Natural Products AKTing on Signal Transduction Pathways- Molecules and Methods

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Summary

Natural products play an important role as source of inspiration for drug discovery and development and as tools for basic research. Isolated natural products that had shown diverse biological activity in previously published tests were analysed for their direct effect on molecular signalling processes, militarinone A from the entomogenous fungus *Paecilomyces militaris* and indolinone from *Isatis tinctoria*.

The fungal alkaloid militarinone A was originally identified in a screening for neurotrophic substances, where it induced neuronal outgrowth in PC12 cells. To uncover the mechanism of this action we studied the cell signalling pathways involved in neuronal spike formation and differentiation in two types of neuronal cells (PC12 and N2a) and the interaction of militarinone A with associated pathways. The increased neuronal outgrowth could not be confirmed as a general activity of militarinone A, as this effect was only transiently seen in PC12 cells and all other cell lines tested underwent apoptosis within 24h. We propose that this difference is due to varying constitutive levels of p53.

Furthermore, an alkaloid from the traditional European medicinal plant *Isatis tinctoria* was analysed. *Isatis tinctoria* contains several known anti-inflammatory components, namely, tryptanthrin, indirubin, and indolinone. In a previous study, indolinone was shown to inhibit degranulation of mast cells and this anti-allergic effect of indolinone should be further characterised. We confirmed the initially observed stabilising effect on mast cells of a different species and in a different assay set-up and showed that indolinone efficiently blocked $PtdInsP_3$ production due to inhibition of all class I PI3-kinases, therefore preventing activation of Akt and subsequent mast cell degranulation. The concentrations necessary to obtain the observed effect *in vitro*, however, were too high to consider *in vivo* testing.

Since mast cell degranulation depends on phosphoinositide signalling we studied phosphosinositide levels in cells upon stimulation. For this purpose, we developed a method that allows individual analysis of all phosphoinositides, including all PtdInsP- and $PtdInsP_2$-isomers. This novel method, based on ion-pair chromatography and ESI-MS detection, offers substantial perspectives for application in phosphoinositide-signalling research as it allows relative quantification of all the different PIs in cells.

Zusammenfassung

Naturstoffe spielen eine wichtige Rolle für die Entwicklung neuer Medikamente als Leitstrukturen sowie als Hilfsmittel in der Grundlagenforschung. In vorangehenden Arbeiten haben zwei unlängst isolierte Naturstoffe, Militarinon A und Indolinon, biologische Aktivitäten gezeigt. Diese Aktivitäten wurden auf der Ebene der Signaltransduktionswege in dieser Arbeit genauer untersucht.

Das aus dem entomogenen Pilz *Paecilomyces militaris* stammende Alkaloid Militarinon A, wurde ursprünglich in einem Screening für neurotrophe Aktivität entdeckt, bei dem es die Bildung neuronaler Auswüchse in PC12 Zellen stimulierte. Diese induzierte Bildung von Dendriten konnte jedoch nur für diese spezifische Zelllinie nachgewiesen werden, während andere Zellen Apoptose eingingen. Wir konnten zeigen, dass diese unterschiedlichen Reaktionen auf verschiedenen konstitutiven Expressionslevel von p53 beruhen.

Des weiteren wurde die Wirkungsweise von Indolinon, einem Alkaloid aus der traditionellen, europäischen Heilpflanze *Isatis tinctoria*, in dieser Arbeit analysiert. *Isatis tinctoria* enthält einige bekannte, entzündungshemmende Substanzen wie Tryptanthrin, Indirubin und Indolinon. In einer früheren Studie wurde gezeigt, dass Indolinon die Degranulierung von Mastzellen hemmt und diese anti-allergische Wirkung sollte nun näher untersucht werden. Die Mastzellstabilisierende Wirkung von Indolinon konnte bestätigt werden. Wir zeigten, dass Indolinon die Bildung von $PtdInsP_3$ durch Hemmung aller Klasse I PI3-Kinasen effizient inhibiert. In Folge dessen werden die Phosphorylierung von Akt sowie die Degranulierung verhindert. Die für eine deutliche Wirkung *in vitro* benötigten Konzentrationen waren jedoch zu hoch um für eine *in vivo*-Anwendung in Frage zu kommen.

Da die Degranulierung von Mastzellen abhängig von Menge und Phosphorylierungsgrad von Phosphatidylinositolen (PIs) ist, haben wir deren Level in stimulierten Zellen untersucht. Dazu wurde eine analytische Methode entwickelt, durch die alle Phosphphatidylinositole aufgetrennt und identifiziert werden können, insbesondere auch die PtdInsP- und $PtdInsP_2$-Isomere. Diese neue Methode, basierend auf Ionen-Paar Chromatographie und ESI-MS Detektion, eröffnet neue Perspektiven in der Erforschung der Phosphoinositol-Signaltransduktionswege, da eine relative Quantifizierung einzelner PIs möglich ist.

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Abbreviations

A3AR	A3 adenosine receptor
AD	Alzheimer's disease
Ade	Adenosine
AIF	Apoptosis inducing factor
AP-1	Activator protein 1
Apaf-1	Apoptotic protease-activating factor-1
ARE	Antioxidant responsive element
AS160	Akt substrate of 160 kDa
ATP	Adenosine-5'-triphosphate
BH	Bcl-2 homology
BMMC	Murine bone marrow derived mast cells
BPC	Base peak chromatogram
BSA	Bovine serum albumin
BTK	Bruton’s tyrosine kinase
CDK2	Cyclin-dependent-kinase 2
c-FLIP	Cellular caspase-8 (FLICE)-like inhibitory protein
CMT	Charcot-Marie-Tooth
CNS	Central nervous system
DAG	Diacylglycerol
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMHA	N,N-dimethy-hexylamine
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
EIC	Extracted ion chromatogram
ELSD	Evaporative light scattering
EMSA	Electrophoretic mobility shift assay
ESI	Electrospray ionisation
FACS	Flow cytometry (fluorescence activated cell sorting)
FADD	Fas-associated death domain
FasL	Fas-ligand
FCS	Foetal calf serum
FDA	U.S. Food and Drug Administration
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1 beta-converting enzyme
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FoxO1	Forkhead box O1
FRAP	FKBP12-rapamycin-associated protein
GPCR	G-protein-coupled receptor
GSK3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
HIV	Human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	High performance liquid chromatography

HS	Horse serum
HTS	High throughput screening
IAP	Inhibitor of apoptosis
IκB	Inhibitor of NFκB
IKK	IκB-kinase
IL2	Interleukin 2
IL3	Interleukin 3
IMDM	Iscove's modified Dulbecco's Medium
IP_3	Inositol-(1,4,5)-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LC	Liquid chromatography
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MiliA	Militarinone A
MOMP	Mitochondrial outer membrane permeabilisation
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NAC	N-acetyl-L-cysteine
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
OCRL	Occulocerebrorenal syndrome of Lowe
OTC	Over-the-counter
PDK1	3-phosphoinositide dependent protein kinase-1
PFTa	Pifithrin a
PH	Pleckstrin-homology
PI	Phosphoinositide
PI3-K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate-13-acetate
PP	Protein serine/threoinine phosphatase
PtdIns	Phosphatidylinositol
PtdIns(3,4)P_2	Phosphatidylinositol-3,4-bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol-3,5-bisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
PtdIns3P	Phosphatidylinositol-3-phosphate
PtdIns4P	Phosphatidylinositol-4-phosphate
PtdIns5P	Phosphatidylinositol-5-phosphate
PtdInsP_3	Phosphatidylinositol-3,4,5-trisphosphates
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
ROS	Reactive oxygen species
RP	Reversed phase
S1P	Sphingosine-1-phosphate
SERCA	Sarco/endoplasmatic reticulum Ca^{2+} -ATPase
SHIP	SH2-containing inositol 5-phosphatase

Smac	Second mitochondrial-derived activator of caspases
SphK	Sphingosine kinase
TNF	Tumour necrosis factor
TNFR	TNF-receptor
TOR	Target of rapamycin
TORC1	mTOR complex 1
TORC2	mTOR complex 2
TPA	4β-12-O-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis ligand
v-FLIP	Viral caspase-8 (FLICE)-like inhibitory protein
wort	Wortmannin

A. INTRODUCTION

1 Natural Products in Research and Drug Discovery

˝Embrace the Challenge˝
-Teachings of Anusara Yoga

The therapeutic use of plants is as old as human civilisation. Even today plants remain the primary sources of health care for most people in the world. It is estimated that around 80% of the world's population rely mainly on traditional medicine for their primary health care (Wang, 2008). Over the centuries, natural products have provided a tremendous amount of substances that serve as medicine or as lead structure for drug development. Around one third of the currently marketed drugs have structures that are related or derived from natural products (Onaga, 2001).

1.1 History

Natural products have been the first and, for a long time, sole source of medicine. The exploitation of traditional medicines for the development of modern drugs produced the first commercially available pure drug substances. The isolation of morphine from the opium latex by the German Pharmacist Sertürner in 1805 could be seen as the start of pharmaceutical natural product research. Shortly thereafter the isolation of many other substances followed, atropine in 1819, quinine and caffeine in 1820, and digitoxin in 1841 (Potterat and Hamburger, 2008). Quinine was isolated in 1820 from the bark of several *Cinchona* species that have been used by Peruvian Indians to treat shivering and malarial fevers (Corson and Crews, 2007; Greenwood, 1992). In 1826, quinine and morphine became the first commercially available pure natural compounds produced by Caventou and Merck, respectively (Newman, 2000; Potterat and Hamburger, 2008).

The synthetic modification of salicylic acid to acetylsalicylic acid in 1897, and the modification of morphine into diacetylmorphine (heroin) in 1898 by Hoffmann at Bayer AG produced the first semi-synthetic natural-product derived drugs (Schmidt, 2008; Sneader, 2000).

The progression of the role of natural products in drug discovery (Figure 1) proceeded from the traditional use of whole plants and plant extracts to the isolation and identification of the active principle pure compounds, to the application of derivatised, optimised molecules, and finally to the use of natural products as leads for medicinal chemistry.

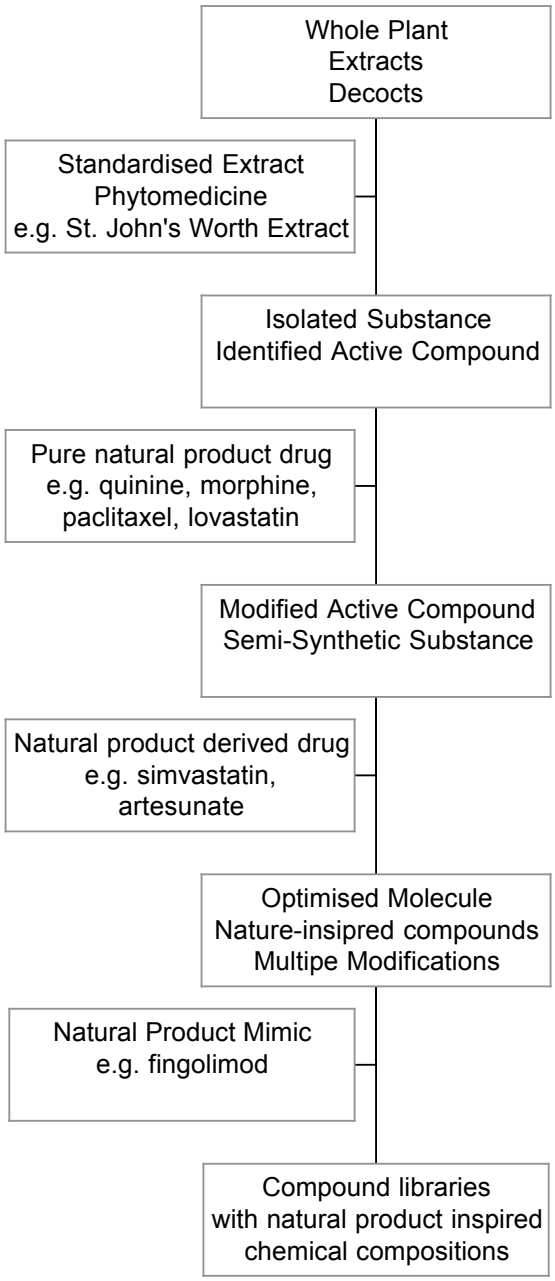

Figure 1: Diagram describing the various roles of natural products in drug discovery and development

1.2 Today

After decades of very successful drug discovery and development, the pharmaceutical industry downscaled natural product research in the late 1990s in favour of automated high throughput screening (HTS) of compound libraries (Baker, 2007). Compound libraries assembled with the aid of combinatorial chemistry were thought to produce more hits than 'old fashioned' natural products. Despite this decline in the use of natural products in drug discovery, newly marketed drugs derived from natural compounds hold about the same share as before (Newman, 2003).

The numerous and successful discoveries of compounds in the early times of modern drug discovery were quite exclusively based on the traditional use of the plant (Rishton, 2008). Of all known organic molecules, only 1% are natural products, 99% are synthetic (von Nussbaum, 2006), but more than one third of all drug sales are based on natural products (Newman, 2003). How can these striking numbers be explained? Evolutionary selection is the answer; nature's own high-throughput screening has optimized these biologically active compounds (Paterson and Anderson, 2005). Especially the numerous compounds with antibacterial activity do not surprise, as fighting for space and resources, and against other organisms, plays a pivotal role in survival.

Early attempts to apply HTS to botanical extracts were faced with many difficulties. With the introduction of biochemical assays in the 90s, the screening process had shifted from functional cellular assays to cell-free biochemical assay formats, which are very sensitive and prone to artefacts. The typically coloured plant extracts are not compatible with such screening assays due to interference with detection caused by colour, fluorescence, or quenching effects of components in the extract. Moreover the complexity of extracts potentially induces aggregation of components, chemical reactions within the assay or difficulties of solubility in assay buffer (Rishton, 2008). Pre-fractionation or purification to reduce the chemical complexity of the extracts needs to be implemented before HTS can be performed. This is time-consuming and laborious and generally reduces the attractiveness of screening of natural compounds. Another approach is to use the power of combinatorial chemistry in combination with knowledge on active natural products and create a library that extends upon the structural properties of known natural compounds. This strategy

generates libraries with enhanced specificity and selectivity (Koehn and Carter, 2005).

1.3 Marketed Drugs

Here I briefly describe selected examples of successfully marketed drugs that are natural compounds, derivatives of natural compounds, or synthetic molecules for which the lead was a natural product. The focus here is on some more recent drugs that had a major impact on human lives, while the more historical and well-known examples such as morphine, penicillin, quinine, streptomycin and others are left out.

1.3.1 Lovastatin (FDA Approval 1987)

Since the discovery of a correlation between high cholesterol levels and coronary heart disease in the 1950s, the lowering of high cholesterol levels with drugs has been pursued (Kannel, 1995; Keys, 1984). The cholesterol biosynthesis is a complex process involving more than 30 enzymes and was discovered during the 1950s and 60s (Russell, 1992). HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase is the rate-limiting enzyme of the biosynthetic pathway and hence most suitable for inhibition. Furthermore, when HMG-CoA reductase is inhibited an alternative pathway for degradation of the substrate is available which prevents accumulation of HMG-CoA.

A potent inhibitor of HMG-CoA reductase, compactin (later additionally named mevastatin (Figure 2)), was found in a broth of *Penicillium citrinum* (Endo, 1976). Soon after, in 1978, another HMG-CoA reductase inhibitor was discovered in the Merck laboratories from *Aspergillus terreus* and named mevilonin (lovastatin (Figure 2)) (Alberts, 1980). Compactin was highly effective in lowering plasma cholesterol in animal models as well as patients with hypercholesterolaemia (Kuroda, 1979; Mabuchi, 1981; Mabuchi, 1983; Tsujita, 1979; Watanabe, 1981). However, compactin was withdrawn from clinical trials in 1980 due to unpublished reasons (Tobert, 2003). Because of the structural similarity between compactin and lovastatin, clinical studies with lovastatin had to be stopped as well. After additional animal studies and some investigation in small-scale high-risk patient studies, clinical development was re-launched in 1983 until, finally, in 1987 the FDA (U.S. Food and Drug Administration) approval for lovastatin was obtained (Illingworth and Sexton, 1984; Thompson, 1986). Soon after, other statins from microbial sources were

released. Simvastatin (Figure 2) entered the market in 1988, it is semi-synthetically derived from lovastatin introducing a minor side chain modification. In 1991 pravastatin followed, with a modification in the side chain ring. Later, synthetically designed products with different chemical structures followed; fluvastatin in 1994, atrovastatin in 1997, cerivastatin in 1998 and rosuvastatin in 2003.

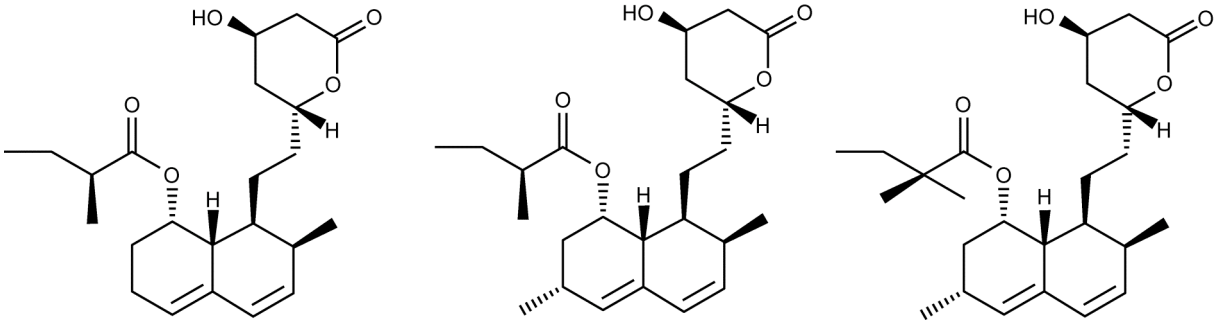

Figure 2: Structures of compactin, mevilonin and simvastatin

The mechanism of action of statins goes beyond blocking cholesterol biosynthesis. Inhibition of HMG-CoA reductase reduces levels of mevalonate, which in turn leads to upregulation of low-density lipoprotein (LDL) receptors on hepatocytes. The upregulation of LDL receptors increases the uptake of LDL from blood, the major marker of elevated cholesterol levels (Brown and Goldstein, 1980; Reihner, 1990).
With the discovery of lovastatin it was for the first time possible to achieve large reductions in plasma cholesterol of up to 40% (Tobert, 1982). The treatments formerly available were all of limited efficacy or tolerability. The bile acid sequestrants are moderately effective and poorly tolerated due to gastrointestinal side effects whereas fibrates produce a rather small reduction in LDL-cholesterol but are well tolerated and widely used. The statins revolutionised the treatment of hypercholesterinaemia and annual sales are > 15 billion US $ (Downton and Clark, 2003). In 2001, however, cerivastatin, only introduced in 1998, had to be withdrawn from the market due to severe side effects of rhabdomyolysis which occurred in concomitant use with gemfibrozil (Furberg and Pitt, 2001). The mechanism for this side effect still remains elusive but further studies demonstrated the safety of other statins. Nevertheless myalgia as a side effect under statin therapy occurs but seldom develops into severe myolysis.

The statins became the most effective drugs so far for preventing and halting arteriosclerosis. Despite the wide use of these drugs it is believed that they are underutilised in patients who are free of symptoms with only moderately elevated cholesterol levels. This led to the decision to approve simvastatin as an 'over-the-counter' (OTC) medicine, available without prescription, in the UK in July 2004 (Link, 2004; Roberts, 2004).

1.3.2 Paclitaxel (FDA Approval 1992)

Plants have a long history in the use of cancer treatment. The first plant derived drug to treat cancer was the *Vinca* alkaloid vincristine, which was approved for clinical use in 1963. A more recent discovery of a plant-derived chemotherapeutic agent was paclitaxel (Figure 3) from *Taxus brevifolia* bark (Wani, 1971). Paclitaxel was shown to stabilise microtubule assembly, whereas *Vinca* alkaloids and colchicin prevent the assembly of microtubules (Schiff, 1979; Schiff and Horwitz, 1981). Even in absence of essential GTP, paclitaxel promotes microtubule assembly. Although paclitaxel shows no structural resemblance to GTP, it is able to interact specifically with the β-subunit of microtubules, a region that is associated with GTP binding and hydrolysis (Snyder, 2001). The stabilisation of microtubules by paclitaxel forces the tumour cell into multiple DNA replication cycles that eventually initiate apoptosis (Stewart, 1999).

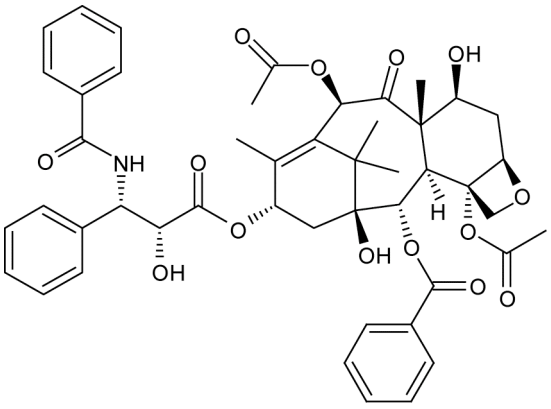

Figure 3: Structure of paclitaxel

Clinical trials with paclitaxel were started in the early 1980s, and FDA approval for treatment of refractory ovarian cancer was granted in 1992. Since the introduction of paclitaxel to the treatment of ovarian cancer the survival rate has more than doubled (Crown and O'Leary, 2000), and further applications have been approved since.

Today, paclitaxel is also used in the treatment of breast and colon cancers as well as Kaposi's sarcomas of HIV infected patients (Oberlies and Kroll, 2004).
Preparation of sufficient amounts of paclitaxel to launch clinical studies was nearly impossible, as isolation from the bark results in very low yields and excoriation causes the trees to die. In 1986, the precursor deacetyl baccatin III was isolated from the needles of *Taxus baccata,* and the semi-synthetic approach lead to the production of sufficient amount of paclitaxel from renewable sources (Gueritte-Voegelein, 1991). Furthermore, semisynthesis enabled the creation of an analogue of paclitaxel, docetaxel (Bissery, 1991), which entered the market in 1996.
From the perspective of both basic science and clinics, paclitaxel has led to significant progress in understanding and treating cancer.

1.3.3 Sirolimus, Rapamycin (FDA Approval 1999)

In this section, the clinical aspects of sirolimus are described, whereas the molecular and signalling-related aspects are discussed later in this chapter.
Rapamycin (Figure 4) was discovered in the 1970s as a potent antifungal metabolite of the bacterial strain *Streptomyces hygroscopicus* and was named after the origin of the sample, the Easter Island Rapa Nui (Vezina, 1975). Its clinical development as an antifungal drug was stopped when its strong antiproliferative and immunosuppressive effects were detected (Chang, 1991; Eng, 1984). The substance fell into oblivion until studies on the mechanism of action led to the identification of its target (target of rapamycin, TOR) in 1992 (Heitman, 1992). Later, the compound was also named sirolimus due to its structural similarities with tacrolimus, which was discovered in 1987.
The immunosuppressive activity of rapamycin is due to its blocking of interleukin 2 (IL2) mediated T-cell proliferation and activation (Dumont, 1990) thereby preventing allograft rejection after organ transplantation. The combination of rapamycin with calcineurin inhibitors such as cyclosporine A or tacrolimus results in significant synergistic effects that improve the prevention of organ rejection (Kahan, 1998). In 1997 rapamycin/sirolimus obtained FDA approval for preventing host-rejection of kidney-transplants. Further studies address the use of rapamycin in autoimmune diseases such as psoriasis, multiple sclerosis or rheumatoid arthritis (Foroncewicz, 2005; Tsang, 2007) and are still ongoing. The additional inhibitory effects of rapamycin on the proliferation of vascular smooth muscle cells led to the

development of rapamycin as antirestenosis drug, and coronary-artery stents releasing rapamycin are approved in surgery since 2003 (Morice, 2002).

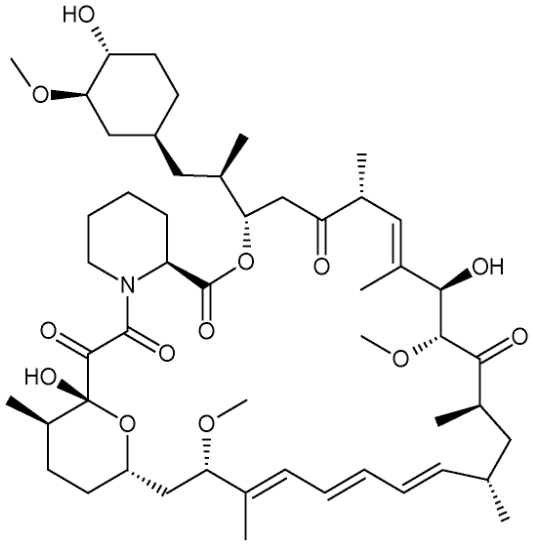

Figure 4: Structure of rapamycin

1.3.4 Artemisin (Swissmedic Approval 2000, no FDA Approval)

The Chinese medicinal herb qing hao (*Artemisia annua*) was traditionally used to reduce fever and, in 1596, was mentioned for the first time to treat malaria (Klayman, 1985). In 1972, Chinese scientists managed to isolate the active principle of the herb and called it qinghaosu, meaning 'the active principle of qing hao' (1979), named artemisinin (Figure 5) for the Western world. The structure was elucidated in 1980 and revealed to be a sesquiterpene structure with an unusual endoperoxide group (Acton and Klayman, 1985). The high lipophilicity of artemisinin made administration as a drug difficult; therefore, various derivatives were synthesised, including arthemether, arteether and artesunate (Figure 5).

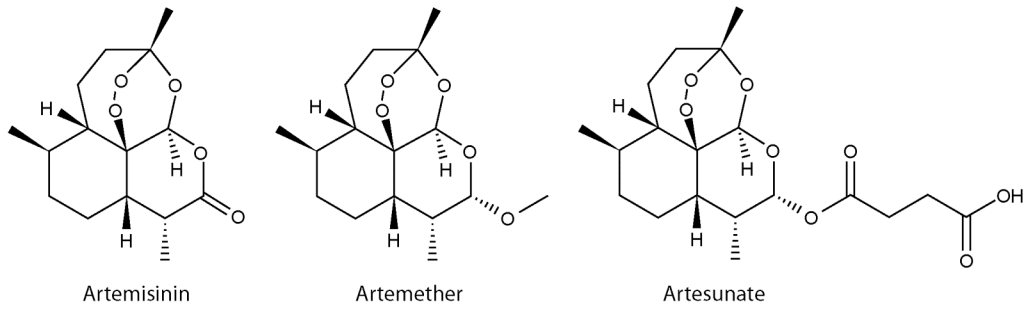

Figure 5: Structures of artemisinin, artemether, and artesunate

The biological activity of the artemisinins depends on the cleavage of the peroxide bond after contact with iron-II-hem within the parasite. The generated free radical alkylates the hem molecule or parasite proteins (Bhisutthibhan, 1998; Olliaro, 2001). Inhibition of the sarco/endoplasmatic reticulum Ca^{2+} -ATPase (SERCA) has been proposed as an additional target (Eckstein-Ludwig, 2003). The active metabolite dihydroartemisinin kills nearly all asexual stages of parasite lifecycle in the blood, and also affects the gametocytes, which are responsible for the infection of the Anopheles mosquito and transmit the disease. Furthermore the artemisinins act faster than any other antimalarial drug with a fever and parasite clearance time of less than two days (Wiesner, 2003). However, due to the short plasma half-life of these drugs therapy needs to be continued for 5-7 days, or needs to be combined with other antimalarial drugs (White, 2008). The combination usually applied is artesunate-lumefantrine. In 2000 Swissmedic approved the drug for sale under the name Riamet; in other countries it is sold as Coartem. At the moment, Novartis is still awaiting FDA approval for Coartem.

1.4 Promising Research

The examples discussed here were selected for their uniqueness, as they all represent the first substances in clinical development with their respective mode of action. Mostly the understanding of their molecular target has evolved concurrently with the discovery of the substance and the subsequent studies of the pharmacological and clinical effects.

1.4.1 Fingolimod

Fingolimod is a synthetic compound inspired by the structure of myriocin (Figure 6), which is produced by the fungus *Isaria sinclarii* (Fujita, 1994). Myriocin is a structural analogue of sphingosine, a C18 amino alcohol, which is a part of sphingolipids. *In vivo* myriocin caused severe intoxication resulting in death of the animals. Because of potent immunosuppressive activity *in vitro*, synthetic modifications were introduced to reduce toxicity (Chiba, 1996). Fingolimod (FTY720 (Figure 6)) is such a synthetic analogue that only obtains its immunosuppressive activity after phosphorylation by sphingosine kinase 2 *in vivo* (Suzuki, 1996; Zemann, 2006). The biologically active fingolimod-phosphate binds to four of the five known sphingosine-1-phosphate (S1P)

receptors, all but $S1P_2$ (Rosen and Goetzl, 2005). Binding of fingolimod-P results in internalisation and degradation of the $S1P_1$ receptor, thus depriving the cell of the necessary signals for cell motility and chemotaxis, and leading to down regulation of the receptor (Cyster, 2005). Therefore, circulating lymphocytes are retained in lymph nodes, resulting in reduction of peripheral lymphocytes (Mandala, 2002).
Clinical trials of fingolimod for suppression of transplant rejection were discontinued in 2006 due to a lack of advantage over the existing treatments (Salvadori, 2006). However, trials for treatment of relapsing multiple sclerosis still are ongoing and preliminary data looks very promising (Kappos, 2006).

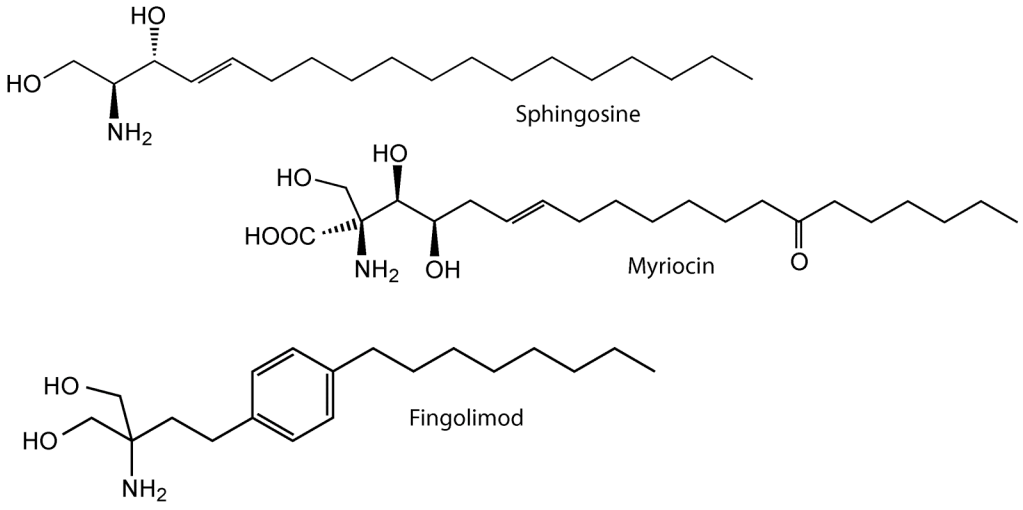

Figure 6: Structures of sphingosine, myriocin and fingolimod

1.4.2 Gossypol

Gossypol (Figure 7) is a polyphenolic compound isolated from cottonseeds or crude cotton oil that was described and named already in 1899 by Marchlewski (Withers and Carruth, 1915). Of the two enantiomers of gossypol, (-)-gossypol and (+)-gossypol only (-)-gossypol shows biological activity *in vivo*, although this was only discovered after separation of the two isomers succeeded in 1985 (Zheng, 1985). The toxic effects of gossypol, however, are long known and for that reason cottonseeds and cotton oil cannot be used for animal feeding or aliment. As a result of a general survey of public health in Chinese rural communities in the 1950s the use of crude cotton oil was linked with reduced male fertility (Kong, 1986). Clinical studies for a male contraceptive agent with over 8000 precipitants showed an overall antifertility effect of 99.9 % (1980). Despite these promising initial results clinical

studies as male contraceptive were discontinued in 1998 due to sustained infertility after prolonged use of gossypol (Porat, 1990). One possible explanation for the inhibition of spermatogenesis is the suppression of telomerase activity by gossypol (Moon, 2008). The reverse transcriptase telomerase is only found in embryonal, gonadal and cancer cells, as it enables unlimited cell division (Mego, 2002). Telomerase inhibition, however, is an interesting target in cancer therapy and gossypol was therefore tested in several cancer models as well as in vivo against diffuse large cell lymphoma, head and neck squamous cell carcinoma, and breast cancer (Mohammad, 2005; Van Poznak, 2001; Wolter, 2006).

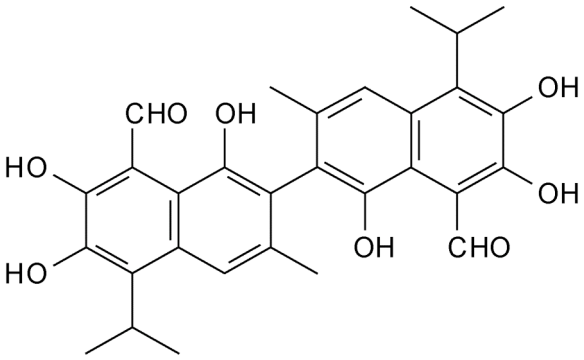

Figure 7: Structure of gossypol

In a screening of a small natural product library gossypol showed potent inhibition of the antiapoptotic proteins Bcl-2 and Bcl-x_L (Kitada, 2003). It binds to the BH3 binding domain of the antiapoptotic Bcl-2 family members, where the pro-apoptotic Bcl-2 family member Bid would bind to induce apoptosis. Gossypol is the first substance found to mimic BH3-binding to Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 and serves now as lead substance for further development of small inhibitory molecules (Marzo and Naval, 2008). Currently, gossypol is evaluated as the first Bcl-2 inhibitor in clinical trials against various cancers.

1.4.3 Bryostatin-1

The macrocyclic lactone bryostatin-1 (Figure 8) was isolated and identified in 1982 by Pettit et al. (Pettit, 1982) from the marine 'moss-animal' (Ectoprocta or Bryozoa) *Bugula neritina*, which was collected in the Gulf of Mexico in 1968. Bryozoans are aquatic colonial animals that are abundant in marine enviroment. Already in 1970 the antineoplastic effect of *Bugula neritina* extract was shown to lead to prolonged survival in a leukaemia mouse model (Pettit, 1970). Like the phorbol esters,

bryostatin-1 can be a potent activator of protein kinase C (PKC) (Berkow and Kraft, 1985). Prolonged exposure to bryostatin-1, however, induces PKC inhibition by degradation and subsequent downregulation of PKC (Isakov, 1993). Furthermore, bryostatin-1 inhibits phorbol ester induced tumourgenesis and differentiation of promyelocytic leukaemia cells (Hennings, 1987; Kraft, 1987). Bryostatin-1 was shown to bind to the 'phorbol ester receptor', which means the diacylglycerol (DAG)- binding pocket of PKC (de Vries, 1988) and, when bound to PKC, induces PKC-degradation by ubiquitination (Lee, 1996a; Lee, 1996b).

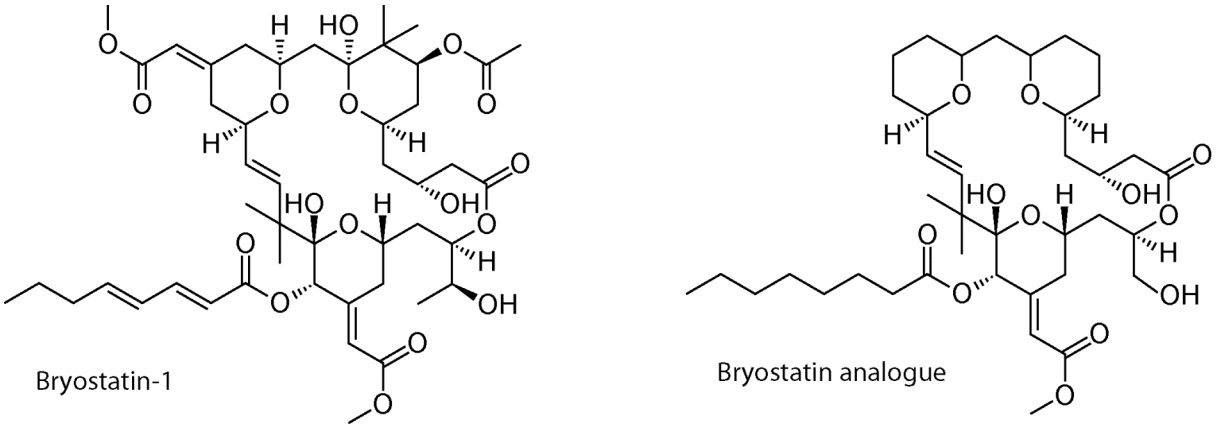

Figure 8: Structures of bryostatin-1 and its analogue, synthesised by Wender 2004.

Clinical phase II studies with single-agent bryostatin-1 have been conducted for melanoma, renal cell carcinoma, colorectal cancer, and non-Hodgkin's lymphoma, mostly with disappointing results (Kortmansky and Schwartz, 2003). But co-administration of bryostatin-1 with other cytotoxic agents produced promising results; particularly the combinations of bryostatin-1/paclitaxel and bryostatin-1/temsirolimus proved to be effective and are currently evaluated in clinical trials (Ku, 2008).

Development of analogues of bryostatin-1 (Figure 8) by Wender et al. (Wender, 2004) led to simplifications of parts of the structure that allow large-scale synthesis but preserve the crucial parts for bioactivity (Paterson and Anderson, 2005). Additionally, these studies created a better understanding of the structure-activity relationship of PKC inhibition and most likely will generate bryostatin analogues for clinical development.

Besides development as an anticancer drug bryostatin-1 is also under investigation as a central nervous system (CNS) drug (Sun and Alkon, 2006). Numerous reports imply a critical role of PKC malfunctions in the development of Alzheimer's disease (AD) (Cole, 1988; Favit, 1998; Lee, 2004). The PKC modulating effects of bryostatin-

1 seems to promote memory-enhancing and mood regulation effects (Sun and Alkon, 2005). Although passage of Bryostatin-1 through the blood-brain barrier was not determined in this study, previous studies with mice indicate that bryostatin-1 can pass across the blood brain barrier, but the brain levels of the drug were much lower than plasma levels (Zhang, 1996). Development of analogues of bryostatin-1 may result in compounds with improved CNS permeability and that could produce the desired effects at much lower doses.

