The effects of the lichen metabolites usnic acid and protolichesterinic acid on energy and lipid metabolism in cancer cells

Margrét Bessadóttir

Thesis for the degree of Philosophiae Doctor

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Áhrif fléttuefnanna úsnínsýru og prótólichesterínsýru á orku- og fituefnaskipti í krabbameinsfrumum

Margrét Bessadóttir

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Ágrip

Um helmingur krabbameinslyfja sem komið hafa á markað síðustu áratugi á rætur sínar að rekja til náttúrunnar. Fléttur, sem eru sambýli sveppa og þörunga eða blábaktería, hafa verið notaðar í alþýðulækningum við ýmiss konar kvillum frá örófi alda. Fyrri rannsóknir hafa sýnt að tvö hrein annars stigs efni, úsnínsýra (UA) og prótólichesterínsýra (PA) einangruð úr fléttunum hreindýramosa (*Cladonia arbuscula)* og fjallagrösum *(Cetraria islandica)* hafa margvísleg líffræðileg áhrif á frumur. UA er prótónuskutla og getur haft áhrif á himnuspennu í hvatberum og hindrað fjölgun og vöxt ýmissa krabbameinsfrumulína. PA er 5- og 12-lípoxygenasa hindri og hefur fjölgunarhemjandi áhrif á ýmsar gerðir krabbameinsfruma en lítil áhrif á eðlilegar frumur. Markmið þessa verkefnis var að kanna hvernig fjölgunarhemjandi áhrifum þessara efna er miðlað. Notaðar voru krabbameinsfrumulínur sem eru upprunnar úr brjósti, brisi og mergæxli ásamt eðlilegum bandvefsfrumum. Fjölbreyttum aðferðum var beitt við rannsóknir á áhrifum fléttuefnanna á ýmsa ferla og frumulíffæri í frumunum.

Niðurstöðurnar gefa til kynna að UA skutli prótónum yfir himnur hvatbera og lýsósóma í brjóstakrabbameinsfrumum og trufli þannig sýrustigul sem leiðir til minnkunar í orkubúskap frumunnar. Fruman fær því ekki þá orku og efni sem hún þarf til að vaxa og fjölga sér. Einnig getur minnkun í orkubúskap haft áhrif á innanfrumu boðleiðir og hvatt til sjálfsáts. Meðhöndlun með UA leiðir til myndunar sjálfsátsbóla en ekki verður niðurbrot á innihaldsefnum frumunnar í lýsósómum líklega vegna truflunar á sýrustigli. Sjálfsátið nær því ekki að ganga alla leið. Líklegt er að fjölgunarhemjandi áhrif UA skýrist aðallega af prótónuskutlu eiginleikum þess.

Rannsóknir á lípíð efnasambandinu PA gefa til kynna að vaxtarhemjandi áhrifum þess á bris- og mergæxlisfrumur sé ekki miðlað með hindrun á lípoxygenasa. Líklegt er að PA hafi áhrif snemma í stjórnun frumuhringsins og hindri mögulega beint DNA polýmerasa. PA hefur svipaða efnabyggingu og þekktur fitusýrusynþasa (FASN) hindri og niðurstöðurnar gefa til kynna að PA hindri FASN í brjóstakrabbameinsfrumum sem leiðir til minnkunar í umritun á HER2 viðtakanum og síðkominna áhrifa á vaxarboðleiðir í frumunni. Áhugavert er að sjá að verkunarmáti PA í krabbameinsfrumum virðist vera mun flóknari og fjölþættari en hjá UA og tengist að öllum líkindum efnafræðilegum eiginleikum fléttuefnisins að einhverju leyti.

Niðurstöður samvirknitilrauna með fléttuefnin og þekkt krabbameinslyf gefa til kynna að samvirknin byggist bæði á sérkennum hvers krabbameins og

mismunandi áhrifum þeirra á orku og efnaskipti í frumunni. Líklegt er að samvirknin geti einnig byggst á upptöku og dreifingu efnanna og áhrifum þeirra á boðleiðir í frumunni.

Lykilorð: Fléttuefni, krabbamein, prótónuskutla, sjálfsát, fitusýrusynþasi.

Abstract

Around half of all chemotherapeutic agents that have been marketed over the past decades are natural products or directly derived from them. Lichens, formed by symbiosis between fungi and green algae or cyanobacteria, have been used traditionally for centuries to treat various disorders. Previous data have shown that two pure secondary lichen metabolites, usnic acid (UA) and protolichesterinic acid (PA) isolated from *Cladiona arbuscula* and *Cetraria islandica*, respectively can exhibit several biological activities on cells. UA shuttles protons across membranes and affects the mitochondrial membrane potential. UA also has anti-proliferative and growth inhibitory effects on several cancer cell lines. PA inhibits 5- and 12-lipoxygenase and has anti-proliferative effects on several cancer cells lines without effecting normal skin fibroblast. The main aim of this project was to try to understand the mechanisms that lie behind the anti-proliferative effects of the two lichen compounds. Breast-, pancreatic and multiple myeloma cancer cell lines along with normal fibroblasts were used for these studies. Various methods were used to estimate the effects of the two compounds on several pathways and cellular organelles.

The results indicate that UA shuttles protons across mitochondrial and lysosomal membranes in breast cancer cells which disrupts the pH gradient. This can lead to decrease in cellular energy and therefore affect the ability of the cell to grow and proliferate. Also, a decrease in cellular energy can affect intracellular signalling pathways and initiate autophagy. Treatment with UA triggers formation of autophagosomes, however no degradation of the cytoplasmic contents occurs in the lysosomes, likely because of deregulated pH. Therefore the autophagic process is not completed. It is likely that the anti-proliferative effects of UA can be explained by its proton shuttling effects.

Studies on the lipid compound PA indicate that its anti-proliferative effects are not associated with its lipoxygenase-inhibitory activity in pancreatic and myeloma cancer cells. It is likely that PA is affecting the cells at an early stage in the cell cycle possibly through direct inhibition of DNA polymerase. The chemical structure of PA is similar to a known fatty acid synthase (FASN) inhibitor and the results indicate that PA inhibits FASN in breast cancer cells leading to transcriptional repression of the HER2 receptor and secondary effects on major signalling pathways. Interestingly, the mode of action of PA seems to be more complex and diverse than for UA and is probably linked to its chemical properties.

Results on synergism between the two lichen compounds and known cancer drugs suggest that the synergistic effects of these compounds are dependent on the characteristics of the cancer and how the compounds affect cellular metabolism. It is likely that the synergism is also based on the uptake and distribution of the compounds and their effects on cell signalling pathways.

Keywords: Lichen metabolites, cancer, proton shuttle, autophagy, fatty acid synthase.

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Finally I want to thank my parents, my precious, Katla and Ása Kristín and my family and friends for endless support throughout this long journey.

Contents

List of abbreviations

5-FU –fluorouracil

AA – arachidonic acid

ADCs – antibody-drug conjugates

AMPK – AMP-activated protein kinase

ATG –autophagy-related gene

CDK –cyclin dependent kinase

CI –combination index

COX – cyclooxygenase

CPT-1 – carnitine palmitoyl-transferase I

CQ – chloroquine

CYP450 –cytochrome P450

DSB –double strand break

EETs – epoxyeicosatrienoic acids

EFGR – epidermal growth factor receptor

ERK1 – extracellular signal-regulated kinases 1

ERK2 – extracellular signal-regulated kinases 2

FASN –fatty acid synthase

FDA - US food and drug administration

HER2 – human epidermal growth factor receptor 2

HETEs – hydroxyeicosatetraenoic acids

HPETEs – hydroxyperoxy-eicosatetraenoic acids

LC3-I – microtubule-associated protein 1A/1B-light chain 3

LOX – lipoxygenase

mAb – monoclonal antibody

MAPKs –mitogen-activated kinases (MAPKs)

MEK1 –mitogen-activated protein kinase kinase 1

MEK2 – mitogen-activated protein kinase kinase 2

MIC – minimum inhibitory concentrations

MM –multiple myeloma

mTORC1 – mammalian target of rapamycin complex 1

NADH –reduced nicotinamide adenine dinucleotide

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NMR – nuclear magnetic resonance

PA – (+)-protolichesterinic acid

PCD –programmed cell death

PCNA – proliferating cell nuclear antigen

pH_e – extracellular pH

pHi –intracellular pH

PI3K – phosphatidylinositol-3-kinase

Pol β – polymerase beta

Pol δ – polymerase delta

PS –phospatidylserine

PTEN – phosphatase and tensin homologue deleted on chromosome 10

SRB – sulforhodamine B

STAT3 – signal transducer and activator of transcription 3

TCM – traditional Chinese medicine

TUNEL – terminal dexoynucleotidyl transferase assay

UA – (+)-usnic acid

List of figures

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List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Proton-shuttling lichen compound usnic acid affects mitochondrial and lysosomal function in cancer cells.** Bessadóttir M, Egilsson M, Einarsdóttir E, Magnúsdóttir IH, Ögmundsdóttir MH, Ómarsdóttir S, Ögmundsdóttir HM. PLoS One. 2012: 7(12):e51296. Doi:10.1371/journal.pone.0051296. Epub 2012 Dec 5.

II. **Effects of anti-proliferative lichen metabolite, protolichesterinic acid on fatty acid synthase, cell signalling and drug response in breast cancer cells.** Bessadóttir M, Skúladóttir EÁ, Gowan S, Eccles S, Ómarsdóttir S, Ögmundsdóttir HM. Phytomedicine 21 (2014), pp. 1717-1724. DOI information: 10.1016/j.phymed.2014.08.006.

III. **Anti-proliferative and pro-apoptotic effects of lichen-derived compound protolichesterinic acid are not mediated by its lipoxygenase-inhibitory activity.** Bessadóttir M, Eiríksson FF, Becker S, Ögmundsdóttir MH, Ómarsdóttir S, Thorsteinsdóttir M, Ögmundsdóttir HM. Submitted for publication in Prostaglandins Leukotrienes and Essential Fatty Acids (PLEFA).

Declaration of contribution

With the guidance and supervision of Prof. Helga M. Ögmundsdóttir and Prof. Sesselja Ómarsdóttir, Margrét Bessadóttir planned and carried out the experiments presented in the thesis. Margrét Bessadóttir, Helga M. Ögmundsdóttir and Sesselja Ómarsdóttir designed the studies behind following papers.

Paper I: Margrét Bessadóttir performed the ATP measurements, the Western blotting, plasmid transfection, fluorescent acidotropic probing, and immunocytochemistry experiments; Már Egilsson performed the electron microscopy experiments; Eydís Einarsdóttir performed the immunostaining shown in fig. S1A-B; Íris H. Magnúsdóttir contributed to the Western blotting. Margrét Bessadóttir and Helga M. Ögmundsdóttir wrote the manuscript, Sesselja Ómarsdóttir and Margrét H. Ögmundsdóttir contributed to the writing of the manuscript.

Paper II: Margrét Bessadóttir performed the isolation and structural identification of the lichen compound, the cell viability assay and the immunofluorescence staining. Sharon Gowan assisted with the measurements in the MSD assay. Edda Á. Skúladóttir performed the synergism assay. Margrét Bessadóttir, Sesselja Ómarsdóttir and Helga M. Ögmundsdóttir wrote the manuscript.

Paper III: Margrét Bessadóttir performed the cell viability assay and the immunofluorescence staining. Stefan Becker performed the TUNEL and annexin V/Propidium iodide double stainings: Finnur F. Eiríksson performed the extraction of lipids and mass spectrometry measurements. Margrét Bessadóttir, Finnur F. Eiríksson and Helga M. Ögmundsdóttir wrote the manuscript.

1 Introduction

1.1 Natural product – history and drug discovery

Natural products and their derivatives play a dominant part in the development of new compounds for medicinal purposes (Newman and Cragg, 2012). A record which dates from 1500 BCE documents over 700 drugs, most of them derived from plants, and many of them are still used today against various disorders (Borchardt, 2002). In recent time, natural products have continued to be important sources for drug discovery and inspiration for development of semi-or total synthesis of effective new drugs (Cragg et al., 2009; Newman and Cragg, 2012). From the year 2005 to 2010, 17 natural-product-based drugs were approved by the US food and drug administration (FDA) for marketing (Mishra and Tiwari, 2011). The therapeutic area of cancer research benefits from the molecular diversity of natural compounds and over the past 70 years about 50% of small molecules identified in this area are natural products or directly derived from them (Newman and Cragg, 2012). It is therefore important to expand research on natural products and combine different methodologies to discover novel agents and drug entities.

1.2 Lichens – potential source of drug therapies

Lichens have been used for several diverse purposes over the ages, in cosmetics, as dyes, for the evaluation of air pollution, as well as for dietary and medicinal purposes (Shukla et al., 2010). Lichens are formed by symbiosis between fungi and a photosynthetic partner (green algae in 90% of lichens) and/or cyanobacteria and sometimes non-photosynthetic bacteria (Grube and Spribille, 2012; Selbmann et al., 2010). Lichens are distributed worldwide and in most habitats. They cover about 8% of the earth's land surface and the lichen flora is estimated to include approximately 18.500 species (Feuerer and Hawksworth, 2007; Vernon, 1995). Because of their ability to tolerate adverse atmospheric conditions and their symbiotic relationship, lichens can produce a variety of compound classes (Boustie et al., 2011; Lawrey, 1986). These compounds have a role in protecting the lichens against diverse consumers and defend them against environmental stress like UV radiation. They also take part in regulating cell metabolism e.g. by increasing the permeability of the cell wall of the phycobionts to enhance flow of nutrients. Lichens produce two different types of metabolites, primary and secondary. Primary metabolites are often water soluble and take part in

cellular metabolism and structural functions and are synthesized by both symbionts independently (Nash, 1996; Shrestha and St Clair, 2013). Secondary metabolites are thought to be mainly produced by the fungi and are biosynthetically derived from the mevalonate, shikimate and acyl-polymalonate pathways and consist of aliphatic, cycloaliphatic, aromatic and terpen compounds (Figure 1) (Boustie and Grube, 2005; Huneck, 1999).

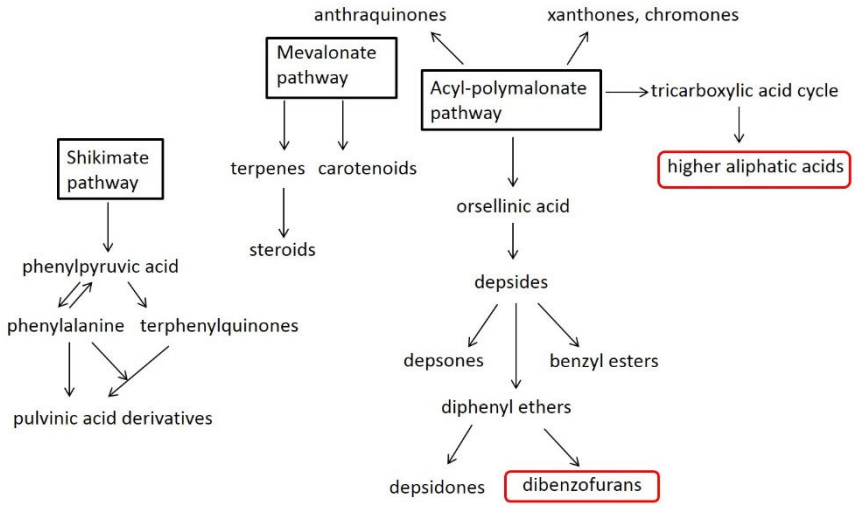


Figure 1. Biosynthetic pathways of lichen secondary metabolites.

The red boxes indicate the two chemical classes that are mentioned in this project. The figure is based on (Molnar and Farkas, 2010).

Over 1050 secondary lichen metabolites have been reported using various analytical methods (Molnar and Farkas, 2010; Stocker-Worgotter, 2008). Several secondary lichen metabolites have been demonstrated to have various possible biological functions including anti-bacterial, anti-inflammatory, anti-proliferative, anti-viral and antioxidant activity (Shrestha and St Clair, 2013). However, there is a lack of interest in lichen metabolites within the pharmaceutical industry. This is due to their slow growth rate and because of how difficult it is to propagate lichens in culture and synthesize their compounds. In addition, the typical lichen compounds are usually not produced in cultures, therefore further development of techniques for synthesizing these compounds is needed (Miao et al., 2001; Shrestha and St Clair, 2013).

1.2.1 Usnic acid – biological activities

Usnic acid (UA) [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-furandione; $C_{18}H_{16}O_7$] is a weak lipophilic acid (pK_a 4.4; 8.8 and 10.7). UA was first described in detail by a German scientist W. Knop in the year 1844. It is widely distributed in various species of lichens including *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae) and other lichen genera. The biosynthesis of UA is derived from the acyl-polymalonate pathway and it occurs in two enantiomeric forms, depending on the projection of the angular methyl group at the chiral 9b position (Figure 2) (Ingolfsdottir, 2002).

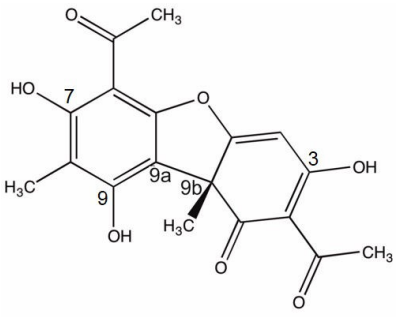


Figure 2. Chemical structure of (+)-usnic acid.

The two enantiomeric forms of UA depend on the projection of the angular methyl group at the chiral 9b position. The 3-OH group has the strongest acidic character, pK_a 4.4. The acidity of the phenolic 9-OH is pK_a 8.8 and the phenolic 7-OH is weakly acidic, pK_a 10.7.

Most of its biological activity has been associated with the (+) - enantiomer. UA is one of the lichen metabolites that have been most extensively studied and is now commercially available (Muller, 2001; Yellapu et al., 2011). In the 1950s Josef Klosa described that UA had anti-bacterial activity against tuberculosis bacilli and gram-positive organisms. Since then UA has been shown to be effective against several pathogenic bacteria (Ingolfsdottir, 2002; Shrestha and St Clair, 2013). Recent research showed anti-bacterial effects of UA *in vitro* on two gram positive strains, *S.aureus* and *B.subtilis,* with minimum inhibitory concentrations (MIC) 1.2 µg/mL and 5.6 µg/mL, respectively. MIC value after ampicillin treatment was 0.4 µg/mL (Paudel et al., 2010). Interestingly, the interaction of UA in combination with five different antibiotics against methicillin resistant clinical isolates of *S.aureus* has recently been evaluated. Result showed that UA, in combination with

gentamicin, gave synergistic action. These are promising results which could help in the battle against antibiotic resistance (Segatore et al., 2012).

UA has also been shown to have anti-tumour activity. The first experiments showing this effect were reported about four decades ago showing reduction of lung carcinoma growth in mice upon treatment with UA (Kupchan and Kopperman, 1975). A few years later a study on structure-activity relationship of UA indicated a link between its cytotoxicity and lipophilic properties (Takai et al., 1979). In recent years it has been shown that UA affects several cancer cell lines. Loss of viability/cytotoxicity has been detected in leukemia, lung, colon ovarian and breast cancer cells (Backorova et al., 2011; Bezivin et al., 2004; Brisdelli et al., 2013; Koparal et al., 2006). However, the mechanism behind the biological activity of UA on cancer cells is mostly unknown. UA has been shown to induce apoptosis in murine lymphocytic leukemia cells (Bazin et al., 2008; Bezivin et al., 2004). However, UA did not show any effect on p53 transcriptional activity and is therefore not proposed to be involved in DNA damage (Mayer et al., 2005). A recent study, from our laboratory, showed that UA inhibited cell entry into S phase in breast and pancreatic cells, caused growth inhibition and loss of mitochondrial membrane potential (Einarsdottir et al., 2010). A previous study has reported that UA caused proton leakage by diffusion through mitochondrial membranes (Joseph et al., 2009). In mouse liver cells UA has been shown to disturb the normal metabolic processes of the cell by uncoupling oxidative phosphorylation in mitochondria and by activation of oxidative stress (Abo-Khatwa et al., 1996; Han et al., 2004). A study on human colon cells also indicated a great loss in the mitochondrial membrane potential as well as caspase-3 activation after UA treatment (Backorova et al., 2012). UA induced apoptosis through depolarization of the mitochondrial membrane potential in lung carcinoma cells and induced G0/G1 cell cycle arrest through modulation of cell cycle regulators (Singh et al., 2013b). In addition to growth inhibitory/anti-proliferative and anti-bacterial effects of UA it is proposed to have other biological effects such as anti-pyretic and analgesic effects (Okuyama et al., 1995), anti-viral activity (Sokolov et al., 2012; Yamamoto et al., 1995) and anti-inflammatory effects (Jin et al., 2008; Vijayakumar et al., 2000).

The use of commercial herbal products against various conditions has increased over the past decades. However, data on their effectiveness and safety are often lacking and some of the herbal medicine products have been associated with hepatotoxicity (Bunchorntavakul and Reddy, 2013). Several reports have indicated toxic effects on the liver after intake of non-

prescription pure UA (Pure Usnic Acid, Industrial Strength™; 500 mg a day) or weight loss supplements containing UA (Durazo et al., 2004; Favreau et al., 2002; Pramyothin et al., 2004; Sanchez et al., 2006; Yellapu et al., 2011). More systematic and thorough studies on the pharmacokinetic of UA are needed to evaluate its adverse and toxicological effects.

1.2.2 Protolichesterinic acid – biological activities

Iceland moss, *Cetraria islandica* has been used traditionally to treat bronchial and inflammatory conditions and for treatment of gastritis and duodenal ulcers (Ingolfsdottir et al., 1994). (+)-Protolichesterinic acid (PA) [(2S)-2β-Tridecyl-4-methylene-5-oxotetrahydrofuran-3α-carboxylic acid; $C_{19}H_{32}O_4$] is an aliphatic α-methylene γ-butyrolactone and was reported by Otto Sticher in 1965 to be a component of *Cetraria islandica* (Sticher, 1965) (Figure 3). PA is considered to be the major biologically active second metabolite isolated from the lichen but can rearrange into its tautomeric form, lichesterinic acid because of its chemical instability (Muller, 2001).

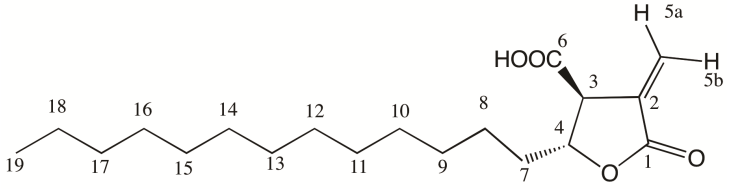


Figure 3. Chemical structure of (+)-protolichesterinic acid.

PA has been reported to have anti-bacterial activity, specifically against *Helicobacter pylori* (Ingolfsdottir et al., 1997) and mycobacteria (Ingolfsdottir et al., 1998) supporting the traditional use of Iceland moss for treating peptic ulcers and chest conditions. PA has also been shown to have an effect on several other types of bacteria (Turk et al., 2003) and to exhibit anti-fungal activity (Goel et al., 2011). In addition, our laboratory has shown that PA has anti-proliferative effects on several types of cancers cells, without affecting normal skin fibroblasts (Haraldsdottir et al., 2004; Ogmundsdottir et al., 1998). The mechanism of this anti-proliferative activity of PA is however largely unknown. However, a recent study on the effects of PA and synthetic analogues revealed that the α-methylene γ-lactone structural motif is important for the *in vitro* toxicity of these compounds in melanoma cell line (Le Lamer et al., 2014). It has been suggested that cross-talk between intrinsic and extrinsic apoptotic pathways in HeLa cells could explain in part the anti-proliferative effects of PA (Brisdelli et al., 2013). In prostate cancer cells the anti-proliferative effects also appear to be mediated through

induction of apoptosis and in part via the inhibition of Hsp70 expression, high expression of Hsp70 may take part in tumour formation and resistance to chemotherapy (Russo et al., 2012). PA has been shown to inhibit human immunodeficiency virus-1 reverse transcriptase through non-specific binding to the enzyme at non-substrate binding sites and to inhibit DNA polymerase beta (Pol β) (Pengsuparp et al., 1995) which could imply that the anti-proliferative effects of PA could be mediated through direct effects on DNA polymerase. In addition PA was shown to inhibit DNA ligase I, which catalyses the joining of DNA strands, however, in order to estimate the mode of inhibition mediated by PA, enzyme-kinetic studies are needed (Tan et al., 1996). The traditional use of Iceland moss, throughout centuries, to treat bronchial and inflammatory conditions also led to experiments on the effects of PA on lipoxygenase (LOX) activity. Leukotrienes, products of the LOX pathway, are involved in a number of homeostatic biological functions and inflammation (Janakiram et al., 2011; Pidgeon et al., 2007). Result showed that PA was a moderate inhibitor of 5-LOX from porcine leukocytes (Ingolfsdottir et al., 1994) and from bovine leukocytes (Kumar and Muller, 1999), respectively. PA also exhibited inhibitory activity on platelet-type 12(S)-LOX using human platelets as a source of the enzyme system (Bucar et al., 2004).

1.3 Cancer

Cancer arises from dynamic changes in the genome which can lead to the progressive conversion of normal cells into cancer cells. Formation of a malignant tumour can occur if the cells fail to control the cell growth through the critical equilibrium that needs to exist between proliferation, differentiation and death. More than 100 distinct types of cancer and subtypes of tumours exist in different tissues and organs (Hanahan and Weinberg, 2000). To understand the fundamental processes underlying the variety of neoplastic diseases six important hallmarks in cell physiology of malignant transformation have been proposed which include: sustained proliferative signals; evading growth suppressors; activation of invasion and metastasis; enabling replicative immortality; induction of angiogenesis and resistance on cell death. There is increasing evidence that two additional emerging hallmarks can be involved in cancer. The first is the capability of the cancer cell to reprogram energy metabolism and the second is allowing the cancer cells to avoid immunological destruction. In addition two enabling characteristics, that can be causally associated with the hallmarks, have also been proposed, they are genome instability and mutation and tumour-

promoting inflammation (Hanahan and Weinberg, 2011). The abilities of these hallmarks of cancer are believed to be shared in common by all types of tumours even though the paths that the cell chooses to become malignant are very variable (Hanahan and Weinberg, 2000).

1.3.1 Breast cancer

Breast cancer is the most common cancer diagnosed in women and accounts for 22% of all female cancer worldwide and 29.5% of all female cancers in Iceland (Jónasson and Tryggvadóttir, 2012; Oldenburg et al., 2007). Factors that are involved in the cause of breast cancer are both genetic and lifestyle/environmental. Female gender, increasing age, reproductive factors and family history are the most important risk factors (Draper, 2006; Oldenburg et al., 2007; Tryggvadottir et al., 2006). Extensive studies, organized screening and progress in chemotherapy are important aspects that have led to reduction in mortality of breast cancer patients (Howard and Bland, 2012). Stimulation of cell growth, division and survival in breast cancers is incited by mutations in proto-oncogenes, genes that control normal growth, or loss of function in tumour suppressor genes. As a result of these mutations various pathways become deregulated. Among oncogenes that are frequently deregulated in breast cancers are the human epidermal growth factor receptor 2 (HER2), PIK3CA, MYC and CCND1 (Fanale et al., 2012). Family history is one of the risk factors for breast cancer. Ten tumour suppressor genes have been linked to hereditary breast cancer and studies have shown that mutations in the major breast cancer susceptibility genes, BRCA1 and BRCA2, are associated with a significantly increased risk for breast and ovarian cancers (Fanale et al., 2012; Lee and Muller, 2010; Tryggvadottir et al., 2003). A recent prospective analysis indicates that the average cumulative risk by age 70 years for BRCA1 and BRCA2 carriers is 60% and 55% for breast cancer and 59% and 16.5% for ovarian cancer, respectively (Mavaddat et al., 2013). It is estimated that susceptibility genes explain 20-25% of the risk of developing breast cancer, and probably more genes are still to be found. This indicates that many low-penetrating genes are working together and their combined effects could be increasing the risk of breast cancer (Fanale et al., 2012; Oldenburg et al., 2007).

Human breast tumours can be classified into four distinct subtypes by gene expression patterns. The luminal subgroup, associated with highly expressing genes of the breast luminal cells; the basal-like subgroup, expressing genes typically in breast basal epithelial cells; the third subgroup overexpresses the HER2; and the fourth group is classified as normal-like breast tumours,

tumours that cluster with normal breast samples (Perou et al., 2000). These classifications and further understanding of the diversity of both natural history and responsiveness to treatment is necessary for development of new drug targets (den Hollander et al., 2013). Surgery, radiation therapy, chemo-, hormone- and targeted therapy are the general treatment options for breast cancer. Antracyclines (doxorubicin) and taxanes (paclitaxel) are important drugs and are used in chemotherapy alone or in combination with other drugs, like anti-metabolites ((fluorouracil (5-FU)) and alkylating agents (cyclophosphamide). Hormone treatment with e.g. estrogen receptor antagonist (tamoxifen) is commonly used as an adjuvant therapy to reduce the risk of relapse of the cancer after surgery. Different treatment approaches can be used depending on the subtype and stage of the breast cancer (American Cancer Society, 2014). Drug resistance is an increasing problem among breast cancer patients. For example, in patients with overexpression of HER2, resistance to targeting agent, trastuzumab, commonly occurs within one year of treatment (Nahta et al., 2006). This emphasizes the need for further studies on the potential benefits of combinational therapy as a first line treatment; this will be discussed later in the thesis.

1.3.2 Pancreatic cancer

Pancreatic cancer is a disease with poor prognosis, which is difficult to diagnose, and 5-year survival rate is less than 5% (Furuse and Nagashima, 2012; Hidalgo, 2010). Pancreatic cancer accounts for around 2% of all cancers in men and women in Iceland (Jónasson and Tryggvadóttir, 2012). The causes of pancreatic cancer are unknown, smoking is a risk factor, and a relationship between diabetes and pancreatic cancer has been reported (Ben et al., 2011). Other environmental factors have been less studied (Hassan et al., 2007). The KRAS2 oncogene is mutated in approximately 90% and TP53 is abnormal in 50-75% of malignant tumours which can lead to constant activation of signalling pathways and genomic instability (Hidalgo, 2010). It is estimated that family history explains 5-10% of pancreatic cancer (Shi et al., 2009). Some crucial mutations in BRCA1/2, PALB2, ATM and other genes are the cause of pancreatic cancer in some patients with family history (Fendrich et al., 2014; Lucas et al., 2013; Solomon et al., 2012). In sporadic pancreatic cancer the genetic basis is extremely complex and heterogeneous, but there are indications that common variants in BRCA2 and MAP2K4 could be linked to sporadic pancreatic cancer (Hidalgo, 2010; Huang et al., 2013). The main treatment options for pancreatic cancer are surgery, ablative techniques such as using radiofrequency or microwave thermotherapy to destroy the tumour, chemotherapy and radiation therapy

(American Cancer Society, 2014). A nucleoside analogue (gemcitabine) is often used in the treatment of pancreatic cancer. Combination treatment with other cytotoxic drugs such as 5-FU, cisplatin and others have failed to significantly improve the overall survival of patients (Berlin et al., 2002; Heinemann et al., 2006). More recent studies have however revealed that treatment with gemcitabine and erlotinib, a tyrosine kinase inhibitor, can improve outcomes in pancreatic patients (Yang et al., 2013). It is difficult to diagnose pancreatic cancer at an early stage and resistance to chemotherapy is common. Therefore, over the past years the mortality rate has not improved. There is a critical need for discovery of novel therapeutic targets for treatment of pancreatic cancer and strategy development for early detection of the disease (Hidalgo, 2010).

1.3.3 Multiple myeloma

Multiple myeloma (MM) is the second most common haematological cancer and accounts for 0.8% of all diagnosed cancer in the world (Parkin et al., 2005) and for approximately 1.3% of all cancers in Iceland (Jónasson and Tryggvadóttir, 2012). MM arises when plasma cells, that generally produce a monoclonal immunoglobulin, become malignant and accumulate in the bone marrow (Smith and Yong, 2013). It can be classified by genetic abnormalities into two categories, IgH translocations and genomic imbalances, both containing genetic factors that count for unfavourable prognosis of the disease (Bergsagel et al., 2013). Due to the rarity of MM it is hard to define the strength of environmental and hereditary genetic factors in development of the disease (Alexander et al., 2007). MM remains an incurable disease even though the overall survival has improved over the past 10 years mostly because of introduction of high dose chemotherapy and stem-cell rescue along with new therapeutic agents (Bergsagel et al., 2013). Combination therapy has also been shown to be beneficial in patients with relapsed disease (Richardson et al., 2005) and the first reversible proteasome inhibitor approved by the FDA in 2003 for treatment of MM, bortezomib, displays a wide synergistic effect with other cancer agents (Romano et al., 2013).

1.4 Deregulated control of proliferation and survival in cancer cells

1.4.1 Cell cycle control

The cell cycle is a series of events that involve replication of DNA, division of the nucleus and partitioning of the cytoplasm to generate two daughter cells. Between nuclear division (M-phase) and DNA synthesis (S-phase) is a gap

called G1 and another gap, G2, is placed between S and M phase. In those gap periods repairs of replication errors and DNA damage can take place (Massague, 2004). DNA is synthesized from nucleosides during the S-phase of the cell cycle. The polymerase delta (Pol δ) is an essential protein which participates in the replicative DNA synthesis process. Pol δ is stimulated by proliferating cell nuclear antigen (PCNA) to act as a primary lagging strand replicase (Prindle and Loeb, 2012). It is possible to quantify DNA synthesis accurately and estimate cell proliferation by measuring uptake of ^{3}H-tymidine that occurs in DNA but not in RNA (Tehrani and Shields, 2013). To maintain genome stability between generations the cell uses several DNA damage response mechanisms such as DNA repair, cell cycle checkpoint arrest and apoptosis (Jeggo and Lobrich, 2006). Homologous recombination and DNA non-homologous end-joining represent the two main mechanisms for repair of DNA double strand break (DSB) in mammalian cells (Hefferin and Tomkinson, 2005). The cell cycle checkpoints at the G1/S, G2/M control DNA damage through a complex signalling network either by synergism with DNA repair mechanisms to maintain chromosomal stability or by initiating the arrest or delay of cell cycle progression. Thus, the delay creates time for repairing DNA damage and replication errors or prevents proliferation of damaged cells (Lukas et al., 2004; Massague, 2004). By using a fluorochrome such as propodium iodide that binds and labels DNA and examine the stained cell with flow cytometry it is possible analyse the cell cycle parameters of dividing cells (Riccardi and Nicoletti, 2006).

The specific phases of the cell cycle are regulated by several of molecular effectors such as cyclin dependent kinases (CDK) and cell cycle inhibitors. A major cell cycle restriction point (R) is at the end of the G1 phase and after that point there is no further need for extracellular proliferation stimulants (Figure 4).

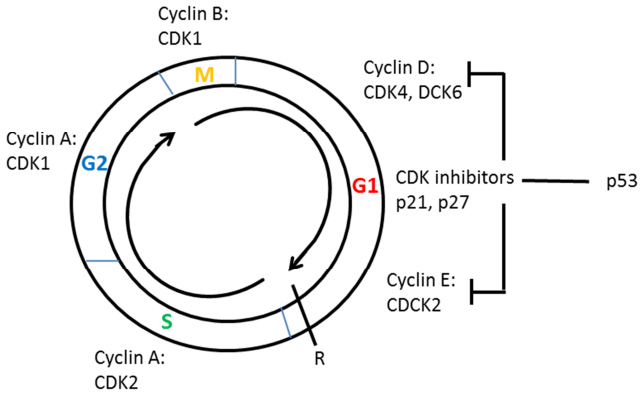


Figure 4. Cell cycle control regulators.

The figure is based on (Dehay and Kennedy, 2007).

The expression of p21, a CDK inhibitor, is regulated by p53 and it has been suggested that p21 is activated as a reaction to DSB resulting in inhibition of entry from G1 into S phase (Abukhdeir and Park, 2008; Lukas et al., 2004; Massague, 2004). The expression of p21 in human cancers depends on cellular context indicating that it can both serve as a tumour suppressor or as an oncogene (Abbas and Dutta, 2009).

