Quantitative analysis of eicosanoids and other oxylipins – Investigation of oxidative stress and inflammation by means of targeted metabolomics of oxylipins in cell culture

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What you will learn in this chapter

- Application of a quantitative targeted metabolomics approach to lipid mediators as an important complement to non-targeted metabolomics and semiquantitative methods described in this book
- Concept of quantitative LC-MS analysis using external calibration and internal standards
- Strategies for the analysis of low abundant endogenous compounds/lipids
- Basic cell culture strategies
- Evaluation of oxidative stress based on lipid peroxidation products
- Key lipid mediators formed during inflammation in the cyclooxygenase and lipoxygenase pathways of the arachidonic acid cascade and their pharmacological modulation
- Lipid mediators are functional markers with dual function reflecting both non-enzymatic formation by autoxidation and enzymatic formation as cellular response

Abstract

Eicosanoids and other oxylipins are oxygenation products from polyunsaturated fatty acids (PUFA). They can be formed non-enzymatically by (lipid) autoxidation as well as by specific enzymatic reactions. In mammals, enzymatic formation comprises three main pathways catalyzed by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 monooxygenases (CYP). Several of the oxylipins are highly potent lipid mediators, playing a key role in the regulation of pain, fever and inflammation. Thus, oxylipin formation is a major drug target. In fact, common pharmaceuticals being sold without prescription, nonsteroidal anti-inflammatory drugs (NSAID), directly target COX.

In this chapter, a detailed protocol is described to investigate oxylipin formation during autoxidation and upon an inflammatory stimulus in cell culture. The low abundant lipid mediators are quantified by means of targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS). The experiments demonstrate that oxylipins are markers of cell biology, reflecting the redox status/oxidative stress on the one hand and the cellular inflammatory response on the other.

Based on a selected set of oxylipins the students learn how to set up a quantitative LC-MS/MS method and apply it to biological samples. With the detailed protocol, cell culture experiments can be rapidly set up in the laboratory to investigate the effect of oxidative stress or inflammatory stimuli in cells. The strategy also allows to test new compounds for their efficacy to reduce oxidative stress or to alleviate proinflammatory lipid mediator formation. Thus, the described procedures facilitate the implementation of individual research projects in advanced practical student courses.

Overall, the experiments lead to a comprehensive understanding of quantitative LC-MS/MS as well as basic cell culture experiments to study the bioactivity of new compounds. The investigation and interpretation of the results enables to understand oxylipin formation and their role in biology.

Keywords

oxidative stress, inflammation, oxylipins, eicosanoids, LC-MS, quantitative analysis, cell culture, arachidonic acid cascade, leukotrienes, prostaglandins, isoprostanes

Abbreviations

4-HNE	4-hydroxy-2-nonenal
ARA	arachidonic acid (C20:4 n6)
BCA	bicinchoninic acid
BHT	butylated hydroxytoluene
CE	collision energy
COX	cyclooxygenase
CXP	collision exit potential
CYP	cytochrome P450 monooxygenase
DHA	docosahexaenoic acid (C22:6 n3)
DiHETE	dihydroxy eicosatetraenoic acid
DiHETrE	dihydroxy eicosatrienoic acid
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
DP	declustering potential
EDTA	ethylenediaminetetraacetic acid
EP	entrance potential
EPA	eicosapentaenoic acid (C20:5 n3)
EpETrE	epoxy-eicosatrienoic acid
ESI	electrospray ionization
EtOH	ethanol
FCS	fetal calf serum
GIT	gastrointestinal tract
HAc	acetic acid
HETE	hydroxy-eicosatetraenoic acid
HHT/HHTrE	hydroxy heptadecatrienoic acid
HpETE	hydroperoxyeicosatetraenoic acid; hydroperoxy-ARA
IC50	inhibitory concentration 50%
IS	internal standard
IsoP	isoprostane
KOH	potassium hydroxide
LC	liquid chromatography
LLOQ	lower limit of quantification
LOD	limit of detection
LOX	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
LTA_4	5*S*-*trans*-5,6-oxido-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid
LTAH	leukotriene A4 hydrolase
LTB_4	5*S*,12*R*,6*Z*,8*E*,10*E*,14*Z*-dihydroxy-eicosatetraenoic acid
m/z	mass to charge ratio
MDA	malondialdehyde
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
n3-PUFA	omega-3 polyunsaturated fatty acid
n6-PUFA	omega-6 polyunsaturated fatty acid
NSAID	non-steroidal anti-inflammatory drug
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
$PGF_{2\alpha}$	9α,11α,15S-trihydroxy-prosta-5*Z*,13*E*-dien-1-oic acid
PUFA	polyunsaturated fatty acid
ROOH	organic hydroperoxide
RP	reversed phase
rpm	rounds per minute
RPMI	Rosewell Park Memorial Institute
RSD	relative standard deviation
S/N	signal-to-noise ratio
SD	standard deviation
SPE	solid phase extraction
SRM	selected reaction monitoring
t-AUCB	*trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid
t-BOOH	*tert*-butyl hydroperoxide
TGF-β1	transforming growth factor-β1
t_R	retention time
TX	thromboxane
ULOQ	upper limit of quantification
UPLC	ultra high performance liquid chromatography

1 Introduction

Polyunsaturated fatty acids (PUFA) occur in all biological systems and are major constituents of the polar lipids in the cell membrane. These PUFA act as precursors of signaling molecules – following a concept widely used by biological systems: The generation of signaling molecules from an abundant species of a major class of biomolecules such as, e.g. amino acids (serotonin, epinephrine, histamine, thyroxine, etc.) or cholesterol (cortisol, testosterone, estradiol, vitamin D, etc.). The signaling molecules resulting from PUFA – lipid mediators – comprise a multitude of different oxygenated PUFA. A challenge for analytical methods is that the concentration of the signaling molecules is low, and orders of magnitude lower than those of their precursors. Targeted analysis of lipid mediators is currently carried out by LC-MS/MS as summarized in (1, 2) allowing the selective and sensitive detection and quantification of multiple analytes in parallel.

Lipid mediators are formed from PUFA by oxidation leading to eicosanoids (oxygenated C20 PUFA) and other oxylipins (all oxygenated PUFA). Because arachidonic acid (ARA, C20:4, n6) is the major n6-PUFA occurring in mammalian cells, it plays a key role in the formation of highly biologically active lipid mediators (3). Though this chapter focusses on ARA-derived eicosanoids

(which are best investigated), it should be noted that oxylipins can be formed from all PUFA. This is why the diet, i.e. the intake of long-chain n3-PUFA, such as EPA and DHA, directly modulates the oxylipin formation and pattern in the organism (4).

PUFA bearing one or more 1,5 pentadiene systems (two double bounds with a methylene group (CH_2) in between) can easily be chemically oxidized by free radicals and singlet oxygen (5, 6). Initially hydroperoxides (ROOH) are formed reacting further to a plethora of oxidation products. A break in the carbon chain leads to aldehydes, which are the cause of rancidity in food (plant oils, potato chips, etc.) or reactive markers (e.g. malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE)) of oxidative stress. This oxidative stress is characterized by a misbalance of radical production and removal processes in cells and associated with several diseases (7-9) – even though it is often unclear if it is its cause or the consequence. In order to investigate oxidative stress in cells, tissues and the whole organism stable products are needed.

Oxylipins can serve here as ideal biomarkers: Reduction of initially formed hydroperoxides (ROOH, Hp) – which takes place rapidly in the cell through the action of glutathione reductase – leads to hydroxy-PUFA, in case of ARA particularly 5-, 9-, 11- and 15-hydroxy-eicosatetraenoic acid (HETE) (Fig. 1), whereof those which are not enzymatically formed can be used as marker of oxidative stress. More specifically, cyclic stable reaction products formed *via* bicycloendoperoxide intermediates from the initial peroxy-fatty acids – so-called isoprostanes (IsoP) – are established biomarkers for oxidative stress, induced e.g. by smoking, cardiovascular or neurological diseases (10, 11).

In the first experiment of this chapter, oxidative stress is induced in a cell line by incubation with *tert*-butyl hydroperoxide (*t*-BOOH), directly inducing lipid peroxidation. This leads to a time- and dose-dependent increase of isoprostanes demonstrating its applicability to monitor oxidative stress in biological systems. Analysis by LC-MS/MS using the targeted oxylipin metabolomics approach additionally enables the simultaneous quantification of hydroxy-PUFA, i.e. HETEs derived from hydroperoxy-PUFA (HpETE), which can be formed by autoxidation as well as by enzymes (see below). Moreover, the effect of oxidative stress on epoxy-PUFA, i.e. EpETrE is explored. Though epoxidation of double bounds is not a dominating reaction in the course of autoxidation, we recently uncovered that the ratio of the stereoisomers (*cis*- and *trans*-epoxy-PUFA) is a new marker of oxidative stress (12). Based on the evaluation of these three classes of oxylipins the power of quantitative oxylipin metabolomics to assess oxidative stress is demonstrated and enables to learn about the biology of (non-enzymatic) oxylipin formation by correlating the different results.

Conversion of PUFA by the enzymes of the ARA-cascade leads to similar oxygenation products. However – as for all enzymatic catalysis – the reactions are much more selective and give rise to specific products (3). Though some of these products can also be formed by non-enzymatic reactions, the product resulting from enzymatic conversion is just one of a large number of possible regio- and stereoisomers (e.g. prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)) or even not formed without a specific enzyme which is highlighted in the second experiment for leukotriene B_4 (LTB_4).