1.5 Natural products as research tool

The elucidation of signal transduction pathways uses several tools of molecular biology, such as gene knockdown, overexpression of proteins, and the use of specific inhibitors of certain signalling molecules. All have been helpful to trace the function of pathways *in vivo* (Levine, 2007). Especially inhibitors of specific signal transduction molecules have offered opportunities for studying the signal transduction mechanisms. The example of rapamycin, its biological activity, the detection of its target mTOR, the mammalian target of rapamycin, and the following identification of a novel signalling cascade involved in fundamental processes of growth and development, shows the value of natural products like rapamycin as research tool.

1.5.1 Rapamycin

Rapamycin (also named sirolimus) was discussed as an immunosuppressive treatment earlier in this chapter (Chapter 1.3.3; Figure 4). Here the impact of the discovery of rapamycin on biological research shall be highlighted.

The target of rapamycin (TOR) was identified in the budding yeast *Saccharomyces cervisiae* in the 1990s (Heitman, 1992), and subsequent studies in mammalian cells led to the identification of the mammalian TOR (mTOR) (Sabers, 1995). Since several groups cloned the gene at about the same time, TOR is also known as FRAP (FKBP12-rapamycin-associated protein), RAFT (rapamycin and FKBP12 target), RAPT (rapamycin target), and SEP (sirolimus effector protein) (Fingar and Blenis, 2004). TOR is a 290 kDa large member of the PI3K-kinase-related-kinase (PIKK) superfamily and is 40-60% identical amongst mammals, flies, worms and yeast (Wullschleger, 2006). Two different TOR complexes are formed, in yeast containing two different TORs, TOR1 and TOR2. In mammals mTOR is associated with raptor forming the mTORC1 complex or with rictor, forming the mTORC2 complex. In cells

rapamycin forms a complex with a cofactor, FKBP12, and binds to TOR resulting in the inhibition of TOR. In mammalian cells rapamycin only inhibits the mTORC1 complex, but not the mTORC2.

Rapamycin treatment results in cell cycle arrest in late G1 phase (Dumont and Su, 1996), because mTOR initiates the signal for translation of key mRNAs required for cell cycle progression from G1 to S phase. In addition, rapamycin blocks cyclin-dependent activation and accelerates the turnover of cyclin D, resulting in growth arrest in G1 phase of the cell cycle (Rowinsky, 2004).

1.5.2 Wortmannin

The fungal metabolite wortmannin (Figure 9) was isolated from *Talaromyces wortmanni*, a *Penicillium* strain, in 1957 by Brian and Norris (Brian, 1957) and the structural determination as a furanosteroid followed in 1968 (MacMillan, 1968). Wortmannin was found to inhibit phosphoinositide 3-kinases (PI3-kinases), where it binds colvalently to the p110α subunit of the PI3-kinase into the ATP-binding site and blocks it with an IC_{50} of 5 nM (Wipf and Halter, 2005; Wymann, 1996). Other potential targets as protein kinases remain fairly unaffected in these concentrations (Bain, 2007). Therefore, the compound has been a useful tool for investigations of signal-transduction pathways involving PI3-kinase activity (Cardenas, 1998). Due to the high toxicity the clinical use of wortmannin never became possible, and due to lack of isoform specificity all essential PI3-kinase isoforms are equally inhibited. Isoform specific inhibitors of PI3-kinase γ are in clinical development.

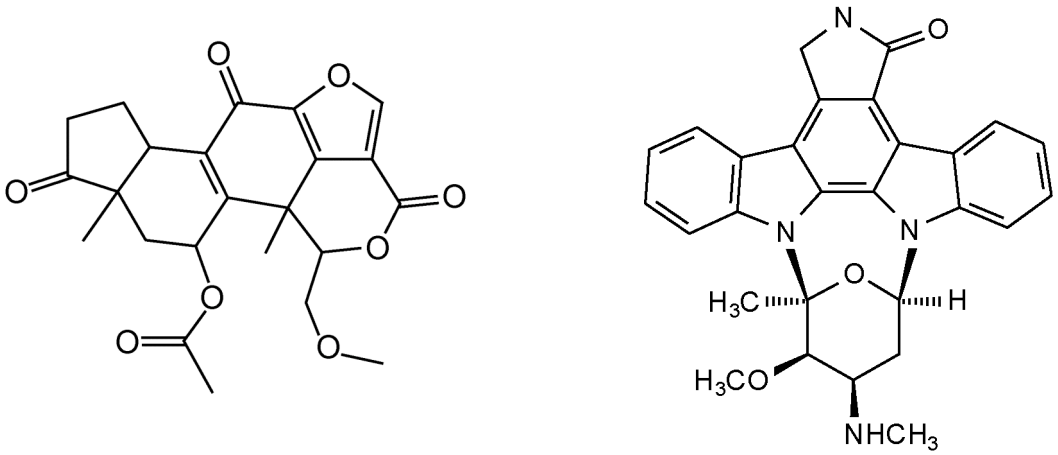

Figure 9: Structures of wortmannin (left) and staurosporine (right)

1.5.3 Staurosporine

Staurosporine (Figure 9) is an alkaloid isolated from a *Streptomyces* strain that was discovered in 1977 in a screening for PKC inhibitors (Omura, 1977). The compound turned out to be a potent, but not selective, inhibitor of protein kinases, competing with ATP for binding (Lamers, 1999). Staurosporin shows nanomolar activity against many protein kinases and has become the 'lead' inhibitor for the design of protein kinase inhibitors. Various analogues have been synthesised to obtain better selectivity, but the precise mechanisms to achieve selectivity remains elusive. The recently synthetically derived analogue of staurosporine, Enzastaurin (LY 315615) has now entered Phase III clinical trials for prevention of relapse in patients with some specific tumours (Butler and Newman, 2008; Graff, 2005). Staurosporine was also found to be a potent inducer of apoptosis through caspase-dependent, as well as independent pathways (Belmokhtar, 2001). It has been shown to induce apoptosis in all cell types tested to date and therefore became a widely employed inducer of mitochondria-dependent apoptosis in research (Kruman, 1998; Leist and Jaattela, 2001).

1.5.4 Phorbol Esters

Phorbol esters (Figure 10) activate PKC in a DAG-mimicking manner. The tumour promoting activity of Croton oil from *Croton tiglium* has been observed by Berenblum in 1941 (Berenblum, 1941) and was linked to PKC in 1988 (Nishizuka, 1988). The widely used active phorbol ester TPA (4β-12-O-tetradecanoylphorbol-13-acetate, also known as PMA (phorbol 12-myristate-13-acetate)) acts as an analogue of the natural PKC substrate, DAG, but is a much more potent activator of PKC. Prolonged incubation with phorbols, however, results in down-regulation of PKC (Silinsky and Searl, 2003).

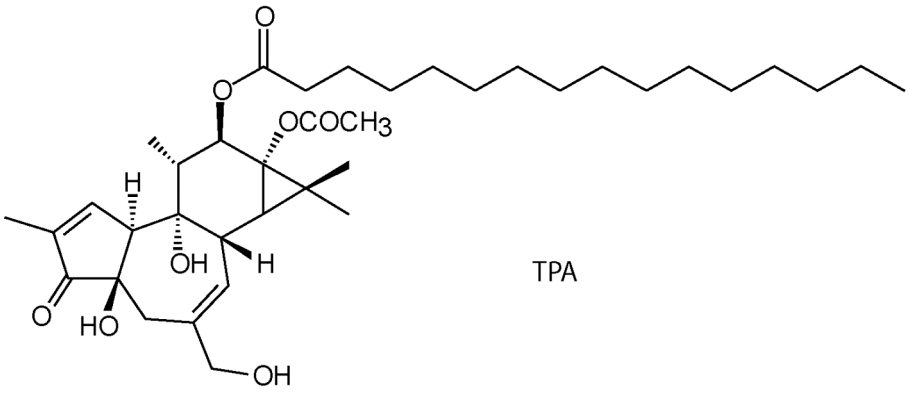

Figure 10: Structure of the phorbol ester 4β-12-O-tetradecanoylphorbol-13-acetate (TPA)

In vivo the phorbols do not induce tumour formation but promote tumour growth following exposure to carcinogens. Thus, they can be characterised as co-carcinogenics (Goel, 2007).

1.5.5 Okadaic Acid

Okadaic acid (Figure 11) is a marine natural product isolated from the sponge *Halichondria okadai* (Tachibana, 1981) that shows cytotoxic activity against several carcinoma and leukaemia cell lines. It was subsequently found that okadaic acid possesses a tumour promoting activity similar to phorbol esters (Nishizuka, 1984). But in 1988 okadaic acid was found to inhibit protein serin/threonine phosphatases (Bialojan and Takai, 1988) and not PKC. The serine/threonine phosphatase PP2A, however, is physically associated with PKCα, maintaining it in a hypo-phosphorylated state and, when inhibited, leads to hyper-activity of PKC (Boudreau and Hoskin, 2005). Okadaic acid has since been used as a key laboratory tool for identifying and studying the events associated with protein serine/threonine phosphatase (PP) inhibition, especially of the two major members PP1 and PP2A (Gehringer, 2004). For clinical application, however, okadaic acid lacks sufficient specificity and shows tumour-promoting activity.

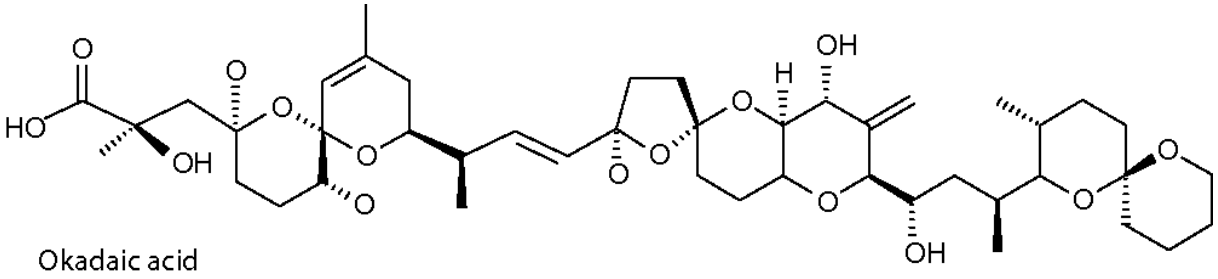

Figure 11: Structure of the marine phosphatase inhibitor okadaic acid

1.6 References

Acton, N., and Klayman, D.L. (1985). Artemisitene, a new sesquiterpene lactone endoperoxide from *Artemisia annua*. Planta Med *51*, 441-442.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E.*, et al.* (1980). Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc Natl Acad Sci U S A *77*, 3957-3961.

Anonymous (1979). Antimalaria studies on Qinghaosu. Chin Med J (Engl) *92*, 811-816.

Anonymous (1980). Gossypol as an oral contraceptive for men. J Med Soc N J *77*, 50.

Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C.J., McLauchlan, H., Klevernic, I., Arthur, J.S., Alessi, D.R., and Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J *408*, 297-315.

Baker, D.D., Chu, M., Oza, U., and Rajgarhia, V. (2007). The value of natural products to future pharmaceutical discovery. Nat Prod Rep *24*, 1225-1244.

Belmokhtar, C.A., Hillion, J., and Segal-Bendirdjian, E. (2001). Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. Oncogene *20*, 3354-3362.

Berenblum, I. (1941). The Cocarcinogenic Action of Croton Resin. Cancer Research *1*, 44-48.

Berkow, R.L., and Kraft, A.S. (1985). Bryostatin, a non-phorbol macrocyclic lactone, activates intact human polymorphonuclear leukocytes and binds to the phorbol ester receptor. Biochem Biophys Res Commun *131*, 1109-1116.

Bhisutthibhan, J., Pan, X.Q., Hossler, P.A., Walker, D.J., Yowell, C.A., Carlton, J., Dame, J.B., and Meshnick, S.R. (1998). The Plasmodium falciparum translationally controlled tumor protein homolog and its reaction with the antimalarial drug artemisinin. J Biol Chem *273*, 16192-16198.

Bialojan, C., and Takai, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J *256*, 283-290.

Bissery, M.C., Guenard, D., Gueritte-Voegelein, F., and Lavelle, F. (1991). Experimental antitumor activity of taxotere (RP 56976, NSC 628503), a taxol analogue. Cancer Res *51*, 4845-4852.

Boudreau, R.T., and Hoskin, D.W. (2005). The use of okadaic acid to elucidate the intracellular role(s) of protein phosphatase 2A: lessons from the mast cell model system. Int Immunopharmacol *5*, 1507-1518.

Brian, P.W. (1957). The effects of some microbial metabolic products on plant growth. Symp Soc Exp Biol *54*, 166-182.

Brown, M.S., and Goldstein, J.L. (1980). Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J Lipid Res *21*, 505-517.

Butler, M.S., and Newman, D.J. (2008). Mother Nature's gifts to diseases of man: the impact of natural products on anti-infective, anticholestemics and anticancer drug discovery. Prog Drug Res *65*, 1, 3-44.

Cardenas, M.E., Sanfridson, A., Cutler, N.S., and Heitman, J. (1998). Signal-transduction cascades as targets for therapeutic intervention by natural products. Trends Biotechnol *16*, 427-433.

Chang, J.Y., Sehgal, S.N., and Bansbach, C.C. (1991). FK506 and rapamycin: novel pharmacological probes of the immune response. Trends Pharmacol Sci *12*, 218-223.

Chiba, K., Hoshino, Y., Suzuki, C., Masubuchi, Y., Yanagawa, Y., Ohtsuki, M., Sasaki, S., and Fujita, T. (1996). FTY720, a novel immunosuppressant possessing unique mechanisms. I. Prolongation of

skin allograft survival and synergistic effect in combination with cyclosporine in rats. Transplant Proc *28*, 1056-1059.

Cole, G., Dobkins, K.R., Hansen, L.A., Terry, R.D., and Saitoh, T. (1988). Decreased levels of protein kinase C in Alzheimer brain. Brain Res *452*, 165-174.

Corson, T.W., and Crews, C.M. (2007). Molecular understanding and modern application of traditional medicines: triumphs and trials. Cell *130*, 769-774.

Crown, J., and O'Leary, M. (2000). The taxanes: an update. Lancet *355*, 1176-1178.

Cyster, J.G. (2005). Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol *23*, 127-159.

de Vries, D.J., Herald, C.L., Pettit, G.R., and Blumberg, P.M. (1988). Demonstration of sub-nanomolar affinity of bryostatin 1 for the phorbol ester receptor in rat brain. Biochem Pharmacol *37*, 4069-4073.

Downton, C., and Clark, I. (2003). Statins--the heart of the matter. Nat Rev Drug Discov *2*, 343-344.

Dumont, F.J., Staruch, M.J., Koprak, S.L., Melino, M.R., and Sigal, N.H. (1990). Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. J Immunol *144*, 251-258.

Dumont, F.J., and Su, Q. (1996). Mechanism of action of the immunosuppressant rapamycin. Life Sci *58*, 373-395.

Eckstein-Ludwig, U., Webb, R.J., Van Goethem, I.D., East, J.M., Lee, A.G., Kimura, M., O'Neill, P.M., Bray, P.G., Ward, S.A., and Krishna, S. (2003). Artemisinins target the SERCA of Plasmodium falciparum. Nature *424*, 957-961.

Endo, A., Kuroda, M., and Tanzawa, K. (1976). Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. FEBS Lett *72*, 323-326.

Eng, C.P., Sehgal, S.N., and Vezina, C. (1984). Activity of rapamycin (AY-22,989) against transplanted tumors. J Antibiot (Tokyo) *37*, 1231-1237.

Favit, A., Grimaldi, M., Nelson, T.J., and Alkon, D.L. (1998). Alzheimer's-specific effects of soluble beta-amyloid on protein kinase C-alpha and -gamma degradation in human fibroblasts. Proc Natl Acad Sci U S A *95*, 5562-5567.

Fingar, D.C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene *23*, 3151-3171.

Foroncewicz, B., Mucha, K., Paczek, L., Chmura, A., and Rowinski, W. (2005). Efficacy of rapamycin in patient with juvenile rheumatoid arthritis. Transpl Int *18*, 366-368.

Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Chiba, K., Hoshino, Y., and Okumoto, T. (1994). Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. J Antibiot (Tokyo) *47*, 208-215.

Furberg, C.D., and Pitt, B. (2001). Withdrawal of cerivastatin from the world market. Curr Control Trials Cardiovasc Med *2*, 205-207.

Gehringer, M.M. (2004). Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. FEBS Lett *557*, 1-8.

Goel, G., Makkar, H.P., Francis, G., and Becker, K. (2007). Phorbol esters: structure, biological activity, and toxicity in animals. Int J Toxicol *26*, 279-288.

Graff, J.R., McNulty, A.M., Hanna, K.R., Konicek, B.W., Lynch, R.L., Bailey, S.N., Banks, C., Capen, A., Goode, R., Lewis, J.E.*, et al.* (2005). The protein kinase Cbeta-selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. Cancer Res *65*, 7462-7469.

Greenwood, D. (1992). The quinine connection. J Antimicrob Chemother *30*, 417-427.

Gueritte-Voegelein, F., Guenard, D., Lavelle, F., Le Goff, M.T., Mangatal, L., and Potier, P. (1991). Relationships between the structure of taxol analogues and their antimitotic activity. J Med Chem *34*, 992-998.

Heitman, J., Movva, N.R., and Hall, M.N. (1992). Proline isomerases at the crossroads of protein folding, signal transduction, and immunosuppression. New Biol *4*, 448-460.

Hennings, H., Blumberg, P.M., Pettit, G.R., Herald, C.L., Shores, R., and Yuspa, S.H. (1987). Bryostatin 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin. Carcinogenesis *8*, 1343-1346.

Illingworth, D.R., and Sexton, G.J. (1984). Hypocholesterolemic effects of mevinolin in patients with heterozygous familial hypercholesterolemia. J Clin Invest *74*, 1972-1978.

Isakov, N., Galron, D., Mustelin, T., Pettit, G.R., and Altman, A. (1993). Inhibition of phorbol ester-induced T cell proliferation by bryostatin is associated with rapid degradation of protein kinase C. J Immunol *150*, 1195-1204.

Kahan, B.D., Podbielski, J., Napoli, K.L., Katz, S.M., Meier-Kriesche, H.U., and Van Buren, C.T. (1998). Immunosuppressive effects and safety of a sirolimus/cyclosporine combination regimen for renal transplantation. Transplantation *66*, 1040-1046.

Kannel, W.B. (1995). Range of serum cholesterol values in the population developing coronary artery disease. Am J Cardiol *76*, 69C-77C.

Kappos, L., Antel, J., Comi, G., Montalban, X., O'Connor, P., Polman, C.H., Haas, T., Korn, A.A., Karlsson, G., and Radue, E.W. (2006). Oral fingolimod (FTY720) for relapsing multiple sclerosis. N Engl J Med *355*, 1124-1140.

Keys, A., Menotti, A., Aravanis, C., Blackburn, H., Djordevic, B.S., Buzina, R., Dontas, A.S., Fidanza, F., Karvonen, M.J., Kimura, N.*, et al.* (1984). The seven countries study: 2,289 deaths in 15 years. Prev Med *13*, 141-154.

Kitada, S., Leone, M., Sareth, S., Zhai, D., Reed, J.C., and Pellecchia, M. (2003). Discovery, characterization, and structure-activity relationships studies of proapoptotic polyphenols targeting B-cell lymphocyte/leukemia-2 proteins. J Med Chem *46*, 4259-4264.

Klayman, D.L. (1985). Qinghaosu (artemisinin): an antimalarial drug from China. Science *228*, 1049-1055.

Koehn, F.E., and Carter, G.T. (2005). The evolving role of natural products in drug discovery. Nat Rev Drug Discov *4*, 206-220.

Kong, Y.C., Xie, J.X., and But, P.P. (1986). Fertility regulating agents from traditional Chinese medicines. J Ethnopharmacol *15*, 1-44.

Kortmansky, J., and Schwartz, G.K. (2003). Bryostatin-1: a novel PKC inhibitor in clinical development. Cancer Invest *21*, 924-936.

Kraft, A.S., Appling, C., and Berkow, R.L. (1987). Specific binding of phorbol esters to nuclei of human promyelocytic leukemia cells. Biochem Biophys Res Commun *144*, 393-401.

Kruman, I., Guo, Q., and Mattson, M.P. (1998). Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. J Neurosci Res *51*, 293-308.

Ku, G.Y., Ilson, D.H., Schwartz, L.H., Capanu, M., O'Reilly, E., Shah, M.A., Kelsen, D.P., and Schwartz, G.K. (2008). Phase II trial of sequential paclitaxel and 1 h infusion of bryostatin-1 in patients with advanced esophageal cancer. Cancer Chemother Pharmacol *62*, 875-880.

Kuroda, M., Tsujita, Y., Tanzawa, K., and Endo, A. (1979). Hypolipidemic effects in monkeys of ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Lipids *14*, 585-589.

Lamers, M.B., Antson, A.A., Hubbard, R.E., Scott, R.K., and Williams, D.H. (1999). Structure of the protein tyrosine kinase domain of C-terminal Src kinase (CSK) in complex with staurosporine. J Mol Biol *285*, 713-725.

Lee, H.W., Smith, L., Pettit, G.R., and Bingham Smith, J. (1996a). Dephosphorylation of activated protein kinase C contributes to downregulation by bryostatin. Am J Physiol *271*, C304-311.

Lee, H.W., Smith, L., Pettit, G.R., Vinitsky, A., and Smith, J.B. (1996b). Ubiquitination of protein kinase C-alpha and degradation by the proteasome. J Biol Chem *271*, 20973-20976.

Lee, W., Boo, J.H., Jung, M.W., Park, S.D., Kim, Y.H., Kim, S.U., and Mook-Jung, I. (2004). Amyloid beta peptide directly inhibits PKC activation. Mol Cell Neurosci *26*, 222-231.

Leist, M., and Jaattela, M. (2001). Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol *2*, 589-598.

Levine, A.J., Hu, W., Feng, Z., and Gil, G. (2007). Reconstructing signal transduction pathways: challenges and opportunities. Ann N Y Acad Sci *1115*, 32-50.

Link, M. (2004). Statin switch to OTC. Nat Rev Drug Discov *3*, 641-642.

Mabuchi, H., Haba, T., Tatami, R., Miyamoto, S., Sakai, Y., Wakasugi, T., Watanabe, A., Koizumi, J., and Takeda, R. (1981). Effect of an inhibitor of 3-hydroxy-3-methyglutaryl coenzyme A reductase on serum lipoproteins and ubiquinone-10-levels in patients with familial hypercholesterolemia. N Engl J Med *305*, 478-482.

Mabuchi, H., Sakai, T., Sakai, Y., Yoshimura, A., Watanabe, A., Wakasugi, T., Koizumi, J., and Takeda, R. (1983). Reduction of serum cholesterol in heterozygous patients with familial hypercholesterolemia. Additive effects of compactin and cholestyramine. N Engl J Med *308*, 609-613.

MacMillan, J., Vanstone, A.E., and Yeboah, S.K. (1968). The structure of wortmannin, a steroidal fungal metabolite. Chemical Communications (London), 613-614.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C.*, et al.* (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science *296*, 346-349.

Marzo, I., and Naval, J. (2008). Bcl-2 family members as molecular targets in cancer therapy. Biochem Pharmacol *76*, 939-946.

Mego, M. (2002). Telomerase inhibitors in anticancer therapy: gossypol as a potential telomerase inhibitor. Bratisl Lek Listy *103*, 378-381.

Mohammad, R.M., Wang, S., Aboukameel, A., Chen, B., Wu, X., Chen, J., and Al-Katib, A. (2005). Preclinical studies of a nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-X(L) [(-)-gossypol] against diffuse large cell lymphoma. Mol Cancer Ther *4*, 13-21.

Moon, D.O., Kim, M.O., Choi, Y.H., Lee, H.G., Kim, N.D., and Kim, G.Y. (2008). Gossypol suppresses telomerase activity in human leukemia cells via regulating hTERT. FEBS Lett *582*, 3367-3373.

Morice, M.C., Serruys, P.W., Sousa, J.E., Fajadet, J., Ban Hayashi, E., Perin, M., Colombo, A., Schuler, G., Barragan, P., Guagliumi, G*., et al.* (2002). A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. N Engl J Med *346*, 1773-1780.

Newman, D.J., Cragg, G.M., and Snader, K.M. (2000). The influence of natural products upon drug discovery. Nat Prod Rep *17*, 215-234.

Newman, D.J., Cragg, G.M., and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. J Nat Prod *66*, 1022-1037.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature *308*, 693-698.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature *334*, 661-665.

Oberlies, N.H., and Kroll, D.J. (2004). Camptothecin and taxol: historic achievements in natural products research. J Nat Prod *67*, 129-135.

Olliaro, P.L., Haynes, R.K., Meunier, B., and Yuthavong, Y. (2001). Possible modes of action of the artemisinin-type compounds. Trends Parasitol *17*, 122-126.

Omura, S., Iwai, Y., Hirano, A., Nakagawa, A., Awaya, J., Tsuchya, H., Takahashi, Y., and Masuma, R. (1977). A new alkaloid AM-2282 OF Streptomyces origin. Taxonomy, fermentation, isolation and preliminary characterization. J Antibiot (Tokyo) *30*, 275-282.

Onaga, L. (2001). Cashing in on nature's pharmacy: Bioprospecting and protection of biodiversity could go hand in hand. EMBO Rep *2*, 263-265.

Paterson, I., and Anderson, E.A. (2005). Chemistry. The renaissance of natural products as drug candidates. Science *310*, 451-453.

Pettit, G.R., Day, J.F., Hartwell, J.L., and Wood, H.B. (1970). Antineoplastic components of marine animals. Nature *227*, 962-963.

Pettit, G.R., Herald, C.L., Doubek, D.L., Herald, D.L., Arnold, E., and Clardy, J. (1982). Isolation and structure of bryostatin 1. Journal of the American Chemical Society *104*, 6846-6848.

Porat, O. (1990). Effects of gossypol on the motility of mammalian sperm. Mol Reprod Dev *25*, 400-408.

Potterat, O., and Hamburger, M. (2008). Drug discovery and development with plant-derived compounds. Prog Drug Res *65*, 45, 47-118.

Reihner, E., Rudling, M., Stahlberg, D., Berglund, L., Ewerth, S., Bjorkhem, I., Einarsson, K., and Angelin, B. (1990). Influence of pravastatin, a specific inhibitor of HMG-CoA reductase, on hepatic metabolism of cholesterol. N Engl J Med *323*, 224-228.

Rishton, G.M. (2008). Natural products as a robust source of new drugs and drug leads: past successes and present day issues. Am J Cardiol *101*, 43D-49D.

Roberts, W.C. (2004). Over-the-counter statin drug. Am J Cardiol *94*, 1362.

Rosen, H., and Goetzl, E.J. (2005). Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat Rev Immunol *5*, 560-570.

Rowinsky, E.K. (2004). Targeting the molecular target of rapamycin (mTOR). Curr Opin Oncol *16*, 564-575.

Russell, D.W. (1992). Cholesterol biosynthesis and metabolism. Cardiovasc Drugs Ther *6*, 103-110.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and Abraham, R.T. (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J Biol Chem *270*, 815-822.

Salvadori, M., Budde, K., Charpentier, B., Klempnauer, J., Nashan, B., Pallardo, L.M., Eris, J., Schena, F.P., Eisenberger, U., Rostaing, L.*, et al.* (2006). FTY720 versus MMF with cyclosporine in de novo renal transplantation: a 1-year, randomized controlled trial in Europe and Australasia. Am J Transplant *6*, 2912-2921.

Schiff, P.B., Fant, J., and Horwitz, S.B. (1979). Promotion of microtubule assembly in vitro by taxol. Nature *277*, 665-667.

Schiff, P.B., and Horwitz, S.B. (1981). Taxol assembles tubulin in the absence of exogenous guanosine 5'-triphosphate or microtubule-associated proteins. Biochemistry *20*, 3247-3252.

Schmidt, B., Ribnicky, D.M., Poulev, A., Logendra, S., Cefalu, W.T., and Raskin, I. (2008). A natural history of botanical therapeutics. Metabolism *57*, S3-9.

Silinsky, E.M., and Searl, T.J. (2003). Phorbol esters and neurotransmitter release: more than just protein kinase C? Br J Pharmacol *138*, 1191-1201.

Sneader, W. (2000). The discovery of aspirin: a reappraisal. Bmj *321*, 1591-1594.

Snyder, J.P., Nettles, J.H., Cornett, B., Downing, K.H., and Nogales, E. (2001). The binding conformation of Taxol in beta-tubulin: a model based on electron crystallographic density. Proc Natl Acad Sci U S A *98*, 5312-5316.

Stewart, Z.A., Mays, D., and Pietenpol, J.A. (1999). Defective G1-S cell cycle checkpoint function sensitizes cells to microtubule inhibitor-induced apoptosis. Cancer Res *59*, 3831-3837.

Sun, M.K., and Alkon, D.L. (2005). Dual effects of bryostatin-1 on spatial memory and depression. Eur J Pharmacol *512*, 43-51.

Sun, M.K., and Alkon, D.L. (2006). Bryostatin-1: pharmacology and therapeutic potential as a CNS drug. CNS Drug Rev *12*, 1-8.

Suzuki, S., Enosawa, S., Kakefuda, T., Amemiya, H., Hoshino, Y., and Chiba, K. (1996). Long-term graft acceptance in allografted rats and dogs by treatment with a novel immunosuppressant, FTY720. Transplant Proc *28*, 1375-1376.

Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y., and Schmitz, F.J. (1981). Okadaic acid, a cytotoxic polyether from two marine sponges of the genus Halichondria. Journal of the American Chemical Society *103*, 2469-2471.

Thompson, G.R., Ford, J., Jenkinson, M., and Trayner, I. (1986). Efficacy of mevinolin as adjuvant therapy for refractory familial hypercholesterolaemia. Q J Med *60*, 803-811.

Tobert, J.A. (2003). Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. Nat Rev Drug Discov *2*, 517-526.

Tobert, J.A., Hitzenberger, G., Kukovetz, W.R., Holmes, I.B., and Jones, K.H. (1982). Rapid and substantial lowering of human serum cholesterol by mevinolin (MK-803), an inhibitor of hydroxymethylglutaryl-coenzyme A reductase. Atherosclerosis *41*, 61-65.

Tsang, C.K., Qi, H., Liu, L.F., and Zheng, X.F. (2007). Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Discov Today *12*, 112-124.

Tsujita, Y., Kuroda, M., Tanzawa, K., Kitano, N., and Endo, A. (1979). Hypolipidemic effects in dogs of ML-236B, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Atherosclerosis *32*, 307-313.

Van Poznak, C., Seidman, A.D., Reidenberg, M.M., Moasser, M.M., Sklarin, N., Van Zee, K., Borgen, P., Gollub, M., Bacotti, D., Yao, T.J*., et al.* (2001). Oral gossypol in the treatment of patients with refractory metastatic breast cancer: a phase I/II clinical trial. Breast Cancer Res Treat *66*, 239-248.

Vezina, C., Kudelski, A., and Sehgal, S.N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot (Tokyo) *28*, 721-726.

von Nussbaum, F., Brands, M., Hinzen, B., Weigand, S., and Häbich, D. (2006). Antibacterial Natural Products in Medicinal Chemistry - Exodus or Revival? Angewandte Chemie International Edition *45*, 5072-5129.

Wang, Y. (2008). Needs for new plant-derived pharmaceuticals in the post-genome era: an industrial view in drug research and development. Phytochemistry Reviews *7*, 395-406.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and McPhail, A.T. (1971). Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc *93*, 2325-2327.

Watanabe, Y., Ito, T., Saeki, M., Kuroda, M., Tanzawa, K., Mochizuki, M., Tsujita, Y., and Arai, M. (1981). Hypolipidemic effects of CS-500 (ML-236B) in WHHL-rabbit, a heritable animal model for hyperlipidemia. Atherosclerosis *38*, 27-31.

Wender, P.A., Baryza, J.L., Brenner, S.E., Clarke, M.O., Craske, M.L., Horan, J.C., and Meyer, T. (2004). Function oriented synthesis: the design, synthesis, PKC binding and translocation activity of a new bryostatin analog. Curr Drug Discov Technol *1*, 1-11.

White, N.J. (2008). Qinghaosu (artemisinin): the price of success. Science *320*, 330-334.

Wiesner, J., Ortmann, R., Jomaa, H., and Schlitzer, M. (2003). New antimalarial drugs. Angew Chem Int Ed Engl *42*, 5274-5293.

Wipf, P., and Halter, R.J. (2005). Chemistry and biology of wortmannin. Org Biomol Chem *3*, 2053-2061.

Withers, W.A., and Carruth, F.E. (1915). Gossypol--a Toxic Substance in Cottonseed. a Preliminary Note. Science *41*, 324.

Wolter, K.G., Wang, S.J., Henson, B.S., Wang, S., Griffith, K.A., Kumar, B., Chen, J., Carey, T.E., Bradford, C.R., and D'Silva, N.J. (2006). (-)-gossypol inhibits growth and promotes apoptosis of human head and neck squamous cell carcinoma in vivo. Neoplasia *8*, 163-172.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Wymann, M.P., Bulgarelli-Leva, G., Zvelebil, M.J., Pirola, L., Vanhaesebroeck, B., Waterfield, M.D., and Panayotou, G. (1996). Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. Mol Cell Biol *16*, 1722-1733.

Zemann, B., Kinzel, B., Muller, M., Reuschel, R., Mechtcheriakova, D., Urtz, N., Bornancin, F., Baumruker, T., and Billich, A. (2006). Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. Blood *107*, 1454-1458.

Zhang, X., Zhang, R., Zhao, H., Cai, H., Gush, K.A., Kerr, R.G., Pettit, G.R., and Kraft, A.S. (1996). Preclinical pharmacology of the natural product anticancer agent bryostatin 1, an activator of protein kinase C. Cancer Res *56*, 802-808.

Zheng, D.K., Si, Y.K., Meng, J.K., Zhou, J., and Huang, L. (1985). Resolution of racemic gossypol. J Chem Soc Chem Commun, 168-169.

2 Signal transduction pathways

˝Flowing with Grace˝
-Teachings of Anusara Yoga

2.1 Apoptosis

The term apoptosis was introduced in a paper by Kerr, Wyllie, and Currie in 1972 (Kerr, 1972) to describe a form of programmed cell death with distinct morphological features such as cell shrinkage, membrane ruffling (also named membrane blebbing), chromatin condensation, and DNA fragmentation (Rich, 1999). In multicellular organisms the process of apoptosis is fundamental for development and maintenance of homeostasis, when excessive, infected or aged cells need to be eliminated (Movassagh and Foo, 2008). In a healthy human adult around 10 million cells per day undergo apoptosis (Curtin and Cotter, 2003). Apoptosis is a active cellular process, in contrast to necrosis, in which cell death is uncontrolled and leads to loss of membrane integrity, swelling and disruption of the cells with subsequent inflammatory response in the surrounding.

There are two major apoptosis inducing pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway is initiated by extrinsic signals leading to activation of death receptors, such as Fas (also named CD95 or Apo-1), tumour necrosis factor receptor (TNFR), and TRAIL-receptor (TNF-related apoptosis ligand). The intracellular death domain recruits and activates caspase-8, which leads to subsequent activation of downstream caspases (Lavrik, 2005). Whereas the intrinsic pathway involves non-receptor-mediated intrinsic signals induced by DNA damage, growth factor deprivation, or oxidative stress that lead to activation of the Bcl-2 family proteins and results in release of cytochrome c from the mitochondria and consequential activation of caspases (Figure 12). The pathways of apoptosis converge on the activation of caspases and influence each other (Igney and Krammer, 2002).

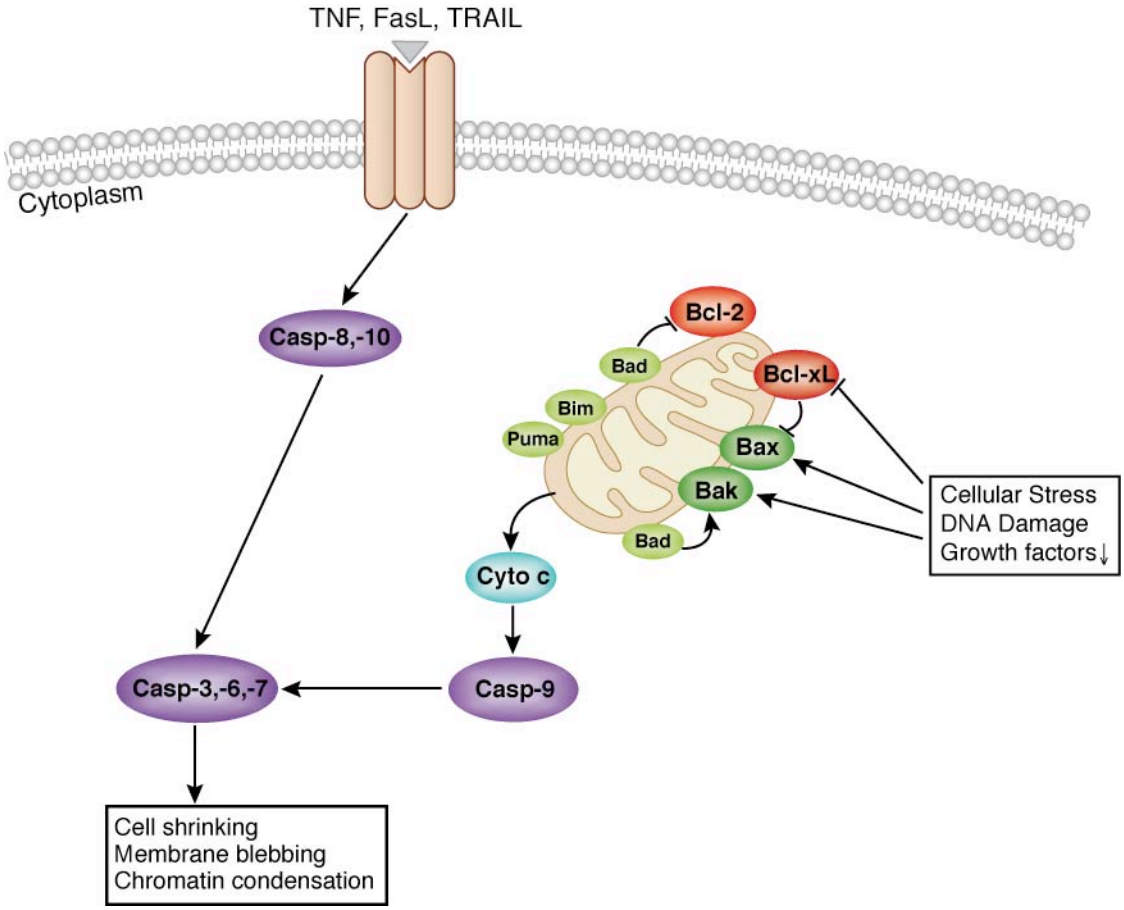

Figure 12: Simplified diagram of apoptosis pathways (built with templates from www.cellsignaling.com).