1.4.2 Apoptosis – programmed cell death

The balance of cell growth and cell death is regulated mainly by programmed cell death (PCD) and autophagy (Liu et al., 2011). The best studied form of PCD is called apoptosis. PCD can be mediated through the mitochondrial pathway (the intrinsic pathway), or triggered by receptor signalling (the extrinsic pathway). The sensitivity towards apoptosis is cell line dependent (Jeggo and Lobrich, 2006; Ouyang et al., 2012). The nuclear transcription factor p53 and mitochondrial proteins like the Bcl-2 family include key regulators of the intrinsic apoptosis pathway. Post-translational modifications of the anti-apoptotic members of the Bcl-2 family occur following a death signal, they become activated and are translocated to the mitochondria which become permeable leading to release of cytochrome *c* into the cytosol. Caspases become activated and apoptosis is triggered (Ouyang et al., 2012). The extrinsic apoptotic pathway is induced through transmembrane receptor of the tumour necrosis factor family leading to caspase activation and onset of apoptosis (Evan and Vousden, 2001).

In early apoptotic cells phospatidylserine (PS) from the inner side of the plasma membrane is translocated to the outer layer of the membrane, which still remains intact. Measurements of Annexin V, a Ca^{2+} dependant phospholipid binding protein with high affinity for PS, is a valid method to detect early apoptosis and discriminate between apoptotic and necrotic cells, which lose their cell membrane integrity (Vermes et al., 1995). Based on the principle that DNA of apoptotic cell becomes fragmented and nuclear DNA content is then lost, PI staining, mentioned above, is also useable to estimate apoptosis. However not all apoptotic cells undergo these DNA changes and thus to confirm apoptosis, methods such as terminal dexoynucleotidyl transferase assay (TUNEL) which demonstrates specifically DNA breaks by labelling the terminal end of nucleic acids, can be applied (Riccardi and Nicoletti, 2006).

1.4.3 Signalling pathways

Deregulation of signalling pathways to maintain chronic proliferation is one of the fundamental characteristics of cancer cells. In normal cells transmembrane receptors can bind to different signalling molecules such as growth factors, cytokines and hormones and transmit signals into the cell. In contrast to normal cells, tumour cells are not dependent on exogenous growth stimulation, they can generate many of their own growth signals (Hanahan and Weinberg, 2000). The RAS proteins were among the first proteins that were identified as cell growth regulating proteins and shown to be constitutively active in cancers because of point mutations in their coding sequences (Barbacid, 1987). RAS gene mutations can be found in various of cancers, e.g. pancreatic cancer, colon cancer and multiple myeloma, however, their frequency varies between cancers (Bezieau et al., 2001; Bos, 1989). Activated RAS contributes to stimulation of protein serine/threonine kinase RAF, activated RAF then phosphorylates and activates many signalling pathways. Among them are the mitogen-activated protein kinases (MAPKs) including the mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2) and extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) which are transported to the nucleus. ERK can phosphorylate transcriptional regulators that express important cell-cycle regulatory proteins such as D-type cyclins (see Fig. 4) which allow the progression of cells from the G0/G1 to S phase (Downward, 2003). The MAPK pathway is deregulated in approximately 30% of all human cancers, it initiates diverse cellular responses and abnormalities and can play a fundamental role in the progression of cancer (Dhillon et al., 2007).

Growth factor receptors are also often overexpressed in cancers and they contain intracellular tyrosine kinase domain that generate signals that are important for cell communications and regulation of cellular function. The HER2 receptor is well studied and is overexpressed in 20-25% of invasive breast cancer and linked to poor prognosis (Hanahan and Weinberg, 2011; Slamon et al., 1987). A dimerization of the receptors with another member of the HER family is required for the function of HER2 which then leads to intracellular responses through several phosphorylation events and protein interactions which regulates cellular activities such as cell proliferation, differentiation and motility through various signalling pathways. The phosphatidylinositol-3-kinase (PI3K)/AKT and the RAS/RAF/MAPK signalling pathways are well studied in HER2 activation and in normal cells these signals are tightly controlled and homeostasis of cell number is maintained (Akinleye et al., 2013; Hanahan and Weinberg, 2011; Pohlmann et al., 2009).

The PI3K/AKT signalling pathway is a key regulator of cell proliferation and survival and is frequently deregulated in human cancers. PI3K transfers signals from cytokines, growth factors and oncoproteins including RAS. AKT is then activated by phosphorylation at the plasma membrane. The tumour suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a crucial negative regulator of PI3K/AKT signalling (Crowell et al., 2007). Deregulation of the AKT pathway can occur through mutation or amplification of PI3K, loss of PTEN function and activation or mutation of receptor kinases such as HER2 or oncogenes such as RAS (Altomare and Testa, 2005). Various effectors mediate the results of AKT activation, and one of them is mammalian target of rapamycin complex 1 (mTORC1). mTORC1 regulates cell growth by modulating many processes including protein synthesis and autophagy, (see later) and plays an important role in several pathways that have been linked to cancer (Crowell et al., 2007; Sabatini, 2006).

1.4.4 Cell signalling pathways as a therapeutic target

Almost a century ago the scientist Paul Ehrlich first described the concept of “magic bullet” and drug targeting. It was not until 50 years later, in 1958 that this concept was used when methotrexate was linked to an antibody for treatment against leukemia (Perez et al., 2013). In 1986 the first monoclonal antibody (mAb) drug was approved by the FDA and in 1998 trastuzumab was marketed as a treatment for metastatic breast cancer. Trastuzumab targets epitopes in the HER2 extracellular domain leading to a down-regulation of the protein, in addition trastuzumab is suggested to inhibit transcription and

decrease the levels of a constitutively active truncated form of HER2 (p95-HER2) (Blair et al., 2014; Incorvati et al., 2013). Another mAb drug, pertuzumab, which blocks heterodimerization of HER2 and HER3 has also shown promising synergistic effects with trastuzumab against breast cancer and is being evaluated further (Gianni et al., 2012). By increased knowledge in technology and the development of highly selective targeted therapy agents, antibody-drug conjugates (ADCs) became an interesting approach in drug development. ADCs are based on the advantages of the target specificity of mAbs to deliver cytotoxic drugs selectively to tumour cells. In 2013 the FDA approved anti-HER2 ado-trastuzumab emtansine, Kadcyla®, which combines trastuzumab with a potent anti-microtubule cytotoxic agent, against HER2-positive breast cancer. More similar agents are currently in clinical trials (Perez et al., 2013). During the 1990s there was also an increasing interest in tyrosine kinase inhibitors as potential targeting agents. One of them, imatinib, was found to selectively inhibit the enzymatic action of the BCR-ABL fusion protein thus affecting cellular growth and inducing apoptosis. In 2001 imatinib was approved by the FDA against chronic myeloid leukemia (Goldman and Melo, 2003; Lambert et al., 2013). Further studies on tyrosine kinase inhibitors led to the development of a dual epidermal growth factor receptor (EGFR)/HER2 inhibitor lapatinib. By inhibiting HER2 it also supresses MAPK/ERK1/2 and PI3K/AKT pathways and is used in combination with other cancer drugs against HER2 positive breast cancer (Geyer et al., 2006). In addition to targeted therapies such as mAbs, ADCs and tyrosine kinase inhibitors, there are a number of MEK and AKT specific inhibitors that have been developed (Akinleye et al., 2013; Crowell et al., 2007). One selective inhibitor of MEK1/2, trametinib, has shown significant clinical efficacy in melanoma, and is currently being assessed by the FDA, and others are in clinical development (Kim et al., 2013). The PI3K pathway is complex and cross-talks with various other signalling pathways. A pan-class I PI3K inhibitor is being tested in combination with other cancer treatments in breast cancer patient with overexpression in the HER2 receptor. Several other PI3K inhibitors are being evaluated as a treatment for different types of cancers. The mTORC1 inhibitor everolimus is approved and used in cancer treatment (Massacesi et al., 2013).

1.5 Deregulation of metabolism in cancer cells

1.5.1 pH

The metabolism of cancer cells is diverse and complicated, and it is important to gain an understanding of its role in the biology of tumour development and behaviour in order to develop new effective drugs (Izumi et al., 2003). Changes in cellular metabolism is one of the hallmarks of cancer (Hanahan and Weinberg, 2011) and there is evidence that these changes are linked to every major oncogene or tumour suppressor gene (Bensinger and Christofk, 2012). Regulation of pH is critical for various cellular functions in normal cells and there are strong indications that pH is deregulated in cancer cells, where intracellular pH (pH_i) is generally higher than the extracellular pH (pH_e) while in normal cells the gradient is vice versa. Increased expression and/or activity of V-ATPases, that are located intracellularly and in the plasma membrane in cancer cells (Hinton et al., 2009) and the stimulation of the plasma membrane Na^+/H^+ exchanger NHE1 (Harguindey et al., 2005) maintain a higher pH_i. Monocarboxylate transporters are also important cellular pH regulators in cancer where their expression is increased; their role is to export accumulating acids and promote the conversion of pyruvate to lactate (Pinheiro et al., 2010). Higher pH_i enables the cancer cell to increase growth factor-independent proliferation, migration and invasion and to avoid apoptosis. The cell then becomes adapted to these conditions (Webb et al., 2011). However there are also strong indications that deregulation in pH in cancer cell can affect drug action (Gerweck et al., 2006). One example is the weak base doxorubicin, used to treat several cancers. Higher concentrations of weak bases are in neutral form in the high pH cytoplasmic state in cancer cell, meaning that the drug can permeate across the membrane and out of the cell. In non-tumour cells weak bases can accumulate inside of the cell and cause toxic effects because the situation is reversed. It is therefore possible that weak acids, such as UA, could benefit from the abnormal pH in cancer cells, higher concentrations become neutral at low pH and the drug accumulates inside the cancer cells and destroys them with little effects on normal cells (Webb et al., 2011). Weak acids could therefore be better drug candidates for therapeutic use than weak bases (Figure 5).

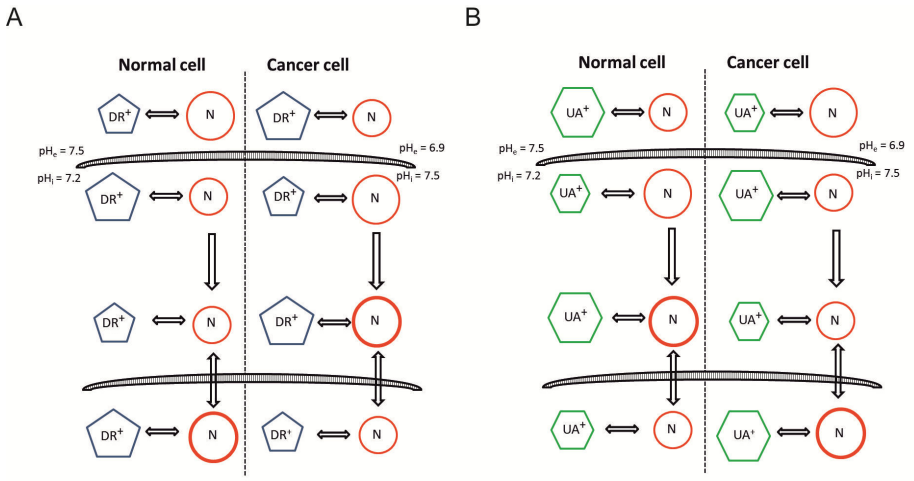


Figure 5. Effects of deregulated pH on drug distribution in cancer cells.

Weak bases accumulate inside normal cells. Weak acids accumulate inside cancer cells. (A) In normal cells, weak bases such as doxorubicin accumulate at higher concentration inside the cells than in extracellular space. That can cause toxic effects in healthy tissues. In cancer cells, were pH is deregulated, less doxorubicin can permeate across the membrane leading to a lower concentration of the drug inside the cancer cells. (B) Weak acids such as UA, become neutral at lower pH and therefore accumulate in extracellular space in normal cell, however, higher concentrations are obtained intracellular in cancer cells. N; neutral form, DR^+; protonated form of doxorubin, UA^+; protonated form of UA. pH_e,;extracellular pH, pH_i,;intracellular pH. The figures are based on (Webb et al., 2011).

Treatment with UA shows significant induction of genes that are linked to complexes I through IV of the electron transport chain, this could be a compensating mechanism to conserve the proton gradient across the inner mitochondrial inner membrane (Joseph et al., 2009; Webb et al., 2011). By increased knowledge of deregulated pH in cancer cells it might be possible to develop agents that could be effective against various classes of cancer.

1.5.2 Autophagy

Autophagy, or cellular self-digestion, is an evolutionarily conserved mechanism by which cells consume parts of themselves to survive starvation and stress (Amaravadi et al., 2011; Mizushima et al., 2008). The principal role of autophagy is to protect organisms against e.g. infections, ageing, neurodegeneration, heart diseases and cancer. Another important role of

autophagy is to maintain cellular energy levels by degrading and recycling cellular components (Levine and Kroemer, 2008). Autophagy was first genetically defined in yeasts, and since then 31 autophagy-related genes (ATG) have been identified (Mizushima, 2007). Many of them are conserved in mammalian cells, 16 ATG have been identified so far, *beclin 1* being the most studied one (Liu and Ryan, 2012). Autophagy is mediated by formation of an autophagosome which forms a double membrane that fuses with the endo-lysosomal system resulting in degradation of the cytoplasmic contents of the autophagic vacuoles and inner membranes by the lysosomal hydrolases (Amaravadi et al., 2011; Mizushima, 2007). It was first described morphologically in kidney cells in new-born mice in 1957 and was first thought to be a nonspecific process. Further studies have revealed that autophagy plays a vital role in the cellular homeostatic function and is regulated by the availability of nutrients, growth factors and hormones. Additionally, stress also regulates autophagy, an increase in damaged organelles, intracellular pathogens or stress in the endoplasmic reticulum can induce autophagy through different pathways that are not activated by starvation (Amaravadi et al., 2011; Chen and White, 2011). The process of autophagy generally includes four steps, initiation, nucleation, maturation of autopagic vacuoles and finally fusion and degradation of intracellular contents (Amaravadi et al., 2011). The mTORC1 pathway is a major regulator of autophagy and controls protein synthesis, cell division and cell metabolism in a nutrient and growth factor sensitive manner as well as during stress (Chen and White, 2011; Jewell et al., 2013). The energy sensor AMP-activated protein kinase (AMPK), which is activated when intracellular adenosine triphosphate (ATP) declines, signals to mTORC1 (Shackelford and Shaw, 2009). The fundamental machinery of autophagy involves several of the ATG proteins that come together to trigger formation of the autophagosome. ULK1/ULK2, a protein kinase, receives signals from mTORC1 and initiates formation of the phagophore, a membrane structure. In the next step, nucleation, *beclin 1* and more ATG genes are recruited for closure of the autophagosome and later for fusion of autophagosome with lysosomes. In the maturation process the cytoplasmic ubiquitin-like protein ATG8/microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-II which is recruited to autophagosomal membranes and incorporated into the lipid bilayer. In order to reach the end step of autophagy the autophagic flux must be completed; substrates must be delivered and degraded inside the lysosome. The role of the autopagic cargo such as p62, is to bind to ubiquitinated proteins, like LC3, and target them for degradation in the lysosome (Amaravadi et al.,

2011; Chen and White, 2011; Liu and Ryan, 2012). The contents of autophagic vesicles coalesce and are degraded by lysosomal hydrolases and contents released to the cytosol. The acidic environment of lysosomes is essential for those final steps of autophagy, which are mediated by UVRAG, RAB7A and the lysosomal protein LAMP2 and involve the vacuolar H^+ ATPase (Figure 6) (Amaravadi et al., 2011; Chen and White, 2011; Liu and Ryan, 2012).

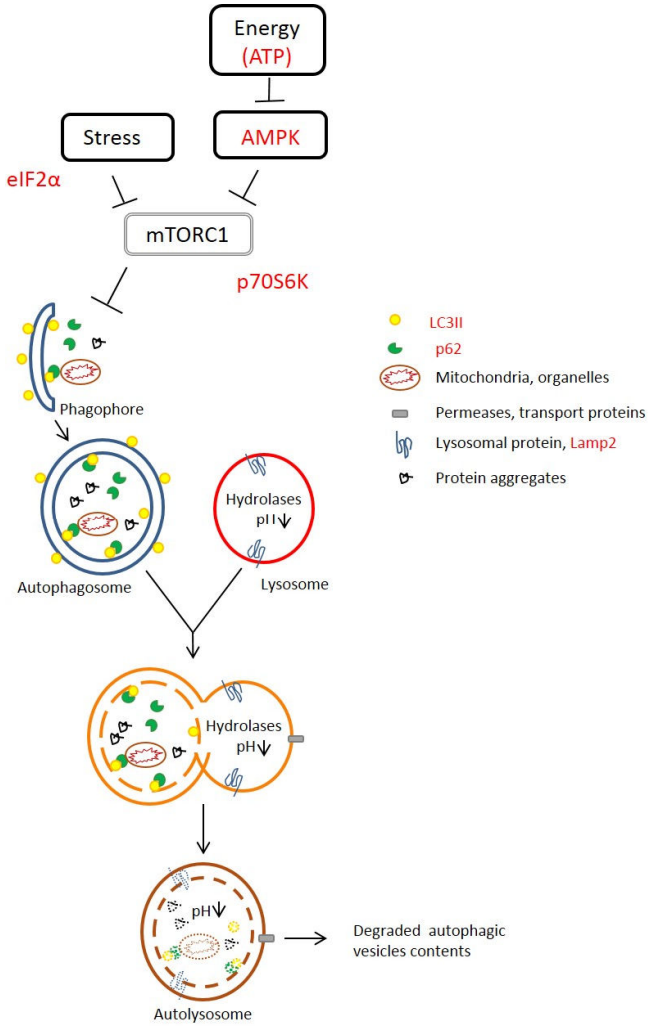


Figure 6. Simplified schematic picture of autophagy.

Red color indicates the factors studied in this project. The figure is based on (Chen and White, 2011).

1.5.2.1 *Role of autophagy in cancer*

Autophagy can both promote tumour cell survival and contribute to tumour cell death and it is likely that the function depends on tumour stage, cellular context and tissue of origin but its role is incompletely understood (Lozy and Karantza, 2012; Rosenfeldt and Ryan, 2011). Increased autophagy can therefore reverse the neoplastic phenotype or protect the cancer cells against metabolic stress including hypoxia and anti-cancer agents (Vazquez-Martin et al., 2009b). The *beclin 1* is classified as a tumour suppressor gene (Liu and Ryan, 2012). Allelic loss of *beclin 1* has been described in human cancers such as breast (Futreal et al., 1992) and prostate cancer (Gao et al., 1995). In addition, tumour-associated mutations and deletions have been reported in several other autophagy regulators (Liu and Ryan, 2012). Autophagy can be stimulated by tumour suppressor genes that are connected to the mTORC1 pathway and its downstream targets p70S6K and 4EBP1, including PTEN and TSC1/2. The mTORC1 pathway is commonly deregulated in human cancers (Guertin and Sabatini, 2007; Levine and Kroemer, 2008; Sarbassov et al., 2005). Also, the adaptor protein p62, that links LC3 with misfolded proteins, is frequently upregulated in cancers. It is thought to contribute to tumour progression (Mathew et al., 2009) and a recent study indicates that p62 accumulates in benign liver tumours formed in a mouse models and reduction in tumour size is seen after p62 deletion (Takamura et al., 2011).

1.5.2.2 *Autophagy as a potential therapeutic target*

Autophagy can be upregulated or suppressed by several therapeutic agents and therefore plays a complex and paradoxical role in cancer therapy (Liu and Ryan, 2012). Autophagy can promote survival of cancer cells during treatment, and therefore induce drug resistance to specific drugs. This has been shown in breast cancer cells after treatment with the DNA-damaging agent camptothecin, where cell death was delayed because of induction of autophagy (Abedin et al., 2007). Autophagy has also been shown to have a cytoprotective role in oesophageal cancer cells treated with 5-FU where chemosensitive cells underwent apoptosis and chemoresistant populations underwent autophagy. Specific inhibition of early autophagy, increased the effects of 5-FU (O'Donovan et al., 2011). A recent study also showed upreglation of autophagy as a protective response to growth inhibitory effects of the anti-HER2 drug trastuzumab in HER2-overexpressing cancer cells. These results indicate that treatment with selective autophagy inhibitors in combination with known cancer drugs could be an efficient way to enhance

therapeutic benefits and avoid resistance (Vazquez-Martin et al., 2009b).

Induction of autophagy does not only have a cytoprotective role it can also act as a pro-death mechanism resulting in destruction of cancer cells. A recent study showed that induction of autophagy by triptolide, an active compound extracted from traditional Chinese medicinal herb, caused cell death in pancreatic cancer cells (Mujumdar and Saluja, 2010). Rapamycin and its derivatives are the most established mTOR inhibitors. They can also act as inducers of autophagy in cancer cells where the autophagic pathway is crucial for the rapamycin mediated anti-cancer activity. In malignant glioma cells, autophagy was shown to be the primary mediator of the rapamycin's anti-proliferative effects (Iwamaru et al., 2007).

Chloroquine (CQ) (Figure 7) has been shown to inhibit autophagy. CQ has been used for decades to treat or prevent malaria, and it has been indicated that its activity could be through the effect on lysosomal enzyme (Homewood et al., 1972).

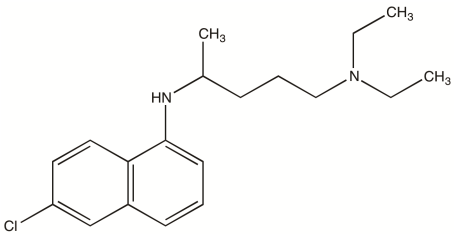


Figure 7. Chemical structure of chloroquine.

CQ is a weak base and when it enters the lysosome it becomes protonated, because of low inside pH. CQ accumulates inside the lysosomes leading to disruption in lysosomal function. The later steps of autophagy are therefore hindered, contents of the autophagic vacuole are not degraded and no energy is provided for the cell (Amaravadi et al., 2011; Kimura et al., 2013). This ability of CQ has led to further research on CQ in cancer treatment. Currently, multiple clinical trials using treatment with CQ or hydrochloroquine alone or in combination with other cancer drugs are ongoing to test if inhibition of autophagy can increase the potency of cancer therapy (Amaravadi et al., 2011). Recent results indicate that the effects of combination treatment CQ and a PI3K-mTOR inhibitor, on cell death in glioma cells, are associated with the ability of CQ to inhibit autophagy (Fan et al., 2010). However, there is also evidence that the combinatory effect of CQ with a PI3K and mTOR inhibitors, in mouse mammary breast cancer cell lines, is independent of autophagy inhibition (Maycotte et al., 2012). This

could indicate that the effects of CQ, when in combination with other drugs, might be mediated by mechanisms other than its inhibition of autophagy.

1.5.3 Mitochondria

In mitochondria carbohydrates and fatty acids are degraded to generate metabolic energy. In the presence of oxygen glucose is metabolized to carbon dioxide by oxidation of pyruvate in the mitochondrial tricarboxylic cycle which generates nicotinamide adenine dinucleotide (NADH). The high energy electrons from NADH are then transferred to molecular oxygen, a proton gradient across the inner membrane is established and the stored energy is used to drive ATP synthesis, the major source of cellular energy. Under anaerobic conditions, normal cells largely reduce pyruvate into lactate in the cytoplasm thus completing the glycolytic cycle (Cantor and Sabatini, 2012; Geoffrey M. Cooper, 2004; Vander Heiden et al., 2009). In 1926 Otto Warburg discovered that ATP production of cancer cells was mainly through metabolism of glucose to lactate even in the presence of abundant oxygen and suggested that cancer was caused by diminished mitochondrial metabolism. Further studies indicated that mitochondrial function was not impaired in most cancer cells, however the fact remains that cancer cells do upregulate glucose metabolism and Warburg's observation is widely used to differentiate between normal and tumour tissue by using emission tomography to image the uptake of radioactive glucose derivatives and use it as a marker for uptake of glucose in the tissue (Gogvadze et al., 2008; Jang et al., 2013; Som et al., 1980).

There is still much to learn about the link between metabolism and proliferation. Whether this metabolic switch from oxidative phosphorylation to less efficient metabolism of aerobic glycolysis in cancer cells is a cause or a consequence of malignant transformation remains an open question but can possibly be explained by two factors. One is that in proliferating cells there is evidence that ATP may never be limiting factor, and the ineffective ATP production is only a problem when resources are limited. The other reason could be that proliferating cells need to maintain high levels of glycolytic intermediates to support biomass accumulation and redox maintenance (Cantor and Sabatini, 2012; Vander Heiden et al., 2009). Down-regulation of the catalytic subunit of the mitochondrial H^+-ATP synthase can occur in e.g. in human lung and breast carcinomas (Cuezva et al., 2004; Isidoro et al., 2005) and inhibition of oxidative phosphorylation in lung cancer cells and carcinomas increases the aerobic glycolysis thus indicating that if energy production from the mitochondria is inhibited cells can become glycolytic.

However, the cancer cells are not able to upregulate the oxidative phosphorylation when glycolysis is supressed (Lopez-Rios et al., 2007; Wu et al., 2007).

In addition to the role of mitochondria in a variety of physiological processes through production of ATP and regulation of cell signalling pathways they have a specific role in apoptotic cell death. The mitochondria release proteins, in response to cell death signals, into the cytosol due to an increase in permeability of the outer mitochondrial membrane (Ulivieri, 2010), as discussed previously in the apoptosis chapter. Agents that suppress mitochondrial respiration or uncouple oxidative phosphorylation have been shown to stimulate cell death, and could therefore be a promising target for development of new anti-cancer agents (Gogvadze et al., 2009).

1.5.4 Lipid metabolism

Lipids are found in almost all forms of life and can be divided into three main categories, isoprenoids, polyketides and fatty acids. Fatty acids consist of a carboxyl acid with a long aliphatic chain which can exist as a free form or bound to a variety of compound such as proteins and carbohydrates. (Abramson, 2011; Liu et al., 2010).

1.5.5 Fatty acid synthase (FASN)

Free fatty acids play a key role in several cellular functions, providing substrate for cell energy metabolism and serving as an important building block for bilayer cell membranes and intracellular second messengers. They also recruit proteins that are involved in signal transduction. Free fatty acids can either be obtained from the diet through the circulation or derived from *de novo* synthesis catalysed by a multifunctional lipogenic enzyme, fatty acid synthase (FASN), in lipogenic tissues such as liver, lactating breast and adipose tissue (Liu et al., 2010) (Figure 8).

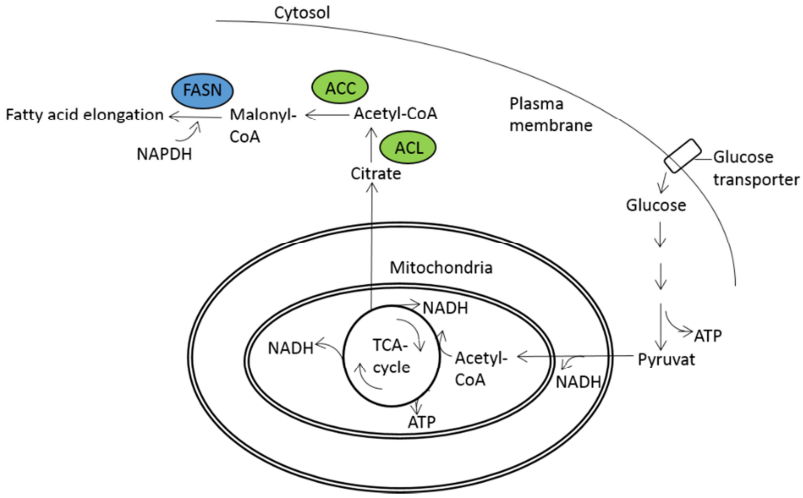


Figure 8. Overview of fatty acid biosynthesis.

Glucose enters the cell and can proceed through glycolysis to produce pyruvate which is converted to acetyl-CoA that enters the tricarboxylic acid (TCA) cycle and produces ATP and NADH molecules. NADH is used in mitochondrial oxidative phosphorylation for ATP production. Citrate is formed in the TCA cycle and transported out of the mitochondria and broken down by ATP citrate lyase (ACL). Acetyl-CoA can be converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). The multifunctional enzyme fatty acid synthase (FASN) catalyses synthesis and elongation of fatty acid chains. The figure is based on (Buchakjian and Kornbluth, 2010).

In 1994 Kuhajda and colleagues showed that a protein that was identical to FASN was linked to poor prognosis in breast cancer (Kuhajda et al., 1994). Further studies reported that upregluation of FASN occurs in a variety of cancers such as breast (Vazquez-Martin et al., 2008; Wang et al., 2001), prostate (Migita et al., 2009) and colon cancers (Notarnicola et al., 2012). The mechanism behind the FASN overexpression has not been fully explained. It is a common characteristic in sex steroid related tumours such as breast carcinoma and it has been shown that synthetic progestins can activate FASN expression (Menendez et al., 2005b). Signalling through the HER2 receptor also plays an important role shown by connection between the FASN and HER2 genes (Kumar-Sinha et al., 2003) and an association between the circulating levels of FASN in the serum and the HER2 extracellular domains levels in the blood of metastatic breast cancer patients has been observed (Vazquez-Martin et al., 2009a).

HER2 is overexpressed in 20-25% of invasive breast cancer and is linked to poor prognosis (Slamon et al., 1987). It has also been shown that FASN

expression is significantly higher in those tumours (Vincent-Salomon et al., 2008). The increased expression of FASN in HER2-overexpressing cells is suggested to be mediated through the MAPK and PI3K signalling cascades (Yang et al., 2002). Furthermore, FASN is directly activated by HER2-mediated phosphorylation of FASN (Jin et al., 2010).

As mentioned above, cancer cells are dependent on *de novo* synthesis of fatty acids making FASN inhibitors suitable candidates for cancer treatment with good therapeutic index. Cerulenin was the first characterized FASN inhibitor derived from the fungus *Cephalosporum caerulens* (Omura, 1976). Cerulenin was shown to trigger apoptosis in cancer cells through FASN inhibition and delay disease progression in a xenograft model (Kuhajda et al., 1994; Pizer et al., 1996). Clinical applications were, however, limited because of the chemical instability of cerulenin due to its reactive epoxide ring structure which may interact with other cellular processes (Lupu and Menendez, 2006). C75, a related analogue of cerulenin was synthesized by eliminating the reactive epoxide ring, resulting in increased chemical stability and specificity (Kuhajda et al., 2000) (Figure 9).

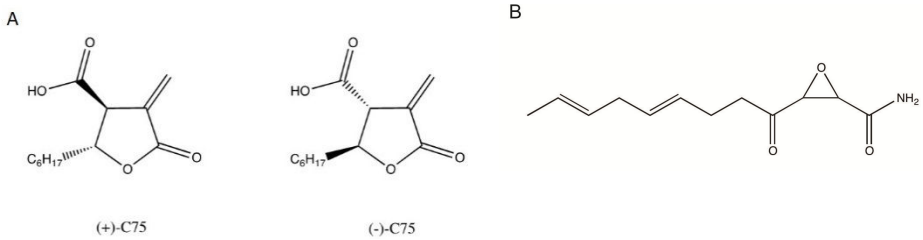


Figure 9. Chemical structure of (A) (±)-C75 and (B) cerulenin.

C75 and other related α-methylene-γ-butyrolactones have been shown to inhibit FASN in several types of cancer cells (Pizer et al., 2000; Wang et al., 2005) and this is associated with activation of apoptotic pathways (Menendez et al., 2004b). These FASN inhibitors also interact with another lipid target, activating directly carnitine palmitoyl-transferase I (CPT-1), the rate-limiting enzyme for mitochondrial fatty acid oxidation, which increases energy production and causes weight loss (Kuhajda et al., 2005). In addition FASN inhibitors can block a novel hypothalamic mechanism causing decrease in food consumption (Mobbs and Makimura, 2002). Recently results suggest that the (-)-C75 enantiomer inhibits FASN (IC_{50} = 460 μM) and has cytotoxic effects on tumour cells but does not affect food intake. The (+)-C75 is less effective against FASN (IC_{50} > 5000 μM) and causes weight loss likely through inhibition of CPT-1 (Makowski et al., 2013). C93, an analogue

structurally related to C75, could also be a promising compound because it can inhibit tumour growth in a lung cancer model without causing weight loss (Orita et al., 2008). The lichen compound PA is structurally similar to C75, the only difference is the length of the alkyl side chain. A synthetic racemic mixture of PA, named C83, has been shown to inhibit FASN in breast cancer cells (Kuhajda et al., 2000) and our preliminary data indicate that (+)-PA may inhibit FASN in breast cancer cells (unpublished data). Orlistat, a reduced form of the natural product lipstatin currently marketed as an anti-obesity medication, and (-)-epigallocatechin 3-gallate a polyphenol derived from green tea have also been found to inhibit FASN and to have anti-tumour activity in several cancers without effecting normal cells. However, these compounds have poor oral bioavailability and solubility and their use in cancer chemotherapy is very limited (Liu et al., 2010; Yang et al., 2009). New synthetic polyphenolic compounds that inhibit FASN have been shown to induce apoptosis in HER2 overexpressing breast cancer cells by blocking HER2 activation and its downstream pathways ERK and AKT, without stimulation of CPT-1, making them a promising new therapeutic approach for treatment of cancer (Puig et al., 2009; Turrado et al., 2012). The sensitivity of cancer cells towards anti-proliferative and pro-apoptotic effects of FASN blockade is suggested to be more dependent of expression levels of HER2 than high levels of FASN expression/activity (Menendez et al., 2005a). However, when FASN is pharmacologically inhibited overexpression of HER2 is also transcriptionally repressed (Menendez et al., 2005c).

1.5.6 Lipoxygenase (LOX)

Phospholipids are the dominant lipids in bio-membranes. Arachidonic acid (AA), a long chain fatty acid (20:4), can be released from cellular membranes by phospholipase A_2 following a variety of stimuli. AA can be further metabolized by three different enzyme classes: LOX, cyclooxygenases (COX) and cytochrome P450 (CYP450). (Funk, 2001; Janakiram et al., 2011). COX enzymes catalyse the rate-limiting steps in the biosynthesis of prostaglandins (PG) and thromboxanes from AA and CYP450 converts AA to epoxyeicosatrienoic acids (EETs) or hydroxyeicosatetraenoic acids (HETEs). When AA is metabolized by LOX enzymes it generates the biologically active metabolites hydroxyperoxy-eicosatetraenoic acids (HPETEs), which can be further reduced to the corresponding HETEs or it can be converted into leukotrienes by 5-LOX that mediate inflammatory responses (Cathcart et al., 2011; Pidgeon et al., 2007; Schneider and Pozzi, 2011) (Figure 10).

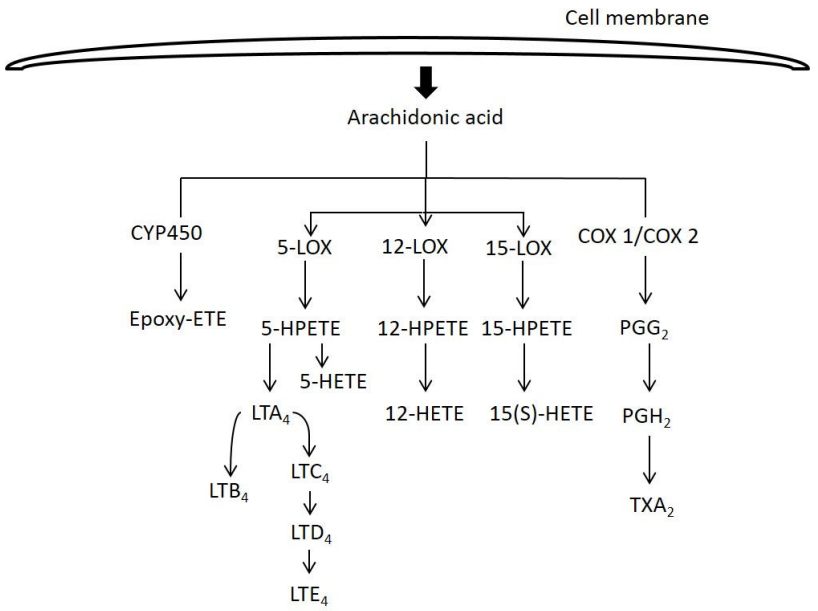


Figure 10. Arachidonic acid cascade.