Three (super)families of enzymes catalyze the formation of eicosanoids and other oxylipins. By far the best investigated is the cyclooxygenase (COX) pathway. COX generates an unstable

bicycloendoperoxide-peroxy product (PGG_2) from ARA, which is reduced to the unstable PGH_2 which can be further converted by specific synthases (and in case of PGE_2, PGD_2 and TXB_2 also non-enzymatically) to prostaglandins (PG), thromboxane (TX) and prostacyclin (10, 13). The latter are regulators of, e.g. pain, inflammation, fever and blood coagulation. Thus, COX is a major target of widely used pharmaceuticals involving the best-selling over-the-counter drugs aspirin, diclofenac and ibuprofen, summarized as non-steroidal anti-inflammatory drugs (NSAIDs). Two isoforms of COX exist: COX-1 being constitutively expressed, e.g. in the gastrointestinal tract (GIT), and the inducible COX-2 playing a key role in inflammation. In the second experiment a monocytic cell line (THP-1), which is differentiated to a macrophage-like phenotype is used. Here, an inflammatory response, and thus induction of COX is elicited by lipopolysaccharide (LPS), a constituent of the cell wall of Gram-negative bacteria. This is assessed based on a dramatic increase in the production of PGE_2, a major prostanoid formed during acute inflammation, as well as two non-enzymatically formed PGH_2 breakdown products: 12-HHTrE and TXB_2 (14). The experimental setting also allows the testing of common drugs, enabling to investigate hands-on the mechanism and biological effect of drugs known from daily life.

Different hydroxy-PUFA are formed (through reduction of initially generated hydroperoxides) as side products of COX activity, i.e. 11-HETE and 15 HETE (15, 16). Again, it is a key learning for the interpretation of data from metabolomics to evaluate and interpret the levels of these oxylipins, which can be formed *via* different enzymatic as well as non-enzymatic pathways. A highly interesting extension of the experiment could be the investigation of aspirin, irreversibly blocking prostanoid formation, while COX-2 is still giving rise to 15-H(p)ETE (15, 17, 18).

The second pathway of the ARA-cascade is catalyzed by several lipoxygenases, forming in the first step positional and stereospecific hydroperoxides, which can be reduced to hydroxy-PUFA, HETE in the case of ARA. The nomenclature of the enzymes originates from the position where the hydro(peroxy) group is inserted within the ARA molecule. Two 12-LOX, two 15-LOX and the 5-LOX are found in humans (19).

In the second experiment only the 5-LOX pathway is investigated giving rise to physiologically active leukotrienes: The initially formed 5-hydroperoxy fatty acid is converted by 5-LOX to the unstable epoxide LTA_4 (Fig. 1). Similar to PGH_2 in the prostanoid formation pathway, this intermediate is then transformed by specific enzymes. The LTC_4 synthase reduces the epoxy group forming a glutathione conjugate, which causes muscle contraction of the smooth muscles playing a key role in the regulation of the lung function (not shown). Hydrolysis by leukotriene A_4 hydrolase (LTAH) leads to LTB_4 a highly potent chemoattractant for neutrophils. This means, if this compound is released by a given cell, it attracts neutrophils to come to the site increasing the (local) inflammatory response. In this experiment, the formation of LTB_4 is analyzed in THP-1 cells. Moreover, in parallel also the isomers of LTB_4 (*trans*- and *epi*-isomers of LTB_4 as well as 5,6-DiHETE isomers) are monitored which are chemical breakdown products of the unstable LTA_4 formed when the short-lived (about 20 s in a biological setting) LTA_4 is not timely converted by LTAH.

The third pathway of the ARA-cascade is catalyzed by cytochrome P450 monooxygenase (CYP) enzymes giving also rise to highly potent lipid mediators (20, 21). Terminal hydroxylation results in formation of 20-HETE – a hydroxy-PUFA that cannot be generated by autoxidation – playing a

key role in the regulation of blood pressure. Moreover, epoxy-PUFA (Fig. 1) are formed acting as potent anti-inflammatory and vasodilatory mediators. Investigating the formation of these mediators is beyond the scope of the current educational experiments (for an example for the investigation of the modulation of the CYP pathway by phytochemicals see (22)).

It should be noted that the experiments carried out in this chapter only highlight a few of the large number of oxylipins formed from ARA and all other PUFAs, which can be in parallel investigated using the described methodology (for the methods please refer to (14, 23-25).

With the application of quantitative targeted oxylipin metabolomics for the evaluation of oxidative stress, the experiment demonstrates how oxylipins can be used as markers for this (patho)physiological condition.

In the second set of experiments, oxylipins are monitored as active mediators of inflammation, generated by COX-2 and 5-LOX. It is demonstrated how these oxylipins can be modulated by stimuli and how their formation can be blocked by drugs. Here, targeted metabolomics serves as indispensable tool to monitor the target engagement of the drugs.

Chemicals and materials

All chemicals and materials needed for the experiments are listed the supplementary material (Table S1). Preparation of solutions for cell culture, SPE and LC-MS/MS analysis is described in the supplementary material.

Step-by-step protocol starts

2 Step-by-step protocol

2.1 Cell cultivation: Maintaining cells in culture

Thawing of cells stored in cryo tubes in liquid nitrogen

a. Gently thaw the cell pellet in the cryo tube in a water bath (37 °C, approx. 2 min)
b. Transfer the cells under sterile conditions to a 15 mL conical centrifuge tube containing 10 mL warmed medium (see sections 2.2 and 2.3 for medium composition)
c. Centrifuge (5 min, room temperature, 600 × g)
d. Remove the medium
e. Add 7 mL (for HCT-116) or 5 mL (for THP-1) fresh medium and resuspend the cells thoroughly
f. Seed/transfer the cells:
    i. For HCT-116 (adherent): Seed in a T25 flask (25 cm^2) for adherent cells
    ii. For THP-1 (suspension cells): Seed in a 22.1 cm^2 dish for suspension cells

g. Check growth and morphology of the cells under the microscope the following days, replace the medium with fresh if needed and transfer cells to bigger dishes when almost confluent

NOTE: Use the cells only for at most 10 passages after defrosting, enabling the replication of experiments from frozen cell stocks (at comparable passage number).

Check absence of mycoplasmas (every second month): These are small bacteria which can contaminate the cell culture and alter the biology of the test system. To verify their absence in the cell culture, collect an aliquot of the cell culture medium from nearly confluent cells (right before transfer) and determine mycoplasmas using, e.g. a commercial mycoplasma detection kit for conventional PCR.

Generation of a lab stock of frozen cells

a. Transfer cells from the dish to a conical centrifuge tube
   i. For HCT-116 (adherent cells): Detach cells from the dish using trypsin (as described in section: 2.2 “Cell culture: oxidative stress”, step 3. a. – g)
   ii. For THP-1 (suspension cells): Gently resuspend cells before transfer
b. Determine the cell number in the suspension, e.g. with a Neubauer chamber
c. Centrifuge (5 min, room temperature, 600 × g)
d. Remove the medium and resuspend the cells in cold fetal calf serum (FCS) containing 10% DMSO leading to 2×10^6 cells/mL (HCT-116) or 10×10^6 cells/mL (THP-1)
e. Transfer 1 mL in cryo tubes and freeze at -80 °C overnight using a cell freezing container (e. g. “Mr. Frosty”) allowing a freezing rate of 1 °C/min which is the optimal condition to conserve cells
f. Transfer the cryo tubes to liquid nitrogen storage

   NOTE: Check the viability of the cells in an aliquot of the generated frozen cell pellet by performing the thawing steps described under section 2.1 a – g.

2.2 Cell culture assay: Oxidative Stress in HCT-116 cells

1. The adherent human colorectal carcinoma cell line HCT-116 (obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany)) is cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin (P/S, 2%) and 2 mM L-glutamine (1%) in a humidified incubator at 37 °C and 5% CO_2 in 10 cm dishes (60.1 cm^2) for adherent cells

Maintaining the cell culture: Transfer cells every 2-3 days

2. Replace medium every 2 days (if no cell passaging is carried out, otherwise see step 3.)
    a. Carefully remove medium (e.g. using a membrane pump and pipette)
    b. Replace with fresh warmed medium
3. Passaging every 2-3 days (Monday, Wednesday, Friday)
    a. Carefully remove medium
    b. To detach the cells, add 1.5 mL trypsin (0.25% trypsin in PBS-EDTA) to the cells, swivel the dish to wet the surface completely and remove the trypsin directly
    c. Add 1.5 mL trypsin (0.25% trypsin in PBS-EDTA), let it act while swiveling the dish for 30 s and remove again
    d. Incubate the cells on the dish without liquid for 1.5 min at 37 °C
    e. Tap the dish e.g. against the palm of the hand or the bench and observe if the cells are detaching and moving from the surface of the dish. If the cells do not move, incubate for another 10 s at 37 °C and check again. Repeat in steps of 10 s until the cells are detaching

NOTE: If protein expression levels are intended to be determined the use of trypsin for detachment of the cells should be omitted to avoid premature protein digestion. For this, scraping is recommended to detach the cells from the surface of the dish (see section: 2.3 "Cell culture: inflammatory response", step 4. a).

    f. Add 10 mL fresh warmed medium and flush the surface of the dish thoroughly to detach and remove all cells from the surface of the dish
    g. Transfer the cells and medium to a conical centrifuge tube
    h. Determine the cell number
    i. Seed 1×10^6 (on Monday and Wednesday) or 0.8×10^6 (on Friday) cells in a total of 10 mL fresh medium in a new dish 10 cm (60.1 cm^2) for adherent cells
4. Incubation with *tert*-butyl hydroperoxide (*t*-BOOH)
    a. Seed 2×10^6 cells in a total of 10 mL fresh medium in a new dish 10 cm (60.1 cm^2) for adherent cells
    b. After 24 h of growth remove the medium, add fresh medium (without FCS) and add 10 µL *t*-BOOH (in water). For dose- and time-dependent investigation of the effects recommended final concentrations in the dishes (60.1 cm^2 for adherent cells, 10 mL medium) are 50 µM and 200 µM *t*-BOOH for an incubation time of 30 min, 1 h and 2 h.
5. Harvest cells using trypsin as described in step 3. a – e.

a. Add 5 mL cold PBS + 5% FCS to the detached cells in suspension and transfer to a 15 mL conical centrifuge tube
b. Centrifuge (5 min, 4 °C, 200 – 600 × g)
c. Remove the supernatant liquid
d. Resuspend the cell pellet in 1 mL cold PBS and transfer to a 1.5 mL reagent tube yielding a pellet of approx. 4 – 8 x 10^6 cells. The 15 mL tube should be rinsed with additional ~ 300 µL cold PBS, which are transferred to same 1.5 mL reagent tube.
e. Centrifuge (5 min, 4 °C, 600 – 1000 × g) and remove the liquid

6. Freeze cell pellet at -80°C until analysis
7. Exclude cytotoxic effects of *t*-BOOH at the used concentrations and incubation times e.g. by resazurin (Alamar Blue) assay (26) and lactate dehydrogenase assay (27, 28).