2.1.1 Bcl-2 family

The first protein identified to be involved in apoptosis was Bcl-2, initially characterised as proto-oncogene in human lymphoma cells (hence the encoding gene was named B-cell lymphoma-2*, bcl-2*) (Tsujimoto and Croce, 1986; Vaux, 1988). To date about 20 Bcl-2 family members are identified and can be divided into two functional groups with either anti- or pro-apoptotic activity. The anti-apoptotic proteins Bcl-2, Bcl-xL, Bcl-w, and others contain at least four highly conserved bcl-2 homology (BH) domains. Bcl-2 is exclusively found associated with intracellular membranes, including the outer mitochondrial membrane, the endoplasmatic reticulum and the nuclear envelope (Krajewski, 1993). Whereas Bcl-xL is additionally found soluble in the cytosol, and translocation from the cytosol to the mitochondrial outer membrane is induced during apoptosis (Hsu, 1997). The pro-apoptotic subfamily can be further divided into two groups, the multidomain proteins (or BH123), including Bax, Bak,

and others, and the BH3-only proteins, that contain only one BH3 motif such as Bid, Bim, Bad, PUMA, Noxa, and some more (Figure 13) (Antonsson, 2004).

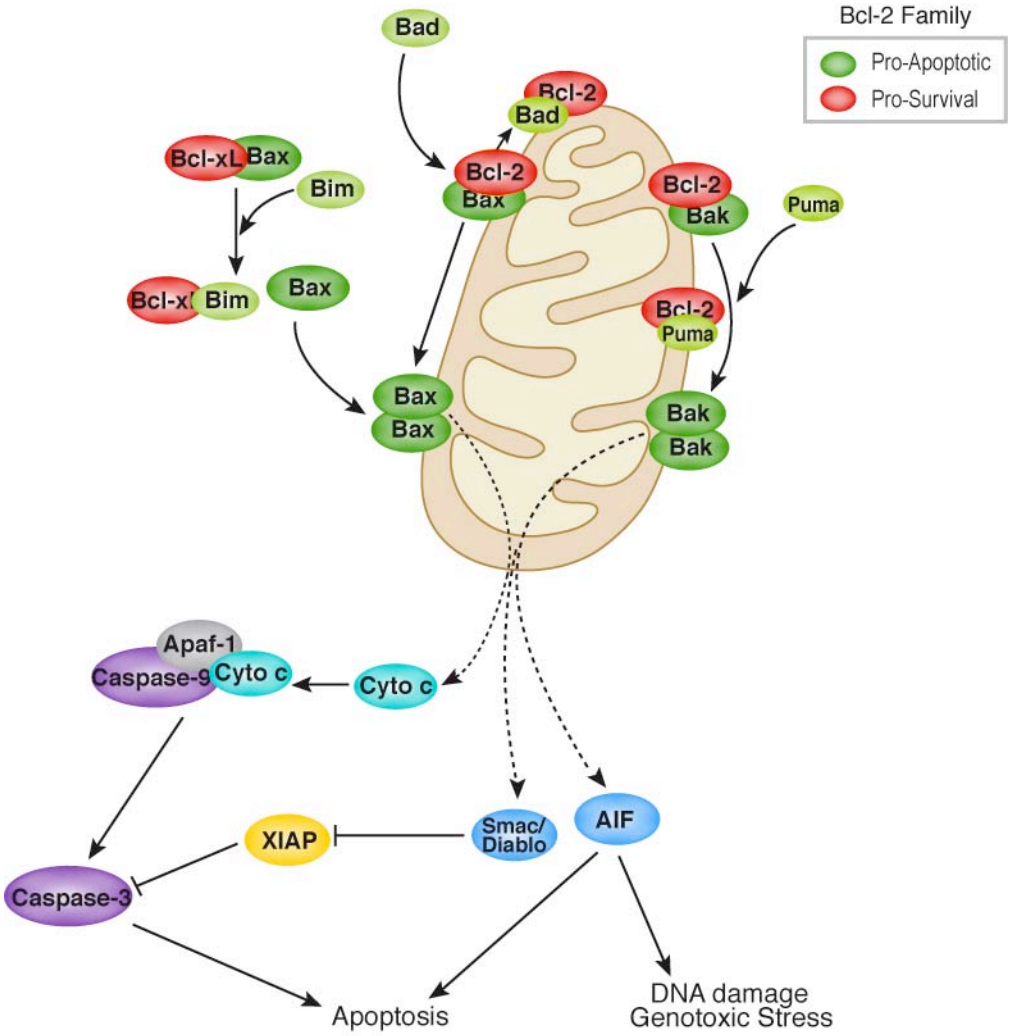

Figure 13: Mitochondrial pathway of apoptosis (modified from a model on www.cellsignaling.com).

The BH3-only proteins are pro-apoptotic and function as initial sensors of apoptotic signals, activating the pro-apoptotic family members. The anti-apoptotic family members, such as Bcl-2 and Bcl-xL bind to and thereby inhibit the pro-apoptotic family members Bax and Bak. Activation of the BH3-only proteins results in liberation of Bax and Bak and thus initiation of apoptosis. The precise mechanism for the activation of Bax and Bak remains unclear and 'constitutes the holy grail of apoptosis research' (Youle and Strasser, 2008). Liberation of Bax and Bak from binding to their anti-apoptotic inhibitors Bcl-2 and Bcl-xL leads to several conformational changes and results in homo-oligomerisation (Chipuk, 2006; Newmeyer and Ferguson-Miller, 2003). These oligomers are believed to build pores in the outer mitochondrial membrane and induce the mitochondrial outer membrane permeabilisation (MOMP),

which leads to the release of cytochrome c and other proteins residing in the mitochondrial intermembranous space, such as apoptosis inducing factor (AIF) and Smac/DIABLO, an inhibitor of IAPs (inhibitors of apoptosis) (Green and Kroemer, 2004). Progression of apoptosis through activation of caspases, mitochondrial decomposition and initiation of DNA fragmentation result from these released molecules (Figure 13).

2.1.2 Cytochrome c

Cytochrome c is a haem-containing protein that participates in the mitochondrial electron-transport chain, using its heam group to shuttle electrons. However, upon activation of the intrinsic pathway and MOMP, cytochrome c is released to the cell plasma where it binds to apoptotic protease-activating factor-1 (Apaf1) that oligomerises and forms a complex, called apoptosome, which recruits and activates procaspase-9 (Li, 1997). Active caspase-9 in turn activates downstream effectors, such as caspase-3 and -7 that lead to execution of apoptosis (Figure 13).

2.1.3 Caspases

Caspases are cysteinyl-aspartic acid proteases that play a key role in apoptotic cell death and are normally present in healthy cells as inactive precursor zymogens. To date 11 caspases have been identified in humans (Degterev, 2003). Three groups of caspases can be formed: the cell death initiators, caspase -8, -9, -2, and -10, the cell death executors, caspases-3, -6, and -7, and the inflammatory caspases, not involved in apoptosis (caspase-1, -4, -5, -11, -12, -13, -14) (Rupinder, 2007).

The extrinsic, death receptor induced apoptosis pathway activates primarily caspase-8. Ligation of death receptors (Fas, TNFR, TRAILR) induces conformational changes and recruits adaptor proteins such as Fas-associated death domain (FADD) to the receptor. There, together with procaspase-8, a complex is formed, called death inducing signalling complex (DISC) that leads to activation of caspase-8 (Figure 14). Caspase-8 in turn activates the executive caspase-3 that directly triggers chromatin condensation, cleavage of diverse cellular proteins and DNA fragmentation (Porter and Janicke, 1999). Furthermore caspase-8 can activate the BH3-only protein Bid, which leads to its mitochondrial translocation and to activation of the intrinsic pathway (Luo, 1998).

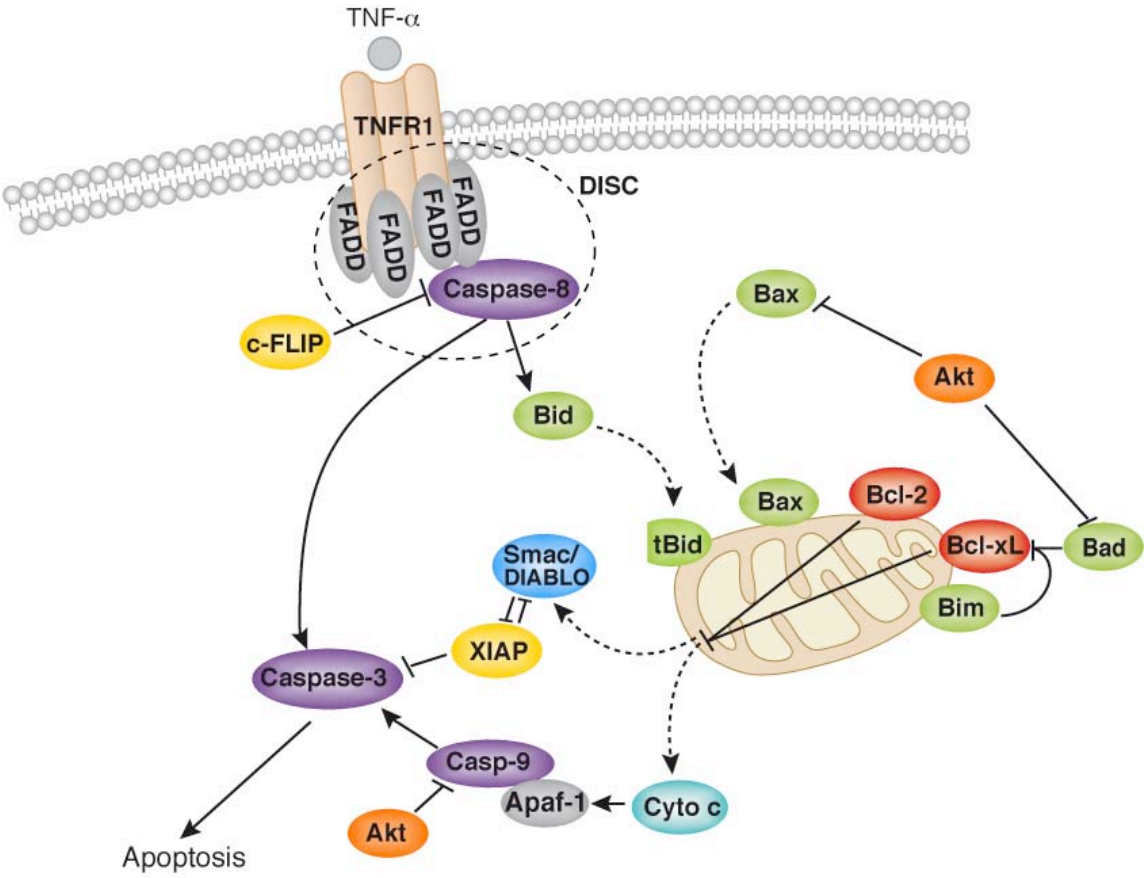

Figure 14: Extrinsic pathway and possible points of inhibition (Figure constructed with parts from www.cellsignaling.com).

2.1.4 IAPs

Although the major way to regulate caspase activity is by proteolytic cleavage, there are other regulatory pathways. Most importantly the inhibitor-of-apoptosis proteins (IAPs) that are able to sequester and inactivate pro-caspases and caspases (LaCasse, 1998). To date eight human IAPs have been identified, including XIAP, cIAP1, cIAP2, and Survivin. Activity of IAPs, however, is also finely regulated during apoptosis. The 'second mitochondrial-derived activator of caspases' (smac, also called DIABLO, 'direct IAP binding protein with low pI') is released along with cytochrome c from the mitochondria upon apoptotic stimuli and binds to IAPs in a manner that liberates caspases from IAP (Figure 14) (Adrain, 2001; Salvesen and Duckett, 2002).

2.1.5 c-FLIP

Cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIP) is a protein associated with the DISC, downstream of Fas, TRAIL or TNF receptors (Hyer, 2006; Jin, 2005) and is

a main regulator of caspase-8 activity (Figure 14). c-FLIP exhibits a dual function of inhibition or activation of caspase-8 activation downstream of death receptors. In low, physiological concentrations (1% of caspase-8 content) there is no explicit inhibition of caspase-8 activation apparent. In higher concentrations, however, c-FLIP inhibits homodimer formation of caspase-8 and hence prevents activation (Lamkanfi, 2007). The induced signal leads then to activation of the NFκB pathway and therefore to pro-survival pathways. Several retroviruses (best characterised in Herpes virus -8) express FLIP (v-FLIP), which detracts the cell from apoptosis and promotes survival of virus-infected cells (Thome, 1997).

2.1.6 AIF

In addition to proteins that directly activate, mitochondria release proteins unrelated to caspase signalling, such as the apoptosis-inducing factor AIF. AIF is a mitochondrial flavoprotein that is localised in the mitochondrial intermembranous space. In healthy cells, AIF is required for efficient oxidative phosphorylation. Upon apoptotic insult it is released to the cytosol (Figure 13) and eventually imported into the nucleus, where it binds to DNA and induces caspase-independent cell death resulting in chromatin condensation and DNA degradation (Galluzzi, 2008). In fact, AIF is believed to play a major role in caspase-independent cell death (Joza, 2001). Mitochondrial AIF, however, has been shown to contribute to cell survival by protecting cells against harmful oxidative damage but in response to severe stress it predisposes them to more efficient death (Porter and Urbano, 2006). AIF expression levels in cells were shown to be regulated by the tumour suppressor p53 (Stambolsky, 2006).

2.1.7 p53

The tumour suppressor p53 is one of the most mutated genes in human cancers (Vousden and Lu, 2002). Extrinsic or intrinsic stress, such as irradiation, reactive oxidative species (ROS), and DNA damage activates p53. Depending on cell type, cell environment and the kind of stress, activation of p53 leads to cell cycle arrest, DNA repair, senescence, differentiation or apoptosis (Vousden and Lu, 2002). In most cases, however, induction of p53 leads to an irreversible inhibition of cell growth and apoptosis. Both, loss or gain of p53 function result in aberrant cell growth, hence, the cellular expression and the activity of p53 are tightly regulated. The p53 protein

has a very short half-life and is only present at extremely low levels within the cell (Pietsch, 2008). Control of the function of p53 is possible at different levels and the most effective is the control of its plasma levels by Mdm2. Mdm2 is a ubiquitin ligase that binds to p53 and targets its proteosomal degradation, Mdm2 itself is a transcriptional target of p53, creating a autoregulatory negative feedback loop (Harris, 2005). The activation and stabilisation of p53 is generally associated with inhibition of the function of Mdm2 (Figure 15). Different stress signals allow p53 to escape Mdm2-mediated protein degradation and to become active. Activation of p53 induces transcription of a myriad of proteins, such as the Bcl-2 proteins Bax, Puma, and Noxa, the death receptor Fas, caspases-1 and -6, PTEN and many more (Riley, 2008).

Apart from its functions as a transcription factor, p53 directly activates the apoptotic machinery by translocation to the mitochondria (Caelles, 1994). There, p53 seems to function analogous to BH3-only members, resulting in oligomerisation of Bak and Bax and leading to cytochrome c release from the mitochondria (Leu, 2004). This role of p53, however, has not been studies as extensively as its role as transcription factor and its relevance it still discussed controversially (Pietsch, 2008).

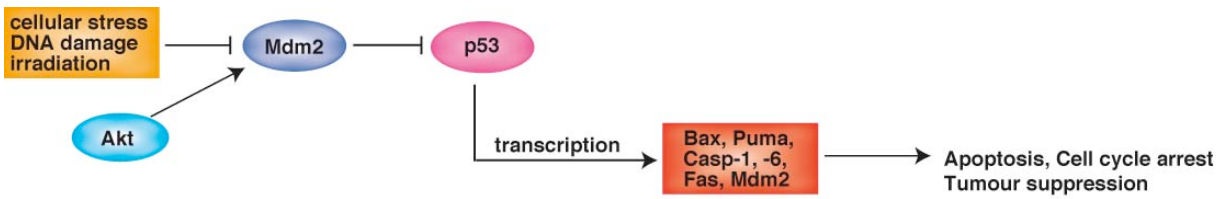

Figure 15: Regulation of p53

2.1.8 The NFκB pathway

Tumour necrosis factor (TNF) is a multifunctional pro-inflammatory cytokine mainly produced by macrophages (Wajant, 2003). Besides its function as a death receptor, TNFR also activates NFκB and c-Jun N-terminal kinase (JNK) pathways. Unlike the other death receptors, Fas and TRAILR, activation of TNFR does not spontaneously induce cell death as, the simultaneous NFκB-activation promotes a strong pro-survival signalling pathway and targets several anti-apoptotic proteins such as Bcl-xL, IAP, XIAP, and FLIP (Wajant, 2003). Apoptosis is only induced when NFκB activation is blocked (Wang, 1996).

In absence of an activating signal NFκB is sequestered in an inactive form in the cytoplasma by its inhibitor IκB (inhibitor of NFκB). In response to extracellular stimuli the IKK-complex (IκB-kinase-complex) is activated and phosphorylates IκB (Figure 16). Phosphorylated IκB can no longer inhibit NFκB and gets degraded. Once freed from IκB, NFκB translocates into the nucleus, where it induces transcription of a range of genes that confer resistance to death-inducing signals (Li and Lin, 2008).

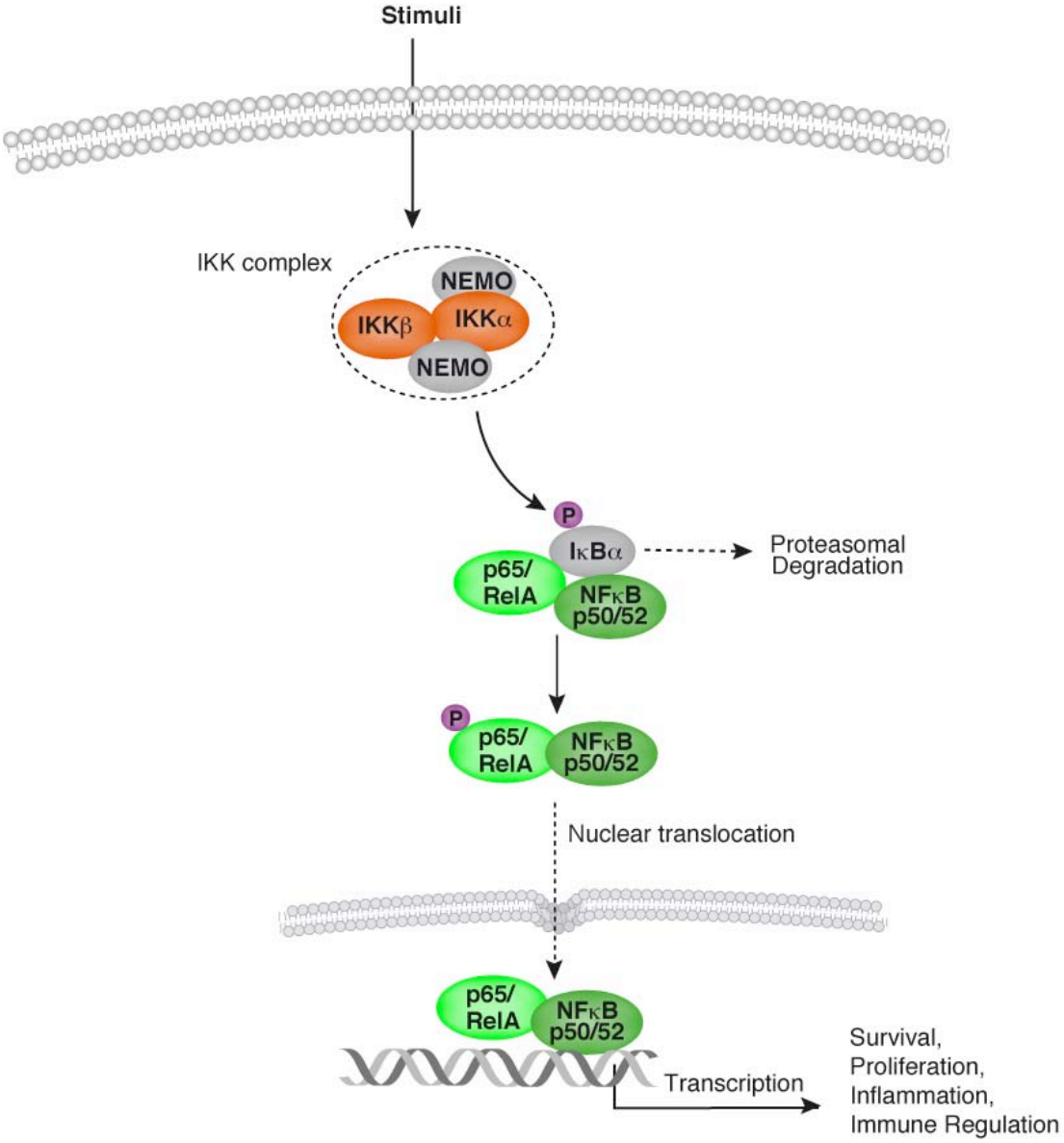

Figure 16: Regulation of NFκB. Cytosolic NFκB is inhibited by IκB, which is degraded upon activation and NFκB can translocate to the nucleus to induce transcription of various proteins.

2.1.9 Other cellular stress responses involved in apoptosis

JNK signalling has been implicated in cellular stress-induces apoptosis by the fact that it is able to influence the activity of p53. Depending on the cellular context, JNK

either destabilises p53 by promoting its degradation or stabilises p53 by phosphorylation (Fuchs, 1998a; Fuchs, 1998b).

Sphingosine and ceramide belong to a group of lipids (sphingolipids) that are abundant in membranes and are gaining recognition as important signalling mediators. In healthy cells, sphingomyelin is found predominantly in the outer leaflet of the plasma membrane. Under stress conditions, however, sphingomyelin turnover is induced and leads to increased plasma levels of sphingosine and ceramide. Sphingosine can then be phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase (SphK). The discovery that S1P regulates cell growth and suppresses apoptosis triggered the interest in this bioactive lipid mediator. Five GPCR coupled receptors have been found to mediate the effects of S1P and stimulating various pathways; JNK, ERK (extracellular signal-regulated kinase), Rac, Rho, and PI3-kinase/Akt (Takabe, 2008). The first S1P analogue, fingolimod, is in clinical development and has been described earlier in this introduction.

2.1.10 Disease as a consequence of dysregulated apoptosis

Development of cancer is a multistep process that involves genetic alterations resulting in unlimited growth of cells. Prominent mechanisms of acquiring resistance to apoptosis include overactivation of anti-apoptotic proteins or mutation of pro-apoptotic proteins. Examples of dysregulation of anti-apoptotic proteins include Bcl-2 and Bcl-xL, IAPs, members of the PI3-kinase-Akt pathway and NFκB pathway. Inactivation of pro-apoptotic signals, on the other hand, is often caused by mutational changes of p53, Bax, and Apaf-1 and loss of expression of death receptors. The resistance of cancer cells to apoptosis hinders the success of cancer therapy and dependant of the kind of mutation therapy needs to be adjusted. Personalised cancer therapy seems the obvious strategy to pursue, as the type of mutation is fundamental for successful therapy.

Cancer treatments that specifically target the altered apoptosis pathways are currently under development. Either by inhibition of the anti-apoptotic members or activation of pro-apoptotic pathways, cell death is stimulated in cancer cells. For example the earlier mentioned molecule gossypol, a BH3-mimicking inhibitor of Bcl-2 and Bcl-xL. Inhibition by antisense nucleotide is another strategy, oblimersen, a Bcl-2 antisense oligonucleotide is currently in phase III studies and could reach the market soon. Several agonist antibodies that target death receptors and induce apoptosis

are in clinical studies phase I or II, for example mapatumumab and apomab. The proteasome inhibitor bortezomib (Velcade) blocks proteosomal degradation of proteins and activates caspase-8 and -9 in vivo, due to inhibition of the NFκB pathway. Velcade has been approved by the FDA in May 2003 for treatment of myeloma and has become the first anti-cancer drug on the market to target the proteasome and thereby, also the first to target NFκB activation (Folmer, 2008).

In neurodegenerative disease the apoptotic pathways are altered in the other direction and too much apoptosis takes place (Jin, 2005). Neurons naturally possess very strong anti-apoptotic factors that help them to survive for the lifetime of the organism. In neurodegenerative diseases such as AD and Parkinson's disease p53 activation plays a critical role in cell death. The p53 inhibitor pifithrin was shown to inhibit in vitro amyloid peptide induced neuronal death and protect from neuronal loss in a Parkinson mouse model (Duan, 2002).

2.2 PI3K/PIPs/Akt signalling pathway

The serine/threonine kinase Akt, also known as protein kinase B (PKB), plays a central regulatory role in diverse cellular processes such as homeostasis, survival, cell cycle progression, and metabolism. Aberrant Akt signalling is observed in cancer development, insulin resistance and many other pathological conditions. As a general mechanism, activation of Akt is induced via surface receptor stimulation, where activated PI3-kinases catalyse the phosphorylation of membrane-bound phosphoinositides, thus creating docking sites for signalling proteins like Akt (Figure 17). These signalling proteins coordinate complex events that lead to changes in cell metabolism, cell growth, cell movement and cell survival (Cantley, 2002).

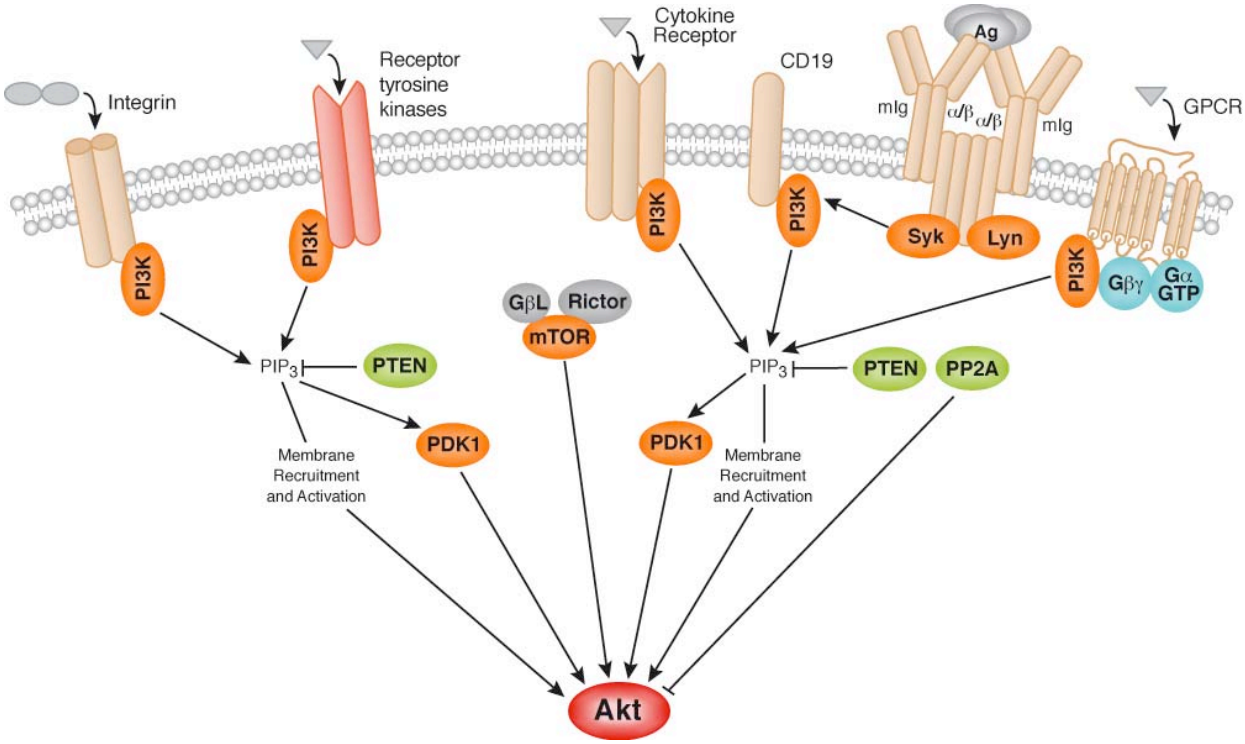

Figure 17: Diagram of the activation of Akt.(modified from www.cellsignaling.com)

2.2.1 Akt pathway

The Akt pathway is activated by receptor tyrosine kinases, B- and T-cell receptors, cytokine receptors, or G-protein-coupled receptors (GPCRs) that induce production of phosphatidylinositol-3,4,5-trisphosphates ($PtdInsP_3$) by PI3-kinases (phosphoinositide 3-kinases). These lipids serve as membrane docking sites for proteins that harbour pleckstrin-homology (PH) domains, including Akt and its upstream activator PDK1, thereby recruiting them to the plasma membrane. PDK1 phosphorylates Akt at Thr308, whereas the additional phosphorylation at Ser473 is

accomplished by the mTOR complex 2 (TORC2) that further increases Akt activity (Huang and Manning, 2009). Once active, Akt regulates a number of downstream targets (Figure 18). Three mammalian isoforms of Akt have been identified, Akt1, Akt2, and Akt3. They share a high degree of structural similarity and more than 80% sequence homology. Each isoform plays an unique as well as a common role in cells (Parcellier, 2008).

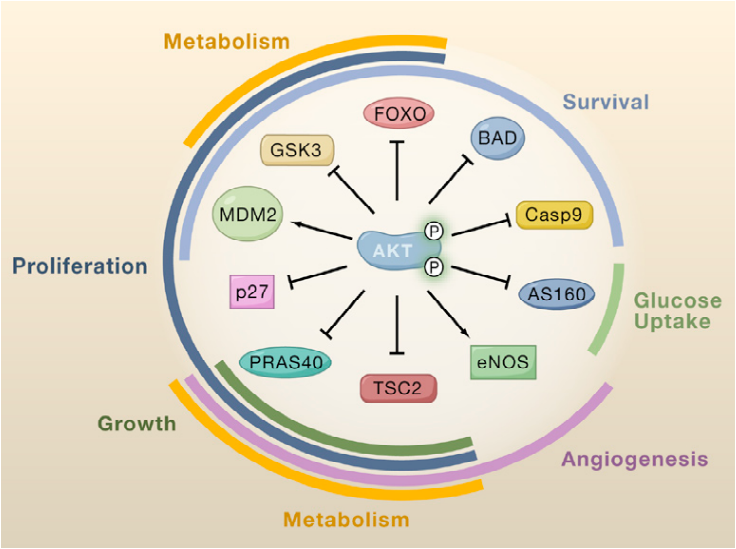

Figure 18: Effects of Akt activation on different cellular functions (Huang and Manning, 2009).

Akt enhances the survival of cells by blocking the function of proapoptotic proteins such as inactivation of Bad, a BH3-only protein, by phosphorylation. This prevents binding of Bad to its target proteins Bcl-2 and Bcl-xL and hence promotes cell survival. Akt has also been reported to directly phosphorylate capsase-9 and thereby blocking its activation (Cardone, 1998). Furthermore Akt inhibits the transcription factor FoxO1 (forkhead box O1) that induces transcription of Bim, another BH3-only protein stimulating cell death. A further target of Akt that promotes cell survival is Mdm2, the negative regulator of p53, leading to translocation of Mdm2 into the nucleus where it inhibits p53 transcriptional activity (Figure 15).

The impact of Akt on cell growth is mainly due to activation of the mTOR complex 1 (TORC1), which is regulated by both nutrient and growth factor signalling. TORC1 regulates protein synthesis by initiation of translation and biogenesis of ribosomes (Wullschleger, 2006). The enhanced sensitivity of cancer cells exhibiting oncogenic activation of the Akt-pathway to the mTOR inhibitor rapamycin, illustrates the importance of TORC1 activation downstream of Akt (Sabatini, 2006).

Cell proliferation is stimulated by Akt through inhibitiory phosphorylation of the cyclin-dependent kinase (CDK) inhibitors p21 and p27. Akt thus prevents translocation of p21 and p27 to the nucleus and attenuates their cell-cycle inhibitory effects (Liang and Richardson, 2003). Moreover, inhibitory phosphorylation of the glycogen synthase kinase 3 (GSK3) by Akt leads to cell cycle progression, as inactivation of GSK3 rescues β-catenin from degradation. β-catenin induces transcription of cyclin D1, which induces cell cycle progression (Osaki, 2004).

Akt signalling also takes part in the regulation of nutrient uptake and metabolism. Upon stimulation with insulin, for instance, the glucose transporter Glut4, most likely through interaction with AS160 (Akt substrate of 160 kDa), translocates to the cellular membrane and enhances glucose uptake (Sano, 2003). Furthermore, the Akt-mediated phosphorylation and inhibition of GSK3 stimulates glycogen synthesis.

The Akt pathway interconnects with many other important pathways in the cell. Several studies have demonstrated that Akt signalling can activate NFκB upon a variety of stimuli, for instance upon stimulation with platelet-derived growth factor. Although there are likely to be multiple levels of crosstalk between Akt and NFκB, one mechanism consists in phosphorylation of IKK by Akt and subsequent activation of NFκB. Furthermore, activation of Akt influences other pathways such as ERK, JNK, and MAPK, as their pathways converge upstream.

2.2.2 The phosphoinositide and phosphoinositide-kinases pathways

Hokin and Hokin (1964) have first demonstrated phospholipid turnover in response to cell stimulation (Hokin and Hokin, 1964). Since then research in this field has yielded many mechanistic insights of phosphoinositide cell signalling.

Phosphoinositides (PIs) are inositol-containing glycerophospholipids that can be reversely phosphorylated in several positions on the inositol ring (Figure 19).

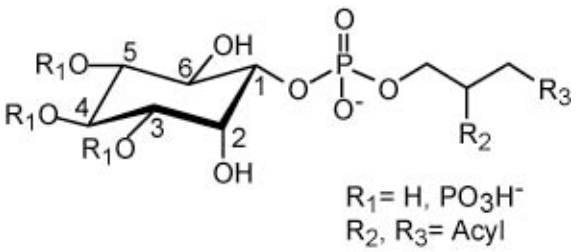

Figure 19: Structure of PIs

The family of PIs consists of the mutual non-phosphorylated precursor phosphatidylinositol (PtdIns) and seven members with different phosphorylation

patterns on the inositol ring, where the 3-, 4-, and 5- positions can be phosphorylated by specific kinases (Figure 20) (Wymann and Pirola, 1998). These phosphoinositide-kinases (PI-kinases) are categorised into three families, depending on the position of the phosphorylation on the inositol ring: phosphoinositide 3-kinases (PI3-kinases), phosphoinositide 4-kinases (PI4-kinases or PIP4-kinases), and phosphoinositide 5-kinases (PI5-kinases or PIP5-kinases).

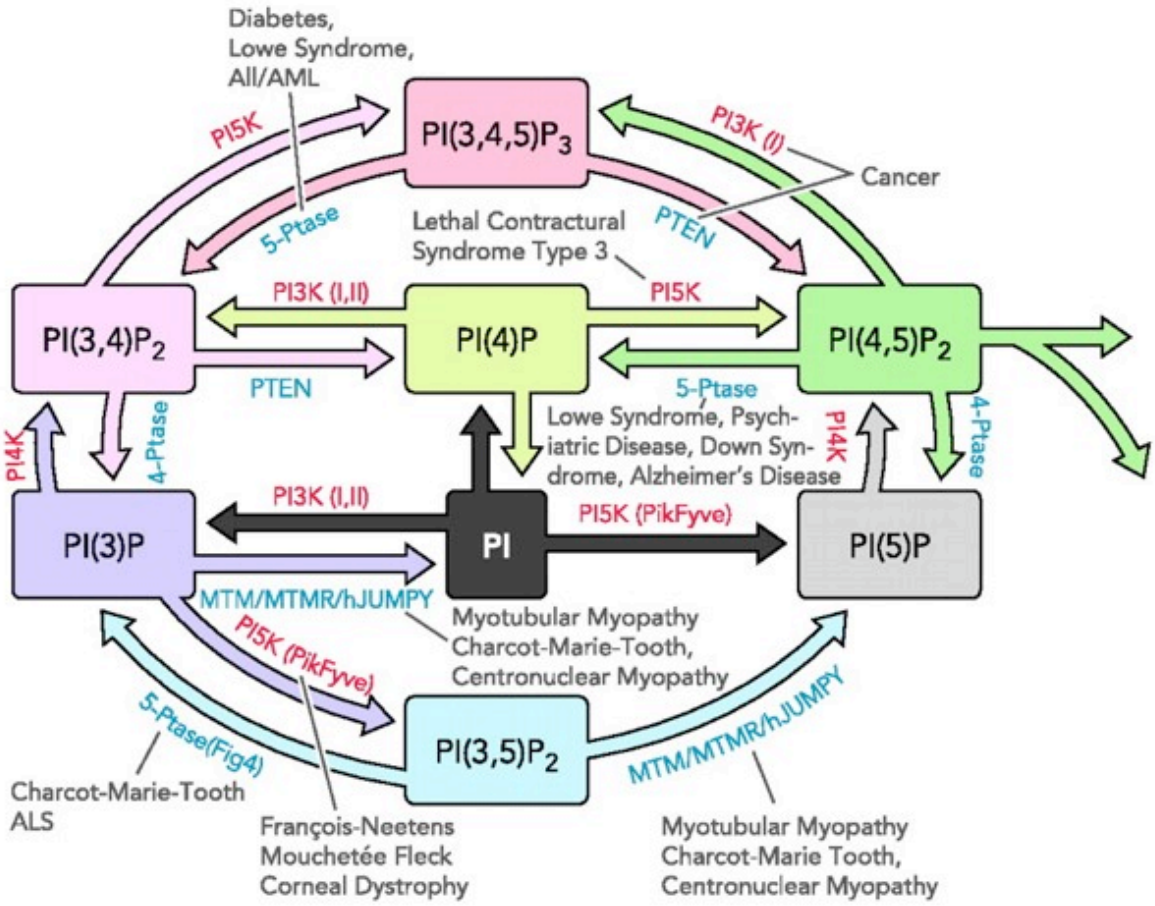

Figure 20: Interconversions of all the PIs, with indication of the respective enzyme and the disease resulting when proper function of the enzyme fails (figure from McCrea and De Camilli, 2009).

2.2.3 Phosphoinositide-kinases

The best characterised amongst them are the PI3-kinases that are further grouped into class I, II, and III, depending on their subunit structure, substrate specificity, and type of activation.