LT; leukotriene, PG; prostaglandin, TX; thromboxane. The figure is based on (Janakiram et al., 2011).

Both COX and LOX pathways have been linked to cancer and the CYP450 pathway has also been implicated in carcinogenesis, however its precise role needs to be further elucidated (Panigrahy et al., 2010). COX-2 has been shown to be overexpressed in several types of tumours and emerging data suggest that COX-2 contribution to cancer is through increased production of prostaglandins, angiogenesis, inhibition of apoptosis and invasion of cancer cells (Khan and Lee, 2011; Wang and Dubois, 2010). The expression of LOX enzymes in cancer is believed to be regulated by oncogenes and tumour suppressor and studies on the association between various LOX isoforms and the control of cancer development and progression have increased over the past years. LOX enzymes are classified into 5-,8-,9-,12- and 15-LOX depending on the position of inserted oxygen. 5-LOX is the main isoform associated with the formation of HPETEs (Aparoy et al., 2012; Guo and Nie, 2012). 5- and 12-LOX are often constitutively expressed in various epithelial cancers such as colon (Ohd et al., 2003), prostate (Gupta et al., 2001), oesophageal (Chen et al., 2004) and breast cancer (Jiang et al., 2003). Emerging data indicate that by inhibiting LOX isoforms it is possible to supress proliferation and induce apoptosis in a variety of tumour cells. These effects are thought to be mediated through cell cycle arrest at G_1/S phase,

induction of cyclin-dependent kinase inhibitors and through inhibition of major cell signalling pathways, AKT and MAPK as well as inhibition of the transcription factor NF-κB (Pidgeon et al., 2007). Several pancreatic cell lines have been shown to express 5-LOX both at transcriptional and translational levels in contrast 5-LOX mRNA levels were not detectable in normal pancreatic ductal cells. Overexpression of 5-LOX in pancreatic cancer has been characterized by poor prognosis and a lack of response to conventional therapy (Ding et al., 2003; Hennig et al., 2002).

1.5.6.1 *LOX inhibitors*

The association of products from the 5-LOX pathway in various diseases makes it an interesting therapeutic target. Several 5-LOX inhibitors are currently being tested and show promising results e.g. on osteoarthritis (Williams and Spector, 2009) and atherosclerosis (Tardif et al., 2010). The crystal structure of 5-LOX has recently been elucidated (Gilbert et al., 2011) making it possible to predict more about how strong the binding affinity is and how the inhibitors function which could contribute to the identification of new classes of 5-LOX inhibitors with fewer or no side effects (Aparoy et al., 2012). LOXs could be promising targets for cancer treatment, however, no 5-LOX inhibitor has been developed yet for clinical use in cancer (Aparoy et al., 2012). Zileuton is the only clinically available 5-LOX inhibitor and is used to decrease symptoms of asthma (Drazen et al., 1999). Zileuton has been shown to have inhibitory effect on oesophageal and oral carcinogenesis in rat models and human tissues and to prevent oral cancer in hamster models (Chen et al., 2004; Li et al., 2005). In contrast, Fischer and colleagues showed that zileuton did not have any anti-proliferative or cytotoxic effects in tested cell lines, e.g. from pancreatic cancer, and that the cytotoxic and anti-proliferative effects of certain 5-LOX inhibitors were independent of suppression of 5-LOX activity and the concentration needed for anti-proliferative effects exceeded those needed for suppression of 5-LOX. The association between 5-LOX and cancer cell viability needs to be further elucidated (Fischer et al., 2010).

1.6 Cancer drug resistance

The development of cancer drugs over the past decades has led to improved response rate and reduced side effects of cancer therapeutic agents. There has been rapid increase in specificity from using general cytotoxic agents such as nitrogen mustards in the 1960s to the use of anti-cancer drugs and targeted therapy alone or in various combinations (Gottesman, 2002).

However, drug resistance is a major problem and limits the effectiveness of chemotherapy. Large tumours can be genetically diverse and therefore it is possible that a small part of cells within the tumour are resistant to the drug and will survive the therapy and proliferate rapidly (Bozic et al., 2013). Resistance can be divided into two categories; intrinsic resistance, where resistant mediating factors exists in the tumour cells before they are treated and the treatment is inactive, or acquired resistance were the cells initially respond to treatment but become resistant over time (Holohan et al., 2013). The acquired resistance can occur at many levels, it can be caused by mutations in the cancer cell or through different adjustment responses such as an increase in drug efflux or in alterations of the therapeutic target, or by activation of compensatory signalling pathways and evasion of apoptosis. In addition many tumours are genetically and molecularly heterogeneous as mentioned above, which could contribute to resistance (Holohan et al., 2013; Longley and Johnston, 2005).

As discussed earlier in this thesis, autophagy can have tumour-promoting and tumour-supressing properties and it is controversial whether induction of autophagy leads to cell death or survival. It has been suggested that activation of autophagy can protect cancer cells from targeted therapy in e.g. in breast (Vazquez-Martin et al., 2009b; Zou et al., 2012) and prostate cancer cells (Zhu et al., 2010) and that it is likely that inhibition of autophagy can sensitize the resistant cancer cell to anti-cancer drugs. However, more studies on the ability of autophagy inhibitors to overcome resistance are needed (Hu et al., 2012; Sasaki et al., 2010).

1.6.1 Breast cancer

HER2 targeted treatment, with drugs like trastuzumab and lapatinib, has improved outcome in breast cancer patients with HER2 overexpression, however not all patient respond to treatment and approximately 15% of patients relapse after treatment indicating resistance to the drug (Mohd Sharial et al., 2012; Nahta et al., 2006). The proposed resistance mechanisms of the anti-HER2 drug trastuzumab include constitutive activation of the PI3K pathway by HER2 or other HER family members or as a result of loss of PTEN or mutations in PI3KCA. Dimerization with other receptors and increased production of p95-HER2 can also lead to resistance (Lim et al., 2013). A recent study has indicated that loss of PTEN does not result in lapatinib resistance. It is more likely that activation of the PI3K and mTOR pathways can contribute to resistance suggesting that it could be beneficial to use combination or sequential therapy with lapatinb and mTOR-

targeting drugs to overcome lapatinib resistance (Jegg et al., 2012; Sahin et al., 2014). Current strategies that have been suggested to overcome resistance to HER2 targeted therapy include combining an anti-HER2 agent with other chemotherapeutic agents (Cameron et al., 2010), combining HER2 therapies or change to another HER2-targeted therapy (Baselga et al., 2012; Blackwell et al., 2012; Swain et al., 2013) or using inhibitors against alternative signalling pathways such as PI3K, AKT and mTOR (Mohd Sharial et al., 2012). A recent study in mouse models bearing aggressive mammary tumours showed that by using marker-guided targeted therapies in a sequential order it is possible to prolong survival of the mice with minimum toxicity effects. The treatment starts with lapatinib and trastuzumab, followed by treatment with trastuzumab and mTOR inhibitor and lastly all three agents are combined (Sahin et al., 2014). There is also a need for further studies on the side effects of HER2 targeted therapies and for biomarkers that can identify patients that benefit the most from dual targeting (Kumler et al., 2014).

1.6.2 Pancreatic cancer

Alterations in key pathways that are linked to apoptosis and involved in cell cycle control are suggested to be tightly connected to the ability of cancer to develop resistance against chemotherapy. Activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) can supress cell death pathways by activating several anti-apoptotic genes such as Bcl-2 (Tamburrino et al., 2013). NF-κB has been shown to be constitutively activated in pancreatic adenocarcinomas and pancreatic cancer cell lines (Wang et al., 1999). It has been shown that resistance against gemcitabine is decreased when NF-κB is inhibited in pancreatic cells (Arlt et al., 2003) indicating a link between cancer drug resistance and NF-κB activation. Targeting NF-κB for cancer therapy is a challenging task, however, very few inhibitors have reached clinical trials yet (Tamburrino et al., 2013).

1.7 Complementary drug therapy – synergism

Combinational approach in drug therapy has been known in most of the world's ancient medicine systems for centuries. The use of traditional Chinese medicine (TCM) is based on 5000-years old history of combining medicinal herbs. In cancer treatment TCM has been used as an adjuvant therapy to minimize the side effects and resistance to chemo-and radiotherapy (Singh et al., 2013a). A recent study revealed that combination therapy including chemotherapy, radiation therapy and a cocktail of Chinese

herbs showed positive clinical outcome in pancreatic patients with liver metastasis (Ouyang et al., 2011). Many natural compounds can be tracked back to ancient medicine systems. One of them, resveratrol, a compound found e.g. in grapes and berries has been shown to exhibit various biological activities. It has proposed chemo-preventive effects e.g. in skin (Aziz et al., 2005) and prostate cancers (Harper et al., 2007) as well as acting as a chemo-sensitizer in various cancer cell lines, such as in breast and pancreatic cancers, by enhancing the effect of doxorubicin- or cisplatinum-induced apoptosis (Fulda and Debatin, 2004). It is important to investigate further the use of secondary metabolites e.g. derived from plants or herbs such as resveratrol, UA and PA in combination with other known cancer drugs because of their potential ability to synergistically or additively enhance the effects of cancer drugs and minimize the toxicity and side effects of chemotherapy.

The future of cancer therapy lies in intelligent personalized and tumour-specific cancer therapy where sequencing of the genome will play an important role. Recently Bozic and colleagues showed that only one genetic alteration, within the 6.6 billion base pairs in the human cell genome is needed to contribute to resistance to two targeted drugs (Bozic et al., 2013). This emphasizes the need of thorough studies to find strategies to overcome resistance against anti-cancer drugs and targeted therapy. Future researches will aim at investigating the use of multiple therapies, that target independent pathways as first line treatment, (Blair et al., 2014; Bozic et al., 2013; Holohan et al., 2013).

2 Aim of the study

The general aim of this research project was to gain further understanding of the pathways that are involved in mediating the reported anti-proliferative effects of the two lichen compounds, usnic acid (UA) and protolichesterinic acid (PA).

The aim can be divided into three specific aims reflected in paper/manuscript or additional data in this thesis:

- To explore the effects of UA on two cell organelles, mitochondria and lysosomes in breast cancer cells, with focus on autophagy. Paper #1.

- To investigate the effects of PA on proliferation in different cancer cell types. To explore the effect of PA on growth factor signalling and lipid metabolism in pancreatic, myeloma and breast cancer cells. Paper #2 and #3 manuscript.

- To evaluate drug interactions between the two lichen compounds and known cancer drugs. Paper #2 and additional data.

3 Materials and methods

3.1 Lichen materials and general experimental procedures

The lichens *Cladonia arbuscula* (Wallr.) Rabenh. (Cladoniaceae) and *Cetraria islandica* L. (Ach.) were collected at Alftanes in 2007 and Jokuldalsheidi in 2004, respectively. The lichen material was identified by Dr. Hordur Kristinsson, lichenologist, and voucher specimens (*C.arbuscula:* LA-31129 and *C.islandica:* LA-31128) are deposited at the Icelandic Natural History Museum, Akureyri. Solvent for extraction and HPLC grade solvents for chromatography were purchased from Sigma-Aldrich. Nuclear magnetic resonance (NMR) spectra using $CDCl_3$ as a solvent were recorded on a Bruker 400 spectrometer (5 mm BB-1H/D probe-head) at 25°C using TMS as an internal standard.

3.1.1 Isolation and identification of usnic acid

Usnic acid (UA) was isolated from *Cladonia arbuscula* (Wallr.) Rabenh. (Cladoniaceae). Isolation and identification was performed as described (Einarsdottir et al., 2010). Briefly, dried and pulverised *C.arbuscula* was extracted with light petroleum in a Soxhlet apparatus for 18 hours and the precipitates collected and recrystallized from ethanol. The active compound was identified as the known compound (+)-UA (UA). Purity of UA was analysed in collaboration with Eydis Einarsdottir by analytical HPLC on a RP (G.L Sciences, Inc., Herbal medicine, C-18, 4.6 mm x 250 mm), a solvent system of MeOH:H_2O:H_3PO_4 (80:20:0.9) flow rate of 1.5 mL/min, UV-detection at 235 nm at 25°C and was shown to be 97% pure. Isolation of pure UA was performed as needed and stability of the compound tested regularly by analytical HPLC. UA was dissolved in DMSO (Merck) diluted for use in tissue culture medium. All tests include controls where the highest equivalent concentration of DMSO.

3.1.2 Isolation and identification of protolichesterinic acid

The air-dried powdered Iceland moss material was extracted in a Soxhlet apparatus with petroleum ether for 16 hours to obtain crude extract. The organic extract was evaporated to dryness. For isolation of a pure compound, preparative HPLC (Dionex 3000 Ultimate; pump, UV-VIS detector) was connected to (C18 column, 250 x 21.1 mm, 5µm, Phenomenex Luna) eluting with ACN:H_2O:CH_2O_2 (90:10:0.01), a flow rate of 5 mL/min, detection at 210 nm at room temperature. The active compound (t_R = 42 min) was identified as the known compound (+)-protolichesterinic acid (PA). Purity of PA acid was

analysed in collaboration with Eydis Einarsdóttir by analytical HPLC on a RP column (G.L Sciences, Inc., Herbal medicine, C-18, 4.6 mm x 250 mm), a solvent system of ACN:H_2O:CH_2O_2 (90:10:0.01) flow rate of 1 mL/min, UV-detection at 210 nm at 25°C and was shown to be 97% pure. Melting point and optical rotation were also performed in collaboration with Eydis Einarsdottir as described in paper #2. Isolation of pure PA was performed as needed and stability of the compound tested regularly by analytical HPLC. PA was dissolved in ethanol absolute puriss (Redel-de Haën) and diluted for use in tissue-culture medium. All tests include controls where the highest equivalent concentration of ethanol was used.

3.2 Cancer cell lines culture and processing

The breast cancer cell lines T-47D (CRL-2865), MCF7 (HTB-22), SK-BR-3 (HTB-30) the pancreatic cell lines Capan-2 (HTB-80) and AsPC-1 (CRL-1682), the multiple myeloma cell lines RPMI 8226 (CCL-155) and U266 (TIB-196), the ovarian cancer cell lines SK-OV-3 (HTB-77), the colon carcinoma cell line HCT 116 (CCL-247) and the glioblastoma cell line U-87 MG (HTB-14) were obtained from the American Type Culture Collection. The ovarian cell line A2780 (93112519) was purchased from European Collection of Cell Culture. Primary human fibroblasts were cultured from normal skin biopsies, maintained in RPMI-1640 (GIBCO™), and used in passage 6-13 (National Bioethics Committee permission VSNb2006020001/03-16; informed consent obtained). Cell lines were maintained in appropriate culture medium according to manufacturer's protocol. RPMI-1640 (GIBCO™) (T-47D, MCF7, Capan-2, AsPC-1, RPMI 8226, U266, SK-OV-3, A2780), McCoy's (GIBCO™) (SK-BR-3) or DMEM (GIBCO™) (U-87 MG, HCT 116), containing 0.5% penicillin and streptomycin (GIBCO™) and 10% heat-inactivated fetal bovine serum (FBS; GIBCO™) with T-47D receiving additionally 0.01 mg/mL insulin (Sigma). Cells were seeded at an appropriate number to exceed 70-80% confluence after 24 hour culture and treated with various concentrations of the tested compounds, lichens metabolites, metformin (Sigma), zileuton (Cayman), baicalein (Sigma-Aldrich), lapatinib (Laboratories), doxorubicin (Actavis) and solvent control and then the cells were incubated under standard conditions for different time periods. For the induction of autophagy by nutrient deprivation, cells were incubated with Hank's solution (Sigma) for 40 min or 2 hours. To synchronize the cells before certain experiment setups cells were incubated without FBS in their medium for 18 hours. For quantification of lipids and PA, cells were stimulated with 30μM arachidonic acid (Cayman Chemicals) for 30 min after treatment with PA or other

substances, both cells and medium were collected and flash frozen in liquid nitrogen and stored at -80°C. Protein content was quantified spectrometrically using Bradford reagent (Sigma).

3.3 Cell viability screening assays

Crystal violet assay and colorimetric cytotoxicity assay using sulforhodamine B (SRB) were used to determine the half maximal tolerated dose, $IC_{50,}$ of the lichen compound PA for several cancer cell lines. Cells (4000-10.000) were seeded in 96-well plates and cultured at standard conditions for 72 hours. PA was added after 24 hours along with solvent controls with equivalent concentrations of ethanol, at final volume of 200 µL. For the Crystal violet assay cells were stained with 100 µL of 0.25% Crystal violet solution (Reagenzien, Merck, Darmstadt, Germany) for 10 min. Plates were washed with H_2O, dried out and then 100 µL of 33% acetic acid added and the absorbance measured at 490 nm wavelength using a spectrophotometer (SpectraMax Plus384, Molecular Devices Corporation). For the SRB assay cells were fixed with 100 µL of 10% trichloroacetic acid and incubated at room temperature for 30-60 min. Plates were washed with H_2O, dried and then stained with 100 µL of sulforhodamine B (Sigma) for at least one hour. Then the plates were washed, dried and stain re-solubilized with 100 µL of 10mM Tris base (pH10) and the absorbance measured at 570 nm wavelength using a colorimetric plate reader (Spectramax 340, Molecular Devices Corporation). The experiments were performed in triplicate and repeated two to five times. Statistical analysis of IC_{50} values was performed using Graph prism software Inc, La Jolla LA (USA) using non-linear regression (variable slope) equation.

3.4 Estimation of levels of ATP

Cells were detached by trypsinization, a small aliquot removed for counting and harvested using 0.5M perchloric acid (Merck) for 10 min at 4°C. After centrifugation 10 µL of the supernatant were mixed with 1 mL of distilled water. Bioluminescence was assayed using 75 µL luciferase reagent (Promega) which lysed the cells and provided the substrate luciferin. Luminescence was measured in a luminometer (Turner TD 20/20) and expressed as luminescence/cell. The experiments were repeated six times.

3.5 Electron microscopy

Was performed by Már Egilsson according to standardized protocol as described in paper #1.

3.6 Western blotting

Cells were harvested and lysed with RIPA buffer. Protein content was quantified spectrometrically using Bradford reagent (Sigma). Proteins were separated on NuPAGE 10% Bis-Tris Mini Gels and transferred to 0.2 mM polyvinylidene difluoride membrane by electroblotting. Membranes were probed with anti-phospho-AMPK (Thr172) rabbit IgG monoclonal antibodies (Cell Signaling), anti-p62 (SQSTM1), rabbit polyclonal antibody (Enzo), anti-LC3B (D11), rabbit IgG monoclonal antibody (Sigma), anti-phospho-eIF2α (Ser51) rabbit polyclonal antibody (Cell Signaling) or anti-G3PDH rabbit anti-human polyclonal antibody (R&D Systems). Secondary antibody used was goat anti-rabbit IgG/HRPlinked (Cell Signaling) and secondary antibody conjugated to IRDye-680 or 800 (Metabion). Proteins were visualized by the enhanced chemiluminescence detection kit (GE Healthcare) and the signal was detected using a high performance chemiluminescence film (GE Healthcare) or detected by Odyssey infrared imaging system. The experiments were repeated three times.

3.7 MSD analysis

Cells were washed with TBS, harvested and lysed with RIPA buffer (without SDS). Protein content was quantified spectrometrically using Bradford reagent (Sigma). MSD 96-well multisport assays (Meso Scale Discovery, Rockville, MD, USA) were carried out according to the manufacturer's protocol with minor modifications. Briefly, ERK1/2 duplex) and phospho(Ser^{473})/total AKT plates (were blocked (MSD blocking solution, as recommended by the manufacturer, plus 0.1% BSA) for 1 hour at room temperature with shaking and washed four times with Tris-buffered saline with 0.1% Tween-20. 2.5 or 20 micrograms of protein were added to the ERK1/2 or AKT(473) plates respectively, in duplicate wells and incubated overnight at 4°C. Plates were washed as previously; then 25 µL of detection antibody was added and incubated at room temperature for 2 hours with shaking. Plates were washed four times with Tris-buffered saline with 0.1% Tween-20 as before, 150 µL of read buffer was added, and the plates were analysed on a SECTOR™ 6000 instrument (MSD). The two additional spots in each well coated with BSA were used to correct for the background and for any effects of the lysis buffer. The experiments were repeated three times. Statistical analysis of the data was performed by Graph Prism software (GraphPad prism software, Inc., La Jolla, LA, USA) using unpaired t-test with Welch's corrections.

3.8 Immunocytochemistry

3.8.1 Immunofluorescence staining

Immunofluorescence staining was performed using two different staining methods based on fixation in paraformaldehyde or in methanol. Cell were harvested and fixed in 4% paraformaldehyde (Sigma), blocking was performed with IFF (PBS + 1% BSA + 2% FBS) and cell stained with LAMP2, mouse IgG1 monoclonal antibody (H4b4, obtained from University School of Medicine, Baltimore), Fatty Acid Synthase (C20G5), rabbit IgG monoclonal antibody (Cell Signaling), HER2/ErbB2 (29D8), rabbit IgG monoclonal antibody (Cell Signaling), p21 polyclonal antibody (Abcam), and PCNA (PC10), mouse monoclonal antibody (Santa Cruz Biotechnology) followed by Alexa Fluor green 488 goat anti-rabbit IgG antibody (Invitrogen), Alexa fluor red goat anti-rabbit IgG antibody (Invitrogen) Alexa fluor red 546 goat anti-mouse IgG2a antibody (Molecular Probes) For nuclear staining TO-PRO-3 iodide (Invitrogen) was used. Cells were fixed with methanol (Sigma) for 10 min at 20°C. Blocking was performed with PBS + 10% FBS and cells stained with anti-LC3B (D11), rabbit IgG monoclonal antibody (Sigma), anti-5-LOX, rabbit polyclonal antibody (Cayman) and anti-12-LOX (murine leukocyte), rabbit polyclonal antiserum (Cayman) followed by Alexa Fluor green 488 goat anti-rabbit IgG antibody (Invitrogen). For nuclear staining TO-PRO-3 iodide (Invitrogen) was used. The stained cells were visualized and photographed under a confocal microscope (Zeiss, LSM 5 Pascal).

3.8.2 Immunoperoxidase staining

For the immunoperoxidase staining cells were fixed with methanol (Sigma) for 5 min at -20°C and stained with anti-phospho-p70S6 kinase (Thr389; 108D2), rabbit IgG, monoclonal antibody (Cell Signaling), anti-LC3B (D11), rabbit IgG monoclonal antibody (Sigma) and anti-p62 (SQSTM1), rabbit polyclonal antibody (Enzo) followed by incubation with monoclonal mouse anti-rabbit immunoglobulins IgG1k (Dako), polyclonal rabbit anti-mouse immunoglobulins IgG (Dako), PAP, horseradish peroxidase and mouse monoclonal anti-horseradish immunocomplexes, IgG1 (Dako) and DAB tablets, chromogen (Dako). The stained cell were visualized and photographed under a light microscope (Leica DMI 3000B). The experiments were repeated three times.

3.9 LysoTracker staining

The tissue culture medium was replaced by pre-warmed (37°C) 75nM Lysotracker Red DND-99 (Invitrogen) and cells incubated at 37°C for 1 hour. Loading solution was then washed of and replaced by fresh medium and the stained cells were visualized and photographed under fluorescence microscope (Leica DMI 3000B). Lysotracker is a fluorescent acidotropic probe for labelling and tracing acidic organelles in cells. The protonated form of this probe accumulates in acidic compartment, where it forms aggregates that fluoresce bright red.

3.10 Quantification of images by Image J

For the LC3 staining experiments in T47-D, MCF7 and fibroblasts 3-6 pictures were taken for each sample and the experiment was performed three times. For the quantification of the LC3 puncta the brightness/contrast were adjusted for each image, using the same threshold throughout all the experiments. A binary contrast enhancement was used for counting of particles. LC3 puncta, of particle size 3-50 pixels with outlines shown, were analysed and counted. Cells were counted manually and data represented as LC3 puncta/cell. For the lysotracker staining experiments 3-6 pictures were taken for each sample and the experiment was performed three times. Lysotracker mean intensity was quantified by image J. The same limit to threshold was set for all images to highlight the area to analyse. Measurements give intensity measurements in just the threshold area. Cells were counted manually and data represented as mean intensity (fluorescence value) per cell. For FASN/HER2 staining experiments in SK-BR-3 cells, 3-6 pictures were taken for each sample and the experiment was performed five times. In T-47D cells 3-6 pictures were taken and the experiment was performed three times. FASN/HER2 images were converted to 8 bit grey scale and mean gray value measured, which is the sum of the gray values of the entire pixel in the selection divided by the number of pixels, reported in calibrated units. Data was represented as mean gray value. For analysis of images statistical comparisons of mean values were performed using two sided analysis of variance (ANOVA), including treatment and number of run as factors, followed by a post.hoc comparison using Tukey HSD, carried out with R© (RGui 64bit, version 2.15.2). p values of < 0.05 were considered statistically significant.

3.11 Transfection with plasmid construct

The plasmid mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3) construct was kindly provided by Prof. Kevin Ryan, Beatson Institute, University of Glasgow, with permission from Prof. Tamotsu Yoshimori, Osaka University (Kabeya et al., 2000; Kimura et al., 2007). Calcium/manganese based (CCMB) transformation of DH10B strains of E.coli was used as previously described (Hanahan et al., 1991). Transfection was performed using TransPass D2 (BioLabs) according to the manufacturer's protocol. After transfection cells were exposed to test substances or deprived of nutrients as described above. Cells were harvested and fixed in 4% paraformaldehyde (Sigma), and visualized and photographed under a confocal microscope (Zeiss, LSM 5 Pascal). The experiments were repeated three times.

3.12 Apoptosis assays

3.12.1 TUNEL staining - PI staining

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling) assay was performed to detect late apoptosis and to do a cell cycle analysis, using propidium iodie (PI) staining in treated cells. The assay was performed by Guðleif Harðardóttir, Jenný Björk Þorsteinsdóttir and Stefan Becker according to the manufacturer's instructions as described in paper #2. The experiments were repeated three times. Statistical analysis was performed using Graph prism software Inc, La Jolla LA (USA) using two sided ANOVA, followed by a post. hoc comparison using Bonferroni correction for apoptotic results, and Chi square and Fisher's exact test for the cell cycle results. p values of < 0.05 were considered statistically significant.

3.12.2 Annexin V/Propidium iodide double staining

FITC Annexin V in combination with PI staining was performed on non-permeabilized cells to detect early apoptotic or necrotic processes. The assay was performed according to the manufacturer's protocol by Stefan Becker as described in paper #3 manuscript. The experiments were repeated three times. Statistical analysis was performed using Graph prism software Inc, La Jolla LA (USA) using Chi square and Fisher's exact test. p values of < 0.05 were considered statistically significant.

3.13 Synergism

Synergism analysis was performed in collaboration with Edda Ásgerður Skúladóttir and Sindri Baldursson according to standardized protocol described in paper #2. The experiments were performed in triplicate and repeated at least three times. Calculations of the combination index, CI, were done in the CalcuSyn software, version 2.1 (Biosoft, Cambridge, UK). This software is based on Chou's and Talalay's ideas on the combination index as a criterion for synergy or antagonism between two or more drugs (Chou, 2006).

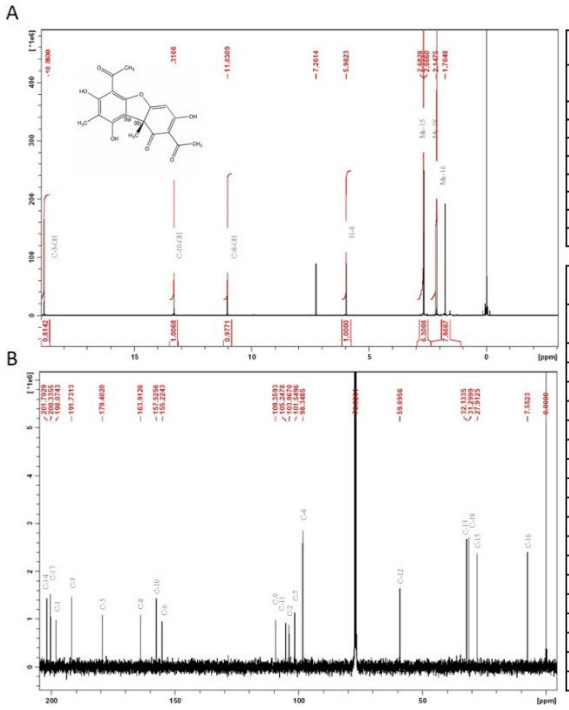
4 Results and discussion

In this chapter the data will be discussed according to published papers and manuscript with additional data added when appropriate.

4.1 Usnic acid affects mitochondrial and lysosomal function in breast cancer cells

4.1.1 Identification of (+)-usnic acid (UA)

After isolation of (+)-usnic acid (UA) from the lichen *C.arbuscula,* identification was performed by NMR spectroscopy and observed values compared to reported data (Ingolfsdottir et al., 1998) (Figure 11). The purity was tested with analytical HPLC method and was shown to be 97%. UA was stored in glassware protected from light and a stock solution (5 mg/mL; diluted in DMSO) was prepared and stored at 4°C.



^{1}H	Usnic acid	(Ingolfsdottir et al, 1998)
	ppm ($CDCl_3$)	ppm (C_6D_6)
Me-13 (s, 3H)	1.76	1.08
Me-16 (s, 3H)	2.15	2.45
Me-15 (s, 3H)	2.68	2.28
Me-18 (s, 3H)	2.66	2.28
H-4 (s, 1H)	5.98	5.35
C-10-OH (s, 1H)	13.32	13.94
C-8-OH (s, 1H)	11.03	11.25
C-3-OH (s, 1H)	18.85	19.21

^{13}C	Usnic acid	(Ingolfsdottir et al, 1998)
	ppm ($CDCl_3$)	ppm (C_6D_6)
C-1	198.1	198.0
C-2	104.0	104.0
C-3	191.7	191.0
C-4	98.3	98.0
C-5	179.4	179.0
C-6	155.2	155.0
C-7	101.5	101.0
C-8	163.9	163.0
C-9	109.4	109.0
C-10	157.5	157.0
C-11	105.2	105.0
C-12	59.1	59.0
C-13	32.1	32.0
C-14	201.8	202.0
C-15	27.9	27.0
C-16	7.6	7.5
C-17	200.3	200.0
C-18	31.3	31.0

Figure 11. ^{1}H-NMR and ^{13}C-NMR spectra of UA in $CDCl_3$.

(A) 400 MHz ^{1}H-NMR spectra of UA in $CDCl_3$. (B) 100 MHz C^{13}-NMR spectra. Signals are compared with reported data in table to the right.

4.1.2 Effects of UA on mitochondria in breast cancer cells

UA has been shown to exhibit anti-proliferative and growth inhibitory effects on several cancer cell lines including the breast cancer cell lines SK-BR-3 and T-47D using different cell viability and cytotoxicity approaches (Backorova et al., 2011; Einarsdottir et al., 2010). Before further analysis of the effects of UA on SK-BR-3 and T-47D cells, their viability was assessed after treatment with UA and doxorubicin (for synergism testing, see later) using Crystal violet staining. T-47D was more sensitive to both UA and doxorubicin compared to SK-BR-3. The results are shown in table 1, expressed as IC_{50} taking account of individual solvent effects as appropriate.

Table 1. Effects of UA and doxorubicin on breast cancer cell lines.

Cell lines	UA (µg/mL)	Doxorubicin (nM)
SK-BR-3	7.9 ± 0.4	63.3 ± 6.5
T-47D	6.7 ± 0.8	45.2 ± 3.8

Results are presented as IC_{50} values in µg/mL ± s.e.m.

Previous data have indicated that UA treatment affects the mitochondrial membrane potential and causes late necrosis but no apoptosis (Einarsdottir et al., 2010). No cytochrome *c* release or cleaved caspase-3 products were detectable after UA treatment of T-47D breast cancer cells (performed by Eydis Einarsdóttir Figure S1A-B in paper #1). For further investigation of the effects on mitochondria, ATP levels were evaluated and phosphorylation of AMPK analysed in T-47D cells. Cells were treated with UA for 24 hours and before the bioluminescence measurements the cells were counted manually in order to take into consideration the anti-proliferative effects of UA, which causes decrease in cell number. Results indicate that ATP levels are decreased after UA treatment, followed by an increase in phosphorylation of the energy sensor AMPK, as expected (Figure 1 in paper #1). These results support previous findings that UA does not cause apoptosis and the mitochondrial membrane stays intact although the pH gradient is disrupted when protons are shuttled across the membrane. The deregulation of pH is likely to cause a loss of mitochondrial membrane potential resulting in a decrease in cellular energy.

4.1.3 Autophagy in breast cancer cells after UA treatment

Having shown that UA affects cellular energy and triggers sensing mechanisms it would be expected that the cell would respond by signalling to the mTOR1 pathway and initiate the onset of autophagy (Amaravadi et al., 2011) (Figure 6). Among downstream targets of mTORC1 are p70S6K and 4EBP1, which have an important role in cell-cycle control and proliferation (Workman et al., 2014). Treatment with UA for 24 hours caused a marked decrease in immunoperoxidase staining of phosphorylated p70S6K (Figure 5A in paper #1). Furthermore an induction of cellular stress was detected after 6 hour treatment with UA (Figure 5B in paper #1) which could be linked to disturbed cellular energy balance in the cell.

To evaluate further the effects of UA on autophagy, electron microscopy images of T-47D after treatment with UA (2.5 and 5.0 µg/mL) were analysed. They revealed an increased presence of autophagic vacuoles, with double membrane characteristics compared with control, indicating initiation of autophagy (Figure 2 in paper #1). In the maturation process of autophagy LC3 II is incorporated into the lipid bilayer of the autophagosomes, making LC3 a suitable marker for autophagy (Amaravadi et al., 2011). Analysis of LC3 puncta by immunofluorescence staining was performed in two breast cancer cell lines, T-47D and MCF7 and in normal fibroblast. Cells were treated with UA for 2 and 24 hours or with the anti-diabetic drug metformin for 2 hours. Metformin decreases mitochondrial respiration and activates the AMPK indirectly (Rena et al., 2013) and has been shown to inhibit growth in breast cancer cell through the AMPK pathway (Zakikhani et al., 2006). For comparison cells were also starved by incubation for 40 min in Hank's balanced salt solution. By converting a greyscale imaging to a black and white image (binary) and defining a greyscale cut-off it was possible to count puncta in the image and divide by cell number to get quantification. No effects were seen after treatment with UA for 2 hours in any of the three cell lines; only metformin treatment showed increase after treatment for 2 hours in T-47D cells, which was no longer present after treatment for 24 hours. Metformin has been shown to stimulate AMPK already after one hour (Zhou et al., 2001). Visual inspection suggested that autophagosomes were present in starved cell, however the puncta were difficult to count and the cells did not tolerate this harsh treatment (Figure 3A-B in paper #1). In contrast, significant increase in LC3 puncta were seen in both breast cancer cell lines after 24 hour treatment with UA and these findings were further confirmed by an immunoperoxidase staining of LC3 in T-47D cells and induction of LC3 I with formation of LC3 II detected by Western blotting (Figure 12A-C).

