$ gene transcription.

AVX inhibitors block cytokine-stimulated NF-κB activity in mesangial cells

As it is well known that the transcription factor NF-κB is crucially involved in $sPLA_2$ gene transcription under inflammatory conditions (Walker *et al*., 1995), we further studied whether the AVX inhibitors had an effect on NF-κB activation. NF-κB activation was measured indirectly by Western blot analysis of the inhibitor of κB (IκB) protein expression. Short-term stimulation of cells for 15 min with IL-1β revealed a marked degradation of IκB protein (Figure 6), which is a key event in NF-κB activation that releases NF-κB from its sequestration in the cytoplasm and allows its nuclear translocation. This down-regulating effect of IL-1β on IκB was reverted by the IκB inhibitor Bay11-7085, but also by AVX001 and AVX002 (Figure 6).

AVX001 and AVX002 do not act through PPARγ activation

As AVX001 and AVX002 are structurally derived from ω3-fatty acids, and it has been reported previously that ω3-PUFAs are ligands and activators of PPAR (Stahl *et al*., 2010), which has been attributed an anti-inflammatory effect (Straus and Glass, 2007), we further tested whether the reducing effect of AVX compounds on PGE_2 is mediated through PPARγ activation. Indeed, we found that in the presence of the PPARγ antagonist G3335, the AVX001 and AVX002 effects were partially reverted (Figure 7). However, the IL-1β effect was also increased to the same extent in the presence of G3335 (Figure 7), supporting the conclusion that there is a general stimulating effect of the PPARγ antagonist on the PGE_2 pathway rather than a specific antagonistic effect on the AVX action.

Discussion

In the past few years, ω3-PUFAs have attracted a lot of interest due to accumulating evidence for their beneficial effects in various inflammatory diseases. These include rheumatoid arthritis, asthma, colitis, atherosclerosis, neurodegenerative

