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The role of 15-Lipoxygenase-1 in pancreatic carcinogenesis

Referees: Dr. S. Wiemann

Prof. Dr. W.Buselmaier

All truths are easy to understand once they are discovered; the point is to discover them.

Galileo Galilei

02/15/1564 - 01/08/1642

Italian astronomer, philosopher, and physicist

Für
Markus

Das Pankreaskarzinom ist bei Frauen und Männern die 4. häufigste Krebstodesursache und besitzt eine der schlechtesten Prognosen unter den malignen Erkrankungen. Gezeigt wurde, dass die ungesättigten Fettsäuren Arachidon- und Linolsäure Wachstum und Entstehung von Pankreaskarzinomen fördern. Zu den Arachidonsäure metabolisierenden Enzymen zählen die Cyclooxygenasen und Lipoxygenasen. Besonders letztere sind bei der Tumorgenese des Pankreas involviert.

15-LOX-1 wird nur im normalen Gewebe aber nicht im Tumorgewebe oder in Tumorzellen exprimiert. Dies konnte sowohl auf mRNA also auch Proteinebene nachgewiesen werden. Auch in immunhistochemischen Färbungen konnte eine deutliche Abgrenzung der 15-LOX-1 Expression zwischen normalem und erkranktem Gewebe nachgewiesen werden. Zusätzlich konnte die Expression der 15-LOX-1 in normalen, selbst isolierten und kultivierten Gangzellen nachgewiesen werden. Bei der Behandlung dieser normalen duktalen Zellen mit dem 15-LOX-1 Inhibitor, Kaffeesäure, konnte eine Zunahme der Proliferation um ca. 60% nachgewiesen werden.

Eine Überexpression der 15-LOX-1 in zwei verschiedenen Tumorzelllinien führte zu einem verminderten Wachstum von bis zu 50%. Auch die exogene Behandlung mit dem Substrat Arachidonsäure und dem Produkt 15-HETE führte zu einem verminderten Wachstum. Anders verhielt es sich für das Substrat Linolsäure und das Produkt 13-HODE. Hier konnte kein Unterschied in der Proliferation beobachtet werden. Auf Zellzyklusebene konnte mittels FACS kein Unterschied zwischen 15-LOX-1 überexprimierenden und Mock Zellen beobachtet werden. Jedoch konnte in weiteren Versuchen eine gesteigerte Apoptoserate in den 15-LOX-1 exprimierenden Zellen nachgewiesen werden.

Mit Hilfe des Histondeacetylaseinhibitors, Natriumbutyrat, konnte die Expression der 15-LOX-1 in beiden getesteten Tumorzelllinien nachgewiesen werden. Zudem wurde eine drastische Abnahme in der Proliferation beobachtet. Zusätzlich konnte gezeigt werden, dass das anti-metastatische Protein KAI1 durch die Behandlung mit Natriumbutyrat wieder hergestellt werden konnte. Jedoch konnte kein Einfluss von Natriumbutyrat auf die Expression des pro-metastatischen Protein S100A4 beobachtet werden.

Diese Ergebnisse lassen darauf schließen, dass 15-LOX-1 eine pro-apoptotische Wirkung hat und evtl. auch die Metastasierung durch den Verlust der 15-LOX-1 beeinflusst wird.

The results from epidemiological and animal studies suggest that a high fat consumption is associated with an increased incidence and growth of tumors at several specific organ sites, including pancreas, colon, breast and prostate. Recently, the important role of lipoxygenase pathways in fat metabolism and in the regulation of pancreatic cancer cell proliferation and survival was identified. The arachidonic and linoleic acid metabolizing 15-lipoxygenase-1 (15-LOX-1) acts anti-tumorigenic in colon, esophageal and gastric cancers. However, since nothing is known about 15-LOX-1 in pancreatic cancer, this was investigated in the present study. Expression of 15-LOX-1 was investigated by RT-PCR and western blotting in human pancreatic cancer cell lines and by immunohistochemistry in human pancreatic cancer tissues. Cell proliferation was analyzed in 15-LOX-1 overexpressing pancreatic tumor cell lines and after treatment with both the substrates and products. Restored 15-LOX-1 expression after sodium butyrate treatment was analyzed by western blot analysis. RT-PCR and western blotting showed absence or very weak expression of 15-LOX-1 in all pancreatic cancer cell lines tested. 15-LOX-1 was strongly stained in normal ductal cells, tubular complexes and centroacinar cells, but no staining was seen in islets, cancer cells, PanIN lesions, or in tumor cells in lymph node metastases. Over-expression of 15-LOX-1 in pancreatic tumor cells or treatment with its downstream metabolite 15-S-HETE resulted in decreased cell growth whereas 13-S-HODE did not result in any altered proliferation. Treatment with the substrate AA reduced cell proliferation significantly though no effect was observed after LA treatment.

On cell cycle progression level no difference between 15-LOX-1 expressing and mock cells was detectable. Though a clear increase in apoptosis was detected in 15-LOX-1 over-expressing cells.

A re-establishment of 15-LOX-1 in pancreatic tumor cell lines could be affirmed on protein level, further sodium butyrate incubation resulted in a strong regression of cell proliferation. Additionally, NaBu incubation restored expression of the anti-apoptotic protein KAI1 whereas no effect on protein expression level was detectable for the pro-metastatic protein S100A4.

This study shows that expression of the anti-tumorigenic 15-LOX-1 is suppressed in pancreatic cancer and already in PanINs. These findings provide evidence that loss of 15-LOX-1 may play an important role in pancreatic carcinogenesis, possibly as a tumor suppressor gene. Lipoxygenases are attractive targets for the prevention and treatment of pancreatic cancer. Induction of 15-LOX-1 expression should be harmless for normal cells and, therefore, may be a valuable new anti-tumorigenic tool in the fight against pancreatic cancer.

A	adenine
AA	arachidonic acid
ALA	α-linolenic acid
Akt	protein kinase B
ATP	adenosine triphosphate
Bcl-2	B-cell leukemia/lymphoma 2
bp	base pairs
BRCA2	breast cancer 2
BSA	bovine serum albumin
CA	caffeic acid
cAMP	cyclic adenosinmonophosphate
cDNA	copy desoxyribonucleic acid
cFLIP	Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein
CK 19	cytokeratine 19
CoA	coenzyme A
COX	cyclooxygenase
COXIB	COX selective inhibitor
$cPLA_2$	cytosolic phospholipase A2
C	cytosine
CD9	cluster of differentiation 9
CYP	cytochrome P450
DB	dynabeads
DHA	docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
DNA	desoxyribonucleic acid
dNTP	desoxy – nucleotidetriphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylendiamintetraacetat
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EPA	eicosapentaenoic acid
ER	endoplasmatic reticulum
ERK	extracellular-signal regulated kinase
EtBr	ethidium bromide

EtOH	ethanol
FA	fatty acid
FAMMM	familial atypical multiple mole melanoma syndrome
FAS	CD95, apoptotic death receptor
FBS	fetal bovine serum
FLAP	5-LOX activating protein
GI	gastrointestinal
GTP	guanosintriphosphate
G	guanine
h	hour
‘	Minute
H_2O	water
HEPES	4-(2-hydroxyethyl)-1-piperazin-ethanesulfonic acid
HETE	hydroxyeicosatetraenoic acid
HNPCC	hereditary non-poliposis colorectal carcinoma
HODE	hydroxyoctadecadienoic acid
HPETE	hydroxyperoxyeicosatetraenoic acid
HRP	horseradish peroxidase
IHC	immunohistochemistry
JNK	c-Jun N-terminal kinase
KAI1	Kangai 1, anti-metastatic protein
KB	kilo base
KCL	kalium chloride
kDa	kilo Dalton
K_m	Michaelis Menten constant
K-ras	oncogene
LA	linoleic acid
LOX	lipoxygenase
MAPK	mitogen activated protein kinase
Mb	mega base
MEK1/2	MAP kinase kinase and ERK activator kinase
MeOH	methanol
$MgCl_2$	magnesium chloride
MEq/ l	milliequivalents of solute per litre
mM	milli molar
mRNA	messenger ribonucleic acid

MUC	mucin marker
mw	molecular weight
NaBu	sodium butyrate
NaCl	sodium chloride
NADH	nicotinamidadenindinucleotide
NDGA	nordihydroguaiaretic acid
NF-κB	nuclear factor κB
Non-RT	non-reverse transcribed
NSAIDs	non-steroidal anti-inflammatory drugs
Nu-PAGE	Invitrogen western blot apparatus
p16	tumor suppressor gene
p21	cip/WAF, cell cycle inhibitor
p53	tumor suppressor gene
PanINs	Pancreatic Intraepithelial Neoplasias
$PBS^{-/-}$	phosphate buffered Saline without $CaCl_2$ und $MgCl_2$
PCD	programmed cell death
PCR	polymerase chain reaction
PD98	PD98059 ERK inhibitor
PDAC	pancreatic ductal adenocarcinoma
Pen/Strep	Penicillin/Streptomycin, antibiotic
PKC	protein kinase C
PLA2	phospholipase 2
PMSF	Phenylmethanesulfonylfluoride
PGE_2	prostaglandine E_2
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonylfluoride
PPARs	peroxisome-proliferator-activated-receptors
PS	phosphatidylserine
PTEN	Phosphatase and Tensin homolog deleted on chromosome Ten
PUFAs	polyunsatturated fatty acids
p-value	$p \leq 0.05$ is significant
RAC	small GTPase
Rho	member of GTP-protein binding protein familiy
RISC	RNA-induced silencing complex
RNA	ribonucleic acid

RNAi	RNA interference
rpm	rounds per minute
RPMI	cell culture medium
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
S100A4	pro-metastatic protein
SAHA	Suberoylanilide hydroxamic acid
SDS	sodium dodecyl sulfate
SIR	standardized incidence ratio
STK1	serine/threonine protein kinase 1
TBE	Tris, boric acid, EDTA buffer
TBS	Tris buffered Saline
TGF-β	transforming growth factor β
T	thymine
T_m	melting temperature
TM	transmembrane
TNF-α	tumor necrosis factor α
TRAIL	tumor necrosis factor-related apoptosis inducing ligand
TTBS	Tris buffered saline with 0.05 %Tween 20
VEGF	vascular endothelial growth factor
WST-1	proliferation assay

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A: Introduction

1. The Pancreas

1.1 Anatomy and Physiology

The pancreas is an elongated, tapered organ located across the back of the abdomen and lying behind the stomach. It is made up of glandular tissue and a system of ducts. Anatomically, it is divided into head, body, and tail. The head is the broadest part of the gland. It is located in the right part of the abdomen nestled in the curve of the duodenum. The body links the part of the head and tail. The tail is the thin tip of the gland in the left part of the abdomen in close proximity with the spleen. The pancreas consists of two different tissue types: exocrine and endocrine [1].

1.1.1 Exocrine pancreas

The exocrine tissue consists of acinar cells, which produce bicarbonate ions (HCO^{3-}) and different digestive enzymes such as amylase and trypsinogen. These enzymes remain inactive until they reach the duodenum. They degrade carbohydrates, triglycerides, proteins, acids and vitamins in the duodenum [2]. Their secretion is regulated by several peptides released from the gastrointestinal tract. The exocrine tissue also secretes bicarbonate to neutralize gastric acid in the duodenum.
The other part of the exocrine system consists of ductal cells. These cells secrete fluids and electrolytes.

1.1.2 Endocrine pancreas

The endocrine tissue consists of the islets of Langerhans which are divided into three main cell types: α-cells are responsible for the production of glucagon, an opponent of insulin that is produced by the β-cells. δ-cells secrete somatostatin which inhibits many digestive functions.

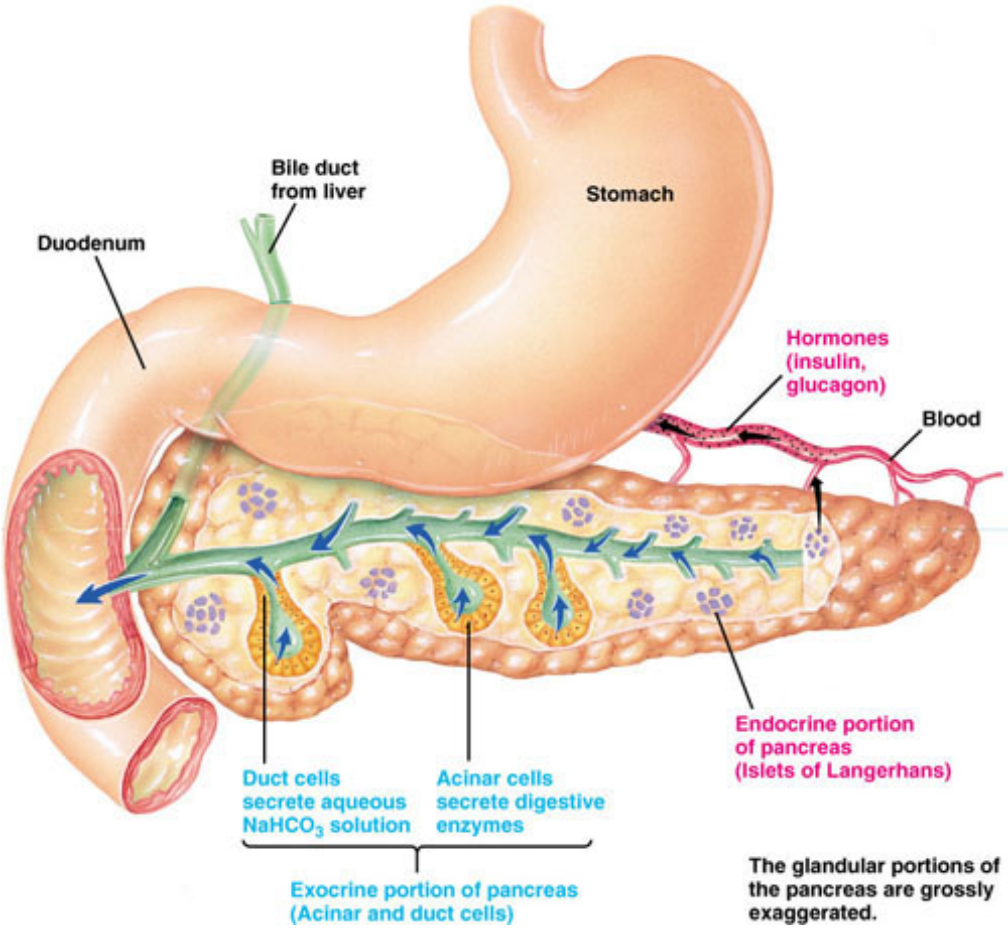


Fig 1: The Pancreas

Picture taken from Books/ Cole Thomson Learning

1.2 Pancreatic secretion

1.2.1 Bicarbonate Secretion

The pancreas secretes an isoosmotic juice at a daily rate of 1500-3000 ml. The pH of the juice ranges between 8.0 and 8.5. The juice contains enzymes for fat, carbohydrate, and protein digestion, water and electrolytes. The total concentration of the major anions Cl^- and HCO_3^- approaches 150 mEq/l. The high pH of the juice after a meal neutralizes acidic gastric chyme and raises duodenal contents to an optimal pH for enzymatic digestion. Pancreatic juice also contains Ca^{2+} and traces of Mg^{2+}, HPO_4^{2-} and SO_4^{2-}, although the role of theses electrolytes is not known [2].

1.2.2 Enzyme Secretion

Four classes of enzymes are secreted by the pancreas:

- Proteolytic: trypsin and chymotrypsin belong to endopeptidases which are secreted as zymogens. Both are activated by enterokinases that are secreted by the mucosa of the proximal intestine. After activation trypsin activates further zymogens.
- Lipolytic: The major component is the lipase which is secreted as an active enzyme. This enzyme hydrolyzes triglycerides to diglycerides, monoglycerides and fatty acids.
- Carbohydrate-hydrolyzing: amylase is a member of this family. It hydrolyzes starch to maltose, maltotrioses and dextrins.
- Nucleotic enzymes: these enzymes hydrolyze the phosphodiester bonds that link nucleotides in nucleic acid [2].

1.2.3 Regulation of Pancreatic Secretion

There are two patterns of pancreatic secretion. The first pattern is basal secretion, which is punctuated every 1 to 2 hours by bursts of increased bicarbonate and enzyme secretion that last 10 to 15 minutes. The second pattern is the postprandial (after meal) stage, which results from complex interaction of neural and hormonal mechanisms [2].
Pancreatic secretion is regulated by several peptides that are released from the gastrointestinal tract, like secretin and cholecystokinin (CCK). These enzymes stimulate pancreatic secretion acting as a counterpart to somatostatin.
All in all about 20 digestive enzymes and cofactors are secreted by the pancreas. These enzymes account for most of the intraluminal digestion of dietary proteins, triglycerides and carbohydrates. Even some vitamins (such as A and B_{12}) are cleaved by these enzymes.

1.3 Pancreatic tumors

The normal pancreas is composed of three major differentiated cell phenotypes. The cell lineages consist of acinar cells, ductal cells, and islet cells [3, 4]. Ductal markers include a secretory component [5, 6], carbonic anhydrase II, and cytokeratin 19 (CK19). Islet cell markers include chromogranin A, insulin, glucagon and somatostatin. Markers for acinar cells comprise trypsin, amylase and lipase. The majority of pancreatic tumors have morphologic characteristics of ductal epithelium, containing mucin and lacking secretory or zymogen granules [7, 8]. Pancreatic tumors can be divided into benign and malignant tumors occurring either from the endocrine or the exocrine part of the pancreas. Benign tumors are very rare and mostly originate from the endocrine system. Benign tumors occurring in the exocrine part of the pancreas include cystic neoplasias such as serous cystadenoma, mucinous cystadenoma and intraductal papillary mucinous neoplasia (IPMN).
The most common type of malignant pancreatic tumors is the ductal adenocarcinoma (90%). Further exocrine malignant tumors like the squamous cell carcinoma, the acinar cell carcinoma and the cystadenocarcinoma are rare. Mucinous cystadenoma progress to mucinous cystadenocarcinoma and IPMNs develop from adenoma and borderline lesions finally to a malignant phenotype like an invasive ductal adenocarcinoma. Tumors also might appear in the connective tissue of the pancreas. They are called leiyomyoma or histiocytoma. Metastases of pancreatic cancer are most often found in local lymph nodes, liver, peritoneum, adrenal glands, lung, kidney, spleen and bone [9].

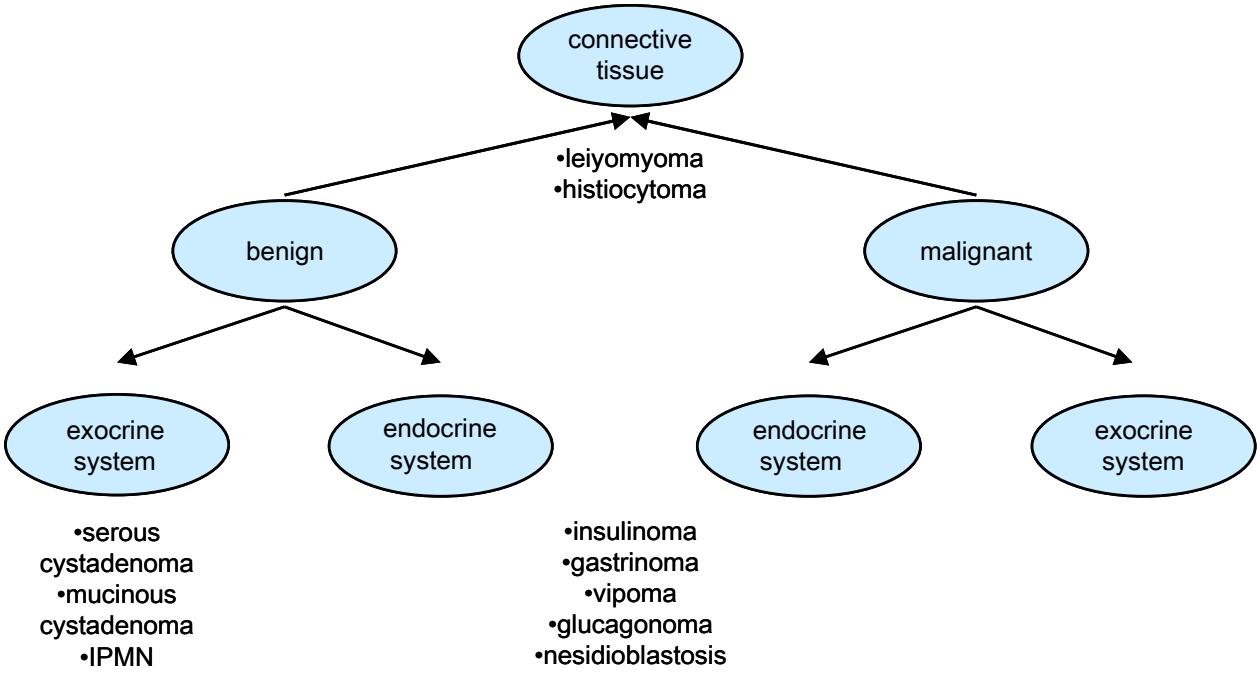


Fig 2: Classification of pancreatic tumors

1.3.1 Epidemiology and prognosis

Epidemiology

Pancreatic cancer is the tenth leading cancer disease in men and the ninth leading in women. Because of late diagnosis it is the fourth leading cause of cancer death in male and the fifth leading cause of cancer death in women [10] . During the last decade, the combined death rate of all cancers decreased by 1.5% per year among men and 0.8% per year among women. The mortality rate of pancreatic cancer, however, did not decrease compared to cancers of the lung, colon, prostate and breast. In the US, 32,300 people died from pancreatic cancer and 33,730 new cases arose in 2006 [10]. Within the last three decades, the Five-year Survival Rate has only increased about 2% up to 5%. Since 1930, the ratio of incidence to mortality has not declined. In contrast, it has increased in Japanese and African Americans over the last decades [11-15]. In Europe, the highest mortality rates occur in Austria and Sweden (around 11 per 100 000/year). The corresponding ratios in Southern Europe (Spain, Portugal, and Greece) are generally lower. Mortality rates in Japan are now similar to rates in Western countries [16].

Survival

Every year 10,000 people are diagnosed with pancreatic cancer in Germany. Males are more often affected than women. Today's surgical methods and chemotherapies are not efficient for many patients [17]. The abysmal prognosis of pancreatic cancer is caused by the lack of therapeutic options and the late diagnosis. Only 9 - 20% of patients proceed to surgical resection and even then these patients have a poor long-term survival [18]. The median survival time is about 19 months when patients receive adjuvant chemotherapy [19]. 24 months after surgery 95% of patients have recurrent disease [18].

From the EUROCARE study, based on 31,312 European cases, survival at 1, 3 and 5 years was 16, 5, and 4%, respectively [20].

Five-year survival was better in patients with 15-44 years of age, with 15% compared to 6% or less for the 45 year old patients and above. Over time there were no changes in the 1, 3 and 5-year relative survival rates of pancreatic cancer.

Thus, there is need for identifying new targets for early prevention, diagnosis, and treatment of pancreatic cancer.

1.3.2 Development of pancreatic ductal adenocarcinoma (PDAC)

Based on clinical and molecular studies, pancreatic ductal adenocarcinoma seems to follow a multi-step progression sequence from low-grade dysplasia to high-grade dysplasia and eventually to invasive carcinoma. However, it was difficult to compare studies investigating dysplasia in pancreatic ducts due to the lack of consensus regarding the terminology and criteria for grading. Some reports of precursor lesions suggested that these definitions do not encompass the full spectrum of precursors of ductal carcinoma, and these issues were the subject of the "Forum on Carcinoma In Situ of the Pancreas" held in Japan in 2002. After this forum, it became clear that the existing definitions of pancreatic intraepithelial neoplasias (PanINs) needed to be revised and expanded. Participants of the Pancreatic Cancer Think Tank and the Forum gathered together at a meeting on precursor lesions of pancreatic cancer in 2003, and an international consensus on the diagnostic criteria for PanINs was created [21]. Grading of PanINs follows a progressive increase of cytological and architectural atypia from PanIN-1A to PanIN-1B characterized by increased crowding of cells with early papillary projections, to PanIN-2 characterized by similar architectural appearance with PanIN-1B but with mild to moderate nuclear atypia, and to PanIN-3 which resemble carcinoma, at a cytological level, demonstrating severe atypia, necrosis, tufting and mitotic activity.

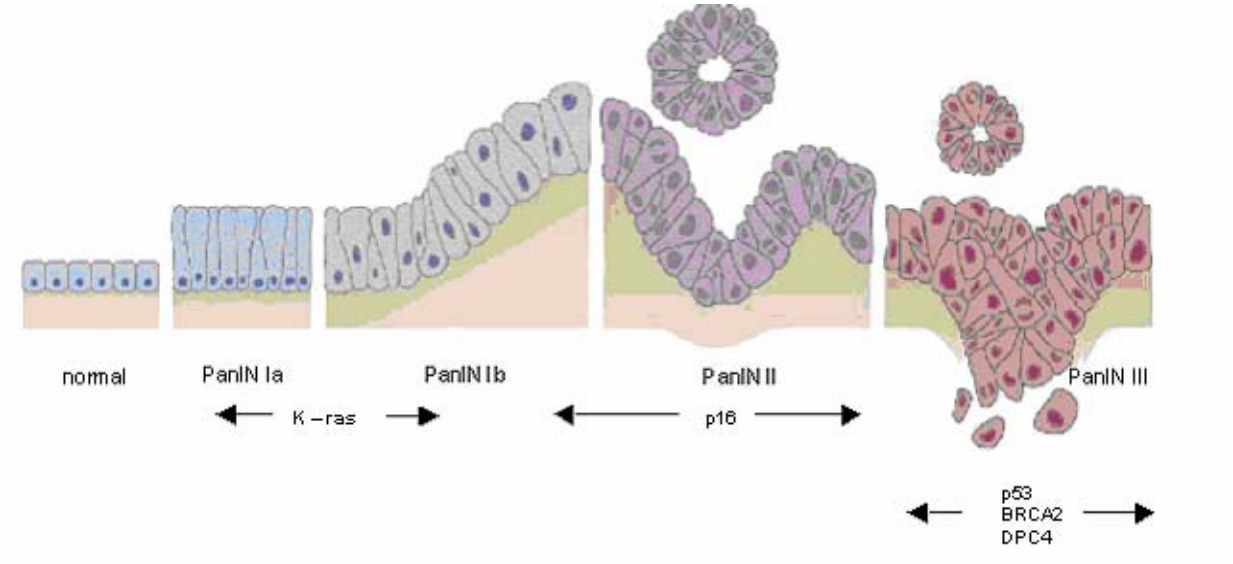


Fig 3: Process of PDAC development

Many stimuli like the oncogene K-ras or cytokines that are involved in inflammation lead to increased cell proliferation and DNA damage. Some checkpoints during cell cycle or senescence are necessary to control normal cell fate and activate apoptosis when necessary. If cell cycle controlling proteins like the tumor suppressor gene products p16 or p53 are abrogated as indicated in this figure, cells are not able to undergo apoptosis which may prevent genetically damaged cells to be removed. Instead, genomic changes accumulate and cause the different grades of PanINs when PDAC is developing [22] (slightly modified by author).

Molecular evidence also supports that this progression is characterized by gradual accumulation of genetic alterations in cancer-associated genes. It leads to inactivation of tumor suppressor genes as well as over-expression or aberrant activation of oncoproteins. These alterations can be categorized as 'early' such as K-ras mutation, HER-2/neu, prostate stem cell antigen, MUC5 and fascin over-expression; 'intermediate' such as p16 inactivation, MUC1 and cyclin D1 over-expression; and finally as 'late' such as p53 deletion, BRCA2 mutation, and over-expression of Ki-67, 14-3-3σ and mesothelin [23]. There are other proteins e.g. 5-lipoxygenase (5-LOX), E-cadherin, β-catenin (cadherin), which are supposed to play a role in the development of PanINs and thereby PDAC [22, 24]. Gene expression studies also revealed a frequent up-regulation of extrapancreatic foregut markers in early PanIN lesions (pepsinogen C, MUC6, KLF4, and TFF19 Sox-2, gastrin, HoxA5, GATA4/5/6, villin and forkhead 6 (Foxll)) and suggest that PanIN development may involve Hedgehog-mediated conversion to a gastric epithelial differentiation program.