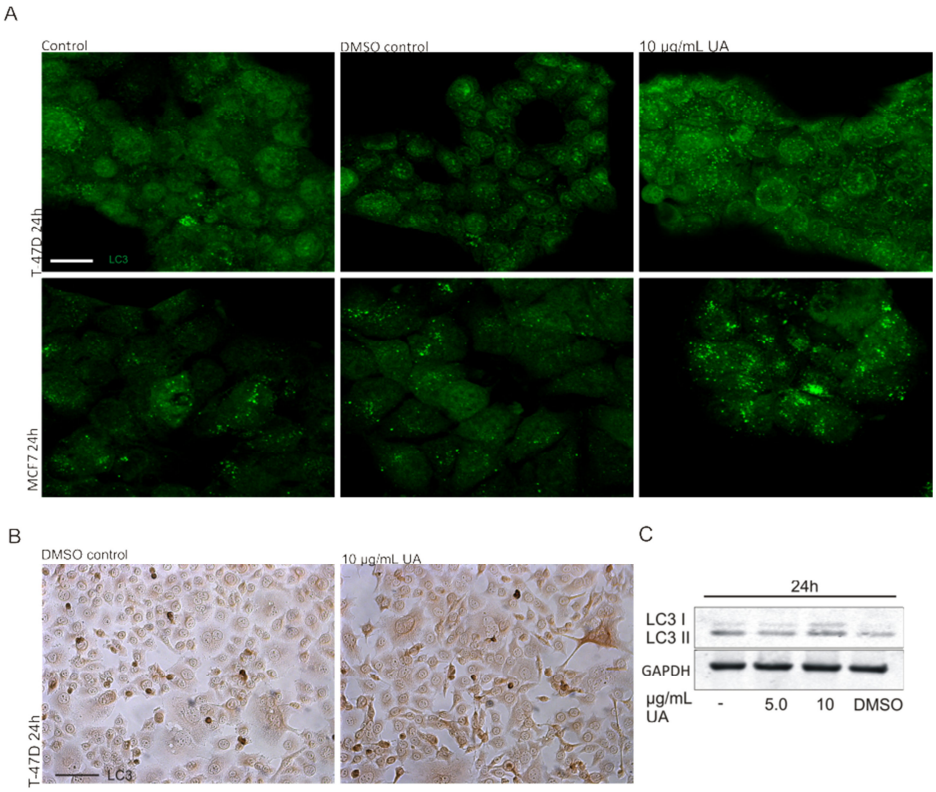


Figure 12. Dectection of LC3 in breast cancer cells after UA treatment.

(A) An increase in LC3 puncta was observed by immunofluorescence in T-47D and MCF7 cells after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 20 µm and applies to all panels. (B) An increase in LC3 staining was observed by immunoperoxidase staining in T-47D after treatment with UA (10 µg/mL) for 24 hours. (C) Increase in LC3 I and LC3 II, verified by Western blotting, was detected in T-47D cells after treatment with UA (10 µg/mL) for 24 hours.

4.1.4 Autophagic flux and lysosomal acidification in breast cancer cells after UA treatment

Autophagic flux is the term used to describe the dynamic process of synthesis of autophagosomes, maturation by fusion with lysosomes and degradation of substrates inside the autophagolysosome (Mizushima et al., 2010). To investigate if the formation of autophagic vacuoles was followed by autophagic flux the levels of p62, an autophagosomal cargo, were estimated after treatment with UA. It has been suggested that one of the roles of p62 is to bind to ubiquitinated protein, like LC3, and target them for degradation by autophagy. If autophagic flux is increased the concentration of p62 has been

shown to diminish as it is degraded in the process (Zheng et al., 2009). No degradation of internalized proteins was observed after UA treatment for 24 hours (5.0 and 10 µg/mL) in T-47D and MCF7 cells, (Figure 4A in paper #1) suggesting a block in autophagosomal maturation. To evaluate further the effects of UA on lysosomes, a lysosomal marker was used, which labels and tracks acidic organelles in cells. A marked diffuse increase in lysotracker staining was observed after UA treatment for 24 and 72 hours (Figure 13).

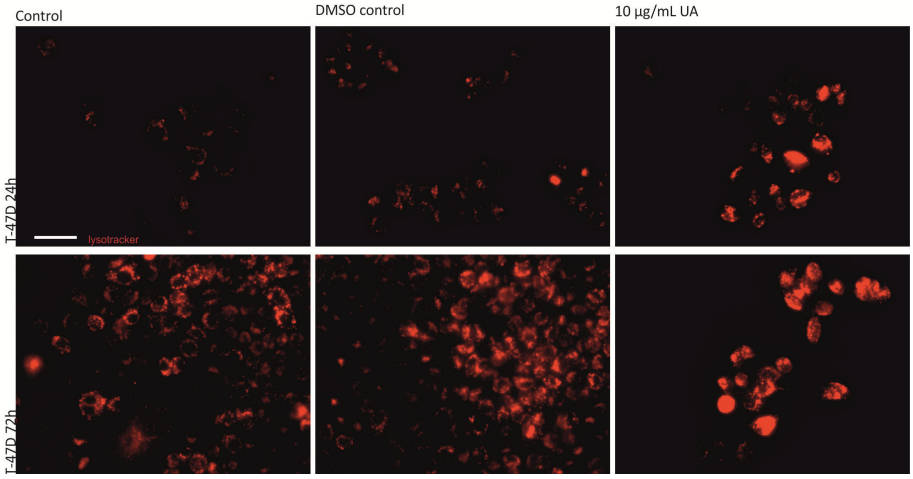


Figure 13. Detection of lysostracker staining in breast cancer cells after UA treatment.

Lysotracker, detected by fluorescence microcopy, shows diffuse staining in T-47D cells after treatment wiht UA (10 mg/mL; DMSO 0.2%) for 24 and 72 hours. The scale bar shown represents 100 µm and applies to all panels.

A staining pattern like this has been interpreted as lysosomal dilatation observed in cells treated with CQ and furthermore, staining with Lamp1, a lysosomal membrane protein, showed similar dilatation pattern (Yoon et al., 2010). In contrast, immunostaining for the lysosomal protein Lamp2, after treatment with UA, showed no morphological changes and no difference was observed between treated and untreated cells (Figure 4D in paper #1). It is possible the lysotracker is staining outside the lysosome and the retention of the dye inside of lysosomes is dependent on acidic pH (Griffiths et al., 1988). The diffuse lysotracker staining could therefore be explained by the proton shuttling properties of UA across lysosomal membranes, in a similar way as occurs across the mitochondrial membrane (Joseph et al., 2009). To follow up on autophagosome maturation after UA treatment a plasmid construct, tfLC3 (mRFP-GFP-LC3 tandem-tagged fluorescent protein) was transfected

into T-47D cells. The GFP-LC3 loses fluorescence because of the acidity of lysosomes while the mRFP-fluorescence remains stable (Kabeya et al., 2000; Kimura et al., 2007). Results showed that the green GFP fluorescence had faded while the red mRFP fluorescence was stable in starved cells, indicating acidic conditions and degradation by lysosomal hydrolases as expected. However, strong signals of both GFP and mRFP fluorescence were observed after UA treatment, which indicates disruption of autophagolysosomal acidification and impaired degradative lysosomal conditions (Figure 14).

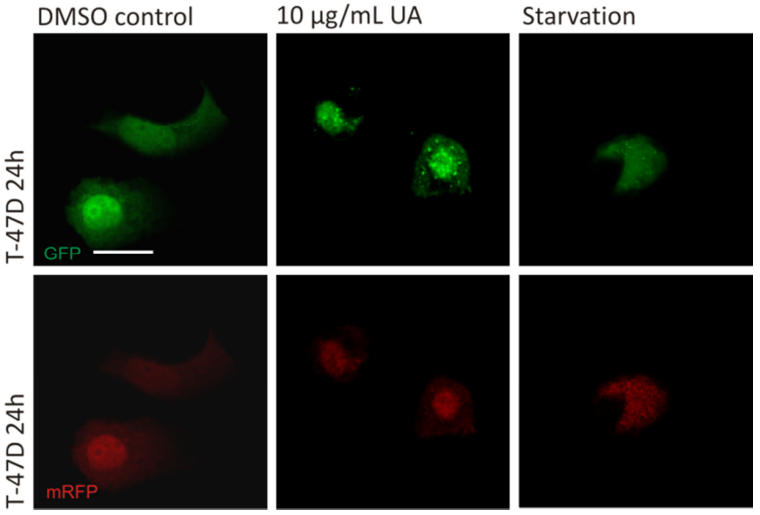


Figure 14. Detection of autophagolysosomal acidification in breast cancer cells after UA treatment.

A plasmid expressing mRFP-GFP-LC3 was transfected into T47D cells. Lack of autophagolysosomal acidification was seen after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 hours by detection of distinct GFP puncta. The scale bar shown represents 20 µm and applies to all panels.

Taken together these results indicate that the proton shuttling effects of UA can operate at two organelles, mitochondria and lysosomes in breast cancer cells disrupting the pH gradient in those two organelles (Figure 15). UA triggers formation of autophagosomes, however the cancer cells fail to complete autophagy, which is likely to cause the increase in accumulation of autophagic vacuoles and the retention of undegraded p62.

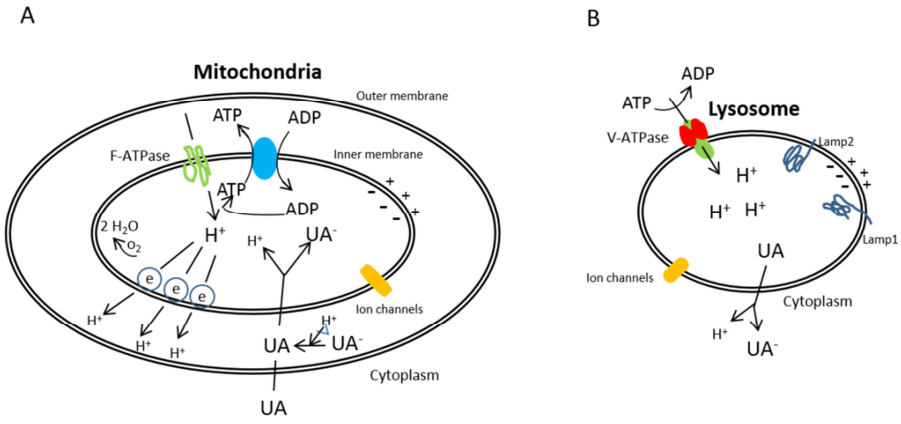


Figure 15. The proton shuttling effects of UA in breast cancer cells.

UA shuttles protons across (A) mitochondria and (B) lysosome membranes in breast cancer cells.The figures are based on (Casey et al., 2010; Settembre et al., 2013).

4.2 Protolichesterinic acid affects proliferation, growth factor signalling, and lipid metabolism in different types of cancer cells

4.2.1 Identification of (+)-protolichesterinic acid (PA)

After isolation and purification of PA, identification of PA was performed by NMR spectroscopy and observed values compared to reported data (Figure S1 in appendix I). Purity of PA was analysed by analytical HPLC and PA shown to be 97% pure (Figure S2 in appendix I). PA was stored in glassware and stock solution (5 mg/mL; diluted in EtOH) was prepared and stored at 4°C.

4.2.2 Effects on proliferation in different types of cancer cell lines after PA treatment

PA has been shown to exhibit anti-proliferative effects on several types of cancer cells; no effects were seen on normal skin fibroblasts (Brisdelli et al., 2013; Haraldsdottir et al., 2004; Ogmundsdottir et al., 1998). In this study, seven other cancer cell lines were added to those previously tested. Because of the structural similarity of PA and the FASN inhibitor C75 and our preliminary results on FASN inhibition in breast cancer cells, the effects of PA on cell viability were tested on the FASN- and HER2-overexpressing breast cell line SK-BR-3 (Yoon et al., 2007). The relationship between FASN and

HER2 acts in both directions. Transcription of HER2 can be repressed through inhibition of FASN (Menendez et al., 2005c). If HER2 is overexpressed an increase is observed in FASN expression by stimulating signalling cascades (Yang et al., 2002) and FASN is directly activated (Jin et al., 2010). The dual EGFR/HER2 tyrosine kinase inhibitor lapatinib was therefore included for comparison and to test for possible synergistic effects.

PA has also been shown to have inhibitory activity on 5- and 12-LOX (Bucar et al., 2004; Ingolfsdottir et al., 1994) and LOX pathways have been associated particularly with development of pancreatic cancer. The AsPC-1 cell line has upregulated levels of 5-and 12-LOX (Ding et al., 1999; Hennig et al., 2002), and was therefore added to previously tested pancreatic cancer cell lines. Viability of SK-BR-3 and AsPC-1 after PA treatment cells was assessed using Crystal violet staining.

With reference to the function of 5-LOX in leukotriene production by inflammatory cells (Funk, 2001), expression in lymphocytes (Jakobsson et al., 1992) and reported effects of LOX inhibitors on cell lines from leukemia cells (Anderson et al., 1996) two myeloma cell lines that grow in suspension; U266 and RPMI 8228 were tested by uptake of ^{3}H-tymidine after PA treatment to estimate cell proliferation. In addition, colorimetric cytotoxicity assay based on the use of SRB was used to estimate proliferation in two ovarian cancer cell lines, A2780 and SK-OV-3 and a colon cancer cell line, HTC116 after PA treatment.

The results are shown in table 2, expressed as IC_{50} taking account of individual solvent effects as appropriate. PA showed inhibitory effects on cell viability in SK-BR-3 cancer cells to similar extend as has been previously shown in T-47D cells. The T-47D cancer cell line was included for comparison because it is a p53 mutated breast cancer cell line (Casey et al., 1991) and does not overexpress FASN and HER2. The SK-BR-3 cells were more sensitive to lapatinib, as expected because of the overexpression of FASN and HER2. The pancreatic, colon and the two ovarian cancer cell line were less sensitive to PA compared to both myeloma and breast cancer cells. Some of the difference in IC_{50} values between cell lines might be attributable to different sensitivities of the three methods used.

Table 2. Effects of PA and lapatinib on several different cancer cell lines.

Cancer type	Cell lines	Relevant characteristics	PA (μg/mL)	Lapatinib (μM)
Breast cancer	SK-BR-3	Overexpr.HER2	3.5 ± 0.1	1.6 ± 0.3
	T-47D	p53 mutated	3.8 ± 0.5	6.4 ± 0.7
Ovarian cancer	SK-OV-3		6.1 ± 0.6	
	A2780		6.6 ± 1.2	
Multiple myeloma	U266	STAT3 activ.	3.5 ± 0.7	
	RPMI 8226	ERK1/2 activ.	1.8 ± 0.2	
Pancreatic cancer	AsPC-1	Overexpr. 5-12 LOX	7.7 ± 0.6	
Colon cancer	HCT 116		7.6 ± 1.0	

Results are presented as IC_{50} values in μg/mL ± s.e.m.

4.2.3 Cell cycle analysis and effects on programmed cell death after treatment with PA in pancreatic and myeloma cancer cells

The previously detected inhibitory effects of PA on various cell lines by thymidine uptake led to further evaluation of DNA cellular content after treatment with PA for 24 hours. The pancreatic cancer cell line AsPC-1 was chosen for cell cycle analysis after PA treatment. Furthermore, the pancreatic cell line AsPC-1, and the RPMI-8226 myeloma cell line were used for detection of apoptosis in order to estimate if the decrease in cell viability after PA treatment could be attributed to induction of apoptosis. Results indicate that cell cycle arrest occurs in G1 phase after PA treatment compared to control, detected by PI staining and flow cytometry. The difference was shown to be statistically significant (Figure 16A). This supports previous data in myeloma cells by thymidine uptake and implies that the effects of PA are occurring at an early stage in the cell cycle.

No apoptosis was detected in AsPC-1 cells estimated with TUNEL, and these results were further confirmed by Annexin V staining, revealing no effect on

early apoptosis. In contrast, very marked early as well as late apoptosis was seen in the RPMI 8226 myeloma cells after PA treatment compared to control. The results were shown to be statistically significant (Figure 16B and C).

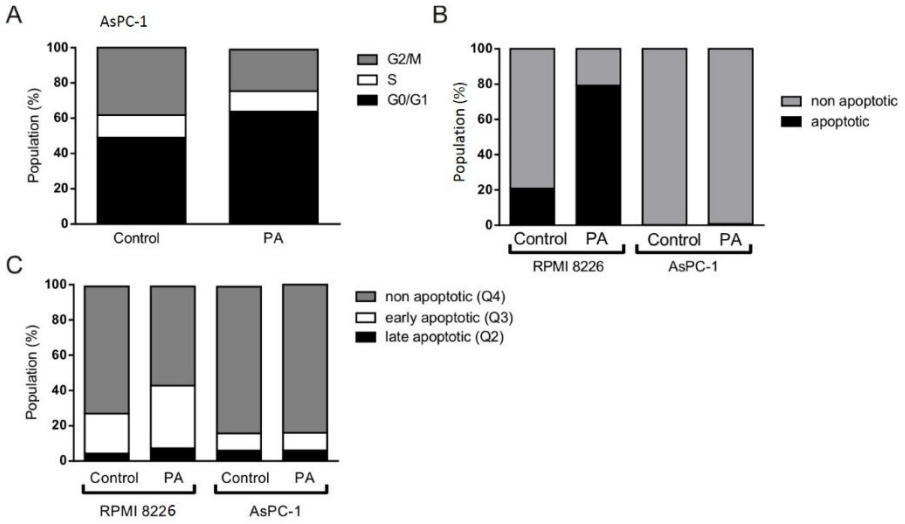


Figure 16. Effect of PA treatment on the cell cycle and apoptosis in myeloma and pancreatic cancer cells.

(A) Cell cycle analysis, detected by PI staining, in AsPC-1 cell line after treatment with PA (8 µg/mL) for 24 hours indicated cell cycle arrest in G1 phase compared with control. The figure shows proportional distribution of cells according to phase of cell cycle. (B) Treatment with PA (8 µg/mL) for 24 hours indicated late apoptosis in RPMI 8226, detected by TUNEL. No effects were seen on AsPC-1 cells. (C) Treatment with PA (10 µg/mL) for 24 hours indicated early as well as late apoptosis in myeloma cells, detected by Annexin V staining. No effects were seen on the AsPC-1 cells. Data in all figures presented as the mean of three separate experiments.

This indicates that the pro-apoptotic effects of PA are likely to be cell-line dependent as has been recently described in various other cancer cell lines (Brisdelli et al., 2013).

4.2.4 Lipoxygenase expression and cell cycle control in pancreatic cancer cells

The pancreatic cancer cell line AsPC-1 was used for further analysis of LOX expression and cell cycle control. Preliminary data have indicated that cellular

expression and localization of LOX enzymes in pancreatic and breast cancer cell lines may change according to stage in the cell cycle (Haraldsdóttir, S and Tómasdóttir, M, unpublished data). It has also been reported that depletion or deletion of serum can lead to nuclear export of 5-LOX and that addition of serum leads to nuclear accumulation of 5-LOX in various types of leukocytes and cell lines (Flamand et al., 2009). To approach the question whether the anti-proliferative effects of PA were associated with its LOX-inhibitory effects the AsPC-1 cell line was used for immunofluorescence detection of LOX and examination by confocal microscopy. This was not feasible using the non-adherent myeloma cells. The cells were synchronized and arrested by incubation in serum-free medium for 18 hours, then the cells were incubated again with serum, with or without PA, and allowed to re-enter the cell cycle. If there is an association between LOX inhibitory effects and anti-proliferative effects it would be expected to be related to variation in the cellular expression of LOX enzymes linked to the phases of the cell cycle. After synchronization there was a marked reduction of 5- and 12-LOX expression and nuclear export of 5-LOX was detected compared with control cells kept in serum-containing-medium. After incubation with serum-containing-medium for 3 hours the 5- and 12-LOX reappeared and were expressed in the nucleus. This confirmed previously obtained results from our laboratory and others and indicates that the products of 5- and 12-LOX enzymes are involved in the progression of the cell cycle. If the anti-proliferative activity of PA is mediated by the LOX-inhibitory effects a compensatory increase in expression of LOX enzymes would be expected to occur. However, reappearance of LOX expression was not affected after treatment with PA for 3 hours indicating either that PA has no significant LOX-inhibitory effects in the cell at that time point or the cells use other pathways to generate biologically active metabolites HETEs and a feedback response of the LOX enzymes is not stimulated (Figure 17).

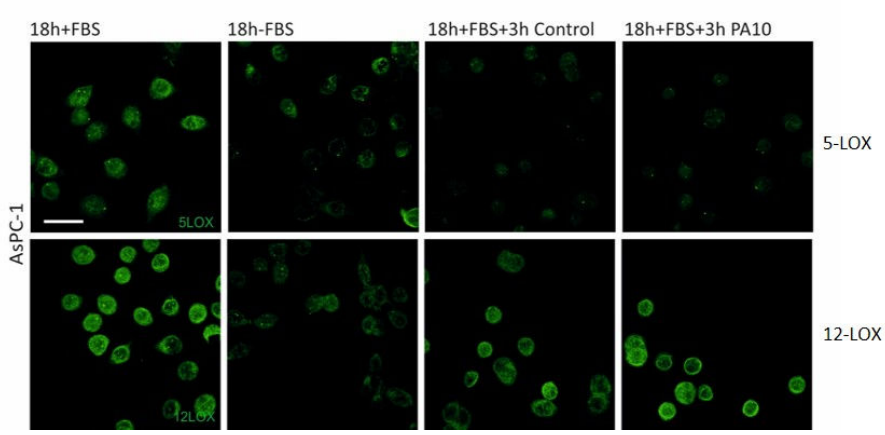


Figure 17. Expression of 5- and 12-LOX in pancreatic cancer cells related to cell cycle and effects of PA.

Immunofluorescence staining showed a marked reduction of 5- and 12-LOX expression and nuclear export of the 5-LOX enzyme in AsPC-1 cells after 18 hours of deprivation of serum followed by re-appearance upon adding serum. PA had no effect on reappearance for the first 3 hours. The scale bar shown represents 20 μm and applies to all panels.

The effects of PA after longer exposure beyond the G1- and S-phases of the cell cycle were also explored. The cells were treated with PA for 24 hours and immunofluorescence staining performed as above. Treatment with PA showed increased staining of 5-LOX and 12-LOX outside the nucleus compared with controls where staining was mostly localized in the nucleus. Treatment with PA also resulted in morphological changes of the AsPC-1 cells, this needs to be further investigated. Exposure to the 5-LOX inhibitor zileuton had no effects on 5-LOX expression (Figure 4A-B in paper #3 manuscript).

Taken together, LOX expression is cell-cycle dependent in AsPC-1 cancer cells as has been indicated in before by Flamand and colleges in leukocytes and other cell lines (Flamand et al., 2009). However, the effects of PA on LOX localization are only observable beyond the early phases of the cell cycle implying that the anti-proliferative effects of PA are not likely to be mediated directly through LOX inhibitory effects.

4.2.5 Expression of FASN and HER2 after treatment with PA in breast cancer cells

To follow up on previous data on FASN inhibition after PA treatment and to estimate and compare the effects of PA on FASN and HER2 expression, immunofluorescence staining was performed after treatment with PA and lapatinib for 24 hours. Lapatinib is a dual EGFR/HER2 inhibitor and by inhibiting HER2 it can supress its major downstream signalling pathways. Lapatinib is used in combination with other chemotherapeutics against HER2 positive breast cancer (Geyer et al., 2006). Treatment with lapatinib showed increased expression of HER2 and FASN in both of the breast cancer cell lines (Figure 18A-B). It is likely when signalling is inhibited through the HER2 receptor, as with lapatinib, the cells respond by increasing the expression of the receptor. Signalling through the HER2 receptor has been shown to have an important in mediating the overexpression of FASN (Kumar-Sinha et al., 2003) and there is an association between the circulating levels of extracellular FASN and HER2 in peripheral blood of HER2-overexpressing breast cancer patients. (Vazquez-Martin et al., 2009a). Therefore the increase in FASN expression after lapatinib treatment can be interpreted in a similar manner as the effects on HER2 expression. However, in HER2 overexpressing cancer cell line SK-BR-3 treatment with PA for 24 hours showed increased expression of FASN and decreased expression of HER2. No effects were seen on the FASN and HER2 expression in the T-47D cells.

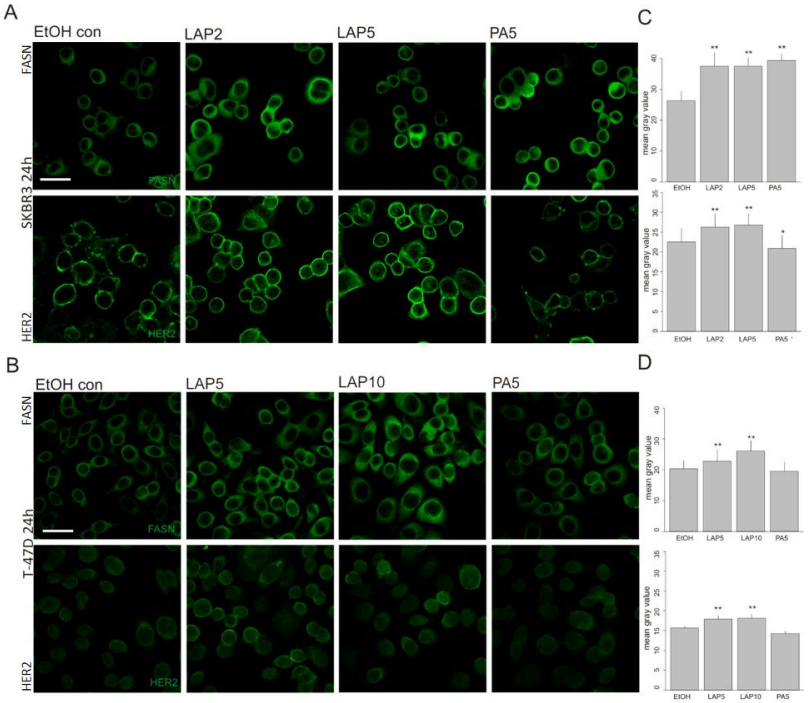


Figure 18. Effects on FASN and HER2 expression in breast cancer cells after treatment with PA.

(A) Increased expression of FASN and HER2 was detected by immunofluorescence staining in SK-BR-3 cells after treatment with lapatinib for 24 hours. An increase in FASN expression was also detected after treatment with PA for 24 hours but this was associated with a decrease in HER2 expression. (B) After treatment with lapatinib for 24 hours an increased expression of FASN was detected in T-47D cells. No effects were seen after PA treatment. The scale bar represents 20 μM and applies to all panels. (C-D). Data was represented as mean gray value of each group compared with EtOH control. Error bars indicate standard error of the mean, *p<0.05 **p<0.001.

Previous data from Menendez and colleagues indicate that treatment with FASN inhibitor, orlistat, in SK-BR-3 cells leads to accumulation of the DNA-binding protein PEA3, which is a member of a transcription family that binds to the HER2 gene promoter, and down-regulates its activity. Furthermore, it was shown that the PEA3 binding site on the HER2 promoter is necessary for transcriptional repression of HER2 by orlistat treatment (Menendez et al., 2005c). Therefore, results shown in figure 18A, indicate that the possible FASN inhibitory effects of PA lead to a compensatory increase in expression

of FASN. However, the decrease in HER2 expression is likely to be secondary repressed transcription of the receptor.

4.2.6 Effects on AKT and ERK1/2 signalling pathways in breast cancer cells after PA treatment

As FASN inhibitors are known to affect the HER2 receptor and the expression of FASN in cancer cells occurs through the signalling pathways PI3K/AKT/mTOR and MEK1/2/ERK1/2 (Puig et al., 2009) the effects of PA on the HER2 downstream signalling transduction pathways ERK1/2 and PI3K/AKT were estimated after PA treatment for 24 hours. Previous testing in, colon and ovarian cancer cells and glioblastoma cells indicated that treatment with PA for 2 and 6 hours did not have any effects on these pathways, therefore exposure of PA for 24 hours was tested in the two breast cancer cell lines. Results show that the phospho/total ERK1/2 and AKT ratio was significantly decreased in SK-BR-3 cells after treatment with PA for 24 hours. No effects were seen on T-47D cells (Figure 4 in paper #2). As PA affected ERK and AKT in SK-BR-3 cells but not in T-47D cells it is likely that the effect is secondary due to the decreased expression of HER2 rather than directly mediated through the signalling pathways, see Fig. 18A and C.

Taken together results show that PA leads to a compensatory effect on FASN expression in SK-BR-3 cells that overexpress HER2 and FASN which corresponds to previous results on PA having FASN inhibitory activity. At the same time a decrease is seen in expression of HER2 which is most likely through transcriptional repression, indicating that the effects on HER2 are secondary. The effects of PA on ERK1/2 and AKT signalling pathways are likely to be a consequence of reduced expression of the HER2 receptor rather than direct effect on the signalling pathways (Figure 19).

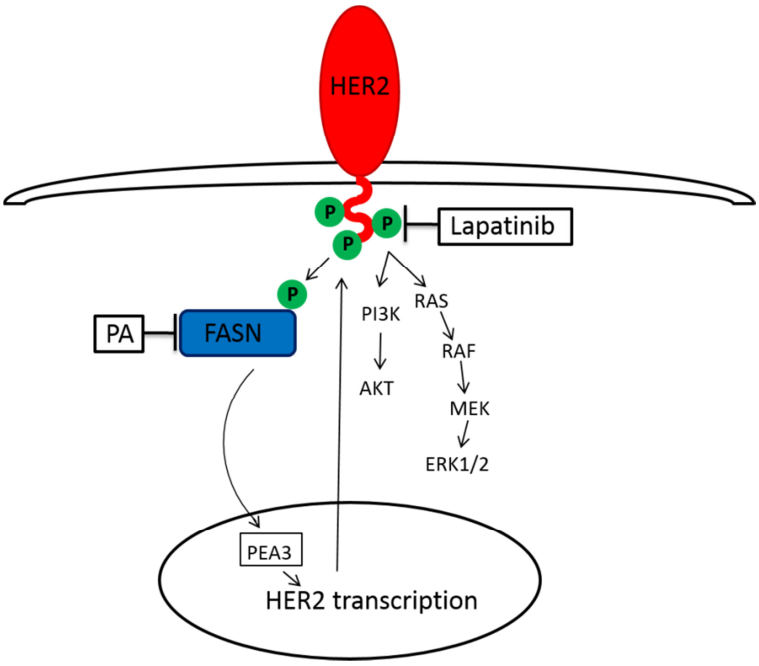


Figure 19. Proposed mechanism of PA in breast cancer cells.

PA primarly inhibits FASN activity in breast cancer cells, effects on HER2 expression and major cell signalling pathways are secondary and likely through transcriptional repression of HER2. The figure is based on (Murphy and Morris, 2012).

4.2.7 Effects of PA treatment on DNA polymerase in pancreatic cancer cells

It is likely that PA has a broad range of activity and can therefore affect cancer cells through several different mechanisms. Almost two decades ago Thitima Pengsuparp and colleagues showed, with enzyme reaction measurements, that PA inhibited DNA Pol β (Pengsuparp et al., 1995). Our current data on the effects of PA on multiple myeloma and pancreatic cancer cells by thymidine uptake and PI staining, also indicate that the effects of PA are occurring at an early stage in the cell cycle. The question therefore arose, whether the effects of PA could be mediated by direct inhibition of DNA synthesis rather than through effects on cell cycle control. DNA Pol δ is stimulated by direct binding to PCNA which is an important protein in many cellular processes such as DNA replication, damage repair and cell cycle progression (Prindle and Loeb, 2012). PCNA proteins are found both in the cytoplasm and the nucleus but its functions are thought to be mainly carried out by the proteins in the nucleus. A role of cytoplasmic PCNA in the regulation of oncogenes, the glycolysis pathway and cytoskeleton integrity has also been suggested (Naryzhny and Lee, 2010). It has also been

indicated that when the CDK inhibitor p21 is activated as a response to DNA damage, it acts by interrupting PCNA interaction with DNA repair molecules and participates in PCNA degradation (Jung et al., 2010; Lukas et al., 2004).

In breast cancer cells it has been shown that p21 is primarily expressed in the cytoplasmic fraction both in non-starved cells and under starvation, however, treatment with the cytotoxic agent etoposide shows more expression in the nucleus (Braun et al., 2011). In human amnion cells the expression of PCNA was shown to be low in the G1 and G2 phases, but in S phase the expression is increased and localized mainly in the nucleus (Fukami-Kobayashi and Mitsui, 1999).

To initiate cell cycle arrest in G1 phase the pancreatic cell line AsPC-1 was exposed to Hank's balanced solution for 2 hours prior to treatment with PA. The cells were then incubated again with serum, with or without PA for 3 hours, and allowed to re-enter the cell cycle. Preliminary results show that after exposure to Hank's solution for 2 hours an increased expression of p21 was observed compared to control which could be due to induced activation of p53 as a response to starvation (Shi et al., 2012) and as a result of that expression of p21 is increased. An increase in cytoplasmic PCNA expression was also detected which could imply that PCNA proteins accumulate when cells arrest in G1 phase and cannot enter S phase (Vriz et al., 1992). Upon addition of serum, nuclear expression was seen in occasional cells in control cultures. However, the cytoplasm of AsPC-1 is relatively small compared to the nucleus which makes detection of intracellular localisation difficult and to confirm this preliminary finding, other cell types have to be tested. At this time p21 was still expressed suggesting that progression through the cell cycle might still have been inhibited. If PA was present during this recovery from starvation, a decrease in PCNA expression was observed and no nuclear expression was detected. This can be interpreted as lack of recruitment to DNA synthesis (Figure 20). Further and more exclusive testing is needed to evaluate the effects of PA on DNA polymerase activity.

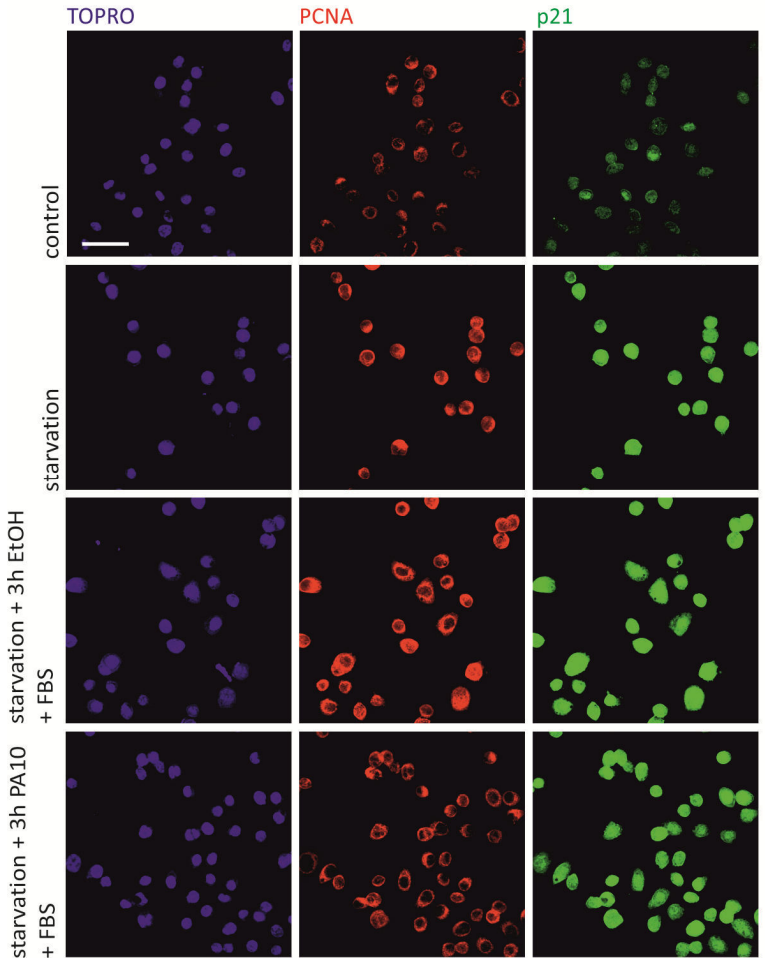


Figure 20. Expression of PCNA and p21 in pancreatic cancer cells related to starvation and treatment with PA.

Immunofluorescence staining in AsPC-1 cells showed a marked increase of p21 and PCNA expression cells after 2 hours of starvation. A decrease in PCNA expression was observed following PA treatment and addition of serum for 3 hours. The expression of p21 was not decreased. The scale bar shown represents 20 µm and applies to all panels.