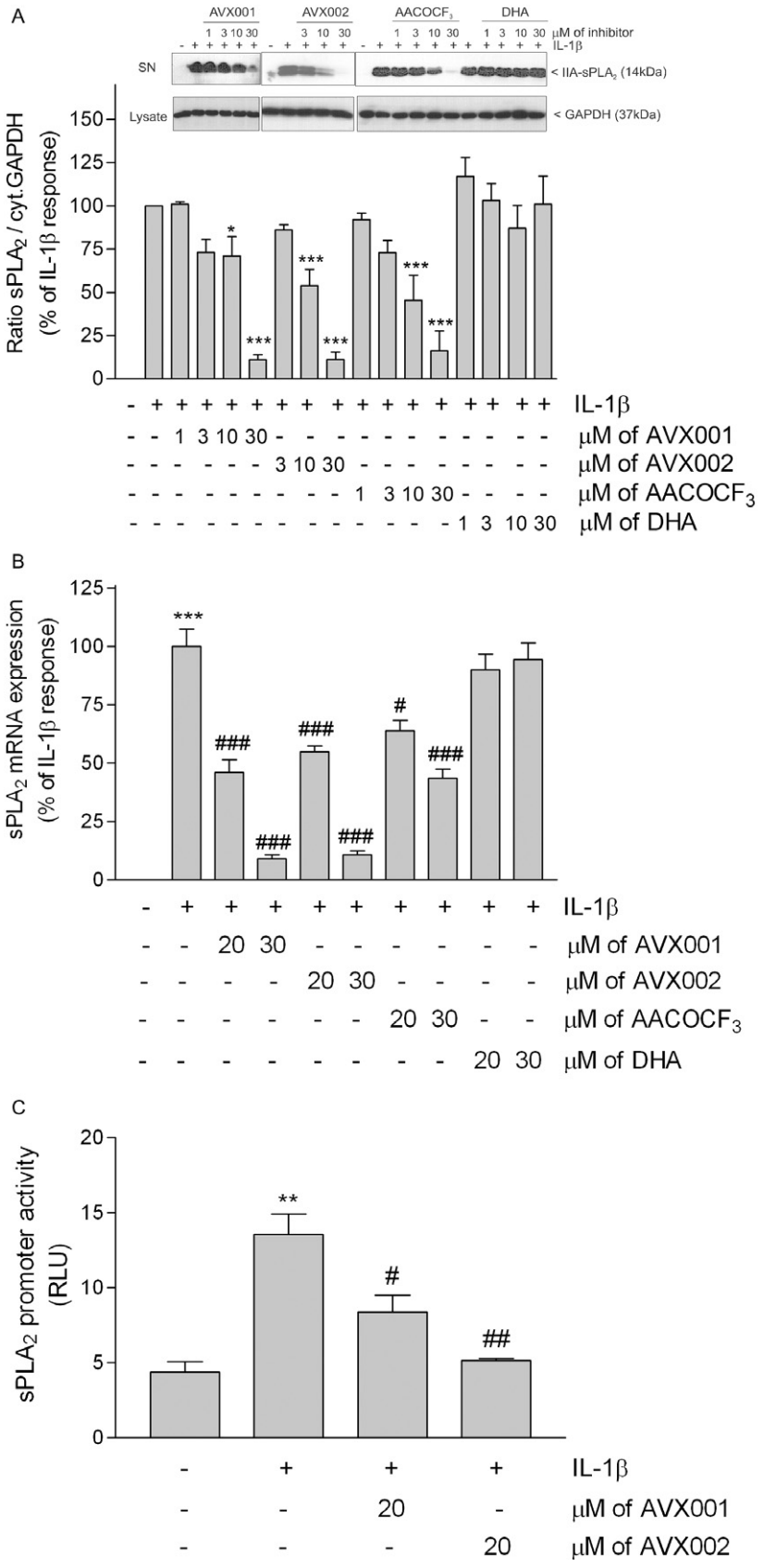
A
AVX001
AVX002
AACOCF3
DHA
μM of inhibitor
IL-1β
SN
< IIA-sPLA2 (14kDa)
Lysate
< GAPDH (37kDa)
Ratio sPLA2 / cyt.GAPDH (% of IL-1β response)
IL-1β
μM of AVX001
μM of AVX002
μM of AACOCF3
μM of DHA
B
sPLA2 mRNA expression (% of IL-1β response)
C
sPLA2 promoter activity (RLU)

Figure 5

Effect of AVX001 and AVX002 on IL-1β-stimulated sPLA$_2$ protein (A), mRNA expression (B) and promoter activity (C) in mesangial cells. Quiescent cells were stimulated with either DMEM (-), or IL-1β (1 nM) in the absence (-) or presence of the indicated concentrations of AVX001, AVX002, AACOCF$_3$ and DHA (all inhibitors were added as a pretreatment for 90 min before stimulation). (A) Supernatants were taken for protein precipitation, separated by SDS-PAGE, transfered to membranes and subjected to Western blot analysis using a monoclonal antibody against rat sPLA$_2$. Corresponding cell lysates were stained for GAPDH and used for normalization. Bands were densitometrically evaluated and the ratio between secreted sPLA$_2$ and cytosolic GAPDH was calculated. Results are expressed as % of IL-1β stimulation. Data are means ± SD (n = 3–5). The inset shows representative Western blots of sPLA$_2$ (upper panels) from supernatants (SN) and GAPDH (lower panels) from cell lysates. (B) Cells were taken for RNA extraction and subjected to quantitative PCR analysis of rat IIA-sPLA$_2$ and 18S RNA. ΔΔCt values were calculated and results are expressed as % of maximal IL-1β-stimulated response and are means ± SD (n = 3–5). (C) Cells were transfected with the sPLA$_2$ promoter construct plus a plasmid coding for *Renilla luciferase*. After transfection, cells were stimulated for 24 h with vehicle (-), IL-1β (1 nM), or IL-1β in the absence or presence of AVX001 or AVX002 (both at 20 μM). Both inhibitors were added as a pretreatment 90 min before stimulation. sPLA$_2$ promoter activity was calculated and results are expressed as relative luciferase units (RLU) and are means ± SD (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ considered statistically significant when compared with the control values; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the IL-1β-stimulated values.