PI3-kinases class I. The class I PI3-kinases are heterodimeric proteins that catalyse the formation of phosphatidylinositol-3,4,5-trisphosphate ($PtdInsP_3$) from phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$). They all consist of a regulatory

and a catalytic subunit and are subdivided into class IA, that contain the catalytic subunits p110α, β, or δ, and the class IB that contains the catalytic subunit p110γ (hence called PI3-kinase γ) (Vanhaesebroeck, 1997). The class IA PI3-kinases are downstream of receptor tyrosine kinases and can be activated by growth factor or insulin stimulation, cytokine signalling, or antibody binding, whereas the class IB is downstream of GPCRs and is activated by Gβγ subunits (Karataeva and Nevinsky, 2007). Although the two classes couple to different surface receptor systems, they share the small GTPase Ras as an interacting partner (Wymann and Pirola, 1998).

PI3-kinases class II. Class II PI3-kinases are monomers of relatively large molecular mass (170-220 kDa) and comprise three catalytic isoforms (C2α, C2β, and C2γ). Unlike class I and III, class II contains no regulatory protein. They mainly catalyse the production of PtdIns3P from PtdIns and PtdIns(3,4)P_2 from PtdIns4P (Falasca and Maffucci, 2007; McCrea and De Camilli, 2009).

PI3-kinase class III. Class III PI3-kinases have a substrate specificity restricted to PtdIns. These PI3-kinases are homologous to Vps34 (vacuolar protein sorting 34), the only PI3-kinase in yeast. Vps34 is closely associated with Vps15, which has been described as a Vps34 regulatory subunit and was therefore named p150 in mammals, analogous to the class I regulatory subunits (Backer, 2008; Vanhaesebroeck, 1997).

PI4-kinases. Two types of PtdIns 4-kinase have been identified. One that only catalyses PtdIns to PtdIns4P, called PI4-kinase, and one that catalyses PtdInsPs to PtdIns(3,4)P_2 or PtdIns(4,5)P_2, respectively, called PIP4-kinase (Jones, 2000; Rusten and Stenmark, 2006).

PI5-kinases. There are two types of PI5-kinase. A characteristic feature is that they only phosphorylate PtdInsPs to PtdInsP_2. PIKfyve converts PtdIns3P to PtdIns(3,5)P_2, while PIP5-kinase phospohorylates PtdIns4P to PtdIns(4,5)P_2 (Krauss and Haucke, 2007). To date no PI5-kinase is known that phosphorylates PtdIns to PtdIns5P.

In summary, the PI3-kinases have been well investigated, but little is known about the other PI-kinases. Studies on PI4- and PI5-kinases are few and their biological significance not fully understood.

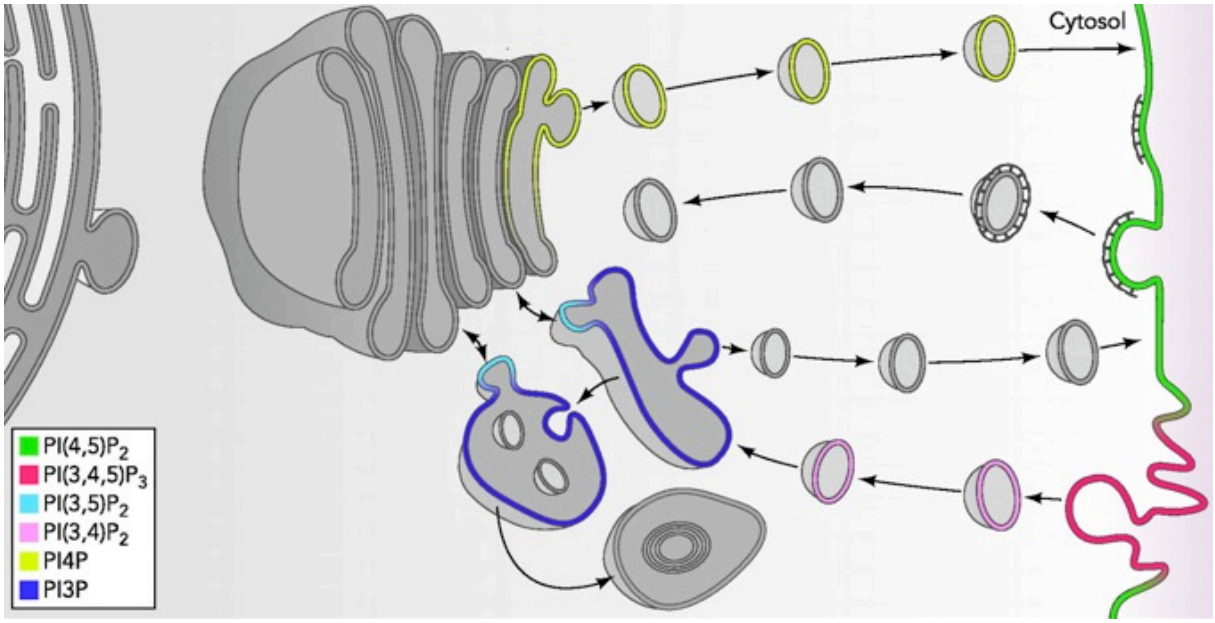

Figure 21: Localisation of the specific PtdIns at different membranes in the cell (figure from McCrea and De Camilli, 2009).

2.2.4 Roles of the different phosphoinositides

PtdIns is the most abundant PI and together with PtdIns4P and PtdIns(4,5)P_2 they build the so called canonical pathway. These PIs are kept at constant levels at the plasma membrane, whereas the other PIs are considered to be low-abundant signalling molecules that transiently appear upon specific stimulation. Furthermore PtdIns(4,5)P_2 serves as precursor of the intracellular second messengers DAG and inositol-(1,4,5)-trisphosphate (IP_3), that are formed through phospholipase C (PLC) mediated hydrolysis of PtdIns(4,5)P_2. DAG binds and regulates protein kinase C (PKC) and a variety of other effectors, whereas IP_3 triggers calcium release from the endoplasmic reticulum. PtdIns(4,5)P_2 can also be cleaved by phospholipase A_2 (PLA_2) to generate arachidonic acid, the precursor of inflammatory eicosanoids (McCrea and De Camilli, 2009).

PtdInsP$_3$. The generation of PtdInsP_3 stimulates numerous signalling pathways that are involved in cell proliferation, metabolism, motility and immune responses. PtdInsP_3 recruits PH domain-containing proteins such as Akt, PDK1, PLC, BKT (bruton's tyrosine kinase), and GTPases-activating factors, to the membrane where they build signalling complexes to produce a specific signal in the cell (Sasaki, 2007). More than 250 proteins in humans have been found to contain a PH binding domain and are therefore potential binding partners of PtdInsP_3. However, the most

important effector is Akt. 'Not all PI3-kinase signalling is mediated via Akt, but clearly a major part of it' (Downward, 2004). $PtdInsP_3$ levels resulting after activation of PI3-kinses class I are tightly regulated by the action of phosphatases such as PTEN (phosphatase and tensin homologue deleted on chromosome ten) and SHIP (SH2-containing inositol 5-phosphatase) that dephosphorylate $PtdInsP_3$ to $PtdIns(3,4)P_2$ and $PtdIns(4,5)P_2$, respectively (Figure 20). Deregulated $PtdInsP_3$ signalling is tightly associated with tumour development, either through mutational changes in receptors that result in constant activation of PI3-kinases or by mutational changes of PTEN and hence impaired shut-down of the signal.

$PtdInsP_2$. The bisphosphorylated PIs, $PtdIns(3,4)P_2$, $PtdIns(3,5)P_2$, and $PtdIns(4,5)P_2$ have distinct functions in cell signalling. $PtdIns(3,4)P_2$ is responsible for signal-extension after $PtdInsP_3$ induction since some of the $PtdInsP_3$ binding proteins also recognise $PtdIns(3,4)P_2$, namely PDK1 and Akt. The 5-phosphtase SHIP, responsible for dephosphorylation of $PtdInsP_3$ to $PtdIns(3,4)P_2$ has important roles in cells of the immune system and in blood platelets (Blero, 2007). SHIP1 deficient mice display overactive immune responses and platelet clotting. SHIP2 is important for control of insulin sensitivity (Sasaoka, 2006).
$PtdIns(3,5)P_2$ has a role in membrane and protein trafficking (Figure 21) and is believed to be only produced via PtdIns3P and subsequent 5-phosphrylation (Michell, Lemmon, Dove, 2006). Besides its function as a precursor of $PtdInsP_3$, DAG, and IP_3, $PtdIns(4,5)P_2$ has some specific functions in regulation of the actin cytoskeleton, exo- and endocytoic processes. $PtdIns(4,5)P_2$ is associated with small GTPases and participates in recruitment and activation of a wide variety of regulatory proteins (Di Paolo and De Camilli, 2006).

PtdInsP. The phosphoinositide monophosphates were long thought to be mere intermediate metabolites in the pathway. In yeast the only PI3-kinase Vps34 is responsible for the production of PtdIns3P and plays a critical role in the sorting of proteins from the golgi to the vacuole (Pendaries, 2005). In mammals it is involved in vesicular trafficking (Wurmser, 1999). Besides being an intermediate product on the way to $PtdIns(4,5)P_2$, PtdIns4P is enriched at the Golgi membrane and targets Golgi-associated proteins (Figure 21) (Pendaries, 2005). The role of PtdIns5P as signalling molecule just emerges, as it is the most recently discovered inositol lipid (Rameh,

1997). It was shown that PtdIns5P levels rise upon thrombin stimulation in platelets (Morris, 2000) and during osmotic stress in mammalian cells (Sbrissa, 2002).

2.2.5 PI3-kinase/Akt signalling in disease

PI3-kinases were originally discovered because of their association with viral oncoproteins (Cantley, 1991) and increased PI3-kinase signalling has since then been found in many cancers. Abberant PI3-kinase/Akt activation occurs in cancer through a variety of mechanisms. These include mutations or amplification of upstream regulators such as receptors and PI3-kinases, inactivation of PTEN, or amplification of Akt. Mutational activation of PI3-kinase IA is found in 15% of all tumour types, suggesting that this may be the most commonly mutated kinase (Crabbe, 2007). The *PTEN* gene is commonly inactivated in cancer cells and is the second most mutated tumour suppressor gene after p53 (Trotman and Pandolfi, 2003). Another negative regulator of $PtdInsP_3$ is SHIP, and the isoform SHIP2 has been implicated in development of diabetes. SHIP2 is responsible for the control of the cellular response to insulin. Elevated levels of SHIP2 expression are found in cells that show insulin resistance.

A role of PtdIns(4,5)P_2 in the early onset of Alzheimer's Disease in Down Syndrome has been suggested (Voronov, 2008). Synaptojanin I, a PtdIns(4,5,)P_2 phosphatase plays a critical role in synaptic transmission and is located in the chromosome 21 region that is triplicated in Down Syndrome. Accordingly levels of synaptojanin I are increased and might add to the early development of AD (McCrea and De Camilli, 2009). Furthermore some hereditary diseases were linked with genetic defects in the PI-signalling system (Figure 20). Lowe syndrome is a X-linked condition that results in severe mental retardation, growth defects, and eye defects. It is caused by mutations in the inositol-5-phosphatase OCRL (occulocerebrorenal syndrome of Lowe) that dephosphrylates PtdIns(4,5)P_2 to PtdIns4P. The cellular causes of the varied disease phenotypes are not yet understood (Skwarek and Boulianne, 2009). Another disease cause by impaired PI metabolism is Charcot-Marie-Tooth (CMT) disease that affects peripheral nerves and is the most common inherited neurological disorder. Several different subtypes of CMT disease are due to mutations in lipid phosphatases involved in dephosphorylation of PtdIns(3,5)P_2 at either the 3- or 5-position (Robinson, 2008).

Targeting of the PI3-kinase pathway is aimed at since the important role of this pathway has been recognised. Various molecules are in clinical development, the Akt inhibitor perifosine, PI3-kinase inhibitors, and already in clinical use, the mTOR inhibitor rapamycin (Yap, 2008). Unfortunately selective inhibition of the PI3-kinases has not been achieved yet. The non-selective PI3-kinase inhibitor wortmannin and its role as a model substance in molecular biology were discussed in chapter 1.5.2. Selective inhibitors of PI3-kinase gamma were once thought to become the 'aspirine of the 21st century' (Ruckle, 2006) but no sufficiently selective inhibitor has been found yet to fulfil that prophecy.

2.3 References

Adrain, C., Creagh, E.M., and Martin, S.J. (2001). Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. Embo J *20*, 6627-6636.

Antonsson, B. (2004). Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. Mol Cell Biochem *256-257*, 141-155.

Backer, J.M. (2008). The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem J *410*, 1-17.

Blero, D., Payrastre, B., Schurmans, S., and Erneux, C. (2007). Phosphoinositide phosphatases in a network of signalling reactions. Pflugers Arch *455*, 31-44.

Caelles, C., Helmberg, A., and Karin, M. (1994). p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature *370*, 220-223.

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281-302.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., and Reed, J.C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.

Chipuk, J.E., Bouchier-Hayes, L., and Green, D.R. (2006). Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ *13*, 1396-1402.

Crabbe, T., Welham, M.J., and Ward, S.G. (2007). The PI3K inhibitor arsenal: choose your weapon! Trends Biochem Sci *32*, 450-456.

Curtin, J.F., and Cotter, T.G. (2003). Apoptosis: Historical perspectives. Essays Biochem *39*, 1-10.

Degterev, A., Boyce, M., and Yuan, J. (2003). A decade of caspases. Oncogene *22*, 8543-8567.

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. Nature *443*, 651-657.

Downward, J. (2004). PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol *15*, 177-182.

Duan, W., Zhu, X., Ladenheim, B., Yu, Q.S., Guo, Z., Oyler, J., Cutler, R.G., Cadet, J.L., Greig, N.H., and Mattson, M.P. (2002). p53 inhibitors preserve dopamine neurons and motor function in experimental parkinsonism. Ann Neurol *52*, 597-606.

Falasca, M., and Maffucci, T. (2007). Role of class II phosphoinositide 3-kinase in cell signalling. Biochem Soc Trans *35*, 211-214.

Folmer, F., Jaspars, M., Dicato, M., and Diederich, M. (2008). Marine natural products as targeted modulators of the transcription factor NF-kappaB. Biochem Pharmacol *75*, 603-617.

Fuchs, S.Y., Adler, V., Buschmann, T., Yin, Z., Wu, X., Jones, S.N., and Ronai, Z. (1998a). JNK targets p53 ubiquitination and degradation in nonstressed cells. Genes Dev *12*, 2658-2663.

Fuchs, S.Y., Adler, V., Pincus, M.R., and Ronai, Z. (1998b). MEKK1/JNK signaling stabilizes and activates p53. Proc Natl Acad Sci U S A *95*, 10541-10546.

Galluzzi, L., Joza, N., Tasdemir, E., Maiuri, M.C., Hengartner, M., Abrams, J.M., Tavernarakis, N., Penninger, J., Madeo, F., and Kroemer, G. (2008). No death without life: vital functions of apoptotic effectors. Cell Death Differ *15*, 1113-1123.

Green, D.R., and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. Science *305*, 626-629.

Harris, L.C. (2005). MDM2 splice variants and their therapeutic implications. Curr Cancer Drug Targets *5*, 21-26.

Hokin, L.E., and Hokin, M.R. (1964). The Incorporation of 32p from Triphosphate into Polyphosphoinositides (Gamma-32p)Adenosine and Phosphatidic Acid in Erythrocyte Membranes. Biochim Biophys Acta *84*, 563-575.

Hsu, Y.T., Wolter, K.G., and Youle, R.J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A *94*, 3668-3672.

Huang, J., and Manning, B.D. (2009). A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans *37*, 217-222.

Hyer, M.L., Samuel, T., and Reed, J.C. (2006). The FLIP-side of Fas signaling. Clin Cancer Res *12*, 5929-5931.

Igney, F.H., and Krammer, P.H. (2002). Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer *2*, 277-288.

Jin, F., Liu, X., Zhou, Z., Yue, P., Lotan, R., Khuri, F.R., Chung, L.W., and Sun, S.Y. (2005). Activation of nuclear factor-kappaB contributes to induction of death receptors and apoptosis by the synthetic retinoid CD437 in DU145 human prostate cancer cells. Cancer Res *65*, 6354-6363.

Jones, D.H., Morris, J.B., Morgan, C.P., Kondo, H., Irvine, R.F., and Cockcroft, S. (2000). Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment. J Biol Chem *275*, 13962-13966.

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.Y., Ravagnan, L.*, et al.* (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature *410*, 549-554.

Karataeva, N.A., and Nevinsky, G.A. (2007). Enzymes phosphorylating lipids and polysaccharides. Biochemistry (Mosc) *72*, 367-379.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., and Reed, J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res *53*, 4701-4714.

Krauss, M., and Haucke, V. (2007). Phosphoinositides: regulators of membrane traffic and protein function. FEBS Lett *581*, 2105-2111.

LaCasse, E.C., Baird, S., Korneluk, R.G., and MacKenzie, A.E. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. Oncogene *17*, 3247-3259.

Lamkanfi, M., Festjens, N., Declercq, W., Vanden Berghe, T., and Vandenabeele, P. (2007). Caspases in cell survival, proliferation and differentiation. Cell Death Differ *14*, 44-55.

Lavrik, I., Golks, A., and Krammer, P.H. (2005). Death receptor signaling. J Cell Sci *118*, 265-267.

Leu, J.I., Dumont, P., Hafey, M., Murphy, M.E., and George, D.L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol *6*, 443-450.

Li, H., and Lin, X. (2008). Positive and negative signaling components involved in TNFalpha-induced NF-kappaB activation. Cytokine *41*, 1-8.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479-489.

Liang, S.X., and Richardson, D.R. (2003). The effect of potent iron chelators on the regulation of p53: examination of the expression, localization and DNA-binding activity of p53 and the transactivation of WAF1. Carcinogenesis *24*, 1601-1614.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell *94*, 481-490.

McCrea, H.J., and De Camilli, P. (2009). Mutations in phosphoinositide metabolizing enzymes and human disease. Physiology (Bethesda) *24*, 8-16.

Morris, J.B., Hinchliffe, K.A., Ciruela, A., Letcher, A.J., and Irvine, R.F. (2000). Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay. FEBS Lett *475*, 57-60.

Movassagh, M., and Foo, R.S. (2008). Simplified apoptotic cascades. Heart Fail Rev *13*, 111-119.

Newmeyer, D.D., and Ferguson-Miller, S. (2003). Mitochondria: releasing power for life and unleashing the machineries of death. Cell *112*, 481-490.

Osaki, M., Oshimura, M., and Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis *9*, 667-676.

Parcellier, A., Tintignac, L.A., Zhuravleva, E., and Hemmings, B.A. (2008). PKB and the mitochondria: AKTing on apoptosis. Cell Signal *20*, 21-30.

Pendaries, C., Tronchere, H., Racaud-Sultan, C., Gaits-Iacovoni, F., Coronas, S., Manenti, S., Gratacap, M.P., Plantavid, M., and Payrastre, B. (2005). Emerging roles of phosphatidylinositol monophosphates in cellular signaling and trafficking. Adv Enzyme Regul *45*, 201-214.

Pietsch, E.C., Sykes, S.M., McMahon, S.B., and Murphy, M.E. (2008). The p53 family and programmed cell death. Oncogene *27*, 6507-6521.

Porter, A.G., and Janicke, R.U. (1999). Emerging roles of caspase-3 in apoptosis. Cell Death Differ *6*, 99-104.

Porter, A.G., and Urbano, A.G. (2006). Does apoptosis-inducing factor (AIF) have both life and death functions in cells? Bioessays *28*, 834-843.

Rameh, L.E., Tolias, K.F., Duckworth, B.C., and Cantley, L.C. (1997). A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature *390*, 192-196.

Rich, T., Watson, C.J., and Wyllie, A. (1999). Apoptosis: the germs of death. Nat Cell Biol *1*, E69-71.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Robinson, F.L., Niesman, I.R., Beiswenger, K.K., and Dixon, J.E. (2008). Loss of the inactive myotubularin-related phosphatase Mtmr13 leads to a Charcot-Marie-Tooth 4B2-like peripheral neuropathy in mice. Proc Natl Acad Sci U S A *105*, 4916-4921.

Ruckle, T., Schwarz, M.K., and Rommel, C. (2006). PI3Kgamma inhibition: towards an 'aspirin of the 21st century'? Nat Rev Drug Discov *5*, 903-918.

Rupinder, S.K., Gurpreet, A.K., and Manjeet, S. (2007). Cell suicide and caspases. Vascul Pharmacol *46*, 383-393.

Rusten, T.E., and Stenmark, H. (2006). Analyzing phosphoinositides and their interacting proteins. Nat Methods *3*, 251-258.

Sabatini, D.M. (2006). mTOR and cancer: insights into a complex relationship. Nat Rev Cancer *6*, 729-734.

Salvesen, G.S., and Duckett, C.S. (2002). IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol *3*, 401-410.

Sano, H., Kane, S., Sano, E., Miinea, C.P., Asara, J.M., Lane, W.S., Garner, C.W., and Lienhard, G.E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem *278*, 14599-14602.

Sasaki, T., Sasaki, J., Sakai, T., Takasuga, S., and Suzuki, A. (2007). The physiology of phosphoinositides. Biol Pharm Bull *30*, 1599-1604.

Sasaoka, T., Wada, T., and Tsuneki, H. (2006). Lipid phosphatases as a possible therapeutic target in cases of type 2 diabetes and obesity. Pharmacol Ther *112*, 799-809.

Sbrissa, D., Ikonomov, O.C., Deeb, R., and Shisheva, A. (2002). Phosphatidylinositol 5-phosphate biosynthesis is linked to PIKfyve and is involved in osmotic response pathway in mammalian cells. J Biol Chem *277*, 47276-47284.

Skwarek, L.C., and Boulianne, G.L. (2009). Great expectations for PIP: phosphoinositides as regulators of signaling during development and disease. Dev Cell *16*, 12-20.

Stambolsky, P., Weisz, L., Shats, I., Klein, Y., Goldfinger, N., Oren, M., and Rotter, V. (2006). Regulation of AIF expression by p53. Cell Death Differ *13*, 2140-2149.

Takabe, K., Paugh, S.W., Milstien, S., and Spiegel, S. (2008). "Inside-out" signaling of sphingosine-1-phosphate: therapeutic targets. Pharmacol Rev *60*, 181-195.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J.L., Schroter, M.*, et al.* (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature *386*, 517-521.

Trotman, L.C., and Pandolfi, P.P. (2003). PTEN and p53: who will get the upper hand? Cancer Cell *3*, 97-99.

Tsujimoto, Y., and Croce, C.M. (1986). Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci U S A *83*, 5214-5218.

Vanhaesebroeck, B., Leevers, S.J., Panayotou, G., and Waterfield, M.D. (1997). Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci *22*, 267-272.

Vaux, D.L., Cory, S., and Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature *335*, 440-442.

Voronov, S.V., Frere, S.G., Giovedi, S., Pollina, E.A., Borel, C., Zhang, H., Schmidt, C., Akeson, E.C., Wenk, M.R., Cimasoni, L.*, et al.* (2008). Synaptojanin 1-linked phosphoinositide dyshomeostasis and cognitive deficits in mouse models of Down's syndrome. Proc Natl Acad Sci U S A *105*, 9415-9420.

Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat Rev Cancer *2*, 594-604.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. Cell Death Differ *10*, 45-65.

Wang, C.Y., Mayo, M.W., and Baldwin, A.S., Jr. (1996). TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science *274*, 784-787.

Wullschleger, S., Loewith, R., and Hall, M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Wurmser, A.E., Gary, J.D., and Emr, S.D. (1999). Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. J Biol Chem *274*, 9129-9132.

Wymann, M.P., and Pirola, L. (1998). Structure and function of phosphoinositide 3-kinases. Biochim Biophys Acta *1436*, 127-150.

Yap, T.A., Garrett, M.D., Walton, M.I., Raynaud, F., de Bono, J.S., and Workman, P. (2008). Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol *8*, 393-412.

Youle, R.J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol *9*, 47-59.

3 Aim of the thesis

The importance of natural products as sources of inspiration for drug discovery and development and as tools for basic research is undisputed. The combination of traditional knowledge and modern scientific research has been a successful strategy to identify new active substances that might serve as lead structures for drug discovery, and natural products have offered numerous opportunities to study unknown cellular mechanisms.

The exploitation of natural products is limited by the fact that the modes of action of numerous compounds are poorly understood. The potential of a given compound can only be assessed if sufficient information is available regarding their molecular targets. The natural products analysed during this thesis have both some background in traditional medicine and have been identified with the aid of functional assays. In order to assign these observed effects to an interaction on the molecular level, we studied associated signal transduction pathways in cells, namely:

(i) Phosphorylation of proteins
(ii) Activation of transcription factors
(iii) Concentration of second messengers (i.e. phosphoinositides)
(iv) Localisation of the substance within the cell

In the first part of this thesis the molecular effects of the fungal alkaloid militarinone A were studied. In an earlier screening for neurotrophic substances, the extract of the entomogenous fungus *Paecilomyces militaris* had been shown to induce increased spike formation in PC12 cells (Schmidt, 2003), and militarinone A had been subsequent identified as the active metabolite via activity guided fractionation (Figure 22) (Schmidt, 2002).

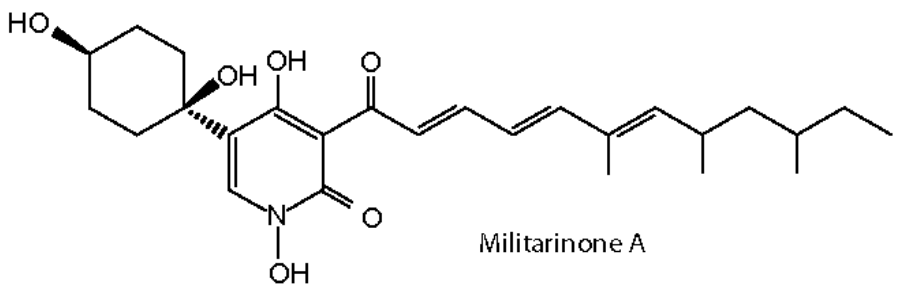

Figure 22: Structure of militarinone A.

To uncover the mechanism of this activity we studied the cell signalling pathways involved in neuronal spike formation and differentiation in two types of neuronal cells (PC12 and N2a) and the interaction of militarinone A with associated pathways.

In the second part, an alkaloid from the traditional European medicinal plant woad was analysed. Woad (*Isatis tinctoria*) contains several known anti-inflammatory components, namely, tryptanthrin, indirubin, and (*E,Z*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (indolinone (Figure 23)). The effects of tryptanthrin as a potent inhibitor of COX-2 (Danz, 2002), 5-LOX (Oberthur, 2005), and of nitric oxide production catalyzed by inducible NO synthase (iNOS) (Ishihara, 2000) are well characterised and account for a good part of the anti-inflammatory activity of the plant extract. Indirubin is used in Chinese medicine (from *isatis indigotica*) as an anti-leukemia treatment and has been shown to inhibit cyclin-dependent-kinase 2 (CDK2) (Hoessel, 1999) while indolinone was shown to inhibit degranulation of mast cells (Rüster, 2004).

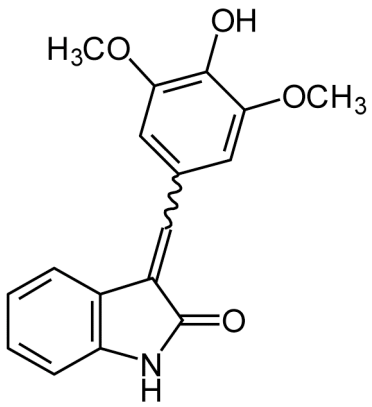

Figure 23: Structure of indolinone.

The anti-allergic activity of indolinone should be further characterised. Given the structural similarities with indolin-2-one derived kinase inhibitors, we focussed on signalling pathways associated with degranulation to determine the mechanism and point of action of indolinone.

During the studies on indolinone, measurements of intracellular $PtdInsP_3$ (Figure 19) were initially performed with the current standard methodology that includes radioactive labelling with $^{32}P_i$, ion-exchange HPLC and flow scintillation analysis (Dove, 1997; Laffargue, 2002). The method proved to be laborious, time-consuming and potentially hazardous. This led us to develop a non-radioactive method for

analysis of phosphoinositides based on chromatographic separation and mass spectrometry detection. This method would allow analysis of all phosphoinositides without the need for radioactive labelling and should be of interest for many other laboratories, as it offers new perspectives in detection and relative quantification of phosphoinositides.

3.1 References

Danz, H., Stoyanova, S., Thomet, O.A., Simon, H.U., Dannhardt, G., Ulbrich, H., and Hamburger, M. (2002). Inhibitory activity of tryptanthrin on prostaglandin and leukotriene synthesis. Planta Med *68*, 875-880.

Dove, S.K., Cooke, F.T., Douglas, M.R., Sayers, L.G., Parker, P.J., and Michell, R.H. (1997). Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. Nature *390*, 187-192.

Hoessel, R., Leclerc, S., Endicott, J.A., Nobel, M.E., Lawrie, A., Tunnah, P., Leost, M., Damiens, E., Marie, D., Marko, D*., et al.* (1999). Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat Cell Biol *1*, 60-67.

Ishihara, T., Kohno, K., Ushio, S., Iwaki, K., Ikeda, M., and Kurimoto, M. (2000). Tryptanthrin inhibits nitric oxide and prostaglandin E(2) synthesis by murine macrophages. Eur J Pharmacol *407*, 197-204.

Laffargue, M., Calvez, R., Finan, P., Trifilieff, A., Barbier, M., Altruda, F., Hirsch, E., and Wymann, M.P. (2002). Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. Immunity *16*, 441-451.

Oberthur, C., Jaggi, R., and Hamburger, M. (2005). HPLC based activity profiling for 5-lipoxygenase inhibitory activity in Isatis tinctoria leaf extracts. Fitoterapia *76*, 324-332.

Rüster, G.U., Hoffmann, B., and Hamburger, M. (2004). Inhibitory activity of indolin-2-one derivatives on compound 48/80-induced histamine release from mast cells. Pharmazie *59*, 236-237.

Schmidt, K., Gunther, W., Stoyanova, S., Schubert, B., Li, Z., and Hamburger, M. (2002). Militarinone A, a neurotrophic pyridone alkaloid from Paecilomyces militaris. Org Lett *4*, 197-199.

Schmidt, K., Li, Z., Schubert, B., Huang, B., Stoyanova, S., and Hamburger, M. (2003). Screening of entomopathogenic Deuteromycetes for activities on targets involved in degenerative diseases of the central nervous system. J Ethnopharmacol *89*, 251-260.

B. PUBLICATIONS

˝It's a wild dance floor there at the molecular level˝
- Roald Hoffmann in 'Designing the molecular world' by Philip Ball (p.83)

1 Promotion of cell death or neurite outgrowth in PC12 and N2a cells by the fungal alkaloid militarinone A depends on basal expression of p53

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ORIGINAL PAPER

Promotion of cell death or neurite outgrowth in PC-12 and N2a cells by the fungal alkaloid militarinone A depends on basal expression of p53

Peter Küenzi · Sabine Kiefer · Anna Koryakina · Matthias Hamburger

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Abstract The fungal alkaloid militarinone A (MiliA) was recently found to stimulate neuronal outgrowth in PC-12 cells by persistant activation of pathways that are also involved in NGF-mediated differentiation, namely the PI3-K/PKB and the MEK/ERK pathways. Application of equal concentrations of MiliA to other cells such as the murine neuroblastoma cell line N2a resulted in immediate onset of apoptosis by nuclear translocation of apoptosis inducing factor (AIF), activation of caspases and c-Jun/AP-1 transcription factor without an intermediate differentiated phenotype, although minor transient phosphorylation of PKB and MAPK as well as activation of NF-κB were also observed. Translocation of AIF was preceded by p53 phosphorylation at Ser15 and blocked by pifithrin α, a known inhibitor of p53-transcriptional activity. We here show that both cell types activate the same pathways albeit in different time scales. This is mainly due to contrasting basal expression levels of p53, which in turn regulates expression of AIF. In PC-12 cells, continuous activation of these pathways after prolonged treatment with 40 μM MiliA first led to up-regulation of p53, phosphorylation of p53, release of AIF from mitochondria and its translocation into the nucleus. Additionally, also activation of the c-Jun/AP-1 transcription factor was observed, and PC-12 cells subsequently underwent apoptosis 48–72 h post-treatment. We report that similar pathways working on different levels are able to initially shape very divergent cellular responses.

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Introduction

Neurotrophic factors are capable of preventing apoptosis in neuronal cells, and exogenous supplementation with such factors have been proposed as a new disease-modifying strategy in the treatment of neurogenerative diseases. However, clinical trials have been disappointing so far due to difficulties in delivery of these glycoproteins into the central nervous system [1]. Small non-peptidic molecules with neurotrophic properties, and capable of penetrating the blood–brain barrier, have been proposed as an alternative [2]. In the course of a screening for such molecules we discovered that the fungal alkaloid militarinone A (MiliA) induced neurite extension in PC-12 cells [3]. Due to its amphiphilic properties MiliA was rapidly integrated into the membranous fraction where it triggered PI3-K/PKB- and ERK1/2-dependent differentiation [4, 5]. PKB promotes survival of a variety of neuronal cells and has been shown to be required for neuronal outgrowth [6, 7]. In addition, phosphorylation and activation of the MAPKs ERK1/2 [8] have also been connected with neuronal differentiation [9], albeit its function is not fully clear.

Apoptosis inducing factor (AIF) is a mitochondrial flavoprotein that is mainly located in the mitochondrial intermembrane space. In healthy cells, AIF is required for efficient oxidative phosphorylation. Upon apoptotic insult, it translocates to the cytosol and subsequently into the nucleus where it eventually binds to DNA and induces caspase-independent cell death [10–12]. The concept that AIF is able to induce apoptotic cell death independent of caspases is supported by a series of experiments where

programmed cell death was executed even in presence of chemical caspase inhibitors such as Z-VAD-Fmk (zVAD) or BAF. Moreover, AIF translocation was also observed in mice in the absence of caspase activation [13], and the biochemical changes were consistent with those described in many caspase-dependent cell death models [14]. Contrasting with its death-inducing properties after nuclear translocation, mitochondria-located AIF has been shown to have a protective role in healthy and in tumour cells [11, 15]. Mitochondrial AIF is even thought to contribute to cell survival by protecting cells against harmful oxidative damage in the absence of deleterious stress while predisposing them to a more efficient killing in response to severe stress. Recently, it has been reported that the *aif* gene is a transcriptional target of basal levels of p53 [16].

p53 is a key tumour suppressor protein that is stabilised and phosphorylated due to genotoxic stress and is negatively controlled by Mdm2 which in turn is positively controlled by PI3-K-PKB signalling [17, 18]. Once activated, p53 is able to promote cell-cycle arrest, apoptosis, differentiation, senescence or DNA repair [19, 20]. It may translocate to the nucleus where it regulates a plethora of genes eventually leading to the expression of Bax, Bid, FAS and redox-related genes. Therefore, a model was proposed in which p53 initiates apoptosis through the regulation or generation of reactive oxygen species (ROS) [21].

ROS are by-products of aerobic oxidation, mediate toxicity but also work as signalling molecules [22]. Among others, ROS activate diverse transcription factors such as AP-1, antioxidant responsive element (ARE) and NF-κB [23–25]. AP-1 describes a group of related members of the Jun/Fos family and is involved in various cellular processes depending on the composition of AP-1 dimers and on the cell type. It is commonly thought that in neuronal cells, c-jun has pro-apoptotic function [26], but a simple increase of c-jun concentration was considered to be insufficient to efficiently trigger neuronal cell death [27]. The contribution of AP-1 to survival, apoptosis or differentiation is strictly depending on the cellular situation at a given time point. ARE is important for cell defence under conditions of oxidative stress [24], and NF-κB is a multifunctional transcription factor mainly involved in promoting cell survival [28].

Decline of mitochondrial integrity is considered to be of major importance in generation of oxidative stress and induction of apoptosis through the release of AIF and cytochrome c [14, 29]. It is widely accepted that Bcl-2 proteins such as Bax or Bad are able to disturb the outer mitochondrial membrane leading to the release of these small molecules, and that other Bcl-2 proteins such as Bcl-x_L or Bcl-2 itself are capable to prevent this.

We report here that the small non-peptidic molecule MiliA is capable of inducing signalling complexes such as PI3-K/PKB, MEK/ERK, p53 and accumulation of ROS that together contribute to neurite extension or/and apoptosis depending on the basal expressional level of p53.

Materials and methods

General remark

All experiments have been successfully reproduced at least three times.

Materials and cell culture

Rat PC-12 cells were a kind gift from Prof. Anne Eckert (Neurobiology Research Laboratory, University Clinic for Psychiatry, Basel, Switzerland). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, Buchs, Switzerland) supplemented with 5% heat-inactivated foetal calf serum (FCS, Amimed, Basel, Switzerland), 10% heat-inactivated horse serum (Amimed, Basel, Switzerland), 100 U/ml penicillin/streptomycin (Invitrogen, Basel, Switzerland), and 2 mM L-glutamine (Invitrogen, Basel, Switzerland). Murine Neuro 2a (N2a) cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Both cell types were grown in humified atmosphere containing 5% CO_2. PC-12 cells stably overexpressing TrkA were a kind gift from Prof. Frank-D. Böhmer, (Institute of Molecular Cell Biology, Jena, Germany) and were treated as described [3]. The following chemicals have been used: LY294.002, zVAD, pifithrin α (PFTα) (all from Alexis Corporation, Lausen, Switzerland); *N*-Acetyl-L-cysteine (NAC) and nerve growth factor (NGF) (both from Sigma-Aldrich, Buchs, Switzerland). All chemical inhibitors were added 30 min prior to stimulation.

Western blot

Immunoblot analysis was performed according to standard procedures essentially as described [30]. Nitrocellulose membranes were blocked and incubated overnight at 4°C with specific primary Ab diluted in blocking buffer (5% BSA in TBS-Tween): anti-PKB 1:1000; anti-Phospho-PKB (Ser473) 1:1000; anti-ERK1/2 1:2000; anti-Phospho-ERK1/2 (Thr202/Tyr204) 1:1000; anti-AIF 1:2000; anti-Phospho-p53 (Ser15) 1:2000 (all rabbit polyclonal); mouse monoclonal anti-p53 1:1000 (all Cell Signaling Technology, Beverly, MA, USA); goat polyclonal anti-Actin 1:4000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Specific bands were tagged using specific, HRP conjugated secondary Abs and detected using the ECL Plus System (GE Healthcare, Little Chalfont, UK).