4.3 Synergism between UA and PA and known cancer drugs in breast cancer cells

Synergistic effects can be obtained by combination therapy, thus lower doses can be used and thereby side effects and toxicity of chemotherapy can be minimized. Doxorubicin alone or in combination with other cancer drugs is widely used to treat various types of cancers such as breast cancer. The basic mechanism of doxorubicin effects is stopping DNA replication. Its accumulation and distribution in the cell is also pH-dependant (Webb et al., 2011; Yang et al., 2014). Studies have also shown that combination therapy with FASN inhibitors and other cancer drugs show synergistic effects in breast cancer cells (Menendez et al., 2004a; Vazquez-Martin et al., 2007). To explore further the effects of combining lichen metabolites with known cancer drugs the synergistic effects of PA and UA in combination with the anti-HER2 drug; lapatinib and doxorubicin were assessed using the medium-effect analysis of Chou and Talalay. Combination index (CI) quantitatively defines synergism (CI < 1), additive effects (CI = 1) and antagonism (CI > 1) (Chou, 2006). Concentrations around the IC_{50} values of PA, UA and doxorubicin from previous experiments, were used in combination with concentrations well below the IC_{50} of lapatinib (Table 3).

Table 3. Effects of PA, UA lapatinib and doxorubicin on two breast cancer cell lines.

Cancer type	Cell lines	PA (µg/mL)	Lapatinib (µM)	UA (µg/mL)	Doxorubicin (nM)
Breast cancer	SK-BR-3	3.5 ± 0.1	1.6 ± 0.3	7.9 ± 0.4	63.3 ± 6.5
	T-47D	3.8 ± 0.5	6.4 ± 0.7	6.7 ± 0.8	45.2 ± 3.8

Results are presented as IC_{50} values in µg/mL ± s.e.m.

Results, shown in figure 21A, indicate a synergism between PA (5 µg/mL) and lapatinib at two concentrations (0.01 µM; CI = 0.631 and 0.10 µM; CI = 0.713) in SK-BR-3 cells. However, at lower concentrations of PA antagonist effects are observed with lapatinib. This underscores that the effect of PA is complex and that its function needs to be explored in a dose dependent manner. In T-47D cells, weak synergism between PA (1 µg/mL; 5 µg/mL) and lapatinib was observed (0.10 µM; CI = 0.810 and 0.840) (1.00 µM; CI = 0.760 and 0.794), respectively. No synergistic effects were detected in either cell line when PA was combined with doxorubicin (Figure 21).

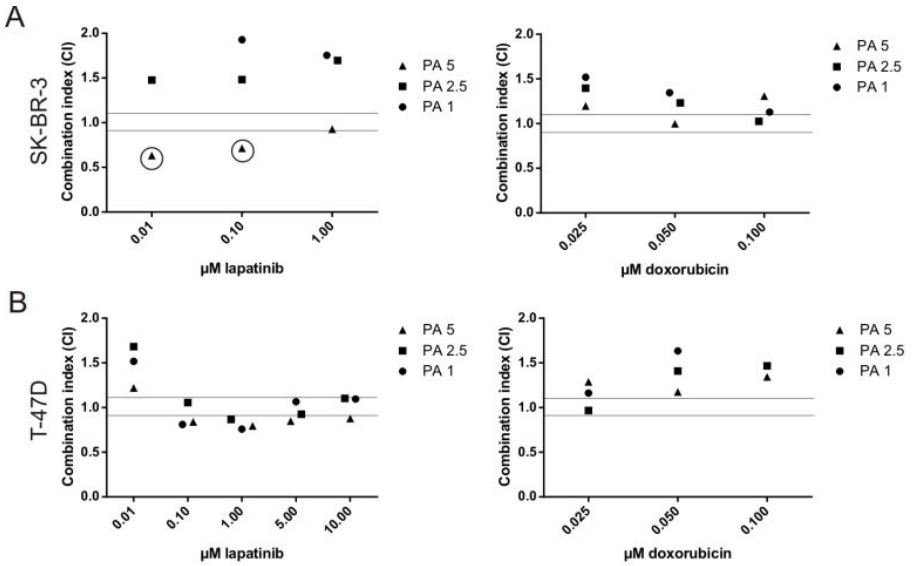


Figure 21. Synergism analysis of the interaction between PA and lapatinib and PA and doxorubicin in breast cancer cells.

(A) Combined treatment with PA (5 µg/mL) and lapatinib (0.01 µM) showed synergistic effects in HER2 overexpressing SK-BR-3 cancer cells, assessed using the medium-effect analysis of Chou and Talalay. Moderate synergism was observed after treatment with PA (5 µg/mL) and lapatinib (0.10 µM). (B) Weak synergism or additive interactions were seen after treatment with PA and lapatinib at various concentrations in T-47D cells. No synergistic effects were observed after combined treatment with PA and doxorubicin in either of the cell lines. Lines in the figures represent CI = 0.90 and CI = 1.10.

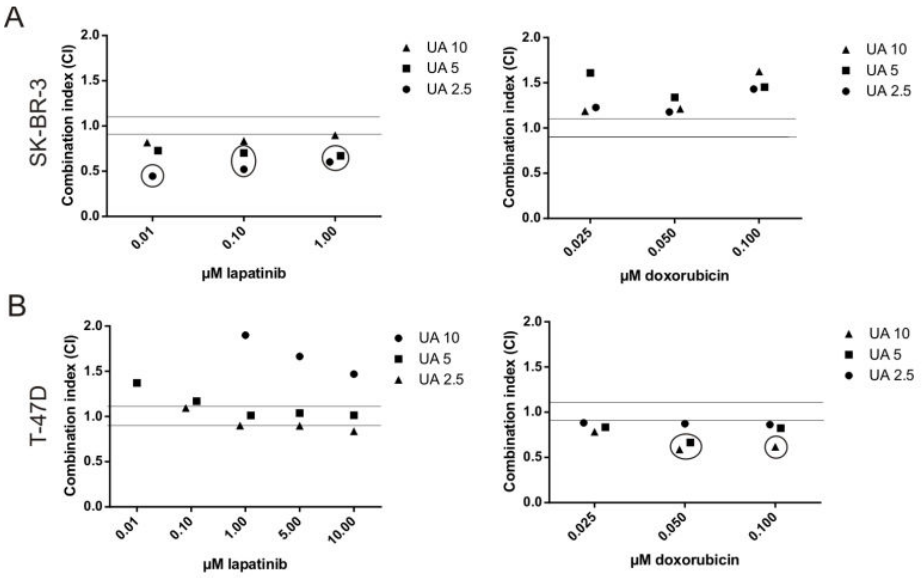


Figure 22. Synergism analysis of the interaction between UA and lapatinib and UA and doxorubicin in breast cancer cells.

(A) Combined treatment with UA (2.5 and 5 µg/mL) and lapatinib (0.01 and 0.10 µM) showed synergistic effects in HER2 overexpressing SK-BR-3 cancer cells, assessed using the medium-effect analysis of Chou and Talalay. No synergistic effects were observed after combined treatment with UA and doxorubicin (B) In T-47D cells combination treatment with UA (5 and 10 µg/mL) and doxorubicin (0.05 and 0.10 µM) indicated synergistic effects. Lines in the figures represent CI = 0.90 and CI 1.10.

Combined treatment with UA and lapatinib indicated synergism between two lower concentrations of UA (2.5 and 5 µg/mL) and all tested concentrations of lapatinib in SK-BR-3 cells (UA 2.5 µg/mL: 0.01 µM; CI = 0.446, 0.10 µM; CI = 0.521 and 1.00 CI = 0.603 UA 5.0 µg/mL: 0.10 µM; CI = 0.703, 1.00 µM; CI = 0.671). No synergistic effects were seen after UA and doxorubicin treatment (Figure 22A). However, in T-47D cells, a synergism was observed after treatment with UA (5 and 10 µg/mL) and doxorubicin (0.05 µM; CI = 0.665, CI = 0.588; 0.10 µM; CI = 0.618). No synergistic effects were observed after UA and lapatinib treatment (Figure 22B). Our focus has been on analysing the synergistic effects but it would be interesting to explore further the antagonist effects that we have also detected.

Synergism between PA and lapatinib is restricted to SK-BR-3 that overexpress HER2 supporting the hypothesis that this is likely to be dependent on FASN inhibition and the transcriptional repression of HER2.

UA shuttles protons across membranes and affects pH balance and energy status. Combined treatment with UA and lapatinib shows synergistic effects with lapatinib in SK-BR-3 cells and with doxorubicin in T-47D cells which could indicate that synergistic effects of these lichen compounds are dependent on characteristics of the cancer cells, metabolic effects of the lichen compounds a mode of action and distribution of the drugs.

5 General discussion

Previous work and data from this thesis

In previous studies from this laboratory the lichen compound UA has been shown to exhibit anti-proliferative and growth inhibitory effects on several cancer cell lines. Loss of mitochondrial membrane potential was observed after UA treatment and late necrosis was detected but no apoptosis. In this project data show that autophagy is induced after exposure to UA but is not completed, lysosomal acidification is reduced and degradative lysosomal conditions are impaired. Cellular energy and stress mechanism are affected indicating that UA-induced autophagy is mediated through both starvation and stress pathways. The fundamental activity of UA is its proton shuttling effect which can operate at two organelles, mitochondria and lysosome. The deregulation in pH is likely to be the cause of the disruption of lysosomal function and the loss in mitochondrial membrane potential leading to decreased cellular energy.

The lichen compound PA has previously been shown to have anti-proliferative effects on several cancer cell lines, without affecting normal skin fibroblasts, and to inhibit 5- and 12-LOX pathways. In this project data show that PA, which is a lipid compound, can be detected inside pancreatic cancer cells (Figure 5 in paper #3 manuscript) indicating that it enters into the cells. Results suggest that the pro-apoptotic and anti-proliferative effects of PA are not mediated by its LOX-inhibitory effects in pancreatic and myeloma cancer cells. Data also suggest that the anti-proliferative effects of PA are likely to occur at an early stage in the cell cycle and possibly through direct inhibition of DNA polymerase. Previous work in the laboratory had indicated that PA inhibited FASN activity in breast cancer cells. Data from this thesis correlate with previous findings suggesting that PA is a direct inhibitor of FASN leading to transcriptional repression of the HER2 receptor in breast cancer cells. Observed effects on major signalling pathways are probably a consequence of the reduced HER2 expression.

Results on synergism between the two lichen compounds and known cancer drugs suggest that the synergistic effects of these compounds are dependent on the characteristics of the cancer cells and how the compounds affect cellular metabolism. It is likely that their effects on cell signalling pathways, uptake and distribution of drugs in the cell can also play an important role.

In order to explore if the anti-proliferative effects of PA were mediated through cell signalling pathways, a few major pathways were tested after PA

treatment in cancer cell lines derived from different origins. Initial experiments where cells where treated with PA for 2 hours showed no effects on the two major signalling pathways ERK1/2 and AKT in ovarian cancer cells and glioblastoma cells, however PA treatment for 24 hours revealed decreased levels of phosphorylated/total ERK1/2 in those two cell lines and in one of two tested myeloma cell lines. Interestingly, levels of phosphorylated/total ERK1/2 were increased in pancreatic cancer cells. An increase in the levels of phosphorylated/total AKT was also observed in the ovarian and glioblastoma cancer cells after 24 hours of treatment but no effects were seen in pancreatic cancer cells. In addition, unpublished data from Marleen Georgi (Diploma thesis: Effects of the lichen compound protolichesterinic acid on proliferation, survival and STAT3 activity of human cancer cells) showed that PA treatment did not affect activation of signal transducer and activator of transcription 3 (STAT3) in the two tested myeloma cell lines. These results suggest and correlate with results presented in this thesis that the effects of PA on cell signalling occur late and are cell type-dependent and therefore the anti-proliferative effects of PA are not likely to be primarily mediated through inhibition of signalling from growth factor receptors, rather that the observed effects on cell signalling are secondary.

In order to explore the effects of the two lichen compound on cell cycle control, PA and UA were screened for inhibitory activity on cyclin dependent kinase 5 or on the dual tyrosine-phosphorylation-regulated kinase 1A, which are involved in regulation processes in the development of the central nervous system and are suggested to play a role in regulating cell proliferation (Dehbi et al., 2014). PA and UA did not show any inhibitory effects on these kinases. The screening assays were performed by Dr. Laurent Meijer and colleagues at the French National Centre for Scientific Research. These results were not followed further, the focus was rather set on investigating in more detail other potential mechanisms of action of these compounds.

Comparison with previous reports on UA, PA and related compounds.

UA has been shown to induce apoptosis in leukemia cells (Bazin et al., 2008; Bezivin et al., 2004) in colon and ovarian (Backorova et al., 2012) and in lung cancer cells (Singh et al., 2013b). However, previous results from our laboratory and from this thesis indicate that UA does not induce apoptosis in breast and pancreatic cancer cells and that pancreatic cancer cells undergo late necrosis after UA treatment (Einarsdottir et al., 2010). Induction of necrosis after UA treatment has also been shown in mouse hepatocytes (Han

et al., 2004). These results indicate that the effects of UA on cell death appear to be cell-line-dependent and are likely to be triggered through various pathways. In the same study Han and colleagues show that UA causes drop in ATP levels and suggest that UA inhibits complex III or other components in the mitochondrial respiratory chain (Han et al., 2004). In 2009 a paper came out indicating that UA affects several genes encoding proteins for complexes I-IV of the mitochondrial electron transport chain. UA was shown to cause proton leakage through mitochondrial membranes and the authors suggest that up-regulation of complexes I-IV occurred as a compensatory mechanism to sustain the proton gradient necessary to maintain ATP synthesis by complex V (Joseph et al., 2009). Data from this thesis suggest that effects on the respiratory chain are an indirect consequence of the proton shuttling properties of UA. The proton gradient across mitochondrial membranes becomes deregulated causing decrease the membrane potential. In addition data presented in this thesis suggest that UA also shuttles protons through lysosomal membranes in breast cancer cells and can therefore disturb the pH gradient in both these organelles. Furthermore UA induces autophagy but because of deregulated pH gradient autophagy cannot be completed. The effects of UA *in vivo* have been less studied than its *in vitro* effects, however, UA has been shown to inhibit tumour growth in Sarcoma 180-bearing mice (Ribeiro-Costa et al., 2004) and in a mouse breast xenograft model (Song et al., 2012).

Available reports show that PA has mostly been investigated in simple screening studies along with several other lichen compounds against e.g. anti-bacterial, anti-fungal and anti-trypanosomal activity (Goel et al., 2011; Igoli et al., 2014; Turk et al., 2003). PA has been included in a study exploring the effects on FASN inhibition where a synthetic racemic mixture of PA (ID_{50} = 3.9 µg/mL) was shown to inhibit FASN in breast cancer cells to a similar extent as a known FASN inhibitor cerulenin (ID_{50} = 3.3 µg/mL) (Kuhajda et al., 2000). Previous data from our laboratory and from this thesis also imply that PA inhibits FASN in breast cancer cells.

Our preliminary data indicate that PA could possibly have direct inhibitory effects on DNA polymerase. Fifteen DNA polymerases are encoded in the genome and they are essential for DNA replication. Targeting those enzymes could therefore be a novel cancer therapeutic strategy (Lange et al., 2011). Several small-molecular inhibitors of Pol β have been identified. Recently it was shown that one derivative of kohamaic acid which is a sesterterpenic acid derivative that was first isolated from the marine sponge *Ircinia* sp. exhibited strong inhibitory effect on Pol β and inhibited growth of leukemia

cells. Moreover, the length of the aliphatic side chain was important for the inhibition (Mizushina et al., 2009). Interestingly, PA has an aliphatic chain length of 13 carbon. It has also been indicated that treatment with conjugated eicosapentaenoic acid, which inhibits DNA polymerases β,δ and ε, suppresses cancer cell growth and enhances the radiosensitivity of human colon carcinoma cells (Kumamoto-Yonezawa et al., 2010). Specific inhibitors of DNA polymerase could therefore be a novel approach as an adjuvant therapy for DNA damaging radiation or chemotherapy. It is therefore of interest to investigate further the effects of PA on DNA polymerase inhibition.

Mechanism of activity of UA and PA

Lichen metabolites have been shown to have various possible biological functions (Shrestha and St Clair, 2013) which indicates that their mode of action is diverse and it is likely that they can affect several different pathways at the same time rather than target one specific pathway. In addition to previous reported data results from this thesis point to the direction of one main mechanism of action of the lichen compound UA on cancer cells. One can speculate if effects of UA on various pathways and organelles proposed in this thesis could all be explained by the proton shuttling effects of UA through mitochondrial and lysosomal membranes. It is likely that when protons are shuttled across mitochondrial membrane the pH gradient becomes deregulated and causing a loss in mitochondrial membrane potential which then results in a decrease in cellular energy. Lower levels of ATP are detected followed by an increase in phosphorylation of the energy sensor AMPK. This initiates signalling to inhibit cell growth and cell cycle progression (Shackelford and Shaw, 2009) and the loss of cellular energy induces cellular stress. Suppression of the mTORC1 signalling pathway activates autophagy (Amaravadi et al., 2011). However autophagy is not completed due to the disruption of pH gradient in the lysosome which occurs in a similar way as in the mitochondria, through proton-shuttling properties of UA. It is therefore very likely that the proton-shuttling effects of UA can explain its effects directly or indirectly on various pathways in cancer cells.

The lichen compound PA has been less studied, however, data indicate that the mode of action of PA in cancer cells is far more diverse and complex than for UA. No clear single underlying mechanism seems to explain the effects of PA on cancer cells. Data from this thesis imply that the effects of PA are cell-line-dependant and therefore the anti-proliferative effects of PA can be mediated through different pathways in cancer cells derived from different origin. Data also suggest that the LOX-inhibitory and anti-proliferative effect

of PA are not linked. A study by Fischer and colleagues indicates that the cytotoxic and anti-proliferative effects of certain 5-LOX inhibitors are independent of suppression of 5-LOX activity (Fischer et al., 2010) and data from this thesis also suggest that the anti-proliferative effects of PA are not related to it LOX-inhibitory effects in pancreatic cancer cells. It would be very interesting to follow up on the LOX-inhibitory effects of PA in relation to inflammatory diseases.

PA has similar chemical structure as a known FASN inhibitor, C75 (Kuhajda et al., 2000). The primary effect of PA in breast cancer cells that are driven by overexpression of FASN and HER2 is likely due to a direct inhibition of FASN leading to decreased cell viability and therefore it is possible that the effects of PA on FASN is related to its chemical structure. To explore further whether PA directly inhibits FASN it will be important to monitor the effects of PA in cells where FASN has been knocked down e.g. using siRNA.

Currently available methods for testing FASN inhibition are not optimal. The FASN enzyme is not commercially available and our previous study in the laboratory showed that FASN isolated from rat liver is rapidly degraded and therefore not suitable for investigation. FASN activity has been estimated by the indirect method of measuring the decrease of oxidation of NADPH after malonyl-CoA has been added (Puig et al., 2008). Measuring cellular uptake of ^{14}C-acetate can be used as an initial screen and this can be refined by assaying ^{14}C-acetate incorporation into total cellular lipids (Little et al., 2007). Therefore, to confirm that PA inhibits FASN further investigations are needed. With increasing knowledge in lipid analysis by mass spectrometry, it should become possible to use that sensitive technique in targeted lipid analysis for direct quantification of synthesised lipids (Murphy and Gaskell, 2011).

In cancer cells that do not depend on FASN overexpression PA is likely to mediate its anti-proliferative effects through other mechanism. One possible mechanism could be a direct inhibition in DNA polymerase that indicate that PA affects the cells at an early stage in the cell cycle. Further studies are needed to determine the proposed mechanism of action of PA. Interestingly, these two lichen compound therefore seem to have very different mode of action in cancer cells. UA most likely affects cell through one main mechanism, its proton shuttling effects, while the effects of PA are likely to be more complex and diverse and to be related to its chemical structure.

Potential for drug development

Lichens grow slowly and are difficult to propagate in culture and it can be complicated to synthesize their compounds (Shrestha and St Clair, 2013). However, in 2009 Drew J. Hawranik and colleagues synthesized UA from commercially available starting material in two steps which could be a driving force for further biosynthetic studies of UA (Hawranik et al., 2009). It is also possible to synthesize PA and analogues with simple approaches (Kuhajda et al., 2000; Le Lamer et al., 2014). Another limiting factor of working with compounds derived from nature is their poor solubility which can hinder further pharmacological testing. It has been shown that the use of the complex agent 2-hydroxypropyl-beta-cyclodextrin and UA can improve the delivery of the compound into leukemia cells (Kristmundsdottir et al., 2002) and encapsulation of UA into nanocapsules has been shown to increase anti-tumour activity in Sarcoma-bearing mice compared to standard UA treatment and considerably reduce the hepatotoxicity of UA (da Silva Santos et al., 2006). It has also been shown that by conjugating UA to a polyamine chain it is possible to increase the cytotoxic activity in several cancer cell lines (Bazin et al., 2008). An essential step in further pharmacological investigations of nature-derived compounds like UA and PA is the establishment of their adverse effects and thorough studies on the hepatotoxicity of UA and the possible weight loss toxicity of PA related to FASN inhibition (Bunchorntavakul and Reddy, 2013; Makowski et al., 2013).

Taken together, data from this thesis along with previously reported data strongly indicate that the anti-proliferative activity of these two lichen compounds is diverse and is mediated through various mechanisms and is most likely cell-dependent rather than targeting one specific pathway.

Potential for synergistic action.

Synergism with known cancer drugs presents another possibility for therapeutic use of natural compounds such as UA and PA. The proposed mechanism for the synergism between lapatinib and PA suggested from this thesis involves inhibition of HER2 activation by lapatinib, leading to a decrease in FASN activation through inhibition of phosphorylation by activated HER2 (Jin et al., 2010). If PA is inhibiting FASN it would result in a decrease in HER2 expression on the cell surface, therefore, a lower concentration of lapatinib would be needed to inhibit HER2 activity in the SK-BR-3 breast cancer cells. The synergism of UA and doxorubicin and lapatinib could be explained by its effects on pH balance and energy status in the cancer cell. The distribution and accumulation of doxorubicin is pH-

dependant (Webb et al., 2011) and therefore UA could be acting as a chemo-sensitizer by enhancing the effects of doxorubicin-induced apoptosis in the T-47D breast cancer cells. The effects of UA on energy status could then explain the synergism between UA and lapatinib. The cell needs energy to proliferate, UA affects ATP levels and AMPK and decreases cellular energy, therefore, a lower concentration of lapatinib is needed to exhibit cytotoxic effects and synergism between lapatinib and UA is observed in SK-BR-3 cells.

The future in cancer therapy lies in using combinational approaches rather than targeted therapies to avoid resistance and compensatory effects of other mechanism to survive treatment. These two lichen compound could be novel candidates for further testing in combinational therapy because of the diverse mode of action of these compounds.

6 Conclusions

The main focus of this research project was to try to understand the mechanisms that lie behind the reported anti-proliferative effects of the two lichen compounds UA and PA. It is important to gain understanding of the pathways that are involved in mediating the anti-proliferative effects of the lichen compounds in order to take rational steps in studying these compounds.

The dibenzo-furandione compound UA shuttles protons across mitochondria and lysosomal membranes. Results from this thesis strongly indicate that the proton-shuttling effect of UA is the main mode of action that mediates the anti-proliferative effects on several types of cancer cells. The effect of the aliphatic α-methylene γ-butyrolactone compound PA on cancer cells is likely to be more complex and diverse and related to its chemical properties. Data from this thesis indicate that the anti-proliferative effects of PA can be cell-type-dependant and affect the cells at an early stage in the cell cycle, possibly through direct inhibition of DNA polymerase. The inhibitory effect of PA on FASN in breast cancer cells is likely to be related to its chemical structure. In addition, results show that combined treatment with the lichen compounds and known cancer drugs exhibits synergistic effects that opens new possibilities for therapeutic use of these natural compounds against cancer.

Individualized and tumour-specific therapy based on the combined use of targeted and conventional drugs as a first line treatment is a future goal in cancer therapy. The use of natural compounds like UA and PA in combinational therapy could be a novel therapeutic approach. Basic science is fundamental in the long pathway towards discovery of new drugs and it is important to bear in mind that only a small proportion of compounds that are discovered as potential drug candidates reach the preclinical stage of drug discovery. From approximately 5-10 thousand drug candidates that reach the drug and development process one drug is approved by the FDA. In addition the road towards identification of a new drug take approximately 10-15 years and is extremely costly (Pharmaceutical Research and Manufacturers of America (PhRMA), 2014). It has been shown that up to 70% of drug candidates never pass through the toxicity testing in animals during the early research phase. During drug development most of the drugs are withdrawn from clinical testing in phase II and III (Silber, 2010). Nevertheless with good collaboration of the best scientific minds from academic laboratories and the efforts of the industry, the best available technology, a lot of positivity and

persistence and a little bit of luck it is possible to bring basic science discoveries to important new drugs to keep up the battle against diseases.

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Paper I

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Proton-Shuttling Lichen Compound Usnic Acid Affects Mitochondrial and Lysosomal Function in Cancer Cells

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Abstract

The lichen compound usnic acid (UA) is a lipophilic weak acid that acts as a proton shuttle and causes loss of mitochondrial inner membrane potential. In the current study we show that UA treatment induced the formation of autophagosomes in human cancer cells, but had minimal effects on normal human fibroblasts. However, autophagic flux was incomplete, degradation of autophagosomal content did not occur and acidification was defective. UA-treated cells showed reduced ATP levels and activation of AMP kinase as well as signs of cellular stress. UA is thus likely to trigger autophagosome formation both by energy depletion and stress conditions. Our findings indicate that the H^+-shuttling effect of UA operates not only in mitochondria as previously shown, but also in lysosomes, and have implications for therapeutic manipulation of autophagy and pH-determined drug distribution.

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Introduction

Lichens, the symbiosis between a fungal partner and a photobiotic microorganism, are found all around the world and give rise to a large number of unique secondary metabolites [1]. The dibenzofuran derivative, usnic acid (UA) is a known secondary metabolite and has been studied to some extent [2]. A wide range of biological activities has been reported for usnic acid, e.g. anti-microbial, anti-viral, anti-pyretic, anti-inflammatory and analgesic effects [2]. Anti-tumor activity of UA was first reported three decades ago in lung carcinoma in mice and in P388 leukemia [3,4]. It has furthermore been shown that usnic acid has anti-mitotic effects on human cancer cell lines [5] and causes a loss of viable cells in leukemia, lung and breast cancer cells [6,7]. However, exposure to UA does not activate p53 and has not been proposed to be involved in DNA damage [8].

UA is a lipophilic weak acid (p*K*a 4.4) that can cause proton leakage by diffusing through mitochondrial membranes [9]. In mouse liver cells usnic acid disrupts the normal metabolic processes of cells by uncoupling oxidative phosphorylation in mitochondria and by activating oxidative stress [10]. Mitochondria play an important role in the regulation of cell death pathways and mitochondrial changes have been described in cancer cells, including increased stability, thus inhibiting the release of cytochrome *c* and preventing induction of apoptosis [11]. Our previous study showed that UA treatment causes loss of mitochondrial membrane potential in two different cancer cell lines [12]. Interestingly, it has been shown that changes in mitochondrial membrane potential can lead to the onset of autophagy [13].

Autophagy is a process that can both aid cancer cell survival during nutrient shortage but can also promote cancer cell death. The molecular pathways that determine this dual role remain obscure and it is likely that the function of autophagy in cancer depends on tumor stage, cellular context and tissue of origin [14,15]. More than 30 different protein encoding genes, known as autophagy-related genes (ATG), have been identified and studies in mouse models have shown that macroautophagy is essential for maintenance of cellular homeostasis in many tissues [16,17]. Autophagy can be triggered by nutrition depletion or metabolic stress and can vary depending on the demand for substrate degradation and stimulus. The energy sensor AMP kinase signals to the mammalian target of rapamycin complex 1 (mTORC1), a major regulator of autophagy, which directly controls protein synthesis and anabolic processes in a nutrient-sensitive manner. Starvation-induced autophagic vesicles are formed, which are likely to contain free cytosol [18,19]. Additionally, other stress conditions such as damaged organelles, intracellular pathogens or stress in the endoplasmic reticulum can induce autophagy through different pathways from those activated by starvation [19]. The maturation process, the final step of autophagy, involves delivery and degradation of autophagic cargo. Fusion occurs with lysosomes, and autophagic vesicles coalesce and contents are degraded. The acidic environment of lysosomes is essential for the final steps of autophagy, and by disrupting the vacuolar H^+ ATPase, which is involved in acidifying lysosomes, the completion of autophagy can be inhibited [15,19].

The aim of present study was to explore further the consequences of loss of mitochondrial membrane potential induced by UA. We asked whether this caused release of cytochrome *c* and triggered apoptosis. Loss of membrane potential

and the property of UA to shuttle protons across membranes would be expected to lead to a decline in ATP production by mitochondria [9]. We found that UA treated cancer cells had decreased ATP levels and increased phosphorylation of AMP kinase. Interestingly, UA triggered autophagy but without degradation of autophagosomal content, suggesting a disruptive effect on autophagolysosomal acidification. Our results indicate that the induction of autophagy was mediated by a combination of response to nutrient shortage as well as cellular stress.

Materials and Methods

Plant Material, Cell Culture and Exposure to Test Substances

(+)-Usnic acid (97%) was isolated from *Cladonia arbuscula* (Wallr.) Rabenh. (Cladoniaceae) collected in open country in Iceland, not privately owned. Isolation and identification was performed as described [12]. The substance was dissolved in dimethyl sulfoxide (DMSO; Merck, 2951) and diluted for use in tissue-culture medium. All tests included controls where the highest equivalent concentration of DMSO was used. The breast cancer cell lines T47D and MCF7 and the pancreatic cancer cell line Capan-2 were obtained from the American Type Culture Collection (ATTC) through LGC Promochem. T47D contains a single mutated copy of p53 [20], but Capan-2 and MCF7 are homozygous for wild-type p53 [21]. MCF7 is estrogen receptor positive [22]. Primary human fibroblasts were cultured from normal skin biopsies and used in passage 6–13 (National Bioethics Committee permission VSNb2006020001/03-16; informed consent obtained). All cell lines were maintained in RPMI-1640 tissue culture medium (GIBCO^{TM}, 52400), containing 0.5% penicillin and streptomycin (GIBCO^{TM}, 15140-148) and 10% heat-inactivated fetal bovine serum (FBS; GIBCO^{TM}, 10270) with T47D receiving additionally 0.01 mg/mL insulin (Sigma, I1882) and subcultured following detachment by trypsin (0.25% Trypsin/EDTA, Difco^{TM}, 215240) as appropriate. Cells were seeded at an appropriate number to exceed 70–80% confluence after 24 hour culture. (+)-Usnic acid (5 or 10 μg/mL), metformin (10 mM; Sigma, D150959) and solvent control were added and the cells were incubated under standard conditions, for different time periods. For the induction of autophagy by nutrient deprivation, cells were incubated with Hank's solution (Sigma, H9394) for the last 40 min of the incubation time.

Estimation of Levels of ATP

Cells were detached by trypsinization, a small aliquot removed for counting and harvested using 0.5 M perchloric acid (Merck, 1.00519) for 10 min at 4°C. After centrifugation 10 μL of the supernatant were mixed with 1 mL of distilled water. Bioluminescence was assayed using 75 μL luciferase reagent (Promega, FF2021) which lysed the cells and provided the substrate luciferin. Luminescence was measured in a luminometer (Turner TD 20/20) and expressed as luminescence/cell.

Electron Microscopy

After 24 hours' incubation time at standard conditions cells were harvested by trypsinization and fixed in 1 mL of glutaraldehyde (Ted Pella Inc, 18426) solution for 60 min at room temperature, then centrifuged and stored at 0–5°C for 24 hours. After removing the glutaraldehyde, two drops of a 2% gelatinized solution (Ted Pella Inc, 19225) of distilled water were added to the cell pellet, carefully mixed and stored at 0–5°C for 24 hours. The samples were then washed twice with PBS and osmium tetroxide (Ted Pella Inc, 18463) added to each sample for one hour and washed again with PBS. Samples were cut to 2–5 mm pieces under a macroscope using razor blades. The pieces were dehydrated using ethanol (Merck, 64271D) at increasing concentrations, under rotation. Epoxy-resin (Ted Pella Inc, 18300) was added, first at 1:1 (vol:vol) with 99% ethanol (Merck, 64271) for one hour, then twice resin only for one hour each time. The moulds were then placed in an oven at 70°C for 24 hours. The resin was then sliced with a glass knife (thickness about 0.5 μm) and stained with toluidine blue for selection of samples for sectioning with a diamond knife (70–100 nm thickness). The samples were placed on a copper frame before staining with a 0.06 g/mL lead-citrate solution (Ted Pella Inc, 19314) and were visualized using a Philips EM300 electron microscope. Images projected were developed using standard procedures for photographing. The developed film was scanned into a computer with a Nikon Coolscan V ED.

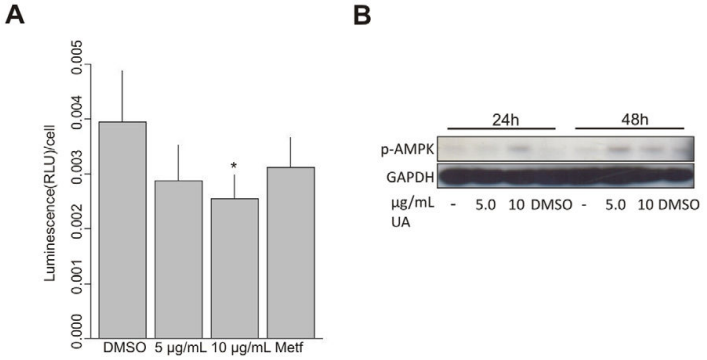

Figure 1. UA caused decline in cellular ATP and activation of AMP kinase. (A) Levels of ATP, measured in a luminometer, were decreased in T47D cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24 hours. Data are presented as luminescence/cell of each group compared with DMSO control. Error bars indicate standard error of the mean, *$p<0.05$. (B) Phosphorylation of AMP kinase, verified by Western blotting, was detected in T47D cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24 and 48 hours.
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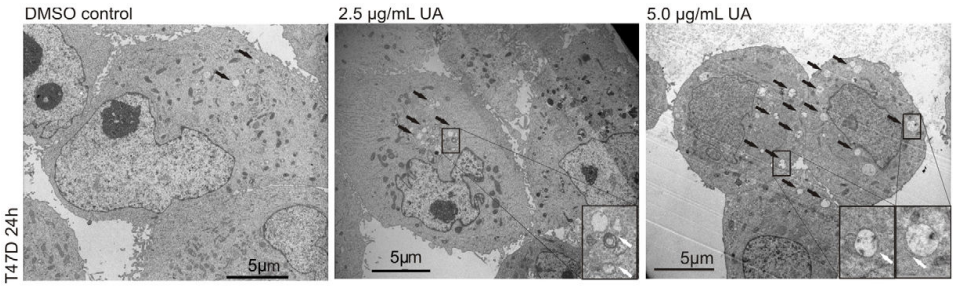

Figure 2. UA induced formation of autophagosome vacuoles. Induction of autophagic vacuoles, with double membranes characteristic of autophagosomes, was detected by electron microscopy in T47D cells after treatment with UA (2.5 and 5.0 µg/mL; DMSO 0.1%) for 24 hours. Black arrows indicate autophagic vacuoles, white arrows indicate double membrane formation.
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Immunocytochemistry

For immunofluorescence staining, cells were harvested and fixed in 4% paraformaldehyde (Sigma, P6148) and stained with anti-cytochrome *c*, mouse IgG2a monoclonal antibody (Abcam, ab110325), cleaved caspase-3, rabbit polyclonal antibody (Cell Signaling, 9661) or LAMP2, mouse IgG1 monoclonal antibody (H4b4, obtained from University School of Medicine, Baltimore), followed by Alexa Fluor red 546 goat anti-rabbit IgG antibody (Invitrogen, A11010), Alexa Fluor green 488 goat anti-rabbit IgG antibody (Invitrogen, A11070) or Alexa fluor red 546 goat anti-mouse IgG_{2a} antibody (Molecular Probes, A11018) For nuclear staining TO-PRO-3 iodide (Invitrogen, T3605) was used. For the LC3 detection the cells were fixed with methanol (Sigma, 34860) for 10 min at -20°C and stained with anti-LC3B (D11), rabbit IgG monoclonal antibody (Sigma, L7543) followed by Alexa Fluor green 488 goat anti-rabbit IgG antibody (Invitrogen, A11070). The stained cells were visualized and photographed under a confocal microscope (Zeiss, LSM 5 Pascal). For the immunoperoxidase staining cells were fixed with methanol (Sigma, 34860) for 5 min at -20°C and stained with anti-phospho-p70S6 kinase (Thr389; 108D2), rabbit IgG, monoclonal antibody (Cell Signaling, 9234), anti-LC3B (D11), rabbit IgG monoclonal antibody (Sigma, L7543) and anti-p62 (SQSTM1), rabbit polyclonal antibody (Enzo, PW9860) followed by incubation with monoclonal mouse anti-rabbit immunoglobulins IgG1κ (Dako, M0737), polyclonal rabbit anti-mouse immunoglobulins IgG (Dako, Z0259), PAP, horseradish peroxidase and mouse monoclonal anti-horseradish immunocomplexes, IgG1 (Dako, P850) and DAB tablets, chromogen (Dako, S3000). The stained cells were visualized and photographed under a under light microscope (Leica DMI 3000B).