1.3.3 Molecular genetics of PDAC

The molecular analysis of evolving PDAC has provided an outline of genetic lesions, often implicating known cancer genes and classical cancer signalling cascades. In the following, several genes involved in the development of pancreatic cancer will be described:

The K-ras oncogene and its signaling pathways

K-ras is a member of the Ras family of GTP-binding proteins that mediate a wide variety of cellular functions including proliferation, differentiation, and survival [25, 26]. Although, K-Ras is a GTPase, its intrinsic activity is inefficient and requires GTPase activating proteins (GAPs) to promote GTP hydrolysis and attenuate downstream signaling. Activating K-ras point mutations in codon 12 (GGT to GAT) result in substitution of glycine with aspartate, thereby preventing the intrinsic and GAP-catalyzed hydrolysis of GTP. These mutations are the first known genetic alterations, occurring sporadically in normal pancreas. They are detected in ~30% of early neoplasms with the frequency rising to nearly 100% in advanced PDAC [27, 28]. Activated K-Ras engages multiple effector pathways, mainly the Raf-mitogen-activated protein kinase (MAPK) and phophoinositide-3-kinase (PI3K) pathways.

The Raf-MAPK pathway

The Raf family of serine-threonine kinases, which are bound by activated Ras, lead to MAPK/ERK kinase activation through a series of phosphorylation events resulting in proliferation [29]. The importance of Raf in cancer was obvious after the identification of activating B-Raf mutations in many malignancies, including melanoma, papillary thyroid, colorectal, and serous ovarian cancers. B-Raf mutations are rare in PDAC but ~33% of the histologically distinct pancreatic medullary carcinomas, a recently described rare variant of pancreatic adenocarcinoma, carry this mutation [30]. An analysis of nine pancreatic cancer tissues revealed JNK activation in all tumor samples [31]. Inhibition of MAPK results in decreased proliferation of PDAC cell lines and cell cycle arrest.

Phosphoinositide 3-kinase (PI3K)

The PI3K signaling pathway, which can be activated by Ras [32], regulates cell survival, size, and proliferation via several downstream effectors including Akt, p70-S5K and the small GTPase RAC [33, 34]. Mutations in the PI3K pathway that are common in other cancer types, including activating mutations of the catalytic subunit of PI3K and loss-of-function mutants of the PTEN tumor suppressor, have not been commonly observed in PDAC [35, 36]. However, there are reports of decreased PTEN expression in PDAC, possibly due to promoter hypermethylation [37]. The downstream effector Akt2 is amplified in 10%-20% of PDAC, providing genetic evidence for a role of this pathway in this tumor type [38-41]. Additionally, inhibitors of PI3K appear to increase the sensitivity of PDAC cell lines to chemotherapy as well as TNF-α-induced apoptosis and diminish serum-induced cell proliferation [42-44].

Nuclear factor κB (NFκB)

The NFκB transcription factor may be another important downstream mediator of mutated K-ras signaling in PDAC [45]. Activation of this pathway occurs in response to a variety of cell stress through stimulation by pro-inflammatory cytokines and growth factors, and is known to regulate the immune response, apoptosis, and many other processes [46-48]. Constitutive activation is observed in many cancers, where it is thought to contribute to cell survival, angiogenesis, and invasion [49]. Most primary pancreatic cancers and cell lines, but not normal pancreas specimens, show constitutive NFκB activation [50, 51]. The NFκB pathway may also contribute to the prominent chemoresistance of PDAC [52] perhaps via its capacity to up-regulate Bcl-2 and Bcl-xl, as well as many other anti-apoptotic proteins.

p53 tumor suppressor gene

The p53 tumor suppressor gene is mutated, generally by missense alterations of the DNA-binding domain, in >50% of PDAC cases [28]. Consistent with an advanced malignant progression, p53 mutation appears in later-stage PanINs that have acquired significant features of dysplasia [53, 54]. Loss of p53 function could serve to enable the growth and survival of cells harboring pro-carcinogenic chromosomal aberrations [55].

$p16^{INK4A}$-Rb

$P16^{INK4A}$-Rb is a tumor suppressor system, which is critically involved in the molecular regulation of oncogene-induced premature senescence. The INK4A protein inhibits cell-cycle progression as an inhibitor of the cyclin D/ cyclin-dependent kinase 4/6 complex, indirectly influencing the activation status of the Rb protein. Inactivation of INK4A occurs in up to 95% of ductal pancreatic cancers, thus suggesting that the overcoming of the $P16^{INK4A}$-Rb security program is absolutely necessary in PDAC [28, 56-58].

TGF-β

TGF-β is the prototypic member of a super-family of secreted proteins. These growth factors signal through serine/threonine kinase receptor complexes. Upon ligand binding they, phosphorylate receptor-regulated Smad proteins that regulate a variety of cellular functions including proliferation, differentiation, migration, and apoptosis. In numerous epithelial cell lines and in epithelial tissue in vivo, TGF-β exerts a growth inhibitory program that involves modulation of cell cycle regulators, including induction of $p15^{INK4B}$ and $p21^{CIP1}$ expression and repression of c-myc, as well as induction of the apoptotic machinery [59]. Likewise, elevations in TGF-β signaling inhibit epithelial cancer initiation in vivo, and lesions in this pathway promote intestinal, ovarian, and pancreatic tumorigenesis. On the other hand, TGF-β promotes proliferation and transformation of fibroblasts and the epithelial-to-mesenchymal transition in breast and skin cancer by which advanced carcinomas lose their differentiated features and acquire a highly aggressive, invasive phenotype [60-63]. TGF-β family ligands are expressed at elevated levels in PDAC cells [64] relative to normal pancreas and may help to promote the characteristic desmoplastic response of this malignancy as suggested from xenograft studies [65]. TGF-β signaling may also contribute to tumorigenesis in an autocrine manner since PDAC often over-express the type II TGF-β receptor relative to normal pancreas [66]. Furthermore, antibodies to TGF-β inhibit the invasion of PDAC cell lines in vitro [67].

The BRCA2 tumor suppressor

Inherited BRCA2 mutations are typically associated with familial breast and ovarian cancer syndrome, but also carry a significant risk for the development of pancreatic cancer. One study estimated that ~17% of pancreatic cancers occurring in a familial setting harbor mutations in this gene [68]. BRCA2 is known to play a critical role in the maintenance of genomic stability by regulating homologous recombination-based DNA repair processes [69]. The fact that BRCA2 is selectively mutated late in tumorigenesis likely reflects the need for DNA damage response pathways to be inactivated first, so that the genetic damage incurred can be tolerated.

1.3.4. Apoptosis resistance in pancreatic cancer cell lines

The apoptosis-sensing, -inducing, and executing machinery is regulated at multiple levels, whereby every level can be disturbed in cancer cells, leading to an apoptosis-resistant phenotype [70]. In pancreatic cancer cells, the executive machinery is intact, and resistance mechanisms have evolved that work especially at the death-receptor level, mitochondrial level, and caspase-inhibitor level [71, 72]. Activation of apoptosis is triggered by two pathways [73]: the extrinsic and the intrinsic pathway. The extrinsic pathway is activated by the binding of the so-called death receptors, such as FAS/CD95, tumor necrosis factor (TNF) receptor, or TRAIL receptor, through their ligands, FAS-L, CD95L, TNF-α, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Most pancreatic cancer cells are resistant toward CD95L, TRAIL, or TNF-α-mediated apoptosis, although they express the corresponding death receptors [74, 75].

Upon binding to these receptors, the death-inducing signaling complex (DISC) is formed, and the apical inducer caspase, caspase 8, is activated. Active caspase 8 leads to cleavage of the executioner caspases (caspase 3, caspase 6, and caspase 7) whose activity is responsible for the typical morphological and biochemical changes of apoptosis [76]. C-Flip$_L$ (caspase 8 homologue) is a potent inhibitor of caspase 8 activation and determines death-receptor induced sensitivity [77]. C-Flip$_L$ is overexpressed in pancreatic cancer and is consequently one limiting factor for the therapeutic application of death receptor-based therapies [78, 79].

The central player of the intrinsic pathway is the mitochondrium, which triggers apoptosis upon lack of survival factors, stress or cellular injury. The alteration of the cellular situation are sensed by the proapoptotic BH3-only Bcl-2 family members, such as Bim, Bid, Bad, Bmf, Blk, Hrk, Noxa, or Puma. Once activated, BH3-only proteins act to antagonize the prosurvival functions of Bcl-2 or its homologues Bcl$_{xl}$ or Mcl-1. This leads to permeabilization of the

mitochondrial membrane by Bax-like proteins [80]. The permeabilization promotes the release of death factors, such as cyctochrome c, from the intramembraneous mitochondrial space [81].

At the mitochondrial level, a deregulated expression of members of the Bcl-2 family was shown in PDAC. In contrast to Bcl-2, whose expression is not significantly altered, Bcl_{xl} is overexpressed, thus suggesting an important role for this pro-survival Bcl-2 family member in apoptosis resistance [82-84].

The third level of apoptosis resistance is conferred by the inhibitor of apoptosis protein (IAP) family, including cIAP1, cIAP2, Xiap, and Survivin. One molecular action of this protein family is to inhibit executioner caspase activity [85]. At least for the family members Xiap and Survivin, increased protein expression could be shown [79, 86, 87]. It is interesting to note that nearly all molecules that mediate apoptosis resistance in pancreatic cancer cells are controlled by the transcription factor family NF-κB (already introduced). Recently, NF-κB has been shown to be constitutively activated in PDAC [51, 88]. In summary, pancreatic cancer cells have evolved multiple anti-apoptotic strategies which are possible targets for effective novel chemotherapeutics. Recently, numerous apoptosis-inducing agents, e.g. histone deacetylase inhibitors have been shown to have potent activities against various types of cancer cells.

1.3.5 Metastasis

After the initial neoplastic transformation, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential [89]. The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate-limiting for further growth. As the tumor grows and the central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply (angiogenesis).

Invasive tumor cells down-regulate cell-cell adhesion by modulating the expression of cadherins, alter their attachment to the extracellular matrix by changing integrin expression profile and proteolytically alter the matrix by secretion of matrix metalloproteinases [90]. These changes result in increased cell motility. Invasive cells can detach from the primary tumor and create defects in the extra-cellular matrix, thus accomplishing stromal invasion. Once the tumor cells have reached the vascular or lymphatic compartment, they must survive a variety of hemodynamic and immunologic challenges. However, some tumor cells evade the immune surveillance by a variety of mechanisms such as down-regulation of MHC I [91] and secretion of Fas ligand [92]. After survival in the circulation, tumor cells must arrest in distant organs or lymph nodes. Arrested tumor cells extravasate before

proliferating. After extravasation, tumor cells migrate to a local environment more favorable for their continued growth. The process of tumor metastasis requires a coordination of the actions of many positive and negative factors. The fact that fusing a non-metastatic cell with a highly metastatic cancer cell results in suppression of metastatic ability of the tumor cell leads to the hypothesis that tumor metastasis is negatively regulated by tumor metastasis suppressor genes [93]. One of these genes is KAI1. The KAI1 gene was isolated originally as a prostate-specific tumor metastasis suppressor gene [94]. It is located in the p11.2 region of chromosome 11 [95]. KAI1 is a typical type III integral membrane protein that belongs to a structurally distinct family of membrane glycoproteins (tetraspanins) [96]. These proteins are characterized by the presence of 4 highly conserved hydrophobic domains, which are presumed to span the cell membrane, and one large extracellular N-glycosylated domain. They function via cell-cell and cell-extracellular matrix interactions, thereby potentially influencing the ability of cancer cells to invade tissue and to metastasize [94]. KAI1 associates with other tetraspanins such as CD9, CD63, and CD81 in the plasma membrane and forms transmembrane complexes [97]. KAI1 is down-regulated during tumor progression in prostate [94], lung [98], breast [99], bladder [100], and pancreatic cancers [96, 101].

Another protein that is not less important than Kai1 for metastasis is S100A4, also called mts1, p9Ka, calvasculin, CAPL, pEL98. It is a member of a family containing 16 S100 calcium-binding proteins, that all have in common a functional EF-hand domain that mediates their activity [102, 103]. S100A4 is thought to promote metastasis. In fact, non-metastatic tumor cell lines that were transfected with S100A4 have a higher incidence of metastasis and show increased motility [104]. Rosty et al confirmed the over-expression of S100A4 in pancreatic carcinoma cell lines. Expression of S100A4 is associated with poor differentiation of the PDAC. Hypomethylation of CpG sites in the promoter has been found to be associated with the over-expression of S100A4 in colorectal cancer, lymphoma and PDAC cell lines. It is suggested that the majority of pancreatic carcinomas undergo selection for hypomethylation and overexpression of S100A4.

1.3.6 Risk factors

- Familial pancreatic cancer

It has been estimated that between 5 and 10% of pancreatic cancer cases are due to hereditary factors. Four case-control studies and 1 cohort study reported 3 to 5-fold higher risk among individuals with close relationship to a diseased person. No single pancreatic cancer gene has been reported to date, but a number of inherited cancer syndromes have been associated with increased PDAC cases. Among them are familial breast cancer (BRCA2 mutation), familial atypical multiple mole melanoma syndrome (FAMMM), hereditary pancreatitis, hereditary non-polyposis colorectal carcinoma (HNPCC), and Peutz-Jeghers syndrome [105].

- Tobacco

Elevated mortality rates of pancreatic cancer among smokers have been reported consistently. Relative risk for pancreatic cancer have generally ranged between 1.5 and 3 for current smokers versus non-smokers, and dose-response relationships between increasing dose or duration of cigarette smoking and pancreatic cancer have been observed in a number of studies [105]. Carcinogens from cigarette smoke such as aromatic and heterocyclic amines may be responsible for this activity.

- Aspirin and NSAID use

Data from experimental studies suggest that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit pancreatic cancer [105]. Two case-control studies reported no association between aspirin or NSAID use or duration of use and the risk of pancreatic cancer [106, 107]. In a large case-control study, a statistically significant 49% increase in risk was observed among patients with 7 or more prescriptions as compared to patients with none [108]. Thus, whether or not aspirin use is rather protective against or is a risk factor for pancreatic cancer remains controversial.

- Chronic pancreatitis

Individuals with chronic pancreatitis have an increased risk of pancreatic cancer as compared to healthy populations. In a multinational cohort study a standardized incidence ratio (SIR=16.5) was observed. SIR is the ratio of the observed to the expected new cases of cancer. Two other studies have also reported a high risk of pancreatic cancer in patients with chronic pancreatitis when compared to the general population SIR=19 [109] and SIR=18.5 [110]. Others have argued that chronic pancreatitis is not likely to be causally related to pancreatic cancer based on their observations in 2 cohorts [111, 112]. On the other hand, acute pancreatitis does not appear to be related to an increased risk of pancreatic cancer. Therefore, it is likely that the prolonged inflammation observed in chronic pancreatitis is responsible for the initiation or progression of pancreatic cancer.

- Cholecystectomy and cholelithiasis

A number of case-control studies reported elevated pancreatic cancer risk among individuals with a history of gallbladder disease (cholelithiasis) [113-115] or among individuals who had their gallbladder removed (cholecystectomy) [113, 116]. However, other studies could not confirm these results [117-119]. Considering the existing data, it is not clear whether or not cholecystectomy and cholelithiasis have an impact on pancreatic carcinogenesis.

- Diabetes mellitus

Late-onset diabetes (type II) has been consistently associated with an elevated risk of pancreatic cancer [105]. However, the causal relationship has been questioned, given that diabetes also could be a consequence of pancreatic cancer. Enhanced risk may be due to elevated glucose concentration, hyperinsulinemia and gradual impaired glucose tolerance. In a case-control study, diabetics for more than 5 years before diagnosis had a 2-fold increased risk of pancreatic cancer, though those receiving insulin had a 6-fold increase in risk compared to those getting oral antidiabetics.

The majority of diabetes associated with pancreatic cancer is diagnosed either concomitantly with the cancer or during the two years before the cancer is found [120]. 71% of the glucose intolerance found in pancreatic cancer patients is unknown before the cancer is diagnosed [121]. Therefore, recently-developed glucose intolerance or diabetes may be a consequence of pancreatic cancer and important as an early sign of the disease. Several studies have demonstrated that diabetes in pancreatic cancer patients is characterized by peripheral insulin resistance [120-122]. Insulin resistance is also found in non-diabetic or glucose intolerant pancreatic cancer patients, though to a lesser degree [122]. These data suggest that pancreatic tumors are causally related to the insulin resistance and diabetes seen in pancreatic cancer patients. Basso *et al* found a 2030 MW peptide (S100A8 N-terminal peptide) in sera from pancreatic cancer patients that they considered to be a putative pancreatic cancer associated diabetogenic factor [123]. This peptide impairs the catabolism of glucose by myoblasts in vitro and may cause hyperglycemia in vivo assuming that its identification in biological fluids might be helpful in diagnosing pancreatic cancer in patients with recent onset diabetes mellitus [124].

- Dietary factors

The pancreas is intimately related to digestion and absorption of nutrients, hence it is consequential to place diet among the possible causal factors for PDAC. However, the pancreas is never exposed to ingested or absorbed foods, directly or indirectly. Therefore, the effects of diet on carcinogenesis in the pancreas must be via changes in the internal metabolic environment of that organ, exposure to blood-circulation, or even both.
The results from epidemiological and animal studies suggest that a high fat consumption is associated with an increased incidence and growth of tumors at several specific organ sites including pancreas, colon, breast and prostate [17]. In these tissues, it has been demonstrated that linoleic acid promotes carcinogenesis.

Coffee: Out of 13 cohort studies, only 3 reported an elevated cancer risk for higher coffee intake [125-127]. Based on the existing literature in 1991, the International Agency for Research on Cancer (IARC) concluded, that there was little evidence to support a causal relation between coffee and risk of pancreatic cancer [128].

Alcohol: Ten prospective studies have examined the influence of alcohol intake in non-alcoholic populations [125-127, 129-135]. Overall, and taking into account additional data from other case-control studies, it appears unlikely that alcohol plays a major role in pancreatic cancer.

Fatty acids: Some studies suggest that high sugar intake together with high consumption of fatty acids (FAs) like saturated FAs ($p=0.01$), such as palmitic acid ($p=0.02$), stearic acid ($p=0.04$) and monounsaturated FAs ($p=0.02$), such as oleic acid ($p=0.04$) increase the risk of pancreatic cancer [136]. Recent reviews pointed out the important role of lipoxygenase pathways (see 2.0.) in fat metabolism and in the regulation of pancreatic cancer cell proliferation and survival.

2. Fatty acids and lipoxygenases

Although the role of individual fatty acids in human cancer risk has been poorly investigated up to now, some recent epidemiological and experimental data have linked a high dietary intake of ω-6 polyunsaturated fatty acids (PUFAs) such as linoleic acid (C18:2), especially in association with a low intake of ω-3 PUFAs such as docosahexaenoic acid (C22:6), to increased risks for cancers of the breast, colon and, possibly, prostate [137-139]. ω-6 PUFAs enhance tumorigenesis and metastasis in experimental animals by several mechanisms, whereas ω-3 PUFAs can inhibit the growth of initiated cancer cells [140]. Furthermore, persistent oxidative stress, often involving enhanced peroxidation of PUFAs in cell membranes by intracellularly produced *O*- and *N*-centered free radicals and altered cellular redox potential are now known to enhance the development of malignant diseases. Thus, the carcinogenic process could be initiated and/or accelerated by lipid peroxidation-induced DNA and protein damage. The presence of exocyclic DNA adducts together with oxidized DNA base damage has been reported in human tissues and cells [140].

2.1 Fatty acids

A typical fatty acid consists of an unbranched hydrocarbon chain with a carboxyl group at one end. Most naturally occurring fatty acids have an even number of carbons, with chain length of 16 and 18 being the most common.

2.1.1 Saturated fatty acids

Saturated fatty acids do not contain any double bonds or other functional groups along the chain. Of the fatty acids listed, acetic acid does not occur in natural fats and oils, but vinegar contains approximately 5% acetic acid. Butyric acid is also uncommon in natural fats other than milk fat. Myristic acid is a major fatty acid in nutmeg, coconut, and palm kernal oil; palmitic acid and stearic acid are the most abundant saturated fatty acids in animal and human fat, accounting for 30% to 40% of the fatty acids in adipose tissue [141].

2.1.2 Monounsaturated fatty acids

Monounsaturated fatty acids have one carbon-carbon double bound. There is no free rotation around the double bond, and the substituents are fixed in *cis* or *trans* configuration. The most common fatty acid in animals is oleic acid.

2.1.3 Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) carry more than one double bound. Two types of PUFAs are distinguished. Omega-3 fatty acids have a double bond three carbons away from the terminal methyl carbon, whereas omega-6 fatty acids have a double bond six carbons away from the terminal methyl carbon [141].

Omega-3 fatty acids

Important omega-3 fatty acids in nutrition are: α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). The human body cannot synthesize omega-3 fatty acids *de novo*, but can convert the omega-3 fatty acid ALA to other omega-3 fatty acids through chain elongation and desaturation. Therefore, ALA is an essential nutrient which must be obtained from food. ALA (ω-3) is found in dark green leafy plants and in linseed, rapeseed, walnut and blackcurrant oils. Deep cold-water fatty fish are rich sources of ω-3 fatty acids (EPA and DHA) and the marine food chain is based on ω-3 fatty acids, which are present in the plankton and algae [140].

Omega-6 fatty acids

Important omega-6 fatty acids in nutrition are: Linoleic acid, Gamma-linolenic acid, Eicosadienoic acid, Dihomo-gamma-linolenic acid, Arachidonic acid, Docosadienoic acid Adrenic acid, Docosapentaenoic acid, and Calendic acid. Linoleic acid, the essential ω-6 fatty acid is found in vegetable seeds and oils such as those from safflower, sunflowers, soybeans and corn.

2.1.4 Polyunsaturated fatty acids and cancer

The relative contribution of various fatty acids to dietary intake differs widely among countries, as do the incidences of many cancers. In Italy and Spain, for example, where olive oil is the predominant edible oil, the incidence of breast cancer is lower than that in North America and Northern Europe [142], and Greek women, who consume 42% of their energy supply as fat, mainly from olive oil have significantly lower rates of breast cancer than women in the USA, whose energy intake from fat is ~35% from different origin. Eskimos, who eat fish and meat from marine mammals rich in ω-3 fatty acids, and Japanese fishermen, who have the highest consumption of fish per head in the world, have high blood levels of long chain ω-3 PUFAs such as EPA and low rates of cancers of the breast and colon, despite their overall high fat consumption. Fay conducted a meta-analysis of data on mammary tumor incidence

extracted from 97 reports of experiments involving over 12,800 mice and rats to study the effects of saturated and monounsaturated fats and ω-6 PUFAs and ω-3 PUFAs [143]. The results indicated that ω-6 PUFAs have a strong and saturated fats a weaker tumor-enhancing effect, whereas the ω-3 PUFAs have a small, statistically non-significant protective effect; while monounsaturated fats had no significant effect. ω-6 PUFAs had a stronger tumor-enhancing effect when they represented <4% of total calories, but the effect was still stronger than that of saturated fat when they represented >4% of the caloric intake. Karmali first reported a difference in the effects of ω-3 and ω-6 PUFAs on the growth of transplantable mammary tumors, in which the tumor-promoting activity of ω-6 PUFAs was abrogated by competitive inhibition by ω-3 PUFAs [144, 145]. Several studies subsequently showed that diets containing corn oil, with high levels of ω-6 PUFAs such as linoleic acid, enhance breast and colon tumorigenesis in rodents, whereas fish oil, which is rich in the ω-3 PUFAs eicosapentaenoic acid and docosahexaenoic acid, reduces carcinogenesis [146]. There is now also evidence that consumption of ω-3 PUFAs is associated with reduced mortality from cardiovascular disease [147].

Mechanisms in fat-related carcinogenesis

The existence of a causal relationship between a high dietary fat intake and increased cancer risk has been controversial for a long time, partly because of the lack of consensus on the mechanisms of action of dietary fat in mammalian cells. Dietary fats, specifically ω-6 and ω-3 PUFAs, affect a variety of steps in the multistage carcinogenesis process, adding further weight to a causal relationship. The effects may be direct or indirect and include: (i) peroxidation of conjugated double bonds in PUFAs, leading to persistent oxidative stress and generation of reactive lipid peroxidation products (malondialdehyde, 4-hydroxyalkenals), which can induce DNA damage; (ii) conversion of essential fatty acids to eicosanoids by cyclooxygenases and lipoxygenases, short-lived hormone-like lipids derived primarily from dietary arachidonic acid; (iii) interaction of fatty acids with signal transduction pathways leading to altered gene expression; (iv) effects on membrane (lipid)-bound enzymes such as cytochrome P450 (CYP), (v) structural and functional changes in cell membranes resulting in alterations of hormone and growth factor receptors [140].

Effects on cell proliferation and signal transduction

Fatty acids may regulate cellular functions by affecting the expression or activity of genes in the signal transduction pathway related to the control of cell growth and apoptosis. High intake of ω-6 PUFAs induces various physiological and metabolic effects [146, 148] (i) increased ornithine decarboxylase activity in colonic mucosa, resulting in enhanced epithelial polyamine levels and increased colonic crypt cell proliferation; (ii) enhanced activities of protein kinases like protein kinase C in rodent mammary gland and an increased number of oestrogen receptor binding sites [149], (iii) increased prostaglandin concentrations. Prostaglandins, thromboxanes, leukotrienes and hydroxyperoxy and hydroxy fatty acids (collectively referred to as eicosanoids) are produced by cyclooxygenases and lipoxygenases and are involved in tumor initiation and promotion, cell proliferation, tissue invasion and metastatic spread. Tumor cells produce larger amounts of eicosanoids than their normal cell counterparts and eicosanoids ultimately derived from arachidonic and linoleic acid (ω-6 eicosanoids) have been linked to increased growth and metastasis. The finding that oleic acid and ω-3 PUFAs, specifically eicosapentanoic acid, block the desaturase reaction, the first step from linoleic acid to eicosanoids, may partially explain their inhibitory effects on tumorigenesis. Indeed, a stepwise reduction in eicosapentaenoic acid concentration was seen in diseased mucosa from benign adenoma to the most advanced colon cancer, indicating that a decline in the ω-3 : ω-6 ratio may participate in the early phases of human carcinogenesis [150].

2.2 Eicosanoid biosythesis

The oxidative metabolism of arachidonic acid through cyclooxygenase, lipoxygenase, or P450 epoxygenase pathways leads to the formation of various bioactive eicosanoids. In mammalian cells, eicosanoid biosynthesis is usually initiated by the activation of phospholipases A2 and the release of arachidonic acid (AA) from membrane phospholipids. Cyclooxygenases (COXs) and lipoxygenases (LOXs) subsequently transform AA into prostaglandins, thromboxane, and leukotrienes. Eicosanoid production is considerably increased during inflammation. Both COX and LOX pathways are of particular clinical relevance. The COX pathway is the major target for NSAIDs, the most popular medications used to treat pain, fever, and inflammation. Although, their anti-inflammatory effects are well known, their long-term use is associated with gastrointestinal (GI) complications such as ulceration of the gastric epithelium. In 1991, it was discovered that COX exists as two distinct isozymes, COX-1 and COX-2, of which COX-2 is the inducible isoform expressed at sites of

inflammation and produces pro-inflammatory eicosanoids. For this reason, COX-2 selective inhibitors (COXibs) have been developed as anti-inflammatory agents to minimize the risk of GI toxicity which is known to be primarily caused by COX-1-derived prostaglandins. Recently, some COX-2 selective inhibitors have shown adverse cardiovascular side-effects, resulting in the withdrawal of rofecoxib and valdecoxib from the market. [151].

2.3 Lipoxygenases

Lipoxygenases (LOXs) are a family of monomeric non-heme, non-sulfur iron dioxygenases, which catalyse the conversion of polyunsaturated fatty acids into conjugated hydroperoxides. Unsaturated fatty acids are absent in most bacteria and thus LOXs are also absent in typical prokaryotes. LOXs are widely expressed in plant and animal cells, sometimes at high level, and their activity may initiate the synthesis of signaling molecules or may induce structural or metabolic changes in the cell membrane. Mammalian LOXs have been implicated in the pathogenesis of several inflammatory conditions such as arthritis, psoriasis and bronchial asthma [152]. They are also thought to have a role in atherosclerosis, brain aging, HIV infection, kidney disease and cancer. In plants, LOXs favour germination, participate in the synthesis of traumatin and jasmonic acid, and are involved in the response to abiotic stress [153]. The phylogenetic tree shows that plant and animal LOXs are separate branches, each forming several subgroups [154].