QUESTION (1): Why is it important to determine the cytotoxicity of the test compound at the used concentration? Which parameters for different cytotoxic endpoints can be assessed?

2.3 Cell culture assay: Inflammatory response in THP-1 cells

1. The monocytic cell line THP-1 (obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany)) is cultivated in suspension in bicarbonate buffered RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin (P/S, 2%) and 2 mM L-glutamine (1%) in a humidified incubator at 37 °C and 5% CO_2 in 10 cm dishes (58.8 cm^2) for suspension cells
2. *Maintaining the cell culture:* Transfer cells every 4-5 days (Monday and Friday):
   a. Gently resuspend cells in the dish and transfer cells and medium to a conical centrifuge tube
   b. Determine the cell number
   c. Seed 1 × 10^6 cells (on Monday and let them grow till Friday) or 1.8 ×10^6 cells (on Friday and let them grow till Monday) in a total of 10 mL fresh medium in new 10 cm dishes (58.8 cm^2) for suspension cells
3. Differentiation and incubation with test compounds:
   a. For differentiation of cells, prepare medium by adding 10 µL of 50 µM vitamin D3 (in DMSO) and 10 µL of 1 µg/mL TGF-β1 (in PBS) to 10 mL RPMI 1640 medium resulting in 50 nM vitamin D3 (0.1% DMSO) and 1 ng/mL TGF-β1. Seed cells at densities of 0.125 × 10^6 cells/mL in 10 mL of this medium in 10 cm dishes (60.1 cm^2) for adherent cells as cells will become partially adherent during differentiation. Allow the cells to differentiate for 72 h

b. For incubation with test compounds, replace cell culture medium 7 h before the end of the differentiation (65 h after the start of differentiation) with serum-free 50 mM TRIS buffered RPMI medium (2% P/S, 1% L-glutamine):
    i. Transfer non-attached cells and medium to a conical centrifuge tube and add directly 5 mL serum-free 50 mM TRIS buffered RPMI medium (2% P/S, 1% L-glutamine) to the dish
    ii. Centrifuge the tube (5 min, room temperature, 500 × g) and remove the medium
    iii. Add 5 mL serum-free 50 mM TRIS buffered RPMI medium (2% P/S, 1% L-glutamine) to the cell pellet, resuspend cells and transfer back to the dish
    iv. Add 10 µL of the test compound (pharmacological drug/inhibitor) in DMSO (e.g. add 10 µL 100 µM indomethacin yielding a final concentration of 100 nM) or DMSO (0.1%) as control. Suggestions for possible test compounds are summarized in Tab. 1.
d. After 1 h of preincubation, add 10 µL 1 mg/mL LPS (in PBS) to the medium resulting in 1 µg/mL LPS (for control add 10 µL PBS) for 6 h

QUESTION (2): Which control incubations should be carried out to allow meaningful interpretation? Which aspects should be considered when doing control incubations?

4. Harvest all adherent and non-adherent cells:
    a. Scrape the cells from the dish in the culture medium with a cell scraper and thoroughly flush the dish with the suspension
    b. Transfer cells and medium to a 15 mL conical centrifuge tube and place in ice
    c. Centrifuge (5 min, 4 °C, 200 – 600 × g)
    d. Remove the medium
    e. Add 5 mL PBS and resuspend thoroughly
    f. Centrifuge and remove supernatant again
    g. Resuspend cell pellet in 1 mL PBS (containing 1% protease inhibitor mixture with AEBSF, 129 Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin A, if proteomics analysis with the cell pellet is intended to be carried out)
    h. Transfer to a 1.5 mL reagent tube yielding a pellet of approx. 6 – 9 x 10^6 cells
    i. Centrifuge (5 min, 4 °C, 500 × g) and remove the supernatant
    j. Freeze the cell pellet at -80 °C until analysis

5. Exclude cytotoxic effects of the test compounds at the used concentrations by resazurin (Alamar Blue) assay (26) and lactate dehydrogenase assay (27, 28).

2.4 Sample preparation

NOTE: Work on ice and store samples on ice during sample preparation to minimize artificial oxylipin formation.

Free and total oxylipins, the protein concentration as well as total fatty acyls can be determined from a single cell pellet.

1. Resuspend cell pellets in an exact volume (300-500 µL) PBS (containing 1% protease inhibitor mixture with AEBSF, 129 Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin A, if proteomics analysis with the cell pellet is intended to be carried out) and add 10 µL inhibitor/antioxidant solution (0.2 mg/mL BHT, 100 µM of the COX inhibitor indomethacin, 100 µM of the soluble epoxide hydrolase inhibitor ***t****rans*-4-[4-(3-**A**damantan-1-yl-**u**reido)-**c**yclohexyloxy]-**b**enzoic acid (*t*-AUCB) and 100 µM of the 15-LOX inhibitor BLX3887 in methanol (MeOH))
2. Sonicate (keep the tube in ice during the sonication)
    a. small ultrasonic tip (1 mm): amplitude 100%, unpulsed, cycle 1, 8 – 10 s
    b. ultrasonic tip (3 mm): level 2, 20% output, unpulsed, 8 – 10 s

    Repeat sonication for another 10 s if cell suspension doesn't look clear when viewed towards the light
3. Determine protein content in an aliquot of the sonicate (use an exact volume of 10 – 30 µL for an appropriate dilution) *via* bicinchoninic acid (BCA) assay (29) allowing normalization of the oxylipin concentration to the protein content. (Expected protein concentration in the sonicate: for HCT-116 cells 3 – 6 mg/mL, for THP-1 cells 0.4 – 2 mg/mL). If the cell number of the pellet is known the oxylipin concentration can alternatively be normalized on the cell number.

    For an assay as carried out in our lab with a working range of 0.1 – 1 mg/mL BSA the following dilutions are suggested for the BCA assay if the cell pellet is resuspended in 500 µL PBS (with inhibitors) for sonication:
    a. For HCT-116 cells an 1+4 dilution
    b. For THP-1 cells an 1+2 dilution
4. The homogenous cell lysate can be split to analyze free and total (the sum of free and esterified) oxylipins from the same cell pellet depending on the scientific question. Based

on that the volume of this step should be decided, e.g. if higher sensitivity is needed for free or total oxylipins.

If both free and total oxylipins are analyzed use 350 µL of the sonicate for the determination of free oxylipins and 100 µL of the sonicate for the determination of total oxylipins and the rest for protein determination *via* BCA. Otherwise select volumes to achieve best sensitivity for the scientific question.

QUESTION (3): With the described methodology it is possible to analyze free and total (the sum of free and esterified) oxylipins. Can you give examples in which experimental setups the analysis of free or total oxylipins makes more sense and why? For the two experiments described here is the analysis of free or total oxylipins more meaningful to characterize the resulting biological effect?

a. <u>For free oxylipins:</u> Add the 2.8-fold volume of ice-cold methanol and 10 µL internal standards (IS) (100 nM of $^2H_4$-PGE$_2$, $^2H_4$-TxB$_2$, $^2H_4$-LTB$_4$, $^2H_8$-5-HETE, $^2H_8$-15-HETE, $^2H_{11}$-8(9)-EpETrE, $^2H_{11}$-14(15)-EpETrE) using a repeating syringe dispenser ("Hamilton-Repeater") (500 µL syringe)

b. <u>For total oxylipins:</u> Add 400 µL ice-cold isopropanol and 10 µL internal standards (IS) ($^2H_4$-15-F$_{2t}$-IsoP, $^2H_{11}$-5-(*R,S*)-5-F$_{2t}$-IsoP, $^2H_8$-5-HETE, $^2H_8$-15-HETE, $^2H_{11}$-8(9)-EpETrE, $^2H_{11}$-14(15)-EpETrE) using a Hamilton-Repeater (500 µL syringe)

5. Vortex samples thoroughly and precipitate proteins by freezing at -80 °C for at least 30 min

Possible Break for hours up to several days

6. When samples are taken from -80 °C freezer, leave them at room temperature for 1-2 min, then vortex briefly
7. Centrifuge (10 min, 4 °C, 20 000 x g)

OPTION: The pellet is discarded or can be used to determine protein levels using targeted LC-MS/MS based proteomics analysis as described in (14). Therefore, we recommend to centrifuge with a slightly lower acceleration and to store the pellet at -80 °C until proteomics analysis.

8. The supernatant serves as sample for oxylipin analysis:
   a. <u>For free oxylipins:</u> The sample can be loaded directly onto the prepared SPE cartridge (see Section 2.5 "Solid Phase Extraction", step 3)
   b. <u>For total oxylipins</u> a hydrolysis step is required
9. Hydrolysis for total oxylipins:
   a. Transfer supernatant into a 1.5 mL reagent tube
   b. Add 100 µL 0.6 M KOH in MeOH/H_2O (75/25, *v/v*)

c. Vortex
d. Hydrolyze sample for 30 min at 60 °C using a pre-heated shaker (500 rpm)
e. After hydrolysis, cool sample immediately on ice and neutralize by adding 20 µL acetic acid (HAc, 25% in water)
f. Vortex
g. Centrifuge sample very briefly in order to collect the liquid on the bottom of the reaction tube

OPTION: Fatty acyls can be determined in an aliquot of the sample according to (30). For free fatty acyls use an appropriate aliquot of the sample before hydrolysis (e.g. dilute 50 µL + 50 µL EtOH). For total fatty acyls use an appropriate aliquot of the hydrolyzed sample (e.g. dilute 20 µL + 180 µL EtOH for low abundant fatty acyls and 10 µL + 490 µL EtOH for high abundant fatty acyls).