Immunofluorescence microscopy and light microscopy

PC-12 and N2a cells were fixed in 4% formaldehyde in PBS for a minimal 15 min at 4°C, permabilised with 0.2% Triton-X (prepared in PBS containing 10% heat-inactivated FCS) and then incubated with primary Ab. The Abs were diluted in PBS containing 10% heat-inactivated FCS and used at the following conditions: rabbit polyclonal anti-AIF (diluted 1:50), rabbit anti-Phospho-p53 (Ser15) (diluted 1:100), mouse monoclonal anti-p53 (diluted 1:200) (all from Cell Signaling CST). Subsequently the cells were washed thrice with PBS and incubated for 1 h with affinity-purified Alexa-Fluor 488 goat anti-rabbit IgG (H + L) or Alexa-Fluor 488 goat anti-mouse IgG (H + L) (both from Invitrogen-Molecular Probes, Basel, Switzerland), respectively. Nuclei were counterstained with Dapi (diluted 1:4000 in PBS) (from Invitrogen-Molecular Probes, Basel, Switzerland). Cells plated on coverslips were mounted on glass slides and visualised by confocal microscopy (Leica DM RXE scanning confocal microscope) using Leica confocal software, version 2.5 (Leica Microsystems, Heidelberg, Germany) or on a fluorescence microscope (Axiophot, Carl Zeiss AG, Jena, Germany) using the analySIS Software (Soft Imaging Systems, Münster, Germany). Identical exposure times were used across conditions.

Light microscopy pictures were taken on a Leitz Laborlux (Ernst Leitz, Wetzlar, Germany) equipped with a Leica DC200 Camera (Leitz Microsystems AG, Glattbrugg, Switzerland) and analysed with Leica DC Viewer Software version 3,2,0,0 (Leica Microsystems AG, Heerbrugg, Switzerland).

Flow cytometry and measurement of membrane potential

To detect caspase activation, staining with FITC-valyl-alanyl-aspartic acid fluoromethyl ketone (FITC-VAD-fmk; Promega) was carried out as described [30]. Annexin V staining of exposed membrane phosphatidylserine was carried out using the Annexin V assay kit (Roche Diagnostics, Rotkreuz, Switzerland) following the manufacturer's protocol. Quantification of loss of membrane potential was determined by incubating 1×10^6 cells with 2 nM $DiOC_6(3)$ in serum-reduced media at 37°C for 30 min. After start of treatment, aliquots were taken at time points indicated, diluted in PBS and subjected to flow cytometry. FACS Analysis was performed on a Dako CyAn™ ADP LX 7 using Summit software (DakoCytomation, Fort Collins, CO, USA).

Measurement of intracellular ROS

The accumulation of ROS in PC-12 and N2a cells was determined by analysis of dichlorofluorescein fluorescence. Cells were plated in serum-containing DMEM into 96-well plates and allowed to adhere overnight at 37°C. After washing twice with PBS, 2,7-dichlorofluorescein diacetate (DCFH-DA; Invitrogen-Molecular Probes, Basel, Switzerland) was applied at 10 μM in PBS for 45 min at 37°C. After removal of the supernatant, cells were re-suspended in serum-containing DMEM including drugs or vehicle only and incubated for 60 min at 37°C. DCF fluorescence was quantified (excitation: 492 nm; emission: 535 nm) using a Chameleon microplate reader (Hidex Oy, Turku, Finland).

Alternatively, cells were left to grow on coverslips and subsequently loaded with 5 μM DCFH-DA for 30 min. After incubation with drugs in serum-containing DMEM, the cells were fixed with MeOH at −20°C for 5 min and analysed by immunofluorescence microscopy as described above. Identical exposure times were used across conditions.

Preparation of nuclear extracts, electrophoretic mobility shift assay and supershift

Cells were collected and washed with ice-cold PBS (pH 7.4) and lysed in a hypotonic buffer (10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40) containing protease inhibitors (1 mM PMSF, 1× Complete® Protease Inhibitors (Roche Diagnostics, Rotkreuz, Switzerland)). The nuclei were collected by centrifugation at 800*g* for 5 min at 4°C and washed once in ice-cold hypotonic buffer without NP-40. Nuclei were subsequently re-suspended in nuclear buffer (250 mM Tris–HCl, pH 7.8, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40) containing protease inhibitors (1 mM PMSF, 1× Complete® Protease Inhibitors). Nuclear membranes were disrupted by freeze-thawing followed by centrifugation at 13,200*g* for 30 min. The supernatant (nuclear extract) was collected and the protein concentration was measured using Coomassie® Protein Assay Reagent (Sigma-Aldrich, Buchs, Switzerland). The binding reactions were carried out as described [31]. Briefly, nuclear extracts containing 3 μg nuclear proteins were incubated for 20 min with ^{32}P-radiolabelled oligonucleotides in 20 μl reaction buffer. For supershift analysis, 1 μg of anti-c-jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubated on ice for another 30 min. Nuclear protein-oligonucleotide and antibody-protein-oligonucleotide complexes, respectively, were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel in 0.25× TBE buffer at 20 V/cm for 35 min at room temperature. The gel was dried and autoradiographed on an intensifying screen at −80°C. Double stranded, palindromic oligonucleotides containing the consensus sequences for ARE (5′-AAA TGG CAT TGC TAA TGG TGA CAA AGC AAC T-3′) [32], NF-κB (5′-AGT

TGA GGG GAC TTT CCC AGG C-3′) [31] or AP-1 (5′-GAA TCG AGC ATG AGT CAG ACA CA-3′) [33] were used.

Results

We previously reported [3] that addition of 40 μM MiliA to PC-12 cells for 24 h after pre-treatment with NGF [34] induced neurite outgrowth which was attributed to the NGF-dependent up-regulation of its receptor TrkA. However, pre-treatment with a commercially available TrkA-inhibitor that was able to prevent NGF-induced phosphorylation of PKB and MAPKs, did not prevent MiliA-dependent phosphorylation of these proteins (Fig. 1a) and suggested that phosphorylation of TrkA was not involved in MiliA-induced neurite extension. When applying 40 μM MiliA to PC-12 cells stably over-expressing TrkA (TA) and naïve PC-12, we observed similar neuronal outgrowth in both cases (Fig. 1 and [3]. In addition, phosphorylation of PKB and MAPKs in TA (not shown) and naïve PC-12 was found (Fig. 1c).

Since up-regulation of the PKB and MAPK-pathways was independent of TrkA, we wanted to know if MiliA-induced phosphorylation of these signalling molecules occurred also in other neuronal cell-lines. Treatment of N2a cells with 40 μM MiliA led to a down-regulation of the endogenous PKB and MAPK phosphorylation (Fig. 1 e). This was accompanied by a marked loss of protein content from samples treated with MiliA compared to vehicle-treated controls (not shown). Moreover, light microscopy revealed that most of the cells had lost adherence, were oddly shaped or had crumbled (Fig. 1d).

To discriminate between necrotic and apoptotic cell death, we double-stained N2a cells treated with 40 μM MiliA or vehicle only with Annexin V/PI. The overwhelming part of cell death observed was due to apoptotic mechanisms (Fig. 2a) becoming evident as early as 2 h post-treatment. Additionally, chromatin condensation

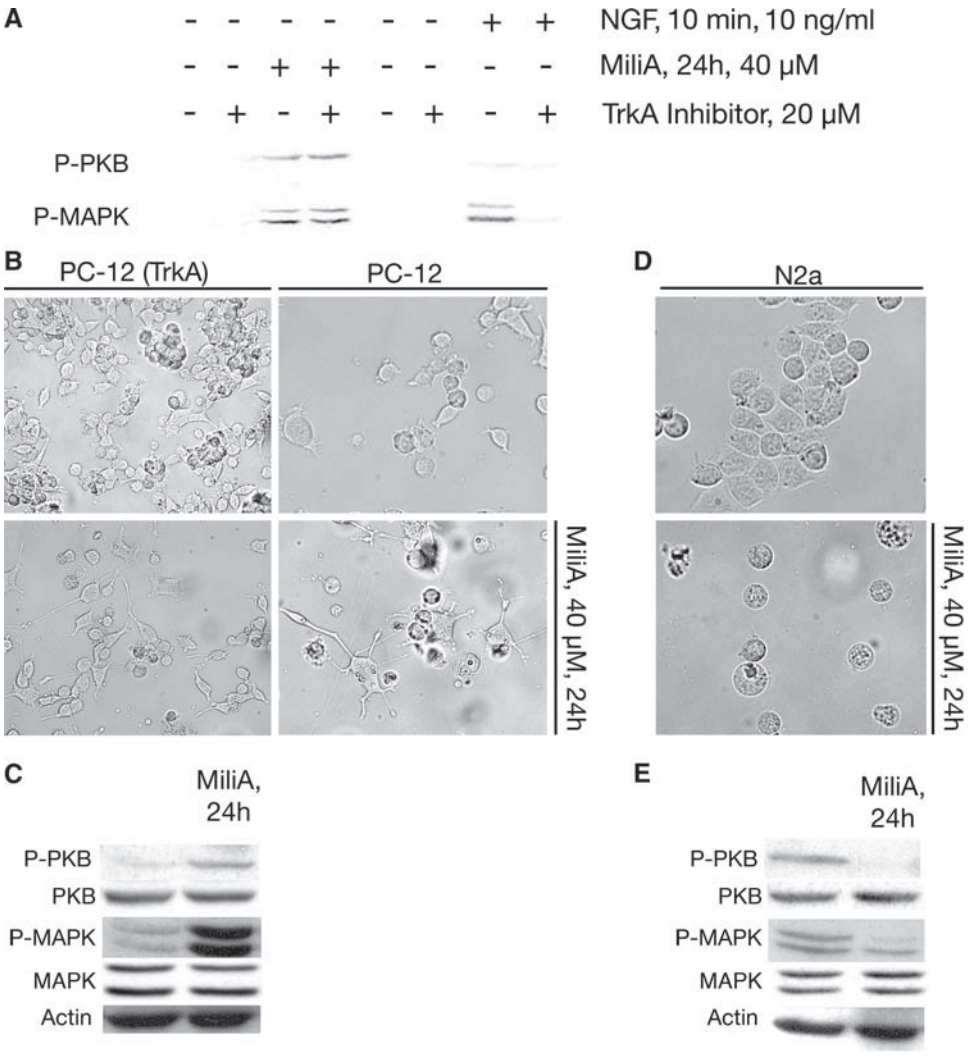


Fig. 1 40 μM MiliA induced TrkA-independent neurite extension in PC-12, but immediate apoptosis in N2a cells. (**a**) Inhibition of TrkA blocked NGF-dependent but not MiliA-dependent phophorylation of PKB and ERk1/2. (**b**) PC-12 cells stably transfected with TrkA (left panels) and naïve PC-12 (right panels) showed similar neuronal outgrowth after 24-h treatment with MiliA. (**c**) Phosphorylation of PKB (Ser473) and MAPKs (ERK1/2; Thr202/Tyr204) was clearly detected after 24 h in PC-12 cells. (**d**) N2a cells lost adherence and normal shape upon addition of MiliA and (**e**) no additional phosphorylation of PKB (Ser473) nor MAPKs (Thr202/Tyr204) could be detected after 24 h

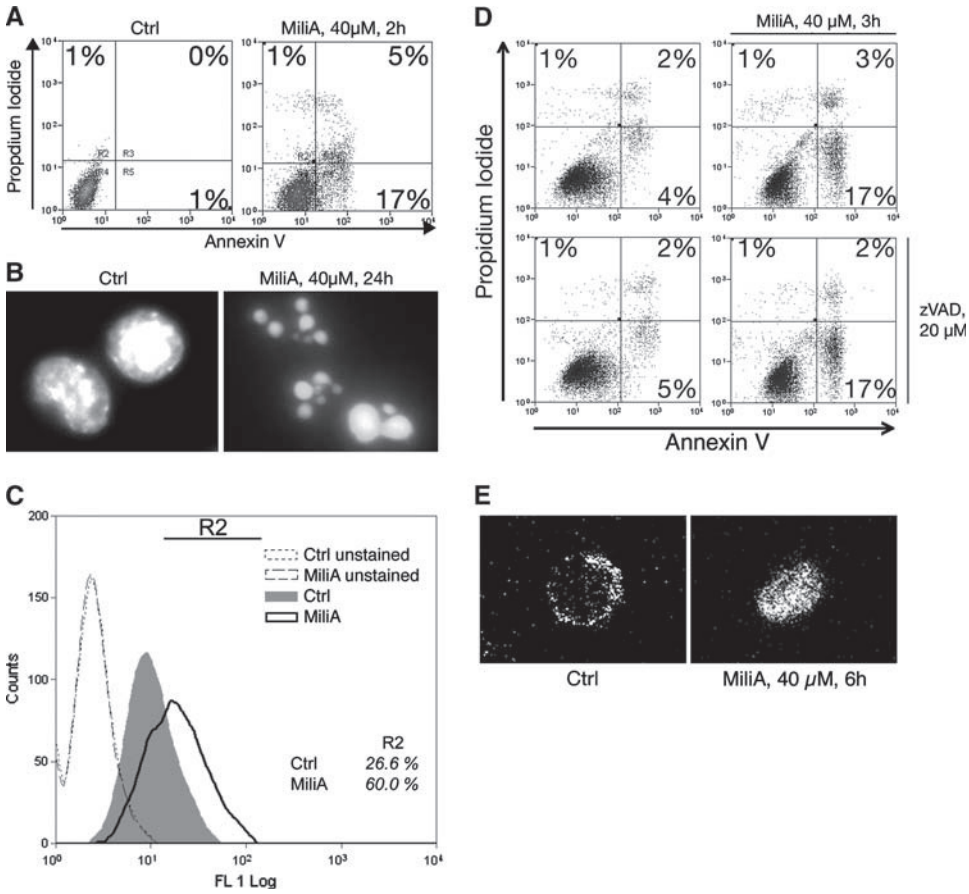


Fig. 2 Treatment with 40 μM MiliA led to caspase-dependent as well as -independent apoptosis in N2a cells. (**a**) Early signs of apoptosis by binding of Annexin V to the cell surface were detected as early as 2 h after treatment with MiliA (40 μM). (**b**) Late signs for programmed cell death such as chromatin condensation were observed in cultures treated with 40 μM MiliA for 24 h. (**c**) General activation of caspases was detected by FACS analysis of FITC-VAD-Fmk-stained cells after 24 h. (**d**) Co-treatment with the general caspase inhibitor zVAD (20 μM) led to only a marginal reduction of programmed cell death after 3 h as shown by Annexin V/PI staining. (**e**) Release and nuclear translocation of AIF was observed in cells treated for 4 h or longer, as shown by confocal immunofluorescence analysis

occurred in cells treated with MiliA for 8 h or longer (Fig. 2b), as visualised in N2a cells stained with Dapi. To test if caspases were involved in the execution of the cellular death observed, MiliA-treated N2a cells were stained with the FITC tagged form of the general caspase inhibitor zVAD and submitted to FACS-analysis: A distinct population showing activation of caspases roughly doubled from 26.6 to 60% 24 h post-treatment (Fig. 2c). We tested in a next step whether pre-treatment with zVAD (20 μM) would be able to suppress onset of apoptosis, but addition of zVAD only slightly diminished the extent of Annexin V-positive cells by $-2.3 \pm 8.7\%$ (data calculated from six independent experiments), and a complete inhibition was never observed after 3 h of treatment with 40 μM MiliA (Fig. 2d). Besides, co-incubation of N2a cells with zVAD and MiliA did not slow the progress of apoptosis, and a phenotype showing neurite extension was observed neither in absence nor in presence of zVAD. As caspase-independent pathways could in addition be activated by administration of MiliA, we checked for nuclear translocation of AIF, a protein normally residing in the mitochondrial intermembrane space [10]. In N2a cells, AIF translocation was detected by confocal immunofluorescence microscopy as early as 4–6 h post-treatment (Fig. 2e). When we checked for simultaneous release of cytochrome c, another pro-apoptotic mitochondrial protein [35], we failed to detect any significant change in localisation (not shown). These findings suggested a specific release of AIF.

As PC-12 showed neurite extension 1 day after addition of 40 μM MiliA, we investigated the effect of prolonged MiliA-treatment on PC-12 cells. Treatment for 1 day only slightly elevated the quantity of Annexin V-positive and Annexin V/PI double-positive cells, but these numbers were substantially increasing after 3 days (Fig. 3a). However, also PI-positive cells were observed and suggested additional necrotic mechanisms. Next we looked for caspase-activation using FACS-analysis of FITC-VAD-Fmk-stained cells but failed to detect any activation 1 day post-treatment (Fig. 3b). Even after 2 days post-treatment, no activation was observed (not shown). However, most of cells showed clear nuclear translocation of AIF between 2 and 3 days post-treatment (Fig. 3c) and analogous to the situation in N2a cells, no significant change in cytochrome c localisation could be detected.

Militarinone A has been reported to rapidly integrate into the membranous fraction [3] due to its amphiphilic properties [36], whereas release into the cytoplasm was markedly slower. As both PC-12 and N2a cells showed release of AIF, we isolated whole, viable mitochondria

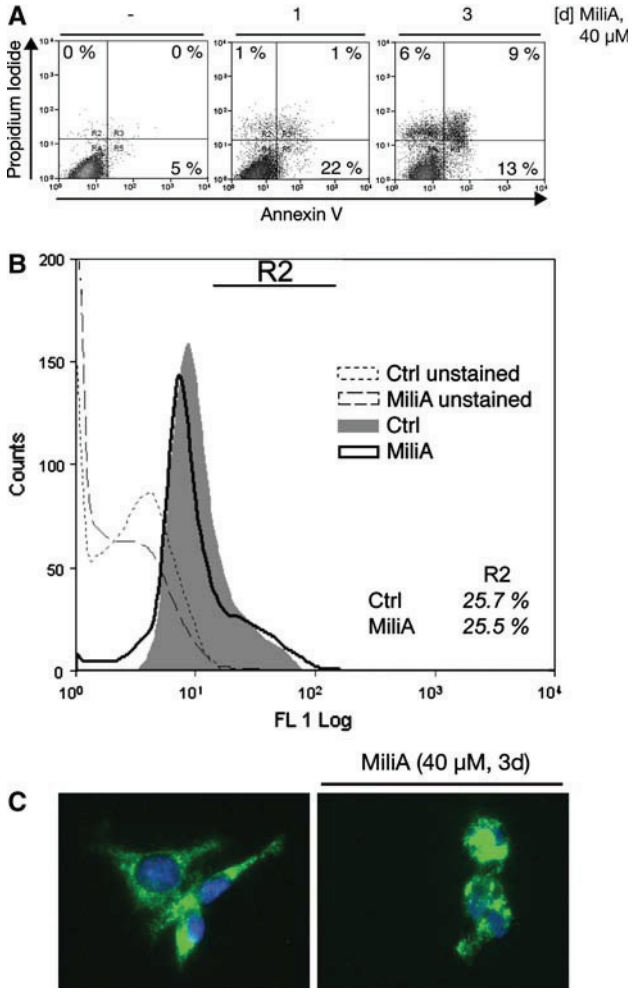


Fig. 3 PC-12 cells undergo homologous apoptosis as N2a cells upon prolonged treatment with 40 µM MiliA. (**a**) Early signs for apoptosis were detected after 1 day and became more pronounced after 3 days, as shown by binding of Annexin V to the cell surface and FACS Analysis in PC-12 cells. (**b**) General activation of caspases was not detected by FACS analysis of FITC-VAD-Fmk stained cells after 1 day. (**c**) Translocation of AIF (green) into the nucleus (blue) in PC-12 cells after a 3 day-treatment with MiliA (40 µM)

from both cell lines to check for membranal integration of MiliA. This rapidly occurred between 30 and 120 s after addition of the compound and was indistinguishable from integration into the cellular membrane (not shown) in both cell types. This observation was unexpected given that N2a showed release of AIF and early apoptosis whereas PC-12 displayed neurite outgrowth and a late onset of cell death. As loss of membrane potential is a sign for the de-stabilisation of mitochondria and a pre-requirement for AIF-release [37], we expected to see a rapid depolarisation in N2a, and a significantly slower effect in PC-12. Loss of membrane potential, however, took place at comparable speed in both cell lines (Fig. 4a). We therefore postulated that PC-12 cells would notwithstanding be less prone to the release of ROS from mitochondria due to loss of membrane potential and we visualised the occurrence of ROS-derived oxidation by measurement of DCF fluorescence via immunofluorescence microscopy. But again, both cell lines reacted in a similar manner to the administration of MiliA (Fig. 4b). When we quantitatively measured the production of ROS we found no significant difference between PC-12 and N2a cells (Fig. 4c). Moreover, the production of ROS by 40 µM MiliA was comparable to administration of 100 µM H_2O_2 and could be efficiently blocked by addition of 15 mM NAC. Given the fact that between depolarisation of the mitochondria and release of ROS, respectively, and induction of cellular death passed several hours (N2a) or even 1–3 days (PC-12), we assume that the release of ROS resulted in activation of signalling pathways not directly involved in apoptosis, as suggested by other publications [38].

To test the involvement of ROS-production in the propagation of apoptosis and neurite extension, respectively, we co-treated both cell-lines with 40 µM MiliA and 15 mM NAC. PC-12 cells remained unaffected (not shown) and N2a cells showed an apoptotic phenotype indistinguishable from MiliA-only treated cells (Fig. 4d). Even though NAC blocked accumulation of ROS (Fig. 4c), the compound did not rescue N2a cells from cell death.

Neither loss of membrane potential nor increase of ROS-levels sufficiently explained the marked difference in reaction of PC-12 and N2a cells to MiliA treatment. Therefore we tested for DNA-binding activities of transcription factors that are reportedly activated by ROS, and bind to consensus sequences of ARE, NF-κB and AP-1 [23–25]. ARE activity raised rapidly and transiently in N2a and PC-12 after addition of MiliA, reaching its peak after 1 h (Fig. 5a) as shown by electrophoretic mobility shift assay (EMSA). DNA-binding activity of AP-1 (Fig. 5b) and NF-κB (Fig. 5c), on the other hand, were differentially regulated in the two cell lines. N2a showed rapid induction of NF-κB and AP-1-binding, peaking between 2 and 4 h. Thereafter, no further activity could be detected. In PC-12 cells, binding of these two transcription factors was more pronounced but only observed from 24–48 h post-treatment.

To test if AP-1 and NF-κB were activated by ROS, we incubated N2a and PC-12 cells pre-treated with 15 mM NAC with MiliA for 4 and 24 h, respectively, and performed EMSA. Only the NF-κB DNA-binding activity was successfully inhibited by NAC in both cell lines (Fig. 5d), whereas AP-1 remained unaffected (not shown). These experiments suggested that ROS would not directly be involved in MiliA-dependent apoptosis but could contribute to initial survival mechanisms mainly through NF-κB activation.

p53 plays an important role in the management of intracellular stress and regulates basal levels of AIF [16].

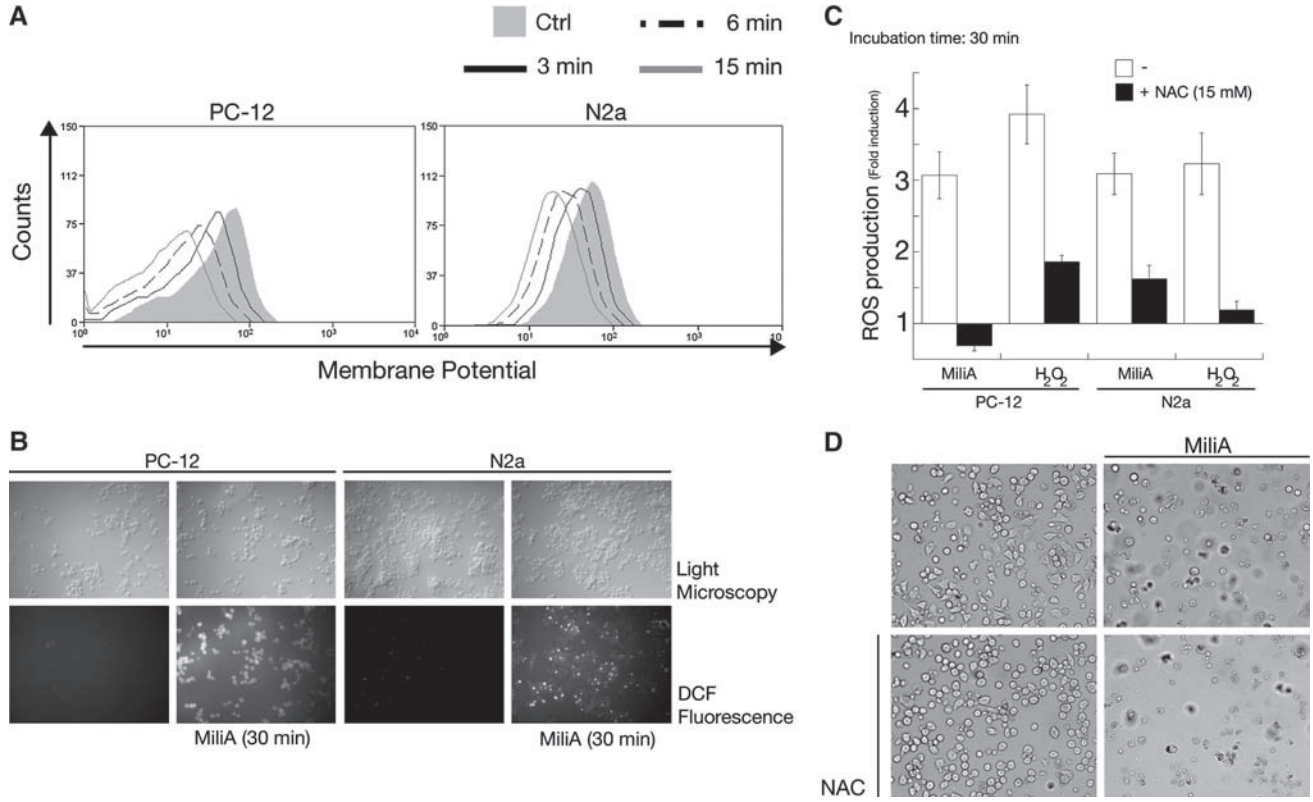


Fig. 4 Immediate loss of membrane potential and release of ROS were detected in both cell lines after addition of 40 μM MiliA. (**a**) Loss of membrane potential was observed shortly after addition of MiliA in PC-12 and N2a. (**b**) ROS production in both cell lines, as shown by immunofluorescence analysis of DCF. (**c**) Production of ROS quantitatively measured by DCF fluorescence in a multiplate reader. Data shown were calculated from three independent experiments and normalised to untreated cells. (**d**) N2a cells treated with 40 μM MiliA overnight underwent apoptosis and were not rescued by addition of NAC (15 mM)

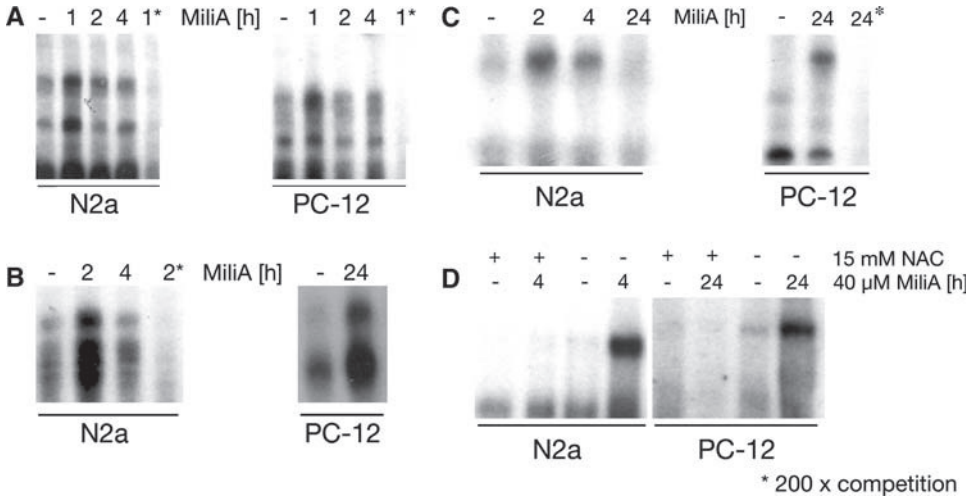


Fig. 5 Activation of the ARE, AP-1 and NF-κB transcription factors after addition of 40 μM MiliA in both cell lines as revealed by EMSA. DNA-binding activity of NF-κB was completely abolished by addition of 15 mM NAC. (**a**) Increased transient binding to the ARE consensus sequence was observed as early as 1 h post-treatment that declined thereafter. There was no obvious difference in ARE DNA-binding activity between PC-12 and N2a cells. (**b**) AP-1 and (**c**) NF-κB were transiently activated in N2a cells peaking at around 2 h post-treatment. PC-12 cells on the other hand showed more persistant DNA-binding activities that began 24 h after start of treatment.(**d**) Addition of 15 mM NAC completely inhibited MiliA-dependent NF-κB DNA-binding activity in both cell lines

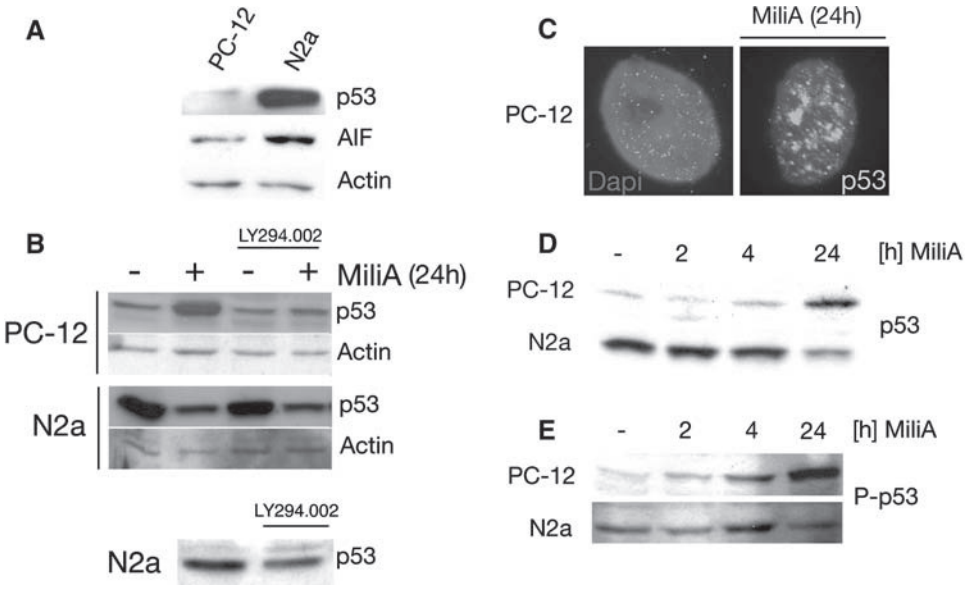


Fig. 6 N2a showed higher expression of p53 and AIF compared to PC-12 cells, and stabilisation of p53 was positively regulated by PI3-K. (**a**) Protein levels of p53 and AIF were higher in N2a than in PC-12 cells. (**b**) Co-treatment with 50 μM LY294.002 efficiently inhibited MiliA-dependent stabilisation (40 μM, 24 h) of p53 in PC-12 but did not block down-regulation in N2a cells. It did, however, slightly diminish basal expression of p53 in N2a cells. (**c**) p53 was almost exclusively localised in the nucleus of PC-12 cells before and after treatment with MiliA for 24 h (40 μM). (**d**) MiliA-dependent down-regulation of p53 in N2a cells did not take place before 4 h post-treatment. (**e**) Phosphorylation of p53 at Ser15 in response to treatment with MiliA (40 μM)

AIF appears to play a dual role: it protects cells against oxidative damage when located in the mitochondria, but increases the chance to succumb to cell death in response to severe stress and subsequent translocation of AIF. As there was a distinct release and nuclear translocation of AIF in both cell lines after addition of 40 μM MiliA, we analysed the expression of p53. N2a cells showed a high basal expression of p53 compared to PC-12 cells and a higher expression of AIF (Fig. 6a). Treatment of PC-12 with MiliA lead to up-regulation of p53, which was almost exclusively localised in the nucleus, whereas N2a cells showed a down-regulation 24 h post-treatment (Fig. 6b, c). The stabilisation of p53 in PC-12 cells was unexpected, since concommittant activation of PKB occurred (Fig. 1c). PKB has been reported to negatively regulate p53 through Mdm2 [17]. We, however, found that phosphorylation of PKB positively regulated p53, as co-treatment with LY294.002, a PI3-K-inhibitor, efficiently blocked PKB phosphorylation and stabilisation of p53 (Fig. 6b). A similar finding was previously published for p53 activation by cisplatin that was equally blocked by the PI3-K-inhibitor [39]. LY294.002, however, did not interfere with the MiliA-dependent down-regulation but led to a slightly diminished expression of basal p53 in untreated N2a cells after incubation with LY294.002 for 1 day (Fig. 6b).

As we had suggested that high p53 expression in N2a cells might be responsible for the sensitisation towards apoptosis induced by MiliA, it was surprising to find p53 down-regulation after 24 h in N2a. However, this down-regulation only occurred after induction of apoptosis. A time course experiment of p53 expression did neither show up- nor down-regulation within 4 h post-treatment (Fig. 6 d). To analyse activation of p53, we used an antibody directed against the phosphorylated form of p53 (Ser15), which was shown to be essential for its pro-apoptotic activity [40, 41]. We, however, only found a significant increase 4 h after addition of 40 μM MiliA, essentially before AIF translocation was observed (Figs. 6e and 2e). In PC-12 cells, stabilisation and activation by phosphorylation of p53 was not observed before 24 h post-treatment, but well before translocation of AIF could be detected (Figs. 6e and 3c).

To check if activation of p53 was indeed responsible for release of AIF and its nuclear translocation, we added MiliA to cells pre-treated for 30 min with PFTα, a known inhibitor of p53-transcriptional activity [42]. Consequently, localisation of AIF remained unchanged in PFTα pre-treated cells upon administration of MiliA in both cell types after 6 h (N2a; Fig. 7a) and 3 days (PC-12; Fig. 7c), respectively. Further, we checked if induction of apoptosis could be blocked by pre-treatment with PFTα and/or PFTα/zVAD combined treatment, which in fact was the case: PFTα alone or in combination with zVAD efficiently blocked initiation of apoptosis as measured by FACS of Annexin V/PI-double stained cells in both N2a (Fig. 7b) and PC-12 cells (Fig. 7d). zVAD (20 μM) alone, however,

only showed marginal reduction of Annexin V-positive cells in N2a and no reduction in PC-12. This is in agreement with the finding that PC-12 showed no caspase-activation in our assays in response to MiliA (Fig. 3b). Even application of up to 100 μM zVAD did not block onset of programmed cell death in neither cell line (not shown).

As LY294.002 blocked stabilisation of p53, we tested if treatment with the inhibitor 30 min prior to addition of MiliA would reduce the extent of cell death in N2a. Co-treatment with LY294.002 efficiently inhibited binding of Annexin-V after 3 h, and only a fraction of the cells lost their shape and adherence after 24 h (Fig. 8a, b).

As treatment with LY294.002 slightly reduced basal levels of p53 in N2a cells (Fig. 6b), we investigated whether phosphorylation at Ser15 of p53 would be repressed as well. Use of an antibody directed against P-p53 in immunofluorescence microscopy showed that activation of p53 by 40 μM MiliA was indeed affected by pre-treatment with LY294.002 (Fig. 8c). 95.5 ± 4.1% of MiliA-treated cells showed phosphorylation of p53 in immunofluorescence microscopy vs. 55.2 ± 4.2% in MiliA/LY294.002 co-treated cells (data calculated from three independent experiments). This reduction was confirmed by analysis of protein samples by SDS-PAGE (Fig. 8d) and was obviously sufficient to prevent immediate onset of apoptosis. However, MiliA-treated N2a cells in presence of LY294.002 succumbed to cell death between 24 and 48 h post-treatment (not shown).

Given that pre-treatment with LY294.002 prolonged survival of MiliA-treated N2a cells, we also applied the PI3-K-inhibitor to PC-12 cells to see if neurite extension and/or late apoptosis would be inhibited. However, PC-12 cells died within 24 h after addition of 50 μM LY294.002 in presence, but also in absence of 40 μM MiliA (not shown). Therefore we continued with N2a cells and observed postponed MiliA-dependent activation of the AP-1 transcription factor in presence of LY294.002 24 h after start of treatment. Interestingly, DNA-binding activity of AP-1 was not detected in MiliA-only treated cells at this time point (Figs. 9a and 5b). MiliA-dependent NF-κB DNA-binding activity on the other hand remained unchanged in presence or absence of LY294.002 (Fig. 5c and not shown). We therefore tested the hypothesis, that raised AP-1 DNA-binding activity is a pro-apoptotic event in this context [43]. As binding of c-jun to AP-1 consensus sequences is considered to be pro-apoptotic [26], we performed supershift analysis using a specific antibody directed against c-jun. We found c-jun-binding in both in MiliA-treated N2a after 4 h as well as in PC-12 after 24 h (Fig. 9b). In addition, the same supershift was also observed in N2a cells co-treated with LY294.002 and MiliA after 24 h (not shown).