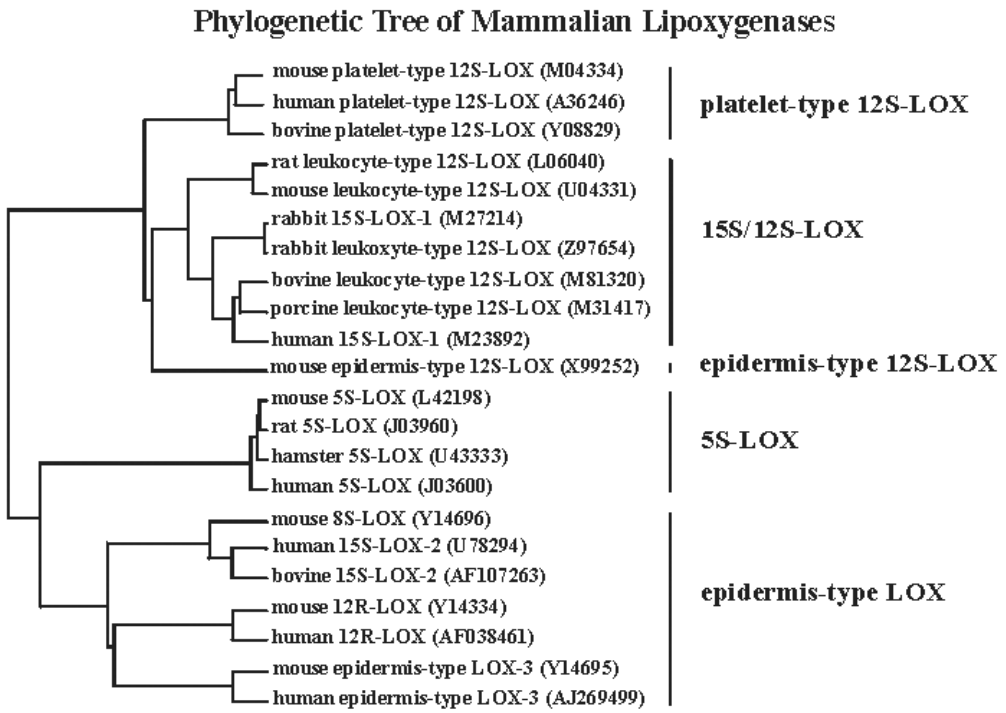


Fig 4: The phylogenetic tree of mammalian lipoxygenases

When arachidonic acid (AA) is the substrate, different LOX isozymes can insert molecular oxygen at carbons 5, 8, 12 or 15. Accordingly, they are designated 5-, 8-, 12- or 15-lipoxygenases [152, 155-159]. The primary products are 5*S*-, 8*S*-, 12*S*-, or 15*S*-hydroxyperoxyeicosatetraenoic acids (5-, 8-, 12-, or 15-HPETE), which are reduced by glutathione peroxidase to the corresponding hydroxy forms (5-, 8-, 12-, or 15-HETE) or metabolized to hepoxilins, lipoxilins or leukotrienes. Linoleic acid (LA) and linolenic acid are also substrates of LOXs.

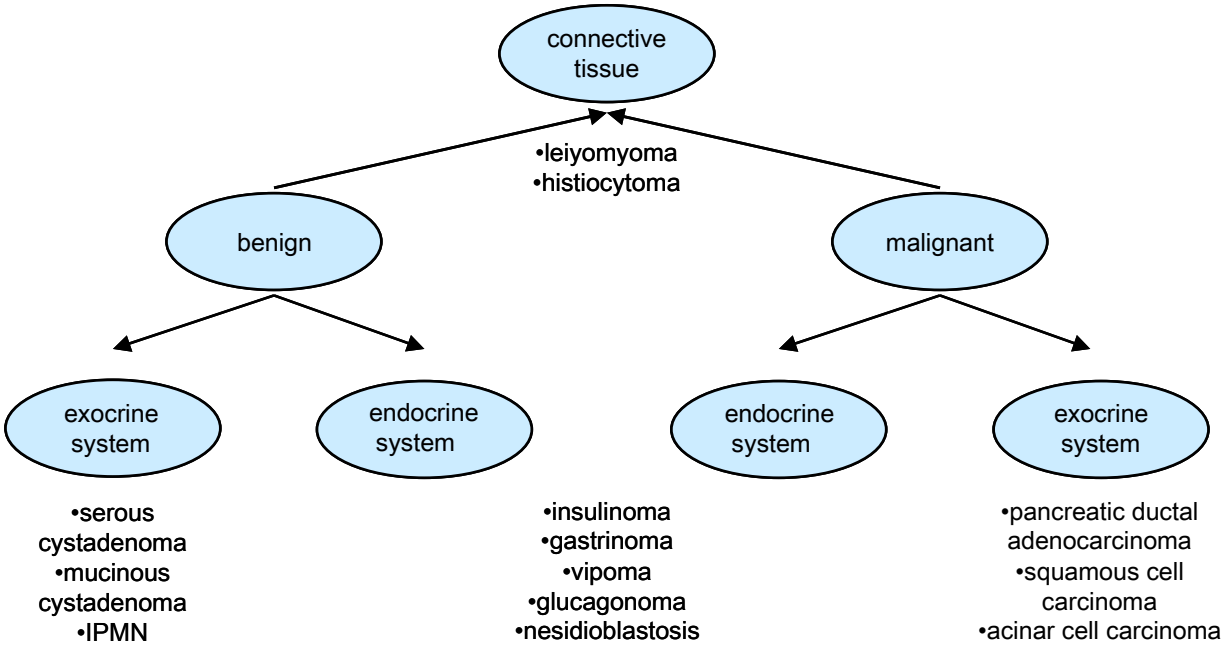


Fig 5: Metabolism pathway of arachidonic and linoleic acid

The generation of LOX-derived metabolites is under a complex set of controls: Firstly, their synthesis from AA is initiated by cytosolic phospholipase A2 (cPLA2). cPLA2 is calcium-dependent and is translocated to the nuclear membrane in several cells stimulated with IgE or the calcium ionophore A23187 [160]. Another critical aspect of LOXs control is its capability to bind proteins (e.g. 5-LOX binding the five-LOX-activating-protein FLAP). Phosphorylation via MAPK-activated protein kinase-2 is critical in controlling targeting and activation of these enzymes [161]. Therefore, the activation of LOX activity is generally rapid and transient, a property that is well adapted to their role in infection or injury, which needs urgent and time-limited generation of proinflammatory molecules. A constitutive LOX expression, on the other hand, seems to be involved in the pathogenesis of several diseases, including asthma [162], ulcerative colitis [163], psoriasis [164], artherosclerosis [165, 166], and cancer [166]. The constitutive LOX activity was shown to influence the proliferative rate of cells, their apoptosis resistance/sensitivity or their senescence response [167].

2.3.1 Lipoxygenase gene structure and chromosomal organisation

In humans, there exist six functional *LOX* genes and at least three pseudogenes. The LOX genes are similar in size (7-21 kb) and lie on the short arm of chromosome 17 within a span of 3-4 Mb, with the exception of the *5-lipoxygenase* gene which is much larger (82 kb) and is located on the human chromosome 10q11.2. [168]. The genes of the epidermis-type LOX including 15-LOX-2, 12R-LOX and eLOX-3 are clustered within a short region of 100 kb on 17p13.1 suggesting possible formation by gene duplication. The same genomic organisation of the epidermis-type LOX enzymes is also observed at the central region of mouse chromosome 11.

2.3.2 Lipoxygenase X-ray crystal structure

LOXs consist of the N-terminal eight stranded anti-parallel β-barrel domain and the larger catalytic domain formed predominantly by α-helices (multi-colours). The β-barrel (dark blue) is not essential for catalysis; it is structurally related to the C2-like domains in lipases, and is thought to be implicated in membrane interactions and acquisition of substrate [169, 170]. The catalytic C-terminal part contains the catalytic iron, and four highly conserved histidine residues and a C-terminal isoleucine which bind the central iron atom. The iron ligands are in an octahedral arrangement with one position “open” and with sufficient space to allow for the approach of substrate [171].

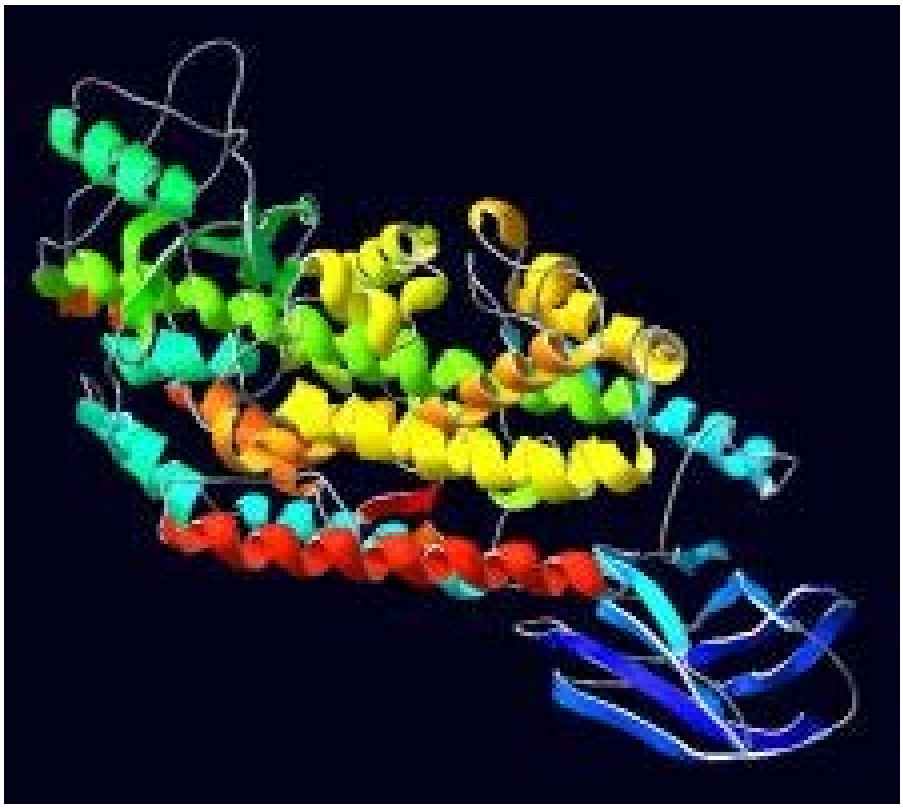


Fig 6: h-15-lox [98]

http://www.dkfz.de/spec/lox-db/gallery.

2.3.3 The substrate-product transformation

A fundamental principle of LOX catalysis is that the reaction is initiated by stereospecific removal of a hydrogen from the CH_2 group between two *cis* double bounds, and is completed by the insertion of O_2 into the position +2 or -2 on the opposite face of the removed hydrogen [172]. This antarafacial relationship between hydrogen abstraction and oxygenation is a universal facet of LOX catalysis, counting for the synthesis of *R* or *S* hydroperoxide products [173, 174]. Coffa *et al* described a model that can rationalize all known possibilities, where the more superficially located end of the reacting pentadiene is always associated with *R* chirality oxygenation, whereas *S* chirality oxygenation always occurs on the end of the pentadiene, lying deeper in the active site pocket.
Some individual LOX enzymes synthesize two regioisomeric hydroperoxide products. Prototypical examples of such dual positional specificity LOXs are the enzymes 15-LOX-1 and the "leukocyte-type" 12-LOX [175, 176].

2.3.4 15-lipoxygenase-1

15-LOXs can be sub-classified according to their enzymatic characteristics and specificty of tissue distribution into 15-LOX-1 and 15-LOX-2. 15-LOX-1 is expressed in reticulocytes, eosinophiles, macrophages, tracheobronchial epithelial cells, skin, and colon [177, 178] and converts linoleic acid preferentially to 13(S)-hydroxyoctadecadienoic acid (13-HODE) and arachidonic acid to 15(S)-hydroxyeicosatetraeonic acid (15-HETE), respectively [179]. On the other hand, 15-LOX-2 expression is detected in skin, prostate, lung, , and cornea but not in colon [180], and it preferentially converts arachidonic acid but also linoleic acid. The enzyme has been proposed to be involved in various physiological and pathophysiological activities such as inflammation, atherosclerosis, cell maturation, and tumorigenesis [181-183].
Cytokines stimulate the expression of 15-LOX-1 through activation of the transcription factor stat6. Additional mechanisms for transcriptional controlling of 15-LOX expression involve promoter methylation as the promoter region of the 15-LOX-1 gene is GC-rich [56, 182, 184].
15-LOX-1 furthermore can be regarded as a prooxidant enzyme generating oxidative stress for the following reasons: (i) unlike other groups of mammalian LOXs, this enzyme is capable of catalysing enzymatic lipid peroxidation in biomembranes and lipoproteins, (ii) it is a source of free radicals which can in turn initiate non-enzymatic lipid peroxdation and other harmful oxidative processes, (iii) LOX-catalysed reactions are favoured when the hydroxyperoxide tone of the cells is increased [185].

2.3.5 Physiological functions of lipoxygenases

Role of LOX in inflammation:

A) proinflammatory: 5-LOX products such as the leukotrienes (LTs) are components of a proinflammatory cascade. LTB4 is a potent chemoattractant for neutrophils, eosinophils, monocytes. Cysteinyl LTs are potent bronchoconstrictors
B) anti-inflammatory: Lipoxins and the recently discovered derivatives of omega-3 PUFAs, the resolvins and protectins are important mediators during the resolution phase of inflammation controlling duration and magnitude of the inflammatory reaction [186].

Role of LOX in apoptosis:

LOX products have been shown to induce programmed cell death (PCD) in human T-cells [187], neutrophils [188], PC12h cells [189], and Jurkat cells [190]. Several studies have shown the proapoptotic activity of 5-LOX [191, 192], of leukocyte type 12-LOX [193, 194], and of 15-LOX-1 [178, 195] and have identified molecular targets for LOX-mediated induction of apoptosis. However, also anti-apoptotic effects of LOXs have been reported, mainly based on the observation that LOX inhibitors, most often nordihydroguaiaretic acid (NDGA) and MK886, exhibited pro-apoptotic activity [46, 196-198]. Interestingly, in other cellular types NDGA or MK886 protected against apoptosis [191, 194, 195, 199-202], or induced PCD in cell types completely devoid of LOX activity [203-205].

2.3.6 Lipoxygenases and cancer

LOXs have been found to be expressed in some types of cancer cells/tissues, including colon, lung, pleural, breast, prostate, bone, brain, skin, and pancreas [206-210], and their aberrant activity has been associated with several aspects of tumorigenesis, including stimulation of cancer-cell proliferation, induction of genotoxicity, suppression of apoptosis, and increased tumor angiogenic and metastatic potentials. HETEs can activate certain isoforms of protein kinase C (PKC) directly or indirectly by incorporating into membrane phospholipids, which then generate HETE-containing diacylglycerol species to activate PKC [165]. They can also activate several MAPKs and thereby activate key transcription factors that stimulate DNA synthesis and proliferation of cancer cells (Ding 2003). Some LOXs metabolites (e.g. 15-HETE) have also been shown to directly stimulate the transcription of

target genes apparently via activation of nuclear receptors, such as peroxisome-proliferator-activated-receptors (PPARs) [211].

Human cancer tissues display an enhanced expression of 5-LOX and 12-LOX. At least for human prostate, lung and pancreatic cancer, very low or undetectable 5-LOX as well as 12-LOX expression is observed in normal cells or tissues, whereas high levels of 5-LOX as well as 12-LOX expression and activity are detected in corresponding transformed tissues [208, 212, 213]. 5-LOX inhibitors have chemopreventive effects in animal lung carcinogenesis [214]. Blockade of 5-LOX inhibits prostate cancer cell proliferation while 5-HETE stimulates prostate cancer cell growth [215]. Furthermore, the FLAP inhibitor, MK886 exerts similar growth inhibitory effects on cancer cell proliferation to 5-LOX enzyme inhibitors [216].

Similar effects were observed for 12-LOX inhibitors which induced apoptosis whereas 12-HETE prevented apoptosis in prostate cancer cells [217]. Intriguingly, the degree of 12-LOX expression in human prostate cancer correlated with the tumor grade and stage, i.e. 12-LOX expression level is higher in metastatic prostate cancers than in nonmetastatic ones [212]. The role of 15-LOX-1 expression and activity in cancer development, however, is unclear and controversial [166]. Several studies suggest that the 15-LOX-1 product, 13-HODE enhanced colonic tumorigenesis. Moreover, 13-HODE enhanced cell proliferation and potentiated the mitogenic response to EGF in different cell types [218, 219]. Yoshinaga et al reported that over-expression of 15-LOX-1 induced extracellular signal-related kinase 1/2 (ERK1/2) phosphorylation, decreased p21 (cip/WAF1) expression, and increased colon cancer cell growth [220]. Eling et al reported that the anti-proliferative effect of 15-LOX-1 in colorectal cancer cells was associated with phosphorylation of p53 [221]. In contrast, Lippmann et al reported that 13-HODE enhanced apoptosis and cell cycle arrest in colorectal cancer cells [222].

These data suggest that distinct LOXs, the expression of which is lost during progression of cancer, may exhibit antitumorigenic activities, while other isoforms that are preferentially expressed during carcinogenesis may exert protumorigenic effects.

2.4 Aim

Pancreatic cancer is the tenth leading cancer disease in men and the ninth leading in women. Because of late diagnosis it is the fourth leading cause of cancer death in male and the fifth leading cause of cancer death in women. Numerous clinical data on LOX expression in human tumors and in animal models indicate a antipodal role of LOX metabolism in carcinogenesis. In fact, different LOX isoforms exhibit either pro-tumorigenic or anti-tumorigenic activities and modulate the tumor response in a tissue-specific manner. The data suggest that the 5- and platelet-type 12-lipoxygenase exhibit pro-carcinogenic activities.
In contrast, 15-LOX-1 is down-regulated in human esophageal, gastric, colorectal and breast carcinomas. In the past, conflicting data on 15-LOX-1 expression in colorectal carcinomas were published since one group described elevated levels of 15-LOX-1 in human colon tumors. To date, nothing is known about the role of 15-LOX-1 in pancreatic cancer. Therefore, in the present study the expression of 15-LOX-1 in normal and pathologic pancreatic tissues and the effect of 15-LOX-1 expression on pancreatic cancer cell proliferation will be investigated, as well as cell cycle analysis and measurement of apoptosis will be performed. Further, the effect of both, substrates and products, on cell proliferation will be analyzed.
In addition, different HDAC inhibitors will be used to restore 15-LOX-1 expression in pancreatic cancer cell lines and cell proliferation will be determined.

B: Materials and methods

3. Materials

3.1 Equipment, apparatures

-80°C freezer	Heraeus AG, Zürich, Switzerland
-20°C freezer	Liebherr; Ochsenhausen
37°C, 5% CO_2 incubator	Sanyo Scientific, Bensenville, IL, USA
4°C fridge	Liebherr, Ochsenhausen
Analysis balance	Labtronics Inc., Gouda, Netherlands
Autoclave Vakulab HP	Medizin Mechanik GmbH München
Balance BL 6100	Zeiss, Goettingen
Bio-photometer	Eppendorf AG, Hamburg
Centrifuge Z33M	Hermle, Wiesloch
CO_2 incubator	Sanyo Scientific, Bensenville, IL, USA
Dynal sample mixer	Invitrogen, Karlsruhe
Guava PCA	Guava Technologies, Hayward, CA, USA
Heating water bath	Lauda, Groton, CT, USA
Ice machine	West End Lumber, Lorain, CL, USA
Kodak biomax x-ray cassette	Eastman Kodak Company, Rochester, USA
Magnet stirrer	Jahnke & Kunkel , Staufen i. B
Magnet particle concentrator (MPC)	Invitrogen, Karlsruhe, germany
Microplate Elisa reader	Thermo Electron GmbH, Karlsruhe
Microscope	Leica, Bensheim
Microwave	Quelle, Fürth
Nano-Drop	Kisker-Biotech, Steinfurt
NuPage western apparatus	Invitrogen, Karlsruhe
pH-analyser pH 330	Fischer BioBlock Scientific, Illirch Cedex
Power supply	Biotec Fischer, Reiskirchen
Shaker	Heidolph, Darmstadt
Shaker for Western Blot analysis	Neolab, Heidelberg
Sterilgard Hood	Kojair, Uilppula, Finland
Table centrifuge	Eppendorf, Hamburg
Ultrasound, sonoplus	Bandelin, Berlin
Vortex	Heidolph, Schwallbach

Water filter system	Millipore, Schwallbach
Water bath	Köttermann, Uetze/Hänigsen
x-ray developer	Protec, Oberstenfeld

3.2 Plastic- and glas ware

24-well plates	Greiner, bio-one, Frickenhausen
50 ml/ 15 ml Falcontubes	Greiner, bio-one, Frickenhausen
96-well plates	Greiner, bio-one, Frickenhausen
Bis-Tris gels (10%)	INVITROGEN, Karlsruhe
Blotting Paper	Schleicher & Schuell, Dassel
Cell culture flasks (T25, T75)	Greiner, bio-one, Frickenhausen
Cell scraper	Greiner, bio-one, Frickenhausen
Collagen I type 60mm dishes	Greiner, bio-one, Frickenhausen
Eppendorf® 1,5ml Tubes	Eppendorf AG, Hamburg
Films for western	Fujifilms, Germany
filter tips	CLP, San Diego, CA, USA
Multistepper	Eppendorf, Hamburg
Nitrocellulose membrane	BioRad, Hercules, CA, USA
Parafilm	Pechiney plastic packaging, Chicago, USA
Pasteur pipettes plastic	BD Biosciences, Heidelberg
PCR- tubes	Brand, Wertheim
Petridishes	BD Bioscience, Heidelberg
Pipetman® (1000 µl, 200 µl, 20 µl, 10 µl)	Gilson, Bad Camberg
Pipettus	Hirschmann, Eberstadt
Plastic cuvettes	Eppendorf, Hamburg
Plastic pipettes Cellstar® 10ml	Greiner, bio-one, Frickenhausen
Plastic pipettes Costar® (2 ml, 5 ml, 25 ml)	Corning Inc., Corning, NY, USA

3.3 Chemicals

Aceton, HPLC-grade	Fischer Scientific, Leicestershire,UK
Acetic acid, HPLC-grade	Fluka, Neu-Ulm
Agarose	Sigma-Aldrich, München
Aprotinin	Sigma-Aldrich, München
ε-Amino-n-Capronsäure	Sigma-Aldrich, München
β-mercaptoethanol	Sigma-Aldrich, München
Arachidonic acid	Cayman Chemicals, Michigan, USA
Boric acid	CALBIOCHEM (Merck KG), Darmstadt
Bovine serum albumin, BSA	Roth, Karlsruhe
Carbenicillin	Hybaid, Heidelberg
Chloroforme	J.T. Baker, Deventer, Netherlads
Dichlormethan, HPLC-grade	Roth, Karlsruhe
Dimethylsulfoxid	Sigma, Taufkirchen
DL-Dithiothreitol, DTT	Sigma-Aldrich, München
Dynabeads Pan Mouse IgG	Invitrogen, Karlsruhe
4-(2-hydroxyethyl)-1-piperazin-ethanesulfonic acid, HEPES	Sigma-Aldrich, München
Ethanol, HPLC-grade	Merck Biosciences, Schwalbach
Ethanol, EtOH (96%)	Roth, Karlsruhe
Ethidium bromide, EtBr	Merck, Darmstadt
Ethylenediaminetetraacetic acid, EDTA	Roth, Karlsruhe
Glycerin	Merck Biosciences, Schwalbach
Glycin	Roth, Karlsruhe
H_2O (MQ)	Millipore, Schwallbach
Hematoxylene	Merck, Darmstadt
15-S-HETE	Cayman Chemicals, Michigan, USA
13-S-HODE	Cayman Chemicals, Michigan, USA
Hydrochlorid acid, HCl	Merck, Darmstadt
Hydrogen peroxide, H_2O_2	Roth, Karlsruhe
Leupeptin	Sigma-Aldrich, München
Linoleic acid	Cayman Chemicals, Michigan, USA
Luria-Agar	Gibco, Karlsruhe
Luria-Broth base	Gibco, Karlsruhe
Magnesium chloride, $MgCl_2$	Merck, Darmstadt

Methanol, MeOH	Roth, Karlsruhe
Methanol, HPLC-grade	Roth, Karlsruhe
Milk powder	Fluka, Neu-Ulm
Phosphate buffered saline (PBS)	Biochrome AG, Berlin
Sodium hydroxide	Merck, Darmstadt
Tris	Roth, Karlsruhe
Tween-20	Merck Biosciences, Schwallbach

3.4 Kits

BCA protein assay kit	Pierce, Rockford, USA
cDNA Synthesis Kit	Roche, Mannheim
GUAVA Check kit	Guava Technologies, Hayward, CA, USA
GUAVA Nexin kit	Guava Technologies, Hayward, CA, USA
GUAVA ViaCount kit	Guava Technologies, Hayward, CA, USA
Lipofectamine2000	Invitrogen, Karlsruhe
RNeasy kit	Qiagen, Hilden
RQ1 RNase-Free DNase	Promega, Mannheim
WST-1 assay	Roche, Mannheim

3.5 Primary antibodies

15-LOX-1 anti serum	Roche (M.Mulkins), Bioscience, CA, USA
CD90 fibroblast	Invitrogen, Karlsruhe
KAI 1	Santa Cruz, CA, USA
S100A4	Dako, Hamburg
Phospho p42/44	Cell signaling, MA, USA
P42/44	Cell signaling, MA, USA
Phospho MEK1/2	Cell signaling, MA, USA
MEK1/2	Cell signaling, MA, USA

3.6 Secondary Antibodies

Goat-anti-rabbit	Santa Cruz, CA, USA
Donkey-anti-mouse	Santa Cruz, CA, USA
Donkey-anti-goat	Santa Cruz, CA, USA

3.7 Ladders

PageRuler™ #SM0671,	
Prestained protein ladder	Fermentas, St. Leon-Rot
1kB DNA ladder	Fermentas, St. Leon-Rot

Bacteria

One Shot™ TOP10 (E.coli)	Invitrogen, Karlsruhe

3.8 Reagents and buffers for western blot analysis

ECL reagent for western	Amersham Biosciences, Freiburg
LDS sample buffer	INVITROGEN, Karlsruhe
MES SDS Running buffer	INVITROGEN, Karlsruhe
Reducing agent	INVITROGEN, Karlsruhe
Stripping buffer	(see 2.2.6)
Transfer buffer	INVITROGEN, Karlsruhe
PBST	(see 2.2.6)
PBSM	(see 2.2.6)

3.9 PCR reagents

Buffer (10x) cont. $MgCl_2$	QBIOgene, Illkirch, France
dNTP's (10mM)	QBIOgene, Illkirch, France
H_2O	Millipore, Schwallbach
Primer (see table 3)	Invitrogen, Paisley, Great Britain
Taq-Polymerase	QBIOgene, Illkirch, France

3.10 Reagents for electrophoresis

Loading dye	Fermentas, St. Leon-Rot
TBE buffer	(see 2.2.4)

3.11 Cell culture

0.25% Trypsin-EDTA	INVITROGEN, Karlsruhe
DMEM (4500 mg/ml Glucose)	GIBCO, Karlsruhe
EMEM	
RPMI 1640 medium	GIBCO, Karlsruhe
Dulbecco's PBS$^{-/-}$	PROMOCELL, Heidelberg
FBS	PAN Biotech, Aidenbach
Penicillin-streptomycin solution	GIBCO, Karlsruhe
Sodium pyruvate solution (100 mM)	GIBCO, Karlsruhe

3.12 Cell lines

Tab 1: Cell lines used in this study described by source, histology and grade of the primary tumor.