2.5 Solid Phase Extraction (SPE)

1. Prepare SPE cartridges (anion exchange Bond Elut Certify II SPE cartridges 200 mg, 3 mL, Agilent, Waldbronn, Germany) by washing with
   a. One column volume of ethyl acetate/*n*-hexane (75/25, *v/v*) containing 1% HAc
   b. One column volume of MeOH
   c. One column volume 0.1 M disodium hydrogen phosphate (Na_2HPO_4) buffer in water/MeOH (95/5, *v/v*) (adjusted to pH 6.0 with HAc). Close the valve when the solution is 2-3 mm above the stationary phase
2. Add 2.0 mL 0.1 M Na_2HPO_4 buffer in water (adjusted to pH 6.0 with HAc) to the cartridges
3. Load samples with a pasteur pipette and mix thoroughly with the buffer

   NOTE: The content of organic solvent on the cartridge should be kept below 16% to prevent breakthrough/elution of the analytes during the loading step, if necessary the volume of the buffer needs to be adjusted or the sample needs to be evaporated appropriately.
4. Check pH using pH stripes (5.1-7.2 scale); only if necessary, carefully adjust pH to 6.0 with diluted HAc (if HAc has to be added, only few µl are needed)
5. Open valves and let samples run by gravity until completely sunk into the stationary phase
6. Wash with
   a. One column volume water
   b. One column volume MeOH/H_2O (50/50, *v/v*)
7. Dry samples with vacuum:

a. Close valves of all cartridges and create stable -200 mbar negative pressure within the manifold
b. Open valves of two or three samples for drying the cartridges
NOTE: Drying of the cartridges can be verified by putting a pasteur pipette cone on top of the cartridges (cone should tighten)
c. Close valves after 30 s (it is not critical if samples dry a few seconds longer, however, do not dry them longer than 1 min)
d. Repeat drying step for all samples in pairs of three or two

8. Elute analytes with 2.0 mL of 75/25 *(v/v)* ethyl acetate/*n*-hexane with 1% HAc by gravity in glass tubes containing 6 µL 30% glycerol in MeOH (a dispenser resistant to organic solvents can be used to measure the eluent volume). Remove last drops of eluent from stationary phase by applying positive pressure with the pasteur pipette cone at the top of the cartridge
9. Evaporate samples to dryness using a vacuum centrifuge (1 mbar, 30 °C, ~60 min)
10. Reconstitute samples in 50 µL MeOH using a Hamilton-Repeater (use 2.5 mL syringe) and dissolve samples by sonication and vortexing
NOTE: Inclusion of one or more secondary internal standards in the reconstitution solvent enables to calculate the extraction efficiency of the sample preparation (see section 3.1) (for examples of secondary internal standards see (25)).

QUESTION (4): How would you select a secondary internal standard?

11. Transfer samples completely into 1.5 mL reagent sample tubes
12. Freeze samples at -80 °C for at least 30 min

Possible break: Reconstituted samples can be stored for at least 2 months at -80 °C with only slight changes in the oxylipin pattern (< 20% for most analytes, CAVE: quantification of isoprostanes might be impaired)

13. Centrifuge (10 min, 4 °C, 20 000 x g)
14. Transfer clear (!) supernatant into vial with insert. Centrifuge samples again if supernatant is not completely clear

2.6 LC-ESI(-)-MS/MS analysis

The analysis is carried out using reversed-phase (RP) LC-MS/MS on a triple quadrupole instrument operated in selected reaction monitoring mode (SRM frequently termed MRM) following negative electrospray ionization (ESI).

The selection of transitions, as well as electronical parameters and source settings requires extensive optimization, which is described in detail in (23, 24).

An excellent chromatographic separation is also mandatory for oxylipin analysis because several oxylipins cannot be separated by MS/MS (a list of typical critical separation pairs can be found in the supplementary information of (31).

Here, we describe an optimized method using a liquid chromatography system composed of a 1290 Infinity LC system (Agilent, Waldbronn, Germany) with autosampler, binary pump and column oven coupled to a QTRAP mass-spectrometer (Sciex, Darmstadt, Germany). For data acquisition and instrument control Analyst Software and for integration and quantification Multiquant Software is used.

However, when using (slightly) different instrumentation, the LC-MS/MS parameters need to be adapted to the used system.

1. Inject (5 µL) the samples into the LC-MS/MS system (keep samples in a 4 °C cooled autosampler until injection)
2. For liquid chromatography an UPLC system is needed as a backpressure of about 600 bar is reached during the analysis. Separate analytes on a Zorbax Eclipse Plus C-18 reversed-phase column (2.1 × 150 mm, particle size 1.8 µm; RRHD; Agilent, Waldbronn, Germany) equipped upstream with an inline filter (0.3 µm, 1290 infinity II in-line filter, Agilent, Waldbronn, Germany) and a SecurityGuard Ultra C18 cartridge as pre-column (2.1 x 2 mm, Phenomenex, Aschaffenburg, Germany) at 40 °C and a flow rate of 0.3 mL/min
3. For chromatographic separation use the following binary gradient with solvent A 0.1% HAc/solvent B (95/5, *v/v*), and solvent B acetonitrile/MeOH/HAc (800/150/1, *v/v/v*): 0-1.0 min isocratic 21% B, 1.0-1.5 min linear from 21% B to 26% B, 1.5-10.0 min linear from 26% B to 51% B, 10.0-19 min linear from 51% B to 66% B, 19-25.1 min linear from 66% B to 98% B, 25.1-27.6 min isocratic 98% B, 27.6-27.7 min linear from 98 B to 21% B followed by reconditioning for 3.8 min. During the first 2 min and the last 6 min of each run, the LC flow is directed to waste using the 2-position-6-port valve integrated in the MS
4. For mass spectrometric detection use negative electrospray ionization (ESI-) with the following source settings: ion-spray voltage: -4500 V, curtain gas (N_2): 35 psi, nebulizer gas (gas 1, zero air): 60 psi generated with a zero air generator, and drying gas (gas 2, zero air): 60 psi at a temperature of 475 °C. The offset of the sprayer is 0.250 cm for the vertical axis and 0.550 cm for the horizontal axis, the electrode protrusion is approx. 1 mm
5. For detection of the analytes use scheduled selected reaction monitoring mode (SRM) with nitrogen as collision gas (set to “high”, 12 psi) and a detection window of ±22.5 s around the expected retention time and a cycle time of 0.4 s

6. For each analyte use the optimized compound-specific parameters. Optimized parameters for the selected analytes evaluated within the experiments are summarized in Tab 2. A list of all covered analytes and their mass spectrometric parameters can be found in (14)
7. For calibration, mix stock solutions of the individual authentic standards (in MeOH) and dilute in glass volumetric flasks (5 – 100 mL) with MeOH at 10 concentration levels (0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 100 and 500 nM), each with 20 nM of the internal standards. An appropriate procedure is described in detail in (14, 25)

   NOTE: For evaluating the extraction efficiency of the sample preparation based on secondary internal standards a secondary calibration curve should be prepared (covering 20 – 120% recovery of IS1).
8. Analyze calibration standards with the same method as samples

Step-by-step protocol ends

3 Data analysis/interpretation

In contrast to non-targeted metabolomics, with targeted metabolomics the metabolites in the biological samples are quantified. Thus, the key step is the calculation of concentrations of the analyzed compounds. Similar to all other chromatography-mass spectrometry-based quantitative methods used in food, pharmaceutical, forensic or environmental chemistry and other fields the concentration is calculated based on external calibration. In order to compensate for losses occurring by sample preparation, matrix effects and instability of the MS-signal internal standards (IS) are used. Thus, instead of the peak area of the analytes, the peak area ratio of the analyte to its assigned internal standard is used.

QUESTION (5): What is ion suppression occurring in ESI-MS and how do you detect it? Why are isotopically labeled standards the only way of allowing a robust quantification of analytes in biological samples with varying matrix (matrix = all other compounds in the sample)?

3.1 Quantification by external calibration using internal standards

1. Integrate peak areas of analytes and internal standards
2. Determine areas of the analytes and corresponding internal standards (Tab. 2) and calculate area ratios

3. Determine the limit of detection (LOD; S/N ≥ 3) and the lower limit of quantification (LLOQ; S/N ≥ 5 and accuracy of the calibration level 80-120%, see step 4 c) based on the signal-to-noise ratio of the peak
4. Determine the calibration curve
    a. Plot the peak area ratio at the individual calibration levels against the respective concentration and determine the calibration curve using linear least square regression (weighting: 1/x or $1/x^2$)
    b. Verify the absence of signal saturation: If the signal of the highest calibration level is below the linear calibration curve, this concentration is not within the linear range, but above the upper limit of quantification (ULOQ) and should be removed from the calibration. Otherwise the highest injected calibration level limits the upper linear range, do not extrapolate
    c. Use the calibration curve to calculate the concentration of the calibration levels. Evaluate the quality of the calibration curve by determination of the accuracy of the determined *vs*. the theoretical concentration at each calibration level. For all calibration levels the accuracy should be within 100 ± 15%, for the LLOQ 100 ± 20%. Otherwise repeat the calibration

NOTE: Quantification can also be performed without weighting, however using the suggested 1/x or $1/x^2$ weighting more accurate results will be obtained at low concentrations

OPTION: For evaluation of sample preparation – more specifically of the extraction efficiency – determine a second calibration curve using the area ratio of internal standards added at the beginning of sample preparation to assigned secondary internal standards (IS2) used for sample reconstitution. Only samples with a sufficient predefined extraction efficiency should be further evaluated.