Discussion

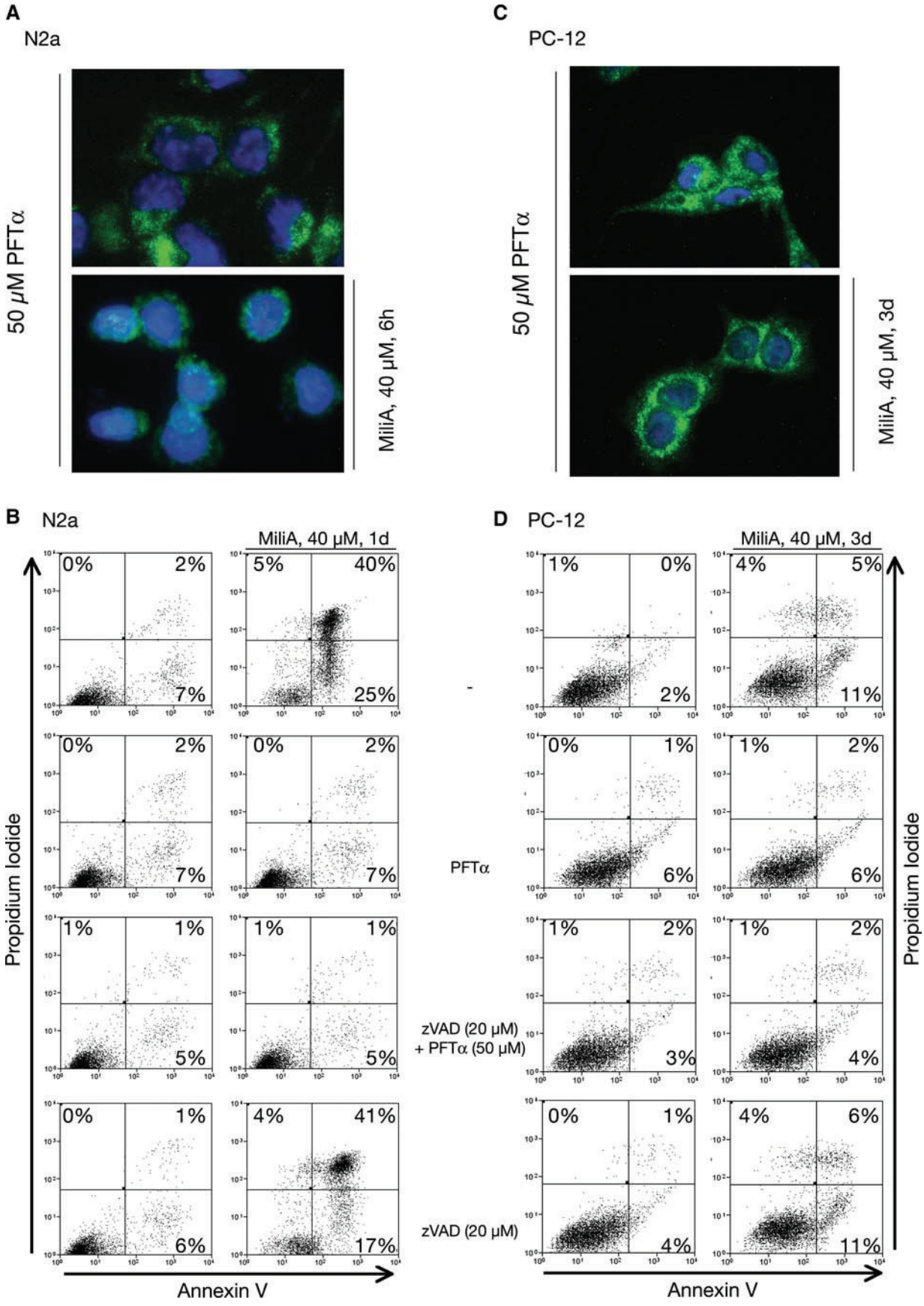
In this study we present evidence that a simple molecule by activating identical pathways is able to trigger events as diverse as neurite extension and apoptosis.

Differentiation is a complex process and involves diverse signalling pathways. In PC-12 cells, neuronal differentiation is usually initiated by the binding of NGF to TrkA and involves the subsequent activation of PI3-K/PKB, PKCs, Ras and MEK/ERK [5]. MiliA, a pyridone alkaloid from *Paecilomyces militaris* [36] basically does the same: it activates PKB and ERK1/2 [3] but circumvents the need for TrkA. This results in neurite extension in PC-12, and apoptosis in N2a cells. The outcome is similar to that reported for geldanamycin, an Hsp90 inhibitor, which provoked programmed cell death in PC-12 and neurite extension in N2a cells [44]. In another study using a MiliA-related compound, (+)-*N*-Deoxymilitarinone A, cytotoxic effects were observed in the human neuroblastoma cell line IMR-32, and neurite extension in PC-12 cells [45].

We report that MiliA is capable of initially driving two basically independent signalling pathways. First, it provides NGF-like activity, leading to neurite extension. The mechanism for activation of PI3-K/PKB and MEK/ERK1/2, however, remains elusive. As MiliA showed high affinity for membraneous compartments, we investigated possible stimulation of the small G-proteins Ras and Rac which are activated at the cytoplasmic side of the cellular membrane [9, 46, 47] but could not detect any consisting and specific activation neither at early nor late time points (data not shown).

By additional insertion into mitochondrial membranes, MiliA disturbed their integrity, which resulted in loss of membrane potential, release and accumulation of ROS, in both cell lines at comparable speed. Since addition of NAC blocked increase of ROS in PC-12 and N2a, but not the apoptotic pace, we assume that the release of oxidative molecules from mitochondria did not trigger apoptosis by itself. Furthermore, NAC did not inhibit cellular death in N2a and blocked NF-κB-activation suggested that accumulation of ROS was involved in defence and survival mechanisms. The subsequent release of AIF and its translocation to the nucleus was fast in N2a but delayed in PC-12, and suggests involvement of additional elements that control release of molecules at mitochondrial level [48]. This was corroborated by the fact that we were unable to detect co-release of cytochrome C. How exactly MiliA induces loss of membrane potential, a necessary pre-requisite for subsequent release of AIF [37], needs further investigation.

Second, it activates p53-dependent apoptosis [19, 20] mainly through a caspase-independent mechanism involving nuclear translocation of AIF, a molecule that is


A
N2a
50 μM PFTα
MiliA, 40 μM, 6h
C
PC-12
50 μM PFTα
MiliA, 40 μM, 3d
B N2a
MiliA, 40 μM, 1d
0% 2% 7%
5% 40% 25%
0% 2% 7%
0% 2% 7%
1% 1% 5%
1% 1% 5%
0% 1% 6%
4% 41% 17%
Propidium Iodide
Annexin V
D PC-12
MiliA, 40 μM, 3d
1% 0% 2%
4% 5% 11%
PFTα
0% 1% 6%
1% 2% 6%
zVAD (20 μM) + PFTα (50 μM)
1% 2% 3%
1% 2% 4%
zVAD (20 μM)
0% 1% 4%
4% 6% 11%
Propidium Iodide
Annexin V

◀ **Fig. 7** MiliA-dependent Apoptosis and nuclear translocation of AIF is blocked by PFTα, an inhibitor of p53 transcriptional activity. 50 μM PFTα, 20 μM zVAD or PFTα and 20 μM zVAD were added 30 min prior to the administration of 40 μM MiliA. (**a**, **c**) N2a and PC-12 cells were incubated with 50 μM PFTα prior to addition of 40 μM MiliA or vehicle only for 6 h (N2a) and 3 days (PC-12), respectively. The cells were subsequently fixed, stained and analysed for AIF localisation (green) by immunofluorescence microscopy. The DNA was counterstained using Dapi (shown in blue). (**b**, **d**) FACS analysis of Annexin V/PI double stained cells revealed a clear reduction of Annexin V- and Annexin V/PI-positive N2a (**b**) or PC-12 (**d**) cells when co-treated with PFTα or PFTα/zVAD plus MiliA for one (N2a) and 3 days (PC-12), respectively. Addition of zVAD alone prior to MiliA-treatment only resulted in a minor reduction of Annexin V-positive cells

positively regulated by basal levels of p53 [16]. N2a showed higher basal levels of p53 and AIF and underwent apoptosis within a few hours whereas PC-12 had a low p53-expression and first reacted with up-regulation of protein-levels before execution of programmed cell death. In the course of MiliA-induced signalling we found additional phosphorylation of p53 at Ser15, which is essential for its pro-apoptotic function [40, 41]. Using PFTα, a compound that does not inhibit p53 phosphorylation but its transcriptional activity, abolished MiliA-dependent AIF translocation and initiation of apoptosis. This suggests that p53 was involved in regulation of AIF-expression and localisation.

However, these pathways described are not independent but interact with each other. Whereas inhibition of ERK1/2-signalling by the MEK-inhibitor UO126 had not specific effect (data not shown), blocking of the PI3-K/PKB pathway by LY294.002 suppressed MiliA-dependent early execution of apoptosis in N2a cells. This finding is in contrast to reports showing that inhibition of PI3-K would lead to stabilisation of p53 and facilitated induction of apoptosis [17, 49]: rather on the contrary, basal expression as well as phosphorylation at Ser15 were moderately repressed. It is, however, in agreement with reports that showed inhibition of cisplatin-induced, p53-dependent apoptosis by LY294.002 [39]. Although the PI3-K/PKB-pathway is mainly known for its pro-survival activity [50], it contributes in this complex context to apoptosis-induction. Additionally, it postponed MiliA-dependent AP-1 DNA-binding activity.

A differentiated phenotype in N2a cells could not be observed, which is likely due to the inhibition of PKB-derived signalling. Unfortunately, this assumption could not be verified in PC-12 cells as they fatally reacted to PI3-K-inhibition as described.

It seems clear that the two cell-lines undergo apoptosis through the same mechanisms once they are activated to a similar extent. Further, it seems to be clear that MiliA-dependent ROS-production does not play a decisive role as

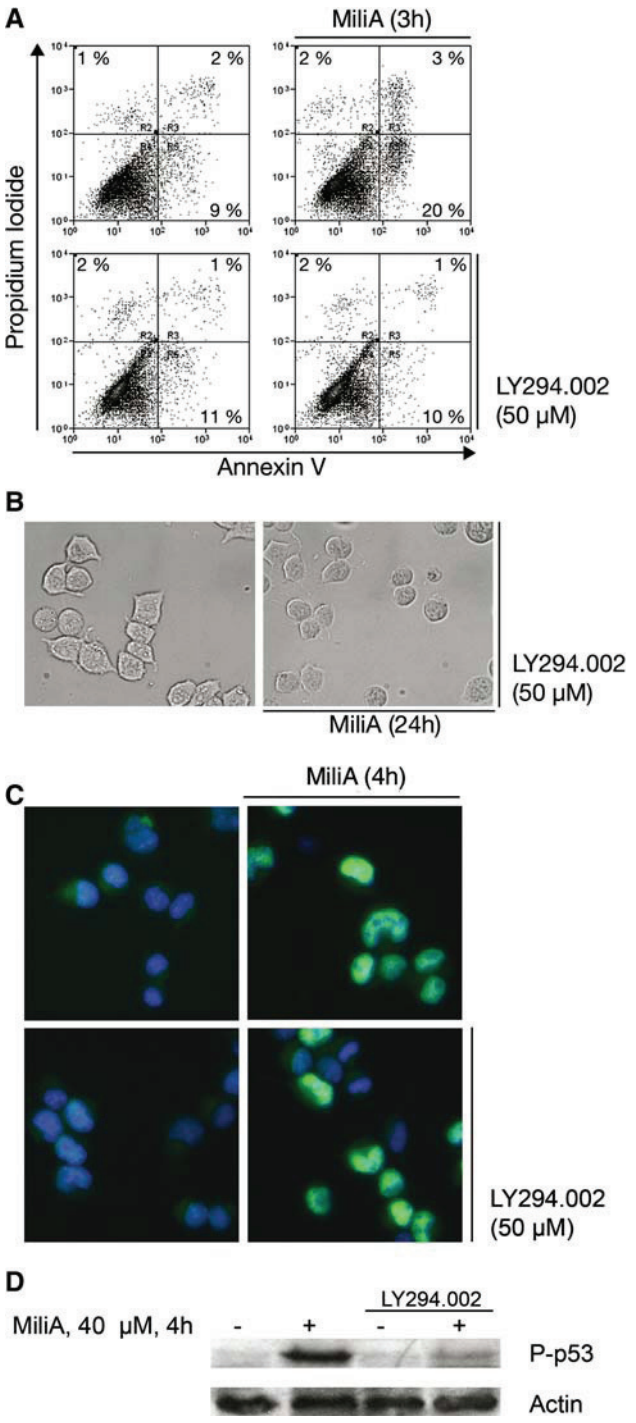


Fig. 8 Inhibition of PI3-K by LY294.002 blocks immediate onset of apoptosis in N2a cells and partly phosphorylation of p53 at Ser15. (**a**) Co-treatment with 40 μM MiliA (3 h) and 50 μM LY294.002 led to clearly reduced surface binding of Annexin V compared to the vehicle-treated controls. (**b**) LY294.002 efficiently inhibited late onset of cell death in N2a induced by 40 μM MiliA. (**c**, **d**) There was incomplete but significant reduction in p53 phosphorylation in LY294.002 and MiliA co-treated N2a cells after 4 h as shown by immunofluorescence microscopy (**c**) and SDS-PAGE (**d**)

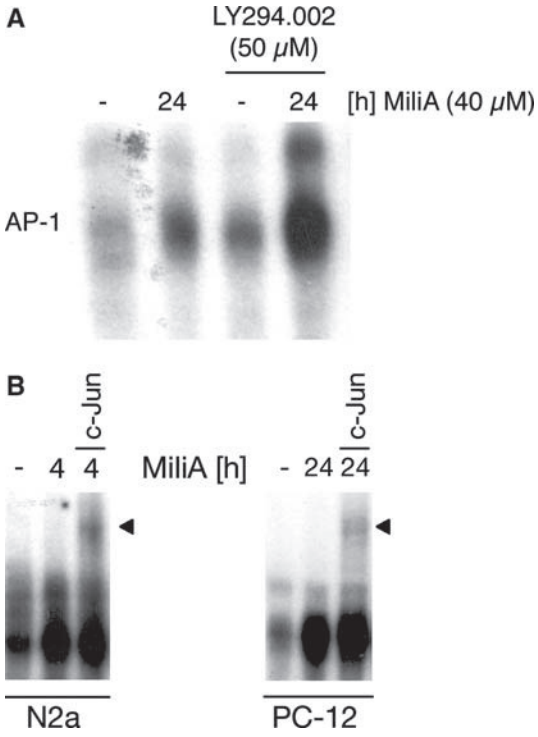


Fig. 9 LY294.002-dependent survival of MiliA-treated N2a cells leads to postponed AP-1 DNA-binding activity. (**a**) AP-1 DNA-binding activity occurs 24 h post-treatment in presence of LY294.002. (**b**) Binding of c-Jun to the AP-1 consensus sequence occurred both in N2a and PC-12 cells due to MiliA administration as visualised by supershift assays

both showed almost identical ROS-up-regulation. We nevertheless assume that it was involved to a certain degree. ROS also act as secondary messengers in intracellular signal transduction and help to induce or maintain the oncogenic phenotype of cancer cells [51]. Treatment with MiliA may just have driven the delicate balance over the edge. Preliminary data suggest that MiliA-treated primary cells (bovine lymph node cells and murine bone marrow mast cells) did not undergo cellular death even after a week's incubation. All cancer or cancerous cell lines tested on the other hand (PC-12, N2a, IMR-32, Jurkat T-cells, *Theileria parva*-infected T-cells [52]) died between 8 and 72 h after start of treatment (P. Küenzi and M. Hamburger, unpublished results).

Conclusion

Militarinone A induces neurite extension in PC-12 cells by persistent activation of pathways that are also involved in NGF-mediated differentiation, namely the PI3-K/PKB and MEK/ERK pathways. The continuous activation of these pathways finally leads to up-regulation of p53, release of AIF from mitochondria, and activation of the c-Jun/AP-1 transcription factor that has also been described as a "killer on the transcriptional leash" [43]. Application of MiliA to N2a cells, however, resulted in rapid onset of apoptosis by nuclear translocation of AIF, activation of caspases and c-Jun/AP-1. The main difference between the two cell types was the basal expression of p53, being high in N2a and low in PC-12. MiliA induced further stabilisation and activation of p53 as well as p53-dependent release and nuclear translocation of AIF in PC-12, which eventually resulted in apoptosis. MiliA induced the self-same pathways in both cell lines, initially leading to diverse, but finally to identical results.

References

1. Blesch A (2006) Neurotrophic factors in neurodegeneration. Brain Pathol 16:295–303
2. Pollack SJ, Harper SJ (2002) Small molecule Trk receptor agonists and other neurotrophic factor mimetics. Curr Drug Targets CNS Neurol Disord 1:59–80
3. Riese U, Ziegler E, Hamburger M (2004) Militarinone A induces differentiation in PC12 cells via MAP and Akt kinase signal transduction pathways. FEBS Lett 577:455–459
4. Frebel K, Wiese S (2006) Signalling molecules essential for neuronal survival and differentiation. Biochem Soc Trans 34:1287–1290
5. Vaudry D, Stork PJS, Lazarovici P, Eiden LE (2002) Differentiation pathway in PC12 Cells. Sci. STKE. http://stke.sciencemag.org/cgi/cm/stkecm;CMP_8038
6. Philpott KL, McCarthy MJ, Klippel A, Rubin LL (1997) Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. J Cell Biol 139:809–815
7. Kimura K, Hattori S, Kabuyama Y et al (1994) Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. J Biol Chem 269:18961–18967
8. Rubinfeld H, Seger R (2005) The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol 31:151–174
9. Kimmelman AC, Nunez Rodriguez N, Chan AM (2002) R-Ras3/M-Ras induces neuronal differentiation of PC12 cells through cell-type-specific activation of the mitogen-activated protein kinase cascade. Mol Cell Biol 22:5946–5961
10. Modjtahedi N, Giordanetto F, Madeo F, Kroemer G (2006) Apoptosis-inducing factor: vital and lethal. Trends Cell Biol 16:264–272
11. Porter AG, Urbano AG (2006) Does apoptosis-inducing factor (AIF) have both life and death functions in cells? Bioessays 28:834–843
12. Cregan SP, Dawson VL, Slack RS (2004) Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 23:2785–2796
13. Cande C, Cohen I, Daugas E et al (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84:215–222
14. Leist M, Jaattela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol 2:589–598
15. Urbano A, Lakshmanan U, Choo PH et al (2005) AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. Embo J 24:2815–2826
16. Stambolsky P, Weisz L, Shats I et al (2006) Regulation of AIF expression by p53. Cell Death Differ 13:2140–2149

17. Mayo LD, Donner DB (2002) The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. Trends Biochem Sci 27:462–467
18. Wymann MP, Zvelebil M, Laffargue M (2003) Phosphoinositide 3-kinase signalling—which way to target? Trends Pharmacol Sci 24:366–376
19. Vousden KH, Lu X (2002) Live or let die: the cell's response to p53. Nat Rev Cancer 2:594–604
20. Hofseth LJ, Hussain SP, Harris CC (2004) p53: 25 years after its discovery. Trends Pharmacol Sci 25:177–181
21. Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B (1997) A model for p53-induced apoptosis. Nature 389:300–305
22. D'Autreaux B, Toledano MB (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 8:813–824
23. Gloire G, Legrand-Poels S, Piette J (2006) NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 72:1493–1505
24. Lyakhovich VV, Vavilin VA, Zenkov NK, Menshchikova EB (2006) Active defense under oxidative stress. The antioxidant responsive element. Biochemistry (Mosc) 71:962–974
25. Nel A, Xia T, Mädler L, Li N (2006) Toxic potential of materials at the nanolevel. Science 311:622–627
26. Hess J, Angel P, Schorpp-Kistner M (2004) AP-1 subunits: quarrel and harmony among siblings. J Cell Sci 117:5965–5973
27. Rossler OG, Steinmuller L, Giehl KM, Thiel G (2002) Role of c-Jun concentration in neuronal cell death. J Neurosci Res 70:655–664
28. Karin M, Lin A (2002) NF-kappaB at the crossroads of life and death. Nat Immunol 3:221–227
29. van Delft MF, Huang DC (2006) How the Bcl-2 family of proteins interact to regulate apoptosis. Cell Res 16:203–213
30. Kuenzi P, Schneider P, Dobbelaere DA (2003) *Theileria parva*-transformed T cells show enhanced resistance to Fas/Fas ligand-induced apoptosis. J Immunol 171:1224–1231
31. Heussler VT, Kuenzi P, Fraga F, Schwab RA, Hemmings BA, Dobbelaere DA (2001) The Akt/PKB pathway is constitutively activated in Theileria-transformed leucocytes, but does not directly control constitutive NF-kappaB activation. Cell Microbiol 3:537–550
32. Nguyen T, Sherratt PJ, Pickett CB (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 43:233–260
33. Botteron C, Dobbelaere D (1998) AP-1 and ATF-2 are constitutively activated via the JNK pathway in *Theileria parva*-transformed T-cells. Biochem Biophys Res Commun 246:418–421
34. Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73:2424–2428
35. Gogvadze V, Orrenius S, Zhivotovsky B (2006) Multiple pathways of cytochrome c release from mitochondria in apoptosis. Biochim Biophys Acta 1757:639–647
36. Schmidt K, Gunther W, Stoyanova S, Schubert B, Li Z, Hamburger M (2002) Militarinone A, a neurotrophic pyridone alkaloid from *Paecilomyces militaris*. Org Lett 4:197–199
37. Ly JD, Grubb DR, Lawen A (2003) The mitochondrial membrane potential (deltapsi(m)) in apoptosis: an update. Apoptosis 8:115–128
38. Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5:415–418
39. Bar J, Lukaschuk N, Zalcenstein A, Wilder S, Seger R, Oren M (2005) The PI3K inhibitor LY294002 prevents p53 induction by DNA damage and attenuates chemotherapy-induced apoptosis. Cell Death Differ 12:1578–1587
40. Bouleau S, Parvu-Ferecatu I, Rodriguez-Enfedaque A et al (2007) Fibroblast growth factor 1 inhibits p53-dependent apoptosis in PC12 cells. Apoptosis 12:1377–1387
41. Nakanishi M, Ozaki T, Yamamoto H et al (2007) NFBD1/MDC1 associates with p53 and regulates its function at the crossroad between cell survival and death in response to DNA damage. J Biol Chem 282:22993–23004
42. Komarov PG, Komarova EA, Kondratov RV et al (1999) A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 285:1733–1737
43. Franzoso G, Zazzeroni F, Papa S (2003) JNK: a killer on a transcriptional leash. Cell Death Differ 10:13–15
44. Lopez-Maderuelo MD, Fernandez-Renart M, Moratilla C, Renart J (2001) Opposite effects of the Hsp90 inhibitor Geldanamycin: induction of apoptosis in PC12, and differentiation in N2A cells. FEBS Lett 490:23–27
45. Cheng Y, Schneider B, Riese U, Schubert B, Li Z, Hamburger M (2006) (+)-*N*-Deoxymilitarinone A, a neuritogenic pyridone alkaloid from the insect pathogenic fungus *Paecilomyces farinosus*. J Nat Prod 69:436–438
46. Symons M, Takai Y (2001) Ras GTPases: singing in tune. Sci STKE 2001: PE1
47. Wennerberg K, Rossman KL, Der CJ (2005) The Ras superfamily at a glance. J Cell Sci 118:843–846
48. Xu J, Ji LD, Xu LH (2006) Lead-induced apoptosis in PC 12 cells: involvement of p53, Bcl-2 family and caspase-3. Toxicol Lett 166:160–167
49. Sabbatini P, McCormick F (1999) Phosphoinositide 3-OH kinase (PI3K) and PKB/Akt delay the onset of p53-mediated, transcriptionally dependent apoptosis. J Biol Chem 274:24263–24269
50. Song G, Ouyang G, Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 9:59–71
51. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84
52. Dobbelaere DA, Kuenzi P (2004) The strategies of the Theileria parasite: a new twist in host-pathogen interactions. Curr Opin Immunol 16:524–530

2 (*E,Z*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone blocks mast cell degranulation

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Abstract

(*E,Z*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (indolinone) is an alkaloid that has been identified as a pharmacologically active compound in extracts of the traditional anti-inflammatory herb *Isatis tinctoria.* Indolinone has been shown to inhibit compound 48/80-induced mast cell degranulation in vitro. We further studied the effects of indolinone on some of the signal transduction molecules that are responsible for degranulation. Stimulation of mast cells with adenosine resulted in phosphorylation of PKB, MEK, MAPK, and PKC. Pre-treatment with indolinone inhibited phosphorylation of PKB, MEK and MAPK but phosphorylation of PKC remained unaffected. Adenosine-induced production of the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) was blocked after pre-treatment with indolinone, and an analysis of kinase activity revealed inhibition of all class I PI3-K. Fluorescence microscopy showed that indolinone was equally distributed in the cytoplasm and that cellular uptake was terminated within minutes. These findings suggest that indolinone acts via inhibition of PI3-K, abolishing subsequent intracellular signalling events and ultimately blocking mast cell degranulation.

Abbreviations: BMMC, murine bone marrow derived mast cells; DNP, dinitrophenyl; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; PI3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PKC, protein kinase C; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphophate;

Introduction

Isatis tinctoria (woad) is an ancient European dye and medicinal plant. It has traditionally been used as a source of indigo for blue dye and as anti-inflammatory medicine [1]. In China, the closely related *Isatis indigotica* is monographed in the Chinese Pharmacopoeia where it is indicated as a treatment for inflammation. Natural indigo, obtained by processing of various indigo plants, is used in traditional Chinese medicine to treat inflamed skin, intestines or mucosa [2]. The anti-inflammatory and anti-allergic potential of lipophilic *Isatis tinctoria* leaf extracts was confirmed some years ago in a pharmacological profiling involving some 20 clinically relevant targets [1,3], and later in several in vivo models for inflammation and allergy, and in a clinical pilot study for topical application [4-6].

Several pharmacologically active constituents of woad have been identified, such as tryptanthrin, indirubin, α-linoleic acid, and (*E,Z*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (indolinone). Tryptanthrin is a potent inhibitor of COX-2 [7] and 5-LOX [8], and of nitric oxide (NO) production catalyzed by inducible NO synthase (iNOS) [9]. Indirubin is used in Chinese medicine as an antileukemia medicine and has been shown to inhibit cyclin-dependent-kinase 2 (CDK2) [10]. The lipid constituent α-linolenic acid inhibited 5-LOX [8] and seems to act as a competitor of arachidonic acid and thus reduces the inflammatory response of the cells [11]. Indolinone (Fig. 1) inhibited compound 48/80-induced histamine release from rat peritoneal mast cells [12].

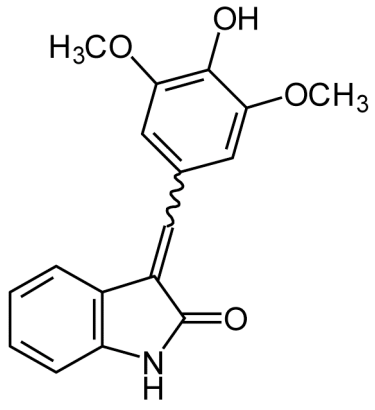

Fig. 1 - Chemical Structure of indolinone. Structure of (*E,Z*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (indolinone).

Indolinone derivatives have been tested in disease models for multiple sclerosis [13], cancer [14], HIV [15], and infection [16], and an indolinone derivative, sunitinib, was recently approved as tyrosine kinase inhibitor for treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumours [17]. The indolin-2-one scaffold seems to exhibit some general kinase inhibitor activity. Substituted indolin-2-ones occupy the ATP-binding site and act as ATP-mimetic inhibitors [18]. This suggested that the effects of indolinone could be due to kinase-inhibitory properties.

Although many cells are involved in allergy and inflammation, mast cells play an important role as initial effectors due to their localisation in the tissue. By release of pro-inflammatory molecules, mast cells initialise processes that result in early and late phase allergic reactions [19]. They are implicated in many diseases, such as allergy and asthma, gastrointestinal disorders, rheumatic diseases and multiple sclerosis [20,21]. Degranulation of mast cells requires binding of IgE antibodies to the high affinity IgE receptor (FcεRI) for activation and crosslinking of these antibodies by subsequent binding of an antigen. The crosslinking induces intracellular signalling events that lead to activation of the protein kinase Lyn that in turn phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) and Syk kinases. Class IA PI3-K recognise phosphorylated ITAMs as docking sites, which leads to the production of PtdIns(3,4,5)P_3 that recruits PKB (also known as Akt), leading to its activation and initiates a signalling cascade involving Bruton's tyrosine kinase (Btk) and Phospholipase Cγ and the Raf-MEK-MAPK pathway. This eventually induces opening of plasma membrane calcium channels leading to degranulation [22]. However, the FcεRI-mediated mast cell response is limited by subsequent de-phosphorylation of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by SH2-containing inositol 5'-phosphatase (SHIP). To circumvent the control of SHIP, murine mast cell responses are intensified in vitro and in vivo by autocrine signals depending on functional class IB PI3-K such as adenosine, acting through the A3 adenosine receptor (A3AR) [23, 24, 25, 26]. The stimulation of A3AR exclusively activates PI3-Kγ that transiently increases levels of the lipid second messenger PtdIns(3,4,5)P_3, resulting in signal-amplification following FcεRI activation, which was absolutely necessary to sustain calcium influx and degranulation [27,28].

Mast cell stabilisers such as disodium cromoglycate inhibit degranulation and late phase allergic reactions [19]. Other anti-inflammatory, anti-allergic or

immunosuppressive drugs such as corticosteroids or calcineurin-antagonists inhibit degranulation as well but additionally have many other effects [29].
The aim of this study was to elucidate whether indolinone interacts with the pathways that lead to mast cell activation and degranulation and at what level this interaction occurs.

Methods

Chemicals and Cell Culture

Murine bone marrow derived mast cells (BMMC) were cultured in Iscove's modified Dulbecco's medium (IMDM; Sigma- Aldrich, Buchs, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS; Amimed, Basel, Switzerland), 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine (both from Invitrogen, Basel, Switzerland). Cells were grown in humified atmosphere containing 5% CO_2 and maintained with 2 ng/ml recombinant murine interleukin-3 (IL3; PeproTech EC Ltd, London, UK). Chemicals used were: stem cell factor (SCF; Biosource, Invitrogen, Basel, Switzerland), adenosine (Ade; Sigma-Aldrich, Buchs, Switzerland), LY294002 (Alexis Corporation, Lausen, Switzerland), and wortmannin (Wort; Sigma-Aldrich). All solvents used were from Scharlau (Barcelona, Spain).
Indolinone was synthesised according to the procedure of Sun et al. [18]. The ratio of slowly interconverting *E* and *Z* isomers was determined as 81:19 by HPLC [12]. Indolinone showed fluorescence when excited at 488 nm or 405 nm, with an emission maximum at 499 nm. The absorption maxima are at 256 and 380 nm.

Western Blot

Immunoblot analysis was performed according to standard procedures. Equal amounts of cellular protein were separated on SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked and incubated overnight at 4°C with specific primary antibody diluted in blocking buffer (5% milk powder in TBS-Tween): anti-phospho-PKB (Thr308) 1:1000; anti-phospho-MAPK1/2 (Thr202/Tyr204) 1:1000; anti-phospho-MEK1/2 (Ser 217/221) 1:1000, anti-phospho-PKC (pan, γ Thr514; all Cell Signaling Technology, Beverly, MA); goat polyclonal anti-Actin 1:4000 (Santa Cruz Biotechnology, Santa Cruz, CA). Specific bands were

tagged with HRP conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) and detected using enhanced chemiluminescence (ECL Plus System, GE Healthcare, Little Chalfont, UK).

Flow Cytometry

Analysis of degranulation was performed with annexin V staining of mast cells [30,31]. Mast cells were sensitised with anti-DNP-IgE (Santa Cruz Biotechnology, Santan Cruz, CA) overnight and incubated with adenosine and DNP-albumin (Sigma-Aldrich, Buchs, Switzerland) for 20 min. Then, annexin V-Cy5 (Abcam, Cambridge, UK) was diluted according to the manufacturer's protocol and the cells were incubated for 10 min. Fluorescence was measured in FL4 (excitation 635 nm, emission 680/30 nm).

Measurement of intracellular indolinone was conducted in the violet 2 channel (FL7) (excitation 405 nm, emission 450/50 nm). Cells were analysed directly from medium that was diluted with PBS and indolinone (50 µM) was added during data acquisition. FACS analysis was performed on a Dako CyAn ADP LX 7 running Summit software (DakoCytomation, Glostrup, Denmark).

Fluorescence Microscopy

BMMC cells were suspended in PBS and left to adhere to glass coverslips and fixed in 4% formaldehyde in PBS for a minimum of 15 min at 4 °C. Nuclei were stained with DRAQ5 (Alexis Corporation, Lausen, Switzerland) according to the manufacturer's protocol. Cells plated on coverslips were mounted on glass slides with Fluorescent Mounting Medium (DakoCytomation, Glostrup, Denmark) and visualised by confocal microscopy (Leica DM RXE scanning confocal microscope) using Leica confocal software, version 2.5 (Leica Microsystems, Heidelberg, Germany).

Phosphatidylinositol (3,4,5)-trisphosphate levels

Levels of intracellular phosphatidylinositides were determined essentially as described [28,32]. Briefly, 4 million cells were incubated with 500 µCi [$^{32}P_i$] for 60 min at 37°C. After removal of non-incorporated [$^{32}P_i$], cells were treated with inhibitor for 30 min at 37°C and stimulated with adenosine for 30 s. The reaction was stopped by addition of 3 ml methanol/ chloroform (2:1, v/v, with 3 mM BHT and 100 µM NaF).

Phospholipids were extracted with 2.1 ml HCl 2.4 M and 2.1 ml chloroform and the organic phase was transferred to a tube with 1 ml methanol/ 0.5 M HCl and extracted again. The lower phase was evaporated by nitrogen stream and transferred to deacylation. Dried samples were incubated with methylamine solution in water/ methanol/ butanol (43: 46: 11) at 53° C for 50 min, all solvent was evaporated under vacuum, and then extracted with a mixture of butanol/ light petroleum/ ethyl formiate (20:4:1) and water [33]. The water-phase was dried in vacuum and analysed by HPLC analysis on a Partisphere SAX (10 µm, 4.6 mm x 250 mm; Alltech, Deerfield, IL) column. A gradient of water and 1M $NH_4H_2PO_4$, pH 3.8 was developed as described in [28]. Eluents from the column were examined online with a FLO-ONE A500 β-detector (Packard, Perkin Elmer, Monza, Italy).

Kinase assay

Appropriate concentrations of PI3-K isoforms p110 α, β, γ GST, γ tr, and δ (fused to interSH2 domains, a kind gift from Novartis, Basel, Switzerland) were mixed with 10 µg/ml L-α-phosphatidylinositol (Sigma-Aldrich, Buchs, Switzerland) dissolved in 0.3% octylglucoside kinase buffer (10 mM Tris HCl, 3 mM $MgCl_2$, 50 mM NaCl, 0.8 mM CHAPS, 1 mM DTT). After 5 min pre-treatment with indolinone or DMSO, 2 µM ATP (Roche, Basel, Switzerland) dissolved in kinase buffer were added to start the reaction. Reactions were incubated for 30-90 min, depending on the kinase isoform. Kinase Glo (Promega, Madison, WI) was added to each well to start the luciferase reaction. After 15 min incubation at room temperature, plates (white 96 well luciferase plates (Berthold, Bad Wildbad, Germany) were read out in a luminometer (Centro LB 960, Berthold) with an integration time of 0.5 s. Samples were run in duplicate.

Results

Inhibition of degranulation in murine bone marrow derived mast cells

Indolinone had been previously shown to inhibit compound 48/80-induced histamine release in rat peritoneal mast cells [12]. We reconfirmed this effect in antigen-stimulated degranulation of murine bone marrow derived mast cells (BMMC), to demonstrate that this effect was neither species-dependent nor assay-specific.

BMMC were sensitised overnight with anti DNP-IgE (100 ng/ml) and indolinone (50 µM) was added 30 min before stimulation where applicable. Degranulation was simultaneously induced with the antigen DNP-albumin (5 ng/ml) and adenosine (2 µM), as stimulation with antigen DNP-albumin alone only resulted in week responses. Indolinone pre-treatment reduced the number of degranulated cells from 63.1% ± 8.7% to 23.5% ± 4.6%, only just above Ctrl level (15.0% ± 0.1%) (Fig. 2A & B). Even though we used a different assay setup, another kind of cells, and changed the type of degranulation-inducing agents, indolinone clearly inhibited degranulation in BMMC. Due to interference with indolinone fluorescence occurring at all wavelengths when excited with 488 or 405 nm, typical hexosaminidase-release measurement had to be abandoned. Instead, degranulation was measured using Cy5-conjugated annexin V. Annexin V-Cy5 was exited at 635 nm and emission was measured at 680/30 nm.

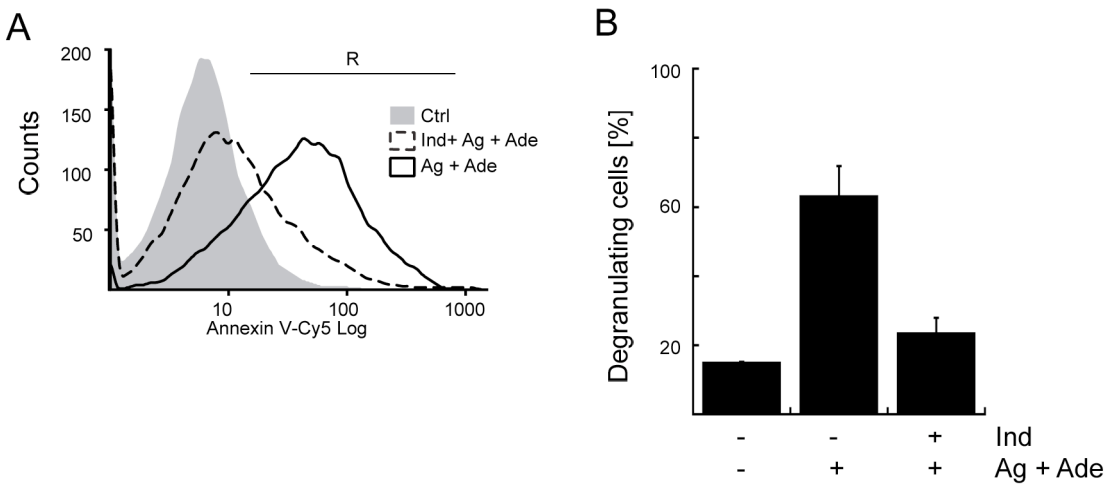

Fig. 2 - Degranulation of BMMC. (A) Mast cells were sensitised with IgE antibodies over night and subsequently challenged with antigen (Ag) and 5 µM adenosine. Indolinone (50 µM) was added to the cells 30 min before challenge. Cells were stained with annexin V-Cy5 and degranulation was measured by flow cytometry. Shown is a typical experiment. Region R was determined as approx. 15% degranulation of control cells. (B) Histogram of cells in Region R of three independent experiments. Shown are the mean ± S.E.: Ctrl: 15.0% ± 0.1%; Ag + Ade: 63.1% ± 8.7%; Ind + Ag + Ade: 23.5% ± 4.6%.

Reduced phosphorylation of selected signal transduction molecules involved in degranulation

Signals that lead to degranulation involve phosphorylation of several signalling proteins such as PKB, MEK and MAPK. Furthermore, involvement of PKC has been

suggested [34-36]. Therefore, we checked for phosphorylation of these proteins upon stimulation with adenosine and analysed for differences between cells pre-treated with indolinone or untreated controls. Stimulation of BMMC with 5 µM adenosine for 30 s resulted in phosphorylation of PKB (Thr308), MEK1/2 (Ser 217/221), MAPK1/2 (Thr202/Tyr204), and PKC (pan, γ Thr514). Pre-treatment with 50 µM indolinone 30 min prior to stimulation diminished the phosphorylation of PKB, MEK, and MAPK, whereas phosphorylation of PKC was not affected (Fig. 3A). Pre-treatment with the PI3-K inhibitor wortmannin also blocked phosphorylation of PKB, MEK, and MAPK. Quantitative analysis by flow cytometry of adenosine-induced PKB phosphorylation showed a dose-dependent inhibition after indolinone pre-treatment. Phosphorylation of PKB was almost completely blocked by 50 µM indolinone in respect to LY pre-treatment, and partially by 25 µM indolinone (Fig. 3B).