Name	Source of tumor cells	Histology and grade of primary tumor
AsPC-1	Ascites	PDAC, G2
Capan I	liver metastasis	PDAC, G1
Capan II	primary tumor	PDAC, G1
MiaPaCa2	primary tumor	PDAC, G3
Panc-1	primary tumor	PDAC, G3
S2-O13	Liver metastasis	
SU8686	Liver metastasis	PDAC, G2/ G3
T3M4 (Panc-89)	lymph node metastasis	PDAC, G2
HEK 293	Human embryonic kidney	
normal ductal cells	pancreas donor	

3.13 Software

Microsoft ® Excel 2003	Microsoft Corp, Albuquerque, NM, USA
Microsoft ® PowerPoint 2003	Microsoft Corp, Albuquerque, NM, USA
Microsoft ® Word 2003	Microsoft Corp, Albuquerque, NM, USA
NanoDrop 3.1.0	Coleman Technologies Inc., MO, USA
EndNote X.0.2	Thomson ResearchSoft, CA, USA
Millenium HPLC software	Waters, MA, USA
World Wide Web	www.google.com
	www.ncbi.nml.nhi.gov
	www.pathology.jhu.edu

4. Methods

4.1 DNA preparation and isolation

4.1.1 Transformation of competent cells

50 µl competent cells (One Shot™ TOP10) were thaw rapidly, mixed with 1 µl DNA (50 ng/µl) and placed on ice for 10 min. Heat shock was carried out by placing tubes into a 42˚C water bath for 45 sec and then incubated on ice for 2 min. 900 µl SOC medium were added to each tube and placed in a shaker at 250 rpm for 1 hour at 37˚C. Finally the mix was plated on carbenicillin containing LB plates. Colonies were grown for 12-16 hours at 37°C.

4.1.2 DNA isolation

A single colony from a freshly streaked selective plate was picked and a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic was inoculated and incubated for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm). The starter culture was diluted 1/500 to 1/1000 into selective LB medium. The bacterial cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml Buffer P1. 10 ml Buffer P2 were added, and mixed thoroughly by vigorously inverting and incubated at room temperature for 5 min. 10 ml chilled Buffer P3 were added to the lysate, and mixed immediately and
thoroughly by vigorously inverting 4–6 times. The lysate was poured into the barrel of the QIAfilter Cartridge and incubate at room temperature for 10 min. The QIAGEN-tip 500 were equilibrated by applying 10 ml Buffer QBT. The cell lysate was filtered into
the previously equilibrated QIAGEN-tip. The cleared lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 x 30 ml Buffer QC. DNA was eluted 15 ml Buffer QF and precipitated by adding 10.5 ml (0.7 volumes) room-temperature isopropanol. Finally mixed and centrifuged immediately at ≥15,000 x *g* for 30 min at 4°C, the supernatant was decanted carefully. The DNA pellet was washed with 5 ml of room-temperature 70% ethanol, and centrifuged at ≥15,000 x *g* for 10 min. The supernatant was decanted carefully. The pellet was air-dried for 5–10 min, and re-dissolved in a suitable volume of EB-buffer.

4.1.3 Restriction analysis

Isolated DNA was analyzed by restriction enzymes. For the 15-LOX-1 plasmid (2042) Bg1II and EcoRI were used. The final mix contained 1 µl buffer H, 0,5 µl of each enzyme, 1 µl DNA and 7 µl H_2O. The mix was incubated for 1 hour at 37°C and then analyzed on an agarose gel.

4.2 RNA isolation and cDNA preparation

4.2.1 RNA isolation

Total RNA from all cultured cell lines was isolated using the RNeasy Kit. Cells were seeded in 10 cm dishes and grown to 90% confluence. Before starting, 10 µl of β-mercaptoethanol was added to 1 ml RLT buffer (RLT*). Then medium was aspirated and 350 µl RLT* buffer were added to each 10 cm dish. Cells were scraped and pipetted into a 1.5 ml Eppendorf tube. Afterwards the lysates were passed at least 5 times trough a needle (0.9 mm diameter) fitted to an RNase-free syringe. 350 µl EtOH (70%) were added, mixed well and the lysate pipetted onto RNeasy columns placed in a 2 ml collection tube. After centrifugation at room temperature (RT), for 15 s at 10,000 rpm, the flow through was discarded and 700 µl of RW1 buffer were added and the columns centrifuged again. The column was now placed in a new collection tube and centrifuged again. After this step 500 µl RPE buffer were added and the columns centrifuged for 15 s at 10,000 rpm. Then again 500 µl RPE buffer were added and the columns were centrifuged for 2 min at 10,000 rpm. The flow-through was discarded and the columns centrifuged for 1 min at 13,200 rpm. Finally the columns were placed in 1.5 ml tubes and 40 µl of RNase-free water were added directly onto the membrane in the column and centrifuged for 1 min at 10,000 rpm. This step was repeated using the flow through.
Concentration of cDNA in aqueous solutions was measured using the NanoDrop spectrophotometer. 1 µl of each sample was pipetted onto the reading area. Absorption was measured at 260 nm against EB-buffer which served as reference. Simultaneously absorption at 280 nm was measured and the ratio A_{260}/ A_{280} was calculated. The ratio gives the purity of each sample. Normal values for DNA are between 1.8 and 2. For DNA free RNA the ratio should be between 1.9 and 2.1.

4.2.2 Reverse transcriptase-PCR

To ensure purity, isolated RNA was treated with DNase I to get rid of contaminating genomic DNA. Final reaction volume was 10 µl, containing 3 µg RNA, 3 µl DNase I, 1 µl reaction buffer and MQ-H_2O. The mix was incubated at 37°C for 30 minutes, afterwards 1 µl of stop solution was added and incubated at 65°C for 10 min.

The isolated RNA was reverse transcribed into cDNA. The total reaction volume was 21.8 µl: 10 µl DNase I treated total RNA, 2 µl reaction buffer, 2 µl $MgCl_2$, 2 µl dNTP-Mix, 2 µl Oligo-$p(dN)_6$ Primer, 1 µl RNase Inhibitor, 0.8 µl AMV *reverse transcriptase* and 2 µl RNase/DNase-free H_2O. The thermocycler protocol for the RT-PCR is: one cycle at 25°C for 10 min, 42°C for 60 min, one cycle at 99°C for 5 min and one last cycle at 4°C for 5 min (see fig. 13).

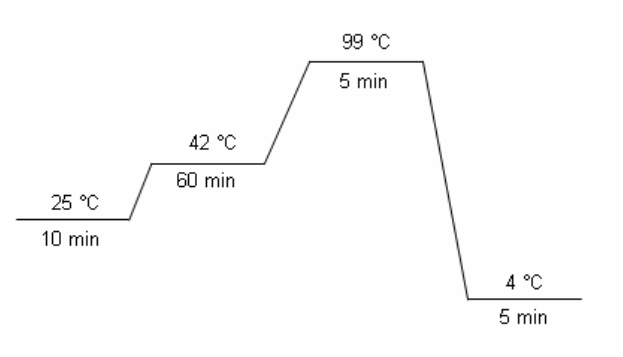


Fig 6: Reaction profile of a reverse transcription polymerase chain reaction.

4.2.3 Electrophoresis for 15-LOX-1 and actin

Polymerase chain reaction (PCR) is used to amplify a region of cDNA to identify the mRNA expression level of a gene.

For amplifying mRNA of interest, first primers have to be constructed which are specific. After this the melting temperature (T_m) is calculated with following formula:

$$T_m = 2 \cdot (A + T) + 4 \cdot (G + C)$$

It is proposed to use a T_m about 3 - 5°C below the calculated temperature.

Of the obtained cDNA 1 µl was used for 15-LOX-1 PCR using specific primers (see Tab. 4). These primers amplify a region which is specific for the protein that is encoded by the corresponding gene. 0.1 µl Taq-polymerase, 2.5 µl dNTP's (2.5 mM), 2.5 µl $MgCl_2$ (50 mM), 2.5 µl reaction buffer(10x), 1 µl (10 pM) primers and x µl MQ-H_2O and 1 µl cDNA were used up to 25 µl.

The PCR profile was 1 cycle at 95°C for 5 min; 94°C for 1.5 min; 56°C, 1.5 min and 72°C, 2.5 min for 36 cycles and one cycle at 72°C for 10 min the final step is the incubation at 4°C (see fig. 6).

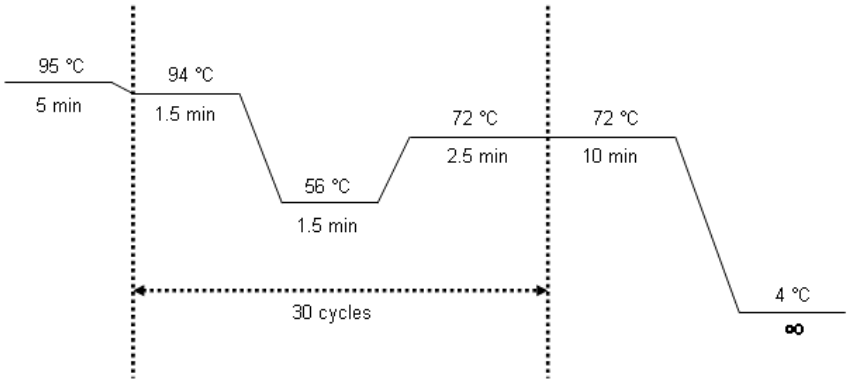


Fig 7: Reaction profile of polymerase chain reaction to detect *15-LOX-1* mRNA in pancreatic cancer cell lines

The final PCR product was separated on 1% agarose gel containing 0.00001% ethidium bromide (10 mg/ml). For this 1.0 g of agarose were solved in 100 ml of 1x TBE buffer (10x TBE: 2.5 M Tris, 2.22 M boric acid, 0.32 g EDTA, ad MQ-H_2O to 1000 ml), boiled in a microwave and after slight cooling EtBr was added. The mixture was then allowed to cool down in a gel mould with combs to produce the pockets. After polymerisation combs were removed and the gel was set into an electrophoresis chamber and submerged with 1x TBE buffer. 1 µl loading dye was added to the samples and 10 µl of each sample and 8 µl of the 100bp DNA ladder were pipetted into each pocket. The electrophoresis was carried out at a voltage of 100 V for 1 hour. The PCR product was visualized using UV light and photographed.

Tab 2 : Specific primers

	5' Primer	3' Primer	Product size
15-LOX-1 (human)	5'- CATCTATCGGTATGTGGA -3'	3- GAAGTTGGGCAGTGTC -5'	340 bp
β-actin (human)	5'- CTTCCTGGGCATGGAGTCCT -3'	3'- CCGCCGATCCACACAGAGTA -5'	182 bp

4.3 Cell culture

4.3.1 Cell lines

Pancreatic tumor cell lines including AsPC-1, Capan I, Capan II, MiaPaCa-2, Panc-1, SU8686 and T3M4 were established from patients with pancreatic adenocarcinoma and cultured in RPMI 1640 supplemented with 10% FBS and 1% Penicillin-streptomycin. S2-O13 cell line was cultured in DMEM containing 10% FBS and 1% Pen/Strep. HEK293 cells were maintained in EMEM supplemented with 10% FBS, 1% Penicillin-streptomycin, and 1% L-glutamine (200 mM). All cell lines except S2-O13 were purchased from the American Type Culture Collection (ATCC, Rockville, MA, USA). S2-O13 cells were provided by Dr. M.A.Hollingsworth (Eppley Cancer Institute, Omaha, NE, USA).
Cells were grown as monolayers in a humidified atmosphere of 5% CO_2 at 37°C. Adherent cells were detached by trypsinisation with 1 ml trypsin-EDTA solution for a T75 flask after washing with 5 ml PBS. Cells were cultured in a volume of 10 ml in T75 flasks. For petri-dishes (d = 10 cm) 3 ml PBS and 0,5 ml trypsin-EDTA solution were used and the final volume of medium was 10 ml.

4.3.2 Ductal cell isolation

After obtaining the ethical approval, normal ducts isolated from patients were cut into 1-2 mm pieces and plated onto collagen I coated 60 mm dishes. After 5 to 6 days the tissue pieces were removed and medium was replaced. To remove interfering fibroblasts dynabeads (DB) were used. Before starting the beads were coupled to the fibroblast antibody CD92. Dynabeads (1 ml) were resuspended in 1 ml PBS and then placed in the magnetic particle concentrator (MPC) for 2 min. This step was repeated twice and the beads finally were resuspended in 1 ml PBS. Per 25 µl DB 1 µg primary antibody were used. After adding the antibody to the beads the mix was incubated for 1 hour at 18-25°C gently tilting and rotating. After applying to the MPC the supernatant was discarded. The beads were resuspended in 2 ml PBS and again placed on the MPC. Finally, the beads were mixed into 1 ml PBS. At least 4 DB per targeted cell and > 1 x 10^7 (25 µl) beads were used. After trypsinization cells were incubated with the coated beads for 30 min at 4°C gently tilting and rotating. The tube was afterwards placed in the MPC and left there for 2-3 min. Finally, the purified ductal cells, comprised in the supernatant, were placed onto collagen I coated 60 mm dishes.

4.3.3 Stable transfection

The plasmids of pcDNA3.1 and pcDNA3.1-15-LOX-1 containing the coding region of human 15-LOX-1 were transfected into MiaPaCa2 and S2-O13 cells according to the manufacturer's protocol using Lipofectamine2000 (Invitrogen, Karlsruhe, Germany).
One day before transfection 2 x 10^6 cells were seeded in 10 ml of growth medium into 100 mm dishes. At 90% confluence medium was replaced by 15 ml medium without serum. 24 µg DNA were diluted in 1.5 ml serum-free medium and mixed. 60 µl Lipofectamine2000 were mixed with 1.5 ml serum-free medium and incubated for 5 min at RT. After incubation Lipofectamine2000 mix was added to the DNA mix and incubated for 20 min at RT. The 3 ml of complexes were added to the plate.
After 24 hours fresh medium was added to the cells containing the selection reagent G418 (100 µg/ml). Selection was continued for one week, with the medium being refreshed every alternate day. After one week cells were maintained at 20 µg/ml G418. Clones were isolated and 15-LOX-1 over-expression was confirmed by western blot analysis and activity assay.

4.3.4 Transient transfection

Transient transfection of HEK293 cells was carried out to generate positive controls for western blot analysis. The plasmid of pcDNA3.1-15-LOX-1 containing the coding region of human 15-LOX-1 was transfected into HEK293 cells according to the manufacturer's protocol using Lipofectamine2000 (Invitrogen, Karlsruhe, Germany).
One day before transfection 2 x 10^6 cells were seeded in 10 ml of growth medium into 100mm dishes. At 90% confluence medium was replaced by 15 ml medium without serum. 24 µg DNA were diluted in 1.5 ml serum-free medium and mixed. 60 µl Lipofectamine2000 were mixed with 1.5 ml serum-free medium and incubated for 5 min at RT. After incubation Lipofectamine2000 mix was added to the DNA mix and incubated for 20 min at RT. The 3 ml of complexes were added to the plate. After 24 hours the cells were washed with PBS and removed from the plate using a cell scraper and centrifuged for 10 min at 1200rpm at RT. The pellet was lysed in 500 µl lysis buffer (50 mM Tris pH 7.4; 1 mM EDTA; 1 mM EGTA; 1 mM leupeptin) and sonicated 3 times 10 sec, using ultrasound. Protein concentration was measured using the BCA assay.
MiaPaCa2 was transiently transfected with the pcDNA3.1-15-LOX-1 plasmid following the protocol described above. Proteins were isolated 1h, 6h, 24h, 48h, 72h, and 96h after transfection. As negative control non-transfected cells were used for western blot analysis.

4.5 Protein expression analysis

4.5.1 Measurement of protein concentrations using BCA assay

The protein concentration was measured using the BCA assay. Reagents A and B were diluted 50:1 and 200 µl of this were pipetted into each well of a 96-well plate. Four Standards were used (2 µg/µl, 1 µg/µl, 0.5 µg/µl and 0.25 µg/µl). 5 µl of each sample and standard were pipetted in duplicates. The plate was incubated at 37°C for 30 min and protein concentration was measured at 570 nm using a photometer and calculated using linear equation and a standard.

In cases of low protein concentrations EtOH-precipitation or SpeedVac system were used to concentrate the proteins. For EtOH-precipitation 100 µl sample were diluted in 1 ml EtOH, incubated for 5 min in $N_{2\ liq}$, centrifuged at 13,000 rpm for 10 min and then dissolved in 25 µl MQ-H_2O. After this, concentration was calculated according to the used µl of sample and MQ-H_2O. Most often SpeedVac system was used to vaporize the water from the sample and get higher concentrated proteins.

4.5.2 Western blot analysis for 15-lipoxygenase-1 (15-LOX-1) and MAPK

Western blot analysis was carried out to determine the protein expression of 15-LOX-1 in pancreatic tumor cell lines. Proteins were prepared as mention above.

The NuPAGE® System for Western Blot analysis is based upon a Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel, with a separating gel that operates at pH 7.0. As NuPAGE® Novex Bis-Tris gels do not contain SDS, they are formulated for denaturing gel electrophoresis applications only. For this, samples were mixed with LDS sample buffer (lithium dodecyl sulfate) and reducing agent and denatured at 70°C for 10 min to unfold the proteins. DTT (dithiothreitol) which is comprised in the reducing agent prevents the reformation of disulfide bonds. LDS besieges the protein with a negative charge. In an electric field the proteins are moving towards the anode (+) and are separated by their molecular weight (mw).

30 µg protein lysate of each pancreatic cancer cell line was mixed with 1 µl Reducing agent, 2.5 µl LDS Sample Buffer and MQ-H_2O up to 10 µl. 5 µl prestained protein ladder and 10 µl of each sample were separated by SDS-PAGE. MES buffer was used and an electric current of 200 V was applied for 35 min. Proteins were transferred to nitrocellulose membranes by electroblotting using a Nu-PAGE transfer blotting apparatus at 30 V for 2 hours.

Nitrocellulose membranes were subsequently blocked in 1x PBS containing 5% non-fat milk and 0.05% Tween 20 (PBSTM) overnight at 4°C and then treated with 15-LOX-1 antibody or

the different MAPK antibodies (see table) diluted in PBSTM for 1 hour. After six times 10 min washing in PBSTM, the membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (1:2000) for 1 hour at RT. After 3 x 10 min washing with PBST, 15-LOX-1 expression was detected by ECL (enhanced chemiluminescence) treatment (1 min) and light emission was detected on a x-ray film after 30 min of exposure.
To confirm equal loading the membrane was stripped in 0.2 M Glycin containing 0.05% Tween20 for 20 min at 80°C. After 1 hour blocking, membrane was incubated with the first antibody β-actin (1:5000) for 1 hour at RT. After 3 x 10 min washing and 1 hour blocking the membrane was incubated with HRP secondary anti-goat antibody for 1 hour. After 3 x 10 min washing detection was carried out as described above.

Tab 3: List of used antibodies

1. Antibody	Dilution	2.Antibody	Dilution
15-LOX-1	1:10000	Anti-rabbit	1:2000
Phospho p42/44	1:1000	Anti-rabbit	1:2000
P42/44	1:1000	Anti-rabbit	1:2000
Phospho MEK1/2	1:1000	Anti-rabbit	1:2000
MEK1/2	1:1000	Anti-rabbit	1:2000
Actin	1:2000	Anti-goat	1:2000

4.6 LOX activity assays

LOX activity was quantified by measuring HETE formation in cell-free protein extracts. 250 µg cytosolic protein homogenates were incubated with 100 µM arachidonic acid for 15 min at 37°C. The incubations were terminated by addition of 40 µl of 1 M sodium formate buffer (pH 3.1) and products were extracted with a modification of the Bligh and Dyer procedure as described (36). Products were analyzed by Reverse-phase-HPLC on a 5 µm YMC-Pack ODS-AM column (25 x 0.46 cm, YMC Europe, Schermbeck, Germany) with a 1 cm guard column using the solvent system of methanol/water/acetic acid (82/18/0.01 by volume) and a flow rate of 0.5 ml/min. Elution was monitored at 236 nm with a Bio-Tek Kontron 540 diode array detector. The products were identified by comparing retention times and peak areas with those of authentic external 15-S-HETE standard (Reatec, Weiterstadt, Germany). The retention time for 15-S-HETE was 28.0 min.

4.7 Cell proliferation analysis

4.7.1 WST-1 assay

Cell proliferation was measured using the WST-1 assay. This is a tetrazolium salt which is cleaved by mitochondrial succinate dehydrogenase to build formazan. The overall activity of mitochondrial dehydrogenases is associated with cell viability. The more products are built the more proliferation takes place. The advantage of WST-1 in contrast to MTT, XTT or MTS is that the cleavage products are water soluble and can easily be measured. 1000 cells of each cell line were plated into six wells of four 96-well plates using 100 µl/well cell culture medium. For each treatment triplicates were carried out. After 24 hours treatment was started and proliferation measured, comparing treated vs solvent treated cells, 24 hours after induction up to 120. 72 hours after treatment medium was changed.10 µl of the ready-to-use WST-1 reagent were added every 24 hours up to 120 hours to the cells and incubated for 1 hour at 37°C and 5% CO_2 according to the manufacturer's protocol. Absorbance was measured in a microplate reader at a wavelength of 450 nm.

4.7.2 GUAVA ViaCount

The GUAVA® PCA™ is a desktop cell analysis system with a wide range of possible cellular assays like cell counting, viability, TUNEL assay, Multicaspase assay, Nexin assay, cell cycle analysis etc. GUAVA ViaCount analyzes the fluorescence of cells stained with the ViaCount reagent and quantitates the number of viable and non-viable cells in each sample. The ViaCount reagent contains DNA-binding dyes and stains viable and non-viable cells in a different way corresponding to their permeability to this reagent. Stained nucleated events are counted and the forward scatter properties are used to distinguish free nuclei and cellular debris from cells to determine an accurate cell count.
Before starting experiments GUAVA Check was carried out with supplemented beads as described in the manufacturer's protocol. At starting point 3 x 10^4 cells/ml for MiaPaCa2 and S2-O13 were seeded in triplicates into 6-well plates. After 24 hours cells were treated. Duplicates of each well were counted every 24 hours up to 120 hours using the GUAVA cell counter. 72 hours after treatment medium was changed. As negative controls the appropriate solvents served.

Proliferation assay setups:

- Comparison of 15-LOX-1 vs moc transfected cells:

50000 cells each were seeded into 60mm dishes with a final volume of 2ml. For each counting triplicates were performed. Proliferation was determined 24 hours up to 120 hours after seeding without any further treatment.

- Exogenous treatment with 15-LOX-1 substrates and products:

15-LOX-1 and mock cells were seeded in serum-containing medium at a concentration of 5 x10^4 cells into three 60mm dishes with a final volume of 2 ml. After 24 hours treatment was started in serum-free medium.

- Treatment with HDAC inhibitors

15-LOX-1 and mock cells were seeded in serum-containing medium at a concentration of 5 x10^4 cells into three 60mm dishes with a final volume of 2 ml. After 24 hours treatment was started in serum-containing medium.

- Treatment with caffeic acid

Normal ductal cells were seeded in serum-containing medium at a concentration of 1000 cells/ well into a 96 well plate. After 24 hours treatment was started in serum-containing medium.

4.8 Apoptosis measurement

4.8.1 Nexin Assay (Apoptosis Assay)

In this assay cells can be differentiated in viable, early and late apoptotic cells. Also cell debris can be detected. The principle of this test follows the externalization of phosphatidylserine (PS) during apoptosis of cells. PS is normally localized on the inner side of the cell membrane and moves to the outer surface when cells undergo apoptosis. In late stages of apoptosis cell membranes loose their integrity and begin to brake down. Annexin V is a Ca^{2+} dependent phospholipid binding protein which binds PS with a high affinity when it is located on the outer surface of the cell membrane. Nexin 7-AAD is a viability stain and used as an indicator of membrane structural integrity in this assay. 7-AAD binds to nuclear material which is released when the membrane breaks down. Cells were washed, trypsinized and centrifuged at 4°C, 350 x g for 10 min. After decanting the supernatants cells were resuspended in 1 ml ice cold 1x Nexin buffer, centrifuged again and resuspended in 1 ml 1x Nexin buffer. 5 µl Annexin V-PE and 5 µl Nexin 7-AAD were added to 40 µl of each cell suspension and incubated on ice for 20’ protected from light. Then 450 µl 1x Nexin buffer were added and the samples were measured using the GUAVA PCA.

4.9 Statistical analysis

Statistical analysis to determine significance of all experiments was carried out using SigmaPlot and the unpaired t-test. Counting of statistical significance was always performed against the values of the corresponding negative control. The p-value is a probability with a value ranging from zero to one. In this work all p-values ≤ 0,05 were determined significant.

5. 15-LOX-1 expression in pancreatic cancer cells

5.1 mRNA expression

Preliminary data showing by IHC that 15-LOX-1 expression may be lost during pancreatic carcinogenesis. Western blotting revealed no or very weak expression of 15-LOX-1 in all pancreatic cancer cell lines tested compared to mononuclear cells (see discussion).
To confirm these data, 15-LOX-1 mRNA expression was analyzed by RT-PCR in various human pancreatic cancer cell lines. As shown in Fig. 8a, 15-LOX-1 mRNA expression was below the level of detection in all cancer lines tested including MiaPaCa2, Panc-1, S2-O13, AsPC-1, Capan I, and Capan II. HEK293 cells transiently transfected with a 15-LOX-1 expression plasmid were used as a positive control. As negative controls RNA from HEK293 cells and water were used. The primers for 15-LOX-1 amplify a product of 340bp (Fig. 7a). Quality of the used cDNA was confirmed by actin PCR, amplifying a product of 234 bp (Fig. 7b).

Fig. 7: 15-LOX-1 expression in pancreatic cancer cell lines tested on mRNA level

Total RNA was isolated from various human pancreatic cancer cell lines and from HEK293 cells transiently transfected with a 15-LOX-1 expressing plasmid. RNA was reverse-transcribed to cDNA and PCR was run with specific primers for 15-LOX-1 **(a)** and human β-actin **(b)**, as described in material and methods. Lane 1: positive control, lane 2: HEK293, lane 3: non-RT control, lane 4: MiaPaCa2, lane 5: Panc-1, lane 6: S2-O13, lane 7: AsPC-1, lane 8: Capan I, lane 9: Capan II, lane 10: H_2O

5.2 Protein expression

As no 15-LOX-1 expression could be detected on mRNA level, expression on protein level was analyzed for verification of the obtained results. Before screening pancreatic cancer cell lines and normal duct cells for 15-LOX-1 expression, anti-sera directed against individual LOX isoforms were analyzed with regard to their cross-reactivities with other members of the LOX family. The specificities of the used anti-sera could be ascertained clearly (Fig. 8). With one exception, the anti-sera exhibited mono-specificities for the respective LOX isoforms as shown for 15-LOX-1, eLOX3, 5-LOX, 15-LOX-2 and 12R-LOX. Only the antiserum directed against p12-LOX showed cross reactivity with 15-LOX-2.

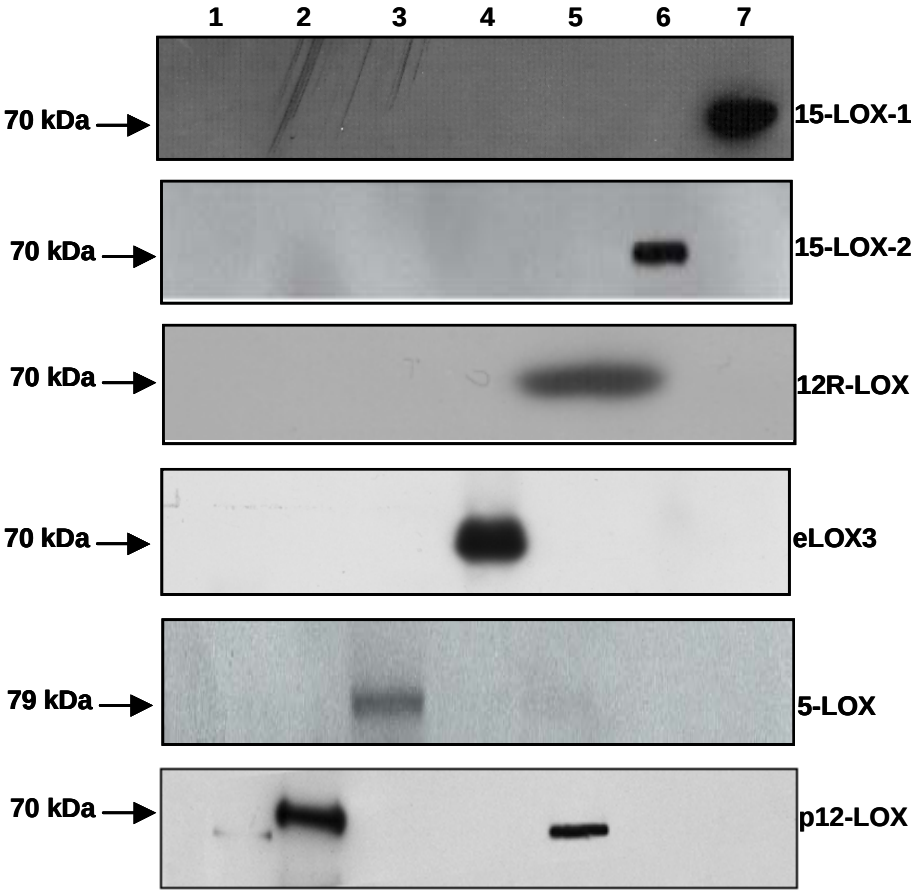


Fig. 8: Cross reactivity screening of the different LOX antibodies

Cross reactivity of the LOX antibodies was checked using western blot analysis. Cell homogenate protein (30 µg) from sonicated HEK293 cells transiently transfected with different LOX constructs were separated on a 10% SDS-PAGE, blotted onto nitrocellulose membrane and incubated with antibodies against the different LOX isoforms as indicated.