5. For quantification, calculate the oxylipin concentration in the vial based on the analyte to corresponding IS area ratio in the samples using the external calibration of the respective authentic standards (see step 4). Only determine concentrations between the LLOQ and the highest calibration point/ULOQ to ensure reliable quantification (as indicated in (32)). Suggested unit: nmol/L (= nM) or pmol/mL
6. Taking the determined protein concentration, the volumes used for homogenization and oxylipin analysis and the reconstitution volume after SPE into account calculate the concentration of oxylipins in the cells, e.g. in pmol/mg protein (14). Alternatively, the oxylipin concentration can be calculated based on the number of cells, eg in fmol/10^6 cells (12)

7. For epoxy-fatty acid regioisomers, two peaks can be detected with *trans*-epoxy-PUFA isomers eluting 0.14 – 0.3 min after their corresponding *cis*-isomers
    a. Characterize *trans*-epoxy-PUFA based on retention time and identical MS-fragmentation pattern as described (12, 33-35).
    b. For the individual regioisomers determine the *trans/cis*-epoxy-PUFA ratio

3.2 Evaluation of the quantitative data

1. Keep in mind what is (your/the) hypothesis of the experiment: Which oxylipins do you expect to be up/down regulated by the stimuli used? Which experimental groups are you going to select as controls to compare the impact of the stimuli?

NOTE: Further data evaluation and statistical analyses can be performed using e.g. GraphPad Prism, R, origin, SPSS or a similar software. Data evaluation can also be carried out using Microsoft Excel, however, it has limited functions for statistical analysis.

2. For each set of samples (the replicates of the same treatment) calculate the mean and the standard deviation (SD) and relative standard deviation (RSD).

NOTE: For specific pairs of oxylipins where a ratio is biologically meaningful to be calculated (e.g. the ratio between cis- and trans epoxy PUFA), calculate the ratio for each sample. Then the mean and SD is calculated leading to a higher precision.

- Plot the results in diagrams allowing to compare the concentration of individual oxylipins or ratio of oxylipin pairs with the controls (mean ± SD) (examples can be found in (12, 14)).
    - HCT-116/*t*-BOOH: Compare the different *t*-BOOH concentrations against the control without *t*-BOOH. Which oxylipins are elevated, which are not changed? Is it possible to deduce a dose- and time-dependent effect of *t*-BOOH incubation? Correlate different oxylipins which may result from non-enzymatic conversion against each other.
    - THP-1/LPS: Which oxylipins are not affected and which are elevated in incubations with LPS (positive control) *vs.* without LPS (negative control)? Can the test compounds block the LPS-induced formation of oxylipins? Are there concentration-dependent effects of the test compounds?

3.3 Interpretation and learnings

Oxidative stress: Oxidative stress leads to the formation of reactive oxygen species. Among other cellular biomolecules, lipids are oxidized by radical chain reactions.

Both, living cells as well as in foods (e.g. plant oils) particularly unsaturated fatty acids are prone to oxidation, a process called aut(o)oxidation. Here, initially hydroperoxy radicals are formed which further react to volatile aldehydes such as MDA but also to the oxylipins measured in this experiment. The formation of hydroxy-PUFA from hydroperoxy-PUFA occurs by reduction, e.g. catalyzed by cellular glutathione peroxidases. The level of hydroxy-PUFA thus should be analyzed as potential marker of oxidative stress. Besides hydroxy-PUFA also other oxylipins are elevated by oxidative stress. With the targeted oxylipin metabolomics approach several different oxylipin classes are analyzed in parallel.

In this experiment, different oxylipin classes should be evaluated with respect to their changes during *t*-BOOH-induced oxidative stress. Based on the literature (10-12, 23), understand how prostanoid-like isoprostanes are formed during oxidative stress, and suggest potential mechanisms how *trans*-epoxy-PUFA can be formed. Evaluate which oxylipin class, and which isomers within this class are strongest elevated by *t*-BOOH-induced oxidative stress. Suggest which (set of) oxylipins could be used to assess autoxidation in diseases associated with oxidative stress and how these experiments/sampling could be carried out.

The first set of experiments demonstrates that PUFA are non-enzymatically oxidized to oxylipins. However, oxylipins are also formed by specific enzymes giving rise to highly potent lipid mediators. In the experiments with THP-1/LPS the focus is set on the 5-LOX- and COX-derived oxylipins, which are formed in macrophage-like cells upon an inflammatory stimulus.

Question (6): What is LPS and how does it elicit an inflammatory stimulus in mammalian cells?

The 5-LOX- and COX-derived oxylipins and their modulation by LPS treatment should be evaluated. Is there an overlap to oxylipins formed by autoxidation?

5-LOX: Which oxylipins can be used to monitor 5-LOX activity? What is the difference between the enzymatic and the non-enzymatic LTA_4 products (Fig. 1)? How can the level of the different LTB_4 isomers be interpreted with respect to the LTAH activity in the cells?

COX: Inflammation is a major target of today's pharmaceuticals. Among the pharmaceuticals sold over-the-counter, NSAIDs (ibuprofen, indomethacin, diclofenac) are under the top sellers in pharmacies in Europe and worldwide. These compounds directly target COX enzymes and elicit their intended effects by reducing the formation of pain-mediating and fever-causing prostanoids.

However, these compounds block both COX-1 and COX-2 activity, causing severe side effects, such as ulcers in the gastrointestinal tract by COX-1 inhibition. In order to selectively block COX-2, selective inhibitors have been developed such as celecoxib. The strongest agent to dampen inflammation are steroids, such as the synthetic glucocorticoid dexamethasone, which is e.g. used in severe Covid-19.

In the experiments these two classes of drugs are tested for their effects on the inflammatory response. What are the similarities, what are the differences in the effects of the compounds (classes) on the oxylipin formation particularly based on COX activity in the employed model of human macrophages? Also, compare the concentration range in which the compounds are active. If it is possible to analyze a sufficient number of different concentrations, calculate a dose-response curve and compare the IC_{50} values. Based on the potency and selectivity on 5-LOX, COX(s) and autoxidation, this assay also allows to evaluate the inhibitory activity of new compounds.

Take home message starts

4 Take home message

- Oxylipins are formed non-enzymatically by (lipid) autoxidation as well as by specific enzymatic reactions. In mammals, enzymatic formation comprises three main pathways catalyzed by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 monooxygenases. The initial products are further converted by several other enzymes forming specific lipid mediators with distinct biological activity.
- Multiple oxylipins can be formed by several pathways. For example, 15-HETE can be formed by LOX and as side product by COX, and also as minor product by CYP. Moreover, 15-HETE is a major product of autoxidation.
- Autoxidation leads to a multitude of different oxylipins: Hydroxy fatty acids such as 15-HETE, *trans*-epoxy-fatty acids as well as isoprostanes serve as marker of oxidative stress. Thus, the parallel analysis of different oxylipins can serve together as markers of oxidative stress (see (12) for further information).
- Several oxylipins are highly potent lipid mediators, playing a key role in the regulation of pain, fever and inflammation. Thus, oxylipin formation is a major drug target, e.g. for COX-inhibiting non-steroidal anti-inflammatory drugs (NSAID) as shown in the inflammatory assay.

- Oxylipins occur in biological samples in low concentrations, in cells a range of pmol to nmol per mg protein can be expected. Targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) is currently the best-suited method to quantify the low abundant lipid mediators. Quantification is carried out by external calibration with internal standards. In the experiments it becomes clear, that basic method validation is indispensable including the definition of upper and lower limits of quantification, accuracy and recovery rates.

Take home message ends.

Tables and Figures

Table 1: Suggested test compounds and concentrations for the incubation to assess inflammatory response in THP-1 cells.

Test compounds	Concentration range
Dexamethasone	10 pM – 1 µM
Indomethacin	1 nM – 10 µM
Celecoxib	1 nM – 10 µM

Table 2: Parameters for the targeted oxylipin metabolomics LC-ESI(-)-MS/MS anaylsis of selected oxylipins covered in the experiments. Shown are the mass transitions for quantification in scheduled SRM mode (*m/z* of precursor and fragment ion). The instrument settings (declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP)) are shown for a Sciex 5500 QTrap instrument. Moreover, the internal standard (IS) for each analyte, as well as the retention time (tR), limit of detection (LOD), the calibration range (lower limit of quantification (LLOQ), upper limit of quantification (ULOQ)) of our method are provided as orientation for method development.

analyte	**mass transition**		**mass parameters**				**internal standard**	**t_R**	**calibration range**		
	Q1	**Q3**	**DP**	**EP**	**CE**	**CXP**			**LOD**	**LLOQ**	**ULOQ**
			[V]	**[V]**	**[V]**	**[V]**		**[min]**	**[nM]**	**[nM]**	**[nM]**
15-F_{2t}-IsoP (8-*iso*-$PGF_{2\alpha}$)	353.1	193.1	-95	-10	-34	-8	$^2H_4$-8-*iso*-$PGF_{2\alpha}$	7.58	0.10	0.25	500
TxB_2	369.2	169.1	-80	-10	-24	-7	$^2H_4$-TxB_2	7.68	0.25	0.50	1000
5(*R,S*)-5-F_{2t}-IsoP (5-i$PF_{2\alpha}$-VI)	353.2	114.8	-85	-10	-27	-8	$^2H_4$-8-*iso*-$PGF_{2\alpha}$	8.07	0.1	0.25	500
PGE_2	351.2	271.3	-80	-10	-23	-6	$^2H_4$-PGE_2	8.91	0.25	0.50	750
6-*trans*-LTB_4	335.2	195.1	-80	-10	-22	-9	$^2H_4$-LTB_4	13.36	0.1	0.25	250
6-*trans*-12-*epi*-LTB_4	335.2	195.1	-85	-10	-20	-9	$^2H_4$-LTB_4	13.51	0.1	0.25	500
LTB_4	335.2	195.1	-80	-10	-22	-9	$^2H_4$-LTB_4	13.83	0.05	0.1	500