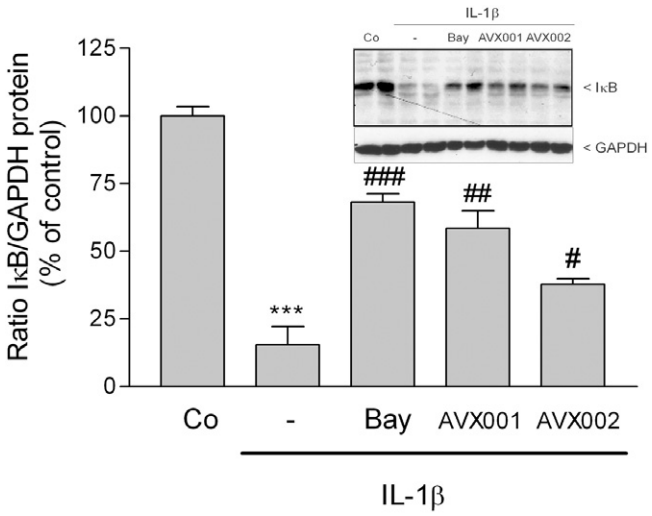

Figure 6

Effect of AVX inhibitors on IL-1β-stimulated NF-κB activation in mesangial cells. Quiescent cells were stimulated for 15 min with either vehicle (Co), or IL-1β (1 nM) in the absence (-) or presence of Bay11-7085 (Bay, 10 μM), AVX001 or AVX002 (both at 30 μM), pretreated for 90 min. Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subjected to a Western blot analysis using a polyclonal antibody against IκBα (inset, upper panel) and GAPDH (inset, lower panel). Bands corresponding to IκB and GAPDH were densitometrically evaluated and the ratio between IκB and GAPDH was calculated for each sample. Results are depicted as % of control stimulations and are means ± SD (n = 3). $^{***}P < 0.001$ considered statistically significant when compared with the control values; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the IL-1β-stimulated values. The inset shows a representative experiment in duplicates.

diseases and even certain forms of cancer (Calder, 2005; Calon and Cole, 2007; Edwards and O'Flaherty, 2008).

Various possibilities for the mechanism of these anti-inflammatory effects were forwarded. For example, an increased incorporation of ω3-PUFA species into phospholipids may occur at the expense of arachidonic acid. Consequently, they replace arachidonic acid as a substrate for COXs and lipoxygenases resulting in reduced formation of PGE$_2$ (Bagga *et al.*, 2003), TXA$_2$ (Krämer *et al.*, 1996), LTB$_4$ and LTE$_4$ (Whelan *et al.*, 1991). Instead, an increased generation of other less active PG and LT subspecies occurs (Terano *et al.*, 1986; Krämer *et al.*, 1996; Li *et al.*, 1997; Bagga *et al.*, 2003). Furthermore, it was proposed that ω3-PUFA may change the membrane lipid composition and thereby directly affect the functions of immune cells including the phagocytotic activity of macrophages, T cell signalling and proliferation, and antigen presentation activity of dendritic cells (Costa Rosa *et al.*, 1996; Sanderson *et al.*, 1997; Calder 2008).

Various previous studies have shown that ω3-PUFAs are able to down-regulate the gene transcription of pro-inflammatory and growth-promoting genes by interfering with transcription factors. In this regard, Liu *et al.*, (2001) showed that in a mouse epidermal cell system, ω3-PUFAs, but not the ω6-arachidonic acid, effectively inhibited growth factor-triggered activator protein-1 transactivation. Moreover, ω3-PUFAs down-regulated cytokine-triggered NF-κB activation and subsequent gene transcription in endothelial cells (McGuinness *et al.*, 2008) as well as in macrophages (Lo *et al.*, 1999). However, the direct PUFA target was not addressed in any of these studies.