Lane 1: negative control, lane 2: p12-LOX, lane 3: 5-LOX, lane 4: eLOX3, lane 5: 12R-LOX, lane 6: 15-LOX-2, lane 7: 15-LOX-1.

Normal duct biopsies were taken from different patients who underwent pancreatic surgery. The ducts were placed onto collagen type I coated dishes and cultured for two weeks at 37°C and 5% CO_2. In Figure 9, epithelial cells grown out of the biopsies are shown. These epithelial cells were isolated and protein was extracted for western blot analysis.

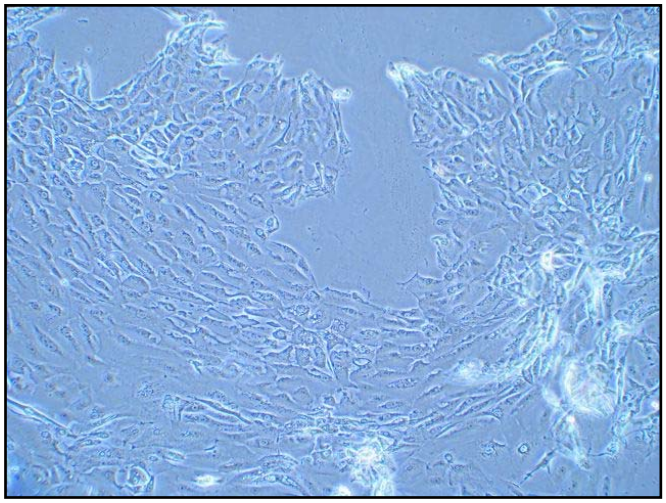


Fig. 9: Duct cells

After 2 weeks of cultivation the out-grown cells were analyzed using a light microscope to verify fibroblast free cultures. No contamination was detectable in the selected sample.

The isolated normal duct cells were first screened for the duct marker cytokeratin 19 (CK19) to verify the ductal origin. As positive controls two pancreatic ductal adenocarcinoma cell lines were used. As shown in Figure 10a the duct cells expressed CK19 in accordance with their ductal origin. Furthermore, 15-LOX-1 expression was detected in these freshly isolated normal duct cells (Fig. 10b).

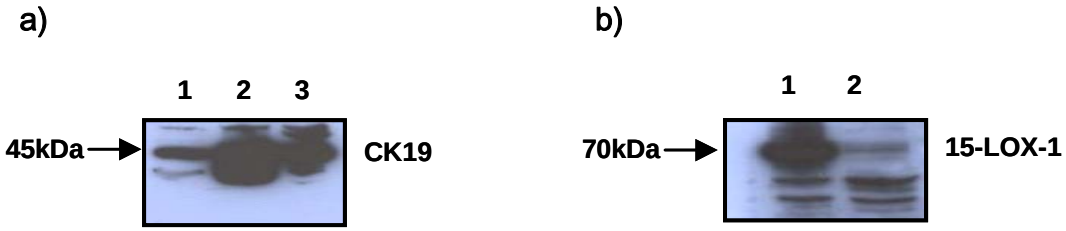


Fig. 10: Western blot detection of 15-LOX-1 in normal duct cells

30 µg total protein lysates from cells were separated on 10% SDS-PAGE and immunoblotting was performed using specific antibodies against **(a)** CK19 (1:1000), lane 1: MiaPaCa2, lane 2: S2-O13, lane 3: normal duct cells and **(b)** 15-LOX-1 (1:10000), lane 1: positive control, lane 2: normal duct cells.

After detection of 15-LOX-1 expression in normal duct cells, expression in pancreatic cancer cell lines was analyzed. On the protein level no 15-LOX-1 expression was detectable in all tested cell lines (Fig. 11). Thus, the lack of 15-LOX-1 expression on mRNA and protein level in all tested pancreatic cancer cell lines indicates that the expression of this LOX isoform is lost during pancreatic carcinogenesis. The normal bovine duct cell line AMA did not show any 15-LOX-1 expression, which might be due to non-reactivity with the antiserum.

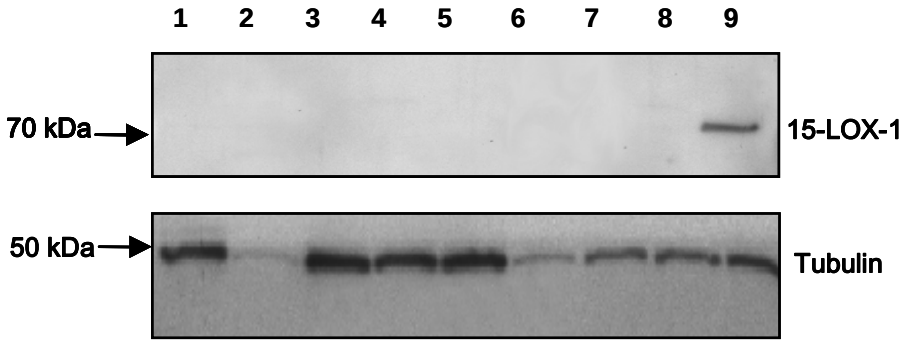


Fig. 11: 15-LOX-1 expression in pancreatic cancer cells

30 µg total protein lysates from cells were separated on 10% SDS-PAGE and immunoblotting was performed using specific antibodies against 15-LOX-1 and γ-tubulin. Equal loading was determined by γ-tubulin expression. Lane 1: negative control, lane 2: Capan I, lane 3: Capan II, lane 4: AsPC-1, lane 5: Panc-1, lane 6: AMA, lane 7: S2-O13, lane 8: T3M4, lane 9: positive control.

5.3 Caffeic acid increases cell proliferation in normal duct cells

Caffeic acid (CA) has been reported to be a selective inhibitor for 15-LOX-1 (see introduction). Treatment of colon cancer cells with doses of 2.2 µM and 4.4 µM caffeic acid that inhibited 15-LOX-1 activity reversed the 15-LOX-1 induced growth inhibitory effect significantly. Therefore, it was checked if caffeic acid increased cell proliferation in primary duct epithelial cells that express 15-LOX-1 (see Fig. 10) at similar dose ranges. CA was indeed able to increase cell proliferation in a dose-dependent manner about 70% after 48 hours ($p<0.001$) and about 80% after 72 hours ($p<0.001$) compared to untreated cells at a concentration of 10 µM. 4.4 µM caffeic acid showed a significant increase comparable to that of 10µM only after 72 hours ($p<0.001$), while 2,2 µM had no effect at all (Fig. 12).

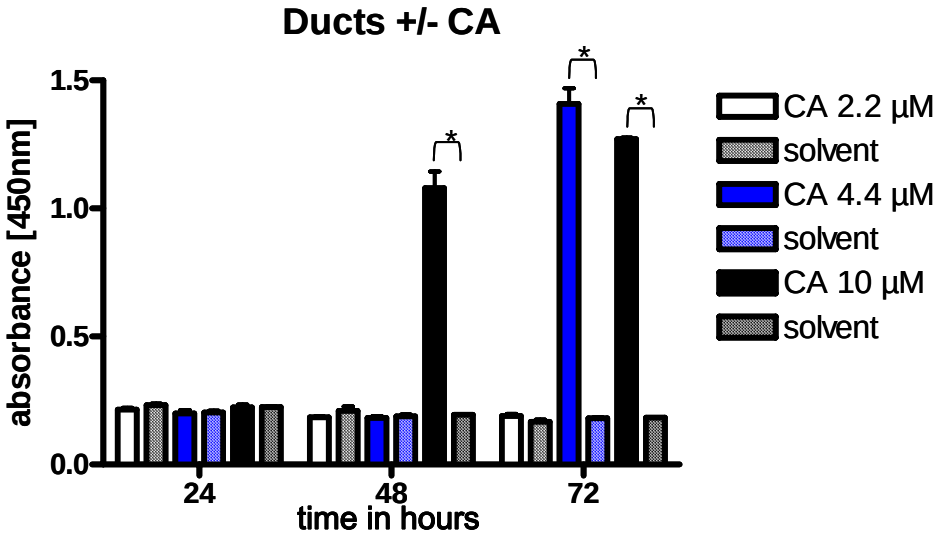


Fig. 12: CA treatment leads to increased cell proliferation

WST-1 assay: 1000 cells/ 100 µl of normal duct cells were seeded into 96 well plates. After 24 hours the medium was replaced by medium supplemented with 2. 2µM, 4.4 µM, or 10 µM CA or the appropriate solvent. At the mentioned time points 10µl of the ready-to-use WST-1 reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2 proliferation was measured at 450 nm using an ELISA reader.

6. Establishment of 15-LOX-1 over-expressing cells

To investigate the function of 15-LOX-1 in human pancreatic cancer cells, 15-LOX-1 over-expressing cells were established. MiaPaCa2, S2-O13 and Panc-1 were transfected with 15-LOX-1 expressing constructs and all clones stably expressing 15-LOX-1 were selected. Western blot analysis demonstrated high level of 15-LOX-1 expression in MiaPaCa2 clone 13, S2-O13 clone 8 (Fig. 13 lane 3 and 5) compared to their Mock transfected clones. No over-expressing clone could be derived from Panc-1 cells (Fig. 13 lane 7).

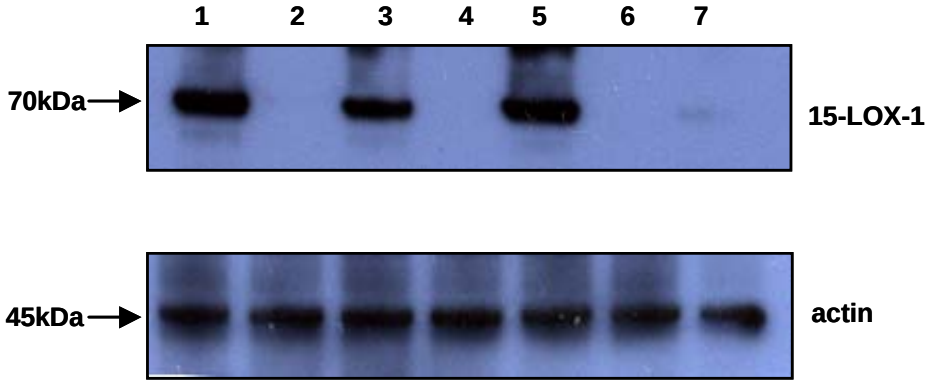


Fig. 13: 15-LOX-1 expression in 15-LOX-1 transfected cells compared to their Mock cells

30 µg total protein lysates from cells were separated on 10% SDS-PAGE and immunoblotting was performed using specific antibodies against 15-LOX-1 and β-actin. **a)** Clear expression was detected in MiaPaCa2-15-LOX-1 (cl 13) and S2-O13-15-LOX-1 (cl 8). **b)** Equal loading was determined by actin expression. Lane 1: positive control, lane 2: MiaPaCa2, lane 3: MiaPaCa-15-LOX-1 (cl 13), lane 4: S2-O13, lane 5: S2-O13-15-LOX-1 (cl 8), lane 6: Panc-1, lane 7: Panc-1-15-LOX-1 (cl 1).

6.1 15-LOX-1 enzyme activity in stably transfected MiaPaCa2 and S2-O13 cells

To assure the activity of the transfected 15-LOX-1 cDNA the enzymatic activity of 15-LOX-1 was determination by 15-S-HETE formation in protein lysates of MiaPaCa2 and S2-O13 cells upon incubation with arachidonic acid (AA). In both 15-LOX-1 expressing cell lines the production of 15-S-HETE was detectable (Fig. 14a&b), while no 15-S-HETE production was observed in Mock transfected clones (Fig. 14c&d).

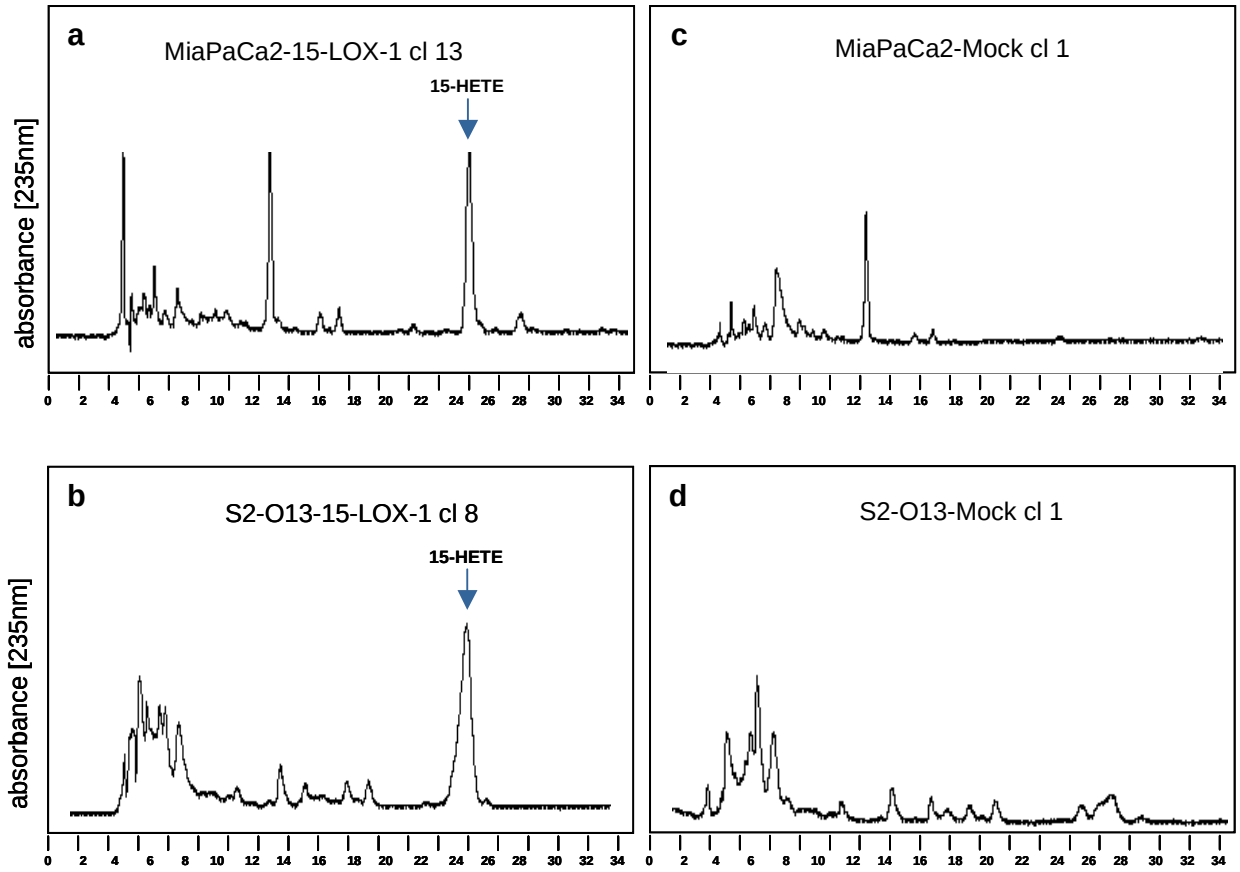


Fig. 14: Activity assay for 15-LOX-1 over-expressing cells

RP-HPLC analysis of the products formed in cell extracts from stably transfected 15-LOX-1 expressing cell lines. Cell homogenates were incubated with 10 µM AA for 45 min and products were extracted with methanol-dichloromethane (1:1, v:v), dried under vacuum, re-dissolved in methanol-water-acetic acid (82:18:0.01, v:v), injected on a 5 µM YMC-Pack ODS-AM column, and eluted at 0.5 ml/min. The eluate was monitored at 235nm. The retention time of 15-S-HETE used as standard was 24.5 min. After lipid extraction 15-S-HETE was detected in the 15-LOX-1-over-expressing cells (**a&b**) but not in the Mock transfected ones (**c&d**). This result could be confirmed for MiaPaCa2 and S2-O13.

7. Functional role of 15-LOX-1 expression

7.1 Effect of 15-LOX-1 on cell growth

When compared with Mock transfected cells, the proliferation of 15-LOX-1 over-expressing MiaPaCa2 and S2-O13 cells was significantly inhibited , as measured by both WST-1 assay and cell counting. Highly significant differences in the cell numbers were observed at 96 and 120 hours after seeding determined by the WST-1 assay (MiaPaCa2: ~55%; 20%; S2-O13: 40%; 15%, respectively). Cell counting also revealed a significant decrease of the cell numbers after 96 hours up to 120 hours for recombinant MiaPaCa2 (~50%; 60%) and S2-O13 cells (~30%; 40%). WST-1 results are shown in Figure 15a&c and cell counting results in Figure 15b&d.

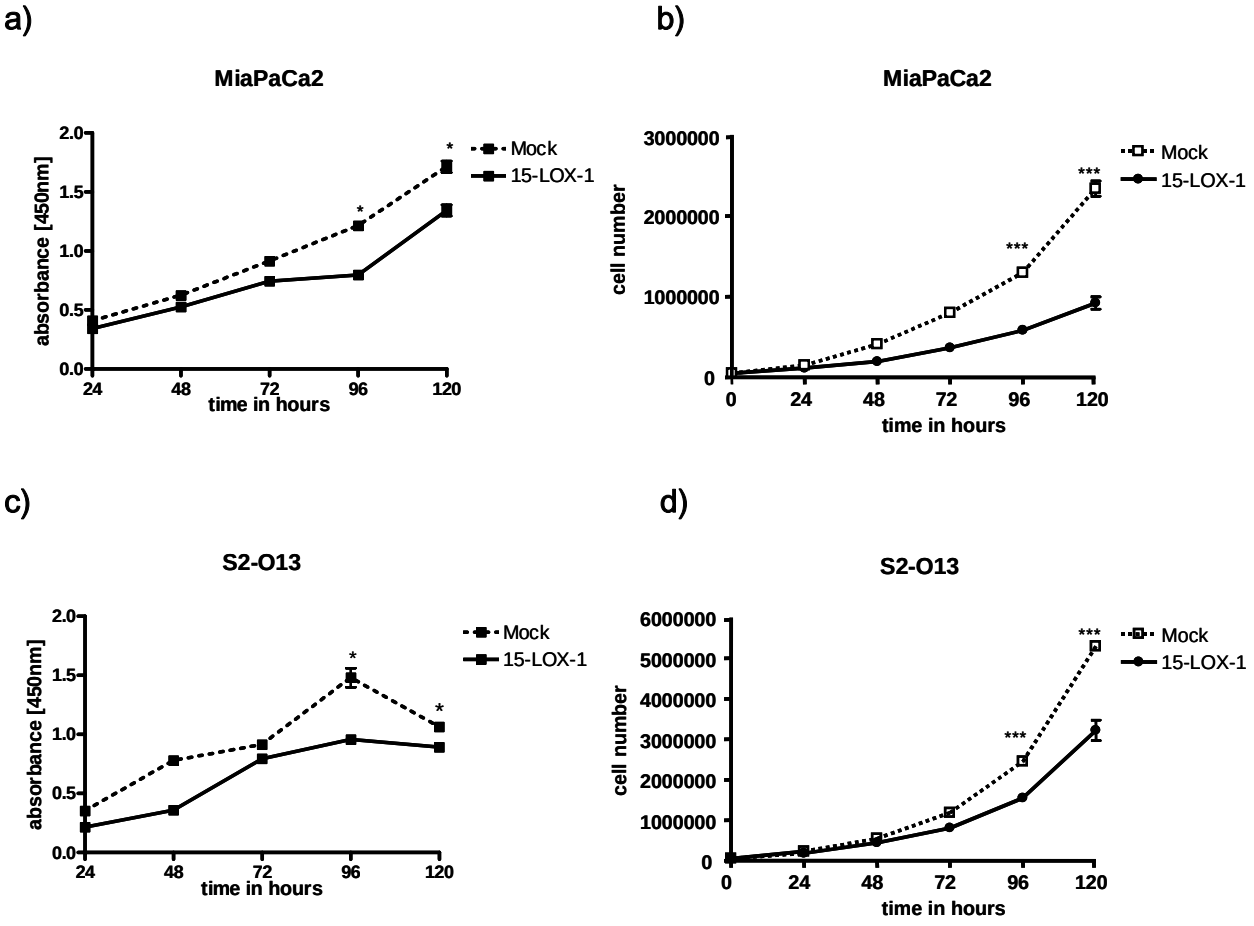


Fig. 15: 15-LOX-1 expression inhibits cell growth in MiaPaCa2 and S2-O13

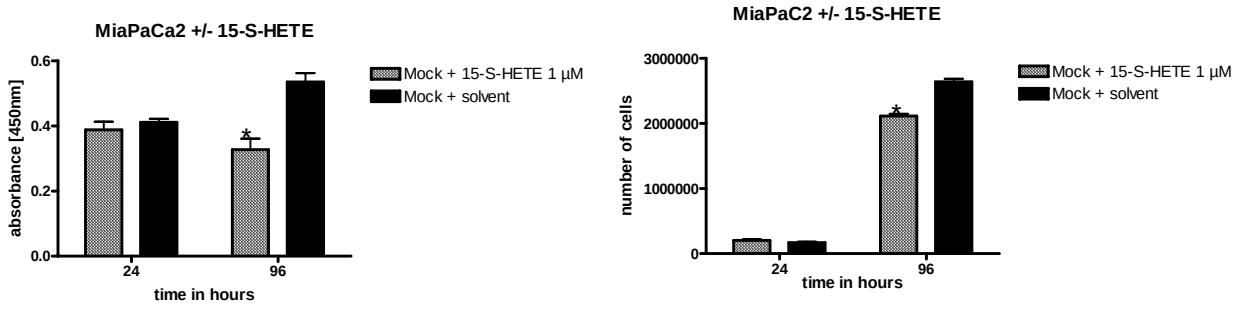
a&c WST-1 assay: 1000 cells/ 100 µl were seeded into 96 well plates. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader. Growth inhibition was significant at 96 and 120 hours in MiaPaCa2 ($p<0.001$ and $p<0.001$) and S2-O13 ($p<0.001$ and $p<0.001$). **b&d** Cell counting: Cells were plated at a concentration of 25,000 cells/ ml with a final volume of 2 ml into 60 mm dishes. The number of living cells was counted at indicated time points using the GUAVA–PC. Growth inhibition was significant at 96 and 120 hours in MiaPaCa2 ($p<0.001$ and $p<0.001$) and S2-O13 ($p<0.001$ and $p<0.0001$).

7.2 Effect of LOX products on cell growth

7.2.1 Exogenous 15-S-HETE treatment

15-LOX-1 has been shown to accept both AA and LA as substrates with comparable efficiency. To examine whether 15-LOX-1 mediated growth inhibition was due to the LOX products the effects of exogenous treatment with 15-S-HETE and 13-S-HODE on cell growth were analyzed. MiaPaCa2 and S2-O13 cell lines were exogenously treated with its AA derived product 15-S-HETE. While, 24 hours after treatment of MiaPaCa2 no significant decrease in proliferation was detected for MiaPaCa2 ($p=0,449$) determined by WST-1 and cell counting ($p=0,168$), at 96 hours time point a significant decrease in proliferation of ~40% was measurable for MiaPaCa2 (Fig. 16a) determined by WST-1. Similar effect was determined by cell counting, though measured decrease was only about 20%. ($p<0,008$ and $p<0,001$). Same effect was observed in S2-O13 (Fig. 16b) exhibiting a decreased proliferation ~20 -30% determined by WST-1 and cell counting ($p<0,02$ and $p<0,001$).

a)



b)

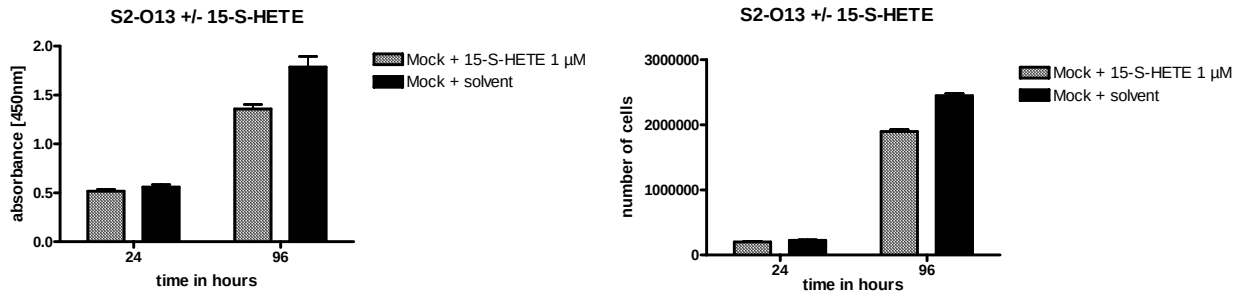


Fig 16: Effect of exogenous 15-S-HETE (1 µM) on proliferation of MiaPaCa2 and S2-O13 cells

WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 and S2-O13 were seeded into 96well plates. After 24 hours the medium was replaced by serum-free medium supplemented with 1 µM 15-S-HETE. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader. GUAVA cell counting: cells were plated at a concentration of 25,000 cells/ ml with a final volume of 2 ml into 60 mm dishes. After 24 hours the medium was replaced by serum-free medium supplemented with 1 µM 15-S-HETE. The number of living cells were counted at indicated time points using the GUAVA cell counter.

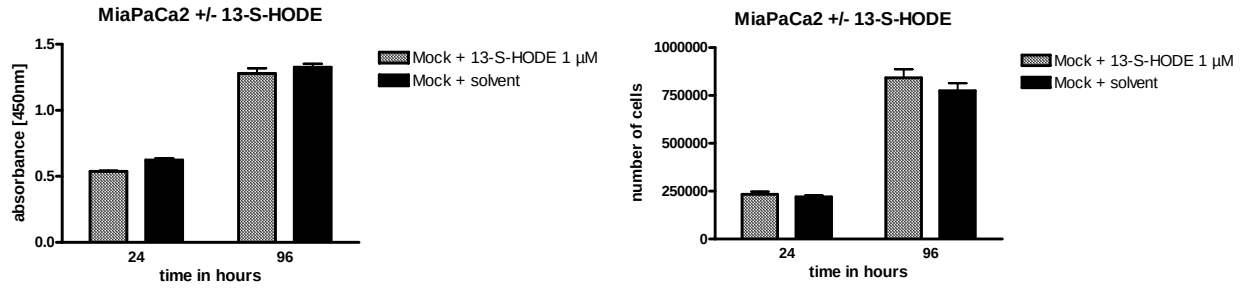
7.2.2 Exogenouse 13-S-HODE treatment

15-LOX-1 is known to metabolize AA but also LA to form 13-S-HODE.

Parental MiaPaCa2 and S2-O13 cell lines were exogenously treated with 13-S-HODE. The incubation with 1 µM of this metabolite had no significant effect on proliferation in both cell lines. For MiaPaCa2, 24 hours after treatment a slight decrease in proliferation (~ 25%) was detected (MiaPaCa2: p=0.002) determined by WST-1 however, this effect could not be verified by cell counting (p=0.424). In addition, 96 hours after treatment no significant decrease in proliferation was measurable for MiaPaCa2 (Fig. 17a) using WST-1 and cell counting (p=0.365 and p=0.279).