Western Blot Analysis

Cells were harvested and lysed with RIPA buffer. Protein content was quantified spectrometrically using Bradford reagent (Sigma, B6916). Proteins were separated on NuPAGE 10% Bis-Tris Mini Gels and transferred to 0.2 µM polyvinylidene difluoride (PVDF) membrane by electroblotting. Membranes were probed with anti-phospho-AMPKα (Thr172) rabbit IgG monoclonal antibodies (Cell Signaling, 4188), anti-p62 (SQSTM1), rabbit polyclonal antibody (Enzo, PW9860), anti-LC3B (D11), rabbit IgG monoclonal antibody (Sigma, L7543), anti-phospho-eIF2α (Ser51) rabbit polyclonal antibody (Cell Signaling, 9721) or anti-G3PDH rabbit anti-human polyclonal antibody (R&D Systems, 2275-PC-1). Secondary antibody used was goat anti-rabbit IgG/HRPlinked (Cell Signaling, 7074S) and secondary antibody conjugated to IRDye-680 or 800 (Metabion, 68021). Proteins were visualized by the enhanced chemiluminescence (ECL) detection kit (GE Healthcare, RPN2132) and the signal was detected using a high performance chemiluminescence film (GE Healthcare, 91415) or detected by Odyssey infrared imaging system.

Visualization of Lysosomes by LysoTracker Probes

The tissue culture medium was replaced by pre-warmed (37°C) 75 nM Lysotracker Red DND-99 (Invitrogen, L7528) and cells incubated at 37°C for 1 h. Loading solution was then washed of and replaced by fresh medium and the stained cells were visualized and photographed under fluorescence microscope (Leica DMI 3000B). Lysotracker is a fluorescent acidotropic probe for labeling and tracing acidic organelles in cells. The protonated form of this probe accumulates in acidic compartments, where it forms aggregates that fluoresce bright red.

Transfection with tfLC3 Construct

The plasmid mRFP-GFP tandem fluorescent-tagged LC3 (tfLC3) construct was kindly provided by Prof. Kevin Ryan, Beatson Institute, Univeristy of Glasgow, with permission from Prof. Tamotsu Yoshimori, Osaka University [23,24]. Calcium/manganese based (CCMB) transformation of DH10B strains of E.coli was used as previously described [25]. Transfection was performed using TransPass D2 (BioLabs, M2554S) according to the manufacturer's protocol. After transfection cells were exposed to test substances or deprived of nutrients as described above. Cells were harvested and fixed in 4% paraformaldehyde (Sigma, P6148), and visualized and photographed under a confocal microscope (Zeiss, LSM 5 Pascal).

Statistical Analyses

Statistical comparisons of mean values were performed using two sided analysis of variance (ANOVA), including the treatment and number of run as factors, followed by a post. hoc comparison using Tukey HSD. p values are described in the text at appropriate points. On all figures, * and ** indicate $p<0.05$ and $p<0.001$ respectively. Images and data shown are representative of what was observed in at least three separate experiments.

Results and Discussion

The intrinsic pathway of apoptosis is triggered by opening of pores into the outer mitochondrial membrane leading to release of cytochrome *c* into the cytosol and activation of the caspase cascade

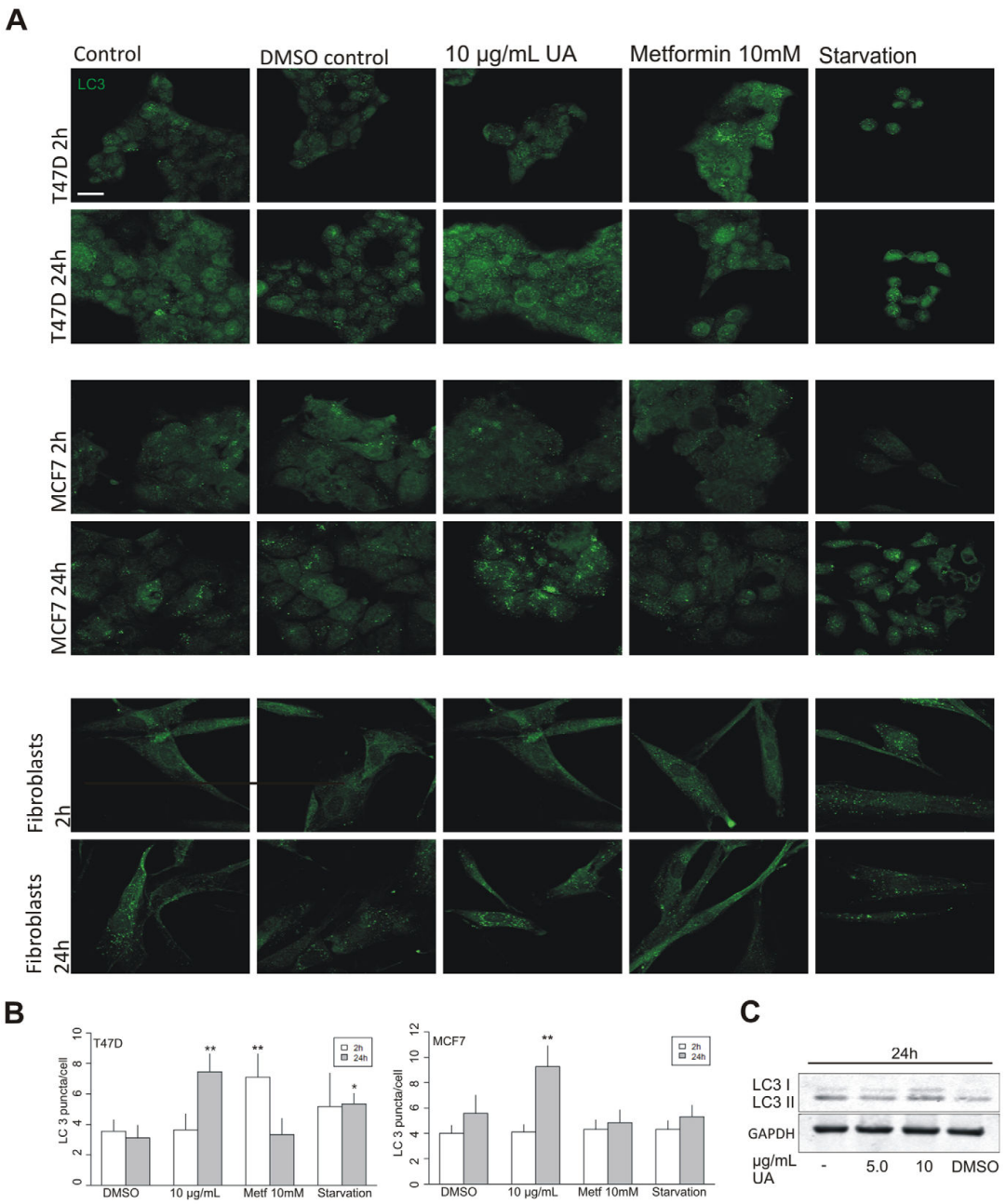

Figure 3. UA induced formation of LC3 puncta. (A) An increase in LC3 puncta was detected, by immunofluorescence in T47D and MCF7 cells after treatment with UA (10 µg/mL) for 24 hours. No effect was seen in normal fibroblasts. The scale bar shown represents 20 µm and applies to all panels. (B) LC3 puncta per cell were counted and quantified by ImageJ and data represented as LC3 puncta/cell of each group compared with DMSO control. Error bars indicate standard error of the mean, *$p<0.05$, **$p<0.001$. (C) Increase in LC3 I and LC3 II, verified by Western blotting, was detected in T47D cells after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 hours.
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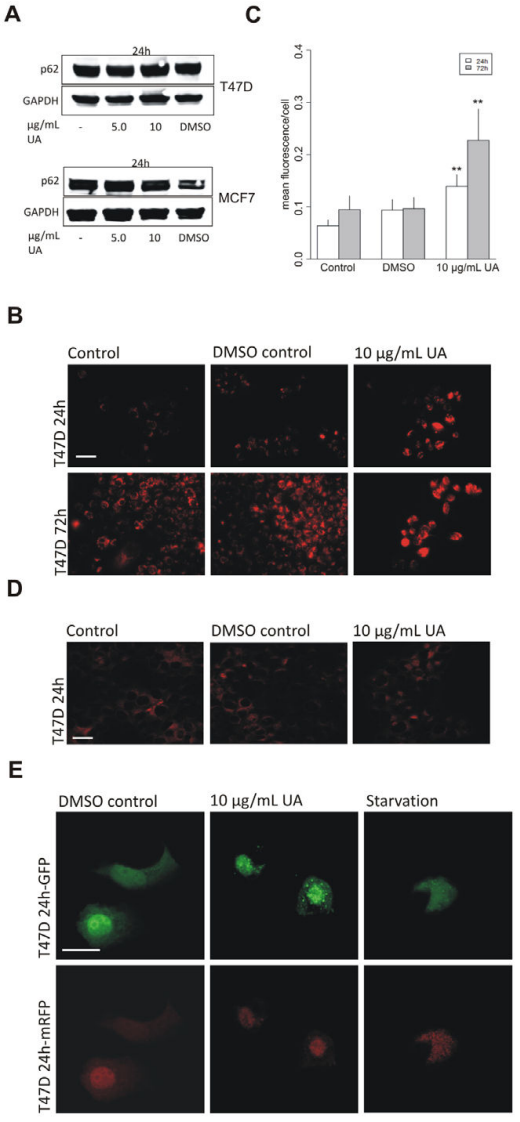

Figure 4. UA-induced formation of autophagosomal vacuoles was not followed by autosomal maturation and substrate degradation. (A) No degradation of p62 was detected, by Western blotting, in T47D and MCF7 cells after treatment with UA (5.0 and 10 µg/mL, 24 h; DMSO 0.1%). (B) Lysotracker, detected by fluorescence microscopy, shows diffuse staining in T47D cells after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 h and 72 h. (C) Lysotracker intensity per cell was quantified by ImageJ and data presented as mean fluorescence value of each group compared to DMSO control. Error bars indicate standard error of the mean, **$p<0.001$. The scale bar shown represents 100 µm and applies to all panels. (D) No effects on Lamp2 immunostaining were detected, after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 100 µm and applies to all panels. (E) A plasmid expressing mRFP-GFP-LC3 was transfected into T47D cells. Lack of autophagolysosomal acidification was seen after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 hours by detection of distinct GFP puncta. The scale bar shown represents 20 µm and applies to all panels.
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[26]. To follow up on our previous work on the effects of UA on mitochondrial membrane potential [12] we investigated cytochrome *c* leakage and cleavage of caspase-3 by immunostaining in the breast cancer cell line T47D and the pancreatic cell line Capan-2. No cytochrome *c* release or cleaved caspase-3 products were detectable after treatment with usnic acid (10 µg/mL) after 24, 48 and 72 hours (data shown for 72 hours; Fig. S1A and Fig. S1B). These results support our previous data that UA causes late necrosis but no apoptosis [12], and indicate that, although the mitochondrial pH gradient is disrupted, the mitochondria themselves are intact.

It has been reported that usnic acid causes uncoupling of mitochondria [27], inhibits mitochondrial respiration and causes a drop in ATP levels in murine hepatocytes [10]. Gene expression data from microarray analysis has strengthened the suggestion that usnic acid shuttles protons against the gradient created by the mitochondrial electron transport, as it leads to induction of genes associated with complexes I-IV of the electron transport chain [9]. To investigate this further, ATP levels were evaluated and phosphorylation of AMP kinase analyzed in T47D cells. Results indicate that UA treatment leads to decreased cellular levels of ATP after 24 hour treatment (5.0 µg/mL $p = 0.0523$, 10 µg/mL $p = 0.010$). As expected, the decreased levels of ATP were associated with increased phosphorylation of AMP kinase after both 24 and 48 hour treatment (10 µg/mL) (Fig. 1A and B).

This decline in cellular energy levels and triggering of the sensing mechanism would be expected to induce autophagy. Electron microscopy analysis of T47D cells treated with UA (2.5 and 5.0 µg/mL) for 24 hours indicated more marked presence of autophagic vacuoles, with double membranes characteristic of autophagosomes, compared with control (Fig. 2). These results were followed up by analysis for LC3 puncta by immunofluorescence, and an estimation of the abundance of autophagosomes, at different time points and treatments of three cell types (Fig. 3A, Fig. 3B and Fig. S2A–C). No effects were seen after treatment with UA (10 µg/mL) for 2 hours in any of the three cell lines, but an increase was observed after treatment with the anti-diabetic drug metformin in the T47D breast cancer cell line, which was no longer present following prolonged treatment. Metformin has previously been shown to stimulate AMP kinase already after one hour [28]. After 24 hours of treatment with UA (10 µg/mL) a significant increase in LC3 puncta was evident compared with controls in the two breast cancer cell lines. Immunoperoxidase staining of T47D cells also showed increased presence of LC3 puncta after UA treatment (Fig. S2D). These findings were further confirmed by observing an Increase in LC3 I and LC3 II by Western blotting (Fig. 3C). The effects on normal skin fibroblasts were not marked. For comparison, cells were starved by 40 min incubation in nutrient-free Hank's balanced solution. Although visual inspection suggested presence of autophagosome formation in starved cells (Fig. 3A), LC3 puncta were difficult to count and this harsh treatment was not well tolerated by the cells.

Having observed an increase in LC3 puncta after treatment with UA, we investigated whether the formation of autophagic vacuoles was followed by autophagic flux. The levels of the autophagosomal cargo p62 were evaluated after exposure to UA. The concentration of p62 has been shown to diminish if autophagic flux is increased as it is degraded in the process [29]. Formation of autophagosomes as a result of treatment with UA after 24 hours (5.0 and 10 µg/mL) in T47D and MCF7 cells (Fig. 4A and Fig. S3) was not followed by degradation of

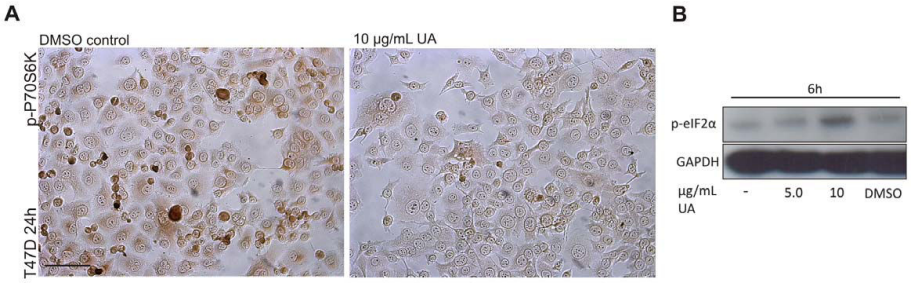

Figure 5. Formation of autophagosomes following UA treatment is likely to be induced through two different pathways. (A) A decrease in p-P70S6K was detected, by immunoperoxidase staining, after treatment with UA (10 µg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 100 µm and applies to both panels. (B) An increase in p-eIF2α was detected, after treatment with UA (10 µg/mL; DMSO 0.2%) for 6 hours.
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internalized protein. The absence of p62 degradation at 24 hours suggests a disruption of lysosomal acidification and autophagolysosome maturation which could be caused by the proton shuttling properties of usnic acid across the lysosomal membrane, as seen in depolarization of the mitochondria [9,10].

To evaluate further the effects of UA on lysosomes we used the lysosomal marker lysotracker in T47D cells which labels and tracks acidic organelles in cells. Results revealed very marked diffuse increase in lysotracker staining in T47D cells after UA treatment for 24 and 72 hours (Fig. 4B and Fig. 4C). This staining pattern has been interpreted as lysosomal dilatation as caused by treatment with chloroquine, which accumulates inside lysosomes. In cells treated with chloroquine, immunostaining for the lysosomal membrane protein Lamp1 copied the pattern obtained with lysotracker, thus confirming lysosomal dilatation [30]. In contrast, in our experiments, immunofluorescence staining for the lysosomal protein Lamp2 showed no morphological changes and no difference was observed between treated and untreated cells (Fig. 4D). This indicates that the lysotracker was staining outside the lysosome and could be explained by that fact that the retention of the dye inside of lysosomes depends on acidic pH [31]. The diffuse lysotracker staining following UA treatment might thus be due to protons being shuttled out of the lysosome, in a similar way as occurs across the mitochondrial membrane.

To explore autophagosome maturation following UA treatment, we utilized a plasmid construct, tfLC3 (mRFP-GFP-LC3 tandem-tagged fluorescent protein), with which we transfected the T47D cells. This method has previously been used to follow the autophagic maturation process. The GFP-LC3 loses fluorescence due to lysosomal acidity while the mRFP fluorescence is stable [23,24]. Results showed that in starved cells GFP fluorescence was attenuated implying acidic conditions and degradation by lysosomal hydrolases, whereas mRFP fluorescence remained stable. After treatment with UA for 24 hours, GFP, as well as mRFP fluorescence was observed indicating disruption of autophagolysosomal acidification and impaired degradative conditions after treatment with UA (Fig. 4E). The failure of these cells to complete autophagy could contribute to the accumulation of autophagic vacuoles and retention of undegraded p62.

One of the adaptive features of most cancers is dysregulated pH. In normal cells intracellular pH is lower than the extracellular pH. In cancer cells the gradient is reversed creating a favorable environment for metastatic progression. Higher intracellular pH is maintained because of increased H^+ efflux due to changes in the expression and/or activity of plasma membrane pumps and transporters [32]. The pH gradient in tumor cells is beneficial for the cellular accumulation of weak acids, such as usnic acid, causing weak acids to be mainly neutral at low pH and facilitating their transfer across the membrane. Treatment with UA shows significant induction of genes that are connected to complexes I through IV of the electron transport chain, which could be a compensating mechanism to preserve the proton gradient across the mitochondrial inner membrane [9,32].

Studies of several inherited syndromes that predispose to various types of tumors and carcinomas have led to the identification of the mTOR pathway as a regulator of autophagy. Among downstream targets of mTORC1 are p70S6K and 4EBP1, which have an essential role in cell-cycle control and proliferation [33,34]. In Fig. 1B we show that UA activates AMP-kinase, which signals to the mTORC1 complex. To explore downstream targets of mTORC1 we analyzed the effects in UA-treated cells on phosphorylated p70S6K by immunoperoxidase staining. The results showed a marked decrease in staining after treatment for 24 hours (Fig. 5A). Cells respond to nutrient shortage by inducing autophagy but this process can also be triggered by cellular stress [19]. To explore if UA could be triggering autophagy by other mechanism we tested for evidence of cellular stress in T47D cells after treatment with UA (10 µg/mL) for 6 hours. Increased phosphorylation of eIF2α, which is one of the recognized signs of ER stress, was detected (Fig. 5B).

The effects of usnic acid on autophagy can be compared with those described for the anti-malarial drug chloroquine which is currently in clinical trials in combination with anticancer regimens [19]. Chloroquine is a weak base (p*K*a 8.5) and accumulates inside lysosomes which become distended and dysfunctional, blocking autophagic flux [19]. In contrast, usnic acid is a weak acid that shuttles protons across membranes, thus increasing lysosomal pH, as shown by retention of the GFP signal, but lysosomal shape was not affected (LAMP2 staining). ER stress is induced by proteasome inhibition and ER-associated autophagy is therefore particularly relevant for cancer therapy with proteasome inhibitors. It has been shown that combining chloroquine with the proteasome inhibitor bortezomib increases tumor cell death *in vitro* and *in vivo* [35]. Cellular uptake and intracellular distribution of drugs is affected by pH [32,36]. Chloroquine can e.g. prevent intracellular sequestration in lysosomes [36] but has no effect on mitochondrial accumulation of daunorubicin, suggesting that the compound does not affect mitochondrial pH [37]. As usnic acid affects pH in lysosomes and mitochondria it is predicted to influence intracellular drug distribution.

In conclusion, our previous study has shown that UA causes loss of mitochondrial membrane potential. In the current study, we have shown that this does not lead to release of cytochrome *c* and triggering of apoptosis. The H^+ shuttling effect of UA operates at two organelles, mitochondria and lysosomes and its effect on autophagosome formation is likely to be triggered both by nutrition depletion and stress conditions. Autophagic flux is however incomplete and degradation of autophagosomal content does not occur. Our findings have implications for therapeutic manipulation of autophagy and pH-determined drug distribution.

Supporting Information

Figure S1 UA does not cause apoptosis. (A) Cytochrome c leakage was not detectable, by immunofluorescense in T47D and Capan-2 cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24, 48 and 72 hours. (B) No cleavage products of Caspase-3 were detectable after treatment with UA (10 μg/mL; DMSO 0.2%) after 24, 48 and 72 hours. The scale bar shown represents 20 μm and applies to all panels.
(TIF)

Figure S2 UA induces formation of autophagosome vacuoles. LC3 puncta per cell were counted and quantified by ImageJ and data presented as 95% family-wise confidence level. (A) T47D cells treated with UA for 2 and 24 hours. (B) MCF7 cells treated with UA for 2 and 24 hours. (C) Normal human fibroblasts treated with UA for 2 and 24 hours. (D) An increase in LC3 immunoperoxidase staining was detected, in T47D cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 100 μm and applies to both panels.
(TIF)

Figure S3 UA does not lead to degradation of p62. No decrease in p62 immunoperoxidase staining was detected, in T47D cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 100 μm and applies to both panels.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HMO MB MHO. Performed the experiments: MB ME IHM EE. Analyzed the data: MB ME. Contributed reagents/materials/analysis tools: SO. Wrote the paper: MB HMO MHO.

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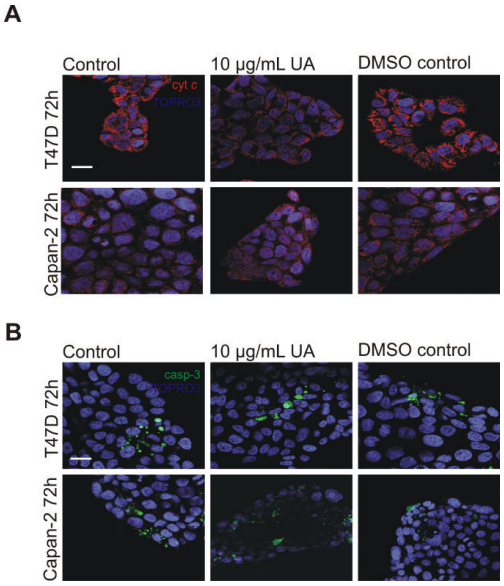
Bessadottir et al., Supplementary Figure 1



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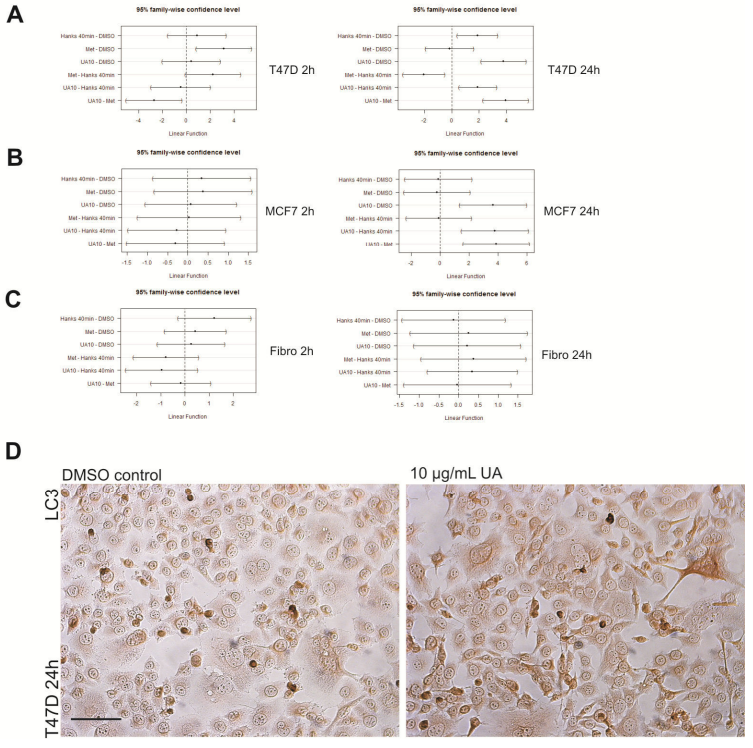

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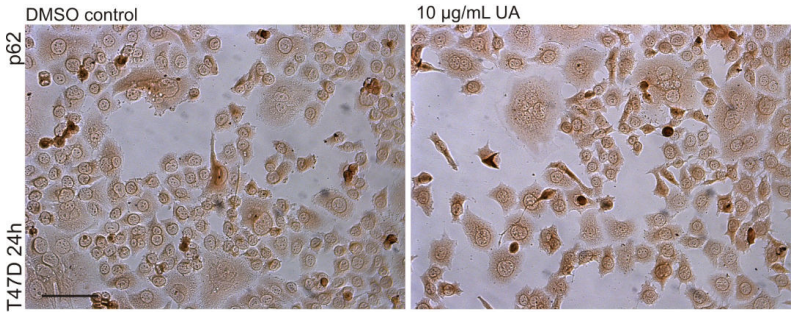

Supplementary Figure Legends

Supplementary Figure 1. UA does not cause apoptosis. (A) Cytochrome c leakage was not detectable, by immunofluorescense in T47D and Capan-2 cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24, 48 and 72 hours. (B) No cleavage products of Caspase-3 were detectable after treatment with UA *(10 μg/mL;* DMSO 0.2%*) after 24, 48 and 72 hours.* The scale bar shown represents 20 μm and applies to all panels.

Supplementary Figure 2. UA induces formation of autophagosome vacuoles. LC3 puncta per cell were counted and quantified by ImageJ and data presented as 95% family-wise confidence level. (A) T47D cells treated with UA for 2 and 24 hours. (B) MCF7 cells treated with UA for 2 and 24 hours. (C) Normal human fibroblasts treated with UA for 2 and 24 hours. (D) An increase in LC3 immunoperoxidase staining was detected, in T47D cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 100 μm and applies to both panels.

Supplementary Figure 3. UA increases levels of p62. (A) An increase in p62 immunoperoxidase staining was detected, in T47D cells after treatment with UA (10 μg/mL; DMSO 0.2%) for 24 hours. The scale bar shown represents 100 μm and applies to both panels.

Paper II

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Effects of anti-proliferative lichen metabolite, protolichesterinic acid on fatty acid synthase, cell signalling and drug response in breast cancer cells

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ABSTRACT

Background: The lichen compound (+)-protolichesterinic acid (+)-PA, isolated from Iceland moss, has anti-proliferative effects on several cancer cell lines. The chemical structure of (+)-PA is similar to a known fatty acid synthase (FASN) inhibitor C75.
Aims: To test whether the anti-proliferative activity of (+)-PA is associated with effects on FASN and HER2 (human epidermal growth factor receptor 2) and major signalling pathways. Synergism between (+)-PA and lapatinib, a HER2 active drug, was also evaluated.
Materials and methods: Pure compound was isolated by preparative high-performance liquid chromatography (HPLC) and purity of (+)-PA analyzed by analytical HPLC. Cell viability was assessed using Crystal violet staining. FASN and HER2 expression was estimated by immunofluorescence. The Meso Scale Discovery (MSD)® assay was used to measure activation of ERK1/2 and AKT. Synergism was estimated by the CalcuSyn software.
Results: Treatment with (+)-PA increased FASN expression in SK-BR-3 cells, which overexpress FASN and HER2, implying a compensatory response to inhibition of FASN activity. HER2 expression was decreased suggesting secondary downregulation. ERK1/2 and AKT signalling pathways were inhibited, probably due to reduced levels of HER2. No effects were observed in T-47D cells. Synergism between (+)-PA and lapatinib was observed in the SK-BR-3 cells.
Conclusion: Results suggest that the primary effect of (+)-PA is inhibition of FASN activity. Synergistic effects with lapatinib were seen only in SK-BR-3 cells, and not T-47D cells, further supporting the notion that (+)-PA acts by inhibiting FASN with secondary effects on HER2 expression and signalling. (+)-PA could therefore be a suitable agent for further testing, alone or in combination treatment against HER2-overexpressing breast cancer.

Introduction

Lichens are formed by symbiosis between fungi, algae and/or cyanobacteria and are found widely around the world (Shrestha and St Clair 2013). Iceland moss, *Cetraria islandica* has been used traditionally to treat chest conditions and for relief of stomach ulcers (Ingolfsdottir et al. 1994). (+)-Protolichesterinic acid ((+)-PA)) was reported by Sticher in 1965 to be a component of *Cetraria islandica* and is considered to be one of its major biologically active secondary metabolites (Sticher 1965) (Fig. 1).

PA is produced by several lichen species, in either (+) or (−) form, however, many reports do not state which form was tested. (+)-PA has anti-bacterial activity, specifically against *Helicobacter pylori* (Ingolfsdottir et al. 1997) and mycobacteria (Ingolfsdottir et al. 1998) supporting the traditional use of Iceland moss for treatment of peptic ulcers and chest conditions. A role for (+)-PA in mediating anti-inflammatory effects is suggested by its ability to inhibit 5- and 12 lipoxygenase (LOX) (Bucar et al. 2004; Ingolfsdottir et al. 1994; Kumar and Muller 1999). *In vitro* studies also showed (+)-PA to be a potent inhibitor of the DNA polymerase activity of human immunodeficiency virus-1 reverse transcriptase (HIV-1 RT)

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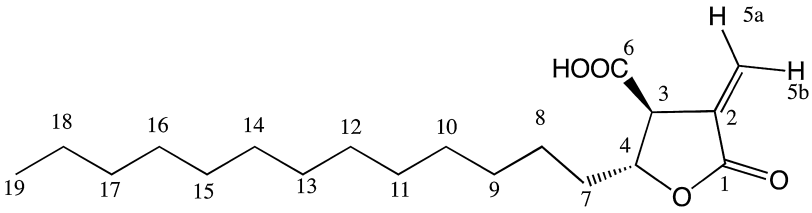

Fig. 1. Chemical structure of (+)-protolichesterinic acid.

(Pengsuparp et al. 1995). PA has anti-proliferative effects on several types of cancer cells; no effects were seen on normal skin fibroblasts (Brisdelli et al. 2013; Haraldsdottir et al. 2004; Ogmundsdottir et al. 1998). In our recent studies we found that the anti-proliferative effect of (+)-PA is not likely to be related to its LOX-inhibitory activity in pancreatic cancer and multiple myeloma cells (Bessadóttir et al., in manuscript). Others have suggested that the anti-proliferative effects of PA are mediated *via* the inhibition of Hsp70 expression in prostate cancer cells (Russo et al. 2012). Pro-apoptotic effects of PA are inconsistent and likely to be cell line dependent (Brisdelli et al. 2013; Bessadóttir et al., in manuscript).

Type I fatty acid synthase (FASN) is a multifunctional lipogenic enzyme which catalyses the synthesis of free fatty acids. Its expression is controlled mainly by nutritional signals (Abramson 2011). In 1994 Kuhajda and colleagues reported that a protein (oncogenic antigen-519) linked to poor prognosis in breast cancer was identical to FASN (Kuhajda et al. 1994). Since then FASN has been shown to be up-regulated in a variety of cancers such as breast (Vazquez-Martin et al. 2008; Wang et al. 2001), prostate (Migita et al. 2009) and colon (Notarnicola et al. 2012). The mechanism behind the FASN overexpression is not completely understood but sex steroid hormones and their receptor (Chalbos et al. 1987; Menendez et al. 2005b) as well as the human epidermal growth factor receptor 2 (HER2) (Kumar-Sinha et al. 2003; Vazquez-Martin et al. 2009) have been shown to have an important role involving the mitogen activated protein (MAP) kinase and phosphatidylinositol (PI) 3-kinase signalling cascades (Yang et al. 2002). FASN expression is significantly higher in breast tumours that overexpress HER2 (Vincent-Salomon et al. 2008), which is overexpressed in 20–25% of invasive breast cancer and is linked to poor prognosis (Slamon et al. 1987).

Cerulenin was the first characterized FASN inhibitor derived from the fungus *Cephalosporum caerulens* (Omura 1976). FASN inhibition was shown to be cytotoxic to cancer cells by triggering apoptosis (Kuhajda et al. 1994; Pizer et al. 1996), however clinical applications were limited because of the chemical instability of cerulenin due to its reactive epoxide ring structure (Lupu and Menendez 2006). The related synthetic analogue, C75 lacked the reactive epoxide ring, resulting in increased chemical stability and specificity (Kuhajda et al. 2000) (Fig. 2). C75 and other related α-methylene-γ-butyrolactones can inhibit FASN in several types of cancer cells (Pizer et al. 2000; Wang et al. 2005) and this is

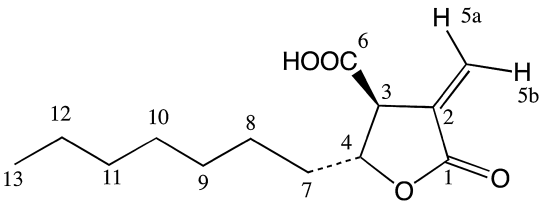

Fig. 2. Chemical structure of (±)-C75.

associated with activation of apoptotic pathways (Menendez et al. 2004b).

Weight loss induced by these FASN inhibitors has hindered further clinical development (Liu et al. 2010), however, C93, an analogue structurally related to C75, inhibits tumour growth in a lung cancer model without causing body weight loss (Orita et al. 2008) making it a promising compound for further development. PA is structurally similar to C75, the only difference is the length of the alkyl side chain. Kuhajda and colleagues showed that C83, a synthesized racemic mixture of PA, inhibits purified mammalian FASN in breast cancer cells (Kuhajda et al. 2000) and our preliminary data indicate that (+)-PA may inhibit FASN in breast cancer cells (unpublished data). Orlistat, a reduced form of the natural product lipstatin currently marketed as an anti-obesity medication, and (-)-epigallocatechin 3-gallate (EGCG) a polyphenol derived from green tea have also been found to exhibit anti-tumour activity in several cancer cells. However, these compounds have poor oral bioavailability and solubility making their effectiveness *in vivo* limited (Liu et al. 2010; Yang et al. 2009). New synthetic polyphenolic compounds that inhibit FASN have been shown to induce apoptosis in HER2-overexpressing breast cancer cells and interfere with the ERK and AKT pathways without causing body weight loss (Puig et al. 2009; Turrado et al. 2012). The sensitivity of cancer cells towards anti-proliferative and pro-apoptotic effects of FASN blockade is suggested to be more dependent on expression levels of HER2 than high levels of FASN expression/activity (Menendez et al. 2005a). However, it has been suggested that when FASN is pharmacologically inhibited, overexpression of HER2 is also transcriptionally repressed (Menendez et al. 2005c).