Intriguingly, it was recently shown that ω3-PUFAs can act as ligands of PPARs and thereby can modulate a multitude of pro- and anti-inflammatory genes (Forman *et al.*, 1997; Price *et al.*, 2000; Stahl *et al.*, 2010). In fact, the appreciated lipid-lowering effect of fish oil supplementation (McKenney and Sica, 2007) is mainly explained by the action of ω3-PUFAs or metabolites as PPAR agonists (Forman *et al.*, 1997; Xu *et al.*, 1999). ω3-PUFAs can also act as ligands for the orphan receptor GPR120 and it was shown that this action contributes to its anti-inflammatory and insulin-sensitizing effects in macrophages (Oh *et al.*, 2010).

Our data now clearly show that certain ω3-PUFA derivatives, such as AVX001 and AVX002, can also directly inhibit cPLA$_2$ *in vitro* as well as in intact cells. As a consequence, they are able to down-regulate cytokine-stimulated PGE$_2$ formation in mesangial cells and thereby reduce an inflammatory reaction. Mechanistically, we further showed that cPLA$_2$ is involved in the cross-regulation of sPLA$_2$-IIA expression via NF-κB activation. This is suggested by our findings that the direct cPLA$_2$ inhibitors AVX001 and AVX002 not only down-regulated cytokine-induced sPLA$_2$ promoter activities (Figure 5C), mRNA expression (Figure 5B), protein expression (Figure 5A) and subsequent activity (Figure 4), but also, as a more upstream event, blocked IκB degradation and subse-

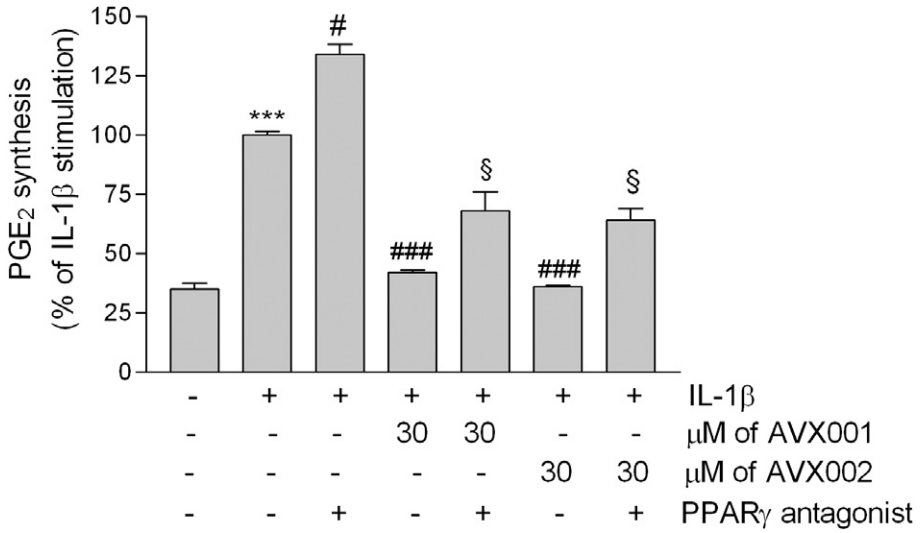

Figure 7

Effect of the PPARγ antagonist G3335 on AVX001 and AVX002 reduced PGE_2 formation in mesangial cells. Quiescent cells were stimulated for 24 h with either DMEM (-), and IL-1β (1 nM) in the absence (-) or presence of 30 μM AVX001 or AVX002, in the absence or presence of 10 μM of G3335. The inhibitors were all added as a pretreatment for 90 min before stimulation. Supernatants were collected and taken for PGE_2 quantification by using an ELISA. Data are expressed as % of maximal IL-1β-stimulated PGE_2 and are means ± SD ($n = 3$). ***$P < 0.001$ considered statistically significant when compared with the control values; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ when compared with the IL-1β-stimulated values; $^{\S}P < 0.05$ when compared with the corresponding AVX001 or AVX002 values.

quent NF-κB activation (Figure 6). Notably, higher concentrations of AVX001 and AVX002 were needed to down-regulate the $sPLA_2$ protein and mRNA expressions compared with the reduction of PGE_2. Their more potent effect on PGE_2 formation than on $sPLA_2$ expression may be derived from the dual action of AVX compounds, that is, the direct inhibition of $cPLA_2$ (this enzyme accounts for approx. 20% of the cytokine-induced PGE_2 formation according to Pfeilschifter *et al.*, 1993), and the partial down-regulation of $sPLA_2$ expression (this enzyme accounts for the remaining 80% of cytokine-induced PGE_2 formation according to Pfeilschifter *et al.*, 1993).