The same effect was observed in S2-O13 (Fig. 17b) cells at 24 hours past treatment (p=0.852 and p=0.424) analyzed by WST-1 and cell counting. Even after 96 hours no effect was measurable determined by WST-1 and cell counting (p=0.436 and p=0.279). WST-1 (right graphs) and cell counting (left graphs).

a)



b)

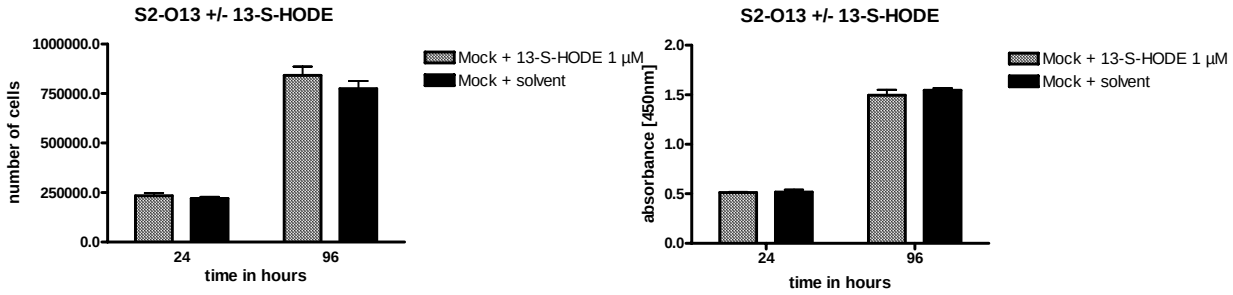


Fig. 17: Effect of exogenous 13-S-HODE (1µM) treatment on proliferation of MiaPaCa2 and S2-O13 cells

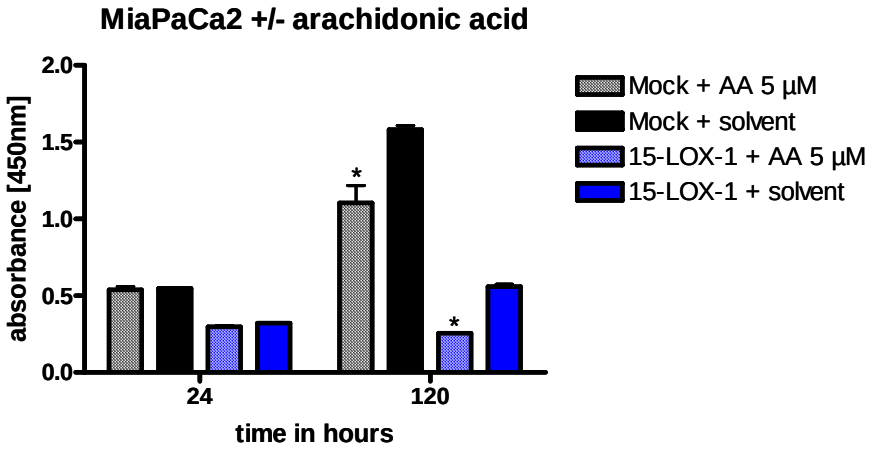
WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 (a) and S2-O13 (b) were seeded into 96 well plates. After 24 hours the medium was replaced by serum-free medium supplemented with 1 µM 13-S-HODE. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader. Cell counting with GUAVA-PC: cells were plated at a concentration of 25,000 cells/ ml with a final volume of 2 ml into 60 mm dishes. After 24 hours the medium was replaced by serum-free medium supplemented with 1 µM 13-S-HODE. The number of living cells were counted at indicated time points using the GUAVA cell counter.

7.3 Stimulation of growth inhibition by 15-LOX-1 substrates

7.3.1 Exogenous AA inhibits cell growth

The AA derived product 15-S-HETE revealed a proliferation inhibitory activity. Treatment with AA was performed to examine if the same effect could be achieved or if the growth inhibitory effect could still be enhanced. After 24 hours treatment no significant effect of exogenous AA on growth was observed in Mock and 15-LOX-1 cells. After 120 hours, however, 15-LOX-1 over-expressing cells showed a decreased proliferation of about 60% as determined by WST-1 ($p<0.001$). In Mock cells, proliferation decreased about 25% ($p=0.015$). Similarly, a decreased proliferation (~ 30%) was detected in S2-O13-15-LOX-1 over-expressing cells, 120 hours after exogenous AA treatment ($p=0.007$). In contrast, no effect on growth was observed in Mock transfected cells (Fig. 18).

a)



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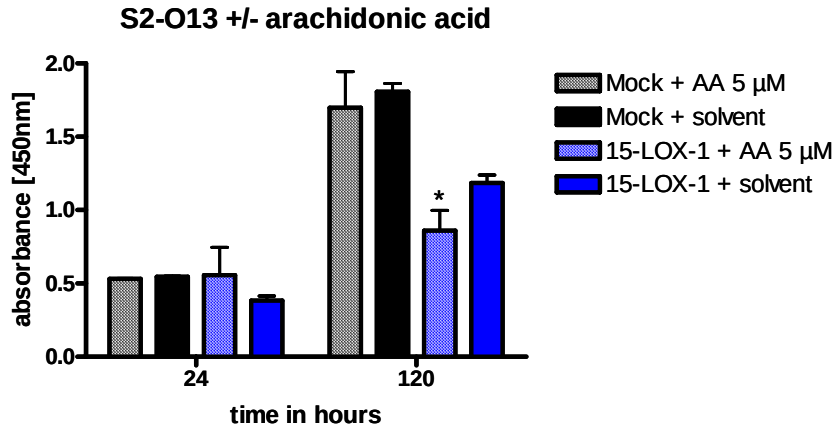
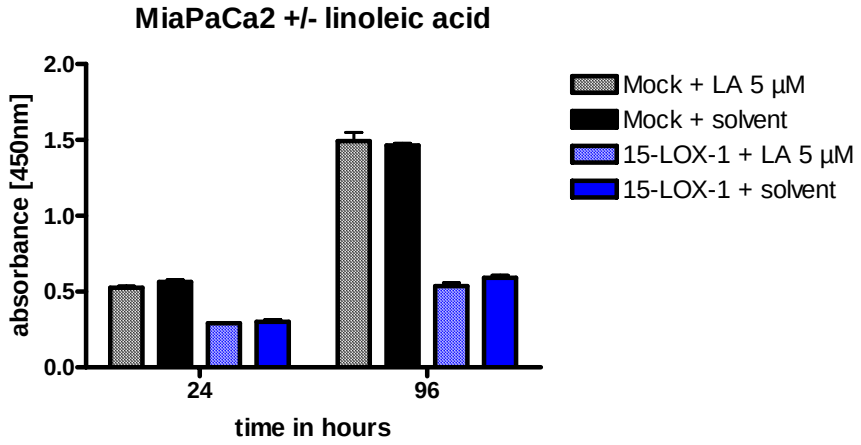


Fig. 18: Effect of 5 µM exogenous arachidonic acid on proliferation of treatment for MiaPaCa2 and S2-O13 Mock transfected and 15-LOX-1 over-expressing cells. WST-1 assay: carried out as described under Fig. 15.

7.3.2 Treatment with the 15-LOX-1 substrate linoleic acid

Treatment of MiaPaCa2 with LA did not show any inhibition in cell proliferation in Mock and 15-LOX-1 transfected cells. Proliferation was measured at two different time points after treatment with 5 µM LA. No significant decrease was observed. Even after 24 hours and 120 hours of treatment (Fig.19a). These results were confirmed for recombinant S2-O13 cells (Fig. 19b).

a)



b)

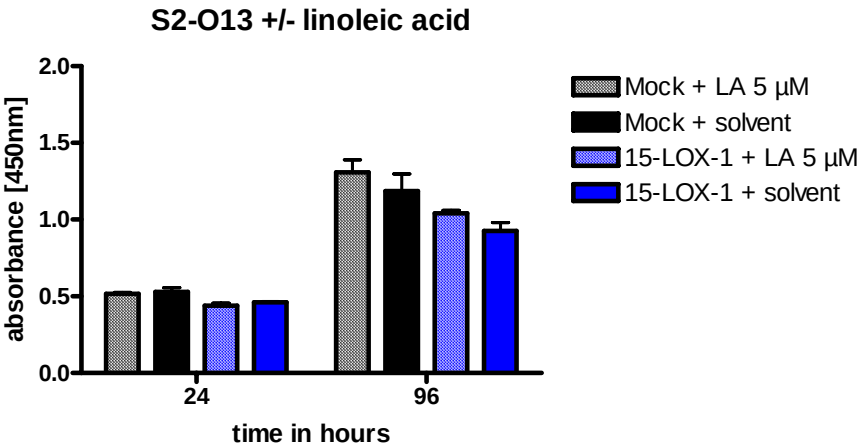


Fig. 19: Proliferation analysis after 5 µM exogenous linoleic acid treatment for MiaPaCa2 and S2-O13 transfected cells

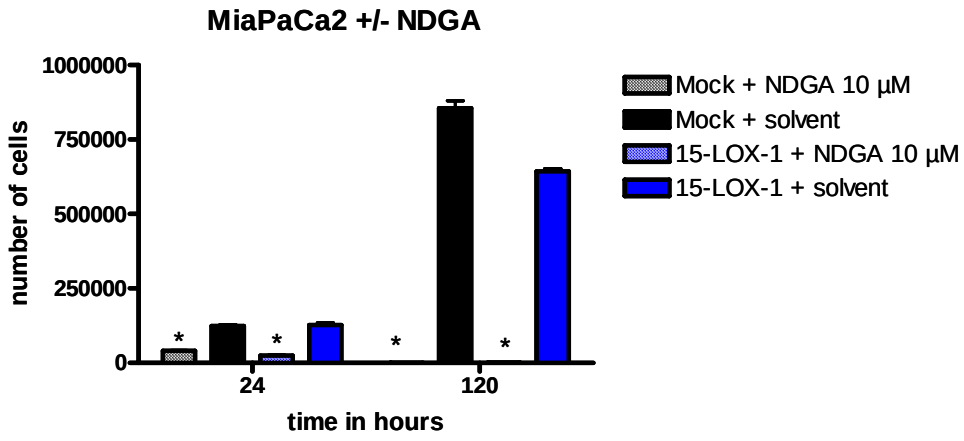
WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 or S2-O13 were seeded into 96 well plates. After 24 hours the medium was replaced by serum-free medium supplemented with 5 µM LA. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader.

8. LOX inhibitors reduce cell proliferation

8.1 Effect of NDGA on cell growth

The previous results indicate that 15-LOX-1 and its AA-derived product 15-S-HETE inhibit growth of pancreatic cancer cells. Studies using the general LOX inhibitor NDGA and the more specific LOX inhibitor baicalein were performed to evaluate if inhibition of LOX activity may reverse the 15-LOX-1 induced growth inhibition. At 24 hours decrease in Mock cells was about 40% and in 15-LOX-1 transfected cells about 75% in proliferation (Fig. 20a) using cell counting ($p<0.001$ and $p=0.001$, respectively). Inhibition of cell growth in 15-LOX-1 cells was about 90% ($p<0.001$). Similar, significant decrease (~90%) was observed after 120 hours in Mock cells ($p<0.001$).

a)



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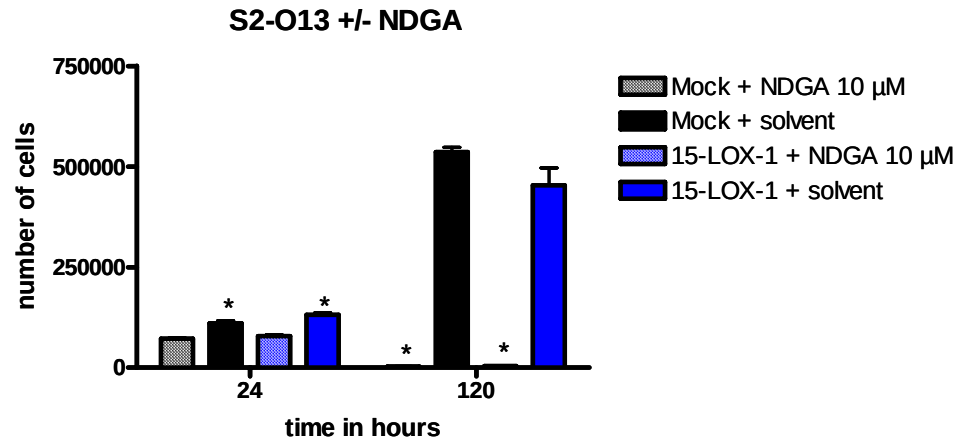


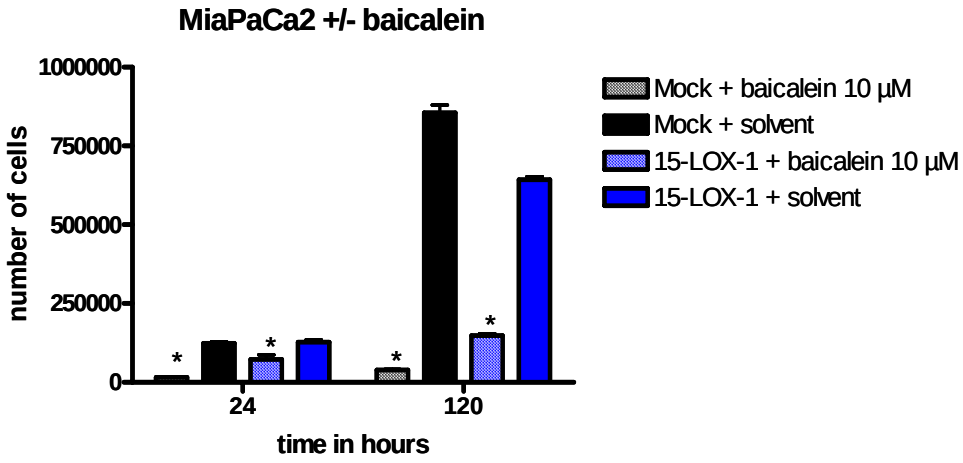
Fig. 20: Effect of LOX inhibitor NDGA on cell growth in MiaPaCa2 and S2-O13

GUAVA cell counting: cells were plated at a concentration of 25,000 cells/ ml with a final volume of 2 ml into 60 mm dishes. After 24 hours the medium was replaced by serum-free medium supplemented with 10 µM NDGA. Cell counting was carried out at the indicated time points.

8.2 Baicalein inhibits cell proliferation

Likewise, treatment of MiaPaCa2 with baicalein resulted in the inhibition of cell growth. Proliferation of Mock cells was decreased by about 90% ($p<0.002$) and that of 15-LOX-1 cells by about 60% ($p=0.007$) after 24 hours. At 120 hours after treatment, proliferation of Mock cells and 15-LOX-1 cells was suppressed by about 80% ($p<0.001$) and 60%, ($p<0.001$), respectively (Fig. 21a). Treatment of S2-O13 with baicalein also resulted in a strong inhibition of cell growth (Fig. 21b).

a)



b)

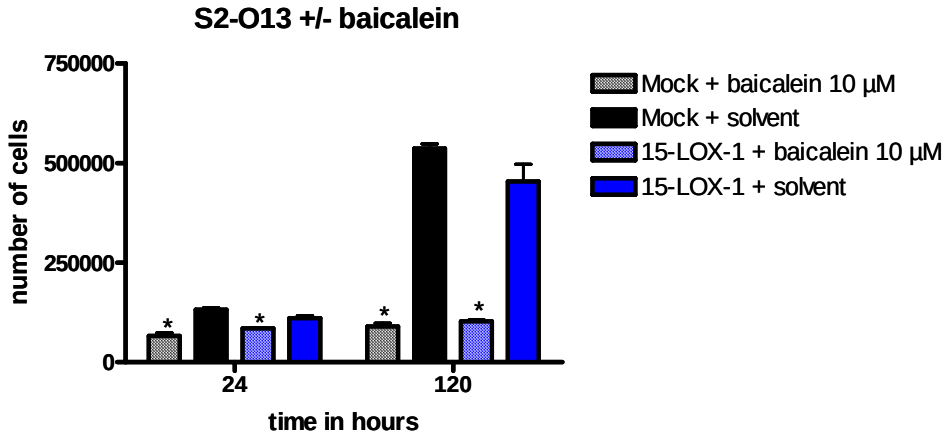


Fig. 21: Effect of LOX inhibitor baicalein on cell growth in MiaPaCa2 and S2-O13 cells

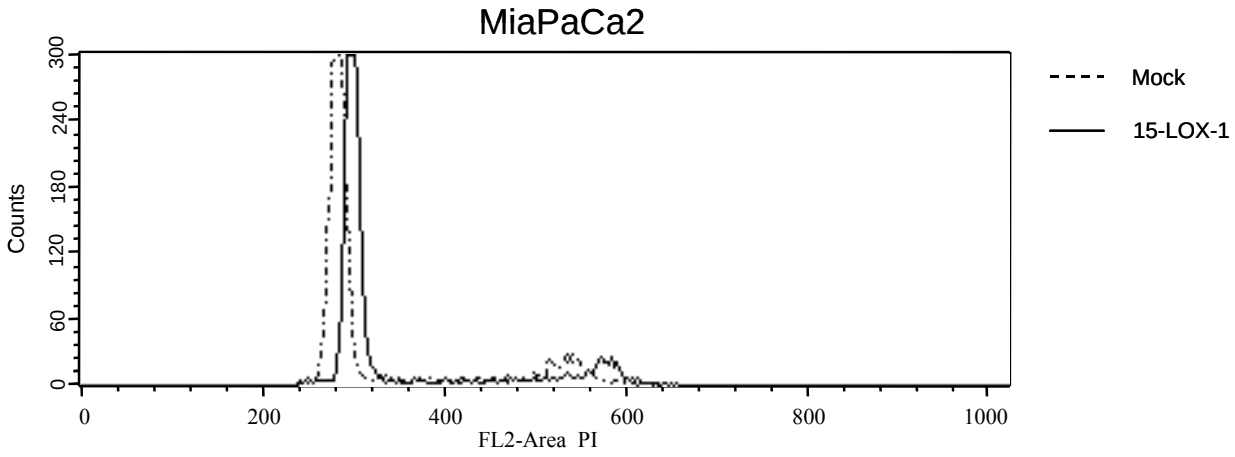
GUAVA cell counting: cells were plated at a concentration of 25,000 cells/ ml with a final volume of 2 ml into 60 mm dishes. After 24 hours the medium was replaced by serum-free medium supplemented with 10 µM baicalein. The number of living cells were counted at indicated time points using the GUAVA-PC.

9. Mechanisms involved in 15-LOX-1-mediated growth inhibition

9.1 Effect of 15-LOX-1 expression on cell cycle

As shown in Fig. 23 over-expression of 15-LOX-1 led to decreased proliferation as compared to Mock transfected cells. To investigate the molecular mechanism involved in 15-LOX-1 mediated growth inhibition cell cycle analysis were carried out. As shown in Figure 22 a&b no difference in cell cycle progression could be observed in 15-LOX-1 over-expressing MiaPaCa2 and S2-O13 cells as compared to control cells.

a)



b)

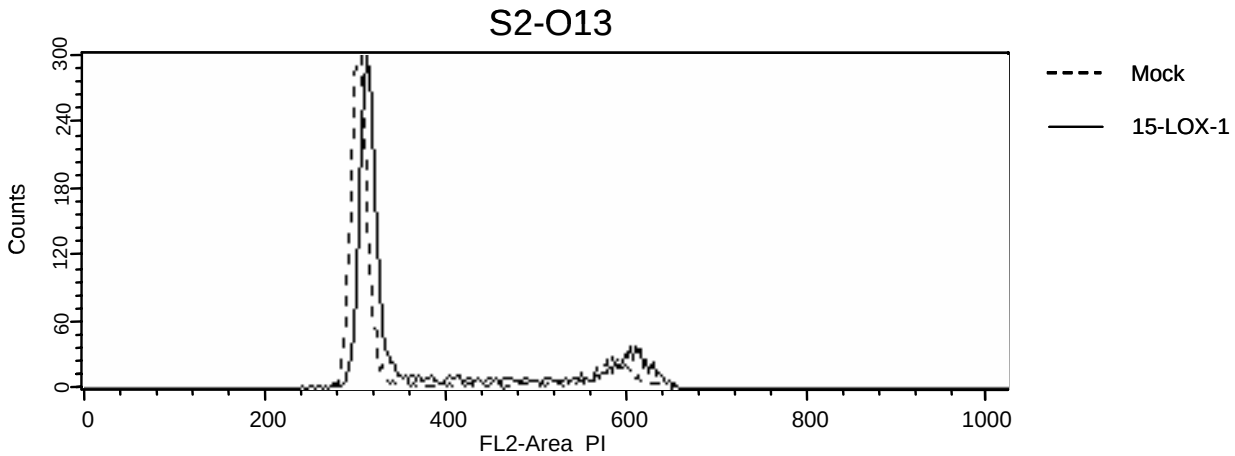


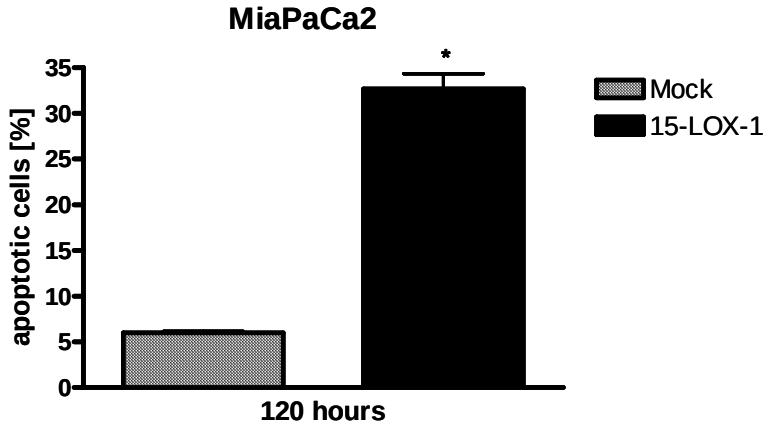
Fig. 22: Cell cycle analysis for 15-LOX-1 over-expressing cells

Cell cycle analysis was carried out using FACS analysis. 120 hours after seeding cells were prepared as described in material and methods. As shown for both cell lines **a)** MiaPaCa2 and **b)** S2-O13 no difference in cell cycle progression could be detected.

9.2 Increased apoptosis in 15-LOX-1 over-expressing cells

As no difference in cell cycle progression was detected in 15-LOX-1 over-expressing cells compared to Mock cells, the rate of apoptosis was determined (Fig. 23a). The comparison of MiaPaCa2-15-LOX-1 with MiaPaCa2-Mock cells showed an increase of apoptosis up to ~25% at 120 hours after seeding. In S2-O13 cells the rate of apoptosis was only marginally increased by about 8% (Fig. 23b).

a)



b)

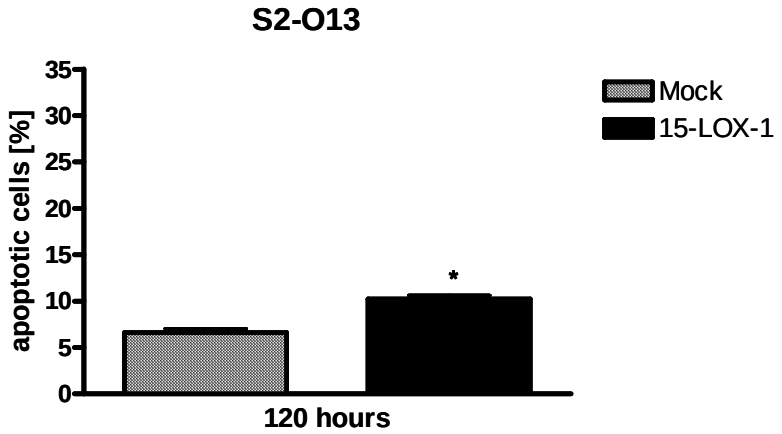


Fig. 23: Determination of apoptosis in 15-LOX-1 over-expressing cells

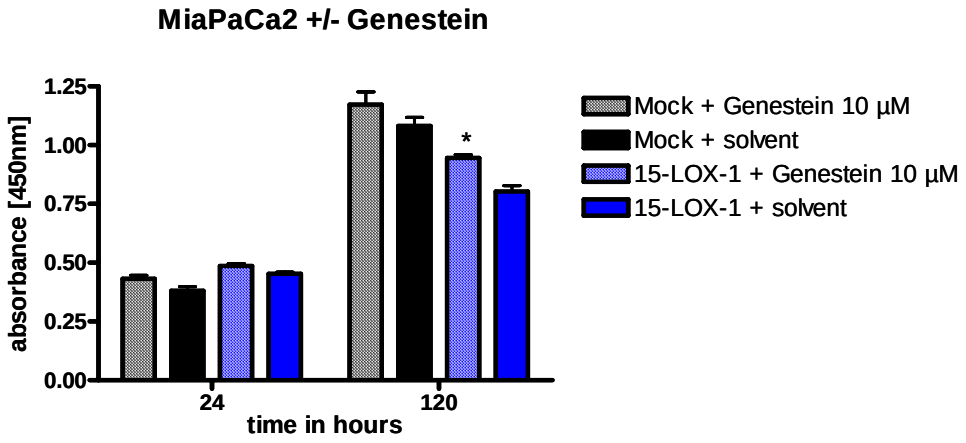
Apoptosis was measured using the GUAVA Nexin assay according to manufacturers protocol described in material and methods. The assay was performed with cells grown for 120 hours. Cells were trypsinised washed and stained with 7-AAD and Annexin V. After 20 min incubation cells were measured using the GUAVA-PC. 15-LOX-1 expressing cells were compared to Mock cells. In **a)** MiaPaCa2 and **b)** S2-O13 significant increase of apoptosis could be observed in 15-LOX-1 transfected cells ($p<0.001$ and $p<0.001$).

9.3 Analysis of signaling pathway potentially involved in 15-LOX-1 mediated growth inhibition

9.3.1 Genestein

To delineate potential signaling cascades involved in 15-LOX-1 effected growth inhibition in pancreatic cancer cells, pharmacologic kinase inhibitors were used. In a first set of experiments 15-LOX-1 over-expressing cells and Mock control cells were incubated in medium containing 10 µM genestein, a specific tyrosine kinase inhibitor (Fig. 24a&b). While no effect on growth was observed in Mock cells. A clear reversion of 15-LOX-1-induced growth inhibition was observed in 15-LOX-1 over-expressing MiaPaca2 and S2-O13 cells as determined by WST-1 (p=0.006) and cell counting (p=0.004) at 120 hours after treatment.

a)



b)

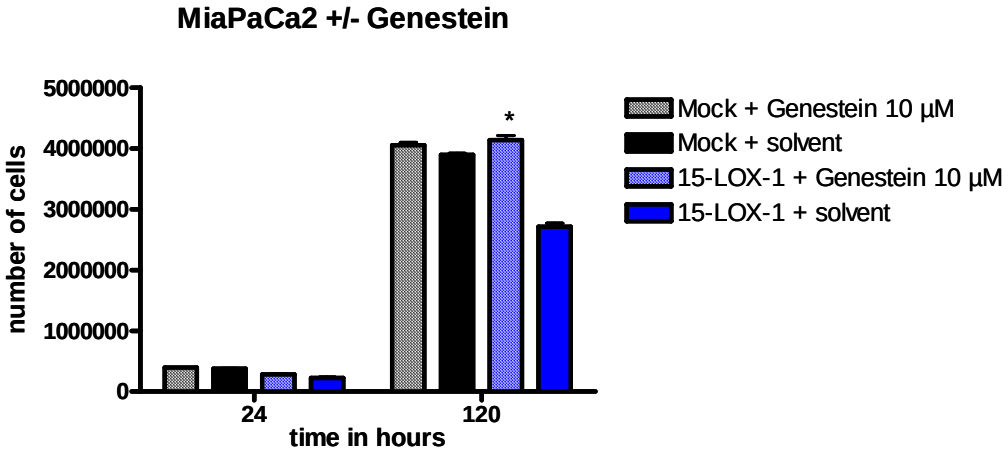
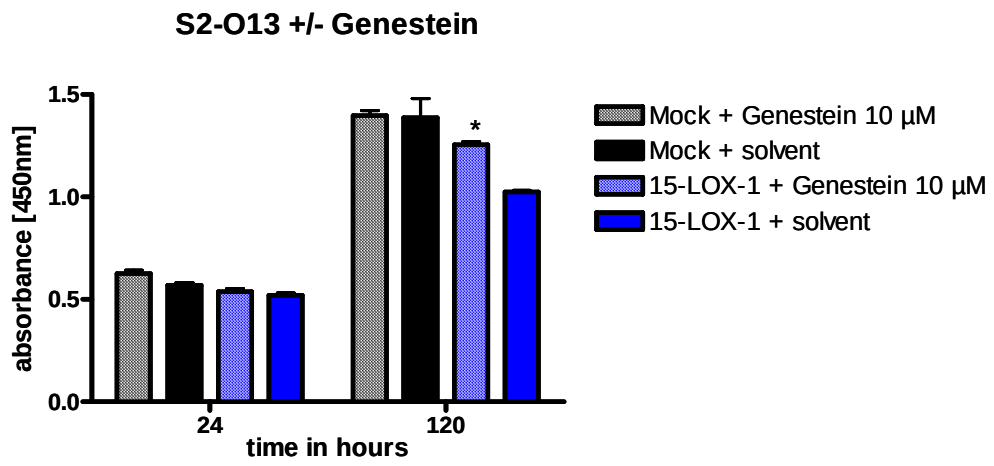


Fig. 24: Proliferation analysis for MiaPaCa2 after treatment with the MAPK inhibitor genestein

a) WST-1 assay: carried out as described under Fig. 15 **b)** GUAVA cell counting: carried out as described under Fig. 15.