5(*S*),12(*S*)-DiHETE	335.2	195.1	-80	-10	-21	-8	$^{2}H_{4}$-LTB$_{4}$	14.40	0.025	0.05	500
12-HHTrE	279.1	179.0	-70	-10	-15	-8	$^{2}H_{11}$-11,12-DiHETrE	15.62	0.25	0.5	500
5(*S*),6(*R*)-DiHETE (ARA)	335.2	115.1	-70	-10	-20	-8	$^{2}H_{11}$-11,12-DiHETrE	17.33	0.020	0.039	390
5(*S*),6(*S*)-DiHETE (ARA)	335.2	115.1	-70	-10	-20	-8	$^{2}H_{11}$-11,12-DiHETrE	17.80	0.022	0.045	223
15-HETE	319.2	219.2	-80	-10	-18	-8	$^{2}H_{8}$-15-HETE	20.08	0.11	0.22	220
11-HETE	319.2	167.2	-80	-10	-21	-7	$^{2}H_{8}$-12-HETE	20.68	0.022	0.044	219
9-HETE	319.2	167.2	-80	-10	-21	-7	$^{2}H_{8}$-5-HETE	21.45	0.27	0.4	265
5-HETE	319.2	115.2	-80	-10	-19	-7	$^{2}H_{8}$-5-HETE	21.74	0.018	0.035	350
14(15)-EpETrE	319.2	219.2	-90	-10	-15	-4	$^{2}H_{11}$-14(15)-EpETrE	22.45	0.1	0.25	500
trans-14(15)-EpETrE	319.2	219.2	-90	-10	-15	-4	$^{2}H_{11}$-14(15)-EpETrE	22.60		relative quantification based on 14(15)-EpETrE	
11(12)-EpETrE	319.2	167.2	-85	-10	-16	-7	$^{2}H_{11}$-8(9)-EpETrE	22.98	0.05	0.1	500
trans-11(12)-EpETrE	319.2	167.2	-85	-10	-16	-7	$^{2}H_{11}$-8(9)-EpETrE	23.13		relative quantification based on 11(12)-EpETrE	
8(9)-EpETrE	319.2	155.2	-90	-10	-16	-6	$^{2}H_{11}$-8(9)-EpETrE	23.16	0.25	0.5	500
trans-8(9)-EpETrE	319.2	155.2	-90	-10	-16	-6	$^{2}H_{11}$-8(9)-EpETrE	23.31		relative quantification based on 8(9)-EpETrE	
$^{2}H_{4}$-8-*iso*-PGF$_{2\alpha}$	357.2	196.8	-75	-10	-34	-8	*internal standard*	7.55			
$^{2}H_{4}$-TxB$_{2}$	373.3	173.2	-85	-10	-23	-8	*internal standard*	7.66			
$^{2}H_{4}$-PGE$_{2}$	355.2	275.3	-80	-10	-24	-6	*internal standard*	8.88			
$^{2}H_{11}$-8,12-*iso*-iPF$_{2\alpha}$-VI	364.2	320.1	-90	-10	-30	-8	*internal standard*	10.01			
$^{2}H_{4}$-LTB$_{4}$	339.2	197.2	-80	-10	-22	-9	*internal standard*	13.76			
$^{2}H_{8}$-15-HETE	327.2	226.0	-90	-10	-18	-8	*internal standard*	19.88			
$^{2}H_{8}$-12-HETE	327.2	184.2	-85	-10	-20	-8	*internal standard*	20.93			

$^{2}H_{8}$-5-HETE	327.2	116.1	-80	-10	-19	-8	*internal standard*	21.60	
$^{2}H_{11}$-14(15)-EpETrE	330.2	219.3	-90	-10	-16	-4	*internal standard*	22.32	
$^{2}H_{11}$-8(9)-EpETrE	330.2	155.0	-80	-10	-16	-7	*internal standard*	23.05	
Aleuritic Acid	303.1	268.8	-75	-10	-41	-10	*secondary internal standard*	5.39	
(1-(1-(Ethyl-sulfonyl)piperidin-4-yl)-3-(4-(trifluoromethoxy) phenyl)urea)	394.0	176.0	-100	-10	-21	-10	*secondary internal standard*	10.69	
12-oxo Phytodienoic acid (OPDA)	291.1	165.0	-90	-10	-27	-10	*secondary internal standard*	15.56	
12-[[(tricyclo[3.3.1.13,7]dec-1-ylamino)carbonyl]amino]-dodecanoic acid (AUDA)	391.0	240.1	-100	-10	-25	-10	*secondary internal standard*	19.66	

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Figure Captions

Fig. 1: Simplified overview of oxylipin formation. Shown are the main pathways of arachidonic acid-derived eicosanoid formation by COX, LOX and CYP as well as non-enzymatic autoxidative formation. Formation of enzymatic products is illustrated by green arrows, autoxidation by orange arrows, black arrows depict chemical non-enzymatic breakdown. Furthermore, modulation of eicosanoid products by the differentiation and stimulation used in the experiment to investigate inflammatory response (THP-1 assay) is indicated.

Fig. 2: Workflow of cell incubation and sample preparation. Shown is the incubation strategy used in the experiments in this chapter: Left: Induction of oxidative stress by *t*-BOOH in adherent HCT-116 cells; Right: Differentiation and compound testing in the context of inflammation induced by LPS using the monocytic cells THP-1. Bottom: Sample preparation strategy for analysis of free and esterified oxylipins. The possible parallel analysis of protein levels as well as fatty acyls is highlighted.

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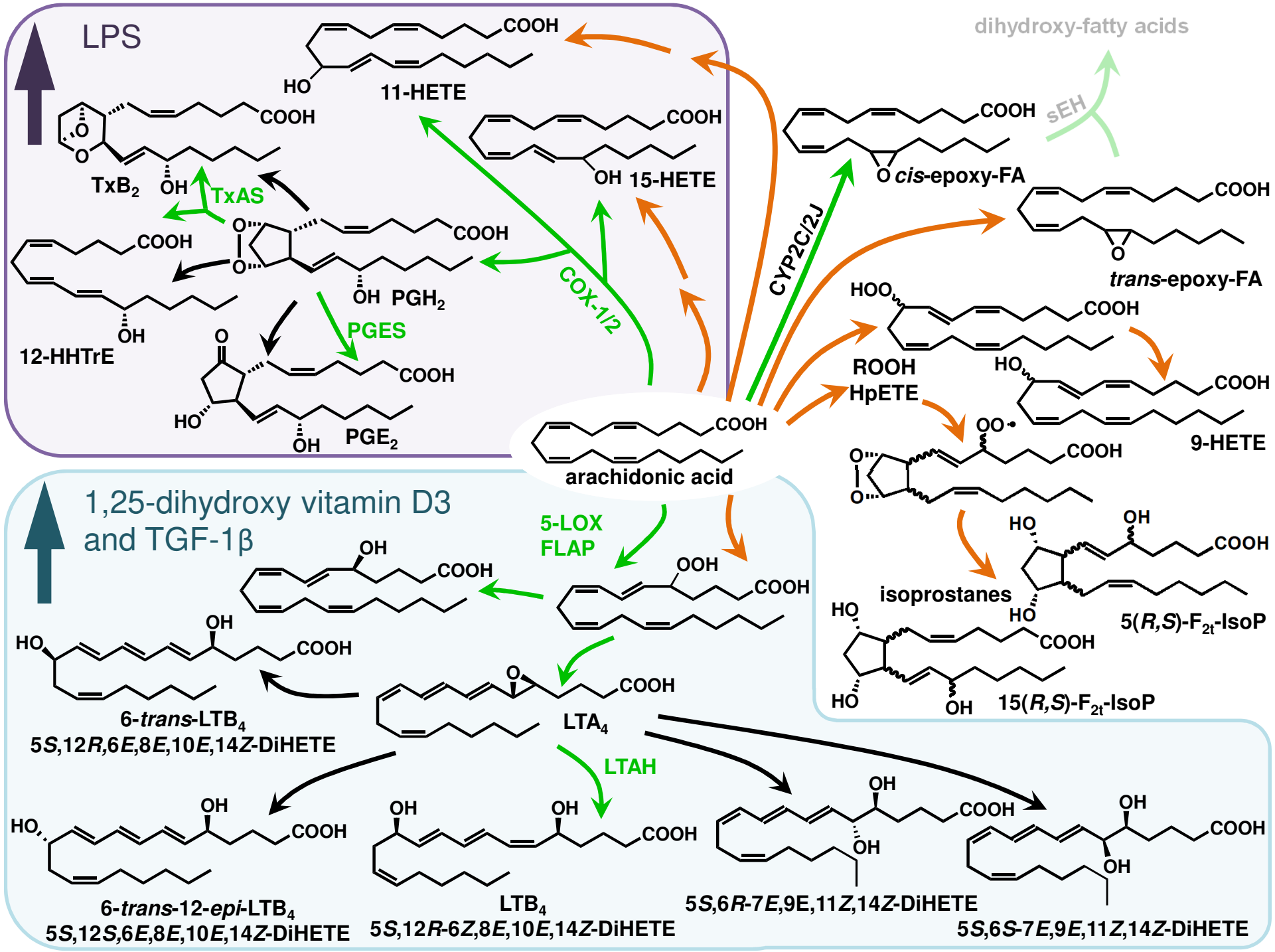
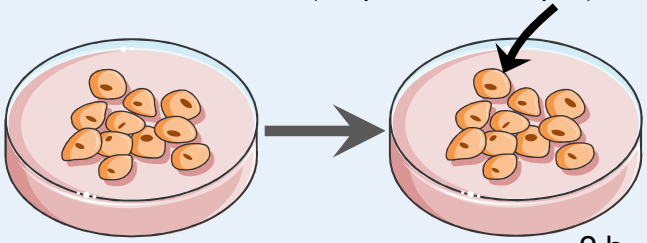