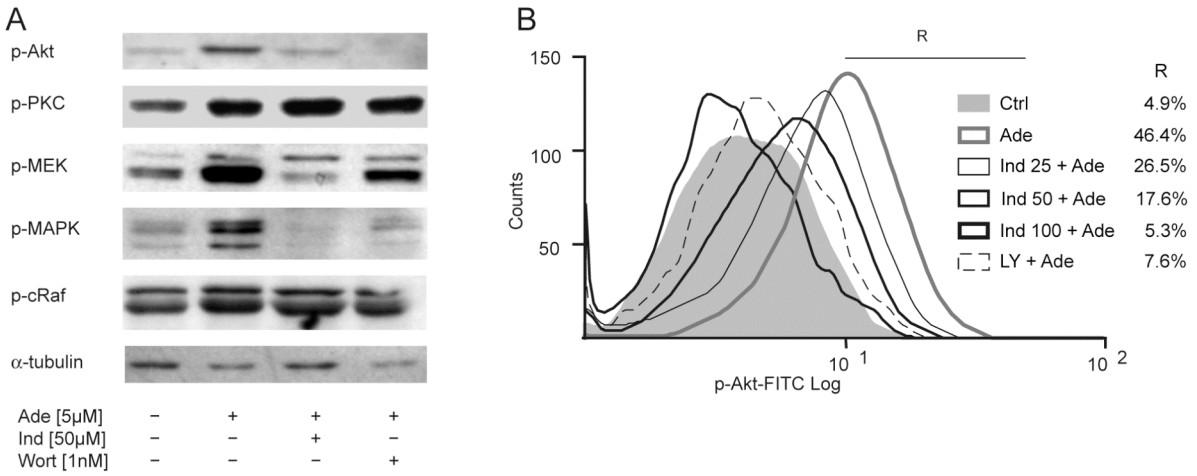

Fig. 3 - Phosphorylation of important signalling molecules involved in degranulation, analyzed by western blot and flow cytometry. (A) Equal amounts of cellular protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed using specific antibodies. α-tubulin was used as an internal loading control. BMMC cells (0.5 x 10^6) were starved in 2% serum for 4 h, treated with indolinone or inhibitor (Wort) for 30 min, and then stimulated with adenosine (Ade) for 30 s. Untreated cells were used as control. Shown are typical results from at least three independent experiments. (B) Flow cytometry analysis of PKB-phosphorylation. Cells were fixed and stained using specific antibodies, followed by flow cytometry analysis of 10'000 cells per sample in the FITC channel (FL1). BMMC cells (0.5 x 10^6) were starved in 2% serum for 4 h, treated with indolinone (25, 50, and 100 µM) or LY (50 µM) for 30 min, and then stimulated with adenosine (5 µM) for 30 s. Shown is a typical result from at least three independent experiments.

Adenosine-induced rise of PtdIns(3,4,5)P_3 levels inhibited by indolinone

Generation of PtdIns(3,4,5)P_3 by PI3-K induces translocation of PKB towards the membrane and its binding to PtdIns(3,4,5)P_3 via the pleckstrin homology domain [37]. Eventually, PDK1 [38] and mTORC2 [39] phosphorylate PKB at Thr308 and Ser473, respectively.

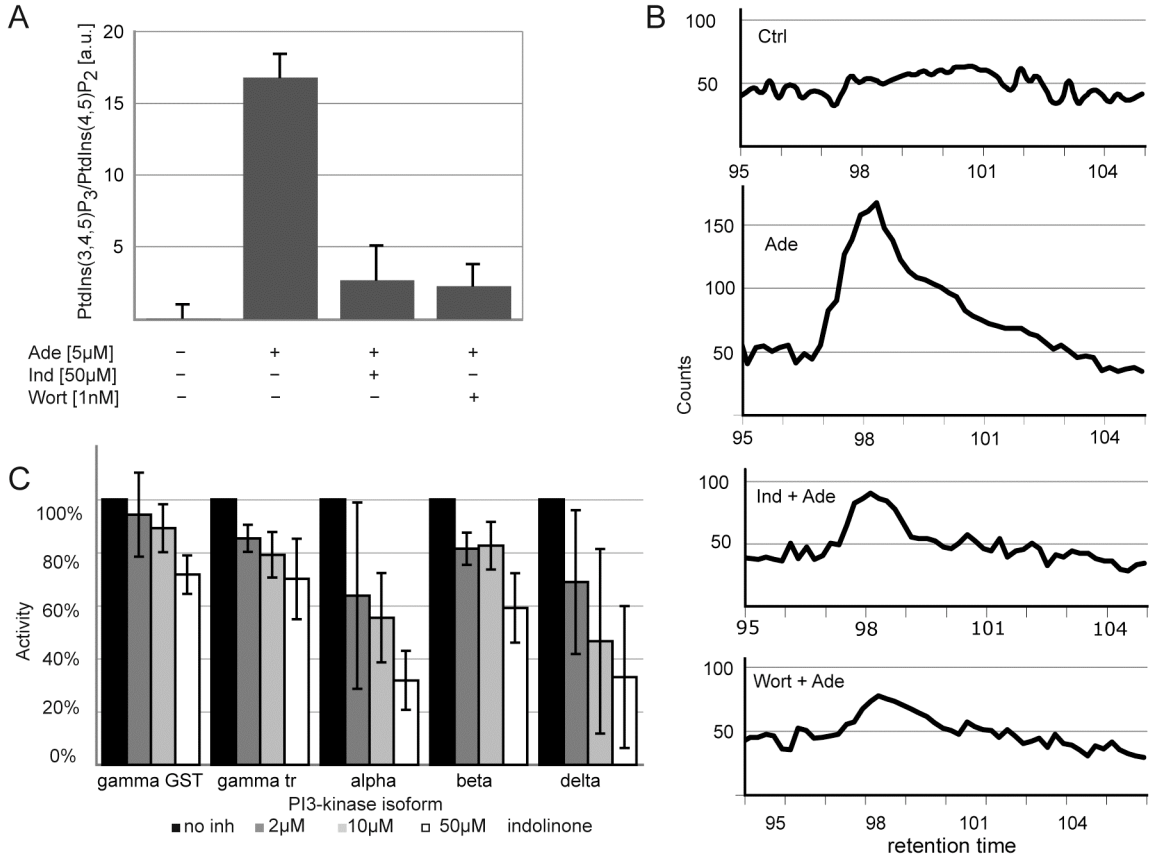

Fig. 4 - Analysis of PI(3,4,5)P_3 levels in BMMC cells and activity of PI3-K class IA and IB isoforms. (A) BMMC cells were labelled with $^{32}P_i$, and phospholipids were extracted, deacylated, and separated by HPLC. Peaks of interest were integrated and PtdIns(3,4,5)P_3 levels expressed in relation to the integral of PtdIns(4,5)P_2. BMMC cells (4 x 10^6) were starved in 2% serum for 4 h, treated with indolinone or wortmannin for 30 min, and then stimulated with adenosine for 30 s. Shown are the mean ± S.E. of three independent experiments. (B) Chromatograms of PtdIns(3,4,5)P_3 analysis, recorded by flow scintillation from a typical experiment. (C) PI3-Ks were co-incubated with ATP and either indolinone in different concentrations or DMSO as a control. Kinase activity was determined as consumption of ATP and residual ATP was measured by luciferase reaction. Samples were measured as duplicates in three independent experiments. Kinase activity of the control was defined as 100%.

We analysed PtdIns(3,4,5)P_3 levels in BMMC to determine whether inhibition of PKB-phosphorylation by indolinone was due to reduced PtdIns(3,4,5)P_3 production. Stimulation of BMMC with adenosine results in elevated PtdIns(3,4,5)P_3 levels and

preliminary experiments showed that PtdIns(3,4,5)P_3 levels reached a maximum 30 s post treatment. PtdIns(3,4,5)P_3 levels were quantified relative to PtdIns(4,5)P_2 that was used as internal standard. This ratio increased 15-fold after stimulation with adenosine. Pre-treatment with indolinone significantly reduced this increase to less than 5-fold, comparable to pre-treatment with wortmannin (Fig. 4A and 4B).

Inhibition of PI3-Ks class IA and IB by indolinone

The activity of all PI3-Ks class IA and IB isoforms (α, β, γ, δ), responsible for the generation of PtdIns(3,4,5)P_3, was tested in an in vitro assay after addition of different concentrations of indolinone. The γ isoform was tested in two different ways, as the complete GST tagged enzyme and as a trunctuated form with 144 amino acids less. We expected reduced kinase activity as the reason for the blocked increase of PtdIns(3,4,5)P_3 levels. Indeed, indolinone inhibited all PI3-K isoforms to some extent (Fig. 4C). When incubated with 50 µM indolinone the α and the δ isoform were inhibited to less than 50%, whereas the other isoforms still showed activities over 60%. Degranulation in BMMC is predominantly induced by PI3-Kγ and we expected a clear inhibition by indolinone.

Localisation of indolinone in BMMC

Due to the fluorescent properties of indolinone itself, we were able to visualise its localisation in cells directly and monitor its uptake into the cell by flow cytometry. Although we had expected to see some accumulation at the membrane, analysis of indolinone-stained cells by confocal microscopy (Fig. 5A) showed equal distribution throughout the cytoplasm, and no staining of the nucleus. Administration of indolinone during flow cytometry measurement produced an instant increase in fluorescence. After less than 3 min no further increase was detected and fluorescence persisted for the duration of the measurement (15 min). This showed that indolinone was immediately taken up into the cells and retained in the cytosol (Fig. 5B and 5C). We further tested if BMMCs would regain their ability to degranulate after removal of indolinone and incubation in indolinone-free media for two hours, which was not case (Fig. 5D and 5E).

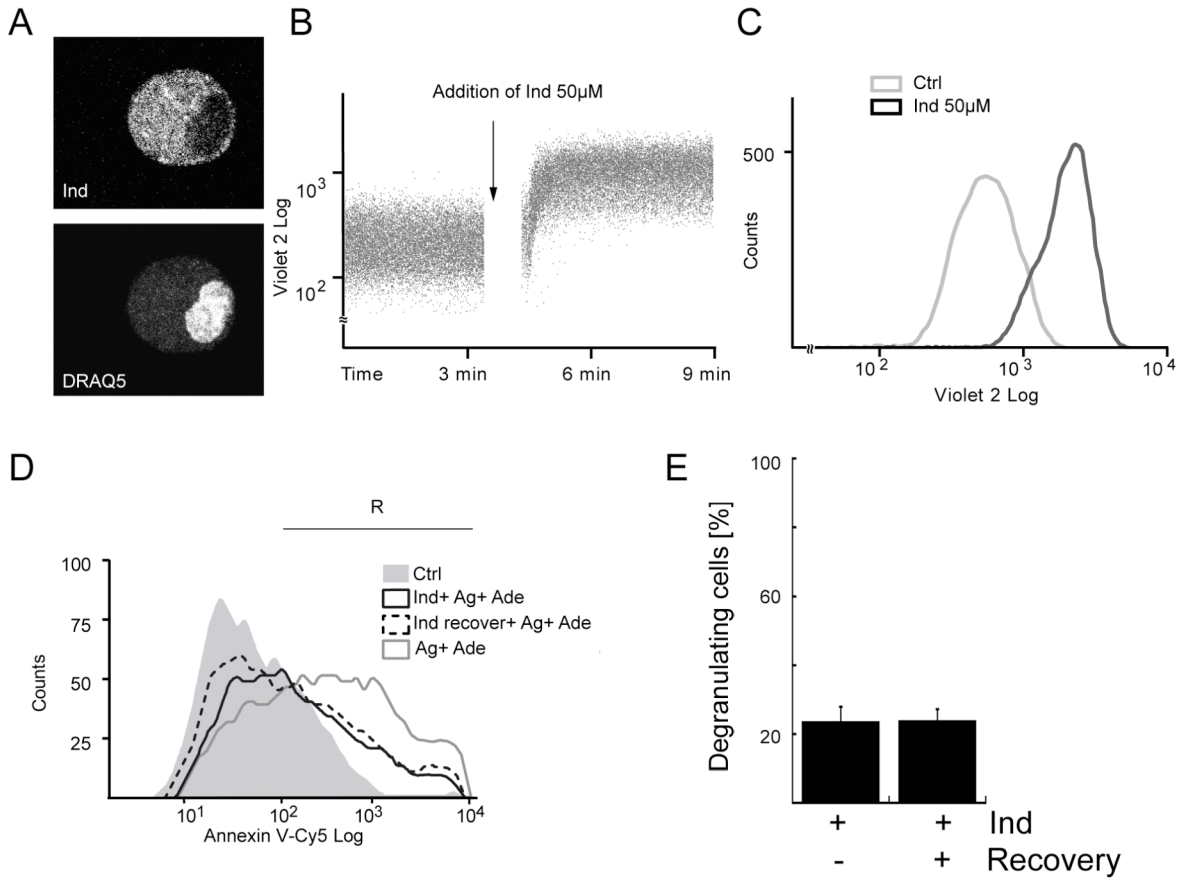

Fig. 5 - Appearance and localization of indolinone in BMMCs. Due to the fluorescent properties of indolinone itself, it was possible to monitor its appearance and localisation in the cell. (A) BMMC were fixed, stained with indolinone and DRAQ5 and mounted for fluorescence microscopy. The upper panel illustrates localisation of indolinone in the cell, the lower panel shows DRAQ5 staining of the nucleus. (B+C) Flow cytometry analysis of BMMCs. (B) Administration of 50 µM indolinone during ongoing measurement. (C) Fluorescence of BMMC before and after addition of 50 µM indolinone (Ind). (D) Mast cells were sensitised with IgE antibodies over night and subsequently challenged with antigen (Ag) and 5 µM adenosine (Ade). 50 µM Indolinone (Ind) was added to the cells for 30 min. Cells were subsequently washed three times and left for 2 hours or analysed immediately. Cells were stained with annexin V-Cy5 and degranulation was measured by flow cytometry. Shown is a typical experiment. Region R was determined as approx. 15% degranulation of control cells. (E) Histogram of cells in Region R of three independent experiments. Shown are the mean ± S.E.: Ind + Ag + Ade: 23.5% ± 4.6%; Ind + recovery + Ag + Ade: 23.9% ± 3.4%.

Discussion

The only drugs in clinical use that directly inhibit mast cell degranulation are disodium cromoglycate and its derivative nedocromil [19]. Cromoglycate was developed from khellin, a furanochromone from the anti-allergic plant *Ammi visnaga*. It is a mast cell stabilising agent that has been shown to block ion-channels. However, cromoglycate likely has additional effects and its mode of action is still not very clear [40]. Various other agents have been used experimentally to inhibit degranulation [29], but were not applicable for clinical use. The most recent approaches include Syk kinase inhibitors [41,42] and anti IgE antibodies. One such anti IgE antibody, Omalizumab (Xolair), has recently reached the market [43,44].

Extracts of *Isatis tinctoria* have traditionally been used in the treatment of anti-inflammatory complaints. Previous studies assigned specific facets of the in vitro pharmacological spectrum of the extract to specific compounds [7,8] in this complex multi-component mixture. In the model of compound 48/80 stimulated mast cell degranulation, indolinone was significantly more potent than disodium cromoglycate (IC50 of 15 µM (0.0045 µg/ml) vs 1.5 mM). In an earlier study *Isatis* extract had an IC50 of 2.3 µg/ml in this assay [3]. Given that indolinone is a minor constituent in the extract (typical concentrations of 0.04%) [45], it is likely that other components of the extract contribute to its mast cell stabilising activity.

Marquardt et al. showed suppressed degranulation of BMMCs after addition of the PI3-K inhibitor wortmannin [46]. Kinase activity of all PI3-K class I isoforms was inhibited by indolinone. Hence, it can be assumed that degranulation of BMMC was impaired due to PI3-K inhibition. All PI3-K-subtypes were blocked by indolinone to some extent, but none of them substantially preferred. PI3-K isoforms important for mast cell activation are p110γ and δ. Downstream of the high-affinity IgE receptor FcεRI is the tyrosine kinase-activated p110δ and downstream of the adenosine receptor the GPCR-activated p110γ [47].

Consequently, phosphorylation of PKB, MEK, and MAPK was blocked due to inhibition of the upstream PI3-Ks. Adenosine-dependent activation of PKC, however, was reported to be independent of PI3-K activity and mediated through PLC only [48,49]. Our findings agree with that model, since adenosine-induced phosphorylation of PKC persisted after indolinone pre-treatment. Furthermore, the

intact PKC phosphorylation revealed that indolinone was not an unspecific inhibitor of phosphorylation but rather blocked all events downstream of the PI3-K.

The calculated distribution coefficient (clogD) of indolinone at pH 7 is 2.28, and polar surface area (PSA) is 67.8 $Å^2$. These physico-chemical properties explain its rapid cellular uptake, its distribution in the cytoplasma, and its ability to persist within and retain its inhibitory role.

Even though the point of action is supposedly localised near or at the cellular membrane, equal distribution in the cytoplasm may confer some advantage since the inactive proteins are usually located in the cytoplasm and recruited to the membrane upon induction.

In this study we have shown that indolinone blocks antigen-induced mast cell degranulation, decreases adenosine-stimulated production of PtdIns(3,4,5)P_3, and inhibits activity of all PI3-K class I isoforms. The findings corroborate earlier findings with the lipophilic *Isatis* extract and thus contribute to a better understanding of molecular modes of action and synergistic effects of its constituents.

References

[1] Danz H. Untersuchungen zur antiinflammatorischen Wirkung und zur Analytik von Tryptantrin in *Isatis tinctoria* L. Biologisch-Pharmazeutische Fakultät. Jena: Friedrich-Schiller University, 2000.

[2] Stöger E. Arzneibuch der chineischen Medizin: Wissenschaftliche Verlagsgesellschaft.

[3] Hamburger M. Isatis tinctoria - from the rediscovery of an ancient medicinal plant towards a novel anti.inflammatory phytopharmaceutical. Phytochem Rev 2002;1:333-44.

[4] Heinemann C, Schliemann-Willers S, Oberthur C, Hamburger M, Elsner P. Prevention of experimentally induced irritant contact dermatitis by extracts of Isatis tinctoria compared to pure tryptanthrin and its impact on UVB-induced erythema. Planta Med 2004;70 (5):385-90.

[5] Recio MC, Cerda-Nicolas M, Hamburger M, Rios JL. Anti-arthritic activity of a lipophilic woad (Isatis tinctoria) extract. Planta Med 2006;72 (8):715-20.

[6] Recio MC, Cerda-Nicolas M, Potterat O, Hamburger M, Rios JL. Anti-inflammatory and antiallergic activity in vivo of lipophilic Isatis tinctoria extracts and tryptanthrin. Planta Med 2006;72 (6):539-46.

[7] Danz H, Stoyanova S, Wippich P, Brattstrom A, Hamburger M. Identification and isolation of the cyclooxygenase-2 inhibitory principle in Isatis tinctoria. Planta Med 2001;67 (5):411-6.

[8] Oberthur C, Jaggi R, Hamburger M. HPLC based activity profiling for 5-lipoxygenase inhibitory activity in Isatis tinctoria leaf extracts. Fitoterapia 2005;76 (3-4):324-32.

[9] Ishihara T, Kohno K, Ushio S, Iwaki K, Ikeda M, Kurimoto M. Tryptanthrin inhibits nitric oxide and prostaglandin E(2) synthesis by murine macrophages. Eur J Pharmacol 2000;407 (1-2):197-204.
[10] Hoessel R, Leclerc S, Endicott JA, Nobel ME, Lawrie A, Tunnah P, Leost M, Damiens E, Marie D, Marko D, Niederberger E, Tang W, Eisenbrand G, Meijer L. Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat Cell Biol 1999;1 (1):60-7.
[11] Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr 2002;21 (6):495-505.
[12] Ruster GU, Hoffmann B, Hamburger M. Inhibitory activity of indolin-2-one derivatives on compound 48/80-induced histamine release from mast cells. Pharmazie 2004;59 (3):236-7.
[13] Bouerat L, Fensholdt J, Liang X, Havez S, Nielsen SF, Hansen JR, Bolvig S, Andersson C. Indolin-2-ones with high in vivo efficacy in a model for multiple sclerosis. J Med Chem 2005;48 (17):5412-4.
[14] Kaur K, Talele TT. 3D QSAR studies of 1,3,4-benzotriazepine derivatives as CCK(2) receptor antagonists. J Mol Graph Model 2008;27 (4):409-20.
[15] Boechat N, Kover WB, Bongertz V, Bastos MM, Romeiro NC, Azevedo ML, Wollinger W. Design, synthesis and pharmacological evaluation of HIV-1 reverse transcriptase inhibition of new indolin-2-ones. Med Chem 2007;3 (6):533-42.
[16] Bouchikhi F, Rossignol E, Sancelme M, Aboab B, Anizon F, Fabbro D, Prudhomme M, Moreau P. Synthesis and biological evaluation of diversely substituted indolin-2-ones. Eur J Med Chem 2008;43 (11):2316-22.
[17] Atkins M, Jones CA, Kirkpatrick P. Sunitinib maleate. Nat Rev Drug Discov 2006;5 (4):279-80.
[18] Sun L, Tran N, Tang F, App H, Hirth P, McMahon G, Tang C. Synthesis and biological evaluations of 3-substituted indolin-2-ones: a novel class of tyrosine kinase inhibitors that exhibit selectivity toward particular receptor tyrosine kinases. J Med Chem 1998;41 (14):2588-603.
[19] Lüllmann H, Wehling M, Mohr K. Pharmakologie und Toxikologie Arzneimittelwirkungen verstehen - Medikamente gezielt einsetzen. Stuttgart: Georg Thieme, 1999.
[20] Kopec A, Panaszek B, Fal AM. Intracellular signaling pathways in IgE-dependent mast cell activation. Arch Immunol Ther Exp (Warsz) 2006;54 (6):393-401.
[21] Theoharides TC, Kempuraj D, Kourelis T, Manola A. Human mast cells stimulate activated T cells: implications for multiple sclerosis. Ann N Y Acad Sci 2008;1144:74-82.
[22] Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997;77 (4):1033-79.
[23] Marquardt DL, Parker CW, Sullivan TJ. Potentiation of mast cell mediator release by adenosine. J Immunol 1978;120 (3):871-8.
[24] Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. Nat Rev Immunol 2006;6 (3):218-30.
[25] Ramkumar V, Stiles GL, Beaven MA, Ali H. The A3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. J Biol Chem 1993;268 (23):16887-90.

[26] Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH, Jacobson MA. Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. J Biol Chem 2000;275 (6):4429-34.
[27] Schulte G, Fredholm BB. Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 2003;15 (9):813-27.
[28] Laffargue M, Calvez R, Finan P, Trifilieff A, Barbier M, Altruda F, Hirsch E, Wymann MP. Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. Immunity 2002;16 (3):441-51.
[29] Ludowyke R, Lagunoff D. Drug inhibition of mast cell secretion. Prog Drug Res 1985;29:277-301.
[30] Demo SD, Masuda E, Rossi AB, Throndset BT, Gerard AL, Chan EH, Armstrong RJ, Fox BP, Lorens JB, Payan DG, Scheller RH, Fisher JM. Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin-V binding assay. Cytometry 1999;36 (4):340-8.
[31] Windmiller DA, Backer JM. Distinct phosphoinositide 3-kinases mediate mast cell degranulation in response to G-protein-coupled versus FcepsilonRI receptors. J Biol Chem 2003;278 (14):11874-8.
[32] Serunian LA, Auger KR, Cantley LC. Identification and quantification of polyphosphoinositides produced in response to platelet-derived growth factor stimulation. Methods Enzymol 1991;198:78-87.
[33] Clarke NG, Dawson RM. Alkaline O leads to N-transacylation. A new method for the quantitative deacylation of phospholipids. Biochem J 1981;195 (1):301-6.
[34] Kalesnikoff J, Galli SJ. New developments in mast cell biology. Nat Immunol 2008;9 (11):1215-23.
[35] Klemm S, Ruland J. Inflammatory signal transduction from the Fc epsilon RI to NF-kappa B. Immunobiology 2006;211 (10):815-20.
[36] Tan SL, Parker PJ. Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem J 2003;376 (Pt 3):545-52.
[37] Brazil DP, Park J, Hemmings BA. PKB binding proteins. Getting in on the Akt. Cell 2002;111 (3):293-303.
[38] Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 1997;7 (4):261-9.
[39] Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307 (5712):1098-101.
[40] Kay AB, Walsh GM, Moqbel R, MacDonald AJ, Nagakura T, Carroll MP, Richerson HB. Disodium cromoglycate inhibits activation of human inflammatory cells in vitro. J Allergy Clin Immunol 1987;80 (1):1-8.
[41] Masuda ES, Schmitz J. Syk inhibitors as treatment for allergic rhinitis. Pulm Pharmacol Ther 2008;21 (3):461-7.
[42] Matsubara S, Li G, Takeda K, Loader JE, Pine P, Masuda ES, Miyahara N, Miyahara S, Lucas JJ, Dakhama A, Gelfand EW. Inhibition of spleen tyrosine kinase prevents mast cell activation and airway hyperresponsiveness. Am J Respir Crit Care Med 2006;173 (1):56-63.
[43] Chang TW, Shiung YY. Anti-IgE as a mast cell-stabilizing therapeutic agent. J Allergy Clin Immunol 2006;117 (6):1203-12; quiz 13.

[44] Gomez G, Jogie-Brahim S, Shima M, Schwartz LB. Omalizumab reverses the phenotypic and functional effects of IgE-enhanced Fc epsilonRI on human skin mast cells. J Immunol 2007;179 (2):1353-61.
[45] Mohn T, Potterat O, Hamburger M. Quantification of active principles and pigments in leaf extracts of Isatis tinctoria by HPLC/UV/MS. Planta Med 2007;73 (2):151-6.
[46] Marquardt DL, Alongi JL, Walker LL. The phosphatidylinositol 3-kinase inhibitor wortmannin blocks mast cell exocytosis but not IL-6 production. J Immunol 1996;156 (5):1942-5.
[47] Ali K, Camps M, Pearce WP, Ji H, Ruckle T, Kuehn N, Pasquali C, Chabert C, Rommel C, Vanhaesebroeck B. Isoform-specific functions of phosphoinositide 3-kinases: p110 delta but not p110 gamma promotes optimal allergic responses in vivo. J Immunol 2008;180 (4):2538-44.
[48] Kuehn HS, Gilfillan AM. G protein-coupled receptors and the modification of FcepsilonRI-mediated mast cell activation. Immunol Lett 2007;113 (2):59-69.
[49] Vosseller K, Stella G, Yee NS, Besmer P. c-kit receptor signaling through its phosphatidylinositide-3'-kinase-binding site and protein kinase C: role in mast cell enhancement of degranulation, adhesion, and membrane ruffling. Mol Biol Cell 1997;8 (5):909-22.

3 Separation and Detection of all Phosphoinositide Isomers

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Abstract

Phosphoinositides (PIs) play fundamental roles as signalling molecules in numerous cellular processes. Direct analysis of PIs is typically accomplished by metabolic labelling with ^{3}H-inositol or inorganic ^{32}P followed by deacylation, ion-exchange chromatography, and flow scintillation detection. This analysis is laborious, time consuming, and involves massive amounts of radioactivity. To overcome these limitations we established a non-radioactive LC-ESI/MS assay for the separation and analysis of deacylated PIs that allows discrimination of all isomers without the need for radioactive labelling. We applied the method to various cell types to study the PI levels upon specific stimulation.

1. Introduction

Biology of phosphoinositides

The inositol containing glycerophospholipids, collectively known as phosphoinositides (PIs), play a fundamental role in diverse cellular functions such as cell growth and differentiation, motility, calcium mobilisation and oncogenesis [1,2]. The family of the phosphoinositides consists of the mutual non-phosphorylated precursor phosphatidylinositol (PtdIns) and seven members with different phosphorylation patterns on the *myo*-inositol ring, where the 3-, 4-, and 5-positions can be phosphorylated by specific kinases (Fig. 1 A).

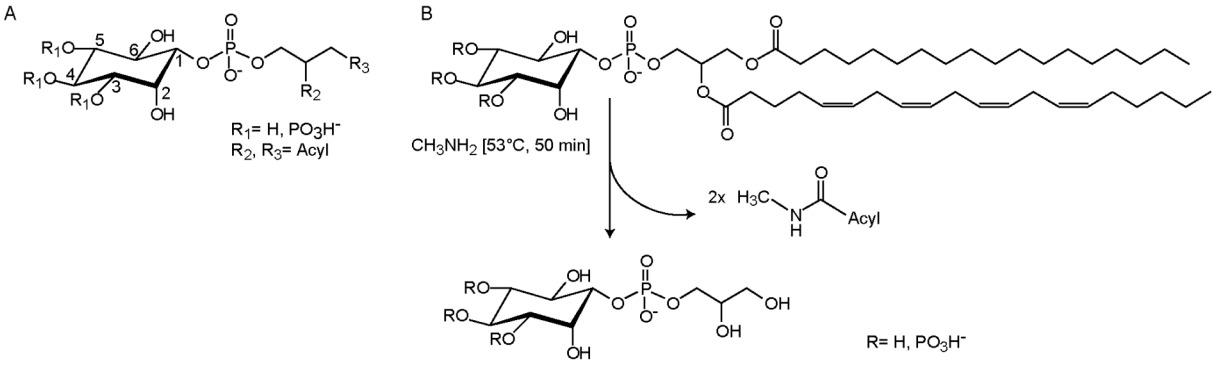

Fig. 1. A. General structure of all phosphoinositides, numbering of the ring is indicated and shows the positions of the phosphorylations. B. Structure of a typical PI, sn-1-stearoyl-2-arachidonyl-phosphatidylinositol and the performed deacylation step, resulting in cleavage of the lipid moiety.

PtdIns and its phosphorylation products phosphatidylinositol-4-phosphate (PtdIns4*P*) and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) form the so called canonical pathway [3] and are believed to be kept at constant levels at the plasma membrane. The other PIs are considered to be low-abundant signalling molecules that transiently appear upon stimulation. Stimulation with growth factors or insulin leads to increased PtdIns(3,4,5)P_3 levels, which in turn produces specific cellular responses. The bisphosphorylated PIs, PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,5)P_2 play distinct roles in signal prolongation after PtdIns(3,4,5)P_3 inducing stimuli, regulation of the actin cytoskeleton and vesicle transport, respectively [4-8]. Phosphoinositides monophosphates were long thought to be mere intermediates in the pathway but are now recognised to possess specific functions themselves in protein sorting, vesicular trafficking and in osmotic stress response [9-12].

Detection of PIs

Analysis of PIs has been achieved in several ways. Most frequently, metabolic radioisotope labelling with inorganic ^{32}P or ^{3}H-inositol, lipid extraction, chromatographic separation and radiographic analysis of phosphoinositides is performed [13-15]. Metabolic labelling involves very high doses of radioactivity (GBq), long labelling times, and only detects the turnover of PIs, whereas dormant pools of PIs remain unlabelled. More recent approaches include fluorescent-labelled binding proteins for specific PIs, and antibodies directed against PIs [16,17]. However, differentiation of all mono- and bisphosphorylated positional isomer PIs, has not been achieved yet.

Two fundamentally different approaches have been pursued in PI detection: (i) a comprehensive profiling of intact PIs [18-21], and (ii) head group analysis after cleavage of the lipid moieties [22,23]. The first approach used in lipidomics leads to a highly complex picture due to a plethora of closely related molecules that only vary in their lipid moieties. Separation and detection of the head groups following deacylation (Fig. 1 B) is a much more suitable approach if the focus lies on the signalling functions of PIs. Nonetheless, analysis of the low abundant PIs remains highly challenging.

As alternatives to radioisotope labelling, analysis by mass spectrometry [24], suppressed conductivity detection [25] and evaporative light scattering detection (ELSD) [26] have been used. Unfortunately, none of these allowed a discrimination of all isomers. An LC-ESI/MS method for separation of deacylated PtdInsP_2 isomers on a cyclodextrin column has been recently published [14]. However, chromatography suffered from poor peak shape and co-elution of PtdIns(3,5)P_2 and PtdIns(3,4,5)P_3.

Since all of the PIs can be interconverted by specific kinases and phosphatases, inhibition, stimulation, modification, or deletion of one of these enzymes may have profound implications on the biological response. Therefore the separation and simultaneous quantitative detection of all PIs are of major importance for a better understanding of their biological roles.

We have developed and describe here an LC-ESI/MS method for headgroup analysis of all PIs that allows a complete separation of PtdIns$P$ and PtdInsP_2 isomers using a polar endcapped RP-18 column and a volatile ion-pairing reagent. We also show selected applications of our method to PI analysis in relevant cellular models.

2. Experimental

2.1. LC-MS instrumentation

HPLC separation was carried out on an Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, and column thermostat (Agilent Technologies, Waldbronn, Germany). A liquid handler 215 (Gilson, Mettmenstetten, Switzerland) was used as autosampler. The HPLC was coupled to an Esquire 3000 ion trap mass spectrometer equipped with an electrospray (ESI) interface (Bruker Daltonics, Bremen, Germany).

Data acquisition and processing was performed using HyStar 3.0 software from Bruker Daltonics.

2.2. LC Method

2.2.1. Ion-pair chromatography

N, N-Dimethylhexylamine (DMHA; Acros, Thermo Fisher, Wohlen, Switzerland) was used as ion-pair reagent [27]. Mobile phase A consisted of 5 mM DMHA in water adjusted to pH 7.0 with acetic acid (Sigma-Aldrich, Buchs, Switzerland) and mobile phase B of acetonitrile or methanol with 5 mM DMHA and an equal amount of acetic acid. All solvents were from Scharlau (Scharlau, Barcelona, Spain).

2.2.2. Columns

Various columns were tested for suitability in phosphoinositide analysis, including Atlantis C18 (150 x 4.6 mm, 5 µm) and T3 (150 x 3.5 mm, 3 µm; Waters, Baden, Switzerland), Nucleosil C100 (250 x 4.6 mm, 5µm; Macherey-Nagel, Düren, Germany), LiChrosphere Diol (125 x 4.0 mm, 5 µm; Merck, Darmstadt, Germany) and Aqua C18 (250 x 4.6 mm, 5 µm and 75 x 2.0 mm, 3µm; Phenomenex, Torrance, CA).

2.2.3. Separation of phosphoinositides

Method 1

Separation of deacylated PIs with different numbers of phosphorylations; PtdIns, PtdIns*P*, PtdInsP_2, and PtdInsP_3, was achieved by ion pair chromatography on a modified RP-column (Aqua, Phenomenex C18 125 Å, 75 x 2.0 mm, 3 µm). A gradient from mobile phase B (acetonitrile) 0.1% to 50% in 25 min and a wash step (50% B to

100% B in 3 min, 100% B for 12 min, 100% B to 0.1% B in 5 min, 0.1 % B for 5 min) was applied.

Method 2

Separation of deacylated PIs with different numbers of phosphorylation plus additional separation of phosphoinositides bisphosphate isomers PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3,4)P_2, was achieved by ion pair chromatography on a modified RP-column (Aqua, Phenomenex C18 125 Å, 250 x 4.6 mm, 5 µm). A gradient from mobile phase B (acetonitrile) 15% to 35% in 40 min followed by a wash sequence (35% B to 100% B in 2 min, 100% B for 15 min, 100% B to 15% B in 3 min, 15 % B for 5 min) was applied.

Method 3

Separation of PtdIns(3)*P*, PtdIns(4)*P*, and PtdIns(5)*P*, additionally to separation of all other PIs, was performed with methanol as mobile phase B and a gradient from 15% to 50% in 60 min, followed by a wash step (50% B to 100% B in 2 min, 100% B for 15 min, 100% B to 15% B in 3 min, 15 % B for 5 min).

2.3. Mass spectrometry

Negative ion LC–MS spectra on the ion trap instrument were recorded after optimization of settings, under ion charge control conditions (ICC 20000) at a scan speed of 13000 m/z/s, using a gauss filter width of 0.2 m/z. Nitrogen was used as a drying gas at a flow rate of 10 L/min and as a nebulizing gas at a pressure of 30 psi. The nebulizer temperature was set to 300°C. Spectra were recorded in the range of m/z 200–600 in negative mode. Capillary voltage was at 4500V, endplate offset at −500V, capillary end voltage at −115.0V, skimmer voltage −40.0V and trap drive at 53.4.

2.4. Flow scintillation analysis

Levels of radioactively labelled intracellular phosphatidylinositides were determined essentially as described [28]. Briefly, 4 million cells were incubated with 500 µCi $^{32}P_i$ for 60 min at 37°C. After removal of non-incorporated $^{32}P_i$, cells were extracted as described below. Eluents from the column were splitted and examined online with a FLO-ONE A500 β-detector (Packard, Perkin Elmer, Monza, Italy).

2.5. Chemicals and cell culture

Murine bone marrow cells were cultured in Iscove's Modified Dulbecco's medium (IMDM; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (FCS; Amimed, Basel, Switzerland), 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine (both from Invitrogen, Basel, Switzerland). Cells were grown in humified atmosphere containing 5% CO_2 and maintained with 2 ng/ml recombinant murine interleukin-3 (IL3; PeproTech EC Ltd, London, UK).

Human embryonic kidney cells HEK 293 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine. Cells were transfected with jetPEI cationic polymer transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturers instructions. 24 h before transfection, cells were plated at 10^6 cells/ 25 cm^2 flask, then transfected with 2.6 µg GST-Vps34 and 0.4 µg Myc-S6K. 30 h after transfection, cells were starved over night and experiments were performed the following day.

Platelets were isolated from blood of healthy donors. Blood samples were mixed with acid citrate dextrose ACD (10.1 mM glucose, 30µM citric acid, pH 6.5 in 0.9% NaCl, all from Sigma-Aldrich) and centrifuged for 5 min at 1000g [29]. Platelet rich plasma was collected and washed in PBS.

Chemicals used for experiments were: Adenosine (Ade), N-Formyl-Met-Leu-Phe (fMLP), wortmannin (wort) (all from Sigma-Aldrich).

Phosphoinositide standards used were: Phosphoinositides sodium salt from bovine brain (Sigma-Aldrich), PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_2 as 1,2-dioctanoyl-*sn*-glycero-3-phosphoinositolphosphates ammonium salt and PtdIns3*P* and PtdIns5*P* as 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)- *sn*-glycero-3-phosphoinositolphosphates ammonium salt from Avanti Polar Lipids (Avanti Polar Lipids, Alabaster, AL).