Likewise, S2-O13-15-LOX-1 cells and Mock cells were treated with 10 µM genestein. Proliferation was measured at two different time points using WST-1 (Fig. 25a) and cell counting (Fig. 25b). In Mock cells, cell growth after 24 hours and 120 hours (p=0.241 and p=0.099) was not affected. In S2-O13-15-LOX-1 cells the 15-LOX-1 induced growth inhibition was not reversed at 24 hours However, a clear reversion (~30%) was observed at 120 hours after treatment (WST, p=0.006; cell counting, p=0.004).

a)



b)

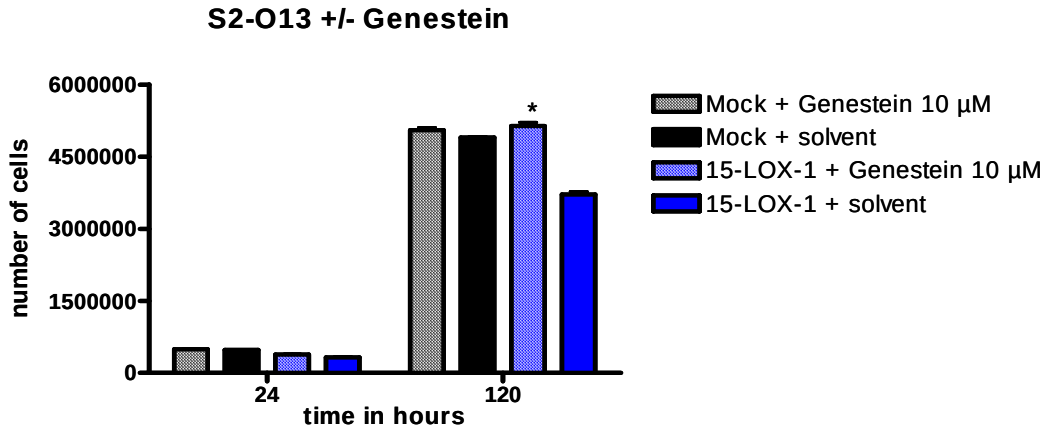


Fig. 25: Proliferation analysis for S2-O13 after treatment with the MAPK inhibitor genestein

a) WST-1 assay: 1000 cells/ 100 µl of S2-O13 were seeded into 96 well plates. After 24 hours the medium was replaced by medium supplemented with 10 µM genestein. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader.

b) GUAVA cell counting: cells were plated at a concentration of 25,000 cells/ ml with a final volume of 2 ml into 60 mm dishes. After 24 hours the medium was replaced by medium supplemented with 10 µM genestein. The number of living cells were counted at indicated time points using the GUAVA cell counter.

9.3.2 Effect of MEK1/2 inhibitor on cell proliferation

Next, the Mitogen-activated kinases (MAPK) signaling pathway was addressed by using specific inhibitors for MEK1/2 and ERK. Treatment of transfected MiaPaCa2 cells with these inhibitors did not reverse the LOX-induced growth inhibition but in contrast induced growth suppression by itself. Suppression of growth in 15-LOX-1 over-expressing MiaPaCa2 cells was about 20% (p=0.02; Fig. 26).

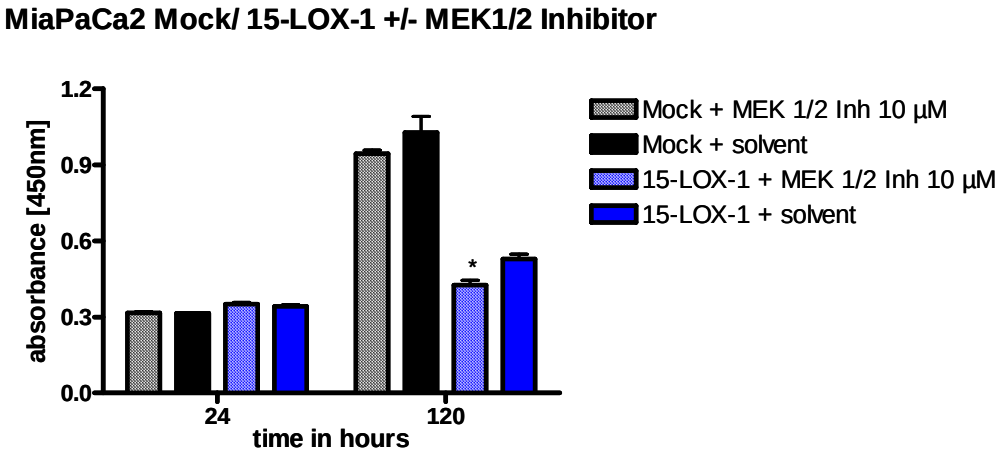


Fig. 26: Proliferation analysis for MiaPaCa2 after treatment with the MAPK inhibitor MEK1/2

WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 were seeded into 96well plates. After 24 hours the medium was replaced by medium supplemented with either 10 µM MEK1/2 inhibitor or solvent. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader.

9.3.3 Effect of PD98 on cell proliferation

Similarly, treatment of MiaPaCa2-Mock and MiaPaCa2-15-LOX-1 with the ERK inhibitor PD98 showed only a minor decrease in Mock (~20%) as well as in 15-LOX-1 (~10%) cells after 24 hours (MiaPaCa2-Mock $p<0.001$; MiaPaCa2-15-LOX $p=0.009$). After 120 hours of incubation the inhibitory effect of PD98 increased in 15-LOX-1 cells (~20%) and even stronger in Mock cells (~40%). The observed inhibition (Fig. 27) of cell growth after 129 hours was statistically significant for 15-LOX-1 ($p<0.001$) and Mock cells ($p=0.009$).

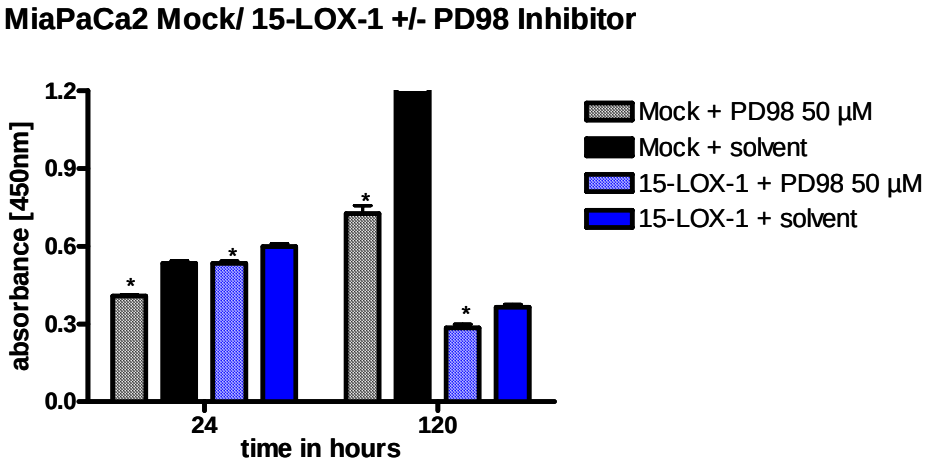


Fig. 27: Proliferation analysis for S2-O13 after treatment with the MAPK inhibitor PD98

WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 were seeded into 96 well plates. After 24 hours the medium was replaced by medium supplemented with 50 µM PD98 or solvent. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader.

10. HDAC inhibitors

10.1 Effect of different HDAC inhibitors on pancreatic tumor cell proliferation

It is known that HDAC inhibitors inhibit cell proliferation in several cancer cell lines (e.g. lung cancer and pancreatic cancer cells) via induction of apoptosis (see introduction). Additionally, it has been described that HDAC inhibitors do restore 15-LOX-1 expression human colon cancer cells (see introduction). MiaPaCa2 and S2-O13 cell lines were treated with the HDAC inhibitors sodium butyrate (NaBu), SAHA, and Trichostatin A (TA) to prove that the inhibitory effect could also be observed in these pancreatic cancer cell lines. Already after 24 hours of treatment MiaPaCa2 (Fig. 28) showed a strong decrease in cell proliferation, which lasted up to 120 hours, when treated with 5mM NaBu (~ 70%), 2.5 µM SAHA (~ 100%), and TA (~ 100%). Similar observations were made for S2-O13.

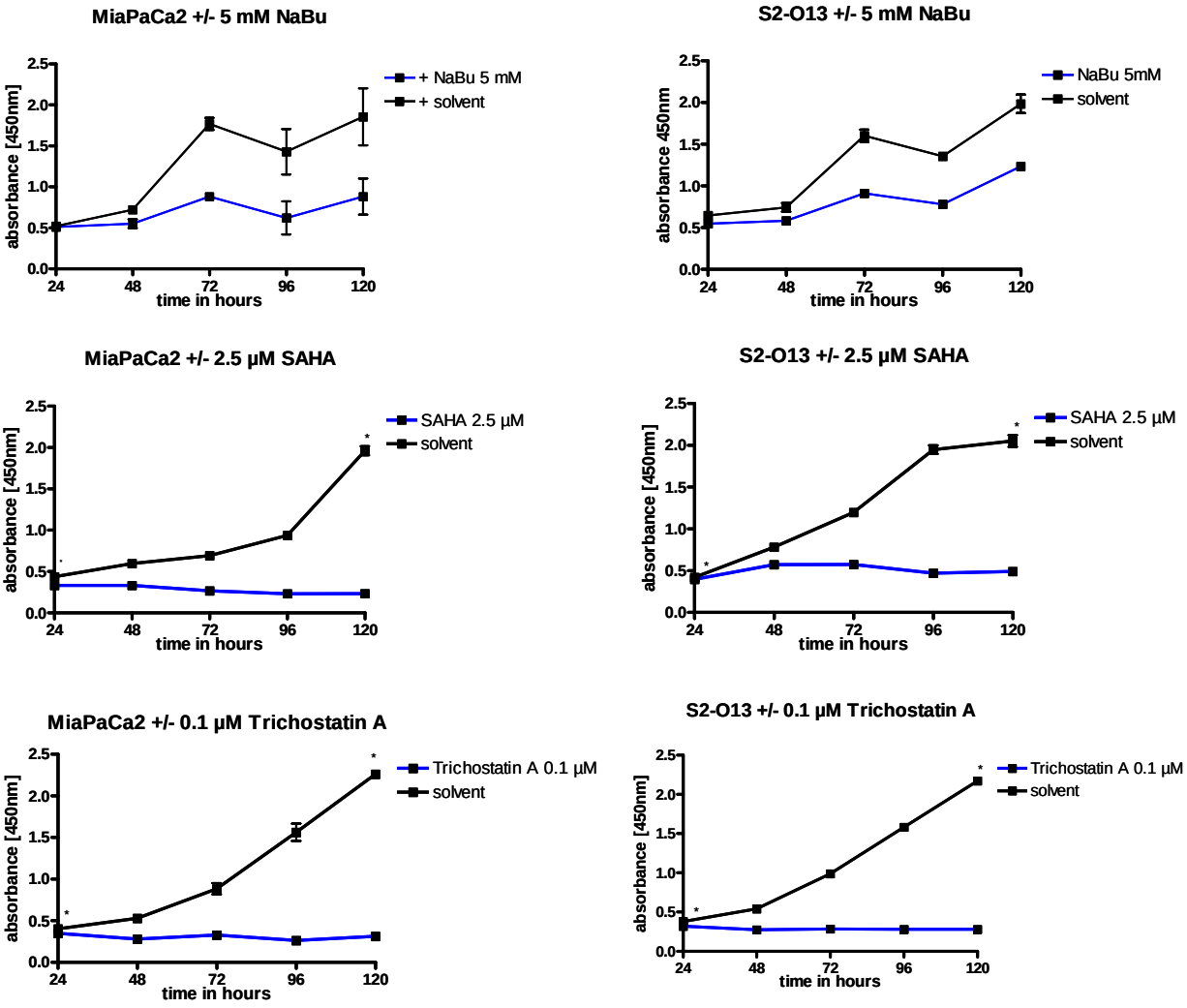


Fig. 28: Proliferation analysis after NaBu treatment for MiaPaCa2 and S2-O13

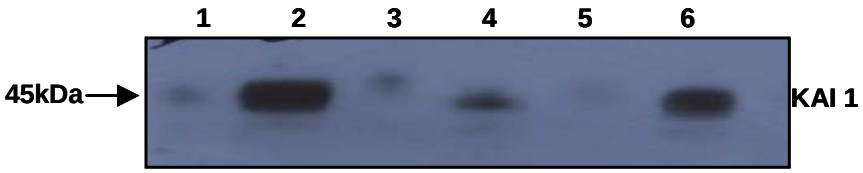
a) WST-1 assay: carried out as described under Fig. 15.

11. NaBu alters metastatic protein expression

11.1 NaBu restores expression of the anti-metastatic protein KAI1

Previously, it has been described that NaBu not only induces apoptosis but also restores the expression of KAI1, an anti-metastatic protein in prostate cancer cells (see introduction). To date no such findings were made in pancreatic cancer cells (see introduction). First, several pancreatic cancer cells were screened for KAI1 expression. As shown in Figure 29a, three (Capan I, Panc-1, and T3M4) out of six tested cell lines expressed the anti-metastatic protein and were used as positive controls in the subsequent induction experiments with the KAI1 negative cell lines MiaPaCa2 and S2-O13. In MiaPaCa2, six hours after treatment a slight induction of KAI1 protein expression was detectable which became stronger after 24 and 48 hours (Fig. 29b).

a)



b)

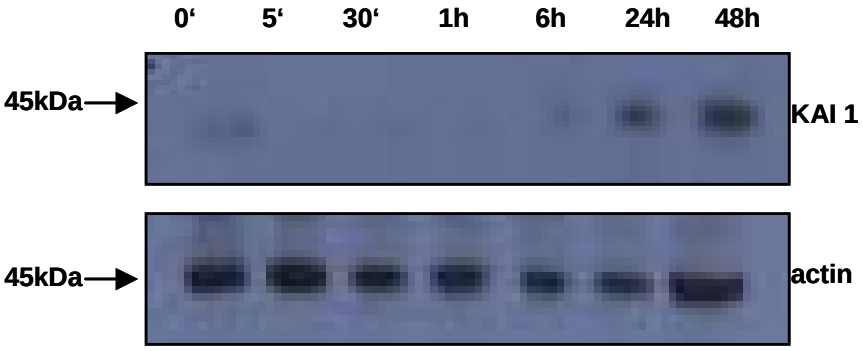


Fig. 29a&b: KAI1 induction after NaBu treatment in MiaPaCa2

Western blot analysis was carried out for MiaPaCa2. Cells were seeded and 24 hours later treated with 5 mM NaBu. Protein lysates were prepared after different incubation times. 30 µg lysates were analyzed using 10% SDS-PAGE and immunoblotted onto nitrocellulose membrane. After incubation with the specific KAI1 antibody (1:1000) KAI expression in different pancreatic cancer cell lines was detected **(a)**. Lane 1: AsPC-1, lane 2: Capan I, lane 3: Capan II, lane 4: Panc-1, lane 5: S2-O13, lane 6: T3M4. **b)** Clear KAI1 expression could be detected after 6 hours up to 48 hours. Equal loading was verified by β-actin (1:5000).

In S2-O13 cells, KAI1 expression started about one hour after treatment with NaBu and increased with time. After 48 hours the intensity of KAI1 expression decreased (Fig. 29c).

c)

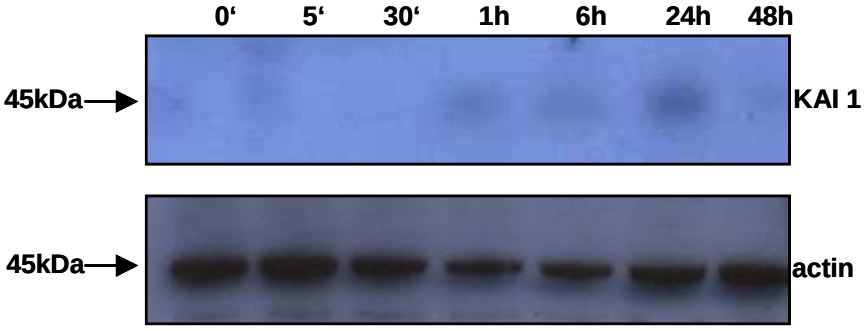


Fig. 29c: KAI1 induction after NaBu treatment in S2-O13

Western blot analysis was carried out for S2-O13. Cells were seeded and 24 hours later treated with 5 mM NaBu. Protein lysates were prepared after different incubation times. 30 µg lysates were analyzed using 10% SDS-PAGE and immunoblotted onto nitrocellulose membrane. After incubation with the specific KAI1 antibody (1:1000) slight KAI1 expression could be detected after 1 hour up to 24 hours. No signal could be detected after 48 hours. Equal loading was verified by β-actin (1:5000).

11.2 NaBu has no effect on the pro-metastatic protein S100A4

On the other hand, the pro-metastatic protein S100A4 was reported to be expressed in pancreatic tumor cell lines (see introduction). This was found to be true also for MiaPaCa2 and S2-O13 cells (Fig. 30a&b). Western blot analysis was carried out to analyze whether or not NaBu can downregulate S100A4 expression concomitant with up-regulation of the anti-metastatic protein KAI1. Figure 32 documents that NaBu has no effect on the expression of S100A4 in MiaPaCa2.

a)

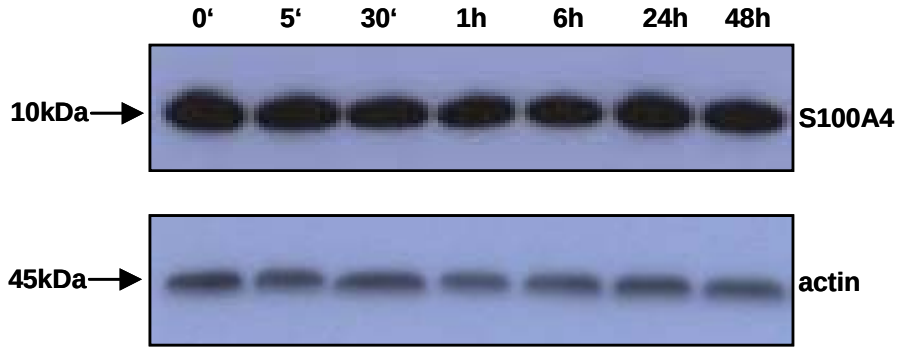


Fig. 30a: S100A4 expression MiaPaCa2

Western blot analysis was carried out for MiaPaCa2. Cells were seeded and 24 hours later treated with 5 mM NaBu. Protein lysates were prepared after different incubation times. 30 µg lysates were analyzed using 10% SDS-PAGE and immunoblotted onto nitrocellulose membrane. After incubation with the specific S100A4 antibody (1:1000) no altered expression could be detected. Equal loading was verified by β-actin (1:5000).

b)

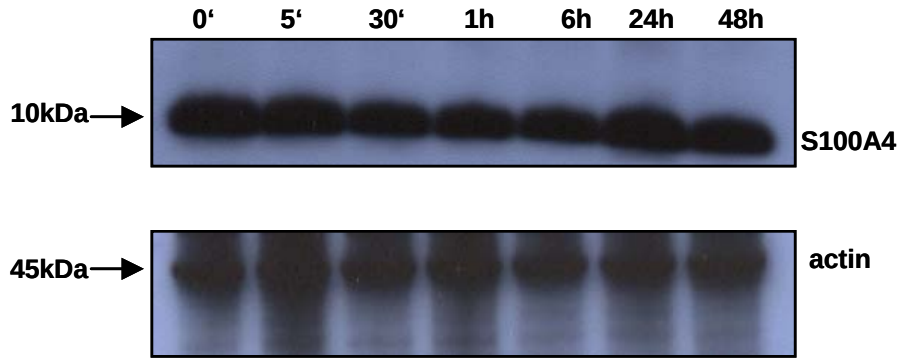


Fig. 30b: S100A4 expression S2-O13

Western blot analysis was carried out for S2-O13. Cells were seeded and 24 hours later treated with 5 mM NaBu. Protein lysates were prepared after different incubation times. 30 µg lysates were analyzed using 10% SDS-PAGE and immunoblotted onto nitrocellulose membrane. After incubation with the specific S100A4 antibody (1:1000) no altered expression could be detected. Equal loading was verified by β-actin (1:5000).

12. Restoration of 15-LOX-1 expression by NaBu

As shown in Fig. 28, growth of pancreatic tumor cells can be inhibited by different HDAC inhibitors and NaBu does also influence the expression of the anti-metastatic protein KAI1. Moreover, HDAC inhibitors such as NaBu were shown to be able to restore 15-LOX-1 expression in different cancer cells, including colorectal and lung cancer cells (see introduction). To prove if this observation can also be made in pancreatic cancer cells, MiaPaCa2 cells (Fig. 31a) and S2-O13 cells (Fig. 31b) were treated with the HDAC inhibitor NaBu. In MiaPaCa2 cells, NaBu could indeed induce the expression of 15-LOX-1, which could be detected after 6 hours of induction and steadily increased up to 48 hours.

a)

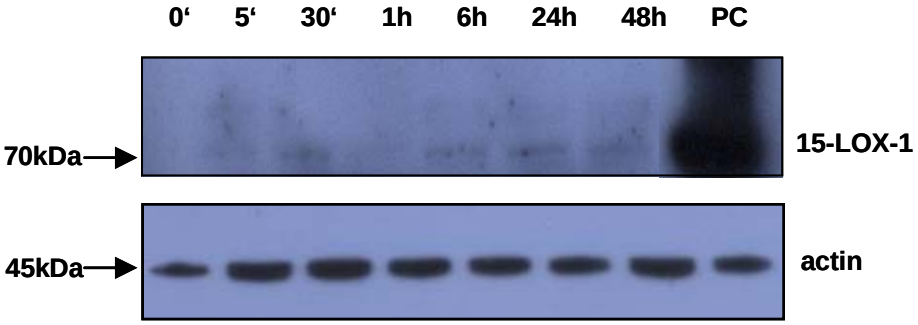


Fig. 31a: 15-LOX-1 induction in MiaPaCa2 after NaBu treatment

Western blot analysis was carried out for MiaPaCa2. Cells were seeded and 24 hours later treated with 5mM NaBu. Protein lysates were prepared after different incubation times. Cell homogenate proteins (30µg) from sonicated MiaPaCa2 cells treated with 5mM NaBu were separated on a 10% SDS-PAGE, blotted onto nitrocellulose membrane and incubated with antibodies against 15-LOX-1 (1:10000) and β-actin (1:5000). Clear signal could be seen for 15-LOX-1 6 hours after incubation remaining stable up to 48 hours. Equal loading was verified by β-actin.

In S2-O13 cells 15-LOX-1 expression was already induced after 30 min and could be observed up to 48 hours after treatment. In general, the induction of 15-LOX-1 expression was not as strong as in MiaPaCa2, but occurred earlier.

b)

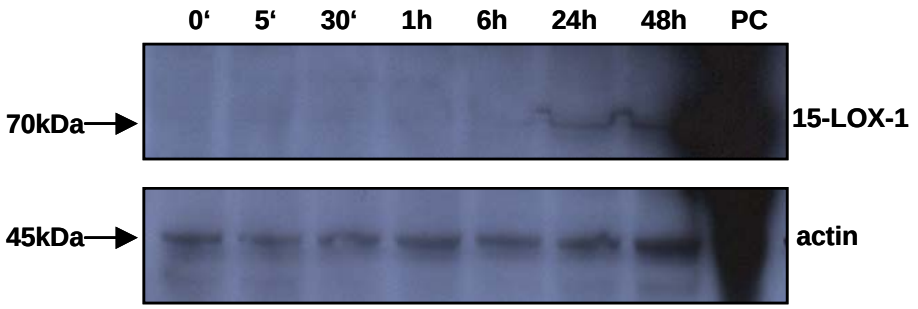


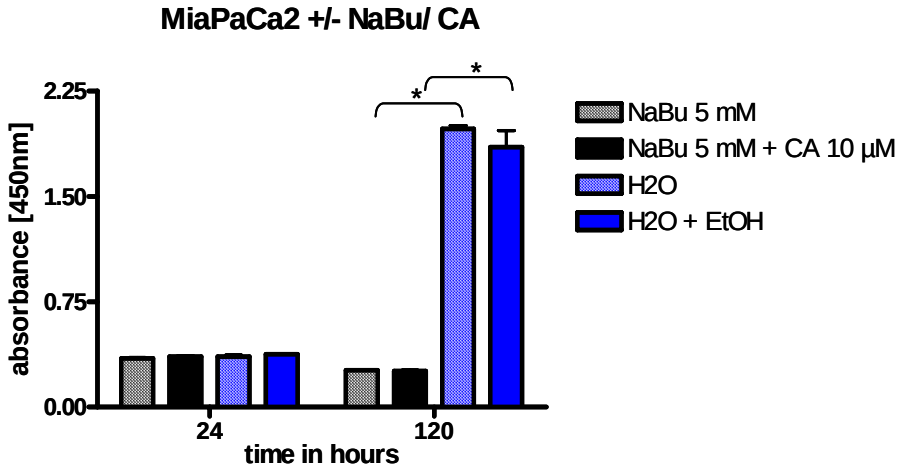
Fig. 31b: 15-LOX-1 induction in S2-O13 after NaBu treatment

Western blot analysis was carried as described under Fig. 31a.