LPS
11-HETE
15-HETE
TxB2
TxAS
PGH2
12-HHTrE
PGES
PGE2
COX-1/2
CYP2C/2J
dihydroxy-fatty acids
sEH
cis-epoxy-FA
trans-epoxy-FA
ROOH
HpETE
9-HETE
arachidonic acid
isoprostanes
5(R,S)-F2t-IsoP
15(R,S)-F2t-IsoP
1,25-dihydroxy vitamin D3
and TGF-1β
5-LOX
FLAP
6-trans-LTB4
5S,12R,6E,8E,10E,14Z-DiHETE
LTA4
LTAH
6-trans-12-epi-LTB4
5S,12S,6E,8E,10E,14Z-DiHETE
LTB4
5S,12R-6Z,8E,10E,14Z-DiHETE
5S,6R-7E,9E,11Z,14Z-DiHETE
5S,6S-7E,9E,11Z,14Z-DiHETE

Cell culture: oxidative stress

HCT-116

induction of oxidative stress
tert-butyl hydroperoxide
(50 µM and 200 µM)



24 h — 2 h — 1 h — 0.5 h

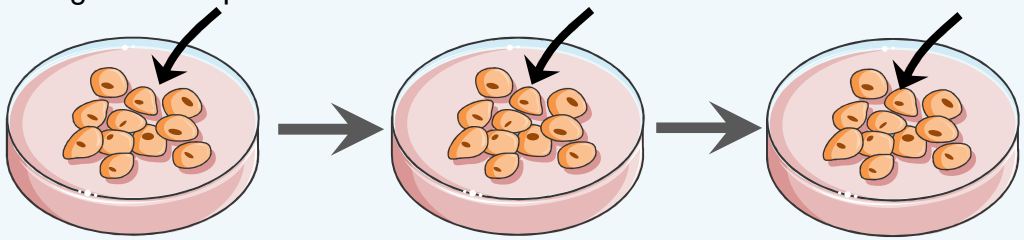
Cell culture: inflammatory response

Monocytic cells: THP-1

vitamin D3 (50 nM 0.1% DMSO),
1 ng/mL TGF-β1 for 65 h

test compound

1 µg/mL LPS



72 h
65 h — 1 h — 6 h

Sample preparation: oxylipin analysis

harvest

resuspension
(PBS),
sonication

total oxylipins (free and esterified)

free oxylipins

protein precipitation
(isopropanol, -80 °C)
centrifugation

protein precipitation
(MeOH, -80 °C)
centrifugation

aliquot for free **fatty acyl** analysis (LC-MS/MS)

aliquot for total **fatty acyl** analysis (LC-MS/MS)

supernatant: alkaline hydrolysis
KOH, 30 min, 60 °C
neutralization (HAc)

SPE
(mixed mode C8
/anion-exchange)

supernatant

RP-LC-ESI(-)-MS/MS

aliquot for protein determination (**BCA-assay**)
→ normalization

protein pellet for **proteomics** analysis (LC-MS/MS)

Supplementary Information

Quantitative analysis of eicosanoids and other oxylipins

–

Investigation of oxidative stress and inflammation by means of targeted metabolomics of oxylipins in cell culture

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Preparation of solutions

Solutions for cell culture experiments

- **DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin (P/S, 2%) and 2 mM L-glutamine** (for adherent HCT-116 cells)
  For 1 L dissolve 3.7 g $NaHCO_3$ and 10 g DMEM (powder 1000 mg/L glucose and L-glutamine, without sodium bicarbonate) in 900 mL water (ultrapure 18 MΩ*cm), adjust pH to 6.8 and fill it up to 1 L. Sterile filtrate the solution. Add 100 mL FCS, 20 ml P/S and 10 mL 200 mM L-glutamine to 870 mL prepared DMEM.

- **RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin (P/S, 2%) and 2 mM L-glutamine (1%)** (for maintaining suspension cell line THP-1)
  For 1 L add 100 mL FCS, 20 ml P/S and 10 mL 200 mM L-glutamine to 870 mL RPMI 1614 (without L-glutamine, with sodium bicarbonate, liquid).

- **Serum-free 50 mM TRIS buffered RPMI medium (2% P/S, 1% L-glutamine)**
  For 1 L add 10.39 g RPMI 1614 (with L-glutamine, without sodium bicarbonate, powder) and 6.05 g TRIS to 0.9 L water (ultrapure 18 MΩ*cm) and adjust pH to 7.4 with HCl. Sterile filtrate the solution. Add 20 mL P/S to 980 mL prepared medium.

- **FCS with 10% DMSO**
  Add 1 mL DMSO to 9 mL FCS.

- **Phosphate buffered saline (PBS)**
  For 1 L add 5.84 g NaCl, 0.34 g KCl, 0.99 g Na_2HPO_4 (anhydrous), 0.41 g KH_2PO_4 (anhydrous) to 500 mL water (ultrapure 18 MΩ*cm) in a beaker. Dissolve the salts and adjust pH to 7.4 by addition of HCl or NaOH. Transfer the solution to a 1 L volumetric flask and fill it up to the mark with water (ultrapure 18 MΩ*cm). Sterile filtrate the solution.

- **PBS-EDTA (500 mM EDTA in PBS)**
  For 1 L add 5.84 g NaCl, 0.34 g KCl, 0.99 g Na_2HPO_4 (anhydrous), 0.41 KH_2PO_4 (anhydrous) and 0.2 g EDTA-disodium salt (dihydrate) to 500 ml water (ultrapure 18 MΩ*cm) in a beaker. Dissolve the salts and adjust pH to 7.4 by addition of HCl or NaOH. Transfer the solution to a 1 L volumetric flask and fill it up to the mark with water (ultrapure 18 MΩ*cm). Sterile filtrate the solution.

- **Trypsin (0.25% trypsin in PBS-EDTA)**
  Add 100 mL 25 g/L trypsin to 900 mL PBS-EDTA.

- **PBS with 5% FCS**
  Add 5 mL FCS to 95 mL PBS.

- **PBS + 1% protease inhibitor**
  Add 100 µl protease inhibitor mixture to 10 mL PBS.

- **vitamin D3**
  Stock: 50 mM in DMSO.
  Dilution for experiments: 50 µM in DMSO. Add 10 µL 50 mM vitamin D3 to 90 µL DMSO. Add 10 µL 5 mM vitamin D3 to 990 µL DMSO. Store aliquots à 200 µL.

- **TGF-β1**
  Stock: 0.1 mg/mL in 10 mM citric acid.
- *Dilution for experiments*: 1 µg/mL in 0.1% BSA in PBS. Add 20 µL 0.1 mg/mL TGF-β1 to 1980 µL 0.1% BSA in PBS. Store aliquots à 200 µL.

- **LPS**
  Stock: 10 mg/mL in PBS.
  Dilution for experiments: 1 mg/mL in PBS. Add 300 µL 10 mg/mL LPS to 2.7 mL PBS. Store aliquots à 500 µL.

- **Test compounds**, e.g. indomethacin, dexamethasone, celecoxib
  Prepare suitable concentration in DMSO

Solutions for SPE

- **Antioxidant-Mixture** (0.2 mg/mL BHT, 100 µM sEHi (*t*-AUCB), 100 µM indomethacin, 100 µM BLX3887 in MeOH):
  a) BHT (0.2 mg/mL): Dissolve 10 mg BHT in 20 mL MeOH (LC-MS grade)
  b) *t*-AUCB: Dissolve 4.13 mg *t*-AUCB in 1 mL DMSO for a 10 mM stock
  c) Indomethacin: Dissolve 3.58 mg Indomethacin in 1 mL DMSO for a 10 mM stock
  d) BLX3887: Dissolve 3.085 mg BLX3887 in 1 mL DMSO for a 10 mM stock
  Add inhibitors indomethacin, BLX3887 and *t*-AUCB to BHT solution (final inhibitor concentration 100 µM):
  1940 µL BHT (0.2 mg/mL)
  + 20 µL 10 mM *t*-AUCB
  + 20 µL 10 mM indomethacin
  + 20 µL 10 mM BLX3887

- **Buffer for conditioning of SPE cartridges: 0.1 M Na_2HPO_4/HAc (pH 6.0) in H_2O/MeOH (95/5, *v/v*)**
  Dissolve 1.78 g disodium phosphate dihydrate in ~95 mL H_2O/MeOH (95/5, *v/v*), add 450 µL concentrated HAc and fill to 100 mL with H_2O/MeOH (95/5, *v/v*). Adjust pH carefully to 6.0 with concentrated HAc. Use ultrapure 18 MΩ*cm water and LC-MS grade MeOH for H_2O/MeOH (95/5, *v/v*).

- **Buffer for sample loading (SPE): 0.1 M Na_2HPO_4/HAc (pH 6.0) in H_2O**
  Same procedure as above, but dissolve disodium phosphate dihydrate in ultrapure water (18 MΩ*cm).

- **Ethylacetate/*n*-hexane (75/25, v/v) with 1% HAc**
  Mix 750 mL ethyl acetate with 250 mL *n*-hexane, discard 10 mL (measuring cylinder) and add 10 mL HAc (LC-MS grade).

- **0.6 M KOH in MeOH/H_2O (75/25, *v/v*)**
  Add ~20 mL MeOH/H_2O (75/25, *v/v*) to 1.98 g potassium hydroxide (85%) and dissolve (CAVE: exothermic reaction, work on ice). Following complete dissolution, fill up to 50 mL with MeOH/H_2O (75/25, *v/v*). Use ultrapure 18 MΩ*cm water and LC-MS grade MeOH for MeOH/H_2O (75/25, *v/v*).