It is also worth noting that we previously reported that in the human keratinocyte cell line HaCaT, three previously known $cPLA_2$ inhibitors, $AACOCF_3$, MAFP and the trifluoromethyl ketone analogue of EPA ($EPACOCF_3$), all inhibited TNFα-induced NF-κB activation and ICAM-1 expression (Thommesen *et al.*, 1998). The reduced NF-κB activity could be reversed by the addition of an excess of exogenous arachidonic acid (Anthonsen *et al.*, 2001), which further corroborates the involvement of $cPLA_2$ in NF-κB activation. Additional evidence that $cPLA_2$ is positively regulating NF-κB activation was also presented by Camandola *et al.*, (1996). These authors showed that in promonocytic U937 cells, arachidonic acid stimulated NF-κB activation by a mechanism involving the metabolization of arachidonic acid to PGs and LTs.

However, when considering the fact that ω3-PUFAs can act as PPAR agonists, and the fact that the IIA-$sPLA_2$ gene contains functional PPAR binding elements in its promoter sequence (Scholz-Pedretti *et al.*, 2002), and in view of the recent report that parts of the anti-inflammatory effect of PPARs may be mediated by direct negative interference with transcription factors such as NF-κB, AP-1 and C/EBP (Genolet *et al.*, 2004), it is possible that the observed reducing effect of the AVX compounds on PGE_2 formation reported in this study also involves additional mechanisms besides the direct inhibition of $cPLA_2$ such as PPAR activation. We therefore used a PPARγ antagonist to see whether the AVX001- and AVX002-mediated effects on PGE_2 could be reversed. Indeed, the down-regulated PGE_2 formation was increased in the presence of a PPARγ antagonist (Figure 7). However, as the IL-1β-triggered PGE_2 formation was also increased by the PPARγ antagonist, we concluded that there is more a general PGE_2 stimulating effect induced by PPARγ antagonism than a specific effect on AVX action.

PLA_2 activation is the rate-limiting step in the generation of eicosanoids. It is also the initial step in the generation of PAF, which is a further potent inflammatory mediator. Therefore, the pharmacological inhibition of PLA_2 is considered an attractive target to block inflammatory processes and should, at least theoretically, be a more effective approach than blocking one of the downstream enzymes like the COXs. Unfortunately, the development of PLA_2 inhibitors has been hampered by the fact that too many subtypes of PLA_2 exist, that are involved in a redundant manner in the inflammatory reaction (Murakami and Kudo, 2004; Cummings, 2007; Lambeau and Gelb, 2008).

Very few PLA_2 inhibitors have been developed which also exerted *in vivo* efficacy in various inflammatory animal models (reviewed in Huwiler and Pfeilschifter, 2009). These include an inhibitor of IIA-$sPLA_2$, LY311727 (Schevitz *et al.*, 1995), which proved effective in a rat model of inflammatory pain (Svensson *et al.*, 2005). The synthesis of novel IIA-$sPLA_2$ inhibitors that also exert *in vivo* activity and reduce carrageenan-induced oedema formation in rats with a similar

potency to indomethacin, at a dose of 10 mg·kg^{-1}, has recently been described (Boukli *et al.*, 2008).