12.1 Caffeic acid does not reverse the growth inhibitory effects of NaBu and SAHA

In order to study, whether the NaBu-induced inhibition of growth (Fig. 28) was causally related with the induction of 15-LOX-1, NaBu-treated cells were treated simultaneously with the 15-LOX-1 inhibitor CA. As shown in Figure 32 the inhibitory effect of 5 mM NaBu could not be reversed by inhibition of the 15-LOX-1 activity with 10 µM CA in both, MiaPaCa2 cells (Fig. 32a) and S2-O13 cells (Fig. 32b). No significant increase in cell proliferation was detectable for MiaPaCa2 (p=0,242 and p=0.585) and S2-O13 (p=0.842 and p=0.711) measured 24 and 120 hours after induction.

a)



b)

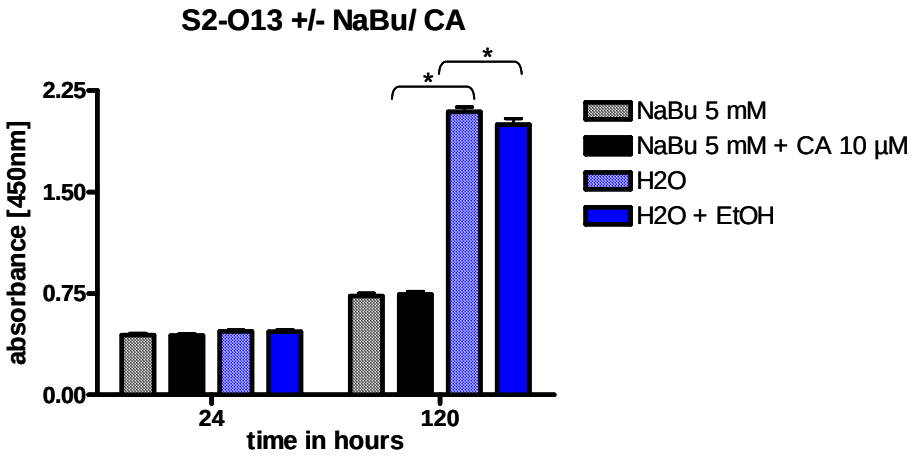
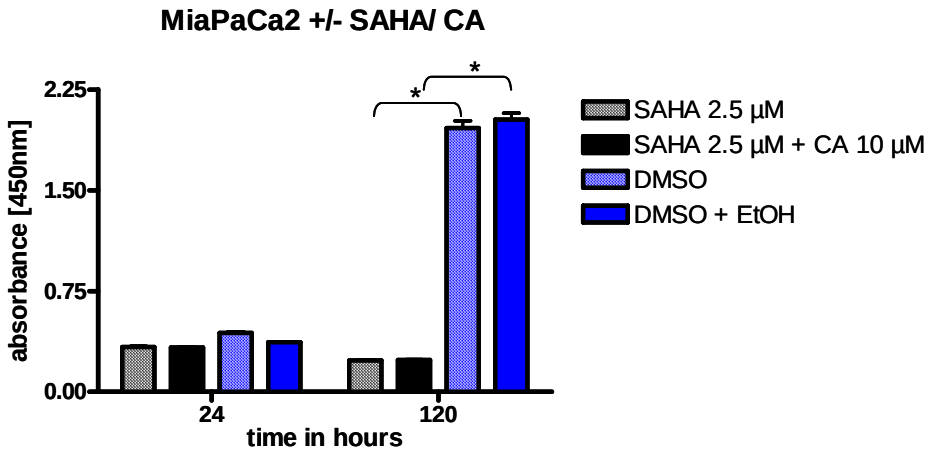


Fig. 32: Proliferation assay for MiaPaCa2 and S2-O13 after NaBu and CA treatment

WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 and S2-O13 cells were seeded into 96 well plates. After 24 hours the medium was replaced by medium supplemented with 5 mM NaBu and 10 µM CA or the appropriate solvent. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader.

Likewise, SAHA (2.5 µM) effected inhibition of cell proliferation could not be overcome by treatment with the 15-LOX-1 inhibitor CA at any time point in both, MiaPaCa2 cells (Fig. 33a) and S2-O13 cells (Fig. 33b). No significant effect could be determined after 24 and 120 hours for MiaPaCa2 cells (p=0.717 and p=0.579). The same result was observed for S2-O13 after 24 hours (p=0.582). After 120 hours, however, a significant increase of proliferation was measured in S2-O13 cells (p=0.001).

a)



b)

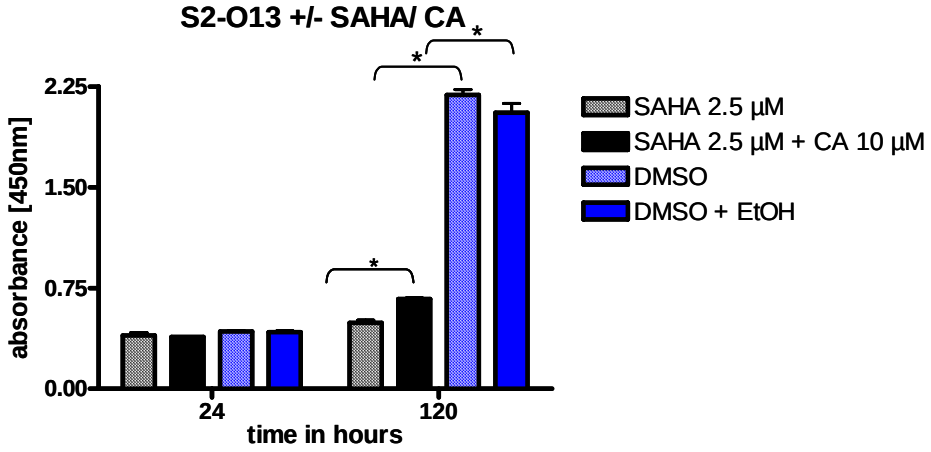


Fig. 33: Proliferation assay for MiaPaCa2 and S2-O13 after SAHA and CA treatment

WST-1 assay: 1000 cells/ 100 µl of MiaPaCa2 and S2-O13 cells were seeded into 96 well plates. After 24 hours the medium was replaced by medium supplemented with 2.5 µM SAHA and 10 µM CA or the appropriate solvent. At the mentioned time points 10 µl of the ready-to-use reagent were added to the cells. After 1 hour incubation at 37°C and 5% CO_2, proliferation was measured at 450 nm using an ELISA reader.

13. Discussion

At present, there is no effective therapy for pancreatic cancer patients. Therefore, prevention or early detection before this cancer has become invasive is thought to be an alternative strategy to cope with this situation. Even small resectable tumors recur or have metastasized in most cases within 2 years after primary surgery. Chemotherapy only prolongs life by a matter of weeks, maintaining the unchanged abysmal prognosis of pancreatic cancer.

The development of pancreatic cancer is known to be closely associated with inflammatory processes. The observation of a connection between inflammation and cancer in general goes back to Virchow in 1863, when he noted a “lymphoreticular infiltrate” in neoplastic tissues [223]. He suggested the origin of cancer in the context of chronic inflammation. Inflammatory cells and cytokines more likely contribute to tumor growth and progression than to mount the host anti-tumor response [223]. In addition, pancreatic cancer is characterized by a strong desmoplastic reaction and in 1986 Dvorak pointed out that wound healing and tumor stroma formation share many important properties. He suggested that tumors are wounds that do not heal [223]. At the same time observations were made, showing that intake of NSAIDs not only reduces inflammation and pain but also decreases the risk of cancer [224], in particular colon cancer [223]. Therefore, the eicosanoid pathway that is targeted by NSAIDs seems to play an important role in tumor development, growth and progression. The eicosanoid pathway can be divided into the cyclooxygenase (COX), the P450, and lipoxygenase (LOX) pathway. Cyclooxygenases (COX) are the rate-limiting enzymes in prostaglandin synthesis and it is well accepted that the inducible isoform COX-2 plays an important role in carcinogenesis [225]. Similarly, LOXs are reported to play an important role during tumourigenesis of different organ sites. Six human LOXs are characterized to date. Among the enzymes that are expressed in PanINs and pancreatic adenocarcinomas are 5-LOX and COX-2. Recently, 5-LOX has been suggested to be a tumor promoter in pancreatic carcinogenesis [226, 227]. Different studies using reverse transcriptase-polymerase chain reaction (RT-PCR) revealed expression of 5-LOX and 12-LOX mRNA in all commonly used pancreatic cancer cell lines, including Panc-1, AsPC-1 and MiaPaCa2, but not in normal pancreatic ductal cells [228]. Similar results were obtained on protein level [227]. 5-LOX up-regulation in human pancreatic tissues was verified by immunohistochemistry, which resulted in intense positive staining in cancer cells and premalignant lesions [227].

There is also RT-PCR evidence for 12-LOX mRNA expression in pancreatic cancer cell lines [228]. Anderson et al. showed that pancreatic cancer cell proliferation was inhibited by treatment with MK886, which is known to antagonize the sequestration of arachidonic acid by

the 5-LOX activating protein (FLAP) thereby suppressing 5-LOX activity [219, 227-235]. Ding et al. described that a LOX inhibitor, that was extracted from the sea cucumber attenuates pancreatic cancer development [236].

The activities of 5-LOX and 12-LOX are obviously associated with pro-tumorigenic effects on different tumor cells. On the other hand the role of 15-LOX-1 has been discussed controversially [236]. Expression of 15-LOX-1 has been associated with anti-tumorigenic effects in the colon but pro-tumorigenic effects in the prostate, indicating tissue selectivity for this LOX [221, 237]. In the pancreas, Hennig et al. showed that 15-LOX-1 protein was expressed in normal prancreatic tissue showing up as strong granular cytoplasmic staining in normal duct cells, pseudoducts, and centroacinar cells while no staining was seen in islets when analyzed immunohistochemically. Cancer cells or cells of PanIN lesions did not express 15-LOX-1. In addition, no or very weak expression of 15-LOX-1 was reported for pancreatic cancer cell lines tested in this report as compared to 15-LOX-1-positive mononuclear cells [238].

As nothing is known about the role of 15-LOX-1 in pancreatic carcinogenesis its potential involvement in pancreatic carcinogenesis was object of this study.

13.1 Expression of 15-LOX-1 mRNA and protein in pancreatic cancer cell lines

The first step was to determine the mRNA and protein levels of 15-LOX-1 in several cancer cell lines from primary prancreatic tumors (Capan II, MiaPaCa-2, Panc-1), malignant ascites (AsPC-1), and liver metastases (S2-O13, Capan I). In fact, pancreatic cancer is known to metastasize in local lymph nodes, liver, peritoneum, adrenal glands, lung, kidney, spleen and bone [2, 109]. Therefore, S2-O13 and Capan I were used to investigate whether or not metastatic and primary tumor cell lines differ in their 15-LOX-1 expression pattern.

On mRNA and protein level, no expression of 15-LOX-1 mRNA and protein was detectable in any of the tested cell lines which is in agreement with the loss of 15-LOX-1 expression in pancreatic cancer tissue [238]. In contrast, primary normal pancreatic ducts expressed 15-LOX-1 protein thereby recapitulating the in vivo situation [239]. Similar observations were reported by Shureiqi et al. which showed the expression of 15-LOX-1 in normal colonic epithelial cells but not colon carcinoma cell lines [240].

These results led us to the conclusion that 15-LOX-1 expression is lost during pancreatic carcinogenesis pointing to an anti-tumorigenic rather than a pro-tumorigenic function of this LOX isozyme in pancreatic carcinogenesis.

13.2 The functional role of 15-LOX-1

To analyze the role of 15-LOX-1 for pancreatic carcinogenesis, different 15-LOX-1 over-expressing cell lines were established. Comparing to mock transfected cells a clear inhibition of proliferation was detectable in two 15-LOX-1 over-expressing cell lines. Similar observations have been already reported for cancer cells of the colon [241], esophagus [166], stomach [242], and prostate [180, 243].

Further experiments were carried out to examine which of the two 15-LOX-1 metabolites, 15-S-HETE or 13-S-HODE, mediated the observed decrease of proliferation in pancreatic cancer cells. Exogenous treatment with 1 µM 15-S-HETE led to decreased proliferation. Chen et al. showed a similar inhibitory effect of 15-S-HETE in colorectal cancer cells, though the concentrations used in this study ranked between 30 µM and 100 µM [244]. Likewise, Hsi et al. demonstrated a decreased proliferation in colorectal cancer cells that were treated with 15-S-HETE in the dose range of 0.1 µM to 50 µM [245]. In contrast, 13-S-HODE did not show any altered proliferation in our pancreatic cancer cell lines at 1 µM concentration. Similar results were described by Shureiqi et al. in colon cancer cells, but they observed a decreased proliferation at higher concentrations of 13-S-HODE (50 µm up to 150 µM) [240]. In contrast, Hsi et al. showed a proliferation stimulating effect of 13-S-HODE in colon cancer cells when used at concentrations of 0.1 µM, 1 µM and 5 µM. An inhibitory effect was shown for higher concentrations (10 µM and 50 µM). These data suggest that the effect of 13-S-HODE on the proliferation of colon cancer cells depends on the dose of this agent [240].

The question then is which mechanisms are involved in the growth inhibitory activity of 15-S-HETE in pancreatic cancer cell lines. In colon cancer cells, 15-S-HETE was found to activate PPARγ. This pathway finally leads to increased apoptosis in colon cancer cells. Whether or not this pathway is also true for pancreatic cells remains to be established. Interestingly, Shureiqi et al. showed that the pro-apoptotic effect of 13-S-HODE in colon cancer cells was associated with the down-regulation of PPARδ [241]. This effect could only be observed at low concentrations of 13-S-HODE that were not used in the study with pancreatic cancer cell lines.

Incubating the 15-LOX-1 over-expressing cells with 5 µM AA also reduced proliferation significantly. As AA is converted to 15-S-HETE the inhibitory effect might be due to 15-S-HETE accumulation and thereby the subsequent of downstream pathways. Furthermore, Monjezeb et al. showed that exogenous AA per se and inhibitors of AA metabolism that lead to the cellular accumulation of free AA were cytotoxic to the colon cancer cell line, HCT-116 [246]. Additionally, exogenous AA and triacsin C, an inhibitor of AA acylation, induced apoptosis via caspase-3 activation in a transcription-dependent manner.

Gene array analysis revealed that both exogenous AA and triacsin C alter the expression of similar genes in HCT-116 cells. For example, both down-regulate several genes with well-documented roles in cell survival and apoptotic resistance [246]. Thus, AA-induced inhitibion of cell proliferation in 15-LOX-1 over-expressing cells can occur through pathways that are dependent or independent of LOX activity. Treatment with 5 µM AA did not show any difference in mock cells. A potential inhibitory activity of AA could be compensated by COX-2-dependent synthesis of PGE_2 which has been shown to stimulate proliferation through stimulation of growth-related genes and proliferation via PI3K signaling and NF-κB translocation to the nucleus [247]. LA can also be metabolised by 15-LOX-1 and was therefore analysed as well. LA (1 µM) showed no effect. This result is conceivable with the observation that 13-S-HODE in the concentration used in my experiments does not inhibit cell growth in the pancreatic cell lines that were investigated.

Causes for decreased cell proliferation may either be settled in modified cell cycle progression and/ or increased rate of apoptosis. Kim et al. reported that over-expression of 15-LOX-1 induces growth arrest through phosphorylation of p53 in human colorectal cancer cells [221]. However, in my study no altered cell cycle progression was observed for 15-LOX-1 over-expressing cells. On the other hand, LOX products have been shown to induce programmed cell death (PCD) in human T-cells [187], neutrophils [188], PC12h cells [189], and Jurkat cells [190]. Several other studies have shown the pro-apoptotic activity of 5-LOX [191, 192], of leukocyte type 12-LOX [193, 194], and of 15-LOX-1 [178, 195] and have identified molecular targets for LOX-mediated induction of apoptosis. However, also anti-apoptotic effects of LOXs have been reported, mainly based on the observation that LOX inhibitors, most often nordihydroguaiaretic acid (NDGA) and MK886, exhibited pro-apoptotic activity [46, 196-198]. Interestingly, in other cellular types NDGA or MK886 protected against apoptosis [191, 194, 195, 199-202], or induced PCD in cell types completely devoid of LOX activity [203-205]. In this context, it should be recalled that most LOX inhibitors used in PCD studies, including NDGA, act by reducing the active Fe^{3+} enzyme to the inactive Fe^{2+} form. NDGA also blocks voltage activated calcium currents, inhibits P450 monooxygenase activity and acts as a general radical scavenger [201]. On the other hand, MK886, which inhibits 5-LOX by blocking substrate sequestration through interaction with the 5-LOX activating protein (FLAP), can also induce PCD via a caspase-3-dependent pathway, which is related to increased expression of the proapoptotic protein Bcl-x_L but unrelated to 5-LOX activity [204]. Moreover, it is known that some LOX products (namely, 12- or 15-S-HETE, but not their metabolic precursors 12- or 15-HPETE) may protect cells from apoptosis, whereas others (5-HETE) are toxic [197, 198, 217]. Maccarrone et al. reported that, within the physiological range, 15-LOX-1 dose-dependently increased the oxidative index of leukaemia cells (up to threefold over the control) and the uncoupling of mitochondria, both indicative of

the disruption of membrane integrity. Remarkably, incubation of mitochondria with 15-LOX-1 also caused a dose-dependent release of cytochrome c (up to fivefold over the control), a critical trigger of apoptosis induced by several unrelated stimuli [248]. Taken together, LOXs are able to disrupt mitochondrial integrity and to trigger cytochrome c release, thus giving a biochemical explanation for the observation that activation of these enzymes is critical in different models of apoptosis. In this study, an increased rate of apoptosis in pancreatic cancer cell lines over-expressing 15-LOX-1 cell was observed by using a Nexin assay. Other studies also support the view that 15-LOX-1 is a critical component of induction of apoptosis in cancer cells. As an example, Wu et al. reported that 15-LOX-1 mediates apoptosis induced by COX-2 inhibitors in gastric cancer [242] and Shureiqi et al. described that NSAIDs, the non-selective inhibitors of COX induce apoptosis in esophageal and colorectal cancer cells by restoring 15-LOX-1 expression [195, 249]. It has further been published that administration of the COX-2-selective inhibitor celecoxib restores 15-LOX-1 protein expression and induces apoptosis in colorectal cancer [250]. Yu et al. published that conditional, highly selective induction of 15-LOX-1 caused an inhibition of ribonucleotide reductase activity, cell cycle arrest in G_1, impairment of anchorage-independent growth, and accumulation of the pro-apoptotic protein BAX [251].

Possible mechanisms underlying 15-LOX-1 induced growth inhibition in colon cancer were identified by Zuo et al. They showed that (a) 15-LOX-1 catalyzed formation of 13-S-HODE promoted PPAR-γ activity, (b) PPAR-β/δ expression suppressed PPAR-γ activity in models of both loss and gain of PPAR-β/δ function, (c) 15-LOX-1 activated PPAR-γ by down-regulating PPAR-β/δ, and (d) 15-LOX-1 expression induced apoptosis in colon cancer cells via modulating PPAR-β/δ-mediated suppression of PPAR-γ. These findings elucidate a novel mechanism of signaling by natural ligands of PPARs, which involves the modulation of the interaction between PPAR-β/δ and PPAR-γ [252]. Based on these findings further investigations of 15-LOX-1 mediated growth inhibition in pancreatic cancer can be carried out. Preliminary data obtained in this study indicate that 15-LOX-1 expression is linked to dephosphorylation of ERK and MEK, leading to inactivation of this MAP kinase pathway molecules. It is well known that most human pancreatic cancers harbor mutations in the K-ras oncogene [253, 254]. Ras has been shown to mediate activation of various downstream targets, including the family of the MAPKs. One of the three well-defined MAPK pathways is the ERK pathway. ERK is involved in cell proliferation and transformation [255-258]. The relation between ERK and apoptosis has already been described by several groups. Anderson et al. and Xia et al. showed that ERK1/2 activation promotes the anti-apoptotic functions of Bcl-2 and cell survival in neuronal PC12 cells [259, 260]. Berra et al. further could show that inhibition of basal ERK1/2 activity was sufficient to trigger apoptosis in HeLa cells [261]. Studies for pancreatic cancer cells have been carried out by Boucher et al.[262].

They demonstrated that MEK/ ERK signalling pathway regulates the expression of Bcl-2, Bcl-x_L, and Mcl-1and promotes survival of human pancreatic cancer cells. Similar to this study, they used MiaPaCa2, a cell line which is known to exhibit activating mutations in the small GTPase K-ras [263]. Bocher et al. showed that inhibition of basal ERK activities causes (1) a G1 arrest; (2) a down-regulation of the expression levels of anti-apoptotic homologs; (3) a promotion of caspase-3 and caspase-8 activities; (4) a stimulation of PARP cleavage; and (5) a programmed cell death by apoptosis. In this study I could show that 15-LOX-1 is related to apoptosis, and preliminary data indicated that 15-LOX-1 on the same time inhibits phosphorylated MEK/ ERK (data not shown). Taken together I postulate following pathway for 15-LOX-1 or 15-S-HETE induced apoptosis.

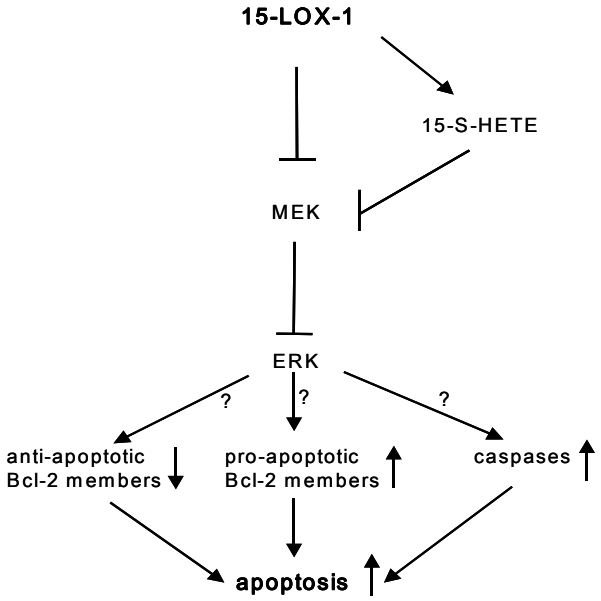


Fig 34: Postulated 15-LOX-1 induced apoptosis pathway

In contrast, Yoshinaga et al. described that over-expression of 15-LOX-1 induced extracellular signal-related kinase (ERK) 1/2 phosphorylation, decreased p21 (Cip/WAF 1) expression, and increased HCT-116 cell growth. Treatment with NDGA decreased ERK 1/2 phosphorylation, and increased p21 (Cip/WAF 1) expression in 15-LOX-1 over-expressing HCT-116 cells. In these colon carcinoma cells the 15-LOX-1 effects also were shown to be mediated via the LA product 13-S-HODE and the results support the hypothesis that 15-LOX-1 may have "pro-neoplastic" effects during the development of colorectal cancer [220]. Opposing effects of 15-S-HETE and 13-S-HODE on MAPK signaling in prostate cancer cells was reported by His et al. 13-S-HODE up-regulated MAP kinase and subsequentely increased PPARγ phosphorylation which was linked to a decreased PPARγ transcriptional activity and subsequently to enhanced proliferation, whereas 15-S-HETE down-regulated MAP kinase, decreased PPARγ phosphorylation which was linked to increased differentiation.

13.3 Effect of LOX and HDAC inhibitors

In this study, it was shown that LOX inhibitors like NDGA and baicalein did not reverse the 15-LOX-1 mediated growth inhibition but on the same time did inhibit cell proliferation per se to add up to about 100% in pancreatic tumor cell lines. Several groups already published same effect for NDGA in lympholeukemia cells [264], breast cancer cells [265], urological cancers [266], and several other cancers [267]. Similar results have been published for the treatment with baicalein. The 12-LOX-selective inhibitor baicalein significantly inhibited proliferation in cultures of the epidermoid carcinoma cells A431 as described by Kudryavtsev et al. [268]. Growth inhibition post baicalein treatment has also been described in human prostate cancer cells [269] as well as in pancreatic cancer xenografts [270]. In consideration of the fact that LOX inhibitors by themselves inhibit proliferation of several tumor cells it is conceivable that 15-LOX-1 mediated growth inhibition can not be reversed by these inhibitors.

Not only LOX inhibitors but also histone deacetylase (HDAC) inhibitors have been shown to be potent inducers of growth arrest, differentiation, and/or apoptotic cell death in transformed cells *in vitro* and *in vivo.* Target of the inhibitor action is the catalytic site of HDAC that has a tubular structure with a zinc atom at its base. The HDAC inhibitors, such as sodium butyrate (NaBu), suberoylanilide hydroxamic acid (SAHA), and Trichostatin A (TA), fit into this structure, in the case of SAHA, with the hydroxamic moiety of the inhibitor binding zinc. HDAC inhibitors cause acetylated histones to accumulate in both tumor and normal tissues. In general, chromatin fractions enriched in actively transcribed genes are also enriched in acetylated core histones, whereas silent genes are associated with nucleosomes with a low level of actetylation. HDACs also can acetylate proteins other than histones [271]. HDAC inhibitors unexceptionally inhibit proliferation of transformed cells in culture, and a subset has been shown to inhibit tumor growth in animal models [271]. They perform this activity by inducing growth arrest, differentiation, and/or apoptotic cell death in a wide variety of cultured transformed cells, including neuroblastoma, melanoma, and leukemia cells, as well as cells from breast, prostate, lung, ovary, colon [272-281] and pancreatic cancers [282]. Natoni et al. revealed that sodium butyrate sensitizes human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathway [282]. Additionally, Farrow et al. showed that pancreatic cancer invasion was inhibited by sodium butyrate [283]. Similar to Natoni's results, growth inhibition after NaBu treatment in both tested cell lines was observed in this study. Furthermore, inhibition in cell growth was also achieved after treatment with two other HDAC inhibitors, namely Trichostatin A and SAHA supporting similar observations that were made previously in other models. Desai et al. showed that SAHA markedly inhibited tumor growth in a mouse lung cancer model [284]. Kuefer et al. reported growth inhibition and apoptosis in

human prostate cancer *in vivo* after sodium butyrate and tributyrin treatment [285]. Beside growth inhibitor properties, HDAC inhibitors have also been published to restore 15-LOX-1 expression in colon cancer [286, 287]. Taken together, 15-LOX-1 may play a critical role in the control of epithelial cell growth and the loss of 15-LOX-1 expression and activity might contribute to the progression of cancer. In addition it was shown recently that down-regulation of 15-LOX-1 is an early event in the adenoma to carcinoma sequence both in colonic and gastric cancer development [240, 242]. This holds also true for pancreatic carcinogenesis in that 15-LOX-1 expression has already been lost in advanced PanIN 2 and -3 lesions that are regarded as precursor lesions for ductal adenocarcinoma as well as in cancer cells but not in PanIN-1 lesions. It is widely accepted that PanIN-1 lesions that are also found in many non-malignant pancreatic tissues do not yet fulfill the conditions of a premalignant lesion. Therefore, the switch to develop pancreatic cancer may happen at PanIN-2 stage, concomitant with the loss of 15-LOX-1 expression [238]. Thus, the modulation of 15-LOX-1 expression may be an attractive measure for the prevention and treatment of early pancreatic cancer. There are several pathways that are involved in the regulation of 15-LOX-1 expression. COX-2 inhibitors induce 15-LOX-1 expression, via down-regulation of the transcription factor GATA-6 [288]. Recently, Shureiqi et al. showed that GATA-6 is overexpressed in in vivo and contributes to silencing 15-LOX-1 in vitro in human colon cancer. Moreover, GATA-6 knockdown though being insufficient by itself to restore 15-LOX-1 expression, contributes significantly to the restoration of 15-LOX-1 expression [289]. Furthermore, an important role for the cGMP/PkG pathway in the restoration of 15-LOX-1 activity was suggested [290]. In addition, IL4 & IL13 were shown to stimulate the expression of 15-LOX-1, which than can induce p53 expression and phosphorylation/activation [56, 221]. Other research groups found the 15-LOX-1 promoter completely methylated in cells that are unable to express this enzyme and that 5-aza-2-deoxycytidine (5-Azadc), a potent methyltransferase inhibitor (MTI), induces the expression of 15-LOX-1 in human colorectal cancer cells [291]. Reversing epigenetic silencing, e.g. with an MTI as 5-Azadc, has become a new and important target approach for cancer prevention and therapy [291]. Another possibility to induce 15-LOX-1 expression is the use of sodium butyrate, a HDAC inhibitor [286]. In agreement with published data I could show in this study that 15-LOX-1 expression could be restored after treatment with the HDAC inhibitor NaBu. Whether SAHA has the same effect on 15-LOX-1 expression has yet to be confirmed. Besides the restoration of 15-LOX-1, up-regulation of KAI1, an anti-metastatic protein, could be demonstrated in this study. Similar findings have been described by Joseph et al. in prostate cancer cells [292]. It remains to be shown if both anti-cancer proteins are co-regulated upon induction of 15-LOX-1 expression.

In future experiments, the anti-tumorigenic function of 15-LOX-1 in human pancreatic cancer has to be subject to a detailed mechanistic analysis in order to provide the foundation for the development of preventive measures or the intervention in early stages of pancreatic cancer To this end, cell culture models with inducible 15-LOX-1 expression will be established. Moreover, an orthotopic mouse model will be developed to verify the postulated anti-tumor activity of 15-LOX-1. Such an experiment will also shed light on the potential anti-metastatic activity of 15-LOX-1 in pancreatic carcinogenesis in vivo.

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