- **MeOH/H_2O (50/50, *v/v*)**
  Use LC-MS grade MeOH and ultrapure 18 MΩ*cm water.

Eluents for LC-MS/MS analysis

- **Aqueous Eluent (A)**
  Add 50 mL organic eluent (B) and 950 µL HAc to 950 mL water. Use LC-MS grade HAc and ultrapure 18 MΩ*cm water.

- **Organic Eluent (B)**
  Add 150 mL MeOH and 1 mL HAc to 800 mL ACN. Use LC-MS grade MeOH, ACN and HAc as well as ultrapure 18 MΩ*cm water.

Table S1: Materials and chemicals used for the described experiments together with information on suggested suppliers and article number.

	specification	supplier	article number
Materials - for cell culture			
cryo tube	2 ml	Greiner Bio-One (Solingen, Germany)	126263
conical centrifuge tube	15 ml	Sarstedt (Nümbrecht, Germany)	62.554.502
	50 ml		62.547.254
serologic pipette	10 ml	Sarstedt (Nümbrecht, Germany)	86.1254.001
	25 ml		86.1685.001
pasteur pipette		VWR (Darmstadt, Germany)	612-1701
Alternative: pasteur pipette		Carl Roth (Karlsruhe, Germany)	4518,1
dishes for thawing (adherent cells)	T25 flask, 25 cm^2 (92 x 51 x 29 mm), 3 - 15 ml	Techno Plastic Products (Trasadingen, Switzerland)	90025
dishes for maintainance (adherent cells)	60.1 cm^2, (ID x H, 87 cm x 21 mm)	Techno Plastic Products (Trasadingen, Switzerland)	93100
dishes for thawing (TC tested, suspension cells)	22.1 cm^2 (ID x H, 60 x 15 mm), 5 ml	Sarstedt (Nümbrecht, Germany)	83.3901.500
dishes for differentiation (TC tested, suspension cells)	58.8 cm^2 (ID x H, 100 x 20 mm), 13 ml	Sarstedt (Nümbrecht, Germany)	83.3902.500
cell scraper	Length 195 mm, Width of Blade 14 mm, PP	Techno Plastic Products (Trasadingen, Switzerland)	99010
1.5 ml reagent tube		Sarstedt (Nümbrecht, Germany)	72,706
2 ml reagent tube		Sarstedt (Nümbrecht, Germany)	72,691
cell freezing container		Thermo Fisher Scientific (Darmstadt, Germany)	5100-0036
Materials - for SPE and LC-MS analysis			
Autosampler vials	1.5 ml short thread vial (9-425), 32 x 11.6 mm, amber glass	IVA Analysentechnik (Meerbusch, Germany)	70911302
Inserts for autosampler vial	0.1 ml micro insert, 31 x 6 mm, 15 mm tip, clear glass	IVA Analysentechnik (Meerbusch, Germany)	70906500
Screw caps for autosampler vial	9 mm PP short thread cap, black with 6 mm hole, silicon white/PTFE red, 45° shore A	IVA Analysentechnik (Meerbusch, Germany)	71509326
Glass test tubes	70 x 10 mm	LAT (Labor und Analysentechnik) (Garbsen, Germany)	11 18 14004
Hamilton syringe 500 µL	gastight, 22/51mm/pst.3	Hamilton Germany GmbH (Gräfelfing, Germany)	1750 LTN
Hamilton syringe 2.5 mL	gastight, 22/51mm/pst.3	Hamilton Germany GmbH (Gräfelfing, Germany)	1002 LTN
Hamilton repeating dispenser		Hamilton Germany GmbH (Gräfelfing, Germany)	PB600-1
Pipette Tips (20 µL)		StarLab (Hamburg, Germany)	S1110-3000
Pipette Tips (200 µL)		StarLab (Hamburg, Germany)	S1113-1006
Pipette Tips (1000 µL)		StarLab (Hamburg, Germany)	S1111-6000
Alternative pipette tips: 20 µl		Sarstedt (Nümbrecht, Germany)	70.1114.100
300 µl		Sarstedt (Nümbrecht, Germany)	70,3040
1000 µl		Sarstedt (Nümbrecht, Germany)	70,3050
SPE cartridges	Bond Elut Certify II 200mg 3ml; 50/pk	Agilent (Waldbronn, Germany)	12102080
pH sticks	pH-Fix 5.1-7.2; 100/pk	Macherey-Nagel (Dueren, Germany)	92140
Chemicals - for cell culture			
DMEM	1000 mg/L glucose and L-glutamine, without sodium bicarbonate	Sigma/Merck (Taufkirchen, Germany)	D5523
RPMI 1640	with sodium bicarbonate, without L-glutamine, sterile-filtered (liquid)	Sigma/Merck (Taufkirchen, Germany)	R0883
RPMI 1640	without sodium bicarbonate, with L-glutamine (powder)	Thermo Fisher Scientific/Life Technologies (Darmstadt, Germany)	31800089
Sodium chloride (NaCl)	99.5%, 1 kg	Grüssing GmbH (Filsum, Germany)	121221000
Potassium chloride (KCl)	≥99,5 %, 1 kg	Carl Roth (Karlsruhe, Germany)	6781,1
di-Sodium hydrogen phosphate (Na_2HPO4, anhydrous)	≥99 %, 1 kg	Carl Roth (Karlsruhe, Germany)	P030.2
Potassium dihydrogen phosphate (KH_2PO_4)	≥99 %, 1 kg	Carl Roth (Karlsruhe, Germany)	3904,1
FBS Superior	fetal bovine serum superior standardized tested for virus and mycoplasma, testedd for endotoxin	Biochrom GmbH (Berlin, Germany)	S 0615
Penicillin/Streptomycin (P/S)	5000 units Penicillin, 5 mg streptomycin/ml; 100 ml	Sigma/Merck (Taufkirchen, Germany)	P4458
L-Glutamine	200 mM, solution, sterile-filtered, BioXtra; 100 ml	Sigma/Merck (Taufkirchen, Germany)	G7513
Tris(hydroxymethyl)aminomethane (TRIS)	≥ 99.9%; 1 kg	Carl Roth (Karlsruhe, Germany)	5429,3
Trypsin from porcine pankreas	25 g/L in 0.9 % NaCl sterile-filtered, BioReagent; 100 ml	Sigma/Merck (Taufkirchen, Germany)	T4549
Ethylenediaminetetraacetic acid (EDTA)	disodium salt dihydrate, ≥ 99%, p.a, ACS; 250 g	Carl Roth (Karlsruhe, Germany)	8043,1
Dimethylsulfoxide (DMSO)	≥ 99.8%, p.a; 1 L	Carl Roth (Karlsruhe, Germany)	4720,1
Mycoplasma test	Venor GeM OneStep	Minerva biolabs (Berlin, Germany)	11-0825
Transforming growth factor-β1 (TGF-β1)	HEK293 cells derived recombinant human TGF-β1	PeproTech (Hamburg Germany)	100-21
Calcitriol (1α,25-dihydroxy cholecalciferol)	≥ 97%; 5 mg	Cayman Chemical; local distributor biomol (Hamburg, Germany)	71820
Lipopolysaccharide (LPS) from *E. coli* (0111:B4)	purified by phenol extraction; 25 mg	Sigma/Merck (Taufkirchen, Germany)	L2630
Protease inhibitor mix	AEBSF, 129 Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin A	SERVA Electrophoresis GmbH (Heidelberg, Germany)	39102,01
Resazurin·Na-salt	p.a	SERVA Electrophoresis GmbH (Heidelberg, Germany)	34226,02
Dexamethasone	≥ 98%	Cayman Chemical; local distributor biomol (Hamburg, Germany)	11015
Indomethacin	≥99%; 1 g	Cayman Chemical; local distributor biomol (Hamburg, Germany)	70270
Celecoxib	≥ 98%	Sigma/Merck (Taufkirchen, Germany)	SML3031
tert-butyl hydroperoxide (*t*-BOOH)	70 wt. % in H_2O	Sigma/Merck (Taufkirchen, Germany)	458139
Chemicals - for SPE and LC-MS analysis			
Butylated hydroxytoluene (BHT)	≥ 99%; 1kg	Sigma/Merck (Taufkirchen, Germany)	W218405
trans-4-[4-(3-Adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB)	≥ 90%;10 mg	Cayman Chemical; local distributor biomol (Hamburg, Germany)	16568
15-LOX-1 inhibitor BLX3887	≥ 95%	Cayman Chemical; local distributor biomol (Hamburg, Germany)	27391
Acetic acid	99.7%, ACS reagent, Acros; 500 mL	Thermo Fisher Scientific (Darmstadt, Germany)	423225000
Disodium hydrogen phosphate dihydrate	≥ 99.0 %	Carl Roth (Karlsruhe, Germany)	4984,1
Ethyl acetate	HPLC grade, certified acidity < 0.00003 MEQ/g; 2.5 L	Fisher Scientific (Schwerte, Germany)	E/0906/17
n-Hexane	Rotisolv HPLC; 2.5 L	Carl Roth (Karlsruhe, Germany)	7339,1
Alternative: n-Hexane	HPLC grade; 2.5 L	Fisher Scientific (Schwerte, Germany)	H/0406/17
Glycerol	99.5%, water free; 1L	Sigma/Merck (Taufkirchen, Germany)	G7893
Potassium Hydroxide	85%, for analytical purposes; 1 kg	Grüssing GmbH (Filsum, Germany)	120381000
Isopropanol	Optima LC/MS Grade; 2.5 L	Fisher Scientific (Schwerte, Germany)	A461-212
Methanol	Optima LC/MS Grade; 4 L	Fisher Scientific (Schwerte, Germany)	10402824
Acetonitrile	HPLC-MS Grade; 2.5 L	Fisher Scientific (Schwerte, Germany)	A/0638/17
Acetic acid	Optima LC/MS Grade; 50 mL	Fisher Scientific (Schwerte, Germany)	A113-50