The monoclonal antibodies trastuzumab and pertuzumab, the antibody-drug conjugate (ADC) ado-trastuzumab emtansine, T-DM1, which combines trastuzumab with a potent anti-microtubule cytotoxic agent and the small-molecular tyrosine kinase inhibitor lapatinib are approved for HER2-targeted therapy of breast cancer (Esteva et al. 2010; Perez et al. 2014). Trastuzumab binds with high affinity to the extracellular domain of HER2 and is highly effective, but the majority of patients become resistant to trastuzumab within one year of treatment (Nahta et al. 2006). Lapatinib binds to the ATP-binding pocket of and inhibits epidermal growth factor receptor (EGFR) and HER2 and inhibits self-phosphorylation and downstream ERK1/2 and AKT which leads to growth arrest and/or apoptosis (Xia et al. 2002) and is active in trastuzumab-resistant HER2-positive cells (Konecny et al. 2006). Cardiotoxicity is a known adverse effect of HER2-targeted therapies, but the rate is lower for novel agents, including lapatinib, than trastuzumab (Sendur et al. 2013). Current strategies suggested for overcoming resistance include combining an anti-HER2 agent with other chemotherapeutic agents (Cameron et al. 2010), switching to another HER2-targeted therapy, combining HER2 inhibitors (Baselga et al. 2012; Blackwell et al. 2012; Swain et al. 2013) or employing inhibitors against alternative signalling pathways such as PI3K, AKT and mTOR (Mohd Sharial et al. 2012). There is evidence of synergistic down-regulation of HER2 after treatment with FASN inhibitors and trastuzumab in HER2 overexpressing and

trastuzumab-resistant breast cancer cells (Menendez et al. 2004c; Vazquez-Martin et al. 2007). These data indicate that FASN participates in regulating metabolism, proliferation and survival in HER2 overexpressing cancer cells and that combined treatment with FASN and HER2 inhibitors could be a novel and useful approach to treat HER2 overexpressing human breast cancer.

The aim of the present study was to test whether the antiproliferative activity of the lichen-derived substance (+)-PA is associated with effects on FASN and HER2, previously shown to be linked in breast cancer cell lines. Furthermore, we wished to determine whether such effects are associated with major signalling pathways, specifically ERK1/2 and AKT. Finally, with reference to the interaction between FASN and HER2 and previous evidence of synergism between FASN inhibitors and a HER2-active drug, we tested for synergism between (+)-PA and the HER2-tyrosine kinase inhibitor lapatinib.

Materials and methods

Plant material and general experimental procedures

The lichen, *Cetraria islandica* L. (Ach.), was collected at Jokuldalsheidi in the East part of Iceland in July 2004. The lichen material was identified by Dr. Hordur Kristinsson, lichenologist, and a voucher specimen LA-31128, is deposited at the Icelandic Natural History Museum, Akureyri. Solvent for extraction and high-performance liquid chromatography (HPLC) grade solvents for chromatography were purchased from Sigma–Aldrich. Melting point was determined on a Büchi 510 melting point apparatus. Optical rotation, was determined using C. Zeiss 58558 equipment. Nuclear magnetic resonance (NMR) spectra using $CDCl_3$ as a solvent were recorded on a Bruker 400 spectrometer (5 mm BB-1H/D probe-head) at 25 °C and compared to literature data. The chemical and physical data of PA are in accordance with those of the reference of Horhant et al. (2007) (S1).

Isolation and identification of protolichesterinic acid

The air-dried, powdered Iceland moss material was extracted in a Soxhlet apparatus with petroleum ether for 16 h to obtain crude extract. The organic extract was evaporated to dryness. For isolation of a pure compound, preparative HPLC (Dionex 3000 Ultimate; pump, UV-VIS detector) was connected to a column (C18 column, 250 × 21.1 mm, 5 μm, Phenomenex Luna) eluting with ACN:H_2O:CH_2O_2 (90:10:0.01), a flow rate of 5 ml/min, detection at 210 nm at room temperature. The active compound (t_R = 42.0 min) was identified as the known compound (+)-protolichesterinic acid (+)-(PA). Purity of (+)-PA was analyzed by analytical HPLC on a reversed phase column (G.L Sciences, Inc., Herbal medicine, C-18, 4.6 mm × 250 mm), a solvent system of ACN:H_2O:CH_2O_2 (90:10:0.01) flow rate of 1 ml/min, UV-detection at 210 nm at 25 °C and shown to be 97% pure (S2). The melting point was determined to be 106 °C and the optical rotation $[\alpha]_D^{20}$ °+12 ($CHCl_3$, c 0.6) which is comparable to literature data (Horhant et al. 2007). PA was dissolved in ethanol absolute puriss (Redel-de Haën) and diluted for use in tissue-culture medium. All tests include controls where the highest equivalent concentration of ethanol was used.

Cell culture and test substances

The human breast cancer cell lines SK-BR-3 and T-47D were obtained from the American Type Culture Collection (ATCC) through LGC Promochem. T-47D was maintained in RPMI-1640 tissue culture medium (GIBCO™) and SK-BR-3 in McCoy's (GIBCO™) all containing 0.5% penicillin and streptomycin (GIBCO™) and 10% heat-inactivated foetal bovine serum (FBS; GIBCO™) with T-47D receiving additionally 0.01 mg/mL insulin (Sigma, I1882). Cells were subcultured following detachment by trypsin (0.25% Trypsin/EDTA, Difco™) as appropriate and seeded to reach 70–80% confluence after 24 h culture. The lichen compounds, lapatinib (LC Laboratories) and solvent control were added in various concentrations and the cells were incubated under standard conditions, for different time periods.

Cell viability assay

Crystal violet staining was used for the estimation of IC_{50} values for the breast cancer cell lines as previously described (Vidarsdottir et al. 2012) with minor modifications. Briefly, cells were seeded and medium was changed after 24 h, compounds added and incubated for 72 h. Absorbance was measured at 570 nm wave lengths using a spectrophotometer (SpectraMax Plus 384, Molecular Devices Corporation). Statistical analysis was performed using software from GraphPad Prism version 6.01, using non-linear regression (variable slope) equation. Data shown are representative of what was observed in at least three separate experiments.

Immunocytochemistry

For immunofluorescence staining, cells were harvested and fixed in 4% paraformaldehyde (Sigma) and stained with fatty acid synthase, rabbit IgG monoclonal antibody (C20G5) (Cell Signalling), HER2/ErbB2, rabbit IgG monoclonal antibody (29D8) (Cell Signalling), followed by Alexa Fluor green 488 goat anti-rabbit IgG antibody (Invitrogen). For nuclear staining, TO-PRO-3 iodide (Invitrogen) was used. The stained cells were visualized and photographed under a confocal microscope (Zeiss, LSM 5 Pascal).

Image J quantification of images and statistical analysis

Images were converted to 8 bit grey scale and mean grey value measured, which is the sum of the grey values of the entire pixel in the selection divided by the number of pixels, reported in calibrated units. For analysis of images statistical comparisons of mean values were performed using two-sided analysis of variance (ANOVA), including treatment and number of run as factors, followed by a *post.hoc* comparison using Tukey HSD, carried out with RGui 64bit, version 2.15.2. In all figures, * and ** indicate $p < 0.05$ and $p < 0.001$ respectively. Images and data shown are representative of at least three separate experiments.

Meso Scale Discovery (MSD)® assay

Cells were washed with tris-buffered saline (TBS), harvested and lysed with RIPA buffer (without SDS). Protein content was quantified spectrophotometrically using Bradford reagent (Sigma). Meso Scale Discovery (MSD) 96-well multispot assays were carried out according to the manufacturer's protocol with minor modifications. Briefly, ERK1/2 (duplex) and phospho (Ser^{473})/total AKT plates were blocked (MSD blocking solution, as recommended by the manufacturer, plus 0.1% bovine serum albumin (BSA)) for 1 h at room temperature with shaking and washed four times with TBS with 0.1% Tween-20. Protein added at 2.5 or 20.0 μg to the ERK1/2 or phospho (Ser^{473})/total AKT plates, respectively, in duplicate wells and incubated overnight at 4 °C. Plates were washed as previously; then 25 μl of detection antibody was added and incubated at room temperature for 2 h with shaking. Plates were washed four times with TBS with 0.1% Tween-20 as before, 150 μl of read buffer was added, and the plates were analyzed on a SECTOR™ 6000 instrument (MSD). The two additional spots in each well coated with BSA were used to correct for the background and for any effects of the lysis buffer. Data were analyzed using software from GraphPad

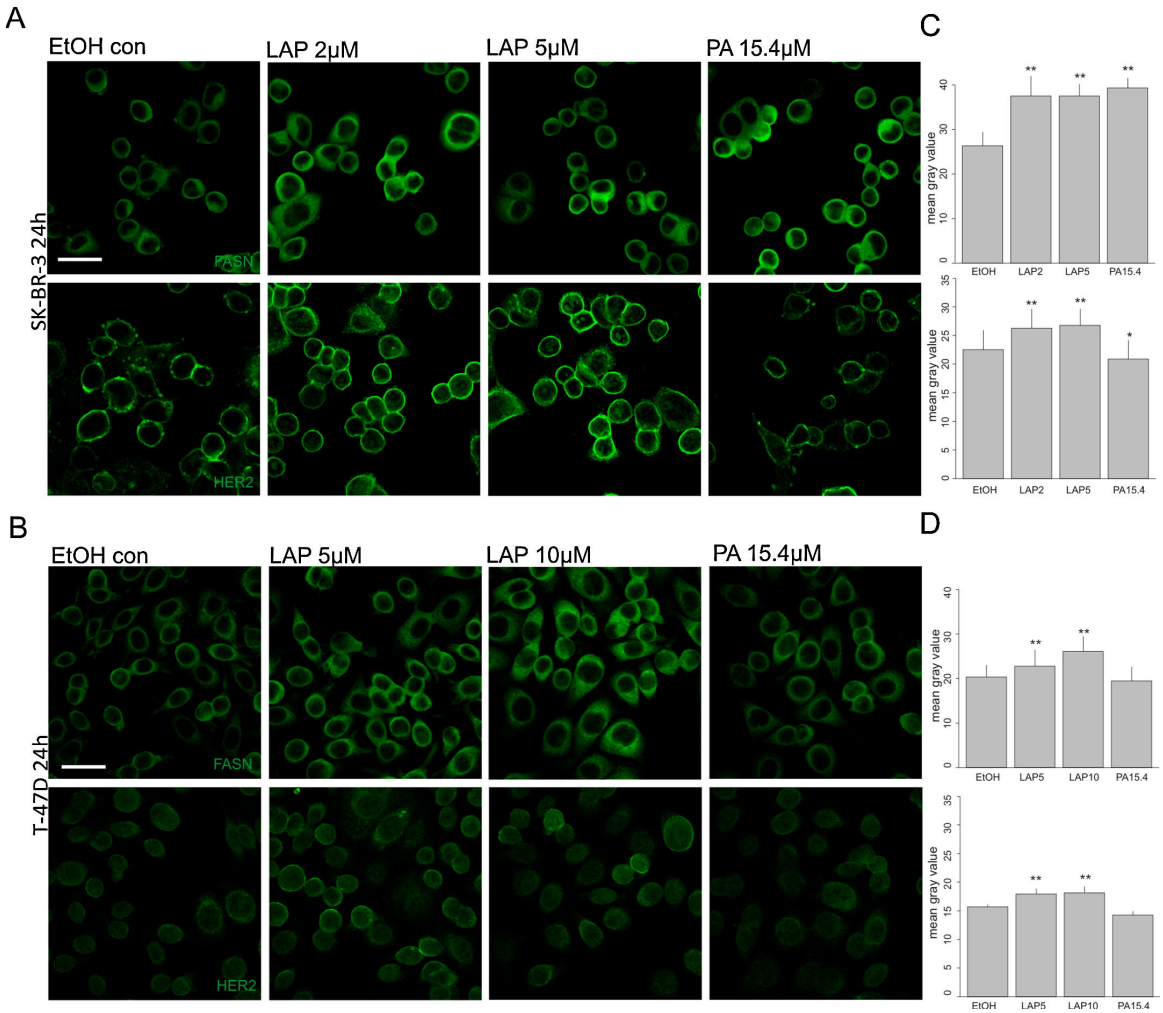

Fig. 3. (A) Increased expression of FASN and HER2 was detected in SK-BR-3 cells after 24 h of lapatinib (2 and 5 μM) treatment. An increase in FASN expression was also detected after treatment with (+)-PA (15.4 μM) for 24 h but this was associated with a decrease in HER2 expression. The scale bar shown represents 20 μm and applies to all panels. (B) Increased expression of FASN and HER2 was detected in T-47D cells after 24 h of lapatinib (5 and 10 μM) treatment. No effects were seen after (+)-PA (15.4 μM) treatment. (C and D) Images were quantified by ImageJ and data represented as mean grey value of each group compared with EtOH control. Error bars indicate standard error of the mean, $^{*}p < 0.05$, $^{**}p < 0.001$.

Prism version 6.01, using unpaired *t*-test with Welch's corrections. Data shown are representative of at least three separate experiments.

Synergism assay

Synergism was estimated following incubation under standard conditions with different combinations of concentrations of the compounds. The same experimental setup was used as for the cell viability assay described above. Calculations of the combination index, CI, were done in the CalcuSyn software version 2.1. This software is based on Chou's and Talalay's description of the combination index as a criterion for synergism or antagonism between two or more drugs (Chou 2006). Data shown are representative of at least three separate experiments.

Results and discussion

(+)-PA affects cell viability in two breast cancer cell lines

With reference to known anti-proliferative effects of (+)-PA on cancer cells (Haraldsdottir et al. 2004; Ogmundsdottir et al. 1998), the structural similarity of (+)-PA and C75 and our preliminary results on FASN inhibition in breast cancer cells, the effects of (+)-PA on cell viability were tested on the SK-BR-3 cell line which overexpresses FASN and HER2 (Yoon et al. 2007). The relationship between FASN and HER2 acts in both directions. Inhibition of FASN can repress transcription of HER2 (Menendez et al. 2005c). HER2 overexpression increases the expression of FASN by stimulation of signalling cascades (Yang et al. 2002) and activates FASN directly (Jin et al. 2010). We therefore included the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib in our experiments for comparison

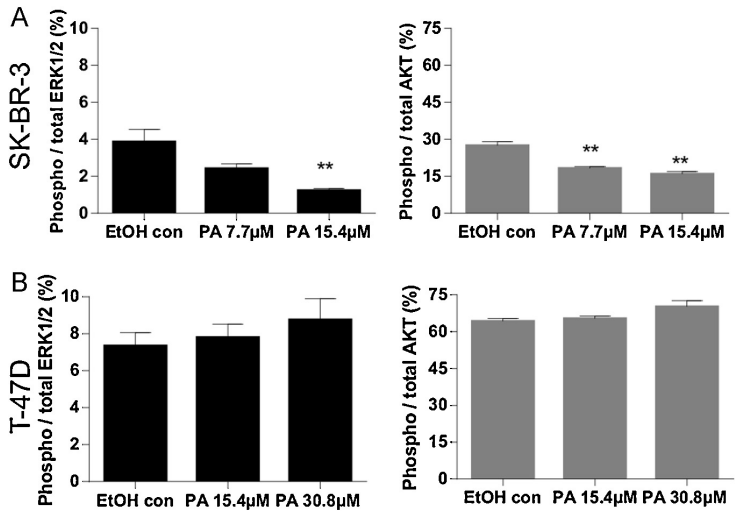

Fig. 4. (A) Decrease in phospho/total ERK1/2 and AKT ratio was detected after 24 h treatment with (+)-PA (7.7 μM and 15.4 μM) in SK-BR-3 cancer cells. The difference is statistically significant. (B) No effects were seen in T-47D cancer cells. Error bars indicate standard error of the mean, ($^{*}p < 0.05$, $^{**}p < 0.001$).

and to test for possible synergistic effects. The effects of lapatinib were estimated in SK-BR-3 and T-47D breast cancer cell lines. T-47D is p53-mutated (Casey et al. 1991) and does not express FASN and HER2, and was therefore included for comparison. The results are shown in Table 1, expressed as IC_{50}, taking account of individual solvent effects as appropriate. (+)-PA showed inhibitory effects on cell viability in both cell lines to a similar extent (IC_{50} = 10.8 μM for SK-BR-3 and IC_{50} = 11.7 μM for T-47D). The SK-BR-3 cells were more sensitive to lapatinib, IC_{50} = 1.6 μM, compared to 6.4 μM in T-47D as expected because of the overexpression of HER2 and FASN in the SK-BR-3 cells. Lapatinib showed stronger effects on cell viability in these two breast cancer cell lines compared to PA.

FASN expression is increased in SK-BR-3 cancer cells after treatment with (+)-PA: effects on HER2 are likely to be secondary

In order to estimate and compare the effects of (+)-PA on FASN and HER2 expression in these two cell lines, immunofluorescence staining for FASN and HER2 was performed after treatment with (+)-PA and lapatinib for 24 h (Fig. 3).

Treatment with lapatinib (2, 5 and 10 μM) was associated with increased expression of HER2 and FASN in both cancer cell lines. When signalling is inhibited through the HER2 receptor, as with lapatinib treatment, it is likely that the cells respond by increasing the expression of the receptor. As signalling through the HER2 receptor has been shown to play important role in activation of FASN (Kumar-Sinha et al. 2003) the increase in FASN expression can be interpreted in a similar manner. In the HER2-overexpressing cancer cell line SK-BR-3, treatment with (+)-PA (15.4 μM) led to increased expression of FASN and decreased expression of HER2.

Table 1
Effects of protolichesterinic acid and lapatinib on cell viability in two breast cancer cell lines.

Cell lines	(+)-Protolichesterinic acid (μM)	Lapatinib (μM)
SK-BR-3	10.8 ± 0.3	1.6 ± 0.3
T-47D	11.7 ± 1.5[a]	6.4 ± 0.7

Results are presented as IC_{50} values in μM ± SEM.
[a] Haraldsdottir et al. (2004).

(+)-PA did not affect expression of FASN or HER2 in the T-47D cancer cells. These results indicate that the possible FASN inhibitory effects of (+)-PA are dependent on the level of FASN expression in the cells and that it is likely that (+)-PA is primarily inhibiting FASN activity, leading to a compensatory increase in expression of FASN. However, the decrease in HER2 expression could be secondary through repressed transcription of the receptor, as has been indicated before (Menendez et al. 2005c).

(+)-PA affects the ERK1/2 and AKT signalling pathways via a decrease in HER2 expression

As FASN inhibitors are known to affect the HER2 receptor (Puig et al. 2009), we estimated the effects of (+)-PA on the HER2 downstream signal transduction pathways ERK1/2 and PI3K/AKT.

After treatment with (+)-PA (7.7 μM and 15.5 μM) for 24 h the phospho/total ERK1/2 and AKT ratio was significantly decreased in SK-BR-3 cancer cells (Fig. 4A), but no effects were seen on the T-47D cells (Fig. 4B). As (+)-PA affected ERK and AKT in SK-BR-3 cells but not in T-47D cells the effect is likely to be secondary to the decreased expression of HER2 rather than mediated directly through the signalling pathways (Fig. 3A and C).

Combined effects of (+)-PA and lapatinib in SK-BR-3 cancer cells

It has been reported that FASN inhibitors in combination with other cancer drugs can exhibit synergistic interactions (Menendez et al. 2004a; Vazquez-Martin et al. 2007). The combined effect of (+)-PA and lapatinib was analysed using the medium-effect analysis of Chou and Talalay. This method allows the characterization of drug interactions with a single number, the combination index (CI) where the CI > 1 indicates antagonism, CI = 1 additivity or CI < 1 synergism (Chou 2006). Concentrations around the IC_{50} values of (+)-PA, from previous experiments, were used in combination with concentrations of lapatinib that were well below the IC_{50} levels (Table 1). Results indicate a synergism (according to Chou) between (+)-PA (15.4 μM) and lapatinib at two concentrations (0.01 μM; CI = 0.631 and 0.10 μM; CI = 0.713) in SK-BR-3 cells (Fig. 5A). In T-47D cells, weak synergism between (+)-PA 3.1 μM; 15.4 μM) and lapatinib was seen (0.10 μM; CI = 0.810 and 0.840) (1.00 μM;

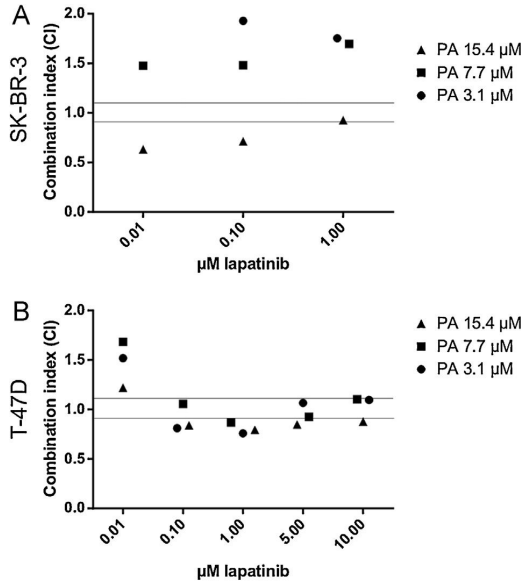

Fig. 5. Analysis of the interaction between (+)-PA and lapatinib in SK-BR-3 and T-47D breast cancer cells according to the median-effect plot method of Chou and Talalay. Lines in the figures represent CI = 0.90 and CI = 1.10 (A) Combined treatment with (+)-PA (15.4 μM) and lapatinib (0.01 μM) show synergistic effects in the HER2 overexpressing SK-BR-3 cancer cells (CI = 0.631), moderate synergism is observed after treatment with (+)-PA (15.4 μM) and lapatinib (0.10 μM) (CI = 0.713). (B) Weak synergism or additive interactions were observed after combined treatment with (+)-PA and lapatinib at various concentrations in T-47D cancer cells.

CI = 0.760 and 0.794), respectively. Additive effects were observed between (+)-PA 7.7 μM and lapatinib (0.10 μM; CI = 1.056) (Fig. 5B). These results suggest that combined treatment with (+)-PA leads to reduction in the required dose of lapatinib to achieve effect in SK-BR-3 HER2 overexpressing cancer cells.

Conclusions

PA has a broad range of activities, both supported by the traditional use of Iceland moss and various studies on the effect of PA on several pathogens, LOX enzymes and more recently on cell viability in cell lines derived from different cancer types. Here we show that (+)-PA has anti-proliferative effects on the breast cancer cell line SK-BR-3 in addition to other cancer cell lines previously tested (Haraldsdottir et al. 2004). Preliminary data indicate FASN-inhibitory effects of (+)-PA on breast cancer cells. Our current results are consistent with (+)-PA having FASN inhibitory activity leading to a compensatory effect on FASN expression in cancer cells that overexpress HER2 and FASN. At the same time a decrease is observed in HER2 expression most likely *via* transcriptional repression. This implies that the effects on HER2 are secondary and the observed inhibitory effects of (+)-PA on ERK1/2 and AKT signalling pathways are most likely a consequence of reduced expression of the HER2 receptor rather than a direct effect on the signalling pathways. In addition, we show that synergism between of (+)-PA and lapatinib is restricted to SK-BR-3 cells supporting the hypothesis that this is likely to be dependent on FASN inhibition and the transcriptional repression of HER2.

In cancer treatment drug resistance is a major problem and limits the effectiveness of chemotherapy. Large tumours can be genetically diverse and therefore it is possible that a small part of cells within the tumour can be resistant to the drug and will survive therapy and continue to grow (Bozic et al. 2013). Several strategies have been suggested to overcome resistance to HER2-targeted therapy in order to block by-pass mechanism or induce synergistic effect between anti-cancer agents (Blackwell et al. 2012; Mohd Sharial et al. 2012; Xia et al. 2002). It has been proposed that the use of combined therapy of two agents from the beginning of cancer treatment gives more hope for cure compared to the traditional sequential approach (Blair et al. 2014; Bozic et al. 2013). Interestingly, a recent study indicates that by using targeted therapies in a sequential order, starting with two anti-HER2 drugs, followed by treatment with an anti-HER2 drug and a PI3K/mTOR inhibitor and lastly combining all three agents can prolong survival and minimize toxic effects in mouse models bearing aggressive breast tumours (Sahin et al. 2014). Future research will aim at investing the use of combinational therapies and our current results indicate that (+)-PA could be a suitable agent for further testing, alone or in combination treatment against HER2-overexpressing breast cancer.

Supplementary content

^{1}H and ^{13}C NMR spectra of (+)-protolichesterinic acid, analytical HPLC chromatogram of protolichesterinic acid.

Conflict of interest

None of the authors have financial relationships with a commercial entity that has an interest in the content of this paper.

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Paper III

Anti-proliferative and pro-apoptotic effects of lichen-derived compound protolichesterinic acid are not mediated by its lipoxygenase-inhibitory activity

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Abstract

Lipoxygenases (LOXs) and their products are involved in biological functions including inflammation, and have been associated with carcinogenesis. Protolichesterinic acid (PA), a lichen metabolite, is known to inhibit 5- and 12-LOX activity and exhibit anti-proliferative effects on various cancer cell lines. In the current study PA was shown to have anti-proliferative effects on multiple myeloma and pancreatic cancer cells but apoptosis was induced only in multiple myeloma cells. Cell-cycle-dependent LOX expression was observed but effects of PA on LOX sub-cellular localization were only evident beyond the early phases of the cell cycle. Measurements with mass spectrometry showed that PA entered the pancreatic cancer cells, however effects on LOX metabolites were only evident after treatment with concentrations exceeding those having anti-proliferative effects; no effects were measurable on myeloma cells. Results indicate that the anti-proliferative and pro-apoptotic effects of PA are not likely to be mediated directly through inhibition of LOX activity.

Key words: Protolichesterinic acid, lipoxygenase, cancer, mass spectrometry.

1. Introduction

Arachidonic acid (AA) is the source of several bio-active lipids and is released from cellular membranes by phospholipase A_2 following a variety of stimuli. Three distinct enzyme classes can metabolize AA: lipoxygenases (LOX), cyclooxygenases (COX) and cytochrome P450 (CYP450). Products of the COX and LOX pathways, prostaglandins (PG) and leukotrienes (LT), respectively are involved in a number of homeostatic biological functions and inflammation. COX enzymes catalyse the rate-limiting steps in the biosynthesis of PGs and thromboxanes from AA. LOX enzymes metabolise AA to generate the biologically active metabolites hydroxyperoxy-eicosatetraenoic acids (HPETEs). HPETEs are then further reduced to the corresponding 5HETEs or converted to LTs by 5-LOX. The most studied LOX enzymes are 5-, 12-LOX and 15-LOX-1. The CYP450 system converts AA to epoxyeicosatrienoic acids (EETs) or hydroxyeicosatetraenoic acids (HETEs) [1-3]. Both COX and LOX pathways have been associated with various aspects of cancer biology [4]. The CYP450 pathway has also been implicated in carcinogenesis but the role of CYP-derived eicosanoids has not been studied in great detail [5].

Considerable progress has been made in recent years in lipid analysis. One of the most important discoveries for analysis of lipids by mass spectrometry was the innovation of the soft ionization electrospray (ESI) by Fenn and colleagues [6]. Ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) is a highly specific and sensitive technique that can be used in targeted lipid analysis and quantification of lipid metabolites [7].

The potential role of LOX enzymes in carcinogenesis and cancer progression was first noted two decades ago. The evidence was partly based on expression of the enzymes in various types of cancer [8-11] and partly on the effects of LOX inhibitors in cell culture, indicating cell cycle arrest at G_1/S phase and induction of apoptosis [1]. Several pancreatic cell lines have been shown to express 5-LOX both at

transcriptional and translational levels. In contrast, 5-LOX mRNA was undetectable in normal pancreatic ductal cells [12]. Overexpression of 5-LOX in pancreatic cancer is associated with poor prognosis and a lack of response to conventional therapy [12, 13]. One clinical trial has shown that treatment with a 5-LOX, CV6504 inhibitor appeared to delay disease progression and was well tolerated in patients with advanced pancreatic cancer [14]. Inhibition of 5-LOX has been shown to reduce proliferation and induce programmed cell through caspase activation in leukemic cell lines [15], whereas cell death in pancreatic cancer cells appeared to be mediated by effects on mitochondrial membrane potential [16]. Several 5-LOX inhibitors are currently being tested, however no 5-LOX inhibitor has been developed for clinical use in cancer, although some of the inhibitors are undergoing clinical trials for various other diseases [17]. Zileuton, the only clinically available 5-LOX inhibitor, used to decrease symptoms of asthma [18] has been reported to have inhibitory effects on esophageal and oral carcinogenesis [10, 19]. In contrast, Fischer and colleagues demonstrated that zileuton did not have any anti-proliferative or cytotoxic effects in tested cell lines and that the cytotoxic and anti-proliferative effects of certain 5-LOX inhibitors were independent of suppression of 5-LOX activity, thus concentrations of 5-LOX inhibitors required to induce anti-proliferative effects exceeded those needed for suppression of 5-LOX. The role of 5-LOX overexpression in tumour cell viability therefore remains unclear and requires further elucidation [20]. Specific inhibitors of 12-LOX have also been identified and have been shown to induce apoptosis e.g. in prostate [21] and pancreatic cancer cells [22] but have not been developed for clinical use.

The lichen metabolite protolichesterinic acid (PA) is considered to be one of the major biologically active secondary metabolite of the Iceland moss *Cetraria islandica* which has been used traditionally to treat bronchial and inflammatory conditions [23, 24]. Previous studies have shown that PA inhibited 5-LOX from porcine leucocytes *in vitro*

(IC_{50} = 6.5 µg/mL) [23] and also exhibited inhibitory activity on platelet-type 12(S)-LOX using human platelets as a source of the enzyme (IC_{50} = 25.0 µg/mL) [25].

Further studies of this LOX-inhibitory lichen metabolite revealed anti-proliferative effects against a variety of malignant cell lines and mitogen-stimulated lymphocytes. Normal fibroblasts were not affected [26, 27]. The aim of the present study was therefore, to investigate if the LOX-inhibitory effects of the lichen compound PA are associated with anti-proliferative and pro-apoptotic effects. We explored the effects of PA on the cell cycle and tested for induction of programmed cell death. We used ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) to detect intracellular PA and measure directly the effects on formation of LOX metabolites in cancer cells lines. Furthermore, we tested if the sensitivity to the anti-proliferative effects of the lichen metabolite was associated with expression and cellular localization of 5-and 12-LOX after treatment with PA.

2. Materials and methods

2.1 Plant material, cell culture and test substances

The lichen *Cetraria islandica* L. (Ach) was collected in open country in Iceland, not privately owned. The lichen material was identified by Dr. Hordur Kristinsson, lichenologist, and a voucher specimen LA-31128, is deposited at the Icelandic Natural History Museum, Akureyri. Isolation and identification of (+)-protolichesterinic acid (PA) was performed as described (Bessadottir et al 2014). The structure of PA was further confirmed by UPLC – Quadrupole Time of Flight mass spectrometer (Fig. S1). PA was dissolved in ethanol absolute puriss (Redel-de Haën) and diluted for use in tissue-culture medium. All tests include controls where the highest equivalent concentration of ethanol was used.

Cell lines were obtained from the American Type Culture Collection (ATTC) through LGC Promochem. RPMI 8226 and U266 (human multiple myeloma cell lines), were maintained as suspension, AsPC-1 (pancreatic carcinoma cell line) grew adherent. All cell lines were cultured in RPMI-1640 tissue culture medium (GIBCO™, 52400), containing 0.5% penicillin and streptomycin (GIBCO™, 15140-148) and 10% heat-inactivated fetal bovine serum (FBS; GIBCO™, 10270). The cells were incubated under standard conditions (95% humidity, 37°C, and 5% CO_2) and sub-cultured following detachment by trypsin (0.25% Trypsin/EDTA, Difco™, 215240) as appropriate. Cells were seeded at an appropriate number to exceed 70-80% confluence after 24 hour culture. The lichen compound, the 5-LOX inhibitor zileuton (Cayman Chemical, 0006967), the 12-LOX inhibitor baicalein (Sigma-Aldrich, 465119) and solvent control were added in various concentrations and the cells were incubated under standard conditions, for different time periods. For synchronization in G1 the cells were incubated in serum-free medium for 18 hours. For quantification of lipids and PA, cells were stimulated with 30 μM arachidonic acid (Cayman Chemicals, 90010) for 30 min after treatment with PA or other substances, both cells and medium

collected and flash frozen in liquid nitrogen and stored at -80°C. Protein content was quantified spectrometrically using Bradford reagent (Sigma, B6916).

2.2 Cell viability assays

The ^{3}H-thymidine uptake assay was performed as previously described [26] to estimate survival and determine the half maximal tolerated dose IC_{50} of the metabolites for the multiple myeloma cell lines, that grow in suspension. Crystal violet staining was used for the estimation of IC_{50} values for the adherent AsPC-1 cell line as previously described [28]. Statistical analysis was performed using Graph prism software Inc, La Jolla LA (USA) using non-linear regression (variable slope) equation.

2.3 TUNEL staining – PI staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed to detect late apoptosis combined with cell cycle analysis, using propidium iodide (PI) staining. The assay was performed according to the manufacturer's instructions using Apo-Direct™Kit (BD Pharmingen™, 556381) and flow cytometry (FACSCalibur,Becton-Dickinson, Franklin Lakes, NJ). Data were analysed using FCS Express Plus 4. Statistical analysis was performed using Graph prism software Inc, La Jolla LA (USA) using two sided analysis of variance (ANOVA), followed by a post hoc comparison using Bonferroni correction for apoptotic results, and Chi square and Fisher's exact test for the cell cycle results. In all figures, * and ** indicate $p<0.05$ and $p<0.001$ respectively. Data shown are representative of what was observed in at least three separate experiments.

2.4 Annexin V/Propidium iodide double staining

FITC Annexin V in combination with PI staining was performed on non-permeabilized cells to detect early apoptotic or necrotic processes. The assay was performed according to the manufacturer's protocol using FITC Annexin V apoptosis detection kit I (BD Pharmingen™, 556547). Data were collected by flow cytometry (FACSCalibur)

and analyzed using FlowJo software (Tree Star, Inc,. Ashland, OR, USA). Statistical analysis was performed using Graph prism software Inc, La Jolla LA (USA) using Chi square and Fisher‘s exact test. In all figures, * and ** indicate $p<0.05$ and $p<0.001$ respectively. Data shown are representative of what was observed in at least three separate experiments.

2.5 Immunocytochemistry

For immunofluorescence staining the cells were fixed with methanol (Sigma, 34860) for 10 min at -20°C and stained with rabbit polyclonal antibody against 5-LOX (Cayman Chemical, 160402) and 12-LOX (Cayman Chemical, 160304) followed by Alexa Fluor green 488 goat anti-rabbit IgG antibody (Invitrogen, A11070). For nuclear staining TO-PRO-3 iodide (Invitrogen, T3605) was used. The stained cells were visualized and photographed under a confocal microscope (Zeiss, LSM 5 Pascal).

2.6 Extraction of lipids – sample preparation

For analysis of lipids released into the medium, supernatants were collected from test and control cultures in 12-well plates. Sirocco™ Protein precipitation in 96-well plate was used for sample preparation of secreted lipids from medium. First, the methanol-containing internal standards; 12(S)-HETE-d_8 and 5(S)-HETE were added to the Sirocco plate, after which the sample was added. The sample was collected by applying vacuum and analysed by UPLC-MS/MS. For analysis of intra-cellular lipids and PA, liquid-liquid extraction was used. Hexane:ethyl acetate (1:1,v/v) was added to the sample and vortex mixed, samples were then centrifuged. The upper organic phase was collected and was pooled from two extractions. The solvent was evaporated under a stream of nitrogen. Samples were then reconstituted in 50% acetonitrile and analysed by UPLC-MS/MS.