Also, the cPLA$_2$ inhibitors MAFP and AACOCF$_3$ were shown to reduce thermal hyperalgesia induced by carrageenan- or formalin-induced flinching in rats (Lucas *et al.*, 2005), suggesting that not only the IIA-sPLA$_2$, but also the cPLA$_2$, is involved in the molecular mechanisms of nociception. Furthermore, it was shown that AACOCF$_3$ reduced chronic inflammatory responses in mice; AACOCF$_3$ given i.p. inhibited phorbol ester-induced chronic ear oedema (Malaviya *et al.*, 2006). Oral application of AACOCF$_3$ also prevented the development of airway hyperresponsiveness in a mouse asthma model (Malaviya *et al.*, 2006) and reduced acute lung injury induced by septic syndrome in mice (Nagase *et al.*, 2003). AACOCF$_3$ also has beneficial effects in a mouse model of experimental autoimmune encephalomyelitis, which is an inflammatory demyelinating disease of the CNS that results in CNS lesions (Kalyvas and David, 2004). However, due to the fact that AACOCF$_3$ is not exclusively selective for the cPLA$_2$ (Ackermann *et al.*, 1995) and, additionally, a cell lytic activity has been reported (Risse *et al.*, 2002), the various *in vivo* activities of AACOCF$_3$ must be viewed with caution. Recently, second-generation cPLA$_2$ inhibitors have become available such as 2-oxoamides, which exert potent cPLA$_2$ inhibitory effects *in vitro* and have proved effective in first *in vivo* models of inflammation and pain (Kokotos *et al.*, 2004). All these data strongly suggest that cPLA$_2$ is a valid target to treat inflammatory diseases, and, therefore, the development of novel, more selective cPLA$_2$ inhibitors, is of utmost importance.

AACOCF$_3$ has the characteristic of being a slow binding inhibitor of cPLA$_2$ (Street *et al.*, 1993). The trifluoromethyl ketone group turned out to be especially important for inhibition since substitution of this group by either CONH$_2$, CHO, COCH$_3$ or CH(OH)CF$_3$ led to a loss of inhibitory potency (Street *et al.*, 1993). Holmeide and Skattebol (2000) speculated that the replacement of the methylene group β to the carbonyl group of the ketone with a sulphur atom would make the carbonyl carbon more electrophilic, and consequently, the molecule a more potent inhibitor of cPLA$_2$. This assumption is indeed confirmed by our study that showed a more potent inhibitory effect of the sulphur-containing AVX001 and AVX002 when compared to the AACOCF$_3$, which originally was reported to inhibit cPLA$_2$ by 78% at 1.6 mol% of AACOCF$_3$ in the cPLA$_2$ assay system (Riendeau *et al.*, 1994). A further point that needs attention is the different potencies of AVX001 and AVX002 in a cell-free *in vitro* system compared with their potencies in a cellular system. Riendeau *et al.* (1994) already highlighted the issue that in a cellular system, higher concentrations of AACOCF$_3$ were needed when compared with the *in vitro* system. This is due to the fact that AACOCF$_3$ is a slow binding inhibitor of cPLA$_2$, meaning that it takes many minutes to exert its full inhibitory potency (Street *et al.*, 1993). As arachidonic acid release in cells is a rapid process taking place within minutes, relatively high concentrations are required to inhibit cPLA$_2$. A second point concerns its stability. Riendeau *et al.* (1994) showed the conversion of the ketone of AACOCF$_3$ to its non-inhibitory alcohol in cells, which may become particularly relevant in long-term stimulation settings and could reduce the inhibitory potential. Furthermore, PUFAs are known to be easily oxidized, which can additionally reduce their inhibitory effect. Similar explanations may also be true for the AVX001 and AVX002, although the metabolism of these compounds *in vivo* is still unknown.

In summary, in this study we have described two novel cPLA$_2$ inhibitors, the ω3-PUFA derivatives AVX001 and AVX002, which are effective at reducing inflammatory PGE$_2$ synthesis in mesangial cells. These two compounds are certainly very promising candidates to be tested in *in vivo* disease models, such as in models of chronic inflammatory kidney disease. Also, further studies are needed to characterize the pharmacokinetic properties of these novel cPLA$_2$ inhibitors.

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Statement of conflict of interest

BJ is a stockholder of Avexxin AS.

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