2.6. Extraction, deacylation, and sample preparation

Extraction of PIs was adapted from Ogiso [24], a modified acidic Bligh-Dyer extraction [30] with addition of NaCl to the aqueous phase, which helps to reduce loss of PIs. Briefly, ca. 10^6 cells were extracted with 2 ml Methanol, 2 ml 1 M HCl, 0.15 ml 2 M NaCl and 2 ml chloroform (Solvents from Scharlau, other reagents from Sigma-Aldrich). Methanol was supplemented with PhosSTOP (Roche, Basel,

Switzerland), 1 mM NaF, 3 mM BHT, and 0.5 mM phosphatidic acid (all Sigma-Aldrich). The two phases were mixed well and centrifuged shortly for separation. The lower organic phase was removed, evaporated by nitrogen stream and transferred to deacylation (Fig. 1 B). Dried samples were incubated with methylamine solution in water/methanol/n-butanol (43: 46: 11) at 53° C for 50 min, all solvent was evaporated under vacuum, and then extracted with a mixture of n-butanol/petrol ether/ethyl formiate (20:4:1) and water [31]. The water-phase was dried in vacuum and the samples were dissolved in 40 µl of solvent A for LC-MS analysis.

3. Results and discussion

3.1. Separation of phosphoinositides in order of increasing phosphorylation

Separation of anionic or phosphorylated compounds is typically achieved by ion-exchange chromatography. However, typical ion-pairing reagents are not volatile and, hence, not compatible with LC-MS. We tested several volatile and MS-compatible ion-pairing reagents, such as formic acid, ammonium formiate and N,N-dimethyl-hexylamine (DMHA) and applied them on various columns (Nucleosil C100, LiChrospher Diol, and Phenomenex Aqua C18). The only acceptable separation of a phosphoinositide reference mixture was achieved on a short (75 mm) Phenomenex Aqua column with the addition of DMHA. Subsequently, we tested different gradient profiles, column temperatures, pH and concentrations of DMHA to optimize separation. Column temperature had a slight impact, and the best separation was obtained at 15°C. In contrast, pH of the mobile phase was critical. Best results were obtained at pH 7, while lower pH values lead to peak tailing and split peaks and higher pH resulted in shorter retention times. Increase of DMHA concentration from 5 mM to 10 mM and 20 mM did not enhance the quality of the separation. A water-acetonitrile gradient was applied and the final gradient program was 0.1%-50% ACN (containing 5 mM DMHA) in 25 minutes, leading to the separation of a PI standard mixture shown in Fig. 2 A. Peaks shown resulted from 0.1 μg of deacylated PtdIns(3,4,5)P_3 standard mixed with 4 μg of deacylated phosphoinositde extract (mixture of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2).

Separation of a mixture of PIs standards was also achieved under isocratic conditions (27% ACN and 73% water (5 mM DMHA)) but separation of biological samples, however, could not be achieved under these conditions, probably due to interference with the biological matrix.

To test the applicability of our method to biological samples, we analysed mast cell extracts. Mast cells are known to produce large amounts of PtdIns(3,4,5)P_3 upon activation that can be provoked *in vitro* by stimulation with adenosine [28]. Murine bone marrow derived mast cells (BMMCs) were stimulated with 5 μM adenosine for 30 sec, the lipids were extracted, deacylated and transferred to LC-MS analysis, where phosphoinositides were separated with method 1. Our method clearly succeeded in reproducing the increased amounts of PtdIns(3,4,5)P_3 upon stimulation

of mast cells with adenosine, whereas peaks of PtdIns and PtdIns*P* remained constant (Fig. 2 B, C).

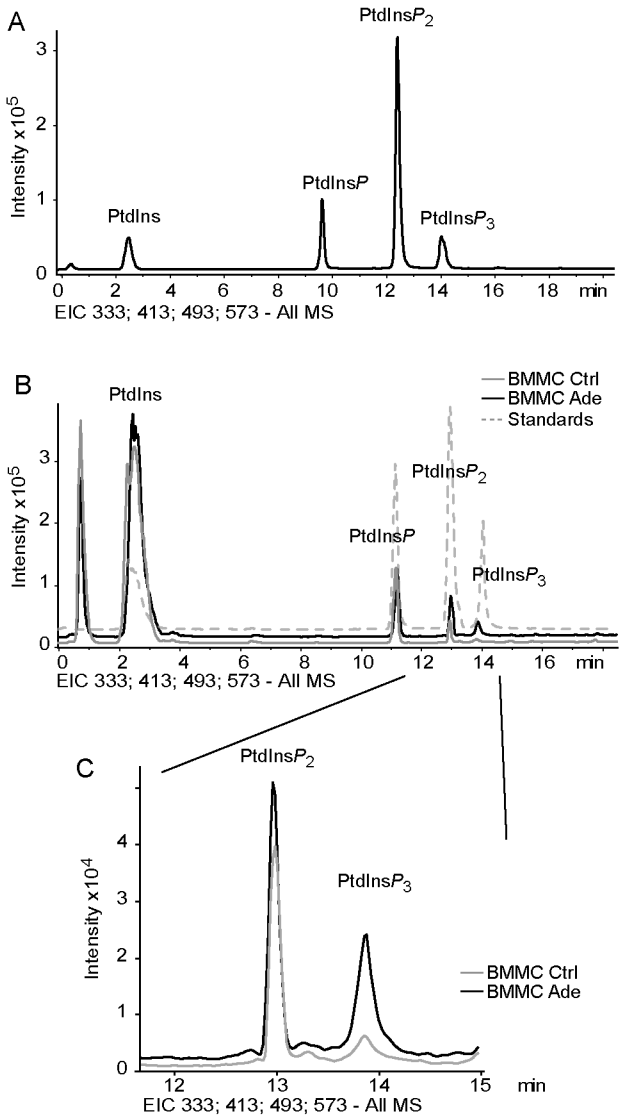

Fig. 2. A. Extracted ion chromatogram (EIC) of deacylated phosphoinositide standards (PtdIns, PtdIns*P*, PtdInsP_2, and PtdInsP_3) mixture. B. EIC of cell samples from 4 mio BMMC and standards. Cells were stimulated with Adenosine (Ade) 5 µM for 30 s to induce production of PtdInsP_3. C. Peaks of PtdInsP_2 and PtdInsP_3 from control cells in relation to stimulated cells, levels of PtdInsP_3 increased after stimulation with Adenosine. Column: Phenomenex Aqua C18 (75 x 2 mm, 3 µm). Solvent A: H_2O/5 mM DMHA, Solvent B: Acetonitrile/5mM DMHA. Gradient: 0.1% B to 50% B in 25 min.

3.2. Regioisomer separation of phosphatidylinositol bisphosphates

To achieve separation of PtdInsP_2 regioisomers various columns were tested, including Nucleosil C100, Atlantis C18, Atlantis T3, and Phenomenex Aqua C18. The separation was only achieved on a 250 mm long Phenomenex Aqua column with application of a water-acetonitrile gradient, under addition of 5 mM DMHA as ion-pair

reagent (method 2). A mixture of standards of all PtdInsP_2 isomers was separated in the elution order of PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,5)P_2 (Fig. 3 A, B). Peaks shown resulted from 0.1 μg of deacylated PtdIns(3,5)P_2 and PtdIns(3,4)P_2 standard mixed with 4 μg of deacylated phosphoinositde extract (mixture of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2). Several other solvent mixtures and addition of modifiers were tested. A gradient of water-methanol (method 3) resulted in separation as well, producing a change in elution order to PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(4,5)P_2 (Fig. 3 C, D).

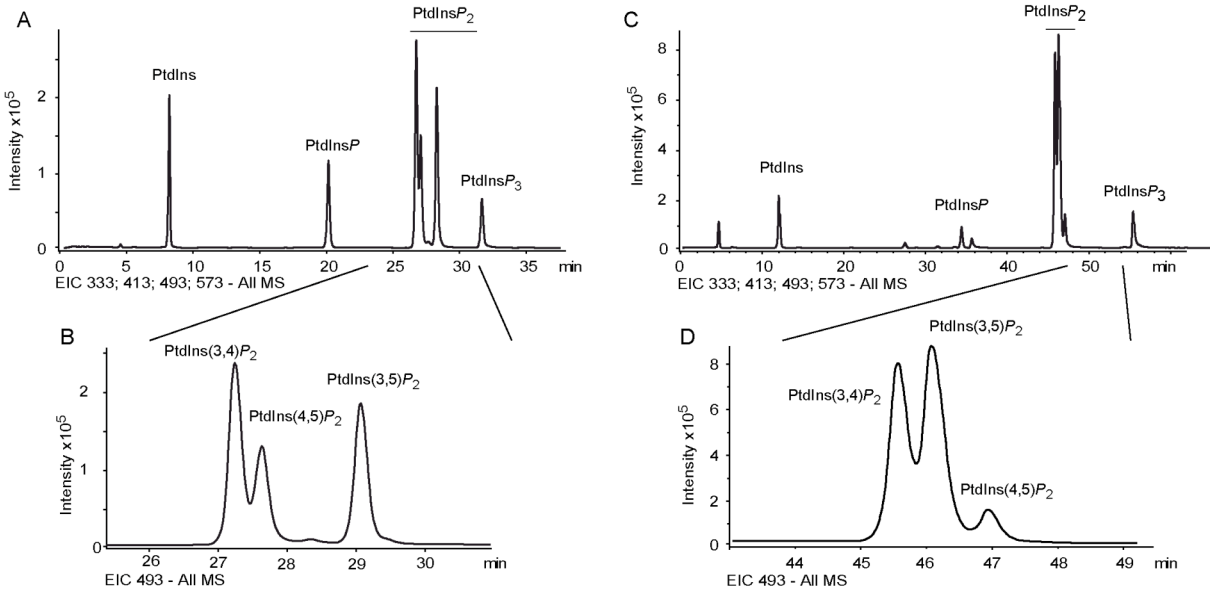

Fig. 3. A + B. Separation of a standard mixture of PIs containing all PtdInsP_2 regioisomers B. Elution of the isomers of PtdInsP_2 in following order: PtdIns(3,4)P_2, PtdIns(4,5)P_2, and PtdIns(3,5)P_2. Column: Phenomenex Aqua C18 (250 x 4.6 mm, 5 µm). A: H2O, 5 mM DMHA, B: Acetonitrile, 5mM DMHA. Gradient: 15% B to 35% B in 40 min. C + D. Separation of a standard mixture of PIs containing all PtdInsP_2 regioisomers D. Separation of PtdInsP_2 isomers in sequence of PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(4,5)P_2. Column: Phenomenex Aqua C18 (250 x 4.6 mm, 5 µm). A: Methanol/5 mM DMHA, B: Acetonitrile/5 mM DMHA. Gradient: 15% B to 50% B in 60 min.

As a practical example for the analysis of PtdIns(3,5)P_2 in biological samples, we selected Vps34 transfected HEK 293 cells under hyperosmolar stress [22,25]. The PI3-kinase Vps34 is known to stimulate osmotic stress related production of PtdIns(3,5)P_2 in yeast [22]. HEK Vps34 were incubated in medium supplemented with 1M NaCl solution for 10 minutes inducing stimulation of Vps34 and generation of PtdIns3*P* that lead to production of PtdIns(3,5)P_2. As can be seen in Fig. 4 A + B, the transfection with Vps34 already induced some production of PtdIns(3,5)P_2, which was then further increased upon NaCl hyperosmotic stimulation.

Analysis of PtdIns(3,4)P_2 in biological samples was performed with human blood platelets. In comparison to many other cell models, platelets produce PtdIns(3,4)P_2 in relatively large amounts upon activation. PtdIns(3,4,5)P_3 is degraded to PtdIns(3,4)P_2 by the 5-phosphatase SHIP1 [32]. PtdIns(3,4)P_2 is responsible for the persistence of the signal induced by PtdIns(3,4,5)P_3 [8,33]. For detection of PtdIns(3,4)P_2, platelets were stimulated with fMLP for 90 s. This resulted in elevated amounts of PtdIns(3,4)P_2 which were not present in control cells (Fig. 4 C, D).

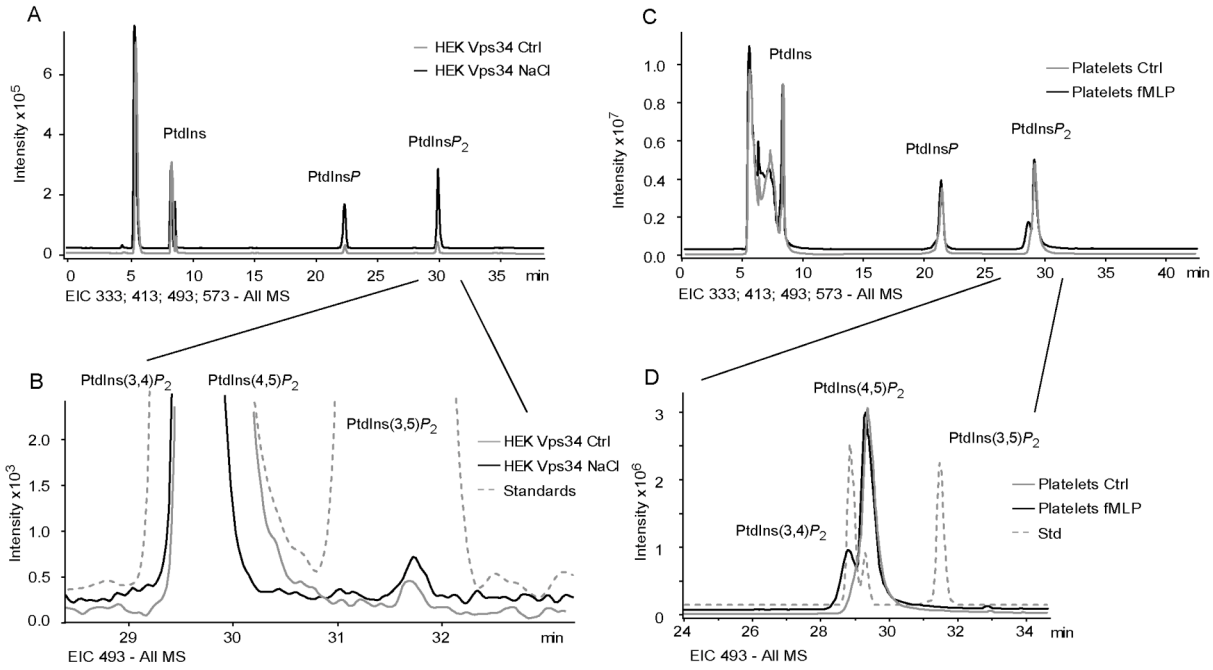

Fig. 4. Analysis of phosphoinositide cell samples A + B. Extracted ion chromatograms (EIC) of HEK Vps34 cell samples showed increased amounts of PtdIns(3,5)P_2 , that were further increased by stimulation with 1M NaCl for 10 minutes. C + D. EIC of lipids extracted from platelets, control sample and stimulated with fMLP for 1 min. Stimulation induced generation of PtdIns(3,4)P_2 that was not present in the control sample. Column: Phenomenex Aqua C18 (250 x 4.6 mm, 5 µm). A: H2O, 5 mM DMHA, B: Acetonitrile, 5mM DMHA. Gradient: 15% B to 35% B in 40 min.

3.3. Regioisomer separation of phosphatidylinositol monophosphates

Separation of the mono-phosphorylated isomers was only achieved with methanol as organic modifier (Fig. 5 A, B), while use of acetonitrile resulted in co-elution of PtdIns3*P* and PtdIns5*P*.

We tested the practical applicability of these conditions with Vps34 transfected HEK cells. Vps34 is stimulated by amino acid addition through a yet unknown mechanism [34]. Starvation and subsequent amino acid supplementation stimulates Vps34 and induces the generation of PtdIns3*P* [35,36]. HEK Vps34 cells were serum and amino

acid starved for 12 h and 2 h, respectively, stimulated for 30 min by addition of serum and amino acids, prior to extraction of lipids, deacylation and analysis. The stimulation with serum and amino acids induced synthesis of PtdIns3*P*, which, in contrast, was inhibited by incubation with the pan-PI3-kinase inhibitor wortmannin 15 min prior to and during stimulation with amino acids and serum (Fig. 5 C, D).

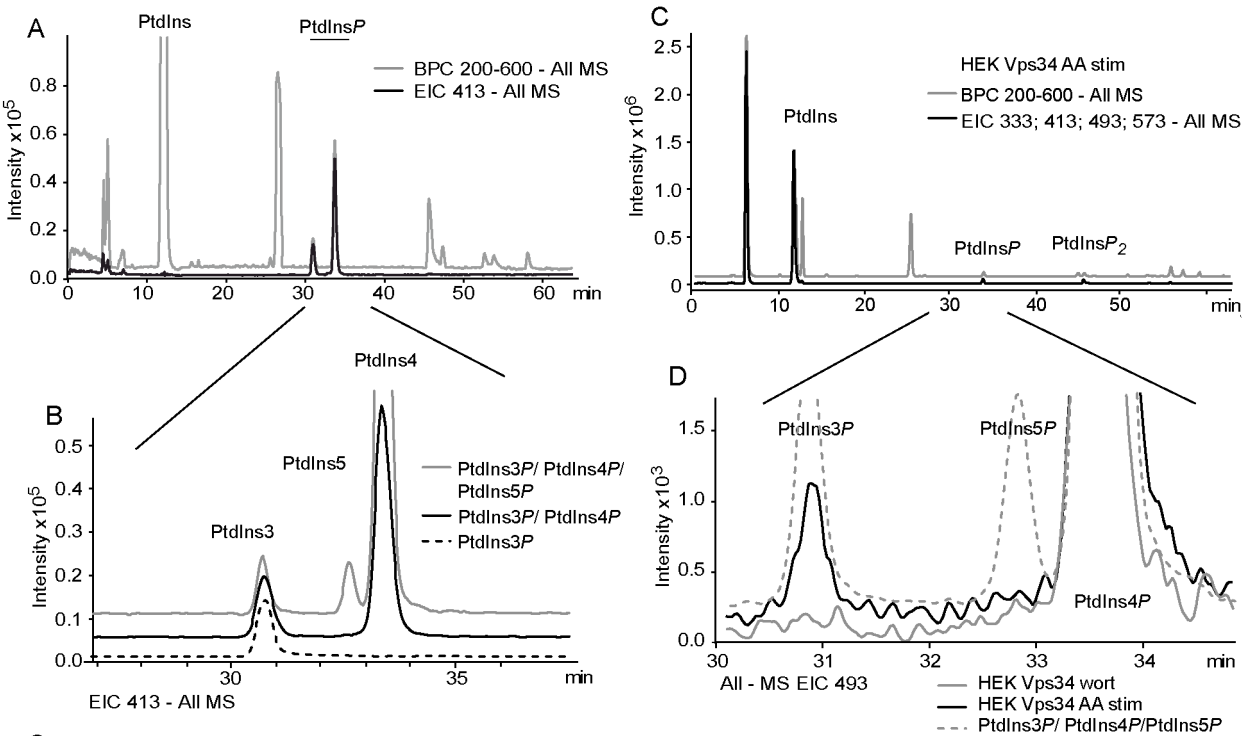

Fig. 5. A + B. Extracted ion chromatogram (EIC) and base peak chromatogram (BPC) of mixed standards of phosphoinositide monophosphates separated according to method 3. Separation of PtdIns3*P*, PtdIns4*P*, and PtdIns5*P* was achieved. C. BPC and EIC of a HEK Vps34 cell sample. D. EIC of HEK Vps34 cell samples that were amino acid stimulated and incubated with wortmannin 15 min prior to and during amino acid stimulation. The increase in PtdIns3*P* following amino acid starvation and subsequent stimulation was blocked by addition of wortmannin. Column: Phenomenex Aqua C18 (250 x 4.6 mm, 5 µm). A: Methanol/5 mM DMHA, B: Acetonitrile/5 mM DMHA. Gradient: 15% B to 50% B in 60 min.

3.4. Comparison with radiolabelling method

For comparison with the standard detection method of scintillation analysis, we applied radiolabelled samples to the newly developed HPLC method combined with subsequent scintillation analysis. This also gave a reconfirmation of the peaks measured with MS. The large loop size of 1 ml within the flow scintillation analyser and a flow rate of only 0.5 ml/min resulted in significant peak broadening and asymmetry. Nonetheless, peaks of the major PIs could be detected (Fig. 6 A, B). A

radioactive labelled cell sample, and a labelled standard of PtdIns3*P* showed all major PI peaks at the same retention times as when detected with MS (Fig. 6 C). Furthermore it showed a different retention time than the PtdIns4*P* peak of the cell sample.

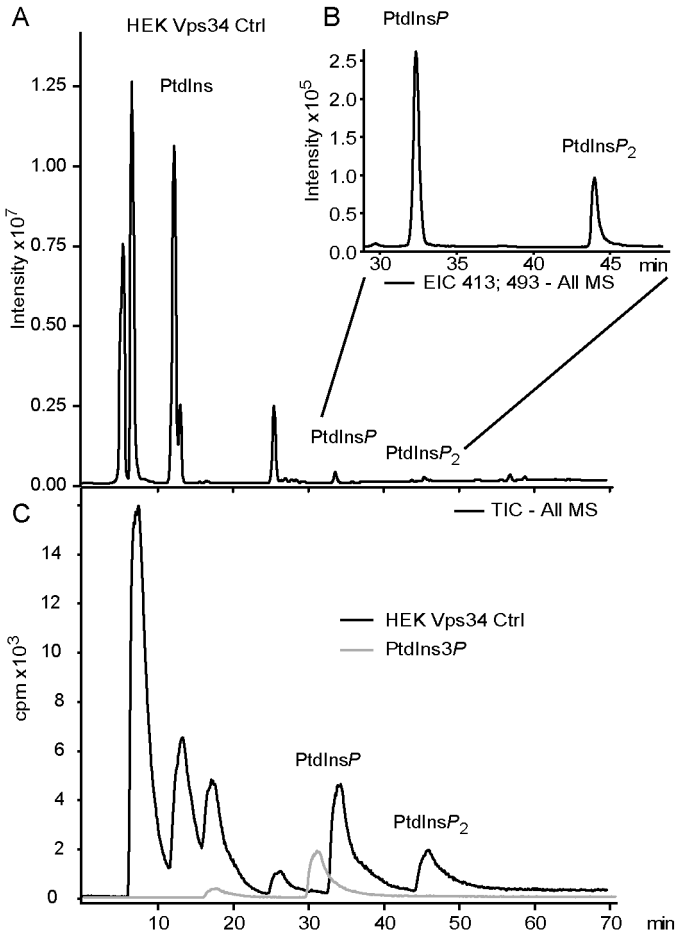

Fig. 6. A + B. Total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of a HEK Vps34 control cell sample separated according to method 3. C. Chromatogram of parallel online flow scintillation analysis of the same HEK Vps34 cell sample and a standard of PtdIns3*P*. Column: Phenomenex Aqua C18 (250 x 4.6 mm, 5 µm). A: Methanol/5 mM DMHA, B: Acetonitrile/5 mM DMHA. Gradient: 15% B to 50% B in 60 min.

3.5. Discussion

The separation of PIs differing in degree of phosphorylation was successfully achieved with method 1 and was successfully applied to analysis of phosphoinositides in cell samples. The choice of a polar endcapped RP column and an appropriate volatile ion-pairing reagent was decisive for good separation and highly symmetrical peak shapes. For analysis of PtdIns(3,4,5)P_3 this method offers a good alternative to currently used HPLC methods using radiolabelling and ion-

exchange columns. The short analysis time facilitates handling of large sample numbers.

A satisfactory separation of PtdInsP_2 isomers was achieved on the same column type. However, maximum column length was needed to obtain the required separation performance. This analytical method offers new perspectives for research on phosphoinositide-signalling. The low abundant PtdInsP_2 isomers can be separated with method 2 and analysed without the need for radioactive labelling.

Furthermore a complete separation of PtdIns3*P*, PtdIns4*P*, and PtdIns5*P* was achieved by applying methanol instead of acetonitrile as solvent B (method 3). This isomer separation has not been possible before. Also the PtdInsP_2 isomers were separated with method 3. However, the changed elution order as compared to method 2 precludes a complete separation of the highly abundant PtdIns(4,5)P_2 from the minor PtdIns(3,5)P_2. Therefore, analysis of PtdIns(3,5)P_2 should be performed with method 2. Depending on the research focus the suitable method can be applied and produces better separation of isomers than what was possible before.

Separation of PtdInsP_2 isomers has been shown before on a cyclodextrin column [14] but produced co-elution of PtdIns(3,4,5)P_3 and PtdIns(3,5)P_2. Furthermore separation of PtdIns*P* isomers was neither shown nor discussed in that publication. Compared to a cyclodextrin column the polar endcapped RP-column is more robust, offers superior separation performance for PIs, and is easier to use with respect to predictability of chromatographic behaviour.

All methods presented here were tested in relevant biological samples. However, the extraction procedure of PIs still remains a major concern. As extensively discussed by Ogiso et al. [24], recovery rates of PIs are generally poor and decrease with increasing phosphorylation. Due to the amphiphilic properties these lipids are difficult to extract. The ionic headgroup adsorbs easily to glass surfaces, whereas the lipid moiety adsorbs to plastic. Glass surfaces adsorption can be prevented by silanized glassware, plastic surfaces can be pretreated with lipids as adsorption protectants. We used plastic tubes, lipids as adsorption protectants, phosphatase inhibitors to prevent artefactual changes in PI patterns, and were able to significantly increase the recovery of analytes. However, further optimization of the extraction protocol will be needed in the future to enable a quantitative recovery of deacylated PIs and truly quantitative analysis.

Parallel detection with online flow scintillation and mass spectrometry showed the comparability of the methods and, at the same time, the significantly better peak shape achieved in LC-MS. There are numerous other drawbacks with radiolabelling, such as the potential harmfulness of radioisotopes for the user and the environment, the short half-life of $^{32}P_i$, a need for high doses per experiment, high costs, and limited applicability in long-term experiments due to loss of signal and radioactivity-related degradation of biomolecules.

The LC-MS methods presented here offer a superior approach for analysis of intracellular PI-levels including differentiation of PtdIns*P* and PtdInsP_2 isomers.

4. Literature

[1] L.C. Skwarek, G.L. Boulianne, Dev Cell 16 (2009) 12.
[2] M.P. Wymann, R. Schneiter, Nat Rev Mol Cell Biol 9 (2008) 162.
[3] B. Payrastre, K. Missy, S. Giuriato, S. Bodin, M. Plantavid, M. Gratacap, Cell Signal 13 (2001) 377.
[4] I.H. Batty, J. van der Kaay, A. Gray, J.F. Telfer, M.J. Dixon, C.P. Downes, Biochem J 407 (2007) 255.
[5] M.J. Clague, S. Urbe, J. de Lartigue, Exp Cell Res (2008).
[6] D. Manna, A. Albanese, W.S. Park, W. Cho, J Biol Chem 282 (2007) 32093.
[7] R.H. Michell, V.L. Heath, M.A. Lemmon, S.K. Dove, Trends Biochem Sci 31 (2006) 52.
[8] G. Di Paolo, P. De Camilli, Nature 443 (2006) 651.
[9] J.B. Morris, K.A. Hinchliffe, A. Ciruela, A.J. Letcher, R.F. Irvine, FEBS Lett 475 (2000) 57.
[10] C. Pendaries, H. Tronchere, C. Racaud-Sultan, F. Gaits-Iacovoni, S. Coronas, S. Manenti, M.P. Gratacap, M. Plantavid, B. Payrastre, Adv Enzyme Regul 45 (2005) 201.
[11] D. Sbrissa, O.C. Ikonomov, R. Deeb, A. Shisheva, J Biol Chem 277 (2002) 47276.
[12] A.E. Wurmser, J.D. Gary, S.D. Emr, J Biol Chem 274 (1999) 9129.
[13] H. Guillou, L.R. Stephens, P.T. Hawkins, Methods Enzymol 434 (2007) 117.
[14] P.T. Ivanova, S.B. Milne, M.O. Byrne, Y. Xiang, H.A. Brown, Methods Enzymol 432 (2007) 21.
[15] K.R. Auger, L.A. Serunian, S.P. Soltoff, P. Libby, L.C. Cantley, Cell 57 (1989) 167.
[16] H. Hama, J. Torabinejad, G.D. Prestwich, D.B. DeWald, Methods Mol Biol 284 (2004) 243.
[17] T.E. Rusten, H. Stenmark, Nat Methods 3 (2006) 251.
[18] S.B. Milne, P.T. Ivanova, D. DeCamp, R.C. Hsueh, H.A. Brown, J Lipid Res 46 (2005) 1796.
[19] M. Pulfer, R.C. Murphy, Mass Spectrom Rev 22 (2003) 332.

[20] M.R. Wenk, L. Lucast, G. Di Paolo, A.J. Romanelli, S.F. Suchy, R.L. Nussbaum, G.W. Cline, G.I. Shulman, W. McMurray, P. De Camilli, Nat Biotechnol 21 (2003) 813.
[21] A.D. Postle, D.C. Wilton, A.N. Hunt, G.S. Attard, Prog Lipid Res 46 (2007) 200.
[22] S.K. Dove, F.T. Cooke, M.R. Douglas, L.G. Sayers, P.J. Parker, R.H. Michell, Nature 390 (1997) 187.
[23] M. Laffargue, J.M. Ragab-Thomas, A. Ragab, J. Tuech, K. Missy, L. Monnereau, U. Blank, M. Plantavid, B. Payrastre, P. Raynal, H. Chap, FEBS Lett 443 (1999) 66.
[24] H. Ogiso, R. Taguchi, Anal Chem (2008).
[25] C. Nasuhoglu, S. Feng, J. Mao, M. Yamamoto, H.L. Yin, S. Earnest, B. Barylko, J.P. Albanesi, D.W. Hilgemann, Anal Biochem 301 (2002) 243.
[26] T. Gunnarsson, L. Ekblad, A. Karlsson, P. Michelsen, G. Odham, B. Jergil, Anal Biochem 254 (1997) 293.
[27] R. Tuytten, F. Lemiere, W.V. Dongen, E.L. Esmans, H. Slegers, Rapid Commun Mass Spectrom 16 (2002) 1205.
[28] M. Laffargue, R. Calvez, P. Finan, A. Trifilieff, M. Barbier, F. Altruda, E. Hirsch, M.P. Wymann, Immunity 16 (2002) 441.
[29] B. Dewald, M. Baggiolini, Biochim Biophys Acta 888 (1986) 42.
[30] E.G. Bligh, W.J. Dyer, Can J Biochem Physiol 37 (1959) 911.
[31] N.G. Clarke, R.M. Dawson, Biochem J 195 (1981) 301.
[32] M.P. Gratacap, S. Severin, G. Chicanne, M. Plantavid, B. Payrastre, Adv Enzyme Regul 48 (2008) 240.
[33] A. Sorisky, W.G. King, S.E. Rittenhouse, Biochem J 286 (Pt 2) (1992) 581.
[34] P. Gulati, G. Thomas, Biochem Soc Trans 35 (2007) 236.
[35] T. Nobukuni, M. Joaquin, M. Roccio, S.G. Dann, S.Y. Kim, P. Gulati, M.P. Byfield, J.M. Backer, F. Natt, J.L. Bos, F.J. Zwartkruis, G. Thomas, Proc Natl Acad Sci U S A 102 (2005) 14238.
[36] M.P. Byfield, J.T. Murray, J.M. Backer, J Biol Chem 280 (2005) 33076.

C. CONCLUSIONS

˝When moving forward towards the discovery of the unknown,
the scientist is like a traveller who reaches higher and higher summits
from which he sees in the distance new countries to explore˝
– Louis Pasteur, Free lance of Science, Chapter III (p.87)

Isolated natural products that had shown diverse biological activity in previously published tests, namely militarinone A and indolinone were analysed for their direct effect on molecular signalling processes. Whereas militarinone A had initially shown neurotrophic activity in PC12 cells, indolinone pronouncedly blocked degranulation of rat peritoneal mast cells. Degranulation is dependent on phosphoinositide signalling. This eventually prompted us to have a closer look at phosphoinositide-levels in living cells and to develop an improved analysis that offers significant advantages over the current approach using radioactive labelling and ion-exchange HPLC.

Published results of militarinone A reporting it as a neurotrophic substance were found to describe an intermediate phenomenon restricted to PC12 cells, since other cell lines underwent apoptosis within 24h. We propose that this difference is due to varying constitutive levels of p53. Hence, increased neuronal outgrowth could not be confirmed as a general activity of militarinone A, as this effect was strongly dependent on the cell type. Although the exact mechanism of action and the direct target of militarinone A could not be determined, the discovery of militarinone A as an apoptosis-inducing agent interfering with p53 transcriptional activity may open a new area for further studies. Regulation of p53 and the plethora of factors influencing its activity are still not fully understood. Of particular interest are preliminary data indicating that primary cells (bovine lymph node cells and BMMC) do not undergo apoptosis even after incubation for one week with militarinone A, whereas all cancer or cancerous cells tested (PC12, N2a, IMR-32, Jurkart T-cells, *Theileria parva*-infected T-cells) died within 72 hours at the latest. This suggests that transformed cells might be more sensitive to p53-dependent apoptosis, which is supported by the fact that p53 is mutated in 50% or more of all cancers known.

The study on militarinone A clearly demonstrates the importance of selecting appropriate assays for screening and subsequent bioactivity tests. Once an active extract or substance has been identified its activity should be assessed in various cells lines of different species to verify the observed effect. Testing of newly identified natural products on a subset of defined cells would yield a more comprehensive picture of their bioactivity profile that would allow a better comparison of different compounds. If all data were fed into a single database, eventually a system biology or '-omics' –type activity picture would emerge.

In the study on indolinone, we confirmed the initially observed stabilising effect on mast cells of a different species and in a different assay set-up. We showed that indolinone efficiently blocked $PtdInsP_3$ production due to inhibition of all class I PI3-kinases, therefore preventing activation of Akt and subsequent mast cell degranulation. The concentrations necessary to obtain the observed effect *in vitro*, however, were too high to consider *in vivo* testing.

Since mast cell degranulation depends on phosphoinositide signalling we studied phosphosinositide levels in living cells upon stimulation. For this purpose, we developed a method that allows individual analysis of all phosphoinositides, including all PtdInsP- and $PtdInsP_2$-isomers. This novel method based on ion-pair chromatographic separation and ESI-MS detection offers substantial perspectives for application in phosphoinositide-signalling research as it allows relative quantification of all the different PIs in cells.
Phosphoinositides are upstream members of various important signalling cascades such as the Akt-, PKC-, and PLC-pathway and, hence, involved in a multiplicity of cellular processes. While the importance of $PtdInsP_2$ and $PtdInsP_3$ in signalling has long been recognized, PtdInsP has received little attention only. Our LC-MS method facilitates PI analysis and will contribute to the further elucidation of their role in cellular signalling.

In contrast to the industrial drug discovery approach, where chemical libraries are screened for molecules with activity in a given assay, the strength of natural product discovery lies in identification of new mechanisms or drug targets. Assignment of an observed effect to a defined molecular interaction is extremely challenging. In the

study on militarinone A we could not determine the specific interaction and mechanism, although we have elucidated parameters involved in its activity. However, the concept of a step-by-step examination of a signalling pathway associated with an observed effect (degranulation) and determination of the point of action was successfully applied in the case of indolinone. This strategy could be analogously applied for other natural products with preliminary assessed specific activity but unknown mechanism of action.

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Zwei Dinge sind zu unserer Arbeit nötig:
Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen
-Albert Einstein

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E. CURRICULUM VITAE

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Publications

Kuenzi P, Kiefer S, Koryakina A, Hamburger M. Promotion of cell death or neurite outgrowth in PC-12 and N2a cells by the fungal alkaloid militarinone A depends on basal expression of p53. Apoptosis 2008;13 (3):364-76.

Koryakina A, Aeberhardt J, Kiefer S, Hamburger M, Kuenzi P. Regulation of secretases by all-trans retinoic acid. FEBS 2009; 276: 2645-55.

Kiefer S, Mertz A, Koryakina A, Hamburger M, Kuenzi P. Mast Cell Degranulation. Biolochemical Pharmacology. Submitted.

Kiefer S, Rogger J, Koryakina A, Hamburger M, Kuenzi P. Non-Radioactive Measurement of all Phosphatidylinositolphosphates. Journal of Chromatography A. Submitted.

Koryakina A, Meoli D, Kiefer S, Moes S. Jenö P, Hamburger M, Kuenzi P. ADAM17 binds to alpha- and beta-tubulin during mitosis. In Preparation.

Presentations

S. Kiefer, P. Küenzi, A. Koryakina and M. Hamburger. “The Fungal Metabolite Militarinone A Induces Cell Death and Differentiation” 4th EMBO Meeting on Cellular Signaling and Molecular Medicine, Dubrovnik, Croatia, June 15-17, 2006 (poster presentation)

S. Kiefer, P. Küenzi, T. Mohn and M. Hamburger. “Non-Radioactive Measurement of Phosphatidylinositol (3,4,5) Trisphosphate” Life Science 2007, Glasgow, UK, July 8-12, 2007 (poster presentation)

P. Küenzi, S. Kiefer, A. Koryakina, M. Hamburger. “The fungal metabolite militarinone A induces cells death and differentiation” 55th Annual Meeting and International Congress of the Society for Medicinal Plant Research, Graz, Austria, Sept 2 - 6, 2007 (poster presentation)

S. Kiefer, P. Küenzi, T. Mohn and M. Hamburger. “Non-Radioactive Measurement of Phosphatidylinositol (3,4,5) Trisphosphate” Bio Valley Science Day 2007 Basel, Switzerland, October 23, 2007 (poster presentation)

S. Kiefer, J. Rogger, P. Küenzi, M. Hamburger. “Analysis of Phosphatidylinositides by LC-ESI/MS” FEBS/ESF Workshop, Oslo, Norway, September 25-28, 2008 (poster presentation)

S. Kiefer, P. Küenzi, M. Hamburger. “Effects of 1,3-Dihydro-3-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2 H-indol-2-one (Indolinone) from Isatis tinctoria on Mast Cell Degranulation” Swiss Pharma Science Day 2008, October 9, 2008, University of Bern (poster presentation)

S. Kiefer, P. Küenzi, M. Hamburger “(E,Z)-3-(3’,5’-dimethoxy-4’-hydroxy-benzylidene)-2-indolinone blocks mast cell degranulation” USGEB Annual Meeting 2009, Interlaken, January 29/30 , 2009 (poster presentation)