2.7. Ultra performance liquid chromatography-tandem mass spectrometry

The quantification of 5-HETE, 12-HETE and PA was performed with a Waters Acquity UPLC system, coupled to electrospray ionization on a Waters Quattro Premier XE triple quadrupole mass spectrometer (Micromass, Manchester, UK). The analytical column used was an ACQUITY UPLC BEH C18 (2.1 mm x 100 mm i.d.; 1.7 µm) (Waters corp., Milford, MA, USA), maintained at 60ºC in a column oven. The gradient system mobile phase consisted of A: 0.1% formic acid and B: 90% methanol/10% isopropanol, at a flow rate of 0.45 mL/min. The injection volume was 15 µL. The mass spectrometer was optimized for analysing lipids using multiple reactions monitoring (MRM) in the negative electrospray ionization (ESI) mode. Data acquisition was carried out using MassLynx 4.1 software (Micromass, Manchester, UK). Statistical analysis was performed using Graph prism software Inc, La Jolla LA (USA) using two-tailed paired t-test. In the figure, * indicates $p<0.05$. Data shown are representative of what was observed in at least three separate experiments.

3. Results

3.1 PA affects cell viability of two myeloma cell lines and a pancreatic cell line

With reference to known anti-proliferative effects of PA on cancer cells and activated lymphocytes [26, 27], the known function of 5-LOX in LT production by inflammatory cells [29], expression in lymphocytes [30], and reported effects of LOX inhibitors on cell lines from leukemia cells [15], the effect of PA on proliferation was tested on two myeloma cell lines; U266 and RPMI 8226, by uptake of ^{3}H-thymidine. The 5-LOX inhibitor zileuton was used for comparison as before [25]. LOX pathways have been associated particularly with development of cancer of the pancreas. We therefore added the AsPC-1 cell line to those previously tested [26], because of its reported overexpression of 5- and 12-LOX [12, 31]. Viability of this adherent cell line was assessed using Crystal violet staining. The results are shown in Table 1, expressed as IC_{50}, taking account of individual solvent effects as appropriate.

Table 1. Anti-proliferative effects of protolichesterinic acid and zileuton two multiple myeloma (RPMI 8226 and U266) and pancreatic cancer cells (AsPC-1).

Cell lines	Protolichesterinic acid	Zileuton
RPMI 8226	1.8 ± 0.2	22.6 ± 13.9
U266	3.5 ± 0.7	27.9 ± 14.2
AsPC-1	7.7 ± 0.6	NA

Results are presented as IC_{50} values in µg/mL ± s.e.m.

PA inhibited cell proliferation and viability in both myeloma cells to a similar extent (IC_{50} = 1.8 µg/mL for RPMI 8226 and IC_{50} = 3.5 µg/mL for U266). The IC_{50} value for the pancreatic cell line AsPC-1 was higher than for the myeloma cell lines (IC_{50} = 7.7); this difference might to some extent be attributable to different sensitivities of the two methods used. Zileuton showed anti-proliferative effects only at concentrations that are much higher than the reported 5-LOX inhibitory concentration [32]. The cell lines, RPMI 8226 and AsPC-1, were selected for further studies.

3.2 PA induces cell cycle arrest at G1 phase in pancreatic cancer cell line

Having observed inhibitory effects of PA on the two myeloma cell lines by thymidine uptake, evaluation of DNA cellular content was performed using PI staining and flow cytometry after 24 hour treatment with PA in AsPC-1 cells. Cell cycle arrest occurred in G1 phase after PA treatment (Fig.1A and B), supporting previous data in myeloma cells by thymidine uptake. Taken together these results imply that the effects of PA manifest at an early stage in the cell cycle.

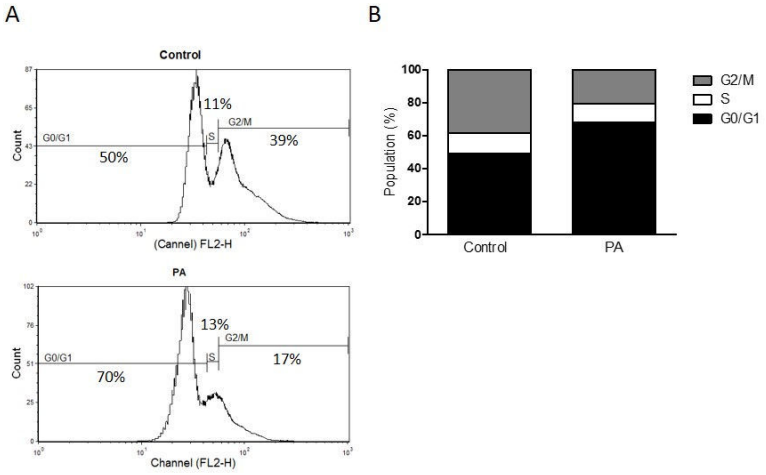

Fig. 1. Cell cycle analysis. (A) Cell cycle analysis in AsPC-1 cell line after treatment with PA (8 μg/mL) for 24 hours indicated cell cycle arrest in G1 phase after treatment compared with control. The difference is statistically significant ($p<0.001$). (B) Proportional distribution of cells according to phase of cell cycle. Data presented as the mean of three of separate experiments.

3.3 PA induces programmed cell death in multiple myeloma cell line but not in pancreatic cancer cell line

To test further if the decrease in cell viability could also be attributed to induction of apoptosis, the effects of PA (8 μg/mL) on RPMI 8226 and AsPC-1 cell lines were estimated using TUNEL assay. Very marked apoptosis was seen in the RPMI 8226

after PA treatment for 24 hours. In contrast, no apoptosis was seen in AsPC-1 cells (Fig. 2A).

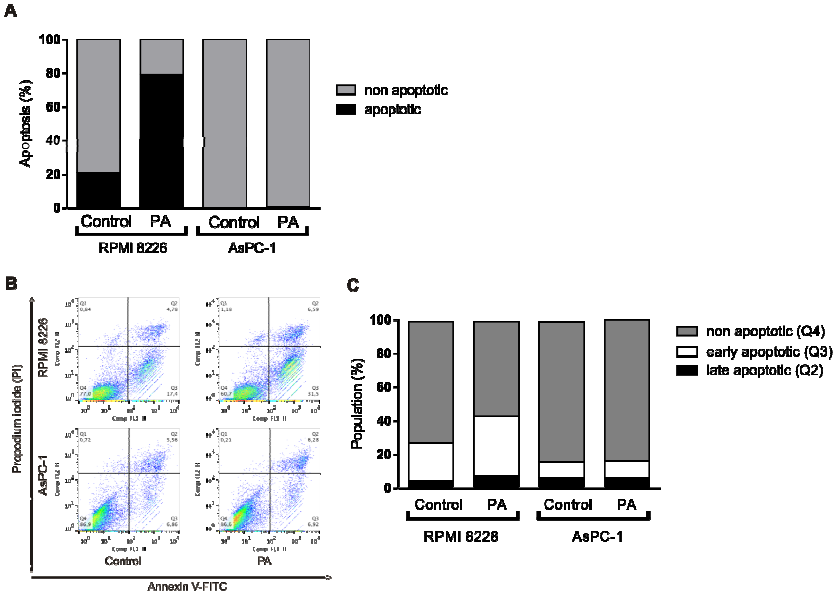

Fig. 2. Detection of apoptosis by DNA fragmentation and Annexin V expression. (A) Treatment with PA (8 µg/mL) for 24 hours induced late apoptosis in RPMI 8226 cell line as indicated by the TUNEL assay. Results were statistically significant ($p<0.001$). No effects were seen on AsPC-1 cells. (B) Annexin V staining in RPMI 8226 and AsPC-1 cell lines after treatment with PA (10 µg/mL) for 24 hours revealed an increased percentage of treated cells undergoing early and late apoptosis in comparison with control cells in the myeloma cells. The difference was statistically significant ($p<0.001$). No effects were seen on the AsPC-1 cells. (C) Data presented as a mean of three of separate experiments.

The effects of PA on cell death were further tested by Annexin V staining in RPMI 8226 and AsPC-1 cells. This revealed early as well as late apoptosis after treatment with PA (10 µg/mL) for 24 hours in the myeloma cell line; no effects were seen on the AsPC-1 cells (Fig. 2B and C). Further testing with PA at a higher concentration in the AsPC-1 cell line (20 µg/mL for 24 hours, one experiment) showed no effect. These

results agree with the TUNEL results above and indicate that AsPC-1 cells do not undergo apoptosis after treatment with PA. The pro-apoptotic effects of PA are likely to be cell-line dependent as has recently been described in several other cancer cell lines [33].

3.4 Cell-cycle dependent changes in LOX expression are not affected by PA

Next we addressed the question whether the anti-proliferative effects of PA were associated with its LOX-inhibitory activity. This was first approached by add-back experiments using 5-HETE and 12-HETE along with PA but results were inconclusive (data not shown). Our preliminary data show that cellular expression and localization of 5- and 12-LOX enzymes changes according to stage in the cell cycle in breast and pancreatic cancer cell lines and we have noted both cytoplasmic and nuclear localization in tissue samples from breast, stomach and pancreatic carcinomas (Haraldsdóttir, S and Tómasdóttir, M, unpublished data). Work by Flamand and colleagues also indicated that depletion or omission of serum leads to nuclear export of 5-LOX and that the addition of serum is followed by nuclear accumulation of 5-LOX in leukocytes [34]. We therefore decided to investigate expression of LOX enzymes in relation to progression through the cell cycle by immunofluorescence staining. To do this we used the AsPC-1 cell line and examination by confocal microscopy, as this was not feasible using the non-adherent myeloma cells. Cells were synchronized and arrested by incubation in serum-free medium for 18 hours. After synchronization there was a marked reduction of 5- and 12-LOX expression, compared with control cells kept in serum-containing medium for 18 hours. When the cells were again incubated with serum and allowed to re-enter the cell cycle, 5- and 12- LOX reappeared and were both expressed in the nucleus. This confirmed previously obtained results by us and others and indicates that 5- and 12-LOX enzymes are involved in the progression of the cell cycle. We then tested whether cell-cycle-related changes in LOX expression could be affected by PA. If the anti-proliferative activity is mediated by the

LOX-inhibitory effect the cells would be expected to respond by a compensatory increase in expression of the LOX enzymes. The reappearance of LOX expression upon re-entry into the cell cycle was not affected by PA for the first 3 hours after re-introducing serum indicating either that PA has no significant LOX-inhibitory effects in the cells at that time point or cells can use other pathways than LOX to generate HETE and a feedback response on LOX levels is not induced (Fig.3).

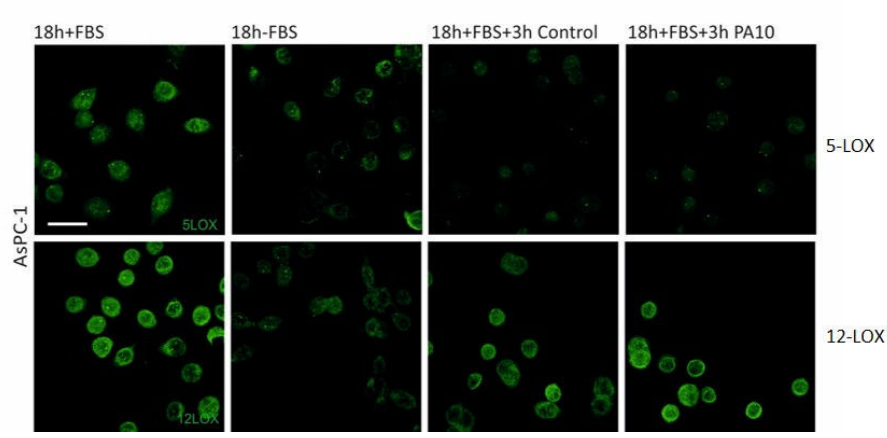

Fig. 3. Expression of 5- and 12-LOX in AsPC-1 cells related to cell cycle and effects of PA. Immunofluorescence staining showed a marked reduction of 5- and 12-LOX expression in AsPC-1 cells after 18 hours of deprivation of serum followed by reappearance upon adding serum. PA had no effect on reappearance for the first 3 hours. The scale bar shown represents 20μm and applies to all panels.

To explore further the effects of PA after longer exposure beyond the G1- and S-phases of the cell cycle, cells were treated for 24 hours and immunofluorescence staining for 5- and 12-LOX in AsPC-1 was performed as above (Fig. 4A). In the untreated cells both 5- and 12-LOX were seen localized to the nucleus. Exposure to the 5-LOX inhibitor zileuton had no effect on 5-LOX expression but PA-treated cells showed increased staining of 5-LOX and 12-LOX outside the nucleus. Treatment with

PA for 24 hours also resulted in morphological changes of the AsPC-1 cell line (Fig. 4B).

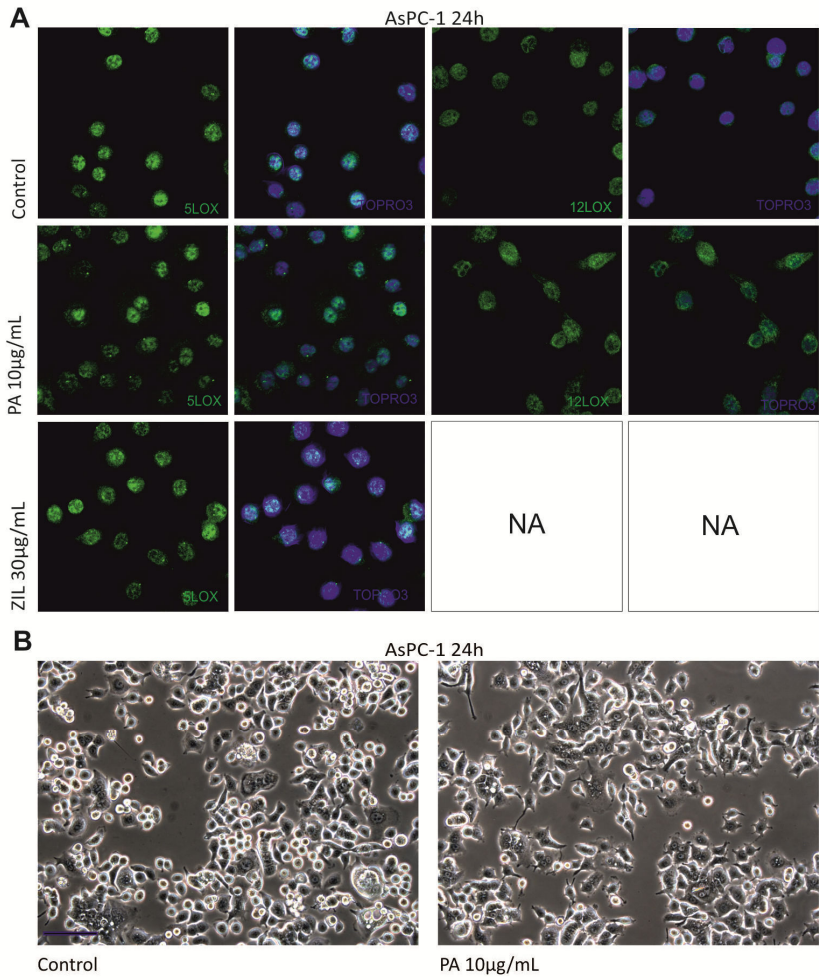

Fig. 4. Effects of PA on localization of 5- and 12-LOX in non-synchronized AsPC-1 cells. (A) Immunofluorescence staining of 5- and 12-LOX after 24 hours treatment with PA in non-synchronized AsPC-1 cells revealed increased staining outside the nucleus compared with localized nuclear staining in controls. The scale bar shown represents 20 µm and applies to all panels. (B) A morphological analysis under a phase-contrast microscope of live non-synchronized AsPC-1 cells showed a change to a star-like shape after treatment

with PA for 24 hours. The scale bar shown represents 100 µm and applies to both panels.

Taken together, LOX expression is cell-cycle-dependent but the effects of PA on LOX localization are only evident beyond the early phases of the cell cycle implying that the anti-proliferative effects of PA are not likely to be mediated directly through LOX-inhibitory effects.

3.5 PA enters pancreatic cancer cells

One reason for the observed lack of effect of PA on cell-cycle-related changes in 5- and 12-LOX expression could be that PA failed to gain entry into the cells and thus affect LOX activity intra-cellularly. This was studied by analysing the amount of PA in the cell lysates by an UPLC-MS/MS method and by quantifying the change in HETE production directly. The results show that PA enters the cells. Concentrations of 2.2, 3.8, 5.2 and 13.7 ng/mL were measured in cell lysates isolated from AsPC-1 after treatment with PA 5, 10, 20 and 30 µg/mL for 8 hours, respectively (Fig.5). There was thus a clear dose-dependent content of PA in the AsPC-1 cells after treatment with different concentrations implying that PA was located in the cell and can therefore have intracellular effects.

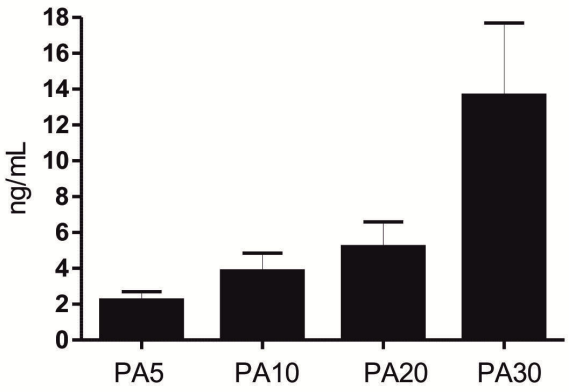

Fig. 5. Entry of PA into AsPC-1 cells. PA was present in cell lysates from AsPC-1 after treatment with PA for 8 hours, showing dose-dependent increase indicating that PA enters into the cell (PA 5, 10, 20 and 30 µg/mL). Data presented as a mean of three of separate experiments. Error bars indicate standard error of mean.

3.6 PA affects 5- and 12-HETE production in pancreatic cells at high concentrations, no effects observed on myeloma cells

To test the direct effects of PA on 5- and 12-HETE production, AsPC-1 cells were exposed to PA (5, 10 and 20 µg/mL), zileuton (10, 20 and 30 µg/mL) and the 12-LOX-inhibitor baicalein (10 and 20 µg/mL) for 8 hours and the production of 5- and 12-HETE measured. LTs are released from cells and take part in paracrine and autocrine signalling [35]. Nevertheless, LT synthesis is not initiated at the plasma membrane as was originally believed; rather, it begins at the nuclear membrane [36]. We therefore estimated the concentration of 5- and 12-HETE both in cell lysates and supernatant medium. Much lower levels (approximately thirty times) were detectable in lysates than medium indicating immediate release from the cells.

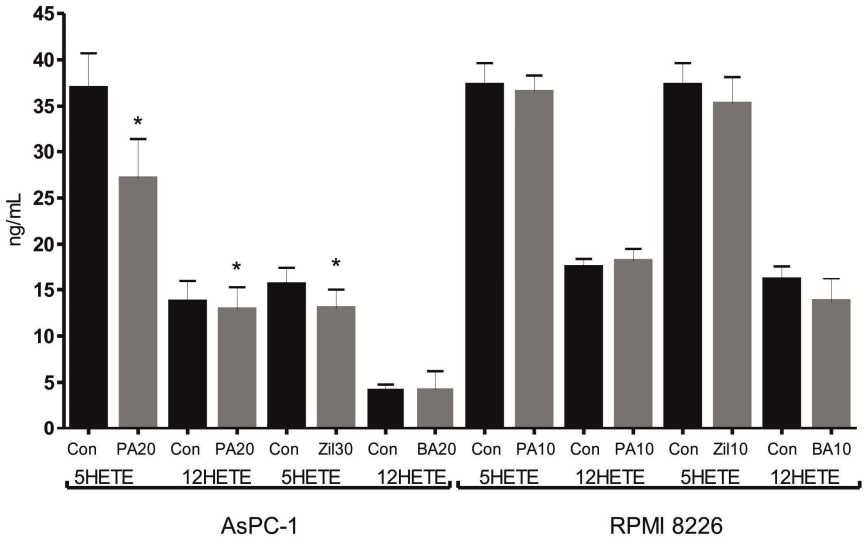

Fig. 6. Effects of PA and LOX-inhibitors on release of 5- and 12-HETE into medium estimated by the UPLC-MS/MS method. Synchronized pancreatic cancer cells (AsPC-1) were treated for 8 hours with PA 20 µg/mL, zileuton 30 µg/mL or baicalein 20 µg/mL. Significant reductions were seen in production of 5- and 12-HETE in PA-treated cells and 5-HETE in zileuton-treated cells. RPMI 8226 myeloma cells were treated with PA, zileuton or baicalein at 10 µg/mL. No effects were seen on release of 5- or 12-HETE. Error bars indicate standard error of the mean (*p<0.05).

Effects of PA were only observed at the highest concentration, showing that 5- and 12-HETE production was significantly decreased in medium after treatment for 8 hours (Fig.6). It should be noted that this concentration is higher than the reported IC_{50} value for 5-LOX inhibition and anti-proliferative effects but slightly under the IC_{50} value for 12-LOX inhibition. Small but significant effects were observed for 5-HETE after exposure to zileuton at 30 µg/mL, a concentration much higher than the reported IC_{50}

value of the inhibitor. No effects were seen after exposure to baicalein. Cell viability of AsPC-1 was not affected by the high concentration after 8 hours of treatment as indicated by microscopic observation and measurement of protein content. The same type of experiment was performed using the RPMI 8226 cell line but effects on 5- and 12-LOX were not observed after PA treatment up to 10 µg/mL. Treatment with PA 8 µg/mL causes apoptosis in the RPMI 8226, see fig.2, therefore it was not relevant to test higher concentrations of PA.

Taken together, these results imply that the inhibitors are not as active as expected in the intracellular environment, as effects on HETE production were only detected at high concentrations of inhibitors. It is also possible that the cells use other pathways, such as CYP450 to generate HETE, when LOX is inhibited, which could be further explored using CYP450 inhibitors.

4. Discussion

The lichen, *Cetraria islandica*, has been used traditionally over the past centuries to treat various conditions. The pure compound PA, isolated from the lichen, inhibits 5- and 12-LOX [23] and has anti-proliferative effects on various cancer cell lines [26, 27].

In this study we show that PA enters into cancer cells implying that PA can be expected to have intracellular effects. Indeed, PA-treated pancreatic cancer cells showed measurably reduced production of 5- and 12-HETE. However, this effect was only observed at very high concentrations. These concentrations were above those inducing anti-proliferative effects in the pancreatic cancer cell line and cell-cycle related changes in expression of LOX enzymes were not affected by PA. In the myeloma cells PA induced cell death occurred at concentrations that had no effect on HETE production. The anti-proliferative and pro-apoptotic effects are thus not likely to be mediated directly through inhibition of LOX activity.

In 1996 Tisdale and colleagues showed that treatment with the 5-LOX inhibitors BWA4C and BWB70C inhibited growth of murine adenocarcinomas both *in vitro* and

in vivo, whereas zileuton was less effective. All three agents caused decrease in 5-HETE production. A decrease in 12-HETE formation was seen for the former two agents but not for zileuton. From these results the authors suggested that the inhibitory effect on cell growth could result from an imbalance in metabolism of AA connected to 5-,12- and 15-lipoxygenase pathways [37]. The beneficial effects of zileuton in oral carcinogenesis have been linked to reduced formation of LTs as a result of inhibition of 5-LOX [19]. These *in vivo* effects are likely to be mediated by anti-inflammatory effects. The question also remains if the effects of zileuton are mediated only through the inhibition of 5-LOX or related to the fact that zileuton can inhibit the release of AA which could have numerous effects on target cancer cells [38].

Several reports have been published on anti-proliferative effects of 5-LOX inhibitors. Thus, MK886 was shown to inhibit proliferation of colon [39] and lung cancer cells [40]. The 12-LOX inhibitor baicalein has also been found to induce apoptosis in prostate [21] and pancreatic cancer cell lines [22]. Subsequently, other reports cast some doubt on a direct connection of anti-proliferative activities with LOX-inhibitory effects of substances. The growth-inhibitory effect of the 5-LOX inhibitor, Rev-5901 on NIH-3T3 cells was independent of 5-LOX expression of the cells [41] and in 5-LOX-deficient HeLa carcinoma cells the effects of LT-synthesis inhibitors were concluded to be 5-LOX independent [42]. Three common 5-LOX inhibitors were also shown to induce cytotoxic and anti-proliferative effects in pancreatic cancer cells irrespective of expression of 5-LOX, at concentrations that highly exceeded those for 5-LOX inhibitory effect, and Zileuton did not affect cell viability [20]. In addition, it has been suggested that baicalein is not a selective human platelet 12-LOX inhibitor but also inhibits reticulocyte 15-LOX-1 [43]. A number of plant metabolites including resveratrol have been shown to exhibit anti-proliferative effects and suppress expression of e.g. 5-LOX in various of cancer cells [44]. However, it is important to bear in mind that

natural compounds such as resveratrol and curcumin have been shown to have a wide spectrum of activity rather than being targeted agents [45, 46].

To our knowledge the effects of LOX inhibitors on cancer cells have not been assessed previously by direct measurements of the metabolites 5- and 12-HETE by UPLC-MS/MS, however LC/-MS/MS has been used to measure 5- and 12-HETE in murine retina and human vitreous samples [47]. Previous studies have reported measurements of 5- HETE and AA by radioactivity using a liquid scintillation counter or by using HPLC and radioactivity-monitoring [23, 32]. The methods described here yield exact quantitative results. The availability of such methods could therefore be useful for assessing activities of new LOX inhibitors that can be anticipated following the elucidation of the crystal structure of LOX enzymes [48].

In conclusion, our previous studies have shown that PA exhibits inhibitory effects on 5- and 12-LOX and has anti-proliferative effects on several cancer cell lines. In the current study we have shown the PA can also induce programmed cell death in one cell type but fails to do so in another. Overall, the results imply that the anti-proliferative and pro-apoptotic effects are not mediated by the LOX-inhibitory effects. Although PA enters the cells and could be shown to affect intracellular production of LOX-derived 5- and 12-HETE, this occurred only at concentrations well beyond those having anti-proliferative or pro-apoptotic effects. Furthermore, cell-cycle-induced changes in expression and localization of 5- and 12 LOX were not affected by PA treatment. Our findings emphasize the importance of direct quantification of the products when evaluating the cellular effects of LOX inhibitors. Although LOX inhibitors do not affect the production of LOX pathway metabolites in cancer cells at relevant concentrations, according to our results, the fact remains that some of them exert significant anti-proliferative as well as pro-apoptotic effects. The mechanisms

remain to be elucidated and it should be kept in mind that natural compounds frequently exert a broad range of activity.

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Bessadottir et al., Supplementary Figure 1

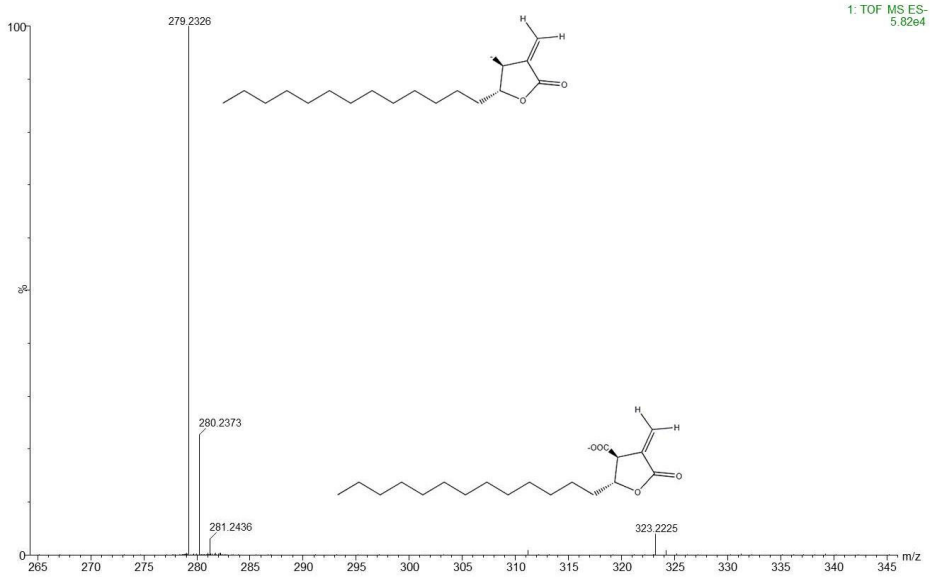
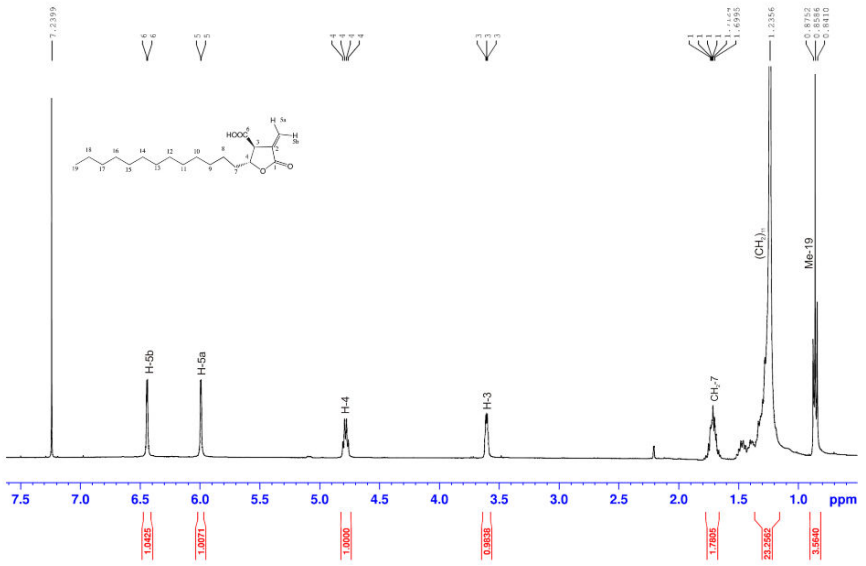


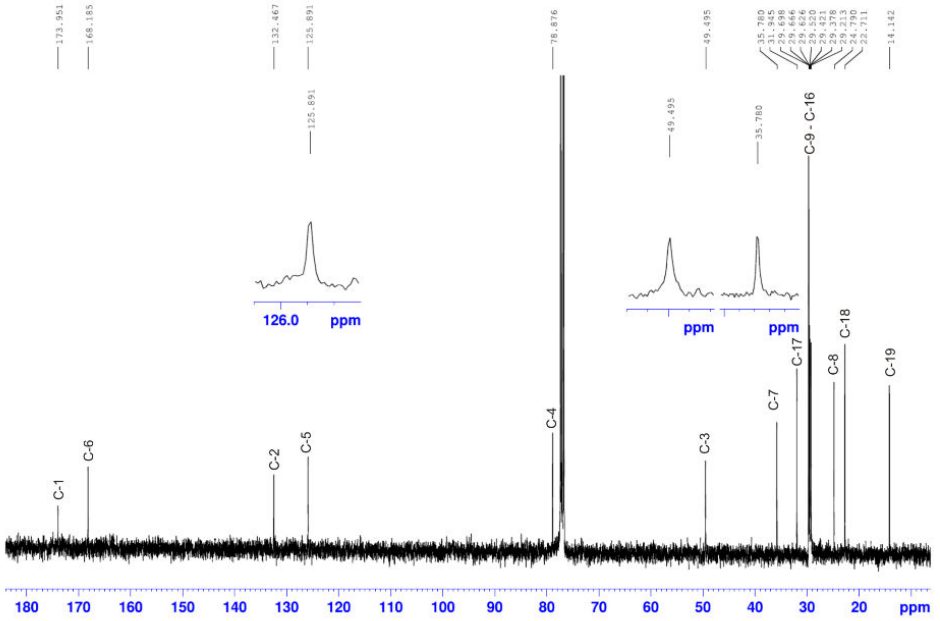
Fig. S1. Mass spectrum of (+)-protolichesterinic acid. Mass spectrum shows deprotonated molecule $[M-H]^-$ with an m/z of 323.2225 corresponding to the elemental composition $C_{19}H_{32}O_4$ (mass error of +0.9 ppm). A fragment ion is also detected where the carboxyl group breaks off resulting in decarboxylated protolichesterinic acid $[M-COOH]^-$ corresponding to the elemental composition $C_{18}H_{32}O_2$ (mass error of +0.7 ppm).

Appendix

S1. ^{1}H NMR spectrum of (+)-protolichesterinic acid ($CDCl_3$ 400 MHz)



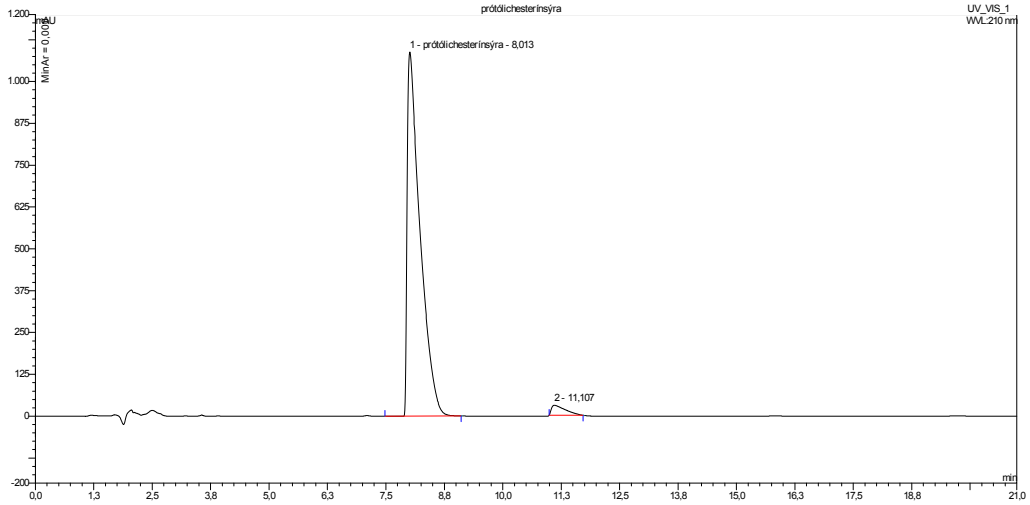
^{13}C NMR spectrum of (+)-protolichesterinic acid ($CDCl_3$ 100 MHz)



^{1}H	(+)-protolichesterinic acid	(Horhant et al 2007)
	ppm ($CDCl_3$)	ppm ($CDCl_3$)
Me-19 (t, 3H)	0.86	0.88
$(CH_2)_{11}$ (bs, 22H)	1.24-1.50	1.25 – 1.52
CH_2-7 (m, 2H)	1.70 – 1.74	1.70 – 1,.8
H-3 (ddd, 1H)	3.60	3.63
H-4 (ddd, 1H)	4.78	4.81
H-5a (d, 1H)	6.00	6.02
H-5b (d, 1H)	6.44	6.46

^{13}C	(+)-protolichesterinic acid	(Horhant et al 2007)
	ppm ($CDCl_3$)	ppm ($CDCl_3$)
C-1	174.0	174.1
C-2	132.5	132.4
C-3	49.5	49.5
C-4	78.9	79.0
C-5	125.9	125.9
C-6	168.2	168.3
C-7	35.8	35.7
C-8	24.9	24.8
C-9 – C-16	29.2 ; 29.7	29.2 ; 29.7
C-17	32.0	31.9
C-18	22.7	22.7
C-19	14.1	14.1

S2. Analytical HPLC chromatograph of (+)-protolichesterinic acid



Retention time (min)	Area under curve (mAU)	Realtive purity (%)
8.01 (+)-protolichesterinic acid	345.75	97.09
11.07 lichesterinic acid	10.36	2.91
Total	356.11	100

Column: RP column (G.L Sciences, Inc., Herbal medicine, C-18, 4.6 mm x 250 mm)

Solvent system: ACN:H_2O:CH_2O_2 (90:10:0.01)

Flow rate:1 mL/min,

UV-detection: 210 nm at 25°C